# Targeting Heat Shock Proteins for Immunotherapy in Multiple Myeloma: Generation of Myeloma-Specific CTLs Using Dendritic Cells Pulsed with Tumor-Derived gp96

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**Abstract**

**Purpose:** To develop effective immunotherapies for patients with multiple myeloma, it is important to use novel tumor antigens. Recent studies in solid tumors show that tumor-derived heat shock proteins (Hsp) can be used as immunogen; however, no such study has yet been reported in multiple myeloma.

**Experimental Design:** We examined whether myeloma-derived Hsp gp96 can be used as a myeloma antigen. Specific CTL lines were obtained after repeatedly stimulating T cells with autologous, HLA-A*0201$^+$ dendritic cells pulsed with gp96 derived from HLA-A*0201$^+$ human myeloma cell line (HMCL) U266 or primary myeloma cells.

**Results:** These T cells lysed not only gp96-pulsed dendritic cells, U266, and other HLA-A*0201$^+$ HMCLs IM-9 and XG1 but also effectively killed HLA-A*0201$^+$ primary myeloma cells from patients. No killing was observed against unpulsed dendritic cells, dendritic cells pulsed with control gp96, HLA-A*0201$^-$ HMCLs, and primary myeloma cells, or HLA-A*0201$^+$ nonmyeloma cells. Cytotoxicity was mainly MHC class I/HLA-A*0201 restricted, suggesting that the CTLs recognized gp96-chaperoned peptides on HLA-A*0201 that were derived from shared myeloma antigens and that myeloma cells naturally present these peptides in the context of their surface MHC molecules. Upon antigen stimulation, these T cells secreted IFN-$\gamma$ and tumor necrosis factor-$\alpha$, indicating that they belong to type 1 T-cell subsets.

**Conclusion:** These results show that these T cells are potent CTLs that are able to effectively lyse myeloma cells but not normal blood cells and also suggest that Hsps from allogeneic tumor cells may be used as vaccines to immunize patients.

Multiple myeloma is still a fatal hematologic malignancy, characterized by an accumulation of, in most cases, monoclonal malignant plasma cells in the bone marrow of patients (1). Clearly, there is a need for new treatments to stabilize or even eradicate minimal residual disease achieved after the treatment with high-dose chemotherapy and stem cell transplantation. Immunotherapy may be an appropriate means to control residual disease as well as to provide an alternative treatment modality to conventional chemotherapy for patients with multiple myeloma. Currently, immunotherapy trials in multiple myeloma have employed idiotype proteins isolated from the serum of patients (2), and there is evidence to suggest that idiotype-specific T cells are either tolerized or deleted *in vivo* and that idiotype-derived immunodominant tumor peptides may not exist in all patients (3, 4). To develop more effective immunotherapy strategies for patients with multiple myeloma, there is an urgent need for identifying and using other potent myeloma antigens that also are shared among patients with multiple myeloma.

Heat shock proteins (Hsp) are a large family of both inducibly and ubiquitously expressed protein chaperones involved in assisting protein folding and unfolding in cells. They include a number of different molecular weight class families, such as Hsp27, Hsp70, and Hsp90. Hsps are among the most conserved proteins known in phylogeny with respect to both structure and function (5, 6). The realization of the immunologic significance of Hsps came from the observation that tumor cell–derived Hsps could immunize against tumors, which is attributed to Hsp-chaperoned peptides derived from tumor antigens (7–9). Recent studies in solid tumors have shown that tumor-derived Hsps, such as Hsp70 and gp96, are immunogenic and potent in stimulating the generation of tumor-specific CTLs (10, 11). Hsp70- and gp96-based vaccines have been tested in early-phase clinical trials in solid tumors as well as in lymphoma and leukemias; all showed minimal

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toxicity and potential efficacy (12–14). Phase III clinical studies using tumor-derived Hsp70 or gp96 as vaccines are ongoing for melanoma and renal cell carcinoma (15, 16). Nevertheless, there is no report whether myeloma-derived Hsps could be used as tumor antigens for immunotherapy in multiple myeloma. Considering the diverse biology of different tumors, which precludes any tumor type(s) or approach from serving as a dominant paradigm for the development of immunotherapies across a broad spectrum of cancers, preclinical studies are much needed to explore the usefulness and feasibility of using myeloma-derived Hsps for immunotherapy in multiple myeloma.

The aim of this study was to investigate whether myeloma cell–derived Hsp-peptide complexes (hereafter referred to as Hsps or gp96 for simplicity) can be used as myeloma-associated antigens. Myeloma-derived gp96 was obtained and used to pulse dendritic cells. With the use of autologous, gp96-pulsed mature dendritic cells, specific CTL lines were generated from HLA-A*0201$^+$ healthy individuals and myeloma patients. Our studies show that the specific CTLs are able to lyse myeloma tumor cells but not normal blood cells in a MHC class I–restricted and/or HLA-A*0201-restricted manner and thus provide a rationale for gp96-based immunotherapy in multiple myeloma.

## Materials and Methods

***Reagents.*** Phycoerythrin- or FITC-conjugated monoclonal antibodies (mAb) against HLA-ABC, HLA-DR, CD1a, CD3, CD4, CD8, CD14, CD19, CD40, CD54, CD80, CD83, CD86, CD138, IFN-γ, interleukin-4 (IL-4), tumor necrosis factor-α (TNF-α), and mouse immunoglobulin isotype controls were purchased from PharMingen (San Diego, CA). Anti-CD19 and anti-CD138 antibody–conjugated microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Mouse mAbs anti-human IFN-γ and IL-4 and biotinylated antibodies against these cytokines (for the enzyme-linked immunospot assays) were purchased from Mabtech AB (Nacka, Sweden), and recombinant cytokines, including IL-1β, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, IL-2, IL-7, and IL-15 were purchased from R&D Systems (Minneapolis, MN). The avidin-biotin complex (ABC) kit was purchased from Vector Laboratories, Inc. (Burlingame, CA). $^3$[H]thymidine, $^{51}$[Cr]sodium chromate, and Ficoll-Hypaque were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

***Myeloma and other tumor cells.*** Five human myeloma cell lines (HMCL) were used. HLA-A*0201$^+$ U266, IM-9, and XG1 and HLA-A*0201$^-$ ANBL-6 were purchased from the American Type Culture Collection (Rockville, MD). ARP-1 cells (HLA-A*0201$^-$) were kindly provided by Dr. J. Epstein at the University of Arkansas for Medical Sciences (Little Rock, AR). As a control, a nonmyeloma cell line A375, which are HLA-A*0201$^+$ melanoma cells, was also purchased from the American Type Culture Collection. All cell lines were maintained in RPMI 1640 supplemented with 10% FCS. Primary myeloma plasma cells were isolated from myeloma patients' bone marrow aspirates by density centrifugation and subsequent sorting, as previously described (17). Aliquots of purified myeloma cells were cryopreserved in liquid nitrogen until use.

***Immunohistochemistry.*** Formalin-fixed, paraffin-embedded sections of bone marrow biopsy from myeloma patients were deparaffinized with xylene and rehydrated to water through a graded alcohol series. Heat-induced antigen retrieval was done before immunohistochemical staining. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. Malignant plasma cells were identified by morphologic assessment and by immunohistochemistry using an anti-CD138 antibody (B-B4, Serotec, Oxford, United Kingdom). Expression of gp96 was detected using a mAb (SPA-850, 1:30 dilution, Stressgen Biotechnologies, Victoria, British Columbia, Canada). Detection of signal was achieved using the LSAB$^+$ kit (DAKO, Carpinteria, CA) containing secondary biotinylated antibody and streptavidin/horseradish peroxidase complex, according to the manufacturer's recommendations. Chromagen 3,3′-diaminobenzidine/$H_2O_2$ (DAKO) was used, and slides were counterstained with hematoxylin. All slides were observed with light microscopy (Olympus America), and images were captured with a SPOT RT camera (Diagnostic Instruments, Burlingame, CA).

***Preparation of gp96.*** gp96 was purified from HLA-A*0201$^+$ U266 cells, the melanoma cell line A375, primary myeloma cells, and peripheral blood mononuclear cells (PBMC) as described before (18, 19). After sterile filtration, the preparations were aliquoted and stored at −20°C until use. Preparations were considered to be of acceptable biochemical quality only if all of the following three conditions (18, 19) were met: (*a*) the major protein band on SDS-PAGE was 96 kDa; (*b*) this band could be immunoblotted with an anti-gp96 mAb; and (*c*) any other bands detected by SDS-PAGE could be immunoblotted with the anti-gp96 mAb. Figure 1A and B shows a representative preparation of gp96 purified from U266 cells. No lipopolysaccharide contamination was detected in gp96 preparation according to the standard assay.

***Generation of dendritic cells.*** Monocyte-derived dendritic cells were generated from PBMCs using standard protocols (20–22). PBMCs from peripheral blood of HLA-A*0201$^+$ blood donors or myeloma patients were allowed to adhere in culture flasks for 2 hours, and nonadherent cells were removed and cryopreserved in aliquots for further use. The adherent cells were cultured in Aim-V medium supplemented with granulocyte macrophage colony-stimulating factor (100 ng/mL) and IL-4 (100 ng/mL) in a humidified incubator at 37°C with 5% $CO_2$, with further addition of cytokines every other day when 50% of the medium was replaced with fresh medium containing the cytokines. After 7 days of culture, cells were harvested and phenotypically characterized to ensure that they met the typical phenotype of immature dendritic cells (20, 21): CD3$^-$, CD14$^-$, CD19$^-$, CD83$^\pm$, CD1a$^{2+}$, CD40$^{2+}$, CD54$^{2+}$, CD80$^+$, CD86$^+$, HLA-DR$^+$, HLA-ABC$^{2+}$.

***Generation of gp96-specific T cell lines.*** Gp96-specific T cells were generated from PBMCs of HLA-A*0201$^+$ blood donors and myeloma patients by repeated stimulation of autologous T cells with gp96-pulsed mature dendritic cells. Briefly, immature dendritic cells were incubated

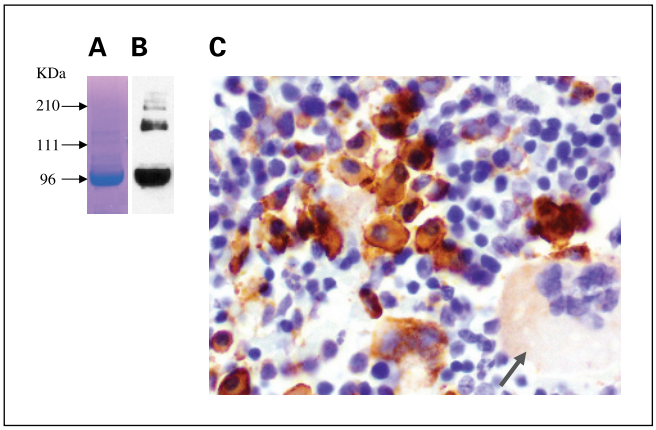


**Fig. 1.** Expression of gp96 and purity of isolated gp96 from myeloma cells. *A,* SDS-PAGE showing a major protein band at 96 kDa. *B,* Western blot showing that the band was immunoblotted with the anti-gp96 mAb; dimers of gp96 (18), which were immunoblotted by the mAb. *C,* immunohistochemical staining of gp96 in myeloma cells but not in normal hematopoietic cells in the bone marrow biopsy of myeloma patients. Representative of four independent experiments. The green arrow indicates a megakaryocyte.

for 24 hours with purified gp96 at a final concentration of 10 μg/mL at 37°C in 5% $CO_2$ followed by addition of TNF-α (10 ng/mL) and IL-1β (10 ng/mL) for 48 hours to induce dendritic cells to maturation. The nonadherent cells of the autologous PBMCs ($2 \times 10^6$/mL; used as T-cell population) were then cocultured in Aim-V medium supplemented with 10% pooled human serum (T-cell medium), with gp96-pulsed mature dendritic cells ($2 \times 10^5$/mL) in 50-mL tissue culture flasks at 37°C in 5% $CO_2$. The T cells were restimulated with gp96-pulsed autologous mature dendritic cells every 2 weeks, and the cultures were fed every 5 days with fresh medium containing recombinant IL-2 (20 IU/mL) and IL-7 (5 ng/mL). The induction of gp96-specific T cells was monitored biweekly using a T-cell proliferation assay (see below). After four to five cycles of *in vitro* stimulation and selection, T cell lines were established; expanded in T cell medium containing IL-2 (30 IU/mL), IL-7 (5 ng/mL), and IL-15 (5 ng/mL) for 2 weeks; and subjected to functional tests.

***Proliferation assays.*** T cells ($5 \times 10^4$/100 μL/well) were seeded into 96-well, U-bottomed tissue culture plates (Corning, Inc., Corning, NY) in T-cell medium. Varying numbers of autologous mature dendritic cells pulsed with or without gp96 were added to the plates and cultured for 4 days at 37°C in 5% $CO_2$. T-cell proliferation was measured after overnight incubation with $^3$[H]thymidine (0.5 μCi/well). Results from triplicate cultures are given as the arithmetic mean counts per minute.

In some experiments, cultured T cells were labeled with 5(6)-carboxyfluorescein diacetate succinimidyl ester (5 μmol/L; Molecular Probes, Eugene, OR) for 10 minutes at 37°C. After washing, the labeled T cells were seeded into 96-well, U-bottomed plates and incubated with various stimulatory cells for 7 days. Flow cytometry analysis was used to detect dilution of 5(6)-carboxyfluorescein diacetate succinimidyl ester, which represents T-cell proliferation (23).

***Cytotoxicity assay.*** The standard 4-hour $^{51}$Cr release assay was done to measure cytolytic activity of the T cells. Target cells included autologous dendritic cells pulsed with gp96 derived from various cells, unpulsed dendritic cells, U266, ARP-1, A375, and primary myeloma cells isolated from patients with multiple myeloma. Cells were separated by centrifugation using a Ficoll-Hypaque gradient to remove dead cells, and live cells were incubated with 100 μCi of $^{51}$Cr-sodium chromate for 1 hour. After extensive washing, the labeled cells ($1 \times 10^4$ target cells/well) were incubated for 4 hours with various numbers of T cells in 96-well, U-bottomed plates in T cell medium. All assays were done in triplicate. Results are shown as mean percentage. $^{51}$Cr release is calculated as follows: [(sample counts − spontaneous counts) / (maximum counts − spontaneous counts)] × 100%. Spontaneous release was <20% of the maximum $^{51}$Cr uptake.

To determine whether the cytolytic activity was restricted by MHC class I or II molecules, 20 μg/mL mAb against HLA-ABC (W6/32) or HLA-A2 (BB7.2; both were purchased from Serotec), HLA-DR (B8.12.2; Immunotech, Marseilles, France), or an isotopic control mouse IgG (Immunotech) were added to the cultures at the start of the assay.

***Inhibition of perforin-mediated and Fas-mediated pathways of cytolysis.*** Concanamycin A (Sigma, St. Louis, MO), an inhibitor of vacuolar type $H^+$ ATP (ATPase), or Brefeldin A (Sigma) were used as selective inhibitors of perforin-mediated and Fas-mediated cytotoxicity (24, 25), respectively. Effector cells were pretreated with 100 nmol/L concanamycin A or 10 μmol/L Brefeldin A for 2 hours and assayed for cytotoxicity in the presence of the drugs.

***Enzyme-linked immunospot assay.*** Detailed methods of the enzyme-linked immunospot assay for the enumeration of antigen-specific, cytokine-secreting cells have been described previously (26, 27). Spots corresponding to the cytokine-secreting cells were enumerated under a dissection microscope (Stemi SV6; Zeiss, Jena, Germany). All samples were run in duplicate. Data are expressed as the mean number of cytokine-secreting cells per $10^4$ T cells.

***Immunophenotyping and intracellular cytokine staining.*** Phycoerythrin- or FITC-conjugated mAbs were added to cell pellets, incubated for 30 minutes on ice, and washed thrice before analysis. Intracellular cytokine staining was done using the Cytofix/Cytoperm kit (BD Biosciences, San Diego, CA) according to the manufacturer's recommendations. Samples were analyzed using a FACSCalibur flow cytometer and CELLquest software.

***Statistical analysis.*** Student's *t* test was used to compare various experimental groups. $P < 0.05$ was considered statistically significant. Unless otherwise indicated, means and SD are shown.

## Results

***Generation of gp96-pulsed mature dendritic cells.*** Immunohistochemistry was used to examine the expression pattern of gp96 in myeloma cells versus normal hematopoietic cells in the bone marrow of patients with multiple myeloma. Bone marrow biopsy sections of myeloma patients were stained with a mAb specific for gp96. As shown in Fig. 1C, it seemed that gp96 was preferentially expressed in myeloma plasma cells. Normal hematopoietic cells were not stained.

To generate gp96-specific T cells from HLA-A*0201$^+$ blood donors or myeloma patients, autologous dendritic cells were used as antigen-presenting cells (APC). To maximize the stimulatory capacity of dendritic cells, we generated gp96-pulsed mature dendritic cells by pulsing immature dendritic cells with gp96 for 24 hours and then inducing dendritic cells to maturation with cytokines IL-1β and TNF-α. To ensure the quality of these cells, flow cytometry was employed to examine dendritic cell–related surface molecules on these cells. As shown in Fig. 2, immature dendritic cells expressed modest levels of CD83, CD80, CD86, and HLA-DR and high levels of CD1a, CD40, CD54, and HLA-ABC. Incubation of immature dendritic cells with gp96 alone for 24 hours, or with TNF-α and IL-1β for 48 hours resulted in significantly up-regulated expression of CD83, CD54, CD80, CD86, HLA-DR, and HLA-ABC molecules, indicating maturation and activation of dendritic cells. Moreover, gp96-pulsed, IL-1β-activated, and TNF-α-activated dendritic cells expressed the highest levels of these molecules.

***Generation of gp96-specific T cell lines.*** After four to five rounds of T-cell stimulation with gp96-pulsed mature dendritic cells, specific T cell lines, which consisted of both CD4$^+$ (20-30%) and CD8$^+$ (70-80%) T cells, were generated and propagated from PBMCs of HLA-A*0201$^+$ donors and myeloma patients. Proliferation assays were employed to detect a specific T-cell response. The T cell lines proliferated in response to autologous dendritic cells pulsed with U266-derived gp96 (DC-gp96M; $P < 0.01$, compared with controls; Fig. 3A) and to HLA-A*0201$^+$ U266 cells ($P < 0.01$, compared with that of ARP-1; Fig. 3B). The T cells responded weakly to HLA-A*0201$^-$ HMCL ARP-1. No proliferative response was observed when the T cells were challenged with unpulsed dendritic cells (dendritic cell control), and dendritic cells pulsed with gp96 derived from normal PBMCs (DC-gp96N) of HLA-A*0201$^+$ individuals. The same results were also obtained by using a 5(6)-carboxyfluorescein diacetate succinimidyl ester–labeling assay to measure T-cell proliferation (Fig. 3C). It is evident that in response to dendritic cells pulsed with myeloma cell–derived gp96, but not to unpulsed dendritic cells or dendritic cells pulsed with gp96 derived from normal PBMCs, significant 5(6)-carboxyfluorescein diacetate succinimidyl ester dilution or T-cell proliferation was observed. These results suggest that the T cell lines recognized HLA-A*0201$^+$-restricted, myeloma-derived gp96.

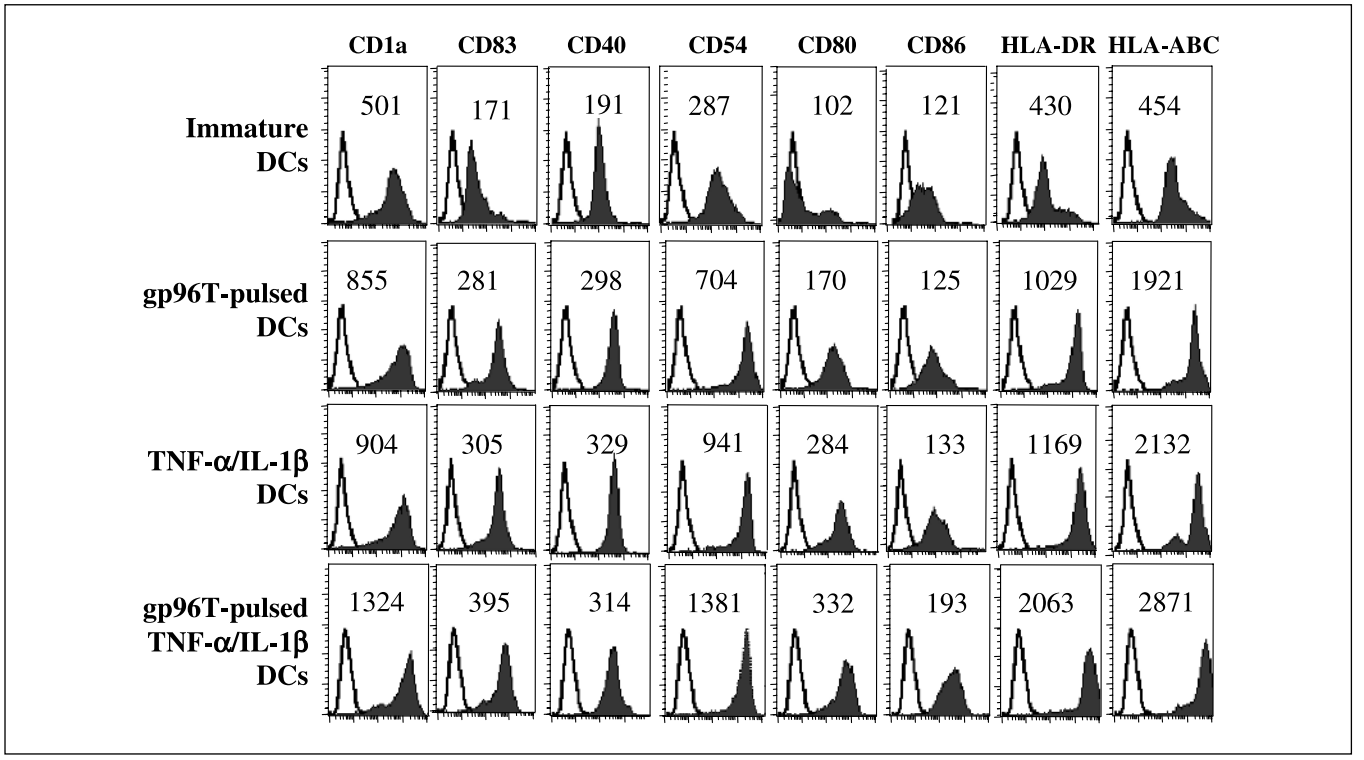


**Fig. 2.** Phenotype of monocyte-derived dendritic cells (*DC*). Immature dendritic cells on day 7, dendritic cells after cultured with U266-derived gp96 (*gp96T*) for additional 24 hours, mature dendritic cells induced by TNF-α and IL-1β for 48 hours, and activated and mature dendritic cells after pulsing with U266-derived gp96 for 24 hours and inducing maturation with TNF-α and IL-1β for additional 48 hours. Values above the curves are mean fluorescence intensity. Representative of four independent experiments.

***Cytolytic activity of the T cells against gp96-pulsed antigen-presenting cells.*** To examine cytotoxicity of these T cells, a standard 4-hour $^{51}$Cr release assay was employed. In our first experiments, autologous dendritic cells were used as target cells. As shown in Fig. 4A, these T cell lines specifically lysed autologous dendritic cells pulsed with U266-derived gp96 (gp96M; $P < 0.01$, compared with controls) but not unpulsed dendritic cells or dendritic cells pulsed with control gp96 [purified from HLA-A*0201$^+$ PBMCs (gp96N) or A375 cells (gp96T)]. Similarly, these T cells also lysed HLA-A*0201$^+$ EBV-transformed B cells pulsed, but not unpulsed, with (U266) myeloma-derived gp96 ($P < 0.05$; Fig. 4B). Hence, the results indicate that these T cells were potent CTLs.

We next examined whether the T cells were cytolytic to normal blood cells. In these experiments, mature dendritic cells, purified blood B cells (using anti-CD19 antibody-coated

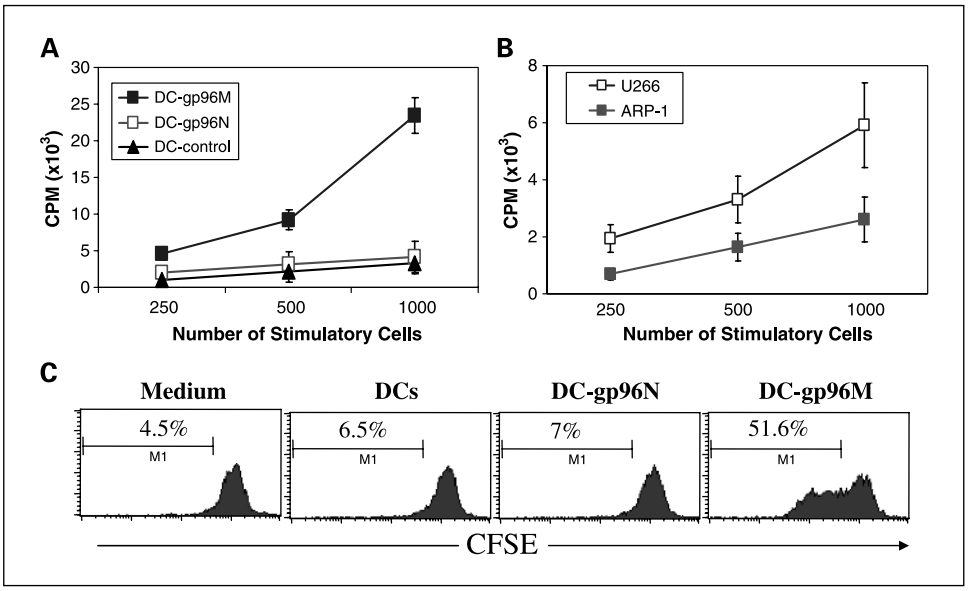


**Fig. 3.** Proliferative responses of gp96-specific T cell lines. *A*, T-cell proliferation in response to autologous dendritic cells (DC) pulsed with U266 cell – derived gp96 (gp96M) or with normal PBMC-derived gp96 (gp96N), and to unpulsed dendritic cells. *B*, T-cell proliferation in response to HMCLs U266 or ARP-1, measured by $^3$[H]thymidine incorporation [counts per minute (*CPM*)]. Points, mean of cell lines from four donors; bars, SD. Similar results were also obtained with T cell lines from two myeloma patients. *C*, T-cell proliferation measured by CFSE dilution, induced by unpulsed autologous dendritic cells, dendritic cells pulsed with U266-derived gp96 (*gp96M*), or dendritic cells pulsed with normal PBMC-derived gp96 (*gp96N*). Representative of three independent experiments.

microbeads) or EBV-transformed B cells, and PBMCs from autologous, HLA-A*0201$^+$ individuals were used as target cells. As shown in Fig. 4C, although strong killing was noted with dendritic cells pulsed with U266-derived gp96, no cell death was observed in unpulsed dendritic cells, B cells or EBV-transformed B cells, or PBMCs. These results indicate that the T cells recognized myeloma-derived antigenic peptides.

To determine MHC restriction of these T cell lines, we examined the inhibitory effects of anti-MHC antibodies on their cytolytic activity. As shown in Fig. 4D, about 60% inhibition of T cell–mediated cytotoxicity was observed with mAb against HLA-ABC (MHC class I; $P < 0.01$, compared with control) and >50% inhibition with mAb against HLA-A2 molecules ($P < 0.01$). In cultures with addition of mAb against HLA-DR (MHC class II), about 25% inhibition was noted ($P < 0.05$). No inhibitory effect was observed with the isotype control IgG. The results show that the cytotoxicity was mainly attributed to MHC class I (HLA-A2)–restricted CD8$^+$ T cells.

***Cytolytic activity of the T cells against myeloma tumor cells.*** We examined the cytolytic activity of the CTLs against myeloma cells, including HMCLs and primary myeloma cells isolated from patients with multiple myeloma. As shown in the representative experiments, the CTLs killed U266 cells, from which tumor-derived gp96 was obtained and other HLA-A*0201$^+$ HMCLs IM-9 and XG1 ($P < 0.01$ to $P < 0.05$; Fig. 5A and B) but not HLA-A*0201$^-$ ARP-1 and ANBL-6 nor HLA-A*0201$^+$ nonmyeloma A375 cells. No killing was observed against the K562 cell line, thus excluding the possibility that natural killer cells contributed to cytotoxicity.

We next evaluated the cytolytic effects of the CTLs on primary myeloma cells isolated from patients. As shown in Fig. 5C, CTLs generated from a HLA-A*0201$^+$ myeloma patient 1 effectively lysed the autologous primary myeloma cells and myeloma cells from an allogeneic, HLA-A*0201$^+$ patient 3 ($P < 0.05$ to $P < 0.01$, compared with other patients) but not myeloma cells from HLA-A*0201$^-$ patients 2 and 4. Similar results were also obtained with CTLs generated from patient 3 (data not shown). These patient-derived CTLs killed HLA-A*0201$^+$ but not HLA-A*0201$^-$ HMCLs or nonmyeloma A375 cells (data not shown). Furthermore, CTLs generated from HLA-A*0201$^+$ healthy donors (induced by autologous dendritic cells pulsed with U266-derived gp96) were also able to kill HLA-A*0201$^+$ but not HLA-A*0201$^-$ primary myeloma cells from the patients (Fig. 5D). Taken together, these results showed that these CTL lines were not only able to lyse tumor-derived gp96-pulsed APCs but also myeloma cells, including primary myeloma cells from HLA-A*0201$^+$ patients, indicating that the T cells recognized myeloma-derived, gp96-chaperoned peptides that were naturally presented on HLA-A*0201 molecules and shared among patients.

To examine whether the same T cells mediated the killing of both gp96-pulsed dendritic cells and myeloma cells, a cold target inhibition assay was done. In these experiments, the T cells were first incubated for 2 hours at 37°C with cold/unlabeled autologous dendritic cells pulsed with U266-derived gp96 or with myeloma tumor cells. As shown in Fig. 5E, preincubating T cells with dendritic cells pulsed with U266-derived gp96, but not with unpulsed dendritic cells, significantly inhibited the killing of $^{51}$Cr-labeled U266 ($P < 0.05$). On the other hand, preincubation of T cells with U266, but not with ARP-1, resulted in reduced lysis of $^{51}$Cr-labeled dendritic cells pulsed with U266-derived gp96 ($P < 0.01$; Fig. 5F). The results show that the same CTLs killed both gp96-pulsed dendritic cells and myeloma cells.

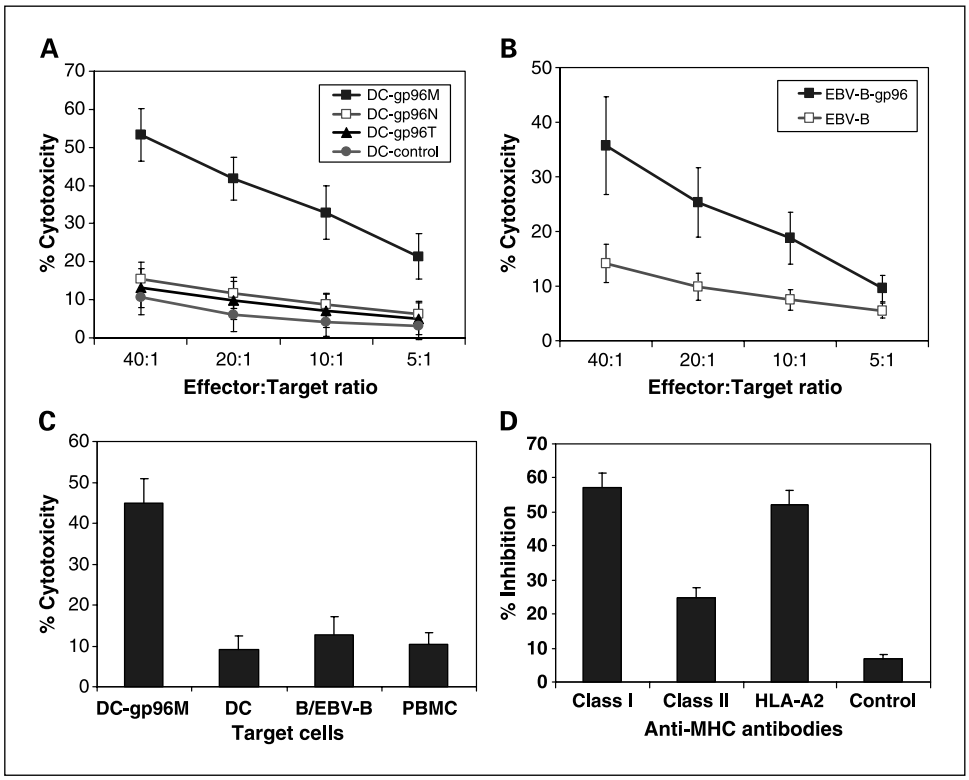


**Fig. 4.** Cytotoxicity of gp96-specific T cell lines against APCs. *A,* against dendritic cells (DC) pulsed with U266-derived gp96 (gp96M), dendritic cells pulsed with gp96 derived from normal PBMCs (gp96N) or from A375 (gp96T), or unpulsed dendritic cells. *B,* against EBV-transformed B cells pulsed with or without U266-derived gp96. *C,* against autologous, normal dendritic cells, purified B cells or EBV-transformed B cells, or PBMCs. Dendritic cells pulsed with U266-derived gp96 served as positive control. *D,* inhibition of T cell–mediated cytotoxicity by mAbs against MHC class I (HLA-ABC) or class II (HLA-DR), HLA-A2, or an isotypic control IgG. *C* and *D,* E/T = 20:1. Columns, mean of one representative of three experiments done using a T cell line from a donor; bars, SD. Similar results were obtained with T cell lines from other three donors and two myeloma patients.

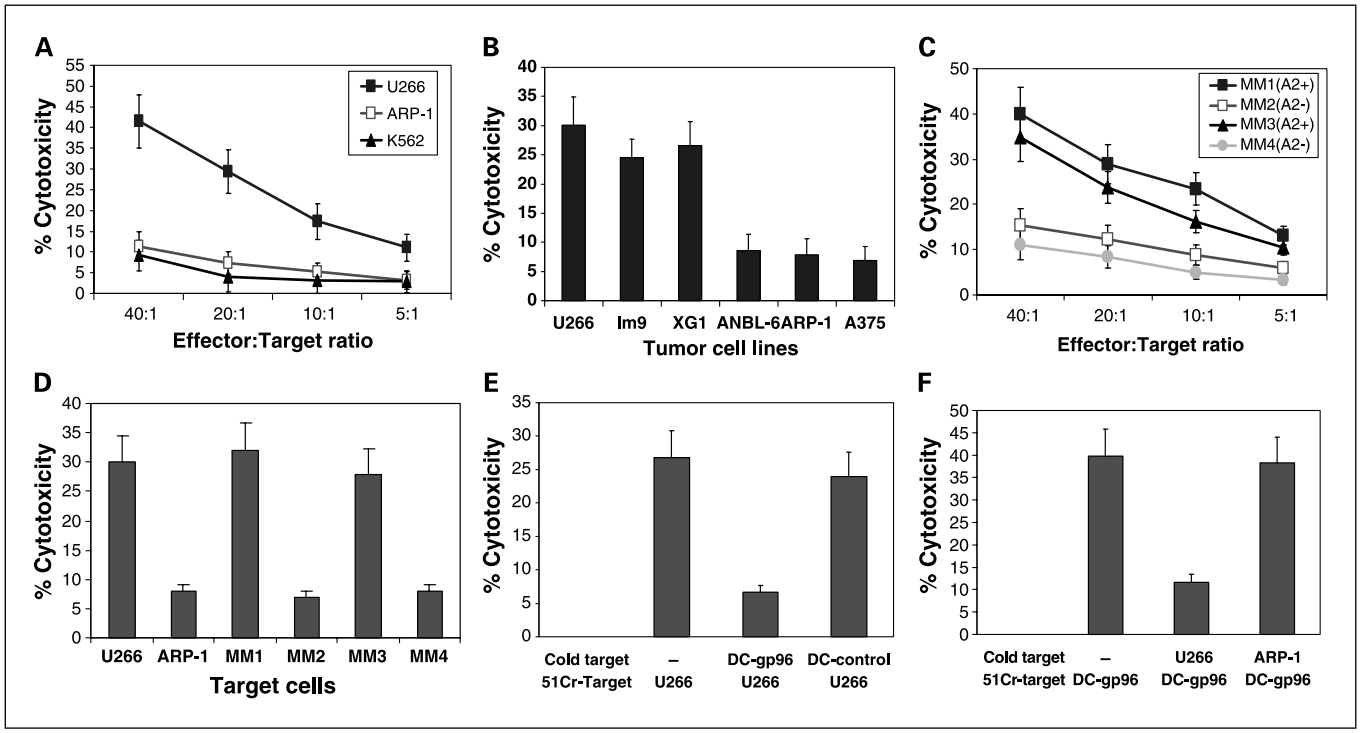


**Fig. 5.** Cytotoxicity of gp96-specific T cell lines against myeloma cells. *A,* against HMCLs U266 and ARP-1. K562 was included to exclude natural killer cell activity. *B,* against different HMCLs, including HLA-A*0201$^+$ HMCLs U266, IM-9, and XG1; HLA-A*0201$^+$ A375 cells; and HLA-A*0201$^-$ HMCLs ANBL-6 and ARP-1. Representative of a T cell line from a blood donor stimulated with autologous dendritic cells (DC) pulsed with U266-derived gp96. The effector/target ratio was 20:1. Similar results were obtained with T cell lines from other three donors (with U266-derived gp96) and two myeloma patients (with autologous myeloma plasma cell-derived gp96). *C,* against primary myeloma cells from four patients with multiple myeloma. Patients 1 and 3 were HLA-A*0201$^+$ (A2+), and patients 2 and 4 were HLA-A*0201$^-$ (A2–). Representative experiments using the T cell line from patient 1 stimulated by autologous dendritic cells pulsed with myeloma plasma cell – derived gp96. Similar results were also obtained with the T cell line from patient 3. *D,* against primary myeloma cells by a T cell line from a blood donor stimulated by autologous dendritic cells pulsed with U266-derived gp96. The effector/target ratio was 20:1. Similar results were also obtained with T cell lines from other three donors (with U266-derived gp96). Cold target inhibition assay showing that (*E*) preincubation of the T cells with gp96-pulsed dendritic cells, but not unpulsed dendritic cells, inhibited the killing of labeled HMCL U266, and (*F*) preincubation of HMCL U266, but not ARP-1 cells, inhibited the killing of labeled gp96-pulsed dendritic cells. The effector/target ratio was 20:1. Columns, mean of one representative of three experiments done; bars, SD.

***Cytotoxicity of the CTLs was mediated by the perforin exocytosis pathway.*** Ninety percent inhibition of T cell – mediated cytotoxicity was observed with concanamycin A ($P < 0.01$), whereas no significant inhibitory effect was noted with Brefeldin A, indicating that the Fas/FasL system was not the main pathway of cytotoxicity mediated by the CTLs. This result was supported by flow cytometry analysis showing low or absent FasL expression by the T cells (data not shown). Treatment of the T cells with Brefeldin A or concanamycin A did not induce apoptosis in the cells (data not shown). Taken together, these results indicate that the cytotoxic function of gp96-specific CTLs was mediated by the perforin-dependent pathway.

***Cytokine expression and production of the CTLs.*** Two independent methods were employed to examine the cytokine expression profile of the T cells. Figure 6A shows a representative experiment of intracellular cytokine staining for IFN-γ, TNF-α, and IL-4 expression in a specific CTL line. Upon restimulation with dendritic cells pulsed with tumor-derived gp96, but not with unpulsed dendritic cells or dendritic cells pulsed with normal PBMC-derived gp96, high portions (34-40%) of the CD8$^+$ T cells expressed IFN-γ and TNF-α. IL-4-expressing CD8$^+$ T cells were very few (0.2%). To detect cytokine secretion, an enzyme-linked immunospot assay was used to enumerate IFN-γ-secreting and IL-4-secreting cells. After rechallenging with dendritic cells pulsed with tumor-derived gp96, or with U266 and HLA-A*0201$^+$ primary myeloma cells, high numbers of IFN-γ-secreting cells were detected ($P < 0.01$), whereas the numbers of IL-4-secreting cells remained low and unchanged (Fig. 6B). Other stimulatory cells, including unpulsed dendritic cells, and HLA-A*0201$^-$ ARP-1, and primary myeloma cells, did not increase the number of cytokine-secreting cells. Therefore, these T cells expressed and secreted predominantly IFN-γ and TNF-α and were thus type 1 T cells (28, 29).

## Discussion

Although immunotherapy combined with high-dose chemotherapy holds great promise for the treatment of multiple myeloma, clinical studies have not yet delivered the expected results. In addition to optimizing immunotherapy methods, there is an urgent need for novel myeloma antigens that can be used as universal antigens to efficiently stimulate anti-myeloma CTL responses in the majority of patients. In the present study, we chose to target Hsps in myeloma cells, because previous studies in solid tumors (12 – 14) and in other hematologic malignancies (15, 16) have shown the usefulness and efficacy of tumor-derived Hsps in generating tumor-specific immune responses. However, there is no such a report in multiple myeloma. Considering the diverse biology of different tumors,

preclinical studies are much needed in multiple myeloma to explore the usefulness and feasibility of using myeloma-derived Hsps for immunotherapy in myeloma patients.

With the help of autologous dendritic cells, we successfully generated gp96-specific CTL lines *in vitro* from HLA-A*0201$^+$ blood donors and myeloma patients. We were able to obtain specific CTLs from all these individuals except that cycles of *in vitro* stimulation with gp96-pulsed dendritic cells were different them. The CTLs had cytolytic activity against gp96-pulsed dendritic cells, U266 from which the tumor-derived gp96 was obtained, and other HLA-A*0201$^+$ HMCLs and also effectively lysed primary myeloma plasma cells from patients in a MHC class I–restricted and/or HLA-A*0201-restricted manner. These CTLs did not display cytolytic activity towards normal blood cells including B cells and PBMCs, indicating that these CTLs may be promising effector cells for immunotherapy in multiple myeloma.

Hsp-based vaccines are personalized vaccines that carry the fingerprint of a given tumor, circumventing the need to identify tumor antigens for each individual cancer. It is likely that Hsps chaperone peptides from endogenously degraded proteins, including both normal cell proteins and tumor-associated antigens (30, 31). Thus, the first question was whether Hsps could stimulate the generation of autoreactive T cells. For this purpose, we tested the CTLs against normal blood cells, including dendritic cells, B cells, and PBMCs, and we found no evidence that these CTLs were reactive to these normal cells. Second, we asked whether there were shared myeloma antigens among tumor cells from different patients. In this study, we focused on tumor antigen-derived peptides carried on gp96 that were presented by HLA-A*0201 molecules. The finding that U266-derived and primary myeloma cell–derived gp96-specific CTLs recognized and lysed allogeneic HLA-A*0201$^+$ but not HLA-A*0201$^-$ HMCLs and primary myeloma cells supports the hypothesis that there are shared tumor antigens. Thus, it may be possible to use Hsps from allogeneic tumor cells as vaccines to immunize MHC-matched or partially matched patients. This is an important result and deserves further investigation.

Classically, CTLs recognize antigens localized in the cytoplasm of target cells that are processed and presented as peptide complexes with MHC class I molecules (32). However, there is evidence for an exogenous pathway whereby antigens that are not expected to gain access to the cytoplasm are presented on MHC class I molecules (32, 33). One of the best examples is cross-priming, in which antigens from donor cells are acquired by bone marrow–derived host APCs and presented on MHC class I molecules (33). Recently, studies have shown that human immature dendritic cells efficiently phagocytose apoptotic cells and cross-present viral, tumor, and self-antigens to CD8$^+$ T cells (34, 35). This mechanism had been used in our study to generate myeloma-specific CTLs *in vitro* following stimulation with mature dendritic cells pulsed with tumor-derived gp96. Because immature dendritic cells are highly effective in taking up and processing antigens compared with mature dendritic cells, we used immature dendritic cells to pulse with gp96 and induced dendritic cells to maturation after antigen pulsing. Generation of antitumor immunity by dendritic cells is intimately linked to dendritic cell maturation stage (36), and recent reports have shown that APCs, such as dendritic cells and monocytes, can internalize Hsps spontaneously by receptor (such as CD91)–mediated endocytosis and direct chaperoned proteins/peptides into the intracellular pathway for MHC class I–restricted presentation to CD8$^+$ T cells, concomitant with the induction of dendritic cell maturation and cytokine secretion (37, 38). Indeed, our results also showed that gp96 alone could activate immature dendritic cells. However, the most mature or activated dendritic cells were obtained after pulsing with gp96 and inducing maturation with TNF-α and IL-1β (Fig. 2). These dendritic cells were used in our study to generate gp96-specific CTLs.

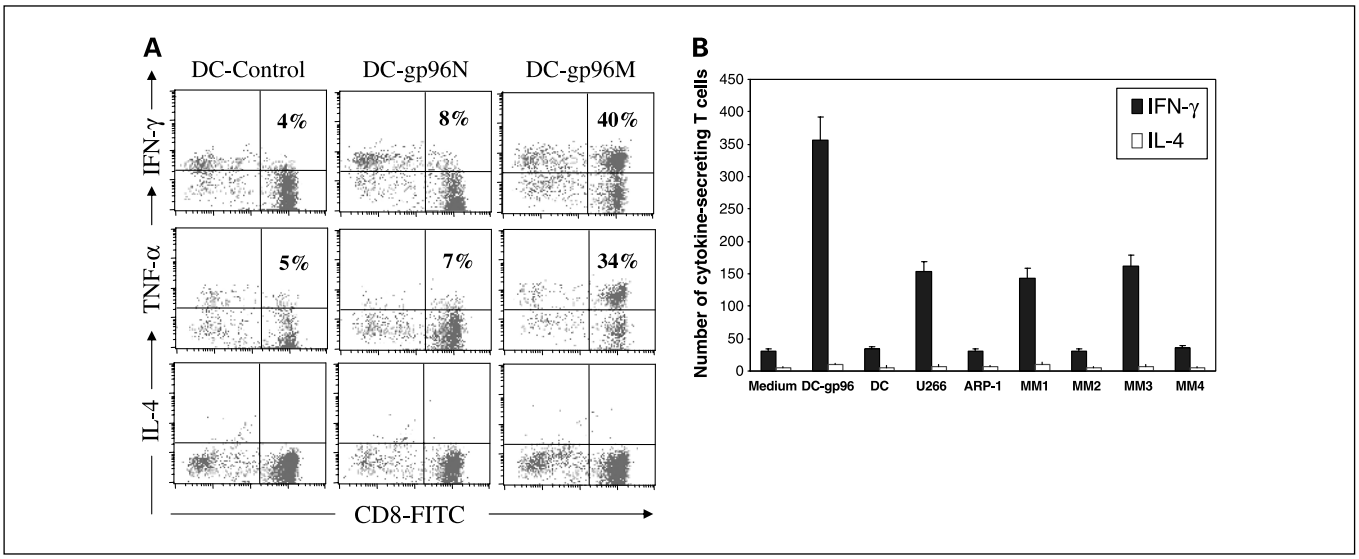


**Fig. 6.** Cytokine expression profile of the T cells. *A,* intracellular staining showing the percentage of cytokine (IFN-γ, TNF-α, and IL-4)–expressing CD8$^+$ T cells after restimulation with dendritic cells (*DC*) pulsed with U266-derived gp96 (*DC-gp96M*) or that from normal PBMCs (*DC-gp96N*), and unpulsed dendritic cells. *B,* enzyme-linked immunospot assay showing the number of cytokine (IFN-γ and IL-4)–secreting cells per $10^4$ T cells in medium, or induced by dendritic cells pulsed with gp96, unpulsed dendritic cells, HMCLs U266 or ARP-1, HLA-A*0201$^+$ primary tumor cells from patient 1 (*MM1*) and patient 3 (*MM3*), and HLA-A*0201$^-$ primary myeloma cells from patient 2 (*MM2*) and patient 3 (*MM4*). Columns, mean of one representative of three experiments done using a T cell line from a donor; bars, SD. Similar results were obtained with T cell lines from other three donors and two myeloma patients.

The ability of Hsps to facilitate the cross-presentation of MHC class I – restricted epitopes and to prime $CD8^+$ cell effector responses is well established (31). Interestingly, recent studies have also shown that Hsp-peptide complexes can also lead to antigen presentation on MHC class II molecules, thus activating $CD4^+$ T cells (39, 40). In line with these findings, our study also showed that we might have induced a $CD4^+$ T-cell response specific for MHC class II – restricted peptides that were chaperoned by gp96. This is supported by the facts that our T cell lines contained both $CD4^+$ and $CD8^+$ T cells, and that the T-cell response was inhibited by mAbs against MHC class I and II molecules. It is possible that uptake of gp96-peptides complexes led to antigen presentation on both MHC class I and class II molecules on mature dendritic cells, thus activating $CD8^+$ CTL as well as $CD4^+$ T cells. Hence, further studies are warranted to examine the involvement of MHC class II and peptides in activating specific $CD4^+$ T cells.

In conclusion, our study shows that myeloma-specific CTLs can be generated by stimulating T cells with dendritic cells pulsed with myeloma-derived Hsp gp96. These CTLs may be promising effector cells for immunotherapy in multiple myeloma because they are potent and specific CTLs, able to specifically and effectively kill myeloma cells, including primary myeloma cells, but not normal blood cells. Our study also indicates that there are shared myeloma antigens among patients, and that allogeneic Hsps may be used as vaccines to immunize MHC-matched or partially matched patients. Thus, our study is the first to provide strong and direct evidence to support the application of gp96-based immunotherapy in multiple myeloma.

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