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Immunization with heat shock protein 105-pulsed dendritic cells leads to tumor rejection in mice ☆

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Abstract

Recently, we reported that heat shock protein 105 (HSP105) DNA vaccination induced anti-tumor immunity. In this study, we set up a preclinical study to investigate the usefulness of dendritic cells (DCs) pulsed with mouse HSP105 as a whole protein for cancer immunotherapy in vivo. The recombinant HSP105 did not induce DC maturation, and the mice vaccinated with HSP105-pulsed BM-DCs were markedly prevented from the growth of subcutaneous tumors, accompanied with a massive infiltration of both $CD4^+$ T cells and $CD8^+$ T cells into the tumors. In depletion experiments, we proved that both $CD4^+$ T cells and $CD8^+$ T cells play a crucial role in anti-tumor immunity. Both $CD4^+$ T cells and $CD8^+$ T cells specific to HSP105 were induced by stimulation with HSP105-pulsed DCs. As a result, vaccination of mice with BM-DCs pulsed with HSP105 itself could elicit a stronger tumor rejection in comparison to DNA vaccination.

Keywords: Heat shock protein 105; Cancer antigen; Dendritic cells; Th; CTL

Heat shock proteins (HSPs) are soluble intracellular proteins, which are ubiquitously expressed, and their expression can be induced at much higher levels as a result of heat shock or other forms of stress. HSPs have essential functions in the regulation of protein folding, conformation, assembly, and sorting. HSPs have been shown to be molecular chaperones that function to maintain the native conformational states of proteins and prevent protein–protein aggregation [1]. HSPs can also induce the response of antigen-specific effector $CD8^+$ T cells which can protect hosts from both infection and tumor challenge [2]. Srivastava and co-workers [3,4] led to a proposal that the tumor-derived HSP–peptide complex elicits a protective immunity that is specific to a particular cancer, while HSPs derived from normal tissues did not elicit any protective immunity to the cancers tested. Immunotherapeutic clinical trials targeted at autologous tumor-derived gp96–peptide complexes are still ongoing in metastatic melanoma and colorectal carcinoma patients [5].

Dendritic cells (DCs) are powerful antigen-presenting cells (APCs) that are considered to be potent immunotherapeutic agents to promote the host immune response against tumor antigen. DCs become efficient tumor vaccines when pulsed with synthetic or natural tumor-derived peptides, transduced with tumor-derived RNA or vectors

☆ *Abbreviations:* BM-DC, bone marrow-derived DC; HSP105, heat shock protein 105; Th, helper T cell; CTL, cytotoxic T lymphocyte; MHC, major histocompatibility complex; C26 (C20), colon 26 clone 20.

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encoding for tumor-associated proteins, or directly fused to or incubated with tumor cells [6]. For effective induction of cytotoxic T lymphocytes (CTLs) by vaccination, ''Cross-presentation'' mediated by DCs often plays an important role. Such cross-presentation includes the antigen presentation of exogenous antigens by major histocompatibility complex (MHC) class I molecules as well as by MHC class II molecules [7,8]. HSP-chaperoned peptides were cross-presented by the MHC class I molecules of the DCs several 100-fold more efficiently than unchaperoned peptides [9]. In addition, CD91, also called α_2-macroglobulin receptor is expressed on DCs and has been shown to act as one of the receptors for HSP-chaperoned peptides to efficiently incorporate the HSP–peptide complexes [10].

We earlier reported that heat shock protein 105 (HSP105) was overexpressed in a variety of human cancers but it is not expressed in normal tissue except for the testes [11,12], thus suggesting that HSP105 itself may be a potential candidate as a target antigen for cancer immunotherapy. The amino acid sequences and expression patterns of HSP105 are very similar between humans and mice. HSP105 has been found to be immunogenic in mice and an effective anti-tumor immunity has been observed after *HSP105* DNA vaccination [13]. In the present study, we set up a preclinical study to investigate the usefulness of HSP105 as a target for cancer immunotherapy using DCs. It has been reported that HSPs can induce DC maturation and activation as determined by the upregulation of MHC class II and CD86 molecules, the secretion of IL-12 and TNFα [14,15], and migration into draining lymphoid organs [16]. On the contrary, some investigators reported that HSP-mediated maturation of DCs was caused by contaminating lipopolysaccharide (LPS) fraction because endotoxin-free HSP70 failed to induce DC maturation [17]. We herein show that the highly purified HSP105 did not induce DC maturation and that the immunization of HSP105-pulsed DC led to the tumor rejection of melanoma and colorectal cancer in mice. These findings suggested that HSP105 itself could be a valuable tumor-associated antigen applicable for DC-based immunotherapy of tumors overexpressing it.

Materials and methods

Cell lines and mice. A subline of BALB/c-derived colorectal cancer cell line Colon 26, C26 (C20), was provided by Dr. Kyoichi Shimomura (Astellas Pharmaceutical Co., Tsukuba, Japan). Other cancer cell lines were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). All these cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum at 37 °C in a humidified 5% CO_2 atmosphere. We used the B16-F10 melanoma cell line syngeneic to C57BL/6 mice and C26 (C20) for the tumor challenge. Female 6- to 8-week-old C57BL/6 mice (H-2^b) and BALB/c mice (H-2^d) were purchased from Charles River Japan (Yokohama, Japan). These mice were kept under specific pathogen-free conditions. These experiments were approved by the Animal Research Committee of Kumamoto University.

Production of recombinant proteins. We produced highly purified recombinant mouse HSP105 from the *Escherichia coli* strain BL21 cells transduced with the mouse *HSP105* gene expression vector, as described previously [18]. Purified proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and Coomassie brilliant blue (CBB)-stained bands were quantified by densitometry. Thereafter, by using affinity chromatography on a polymyxin B agarose gel (Sigma Chemical Co., St. Louis, MO), the endotoxin levels were decreased. We also produced highly purified recombinant myelin basic protein (MBP) as described previously [19]. Both recombinant HSP105 and MBP were estimated to be almost endotoxin free by using Limulus amebocyte lysate assay kit (BioWhittaker, Walkersville, MD), and endotoxin contents in these two materials were below 10 endotoxin U/mg.

Immunizations and tumor challenge. Bone marrow-derived DCs (BM-DC) were prepared as described previously [20]. BM-DCs were pulsed with 2 μg/ml HSP105 at 37 °C for 16 h, non-adherent and loosely adherent proliferating DCs were collected and used as HSP105-pulsed BM-DC. In tumor prevention experiments, mice were intraperitoneally inoculated with HSP105-pulsed BM-DC (5×10^5) suspended in 200 μl PBS on days −14 and −7. In parallel, groups of mice were injected with BM-DC alone or PBS as controls. Tumor challenge was initiated by subcutaneous injection with B16-F10 cells (1×10^4) or C26 (C20) cells (3×10^4) suspended in 100 μl HBSS (Gibco, Grand Island, NY) in shaved right flanks on day 0. Tumor occurrence was observed twice a week. The tumor size was evaluated by measuring two perpendicular diameters using calipers.

Flow cytometric analysis. Staining of cells and analysis on a flow cytometer (FACScan; BD Biosciences) were done as described previously [21]. Antibodies and reagents used for staining were as follows: FITC-conjugated anti-I-A^b (clone 28-16-8S; mouse IgG2a; Caltag, Burlingame, CA), R-PE-conjugated anti-mouse CD80 (clone RMMP-1; rat IgG2a; Caltag), R-PE-conjugated anti-mouse CD86 (clone RMMP-2; rat IgG2a; Caltag), FITC-conjugated anti-mouse CD4 (clone L3T4; rat IgG2a; BD PharMingen, San Diego, CA), FITC-conjugated anti-mouse CD8 (clone Ly-2; rat IgG2a; BD PharMingen), FITC-conjugated mouse IgG2a control (clone G155-178; BD PharMingen), and R-PE-conjugated rat IgG2a control (clone LO-DNP-16; Caltag).

Depletion of $CD4^+$ T cells and $CD8^+$ T cells in mice. Rat monoclonal antibodies (mAbs) GK1.5 specific to mouse CD4 and 2.43 specific to mouse CD8 were used to deplete $CD4^+$ T cells and $CD8^+$ T cells in vivo, respectively. The mice were injected with ascites (0.1 ml/mouse) from hybridoma-bearing nude mice intraperitoneally on days −18, −15, −11, −8, −4, and −1 and the tumor cells were inoculated on day 0. Normal rat IgG (Sigma, St. Louis, MO; 200 μg/mouse) was used as a control. The depletion of T cell subsets was monitored by a flow cytometric analysis, which showed more than a 90% specific depletion in the number of splenocytes.

Immunohistochemical analysis. Immunohistochemical detection of HSP105 was done as previously described [11,12]. Rabbit polyclonal anti-human HSP105 (Santa Cruz, Santa Cruz, CA) was used as the primary antibody in this study. Immunohistochemical staining of CD4 and CD8 was done as previously described [22]. For the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) method, we used ApopTag Fluorescein In Situ Apoptosis Detection Kits (Serologicals Corporation, Norcross, GA).

Induction of $CD4^+$ T cells and $CD8^+$ T cells specific to HSP105. The mice were inoculated intraperitoneally with HSP105-pulsed BM-DC on days −14 and −7. Spleen cells were harvested on day 0, and $CD4^+$ T cells and $CD8^+$ T cells were purified using the magnetic cell sorting system (MACS) with anti-mouse CD4 (L3T4) mAb and anti-mouse CD8α (Ly-2) mAb, respectively. The purity of these T cell subsets exceeded 95% by a flow cytometric analysis. $CD4^+$ T cells or $CD8^+$ T cells (3×10^5/well) were separately incubated in RPMI 1640 medium supplemented with 10% horse serum, IL-2 (100 U/ml), and 2-ME (50 μM) together with the irradiated (4500 Gy) HSP105-pulsed BM-DC in 24-well culture plates. BM-DCs (3×10^4/well) pulsed with 2 μg/ml HSP105 for 16 h were irradiated (4500 Gy) and added to the culture wells for the restimulation once a week. After the third restimulation in vitro, both proliferation and cytotoxicity assays were performed as described previously [23]. For the

control of ^{51}Cr-release assay, CD8$^+$ T cells isolated from the mice immunized with BM-DCs alone were restimulated in vitro with BM-DCs alone once a week and used as effector cells.

ELISPOT assay. HSP105-specific IFN-γ production of T cells was quantified using the appropriate ELISPOT kit (BD Biosciences, San Diego, CA) according to the manufacturer's instructions. CD4$^+$ T cells or CD8$^+$ T cells were incubated with the BM-DC alone, BM-DCs pre-pulsed with HSP105, or BM-DCs pre-pulsed with myelin basic protein (MBP) as a control at 37 °C for 24 h. Each BM-DC was pre-pulsed with 2 μg/ml protein at 37 °C for 16 h. The spots were automatically counted and subsequently analyzed using the Eliphoto system (MINERVA TECH, Tokyo, Japan).

Statistical analysis. The statistical significance of the differences in the findings between the experimental groups was determined by Student's *t* test. The overall survival rate was calculated using the Kaplan–Meier method, and statistical significance was evaluated using Wilcoxon's test. A value of $P < 0.05$ was considered to be statistically significant.

Results

HSP 105 does not induce maturation of DCs

To analyze the direct effect of HSP 105 used in this study on BM-DCs, BM-DCs were incubated with HSP105, LPS as a positive control, and left untreated for 16 h. As shown in Fig. 1, no significant difference was observed in the levels of surface expression of CD80, CD86, and I-A^b between untreated BM-DCs and HSP105-pulsed BM-DCs. Moreover, HSP105-pulsed BM-DCs microscopically did not show any morphological changes in comparison to the untreated BM-DC. On the contrary, LPS-pulsed BM-DCs exhibited markedly increased expression of these three molecules. Although it is reported that HSPs could induce DC maturation and activation [14–16], the recombinant HSP105 used in this study including little LPS (below 10 endotoxin U/mg) did not show such activity. Thereafter, we evaluated the antigenicity of HSP105 to induce anti-tumor immunity.

The HSP105-pulsed BM-DC vaccine induced anti-tumor immunity against the lethal challenge of B16-F10 and C26 (C20)

We recently reported that mouse HSP105 was also overexpressed in the liver metastasis of C26 (C20) cells, and lung metastase of the B16-F10 cells, and that *HSP105* DNA vaccination inhibited the growth of these tumors [13]. In this study, we investigated the effects of HSP105 vaccination based on DCs on the growth of B16-F10 and C26 (C20) tumor cells in vivo. The objective was to determine whether prophylactic vaccination induced significant immunity against tumor growth and a prolonged survival. The protocol of vaccination in this study is shown in Fig. 2A. The results shown in Fig. 2B demonstrate that immunization with HSP105-pulsed BM-DC markedly inhibited the growth of B16-F10 tumors in comparison to other groups ($P < 0.01$). As shown in Fig. 2C, five of eight (62.5%) mice immunized with HSP105-pulsed BM-DC remained tumor free and survived for 100 days after the tumor challenge. In contrast, the mice vaccinated with BM-DC alone (12.5%) or PBS (0%) showed little protection against the growth of B16-F10 tumor in comparison to the observations in mice treated with HSP105-pulsed

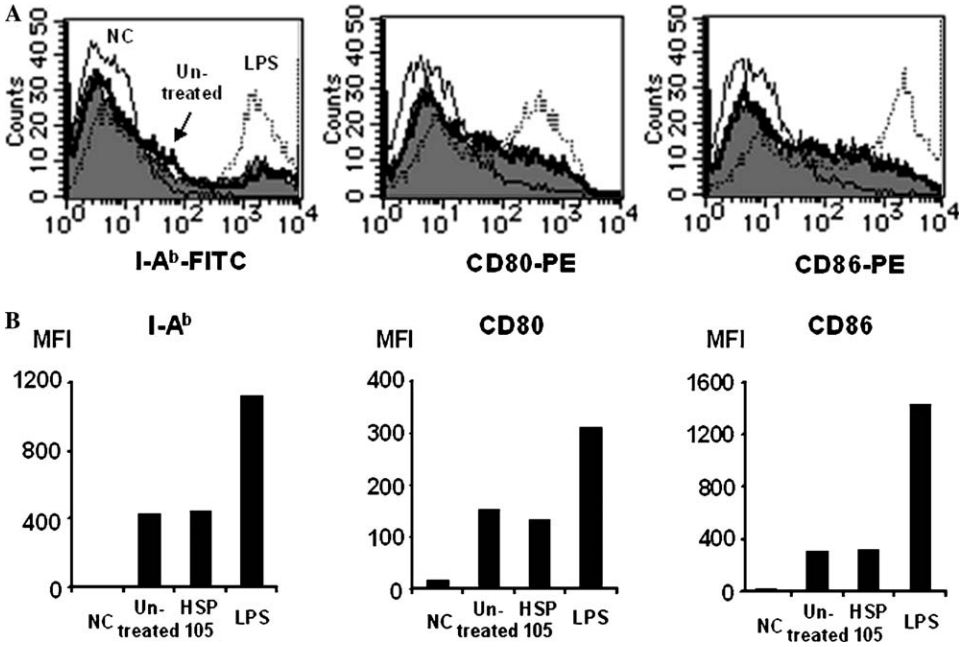

Fig. 1. Expression levels of cell surface I-A^b, CD80, and CD86 on BM-DCs, HSP105-pulsed BM-DCs, and LPS-pulsed BM-DCs were analyzed by flow cytometric analysis. BM-DCs were pulsed with 2 μg/ml HSP105, 1 μg/ml LPS or left untreated for 16 h. (A) The expression levels in HSP105-pulsed BM-DCs (filled histogram), LPS-pulsed BM-DCs (dotted line), and untreated BM-DCs (thick line), and the profiles of cells treated with isotype matched Ig as a negative control for staining (thin line). (B) The mean fluorescence intensity (MFI) of I-A^b, CD80, and CD86 staining in the cells. The results are representative of three independent experiments with similar results.

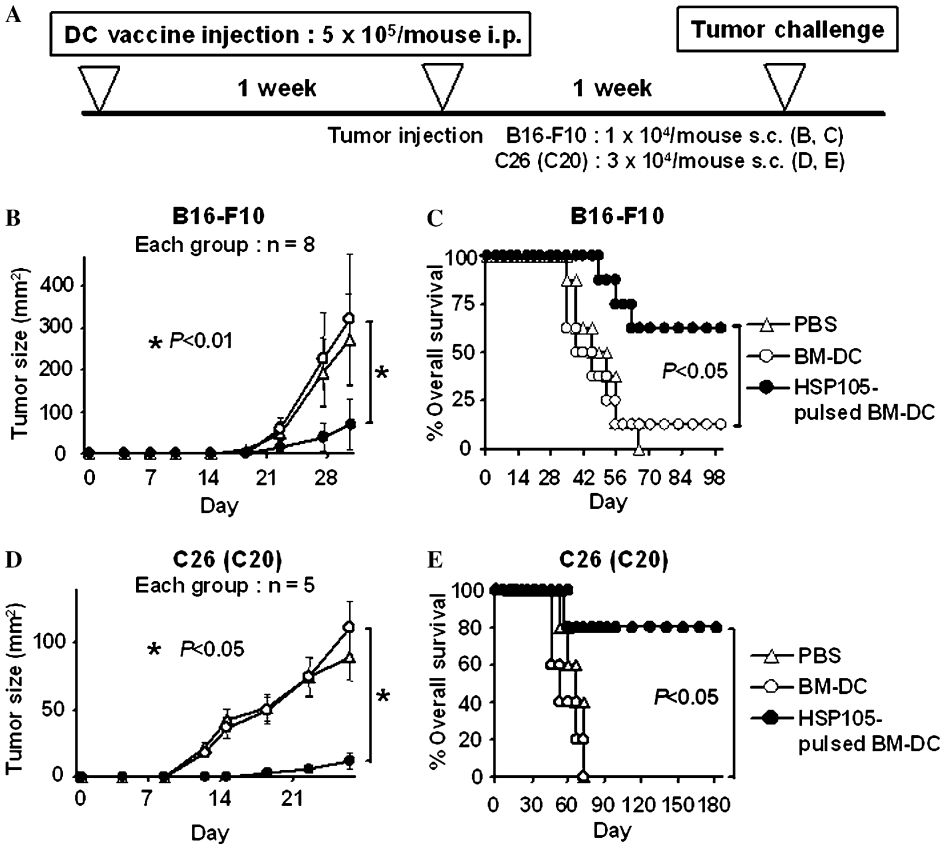

Fig. 2. Protection against tumor growth of B16-F10 and C26 (C20) cells by immunization with HSP105-pulsed BM-DC vaccine. (A) Protocol of the vaccination. The mice were immunized with PBS, BM-DC alone, and HSP105-pulsed BM-DC on 14 and 7 days before the tumor challenge. Seven days after the second immunization, the mice were challenged with B16-F10 cells s.c. (B,C), or C26 (C20) cells s.c. (D,E). (B,D) The tumor size was evaluated by measuring two perpendicular diameters. The result is presented as the mean area of tumor ± SE, and we evaluated statistical significance of the differences between each group using the unpaired Student's *t* test. (C,E) The mice in each group were observed for their survival period. The statistical significance of the differences between each group was evaluated using Wilcoxon's test.

BM-DC ($P < 0.05$). Similar results were observed in a prophylactic immunotherapy model using C26 (C20). Four of five (80%) mice immunized with HSP105-pulsed BM-DC completely rejected the C26 (C20) (3×10^4) cells (Figs. 2D and E), whereas tumors grew rapidly and all five mice died within 70 days in control mice treated with PBS or BM-DC alone. These results suggest that the HSP105-pulsed BM-DC vaccine is a potent vaccine that can efficiently induce specific anti-tumor immunity.

Both $CD4^+$ T cells and $CD8^+$ T cells are required for anti-tumor immunity

To determine the role of $CD4^+$ T cells and $CD8^+$ T cells in the protection against B16-F10 and C26 (C20) tumor cells induced by HSP105 vaccination, we depleted mice of $CD4^+$ T cells or $CD8^+$ T cells by the treatment with anti-CD4 or anti-CD8 mAb in vivo, respectively. During the depletion procedure, the mice were immunized with HSP105-pulsed BM-DC vaccine and challenged with B16-F10 or C26 (C20) cells (Fig. 3A). In both B16-F10 and C26 (C20) models, mice depleted of $CD4^+$ T cells, and $CD8^+$ T cells developed aggressively growing tumors after the challenge in comparison to the findings in control mice treated with rat IgG ($P < 0.05$) (Figs. 3B and D). The mice depleted of $CD4^+$ T cells or $CD8^+$ T cells all died by 52–65 days, whereas more than 50% of the control mice survived for 70 days ($P < 0.05$) (Figs. 3C and E). These results suggest that both $CD4^+$ T cells and $CD8^+$ T cells play crucial roles in the protective anti-tumor immunity induced by the HSP105-pulsed BM-DC vaccine.

Vaccination of HSP105-pulsed BM-DCs induced infiltrations of both $CD4^+$ T cells and $CD8^+$ T cells into tumor cells, but not into normal organs

Four of five (80%) mice immunized with the HSP105-pulsed BM-DCs completely rejected challenges of C26 (C20) cells (3×10^4) (Fig. 2E). To ascertain whether these rejections were induced by $CD4^+$ T cells or $CD8^+$ T cells, the subcutaneous inoculation of many C26 (C20) cells (1×10^6) into the right flank was done at 7 days after the second vaccination. After tumor formation, we removed the tumor and immunohistochemically stained it using anti-CD4 mAb, anti-CD8 mAb, and the TUNEL method. The infiltration of $CD4^+$ T cells and $CD8^+$ T cells into C26

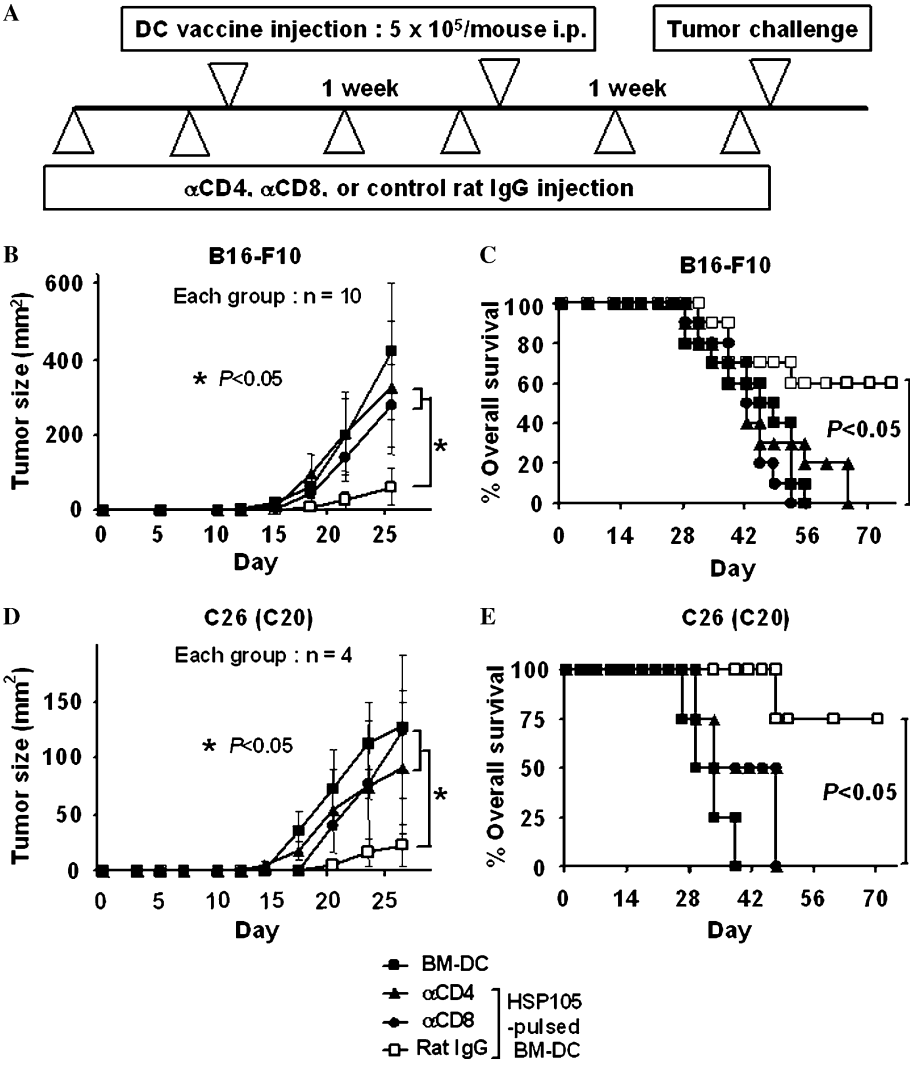

Fig. 3. Both $CD4^+$ and $CD8^+$ T cells are involved in the antitumor immunity elicited by the HSP105-pulsed DC vaccine. (A) Protocol for the vaccination and the depletion of T cells. C57BL/6 mice and BALB/c mice were challenged s.c. with B16-F10 cells and C26 (C20) cells, respectively. (B,D) The tumor size was evaluated by measuring two perpendicular diameters. The result is presented as the mean area of tumor ± SE, and we evaluated the statistical significance of the differences between each group using the unpaired Student's *t* test. (C,E) The mice in each group were observed for their survival period. The statistical significance of the differences between each group was evaluated using Wilcoxon's test.

(C20) tumors and some apoptotic C26 (C20) tumor cells were observed in the mice vaccinated with HSP105-pulsed BM-DCs, but never in the mice vaccinated with unpulsed BM-DCs (Fig. 4A). These results suggest that HSP105-pulsed BM-DCs have the potential to sensitize many HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells to kill C26 (C20) tumor cells.

We evaluated the risk of autoimmunity by immunization against self-HSP105. Both BALB/c and C57BL/6 mice immunized with HSP105-pulsed BM-DC were apparently healthy without any abnormality such as dermatitis, arthritis, or neurological disorders. The tissues of the mice immunized with HSP105-pulsed BM-DC were histologically examined. The brain, liver, heart, kidneys, and spleen had normal structures and did not show any pathological changes suggestive of an immune response, such as the infiltration of $CD4^+$ T cells and $CD8^+$ T cells or tissue destruction and repair. Although we used female mice for the experiments described above, we also immunized male mice with HSP105-pulsed BM-DC to ascertain whether immunization with HSP105-pulsed BM-DC induced autoimmunity in the testis in which HSP105 is strongly expressed. However, no sign of autoimmunity was observed in the testis (Fig. 4B).

Induction of HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells by immunization with HSP105-pulsed BM-DC

$CD4^+$ T cell lines specific to HSP105 were established from spleen cells derived from mice vaccinated with HSP105-pulsed BM-DC. $CD4^+$ T cells were separated from spleen cells and the purity of these cells was more than 95% by flow cytometric analysis. These cells were restimulated with irradiated and HSP105-pulsed DCs once

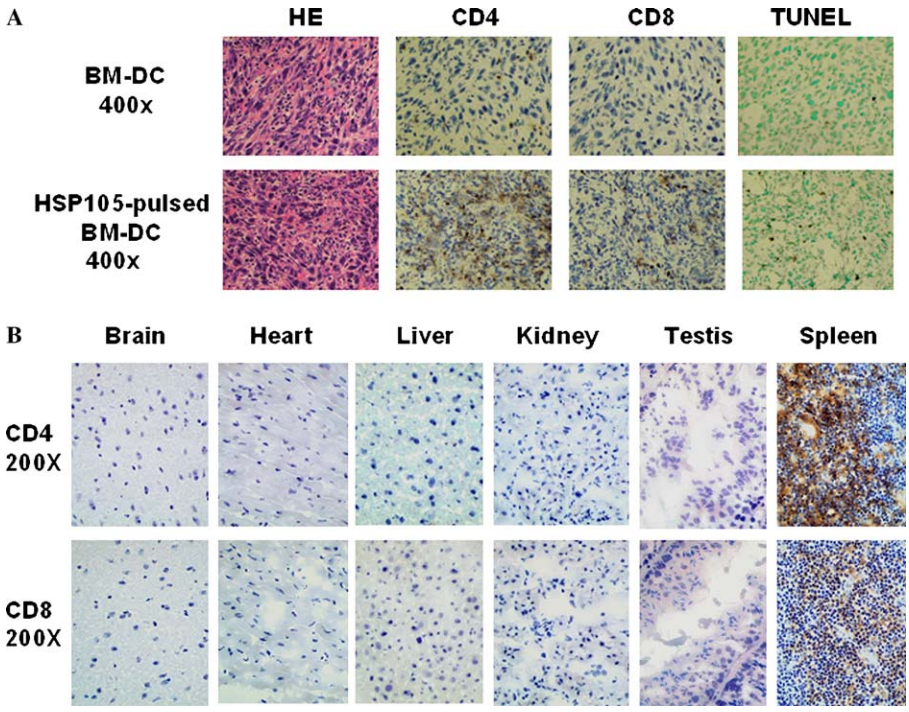

Fig. 4. Vaccination of HSP105-pulsed BM-DCs induced infiltrations of both $CD4^+$ T cells and $CD8^+$ T cells into C26 (C20) tumor and induced the apoptosis of C26 (C20) tumor cells. (A) C26 tumors removed from the mice vaccinated with BM-DCs or HSP105-pulsed BM-DCs were analyzed using immunohistochemical staining with anti-CD4 mAb, anti-CD8 mAb, and the TUNEL method on 4 days after the inoculation of tumor cells (1×10^6). (B) Normal tissue specimens of mice vaccinated with HSP105-pulsed BM-DCs were examined histologically and immunohistochemically. Objective magnification was 200×. The spleen was used as a positive control for the staining of both $CD4^+$ and $CD8^+$ cells.

a week. After three restimulations, both an ELISPOT assay and a proliferation assay were performed. The ELISPOT assay showed that HSP105-sensitized $CD4^+$ T cells produced IFN-γ in response to BM-DCs prepulsed with HSP105 but not an irrelevant MBP (Fig. 5A). As shown in Fig. 5B, HSP105-sensitized $CD4^+$ T cells proliferated in the presence of BM-DCs prepulsed with HSP105 but not MBP. These observations clearly indicated that HSP105-specific $CD4^+$ T cells were included in the T cell line.

We investigated whether HSP105-specific $CD8^+$ T cells were also induced with HSP105-pulsed DC vaccination. $CD8^+$ T cells were purified (>95%) from spleen cells of vaccinated mice and restimulated with irradiated and HSP105-pulsed DCs once a week. After three restimulations, the ELISPOT assay and 6 h ^{51}Cr-release assay were performed to detect the HSP105-specific CTL responses (Figs. 5C and D). The $CD8^+$ T cell line exhibited a HSP105-specific production of IFN-γ in an ELISPOT assay when cells were stimulated with BM-DCs prepulsed with HSP105 but not MBP ($P < 0.01$), however, the number of spots was smaller than that of $CD4^+$ T cells (Fig. 5C). $CD8^+$ T cells immunized with HSP 105-pulsed DC demonstrated a significant cytolytic activity against the B16-F10 cells pretreated with IFN-γ to induce the expression of MHC class I molecules on the cell surface, whereas $CD8^+$ T cells from mice immunized with BM-DC alone revealed little cytolytic activity ($P < 0.005$) (Fig. 5D). The induction of HSP105-specific $CD8^+$ T cells by the immunization in vivo with HSP105-pulsed BM-DC and the stimulation of the $CD8^+$ T cell line in vitro with the HSP105-pulsed BM-DC strongly suggested that these HSP105-specific $CD8^+$ T cells were induced by the cross-presentation of HSP105 by BM-DCs.

Discussion

HSPs are classified into several families based on their apparent molecular weights, such as HSP105/110, HSP90, HSP70, HSP60, HSP40, and HSP27 [24]. HSP105 consists of HSP105α and HSP105β. HSP105α is a constitutively expressed 105-kDa HSP that is induced by a variety of stresses, whereas HSP105β is a 90-kDa truncated form of HSP105α that is specifically induced by heat shock at 42 °C [24]. In this study, we used the mouse HSP105α protein. The cDNA sequence of murine HSP105 is almost the same as that of the Chinese hamster HSP110 [25,26], so HSP105 belongs to a member of the HSP105/110 family. We recently reported by the immunohistochemical analysis that HSP105 is overexpressed in a variety of human tumors [12], the liver metastasis of the C26 (C20) cells in the BALB/c mice, and lung metastasis of the B16-F10 cells in the C57BL/6 mice [13]. We examined the expression of HSP105 in the mouse cancer cell lines using a Western blotting analysis and found that HSP105 was strongly expressed in all 7 mouse cell lines tested (data not shown).

Many studies have shown that certain HSPs purified from a tumor can function as an effective vaccine against the same

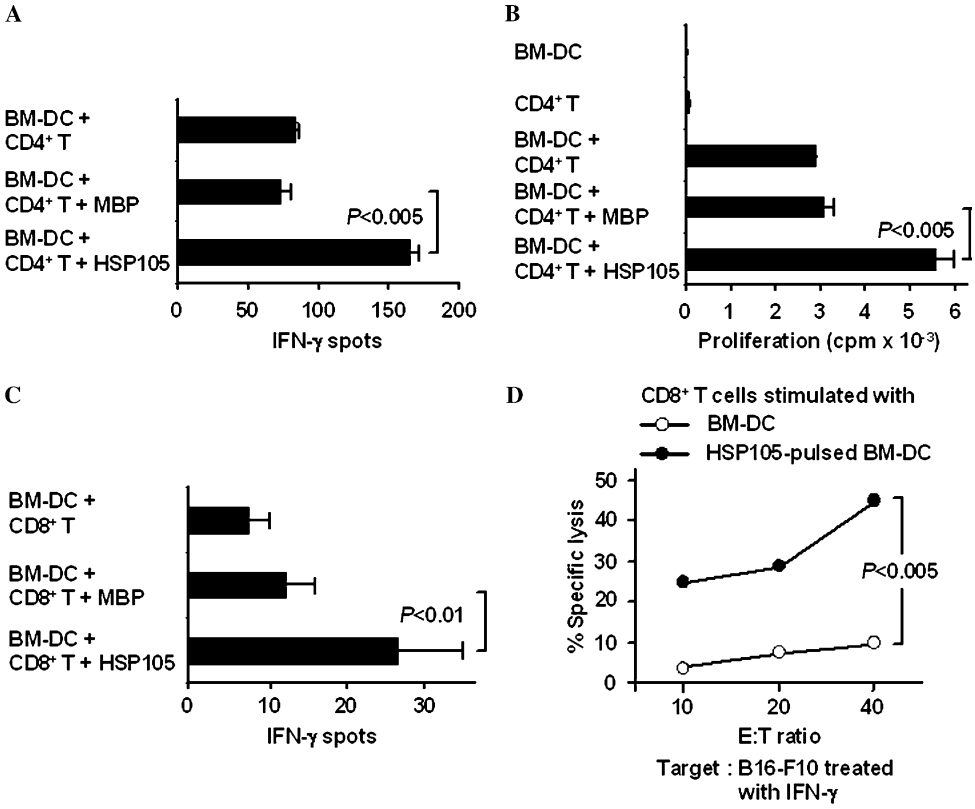

Fig. 5. Induction of HSP105-specific $CD4^+$ T cells and $CD8^+$ T cells by stimulation with HSP105-pulsed BM-DCs. (A) An ELISPOT assay for IFN-γ production by $CD4^+$ T cell lines stimulated with HSP105 protein-pulsed BM-DCs. $CD4^+$ T cells derived from the mice vaccinated with HSP105-pulsed BM-DC were stimulated in vitro with HSP105-pulsed BM-DC three times. For the ELISPOT assay, these $CD4^+$ T cells were co-cultured with BM-DC prepulsed with HSP105, MBP, or unpulsed BM-DC for 24 h. (B) Cell proliferation of $CD4^+$ T cell lines stimulated with HSP105-pulsed BM-DCs was determined by measuring [^{3}H]thymidine incorporation. $CD4^+$ T cells were co-cultured with BM-DC prepulsed with HSP105, MBP, or unpulsed BM-DC for 72 h. (C) An ELISPOT assay for IFN-γ production by $CD8^+$ T cell lines stimulated with HSP105-pulsed BM-DCs. $CD8^+$ T cells derived from mice vaccinated with HSP105-pulsed BM-DC were stimulated with HSP105-pulsed BM-DC three times in vitro. For the ELISPOT assay, these $CD8^+$ T cells were co-cultured with BM-DC prepulsed with HSP105, MBP, or unpulsed BM-DC for 24 h. (D) $CD8^+$ T cells stimulated with HSP105-pulsed BM-DC or BM-DC alone (control) were examined for their CTL activity against B16-F10 cells treated with IFN-γ (10^3 U/ml) using 6 h ^{51}Cr-release assay. The results were analyzed using the mean values of a triplicate or a quadruplicate assay. The data shown in A–D are each representative of three independent experiments with similar results.

tumor by stimulating T cells with tumor-specific peptides bound to HSPs. Subjeck and co-workers [27,28] reported that tumor-derived HSP110–peptide complexes also stimulated tumor immunity as other HSP families did in mice. Despite studies establishing a chaperoning effect of HSPs, one impediment to the full-fledged acceptance of HSPs as peptide-transporting vehicles is the lack of mass spectrometric data directly identifying HSP-associated peptides [29]. Stress-inducible proteins can be recognized by natural killer cells and CTLs as whole antigens expressed on the surface of stressed cells in humans [30]. Proteins dramatically upregulated or modified under stressful conditions should lead to increased presentation as do peptides presented by HLA class I molecules. About 25 HSP-derived peptides bound by HLA class I molecules have been identified through mass spectrometry [30]. Cancer patients have been reported to possess CTLs specific to HSP60-derived peptide [31], while HLA-A*0201-restricted HSP70-derived CTL epitopes have been identified in both an HLA-A*0201 transgenic mouse model and in humans [32]. In this study, although we did not identify HSP105-derived epitope peptides for $CD4^+$ T cells and $CD8^+$ T cells, we did prove that HSP105 itself could induce both $CD4^+$ Th-cells and $CD8^+$ CTLs specific to HSP105 as a cancer antigen. Contrary to our findings, however, Subjeck and co-workers [28] reported that HSP110 immunization did not elicit anti-tumor immunity. This discrepancy could be attributed to the difference in the methods of immunization.

It has been reported that HSPs can induce the maturation and activation of DCs as determined by upregulation of MHC class II and CD86 molecules, secretion of the IL-12 and TNFα [14,15]. However, HSP105-pulsed BM-DCs did not show any changes in comparison to the untreated BM-DC, thus suggesting that HSP105 did not induce DC maturation and activation. It is unlikely that HSP105 brought tumor-derived peptides into the culture system, because the HSP105 used in this study was the recombinant protein produced in *E. coli*. Furthermore, we recently identified HSP105-derived CTL epitopes restricted by HLA-A*0201 or -A*2402 using HLA

transgenic mouse model (unpublished data). These results also supported that HSP105 served not as a mediator for maturation of DCs, but as a cancer antigen eliciting tumor immunity.

The results of the T cell depletion study showed that the depletion of either $CD4^+$ T cells or $CD8^+$ T cells abrogated the anti-tumor immune response induced by the HSP 105-pulsed BM-DC vaccine, and that both $CD4^+$ and $CD8^+$ T cells play crucial roles in the protective anti-tumor immunity. $CD8^+$ T cells are thought to serve as the dominant effector cell mediating tumor killing, in contrast, $CD4^+$ T cells are thought to have an indirect role in providing help to CTL as well as a direct role in tumor rejection [33]. It is interesting that B16-F10 tumor cells that lack MHC class I were killed in in vivo study. We suppose that $CD4^+$ T cells may have an important role in this case. Peptides derived from HSP105 bound by MHC class II on the surface of HSP105-pulsed BM-DCs activate $CD4^+$ T cells. The activated $CD4^+$ T cells can secrete IFN-γ upon stimulation with tumor local DCs presenting tumor-derived HSP105 peptides, which contribute not only to activation of $CD8^+$ T cells but also to restoration of MHC class I expression in B16-F10 cells. The activated HSP105 specific $CD8^+$ T cells can recognize the peptides derived from HSP105 in the context of MHC class I and kill the B16-F10 cells.

In the field of cancer immunotherapy, most enthusiasm has been directed toward the use of various cancer vaccines; peptide vaccines alone, peptide plus cytokines, vaccination either with recombinant virus or with naked DNA encoding tumor antigen, and peptide pulsed on DCs [34]. DCs represent the most potent antigen presenting cells and also play an important role in the induction of specific T cell response [35]. Peptides pulsed on DCs have been reported to be the most effective vaccine in comparison to DNA vaccine or peptide–adjuvant mixture [36]. In this study, 62.5% and 80.0% of the mice immunized with HSP105-pulsed BM-DC completely rejected B16-F10 cells and C26 (C20) cells, respectively. On the other hand, only 50.0% of the mice immunized with the *HSP105*-DNA vaccine rejected these tumor cells in our previous study [13]. Although a further comparative analysis of the vaccination properties of these two strategies is required, our results suggested that protein-pulsed DCs are a more powerful vaccine than the DNA vaccine.

In this study, we used BM-DCs pulsed with HSP105 but not with HSP105-derived peptide as a cancer vaccine. We think that protein-pulsed DCs thus have an advantage over peptide-pulsed DCs. DCs are the major cell type known for its capacity to cross-present antigens [37]. In this study, HSP105-sensitized $CD8^+$ T cells responded to HSP105 in vitro by the stimulation of purified $CD8^+$ T cells with HSP105-pulsed DCs. This result strongly suggested that the HSP105-specific $CD8^+$ T cells were activated via the cross-presentation of HSP105 by BM-DCs. Although it became evident that gp96- and HSP70-chaperoned peptides can be presented to CTLs by DCs in the context of MHC class I molecules [38,39], we herein provide the first evidence that HSP itself can be cross-presented to CTLs by DCs. HSP105-pulsed DC can present peptides derived from exogenously added HSP105 in the context of not only MHC class II molecules on the surface of DCs to activate $CD4^+$ T cells, but also MHC class I molecules by cross-presentation to activate $CD8^+$ T cells. We herein showed the induction of specific $CD4^+$ T cells and $CD8^+$ T cells in vivo by stimulation with HSP105-pulsed DCs. The application of the peptide-pulsed DC as potential vaccine is limited to patients with the appropriate HLA alleles. To circumvent this limitation, we have used HSP105-pulsed DC to induce a HSP105 specific T cell response. HSP105-pulsed DCs offer the advantage of potentially presenting multiple immunogenic T cell epitopes without the need of prior knowledge of the individual patient's HLA type.

The mechanism of action of HSP105-pulsed BM-DCs injected intraperitoneally is still unclear. We think that DCs injected in the abdominal cavity might immigrate into mesenteric lymphatic vessels. Some DCs stay in mesenteric lymph nodes, others circulate in the blood via the thoracic duct and finally reach the spleen and bone marrow. Recent experimental evidence suggested that peripheral DCs migrate through the lymphatic vessels to the blood [40]. Although the present study showed that intraperitoneal injection of DCs induced an effective anti-tumor immunity in mice, comparison of effectiveness to other routes of immunization with DCs, such as intravenous, subcutaneous, and intranodal, remains to be investigated.

In conclusion, our results indicate that HSP105 itself is a tumor rejection antigen which may possibly be useful for cancer immunotherapy, and that HSP105-pulsed BM-DC vaccinations can prime HSP105-specific T cells in vivo, to prevent the subcutaneous growth of B16-F10 and C26 cancer cells expressing HSP105, without inducing autoimmune destruction. Our findings suggest that HSP105-pulsed BM-DC vaccination is a novel strategy for the prevention of cancer in patients treated surgically, who are at high risk for a recurrence of the cancer. Because of the overexpression of HSP105 in a variety of human tumors [12], clinical trial of immunotherapy targeted against HSP105 may well be applicable to various cancers.

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