The Journal of Immunology

Synergistic Induction of Antigen-Specific CTL by Fusions of TLR-Stimulated Dendritic Cells and Heat-Stressed Tumor Cells

Shigeo Koido, Eiichi Hara, Sadamu Homma, Makoto Mitsunaga, Akitaka Takahara, Eijiro Nagasaki, Hidejiro Kawahara, Michiaki Watanabe, Yoichi Toyama, Satoru Yanagisawa, Susumu Kobayashi, Katsuhiko Yanaga, Kiyotaka Fujise, Jianlin Gong and Hisao Tajiri

J Immunol 2007; 179:4874-4883; ;
doi: 10.4049/jimmunol.179.7.4874
http://www.jimmunol.org/content/179/7/4874

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

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

Synergistic Induction of Antigen-Specific CTL by Fusions of TLR-Stimulated Dendritic Cells and Heat-Stressed Tumor Cells[1]

Shigeo Koido,[2]*† Eiichi Hara,¶ Sadamu Homma,‡ Makoto Mitsunaga,* Akitaka Takahara,‡ Eijiro Nagasaki,‡ Hidejiro Kawahara,§ Michiaki Watanabe,§ Yoichi Toyama,§ Satoru Yanagisawa,§ Susumu Kobayashi,§ Katsuhiko Yanaga,§ Kiyotaka Fujise,*† Jianlin Gong,‖ and Hisao Tajiri*

Dendritic cell (DC)/tumor cell fusion cells (FCs) can induce potent CTL responses. The therapeutic efficacy of a vaccine requires the improved immunogenicity of both DCs and tumor cells. The DCs stimulated with the TLR agonist penicillin-killed *Streptococcus pyogenes* (OK-432; OK-DCs) showed higher expression levels of MHC class I and II, CD80, CD86, CD83, IL-12, and heat shock proteins (HSPs) than did immature DCs. Moreover, heat-treated autologous tumor cells displayed a characteristic phenotype with increased expression of HSPs, carcinoembryonic Ag (CEA), MUC1, and MHC class I (HLA-A2 and/or A24). In this study, we have created four types of FC preparation by alternating fusion cell partners: 1) immature DCs fused with unheated tumor cells; 2) immature DCs fused with heat-treated tumor cells; 3) OK-DCs fused with unheated tumor cells; and 4) OK-DCs fused with heat-treated tumor cells. Although OK-DCs fused with unheated tumor cells efficiently enhanced CTL induction, OK-DCs fused with heat-treated tumor cells were most active, as demonstrated by: 1) up-regulation of multiple HSPs, MHC class I and II, CEA, CD80, CD86, CD83, and IL-12; 2) activation of $CD4^+$ and $CD8^+$ T cells able to produce IFN-γ at higher levels; 3) efficient induction of CTL activity specific for CEA or MUC1 or both against autologous tumor; and 4) superior abilities to induce $CD107^+$IFN-γ^+CD8^+ T cells and $CD154^+$ IFN-γ^+CD4^+ T cells. These results strongly suggest that synergism between OK-DCs and heat-treated tumor cells enhances the immunogenicity of FCs and provides a promising means of inducing therapeutic antitumor immunity. *The Journal of Immunology,* **2007, 179: 4874–4883.**

Dendritic cells (DCs)[3] are potent APCs that have been used in cancer vaccines because of their ability to initiate $CD8^+$ CTL-mediated immune responses (1). Tumor-associated Ags (TAAs) presented by DCs are needed to evoke tumor-specific immune responses. Various strategies have been developed to deliver TAAs into DCs with tumor RNA, tumor lysates, or apoptotic tumor cells to elicit and boost antitumor immune response (2–5). An alternative strategy for inducing antitumor immunity is the use of fusion cells (FCs) derived from DCs and tumor cells. In this approach, TAAs, including both known and unidentified Ags are delivered to DCs, processed, and presented through both MHC class I and II pathways in the context of costimulatory molecules (6). We have previously reported that immature (Imm) DCs (Imm-DCs) fused with autologous tumor cells can induce Ag-specific polyclonal CTLs in vitro (7–11). Although immunization with DC/tumor cell FCs in patients with cancer has been associated with immunological responses in phase I clinical trials, early clinical trials have shown only limited success (12–17). Because this DC/tumor cell FC approach was developed in animal studies, many adjuvants, including IL-2, IL-12, IL-18, and synthetic oligodeoxynucleotides (ODNs) containing specific bacterial unmethylated CpG motifs (CpG ODNs), have been used to enhance the ability of DC/tumor cell FC vaccines to evoke antitumor immune responses (18–22). These results suggest that an adjuvant is needed to enhance antitumor immunity when DC/tumor cell FC vaccines are used to treat cancer in human patients.

Effective adjuvants for enhancing the induction of Ag-specific CTLs are heat-shock proteins (HSPs), to which the ability of heat-treated tumor cells to enhance immunogenicity has been attributed (23–28). The HSP family includes both constitutively expressed proteins (HSC) and proteins environmentally induced under stress, including heat-treatment. The HSPs are chaperon proteins that can carry peptides, including antigenic peptides. The HSP/peptide complexes can be taken by DCs through receptors and presented in MHC class I and II molecules on DCs (23, 26, 29). This phenomenon leads to activation of maturation and

*Division of Gastroenterology and Hepatology, Department of Internal Medicine, †Institute of Clinical Medicine and Research, ‡Department of Oncology, Institute of DNA Medicine, and §Department of Surgery, Jikei University School of Medicine, Tokyo, Japan; ¶Saitama Cancer Center Research Institute for Clinical Oncology, Saitama, Japan; and ‖Department of Medicine, Boston University School of Medicine, Boston, MA 02118

Received for publication April 11, 2007. Accepted for publication July 25, 2007.

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-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, a Grant-in-Aid of the Japan Medical Association, the Takeda Science Foundation, the Pancreas Research Foundation of Japan, the Jikei University Research Fund, the Promotion and Mutual Aid Corporation for Private School of Japan, and the Science Research Promotion Fund.

[2] Address correspondence and reprint requests to Dr. Shigeo Koido, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8564 Japan. E-mail address: shigeo_koido@jikei.ac.jp

[3] Abbreviations used in this paper: DC, dendritic cell; TAA, tumor-associated Ag; FC, fusion cell; Imm, immature; MFI, mean fluorescence intensity; Imm/HS-FC, Imm-DC fused with heat-treated tumor cell; OK-DC, OK-432-stimulated DC; OK-FC, OK-DC fused with tumor cell; OK/HS-FC, OK-DC fused with heat-treated tumor cell; CEA, carcinoembryonic Ag.

representation of peptide Ag cargo of HSPs by DCs, with migration to draining lymph nodes, where DCs present Ags to naive T cells and initiate CTL responses (26). Moreover, autologous HSP peptide vaccines have induced CTLs against autologous tumor cells in several trials in patients with cancer (30).

Ligands for TLR are potent stimuli for DC maturation. The triggering of TLR by pathogen-associated molecular patterns potently induces DC maturation, leading to the production of proinflammatory cytokines and the up-regulation of costimulatory molecules (31, 32). Therefore, TLR-mediated signals play an important role in the induction and regulation of antitumor immune responses (31, 32). OK-432, a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes* (group A), has been used without apparent side effects for >20 years as an adjuvant for patients with cancer (33–35). OK-432 activates neutrophils, macrophages, lymphocytes, and NK cells by inducing multiple cytokines, such as IL-12 and IFN-γ, and polarizes the T cell response to a helper Th1-dominant state (33–35). Moreover, OK-432 promotes functional maturation of DCs through the TLR4 and β_2 integrin system to enhance Ag-specific CTL responses to a greater extent than does a previously reported mixture (consisting of TNF-α, IL-1β, IL-6, and PGE_2) or LPS (34–40). We have also reported that OK-432 promotes fusion efficiency and the induction of tumor-specific CTL by FCs (41).

We are attempting to produce a more effective DC/tumor cell FC-based vaccine. We have generated FC-based vaccination strategies strong enough to circumvent tumor escape, which is, at least in part, due to the poor immunogenic nature of the tumor cells. In this study, we have created four types of FC preparation by alternating FC partners: 1) Imm-DCs fused with unheated tumor cells (Imm-FCs); 2) Imm-DCs fused with heat-treated tumor cells (Imm/HS-FCs); 3) DCs stimulated with OK-432 (OK-DCs) and fused with unheated tumor cells (OK-FCs); and 4) OK-DCs fused with tumor cells that have been stressed by heat treatment before fusion (OK/HS-FCs). We show that Imm/HS-FCs are better able than Imm-FCs to up-regulate expression of TAAs (carcinoembryonic Ag (CEA) and MUC1), HLA-ABC (HLA-A2 and/or A24), HLA-DR, costimulatory molecules (CD80 and CD86), and the DC maturation marker CD83 and thus enhance their immunogenicity. Although OK-FCs induce superior CTL activity in vitro, OK/HS-FCs were the most effective inducers of Ag-specific CTL activation in the present study. We conclude that fusions of OK-DCs and heat-treated tumor cells can serve as the basis for a fusion-based vaccine in patients with cancer and may provide a platform for adoptive immunotherapy.

Materials and Methods

Cell lines

Colorectal carcinoma cell lines (COLP-2, COLP-12, and COLM-6) and ovarian cancer cell line (OVAP-1) were established and maintained in TIL Media I medium (Immuno-Biological Laboratories) (42). K562 and T2 cells (American Type Culture Collection) were maintained in DMEM and IMDM medium, respectively. All medium were supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Generation of DCs

Monocyte-derived Imm-DCs generated from PBMCs (obtained with informed consent and the approval by our institutional review board) were cultured for 6 days in RPMI 1640 medium containing 1% heat-inactivated autologous serum, 1000 U/ml GM-CSF (PeproTech), and 500 U/ml IL-4 (Diaclone Research) (43). OK-DCs were generated by treating Imm-DCs with 0.1 KE/ml (0.1 KE equals of 0.01 mg of dried streptococci) OK-432 (Chugai Pharmaceutical) for 1 day and were then used for fusion.

Preparation of autologous tumor cells

Specimens from resected tumors (obtained with the approval of our institutional review board) were isolated and maintained in TIL Media I medium with 10% heat-inactivated autologous serum, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. To prepare heat-treated autologous colorectal carcinoma cells, the cells were cultured at 42°C for 3 h, followed by recovery for 24 h at 37°C.

Phenotype analysis

Cells were incubated with FITC-conjugated Abs against-MUC1 (HMPV; BD Pharmingen), CEA (B1.1; BD Pharmingen), MHC class I (W6/32), MHC class II (HLA-DR), B7-1 (CD80), B7-2 (CD86) (BD Pharmingen), HLA-A2 or HLA-A24 (One Lambda). For dual expression, cells were incubated with FITC-conjugated Abs against CEA or MUC1 and PE-conjugated Abs against HLA-DR, CD80, CD86, or CD83. For HSP expression, cells were permeabilized, incubated with PE-conjugated Abs against HSP70 (K-20; Santa Cruz Biotechnology), HSP90α/HSP90β (N-1; Santa Cruz Biotechnology), or HSC70 (K-19; Santa Cruz Biotechnology), and analyzed with a FACS (FACScan; BD Immunocytometry Systems).

Fusion of DCs and colorectal carcinoma cells

Imm-DCs or OK-DCs were fused with autologous colorectal carcinoma cells in the presence of 50% polyethylene glycol (Sigma-Aldrich) (11). After fusion, Imm-FCs and Imm/HS-FCs were cultured with 500 U/ml GM-CSF and 250 U/ml IL-4 for 3 days. OK-FCs and OK/HS-FCs were cultured for 2 days with 500 U/ml GM-CSF, 250 U/ml IL-4, and 0.1 KE/ml OK-432 and maintained with 500 U/ml GM-CSF and 250 U/ml IL-4 for an additional day.

T cell proliferation assay

T cells were cultured with DCs, heat-treated autologous tumor cells, DCs mixed with tumor cells, or four types of FC preparation at a ratio of 10:1 for 3 days in the absence of IL-2. T cells were purified with nylon wool and cultured for an additional 3 days in the presence of human rIL-2 (10 U/ml; Shionogi Company). On day 6, T cells were cultured in 96-well U-bottom culture plates. Dye solution was added, and the cultures were incubated for 4 h and assessed with Cell Titer 96 Nonradioactive Cell Proliferation Assay kit (Promega).

ELISA

The FC preparations, DCs, and heated or unheated autologous colorectal carcinoma cells were cultured (5×10^4 cells/well) for 3 days. Supernatants from these samples were tested for IL-12p70 (BD Pharmingen) or HSP70 (Nventa Biopharmaceuticals) or both by means of ELISA. To assess the production of IFN-γ in T cells, FC preparations were cocultured with T cells. The T cells were purified on day 3 and tested for IFN-γ production by means of ELISA on day 6.

IFN-γ or IL-10 production of $CD4^+$ and $CD8^+$ T cells

Stimulated T cells were incubated with each cytokine-catching reagent for 5 min at 4°C, 10 ml of prewarmed complete medium was added, and the culture was incubated for 45 min. Cells were then stained with PE-conjugated anti-IFN-γ or IL-10 detection Ab and FITC-conjugated anti-CD4 or CD8 mAb with a secretion assay kit for each cytokine (Miltenyi Biotec).

Pentameric assay

Complexes of PE-HLA-A2-MUC1 pentamer (950–958, STAPPVHNV), HLA-A2-CEA pentamer (571–579, YLSGANLNL), or HLA-A24-CEA pentamer (652–660, TYACFVSNL) or irrelevant pentamer (Proimmune) were used. Stimulated T cells were incubated with PE-conjugated pentamer and FITC-conjugated anti-CD8 mAb. The pentameric staining was performed according to the manufacturer's instructions. Briefly, FC preparations were washed twice and cocultured with autologous T cells at a ratio of 1:10 in the absence of human IL-2 for 3 days. From day 4, a low dose of human IL-2 (10 U/ml) was added to the cell culture, which was maintained until day 7. The purified T cells were incubated with PE-conjugated pentamer for 10–15 min at room temperature in the dark. After being washed with PBS, the T cells were incubated with FITC-conjugated anti-CD8 mAb for 20 to 30 min at 4°C in the dark. Cells were washed, fixed, and analyzed with FACScan and CellQuest software (BD Biosciences). The reactivity of $CD8^+$ T cells to MUC1 or CEA or both are shown as the percentage of the total population of $CD8^+$ T cells that were double positive ($CD8^+$pentamer$^+$).

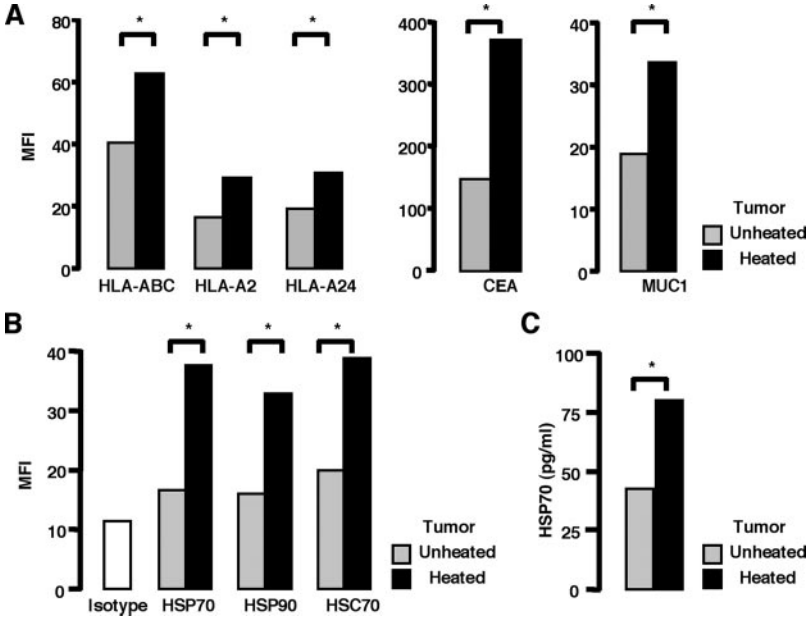

FIGURE 1. Phenotypic analysis and functional characterization of heat-treated autologous tumor cells. *A*, MFIs of HLA-ABC, -A2, -A24, CEA, and MUC1in heat-treated tumor cells and unheated tumor cells from all eight patients were analyzed. *B*, MFIs of intracellular HSP70, HSP90, and HSC70 in heat-treated and unheated tumor cells from seven patients (patients 1–3 and 5–8) were analyzed. *C*, Mean averages of HSP70 production by heat-treated and unheated tumor cells from two patients (patients 2 and 3) were analyzed. ∗, Significant differences.

Cytotoxicity assays

The same numbers of four types of fused cells were cocultured with autologous T cells at a ratio of 1:10 in the absence of human IL-2 for 3 days. The number of fused cells in the FC preparations is described on the basis of the number of cells that coexpressed HLA-DR and CEA. From day 4, a low dose of human IL-2 (10 U/ml) was added to the cell culture, which was maintained until day 8. OK-DCs alone, heated tumor cells alone, and OK-DCs mixed with heated tumor cells were used as controls. The cytotoxicity assays were performed by means of flow cytometry CTL assays using Active Caspase-3 Apoptosis kit I (BD Pharmingen) (44, 45). In certain experiments, target cells were preincubated with anti-MHC class I mAb (W6/32; 1/100 dilution) or control IgG for 30 min at 37°C before the addition of effector cells. The percentage of cytotoxicity (mean ± SD of three replications) was determined with the following equation: percentage of caspase-3 staining = ((caspase-3$^+$PKH-26$^+$ cells)/(caspase-3$^+$PKH-26$^+$ + caspase-3$^-$PKH-26$^+$)) × 100.

Autologous tumor-specific CD4$^+$ and CD8$^+$ T cells with IFN-γ profiles

Stimulated T cells were incubated with autologous tumor at a ratio of 1:5. FITC-conjugated anti-CD154 mAb (TRAP-1; BD Pharmingen) (46, 47) or FITC-conjugated anti-CD107a (H4A3; BD Pharmingen) and CD107b mAb (H4B4; BD Pharmingen) (48, 49) were added to the cells during stimulation. After the first hour of incubation, 10 μg/ml brefeldin A (Sigma-Aldrich) and 6 μg/ml Golgi Stop (BD Pharmingen) were added. The cells were incubated for an additional 4 h and stained with PE-Cy-5-conjugated anti-CD4 mAb (RPA-T4; BD Pharmingen) or PE-Cy-5-conjugated anti-CD8 mAb (HIT8A; BD Pharmingen). The cells were washed, fixed, permeabilized, and stained with PE-conjugated anti-IFN-γ mAb (Miltenyi Biotec).

Statistical analysis

The Student *t* test was used to compare various experimental groups. A p value <0.05 was considered to be statistically significant.

Results

Characteristic phenotype of OK/HS-FCs

Autologous colorectal carcinoma cells isolated from all patients expressed high levels of CEA, MUC1, and HLA-ABC molecules, but not HLA-DR, CD80, CD86, or CD83 molecules (Fig. 1*A* and Ref. 41). Autologous colorectal carcinoma cells were consequently

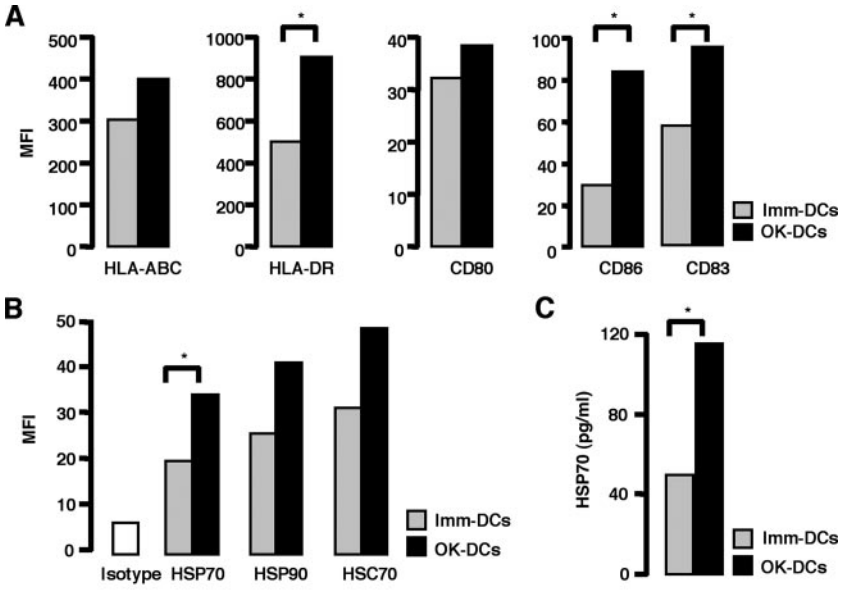

FIGURE 2. Phenotypic analysis and functional characterization of OK-DCs. *A*, Imm-DCs were stimulated by OK-432 for 3 days (OK-DCs). MFIs of HLA-ABC, -DR, CD80, CD86, and CD83 in OK-DCs and Imm-DCs from five patients (patients 1, 2, 3, 5, and 6) were analyzed. *B*, MFIs of intracellular HSP70, HSP90, and HSC70 in OK-DCs and Imm-DCs from three patients (patients 2, 3, and 8) were analyzed. *C*, Mean HSP70 production by OK-DCs and Imm-DCs from two patients (patients 2 and 3) was analyzed. ∗, Significant differences.

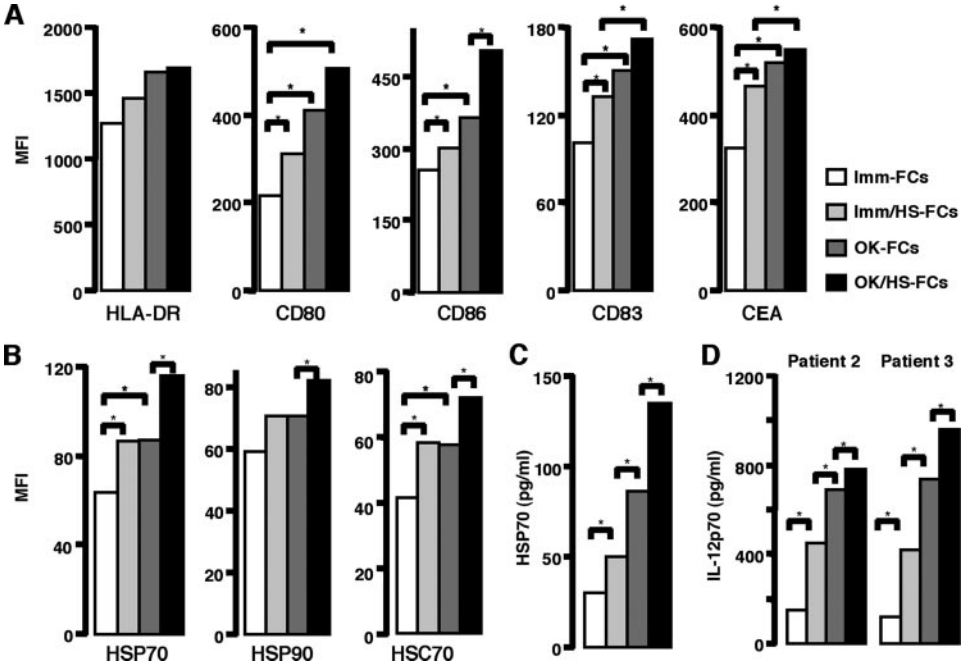

FIGURE 3. Phenotypic analysis and functional characterization of four types of FC preparation. *A*, Four types of FC preparation from eight different patients were incubated with FITC-conjugated anti-CEA and PE-conjugated Abs against HLA-DR, CD80, CD86, or CD83 and analyzed with two-color flow cytometry. The FCs were isolated with FACS analysis, in which fused cells were demonstrated as double-positive cells. MFIs of indicated molecules in double-positive populations from eight different patients were analyzed. *B*, FC preparations were incubated with FITC-conjugated Abs against CEA and PE-conjugated Abs against HSP (HSP70, HSP90, or HSC70). Mean MFIs of HSPs (HSP70, HSP90, or HSC70) in double-positive populations from four patients (patients 1, 3, 6, and 7) were analyzed. *C*, Mean HSP70 production by four types of FC preparation from patient 3 were analyzed with ELISA. *D*, Mean IL-12p70 production by four types of FC preparation from two patients (patients 2 and 3) were analyzed with ELISA. ∗, Significant differences.

heat-treated before fusion. Heat-treatment of tumor cells resulted in up-regulation of the mean fluorescence intensity (MFI) of intracellular multiple HSPs (HSP70, HSP90, and HSC70) and production of extracellular HSP70, all of which may supply danger signals that are critical for the generation of antitumor immunity (Fig. 1, *B* and *C*). Flow cytometric analysis of intracellular HSPs is a rapid, sensitive, quantitative, and more-reproducible method than Western blotting and is particularly suited for determining protein levels in individual cells (50, 51). Moreover, heat-treated tumor cells displayed a characteristic phenotype with higher levels of TAAs (CEA and MUC1) and HLA-ABC (HLA-A2 and/or A24) (Fig. 1*A*). These observations suggest a new approach for increasing the immunogenicity of tumor cells in a FC-based vaccine.

Monocyte-derived Imm-DCs from patients with colorectal carcinoma were generated in the presence of GM-CSF and IL-4. The maturation of DCs was effectively induced by exposure to OK-432 for 3 days. As compared with Imm-DCs, OK-DCs expressed higher levels of HLA-ABC (HLA-A2 and A24), HLA-DR, costimulatory molecules, CD86 and the maturation marker, CD83 (Fig. 2*A*). Interestingly, exposure of Imm-DCs to OK-432 was also associated with up-regulation of intracellular HSPs (HSP70, HSP90, and HSC70), extracellular HSP70, and IL-12p70 (Fig. 2, *B* and *C*, and Ref. 41).

We have created four types of FC preparation: 1) Imm-FCs, 2) Imm/HS-FCs, 3) OK-FCs, and 4) OK/HS-FCs. OK-FCs exhibited up-regulation of the MFIs of CEA, costimulatory molecules (CD80 and CD86), the DC maturation marker CD83, and HSPs (HSP70 and HSC70) compared with Imm-FCs or Imm/HS-FCs. However, OK/HS-FCs exhibited significantly higher MFI per FC of CEA, CD86, HSP70, HSP90, and HSC70, as determined with FACS analysis in which FCs were isolated as double-positive populations (Fig. 3, *A* and *B*). Furthermore, OK/HS-FCs showed significantly higher dual expression of

Table I. *Percentage of DC/tumor FCs*[a]

Patient List	HLA-A2	HLA-A24	Imm-FCs	Imm/HS-FCs	OK-FCs	OK/HS-FCs
1	−	+	39.00	42.08	48.20	50.64
2	+	+	52.48	59.34	64.74	71.00
3	+	+	33.40	54.24	55.10	57.60
4	+	−	46.56	49.54	53.54	57.50
5	−	−	39.06	48.12	49.45	55.96
6	−	−	42.96	58.10	63.72	72.66
7	+	+	72.22	80.50	87.14	92.74
8	−	+	48.24	55.11	59.51	65.82
Mean±SD			46.74 ± 11.93	55.88 ± 11.45	61.71 ± 12.63	65.49 ± 13.42

[a] Note. %, Percent of cells positive for CEA and HLA-DR. Percentage of cells positive for CEA and HLA-DR in OK/HS-FCs is statistically significant compared with those from Imm-FCs, Imm/HS-FCs, or OK-FCs ($p < 0.05$).

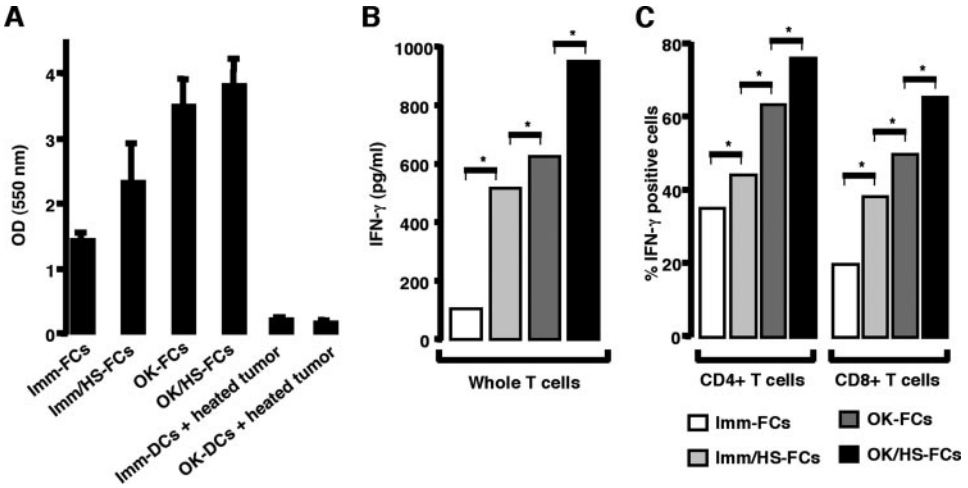

FIGURE 4. Proliferation and activation of $CD4^+$ and $CD8^+$ T cells stimulated by four types of FC preparation. *A*, T cells from patient 8 were cocultured with four types of FC preparation, Imm-DCs mixed with heated tumor cells, or OK-DCs mixed with heated tumor cells, and T cell proliferation assay was performed. For each group, the mean ± SD of three experiments is shown. Similar results were obtained in three different experiments. *B*, Mean IFN-γ production by T cells from six patients (patients 1–5 and 7) cocultured with four types of FC preparation was analyzed with ELISA. *C*, Percentages of IFN-γ-positive $CD4^+$ or $CD8^+$ T cells after stimulation by four types of FC preparation from eight different patients were assessed. ∗, Significant differences.

both CEA and HLA-DR (Table I). These findings suggest the synergism between OK-DCs and heat-treated tumor cells enhances Ag presentation.

Production of IL-12p70 and HSP70 by OK/HS-FCs

To critically assess the characteristic function of OK/HS-FCs, the production of IL-12p70 and HSP70 in supernatants from FC preparations was analyzed with ELISA. Imm/HS-FCs produced higher levels of HSP70 and IL-12p70 than did Imm-FCs (Fig. 3, *C* and *D*). Moreover, OK-FCs produced higher levels of IL-12p70 and HSP70 than did Imm-FCs or Imm/HS-FCs (Fig. 3, *C* and *D*), suggesting that OK-432 promotes IL-12p70 and HSP70 production in FC preparations. We consistently observed significantly increased production of both IL-12p70 and HSP70 by OK/HS-FCs (Fig. 3*, C* and *D*), indicating that both OK-DCs and heat-treated tumor cells are involved in the potent activation of DC/tumor cell FCs.

Enhanced T cell activation by OK/HS-FCs

To examine the ability of FC preparations to stimulate T cells, autologous T cells were stimulated with four types of FC preparation, Imm-DCs mixed with heated tumor cells, or OK-DCs mixed with heated tumor cells in the presence of low-dose human IL-2 (10 U/ml) from day 4. Although coculture of autologous T cells with OK-FCs enhanced T cell proliferation to a greater degree than did coculture with Imm-FCs or Imm/HS-FCs, OK/HS-FCs stimulated T cell proliferative responses more effectively (Fig. 4*A*). In contrast, there was no proliferation of T cells stimulated with an unfused mixture of both DCs and heated tumor cells (Fig. 4*A*).

Next, we used an IFN-γ production assay to verify that both $CD4^+$ and $CD8^+$ T cells can be primed to produce IFN-γ after stimulation with DC/tumor cell FCs. Imm/HS-FCs enhanced the production of IFN-γ in both $CD4^+$ and $CD8^+$ T cells to a greater extent than did Imm-FCs. Although OK-FCs stimulated production of IFN-γ by both $CD4^+$ and $CD8^+$ T cells to a greater extent than did Imm/HS-FCs, OK/HS-FCs stimulated IFN-γ most effectively (Fig. 4, *B* and *C*). In contrast, there was little, if any, IFN-γ production by T cells cocultured with OK-DCs mixed with heat-treated autologous colorectal carcinoma cells (data not shown). In

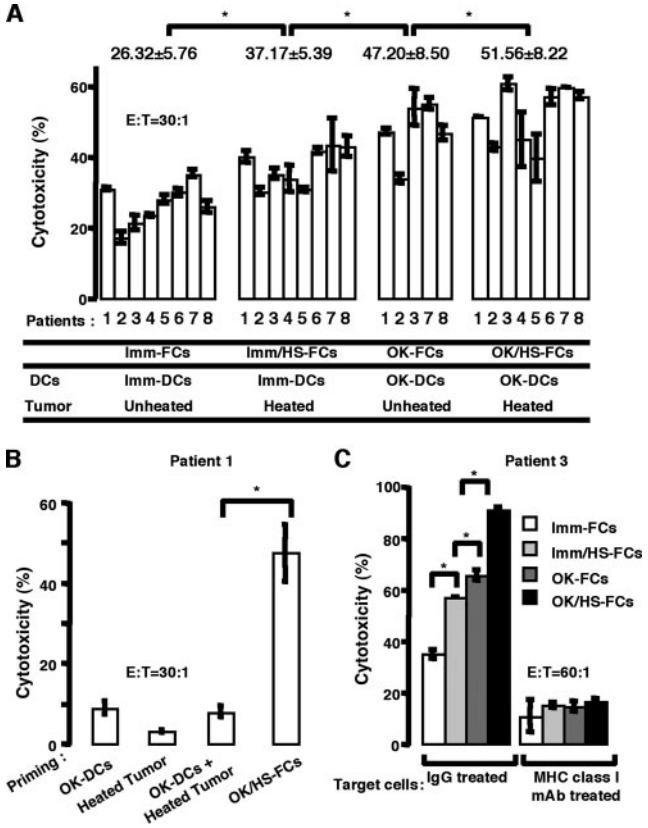

FIGURE 5. Induction of autologous tumor cell-specific CTL responses by four types of FC preparation. *A*, T cells stimulated by four types of FC preparation were cocultured with PKH-26-labeled tumor cells at a ratio of 30: 1. T cells from three patients (patients 4–6) stimulated by OK-FCs had not been determined for CTL assays. *B*, T cells (patient 1) stimulated by OK/HS-FCs, OK-DCs, heated tumor cells, or OK-DCs mixed with heated tumor cells were cocultured with PKH-26-labeled tumor cells at a ratio of 30: 1. *C*, T cells (patient 3) stimulated by four types of FC preparation were cocultured with PKH-26-labeled tumor cells at a ratio of 60: 1. Target cells were preincubated with control IgG (*left panel*) or anti-MHC class I mAb (W6/32; 1/100 dilution; *right panel*). Percentage of cytotoxicity (mean ± SD of three replications) was determined by flow cytometry CTL assays. ∗, Significant differences.

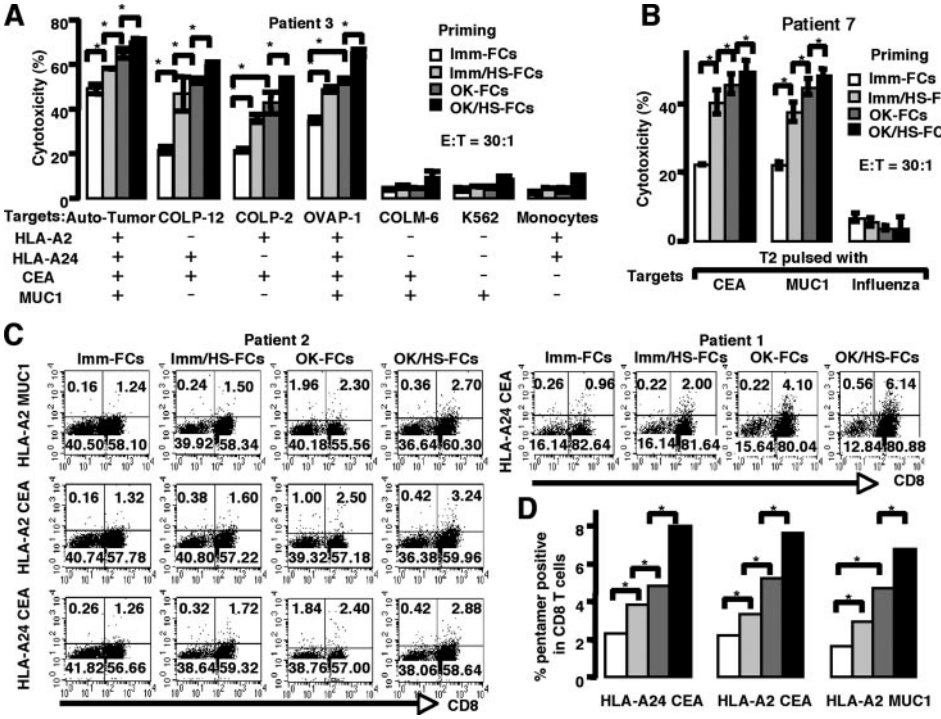

FIGURE 6. Induction of CEA- and MUC1-specific CTL responses restricted in HLA-A2 and A24 by four types of FC preparation. *A*, T cells from patient 3 (HLA-A2$^+$/A24$^+$) stimulated by four types of FC preparation were incubated with PKH-26-labeled autologous tumor cells, allogeneic cell lines, or autologous monocytes. *B*, T cells from patient 7 (HLA-A2$^+$/A24$^+$) stimulated by four types of FC preparation were incubated with PKH-26-labeled T2 cells pulsed with CEA, MUC1, or influenza peptides. Percentage of cytotoxicity (mean ± SD of three replications) was determined. *C*, T cells from two patients (patients 1, HLA-A2$^-$/A24$^+$, and 2, HLA-A2$^+$/A24$^+$) stimulated by four types of FC preparation were examined by pentameric assay. *D*, CD8$^+$ T cells obtained from six different patients (patients 1–4, 7, and 8) were stimulated by four types of FC preparation and analyzed. CD8$^+$ T cell reactivity to MUC1 or CEA or both are shown as the percentage of double-positive populations (CD8$^+$ pentamer$^+$) among all CD8$^+$ T cells. ∗, Significant differences.

addition, the low levels of IL-10 production by both CD4$^+$ and CD8$^+$ T cells stimulated by OK/HS-FCs did not impair the production of IFN-γ (data not shown). Although Imm/HS-FCs or OK-FCs could stimulate both CD4$^+$ and CD8$^+$ T cells, by themselves they could not induce full maturation of FCs. Taken together, our results show that both OK-DCs and heat-treated tumor cells contribute to enhance their ability through a fusion process to stimulate both CD4$^+$ and CD8$^+$ T cells to produce high levels of IFN-γ and may enable stronger Th1 and CTL responses to be generated.

A synergistic augmentation of CTL generation against autologous colorectal carcinoma cells by OK/HS-FCs

To evaluate the functional significance of FC preparations on a per FC basis, autologous T cells from patients with colorectal carcinoma were stimulated with the same number of fused cells that coexpressed both HLA-DR and CEA in all four types of FC preparation in the same set of experiments. Cytotoxicity was assessed with flow cytometry CTL assays that were predicated on measurement of CTL-induced caspase-3 activation in target cells through detection of specific cleavage of fluorogenic caspase-3 (44, 45). Although CTL activity against individual autologous colorectal carcinoma cells (HLA-ABC$^+$) was induced by Imm-FCs in all cases examined, Imm/HS-FCs more effectively primed T cells to differentiate into CTLs able to kill autologous targets (Fig. 5*A*). Although OK-FCs more effectively induced CTL generation than did Imm/HS-FCs or Imm-FCs, the number and functional activity of CTLs in T cells stimulated by OK/HS-FCs were significantly increased (Fig. 5*A*). In contrast, there was minimal lysis of autologous colorectal carcinoma cells by T cells stimulated with OK-DCs, heated tumor cells, or an unfused mixture of both (Fig. 5*B*). In addition, lysis was inhibited by preincubation of target cells with an anti-HLA-ABC mAb, indicating restriction by MHC class I (Fig. 5*C*). Significantly, the synergism between OK-DCs and heat-treated tumor cells could markedly augment CTL responses through two pathways.

Enhanced CEA- and MUC1-specific CTL responses in HLA-A2 and/or A24 restriction elements

To assess the specificity for CEA or MUC1 or both and the HLA restriction element of CTLs induced by the four types of FC preparation, the CTL assay was performed with multiple targets. The CTLs induced by all four types of FC preparation lysed not only autologous colorectal carcinoma cells but also semiallogeneic colorectal carcinoma cell lines COLP-2 and COLP-12, which endogenously expressed CEA or MUC1 or both with a partially matched HLA-restricted element (HLA-A2 and/or A24) (Fig. 6*A*). In addition, there was no lysis of COLM-6 (HLA-A2$^-$/A24$^-$, CEA$^+$ and MUC1$^+$) by T cells stimulated by FC preparations from patients (HLA-A2$^+$ and A24$^+$, CEA$^+$ and MUC1$^+$), suggesting that HLA-A2 and A24 were the restriction elements of CTLs (Fig. 6*A*). Moreover, autologous T cells (HLA-A2$^+$) stimulated by FC preparations lysed T2 cells pulsed with CEA or MUC1 peptides but not T2 cells pulsed with control influenza matrix peptides, confirming that CTLs specific for CEA or MUC1 or both were HLA-A2 restricted (Fig. 6*B*). Interestingly, CTLs primed with Imm/HS-FCs could recognize targets expressing CEA or MUC1 or both more efficiently in an HLA-A2- and/or A24-restricted manner than could CTLs primed with Imm-FCs (Fig. 6, *A* and *B*). Importantly, although OK-FCs further enhanced CTL activity specific for CEA or MUC1 or both, OK/HS-FCs were the most potent inducers of Ags-specific CTL responses in vitro (Fig. 6, *A* and *B*). These results suggest that fusions of OK-DCs and

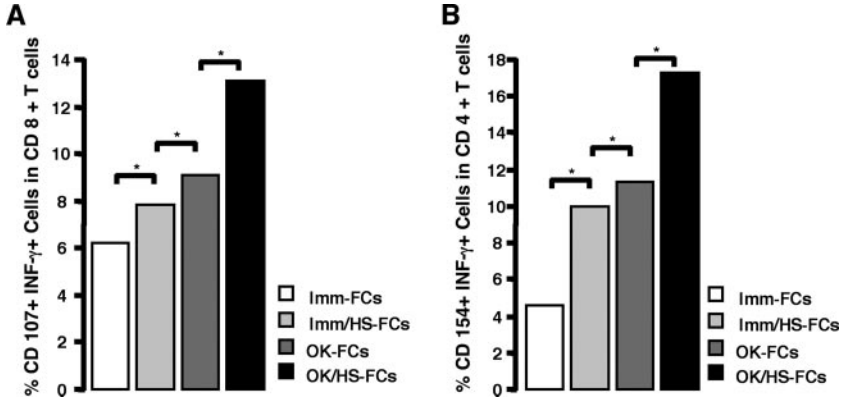

FIGURE 7. Expression of CD107 and CD154 in tumor-specific $CD4^+$ and $CD8^+$ T cells with IFN-γ profiles. *A*, T cells from four patients (patients 3 and 6–8) were stimulated by four types of FC preparation, and the capacity of $CD8^+$ T cells to degranulate upon autologous tumor cells encounter was assessed. The percentages of $CD107^+IFN\text{-}\gamma^+$ cells in $CD8^+$ T cells were examined. *B*, T cells from four patients (patients 3 and 6–8) were stimulated by four types of FC preparation and the capacity of $CD4^+$ T cells highly specific for autologous tumor cells was assessed. ∗, Significant differences.

heat-treated tumor cells might markedly increase therapeutic effects.

Next, to evaluate the reactivity of $CD8^+$ T cells to CEA and MUC1, we analyzed the ability to bind CEA and MUC1 peptide-MHC class I pentamers. A pentameric assay of soluble class I MHC-peptide complexes was used to detect Ag-specific CTLs induced by DC/tumor cell FC preparations (42). As shown in Fig. 6 *C* and *D*, after one cycle of stimulation with Imm-FCs, 2.32 and 2.20% of $CD8^+$ T cells were specific for CEA in HLA-A2- and A24-restrictive manners, respectively. Importantly, when autologous T cells were stimulated with OK/HS-FCs, the percentage of $CD8^+$ T cells specific for CEA increased to 8.00 and 7.67% in HLA-A2- and A24-restricted manners, respectively. The frequency of MUC1 pentamer-binding $CD8^+$ T cells among all $CD8^+$ T cells increased to 6.75% (Fig. 6*D*). In contrast, $CD8^+$ T cells binding control epitope pentamer were not detected (data not shown). Moreover, CTLs specific for CEA and MUC1 were not detected after T cells were stimulated by an unfused mixture of OK-DCs and heat-treated tumor cells (data not shown). These results suggest that OK/HS-FCs are most efficient in priming CEA- and MUC1-specific CTLs in HLA-A2 and/or A24 restriction elements.

Degranulation and IFN-γ production upon autologous tumor encounter

Degranulation of CD107 a and b is a requisite process of perforin/granzyme-dependent lytic fashions mediated by responding Ag-specific CTLs (48, 49). Measurement of CD107a and b expression with FACS can be combined with intracellular IFN-γ staining to more completely assess the functionality of $CD8^+$ T cells expressing cognate TCRs (48, 49). Although $CD8^+$ T cells primed with Imm-FCs, Imm/HS-FCs, or OK-FCs could degranulate, stimulation of T cells with OK/HS-FCs significantly increased the frequency of CD107-expressing $CD8^+$ T cells producing high levels of IFN-γ ($CD107^+$ IFN-γ^+ $CD8^+$) following stimulation with autologous tumor cells (Fig. 7*A*). In contrast, $CD8^+$ T cells could not degranulate when T cells were cocultured with an unfused mixture of OK-DCs and heat-treated tumor cells (data not shown). Therefore, our data indicate that OK/HS-FCs can induce CTL responses specific for autologous tumors.

Autologous tumor-specific $CD4^+$ T cells with IFN-γ profiles

Autologous tumor-induced de novo CD154 expression is highly sensitive for tumor-specific Th cells (46, 47). The coupling of CD154 expression with multiplexed measurements of IFN-γ production provides a greater level of detail for the study of tumor-specific $CD4^+$ T cell responses (46, 47). When exposed to autologous colorectal carcinoma cells, most $CD4^+$ T cells induced by all four types of FC preparation could produce IFN-γ. Interestingly, 17.29% of $CD4^+$ T cells generated by OK/HS-FCs in vitro were IFN-γ-producing tumor-specific T cells ($CD154^+IFN\text{-}\gamma^+CD4^+$), a much higher percentage than with Imm-FCs, Imm/HS-FCs, or OK-FCs (Fig. 7*B*). In contrast, CD154 was not expressed on $CD4^+$ T cells when T cells were cocultured with an unfused mixture of both OK-DCs and heat-treated tumor cells (data not shown). These results indicate that OK/HS-FCs are the most potent inducers of tumor-specific $CD4^+$ T cells, which are essential for the induction of augmented CTL responses against autologous tumor cells.

Discussion

The data presented here clearly show that OK/HS-FCs are better able than other FC preparations to generate CTLs against autologous colorectal carcinoma cells in vitro. Antitumor immunity is strongly enhanced when FCs are accompanied by both OK-432-stimulated DCs and heat-treated tumor cells.

We have demonstrated here that heat-treated colorectal carcinoma cells display a characteristic phenotype with increased expression of TAAs (CEA and MUC1), MHC class I molecules (HLA-A2 and/or A24), and multiple intracellular HSPs (HSP70, HSP90 and HSC70) in all cases examined. Intracellular HSPs play an important role as molecular chaperones in cellular protein-folding pathways (26). Both HSP70 and HSP90 can facilitate the presentation of antigenic peptides on MHC class I molecules, but HSC70 can facilitate TAA processing and presentation on MHC class II molecules (52). Recent studies have shown that cross-priming is based on the transfer of proteasome substrates that are transcriptionally up-regulated by heat treatment in human cells (53, 54). This concept offers additional effects by which heat treatment might enhance Ag processing and presentation in MHC class I and II molecules on the surfaces of FCs. In contrast, extracellular HSPs act as chaperon peptides and interact with DCs in a receptor-mediated manner, leading to maturation as well as proinflammatory responses, all of which are likely to be key danger signals to the immune system (26, 55).

In this study, DCs/heat-treated tumor cell FCs enhanced immunogenicity more strongly than did FCs prepared with unheated tumor cells. The increased expression of intracellular HSPs (HSP70 and HSC70) and an extracellular HSP70 by Imm/HS-FCs may play a critical role in enhancing the immunogenicity of FCs. Therefore, we investigated the effects of up-regulated HSPs on the function of FCs and found that Imm/HS-FCs exhibited an improved immunostimulatory phenotype with higher levels of IL-12p70 production than did Imm-FCs. IL-12 is an important proinflammatory cytokine secreted by DCs and determines Th1/Th2 polarization. Up-regulation of HSPs in tumor cells provides proper

costimulation through fusion process and can be expected to be involved in polarizing the T cell responses to a Th1-dominant state. A recent study that has found that HSP70-peptide complexes derived from DCs/tumor cell FCs in mice have increased immunogenicity and are able to carry an increased repertoire of antigenic peptides (56) supports our finding that up-regulated HSPs in FCs enhance FC immunogenicity.

Although the role of HSPs in the enhancement of antitumor responses is generally acknowledged, little is known about how the combination of HSPs and TLR ligands affects the induction of antitumor immunity. Ag presentation by DCs, in the absence of proper costimulation, induces tolerance (1). In particular, recent studies suggest that TLR-agonist CpG ODNs or conserved pathogen-associated molecular patterns, such as OK-432, start the DC maturation process, which is a critical event in the induction of full effector function in T cells (20, 31, 32, 35, 41, 57, 58). Therefore, we next investigated whether DCs activated by a combination of TLR ligation and up-regulated HSPs enhance the immunogenicity of FCs. We used the TLR4-agonist OK-432 to stimulate Imm-DCs, which were then fused with heat-treated tumor cells. Because OK-432 is an agent of good manufacturing practice grade and has been widely used in patients with cancer (33–37), an important aspect of our work is its potential clinical relevance. Interestingly, OK/HS-FCs expressed significantly higher levels of CD86 and intracellular HSPs (HSP70, HSP90, and HSC70). Moreover, OK/HS-FCs more efficiently produced IL-12p70 and HSP70. Our present observations indicate that OK-FCs and Imm/HS-FCs still have insufficient capacity as APCs and that the combination of OK-432 and up-regulated HSPs has favorable characteristics for strongly activating FCs in vitro. Interestingly, intracellular and extracellular levels of HSP70 were higher with OK-DCs than with Imm-DCs and might also be associated with enrichment of HSPs in OK/HS-FCs. Recent reports have demonstrated that the TLR9-mediated activation of DCs by CpG ODNs increases the intracellular levels of HSPs, particularly those of HSP70 (59, 60). OK-432 may be associated with the up-regulation of HSP70 in DCs via TLR4, because OK-432 induces immune responses via TLR4 (35). Moreover, HSP-mediated activation of DCs depends on the presence of functional TLR2 and TLR4 molecules to transduce their proinflammatory signals (26). The intervention strategy in the combination of OK-432 and up-regulated HSPs may act synergistically through the fusion process and result in efficient Ag processing and presentation in the context of up-regulated MHC and costimulatory molecules. Our findings are consistent with those of a recent study that has found that pathogen-associated molecules cooperate efficiently with damage-associated molecules, such as HSPs, in activating DCs (58).

Surveillance by the innate immune system plays an important role in resistance to cancer. However, once established, augmented CTL induction is particularly important for eradicating tumor cells. Both OK-432 and HSPs are putative ligands of the TLR, stimulate DCs rapidly, and elicit a Th1-promoting phenotype (61, 62), all of which are essential for exhibiting capacities to prime naive T cells and develop antitumor CTL responses. Our previous study, which found that depletion of $CD4^+$ T cells significantly impairs CTL induction by DCs/tumor FCs (11, 41, 63, 64), indicates that efficient CTL generation requires the help of $CD4^+$ T cells. Our present study has shown that OK/HS-FCs are superior to other FC preparations for expanding the population of both $CD4^+$ and $CD8^+$ T cells that produce high levels of IFN-γ. Although OK-FCs can enhance the efficacy of CTL induction against autologous tumor cells, CTL activity induced by OK/HS-FCs was significantly higher, on a per FC basis, than was that induced by other types of FC preparations in the same set of experiments. Factors that may play important synergistic roles in generating augmented CTL activity include: 1) enhanced expression of CD86, 2) increased production of IL-12p70 and HSP70, and 3) enrichment of intracellular HSPs in OK/HS-FCs.

To confirm the functional significance of tumor-specific $CD8^+$ and $CD4^+$ T cells stimulated with OK/HS-FCs, we measured degranulation by $CD8^+$ T cell surface modulation of lytic granules (CD107a and b) and CD154 expression on $CD4^+$ T cells that produce IFN-γ. Stimulation of the TCRs on $CD8^+$ T cells with autologous tumor cells resulted in degranulation and the release of CD107a and b, which became transiently mobilized to the cell surface (48, 49, 65). We found that stimulation of T cells with OK/HS-FCs markedly increased the population of $CD107^+$IFN-γ^+CD8^+ T cells, compared with those induced by Imm-FCs, Imm/HS-FCs, or OK-FCs. Moreover, we observed a substantial population of responding $CD8^+$ T cells that produced IFN-γ without degranulation ($CD107^-$IFN-γ^+CD8^+ T cells). Although functional heterogeneity exists in the population of responding $CD8^+$ T cells, $CD8^+$ T cells that produce IFN-γ and degranulate should be able to kill targets (48, 49). Therefore, OK/HS-FCs might be used to stimulate tumor-specific $CD8^+$ T cells.

Importantly, there is increasing evidence that $CD4^+$ T cells play an important role in helping generate antitumor immunity (66). Recent reports have described a new assay that enables assessment of Th cells highly specific for tumor cells on the basis of CD154 expression, which plays a fundamental role in the interaction between $CD4^+$ T cells and DCs (46, 47). Stimulation of T cells with OK/HS-FCs resulted in significantly increased populations of $CD4^+$ T cells producing IFN-γ and expressing CD154, compared with Imm-FCs, Imm/HS-FCs, or OK-FCs. Therefore, our findings confirm the notion that OK/HS-FCs have more favorable characteristics to induce tumor-specific CTLs and polarize T cells to a Th1-dominant state, all of which are critical in suppressing tumors.

Furthermore, we have assessed the Ag-specific CTL activation by means of CTL assays using many targets and MHC pentamer assays. CEA- and MUC1-specific CTLs were induced simultaneously by OK-FCs at higher frequency, than by Imm-FCs or Imm/HS-FCs. These results indicate that the fusion process (41) and heat-treatment, in the absence of stimuli such as OK-432, induce insufficient FC activation and initiate TAA-specific CTL responses. Heat-treated tumor cells are an effective source of TAAs for a FC-based vaccine. Our findings are consistent with those of another study demonstrating that the increased transcription and translation of multiple TAAs by heat treatment results in enhanced cross-priming by DCs via up-regulation of both HSPs and TAAs (67). Importantly, OK/HS-FCs are the most potent inducers of TAA-specific polyclonal CTL responses in HLA-A2- and/or A24-restricted manners in this experimental setting. The augmented TAA-specific CTL responses by OK/HS-FCs are due in part to the increased expression of costimulatory molecules and antigenic peptides on the MHC molecules of FCs. These findings support the potential for OK/HS-FCs to boost TAA-specific CTL effector functions in vitro. The mechanisms behind the synergistic effect described here may be based in part on the different intracellular pathways of TLR triggered by OK-432 and HSP (26, 35). Importantly, these synergistic strategies of two pathways do not exclude the functional overlap between them through the fusion process.

Ex vivo induction and expansion of TAA-specific CTLs for adoptive immunotherapy are the subject of intensive investigation. Coculture of T cells with OK/HS-FCs in the presence of low-dose human IL-2 (10 U/ml) markedly increases the magnitude of FC-elicited CTL responses against autologous colorectal carcinoma cells in vitro, indicating that synergism between OK-432 and heat treatment remains even with low doses of IL-2. Our results clearly

show that this synergistic effect of OK-432 and HSPs in OK/HS-FCs has an important effect on efficient TAA-specific CTL induction and expansion in vitro.

In conclusion, our results demonstrate that effective CTLs can be induced by fusions of OK-DCs and heat-treated tumor cells. Incorporating OK-DCs and heat-treated tumor cells in the activation regimen may increase the immunogenicity of a DC/tumor cell FC-based vaccine and increase the opportunities for efficient presentation of HSP-chaperoned epitopes to T cells. This alternative approach is simple and may be a highly promising strategy for improving DC/tumor cell FC-based vaccination.

Disclosures

The authors have no financial conflict of interest.

References

1. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9: 271–296.
2. Nestle, F. O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg, and D. Schadendorf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med.* 4: 328–332.
3. Koido, S., M. Kashiwaba, D. Chen, S. Gendler, D. Kufe, and J. Gong. 2000. Induction of antitumor immunity by vaccination of dendritic cells transfected with MUC1 RNA. *J. Immunol.* 165: 5713–5719.
4. Paglia, P., C. Chiodoni, M. Rodolfo, and M. P. Colombo. 1996. Murine dendritic cells loaded in vitro with soluble protein prime cytotoxic T lymphocytes against tumor antigen in vivo. *J. Exp. Med.* 183: 317–322.
5. Russo, V., S. Tanzarella, P. Dalerba, D. Rigatti, P. Rovere, A. Villa, C. Bordignon, and C. Traversari. 2000. Dendritic cells acquire the MAGE-3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response. *Proc. Natl. Acad. Sci. USA* 97: 2185–2190.
6. Gong, J., D. Chen, M. Kashiwaba, and D. Kufe. 1997. Induction of antitumor activity by immunization with fusions of dendritic and carcinoma cells. *Nat. Med.* 3: 558–561.
7. Gong, J., N. Nikrui, D. Chen, S. Koido, Z. Wu, Y. Tanaka, S. Cannistra, D. Avigan, and D. Kufe. 2000. Fusions of human ovarian carcinoma cells with autologous or allogeneic dendritic cells induce antitumor immunity. *J. Immunol.* 165: 1705–1711.
8. Gong, J., D. Avigan, D. Chen, Z. Wu, S. Koido, M. Kashiwaba, and D. Kufe. 2000. Activation of antitumor cytotoxic T lymphocytes by fusions of human dendritic cells and breast carcinoma cells. *Proc. Natl. Acad. Sci. USA* 97: 2715–2718.
9. Gong, J., S. Koido, Y. Kato, Y. Tanaka, D. Chen, A. Jonas, I. Galinsky, D. DeAngelo, D. Avigan, D. Kufe, and R. Stone. 2004. Induction of antileukemic cytotoxic T lymphocytes by fusion of patient-derived dendritic cells with autologous myeloblasts. *Leuk. Res.* 28: 1303–1312.
10. Koido, S., N. Nikrui, M. Ohana, J. Xia, Y. Tanaka, C. Liu, J. Durfee, A. Lerner, and J. Gong. 2005. Assessment of fusion cells from patient-derived ovarian carcinoma cells and dendritic cells as a vaccine for clinical use. *Gynecol. Oncol.* 99: 462–471.
11. Koido, S., E. Hara, A. Torii, S. Homma, Y. Toyama, H. Kawahara, M. Ogawa, M. Watanabe, K. Yanaga, K. Fujise, et al. 2005. Induction of antigen-specific CD4 and CD8 mediated T cell responses by fusion of autologous dendritic cells and metastatic colorectal cancer cells. *Int. J. Cancer* 117: 587–595.
12. Kikuchi, T., Y. Akasaki, M. Irie, S. Homma, T. Abe, and T. Ohno. 2001. Results of a phase I clinical trial of vaccination of glioma patients with fusions of dendritic and glioma cells. *Cancer Immunol. Immunother.* 50: 337–344.
13. Homma, S., K. Matai, M. Irie, T. Ohno, D. Kufe, and G. Toda. 2003. Immunotherapy using fusions of autologous dendritic cells and tumor cells showed effective clinical response in a patient with advanced gastric carcinoma. *J. Gastroenterol.* 38: 989–994.
14. Kikuchi, T., Y. Akasaki, T. Abe, T. Fukuda, H. Saotome, J. L. Ryan, D. W. Kufe, and T. Ohno. 2004. Vaccination of glioma patients with fusions of dendritic and glioma cells and recombinant human interleukin 12. *J. Immunother.* 27: 452–459.
15. Avigan, D., B. Vasir, J. Gong, V. Borges, Z. Wu, L. Uhl, M. Atokins, J. Mier, D. McDermott, T. Smith, et al. 2004. Fusion cell vaccination of patients with metastatic breast and renal cancer induces immunological and clinical responses. *Clin. Cancer Res.* 10: 4699–4708.
16. Homma, S., T. Kikuchi, N. Ishiji, K. Ochiai, H. Takeyama, H. Saotome, Y. Sagawa, E. Hara, D. Kufe, J. L. Ryan, and G. Toda. 2005. Cancer immunotherapy by fusion of dendritic and tumor cells and rh-IL-12. *Eur. J. Clin. Invest.* 35: 279–286.
17. Homma, S., Y. Sagawa, T. Ito, T. Ohno, and G. Toda. 2006. Cancer immunotherapy using dendritic/tumor-fusion vaccine induces elevation of serum antinuclear antibody with better clinical responses. *Clin. Exp. Immunol.* 144: 41–47.
18. Gong, J., S. Koido, D. Chen, Y. Tanaka, L. Huang, D. Avigan, K. Anderson, T. Ohno, and D. Kufe. 2000. Immunization against murine multiple myeloma with fusions of dendritic and plasmacytoma cells is potentiated by interleukin 12. *Blood* 99: 2512–2517.
19. Iinuma, T., S. Homma, T. Noda, D. Kufe, T. Ohno, and G. Toda. 2004. Prevention of gastrointestinal tumors based on adenomatous polyposis coli gene mutation by dendritic cell vaccine. *J. Clin. Invest.* 113: 1307–1317.
20. Hiraoka, K., S. Yamamoto, S. Otsuru, S. Nakai, K. Tamai, R. Morishita, T. Ogihara, and Y. Kaneda. 2004. Enhanced tumor-specific long-term immunity of hemagglutinating virus of Japan-mediated dendritic cell-tumor fused cell vaccination by coadministration with CpG oligodeoxynucleotides. *J. Immunol.* 173: 4297–4307.
21. Suzuki, T., T. Fukuhara, M. Tanaka, A. Nakamura, K. Akiyama, T. Sakakibara, D. Koinuma, T. Kikuchi, R. Tazawa, M. Maemondo, et al. 2005. Vaccination of dendritic cells loaded with interleukin-12 secreting cancer cells augments in vivo antitumor immunity: characteristics of syngeneic and allogeneic antigen-presenting cell cancer hybrid cells. *Clin. Cancer Res.* 11: 58–66.
22. Iinuma, H., K. Okinaga, R. Fukushima, T. Inaba, K. Iwasaki, A. Okinaga, I. Takahashi, and M. Kaneko. 2006. Superior protective and therapeutic effects of IL-12 and IL-18 gene-transduced dendritic neuroblastoma fusion cells on liver metastasis of murine neuroblastoma. *J. Immunol.* 176: 3461–3469.
23. Lindquist, S., and E. A. Craig. 1988. The heat-shock proteins. *Annu. Rev. Genet.* 22: 631–677.
24. Srivastava, P. K. 2005. Immunotherapy for human cancer using heat shock protein-peptide complexes. *Curr. Oncol. Rep.* 7: 104–108.
25. Lee, K. P., L. E. Raez, and E. R. Podack. 2006. Heat shock protein-based cancer vaccines. *Hematol. Oncol. Clin. North Am.* 20: 637–659.
26. Calderwood, S. K., J. R. Theriault, and J. Gong. 2005. How is the immune response affected by hyperthermia and heat shock proteins? *Int. J. Hyperthermia* 21: 713–716.
27. Schild, H., D. Arnold-Schild, E. Lammert, and H. G. Rammensee. 1999. Stress proteins and immunity mediated by cytotoxic T lymphocytes. *Curr. Opin. Immunol.* 11: 109–113.
28. Tamura, Y., P. Peng, K. Liu, M. Daou, and P. K. Srivastava. 1999. Immunotherapy of tumors with autologous tumor-derived heat shock protein preparations. *Science* 278: 117–120.
29. Georgopoulos, C., and E. A. Welch. 1993. Role of the major heat shock proteins as molecular chaperones. *Annu. Rev. Cell Biol.* 9: 601–634.
30. Parmiani, G., A. Testori, M. Maio, C. Castelli, L. Rivoltini, L. Pilla, F. Belli, V. Mazzaferro, J. Coppa, R. Patuzzo, et al. 2004. Heat shock proteins and their use as anticancer vaccines. *Clin. Cancer Res.* 10: 8142–8146.
31. Kaisho, T., and S. Akira. 2003. Regulation of dendritic cell function through Toll-like receptors. *Curr. Mol. Med.* 3: 373–385.
32. Warger, T., P. Osterloh, G. Rechtsteiner, M. Fassbender, V. Heib, B. Schmid, E. Schmitt, H. Schild, and M. P. Radsak. 2006. Synergistic activation of dendritic cells by combined Toll-like receptor ligation induces superior CTL responses in vivo. *Blood* 108: 544–550.
33. Shitara, K., O. Ichimura, T. Mitsuno, and T. Osawa. 1985. Natural killer (NK) cell activating factor released from murine thymocytes stimulated with an antitumor streptococcal preparation, OK-432. *J. Immunol.* 134: 1039–1047.
34. Kuroki, H., M. Morisaki, K. Matsumoto, H. Onishi, E. Baba, M. Tanaka, and M. Katano. 2003. Streptococcal preparation OK-432: a new maturation factor of monocyte-derived dendritic cells for clinical use. *Cancer Immunol. Immunother.* 52: 561–568.
35. Okamoto, M., T. Oshikawa, T. Tano, S. U. Ahmed, S. Kan, A. Sasai, S. Akashi, K. Miyake, Y. Moriya, Y. Ryoma, et al. 2006. Mechanism of anticancer host response induced by OK-432, a streptococcal preparation, mediated by phagocytosis and Toll-like receptor 4 signaling. *J. Immunother.* 29: 78–86.
36. Itoh, T., Y. Ueda, K. Okugawa, H. Fujiwara, N. Fujii, T. Yamashita, H. Fujiki, S. Harada, T. Yoshimura, and H. Yamagishi. 2003. Streptococcal preparation OK 432 promotes functional maturation of human monocyte-derived dendritic cells. *Cancer Immunol. Immunother.* 52: 207–214.
37. Yamanaka, R., J. Homma, N. Yajima, N. Tsuchiya, M. Sano, T. Kobayashi, S. Yoshida, T. Abe, M. Narita, M. Takahashi, and R. Tanaka. 2005. Clinical evaluation of dendritic cell vaccination for patients with recurrent glioma: results of a clinical phase I/II trial. *Clin. Cancer Res.* 11: 4160–4167.
38. Ono, T., M. Harada, A. Yamada, M. Tanaka, Y. Takao, Y. Tanaka, T. Mine, K. Sakamoto, T. Nakashima, and K. Itoh. 2006. Antitumor effects of systemic and local immunization with a CTL-directed peptide in combination with a local injection of OK-432. *Clin. Cancer Res.* 12: 1325–1332.
39. Nakahara, S., T. Tsunoda, T. Baba, S. Asabe, and H. Tahara. 2003. Dendritic cells stimulated with a bacterial product, OK-432, efficiently induce cytotoxic T lymphocytes specific to tumor rejection peptide. *Cancer Res.* 63: 4112–4118.
40. Fujimoto, T., R. B. Duda, A. Szilvasi, X. Chen, M. Mai, and M. A. O' Donnell. 1997. Streptococcal preparation OK-432 is a potent inducer of IL-12 and a T helper cell I dominant state. *J. Immunol.* 158: 5619–5626.
41. Koido, S., E. Hara, S. Homma, A. Torii, M. Mitsunaga, M. Yanagisawa, Y. Toyama, H. Kawahara, M. Watanabe, S. Yoshida, et al. 2007. Streptococcal preparation OK-432 promotes fusion efficiency and enhances induction of antigen-specific CTL by fusions of dendritic cells and colorectal cancer cells. *J. Immunol.* 178: 613–622.
42. Koido, S., E. Hara, S. Homma, A. Torii, Y. Toyama, H. Kawahara, M. Watanabe, K. Yanaga, K. Fujise, H. Tajiri, et al. 2005. Dendritic cells fused with allogeneic colorectal cancer cell line present multiple colorectal cancer-specific antigens and induce antitumor immunity against autologous tumor cells. *Clin. Cancer Res.* 11: 7891–7900.
43. Berger, T. G., B. Feuerstein, E. Strasser, U. Hirsch, D. Schreiner, G. Schuler, and B. Schuler-Thurner. 2002. Large-scale generation of mature monocyte-derived dendritic cells for clinical application in cell factories. *J. Immunol. Methods* 268: 131–140.
44. Liu, L., A. Chahroudi, G. Silvestri, M. E. Wernett, W. J. Kaiser, J. T. Safrit, A. Komoriya, J. D. Altman, B. Z. Packard, and M. B. Feinberg. 2002. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. *Nat. Med.* 8: 185–189.

45. Jerome, K. R., D. D. Sloan, and M. Aubert. 2003. Measuring T-cell-mediated cytotoxicity using antibody to activated caspase 3. *Nat. Med.* 9: 4–5.
46. Chattopadhyay, P. K., J. Yu, and M. Roederer. 2005. A live-cell assay to detect antigen-specific $CD4^+$ T cells with diverse cytokine profiles. *Nat. Med.* 11: 1113–1118.
47. Frentsch, M., O. Arbach, D. Kirchhoff, B. Moewes, M. Worm, M. Rothe, A. Scheffold, and A. Thiel. 2005. Direct access to $CD4^+$ T cells specific for defined antigens according to CD154 expression. *Nat. Med.* 11: 1118–1124.
48. Barry, M., and R. C. Bleackley. 2002. Cytotoxic T lymphocytes: all roads lead to death. *Nat. Rev. Immunol.* 2: 401–409.
49. Betts, M. R., J. M. Brenchley, D. A. Price, S. C. De Rosa, D. C. Douek, M. Roederer, and R. A. Koup. 2003. Sensitive and viable identification of antigen-specific $CD8^+$ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* 281: 65–78.
50. Cronje, M. J., M. Snyman, L. Bornman, and I. E. Weir. 2003. A rapid and reliable flow cytometric method for determining Hsp70 levels in tobacco protoplasts. *Methods Cell. Sci.* 25: 237–246.
51. Bachelet, M., E. Mariethoz, N. Banzet, E. Souil, F. Pinot, C. Z. Polla, P. Durand, I. Bouchaert, and B. S. Polla. 1998. Flow cytometry is a rapid and reliable method for evaluating heat shock protein 70 expression in human monocytes. *Cell Stress Chaperones* 3: 168–176.
52. Dai, S., T. Wan, B. Wang, X. Zhou, F. Xiu, T. Chen, Y. Wu, and X. Cao. 2005. More efficient induction of HLA-A*0201-restricted and carcinoembryonic antigen (CEA)-specific CTL response by immunization with exosomes prepared from heat-stressed CEA-positive tumor cells. *Clin. Cancer Res.* 11: 7554–7563.
53. Norbury, C. C., S. Basta, K. B. Donohue, D. C. Tscharke, M. F. Princiotta, P. Berglund, J. Gibbs, J. R. Bennink, and J. W. Yewdell. 2004. $CD8^+$ T cell cross-priming via transfer of proteasome substrates. *Science* 304: 1318–1321.
54. Callahan, M. K., E. A. Wohlfert, A. Menoret, and P. K. Srivastava. 2006. Heat shock up-regulates lmp2 and lmp3 and enhances presentation of immunoproteasome-dependent epitopes. *J. Immunol.* 177: 8393–8399.
55. Feng, H., Y. Zeng, M. W. Graner, and E. Katsanis. 2002. Stressed apoptotic tumor cells stimulate dendritic cells and induce specific cytotoxic T cells. *Blood* 100: 4108–4115.
56. Enomoto, Y., A. Bharti, A. A. Khaleque, B. Song, C. Liu, V. Apostolopoulos, P. X. Xing, S. K. Calderwood, and J. Gong. 2006. Enhanced immunogenicity of heat shock protein 70 peptide complexes from dendritic cell-tumor fusion cells. *J. Immunol.* 177: 5946–5955.
57. Munz, C., R. M. Steinman, and S. Fujii. 2005. Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity. *J. Exp. Med.* 18: 203–207.
58. Warger, T., N. Hilf, G. Rechtsteiner, P. Haselmayer, D. M. Carrick, H. Jonuleit, P. von Landenberg, H. G. Rammensee, C. V. Nicchitta, M. P. Radsak, and H. Schild. 2006. Interaction of TLR2 and TLR4 ligands with the N-terminal domain of Gp96 amplifies innate and adaptive immune responses. *J. Biol. Chem.* 281: 22545–22553.
59. Aneja, R., K. Odoms, K. Dunsmore, T. P. Shanley, and H. R. Wong. 2006. Extracellular heat shock protein-70 Induces endotoxin tolerance in THP-1 cells. *J. Immunol.* 177: 7184–7192.
60. Kuo, C. C., S. M. Liang, and C. M. Liang. 2006. CpG-B oligodeoxynucleotide promotes cell survival via up-regulation of HSP 70 to increase Bcl-x_L and to decrease apoptosis-inducing factor translocation. *J. Biol. Chem.* 281: 38200–38207.
61. Flohe, S. B., J. Bruggemann, S. Lendemans, M. Nikulina, G. Meierhoff, S. Flohe, and H. Kolb. 2003. Human heat shock protein 60 induces maturation of dendritic cells versus a Th1-promoting phenotype. *J. Immunol.* 170: 2340–2348.
62. Okamoto, M., S. Furuichi, Y. Nishioka, T. Oshikawa, T. Tano, S. U. Ahmed, K. Takeda, S. Akira, Y. Ryoma, Y. Moriya, et al. 2004. Expression of Toll-like receptor 4 on dendritic cells is significant for anticancer effect of dendritic cell-based immunotherapy in combination with active component of OK-432, a streptococcal preparation. *Cancer Res.* 64: 5461–5470.
63. Koido, S., Y. Tanaka, D. Chen, D. Kufe, and J. Gong. 2002. The kinetics of in vivo priming of CD4 and CD8 T cells by dendritic/tumor fusion cells in MUC1-transgenic mice. *J. Immunol.* 168: 2111–2117.
64. Tanaka, Y., S. Koido, M. Ohana, C. Liu, and J. Gong. 2005. Induction of impaired antitumor immunity by fusion of MHC class II-deficient dendritic cells with tumor cells. *J. Immunol.* 174: 1270–1280.
65. Mittendorf, E. A., C. E. Storrer, C. D. Shriver, S. Ponniah, and G. E. Peoples. 2005. Evaluation of CD107 cytotoxic assay for the detection of cytolytic $CD8^+$ cells recognizing HER2/*neu* vaccine peptides. *Breast Cancer Res. Treat.* 92: 85–93.
66. Toes, R. E., F. Ossendorp, R. Offringa, and C. J. Melief. 1999. CD4 T cells and their role in antitumor immune responses. *J. Exp. Med.* 189: 753–756.
67. Shi, H., T. Cao, J. E. Connolly, L. Monnet, L. Bennett, S. Chapel, C. Bagnis, P. Mannoni, J. Davoust, A. K. Palucka, and J. Banchereau. 2006. Hyperthermia enhances CTL cross-priming. *J. Immunol.* 176: 2134–2141.