Streptococcal Preparation OK-432 Promotes Fusion Efficiency and Enhances Induction of Antigen-Specific CTL by Fusions of Dendritic Cells and Colorectal Cancer Cells[1]

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Dendritic/tumor fusion cell (FC) vaccine is an effective approach for various types of cancer but has not yet been standardized. Antitumor activity can be modulated by different mechanisms such as dendritic cell (DC) maturation state. This study addressed optimal strategies for FC preparations to enhance Ag-specific CTL activity. We have created three types of FC preparations by alternating fusion cell partners: 1) immature DCs fused with autologous colorectal carcinoma cells (Imm-FCs); 2) Imm-FCs followed by stimulation with penicillin-inactivated *Streptococcus pyogenes* (OK-432) (Imm-FCs/OK); and 3) OK-432-stimulated DCs directly fused to autologous colorectal carcinoma cells (OK-FCs). Both OK-FCs and Imm-FCs/OK coexpressed the CEA, MUC1, and significantly higher levels of CD86, CD83, and IL-12 than those obtained with Imm-FCs. Short-term culture of fusion cell preparations promoted the fusion efficiency. Interestingly, OK-FCs were more efficient in stimulating $CD4^+$ and $CD8^+$ T cells capable of high levels of IFN-γ production and cytolysis of autologous tumor or semiallogeneic targets. Moreover, OK-FCs are more effective inducer of CTL activation compared with Imm-FCs/OK on a per fusion cell basis. The pentameric assay confirmed that CEA- and MUC1-specific CTL was induced simultaneously by OK-FCs at high frequency. Furthermore, the cryopreserved OK-FCs retained stimulatory capacity for inducing antitumor immunity. These results suggest that OK-432 promotes fusion efficiency and induction of Ag-specific CTL by fusion cells. We conclude that DCs fused after stimulation by OK-432 may have the potential applicability to the field of antitumor immunotherapy and may provide a platform for adoptive immunotherapy in the clinical setting. *The Journal of Immunology,* **2007, 178: 613–622.**

Dendritic cells (DCs)[3] are specialized APCs and attractive vectors for cancer immunotherapy (1). Both immature and mature DCs are capable of processing and presenting MHC-peptide complexes to T cells. However, mature DCs are significantly better at CTL induction due to higher expression of HLA and costimulatory molecules, while presentation of Ags by immature DCs, in absence of proper costimulation, may lead to tolerance induction. After Ag uptake and inflammatory stimulation, immature DCs in peripheral tissues undergo a maturation process characterized by the up-regulation of costimulatory molecules, chemokine receptors such as CCR7 and secretion of cytokines. During this process, mature DCs migrate to the regional lymph nodes, where they act as effective inducers for primary responses of Ag-specific naive T cells (2, 3). The uses of DCs as a booster of antitumor responses have been considered a promising strategy for cancer vaccine (1). Different strategies have been developed to load DCs with tumor Ags, tumor RNA, tumor lysates, and apoptotic tumor cells (4–7). An alternative approach in the development to the induction of antitumor immunity is through the use of fusion cells (FCs) between DCs and tumor cells (8). In this fusion approach, a broad spectrum of tumor Ags, including both known and unidentified, are endogenously processed and presented by MHC class I and II pathways in the context of the potent immune-stimulatory machinery of the DCs and induce polyclonal CTL responses (8–12). This strategy has been generated and successfully used as vaccines in the murine models and induction of CTL responses in preclinical human studies (9, 10, 12–14). We have reported that fusions of monocyte-derived immature DCs and autologous breast cancer, ovarian cancer, acute myeloid leukemia, or colorectal cancer cells induce antitumor immune responses against autologous tumor in vitro (10, 12–16). In phase I clinical trials in patients with melanoma, glioma, gastric, breast, and renal cancer, vaccination with DCs/tumor FCs was associated with immunologic and clinical responses (17–22). Although the DCs/tumor fusion strategy has enormous potential in mice study, results from early clinical trails have been unsatisfactory (8–11, 17–22). In all DC-based strategies, the results of immune responses in the vaccine-treated patients with cancer may be correlated with the characteristics of the DCs used as a vaccine. There are questions that need to be addressed

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[3] Abbreviations used in this paper: DC, dendritic cell; FC, fusion cell; Imm-DC, immature DC; Imm-FC, immature FC; MFI, mean fluorescence intensity; OK-FC, OK-432-stimulated DCs directly fused to autologous colorectal carcinoma cell; PEG, polyethylene glycol; TAA, tumor-associated Ag.

if this DC/tumor fusion approach is to become effectively used on clinical trials.

Antitumor activity can be modulated by different mechanisms such as the status of DCs differentiation. DCs have several functions in innate and adaptive immunity. In peripheral lymphoid organs immature DCs are incapable of mobilizing CTL responses and have been reported to induce tolerance. In contrast, if a stimulus for DCs activation is coadministered with Ags, mature DCs induce an effective antitumor immunity (23). The classical culture system of monocyte-derived immature DCs is based on a culture of peripheral blood monocyte in medium containing GM-CSF and IL-4. For in vivo vaccination, the potency of tumor cells fused with immature DCs vs mature DCs still has to be established in carefully designed clinical trials. Mature DCs have an enhanced capacity to stimulate naive T cells as a result of up-regulated MHC and costimulatory molecules. Maturation of DCs can be influenced by LPS, PGE_2, CD40L, and the reported mixture of cytokines (consisting of TNF-α, IL-1β, IL-6, and PGE_2) (24, 25). However, clinical use of LPS is limited due to potential toxicity. OK-432, penicillin-inactivated and lyophilized preparation of the low-virulence strain (Su) of *Streptococcus pyogenes* (group A), is one of the biological response modifiers and a good manufacturing practice grade agent. For >20 years, OK-432 has been widely used safely in Japan for patients with cancer (25–28). OK-432 has been shown to activate neutrophils, macrophages, lymphocytes, and NK cells by inducing multiple cytokines such as IL-12 and IFN-γ and polarize the T cell response to a Th1-dominant state (25–30). Recently, it has been demonstrated that OK-432 modulates DCs maturation through TLR4 and β_2 integrin system to enhance Ag-specific CTL responses (30–33). OK-432 promotes more functional maturation of DCs than that obtained with either LPS or a standard mixture of cytokines (TNF-α, IL-1β, IL-6, and PGE_2), as demonstrated by significant increase of IL-12 and IFN-γ, and enhancement of CTL activity (27, 30). In addition, it has been reported that microbial induction of DCs maturation by TLR4 is important for abrogating the activity of regulatory T cells (34). Blockade of such regulatory T cell activity may be critical for the induction of effector T cell function. Therefore, we used OK-432 to stimulate immature DCs and examined which differentiation state of DCs is powerful tool for fusion-based immunotherapy.

Present studies demonstrate three types of FC preparations: 1) immature DCs fused with autologous colorectal carcinoma cells (Imm-FCs); 2) Imm-FCs followed by stimulation with OK-432 (Imm-FCs/OK); and 3) OK-432-stimulated DCs directly fused to autologous colorectal carcinoma cells (OK-FCs). We show that OK-FCs are superior to Imm-FCs/OK or Imm-FCs, as demonstrated by enhanced fusion efficiency, expression of significantly higher levels of CD86 and CD83 molecules on a per FC basis, higher levels of IL-12p70 production in fusion cells, proliferation of both $CD4^+$ and $CD8^+$ T cells capable of IFN-γ production, and significant enhanced cytolysis against autologous tumor. These findings suggest that OK-FCs might be more effective inducer of CTL activation compared with Imm-FCs/OK on a per fusion cell basis. Moreover, we demonstrate that OK-FCs can be cryopreserved and nevertheless retains its stimulatory capacity for inducing Ag-specific CTL in vitro.

Materials and Methods

Cell lines

Colorectal carcinoma cell lines obtained from primary lesion (COLP-2 and COLP-12), hepatic metastasis lesion (COLM-6), and ovarian cancer cell line (OVAP-1) were established in Saitama Cancer Center (16). K562 and T2 cells were from American Type Culture Collection. COLP-2, COLP-12, COLM-6, and OVAP-1 were maintained in TIL media I medium (IBL); K562 and MCF-7 were maintained in DMEM; T2 cells were maintained in IMDM. All media were supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.

Generation of monocyte-derived DCs

Monocyte-derived immature DCs from patients with colorectal carcinoma (Institutional Review Board) were generated essentially as described previously (16). In brief, PBMCs were prepared from whole blood of patients with colorectal carcinoma (obtained with following informed consent and approved by our Institutional Review Board) by Ficoll density-gradient centrifugation. PBMCs were resuspended in tissue culture flask in RPMI 1640 medium containing 1% heat-inactivated autologous serum. After incubation for 60 min at 37°C to allow for adherence, nonadherent cells were removed, and adherent cells were cultured in the presence of 1% heat-inactivated autologous serum/RPMI 1640 medium overnight. The loosely adherent cells were collected on the next day and placed in RPMI 1640 medium containing 1% heat-inactivated autologous serum, 1000 U/ml GM-CSF (PeproTech), and 500 U/ml IL-4 (IL-4; Diaclone Research) for 6 days. On day 6, a part of the immature DCs harvested from the nonadherent and loosely adherent cells were used for fusion. Alternatively, instead of harvesting the DCs at their immature stage for fusion, 0.1 KE/ml (0.1KE equals of 0.01 mg of dried streptococci) OK-432 (Chugai Pharmaceutical) was added to the culture on day 5, and after overnight culture, OK-432-stimulated DCs were used for fusion. The firmly adherent monocytes were harvested after treatment with trypsin and used as an autologous target for the CTL assays.

Preparation of autologous colorectal carcinoma cells

Specimens from resected colorectal carcinoma metastatic lesions in the liver ($n = 4$), lungs ($n = 1$), lymph nodes ($n = 1$), or primary lesion ($n = 2$) were obtained with the approval of our institutional review board. The tumor tissues were digested with collagenase (1 mg/ml). Colorectal carcinoma cells were isolated and maintained in TIL media I medium (IBL) supplemented with 10% heat-inactivated autologous serum, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin in an appropriate flask based on volume and use as fusion cell preparations and targets for CTL assays.

Fusions of DCs and colorectal carcinoma cells

Immature DCs (Imm-DCs) and OK-432-stimulated DCs were harvested and washed once with RPMI 1640 and mixed with autologous colorectal carcinoma cells at a ratio of 10:1 in serum-free prewarmed RPMI 1640 medium. Mixed cell pellets were gently resuspended in prewarmed 50% polyethylene glycol (PEG; m.w. = 1450)/DMSO solution (Sigma-Aldrich; 1 ml per 5×10^6 cells) for 3–5 min at room temperature. Subsequently, serum-free prewarmed medium was added slowly to dilute the PEG. Cell pellets obtained after centrifuge at 1000 rpm were resuspended in prewarmed RPMI 1640 medium supplemented with 10% autologous heat-inactivated serum and 500 U/ml GM-CSF and 250 U/ml IL-4. We prepared three types of fusion cell preparations: 1) Imm-DCs were fused with autologous colorectal carcinoma cells (Imm-FCs); 2) Imm-FCs followed by stimulation with OK-432 (Imm-FCs/OK); and 3) Imm-DCs were stimulated by OK-432 overnight and directly fused to autologous colorectal carcinoma cells (OK-FCs). To generate the fused cells in high yield, Imm-FCs were cultured with 500 U/ml GM-CSF and 250 U/ml IL-4 in a 5% CO_2 atmosphere at 37°C for 3 days. To prepare Imm-FCs/OK, Imm-FCs were cultured for 3 days with 500 U/ml GM-CSF, 250 U/ml IL-4, and 0.1 KE/ml OK-432. OK-FCs were cultured with 500 U/ml GM-CSF, 250 U/ml IL-4, and 0.1 KE/ml OK-432 for 2 days. After an additional 1 day of culture with 500 U/ml GM-CSF and 250 U/ml IL-4, OK-FCs were harvested. DCs/tumor fusion cell preparations were selected and purified by gentle pipetting and firmly attached tumor cells were discarded (12).

ELISA

To assess the production of IL-12p70 in three types of fusion cell preparations, these samples were cultured in 48-well plates (5×10^4 cells/well) in 500 U/ml GM-CSF and 250 U/ml IL-4 with or without 0.1 KE/ml OK-432 for 3 days at 37°C. Supernatants from these samples were collected and tested for IL-12p70 production by ELISA (BD Pharmingen) according to manufacture's instructions. Moreover, to assess the production of IFN-γ in T cells, the cultured fusion cell preparations were washed twice and cocultured with T cells (1×10^5 cells) at a ratio of 1:10 in 48-well plates at 37°C for 3 days in the absence of human IL-2. T cells were purified by nylon wool and cultured for 3 days in the presence of low-dose of human IL-2 (10 U/ml) (Shionogi). Supernatants from these

samples were collected and tested for IFN-γ production by ELISA (BD Pharmingen) according to manufacture's instructions.

Phenotype analysis

Cells were washed and incubated with FITC-conjugated Ab 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), CD83, CCR7 (BD Pharmingen), HLA-A2, or HLA-A24 (One λ) for 1 h on ice. After washing with cold PBS, cells were fixed with 2% paraformaldehyde. For analysis of dual expression, cells were incubated with FITC-conjugated anti-CEA or MUC1, washed with PBS, and then incubated with PE-conjugated anti-HLA-DR, CD80, CD86, CD83, or CCR7 for 1 h at 4°C. Cells were washed, fixed, and analyzed by FACScan (BD Biosciences) with CellQuest analysis software.

T cell purification

$CD4^+$ and $CD8^+$ T cells were isolated from purified whole T cells using MACS CD4 and CD8 Microbeads, respectively, according to the manufacturer's instructions (Miltenyi Biotec) on day 0. The purity of selected T cell populations was in all cases >97% by flow cytometry analysis using FACScan.

CFSE labeling of T cells

Purified whole T cells, $CD4^+$ T cells, and $CD8^+$ T cells were washed and resuspended in serum-free medium at a concentration of 1×10^7 cells/ml in a 0.5 μM solution of CFSE (Molecular Probes). After 30 min incubation at 37°C, FCS was added to concentration of 0.5%, and the CFSE-labeled T cells were washed three times with PBS.

T cell proliferation assay

CFSE-labeled T cells were cultured in complete RPMI 1640 medium with DCs, colorectal carcinoma cells, DCs mixed with colorectal carcinoma cells, or three types of fusion cell preparations at a ratio 10:1 in the absence of human IL-2 for 3 days. To stimulate and proliferate Ag-specific T cells, a low dose of human IL-2 (10 U/ml) was added to the cell culture on day 4. On day 6, after being passed through nylon wool, CFSE-labeled T cells were analyzed by single-color FACScan analysis using Modfit LT cell cycle analysis software (Verity Software House). To assess the proliferation ability, autologous T cells were cultured in 96-well U-bottom culture plates at indicated numbers/well. Dye solution was added to each well and incubated for 4 h according to the Protocol of Cell Titer 96 Nonradioactive Cell Proliferation Assay kit (Promega). For measurement, we used the Microplate Imaging System (Bio-Rad) at an OD of 550 nm.

IFN-γ production during division of $CD4^+$ and $CD8^+$ T cells stimulated by three types of fusion cell preparations

To determine the IFN-γ production during division of T cells, intracellular staining of IFN-γ was performed. Fusion cell preparations were washed twice to remove OK-432 and cocultured with CFSE-labeled T cells at a ratio of 1:10 in the complete RPMI 1640 medium in the absence of human IL-2 for 3 days. To maintain Ag-specific T cells, a low dose of human IL-2 (10 U/ml) was added to the cell culture on day 4. DCs mixed with colorectal carcinoma cells, DCs alone, or colorectal carcinoma cells alone were used as controls. On day 6, CFSE-labeled T cells were harvested by nylon wool separation for analysis of IFN-γ production during T cell division using IFN-γ secretion assay kit according to the manufacturer's instructions (Miltenyi Biotec). Briefly, T cells were washed with cold PBS, incubated with IFN-γ catching reagent for 5 min at 4°C, and 10 ml of prewarmed complete medium was added with shaking and cultured for 45 min at 37°C. After incubation, cells were washed with cold PBS and stained with PE-conjugated IFN-γ detection Ab for 20 min on ice. T cells were washed, fixed, and analyzed by two-color FACScan analysis using CellQuest analysis software (BD Biosciences).

IFN-γ and IL-10 production of $CD4^+$ and $CD8^+$ T cells by three types of fusion cell preparations

Fusion cell preparations were washed twice to remove OK-432 and cocultured with T cells at a ratio of 1:10 in the complete RPMI 1640 medium in the absence of human IL-2 for 3 days. A low dose of human IL-2 (10 U/ml) was added to the cell culture on day 4. DCs mixed with colorectal carcinoma cells, DCs alone, or colorectal carcinoma cells alone were used as controls. On day 6, T cells were harvested by nylon wool separation for analysis of IFN-γ and IL-10 production using each cytokine secretion assay kit according to the manufacturer's instructions (Miltenyi Biotec). IFN-γ or IL-10 stained T cells were washed, stained with FITC-anti-CD4 mAb or FITC-anti-CD8 mAb, fixed, and analyzed by two-color FACScan analysis using CellQuest analysis software (BD Biosciences).

Pentameric assays

Pentameric assays of soluble class I MHC-peptide complexes were used to detect Ag-specific CTL activity induced by three types of fusion cell preparations (16). Complexes of PE-labeled HLA-A2-MUC1 pentamer (950-958, STAPPVHNV), HLA-A2-CEA pentamer (571-579, YLSGANLNL), HLA-A24-CEA pentamer (652-660, TYACFVSNL), or irrelevant pentamer were used (Proimmune). The pentameric staining was performed according to the manufacturer's instructions. Briefly, fusion cell preparations were washed twice to remove OK-432 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 and maintained until day 7. DCs alone, colorectal carcinoma cells alone, and DCs mixed with colorectal carcinoma cells were used as controls. Then the purified T cells were incubated with PE-conjugated pentamer for 10–15 min at room temperature in the dark. After washing with PBS, FITC-anti-CD8 mAb was incubated for 20–30 min at 4°C in the dark. Cells were washed, fixed, and analyzed by FACScan using CellQuest analysis software (BD Biosciences). $CD8^+$ T cell reactivity to MUC1 and/or CEA was shown in the percentage of double-positive population ($CD8^+$pentamer$^+$) in the total $CD8^+$ T cells.

Cytotoxicity assays

Fusion cell preparations were washed twice to remove OK-432 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 and maintained until day 7. DCs alone, colorectal carcinoma cells alone, DCs mixed with colorectal carcinoma cells, and OK-432-stimulated monocytes fused with colorectal carcinoma cells were used as controls. The cytotoxicity assays were performed by Flow-cytometry CTL assays using Active Caspase-3 Apoptosis kit I (BD Pharmingen) (35, 36). T2 cells were pulsed overnight with 10 μg/ml HLA-A2-MUC1 peptide (STAPPVHNV), HLA-A2-CEA peptide (YLSGANLNL), or control HLA-A2-influenza matrix peptide (GILGFVFTL) (Proimmune). The target cells, including autologous colorectal carcinoma cells, allogeneic tumor cell lines, autologous monocytes, T2 cells pulsed with peptides, and NK-sensitive K562 cells, were labeled with the red fluorescence dye PKH-26 (Sigma-Aldrich). After washing with PBS, PKH-26-labeled target cells were cultured with T cells for 2 h at 37°C in 96-well, V-bottom plates. In certain experiments, PKH-26-labeled 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 addition of effector cells. Cells were washed, fixed with Cytofix/Cytoperm Solution (BD Pharmingen) and then washed with Perm/Wash Buffer (BD Pharmingen). Cells were incubated with FITC-anti-human Active Caspase-3 substrate (BD Pharmingen) for 30 min at room temperature, followed by two washes with perm/wash buffer. The percentage of caspase-3-positive cells in PKH-26-labeled target cell population was determined by the following calculation: percentage of caspase-3 staining = ((caspase-3^+PKH-26^+ cells)/(caspase-3^+PKH-26^+ cells + caspase-3^-PKH-26^+ cells)) × 100.

Cryopreservation of OK-FCs

Cryopreservation was performed as described previously (37). OK-FCs were resuspended in cryotubes at a concentration of 1×10^7 per ml in autologous serum supplemented with 10% DMSO (Sigma-Aldrich). Cells suspension were slowly frozen to −80°C. After 3 mo, frozen cells were thawed quickly in 37°C water bath and washed. Thawed cells were incubated for 1–2 h at 37°C before additional experiments in cell culture dish containing prewarmed 10% autologous medium of 500 U/ml GM-CSF and 250 U/ml IL-4.

Statistical analysis

Results are expressed as mean ± SD as indicated in the legends. Student's *t* test was used to examine the significance of the data, and p values <0.05 were considered to be statistically significant.

Results

Characterization of three types of fusion cell preparations generated by immature DCs or OK-432-stimulated DCs with autologous colorectal carcinoma cells

Monocyte-derived autologous immature DCs from patients with colorectal carcinoma were generated in the presence of GM-CSF

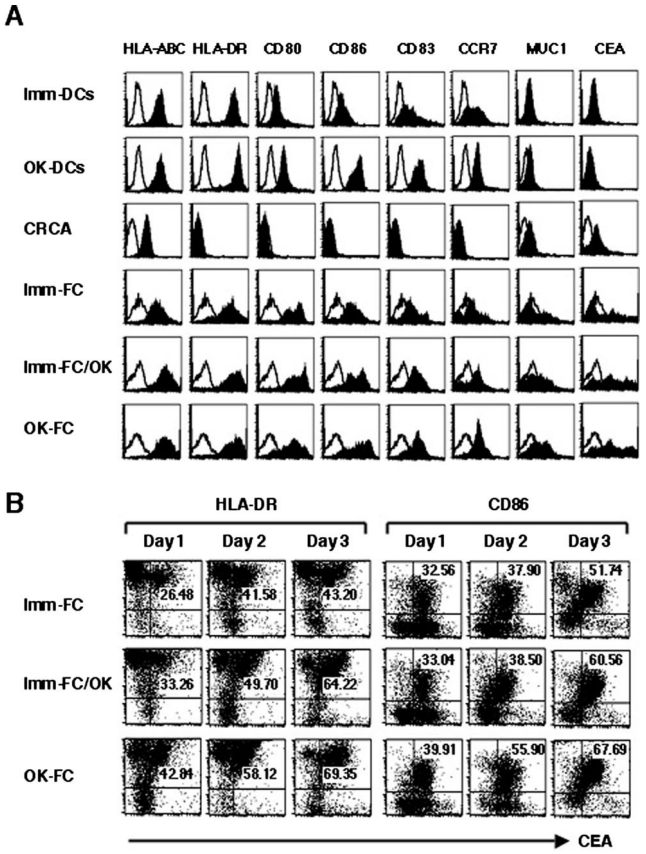

FIGURE 1. Phenotypic analysis of autologous colorectal carcinoma cells fused to autologous DCs. *A*, Autologous Imm-DCs and Imm-DCs stimulated with OK-432 for 3 days (OK-DCs) from patient 3, autologous colorectal carcinoma cells (CRCAs), and three types of fusion cell preparations were analyzed by flow cytometry for expression of the indicated Ags (solid area). *B*, Three types of fusion cell preparations from patient 3 were analyzed by two-color flow cytometry for expression of CEA and HLA-DR and CD86 on days 1, 2, and 3.

and IL-4. The immature DCs displayed a characteristic phenotype with expression of HLA-ABC, HLA-DR, costimulatory molecules (CD80 and CD86), but low levels of the maturation marker, CD83, the chemokine receptor, CCR7, and not CEA (Fig. 1*A*). In this study, DC maturation was effectively induced by exposure to OK-432 for 3 days and was associated with increased expression of HLA-DR, CD80, CD86, CD83, and CCR7. By contrast, all the colorectal carcinoma cells isolated from patients expressed high levels of CEA, MUC1, HLA-ABC, but not HLA-DR, CD80, CD86, and CD83 molecules (Fig. 1*A*). Fusion of DCs to autologous colorectal carcinoma cells coexpressed the CEA and MUC1 Ags, HLA-ABC, HLA-DR, CD80, CD86, and CD83 molecules. The fusion efficiency was determined by dual expression of tumor marker, CEA, and DC marker, HLA-DR. Two-color flow cytometry demonstrated that 36.07 ± 14.78% of Imm-FCs, 42.55 ± 19.30% of Imm-FCs/OK, and 69.59 ± 9.56% of OK-FCs dual-expressed both CEA and HLA-DR on day 3 (Fig. 1*B* and Table I). Both OK-FCs and Imm-FCs/OK, as compared with Imm-FCs, demonstrated an increased expression of CD86 and CD83 (Fig. 1*A*). Fusions of immature DCs and autologous colorectal carcinoma cells resulted in slightly increased expression of CD80, CD86, and CD83, suggesting that DCs activation and maturation was induced by fusion process. Unfused immature DCs or OK-432-stimulated DCs that were exposed to PEG for 3–5 min exhibited similar levels of CD80, CD86, CD83, and CCR7 compared with unexposed DCs (data not shown). Both OK-FCs and Imm-FCs/OK, when compared with Imm-FCs, strongly increased expression of the chemokine receptor, CCR7 (Fig. 1*A*). Mean fluorescence intensity (MFI) of HLA-DR, CD80, CD86, CD83 and CCR7 by the fusion cell preparations was determined by FACS analysis in which fusion cells were isolated by gating cells that coexpressed both HLA-DR and CEA. OK-FCs, as compared with Imm-FCs/OK or Imm-FCs, demonstrated a significant increase of MFI of CD86 and CD83 on a per fusion cell basis (Fig. 2, *A* and *B*). Moreover, OK-FCs significantly dual-expressed both CEA and HLA-DR than those obtained from Imm-FCs/OK or Imm-FCs, therefore, could be optimal for CTL stimulation in vitro (Table I).

Table I. *Percentage of DC tumor fused cells*[a]

Patient List	Imm-FC	Imm-FCs/OK	OK-FC
1	28.12	30.26	52.50
2	34.82	41.06	71.68
3	43.20	64.22	69.35
4	41.68	63.76	66.52
5	18.84	21.10	57.08
6	43.26	63.38	80.24
7	19.64	26.42	ND
8	56.66	60.80	ND
Mean ± SD	36.07 ± 14.78	42.55 ± 19.30	69.59 ± 9.56

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

Expression kinetics of Ags in three types of fusion cell preparations

For clinical use of DCs/tumor fusion cells, it is important to investigate the expression kinetics of CEA, HLA-DR, or CD86 in fusion cell preparations. Three types of fusion cell preparations were cultured for various days and dual expression of these Ags was determined. On day 1 culture, 26.48% of Imm-FCs, 33.26% of Imm-FCs/OK, and 42.84% of OK-FCs dually expressed both CEA and HLA-DR. After cultured for 3 days, the double-positive cells increased to 43.20, 64.22, and 69.36%, respectively (Fig. 1*B*). These fusion cell preparations continued to express CEA, MUC1, CD80, CD86, CD83, and CCR7 for several days after fusion (data not shown). Because the levels of fusion efficiency are closely correlated with antitumor immunity in mice study (our unpublished data), OK-FCs can be harvested on 3 days after fusion process to stimulate autologous T cells in vitro.

IL-12p70 production in three types of fusion cell preparations

To assess the production of IL-12p70 in three types of fusion cell preparations, supernatants from these samples were harvested and analyzed by ELISA. IL-12p70 production in OK-FCs as well as Imm-FCs/OK was observed at higher levels than that in Imm-FCs, suggesting that OK-432 might promote IL-12p70 production in fusion cell preparations (Fig. 2*C*).

T cell proliferation and activation by three types of fusion cell preparations

To compare the stimulating ability, autologous T cells were cocultured with three types of fusion cell preparations. DCs mixed with colorectal carcinoma cells, colorectal carcinoma cells alone, or DCs alone had no effect on proliferation of autologous T cells (Fig. 3*A*). The profile of T cell divisions that we observed when T cells were cocultured with Imm-FCs was drastically weak in presence of low-dose human IL-2 (10 U/ml) compared with Imm-FCs/OK

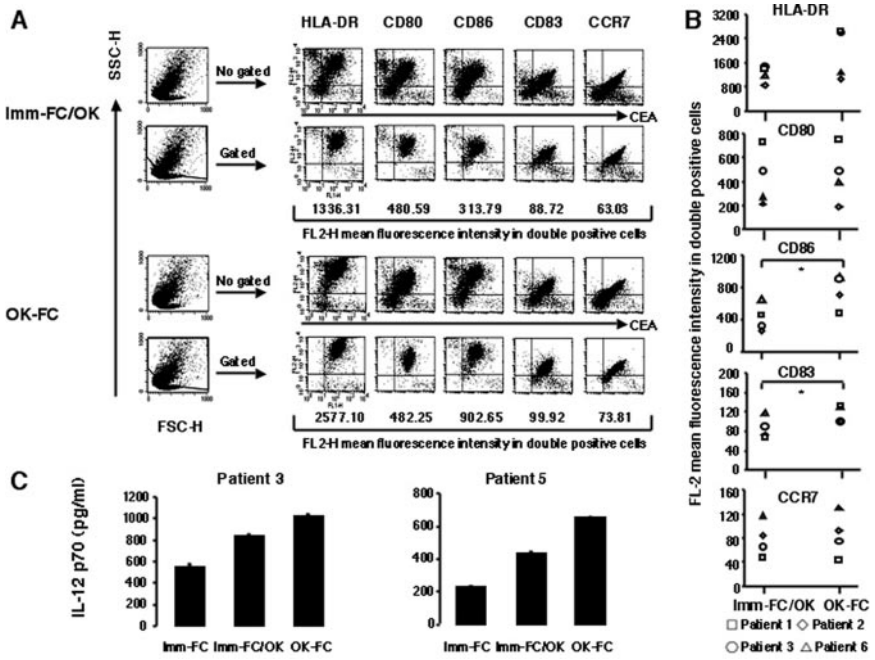

FIGURE 2. Functional analysis of autologous colorectal carcinoma cells fused to autologous DCs. *A* and *B*, Fusion cell preparations were incubated with FITC-conjugated anti-CEA and PE-conjugated anti-HLA-DR, CD80, CD86, CD83, or CCR7. The dot plots show forward and side scatter profiles of fusion cell preparations. MFI of HLA-DR, CD80, CD86, CD83, and CCR7 by the fusion cell preparations was determined by FACS analysis in which fusion cells were isolated by gating cells that coexpressed both HLA-DR and CEA (*upper right*). MFI of HLA-DR, CD80, CD86, CD83, and CCR7 on a per fusion cell basis from four patients (patients 1, 2, 3, and 6) were analyzed. The significant difference is indicated with an asterisk (*). *C*, Fusion cell preparations from two patients (patients 3 and 5) were cultured for 3 days, and supernatants from these samples were harvested and IL-12p70 production was analyzed in a standard ELISA.

or OK-FCs (Fig. 3, *A* and *D*). Furthermore, to directly compare the proliferative responses of T cells stimulated by three types of fusion cell preparations, we made use of CFSE-labeled T cells. Because CFSE is partitioned equally during cell division (38, 39), this technique can monitor T cell division and determine the relationship between T cell division and differentiation. CFSE-labeled

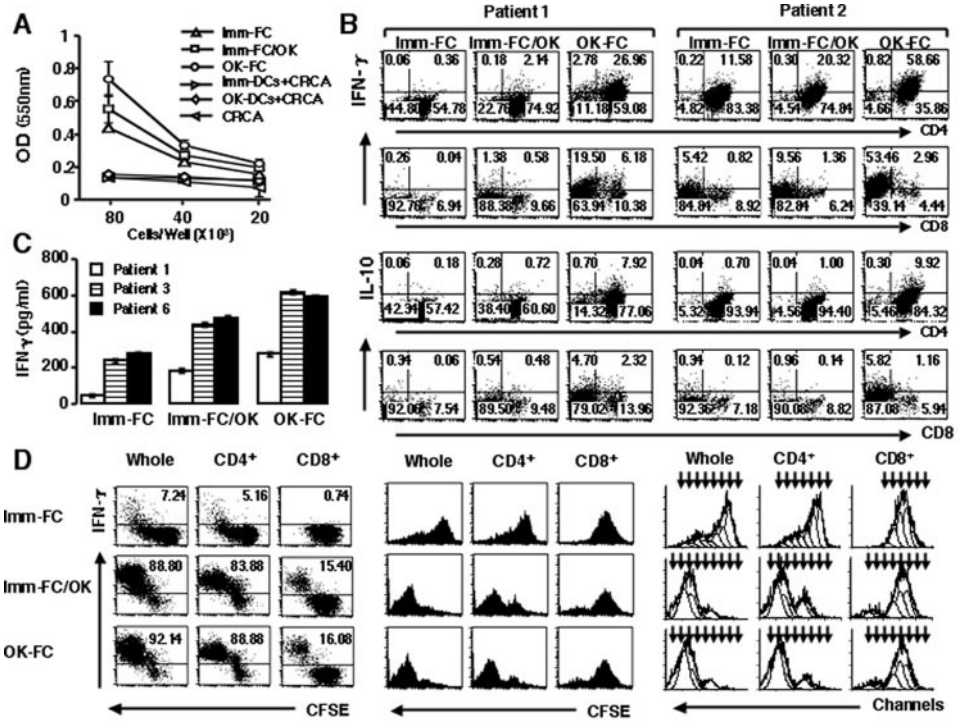

FIGURE 3. Proliferation and activation of $CD4^+$ and $CD8^+$ T cells stimulated by three types of fusion cell preparations. *A*, T cells from patient 5 were cocultured with three types of fusion cell preparations at a ratio of 10:1. As a control, T cells were also stimulated by DCs mixed with autologous colorectal carcinoma cells, autologous colorectal carcinoma cells (CRCAs) alone, or DCs alone. T cell proliferation was performed in indicated numbers of T cells by the Cell Titer 96 Nonradioactive Cell Proliferation Assay kit according to the protocol. Similar results were obtained from T cells stimulated by fusion cell preparations in three independent experiments. *B*, T cells from two patients (patients 1 and 2) incubated with three types of fusion cell preparations were assessed for the cytokine production using human IFN-γ and IL-10 production assay kits according to the manufacturer's protocol. Similar results were obtained in individual experiments using T cells derived from all different patients. *C*, T cells from three patients (patients 1, 3, and 6) were cocultured with three types of fusion cell preparations at a ratio of 10:1. Supernatants were harvested and IFN-γ production was analyzed in a standard ELISA. *D*, CFSE-labeled whole T cells, $CD4^+$, and $CD8^+$ T cells from patient 6 were cocultured with three types of fusion cell preparations at a ratio of 10:1. CFSE-labeled T cells were assessed for the cytokine production using human IFN-γ production assay kits, according to the manufacturer's protocol. Similar results were obtained in individual experiments using T cells derived from three different patients examined.

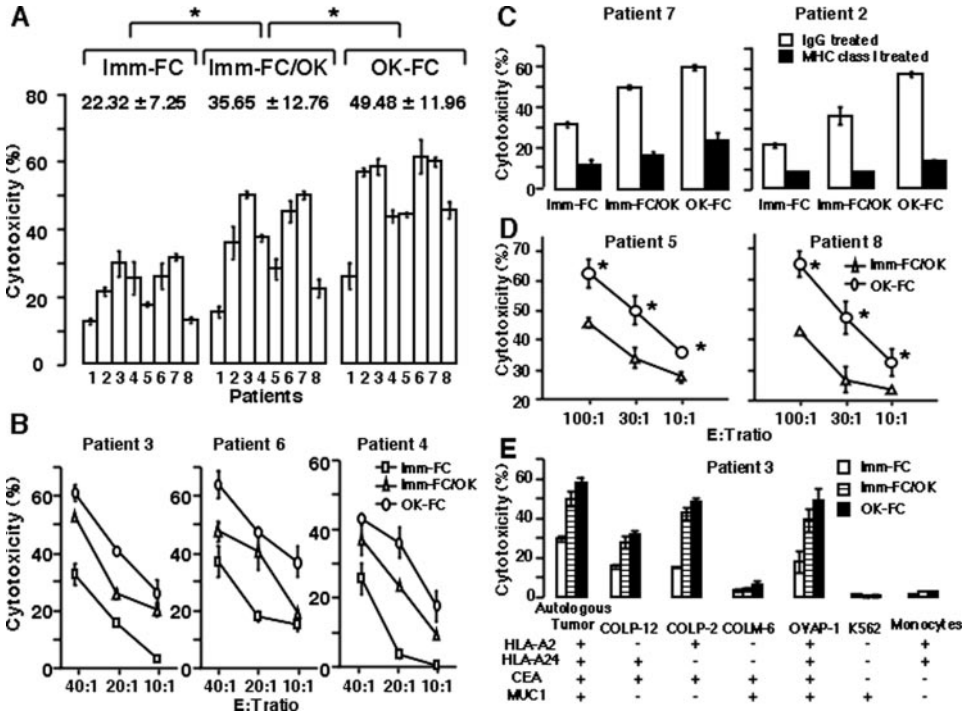

FIGURE 4. Induction of CTL responses by three types of fusion cell preparations against autologous colorectal carcinoma cells. *A*, T cells obtained from eight different patients stimulated by three types of fusion cell preparations were incubated with PKH-26-labeled autologous colorectal carcinoma cells at a ratio of 40:1. *B*, T cells from three patients (patients 3, 4, and 6) were cocultured with three types of fusion cell preparations for 7 days. T cells were incubated at the indicated E:T ratio with PKH-26-labeled autologous colorectal carcinoma cells. *C*, Autologous colorectal carcinoma cells were preincubated with control IgG (□) or anti-MHC class I mAb (W6/32; 1/100 dilution, ■). T cells from two patients (patients 2 and 7) were cocultured with PKH-26-labeled autologous colorectal carcinoma cells at a ratio of 40:1. *D*, The number of fused cells in the fusion cell preparations was described based on the number of cells that coexpressed HLA-DR and CEA. Autologous T cells from two patients (patients 5 and 8) stimulated by the same numbers of fused cells in both OK-FCs and Imm-FCs-OK were incubated with PKH-26-labeled autologous colorectal carcinoma cells at the indicated E:T ratio. *E*, T cells from patient 3 (HLA-$A2^+$/$A24^+$) stimulated by three type of fusion cell preparations were incubated with PKH-26-labeled autologous colorectal carcinoma cells, autologous monocytes, allogeneic colorectal carcinoma cell lines (COLM-6, COLP-2, or COLP-12), ovarian cancer cell line (OVAP-1), or K562 at a ratio of 40:1. Percentage cytotoxicity (mean ± SD of three replicates) was determined by flow cytometry CTL assays. The significant difference is indicated with an asterisk (∗).

whole T cells, purified $CD4^+$ T cells, and $CD8^+$ T cells were cocultured with three types of fusion cell preparations in the absence of human IL-2 for 3 days. On day 4, T cells were cultured in the presence of low-dose of human IL-2 (10 U/ml) to maintain CTL. As shown in Fig. 3*D*, we detected strong T cell proliferation by OK-FCs as well as Imm-FCs/OK. This extensive T cell proliferation was demonstrated by the few undivided T cells left and from proper accumulation of activated T cells, as shown by the increase in T cell counts correlating with the decrease in CFSE label for each division. Moreover, purified $CD4^+$ T cells and $CD8^+$ T cells were strongly stimulated by OK-FCs as well as Imm-FCs/OK even in the presence of low-dose human IL-2 (Fig. 3*D*). Both OK-FCs and Imm-FCs/OK can initiate $CD4^+$ and $CD8^+$ T cells simultaneously and induce strong T cell proliferation.

IFN-γ and IL-10 production of $CD4^+$ and $CD8^+$ T cells by three types of fusion cell preparations

To compare the T cell activation by three types of fusion cell preparations, we performed IFN-γ production by intracellular staining and ELISA. Not only did OK-FCs or Imm-FCs/OK more vigorously induce $CD4^+$ and $CD8^+$ T cell proliferation, but also they were superior in producing IFN-γ in both $CD4^+$ and $CD8^+$ T cells from their 2–3 rounds of division (Fig. 3, *B–D*). In contrast, there was little, if any, IFN-γ production from T cells cocultured with mixtures of DCs with colorectal carcinoma cells, DCs alone, or colorectal carcinoma cells alone (data not shown). The low levels of IL-10 production in both $CD4^+$ and $CD8^+$ T cells stimulated by OK-FCs or Imm-FCs/OK did not impair the production of IFN-γ (Fig. 3*B*).

Induction of CEA- and MUC1-specific CTL responses by three types of fusion cell preparations

To investigate the Ag-specific CTL induction capacity by three types of fusion cell preparations, cytotoxicity assays were performed. Flow cytometry CTL assays were predicated on measurement of CTL-induced caspase-3 activation in target cells through detection of the specific cleavage of fluorogenic caspase-3 (35, 36). In all cases examined, OK-FCs were able to induce markedly potent CTL responses against autologous colorectal carcinoma, compared with Imm-FCs or Imm-FCs/OK in this experimental setup (Fig. 4, *A* and *B*). In addition, preincubation of the target cells with an anti-HLA-ABC mAb inhibited the lysis, indicating restriction by MHC class I (Fig. 4*C*). To determine whether OK-FCs is more effective inducer of CTL activation on a per fusion cell basis, autologous T cells were stimulated with same number of fused cells in both OK-FCs and Imm-FCs-OK. The number of fused cells in the fusion cell preparations was described based on the number of cells that coexpressed HLA-DR and CEA in the fusion cell preparations. OK-FCs from two patients (patients 5 and 8) were more efficient in CTL activation against autologous colorectal carcinoma, compared with Imm-FCs/OK in this setting (Fig.

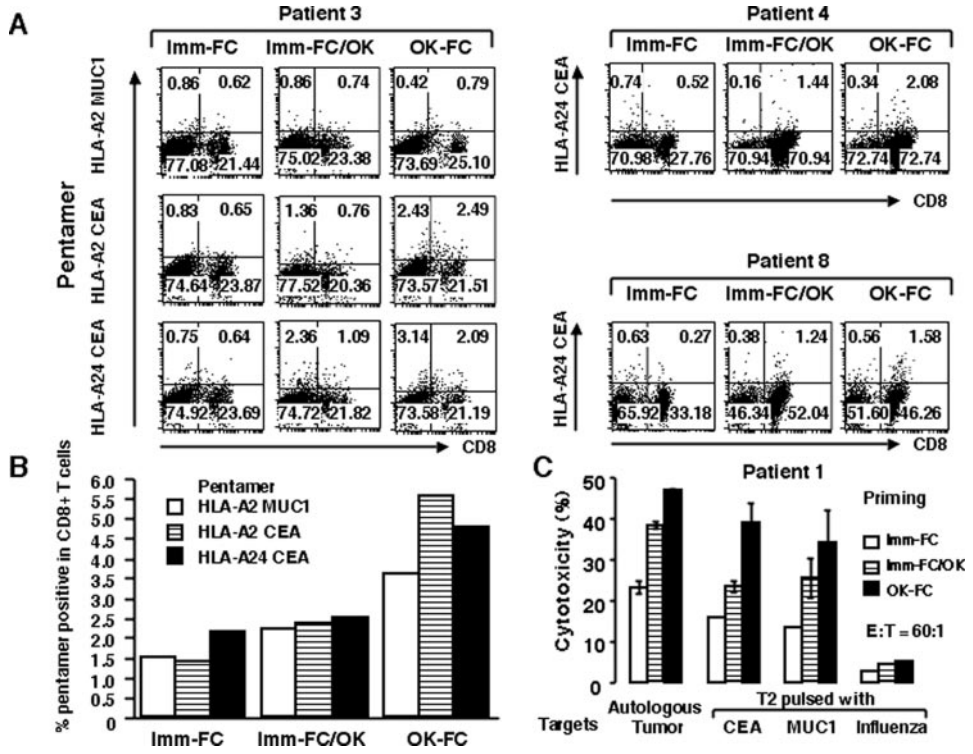

FIGURE 5. Induction of CEA- and MUC1-specific CTL restricted in HLA-A2 and -A24 by three types of fusion cell preparations. *A*, T cells from three patients (patients 3, 4, and 8) stimulated by three types of fusion cell preparations were examined by HLA-A2/MUC1, HLA-A2/CEA, and/or HLA-A24/CEA pentameric assay. *B*, $CD8^+$ T cells obtained from seven different patients (HLA-$A2^+$ and/or $A24^+$) stimulated by three types of fusion cell preparations were analyzed by staining HLA-A2/MUC1, HLA-A2/CEA, and/or HLA-A24/CEA pentameric assay. $CD8^+$ T cell reactivity to MUC1 and/or CEA was shown in the percentage of double-positive population ($CD8^+$pentamer$^+$) in the total $CD8^+$ T cells. *C*, T cells from patient 1 (HLA-$A2^+$/$A24^+$) stimulated by three types of fusion cell preparations were incubated with PKH-26-labeled T2 cells pulsed with CEA peptides, MUC1 peptides, or influenza matrix peptide, at a ratio of 60:1. Percentage cytotoxicity (mean ± SD of three replicates) was determined by flow cytometry CTL assays.

4*D*). In addition, CTL induced by OK-FCs (HLA-$A2^+$/$A24^+$) lysed not only autologous colorectal carcinoma but also colorectal carcinoma cell lines COLP-2 (HLA-$A2^+$/$A24^-$) and COLP-12 (HLA-$A2^-$/$A24^+$), endogenously expressing both CEA and MUC1 in an Ag-specific and HLA-A2 and/or HLA-A24 restriction fashion (Fig. 4*E*), suggesting an additive effect of CTL. Moreover, there was no lysis against COLM-6 (HLA-$A2^-$/$A24^-$, CEA^+, and $MUC1^+$), confirming that HLA-A2 and HLA-A24 were restriction element of CTL (Fig. 4*E*). By contrast, there was minimal lysis of autologous colorectal carcinoma cells by T cells stimulated by

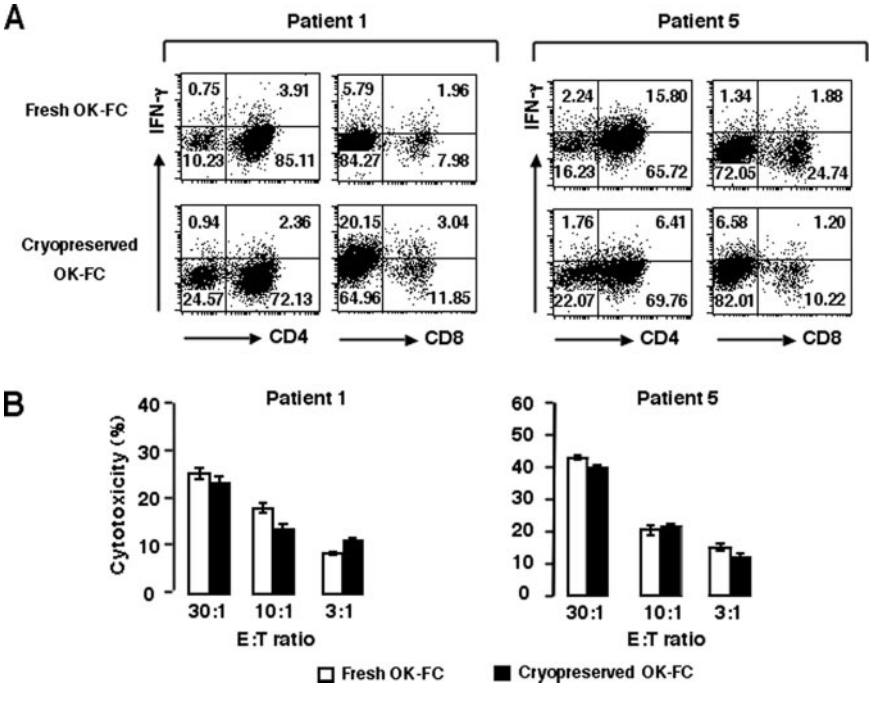

FIGURE 6. Evaluation of the cryopreserved OK-FCs. *A*, OK-FC from two patients (patients 1 and 5) were frozen at −80°C. After 3 mo, frozen fusion cell preparations were thawed at 37°C and washed once. Thawed cells were incubated for 1–2 h at a 37°C incubator to recover. These cells or freshly prepared OK-FC were incubated with autologous T cells at a ratio of 1:10. T cells were assessed for the IFN-γ production using human IFN-γ production assay kits according to the manufacturer's protocol. *B*, T cells stimulated by the cryopreserved or freshly prepared OK-FC were incubated with PKH-26 labeled autologous colorectal carcinoma cells at a ratio of 60:1. Percentage cytotoxicity (mean ± SD of three replicates) was determined by flow cytometry CTL assays.

DCs mixed with autologous colorectal carcinoma cells, fusions cells generated by OK-432-stimulated monocytes and autologous colorectal carcinoma cells, DCs alone, or autologous colorectal carcinoma cells alone (data not shown). Pentametric assay also demonstrated the characteristics of CTL induced with three types of fusion cell preparations. After stimulation with OK-FCs, CEA-specific activity restricted by HLA-A2 and HLA-A24 was observed in 5.55 $\pm$ 3.59 and 4.77 $\pm$ 3.03% of $CD8^+$ T cells, respectively, that was much higher than those obtained with Imm-FCs (Fig. 5, *A* and *B*). Moreover, CEA- and MUC1-specific CTL restricted by HLA-A2 were confirmed by CTL assays using T2 target cells. T2 cells pulsed with CEA or MUC1 peptides were more recognized by T cells stimulated with OK-FCs (Fig. 5*C*).

Stimulatory capacity of the cryopreserved OK-FCs

To assess the stimulatory capacity of the cryopreserved OK-FCs, we examined whether there was a difference in T cell activation between the freshly prepared and the cryopreserved OK-FCs. The activation of T cells induced by the cryopreserved OK-FCs from two patients (patients 1 and 5) resulted in the production of IFN-γ at a similar level (Fig. 6*A*). Moreover, there was no significant reduction in the lytic activity against autologous colorectal carcinoma cells when T cells were stimulated with the cryopreserved OK-FCss in vitro (Fig. 6*B*). Taken together, OK-FCss can be cryopreserved and used as a fusion-based vaccine to induce CTL responses against autologous colorectal carcinoma.

Discussion

DCs have several functions in innate and adaptive immunity. In peripheral lymphoid organs immature DCs are incapable of mobilizing CTL responses and have been reported to induce tolerance. In contrast, if a stimulus for DCs activation is coadministered with Ags, mature DCs induce an effective antitumor immunity (23). DCs/tumor fusion-approach is an effective approach, because multiple tumor-associated Ags (TAAs), including those known and unidentified, are processed endogenously and presented by MHC class I and II pathways with ability to migrate to draining lymph nodes (8, 11, 12). The impact of activated DCs/tumor fusion cells on CTL responses has so far not been systematically examined. In this study, to investigate which differentiation state of DCs is most amenable for fusion vaccine, we used OK-432 to induce DCs stimulation and conducted a series of experiments using three types of fusion cell preparations: 1) immature DCs fused with autologous colorectal carcinoma cells (Imm-FCs); 2) Imm-FCs followed by stimulation with OK-432 (Imm-FCs/OK); and 3) OK-432-stimulated DCs directly fused to autologous colorectal carcinoma cells (OK-FCss). We demonstrate in this study that OK-FCss are optimal status for a clinical use to induce enhanced antitumor immunity against autologous colorectal carcinoma cells in vitro, as demonstrated by: 1) enhanced fusion efficiency; 2) increased expression of CD86 and CD83 molecules and IL-12p70 production in fusion cell preparations; 3) effective expansion of $CD4^+$ and $CD8^+$ IFN-γ producing T cells; and 4) significantly enhanced Ag-specific CTL activity against autologous colorectal carcinoma cells.

Interestingly, although fusions of autologous colorectal carcinoma cells and immature DCs resulted in the presentation of TAAs and up-regulation of costimulatory molecules such as CD80 and CD86 molecules, OK-FCss exhibited significantly higher levels of MFI of costimulatory (CD86) and maturation molecules (CD83) compared with Imm-FCs or Imm-FCs/OK on a per fusion cell basis. This observation might indicate that the fusion process does affect the maturation capacity and OK-432 has favorable characteristics to induce activation of fusion cells. Short-term culture of fusions resulted in increased expression of TAAs (CEA and MUC1), CD86, and HLA-DR. As this fusion approach not only delivers the TAAs-epitopes but also the genes encoding the TAAs, DCs/tumor fusion cells can continue to produce TAAs for several days after fusion (our unpublished data). Short-term culture may permit the fusion cells sufficient time to display the TAAs in the context of MHC molecules. The fusion efficiency was determined by dual expression of tumor marker, CEA, and DC marker, HLA-DR, on day 3. Interestingly, the fusion efficiency is much higher in OK-FCss compared with Imm-FCs/OK. The fusion approach not only delivers the TAAs but also the mRNA encoding the TAAs. In mRNA electroporation into immature or mature DCs, the differences at the level of translation efficiency of mRNA to protein (47, 48) might be in part associated with the difference in fusion efficiency. Moreover, OK-FC also showed up-regulation of HLA-ABC and DR. Ag presentation machinery is different in immature DCs and mature DCs (42, 43). It has been described that MHC class I-peptide complexes and MHC class I molecules have a longer half-life in mature DCs as compared with immature DCs. Therefore, it might be possible that fusions of colorectal carcinoma cells and DCs at matured state that express more stable MHC class I-peptide complexes resulted in efficient Ag-presentation in the context of costimulatory molecules.

In this experimental setting, autologous T cells were stimulated by three types of fusion cell preparations in the absence of human IL-2 for 3 days and maintained in the presence of low-dose human IL-2 (10 U/ml) from day 4 to expand Ag-specific CTL. Imm-FCs, as compared with OK-FCss or Imm-FCs/OK, were insufficient to induce T cell proliferation, IFN-γ production, and CTL responses in this experimental setup. These results indicate that exogenous IL-2 is essentially required for CTL expansion when T cells are stimulated with Imm-FCs in vitro. OK-FCss as well as Imm-FC/OKs, as compared with Imm-FCs, could efficiently induce the activation and proliferation of CTL. This is consistent with the previous observations that CTL can be maintained in vitro in an IL-2-dependent proliferative state and enhanced by efficient presentation of TAAs (44). Moreover, it has been reported that the role of exogenous IL-2 in potentiating the action of IL-12 derived from OK-432 to promote IFN-γ synthesis in T cells is best appreciated in the context of Ags exposure history (26). The issue of a changing ability to produce IL-12 in DCs at different stage of activation by OK-432 has been addressed previously (25–28, 30). IL-12 has been reported to play an important role in the development of Th1-type T cells and functions as a third signal in reversing tolerance and expanding TAA-specific CTL through IFN-γ production (43, 44). Our previous work has also demonstrated that DCs/plasmacytoma fusions-vaccine alone was insufficient, whereas coadministration of IL-12 resulted in eradication of the disease (47). As activation and expansion of TAAs-specific CTL is in part dependent on IL-12 (45, 46), high levels of IL-12 production in OK-FCss as well as Imm-FCs/OK appear to participate in the augmented IFN-γ production during CTL generation in vitro. High levels of IFN-γ production in both $CD4^+$ and $CD8^+$ T cells stimulated by OK-FCss as well as Imm-FCs/OK might be also due to the fact that these fusion cell preparations express higher levels of costimulatory molecules, leading to an enhanced presentation of TAA-derived epitopes in the context of MHC class I and II and an increased T cell stimulation. In addition, a low level of IL-10 production was detected in both $CD4^+$ and $CD8^+$ T cells stimulated by OK-FCss as well as Imm-FCs/OK. It has been reported that low levels of IL-10 modulates the activated $CD8^+$ T cell proliferation and supports memory-type $CD8^+$ T cells in an Ag-specific manner (48). OK-FCss, as compared with Imm-FCs or Imm-FCs/OK, can

polarize the T cell response to a Th1-dominant state and induce enhanced Ag-specific CTL activity.

It could be argued that higher efficacy of OK-FCss in CTL stimulation may be simply because of the presence of higher number of fused cells in OK-FCss compared with that in Imm-FCs/OK. To determine whether OK-FCss are more effective inducer of CTL activation on a per fusion cell basis, autologous T cells were stimulated with same number of fused cells that coexpressed both HLA-DR and CEA in fusion cell preparations. CTL activity induced by OK-FCss showed significantly higher against autologous colorectal carcinoma cells than that by Imm-FCs/OK in this setting. Moreover, OK-FCss exhibited significantly higher levels of MFI of costimulatory (CD86) and maturation molecules (CD83) compared with Imm-FCs/OK on a per fusion cell basis, leading to an increased T cell stimulatory capacity. These observations suggest that OK-FCss may be more efficient in CTL stimulation and activation against autologous colorectal carcinoma, compared with Imm-FCs/OK in this experimental setup. Higher fusion efficiency of OK-FCss may also participate in augmented CTL activation in vitro. These effects of OK-432 are still not clear and should be further investigated. A recent study that the electroporation of mRNA into mature DCs is more efficient in Ag presentation and CTL induction than that into immature DCs, followed by maturation (40, 41, 49), supports our finding that OK-FCss are potent inducers of Ag-specific CTL responses in vitro.

We have reported that DCs/tumor fusion cells migrate to draining lymph nodes, reside in the T cell area, and function as APCs (11). In this study, OK-FCss, when compared with Imm-FCs, strongly increased expression of the chemokine receptor, CCR7. CCR7 plays a fundamental role in directing matured DCs to the lymph nodes and inducing anti-apoptotic signaling in matured DCs (50). Increased expression of CCR7 in OK-FCss is consistent with their capacity to migrate to draining lymph nodes. These might be associated with activation of $CD4^+$ T cells and induction of Ag-specific CTL.

If the cryopreserved fusion cells retain activities as same as the fresh preparations, it is possible to administrate them immediately to the patients in the case of relapse. However, little is known about the impact on fusion cells of freezing and thawing. In this study, we showed that OK-FCss could be efficiently frozen without loss of Ag presentation and T cell stimulatory capacity inducing CTL responses against autologous colorectal carcinoma cells. The cryopreserved OK-FCss have the potential applicability to the field of antitumor immunotherapy and provide a platform for adoptive immunotherapy in the clinical setting.

In conclusion, although DCs activation and maturation was induced by fusion process, its lower capacity to produce IL-12p70 in Imm-FCs would contribute to the lower efficiency in eliciting antitumor immune responses in this experimental setup. Our findings that DCs fused after maturation by OK-432 can express higher levels of CD86 and CD83 molecules and significantly enhance CTL responses in vitro may help in designing optimal strategies for the therapeutic induction of Th1-type responses and may improve DCs/tumor fusion-based vaccination strategies.

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Disclosures

The authors have no financial conflict of interest.

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