Preparation of fully activated dendritic cells capable of priming tumor-specific cytotoxic T lymphocytes in patients with metastatic cancer using penicillin-killed *streptococcus pyogenes* (OK432) and anti-CD40 antibody

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Abstract. In order to achieve sufficient therapeutic potency, it has been proposed that vaccine therapy with dendritic cells needs to be combined with manipulation of immunological checkpoints, such as inhibition of regulatory T cells and blockade of negative signals, and enhancement of T cell trafficking to tumor sites. In the combinatorial cancer immunotherapy, use of matured/activated dendritic cells (DCs) with more potent antigen presenting capacity seems to be essential for eliciting anti-tumor immune responses. We herein established an *ex vivo* induction strategy for activated DCs capable of eliciting efficient tumor antigen-specific cytotoxic T lymphocytes (CTLs) from patients with metastatic cancer as well as healthy donors. Immature DCs were matured by 48-h culture in the presence of anti-CD40 antibody and penicillin-killed *streptococcus pyogenes* (OK432). Supplementation with both anti-CD40 and OK432 resulted in induction of activated DCs with higher surface expression of CD80, CD83, CD86 and major histocompatibility complex class II antigens, compared with other mature DCs that were induced by the combination of anti-CD40 with tumor necrosis factor-α or lipopolysaccharide. In analysis of the produced cytokine profiles, the activated DCs produced the highest T-helper 1-type cytokines for at least 72 h. Furthermore, the activated DCs, pulsed with tumor-associated antigen peptide, elicited *in vitro* tumor-specific CTLs, but DCs activated with other combinations did not in cancer patients. Therefore, we suggest that the activated DCs studied here might be used as a basic element for the combinatorial cancer immunotherapy.

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Introduction

DCs are professional antigen-presenting cells (APC) capable of priming T cells by presenting antigens and providing activation signals using highly expressed major histocompatibility complex (MHC) molecules, co-stimulating molecules (CD80 and CD86), and IL-12 (1-5). Because of their high efficacy in activating both CTLs and helper T cells, DCs have been used for cancer immunotherapy in which DCs activated CTLs *ex vivo* for adoptive cell transfer or were injected as a vaccine directly into cancer patients (6-17). However, the outcome of the previously reported cancer immunotherapy with DCs was unsatisfactory, except for DC-based vaccines against melanoma (6,16).

In patients with advanced and metastatic cancer, immunity is generally suppressed by factors produced by tumors and tumor-infiltrating cells, i.e. vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-ß and IL-10 (18-22), as the stage of disease advances. Further, regulatory T cells have been detected around tumors (23,24). In order to overcome the immunosuppressive circumstances around tumors, combinatorial cancer immunotherapy in which vaccine therapy with DCs is combined with manipulation of immunological checkpoints, such as inhibition of regulatory T cells and blockade of negative signals, and enhancement of T cell trafficking to tumor sites, was proposed (25-28). We assumed that induction of sufficiently activated DCs with more potent antigen presentation in cancer patients would be important for successful combinatorial cancer immunotherapy.

Many strategies have been proposed to prepare mature DCs. For example, immature DCs that were differentiated from plastic plate-adherent peripheral blood mononuclear cells (PBMCs) by conventional culture in the presence of GM-CSF and IL-4 for 6 or 7 days (4,15) were subsequently cultured in the presence of either lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α, CpG, or CD40 ligands to allow them to differentiate into mature DCs producing Th1-type cytokines (29-33). In addition, OK432 has been reported to be the most potent inducer to obtain mature DCs capable of eliciting CTLs *in vitro* in healthy donors (29,33). Accordingly, in cancer patients with impaired immunity, OK432 would seem to be a promising potent stimulator for inducing activated DCs.

We have also previously established a strategy for preparing mature/activated DCs in a mouse model (34). Immature DCs that were prepared by the conventional method from mouse bone marrow cells using GM-CSF were further cultured in the presence of LPS and antibodies against CD40 and IL-10. The expression of surface molecules (CD86 and MHC class II antigens) and production of cytokines involved in T-cell activation by the induced DCs were significantly increased and maintained for at least 72 h. In contrast, DCs matured using only LPS, CpG or anti-CD40 antibody produced less IL-12. Based on findings in which we successfully obtained mature DCs in a mouse model, we attempted in this study to prepare activated DCs with more potent APC activity than usual from peripheral blood monocytes of patients with metastatic cancer and examine their ability to elicit tumor antigen-specific CTLs.

Materials and methods

Reagents. Human recombinant TNF-α was purchased from Biosource International (Camarillo, CA), and LPS was from Sigma (St. Louis, MO). Human recombinant GM-CSF and OK432 were kindly provided by Kirin Brewery Co. (Osaka, Japan) and Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan), respectively, and human recombinant IL-4 was purchased from R&D Systems Inc. (Minneapolis, MN). Ficoll-Conray Lymphosepal was obtained from Immuno-Biological Laboratories (Gunma, Japan), and $Na_2{}^{51}CrO_4$ was from Perkin Elmer Life Science (Boston, MA).

Antibodies and cells. Anti-HLA-DR (L243) monoclonal antibody was purchased from Serotech Corp. (Cambridge, MA), monoclonal antibodies against CD80 (BB-1), CD83 (HB15e) and CD86 (BU63) were from PharMingen (San Diego, CA) and monoclonal antibody against CD40 (82111) was from R&D Systems Inc. Fluorescence isothiocyanate (FITC)-conjugated anti-mouse IgG antibody was purchased from ICN Pharmaceutical Inc. (Aurora, OH). Human TAP-deficient cell lines, T2, were purchased from American Type Culture Collection (Rockville, MD) and leukemic cell lines, TISI, were kindly provided by Dr I. Kawashima (Takara Shuzo Co. Ltd., Otsu, Japan). Cells were maintained in RPMI-1640 medium (Nakalai Tesque, Kyoto, Japan) containing 10% heat inactivated fetal calf serum (FCS), 2 M L-glutamine, 100 U/ ml penicillin G, 0.1 mg/ml streptomycin, and 0.2 mg/ml amphotericin B (all from Gibco BRL, Tokyo, Japan) at 37˚C in a humidified atmosphere at 5% CO_2.

Synthetic peptides. Mac-2 binding protein (M2BP)-derived CTL epitopes with HLA-A2 binding motifs, $M2BP_{216-225}$ and $M2BP_{241-250}$, were used to induce CTLs by *in vitro* stimulation (35). MAGE3-A2 (FLWGPRALV) and MAGE3-A24 (IMPKAGLLI) peptides were used as control peptides (36,37). The oligopeptides were synthesized commercially by Qiagen (Tokyo, Japan).

Cancer patients and healthy donors. DCs and CTLs were prepared from PBMCs of 3 healthy donors and 3 patients with metastatic cancer. Of healthy donors, two were HLA-A24-positive and the other was HLA-A2-positive. The profiles of the cancer patients were as follows: Patient #1, a 49-year-old female with metastatic breast cancer of the lung and HLA-A2; patient #2, a 64-year-old male with lung cancer, malignant pleural effusion and HLA-A2; patient #3, a 69-year-old female with lung cancer, malignant pleural effusion, metastatic brain tumors and HLA-A24. All of the patients had a grade 1 or 2 performance status score. Approval for the study was obtained from the ethics committee of our university, and informed consent for participation in this study was obtained from all patients and healthy volunteers.

Preparation of DCs. PBMCs ($1x10^7$) that were obtained from peripheral blood by density gradient centrifugation as reported previously (4,15) were suspended in 5 ml of AIM-V medium (Gibco BRL) supplemented with 5% human AB serum (Sigma-Aldrich, Tokyo, Japan) and left in a 6-well culture plate for 2 h. After removal of the non-adherent cells, the adherent cells were cultured in 4 ml of AIM-V supplemented with 5% human AB serum, 1,000 units/ml GM-CSF and 0.2 ng/ml IL-4 at 37˚C. Half of the medium was exchanged for fresh conditioned medium on day 3 and the non-adherent cells were harvested at 6 to 7 days after culture. The cells were resuspended in 3 ml of the medium supplemented with or without 0.1 KE/ml OK432, 20 ng/ml LPS, 100 ng/ml TNF-α or 5 μg/ml anti-CD40 antibody and cultured for 2 or 3 days. The harvested non-adherent cells were morphologically compatible with dendritic cells under a microscope, and approximately 80% of the cells were shown to express CD1a molecules on the surface by flow cytometry in each separation (data not shown). Therefore, the prepared cells were used as DCs for CTL induction in this study.

Flow cytometric analysis of DCs. Mature DCs prepared as described above were examined for the expression of the surface markers, CD80, CD83, CD86 and MHC class II antigens. Cells were incubated with 10 μg/ml primary antibodies on ice for 30 min, washed 3 times in cold PBS and then incubated with FITC-conjugated anti-mouse IgG antibody at 1/1,000 dilution on ice for 30 min. After 3 washes in PBS, surface molecules of the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Data were presented as histograms using CellQuest software (Becton Dickinson).

Cytokine profiles produced by mature DCs. During culture of immature DCs in the presence of either OK432, anti-CD40 antibody, TNF-α or LPS, the culture supernatant was collected from each well every 24 h for 72 h and examined for the

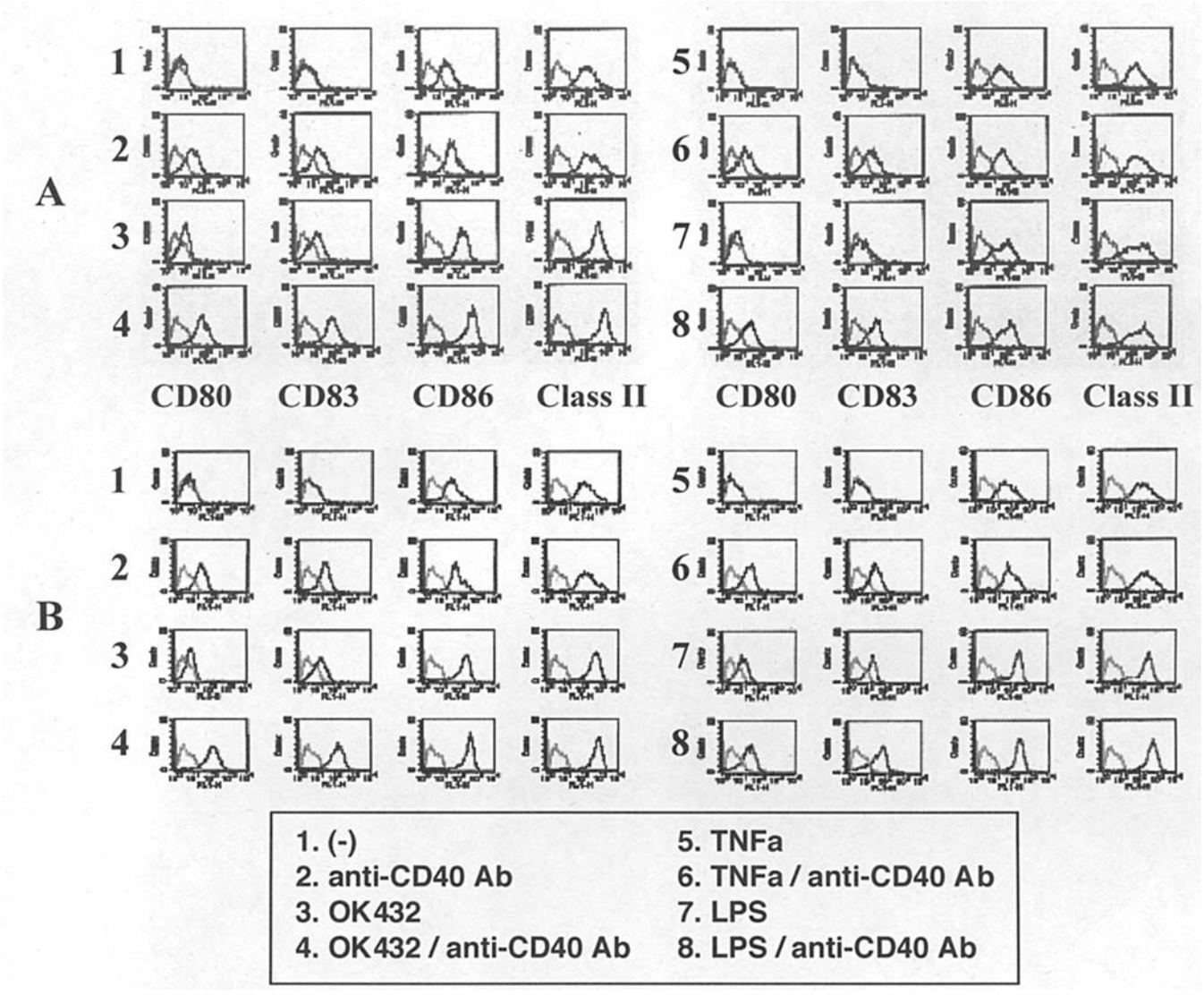

Figure 1. Flow cytometric analysis of surface molecule expression in mature DCs. Peripheral blood monocytes from cancer patients with lung cancer (A) and healthy donors (B) were cultured in the presence of GM-CSF and IL-4 for 7 days. Subsequently, the induced immature DCs were further cultured in the presence of either OK432, TNF-α, LPS or anti-CD40 antibody for 48 h. Expression of CD80, CD83 or CD86 or MHC class II antigens on the surface was examined by FACS. Experiments were performed with peripheral blood monocytes from 3 cancer patients and 3 healthy donors, and repeated twice for each subject.

concentration of interferon (IFN)-γ, IL -10, and IL-12 using an ELISA kit (BioSource International).

Preparation of CTLs. Antigen-specific CTLs were generated from PBMCs of healthy donors or patients with metastatic cancer. CD8-positive T cells purified from PBMCs of cancer patients or healthy donors using magnetic beads coated with an anti-CD8 monoclonal antibody (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) were cultured with autologous DCs that were pulsed with 10 μg/ml antigen peptides in AIM-V medium (Gibco BRL) supplemented with 5% human AB serum followed by 40-Gy irradiation. Two days after culture, 40 U/ml human recombinant IL-2 that was a kind gift of Shionogi Pharmaceutical Co. Ltd. (Osaka, Japan) was added to each well. On days 8 and 15, the responder cells were stimulated with autologous DCs pulsed with the same peptides. Seven days after the last stimulation, the responder cells were examined for their reactivity with the target antigens.

ELISA assay of IFN-γ production. CTLs ($4x10^5$) prepared as described above were incubated with $2x10^5$ T2 (A2-positive) or TISI (A24-positive) cells pulsed with M2BP or control peptides in a 96-well culture plate for 48 h. After incubation, the supernatant was collected from each well, and the amount of IFN-γ released from the CTLs was measured using an IFN-γ ELISA kit (BioSource International).

Cytotoxic assay. The cytotoxic activity of CTLs was determined by using a standard ^{51}Cr-release assay as described previously (38). Briefly, target cells pulsed with peptides were labeled with 50 μCi $Na_2{}^{51}CrO_4$ at 37˚C for 1 h and then incubated with CTLs at various effector/target (E/T) ratios in 96-well culture plates for 4 h. The amount of ^{51}Cr released from lysed target cells in the supernatant was determined by using an LKB-Wallac 1275 Minigamma counter (EG&G Wallac, Turk, Finland). The percentage of specific ^{51}Cr release was calculated using the following formula: % specific lysis

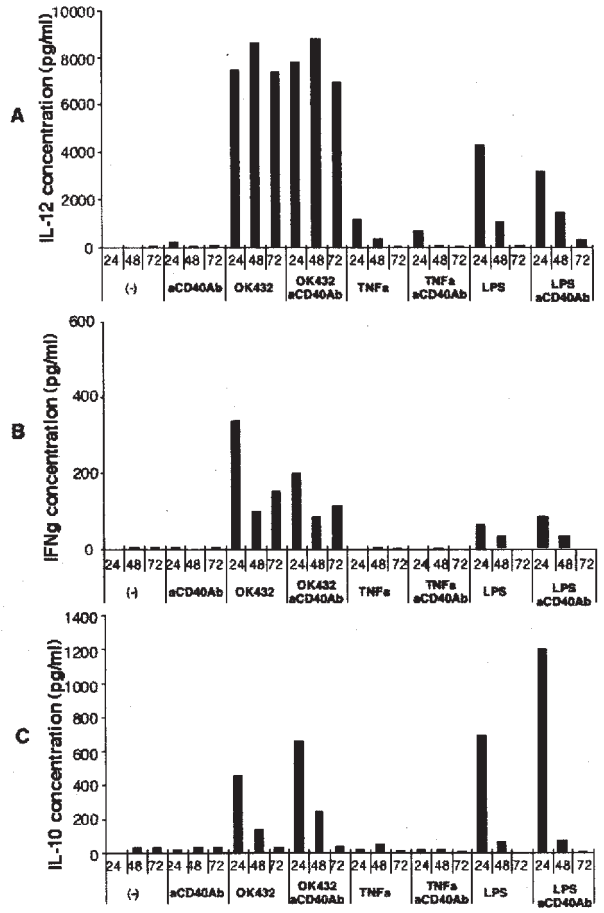

Figure 2. Cytokine production by mature DCs prepared from cancer patients. Immature DCs induced from $1x10^7$ PBMCs of cancer patients by 7-day culture supplemented with GM-CSF and IL-4 were further cultured in 3 ml of AIM-V medium supplemented with 5% human AB serum in the presence of either OK432, TNF-α, LPS or anti-CD40 antibody in a 6-well cell culture plate for 72 h. During the 72-h culture, the culture media were collected and exchanged with fresh medium every 24 h, and measured by ELISA for the amount of IL-12 (A), IFN-γ (B) and IL-10 (C) secreted from DCs. Experiments were performed with peripheral blood monocytes from 3 cancer patients and repeated 3 times for each patient.

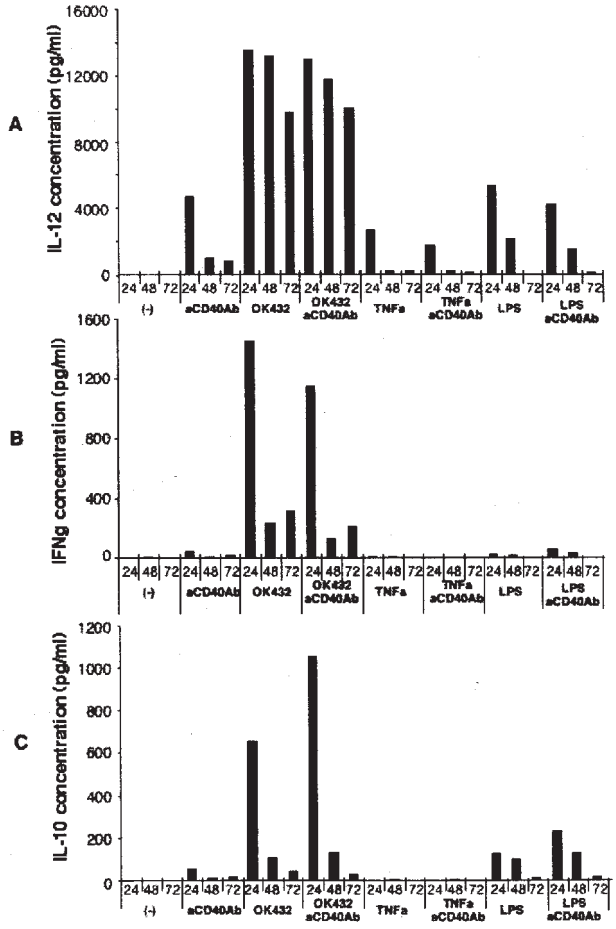

Figure 3. Cytokine production by mature DCs prepared from healthy donors. The culture media from mature DCs prepared from healthy donors were examined by ELISA for the amounts of IL-12 (A), IFN-γ (B) and IL-10 (C) secreted from DCs, as described in Fig. 2. Experiments were performed with peripheral blood monocytes from 3 healthy donors and repeated twice for each donor.

= [(experimental release - spontaneous release) / (maximal release - spontaneous release)] x 100.

Results

Expression of surface markers on mature DCs. Surface expression of CD80, CD83, CD86 and HLA-class II molecules on DCs was studied after 48-h culture of immature DCs in the presence of either OK432, TNF-α, LPS or anti-CD40 antibody. CD80, CD83 and CD86 are involved in transduction of co-stimulatory signals to activate T lymphocytes, and CD83 is a maturation marker of DCs. MHC class II molecules present antigenic epitopes to helper T cells. All of these molecules are essential for priming and activating tumor-specific effector T lymphocytes efficiently (39-43). In flow cytometric analysis, anti-CD40 antibody was shown to up-regulate expression of CD80 and CD83 (5- to 6-fold and 7-fold of the original mean fluorescent intensity, respectively). OK432 increased prominently the expression of CD86 and class II molecules (10-fold and 5-fold, respectively) (Fig. 1A and B), and OK432 up-regulated slightly CD80 and CD83 expression. Using both OK432 and anti-CD40 antibody to allow immature DCs to differentiate showed additive effects on up-regulation of these four molecules, resulting in the highest expression compared with that using other combinations. TNF-α or LPS showed no or modest effects on expression of the molecules on DCs induced from patients with metastatic cancer (Fig. 1A). In DCs induced from healthy donors using the same strategies, the effects of LPS on expression of the surface molecules were nearly compatible with those of OK432 (Fig. 1B). Contrary to our expectations, TNF-α did not affect the expression of any of the molecules on DCs induced from cancer patients or healthy donors.

Cytokine production by mature DCs. To study cytokine profiles produced by DCs during maturation, concentrations of Th1 and Th2 type cytokines (IFN-γ, IL-12 and IL-10, respectively) in the supernatants of DCs were measured every 24 h for 72 h after adding OK432, TNF-α, LPS or anti-CD40 antibody. As a result, DCs from cancer patients that were matured in the presence of OK432 showed a large amount of IL-12 and IFN-γ production and maintained the cytokine production for at least 72 h (Fig. 2A and B). The DCs also produced IL-10

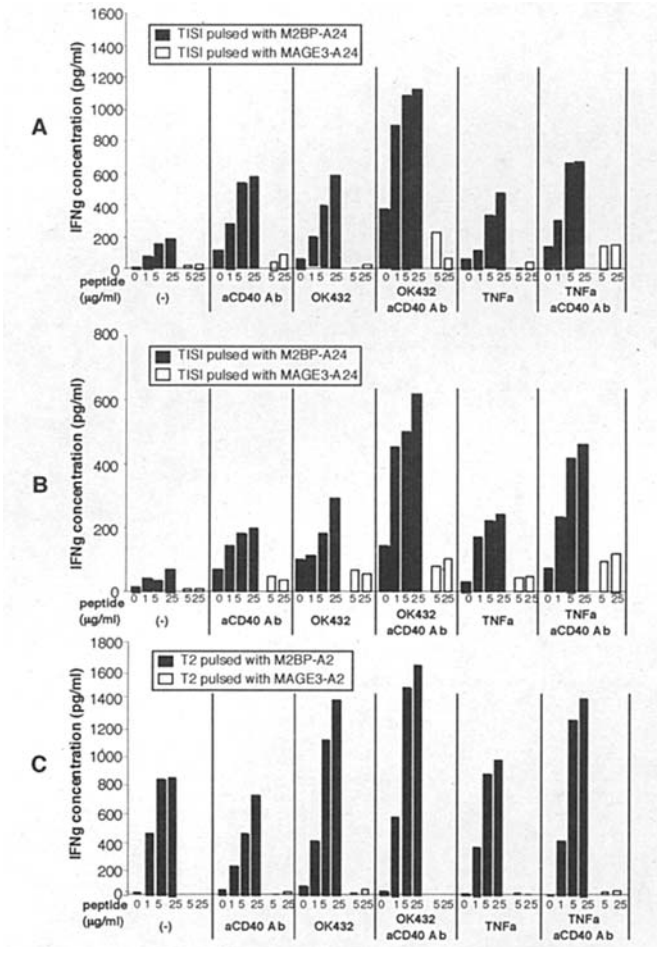

Figure 4. IFN-γ production by CTLs induced from healthy donors using mature DCs. CTLs ($4x10^5$) induced from 3 healthy donors as described in 'Materials and methods' were cultured with $2x10^5$ TISI or T2 cells pulsed with the indicated concentrations of M2BP or the irrelevant peptide, MAGE3, in a 96-well culture plate for 48 h. After the culture, the supernatant was collected from each well, and the amount of IFN-γ released from the CTLs was measured using an IFN-γ ELISA kit. Experiments were repeated twice for each subject.

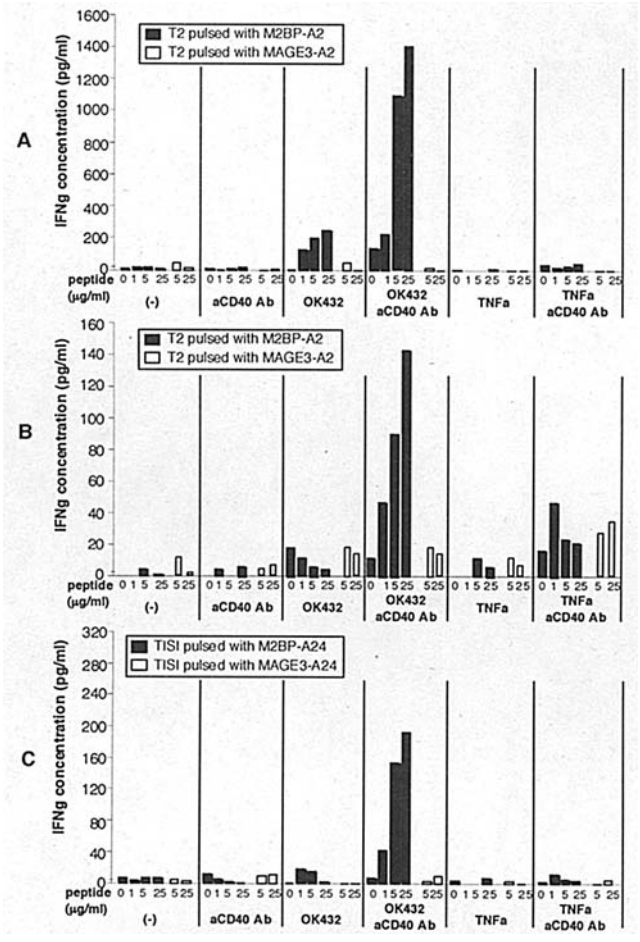

Figure 5. IFN-γ production by CTLs induced from cancer patients using mature DCs. CTLs induced from 3 cancer patients (A, patient #1; B, patient #2; C, patient #3) were cultured with peptide-pulsed TISI or T2 cells as described in Fig. 4. Experiments were repeated twice for each subject.

for the first 24 h but subsequently continued to decrease their IL-10 production for the following 48 h (Fig. 2C). In DCs matured by either TNF-α or LPS, IL-12 and IFN-γ productions were much lower than in DCs matured with OK432 (one fifth to eighth), and the amount of these Th1-type cytokines produced by the DCs decreased gradually to almost undetectable levels in 72 h (Fig. 2A and B). In mature DCs prepared from healthy donors, the produced cytokines and their kinetics were almost compatible with those in DCs from cancer patients (Fig. 3A, B and C). However, the amount of cytokines from the healthy donors was generally twofold higher than that from the cancer patients. These findings suggest that OK432 caused immature DCs to differentiate towards mature DCs showing Th1-type cytokine profiles, and that the mature DCs maintained the cytokine production for at least 72 h even in cancer patients with impaired immunity.

IFN-γ production by CTLs in response to tumor antigens. To assess the ability of DCs to induce tumor antigen-specific CTLs, M2BP-specific CTLs were elicited by *in vitro* stimulation of CD8-positive peripheral blood T lymphocytes from both cancer patients and healthy donors with M2BP peptide-pulsed DCs. All of the CTLs induced using DCs from healthy donors produced IFN-γ in response to M2BP peptides in a dose-response manner (Fig. 4). None of the CTLs responded to the irrelevant peptide, MAGE3. The CTLs that were stimulated by mature DCs activated with OK432 and anti-CD40 antibody showed the highest INFγ production in all of the 3 healthy donors. Regarding DCs from 3 cancer patients, only DCs prepared using both OK432 and anti-CD40 antibody exhibited the ability to elicit CTLs that strongly reacted with M2BP peptides and antigen dose-dependently produced INFγ (Fig. 5). A weak, but detectable, antigen dose-dependent reactivity with M2BP peptides of CTLs induced with DCs that were matured with OK432 alone was observed in one of the 3 patients (Fig. 5A).

Cytotoxic activity of CTLs against antigen-pulsed targets. CTLs used in the IFN-γ production assay as described above were also examined for their cytotoxic activity against M2BP peptide-pulsed target cells. All of the CTLs induced using mature DCs from healthy donors showed specific cytolysis of M2BP peptide-pulsed target cells in an E/T ratio-dependent manner, whereas the CTLs did not lyse targets pulsed with the irrelevant antigen, MAGE3 (Fig. 6A). In contrast, in cancer

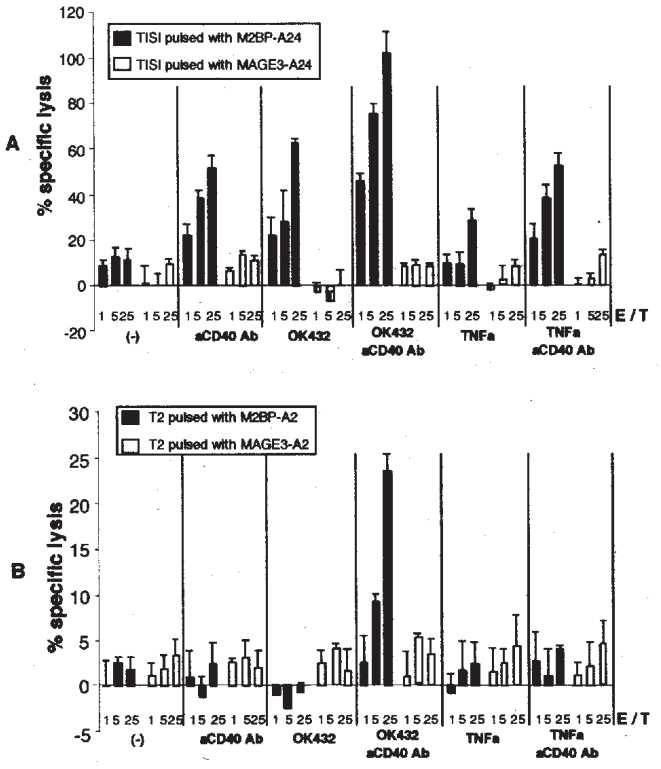

Figure 6. Cytotoxic activity of CTLs induced using mature DCs. CTLs induced from healthy donors (A) or cancer patients (B) were incubated with radiolabeled TISI or T2 cells that were pulsed with 10 μg/ml M2BP or the irrelevant peptide, MAGE3, at various E/T ratios for 4 h. After incubation, the supernatant was collected from each well, and the amount of ^{51}Cr released from lysed targets was measured. Experiments were performed with 3 cancer patients and 2 healthy donors and repeated twice for each subject.

patients only DCs that were matured with both OK432 and anti-CD40 antibody showed the ability to elicit CTLs capable of killing M2BP peptide-pulsed targets specifically (Fig. 6B), although the killing capacity of the primed CTLs was less than that seen in healthy donors. In another two cancer patients, the same tendency was observed, although %killing was lower than that shown here (data not shown).

Discussion

In this study we attempted to prepare mature/activated DCs from peripheral blood monocytes of patients with metastatic lung and breast cancer as well as healthy donors, according to our previous findings in which we successfully obtained mature/activated DCs in a mouse model. As a result, we found that use of both OK432 and anti-CD40 antibody was the most potent combination to establish mature/activated DCs in healthy donors. Furthermore, DCs activated using this combination produced and maintained a large amount of Th1-type of cytokine production for at least 72 h. Importantly, in cancer patients only mature DCs that were stimulated simultaneously with OK432 and anti-CD40 antibody activated CTLs *in vitro*. We presume, therefore, that the DC activation plays an important role in the combinatorial cancer immunotherapy with inhibition of regulatory T cells and blockade of negative signals.

In our clinical study previously reported, the clinical effect of a DC-based vaccine targeting MUC1 against metastatic lung and breast cancers with high expression of MUC1 was significantly superior to the effect against MUC1-negative cancer (median survival: 16.75 versus 3.80 months, p=0.0101) (15). The data suggest that the biological susceptibility of cancer cells to immune responses and the immunogenicity of target antigens expressed on cancer cells are profoundly related to the clinical impact of immunotherapy. However, DC-based vaccines have been generally ineffective in promoting tumor rejection. We suggest that this failure should be attributed to the use of insufficiently matured or activated DCs, when poorly immunogenic TAAs were used as target antigens. For example, if co-stimulatory molecules such as CD80 and CD86 are expressed at low levels on APCs, tumor-specific CTL precursors receive inadequate activation signals and, as a result, become unresponsive or anergic. Indeed, immature DCs and tumor-infiltrated DCs have been shown to be tolerogenic (44,45).

In patients with advanced and metastatic cancer, immunity is generally suppressed mainly by factors produced by cancer cells and tumor infiltrating cells, i.e. IL-6, VEGF, TGF-ß and IL-10 (18-22). These cytokines are involved in suppressing DC function. VEGF has been shown to impair the maturation of DCs, and prostaglandin-E_2 (PGE_2) has been demonstrated to bias DC development toward a type 2 phenotype. IL-10 has also been shown to influence DC recruitment and function in both tumor and non-tumor systems. In cancer patients, therefore, an impaired maturation process of DCs would be one of the most important issues involved in preventing successful immunotherapy against cancer. It has been shown that CD40-ligation provides signals to activate DCs and antagonize the inhibitory effects of IL-10 on the T cell-stimulatory capacity of DCs (46). Furthermore, synergistic pathogen-associated molecular patterns (PAMPs), including LPS and CD40-ligation, provided many more activation signals to DCs *in vivo* than each stimulation by itself (47). These data support the notion that a combination of various stimulations may induce full DC activation and overcome, at least partly, immunosuppressive conditions in cancer patients.

Synergistic PAMPs and CD40-ligation were not the most efficient stimulation for inducing matured DCs in healthy donors, although they had been a potent stimulation in a mouse model. Instead, we have determined that CTLs induced by DCs that were matured with both OK432 and anti-CD40 antibody exhibited the strongest capacity to produce IFN-γ and to lyse the targets in healthy donors, although OK432 has been shown not to interact with TLR (29). Furthermore, only the DCs prepared with the combination of OK432 and anti-CD40 antibody were able to enhance expression of all of CD80, CD83, CD86, and MHC class II molecules and activate tumor-specific CTLs in cancer patients. In mice, the combined stimulation with OK432 and anti-CD40 antibody did not provide more efficient maturation signals to DCs compared with synergistic PAMPs and CD40-ligation. These data suggest differences in signal cross-talk between murine and human DCs. Similarly, IL-10 blockade was not effective in providing full DC maturation in humans, whereas it showed synergistic effect with PAMPs and CD40-ligation in murine DC maturation (unpublished data). Although we can refer to

findings in the mouse model, it is necessary to identify a modified strategy for inducing activated DCs capable of activating tumor-specific CTLs in patients with advanced or metastatic cancer.

Finally, the ability of injected DCs to reach secondary lymphoid organs to elicit T cell responses is considered one of the important factors for the effective use of DC-based vaccines. After subcutaneous injection, only a small fraction of the injected DC population reaches the draining lymph nodes (48). In general, mature DCs migrate to the draining lymph nodes, therefore we assume that the poor migration of DCs in cancer patients may be attributed to the use of insufficiently matured DCs. On the other hand, in mice DC migration into lymph nodes was enhanced by preconditioning of the injection sites with repeated injection of DCs (49). Accordingly, we suggest that when the activated DCs are repeatedly injected at the same sites as a DC-based vaccine, matured DCs effectively migrate to the draining lymph nodes, where CTLs capable of killing tumors are elicited.

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