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ORIGINAL ARTICLE

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Streptococcal preparation OK432 promotes functional maturation of human monocyte-derived dendritic cells

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Abstract Streptococcal preparation OK432 is an immunomodulatory agent extensively used as adjuvant therapy for gastric cancer in Japan. OK432 augments the cytotoxic activity of various effector cells such as lymphocytes, macrophages and (natural killer) NK cells and induces the production of multiple cytokines. Dendritic cells (DC) are professional antigen-presenting cells (APC) that can be used for cancer vaccine therapy. In the present study, we investigated the effect of OK432 on the activation of DC. Here we report that OK432 induced phenotypic and functional maturation of human monocyte-derived DC. In vitro culture of immature DC generated from adherent peripheral blood mononuclear cells (PBMC) using granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) with OK432 at various doses (0.01 to 0.1 KE/ml) for 2 days resulted in increased cell surface expression of CD80, CD83, CD86 and ICAM-1 in a dose-dependent manner. The expression of CD83, a selective marker of mature DC, on DC activated by OK432 (OK-DC) was maximally enhanced after 3 days of incubation. Assay of cytokine production in OK-DC after 2 days in culture revealed that OK432 was a strong inducer of IL-12 and interferon-gamma (IFN-γ). OK432 efficiently augmented the primary allogeneic T-cell responses by DC. This distinct phenotypic profile and allostimulatory capacity of OK-DC was stable for at least 48 h of additional culture in the absence of any cytokines. Moreover, the antiviral cytotoxic T lymphocytes (CTL) response in vitro was also enhanced by the addition of OK432 to the cultures. These findings suggest that OK432 is a potent stimulator of DC, and that stimulated DC are strong inducers of the T helper 1 (Th1)-type response. We conclude that OK-DC are likely candidates for use as an adjuvant for DC-based cancer immunotherapy.

Keywords Dendritic cell · Tumor immunity · Human

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Introduction

Dendritic cells (DC) are professional antigen-presenting cells (APC), which play a critical role in generating primary cytotoxic T-cell responses. Inflammatory signals such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) as well as bacterial products induce migration into secondary lymphoid organs and the maturation of DC. Upon maturation, DC upregulate the major histocompatibility complex (MHC), costimulatory and adhesion molecules and shift to antigen presentation from antigen uptake and processing. DC induce potent antitumor immunity in vitro and in vivo [28] and are currently under active investigation for use in cancer vaccine therapy [13, 27]. In humans, DC can be generated from non-proliferating $CD14^+$ monocytes in peripheral blood by treatment with cytokines, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-4. Monocytes have been used widely as a cell source of DC for experimental and clinical purposes because of their accessibility. The maturation stage of administered DC is one of the critical issues for their clinical application. Mature DC generated by monocyte-conditioned-medium (MCM) efficiently induce antigen-specific T-cell responses [27]. In contrast, immature DC elicit antigen-specific inhibition of effector T-cell function [4]. Therefore the use of mature DC, which can promote T helper 1 (Th1)-type immune responses, is a promising approach for cancer vaccine therapy.

OK432, a penicillin-inactivated, lyophilized preparation of an avirulent human strain of *Streptococcus pyogenes*, has immunomodulatory and potential therapeutic properties for use in cancer treatment as a biologic response modifier. OK432 augments the activity of

neutrophils, macrophages, lymphocytes and natural killer (NK) cells, and induces the production of multiple cytokines including IL-1, IL-2, IL-6, TNF-α, interferon-γ (IFN-γ) and IL-12 [5, 6, 8, 12, 16, 24]. These facts imply that OK432 may have the ability to induce the production of cytokines by DC and to promote the maturation of DC. We found that phenotypical maturation of DC derived from human peripheral blood monocytes (PBMC) was elicited by in vitro stimulation with OK432. We further evaluated cytokine profiles and functional properties of these mature DC treated with OK432 and assessed the implications for cancer immunotherapy. To our knowledge, this is the first report describing DC activation by OK432.

Materials and methods

OK432

OK432, a lyophilized preparation of the Su strain of *S. pyogenes* inactivated with penicillin G was kindly provided by Chugai Pharmaceuticals (Tokyo, Japan). The concentration of OK432 was expressed in units designated as Klinische Einheit (KE; clinical unit), with one KE of OK432 being equivalent to 0.1 mg of dry streptococci. OK432 was diluted in RPMI 1640 at a dose of 1 KE/ml.

PBMC and cell purification

Five healthy volunteers were subjected to leukapheresis after giving their written informed consent. PBMC were then separated from the leukapheresis products by density gradient centrifugation using Ficoll-Paque Plus (Pharmacia Biotech, Sweden). The PBMC obtained were suspended in fetal calf serum (FCS) with 10% DMSO at a concentration of 5×10^7 cells/ml, and divided into vials each containing 2 ml of the suspension. All vials were stored in liquid nitrogen until use. $CD14^+$ monocytes were sorted using monoclonal antibody (mAb) and a FACSvantage (Becton Dickinson, San Jose, Calif.) cell sorter in some experiments.

DC generation and activation by OK432

After thawing, 5×10^7 PBMC were suspended in 20 ml of RPMI 1640 containing HEPES and supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acid solution (Biowhittaker, Walkersville, Md.), 1% heat-inactivated pooled human plasma (Japan Red Cross Society) and 10 μg/ml gentamycin (Fujisawa Pharmaceutical, Osaka, Japan) (complete medium), and plated onto 75-cm^2 cell culture flasks. The flasks were incubated in a 5% CO_2 incubator at 37°C for 2 h, and non-adherent cells were discarded. Adherent cells were cultured in 30 ml of complete medium containing 1,000 U/ml each of recombinant human GM-CSF (rhGM-CSF; Genzyme, Minneapolis, Minn.) and recombinant human IL-4 (rhIL-4; Genzyme). After 7 days of culture, the cultured cells consisted of 60% large cells and 40% small lymphocyte-like cells. DC were then purified from the cultured cells using Human Dendritic Cell Enrichment Cocktail (Stem Cell Technologies, Vancouver, Canada), which is tailored to highly enrich DC using mAb to the following human cell surface antigens: CD3, CD14, CD16, CD19 and CD56. The resulting cell preparation was verified to consist of more than 95% DC as assessed by morphology and FACS analysis. Phenotypic analyses of the DC demonstrated that they expressed HLA-DQ/DP/DR, CD40, CD80 and CD86, but not CD3, CD14, CD16, CD19 and CD56. The contaminating cells that were still detected after purification were exclusively $CD3^+$ lymphocytes.

Five$\times10^5$ DC were cultured in 6-well plates in 3 ml of complete medium with or without OK432. After 48 h, supernatants were recovered and frozen until the determination of cytokine levels. Cells were harvested, washed and used for phenotypic analysis and T-cell proliferation assays.

Immunofluorescent staining and flow cytometric analysis

Phenotypic characterization of cells was carried out by flow cytometry using a FACSCalibur (Becton Dickinson) and CellQuest software. Immunofluorescence staining was performed using the following mAb: CR3/43 mAb for HLA-DP/DQ/DR, FITC-conjugated anti-CD3 mAb (Dako, Denmark), anti-CD14 mAb, CD80 mAb (Pharmingen, San Diego, Calif.), anti-CD83 mAb (Immunotech, Marseille, France), anti-CD86 mAb (Ancell, Bayport, Minn.) and ICAM1 (Takara, Otsu, Japan). Isotype-matched antibodies of irrelevant specificity were used as negative controls.

T-cell proliferation assay

MLR was performed to evaluate the antigen-presenting capacity of DC stimulated with or without OK432 for T-cell proliferation. Responder T-cells isolated using nylon fiber columns (WAKO, Osaka, Japan) were used at 2×10^5 cells per well (96-well flat-bottomed microtiter plates). Stimulator cells were allogeneic DC that had been cultured for 48 h in medium alone or with OK432 (0.1 KE/ml) or with a cocktail of cytokines, i.e. TNF-α (20 ng/ml), IL-1β (10 ng/ml), and IL-6 (100 U/ml). PBMC without any stimulation were used as controls in some experiments. Stimulators were added at various doses after irradiation (25 Gy) and cultures were set up in triplicate and maintained in a humidified atmosphere at 37°C in 5% CO_2 for 5 days. Cultures were pulsed with 1.0 μCi/well of ^{3}H-thymidine for 12 h before harvesting and proliferation (expressed as ^{3}H-thymidine incorporation) was measured by scintillation counting. Supernatants from another similar set of cultures were collected for IFN-γ determinations.

Cytokine assays

Cytokine levels were measured by ELISA. The IL-1β, IL-4, IL-6, TNF-α and GM-CSF ELISA kits were supplied by the Otsuka Pharmaceutical Institute (Tokushima, Japan). The IFN-γ and IL-12p70 kits were purchased from Biosource International (Biosource International, Camarillo, Calif.).

Intracellular cytokine expression

For the detection of cytoplasmic cytokine expression, cells stimulated with PMA (25 μg/ml) and ionomycin (1 mg/ml) for 6 h in the presence of Golgistop (Becton Dickinson) were stained with PerCP-conjugated anti-CD4 mAb, fixed with 4% paraformaldehyde, and treated with permeabilizing solution (Becton Dickinson), and then the fixed cells were stained with PE-conjugated anti-IL-4 mAb and FITC-conjugated anti-IFN-γ mAb for 30 min at room temperature. The percentage of cells expressing cytoplasmic IL-4 or IFN-γ was determined by flow cytometry (FACSCalibur). IgG was used for isotype controls. All mAb and isotype-control IgG were purchased from BD Biosciences (San Diego, Calif.).

Anti-viral CTL induction assay

To quantify the antigen-presenting capacity of DC, a recall cytotoxic T lymphocyte (CTL) assay for detecting flu peptide (RFYIQMCTEL, position 38–47), derived from influenza nucleoprotein restricted with HLA-A24, -specific killer cells was performed. Irradiated DC used as APC (5×10^5 cells/well) were seeded in the wells of 24-well plates and pulsed with 20 μg/ml of peptide with or without OK432 at a concentration of 0.01 KE/ml for 2 h at

37°C. PBMC (5×10^6 cells) collected from HLA-A24-positive volunteers as responders were then added to the well and co-cultured with the peptide-pulsed APC. On days 3 and 5, the cultures were fed with the complete medium containing 20 U/ml IL-2 (Shionogi, Osaka, Japan). On day 7, the cultures were stimulated with autologous peptide-pulsed adherent cells, and fed with IL-2-containing complete medium on days 9 and 12. Effector cells were harvested on day 14 and tested for CTL activity in a ^{51}Cr-release assay using peptide-pulsed TISI cells, an Epstein-Barr virus transformed B cell line expressing HLA-A24 as target cells. The percentage of specific cytotoxicity was determined by calculating the percentage of specific ^{51}Cr-release by the following formula: [(cpm of test sample release–cpm of spontaneous release)/(cpm of maximum release–cpm of spontaneous release)]×100.

Statistical analysis

T-cell proliferation and cytokine data were expressed as mean ± SD. Statistical comparisons were performed using Student's *t*-test for independent samples. All experiments were performed in duplicate or triplicate, and equivalent results were obtained at least twice. Calculations were made using data from representative experiments.

Results

OK432 promotes phenotypic maturation of DC after 48 hours of culture in vitro

First we generated immature DC from human peripheral blood monocytes by the use of IL-4 and GM-CSF using the methods described above. These cells expressed CD14 ^{low}, MHC class II^{high}, CD80^{low}, CD83^{low} and CD86^{high} on their surface and showed a relatively high allo-MLR response (data not shown). To examine the effect of OK432 on the phenotype of immature DC, the cells were cultured with OK432 (0.1 KE/ml), and the expression of surface markers on cultured DC was determined by flow cytometry. The expression of surface markers associated with co-stimulatory and adhesion molecules including CD83, a maturation marker for DC, was upregulated after 48 h of stimulation with OK432 (Table 1). To assess the dose-response relationship, immature DC were cultured with various concentrations of OK432. As shown in Table 2, upregulation of the expression of CD80, CD83 and CD86 was observed in a dose-dependent fashion. To determine the time course of DC maturation induced by OK432, immature DC were cultured with OK432 for various periods and the expression of CD83 was assessed. Fig. 1 shows that the expression of CD83 was maximally enhanced after 3 days of culture with OK432. The viability of DC after OK432-treatment amounted more than 90% in these experiments.

Table 2 Effects of OK432 at various doses on the expression of costimulatory molecules. Immature DC derived from monocytes were cultured with or without various concentrations of OK432. DC were then treated with the stated mAb, and the expression of cell surface molecules was analyzed by FACS. Results are expressed as specific MFI. Data are given as mean ± SD of 2 independent experiments. The results are representative of 2 experiments with similar results

Cell surface expression (MFI)			
OK432 (KE/ml)	CD80	CD83	CD86
0	5.08 ± 0.29	7.19 ± 1.52	49.99 ± 5.27
0.01	10.21 ± 1.72	10.64 ± 1.54	63.65 ± 5.16
0.05	21.81 ± 3.38	19.66 ± 2.75	88.08 ± 7.26
0.1	24.31 ± 3.56	22.81 ± 2.88	94.63 ± 8.65

DC produce multiple cytokines, including the Th1-biasing cytokine IL-12, after activation by OK432

Next we evaluated the effects of OK432 on the production of various cytokines by human DC. In a mouse model, OK432 was found to be a multi-cytokine inducer, especially a strong inducer of IL-12 [5]. As shown in Table 3, the level of IL-1β, IL-6, IL-12, IFN-γ, TNF-α and GM-CSF was increased in the culture supernatants when human monocyte-derived immature DC were stimulated with OK432. Although monocytes also produced these cytokines upon stimulation with OK432, significant differences between DC and monocytes were observed. The levels of TNF-α, IFN-γ and IL-12 produced by DC were significantly higher than the levels produced by the same numbers of monocytes ($P < 0.05$). The production of IFN-γ by DC activated with OK432 was confirmed using intracellular cytokine staining (IFN-γ/CD86) in comparison with DC without OK432. Approximately 90% of DC activated with OK432 were double positive, whereas those without OK432 had less than 10% (data not shown). In contrast, the levels of production of IL-1β and IL-6 by DC were lower than those produced by monocytes ($P < 0.05$). LPS, a

Table 1 Effects of OK432 on DC phenotype. Five×10^5 DC were cultured for 48 h with or without OK432. DC were then stained with different mAb or isotypic controls, and the expression of surface molecules was analyzed by FACS. Results were expressed as specific mean fluorescence intensity (MFI) by subtracting the MFI obtained with the corresponding isotypic control. Data are shown as mean ± SD of 4 independent experiments

Cell surface expression (MFI)						
OK432 (KE/ml)	HLA-class 1	CD14	CD80	CD83	CD86	ICAMI
0	182 ± 9.93	4.65 ± 0.36	5.08 ± 0.29	7.19 ± 1.52	50.0 ± 5.27	61.9 ± 6.28
0.1	171.7 ± 4.53	4.06 ± 0.30	25.27 ± 4.19*	22.82 ± 4.19*	99.63 ± 7.43*	157.85 ± 32.37*

*$P < 0.05$ compared with MFI in the absence of OK432

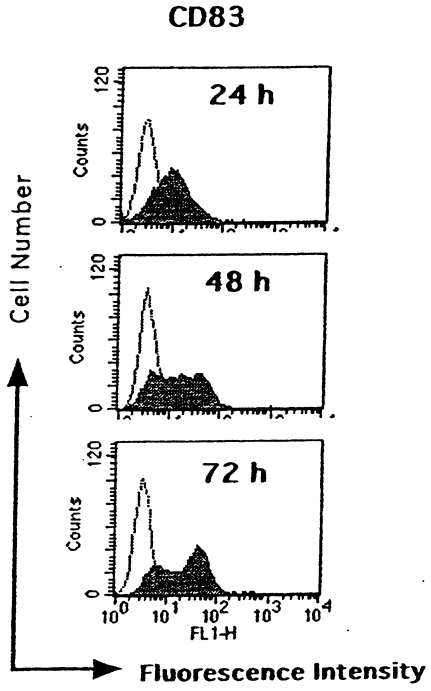

Fig. 1 Kinetics of the expression of costimulatory molecules on the cell surface of DC stimulated with OK432 (0.1 KE/ml). Monocyte-derived DC were cultured with OK432 for 72 h. DC were stained with anti-CD83 mAb followed by FITC-conjugated rabbit anti-mouse IgG, and cell surface expression was analyzed by FACS. One representative example of 3 experiments is shown

bacterial cell wall constituent, induces the production of some regulatory cytokines from DC and is an inducer of IL-12 [29]. The ability of DC stimulated with OK432 to produce IL-12 was equivalent to that of DC stimulated with LPS (data not shown). In brief, among various cytokines produced by DC and monocytes, larger amounts of Th1-biasing cytokines such as IFN-γ, TNF-α and IL-12 were specifically secreted by DC rather than by monocytes when these cells were stimulated with OK432. This indicates that DC are activated by OK432, and activated DC are strong inducers of Th1-biased cytokines.

Medium conditioned by OK432 does not exert the full effects of OK432 on DC maturation

A combination of inflammatory cytokines such as IL-β, IL-6 and TNF-α has been shown to be an activator of DC [19]. Our cytokine assays indicated that the medium of DC stimulated with OK432 contained measurable amounts of these cytokines. This suggested that immature DC might differentiate into mature DC in response to the effects of autocrine cytokines released from DC stimulated with OK432. To address this possibility, conditioned medium harvested from cultures of DC treated with OK432 was used for activation of immature DC, and phenotypic changes were assessed by flow cytometry. DC were stimulated with OK432 (0.1 KE/ml) for 3 days, and then the supernatants were harvested, centrifuged and filtered through 0.22-μm membrane filters (OK432-conditioned medium). Monocyte-derived immature DC generated from 3 different blood donors were cultured in the presence of OK432 (0.1 KE/ml) or in the OK432-conditioned medium for 2 days. As shown in Table 4, the expression of CD80 and CD83 was upregulated in DC stimulated with either OK432 or OK432-conditioned medium. However, the percentages of CD80- and CD83-positive cells were higher in DC stimulated with OK432 than in those stimulated with OK432-conditioned medium in all experiments. These results indicate that cytokines produced by DC upon activation by OK432 play an important role in the maturation of DC. However, OK432-conditioned medium did not exert the full effect of OK432 on DC maturation.

DC activated by OK432 have higher antigen-presenting capacity and promote the differentiation of Th1-type $CD4^+$ T cells

To assess the functional consequences of OK432-mediated maturation of DC, we examined the T cell stimulatory activity of OK432-stimulated DC in primary allogeneic MLR. T cells were cultured with allogeneic DC prepared under different conditions. As shown in Fig. 2A, the proliferation of T cells was significantly increased when they were stimulated with OK432-activated DC (OK-DC) compared to non-stimulated DC. OK-DC tended to have a higher allostimulatory capacity than cytokine-induced mature DC, but the difference was not significant. The production of IFN-γ was also increased more markedly when T-cells were stimulated with OK-DC compared to other APC (Fig. 2B).

The potent stimulatory effect of OK-DC on the generation of functional $CD4^+$ T cells was also demonstrated by investigating intracellular cytokine expression in $CD4^+$ T cells. T cells were co-cultured for 3 days

Table 3 Cytokine production by OK432-treated DC and monocytes. DC and monocytes (Mo) were cultured for 48 h in medium with or without OK432 (0.1 KE/ml). Cytokine concentrations in supernatants were measured by ELISA. Data are given as mean ± SD of 3 independent experiments using blood from different donors

	IL-Iβ	IL-4	IL-6	IL-12	GM-CSF	IFN-γ	TNF-α
DC	213 ± 18	< 20	< 20	296.3 ± 64.2	< 20	195.4 ± 36.2	421.5 ± 65.5
DC + OK	1258 ± 67* **	< 20	5051 ± 950* **	25419.6 ± 1432.6*,**	351.5 ± 95.5*	8380.6 ± 391.4*, **	22541.5 ± 1285.5*, **
Mo + OK	7758 ± 634	< 20	11033.5 ± 1480.5	2532.9 ± 343.1	212 ± 80	< 20	7493 ± 603.5

$P < 0.05$ as compared with the levels of cytokines secreted from DC without OK432*, and Mo with OK 432**

with OK-DC derived from allogeneic donors. Responder cells were harvested and analyzed for intracellular cytokine expression as described in the Materials and methods section. The highest percentage of IFN-γ-producing $CD4^+$ T cells was observed in cultures stimulated with OK-DC (Fig. 3). Thus, IFN-γ-producing $CD4^+$ T cells were efficiently expanded by MLR using OK432-activated allogeneic DC.

Irreversible phenotypic and functional modulation of DC activated by OK432

The combination of GM-CSF and IL-4 induces human $CD14^+$ monocytes to develop some features of DC. However, the cells will revert back to macrophages if the cytokines are removed [25]. Final maturation leads to irreversible expression of MHC class II, CD86 and CD83 [1, 23]. To investigate the stability of the enhanced expression of surface molecules and antigen presenting function of OK-DC, we next carried out a cytokine stripping assay. Immature monocyte-derived DC were cultured for 3 days in complete medium containing IL-4 and GM-CSF with or without OK432 (0.1 KE/ml), then harvested, washed and recultured in the medium without any cytokines. After 2 days of culture, phenotypic expression and MLR were determined. As shown in Fig. 4A, flow cytometric analysis indicated that the re-expression of CD14 on OK-DC was suppressed compared to that on DC without OK432 stimulation. The expression of CD86 and CD83 on OK-DC was preserved at higher levels than on DC without OK432 stimulation. Moreover, high allostimulatory capacity was retained in OK432-stimulated DC after 2 days of culture without cytokines (Fig. 4B). These results suggest that the functional irreversible maturation of DC correlated with phenotypic change was elicited by OK432.

OK432-activated DC augment antiviral CTL response

To improve the efficiency of CTL induction in vitro, SAC-1-activated monocytes have been used as APC [30]. It has also been reported that addition of keyhole limpet hemocyanin (KLH) to cultures enhanced the CTL responses induced by peptide antigen [17]. In such experiments, exogenous protein or bacterial product SAC-1 was considered to be the activator of APC, and may augment CTL responses in vitro. To further evaluate the antigen-presenting capacity, specifically the CTL-induction ability in vitro of OK-DC, we performed CTL induction assays using flu peptide derived from influenza nucleoprotein. Flu-peptide-pulsed DC were cocultured with T cells for 7 days. Responder cells were then restimulated for 7 more days and peptide-specific IFN-γ production was examined on day 14. As shown by the representative results presented in Fig. 5, whereas effector cells stimulated with DC with or without OK432-activation showed a CTL response to flu peptide, peptide-specific cytotoxicity induced by OK432-activated DC was significantly higher than that induced by non-activated DC.

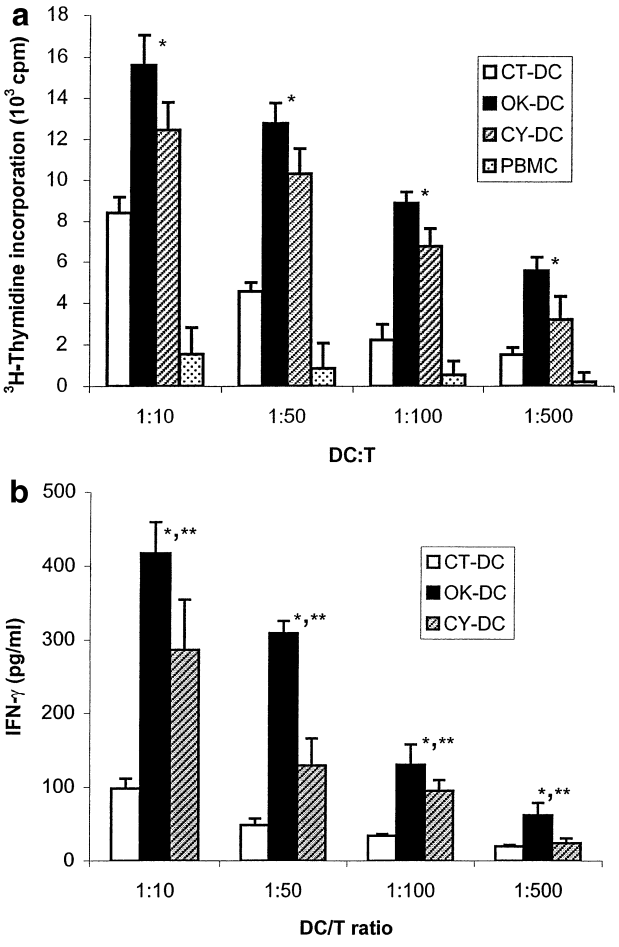

Fig. 2 OK432 enhances the allostimulatory potential of human monocyte-derived DC. DC were incubated in the medium with (OK-DC) or without (CT-DC) OK432 (0.1 KE/ml) for 2 days. DC stimulated with a cocktail of cytokines, i.e. TNF-α, IL-1β and IL-6 (CY-DC), were used as APC as a control for mature DC. PBMC as APC were also used as a control to compare with immature DC. These APC were then washed and irradiated, and varying numbers were added to allogeneic T cells. Five days later, cell proliferation was assessed by ^{3}H-thymidine incorporation (**A**), and culture supernatants were harvested for IFN-γ determination by ELISA (**B**). Cultures were set up in triplicate, and results representing the mean ± SD were obtained from 2 individual experiments. $P < 0.05$ compared with CT-DC*, CY-DC**

Table 4 Effect of OK432 and OK432-conditioned medium on DC phenotype. Five×10^5 DC were cultured for 48 h with OK432 or OK432-conditioned medium (CM). DC were then stained with the stated mAb or isotype controls and the expression of surface molecules was analyzed by FACS. Results are expressed as percentage of positive cells

	CD80		CD83	
Donor	OK432	CM	OK432	CM
1	89.6	75.8	72.3	67.4
2	75.4	72.3	64.2	59.6
3	93.4	91.2	80.6	74.3

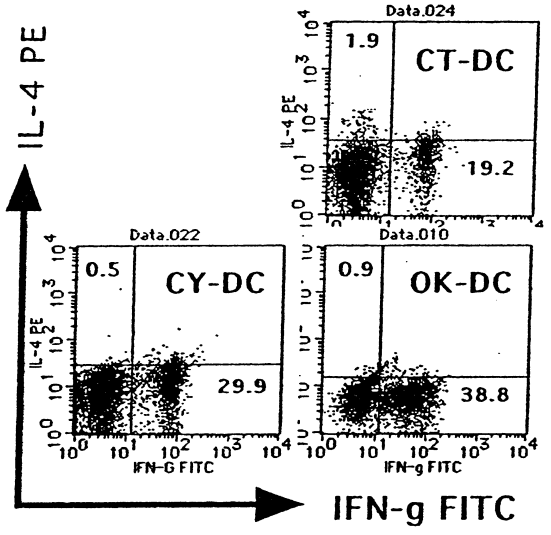

Fig. 3 IFN-γ-producing $CD4^+$ T cells were most markedly expanded in MLR by stimulation with OK432-activated DC. The IFN-γ-producing ability of $CD4^+$ T cells harvested from allogeneic MLR stimulated with immature DC (CT-DC), OK432-activated DC (OK-DC) and cytokine cocktail-induced mature DC (CY-DC) was assessed. Intracellular staining of IL-4 and IFN-γ was carried out as described in Materials and methods. Numbers represent the percentage of cells in each quadrant

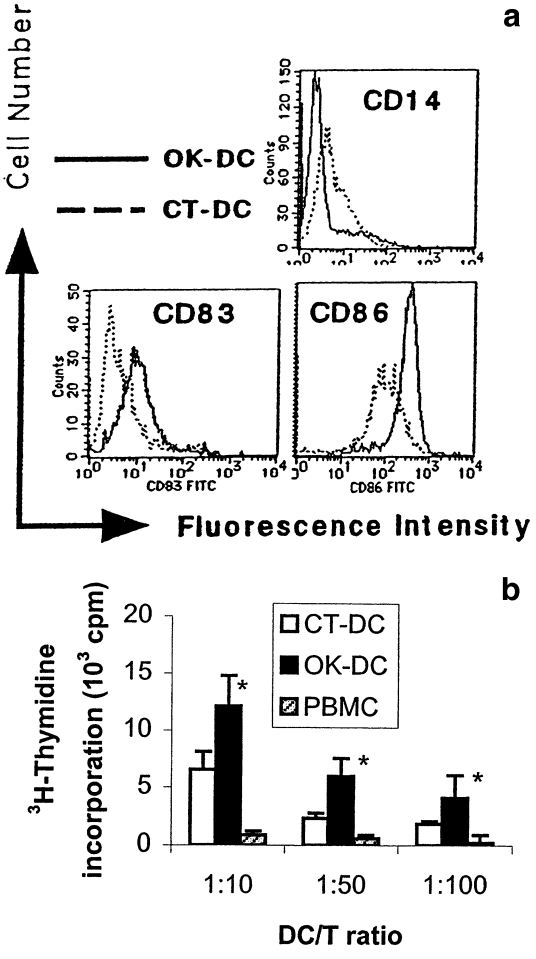

Fig. 4 The maturation-inducing effect of OK432 on monocyte-derived DC is irreversible. Immature monocyte-derived DC were cultured for 3 days in medium containing IL-4 and GM-CSF with (OK-DC) or without (CT-DC) OK432 (0.1 KE/ml). Cells were harvested, washed and recultured in medium without any cytokines. After 2 days of culture, cells were stained with the stated mAb and cell surface expression was analyzed by FACS (**A**). To assess functional properties, a primary allogeneic mixed leukocytes reaction test was performed. Cells harvested after 2 days of reculture were irradiated and added at various doses to allogeneic T cells. PBMC were used as control APC. Five days later, cell proliferation was assessed by ^{3}H-thymidine incorporation (**B**). $P < 0.05$ compared with CT-DC

Discussion

In the present study, we showed that OK432 has immunomodulatory effects that promote Th1-type responses via activation of human monocyte-derived DC. Bacteria have been shown to be potent inducers of DC activation [22]. LPS and *Mycobacterium tuberculosis* activate DC and increase the expression of costimulatory and class I molecules on their surface [7, 29]. DC treated with bacteria secrete elevated levels of inflammatory cytokines, including TNF-α, IL-1β and IL-6, which are thought to be major factors in DC maturation [21].

We compared the effects of OK432 and LPS on DC, and found that there were no significant differences between them in terms of the expression of cell surface phenotypes (class II, CD80, CD83 and CD86) and allostimulatory capacity (MLR; data not shown). Some humoral factors critical for DC maturation such as TNF-α and CD40L have been identified [2, 3]. Monocyte-conditioned medium (MCM) has also been shown to be effective in mediating terminal maturation of DC [19]. Administration of MCM-matured DC proved to be effective in eliciting an antigen-specific immune response in a clinical study [27]. In this system, DC require a number of different cytokines that are released from stimulated monocytes in the conditioned medium for their development. On the other hand, OK432, a bacterial preparation, has been shown to induce multiple cytokines, including IL-1, IL-2 [8], IL-6 [6], IFN-γ [24] and TNF-α [12]. More recently, Fujimoto et al. found that OK432 is a potent inducer of an IL-12 and Th1 dominant state [5]. In their experiments, macrophages purified from mouse spleen cells induced IL-6, IL-10, IL-12 and TNF-α on activation with OK432. We found that OK432 induced measurable IL-1β, IL-6 TNF-α and GM-CSF from human peripheral blood monocyte-derived DC, as expected (Table 3). DC stimulated with OK432 had high levels of expression of costimulatory molecules and a high allostimulatory capacity. Consequently, these results indicate that monocyte-derived DC may differentiate into matured forms in response to exposure to cytokines that they themselves produce upon OK432 stimulation. However, neither a cocktail of cytokines (Fig. 3A and B) nor conditioned medium from

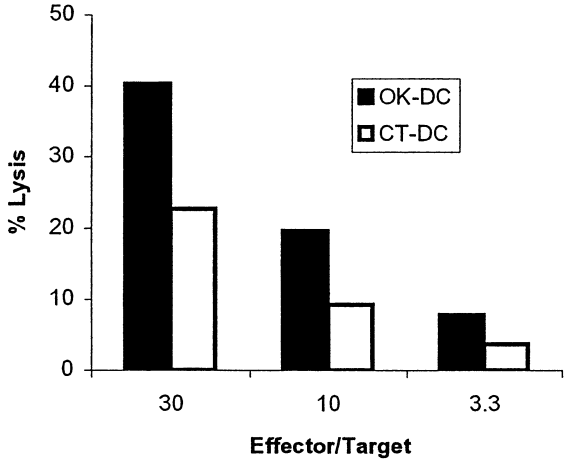

Fig. 5 OK432-stimulated DC have high antiviral CTL-inducing capacity. T cells from an HLA-A24 donor were stimulated with DC pulsed with flu peptide restricted with HLA-A24 in the presence (*solid bars*) or absence (*open bars*) of OK432 (0.01 KE/ml). Effector cells were restimulated with peptide-pulsed monocytes on day 7 and harvested 14 days after the induction for measurement of their cytotoxicity against TISI cells (HLA-A24 positive) pulsed with or without flu peptide in triplicate assays at 3 different effector-to-target ratios, as assessed by ^{51}Cr-release assays. The deviations observed in the triplicate assays were usually $< 10\%$ of the mean values. The background levels of peptide-specific cytotoxicity detected in response to TISI cells without peptide were subtracted to obtain the values shown in the figure

DC treated with OK432 (Table 4) exerted the full effects of addition of OK432 into DC cultures. These findings suggest that OK432 may promote DC maturation not only via autocrine cytokines but also by direct effects of its internalization into DC. Recently, a transducing receptor for LPS was identified in toll-like receptors 2 and 4 [18, 32], and their involvement in LPS-induced activation of DC mediated by NF-κB complex induced by toll-like receptors was shown [20]. Toll-like receptor 4 is expressed in DC [21], and thus this family of receptors might trigger OK432-induced activation of DC. Further studies to clarify the mechanism of OK432-mediated DC maturation are needed.

Myeloid DC are known to induce both Th1- and Th2-type cytokines, depending on the culture environment. One of the discriminative factors of Th1-promoting DC appears to be the level of IL-12 produced. The presence of IFN-γ leads to the development of a Th1-promoting DC subset which produces enhanced levels of IL-12 [10]. PGE_2, in contrast, inhibits the ability of DC to produce IL-12 [9]. LPS, one of the bacterial products, has been shown to stimulate the production of IL-12 and enhance the expression of co-stimulatory molecules. Our findings that OK432 was a strong inducer of IL-12 and DC maturation paralleled the findings about bacterial agents with similar properties, such as LPS [29] and *Mycobacterium tuberculosis* [7]. In cancer vaccine strategies, DC maturation needs to result in development into a Th1-polarizing subset. It has been suggested that OK432 increase the therapeutic potential of the tumor-draining lymph node cells by augmenting their Th1-type response in a mouse model [15]. Local injections of OK432 have been shown to enhance the infiltration of adoptively transferred $CD8^+$ T cells into the tumor site and synergistically induce the local production of Th1-type cytokines [26]. In a previous report, we showed that OK432 mediated the enhancement of IFN-γ production by lymphocytes and its therapeutic benefit in the approach of adoptive immunotherapy [31]. In the current study, OK-DC were shown to have high levels of expression of costimulatory molecules correlated with augmented antigen presenting capacity (Table 1) and to produce high levels of IL-12. The IL-12-inducing capacity of OK-DC was equivalent to that of LPS (data not shown). Moreover, the highest amounts of IFN-γ as well as IL-12 production by T cells were observed when T cells were stimulated with OK-DC rather than with other APC, including mature DC generated using a cytokine cocktail (Fig. 3B). In addition, OK432 augmented the recall antiviral CTL response in vitro. These facts indicate that OK432 can modulate the phenotype and function of DC, and thus that activated DC may induce Th1-type immune responses.

Our results, in addition to the previous reports that have described the therapeutic benefit of OK432 for cancer treatment, suggest that OK432 may be useful for DC-based cancer vaccine therapy. IL-12 has been shown to stimulate the cytolytic activity of NK and LAK cells, IFN-γ production, the development of cellular immunity, and the maturation of Th1 cells, and is thought to be a potent adjuvant for cancer vaccine therapy [11]. Immunization with IL-12-transduced DC induced systemic and therapeutic antitumor immunity [14]. Local production of cytokines such as IL-12 and IFN-γ by OK-DC might augment Th1-type immune responses when they are administered in vivo. We consider that OK432, a good manufacturing products (GMP) grade commercially available immunomodulatory agent with antitumor effects, stimulates the development of the cellular immune response via its action on the secretion of Th1 cytokines, which promote the differentiation of cytotoxic effector cells from DC and can be used as a potent adjuvant for DC-based cancer vaccine therapy. In a previous report, we demonstrated that the response to OK432 (in terms of in vitro cytokine production by lymphocytes) in a patient with advanced gastric cancer was lower than that in a healthy individual [31]. We are planning to investigate the effects of OK432 on DCs derived from cancer patients and the effects of OK-DC in vivo for further development of the use of OK432 as an adjuvant.

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