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T-cadinol and calamenene induce dendritic cells from human monocytes and drive Th1 polarization

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Abstract

T-cadinol and calamenene are sesquiterpenes isolated from the heartwood of *Cryptomeria japonica* and are pharmacologically active substances. Dendritic cells are pivotal in the initiation of adaptive immune responses and are recognized as key to the induction of immune responses to cancer. This study investigated the effects of T-cadinol and calamenene on the phenotypic and functional maturation of human monocyte-derived dendritic cells in vitro. Human monocytes were cultured with recombinant human granulocyte–macrophage colony-stimulation factor (GM-CSF) and recombinant human interleukin-4 (IL-4) for 6 days under standard conditions, followed by another 2 days in the presence of T-cadinol, calamenene, lipopolysaccharide (LPS), CT or nifedipine. Dendritic cells harvested on day 8 were examined using functional assays. The expression levels of CD1a, CD80, CD83, CD86 and HLA-DR on T-cadinol-primed dendritic cells or calamenene-primed dendritic cells were enhanced with a concomitant decrease in endocytic activity. T-cadinol-primed dendritic cells or calamenene-primed dendritic cells also enhanced the T cell stimulatory capacity in an allogeneic mixed lymphocyte reaction, as measured by T cell proliferation. Naïve T cells co-cultured with allogeneic T-cadinol-primed dendritic cells or calamenene-primed dendritic cells at 1:5 dendritic cells/T cell ratio turned into typical Th1 cells which produced large quantities of interferon-gamma (IFN-γ) and released small amounts of IL-4 depending on IL-12 secretion. In contrast, naïve T cells co-cultured with CT-primed dendritic cells turned into Th2 cells. T-cadinol-primed dendritic cells and calamenene-primed dendritic cells expressed the chemokine receptor CCR7 and had a high migration to macrophage inflammatory protein (MIP-3β). Intracellular Ca^{2+} mobilization in T-cadinol-primed dendritic cells and calamenene-primed dendritic cells was induced by MIP-3β. The differentiation and functional maturation of human monocyte-derived dendritic cells were not affected by nifedipine. These results suggest that T-cadinol and calamenene may be used in dendritic cells-based immunotherapy for cancer.

Keywords: Dendritic cell; Sesquiterpene; Th1 response; Cholera toxin; Th2 response

1. Introduction

Dendritic cells are potent APC, which play a central role in bridging innate and acquired immunity via direct cell–cell interactions and/or cytokine production (Banchereau and Steinman, 1998; Lanzavecchia and Sallustoa, 2001; Mellman and Steinman, 2001). Since these dendritic cells functions could be used to induce potent immune responses against certain antigens presented on dendritic cells, clinical application of dendritic cells has been initiated as a cellular immunotherapy against cancer (Steinman and Dhodapkar, 2001). The interaction of T cells with dendritic cells is crucial for directing T cell differentiation towards the Th1 or Th2 type and several factors determine the direction of T cell polarization (Romagnani, 1994; Kuchroo et al., 1995; Lederer et al., 1996; Tao et al., 1997; Iezzi et al., 1999; Tanaka et al., 2000; Vieira et al., 2000; O'garra, 2001). Dendritic cells that generate Th1 responses may be used in clinically applicable therapeutic modalities for cancer (Trinchieri and Scott, 1994) and dendritic cells that generate Th2 responses may be clinically used for transplantation, contact allergy or autoimmune disorders (O'garra and Murphy, 1993). Dendritic cells

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originate from the bone marrow and their precursor's home via the bloodstream to almost all origins, where they can be found as sentinels in an immature state with high endocytic and phagocytic capacity. Dendritic cells migrate to secondary lymphoid organs and reach as fully mature functional stage to recruit rare naïve T cells (Forster et al., 1999). Human monocyte-derived dendritic cells matured by either CD40 ligand (CD40-L), interferon-alpha (IFN-α), tumor necrosis factor-alpha (TNF-α), poly I:C (a synthetic double-stranded RNA), LPS or oligo CpG nucleotides in vitro produce high levels of IL-12 and induce Th1 cells (Cella et al., 1996, 1999; Hartmann et al., 1999). IL-12 plays a central role in the immune system, not only by augmenting the cytotoxic activity of T cells and NK cells and regulating IFN-γ production, but also by the capacity of IL-12 to promote the development of Th 1 cells (Trinchieri and Scott, 1994). A variety of preparations of dendritic cells can stimulate antitumor immunity, including dendritic cells loaded with proteins, dendritic cells fused with tumor cells and dendritic cells transduced with tumor-derived RNA or viral vectors. Analyses of the clinical results suggest that the maturation status of dendritic cells used in such protocols greatly affects the immune response that follows the treatments (Fong and Engelman, 2000). Therefore, it is important to identify factors that might affect the differentiation, maturation and function of dendritic cells.

T-cadinol and calamenene (Fig. 1), which are phytochemically classified as sesquiterpene, are isolated from the black heartwood of *Cryptomeria japonica*. The Japanese cedar, *C. japonica* D. Don (Taxodiaceae), is a widely distributed conifer called sugi in Japanese. This wood is the most popular building material used for Japanese housing. T-cadinol also is isolated from scented myrrh, which is the resin of the plant *Commiphora guidottii* Chiov., Burseraceae (Claeson et al., 1991a). This resin is widely used in Somalian traditional medicine as a remedy for treating various gastrointestinal disorders and diarrhea (Thulin and Claeson, 1991). T-cadinol has been shown to inhibit CT-induced intestinal hypersecretion in mice and electrically induced contractions of the isolated guinea pig ileum (Claeson et al., 1991b). Zygmunt et al. (1993) have shown that T-cadinol is a calcium antagonist at high doses and interacts with dihydropyridine binding sites on the voltage-operated calcium channels. Thus, T-cadinol and related compounds have pharmacologically activity. However, its immunomodulatory properties are still unknown. In the present study, the effects of T-cadinol and calamenene on human dendritic cell differentiation and function were investigated in detail.

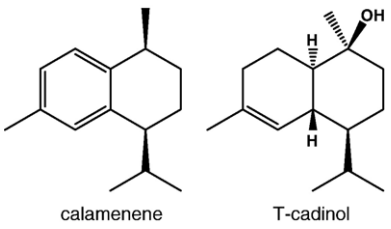

Fig. 1. Structures of T-cadinol and calamenene.

2. Materials and methods

2.1. Culture medium, reagents and monoclonal antibodies

The culture medium used in this study was RPMI 1640 (GIBCO-BRL, NY) supplemented with 10% FCS (Hyclone, Logan, UT), 10 mM Hepes, 2 mM L-gultamine, 1% penicillin–streptomycin (GIBCO-BRL, NY) and 0.1 mM non-essential amino acid. IL-4, GM-CSF and CD40-L were purchased from R&D Systems (Minneapolis, MN). LPS from *Escherichia coli* and nifedipine were purchased from Sigma-Aldrich (St. Louis, MO). CT was purchased from Boehringer Mannheim GmbH (Germany). For flow cytometry, monoclonal antibodies (mAbs) towards the following antigens were purchased from Becton Dickinson (San Jose, CA): anti-CD14-FITC (fluorescent isothiocyanate), anti-CD1a-PE, anti-CD80-PE, anti-CD83-PE, anti-CD86-PE and HLA-DR-FITC. Endotoxin levels in all agents were below 1.0 EU/ml.

2.2. Isolation of T-cadinol and calamenene from the Black Heartwood of C. japonica

T-cadinol and calamenene were prepared as previously described (Arihara et al., 2004). The purity of T-cadinol and calamenene was >99%. T-cadinol and calamenene were dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the culture medium was 0.1%, which had no effect upon the culture and the production of cytokines under the conditions used in this study. The endotoxin in T-cadinol and calamenene was removed using Endo Trap 5/1 (Endotoxin removal system, Profos AG, Regensburg, Germany).

2.3. Generation of monocyte-derived dendritic cells

All cell subsets were isolated from peripheral blood of normal healthy donors. PBMC were first isolated from heparinized whole blood by Ficoll/Isopaque/1.077 g/ml (Pharmacia, Freiburg, Germany) density gradient centrifugation (465×*g*, 45 min, 22 °C) as previously described (Grage-Griebenow et al., 1993). PBMC were further separated into monocytes and lymphocytes by counterflow centrifugation using the JE-6B-elutriator system (Beckman Instruments Inc., Palo Alto, CA) (Grage-Griebenow et al., 1993). The purity of CD14+monocytes was always more than 90%. Monocytes were cultured with GM-CSF (25 ng/ml) and IL-4 (25 ng/ml) in complete RPMI 1640 medium supplemented with 10% FCS medium for 6 days. Dendritic cells were generated by stimulating immature dendritic cells in complete medium containing GM-CSF and IL-4 for additional 2 days with various concentrations of T-cadinol or calamenene, but with LPS (100 ng/ml), CT (10 μg/ml) or nifedipine (0.5 μM). All subsequent tests were performed after harvesting the cells at day 8 and after removing the above factors by extensive washing. The medium was replenished with cytokines every 2 days. To determine the production of IL-6, IL-10 and IL-12p70 by T-cadinol-, calamenene-, LPS-, CT- or nifedipine-primed dendritic cells, dendritic cells (4×10^4 cells/well) were stimulated with

CD40-L (3.0 μg/ml) for 24 h. The cell-free supernatants were collected and frozen at −20 °C until measurement of cytokines using enzyme-linked immunosorbent assay (ELISA).

2.4. Immunophenotype studies

Dual-colour immunofluorescence was performed using the following panel of monoclonal antibodies: PE-conjugated antihuman CD1a, FITC-conjugated antihuman CD14, PE-antihuman CD80, PE-antihuman CD83, PE-antihuman CD86 and FITC-HLA-DR. Negative control was isotype-matched with irrelevant monoclonal antibodies (Becton Dickinson). Cells were re-suspended in staining medium containing PBS, 5% BSA and 0.1% NaN_3, and then fixed with 1% paraformaldehyde. Isotype controls were run in parallel. Cell debris was eliminated from the analysis by forward and side scatter gating. The samples were analyzed on FACSCalibur (Becton Dickinson) with CellQuest software (Becton Dickinson). Ten thousand cells were analyzed per sample. The results were expressed as MFI.

2.5. Endocytic activity

The endocytic activity of dendritic cells was measured as previously described (Kato et al., 2000). FITC-dextran (Sigma-Aldrich, St. Louis, MO) was used to measure mannose receptor-mediated endocytosis. Cells (1×10^5) were incubated with FITC-dextran (200 μg/ml) for 1 h at 37 °C in a water bath or at 4 °C on ice, and then washed extensively with PBS containing 5 mM EDTA and 2% FCS. The samples were analyzed on a FACSCalibur with CellQuest software. Ten thousand cells were analyzed per sample.

2.6. Allogeneic mixed lymphocyte reaction

CD4+ naïve T cells for the allogeneic mixed lymphocyte reaction assay were obtained from allogeneic PBMC using magnetic cell sorting and separation of biomolecules (MACS) beads (Miltenyi Biotec). The purity of isolated cells was >95% of CD4+ naïve T cells as determined by flow cytometry using FACSCalibur. Allogeneic CD4+ naïve T cells (5×10^4 cell/well) were co-cultured in 96-well round-bottomed culture plates with graded doses (2×10^2 to 5×10^4) of irradiated (30 Gy) dendritic cells. After 5 days, cells were pulsed with 1 μCi [^{3}H] methylthymidine per well for 16 h, then harvested and analyzed in a liquid scintillation counter.

2.7. Determination of naïve T cell polarization by dendritic cells

Irradiated (30 Gy) dendritic cells were co-cultured with naïve T cells (2.5×10^5 cell/200 μl) at 1:5 dendritic cells/T cell ratio in 96 well U-bottomed tissue culture plates (Costar, Cambridge, MA). Some cultures were supplemented with neutralizing Abs to block endogenous cytokines: anti-IL-12 (10 μg/ml, R&D Systems). On day 5, cells were washed extensively and expanded with fresh medium containing 10 U/ml of recombinant human IL-2 (IL-2) (Shionogi Pharmaceutical Company, Osaka, Japan). One hundred microliters of culture supernatant was replaced with medium of the same concentration every 3 days. On day 14, cells were washed, counted and T cells (10^6/ml) were re-stimulated for 24 h on plates coated with anti-CD3 (0.2 μg/ml; BD-Pharmingen) and anti-CD28 (2.0 μg/ml; BD-Pharmingen). The cell-free supernatants were collected and frozen at −20 °C until measurement of cytokines using ELISA.

2.8. Intracellular cytokine staining

The intracellular cytokine concentrations of the harvested T cells were measured by FACS analysis, as previously described (Takei et al., 2004). Briefly, T cells (10^6/ml) were stimulated with phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (1 μg/ml) for 5 h at 37 °C in a water bath. Brefeldin A (10 μg/ml) was added during the last 2 h of incubation to prevent cytokine secretion. Cells were collected, fixed with 1% paraformaldehyde, permeabilized with a commercial solution (BD-Pharmingen) and stained with FITC-labelled anti-IFN-γ (IgG_{2a}) and PE-labelled anti-IL-4 (IgG_1) mAbs. The samples were analyzed on a FACSCalibur with CellQuest software. Ten thousand cells were analyzed per sample.

2.9. Transmigration assay

Migration of dendritic cells induced by MIP-3β was measured using a double chamber system as previously described (Jonuleit et al., 1997). Briefly, Transwells of 5 μm pore size filters (Kurabo, Osaka, Japan) were used. MIP-3β was seeded at concentrations of 0.1, 1.0, 10 and 100 nM diluted with 500 μl of culture medium in the lower chambers of 24-well plates. Dendritic cells (1×10^5/100 μl) were then added to the upper chambers of the Transwell plates. Transwell cultures were maintained for 5 h in 5% CO_2 at 37 °C and dendritic cells migrated from the upper wells were counted using a Colter Counter. The results were expressed as net migration percent calculated as: (the number of cells that migrated into the lower chamber containing chemokine − number of cells that migrated in medium alone) / total number of cells loaded in the upper chamber × 100.

2.10. Measurement of intracellular Ca^{2+} concentration

The determination of intracellular Ca^{2+} ($[Ca^{2+}]i$) concentration was carried out as previously described (Takei et al., 1993). Briefly, 2×10^6 cells were incubated for 45 min at 37 °C with 2.5 μM of fura-2/AM in 5 mM HEPES buffer containing 140 mM NaCl, 4 mM KCl, 1.25 mM $CaCl_2$, 1 mM Na_2HPO_4, 1 mM $MgCl_2$, 11 mM glucose and 0.1% bovine serum albumin at pH 7.4. Samples of the cell suspension were placed in the cuvette. Then, MIP-3β (100 nM) was added with a microsyringe directly into the cuvette, without interrupting the recording. $[Ca^{2+}]i$ was monitored with a CAF110 spectrofluorometer (λ_{ex} = 340 nm and 380 nm, λ_{em} = 510 nm; JASCO, Tokyo).

2.11. Reverse transcription–polymerase chain reaction (RT-PCR) for CCR7

Total RNA was extracted from 1 to 2×10^6 dendritic cells using an RNeasy Mini Kit (Qiagen, Germany). The cDNA synthesized from total RNA by Moloney murin leukaemia virus reverse transcriptase (Stratagene, Austin, TX, USA) was subjected to RT-PCR using 30 cycles at 94 °C for 0.5 min, 57.5 °C for 1 min and 72 °C for 1 min for CCR7 (Takara, Tokyo, Japan). The sense and antisense oligonucleotide primers for CCR7 were 5′-CGCGTCCTTCTCATCAGCAA-3′ and 5′-GTCCCGACAGGAAGACCACT-3′, respectively. PCR products were identified by electrophoresis on 2% agarose gels that were photographed.

2.12. Statistical analysis

Statistical significance was determined by Student's unpaired *t*-test. A *p*-value of <0.05 was considered statistically significant. Values are presented as the mean±S.E.M.

3. Results

3.1. Phenotype and endocytic capacity

To study the direct effects of T-cadinol and calamenene on the maturation of sentinel dendritic cells into effector dendritic cells, human monocyte-derived dendritic cells were cultured with T-cadinol or calamenene. Human monocytes were cultured with GM-CSF and IL-4 for 6 days under standard conditions, followed by another 2 days in the presence of various concentrations of T-cadinol or calamenene. LPS and CT were used as a positive control. The resulting populations of dendritic cells were analyzed by flow cytometry. As shown in Table 1, the expression levels of CD1a, CD80, CD83, CD86 and HLA-DR as expressed by MFI on T-cadinol-primed dendritic cells and calamenene-primed dendritic cells were enhanced in a dose-dependent manner. The expression level of CD14 as expressed by MFI on day 8 was low or undetectable. Viability of cells at 10 μM of T-cadinol and calamenene was >95%. From these results, the concentrations of T-cadinol and calamenene were used at 10 μM. The expression levels of CD1a, CD 80, CD83 and HLA-DR as expressed by MFI on LPS (100 ng/ml)-primed dendritic cells and CT (10 μg/ml)-primed dendritic cells were also enhanced (Table 1). This pattern was identical with that of T-cadinol-primed dendritic cells or calamenene-primed dendritic cells. On the other hand, the expression levels of CD1a, CD80, CD83 and HLA-DR as expressed by MFI were not

Table 1
Comparison phenotypes of DC cultured with T-cadinol, calamenene, LPS and CT on day 8

	CD1a	CD80	CD83	CD86	HLA-DR
T-cadinol					
0.1 μM	65±18	110±28	32±12	331±38	834±76
1.0 μM	157±21	159±29	56±18	452±36	921±98
10 μM	183±22	208±32	81±19	586±41	1058±121
Calamenene					
0.1 μM	78±19	105±28	35±13	335±39	956±108
1.0 μM	167±29	158±31	63±16	443±41	1168±231
10 μM	210±31	221±33	109±21	601±38	1267±298
LPS (100 ng/ml)	201±20	232±36	106±20	622±41	1308±299
CT (10 μg/ml)	209±18	228±32	68±8	680±82	1412±389
Nifedipine					
0.5 μM	76±9	52±8	10±2	321±35	598±102
Immature DC	80±10	48±4	9±2	334±28	665±98
Monocyte	5±1	5±2	6±2	11±2	56±3

Data are the mean±S.E.M. of five independent experiments.

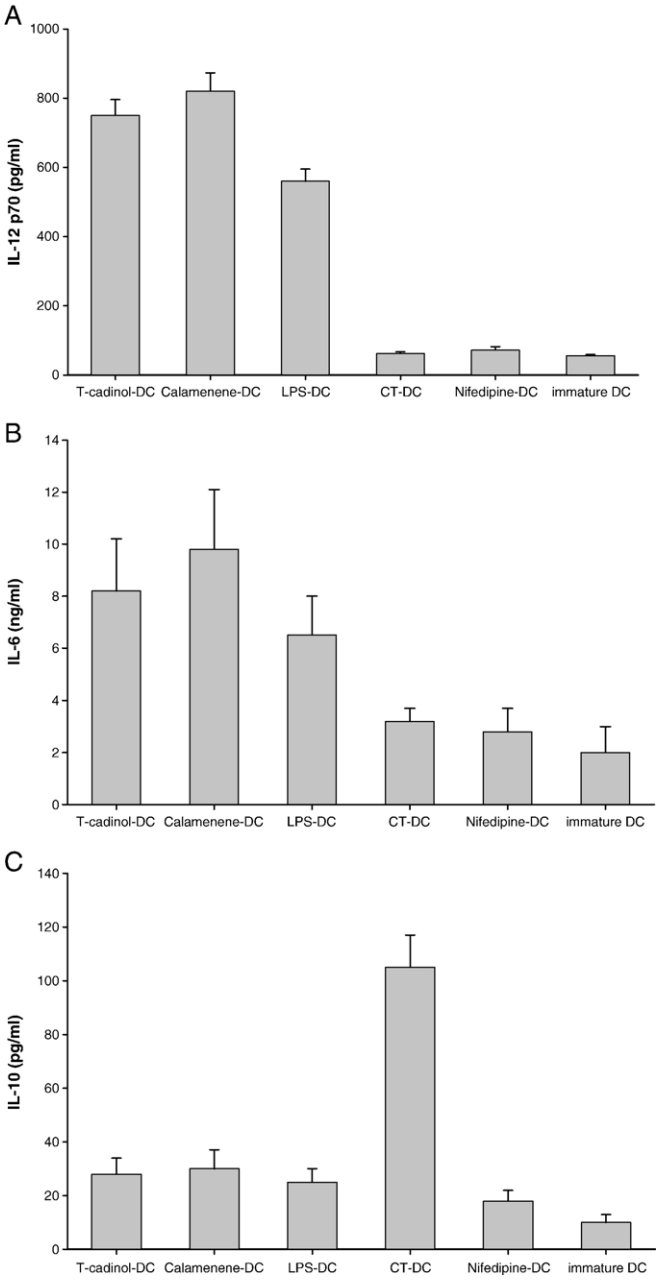

Fig. 2. Cytokine production by CD40-L-stimulated dendritic cells. Dendritic cells were generated by stimulating immature dendritic cells with T-cadinol (10 μM), calamenene (10 μM), LPS (100 ng/ml), CT (10 μg/ml) or nifedipine (0.5 μM). Cells (4×10^4 cell/well) were stimulated with the CD40-L (3.0 μg/ml) for 24 h. After 24 h, the production of IL-12p70 (A), IL-6 (B) and IL-10 (C) was measured by ELISA in culture supernatants. Data are the mean±S.E.M. of five independent experiments.

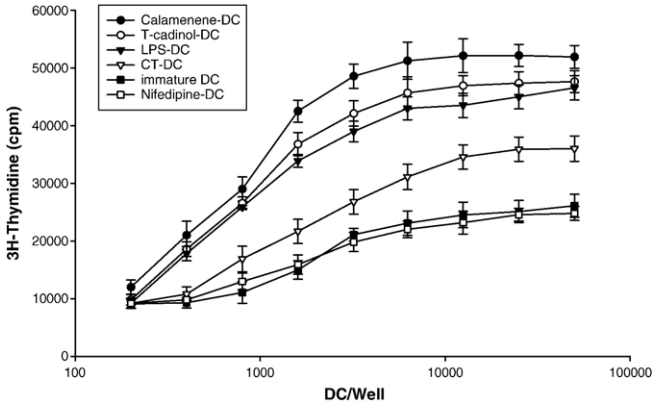

Fig. 3. Allogeneic T cell stimulatory capacity of dendritic cells differentiated with T-cadinol, calamenene, LPS, CT or nifedipine. Naïve T cells (5×10^4 cells/well) were co-cultured with graded doses of T-cadinol-, calamenene-, LPS-, CT-, or nifedipine-primed dendritic cells, and on day 5, [^{3}H]methylthymidine was added 16 h before measurement of the proliferation response. Data are the mean cpm ± S.E.M. of five independent experiments.

affected by the Ca^{2+} antagonist, nifedipine (0.5 μM) (Table 1). As control, immature dendritic cells were generated by cultivating human monocytes with GM-CSF and IL-4 for 8 days. This differentiation process is accompanied by changes in the expression of several surface markers as detailed in Table 1. Immature dendritic cells are efficient in capturing Ag and have a high level of endocytosis. To determine whether mechanisms of Ag capture could also be modulated by T-cadinol or calamenene, the endocytic activity was measured in immature dendritic cells, T-cadinol-, calamenene-, LPS- or CT-primed dendritic cells. FITC-dextran uptake mediated by T-cadinol-, calamenene-, LPS- or CT-primed dendritic cells was significantly lower than immature dendritic cells (data not shown). However, nifedipine-primed dendritic cells had still a high level of endocytic activity (data not shown). These results suggested that dendritic cells differentiated by T-cadinol or calamenene down-regulated their endocytic capacity.

3.2. IL-12p70, IL-6 and IL-10 release by activated dendritic cells

Because the level of IL-12 production by dendritic cells is a major factor driving the development of Th1 cells, we studied the influence of T-cadinol, calamenene, LPS, CT or nifedipine on IL-12 production by dendritic cells. We measured IL-12p70 production in immature dendritic cells and in dendritic cells matured for 2 days in the presence of the above factors after stimulation by CD40-L for 24 h. The production of IL-12p70 by T-cadinol-primed dendritic cells and calamenene-primed dendritic cells was more augmented than that of LPS-primed dendritic cells (Fig. 2A). In contrast, the production of IL-12p70 by CT-primed dendritic cells was low or just detectable (Fig. 2A). IL-6 is an important mediator of a wide range of biologic activities that play a critical role in the induction of proinflammatory and immune responses. Moreover, IL-10 is a pleiotropic cytokine known to have inhibitory effects on the accessory functions of dendritic cells and appears to play a central role in preventing overly pathological Th1 or Th2 responses in a variety of settings. Therefore, we also studied the production of IL-6 and IL-10 by dendritic cells differentiated under the influence of the above factors. The production of IL-6 by T-cadinol-primed dendritic cells and calamenene-primed dendritic cells was higher than that of LPS-primed dendritic cells (Fig. 2B). On the other hand, the production of IL-10 by T-

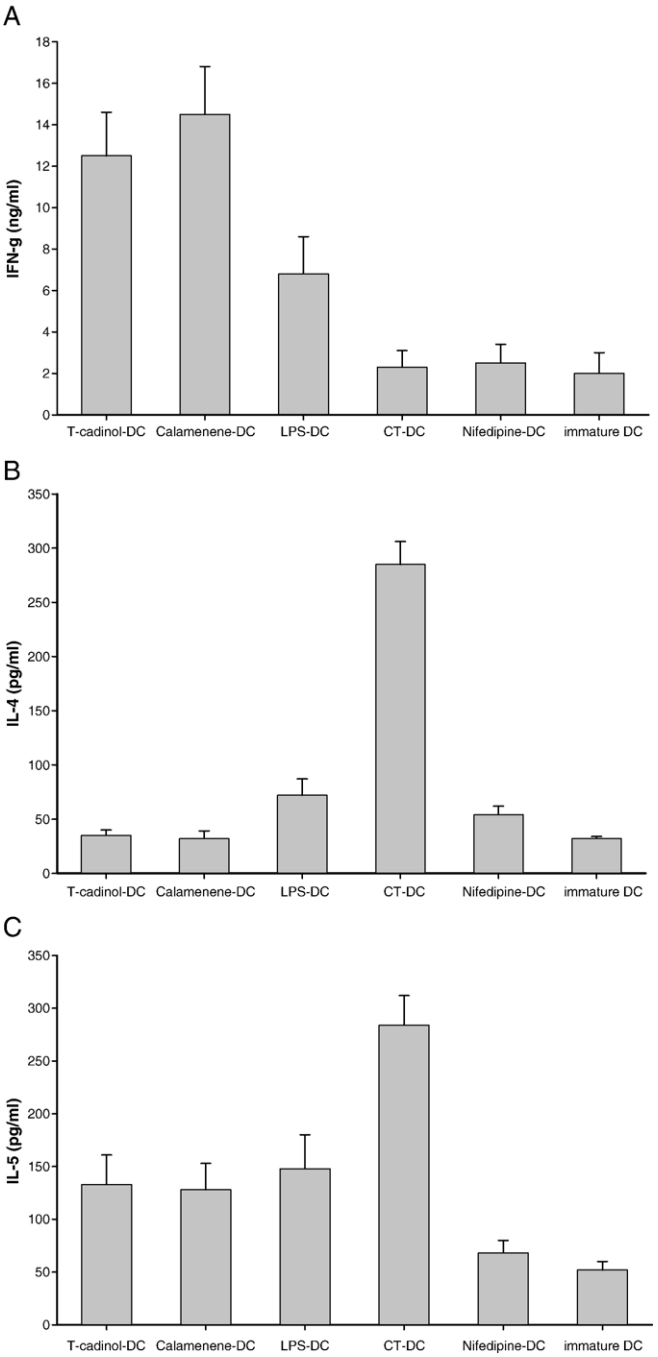

Fig. 4. Measurements of IFN-γ, IL-4 and IL-5 by ELISA in supernatant of naïve T cells stimulated by T-cadinol-, calamenene-, LPS-, CT-, or nifedipine-primed dendritic cells. Allogeneic dendritic cells were co-cultured for 5 days with naïve T cells at 1:5 dendritic cells/T cell ratio. After 9 days of expansion in IL-2, T cells were counted and re-stimulated for 24 h on plates coated with anti-CD3/CD28. IFN-γ (A), IL-4 (B) and IL-5 (C) were measured by ELISA in culture supernatants. Data are the mean ± S.E.M. of five independent experiments.

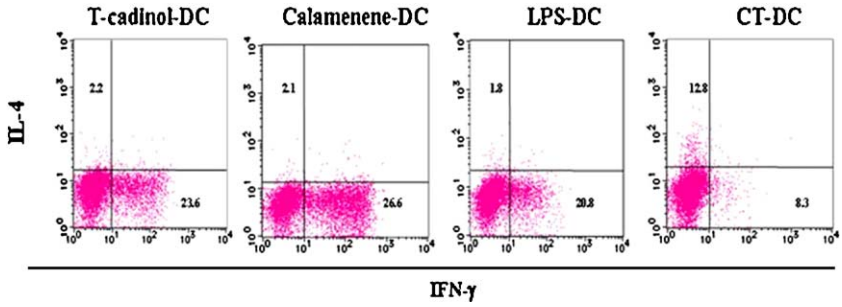

Fig. 5. T-cadinol-, calamenene- or LPS-primed dendritic cells induced the differentiation of naïve T cells to a Th1 response at 1:5 dendritic cells/T cell ratio. After 9 days of expansion in IL-2 expansion, intracellular cytokine (IFN-γ and IL-4) concentrations were measured after re-stimulation with PMA and ionomycin for 5 h. Data are one experiment representative of four independent experiments.

cadinol-, calamenene- or LPS-primed dendritic cells was low (Fig. 2C). Major enhancements of IL-10 production were caused by CT-primed dendritic cells (Fig. 2C). The production of IL-12p70, IL-6 and IL-10 was lower in immature dendritic cells and nifedipine-primed dendritic cells (Fig. 2A–C).

3.3. Immunostimulatory capacity in an allogeneic mixed lymphocyte reaction

We observed that T-cadinol-primed dendritic cells and calamenene-primed dendritic cells expressed increased levels of Ag-presenting and the expression levels of CD80, CD83, CD86 and HLA-DR as expressed by MFI. Therefore, we next compared the capacity of T-cadinol-, calamenene-, LPS-, CT- or nifedipine-primed dendritic cells to stimulate T cells in an allogeneic mixed lymphocyte reaction. Calamenene-primed dendritic cells and T-cadinol-primed dendritic cells showed higher stimulatory efficiency in an allogeneic mixed lymphocyte reaction than LPS-primed dendritic cells or CT-primed dendritic cells (Fig. 3). On the other hand, T cell stimulatory capacity of immature dendritic cells and nifedipine-primed dendritic cells in an allogeneic mixed lymphocyte reaction was low (Fig. 3).

3.4. T-cadinol-primed dendritic cells and calamenene-primed dendritic cells promote the differentiation of naïve T cells into Th1 cells at 1:5 dendritic cells/T cell ratio

We next evaluated the nature of primary allogeneic T cell responses stimulated by T-cadinol-primed dendritic cells and calamenene-primed dendritic cells. Allogeneic T-cadinol-primed dendritic cells or calamenene-primed dendritic cells co-cultured with naïve T cells at 1:5 dendritic cells/T cell ratio secreted sizeable amounts of IFN-γ (Fig. 4A), but little IL-4 (Fig. 4B) and IL-5 (Fig. 4C). Similar results were obtained with LPS-primed dendritic cells (Fig. 4A, B and C). The production of IFN-γ by naïve T cells co-cultured with T-cadinol-primed dendritic cells or calamenene-primed dendritic cells was higher than that of LPS-primed dendritic cells (Fig. 4A). Naïve T cells co-cultured with T-cadinol-, calamenene- or LPS-primed dendritic cells turned into typical Th1 cells, which produced large quantities of IFN-γ and released small amounts of IL-4. In contrast, naïve T cells co-cultured with dendritic cells differentiated with CT turned into Th2 cells producing large quantities of IL-4 and releasing small amounts of IFN-γ (Fig. 4A and B). These responses were confirmed by flow cytometry (Fig. 5). On the other hand, naïve T cells co-cultured with immature dendritic cells or nifedipine-primed dendritic cells

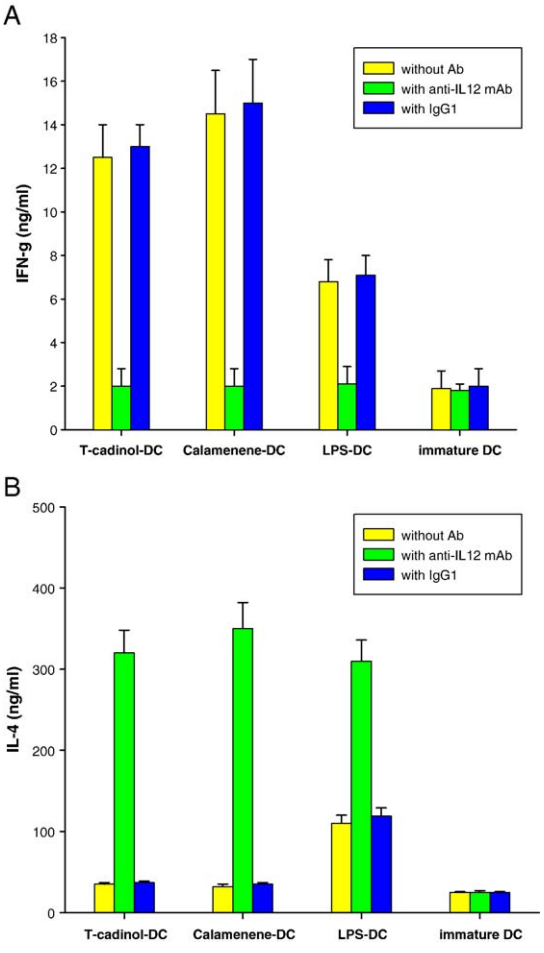

Fig. 6. Effect of anti-IL-12 on Th1 development induced by T-cadinol- or calamenene-primed dendritic cells. Allogeneic dendritic cells were co-cultured with naïve T cells at 1:5 dendritic cells/T cell ratio in the presence of control Ab or anti-IL-12 mAb (10 μg/ml). After 9 days of expansion in IL-2 expansion, T cells were counted and re-stimulated for 24 h on plates coated with anti-CD3/CD28. After 24 h, IFN-γ (A) and IL-4 (B) was measured by ELISA in culture supernatants. Data are the mean ± S.E.M. of five independent experiments.

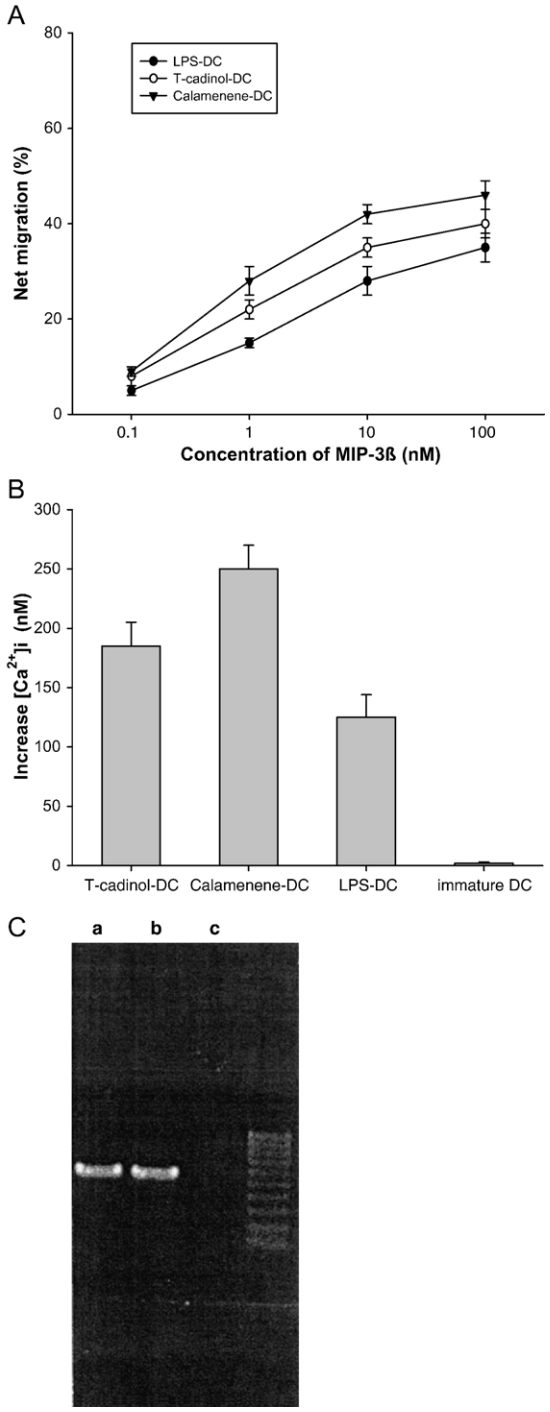

Fig. 7. Chemotaxis and intracellular Ca^{2+} mobilization in response to MIP-3β by T-cadinol-primed dendritic cells or calamenene-primed dendritic cells. (A) T-cadinol-, calamenene- or LPS-primed dendritic cells were prepared and recovered, and their migratory abilities in response to MIP-3β (0.1–100 μM) were determined in vitro. Data are the mean±S.E.M. of five independent experiments. (B) $[Ca^{2+}]i$ mobilization of T-cadinol-, calamenene- or LPS-primed dendritic cells induced by MIP-3β (100 nM). Cells were loaded with fura-2/AM and the ratio of fluorescence at 340 nm and 380 nm was monitor. Data are the mean±S.E.M. of five independent experiments. (C) cDNA prepared from T-cadinol-primed dendritic cells and calamenene-primed dendritic cells was subjected to RT-PCR-specific primer. The PCR products (573 bp) were fractionated on 2% agarose gels and visualized by ethidium bromide staining. (a) T-cadinol-primed dendritic cells, (b) calamenene-primed dendritic cells, (c) immature dendritic cells.

secreted significantly less IFN-γ, IL-4 and IL-5 (Fig. 4A–C). To analyze the contribution of dendritic cells-derived IL-12 on the development of Th1 cells, we tested the effect of a neutralizing anti-IL12 mAb in co-culture experiments, where naïve T cells were co-cultured with T-cadinol-primed dendritic cells or calamenene-primed dendritic cells. In T-cadinol-primed dendritic cells and calamenene-primed dendritic cells, neutralization of IL-12 increased the development of IL-4 producing T cells and dramatically decreased the development of IFN-γ producing T cells (Fig. 6A and B). As shown in Fig. 6A and B, similar results were obtained with LPS-primed dendritic cells. In contrast, the isotype control had no effect (Fig. 6A and B).

3.5. T-cadinol-primed dendritic cells and calamenene-primed dendritic cells are capable of migration in vitro

Because during the maturation process, dendritic cells up-regulate the synthesis of constitutive chemokines and the CCR7 receptor, we measured the migration of T-cadinol-, calamenene- or LPS-primed dendritic cells in response to MIP-3β in vitro. T-cadinol-primed dendritic cells and calamenene-primed dendritic cells had a higher migration response to MIP-3β than LPS-primed dendritic cells (Fig. 7A). Similar results were obtained with intracellular Ca^{2+} mobilization to MIP-3β (Fig. 7B). Migration response and intracellular Ca^{2+} mobilization to MIP-3β were observed with CT-primed dendritic cells (data not shown). On the other hand, migration response and intracellular Ca^{2+} mobilization to MIP-3β in immature dendritic cells (Fig. 7B) and nifedipine-primed dendritic cells (data not shown) were low or just detectable. Additionally, RT-PCR confirmed the up-regulation of the CCR7 receptor in T-cadinol-primed dendritic cells and calamenene-primed dendritic cells (Fig. 7C).

4. Discussion

The present study was performed in order to investigate whether T-cadinol or calamenene can change the phenotype and function associated with dendritic cells differentiation from human monocytes in vitro. The results presented in this paper demonstrated that culture of immature dendritic cells with T-cadinol or calamenene increase cell surface expression of CD80, CD83, CD86 and HLA-DR, while decreasing endocytic activity resulted in cells with a phenotype characterized by efficient Ag-presentation and costimulatory capacity of mature dendritic cells. Functionally, T-cadinol-primed dendritic cells or calamenene-primed dendritic cells have enhanced primary allogeneic T cell stimulatory activity in an allogeneic mixed lymphocyte reaction.

The differentiation of monocytes into dendritic cells has a critical impact on the immune response. Dendritic cells interaction with naïve T cells plays a key role in primary immune responses (Banchereau and Steinman, 1998; Mellman and Steinman, 2001) and the interaction of T cells with dendritic cells is crucial for directing T cell differentiation towards the Th1 or Th2 type (Delespesse et al., 1997). The balance of Th1 and Th2 cells is characterized by different cytokine production and homing capacity (Romagnani, 1994; Lederer et al., 1996;

O'garra, 2001), and strongly depends on the model system used (Tao et al., 1997; Tanaka et al., 2000). Cytokines are most important in the environment response and IL-12 is essential for inducing Th1 polarization and a key cytokine in antitumoral responses (Brunda et al., 1993; Hsieh et al., 1993). IL-12 is made by cell types that can process and present antigen to T cells, such as dendritic cells and macrophages, and additionally by neutrophils (Trinchieri and Scott, 1994). We showed that T-cadinol-primed dendritic cells and calamenene-primed dendritic cells polarized into Th1 via high IL-12p70 secretion upon CD40-L (T cells engagement) stimulation and demonstrated that the production of IFN-γ by naïve T cells co-cultured with T-cadinol-primed dendritic cells or calamenene-primed dendritic cells was affected by the presence of a neutralizing anti-IL-12 mAb. The reduced induction of IFN-γ after incubation with anti-IL-12 mAb indicates that IFN-γ induction is largely dependent on endogenous IL-12. These finding are in good agreement with the strong IFN-γ induction by T-cadinol-primed dendritic cells or calamenene-primed dendritic cells. Similar results were obtained with LPS-primed dendritic cells. IL-6 and IL-12 are two major cytokines that are produced during LPS stimulation and play a central role in host defense. However, the production of IL-12p70 and IFN-γ-producing Th1 cells by T-cadinol-primed dendritic cells or calamenene-primed dendritic cells was more strongly enhanced than that of LPS-primed dendritic cells. One possible explanation for these results is that LPS is a strong inducer of IL-12 and IFN-γ-producing Th1 cells in mouse compared to human. Multiple reagents have been reported to induce maturation of human dendritic cells. Human dendritic cells may more easily induce Th1 cells upon stimulation by poly I:C, dsRNA, TLR7 (R848, ssRNA) or cytokine cocktail containing TNF-α and PGE_2, and these agents have been used for the design of dendritic cells-based vaccines (Whiteside and Odoux, 2004). The Th1 cells that produce IFN-γ have been shown to exert a powerful antitumor effect, whereas a weak Th1 or a Th2 profile may have an opposite effect, that is, down-regulation of innate and acquired antitumor immunity (Brossart et al., 2000). The corollary of these observations is that a Th1 profile may be protective against tumor growth and dissemination. Moreover, a recent study describes mycoplasma as another pathogen involved in dendritic cells activation, resulting in release of IL-12, TNF-α and IL-6 (Salio et al., 2000). T-cadinol-primed dendritic cells and calamenene-primed dendritic cells provide stronger costimulatory signals and/or the proinflammatory cytokines needed for T cell activation and Th1 development. Therefore, it suggests that the effects of T-cadinol and calamenene on the production of IL-12p70 by dendritic cells and strengthening of the Th1 response by naïve T cells might, at least in part, contribute to a potential antitumor effect of T-cadinol and calamenene.

In contrast, CT-primed dendritic cells-induced naïve T cells showed a shift towards Th2 effector cell. CT is a powerful mucosal adjuvant that amplifies B cell and T cell responses to mucosally co-administered antigens, stimulating predominant Th2-type responses (Wilson et al., 1991; Xu-Amano et al., 1993). The Th2 driven response is usually IL-4-dependent (Hsieh et al., 1993). However, Gagliardi et al. (2000) have demonstrated that CT-treated dendritic cells induce Th2 polarization of naïve T cells in vitro is very likely due to the lack of IL-12 production by CT-treated dendritic cells and may be independent of IL-4. We showed that the production of IL-12 by CT-primed dendritic cells that were stimulated CD40-L was low or detectable. Therefore, it suggests that the involvement of these cells in the innate responses to Th1 response-inducing pathogens during the period preceding the initiation of adaptive immunity can create an environment rich in IL-12, which promotes the production of IFN-γ and the outgrowth of Th1 cells. It has been demonstrated that immune balance controlled by cytokines such as IL-10 and IL-12 plays an important role in immune regulation, including antitumor immunity (Trinchieri and Scott, 1994). Recent studies indicated that IL-10 inhibits the IL-12 production by dendritic cells. Our results showed that T-cadinol-primed dendritic cells and calamenene-primed dendritic cells have a decreased ability to produce IL-10, and that these dendritic cells have the potency to induce Th1 cells. On the other hand, the production of IL-10 by CT-primed dendritic cells was high and these dendritic cells polarized into Th2 cells. Therefore, it seems likely that the development of the Th1 response is controlled, at least partly, by IL-10 and IL-12 and may be a crucial polarizing factor for Th1 response development.

Claeson et al. (1991b) have demonstrated that T-cadinol relaxed contractions induced by 60 mM K+ in a dose-dependent manner and T-cadinol is a calcium antagonist, possibly interacting with the dihydropyridine binding sites on the calcium channels. The effects of T-cadinol as a calcium antagonist are at relatively high concentrations (1 mM). To avoid this effect, we used a lower concentration (10 μM) that had no marked differences in the percentage of dead cells. The lower concentration of T-cadinol or calamenene did not influence cell proliferation (Umeyama, A. and Arihara, S., unpublished observation). Moreover, dendritic cells phenotype and the production of cytokines by T cells after being cultured with dendritic cells and naïve T cells were not affected by nifedipine at 0.5 μM. Nifedipine, a dihydropyridine derivate, is a known dihydropyridine-binding site Ca^{2+} antagonist at 0.1–1.0 μM (Gurney et al., 1985). From these results, it seems likely that the effects of T-cadinol and calamenene on dendritic cells maturation process rule out direct regulation of Ca^{2+} mobilization as a mechanism.

One result of dendritic cells maturation and activation demonstrate increased motility and a potential to propensity to migrate towards lymphoid organs. During the maturation process, dendritic cells down-regulate the expression of inflammatory chemokines and their receptors and up-regulate the synthesis of constitutive chemokines and the chemokine receptor CCR7. In contrast, immature dendritic cells can produce inflammatory chemokines and express receptors for inflammatory chemokines (Höuken et al., 2004). We found that T-cadinol-primed dendritic cells and calamenene-primed dendritic cells had high migration, high calcium mobilization in response to MIP-3β and up-regulated the expression of CCR7, suggesting that T-cadinol-primed dendritic cells and

calamenene-primed dendritic cells are likely to be mature dendritic cells that have the potential to migrate in vivo. In most clinical trials using dendritic cells-based immunotherapy, immature monocyte-derived dendritic cells pulsed with tumor antigen peptides were used. However, recent observation suggests that mature dendritic cells could be a better antitumor adjuvant (Nair et al., 2003). Although the effects of T-cadinol and calamenene in vivo and the role of chemokine receptor for clinical application are not known yet, T-cadinol and calamenene appear to be a good factor to induce dendritic cells maturation, or even better in some respect, for the use in clinical dendritic cells therapy to induce strong Th1-type immune responses.

Recent studies suggested that signaling via Toll-like receptors (TLRs), newly identified receptor molecules recognizing many pathogens, are involved in the induction of anticancer immunity (Brightbill et al., 1999; Okamoto and Sato, 2003). Dendritic cells produce IL-12 in response to TLR activation. R-848/Resiquimod (TLR 7 ligand in the mouse and TLR 7/8 ligand in human) synergized with poly (I:C) (TLR 3 ligand) or LPS; (TLR 4 ligand) to produce both high levels of IL-12p70 secretion and IFN-β mRNA accumulation in mouse bone marrow-derived dendritic cells. However, the involvement of TLRs on dendritic cells matured from human monocytes by T-cadinol or calamenene requires further investigation.

5. Conclusion

We have demonstrated that T-cadinol and calamenene regulate differentiation of dendritic cells from human monocytes in combination with GM-CSF and IL-4. Moreover, dendritic cells differentiated with T-cadinol or calamenene enhance the differentiation of naïve T cells towards the Th1 type, which is dependent on IL-12 secretion. Although the molecular events leading to the effects of T-cadinol and calamenene on dendritic cells function remain to be resolved, dendritic cells appear to be potential targets for T-cadinol and calamenene. Further understanding of the mechanisms by which T-cadinol and calamenene modulate dendritic cells function may lead to the development of effective immunotherapy for cancer.

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