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Dendritic cells maturation promoted by M1 and M4, end products of steroidal ginseng saponins metabolized in digestive tracts, drive a potent Th1 polarization

Masao Takei[a,*], Eiichi Tachikawa[b], Hideo Hasegawa[c], Je-Jung Lee[d]

[a]*Division of Cellular Allergology, Research Center Borstel, Parkallee 22 D-23845, Germany*
[b]*Department of Pharmacology, School of Medicine, Iwate Medical University, Uchimaru 19-1, Morioka 020-8505, Japan*
[c]*Fermenta Herb Inc., Motohongo-cho 1-30-15, Hachioji 192-0051, Japan*
[d]*Department of Internal Medicine, Chonnam National University Medical School, Gwangju, Republic of Korea*

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Abstract

Ginseng is a medicinal herb widely used in Asian countries, and many of its pharmacological actions are attributed to the ginsenosides. Dendritic cells (DCs) play a pivotal in the initiation of T-cell-mediated immune responses, making them an attractive cellular adjuvant for use in cancer vaccines. In this study, we investigated whether M1 and M4, end products of steroidal ginseng saponins metabolized in digestive tracts, can drive DCs maturation from human monocytes in vitro. Human monocytes were cultured with GM-CSF and IL-4 for 6 days, followed by another 2 days in the presence of M1, M4 or TNF-α as a maturation stimulus. Stimulation with 20 μM of M1 or M4 increased expression level of CD80, CD83 and CD86 as expressed by mean fluorescence intensity (MFI) and decreased endocytic activity. M4-primed mature DCs also displayed enhanced T cells stimulatory capacity in a MLR, as measured by T cell proliferation. Mature DCs differentiated with M1 or M4 induced the differentiation of naïve T cells towards a helper T cell type 1 (Th1) response at DC/T (1:5) cells ratio depending on IL-12 secretion. In CTL assay, the production of IFN-γ and ^{51}Cr release on M4-primed mature DCs was more augmented than of immature DCs or TNF-α-primed mature DCs. These results suggest that M4 may be used on DC-based vaccines for cancer immunotherapy.

Keywords: Dendritic cells; Ginseng saponins; CTL

1. Introduction

The root of Panax ginseng C.A. Meyer has been widely and well used as an important component of many Chinese prescriptions called ''Kan-Pou medicine'' for more than 2000 years and is now well known as natural medicine throughout the world. It has been used as a medicine to promote perennial youth and long life, for which we do not have alternative, appropriate technical terms in modern medicine. Recently, some reports have shown that the ginseng and its major component (saponines) ameliorate the symptoms and the lesions evoked by stress (anti-stress action), for example, improvements in gastrointestinal symptoms (anorexia, dyspepsia, etc.), gastric ulcer, fatigue, boredom, anxiety and essential hypertension in stressed animals and humans [1–3]. Moreover, Yun [4] has shown that the prolonged administration of ginseng extract significantly inhibited the incidence of hepatoma and also proliferation of pulmonary tumours induced by aflatoxin B1 and urethane. It has been learned that the ginsenosides in ginseng are metabolized by gastric acid and by enzymes in intestinal bacteria when they are orally administered [5,6]. The sugar moieties of saponins are in turn hydrolyzed in the digestive tracts. The ginsenosides are classified in three groups, the protopanaxadiol, the protopanaxatriol and the oleanoic acid groups, on the basis of the chemical structures of their aglycones. Most of the ginsenosides belong to the protopanaxa-type (steroidal

Abbreviations: DC, dendritic cell; CTL, cytotoxic T-lymphocyte assay; MLR, allogeneic mixed lymphocyte reaction; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor-α; GM-CSF, granulocyte-macrophage colony-stimulation factor; IFN-γ, interferon-γ; MFI, mean fluorescence intensity; PE, phycoerythrin; FITC, fluorescent isothiocyanate; EBV, Epstein-Barr virus; Ag, antigen

* Corresponding author. Tel.: +49 4537 188561; fax: +49 4537 188403.
E-mail address: mtakei@fz-borstel.de (M. Takei).

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saponins), and the major metabolites of ginseng including the intermediates are M1, M2, M3, M5 and M12 derived from the protopanaxadiols and are M4 and M11 from the protopanaxatriols. We have recently reported that M4 inhibited the ACh-induced secretion of catecholamines from bovine adrenal chromaffin cells and this inhibition was due to the blockade of Na^+ influx through nicotinic ACh receptor-operated cation channels [7]. Thus, ginseng saponin metabolites have already been proven to be bioactive substances. Dendritic cells (DCs) are potent antigen presenting cells (APC) that play a prominent role in the development of T cell-immune responses [8–10]. In particular, the interaction of T cells with DCs is crucial for directing Th cell differentiation towards the Th1 or Th2 type and several factors determine the direction of T cell polarization [11–17]. DCs that generate Th1 responses may be used in clinically applicable therapeutic modalities for pathologic conditions that are caused by infections or malignant disorders, via secreting interleukin-2 (IL-2) and interferon gamma (IFN-γ) to facilitate T-cell-mediated cytotoxicity [18]. In contrast, DCs that generate Th2 responses may be clinically used in conditions in which Th1 responses are disturbed, e.g., transplantation, contract allergy, or autoimmune disorders, by secreting cytokines, including IL-4, IL-5, IL-6 and IL-10, to help B cells secrete protective antibodies [19]. Therefore, DCs play a key role in the initiation of immune response and are considered promising targets for immunotherapy [9]. One of the most important goal of DCs research is the development of DCs-based strategies for enhancing immune responses against tumours and infectious agents. In vitro-differentiated DCs show functional and phenotypic characteristics of immature DCs and can be further differentiated in vitro into mature DCs with TNF-α, LPS or CD40-L [9]. Thus, for immunotherapeutic applications, it appears crucial to identify factors that might affect the differentiation, maturation, and function of DCs. Because of its wide immunomodulatory properties, DCs might be potential targets for the ginseng and its major components. However, little is know about how the ginseng and its components affect DCs during the local inflammatory response or during the initiation of the immune response. In this study, we investigated the ability of M1 and M4, end products of steroidal ginseng saponins metabolized in digestive tracts, to influence the differentiation of DCs from peripheral blood monocytes.

2. Materials and methods

2.1. Culture medium, regents and monoclonal antibodies

The following culture medium used in this study was serum-free AIM-V medium (Life Technologies, Paisley, UK). Recombinant human IL-4 (IL-4), recombinant human TNF-α (TNF-α), recombinant human granulocyte-macrophage colony-stimulation factor (GM-CSF) and CD40-L were purchased from R&D systems (Minneapolis, MN). For flow cytometry, we used monoclonal antibodies towards the following antigens: anti-CD 14-FITC (fluorescent isothiocyanate), anti-CD1a-PE (phycoerythrin), anti-CD80-PE, anti-CD83-PE, anti-CD86-PE, and HLA-DR-FITC were purchased from Becton Dickinson (San Jose, CA). Endotoxin levels in all agents were quite low (<1.0 EU/ml). The company selling the reagent for removal of endotoxin was Profos AG, Regensburg, Germany.

2.2. Ginseng saponins

The ginseng saponins were supplied by the Korea Tobacco & Ginseng Corp. (Seoul, Republic of Korea). The purities of the ginsenosides were checked by thin-layer chromatography and nuclear magnetic response according to the method of Kawashima and Samukawa and the purity of samples was >95% [20]. The metabolites of the ginseng saponins (M1 and M4) were prepared as previously described [6]. The metabolites were dissolved in dimethylsulfoxide. The concentration of dimethylsulfoxide in the culture medium was 0.1%, which had no effect upon the culture and the productions of cytokines under the conditions used in this study. The endotoxin in the M1 and M4 was removed using Endo Trap 5/1 (Endotoxin removal system, Profos aq, Germany).

2.3. Generation of monocyte-derived DCs

All cell subsets were isolated from peripheral blood of normal healthy donors. Peripheral blood mononuclear cells (PBMC) were first isolated from heparinized whole blood by Ficoll/Isopaque/1.077 g/ml (Pharmacia, Freiburg, Germany) density gradient centrifugation ($465 \times g$, 45 min, 22 °C) as previously described [21]. PBMC were further separated into monocytes and lymphocytes by counter flow centrifugation using the JE-6B-elutriator system (Beckman Instruments Inc., Palo Alto, CA) [21]. 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 serum-free AIM-V medium for 6 days. Mature DCs were generated by stimulating immature DCs in serum-free AIM-V medium containing GM-CSF and IL-4 for additional 2 days with various concentrations of M1 and M4, or for 2 days with TNF-α (25 ng/ml). All subsequent tests were performed after harvesting the cells at day 8 and after removing GM-CSF, IL-4, M1, M4 and TNF-α by extensive washing. The medium was replenished with cytokines every 2 days. Mature DCs (3×10^4 cells) were then prepared on cytospin with centrifugation at 400 revolutions per minute (rpm) for 5 min. These slides were dried, fixed with Carnoy's solution (ethanol: acetic acid: chloroform = 6:1:3), stained with May–Grünwald–Giemsa stain and visualized by light microscopy. To determine the

production of IL-6 and IL-12p70 by M1- or M4-primed DCs, mature DCs (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: phycoerythrin (PE)-conjugated antihuman CD la (Becton Dickinson), fluorescent isothiocyanate (FITC)-conjugated antihuman CD 14 (Becton Dickinson), PE-antihuman CD80 (Becton Dickinson), PE-antihuman CD83 (Becton Dickinson), PE-antihuman CD86 (Becton Dickinson) and FITC-HLA-DR (Becton Dickinson). 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% Para-formaldehyde. Isotype controls were run in parallel. Cell debris was eliminated from the analysis by forward and side scatter gating. The samples were acquired on FACSCan (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson). Ten thousand cells were analyzed per sample. The results were expressed as a mean fluorescence intensity (MFI).

2.5. Allogeneic mixed lymphocyte reaction (allo MLR)

CD4+naïve T cells for the allo MLR assay were obtained from allogeneic PBMC using MACS beads (Miltenyi Biotec). The purity of isolated cells was >95% of CD4+naïve T cells as determined by flow cytometry using FACSCan (Becton Dickinson). Allogeneic CD4+naïve T cells (5×10^4/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) mature DCs. After 5 days, cells were pulsed with 1 uCi [^{3}H]-methylthymidine per well for 16 h, then harvested and analyzed in a liquid scintillation counter. The results were expressed as the mean cpm ± the S.E. of results obtained with five different samples, each performed in triplicate.

2.6. Purification of naïve T cells and DC-T cell cocultures

Naïve CD4+CD45RA+T cells for cytokines and the intracellular cytokines assay were isolated from allogeneic PBMC using MACS beads (Miltenyi Biotec). The purity of isolated cells was >95% for naïve CD4+CD45RA+T cell as determined by flow cytometry using FACSCan (Becton Dickinson). Naïve CD4+Th cells (2.5×10^5 cells/200 μl) were co-cultured with irradiated (30 Gy) mature DCs at 1:5 stimulator (DCs)/responder (T cells) ratio in 98 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 System). On day 5, cells were washed out completely and expanded with fresh medium containing 10 U/ml of human rIL-2 (Shionogi Pharmaceutical Company, Osaka, Japan). One hundred microliters of culture supernatant were replaced with medium of the same concentration every 3 days. On day 14, cells were washed, counted, and T cells (10^6 ml^{-1}) were re-stimulated with anti-CD3 (0.2 μg/ml; BD-Pharmingen) and anti-CD28 (2.0 μg/ml; BD-Pharmingen) for 24 h. The cell-free supernatants were collected and frozen at −20 °C until measurement of cytokines using ELISA.

2.7. Intracellular cytokine staining

The intracellular cytokine concentrations of the harvested T cells were measured by FACS analysis, as previously described [22]. Briefly, T cells (10^6 ml^{-1}) were stimulated with phorbol myristate acetate (PMA) (10 ng/ml) and ionomycin (1 ng/ml) for 5 h in a 37 °C 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-Phrmaingen), and stained with FITC-labelled anti-IFN-γ (IgG2a) and PE-labelled anti-IL-4 (IgG1) mAbs. The samples were acquired on a FACSCan (Becton Dickinson) and analyzed with CellQuest software (Becton Dickinson). Ten thousand cells were analyzed per sample.

2.8. Cytokine measurements

Measurements of IFN-γ, IL-4, IL-5, IL-6, IL-10 and IL-12p70 were determined by ELISA Kit (R&D System). The limits of detection of these ELISAs are as follows: IFN-γ (15.6 pg/ml), IL-4 (0.25 pg/ml), IL-5 (7.8 pg/ml), IL-6 (0.2 pg/ml), IL-10 (7.8 pg/ml) and IL-12p70 (30.2 pg/ml).

2.9. Cytotoxic T-lymphocyte (CTL) assay

Details of the method used in this assay have been previously described [23]. Briefly, immature DCs and mature DCs (5×10^5) cultured from monocytes of HLA-A24$^+$ donors were loaded with 10 μM Epstein-Barr virus (EBV)-derived peptide (TYGPVFMCL) for 2 h at 37 °C in 5% CO_2. EBV-derived peptide can bind to HLA-A2402 as reported by Lee et al. [24]. In 24 well plates, autologous CD8+T cells were co-cultured as effector cells at a ratio of 2:1 with DCs in 2 ml of medium (RPMI 1640:AIM-V = 1:1) containing 10% FCS (Sigma; St. Louis, MO), 1% penicillin-streptomycin, and 0.1 mM nonessential amino acid supplemented with 100 U/ml IL-2 for 10 days; half the medium was changed every 3 days. BEC-2 cell lines (HLA-A2402) and Bamb-2 cell

lines (HLA-A1/A26) were generated from EBV-transformed B-lymphoblastoid cell lines which were generated from an EBV+ healthy donor. The BEC-2 cells and Bamb-2 cells as target cells were loaded at a concentration of 1×10^6 cells/ml with 10 μM EBV peptide and incubated for 2 h at 37 °C in 5% CO_2. Effector cells were co-cultured with target cells at effector-to-target ratios of 2:1, 10:1 and 20:1 in 96 well round-bottomed culture plates (total volume of 200 μl) in duplicate. After overnight incubation at 37 °C in 5% CO_2, IFN-γ concentration of the supernatant was determined by ELISA (R&D System).

2.10. ^{51}Cr release assay

Details of the methods used in this assay have been previously described [25]. Briefly, effectors cells were incubated with ^{51}Cr labelled target cells (1×10^4) at various effector-to-target cell ratios for 6 h, followed by supernatant harvesting for measuring radioactivity using an automatic γ counter. This assay was done in triplicate.

2.11. Statistical analysis

Values are presented as the mean ± the S.E. The Mann–Whitney *U*-test was used to compare values between subgroup using SPSS 10.0 software P values <0.05 were considered statistically significant.

3. Results

3.1. Morphology, phenotype and endocytic capacity

To study the direct effects of M1 and M4 on the maturation of sentinel DCs into effector DCs, human monocytes derived DCs were cultured with M1 or M4. Human monocytes were cultured with GM-CSF and IL-4 for 6 days, followed by another 2 days in the presence of various concentrations of M1 or M4 as a maturation stimulus. As a positive control, human monocytes were cultured with GM-CSF and IL-4 for 6 days, followed by another 2 days in the presence of TNF-α.

With May–Grünwald–Gimsa staining, cells cultured with M1 or M4 showed similar typical features of mature DCs (large, veiled, non-adherent appearance and high motile) (data not shown). These mature DCs showed grossly the same morphology as TNF-α-primed mature DCs. The resulting population of immature DCs and mature DCs were analyzed by flow cytometry. The expression level of CD 14 and CD83 as expressed by MFI on DCs differentiated from human monocytes after culture with GM-CSF and IL-4 at 6 days were 3.1 ± 0.2 and 5.2 ± 1.5, respectively. These results suggested that the majority of the cells at 6 days were immature DCs. The expression level of CD83 as expressed by MFI on mature DCs differentiated from immature DCs after culture with 1, 10 and 20 μM of M1 for 2 days were 48 ± 13, 62 ± 18 and 82 ± 12, respectively. The expression level of CD83 as expressed by MFI on mature DCs differentiated from immature DCs after culture with 1, 10 and 20 μM of M4 for 2 days were 48 ± 4, 69 ± 9, 106 ± 32, respectively. The expression levels of CD80, CD 86 and HLA-DR as expressed by MFI on mature DCs differentiated from immature DCs after cultured with M1 or M4 were enhanced in a dose-dependent manner (Table 1). On the other hand, the expression level of CD83 as expressed by MIF on mature DCs differentiated with TNF-α (25 ng/ml) for 2 days was 82 ± 18. This pattern was identical with that of M1- or M4-primed mature DCs. The expression level of CD 14 as expressed by MFI was low or detectable. Typical data of phenotypes are shown in Fig. 1. From these results, the optimal concentration of M1 or M4 was determined as a dose of 20 μM. Immature DCs are efficient at Ag capture and have a high level of endocytosis. Upon maturation and concomitant with an increase in Ag presenting function, DCs have a reduced capacity for Ag capture via endocytic activity. To determine whether mechanisms of Ag capture could also be modulated by M1 and M4, the endocytic activity was measured in immature DCs, M1-, M4- or TNF-α-primed DCs. FITC-dextran uptake mediated by M1-, M4- or TNF-α-primed DCs was significantly lower than immature DCs (data not shown). These results suggested that mature DCs differentiated by M1 or M4 down-regulated their endocytic capacity.

Table 1
Comparison of phenotypes of mature DCs cultured with M1, M4 or TNF-α on day 8

	CD1a	CD80	CD83	CD86	HLA-DR	CD14
M1						
1 μM	62 ± 19	109 ± 13	48 ± 13	398 ± 12	986 ± 86	48 ± 5
10 μM	198 ± 25	148 ± 7	62 ± 18	458 ± 35	1081 ± 63	49 ± 6
20 μM	183 ± 12	203 ± 14	82 ± 12	560 ± 342	1190 ± 132	45 ± 6
M4						
1 μM	81 ± 32	102 ± 6	48 ± 4	348 ± 38	1056 ± 856	53 ± 4
10 μM	122 ± 18	148 ± 6	69 ± 9	465 ± 15	1189 ± 142	51 ± 8
20 μM	212 ± 40	208 ± 6	106 ±32	592 ± 48	1334 ± 105	50 ± 7
TNF-α	196 ± 48	209 ± 9	82 ± 18	620 ± 43	1395 ± 125	41 ± 4

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

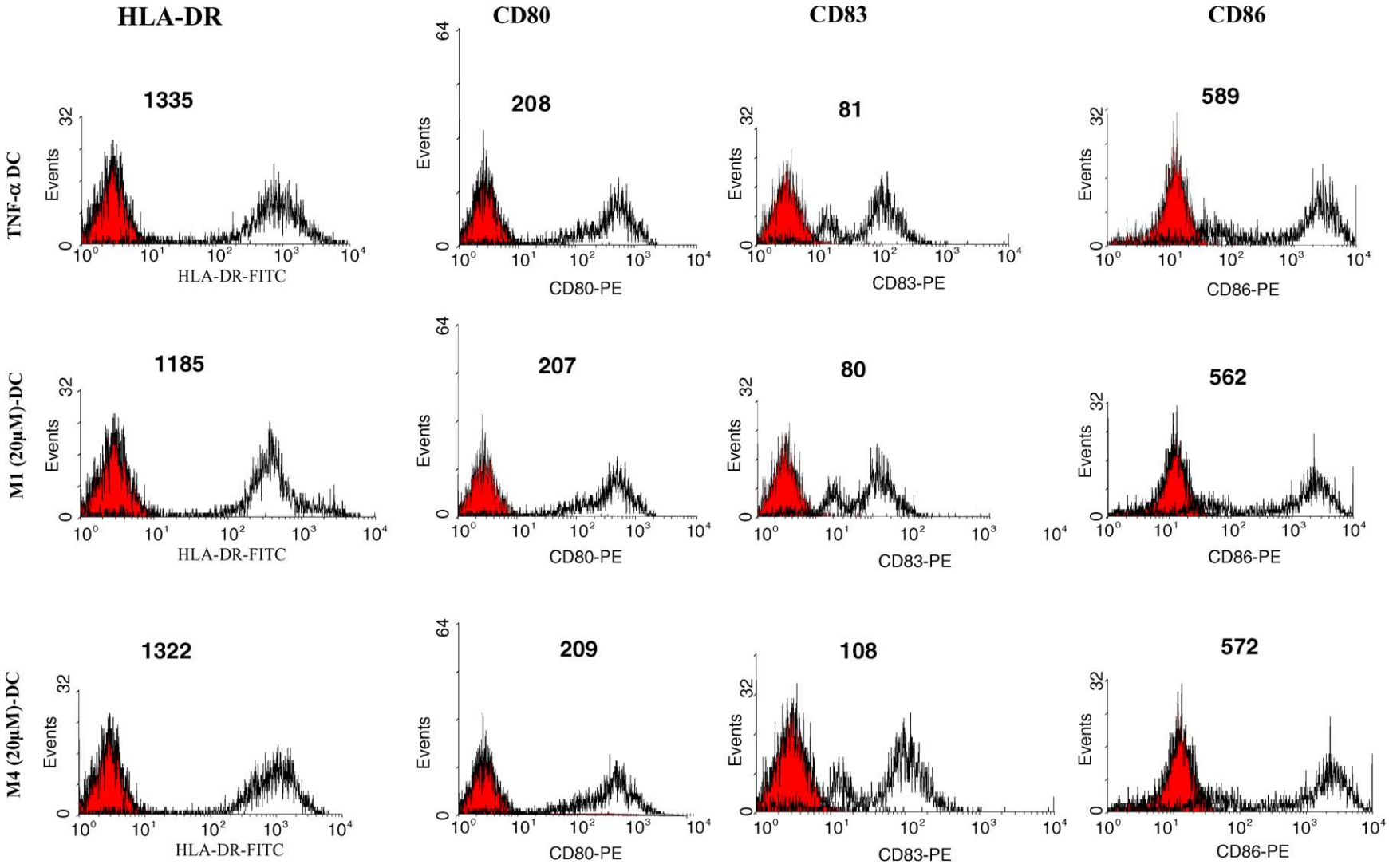


Fig. 1. Phenotype of mature DCs differentiated with TNF-α, M1 or M4. Mature DCs were generated by stimulating immature DCs with M1 (20 μM), M4 (20 μM) or TNF-α (25 ng/ml) and then were stained with FITC or PE-conjugated Abs against HLA-DR, CD80, CD83 and CD86 as described in Section 2. Solid histograms show the back ground staining with isotype-controls mAb, and open histograms represent specific staining of the indicated cell surface markers. The value indicated on the histogram is the MFI of the cells stained with the marker-specific Ab. Data are one experiment representative of five independent experiments.

3.2. IL-12p70 and IL-6 release by activated myeloid DCs

Because the level of IL-12 production by myeloid DCs during activation of naïve Th cells is a major factor driving the development of Th1 cells, we studied whether TNF-α-, M1- or M4-primed DCs are associated with high or low IL-12 production. We measured IL-12p70 and IL-6 production from mature DCs and immature DCs that were stimulated by CD40-L for 24 h. M1- or M4-primed mature DCs enhanced the IL-12p70 production (Fig. 2A). The production of IL-12p70 was consistently higher in M4-primed mature DCs. It is proposed that targeting of IL-6, an immunoregulatory cytokine, may be an attractive treatment for inflammatory diseases. We also studied whether TNF-α-, M1- or M4-primed mature DCs are associated with a high or low IL-6 production. The production of IL-6 by M1- or M4-primed mature DCs after stimulation with CD40-L for 24 h was a higher than that of TNF-α-primed mature DCs (Fig. 2B). On the other hand, the productions of IL-12p70 and IL-6 by immature DCs was a lower (Fig. 2A and B).

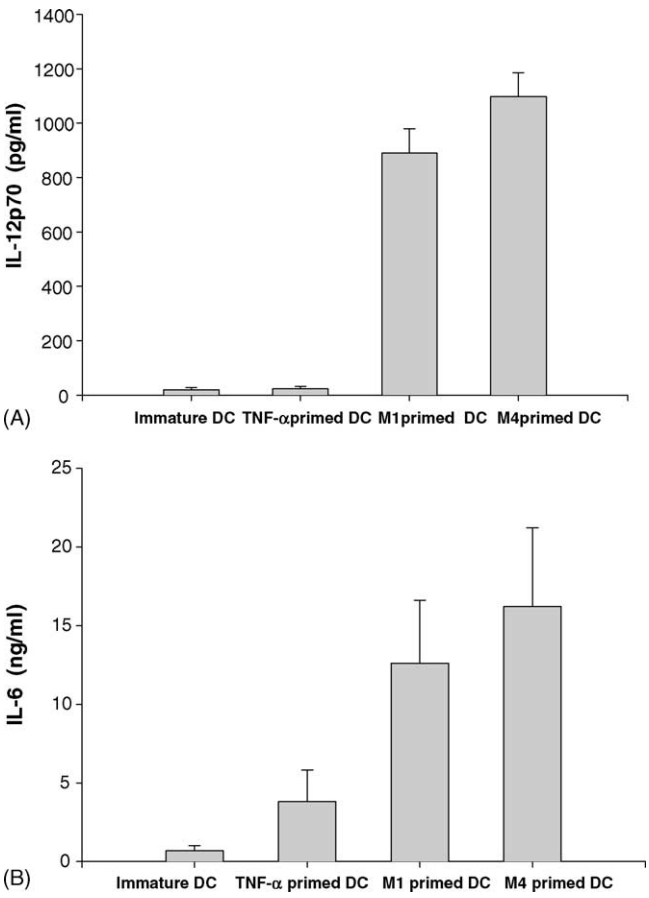


Fig. 2. Cytokine production by CD40 ligand-stimulated DCs. Mature DCs were generated by stimulating immature DCs with M1 (20 μM), M4 (20 μM) or TNF-α (25 ng/ml). As a control, immature DCs were used. Cells (4×10^4 cells/well) were stimulated with the CD40-L (3.0 μg/ml) for 24 h. After 24 h, the production of IL-12p70 (A) and IL-6 (B) were measured by ELISA in culture supernatants. Data are the mean ± S.E. of five independent experiments.

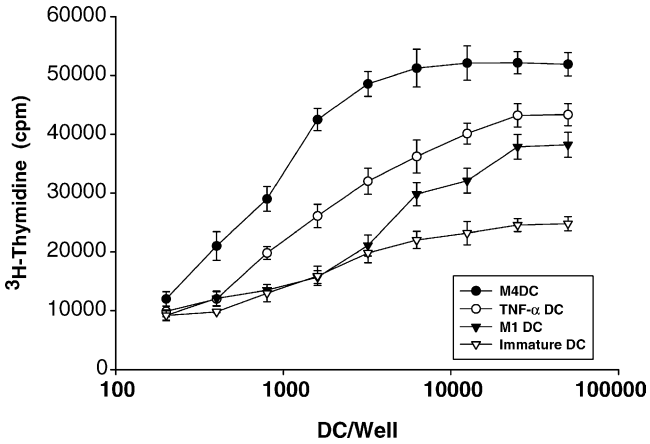


Fig. 3. Allogeneic T-cell stimulatory capacity of mature DCs differentiated with M1, M4 or TNF-α. CD4+naïve T cells (5×10^4 cells/well) were co-cultured with graded doses of mature DCs, and on day 5, [^{3}H]-methylthymidine was added 16 h before measurement of the proliferation response. As a control, immature DCs were used. Data are the mean cpm ± S.E. of five independent experiments.

3.3. Immunostimulatory function in the mixed leukocyte reaction

We observed that M1- or M4-primed DCs expressed increased levels of Ag-presenting and costimulatory molecules. Therefore, we next compared the capacity of M1-, M4- or TNF-α-primed DCs to stimulate T cells in a MLR. Mature DCs differentiated from immature DCs after culture with M4 showed higher stimulatory efficiency in a MLR than TNF-α-primed DCs and immature DCs (Fig. 3).

3.4. M1- and M4-primed mature DCs promote the differentiation of naïve T cells in to Th1 at 1:5 stimulator/responder

We next evaluated the nature of primary allogeneic T cells responses stimulated by mature DCs differentiated with TNF-α, M1 or M4. Naïve CD4+CD45RA+T cells were co-cultured for 5 days with mature DCs at stimulator/responder ratios of 1:5. After 9 days of expansion in IL-2 (10 U/ml), cells were washed, counted, and re-stimulated with anti-CD3 and anti-CD28 for ELISA or PMA and ionomycin for intracellular cytokine staining. T cell-derived cytokines were measured by ELISA and intracellular FACS analysis. In five independent experiments, T cells were co-cultured with allogeneic M4-primed mature DCs at stimulator/responder ratio of 1:5 secreted sizeable amounts of IFN-γ (13.8 ± 3.5 ng/ml) (Fig. 4A), but little IL-4 (39.5 ± 9.8 pg/ml) (Fig. 4B), IL-5 (95.3 ± 7.5 pg/ml) (Fig. 4C) and IL-10 (32.4 ± 7.6 pg/ml) (Fig. 4D). On the other hand, when T cells were co-cultured with allogeneic

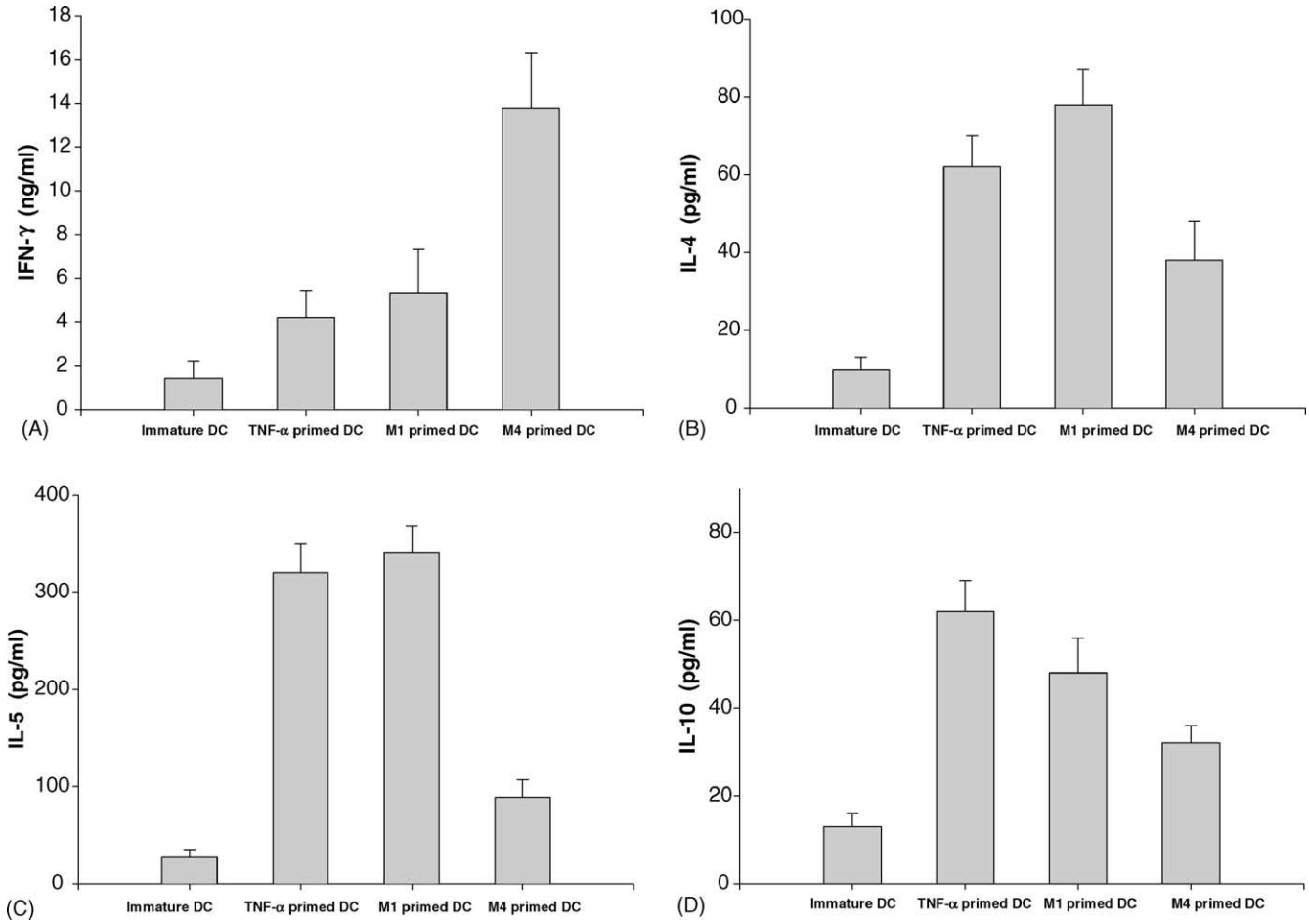


Fig. 4. Measurements of IFN-γ, IL-4, IL-5 and IL-10 by ELISA in supernatant of naïve CD4+CD45RA+T cells stimulated by M1-, M4- or TNF-α-primed mature DCs. Human CD4+CD45RA+naïve T cells were co-cultured for 5 days with allogeneic mature DCs at a high (1:5) stimulator/responder ratio. After 9 days of expansion in IL-2, T cells were counted and re-stimulated with anti-CD3 and anti-CD28 for 24 h. As a control, immature DCs were used. IFN-γ (A), IL-4 (B), IL-5 (C) and IL-10 (D) were measured by ELISA in culture supernatants. Data are the mean ± S.E. of five independent experiments.

immature DCs, they secreted significantly less INF-γ (0.92 ± 0.55 ng/ml). Naïve CD4+T cells were co-cultured with mature DCs differentiated with M4 turned into typical Th1 cells which produced large quantities of IFN-γ and released small amounts of IL-4. This Th1 response was confirmed by flow cytometry. As shown in Fig. 5, M4-primed mature DCs induced more IFN-γ producing cells than IL-4 (24.8% versus 1.8%). The production of IFN-γ and IL-4 by M1-primed mature DCs showed similar results (10.4% versus 1.2%) (Fig. 5). Similar results were obtained with TNF-α-primed mature DCs (Fig. 5). To analyze the contribution of DCs-derived IL-12 to the development of Th1 cells, we tested the effect of neutralizing anti-IL12 mAb in co-cultures of naïve Th cells and M1- or M4-primed mature DCs. Neutralization of IL-12 increased the development of IL-4 producing Th cells and dramatically decreased the development of IFN-γ producing Th cells (Fig. 6A). In contrast, the production of IFN-γ and IL-4 by M1-primed or M4-primed mature DCs was not influenced when IgG1 was used instead of anti-IL-12 mAb (Fig. 6A and B).

3.5. Mature DCs cultured with M4 augment the cytotoxicity of CD8+T cells against BEC-2 target cells

We compared the CTL responses of autologous CD8+T cells supported by mature DCs differentiated with TNF-α, M1 or M4. The production of IFN-γ by M4-primed mature DCs was strongly augmented (Fig. 7A). The production of IFN-γ on M1-primed mature DCs was more augmented than that of immature DCs (Fig. 7A). The production of INF-γ was dependent on the increased number of effector cells. Similar results were obtained with ^{51}Cr release assay to measure specific lysis of target cells (Fig. 7B). On the other hand, production of IFN-γ and ^{51}Cr release was not observed when BamB2 (HLA-A24-) was used as a target cell (negative control) (Fig. 7A and B).

4. Discussion

We analyzed the effects of M1 and M4, end products of steroidal ginseng saponins metabolized in digestive tracts,

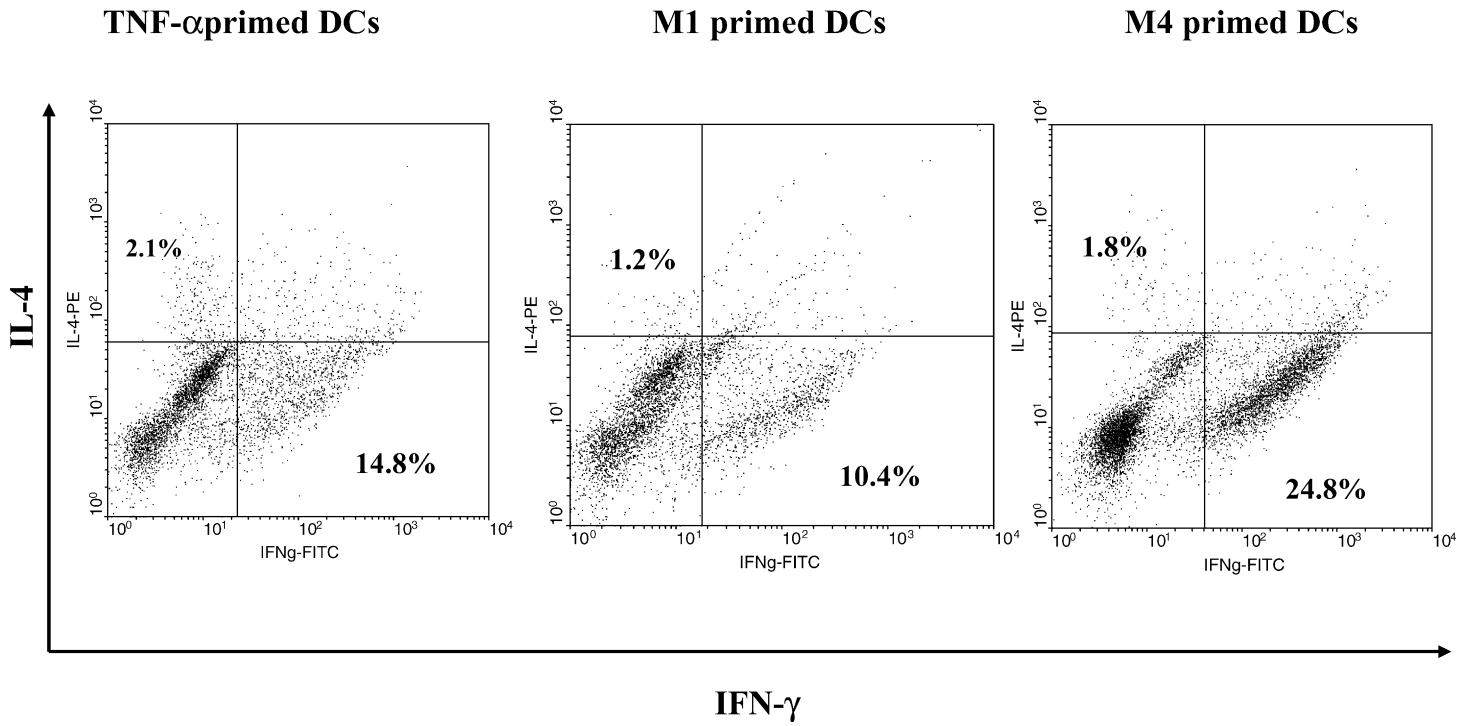


Fig. 5. M1-, M4- and TNF-α-primed mature DCs induced the differentiation of naïve CD4+CD45RA+T cells to a Th1 response at a high ratio of DC/T cells (1:5). After 9 days of expansion in IL-2, intracellular cytokine (IFN-γ and IL-4) concentrations were measured after re-stimulation with PMA and ionomycin for 5 h. Data are one experiment representative of four independent experiments.

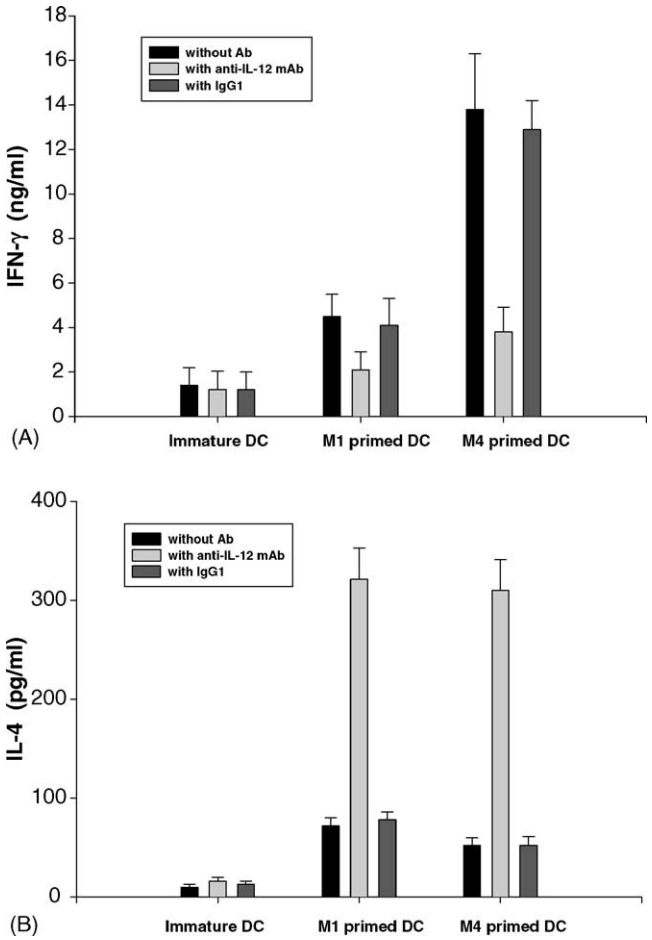


Fig. 6. Effect of anti-IL-12 on Th1 development induced by M1- or M4-primed mature DCs. Human naïve T cells were co-cultured with allogeneic mature DCs at 1:5 stimulator/responder ratio in the presence of control Ab or anti-IL-12 mAb (10 μg/ml). After 9 days of expansion in IL-2, T cells were counted and re-stimulated with anti-CD3 and anti-CD28 for 24 h. After 24 h, IFN-γ (A) and IL-4 (B) was measured by ELISA in culture supernatants. Data are the mean ± S.E. of five independent experiments.

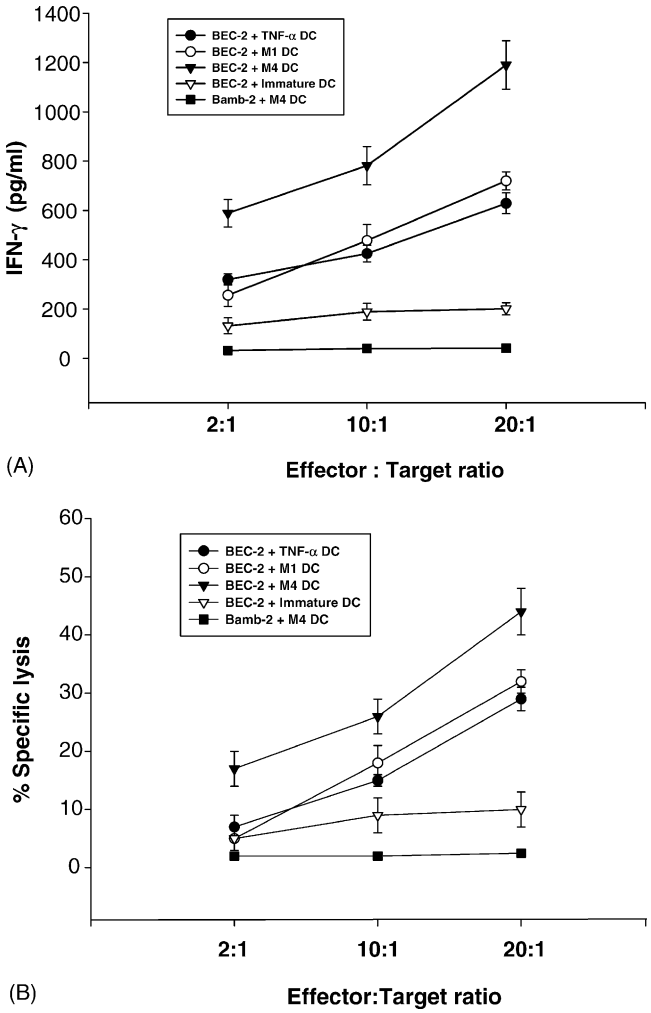
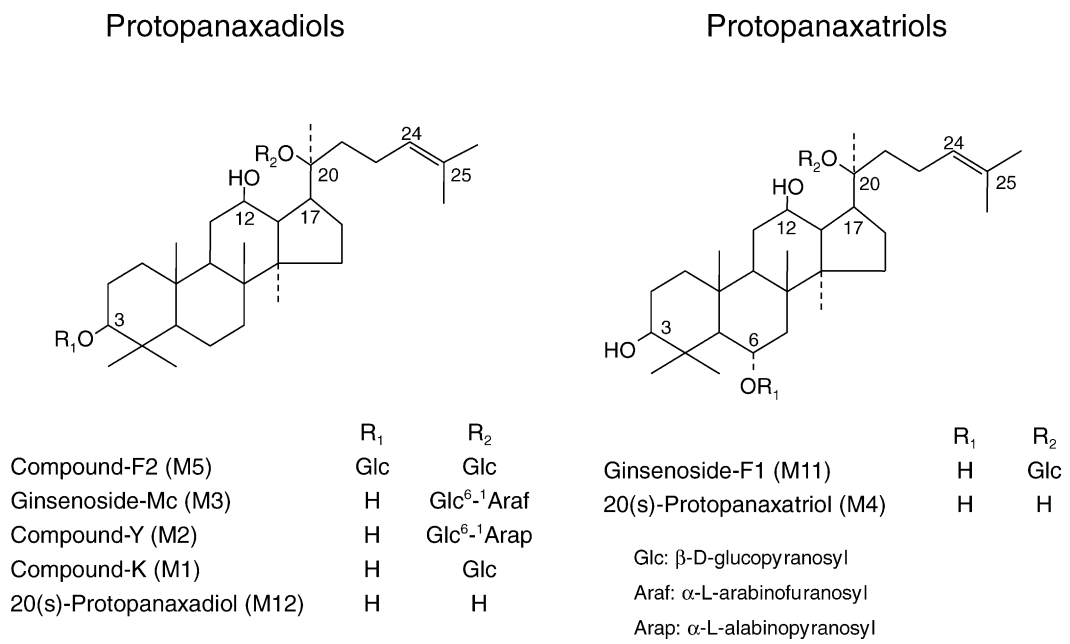


Fig. 7. Autologus CD8+T cells with mature DCs showed higher cytolytic activity against BEC-2 target cells at a high effector-to-target ratio than against Bamb-2 target cells. Autologous CD8+T cells were co-cultured with M1-, M4- or TNF-α-primed mature DCs plus with EBV-derived peptide, expanded with 100 U/ml IL-2, and, on day 10, re-stimulated with BEC-2 or Bamb-2 target cells at various effector-to-target ratios. After overnight culture, the IFN-γ concentration in the supernatant was measured by ELISA (A), and specific lysis was measured by ^{51}Cr release assay (B). Data are the mean ± S.E. of five independent experiments.

on human monocyte-derived DCs. The differentiation of monocytes into DCs has a critical impact on the immune response. During differentiation, DCs up-regulate the expression of MHC class I, MHC class II and costimulatory molecules, and thus increase their efficiency as APC. Equally important, activated DCs released cytokines that can direct the adaptive T cells responses towards either a Th1 or Th2 pattern [16,17]. Here we have demonstrated that culture of immature DCs with M1 or M4 increase cell surface expression of CD80, CD83, CD86 and HLA-DR, while decreasing endocytic activity, resulted in cells with a phenotype characterized by the efficient Ag-presenting and costimulatory capacity of mature DCs. Functionally, M1- or M4-primed mature DCs have enhanced T cells stimulatory activity in a MLR.

DC interaction with naïve T cells plays a key role in primary immune response [8,9] and the interaction of T cells with DCs is crucial for directing Th cell differentiation towards the Th1 or Th2 type [26]. Th1 and Th2 cells represent terminally differentiated effector cells characterized by different cytokine production and homing capacity [15–17]. Th1 development depends on the route of immunization, the nature and the concentration of Ag, and the balance between IL-4 and IL-12 at priming [19]. Langenkamp et al. [27] have shown earlier that maturation of DCs by LPS in vitro primes for a decreased ability to produce IL-12 and that these DCs have the potency to induce mixed populations of Th1 and Th2 cells. Moreover, the balance between Th1 and Th2 cells strongly depends on the model system used, because it varies with Ag level [11], as well as with number [12], and source [13] of APC and is subject to significant donor variability. Mature DCs induced by M1 or M4 at a high ratio (1:5) showed a shift towards Th1 polarization as judged by elevated IFN-γ and less of IL-4



	R_1	R_2
Compound-F2 (M5)	Glc	Glc
Ginsenoside-Mc (M3)	H	Glc^{6}-^{1}Araf
Compound-Y (M2)	H	Glc^{6}-^{1}Arap
Compound-K (M1)	H	Glc
20(s)-Protopanaxadiol (M12)	H	H

	R_1	R_2
Ginsenoside-F1 (M11)	H	Glc
20(s)-Protopanaxatriol (M4)	H	H

Glc: β-D-glucopyranosyl
Araf: α-L-arabinofuranosyl
Arap: α-L-alabinopyranosyl

Fig. 8. Structures of ginseng saponins.

by ELISA and intracellular cytokine staining. M1- or M4-primed mature DCs polarized into Th1 via high IL-12 secretion upon CD40 ligation (T cells engagement), since the production of IFN-γ from Th cells by M1- or M4-primed mature DCs was affected by the presence of neutralizing anti-IL-12 Ab. IL-12 is the critical Th1 polarizing cytokine and a key cytokine in anti-tumoural responses [18,28,29]. On the other hand, the Th2 driven response is usually IL-4 dependent [28]. IL-12 has also antitumour effects of its own for which it is currently under clinical evaluation [30]. Therefore, the finding that M4 can increase IL-12p70 secretion by mature DCs is of major interest, although the rational for ginseng saponins use in the treatment of cancer is still unclear. Although the down-regulation of NF-κB and AP-1 transcription factors has often been hypothesized to explain the anticancer effects of ginseng saponins, there is some evidence that these mechanisms do not clearly account for the anticancer effect of ginseng saponins [31]. 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 antitumour immunity [18]. The Th1 cells that produce IFN-γ have been shown to exert a powerful antitumour effect, whereas a weak Th1 or a Th2 profile may have an opposite effect, that is, down-regulation of innate and acquired antitumour immunity [32]. The corollary of these observations is that a Th1 profile may be protective against tumour growth and dissemination. Wakabayashi et al. [33,34] have demonstrated that the induction of an in vivo anti-metastatic effect by oral administration of ginsenosides may be primarily mediated by their metabolic component M1 and M4. Moreover, Scaglione et al. [35] have investigated immunomodulatory effects of ginseng extract G115 and they have indicated an earlier induction of immune response mediated by their G115 preparation than by the aqueous extract. It seems that TNF-α-primed mature DCs drive the differentiation of naïve T cells towards Th1 cells via an unknown factor, because TNF-α-primed mature DCs did not increase the IL-12 production upon CD40 ligation.

DCs are capable of during CD8 T cell proliferation and CTL responses in the absence of CD4 helper Th cells. CD8 T lymphocytes are activated and thus differentiated into CTL [36].

Activated CTL kills only the antigen-bearing upon specific recognition. Immunotherapy is based on this mechanism. In this context, it is important to know whether M1- or M4-primed DCs enhanced specific CTL responses. We evaluated the CTL response to examine the possible clinical application of our culture system, since mature DCs differentiated with M1 or M4 induced a significant differentiation of naïve T cells towards a helper T cells type I response. As expected from their Th1-polarizing effect, the mature DCs differentiated with M4 induced a stronger CTL response than immature DCs and TNF-α-primed mature DCs. Therefore, we are expecting the possibility of a clinical application. Type I IFN synergizes with IL-12 produced by DCs stimulated via CD40-CD40-L interaction and promotes a potent Th1 polarization. The IL-12 produced by T cell-stimulated DCs may be critical in the generation of cytotoxic CD8+T cell responses, which may eventually lead to clearance of the virus or infectious agent. Indeed, a close interaction between CD8+T cells and DCs is often observed in granulomatous lymphadenitis, as well as a strong signal for the Th1-type cytokine IFN-γ. Recent studies suggested that the signalling via Toll-like receptors (TLRs) which are newly identified receptor molecules recognizing many pathogens, are involved in the induction

of anti-cancer immunity [37]. Seya and Matumoto [38] demonstrated that maturation of DCs and cytokine induction by the cell wall skeleton of Mycobacterium bovis bacillus Calmette-Guerin (BCG-CWS) are induced via both TLR2 and TLR4. However, the involvement of TLRs on M4-primed requires further investigation.

M4-primed mature DCs effected the polarization of T cells and cytotoxic T-lymphocyte stronger than TNF-α-, or M1-primed mature DCs. Recent studies have demonstrated that the ginseng saponins orally administered are promptly metabolized in the digestive tract and absorbed into the circulation [5,6]. The major metabolites of the protopanaxadiol saponin are M1, M2, M3, and M5, and those of the protopanaxatriol are M4 and M11 (Fig. 8). Finally, the protopanaxadiol and the protopanaxatriol saponins and their intermediate metabolites (M2, M3, M5 and M11) are converted into the end products M1 protopanaxadiols and M4 protonanaxatriols, respectively. There is the possibility that the metabolites are the actual active substances of the ginseng saponins, particularly M1 and M4, in vivo.

Yun [4] has demonstrated that Panax ginseng C.A. Meyer has non-organic specific cancer preventive effects against various cancers. About 100 chemotherapeutic agents have been used for histologically different types of cancers in different organs and tissues, and yet there is still no organ-specific drug of choice. Panax ginseng C.A. Meyer has been reported to reduce the human cancer risk of lip, oral cavity, pharynx, larynx, oesophagus, lung, stomach, liver, pancreas, ovary, colon and rectum, and various other cancers by preclinical and epidemiological studies [39]. The effects of M1 and M4 in vivo are not known yet. However, it suggests that the effect of M4 on the production of IL-12p70 by mature DCs and strengthening of the Th1 response by naïve T cells might account, at least in part, for the anti-tumour effect of ginseng.

In conclusion: This is the first study on the effect of ginseng saponins on human monocytes-derived DCs giving new insights into the action of ginseng saponins. Our results suggest that M1 and M4, end products of steroidal ginseng saponins metabolized in digestive tracts, potentially regulate differentiation of DCs from human monocytes in combination with GM-CSF and IL-4 in vitro. Moreover, mature DCs differentiated with M4 enhance the differentiation of naïve T cells towards the Th1 type depending on IL-12 secretion. M4-primed mature DCs produce a stronger CTL response. DCs appear to be a potential target for the immunomodulatory capacity of the ginseng and its component.

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