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Diterpene, 16-phyllocladanol enhances Th1 polarization induced by LPS-primed DC, but not TNF-α-primed DC

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ABSTRACT

16-Phyllocladanol is diterpene isolated form the heartwood of *Cryptomeria japonica*. We demonstrate that the effect of 16-phyllocladanol on the phenotypic and functional maturation of human monocytes-derived DC *in vitro*. Human monocytes were exposed to 16-phyllocladanol alone, or in combination with LPS and thereafter co-cultured with naïve T cells. The expression levels of CD83 and HLA-DR on LPS-primed DC were enhanced by 16-phyllocladanol. 16-Phyllocladanol dose-dependently augmented the T cell stimulatory capacity in an allo MLR to LPS-primed DC and the production of IL-12p70 by LPS-primed DC. The cytokine production by 16-phyllocladanol-primed DC was not inhibited by anti-TLR2 and 4 mAbs. IFN-γ secretion from naïve T cells co-cultured with DC differentiated with LPS was also augmented by 16-phyllocladanol. These results suggest that the enhancement of Th1 cells polarization to LPS-primed DC induced by 16-phyllocladanol via the activation of IL-12p70 and independent on TLR2 or TLR4.

The immune system is confronted with antigens and proteins that have not been encountered previously. Due to their unique antigen processing and presentation and high-level expression of costimulatory and cytokine molecules, dendritic cells (DC) are highly specialized antigen presenting cells (APC) with a unique ability to activate resting T lymphocytes and initiate primary immune responses as to induce peripheral tolerance [1–3]. Upon encountering inflammatory stimuli or pathogens in peripheral tissues, DC become activated and undergo a number of physiological changes are linked to an enhanced ability to activate T cells and to direct the differentiation of T cells into Th1 or Th2 profile [1–4]. Th1 cells responses predominate in organ-specific autoimmune disorders and acute allograft rejection [5,6], and Th2 responses predominate in transplantation tolerance and allergic diseases [7]. Numerous stimuli can mediate DC maturation, the best characterized being TLR ligands and signals such as CD40L delivered by T cells and innate lymphocytes [8]. In addition to their essential role in T cell priming, DC are also involved in innate immunity through the production of cytokines and the activation of NK or NKT cells [9]. Thus, DC play a pivotal role in orchestrating the immune responses.

16-Phyllocladanol are isolated from the black heartwood of *Cryptomeria japonica* and phytochemically classified as diterpenes. Diterpenes have been shown to inhibit JNK1/2 and p38 phosphorylation in LPS-stimulated macrophages and anti-inflammatory effects [10,11]. Furthermore, T-cadinol inhibited choleratoxin-induced intestinal hypersecretion in mice and electrically induced contractions of the isolated guinea pig ileum [12]. We have recently reported that T-cadinol and calamenene differentiated human monocytes to DC, and T-cadinol-primed DC and calamenene-primed DC induced the differentiation of naïve T cells towards Th1 response [13]. Thus diterpenes contain pharmacologically active substance. DC have led to priming naïve T cells use as a cellular platform for vaccination in several encouraging anti-cancer clinical trials with regards to enhanced tumor antigen-specific immune responses [13]. Therefore, it is important to identify factors that might affect the differentiation, maturation and functional of DC. Although some diterpenes have pharmacological activity, little is known about 16-phyllocladanol influences the initiation of the specific immune response at the level of DC, the highly professional APC for T cells. In this study, we investigated the effect of 16-phyllocladanol on human DC differentiation and function in detail.

Materials and methods

Culture medium, reagents, and monoclonal antibodies. The culture medium used in this study was serum-free AIM-V medium (Life Technologies, Paislex, UK). Recombinant human IL-4 (IL-4), recombinant human granulocyte-macrophage colony-stimulation factor (GM-CSF), TNF-α, CD40-L, anti-IL-12 mAb, anti-TLR2 mAb, and anti-TLR4 mAb were purchased from R&D systems (Minneapolis, MN). LPS from *Escherichia coli* were purchased from Sigma–Aldrich (St. Louis, MO). For

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Table 1
Comparison phenotypes of DC cultured with 16-phyllocladanol, LPS or TNF-α on day 8

	CD1a	CD80	CD83	CD86	HLA-DR
16-Phyllocladanol					
0.1 μM	95 ± 18	56 ± 12	16 ± 8	388 ± 39	692 ± 66
1.0 μM	108 ± 39	89 ± 18	29 ± 9	401 ± 40	742 ± 89
10 μM	125 ± 18	109 ± 32	31 ± 16	420 ± 53	782 ± 88
100 μM	124 ± 30	110 ± 28	30 ± 8	418 ± 65	792 ± 109
16-Phyllocladanol (10 μM)					
+LPS	201 ± 22	232 ± 32	112 ± 16	682 ± 32	1386 ± 108
+TNF-α	188 ± 39	189 ± 25	78 ± 12	592 ± 79	998 ± 86
TNF-α (25 ng/ml)	188 ± 38	187 ± 36	71 ± 10	581 ± 87	984 ± 99
LPS (100 ng/ml)	173 ± 21	221 ± 48	88 ± 9	602 ± 49	1006 ± 174
Immature DC	96 ± 17	36 ± 6	9 ± 3	342 ± 28	642 ± 45
Monocyte	8 ± 1	7 ± 2	6 ± 2	12 ± 5	46 ± 6

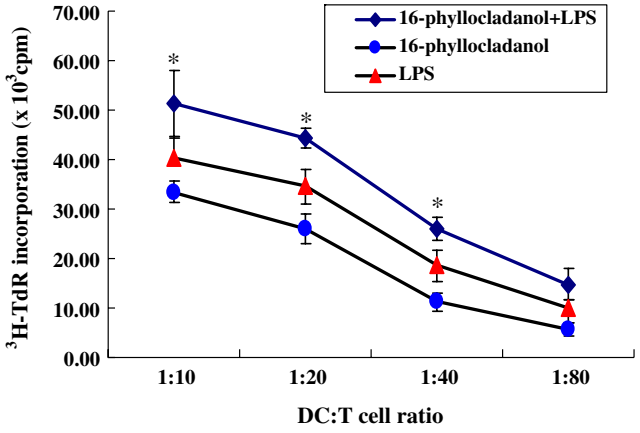

Fig. 1. Allogeneic T cell stimulatory capacity of DC differentiated with 16-phyllocladanol, LPS, TNF-α alone or LPS in the presence of 16-phyllocladanol. Naïve T cells were co-cultured with graded doses of DC, and on day 5, ^{3}[H]methylthymidine was added 16 h before measurement of the proliferation responses. Data are means ± SEM of five-independent experiments. $^*P < 0.05$ compared with LPS-primed DC.

flow cytometry, monoclonal antibodies (mAbs) toward the following antigens were purchased from Becton–Dickinson (San Jose, CA): anti-CD14-FITC (fluorescent isothiocyanate), anti-CD1a-PE (phycoerthrin), anti-80-PE, anti-CD83-PE, anti-CD86-PE, and anti-HLA-DR-FITC. Endotoxin levels in all agents were blow 1.0 EU/ml.

Isolation of 16-phyllocladanol from the Black Heartwood of C. japonica. 16-Phyllocladanol was prepared as previously described [14]. The purity of 16-phyllocladanol was >99%. 16-Phyllocladanol was 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 16-phyllocladanol was removed using End Trap 5/1 (Profos AG, Regensburg, Germany). Leading of LPS concentration below the detection limit of the assay (<0.05 EU/ml).

Generation of monocytes-derived DC. All cell subsets were isolated from human peripheral blood of normal healthy donors and five different 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*g*, 45 min, 22 °C) as previously described [15]. PBMC were further separated into monocytes and lymphocytes by counterflow centrifugation using the JE-6B-elutriator system (Beckman Instruments, Palo Alto, CA) [15]. 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 for 6 days. DC were generated by stimulating immature DC in serum-free AIM-V medium containing GM-CSF and IL-4 additional for 2 days with various concentrations of 16-phyllocladanol, but with LPS (100 ng/ml) or TNF-α (25 ng/ml). All subsequent tests were performed after harvesting the cells at day 8 and after removing GM-CSF, IL-4, 16-phyllocladanol, LPS or TNF-α by extensive washing. The medium was replenished with cytokines every 2 days. To determine the production of IL-12p70 by DC, DC (4×10^4 cell/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).

Immunophenotype studies. Dual-color immunofluorescence flow cytometry was performed as previously described [13].

Allogeneic mixed lymphocyte reaction. The allo MLR assay was carried out as previously [13].

Determination of naïve T cell polarization by DC. Determination of naïve T cell polarization by DC was carried out as previously [13].

Transmigration assay. Transmigration assay was carried out as previously [13].

Statistical analysis. Statistical analysis of the results was performed by ANOVA. Differences were considered statistically significant when *P* value were less than 0.05.

Results and discussion

We first examined the influence of 16-phyllocladanol on differentiation of human monocytes into DC, and phenotypic and func-

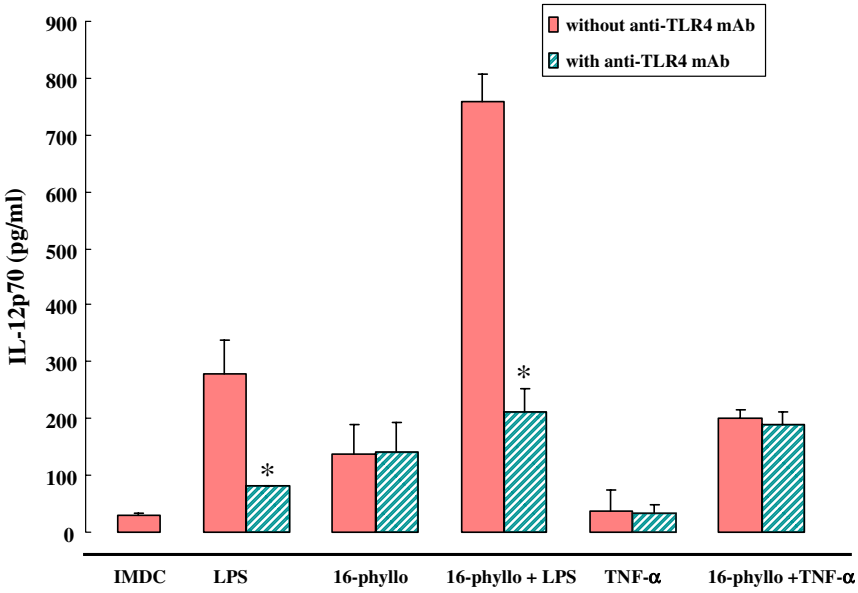

Fig. 2. The production of IL-12p70 by 16-phyllocladanol-primed DC was not inhibited by anti-TLR4 mAb. DC were generated by stimulating immature DC with 16-phyllocladanol (10 μM), LPS (100 ng/ml) or TNF-α (25 ng/ml) alone or together with 16-phyllocladanol (10 μM). Cells were stimulated with the CD40-L (3.0 μg/ml) for 24 h. After 24 h, the production of IL-12p70 was measured by ELISA in culture supernatants. Data are means ± SEM of five-independent experiments. $^*P < 0.05$ compared with without anti-TLR4 mAb. IMDC, immature DC; 16-phyllo, 16-phyllocladanol.

tional DC maturation were analyzed. 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 16-phyllocladanol. The expression levels of CD1a, CD80, CD83, CD86, and HLA-DR as expressed by MFI on 16-phyllocladanol-primed DC were slightly enhanced in a dose-dependent manner (Table 1). Viability of cells at 10 μM of 16-phyllocladanol was >95%. The concentration of 16-phyllocladanol was used at 10 μM for subsequent experiments. The expression level of CD14 as expressed by MFI on day 8 was low or undetectable. The expression levels of CD1a, CD80, CD83, CD86, and HLA-DR as expressed by MFI on LPS (100 ng/ml)-primed DC or TNF-α-primed DC (25 ng/ml) were enhanced. When human monocytes-derived DC were stimulated simultaneously with LPS (100 ng/ml) plus 16-phyllocladanol (10 μM), the presence of 16-phyllocladanol resulted significant up-regulation effect of CD86 and HLA-DR surface expression (Table 1). In contrast, the expression of CD1a, CD80, CD83, CD86, and HLA-DR as expressed by MIF on TNF-α-primed DC were not affected by 16-phyllocladanol (Table 1). As control, immature DC were generated by cultivating human monocytes with GM-CSF and IL-4 for 8 days. It is interesting that 16-phyllocladanol enhanced the differentiation and maturation on LPS-primed DC, but not TNF-α-primed DC. DC maturation induced by LPS depends on signaling by p38 MAPK. In contrast, it seems that the mechanism of DC differentiated by TNF-α are still unknown. Therefore, the maturation of LPS-primed DC enhanced by 16-phyllocladanol probably induces through activation of a p38 MAPK-signaling pathway.

The ability to induce allogeneic T cell proliferation is a functional hallmark of DC *in vitro*. The change in the surface marker expression was also reflected at a functional level, when analyzing the allostimulatory capacity of DC in an allo MLR. Exposure of immature DC to 16-phyllocladanol alone resulted in an enhanced proliferative response of allogeneic naïve T cells (Fig. 1). Simultaneous DC stimulation with LPS plus 16-phyllocladanol appeared to cause enhanced effects (Fig. 1). The effects of 16-phyllocladanol in an allo MLR were dose-dependent (data not shown). On the

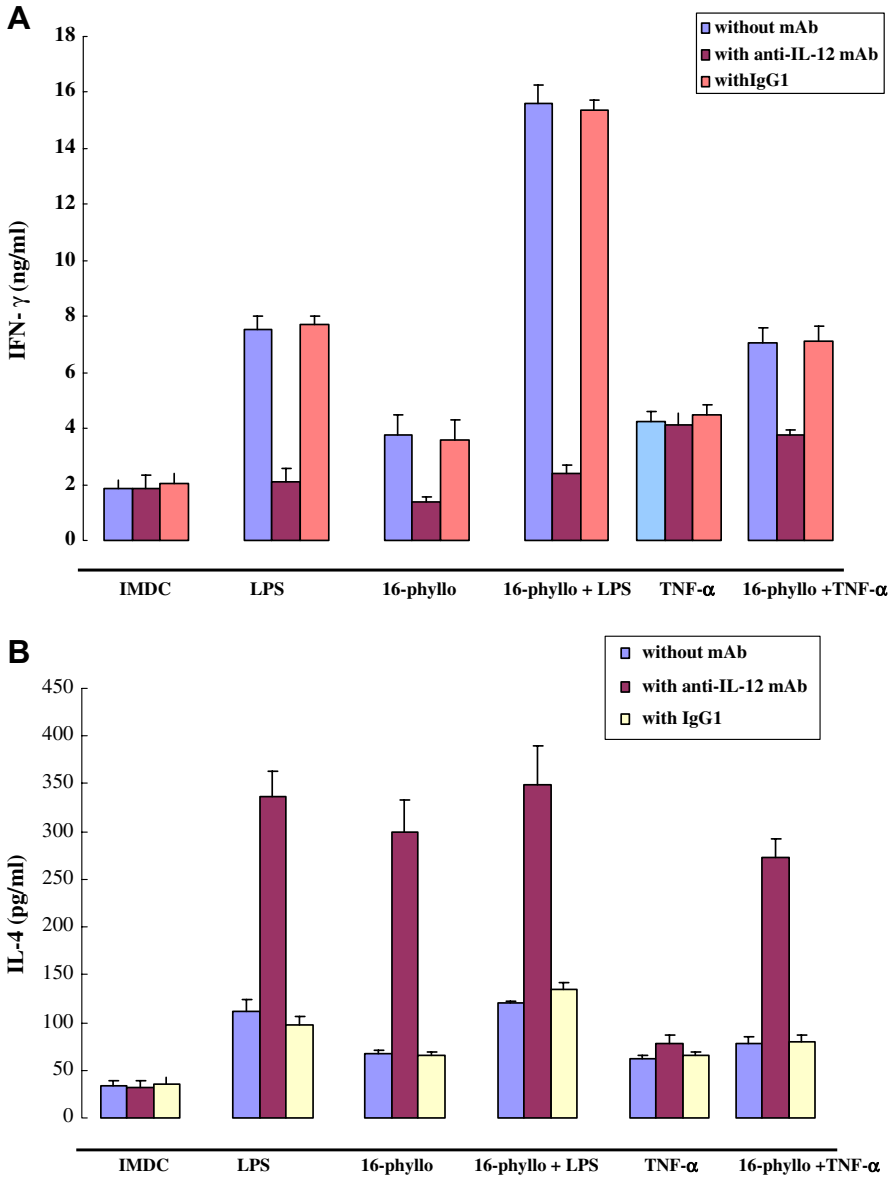

Fig. 3. Immature DC stimulated with LPS in the presence of 16-phyllocladanol increased Th1-polarizing capacity. Allogeneic DC were co-cultured for 5 days with naïve T cells at 1:5 DC/T cell ratio. After 9 days of expansion in IL-2, T cells were counted and re-stimulated for 24 h on plates coated with anti-CD3/CD28. IFN-γ (A) and IL-4 (B) were measured by ELISA in culture supernatants. Data are means ± SEM of five-independent experiments. IMDC, immature DC; 16-phyllo, 16-phyllocladanol.

other hand, 16-phyllocladanol had no influence on an allogeneic T cell proliferation of TNF-α-primed DC (data not shown).

Since DC are the professional effective APC and their secretion of immunoregulatory and proinflammatory cytokines play a crucial role during T cell priming, we then studied whether cytokine production by human monocytes-derived DC is affected by 16-phyllocladanol. We measured the production of IL-10 and IL-12p70 in immature DC (with medium) and in DC matured for 2 days in the presence of the above factors after stimulation by CD40-L for 24 h. Under control conditions (with medium) DC spontaneously produced low levels of IL-10 (6.5 ± 2.3 pg/ml) and IL-12p70 (13.2 ± 4.2 pg/ml). The production of IL-12p70 was consistently higher in LPS-primed DC, whereas the production of cytokine by 16-phyllocladanol-primed DC was low than that of LPS-primed DC (Fig. 2). Interestingly, the production of IL-12p70 by LPS-primed DC was enhanced by 16-phyllocladanol (Fig. 2) in a dose-dependent manner (data not shown). At the concentration of 10 μM, 16-phyllocladanol enhanced the secretion of IL-12p70 by 2- to 3-fold. In contrast, the production of IL-12p70 by TNF-α-primed DC was not significantly enhanced by 16-phyllocladanol at 10 and 100 μM (Fig. 2).

Recent studies have demonstrated that the signaling via TLR which are newly identified receptor molecules recognizing many pathogens, are involved in the induction of anti-cancer immunity [16]. We next studied whether the ability of 16-phyllocladanol to induce IL-12 production in human DC was dependent on TLR2 or TLR4. Before stimulation with 16-phyllocladanol or LPS, monocytes-derived DC were incubated in the presence of the TLR2, TLR4 blocking mAbs or an isotype control (IgG1). The anti-TLR4 mAb blocked the production of IL-12p70 induced by LPS alone or LPS plus 16-phyllocladanol by 30–50% (Fig. 2), whereas the anti-TLR2 mAb was not inhibited (data not shown). In contrast, the response to 16-phyllocladanol alone was not inhibited in the presence of the anti-TLR2 mAb (data not shown) and the anti-TKR4 mAb (Fig. 2). Furthermore, the production of IL-12p70 by TNF-α-primed DC or immature DC was also not affected by the anti-TLR2 mAb (data not shown) and the anti-TLR4 mAb (Fig. 2). IL-10 appears to play a central role in preventing overly pathological Th1 or Th2 responses in a variety of setting. The production of IL-10 by 16-phyllocladanol, LPS or TNF-α was low (data not shown). These cytokine productions were not influenced by the anti-TLR 2 and 4 mAbs (data not shown). In contrast, the production of IL-10 or IL-12p70 by LPS-primed DC was not influenced when IgG1 was added instead of anti-TLR4 mAb (Fig. 2 and data not shown). 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 Th1 cells [17]. IL-12p70 is a key cytokine for the induction of Th1 immune response. Moreover, IL-12 is a produced by macrophages and DC upon Toll-like (TLR) ligation, and in turn, it induces IFN-γ production and Th1 differentiation [9,18].

As the nature of cytokines secreted by DC governs the type of Th response and as 16-phyllocladanol altered the secretion of cytokines by DC, we evaluated the nature of primary allogeneic T cells responses stimulated by LPS-primed DC alone, or combination with 16-phyllocladanol. LPS-primed DC and 16-phyllocladanol-primed DC induced a substantial increase in the secretion of IFN-γ by T cells (Fig. 3A), but little IL-4 (Fig. 3B). As expected, simultaneous DC stimulation with LPS plus 16-phyllocladanol caused a significant enhancement in IFN-γ secretion (Fig. 3A). In contrast, IFN-γ secretion from naïve T cells co-cultured with DC differentiated with TNF-α was not augmented by 16-phyllocladanol (Fig. 3A). To analyzed the contribution of DC-derived IL-12 on the development of Th1 cells, we investigated the effect of a neutralizing anti-IL-12 mAb in co-culture experiments, where naïve T cells were co-cultured with LPS-primed DC plus 16-phyllocladanol. In LPS-primed DC plus 16-phyllocladanol, neutralization of IL-12 increased the development of IL-4 producing T cells and decreased the development of IFN-γ producing T cells (Fig. 3A and B). On the other hand, the production of IFN-γ and IL-4 by naïve T cell co-cultured with TNF-α-primed DC was not influenced by anti-IL-12 mAb and 16-phyllocladanol (Fig. 3A and B). In contrast, the production of IFN-γ and IL-4 by 16-phyllocladanol-primed DC, LPS-primed DC or TNF-α-primed DC was not influenced when IgG1 was used instead of anti-IL-12 mAb (Fig. 3A and B). These results suggest that the function and cytokine production of LPS-primed DC augmented by 16-phyllocladanol is specific action. DC activated by TLR stimulating may induce T cell differentiation toward Th1 by presenting antigens to T cell while promoting Th1-leading situation in the local environment. IFN-γ is a central mediator of cellular Th1 immunity and absolutely required for effective immunity against intracellular pathogens including mycobacteria. Furthermore, IFN-γ plays an important role in the activation of APC leading to both activation of antibacterial cellular responses and enhanced induction of antibacterial lymphocyte responses. Cytokines are the major factors driving the differentiation of Th1 and Th2 cells, and the ability of DC to induce Th1 differentiation has been related to their ability to produce high levels of IL-12p70. There are a number of mechanisms including IL-10 that inhibit IL-12 production [19]. IL-10 can promote Th2 responses that may be inhibitory for Th1 responses and negatively influences the ability of monocytes-derived DC to produce IL-12p70. Therefore, IL-10 might play a key role to response Th1/Th2 cells in inhibiting IL-12p70 production. We recently reported that diterpenes such as Sugiol and Secoferruginol induced the development of Th2 cells via the enhanced expression of OX40L and augmented the Th2 cell polarizing capacity of DC via the inhibition of IL-12p70 [20]. It seems that an Abietane type and a carbonyl group are required to induce Th2 polarization. In contrast, 16-phyllocladanol has Kaurane type and drives Th1 polarization depending on IL-12 secretion. Therefore, opposite effects of these diterpenes may be due to their different structure. However, further experiment is necessary to clarify these points.

Because during the maturation process, DC up-regulate the synthesis of constitutive chemokines, we measured the migration of 16-phyllocladanol-primed DC, LPS-primed DC or TNF-α-primed DC in response to MIP-3β *in vitro*. LPS-primed DC had a higher migration response to MIP-3β than 16-phyllocladanol-primed DC

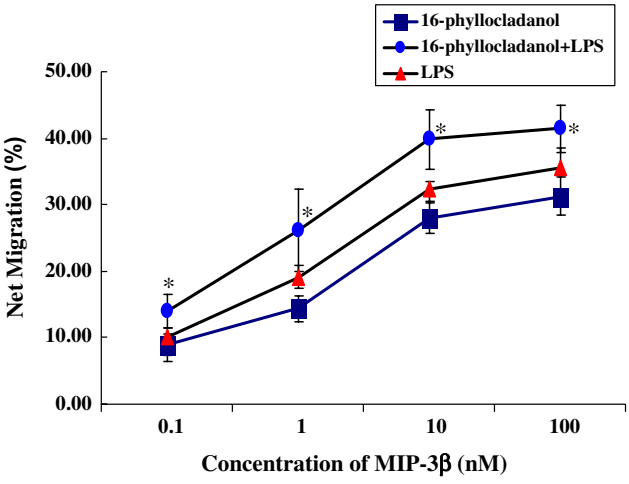

Fig. 4. Chemotaxis in response to MIP-3β by LPS-primed DC enhanced by 16-phyllocladanol. 16-Phyllocladanol-primed DC, LPS-primed DC or TNF-α-primed DC alone or LPS-primed DC in the presence of 16-phyllocladanol prepared and recovered, and their migratory abilities in response to MIP-3β (0.1–100 nM) were determined *in vitro*. Data are means ± SEM of five-independent experiments. $^{*}P < 0.05$ compared with LPS-primed DC. IMDC, immature DC; 16-phyllo, 16-phyllocladanol.

(Fig. 4). Migration of LPS-primed DC in response to MIP-3β was enhanced by 16-phyllocladanol (Fig. 4), whereas that of TNF-α-primed DC was not affected by 16-phyllocladanol (data not shown). The number of DC that are injected, and ultimately the number of DC that migrate to the draining lymph nodes, is likely to affect T cell priming. Antigen-loaded DC might prime T cell responses regardless of the route of injection, but the quality of responses is affected. Recent observation suggests that mature DC could be a better antitumor adjuvant [21,22]. Although the effect of 16-phyllocladanol *in vivo*, and the role of chemokine receptor for clinical application are not known yet, 16-phyllocladanol appear to be a good factor to induce DC, or even better in some respect, for the use in clinical DC therapy to induce strong Th 1-type immune responses. Further understanding of the mechanisms by which 16-phyllocladanol modulate DC function requires investigation.

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