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Relationship between dendritic cells and the D-fraction-induced Th-1 dominant response in BALB/c tumor-bearing mice

Nachi Harada, Noriko Kodama*, Hiroaki Nanba

Department of Microbial Chemistry, Kobe Pharmaceutical University, 4-19-1, Motoyama-kitamachi, Higashinada-ku, Kobe 658-8558, Japan

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Abstract

Dendritic cells (DCs) are known to not only induce the activation of T cells, but are also associated with the differentiation of T cells. The D-fraction, a β-glucan extracted from maitake (*Grifola frondosa*) which expresses anti-tumor effects by establishing a helper (Th)-1 dominance in BALB/c mice, enhanced IL-12p70 production by DCs, when the ratio of $CD8\alpha^+$ DCs to $CD8\alpha^-$ DCs increased. In addition, examination of the tumor rejection effect of D-fraction-stimulated DCs loaded with tumor antigen revealed that tumor growth is inhibited completely by activating $CD4^+$ T cells and $CD8^+$ T cells.

Keywords: Dendritic cells; Polysaccharide; Th-1 dominance

1. Introduction

The dendritic cell (DC) system of antigen-presenting cells (APCs) is essential for the initiation of the primary T cell response [1]. DCs not only stimulate T cells effectively but are also producers of cytokines such as IL-12 and IFN-γ, both of which have important immuno-regulatory functions [2]. In 1986, Mosmann classified $CD4^+$ T cell clones into helper (Th)-1 cells, which produce IL-2, IFN-γ and TNF-β, and introduce cellular immunity to the organism, and Th-2 cells, which produce IL-4, IL-5, IL-6, IL-10 and IL-13, and activate humoral immunity [3,4]. A recent study has shown that many immune responses are controlled by the proportion of Th-1 to Th-2 cells in humans as well as in animals [5]. Disruption of the balance between Th-1 and Th-2 induces an excessive Th-1 or Th-2 dominant response, resulting in various diseases [6]. Therefore, modulating the balance between Th-1 and Th-2 appears important in treating disease. DCs have two subclasses that differ in their CD8α expression and their location in lymphoid organs [7]. $CD8\alpha^+$ DCs, compared with $CD8\alpha^-$ DCs, produce large amounts of IL-12, which is the most important cytokine for induction of Th-1 cells. These subclasses of DCs were reported to involve the activation and differentiation of Th cells by providing costimulatory signals, in which $CD8\alpha^+$ DCs trigger the development of Th-1 cells, while $CD8\alpha^-$ DCs induce a Th-2 response, suggesting that the functions of DCs effect the Th-1/Th-2 balance in immune responses [7–9].

We previously reported that the (1 → 3)-branched (1 → 6)-β-glucan, termed the D-fraction, extracted

* Corresponding author. Tel.: +81-078-441-7567; fax: +81-078-441-7568.
E-mail address: n-kodama@kobepharma-u.ac.jp (N. Kodama).

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from the fruit body of the maitake mushroom (*Grifola frondosa*) is a biological response modifier (BRM) similar to lentinan, which is found in *Letinus edodes* [10–12]. The administration of D-fraction to MM-46 carcinoma-bearing C3H/HeN mice induced a Th-1 dominant response when D-fraction expressed its anti-tumor effects [13]. In a previous study using colon 26 carcinoma-bearing BALB/c mice, D-fraction appeared to promote the differentiation into Th-1 cells of $CD4^+$ T cells through enhancement of IL-12p70 production by APCs when the tumor growth inhibition rate (TIR) was 79% on day 20 after D-fraction administration [14]. However, the nature of the involvement of DCs in a Th-1 dominant response induced by D-fraction is unknown. Therefore, we investigated the roles of splenic and lymph node DCs in colon 26 carcinoma-bearing BALB/c mice administered D-fraction. Furthermore, to elicit both the tumor rejection effect of D-fraction-stimulated DCs through Th cells, and cytotoxic T cells (CTL)-activating action, DCs loaded with tumor antigen from mice administered D-fraction were injected into normal mice before the transplantation of tumor cells.

2. Materials and methods

2.1. Animals

Female BALB/c mice (5 weeks old, Clea Japan Inc.) were had *ad libitum* access to food and water freely until the experiment.

2.2. Preparation of D-fraction

A dried powder prepared from the maitake mushroom was obtained from Yukiguni Maitake Co. Ltd. (Niigata). D-fraction was prepared from the powder as previously described [15]. The polysaccharide concentration was determined by the anthrone method [16].

2.3. Administration of D-fraction

Colon 26 carcinoma cells (1×10^5) were implanted in female BALB/c mice (6 weeks old) in the right axillary region. After 24 h, D-fraction (7.8 mg/kg/day) or saline was administered to the carcinoma-bearing mice intraperitoneally (i.p.) for 19 consecutive days.

2.4. Preparation of whole spleen cell and lymph node cells

On day 20 in mice administered D-fraction or saline, the spleens or the inguinal lymph nodes were extirpated, then spleen cells or lymph node cells were prepared as previously described [13].

2.5. Preparation of DCs

On day 20 in mice administered D-fraction or saline, DCs were prepared from whole spleen cells or lymph node cells using MACS separation columns with CD11c (N418) MicroBeads (Miltenyi Biotec. Co., CA, USA) The percent of MHC II and CD11c expression on the resultant DCs was confirmed by flow cytometric analysis. In the in vivo tumor rejection assay, the term control-DCs describes DCs from tumor-bearing mice administered saline, D-fraction-DCs describes DCs from tumor-bearing mice administered D-fraction, and normal-DCs describes DCs from normal mice.

2.6. In vivo tumor rejection assay

Normal-, control-, or D-fraction-DCs (1×10^6 /mouse) were injected i.p. into normal mice (6 weeks old) 5 times at 7 day intervals. On day 7 after the final sensitization, colon 26 carcinoma cells (1×10^4) were implanted in the normal mice that had been injected with DCs from mice with or without tumors, in the right axillary region and tumor volume then measured every 7 days. On day 28, the tumor was extirpated and weighed to obtain the TIR.

2.7. Determination of cytokine production

A total of 1×10^6 cells/well of whole spleen cells, lymph node cells, or DCs were cultured in RPMI-1640 medium containing 5% FBS with concanavalin A (10 μg/ml) at 37°C for 24 h in 5% CO_2. After the stimulation, levels of IFN-γ, IL-4, IL-12p70, and TNF-α in the culture supernatant were determined using mouse IFN-γ, IL-4, IL-12p70, and TNF-α ELISA kits (Genzyme Co., Minneapolis, MI, USA).

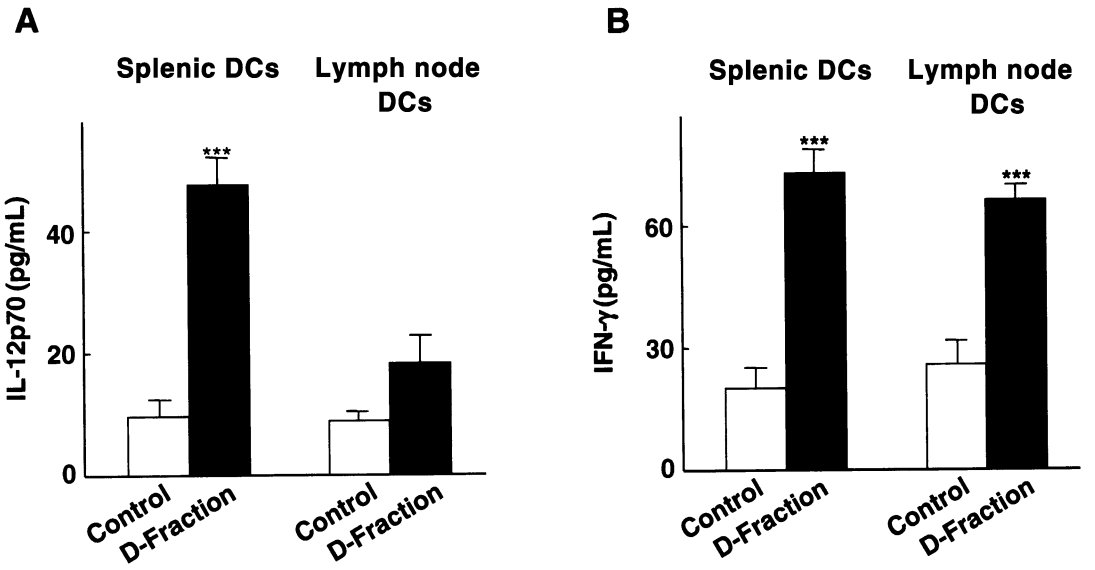

Fig. 1. Effects of D-fraction on the production of IL-12p70 (A) and IFN-γ (B) by splenic and lymph node DCs. Data represent mean ± SEM of four different experiments (four to eight mice/experiment). $***P < 0.001$, compared with the control group (Student's *t*-test).

2.8. Flow cytometry

Flow cytometric analyses were performed as described previously [13]. The following mouse monoclonal antibodies were purchased from Pharmigen Co. (San Diego, CA, USA): FITC-conjugated CD11c, R-PE-conjugated CD28, R-PE-conjugated CD86, Cy-Chrom-conjugated CD4, and Cy-Chrom-conjugated CD8α.

2.9. Statistical analysis

Data represent the mean ± SEM and differences between the control mice group and the D-faction-administered mice group were examined using Student's *t*-test ($***P < 0.001$, $**P < 0.01$, $*P < 0.05$).

3. Results

3.1. Effects of D-fraction on the activation of DCs

The differentiation of Th-1/Th-2 is known to involve cytokines such as IFN-γ and IL-12p70 [17], which are produced by APCs. In particular, IL-12p70 induces differentiation to Th-1 cells, resulting in IFN-γ production by Th-1 cells, which enhances the Th-1 dominant response. In a previous study, D-fraction significantly enhanced the levels of IFN-γ and IL-12p70 production by APCs from whole spleen cells and lymph node cells, suggesting that D-fraction develops the differentiation to Th-1 cells of $CD4^+$ T cells through enhancement of IL-12p70 produced by APCs such as macrophages and DCs [14]. To investigate the involvement of the DC activation by D-fraction in the Th-1 dominant response, the levels of IL-12p70 production by DCs were determined by the ELISA method. As shown in Fig. 1A, D-fraction promoted IL-12p70 production by both splenic and lymph node DCs. IFN-γ is a cytokine known to be not only produced by T cells, macrophages or NK cell, but is also produced by DCs [18]. In addition, the IFN-γ produced by DCs activates the DCs themselves through IFN-γ receptor expressed on the surface of DCs, resulting in enhancement of D-fraction-induced IL-12p70 production. The levels of IFN-γ produced by both splenic and lymph node DCs were increased by D-fraction (Fig. 1B). These results suggest that D-fraction develops the differentiation to Th-1 cells of $CD4^+$ T cells through enhancement of IL-12p70 and IFN-γ produced by activated DCs. In a previous study[14], D-fraction enhanced the CD28/B7 pathways related to T cell activation, via a non-specific

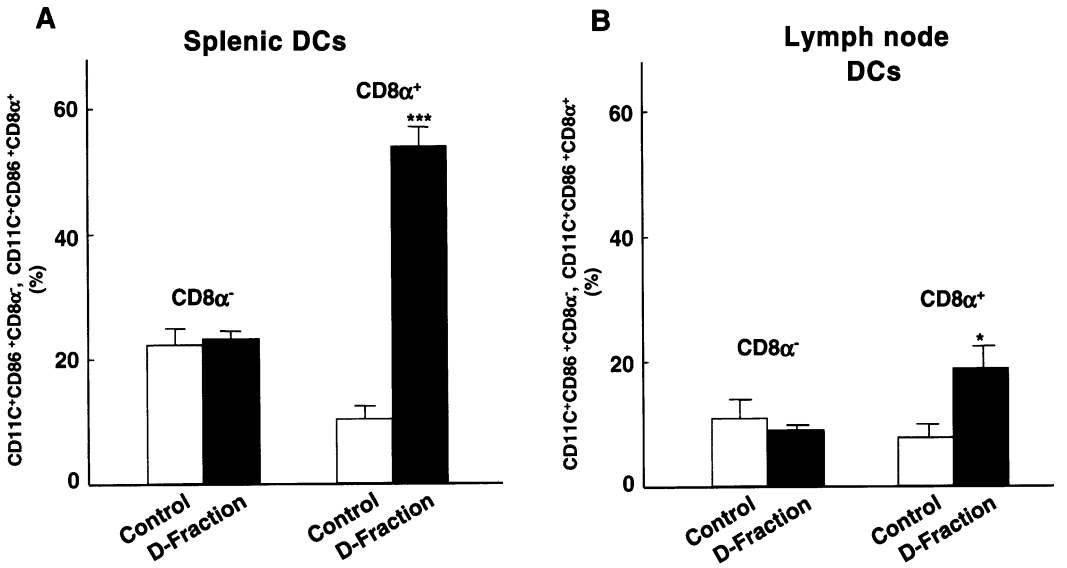

Fig. 2. Effects of D-fraction on CD8α expression in splenic DCs (A) and lymph node DCs (B). Data represent mean ± SEM of four different experiments (four to eight mice/experiment). $*P < 0.05$, $***P < 0.001$, compared with the control group (Student's *t*-test).

signal, resulting from the binding of B7 ligand on the APC with its receptor, CD28, on the T cell [19, 20]. To elicit the activation of DCs by D-fraction, the expression (%) of the CD28 ligands B7.1 (CD80) and B7.2 (CD86) was examined on splenic and lymph node DCs by flow cytometric analysis. The ratio of CD80 and CD86 expression on $CD11c^+$ cells in splenic and lymph node APCs, prepared using MACS separation columns with MHC II (Ia) MicroBeads (Miltenyi Biotec. Co.), were increased 1.5–2.0-fold in both whole spleen cells and lymph node cells by D-fraction (data not shown). These results support the involvement of DCs in the activation of the B7/CD28 pathway in D-fraction-induced immune responses.

3.2. Effects of D-fraction on DC subclasses

The population of splenic DCs appears heterogeneous and includes the $CD8\alpha^+$ and $CD8\alpha^-$ subclasses which direct the differentiation of Th-1 and Th-2 cells, respectively[7–9]. The effects of D-fraction on DC subclasses were investigated by flow cytometric analysis. As shown in Fig. 2, the ratio (%) of $CD11c^+CD86^+CD8\alpha^+$ cells to $CD11c^+CD86^+$ $CD8\alpha^-$ cells in splenic and lymph node DCs increased upon D-fraction administration. These data suggest that activated $CD8\alpha^+$ DCs are involved in priming $CD4^+$ T cells to respond to D-fraction.

3.3. Effects of D-fraction on induction of anti-tumor protective immunity by DCs loaded with tumor antigen

To investigate the effect of D-fraction on protective immunity induced by DCs loaded with tumor antigen, normal-, control-, and D-fraction-DCs were injected i.p. into normal mice (Fig. 3). Control-DCs were capable of inducing protection, with a TIR of 39%, whereas the tumors disappeared completely in the D-fraction-DCs group. These results suggest that antigen-specific DCs express MHC class I and II, indicating the possibility that D-fraction enhanced by DCs loaded with tumor antigen induces both the activation of Th cells and CTL in anti-tumor protective immunity.

3.4. Effects of D-fraction on T cell activation by DCs loaded with tumor antigen

To investigate whether D-fraction enhances the activation of DCs to prime in vivo antigen-specific Th cells ($CD4^+$ T cells) and CTL ($CD8^+$ T cells), the expression of CD28 on T cells, which bind B7

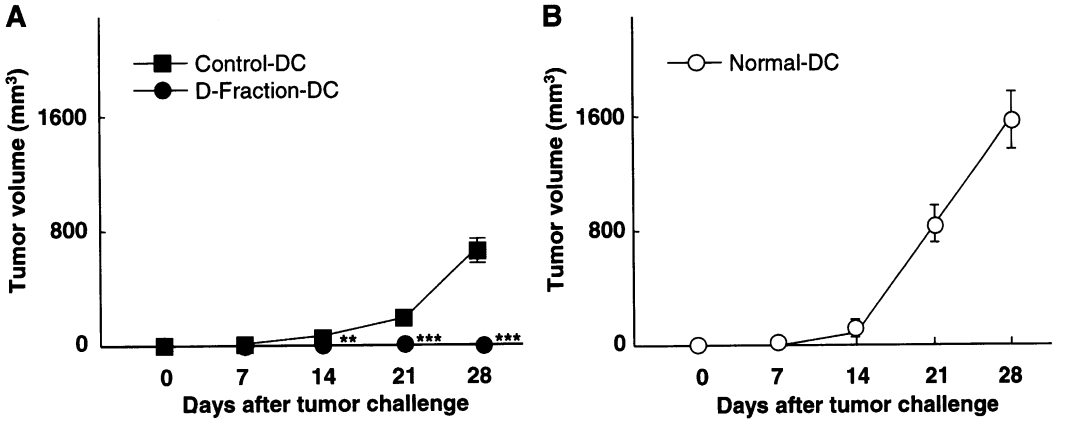

Fig. 3. Effects of D-fraction on the protective immunity induced by DCs (A) loaded with tumor antigen, and (B) without. Data express mean ± SEM of three different experiments (six to eight mice/experiment). $^{**}P < 0.01$, $^{***}P < 0.001$, compared with the control group (Student's *t*-test).

costimulatory molecules on DCs, were examined by flow cytometryic analysis (Table 1). D-fraction-DCs induced CD28 expression to 3.9 times the level of the control-DCs on $CD4^+$ T cells, and at the same time 5.5 times the level of the control-DCs on $CD8^+$ T cells, indicating that D-fraction-DCs enhances $CD4^+$ T cells and $CD8^+$ T cells in generating tumor rejection immunity in vivo. Furthermore, to investigate whether D-fraction-DCs induced a Th-1 dominant response in antigen specific immunity, the production of cytokines by whole spleen cells and lymph node cells was investigated in control- and D-fraction-DCs injected groups. The levels of IFN-γ, a Th-1 cytokine, increased markedly with D-fraction and at the same time IL-4, Th-2 cytokine also increased (Fig. 4A, B). Furthermore, the increase in the IL-12p70 level induced by D-fraction supports the finding that D-fraction-DCs enhance the Th-1 dominant response (Fig. 4C). Furthermore, as shown in Fig. 4D, the level of TNF-α, which is produced by activated macrophages and NK cells and is cytotoxic for tumor cells, increased by D-fraction-DCs injection, indicating that D-fraction enhanced the protective immunity by DCs loaded with tumor antigen through activating macrophages and NK cells.

Table 1
Effect of D-fraction on T cell activation by DCs loaded with tumor antigen

	DCs antigens (%)[a]		
T cells	Normal-DCs	Control-DCs	D-Fraction-DCs
$CD4^+CD28$	1.88 ± 0.61	2.68 ± 0.41	10.35 ± 1.63$^{**\#\#}$
$CD8^+CD28$	0.25 ± 0.02	0.44 ± 0.22	2.41 ± 0.78

[a] % of positive cells was determined by flow cytometric analysis. Data represent mean ± SEM of three different experiments (two mice/experiment). $^{**}P < 0.01$, compared with normal-DCs (Student's *t*-test). $^{\#\#}P < 0.01$, compared with control-DCs (Student's *t*-test).

4. Discussion

Modulating the Th-1/Th-2 balance in immune responses contributes to efficient protection against parasites or infectious diseases. In particular, Th-1 cells are important effectors involved in the eradication of intracellular infectious pathogens, whereas Th-2 cells are efficient in eliminating extracellular parasites. Therefore, identification of the factors that influence the differentiation of distinct Th cell subsets in vivo is of great interest.

We have already reported that the D-fraction, a β-glucan extracted from the maitake mushroom (*G. frondosa*), activates cellular immunity including macrophages, T cells and NK cells and expresses anti-tumor effects [10–12]. In the present study, our data indicate that D-fraction induces a Th-1 dominance in BALB/c mice, in which a Th-2 response is dominant due to the introduction of tumor cells, when a DC subset containing a majority of activated

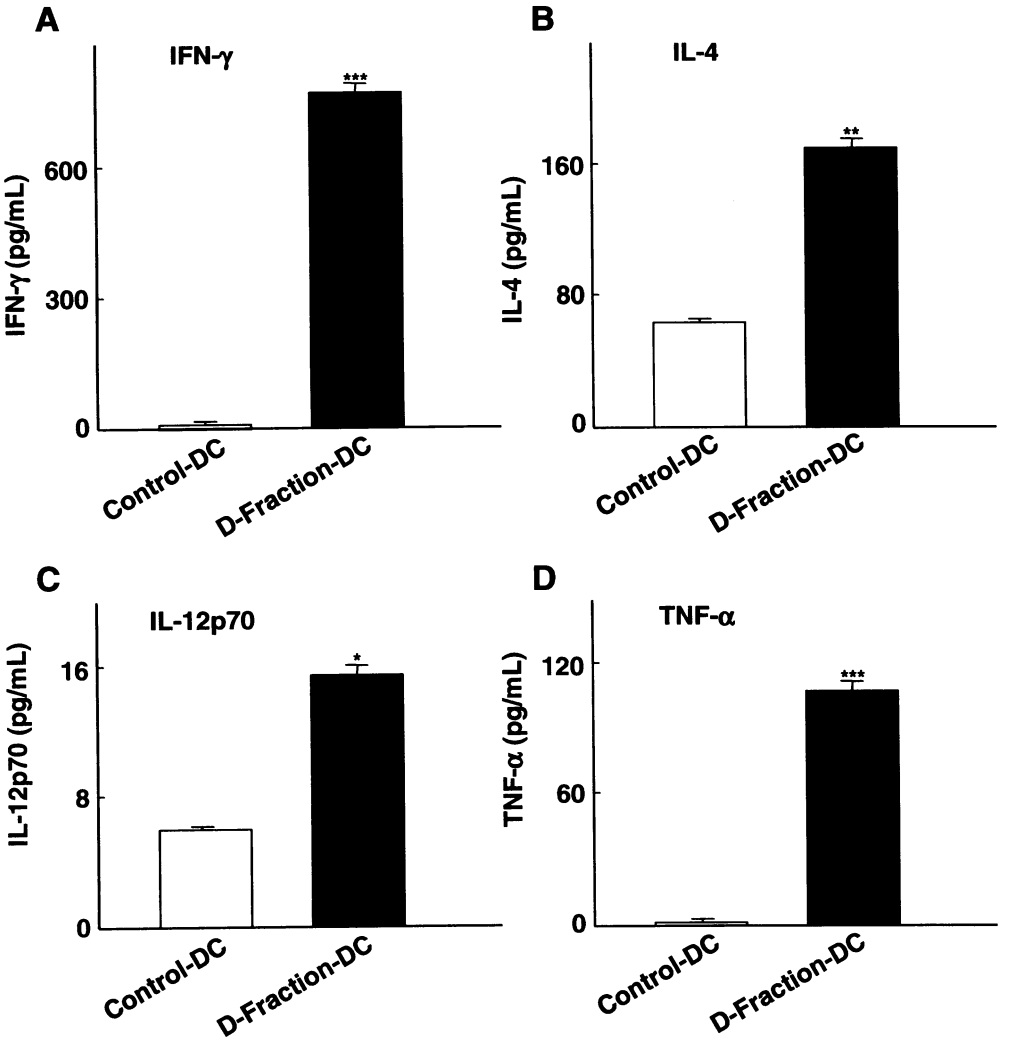

Fig. 4. Effects of D-fraction on the production of IFN-γ (A), IL-4 (B), IL-12p70 (C), and TNF-α (D) by whole splene cells and lymph node cells. Data represent mean ± SEM of three different experiments (two mice/experiment). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, compared with the control group (Student's *t*-test).

$CD8\alpha^+$ DCs shows high IL-12-producing activity (Figs. 1A and 2). Bioactive IL-12 is composed of two subunits, p35 and p40 [21]. In the APC-Th cell interaction, p40 mRNA accumulation in APC has been shown to be up-regulated by stimulation with CD40 ligand (CD40L) on Th cells. The interaction of the MHC class II molecule with TCR evokes an activation signal for p35 mRNA accumulation in APC. Therefore, we speculated that the expression CD40 is induced by D-fraction. In fact, flow cytometric analysis showed an increase in $CD40^+$ cell in DCs activated by D-fraction (data not shown). In addition, D-fraction-stimulated DCs activated T cell subsets of Th cells and CTL (Table 1) when D-fraction enhanced antigen specific protective immunity by DCs loaded with tumor antigen (Fig. 3).

In the present study, we examined the mechanism of the anti-tumor action of D-fraction, including the activation of $DC8\alpha^+$ cells following a Th-1 dominant response. Although the action of D-fraction on DCs and its intracellular signal transduction pathway remain unclear, D-fraction may be a useful stimulator

of DCs, which induce the differentiation of $CD4^+$ T cells to Th-1 cells.

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