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Polyacetylenic Compounds and Butanol Fraction from *Bidens pilosa* can Modulate the Differentiation of Helper T Cells and Prevent Autoimmune Diabetes in Non-Obese Diabetic Mice

Abstract

Compelling evidence suggests that infiltrating $CD4^+$ type I helper T (Th1) cells in the pancreatic islets play a pivotal role in the progression of diabetes in non-obese diabetic (NOD) mice. We demonstrate in the present report that a butanol fraction of *B. pilosa* suppressed the development of diabetes, helped maintain levels of blood sugar and insulin in NOD mice in a dose-dependent manner and elevated the serum IgE levels regulated by Th2 cytokines in NOD mice. Moreover, the butanol fraction inhibited the differentiation of naïve helper T (Th0) cells into Th1 cells but enhanced their transition into type II helper T (Th2) cells using an *in vitro* T cell differentiation assay. Two polyacetylenic compounds, 2-β-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne and 3-β-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne, identified from the butanol fraction also prevented the onset of diabetes like the butanol fraction. The latter compound showed a stronger activity for T cell differentiation than the former. In summary, the butanol fraction of *B. pilosa* and its polyacetylenes can prevent diabetes plausibly via suppressing the differentiation of Th0 cells into Th1 cells and promoting that of Th0 cells into Th2 cells.

Key words

T helper cells · Differentiation · non-obese diabetes · autoimmune diseases · *Bidens pilosa* · Asteraceae

Abbreviations

NOD:	non-obese diabetic
IDDM:	insulin-dependent diabetes mellitus
PBS:	phosphate-buffered saline
PMA:	phorbol 12-myristate 13-acetate
PHA:	phytohemagglutinin from *Phaseolus vulgaris*
IFN:	interferon
IL:	interleukin
LT:	lymphotoxin
TNF:	tumor necrosis factor
HPLC:	high performance liquid chromatography
UV:	ultraviolet
FACS:	fluorescence activated cell sorter
Th:	helper T cells
Th0:	naïve Th
Th1:	type I Th
Th2:	type II Th
i.p.:	intraperitoneal
MTT:	methylthiazoletetrazolium
IC_{50}:	inhibitory concentration 50%
CC_{50}:	cytotoxic concentration 50%

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Introduction

Human type I insulin-dependent diabetes mellitus (IDDM) is caused by the auotoimmune destruction of β cells in the pancreatic islets. NOD mice can spontaneously develop a similar form of autoimmune diabetes, which shares many pathological features with human IDDM and as such serves as an ideal mouse model for IDDM research [1]. Autoimmune diabetes is clinically diagnosed as leukocyte invasion into the pancreatic islets, called insulitis. These leukocytes include T cells ($CD4^+$ and $CD8^+$), B cells, macrophages and NK cells [2]. Several lines of evidence have revealed that $CD4^+$ Th1 cells play a dominant role in the progression of diabetes in NOD mice though other cells are also implicated in this disease [2], [3].

Cytokines have been demonstrated to be key players in the differentiation of Th0 into Th1 or Th2 cells [4]. Th1 cells are characterized by the secretion of Th1 cytokines including interferon (IFN) γ, tumor necrosis factor (TNF) α, interleukin (IL)-2, and lymphotoxin (LT) and cause non-obese diabetes and rheumatoid arthritis. Th2 cells are characterized by the secretion of Th2 cytokines (IL-4, IL-5, IL-13 and/or IL-10) and cause asthma and allergy. It is worthy of note that the Th1 cytokine, IFNγ, enhances Th1 generation but inhibits Th2 generation whereas the Th2 cytokine, IL-4, enhances Th2 generation but inhibits Th1 generation. Therefore, a Th1/Th2 imbalance and various cytokines are thought to be involved in the development of autoimmune diseases [4]. Since Th1 cells have been demonstrated to be implicated in autoimmune diabetes [2], changing the ratio of Th2 to Th1 cells or their cytokines may suppress the disease. Several lines of evidence have shown that systemic administration and/or transgenic expression of IL-4 and/or IL-10 can ameliorate diabetes development and suppress Th1 cell activation [5], [6] whereas introduction of the Th1 cytokine, IFNγ, near pancreatic islets can increase recruitment of islet-reactive $CD4^+$ cells and exacerbate diabetes in NOD mice [7], [8], [9].

Bidens pilosa L., an Asteraceae plant, widely grown in tropical and subtropical areas of the world has been used in various medications, including those for stomach illnesses, infections, malaria and liver disorders [10], [11], [12], [13]. Several constituents isolated from *B. pilosa* have been shown to possess anti-inflammatory, immunosuppressive [14], anti-bacterial [15], anti-malarial [12] and anti-hyperglycemic activities [16], [17], or exhibit inhibitory effects in the prostaglandin synthesis pathway [10].

In this study, we used a T cell differentiation assay to evaluate the immunomodulatory bioactivity of the whole plant extracts of *Bidens pilosa*. We found that a butanol fraction and its two polyynes can suppress the onset of diabetes in NOD mice probably via modulation of Th cell differentiation.

Materials and Methods

Chemicals and cells

Methylthiazoletetrazolium (MTT), phorbol 12-myristate 13-acetate (PMA), phytohemagglutinin (PHA) and ionomycin were purchased from Sigma (MO, USA). Cytokines and antibodies were from R&D systems (MN, USA), RPMI 1640 medium was purchased from Gibco (CA, USA) and human umbilical cord blood cells were obtained from Taipei Medical University Hospital. All other chemicals and solvents used in this study were of HPLC or reagent grade.

Preparation of plant extracts from *B. pilosa* and HPLC profiling

Bidens pilosa var. *radiata* (Asteraceae) was collected and a voucher specimen (no. 0211943) deposited at the Herbarium of National Taiwan University, Taiwan. Crushed whole plants (10.0 kg) were extracted by boiling in water for 2 h, prior to evaporation, suspension in water (1.0 L) and partition with *n*-butanol (1.0 L × 3), yielding two fractions, a water fraction and a butanol fraction (37.7 g). The HPLC profile of the butanol fraction was determined using an RP-18 column [Phenomenex Luna 3μ C18 (2), 150 × 2.0 mm] at a flow rate of 0.2 mL/min, detected at UV 254 nm. The solvent gradient for HPLC was 0.05% TFA/acetonitrile (**B**) in 0.05% TFA/H_2O (**A**): 10 to 19% **B** from 0 to 5 min, 19 to 21% **B** from 5 to 17 min, 21 to 30% **B** from 17 to 23 min, 30 to 35% **B** from 23 to 40 min, 35 to 100% **B** from 40 to 43 min, 100% **B** from 43 to 45 min, and re-equilibration to 10% **B** from 45 to 47 min.

2-β-D-Glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**1**) and 3-β-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne **(2)** were isolated from the *B. pilosa* extract as published elsewhere [16]. The major components of butanol fraction were isolated and identified as 4,5-di-*O*-caffeoylquinic acid (**3**), 3,5-di-*O*-caffeoylquinic acid (**4**), 3,4-di-*O*-caffeoylquinic acid (**5**) by comparing their NMR, MS data and optical rotations with those reported previously [18]. The purities of compounds **1** and **2** are > 95% and > 80%, respectively using ^{1}H-NMR determination and HPLC purification.

T cell isolation, growth, differentiation and intracellular staining

Human umbilical cord blood $CD4^+$ Th0 cells were purified with a MACS column (Miltenyi, CA, USA) and grown in RPMI 1640 medium. Th0 cells (0.5×10^6/mL) were incubated with RPMI medium containing PHA, IL-12 and αIL-4 (Th1 condition) or RPMI medium containing PHA, IL-4 and αIL-12 (Th2 condition). IL-2 was added 48 h later. Plant extracts were incubated with differentiating helper T (Th) cells for 24 h on day 5. For intracellular cytokine staining, T cells were treated with PMA/ionomycin for 4 h plus Golgiplug (BD Biosciences, CA, USA) for 2 h and then subjected to FACS (fluorescence activated cell sorter) analysis.

Mice and diabetes measurement

NOD mice from the Jackson Laboratory (ME, USA) were maintained in the institutional animal facility and handled according to the guidelines of Academia Sinica Institutional Animal Care and Utilization Committee. Marked diabetes in female NOD mice started from 13 weeks of age and the cumulative diabetes incidence was 60% of the total mouse population at 30 weeks of age. Glycosuria and glycemia were monitored at the indicated intervals using Clinistix® and Glucometer Elite® (Bayer, PA, USA), respectively. Blood insulin and IgE concentrations were monitored using an ELISA kit (Crystal, IL, USA).

Results

In this paper, we have tried to identify the immunomodulatory compounds from *B. pilosa*. Therefore, the water and butanol frac-

tions separated from the whole plant extract of *B. pilosa* were subjected to a T cell differentiation assay. The water fraction of *B. pilosa* did not affect Th1 cell differentiation from 0 to 500 μg/mL (Fig. **1A**). In contrast, the butanol fraction showed a 50% inhibition (IC_{50}) to the differentiation of Th0 into Th1 cells at 200 μg/mL and completely stopped the differentiation of Th0 into Th1 cells at 500 μg/mL (Fig. **1B**). These results indicated that the butanol fraction but not the water fraction could inhibit the differentiation of Th0 into Th1 cells. The concentration of the butanol fraction which is toxic to half of the Th1 cells (CC_{50}) was 180 μg/mL, a little lower than its IC_{50} for Th1 cell differentiation (Fig. **1C**). Therefore, the inhibition of Th1 differentiation by the butanol fraction might be partially attributed to cytotoxicity.

Th1 and Th2 cell differentiation has been shown to be cross-regulated [4]. Our data had demonstrated that a butanol fraction of *B. pilosa* could suppress the percentage of IFNγ-producing cells (i.e., Th1 cells) using FACS analysis, we then examined the effect of the butanol fraction on IL-4 producing cells (i.e., Th2 cells). The butanol fraction at 50, 150 and 250 μg/mL increased the percentage of IL-4-producing cells from 11% to 28% (upper panel, Fig. **2**) whereas the butanol fraction at the same doses decreased that of IFNγ-producing cells from 50% to 18% (lower panel, Fig. **2**). Our results indicated that the butanol fraction inhibited the differentiation of Th0 into Th1 cells but promoted that of Th0 into Th2 cells. This observation is in good agreement with the reciprocal antagonism of Th cell differentiation into distinct subsets. In summary, the butanol fraction from *B. pilosa* extract favors the generation of Th2 cells rather than Th1 cells, as evaluated using *in vitro* T cell differentiation assay.

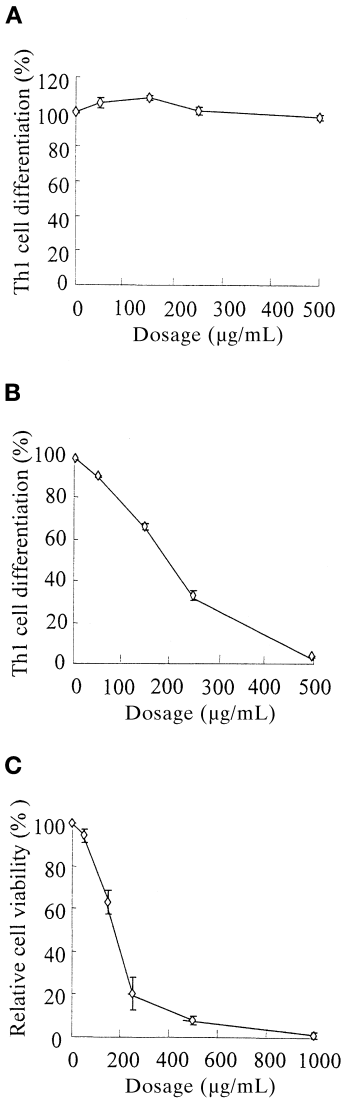

Fig. **1** The butanol fraction can inhibit differentiation of Th0 cells into Th1 cells. **A:** $CD4^+$ T cells were cultured under Th1 condition in the presence of the water fraction of *B. pilosa* at 0, 50, 150, 250 and 500 μg/mL. T cells were analyzed using FACS and the percentage (%) of Th1 cell differentiation was obtained from the ratio of the percentage of IFNγ-producing cells treated with various concentrations of the water fraction to that without any treatment. **B:** The same as **A**, except the butanol fraction was used at concentrations of 0, 50, 150, 250 and 500 μg/mL. **C:** Relative cell viability. The percentage of surviving Th1 cells was determined at 0, 50, 150, 250, 500 and 1000 μg/mL using an MTT test. Data (mean ± SD) are representative of three experiments.

Th1 cells were reported to cause insulitis and diabetes in NOD mice [2]. Our *in vitro* data had indicated that the butanol fraction of *B. pilosa* could suppress the differentiation of Th0 into Th1 cells and preferentially promote that of Th0 into Th2 cells. We reasoned that the butanol fraction may *in vivo* prevent diabetes in NOD mice via down-regulation of Th1 cells or up-regulation of Th2 cells, which antagonize Th1 cell function. To test whether this would be the case, we utilized NOD mice as a Th1-mediated autoimmune disease mouse model to examine the effect of the butanol fraction on the onset of diabetes. Our results indicated that mice with intraperitoneal (*i.p.*) injection of the butanol fraction at 3 mg/kg per dose had a lower diabetes incidence (33%) than control mice (56%), which is a similar incidence as in a prior publication [19]. Likewise, injection of NOD mice with the butanol fraction at 10 mg/kg could stop the initiation of the disease (0%). Thus, the butanol fraction treatment could protect NOD mice from developing diabetes in a dose-dependent manner (Fig. **3A**). We also examined diabetes indicators such as blood glucose and insulin. We found that the butanol fraction treatment at 10 mg/kg protected mice from hyperglycemia and hypoinsulinemia in comparison to control mice (Figs. **3B** and **3C**).

Since the butanol fraction of *B. pilosa* could prevent NOD mice from developing diabetes, we next examined whether the butanol fraction could modulate Th cell differentiation in NOD mice. However, the *in vivo* results obtained from intracellular cytokine staining experiments did not consistently show any significant alteration of Th1 or Th2 cell populations in the spleen or lymph node in the butanol fraction-treated mice (data not shown). Alternatively, Th1 cytokines or Th2 cytokines can be used as indicators to reflect Th1 or Th2 cells, respectively. Furthermore, Th1 or Th2 cytokines control the generation of various types of immunoglobulin (Ig). For instance, the Th1 cytokine, IFNγ, favors IgG2a production while the Th2 cytokine, IL-4, favors IgE production [20]. Therefore, IgG2a and IgE can serve as indirect markers to evaluate the change in the number of Th1 cells or Th2 cells. We next assessed whether the butanol fraction of *B. pilosa* could affect the production of IgG2a and IgE in the serum of NOD mice. The level of serum IgE rose significantly in the mice treated with the butanol fraction of *B. pilosa* as compared to control mice (Fig. **3D**). The level of IgG2a also declined to some extent (data not shown). These findings strongly support the notion that the butanol fraction *in vivo* increases the generation of Th2 cells whose cytokines promote IgE generation but decrease the generation of Th1 cells whose cytokines promote IgG2a generation. Taken together, we propose that the butanol fraction can alleviate Th1 cell-mediated non-obese diabetes via inhibiting differentiation of Th0 cells into Th1 cells and enhancing that of Th0 cells into Th2 cells.

Chemical profiling of the active butanol fraction using HPLC indicated similar compositions of the butanol fraction from different

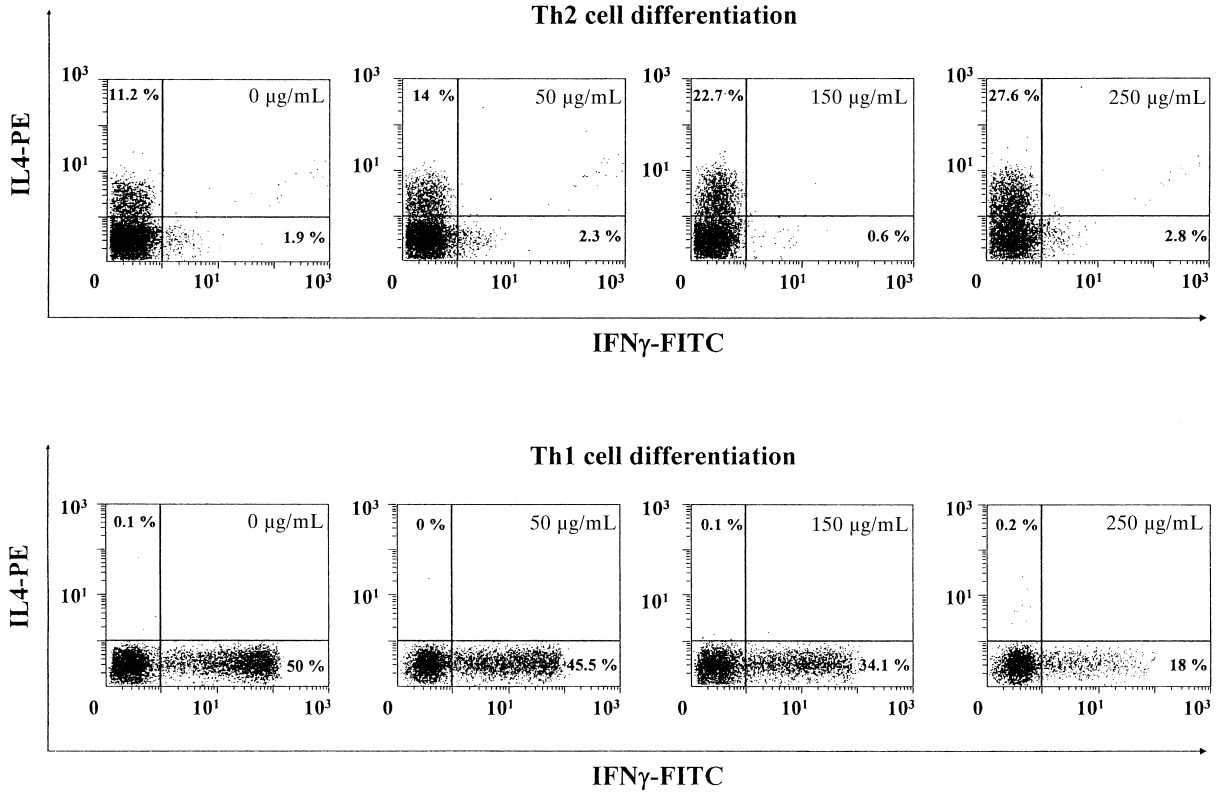

Fig. **2** The butanol fraction can promote differentiation of Th0 cells into Th2 cells. $CD4^+$ T cells were cultured under Th2 conditions in the presence of the butanol fraction at 0, 50,150 and 250 μg/mL. T cells were analyzed using FACS and the percentage of IL-4-producing cells was calculated (Upper panel). Following the same treatment as described for Th1 cell differentiation (Fig. **1**), the percentage of IFNγ-producing cells was calculated (Lower panel). Data are representative of three experiments.

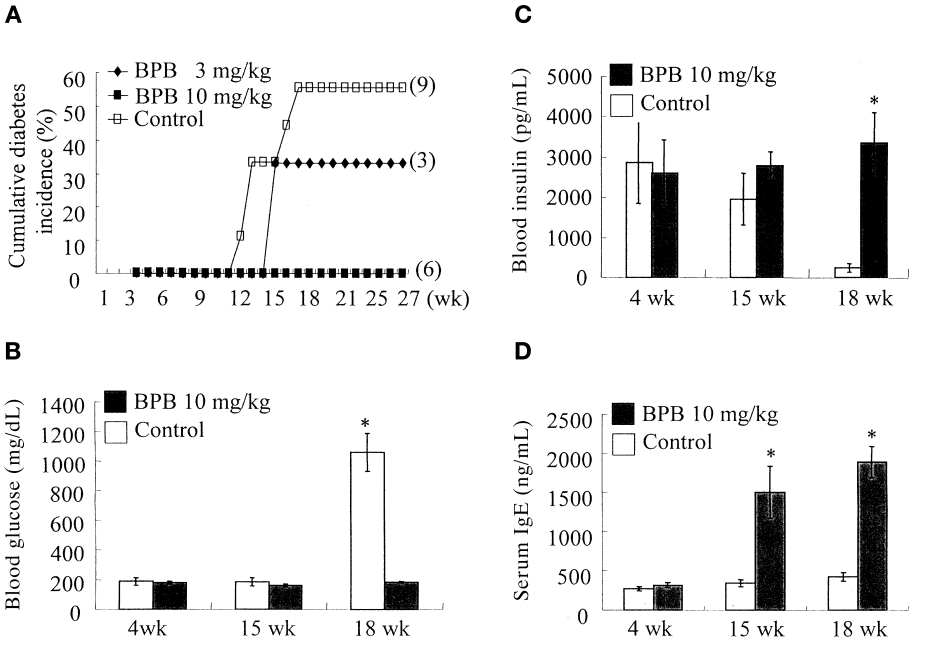

Fig. **3** The butanol fraction can prevent the development of non-obese diabetes. **A:** Cumulative diabetes incidence in female NOD mice. Three groups of female NOD mice (mouse numbers per group are indicated in parenthesis) received *i. p.* injections with the butanol fraction of *B. pilosa* at 3 mg/kg (BPB 3 mg/kg), 10 mg/kg (BPB 10 mg/kg) or PBS (control) 3 times per week from 4 to 27 weeks of age. Urine glucose was monitored using Clinistix at the indicated ages. Mice with 28 mM glucose or more in their urine for two consecutive weeks were considered to be diabetic. **B:** The concentration of blood glucose (mg/dL) of the 10 mg/kg butanol fraction of *B. pilosa*-treated or control mice (4, 15 and 18 weeks of age) from panel **A** was determined using a glucometer. Data (mean ± SD) are representative of three experiments. **C:** The same mice as indicated in panels **A** and **B** had their blood insulin concentrations (pg/mL) determined using an ELISA kit. Data (mean ± SD) are representative of three experiments. **D:** The same mice as indicated in panels **A**, **B** and **C** had their IgE levels in serum (ng/mL) determined using an ELISA kit. Data (mean ± SD) are representative of three experiments and * : $P < 0.05$ by Student T test.

lot-to-lot extract preparations. Two polyacetylenic compounds (**1** and **2**) and three caffeoylquinic acid compounds were then identified from the butanol fraction (Fig. **4**). Compounds **1** and **2** were tested as the active compounds of the butanol fraction and their amounts were determined using HPLC as 1.5 % and 1.1 % (w/w) the dry weight of the fraction, respectively.

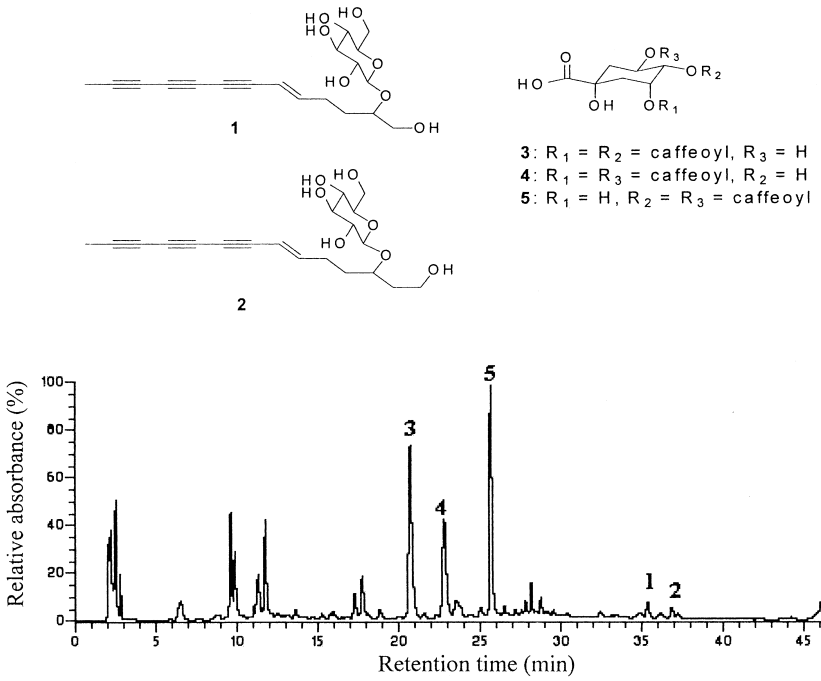

Fig. **4** Chemical profile of the butanol fraction using HPLC analysis. The chemical profile of the butanol fraction was performed using an RP-18 column and detected with a UV detector at 254 nm (see Materials and Methods). (**1**), 2-*β*-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**2**), 3-*β*-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne **(3)**, 4,5-di-*O*-caffeoylquinic acid (**4**), 3,5-di-*O*-caffeoylquinic acid and (**5**) 3,4-di-*O*-caffeoylquinic acid.

We next determined if the aforementioned compounds **1** and **2** have similar effects on the prevention of diabetes as the butanol fraction. We found that treatment with both compounds significantly prevented the onset of diabetes and maintained blood sugar levels in NOD mice (Table **1**). Besides, we demonstrated that compound **2** promoted Th0 differentiation into Th2 cells by 34% but inhibited Th0 differentiation into Th1 cells by 40% at a dose of 15 μg/mL (Fig. **5A**). These results were similar to the data generated from the butanol fraction. In contrast, compound **1** showed, if any, only little inhibition (10%) of the differentiation of Th0 into Th1 cells and little enhancement (8%) of the differentiation of Th0 into Th2 cells at the same dose (Fig. **5B**). Although compound **1** had a lesser effect on the regulation of T cell differentiation, than compound **2**, surprisingly both compounds have a similar preventive efficacy to diabetes development. We therefore provide evidence here that compound **2** as well as compound **1** are the active constituents in the butanol fraction of *B. pilosa* extract which contribute to preventing diabetes.

Discussion

In this study, we found that a polyacetylenic compound and a butanol fraction of *B. pilosa* extract regulated T cell differentiation.

Table **1** Effect of the polyacetylenic compounds on diabetes prevention

Compound **1**	(n = 3)	0% diabetic
Compound **2**	(n = 5)	0% diabetic
Control	(n = 6)	33% diabetic

NOD mice were i.p. injected 3 times per week from 10 to 13 weeks of age with different amounts of compound **1** (37 μg/kg) or compound **2** (45 μg/kg) which were equivalent to the butanol fraction (10 mg/kg). The definition of diabetes is described in the caption of Fig. **3** (n = number of mice).

Meanwhile, the butanol fraction also lowered the diabetic incidence while maintaining normal levels of blood sugar, insulin produced by β cells, and up-regulating IgE in NOD mice. To our knowledge, this is the first report so far to demonstrate that the butanol fraction of *B. pilosa* can effectively prevent IDDM, as evaluated using an NOD mouse model. One possible scenario for the suppression of IDDM may be that the butanol fraction inhibits the generation of Th1 cells and promotes that of Th2 cells infiltrating into the islets of NOD mice as the fraction does *in vitro*. Our results demonstrate that the butanol fraction can up-regulate the production of serum IgE, modulated by Th2 cytokines such as IL-4 and IL-5 and, to some extent, down-regulate that of IgG2a, modulated by the Th1 cytokine, IFNγ, in the mice (data not shown). These results are supported by a previous report summarizing that Ig synthesis from B cells is sophisticatedly controlled by the cytokines produced by various helper T subsets [20]. Therefore, our data suggest that the butanol fraction partially or locally polarizes the differentiation of Th0 cells into Th2 cells, inhibits the differentiation of Th0 cells into Th1 cells and prevents the onset of non-obese diabetes in NOD mice.

A mixture of 2-*β*-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (**1**, Fig. **4**) and 3-*β*-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (**2**, Fig. **4**) from *B. pilosa* have been demonstrated to have a blood sugar lowering effect in type II diabetes (db/db) mice, partly ascribed to the anorexic effect of both polyacteylenic glucosides [16]. However, the mechanism by which both polyacetylenic glucosides affect diabetes is unknown in type II diabetic mice. Here, we demonstrate that compound **2** and compound **1,** isolated from the butanol fraction of *B. pilosa*, can prevent diabetes development in NOD mice although compound **2** is more potent than compound **1** in T cell differentiation. Therefore, we have successfully isolated at least one active compound from *B. pilosa*. More studies are warranted to ascertain the detailed mechanism of the action of both polyynes on IDDM.

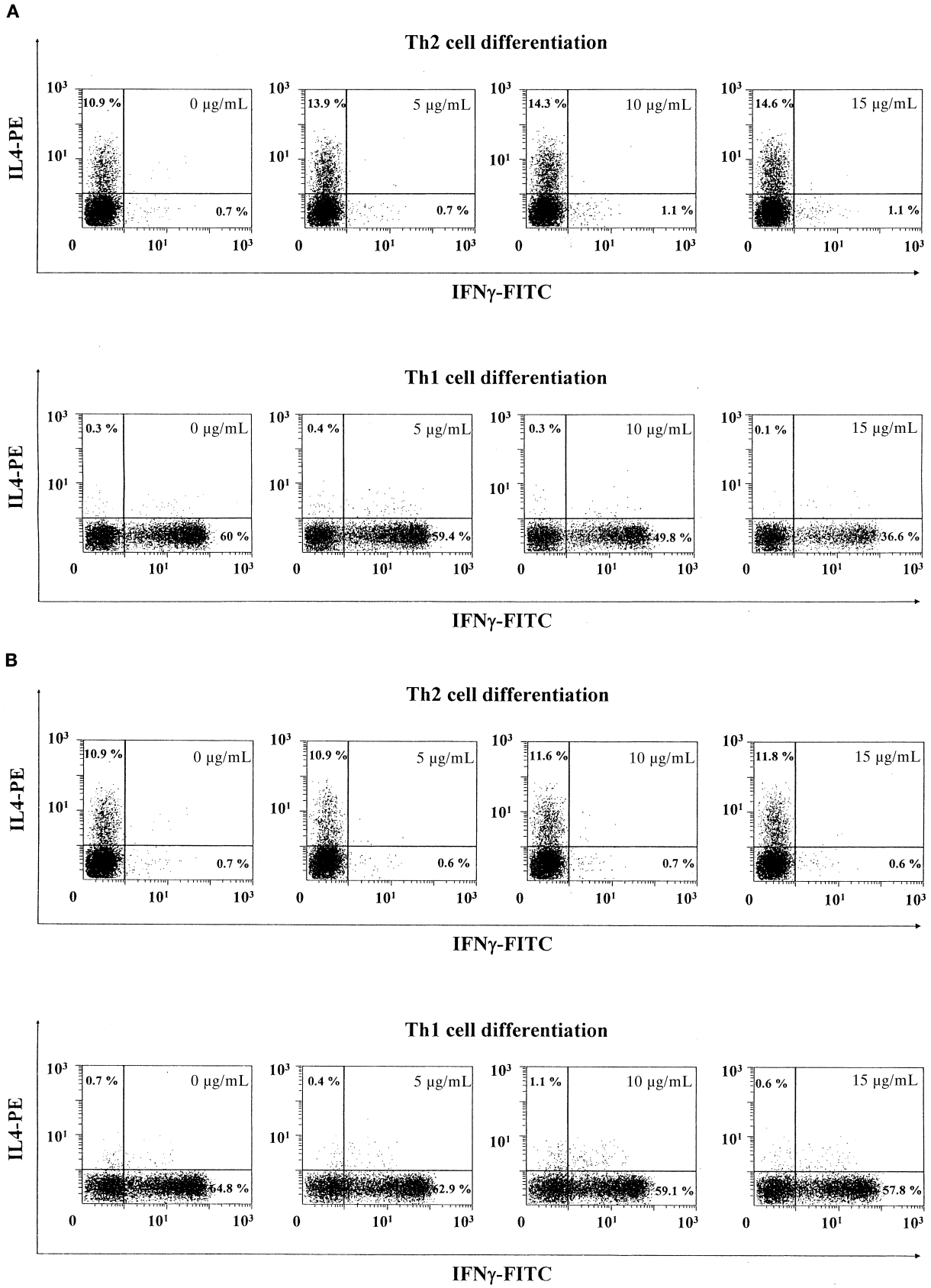

Fig. **5** Polyacetylenic compound can promote differentiation of Th0 cells into Th2 cells but inhibit that of Th0 cells into Th1 cells. **A:** $CD4^+$ T cells were cultured under Th2 (upper panel) or Th1 (lower panel) conditions in the presence of 3-*β*-D-glucopyranosyloxy-1-hydroxy-6(*E*)-tetradecene-8,10,12-triyne (compound **2**) at 0 , 5, 10 and 15 *μ*g/mL. T cells were analyzed using FACS and the percentages of IL4-producing and IFN*γ*-producing cells were calculated, respectively. **B:** Following the same treatment as described above, $CD4^+$ T cells were cultured under Th2 (upper panel) or Th1 (lower panel) conditions in the presence of 2-*β*-D-glucopyranosyloxy-1-hydroxy-5(*E*)-tridecene-7,9,11-triyne (compound **1**) at 0 , 5, 10 and 15 *μ*g/mL. T cells were analyzed using FACS and the percentages of IL4-producing and IFN*γ*-producing cells were calculated, respectively. Data are representative of three experiments.

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