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Cytopiloyne, a Polyacetylenic Glucoside, Prevents Type 1 Diabetes in Nonobese Diabetic Mice[1]

Cicero Lee-Tian Chang,*‡ Shu-Lin Chang,*† Yi-Mei Lee,* Yi-Ming Chiang,* Da-Yung Chuang,*‡ Hui-Kai Kuo,* and Wen-Chin Yang[2]*‡

Some polyacetylenes from the plant *Bidens pilosa* have been reported to treat diabetes. In this study, we report that the cytopiloyne from *B. pilosa*, which is structurally different from the above-mentioned polyacetylenes and inhibits $CD4^+$ T cell proliferation, effectively prevents the development of diabetes in nonobese diabetic mice as evidenced by a normal level of blood glucose and insulin and normal pancreatic islet architecture. Cytopiloyne also suppresses the differentiation of type 1 Th cells but promotes that of type 2 Th cells, which is consistent with it enhancing GATA-3 transcription. Also, long-term application of cytopiloyne significantly decreases the level of $CD4^+$ T cells inside pancreatic lymph nodes and spleens but does not compromise total Ab responses mediated by T cells. Coculture assays imply that this decrease in $CD4^+$ T cells involves the Fas ligand/Fas pathway. Overall, our results suggest that cytopiloyne prevents type 1 diabetes mainly via T cell regulation. *The Journal of Immunology,* 2007, 178: 6984–6993.

Type 1 diabetes is an autoimmune disease resulting from the destruction of insulin-producing pancreatic β cells (1, 2). Patients with type 1 diabetes share many immunopathological and genetic features with NOD mice, a useful mouse model for understanding type 1 diabetes pathogenesis (3, 4). Type 1 diabetes can spontaneously develop in patients or NOD mice. Leukocytes are first found to infiltrate into the pancreatic islets, a condition termed insulitis, and gradually destroy pancreatic islets. Subsequently, a loss of total β cells in the pancreas causes insulin deficiency, leading to diabetes. It has been reported that the β cell death during the disease development was mediated by the Fas ligand (FasL)[3]/Fas and the TNF/TNF receptor pathways. The cytokines (e.g., IFN-γ) produced by the infiltrating leukocytes appeared to affect this disease (5–7). The leukocytes thought to be involved in diabetes include T lymphocytes, B lymphocytes, macrophages, and dendritic cells (8). The importance of $CD4^+$ T cells in the development of type 1 diabetes is supported by findings showing that the adaptive transfer of $CD4^+$ T cells accelerated and provoked this disease in young NOD mice and NOD-SCID mice (9, 10). Moreover, compelling evidence indicates that Th cell differentiation and cytokines are associated with the development of type 1 diabetes (11, 12).

Type 1 Th cells are characterized by the secretion of Th1 cytokines, including IFN-γ, TNF-α, IL-2, and lymphotoxin. Type 2 Th cells are characterized by the secretion of Th2 cytokines (IL-4, IL-5, IL-13, and/or IL-10). During Th cell differentiation, Th1 cells and their cytokines such as IFN-γ enhance Th1 generation but inhibit Th2 generation, whereas Th2 cells and their cytokines such as IL-4 promote Th2 generation but inhibit Th1 generation (13). Some genes known to control T cell differentiation include T-bet (T box expressed in T cells) and Egr for Th1 differentiation and GATA-3, c-Maf, NFAT, Stat6, JunB, NIP45, and Itk for Th2 differentiation (14). However, the mechanistic regulation of these molecules in Th cell differentiation remains elusive. It should also be noted that Th1 cells and their cytokines (e.g., IFN-γ) can promote type 1 diabetes in NOD mice whereas Th2 cells and their cytokines (e.g., IL-4 and IL-10) can suppress this disease in many studies (11, 12). Consistently, shifts from Th1 cells/cytokines to Th2 cells/cytokines by immunomodulatory agents have been reported to protect mice from the development of type 1 diabetes (15). However, most of the above studies have shown relevant but not causative links between such shifts and disease protection. In fact, some studies have pointed out that Th1 to Th2 shifts are a secondary outcome rather than the cause of diabetes protection (16, 17).

Two general strategies for halting the progression of type 1 diabetes are to suppress or eliminate the autoimmunity before it results in overt clinical disease or to use insulin replacement (18) or β cell replacement (19) to replenish insulin. The methods used to suppress autoimmunity against β cells include the use of immunosuppressants, alteration of Th1/Th2 balance, or destruction/depletion of autoreactive immune cells (10, 11, 20). T cells are the main targets of autoimmune suppression or elimination (21). For instance, immunosuppressants such as FK506 (20) can be used to suppress T cell functions and therefore prevent or treat type 1 diabetes. The depletion of $CD4^+$ or $CD3^+$ T cells with anti-CD4 or anti-CD3 Abs, respectively, has also been demonstrated to prevent type 1 diabetes (21–23). In addition, the manipulation of Th1/Th2 cell differentiation has been used to treat autoimmune diseases (24, 25). Nevertheless, too few prophylactic or therapeutic drugs are currently available for type 1 diabetes.

Plants are an extraordinary reservoir for antidiabetic phytochemicals (26, 27). An Asteraceae plant, *Bidens pilosa*, has been used to

*Agricultural Biotechnology Research Center, Academia Sinica, Taiwan, Republic of China; †Department of Biological Science and Technology, National Chiao Tung University, Taiwan, Republic of China; and ‡Department of Veterinary Medicine/Department of Life Science, National Chung Hsing University, Taiwan, Republic of China

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[2] Address correspondence and reprint requests to Dr. Wen-Chin Yang, Agricultural Biotechnology Research Center, Academia Sinica, 128, Academia Road, Section 2, Nankang 115, Taipei, Taiwan, Republic of China. E-mail address: wcyang@gate.sinica.edu.tw

[3] Abbreviations used in this paper: FasL, Fas ligand; AU, arbitrary unit.

treat diabetes in Brazil, Mexico, and Taiwan (28–31). Other and we have shown that two polyacetylenic compounds from this plant prevented and treated type 1 and type 2 diabetes (32–34). In this study, we show that another polyacetylene from *B. pilosa*, cytopiloyne, effectively prevents the development of type 1 diabetes in NOD mice and determine its likely mechanism of action.

Materials and Methods

Cells and mice

EL-4 cells (a mouse T cell line), primary T cells, and primary β cells were grown in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 2-ME (50 μM), sodium pyruvate (1 mM), and glutamate (292 μg/ml). NOD and NOD-SCID mice from The Jackson Laboratory were handled according to the guidelines of the Academia Sinica Institutional Animal Care and Utilization Committee (Taipei, Taiwan). Marked diabetes in our NOD mice was first apparent from ~12 wk of age and the cumulative diabetes incidence in our NOD mouse colonies was 71% in females and 38% in males at 30 wk of age, which are similar to other published rates (35).

Purification of cytopiloyne

Crushed whole *B. pilosa* plants were extracted in methanol for a week, followed by partitioning with ethyl acetate. Cytopiloyne was isolated on an RP-18 HPLC column and its structure was confirmed by NMR spectra using a Bruker DMX-500 spectrometer. Cytopiloyne (purity >95% based on nuclear magnetic resonance determination) was dissolved in DMSO at 20 mg/ml and sterilized with 0.22-μm filters and it tested negative for bacterial growth on agar plates. This stock solution was diluted to the indicated concentrations with RPMI 1640 medium for cell experiments or with PBS for animal experiments. The DMSO dosage in vehicle and cytopiloyne treatment groups was the same.

[^{3}H]Thymidine incorporation assay

Splenic CD4$^+$ T cells purified from prediabetic NOD females (4 wk old) by using MACS columns (Miltenyi Biotec) were cultured with vehicle, IL-2/Con A, or the anti-CD3 Ab in the presence of cytopiloyne for 48 h. After an 18-h [^{3}H]thymidine pulse, the cells were harvested and counted in a PerkinElmer scintillation counter.

Drug administration and diabetes measurement

Female NOD mice received i.p. and i.m. injections of cytopiloyne at 25 μg/kg body weight or 0.2 ml of PBS three times per week from 4 to 30 wk of age unless indicated otherwise. The difference in body weight of cytopiloyne-treated mice and that of PBS-treated mice during the treatment period was not statistically significant. FK506 was administered i.p. at 1 mg/kg body weight in positive control mice, three times per week from 2 to 6 wk of age as published (36). Glycosuria and glycemia in the above mice were monitored every week using a Clinistix strip (Bayer) and an Elite glucometer (Bayer), respectively. Animals whose concentrations of blood glucose were >250 mg/dl for 2 consecutive weeks were considered diabetic. Meanwhile, serum insulin was measured using an ELISA (Crystal Chem).

Immunohistochemistry

Pancreata from NOD females of various groups were snap frozen in OCT compound. Cryosections (5 μm) were collected and fixed with cold acetone for 10 min. Sections were incubated with an anti-insulin Ab plus a HRP-conjugated secondary Ab followed by diaminobenzidine tetrahydrochloride development (Sigma-Aldrich). To double stain the same section, the sections were subsequently incubated with an anti-CD4 Ab plus a HRP-conjugated secondary Ab, developed with use of TrueBlue (Kirkegaard & Perry Laboratories), and mounted with Permount medium. Multiple parallel sections of each pancreas were double-stained and >30 islets per mouse were analyzed by light microscopy. The quantity of CD4$^+$ T cells in the pancreatic islets was quantified from >30 pancreatic sections by the use of AxioVision AC software (Zeiss).

Th cell differentiation

For in vitro Th cell differentiation, splenic CD4$^+$ cells (0.5×10^6/ml) purified from 4-wk-old NOD females on a MACS column were incubated under Th1-inducing conditions (0.1 μg/ml anti-CD3, 0.2 μg/ml anti-CD28, 2 ng/ml IL-12, and 0.5 μg/ml anti-IL-4) or Th2-inducing conditions (0.1 μg/ml anti-CD3, 50 ng/ml IL-4, and 0.5 μg/ml anti-IL-12). IL-2 at 2 ng/ml was added 48 h later. Cytopiloyne at various concentrations was incubated in vitro with differentiating Th cells for 24 h on day 4. After intracellular cytokine staining (BD Biosciences) with anti-IFN-γ-FITC and anti-IL-4-PE, these cells were then subjected to FACS analysis. To evaluate the effect of cytopiloyne on in vivo Th cell differentiation, splenic CD4$^+$ T cells from NOD females that had received vehicle or cytopiloyne for 27 wk (25 μg/kg body weight, three times per week), were treated as for in vitro Th cell differentiation followed by intracellular cytokine staining and FACS analysis.

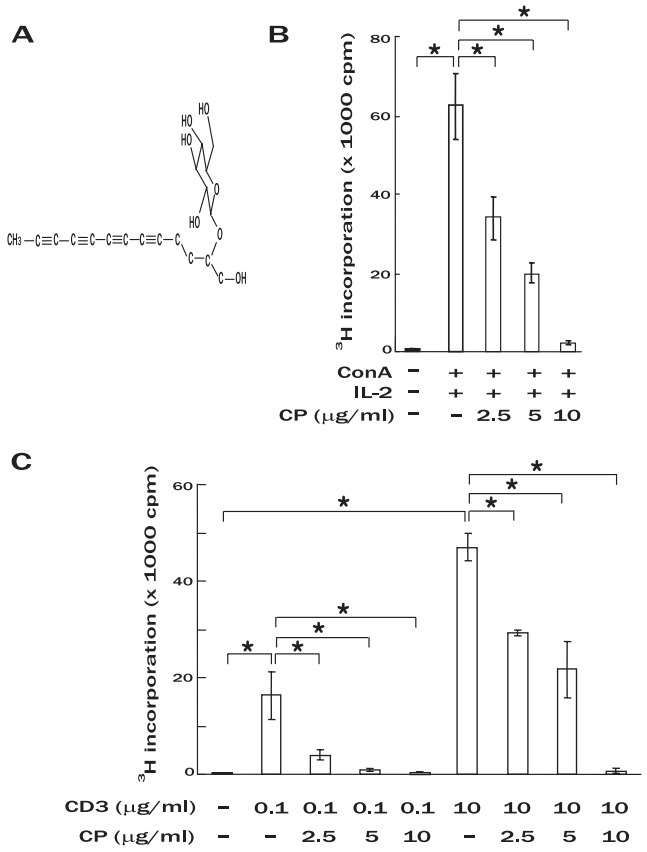

FIGURE 1. Cytopiloyne (CP) inhibits CD4$^+$ T cell proliferation. *A*, The chemical structure of cytopiloyne. *B*, CD4$^+$ T cells purified from NOD mice were left stimulated with vehicle or Con A (125 ng/ml) plus IL-2 (5 ng/ml) in the presence of cytopiloyne at 0, 2.5, 5, or 10 μg/ml for 48 h. [^{3}H]Thymidine incorporation in T cells was determined. *C*, The same assays as *B* except that anti-CD3 Ab was used instead of IL-2 and Con A. Data are expressed as mean ± SE. *, $p < 0.05$ vs control.

ELISA for serum cytokines and antibodies

The levels of serum IFN-γ and IL-4 from 12- or 30-wk-old female NOD mice under different treatments were analyzed on ELISA (eBioscience). To evaluate the T cell-mediated Ab response, NOD females were grouped and injected with cytopiloyne at 25 μg/kg body weight or 0.2 ml of PBS three times per week from 4 to 12 wk of age. These mice were primed with a s.c. injection of 0.2 mg of aluminum hydroxide gel alone or a combination with 0.1 mg of OVA at 10 wk of age. After 2 wk, the sera from the above mice were collected. To determine the levels of OVA-specific antiserum and isotypes, the diluted sera were placed to the OVA-coated, 96-well plates. After extensive washing, peroxidase-conjugated antiserum specific for mouse IgG, IgA, IgM, and IgE (Cappel) and peroxidase-conjugated mAbs specific for mouse IgG2a, IgG2c, IgG1, or IgE (Bethyl Laboratories) were incubated with the plate, followed by color development with tetramethyl benzidine and measurement of the OD at 450 nm.

Dual luciferase reporter assay

EL-4 cells electroporated with an internal control plasmid, pRL-TK, together with pT-bet-Luc or pGATA-3-Luc, were left stimulated with vehicle, PHA (5 μg/ml), or cytopiloyne. Total lysates from the three treatments underwent dual luciferase assays (Promega) as previously described (37).

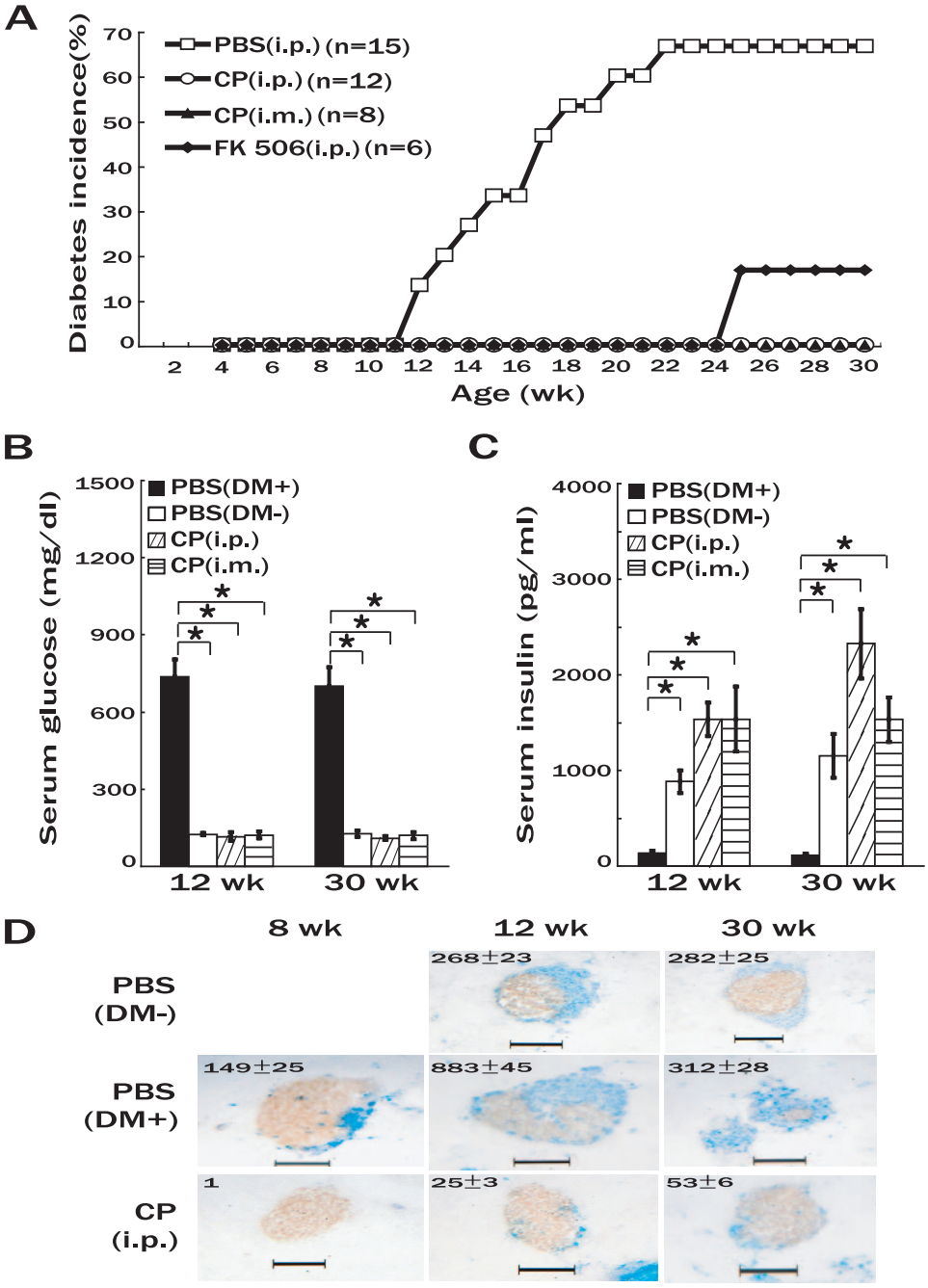

FIGURE 2. Cytopiloyne (CP) prevents the onset of diabetes in NOD mice. *A*, The cumulative diabetes incidence, from birth to 30 wk of age, was monitored in female NOD mice treated with vehicle (PBS; i.p.), cytopiloyne (i.p.), or cytopiloyne (i.m.), or FK 506 (i.p.). The number (*n*) of mice per group is indicated. *B* and *C*, The concentrations of serum glucose (*B*) and serum insulin (*C*) in 12- or 30-wk-old female NOD mice treated with PBS, cytopiloyne i.p., and cytopiloyne i.m. from *A* were determined. Data are expressed as mean ± SE. $*$, $p < 0.05$ vs PBS. *D*, Immunohistochemical images indicated the β cells (brown) and infiltrated $CD4^+$ T cells (blue) in the pancreata of 8-, 12-, and 30-wk-old female NOD mice treated with cytopiloyne (i.p.) or PBS. The ratio of $CD4^+$ T cells, based on blue intensity, was determined and is indicated in the *upper left region* of each image. Scale bars, 100 μm. PBS (DM−) and PBS (DM+) represent the diabetes-free and the diabetic NOD mice, respectively, after receiving PBS injections.

The promoter activity in arbitrary units (AU) was obtained from the ratio of firefly luciferase activity to *Renilla* luciferase activity in the lysate.

Cellularity analysis

Total cells from the pancreatic lymph nodes and spleens of 12- and 30-wk-old NOD females treated i.p. with vehicle or cytopiloyne at 25 μg/kg body weight three times per week for 9 wk (from 4 to 12 wk of age) and 27 wk (from 4 to 30 wk of age) were counted, stained with anti-CD4-FITC, anti-CD8-PE-Texas Red, anti-Mac-1-PE, and anti-B220-PE Abs (BioLegend) and subjected to FACS analysis.

Coculture assay

Splenic $CD4^+$ or $CD8^+$ T cells, purified from 4-wk-old NOD females on MACS columns, were labeled with CFSE for 15 min. Pancreatic islets were isolated from NOD-SCID females that had received either cytopiloyne at 25 μg/kg body weight or 0.2 ml PBS three times per week from 4 to 12 wk of age. CFSE-labeled $CD4^+$ or $CD8^+$ T cells (3×10^4) were cultured alone or with the above islet cells (3×10^4) in the presence of isotype or neutralizing anti-FasL Ab (clone MFL4; BioLegend) for 18 h. Following extensive washing, these cells were stained with propidium iodide (100 μg/ml) and PE-conjugated annexin V and subjected to FACS analysis.

RT-PCR analysis

Total RNA was isolated from the pancreatic islet cells or splenocytes of NOD-SCID females, which had been treated with either cytopiloyne at 25 μg/kg body weight or 0.2 ml of PBS three times per week from 4 to 12 wk of age, by using TRIzol reagents according to the manufacturer's instructions (Invitrogen Life Technologies). The RNA (4 μg) was converted into first-strand cDNA templates using a cDNA synthesis kit (Amersham Biosciences). These cDNA templates were used to perform PCR with FasL primers (5′-AACCAGCCCCTAAACCACAAG-3′ and 5′-TGTCACACAGCAGCCCAAAG-3′), TNF-α primers (5′-TACTGAA CTTCGGGGTGATTGGTCC-3′and5′-CAGCCTTGTCCCTTGAAGAGAA CC-3′), and G3PDH primers (5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′). The PCR products were resolved in DNA gels and visualized with the use of ethidium bromide. The density of the bands from the above gels was measured with a scanning densitometer. AU

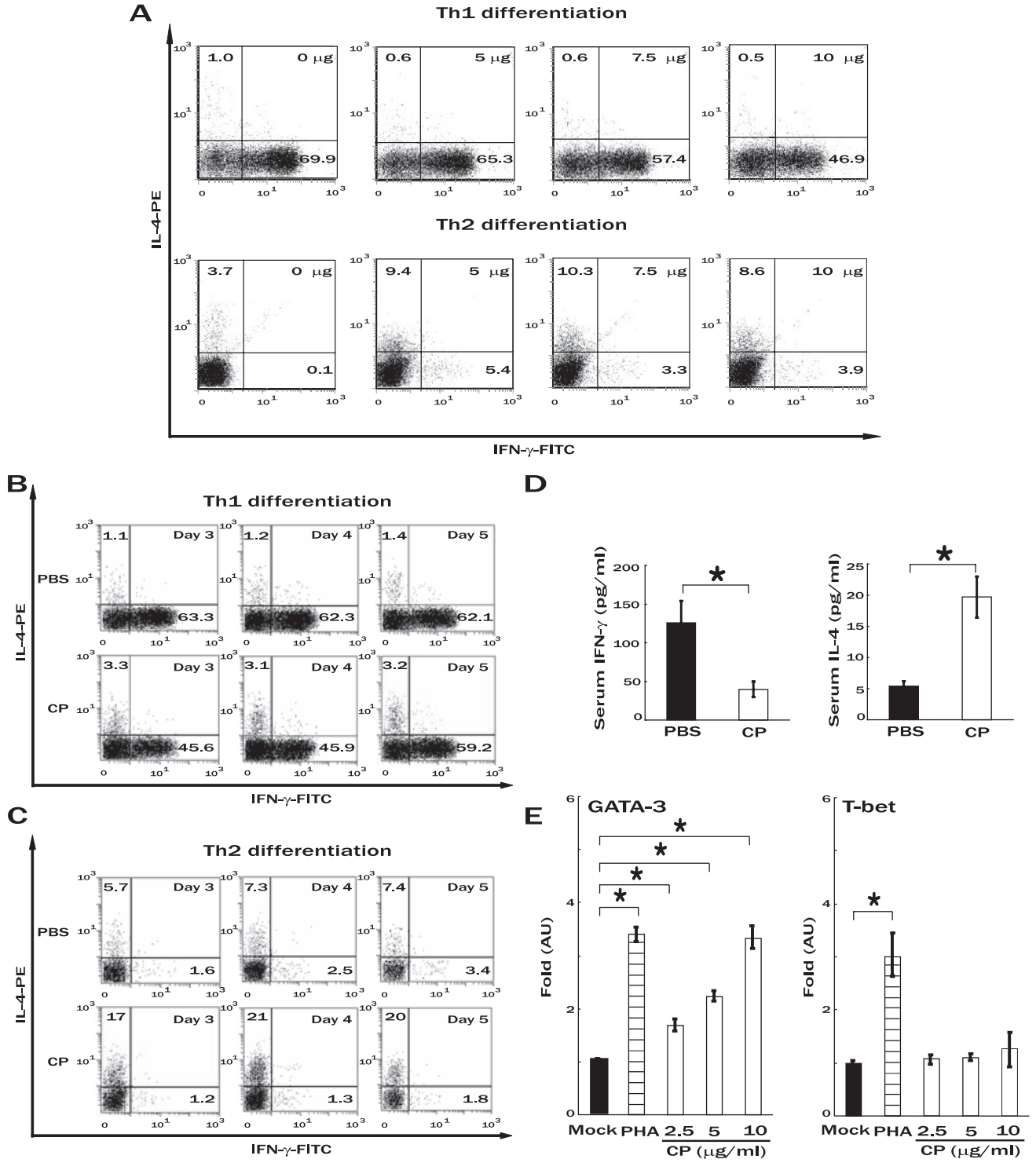

FIGURE 3. Cytopiloyne (CP) modulates Th cell differentiation and cytokine production. *A*, The effect of cytopiloyne on in vitro Th cell differentiation. Splenic CD4$^+$ T cells from NOD mice were in vitro cultured for 4 days under Th1- or Th2-inducing conditions. These cells were treated with cytopiloyne at 0, 5, 7.5, or 10 μg per ml for an additional 24 h and then underwent cytokine profiling analysis with FACS. Percentages of IFN-γ-producing (Th1) cells and IL-4-producing (Th2) cells are indicated in the corresponding quadrants. *B* and *C*, The effect of cytopiloyne on in vivo Th cell differentiation. Splenic CD4$^+$ T cells from NOD mice that had undergone cytopiloyne treatment at 25 μg/kg, 3 times per week, for 27 wk were cultured in vitro under Th1- (*B*) or Th2-inducing (*C*) conditions for 3, 4, and 5 days and then underwent cytokine profiling analysis with FACS. Percentages of IFN-γ-producing (Th1) cells and IL-4-producing (Th2) cells are indicated in the corresponding quadrants. *D*, The serum levels of IFN-γ and IL-4, from 30-wk-old NOD mice ($n = 6$) treated with PBS and cytopiloyne (i.p.) were determined. ∗, $p < 0.05$ vs PBS. *E*, The effect of cytopiloyne on the transcription of GATA-3 and T-bet genes in T cells. EL-4 cells transiently transfected with pGATA-3-Luc (*left panel*) or pT-bet-Luc (*right panel*) and pRL-TK plasmids were left stimulated with vehicle (Mock), PHA (5 μg/ml), or cytopiloyne (2.5, 5, or 10 μg/ml) for 24 h. The activities (in AU) of GATA-3 and T-bet promoters were analyzed. The data expressed as mean ± SE are representative of three independent experiments. ∗, $p < 0.05$ vs mock.

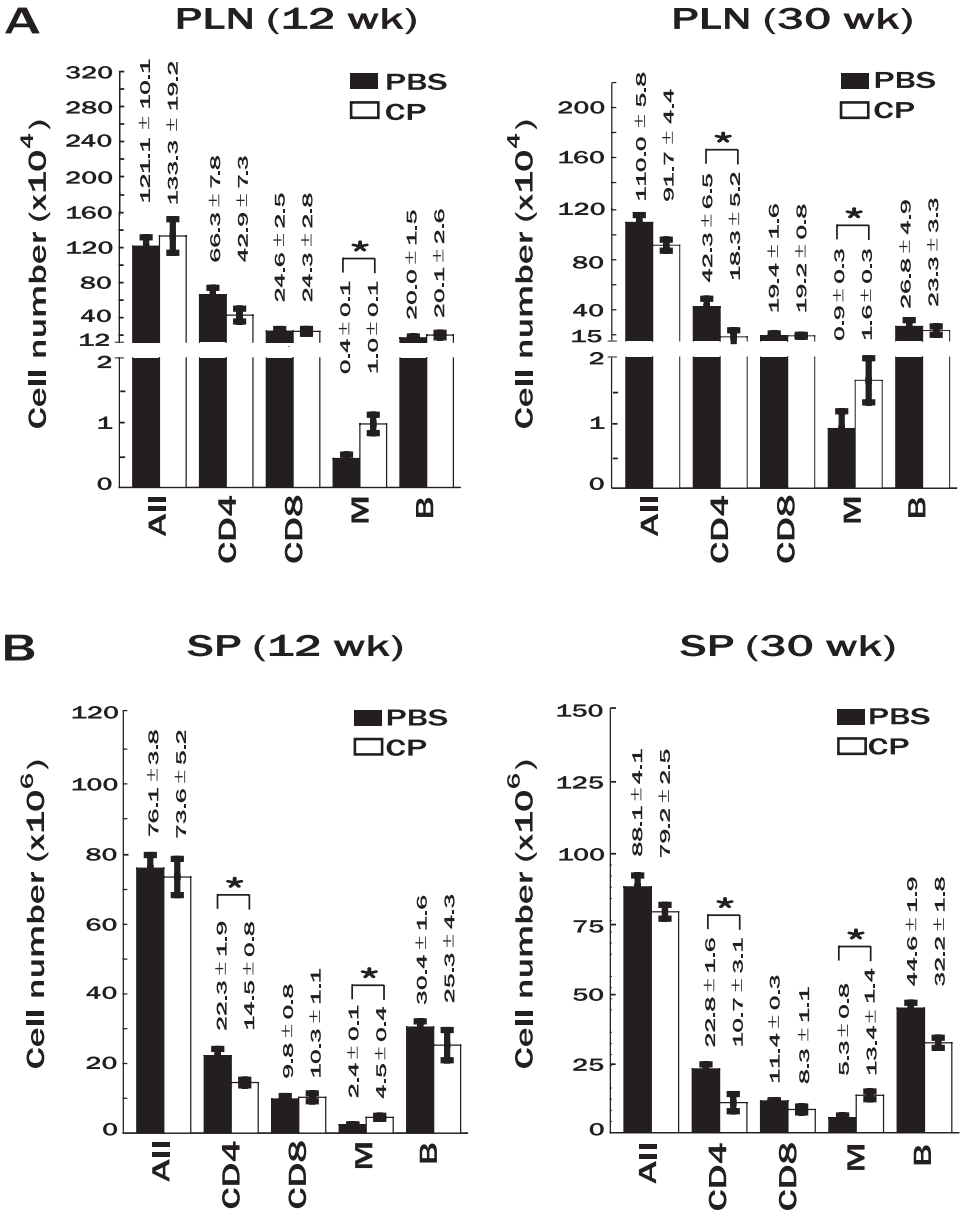

FIGURE 4. Long-term treatment with cytopiloyne (CP) significantly decreases the level of $CD4^+$ T cells but increases that of Mac-1^+ phagocytes in NOD mice. *A*, Cellularity of the pancreatic lymph nodes (PLN) from 12- or 30-wk-old NOD mice with PBS or cytopiloyne treatment. *B*, Cellularity of the spleens (SP) from 12- or 30-wk-old NOD mice ($n = 3$) with PBS or cytopiloyne treatment. All data expressed as mean ± SE are representative of three independent experiments. ∗, $p < 0.05$ vs PBS. All, Total cells from the pancreatic lymph nodes or spleens; CD4, $CD4^+$ T cells; CD8, $CD8^+$ T cells; M, Mac-1^+ cells; and B, B cells.

values were obtained from the ratio of the signal of each band to that of the G3PDH control.

Surface staining in pancreatic islet cells

Pancreatic islet cells were isolated from NOD-SCID females treated with 0.2 ml of PBS or with cytopiloyne at 25 μg/kg body weight three times a week from 4 to 12 wk of age. These cells were stained with anti-FasL, anti-TNF-α, anti-Fas (BioLegend), and anti-insulin Abs (H86; Santa Cruz Biotechnology). FACS analysis was used to determine the mean fluorescence intensity of FasL, TNF-α, and Fas in the insulin-secreting (β) cells.

Statistical analysis

Data from three or more independent experiments are presented as mean ± SE. The difference in diabetes incidence between the groups was analyzed by the Fisher exact probability test. Comparisons between multiple groups were made with ANOVA. Values of $p < 0.05$ were considered significant.

Results

Cytopiloyne inhibits $CD4^+$ T cell proliferation using [^{3}H]thymidine incorporation assays

The polyacetylenic glucoside, used in the study, was isolated from *B. pilosa*, and structurally identified as 2-β-$_D$-glucopyranosyloxy-1-hydroxytrideca-5,7,9,11-tetrayne. For convenience, we hereafter refer to this polyacetylene as cytopiloyne (Fig. 1*A*). To evaluate the role of cytopiloyne in T cells, we first tested whether cytopiloyne could affect $CD4^+$ T cell proliferation. We found that treatment with IL-2/Con A or an anti-CD3 Ab stimulated mouse $CD4^+$ T cell proliferation (Fig. 1, *B* and *C*). These results were consistent with those from a previous publication (38). In contrast, cytopiloyne significantly suppressed mouse $CD4^+$ T cell proliferation induced by IL-2/Con A or anti-CD3 Ab in a dose-dependent manner (Fig. 1, *B* and *C*).

Cytopiloyne can prevent the development of diabetes in NOD mice

$CD4^+$ T cells play an important role in the development of type 1 diabetes (22, 23). Our preliminary data showed that cytopiloyne suppressed $CD4^+$ T cell proliferation. For these reasons, we chose to evaluate the effect of cytopiloyne on diabetes prevention in NOD mice. First, we examined the cumulative incidence of diabetes in NOD mice treated with or without cytopiloyne for 27 wk. Type 1 diabetes spontaneously developed in ~70% of nontreated female NOD mice (Fig. 2*A*) as previously published (35). In

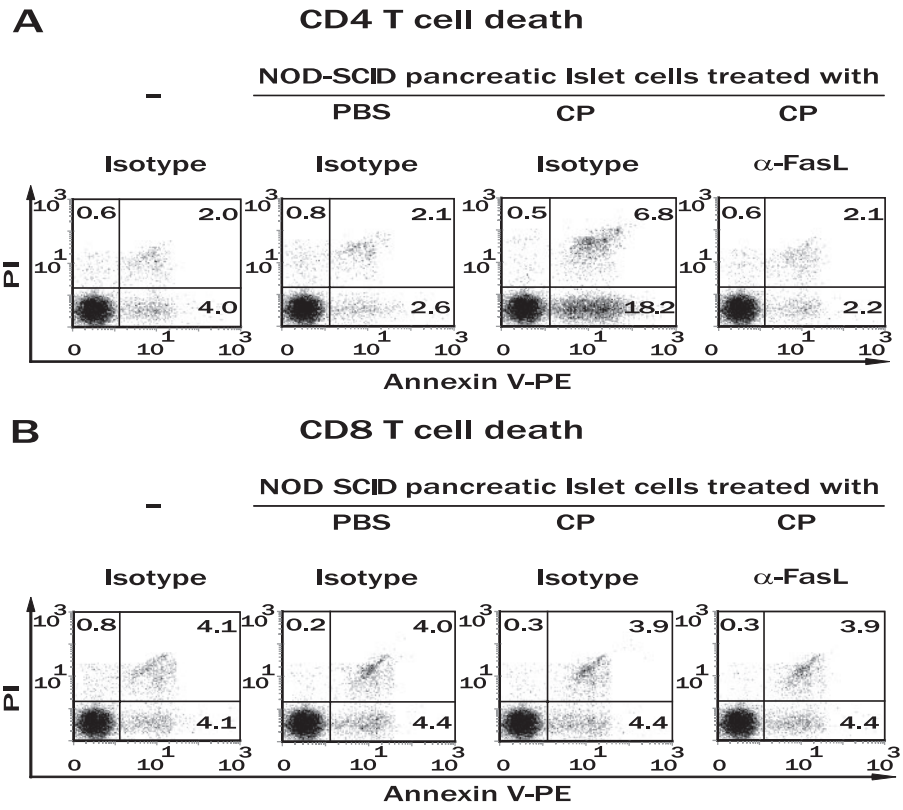

FIGURE 5. Coculture with pancreatic islet cells from cytopiloyne (CP)-treated mice induces apoptosis of $CD4^+$ T cells but not $CD8^+$ T cells. *A*, CFSE-labeled NOD $CD4^+$ T cells were cultured in medium alone or with pancreatic islet cells from NOD-SCID mice treated with either PBS or cytopiloyne in the presence of isotype or anti-FasL Ab for an additional 18 h. After propidium iodide (PI) and annexin V-PE staining, these cells underwent FACS analysis. Data are two-color dot plots of propidium iodide and annexin V gated on $CFSE^+CD4^+$ T cells. Numbers indicate the percentage of cells in each quadrant. *B*, CFSE-labeled NOD $CD8^+$ T cells were cultured in medium alone or with pancreatic islet cells from NOD-SCID mice treated with either PBS or cytopiloyne in the presence of isotype or anti-FasL Ab for an additional 18 h. After propidium iodide and annexin V-PE staining, these cells underwent FACS analysis. Data are two-color dot plots of propidium iodide and annexin V gated on $CFSE^+CD8^+$ T cells. Numbers indicate the percentage of cells in each quadrant.

contrast, an i.p. or i.m. injection of cytopiloyne at 25 μg/kg body weight per dose three times per week completely prevented the development of diabetes in NOD mice aged 30 wk (Fig. 2*A*); thus, the i.p. route was as effective as the i.m. route for cytopiloyne delivery. FK506, a commercial immunosuppressant, has been frequently used to treat type 1 diabetes (20, 36). In our experiments, cytopiloyne was more effective than FK506 in preventing the development of diabetes (Fig. 2*A*). In agreement with the above data, 12- or 30-wk-old NOD mice treated with cytopiloyne maintained normal levels of blood insulin (1,000–2,000 pg/ml) and glucose (<200 mg/dl) as published (39). In contrast, hyperglycemia (blood glucose level >700 mg/dl) and hypoinsulinemia (blood insulin level <135 pg/ml) developed in age-matched control mice (Fig. 2, *B* and *C*).

$CD4^+$ T cell infiltration was barely detected in 8-wk-old NOD mice under cytopiloyne treatment (Fig. 2*D*, *left column*, *bottom row*). However, $CD4^+$ T cells began to infiltrate around the pancreatic islets in the age-matched NOD mice under PBS treatment (Fig. 2*D*, *left column*, *middle row*). Accordingly, pronounced $CD4^+$ T cell infiltration into the pancreatic islets was found in diabetes-free and diabetic 12-wk-old NOD mice under PBS treatment (Fig. 2*D*, *middle column*, *top* and *middle rows*). In marked contrast, only sporadic T cell invasion was found around the pancreatic islets in 12-wk-old NOD mice under cytopiloyne treatment (Fig. 2*D*, *middle column*, *bottom row*). Notably, diabetes developed in 70% of the PBS-treated 30-wk-old NOD mice whose pancreatic islets were severely destroyed (Fig. 2*D*, *right column*, *middle row*). In contrast, diabetes did not develop in the other 30% of PBS-treated 30-wk-old NOD mice with modest T cell infiltration around the pancreatic islets diabetes (Fig. 2*D*, *right column*, *top row*). However, all cytopiloyne-treated 30-wk-old NOD mice were diabetes-free, despite slight T cell infiltration around the circumference of pancreatic islets (Fig. 2*D*, *right column*, *bottom row*). These histopathological data suggest that cytopiloyne strongly inhibits the invasion of $CD4^+$ T cells into the pancreatic islets and thus maintains pancreatic islet integrity in NOD mice.

Cytopiloyne suppresses Th1 cell differentiation and serum IFN-γ level but promotes Th2 cell differentiation and serum IL-4 level in mice

Many immunomodulatory therapeutics have been documented to mediate diabetes protection in NOD mice by skewing Th1 cells/cytokines to Th2 cell/cytokines (15, 40). Therefore, we examined whether cytopiloyne regulated in vitro Th cell differentiation. Cytopiloyne suppressed the differentiation of NOD Th0 cells to Th1 cells in a dose-dependent manner (Fig. 3*A*, *upper row*). In sharp contrast, it promoted the differentiation of NOD Th0 cells to Th2 cells (Fig. 3*A*, *lower row*). To confirm this scenario in vivo, we evaluated Th cell differentiation in NOD mice under cytopiloyne treatment for 27 wk. Cytopiloyne in vivo inhibited Th1 differentiation (Fig. 3*B*) but promoted Th2 differentiation (Fig. 3*C*), similar to its effect on in vitro mouse T cell differentiation (Fig. 3*A*) as well as on human in vitro T cell differentiation (data not shown).

We subsequently assessed the serum level of IFN-γ, a Th1 cytokine, and IL-4, a Th2 cytokine, in NOD mice treated with PBS or cytopiloyne for 27 wk. In line with the role of cytopiloyne in Th cell differentiation, cytopiloyne decreased the level of serum IFN-γ but increased that of serum IL-4 compared with that in controls (Fig. 3*D*). Consistent with IL-4 being important for IgE switching and IFN-γ being essential for IgG2c switching (41), 30-wk-old NOD mice treated with cytopiloyne showed a much higher level of serum IgE and a lower level of serum IgG2c than age-matched PBS-treated controls (data not shown). These findings show that in vivo cytopiloyne regulates Th cell differentiation, cytokine production, and Ab switching.

Cytopiloyne enhances the transcription of GATA-3 but not T-bet genes

To better understand how cytopiloyne regulates Th cell differentiation as well as cytokine expression, we also examined the role of cytopiloyne in the transcriptional regulation of T-bet, a master

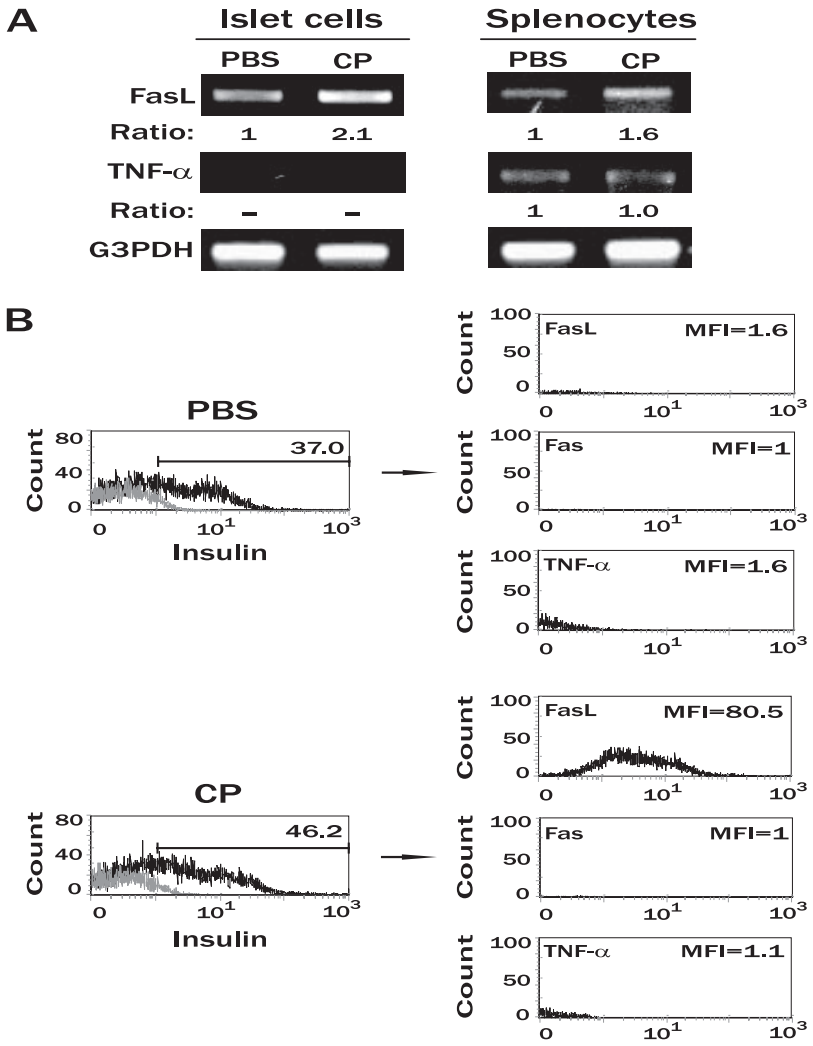

FIGURE 6. Cytopiloyne (CP) up-regulates FasL expression in pancreatic islet cells. *A*, The mRNA levels of FasL, TNF-α, or G3PDH from pancreatic islet cells (*left panels*) or splenocytes (*right panels*) of 12-wk-old NOD-SCID mice, pretreated with vehicle (PBS) or cytopiloyne for 9 wk were determined by RT-PCR. The ratios were obtained by normalizing the signal of FasL or TNF-α bands to that of G3PDH bands. *B*, Pancreatic islet cells from *A* were stained with anti-FasL, anti-TNF-α, anti-Fas, and anti-insulin Abs. The mean fluorescence intensity (MFI) of FasL, TNF-α, and Fas in the insulin-secreting cells was determined by FACS analysis and is indicated in the *upper right region* of each panel.

gene for Th1 cell differentiation, and GATA-3, a master gene for Th2 cell differentiation using dual luciferase assays. Cytopiloyne at 10 μg/ml enhanced GATA-3 transcription 3-fold as compared with controls (Fig. 3*E*). In fact, cytopiloyne stimulated GATA-3 transcription to a similar degree as PHA, a strong T cell activator (Fig. 3*E*, *left panel*). In contrast, cytopiloyne had no significant effect on T-bet transcription compared with controls (Fig. 3*E*, *right panel*). The finding that cytopiloyne enhances GATA-3 expression may explain the preferential role of cytopiloyne in Th2 cell differentiation and IL-4 expression but not Th1 cell differentiation and IFN-γ expression.

Long-term treatment with cytopiloyne depletes CD4$^+$ T cells and increases Mac-1$^+$ cells in the pancreatic lymph nodes and spleens of NOD mice

It is thought that T cell elimination can prevent type 1 diabetes (23). Thus, we examined whether cytopiloyne affected the number of T cells as well as other immune cells in NOD mice treated with PBS or cytopiloyne. Surprisingly, cytopiloyne treatment for 9 wk caused a significant depletion of CD4$^+$ T cells in spleens but an elevation of Mac-1$^+$ cells (namely, macrophages and neutrophils) in the pancreatic lymph nodes and spleens of 12-wk-old NOD mice in contrast to control mice (Fig. 4, *A* and *B*). Moreover, 27 wk of cytopiloyne treatment had an even stronger effect than 9 wk of treatment on CD4$^+$ T cell depletion and the increase in Mac-1$^+$ cells. However, cytopiloyne treatment did not significantly change the number of B cells and CD8$^+$ T cells in 12- and 30-wk-old NOD mice (Fig. 4, *A* and *B*). Of note, the T cell depletion in pancreatic lymph nodes is also in good agreement with a reduced level of CD4$^+$ T cell infiltration and higher islet integrity in the pancreata of NOD mice (Fig. 2*D*).

Pancreatic islet cells from cytopiloyne-treated NOD-SCID mice induce apoptosis of CD4$^+$ T cells but not CD8$^+$ T cells

Our in vivo data showed that cytopiloyne could deplete CD4$^+$ T cells but not CD8$^+$ T cells. However, in vitro treatment with cytopiloyne did not kill CD4$^+$ T cells or CD8$^+$ T cells (data not shown). We therefore hypothesized that cytopiloyne stimulates non-T cells to selectively induce CD4$^+$ T cell death. To test this hypothesis, we examined whether cytopiloyne could induce pancreatic islet cells to kill CD4$^+$ T cells. We first cocultured NOD CD4$^+$ or CD8$^+$ T cells with the pancreatic islet cells of 12-wk-old NOD-SCID mice, which were treated with PBS or cytopiloyne for 9 wk. After a 18-h incubation, the percentage of apoptotic (annexin V$^+$PI$^-$) and necrotic (annexin V$^+$PI$^+$) CD4$^+$ T cells in medium alone and in coculture with the β cells of PBS-treated NOD-SCID mice was $<$4 and 2%, respectively (Fig. 5*A*). Noteworthily, the percentage of apoptotic and necrotic CD4$^+$ T cells in coculture with the pancreatic islet cells of cytopiloyne-treated mice was $\sim$18 and 7%, respectively (Fig. 5*A*). However, an anti-FasL Ab blockade abolished CD4$^+$ T cell apoptosis and necrosis mediated by coculture with the pancreatic islet cells of cytopiloyne-treated mice

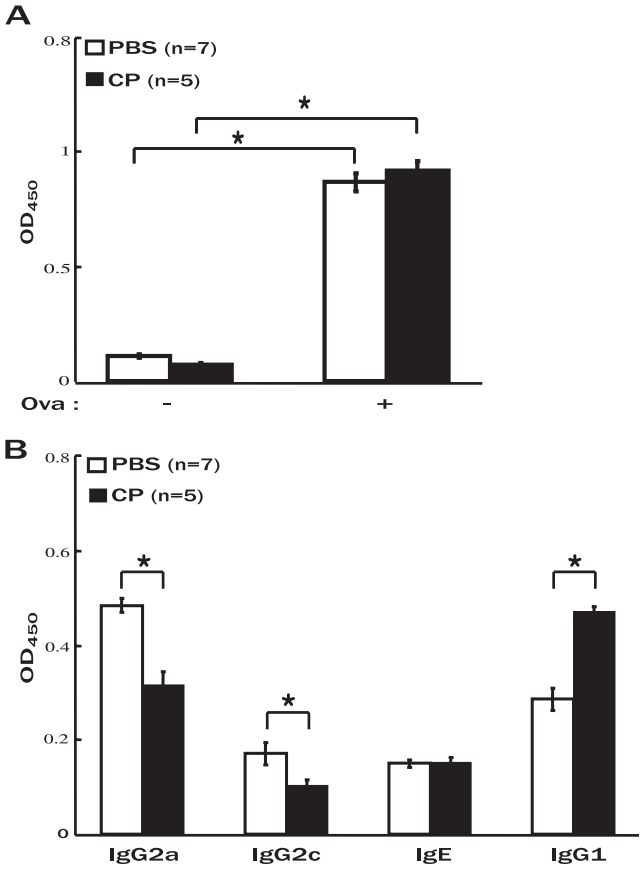

FIGURE 7. Effect of cytopiloyne (CP) on T cell-mediated Ab production. *A*, NOD females were injected with cytopiloyne at 25 μg/kg body weight or 0.2 ml of PBS three times per week from 4 to 12 wk of age. These mice at 10 wk of age were also primed with adjuvant alone or in combination with OVA (Ova). The levels of the whole serum against OVA from the above 12-wk-old females were determined using ELISA kits. The number (*n*) of mice per group is indicated. Data are expressed as mean $\pm$ SE. *, $p < 0.05$ vs PBS. *B*, The levels of OVA-specific isotypes from the above OVA-primed mice in *A* were determined using ELISA kits. The number of mice per group is indicated. Data are expressed as mean $\pm$ SE. *, $p < 0.05$ vs PBS.

(Fig. 5*A*), implying the involvement of the FasL/Fas pathway in the above $CD4^+$ T cell death. In sharp contrast, ~4% of $CD8^+$ T cells were apoptotic and necrotic either in medium or in different cocultures (Fig. 5*B*), which is consistent with the in vivo case in which only the $CD4^+$ T cell level was significantly decreased in pancreatic lymph nodes (Fig. 4*A*). These data suggest that cytopiloyne causes pancreatic islet cell-modulated $CD4^+$ T cell depletion via FasL/Fas interaction.

Cytopiloyne up-regulates FasL expression in pancreatic islet cells

To further elucidate the involvement of the FasL/Fas pathway in pancreatic islet cell-mediated $CD4^+$ T cell death, we examined whether cytopiloyne could affect the expression of two death ligands, FasL and TNF-α, in the pancreatic islet cells and splenocytes of 12-wk-old NOD-SCID mice that had received cytopiloyne or PBS for 9 wk. RT-PCR assays showed that cytopiloyne up-regulated FasL transcription by 2.1-fold in pancreatic islet cells. However, no TNF-α transcription was detected in these cells (Fig. 6*A*, *left panels*). In contrast, cytopiloyne up-regulated FasL transcription by 1.6-fold but had no effect on TNF-α transcription in NOD-SCID splenocytes (Fig. 6*A*, *right panels*). We also evaluated the FasL protein level in the pancreatic β cells of the above NOD-SCID mice. In agreement with FasL mRNA levels, cytopiloyne in vivo increased the FasL protein level by 50-fold in primary β cells (Fig. 6*B*). However, no significant increase in Fas and TNF-α protein levels was found (Fig. 6*B*). These results suggest that $CD4^+$ T cell depletion by cytopiloyne in NOD mice can be partially ascribed to elevated FasL expression in β cells.

Effect of cytopiloyne on T cell-mediated immune response

The above results indicate that cytopiloyne suppressed T cell proliferation and partially depleted $CD4^+$ T cells in the spleens and pancreatic lymph nodes. This raises the possibility that cytopiloyne protects NOD mice from diabetes via a generalized suppression of adaptive immunity. To evaluate whether this was the case, we used OVA as a T cell-dependent Ag to prime NOD mice that had already been treated with cytopiloyne or PBS. We investigated T cell-mediated Ab responses to OVA in 12-wk-old NOD mice. As expected, OVA-primed NOD mice had a much higher anti-OVA antiserum titer than control mice (Fig. 7*A*). However, the level of OVA-specific antiserum from PBS- or cytopiloyne-treated NOD mice was not significantly different 2 wk after OVA priming (Fig. 7*A*). Furthermore, we checked the levels of OVA-specific IgE, IgG1, IgG2a, and IgG2c in the aforesaid sera. NOD mice treated with cytopiloyne showed a significantly lower level of OVA-specific IgG2c and IgG2a but a more elevated level of OVA-specific IgG1 than the age-matched control mice (Fig. 7*B*). In contrast, the level of OVA-specific IgE in both treatments did not significantly change (Fig. 7*B*). The effect of cytopiloyne on Ab switching is consistent with its effect on Th cell differentiation. These findings argued against the role of cytopiloyne in fully suppressing adaptive immunity. Rather, overall the results suggest that cytopiloyne is more immunomodulatory than immunosuppressive.

Discussion

Our results suggest that cytopiloyne is an effective immunomodulatory prophylactic against diabetes development in NOD mice via T cell regulation.

Modulation or intervention of autoimmunity in patients with type 1 diabetes or NOD mice has been indispensable for prophylaxis and therapy for this disease (42, 43). Some traditional immunosuppressants, such as FK506 or cyclosporine A, have been used to prevent or treat type 1 diabetes via the suppression of autoreactive T cells (20, 36, 44, 45). However, these drugs have been gradually abandoned because of their serious side effects, including increased risk of microbial infection and tumorigenesis, toxicity, and reduced immunity (46, 47). In this work, we demonstrate that cytopiloyne prevents type 1 diabetes via T cell intervention by modulating T cell proliferation and differentiation and the partial depletion of $CD4^+$ T cells.

Apparently, cytopiloyne has a different mechanism of action than FK506. Cytopiloyne may have some advantages over FK506, including less toxicity, higher potency, and less immune suppression. Although a detailed drug efficacy comparison between the two compounds for the same treatment period is still required, cytopiloyne (at 25 μg/kg body weight) is more effective therapeutically than FK506 (at 1 mg/kg body weight) in our experimental systems in which FK506 was used in NOD mice from 2 to 6 wk of age, as published earlier (20). Also, cytopiloyne can be used longer than FK506. Cytopiloyne may maintain host immunity to a greater extent than FK506 because cytopiloyne depletes only a portion of $CD4^+$ T cells but increases phagocyte numbers.

Our study showed that cytopiloyne promoted Th2 cell differentiation and cytokine production but suppressed Th1 cell differentiation and cytokine production in NOD mice (Fig. 3) and BALB/c

mice (data not shown), which is consistent with the beneficial effect of cytopiloyne in type 1 diabetes prevention. GATA-3 is known to modulate IL-4 transcription and Th2 cell generation (14). Our results suggest that cytopiloyne increases IL-4 transcription and Th2 differentiation via GATA-3 up-regulation. Although cytopiloyne does not seem to inhibit T-bet expression, it still suppresses Th1 cell differentiation. One explanation could be that Th2 cells and their cytokines, such as IL-4, antagonize Th1 cell differentiation and IFN-γ production (13). Of note, some immunomodulatory agents protect NOD mice from diabetes through shifts from Th1 cells/cytokines to Th2 cells/cytokines (15, 40). In contrast, such shifts may be a consequence but not the cause of diabetes protection mediated by other immunotherapies in different cases (17). Based on our current data, we can conclude that the effect of cytopiloyne on type 1 diabetes prevention is associated with its modulation of the Th1/Th2 balance. To further clarify the cause and effect relationship between Th1 to Th2 shifts and the diabetes prevention mediated by cytopiloyne, the effect of cytopiloyne on diabetes protection in NOD mice deficient in IFN-γ, IL-4, and IL-10 needs to be tested.

T cell depletion with anti-CD3 or anti-CD4 Abs has been used to treat T cell-mediated immune disorders (21–23). Our in vivo treatment with cytopiloyne at 25 μg/kg for 9 wk or more decreases the level of $CD4^+$ T cells but not $CD8^+$ T cells in NOD mice (Fig. 4) and BALB/c mice (data not shown). This depletion should help lower T cell-mediated autoimmune attack on β cells. Furthermore, in vitro incubation with cytopiloyne at 5 μg/ml (200 times the in vivo dosage) did not directly kill $CD4^+$ and $CD8^+$ T cells (data not shown). These data imply that cytopiloyne targets non-T cells, causing them to induce T cell apoptosis. Accordingly, a coculture of pancreatic islet cells from cytopiloyne-treated mice led to the cell death of $CD4^+$ but not $CD8^+$ T cells via up-regulated FasL in β cells (Figs. 5 and 6). These results are consistent with others indicating that $CD4^+$ T cell apoptosis is primarily mediated by the Fas pathway (48). Because $CD4^+$ T cells are depleted in part by cytopiloyne in pancreatic lymph nodes and spleens, cytopiloyne-mediated FasL up-regulation may not be limited to pancreatic β cells. Indeed, cytopiloyne also enhanced FasL expression in NOD-SCID splenocytes (Fig. 6*A*). Therefore, FasL up-regulation by cytopiloyne may be a general event for splenic non-T cells and other cells. The observation that $CD8^+$ T cells were not depleted in cytopiloyne-treated NOD mice (Fig. 4) remains to be further elucidated. $CD8^+$ T cell apoptosis is though to be mediated by the TNF receptor pathway to a greater extent than the Fas pathway (49, 50). Although cytopiloyne up-regulated FasL expression, it did not alter TNF-α expression in pancreatic islet cells and splenocytes (Fig. 6*A*). The low expression level of TNF-α in β cells (Fig. 6*B*) is consistent with the findings of a previous publication (6). These data may explain why cytopiloyne had very little effect on $CD8^+$ T cell death in the pancreatic lymph nodes and spleens of mice.

The FasL/Fas pathway is important in the pathogenesis of type 1 diabetes. Fas expression in β cells, induced by proinflammatory cytokines, is believed to cause β cell death upon encountering FasL-expressing inflammatory cells (5, 7). FasL expression in β cells is thought to promote type 1 diabetes; its exact contribution to disease promotion or protection is still debatable. For example, NOD mice with a FasL point mutation are protected from diabetes compared with standard NOD mice (5). In contrast, islet-specific FasL expression at a high level conferred diabetes protection in transgenic NOD mice, whereas expression at a relatively low level led to diabetes acceleration (5). In addition, different studies have indicated that FasL-expressing non-β cells in the pancreatic islets prevent T cell-mediated β-cell apoptosis (51). The most likely interpretation of these results could be the so-called "kiss of death" mechanism by which FasL expression in β cells or neighboring cells induces T cell death via Fas engagement and this, in turn, prevents β cells apoptosis (51, 52). However, when Fas expression is induced in β cells, coexpression of Fas and its ligand causes β cell death. Our results are consistent with the kiss of death scenario. First, cytopiloyne suppressed the generation of Th1 cytokines, the inducers of Fas expression in β cells (Fig. 3) and, in turn, Fas expression in β cells in NOD mice (data not shown). Second, cytopiloyne can increase FasL protein level up to 50-fold in β cells and perhaps in other cells in NOD-SCID mice (Fig. 6) and up to 30-fold in NOD mice (data not shown). These data may explain why cytopiloyne treatment reduced by half the $CD4^+$ T cells in the pancreatic lymph nodes and spleens of 30-wk-old NOD mice (Fig. 4), prevented β cell death, and inhibited T cell infiltration into pancreatic islets (Fig. 2*D*).

Strikingly, long-term cytopiloyne treatment increased the level of Mac-1^+ cells, including macrophages and neutrophils. We also found that cytopiloyne increased the dextran uptake of macrophages (data not shown). The biological significance of the cytopiloyne-mediated increase in phagocyte number and function is not clear. Compelling evidence has pointed out that macrophages in NOD mice have defects in phagocytosis and apoptotic β cell clearance. These defects are reported to predispose NOD mice to autoimmunity (53, 54). Therefore, the role of cytopiloyne in elevated phagocyte number and function may also be beneficial in type 1 diabetes prevention. As well, the increase in phagocyte number and function may elevate innate immunity against microbes and compensate for a partial loss of $CD4^+$ T cell-mediated adaptive immunity.

Although cytopiloyne decreases T cell proliferation, Th1 differentiation, and T cell number inside lymphoid organs, it does not compromise Ab responses to OVA, a T cell-dependent Ag, and Th2 cell differentiation. The role of cytopiloyne in diabetes protection is not as simple as suppression of the whole immunity. Instead, T cell modulation, such as a decrease in the filtration of T cells into the pancreata and the skewing of Th cell differentiation, may account for diabetes prevention in cytopiloyne-treated NOD mice. However, its effect in treating type 1 diabetes remains elusive. Cytopiloyne might have a therapeutic effect in treating type 1 diabetes in NOD mice in which a certain quantity of β cells in the pancreas are still present. Taken together, our data, for the first time, show that cytopiloyne prevents the development of type 1 diabetes and reveals its likely mechanisms of action. This study not only adds cytopiloyne to the list of biologics for type 1 diabetes but also provides new considerations for the development of drugs for this disease.

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Disclosures

The authors have no financial conflict of interest.

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