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Anti-inflammatory action of *Cudrania tricuspidata* on spleen cell and T lymphocyte proliferation

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Abstract

This study examined whether an extract of *Cudrania tricuspidata* shows anti-proliferative effects in anti-CD3/CD28-mediated spleen and $CD4^+CD25^-$ T cells and decreases the production of the pro-inflammatory cytokines interleukin-2 (IL-2) and interferon-γ (IFN-γ) in anti-CD3/CD28-mediated $CD4^+CD25^-$ T cells. The proliferation of anti-CD3/CD28-mediated spleen cells and $CD4^+CD25^-$ T cells was effectively suppressed by *C. tricuspidata*. This extract, however, did not show cytotoxicity in spleen cells under conditions where the antigen was not stimulated using CCK-8 analysis. *C. tricuspidata* also decreased the production of the pro-inflammatory cytokines IL-2 and IFN-γ by selective inhibition of this extract on proliferating cells in anti-CD3/CD28-mediated $CD4^+CD25^-$ T cells. These results suggest that *C. tricuspidata* may be useful in the treatment of autoimmune diseases and organ transplantation through the inhibitory action of T cells in inflammation.

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Introduction

In South Korea and Oriental countries such as China and Japan, *Cudrania tricuspidata* is used in traditional herbal remedies for inflammation, gastritis, tumours and liver cell damage. Previous investigations have reported that some constituents of *C. tricuspidata* extract show antioxidant activity (Cho et al 2003a, b; Lee et al 2005, 2006), nitric oxide synthase (NOS) and nitric oxide (NO) inhibitory effects on lipopolysaccharide (LPS)-treated macrophages (Seo et al 2000; Kang et al 2002; Park et al 2006), hepatoprotective effects on tacrine-induced cytotoxicity in liver cells (Tian et al 2005) and inhibitory effects on tumour cells (Lee et al 1996; Seo et al 2001; Zou et al 2004; Kim et al 2005). Previous works have reported that biologically active xanthones and flavonoids were isolated from the root bark of *C. tricuspidata* (Lee et al 2005; An et al 2006; Park et al 2006; Seo et al 2007). However, no studies have investigated the effects of this herb on the spleen and the $CD4^+CD25^-$ T cells, which play a crucial role in adaptive immunity.

Activated Th1 cells mainly produce the pro-inflammatory cytokines interleukin (IL)-2 and interferon-γ (IFN-γ) (Constant & Bottomly 1997; Gor et al 2003). IFN-γ promotes the initiation of the autoimmune response such as in rheumatoid arthritis (Asano et al 1996; Chu et al 2000; Dalton et al 2000). In type 1 diabetes, the β-cell mass is reduced by 70–80% at the time of diagnosis, enabling the production of cytokines, such as IL-1β, tumour necrosis factorα (TNF-α) and IFN-γ, by the invading immune cells (Cnop et al 2005). In-vitro exposure of β-cells to IL-1β or to IL-1β+IFN-γ causes functional changes similar to those observed in pre-diabetic patients (Hostens et al 1999). IL-2 can induce T cell expansion and is infused in patients with cancer or AIDS (Pahwa & Morales 1998; Dutcher et al 2002). In contrast, anti-IL-2R antibody is used to inhibit IL-2 signalling for suppressing the rejection of transplanted organs (Morris & Waldmann 2000; Nelson 2004).

Both CD4 and CD8 T cells are involved in graft rejection and autoimmunity (Wang et al 1991, 1996; Hayward et al 1993; Loudovaris et al 1996; Fox et al 2001), although the cells are important mediators of adaptive immunity against pathogens. Therefore, the reduction of side effects of immunosuppressive molecules is important in the care of these diseases. In this study, we investigated whether *C. tricuspidata* can inhibit the proliferation of

anti-CD3/CD28-mediated spleen cells and $CD4^+CD25^-$ T cells in-vitro. We also investigated whether *C. tricuspidata* can decrease the production of the pro-inflammatory cytokines IL-2 and IFN-γ in antigen-activated $CD4^+CD25^-$ T cells.

Materials and Methods

Animals

Male C57BL/6 mice, 8–10 weeks old, were purchased from Charles River Laboratories and cared for and used in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) at the Asan Medical Center and Ulsan University College of Medicine (No. 2007-13-088).

Preparation of crude extract of *C. tricuspidata*

C. tricuspidata was obtained from the Plant Extract Bank of the Korea Research Institutes of Bioscience and Biotechnology. In brief, a trunk of *C. tricuspidata* (30–40 g) was extracted with 200 mL of methanol at 50°C, 1500 psi for 3 days by using an ultrasonic cleaner (Branson Ultrasonics Corporation), and the extracted materials were concentrated with a speed bag (Biotron Corporation, Modul spin 40) at 40°C for 24 h. The *C. tricuspidata* methanol extract (No. 026-021) was dissolved to attain a concentration of 20 mg mL^{-1} in phosphate-buffered saline (PBS), and then diluted to 1–30 μg mL^{-1} for an in-vitro assay with spleen and $CD4^+CD25^-$ T cells.

Cell purification and culture

$CD4^+$ T cells were enriched from spleen and lymph node cells by negative selection, using a $CD4^+$ T cells isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. The isolated $CD4^+$ T cells were incubated for 15 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 antibody (Ab) and PE-conjugated anti-CD25 Ab in PBS, and $CD4^+CD25^-$ T and $CD4^+CD25^+$ T cell populations were isolated by a FACS-Vantage cell sorter (BD Biosciences). The isolated cells were cultured in complete RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM L-glutamine and 55 μM 2-ME at 37°C under 5% CO_2 in humidified air (all purchased from Life Technologies).

Cell proliferation analysis

Spleen and $CD4^+CD25^-$ T cells (3×10^4 cells per well in 96-well U-bottom plates) were activated with 5 μg mL^{-1} coated anti-CD3 (145-2C11; BD Biosciences) and anti-CD28 (37.51; BD Biosciences), respectively, and cultured in the presence or absence of various concentrations of *C. tricuspidata* for 7 days, adding *C. tricuspidata* on alternate days. The cultures were pulsed on day 6 with 1 μCi $[^3H]$-thymidine per well for the last 18 h. After harvesting the cells, $[^3H]$-thymidine incorporation was measured as counts min^{-1} using a β-plate counter (Kruisbeek et al 2004).

Cell viability analysis

The cell viability was determined by a CCK-8 kit (Dojindo Laboratories, Japan) according to the manufacturer's instructions. Briefly, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitropenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (CCK-8) was reduced by dehydrogenases in cells to yield an orange-coloured product (formazan) (Nakagawa et al 2002). The amount of the formazan dye generated by dehydrogenases in cells was directly proportional to the number of living cells. Spleen cells (3×10^4 cells per well in complete RPMI-1640 media in a 96-well U-bottom plate) were cultured in 200 μL medium per well without antigen stimulation in the presence or absence of 30 μg mL^{-1} *C. tricuspidata* for 3 days. CCK-8 (20 μL) was added to each well of the plate and the cells were incubated for 2–3 h. The absorbance was measured at 450 nm using a microplate reader.

Analysis of cytokines

The culture supernatant described above was collected at 2 and 6 days. The level of IL-2 and IFN-γ was determined by ELISA kit (R&D Systems).

GC-MS analysis

GC-MS analysis of the *C. tricuspidata* methanol extract was performed on an Agilent 6890 GC/5973N mass selective detector (MSD) (Agilent Technologies, Palo Alto, CA). A high-resolution capillary column DM-5MS (30 m × 250 μm × 0.25 μm) was used. The oven temperature was initially set at 60°C for 3 min, then increased to 310°C at a rate of 5°C min^{-1}, and was then maintained for 17 min. The front inlet temperature was maintained at 250°C. Split injection was conducted with a split ratio of 10:1, and helium was used as the carrier gas at a rate of 1 mL min^{-1}, with the volume of injection as 1 μL.

Statistical analysis

For the proliferation analysis performed to increase the dose of the herbal extract, the results were analysed by one-way analysis of variance, followed by the Dunnett's multiple comparison test. For the curve plots and cytokine analysis, a two-tailed *t*-test was used. All results were generated using GraphPad Prism software. Asterisks indicate significant differences from the control group ($*P<0.05$, $**P<0.01$, $***P<0.001$).

Results

Inhibitory effect of *C. tricuspidata* on antigen-mediated spleen cell and $CD4^+CD25^-$ T cell proliferation

$CD4^+$ T cells were enriched from the spleen, including lymph node cells, by negative selection and $CD4^+CD25^-$ T cells were isolated from $CD4^+$ T cells by using a FACSVantage cell sorter. The purity of $CD4^+CD25^-$ T cells was 97–99% (Figure 1).

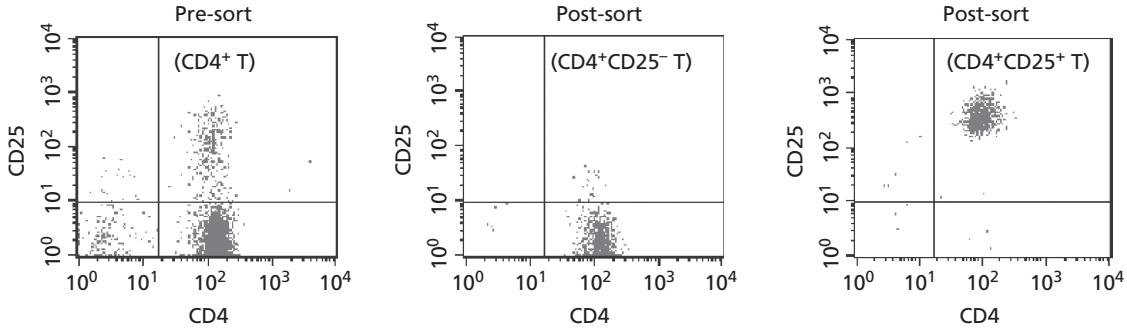

Figure 1 Purification of $CD4^+CD25^-$ T cells from spleen cells. $CD4^+$ T cells isolated from the spleen cells were incubated with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 Ab for 15 min at 4°C and sorted by a FACSVantage cells sorter. The isolated $CD4^+CD25^-$ T and $CD4^+CD25^+$ T cells showed 97–99% and 90–98% purity, respectively. Dot plots show CD4-FITC on the x-axis and CD25-PE on the y-axis.

Although *C. tricuspidata* has been reported to inhibit NOS and NO production in RAW 264.7 cells, little is known about its effects on the inhibition of anti-CD3/CD28-mediated spleen and T cell proliferation. First, we investigated whether *C. tricuspidata* ($30\,\mu g\,mL^{-1}$) showed cytotoxicity against spleen cells incubated without the stimulation of anti-CD3/CD28 for 3 days by using CCK-8 assay. Our results showed that *C. tricuspidata* does not have a cytotoxic effect on spleen cells. Next, we investigated whether it inhibits antigen-mediated spleen cell and T cell proliferation. The results revealed that *C. tricuspidata* effectively inhibited anti-CD3/CD28-mediated spleen cell and $CD4^+CD25^-$ T cell proliferation (Table 1), indicating that *C. tricuspidata* may have potent anti-inflammatory activity in adaptive, as well as innate, immunity.

To further confirm the above results, anti-CD3/CD28-mediated $CD4^+CD25^-$ T cells were incubated for 3–7 days with or without $30\,\mu g\,mL^{-1}$ *C. tricuspidata*. This extract clearly suppressed anti-CD3/CD28-mediated $CD4^+CD25^-$ T cell proliferation on day 7, when the control showed a peak (84% inhibition of proliferation; $P<0.0001$) (Figure 2). On day 5, *C. tricuspidata* showed 70% inhibition of antigen-mediated T cell proliferation in contrast to the control ($P=0.0046$).

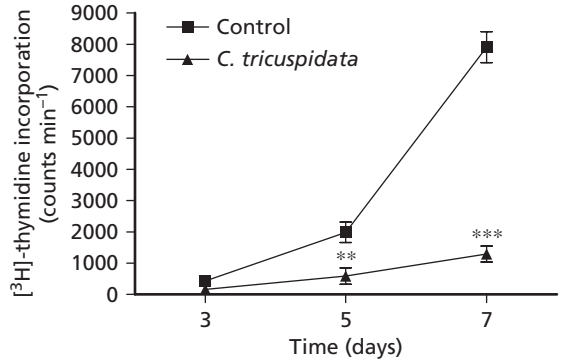

Figure 2 Curve plots of $CD4^+CD25^-$ T cell proliferation generated by counts min^{-1} vs time (days). Anti-CD3/CD28-mediated $CD4^+CD25^-$ T cells were incubated on 96-well plates with or without $30\,\mu g\,mL^{-1}$ *C. tricuspidata*. Changes in the proliferation on day 3–7 are shown. The cultures were pulsed with 1 μCi [^{3}H]-thymidine per well for the last 18 h, and the cells were harvested and counted. Data are presented as the mean ± s.e.m. from three independent experiments. $^{**}P<0.01$, $^{***}P<0.001$ vs control.

Table 1 Inhibitory effect of *C. tricuspidata* on the proliferation of antigen-mediated spleen and $CD4^+CD25^-$ T cells

Treatment	Concentration ($\mu g\,mL^{-1}$)	Proliferation (counts min^{-1})[a]	
		Spleen cells	$CD4^+CD25^-$ T cells
Control	0	2684 ± 194.1	7789 ± 450.6
C. tricuspidata	1	2259 ± 635.3	4511 ± 756.1**
	3	1515 ± 337.6**	3901 ± 391.5**
	10	1495 ± 157.9**	2448 ± 342.3**
	30	673 ± 108.4**	1292 ± 254.8**

Spleen and $CD4^+CD25^-$ T cells were activated with 5 μg mL^{-1} anti-CD3 and anti-CD28, respectively, and incubated in the presence or absence of various concentrations of *C. tricuspidata* for 7 days, which was added on alternate days. [a]On day 6, the cultures were pulsed with 1 μCi [^{3}H]-thymidine per well for the last 18 h, and the cells were harvested and counted. Data are presented as mean ± s.e.m. from four independent experiments. $^{**}P<0.01$ vs control.

Inhibitory effect of *C. tricuspidata* on the production of IL-2 and IFN-γ in antigen-mediated $CD4^+CD25^-$ T cells

We investigated whether *C. tricuspidata* ($30\,\mu g\,mL^{-1}$) can influence the production of the pro-inflammatory cytokines IL-2 and IFN-γ, which are major cytokines secreted by Th1 cells. To that end, $CD4^+CD25^-$ T cells were activated with anti-CD3/CD28 Ab in the presence or absence of *C. tricuspidata* and the level of secreted IL-2 and IFN-γ was analysed on days 2 and 6 by using the ELISA kit. The results showed that *C. tricuspidata* decreased the production of IL-2 ($P=0.0157$) and IFN-γ ($P=0.0234$) in contrast to the control at 6 days (Table 2). This suggests that *C. tricuspidata* may decrease the production of pro-inflammatory cytokines by selective inhibition on proliferating cells. We also assayed whether *C. tricuspidata* can induce, but not influence, the conversion of $CD4^+CD25^-$ T cells to Foxp3-expressing Treg cells (data not shown).

Table 2 Inhibitory effect of *C. tricuspidata* on the production of IL-2 and IFN-γ from antigen-mediated $CD4^+CD25^-$ T cells

Treatment	IL-2 (pg mL^{-1})[a]		IFN-γ (pg mL^{-1})[a]	
	2 days	6 days	2 days	6 days
Control	38.41 ± 10.11	205.3 ± 22.78	41.69 ± 10.6	94.54 ± 12.23
C. tricuspidata	12.35 ± 4.92	96.39 ± 20.16*	34.87 ± 9.90	46.78 ± 7.20*

Anti-CD3/CD28-mediated $CD4^+CD25^-$ T cells were incubated with or without 30 μg mL^{-1} *C. tricuspidata*, which was added on alternate days. [a]The culture supernatant was collected at 2 and 6 days. Data are presented as mean ± s.e.m. from three independent experiments. *$P < 0.05$ vs control.

Volatile components of *C. tricuspidata* methanol extract identified by GC-MS

GC-MS can be applied in the differentiation of various medicinal parts of the *C. tricuspidata* methanol extract. Compound identifications indicated by the library search program as being >90% probable were viewed as likely hits. Table 3 lists the chemical components of the extract. Of the components that were analysed, eight compounds exhibiting anti-inflammatory effects were identified: *p*-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde, scopoletin, 9,12-octadecadienoic acid (Z,Z)-, campesterol, sitosterol, aristolone and olean-12-ene.

Discussion

Activated T cells can cause inflammatory pathologies such as autoimmunity and graft rejection, although these cells are critical for the eradication of intracellular pathogens (Sher & Coffman 1992; O'Garra & Robinson 2004). *C. tricuspidata* is used in herbal remedies against various diseases involving inflammation and tumours in East Asian countries. However, there have been limited reports on the function and action of *C. tricuspidata* in adaptive immunity. In this study, we

Table 3 Volatile compounds identified in *C. tricuspidata*

Retention time (min)	Compound	Quality (%)	Peak area (arbitrary units)
5	2-Furanmethanol	96	1 699 109
7	2,5-Furandione, 3-methyl-	90	2 646 426
12	2,3-Dihydro-3,5-dihydroxy-6-methyl-4*H*-pyran-4-one	93	14 577 422
15	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	91	23 430 954
17	Phenol, 4-(methoxymethyl)-	91	6 279 020
18	*p*-Hydroxybenzyl alcohol	95	12 786 731
18	4-hydroxybenzaldehyde	96	2 126 724
24	4-hydroxyacetyl-2-methoxy-phenol	90	5 042 918
24	Acetaldehyde, (3-chloro-5,5-dimethyl-2-cyclohexen-1-ylidene)-, (E)-	90	3 214 892
29	Syringic acid	95	4 281 274
30	2*H*-1-Benzopyran-2-one, 7-hydroxy-	96	11 348 640
31	Palmitic acid, methyl ester	98	2 584 398
32	Scopoletin	93	1 932 412
32	Palmitic acid	99	26 635 428
35	Methyl linoleate	99	2 158 393
35	9,12,15-Octadecatrienoic acid, methyl ester	99	1 325 842
35	9,12-Octadecadienoic acid (Z,Z)-	98	8 515 023
35	9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-	97	5 725 253
35	9,17-Octadecadienal, (Z)-	93	2 232 359
36	Stearic acid	99	6 341 605
36	Butyl citrate	91	16 288 627
42	Palmitic acid β-monoglyceride	91	5 358 896
50	Lanol	99	3 520 965
50	Vitamin E	97	3 367 460
51	Campesterol	96	3 716 509
52	γ-Sitosterol	99	17 146 568
53	Aristolone	91	40 492 935
53	Lanost-8-en-3-one	91	10 356 153
54	Quinindoline	90	16 171 511
54	Lanosta-8-24-dien-3-ol, acetate, (3β)-	98	13 147 781
55	Olean-12-ene, 3-methoxy-, (3β)-	93	41 362 534

investigated whether *C. tricuspidata* can inhibit the proliferation of antigen-mediated spleen and $CD4^+CD25^-$ T cells, which can accelerate inflammation. We also looked at whether *C. tricuspidata* can decrease the production of the pro-inflammatory cytokines IL-2 and IFN-γ in antigen-mediated $CD4^+CD25^-$ T cells.

Allograft rejection (Hao et al 1987), xenograft rejection (Loudovaris et al 1996) or an autoimmune response (Wang et al 1991) are predominantly initiated by a $CD4^+$ T cell-mediated immune response. The onset of diabetes in NOD mice requires the participation of both CD4 (Shizuru et al 1988; Hayward et al 1993) and CD8 T cells. The control of T cells in these diseases is important for successful treatment. This study suggested that *C. tricuspidata* can effectively suppress anti-CD3/CD28-mediated spleen and $CD4^+CD25^-$ T cell proliferation (Table 1), indicating that *C. tricuspidata* may have potential anti-inflammatory activity in innate and adaptive immunity. To further confirm these results, we incubated antigen-mediated $CD4^+CD25^-$ T cells with or without *C. tricuspidata* for 3–7 days (Figure 2). The results showed that *C. tricuspidata* markedly suppressed anti-CD3/CD28-mediated $CD4^+CD25^-$ T cell proliferation on day 7, where the control showed a peak (84% inhibition of proliferation). However, *C. tricuspidata* is not cytotoxic against spleen cells when incubated for 3 days under conditions wherein anti-CD3/CD28 was not stimulated. We also examined whether *C. tricuspidata* converts anti-CD3/CD28-mediated $CD4^+CD25^-$ T cells into Foxp3-expressing Treg cells, which suppress the function and proliferation of effector T cells. Unfortunately, *C. tricuspidata* had no effect on the conversion of antigen-mediated $CD4^+CD25^-$ T cells into Foxp3-expressing Treg cells (data not shown).

The pro-inflammatory cytokines IL-2 and IFN-γ are mainly produced by Th1 cells. In this study, we showed that *C. tricuspidata* can decrease the production of both IL-2 and IFN-γ by a selective inhibition on proliferating cells in anti-CD3/CD28-mediated $CD4^+CD25^-$ T cells for 6 days (Table 2). Previous studies in a type 1 diabetes model have reported that the exposure of purified human or rodent β-cells to IL-1β alone is not sufficient to induce apoptosis, while IL-1β+IFN-γ induces apoptosis in about 50% of the cell populations after 6–9 days (Eizirik & Mandrup-Poulsen 2001). This result thus shows that *C. tricuspidata* may be useful in the treatment of inflammatory pathologies involving diabetes.

We showed that each of the 8 compounds in the volatile components of the *C. tricuspidata* methanol extract identified by GC-MS analysis was reported to have anti-inflammatory effects (Table 3). However, further studies are required to determine the role of the components from the *C. tricuspidata* methanol extract that are responsible for the anti-inflammatory effects on activated T cells.

Conclusions

This is the first study to report that *C. tricuspidata* can suppress anti-CD3/CD28-mediated spleen and $CD4^+CD25^-$ T cell proliferation. We also showed that *C. tricuspidata* can decrease the production of the pro-inflammatory cytokines IL-2 and IFN-γ by selectively inhibiting the proliferation of anti-CD3/CD28-mediated $CD4^+CD25^-$ T cells. Taken together, our findings suggest that *C. tricuspidata* may be useful in the treatment of inflammatory pathologies involving autoimmune diseases, such as diabetes, and those arising from organ transplantation. However, further studies are necessary to identify the mechanism underlying the inhibition of inflammatory immune cell proliferation and the definite active components in the *C. tricuspidata* extract.

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