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The liquid culture filtrates of *Paecilomyces tenuipes* (Peck) Samson (=*Isaria japonica* Yasuda) and *Paecilomyces cicadae* (Miquel) Samson (=*Isaria sinclairii* (Berk.) Llond) regulate Th1 and Th2 cytokine response in murine Peyer's patch cells in vitro and ex vivo

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Abstract

The effects of liquid culture filtrates of medicinal entomogenous fungi, *Paecilomyces tenuipes* (Peck) Samson (=*Isaria japonica* Yasuda or *Isaria tenuipes*) (PTCF) and *Paecilomyces cicadae* (Miquel) Samson (=*Isaria sinclairii* (Berk.) Llond) (PCCF), on cytokine productions in cultured Peyer's patches (PP) from C57BL/6J mice were investigated in vitro and ex vivo. In an in vitro experiment, PTCF (100 and 10 μg/ml) enhanced the production of T helper 1 (Th1) cytokines, interleukin (IL)-2 and interferon (IFN)-γ, in cultured PP cells stimulated with 5 μg/ml concanavalin A (Con A) but did not influence on the production of T helper 2 (Th2) cytokines, IL-4 and IL-5. PTCF also enhanced the production of granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-10 in the cultured PP cells. While, PCCF enhanced the production of IFN-γ but did not alter the level of IL-2 in the PP cells. In an ex vivo experiment using PP cells removed from the mice after oral treatment of PTCF (10 and 100 mg/kg daily for 7 consecutive days), the production of IL-2 and IFN-γ were increased in response to Con A. On the other hand, orally treated PCCF (10 mg/kg/day) suppressed IL-2 production but did not change the levels of IFN-γ and IL-10 in the isolated PP cells. The flow cytometric analysis revealed that the population of $CD3^+$ cells in the PP cells slightly but significantly increased after oral administration of PCCF. Orally administered PTCF did not change the population of T ($CD3^+$), B ($CD19^+$), T cell subset ($CD4^+$and $CD8^+$) and Th1 (IFN-γ^+) and Th2 (IL-4^+). From PTCF, the fraction rich in proteoglycans was separated as active fraction that stimulates Th1 immune response.

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These results indicate that the mode of action of PTCF and PCCF on mucosal immune response is different and this is contributed to their metabolites. Taken together, there is a possibility of PTCF and PCCF being therapeutic or preventive agents for immune diseases such as cancer, allergy and parasitic disease through activation of mucosal immune response.

Keywords: *Paecilomyces tenuipes*; *Paecilomyces cicadae*; Culture filtrate; Peyer's patch; Cytokine productions; Th1/Th2 balance

1. Introduction

Genus *Cordyceps* (Clavicipitaceae) is an entomogenous fungus, and the representative one is *Cordyceps sinensis* (Berk.) Sacc, which selectively infects bat moth (*Hepialus armoricanus*) larva [1,2]. The combined fruiting body of *C. sinensis* and the host insect is a Chinese traditional medicine (Dong Chong Xia Cao) used mainly as a time-honored tonic [1,2]. *Paecilomycestenuipes* (Peck) Samson (=*Isaria japonica* Yasuda or *Isaria tenuipes*) and *Paecilomyces cicadae* (Miquel) Samson (=*Isaria sinclairii*) are also entomogenous fungi thought to be of the anamorph stage of *Cordyceps* [3]. These fungi including host insects have traditionally been used as health foods for various diseases in Japan, Korea and China [1,2,4]. Myriocin, a sphingosine analog isolated from the culture filtrate of *P. cicadae*, showed inhibitory effect on T cell-dependent immune responses [5]. Recently, we have succeeded in cultivating *P. tenuipes* in a liquid medium and demonstrated that this liquid medium augmented anti-sheep red blood cell IgM plaque-forming cells response upon oral administration in mice [4,6].

The inner surface of intestinal tract possesses a large area of mucosal membranes, and they are continuously exposed to various substances in intestinal lumen [7]. The gut-associated lymphoid tissues exist on the intestinal mucosal site and play an important role in immune system. Peyer's patches (PP) are consider to be lymphoid tissues, where mucosal immune responses such as local IgA production and systemic immunological responses are induced [7]. Antigen presentation in PP is important in determining systemic immune responses including T or B cell-dependent immunity [7,8]. Because the culture filtrate of *P. tenuipes* stimulated immune responses by oral administration [4], it is assumed that the filtrate may affect the function of PP cells. Indeed, orally administrated hot water extract of cultured mycelia of *C. sinensis* increased interleukin (IL)-6 and granulocyte-colony stimulating factor (GM-CSF) production by PP cells in mice [9]. Juzen-taiho-to, one of the Kampo prescriptions, also enhanced the production of these cytokines in PP cells from C3H/HeJ mice [10]. In this study, we therefore studied the effects of culture filtrates of *P. tenuipes* and *P. cicadae* on the production of cytokines that regulate immune responses in cultured PP cells in vitro and ex vivo.

2. Materials and methods

2.1. Animals

Male C57BL/6J mice, 6–10 weeks of age, were purchased from the Japan SLC (Shizuoka, Japan). Mice were housed in groups of five animals in plastic cages with a 12 h light:12 h dark cycle and free access to water and food ad libitum. An adaptation to these conditions of at least 1 week was allowed before the experiment. The experimental procedures complied with the Council for Experimental Animals on Faculty of Pharmaceutical Sciences, Kanazawa University. Mice were sacrificed by an anesthetization with overdose of ether.

2.2. Materials

Beer dry yeast was purchased from Iwaki Pharmaceutical (Tokyo, Japan). RPMI-1640 medium, phosphate-buffered saline (PBS), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Invitrogen Corp. (Carlsbad, CA USA). Concanavalin A (Con A) (type IV) and type I collagenase was from Sigma Chem. (St. Louis, MO). The reagents whose suppliers are not indicated were purchased from Wako Pure Chemical (Tokyo, Japan). For the analysis of T, B and T cell subset ($CD4^+$ and $CD8^+$) in PP cells, the

following monoclonal antibodies (mAb, Beckman Coulter Inc., Hialeah, FL) were used: anti-CD45RA-fluorescein isothiocyanate (FITC) antibodies (RA3-6B2, IgG2a), anti-CD3-FITC antibodies (KT3, IgG2a); anti-CD4-FITC antibodies (YTS191.1, IgG2b), anti-CD8-phycoerythin (PE) antibodies (KT15, IgG2a), anti-IFN-γ-FITC antibodies (XMG1.2, IgG1) and anti-IL-4-FITC antibodies (BVD-24G2, IgG1). The isotype-matched controls used in this experiment were IgG1 conjugated to FITC, IgG2a conjugated to PE, IgG2a conjugated to FITC and IgG2b conjugated to FITC.

2.3. Cultures of P. tenuipes and P. cicadae

Two kinds of fungi collected in natural field were individually cultured in the liquid medium as reported previously [4,6]. In brief, conidiospores isolated from conidium of these fungi were inoculated into the autoclaved culture medium composed of 0.3% (w/v) yeast extract, 0.5% (w/v) glucose and 0.016% (w/v) in a 200 ml flasks, and incubated at 18 °C with an 8 h light:16 h dark rhythms. Typical conidiophores were formed out of the cultivated new hyphae in the artificial medium approximately 80 days after inoculation.

2.4. Preparation of test sample

The hyphae and conidium of both fungi were carefully and completely removed from the cultured medium by filtration (pore size: 0.45 μm) and the filtrates were centrifuged at 120,000 ×*g* for 60 min. The supernatants were lyophilized to give dark-brownish powder (PTCF) and light-brownish powder (PCCF) in yields of 0.2% (w/v) (for PTCF) and 0.4% (w/v) (for PCCF) of filtrates, respectively. PTCF consisted of carbohydrate (76.6%), protein (10.7%), lipid (4.8%) and ash (2.1%). The component amino acid analysis exhibited that PTCF mainly contained glutamic acid (14.6 μg/mg), aspartic acid (14.6 μg/mg), leucine (7.48 μg/mg), phenylalanine (6.64 μg/mg) and tyrosine (6.05 μg/mg). PCCF consisted of carbohydrate (71.1%), protein (18.2%), lipid (3.4%) and ash (1.1%). The component amino acid analysis exhibited that PCCF mainly contained glutamic acid (112.7 μg/mg), proline (46.7 μg/mg), leucine (27.9 μg/mg), serine (20.4 μg/mg) and alanine (19.9 μg/mg).

2.5. Separation of active fraction from PTCF

To analyze active constituents in PTCF, PTCF was dissolved in distilled water (1 g/30 ml) and added to a three-fold volume of ice-cold 50% ethanol. The mixture was allowed to stand at 4 °C overnight. After centrifugation (10,000 ×*g* for 3 h), the supernatant was added to the three-fold volume of 80% ethanol and then centrifuged (10,000 ×*g* for 4 h) at room temperature. The resulted precipitate was dissolved in distilled water and lyophilized to give dark-brownish powder (0.56 g). The lyophilized sample (0.4 g) was subjected to ion-exchange column chromatography on Dowex 50W-X 8 (bed volume=50 ml). The column was successively eluted with H_2O (250 ml), 1N pyridine (500 ml) and 2N NH_4OH (250 ml) to afford 10 fractions (Fr. 1–10) of 60 ml each. A part of the lyophilized active fraction (Fr. 4, 0.5 g) eluted with 1N pyridine was dissolved in 2.5 ml double distilled water, and the insoluble materials were removed by centrifugation. The supernatant was further fractionated by column chromatography on DEAE-cellulose (Cl^- form, Sigma) (bed volume; 30 ml) using NaCl gradient solvent system (0–1.0 M, 500 ml) to give 10 fractions (Fr. 4-1 to 4-10). Each fraction was desalted (MW<1000, PD-10 Desalting Column, Amersham Biosciences, Tokyo, Japan) and lyophilized. The active fraction (Fr.4-3 eluted with 0.3 M NaCl, 0.124 g) was determined to be rich in polysaccharides by phenol–sulfuric method and Lowry–Folin test.

2.6. Peyer's patch (PP) cell preparation [11]

C57BL/6J mice were sacrificed by over dose of ether and the small intestines were placed into petri dish filled with PBS containing penicillin (100 U/ml) and streptomycin (100 μg/ml) on ice. The visible PPs were carefully dissected out from the wall of small intestines using micro scissors under the microscope (10 Peyer's patches were obtained from a mouse), and these tissues were placed in ice cold complete medium RPMI-1640 containing 5% FBS, 50 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin. To obtain a single PP cell suspension, the PPs were digested with type 1 collagenase (70 U/ml) dissolved in same medium, and incubated for 30 min at 37 °C. After filtration through a 75 μm nylon mesh (Becton Dickinson, Oxnard, CA, USA), PP cells were

washed three times with PBS. Cell viability was assessed by trypan blue exclusion. Morphological analysis by characteristic non-specific esterase and Giemsa staining revealed that more than 97% of the cells were lymphoids and less than 1% were monocytes. The PP cells (2×10^6 cells/ml) suspended in the complete medium were seeded in a 24-well tissue culture plate (Becton Dickinson) and were cultured with or without 5 μg/ml concanavalin A (Con A).

2.7. Test sample treatment

PTCF and PCCF were dissolved in PBS. For in vitro assay, various concentrations of test samples were added with or without Con A. For ex vivo assay, each sample was dissolved in water and 0.1 ml/10 g body weight of sample was orally injected into mice once a day for seven consecutive days. Control groups were received a vehicle sterile water instead of test samples.

2.8. Cytokine evaluation

To measure cytokine production in cultured PP cells, culture supernatants were collected at 0, 18, 24, 48, 72 and 96 h and stored at −80 °C before use. The levels IL-2, IL-4, IL-5, IL-10, INF-γ and GM-CSF in the supernatants were measured by enzyme linked immunosorbent assay (ELISA) using commercial kits for these cytokines (Cytoscreen™, BIOSOURCE, Camarillo, CA, USA) according to the manufacture's instruction.

2.9. Flow cytometry

PPs (10 Peyer's patches/mouse) were collected 7 days after oral injection of PTCF or PCCF and dissociated into single-cell suspension by filtrating through a 200 μm nylon mesh. Erythrocytes in PP cells suspensions were lysed in 0.75% ammonium chloride buffer (pH 7.6). The resultant cells were counted and resuspended in PBS. Cells (1×10^6 cells) were stained with fluorochrome directly conjugated with mAb [12]: anti-CD3-FITC, anti-CD45RA-FITC, anti-CD4-FITC/anti-CD8-PE, anti-IFN-γ-FITC and anti-IL-4-FITC or isotype-matched controls. After 60 min incubation at 4 °C, the cells were washed and resuspended in PBS containing 1 mg/ml of propidium iodide. Lymphocyte population in PP cells was analyzed using a flow cytometer (FACScan™, Becton Dickinson, San Jose, CA, USA) as described below. Gates were set by forward and side scatter to delineate leukocytes and exclude dead cells. Their death was confirmed by propidium iodide staining.

2.10. Data analysis for flow cytometry

Data were acquired using Cell Quest TM software (Becton Dickinson). Between 10,000 and 20,000 events were acquired per sample. All data are representative plots derived from a minimum of three independent experiments in which at least three

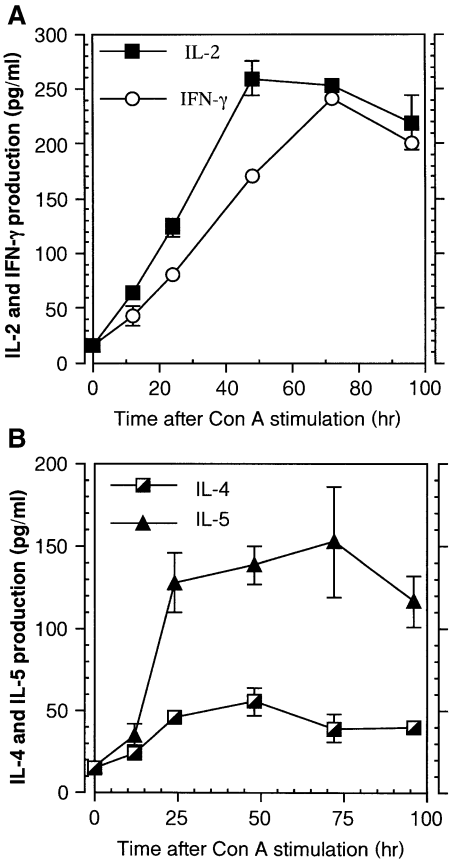

Fig. 1. Time-course kinetics of IL-2, IFN-γ, IL-4 and IL-5 production in cultured PP cells co-stimulation with Con A. PP cells were cultured with 5 μg/ml Con A, and culture supernatants were collected at the indicated time after Con A stimulation. Data are expressed as mean±S.D. of quadruplicate cultures. Results of one experiment repeated twice with similar results.

experimental and three or four control mice were analyzed. Mean FITC- and FTIC/PE-fluorescence intensities were calculated from fluorescence histograms for the gated population.

2.11. Statistical analysis

The mean of the cytokine production and the ratio of the lymphocyte or T lymphocyte subpopulations were considered as a single data point for analysis of results from at least three or four independent experiments. All data are expressed as mean±S.D. Statistical significance was determined by Dunnett's multiple test after one-way analysis of variance (ANOVA) with comparison to a control group, and the differences were considered significant if $p<0.05$.

3. Results

We first measured IL-2, IL-4, IL-5 and IFN-γ production in the PP cells upon non-specific T cell stimulation with Con A. As shown in Fig. 1, these cytokines were produced 12 h after Con A (5 μg/ml) challenge, increased over 72 h and slightly decreased at 96 h ([Fig. 1]). No increment of these cytokines was observed when the PP cells were cultured without Con A (data not shown). On the basis of these data,

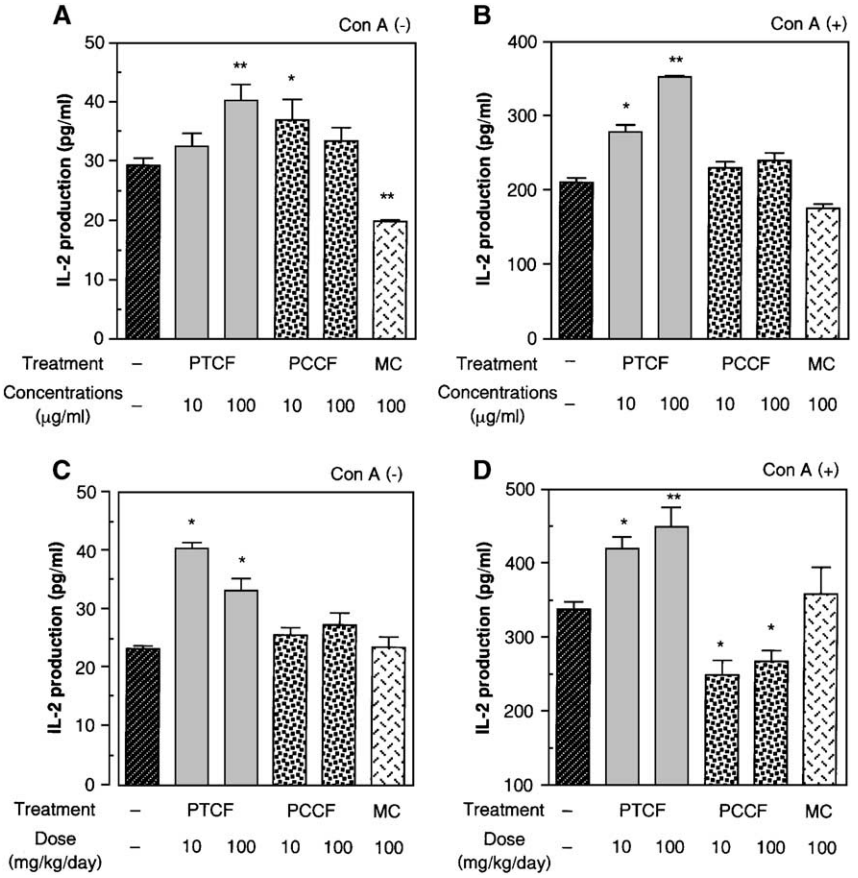

Fig. 2. Effects of PTCF and PCCF on IL-2 production in cultured PP cells in vitro and ex vivo. For in vitro assay (panel A and B), PP cells were directly treated with PTCF or PCCF with or without 5 μg/ml Con A. The culture supernatants were collected at 72 h after Con A stimulation and cytokine production was measured using ELISA. For ex vivo assay (panel C and D), mice were received with PTCF, PCCF or sterile water once a day for 7 consecutive days, and the PP cells were collected and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IL-2 production in cultured supernatants were measured using ELISA kit. All data are expressed as mean±S.D. of quadruplicate cultures, *, $p<0.05$ and **, $p<0.01$ compared with control culture. Results of one experiment repeated twice with similar results. CM: culture medium of PTCF and PCCF.

the effects of PTCF and PCCF on the cytokine production in the PP cells were evaluated 72 h after Con A challenge in both in vitro and ex vivo experiments.

IL-2 and IFN-γ are the major cytokines in cellular immune responses produced by naïve T cells upon activation by professional antigen presenting cells such as dendritic cells and macrophages [13]. As shown in Figs. 2 and 3, PTCF (10 and 100 μg/ml) triggered IL-2 and IFN-γ production in the cultured PP cells without Con A stimulations (Figs. 2A and 3A) and remarkably enhanced the production of IL-2 induced by Con A (Figs. 2B). On the other hand, PCCF (10 and 100 μg/ml) increased the IFN-γ production (Fig. 3A and B) but did not enhance IL-2 production (Fig. 2A and B). The culture medium (CM) itself slightly increased the IFN-γ production in the absence of Con A (Fig. 3B), whose effect was weaker than those of PTCF and PCCF.

Since PTCF stimulated IL-2 and IFN-γ production in the cultured PP cells in vitro, we next examined ex vivo effect of PTCF and PCCF on the cytokine production. Thus, after oral injection of PTCF or PCCF to mice once a day for seven consecutive days, the PP cells were isolated and cultivated to measure the production of IL-2 and IFN-γ. In this experiment,

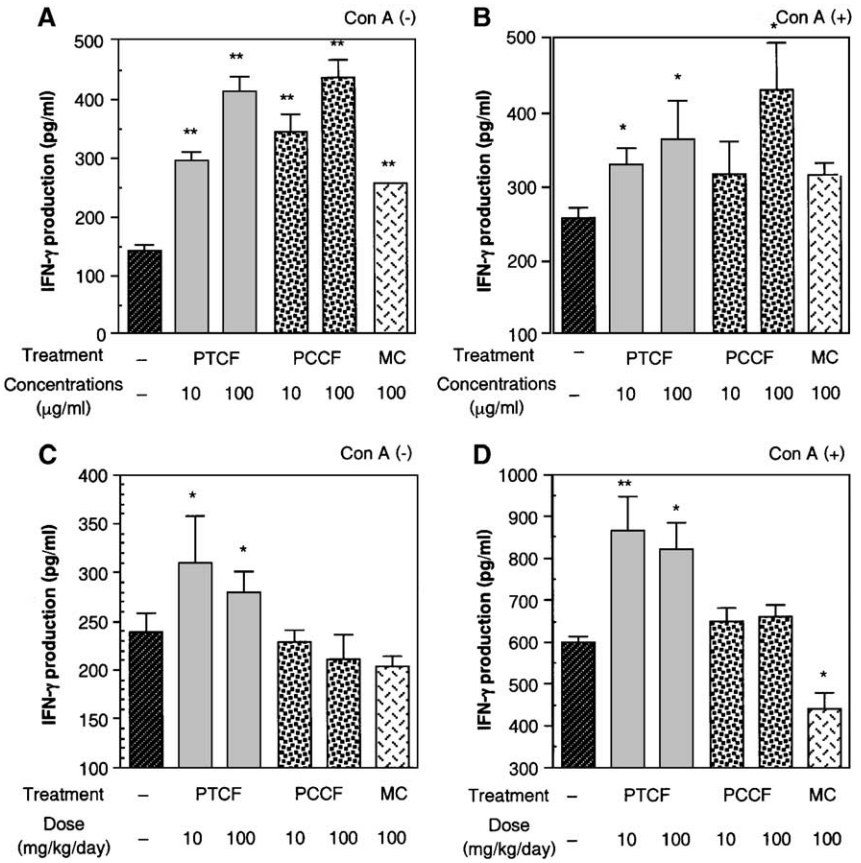

Fig. 3. Effects of PTCF and PCCF on IFN-γ production in cultured PP cells in vitro and ex vivo. For in vitro assay (panel A and B), PP cells were directly treated with PTCF or PCCF with or without 5 μg/ml Con A. The culture supernatants were collected at 72 h after Con A stimulation and cytokine production was measured using ELISA. For ex vivo assay (panel C and D), mice were received with PTCF, PCCF or sterile water once a day for 7 consecutive days, and the PP cells were collected and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IFN-γ production in cultured supernatants were measured using ELISA kit. All data are expressed as mean±S.D. of quadruplicate cultures, *, $p<0.05$ and **, $p<0.01$ compared with control culture. Results of one experiment repeated twice with similar results. CM: culture medium of PTCF and PCCF.

PTCF, PCCF and CM showed no toxicity to mice, which was demonstrated by the fact that the body and liver weights (data not shown) and the activity of plasma hepatic marker enzyme, alanine aminotransferase (ALT, EC 2.6.1.2), did not change (10 mg/kg PTCF: 18.2±1.6 U/ml, n=6; 100 mg/kg PTCF: 15.3±1.1 U/ml, n=6; water only: 15.1±2.9 U/ml, n=4) (ALT data for PCCF and MC are not shown). Production of IL-2 and IFN-γ in the PP cells from PTCF treated mice (10 and 100 mg/kg/day) were much more than those of control mice irrespective of Con A stimulation. PCCF and CM whereas showed no effect on the IL-2 and IFN-γ production (Figs. 2 and 3C, D).

The murine intestine is a highly regulated immune system producing large amounts of regulatory cytokines, with immune responses dominated by Th2 type cytokines [14]. IL-4 and IL-5 are signature cytokines produced by Th2 cells and able to direct developing Th0 cells to differentiate into Th2 cells [15]. We therefore next examined the influence of PTCF and PCCF on IL-4 and IL-5 production in the PP cells in vitro and ex vivo. PTCF did not affect IL-4 production irrespective of Con A stimulation both in vitro and ex vivo (Fig. 4A–D). On the other hand, PCCF reduced Con A-induced IL-4 production in vitro but did not reduce it upon oral treatment (Fig. 4B and D). CM strongly increased Con A-stimulated IL-4 production

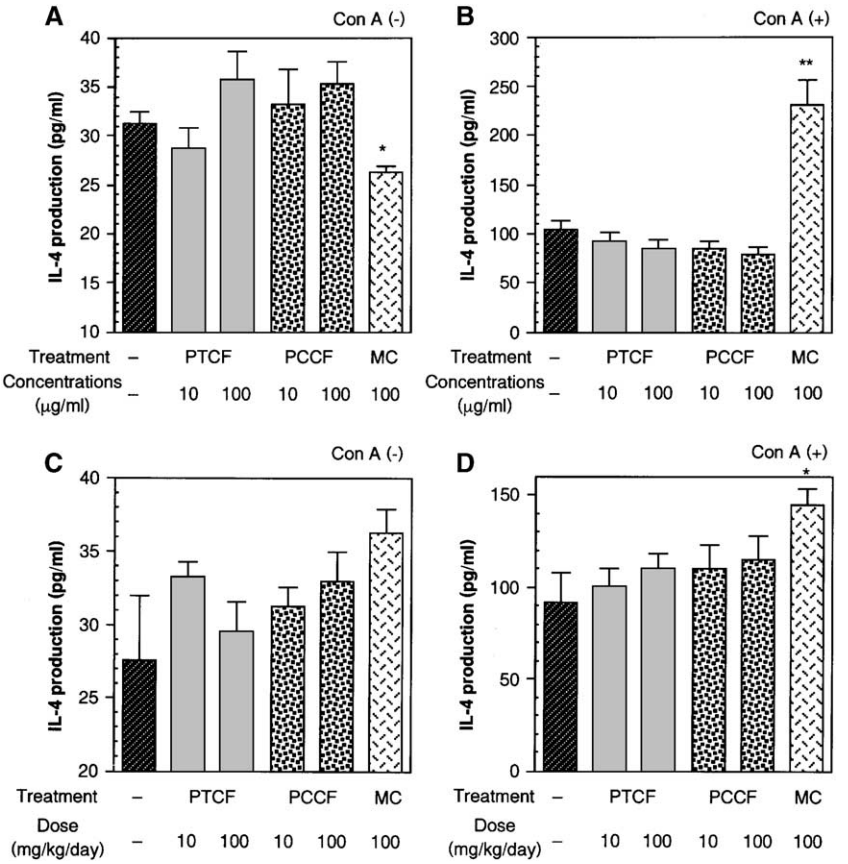

Fig. 4. Effects of PTCF and PCCF on IL-4 production in cultured PP cells in vitro and ex vivo. For in vitro assay (panel A and B), PP cells were directly treated with PTCF or PCCF with or without 5 μg/ml Con A. The culture supernatants were collected at 72 h after Con A stimulation and cytokine production was measured using ELISA. For ex vivo assay (panel C and D), mice were received with PTCF, PCCF or sterile water once a day for 7 consecutive days, and the PP cells were collected and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IL-4 production in cultured supernatants were measured using ELISA kit. All data are expressed as mean±S.D. of quadruplicate cultures, *, p<0.05 and **, p<0.01 compared with control culture. Results of one experiment repeated twice with similar results. CM: culture medium of PTCF and PCCF.

in vitro and moderately increased it ex vivo (Fig. 4B and D).

IL-5 is an important intestinal cytokine with regard to differentiation of IgA-secreting plasma cells [16], and thought to be a Th2-type cytokine [17,18]. PTCF and PCCF at the concentration of 100 μg/ml slightly but significantly triggered IL-5 production in the PP cells irrespective of Con A stimulation in vitro (Fig. 5A). PTCF also increased IL-5 production induced by Con A at the same concentration but PCCF did not affect it (Fig. 5B). Whereas, orally injected PTCF and PCCF did not affect IL-5 production at doses of 10 and 100 mg/kg/day (Fig. 5C and D). The culture medium (CM) itself did not increase IL-5 production both in vitro and ex vivo (Fig. 5A–D).

Koh et al. reported that PP cells from C3H/HeJ mice fed with hot water extract of cultured mycelia of *C. sinensis* (0.5–2.0 g/kg/day for 7 days) produced more GM-CSF and IL-6 than those from untreated normal mice [9]. Hence, we examined the effect of PTCF and PCCF on GM-CSF production in vitro and ex vivo. The levels of GM-CSF in the PP cells incubated with PTCF and PCCF rose 2 or 3 times more than the control (Fig. 6A and B). Same results were obtained in the case of ex vivo study (Fig. 6C and D).

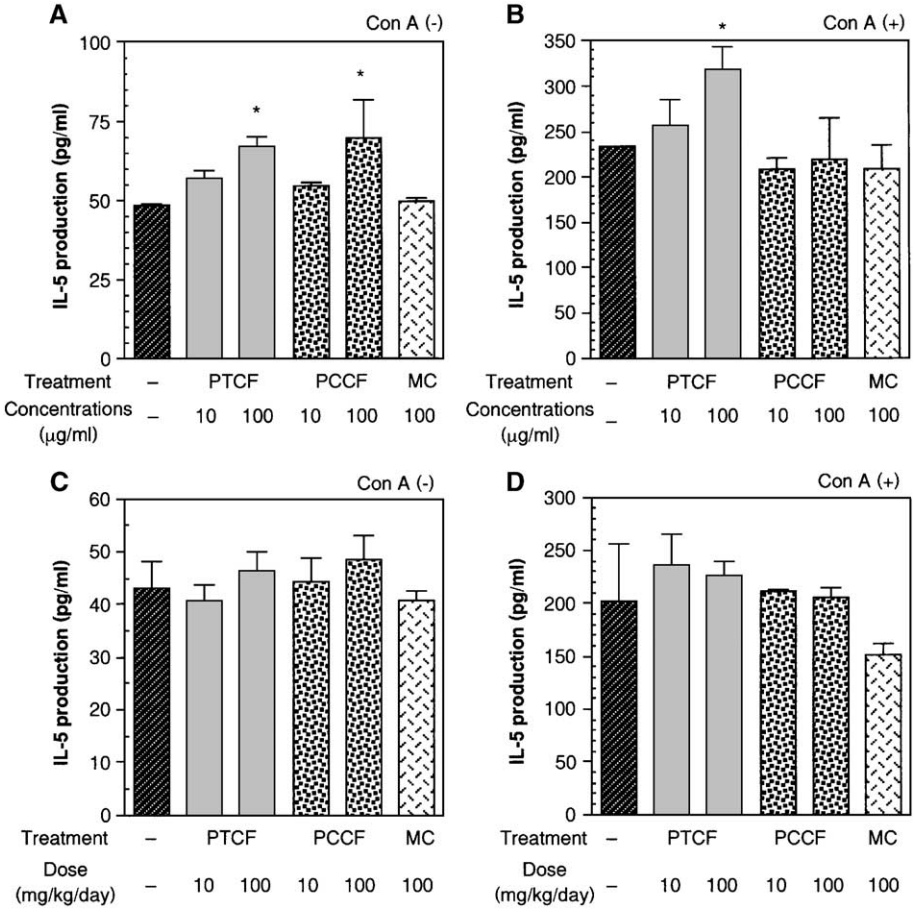

Fig. 5. Effects of PTCF and PCCF on IL-5 production in cultured PP cells in vitro and ex vivo. For in vitro assay (panel A and B), PP cells were directly treated with PTCF or PCCF with or without 5 μg/ml Con A. The culture supernatants were collected at 72 h after Con A stimulation and cytokine production was measured using ELISA. For ex vivo assay (panel C and D), mice were received with PTCF, PCCF or sterile water once a day for 7 consecutive days, and the PP cells were collected and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IL-5 production in cultured supernatants were measured using ELISA kit. All data are expressed as mean±S.D. of quadruplicate cultures, *, $p<0.05$ compared with control culture. Results of one experiment repeated twice with similar results. CM: culture medium of PTCF and PCCF.

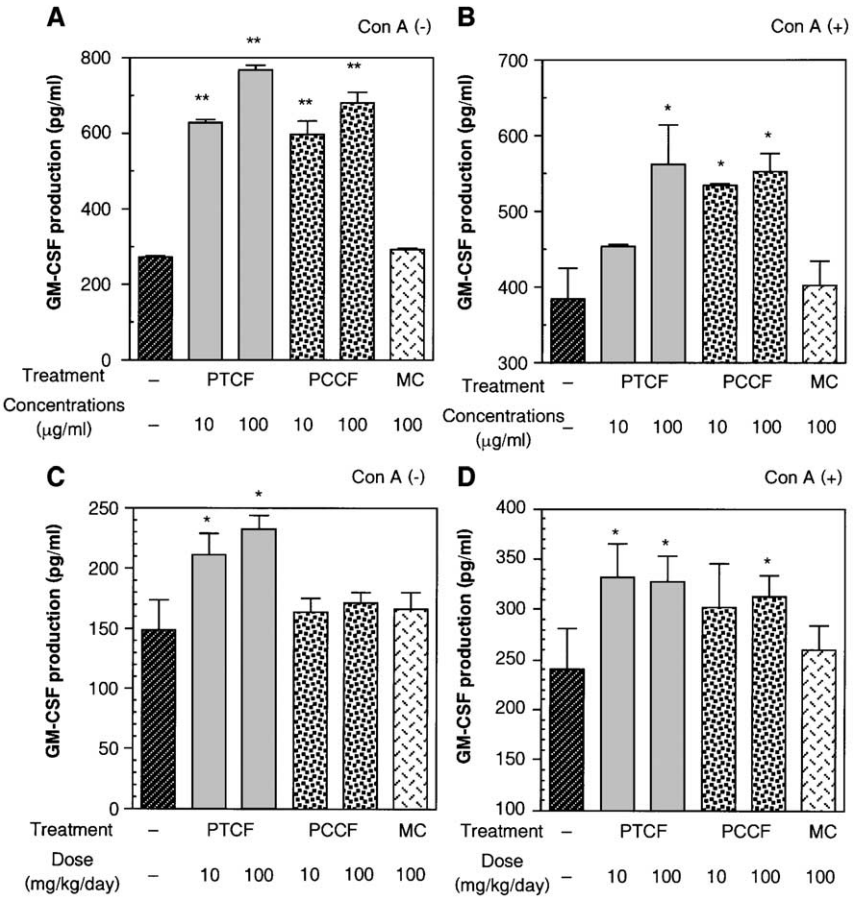

Fig. 6. Effects of PTCF and PCCF on GM-CSF production in cultured PP cells in vitro and ex vivo. For in vitro assay (panel A and B), PP cells were directly treated with PTCF or PCCF with or without 5 μg/ml Con A. The culture supernatants were collected at 72 h after Con A stimulation and cytokine production was measured using ELISA. For ex vivo assay (panel C and D), mice were received with PTCF, PCCF or sterile water once a day for 7 consecutive days, and the PP cells were collected and cultured in the absence or presence of 5 μg/ml Con A for 72 h. GM-CSF production in cultured supernatants were measured using ELISA kit. All data are expressed as mean±S.D. of quadruplicate cultures, *, $p<0.05$ and **, $p<0.01$ compared with control culture. Results of one experiment repeated twice with similar results. CM: culture medium of PTCF and PCCF.

Since IL-10 is a key cytokine produced by a multitude of mucosal immune effector cells and thought to be a Th2 cytokine [20], we next investigated the influence of PTCF and PCCF on IL-10 production in the PP cells in vitro and ex vivo. Both PTCF and PCCF increased the production of IL-10 in the PP cells irrespective of Con A stimulation in vitro (Fig. 7A and B). In the ex vivo experiment, PTCF also accelerated the Con A-stimulated IL-10 production in the PP cells, but PCCF did not accelerate it (Fig. 7D).

To clarify what kinds of T lymphocytes orally treated PTCF and PCCF increased (or decreased) in the PP cells, the PP cells were stained with FITC- or PE-conjugated specific T and B lymphocyte lineage markers and subjected to flow cytometric analysis. We first determined the numbers of PP cells from mice orally treated with PTCF and PCCF. The number of PP cells from normal mice was 17.0 ± 1.3 ($\times10^6$) cells/mouse. PTCF (10 mg/kg/day) and PCCF (10 and 100 mg/kg/day) showed no effect on the number of PP cells, whereas PTCF of 100 mg/kg/day significantly increased the number of PP cells (Table 1). MC gave no effect on the number of PP cells. PP cells are mainly composed of T and B cells, and T cells are

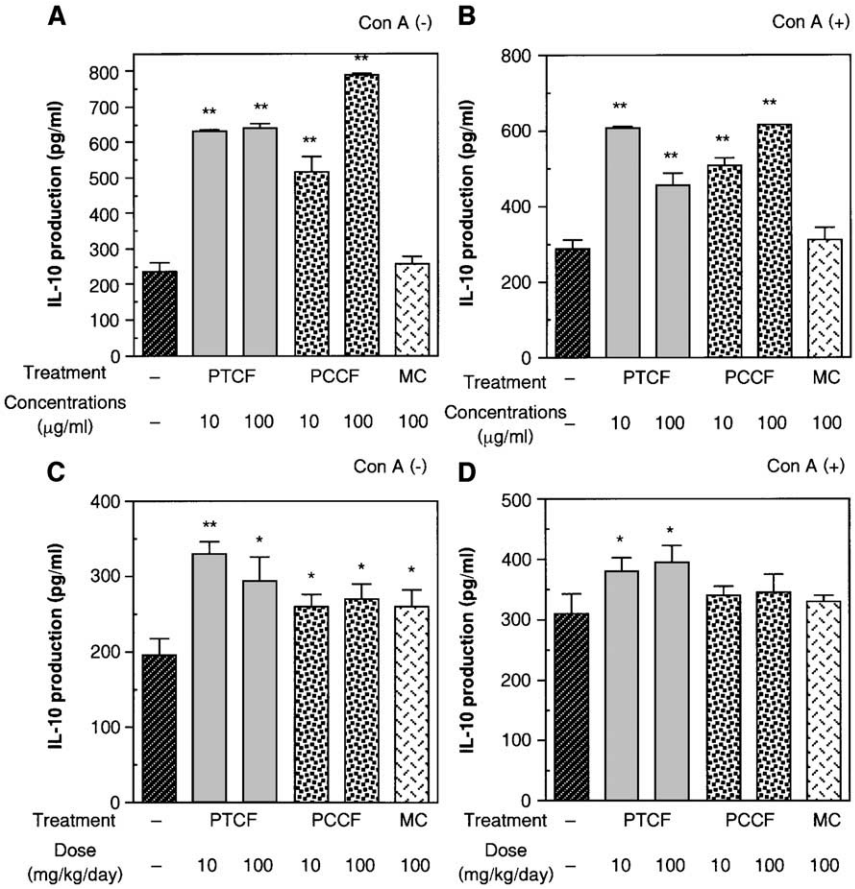

Fig. 7. Effects of PTCF and PCCF on IL-10 production in cultured PP cells in vitro and ex vivo. For in vitro assay (panel A and B), PP cells were directly treated with PTCF or PCCF with or without 5 μg/ml Con A. The culture supernatants were collected at 72 h after Con A stimulation and cytokine production was measured using ELISA. For ex vivo assay (panel C and D), mice were received with PTCF, PCCF or sterile water once a day for 7 consecutive days, and the PP cells were collected and cultured in the absence or presence of 5 μg/ml Con A for 72 h. IL-10 production in cultured supernatants were measured using ELISA kit. All data are expressed as mean±S.D. of quadruplicate cultures, *, $p<0.05$ and **, $p<0.01$ compared with control culture. Results of one experiment repeated twice with similar results. CM: culture medium of PTCF and PCCF.

known to produce various cytokines [19]. Flow cytometric analysis revealed that the population of T ($CD3^+$) and B ($CD\ 19^+$) lymphocytes in normal mice was 14.1% and 84.6%, and the ratio of T cell subpopulation, $CD4^+$ and $CD8^+$, was 15.5% and 5.3%, respectively (Table 2). The proportion of lymphocytes in the PP cells was moderately changed by orally administrated PCCF at doses of 10 and 100 mg/kg/day. That is, PCCF significantly increased the population of $CD3^+$ T lymphocytes, whereas this test sample decreases $CD19^+$ cells by 73.3% at 100 mg/kg/day (Table 2). On the other hand, PTCF did not change the population of T, B cell and T cell subset in PP cells. In addition, the populations of INF-γ and IL-4 positive cells in T lymphocytes were not changed by administration of both samples (Table 2).

In order to isolate the active substance(s) responsible for Th1 activation, the precipitate of PTCF by ethanol was separated into fractions by ion-exchange column chromatography on Dowex 50W-X8. Fig. 8A showed that the stimulatory activity on IL-2 and IFN-γ production in cultured PP cells transferred mainly to Fr. 4 eluted with 1N pyridine, which increased the IL-2 and IFN-γ production co-stimulated with Con A by

Table 1
Cell number of PP in mice orally treated with PTCF or PCCF

Treatment	Dose (mg/kg/day)	Cell numbers ($\times 10^6$ cells/mouse)	*n*
Control	–	17.0±1.3	5
PTCF	10	17.2±1.4	5
	100	18.2±1*	5
PCCF	10	17.3±1.5	5
	100	18.5±2.0	5
CM	100	16.3±3.2	3

PTCF or PCCF were injected orally once a day for 7 consecutive days. PP were collected (10 Peyer's patches/mouse) and counted by hemocytometer with trypan blue stain. Data are expressed as the mean±S.D. of 5 mice. *, $p<0.05$ compared with the control mice. Results of one experiment repeated twice with similar results. CM: culture medium of PTCF and PCCF.

143±15% (10 μg/ml, $p<0.05$) and 284+12 (10 μg/ml, $p<0.05$) (Fig. 8A and B). Fr. 4 was further purified by DEAE-cellulose column chromatography with gradient elution of 0 to 1.0 M NaCl solution. Corresponding 10 fractions (Fr. 4-1 to 4-10) were obtained and their effects on the cytokine productions in PP cells were tested. As shown in Fig. 8C and D, Fr. 4-3 eluted with 0.3 M NaCl showed the most potent stimulatory activity. Fr. 4-3 was determined to consist mainly of polysaccharides (approximately 91%) and protein (approximately 7%) by phenol–sulfuric acid test and Lowry–Folin test, respectively.

4. Discussion

In this study, we demonstrated that PTCF and PCCF modulated the production of IL-2, IFN-γ, GM-CSF and IL-10 in the cultured PP cells in vitro and ex vivo. We previously reported that orally injected PTCF strongly enhanced the splenic anti-sheep red blood cells (SRBC) plaque-forming cell (PFC) at the doses of 3–30 mg/kg/day [4]. The anti-SRBC PFC response is humoral and cellular immune responses requiring activation of B cells, T cells and monocytes [21], and the enhanced cytokines such as IL-2 [22], IFN-γ [23], IL-4, IL-5 and IL-6 [24] correlate with PFC number and IgM titer. In this experiment, PTCF selectively triggered and potentiated IL-2 and IFN-γ production in the PP cells (Figs. 2 and 3). Since these cytokines increased the PFC number in vivo [22,23], orally administered PTCF might enhance the PFC number in part due to enhancement of these cytokines.

Endotoxin has been reported to affect on both mucosal immunity and systemic immune responses [25]. It is very difficult to exclude contaminated endotoxin from traditional medicines, because endotoxin is widely distributed in fungi and plants. To avoid the interference of endotoxin and evaluate the net effect on PP cells of Juzen-taiho-to, one of Kampo prescription, Hong et al. used endotoxin-non-responder C3H/HeJ mice and got good results [10]. Therefore, we used C57BL/6J mice in this experiment, and the results are thought to exhibit the net effects of PTCF and PCCF on PP cells.

PTCF increased the production of IL-2, IFN-γ, GM-CSF and IL-10 but did not affect on the production of IL-4 and IL-5 in vitro. This indicates that some constituents in PTCF have selective immunopotent activities and modulate immune responses in the whole animal. We therefore tried to isolate active constituent(s) from PTCF and found that the fraction rich in proteoglycans showed strong immunopotent activity. This fraction was analyzed to consist mainly of polysaccharides (approximately 91%) and protein (approximately 7%) by phenol–

Table 2
Effects of PTCF and PCCF on PP lymphocyte population in mice

Treatment	Dose (mg/kg/day)	$CD3^+$(%)	$CD19^+$ (%)	$CD4^+$ (%)	$CD8^+$ (%)	IFN-γ^+ (%)	IL-4^+ (%)	*n*
Cotrol	–	14.1±2.2	84.6±3.7	15.5±2.1	5.3±2.4	4.2±0.1	6.7±1.2	6
PTCF	10	14.6±2.9	82.0±4.7	11.7±1.6	4.8±1.3	3.6±0.5	6.6±1.0	5
	100	17.6±3.1	79.8±3.3	13.5±2.7	4.3±1.9	3.2±0.4	7.3±0.3	5
PCCF	10	19.8±1.0*	78.4±1.9	11.6±3.0	7.2±2.5	3.5±0.2	7.3±1.1	6
	100	19.1±1.2*	73.3±1.2*	12.0±2.4	6.0±2.2	3.8±0.5	8.2±1.4	6
CM	100	18.6±2.0*	75.1±2.2*	12.2±1.6	6.3±1.0	4.6±0.3	7.0±1.1	4

PTCF or PCCF were injected orally once a day for 7 consecutive days. PP cells (1×10^6 cells) were collected and stained with lineage specific FITC- or PE-conjugated mAb. Data are expressed as the mean±S.D. of 4–6 mice. *, $p<0.05$ compared with the control mice. Results of one experiment repeated twice with similar results. CM: culture medium of PTCF and PCCF.

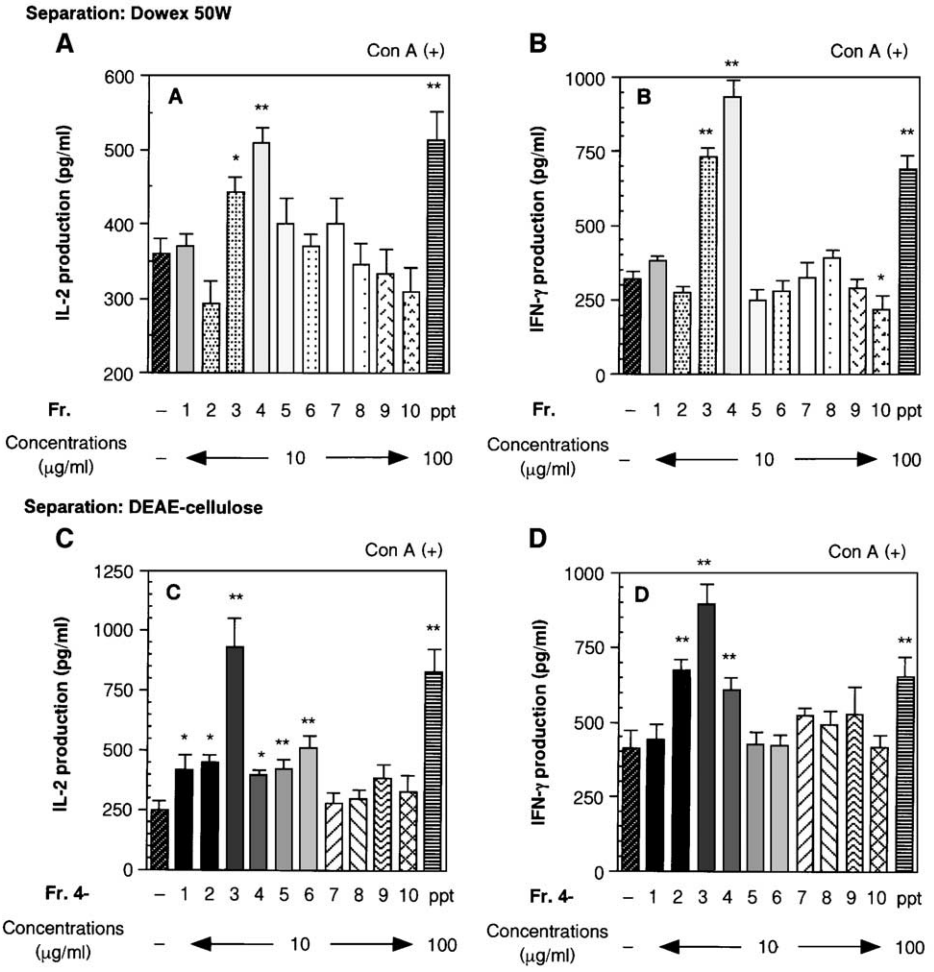

Fig. 8. Effects of fractions purified from PTCF on IL-2 and IFN-γ production in cultured PP cells in vitro. PP cells were directly treated with some fractions of PTCF separated by Dowex 50W X-8 (panel A and B) and further purified bioactive fractions separated by DEAE-cellulose (panel C and D) with 5 μg/ml Con A. The culture supernatants were collected at 72 h after Con A stimulation and cytokine production was measured using ELISA. All data are expressed as mean±S.D. of quadruplicate cultures, *, $p<0.05$ and **, $p<0.01$ compared with control culture. Results of one experiment repeated twice with similar results. Ppt: the extraction purified from PTCF by 80% ethanol precipitation.

sulfuric acid test and Lowry–Folin test, respectively. We recently revealed that PCCF also contained a proteoglycan like PTCF, and the molecular weight of the proteoglycan from PTCF and PCCF was approximately ~35 kD (unpublished data). Further purification and chemical characterization of these proteoglycans as well as evaluation of their inductive effects on Th1 cytokines in PP cells are to be studied.

Orally administrated PCCF significantly suppressed IL-2 production in the PP cells stimulated with Con A (Fig. 3 D). This indicates that the immunosuppressive constituents of PCCF are different from those of PTCF. A sphingosine-like immunosuppressant (ISP-1; myriocin) has been isolated from the culture medium of *P. cicadae* (=*I. sinclairii*) [5], which selectively induced apoptosis in the cultured T cell line, CTLL-2, without suppressing IL-2 production [26,27]. Therefore, low molecular weight immunosuppressive constituent(s) like ISP-1 may be in PCCF. Indeed, PTCF did not influence on IL-2 production in the cultured PP cells stimulated with Con A (Fig 3 B), and the dialysate (MW>7.8 kD)

of PCCF did not suppress IL-2 production in the ex vivo experiment (data not shown).

Orally administered PTCF and PCCF strongly triggered GM-CSF production in the PP cells at lower dosages (10 and 100 mg/kg/day) than those of the hot water extract from mycelia of *C. sinensis* (1.0 and 2.0 g/kg/day) [9]. This indicates that PTCF and PCCF have more potential than the extract of *C. sinensis* to stimulate the production of GM-CSF.

We newly found that the PP cells from mice orally administered with PTCF and PCCF highly produced IL-10 irrespective of Con A stimulation (Fig. 7). IL-10, one of the cytokines produced by Th2, strongly suppresses the activation of monocytes and Th1 lymphocyte immune responses [20]. Since PTCF and PCCF did not inhibit the production of IL-5 and IL-4, that are also Th2 cytokines, (Figs. 4 and 5), there is a possibility to exist some specific stimulatory constituents in PTCF and PCCF. PTCF might modulate the excessive Th1 (IL-2 and IFN-γ)-mediated immune responses by enhancing IL-10 production in vivo.

PP cells are mainly composed of T and B cells, and it is well understood that T cells are source for cytokine production [19]. Orally administrated PTCF did not change the lymphoid proportion in the PP cells (Table 2). This indicates that PTCF directly activated T cells in the PP cells without qualitative change of T cell function. On the other hand, PCCF significantly increased the population of $CD3^+$ T lymphoid and decreased the population of $CD19^+$ B lymphoid, but did not change the PP cell number (Tables 1 and 2). Chiba et al., [28] previously showed that orally administered FTY 720 (1 mg/kg, one shot), a synthetic immunosuppressant derived from ISP-1, augmented the number of T and B cells in peripheral lymph node, mesenteric lymph node and PP within 24 h in rat. Thus, it is possible to discuss that the increment of the number of T cells in PP might cause in part by immunosuppressant(s) such as ISP-1 in PCCF. Further study is required to support this idea.

PTCF and PCCF injected orally for 7 consecutive days selectively modulated the production of T helper 1 cytokines IL-2/IFN-γ as well as GM-CSF and IL-10 but did not show any significant effect to the levels of IL-4 and IL-5. Therefore, PTCF and PCCF did not cause non-specific immune responses like Con A. Further, PTCF protected against 5-fluorouracil-induced immunosuppression in mice [4]. Taken together, PTCF and PCCF have potentialities as therapeutic agents against immune diseases such as cancer, allergy, and parasitic diseases.

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