



## Aqueous extract of *Carica papaya* leaves exhibits anti-tumor activity and immunomodulatory effects

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### ABSTRACT

**Aim of the study:** Various parts of *Carica papaya* Linn. (CP) have been traditionally used as ethnomedicine for a number of disorders, including cancer. There have been anecdotes of patients with advanced cancers achieving remission following consumption of tea extract made from CP leaves. However, the precise cellular mechanism of action of CP tea extracts remains unclear. The aim of the present study is to examine the effect of aqueous-extracted CP leaf fraction on the growth of various tumor cell lines and on the anti-tumor effect of human lymphocytes. In addition, we attempted to identify the functional molecular weight fraction in the CP leaf extract.

**Materials and methods:** The effect of CP extract on the proliferative responses of tumor cell lines and human peripheral blood mononuclear cells (PBMC), and cytotoxic activities of PBMC were assessed by [<sup>3</sup>H]-thymidine incorporation. Flow cytometric analysis and measurement of caspase-3/7 activities were performed to confirm the induction of apoptosis on tumor cells. Cytokine productions by PBMC were measured by ELISA. Gene profiling of the effect of CP extract treatment was performed by microarray analysis and real-time RT-PCR.

**Results:** We observed significant growth inhibitory activity of the CP extract on tumor cell lines. In PBMC, the production of IL-2 and IL-4 was reduced following the addition of CP extract, whereas that of IL-12p40, IL-12p70, IFN- $\gamma$  and TNF- $\alpha$  was enhanced without growth inhibition. In addition, cytotoxicity of activated PBMC against K562 was enhanced by the addition of CP extract. Moreover, microarray analyses showed that the expression of 23 immunomodulatory genes, classified by gene ontology analysis, was enhanced by the addition of CP extract. In this regard, CCL2, CCL7, CCL8 and SERPINB2 were representative of these upregulated genes, and thus may serve as index markers of the immunomodulatory effects of CP extract. Finally, we identified the active components of CP extract, which inhibits tumor cell growth and stimulates anti-tumor effects, to be the fraction with M.W. less than 1000.

**Conclusion:** Since *Carica papaya* leaf extract can mediate a Th1 type shift in human immune system, our results suggest that the CP leaf extract may potentially provide the means for the treatment and prevention of selected human diseases such as cancer, various allergic disorders, and may also serve as immunoadjuvant for vaccine therapy.

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### 1. Introduction

*Carica papaya*, belongs to the family of *Caricaceae*, and several species of *Caricaceae* have been used as remedy against a variety of diseases (Mello et al., 2008; Munoz et al., 2000). Originally derived from the southern part of Mexico, *Carica papaya* is a perennial plant, and it is presently distributed over the whole tropical area. In particular, *Carica papaya* fruit circulates widely, and it is accepted as food or as a quasi drug. Many scientific investigations have been conducted to evaluate the biological activities of various parts of *Carica papaya*, including fruits, shoots, leaves, rinds, seeds, roots or latex.

**Abbreviations:** CP, *Carica papaya*; PBMC, peripheral blood mononuclear cells; APC, antigen-presenting cell; DC, dendritic cell; NK cell, natural killer cell; 7-AAD, 7-amino-actinomycin D; E:T ratio, effector–target ratio; GO, gene ontology.

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The leaves of papaya have been shown to contain many active components that can increase the total antioxidant power in blood and reduce lipid peroxidation level, such as papain, chymopapain, cystatin,  $\alpha$ -tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates (Seigler et al., 2002).

Moreover, *Carica papaya* leaf juice is consumed for its purported anti-cancer activity by people living on the Gold Coast of Australia, with some anecdotes of successful cases being reported in various publications. *Carica papaya* leaf extracts have also been used for a long time as an aboriginal remedy for various disorders, including cancer and infectious diseases.

To examine the potential role of *Carica papaya* as anti-cancer therapy, we analyzed in this report the anti-tumor activity of the aqueous extract of the leaves of *Carica papaya* (CP) against various cancer cell lines, as well as its potential immunomodulatory effects, and attempted to identify the active components.

## 2. Materials and methods

### 2.1. Preparation of botanical extracts

Whole leaves of dried *Carica papaya* (CP) were supplied by Innovatis Pharma Corporation (Takanawa, Tokyo, Japan). 20 g of leaves were boiled in 400 ml of distilled water, and were further heated at 60–70 °C to a concentrated solution (~50 ml). Extracts were subsequently filtered through 0.22  $\mu$ m filters (MILLEX®GP, MILLIPORE, Bedford, MA), and dispensed into individual aliquots and stored at –20 °C. A final volume of 20 ml of extract was derived from 20 g leaves, for the standard concentration of 1 g leaves/ml used for these experiments (1% = 10 mg leaves/ml).

Fractionation of the CP extract was achieved as follows. To fractionate the fraction containing components of greater than M.W. 1000, 1.5 ml of CP extract filled a cellulose membrane tube (Spectra/Por® 7 MWCO 1000, Spectram Laboratories Inc., Rancho Dominguez, CA) along with a large volume of distilled water for more than 10 h. The remaining extract in the tube was collected (0.5 g leaves/ml). To fractionate the fraction containing components of less than M.W. 1000, CP extract filled a cellulose membrane tube as above along with 6 ml of distilled water. The discharged extract in water was collected (0.2 g leaves/ml). Both fractions were filtrated through 0.22  $\mu$ m filter.

### 2.2. Cell lines

T cell lines (Jurkat, Molt-4, CCRF-CEM and HPB-ALL), Burkitt's lymphoma cell lines (Ramos and Raji), a chronic myelogenous leukemia cell line (K562), a cervical carcinoma cell line (Hela), hepatocellular carcinoma cell lines (HepG2 and Huh-7), a lung adenocarcinoma cell line (PC14) a pancreatic epithelioid carcinoma cell line (Panc-1), and mesothelioma cell lines (H2452, H226, and MESO-4) were obtained from RIKEN Cell Bank (Ibaraki, Japan). A T cell line (H9), a plasma cell leukemia cell line (ARH77) and an anaplastic large cell lymphoma cell line (Karpas-299) were obtained from European Collection of Cell Culture (ECACC, Wiltshire, UK). A breast adenocarcinoma cell line (MCF-7) and a mesothelioma cell line (JMN) were obtained from American Type Culture Collection (ATCC, Manassas, VA). A pancreatic adenocarcinoma cell line (Capan1) was kindly provided by Pr. Taketo Yamada, Keio University School of Medicine (Shinjuku, Tokyo).

All cells were cultured in RPMI 1640 medium with L-glutamine supplemented with 10% fetal bovine serum and antibiotics.

### 2.3. Cell viability and proliferative responses

For assays of proliferative responses, cell lines ( $1 \times 10^4$  cells/well) or PBMC ( $2 \times 10^5$  cells/well) were cultured

with or without CP extract at various concentrations (0.0625–2.0%: 0.625–20 mg/ml). All cultures were pulsed with  $^3$ [H]-thymidine (1  $\mu$ Ci/well; PerkinElmer, Boston, MA) for the last 18 h and harvested on a Micro 96™ Cell Harvester. The incorporated radioactivity was measured using a microplate beta counter (Micro  $\beta$  plus, Wallac, Turku, Finland).

For assays of cell viability, cells were stained with FITC-conjugated annexin V (BD Biosciences, San Jose, CA) and 7-amino-actinomycin D (7-AAD, Calbiochem, Darmstadt, Germany). Flow cytometry was performed using FACSCalibur and CellQuest software (BD Biosciences).

### 2.4. Preparation of PBMC

PBMC were isolated from the peripheral blood of healthy volunteers, and were isolated by density-gradient centrifugation. This study was approved by the Institutional Ethics Committee (No. 20-30-1009) and was conducted according to the principles of the Declaration of Helsinki, with each subject being provided with written informed consent.

### 2.5. Determination of cytokine production

PBMC were stimulated ( $2 \times 10^5$ /well) with immobilized anti-CD3 (1  $\mu$ g/ml, OKT3, IgG2a) and soluble anti-CD28 (5  $\mu$ g/ml, 4B10, IgG1) mAbs in the presence or absence of CP extract at the indicated dilutions (0.125–0.5%) in 96-well flat-bottom plate. Supernatants from triplicate cultures were collected after 24 h for assessment of cytokine production by ELISA. IL-2, IL-4, IL-10, IL-12p40, IL-12p70, IFN- $\gamma$ , and TNF- $\alpha$  were measured using BD OptEIA™ ELISA set (BD Biosciences).

### 2.6. Determination of cytotoxic activities

Freshly isolated PBMC ( $2 \times 10^6$ /well) were stimulated as above in the presence or absence of 0.125% CP extract in 24-well plates. After 24 h of culture, viable cells were collected as effector cells using density-gradient centrifugation. As target cells, K562 were labeled with 5  $\mu$ Ci/ml of  $^3$ [H]-thymidine for 6 h at 37 °C prior to assay initiation. Pre-activated PBMC were co-cultured with labeled K562 ( $5 \times 10^3$  cells/well) for 4 h, and then radioactivity level was measured.

### 2.7. Caspase assay

Caspase-3 and -7 activities were measured by using a Caspase-Glo 3/7™ Assay kit (Promega, Madison, WI, USA). Experiments were carried out following the manufacturer's recommended procedures. Briefly, the cells were plated at a concentration of  $1 \times 10^4$  cells/well in white-walled 96-well plates in RPMI1640 containing 5% FCS. Cells were incubated for the indicated time period in the presence of CP extract or etoposide (Sigma) as a positive control. Following incubation over the indicated time period, Caspase-Glo 3/7 reagents were added to the wells in a final volume of 200  $\mu$ l, and subsequently incubated at room temperature for 1 h. Luminescence was measured using GloMax®-Multi Detection System (Promega).

### 2.8. Microarray analysis

Total cellular RNAs from PBMC purified and cultured ( $2 \times 10^6$ ) as above in the presence or absence of 0.125% CP extract were isolated and subjected to a clean-up protocol with RNeasy Mini kits (Qiagen GmbH, Hilden, Germany), according to the manufacturer's recommendation. The quality of total RNA was assessed using an Agilent 2100 Bioanalyzer. First and second strand cDNA

was prepared from a 1 µg RNA template, and then aRNA was synthesized from cDNA by Ambion Amino Allyl aRNA kit (Ambion, Austin, TX, USA). 3D-Gene Human Oligo chip 25K (Toray Res Institute, Kamakura, Japan) were hybridized to Cy5-(purified PBMC) and Cy3-(stimulated PBMC + 0.125% CP extract: CP<sup>+</sup> PBMC) or Cy3-(stimulated PBMC without CP extract: CP<sup>-</sup> PBMC), stained and prepared for each aRNA, and then scanned on a ScanArray Lite (PerkinElmer, MA, USA). To detect the differentially and significantly expressed genes, the raw data were normalized and analyzed using Java TreeView 1.1.6 and Cluster 3.0 software programs (<http://rana.lbl.gov/eisen>).

### 2.9. Quantification of mRNA by real-time PCR

Total cellular RNA from PBMC ( $2 \times 10^6$ ) cultured as above in the presence or absence of 0.06% or 0.125% CP extract was isolated using High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's recommendation. The quality and quantity of total RNA was assessed by Nanodrop spectrophotometer (ND-1000, Thermo Fisher Scientific Inc., MA, USA). cDNA was synthesized from 1 µg of total RNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics). The levels of HPRT, CCL2, CCL7, CCL8, and SERPINB2 expression were detected by RT-PCR using LightCycler<sup>®</sup> TaqMan<sup>®</sup> Master (Roche Diagnostics) with specific primers (Table 1). Reaction condition was 95 °C/10 min; 35 cycles of 95 °C/30 s and 60 °C/20 s. TaqMan<sup>®</sup> probe (Universal ProbeLibrary, Roche Diagnostics) was used to detect specific PCR products. Amplification and detection of TaqMan<sup>®</sup> probe were performed with LightCycler<sup>®</sup> ST300 (Roche Diagnostics). The human HPRT gene was used as a housekeeping reference gene to normalize expression levels between the samples.

### 2.10. Statistical analysis

Statistical analysis was performed by the 2-tailed Student's *t* test for the assays.

## 3. Results and discussion

### 3.1. Inhibition of proliferative response of various tumor cell lines by CP extract

To evaluate the effect of CP extract on tumor cell growth, we initially assessed its effect on the proliferative responses of solid and haematopoietic tumor cell lines. CP extract inhibited the proliferative responses of solid tumor cell lines derived from cervical carcinoma (Hela), breast adenocarcinoma (MCF-7), hepatocellular

carcinoma (HepG2), lung adenocarcinoma (PC14), pancreatic epithelioid carcinoma (Panc-1), and mesothelioma (H2452) in a dose-dependent manner (Fig. 1A). In addition, CP extract inhibited the proliferative responses of haematopoietic cell lines, including T cell lymphoma (Jurkat), plasma cell leukemia (ARH77), Burkitt's lymphoma (Raji), and anaplastic large cell lymphoma (Karpas-299) (Fig. 1B). We also found that proliferation of additional cell lines, derived from various malignant tumor cell lines, was similarly inhibited (data not shown), with no statistically significant differences observed between solid and haematopoietic tumor cell lines.

To determine whether the inhibition of proliferative response was associated with decreased cell viability, we evaluated potential induction of apoptosis and cell death in the T lymphoma cell line Jurkat. As shown in Fig. 1C, the levels of annexin V- or 7-AAD-positive Jurkat cells were increased in a dose-dependent manner (upper panel). The percentage of annexin V-negative cells was shown in line chart (lower panel). To confirm the above results, we measured the activities of caspase-3 and -7, which are effector caspases in inducing cell apoptosis. Fig. 1D shows that activation of caspase-3/7 on Jurkat cells was induced within 24 h of CP extract treatment. These data suggested that one of the mechanisms involved in the inhibition of tumor cell proliferation is associated with the induction of cell death, including apoptosis.

*Carica papaya* is also commonly known as "pawpaw". While there are some published reports regarding the effect of pawpaw as cancer therapy, the plant involved is the North American pawpaw *Asimina triloba* in the family of *Annonaceae*, which is a different species (McLaughlin, 2008). While several extracts from such botanicals as *Astragalus membranaceus* (*Fabaceae*), *Anemarrhena asphodeloides* (*Liliaceae*) and *Stellaria barbata* (*Lamiaceae*) have been demonstrated to exhibit tumor-specific growth inhibitory effect against solid and haematopoietic cell lines (Lin et al., 2003; Takeda et al., 2001), there has been no description of tumor growth inhibitory effect of extracts from any other member of the genus *Caricaceae*.

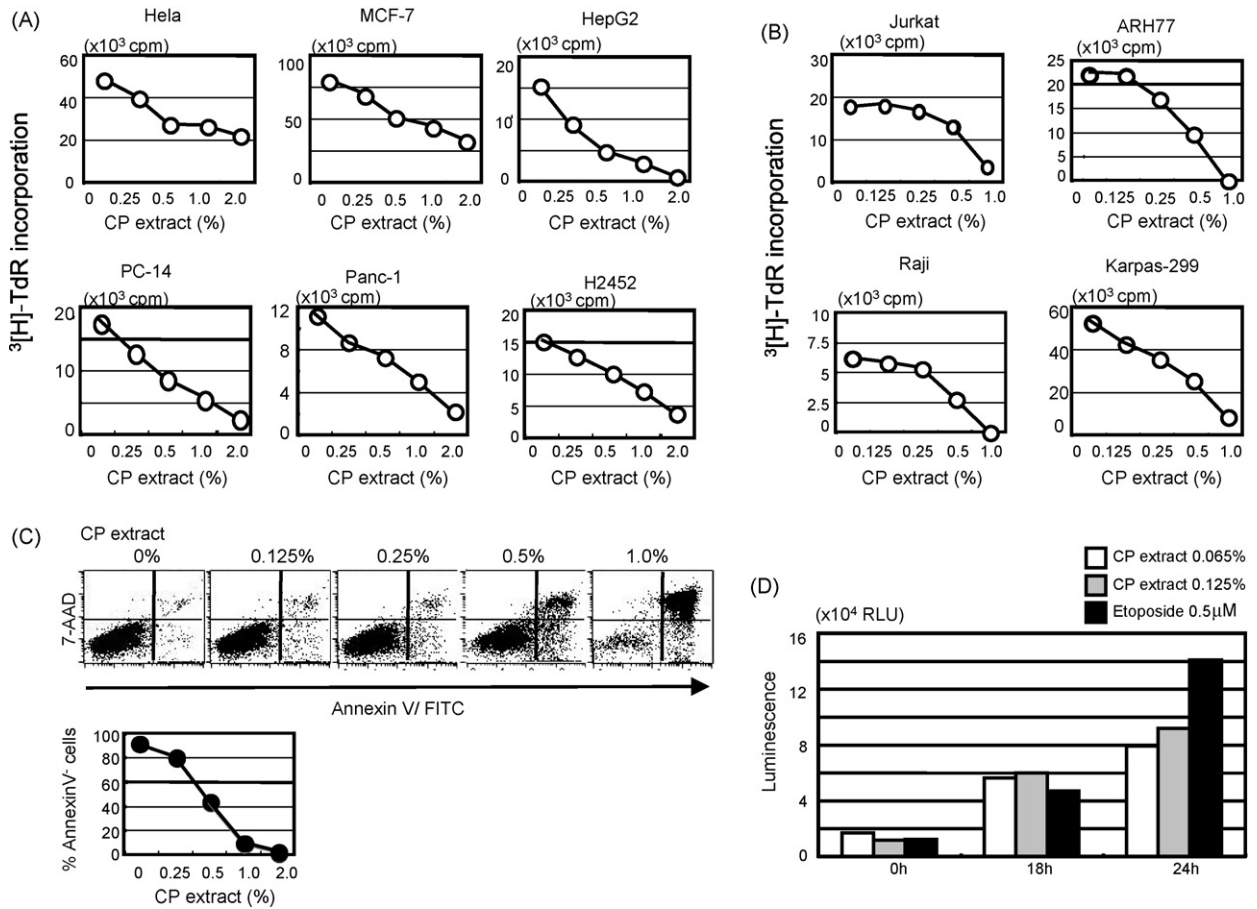
### 3.2. Cytokine production of PBMC induced by CP extract

To evaluate the potential immunomodulatory activities of CP extract on human PBMC, we next assessed the profile of PBMC cytokine production by ELISA. As shown in Fig. 2A, IL-2 and IL-4 production in cultured supernatants was reduced following 24 h of culture with CP extract. Since it is possible that CP extract may induce cell death of PBMC, similar to its effect on tumor cells, we analyzed PBMC cell viability by flow cytometry. Although IL-2 and IL-4 production was decreased in a dose-dependent manner, the percentage of annexin V-negative cells remained unchanged under the same experimental condition (Fig. 2B, a). In addition, we confirmed that <sup>3</sup>[H]-thymidine incorporation of activated PBMC was unaffected following 72 h of culture (Fig. 2B, b). We next examined other Th1 type cytokines that were relevant to anti-tumor immunity, such as IL-12p40, IL-12p70, IFN-γ, or TNF-α. Interestingly, these cytokines were markedly upregulated following the addition of a lower concentration of CP extract (0.125%, Fig. 2C). On the hand, production of IL-5, IL-6, IL-10 and IL-15 was not significantly affected by the addition of CP extract (data not shown).

Of note is that CP extract also had no effect on the viability of unstimulated PBMC (CP control, 96.7% vs. CP 0.2%, 96.5%). Shan et al demonstrated previously that *Cinnamomum cassia* (*Lauraceae*) extract enhanced the proliferative response of PBMC and production of IgG<sub>3</sub> (Shan et al., 1999a). They also reported that extracts from other types of botanicals, including *Astragalus membranaceus*, *Codonopsis pilosula* (*Campanulaceae*), *Epimedium brevicornum* (*Berberidaceae*), *Oldenlandia diffusa* (*Rubiaceae*), *Rhizoma typhonii* (*Araceae*) and *Schisandra chinensis* (*Schisandraceae*),

**Table 1**  
Primers and probes.

HPRT	Forward	5'-tgaccttgatttatttgcatacc-3'
	Reverse	5'-cgagcaagacgttcagctct-3'
	Probe	5'-gctgagga-3'
CCL2	Forward	5'-ttctgtgctgctgctcat-3'
	Reverse	5'-ggggcattgattgcatct-3'
	Probe	5'-cagccacc-3'
CCL7	Forward	5'-tctcagtgtgctgtaaaaactgtgg-3'
	Reverse	5'-ttatacaataccatgaggtaga-3'
	Probe	5'-ctctcccc-3'
CCL8	Forward	5'-ccctcaggagctgctcag-3'
	Reverse	5'-ctccagcctctggataggaa-3'
	Probe	5'-acctgctg-3'
SERPINB2	Forward	5'-catggagcatctcgtccac-3'
	Reverse	5'-actgcattgctcccactt-3'
	Probe	5'-gctccagg-3'



**Fig. 1.** Inhibition of tumor cell line proliferation by CP extract. (A) Solid tumor cell lines were cultured in 96-well flat plates and (B) haematopoietic cell lines were cultured in 96-well round plates for 24 h. CP extract was added at the indicated dilutions (*x*-axis, 1% = 10 mg leaves/ml) at the initiation of culture. Proliferative responses for the final 18 h of 24 h of culture were measured. Data shown were representative of three independent experiments. (C) Involvement of cell death in extract-induced growth inhibition of tumor cells. Jurkat cells were cultured under the same condition as (B). After 24 h of culture, cells were harvested and stained with 7-AAD and FITC-annexin V, and then analyzed by flow cytometry. Data are displayed as dot plot (upper). The percentage of annexin V-negative cells was gated and plotted as line chart (lower). (D) Jurkat cells were cultured as described in Section 2 for 18 h and 24 h. CP extract or etoposide was added at the initiation of culture. Activation of caspase-3/7 was detected using Caspase-Glo® substrate and measured by microplate luminometer. The control luminescence of medium alone was 7000–8000 RLU, and was subtracted from the raw data. (Jurkat alone, 20,000–30,000 RLU).

had no effect on PBMC proliferation. Furthermore, they demonstrated that *Acanthopanax gracilistylus* (Araliaceae) extract inhibited <sup>3</sup>[H]-thymidine incorporation of ConA-activated PBMC following 72 h of culture. Nevertheless, TNF-α was enhanced on monocytes stimulated with LPS for 24 h (Shan et al., 1999b). In our experiments, CP extract reduced the amount of IL-2 and IL-4, whereas production of the anti-tumor related cytokines, such as IL-12p40, IL-12p70, IFN-γ and TNF-α, was enhanced without growth inhibition. Our result showed that CP extract appears to be non-toxic to normal cells as compared to its effect on tumor cell lines.

It is known that p40, a subunit of IL-12p70, is produced by antigen-presenting cells (APCs), such as monocytes or dendritic cells (DCs). It has also been shown that human IL-12p70 production from DCs is dependent on CD40-40L interactions, whereas p40 production is not (Cella et al., 1996). Based on these previous reports, it is conceivable that CP extract may modify not only activation of APCs, but also the interaction of T-APCs or T-B cells; namely, CP extract modifies both innate and acquired immunity.

### 3.3. The effect of CP extract on cytotoxic activity of activated PBMC

Since CP extract enhanced the production of anti-tumor cytokines, such as IL-12p40, IL-12p70, IFN-γ and TNF-α, we

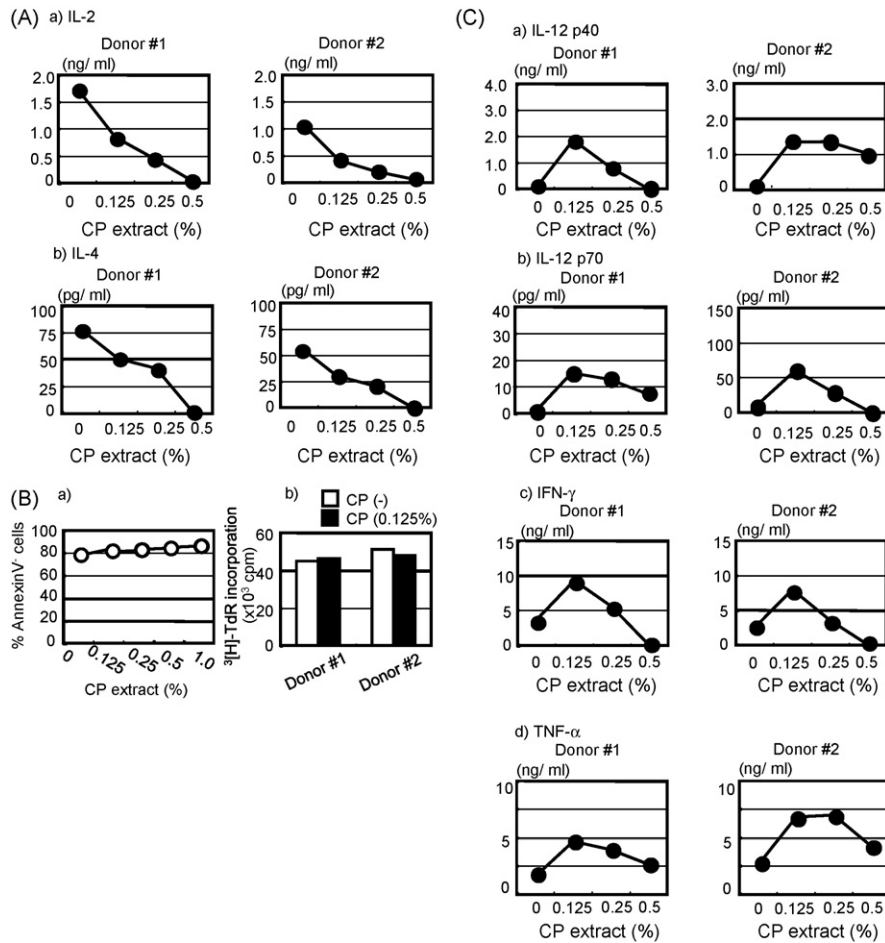
next assessed its effect on the cytotoxic activity of pre-activated PBMC.

As shown in Fig. 3, cytotoxicity of pre-activated PBMC against K562 was significantly enhanced by treatment of CP extracts at 25:1 and 12.5:1 effector–target ratio (*E:T* ratio). In contrast to our data, it was reported previously that the addition of *Acanthopanax gracilistylus* extract in induction phase inhibited CTL and NK cells activities (Shan et al., 1999b). In the case of CP extract, our data suggest that there might be specific relationship between enhanced production of Th1 type cytokines and increased cytotoxicity, since IL-12, TNF-α and IFN-γ are effective factors for inducing cell-mediated cytotoxicity (Foulds et al., 2006), resulting in enhanced anti-tumor immunity (Baxevanis et al., 2000).

### 3.4. Gene profiling by microarray analysis

An important objective of our present study is to define an index of immunomodulatory effects in PBMC following treatment with CP extract. To evaluate this issue, we examined the alteration in gene expression profiling of purified PBMC from three different donors subjected to CD3/CD28-stimulation ± CP extract (CP extract-treated and non-treated).

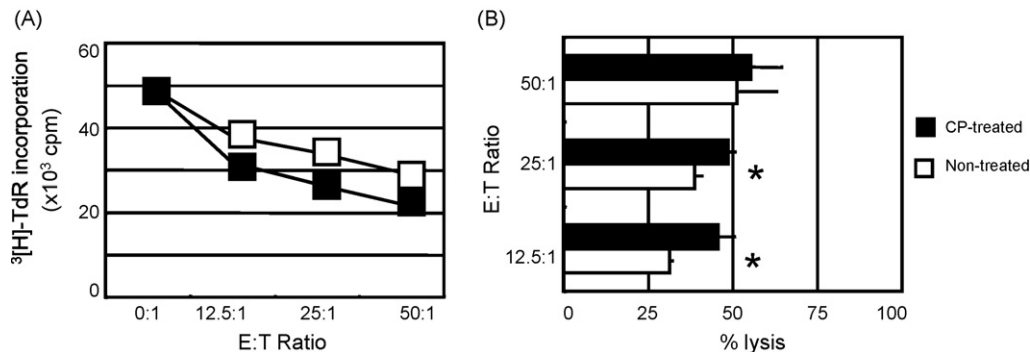
Following 24 h of stimulation, 23 genes were enhanced at least twofold under the condition of CP extract-treated PBMC (Fig. 4A,



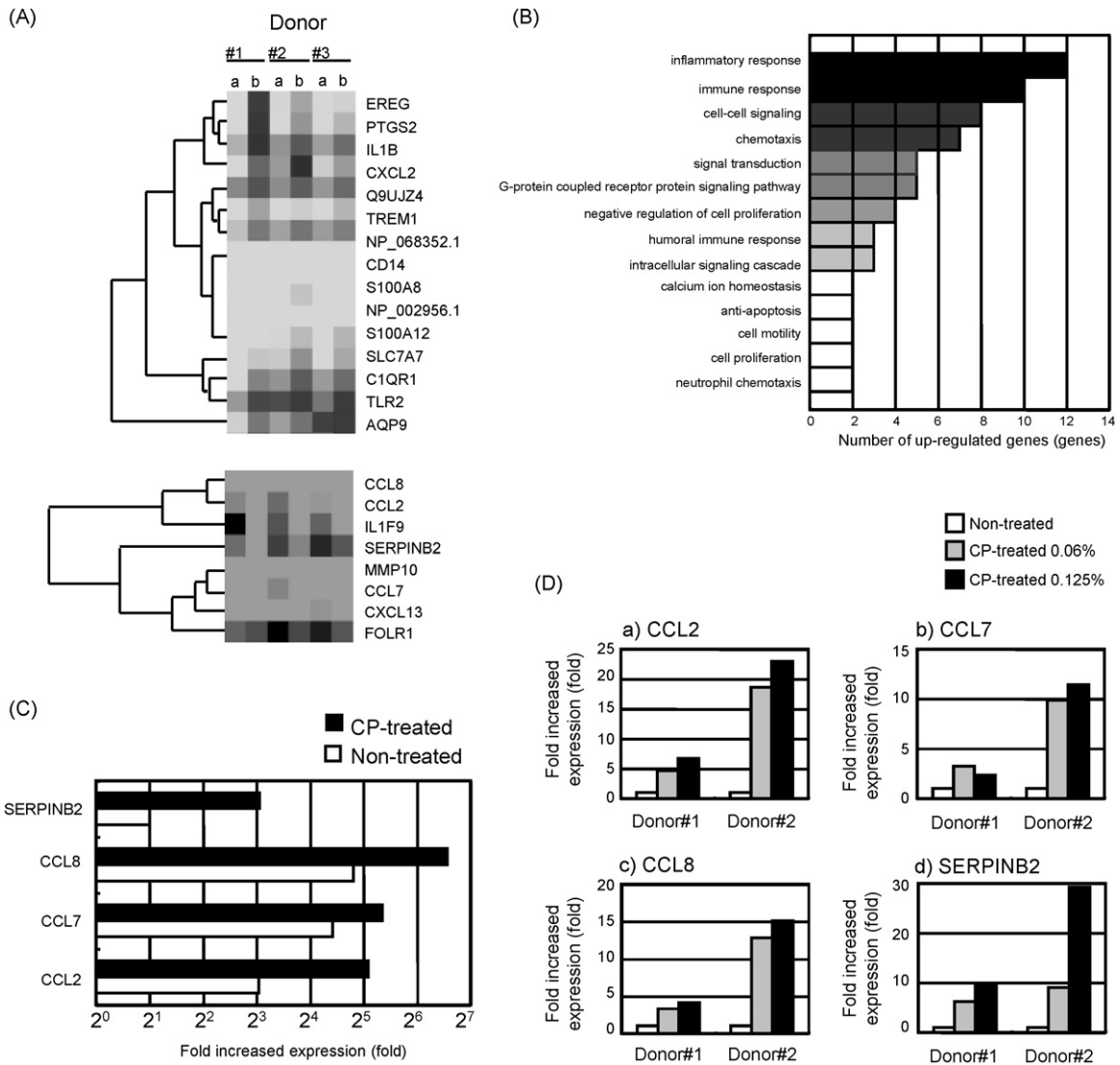
**Fig. 2.** Cytokine production of PBMC following the addition of CP extract. (A) Production of IL-2 and IL-4 in the PBMC culture supernatants following stimulation with anti-CD3 mAb (1  $\mu\text{g/ml}$ ) and anti-CD28 mAb (5  $\mu\text{g/ml}$ ) in the presence of CP extract at the indicated dilutions ( $x$ -axis) for 24 h is demonstrated. Data were from two representative donors of five different donors. (B, a) Cell viability after 24 h culture of (A). Activated PBMC were harvested and stained with FITC-annexin V and 7-AAD, and then analyzed by flow cytometry. The percentage of annexin V-negative cells was plotted. Data were representative of three independent experiments. (B, b) PBMC ( $2 \times 10^5$  cells) were cultured under the same condition as (A) for 72 h. Proliferative responses for the final 18 h of 72 h of culture were measured. Data were expressed in the presence or absence of 0.125% of CP extract, and were from two representative donors of three different donors. (C) Production of IL-12p40, IL-12p70, IFN- $\gamma$ , and TNF- $\alpha$  was assessed under the same condition as described in (A). Data were from two representative donors of five different donors.

lane b). There were no genes that showed twofold lower expression level in CP extract-treated than in non-treated PBMC. Gene ontology (GO) analysis assigned upregulated and downregulated genes to a number of specific biological processes, including inflammation (12 of 23 genes), immune response (10 of 23 genes), chemotaxis (7 of 23 genes) and so on (Fig. 4B). Although we cannot identify presently any downregulated gene with direct relevance to can-

cer, several upregulated genes in our assay have been reported to have a role in cancer biology, with Fig. 4C showing representative genes that were upregulated by the addition of CP extract. To confirm the upregulation of these specific genes, we performed real-time RT-PCR analysis (Fig. 4D). Our data indicated that the relative expression of mRNA of CCL2, CCL7, CCL8, and SERPINB2 was increased among the different donors in a dose-dependent manner.



**Fig. 3.** Effect of CP extract on cytotoxic activity of activated PBMC. PBMC were cultured with anti-CD3 mAb (1  $\mu\text{g/ml}$ ) and anti-CD28 mAb (5  $\mu\text{g/ml}$ ) in the presence (closed symbol) or absence (open symbol) of 0.125% of CP extract for 24 h. Collected cells were then co-cultured with  $^3\text{H}$ -thymidine-labeled K562 for 4 h.  $E:T$  ratio was demonstrated at 12.5:1 to 50:1. (A) Data shown as  $^3\text{H}$ -thymidine incorporation were representative of five independent experiments. The cpm value of K562 alone was plotted at 0:1 ( $E:T$  ratio). (B) Data were shown as means of % lysis  $\pm$  SD from five independent experiments. \* $p < 0.05$ . The level of radioactivity of K562 alone was  $>5000$  cpm in all experiments.



**Fig. 4.** Gene expression profiling by microarray analysis and real-time RT-PCR. PBMC were cultured under the same condition as in Fig. 3. After 24 h of culture, the alteration in gene expression profiling of purified PBMC subjected to CD3/CD28-stimulation ± CP extract cells was evaluated. Human Oligo chip 25K were hybridized to Cy5-(purified PBMC) and Cy3-(stimulated PBMC + 0.125% CP extract: CP<sup>+</sup> PBMC) or Cy3-(stimulated PBMC without CP extract: CP<sup>-</sup> PBMC). (A) Heat maps and dendrograms of the CP extract-induced alteration of 23 gene expression data in CD3/28-stimulated PBMC. (a: CP<sup>-</sup> PBMC and b: CP<sup>+</sup> PBMC) Data were obtained from three different donors. Downregulated genes following stimulation with anti-CD3 and anti-CD28 for 24 h (Upper). Upregulated genes following stimulation with anti-CD3 and anti-CD28 for 24 h (Lower). (B) Classification of the 23 genes based on gene ontology (GO) group in biological processes. (C) Representative genes that were upregulated following the addition of CP extract. x-Axis indicates fold increased expression of genes, both of CP extract-treated (closed column) and non-treated (open column) cells. All data were means of three different donors. (D) Real-time RT-PCR for four genes identified in (C). PBMC were cultured under the same condition as (C) with or without 0.06% or 0.125% of CP extract. x-Axis indicates relative expression level of cultured PBMC. Data shown were representative of four independent experiments from two donors.

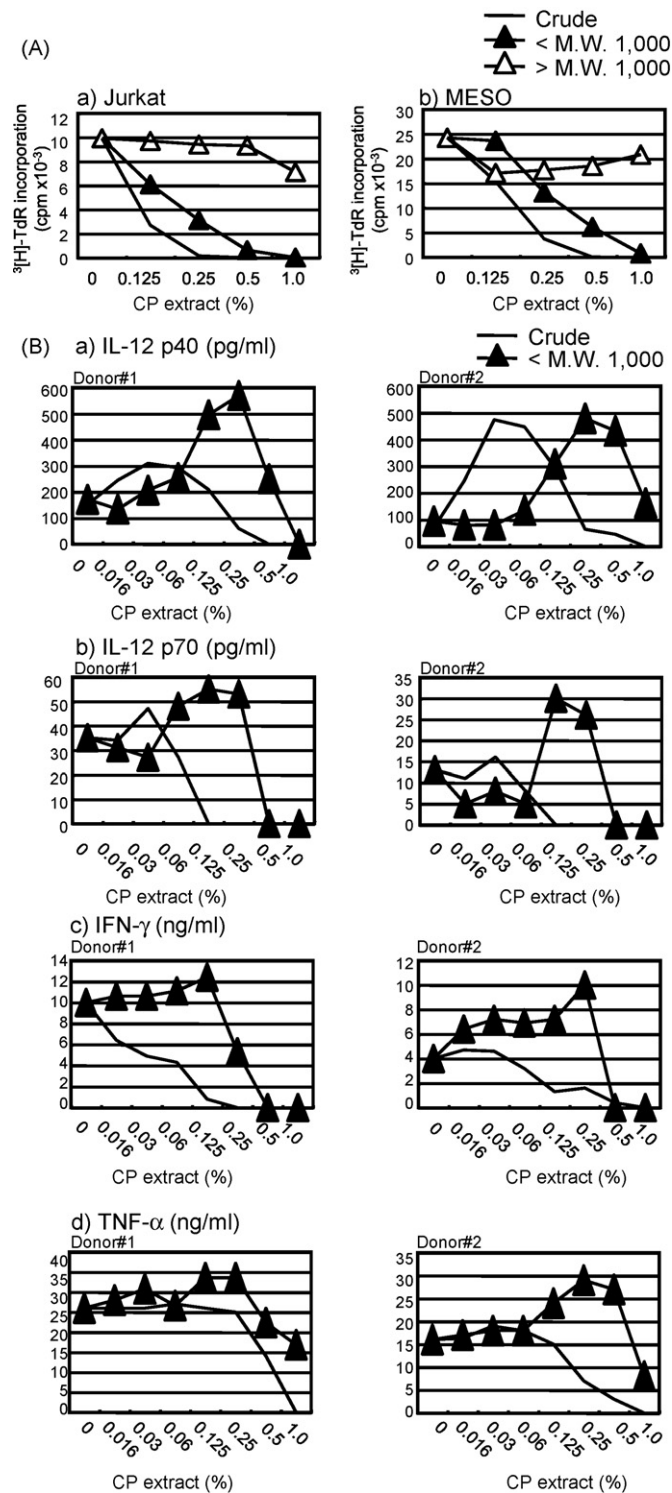
The family of monocyte-chemoattractant proteins, including CCL2 (MCP-1), CCL7 (MCP-3) and CCL8 (MCP-2), are considered to play important roles in the recruitment of monocytes, basophils, plasmacytoid dendritic cells and memory T lymphocytes to sites of inflammation. Their enhanced expression is correlated with various cytokines, such as IL-1β, TNF-α and IFN-γ (Viola and Luster, 2008). In addition, patients with high circulating level of CCL2 had significantly higher survival rate than those with low CCL2 production (Monti et al., 2003). Furthermore, these chemokines are also inducible on tumor cells. It has also been reported that expression of CCL2 or CCL7 on tumor cells is correlated with reduced tumorigenicity through activation of monocytes, T lymphocytes and NK cells (Brown et al., 2007; Wetzel et al., 2007). On the other hand, the serine protease inhibitor SERPINB2, also known as urokinase plasminogen activator inhibitor type 2, is associated with increased survival in patients with breast cancer. Recently, similar observations were demonstrated in other types of cancer, includ-

ing oral, ovarian, gastric, lung, pancreatic, prostate, liver cancers and melanoma (Croucher et al., 2008).

These data imply that the four genes CCL2, CCL7, CCL8 and SERPINB2 found to be upregulated in our estimation of immunomodulatory genes may serve as index markers of the immunomodulatory effects on human PBMC following 24 h of treatment with CP extract.

### 3.5. Identification of the active fraction from CP extract using molecular weight cut off selection

To identify the active components of CP extract, we compared the growth inhibitory effect of crude and fractionated extracts on tumor cells, as well as their immunomodulatory effects on cultured PBMC. We initially performed fractionation of crude CP extract at M.W. 3500, and then we compared growth inhibition of tumor cell lines by crude extract at greater than M.W. 3500, and extract at less



**Fig. 5.** Identification of active fraction from CP extract using molecular weight cut off selection. CP extract were fractionated as described in Section 2. The haematopoietic cell line Jurkat and the solid tumor cell line MESO were cultured under the same condition as in Fig. 1. Whole and fractionated CP extract were added at the indicated dilutions (x-axis) at the initiation of culture. Proliferative responses for the final 18 h of 24 h of culture were measured. Data shown were representative of three independent experiments. (B) Production of IL-12p40, IL-12p70, IFN- $\gamma$ , and TNF- $\alpha$  in the PBMC culture supernatants following stimulation with anti-CD3 mAb (1  $\mu$ g/ml) and anti-CD28 mAb (5  $\mu$ g/ml) in the presence of crude or fractionated CP extract at the indicated dilutions (x-axis) for 24 h was shown. Data shown were from two representative donors of three different donors.

than M.W. 3500. Culturing with the fractionated CP extract of less than M.W. 3500 led to a significant inhibition of tumor cell growth (data not shown). Additionally, when comparing cytokine production of PBMC, we found that production of Th1 cytokines, including IL-12p40, IL-12p70, IFN- $\gamma$ , and TNF- $\alpha$ , was upregulated following the addition of fractionated CP extract of less than M.W. 3500 (data not shown). Focusing on these results, we then fractionated the crude CP extract into a fraction of less than M.W. 2000 and M.W. 1000.

We found that whereas the fractionated CP extract of less than M.W. 1000 markedly inhibited proliferative response of the haematopoietic cell line Jurkat and the solid tumor cell line MESO in a dose-dependent manner, similar to the effect of the crude CP extract, the fraction of greater than M.W. 1000 exhibited only a partial inhibitory effect (Fig. 5A). Furthermore, the production of IL-12p40, IL-12p70, IFN- $\gamma$ , and TNF- $\alpha$  was markedly increased by the addition of the fractionated CP extract of less than M.W. 1000 (Fig. 5B).

These data indicated that the active components of the CP extract with growth inhibitory effect on tumor cells and immunomodulatory effects on cultured PBMC are likely found in the fraction of less than M.W. 1000.

In the present study, we demonstrate that CP extract has several *in vitro* biological effects. It displays anti-proliferative effect on tumor cells, promotes Th1 type cytokine production, enhances cytotoxicity against tumor cells, and upregulates anti-tumor related genes in PBMC. Furthermore, our data suggest that the active components of CP extract should be contained in the fraction with components of less than M.W. 1000.

It is reported that crude extracts from other botanicals, such as *Astragalus membranaceus*, *Anemarrhena asphodeloides*, and *Oldenlandia diffusa*, had anti-tumor effects *in vitro* (Lin et al., 2003; Takeda et al., 2001) and *in vivo* (Gupta et al., 2004; Wong et al., 1996; Yu et al., 2007). In addition, clinical trials have been performed using crude extracts from such botanicals as *Stellaria barbata*, *Punica granatum* (Lythraceae) and *Ganoderma lucidum* (Ganodermataceae), as complementary cancer therapy (Boh et al., 2007; Pantuck et al., 2006; Rugo et al., 2007; Zhao et al., 2007).

Currently, several anti-cancer agents from botanicals have become established therapies in the clinical setting. They can be categorized into four main classes of compounds: vinca alkaloids (vincristine, vinblastine, vindesine, vinorelbine), taxanes (paclitaxel, docetaxel), podophyllotoxin and its derivatives (etoposide, teniposide), and camptothecin and its derivatives (topotecan, irinotecan).

In the leaves of *Carica papaya*, components previously reported to potentially have anti-tumor activity include  $\alpha$ -tocopherol (Ching and Mohamed, 2001), lycopene (van Breemen and Pajkovic, 2008), flavonoid (Miean and Mohamed, 2001), and benzylisothiocyanate (Basu and Halder, 2008). However, our present paper is the first to report the effect of *Carica papaya* extract on enhanced production of Th1 type cytokines from human lymphocytes. We will further fractionate the CP extract in the future to identify more precisely the active components. Our studies suggest that in addition to its anti-tumor effect, the fact that CP extract enhances production of Th1 type cytokines, such as IL-12, IFN- $\gamma$  and TNF- $\alpha$ , raises the possibility that CP extract may contribute to the treatment of Th2-mediated allergic disorders, such as allergic rhinitis and bronchial asthma, or as an adjuvant of various vaccines by inducing a shift from Th2 to Th1 type immune response.

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