

Basic nutritional investigation

## Maitake D-Fraction enhances antitumor effects and reduces immunosuppression by mitomycin-C in tumor-bearing mice

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### Abstract

**Objective:** D-Fraction, a polysaccharide extracted from maitake mushrooms (*Grifola frondosa*), has been reported to exhibit an antitumor effect through activation of immunocompetent cells, including macrophages and T cells, with modulation of the balance between T-helper 1 and 2 cells. We examined whether D-Fraction could decrease the effective dosage of the chemotherapeutic agent, mitomycin-C (MMC), necessary to control carcinoma in mice.

**Methods and results:** We determined that  $0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  was the optimal dosage of MMC because consecutive administration for 17 d resulted in antitumor effects and a survival ratio of 100% in mice bearing mammary cancer cells (MM-46). Although the dosage of MMC was lower than the effective level, spleen weight and total number of nuclear cells in the mouse spleen decreased, indicating that MMC showed immunosuppressive activity. In contrast, the combination of D-Fraction and MMC recovered the decreases in the dose response induced by MMC and inhibited tumor cell growth more than MMC alone. These effects were achieved through increased immunocompetent cell proliferation. We evaluated the expression of CD28 on splenic CD8<sup>+</sup> T cells and the amount of interleukin-12 produced by whole spleen cells including macrophages after administering D-Fraction. The results showed enhancement of the T-helper 1 dominant response.

**Conclusion:** These results suggest that D-Fraction can decrease the effective dosage in tumor-bearing mice by increasing the proliferation, differentiation, and activation of immunocompetent cells and thus provide a potential clinical benefit for patients with cancer. © 2005 Elsevier Inc. All rights reserved.

**Keywords:** polysaccharide; combination treatment; mitomycin-C

### Introduction

None of the currently available anticancer drugs act solely on carcinoma cells. Anticancer drugs are usually extremely toxic and kill malignant and normal cells. The adverse effects of such drugs are greatest on hematopoietic

tissue, gastrointestinal mucosa, gonads, and skin. Injury to hematopoietic tissue causes severe immunosuppression and negatively affects therapy by leaving the host susceptible to infection by opportunistic and pathogenic microorganisms. These limits on the use of chemotherapeutic agents thus constrain their use in effective therapy.

The present study attempts to develop a more potent therapy for carcinoma by combining immunotherapy with chemotherapy. The  $\beta$ -glucans extracted from edible mushrooms have been found to act as biological response mod-

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ifiers that enhance host immune systems. In response to these discoveries, investigators have extracted similar candidate compounds from the fruiting bodies of various basidiomycetes with a view to application in cancer therapy. Lentinan from *Lentinus edodes* [1] and protein-bound polysaccharide K (PSK) (krestin) from *Coriolus versicolor* [2] are typical  $\beta$ -glucans that possess a straight  $\beta$ -1,3 chain and a  $\beta$ -1,6 side chain. A  $\beta$ -glucan (D-Fraction) that we previously extracted from the fruiting bodies of maitake mushrooms (*Grifola frondosa*) with a straight  $\beta$ -1,6 chain and a  $\beta$ -1,3 side chain was found to activate immunocompetent T cells, macrophages, and natural killer cells to a greater extent than lentinan or krestin [3]. The safety of D-Fraction has been confirmed by the Consumer Product Testing Co. (Fairfield, NJ, USA).

The present study investigated the benefits of D-Fraction and mitomycin-C (MMC), an anticancer agent with deleterious side effects, in tumor-bearing mice.

## Materials and methods

### Materials

D-Fraction was prepared as described previously [4] from powdered fruiting bodies of the maitake mushroom (*G. frondosa*; Yukiguni Maitake Co., Ltd., Niigata, Japan).

The ratio (percentage) of contaminating lipopolysaccharide in D-Fraction determined with an Endospecy ES-20S Set (Seikagaku Industry Co., Ltd., Tokyo, Japan) was below 0.0000006%.

### Animals

C3H/HeJ mice (4 wk old, male) that were non-responsive to lipopolysaccharide were provided by Japan Clea Co., Ltd. (Osaka, Japan) and acclimated for 1 wk before experimental use.

### Cells

Mammary cancer cells (MM-46), kindly donated by Dr. Kanki Komiyama of Kitasato Institute for Life Sciences, Kitasato University (Tokyo, Japan) were maintained in the mouse peritoneal cavity before experimental use.

### Administration of MMC and D-Fraction to MM-46 carcinoma-bearing mice

MM-46 carcinoma cells ( $1 \times 10^6$  cells) were implanted into the right axillary region of 5-wk-old male C3H/HeJ mice. Twenty-four hours later, 0.1, 0.25, or 0.5 mg/kg of MMC was administered once a day intraperitoneally for 17 consecutive days ( $n = 3-4$ ). Further, to investigate the effect of D-Fraction on MM-46 carcinoma-bearing mice treated with MMC, we administered the combination ther-

apy of 0.25 mg/kg of MMC and 1.0 mg/kg of D-Fraction for 17 consecutive days and then weighed the spleen and the tumor to determine the tumor inhibition ratio ( $n = 4-19$ ). After 10 d of the 17-d administration period, we examined the activation of splenic macrophages and CD8<sup>+</sup> T cells ( $n = 15$ ) and the production of cytokines such as interleukin-2 (IL-2) and IL-12 by whole spleen cells ( $n = 3-4$ ).

### Preparation of whole spleen cells

Spleens were extirpated after 10 d of MMC (0.25 mg/kg), D-Fraction (1.0 mg/kg), MMC plus D-Fraction (0.25 and 1.0 mg/kg, respectively), or saline administration. Erythrocytes, dead cells, and debris were removed from spleen cell suspensions using a Lympholyte-M Reagent (COSMO Bio Co., Ltd., Tokyo, Japan), and all spleen cells were suspended in RPMI-1640 medium containing 5% fetal bovine serum.

### Flow cytometry

On day 10 after tumor challenge, the ratios of positive major histocompatibility class II (MHC II<sup>+</sup>) cells, CD4<sup>+</sup> T cells, or CD8<sup>+</sup> T cells to whole spleen cells were determined by flow cytometry as described in a previous report [5]. CD19<sup>+</sup> cells were depleted from the whole spleen cells, and then CD19<sup>-</sup>MHC II<sup>+</sup> cells were prepared to detect the activated macrophages using the MACS positive selection system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). To detect the activated CD8<sup>+</sup> T cells, CD19<sup>+</sup> cells and MHC II<sup>+</sup> cells were separated from whole spleen cells, and the remaining cells were analyzed by flow cytometry. The following mouse monoclonal antibodies were purchased from BD Bioscience Pharmingen (San Diego, CA, USA): CD16/CD32 (0.5 mg/mL), fluorescein isothiocyanate-conjugated I-A/I-E (MHC II; 0.5 mg/mL), fluorescein isothiocyanate-conjugated CD86 (B7-2; 0.5 mg/mL), fluorescein isothiocyanate-conjugated CD3 $\epsilon$  (0.5 mg/mL), BD Cy-Chrom-conjugated CD8a (Ly-2; 0.2 mg/mL), Cy-Chrom-conjugated CD4 (L3T4; 0.2 mg/mL), R-phycoerythrin (R-PE)-conjugated CD28 (0.2 mg/mL), and phycoerythrin- and indodicarbocyanin-conjugated CD45R/B220 (0.2 mg/mL). R-PE-conjugated F4/80 antigen was purchased from Serotec Inc. (Raleigh, NC, USA).

### Determination of IL-2 and IL-12

Whole spleen cells ( $1 \times 10^6$  /well) were cultured in RPMI-1640 medium containing 5% fetal bovine serum at 37°C for 24 h in 5% CO<sub>2</sub>. Levels of IL-2 and IL-12 in the culture supernatant were then determined using mouse IL-2 and IL-12 enzyme-linked immunosorbent assay kits (Genzyme Co., Cambridge, MA, USA).

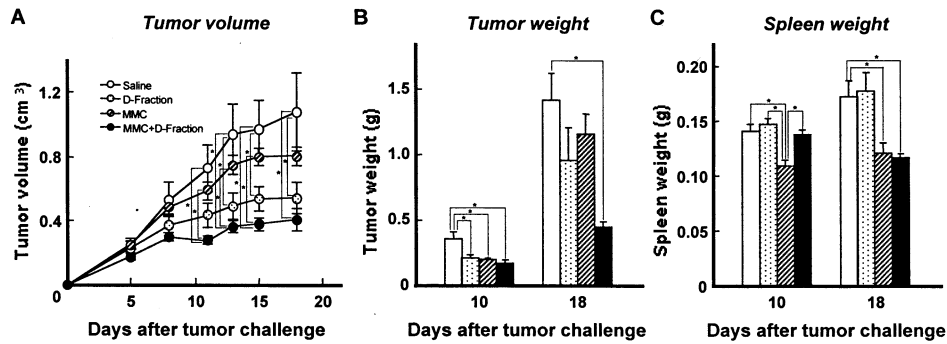


Fig. 1. Antitumor effect of MMC combined with D-Fraction. After tumor challenge, tumor growth was monitored for 17 d by measuring two perpendicular tumor diameters with calipers. (A) Tumor volumes were calculated according to the following formula:  $(\text{tumor volume}) = (\text{longest diameter} \times \text{shortest diameter}^2/2) \text{ cm}^3$ . On days 10 and 18, tumors were weighed to obtain tumor inhibition ratio (B) and spleens were weighed (C). Values represent means  $\pm$  standard errors from three to six mice (A) and of two experiments using 4 to 19 mice (B, C). White bars, saline; dotted bars, D-Fraction; hatched bars, MMC; black bars, MMC plus D-Fraction. Significant differences across the four groups were analyzed by Scheffe's test ( $*P < 0.05$ ). MMC, mitomycin-C.

### Reverse transcriptase polymerase chain reaction

Total cellular RNA was extracted from whole spleen cells by using Cepasol RNA II Super (Nacalai Tesque, Inc., Kyoto, Japan) according to the manufacturer's protocol. RNA (1  $\mu\text{g}$ ) was reverse transcribed to cDNA using oligo(dT) primers and then amplified with a reverse transcriptase polymerase chain reaction kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. The synthetic oligonucleotides were 5'-AGCGGCTGACTGAACTCAGATTGTAG-3' and 5'-GTCACAGTTTTCAGCTGTATAGGG-3' for interferon- $\gamma$  (IFN- $\gamma$ ) and 5'-TCCACGGATGCGACAAAAT-3' and 5'-TGAATCAGGCATCGAAAAG-3' for IL-4. The G3PDH gene with 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCACCACCCTGTTGCTGTA-3' primers served as the control. The expression of IFN- $\gamma$ , IL-4, or G3PDH mRNA was estimated by analyzing the density of bands of electrophoretically separated polymerase chain reaction products.

### Statistical analysis

Values are expressed as means  $\pm$  standard error, and significant differences across the four groups were analyzed with Scheffe's test.

## Results

### Antitumor effects of MMC and survival rate in tumor-bearing mice

Chemotherapeutic agents including MMC, which are cytotoxic to rapidly dividing cells, are associated with suppressed host immune function. Therefore, to decrease the effective dosage, we investigated the survival ratio after 17 consecutive days of MMC administration. On day 18, a

survival rate of 65% was achieved with an MMC dosage of  $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  when the tumor inhibition ratio was 22.2%. At an MMC dosage of  $0.1$  or  $0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ , the survival rate remained at 100% compared with the absence of MMC. However,  $0.1 \text{ mg/kg}$  of MMC had no antitumor effects, unlike the 18.5% of tumor inhibition ratio at  $0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ . These results showed that MMC at a dose of  $0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  was not toxic to the mice but effective against the tumors. Therefore, we used  $0.25 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  of MMC in subsequent experiments.

### Effect of D-Fraction on the growth of MM-46 carcinoma cells in MMC-treated mice

To determine the optimal dosage required in the combination study, we administered  $1.0$  or  $4.0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  of D-Fraction to MM-46 carcinoma-bearing mice. At these dosages, the tumor inhibition ratio values of D-Fraction were 32.9% and above 90%, respectively, on day 18 of tumor challenge. We therefore applied D-Fraction at a dosage of  $1.0 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ . To understand whether D-Fraction enhances the antitumor effect of MMC in mice, we determined the effect of  $0.25 \text{ mg/kg}$  of MMC plus  $1.0 \text{ mg/kg}$  of D-Fraction on MM-46 carcinoma cell growth for 17 d after tumor challenge (Figure 1A). After 5 d, the tumor volume differed between groups treated with MMC and with the combination. By day 10, D-Fraction recovered the decrease in spleen weight induced by MMC to the control level and the 46.6% tumor inhibition ratio of the tumor weight in the MMC group increased to 54.4% in the D-Fraction group (Figure 1B,C). On day 18, MMC decreased the weight of spleen and D-Fraction could not recover this to the control level, indicating that the cumulative dosage of MMC had serious side effects, although the tumor inhibition ratio increased from 18.5% to 68.9%. These results suggest that the antitumor effect of D-Fraction is greater than that of MMC.

Table 1

Effects of combination treatment with MMC and D-Fraction on whole spleen cell number and ratios of MHC II<sup>+</sup> cells, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells<sup>†</sup>

Treatments	Whole spleen cells (×10 <sup>6</sup> cells/mouse)		MHC II <sup>+</sup> cells (%)		CD4 <sup>+</sup> T cells (%)		CD8 <sup>+</sup> T cells (%)	
	Mean ± SE	%	Mean ± SE	%	Mean ± SE	%	Mean ± SE	%
Saline	100.5 ± 23.7	100	29.4 ± 0.9	100	13.3 ± 0.2	100	6.7 ± 0.1	100
D-Fraction	116.2 ± 27.4	116	34.6 ± 0.3	118	14.9 ± 0.4	112	7.1 ± 0.2	106
MMC	58.3 ± 18.1	58	32.3 ± 3.0	110	19.3 ± 0.1	145	8.7 ± 0.4	130
MMC + D-Fraction	92.0 ± 26.3	92	32.7 ± 0.5	111	18.3 ± 0.2	138	8.8 ± 0.2	131

MHC II, major histocompatibility class II; MMC, mitomycin-C; SE, standard error

\* *P* < 0.05, significant with Scheffe's test.

<sup>†</sup> Values are the result of three experiments using 4 to 15 mice.

*Effects of D-Fraction on numbers of whole spleen cells, MHC II<sup>+</sup>, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells in MMC-treated mice*

MMC functions as an anticancer agent by blocking DNA replication. To investigate the effect of MMC on the growth of immunocompetent cells, we counted the total number of cells in whole mouse spleens on day 10 (Table 1). The decrease in cell number elicited by MMC corresponded to the decrease in spleen weight. D-Fraction recovered the decrease caused by MMC. In contrast, the ratios of MHC II<sup>+</sup>, CD4<sup>+</sup> T cells, or CD8<sup>+</sup> T cells to the total number of spleen cells increased in mice treated with MMC alone or in combination with D-Fraction, with no synergism.

*Effect of D-Fraction on CD8<sup>+</sup> T-cell activation in MMC-treated mice*

D-Fraction induces the T-helper 1 (Th1) response and enhances cellular immunity [5,6]. We previously reported that D-Fraction increases CD28 expression on CD8<sup>+</sup> T cells [6]. In this study, we investigated the activation of CD8<sup>+</sup> T cells by analyzing the levels of CD 28 as an active marker (Figure 2). The expression of CD28 on CD8<sup>+</sup> T cells increased slightly more in the MMC-treated group than in the control group. Further, D-Fraction increased CD28 expression in MMC-treated mice to the highest level, indicating that cell-mediated immunity was enhanced by the combination therapy.

*Effect of D-Fraction on the Th1-dominant response in MMC-treated mice*

We measured the production of IL-12, an inducer of the Th1-dominant response, in mice administered MMC and D-Fraction (Figure 3). MMC had no effect on IL-12 productivity or CD86 expression of splenic macrophages compared with control. In contrast, D-Fraction increased IL-12 production and CD86 expression on activated macrophages. These results suggested that D-Fraction enhances the Th1-dependent response in MMC-treated mice through IL-12 produced by activated macrophages.

Activated Th1-type CD4<sup>+</sup> cells produce IL-2 [7,8]. We therefore examined the amount of IL-2 produced by whole spleen cells using an enzyme-linked immunosorbent assay. Figure 4 shows that MMC decreased production of IL-2, whereas MMC increased CD8<sup>+</sup> T-cell activation compared with control, as shown in Figure 2. The mechanism of decreased IL-2 productivity by Th1-type CD4<sup>+</sup> cell is unknown. In contrast, the combination of MMC and D-Fraction produced more of IL-2 than D-Fraction alone, due to the increased ratio of CD4<sup>+</sup> T cells to whole spleen cells (Table 1).

Th1-type CD4<sup>+</sup> cells primarily produce type 1 cytokines such as IL-2 and IFN-γ, whereas Th2-type CD4<sup>+</sup> cells produce those of type 2 such as IL-4, IL-5, and IL-10 [7,8]. To determine the ratio of IFN-γ to IL-4 produced by CD4<sup>+</sup> T cells in mice treated with MMC and D-Fraction, we examined the expression of IFN-γ mRNA and IL-4 mRNA in whole spleen cells (Table 2). D-Fraction in addition to MMC increased the ratio of IFN-γ mRNA to IL-4 mRNA compared with that of the control in contrast to MMC, which decreased the ratio. These results suggested that D-Fraction enhances the Th1-dependent response through

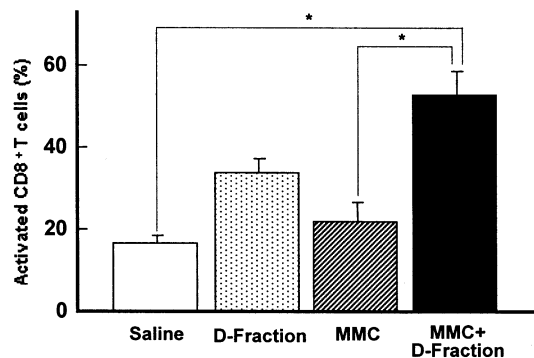


Fig. 2. Effect of MMC plus D-Fraction on CD8<sup>+</sup> T-cell activation. On day 10 after tumor challenge, splenic CD19<sup>-</sup> negative major histocompatibility class II cells were prepared from whole spleen cells and expression ratios (percentage) of CD28 on CD8<sup>+</sup> T cells (CD3<sup>+</sup>CD8<sup>+</sup> cells in lymphocyte gate) were investigated by flow cytometry. Values represent means ± standard errors of three experiments using 15 mice. Significant differences across the four groups were analyzed by Scheffe's test (\**P* < 0.05). MMC, mitomycin-C.

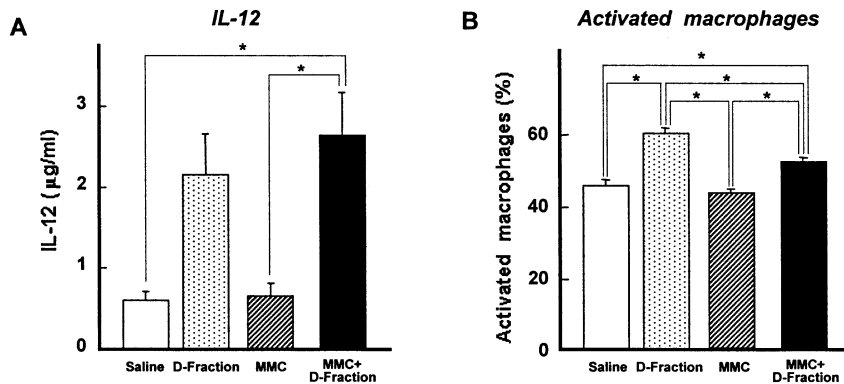


Fig. 3. Effects of MMC combined with D-Fraction on IL-12 production by whole spleen cells (A) and activation of splenic macrophages (B). (A) On day 10 after tumor challenge, levels of IL-12 in culture supernatant from whole spleen cells were investigated by enzyme-linked immunosorbent assay. (B) Splenic CD19<sup>+</sup> positive major histocompatibility class II cells were prepared from whole spleen cells and expression ratios (percentage) of CD86 on macrophages (CD45R/B220<sup>+</sup>F4/80<sup>+</sup> cells in monocyte gate) were investigated by flow cytometry. Values represent means  $\pm$  standard errors of four experiments using three to four mice (A) and of three experiments using 15 mice (B). Significant differences across the four groups were analyzed by Scheffe's test (\* $P < 0.05$ ). IL, interleukin; MMC, mitomycin-C.

IL-12 produced by activated macrophages and increases the production of IL-2 by Th1-type CD4<sup>+</sup> cells that is decreased by MMC.

## Discussion

MMC damages DNA synthesis and suppresses the number of spleen cells, plaque-forming cell production, and the delayed-type hypersensitivity reaction to sheep red blood cells. The present study confirmed that MMC decreases the total number of cells in the spleen (Table 1), the white blood cell count (data not shown), and the amount of IL-2 produced by whole spleen cells (Figure 4). In contrast, MMC increased the ratio of MHC II<sup>+</sup>, CD4<sup>+</sup> T cells, or CD8<sup>+</sup> T cells to the whole spleen cells compared with control (Table 1) and slightly activated CD8<sup>+</sup> T cells (Figure 2). The effects of anticancer drugs on the immune system have generally been thought of as

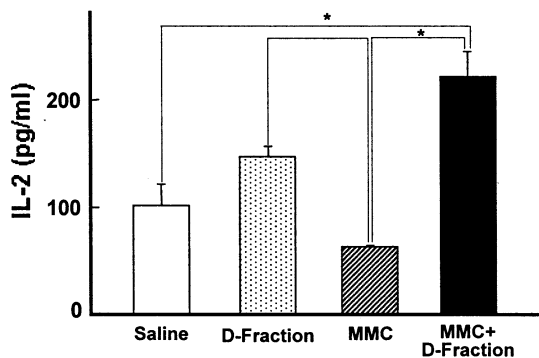


Fig. 4. Effect of MMC plus D-Fraction on IL-2 production by whole spleen cells. On day 10 after tumor challenge, level of IL-2 in culture supernatant from whole spleen cells was investigated by enzyme-linked immunosorbent assay. Values represent means  $\pm$  standard errors of three experiments using 3–4 mice. Significant differences across the four groups were analyzed by Scheffe's test (\* $P < 0.05$ ). IL, interleukin; MMC, mitomycin-C.

suppressive, which is mediated through bone marrow depression with decreased immunocyte production. However, recent evaluations of the effects of chemotherapeutic agents have reported other functional alterations of specific facets of the cellular immune defense system [9–11]. For example, Adriamycin enhances the tumor cell cytotoxicity of macrophages [12] and lymphocytes [13]. In addition, intraperitoneal MMC enhances tumor cell cytotoxicity of peritoneal exudate cells, although the mechanism of the altered macrophage function is unknown [14]. Our results support the notion that MMC modifies the immune system in diverse ways including immunosuppressive and immunostimulatory pathways. D-Fraction produced immunostimulation but no apparent direct antitumor effects as observed in cancer cell lines (unpublished data). It increased the ability of MMC to inhibit the growth of MM-46 carcinoma cells through recovery from MMC-induced immunosuppression resulting from a decrease in the number of immunocompetent cells. In addition, the tumor inhibition ratio of MMC and D-Fraction was higher than that induced by either factor alone. This action was brought about through MMC-induced immunostimulation such as an increase in the ratios of MHC II<sup>+</sup>, CD4<sup>+</sup> T cells, and CD8<sup>+</sup> T cells to whole spleen cells (Table 1) and CD8<sup>+</sup> T-cell activation (Figure 2).

To investigate how the combination of MMC and D-Fraction increased the antitumor effect, we examined the production of IL-12, which induces a Th1-dominant response with macrophage activation. The results indicated that D-Fraction enhanced the Th1-dominant response induced by MMC through IL-12 produced by activated macrophages because MMC alone did not stimulate macrophages (Figure 3). However, MMC decreased the amount of the Th1 cytokine, IL-2 (Figure 4), without decreasing the numbers of activated CD4<sup>+</sup> T cells (data not shown). Further, the ratio of IFN- $\gamma$  mRNA to IL-4 mRNA was decreased compared with the control (Table 2), indicating that MMC shifts the Th1 cytokine response to Th2. However, the Th2-dominant response induces immunity



Table 2

Effects of D-Fraction on the expression of IFN- $\gamma$  mRNA and IL-4 mRNA in MMC-treated tumor-bearing mice.<sup>†</sup>

Treatments	IFN- $\gamma$ mRNA (IFN- $\gamma$ mRNA/G3PDH mRNA)*	IL-4 mRNA (IL-4 mRNA/G3PDH mRNA)*	Ratio (IFN- $\gamma$ mRNA/IL-4 mRNA)
Saline	0.342 $\pm$ 0.032	0.471 $\pm$ 0.112	0.73
D-Fraction	0.480 $\pm$ 0.057	0.212 $\pm$ 0.050	2.26
MMC	0.231 $\pm$ 0.047	1.202 $\pm$ 0.328	0.19
MMC +D-Fraction	0.397 $\pm$ 0.056	0.391 $\pm$ 0.162	1.02

IFN- $\gamma$ , interferon- $\gamma$ ; IL-4, interleukin-4; MMC, mitomycin-C\*  $P < 0.05$ , significant with Scheffe's test.<sup>†</sup> Values are means  $\pm$  standard errors for of three experiments using five mice.

related to antibody production by activated B cells but not the cell-mediated immune response related to cytotoxic T cells. Therefore, the shift of Th1 to Th2 induced by MMC in activated CD8<sup>+</sup> T cells could not be explained (Figure 2). Naive CD8<sup>+</sup> T cells, which are similar to CD4<sup>+</sup> T cells, can differentiate into at least two subsets of cytolytic effector cells with distinct cytokine profiles: cytotoxic-1 (Tc1) cells produce Th1-like cytokines including IL-2 and IFN- $\gamma$ , whereas type 2 CD8<sup>+</sup> T cells (Tc2) produce Th2 cytokines, including IL-4, IL-5, and IL-10 [15,16]. Therefore, the decreased level of IL-2 and the decreased ratio of IFN- $\gamma$  to IL-4 mRNA might be due to the following mechanism. D-Fraction shifts the Th1-Tc2-dominant response induced by MMC to a Th1-Tc1-dominant response, and the increased IL-2 induced in response to D-Fraction is due to the increased ratios of CD4<sup>+</sup> T cells and CD8<sup>+</sup> T cells induced by MMC.

In conclusion, we have demonstrated that D-Fraction can decrease the amount of MMC required to manage a cancer model through enhancement of the Th1-dominant response. Although further studies are required to clarify whether combined therapy using D-Fraction is effective, the present study has indicated a potential benefit when applied with MMC in the therapy of patients with cancer. Our findings raise the possibility that D-Fraction combined with other types of chemotherapy will enhance the outcome and quality of life for patients with cancer.

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