

Original Article



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Conflict of Interest

The authors declare no potential conflicts of interest.

Abbreviations

cDNA, complementary DNA; CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; IFN, interferon; i.p., intraperitoneally; MB-IL-9, membrane-bound form of IL-9; MMC, mitomycin-C; PBS, phosphate-buffered saline; PI, propidium iodide; RPMI, Roswell Park Memorial Institute; s.c., subcutaneously; TAA, tumor-associated antigen; Th, T helper; TNF, tumor necrosis factor; Treg, regulatory T cell

Author Contributions

Formal analysis: Lee H; Investigation: Do Thi VA, Park SM; Writing - original draft: Kim YS.

# Ectopically Expressed Membrane-bound Form of IL-9 Exerts Immune-stimulatory Effect on CT26 Colon Carcinoma Cells

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## ABSTRACT

IL-9 is a known T cell growth factor with pleiotropic immunological functions, especially in parasite infection and colitis. However, its role in tumor growth is controversial. In this study, we generated tumor clones expressing the membrane-bound form of IL-9 (MB-IL-9) and investigated their influences on immune system. MB-IL-9 tumor clones showed reduced tumorigenicity but shortened survival accompanied with severe body weight loss in mice. MB-IL-9 expression on tumor cells had no effect on cell proliferation or major histocompatibility complex class I expression *in vitro*. MB-IL-9 tumor clones were effective in amplifying CD4<sup>+</sup> and CD8<sup>+</sup> T cells and increasing cytotoxic activity against CT26 cells *in vivo*. We also observed a prominent reduction in body weights and survival period of mice injected intraperitoneally with MB-IL-9 clones compared with control groups. Ratios of IL-17 to interferon (IFN)- $\gamma$  in serum level and tumor mass were higher in mice implanted with MB-IL-9 tumor clones than those observed in mice implanted with control cells. These results indicate that the ectopic expression of the MB-IL-9 on tumor cells exerts an immune-stimulatory effect with toxicity. To exploit its benefits as a tumor vaccine, a strategy to control the toxicity of MB-IL-9 tumor clones should be developed.

**Keywords:** Interleukin-9; Toxicity; Colon carcinoma; Membrane-bound; Cancer vaccine

## INTRODUCTION

IL-9, initially termed as p40 and mast cell growth-enhancing activity (MEA), is recognized as a T cell growth factor III (1,2) and acts as a growth factor for murine T and B cell lymphomas *in vivo* (3). T helper (Th) 9 cell is the main cell population producing IL-9 and transforming growth factor (TGF)- $\beta$ , and IL-4 plays a critical role in Th9 induction (4,5). Aside from Th9, Th17 (6), regulatory T cell (Treg) (7), and mast cells (8) also produce IL-9. PU.1 and interferon regulatory factor 4 (IRF4) transcription factors are critical for the development of Th9 cells (9,10).

The role of IL-9 has been explored in immune responses against cancer, but the results are still controversial. Several reports have described the antitumor effect of IL-9 (11-18). Lower expression of IL-9 was associated with colon cancer progression (19), and IL-9 directly inhibited human melanoma cell growth by stimulating apoptotic signal molecules (14) or increasing cytokine production and levels of granzyme/perforin in double-positive T

lymphocytes (13). Studies have reported that Th9 cells exert antitumor effects (12,16,20) via mast cell activation (21-23), prolongation of CD4<sup>+</sup>CD8<sup>+</sup> double-positive T cells (13), and activation of cytotoxic T lymphocytes (CTLs) (11,16). In CT26 colon carcinoma model, IL-9 induces conversion of Treg to Th9 for the inhibition of tumor growth (17), while it is known to play a permissive role for tumor growth environment (24).

The controversial function of IL-9 against cancer was culminated by the findings that IL-9 was characterized as a tumor growth factor in T and B cell lymphomas (3,25) and that IL-9 was a negative prognostic factor in extranodal natural killer (NK)/T cell-cell lymphoma (26), myeloid malignancy, and Hodgkin's lymphoma (27). IL-9 promotes the development of several hematological human tumors (28,29) and enhances Treg function to prevent immunological memory formation (30).

In present study, to investigate the effect of ectopically expressed membrane-bound form of IL-9 (MB-IL-9) on tumor cells, we expressed IL-9 ectopically in CT26 colon cancer cells as a membrane-bound protein and analyzed its effects on immune responses against tumor. The ectopically expressed membrane-bound IL-9 on tumor cells was expected to function as a costimulatory molecule, especially with T cells that are specific to tumor-associated antigens (TAAs) (31). Our goal is to analyze the effect of the MB-IL-9 expressed in tumor cells on immune system and evaluate its effectiveness as a tumor cell vaccine.

## MATERIALS AND METHODS

### Mice and tumor cell lines

Female BALB/c mice (6- to 8-week old) were obtained from the Korea Research Institute of Chemical Technology (Daejeon, Korea). All animal procedures were approved and guided by the Institutional Animal Care and Use Committee of Chungnam National University (approval number: CNU-01020). The murine colon cancer cell line originated from BALB/c mice, CT26, was propagated and maintained in Roswell Park Memorial Institute (RPMI)-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma, St. Louis, MO, USA) in a humidified 5% CO<sub>2</sub> incubator at 37°C. G418 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was used at 0.2 µg/ml concentration as a selective agent for transfections.

### Antibodies and reagents

Anti-mouse IL-9 polyclonal antibody (504802) and fluorescein isothiocyanate (FITC)-conjugated goat anti-hamster (Armenian) IgG (405502) were purchased from Biolegend (San Diego, CA, USA). FITC-conjugated anti-major histocompatibility complex (MHC) class 1 (ab25056) was procured from Abcam (Cambridge, MA, USA). Horseradish peroxidase-conjugated goat anti-mouse (sc-2005) and goat anti-rabbit (sc-1004) IgG antibodies were obtained from Santa Cruz Biotechnology, Inc. FITC-conjugated CD8 (553031) and phycoerythrin-conjugated CD4 (553730) antibodies were supplied by BD Biosciences (San Jose, CA, USA).

### Plasmid construction and transfection

We used complementary DNA (cDNA) library from mouse splenocytes as a template to amplify IL-9 domain of MB-IL-9. To construct the chimeric pcDNA3.1/Mb-IL-9 plasmids, primers specific for IL-9 and tumor necrosis factor (TNF)- $\alpha$  were designed for PCR of

the respective cDNA fragments. *Bam*HI-IL-9 (375 bp)-*Xho*I PCR fragments were digested and used to replace *Bam*HI-IL-17-*Xho*I fragments in MB-IL-17A expression plasmid (32). Briefly, a TNF- $\alpha$  segment (240 bp) containing cytoplasmic and transmembrane regions as well as several extracellular amino acids was ligated with IL-9 cDNA via a small spacer to generate MB-IL-9. The chimeric DNA fragment was subcloned into pcDNA3.1(+) expression vector. The constructs were confirmed by DNA sequencing (Genotech, Daejeon, Korea). CT26 colon cancer cells were transfected with the construct or empty vector using BioT transfection reagent (Morganville Scientific, Morganville, NJ, USA). After 24 h, the cells were plated in the medium containing G418 (1 mg/ml). Drug-resistant colonies were usually visible 2 wk after transfection.

### RT-PCR

To analyze the mRNA level of specific gene, total RNA was isolated with Hybrid-R kit (GeneAll Biotechnology Co., Ltd., Seoul, Korea). RT-PCR reaction was performed using Maxime RT Premix kit (iNtRON Biotechnology, Sungnam, Korea). The semi-quantitative PCR analysis was performed using HSTag premix (GeneAll Biotechnology Co., Ltd.) on a DNA thermal Cycler (Bio-Rad, Hercules, CA, USA). Following primers specific for MB-IL-9 (forward 5' CGCGAATTCAGAGATGCAGCACCCACATGG 3', reverse 5' GCGCTCGAGTCATGGTCGGCTTTTCTGCCT 3'), TNF- $\alpha$  (forward 5' TCAAGCTTATGAGCACAGAAAGC 3', reverse 5' ATTGGATCCCTCCGGCCATAGA 3'), and beta-actin (forward 5' TCACCCACACTGTGCCCATCTACG 3', reverse 5' CATCGGAACCGCTCGTTGCCAATA 3') were used in this study.

### ELISA for IL-9

We used ELISA kit to measure the concentration of IL-9 in culture supernatants and cell pellet. One million cells were incubated in 1 ml of culture medium in 24-well culture plates at 37°C for 24 h. IL-9 level in culture supernatants and cell-associated was measured with an enzyme-amplified sensitive immunoassay kit (KOMA Biotech Inc., Seoul, Korea). To analyze concentration of IL-4, IL-12, IL-17, and interferon (IFN)- $\gamma$  in serum and tumor mass, the suitable ELISA kits were purchased from KOMA Biotech Inc.

### Flow cytometry analysis

Cells were incubated with a primary antibody diluted in a staining buffer (1 $\times$  PBS containing 0.02% sodium azide and 2% FBS) for 1 h at 4°C. After washing off any unbound antibody with the staining buffer, the cells were stained with fluorophore-conjugated secondary antibody for 45 min at 4°C in the dark. After further washing, stained cells were analyzed with a flow cytometer (FACSCalibur; BD Biosciences).

### In vitro cell proliferation assay

Briefly, 1 $\times$ 10<sup>4</sup> cells were plated in 96-well plates. The cells were cultured for 72 h and their proliferation determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (DyneBio, Seoul, Korea).

### Tumor challenge

Syngeneic BALB/c mice (n=5) were challenged with MB-IL-9 tumor clones. Before implantation into mice, the tumor cells were washed twice with PBS and resuspended in PBS. Cells at various concentrations (1 $\times$ 10<sup>6</sup>, 0.5 $\times$ 10<sup>6</sup>, and 0.1 $\times$ 10<sup>6</sup>) in 100  $\mu$ l PBS were subcutaneously (s.c.) injected into the right flank of mice with 1 ml disposable syringe. Tumor size was measured with calipers daily and tumor volume calculated according to the

following formula:  $0.52 \times S^2 \times L$ , where L is the length and S is the width of the tumor. Body weight was also monitored daily. As controls, BALB/c mice (n=5) were challenged with CT26 clone cells transfected with either wild-type or mock vector. At the end of the experiment, the animals were sacrificed in a CO<sub>2</sub>-containing chamber and tumor mass was removed and cultured for further analysis.

BALB/c mice (n=5) were also challenged with one million cells of MB-IL-9 tumor clones intraperitoneally (i.p.) to analyze toxicity. On day 12 after i.p. inoculation, mice from each group were randomly selected and sacrificed by cervical dislocation. To estimate the toxicity of membrane IL-9, their body weights were monitored.

### Mixed cell culture of spleen cells and mitomycin-C (MMC)-inactivated MB-IL-9 tumor clones

Tumor cells were inactivated by treatment with 50 µg/ml MMC (Roche, Indianapolis, IN, USA) for 4 h in a 24-well plate. The cells were extensively washed to remove MMC (thrice in PBS) before next experiment. To prepare spleen cells for the mixed cell culture with MB-IL-9 tumor clones, mice were pre-immunized with MMC-treated parent CT26 cells. Two weeks after the injection, mice were sacrificed to collect spleens. Spleen cell suspension was prepared by grounding spleen in a 70 µm cell strainer (Corning, Corning, NY, USA), followed by treatment with red blood cell lysing buffer (Sigma). The cells were washed in PBS and re-suspended in complete RPMI. To set up a mixed cell culture of splenocytes and tumor cell clones,  $5 \times 10^6$  splenocytes were added to MMC-inactivated tumor clones. At 56 h, wells of the plate were photographed under a microscope. Splenocytes were harvested and assayed for the effector function of CTL.

### Cytotoxicity assay

Ten million parental CT26 cells were incubated in 2.5 µM CFSE containing Dulbecco's phosphate-buffered saline for 20 min in dark. After incubation, the excess of CFSE was washed off by centrifugation of the culture medium (containing 10% FBS) twice. For CTL assay, effector cells and CFSE-labeled target cells were mixed at different ratios (50:1 and 5:1). After 4 h, all cells were harvested and treated with propidium iodide (PI) before analysis with BD FACSCanto machine. CFSE<sup>+</sup> and PI<sup>+</sup> cells were counted as killed cells by cytotoxic effector cells.

### Statistical analysis

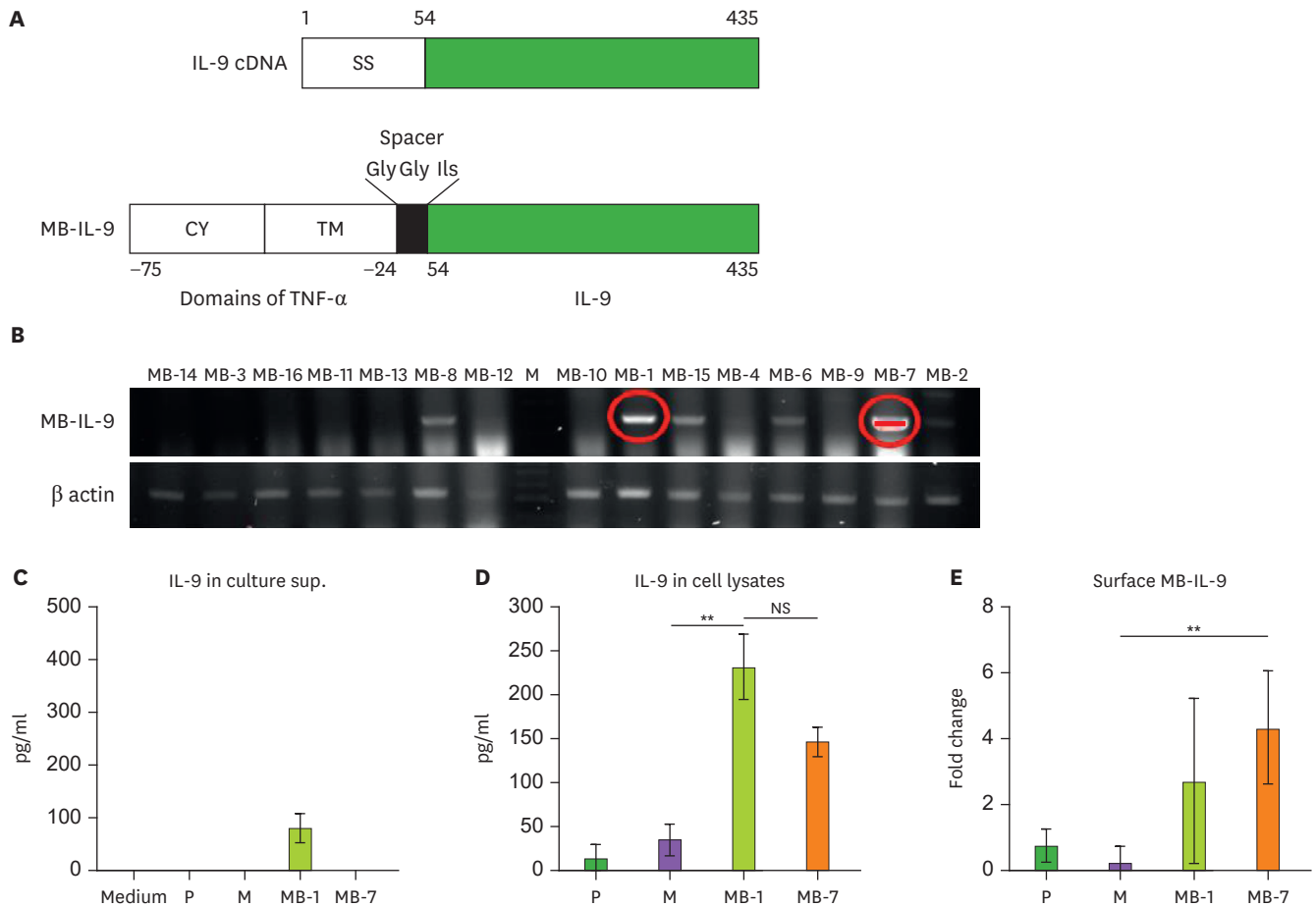
All data were presented as mean ± standard error of means (SEMs, error bars). GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA) was used to analyze 1-way or 2-way ANOVA as indicated in the figure legends. Survival data was analyzed using "Kaplan-Meier Survival Estimates" feature of Origin Pro 8.1 (OriginLab, Northampton, MA, USA).

## RESULTS

### Expression of the MB-IL-9 in CT26 colon cancer cells

To define the role of IL-9 in tumor growth, we adopted murine CT26 colorectal carcinoma cell line as a model system. The cells were transfected with a vector encoding the MB-IL-9. MB-IL-9 protein is a chimeric form of transmembrane/cytoplasmic domains of TNF-α and complete functional IL-9 (from 26 to 158 amino acids) (**Fig. 1A**). Tumor necrosis factor-α-

Antitumor Effect of IL-9



**Figure 1.** Generation of CT26 tumor clones expressing the membrane-bound form of IL-9. (A) The chimeric MB-IL-9 cDNA comprised the cDNA encoding the CY (from -75 to -45) and TM (from -44 to -24) of murine TNF- $\alpha$  and IL-9 cDNA without signal sequence (54-435). A spacer sequence (Gly-Gly-IIs) was inserted between TNF- $\alpha$  and IL-9. (B) MB-IL-9 transfectants were analyzed for IL-9 expression using RT-PCR. Two positive clones of the membrane-bound form (MB-1 and MB-7 clones) were chosen for further experiments. One million cells of MB-IL-9 tumor clones were cultured for 24 h and culture supernatants (C) and cells (D) were separated to quantitate MB-IL-9 protein expression by ELISA. A representative result was presented from more than 3 independent experiments. (E) The expression of IL-9 on cell surface was analyzed by flow cytometry analysis using polyclonal anti-IL-9 antibody. The statistical significance of the differences between mock-vector group vs. MB-IL-9 groups were analyzed by 1-way ANOVA. CY, cytoplasmic domain; TM, transmembrane domain; SS, signal sequence; Medium, complete RPMI medium; P, wild-type CT26; M, mock vector-transfected clone; NS, not significant. \*\*p<0.01.

converting enzyme (TACE) cutting site of the cytoplasmic region of TNF- $\alpha$  was excluded to create a stable membrane-bound form of the chimeric protein (33). Stably transfected clones were selected in G418-containing medium and the mRNA expression of MB-IL-9 was analyzed by RT-PCR (Fig. 1B). Two clones of MB-IL-9 (MB-1 and MB-7) were chosen for further analysis. The wild-type CT26 cells failed to express endogenous IL-9, as evident from RT-PCR analysis (Fig. 1B). As a control, a mock vector-transfected clone was generated.

To quantitate the amount of MB-IL-9 produced in the clones, culture supernatants or cell lysates of MB-IL-9 tumor clones were analyzed by ELISA. High levels of IL-9 were detected in the cell lysates of MB-IL-9 clones (MB-1 and MB-7) but not in culture supernatants (Fig. 1C and D). The expression of the MB-IL-9 was also detected by flow cytometry (Fig. 1E). No significant difference was observed in the expression of L-d MHC class I molecule and growth rate by MTT assay among clones *in vitro* (data not shown).

### Expression of IL-9 induced reduction in tumorigenicity *in vivo*

To analyze whether the expression of the MB-IL-9 in tumor clones affects the tumorigenicity of cancer cells, MB-IL-9 tumor clones were s.c. implanted into syngeneic BALB/c mice. Each of 5 mice per group was challenged with 1, 0.5, or 0.1 million clonal cells per mouse and tumor growth was monitored daily for months. Approximately 2 weeks later, tumor growth was visible in every mouse injected with MB-IL-9 clones as well as in mice injected with mock vector-transfected clone or wild-type CT26 cells. However, the growth of tumor formed by MB-IL-9 tumor clones was retarded as compared with that of tumor from wild-type cells or mock vector-transfected clone (**Fig. 2A**). The size of the tumor mass formed by MB-IL-9 clones was apparently smaller than that formed by control cells (**Fig. 2B**). The mice injected with one million MB-IL-9 cells showed a shorter survival period than control groups (**Fig. 2C**). When we lowered the dosage of MB-IL-9 clone to 0.1 million cells, the tumorigenicity of MB-1 clone was reduced as in other dosages, but the survival period increased. These results imply that MB-IL-9 expression exhibits certain toxicity to mice at higher dose. The tumor mass cells isolated at 30 days after injection retained the expression of MB-IL-9 gene, as observed by RT-PCR analysis (data not shown). To examine the toxic effect of MB-IL-9, the body weight was regularly measured after the implantation of MB-IL-9 clone (0.5 million cells per mouse) (**Fig. 2D**). As expected, the groups implanted with MB-1 or MB-7 clone showed drastic reduction in their body weights as compared with control groups, suggesting that the reduced tumorigenicity of MB-IL-9-expressing tumor clones may result from the strong immune-stimulatory effects accompanied by potential toxicity.

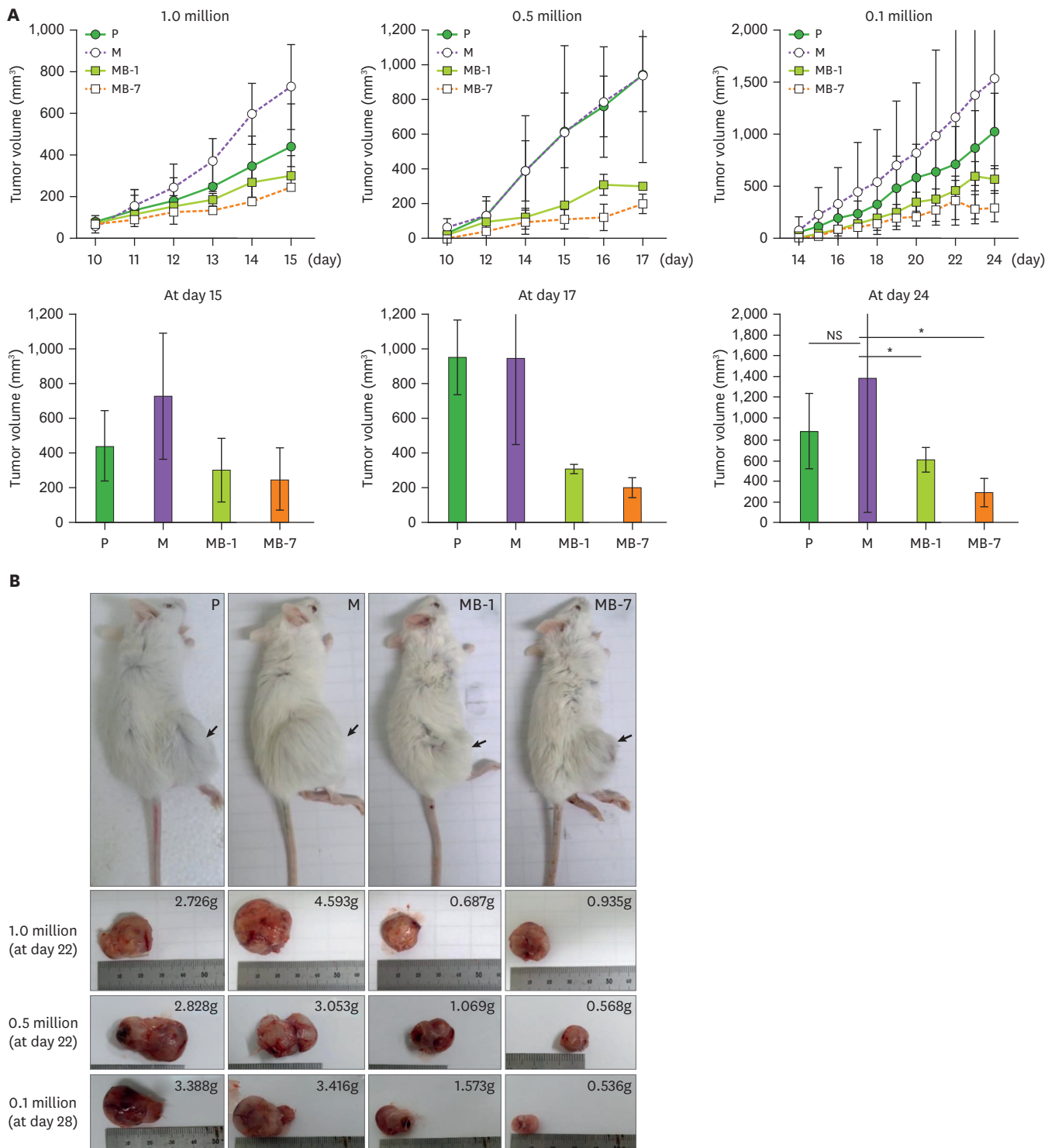
### Spleen cells in MB-IL-9 tumor bearers showed increased population of CD8<sup>+</sup> T lymphocytes

As no difference was observed in the growth rate between MB-IL-9 clones and control cells *in vitro*, the retarded tumor growth *in vivo* was thought to be caused by the stimulation with effector cells against tumor cells. To analyze the immune-stimulatory effect of MB-IL-9 tumor clones, splenocytes from tumor-bearing mice were isolated and stained for the expression CD4 and CD8 molecules. T lymphocyte populations of both CD4<sup>+</sup> and CD8<sup>+</sup> cells increased in mice bearing MB-IL-9 tumor clone, especially in case of MB-1 clone, as compared with mock-vector control mice (**Fig. 3A and B**). These results suggest that the increase in CD4<sup>+</sup> or CD8<sup>+</sup> population was related to the reduced tumorigenicity of MB-IL-9 tumor clones.

### Immune-stimulatory effect of MB-IL-9 tumor clone was superior to that of wild-type CT26 cells or mock vector-transfected clone

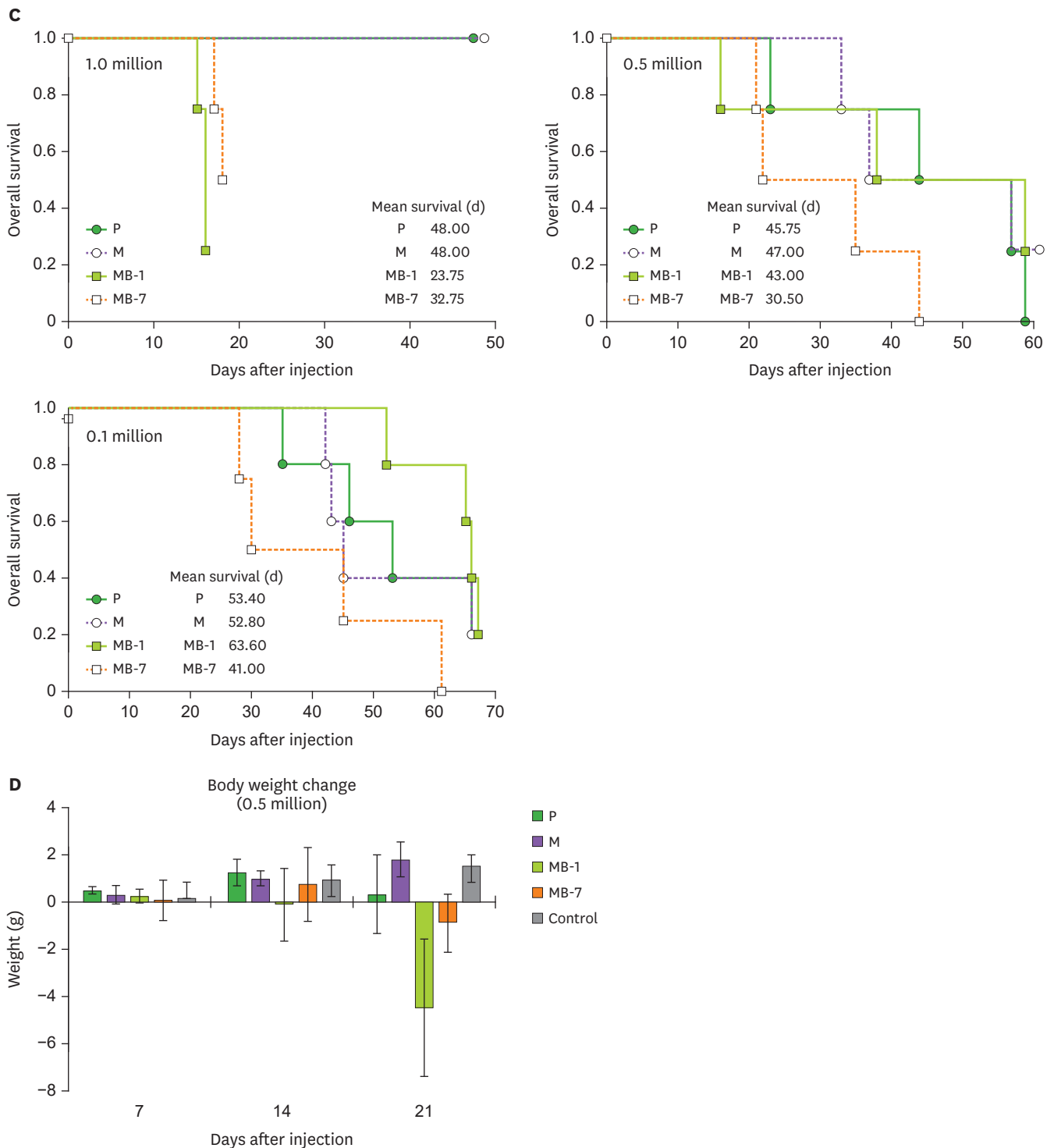
To analyze the cytotoxic stimulatory function of MB-IL-9 clones, mice were pre-immunized with an s.c. injection of MMC-inactivated CT26 cells. After 2 wk, splenocytes were isolated and added on MMC-inactivated MB-IL-9 tumor clones. As shown in **Fig. 4A**, spleen cells cultured with inactivated MB-IL-9 clones behaved differently from those cultured with control cells and exhibited a stimulated pattern, forming lumps 1 day after the co-culture, suggestive of the stimulatory effect of MB-IL-9 clones. At 3 days after co-culture, MMC-inactivated MB-IL-9 clone cells were severely disintegrated and showed dead cell phenotype as compared with the control cells. To analyze the cytotoxic effect of MB-IL-9-activated spleen cells against CT26 tumor cells, *in vitro*-activated splenocytes were harvested after 56 h and CTL assay was performed against CFSE-stained CT26 cells. CFSE- and PI-positive cells increased among spleen cells stimulated with MB-IL-9 clones as compared with those stimulated with control groups (**Fig. 4B**), indicating that MB-IL-9 clones exhibit immune-stimulatory effect.

Antitumor Effect of IL-9



**Figure 2.** Tumor growth and survival of mice implanted with MB-IL-9 tumor clones. (A) BALB/c mice (n=5) were s.c. injected with indicated number (1, 0.5, or 0.1 million) of MB-IL-9 tumor clones (MB-1 and MB-7 clones) and tumor growth monitored. Comparable tumor volume measured at the indicated dates after injection was presented. The statistical significance of the differences between mock-vector group vs. MB-IL-9 groups were analyzed by 1-way ANOVA. (B) One of mice from each group was randomly chosen and sacrificed at indicated dates to visualize tumor mass. The weight of isolated tumor mass was also measured. (C) Survival of mice in each experiment was estimated. Mean survival (days) was indicated insets. Two independent survival experiments were repeated in case of 0.5 and 0.1 million cell injections. One of representative results was presented. (D) Body weights of mice injected with 0.5 million cells of MB-IL-9 tumor clones were simultaneously measured. A group of mice (n=5) with same age was used as control for body weight change. P, wild-type CT26; M, mock vector-transfected clone; NS, not significant. \*p<0.05.

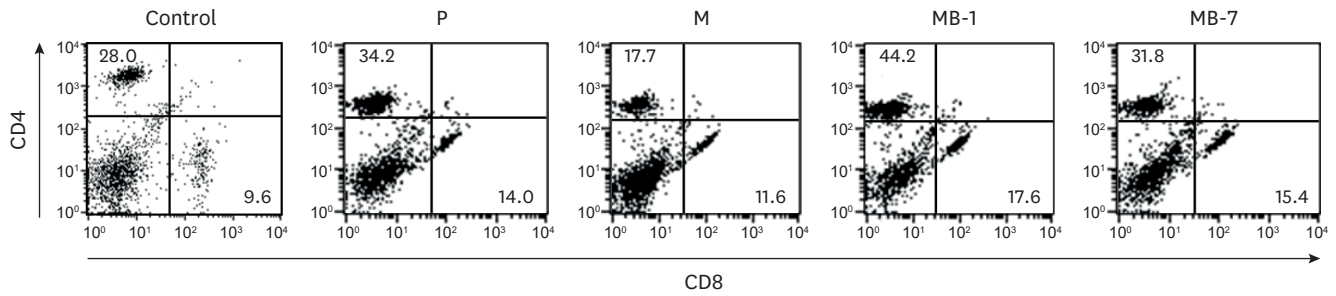
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**Figure 2.** (Continued) Tumor growth and survival of mice implanted with MB-IL-9 tumor clones. (A) BALB/c mice (n=5) were s.c. injected with indicated number (1, 0.5, or 0.1 million) of MB-IL-9 tumor clones (MB-1 and MB-7 clones) and tumor growth monitored. Comparable tumor volume measured at the indicated dates after injection was presented. The statistical significance of the differences between mock-vector group vs. MB-IL-9 groups were analyzed by 1-way ANOVA. (B) One of mice from each group was randomly chosen and sacrificed at indicated dates to visualize tumor mass. The weight of isolated tumor mass was also measured. (C) Survival of mice in each experiment was estimated. Mean survival (days) was indicated insets. Two independent survival experiments were repeated in case of 0.5 and 0.1 million cell injections. One of representative results was presented. (D) Body weights of mice injected with 0.5 million cells of MB-IL-9 tumor clones were simultaneously measured. A group of mice (n=5) with same age was used as control for body weight change. P, wild-type CT26; M, mock vector-transfected clone; NS; not significant. \*p<0.05.



**A** Splenocytes



**B** Relative population of T cell subsets

T cells subset	P	M	MB-1	MB-7
% CD4	33.45±1.06 <sup>a</sup>	16.4±1.83	48.10±5.52 <sup>***b</sup>	32.45±0.92 <sup>***</sup>
% CD8	14.90±1.27	9.50±2.97	17.80±0.28 <sup>**</sup>	15.55±0.21 <sup>**</sup>

**Figure 3.** Effects of MB-IL-9 tumor clones on T lymphocytes subsets in spleens. (A) Two mice per group were implanted with MB-IL-9 tumor clones or control cells (1 million cells), and one of mice per group was sacrificed at day 15 to analyze CD4<sup>+</sup> and CD8<sup>+</sup> T cell subsets. (B) The other mouse of each group was also sacrificed at day 17 for the same analysis, and average proportions of T cell subsets were presented. P, wild-type CT26; M, mock vector-transfected clone. The p-values were \*p<0.05, \*\*p<0.01, \*\*\*p<0.001. Difference alphabets mean follow as; <sup>a</sup>average±standard deviation from tumor bearing mice, <sup>b</sup>statistical significance was assessed by 1-way ANOVA to evaluate differences in T lymphocytes between MB-IL-9 groups and mock controls.

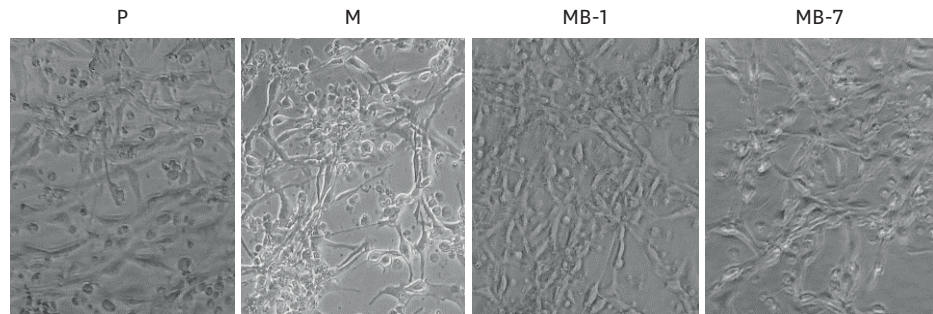
**Reduced survival of mice carrying MB-IL-9 tumor clones may be caused by certain toxicity**

Despite the strong immune-stimulatory effects of MB-IL-9 tumor clones, these clones failed to show advantages with respect to survival, suggestive of the associated toxicity. To confirm the *in vivo* toxicity of MB-IL-9 clones, mice were i.p. injected with one million MB-IL-9 clone cells and their body weights and survival monitored. As expected, from the early days, we observed a prominent reduction in body weights of mice injected with MB-IL-9 clones compared with control groups (Fig. 5A). Consistent with these observations, mice implanted with MB-IL-9 clones showed a shorter survival period as compared with control groups (Fig. 5B). The abdominal cavities of mice injected with wild-type CT26 cells or mock vector-transfected clone showed several growing tumor lumps, whereas no apparent tumor lumps were visible in mice injected with MB-IL-9 clones (Fig. 5C). These results suggest that the shortened survival of mice from MB-IL-9 group is related to the toxicity caused by severe immune-stimulatory effects of MB-IL-9.

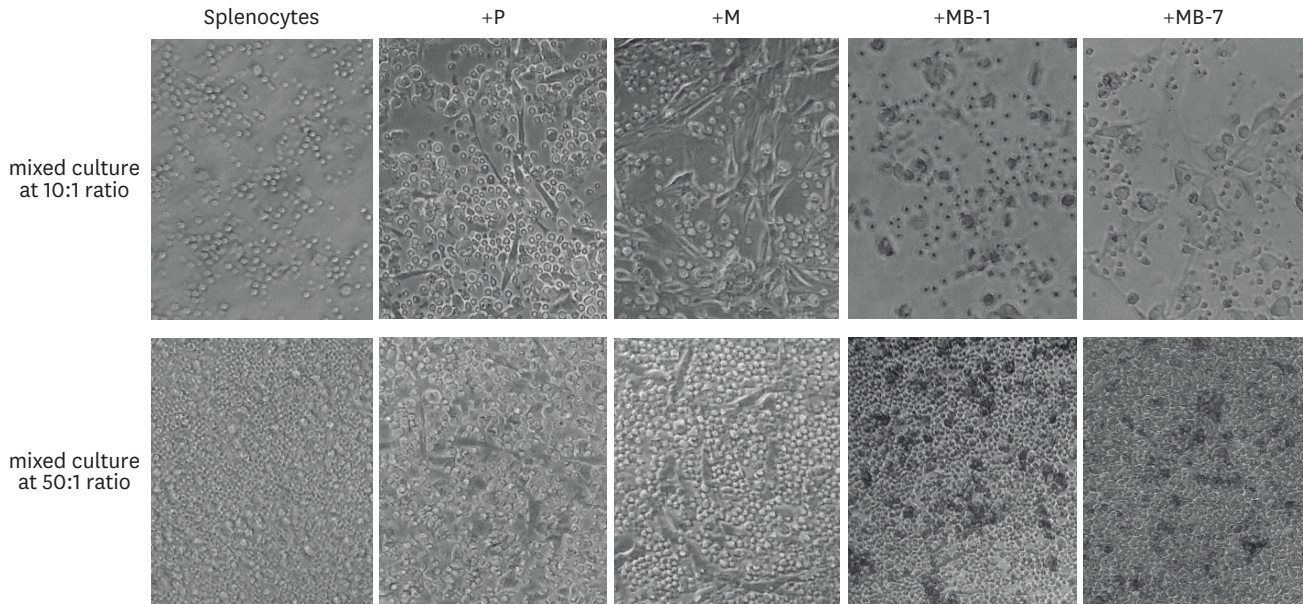
A positive relationship has been reported between serum cytokine levels and their disease outcome in colorectal cancer (34) and inflammation or autoimmune disease setting by the ratio of Th1 (IFN-γ)/Th17 (IL-17) (35,36). To analyze the causes of toxicity, mice were s.c. implanted with 0.5 million cells of MB-IL-9 clones and eye-bled to measure serum concentrations of various cytokines (IL-9, IL-12, IFN-γ, IL-17, and IL-4) with ELISA at 21 days after tumor challenge (Fig. 6A). There was no difference in IL-12 and IL-4, but there were significant increase in IL-17 and decrease in IFN-γ. Similar pattern of cytokine production was observed in the internal milieu of tumor mass (Fig. 6B). Several lines of evidence have suggested that the balance of IL-17 and IFN-γ defines the shift from inflammation to autoimmune response. We observed an increase in IL-17A/IFN-γ ratio in both serum and tumor mass from mice implanted with MB-IL-9 clones (Fig. 6C). Comparison of IL-17A/IFN-γ ratio and survival of mice revealed that a significant increase in the ratio of IL-17 to IFN-γ was in relation to survival of tumor bearing mice.

**A** Splenocytes stimulation

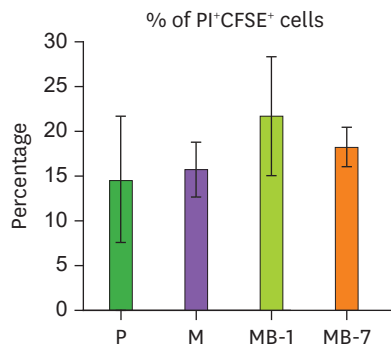
Stimulators only



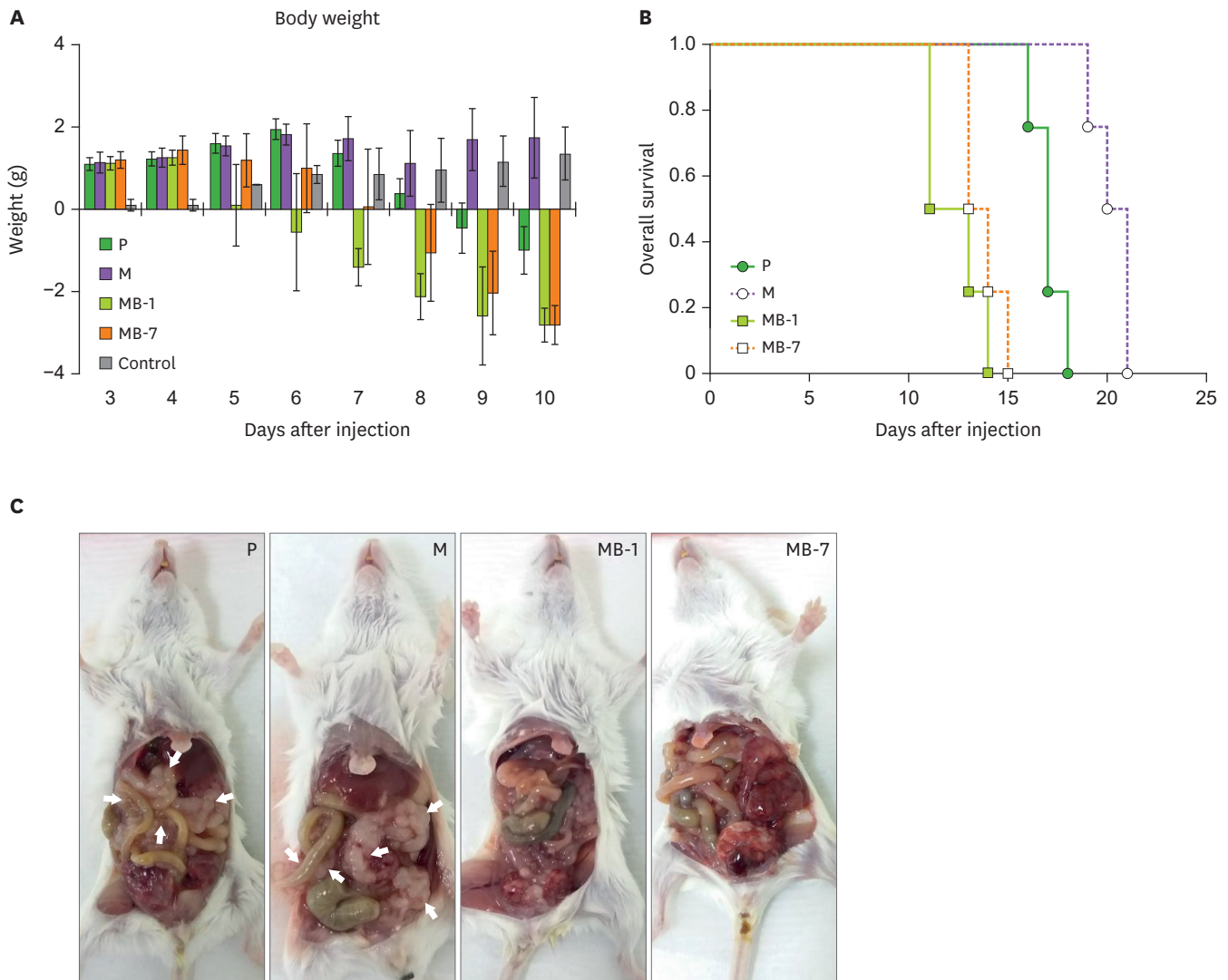
Spleen cells: stimulators



**B** Cytotoxicity assay



**Figure 4.** Cytotoxic activity of spleen cells immunized with MB-IL-9 tumor clones. (A) Splenocytes were isolated from mice pre-immunized with MMC-inactivated CT26 cells and stimulated *in vitro* for 56 h with MMC-inactivated MB-IL-9 clones at different ratios (spleen cells: stimulators=10:1 or 50:1). The morphology of cells in the mixed cell culture was recorded under a microscope. A representative result was shown from 3 independent experiments. (B) Spleen cells activated *in vivo* and *in vitro* were assayed for cytotoxic activity against wild-type CT26 cells. The activated spleen cells were harvested and incubated for 3.5 h with CFSE-stained wild-type CT26 cells at 3 different E:T ratios (1:1, 5:1, 50:1) in triplicate, followed by their treatment with PI before flow cytometry analysis. The ratio of PI<sup>+</sup> cells among CFSE<sup>+</sup> population was estimated cytotoxic activity of splenocytes. A representative result at 50:1 ratio was presented from 2 independent experiments. The p-value between M and MB-1 or MB-7 was less than 0.07 or 0.08, respectively. P, wild-type CT26; M, mock vector-transfected clone.



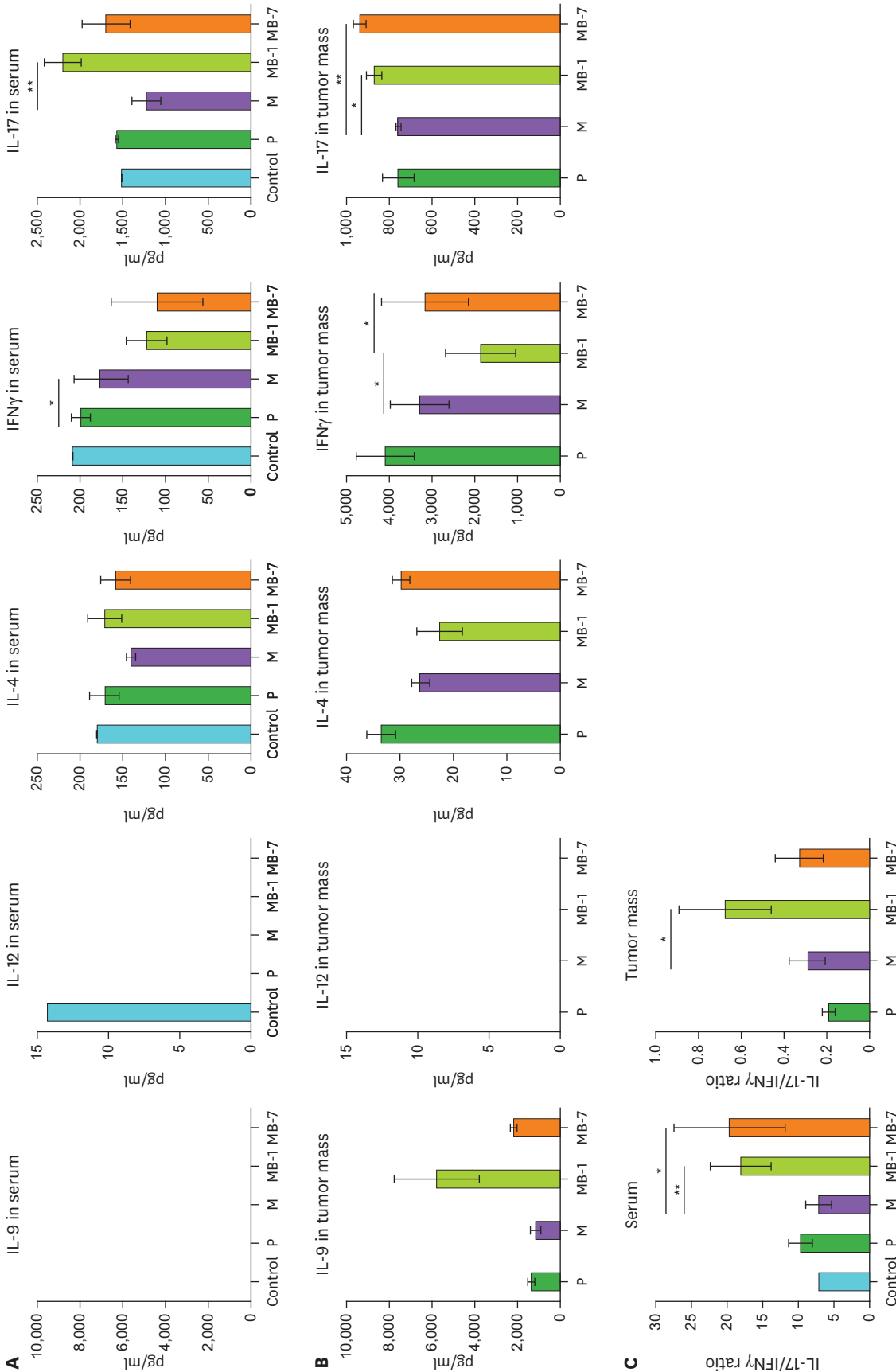
**Figure 5.** Toxicity of MB-IL-9 tumor clones *in vivo*. BALB/c mice (n=5) were i.p. injected with one million MB-IL-9 tumor clones. Body weights (A) and survival (B) of mice were monitored daily. (C) At day 12, one of mice from each group was randomly chosen to sacrifice and the presence of lumps of tumor cells in the abdominal cavity examined. Arrows indicate the lumps of tumor cells. P, wild-type CT26; M, mock vector-transfected clone.

Taken together, the ectopic expression of IL-9 on CT26 colon cancer cells as a membrane-bound protein caused an immune-stimulatory effect, which was accompanied with toxicity. These results suggest the possibility that MB-IL-9 tumor clones may serve as a tool to achieve therapeutic benefit by lowering dose or pre-inactivating the tumor cell vaccine, owing to their strong immune-stimulatory effects.

## DISCUSSION

The expression of cytokine on tumor cells in a membrane-bound form has been used as a strategy to minimize the toxic effect of recombinant cytokines encountered in cancer therapy (31). The membrane-bound form of cytokine may provide signal 2, which is required for the complete activation of lymphocytes. In this study, we evaluate the effect of the

Antitumor Effect of IL-9



**Figure 6.** Levels of cytokines in the serum and tumor mass. At day 21 after the s.c. injection of 0.5 million MB-IL-9 clones per mouse, blood was collected from the eye artery (A) and tumor mass isolated (B) to quantitate various cytokines. To analyze cytokines in tumor mass, 0.5 g of tumor mass from each group was lysed using a lysing buffer. The IL-17/IFN- $\gamma$  ratio was calculated and presented at the lowest panel (C). Cytokines were quantitated by ELISA. The statistical significance of the differences between mock-vector group vs. MB-IL-9 groups were analyzed by 1-way ANOVA. Control; tumor free mice, P; wild-type CT26, M; mock vector-transfected clone. \*p<0.05, \*\*p<0.01.

MB-IL-9 expressed on tumor cells on the immune system to analyze its effectiveness as a tumor cell vaccine. Tumor cell clones engineered to express the MB-IL-9 exhibited reduced tumorigenicity, suggestive of their strong immune-stimulatory effect. However, this effect was accompanied with toxicity, at least at the doses we analyzed.

IL-9 was previously identified as a T cell growth factor. We found amplification of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes in spleens of mice implanted with MB-IL-9 tumor clones. MB-IL-9 tumor clones were effective in stimulating spleen cells from pre-immunized mice; hence, these cells exhibited higher cytotoxic activity against CT26 cells *in vitro*. Functionally, MB-IL-9 tumor clones were effective to activate tumor-specific CTLs *in vitro*. It was reported that Th9 cells induce a strong antitumor CD8<sup>+</sup> CTL activity in pulmonary melanoma model in mice, wherein Th9 cells promoted the survival of dendritic cells (16). In addition, GITR co-stimulation of Th9 cells enhanced CTL induction against melanoma cells (12), suggesting the critical role of dendritic cells in the stimulation of Th9 cells. Another study with melanoma showed that recombinant IL-9 inhibited melanoma tumor growth *in vivo*, and the presence of mast cells was critical for the antitumor effect of IL-9 (11). Thus, the adoptive transfer of CD8<sup>+</sup> IL-9-producing T cells specific to melanoma results in tumor therapeutic effect (15). In CT26 colon cancer model, the cell extract of CT26 cells mixed with adjuvants showed therapeutic effects against CT26 cancer, accompanied with Th9 cell increment in tumor mass (17). In contrast, CT26 colon cancer cells failed to form tumors in IL-9 knockout mice or those treated with IL-9-neutralizing antibody, suggestive of the immune-suppressive function of IL-9 in the tumor model (24). The causes of the opposite results remain elusive.

In our tumor vaccine model with CT26, MB-IL-9 tumor clone itself may act as an antigen-presenting clone, and the MB-IL-9 on tumor cells may substitute the function of Th9. Moreover, only TAA-specific T lymphocytes would be selectively activated because both signal 1 and signal 2 would be supplied at the same time during the interaction of TAA-specific T lymphocytes with MB-IL-9 tumor cells. Despite these assumptions, MB-IL-9 tumor clones displayed unexpected high toxicity, which was apparent in the i.p. implanted experimental setting. In other studies using membrane-bound form of IL-2, IL-4, and IL-12, we failed to observe such toxicity (31,32,37-40). Furthermore, serum levels of IFN- $\gamma$  and IL-17A in mice implanted with MB-IL-9 tumor clones were comparatively different from those observed in mice implanted with control cells. An increment in IL-17A concentration relative to IFN- $\gamma$  was observed both in the serum and in tumor mass of mice implanted with MB-IL-9, but the phenomenon needs further analysis. We propose that the high level of MB-IL-9 on tumor cells may overwhelm normal physiological conditions, or cell adhesion molecules may increase the avidity of cell-cell interaction between T lymphocytes and MB-IL-9 tumor clones beyond the normal physiological level. In fact, we could detect several thousand picogram per milliliter of IL-9 in tumor mass, which is at least thousand times higher than its physiological concentration (Fig. 6) (11). The causes of toxicity need to be further analyzed.

In conclusion, MB-IL-9 molecules on tumor cells play a role in immune-stimulatory function, but MB-IL-9 tumor clones displayed a certain level of systemic toxicity. These findings raise the possibility that MB-IL-9 tumor clones may provide benefits as a tumor cell vaccine if a strategy to minimize the toxicity of MB-IL-9 is developed. With the lower dose of MB-IL-9 tumor clones (0.1 million cells, s.c. injection), an extended survival of mice was observed (Fig. 2C), suggesting that the dosage control may overcome the toxicity problem. Alternatively, the adoption of inactivated MB-IL-9 tumor clones as therapeutic agents would be another choice to obviate the toxicity problem.

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