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Enhancement of antitumor effect by peptide vaccine therapy in combination with anti-CD4 antibody: Study in a murine model

Norihiro Fujinami [a,b], Toshiaki Yoshikawa [a], Yu Sawada [a,c], Manami Shimomura [a], Tatsuaki Iwama [a], Shiori Sugai [a,b], Shigehisa Kitano [a,d], Yasushi Uemura [a], Tetsuya Nakatsura [a,b,*]

[a] Division of Cancer Immunotherapy, Exploratory Oncology Research & Clinical Trial Center National Cancer Center, Kashiwa, Chiba, Japan
[b] Research Institute for Biomedical Sciences, Tokyo University of Science, Japan
[c] Department of Gastroenterological Surgery, Graduate School of Medicine, Yokohama City University, Yokohama, Kanagawa, Japan
[d] Department of Experimental Therapeutics, National Cancer Center Hospital, Tsukiji, Tokyo, Japan

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ABSTRACT

Purpose: The clinical efficacy of cancer peptide vaccine therapy is insufficient. To enhance the anti-tumor effect of peptide vaccine therapy, we combined this therapy with an anti-CD4 mAb (GK1.5), which is known to deplete $CD4^+$ cells, including regulatory T cells (Tregs).

Methods: To determine the treatment schedule, the number of lymphocyte subsets in the peripheral blood of mice was traced by flow cytometry after administration of anti-CD4 mAb. The ovalbumin $(OVA)_{257-264}$ peptide vaccine was injected intradermally and anti-CD4 mAb was administered intraperitoneally into C57BL/6 mice at different schedules. We evaluated the enhancement of OVA peptide-specific cytotoxic T lymphocyte (CTL) induction in the combination therapy using the ELISPOT assay, CD107a assay, and cytokine assay. We then examined the *in vivo* metastasis inhibitory effect by OVA peptide vaccine therapy in combination with anti-CD4 mAb against OVA-expressing thymoma (EG7) in a murine liver metastatic model.

Results: We showed that peptide-specific CTL induction was enhanced by the peptide vaccine in combination with anti-CD4 mAb and that the optimized treatment schedule had the strongest induction effect of peptide-specific CTLs using an IFN-γ ELISPOT assay. We also confirmed that the $CD107a^+$ cells secreted perforin and granzyme B and the amount of IL-2 and TNF produced by these CTLs increased when the peptide vaccine was combined with anti-CD4 mAb. Furthermore, metastasis was inhibited by peptide vaccines in combination with anti-CD4 mAb compared to peptide vaccine alone in a murine liver metastatic model.

Conclusion: The use of anti-CD4 mAb in combination with the OVA peptide vaccine therapy increased the number of peptide-specific CTLs and showed a higher therapeutic effect against OVA-expressing tumors. The combination with anti-CD4 mAb may provide a new cancer vaccine strategy.

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1. Introduction

Cancer peptide vaccine therapy can be used to prolong survival while maintaining the quality of life (QOL) of patients and is expected to prevent recurrence. We previously reported that glypican-3 (GPC3) was a cancer specific antigen [1–3] and identified GPC3-derived peptides capable of inducing peptide-specific cytotoxic T cells (CTLs) [4–6]. A variety of clinical trials of GPC3 peptide vaccine therapy have been performed in hepatocellular carcinoma (HCC) [7–9] and ovarian clear cell carcinoma [10]. We confirmed the safety and immunological efficacy of the vaccine and showed the potential to induce a clinical effect in some patients [7,8,10]. However, the clinical efficacy of cancer peptide vaccine therapy remains insufficient. Therefore, we are attempting to develop effective enhancement methods for peptide vaccine therapy such as intratumoral peptide injection [11] and combination therapy with a

Abbreviations: QOL, quality of life; GPC3, glypican-3; CTL, cytotoxic T lymphocyte; HCC, hepatocellular carcinoma; PD-1, programmed death-1; Treg, regulatory T cell; mAb, monoclonal antibody; TGF-β, transforming growth factor-βl; DC, dendritic cell; OVA, ovalbumin; IFN-γ, interferon-γ; ELISPOT assay, enzyme-linked immunospot assay; MHC, major histocompatibility complex; FITC, fluorescein isothiocyanate; PE, phycoerythrin; FOXP3, forkhead box P3; 7-AAD, 7-amino-actinomycin D; IL-2, interleukine-2; TNF, tumor necrosis factor

* Corresponding author at: Division of Cancer Immunotherapy, Exploratory Oncology Research & Clinical Trial Center, National Cancer Center, 6-5-1 Kashiwanoha, Kashiwa, 277-8577 Chiba, Japan.
E-mail address: tnakatsu@east.ncc.go.jp (T. Nakatsura).

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peptide vaccine and *anti*-programmed death-1 blocking antibody (alphaPD-1 Ab) [12]. Moreover, we believe that it is necessary to develop potent enhancement methods for peptide vaccine therapy.

Several groups have suggested that depletion of $CD4^+$ cells results in strong antitumor effects in tumor-bearing mouse models because of the enhancement of CTL responses. den Boer et al. reported that depletion of $CD4^+$ cells results remarkably increased the number and systemic spread of tumor-specific $CD8^+$ T cells as well as tumor eradication and enhanced survival [13]. Chamoto et al. indicated that combination treatment of carcinoma-bearing mice with regulatory T cell (Treg)-depletion therapy and a Treg-recovery blockade using anti-CD25 monoclonal antibody (mAb) and anti-transforming growth factor-β (anti-TGF-β) mAb was an efficient strategy for inducing strong antitumor immunity and complete rejection of the tumor [14]. Ueha et al. determined that combination treatment with anti-CD4 mAb and immune checkpoint mAbs, particularly anti-PD-1 or anti-PD-L1 mAbs, synergistically suppressed tumor growth and greatly prolonged survival [15].

To enhance the anti-tumor effect of peptide vaccine therapy, we included an anti-CD4 mAb (GK1.5). First, we tracked the changes in the number of each lymphocyte subset in mice peripheral blood by flow cytometry after administration of anti-CD4 mAb in order to determine the best treatment schedule. The enhancement of ovalbumin (OVA) peptide-specific cytotoxic T lymphocyte (CTL) induction in the combination therapy was confirmed using an interferon-γ (IFN-γ) enzyme-linked immunospot assay (ELISPOT assay), CD107a assay, and cytokine assay. Finally, using a murine liver metastatic model, we observed the burden of metastasis was suppressed by the peptide vaccines in combination with anti-CD4 mAb.

2. Materials and method

2.1. Mice

C57BL/6 mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). Mice were maintained under the institutional guidelines set by the Animal Research committee of the National Cancer Center Hospital East. Mice were housed under specific-pathogen-free conditions with a 12-h light cycle and food and water *ad libitum*. Six-to-eight-week-old mice were used in all experiments.

2.2. Cell line

RMA-S cell lines, which have H2-K^b and -D^b as major histocompatibility complex (MHC) class I epitopes, were maintained in our laboratory. RMA-S is an antigen processing-defective cell line; the cells cannot present endogenous antigens with MHC class I epitopes [16]. EL4 and EG7 were maintained in our laboratory. EG7 expresses OVA protein as a model tumor antigen because the full-length OVA gene had been transformed into EL4. EL4 was used as a control target of EG7. B16 and MO4 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer Tohoku University (Sendai, Japan). MO4 expressed OVA protein as a model tumor antigen. B16 was used as a control target of MO4. These cells were maintained *in vitro* in RPMI 1640 supplemented with 10% fetal bovine serum.

2.3. Murine blood sampling

From the tip of the tail vein, 100 μL peripheral blood was collected. The samples were analyzed using a flow cytometer after hemolysis.

2.4. Monoclonal antibodies and chemical reagents

The following mAbs were purchased from BioLegend (San Diego, CA, USA): fluorescein isothiocyanate (FITC)-conjugated Armenian hamster anti-mouse CD3ε mAb (145-2C11), phycoerythrin (PE)-labeled rat anti-mouse CD8α mAb (53-6.7), PE/Cy7-labeled rat anti-mouse CD4 mAb (RM4-5), APC-labeled rat anti-mouse CD25 mAb (3C7), APC-labeled rat anti-mouse CD107a (LAMP-1) mAb (1D4B), APC-labeled rat IgG2a, κ isotype control, APC-labeled rat IgG2b, κ isotype control, APC/Cy7-labeled rat anti-mouse CD45 mAb (30-F11), and PE anti-mouse/rat/human forkhead box P3 (FOXP3) flow kit. Additionally, V500 Rat anti-Mouse CD44 mAb (IM7) and V450 Rat anti-mouse CD62L mAb (MEL-14) were purchased from BD Biosciences (Franklin Lakes, NJ, USA). 7-amino-actinomycin D (7-AAD) viability dye was purchased from Beckman Coulter Inc., (Brea, CA, USA). FcR blocking reagent, mouse, was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

2.5. Flow Cytometry (FCM)

The FCM data were acquired using the FACSCanto II system (BD Biosciences) and analyzed using the Flow-Jo software (Tree Star, Ashland, OR, USA).

2.6. Administration of monoclonal antibody

GK1.5, a rat anti mouse CD4 mAb and LTF-2, a rat isotype control IgG2b were purchased from Bio X Cell. Mice received intraperitoneal injection of 5 mg/kg GK1.5 or LTF-2 as the isotype control on day 0.

2.7. Detection of $CD4^+$ $CD25^+$ $FOXP3^+$ cells after administration of anti-CD4 mAb in EG7-bearing mice

EG7 was implanted subcutaneously in the right flank of mice. Anti-CD4 mAb was administered intraperitoneally into the EG7-bearing mice. The mice were analyzed using flow cytometry on day 1 after administration of anti-CD4 mAb. PE anti-mouse/rat/human FOXP3 Flow Kit was used for FOXP3 intracellular staining according to the manufacturer's instructions.

2.8. Peptide vaccine

H-2K^b-restricted $OVA_{257-264}$ (SIINFEKL) was purchased from AnaSpec, Inc. (Fremont, CA, USA). OVA peptide vaccine consisted of peptide:7% $NaHCO_3$:incomplete Freund's adjuvant (IFA)=1:9:10. Mice were injected intradermally at the base of the tail with the OVA peptide vaccine.

2.9. IFN-γ ELISPOT assay

Effector cells were cocultured with each cancer cell line as a target cell at the indicated effector/target (E/T) ratio. AN IFN-g ELISPOT assay was carried out as previously described [17].

2.10. CD107a assay

Effector cells were isolated using mouse CD8 microbeads (Miltenyi Biotec) from peripheral blood. The $CD8^+$ cells were incubated with cancer cell lines for 3.5 h at 37 °C. APC-conjugated CD107a mAb or isotype control rat IgG2a mAb were incubated in the mixture during the incubation period; after incubation, the cells were stained with additional PE-conjugated anti-CD8 mAb, FITC-conjugated anti-CD3εmAb, and 7-AAD Viability Dye and analyzed using FACSCanto II system and Flow-Jo software.

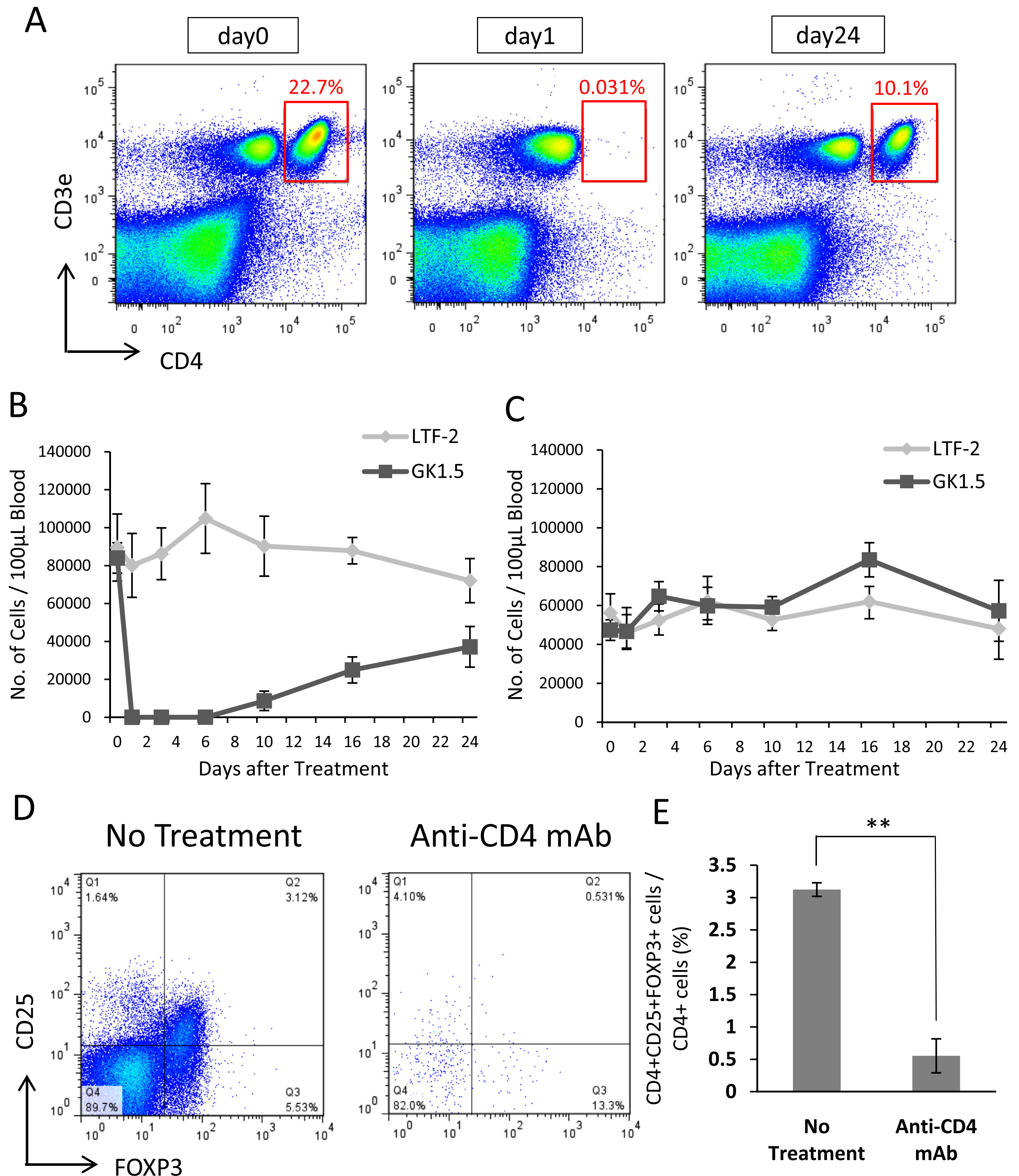

Fig. 1. Number of lymphocyte subsets after administration of anti-CD4 mAb. (A) Representative flow cytometry profiles of $CD4^+$ T cells in peripheral blood on days 0, 1, and 24 after administration of anti-CD4 mAb. (B) Changes in $CD4^+$ T cell counts in the peripheral blood after administration of anti-CD4 mAb (GK1.5) ($n=3$). (C) Changes in $CD8^+$ T cell counts in peripheral blood after administration of anti-CD4 mAb (GK1.5) ($n=3$). (D) Representative flow cytometry profiles of $CD4^+$ $CD25^+$ $FOXP3^+$ cells in splenocytes of EG7-bearing mice on day 1 after administration of anti-CD4 mAb. (E) Statistical analysis of (D) ($n=3$).**$P<0.01$, differences are statistically significant between the two values.

2.11. Cytokine assay

Supernatants of the IFN-γ ELISPOT assay and CD107a assay were collected and interleukine-2 (IL-2) and tumor necrosis factor (TNF) were measured using a Cytometric Bead Array (BD Bioscience) according to the manufacturer's instructions. Samples were analyzed using a FACSCanto II system and the FCAP Array Software 3.0 (BD Bioscience).

2.12. Murine liver metastatic model

A mouse model of liver metastasis was developed by injecting tumor cells into the spleen. We obtained viable single-cell suspensions of subcutaneous tumor cells using a gentleMACS Octo Dissociator with Heaters (Miltenyi Biotec). The single-cell suspensions were injected into the spleen. Mice were anesthetized using isoflurane vaporizer. A small incision was made in the left flank to reveal the spleen. A total of 1×10^6 cells/100 μL of EG7 was injected into the spleen using a 27-gauge needle and the incision was stapled. The spleen weight and the major axis of splenic tumors were estimated as indicators of splenic tumors. The liver weight was estimated as an indicator of hepatic tumors because the number of metastases in the liver could not be counted.

2.13. Immunohistochemical analysis

Immunohistochemical analysis of frozen tissue sections was done as described previously [18] using mAb specific to CD4 (L3T4; BD Pharmingen, San Diego, CA) or CD8(53-6.7; BD Pharmingen). Stained samples were subjected to microscopic analysis on a microscope (EX51 Olympus, Tokyo, Japan).

2.14. Statistical analysis

All data are presented as the mean ± SD. Student's *t* test for comparison of means was used to compare groups. *P* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. Changes in the number of each lymphocyte subset after anti-CD4 mAb administration

To determine the treatment schedule, the number of each lymphocyte subset in the peripheral blood of mice was traced after administration of anti-CD4 mAb using flow cytometry. The subsets of $CD4^+$ T cells were evaluated on day 0, 1, and 24 (Fig. 1A). $CD4^+$ T cell counts were dramatically reduced in mice peripheral blood by the first day after anti-CD4 mAb administration (Fig. 1A and B). Subsequently, $CD4^+$ T cell count gradually increased, recovering to 50% at 24 days after anti-CD4 mAb administration (Fig. 1B). In contrast, $CD8^+$ T cell number did not change after anti-CD4 mAb administration (Fig. 1C). We determined that $CD4^+$ $CD25^+$ $FOXP3^+$ cells disappeared in the splenocytes of EG7-bearing mice on day 1 after anti-CD4 mAb administration. The number of $CD4^+$ $CD25^+$ $FOXP3^+$ cells in spleen of EG7-bearing mice on day 1 after administration of anti-CD4 mAb significantly decreased compared with the untreated group. As $CD4^+$ T cells, including Tregs, had nearly disappeared, we administered the OVA peptide vaccine one day after anti-CD4 mAb administration (Fig. 1D and E).

3.2. Effect of peptide vaccine therapy was enhanced in combination with anti-CD4 mAb

To evaluate the enhancement of OVA peptide-specific CTL induction in the combination therapy, we compared with the schedule I of OVA peptide vaccine alone, the schedule II of combination treatment starting from anti-CD4 mAb administration, and the schedule III of combination treatment starting from OVA peptide vaccination using the IFN-γ ELISPOT assay (Fig. 2A). We then determined that the increase in OVA-specific CTL induction by OVA peptide vaccine in combination with anti-CD4 mAb was higher than that obtained using OVA peptide vaccine alone (Fig. 2B and C). Next, we compared the two combination schedules with anti-CD4 mAb and OVA peptide vaccine (schedules II and III). OVA-specific CTL induction by schedules II and III showed no significant differences. But the mean value of spot number in schedules III was higher than that of spot number in schedules II. From this, we decided to adopt schedules III in subsequent experiments.

3.3. Multi-functionality of peptide-specific CTLs induced by peptide vaccine therapy in combination with anti-CD4 mAb

To determine the multi-function of peptide-specific CTL by combination therapy, we performed a CD107a assay on day 13 and IFN-γ ELISPOT assay on day14 after priming of the OVA peptide vaccine using schedule III of Fig. 2A (Fig. 3A). Perforin and granzyme secretion from $CD107a^+$ cells increased and the production of IL-2 and TNF from these CTLs increased following combined treatment with anti-CD4 mAb and OVA peptide vaccines (Fig. 3B and D). The phenotype of these $CD8^+$ $CD107a^+$ cells were CD44 high CD62L low effector/memory and that of $CD8^+$ $CD107a^-$ cells were included in naïve, central/memory, effector/memory (Fig. 3C). The increase in OVA-specific CTL induction by OVA peptide vaccine in combination with anti-CD4 mAb was detected using an IFN-γ ELISPOT assay, and the production of IL-2 and TNF from these CTLs was detected in the combination schedule (Fig. 3E and F). The results of the IFN-γ ELISPOT assay, CD107a upregulation assay, and cytokine assay indicated that the depletion of $CD4^+$ T cells enhanced the function of $CD8^+$ T cells in the murine peripheral blood and spleen.

3.4. Metastasis was inhibited by peptide vaccine therapy in combination with anti-CD4 mAb

We evaluated whether the number of metastasis was suppressed by the peptide vaccines in combination with anti-CD4 mAb in the murine liver metastatic model. The treatments of schedule III (Fig. 2A) were performed before and after tumor implantation (Fig. 4A). The spleen weight and liver weight of the combination group were significantly lighter than those of the untreated group and group of OVA peptide alone. However, the spleen weight and liver weight of the combination group showed no significant difference from those in the group treated with anti-CD4 mAb alone. The major axis of splenic tumors following combination therapy was significantly shorter than that for the other groups (Fig. 4C).

3.5. The increase of the infiltration of $CD8^+$ CTL into the liver metastasis and the decrease of $CD4^+$T cells in the liver after the administration of anti-CD4 mAb

$CD8^+$ T cells infiltration in the liver metastatic tumors was higher in the combination therapy group than in OVA peptide vaccine alone and untreated group (Fig. 5A). We could not evaluate $CD4^+$ T cells infiltration into the liver metastatic tumors, because EG7 cells were expressing CD4 on the cell surfaces *in vivo* unexpectedly. But $CD4^+$ T cells infiltration in liver surrounding EG7 tumor was fewer in anti-CD4 mAb treatment group (anti-CD4 mAb alone, combination OVA peptide vaccine and anti-CD4 mAb) than in no anti-CD4 mAb treatment group (no treatment, OVA peptide vaccine alone) (Fig. 5B).

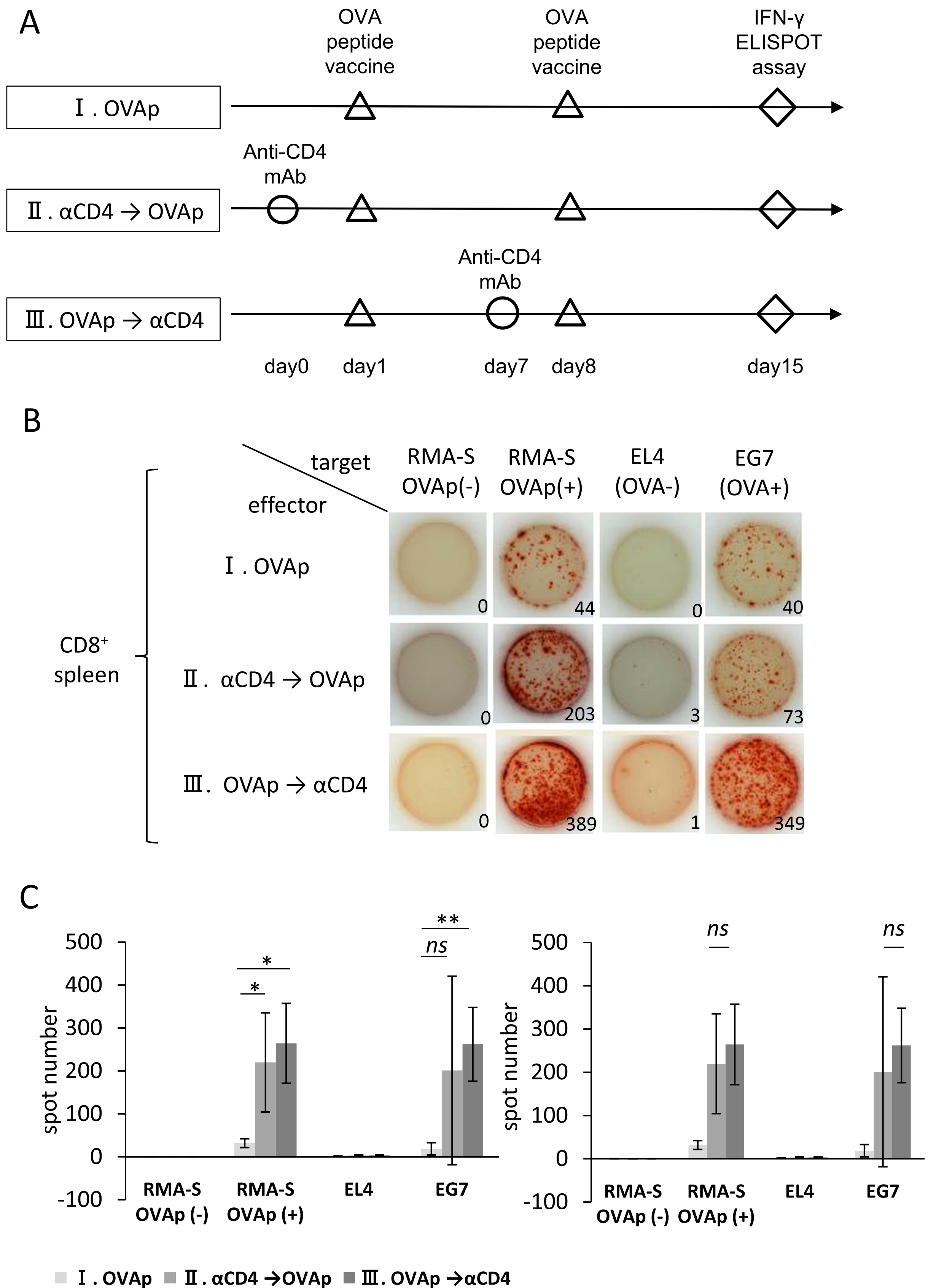

Fig. 2. Comparison of peptide-specific CTL induction by peptide vaccine therapy in combination with anti-CD4 mAb. (A) Schedules for IFN-γ ELISPOT assay. I: OVA peptide vaccine alone, II: Starting from anti-CD4 mAb, and two administrations of OVA peptide vaccine, III: Starting from OVA peptide vaccine, and OVA peptide vaccination after anti-CD4 mAb administration (OVAp: OVA peptide vaccine, αCD4: anti-CD4 mAb). (B) Representative results of IFN-γ ELISPOT assay are shown. Effector cells: $CD8^+$ spleen cells. Groups: I, II, III. Target cells: RMA-S cells, OVA peptide-pulsed RMA-S cells, EL4 cells, EG7 cells. Effector/target ratio=10. (C) Statistical analysis of (B). Comparisons of spot numbers between the group of OVA peptide alone (I) and groups of combination treatment (II or III) [left]. Comparisons of spot numbers between two groups of combination treatment (II and III) [right] ($n=3$).*$P<0.05$,**$P<0.01$, differences are statistically significant between the two value. *ns*, difference was not significant between the two values.

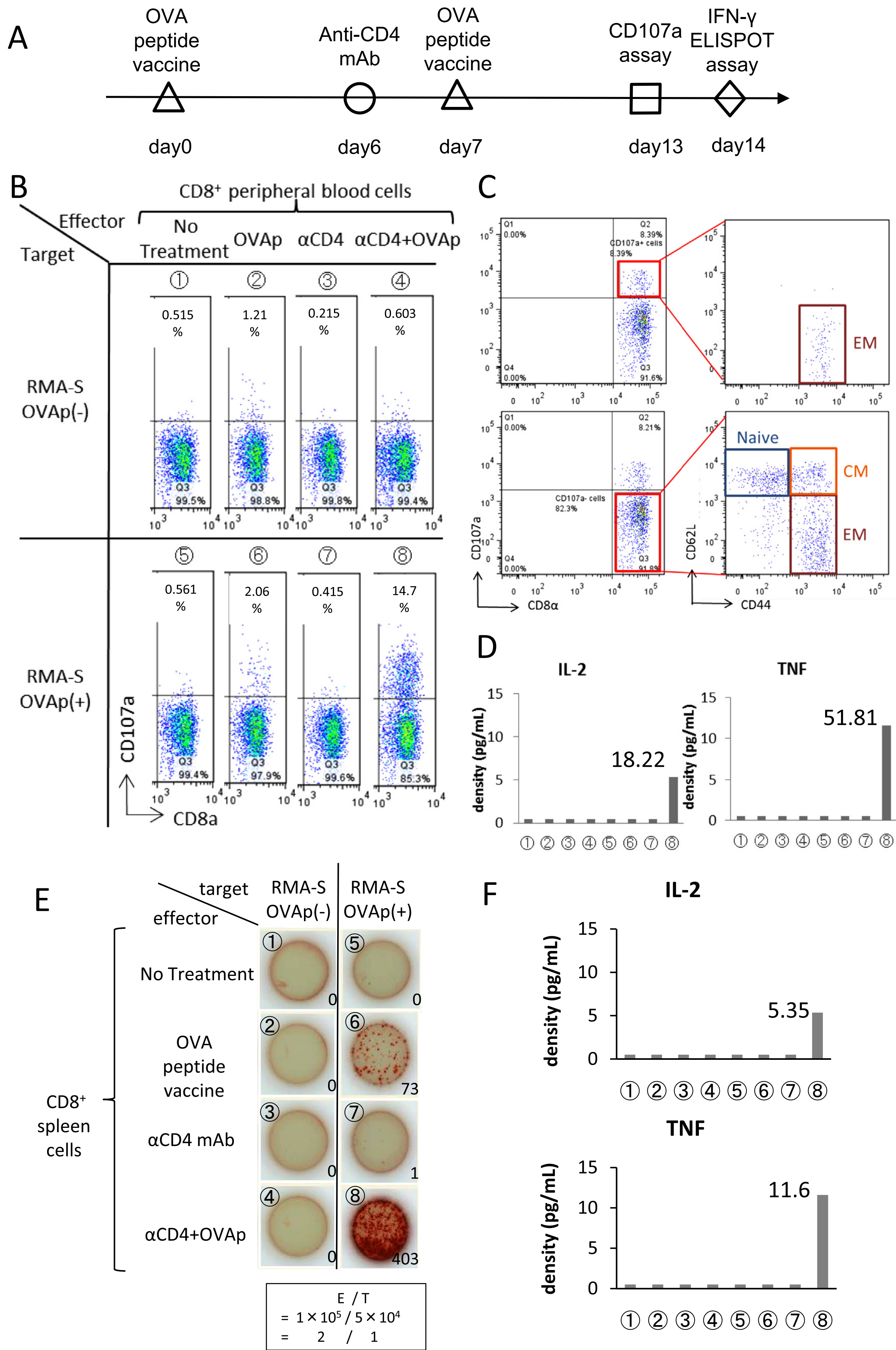

Fig. 3. Multi-function of peptide-specific CTL by peptide vaccine therapy in combination with anti-CD4 mAb. Representative data are shown. (A) Schedule for IFN-γ ELISPOT assay and CD107a assay. On days 0 and 7, mice were injected intradermally at the base of the tail with OVA peptide vaccine. On day 6, mice were injected i.p. with anti-CD4 mAb. On day 13, the CD107a assay was conducted. On day 14, the IFN-γ ELISPOT assay was conducted. (B) CD107a assay of peripheral blood cells. Effector cells: $CD8^+$ peripheral blood cells. Groups: no treatment group, OVA peptide vaccine alone group, anti-CD4 mAb alone group, combination OVA peptide vaccine and anti-CD4 mAb group. Target cells: RMA-S cells, OVA peptide pulsed RMA-S cells. (C) The flow cytometric analysis of effector/memory phenotype of $CD107a^+$ cells and $CD107a^-$ cells in $CD8^+$ T cells. (D) cytokine assay of peripheral blood cells. (E) IFN-γ ELISPOT assay and (F) cytokine assay of splenocytes. Effector cells: $CD8^+$ spleen cells. Groups: no treatment group, OVA peptide vaccine alone group, anti-CD4 mAb alone group, combination OVA peptide vaccine and anti-CD4 mAb group. Target cells: RMA-S cells, OVA peptide pulsed RMA-S cells. Effector/target ratio = 2.

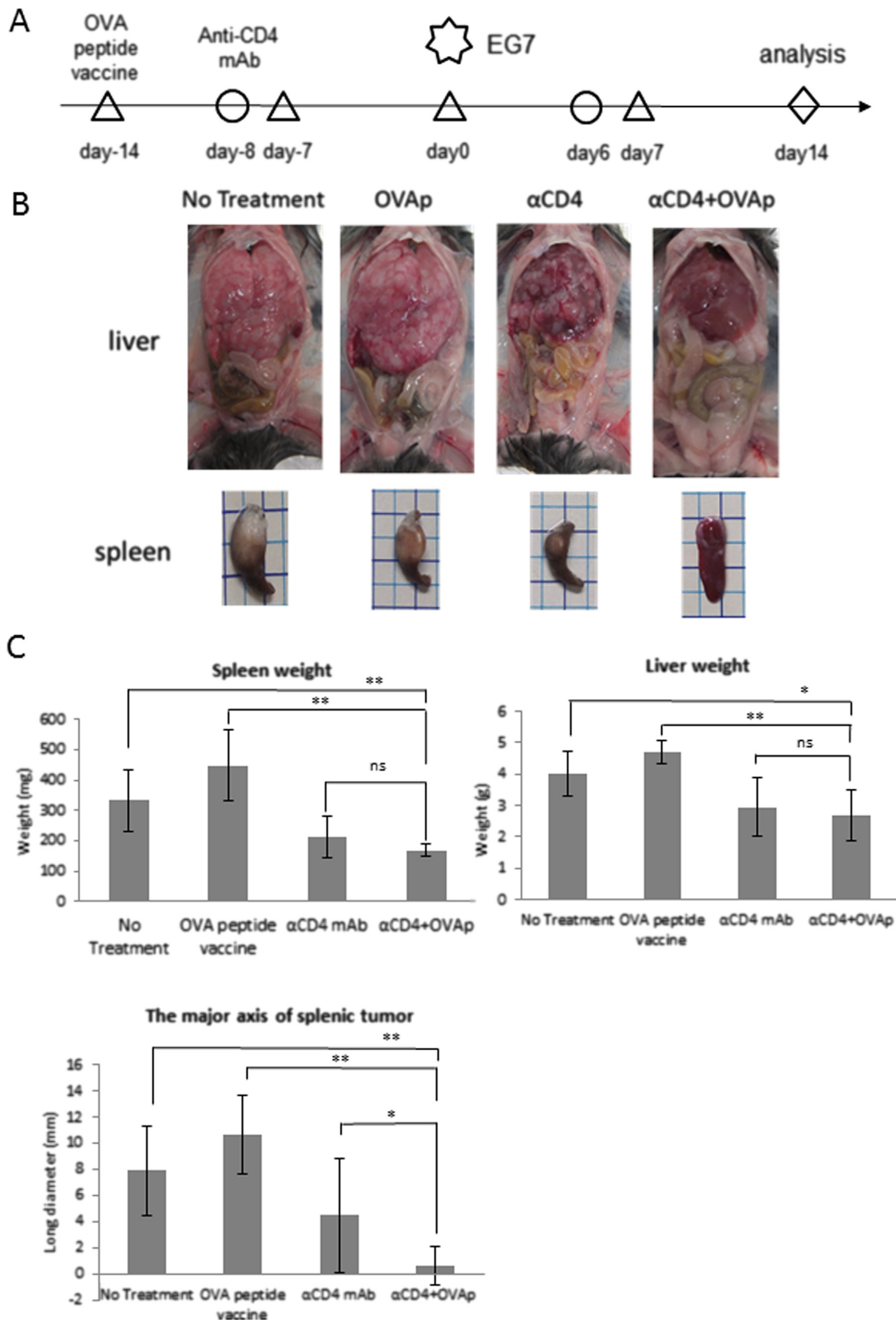

Fig. 4. Suppression of tumor growth in the metastatic tumor model. The protocol for liver metastasis model was indicated in (A). All mice spleens were injected into with 1×10^6 EG7 cells on day 0. On day −14, −7, 0, and 6, mice were injected intradermally at the base of the tail with the OVA peptide vaccine. On days −8 and 6, mice were injected i.p. with anti-CD4 mAb. On day 14, the mice were sacrificed and liver weight, spleen weight, and the major axis of splenic tumor were measured. (B) Representative liver metastasis and splenic tumor in each group. Groups: No treatment group, OVA peptide vaccine alone group, anti-CD4 mAb alone group, combination OVA peptide vaccine and anti-CD4 mAb group (n=5). (C) Statistical analysis of (B). Columns, mean number of liver weight, spleen weight, and major axis of splenic tumor.*$P < 0.05$, **$P < 0.01$, differences were statistically significant between the two values. *ns,* differences were not significant between the two values.

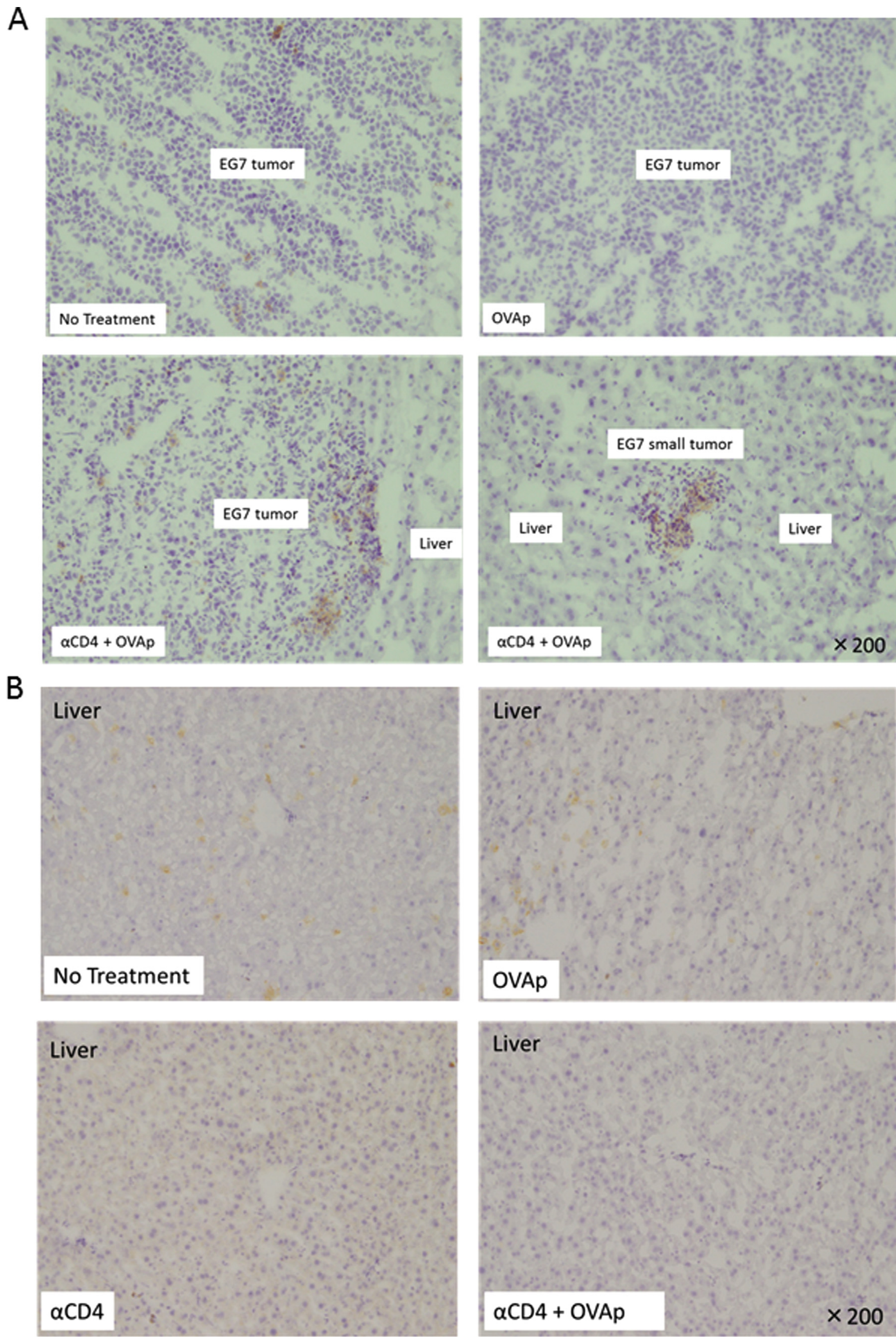

Fig. 5. Immunohistochemical analysis in murine liver. (A) The $CD8^+$ T cells (brown color) infiltration into EG7 tumor in mice (no treatment, OVA peptide vaccine alone, and combination OVA peptide vaccine and anti-CD4 mAb) after the administration of anti-CD4 mAb. (B) $CD4^+$ T cells (brown color) infiltration in liver surrounding EG7 tumor in no anti-CD4 mAb treatment group (no treatment, OVA peptide vaccine alone) and in anti-CD4 mAb treatment group (anti-CD4 mAb alone, combination OVA peptide vaccine and anti-CD4 mAb).

4. Discussion

The aim of this study was to enhance the anti-tumor effect of peptide vaccine therapy by combination with anti-CD4 mAb in a mouse model. In this study, we examined the enhancement of the metastasis inhibitory effect resulting from the use of peptide vaccines in combination with anti-CD4 mAb in a murine liver metastatic model. In this model, $CD4^+$ cells, including Tregs, interfere with CTL induction or its infiltration into the tumor.

Ueha et al. found that anti-CD4 mAb treatment enhanced antitumor $CD8^+$ T cell responses and induced a shift toward type I immunity within the tumor [15]. The immune response is regulated by the Th1/Th2 cytokine balance [19,20]. Nagai et al. suggested that the depletion of $CD4^+$ T cells may result in elimination of Th2 cells despite the abrogation of Th1 cells and induce further activation of $CD8^+$ T cells [21]. We also found that IFN-γ-induced OVA-specific CTL induction increased in the spleen by OVA peptide vaccine in combination with anti-CD4 mAb based on an IFN-γ ELISPOT assay; the production of IL-2 and TNF from these CTLs in the peripheral blood and spleen following the combination therapy were determined using a cytokine assay (Fig. 3C). The depletion of $CD4^+$ T cells induced a shift toward type I immunity in the peripheral blood and spleen, which led to enhancement of the metastasis inhibitory effect by the activation of $CD8^+$ T cells following combination therapy.

Filipazzi et al. reported that the activation of helper $CD4^+$ T cells was necessary for the induction of killer $CD8^+$ T cells [22,23]. Based on the results of the previous study, it was not clear whether helper $CD4^+$ T cells were required for tumor immunity. However, $CD4^+$ T cells may be required for the priming of peptide vaccines, as the frequency of IFN-γ-induced peptide-specific CTLs increased in the murine spleen for schedule III to a greater extent than that in the case of schedule II administered with anti-CD4 mAb (Fig. 2C). Additional studies are required to confirm this.

The efficacy of immunotherapy in mouse models depends on the tumor type. EG7 cells used in this study express CD4 on the cell surface *in vivo*, but not *in vitro* [24]. However, at the beginning of this study, EG7 cells *in vivo* were found to express CD4 on the cell surface, as shown by immunohistochemical staining of CD4 (data not shown). The anti-CD4 mAb (GK1.5) directly killed EG7 cells *in vivo* through complement-dependent cytotoxicity [25], which was considered to be influenced by the result of the murine liver metastatic model. However, we observed metastasis suppression effects following combination therapy in the mouse model using CD4-negative OVA-expressing B16 melanoma, MO4 cells (data not shown). Other tumor cells should be tested in future studies.

In this study, we used OVA as a model antigen, as OVA is thought to be highly immunogenic compared to cancer antigens. We are currently examining targeted cancer antigens such as GPC3 before conducting clinical trials involving combination therapy of the peptide vaccine and anti-CD4 mAb.

The depletion of $CD4^+$ cells may have adverse effects. No serious adverse effects were detected in our mouse model. In addition, no severe adverse effects were observed during phase II clinical trials for T-cell malignancy with long-term administration of humanized anti-CD4 mAbs [26,27]. Autoimmune diseases may occur as result of the continuous reduction of $CD4^+$ cells. However, the depletion of $CD4^+$ cells in this study was transient, not continuous. Although this change was transient, the risk of infectious diseases was increased. Therefore, careful observation of patients after administration of anti-CD4 mAb is required in clinical trials.

Surgery and radiation therapy show excellent local control of tumors, but they are inferior to chemotherapy and immunotherapy for systemic control. In particular, although extremely high-quality radiation therapy such as intensity-modulated radiation therapy or ion radiotherapy has been achieved, radiation enhances metastases under conditions of non-curative doses [28]. Our strategy can suppress the progression of cancer in combination with surgery or radiation therapy to systemically control cancer metastasis and recurrence.

In conclusion, we demonstrated the enhancement of metastasis inhibitory effects following administration of peptide vaccines in combination with anti-CD4 mAb in a murine liver metastatic model. Our results will contribute to the development of cancer treatment.

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Appendix A. Transparency Document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.02.010.

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