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A modulatory effect of L-arginine supplementation on anticancer effects of chemoimmunotherapy in colon cancer-bearing aged mice

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ABSTRACT

Myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) are increased in cancer-bearing aged hosts. Arginase-I in MDSCs degrades L-arginine, an amino acid required for T cell activation and proliferation. In this study, we compared the therapeutic efficacy of 5-fluorouracil (5-FU)/oxaliplatin (L-OHP) and cyclophosphamide (CP) between young and aged colon cancer-bearing mice. Therapy with 5-FU/L-OHP and CP significantly suppressed the *in vivo* growth of CT26 and MC38 colon carcinomas in syngeneic young mice, whereas this effect was attenuated in aged mice. L-arginine monotherapy showed no effect in aged mice. However, additional therapy with anti-programmed cell death (PD)-1 antibody and L-arginine supplementation boosted the effect of chemoimmunotherapy in aged mice, and some mice were cured. During all combination therapy, tumor-specific cytotoxic T lymphocytes (CTLs) were generated from mice with non-progressing tumor, but not from those with progressing tumor. Plasma L-arginine levels were lower in aged than young mice, and chemotherapy tended to decrease the plasma L-arginine levels in aged mice. Compared to young mice, CT26-bearing aged mice decreased arginase activity, arginase-I expression, and the proportion of monocytic MDSCs in tumor tissues, whereas contrasting results were observed in MC38-bearing aged mice. Importantly, the induction of tumor-specific CTLs was impaired at lower doses of L-arginine *in vitro*, and the infiltration of CTLs into CT26 tissues after chemoimmunotherapy was promoted by L-arginine administration *in vivo*. These results indicate that chemoimmunotherapy was less effective in cancer-bearing aged mice, but that L-arginine supplementation can modulate its therapeutic efficacy via its effect on tumor-specific CTLs.

1. Introduction

T cells play a crucial role in antitumor immunity; however, their functions are impaired with aging [1,2]. Given that most cancer patients are aged, T cell impairment must be considered in the treatment of cancer patients. We previously reported that cancer grows more rapidly in aged than young mice [3]. Several mechanisms may account for impaired antitumor T cell responses with aging. Although the age-related increase of interleukin (IL)-6, a proinflammatory cytokine, dampens antitumor immune responses [4], another important mechanism is the increase of immunosuppressive cells, including myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) [5], both of which increase in cancer-bearing [6,7] and aging hosts [8], and represent a challenge in the treatment of cancer patients.

MDSCs suppress antitumor immune responses via mechanisms involving arginase-I, inducible nitric oxide (NO) synthase, and indoleamine 2,3-dioxygenase (IDO) [9,10]. Although L-arginine and L-tryptophan are required for T cell proliferation/activation, arginase-I and inducible NO synthase metabolize L-arginine [11]. In addition, IDO degrades L-tryptophan, resulting in the accumulation of kynurenine, an immunosuppressive metabolite, and leading to T cell dysfunction [9,10]. The degradation of L-arginine by MDSCs leads to reduced expression of the CD3ζ chain, resulting in impaired T cell responsiveness [11,12]. L-arginine is deficient in the cancer-bearing state [13], and L-tryptophan results in the accumulation of immunosuppressive kynurenine; we previously reported that L-arginine supplementation enhances the anticancer T cell response induced by immunomodulating chemotherapy with cyclophosphamide (CP) in young cancer-bearing mice

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[14].

Recent studies have shown that some anticancer agents administered at low or moderate doses exhibit immune-modulating effects in cancer-bearing hosts, and enhance antitumor T cell responses [15,16]. Such effects include regulation of tumor immunogenicity [17], mitigation of immunosuppression mediated by Tregs [18], reduction of MDSCs [19], and mature dendritic cell (DC) induction [20]. A combination of 5-fluorouracil (5-FU) and oxaliplatin (L-OHP) called FOLFOX has been clinically applied as adjuvant chemotherapy for colorectal cancer, as a first-line regimen [21]. However, these drugs have immunomodulating effects; 5-FU can decrease MDSCs [19] and L-OHP can induce immunogenic cancer cell death [22]. In addition, CP can relieve Treg-mediated immunosuppression at low-to-moderate doses [23–25]. Based on these findings, 5-FU/L-OHP appears to suppress tumor growth when combined with anti-programmed cell death (PD)-1 antibody in colon cancer-bearing mice [26]. We also reported that immunogenic chemotherapy with 5-FU/L-OHP and CP in combination with anti-PD-1 antibody induced tumor regression in two mouse colon cancer models [27]. However, these preclinical models using young mice do not necessarily reflect the clinical situation, in which the majority of cancer patients are aged.

L-arginine is necessary during inflammation and wound healing [28]. The tumor-bearing state may be regarded as reflecting chronic inflammation, and cancer-bearing aged hosts, as well as chemotherapy-induced leukopenia, require L-arginine to elicit antitumor T cell responses *in vivo*. Therefore, in this study, we used two different colon cancer models to compare the anticancer effects induced by 5-FU/L-OHP/CP between young and aged mice. Then, we examined antitumor effects upon combination with anti-PD-1 antibody and/or L-arginine supplementation in colon cancer-bearing aged mice.

2. Materials and methods

2.1. Mice and tumor cell lines

Wild-type BALB/c and C57BL/6 female young mice (6–7 weeks old), and retired mice (40 weeks old), were purchased from CLEA Japan Inc. (Tokyo, Japan) and maintained under specific pathogen-free conditions. To prepare aged (60–65 weeks old) mice, retired mice were maintained for an additional 4–5 months. C57BL/6 female aged mice (57 weeks old) were purchased from the Jackson Laboratory Japan (Kanagawa, Japan) and used for experiments. Experiments were conducted in accordance with the ethical guidelines for animal experiments of Shimane University Faculty of Medicine (IZ2-74, IZ2-75, IZ3-97, IZ3-105, IZ3-116, and IZ3-127). CT26 and MC38 are colon carcinomas derived from BALB/c and C57BL/6 mice, respectively. MC38 was kindly provided by Dr. Toshiyasu Ojima of the Department of Surgery, Wakayama Medical University. LuM1, a highly metastatic subline derived from CT26 [29], was kindly provided by Dr. Keizo Takenaga of the Laboratory of Cancer Genetics, Chiba Cancer Center Research Institute. All cell lines were maintained in RPMI 1640 media supplemented with 10% FCS and 20 μg/mL gentamicin (Sigma-Aldrich, St. Louis, MO, USA), at 37°C in an atmosphere with 5% CO_2.

2.2. Reagents

CP was purchased from Shionogi Co. Ltd (Osaka, Japan); 5-FU and L-OHP were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and LCL Laboratories (Tokyo, Japan), respectively. Anti-PD-1 mAb (clone RMP 1-14) and rat IgG were purchased from Bio X Cell Inc. (West Lebanon, NH, USA) and Sigma-Aldrich, respectively. L-arginine was purchased from Sigma-Aldrich and diluted with distilled water (dH_2O). Doxorubicin was purchased from Kyowa Hakko Co. Ltd. (Tokyo, Japan).

2.3. Subcutaneous tumor models

Aged BALB/c mice were injected subcutaneously (s.c.) with 2–5 × 10^5 CT26 cells into the right flank. In the MC38 model, aged C57BL/6 mice were injected s.c. with 5 × 10^5 MC38 cells into the right flank. On day 12, the mice were randomly divided into groups. On days 12 and 20, the mice were injected intraperitoneally (i.p.) with 5-FU (50 mg/kg), L-OHP (6 mg/kg), and CP (50 mg/kg), for a total volume of 200 μL. On days 13 and 21, some mice were injected i.p. with anti-PD-1 mAb (200 μg/mouse), for a total volume of 200 μL. In some experiments, the same volume of rat IgG (200 μg/mouse) was injected i.p. as vehicle control. From day 12, mice were orally administered L-arginine (45 mg/mouse) at a volume of 200 μL. The doses of 5-FU, L-OHP, CP, anti-PD-1 mAb, and L-arginine administered in this study were selected based on previous reports [14,27]. Tumor volume (mm^3) and body weight were measured at 4-day intervals. The tumor volume was calculated as follows: Tumor volume = (Length × $Width^2$) ÷ 2. Mice were considered cured when their tumors were not palpable at least 2 weeks after complete regression following therapy,

2.4. Liver metastasis model

Young and aged BALB/c mice were injected with 2.0 × 10^4 LuM1 cells into the spleen in a volume of 50 μl. On days 8 and 16, 5-FU (50 mg/kg) and L-OHP (6 mg/kg) were injected i.p.. From day 8, L-arginine was orally administered daily at doses of 30 mg and 45 mg for young aged mice, respectively, because the body weights of aged mice were almost 1.5 times higher than those of young mice [3]. The survival of tumor-bearing mice was monitored. In another experiment, mice were euthanized on day 19, and colonies (1 > mm) on the liver surface were counted microscopically.

2.5. Cytotoxicity assay

Two months after complete regression of CT26 or MC38, the spleen was harvested separately from each mouse. After lysing erythrocytes, the spleen cells were cultured individually with the indicated tumor peptide (10 μg/mL) and IL-2 (20 U/mL) for 4 days using RPMI 1640 medium containing with 10% FCS. H-$2L^d$-binding AH1 peptide (SPSYVYHQF) [30] and H-$2K^b$-binding p15E peptide (KSPWFTTL) [31], both of which were derived from the envelope protein (gp70) of an endogenous murine leukemia virus, were used as tumor antigenic peptides for CT26 and MC38, respectively. Both peptides were > 80% pure and were purchased from Invitrogen (Grand Island, NY, USA). Thereafter, cytotoxicity was measured according to a 5-h ^{51}Cr-release assay. To examine tumor-specific CTLs in treating mice, aged BALB/c mice were injected s.c. with 2 × 10^5 CT26 cells into the right flank. On days 12 and 20, the mice were injected i.p. with 5-FU (50 mg/kg), L-OHP (6 mg/kg), and CP (50 mg/kg). On days 13 and 21, some mice were injected i.p. with anti-PD-1 mAb (200 μg/mouse). From day 12, mice were orally administered L-arginine (45 mg/mouse). On day 25, spleen was harvested separately, cultured individually, and the cytotoxicity against CT26 cells was examined, as described above.

2.6. Flow cytometry

To analyze tumor-infiltrating immune cells, tumor tissues were resected from mice individually. The tumors were minced, placed on glass slides, and passed through gauze mesh and nylon mesh. The following mAbs were used: APC-conjugated anti-CD45 mAb (BioLegend, CA, USA), FITC-conjugated anti-Gr-1 mAb (BioLegend), PE-conjugated anti-CD11b mAb (BioLegend), FITC-conjugated anti-CD8 mAb (BioLegend), PE-conjugated anti-CD4 mAb (BioLegend), and PE/cy7-conjugated anti-Ly6C mAb (BioLegend). To assess the proportions of tumor (AH1) peptide-specific $CD8^+$ T cells, the spleen cells from young BALB/c mice, which were immunized with doxorubicin-treated 1 × 10^6

CT26 cells 2 weeks before, were cultured with the AH1 peptide (10 μg/mL) and IL-2 (20 U/mL) at different doses of L-arginine. CT26 cells were inactivated by the treatment with doxorubicin at a dose of 50 μg/mL for 12 h, as reported [3]. Five days later, the cultured cells were stained with a PE-conjugated tetramer of the AH1 peptide and FITC-conjugated anti-CD8 mAb. Analysis was performed using the FACSCalibur (Becton–Dickinson, Franklin Lakes, NJ, USA).

2.7. Protein extraction from tumor tissues, assay of arginase, and immunoblot

Tumor tissue was dissected into cubes of 5 mm in size using a scalpel, inserted into BioMasher III (Nippi, Tokyo, Japan), and left on ice. Next, 150 μL of 0.1% Triton X100, containing a protease inhibitor cocktail (Nacalai Tesque), for assay of arginase activity or 200 μL of RIPA buffer (FUJIFILM Wako Pure Chemical, Osaka, Japan) for immunoblot was added, and samples were homogenized using while cooling on ice. After filtration and centrifugation (12,000 rpm at 4℃ for 15 min), the supernatant was collected, and the protein concentration was quantified. Arginase activity was measured by arginase activity assay kit (AK89; Cosmo Bio Co. Ltd., Tokyo, Japan). Briefly, tumor tissues were homogenized and lysed using BioMasher III with a 0.1% Triton X100 containing a protease inhibitor cocktail (Nacalai Tesque). For immunoblot, equal amounts of protein were resolved on 10% (w/v) SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked and then incubated with the following primary antibodies: anti-arginase-I (sc-271430; SantaCruz Biotechnology). After washing, the membranes were incubated with horse anti-mouse IgG horseradish peroxidase-conjugated secondary antibody (#7076; Cell Signaling Technology). To detect β-actin, peroxidase-conjugated anti-β-actin antibody (#017–24,573; Wako Pure Chemicals) was used. Protein bands were visualized using an Amersham ImageQuant™ 800 Biomolecular Imager (General Electric Company).

2.8. Cell proliferation assay

Spleen cells were harvested from young and aged naïve BALB/c and C57BL/6 female mice. After lysing erythrocytes, spleen cells were cultured in flat 96-well plates pre-coated with anti-CD3 mAb (3 μg/mL, clone 145-2C11, BioLegend) in the presence of soluble anti-mouse CD28 mAb (1 μg/mL, clone 37.51, BioLegend) in RPMI 1640 medium containing the indicated doses of L-arginine. To prepare the indicated doses of L-arginine, the conventional RPMI1640 medium (1150 μM L-arginine) was combined at various ratios with the arginine-free medium RPMI1640 that was purchased from the Research Institute for Functional Peptides (Yamagata, Japan). CT26 and MC38 tumor cells were cultured in flat 96-well plates in RPMI 1640 medium containing the indicated doses of L-arginine. Cell viability was measured using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). Three days later, 10 μL of CCK-8 solution was added to each well and the plates were incubated for 3 h. Absorbance at 450 nm was read using a microplate reader (Beckman Coulter, Brea, CA, USA).

2.9. Plasma L-arginine measurements

Plasma from naïve or CT26-bearing young and aged BALB/c mice, with or without treatment, was prepared individually and stored at −30°C. Plasma L-arginine levels were measured by liquid chromatography–mass spectrometry (LC–MS) (LC-MS8050; Shimadzu, Kyoto, Japan).

2.10. Statistical analyses

Data were analyzed using Student's t-test or the Mann–Whitney U test for two groups, and by analysis of variance (ANOVA) followed by Tukey's post hoc test for more than two groups. Survival data were obtained using the Kaplan–Meier method and evaluated using the log-rank test. $P < 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Antitumor effects induced by combining 5-FU/L-OHP, CP, anti-PD-1 antibody, and L-arginine supplementation in CT26-bearing aged mice

First, we examined the effect of combination therapy, consisting of 5-FU/L-OHP and CP, in CT26-bearing young BALB/c mice. Consistent with our previous study [27], double injections of 5-FU/L-OHP and CP on days 12 and 20 significantly suppressed tumor growth (Fig. 1A, B). By contrast, combination therapy with 5-FU/L-OHP and CP suppressed tumor growth in aged mice, although not significantly (Fig. 1C–E). Although L-arginine supplementation alone showed no effect, combination therapy with 5-FU/L-OHP and CP with L-arginine significantly suppressed CT26 tumor growth ($P < 0.05$ vs. untreated). The addition of anti-PD-1 therapy further enhanced tumor suppression ($P < 0.01$ vs. untreated), and two of five mice were completely cured. Body weight loss, which is considered a systemic adverse event, was not observed in mice in any group (Fig. 1F).

3.2. Effects of L-arginine supplementation on chemoimmunotherapy in CT26-bearing aged mice

Next, we examined whether L-arginine supplementation could enhance the therapeutic efficacy of 5-FU/L-OHP/CP/anti-PD-1 therapy. The 5-FU/L-OHP/CP combination therapy significantly suppressed tumor growth ($P < 0.05$ vs. untreated), whereas no significant difference in tumor size was detected between 5-FU/L-OHP/CP/anti-PD-1 and 5-FU/L-OHP/CP/anti-PD-1/L-arginine therapies (Figs. 2A–C). However, therapy with 5-FU/L-OHP/CP/anti-PD-1/L-arginine induced complete cure in two of six mice. Anti-PD-1 monotherapy showed no effect on the tumor growth of CT26 in aged mice (Fig. 2D). These results show that complete cure was observed only when combined with L-arginine supplementation and suggest a modulatory effect of L-arginine on anticancer effects of chemoimmunotherapy in cancer-bearing aged mice.

3.3. Antitumor effects induced by the combination of 5-FU/L-OHP, CP, anti-PD-1 antibody, and L-arginine supplementation in MC38-bearing aged mice

Next, we performed experiments using MC38-bearing C57BL/6 mice. We examined the effect of combination therapy with 5-FU/L-OHP and CP in MC38-bearing young C57BL/6 mice. Although double injections of 5-FU/L-OHP and CP on days 12 and 20 significantly suppressed tumor growth (Fig. 3A). In addition, double injections of anti-PD-1 antibody on days 13 and 21 significantly suppressed the growth of MC38 in aged C57BL/6 mice (Fig. 3B). Combination therapy with 5-FU/L-OHP and CP failed to suppress tumor growth in aged mice, and additional L-arginine supplementation had no effect (Fig. 3C, D). When anti-PD-1 therapy was added, one of four mice was cured; however, there was no significant difference in tumor growth. No body weight loss was observed (Fig. 3E). Additional supplementation with L-arginine did not enhance the therapeutic efficacy of chemoimmunotherapy in MC38-bearing aged mice (Fig. 3F).

3.4. Generation of colon cancer-specific cytotoxic T lymphocytes (CTLs) from cured and treating aged mice

Next, we examined whether colon cancer-specific CTLs could be induced from cured mice following 5-FU/L-OHP/CP/anti-PD-1/L-arginine supplementation. Two months after tumor regression, spleen cells harvested from CT26-cured BALB/c mice were cultured with the tumor antigen AH1 peptide [30] for 4 days, and their cytotoxicity toward CT26 was examined. Higher levels of cytotoxicity to CT26 were seen in CT26-

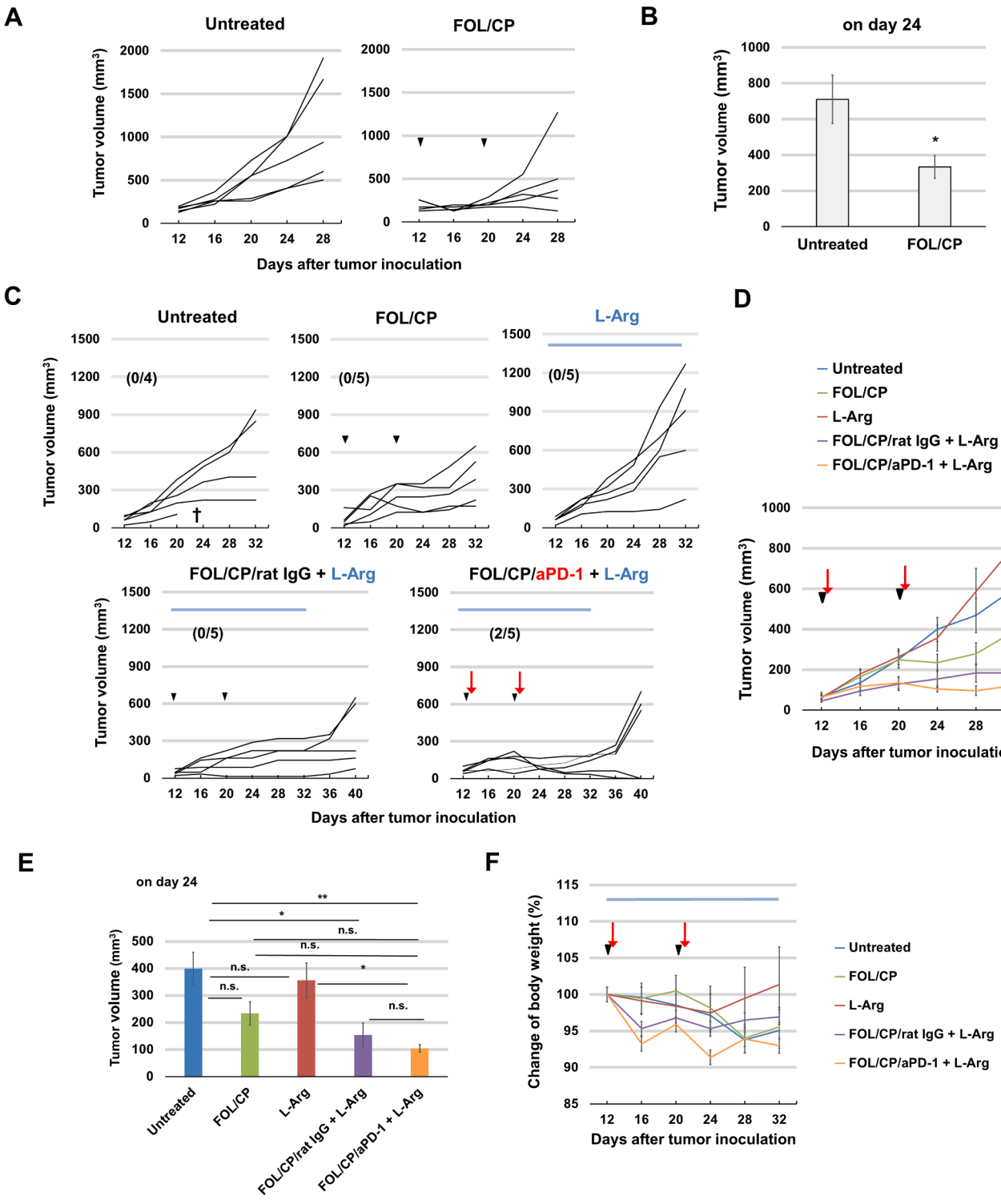

Fig. 1. Antitumor effects induced by combining chemotherapy, anti-PD-1 antibody, and L-arginine supplementation in CT26-bearing aged BALB/c mice. **(A)** Young BALB/c mice were injected subcutaneously (s.c.) with 5×10^5 CT26 cells, into the right flank. On days 12 and 20, 5-FU (50 mg/kg), L-OHP (6 mg/kg), and CP (50 mg/kg) were injected intraperitoneally (i.p.). The tumor volume was measured at 4-day intervals. Lines indicate tumor growth in each mouse. Tumor volume was calculated as (Length × Width^2) ÷ 2. Pointers indicate drug injection. Similar results were obtained in two independent experiments. **(B)** Tumor volumes on day 24. Data are means ± SEM of five mice. *$P < 0.05$ (Mann–Whitney U test). **(C)** Aged BALB/c mice were injected s.c. with 2×10^5 CT26 and treated under the protocol described for (A); however, in some groups, anti-PD-1 antibody or rat IgG (200 μg/mouse) was injected i.p. on days 13 and 21 (red arrows). In addition, L-arginine (45 mg/mouse) was orally administered from day 12 to 32 (blue bars). Numbers in parentheses represent the ratios of cured mice to total mice. Similar results were obtained in two independent experiments. **(D)** Kinetic tumor volumes for each group. Data are means ± SEM of 4′ – 5 mice. **(E)** Tumor volumes on day 24. Data are means ± SEM. *$P < 0.05$, **$P < 0.01$ n.s., not significant. (ANOVA). **(F)** Kinetic body weights for each group. FOL, 5-FU + L-OHP; CP: cyclophosphamide; L-Arg, L-arginine.

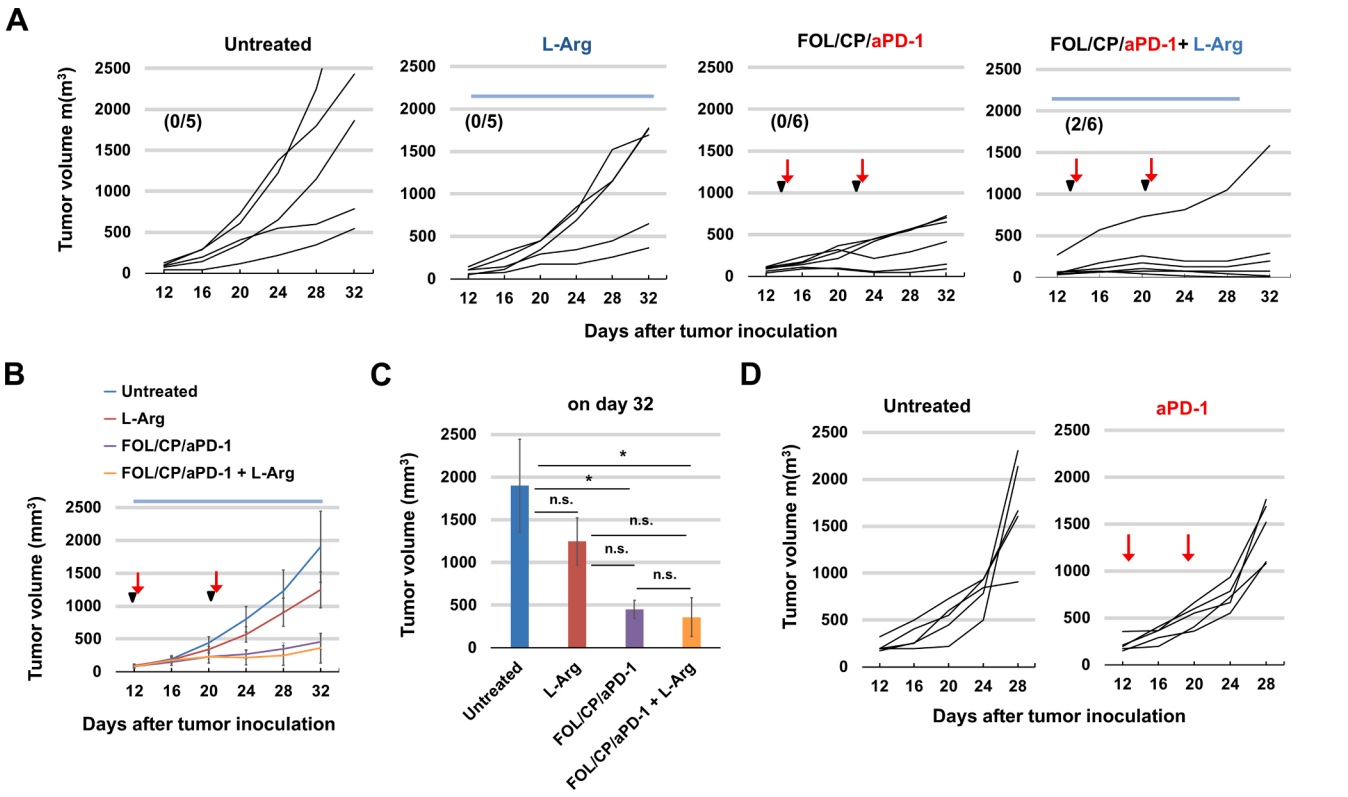

Fig. 2. Effects of L-arginine supplementation on chemoimmunotherapy in CT26-bearing aged mice. (A) Aged BALB/c mice were treated as described for Fig. 1C. Pointers and red arrows indicate the injection of chemotherapeutic drugs and antibodies, respectively. Blue bars indicate L-arginine supplementation. Numbers in parentheses represent the ratios of cured to total mice. Lines indicate tumor growth in each mouse. Similar results were obtained in two independent experiments. **(B)** Kinetic tumor volume of each group. Data are means ± SEM of 5–6 mice. **(C)** Tumor volumes on day 32. Data are means ± SEM. $^*P < 0.05$. n.s., not significant. (ANOVA). **(D)** Anti-PD-1 antibody or rat IgG (200 μg/mouse) was injected i.p. on days 13 and 21 in CT26-bearing aged mice. FOL, 5-FU + L-OHP; CP, cyclophosphamide; L-Arg, L-arginine.

cured aged BALB/c mice; no cytotoxicity was observed in naïve mice (Fig. 4A). Similarly, spleen cells harvested from MC38-cured C57BL/6 mice were cultured with the tumor antigen p15E peptide [31] for 4 days, and cytotoxicity toward MC38 was examined. Higher levels of cytotoxicity to MC38 were seen in MC38-cured aged C57BL/6 mice than naïve mice (Fig. 4B).

We also examined tumor-specific CTL activity of treating CT26-bearing aged mice with all combination therapy. As seen in Fig. 1C (lower right), all combination therapy resulted in two groups, a tumor progressing group and a tumor non-progressing group. To this end, we compared the CTL responses of individually cultured spleen cells from eight mice with progressing (P) and non-progressing (N) tumors during all combined therapy. Four mice showed progressing tumors and four mice non-progressing tumors (Fig. 4C). As a result, although CTL activity was low, spleen cells from the non-progressing (N) group showed higher cytotoxicity than those from the progressing (P) group (Fig. 4D).

3.5. Therapy with 5-FU/L-OHP and L-arginine supplementation was less effective in aged mice with liver metastasis

We next compared the therapeutic efficacy of 5-FU/L-OHP and L-arginine supplementation between young and aged mice in a liver metastasis model. LuM1 is a highly metastatic cell line derived from CT26 that can cause liver metastasis when injected into the spleen [29]. We examined the overall survival of young and aged mice after 5-FU/L-OHP chemotherapy in combination with L-arginine supplementation. In young mice, 5-FU/L-OHP treatment significantly extended overall survival compared with the untreated group ($P < 0.05$), whereas additional L-arginine supplementation further prolonged survival ($P < 0.01$ vs. untreated; Fig. 5A). By contrast, 5-FU/L-OHP treatment failed to significantly extend overall survival compared with the untreated group. Additional L-arginine supplementation prolonged survival compared to the untreated group, but not significantly in aged mice (Fig. 5B). We also examined metastatic colonies in the liver. Untreated aged mice had significantly more metastatic colonies in the liver than young mice (Fig. 5C). Although chemotherapy with or without L-arginine supplementation showed a tendency to decrease the metastatic colonies, statistical significance was not observed. L-arginine alone showed no effect. Representative photos of each group are shown in Fig. 5D, and all photos are shown in Supplementary Fig. 1. We observed that all cancer-bearing mice exhibited bleeding in the peritoneal cavity, which might have influenced their survival.

3.6. Plasma L-arginine and L-tryptophan levels in young and aged CT26-bearing mice

We measured plasma L-arginine and L-tryptophan levels in young and aged naïve and CT26-bearing BALB/c mice using LC–MS. Mice were orally administered L-arginine (30 mg/young mice and 45 mg/aged mouse) continuously from day 12. On days 12 and 20, CT26-bearing mice were injected i.p. with 5-FU (50 mg/kg) and L-OHP (6 mg/kg). Plasma was collected on day 25 after CT26 inoculation. Plasma L-arginine levels decreased in both young and aged CT26-bearing mice, whereas plasma L-tryptophan levels decreased only in aged CT26-bearing mice (Fig. 6A, B). Unexpectedly, L-arginine administration decreased the plasma levels of both L-arginine and L-tryptophan in aged mice. Similarly, 5-FU/L-OHP administration tended to decrease plasma L-arginine levels in CT26-bearing aged mice, but not significantly. L-arginine levels were lower in aged mice treated with or without L-arginine administration (Fig. 6C). Unexpectedly, L-tryptophan levels

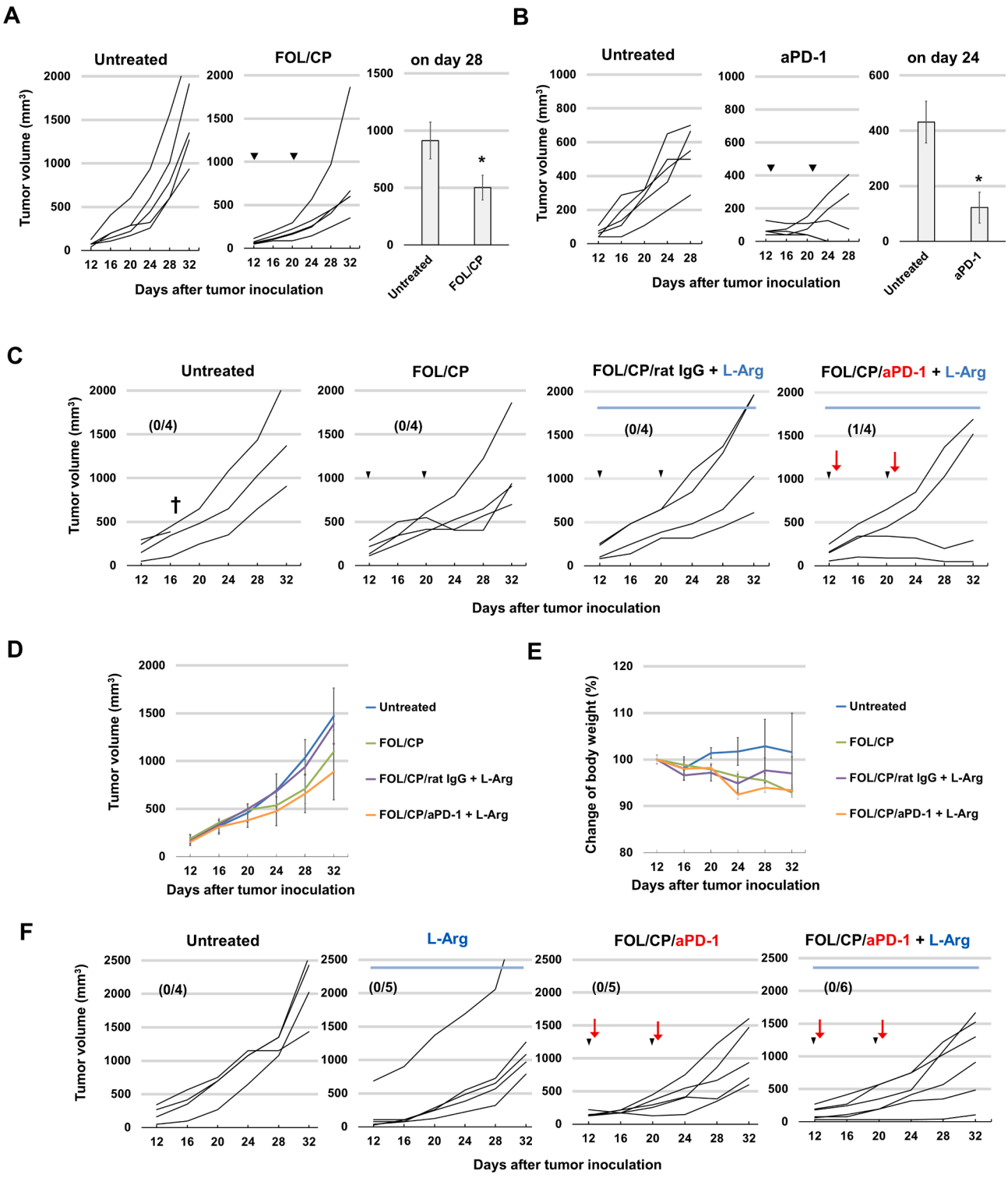

Fig. 3. Antitumor effects induced by combining chemotherapy, anti-PD-1 antibody, and L-arginine supplementation in MC38-bearing aged C57BL/6 mice. **(A)** Young C57BL/6 mice were injected s.c. with 5.0×10^5 MC38 cells, into the right flank. Thereafter, these mice were treated as described for Fig. 1A. *$P < 0.05$ (Mann–Whitney U test). Data are means ± SEM of five mice. **(B)** Anti-PD-1 antibody or rat IgG (200 μg/mouse) was injected i.p. on days 13 and 21 in MC38-bearing aged mice. Red arrows, antibody injection; lines, tumor growth of each mouse. *$P < 0.05$ (Mann–Whitney U test). Data are means ± SEM of five mice. **(C)** Aged C57BL/6 mice were treated as described for Fig. 1C. Pointers and red arrows indicate the injection of chemotherapeutic drugs and antibodies, respectively. Blue bars indicate L-arginine supplementation. Numbers in parentheses represent the ratios of cured to total mice. †: One mouse died. Similar results were obtained in two independent experiments. **(D)** Kinetic tumor volume of each group. Data are means ± SEM of four mice. **(E)** Kinetic body weights of each group. **(F)** Aged C57BL/6 mice were treated as described for Fig. 2A. Pointers and red arrows indicate the injection of chemotherapeutic drugs and antibodies, respectively.

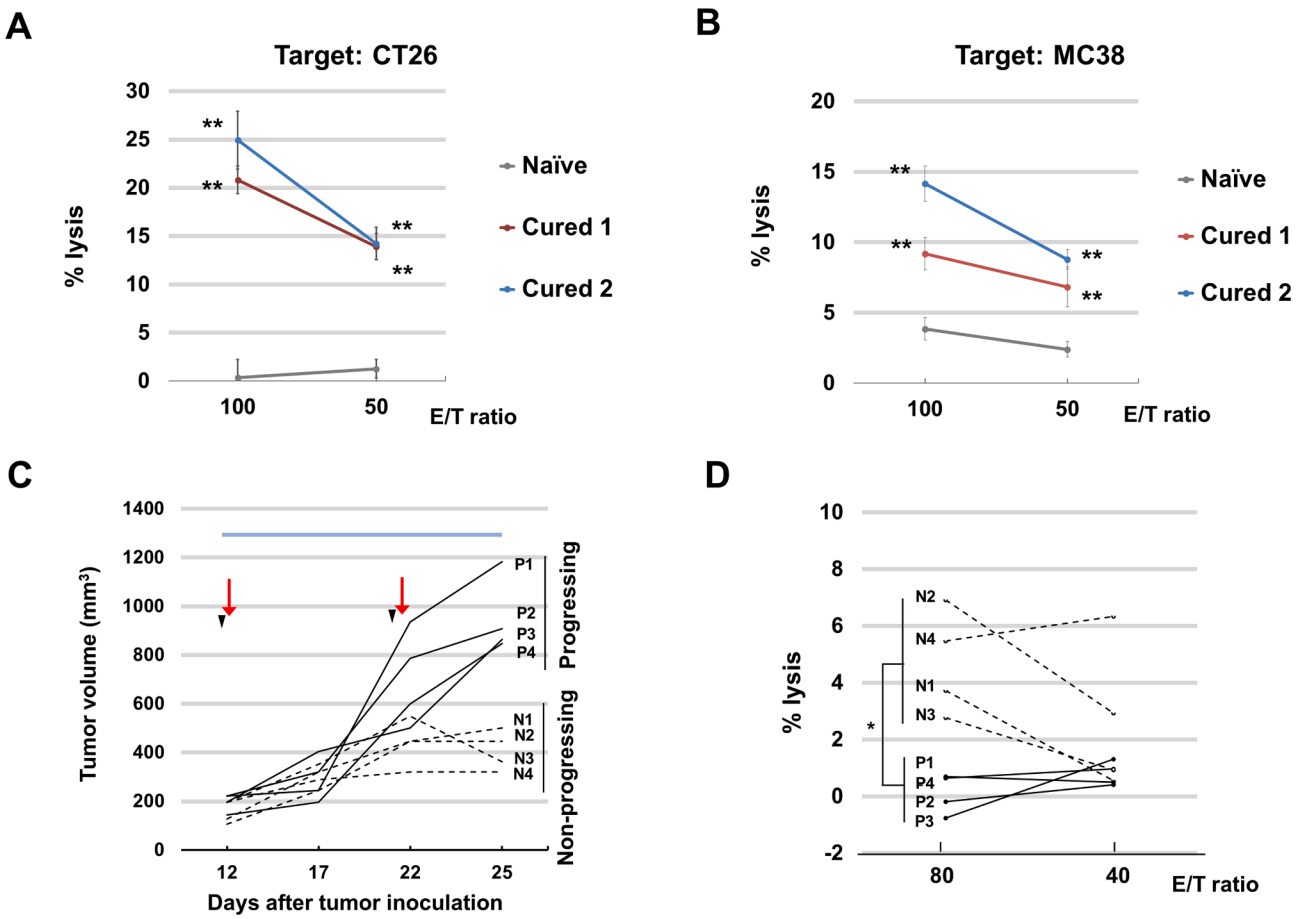

Fig. 4. Generation of cancer-specific cytotoxic T lymphocytes (CTLs) from cured and treating aged mice. (A) Spleen cells from naïve and CT26-cured aged BALB/c mice were harvested and cultured with AH1 peptide in the presence of IL-2 (20 U/mL) for 4 days. The cytotoxicity of these cultured cells against CT26 cells was examined using a 5-h ^{51}Cr-release assay. $^{**}P < 0.01$ vs. the naïve group (Student's *t*-test). **(B)** Spleen cells from naïve and MC38-cured aged C57BL/6 mice were harvested and cultured with p15E peptide in the presence of IL-2 (20 U/mL) for 4 days. The cytotoxicity of the cultured cells against MC38 cells was examined using a 5-h ^{51}Cr-release assay. $^{**}P < 0.01$ vs. the naïve group (Student's *t*-test). **(C)** Aged BALB/c mice were injected s.c. with 2×10^5 CT26 cells. On days 12 and 20, 5-FU (50 mg/kg), L-OHP (6 mg/kg), and CP (50 mg/kg) were injected intraperitoneally (i.p.) (black arrowheads). Anti-PD-1 antibody or rat IgG (200 μg/mouse) was injected i.p. on days 13 and 21 (red arrows). L-arginine (45 mg/mouse) was orally administered from days 12 to 24 (blue bar). Lines indicate tumor growth. **(D)** On day 25, spleen was harvested separately, cultured individually and the cytotoxicity against CT26 cells was examined. $^{*}P < 0.05$ (Mann-Whitney U-test). N, non-progressing: P, progressing.

were higher in CT26-bearing young mice after 5-FU/L-OHP treatment, and were significantly lower in CT26-bearing aged mice treated with 5-FU/L-OHP.

3.7. *Levels of plasma L-arginine, arginase activity, arginase-I expression, and tumor-infiltrating immune cells in tumor tissues of young and aged tumor-bearing mice*

We measured plasma levels of L-arginine in young and aged tumor-bearing BALB/c and C57BL/6 mice (Fig. 7A). The CT26-bearing state decreased the plasma levels of L-arginine in young BALB/c mice. Aged BALB/c mice showed lower levels of L-arginine than young mice, and CT26-bearing aged mice showed a tendency of decreased L-arginine levels, but not significant. In terms of C57BL/6 mice, the plasma levels of L-arginine were decreased only in aged MC38-bearing mice. Next, the arginase activity and the expression of arginase-I of CT26 tumor tissues were examined. As a result, both the arginase activity and the expression of arginase-I protein showed a tendency to decrease in CT26 tissues of aged BALB/c mice (Fig. 7B and Suppl. Fig. 2). By contrast, both of them increased in MC38 tissues of aged C57BL/6 mice.

Next, tumor-infiltrating CD45$^+$ immune cells were examined (Fig. 7C and Suppl. Fig. 3A, B). The infiltrating CD45$^+$ immune cells were higher in MC38 tissues than in CT26 tissues. MDSCs are classified as either Gr-1^{high} Ly6C^{med} granulocytic MDSCs (G-MDSCs) or Gr-1^{med} Ly6C^{high} monocytic MDSCs (M-MDSCs) (G-MDSCs) [9]. Interestingly, the proportions of M-MDSCs in CT26 tissues were significantly suppressed in aged BALB/c mice compared to young mice, whereas those of M-MDSCs were increased in aged C57BL/6 mice. Such changes were not observed in the proportions of G-MDSCs. In terms of T cells, the proportions of CD8$^+$ T cells were significantly suppressed in CT26 tissues of aged BALB/c mice, whereas they increased in MC38 tissues of aged C57BL6 mice. CD4$^+$ T cells increased in tumor tissues from both aged mice.

3.8. *Dependency of in vitro splenic T cell and cancer cell proliferation on L-arginine*

Next, we examined how L-arginine influences *in vitro* proliferation of splenic T cells and cancer cells. Spleen cells from young and aged BALB/c and C57BL/6 mice were cultured *in vitro* with anti-CD3 and anti-CD28 antibodies at the indicated levels of L-arginine. The cell viability of spleen cells from young BALB/c mice started to decrease at a dose of 57.5 (μM) L-arginine, and was higher in aged BALB/c than young mice (Fig. 8A). By contrast, cell viability was lower in aged C57BL/6 than young mice. CT26 and MC38 cell viability began to decrease at an L-arginine dose of 143.8 (μM), with no difference seen between the two cancer cell lines. Cancer cells were less dependent on L-arginine than

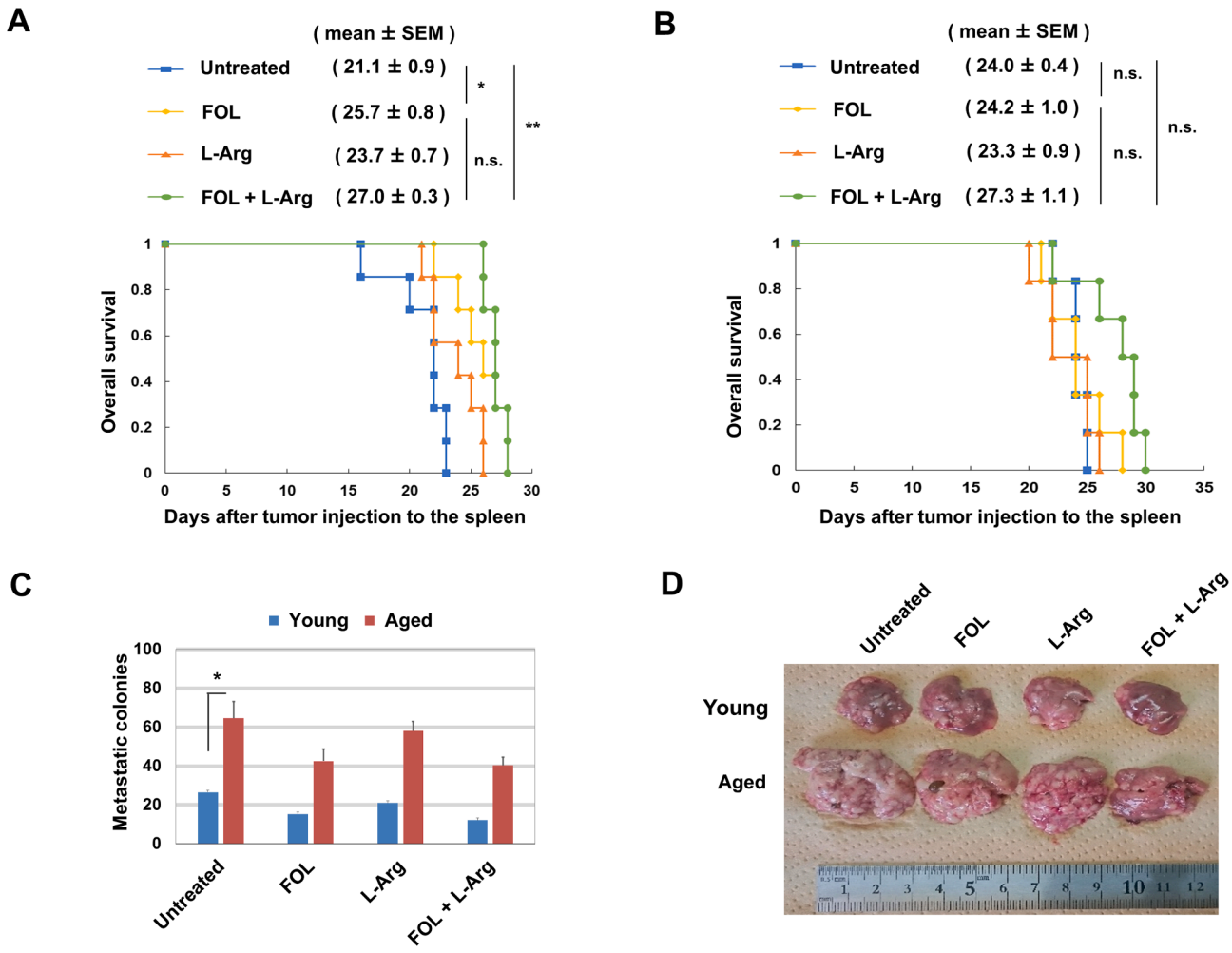

Fig. 5. Antitumor effects of chemotherapy with L-arginine supplementation in aged mice with liver metastasis. (A) Young and **(B)** aged BALB/c mice were injected with 2.0×10^4 LuM1 cells into the spleen. On days 8 and 16, 5-FU (50 mg/kg) and L-OHP (6 mg/kg) were injected i.p. From day 8, L-arginine (30 mg/young mouse and 45 mg/aged mouse) was orally administered daily. Survival was assessed over time and illustrated by Kaplan–Meier plots. $^*P < 0.05$, $^{**}P < 0.01$ (log-rank test). n.s., not significant. **(C)** Young and aged BALB/c mice (n = 6) were injected with 2.0×10^4 LuM1 cells into the spleen and treated by the protocol described above. On day 19, mice were euthanized and colonies (> 1 mm) on the liver surface were counted microscopically. $^*P < 0.05$ (Mann-Whitney U test). **(D)** Representative photographs of liver metastases. FOL, 5-FU + L-OHP; CP, cyclophosphamide; L-Arg, L-arginine.

splenic T cells. In the *in vitro* assays, FCS was added into both media because of its influence on cell proliferation. However, as shown in Fig. 6, the level of L-arginine in mouse plasma was 150–200 μM, suggesting that 10% FCS contains 15–20 μM L-arginine. Therefore, the level of L-arginine in 10% FCS was likely negligible in the *in vitro* culture.

3.9. Effects of L-arginine on tumor-specific CTLs in vitro and in vivo

To elucidate the mechanisms of enhancing antitumor effects by L-arginine administration, we examined effects of L-arginine on tumor-specific CTLs *in vitro* and *in vivo*. In the *in vitro* assay, the induction of tumor-specific (AH1 tetramer$^+$) CTLs and their number were dependent on L-arginine doses (Fig. 8B and Suppl Fig. 4). Lower doses of L-arginine decreased the numbers of tumor-specific CTLs. In addition, analyses on tumor-infiltrating immune cells in CT26-bearing aged mice revealed that chemoimmunotherapy with L-arginine, but not chemoimmunotherapy, significantly increased the proportions of CTLs in tumors ($P < 0.01$ vs. untreated) (Fig. 8C and Suppl Fig. 3C). By contrast, L-arginine administration alone increased the proportions of both MDSCs, whereas combination with chemoimmunotherapy and L-arginine abolished this effect.

4. Discussion

In tumor-bearing aged hosts, Tregs and MDSCs are increased and T cell effector functions are inhibited. Therefore, we compared the therapeutic effects of immunogenic chemotherapy with 5-FU/L-OHP/CP between young and aged mice using two mouse colon cancer models. In both models, therapeutic efficacy of 5-FU/L-OHP/CP was observed in colon cancer-bearing young mice, but not in aged mice. In CT26-bearing aged mice, 5-FU/L-OHP/CP therapy suppressed tumor growth, but not significantly (Fig. 1E). In MC38-bearing aged mice, 5-FU/L-OHP/CP therapy showed no clear anti-tumor effect (Fig. 3C, D). These results led us to further examine the effects of supplementing the combined therapy with L-arginine and/or anti-PD-1 blockade therapy.

We previously reported that L-arginine supplementation significantly augments the antitumor effect of chemotherapy in CT26-bearing young mice and the chemotherapy/anti-PD-1-induced antitumor effect in MC38-bearing young mice [14]. To this end, the primary aim of this study was to determine whether the effect of L-arginine could occur in cancer-bearing aged mice because cancer-bearing young mice do not necessarily reflect the clinical situation in that most cancer patients are aged. As a result, we found impaired antitumor responses in aged mice compared to young mice receiving chemoimmunotherapy and that L-arginine administration just modulated the effect. Although L-arginine

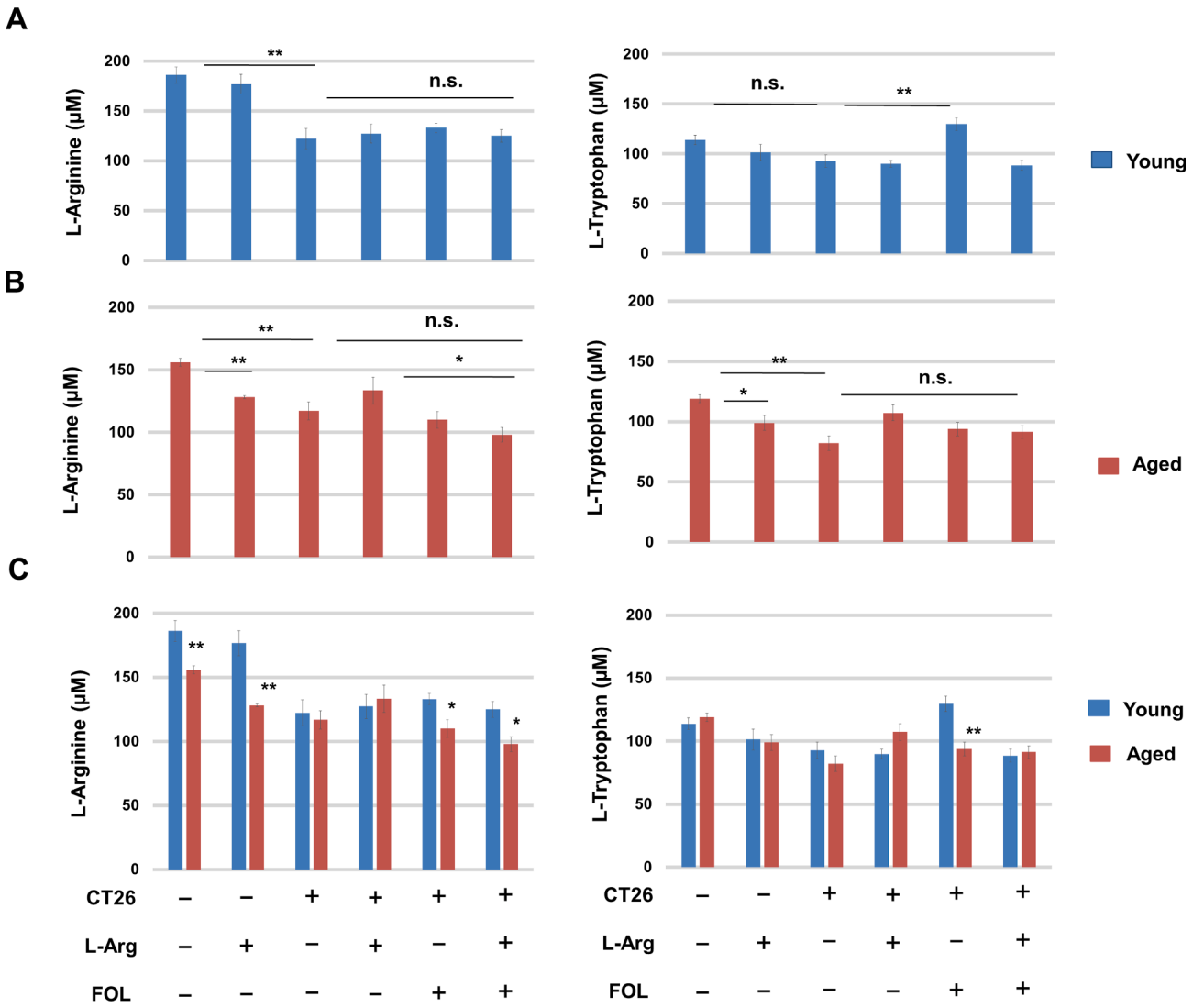

Fig. 6. Plasma L-arginine and L-tryptophan levels in young and aged CT26-bearing mice, with or without chemotherapy or L-arginine supplementation. Young and aged BALB/c mice were injected s.c. with 5.0×10^5 CT26 cells, into the right flank. On days 12 and 20, 5-FU (50 mg/kg) and L-OHP (6 mg/kg) were injected i.p. Oral administration of L-arginine (30 mg/young mouse and 45 mg/aged mouse) was started on day 12 and continued until the mice were sacrificed. On day 25, peripheral blood was collected, and plasma was prepared individually. Plasma L-arginine and L-tryptophan levels of young and aged mice were measured by liquid chromatography–mass spectrometry (**A** and **B**). $^*P < 0.05$, $^{**}P < 0.01$ (ANOVA). n.s., not significant. (**C**) Data for young and aged mice were pooled. Data are means ± SEM of six mice. $^*P < 0.05$, $^{**}P < 0.01$ (Mann-Whitney U test). L-Arg, L-arginine ; FOL, 5-FU + L-OHP.

monotherapy showed no effect on the *in vivo* tumor growth, complete cure was observed only when chemoimmunotherapy was combined with L-arginine supplementation in CT26-bearing aged mice (Figs. 1C and 2A).

Tumor-specific CTLs were generated from both CT26-cured and MC38-cured aged mice (Fig. 4A, B). In support of this finding, we previously reported that combination therapy with 5-FU/L-OHP/CP significantly attenuated tumor growth in CT26-bearing nude mice [27], indicating that tumor-specific CTLs play a key role in therapeutic efficacy. In addition, we showed that spleen cells from the non-progressing group showed higher cytotoxicity against CT26 than did those from the progressing group after all combination therapy (Fig. 4C, D). These results indicate that tumor-specific CTLs and/or precursors were present in tumor-non-progressing mice after combination therapy. These results also suggest that the usefulness of L-arginine supplementation of chemoimmunotherapy in cancer-bearing aged hosts may be due, at least in part, to its effect on CTLs.

Although many treatments for liver metastasis of colorectal cancer have been developed, few patients have achieved a complete response [21]. Using a highly metastatic LuM1 cell line derived from CT26 [29], we compared the therapeutic effect of 5-FU/L-OHP therapy with L-arginine supplementation, with respect to liver metastasis, between young and aged mice (Fig. 5). In young mice, 5-FU/L-OHP therapy significantly extended overall survival ($P < 0.05$ vs. untreated), whereas additional supplementation of L-arginine further prolonged survival ($P < 0.01$ vs. untreated). By contrast, therapy with 5-FU/L-OHP and L-arginine supplementation prolonged survival compared with the untreated control in aged mice (27.3 vs. 24.0 days), albeit not significantly. In addition, untreated aged mice had significantly more metastatic colonies in the liver than young mice (Fig. 5C). Although chemotherapy with or without L-arginine supplementation showed a tendency to decrease the metastatic colonies, statistical significance was not observed. We observed inconsistent results between the overall survival and metastatic colonies in the liver. Because all cancer-bearing mice exhibited bleeding in the peritoneal cavity, this observation could heavily influence on the survival.

L-arginine is a non-essential amino acid widely used as a food additive and dietary supplement; it promotes to the proliferation and

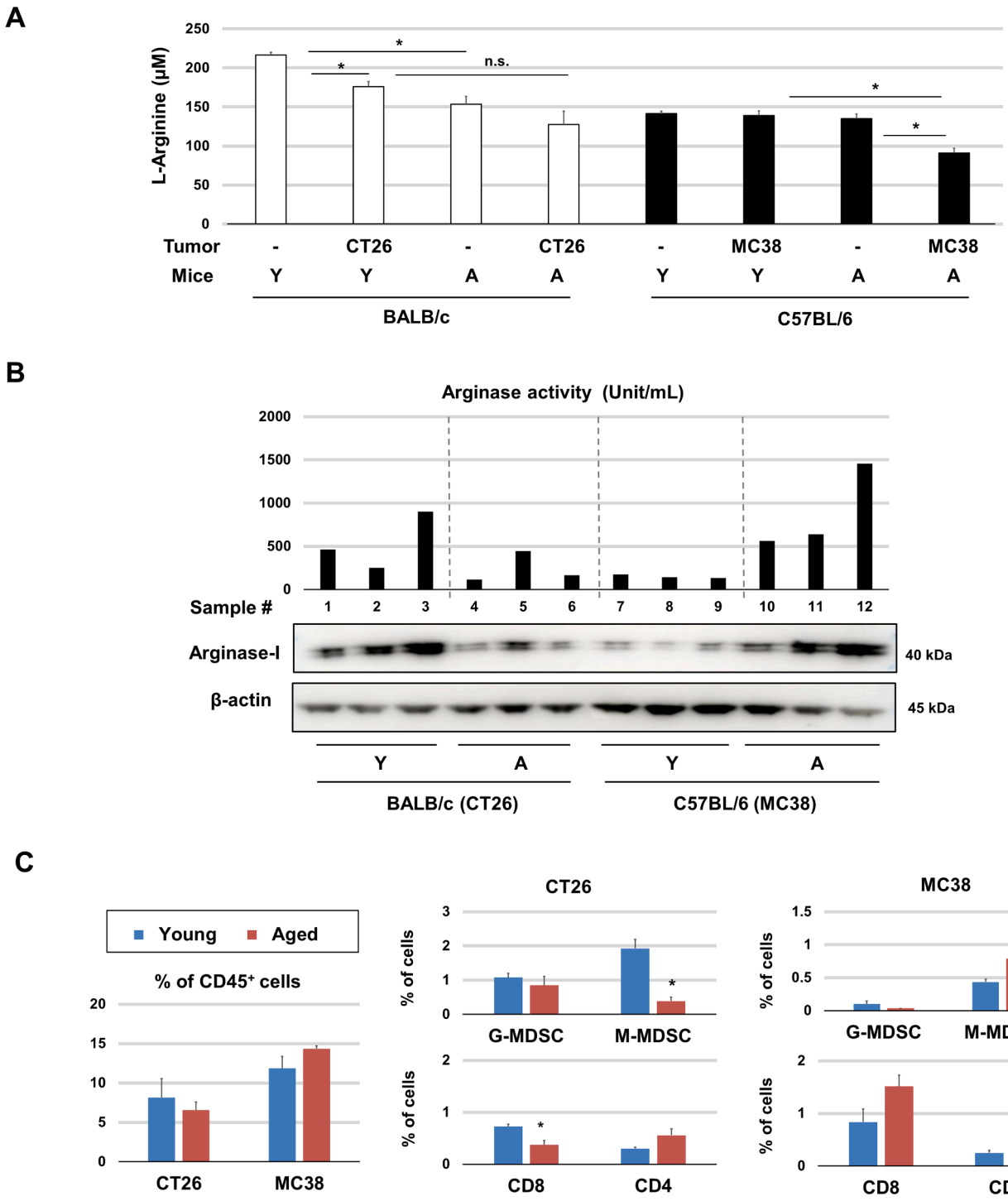

Fig. 7. The plasma levels of L-arginine, arginase activity, arginase-I expression, and tumor-infiltrating immune cells in tumor tissues of young and aged mice. (A) Young and aged BALB/c mice were injected s.c. with 5.0×10^5 CT26 cells, into the right flank. Similarly, Young and aged C57BL/6 mice were injected s.c. with 5.0×10^5 MC38 cells. On day 25, peripheral blood was collected individually, and plasma L-arginine levels were measured. $^*P < 0.05$ (Mann-Whitney U test). n. s., not significant. Data are means ± SEM of four mice. (**B**) Harvested tumor tissues (n = 3) were examined for their arginase activity and the protein expression of arginase-I. β-Actin was used as a control. (**C**) Tumor-infiltrating immune cells, including G-MDSCs, M-MDSCs, $CD8^+$ T cells, and $CD4^+$ T cells, were examined by flow cytometry. Data are means ± SEM of four mice. $^*P < 0.05$ (Mann-Whitney U test).

activation of T cells. MDSCs that increase in cancer-bearing and aged hosts express arginase I, which is an arginine-degrading enzyme, and decompose L-arginine [32,33]. We measured plasma L-arginine and L-tryptophan levels in young and aged mice that were either naïve or CT26-bearing, and received L-arginine supplementation and/or 5-FU/L-OHP chemotherapy (Fig. 6). The CT26-bearing state decreased L-arginine levels similarly in young and aged mice, but that L-tryptophan levels decreased only in aged mice. L-arginine levels of aged mice were lower than those of young mice (Figs. 6C and 7A). We had expected L-arginine supplementation to restore L-arginine levels decreased in the cancer-bearing state; however, no apparent increases were observed. We did not measure the levels of these amino acids in tumor sites. However, given that MDSC-derived arginase and MDSC/tumor-derived IDO degrade L-arginine and L-tryptophan, respectively [9,10], the levels of

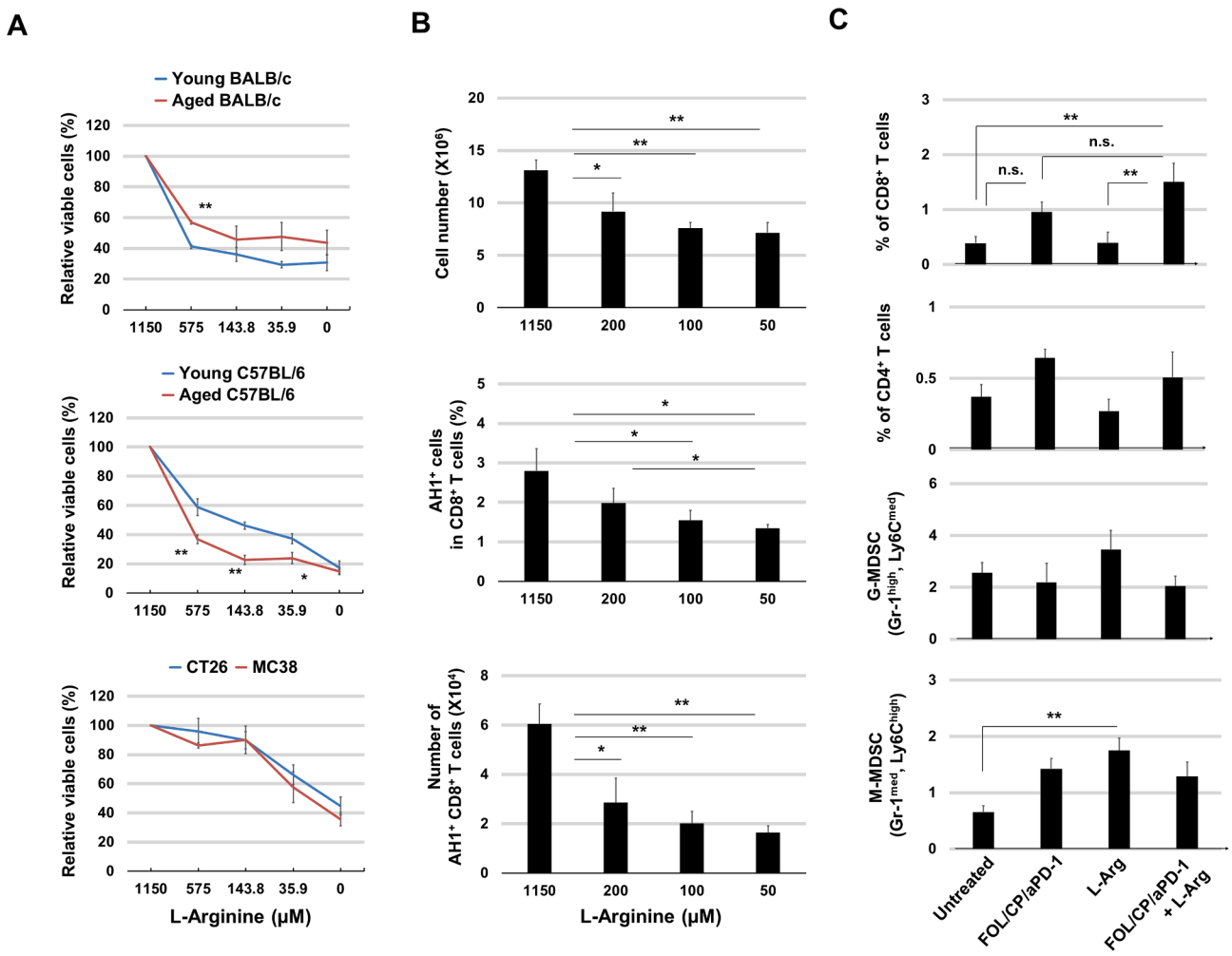

Fig. 8. Effects of L-arginine on tumor-specific CTLs *in vitro* and tumor-infiltrating immune cells *in vivo*. (A) (Upper and Middle) Spleen cells from young and aged BALB/c and C57BL/6 mice were cultured in flat 96-well plates pre-coated with anti-CD3 mAb (3 μg/mL) using RPMI 1640 medium with soluble anti-mouse CD28 mAb (1 μg/mL) and the indicated doses of L-arginine. (Lower) CT26 and MC38 cells were cultured in flat 96-well plates using RPMI 1640 medium with the indicated doses of L-arginine. After 3 days, cell viability was evaluated using Cell Counting Kit-8. Data are means ± SD of four wells. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). **(B)** The spleen cells from young BALB/c mice, which were immunized with doxorubicin-treated 1×10^6 CT26 cells 2 weeks before, were cultured with the AH1 peptide (10 μg/mL) and IL-2 (20 U/mL) at the indicated doses of L-arginine for five days. After staining with a PE-conjugated AH1 peptide tetramer and FITC-conjugated anti-CD8 mAb, flow cytometric analysis was performed. Data are means ± SD of three wells. $*P < 0.05$, $**P < 0.01$ (Student's *t*-test). **(C)** Aged BALB/c mice were injected s.c. with 5×10^5 CT26 cells, into the right flank, and treated with the same protocol described in Fig. 1C. On day 25, tumor-infiltrating immune cells were examined by flow cytometry individually. Data are means ± SEM of five mice. $**P < 0.01$ n.s., not significant. (ANOVA). FOL, 5-FU + L-OHP; CP: cyclophosphamide; L-Arg, L-arginine.

these amino acids could be dissociated between the blood and the tumor site.

In our previous study using CT26-bearing young mice [14], L-arginine administration showed no effect on the proportions of $CD4^+$ T cells, $CD8^+$ T cells, and MDSCs at spleen and tumor sites. Although we did not compare MDSC and Tregs between young and aged mice in this study, we previously compared MDSCs in tumor sites and found that infiltration of $CD45^+$ immune cells was promoted in CT26-bearing aged mice compared to young mice and found no difference in their proportion [3]. In addition, MDSC and Tregs are reportedly increased in cancer-bearing aged mice compared to young mice, as described in Introduction [5–8]. In terms of the mechanism by which L-arginine administration modulated anti-tumor effects in tumor-bearing mice, Cao *et al.* reported that L-arginine administration can decrease MDSC in tumor sites of 4T1 mammary carcinoma-bearing mice [34]. In addition, Geiger *et al.* reported that L-arginine can modulate T cell metabolism from glycolysis to oxidative phosphorylation and enhance their survival in vivo [35]. However, these experiments were done using young mice. In this study, we showed that the induction of tumor-specific (tetramer$^+$) CTLs and their cell numbers were dependent on L-arginine doses *in vitro* (Fig. 8B), and that chemoimmunotherapy with L-arginine, but not chemoimmunotherapy alone, significantly increased the proportion of CTLs in tumors (Fig. 8C). Based on these results, L-arginine administration could promote the induction/proliferation of tumor-specific CTLs and their infiltration into tumor sites.

The viability of tumor cells and splenocytes in young mice is dependent on L-arginine concentration [14,36]. Therefore, we examined the dependency of splenic T cells from young and aged mice and cancer cells on L-arginine concentrations. We found that splenic T cells were more sensitive to L-arginine than colon cancer cells (Fig. 8A). Plasma L-arginine levels ranged from 100 to 150 μM (Fig. 6). As shown in Fig. 8A, these plasma doses had no influence on the proliferation of cancer cells, but heavily influenced that of T cells. Unexpectedly, the L-arginine dependency of splenic T cells from young and aged mice was inconsistent between BALB/c and C57BL/6 mice; splenic T cells from young BALB/c mice were more sensitive to L-arginine deficiency than those from aged BALB/c mice, and vice versa in splenic T cells from C57BL/6 mice. Although we have no explanation for this result, the

patterns of immune responses and proliferative capacity of T cells may explain the difference, as Th1- and Th2-type immune responses are dominant in C57BL/6 and BALB/c mice, respectively [37].

We compared the arginase activity, the expression of arginase-I, and MDSCs in CT26 and MC38 tumor tissues (Fig. 7). Unexpectedly, compared to young mice, both arginase activity and the arginase-I expression were decreased in CT26-bearing aged BALB/c mice. By contrast, they increased in aged MC38-bearing C57BL/6 mice. These results were compatible with the proportion of M-MDSCs in tumor sites. Given that M-MDSCs have a greater suppressive effect than G-MDSCs on antigen-stimulated $CD8^+$ T cells [38], these results indicate that arginase-I is higher in M-MDSCs than in G-MDSCs. On the other hand, MC38 is known as an immunogenic tumor with T cell infiltration [26]. Interestingly, the infiltration of CTLs into MC38 tissues was higher in aged C57BL/6 mice (Fig. 7C). These results suggest that age-associated impairment was less in C57BL/6 mice, compared to BALB/c mice. This may account for a significant response to anti-PD-1 antibody therapy in MC38-bearing aged C57BL/6 mice (Fig. 3B).

In conclusion, we demonstrated that immunogenic chemotherapy was less effective in colon cancer-bearing aged mice, whereas additional therapy with anti-PD-1 antibody and L-arginine enhanced the therapeutic effect, even in aged hosts. Our results suggest that L-arginine supplementation can modulate the therapeutic efficacy of chemoimmunotherapy via its effect on tumor-specific CTLs. Given that L-arginine is safe and inexpensive, it may be a useful supplementary drug in clinical practice.

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Author Contributions

Conceived and designed the experiments: KI, TT, YT, MH. Performed the experiments: KI, HK, YN, MH. Analyzed the data: YI, HK, MH. Wrote the paper: KI, MH.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2022.109423.

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