

Cancer-associated fibroblast-targeted strategy enhances antitumor immune responses in dendritic cell-based vaccine

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Given the close interaction between tumor cells and stromal cells in the tumor microenvironment (TME), TME-targeted strategies would be promising for developing integrated cancer immunotherapy. Cancer-associated fibroblasts (CAFs) are the dominant stromal component, playing critical roles in generation of the pro-tumorigenic TME. We focused on the immunosuppressive trait of CAFs, and systematically explored the alteration of tumor-associated immune responses by CAF-targeted therapy. C57BL/6 mice s.c. bearing syngeneic E.G7 lymphoma, LLC1 Lewis lung cancer, or B16F1 melanoma were treated with an anti-fibrotic agent, tranilast, to inhibit CAF function. The infiltration of immune suppressor cell types, including regulatory T cells and myeloid-derived suppressor cells, in the TME was effectively decreased through reduction of stromal cell-derived factor-1, prostaglandin E₂, and transforming growth factor- β . In tumor-draining lymph nodes, these immune suppressor cell types were significantly decreased, leading to activation of tumor-associated antigen-specific CD8⁺ T cells. In addition, CAF-targeted therapy synergistically enhanced multiple types of systemic antitumor immune responses such as the cytotoxic CD8⁺ T cell response, natural killer activity, and antitumor humoral immunity in combination with dendritic cell-based vaccines; however, the suppressive effect on tumor growth was not observed in tumor-bearing SCID mice. These data indicate that systemic antitumor immune responses by various immunologic cell types are required to bring out the efficacy of CAF-targeted therapy, and these effects are enhanced when combined with effector-stimulatory immunotherapy such as dendritic cell-based vaccines. Our mouse model provides a novel rationale with TME-targeted strategy for the development of cell-based cancer immunotherapy.

Tumor tissues contain heterogeneous cell types consisting of tumor cells and tumor stromal cells. Most tumor stromal cells, such as immune suppressor cells, vascular endothelial cells, lymphatic endothelial cells, and fibroblasts, help to maintain pro-tumorigenic homeostasis in the tumor microenvironment (TME).⁽¹⁾ The interaction between tumor cells and tumor stromal cells affects the aggressive motility of tumor cells, which is responsible for the spread of tumors and metastases. Accumulation of immune suppressor cells including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in the TME is common in human tumor tissues,^(2–4) leading to suppression of antitumor immune responses with a subsequently poor prognosis.^(5–7)

Cancer immunotherapy has been expected to be a promising treatment strategy against solid tumors; however, its benefit remains insufficient to eradicate tumors. The biological complexity of the TME seems to be an obstacle for cancer immunotherapy, suggesting that the simple strategy in which only tumor cells are targeted is hardly adequate to overwhelm the aggressively growing tumor; therefore, a synergistic TME-

targeted strategy should be required for the development of more potent cancer immunotherapy.

Among tumor stromal cell types, cancer-associated fibroblasts (CAFs) are the dominant component in the TME, and play critical roles in promoting tumor progression.⁽⁸⁾ Cancer-associated fibroblasts support tumor growth through secretion of several soluble factors such as epidermal growth factor, monocyte chemoattractant protein-1, hepatocyte growth factor, and stromal cell-derived factor-1 (SDF-1), which contribute to proliferation, invasion, and metastasis of tumor cells.^(9–12) In addition, CAFs have recently been reported to function as modulators of immune systems through secretion of transforming growth factor- β (TGF- β) and other immune suppressive cytokines, creating the milieu in which antitumor immune responses are impaired.⁽¹³⁾ In this context, CAFs have emerged to be novel targets of cancer immunotherapy; however, it remains unclear whether antitumor immune responses would be enhanced by anti-CAF therapy. In this study, we investigated whether a CAF-targeted strategy would improve the locoregional and systemic antitumor immune responses.

As a possible agent for this CAF-targeted strategy, we focused on tranilast, an anti-allergic or antifibrotic agent that has been used clinically. Tranilast is known to suppress proliferation of fibroblasts derived from several types of normal tissues,^(14–17) and decrease release of TGF- β from fibroblasts.⁽¹⁸⁾ We previously reported that tranilast served as a specific inhibitor of CAFs *in vitro*,⁽¹⁹⁾ showing that tranilast at doses under 100 μ M inhibited the proliferation of CAFs and reduced the levels of SDF-1, TGF- β 1, vascular endothelial growth factor (VEGF), interleukin (IL)-6, and prostaglandin E₂ (PGE₂) from CAFs in a dose-dependent manner. However, tranilast exerted no inhibitory effects on immune cells or murine tumor cell lines, such as E.G7, LLC1, and B16, at doses under 100 μ M. The induction of Tregs and MDSCs from their progenitor cells were suppressed in medium in which CAFs had been cultured in the presence of tranilast.

Here we investigated whether *in vivo* inhibition of CAF function would improve locoregional and systemic antitumor immune responses in a tumor-bearing mouse model. Furthermore, we report that a CAF-targeted strategy synergistically enhances multiple types of antitumor immune responses in combination with a tumor-associated antigen (TAA)-loaded dendritic cell (DC)-based vaccine in mice bearing several types of tumors.

Materials and Methods

Mice, cells, and reagents. Female C57BL/6J and SCID mice aged 6 weeks were purchased from Japan SLC (Hamamatsu, Japan) and CLEA Japan (Tokyo, Japan), respectively. The mice were maintained under specific pathogen-free conditions. All mouse experiments were carried out in compliance with the Guidelines for Animal Experimentation from Shiga University of Medical Science (Shiga, Japan).

The mouse lymphoma cell line E.G7 that expresses ovalbumin (OVA) and the natural killer (NK) cell-sensitive cell line YAC-1 were purchased from ATCC (Manassas, VA, USA), and were passaged for fewer than 6 months. The mouse Lewis lung carcinoma cell line LLC1 and the mouse melanoma cell line B16F1 were provided by the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan).

Tranilast (N-[3,4-dimethoxycinnamoyl]-anthranilic acid) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO at a concentration of 25 mM as a stock solution.

Tumor-bearing mouse models and *in vivo* CAF inhibition. Female C57BL/6J mice were inoculated s.c. in the right flank with 5×10^5 tumor cells. Seven days after tumor inoculation, when s.c. tumors had grown to 5–7 mm in diameter, the mice were randomly grouped. To inhibit the function of CAFs, some groups of mice were treated with 100 μ L of 200 μ M tranilast directly into the established tumor every day for 2 weeks. Mice in control groups were given 0.8% DMSO. Along with the DC-based vaccines, some groups of mice were s.c. administered 1×10^6 TAA-loaded DCs suspended in 100 μ L PBS near the tumor on days 7, 13, and 19. Five days after the final administration of tranilast, the tumor tissues, tumor-draining lymph nodes (TDLNs), spleens, and sera were harvested from mice. Subcutaneous lymph nodes at the right flank of normal mice were harvested as a counterpart of TDLNs in tumor-bearing mice and used in the following experiments. Volumes of tumors harvested from the mice were calculated using the following formula: length \times width²/2. Unless specified otherwise, each experimental or control group comprised five mice.

Western blot analysis. Tumors harvested from the mice were lysed with lysis buffer (1 mM EDTA, 20 mM Tris-HCl in

distilled water). The protein of tumor lysate (5 μ g/lane for α -smooth muscle actin [α -SMA] or 75 μ g/lane for SDF-1) was subjected to 7.5% SDS-PAGE and transferred to a PVDF membrane. The membrane was incubated with rabbit polyclonal anti-mouse α -SMA (1:5000; Abcam, Cambridge, UK) or rabbit polyclonal anti-mouse SDF-1 (1:3000; Abcam) antibodies, followed by the standard Western blotting procedure, as described previously.⁽²⁰⁾

Quantification of levels of PGE₂ and TGF- β 1. The levels of PGE₂ and TGF- β 1 in tumor tissues were measured using Parameter Prostaglandin E₂ Assay and TGF- β 1 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), respectively.

Preparation of DC-based vaccine. The DC-based vaccine was prepared as described previously.⁽²¹⁾ Bone marrow cells from the femurs of C57BL/6J mice were cultured for 7 days in the presence of recombinant mouse granulocyte macrophage colony-stimulating factor and IL-4 (R&D Systems) with final concentrations of both of 20 ng/mL. Induced immature DCs were matured in culture for 24 h in the presence of 0.1 KE/mL OK432 (Chugai Pharmaceutical Co., Tokyo, Japan). To prepare the DC-based vaccine, mature DCs were pulsed with 1 μ M TAA-derived MHC class I peptides, SIINFEKL for E.G7, EGSRNQDWL for B16F1, and FEQNTAQP and FEQNTAQA for LLC1,⁽²²⁾ at 37°C for 2 h.

Immunohistochemistry. The tumor tissues were frozen in the optimal cutting temperature compound, sliced, and fixed with ethanol. The sections were incubated with rabbit polyclonal anti-mouse α -SMA antibody (1:200; Abcam) for the detection of CAFs, or anti-mouse Foxp3 antibody (clone, FJK-16s, 1:50; eBioscience, San Diego, CA, USA) for the detection of Tregs, followed by incubation with biotinylated anti-rabbit IgG (1:200 dilution). For the detection of MDSCs, the sections were incubated with FITC-conjugated anti-CD11b (clone, M1/70) and phycoerythrin (PE)-conjugated anti-Gr-1 (clone, Ly-6G) antibodies, and examined using a BX-61 fluorescent microscope (Olympus, Tokyo, Japan).

Flow cytometry. To detect Tregs, cells were stained with PE-conjugated anti-CD4 (clone, L3T4), FITC-conjugated anti-CD25 (clone, 7D4), and allophycocyanin-conjugated anti-Foxp3 (clone, FJK-16s) antibodies using an anti-mouse/rat Foxp3 staining set (eBioscience), according to the manufacturer's instructions. To detect MDSCs, cells were stained with FITC-conjugated anti-CD11b (clone, M1/70; eBioscience) and PE-conjugated anti-Gr-1 (clone, Ly-6G; eBioscience) antibodies.

To evaluate TAA-specific CD8⁺ T cell responses that had been elicited in the mice, TDLN cells or spleen cells were stimulated with or without 1 μ M OVA-derived MHC class I epitope peptide (SIINFEKL) for 16 h. The cells were stained with PE-conjugated anti-CD8 (clone, Ly-2; eBioscience) and FITC-conjugated anti- γ -interferon (IFN- γ) antibodies (clone, XMG1.2; eBioscience), as previously described.⁽²¹⁾

The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The data were visualized as dot blots using CellQuest software (BD Biosciences).

Cytotoxicity assay. Spleen cells were stimulated with 1 μ M OVA-derived MHC class I epitope peptide (SIINFEKL) in the complete medium containing 1 ng/mL recombinant mouse IL-2 (Wako Pure Chemical Industries, Osaka, Japan) for 5 days. Then, the cells were cultured with 1×10^4 E.G7 or YAC-1 cells at various effector/target ratios for 4 h. The amount of lactate dehydrogenase released from lysed target cells in the

supernatants was estimated using a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA).

Statistical analysis. Statistical significance of the differences between groups was analyzed using Student's *t*-test. *P*-values lower than 0.05, 0.01, and 0.001, considered statistically significant, are indicated as *, **, and ***, respectively, in each figure. Representative results from three independent experiments that produced similar results are shown.

Results

Reduction of immune suppressor cells in TME through inhibition of CAF function. To inhibit CAF function *in vivo*, we administered tranilast into the established E.G7 tumors in tumor-bearing mice. Immunohistochemistry of tumor tissues showed that intratumoral administration of vehicle (DMSO) alone did not reduce the number of α -SMA⁺ cells compared with that in the case of PBS treatment alone, whereas the number of α -SMA⁺ cells was significantly reduced after repeated intratumoral administrations of tranilast compared with that

after vehicle treatment ($P < 0.001$) (Figs 1a,S1). Concerning soluble factors secreted from CAFs, the level of SDF-1 in tumor tissues was significantly decreased, associated with reduction of CAFs (anti-CAF vs vehicle, $P = 0.007$) (Figs 1b, S2). Furthermore, levels of immunosuppressive cytokines such as PGE₂ and TGF- β 1 in tumor tissues were also significantly reduced by inhibition of CAF function (anti-CAF vs vehicle, $P < 0.05$, respectively) (Fig. 1c,d).

In the tumor-bearing mice, we examined the infiltration of CD4⁺ cells and CD8⁺ T cells in the TME. The numbers of CD4⁺ cells as well as CD8⁺ cells were significantly increased in tumor tissues of mice treated with anti-CAF therapy compared with that in control mice (anti-CAF vs vehicle, $P < 0.05$, respectively) (Fig. S3). Next, we investigated the association between CAFs and suppressor immune cells in the TME. The number of Foxp3⁺ Tregs was significantly reduced in tumor tissues of mice treated with anti-CAF therapy compared with that in control mice (anti-CAF vs vehicle, $P = 0.0043$) (Fig. 1e). The number of CD11b⁺Gr-1⁺ MDSCs in tumor tissues was also significantly reduced compared with

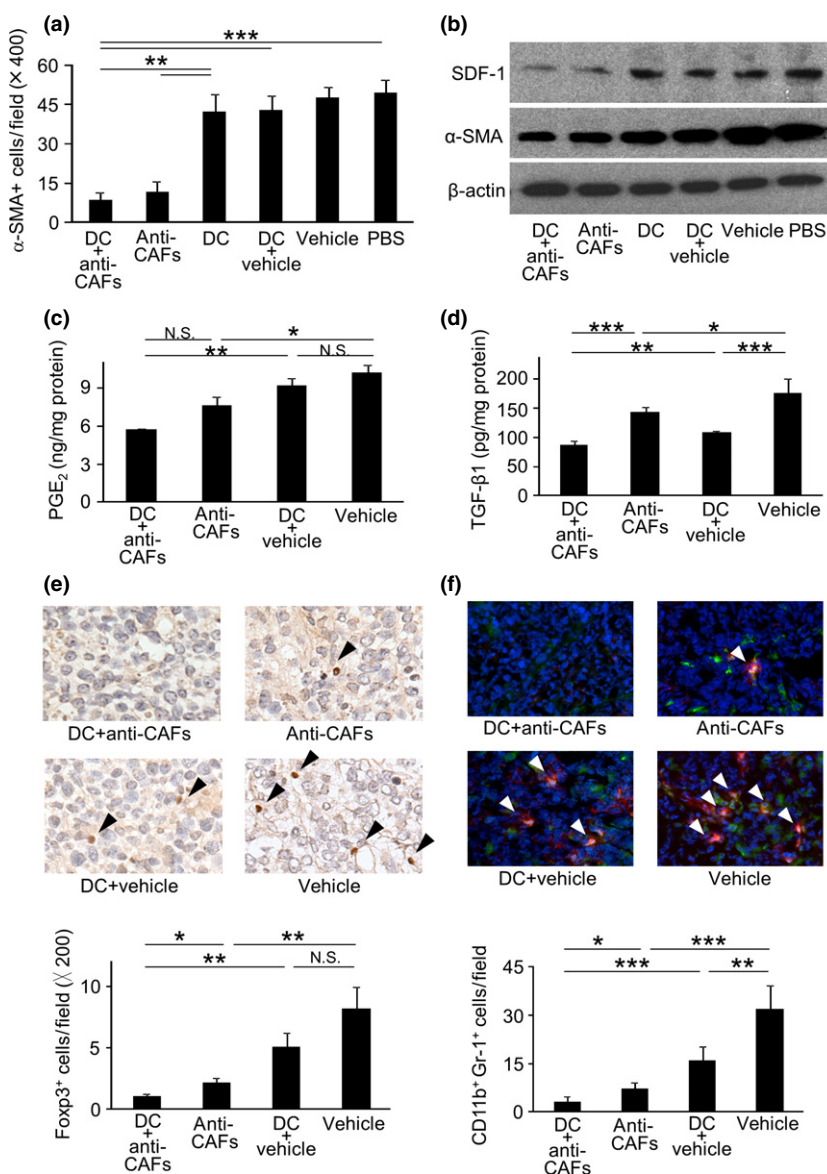


Fig. 1. Reduction of infiltrative suppressor immune cells in tumor microenvironment through inhibition of cancer-associated fibroblast (CAF) function. Mice bearing E.G7 tumors were treated with both tranilast and a dendritic cell (DC)-based vaccine. Five days after the final treatment, tumor tissues were harvested from the mice. (a) Numbers of α -smooth muscle actin (α -SMA)⁺ CAFs in the tumor tissues were determined by immunohistochemical staining. (b) Expression of stromal cell-derived factor-1 (SDF-1) in tumor tissues was analyzed by Western blotting. The levels of prostaglandin E₂ (PGE₂) (c) and transforming growth factor- β 1 (TGF- β 1) (d) in tumor tissues were measured by ELISA. Infiltration of Foxp3⁺ regulatory T cells (black arrowheads) (e) and Gr-1⁺ myeloid-derived suppressor cells (white arrowheads) (f) in tumor tissues was analyzed by immunohistochemical staining. Bars represent mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N.S., not significant.

that in control mice (anti-CAFs vs vehicle, $P < 0.001$) (Fig. 1f). When mice were treated with anti-CAF therapy combined with a TAA-loaded DC-based vaccine, the suppressive effect of anti-CAF therapy on migration of both Foxp3⁺ Treg cells (DC + anti-CAFs vs anti-CAFs, $P = 0.013$) and CD11b⁺Gr-1⁺ MDSCs (DC + anti-CAFs vs anti-CAFs, $P = 0.010$) in the TME was enhanced (Fig. 1e,f). These results indicate that CAFs are critically involved in the migration of immune suppressor cells.

Improvement of antitumor immune responses in TDLNs through inhibition of CAF function. Tumor-draining lymph nodes are critical sites for generation of antitumor immune responses as various soluble factors and immune suppressor cells flowing from the TME into TDLNs regulate the immune system.^(23,24) Indeed, TDLNs of tumor-bearing mice with vehicle treatment contained more immune suppressor cells than did those of normal mice without tumors (Fig. 2a,b). Thus, we examined whether the reduction of immune suppressor cells in the TME by inhibition of CAF function would affect the immune system in TDLNs. Our results revealed that anti-CAF therapy contributed to reduction of CD4⁺CD25⁺Foxp3⁺ Tregs (anti-CAFs vs vehicle, $P < 0.01$) (Figs 2a,S4) and CD11b

+Gr-1⁺ MDSCs (anti-CAFs vs vehicle, $P < 0.001$) (Fig. 2b) in TDLNs.

We then evaluated whether the reduction of suppressor immune cells contributed to activation of TAA-specific CD8⁺ T cells in TDLNs of E.G7-bearing mice. The TAA-specific CD8⁺ T cells that produced IFN- γ in TDLNs were increased in mice treated with anti-CAF therapy alone (anti-CAFs vs vehicle, $P = 0.023$), more significantly in mice that received the DC-based vaccine in combination with anti-CAF therapy (DC + anti-CAFs vs anti-CAFs, $P < 0.001$; DC + anti-CAFs vs DC + vehicle, $P < 0.001$) (Fig. 2c). These results indicated that the reduction of immune suppressor cells elicited enhancement of immune effector cell functions as antitumor immune responses in TDLNs.

Improvement of systemic cellular and humoral antitumor immune responses through anti-CAF therapy. Next, we examined whether systemic immune responses in spleens were improved by inhibition of CAFs, as seen in TDLNs. The percentage of CD4⁺CD25⁺Foxp3⁺ Tregs was reduced in spleens of mice treated with anti-CAF therapy, but with minimal effects (Fig. 3a). In contrast, the number of CD11b⁺Gr-1⁺ MDSCs was significantly reduced in spleens of mice treated

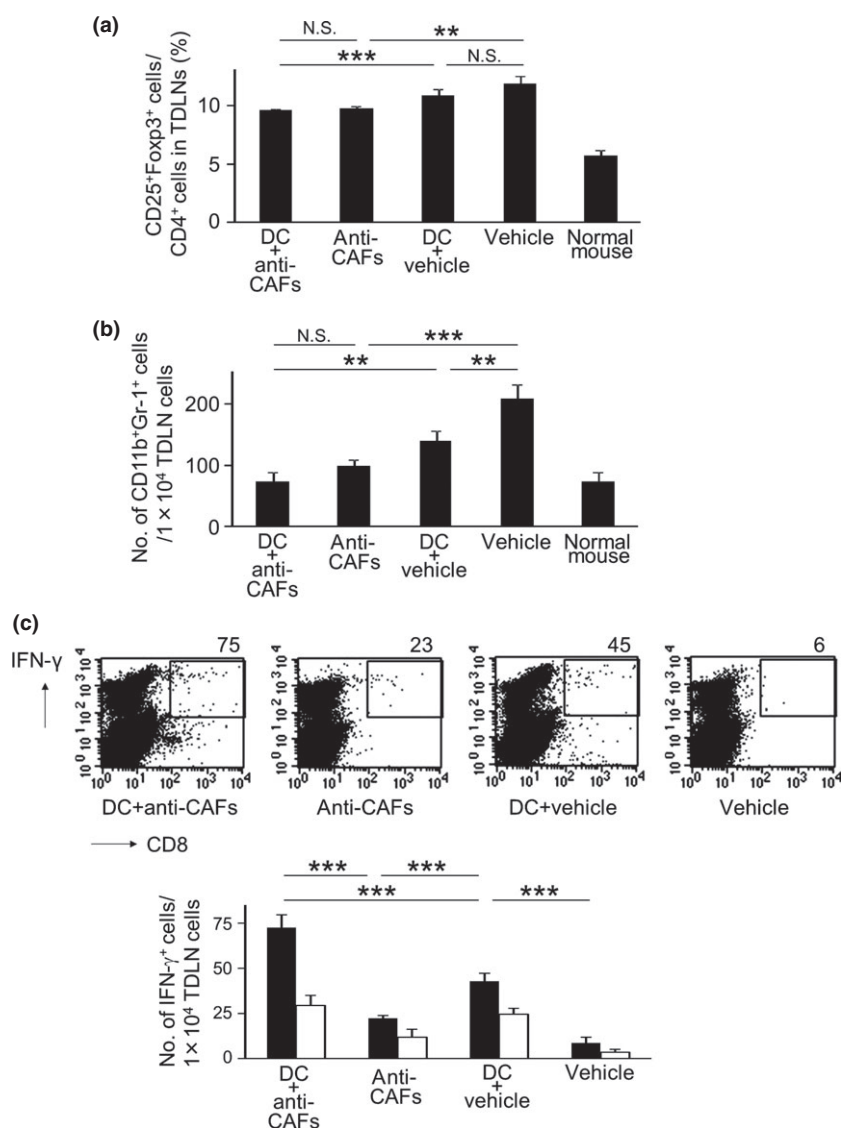


Fig. 2. Improvement of antitumor immune responses in tumor-draining lymph nodes (TDLNs) through inhibition of cancer-associated fibroblast (CAF) function. Mice bearing E.G7 tumors were treated with a dendritic cell (DC)-based vaccine in combination with or without anti-CAF therapy. Five days after the final treatment with tranilast, the populations of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (a) and CD11b⁺Gr-1⁺ myeloid-derived suppressor cells (b) in TDLNs were analyzed by flow cytometry. (c) TDLNs cells were stimulated with ovalbumin (tumor-associated antigen)-derived MHC class I peptide in vitro, then the population of interferon- γ ⁺CD8⁺ T cells was analyzed by flow cytometry (closed square, with tumor-associated antigen stimulation in vitro; open square, control). Bars represent mean \pm SD of three independent experiments. ** $P < 0.01$, *** $P < 0.001$. N.S., not significant.

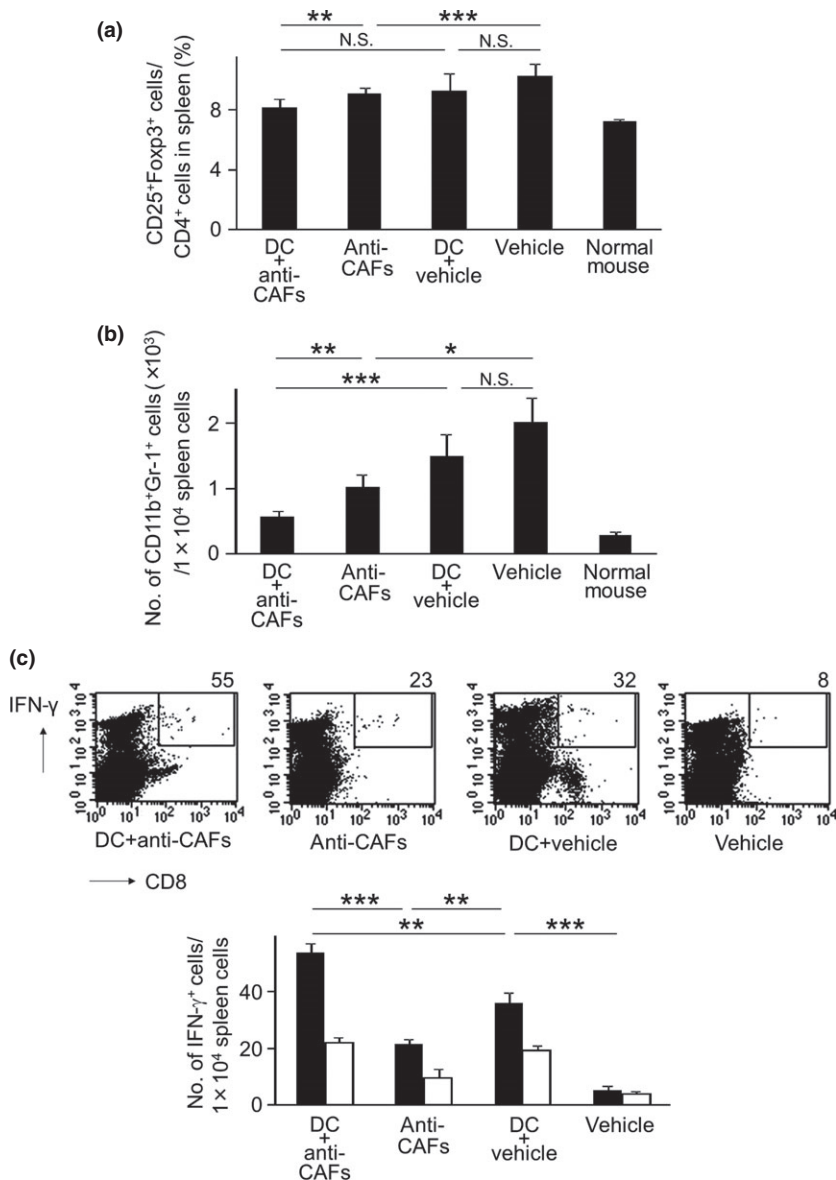


Fig. 3. Improvement of systemic antitumor immune responses associated with inhibition of cancer-associated fibroblast (CAF) function. Mice bearing E.G7 tumors were treated with a dendritic cell (DC)-based vaccine in combination with or without anti-CAF therapy. Five days after the final treatment with tranilast, the populations of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (a) and CD11b⁺Gr-1⁺ myeloid-derived suppressor cells (b) in spleen cells were analyzed by flow cytometry. (c) Spleen cells were stimulated with ovalbumin (tumor-associated antigen)-derived MHC class I peptide in vitro, then the population of interferon-γ⁺CD8⁺ T cells was analyzed by flow cytometry (closed square, with tumor-associated antigen stimulation in vitro; open square, control). Bars represent mean ± SD of three independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. N.S., not significant.

with anti-CAF therapy compared with those in control mice (anti-CAFs vs vehicle, *P* = 0.013) (Fig. 3b). Furthermore, the reduction of CD11b⁺Gr-1⁺ MDSCs in spleens was more significant in mice simultaneously treated with anti-CAF therapy and the DC-based vaccine than in mice treated with anti-CAF therapy alone (*P* = 0.0073).

We also investigated the effects of anti-CAF therapy on TAA-specific CD8⁺ T cell responses in spleens. The number of TAA-specific CD8⁺ T cells that produced IFN-γ in spleens was increased in mice treated with anti-CAF therapy compared with those in control mice (anti-CAFs vs vehicle, *P* < 0.001) (Fig. 3c). The DC-based vaccine activated TAA-specific CD8⁺ T cell responses in spleens (DC + vehicle vs vehicle, *P* < 0.001), whereas the combination of the DC-based vaccine and anti-CAF therapy had a synergistic effect on the activation of TAA-specific CD8⁺ T cells (DC + anti-CAFs vs anti-CAFs, *P* < 0.001; DC + anti-CAFs vs DC + vehicle, *P* = 0.0031). Corresponding to IFN-γ production by CD8⁺ T cells in the spleens, cytotoxic effects against E.G7 tumor cells were enhanced more significantly in mice simultaneously treated

with anti-CAF therapy and the DC-based vaccine than in mice treated with the DC-based vaccine alone (*P* < 0.001) (Fig. 4a).

We investigated whether other components of the immune system were improved in mice treated with anti-CAF therapy. Activity of NK cells was enhanced in mice treated with anti-CAF therapy compared with that in control mice (anti-CAFs vs vehicle, *P* = 0.0053) (Fig. 4b). The DC-based vaccine enhanced NK activity (DC + vehicle vs vehicle, *P* < 0.001) and induced a synergistic effect on activation of NK cells in combination with anti-CAF therapy (DC + anti-CAFs vs DC + vehicle, *P* < 0.001) (Fig. 4b). In terms of humoral immune responses against TAA, anti-OVA antibody levels were determined by ELISA⁽²⁵⁾, revealing that they were increased in sera of mice treated with anti-CAF therapy compared with those in control mice (anti-CAFs vs vehicle, *P* < 0.001) (Fig. 4c). Furthermore, humoral immune responses to the target antigen were effectively enhanced in mice treated with the DC-based vaccine in combination with anti-CAF therapy (DC + anti-CAFs vs DC + vehicle, *P* = 0.048). These results indicated that systemic cellular and humoral antitumor

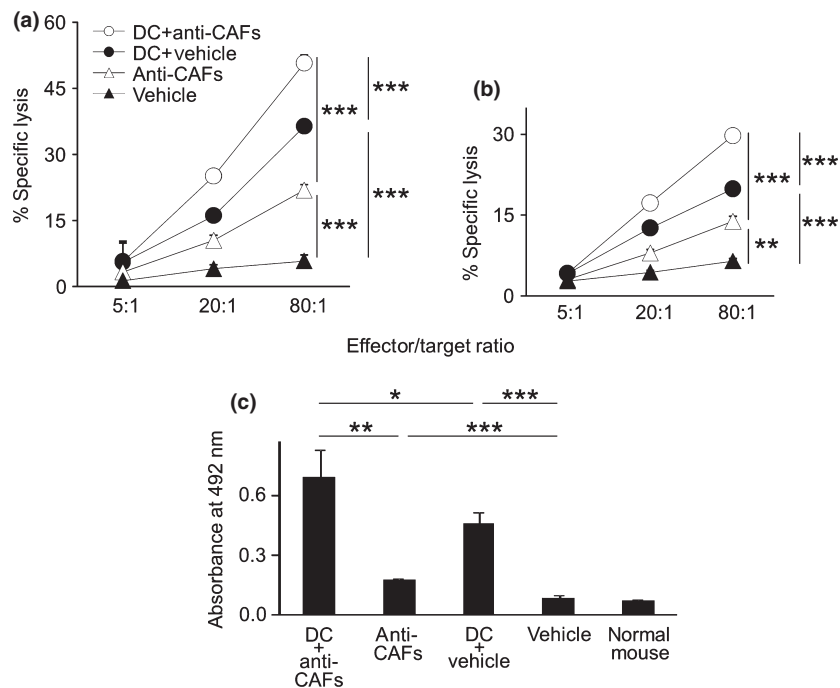


Fig. 4. Enhancement of multiple components of antitumor immune systems by inhibition of cancer-associated fibroblast (CAF) functions *in vivo*. Mice bearing E.G7 tumors were treated with a dendritic cell (DC)-based vaccine in combination with or without anti-CAF therapy. Five days after the final treatment with tranilast, spleens and sera were harvested from the mice. (a) Spleen cells were stimulated with ovalbumin (tumor-associated antigen)-derived MHC class I peptide and interleukin-2 for 5 days *in vitro*, then were cocultured with E.G7 cells for 4 h. The cytotoxicity against E.G7 cells was measured by lactate dehydrogenase release assay. (b) Natural killer activity in spleen cells was evaluated by the lactate dehydrogenase release assay that targeted YAC-1 cells. (c) Levels of anti-ovalbumin antibody production in sera were measured by ELISA. Bars represent mean \pm SD of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N.S., not significant.

immune responses were enhanced by inhibition of CAF function *in vivo*.

Enhancement of DC-based vaccine potency in combination with anti-CAF therapy. We examined whether improved systemic antitumor immune responses contributed to suppression of tumor growth in various tumor-bearing mouse models. In E.G7 tumor-bearing mice, tumor growth was suppressed by anti-CAF therapy (anti-CAFs vs vehicle, $P = 0.012$); in addition, the combination of the DC-based vaccine and anti-CAFs therapy suppressed tumor growth more effectively than did the DC-based vaccine alone (DC + anti-CAFs vs DC + vehicle, $P < 0.001$) (Fig. 5a). As well as in mice bearing B16F1 or LLC1, the DC-based vaccine in combination with anti-CAF therapy had a synergistic suppressive effect on tumor growth (DC + anti-CAFs vs DC + vehicle, $P < 0.001$ or $P = 0.041$, respectively) (Fig. 5b,c).

Finally, to confirm that suppression of tumor growth was due to improved immune responses by anti-CAF therapy, we investigated whether inhibition of CAFs directly contributed to suppression of tumor growth in SCID mice. Proliferation of CAFs was effectively suppressed by anti-CAF therapy in SCID mice bearing E.G7 tumors (anti-CAFs vs vehicle, $P < 0.001$) (Fig. 5d). Although infiltration of Gr-1⁺ cells in tumor tissues was decreased (anti-CAFs vs vehicle, $P = 0.012$) while that of NK cells was increased (anti-CAFs vs vehicle, $P = 0.040$) (Fig. 5e), a suppressive effect on tumor growth was not observed (anti-CAFs vs vehicle, $P = 0.86$) (Fig. 5e). These results suggest that CAFs have little direct supportive effect on tumor growth, but play a critical role in immune modulation in TME and that inhibition of CAF function *in vivo* enhances the potency of DC-based vaccines by dampening the immune suppressive system.

Discussion

In this study, we focused on the supportive role of CAFs in suppression of antitumor immune responses. Inhibition of CAF function reduced the infiltration of immune suppressor cells

into the TME and TDLNs, thereby improving both cellular and humoral antitumor immune responses in combination with TAA-loaded DC-based vaccines.

As a possible agent for a CAF-targeted strategy, we focused on tranilast, an anti-allergic or antifibrotic agent that has been used clinically. Our previous data indicated that tranilast at doses under 100 μM served as a specific inhibitor against CAFs *in vitro*, but exerted no inhibitory effects on immune cells such as CD4⁺, CD8⁺, and NK cells⁽¹⁹⁾ as well as DC (Fig. S6). In addition, tranilast at doses under 100 μM did not affect the cell viability of murine tumor cells, such as E.G7, LLC1, and B16F1.⁽¹⁹⁾ Given that the tranilast concentration in tumor tissues in our mouse models was lower than 50 μM after repeated tranilast treatment (data not shown), it is considered that administration of tranilast into the tumor exerts no direct inhibitory effect on tumor cells. Although tranilast has been reported to exert an inhibitory effect on tumor cells and immune cells, the concentrations of tranilast that had been applied in previous studies were 100–300 μM .⁽²⁶⁾ Given that the C_{max} of tranilast at the therapeutic dose in humans (300 mg/day) is estimated to be 100 μM in peripheral blood,⁽²⁷⁾ the concentrations of tranilast that had been applied in previous studies were quite excessive, indicating that much higher doses of tranilast would be required to exert the inhibitory effect on tumor cells and immune cells.

The interaction between CAFs and immune suppressor cells in the TME needs to be further elucidated for the development of cancer immunotherapy. Our data showed that infiltration of MDSCs into the TME was reduced by anti-CAF therapy, accompanying significant reduction in levels of SDF-1 and PGE₂ in the tumor tissue. As recruitment of MDSCs to the TME are induced by several factors, including stem cell factor, SDF-1, PGE₂, VEGF, and MMP,^(28–31) the decreased levels of SDF-1 and PGE₂ in the TME, caused by inhibition of CAFs' function, could have contributed to the reduced migration of MDSCs into the TME.

The association between CAFs and the induction of Treg cells was reported, demonstrating that TGF- β and VEGF were

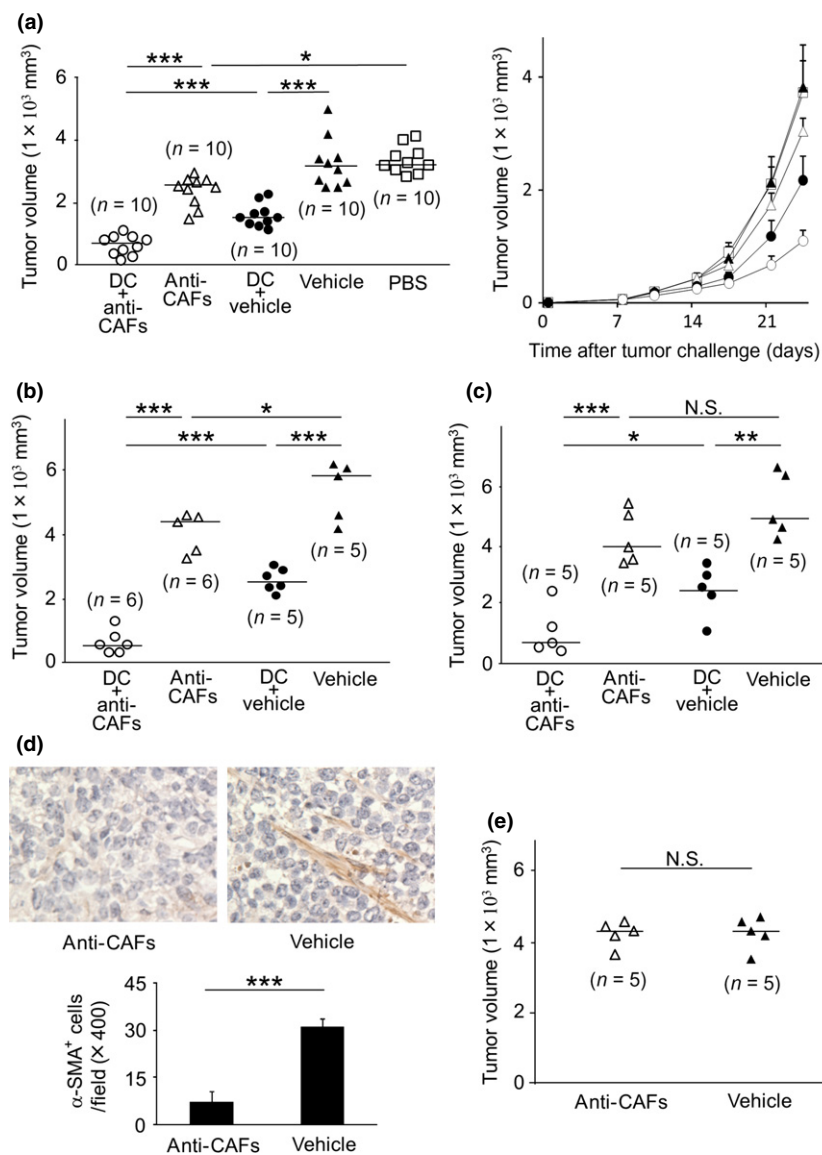


Fig. 5. Enhancement of dendritic cell (DC)-based vaccine potency in combination with anti-cancer-associated fibroblast (CAF) therapy. Mice were inoculated s.c. with (5×10^5) tumor cells. Seven days after tumor inoculation, the mice were treated with a DC-based vaccine s.c. near the tumors on days 7, 13, and 19, in combination with or without intratumoral treatment with tranilast every day for 2 weeks. Twenty-four days after tumor inoculation, volumes of E.G7 (a, left), B16F1 (b), and LLC1 tumors (c) were measured. Tumor volumes in E.G7 tumor-bearing mice were monitored every 3–4 days (a, right). SCID mice bearing E.G7 tumors were administered tranilast into tumors every day for 2 weeks. Five days after the final treatment with tranilast, tumor tissues were harvested from the mice. (d) α -Smooth muscle actin⁺ CAFs in the tumor tissues were evaluated by immunohistochemical staining. (e) Twenty-four days after tumor inoculation, volumes of tumors were measured. Each group had 5–10 mice. Bars indicate the median tumor volume \pm SD of the group. **P* < 0.05, ****P* < 0.001, N.S., not significant.

upregulated in CAFs in the region containing a high number of Treg cells, and these CAFs were more efficient in inducing Treg cells from naïve CD4⁺ T cells.⁽³²⁾ Our previous data showed that TGF- β release from E.G7 cells was not affected by tranilast at doses under 100 μM ;⁽¹⁹⁾ thus, in mice treated with anti-CAF therapy, the decreased level of TGF- β in the TME was induced through anti-CAF therapy. In mice treated with the DC-based vaccine alone, the level of TGF- β was significantly decreased in the TME, which would be caused by the effective suppression of tumor growth through the induction of CTLs. Therefore, in mice treated with the DC-based vaccine in combination with anti-CAF therapy, the level of TGF- β in the TME was significantly decreased, contributing to the effective reduction of Treg cells there. The decreased level of SDF-1 in the TME by inhibition of CAFs would be associated with the reduced migration of Treg cells in the TME, as blocking of the CXCR4–CXCL12 axis was reported to prevent migration of Treg cells into the TME.⁽³³⁾ Taken together, CAFs play critical roles in migration to, and induction of, immune suppressor cells in the TME, thus the immune suppressive TME would be improved by inhibition of CAF

function. In addition, the potency of the DC-based vaccine was synergistically augmented when combined with anti-CAF therapy, contributing to effective suppression of tumor growth and decreased levels of immunosuppressive cytokines in tumor tissues. Therefore, this combination therapy would more effectively improve the immune suppressive TME.

Tumor-draining lymph nodes are primary sites for induction and suppression of antitumor immune responses as tumor tissue-derived soluble factors and tumor-associated immune cells flow into TDLNs, modifying the functional characteristics of downstream TDLNs.^(23,24) Activation of immune suppressor cells in TDLNs induces systemic tolerance against TAA,⁽²⁸⁾ so dampening of immune suppressive responses in TDLNs could improve systemic antitumor immune responses. Our data showed that the improved immune responses in TDLNs by anti-CAF therapy was associated with enhanced systemic TAA-specific CD8⁺ T cell responses, and a combination of the DC-based vaccine and anti-CAF therapy enhanced the potency of the DC-based vaccine. In addition to the CD8⁺ T cell response, the TAA-specific humoral response and NK activity were significantly improved in mice treated with anti-CAF

therapy. It was previously reported that PGE₂ released by CAFs dampened activity of NK cells by downregulation of NKp44 and NKp30,⁽³⁴⁾ and thus NK activity might be recovered by direct inhibition of CAF function. Based on the results of our previous study showing that inhibition of TGF- β function in TDLNs enhanced systemic immune responses,⁽³⁵⁾ immune modulation of TDLNs through inhibition of CAF function might enhance antitumor cellular and humoral immune responses.

Several strategies for targeting CAFs in cancer therapy have been reported. Of the molecules that are expressed in CAFs, fibroblast activation protein (FAP) has emerged as the promising therapeutic target.⁽³⁶⁾ In tumor-bearing animal models, FAP mRNA-transduced DC-based vaccines and DNA vaccines encoding FAP exerted suppressive effects on tumor growth.^(37–40) In terms of immune responses, the previous papers reported that the infiltration of immune suppressor cells was reduced through inhibition of FAP⁺ cells, consistent with our present data. In addition, some published works reported that antitumor CTL responses were enhanced in combination with the cytotoxic chemotherapy.^(38,39) These studies showed that inhibition of CAFs contributed to enhancement of effector cell responses, highlighting the immune suppressive trait of CAFs. The tumor-bearing SCID

mouse model in our study showed that anti-CAF therapy suppressed the proliferation of CAFs but exerted no suppressive effect on tumor growth. These data suggest that simultaneous stimulation of antitumor immune effector responses would be combined to elicit potent antitumor effects in a CAF-targeted strategy.

This study showed the critical roles of CAFs in generating the immunosuppressive and pro-tumorigenic TME through supporting the infiltration of immune suppressor cells. Immune modulation through inhibition of CAF function improved regional and systemic antitumor immune responses, thereby enhancing the potency of the DC-based vaccine immunotherapy. Our mouse models provide a novel rationale with a TME-targeted strategy for the development of cell-based cancer immunotherapy.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Anti-cancer-associated fibroblast effects of tranilast on tumor tissues.

Fig. S2. Reduced level of stromal cell-derived factor-1 (SDF-1) in tumor microenvironment through inhibition of cancer-associated fibroblast function.

Fig. S3. Increased infiltration of CD4⁺ and CD8⁺ cells in tumor microenvironment through inhibition of cancer-associated fibroblast function.

Fig. S4. Reduction of regulatory T cells in tumor-draining lymph nodes through inhibition of cancer-associated fibroblast function.

Fig. S5. Preferable effects of anti-cancer-associated fibroblast therapy in a SCID mouse model.

Fig. S6. Non-inhibitory effects of tranilast on maturation of dendritic cells.