学位論文

Dysfunction of sinus macrophages in tumor-bearing host induces resistance to immunotherapy
（リンパ節洞マクロファージ機能不全による免疫療法抵抗性の誘導）

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ORIGINAL ARTICLE

Dysfunction of sinus macrophages in tumor-bearing host induces resistance to immunotherapy

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Abstract

Sinus macrophages in draining lymph nodes (DLNs) are involved in anti-tumor immune reactions. CD169 (Sialoadhesin, Siglec-1) is expressed on sinus macrophages and is considered a surrogate marker for the immunostimulatory phenotype of macrophages. In this study, the significance of sinus macrophages in immunotherapy was evaluated using mouse models. Treatment with anti-programmed death-ligand 1 (PD-L1) antibody suppressed the subcutaneous tumor growth of MC38 and E0771 cells but was not effective against MB49 and LLC tumors. Decreased cytotoxic T-lymphocyte (CTL) infiltration in tumor tissues and CD169 expression in sinus macrophages were observed in MB49 and LLC cells compared to corresponding parameters in MC38 and E0771 cells. The anti-tumor effects of the anti-PD-L1 antibody on MC38 and E0771 cells were abolished when sinus macrophages in DLNs were depleted, suggesting that sinus macrophages are involved in the therapeutic effect of the anti-PD-L1 antibody. Naringin activated sinus macrophages. Naringin inhibited tumor growth in MB49- and LLC-bearing mice but did not affect that in MC38- and E0771-bearing mice. The infiltration of CTLs in tumor tissues and their activation were increased by naringin, and this effect was impaired when sinus macrophages were depleted. Combination therapy with naringin and anti-PD-L1 antibody suppressed MB49 tumor growth. In conclusion, CD169-positive sinus macrophages in DLNs are critical for anti-tumor immune responses, and naringin suppresses tumor growth by activating CD169-positive sinus macrophages and anti-tumor CTL responses. The activation status of sinus macrophages has been suggested to differ among tumor models, and this should be investigated in future studies.

KEYWORDS
CD169, lymph node, macrophages, naringin, tumor immunity

1 | INTRODUCTION

Recently, immune checkpoint inhibitors (ICIs) have revolutionized cancer treatment. However, because only a limited number of patients benefit from this therapy, the establishment of new treatment strategies, including combination therapy, is desired. It is well known that the abundance of infiltrated CD8-positive cytotoxic T-lymphocytes (CTLs) is associated with high response rates in patients treated with ICIs.[1,2] Draining lymph nodes (DLNs) are important organs in the induction of anticancer immunity and are responseble for an early step in the cancer-immune cycle.[3] In particular, subcapsular and medullary sinus macrophages located at the periphery of lymph nodes are involved in the capture of cancer antigens.[4]

CD169 (Sialoadhesin, Siglec-1) is a receptor expressed on macrophages for sialic acid, which regulates cellular functions in the innate and acquired immune systems by promoting cell–cell interactions through sugar chain recognition.[5] CD169 expression is predominantly observed in the lymphoid organs, and subcapsular and medullary sinus macrophages express CD169. The depletion of CD169-positive macrophages in DLNs reduces the activation and proliferation of CTLs and attenuates the efficacy of cancer vaccines in a mouse model, suggesting that CD169-positive macrophages in DLNs play an important role in cancer immunity.[6] However, its role in ICI efficacy remains unclear. In addition, the number of CD169-positive macrophages in LNs correlates with the number of CD8-positive tumor-infiltrating lymphocytes and is associated with a favorable prognosis in several human cancers,[7–13] suggesting that CD169-positive macrophages are involved in CTL-mediated anti-cancer immunity in humans, as well as in mice. These findings suggest that the induction of CD169-positive macrophages in LNs may be a new therapeutic strategy to enhance cancer immunity in cancer immunotherapy. We previously identified naringin, a natural compound, as a CD169 inducer in human monocyte-derived macrophages.[14] Naringin is a flavonoid glycoside found in citrus fruits, tomatoes, and legumes and has several functional activities, such as anti-oxidative and anti-inflammatory activities.[15] Therefore, we investigated the effects of naringin in murine cancer models and its synergistic effects with ICI therapy.

2 | MATERIALS AND METHODS

2.1 | Preparation of reagents

Anti-programmed death-ligand 1 (PD-L1) antibody and IgG controls were purchased from BioLegend. Diphtheria toxin was obtained from FUJIFILM Wako Pure Chemical Corporation. Naringin and naringenin (Tokyo Chemical Industry) were dissolved in dimethyl sulfoxide at a concentration of 100 mM.

2.2 | Cell lines and cell culture

The cell lines used in this study are listed in Table S1. Cells were maintained in RPMI 1640 medium (FUJIFILM Wako Pure Chemical Corporation) supplemented with 10% FBS and 1% penicillin/streptomycin. Primary murine peritoneal macrophages were obtained from the peritoneal exudates of mice. Peritoneal macrophages were cultured in low-glucose DMEM supplemented with 2% FBS and 1% penicillin/streptomycin.

2.3 | Cell viability assay

To measure cell viability, 1.0×10^4 cells were seeded in 96-well plates containing 100 μL of the medium and treated with the indicated concentrations of naringin (30, 50, 100, 150, and 200 μM) for 24 h. After incubation, 10 μL of the Cell Counting Kit-8 (Dojindo Laboratories) solution was added to the 96-well plates and incubated for 1 h at 37°C. The number of surviving cells was determined by measuring the absorbance at 450 nm. Cell viability was calculated as a percentage of the control value.

2.4 | Animals

C57BL/6 mice and nude mice were obtained from Kyodo. CD169-DTR mice with a C57BL/6 background were kindly gifted by Kenichi Asano (Tokyo University of Pharmacy and Life Sciences). The mice were housed in a temperature-controlled room with a 12-h light/dark cycle.

2.5 | Murine tumor-bearing model

MC38, a murine colon adenocarcinoma cell line (5×10^5 cells/mouse), E0771, a murine breast adenocarcinoma cell line (1.0×10^6 cells/mouse), MB49 a murine bladder Carcinoma cell line (2×10^5 cells/mouse), or lewis lung carcinoma (LLC), a murine lung carcinoma cell line (3×10^5 cells/mouse) cells suspended in 100 μL RPMI 1640 medium were subcutaneously injected into mice. Three days later, an anti-PD-L1 antibody (200 μg) was intravenously administered, and/or naringin (200 mg/kg per day) was mixed with food and administered daily, followed by determination of subcutaneous tumor weight at the end of the study and measurement of the tumor microenvironment in subcutaneous tumor tissues using immunohistological analysis and flow cytometry.

2.6 | Flow cytometry

Mouse peritoneal macrophages and disaggregated cells prepared from mouse tissues were treated with mouse FcR-blocking reagent (Miltenyi Biotec) for 5 min and then incubated with fluorescence-labeled antibodies. The antibodies used are described in Table S2. The stained cell samples were analyzed on a FACSVerse flow cytometer using FACSuite software.

2.7 | Immunohistochemistry

Paraffin-embedded tumor tissue samples were sectioned into 3 μm-thick specimens for immunostaining. The primary antibodies used are described in Table S3. HRP-labeled anti-mouse, anti-rabbit, or anti-goat immunoglobulin antibodies (Nichirei) were used as secondary antibodies. A DAB substrate system (Nichirei) was used to visualize the immunoreactions. For lymphocyte counting, eight non-overlapping high-power fields (400× magnification) were randomly selected in tumor areas without necrosis or hemorrhage, and cell counting was performed using the KEYENCE BZ-X800 software (Keyence).

2.8 | RNA sequencing analysis

Naringin (200 mg/kg per day) was mixed with food and administered daily for 5 days. Lymph nodes were removed and total RNA was isolated by using RNAiso Plus (Takara Bio). RNA sequencing analysis was performed by Amelieff. Briefly, sequencing libraries were crafted using the NEBNext UltraTM RNA Library Prep Kit for Illumina (NEB, Cat. No. 7760), in strict adherence to the manufacturer's guidelines. Subsequent to this, index codes were incorporated to allocate sequences distinctly to each specimen. Concluding the process, libraries underwent purification via the AMPure XP system, and their quality was meticulously evaluated using the Agilent Bioanalyzer 2100 system. The refined libraries were then sequenced on the Novaseq 6000 (Illumina), utilizing a 150-base pair-end read configuration.

2.9 | Statistics

Statistical analyses (Mann–Whitney *U*-test and one-way ANOVA) were performed using GraphPad Prism 7 (GraphPad Software). A p-value <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Dysfunction of sinus macrophages potentially contributes to the resistance to anti-programmed death-ligand 1 therapy

Using an animal model, we assessed whether CD169-positive sinus macrophages in DLNs are involved in the anti-tumor effect of anti-PD-L1 therapy. First, four murine subcutaneous tumor models were established using the MC38, E0771, MB49, and LLC cell lines, and the efficacy of anti-PD-L1 therapy was examined. Tumor growth was suppressed in MC38- and E0771-bearing mice but not in MB49- and LLC-bearing mice (Figure 1A,B and Figure S1). The tumor immune microenvironment was then compared between MC38, E0771, MB49, and LLC tumors. There were many infiltrating CD8- and CD4-positive cells in the tumor tissues implanted with MC38 and E0771 cells, whereas there were few infiltrating CD8- and CD4-positive cells in the tumor tissues implanted with MB49 and LLC cells (Figure 1C). Next, the expression of CD169 in sinus macrophages of DLNs was evaluated. CD169 expression in sinus macrophages was lower in MB49- and LLC-bearing mice than in MC38- and E0771-bearing mice, whereas CD169 expression in the lymph nodes of MC38- and E0771-bearing mice was unchanged compared from that in the non-tumor control model (Figure 1D).

3.2 | CD169-positive sinus macrophages in draining lymph nodes contribute to anti-tumor immune responses in anti-programmed death-ligand 1 therapy

Next, the involvement of CD169-positive sinus macrophages in DLNs in the anti-tumor effects of anti-PD-L1 therapy was investigated using CD169-DTR mice. Following deletion of CD169-positive sinus macrophages in DLNs by diphtheria toxin (DT) injection, the tumor cells were subcutaneously injected (day 0), and anti-PD-L1 antibody was administered on day 2 (Figure 2A). The depletion of macrophages in the DLNs was confirmed by immunohistochemistry (IHC) (Figure 2B). When anti-PD-L1 therapy was performed in MC38- and E0771-bearing mice, the anti-tumor effect was completely abolished in DT-treated mice (Figure 2C), suggesting that the anti-tumor effect of anti-PD-L1 in these tumor models is highly dependent on CD169-positive macrophages.

3.3 | Naringin suppresses MB49 and LLC tumor growth by T lymphocyte-mediated mechanisms

Some natural compounds have been previously identified to stimulate sinus macrophages; therefore, we suggest that these compounds recovered dysfunctional sinus macrophages in the DLNs of tumor-bearing mice. Naringin was selected for use in this study because it is commercially available (Figure 3A). Naringin increased the number of CD169-positive murine peritoneal macrophages under cell culture conditions (Figure 3B). The anti-tumor effects of naringin were tested in tumor-bearing mice (Figure 3C). Before conducting the in vivo study, we examined the direct anti-tumor effect of naringin under in vitro culture conditions. Naringin was not toxic to macrophages or tumor cell lines (Figure S2). Animal studies showed that tumor growth was significantly suppressed by naringin administration in MB49- and LLC-bearing mice but not in MC38- and E0771-bearing mice (Figure 3D and Figure S3). This anti-tumor effect of naringin on MB49 and LLC tumors disappeared in immunodeficient nude mice (Figure 3E).

3.4 | Naringin activates immune responses in the tumor microenvironment and draining lymph nodes

The effect of naringin on T cell infiltration and activation in tumor tissues and DLNs was examined using IHC and flow cytometry.

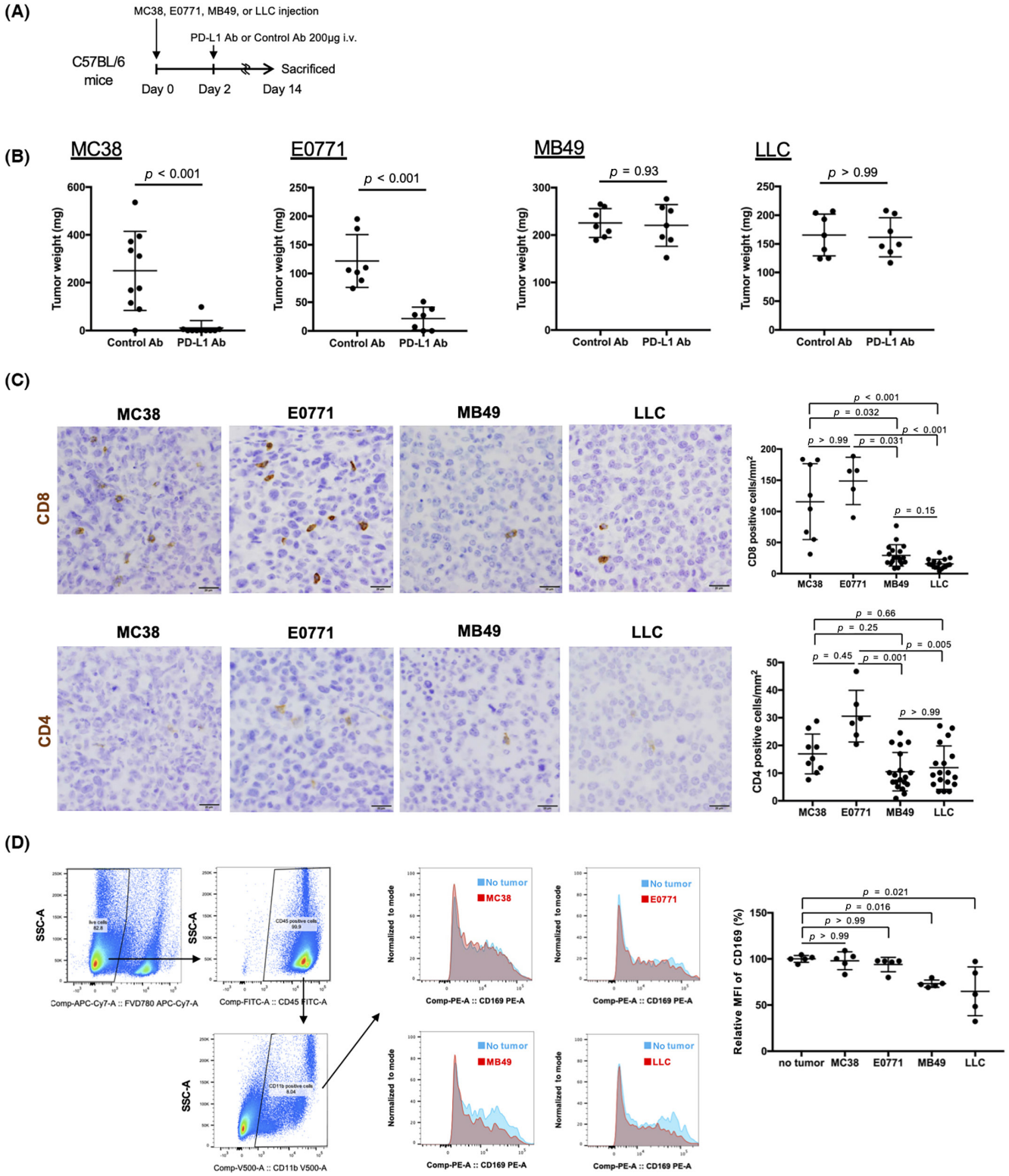

FIGURE 1 Anti-tumor effect of immune checkpoint inhibitors in mouse tumors. (A) The protocol for administration of anti-programmed death-ligand 1 (PD-L1) antibody and implantation of MC38, E0771, MB49, and LLC cells in C57BL/6 mice. (B) Subcutaneous tumor weight was measured after administration of anti-programmed death-ligand 1 (PD-L1) antibody in tumor-bearing mice. (C) Infiltration of CD8- and CD4-positive lymphocytes in subcutaneous tumor tissues was evaluated via immunostaining. (D) Flow cytometry was performed to evaluate CD169 expression levels in macrophages in draining lymph nodes (DLNs). i.v., intravenous injection.

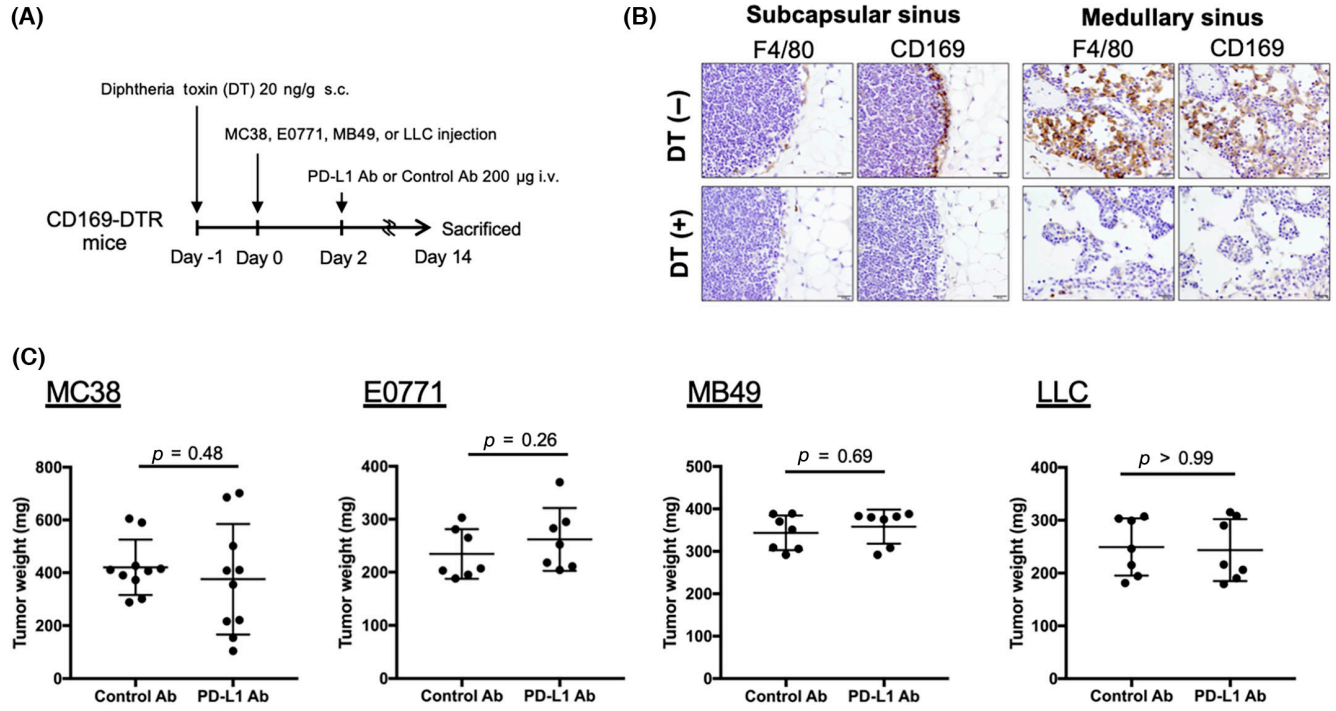

FIGURE 2 Anti-tumor effect of immune checkpoint inhibitors in CD169-DTR mice. (A) The protocol for administration of anti-programmed death-ligand 1 (PD-L1) antibody and implantation of MC38, E0771, MB49, and LLC cells in CD169-DTR mice. (B) Sinus macrophages in diphtheria toxin (DT) (–) and DT (+) mice (no tumor-bearing model) were evaluated by immunostaining of F4/80 and CD169. (C) Subcutaneous tumor weight was measured after the administration of anti-PD-L1 antibody. i.v., intravenous injection; s.c., subcutaneous injection.

Naringin administration enhanced CD8-positive T-cell infiltration in tumor tissues (Figure 4A). Naringin administration increased the number of GzmB-positive T cells (Figure 4B). Ki-67 and PD-1 expression in CD8-positive T cells was elevated by naringin; however, this was not statistically significant (Figure 4B). Naringin administration elevated the number of CD169-positive macrophages in the lymph nodes (Figure 4C). CD69, an activation marker of lymphocytes, was also upregulated in CD4- and CD8-positive T cells in DLNs following naringin administration (Figure 4D). The IHC results showed that PD-L1 expression was enhanced by naringin administration (Figure 4E), and flow cytometry revealed that PD-L1 expression in tumor cells was significantly increased by naringin administration (Figure 4F).

3.5 | CD169-positive sinus macrophages in draining lymph nodes contribute to the anti-tumor effect of naringin

Next, we tested whether CD169-positive sinus macrophages in DLNs were involved in the anti-tumor effect of naringin. After the depletion of CD169-positive sinus macrophages in DLNs due to DT injection in CD169-DTR mice, tumor cells were subcutaneously injected (Figure 5A). The results showed that the anti-tumor effect of naringin was completely abolished by DT treatment (Figure 5B). IHC results indicated that the infiltration of CD8-positive T cells was not altered by naringin treatment (Figure 5C). Moreover, PD-L1 expression did not change in the tumor tissues after naringin administration (Figure 5D). These data demonstrated that CD169-positive sinus macrophages are involved in the anti-tumor effects of naringin in a tumor-bearing mouse model.

3.6 | Naringin administration changes immunotherapy-resistant tumors to immunotherapy-sensitive tumors

As shown in Figure 1, anti-PD-L1 therapy was ineffective in the MB49 and LLC tumor models. Because naringin administration induced T cell infiltration and activation in the tumor tissues of MB49 and LLC tumors with increased PD-L1 expression, we suggest that naringin administration improves anti-PD-L1 therapy against these tumors. Therefore, combined therapy using naringin and anti-PD-L1 antibodies was performed for MB49 tumors (Figure 6A). The results showed that anti-PD-L1 antibody treatment in combination with naringin suppressed tumor growth compared to both the control antibody treatment group and the naringin alone treatment group (Figure 6B and Figure S4). IHC results indicated that the infiltration of CD8-positive T cells was significantly increased by the combined administration of naringin and anti-PD-L1 antibodies (Figure 6C). PD-L1 expression was also enhanced in the tumor tissues after the combined administration

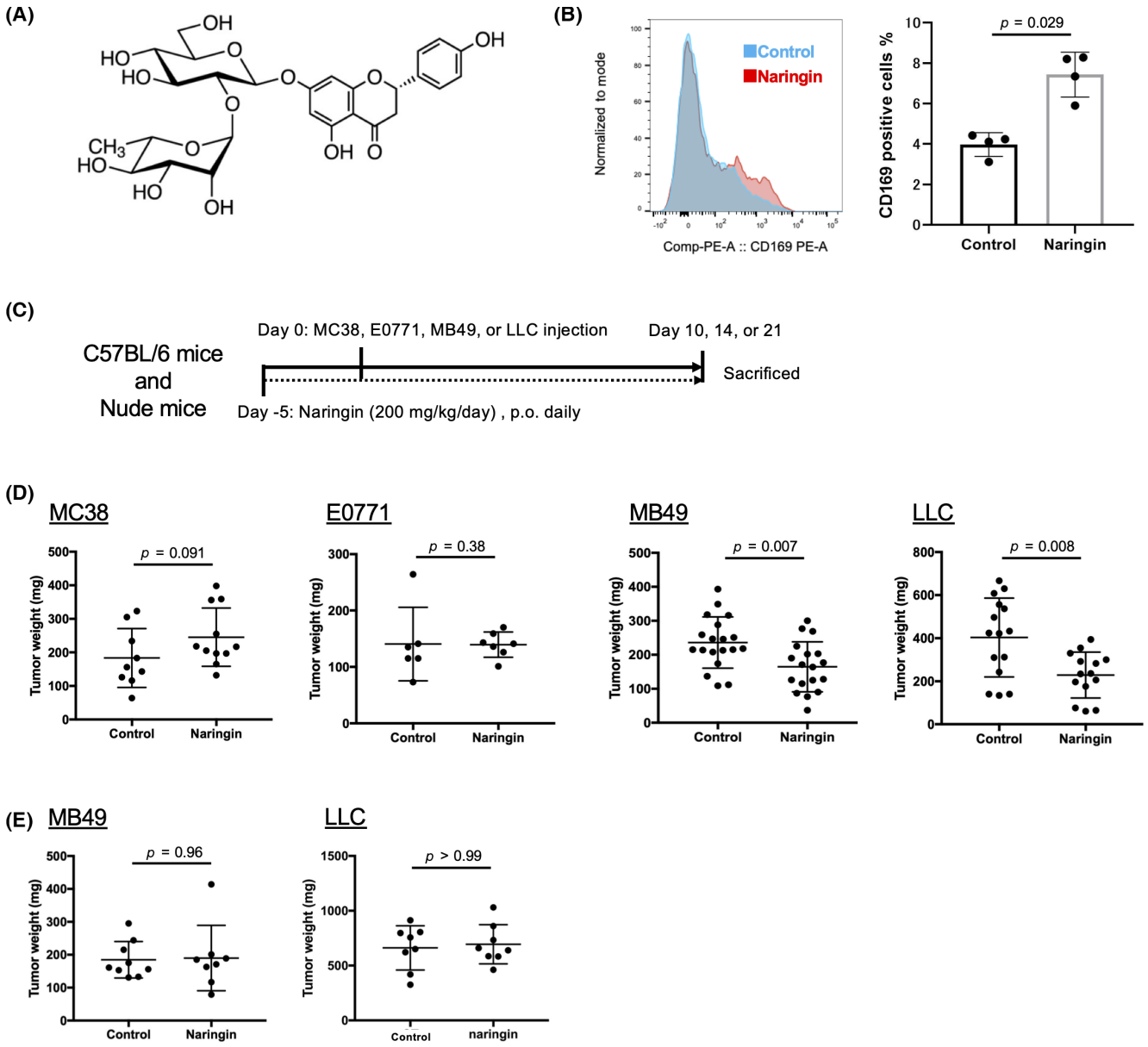

FIGURE 3 Effect of naringin on tumor progression in tumor-bearing mice. (A) The chemical structure of naringin. (B) Mouse peritoneal macrophages were incubated with naringin (30 μM) for 24 h, followed by determination of CD169 expression using flow cytometry. (C) The protocol for administration of naringin and implantation of MC38, E0771, MB49, and LLC cells in C57BL/6 mice. (D) Subcutaneous tumor weight was measured after administration of naringin. (E) Subcutaneous tumor weight in nude mice was measured after administration of naringin. p.o., per os (oral administration).

(Figure 6D). These data demonstrate that combined therapy with naringin and anti-PD-L1 antibody is effective against immunotherapy-resistant tumors.

4 | DISCUSSION

CD169-positive sinus macrophages that phagocytose tumor cells in LNs present tumor antigens directly to CD8-positive lymphocytes and activate them independently of dendritic cells related to antigen presentation.[6] In this study, we investigated the role of CD169-positive sinus macrophages in tumor suppression using ICIs, which have shown beneficial effects in various cancer models. MB49 and LLC tumors were resistant to anti-PD-L1 therapy, whereas MC38 and E0771 tumors were sensitive to anti-PD-L1 therapy. The number of infiltrating lymphocytes was significantly lower in MB49 and LLC tumors than in MC38 and E0771 tumors, indicating that MB49/LLC and MC38/E0771 tumors were immunologically cold and hot tumors, respectively. The depletion of CD169-positive macrophages also induced tumor progression (Figure S5), indicating that CD169-positive macrophages play an important role in activating anti-tumor immunity and suppressing tumor progression. Furthermore, the depletion of CD169-positive macrophages nullified the anti-tumor effect of anti-PD-L1 therapy in MC38 and E0771 tumor-bearing

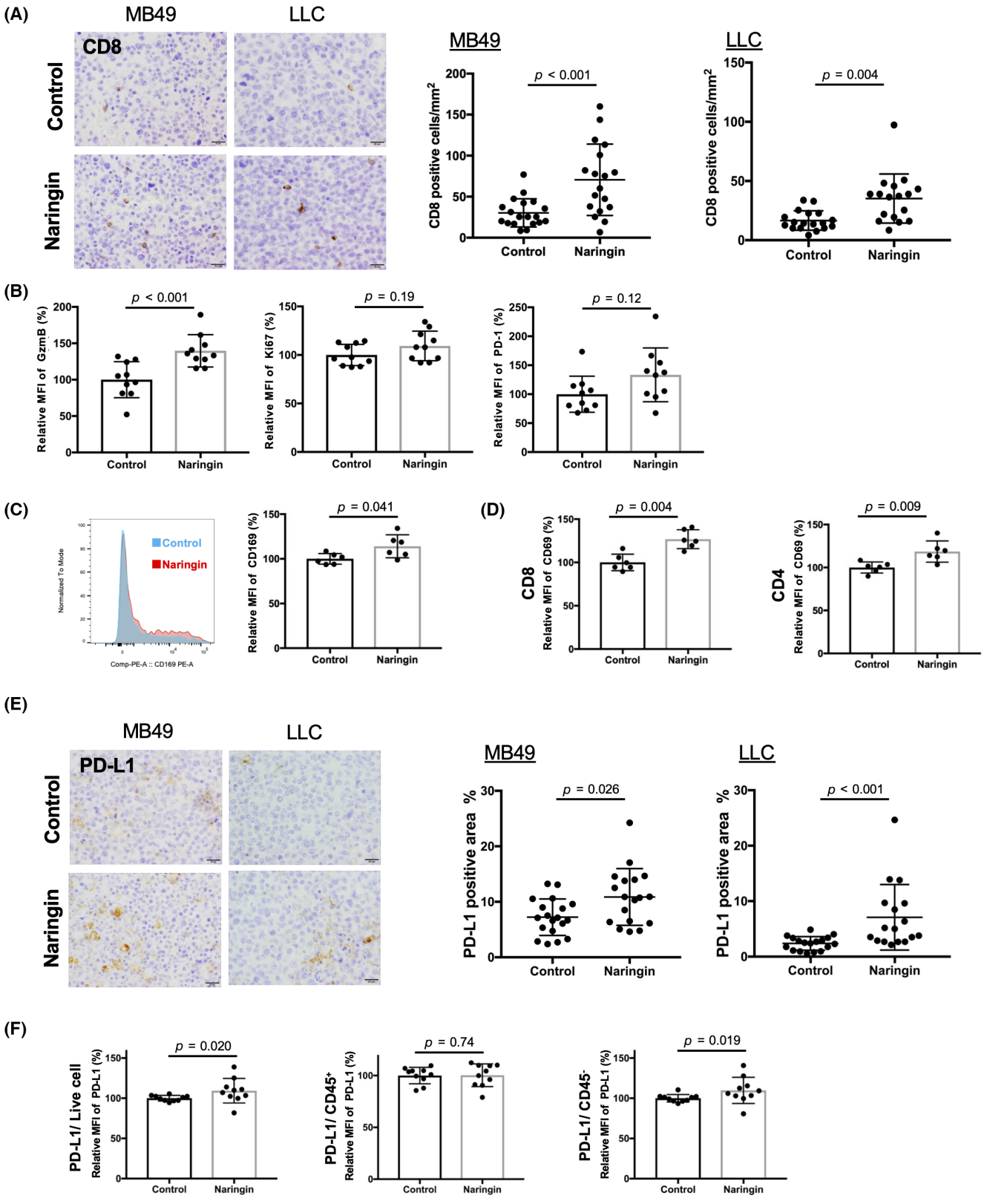

FIGURE 4 The effect of naringin on immune responses in the tumor microenvironment and draining lymph nodes (DLNs). (A) Infiltration of CD8-positive lymphocytes in MB49 and LLC subcutaneous tumor tissues was determined via immunostaining. (B) The expression levels of GzmB, Ki67, and PD-1 in infiltrated $CD8^+$ cells within MB49 tumor tissues were determined via flow cytometry. (C) The expression level of CD169 in sinus macrophages in DLNs of MB49-bearing mice was analyzed using flow cytometry. (D) The expression level of CD69 in lymphocytes in DLNs was also analyzed using flow cytometry. (E) programmed death-ligand 1 (PD-L1) expression level in MB49 and LLC subcutaneous tumors was determined via immunostaining. (F) PD-L1 expression levels in both $CD45^-$ and $CD45^+$ cells in MB49 tumor tissues were determined using flow cytometry.

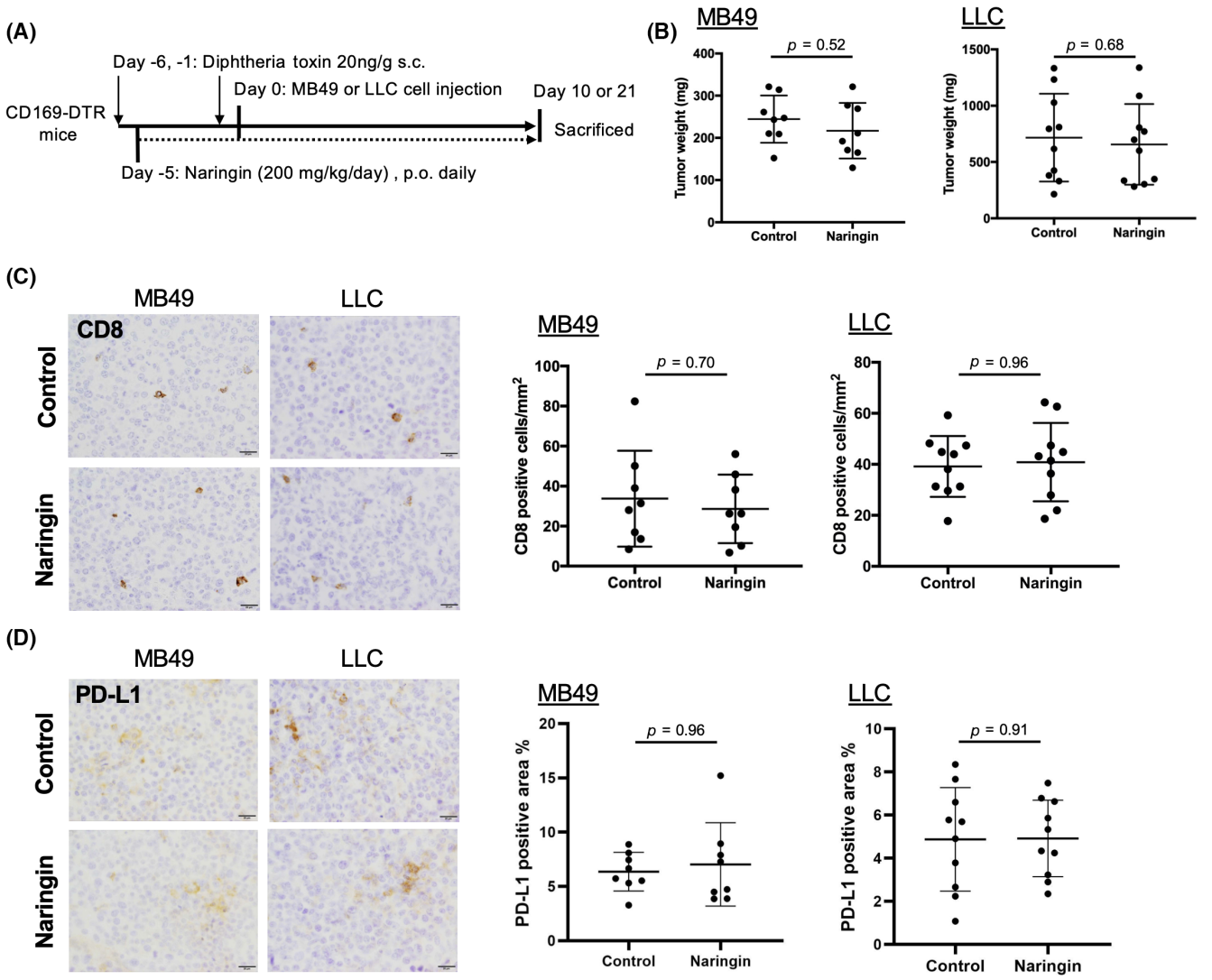

FIGURE 5 Effect of naringin administration on tumor-bearing CD169-DTR mice. (A) The protocol for administration of naringin and implantation of MB49 or LLC cells in CD169-DTR mice. (B) Subcutaneous tumor weight was measured after administration of naringin. (C) Infiltration of CD8-positive cells in subcutaneous tumor tissues was determined by immunostaining. (D) programmed death-ligand 1 (PD-L1) expression level in subcutaneous tumor tissues was determined by immunostaining. p.o., per os (oral administration); s.c., subcutaneous injection.

mouse models that responded to anti-PD-L1 therapy, suggesting that CD169-positive sinus macrophages are associated with the therapeutic effect of ICIs in the early stage of tumor immunity, and inducing CD169-positive macrophages in LNs may enhance the therapeutic effect of ICIs. CD169 expression in sinus macrophages was downregulated in MB49/LLC tumors, suggesting that sinus macrophage dysfunction is involved in immunological responses to tumor cells (Figure 6E).

Anti-PD-L1 antibody had no effect in the MB49 and LLC tumor-bearing mouse models, which originally had few infiltrated CD8 lymphocytes in the tumor tissue, suggesting that the infiltration of tumor tissues by CD8-positive T cells is important for the therapeutic effect of ICIs. Similar results have been observed in real-world clinical practice for cancer treatment. It has been reported that the lack of CD8-positive T cells infiltrating tumor tissues is associated with poor response of cancer patients to ICI therapy.[16] Combination therapy with immunotherapy is known to be effective in improving efficacy[17]; however, more therapeutic approaches are needed, especially for cold tumors. Targeted therapy to reprogram dysfunctional DLNs may be a new approach for improving immunotherapy in immunologically cold tumors.

In this study, we focused on naringin, a natural compound, as a candidate agent that induces the infiltration of CD8-positive T cells into tumor tissues by enhancing tumor immunity. Naringin, a major bioactive polyphenol found in citrus fruits, is beneficial to human health and has been consumed for many years.[18] It was previously identified as an inducer of CD169-expression in sinus macrophages.[14] The administration of naringin to mice also enhanced CD169 expression on macrophages and CD69 expression on lymphocytes in DLNs (Figure 4C,D). As shown in Figure S6, differential gene expression (DGE) analysis also revealed that naringin administration augments the expression of CD169 (Siglec1), a

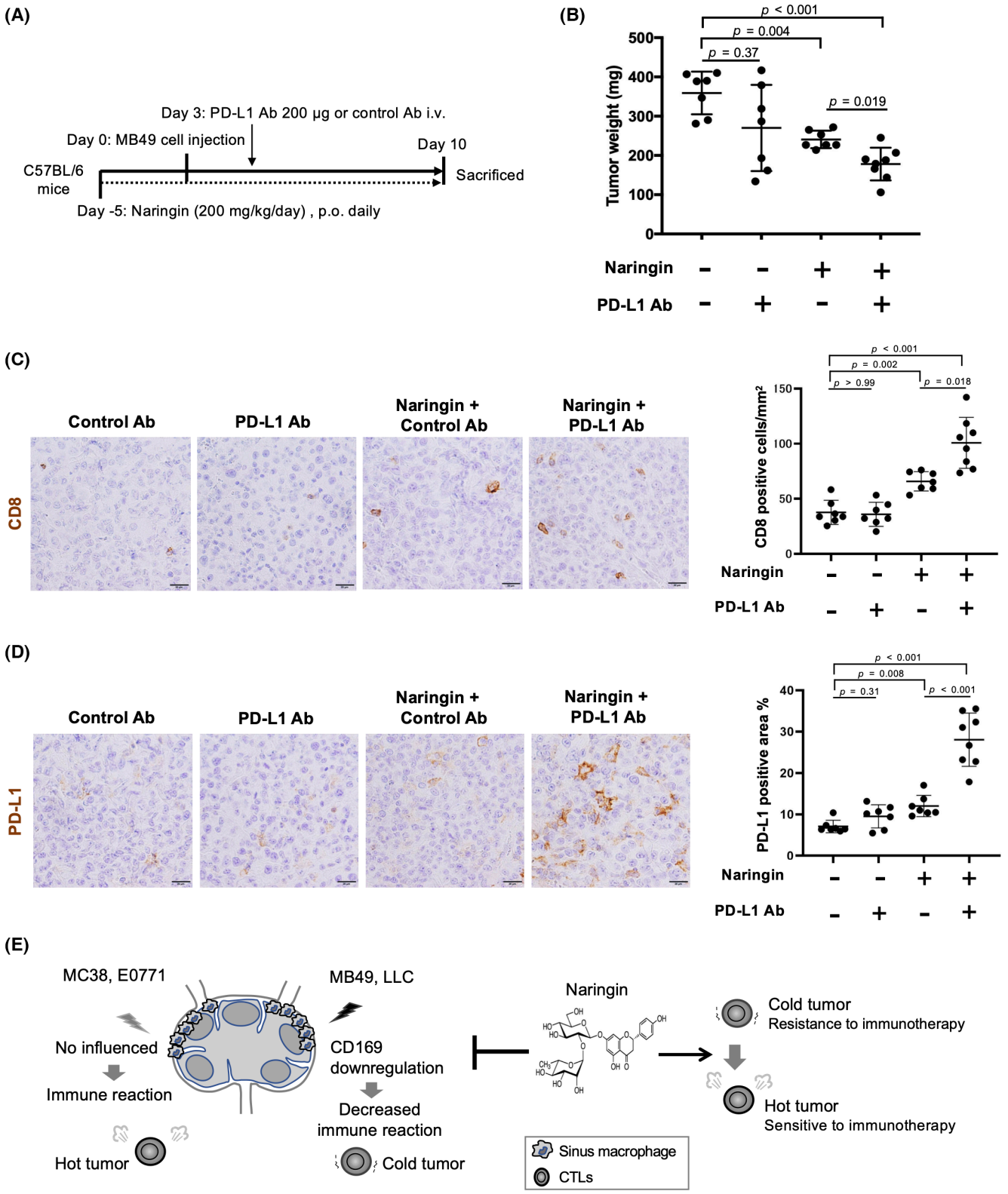

FIGURE 6 Combination effect of naringin and immune checkpoint inhibitors on tumor progression in a tumor-bearing mouse model. (A) The protocol for administration of naringin with anti-programmed death-ligand 1 (PD-L1) antibodies and implantation of MB49 cells in C57BL/6 mice. (B) Subcutaneous tumor weight was measured after administration of naringin and anti-PD-L1 antibodies. (C) The number of infiltrated CD8-positive lymphocytes in tumor tissues was measured by immunostaining. (D) The expression level of PD-L1 in tumor tissues was measured by immunostaining. (E) Scheme of the hypothesis presented in this article. Immune reactions in draining lymph nodes (DLNs) were not affected in MC38/E0771 (hot) tumor models, whereas downregulation of CD169 and decreased immune reaction were observed in tumor models with DLNs bearing MB49/LLC (cold) cells. Naringin blocked the downregulation of CD169 expression and potentially changed the cold tumor resistance to immunotherapy to hot tumor sensitivity to immunotherapy. i.v., intravenous injection; p.o., per os (oral administration).

molecule typically enhanced by type I interferon activation in macrophages. Moreover, there was a noted increase in the expression of Irf7, deemed the central orchestrator of type I interferon. Both DGE analysis and enrichment analyses further highlighted that naringin amplifies the expression of S100a8 and S100a9, markers associated with inflammation in phagocytes, and enhances the expression of Cxcl10 and Ccl20, markers for activated T cells. These findings suggest that naringin may instigate various immune functions, essentially bolstering immunity through the stimulation of immunoreactive sinus macrophages. Furthermore, administration of naringin significantly reduced tumor progression in both MB49- and LLC-bearing tumor models (Figure 3D), whereas it had no effect on tumor progression in either the MC38- or E0771-bearing tumor models (Figure 3D). The number of infiltrated CD8-positive cells significantly increased in tumor tissues of cold tumor (MB49 and LLC)-bearing mice after naringin administration (Figure 4A), whereas there were no CD169-positive macrophages in tumor tissues (unpublished data), indicating that naringin enhances tumor immunity by inducing CD169-positive macrophages in LNs, followed by infiltration of CD8-positive T cells into tumor tissues.

Immunotherapy for cancer has recently received considerable attention. For example, ICIs that target the programmed cell death 1 (PD-1)–PD-L1 pathway have dramatically changed the treatment of various types of cancer.[19,20] However, because the abundance of infiltrated CD8-positive lymphocytes is associated with high response rates in patients treated with ICIs,[1,2] only a limited number of patients benefit from this therapy. However, naringin increased the number of infiltrated CD8-positive T cells in tumor tissues from cold tumor-bearing mice (Figure 4A), and combination therapy with anti-PD-L1 and naringin ameliorated tumor progression (Figure 6), demonstrating that ICIs suppress tumor progression via naringin-induced infiltrating CD8 T cell growth and activation in tumor tissues. This result holds immense potential for application in treatment of cold tumors, for which alternatives involving monotherapy with ICIs are limited. However, one limitation of this study is that the detailed mechanism by which naringin induces CD169-positive macrophages in LNs is unknown because the complex structure of the natural compound makes it difficult to identify its target molecules in macrophages.[21]

This is the first study to demonstrate naringin-induced activation of anti-tumor immunity through the enhancement of CD169-positive macrophages in LNs and promotion of CTL infiltration in tumors. The mechanisms by which each tumor regulates the activation of sinus macrophages should be investigated in future studies. Furthermore, our results suggest that CD169-positive macrophages in LNs are a prognostic marker for tumors and that naringin is a novel potential agent, especially for immune-cold tumors that are refractory to ICI monotherapy.

AUTHOR CONTRIBUTIONS

Toshiki Anami: Data curation; formal analysis; investigation; validation; writing – original draft. **Cheng Pan:** Data curation; formal analysis; investigation; methodology. **Yukio Fujiwara:** Conceptualization; data curation; funding acquisition; investigation; project administration; validation; writing – original draft; writing – review and editing. **Yoshihiro Komohara:** Conceptualization; data curation; funding acquisition; project administration; validation; writing – original draft; writing – review and editing. **Hiromu Yano:** Investigation; methodology. **Yoichi Saito:** Formal analysis; funding acquisition; investigation; validation. **Masamichi Sugimoto:** Conceptualization; writing – original draft. **Daiko Wakita:** Conceptualization; methodology. **Takanobu Motoshima:** Resources. **Yoji Murakami:** Resources. **Junji Yatsuda:** Resources. **Naofumi Takahashi:** Investigation. **Shinya Suzu:** Supervision. **Kenichi Asano:** Resources. **Koji Tamada:** Resources. **Tomomi Kamba:** Resources; supervision.

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CONFLICT OF INTEREST STATEMENT

Masamichi Sugimoto and Daiko Wakita are employees of Chugai Pharmaceutical, which supported a part of this research. Y.F. was provided joint research funds from Chugai Pharmaceutical. K.T. and Y.K. are editorial board members. The other authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed in the current study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: N/A.

Informed Consent: N/A.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: All animal procedures were planned according to the "Animal Research: Reporting of In Vivo Experiments (ARRIVE)" guidelines and were approved by the Animal Research Committee at Kumamoto University (A2019–015).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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