Methyl Gallate Exhibits Potent Antitumor Activities by Inhibiting Tumor Infiltration of CD4+CD25+ Regulatory T Cells

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Methyl Gallate Exhibits Potent Antitumor Activities by Inhibiting Tumor Infiltration of CD4+CD25+ Regulatory T Cells

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CD4+CD25+ regulatory T (Treg) cells play crucial roles in the host response to tumors. Increasing evidence supports the existence of elevated numbers of Treg cells in solid tumors and hematologic malignancies. In this study, the effects of methyl gallate on Treg cells were examined. Methyl gallate inhibited Treg cell-suppressive effects on effector CD4+ T cells and Treg migration toward tumor environment. The expression of Treg surface markers including CTLA-4, CCR4, CXCRI4, and glucocorticoid-induced TNFR was significantly suppressed upon methyl gallate treatment. Furthermore, forkhead box P3 (Foxp3) expression was also significantly decreased by methyl gallate, suggesting that the suppressive effects of methyl gallate on Treg were mediated by decrease of Treg-specific transcription factor Foxp3. In tumor-bearing hosts, methyl gallate treatment substantially reduced tumor growth and prolonged the survival rate. In contrast, nu/nu mice did not show decreased tumor progression in response to methyl gallate. In addition, in tumor-bearing Treg-depleted mice, tumor growth and the survival rates were not changed by methyl gallate treatment, strongly suggesting that the main therapeutic target of methyl gallate in tumor suppression was related to modulation of the CD4+CD25+ T cell functions. In the spleen of tumor-bearing mice, methyl gallate treatment induced a significant decrease in the CD4+CD25+Foxp3high Treg cell population. Especially, the number of tumor-infiltrating CD25+Foxp3high Treg cells was significantly lower in methyl gallate-treated mice. These results suggest that methyl gallate can be used to reverse immune suppression and as a potentially useful adjunct for enhancing the efficacy of immune-based cancer therapy. The Journal of Immunology, 2010, 185: 000–000.

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Abbreviations used in this paper: EGF, enhanced GFP; Foxp3, forkhead box P3; GITR, glucocorticoid-induced TNFR; IGDE, isobutyl gallate-3,5-dimethyl ether; MGDE, methyl gallate-3,5-dimethyl ether; TAA, tumor-associated Ag; Treg, regulatory T.

Copyright © 2010 by The American Association of Immunologists, Inc. 0022-1767/10S16/00 specific immunity, although spontaneous immunologic clearance of cancer is rare. Thus, boosting antitumor immunity with various vaccine strategies is a logical approach to improving current cancer treatments. However, recent evidence has shown that tumors actively defeat TAA-specific immunity. Self-Ags are mechanisms of active immune evasion, and it has been postulated that patients develop tolerance to their TAAs (and hence their tumor) through Treg cells. In support of this supposition, it has been demonstrated that there are consistently fewer Treg cells in tumor-draining lymph nodes than in lymphoid organs unrelated to the tumor during all ovarian tumor stages. These findings suggest that Treg cells may migrate to tumors from lymph nodes. The suppressive effect of Treg on the activation of antitumor effector cells has also been demonstrated in various murine tumor models. These tumor Treg cells are functionally suppressive and able to block tumor-specific immunity, foster tumor growth, and predict poor patient survival (4). Injection of an anti-CD25 mAb led to rejection of different murine tumors; thus, it is important to understand the key role that Treg cells play in blocking the immune response against cancer. Intraperitoneal injection of anti-CD25 Abs effectively depleted Treg in the spleen and tumor-draining lymph nodes up to 3 wk after the last injection. Subsequently, tumor-bearing Treg-depleted mice showed slower tumor growth and prolonged survival.

Several phytochemicals (e.g., polyphenols) found in plants exert antioxidant and anticancer activities, including cell cycle arrest and induction of apoptosis in cancer cells (5). Recently, two gallic acid-derived compounds, isobutyl gallate-3,5-dimethyl ether (IGDE) and methyl gallate-3,5-dimethyl ether (MGDE), were isolated from the alcoholic extract produced by Casearia sylvestris leaves and characterized (6). Gallic acid and other polyphenolic
substances are able to inhibit the growth of cells from several types of tumors. In addition, it has been shown that plants with abundant polyphenolic compounds are able to act through the humoral immune response, as well as the cell response, activating B lymphocytes with subsequent in vitro proliferation of T cells. These chemical properties may be useful indicators for evaluation of the potential of polyphenolic fractions for cancer prevention or treatment and the degree of polymerization related to bioavailability (7). In the current study, we showed that treatment of murine tumors with methyl gallate extracted from Moutan Cortex Radicis enhances antitumor effects through modulation of the function of CD4+CD25+ Treg cells. In vitro, methyl gallate decreased CD4+CD25+ Treg cell migration and reduced the suppressive function of effector T cells. In tumor-bearing animals, treatment with methyl gallate delayed tumor progression and prolonged survival through inhibition of the tumor infiltration of CD4+CD25+ Treg cells.

Materials and Methods

Cells, reagents, and mAbs

The EL-4 mouse lymphoma cell line was obtained from the Korean Cell Line Bank (KCLB 40039; Cancer Research Institute, Seoul, Korea). These cells were then cultured in tissue culture plates (Corning Glass, Comining, NY) in DMEM (Invitrogen, Rockville, MD) supplemented with 10% FBS (HyClone, Logan, UT) and penicillin-streptomycin (Invitrogen). Methyl gallate was purchased from ChromaDex (Irvine, CA) and dissolved in distilled water. PE-labeled anti-mouse CD69 mAb (HI.2F3; hamster IgG), PE-CTLA4 mAb (UC10-4B9; hamster IgG), PE-CXCR3 mAb (CXC3R-173; hamster IgG), PE-CCR5 mAb (HM-CCR5; hamster IgG), PE-CXCR4 mAb (2B11; rat IgG2b), PE-glucocorticoid-induced TNFR associated protein of death domain (GITR) mAb (DTA-1; rat IgG2b), PE-CD8 mAb, and isotype-matched mAbs were purchased from eBioscience (San Diego, CA). In addition, PE-CCR4 mAb (2G12-CCR4; hamster IgG) was obtained from BioLegend (San Diego, CA).

Animals

C57BL/6 mice (6–8 wk of age, weighing 20–25 g) and nu/nu male mice (C.Cg-Foxn1nu/CrljBg) were purchased from Charles River Korea (Seungnam, Seoul, South Korea). Foxp3EGFP^+ C57BL/6 (C.Cg-Foxp3^tm2CAG/Il) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were kept under pathogen-free conditions with air conditioning and a 12-h light/dark cycle. In addition, all mice had free access to food and water during the experiments. This study was approved by the Kyung Hee University Institutional Animal Care and Use Committee (KHUASP(SE)-09-017).

Cell cycle analysis

Cells were plated in six-well culture dishes at concentrations determined to yield 60–70% confluence within 24 h. The cells were then treated with different concentrations of methyl gallate (0–50 μg/ml). After 24 h, the cells were washed twice with PBS and the pellet was fixed with 70% ethanol for 1 h at 4°C. Next, the cells were washed with PBS, resuspended with propidium iodide solution (0.05 mg/ml) containing R-Nase, and incubated at room temperature in the dark for 30 min. The DNA content was then analyzed using a flow cytometer.

CD4+CD25+ Treg and CD4+CD25− T cell isolation

CD4+CD25+ Treg and CD4+CD25− T cells were isolated from spleens obtained from male C57BL/6 or Foxp3EGFP^+ C57BL/6 mice using magnetic bead separation (CD4+CD25+ Treg cell kit; Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, non-CD4+ T cells were depleted using biotinylated Ab mixture and anti-biotin microbeads. CD4+CD25+ T cells were positively selected from CD4+CD25− T cells using PE-labeled anti-CD25 mAb and anti-PE microbeads. The purity of both populations was determined by flow cytometric analysis and routinely reached >93%.

Transwell migration assay

Isolated CD4+CD25+ Treg cells were treated with the indicated concentrations of methyl gallate for 48 h. The culture supernatant of EL-4 lymphoma was then subjected to a migration assay, which was conducted using a QCM chemotaxis 96-well (5 μM) cell migration kit (Millipore, Billerica, MA), according to the manufacturer’s instructions.

In vitro cytotoxicity assay

To determine the cytotoxicity capacity of methyl gallate on CD4+CD25+ Treg cells, the Cell Titer 96 nonradioactive cell proliferation assay was used following the directions provided by the manufacturer (Promega, Madison, WI). Cells were plated at 2 × 10^3/well in a total volume of 100 μl. Cells were then washed twice and cultured in methyl gallate (0, 0.01, 0.1, 1, 10, and 50 μg/ml) containing medium. Cells were incubated for 48 h at 37°C in 5% CO2 incubator. Values for cell viability were then normalized against the value for controls.

In vitro proliferation assay

T cell immunosuppression was tested in a coculture system. CD4+CD25+ T cells (2 × 10^5/ml) were stimulated with 2.5 μg/ml anti-CD3 Ab (clone 145-2C11; BD Biosciences, San Jose, CA) and 1.2 μg/ml anti-CD28 Ab (clone 37.51; BD Biosciences), and then labeled prior to coculture with CFSE (Molecular Probes, Eugene, OR). CD4+CD25+ T cells were treated with methyl gallate for 3 d in the presence of anti-CD3 and anti-CD28 Abs plus 10% (100 ng/ml; BD Bioscience). The two populations were then cocultured for 7 d (8, 9), after which the cells were stained for anti-CD25 PE Ab. Proliferation of the CD4+CD25+ T cells was measured using flow cytometry and reported as a percentage of cells that had undergone at least one division as defined by the CFSE fluorescent intensities.

Real-time PCR analysis

Total cellular RNA was extracted using TRizol reagent (Invitrogen Life Technologies, Carlsbad, CA), according to the manufacturer’s protocols (10), after which the concentration of total RNA was quantified by determining the OD at 260 nm (OD 260). The reaction mixtures were then subjected to 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. Amplification was conducted using the following gene-specific primers: FOXP3-F, 5'-GCC CAA GAT GTG CAC TGA TA-3'; FOXP3-R, 5'-AGC CTG CTC CAT ACC TTG AA-3'. Expression was calculated using the following formula:

\[ \text{Expression} = \frac{\text{target gene}}{\text{reference gene}} \times 2^{\Delta \text{Ct}} \]

where ΔCt = Ct (target gene) - Ct (reference gene).

In vivo tumor model

On day 0, C57BL/6, Foxp3EGFP^+ C57BL/6 or nu/nu male mice were inoculated s.c. in the right flank with 1 × 10^5 EL-4 lymphoma cells. To induce the depletion of CD4+CD25+ Treg cells, anti-CD25 Abs (hybridoma clone 3B3; BD Biosciences) were injected i.p. on days 5, 8, and 11 at 100 μg. After depletion, T cells were labeled with anti-CD4 PE and anti-CD25 allo-Ab mixture and anti-biotin microbeads. CD4+CD25+ T cells were positively selected from CD4+CD25− T cells using PE-labeled anti-CD25 mAb and anti-PE microbeads. The purity of both populations was determined by flow cytometric analysis and routinely reached >93%.

Preparation of single-cell suspension from tumors

At 21 d after inoculation, tumors were removed from the mice and single-cell suspensions were prepared by enzymatic digestion (15). Dissected tumors were then minced into small pieces with a scalpel, after which 0.25-g aliquots were immersed in 10 ml digestion mixture (5% FBS in RPMI 1640, 0.5 mg/ml collagenase A [Sigma-Aldrich, St. Louis, MO], 0.2 mg/ml hyaluronidase type V [Sigma-Aldrich], and 0.02 mg/ml DNase I [Sigma-Aldrich]). This mixture was then incubated at 37°C for 45 min on a rotating platform, after which the resulting cell suspensions were filtered sequentially through 70- and 40-μm cell strainers (BD Biosciences) and washed with 5% FBS in RPMI 1640. Finally, the RBCs were lysed by brief incubation in ammonium chloride solution, and the cell debris was removed by centrifugation.

Flow cytometry analysis

Isolated CD4+CD25+ Treg cells were treated with 50 μg/ml methyl gallate for 72 h and then incubated with an optimal concentration of PE-labeled mAbs for 30 min at 4°C in the dark. Cells were washed three times and resuspended by flow cytometry buffer (PBS with 2% FBS and 0.1% NaN3). Single-cell suspension and splenocytes obtained from Foxp3EGFP^+ C57BL/6 mice were labeled with anti-CD4 PE and anti-CD25 allo-

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Methyl gallate inhibits Treg cell migration

To determine whether the migration of CD4⁺CD25⁺ Treg cells toward tumors was inhibited by methyl gallate, an in vitro migration assay was performed using CD4⁺CD25⁺ Treg cells purified from the splenocytes of mice. Isolated CD4⁺CD25⁺ Treg cells were then treated with methyl gallate for 48 h. A Transwell migration assay revealed that the migration of CD4⁺CD25⁺ Treg cells toward the chamber containing the EL-4 cell supernatants was reduced by up to 31% in response to treatment with methyl gallate at a concentration of 10 μg/ml. The migration of CD4⁺CD25⁺ Treg cells was also reduced by 73% in response to treatment samples were determined by one-way ANOVA or Student’s t test. A p value <0.05 was considered to be statistically significant.

Inhibition of Foxp3 expression by methyl gallate

For assessment of Foxp3 expression by methyl gallate treatment, we used the Foxp3EGFP/C57BL/6 mice initially described by Haribhai et al. (17). These mice were derived with a bicistronic Foxp3 locus that coexpresses the EGFp under control of the endogenous Foxp3 promoter, which enables Foxp3, a key transcriptional factor in CD4⁺CD25⁺ Treg cells, to be analyzed in real time using the EGFP fluorescence signal. To visualize and quantify the degree of Foxp3 expression, flow cytometry analysis was conducted using CD4⁺CD25⁺ Treg cells from Foxp3EGFP mice. The EGFP fluorescence signal decreased by ~86.5% in response to treatment with methyl gallate (Fig. 1D).

We also measured the mRNA expression levels of Foxp3 using real-time PCR. Treatment with 50 μg/ml methyl gallate suppressed Foxp3 expression by 47% (Fig. 1E), which implies that the suppressive effects of methyl gallate on CD4⁺CD25⁺ Treg cells were attributed to decreased Foxp3 expression in CD4⁺CD25⁺ Treg cells.

Methyl gallate inhibits the suppressive effects of CD4⁺CD25⁺ Treg cells

To determine whether methyl gallate could modulate the suppressive effects of CD4⁺CD25⁺ Treg cells on CD4⁺CD25⁻ T cells. CD4⁺CD25⁻ T cells were labeled with CFSE and cocultured with CD4⁺CD25⁺ Treg cells that had been pretreated with methyl gallate. After 7 d, the mixed cells were stained with anti-CD25 Ab, and the percentage of mean fluorescence intensity of responder cells was measured. We found that the suppressive function of methyl gallate–treated CD4⁺CD25⁺ Treg cells was significantly decreased by increasing the proliferation of CD4⁺CD25⁻ T cells (Fig. 1F). These results strongly suggested that methyl gallate has inhibitory effects against CD4⁺CD25⁺ Treg cell functions in vitro.

Methyl gallate does not affect the EL-4 tumor cell cycle

To determine whether methyl gallate had any direct inhibitory effects on EL-4 cells, we conducted tumor cell cycle analysis using various concentrations of methyl gallate (0–50 μg/ml). As shown in Fig. 2, DNA flow cytometry analysis indicated that treatment with methyl gallate did not induce any specific phase arrest of the cell cycle.

Methyl gallate decreases tumor growth in an EL-4 lymphoma model and improves long-term survival rate

As a screening assay, we did migration assay of seven tumor cell lines (CMT, mouse lung adenocarcinoma; M-3, mouse melanoma; HEPA, mouse hepatoma; RAG, mouse adenocarcinoma; MTV, mouse mammary tumor; EL-4, mouse lymphoma; B16, mouse melanoma). Methyl gallate effectively inhibited 30–50% Treg migration in all cell lines. However, EL-4 lymphoma cell line was selected due to the effectiveness of tumor generation in C57BL/6 mouse (data not shown). Subcutaneous EL-4 lymphoma was established in their respective hosts, C57BL/6 mice, by injection of 10⁶ cells/animal. Methyl gallate was injected i.p. three times per week from day 5 after tumor inoculation. All mice in the untreated control group (EL-4) died between 38 and 70 d of tumor implantation, but methyl gallate–treated group showed a survival rate of ~20% at 90 d (Fig. 3A–C).

The effects of methyl gallate on tumor growth and survival in nu/nu and Treg cell-depleted mice

To determine whether there was a possible relationship between Treg cells and methyl gallate in tumor regression and survival, we used T cell–deficient nude mice (nu/nu) and evaluated their antitumor effects. EL-4 lymphoma was established in nu/nu mice by the injection of 10⁶ cells/animal. We did not observe any significant regression or delay in tumor growth in the methyl gallate–treated group when compared with the control group (Fig. 3D). We also did not observe any significant prolongation in the overall survival time in methyl gallate–treated mice (Fig. 3E). We also...
examined the effects of anti-CD25 Ab (PC61) on tumor regression to address the role of Treg cells in tumor growth. Specifically, we injected PC61 for 3 d prior to EL-4 tumor inoculation into B6 mice and found that a single injection of PC61 resulted in a significant inhibition of tumor growth, as previously described by Imai et al. (18). However, as shown in Fig. 3A, methyl gallate does not inhibit tumor growth and survival rate in PC61 treatment, suggesting that tumor regression by methyl gallate was mediated by the modulation of Treg cells (Fig. 3A–C).

Methyl gallate suppresses splenic CD4⁺CD25⁺Foxp3⁺ Treg cells and tumor infiltration of CD4⁺CD25⁺ Treg cells
To evaluate the effects of methyl gallate on CD4⁺CD25⁺ Treg cells in vivo, the expression of Foxp3 in Treg cells was analyzed in splenocytes of tumor-bearing Foxp3EGFP mice. Flow cytometry analysis was conducted using splenocytes from Foxp3EGFP mice sacrificed at 21 d after tumor inoculation. The splenocytes were then immunofluorescently stained with anti-CD4 PE and anti-CD25 allophycocyanin, after which their EGFP signals were measured.
analyzed. The methyl gallate-treated group decreased the percentage of CD4+CD25+ Treg cells from 3.35 ± 0.14% to 3.1 ± 0.15% when compared with the control group; however, this decrease did not reach the level of statistical significance. Conversely, the intensity of Foxp3 green fluorescence decreased significantly by 56.7% in the CD4+CD25+ gated cells (Fig. 4A).

Therefore, treatment with methyl gallate induced a significant decrease of CD4+CD25+Foxp3high Treg cells in the spleens of tumor-bearing mice. To determine the effects of methyl gallate on CD4+CD25+ Treg cell migration, the expression of tumor-infiltrating CD4+CD25+ Treg cells was analyzed in tumors of Foxp3EGFP mice. Flow cytometry analysis was conducted using

FIGURE 2. Cell cycle analysis of EL-4 cells treated with methyl gallate. Cells were cultured with different concentrations of methyl gallate and then with propidium iodide. The DNA content was measured by flow cytometry (A) and analyzed using the ModifiLT Software (VER 3.0, Verity Software House, Topsham, ME). B, The graph shows the percentages of each cell cycle phase from A. G1, S, and G2 indicate the cell cycle phases.
tumors from Foxp3EGFP mice that were sacrificed at 21 d after tumor inoculation (Fig. 4B). The tumor suspensions were immuno-fluorescently stained for anti-CD25 PE and the Foxp3EGFP was then analyzed. Methyl gallate treatment led to a 42.9% decrease in CD25+Foxp3+ Treg cells from 0.35 ± 0.05% to 0.20 ± 0.02% when compared with the control group.

Image analysis of Foxp3EGFP in EL-4 lymphoma
To confirm the effects of methyl gallate on CD4+CD25+ Treg cell migration, confocal microscopy was conducted using cryosectioned lymphoma tissue from Foxp3EGFP mice sacrificed at 21 d after tumor inoculation. The expansion of tumor-infiltrating Foxp3-positive cells decreased in the methyl gallate-treated mice group (Fig. 5A), and the cell numbers of Foxp3-positive cells were also significantly decreased when compared with those in the tumor tissues of normal mice (Fig. 5B).

Discussion
Tumor-induced immune suppression continues to impede the effectiveness of immune-based therapies against cancer (19). Solid tumors use numerous mechanisms designed to evade host antitumor immune response. CD4+CD25+ Treg cells are crucial cellular mediators in immune evasion by tumors (20, 21). Both Treg and effectors can be primed during tumor progression (22–24), and they both accumulate early in tumorigenesis and increase in prevalence with disease progression (25). Many of these mechanisms involve the accumulation of immune-suppressive infiltrates. Elucidation of the mechanisms by which these immune-suppressive phenotypes accumulate in the tumor microenvironment can produce attractive therapeutic targets with which to combat tumor-induced immune suppression. One of the most potent and well-studied immune-suppressive cell types is the Treg. In this study, we provide strong evidence that methyl gallate effectively inhibits accumulation of CD4+CD25+ Treg cells and enhances the antitumor effects that occur in established lymphoma models. In vitro, methyl gallate treatment led to decreased Foxp3 expression on CD4+CD25+ Treg cells and inhibited Treg cell migration toward tumor environmental factor, whereas proliferation of effector T cells cocultured with methyl gallate-treated Treg cells increased. These data suggest that methyl gallate can reduce the functional activity of CD4+CD25+ Treg cells. Phenotypic analysis of CD4+CD25+ Treg cells in methyl gallate treatment revealed a lower expression pattern of CTLA-4, CCR4, CXCR4, and GITR than control. No significant differences were observed for the expression of CD69, CCR5, and CXCR3 between methyl gallate treatment and control. To date, expression of chemokine receptors was associated with cancer metastases, such as CXCR4, CCR7, CXCR5, CXCR3, and CCR5 in various cancers. Among them, CXCR4 seems to be a major metastasis-regulating receptor that uses the lymph node trafficking network, and CCR4+ Treg cells have been shown to infiltrate human tumors and suppress antitumor activity of tumor-infiltrating effector T cells (26).
CD4+CD25+ T cells were found to be positive for Foxp3 and to play a central role in their development and function (32). CD4+CD25+ Treg cells. A significantly lower tumor-infiltrating CD25+Foxp3+ Treg cells. These results demonstrated that methyl gallate can mediate antitumor effects through Treg cells. We also did not observe any significant prolongation in overall survival time or regression in tumor growth when compared with the control group in nude mice. Thus, we have confirmed that methyl gallate is a potent therapeutic agent for T cell-mediated tumor immunity.

CD4+CD25+ Treg cells arise in the thymus, represent 5–10% of CD4+ T cells in the periphery, and are distinguished by the expression of transcription factor Foxp3. Therefore, we used Foxp3EGFP C57BL/6 to access the infiltration of CD4+CD25+ Treg cells. The methyl gallate-treated group showed decreased levels of CD4+CD25+ Treg cells; however, this decrease was not statistically significant. Also, there were some differences in Foxp3 green fluorescence intensity was significantly lower in the CD4+CD25+ gated cells. These results indicated that treatment of methyl gallate induced a significant decrease in splenic CD4+CD25+Foxp3high Treg cells. A significantly lower tumor-infiltrating CD25+Foxp3+ level was detected in methyl gallate-treated mice when compared with the control mice. Studies in recent years have clearly shown that Foxp3 is a functional marker of CD4+CD25+ Treg cells, playing a central role in their development and function (32). CD4+ CD25+ Treg cells account for the vast majority of Foxp3 expression within murine T cells, and Foxp3+–/– mice suffer from an autoimmune pathology that is secondary to a loss of CD4+CD25+ Treg cell function. Therefore, our results suggested that the therapeutic efficacy of methyl gallate occurs via suppression of Foxp3 expression in Treg cells and subsequently inhibits the suppressive and tumor-trafficking abilities of CD4+CD25+ Treg cells.

The findings that methyl gallate did not exert any cytotoxicity against Treg cells in vitro and did not cause liver or kidney dysfunction in vivo (data not shown) reflect the general safety of this phytochemical in this research. However, long-term toxicity study is needed to give methyl gallate to humans. Mechanisms by which methyl gallate might cause potential toxicity effect on other hematopoietic cells are beyond the scope of the present study and merit to be investigated further to make this result to do clinical application.

In summary, the results of this study demonstrated that methyl gallate enhances antitumor effects through suppression of CD4+CD25+ Treg cell functions, delaying tumor growth and substantially prolonging survival in mice with established tumors. Therefore, this treatment strategy could be used as an approach to potentiate the antitumor effects of immune-based cancer therapy.

Disclosures

The authors have no financial conflicts of interest.

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