Gallic acid induces T-helper-1-like T<sub>reg</sub> cells and strengthens immune checkpoint blockade efficacy

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ABSTRACT
Background Foxp3<sup>+</sup> regulatory T (T<sub>reg</sub>) cells facilitate tumor immune evasion by forming a suppressive tumor microenvironment. Therefore, immune therapies promoting T<sub>reg</sub> fragility may greatly enhance immune checkpoint blockade (ICB) efficacy in cancers.

Methods We have screened 2640 compounds and identified the gut microbial metabolite gallic acid, which promotes Foxp3 degradation and T<sub>reg</sub> instability by repressing Usp21 gene transcription. In vivo and in vitro experiments have been performed to explore the roles of Usp21 in T<sub>reg</sub> cells. Importantly, we treated tumor-bearing mice with gallic acid and anti-PD-1 antibody to explore the potential therapeutic value of gallic acid in clinical cancer immunotherapy.

Results Mechanistically, gallic acid prevents STAT3 phosphorylation and the binding of phosphorylated STAT3 to Usp21 gene promoter. The deubiquitinated Usp21 and stabilized PD-L1 proteins boost the function of T<sub>reg</sub> cells. Combination of gallic acid and anti-PD-1 antibody, in colorectal cancer (CRC) treatment, not only significantly dampen T<sub>reg</sub> cell function by impairing PD-L1/PD-1 signaling and downregulating Foxp3 stability, but also promote CD8<sup>+</sup> T cells' production of IFN-γ and limited tumor growth.

Conclusion Our findings have implications for improving the efficacy of ICB therapy in CRC by inducing T-helper-1-like Foxp3<sup>−</sup> T<sub>reg</sub> cells.

INTRODUCTION
During cancer development, cytotoxic T lymphocytes (CTLs) progressively differentiate into a dysfunctional and exhausted state marked by the accumulation of surface inhibitory receptors and reduction in effector functions.1 CD8<sup>+</sup> T cells provide immunity against cancer by recognizing ‘foreign-looking’ antigens presented on the surface of cancerous cells.2 However, when infection persists for a long period, CD8<sup>+</sup> T cells adapt to this stress by desensitizing their T cell antigen receptor (TCR) via the upregulation of inhibitory receptors such as PD-1, TIM-3, LAG-3 and TIGIT, which prevents T cells deterioration caused by overstimulation. This coping mechanism also reduces the ability of CD8<sup>+</sup> T cells to kill tumor cells, produce inflammatory cytokines (such as IFN-γ and TNF), and proliferate and form long-term memory cells.3,4 Recently, several groups have reported that the nuclear factor TOX (thymocyte selection-associated HMG BOX) as the key factor which mediates transcriptional and epigenetic changes, which are critical for the adaptation of CD8<sup>+</sup> T cells to chronic stress.

Recent immune checkpoint blockade (ICB) of PD-1 enhances immune surveillance by reinvigorating exhausted PD-1<sup>+</sup> CTLs to kill tumor cells, which has shown clinical efficacy in a variety of cancer types and even in patients with advanced stages of cancer.4,46 However, only a subset (15%–30%) of patients exhibit durable antitumor immune responses after anti-PD-1 treatment, suggesting the requirement of therapeutic strategies (eg, targeting protumorigenic or immune suppressive cells) to potentiate antitumor immunity.7–21 More importantly, in about 10% of patients with
advanced gastric cancer. PD-1 blockade promotes hyperproliferation of cancer by facilitating the amplification of PD-1+ regulatory T (Treg) cells. Overall, immunotherapy has greatly revolutionized the therapeutic interventions in cancer treatments, but its efficacy remains quite limited in clinical settings.

Treg cells express the key transcription factor Foxp3 and critically maintain immune tolerance in tumor microenvironment (TME) by suppressing antitumor immunity. For instance, tumor infiltrating Treg cells, with upregulated PD-L1 expression, significantly suppress exhausted PD-1+ cytotoxic T cells. In addition, neuropilin-1 (Nrp1) also strengthens the function of intra-tumoral Treg cells. Mechanistically, Nrp1-deficient Treg cells produce interferon-γ (IFN-γ), which drives the fragility of surrounding wild-type (WT) Treg cells in the TME, thus, boosts antitumor immune responses and significantly improves the efficacy of PD-1 blockade therapy. Therefore, immune therapies that directly promote Treg cell fragility may greatly improve the efficacy of ICB efficacy in cancer treatment.

The significant challenge of targeting Treg cells is to specifically modulate their stability and plasticity in TME. Instability of Treg cells, characterized by polyubiquitination-mediated degradation of Foxp3 and acquisition of proinflammatory T-helper-1 (Th1)-like properties, facilitates effective antitumor immunity. By contrast, Treg cells with the suppressive MondoA-like phenotype, leading to interleukin-17A (IL-17A) prominent microenvironment, CD8+ T-cell exhaustion and colorectal carcinogenesis. For these reasons, specific induction of Th1-like Foxp3lo Treg cells may restrain tumor progression by making ICB safer and more effective for cancer immunotherapies.

Tissue-resident Treg cells lacking Usp21 are instable, which confers a Th1-like Foxp3lo phenotype and may further promote antitumor immune responses. In colorectal cancer (CRC), Foxp3lo Treg cells are less immune suppressive, produce IFN-γ and are consistent with better prognosis. Conceivably, induction of Th1-like Foxp3lo Treg cells by targeting Usp21, rather than depletion of total Treg cells, may greatly increase ICB efficacy and prevent colorectal carcinogenesis, but research on this topic has been quite limited.

We have screened 2640 compounds and identified gallic acid, which promotes Foxp3 degradation by suppressing Usp21 gene transcription, leading to the generation of Th1-like Foxp3lo Treg cells. Mechanistically, gallic acid inhibits STAT3 phosphorylation and prevents the binding of phosphorylated STAT3 (p-STAT3) to the promoter of Usp21 gene. We further reveal that Usp21 additionally deubiquinates and stabilizes PD-L1 to strengthen Treg cell function. A combined immunotherapy against CRC, using gallic acid and anti-PD-1 antibody simultaneously, significantly dampens Treg cell function by impairing PD-L1/PD-1 signaling and Foxp3 stability as well as promotes cytotoxic T cells’ expression of IFN-γ, thus, limits tumor growth. Our findings provide a new strategy to improve the efficacy of ICB therapy in CRC treatment by inducing Th1-like Foxp3lo Treg cells.

**METHODS**

**Mice**

In this study, all mouse lines were maintained on a C57BL/6J background. Foxp3cre mice were purchased from the Jackson Laboratory (stock number: 016959, Bar Harbor, USA). Usp21fl/fl mice were described in previous report. To exclude cage effects, heterozygotes (For example, Usp21fl/foxp3creXUsp21fl/foxp3cre) were bred to generate Treg-specific KO mice, Usp21fl/foxp3cre and Foxp3cre (control) mice. Foxp3cre (WT) and Usp21fl/foxp3cre (KO) mice of C57BL/6 strain were maintained in the animal facility of Shanghai Jiao Tong University School of Medicine under specific pathogen-free conditions. Animal experiments were conducted in accordance with institutional guidelines approved by the Institutional Animal Care and Use Committee/Shanghai Jiao Tong University School of Medicine.

**Cell lines**

The C57BL/6 murine colon MC38 adenocarcinoma cell line were routinely cultured at 37°C and 5% CO2 in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), Gibco, ThermoFisher Scientific, Waltham, MA, USA), 1% penicillin, 1% streptomycin and 2 mM glutamine. Human LOVO cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), which were cultured at 37°C and 5% CO2 in DMEM supplemented with 10% FBS, 1% penicillin, 1% streptomycin and 2 mM glutamine. HEC 293T (ATCC, CRL-11268) cells were cultured at 37°C and 5% CO2 in DMEM supplemented with 10% FBS, 1% penicillin, 1% streptomycin and 2 mM glutamine. Cell lines were tested for mycoplasma contamination before use.

**TCGA data analysis**

Survival analysis, using TCGA COAD data, was performed to evaluate the association of individual gene or gene sets derived from specific cell clusters with GC prognosis. The statistical analysis of gene expression and the clinical outcomes were performed by GEPIA2 and Kaplan-Meier plotter. The mean expression of given signatures was grouped into high and low expression groups by the 25th and 75th quantile values.

**Human CRC patients’ specimens**

Blood, paratumor and tumor samples from CRC patients were obtained from the Department of Gastrointestinal Surgery, Renji Hospital, Shanghai Jiao Tong University School of Medicine. The clinical criteria for patient recruitment were as follows: (1) patients had no autoimmune disorders or other primary malignant tumors; (2) patients had not been treated with chemotherapy, radiation, or any other antitumor medicine prior to tumor...
resection; (3) patients had completed clinical information, postoperative pathological diagnoses, and follow-up data. Clinical characteristic of CRC patients can be found in online supplemental table 1. Clinical characteristics of CRC patients can be found in online supplemental table 1. Tumor and paratumor tissues were excised and digested with 2 mg/mL Collagenase D (Sigma-Aldrich, St. Louis, USA Cat#11088882001) and 150 µg/mL DNase I (Sigma-Aldrich, Cat# DN25) at 37°C with shaking at 200 r.p.m for 1 hour. Lymphocytes were further analyzed by flow cytometry (described in detail below).

**Tumor engraftment**

Mice were subcutaneously injected with 2×10³ MC38 colorectal adenocarcinoma cells or B16 melanoma cells or 4T1 mammary carcinoma cells. When palpable tumors were presented, treatment was started as described below and tumor volume was assessed by caliper measurement. Mice were sacrificed when tumor volume reached 1500 mm³. Gallic acid (5 mg/kg, Sigma-Aldrich, Cat#149-91-7) was dissolved in PBS and intraperitoneally injected daily starting on day 7. Starting on day 10, anti-PD-1 antibody (100 µg/injection, BioxCell, New Hampshire, USA, Cat#BE0146) or isotype control (100 µg/injection, BioxCell, Cat#BE0083) were intraperitoneally injected once every 3 days for three times. Mice were next transcardially perfused with heparin (10 U/mL, Sigma-Aldrich, Cat#H3149-25KU) in PBS under anesthesia. Tumors were collected and processed for flow cytometry. In subcutaneous tumor models, fresh tumor tissues were washed three times with RPMI 1640 before cut into small pieces. The specimens were then collected in RPMI 1640 containing 2 mg/mL collagenase D (Roche, Cat#11088882001) and 150 µg/mL DNase I (Sigma-Aldrich, Cat# DN25). The specimens were then mechanically dissociated using the gentle MACS Dissociator (Milltenyi Biotec, Bergisch Gladbach, GER). Dissociated cell suspensions were further incubated for 30 mins at 37°C under continuous rotation and filtered through 70 µm cell strainers. Lymphocytes were further separated by centrifugation through 70 µm cell strainers to obtain cell suspensions. Lymphocytes were further analyzed by flow cytometry.

**Azoxymethane-dextran sodium sulfate-induced colorectal carcinogenesis mice model**

Eight-week-old female mice were intraperitoneally injected with azoxymethane (AOM, 10 mg/kg, Sigma-Aldrich, Cat# A5486). Seven days later, 2% dextran sodium sulfate (DSS, 36-50 kDa, MP Biomedicals, Irvine, USA Cat# 160110) was added to drinking water for 7 days followed by regular drinking water for 2 weeks. This cycle of DSS treatment was repeated twice and mice were euthanized on day 100 after treatment. In the antibody therapy, Foxp3ser and Usp21fl/flFoxp3ser mice were co-housed in the same cage and AOM-DSS were used to induce tumors. Anti-PD-1 (100 µg/injection, BioxCell, Cat# BE0146) or isotype control (100 µg/injection, BioxCell, Cat# BE0083,) antibodies were intraperitoneally injected once every 3 days for five times, starting on day 80. Gallic acid (5 mg/kg, Sigma-Aldrich, Cat#149-91-7) was dissolved in PBS and injected daily starting at the 80th day. To obtain lamina propria lymphocytes, colons were opened longitudinally; washed with Hank’s balanced salt solution; shaken in Hank’s balanced salt solution containing 2 mmol/L EDTA at 37°C to remove epithelial cells, and incubated with collagenase VIII (Sigma-Aldrich, Cat#C5138) and deoxyribonuclease I (Sigma-Aldrich, Cat#DN25) for 40 mins. The supernatants were passed through 70 µm cell strainers. Lymphocytes were further separated by centrifugation with 40%–70% Percoll (GE Healthcare, Chicago, USA, Cat#17-0891-01) gradient and then subjected to flow cytometric analysis.

**Reagents and antibodies**

The following reagents and antibodies were used in this study:

- MG132 (Sigma-Aldrich, Cat#M7449); anti-Flag (Sigma-Aldrich, Cat#F7425); anti-MYC (Sigma-Aldrich, Cat#M449); anti-STAT3 (Cell Signaling Technology, Cat#9138S); anti-phospho-STAT3 (Cell Signaling Technology, Cat#9145S).
- Mouse IgG Isotype control (Cell Signaling Technology, Cat#53484).
- Rabbit IgG Isotype control (Cell Signaling Technology, Cat#2729); anti-Usp21 (Invitrogen, Cat#PA5-110556); anti-beta actin (2D4H5) (ProteinTech, Cat#66009-1-Ig).

**Immunoprecipitation and immunoblot analysis**

HEK293T cells were transfected with indicated vectors and further lysed in 300 µL RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5 mM EGTA, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10 µg/mL aprotinin, 10 µg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) supplemented with protease inhibitor cocktail (1:100, Sigma-Aldrich, Cat#P8340), 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride (PMSF). For immunoprecipitation, cell lysates were cleared by centrifugation and supernatants were immunoprecipitated with the appropriate antibodies using protein A/G-agarose beads at 4°C. After washing, sample-loading buffer was added to the precipitates. Samples were then used for immunoblot analysis.

**Flow cytometry**

To determine cytokine expression, cells were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, Cat#P1585), 1 mM ionomycin (Sigma-Aldrich, Cat#13909), Golgi Stop and Golgi Plug (BD Bioscience, New Jersey, USA, Cat#554724) for 4 hours. At the end of stimulation, cells were stained with fixable viability dye eFlour 780 (eBioscience, San Diego, USA, Cat#65-0865-14). For the analysis of surface markers, cells were stained in PBS containing 2% FBS (Gibco, Cat#10-082-147) with antibodies as indicated. Foxp3 staining was performed according to the manufacturer’s instructions (Transcription Factor Staining Buffer Set, eBioscience, Cat#00-5523-40). For flow cytometry staining, unless...
In vitro expansion of human regulatory T cells

Human PBMCs were obtained from blood samples of healthy donors (Shanghai Blood Center) with ethical approval from Shanghai Blood Center Ethics Committee. Human CD4+CD25lowCD122highCD45RA- naïve CD4+ T cells were sorted by BD FACS Aria II cell sorter and differentiated into inducible T reg cells in X-VIVO (Lonza, Malsersville, USA, Cat#04-418Q) medium supplemented with 10% fetal bovine serum (Invitrogen, Maltham, USA, Cat#04-418Q), 1% Glutamax, 1% sodium pyruvate, 1% minimum essential medium with nonessential amino acids, 1% penicillin-streptomycin, 100 U/mL IL2 (R&D systems, Minneapolis, USA, Cat#202-IL), and 5 ng/mL transforming growth factor-β (R&D Systems, Cat#240-B), in the presence of Dynabeads Human T-activator CD3/CD28 (Gibco, Cat#1132D) at a bead-to-cell ratio of 1:4. Approximately 7 days later, the differentiation efficiency reached at least 90% and could be used for analysis.

In vitro suppressive assay

Human effective CD4+CD25low T cells were labeled with CellTrace Violet and sorted with BD FACS Aria II cell sorter. The labeled Teffs were then cultured alone or mixed, at different ratios, with inducible T reg cells. For T reg suppression assay, inducible T reg cells were first cultured in the presence or absence of 10 μmol/L gallic acid for 24 hours. The treated T reg cells were then cultured with CellTrace Violet-labeled responder T cells in the presence of anti-CD3/CD28 beads for 3 days.

His-ubiquitin pulldown assay

For ubiquitination assay, HEK293T cells were transfected with the indicated plasmids and were treated with 10 μM MG132 for 6 hours before harvesting. Cells were lysed in a pH 8 urea buffer (8 M urea, 100 mM NaH2PO4, 10 mM TRIS (pH 8.0), 0.2% TX-100, 10 mM imidazole and 1 mM N-ethylmaleimide) and incubated with Ni-NTA beads for 2 hours at room temperature. The beads were washed twice in pH 8 urea buffer; twice in pH 6.3 urea buffer (8 M urea, 100 mM NaH2PO4, 10 mM TRIS (pH 6.3), 0.2% TX-100 and 10 mM imidazole); and once in wash buffer (20 mM TRIS (pH 8.0), 100 mM NaCl, 20% glycerol, 1 mM dithiothreitol and 10 mM imidazole). Samples were then used for immunoblotting analysis with indicated antibodies.

ChiP assay

Briefly, $1 \times 10^7$ T reg cells were cross-linked with formaldehyde and the chromatin was sonicated into ~500 bp fragments. After sonication, the chromatin solution (approximately 500 μg) was incubated with ChiP-grade antibodies against p-STAT3 (Tyr705, D3H7, CST) and rabbit IgG overnight at 4°C. The antibody-bound complexes were precipitated, and the DNA fragments extricated from these complexes were purified using a QIAquick PCR Purification Kit (Qiagen, Düsseldorf, Germany). Preimmunoprecipitated input DNA was used as control in each reaction. The purified ChiP DNA samples were analyzed by quantitative real-time PCR (qRT-PCR) with primers listed in online supplemental table 2.

RESULTS

Gallic acid induces Th1-like FOXP3low T reg cells

To induce Th1-like FOXP3low T reg cells, might prevent colorectal carcinogenesis, we first screened 2640 compounds and identified 31 small molecules that could downregulate the protein levels of FOXP3, including the gut microbial metabolite gallic acid (figure 1A). Flow cytometric analysis further confirmed that gallic acid treatment induced the decrease of FOXP3 protein expression (figure 1B,C) and simultaneously production of IFN-γ (figure 1D,E). Interestingly, Foxb3 gene was still actively transcribed after gallic acid treatment (figure 1F), suggesting that gallic acid compromised the stability of FOXP3 protein potentially through post-translational modification.

To investigate how gallic acid modulated FOXP3 protein stability, we next examined the expression of verified E3 ubiquitin ligases and deubiquitinas in
Figure 1  Gallic acid induces Th1-like T<sub>reg</sub> cells by suppressing Usp21 gene transcription. (A) Human iT<sub>reg</sub> cells were polarized from naive CD4<sup>+</sup> T cells and further treated with small molecules (n=2640) for 48 hours. FOXP3 levels were next analyzed by flow cytometry. (B) Representative figure shown the expression of FOXP3 protein in iT<sub>reg</sub> cells treated with or without gallic acid (10µM) for 48 hours (n=4 per group). (C) Mean fluorescent intensity (MFI) of FOXP3 in iT<sub>reg</sub> cells as indicated in (B). (D) Representative figure shown the expression of IFN-γ in FOXP3<sup>+</sup> iT<sub>reg</sub> cells treated with or without gallic acid (10µM) for 48 hours (n=4 per group). (E) Percentages of IFN-γ FOXP3<sup>+</sup> iT<sub>reg</sub> cells as indicated in (D). (F) Relative expressions levels of Foxp3, Rnf31, Mdm2, Stubb1, Cblb, Usp7, Usp21, Usp22 and Usp44 mRNAs in iT<sub>reg</sub> cells treated with or without gallic acid (10µM) for 48 hours (n=3 per group). (G) Representative Western blot shown the expression of USP21, FOXP3, phosphorylated STAT3 (p-STAT3) and total STAT3 proteins in iT<sub>reg</sub> cells treated with indicated doses of gallic acid for 48 hours. (H) ChIP-qPCR analysis of p-STAT3 occupancy on the promoter of Usp21 gene in human iT<sub>reg</sub> cells treated with or without gallic acid (10µM) for 24 hours (n=3 per group). (I) Human iT<sub>reg</sub> cells were treated with or without gallic acid (10µM) for 48 hours. Cells were treated with MG132 for 6 hours before collection. Cell lysates were immunoprecipitated with anti-Ubiquitin antibody and ubiquitinated FOXP3 levels were detected by Western blot. (J) Flow cytometric analysis of CellTrace Violet-labeled effector T cells (T<sub>Teff</sub>) cells cultured with iT<sub>reg</sub> cells that were pre-treated with or without gallic acid (10µM) at the indicated T<sub>Treg</sub>:T<sub>Teff</sub> ratios in the presence of anti-CD3/CD28 beads for 3 days. (K) Percentages of proliferating T<sub>Teff</sub> cells (n=3 for each group). (L) Kaplan-Meier overall survival curve of CRC patients based on Usp21 gene expression (from TCGA). (M) Representative figure shown the expression of FOXP3 and IFN-γ by CD4<sup>+</sup> T cells from peripheral blood mononuclear cells (PBMCs) and single cells of paratumor and tumor tissues of CRC patients, which were treated with or without gallic acid (10µM) for 24 hours (n=6 per group). (N) Percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells as indicated in (M). (O) Percentages of IFN-γ FOXP3<sup>+</sup> T<sub>reg</sub> cells as indicated in (M). Results are representative of two (I) and three (G) independent experiments. Data are mean±SD; NS, not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 as determined by unpaired two-tailed Student’s t-test. CRC, colorectal cancer; p-STAT3, phosphorylated STAT3; q-PCR, quantitative PCR.
T<sub>reg</sub> cells, including Rnf31, Mdm2, Stab1, Chib, Usp7, Usp21, Usp22 and Usp44. We observed that gallic acid specifically suppressed the transcription of Usp21 gene (figure 1F). Taken together, gallic acid potentially induced Th1-like Foxp3<sup>lo</sup> T<sub>reg</sub> cells by dampening the expression of Usp21 gene.

As p-STAT3 drives Usp21 gene transcription, we next tested the effects of gallic acid on STAT3 phosphorylation. Gallic acid inhibited STAT3 phosphorylation at Y705 site (figure 1G) and prevented the binding of p-STAT3 (p-STAT3) to Usp21 gene promoter (figure 1H). These data suggested that gallic acid suppressed Usp21 gene transcription by inhibiting STAT3 phosphorylation.

Gallic acid suppressed Usp21 gene expression and might promote FOXP3 degradation through polyubiquitination. Indeed, we observed higher levels of ubiquitinated FOXP3 in T<sub>reg</sub> cells after gallic acid treatment (figure 1I). Since FOXP3 critically maintains T<sub>reg</sub> cell function, we next examined whether gallic acid perturbed suppressive capacity of T<sub>reg</sub> cells. Using an in vitro suppression assay, we found that gallic acid-treated T<sub>reg</sub> cells had significantly impaired suppressive capacity towards CD4<sup>+</sup>FOXP3<sup>+</sup> effector T (T<sub>eff</sub>) cell proliferation (figure 1J,K). These results collectively suggested that gallic acid promoted FOXP3 degradation and dampened the suppressive function of T<sub>reg</sub> cells.

With respect to CRC, patients with higher levels of Usp21 transcripts in tumor displayed worse overall survival (OS) (figure 1L). Conceivably, gallic acid suppressed Usp21 gene expression and potentially promoted better prognosis of CRC patients. To test the potential effects of gallic acid on CRC, peripheral blood mononuclear cells (PBMCs) and single cells of paratumor and tumor tissues from CRC patients were next treated with gallic acid. Interestingly, gallic acid induced FOXP3 loss by T<sub>reg</sub> cells (figure 1M–N) and simultaneously induced Th1-like T<sub>reg</sub> cells in blood, paratumor and tumor samples, which was characterized by increased percentages of IFN-γ FOXP3<sup>lo</sup> T<sub>reg</sub> cells (figure 1M,O). Overall, these results supported that gallic acid induced Th1-like FOXP3<sup>lo</sup> T<sub>reg</sub> cells by repressing Usp21 gene transcription.

In summary, we found that gallic acid inhibited Usp21 expression by decreasing STAT3 phosphorylation. Gallic acid further dampened FOXP3 stability and induced Th1-like FOXP3<sup>lo</sup> T<sub>reg</sub> cells.

**Gallic acid prevents subcutaneous tumor growth**

As previously outlined, gallic acid induced stable FOXP3<sup>lo</sup> T<sub>reg</sub> cells and might promote antitumor immunity to restrain tumor growth. Thus, we first tested the antitumor effects of gallic acid in subcutaneous tumor models. Indeed, gallic acid greatly suppressed the growth of syngeneic MC38 colorectal adenocarcinoma cells (figure 2A), B16F10 melanoma cells (figure 2E) and 4T1 mammary carcinoma cells (figure 2I). Overall, gallic acid significantly prevented subcutaneous tumor growth.

To investigate in vivo influences of gallic acid on the stability of T<sub>reg</sub> cells and their suppressive capacity towards PD-1<sup>+</sup> cytotoxic T cells, we next analyzed Foxp3 and PD-L1 expression using flow cytometry. Notably, we observed decreased frequencies of Foxp3<sup>+</sup> and Foxp3<sup>+</sup>PD-L1<sup>+</sup> intra-tumoral T<sub>reg</sub> cells on gallic acid treatment (online supplemental figure 4A–F), which was simultaneously accompanied with much lower protein levels of Foxp3 and PD-L1 in T<sub>reg</sub> cells from subcutaneous MC38 tumor (figure 2B–D), B16F10 tumor (figure 2F–H) and 4T1 tumor (figure 2J–L). Taken together, gallic acid restricted subcutaneous tumor growth by inhibiting Foxp3 and PD-L1 expression in intra-tumoral T<sub>reg</sub> cells, which might further promote antitumor immunity.

**Usp21-deficient T<sub>reg</sub> cells prevent subcutaneous tumor growth**

To study the physiological roles of Usp21 in intra-tumoral T<sub>reg</sub> cells, we next generated a subcutaneous MC38 tumor model in Usp2<sup>fl/fl</sup>Foxp3<sup>cre</sup> and Foxp3<sup>−/−</sup> mice. Usp2<sup>fl/fl</sup>Foxp3<sup>−/−</sup> mice displayed attenuated tumor growth, which was characterized by smaller MC38 tumor sizes (figure 3A,B). Compared with Foxp3<sup>−/−</sup> mice, we observed decreased percentages of CD4<sup>+</sup>Foxp3<sup>−</sup> T<sub>reg</sub> cells in Usp2<sup>fl/fl</sup>Foxp3<sup>−/−</sup> mice (online supplemental figure 4G). Moreover, Usp2-deficient T<sub>reg</sub> cells had much lower levels of Foxp3 protein (figure 3C,D) but higher levels of IFN-γ (figure 3F), which significantly promoted IFN-γ expression by CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>eff</sub> cells (figure 3G). Therefore, Usp2-deficient T<sub>reg</sub> cells, expressed lower Foxp3, became Th1-like, which amplified Th1 responses and limited subcutaneous tumor growth.

We next tested whether Usp2 perturbed intra-tumoral T<sub>reg</sub> cell’s expression of PD-L1 protein. Compared with WT T<sub>reg</sub> cells, we observed much lower levels of PD-L1 in Usp2-deficient counterparts (figure 3G), which were characterized by lower percentages of PD-L1<sup>+</sup>Foxp3<sup>−</sup> T<sub>reg</sub> cells (online supplemental figure 4H) and decreased mean fluorescent intensity (MFI) of PD-L1 (figure 3H). Moreover, tumor infiltrating CD8<sup>+</sup> cytotoxic T cells produced higher amounts of IFN-γ in Usp2<sup>fl/fl</sup>Foxp3<sup>−/−</sup> mice (figure 3J,L), while expressed lower levels of PD-1 (figure 3L,K) possibly due to loss of Foxp3 and PD-L1 in Usp2-deficient T<sub>reg</sub> cells. Taken together, these results revealed that Usp2-deficient T<sub>reg</sub> cells amplified Th1 phenotype and cytotoxic T cell responses and prevented subcutaneous MC38 tumor growth.

**Usp21-deficient T<sub>reg</sub> cells prevent colorectal carcinogenesis**

To confirm the role of Usp2-deficient T<sub>reg</sub> cells in CRC progression, we generated an AOM-DSS-induced murine CRC model in Usp2<sup>fl/fl</sup>Foxp3<sup>cre</sup> and Foxp3<sup>−/−</sup> mice. Interestingly, compared with Foxp3<sup>−/−</sup> mice, Usp2<sup>fl/fl</sup>Foxp3<sup>−/−</sup> mice developed less colorectal tumors in middle and distal colon regions (figure 4A,B). Statistical analysis further confirmed that colorectal tumor numbers and sizes dramatically decreased in Usp2<sup>fl/fl</sup>Foxp3<sup>−/−</sup> mice (figure 4C,D). Together, Usp2-deficient T<sub>reg</sub> cells prevent colorectal carcinogenesis in vivo.

In Usp2<sup>fl/fl</sup>Foxp3<sup>−/−</sup> mice, we further observed decreased percentages of CD4<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub> cells (figure 4E, online supplemental figure 4I), with lower levels of Foxp3.
protein (figure 4F). Meanwhile, Usp21-deficient Treg cells expressed much lower levels of PD-L1 (figure 4G, online supplemental figure 4J), suggesting regulatory roles of Usp21 in PD-L1 expression. In parallel with previous results, CD4+Foxp3+ Usp21-deficient Treg cells became Th1-like and produced increased levels of IFN-γ (figure 4H,I). Moreover, tumor infiltrating CD8+ cytotoxic T cells produced higher amounts of IFN-γ and expressed much lower levels of PD-L1 in Usp21fl/flFoxp3cre mice (figure 4J–L), possibly due to impaired suppressive activity of Usp21-deficient Treg cells.21 These findings convinced that Usp21-deficient Treg cells significantly prevented AOM-DSS-induced colorectal carcinogenesis.

**Gallic acid downregulates PD-L1 through inhibiting USP21 mediated deubiquitination**

Previous data suggested that PD-L1 protein level was decreased in gallic acid-treated and Usp21-deficient Treg cells, and we observed that Pdcd1 gene was still actively transcribed (figure 5A), while Usp21 and PD-L1 protein were downregulated in Usp21-deficient Treg cells (figure 5B), suggesting PD-L1 protein stability potentially through post-translational modification. Our data revealed ubiquitination-mediated degradation of PD-L1.

We studied the half-life of PD-L1 using the protein synthesis inhibitor cycloheximide. Ectopically expressed Usp21 significantly extended the half-life of PD-L1 protein (figure 5C). To test whether Usp21 functioned as a direct E3 deubiquitinase of PD-L1, we next carried out binding studies to determine whether PD-L1 could bind to Usp21. Reciprocal immunoprecipitation of FLAG-PD-L1 and MYC-Usp21 revealed an interaction between Usp21 and PD-L1 (figure 5D). We also detected the endogenous interaction between Usp21 and PD-L1 in human Treg cells (figure 5E) and LOVO cancer cells (figure 5F). These data further suggested regulatory roles of Usp21, in Treg cells, were carried out through interacting with PD-L1.

Using a His-ubiquitin pulldown assay, we found that ectopically expressed Usp21, but not the enzymatically inactive mutant C221A, reduced levels of ubiquitinated PD-L1 (figure 5G). To identify the specific lysine residues

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**Figure 2** Gallic acid prevents subcutaneous MC38 tumor growth. (A) MC38 tumor volume of WT mice intraperitoneally treated with or without gallic acid (5 mg/kg) daily, starting at day 7. Tumor volume was measured every 3 or 4 days (n=5 per group). (B) Representative figure shown the expression of Foxp3 and PD-L1 by tumor infiltrating CD4+ T cells in MC38 tumors, as indicated in (A). (C, D) Mean fluorescent intensity (MFI) of Foxp3 (C) and PD-L1 (D) in Treg cells as indicated in (B). (E) B16F10 tumor volume of WT mice intraperitoneally treated with or without gallic acid (5 mg/kg) daily, starting at day 7. Tumor volume was measured every 3 or 4 days (n=5 per group). (F) Representative figure shown the expression of Foxp3 and PD-L1 by tumor infiltrating CD4+ T cells in B16F10 tumors, as indicated in (E). (G, H) MFI of Foxp3 (G) and PD-L1 (H) in Treg cells as indicated in (F). (I) 4T1 tumor volume of WT mice intraperitoneally treated with or without gallic acid (5 mg/kg) daily, starting at day 7. Tumor volume was measured every 3 or 4 days (n=5 per group). (J) Representative figure shown the expression of Foxp3 and PD-L1 by tumor infiltrating CD4+ T cells in 4T1 tumors, as indicated in (I). (K, L) MFI of Foxp3 (K) and PD-L1 (L) in Treg cells as indicated in (J). Data are mean±SD; NS, not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 as determined by unpaired two-tailed Student’s t-test. WT, wild-type.
of PD-L1 that could be deubiquitinated by Usp21, we screened each individual lysine-only mutants of PD-L1 (where all lysines within the intracellular regions of PD-L1 were mutated to arginines with only one lysine untouched) and identified three lysine residues (K271, K280 and K281), which were potential Usp21 targets (figure 5H). We further tested mutants where only three lysine residues (K271, K280 and K281) were retained or mutated into arginines (termed K271/280/281 only and K271/280/281R mutants, respectively, figure 5I). His-ubiquitin pulldown assays further confirmed that Usp21 could deubiquitinate PD-L1 at these three lysine residues and the K271/280/281R construct was unresponsive to Usp21-mediated deubiquitination (figure 5I). His-ubiquitin pulldown assays further confirmed that Usp21 could deubiquitinate PD-L1 at these three lysine residues and the K271/280/281R construct was unresponsive to Usp21-mediated deubiquitination (figure 5I). Furthermore, Usp21 removed K48-linked ubiquitin chain of PD-L1 (figure 5J). These findings demonstrated that Usp21 could stabilize PD-L1 through deubiquitination in Treg cells.

Next, we observed the downregulation of PD-L1 in gallic acid treated Treg, while Pdcd1 gene was still actively transcribed (online supplemental figure 3A). Importantly, ectopic Usp21 expression restores Foxp3 and PD-L1 protein expression in Treg cells during gallic acid treatment (online supplemental figure 3B). Gallic acid treatment significantly shortened the half-life of PD-L1 protein (online supplemental figure 3C), and proteasome inhibition MG132 stabilized PD-L1 during gallic acid treatment (online supplemental figure 3D). Taken together, gallic acid downregulates PD-L1 through inhibiting Usp21 mediated deubiquitination.

Gallic acid strengthens anti-PD-1 efficacy in subcutaneous tumor model

We further asked whether additional gallic acid treatment improved ICB efficacy in a subcutaneous MC38 tumor model. MC38 tumor-bearing mice were treated with isotype control, gallic acid, anti-PD-1 or gallic acid combined with anti-PD-1 antibody. Gallic acid as well as anti-PD-1 administration prevented MC38 tumor growth and reduced tumor sizes (figure 6A,B), while the combined gallic acid and anti-PD-1 treatment more significantly limited MC38 tumor growth (figure 6A,B) and extended the OS of MC38 tumor-bearing mice (figure 6C). These data suggested that gallic acid strengthened anti-PD-1 efficacy.
In parallel with previous findings, gallic acid significantly induced instable Foxp3 and PD-L1 expression by intratumoral T\textsubscript{reg} cells (figure 6D). In detail, we observed lower percentages of Foxp3\textsuperscript{+} or Foxp3\textsuperscript{+}PD-L1\textsuperscript{+} Treg cells (online supplemental figure 4K–L) and decreased MFIs of Foxp3 or PD-L1 among T\textsubscript{reg} cells (figure 6E,F) after gallic acid treatment. Compared with control group, PD-1 blockade slightly decreased Foxp3 and PD-L1 expression by intra-tumoral T\textsubscript{reg} cells, whereas these changes were not statistically significant (figure 6E,F). Moreover, compared with PD-1 blockade group, a combined gallic acid and anti-PD-1 treatment robustly dampened Foxp3 and PD-L1 expression by tumor infiltrating T\textsubscript{eff} cells (figure 6E,F). Together, gallic acid induces the instability of Foxp3 and PD-L1 in intra-tumoral T\textsubscript{reg} cells, while anti-PD-1 antibody does not influence Foxp3 and PD-L1 protein of T\textsubscript{reg} cells.

We next analyzed IFN-\gamma expression by tumor infiltrating CD4\textsuperscript{+}Foxp3\textsuperscript{+} T\textsubscript{reg} cells. Compared with control group, gallic acid or PD-1 blockade significantly increased percentages of IFN-\gamma\textsuperscript{+} T\textsubscript{reg} cells in TME (figure 6D,G). Notably, a combined gallic acid and anti-PD-1 treatment enable intra-tumoral T\textsubscript{reg} cells to produce much more IFN-\gamma (figure 6D,G), when compared with PD-1 blockade group. Therefore, gallic acid strengthened anti-PD-1 efficacy and restored the function of tumor infiltrating T\textsubscript{eff} cells.

Similar phenotypes were observed in intra-tumoral CD8\textsuperscript{+} cytotoxic T cells. Compared with control group, gallic acid or PD-1 blockade significantly increased percentages of CD8\textsuperscript{+}IFN-\gamma\textsuperscript{+} cytotoxic T cells in TME (figure 6H,J), while simultaneously dampened PD-1 expression (figure 6H,J). In addition, a combined gallic acid and anti-PD-1 treatment significantly reinvigorated CD8\textsuperscript{+} T cells to produce higher amounts of IFN-\gamma and express lower levels of PD-1 in MC38 tumor (figure 6H–J). Also, we noticed several labs have revealed that TOX is the key regulator of T cell exhaustion. TCR stimulation induces TOX expression, through the NFAT pathway, which further upregulate inhibitory factors PD-1, TIM3, TIGIT and LAG3, associated with decreased IFN-\gamma and TNF production.\textsuperscript{3,32–34} We have, additionally, measured the protein level of TOX, LAG3 and TNF-\alpha besides IFN-\gamma and PD-1 to explore the function of gallic acid and anti-PD-1 antibody in T cell
exhaustion. The results showed that TOX and LAG3 expression decreased in CD8+ T cells, TNF-α increased in CD8+ T cells after gallic acid treatment and anti-PD-1 antibody treatment (online supplemental figure 5). Taken together, these results suggested that gallic acid could strengthen ICB efficacy in repressing tumor development.
In summary, anti-PD-1 antibody repressed the growth of MC38 subcutaneous tumor growth by restoring the function of exhausted tumor infiltrating CD8^+ T cells. Gallic acid could strengthen ICB efficacy by dampening the expression of PD-L1 and Foxp3 proteins in Treg.

**Gallic acid strengthens anti-PD-1 efficacy in murine CRC model**

We next investigated whether gallic acid might improve ICB efficacy in AOM-DSS-induced CRC. To minimize the potential effects of microbiota differences during ICB therapy, we co-housed mice when inducing CRC following AOM-DSS protocol. CRC-bearing mice were treated with isotype control, gallic acid, anti-PD-1 or gallic acid combined with anti-PD-1 antibody, respectively. Gallic acid or anti-PD-1 administration prevented AOM-DSS-induced colorectal carcinogenesis, while a combined gallic acid and anti-PD-1 treatment more significantly limited CRC progression (figure 7A–C).

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**Figure 6**  Gallic acid strengthens anti-PD-1 efficacy in subcutaneous tumor model. (A) MC38 tumor volume of WT mice intraperitoneally treated with isotype control (100 µg/injection), anti-PD-1 antibody (100 µg/injection), gallic acid (5 mg/kg) or gallic acid (5 mg/kg) plus anti-PD-1 antibody (100 µg/injection). Tumor volume was measured every three or 4 days (n=6 per group). (B) MC38 tumor sizes at day 18 (n=6 per group), as indicated in (A). (C) The survival curve of WT mice in (A). (D) Representative figure shown the expression of Foxp3, PD-L1 and IFN-γ by CD4^+ T cells in MC38 tumors, as indicated in (A). (E) Mean fluorescent intensity (MFI) of Foxp3 in T_reg cells (n=5 per group), as indicated in (D). (F) MFI of PD-L1 in T_reg cells (n=5 per group), as indicated in (D). (H) Representative figure shown the expression of PD-1 and IFN-γ by CD8^+ T cells in colorectal tumors (n=5 per group), as indicated in (A). (I) Percentages of CD8^+ IFN-γ^+ T cells as indicated in (H). Data are mean±SD; NS, not significant; *p<0.05, **p<0.01, ***p<0.001, as determined by unpaired two-tailed Student’s t-test. P values in (C) were determined by log-rank test. WT, wild-type.
Consistently, gallic acid administration significantly induced instability of Foxp3 and PD-L1 in tumor infiltrating Treg cells (figure 7D), which were characterized by decreased percentages of Foxp3+ or Foxp3+PD-L1+ Treg cells (online supplemental figure 4M–N) and lower MFIs of Foxp3 or PD-L1 among Treg cells (figure 7E,F). Compared with control group, PD-1 blockade slightly decreased percentages of Foxp3+ or Foxp3+PD-L1+ intra-tumoral Treg cells (online supplemental figure 4M–N), without affecting MFIs of Foxp3 or PD-L1 (figure 7E,F).
Moreover, compared with PD-1 blockade group, a combined gallic acid and anti-PD-1 treatment robustly dampened Foxp3 and PD-L1 expression by tumor infiltrating Treg cells in CRC (figure 7E,F). Overall, gallic acid strengthened anti-PD-1 efficacy in AOM-DSS-induced CRC by inducing Foxp3<sup>−</sup>PD-L1<sup>−</sup> Treg cells, which might further reinvigorate tumor infiltrating T cell function.

Compared with control group, gallic acid or PD-1 blockade significantly increased percentages of CD8<sup>+</sup>IFN-γ<sub>−</sub> cytotoxic T cells in TME (figure 7G,H), while simultaneously dampened PD-1 expression (figure 7G,I). More importantly, a combined gallic acid and anti-PD-1 treatment significantly promoted CD8<sup>+</sup> T cells to produce higher amounts of IFN-γ in CRC (figure 7H). Consistently, TOX and LAG3 expression decreased in CD8<sup>+</sup> T cells, while TNF-α increased in CD8<sup>+</sup> T cells after gallic acid and anti-PD-1 antibody treatments (online supplemental figure 6). Together, these results demonstrated that gallic acid could strengthen ICB efficacy in AOM-DSS-induced CRC model.

In conclusion, we first screened compounds and identified gallic acid specifically induced Th1-like Foxp3<sup>+</sup>PD-L1<sup>+</sup> Treg cells by suppressing Usp21 gene transcription (online supplemental figure 1). Mechanistically, Usp21 stabilized Foxp3 and PD-L1 through deubiquitination. Of note, Usp21-deficient Treg cells impaired PD-L1/PD-1 signaling and Foxp3 stability, promoted IFN-γ expression by CD8<sup>+</sup> T cells, and limited tumor growth. Next, gallic acid greatly strengthened anti-PD-1 efficacy in both subcutaneous tumor and AOM-DSS-induced CRC models by inducing Th1-like Foxp3<sup>+</sup> Treg cells.

**DISCUSSION**

Our study suggests that intestinal Treg cells are potential therapeutic target for immunotherapies to boost antitumor immunity and strengthen anti-PD-1 efficacy in CRC treatment. Using murine subcutaneous tumor and AOM-DSS-induced CRC models, we clearly demonstrate that gallic acid induces Th1-like Foxp3<sup>+</sup>PD-L1<sup>+</sup> Treg cells, which are less immune suppressive, reinvigorate antitumor T cell responses and ultimately prevent tumor growth. By contrast, Th17-like Treg cells facilitate Th17 responses, which induce exhausted tumor infiltrating CD8<sup>+</sup> T cells and promote colorectal carcinogenesis. Therefore, dysfunctional Th-helper-like Treg cells are heterogeneous and play disparate roles in controlling tumor progression. Here we found that type two cytokines such as IL-4 and type 17 cytokines such as IL-17 have no significant difference after gallic acid and anti-PD-1 antibody treatment. These data suggested that gallic acid induces Th1-like Treg cells specially.

Gallic acid is a gut microbial metabolite and potentially protects against DSS-induced intestinal injuries. Janus kinases (JAKs) phosphorylate STAT3 at Y705 site and thus promote the dimerization and nuclear translocation of STAT3. Nuclear STAT3 dimers further activate the transcription of target gene. Here, we demonstrate that gallic acid inhibits STAT3 phosphorylation at Y705 site and prevents the binding of p-STAT3 to Usp21 gene promoter. However, it remains largely unclear whether and how gallic acid inhibits the kinase activity of JAKs. To really understand whether this is the case, additional kinase activity assays for gallic acid may be required.

Although previous studies have also revealed that gallic acid exhibits anti-carcinogenic effects, the mechanism underlying whether and how gallic acid enhances PD-1 blockade therapy remains largely unclear. Here, we reveal gallic acid treatment significantly dampens Treg cell function by impairing PD-L1/PD-1 signaling and Foxp3 stability, promotes IFN-γ expression by CD8<sup>+</sup> T cells, and thus, prevents colorectal carcinogenesis. Notably, gallic acid enhances the ICB efficacy, in CRC, by targeting Usp21. These results contribute to a better understanding of the roles of Usp21 in tumor infiltrating Treg cells from CRC. Overall, our studies provide a unique molecular mechanism and rationale for combining Usp21 inhibition with PD-1 blockade as an effective immunotherapy for cancer.

Ample studies have demonstrated the regulatory roles of Usp21 in tumor incidence and progression. For instance, Usp21 critically promotes hepatocellular carcinoma development by stabilizing MEK3<sup>40</sup> and drives CRC metastasis by regulating Fra-1. Meanwhile, Usp21 activates Wnt-β-catenin pathway and maintains cancer cell stemness.<sup>41</sup> Here, we newly reveal that Usp21 stabilizes and deubiquitinates PD-L1 at K271, K280 and K280<sup>+</sup> sites. Meanwhile, Usp21 interacts with PD-L1 in human LOVO cancer cells. These results suggest that gallic acid directly dampens PD-L1 expression by tumor cells and further prevents PD-L1-mediated immune tolerance. In addition, IFN-γ has significant antitumor properties, however, it also simultaneously activates PD-L1 expression to promote tumor resistance to PD-1 blockade immunotherapy.<sup>42 43</sup> Conceivably, gallic acid may simultaneously overcome the pro-tumorigenic effects of IFN-γ by promoting PD-L1 degradation, leading to improved ICB efficacy in cancer immunotherapy.

The TME harbors cancer cells and other cells that contribute to tumor development and progression. Consequently, targeting and manipulating the cells in the TME during cancer treatment can help control malignancies and achieve positive health outcomes. We had demonstrated that gallic acid represses CRC development by targeting Treg cells. However, it remains unclear whether gallic acid mediated effects through Treg cell independent mechanisms. Our unpublished data revealed that gallic acid treatment downregulates PD-L1 protein level in human CRC (LOVO) cells, however, gallic acid does not influence the proliferation and migration of tumor cells. Further works should be focus on the additional roles for gallic acid in non-Treg cells.

Our study originality revealed that gallic acid inhibits Usp21 expression by decreasing STAT3 phosphorylation, further dampens FOXP3 stability and induces Th1-like FOXP3<sup>+</sup> Treg cells. Our results elucidate the mechanisms for gallic acid to repress CRC development and
strengthen anti-PD-1 blockade efficacy. However, the underlying mechanisms for gallic acid to inhibit STAT3 phosphorylation need to be further explored. Moreover, we will focus on clarifying the functions of gallic acid and the homeostasis of intestinal flora it associated with.

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Contributors BD and YL designed the study. BD and BY performed the experiments, and analyzed the data. WZ and YG performed the experiments. YZ provided reagent and expertise. YG provided clinical samples. BL and YL supervised experiments, and analyzed the data. WZ and YG performed the experiments. YZ

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Correction: Gallic acid induces T-helper-1-like Treg cells and strengthens immune checkpoint blockade efficacy


Yuansheng Zang, Yangyang Li and Bin Li have now been listed as co-corresponding authors.

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