

The effect of Curcumin on Multi-Level Immune Checkpoint Blockade and T Cell Dysfunction in Head and Neck Cancer

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Research

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Abstract

Background

Despite recent advances in understanding the complex immunologic dysfunction in the tumor microenvironment (TME), fewer than 20% of patients with head and neck squamous cell carcinoma (HNSCC) respond to immune checkpoint blockade (ICB). Thus, it is important to understand how inhibitory IC receptors maintain the suppressed dysfunctional TME, and to develop more effective combination immunotherapy. This study evaluated the immune-modulating effects of Curcumin, which has well-established anti-cancer and chemopreventive properties, and its long-term safety as a phytochemical drug.

Methods

We carried out the western blot and small interfering RNA (siRNA) transfection assay

to evaluate the effects of Curcumin on IC ligands and IC ligands function in HNSCC. Through T-cell cytotoxicity assay and measurements of cytokine secretion, we assessed the effects of combination of Curcumin with programmed death-ligand 1 (PD-L1) Ab on cancer cell killing. Flow cytometry were used to analyze the effects of Curcumin on the expression of programmed cell death protein 1 (PD-1) and T-cell immunoglobulin and mucin-domain3 (TIM-3) on CD4, CD8 and Treg. Immunofluorescence, immunohistochemistry and western blot were used to detecte the cytokine (IFN- γ , Granzyme B), IC receptors (PD-1 and TIM-3) and its ligands (PD-L1, PD-L2, Galectin-9) in xenograft mouse model and 4-nitroquinoline-1-oxide (4-NQO) oral cancer model.

Results

We found that Curcumin decreased the expression of IC ligands such as PD-L1, PD-L2, and Galectin-9 in HNSCC, leading to regulation of epithelial-to-mesenchymal transition-associated tumor invasion. Curcumin also effectively restored the ability of CD8⁺ cytotoxic T cells to lyse cancer cells. To evaluate the effect of Curcumin on the TME further, the 4-NQO oral cancer model was used. Curcumin increased T-cell proliferation, tumor-infiltrating lymphocytes (TILs), and effector cytokines, and decreased the expression of PD-1, TIM-3, suppressive IC receptors and their ligands (PD-L1, PD-L2, and Galectin-9) in the TME, implying reinvigoration of the exhausted CD8⁺ T cells. In addition, Curcumin inhibited expression of CD4⁺CD25⁺FoxP3⁺ Treg cells as well as PD-1 and TIM-3.

Conclusions

These results show that Curcumin reinvigorates defective T cells via multiple (PD-1 and TIM-3) and multi-level (IC receptors and its ligands) IC axis suppression, thus providing a rationale to combine Curcumin with conventional targeted therapy or ICB as a multi-faceted approach for treating patients with HNSCC.

Background

Head and neck squamous cell carcinoma (HNSCC), which accounts for more than 6% of all cancers, kills thousands of people every year worldwide[1–5]. Many traditional strategies have been designed to control cancer invasion, including surgery, chemotherapy, radiation therapy, and targeted therapy[6, 7]. However, the overall 5-year survival rate has not improved over the past three decades due to the lack of effective therapeutic options for HNSCC patients with recurrent disease^[3, 8, 9]. In addition to traditional standard strategies including targeted therapy, a better understanding of immune dysfunction in tumorigenesis and the progression of various cancers including HNSCC has recently led to the promising novel cancer therapy of immune checkpoint blockade (ICB) with IC inhibitors such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and programmed death ligand-1 (PD-L1)[10, 11]. A few clinical trials have reported favorable results in patients with recurrent/metastatic HNSCC compared with standard treatment, opening a new avenue for cancer research[12].

Immunotherapies targeting the PD-1/PD-L1 axis have been the most widely investigated and are approved for various solid tumors including HNSCC[12–15]. Unfortunately, contrary to initial expectations, anti-PD1/PD-L1 therapy has shown limited response rates[12, 14–16]. Many patients suffer from primary resistance to IC inhibitors and do not respond to blockade of PD-1/PD-L1 signaling, and many responders develop acquired resistance after initial responses. Ferris *et al.*[14] reported that the overall response rate of anti-PD-1 treatment for recurrent HNSCC with the progression of disease within 6 months after platinum-based chemotherapy setting was 13.3%, although 57.3% of the patients showed high PD-L1 expression, implying that PD-L1 expression does not guarantee a clinical response. Although the mechanisms underlying the limited clinical response or resistance are largely unknown, it has been suggested that compensatory additional inhibitory signaling other than the PD-1/PD-L1 axis is the major cause of acquired resistance to ICB.

Curcumin, also known as diacetylmethane, belongs to a chemical class of polyphenols derived from the rhizomes of *Curcuma longa* L. (turmeric). It has garnered increasing attention in the past two decades because of its chemopreventive potential due to various bio-functional properties such as anti-oxidant, anti-inflammatory, and anti-cancer effects; genetic and epigenic modulatory effects[17]; safety and easy accessibility; and important roles in the prevention and treatment of various illnesses ranging from cancer to autoimmune[18], neurological[19], and cardiovascular diseases[20], and diabetes[21, 22]. Moreover, recent evidence implies that Curcumin may relieve T-cell-mediated adaptive immune dysfunction[18, 23], indicating its potential effect on the suppressive tumor microenvironment (TME).

Therefore, we hypothesized that Curcumin has immunomodulatory effects on the HNSCC microenvironment and investigated how it affects the expression of IC proteins in HNSCC and its microenvironment.

Materials And Methods

Cell lines and reagents

The human HNSCC cell lines SNU1076 (larynx), SNU1041 (hypopharynx), and FaDu (hypopharynx) were obtained from the Korean Cell Line Bank (Seoul, South Korea). Another HNSCC cell line SCC15 (oral tongue) was kindly donated by Prof. Kim (Ajou University, Suwon, South Korea). Primary human fibroblasts (hFB), kindly donated by Professor Lee (Chungnam National University, Daejeon, South Korea), were used as normal epithelial cells. SNU1076 and SNU1041 cell lines were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA). SCC15 cells were maintained in Dulbecco's modified Eagle medium/Ham's nutrient mixture F-12 (DMEM/F12; Gibco). Normal hFB and FaDu cells were cultured in high-glucose DMEM (Gibco). All cell lines were supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin–streptomycin (Gibco). All cell lines were grown at 37°C under an atmosphere of humidified air with 5% CO₂.

Cell proliferation assay

SNU1041 and SCC15 cells were seeded into 96-well plates at a density of 10,000 cells per well in 100 µL medium. The next day, the cells were treated with various concentrations of Curcumin (0–100 µM; Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Cell viabilities were measured using the WST-1 cell proliferation reagent (Roche Diagnostics Corp., Indianapolis, IN, USA) as previously described[24]. The optical density of each culture well was measured at 450 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

Small interfering RNA (siRNA) transfection

Cells were seeded at a density of 2×10^5 /well in six-well plates and then cultured overnight to achieve 60–70% confluence. The next day, transient transfection was performed using Lipofectamine RNAi MAX reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's standard protocol. PD-L1 small interfering RNA (siRNA) (sc-39699) was a pool of three different siRNA duplexes: sc-39699A: (sense: 5'-GUA GAG UAU GGU AGC AAU Att-3'; antisense: 5'-UAU UGC UAC CAU ACU CUA Ctt-3'), sc-39699B: (sense: 5'-CAA GCG AAU UAC UGU GAA Att-3'; antisense: 5'-UUU CAC AGU AAU UCG CUU Gtt-3'), sc-39699C: (sense: 5'-GGA GAA UGA UGG AUG UGA Att-3'; antisense: 5'-UUC ACA UCC AUC AUU CUC Ctt-3'). PD-L2 siRNA (sc-39701) was a pool of three different siRNA duplexes: sc-39701A: (sense: 5'-CAG UAC CAA UGC AUA AUC Att-3'; antisense: 5'-UGA UUA UGC AUU GGU ACUGtt-3'), sc-39701B: (sense: 5'-CAA GUA CCU GAC UCU GAA Att-3'; antisense: 5'-UUU CAG AGU CAG GUA CUU Gtt-3'), sc-39701C: (sense: 5'-CUA CUG CAC UUU ACA GAA Utt-3', antisense: 5'-AUU CUG UAA AGU GCA GUA Gtt-3'). Galectin-9 siRNA (sc-35444) was a pool of three different siRNA duplexes: sc-35444A: (sense: 5'-GCU UCA GUG GAA AUG ACA Utt-3'; antisense: 5'-AUG UCA UUU CCA CUG AAG Ctt-3'), sc-35444B: (sense: 5'-GGA UCC UCU UCG UGC AGU Att-3'; antisense: 5'-UAC UGC ACG AAG AGG AUC Ctt-3'), sc-35444C: (sense: 5'-CCU CUC UGA CCU UUA ACC Utt-3'; antisense: 5'-AGG UUA AAG GUC AGA GAG Gtt-3') or negative control siRNA (#SN-1003) was acquired from Bioneer (Daejeon, Korea). The medium was changed after 6–8 h, and transfected cells

were incubated at 37°C for an additional 48 h. Western blotting was performed to evaluate the efficiency and efficacy of the siRNA knockdown.

Western blot analysis

Cells were rinsed with phosphate-buffered saline (PBS) and lysed in RIPA lysis buffer containing 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0, and a protease inhibitor cocktail (pH 7.4; Roche Applied Science, Vienna, Austria). The mixture was centrifuged at 13,000 rpm for 20 min at 4°C. Then protein concentration was determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Waltham, MA, USA). For immunoblotting, the proteins were denatured by boiling. Equal amounts of protein were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electrotransferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% skim milk for 1 h at room temperature. After being washed with Tris-buffered saline, 0.1% Tween 20 (TBST), membranes were incubated with primary anti-human antibodies (Abs) at 4°C. The next day, after four washes with TBST, the membranes were incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary Ab (1:1000; Cell Signaling Technology Inc, Danvers, MA, USA) for 1 h at room temperature. After another four washes, the proteins were visualized by enhanced chemiluminescence (Bio-Rad, Hercules, CA, USA).

Cell invasion (transwell) assay

Cell invasion were determined using transwell assay. Briefly, transwell membranes (24-well; Costar, Cambridge, MA, USA) were coated with Matrigel for 6 h for the invasion assay. SNU1041 and SCC15 cells were transfected for 48h before the transwell assay. After 48h, control or immune checkpoint proteins siRNA-transfected cells (2×10^5 in 100 μ L serum-free medium) were added to the upper chamber. Next, a 600 μ L medium containing 10% fetal bovine serum was added to the lower chamber. The chamber was then incubated for 24-48 h in 5% CO₂ at 37°C. Finally, the cells adhering to the upper surface of the membrane were removed with a cotton swab. The invasion cells, which adhered to the lower surface, were stained with 0.1% Crystal Violet and counted in four representative fields by light microscopy (200 \times magnification). The experiments were repeated three times. The unpaired Student's t-test was used for statistical analysis.

T-cell cytotoxicity assay and measurements of cytokine secretion

Geißler et al.[25] reported that tonsils from patients with chronic tonsillitis harbored more PD-1⁺ T-cells, pointing to T-cell exhaustion being due to chronic infection and showing dampened function of T cells compared to peripheral T cells. Therefore, to better mimic the immunologically suppressive TME of HNSCC, we designed T-cell cytotoxic assay using tonsil derived T-cells obtained from surgical specimen of a patient who underwent tonsillectomy for chronic tonsillar hypertrophy at the Department of Otolaryngology-Head and Neck Surgery of Chungnam National University Hospital instead of T-cell obtained from peripheral blood mononuclear cells (PBMC). This study was approved by the Institutional Review Board of Chungnam National University College of Medicine (Jung-Gu Daejeon, Korea, IRB

number: CNUH 2018-06-021). CD8⁺ T-cells were separated by human CD8⁺ T-cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). CD8⁺ T-cells were activated with CD3 antibody (1 µg/ml), CD28 antibody (1 µg/ml) and IL-2 (10 ng/ml). To study the mechanism of combined blockade, SNU1041 cells were treated with isotype control antibody, Curcumin (20 µM), PD-L1 antibody (clone 29E.2A3 [10 µg/ml]), Curcumin (20 µM) combined with PD-L1 antibody (clone 29E.2A3 [10 µg/ml]). After 72h of incubation, supernatants were harvested and IFN-γ and Granzyme B were measured by Human IFN-γ and Granzyme B ELISA Kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions. For the T-cell cytotoxicity assay, each well was washed with PBS twice to remove T-cells, and then the living tumor cells were fixed and stained with crystal violet solution. The crystal violet was dissolved with 1% SDS and absorbance were measured at 540 nm using an ELISA reader to quantify tumor cell viability.

Immunohistochemistry

The 4-µm paraffin-embedded tissue samples were soaked first in xylene to remove the paraffin wax and then sequentially in solutions of 100%, 90%, 80%, and 70% ethanol for rehydration, then heated in 1x sodium citrate buffer, PH 6.0 for antigen retrieval. For single immunostaining, endogenous peroxidase activity was blocked in a 1% hydrogen peroxide solution (Sigma-Aldrich, St. Louis, MO, USA) in PBS with 0.3% Triton X-100 for 30 min at room temperature. Nonspecific binding (2% BSA) was blocked. The sections were incubated with the indicated antibodies at 4°C in a humidified box and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody. Finally, 3,3' diaminobenzidine (DAB; DAKO) was used to detect these labeled antibodies. The nucleus was stained with hematoxylin. After rinsing with PBS, the samples were mounted using Permount™ Mounting Medium (Fisher Chemical, Fair Lawn, NJ, USA).

Immunofluorescence

The paraffin-embedded tissue samples were deparaffinized, rehydrated, and heated in antigen retrieval Citra solution (pH 6; BioGenex Laboratories, San Ramon, CA, USA). After blocked with 2% bovine serum albumin in PBS buffer for 1 hour, the slides were stained with the CD8 (Biorbyt, San Francisco, CA), PD-1 (Thermo Fisher, Waltham, MA, USA), TIM-3 (Bio-Rad, Hercules, California, USA) primary antibody at 4°C overnight. The next day, incubated the slides with fluorochrome-conjugated secondary antibodies (Alexa 594 anti-rabbit, Alexa 488 anti-mouse, Alexa 647 anti-Rat, Invitrogen, Carlsbad, CA, USA). After rinsing with PBS, slides were mounted with Vectashield antifade mounting medium with 4', 6-diamidino-2-phenylindole (DAPI).

Abs and flow cytometry

Spleen and blood samples were harvested from mice, and single-cell suspensions were stained with the following conjugated Abs: CD3-APC/Cyanine 7, CD4-PerCP, PD-1-PE, TIM-3-Brilliant Violet 421, CD25-APC (all from Biolegend, San Diego, CA, USA), CD8-FITC (BD, Bioscience, San Jose, CA, USA), FoxP3-FITC (Invitrogen, Carlsbad, CA, USA). Intracellular staining of FoxP3 was performed as follows: cells from the spleen were first stained with surface marker Abs, and then fixed in fixation/permeabilization buffer

(eBioscience, San Diego, CA, USA), washed, and finally stained for intracellular antigens in 1X permeabilization buffer. Cells were analyzed on a BD LSRFortessa™ X-20 (BD Biosciences, San Jose, CA, USA), and data analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Xenograft tumor models

Six-week-old male nude mice were obtained from Orient Bio (Seongnam, South Korea). Mice were used in accordance with the guidelines of the Institutional Animal Care and Use Committee of Chungnam National University, which approved of the animal research (Daejeon, South Korea). Mice were injected subcutaneously with SCC15 cells (1×10^7 in 100 μ l PBS). After tumors reached 40mm³ (day 0), Curcumin (0, 50 mg/kg) was administered every other day through intraperitoneal injections. After 22 days, the mice were sacrificed, and the tumor was harvest. Tumor volumes were calculated according to the following formula: tumor volume (mm³) = (length) \times (width)² \times 0.5, and the tumor weights were recorded.

4-NQO-induced oral tumorigenesis model

The carcinogen 4-nitroquinoline 1-oxide (4-NQO) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO at 50 mg/ml to create a stock solution, which was stored at -20°C and diluted to a final concentration of 50 μ g/ml. To avoid decomposition of 4-NQO, light was avoided.. For the malignant transformation of the oral mucosa model, a total of 44 female C57BL/6 mice (Narabiotech, Korea), six-week-old, and weighing 18 to 20 g were used for the studies with 4-NQO. For the drinking water method, the 4-NQO stock solution was diluted in the drinking water for mice. Fresh 4-NQO water was supplied every week. After 16 weeks, the drinking water was switched to distilled water, and then the mice were beginning to treat with corn oil or Curcumin 50 mg/kg by oral gavage for consecutive 6 weeks. The mice were analyzed for oral lesions and weighed at different times for up to 22 weeks. All the experiments were conducted in accordance with the approval of the Institutional Animal Care and Use Committee (IACUC) at Chungnam National University (Daejeon, South Korea).

Statistical analysis

Statistical analyses were performed using SPSS 22 (version 22.0.0.0, International Business Machines, Armonk, NY, USA) and GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA). The unpaired Student's t-test or one-way ANOVA was used for statistical analysis. Data from three independent experiments were expressed as the mean \pm standard deviation. $P < 0.05$ was considered to indicate a statistically significant difference. (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Results

Curcumin reduces the expression of IC proteins (PD-L1, PD-L2, and Galectin-9) which promote cell viability and cell invasion in HNSCC cell lines

To investigate the involvement of IC proteins on the anti-cancer effect of Curcumin, we first examined the expression of IC proteins in HNSCC cell lines. Cell lysates were prepared from human fibroblast-cells (hFB) and four HNSCC cell lines (FaDu, SNU1041, SNU1076, and SCC15). The expression of IC proteins PD-L1, PD-L2, and Galectin-9 was upregulated in HNSCC cell lines compared with the human fibroblasts (Fig. S1A). We initially sought to evaluate the effect of Curcumin on cell viability on HNSCC cell lines. SNU1041 and SCC15 cells were treated with increasing doses of Curcumin for 24h and cell viability was analyzed by WST-1 assay. Data analysis revealed that Curcumin inhibited the cell viability of SNU1041 and SCC15 cell line in a dose-dependent manner (Supplementary Fig. S1B, C). Next, to determine the influence of Curcumin on IC proteins, SNU1041 and SCC15 cells were treated with different concentrations of Curcumin for 24h. The treatment of HNSCC cells with Curcumin resulted in dose and time-dependent decreases in IC protein expression in both cell lines (Fig. 1A-D). The finding that the expression of IC proteins was higher in HNSCC cell lines than in normal cell lines led us to hypothesize that these IC proteins may drive cell growth. To test this hypothesis, we performed a proliferation assay after downregulation of IC proteins in SNU1041 and SCC15 cell lines. PD-L1, PD-L2, and Galectin-9 knockdown cells showed markedly decreased cell viability in both cell lines, indicating the contribution of IC proteins to HNSCC cancer cell viability (Fig. 1E-G). Cell invasion is crucial step in tumor metastasis. To investigate whether IC proteins affected the malignant behavior of HNSCC, we did the transwell assay. Significant inhibition of invasion was observed after knockdown of IC proteins by siRNA compared with the control group (Fig. 1H-J). These results clearly show that IC proteins PD-L1, PD-L2, and Galectin-9 positively promote the invasion of HNSCC cells. The epithelial-mesenchymal transition (EMT), which refers to changes in cell phenotype from epithelial to mesenchymal morphology, is an essential process during the initiation and progression of tumorigenesis and metastasis[26]. To investigate whether IC proteins regulate the EMT, we examined EMT-related proteins (E-cadherin, N-cadherin, Vimentin, and Slug) by western blotting. Knockdown of IC proteins by siRNA significantly suppressed the EMT, as evidenced by upregulating the expression of epithelial marker (E-cadherin) and downregulating the expression of mesenchymal marker (N-cadherin, Vimentin), transcription factor (Slug) (Fig. 1K-M). Together, these data imply that IC proteins PD-L1, PD-L2, and Galectin-9 regulate cancer cell metastasis by affecting the actions of EMT-related genes.

Combination of Curcumin and PD-L1 Ab potentiates the cytotoxic effect of CD8⁺ T-cells and has an additive effect on IFN- γ and Granzyme B secretion of T cell

Curcumin treatment enhances the ability of effector T cells to kill cancer cells[23]. To determine whether Curcumin can enhance the impact of CD8⁺ T cells on immune-mediated cytotoxicity, CD8⁺ T cells were isolated from tonsil tissue. CD8⁺ T-cells were activated by CD3, CD28 and IL-2 (Fig. 2A) and incubated with SNU1041 cells pretreated with Curcumin for 24 h. Activated CD8⁺ T-cell and SNU1041 cells were co-cultured for another 3 days. Although activated CD8⁺ T cells could inhibit tumor cell growth in the absence of Curcumin, Curcumin-treated groups showed significant inhibitory effects on tumor growth. Simultaneously, the combination of Curcumin with PD-L1 Ab showed additional treatment efficacy compared with control, Curcumin treatment, or PD-L1 Ab group (Fig. 2B). These results showed that the

combination of Curcumin with PD-L1 Ab potentiates the effect of CD8⁺ T-cells-mediated cancer cell killing. Moreover, combined PD-L1 blockade and Curcumin treatment resulted in increase the secretion of IFN- γ , Granzyme B compare with the other three groups (Fig. 2C). To avoid confusion between Curcumin's anti-tumor growth effect, PD-1/PD-L1 mediated tumor immune escape, and CD8⁺ T cell killing effect, we calculated the difference between these groups. These results show that the combination of Curcumin with PD-L1 Ab potentiates the effect of CD8⁺ T cells-mediated cancer cell killing and increased the secretion of IFN- γ and Granzyme B.

Administration of 4-NQO induces an aggressive oral squamous carcinoma and overexpression of PD-1 and TIM-3 in peripheral lymphatic tissue in vivo

To evaluate the effect of the Curcumin on the anti-tumor immunity response *in vivo*, we established the 4-NQO-induced carcinogenesis model in immunocompetent C57BL/6 mice. The 4-NQO oral cancer model is well-established and mimics the pathology of human oral cancer[27-29]. In this study, to induce tumorigenesis in the mouse oral cavity, C57BL/6 mice were given 4-NQO (50 $\mu\text{g}/\text{mL}$) in drinking water for 16 consecutive weeks (or water in the absence of 4-NQO as a control) and then regular water until week 22 (Fig. 3A). The size and number of these lesions continued growing progressively even after 4-NQO withdrawal. Representative pictures of oral lesions and hematoxylin and eosin (H&E) staining showed that at week 22, all 4-NQO-treated mice (100%) developed at least one or more large oral squamous cell carcinoma, supporting the usefulness of this chemical carcinogenesis model for investigating the development of oral cancer (Fig. 3B, C)[30]. We first examined the potential role of the immunosuppressive PD-1 and TIM-3 signaling in oral carcinogenesis model in immunocompetent C57BL/6 mice. Flow cytometry analysis of the spleen and blood from 4-NQO-treated mice revealed that the expression of PD-1 and TIM-3 were upregulated on both CD4⁺ T-cells and CD8⁺ T-cells compared to the control group (Fig. 3D, E). Regulatory T-cells (Tregs) are a subpopulation of T-cells that suppress the immune response in cancer cells. Flow cytometry analysis of the spleen showed that the percent of Tregs in HNSCC mice was increased in the 4-NQO-treated group (Fig. 3F). At the same time, the percentage of PD-1⁺ and TIM-3⁺ Treg was also upregulated in the 4-NQO-treated group (Fig. 3G). These data imply that overexpression of PD-1 and TIM-3 on CD4⁺ T-cells, CD8⁺ T-cells and Tregs may positively enhance the immunosuppressive activity of tumors.

Curcumin reduces tumor formation and growth in 4-NQO oral carcinogenesis model and inhibits IC proteins (PD-L1/PD-L2/Galectin-9) in xenograft mouse model

To investigate the impact of Curcumin on immunocompetent mice, C57BL/6 mice were exposed to 4-NQO for 16 weeks and then treated with Curcumin 50 mg/kg by oral gavage for consecutive 6 weeks (Fig. 4A). Mice treated with Curcumin had increased weight and overall survival compared to the control group (Fig. 4B, C). Representative images of the oral lesions in the control and Curcumin-treated groups showed that Curcumin treatment led to a noticeable reduction in tumor size and the number of 4-NQO-induced lesions (Fig. 4D), accompanied by a less malignant and invasive phenotype in the lesions compared with the control group. The tissue sections were stained with H&E for detailed histopathological analysis.

Curcumin-treated mice had less-invasive oral carcinoma lesions, but more low-grade dysplasia in their tongues than the control group (Fig. 4E). At the same time, Curcumin treatment reduced both the number and size of lesions induced by 4-NQO (Fig. 4F, G). Our findings demonstrated that Curcumin had growth inhibitory effects and led to less tumor formation and a transformative phenotype in the 4-NQO-induced model. Moreover, we established an HNSCC xenograft mouse model using SCC15 cell lines. The tumor volumes were significantly decreased after Curcumin treatment (Supplementary Fig. S2A). The expression of IC proteins PD-L1, PD-L2, and Galectin-9 in tumor tissue from the Curcumin group was significantly downregulated compared with the control group (Supplementary Fig. S2B). We also confirmed the downregulated expression of IC proteins in the Curcumin group by western blotting (Supplementary Fig. S2C). Our findings collectively reveal that Curcumin not only regulates tumor sensitivity to immune cell-mediated tumor killing through the suppression of PD-L1 expression but also inhibits the expression of PD-L2 and Galectin-9 in HNSCC cells.

Curcumin restores effector T cells by modulating the expression of PD-1 and TIM-3 on CD4⁺ or CD8⁺ T cells or CD4⁺CD25⁺FoxP3⁺Tregs in 4-NQO oral carcinogenesis model

To further explore the effects of Curcumin on the T lymphocyte subpopulation in the immune system, we collected cells from the spleen and blood of 4-NQO-induced tumor-bearing mice. Curcumin treatment increased the proportion of CD4⁺ and CD8⁺ T cells in the spleen and blood, which increased the immune response to tumors (Fig. 5A, B). PD-1 and TIM-3 on the surface of immune cells are IC molecules that mediate the immune escape of tumor cells. We investigated the expression of PD-1 and TIM-3 on CD4⁺ T cells and CD8⁺ T cells by flow cytometry. Curcumin treatment led to a substantial reduction in PD-1⁺CD4⁺ T cells in both the blood and spleen and a decreased percentage of TIM-3⁺CD4⁺ T cells in the blood (Fig. 5C, D). In addition, Curcumin-treated mice also had a significantly decreased percentage of PD-1⁺ CD8⁺ T and TIM-3⁺CD8⁺ T cells in both blood and the spleen (Fig. 5E, F). These findings imply that Curcumin can restore effector T cells by modulating the expression of PD-1 and TIM-3 on CD4⁺ or CD8⁺ T-cells. Next, we determined the role of Curcumin in the 4-NQO-induced anti-tumor immune response through the regulation of Tregs, which play roles in suppressing the immune response. We examined the population of Tregs in each group by flow cytometry. The population of CD25⁺FoxP3⁺ in CD4⁺ T cells was significantly suppressed in Curcumin-treated mice compared with control mice (Fig. 5G). These results imply that Curcumin can effectively inhibit tumor growth by downregulating Tregs in mice. FoxP3⁺ Tregs co-expressing PD-1 and TIM-3 are highly immunosuppressive, including a specialized subset of tissue Tregs in breast cancer models[31]. We investigated whether the expression of PD-1 and TIM-3 on Tregs was also reduced by Curcumin treatment. The percentage of PD-1⁺ and TIM-3⁺ on Tregs was significantly decreased in the Curcumin treatment group (Fig. 5H, I). These data indicate that Curcumin reduces not only Tregs itself but also decreases the expression of IC receptors PD-1 and TIM-3 on Tregs, implying that Curcumin may be an alternative treatment for Treg-mediated immunosuppression in HNSCC.

Curcumin suppresses the expression of IC proteins and promotes the expression of IFN- γ and Granzyme B in 4-NQO oral carcinogenesis model

We assessed the expression of IC proteins PD-L1, PD-L2, and Galectin-9 in 4-NQO-induced tongue cancer tissues by western blotting and immunohistochemistry analysis. The expression of PD-L1, PD-L2, and Galectin-9 in the Curcumin group was downregulated compared with the control group in tumor tissues (Fig. 6A, B). Next, we examined the effects of Curcumin treatment on locoregional immunity *in vivo*. The confocal assay showed that the Curcumin group led to a decreased percentage of CD8⁺ PD-1⁺ and CD8⁺ TIM-3⁺ in TILs compared with the control group (Fig. 6C). We also analyzed the expression of IFN- γ , Granzyme B, which are produced by cytotoxic T cells. We observed a significant increase in the expression of IFN- γ , Granzyme B in Curcumin-treated mice compared with the control group (Fig. 6D). These data show that Curcumin therapy not only effectively reduces the expression of IC molecules on cancer cells but also upregulates T-cell populations in peripheral tissues *in vivo*, indicating both locoregional and systemic immune activation in mice with carcinogen-induced early lesions.

Discussion

Recurrence is a very challenging issue in cancer treatment, and one of the reasons that cancer treatment is so difficult and can have a poor prognosis[32–34]. Recently, the Korean Society of Thyroid-Head and Neck Surgery (KSTHNS) developed the guideline about surgical treatment of HNSCC to improve the patients' survival[35]. The reasons for HNSCC recurrence are mainly due to its high propensity for intrinsic, spatial, and acquired resistance to chemotherapeutic agents, radiotherapy, and anti-epidermal growth factor receptor (EGFR) mAb[36, 37]. Although ICB with PD-1/PD-L1 blockade is an alternative for overcoming recurrence and resistance that has shown great promise for the treatment of a variety of advanced cancers including HNSCC, a significant durable response is limited to a minority of patients, and eventually, most of these patients experience relapse of the disease[14]. While the mechanism underlying resistance to ICB has not been fully elucidated, accumulating evidence implies that immunosuppressive signaling receptors such as TIM-3, which can impair cytotoxic T-cell functionality, play an important role in acquired resistance[31, 38]. Therefore, combination strategies, not only ICB with conventional standard therapies such as cytotoxic or targeted agents and radiotherapy but also multiple IC inhibitors and their ligands in the TME, have been tested in various cancer subtypes[39]. Preliminarily, the combination has increased the response rate in a few clinical trials, but the immune-related adverse events commonly associated with checkpoint blockers, such as diarrhea, colitis, myocarditis and endocrine disorders, are more severe in these patients[40–42].

Curcumin is one of the best characterized chemopreventive agents. It has strong anti-oxidative, anti-inflammatory and anti-septic properties and has been widely used for a long time in traditional medicine, implying that similar to many phytochemicals, it is safe in the human body and side effects are rare[22]. Recently, Curcumin has emerged as a potent anti-cancer agent that targets several biological pathways and processes in various cancers, including mutagenesis, cell cycle, oncogene expression, angiogenesis, metastasis, and cell death signaling such as apoptosis and autophagy without adverse effects on normal tissue[22]. Moreover, in combination with conventional anti-cancer therapies such as chemotherapy and radiation, Curcumin enhances efficacy by sensitizing cancer cells to their cytotoxic effects and reduces

treatment-associated side effects including cardio-, hepato-, nephro- and neuroprotective properties by balancing reactive oxygen species or inflammatory reactions[18–23]. In addition to its direct effects on cancer cells described above, emerging evidence has shed light on the immune-modulating effects of Curcumin that may play a role in its anti-tumor effects[23]. Although, the immune-modulatory actions of Curcumin have been shown in a wide range of inflammatory and autoimmune diseases such as arthritis, colitis and hepatitis, its immunomodulatory capacity in the TME has emerged relatively recently. Research has shown that Curcumin not only enhances tumor antigen-specific T cells via reversal of tumor-induced immunosuppression but also enhances cytotoxic T cells by acting directly on immune cell dysfunction, which is one of the major mechanisms of tumor escape from immune surveillance via the signal transducer and activator of transcription 3 and nuclear factor kappa B (NF- κ B) signaling pathways[23, 43, 44]. Lim *et al.*[45] showed inhibition of inflammation-mediated PD-L1 expression by Curcumin, and Liao *et al.*[46] showed decreased PD-L1 expression following immunosuppression in cell populations, such as Tregs and myeloid-derived suppressor cells, in a murine oral cancer model after Curcumin treatment. Hayakawa *et al.*[44] showed that Curcumin increases the induction of tumor antigen-specific T cells by restoring T-cell stimulation, implying that the combination of PD-1/PD-L1 Ab is attractive for the development of effective ICB. Given the various biologic effects, we hypothesized that Curcumin, as well as PD-L1, may affect the simultaneous expression of other IC proteins on immune cells and its ligands on tumor cells. We hypothesized that Curcumin could be an alternative to overcoming disease progression or relapse due to resistance to immunotherapy. To this end, we evaluated the effects of Curcumin on the expression of multiple IC ligands on tumor cells.

We demonstrated that PD-L1, PD-L2, and Galectin-9 induced HNSCC cell invasion via EMT activation, indicating the intrinsic roles of these IC ligands in HNSCC independent of the interaction with immune cells. In addition, by showing that Curcumin simultaneously inhibits PD-L1, PD-L2, and Galectin-9, for the first time, we show that Curcumin can simultaneously inhibit IC ligands other than PD-L1. Our data have clinical significance in that recent advances have revealed that bidirectional regulation may exist between EMT status and IC ligands, especially PD-L1 expression that ultimately leads to tumor immune escape. Our data also indicate that PD-L1 signaling plays an important role in the maintenance of EMT status in HNSCC, in accordance with similar reports in solid tumors such as renal cell carcinoma, breast cancer, hepatocellular carcinoma, esophageal cancer, and glioblastoma[47, 48]. Although far less investigated than PD-L1, PD-L2 reportedly contributes to T-cell exhaustion by interacting with the PD-1 receptor, implying functional relevance to the TME. A few clinical papers have shown that PD-L2 expression is independently associated with clinical response in anti-PD-1-treated patients, indicating that the effect of ICB may be related partly to blockade of PD-1/PD-L2 interactions. Therefore, targeting both PD-1 ligands may provide additional clinical benefit[49]. The effect of PD-L2 intrinsic signaling on the tumor itself has been much less investigated than that of PD-L1[50]. However, recently Ren *et al.*[51] suggested that PD-L2 intrinsically promotes tumor invasion and metastasis via RhoA and autophagy pathways. Consistent with the data, we demonstrated that Curcumin can reduce intrinsic PD-L2 expression independent of immune cells, thereby inhibiting the EMT in HNSCC cells. Although PD-L2 expression in HNSCC tumor tissue has been shown[52], we report for the first time the association of PD-L2 with the EMT in HNSCC.

Our data also delineate the role of PD-L2 as an immunosuppressive and cancer-promoting signaling molecule. In this study, we showed that suppression of Galectin-9 decreased the EMT in HNSCC cells. However, because the role of Galectin-9 apart from tumor immune escape has not yet been studied in HNSCC[53], further validation and mechanistic studies about associated molecular pathways are needed. Our findings support previous findings that Galectin-9 expression in solid tumors may be linked to tumor cell adhesion or metastasis in various cancers[54, 55], and Galectin-9 is expressed significantly more in advanced cancer stages compared to early stages and expressed by tumor-infiltrating lymph nodes[55, 56]. Moreover, mechanisms underlying the regulation of multiple IC ligands are need more detailed study.

The nude mouse model was used to investigate the effect of Curcumin on the expression of ligand in HNSCC cells independently of immune cells. In this HNSCC xenograft model, we do not think that Curcumin can inhibit tumor progression by targeting these molecules alone, as its anti-cancer properties are through other multiple signaling pathways[22]. However, the *in vivo* model validates the IC ligand-inhibiting effects of Curcumin evident in the *in vitro* results.

To further evaluate the effects of Curcumin on the TME with regard to ICB, we used the 4NQO-induced syngenic murine tongue squamous cell carcinoma model, which mimics the carcinogenesis of HNSCC. As expected, the model showed a significantly immune-suppressive TME, consisting of increased expression of inhibitory IC proteins such as PD-1 and TIM-3 in lymphocytes. The increase in both CD25⁺FoxP3⁺ Treg cells and PD-1⁺ and TIM-3⁺ Treg expression is associated with more suppressive activity[57]. Curcumin treatment not only reduces the size and number of lesions but also may reduce the risk of invasive cancer and increase the proportion of precancerous lesions, implying that Curcumin functions as a chemopreventive agent that inhibits cancer initiation and may also reduce cancer progression[58].

Our study differs from previous studies in that it focused on the effects of inhibiting multiple IC proteins via not only receptors on T cells but also its ligands on tumor cells simultaneously, as a mechanism to restore CD8⁺ T-cell dysfunction. Given that exhausted T cells exhibit defective proliferative capacities and cytokine production, and inert lytic function[31], Curcumin treatment successfully reinvigorates T cells of the HNSCC TME in that it increases total CD4⁺ and CD8⁺ cells in the periphery implying T-cell proliferation; CD8⁺ TILs are a subset of T cells that directly target tumor cells, and are a good prognostic factor in all HNSCC[59]. Also, Curcumin treatment increases the secretion of cytokines such as IFN- γ and Granzyme B, reflecting the effector functions of cytotoxic T cells. Sakuishi *et al.*[38] reported that TIM-3⁺PD-1⁺ TILs exhibit the most severe exhausted phenotype as defined by the failure to proliferate and produce IL-2, TNF α , and interferon gamma (IFN- γ); thus, combined targeting of the TIM-3 and PD-1 pathways is more effective in controlling tumor growth than targeting either pathway alone. Some studies have reported inhibition of PD-L1 expression by Curcumin as described above, but this is the first study of the effect of Curcumin on the Galectin-9/TIM-3 axis in solid tumors.

Moreover, we demonstrated that Curcumin-induced restoration of T-cell function may be due to the effect of Curcumin inhibiting the PD-1 and TIM-3 axis in CD4⁺ and CD8⁺ T-cells not only at the tumor site (TILs)

but also in the periphery (blood and spleen). These data highlight the clinical usefulness of Curcumin in upregulating TIM-3, which is a well-known regulator of CD8⁺ T-cell exhaustion[38] and has been shown to mediate adaptive resistance to anti-PD-1 in models of non-small cell lung cancer and HNSCC[60]. Jie *et al.*[61] showed that the frequency of PD-1⁺ and TIM-3⁺ cells was significantly increased on CD8⁺ TILs after cetuximab treatment, which is an mAb to EGFR and the most well-known molecular target agent in HNSCC. The PD-1 and TIM-3 axis is associated with resistance to chemotherapy. The combination of cetuximab with PD-1 or TIM-3 should be considered to improve clinical outcomes for HNSCC patients. In line with these data, clinical trials are currently underway to elucidate the role of combined blockade of TIM-3 and PD-1/PD-L1 in various advanced and/or metastatic solid tumors (Registration Nos. NCT03680508, NCT03708328, and NCT03961971 at ClinicalTrials.gov). Mechanistically, CD8⁺ T cells that express TIM-3 have diminished proliferation capacity, reduced IL-2 and IFN- γ expression, and reduced perforin and/or Granzyme B secretion compared to CD8 T cells that express PD-1[62, 63]. Thus, increased TIM-3 expression after anti-PD-1/PD-L1 treatment may serve as a compensatory mechanism of T-cell exhaustion[38]. Also, we showed PD-1 and TIM-3 inhibition on peripheral Treg cells by Curcumin treatment. This is the first study demonstrating the effect of Curcumin on PD-1 and TIM-3 expression on FoxP3⁺ Treg cells. These data are supported by previous studies showing that the majority of intratumoral CD25⁺FoxP3⁺ Tregs express TIM-3. Importantly, TIM-3⁺ Tregs express higher levels of IL-10 compared to TIM-3⁻ Tregs and a higher capacity for inhibiting IFN- γ and TNF α secretion by effector T-cells[64–67].

We do not consider Curcumin's inhibitory effects or binding affinity for IC proteins to be superior to those of specifically engineered mAbs. However, we showed that Curcumin can simultaneously inhibit both PD-1 and TIM-3 expression, which is important for the reinvigoration of exhausted T cells and an important adaptive escape mechanism from anti-PD-1 inhibition, respectively. Another advantage of Curcumin shown in our study is that Curcumin inhibits not only IC proteins on T-cell subsets but also its ligands on tumor cells, whereas existing ICB acts on either the ligand (of a tumor cell or antigen-presenting cell) or IC protein of immune cells. Although additional large-scale randomized clinical trials are needed, our study provides a rationale for combining Curcumin with conventional standard therapeutic modalities including approved IC inhibitors such as nivolumab, pembrolizumab, and durvalumab. This strategy may provide multi-faceted, sustained anti-cancer effects in patients with limited responses to ICB, and even those who fail to respond to ICB and molecular targeted therapy.

Conclusion

In conclusion, we showed that Curcumin restores T-cell dysfunction via multiple and multi-level IC suppression. Given the adverse reactions caused by ICB blockade by specific mAbs, the well-established safety of Curcumin as a phytochemical and its anti-cancer effects underscore the potential benefits of Curcumin when used as a combination therapy with conventional targeted agents including ICB or in post-treatment chemoprevention.

Abbreviations

TME Tumor microenvironment

HNSCC Head and neck squamous cell carcinoma

ICB Immune checkpoint blockade

PD-L1 Programmed death-ligand 1

TILs Tumor-infiltrating lymphocytes

PD-1 Programmed cell death protein 1

TIM-3 T-cell immunoglobulin and mucin-domain 3

CTLA-4 Cytotoxic T-lymphocyte-associated protein 4

PBMC Peripheral blood mononuclear cells

hFB human fibroblast-cells

EMT Epithelial-mesenchymal transition

Tregs Regulatory T-cells

4-NQO 4-nitroquinoline 1-oxide

KSTHNS Korean Society of Thyroid-Head and Neck Surgery

EGFR Epidermal growth factor receptor

NF- κ B Nuclear factor kappa B

IFN- γ Interferon gamma

Declarations

Ethics approval and consent to participate

Studies involving human samples were reviewed and approved by the Institutional Review Board of Chungnam National University College of Medicine (Jung-Gu Daejeon, Korea, IRB number: CNUH 2018-06-021).

Consent for publication

All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this article and its supplementary information files.

Competing interests

The authors declare no competing financial interests.

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Authors' contributions

JC and BK conceived and designed the research. LL and ML performed the research. SJ, CO, HW and YJ analyzed the data. YP, HK realized Figures and Tables. LL, BK and JC wrote the manuscript. All authors read and approved the final manuscript.

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Figures

Figure 1.

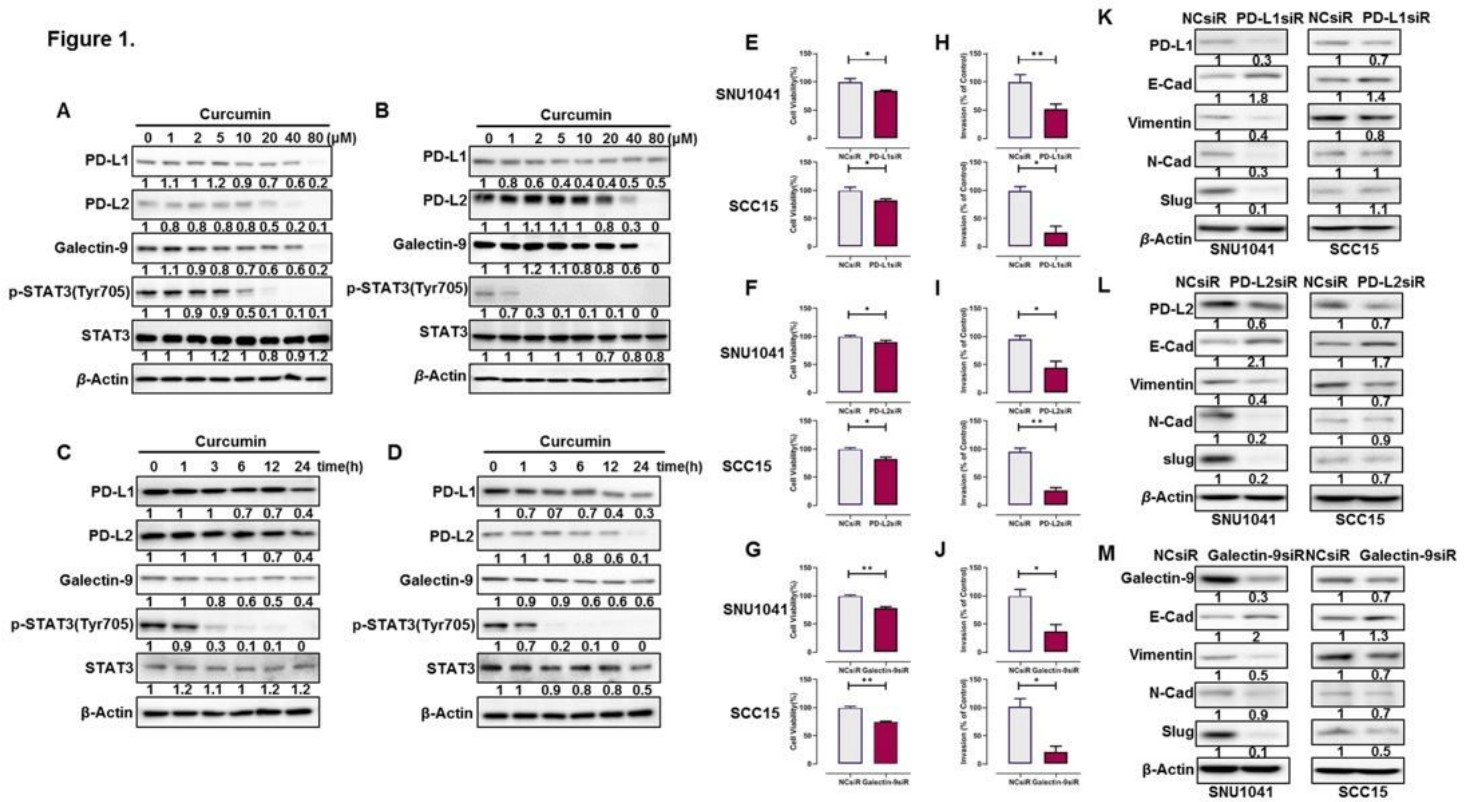


Figure 1

Curcumin suppresses IC proteins expression in a dose and time-dependent manner which promote the proliferation, invasion and regulates epithelial to mesenchymal transition (EMT) of HNSCC cell lines. SNU1041 (A) and SCC15 (B) cells were treated with different concentration of Curcumin (0-80 μM) for 24h. Also, SNU1041 (C) and SCC15 (D) cells were treated with 40 μM of Curcumin at different times (0-24 h). The expression of immune checkpoint proteins was detected by western blot assay. (E-G) SNU1041 and SCC15 cells were transfected with siRNA of PD-L1, PD-L2, and Galectin-9 or negative control siRNA for 48 h, cell viability was determined by WST-1 assay. (H-J) After 48h of transfection, the cells were allowed to migrate for 48h in chambers with matrigel (cell invasion). Stained cells were counted under a light microscope. (K-M) Also, after 48h of transfection, the expression of, E-cadherin, N-cadherin, Vimentin, and Slug was examined by western blot assay. The data represent the means ± standard deviation of three independent experiments. * P<0.05, ** P<0.01, and *** P<0.001.

Figure 2.

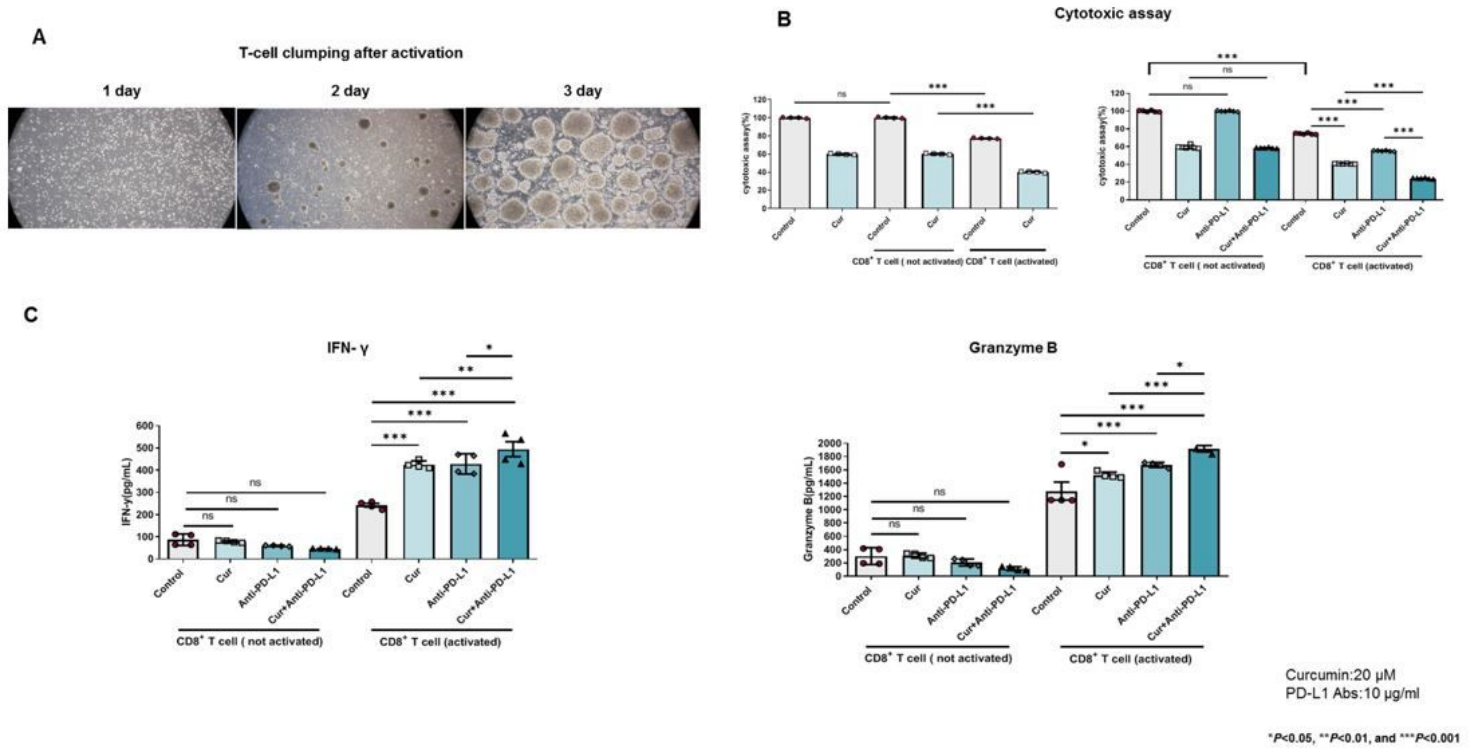


Figure 2

The effects of combination of Curcumin with PD-L1 Ab on CD8+ T-cell-mediated cancer cell killing and cytokines secretion. CD8+ T-cells were separated from the human sample. (A) The Representative pictures of T cell clumping after activation. (B) SNU1041 cells pretreated with Curcumin were co-cultured with or without CD8+ T-cells (Left). SNU1041 cells were treated with Curcumin (20 μ M), PD-L1 antibody (10 μ g/ml), Curcumin (20 μ M) combined with PD-L1 antibody (10 μ g/ml). The difference in residual tumor cells survival rate between T-cells (+) and T-cells (-) (Right). The crystal violet was dissolved with 1% SDS and absorbance was measured at 540 nm to quantify tumor cell viability. (C) The cytokine IFN- γ , Granzyme B secretion were measured by the ELISA kit. The data were represented as mean \pm SEM.

*P<0.05, **P<0.01, and ***P<0.001.

Figure 3.

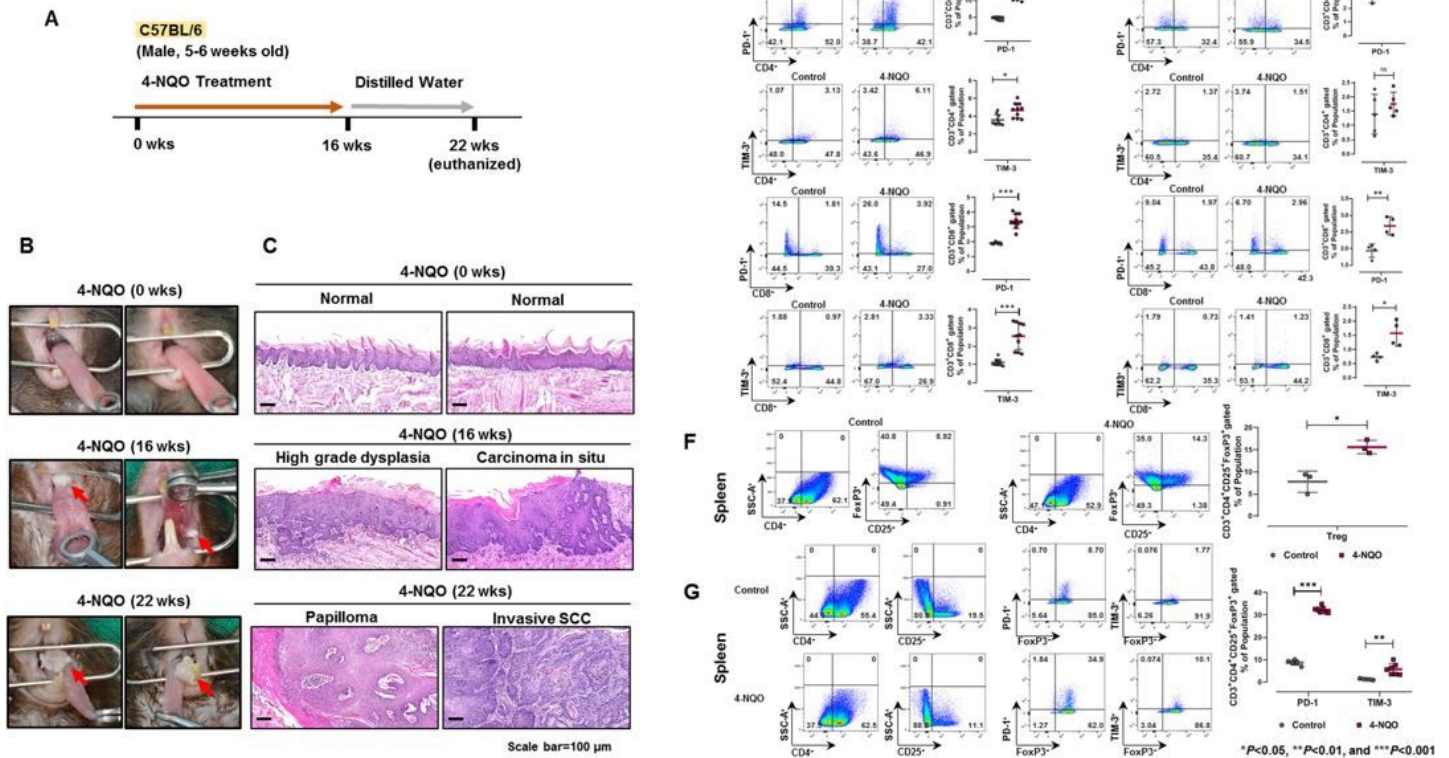


Figure 3

Tumor formation induced by 4-NQO and upregulation of PD-1 and TIM-3 in 4-NQO treated mice group. (A) C57BL/6 mice were administered either water (control) or 4-NQO (50 μ g/ml) in the drinking water containing 1% sucrose for 16 weeks. Then all mice were reverted to regular water until 22 weeks. (B, C) Representative oral lesions and H&E staining in control and 4-NQO treated group mice (0 weeks, 16 weeks, 22 weeks). (D, E) Spleen and blood samples from control and 4-NQO treated mice were analyzed by flow cytometry. Representative images of PD-1 and TIM-3 on CD4⁺ and CD8⁺ T-cells in control and 4-NQO treated mice group. (F, G) Representative flow cytometry images of CD4⁺CD25⁺FoxP3⁺ Treg and percent of PD-1⁺ and TIM-3⁺ in the CD4⁺CD25⁺FoxP3⁺ Treg population from control and 4-NQO-treated mice. The data were represented as mean \pm SEM. *P<0.05, **P<0.01, and ***P<0.001.

Figure 4.

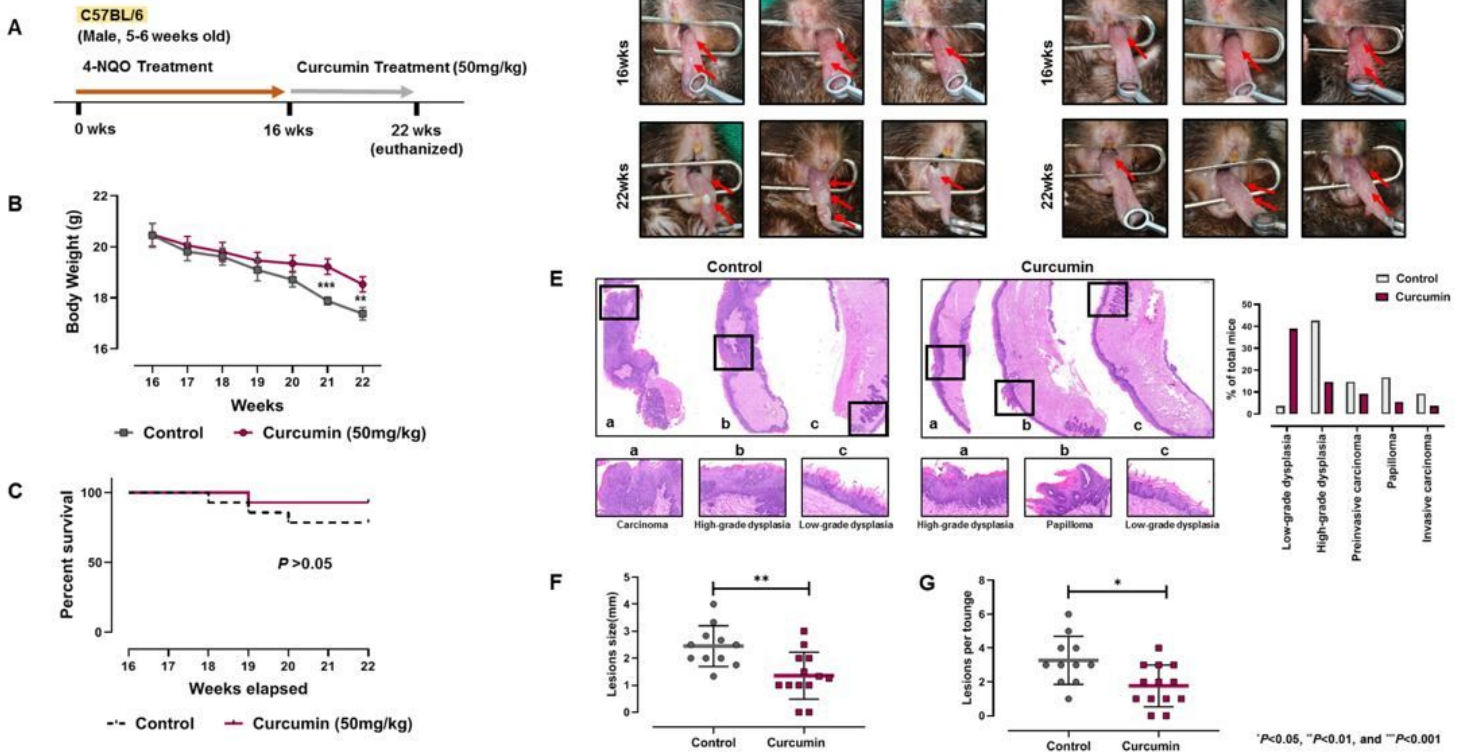


Figure 4

Curcumin induces prolonged survival of the C57BL/6 mice and reduces the tumor burden in the 4-NQO-induced oral carcinogenesis model. (A) Experimental scheme. C57BL/6 mice were administered 4-NQO (50 μ g/ml) in the drinking water for 16 consecutive weeks. At the end of 4-NQO exposure, mice were daily treated with either vehicle (control) or Curcumin by oral gavage for 6 consecutive weeks. (B) Control and Curcumin-treated groups were weighed every other day. (C) The survival rate of control and Curcumin-treated group. (D, E) Representative oral lesions and H&E staining of control and Curcumin-treated group mice at 16 weeks and 22 weeks. Tumor lesions were classified into low-grade dysplasia, high-grade dysplasia, preinvasive carcinoma, papilloma, invasive carcinoma. (F, G) Quantitative analysis of the lesion per tongue and lesion size (mm) at the end of Curcumin treatment (week 22). The data were represented as mean \pm SEM. *P<0.05, **P<0.01, and ***P<0.001.

Figure 5.

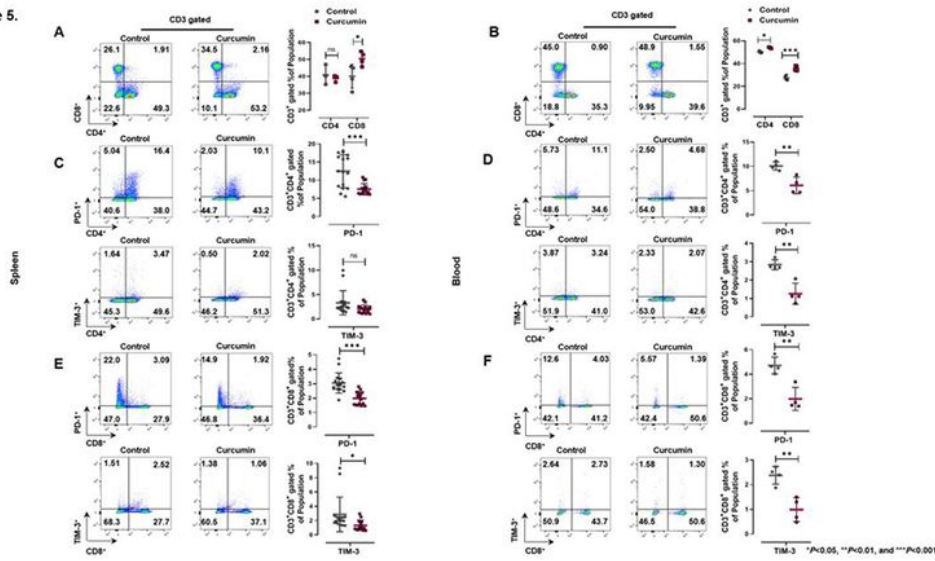


Figure 5.

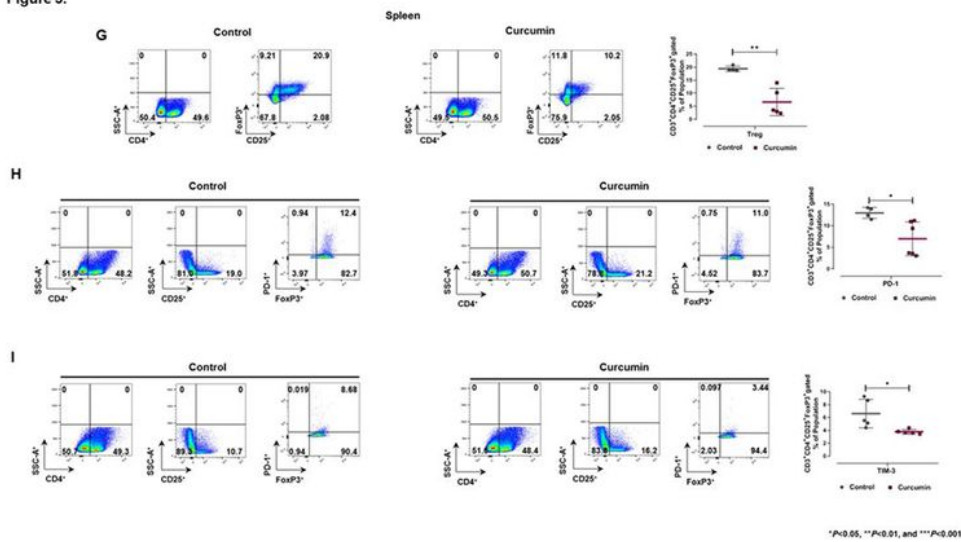


Figure 5

Curcumin reduces the expression of PD-1 and TIM-3 on CD4⁺, CD8⁺ T cells and Tregs in both blood and spleen. (A, B) Flow cytometric dot plot presentation shows CD4⁺ and CD8⁺ T-cell populations in the spleen and blood. (C-F) Representative flow cytometry images of PD-1 and TIM-3 expression on CD4⁺ and CD8⁺ T-cells in blood and spleen. (G) Representative images of CD25⁺FoxP3⁺ Tregs in the CD4⁺ T-cell population from the spleen. (H, I) Percent of PD-1⁺ and TIM-3⁺ cells in the CD4⁺CD25⁺FoxP3⁺ Treg

population from control and Curcumin treated mice. The data were represented as mean \pm SEM. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Figure 6.

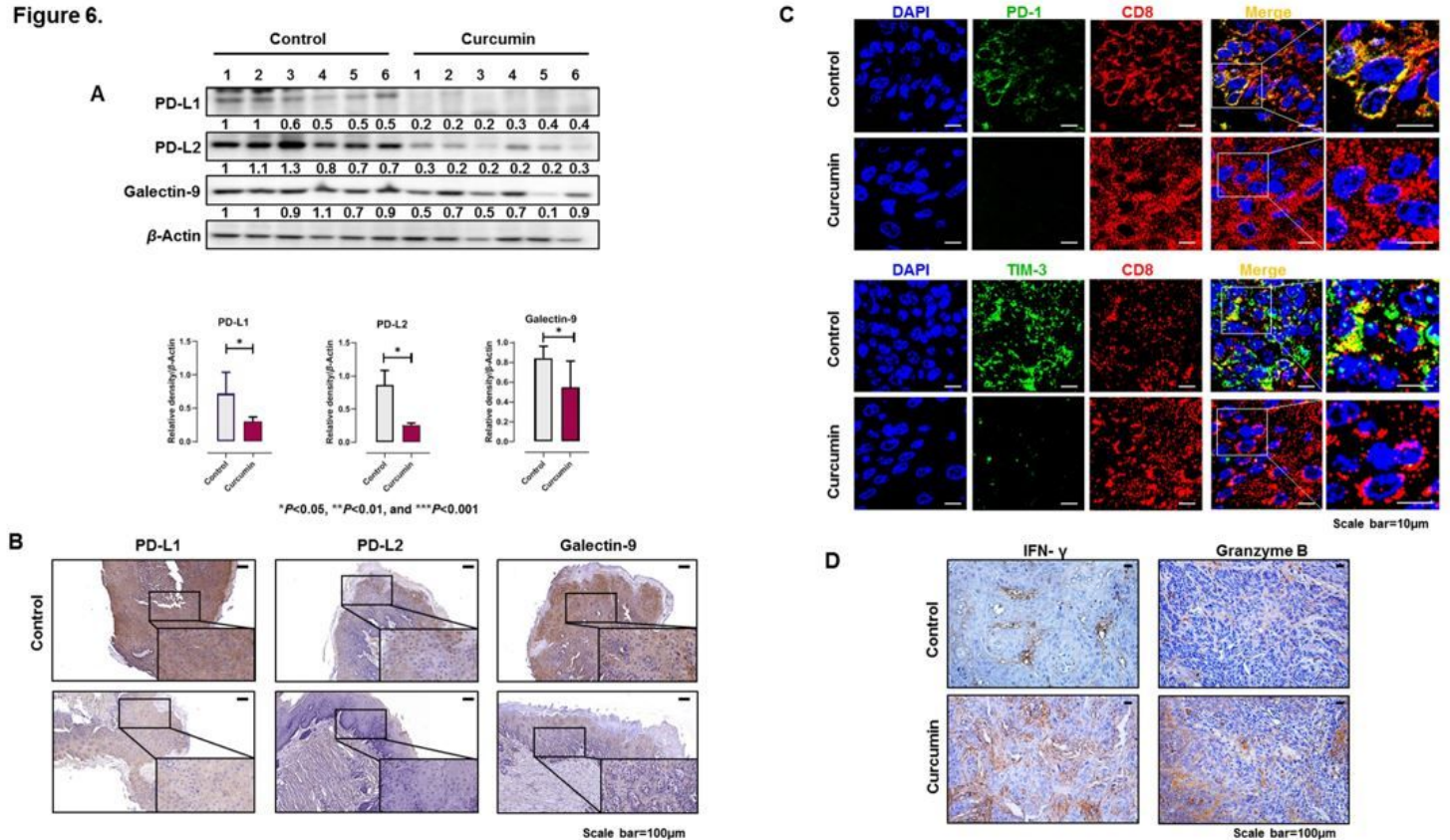


Figure 6

Curcumin downregulates the expression of IC ligands and its related receptors in TIL and increases the expression IFN- γ and Granzyme B. (A, B) The expression of IC ligands PD-L1, PD-L2, and Galectin-9 assessed from the cancer tissues of control and Curcumin-treated group by western blot assay and immunohistochemical staining. (C) Confocal assay of CD8+ PD-1+ and CD8+ TIM-3+ in TIL from control and Curcumin group. (D) Immunohistochemical images for IFN- γ and Granzyme B from the cancer tissue of control and Curcumin-treated groups. All data were represented as mean \pm SEM. Statistical significance was analyzed using Student's t-tests. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Supplementary Files

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