Intratumoral Delivery of Chlorine Dioxide Exploits its ROS-like Properties: A Novel Paradigm for Effective Cancer Therapy

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Highlights

- Chlorine dioxide: safe and effective, exhibits ROS-like properties for cancer elimination and tissue regeneration.
- Intratumoral delivery of chlorine dioxide: targets and eliminates cancer cells without promoting drug resistance.
- Chlorine dioxide stimulates an immune response against cancer, enhancing therapeutic potential.
- Intratumoral injections of chlorine dioxide: innovative strategy for effective cancer therapy.

Abstract

Reactive Oxygen Species (ROS) are potent oxidizing compounds renowned for their ability to eradicate cancer cells and facilitate tissue regeneration. In our research, we have discovered chlorine dioxide (CD), a substance that exhibits ROS-like properties and can be safely administered as a medicinal agent in the human body. Our study focuses on the utilization of intratumoral delivery of CD as a novel and efficacious approach for cancer therapy. Through intratumoral injections, CD selectively targets and eliminates cancer cells without promoting drug resistance. Furthermore, CD has demonstrated the capacity to elicit an immune response against cancer, thereby augmenting its therapeutic potential. We propose a groundbreaking paradigm for cancer treatment, employing intratumoral injections of CD, which holds great promise in prolonging the survival of cancer patients and transforming cancer into a manageable chronic condition. This approach harnesses the ROS-like properties of CD to effectively eradicate cancer cells while fostering tissue regeneration. By capitalizing on the unique characteristics of CD, we present a new avenue for enhancing patient outcomes in cancer therapy. Further research is warranted to optimize the administration protocols and dosage of CD, as well as to explore

potential synergistic effects with other therapeutic agents. With ongoing investigation, the intratumoral delivery of CD represents a promising and innovative strategy for achieving effective cancer therapy.

Keywords

Reactive Oxygen Species, chlorine dioxide, cancer cells, tissue regeneration, intratumoral delivery, drug resistance, immune response.

Abbreviations

SOD- Superoxide dismutase
ROS - Reactive Oxygen Species
PDT - Photodynamic therapy
CD - Chlorine dioxide
DAMPs - Death-associated molecular patterns
HCMs - Human cardiac myocytes
HUVECs - Human vascular endothelial cells
NSCLC - non-small cell lung cancer
LT - Large tumor
ST - Small tumor

Introduction

Certain natural compounds or drugs that target Superoxide dismutase (SOD) possess a unique capability to selectively eradicate cancer cells by stimulating the generation or accumulation of Reactive Oxygen Species (ROS) [1, 2]. Furthermore, the body's neutrophils can also generate ROS, which contributes to the destruction of cancer cells [3]. Certain drugs can selectively enhance the levels of ROS in cancer cells, exhibiting long-term inhibitory effects on cancer. For example, the combination of metformin-induced ROS upregulation and apigenin amplification results in significant anticancer activity while preserving the integrity of normal cells [4]. Photodynamic therapy (PDT) is a treatment method that utilizes light to activate a photosensitizer within the body, resulting in the production of singlet oxygen ($^1O_2$). This sudden increase in oxygen levels induces toxicity in tumor cells, leading to their demise through apoptosis or necrosis. PDT not only directly targets and eradicates the tumor but also stimulates the release of cell death-associated molecular patterns (DAMPs) by dendritic cells, activating the immune system's antigen-presenting response and promoting an anti-tumor immune response [5].

Based on our hypothesis, we propose that the exogenous supplementation of ROS or their analogs could effectively eradicate tumors and, similar to PDT, elicit an antitumor immune response. In this study, we have selected chlorine dioxide (CD) as a ROS-like oxidant to evaluate its potential in cancer therapy. CD is widely acknowledged as a potent oxidizing agent with disinfectant properties, distinguished by its molecular formula ClO_2. Its applications encompass various domains, including water treatment, food processing, medical hygiene, and environmental cleaning. Notably, CD exhibits rapid destruction of cell membranes and DNA in bacteria, viruses, and other microorganisms, thereby effectively eliminating pathogens and contaminants. Remarkably, at lower concentrations of 0.25 mg/L, CD can eradicate 99% of E. coli (15,000 cells/mL) within a mere 15 seconds [6]. Furthermore, studies have demonstrated that CD and

hydrogen peroxide demonstrate comparable efficacy in inducing cell death in human gingival fibroblasts [7].

ROS are generated during cellular respiration for ATP production [8]. However, the dispersed nature of ROS production in the body and the limited capacity to rapidly eliminate a large number of cells, especially within tumor tissues, present significant challenges. These challenges are primarily attributed to tumor hypoxia, characterized by low oxygen levels in the tumor microenvironment [9]. To establish a novel paradigm for cancer treatment, it is crucial to elevate the concentration of CD to a level that can effectively eradicate substantial tumor masses. This concentration exceeds the typical endogenous ROS production in the body. Furthermore, considering the short half-life of CD as an oxidizing agent in body tissues and the imperative of minimizing systemic side effects, intratumoral administration of CD has been selected as the preferred approach to achieve our objective.

Materials and methods

Cell Culture

An MTT assay was conducted to assess the inhibitory effect of a CD solution on the survival rate of MCF-7 breast cancer cells, human cardiac myocytes (HCM), and human vascular endothelial cells (HUVECs). Additionally, the MTT assay was utilized to determine the survival inhibition rate of ten non-small cell lung cancer (NSCLC) cell lines, namely H2110, H1975, H650, H1623, H2126, HCC827, A549, H810, H1048, and H1355. To evaluate apoptosis or necrosis in MCF-7 breast cancer cells and human cardiac myocytes, flow cytometry with Annexin-V PI double staining was employed. The cells were incubated with varying concentrations of CD for 24 hours at 37°C, harvested, and washed with DMEM. Subsequently, the cells were resuspended in annexin V-FITC and PI staining solution, followed by a 15-minute incubation in the dark at room temperature. After the addition of binding buffer, the stained cells were analyzed using a FACSCalibur flow cytometer, with FITC fluorescence measured between 515 and 545 nm and PI fluorescence measured between 564 and 606 nm.

Reagent

CD solution at concentrations of 8mg/mL, 13mg/mL, and 15mg/mL was obtained from Beijing Wanbincell Biotechnology Co., Ltd.

Animal Models

C57BL/6 and Balb/c mice were obtained from the China Experimental Center for Food, Drugs, and Biological Products. All experiments were conducted with the approval of the The Cancer Institute and Hospital, Chinese Academy of Medical Sciences.

Mouse Safety Studies

C57BL/6 mice were administered a single intraperitoneal injection of 0.3 mL of a CD solution (7.5 mg/mL), which was determined to be well-tolerated. Similarly, a single dose of 0.5 mL of a CD solution (1 mg/mL) was found to be safe for C57BL/6 mice. However, when a single dose of 0.2 mL of a CD solution (7.5 mg/mL) was injected into the caudal vein, tail loss was observed at

the injection site, potentially due to CD-induced damage to blood vessels and subsequent ischemic necrosis. On the other hand, intracranial injection of a single dose of 0.02 mL of a CD solution (1.5 mg/mL) was deemed safe for C57BL/6 mice.

CD simulated the impact of ROS on healthy tissues.

Two groups of female C57BL/6 mice, aged 9-10 weeks, were randomly assigned into two groups, with each group consisting of four mice (n=4). The first group received a subcutaneous injection of 0.3 mL of a CD solution with a concentration of 7.5 mg/mL, while the second group received a subcutaneous injection of 0.3 mL of a CD solution with a concentration of 15 mg/mL. The injection sites were monitored daily, and the extent of injury was assessed and recorded using an injury score. The injury score was determined by multiplying the area of the lesion by the severity of the lesion.

Effect of CD on Tissue Regeneration in Mice

Twenty female C57BL/6 mice (9-10 weeks old) were anesthetized with halothane and had their tails severed 2 cm from the base. The mice were randomly divided into 5 groups, each consisting of 4 mice. Group 1 immersed their tail wounds in a 15mg/mL CD solution for 1 minute and received daily treatment with the same solution. Group 2 immersed their tail wounds in physiological saline solution for 1 minute and received daily treatment with the same solution. Group 3 immersed their tail wounds in a 15mg/mL CD solution for 10 minutes daily for 8 consecutive days, and then on days 10, 14, and 18. Group 4 immersed their tail wounds in a 1.5% hydrogen peroxide solution for 10 minutes daily for 8 consecutive days, and then on days 10, 14, and 18. The progress of wound healing and tail severance wound scores were recorded. Group 5 immersed their tail wounds in a 15mg/mL CD solution for 10 minutes on days 1, 4, 8, 12, 16, and 20. Wound healing progress was assessed using a scale ranging from 0 (fully healed) to 90 (exudate or crusting area).

Inhibition of Lewis Lung Cancer in Mice by Intrapericardial Injection of CD Solution

A total of ten male C57BL/6 mice, aged 9-10 weeks, were included in the study. The mice were subcutaneously inoculated with 4×10^6 LLC cells in the right axillary region and subsequently divided into two groups, with ten mice in each group. Starting from the 4th day after cell inoculation, the CD solution was intratumorally injected at a dosage of 0.2mL per mouse (8 mg/mL) every other day for a total of three doses. From the second injection onwards, the dosage was increased to 0.3mL per mouse. The control group received intratumoral injections of PBS injection solution. The experimental period lasted for 10 days, after which all animals were humanely euthanized two days following the administration of the final dose in the experimental group. Tumor weight was measured after recording the animals' body weight, and the tumor suppression rate was calculated. Tumor length and width were regularly measured to accurately calculate tumor volume.

Inhibition of B16 Melanoma Growth in C57BL/6 Mice by Intratumoral Injection of CD Solution.

Each male C57BL/6 mouse, aged 9-10 weeks, was subcutaneously inoculated with 2×10^6

cells in the right axillary region. Subsequently, the mice were randomly divided into three groups, with ten mice in each group. On the 8th day after the inoculation of B16 cancer cells, the intratumoral injection group received CD solution intratumorally. The CD solution was administered at a dosage of 0.2mL (13mg/mL) every 4 days for a total of three doses. On the 2nd day after the inoculation of melanoma, the mouse tail vein injection group received CD solution via the tail vein. The CD solution was injected at a dosage of 0.2mL (1.5 mg/mL) every 4 days for a total of five doses. The tumor model group served as the control and received intratumoral injections of PBS injection solution for observation purposes. The experiment was conducted over a period of 20 days. Four animals from each group were humanely euthanized four days after the last dose, and their tumor weight was measured after recording their body weight in order to calculate the tumor suppression rate. On the 20th day, the remaining mice in each group were tested for cytokines.

Inhibition of Melanoma B16 Metastasis in C57BL/6 Mice by CD Solution.

Each male C57BL/6 mouse, aged 9-10 weeks, was subcutaneously inoculated with 2×10^6 B16 cells in the right axillary region and 1×10^6 B16 cells in the tail vein. Subsequently, the mice were randomly divided into three groups, with 9 mice in each group. The groups consisted of a tumor model control group, which received intratumoral injections of PBS solution; an intratumoral administration group in the axillary region; and an intratumoral administration group in the axillary region with daily 2-hour inhalation treatment for the tumor-bearing mice.

The administration of CD solution began on the 7th day after melanoma inoculation. The intratumoral injections of CD solution were administered at a volume of 0.2mL (15 mg/mL) every 4 days for a total of 4 doses. In the inhalation group, the mice received intratumoral injections of CD solution at the same dosage and frequency, along with 2 hours of daily inhalation treatment. For the inhalation treatment, 20mL of 15 mg/mL CD solution was placed in the mouse cage for natural evaporation and inhalation.

On the 17th day, three mice from each group underwent HE staining of the tumor tissue and lungs. On the 21st day, the lungs were harvested, and the pulmonary metastatic foci were counted.

Inhibitory Effect of Intratumoral CD Solution on 4T1 Breast Cancer Cell Transplantation in Balb/c Mice

A total of 32 female Balb/c mice, aged 9-10 weeks, were utilized for this experiment. They were divided into four groups, each consisting of eight mice. The large tumor group (LT group) received a subcutaneous inoculation of 4×10^5 4T1 cells in the right axillary region and contralateral mammary pad. Subsequently, they were randomly assigned to two groups: the control group (PBS+LT) and the treatment group (CD+LT), with eight mice in each group. Similarly, the small tumor group (ST group) received a subcutaneous inoculation of 2×10^5 4T1 cells in the right axillary region and contralateral mammary pad. They were then divided into the control group (PBS+ST) and the treatment group (CD+ST), with eight mice in each group.

Starting from the 15th day after inoculation, groups 2 and 4 (CD+LT and CD+ST) received intratumoral injections of a CD solution with a concentration of 15 mg/mL. A total volume of 0.2 mL (15 mg/mL) was administered every 3 days for a total of 6 doses. The mammary pad tumors in the control groups (PBS+LT and PBS+ST) were observed without any treatment (Untreated). On

the 27th day, partial immune cells were tested, and on the 33rd day, the animals were euthanized. The lungs were harvested for counting pulmonary metastasis, as well as for the detection of serum and axillary tumor cytokines.

On the 27th day of the 4T1 model, blood and spleen samples were collected from 3 mice in each group, totaling 12 mice. Whole blood samples were collected from 12 mice, and spleen samples were collected from the remaining 12 mice. The spleen suspended cells were prepared for the detection of CD3, CD4, CD8, and CD335 using four-color staining, as well as CD11b, Gr-1, and F4/80 using three-color staining. Fresh anticoagulated peripheral blood from the mice (spleen suspended cells) was added to the bottom of the flow cytometry sample tube, and an appropriate amount of fluorescently labeled antibodies was added, including CD3e PerCP-Cy5.5, CD4 PE, CD8a FITC, and CD335 (NKp46) APC (Biolegend), as well as Gr-1 FITC, F4/80 PE, and CD11b APC (Biolegend). Analysis was performed using a flow cytometer (BD).

On the 33rd day of the 4T1 model, blood and axillary tumor samples were collected from 5 mice in each group, totaling 20 mice (Untreated). ELISA was used to measure the levels of IL-6, TNF-α, INF-γ, and CTLA-4 in the serum and tumor samples.

Statistics

Statistical analyses were performed using Origin 9.0 software. When the transformed data showed significant variation among treatments, differences among populations and treatments were assessed using the nonparametric Mann-Whitney test. However, in most cases, no significant variation was observed among treatments. In such instances, two-tailed Student's t-tests were used to evaluate differences between two treatments. For comparisons involving more than two treatments, a two-way ANOVA followed by Dunnett's multiple comparisons tests was employed to compare multiple groups with repeated measures.

Results and Discussion

Our initial safety experiment confirmed the safe administration of the CD solution at various concentrations to the mice in this study. CD exhibits potent cytotoxicity against breast cancer cells as well as normal cells, including cardiomyocytes and vascular endothelial cells (Fig. 1A, B). It induces cell death through both apoptotic and necrotic pathways, similar to ROS, which can trigger cell death through necrotic or apoptotic mechanisms [10], regardless of the cell type (Fig. 1C).

Due to the non-selective cytotoxicity of CD towards the cells it encounters, systemic administration of CD as a drug is not feasible. Therefore, intratumoral administration is a suitable choice for targeting solid tumors. In our study, we administered CD via subcutaneous injection in the dorsal region of mice, and the results revealed significant damage to normal tissues caused by CD. Injection of CD resulted in severe skin damage in mice and noticeable necrosis of various cell types in the subcutaneous tissue. However, approximately 20 days later, the damaged tissue completely recovered, and the skin returned to its original state (Fig. 2A, B), with successful hair regrowth (Supplementary Fig. S1D). This indicates that the tissue damage caused by CD is not cumulative and that the damaged tissue is capable of complete regeneration.

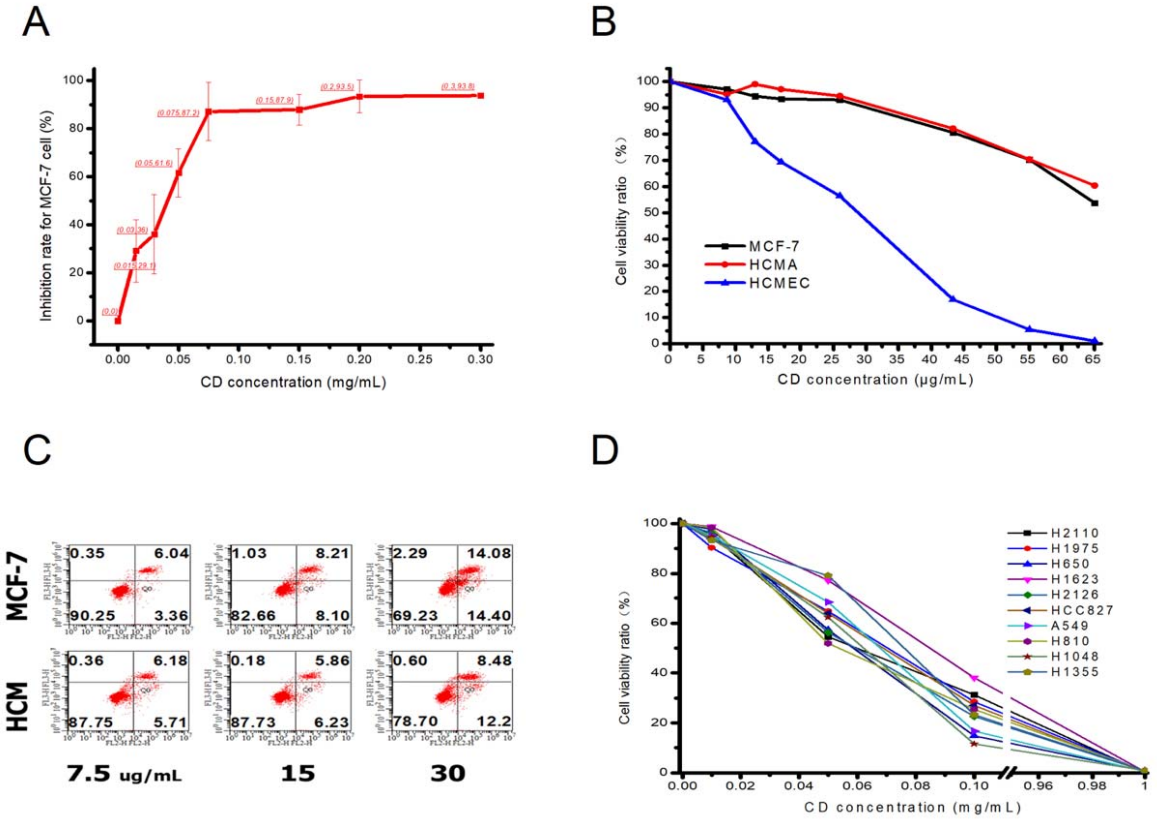

Fig. 1. In vitro cell viability assay of CD. (A) Inhibition of cell survival in MCF-7 human breast cancer cells by CD. (B) Percentage of cell survival inhibition by CD in MCF-7 breast cancer cells, normal human cardiomyocytes (HCM), and human vascular endothelial cells (HCMEA). (C) Representative plots demonstrating the measurement of apoptosis or necrotic pathways in breast cancer cells and cardiomyocytes using flow cytometry after treatment with different concentrations of CD (The lower right quadrant represents early apoptotic cells, and the upper right quadrant represents necrotic or late apoptotic cells). (D) Measurement of cell proliferation in 10 NSCLC cell lines when treated with increasing doses of CD.

Neutrophils have the ability to produce hypochlorous acid, an oxidizing agent similar to ROS, which has been demonstrated to accelerate skin wound healing and promote skin rejuvenation when applied topically [11]. Similarly, hydrogen peroxide, another type of ROS, has been found to facilitate sensory axon regeneration in zebrafish skin [12]. Building upon these findings, we formulated the hypothesis that CD may also possess the capability to promote tissue regeneration. To investigate this hypothesis, we applied CD to wounds created by cutting the tails of mice. Remarkably, both CD treatment and hydrogen peroxide treatment significantly expedited the healing process in tail-ablated mice compared to treatment with physiological saline, resulting in complete wound closure approximately 6 days earlier (Supplementary Fig. S1A-B, E). Prolonged exposure to the CD solution in one group led to significant damage to the excised wound and the surrounding normal skin tissue. However, once the exposure was reduced, the damaged skin rapidly regenerated without impeding the healing of the original wound (Supplementary Fig. S1C). As an oxidant, CD also facilitated the oxidation of cellular tissue debris, thereby promoting the clearance of deposits near the wound (Supplementary Fig. S1F). This mechanism may potentially

serve as a pathway for tissue regeneration..

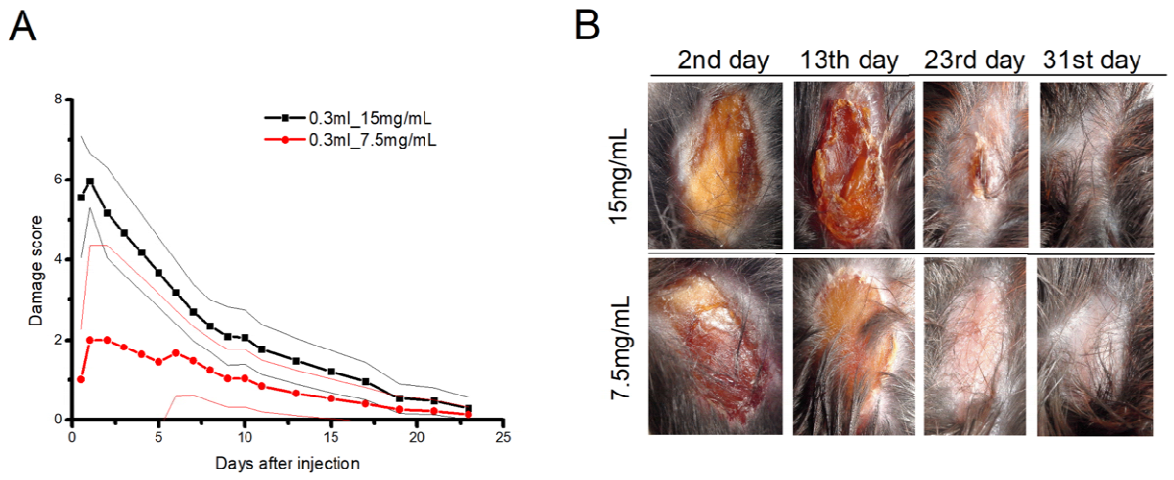

Fig. 2. In vivo tissue damage and regeneration assay of CD. (A) Injection of CD solution at concentrations of 15mg/mL and 7.5mg/mL subcutaneously into the dorsal region of C57BL/6 mice, showing the trend of injury scores (injury recovery) over time. Data presented as mean ± SD (n=4). (B) Representative images of the back skin taken on days 2, 13, and 23 after the start of CD injection. Images selected from each group.

On the fourth day following subcutaneous inoculation of LLC cancer cells in C57BL/6 mice, we initiated intratumoral injection of a lower dose (8mg/mL) of CD. Notably, compared to the control group, the intratumoral injection of CD on the sixth day exhibited a substantial suppression of tumor growth (Fig. 3A; Supplementary Fig. S2B). Furthermore, the CD-injected tumors displayed evident necrosis, indicating a robust necrotic effect on the tumor tissue (Supplementary Fig. S2A). To further investigate the therapeutic potential of CD, we escalated the dosage to 13 mg/mL and additionally employed intravenous injection at a concentration of 1.5 mg/mL in a B16 tumor model. Remarkably, the intratumoral injection group demonstrated significant tumor suppression (74.1% inhibition) on day 4, while the intravenous group exhibited a comparatively weaker but still significant inhibition (34.8% inhibition). Subsequent evaluations revealed that the intratumoral injection group consistently exhibited a higher degree of tumor suppression, whereas the intravenous group failed to exhibit any inhibitory effect (Fig. 3B). Intravenous administration of CD resulted in a reduced tumor burden, suggesting a potential involvement of antitumor immunity, albeit without sustained efficacy. On day 20, IL-1β and VEGF levels were assessed in the mice, revealing no significant differences between the groups (Supplementary Fig. S3A, B).

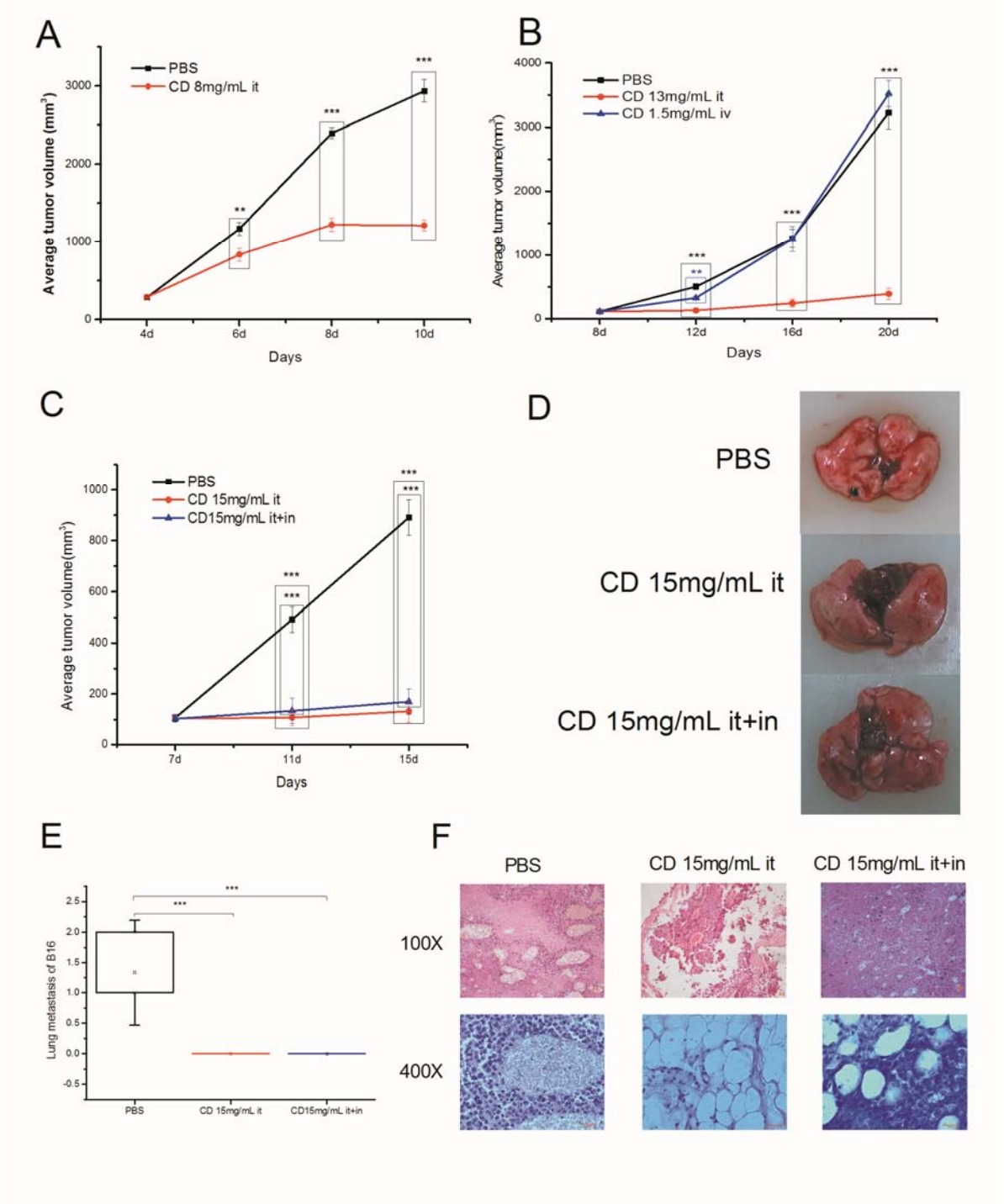

Fig. 3. In vivo anti-cancer assay of CD through intratumoral injection in C57BL/6 mice. (A) Subcutaneous tumor volume of LLC cancer cells. One group received intratumoral injection of PBS as a control, while another group received intratumoral injection of CD (8mg/mL). (**P < 0.01, *** P < 0.001, two-tailed t-test; n = 10 mice per cohort). Error bars represent mean ± SEM. (B) Subcutaneous tumor volume of B16 cells, with one group receiving intratumoral injection of PBS as a control, another group receiving intratumoral injection of CD (13 mg/mL), and a third group receiving intravenous injection of CD (1.5 mg/mL). (**P < 0.01, *** P < 0.001, two-tailed t-test; n = 10 mice per cohort). Error bars represent mean ± SEM. (C) Tumor volume of subcutaneous

and lung metastasis models established by subcutaneous and tail vein injection of B16 cells, with one group receiving intratumoral injection of PBS, another group receiving intratumoral injection of CD (15 mg/mL), and a third group receiving intratumoral injection of CD (15mg/mL) along with daily inhalation of CD gas. (*** $P < 0.001$, two-tailed t-test; n = 9 mice per cohort). Error bars represent mean ± SEM. (D) Lung metastasis nodules counted on day 21 in the subcutaneous and lung metastasis models. (E) Lung metastasis nodules counted on day 21. (*** $P < 0.001$, two-tailed t-test; n = 3 mice per cohort). Error bars represent mean ± SD. (F) Histological images of subcutaneous and lung metastasis models on day 17 stained with H&E (100x and 400x magnification).

In the context of cancer cell death, both apoptosis and necrosis have been shown to elicit anti-cancer immune responses [13, 14]. Based on this knowledge, we hypothesized that direct injection of CD into tumors could induce apoptosis and necrosis of cancer cells, thereby activating the body's anti-tumor immune response. To investigate this hypothesis, we established B16 tumor and B16 lung metastasis models in C57BL/6 mice. Our findings revealed that CD significantly inhibited tumor progression, while daily inhalation of CD gas had minimal impact on subcutaneous tumors (Fig. 3C). On day 21, the mice's lungs were examined to assess the presence of B16 lung metastases. In the control group, each mouse (n=3) exhibited 1-2 lung metastatic foci. However, in both the intratumoral injection of CD and the combination of intratumoral injection and inhalation of CD gas groups (n=5), no lung metastatic foci were observed (Fig. 3D). These results indicate that intratumoral CD injection can directly ablate the tumor and trigger a systemic anti-tumor immune response, leading to significant inhibition of tumor metastasis (Fig. 3E). Histological analysis on the 17th day revealed extensive necrotic centers and destruction of the tumor architecture following CD injection (Fig. 3F). Although inhalation of CD gas caused some lung damage (Supplementary Fig. S4), it did not affect B16 lung metastasis.

In our study, we utilized a mouse model and injected 4T1 tumor cells into the mammary pad and axillary regions of BALB/c mice. Two groups of mice were administered different concentrations of tumor cells: a small tumor group (ST) received 4×105 cells in the mammary pad and 2×105 cells in the axillary region, while a large tumor group (LT) received 4×105 cells in both regions. On the 15th day, CD (15 mg/mL) was administered exclusively into the mammary pad tumors of the experimental group. We observed a significant reduction in tumor volume in the CD-treated groups, particularly in the small tumor group ($P < 0.05$) (Fig. 4A, B). The large tumor group exhibited a 30% inhibition rate on the third day, whereas the small tumor group demonstrated a higher inhibition rate of 72%. The axillary tumors displayed a lower tumor suppression rate due to the absence of direct killing effects from CD. However, at 21 and 27 days, the CD-treated groups exhibited significantly higher inhibition rates compared to the control groups (Fig. 4C, D). These findings suggest that intratumoral CD injection can induce an anti-tumor immune response, although the response may not be sustained. T-cell exhaustion is frequently observed in cancer [15], indicating a general decline in the immune system's ability to combat tumors under various circumstances. On the 27th and 33rd days, lung metastasis was significantly inhibited in the CD-treated groups compared to the control groups. The small tumor group exhibited a slightly delayed response compared to the large tumor group, but the difference was not statistically significant (Fig. 4E, G). These results underscore the potential of intratumoral CD injection as a therapeutic strategy for inhibiting tumor growth and metastasis.

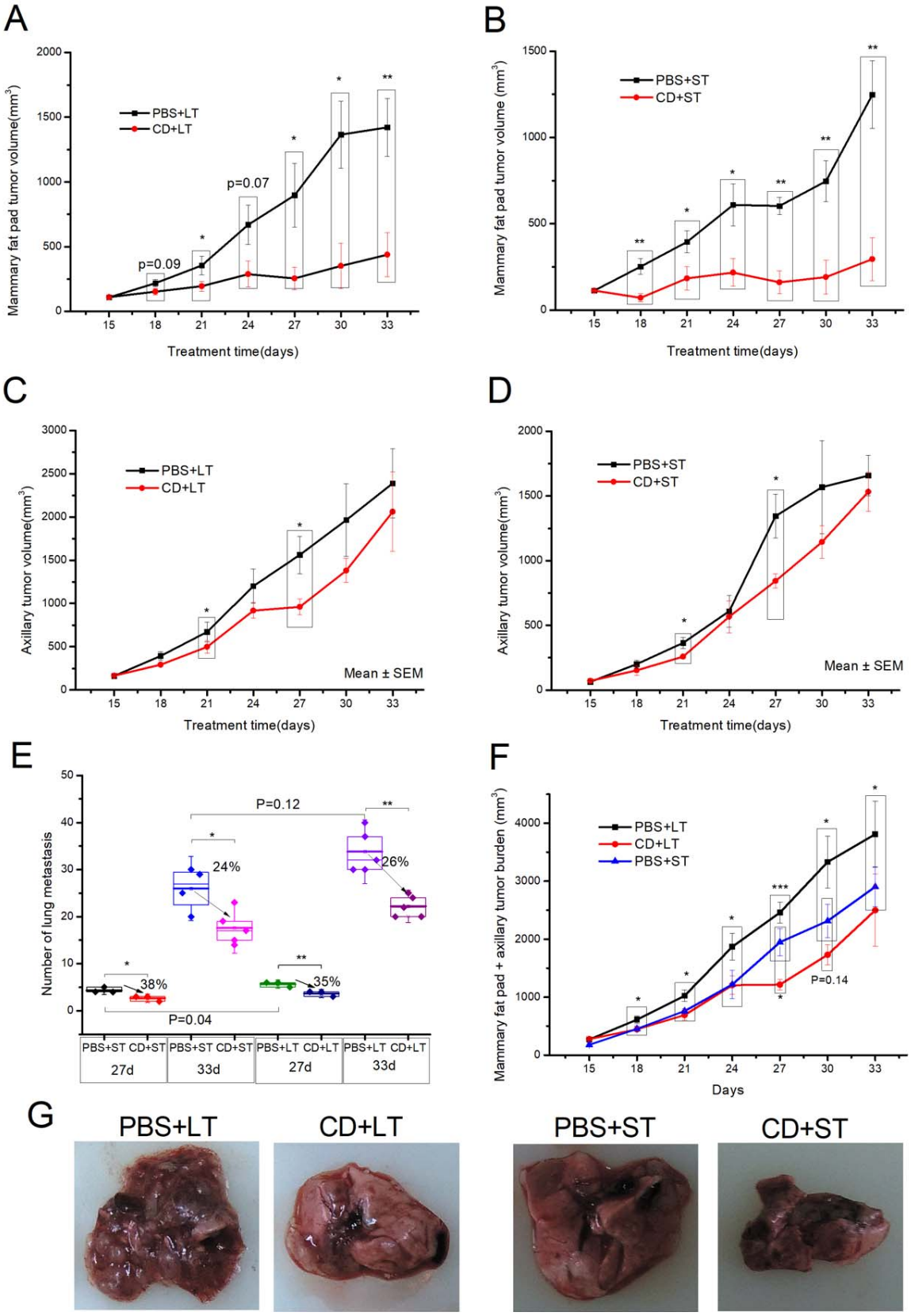

Fig. 4. In vivo anti-cancer assay of CD through intratumoral injection in BALB/c mice. (A) Schematic

representation of the subcutaneous and mammary pad injection of 4T1 tumor cells and the administration of CD. (B) Tumor volume of the large tumor group, with one group receiving intratumoral injection of PBS as a control (PBS+LT) and another group receiving intratumoral injection of CD (15 mg/mL) as a treatment (CD+LT). (*P < 0.05, ** P < 0.01, two-tailed t-test; n = 5-8 mice per cohort). Error bars represent mean ± SEM. (C) Tumor volume of the small tumor group, with one group receiving intratumoral injection of PBS as a control (PBS+ST) and another group receiving intratumoral injection of CD (15 mg/mL) as a treatment (CD+ST). (*P < 0.05, ** P < 0.01, two-tailed t-test; n = 4-8 mice per cohort). Error bars represent mean ± SEM. (D) Tumor volume of the large tumor group in the axilla (no injection). (*P < 0.05, two-tailed t-test; n = 5-8 mice per cohort). Error bars represent mean ± SEM. (E) Tumor volume of the small tumor group in the axilla (no injection). (*P < 0.05, two-tailed t-test; n = 4-8 mice per cohort). Error bars represent mean ± SEM. (F) Overall tumor burden, with the tumor volume of the large tumor group (mammary pad tumor injected with PBS) in the axilla added to represent the tumor burden of the control group, the tumor volume of the large tumor group (mammary pad tumor injected with CD) in the axilla added to represent the tumor burden of the treatment group, and the tumor volume of the small tumor group (mammary pad tumor injected with PBS) in the axilla added to represent the tumor burden of the control group. (*P < 0.05, ***P < 0.001, ANOVA, n = 5-8 mice per cohort). Error bars represent mean ± SEM. (G) Lung metastasis images on day 33, with the number of lung metastatic nodules roughly following the order: PBS+LT > CD+LT ≤ PBS+ST > CD+ST.

The presence of a primary tumor has been shown to decrease immunocompetence, but this can be improved by surgical resection of the primary tumor [16]. In our study, we investigated the effects of intratumoral CD injection for tumor ablation on the growth trajectory of tumors in different sizes. We hypothesized that if intratumoral CD injection can enhance the antitumor immune response, it would result in a significantly lower growth curve in the large tumor treatment group compared to the untreated growth curve of the small tumor group. To assess the overall tumor burden, we combined the mammary pad tumor and axillary tumor measurements. Our results showed that from day 24, the growth curve of the overall tumor burden in the large tumor treatment group deviated from that of the small tumor control group, with a notably lower growth rate. This suggests that intratumoral CD injection not only directly ablates the tumor and restores the dominant immune capability, but also provides additional immune enhancement. However, it is important to note that the immune enhancement observed was transient, as the growth curves of both groups started to converge after 30 days (Fig. 4F). These findings indicate that intratumoral CD injection has the potential to enhance the immune response against tumors, but further studies are needed to understand the duration and sustainability of this immune enhancement.

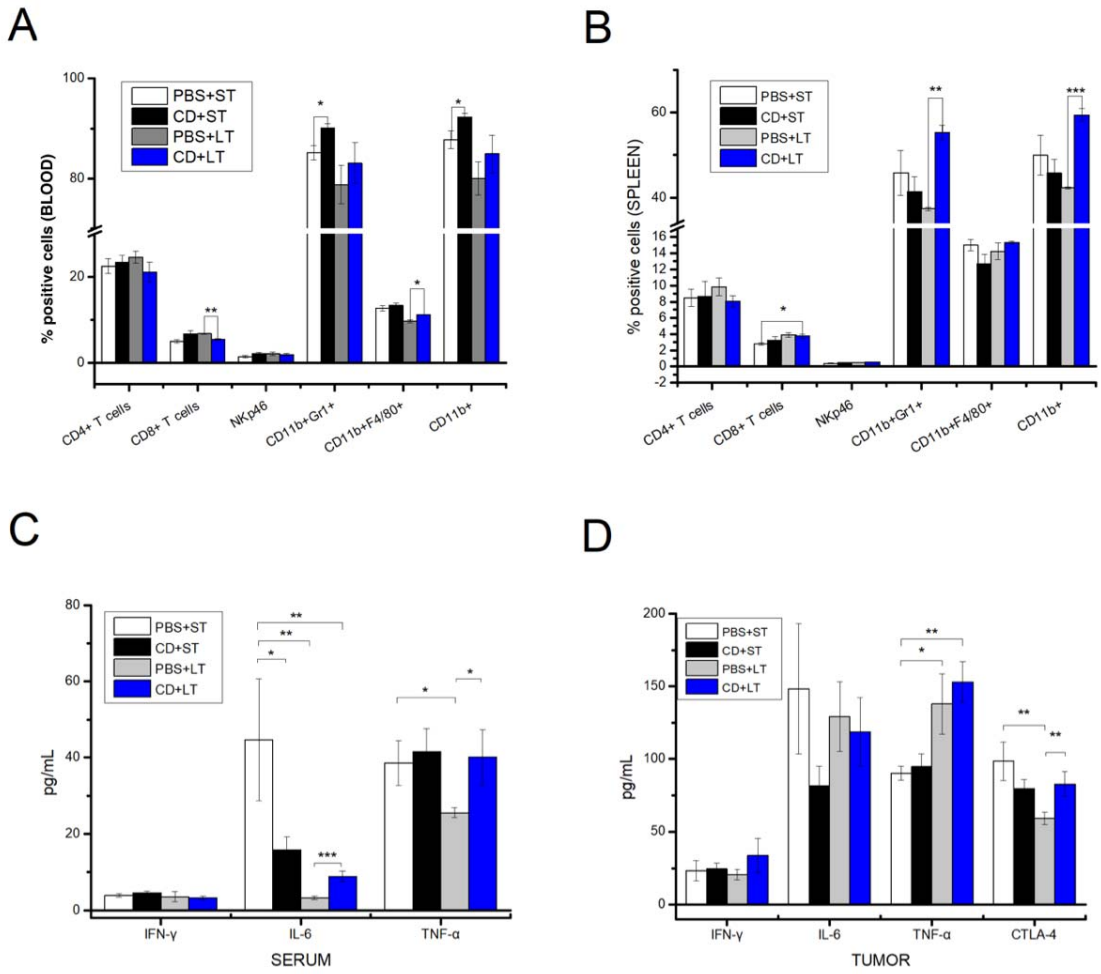

Fig. 5. Comprehensive analysis of the therapeutic effects of intratumoral CD injections in BLAB/c mice inoculated with 4T1 cells. (A) Flow cytometric analysis of plasma samples from mice at day 27, demonstrating quantification of CD4, CD8, NKp46, CD11b+Gr-1+, CD11b+F4/80+, and CD11b+ positive cells. (B) Flow cytometric analysis of spleen samples from mice at day 27, showing quantification of CD4, CD8, NKp46, CD11b+Gr-1+, CD11b+F4/80+, and CD11b+ positive cells. (C) Cytokine analysis of serum samples from mice at day 33. (D) Cytokine analysis of tumor samples from mice at day 33. (A-D) Data are presented as the mean ± SEM. Differences between therapy groups were assessed using two-way ANOVA followed by Dunnett's multiple comparisons tests (B-C: n=3; D-E: n=5).

On day 27, no significant changes were observed in CD4+ T cells and NKp46 cells in the plasma and spleen. However, the treatment group showed a significant increase in CD11b+Gr-1+ myeloid cells compared to the control group (Fig. 5A, B). In the large tumor group, the treatment group exhibited a significant decrease in CD8+ T cells in the plasma compared to the control group. Conversely, in the spleen, the treatment group in the large tumor group demonstrated a significant increase in CD8+ T cells compared to the control group in the small tumor group.

These findings are consistent with the enhanced immune response induced by intratumoral CD injection. It is worth noting that CD11b+Gr-1+ cells possess immunosuppressive properties and contribute to the promotion of cancer metastasis [17], which may explain the transient nature of the immune response stimulated by intratumoral CD injection.

On day 33, cytokine levels in the serum and axillary tumors were evaluated. In the serum, a significant difference in TNF-α levels was observed between the large tumor group and the small tumor group. TNF-α levels increased during the later stages of tumor development, indicating that the antitumor immune response does not decline entirely but continues to rise, albeit at a relatively diminished rate compared to tumor evasion. The decrease in CTLA-4 levels also supports this observation. In the large tumor group, TNF-α levels significantly increased after treatment compared to the control group, consistent with the immune response triggered by intratumoral CD injection (Fig. 5C). Within the large tumor group, treatment with intratumoral CD injection led to a significant increase in CTLA-4 levels, potentially indicating a further reduction in immune capability. Regarding tumor tissue, the changes in TNF-α mirrored those observed in serum TNF-α (Fig. 5D). The alterations in IL-6 also correlated with TNF-α, suggesting potential interaction effects. The absolute value of immune capability in the large tumor group (both control and treatment) surpassed that of the preceding small tumor group. Following treatment in the large tumor group, the relative decrease in IL-6 compared to the control group in the small tumor group may be attributed to the additional immune response stimulated by intratumoral CD injection.

Conclusion

Our study highlights the potential of CD as a versatile therapeutic agent in cancer treatment. CD exhibits properties similar to ROS, such as promoting tissue regeneration and performing comparable functions. Considering the significant role of injury-induced ROS production in tissue regeneration [18], we have confirmed that CD can facilitate tissue regeneration in injured areas, akin to the role of ROS. Tumors can be regarded as non-healing wounds [19], presenting an opportunity to harness CD's regenerative capabilities during cancer treatment to expedite wound healing and enhance patient outcomes.

Compared to conventional cancer treatment methods, CD treatment offers numerous advantages. It significantly reduces disease complications and associated risks, providing a safer and more efficacious alternative. We propose the concept of intratumoral injection of CD, which involves direct tumor ablation without encountering resistance, while concurrently enhancing antitumor immunity. This approach minimizes harm to healthy tissues while harnessing the regenerative potential following tumor ablation. By embracing this comprehensive strategy, we can optimize the outcomes of cancer treatment.

Immune checkpoint inhibitors are catalyzing a revolution in oncology. Numerous clinical trials are currently evaluating evident strategies, specifically intratumoral delivery and tumor tissue-targeted compounds, which have shown potential to enhance local bioavailability, thereby increasing the effectiveness of immunotherapies [20]. As technological advancements unfold, intratumoral administration of CD is emerging as a promising, efficient, and patient-friendly cancer treatment strategy, which may extend patient survival and reduce treatment burden. This strategy has the potential to redefine the management of cancer, likening it to the treatment of chronic conditions. To realize this potential, further research is imperative to fine-tune administration protocols and dosages for CD, investigate synergistic effects with other therapies,

and understand the regenerative and ablative effects on tumor tissue, as well as its capability to elicit a systemic anti-tumor immune response. The ongoing exploration of CD highlights its prospective value as an impactful tool in cancer therapy, signaling new opportunities for enhancing patient care and clinical outcomes.

Author contributions

Xuewu Liu developed and designed the research framework, while Zhaoyang Liu, Xuewu Liu, Jiao Zhang, and Xueyan Liu carried out all the experiments. Xuewu Liu, Shuangning Liu, and Jiao Zhang analyzed the experimental data and drafted the manuscript. Shuangning Liu created the main figures. All authors have reviewed and approved the final version of the manuscript for publication.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

Xuewu Liu is the founder and owner of Beijing Wanbincell Biotechnology Co., Ltd. Jiao Zhang is an employee of Beijing Wanbincell Biotechnology Co., Ltd. Xuewu Liu. and Xueyan Liu are inventors on patent applications (WO2016074203 (A1) and WO2017152718 (A1)) filed by Xuewu Liu related to the use of chlorine dioxide for cancer treatment.

Appendix A. Supplementary

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