Research Article

Lentinan Inhibits Tumor Progression by Immunomodulation in a Mouse Model of Bladder Cancer

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Abstract

Background: Lentinan (LNT), an isolated traditional Chinese herbal component, has antitumor potential. In the current study, the intrinsic mechanism of LNT-induced immunity against bladder cancer was explored in a mouse model. **Methods:** In the mouse model of bladder cancer, we used flow cytometry to detect the LNT caused population changes of T cells, macrophages, MDSC cells, and Treg cells. ELISA was used to evaluate cytokines expression in the supernatant of splenocytes. **Results:** We found that the administration of LNT increased the proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets as well as $CD11b^+F480^+$ macrophages, whereas it diminished the subpopulations of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) and $Gr\text{-}1^+CD11b^+$ myeloid-derived suppressor cells (MDSCs). LNT also upregulated the expression of interferon (IFN)-γ and interleukin (IL)-12, accompanied by a significant reduction in IL-10 and tumor growth factor (TGF)-β ($P < .05$). Our research further confirmed the synergy between LNT and gemcitabine (GEM) to activate immunity and inhibit the growth of bladder tumors in mouse model. **Conclusions:** LNT induced macrophage activation, followed by the enhanced proliferation of $CD4^+$ and $CD8^+$ T cells, and the upregulated expression of IFN-γ and IL-2. Meanwhile, the proportions of MDSCs and Tregs were downregulated, leading to a reduced expression of the anti-inflammatory cytokines IL-10 and TGF-β. The synergy between LNT and GEM provides additional evidence supporting the application of this traditional Chinese herbal component for bladder cancer therapy.

Keywords

lentinan, bladder cancer, immunomodulation, gemcitabine

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Background

Bladder cancer is a prevalent urological malignancy. Early symptoms include painless hematuria, accompanied by urgency, dysuria, frequent urination, or difficulty urinating. The time course of this disease is long, the prognosis is poor, and the patient's quality of life is seriously affected.[1] At present, surgery is the main method for treating bladder cancer, but approximately two-thirds of patients can relapse after surgery.[2,3] Although intravesical drug infusion chemotherapy is used to reduce the recurrence rate, such a treatment can cause bladder irritation symptoms and patients often discontinue treatment due to intolerance. Therefore, the discovery of ways to reduce the symptoms of bladder irritation and improve the quality of life of patients during chemotherapy has become a challenge that requires urgent attention.[4]

Recently, the search for new preventive and therapeutic cancer agents from natural products that have few side effects has attracted growing attention.[5] A wide range of biological effects related to cancer prevention from Shiitake mushrooms, including antioxidant, antimutagenic, antiproliferative, and cell cycle regulation capacities, have been reported in previous studies.[6] Lentinan (LNT) is an isolated polysaccharide extracted from the mycelia of *Lentinula edodes* and was officially approved as an adjuvant therapy for several solid tumors.[7,8] However, little effort has been made to reveal the underlying mechanisms of immunoregulation leading to the antitumor activity of LNT.[9] In addition,

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although several clinical trials of LNT have supported its use for cancer therapy, the role of LNT in the treatment of bladder cancer is still uncertain. In this study, we examined the effects of LNT on antitumor immunity in bladder cancer and its synergistic effect with the chemotherapeutic agent gemcitabine (GEM) on tumor growth.

Methods

Tumor-Bearing Mouse Model

Six-week-old, male C57BL/6 mice with a bodyweight of 18 to 22 g were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). The mice were bred and housed in specific pathogen-free conditions at the Laboratory Animal Center of China Medical University. The mouse bladder cancer cell line MB49, ordered from Sun Yat-sen University (Guangzhou, China), was propagated in RPMI-1640 (Gibco) containing 10% fetal bovine serum (Gibco) in an incubator at 37°C and containing 5% CO_2. To establish the implanted tumor model, 1×10^6 MB49 cells were injected subcutaneously in the dorsal side of C57BL/6 mice. The Animal Care and Use Committee of China Medical University approved all experimental procedures, and every effort was made to minimize animal suffering.

LNT and GEM Treatment

LNT (Shanxi Taisheng Pharmaceutical Co., Ltd., Shanxi, China) and GEM (Eli Lilly France, Fegersheim, France) were dissolved in 0.9% NaCl prior to *in vivo* administration. When the tumors reached 5 to 10 mm in diameter, the mice were randomly subjected to LNT, GEM, or LNT+GEM combination treatment. In different experimental groups, ten mice were treated individually by intraperitoneal injection of 4 mg/kg LNT, 40 mg/kg GEM, or 4 mg/kg LNT + 40 mg/kg GEM, twice a week for 35 days. In the normal control (NC) group, the same volume of saline solution was administered to the control mice.

Measurement of Tumor Size

On day 35, three mice randomly selected from each group (n = 10) were sacrificed by cervical dislocation after chloral hydrate anesthesia. The tumors and spleens were removed, photographed, and measured. The tumor volume (mm^3) was estimated by using the following formula: tumor volume $= \pi/6 \times ab^2$, where a is the long diameter and b is the short diameter, in mm.

Flow Cytometric Analysis

Phenotyping of spleen immunocytes was examined by flow cytometry. Specifically, the excised spleens were cut into small pieces, washed twice with phosphate-buffered saline (PBS), and sliced with forceps and a scalpel. A single-cell suspension of spleen cells was prepared by passing the cells through a 70-μm nylon strainer (BD Biosciences). After rinsing twice with precooled PBS, the single cells suspended in PBS were stained with the following anti-mouse antibodies: CD3-FITC, CD4-PE, CD8-PC5, CD25-FITC, FOXP3-PC5, CD11b-APC, GR-1-FITC, and F4/80-PE (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. The specificity of labeling was confirmed by isotype-matched antibody staining. The labeled cells were analyzed with a FACS Canto II instrument (BD Biosciences, San Diego, CA, USA). A total of 10,000 events acquired in the gate were analyzed by FlowJo v7.6.2 software (Tree Star, San Carlos, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The expression levels of transforming growth factor (TGF)-β, interferon (IFN)-γ, interleukin (IL)-2, and IL-10 in the lysate of the single-cell suspension of splenocytes and tumor tissues were measured by ELISA kits (Sigma-Aldrich Co., St. Louis, MO, USA). All of the procedures were performed in accordance with the manufacturer's instructions. Cytokine concentrations were calculated using the standard regression curve obtained from the values of reference absorption.

Statistical Analysis

GraphPad Prism v7.0 (GraphPad Software Inc.) was used for statistical analysis. Normally distributed measurement data with homogeneity of variance were displayed as the mean ± standard deviation (SD) and analyzed by the unpaired two-tailed Student's test (between two groups) or one-way analysis of variance (among-group comparisons), respectively. The survival curves were calculated by Kaplan–Meier estimates. A value of $P < .05$ indicated a statistically significant difference.

Results

Effects of LNT and GEM on the Immune Cell Subpopulations

As shown in Figure 1A and B, the proportion of $CD3^+CD4^+$ T cells significantly increased after LNT treatment, compared with that in the control group ($P < .05$). In addition, the proportion of $CD3^+CD4^+$ T cells was significantly higher in the combination treatment group than in the GEM group ($P < .05$). As shown in Figure 1C and D, the proportion of $CD3^+CD8^+$ T cells significantly increased after LNT treatment. Meanwhile, the proportion of $CD3^+CD8^+$ T cells was significantly higher in the combination treatment group

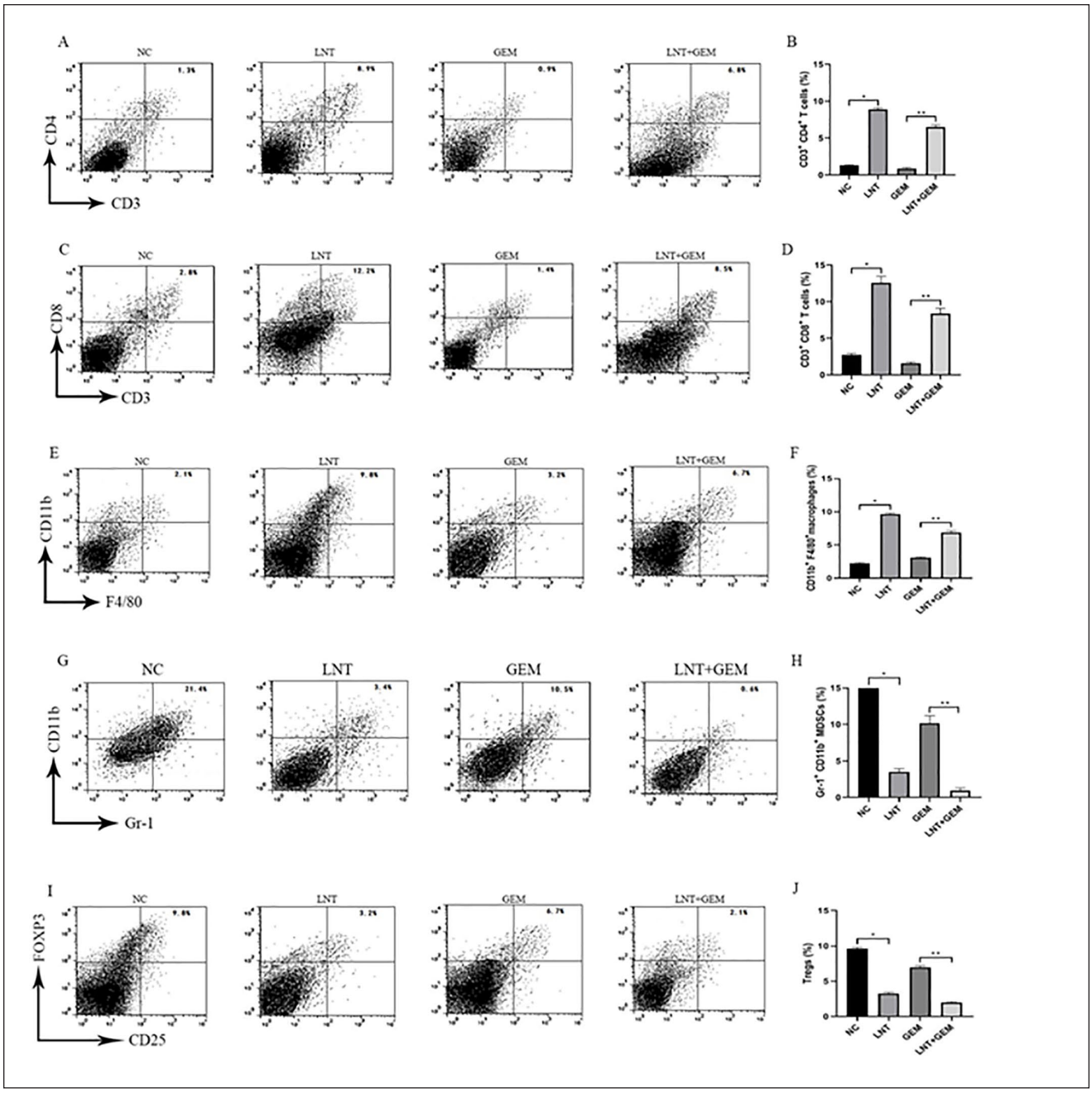

Figure 1. Immunomodulatory effects of LNT on the shift of $CD3^+CD4^+$ T cells (A, B), $CD3^+CD8^+$ T cells (C, D), $CD11b^+$ $F4/80^+$ macrophage cells (E, F), $CD11b^+Gr\text{-}1^+$ MDSC cells (G, H), and $CD4^+CD25^+Foxp3^+$ Treg cells (I, J). Data are shown as means ± SD. *$P < .05$, compared with the control group; **$P < .05$, compared with the GEM treatment group.

than in the GEM group ($P < .05$). Similarly, as shown in Figure 1E and F, the proportion of $CD11b^+F4/80^+$ macrophages significantly increased after LNT treatment, compared with that in the control group ($P < .05$). Furthermore, the proportion of $CD11b^+F4/80^+$ macrophages was significantly higher in the combination treatment group than in the GEM group ($P < .05$). In contrast, as shown in Figure 1G and H, the proportion of $CD11b^+Gr\text{-}1^+$ MDSCs significantly decreased after LNT treatment, compared with that in the control group ($P < .05$). Moreover, the proportion of $CD11b^+Gr\text{-}1^+$ MDSCs in the combination treatment group was significantly lower than that in the GEM group ($P < .05$). As shown in Figure 1I and J, the proportion of $CD4^+CD25^+Foxp3^+$ Tregs also significantly decreased after LNT treatment, compared with that in the control group ($P < .05$). Furthermore, the proportion of $CD4^+CD25^+Foxp3^+$

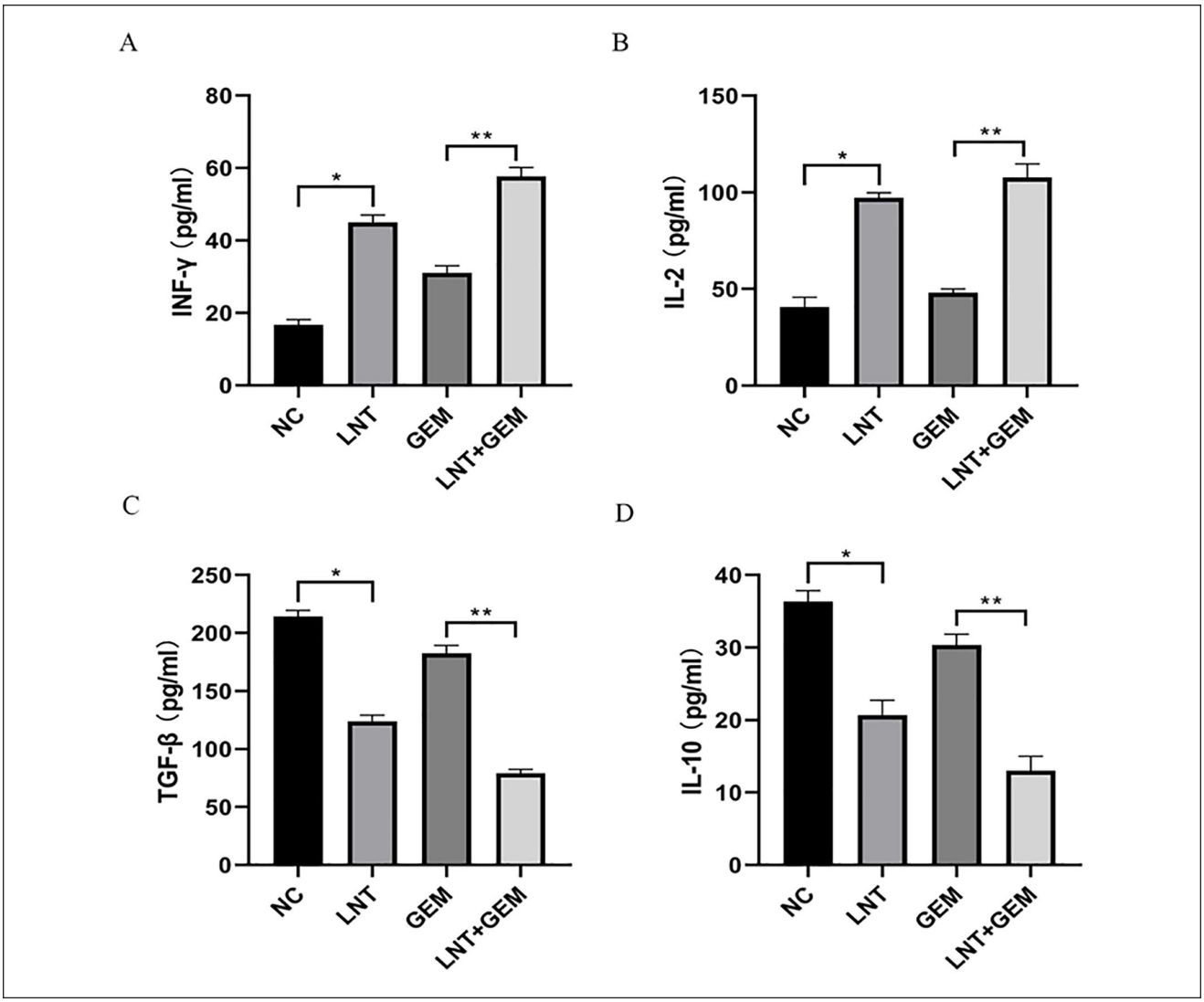

Figure 2. Immunomodulatory effects of LNT on the expression of the cytokines IFN-γ (A), IL-2 (B), TGF-β (C), and IL-10 (D). Data are shown as means ± SD.
*$P < .05$, compared with the control group; **$P < .05$, compared with the GEM treatment group.

Tregs were significantly lower in the combination treatment group than in the GEM group ($P < .05$).

Effects of LNT on the Cytokine Profile

To determine whether LNT improves the immune function of MB49 tumor-bearing mice receiving chemotherapy, the expression levels of IL-2, IFN-γ, IL-10, and TGF-β were measured using their respective ELISA kits. Compared to the control group, the expression of IFN-γ and IL-2 was significantly upregulated by LNT treatment ($P < .05$) (Figure 2A and B). Higher expression levels of IFN-γ and IL-2 also were observed in the combination treatment group, compared to the GEM group (Figure 2A and B). The results also showed that after treatment, the expression changes of IFN-γ and IL-2 in tumor tissues were consistent with the expression changes in splenocyte suspension (Figure 3E and F). In contrast, regardless of the tumor tissue or the spleen cell suspension, the TGF-β level of the LNT group was significantly lower than that of the control group ($P < .05$) (Figures 2C and 3G); and it was also significantly lower in the combination treatment group than in the GEM group ($P < .05$) (Figures 2C and 3G). Finally, the evaluation in spleen cell suspension and tumor tissue showed that compared with the control group and the GEM group, the IL-10 levels of the LNT and LNT + GEM groups were significantly downregulated ($P < .05$) (Figures 2D and 3H).

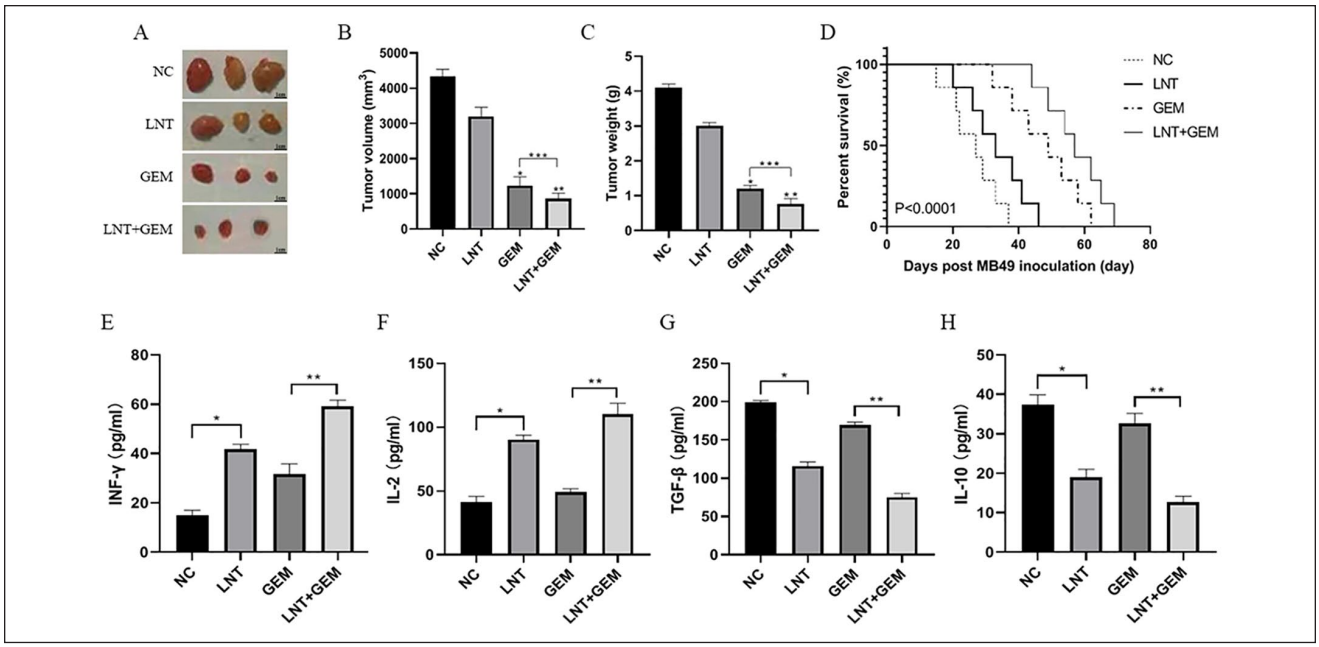

Figure 3. Effects of LNT, GEM, or LNT+GEM on tumor growth *in vivo* and expression of immune-related cytokines in tumor tissues. (A) After 5 weeks of treatment, the mice were sacrificed, and the tumor xenografts were excised and photographed (n = 3) (B) Tumor volume of tumor xenografts at the end of 5 weeks of therapy. *$P < .05$, GEM group vs. NC or LNT group; **$P < .05$, LNT+GEM group vs. NC or LNT group. ***$P < .05$, LNT+GEM group vs. GEM group. (C) Tumor xenograft weights at the end of 5 weeks of therapy. *$P < .05$, GEM group vs. NC or LNT group; **$P < .05$, LNT+GEM group vs. NC or LNT group. ***$P < .05$, LNT+GEM group vs. GEM group. (D) Survival curves for mice administered with different treatments (n = 7). Immunomodulatory effects of LNT on the expression of the cytokines IFN-γ (E), IL-2 (F), TGF-β (G), and IL-10 (H) in tumor tissues. *$P < .05$, compared with the control group; **$P < .05$, compared with the GEM treatment group.

LNT Synergized with GEM to Inhibit Tumor Growth in Mice

To examine the impact of LNT on tumor growth, we used the MB49 bladder cancer transplantation model established in C57BL/6 mice. It is noteworthy that after 5 weeks of treatment, the tumor-bearing mice treated with LNT or GEM alone showed significant tumor growth inhibition (Figure 3A). Within 5 weeks after the injection, the tumor growth was significantly inhibited, resulting in significant differences in tumor size and weight among the four groups on the day of sacrifice (Figure 3B and C). At the same time, the survival of the MB49 tumor-bearing mice was apparently prolonged by LNT or GEM treatment (Figure 3D). In addition, LNT synergized greatly with GEM to enhance the suppression of tumor growth and prolong the survival of the tumor-bearing mice ($P < .05$) (Figure 3).

Discussion

LNT is a polysaccharide isolated from *Lentinus edodes* that has been demonstrated to have a wide range of *in vitro* and *in vivo* bioactivities.[10] Pharmacological studies have shown that LNT has the potential to strengthen immune function by activating T cells and enhancing macrophage phagocytosis.[11] Due to its ability to reduce the side effects of radiotherapy and increase the survival of cancer patients over a 5-year follow-up period, LNT has been approved as an adjuvant treatment for gastric cancer and colon cancer.[12-14] Although the direct antitumor effects of LNT also have been demonstrated in animal models with either primary or implanted tumors, to the best of our knowledge, our data provide the first evidence of the immunotherapeutic potential of LNT in an animal model of bladder cancer.[15,16]

In this research, the increased proportions of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells as well as activated macrophages were confirmed in the splenocytes of MB49 tumor-bearing mice after LNT treatment. In the meantime, LNT induced reduced levels of Tregs and MDSCs. Besides, LNT significantly suppressed the expression levels of the anti-inflammatory cytokines IL-10 and TGF-β, whereas it stimulated the expression levels of the proinflammatory cytokines IFN-γ and IL-2. It was found that the subpopulations of both MDSCs and Tregs were upregulated in the tumor microenvironment, indicating their immunosuppressive role in the immune escape of tumors.[17] Previous studies also have demonstrated that IL-10 and TGF-β produced by MDSCs and Tregs can inhibit the proliferation and activity of T lymphocytes.[18,19] Furthermore, other reports have indicated that LNT can shift the cytokine activities of T helper (Th) cells toward

Th1 cells by altering the equilibrium between reductive and oxidative macrophages.[20] Therefore, it is reasonable to propose that in this bladder cancer model, LNT initially induced macrophage activation, then enhanced the proliferation of $CD4^+$ and $CD8^+$ T cells, and finally upregulated the expression of IFN-γ and IL-2.[21,22] Meanwhile, the percentages of MDSCs and Tregs were downregulated, leading to a reduction of the anti-inflammatory cytokines IL-10 and TGF-β.

It is well known that GEM has a significant inhibitory effect on solid tumors such as bladder cancer. Our research further confirmed that the application of LNT to assist GEM in the treatment of bladder cancer is significantly better than GEM monotherapy. This result may be attributed to the various biological mechanisms by which LNT affects tumor growth, including tumor cell apoptosis induction and tumor angiogenesis inhibition.[23-25] In addition, the safety profile of LNT and its ability to reduce the side effects of chemotherapy provide additional evidence for the development of LNT as an antitumor agent.[26,27] In view of the high costs of chemotherapy and molecularly targeted medications, LNT also has inherent cost-effectiveness benefits.[28]

There is growing evidence supporting the idea that chemotherapies and cancer immunotherapies can be synergized through modulating the activity of different immunocytes or the immunogenicity of cancer cells by immunotherapies as well as inducing immunogenic cell death by chemotherapies.[29] Recently, Galsky and colleagues have proven the efficacy of the combination of the immune checkpoint inhibitor ipilimumab plus GEM and cisplatin in patients with metastatic bladder cancer.[30] In this multicenter phase II study, patients demonstrated an objective response rate of 69% and a complete response rate of 17%. Wang et al also found that *Astragalus* polysaccharides could significantly inhibit the growth of melanoma in a mouse model by reducing the expression of programmed death-ligand 1 in B16-F10 cells.[31] Therefore, the role of LNT on the microenvironment of bladder cancer, especially the immune checkpoint pathways, will be investigated in our lab to explore the mechanism of LNT-induced antitumor immunity and its synergetic effects with chemotherapy.

Conclusion

In summary, LNT can shift the balance between T helper 1 (Th1) and T helper 2 (Th2) cytokine activities toward Th1. The synergy between LNT and GEM can inhibit the growth of bladder tumors in mouse model. As an immunomodulatory, LNT can improve the side effects of GEM treatment in order to achieve the best therapeutic effect.

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Authors' Contributions

MS and YC designed the study. RB and CL analyzed and interpreted the data. MS, BZ, and WZ performed the experiments and drafted the manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethics Approval and Consent to Participate

This study was approved by the Animal Care and Use Committee of China Medical University.

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Availability of Data and Materials

All data generated or analyzed during this study are available upon request.

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