Norcantharidin alleviates cyclophosphamide-induced immunosuppression via circBCL2L1/miR-30c-3-3p/TRAF6 axis

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Abstract

Cyclophosphamide is a widely used antitumor drug, with induced adverse effects, such as intestinal mucosal injury and immunosuppression. Norcantharidin possesses anticancer activity through enhancement of antitumor immunity. We investigated the role of norcantharidin in cyclophosphamide-induced immunosuppression. Mice were treated with cyclophosphamide, and exposed to norcantharidin. Enzyme-linked-immunosorbent serologic assay was performed to assess the levels of immunoglobulin and cytokines in serum, and the splenic T lymphocytes were analyzed by immunohistochemistry. Incubation with norcantharidin increased the serum levels of immunoglobulin G (IgG), interleukin (IL)-12, interferon-gamma (IFN-γ), and IL-6, and enhanced the percentage of CD4⁺ and CD8⁺ T lymphocytes in cyclophosphamide-induced mice. Expression of circBCL2L1 was down-regulated in the spleen of cyclophosphamide-induced mice, while up-regulated by norcantharidin incubation. Norcantharidin attenuated cyclophosphamide-induced up-regulation of miR-30c-3-3p and down-regulation of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) in mice. Over-expression of circBCL2L1 increased serum levels of immunoglobulin and cytokines, and enhanced the percentage of splenic CD4⁺ and CD8⁺ T lymphocytes in cyclophosphamide-induced mice. Moreover, over-expression of circBCL2L1 increased TRAF6 in cyclophosphamide-induced mice through down-regulation of miR-30c-3-3p. Knockdown of TRAF6 attenuated norcantharidin-induced increase of serum levels of IgG, IL-12, IFN-γ, and IL-6, and up-regulation of CD4⁺ and CD8⁺ T lymphocytes in cyclophosphamide-induced mice. Norcantharidin exhibited protective effect against cyclophosphamide-induced immunosuppression in mice through regulation of circBCL2L1/miR-30c-3-3p/TRAF6 axis.

Keywords: norcantharidin; cyclophosphamide; immunosuppression; circBCL2L1; miR-30c-3-3p; TRAF6

Introduction

Immune system is composed of lymphoid organs, including the spleen, lymph nodes, thymus, and bone marrow. These organs create lymphocytes and play an important role in immune response to counteract the effects of harmful stimuli (Nicholson, 2016). Healthy immune system exerts cytotoxic effects on malignant diseases, such as cancer, while the suppression of the immune system is associated with the progression of carcinomas (Whiteside, 2006). Immunomodulatory agents to restore the immune system are regarded as promising strategies to prevent cancers (Gonzalez et al., 2018).
Cyclophosphamide is widely used as an antineoplastic drug because of antireplicative and antimitotic properties (Emadi et al., 2009). Moreover, cyclophosphamide is also used in the treatment of organ transplants and autoimmune diseases through immunosuppressive effect (Ahllmann and Hempel, 2016). A previous study has shown that administration of cyclophosphamide reduced the proliferation of B and T cells, suppressed the activity of cytotoxic T lymphocyte and NK cells, and stimulated the imbalance of type 1 T helper/type 2 T helper (Th1/Th2) cells, thus resulting in sudden immunosuppression in tumor cells (Noh et al., 2019). Amelioration of cyclophosphamide-induced immunosuppression contributed to the clinical benefits of cyclophosphamide-inhibited cancers (Noh et al., 2019).

Norcantharidin is a demethylated compound of cantharidin that exerts anti-tumor effect in a variety of cancers (Chen et al., 2002). For example, norcantharidin stimulated mitochondrial-dependent apoptosis of prostate cancer (Lin et al., 2017), and inhibited tumor growth of glioma (Zheng et al., 2014). Norcantharidin also induced reduction of CD4+/CD25+Foxp3 T cell populations and M2 to M1 polarization in the microenvironment of hepatocellular carcinoma (Lu et al., 2014), and enhanced the antitumor immunity of prostate cancer through increased CD4+ and CD8+ T lymphocytes (Mo et al., 2018). However, the role of norcantharidin in cyclophosphamide-induced immunosuppression remains unknown.

The aim of this study was to investigate immunomodulatory effect of norcantharidin on cyclophosphamide-induced immunosuppressed mice model. The underlying mechanism might provide evidence for the clinical application of norcantharidin in combination therapy with cyclophosphamide to treat cancers.

**Materials and methods**

A total of 60 male Balb/c mice (Huafukang Biological Products Co. Ltd, Beijing, China) were housed in a facility under standard conditions. All experiments were approved by the Ethics Committee of Guizhou Provincial People's Hospital Guiyang, China in accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines. Mice (18–20 g, and 6–8 weeks old) were divided into two groups: control group (n = 6) and cyclophosphamide group (CY; n = 54). Mice in the control group were intraperitoneally injected with normal saline (Sigma-Aldrich, St. Louis, MO, USA) for 10 days. Mice of the cyclophosphamide group were further divided into the following nine groups: cyclophosphamide (CY; n = 6); CY+1-mg/kg norcantharidin (NCTD; n = 6); CY+2-mg/kg NCTD (n = 6); CY+4-mg/kg NCTD (n = 6); CY+4-mg/kg NCTD+sh-NC (n = 6); CY+4 mg/kg NCTD+sh-circBCL2L1 (n = 6); and CY+4-mg/kg NCTD+sh-TRAF6 (n = 6). Mice in the CY group were intraperitoneally injected with 80-mg/kg cyclophosphamide on days 1, 2, 3, and 9 and with normal saline on days 4, 5, 6, 7, 8, and 10 according to a previous study (Yan et al., 2021). Mice in CY+NCTD were intraperitoneally injected with 80-mg/kg cyclophosphamide on days 1, 2, 3, and 9, and with different concentrations of norcantharidin (Sigma-Aldrich) on day 4, 5, 6, 7, 8, and 10. Mice in CY+vector or CY+OE-circBCL2L1 group were intraperitoneally injected with 80-mg/kg cyclophosphamide on day 1, 2, 3, and 9, and with plasmid cloning DNA (pcDNA) empty vector or pcDNA-circBCL2L1 (OE-circBCL2L1) in the tail vein every 2 days for 10 days. Mice in the CY+4-mg/kg NCTD+sh-NC, CY+4-mg/kg NCTD+sh-circBCL2L1, or CY+4-mg/kg NCTD+sh-TRAF6 groups were intraperitoneally injected with 80-mg/kg cyclophosphamide on day 1, 2, 3, and 9; with 4-mg/kg norcantharidin on day 4, 5, 6, 7, 8, and 10; and with sh-NC, sh-circBCL2L1, or sh-TRAF6 in the tail vein every 2 days for 10 days. The pcDNA vectors and small hairpin RNAs (shRNAs) were packaged with lentivirus obtained from RiboBio (Guangzhou, China), and mice were injected with 9×10⁶ international units (IU) of the virus.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

Mice were sacrificed 24 h after the last intraperitoneal injection. The spleen of each mouse was isolated and lysed using TRIzol kit (Life Technologies, Carlsbad, CA, USA) or miRcute microRNA (miRNA) isolation kit (Tiangen, Beijing, China) to isolate total RNAs or small RNAs. The isolated RNAs were synthesized into complementary DNA (cDNAs), and the messengerRNA (mRNA) expressions of circBCL2L1, miR-30c-3-3p, and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) were detected by SYBR Premix Ex Taq (Takara, Dalian, Liaoning, China) or miRcute miRNA qPCR detection kit (Tiangen) according to a previous study (Yan et al., 2021). The following primers were used in this study: circBCL2L1 forward: 5'-GCTGGGAGAGGGGTGT-3' and reverse: 5'-AGGCTTTCTCCCTGCGTC-3', miR-30c-3-3p forward: 5'-GCCGAGAGGAGGTGTTG-3' and reverse: 5'-TCCAGTTTTTTTTTTTTTTTTTTTTTTTT-3', and TRAF6 forward: 5'-ATGATGGAAAAGGAACGGGAAATG-3' and reverse: 5'-GCTGACAGGGAACCTTTAG-3'. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: 5'-ACCTTTCACCTCCTCATCTTT-3', reverse: 5'-AGGTCAACAGACACGTTGTTG-3', and 5.8S rRNA forward: 5'-AUCTCTAGGCGTGTA-3'.
Results

Norcantharidin-mediated circBCL2L1, miR-30c-3-3p, and TRAF6 in cyclophosphamide-induced mice

In order to evaluate immunomodulatory role of norcantharidin, cyclophosphamide-induced immunosuppressive mice were exposed to norcantharidin. Expression of circBCL2L1 was significantly down-regulated in the spleen of cyclophosphamide-induced mice ($P < 0.001$) (Figure 1A), and norcantharidin significantly induced up-regulation of circBCL2L1 in cyclophosphamide-induced mice through a dosage-dependent way ($P < 0.001$) (Figure 1A). Cyclophosphamide significantly increased expression of miR-30c-3-3p ($P < 0.001$) (Figure 1B), while and decreased expression of TRAF6 (Figure 1C) in the mice. However, norcantharidin significantly reduced miR-30c-3-3p ($P < 0.001$) (Figure 1B) and enhanced TRAF6 (Figure 1C) in cyclophosphamide-induced mice. The protein expression of TRAF6 in cyclophosphamide-induced mice was also up-regulated by norcantharidin (Figures 1D1 and D2), suggesting that norcantharidin might mediate cyclophosphamide-induced immunosuppression through circBCL2L1/miR-30c-3-3p/TRAF6 axis.

circBCL2L1 enhanced serum levels of immunoglobulin and cytokines in cyclophosphamide-induced mice

In order to investigate the role of circBCL2L1/miR-30c-3-3p/TRAF6 in cyclophosphamide-induced immunosuppression, mice were injected with OE-circBCL2L1, which increased the expression of circBCL2L1 in the spleen of cyclophosphamide-induced mice (Figure 2A). Over-expression of circBCL2L1 significantly enhanced serum levels of IgG (Figure 2B1), IL-12 (Figure 2B2), IFN-γ (Figure 2B3), and IL-6 (Figure 2B4) in immunosuppressive mice ($P < 0.001$). Moreover, the percentage of CD4$^+$ and CD8$^+$ T lymphocytes in immunosuppressive mice were also enhanced by over-expression of circBCL2L1 (Figure 2C), demonstrating that circBCL2L1 alleviated immunosuppression in cyclophosphamide-induced mice.

circBCL2L1 regulated miR-30c-3-3p and TRAF6

Over-expression of circBCL2L1 significantly down-regulated expression of miR-30c-3-3p ($P < 0.001$) (Figure 3A) and up-regulated TRAF6 (Figure 3B1 and B2) in cyclophosphamide-induced mice. Moreover, immunohistochemical analysis also confirmed the increase of TRAF6 in the spleen of immunosuppressive mice injected with OE-circBCL2L1 (Figure 3C).

Enzyme-linked-immunosorbent serologic assay (ELISA)

The blood samples of mice were harvested by extracting the eyeballs and centrifuged at 1,500 rpm for 30 min to isolate the serum. Levels of immunoglobulin G (IgG), interleukin(IL)-12, interferon-gamma (IFN-γ), and IL-6 were detected by ELISA kits (Lianke Biotechnology Co. Ltd., Hangzhou, China) as mentioned in a previous study (Yan et al., 2021).

Immunohistochemistry

The spleen of each mouse was fixed in 4% paraformaldehyde and embedded in paraffin. The tissues were then cut into 5-µm slices, and the slices were probed with specific antibodies against mouse CD3+, CD4+, or CD8+ (BioLegend, San Diego, CA, USA). The sections were incubated with horseradish peroxidase-conjugated secondary antibody (BioLegend), and then treated with 3,3-diaminobenzidine. Sections were stained with 4′,6-diamidino-2-phenylindole (DAPI), and observed under microscope (Olympus, Tokyo, Japan) according to a previous study (Delcambre, Liu, Herrington, Vallario, & Long, 2016).

Western blot analysis

The spleen of mice was lysed by radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich), and the protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Samples were transferred onto nitrocellulose membranes, and the membranes were blocked in 5% bovine serum albumin (BSA). Membranes were probed with the following specific antibodies: anti-TRAF6 and anti-GAPDH (1:2,000; BioLegend), and anti-CD3$^+$, anti-CD4$^+$, and anti-CD8$^+$ (1:3,000; BioLegend). The membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:4,000), and treated with enhanced chemiluminescence (Sigma-Aldrich) to detect immunoreactivities.

Statistical analysis

All the data with at least triple replicates were expressed as mean ± SEM. Student's $t$-test or one-way analysis of variance was used to determine statistical significance of the differences between various groups using the SPSS software. $P < 0.05$ was considered as statistically significant.
Figure 1. Norcantharidin mediated circBCL2L1, miR-30c-3-3p, and TRAF6 in cyclophosphamide-induced mice. (A) Norcantharidin attenuated cyclophosphamide-induced decrease of circBCL2L1 in the spleen of mice. (B) Norcantharidin attenuated cyclophosphamide-induced increase of miR-30c-3-3p in the spleen of mice. (C) Norcantharidin attenuated cyclophosphamide-induced decrease of TRAF6 mRNA in the spleen of mice. (D) Norcantharidin attenuated cyclophosphamide-induced decrease of TRAF6 protein in the spleen of mice. ***P < 0.001 vs. control. ###P < 0.001 vs. CY (cyclophosphamide). NCTD: norcantharidin.

Figure 2. circBCL2L1 enhanced serum levels of immunoglobulin and cytokines in cyclophosphamide-induced mice. (A) Injection with OE-circBCL2L1 increased expression of circBCL2L1 in the spleen of cyclophosphamide-induced mice. (B) Over-expression of circBCL2L1 enhanced serum levels of IgG (B1), IL-12 (B2), IFN-γ (B3), and IL-6 (B4) in immunosuppressive mice. (C) Over-expression of circBCL2L1 enhanced the percentage of CD4+ and CD8+ T lymphocytes in immunosuppressive mice. ***P < 0.001 vs. CY+vector.
Figure 3. circBCL2L1 regulated miR-30c-3-3p and TRAF6. (A) Over-expression of circBCL2L1 down-regulated expression of miR-30c-3-3p in cyclophosphamide-induced mice. (B) Over-expression of circBCL2L1 up-regulated TRAF6 protein in cyclophosphamide-induced mice. (C) Immunohistochemical analysis confirmed that over-expression of circBCL2L1 up-regulated TRAF6 protein in cyclophosphamide-induced mice. **P < 0.001 vs. CY+vector.

Norcantharidin mediated miR-30c-3-3p and TRAF6 through circBCL2L1

Immunosuppressive mice were injected with sh-circBCL2L1 to investigate effects of norcantharidin/circBCL2L1 on miR-30c-3-3p and TRAF6. Knockdown of circBCL2L1 significantly attenuated norcantharidin-induced increase of circBCL2L1 (P < 0.001) (Figure 4A), decrease of miR-30c-3-3p (Figure 4B), and up-regulation of TRAF6 (Figures 4C, D1, and D2) in the spleen of immunosuppressive mice, indicating that norcantharidin decreased miR-30c-3-3p and increased TRAF6 through up-regulation of circBCL2L1 in immunosuppressive mice.

Norcantharidin attenuated immunosuppression in cyclophosphamide-induced mice through up-regulation of TRAF6

Immunosuppressive mice were injected with sh-TRAF6 to investigate effects of norcantharidin/circBCL2L1/miR-30c-3-3p/TRAFL6 on immunosuppression. Incubation with norcantharidin enhanced serum levels of IgG (Figure 5A1), IL-12 (Figure 5A2), IFN-γ (Figure 5A3), and IL-6 (Figure 5A4) in cyclophosphamide-induced mice, revealing the protective effect of norcantharidin against cyclophosphamide-induced immunosuppression. However, knockdown of TRAF6 attenuated norcantharidin-induced increase of IgG (Figure 5A1), IL-12 (Figure 5A2), IFN-γ (Figure 5A3), and IL-6 (Figure 5A4) in immunosuppressive mice. Additionally, knockdown of TRAF6 also attenuated norcantharidin-induced increase of CD3, CD4, and CD8 proteins in immunosuppressive mice (Figures 5B1–B4), revealing that norcantharidin up-regulated TRAF6 to attenuate cyclophosphamide-induced immunosuppression.

Discussion

Norcantharidin has been demonstrated to induce shift of M2 tumor-associated macrophages into M1 (Lu et al., 2014), and inhibit proliferation of CD4+/CD25+/Foxp3 T cells (Mo et al., 2018) to enhance antitumor immunity. Cyclophosphamide interfered with differentiation and proliferation of macrophages (Liu et al., 2020), and induced increase of CD4+/CD25+/Foxp3 T cells (Audia et al., 2007) to induce immunosuppression. This study found that norcantharidin inhibited cyclophosphamide-induced immunosuppression through increase of percentage of CD4+ and CD8+ T lymphocytes.

Immune organs, such as the thymus and spleen, are responsible for maturation and differentiation of lymphocytes. Cyclophosphamide reduces the indices of the thymus and spleen and inhibits the immune function (Chen et al., 2006). Therefore, cyclophosphamide-induced mice was widely used as immunosuppressive model through inhibition of lymphocyte proliferation and down-regulation of immunoglobulins and cytokines (Yan et al., 2021). In this study, we also established immunosuppressive mice through incubation with cyclophosphamide. Th1 cells secrete pro-inflammatory cytokines,
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Figure 4. Norcantharidin mediated miR-30c-3-3p and TRAF6 through circBCL2L1. (A) Knockdown of circBCL2L1 attenuated norcantharidin-induced increase of circBCL2L1 in the spleen of immunosuppressive mice. (B) Knockdown of circBCL2L1 attenuated norcantharidin-induced decrease of miR-30c-3-3p in the spleen of immunosuppressive mice. (C) Knockdown of circBCL2L1 attenuated norcantharidin-induced increase of TRAF6 mRNA in the spleen of immunosuppressive mice. (D) Knockdown of circBCL2L1 attenuated norcantharidin-induced increase of TRAF6 protein in the spleen of immunosuppressive mice. ***P < 0.001 vs. CY. ###P < 0.001 vs. CY+NCTD+sh-NC.

IFN-γ and IL-2, while Th2 cells secrete anti-inflammatory cytokines, IL-4 and IL-10. Cyclophosphamide injection stimulated increase of IL-4 and IL-10, while reduced IFN-γ and IL-2, to induce immunosuppression (Qi et al., 2018). Suppression of IL-4 and IL-10, and Promotion of IFN-γ and IL-2 attenuated cyclophosphamide-induced immunosuppression (Qi et al., 2018). Moreover, increase of lymphocyte proliferation and numbers of CD4+ and CD8+ T lymphocytes also alleviated the cyclophosphamide-induced immunosuppression (Qi et al., 2018). A previous study has shown that norcantharidin increased levels of pro-inflammatory cytokine, IL-12, and decreased IL-10 to enhance the antitumor effect (Lu et al., 2014). Furthermore, norcantharidin enhanced numbers of CD4+ and CD8+ T lymphocytes (Mo et al., 2018). Results of this study demonstrated that norcantharidin up-regulated serum levels of IgG, IL-12, IFN-γ, and IL-6, and enhanced the expressions of CD3, CD4, and CD8 proteins in cyclophosphamide-induced mice, thereby exerting protective effect against immunosuppression.

Emerging evidence has established that circRNAs were associated with physiological functions of various diseases, including immune diseases (Fang et al., 2021). For example, circMET promoted resistance of hepatocellular carcinoma to anti-PD1 therapy and induced immunosuppression through targeting miR-30-5p (Huang et al., 2020). It has been reported that circBCL2L1, as the ceRNA of miR-30c-3-3p, promoted expression of TRAF6 to enhance antiviral and antibacterial signaling and maintain the homeostasis of innate immune system (Zheng et al., 2021). This study determined that levels of miR-30c-3-3p increased, while that of circBCL2L1 and TRAF6 decreased in the spleen of immunosuppressive mice. However, norcantharidin reduced miR-30c-3-3p and enhanced circBCL2L1 and TRAF6 in immunosuppressive mice. Overexpression of circBCL2L1 enhanced the expression of TRAF6 through down-regulation of miR-30c-3-3p. Moreover, knockdown of circBCL2L1 attenuated norcantharidin-induced decrease of miR-30c-3-3p and increase of circBCL2L1 and TRAF6 in immunosuppressive mice, demonstrating that norcantharidin/circBCL2L1/miR-30c-3-3p/TRAF6 axis was associated with cyclophosphamide-induced immunosuppression.

Functional assays in this study demonstrated that overexpression of circBCL2L1 inhibited cyclophosphamide-induced immunosuppression through up-regulation of serum levels of IgG, IL-12, IFN-γ, and IL-6, and promotion of percentage of CD4+ and CD8+ T lymphocytes. TRAF6 has been identified as an ubiquitin E3 ligase to intermediate various receptors and activation of downstream Nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, thus regulating immune responses (Dainichi et al., 2019). Moreover, TRAF6-mediated signaling, including PI3K, T-cell receptor, toll-like receptor, interferon regulatory
factor, and MAPKs, plays critical roles in development, homeostasis, and activation of various immune cells as well as immune tolerance (Walsh et al., 2015). TRAF6 was associated miRNA-mediated immunosuppression (Qiu et al., 2015), and cyclophosphamide reduced TRAF6 expression in immunosuppressive mice (Yun et al., 2018). Results of this study indicated that knockdown of TRAF6 attenuated norcantharidin-induced increase of serum levels of IgG, IL-12, IFN-γ, and IL-6, and up-regulation of CD3, CD4, and CD8 proteins in immunosuppressive mice, indicating that norcantharidin attenuated cyclophosphamide-induced immunosuppression through up-regulation of TRAF6.

**Conclusion**

Collectively, this study provided the evidence that norcantharidin improved immune functioning through increase of CD4+ and CD8+ T lymphocytes and up-regulation of IgG, IL-12, IFN-γ, and IL-6 in cyclophosphamide-induced immunosuppressive mice. This study established that norcantharidin might be a potent immunomodulatory agent in patients with chemotherapy-induced immunosuppression through regulation of circBCL2L1/miR-30c-3p/TRAF6. However, the role of norcantharidin/circBCL2L1/miR-30c-3p/TRAF6 in humoral and cellular immune functions in cyclophosphamide-induced immunosuppressive mice treatment needs further investigation.
imunosuppressive mice should be investigated in the future research.

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**Competing interests**

The authors had no conflicts of interest to disclose.

**Data availability**

The authors declare that all data supporting the findings of this study are available in the paper, and any raw data are obtained from the corresponding author upon request.

**Author Contributions**

Guochuan Wang and Yali Zhang designed the experiments and Xiaolu Zhou carried them out. Mei Yang analyzed and interpreted the data. Xiaoyu Ma and Xin Liu prepared the manuscript with contributions from all co-authors. All authors have read and approved the final manuscript.

**References**


