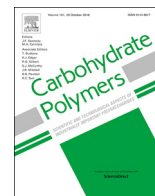




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Sulfated polymannuroguronate TGC161 ameliorates leukopenia by inhibiting CD4⁺ T cell apoptosis



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ABSTRACT

Polysaccharides have aroused considerable interest due to their diverse biological activities and low toxicity. In this study, we evaluated the effect of marine polysaccharide sulfated polymannuroguronate (TGC161) on the leukopenia induced by chemotherapy. It is found that TGC161 ameliorates the leukopenia. Besides, TGC161 would promote CD4⁺ T cell differentiation and maturation in the thymus, but does not have a significant effect on precursor cells in bone marrow. Furthermore, TGC161 inhibits CD4⁺ T cell apoptosis *in vitro*. Collectively, our findings offer a natural and harmless polysaccharide to ameliorate leukopenia.

1. Introduction

Leukopenia patients have a reduced number of white blood cells in their blood stream, which may be caused by different conditions, including viral infection, cancer, genetic and medication conditions. These patients are taking a higher risk of infections, especially for hospital infections and conditional pathogen infections (Christen, Brummendorf, & Panse, 2017). Although many other medical issues usually should be addressed before the white blood cell count can return to normal, medications would be used immediately to boost white blood cells when the count of white cells is very low. This requirement often happens in chemotherapies and severe viral infections. Granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), vitamin B4 and alkylglycerols are the most often used medications to treat leukopenia, which are named as neupogen or leupogen dependent on their functional roles (Iannitti & Palmieri, 2010; Mehta, Malandra, & Corey, 2015; Tomita et al., 2016). However, doctors might decide to stop or delay the treatment instead of administration of these drugs for leukopenia, because of the undergoing side effects of colony stimulating factors, such as fever, bone pain and nausea, or the uncertain outcomes of vitamin B4 and alkylglycerols, if the leukopenia is caused by medications. A more promised medication

is required here.

Cancer is the second leading cause of deaths all over the world (Sultana, Asif, Nazar, Akhtar, & Rehman, 2014), and chemotherapy is still the mostly often used treatment to kill fast-growing cancer cells. It believed that chemotherapy directly destroy tumor cells and has less impact on normal cells, since cancer cells grow and multiply quicker than most cells in the body, but it is undoubted that chemotherapy is also killing other normal cells, particularly for bone marrows and white blood cells, which reduces the number of white blood cells, and was named as chemotherapy induced leukopenia (Nieweg & Van, 1992). Recently, it is found that chemotherapy triggers host immune responses to tumor cells which is essential for tumor killing (Hodge et al., 2013). Herein, leukopenia patients not only bear an increased risk of infection, but also present an unsatisfied outcome during chemotherapies. Medications should be taken to boost white blood cells and tumor cell killing. Nevertheless, colony stimulating factors might make the condition even worse in rare cases. For example, administration of GM-CSF might promote bone metastatic in breast cancer and prostate cancer (Dai et al., 2010). Doctors are also hesitating to use colony stimulation factors because of the risk of over-enhancement of bone marrow cell differentiation (Potosky et al., 2011). Lentinan, an expensive polysaccharides derived from mushrooms in *Lentinus* family, was an

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alternative for some patient (Ren, Perera, & Hemar, 2012; Wasser, 2002). Unfortunately, the responses to lentinan in patients are also unpredictable.

Reduced number of peripheral white blood cells is also a typical symptom in viral infections, such as influenza A infection, coronavirus infection and human immunodeficiency virus (HIV) infection. Since HIV infects CD4⁺ T cells, it induces cellular apoptosis / pyroptosis and leads to immune exhaustion (Doitsh et al., 2014; Selliah & Finkel, 2001). In few of HIV positive patients, viral genomic RNA is nearly undetectable in serum, but the counts of white blood cells are maintaining on a very low level (Omondi et al., 2019; Shen et al., 2015). Herein, drugs for leukopenia, G-CSF, interleukin-12 and others, would be suggested (Maeda, Das, Kobayakawa, Tamamura, & Takeuchi, 2019). Since the underlying mechanism of the chronic reduced CD4⁺ T cells is still unclear, these medications might not give expectable outcomes.

Polysaccharides have aroused considerable interest due to their immunity-enhancing activities (Li et al., 2020; Liu et al., 2016; Su et al., 2019). It is reported that a polysaccharide derived from *Rehmannia glutinosa* significantly stimulates lymphocyte proliferation (Huang et al., 2013). EPS1-1, another polysaccharide from the liquor of *Rhizopus nigricans*, stimulates lymphocyte proliferation and the phagocytic function of peritoneal macrophage to enhance immunity (Yu, Kong, Zhang, Sun, & Chen, 2016). Sulfated polymannuroguluronate (SPMG), a heparin-like sulfated polysaccharide extracted from brown algae, could enhance T cell responses either with or without Concanavalin A stimulation (Miao, Li, Fu, Ding, & Geng, 2005). Here, we found TGC161 which is similar to SPMG, ameliorates leukopenia in the chemotherapy model. The mechanism is that TGC161 promotes CD4⁺ T cell differentiation and maturation in thymus, but has no significant effect on precursor cells in bone marrow. Moreover, TGC161 also inhibits CD4⁺ T cell apoptosis *in vitro*.

2. Materials and methods

2.1. Structural analysis

TGC161 was prepared following the published sulfated polymannuroguluronate preparation procedure with minor modifications (Geng et al., 2003). Briefly, alginate powder was hydrolyzed in 0.5 mol/L HCl at 100 °C for 8 h. Subsequently, the sulfated alginate was prepared by treatment with 0.5 mol/L chlorosulfonic acid for 3 h at 70 °C. The solution was neutralized using 2 mol/L NaOH, and then precipitated with ethanol, and dried. The structure of TGC161 was identified by nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FT-IR) analysis. The ¹H-NMR (500 MHz), ¹³C-NMR (125 MHz), ¹H-¹H correlated spectroscopy (COSY), heteronuclear single quantum correlation (HSQC) and total correlated spectroscopy (TOCSY) of the TGC161 were recorded at 298 K on an Agilent DD2-500 500 MHz spectrometer (Agilent, Santa Clara, US). The FT-IR spectrum was recorded on a Nicolet Nexus 470 FT-IR spectrophotometer (GMI, Ramsey, US) in KBr pellets over a wavelength range of 400-4000 cm⁻¹. The weight-average molecular weight (Mw) was determined by high performance gel permeation chromatography (HPGPC) using a TSK-Gel GMPW column (13 mm, 7.8 mm × 300 mm) on an ultimate 3000 high performance liquid chromatography instrument.

The sulfate content analysis was performed according to the previous method (Xue et al., 2016). In brief, TGC161 was prepared by the oxygen flask combustion (OFC) method. Then, total sulfate content and free sulfate content of TGC-161 were analyzed by ion chromatography. A series of sulfate standard solutions were measured at the same time to generate a standard calibration curve. The concentration of free sulfate and total sulfate could be calculated according to this standard calibration curve. The sulfate content % = $(C_{\text{total sulfate}} - C_{\text{free sulfate}}) / C_{\text{TGC161}} \times 100\%$; $C_{\text{total sulfate}}$, $C_{\text{free sulfate}}$ and C_{TGC161} represented the concentration of total sulfate, free sulfate and TGC161, respectively.

Monosaccharide composition of TGC161 was determined using a 1-phenyl-3-methyl-5-pyrazolone (PMP) pre-column derivatization HPLC method (Wu, Zhao, Ren, Xue, & Guan, 2014, Wu, Zhao, Ren, Xue, Li et al., 2014). The PMP-labeled carbohydrates were separated by a BDS-C18 column (4.6 mm × 250 mm, 5 μm, USA) with 0.1 mol/L phosphate buffer (pH 6.0) and acetonitrile at a ratio of 83:17 (v/v, %) as a mobile phase at a flow rate of 0.8 mL/min.

2.2. Mice and cells

Seven-week-old male C57BL/6 mice were purchased from the Southern Animal Model Center (Shanghai, China). All animals were maintaining in a SPF animal facility in School of Medicine and Pharmacy, Ocean University of China. Mice were housed under the room humidity (55 ± 5%) and temperature (22 ± 2 °C) with free access to water and diets. Mice were injected intraperitoneally with a single dose of carboplatin (60 mg/kg, Selleckchem, Houston, US). TGC161 (400 mg/kg) and alkylglycerols (22.5 mg/kg, Peng-Yao, Yixing, China) were administrated by gavage once a day. All of the procedures were approved by the Committee of Experimental Animals of the Ocean University of China and conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No 85-23, revised 1996).

Spleen and thymus tissues were isolated from 7-week-old C57BL/6 male mice. These tissues were ground into the cell suspension and slowly filtered through 75 μm nylon cell strainers (Corning, Corning, US). All cells were cultured in RPMI 1640 medium (Gibco, Grand Island, US) supplemented with 10 % fetal bovine serum and 100 U/mL penicillin/streptomycin at 37 °C and 5% CO₂ for 2 h. The culture supernatant was slowly aspirated and centrifuged to obtain cell depositions. Acquired cell depositions were re-suspended with fresh medium and planted in 24-well culture plates for 12 h. The above cell cultivation method refers to the Lefort study (Lefort & Kim, 2010). TGC161 was distilled into cell culture medium and filtered through a 0.22 μm membrane (Merk Millipore, Darmstadt, DE) for experimental use. In some experiments, TGC161 (1, 10, 100 μg/mL) was added in thymus cell culture medium, respectively. Harvested cells were saved for following experiments.

2.3. Cytotoxicity assay

Cytotoxicity of TGC161 was evaluated by the resazurin based assay (Sigma-Aldrich, Darmstadt, GER). 293 T (Homo sapiens embryonic kidney) cells were treated with TGC161 at different concentrations (1000, 500, 250, 125, 62.5, 31.3 μg/mL) for 48 h. The absorbance (A) at 544/595 nm was measured using a SpectraMax M3 reader (Molecular Devices, Sonny Wilber, US).

2.4. Blood cell density assay

The experimental mice were euthanized using CO₂. Peripheral blood cell density was analyzed using the ProCyteDx Hematology Analyzer (IDEXX, Westbrook, US) following manufacture's instruction.

2.5. Flow cytometry

All cells were harvested and washed with PBS (Servicebio, WuHan, China), and then stained with flow antibodies. All cells were treated with anti-FcγR III/II (Fc blocking, BD Biosciences, Franklin Lakes, US) in PBS for 30 min. CD3e-FITC was purchased from BD Biosciences (Franklin Lakes, US). CD4-PE, CD8a-APC-Vio770 and CD34-FITC were purchased from Miltenyi Biotec (Bergisch Gladbach, GE). CD19-APC, CD11b-PE-Cyanine7 and CD117-eFluor 450 were purchased from eBioscience (San Diego, US). Ly-6G-Brilliant Violet 510™, CD135-APC, FCgr-Brilliant Violet 510™ and CD127-APC/Cyanine7 were purchased from BioLegend (San Diego, US). All cells were loaded into a BD Aria III

Flow Cytometer (BD Bioscience, Franklin Lakes, US) with 500 μ L PBS suspending. The results were analyzed using FlowJo software (Stanford University, Palo alto, US).

2.6. Complement-dependent cytotoxicity assay

The experiment was performed as previous described (Kourtzelis & Rafail, 2016). Briefly, mice were injected intraperitoneally with CD4 monoclonal antibody (150 μ g/per mouse, BD Bioscience, Franklin Lake, US). Fifteen days later, less than 0.5 % of CD4⁺ T cells in peripheral blood indicated that the model was constructed successfully. CD4⁺ T cells in peripheral blood were detected after continuous gavage administration of TGC161 (400 mg/kg) for 15, 20, and 25 days.

2.7. Cell proliferation and apoptosis analysis

Thymus cells were isolated from C57BL/6 mice and cultured according to the above method in "mice and cells". All cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFDA SE, Beyotime, Shanghai, China) and seeded into 24-well plate for 12 h. Cells were collected and stained with CD4-PE (Miltenyi, Bergisch Gladbach, GER) antibody to detect proliferation by flow cytometry.

Cells were collected and stained with CD4-PE (Miltenyi, Bergisch Gladbach, GER) antibody. AnnexinVand PI were applied (Vazyme Biotech, Nanjing, China) to detect CD4⁺ T cell apoptosis by flow cytometry.

2.8. Western blotting

Cells were harvested and lysed in the homogenization buff ;er (50 mM Tris pH 7.6, 150 mM NaCl, 0.5 % Triton X-100, 1 mM Na₃VO₄, 10 mM NaF, 5 mM Na-pyrophosphate, 10 mM β -glycerophate, PMSF and protease inhibitor cocktails). Equal amounts of total protein were subjected to SDS-PAGE and transferred onto PVDF membranes. After blocking with 5% milk for 1 h at room temperature, membranes were incubated with specific anti-Bcl2 (cat# 15071, 1:1000), anti-caspase 3 (cat# 9665, 1:1000), anti-caspase 8 (cat# 9746, 1:1000) and anti- β -Actin (cat# 3700, 1:1000) from Cell Signaling Technology (Massachusetts, China) overnight at 4 °C. Then the membranes were washed by TBST and immunoblotted with HRP-conjugated secondary antibodies for 1 h at room temperature. All membranes were visualized by using the ECL western blotting reagent (Tanon, Shanghai, China).

2.9. Statistic analysis

The data were presented as mean \pm standard error of the mean (mean \pm SEM). One-way ANOVA was used for comparisons (SPSS 13.0 software) and the difference was considered statistically significant at $P < 0.05$.

3. Results

3.1. Structural characterization of TGC161

The TGC161 is a sulfated polysaccharide prepared by chemical sulfation of low molecular-weight alginate. It is composed of a central backbone of sulfated poly-D-mannuronic acid (M) and sulfated poly-L-guluronic acid (G) (Fig. 1A). TGC161 had no significant inhibition on the growth of 293 T cells at the concentration range of 31.3–1000 μ g/mL (Fig. 1B). The single peak in gel chromatography analysis suggested that the isolated TGC161 is homogeneous (Fig. 1C). The molecular weight (Mw) of TGC161 is 10 kDa as determined by HPGPC (as shown in Table 1). The degree of polymerization (DP) of TGC161 is about 20–30 basing on the molecular weight. The sulfate contents of TGC161 was about 28 %, indicating 0.9 average sulfation in every monosaccharide residue. The ratio of guluronic acid (G) to mannuronic acid

(M) in TGC161 is about 1:7.2. It is calculated according to monosaccharide composition analysis in Fig. 1D. M/G ratio of TGC161 was calculated by the following formula. M/G ratio = Peak area of M / Peak area of G (Wu, Zhao, Ren, Xue, Guan et al., 2014, Wu, Zhao, Ren, Xue, Li et al., 2014).

The structure of TGC161 was established by FT-IR. As shown in Fig. 1C, the bands at 1619 and 1413 cm^{-1} sites are attributed to the asymmetric and symmetric valence vibrations of a carboxyl group (COO^-), respectively. The bands at 895 and 837 cm^{-1} are attributed to the characteristic $\delta\text{C1-H}$ of α -guluronic acid residue and the characteristic $\delta\text{C1-H}$ of β -mannuronic acid residue, respectively. Moreover, the absorption band of 1248 cm^{-1} is attributed to the asymmetric S=O stretching vibration. The band at 1033 cm^{-1} is ascribed to the C–O vibration absorption peaks.

To further verify the structure information of TGC161, NMR analysis was utilized (Fig. S1–S6). The ¹H-NMR and ¹³C NMR data assignments of TGC161 were given in Table 2. The signals at 69.0/4.23, 67.0/4.47, 69.0/3.90 and 71.3/3.65 are attributed to M2, M3, G2, and G3 of TGC161. The signals of 77.3/4.54 (M2-O-S), 73.6/4.78 (M3-O-S), 73.7/3.95 (G2-O-S) and 77.3/3.87 (G2-O-S) indicated that the C2-OH and C3-OH were sulfated. The correlative signal analysis of each hydrogen with carbon in the HSQC spectrum indicated that the carbon and hydrogen signals were ascribed properly (Fig. 1E).

3.2. TGC161 reverses the leukopenia induced by chemotherapy

Mice were injected intraperitoneally with carboplatin to induce leukopenia, and TGC161 was delivered by gavage daily since the next day of carboplatin treatment. Mostly, leukopenia was observed since the third day post carboplatin injection. Here, cell densities of leukocyte and lymphocyte were elevated on the third day after TGC161 treatment (Fig. 2A, B and C). Alkylglycerols which was clinically used in leukopenia patients to promote counts of white blood cell (Oh & Jadhav, 1994), was introduced as a positive control. Noticeably, TGC161 promoted lymphocyte cell density, while alkylglycerols increased neutrophils (Fig. 2B and D). Compared with alkylglycerols, TGC161 increased lymphocyte numbers more efficiently which induced a considerable boost at the third day post treatment (Fig. 2A), but it only slightly increased the percentage of lymphocytes compared with carboplatin treated mice (Fig. 2D). TGC161 didn't promote neutrophils significantly in carboplatin treated mice (Fig. 2A, E). Both alkylglycerols and TGC161 increase lymphocytes to ameliorate leukopenia on the sixth day post administration (Fig. 2F, J). However, TGC161 was able to increase lymphocyte numbers more efficiently because a significant boost was found at third day post treatment in TGC161 treated mice but not in alkylglycerols treated mice. This may be due to the different mechanisms of two drugs. TGC161 ameliorates chemotherapy induced leukopenia by increasing lymphocytes which is essential for tumor cell killing (Ostroumov, Fekete Drimusz, & Woller, 2018; Yuen, Demissie, & Pillai, 2016). Mostly, neutrophils defend against bacterial infection in the body (Yang, Ghose, & Ismail, 2013).

3.3. TGC161 increases CD3⁺CD4⁺ T cells in peripheral blood

To clarify the effect of TGC161 on specific subtype of lymphocytes, cells were stained with different monoclonal fluorescent monoclonal antibodies in FACS analysis. The surface-differentiated molecule CD3 is the hallmark of mature T lymphocytes which were divided into two populations by the cellular markers, CD4 and CD8 (Masopust & Schenkel, 2013). CD4 T cells are helper T population expressing both CD3 and CD4 (Zhou, Chong, & Littman, 2009). CD8 T cells which function in killing target cells are cytotoxic population expressing both CD3 and CD8 (Gerritsen & Pandit, 2016). Consistent with previous studies (Verma, Foster, Horgan, Hughes, & Carter, 2016), T cells (CD3⁺T cells) reduced significantly in cell counts and percentage after carboplatin administration (Fig. 3A). Surprisingly, we found that total T

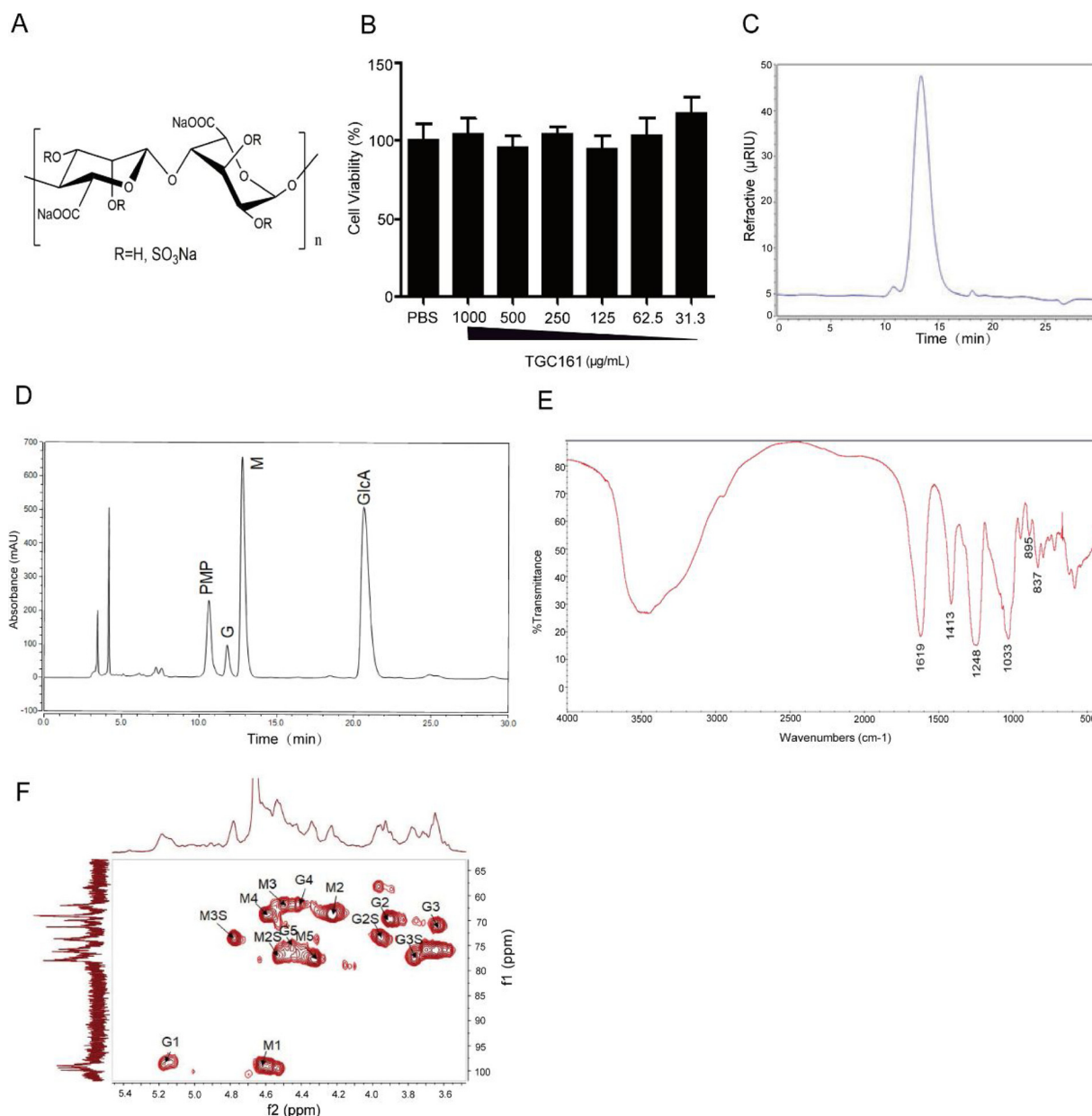


Fig. 1. Structural characterization of TGC161. (A) Chemical structure of TGC161. (B) Cytotoxicity of TGC161 in 293 T cells. 293 T cells were cultured with TGC161 at indicated concentrations for 48 h. Cell viabilities were measured by resazurin assay ($n = 4$). (C) Gel chromatography analysis of TGC161. Analysis was established by a TSK-Gel GMPW column on an Ultimate 3000 high performance liquid chromatographic instrument. (D) Monosaccharide composition of TGC161. (E) The IR spectrum of TGC161. The FT-IR spectrum was recorded in the region between 400 and 4000 cm^{-1} with a KBr pellet. (F) The HSQC spectrum of TGC161. The spectrum of NMR analysis was recorded at room temperature in an Agilent DD2 superconducting NMR spectrometer, operating at 500 MHz.

Table 1
Mw and sulfate contents of TGC161.

Samples	Mw*	Sulfate contents (%)	Ratio of M/G
TGC161	10.4 kDa	28 %	1:7.2

Table 2
 ^1H NMR and ^{13}C NMR signal assignment of TGC161.

	C1/H1	C2/H2 (-SO ₃ H)	C3/H3 (-SO ₃ H)	C4/H4	C5/H5	-COOH
β -Mannuronic acid	99.0/4.62	69.0/4.23 (77.3/4.54)	67.0/4.47 (73.6/4.78)	69.0/4.59	78.0/4.34	163.3/-
α -Guluronic acid	98.7/5.18	69.0/3.90 (73.7/3.95)	71.3/3.65 (77.3/3.87)	66.7/4.43	75.5/4.47	163.3/-

(CD3⁺) and CD4⁺ (CD3⁺CD4⁺) T cells counts increased substantially after TGC161 administration compared with carboplatin (Fig. 3A and B). It indicated that TGC161 would specifically promote CD3⁺CD4⁺ T cell populations in chemotherapy induced leukopenia, which was different from alkylglycerols. B cell, another main component in lymphocytes, also plays an important role in tumor immunity. We were wondering if TGC161 also stimulated B cells as a general immune stimulator. However, no significant difference was observed in TGC161

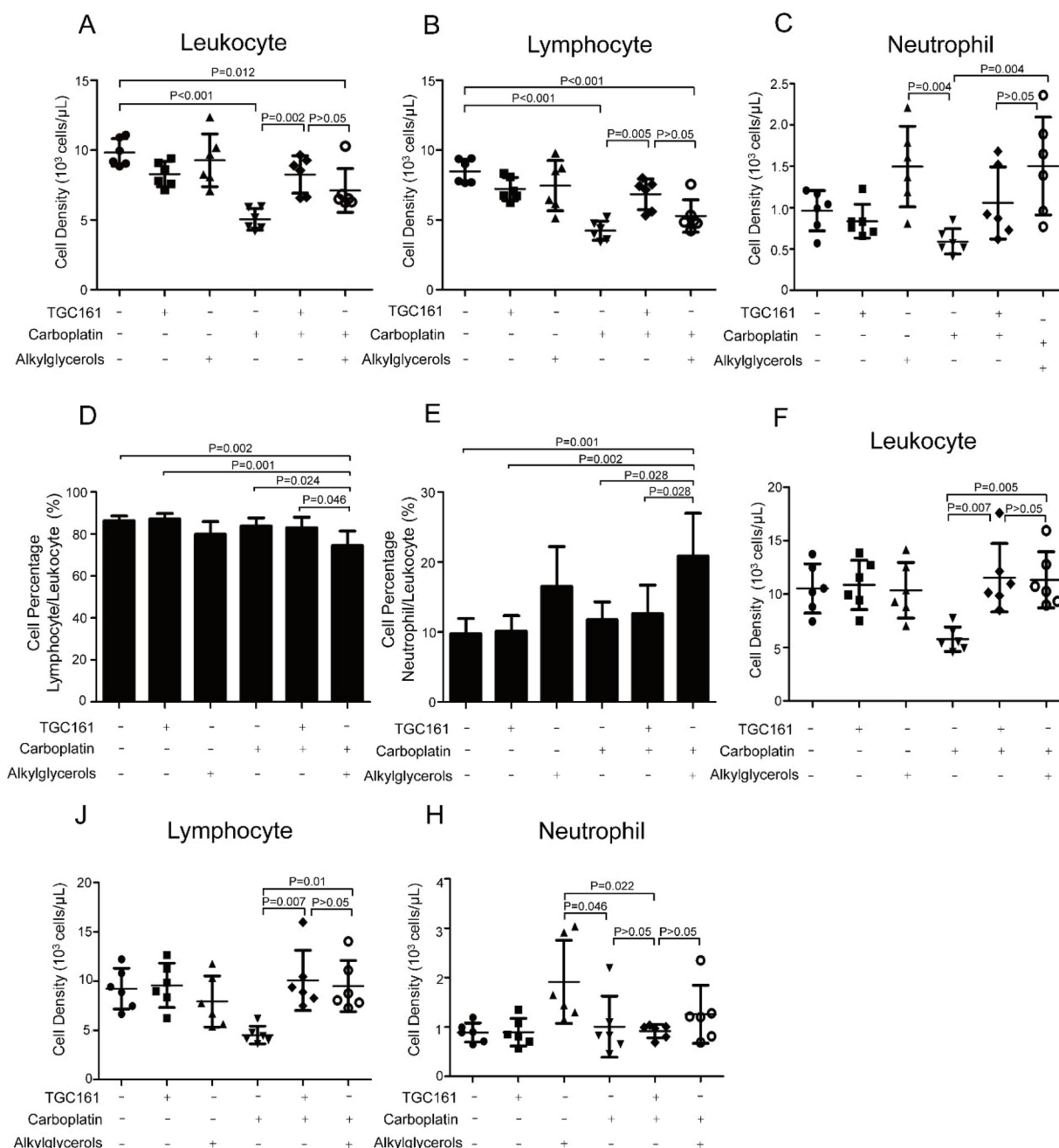


Fig. 2. TGC161 ameliorates chemotherapy induced leukopenia. C57BL/6 male mice were injected intraperitoneally with a single dose of carboplatin (60 mg/kg). TGC161 (400 mg/kg) and alkylglycerols (22.5 mg/kg) were administrated by gavage once a day, respectively (n = 6). (A, B, and C) Cell densities of peripheral leukocytes, lymphocytes and neutrophils on the third day. (D and E) Percentage of lymphocyte and neutrophil in leukocyte on third day. (F, J and H) Cell densities of peripheral blood leukocytes, lymphocytes and neutrophils on sixth day. Data were presented as mean \pm standard error of the mean.

treated and untreated mice. The same conclusion was true when we monitor granulocytes (Fig. 3D, E, I and J). In addition, TGC161 enhanced the percentage of total T and CD3⁺CD4⁺ T cells in leukocytes in contrast with the control group (Fig. 3F and G). It might attribute to the stronger killing/inhibiting effects of carboplatin on B cells and granulocytes than other leukocytes (Bisch et al., 2018; Menetrier Caux, Ray Coquard, & Caux, 2019).

3.4. TGC161 increases CD4⁺ T cell counts in antibody-dependent complement-mediated killing model

Viral infection usually leads to the activation of complement system (Agrawal, Nawadkar, Ojha, Kumar, & Sahu, 2017). Complement

activation might result in both acute and chronic leukopenia (Kociba, 1995). To specify the effects of TGC161 on CD4⁺ T cells, CD4 antibody-dependent complement-mediated killing model was utilized to kill CD4⁺ T cells. Apparently, no or low CD4⁺ T cells were found in peripheral blood when complement system was activated by CD4⁺ antibodies (Fig. 4A). CD4⁺ T cell counts and percentage increased significantly after continuous treatment of TGC161 for 25 days (Fig. 4A, B and C). To sum up, TGC161 raises the CD4⁺ T cell counts in antibody-dependent complement-mediated cytotoxicity killing model.

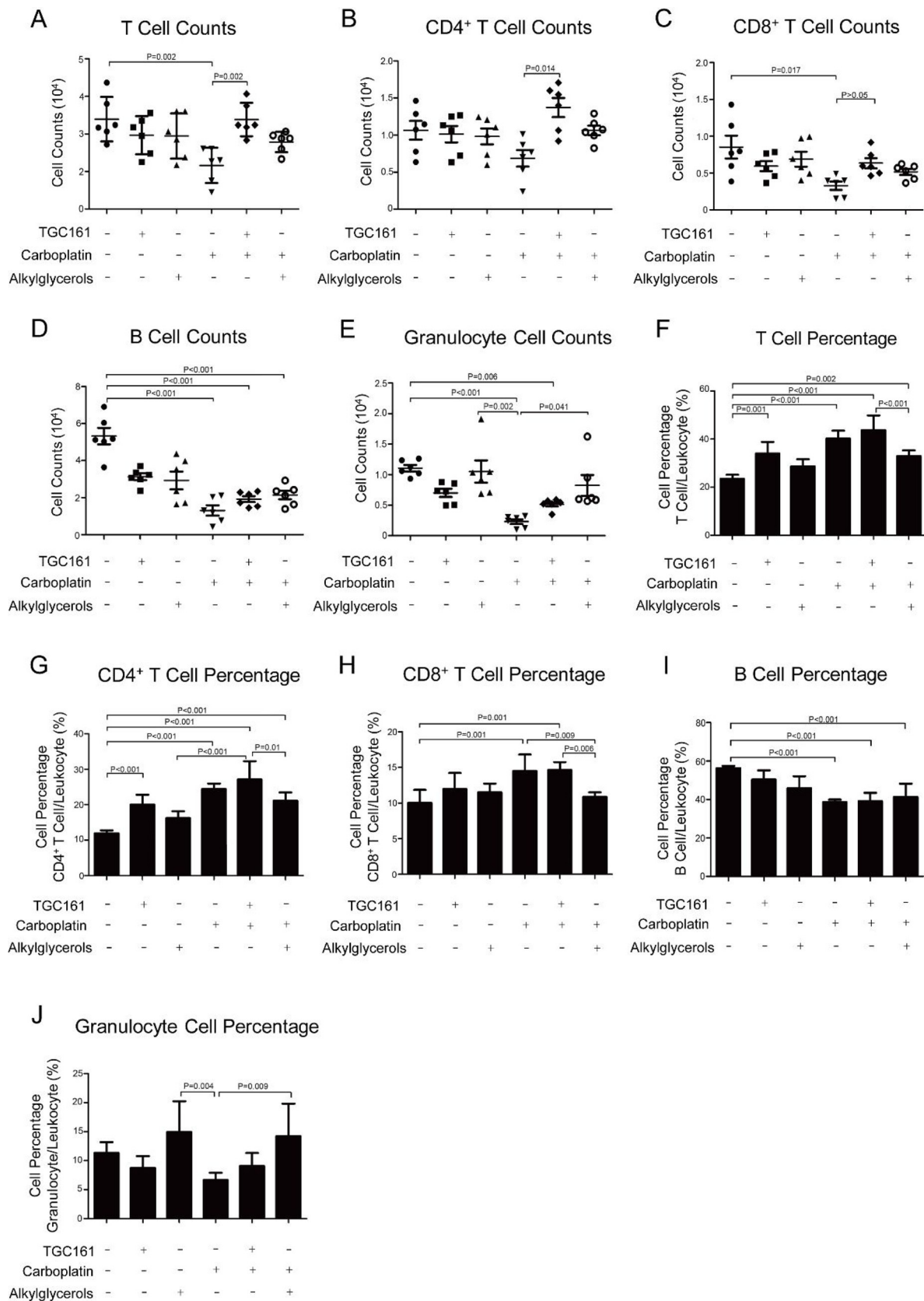


Fig. 3. TGC161 raises CD4⁺ T cell numbers. C57BL/6 mice were treated with carboplatin (60 mg/kg) by intraperitoneal injection. TGC161 (400 mg/kg) and alkylglycerols (22.5 mg/kg) were administered by gavage once a day (n = 6). (A, B, C, D and E) Cell counts of total T cells, CD4⁺ T cells, CD8⁺ T cells, total B cells and granulocytes. (F, G, I and J) Ratio of total T cells, CD4⁺ T cells, CD8⁺ T cells, B cells and granulocytes in leukocytes. Data was presented as mean ± standard error of the mean.

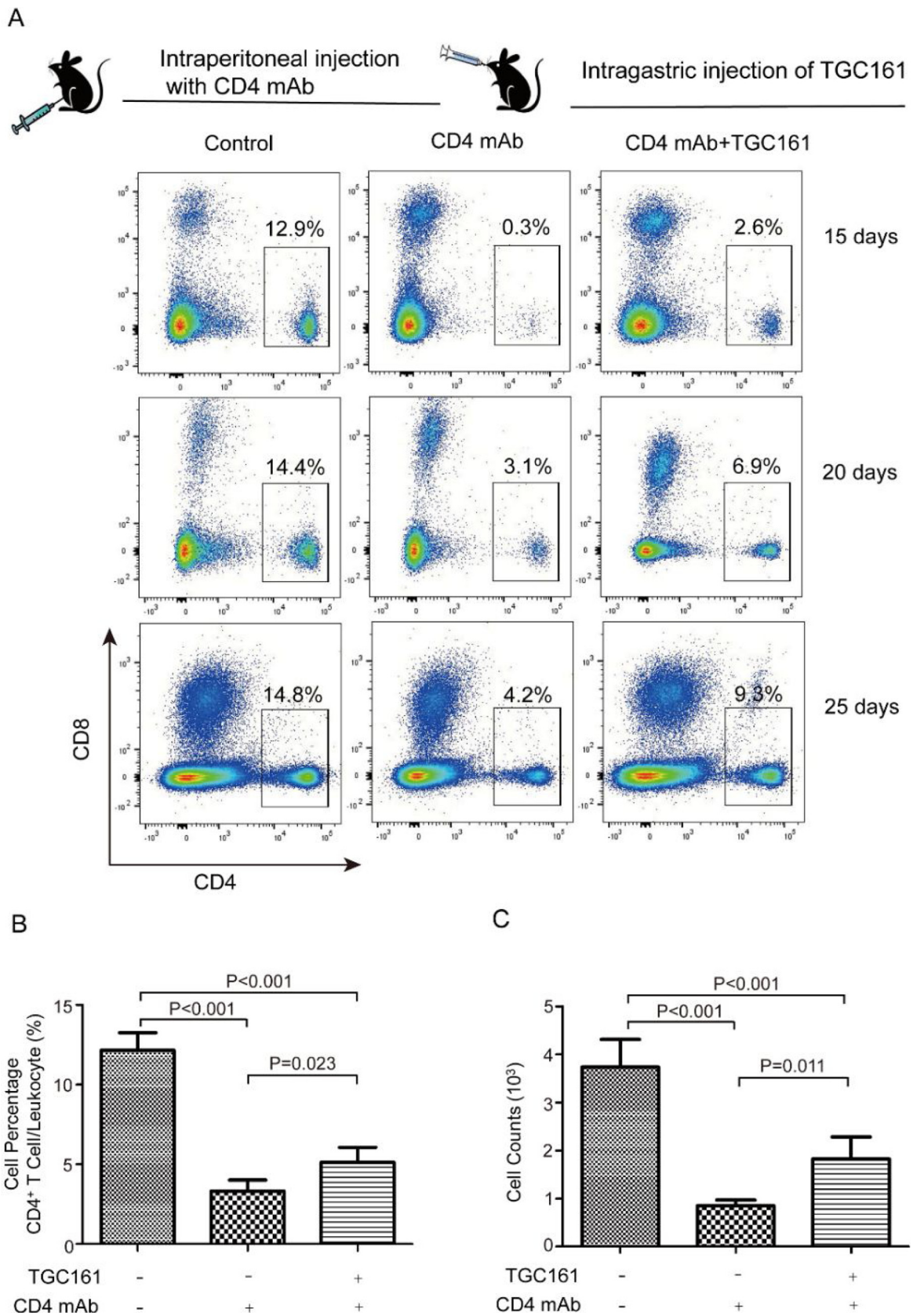


Fig. 4. TGC161 raises CD4⁺ T cell counts in complement-mediated cytotoxicity killing model. Mice were treated with monoclonal CD4 antibodies (150 µg/per mouse) by intraperitoneal injection. TGC161 (400 mg/kg) were administrated by gavage once a day for 15, 20, 25 days (n = 4). (A) CD4⁺ T cell percentage in peripheral blood. (B and C) Analysis of CD4⁺ T cell percentage and counts. Data were presented as mean ± standard error of the mean.

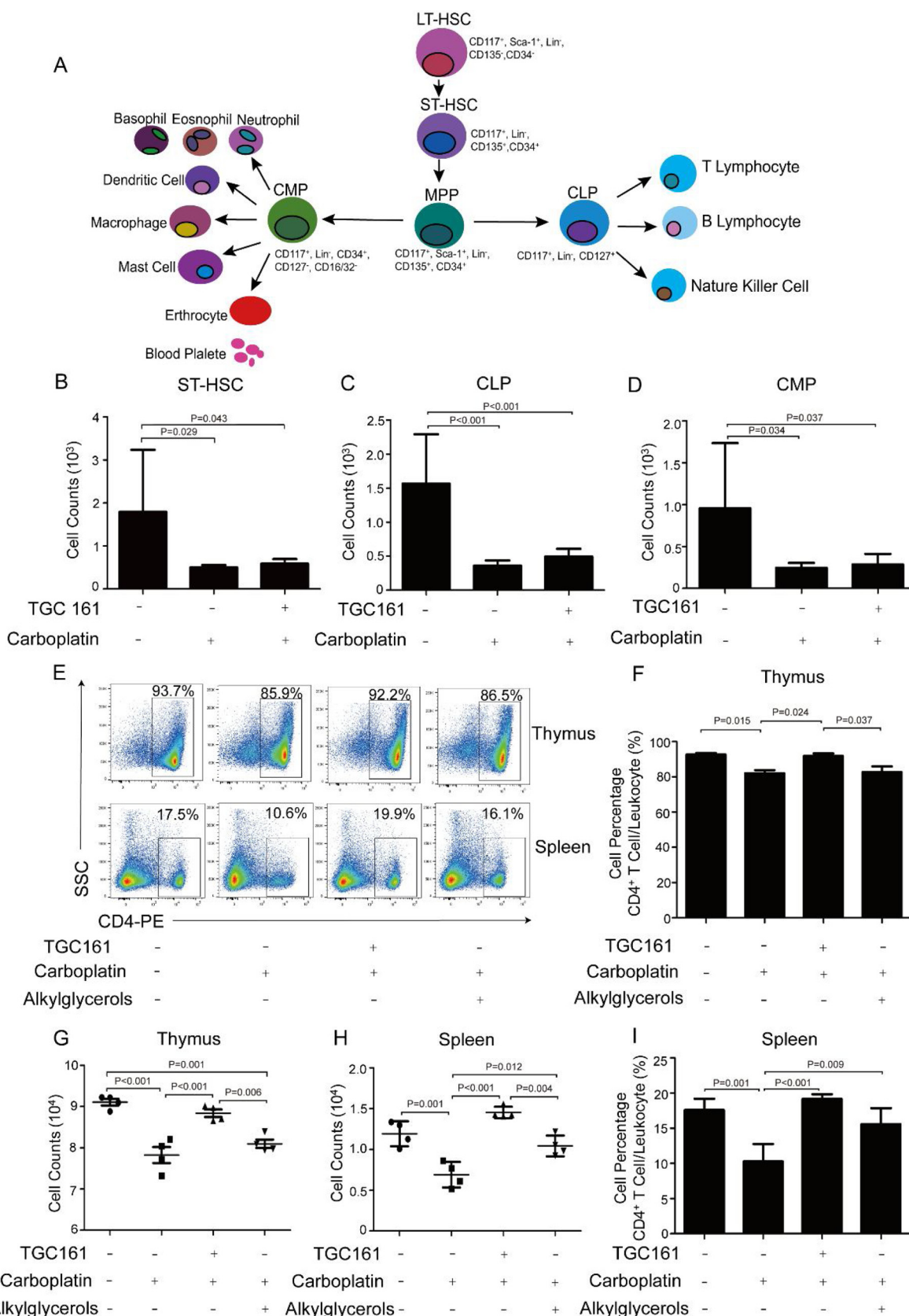


Fig. 5. TGC161 facilitates CD4⁺ T cell differentiation and maturation in thymus, but does not affect precursor cells in bone marrow. C57BL/6 male mice were injected intraperitoneally with a single dose of carboplatin (60 mg/kg). TGC161 (400 mg/kg) and alkylglycerols (22.5 mg/kg) were administrated by gavage once a day, respectively (n = 4). (A) Schematic diagram of HSCs differentiation period axis and associated cell markers. Short-time hematopoietic stem cells (ST-HSC), common myeloid progenitors (CMP), common lymphoid progenitors (CLP) were stained with the corresponding fluorescent antibodies to test by flow cytometry. (B, C and D) Cell counts of ST-HSC, CLP and CMP. (E) The percentage of CD4⁺ T cell in thymus and spleen. (F and G) The analysis of CD4⁺ T cell percentage and counts in thymus. (H and I) The analysis of splenic CD4⁺ T cell percentage and counts. Data were presented as mean ± standard error of the mean.

3.5. TGC161 promotes the CD4⁺ T cell differentiation and maturation in thymus, but has no significant effects on precursor cells in bone marrow

Hematopoietic stem cells (HSCs) produce a variety of immune cell lineage. Since all mature blood cells have a limited life span, the HSCs continuously generate new progenitor cells to maintain enough mature cells in the periphery (Bertrand et al., 2010; De Bruin, Demirel, & Nolte, 2013; De Bruin, Voermans, & Nolte, 2014). Multipotent HSCs differentiate into common lymphoid progenitors (CLP), which then become pre-T cells and move to the thymus (Bertrand et al., 2010; King & Goodell, 2011). Therefore, the effect of TGC161 on T cell differentiation has important significance for further research. ST-HSC, CLP and CMP decreased in the carboplatin-treated group (Fig. 5B–D). But TGC161 did not have significant effects on reversing ST-HSC, CLP and CMP cell counts (Fig. 5B–D).

Positive and negative selection allow T cells to gain MHC restriction and autoimmune tolerance in the thymus (Lang et al., 2013). Then naive T cells derived from thymus stored in the spleen and other immune organs. They play a critical role in the host defense system (Josefowicz, Lu, & Rudensky, 2012; Schwartz, 2003). Thymic and splenic CD4⁺ T cell percentage increased after TGC161 administration (Fig. 5E). TGC161 promoted the thymic and splenic CD4⁺ T cell numbers and percentage, and the difference was statistically significant (Fig. 5F–I). TGC161 increased CD4⁺ T cells in the spleen, which indirectly enhanced cellular immunity. Therefore, TGC161 facilitates the CD4⁺ T cell differentiation and maturation in the thymus, but has no significant effects on precursor cells in bone marrow.

3.6. TGC161 inhibits CD4⁺ T cell apoptosis in vitro

To clarify the effects of TGC161 on CD4⁺ T cell proliferation, CFSE fluorescence intensities of CFSE in CD4⁺ T cells were detected by flow cytometry. TGC161 does not affect the thymus-derived CD4⁺ T cell proliferation (Fig. 6B). However, TGC161 inhibited CD4⁺ T cell apoptosis, especially at the concentration of 10 and 100 µg/mL (Fig. 6C and D).

3.7. TGC161 reduces caspase 8 and caspase 3 cleavage in vitro

The intrinsic and extrinsic apoptosis pathways have been identified the central apoptotic pathway. Intrinsic apoptosis pathway promotes the release of cytochrome c by activating Bcl2 family proteins (Brenner & Mak, 2009; Lindsay, Esposti, & Gilmore, 2011). Extrinsic apoptosis pathway is triggered by signals originating from death receptors on cell surface. Ligands of death receptor characteristically initiate signaling via receptor oligomerization, which results in the recruitment of specialized adaptor proteins and the activation of caspase cascades (Declercq, Vanden Berghe, & Vandenabeele, 2009). Then, the activated caspase 8 directly cleave and activate caspase 3 to deliver apoptosis signal (Kantari & Walczak, 2011). In our studies, TGC161 inhibited caspase 8 and caspase 3 cleavage, but has no significant effect on Bcl2 (Fig. 7A, B). We speculated that low level of caspase 8 and caspase 3 cleavage is indicating the reduced cell apoptosis. Besides, the gray value of cleaved-caspase 8 and cleaved-caspase 3 protein bands were statistically significant (Fig. 7C, D). Taken together, TGC161 may inhibit CD4⁺ T cell apoptosis by decreasing the caspase 3 and caspase 8 cleavage (Fig. 7E).

4. Discussion

Nowadays, leukopenia has aroused considerable research interest. Medications for leukopenia treatment were attracting scientists' and investors' attention. The recently developed anti-tumor medication plinabulin promotes dendritic cell maturation and ameliorates chemotherapy induced neutropenia (Bertelsen et al., 2011), which is attracting tons of investments. In this study, we reported that a

polysaccharide would benefit chemotherapy induced leukopenia patients by increasing number and percentage of CD4⁺ T cells in blood stream. As we know, it is the first report that polysaccharides could selectively stimulate CD4⁺ T cells but only have limited impact on neutrophils.

Some polysaccharides with immune activity have aroused considerable research interest. β-1,3/1,6-glucan derived from *Durivillaea antarctica* can increase macrophage phagocytosis and the pro-inflammatory cytokine secretion *in vivo* (Su et al., 2019). In addition, SPMG, which is very similar to TGC161, can enhance the T cell response without the activator stimulation (Miao et al., 2005). In our study, TGC161 ameliorates chemotherapy induced leukopenia. Besides, TGC161 promotes the CD4⁺ T cell differentiation and maturation in thymus but has less impact on precursor cells. Moreover, TGC161 may reduce caspase 8 and caspase 3 cleavage to down regulate CD4⁺ T cell apoptosis *in vitro*.

In present study, TGC161 increased CD3⁺CD4⁺ T cells to ameliorate the leukopenia (Fig. 3B and G). The percentage of CD3⁺CD8⁺ T cells elevated after TGC161 administration, but the cell numbers did not rise significantly (Fig. 3C and H). CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells derived from CLP and then undergo both negative and positive selection to obtain corresponding subtypes (Zuniga Pflucker, 2004). We speculated that the TGC161 administration may have created a special internal environment that is more conducive for CD3⁺CD4⁺ T cell to survive. We all know that T cells' immune activity is regulated by kinds of cytokines. Interleukin-12 and interferon γ are the critical cytokines to develop Th1 CD4⁺ T cells (Luckheeram, Zhou, & Xia, 2012). Interleukin-10 inhibits CD8⁺ T cell function by improving N-glycan branching to decrease the antigen sensitivity (Smith et al., 2018). Therefore, we hypothesized that TGC161 may affect the secretion of cytokines to increase the number of CD3⁺CD4⁺ T cells in peripheral blood significantly. Exploring the effect of TGC161 on specific cytokines requires us to do further experimental verification. Taken together, our data suggested that TGC161 may be developed as a stimulator to increase CD3⁺CD4⁺ T cell numbers and reverse leukopenia. TGC161 may be more conducive to the improvement of clinical associated diseases caused by low lymphocytes. Compared with alkylglycerols, TGC161 may affect faster in terms of elevating lymphocytes.

In vivo, TGC161 has no significant effects on precursor cells in the bone marrow (Fig. 5B–D). We speculate that TGC161 may not recognize and act on these naive precursor cells, resulting in neither ST-HSC nor CMP being elevated. After positive and negative selection in pre-T cells, double positive (CD4⁺CD8⁺) cells are converted to single positive (CD4⁺CD8⁻ or CD4⁻CD8⁺) cells. Following selection, down-regulation of co-receptor induced either naive CD4 or CD8 single positive cells that exit the thymus and circulate the periphery (Takaba & Takayanagi, 2017; Takada & Takahama, 2015). Data showed that TGC161 promoted the differentiation and maturation of CD4⁺ T cells (Fig. 5). And further research is needed to determine specific isotypes of CD4⁺ T cell which are the TGC161 targeting.

In vitro, we found that TGC161 did not promote the proliferation of thymus-derived CD4⁺ T cells (Fig. 6B). However, it was inconsistent with Miao who reported that TGC161 can promote T cell proliferation (Miao et al., 2005). They used MTT to measure cellular activity and made it as an indicator of cell proliferation. However, we believe that faster cell growth or fewer cell death also show a satisfactory cellular activity. To avoid interference with this factor, we labeled T cells with CFSE and detected fluorescence intensities by flow cytometry. As the cell division rising, the fluorescence intensities of CFSE will decrease. In this way, the cell proliferation ability can be properly determined. Importantly, we were surprised to discover that TGC161 inhibited the CD4⁺ T cell apoptosis (Fig. 6C). Complex apoptosis signal facilitates in the assembly of pro-caspases 8 and 3 and their autoproteolytic activation, which sends the apoptosis signal to the nucleus (Zhang, Zhang, & Xue, 2000). Changes in cleaved caspase 3 and caspase 8 directly reflect whether apoptosis has occurred. TGC161 may inhibit caspase 3 and

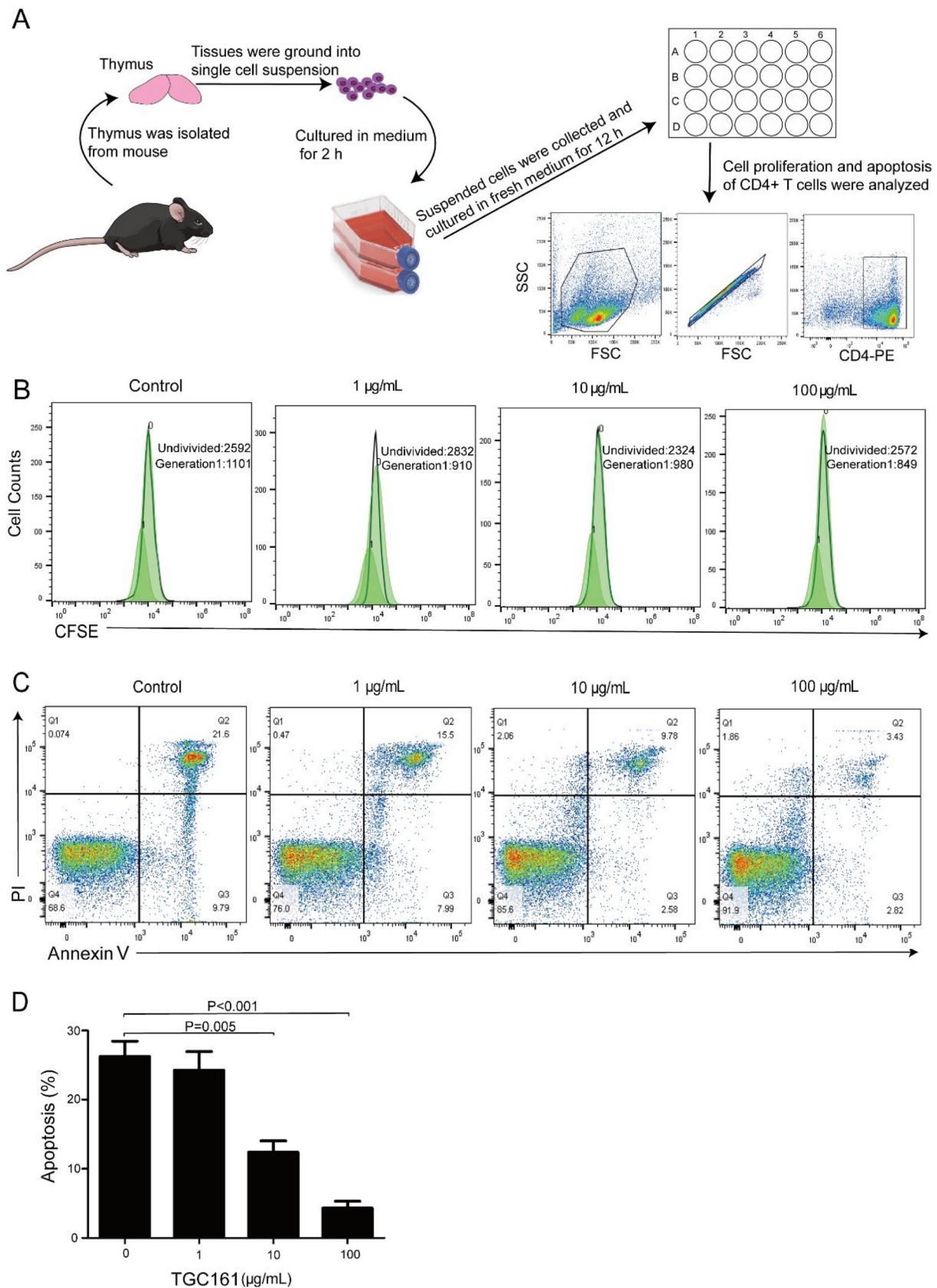


Fig. 6. TGC161 suppresses CD4⁺ T cell apoptosis *in vitro*. Thymus single cell was cultured with TGC161 in different concentration (0,1,10,100 µg/mL) for 12 h and stained with CD4-PE before testing. (A) Schematic diagram isolation and culture of single thymus cells *in vitro*. (B) The proliferation analysis of CD4⁺ T cells. CD4⁺ T cells were marked with CFSE dye. (C) The apoptosis analysis of CD4⁺ T cells. CD4⁺ T cells were marked with AnnexinV and PI dye. (D) The percentage of apoptosis CD4⁺ T cells. Data were presented as mean ± standard error of the mean.

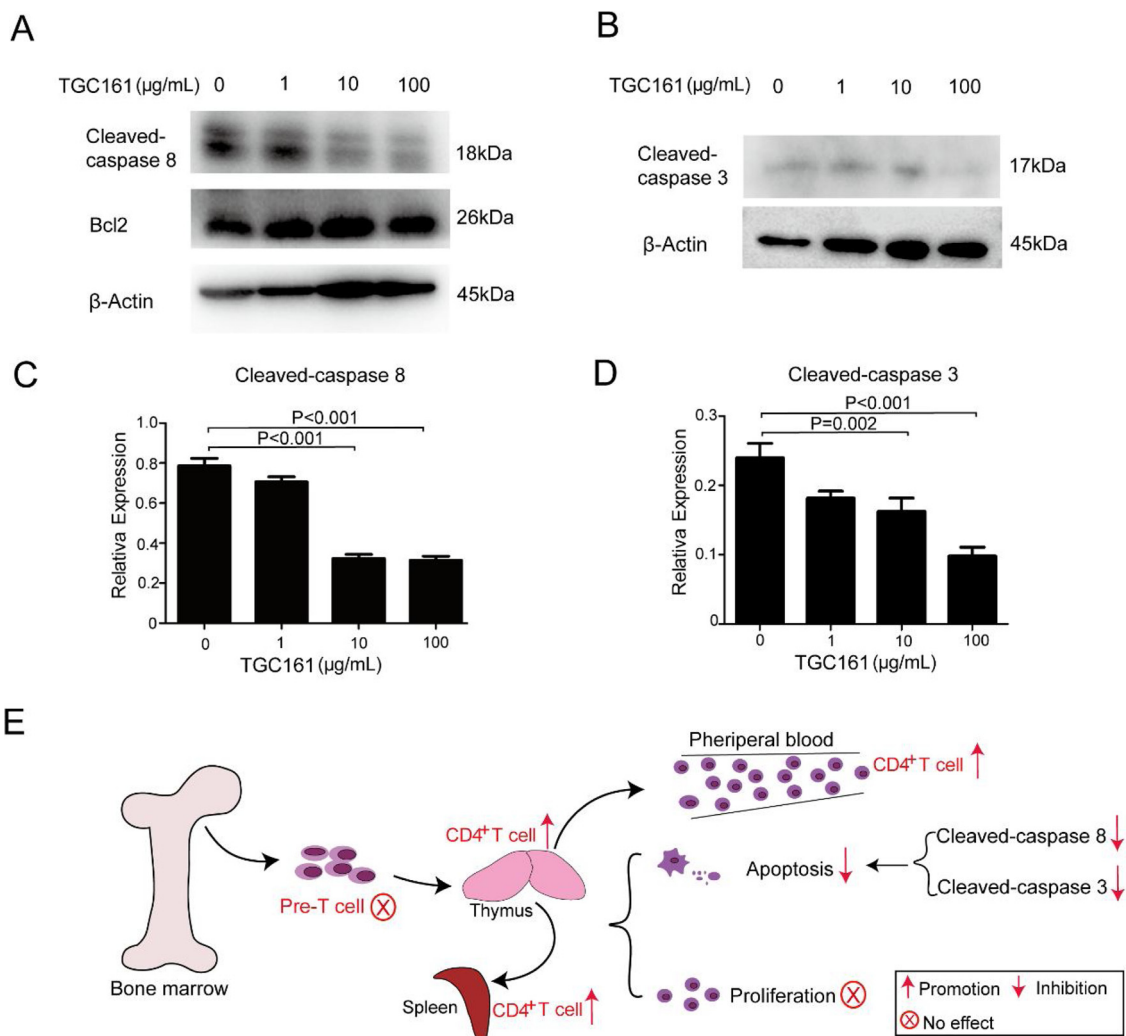


Fig. 7. TGC161 reduces the caspase 8 and caspase 3 cleavage *in vitro*. Single thymic cells were cultured with TGC161 at different concentration (0,1,10,100 µg/mL) for 12 h *in vitro*. Cellular lysates were tested by Western blotting. β -actin was used as an internal control. (A) Cleaved-caspase 8, Bcl-2 and β -actin. (B) cleaved-caspase 3 and β -actin. (C and D) Analysis of cleaved-caspase 8 and cleaved-caspase 3 cleavage by protein bands' gray value. (E) Diagrammatic representation of TGC161 effects on different immune organs and cells.

caspase 8 cleavage, but have no significant difference in Bcl2 (Fig. 7A and B). All these results showed that TGC161 may inhibit CD4⁺ T cell apoptosis to ameliorate leukopenia.

5. Conclusion

In summary, our data suggested that TGC161 ameliorates leukopenia caused by chemotherapy. In addition, TGC161 increases the differentiation and maturation of CD4⁺ T cells in the thymus, but has less impact on precursor cells in bone marrow. Moreover, TGC161 inhibits CD4⁺ T cell apoptosis *in vitro*. This research will help the development of new leukopenia treatment drugs and provide new ideas for clinical treatment.

CRedit authorship contribution statement

Chuanqin Shi: Conceptualization, Resources, Methodology, Data curation, Writing - original draft. **Wenwei Han:** Methodology, Data curation, Validation, Writing - original draft. **Meifang Zhang:** Investigation, Methodology. **Ruochen Zang:** Data curation, Methodology. **Kaixin Du:** Data curation, Methodology. **Li Li:** Software, Methodology. **Ximing Xu:** Supervision, Validation. **Chunxia Li:** Methodology. **Shixin Wang:** Resources. **Peiju Qiu:** Methodology.

Huashi Guan: Methodology, Project administration. **Jinbo Yang:** Software, Supervision. **Shuai Xiao:** Supervision, Writing - review & editing. **Xin Wang:** Project administration, Writing - review & editing.

Declaration of Competing Interest

There are no conflict of interest exists in the present study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.carbpol.2020.116728>.

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