Am J Cancer Res 2023;13(5):1786-1805
www.ajcr.us /ISSN:2156-6976/ajcr0148964

Original Article

Alcohol remodels the immunosuppressive tumor microenvironment by targeting myeloid-derived suppressor cells

Xueyan Li[1,2], Xue Deng[1,2], Xiaoqi Luo[1,2], Jiahui Zhong[2,3], Hui Wu[2,4], Siru Chen[2,3], Jiayi Chen[2,3], Xuhui Huang[1,2], Changjun Wang[1,2]

[1]School of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, Guangdong, China; [2]Department of Traditional Chinese Medicine, Guangdong Provincial People's Hospital (Guangdong Academy of Medical Sciences), Southern Medical University, Guangzhou 519041, Guangdong, China; [3]Guangzhou University of Chinese Medicine, Guangzhou 510006, Guangdong, China; [4]School of Medicine, South China University of Technology, Guangzhou 510000, Guangdong, China

Received January 5, 2023; Accepted April 6, 2023; Epub May 15, 2023; Published May 30, 2023

Abstract: The tumor immunosuppressive microenvironment plays an important role in tumor progression. Alcohol is well-known as a regulator of the immune system and several studies have also reported that chronic alcohol intake can activate the immune system. However, it is unclear whether alcohol can affect liver cancer progression by regulating the immunosuppressive microenvironment. In this study, we investigated the effects of different alcohol concentrations on the growth of liver cancer and tumor immune microenvironment. We examined the growth of tumors in mice provided with water, or alcohol (for 2 weeks before tumor injection, and for 3 weeks after tumor injection). We found that alcohol consumption at 5% and 20% inhibited the growth of subcutaneous tumors in hepatocellular carcinoma-bearing mice, whereas 2% alcohol concentration did not significantly inhibit liver cancer growth. The ratio of myeloid-derived suppressor cells (MDSCs) in peripheral blood and spleen of mice treated with 5% or 20% alcohol for 2 weeks before tumor inoculation was downregulated. After tumor inoculation, the proportion of MDSCs in peripheral blood, spleen, and tumor of mice treated with 5% or 20% alcohol for another 3 weeks also decreased and the proportion of CD4+ T cells and CD8+ T cells increased. In addition, Alcohol consumption of 20% reduced levels of the inflammatory factor IL-6 by inhibiting JAK/STAT3 signaling. These results indicate that chronic alcohol consumption may inhibit the growth of liver cancer by regulating MDSCs.

Keywords: Chronic alcohol consumption, hepatocellular carcinoma, immunosuppression, myeloid-derived suppressor cells

Introduction

The tumor immunosuppressive microenvironment is a crucial factor for the development and metastasis of tumors. It is well recognized that hepatocellular carcinoma (HCC) is a typical inflammation-related cancer and HCC progression is correlated with the infiltration of inflammatory factors and accumulation of immunosuppressive cells. Myeloid-derived suppressor cells (MDSCs), representing a heterogeneous population of myeloid progenitors, are divided into two major groups in humans and mice, namely granulocytic/polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs), classified according to their origin from the granulocytic or monocytic myeloid cell lineages, respectively. MDSCs are one of the leading causes of tumor immunosuppressive microenvironment and serve as powerful pro-inflammatory mediators in suppressing T-cell functions to contribute to immune evasion [1]. However, the effects of chronic alcohol consumption on MDSC recruitment and immunosuppressive function remain poorly defined.

Ethanol (ethylic alcohol, EtOH), in the form of a variety of beverages, is one of the most common active ingredients in the world, along with caffeine [2]. Alcohol is causally linked to upper aerodigestive tract cancers, such as hepatocellular carcinoma [3], but the precise role of alco-

hol affecting the growth of liver cancer requires further research. Several studies presented controversial effects of alcohol consumption on cancer-associated outcomes. Some animal studies showed chronic alcohol consumption accelerated tumor growth, development, and metastases by impairing antitumor immunity [4], while others presented suppression of tumors by alcohol intake via activating immune cells [5, 6]. Alcohol is a small polar organic molecule, which can diffuse through the cell membrane and distribute into all tissues through blood, of which the majority is metabolized in the liver [7]. As the liver is an immune organ itself, EtOH consumption is associated with the activation of both several signaling pathways and the innate immune system, thus initiating a multi-cascade of different biochemical actions and immunological responses. For example, Hsiao-Yen Ma et al. demonstrated that chronic alcohol consumption is associated with a marked increase in the expression of pro-inflammatory IL-17A, which is a tumor-promoting cytokine and critically regulates inflammatory responses in macrophages (Kupffer cells and bone-marrow-derived monocytes) in a hepatocellular carcinoma (HCC) model [8]. In addition, hepatic ethanol metabolism results in the accumulation of ROS which further causes oxidative stress and ER stress, as well as fatty acid overproduction and accumulation. All these factors together stimulate the liver macrophages (Kupffer cells) to produce and release pro-inflammatory cytokines and chemokines, leading to the recruitment of other immune cells and triggering an inflammatory signal cascade [2]. However, how alcohol-mediated inflammatory responses affect the tumor immune microenvironment remains unclear. Thus, exploring the pathogenesis and developing therapeutic targets are urgently warranted.

Therefore, this study was designed to further investigate the effects of chronic alcohol consumption on tumor growth and tumor immune microenvironment. Our results showed that 5% or 20% alcohol intake significantly inhibited hepatocellular carcinoma growth, however, 2% alcohol concentration did not show a tumor-suppressive effect. In addition, 5% or 20% alcohol intake for two weeks before tumor inoculation suppressed MDSCs in blood and spleen and another three weeks of 5% or 20% alcohol consumption also reduced the number of MDSCs with an increase in CD4+ T cells and CD8+ T cells. Also, alcohol consumption of 20% reduced levels of the inflammatory factor IL-6 by inhibiting JAK/STAT3 signaling.

Materials and methods

Cells culture

The H22 hepatoma carcinoma cell line was kindly provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China), and was cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 atmosphere.

Animal model and treatment

Sixteen (16) male BALB/c mice (five weeks old, 14~18 g) were purchased from Guangzhou Carrot Biotechnology Co., LTD (Guangzhou, China), and maintained under specific pathogen-free (SPF) conditions, at 25 ± 1°C, relative humidity of 55 ± 5%, and a 12 h/12 h light/dark cycle. After a 1-week acclimation period, the mice were given free access to either water or 5% volume/volume (v/v) alcohol as the sole drinking fluid. BALB/c mice were divided randomly into two groups: control (water), and alcohol (ethanol for 2 weeks before tumor injection), respectively. Mice were injected subcutaneously with mouse H22 hepatoma carcinoma cells (2 × 10^6) after 2 weeks of water or alcohol intake and were continuously given either water or 5% (v/v) alcohol for another 3 weeks. The length and width of the subcutaneous tumors were measured, and the mice were weighed daily.

Thirty (30) male BALB/c mice (five weeks old, 14~18 g) were purchased from Guangzhou Carrot Biotechnology Co., LTD (Guangzhou, China), and maintained under specific pathogen-free (SPF) conditions, at 25 ± 1°C, relative humidity of 55 ± 5%, and a 12 h/12 h light/dark cycle. After a 1-week acclimation period, the mice were given free access to either water or 2% (v/v) alcohol or 20% (v/v) alcohol as the sole drinking fluid. BALB/c mice were divided randomly into three groups: control (water), 2% alcohol (2% ethanol for 2 weeks before tumor injection), and 20% alcohol (20% ethanol for 2 weeks before tumor injection) respectively. Mice were injected subcutaneously with mouse H22 hepatoma carcinoma cells (3 × 10^6)

after 2 weeks of water or alcohol intake and were continuously given either water or 2% (v/v) alcohol or 20% (v/v) alcohol for another 3 weeks. The length and width of the subcutaneous tumors were measured, and the mice were weighed daily.

MTT assay

5 × 10^3 H22 in 90 μl of culture medium were seeded into 96-well plates. And then 10 μl of various concentrations of ethanol (0%, 0.001%, 0.01%, 0.1%, 1%, 2%, or 2.5%) were added. Each concentration was tested in six wells, and the control group was treated with 10 μl of the plain medium. After 24, 48, or 72 hours of culture, 10 μl of MTT reagent (Beyotime) was added, and the cells were incubated for 4 hours at 37°C in a CO_2 incubator. Then 200 μl DMSO was added for 2 hours. A Multiscan Spectrum (Thermo Scientific) at a 450 nm wavelength was used to detect absorbance values (OD values). Cell survival rate was equal to the value of [(OD of the alcohol group - OD of the blank group)/(OD of the control group - OD of the blank group)] × 100% [9].

Flow cytometry (FCM)

To prepare single-cell suspensions from mouse spleens, each spleen was placed on a 70-μm cell filter and rinsed with PBS while gently grinding the tissues with the inner core of a 1 ml syringe. After centrifugation, the erythrocytes were lysed with Red Blood Cell Lysis Buffer (Biosharp) at 4°C for 5 minutes and washed once with PBS [10]. The peripheral blood was lysed with Red Blood Cell Lysis Buffer (Biosharp) at 4°C for 10 minutes and washed twice with PBS. The tumor tissues were collected, cut into small pieces, and digested in serum-free DMEM medium with 0.1 mg/mL DNase I (Roche) and 1 mg/mL collagenase (Worthington) at 37°C for 30 minutes, and then ground and filtered through a 70-μm cell strainer (Nest) to obtain single-cell suspension [11]. Fc-receptor blocking reagent (Miltenyi Biotec) was used to block the Fc-receptors on cells, following the manufacturer's instructions. Percp/Cy5.5-CD45.2, allophycocyanin (APC)-CD11b, phycoerythrin (PE)-Ly6G, FITC-Ly6C antibodies were used to identify MDSCs and their subgroups. Cytotoxic T lymphocytes (CTL) were detected with PE/Cyanine7-CD8a, APC-CD3, PE-CD4. All samples were detected with BD ARIA III (BD Bio-sciences). The data were analyzed by Flowjo software. Blank and single staining tubes were set up for all tests. All antibodies mentioned were purchased from Biolegend.

Hematoxylin and eosin staining of tumors

Freshly-obtained mouse tumors were fixed in 4% paraformaldehyde, embedded into paraffin, and cut into 4 μm-thick slices. The slices were baked at 60°C for 2 hours. The slices were then dewaxed by dipping them into xylene, anhydrous ethanol, and alcohol orderly. Next, the slices were dipped into hematoxylin reagent (Sangon Biotech) for 5 min, rinsed with distilled water for 5 min, and dehydrated with anhydrous ethanol for 5 min. Finally, the slices were dipped into an eosin dye solution (Sangon Biotech) for 5 min, dehydrated with anhydrous ethanol for 5 min, and sealed with neutral gum. All slides were observed under an inverted microscope and images were collected [10].

Immunohistochemistry of tumors

Fresh tumor tissues were fixed in 4% paraformaldehyde at room temperature. After paraffin-embedding, the tissues were cut into 5 mm-thick sections for immunohistochemistry staining. First, sections were deparaffinized, processed with an AutoFluo Quencher (Solarbio), and blocked with 5% BSA. Next, the sections were incubated with PCNA (1:200; Abcam), α-SMA (1:1000, Abcam), CD8α (1:2000, Abcam), Granzyme B (1:3000, Abcam), Arg1 (1:2000, Abcam) and iNOS (1:2000, Abcam) at 4°C overnight and incubated with anti-IgG-Horseradish peroxidase (HRP) for 2 hours at room temperature. Finally, a DAB Immunohistochemistry Color Development kit (Sangon Biotech) was used for chromogenic reactions [10].

Immunofluorescent tissue staining

Fresh tumor tissues were fixed in 4% paraformaldehyde at room temperature. After paraffin-embedding, the tissues were cut into 5 mm-thick sections. First, tumor tissue sections were permeabilized with 0.4% Triton X-100 for 15 min at room temperature, then were blocked with 10% goat serum for two hours at room temperature and incubated with IL-6 (1:200; catalog 12912, CST) overnight at 4°C. Samples were then washed with PBS and incubated with

appropriate Alexa Fluor 594-conjugated secondary antibodies (CST, USA) for one hour at room temperature. Nuclei were stained with DAPI (Solarbio Biotechnology, China) for 4 min at room temperature. Images were acquired with an FV1000 confocal microscope (Olympus, USA).

WB analysis

Tumor tissue was homogenized in RIPA buffer and a protease inhibitor (Solarbio) mixture. After incubation for 30 minutes on ice, samples were centrifuged at 12000 × g for 30 minutes at 4°C, and supernatants were collected as total protein. Protein concentrations were determined using the bicinchoninic acid assay (Thermo Fisher Scientific). The primary Abs used were against Arg1 (1:5000; catalog ab233548, Abcam), iNOS (1:1000; catalog ab283655, Abcam), IL-6 (1:1000; catalog 12912, CST), P-STAT3 (1:10000; catalog ab76315, Abcam), STAT3 (1:1000; catalog 91397, CST), P-ERK (1:5000; catalog ab76299, Abcam), ERK (1:10000; catalog ab184699, Abcam), GAPDH (1:10000; catalog ab181602, Abcam).

Statistics

Data are presented as mean ± SEM. The statistical significance between the 2 groups was calculated by a 2-tailed t-test, with $P < 0.05$ defined as statistically significant. All statistical analyses were performed using SPSS, version 19.0 (IBM Corp.).

Results

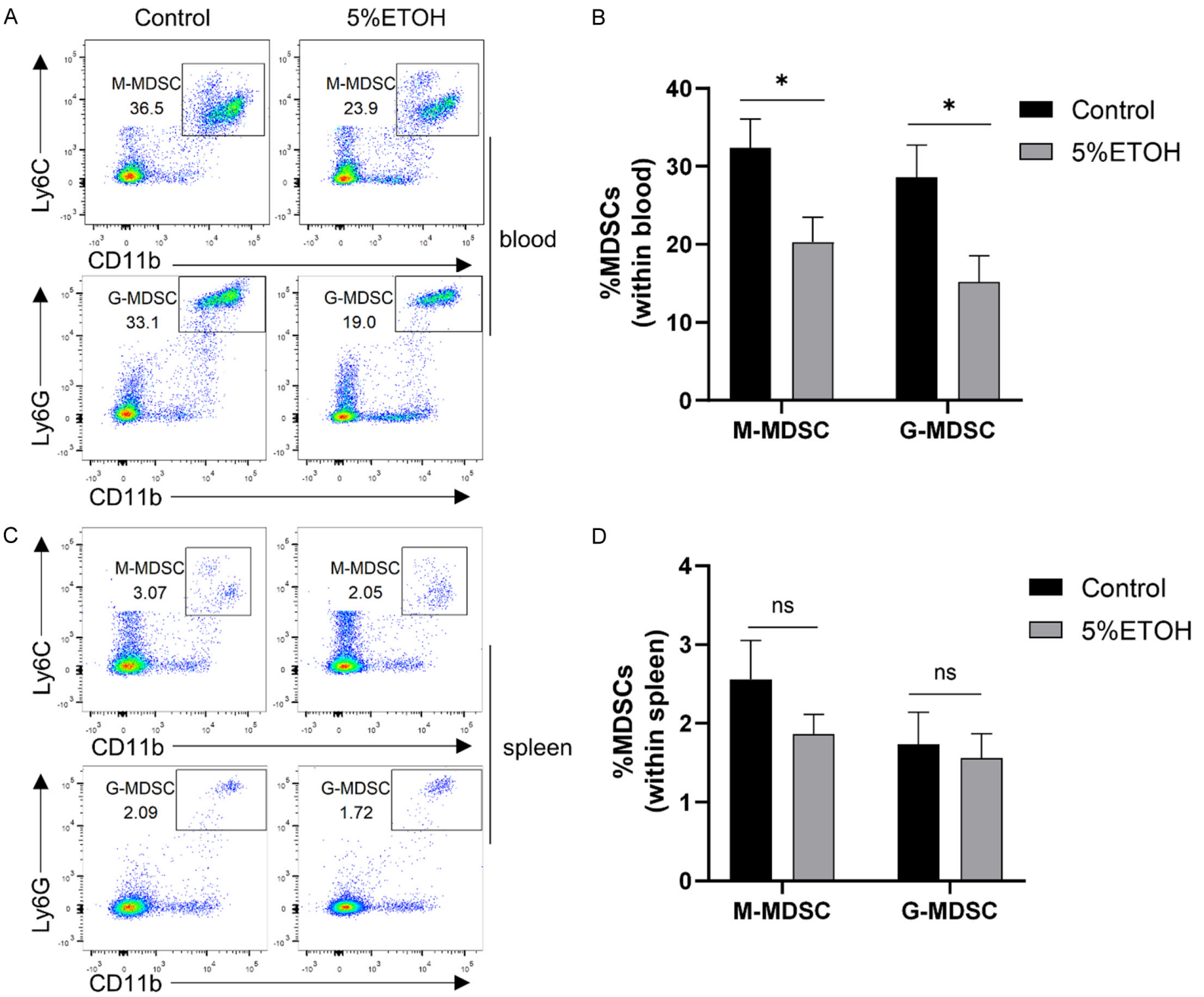
Chronic alcohol consumption activates the immune system by modulating the balance of MDSCs and CD8+ T cells

Alcohol is well-known as a regulator of the immune system. Ethanol exposure might mediate immune responses along a spectrum that spans from pro-inflammatory to anti-inflammatory and from damage to resolution via unidentified mechanisms. Acute ethanol consumption drives the initial pro-inflammatory immune response. Afterward, an anti-inflammatory response would be promoted to protect the host from the systemic cytokine storm [12]. We consider whether alcohol alters host immune function by modulating immune cells. Myeloid-derived suppressor cells (MDSCs), as a heterogeneous population of immature myeloid cells, are one of the major components in the immune suppressive network to both innate and adaptive immune responses [13]. Therefore, we examined if chronic alcohol consumption affects the host immune status by regulating the level of MDSCs. In the present study, 2 weeks of 5% (v/v) alcohol intake significantly decreased the proportions of G-MDSC and M-MDSC cells in the peripheral blood and the proportion of MDSC cells in the spleen showed a slight decline in the drinking mice without a significant difference (**Figure 1A-D**). The main feature of MDSCs is the inhibition of CD8+ T cell function. Therefore, we next detected the proportion of CD8+ T lymphocytes in the blood and spleens. Our results showed that splenic CD8+ T cells were significantly increased in the drinking mice compared to the controls but had no effect on the proportion of CD8+ T cells in the blood (**Figure 1E**, **1F**).

Given that 5% alcohol is consumed in moderate doses, do low and high doses of alcohol regulate the level of MDSCs? To further explore the effect of alcohol consumption on the host immune system, we treated mice with 2% low-dose alcohol and 20% high-dose alcohol respectively, with the same drinking pattern as the 5% alcohol concentration. We found that 20% alcohol treatment significantly decreased the number of M-MDSCs and G-MDSCs in the spleen but has no effect on the number of MDSCs in blood (**Figure 2C-F**). In addition, consistent with previous results, CD8+ T cells in the spleen of mice in the 20% alcohol treated group were significantly higher than those in the control drinking water group (**Figure 2G**, **2H**). However, there was no significant difference in the number of MDSCs and CD8+ T cells in spleens and blood between the control group and the 2% alcohol-treated group (**Figure 2A**, **2B**). Taken together, these results demonstrated that moderate to high-dose alcohol consumption could activate the host immune system by decreasing MDSCs and increasing CD8+ T cells.

Chronic alcohol consumption inhibits the growth of hepatocellular carcinoma

Given that chronic alcohol consumption can affect the host's immune status, we speculate that alcohol consumption may affect hepatocellular carcinoma (HCC) growth by regulating


A
Control
5%ETOH
M-MDSC
36.5
M-MDSC
23.9
Ly6C
CD11b
blood
G-MDSC
33.1
G-MDSC
19.0
Ly6G
CD11b
B
%MDSCs (within blood)
40
30
20
10
0
*
*
Control
5%ETOH
M-MDSC
G-MDSC
C
M-MDSC
3.07
M-MDSC
2.05
Ly6C
CD11b
spleen
G-MDSC
2.09
G-MDSC
1.72
Ly6G
CD11b
D
%MDSCs (within spleen)
4
3
2
1
0
ns
ns
Control
5%ETOH
M-MDSC
G-MDSC

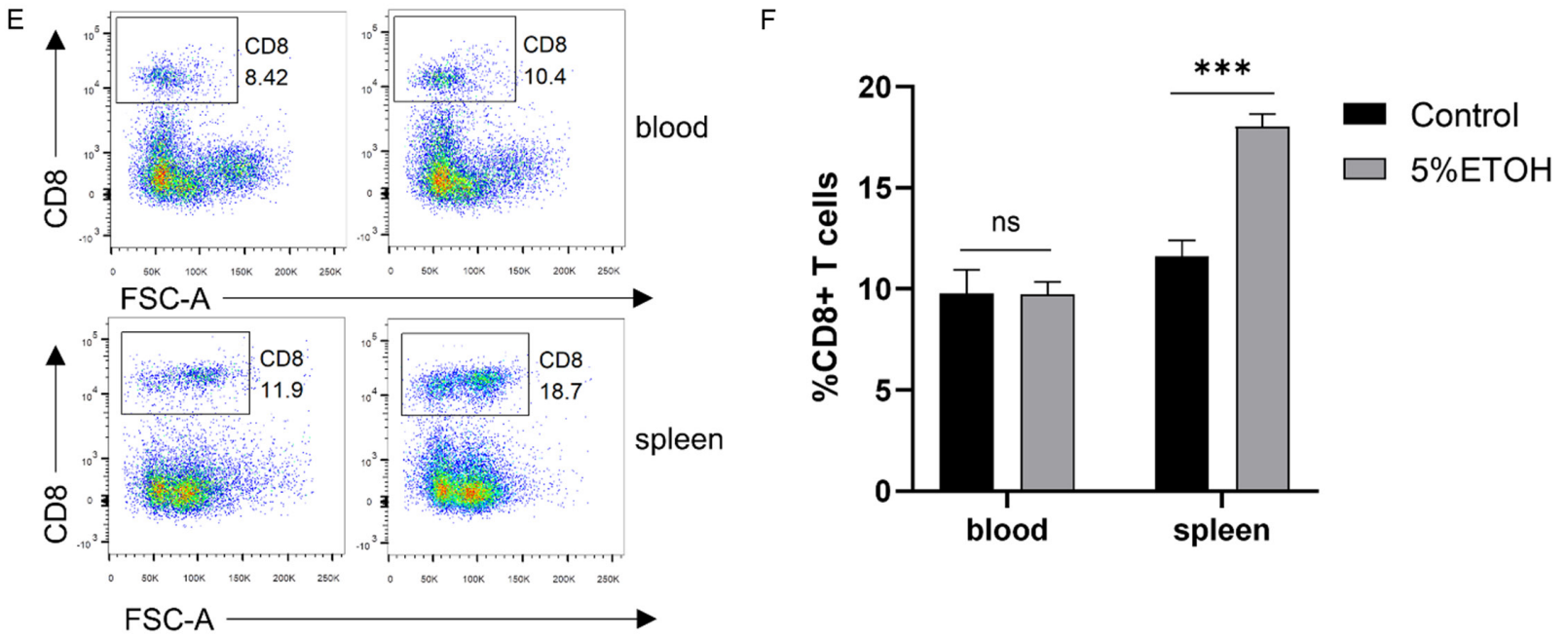
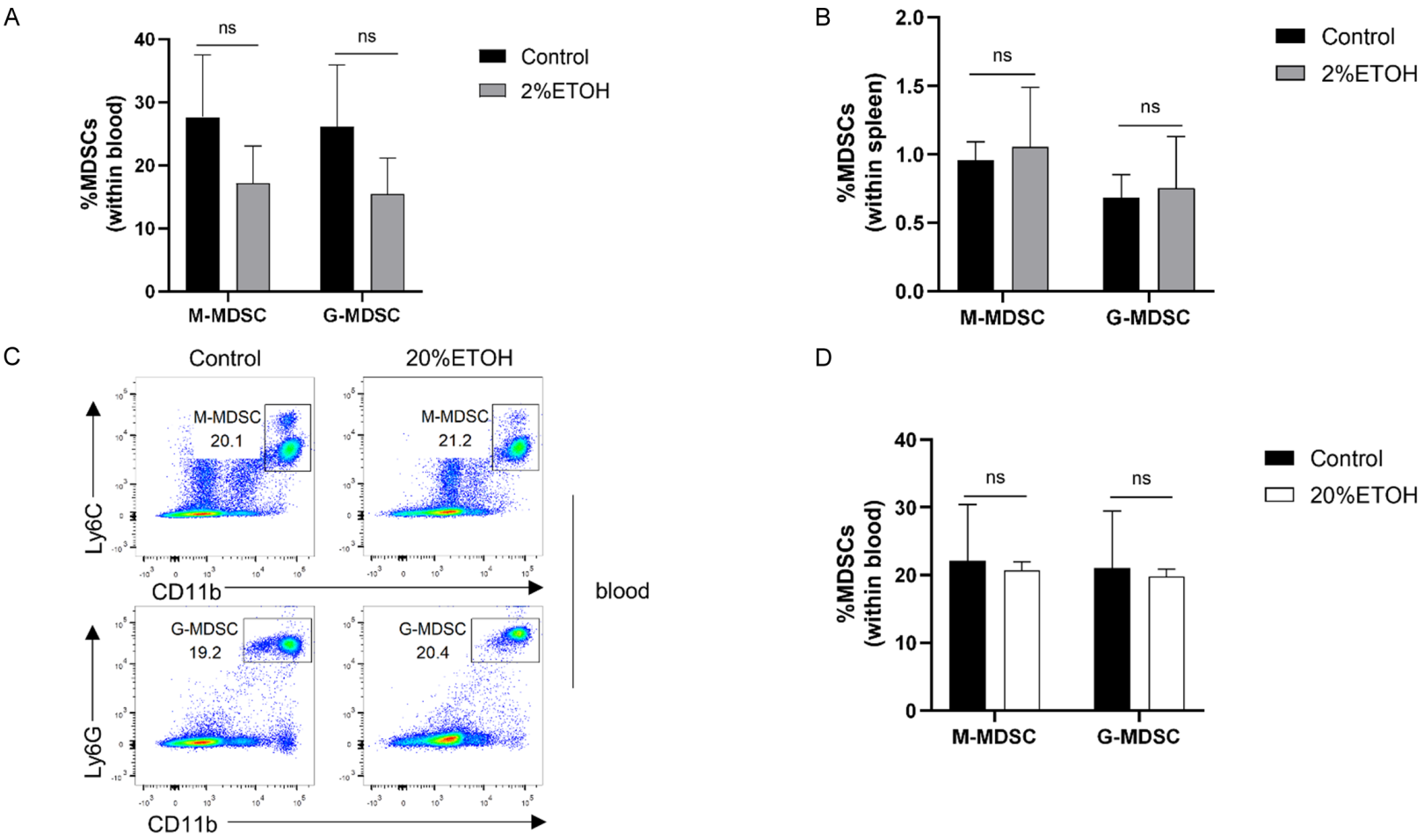


Figure 1. 5% (v/v) alcohol consumption activates the immune system by decreasing the proportion of myeloid-derived suppressor cells (MDSCs). Two groups of 5 weeks male Balb/c mice were fed either water or 5% (v/v) alcohol for 2 weeks. Flow cytometry was performed on the percentage of MDSCs and CD8+ T cells in the blood and spleen. A, B. The proportion of M-MDSCs and G-MDSCs in the blood of the 5% alcohol treatment group was determined (n=3). Representative flow cytometry data and the statistical diagram are shown. C, D. The proportions of M-MDSCs and G-MDSCs in the spleen of the 5% alcohol treatment group were determined (n=3). Representative flow cytometry data and statistical diagrams are shown. E, F. The percentage of CD8+ T cells in blood and spleen were analyzed and shown by flow cytometry data and statistical diagram, respectively (n=3). The statistical diagram is shown. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ns, not significant.


A
%MDSCs (within blood)
ns
ns
Control
2%ETOH
M-MDSC
G-MDSC
B
%MDSCs (within spleen)
Control
2%ETOH
C
Control
20%ETOH
M-MDSC
20.1
M-MDSC
21.2
Ly6C
CD11b
blood
G-MDSC
19.2
G-MDSC
20.4
Ly6G
CD11b
D
%MDSCs (within blood)
Control
20%ETOH

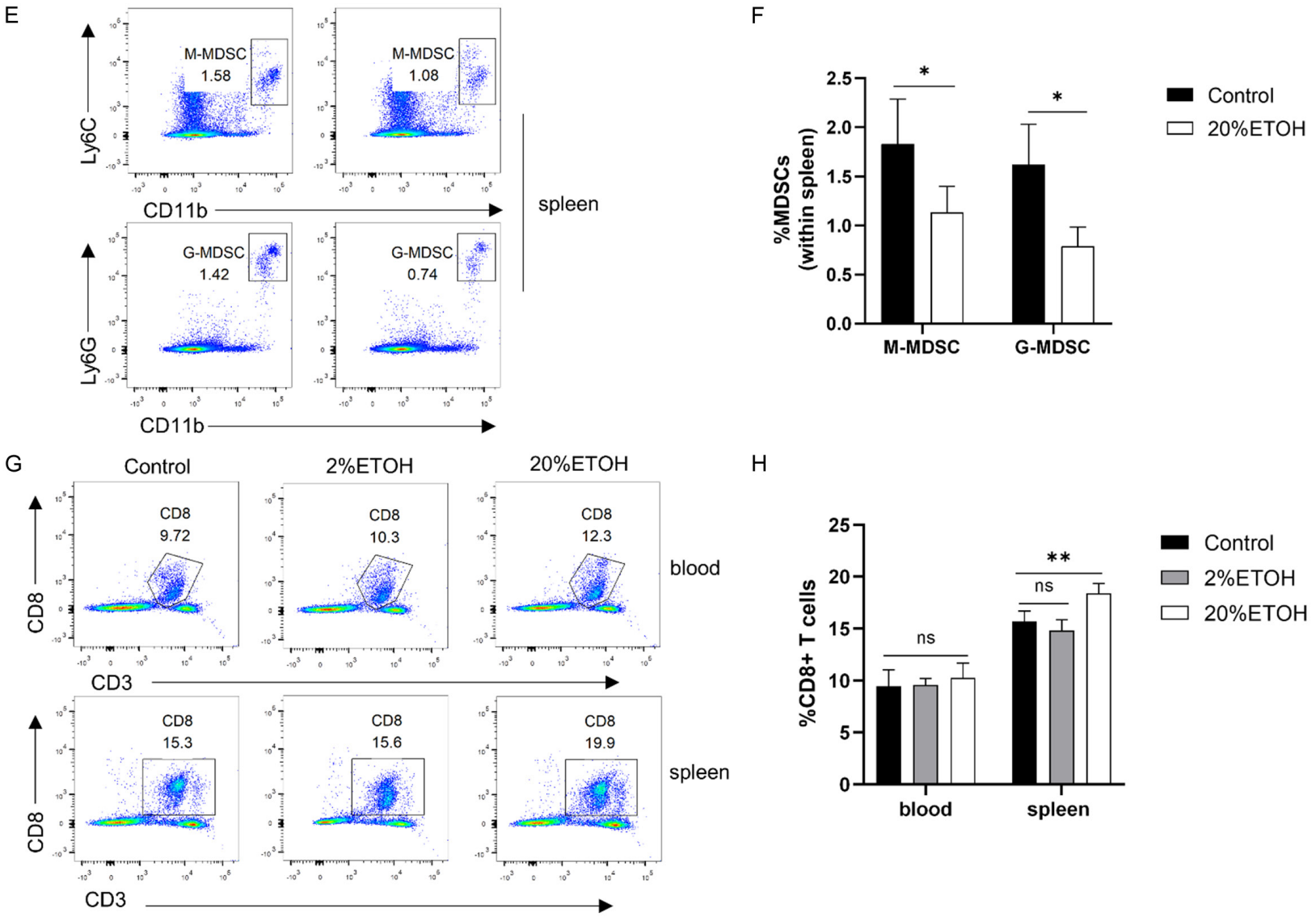


Figure 2. 20% (v/v) alcohol consumption activates the immune system by decreasing the proportion of myeloid-derived suppressor cells (MDSCs). Three groups of 5 weeks male Balb/c mice were fed either water or 2% (v/v) or 20% (v/v) alcohol for 2 weeks. Flow cytometry was performed on the percentage of MDSCs in the blood and spleens. A, B. The proportion of M-MDSCs and G-MDSCs in the blood and spleen of the 2% alcohol treatment group was determined (n=4). The statistical diagram is shown. C-F. The proportion of M-MDSCs and G-MDSCs in the blood and spleen of the 20% alcohol treatment group was determined (n=4). The statistical diagram is shown. G, H. The percentage of CD8+ T cells in blood and spleen of the 2% (v/v) or 20% (v/v) alcohol treatment group were analyzed and shown by flow cytometry data and statistical diagram, respectively (n=4). *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ns, not significant.

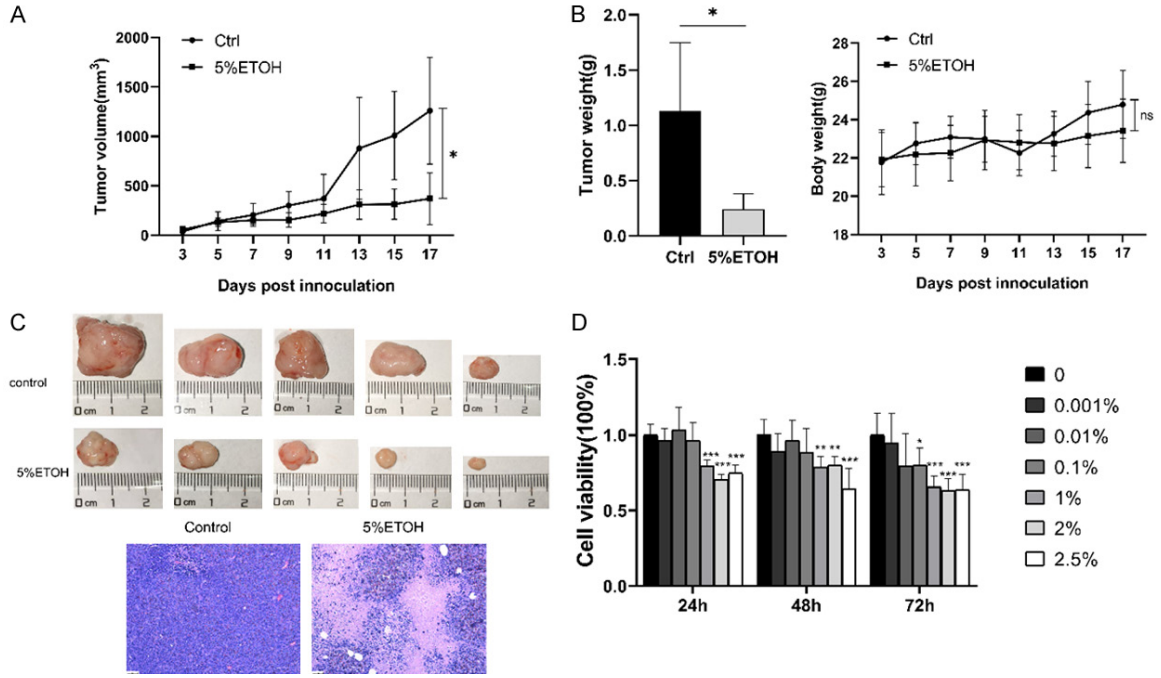


Figure 3. 5% alcohol consumption inhibited the growth of subcutaneous H22 hepatocellular carcinoma. 2 × 10^6 H22 hepatocellular carcinoma cells were injected subcutaneously into the right flank of male BALB/c mice after two weeks of the treatment of 5% (v/v) alcohol or water and continued to drink 5% (v/v) alcohol or water for another 3 weeks (n=5). A. Volume of the subcutaneous tumor was measured every day (n=5). B. Weight of subcutaneous tumor and body weight were shown (n=5). C. Picture of subcutaneous tumor and representative pictures of H&E staining of tumor (× 200 magnification, n=5). D. MTT assay was performed to evaluate the effect of alcohol on the proliferation of H22 cells (n=3). *: P < 0.05; **: P < 0.01; ***: P < 0.001; ns, not significant.

the number of immune cells. To access the effect of chronic alcohol consumption on HCC, a subcutaneous hepatocellular carcinoma model was established. 5% (v/v) alcohol as the sole drinking was given daily for five weeks including two weeks before tumor injection and three weeks after tumor injection. Alcohol consumption significantly inhibited HCC growth as evidenced by tumor volume, tumor weight, tumor images, and histologic findings (**Figure 3A-C**), which somewhat conflicts with previous conclusions in which alcohol is generally considered an independent risk factor for cancer. To clarify the underlying mechanism by which alcohol inhibits the growth of liver cancer, we first explored the direct inhibitory effect of ethanol on mouse liver cancer cells by proliferation assay. Our results demonstrated that H22 cell proliferation was notably inhibited by high concentrations of ethanol (from 1 to 2.5% over 24 hours, 48 hours, and 72 hours) (**Figure 3D**).

Consistent with the above experiments, we also examined the effect of 2% (v/v) alcohol and 20% (v/v) alcohol consumption on liver cancer growth. We observed that 20% (v/v) alcohol treatment inhibited tumor growth within 3 weeks after tumor inoculation and 2% (v/v) alcohol treatment slightly reduced the volume and weight of the tumor without statistically significant difference (**Figure 4A-D**). In addition, 20% (v/v) alcohol treatment inhibited tumor cells proliferation and tumor fibrosis, whereas 2% (v/v) alcohol treatment slightly inhibited tumor cells proliferation and tumor fibrosis characterized by lower PCNA- and α-SMA-positive staining (**Figure 4E**).

Chronic alcohol consumption reduces the proportion of MDSCs after tumor inoculation

To further investigate the effects of chronic alcohol consumption on the tumor immune microenvironment, we examined the proportion of MDSCs in the peripheral blood, spleens, and tumors after tumor inoculation. We found that another three weeks of 5% (v/v) alcohol intake after tumor inoculation also significantly

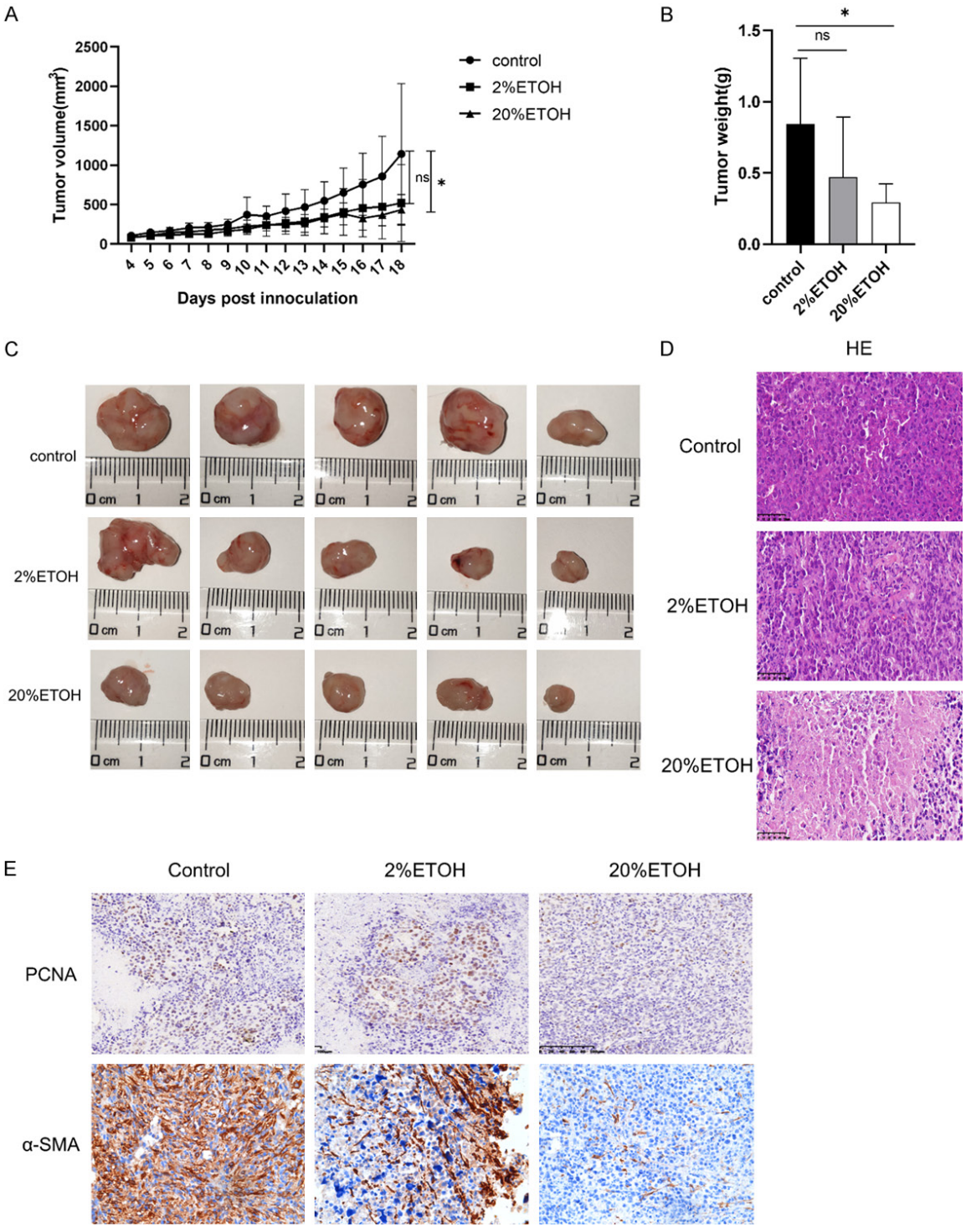


Figure 4. 20% alcohol treatment inhibited the growth of subcutaneous H22 hepatocellular carcinoma. 3 × 10^6 H22 hepatocellular carcinoma cells were injected subcutaneously into the right flank of male BALB/c mice after two weeks of the treatment of 20% (v/v) alcohol or 2% (v/v) alcohol or water and continued to drink 20% (v/v) alcohol or 2% (v/v) alcohol or water for another 3 weeks (n=5). A. Volume of the subcutaneous tumor was measured every day (n=5). B. Weight of the subcutaneous tumor was shown (n=5). C, D. Representative tumor images and photomicrographs of the H&E-stained tumor sections in tumor-bearing mice treated with vehicle or 2% (v/v) alcohol or 20% (v/v) alcohol. E. Representative photomicrographs of PCNA- and α-SMA-stained sections (original magnification, × 40; scale bar: 400 μm). *: P < 0.05; **: P < 0.01; ***: P < 0.001; ns, not significant.

reduced the proportions of G-MDSC and M-MDSC cells in the peripheral blood and spleens (**Figure 5A-D**), and tumor-infiltrating MDSCs showed a slight decline in the drinking mice without significant difference (**Figure 5E**, **5F**). Similarly, G-MDSCs and M-MDSCs in the blood and spleens of mice treated with 20% (v/v) alcohol also reduced significantly in the 3rd week after tumor inoculation, and that of the blood and spleen of 2% alcohol-treated mice did not reach a significant difference though decreasing slightly (**Figure 6A-D**). We also observed a significant reduction in tumor-infiltrating G-MDSCs but not M-MDSCs in tumor-bearing mice treated with 20% alcohol. However, there was no statistically significant difference in the MDSCs within the tumor of mice treated with 2% alcohol (**Figure 6E**, **6F**). The tumor microenvironment plays a key role in cancer development. MDSC cells are the key immune cells responsible for the immunosuppressive tumor microenvironment. Thus, the reduction of MDSC cells likely contributed to inhibiting tumor growth in our mouse models.

Chronic alcohol consumption increased cytotoxic T cell infiltration

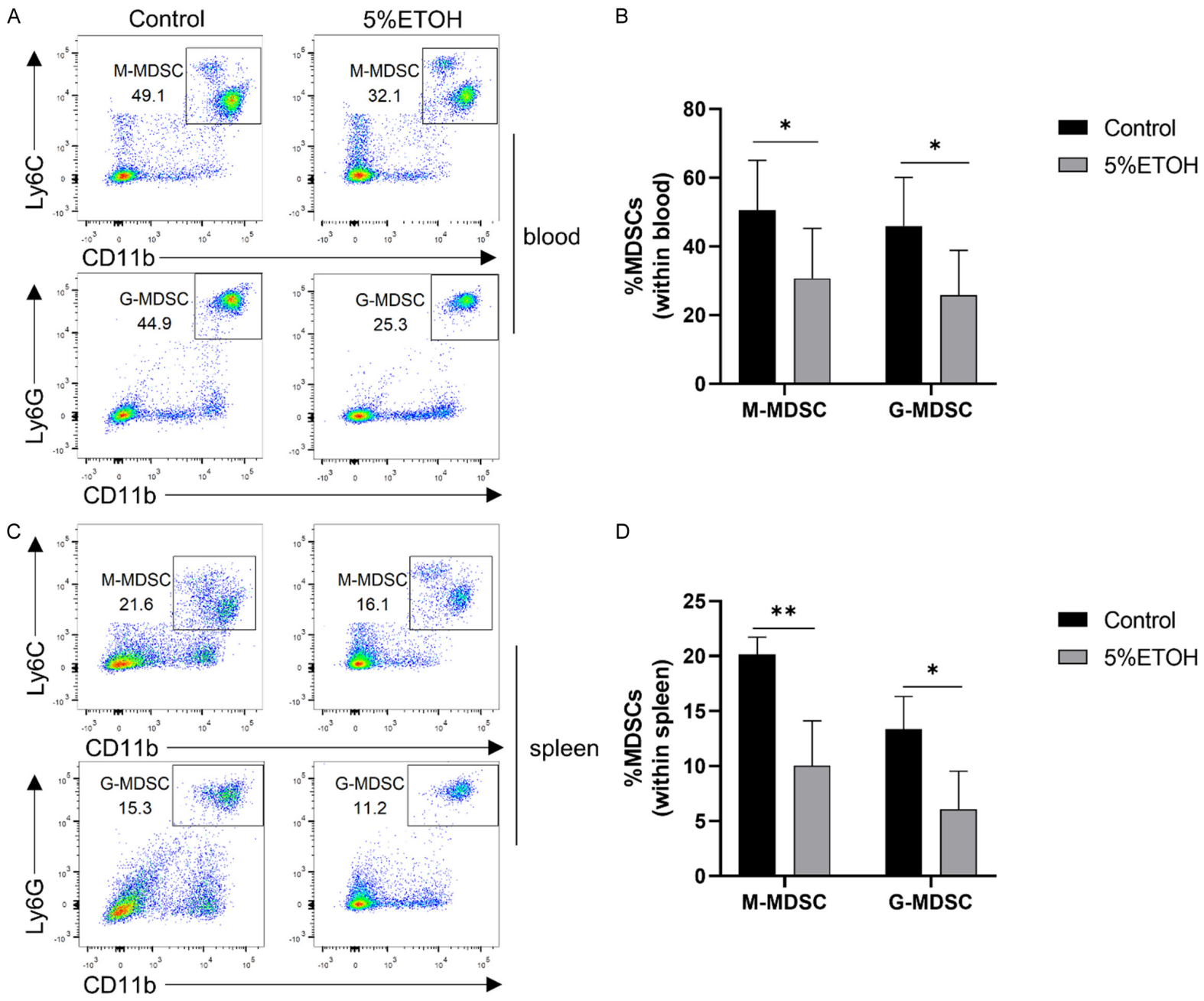
One of the main features of MDSCs as immunosuppressive cells in the tumor microenvironment is to inhibit the function of tumor-killing CD8+ T cells. We found that the number of CD8+ T cells in the spleen and tumor tissue of mice treated with 5% alcohol significantly increased compared to those in the control drinking water group (**Figure 7A**, **7B**). Meanwhile, the results of IHC analysis in the tumor tissue demonstrated a significant increase in the density of CD8+ T cells accompanied by an increase in granzyme B, a marker of immune activation, in the 5% alcohol treatment group (**Figure 7C**, **7D**). Similarly, both CD4+ T cells and CD8+ T cells were also significantly increased in the tumor tissue and the spleen of tumor-bearing mice treated with 20% alcohol (**Figure 7E-H**), and the tumor tissue in mice treated with 20% alcohol showed higher positive staining for CD8 and granzyme B than the control group (**Figure 7I**, **7J**), Although, both CD4+ T cells and CD8+ T cells were also significantly increased in the spleen of tumor-bearing mice treated with 2% alcohol, the proportion of CD8+ T cells infiltrating the tumor did not significantly increase (**Figure 7E-H**) and the tumor tissue in mice treated with 2% alcohol showed no significant difference of positive staining for CD8 and granzyme B compared to those in the control group (**Figure 7I**, **7J**). Our results indicated that chronic alcohol consumption remodels the immunosuppressive tumor microenvironment by reducing the number of immunosuppressive MDSCs and increasing the number of cytotoxic CD8+ T cells.

Chronic alcohol consumption reduced MDSCs expansion by inhibiting IL-6/JAK/STAT3

Emerging data suggested that IL-6 was critical for the induction of MDSCs, and therefore, influenced tumor progression. Thus, in order to elucidate the mechanism underlying MDSCs expansion in tumor-bearing mice, we measured the level of IL-6 production in the tumor tissue. We found a significant decrease in IL-6 production in tumors of 20% alcohol-treated mice, whereas IL-6 production was only slightly decreased in tumor tissues of 2% alcohol-treated mice characterized by lower IL-6-positive staining and decreased expression of IL-6 (**Figure 8A**, **8B**). Next, the expression and phosphorylation of multiple functional proteins induced by IL-6, including JAK/STAT and MAPK signaling pathways, were evaluated by Western blot (**Figure 8B**). The results showed that the phosphorylation level of STAT3 was slightly decreased in the tumor of 2% alcohol-treated mice compared to control mice, whereas the STAT3 phosphorylation was significantly decreased in the tumor of 20% alcohol-treated mice. However, phosphorylated extracellular regulated protein kinases (p-ERK) did not change significantly in 2% or 20% alcohol group compared to the control group (**Figure 8B**). The above data showed that JAK/STAT, rather than MAPK signaling pathway, may participate in IL-6-related MDSCs accumulation.

Discussion

In the current studies, we examined the effect of chronic alcohol consumption on the growth and immune statues of liver cancer using a syngeneic murine model of subcutaneously implanted H22 cells. We observed that 5% or 20% alcohol intake significantly inhibited the growth of subcutaneous H22 liver cancer, whereas 2% alcohol concentration had no inhibitory effect on tumor growth. Additionally, 5% or 20% alcohol concentration decreased the proportion of MDSCs and increased the


A
Control
5%ETOH
M-MDSC
49.1
M-MDSC
32.1
Ly6C
CD11b
blood
G-MDSC
44.9
G-MDSC
25.3
Ly6G
CD11b
B
%MDSCs (within blood)
80
60
40
20
0
*
*
M-MDSC
G-MDSC
Control
5%ETOH
C
M-MDSC
21.6
M-MDSC
16.1
Ly6C
CD11b
spleen
G-MDSC
15.3
G-MDSC
11.2
Ly6G
CD11b
D
%MDSCs (within spleen)
25
20
15
10
5
0
**
*
M-MDSC
G-MDSC
Control
5%ETOH

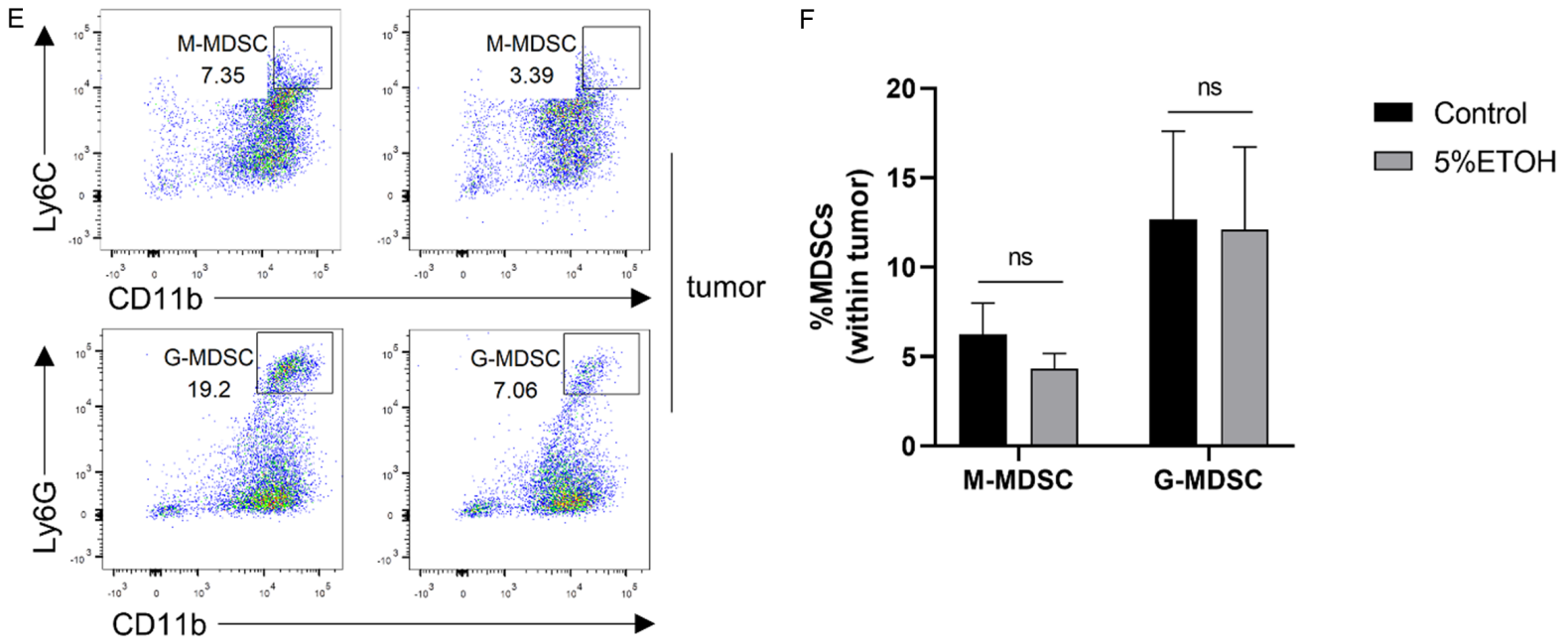
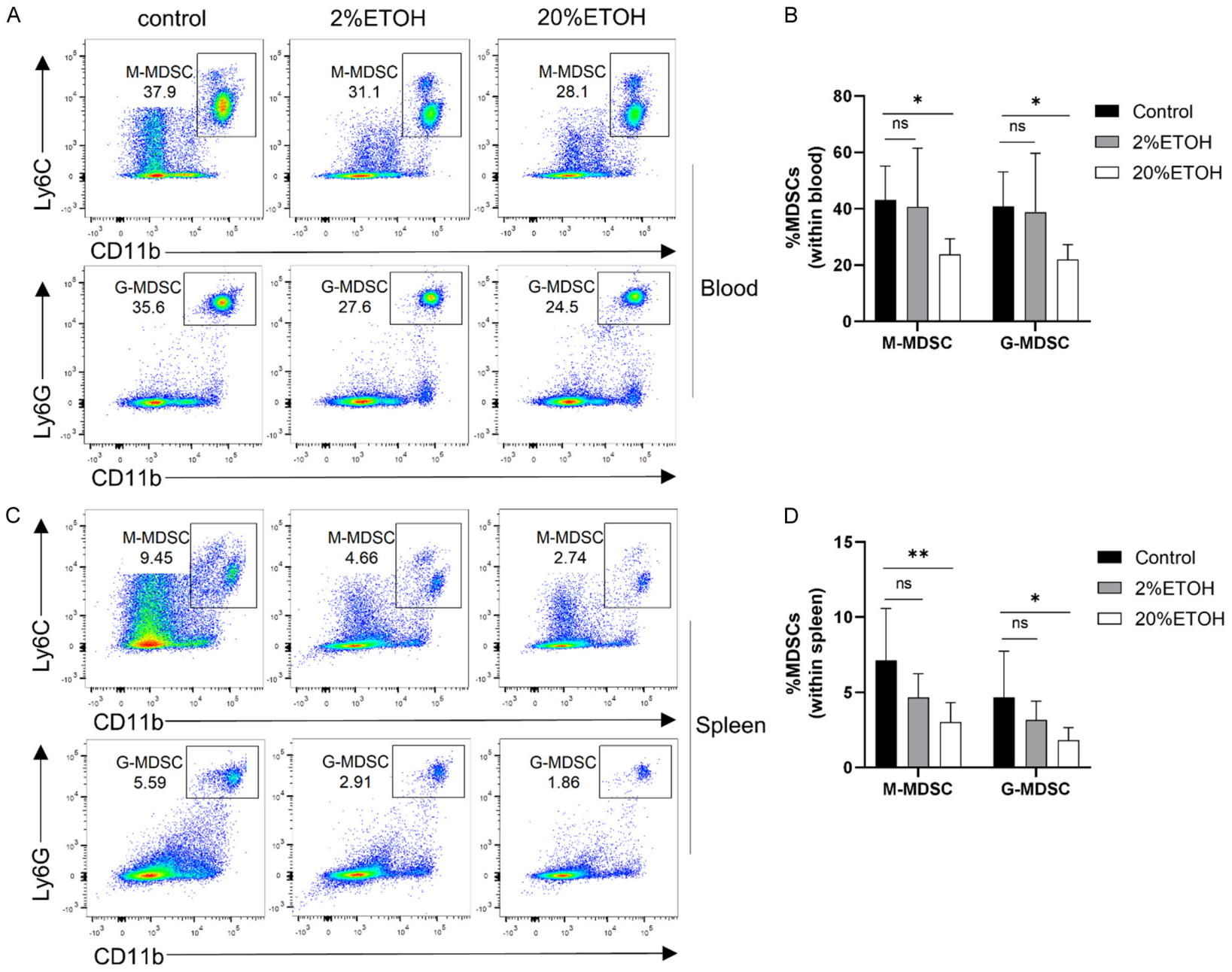


Figure 5. 5% alcohol treatment decreased the proportion of MDSCs in blood and spleens after tumor inoculation. Subcutaneous tumor mouse models were established and administrated as described in **Figure 3**. Flow cytometry was performed on the percentage of MDSCs in blood, spleens, and tumors. A, B. The proportion of M-MDSCs and G-MDSCs in blood was determined (n=6). Representative flow cytometry data and the statistical diagram are shown. C, D. The proportions of M-MDSCs and G-MDSCs in the spleen were determined (n=4). Representative flow cytometry data and the statistical diagram are shown. E, F. The proportions of M-MDSCs and G-MDSCs in the tumor were determined (n=4). Representative flow cytometry data and the statistical diagram are shown. *: $P < 0.05$; **: $P < 0.01$; ns, not significant.


A
control
2%ETOH
20%ETOH
M-MDSC
37.9
M-MDSC
31.1
M-MDSC
28.1
Ly6C
CD11b
G-MDSC
35.6
G-MDSC
27.6
G-MDSC
24.5
Ly6G
CD11b
Blood
B
%MDSCs (within blood)
80
60
40
20
0
*
ns
*
ns
M-MDSC
G-MDSC
Control
2%ETOH
20%ETOH
C
M-MDSC
9.45
M-MDSC
4.66
M-MDSC
2.74
Ly6C
CD11b
G-MDSC
5.59
G-MDSC
2.91
G-MDSC
1.86
Ly6G
CD11b
Spleen
D
%MDSCs (within spleen)
15
10
5
0
**
ns
*
ns
M-MDSC
G-MDSC
Control
2%ETOH
20%ETOH

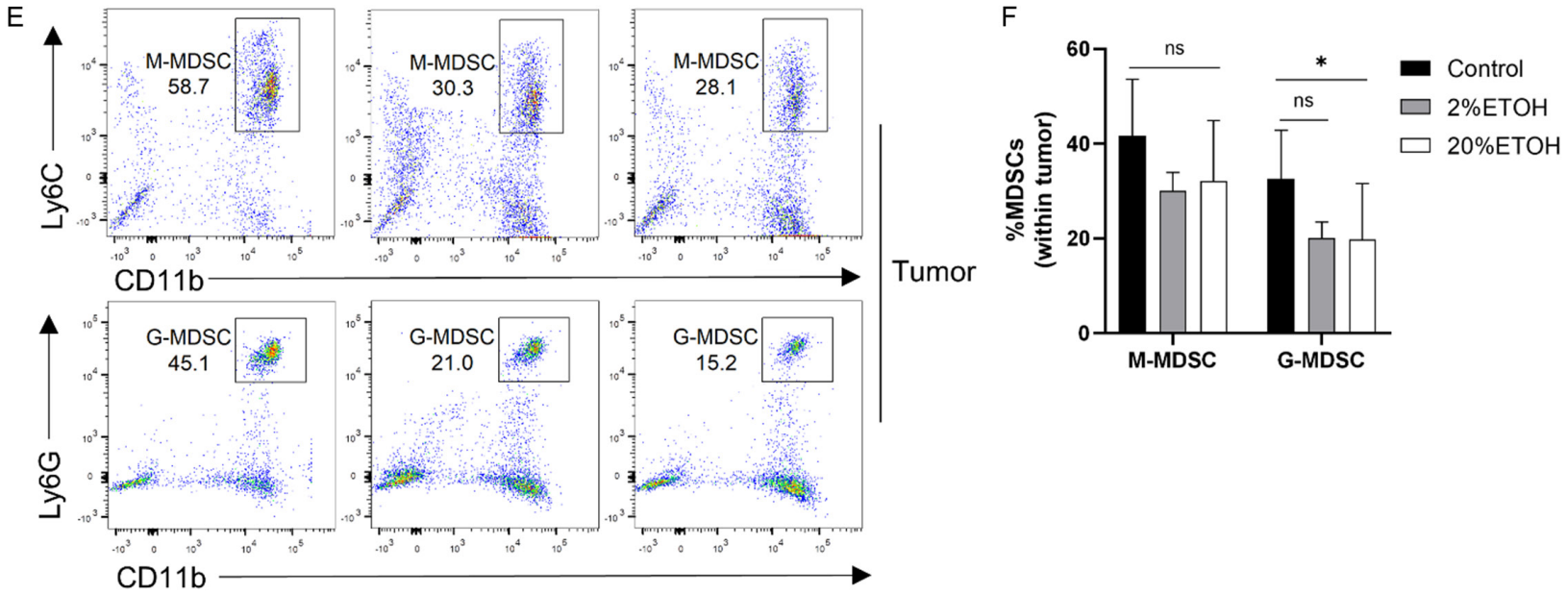
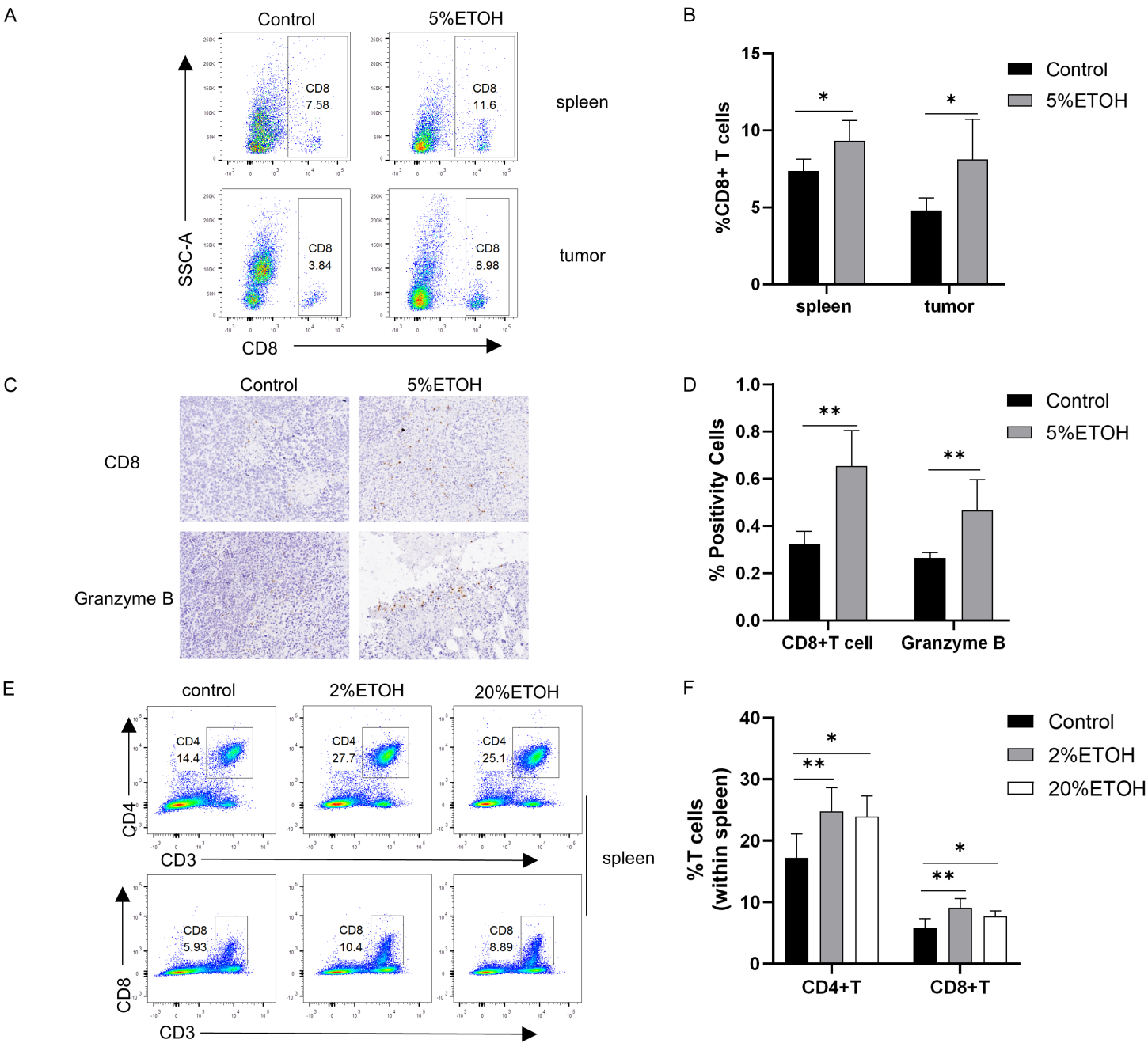


Figure 6. 20% alcohol treatment decreased the proportion of MDSCs in blood, spleens, and tumors after tumor inoculation. Subcutaneous tumor mouse models were established and administrated as described in **Figure 3**. Flow cytometry was performed on the percentage of MDSCs in blood, spleens, and tumors. A, B. The proportions of M-MDSCs and G-MDSCs in blood were determined (n=5). Representative flow cytometry data and statistical diagrams are shown. C, D. The proportions of M-MDSCs and G-MDSCs in the spleen were determined (n=5). Representative flow cytometry data and statistical diagrams are shown. E, F. The proportion of M-MDSCs and G-MDSCs in the tumor was determined (n=5). Representative flow cytometry data and statistical diagrams are shown. *: $P < 0.05$; **: $P < 0.01$; ns, not significant.


A
Control
5%ETOH
CD8
7.58
CD8
11.6
spleen
CD8
3.84
CD8
8.98
tumor
SSC-A
CD8
B
%CD8+ T cells
Control
5%ETOH
spleen
tumor
C
Control
5%ETOH
CD8
Granzyme B
D
% Positivity Cells
Control
5%ETOH
CD8+T cell
Granzyme B
E
control
2%ETOH
20%ETOH
CD4
14.4
CD4
27.7
CD4
25.1
CD4
CD3
spleen
CD8
5.93
CD8
10.4
CD8
8.89
CD8
CD3
F
%T cells
(within spleen)
Control
2%ETOH
20%ETOH
CD4+T
CD8+T

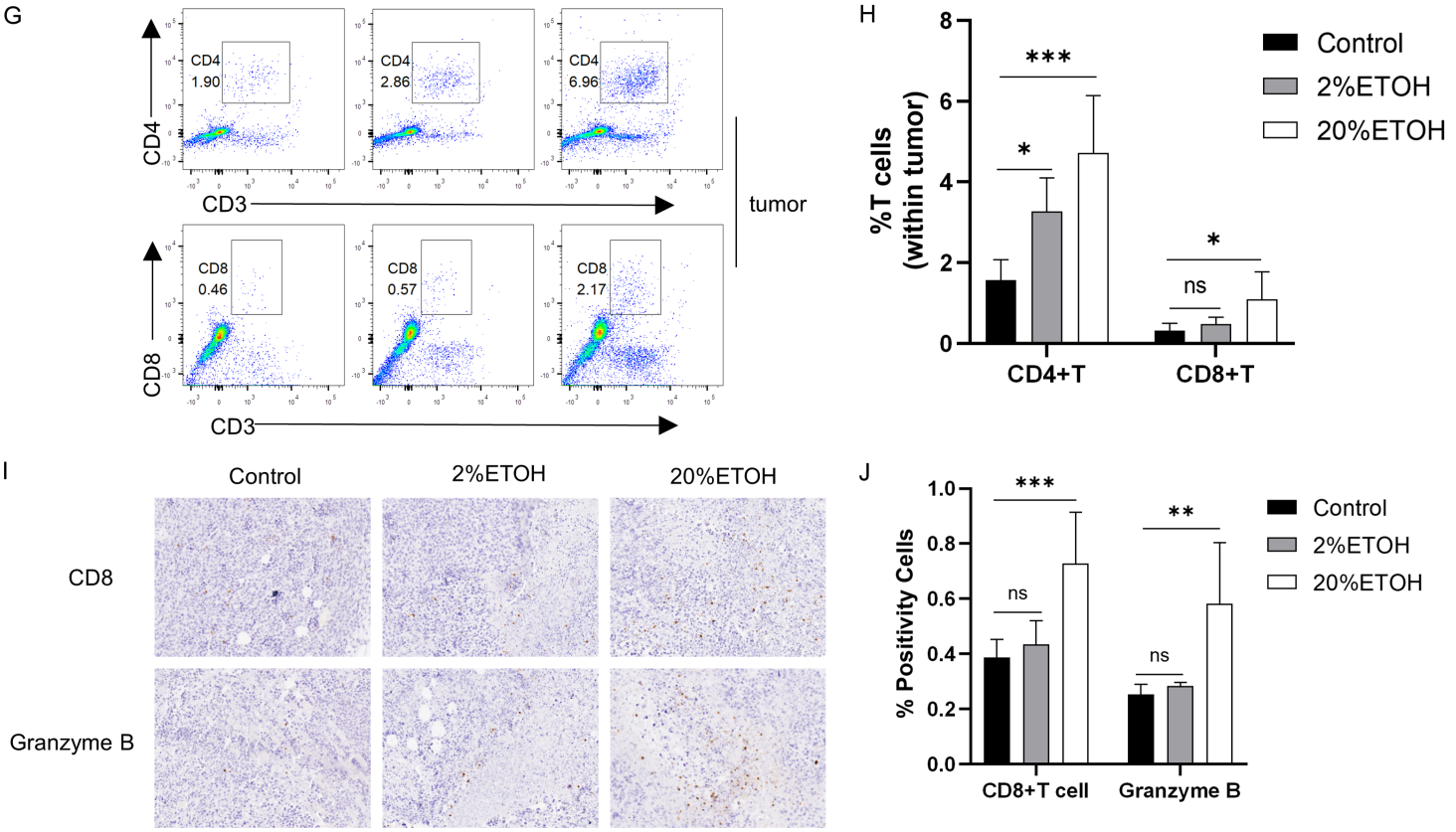


Figure 7. Alcohol treatment increased the proportion of CD8+ T cells in the spleen and tumor after tumor inoculation. Subcutaneous tumor mouse models were established and administrated as described in **Figure 3**. A, B. The proportions of CD8+ T cells in the spleen and tumor of mice treated with 5% alcohol were determined. Representative flow cytometry data and statistical diagrams were shown (n=5). C, D. Representative photomicrographs of CD8- and Granzyme B-stained sections in tumor tissue of mice treated with 5% alcohol (original magnification, × 40; scale bar: 400 μm) and quantification of CD8 and Granzyme B staining presented as the percentage of CD8- and Granzyme B-positive cells (n=5). E, F. The proportions of CD4+ T cells and CD8+ T cells in the spleen of mice treated with 2% or 20% alcohol were determined (n=5). Representative flow cytometry data and statistical diagrams are shown. G, H. The proportion of CD4+ T cells and CD8+ T cells in the tumor of mice treated with 2% or 20% alcohol were determined. Representative flow cytometry data and statistical diagram are shown (n=5). I, J. Representative photomicrographs of CD8- and Granzyme B-stained sections in tumor tissue of mice treated with 2% or 20% alcohol (original magnification, × 40; scale bar: 400 μm) and quantification of CD8 and Granzyme B staining presented as the percentage of CD8- and Granzyme B-positive cells (n=6).

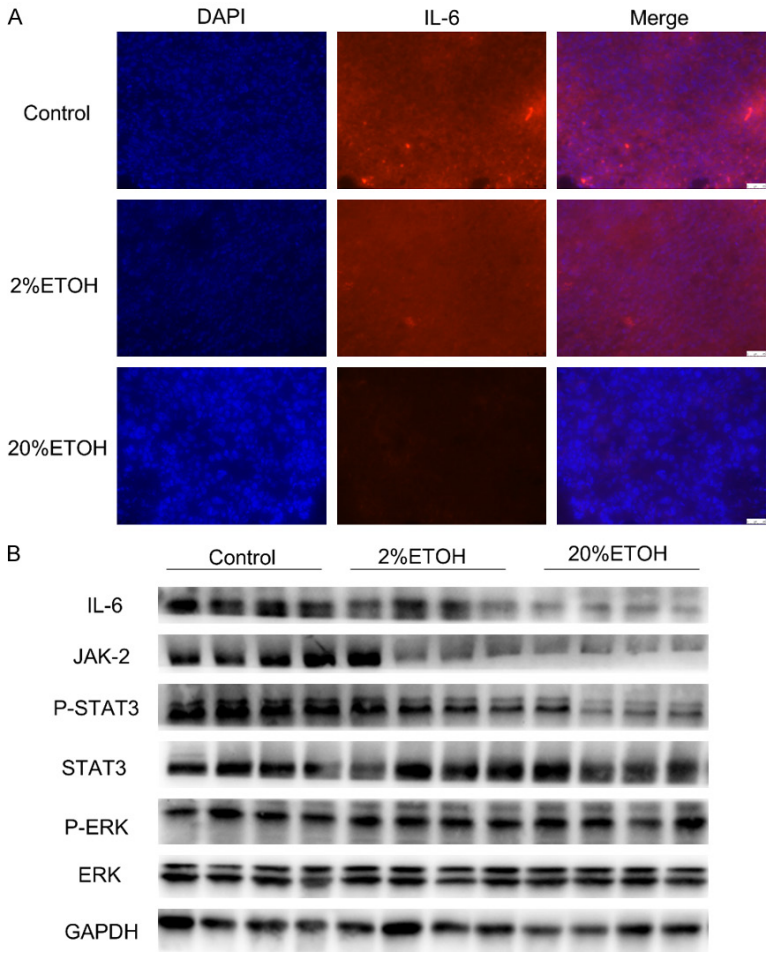


Figure 8. Chronic alcohol consumption may reduce MDSCs expansion by inhibiting IL-6/JAK/STAT3. A. Representative immunofluorescent staining of IL-6 production in the tumor site. B. The expression of IL-6, JAK-2, STAT3, phosphorylated STAT3, ERK, and phosphorylated ERK, in tumor tissues was detected by Western blots. *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$; ns, not significant.

proportion of CD8+ T cells before and after tumor inoculation, which may be associated with the inhibition of tumor growth.

Chronic alcohol intake increases the production of inflammatory cytokines and reactive oxygen stress. Inflammation regulates antitumor immunity and cancer progression [9]. However, the effect of the altered tumor immune status induced by chronic alcohol consumption on tumor growth remains unclear. Numerous preclinical studies suggest that chronic alcohol consumption regulated antitumor immunity but these studies presented controversial effects of alcohol consumption on cancer-associated outcomes. Some studies suggest alcohol promotes tumor growth and suppresses antitumor immunity. Keke Yu et al. reported that ethanol-induced M2 phenotype macrophages promoted tumor immune escape and reinforced the progression and metastasis of Lewis lung carcinoma (LLC) [14]. Zhang et al. found that in B16BL6 melanoma-bearing mice, chronic alcohol consumption impaired distribution and compromised circulation of B cells [15], decreased the percentage and number of NK cells in the peripheral lymph nodes [4], inhibited proliferation of memory T cells, accelerated the decay of IFN-gamma producing CD8(+) T cells, and increased MDSCs [16], all of which could be associated with melanoma progression and reduced survival.

While others suggest that alcohol inhibits tumor growth and improves antitumor immunity. It is reported that chronic alcohol consumption inhibited B16BL6 melanoma lung metastasis by increasing IFN-γ-producing NK, NKT, CD4(+), and CD8(+) T cells [6]. Beth A Vorderstrasse et al. showed that consumption of 18% alcohol significantly suppressed mammary tumor metastasis in a syngeneic tumor transplantation model, whereas consumption of 1% alcohol had no effect and the intermediate concentration (5%) could also reduce the number of metastatic nodules but did not reach statistical significance [5]. Zhang et al. demonstrated that alcohol consumption enhanced the anticancer effect of PD-1 blockade treatment in a murine CT26 colon cancer model while alcohol consumption alone did not affect tumor growth or animal survival [17]. Additionally, chronic alcohol consumption activates the immune system, which leads to the inhibition of subcutaneous melanoma growth and enhances the immune response to immunization with melanoma lysate, however, with tumor progression, alcohol consumption accelerates iNKT cell dysfunction

and compromises antitumor immunity, which leads to decreased survival of melanoma-bearing mice [18]. All of the above indicated that chronic alcohol consumption modulates tumor immunity by affecting the number of immunosuppressive cells and tumor-killing T cells, which may promote or inhibit tumor growth which may depend on the amount and pattern of alcohol consumed or the type and growth stage of the tumor. Our studies revealed that 5% and 20% alcohol intake significantly inhibited the growth of subcutaneous H22 liver cancer, whereas 2% alcohol concentration had no inhibitory effect on tumor growth. One potential explanation for the suppressed tumor growth in the alcohol-consuming mice was that ethanol directly inhibited the growth of H22 tumors. We found that various concentrations of ethanol directly inhibited H22 cell proliferation in vitro and 20% alcohol consumption can inhibit the proliferation of tumor cells in vivo, which is consistent with previous studies in which Hwi-Jin Im et al. demonstrated that MC38 colon cancer cells proliferation was notably inhibited by various concentrations of ethanol [9].

In addition to alcohol directly inhibiting tumor cells, the tumor microenvironment also plays a key role in cancer development and we considered whether the alcohol-associated promotion of liver cancer of H22 cells may be affected by an alteration of the host microenvironment. Cancer cells are under immune surveillance for tumor development, growth, and metastasis. It is well documented that alcohol is well known as an immunosuppressant; however, several studies have also reported that chronic alcohol intake can activate the immune system [18]. In the current study, 2 weeks of 5% and 20% alcohol intake significantly decreased the ratios of MDSCs in the peripheral blood and spleen. Interestingly, Sha Li et al. reported that the MDSCs (G-MDSCs) population in the blood and spleen was significantly increased under acute ethanol exposure [12]. After tumor inoculation, another 3 weeks of alcohol intake also reduced the number of MDSCs. This result somewhat conflicts with previous studies in which chronic alcohol consumption increased MDSCs in melanoma-bearing mice [16], which may be due to the different doses and patterns of drinking. MDSCs, one of the main components of the tumor immunosuppressive microenvironment, play an important role in promoting tumor growth, progression, and metastasis. Our results indicated that alcohol consumption of 5% or 20% may improve antitumor immunity through decreasing the accumulation of MDSCs and increasing tumor-killing CD8+ T cells, thereby exerting antitumor effects.

Previous studies had shown that the expansion and activation of MDSCs were influenced by several different factors, including prostaglandins, stem-cell factor (SCF), M-CSF, IL-6, and granulocyte/macrophage CSF (GM-CSF). IL-6 stimulated the JAK/STAT signaling pathway, which played a crucial role in the amplification and function of MDSCs in multiple tumors [19]. This study found an inhibition of the JAK/STAT signaling pathway during the IL-6-initiated development of MDSCs, which was demonstrated by a significant downregulation of the phosphorylated STAT3 proteins. These findings implied that tumor-derived IL-6 involved in the hyperactivation of the JAK/STAT signaling pathway was the leading cause of the development of competent MDSCs in hepatocellular carcinoma.

In summary, our work demonstrated that alcohol consumption of 5% or 20% inhibits the growth of hepatocellular carcinoma and active antitumor immunity through targeting the MDSCs population and the 2% alcohol concentration did not reduce the number of MDSCs and shows an obvious tumor-suppressing effect.

Acknowledgements

We sincerely thank the Traditional Chinese Pharmacological Laboratory of Southern Medical University (Guangzhou, China). This study was supported by the following foundation projects: The National Natural Science Foundation of China (grant Nos. 81774261, 8197150327, 8207150366, 8217152472, 8227150263), the Guangdong Province Key Field R&D Program (grant No. 2020B1111120001), and the Natural Science Foundation of Guangdong Province (grant No. 2022A1515011260).

Disclosure of conflict of interest

None.

Address correspondence to: Changjun Wang, Department of Traditional Chinese Medicine, Guangdong Provincial People's Hospital (Guangdong Academy of Medical Sciences), Southern Medical

University, Guangzhou 519041, Guangdong, China. E-mail: gzwcj@163.com

References

[1] Veglia F, Sanseviero E and Gabrilovich DI. Myeloid-derived suppressor cells in the era of increasing myeloid cell diversity. Nat Rev Immunol 2021; 21: 485-498.

[2] Nowak AJ and Relja B. The impact of acute or chronic alcohol intake on the NF-κB signaling pathway in alcohol-related liver disease. Int J Mol Sci 2020; 21: 9407.

[3] The Lancet. Alcohol and cancer. Lancet 2017; 390: 2215.

[4] Zhang H, Zhu Z and Meadows GG. Chronic alcohol consumption decreases the percentage and number of NK cells in the peripheral lymph nodes and exacerbates B16BL6 melanoma metastasis into the draining lymph nodes. Cell Immunol 2011; 266: 172-179.

[5] Vorderstrasse BA, Wang T, Myers AK, Wyrick KL and Meadows GG. Alcohol consumption suppresses mammary tumor metastasis in a syngeneic tumor transplantation model. Breast Cancer Res Treat 2012; 136: 729-737.

[6] Zhang H, Zhu Z, McKinley JM and Meadows GG. IFN-γ is essential for the inhibition of B16BL6 melanoma lung metastasis in chronic alcohol drinking mice. Clin Exp Metastasis 2011; 28: 301-307.

[7] Ramadori P, Cubero FJ, Liedtke C, Trautwein C and Nevzorova YA. Alcohol and hepatocellular carcinoma: adding fuel to the flame. Cancers (Basel) 2017; 9: 130.

[8] Ma HY, Yamamoto G, Xu J, Liu X, Karin D, Kim JY, Alexandrov LB, Koyama Y, Nishio T, Benner C, Heinz S, Rosenthal SB, Liang S, Sun M, Karin G, Zhao P, Brodt P, McKillop IH, Quehenberger O, Dennis E, Saltiel A, Tsukamoto H, Gao B, Karin M, Brenner DA and Kisseleva T. IL-17 signaling in steatotic hepatocytes and macrophages promotes hepatocellular carcinoma in alcohol-related liver disease. J Hepatol 2020; 72: 946-959.

[9] Im HJ, Kim HG, Lee JS, Kim HS, Cho JH, Jo IJ, Park SJ and Son CG. A preclinical model of chronic alcohol consumption reveals increased metastatic seeding of colon cancer cells in the liver. Cancer Res 2016; 76: 1698-1704.

[10] Xie Y, Zhang Y, Wei X, Zhou C, Huang Y, Zhu X, Chen Y, Wen H, Huang X, Lin J, Wang Z, Ren Y, Fan B, Deng X, Tan W and Wang C. Jianpi Huayu decoction attenuates the immunosuppressive status of H(22) hepatocellular carcinoma-bearing mice: by targeting myeloid-derived suppressor cells. Front Pharmacol 2020; 11: 16.

[11] Cao M, Huang W, Chen Y, Li G, Liu N, Wu Y, Wang G, Li Q, Kong D, Xue T, Yang N and Liu Y. Chronic restraint stress promotes the mobilization and recruitment of myeloid-derived suppressor cells through β-adrenergic-activated CXCL5-CXCR2-Erk signaling cascades. Int J Cancer 2021; 149: 460-472.

[12] Li S, Wang N, Tan HY, Hong M, Yuen MF, Li H and Feng Y. Expansion of granulocytic, myeloid-derived suppressor cells in response to ethanol-induced acute liver damage. Front Immunol 2018; 9: 1524.

[13] Gabrilovich DI and Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol 2009; 9: 162-174.

[14] Yu K, Yang J, Wang F, Chen L, Lu Y, Luo J and Wang S. Ethanol supports macrophage recruitment and reinforces invasion and migration of Lewis lung carcinoma. Alcohol Clin Exp Res 2014; 38: 2597-2606.

[15] Zhang H, Zhu Z and Meadows GG. Chronic alcohol consumption impairs distribution and compromises circulation of B cells in B16BL6 melanoma-bearing mice. J Immunol 2012; 189: 1340-1348.

[16] Zhang H and Meadows GG. Chronic alcohol consumption enhances myeloid-derived suppressor cells in B16BL6 melanoma-bearing mice. Cancer Immunol Immunother 2010; 59: 1151-1159.

[17] Zhang H, Xia Y, Wang F, Luo M, Yang K, Liang S, An S, Wu S, Yang C, Chen D, Xu M, Cai M, To KKW and Fu L. Aldehyde dehydrogenase 2 mediates alcohol-induced colorectal cancer immune escape through stabilizing PD-L1 expression. Adv Sci (Weinh) 2021; 8: 2003404.

[18] Zhang F, Zhu Z, Meadows GG and Zhang H. Chronic alcohol consumption inhibits melanoma growth but decreases the survival of mice immunized with tumor cell lysate and boosted with α-galactosylceramide. Int Immunopharmacol 2015; 28: 359-368.

[19] An J, Feng L, Ren J, Li Y, Li G, Liu C, Yao Y, Yao Y, Jiang Z, Gao Y, Xu Y, Wang Y, Li J, Liu J, Cao L, Qi Z and Yang L. Chronic stress promotes breast carcinoma metastasis by accumulating myeloid-derived suppressor cells through activating β-adrenergic signaling. Oncoimmunology 2021; 10: 2004659.