Chronic alcohol consumption enhances myeloid-derived suppressor cells (MDSC) in B16BL6 melanoma-bearing mice

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Abstract We previously found that chronic alcohol consumption decreases the survival of mice bearing subcutaneous B16BL6 melanoma. The underlying mechanism is still not completely understood. Antitumor T cell immune responses are important to inhibiting tumor progression and extending survival. Therefore, we examined the effects of chronic alcohol consumption on the functionality and regulation of these cells in C57BL/6 mice that chronically consumed 20% (w/v) alcohol and subsequently were inoculated subcutaneously with B16BL6 melanoma cells. Chronic alcohol consumption inhibited melanoma-induced memory T cell expansion and accelerated the decay of IFN-γ producing T cells in the tumor bearing mice. $Foxp3^{+}CD4^{+}CD25^{+}$ regulatory T cells were not affected; however, the percentage of myeloid-derived suppressor cells (MDSC) was significantly increased in the peripheral blood and spleen. T cell proliferation as determined by carboxyfluorescein succinimidyl ester (CFSE) labeling experiments *in vitro* was inhibited by alcohol consumption relative to control water drinking melanoma-bearing mice. Collectively, these data show that chronic alcohol consumption inhibits proliferation of memory T cells, accelerates the decay of IFN-γ producing $CD8^{+}$ T cells, and increases MDSC, all of which could be associated with melanoma progression and reduced survival.

Keywords Alcohol consumption. MDSC. Melanoma. Memory T cells. Interferon gamma

Introduction

Many dietary and nutritional factors modulate the immune response, both positively and negatively. Alcohol consumption is a dietary component, which when abused is generally immunosuppressive. A causative agent for cancers of the mouth, pharynx and larynx, esophagus, colorectum (men), and breast, alcohol consumption is also emerging as a risk factor for several other types of cancer [1] . Alcohol consumption is associated with enhanced metastasis of colorectal carcinoma to the liver [19] and with decreased survival of lung cancer patients [26]. Survival also is decreased in mice bearing subcutaneous melanoma tumors and consuming high levels of alcohol (20% w/v) [3]. The underlying factors leading to decreased survival in these melanoma-bearing mice are not completely known.

Melanoma cancers are immunogenic and the host immune system plays an important role in controlling tumor growth and metastasis [4, 18]. Results from clinical tumor immunotherapy trials indicate that the number and function of tumor-specific $CD8^+$ T cells are critical to the survival of melanoma patients [32]. Tumors can produce multiple factors that induce specific cell populations that inhibit antitumor immune functions [5, 37]. At least three types of cells, $Foxp3^+$ $CD4^+CD25^+$ regulatory T (Treg) cells, Mac-1^+Gr-1^+ myeloid-derived suppressor cells (MDSC), and tumor associated macrophages (TAM) have been identified as key players that inhibit host antitumor immune activity by suppressing T cell proliferation, cytolytic activity and cytokine production [6, 8, 20, 23].

Previously, we showed in mice not inoculated with melanoma that chronic alcohol consumption decreased the overall number of T cells and that this induced T cell homeostatic proliferation of non-antigen specific T cells expressing the memory phenotype [42]. However, the total numbers of T cells never return to normal levels. Melanoma induces the proliferation of tumor-specific memory T cells. These cells are important to the control of tumor progression and survival in melanoma patients [31, 41]. Because chronic alcohol consumption decreased T cell numbers in the absence of tumor, we examined the hypothesis that this would impact the generation of antigen-specific memory T cells. We found that alcohol consumption inhibited melanoma specific $CD8^+$ T cell expansion, and in addition accelerated loss of interferon (IFN)-γ producing T cells, and increased immunosuppressive MDSC.

Material and methods

Animals and alcohol administration

Animal use and alcohol administration followed previously established procedures [43]. Briefly, female C57BL/6 mice at the age of 6-7 weeks were purchased from Charles River Laboratories (Wilmington, DE). After arrival the mice were single housed in plastic, filter toped cages in the Wegner Hall Vivarium at Washington State University according to the Principles of laboratory animal care (NIH publication No. 85023 revised 1985). The vivarium is accredited by the Association for Assessment and Accreditation

of Laboratory Animal Care and the animal protocol was approved by the Institutional Animal Care and Use Committee. One week after arrival, the mice were randomly divided into two groups. One group of mice continued to drink water and the other was given 20% (w/v) of alcohol as the sole drinking source throughout the experimental period.

Tumor cell culture and inoculation

B16BL6 melanoma cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% of fetal bovine serum (FBS, Equitech-Bio Inc, Kerrville, TX). Cells at 50-70% confluence were harvested and resuspended in calcium-free, magnesium-free phosphate buffered saline to a final concentration of 10^6 cells/ml. After three to six months of consuming alcohol, mice were injected subcutaneously with $2x10^5$ cells into the right hip. They were euthanized at the indicated time points. Experiments were replicated as indicated in the figure legends. Similar results were obtained in mice consuming alcohol for 3-6 months before melanoma inoculation.

Flow cytometry and specific antibodies

The cellular phenotype and expression of melanoma-specific $CD8^+$ T cells, $FoxP3^+$ Treg cells, and IFN-γ producing $CD8^+$ T cells were analyzed by flow cytometry following previously established procedures [42]. The following fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP)-Cy5.5, or PE-Cy5.5 labeled

anti-mouse antibodies were used. Anti-CD11b (M1/70), anti-Ly6C (AL-21), anti-CD4 (L3T4), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD25 (PC-61), anti-CD124-PE (mIL-4R-M1), and anti-IFNγ (XMG1.2) were purchased from BD Biosciences Pharmingen (San Diego, CA). Anti-NK1.1 (PK136), anti-CD3 (145-2C11), anti-FoxP3 (MF-14), Anti-F4/80, anti-Gr-1 (RB6-8C5) were purchased from BioLegend (San Diego, CA).

Leukocyte isolation from blood

Peripheral blood leukocytes (PBL) were isolated with Lympholyte-M (Cedarlane Laboratories Limited, Burlington, NC) as described previously [42]. An appropriate number of isolated cells were suspended in phosphate buffered saline (PBS) +0.1% bovine serum albumin (BSA) for further use.

Splenocyte isolation

Whole spleens were used to isolate lymphocytes using previously established procedures [42]. Total cell number and viability were determined with the Cell Viability Analyzer (Vi-Cell, Beckman Coulter, Fullerton, CA). Cells were resuspended in an appropriate volume of PBS + 0.1% BSA for further use.

Tumor infiltrating lymphocyte (TIL) isolation

Tumors from mice were isolated and washed with ice-cold PBS + 0.1% BSA, passed through a wire mesh screen into ice-cold PBS + 0.1% BSA, and then centrifuged at 4°C for 10 min at 800 x g. The cells were resuspended into 5 ml of warm PBS + 0.1% BSA and layered onto 5 ml of warm Lympholyte-M in a 15 ml tube. The tube was centrifuged at room temperature for 20 min at 800 x g. The TIL at the interface were collected, counted and resuspended in appropriate amount of PBS + 0.1% BSA for further use.

Analysis of melanoma specific $CD8^+$ T cells

Melanoma specific $CD8^+$ T cells were determined by flow cytometry with PE-labeled T3700 tetramer and PE-Cy5-labeled CD8 anti-mouse monoclonal antibody. The H-$2D^b$ binding peptide of mouse gp100 (gp100), $gp100_{25\text{-}33}$: EGSRNQDWL, was synthesized by Celtek Bioscience, LLC (Nashville, TN). The gp100/H-$2D^b$ tetramer (T3700) was synthesized by NIH Tetramer Facility and labeled with PE.

Analysis of Treg Cells

Specific $CD4^+CD25^+$ Treg cells were identified by intracellular staining of FoxP3 according to manufacturer's instructions with modification. Briefly, approximately $5x10^5$ cells were incubated with anti-mouse CD32/16 monoclonal antibody (2.4G2) for 5-10 min. Then appropriate amount of anti-mouse CD4-PerCp and anti-mouse CD25-FITC were added to the cells and incubated on ice for 20 min in a dark chamber. After two washes with cold PBS + 0.1% BSA + 0.09% NaN3 buffer, cells were fixed with 100 ul

of Cytofix/Cytoperm fixation solution (BD Biosciences, San Diego, CA) for 20 min. Cells then were washed with Cytofix/Cytoperm washing buffer twice and stained with appropriate amount of anti-mouse FoxP3 for 30 min. Then cells were washed twice with Cytofix/Cytoperm washing buffer and analyzed by flow cytometry.

Intracellular staining and analysis for IFN-γ

Splenocytes (1 x 10^6 cells) were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 5 hr. Then IFN- γ expression was determined by intracellular staining in $CD8^+$ T cells according to procedures described previously [42].

CFSE labeling and T cell in vitro proliferation assay

Splenocytes ($1x10^7$) in 100 µl of PBS + 0.1% BSA were mixed thoroughly with 900 µl of 2.5 µM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA) in PBS. The mixture was incubated at 37°C for 8 min after which 30 ml of RPMI 1640 medium containing 10% FBS (complete medium) was added to terminate the CFSE staining. The cells were centrifuged for 5 min at 800 x g. The cell pellet was suspended once with complete medium, centrifuged again, and then resuspended in complete medium at 10^8cells/ml. Cells were cultured in anti-mouse CD3 monoclonal antibody coated 24-well plates. Each well contained 2 ml complete medium, 5 µg/ml anti-mouse CD28 monoclonal antibody, and $1x10^6$cells/ml. Cells were cultured at 37°C in an incubator containing 5% CO_2 for 90 hr. Then cells were harvested and washed with ice-cold PBS

+0.1% BSA. Anti-mouse CD32/16 monoclonal antibody was added to the pelleted cells to block non-specific Fcγ receptor binding, stained with anti-mouse CD8-PE/cy5, and then analyzed by flow cytometry [42]. Proliferation in $CD8^+$ cells was determined based on the respective CFSE gated histograms.

Statistical analysis

Data were analyzed except were otherwise indicated with the Microsoft Excel statistical program. The results were expressed as mean ± SD. Significant differences between groups were determined using Student's two-tailed t-test. Values were considered different at $p< 0.05$. The data involving multiple group comparisons in Fig. 1 were analyzed Graph Pad Prism software. Pair-wise comparisons as a function of weeks were determined by Dunnet's Multiple Comparison Test after ANOVA. Values were considered significant at $p<0.05$.

Results

Chronic alcohol consumption inhibits memory phenotype $CD8^+$ T cell expansion in melanoma-bearing mice

We previously found that chronic alcohol consumption increased the percentage of $CD44^{hi}$ $CD8^+$ memory phenotype T cells in mice not inoculated with melanoma by stimulating T cell homeostatic proliferation [42], and expected that tumor-specific

memory T cells would similarly be expanded in mice inoculated with melanoma. To evaluate this possibility, we inoculated mice with B16BL6 melanoma drinking alcohol for three months. Alcohol consumption increased the percentage of memory T cells by 19% in non-tumor injected mice compared to mice drinking water (Fig. 1C). In water drinking mice significant differences in the percentage of memory T cells were observed from 1-3 weeks after tumor inoculation compared to control mice not injected with tumor ($P<0.05$). The peak response was a 2-fold increase at two weeks and this level was maintained at 3 weeks after inoculation. The percentage of memory T cells in alcohol consuming mice was not different from mice not injected with tumor at one week and at three weeks after tumor inoculation ($P>0.05$). A significant increase occurred at week two; however, the percentage of increase was lower than in water drinking mice ($P<0.05$). These results indicate that alcohol consumption impairs tumor-induced memory T cell expansion. In addition these cells decline to control levels at three weeks in the alcohol consuming mice, but not in the water-drinking mice.

Chronic alcohol consumption inhibits tumor specific $CD8^+$ T cell expansion

B16BL6 melanoma cells are immunogenic and they induce tumor specific T cell expansion when inoculated into mice. These cells play essential roles in tumor surveillance and in the inhibition of tumor growth. We used a gp100/H-$2D^b$ (T3700) tetramer to examine the effects of chronic alcohol consumption on B16BL6 melanoma specific $CD8^+$ T cells [25]. We found the melanoma-specific $CD8^+$ T cells, like the memory T cells, reached a peak two weeks after tumor inoculation in both groups and

then decreased at three weeks (Fig. 2B). The percentages were significantly lower in the alcohol consuming compared to the water drinking group at all time. The number of gp100-specific $CD8^+$ T cells was 2.5-fold lower in the spleen of the alcohol consuming mice than the water drinking mice three weeks after tumor inoculation (Fig. 2C).

Chronic alcohol consumption accelerates the decay of IFN-γ producing $CD8^+$ T cells in melanoma-bearing mice

We previously reported that chronic alcohol consumption increased the percentage of IFN-γ producing T cells in non-tumor injected mice [42]. Because of the important role that IFN-γ plays in the anti-tumor immune response to melanoma [7, 14, 24], we examined the effect of alcohol consumption on production of this cytokine in $CD8^+$ T cells from the spleen as a function of time. Similar to our findings in non-tumor injected mice, the percentage of IFN-γ producing $CD8^+$ T cells increased proportionately in alcohol consuming compared to water drinking, melanoma-bearing mice at Days 5 and 11: during the early stages of tumor growth (Fig. 3). However, by day 18 both experimental groups exhibited a significant decline in these cells that continued to Day 26. The rate of decrease in the percentage of IFN-γ producing cells was more rapid in the alcohol consuming mice than in the water drinking mice.

Chronic alcohol consumption increases of MDSC but not Treg cells in melanoma-bearing mice

The results presented above indicate that chronic alcohol consumption inhibits memory and tumor specific T cell proliferation, and accelerates loss of T cells that produce IFN-γ in tumor-bearing mice, suggesting that chronic alcohol consumption facilitates tumor-induced T cell anergy. These results lead us to study further the factors causing T cell dysfunction associated with melanoma. It is well documented that tumors induce specific cell populations that lead to T cell anergy [20]. At least three types of cells are associated with tumor-induced T cell anergy: MDSC, Treg cells, and TAM [6, 17, 20]. We examined if chronic alcohol consumption increased MDSC and Treg cells in melanoma-bearing mice.

MDSC are a heterogeneous cell population composed of immature myeloid cells such as monocytes, granulocytes and dendritic cells at different stages of differentiation [17]. Some MDSC can further differentiate into TAM within the tumor environment [34]. Besides the signature markers, Mac-1 (CD11b) and Gr-1, some of the MDSC also express CD124, which is the interleukin (IL)-4/IL-13 receptor alpha chain. Via this receptor, MDSC can be activated by IL-4 and IL-13 [10]. MDSC activated by IL-13 produces TGF-β to inhibit T cell function [35, 39]. The effects of melanoma on the $CD11b^{+}Gr\text{-}1^{+}$ MDSC are shown in Fig. 4. $Gr\text{-}1^{+}$ cells can be divided into $Gr\text{-}1^{hi}$ and $Gr\text{-}1^{int}$ populations as shown Fig. 4A and 4B. The $CD11b^{+}Gr\text{-}1^{int}$ cells, but not $CD11b^{+}Gr\text{-}1^{hi}$ cells, exhibit T cell suppressor function [45]. We found that chronic alcohol consumption significantly increased the $CD11b^{+}Gr\text{-}1^{int}$ cell population in the PBL from melanoma-bearing mice compared to water drinking mice (Fig. 4C). The $CD11b^{+}Gr\text{-}1^{hi}$ cells were not different between the two groups (data not shown). The percentage of $CD124^{+}$ cells also significantly increased in the MDSC (Fig. 4D).

Treg cells comprise 5-10% of peripheral $CD4^+$ T cells. Phenotypically they express IL-2 receptor alpha (CD25) on the cell surface and FOXp3 intracellularly. Thus, they are defined as $FOXp3^+CD4^+CD25^+$ T cells [9, 13]. Functionally Treg modulate T cell function and play an important role in the control of autoimmune diseases. Additional research also indicates that these cells increase significantly in the lymphoid organs and tumors of tumor-bearing animals and cancer patients, and they facilitate tumor escape from immunosurveillance by inhibiting T cell functions and causing T cell anergy [46]. Thus, we examined if chronic alcohol consumption induced T cell dysfunction in tumor-bearing mice by increasing Treg cells. We found that the percentages of these cells were not different between the two experimental groups in the spleen, PBL or lymph nodes of melanoma-bearing mice (Fig. 5). Thus, it is unlikely that they play an important role in the rapid disintegration of the $CD8^+$T cell-related immune responses in the alcohol consuming mice.

Chronic alcohol consumption compromises anti-CD3 activated T cell proliferation

Since alcohol consumption compromises tumor-induced $CD8^+$ T cell expansion (Fig. 2), we further determined the responsiveness of T cells from the melanoma-bearing mice to T cell receptor stimulation in vitro. We stimulated splenocytes with anti-mouse CD3 and CD28 monoclonal antibodies and determined the decay in the fluorescence intensity of CFSE in the T cells as an indication of cell division. CFSE easily penetrates the cell membrane and gets into the cell. The intracellular esterases cleave the acetate groups to yield the fluorescent carboxyfluorescein molecule. Cell division causes sequential halving of the fluorescence intensity of CFSE. Thus the mean fluorescence intensity

(MFI) reflects the proliferation of the labeled cells. A lower MFI indicates a higher proliferation rate. The results in Fig. 6 indicate that 90 hours after anti-CD3/CD28 stimulation, the MFI of $CD8^+$ T cells is significantly higher in the alcohol consuming, tumor bearing than in the water-drinking, tumor-bearing group (Fig. 6C). The percentage of proliferating $CD8^+$ T cells in the $CD8^+$ T cells of the alcohol-consuming, tumor-bearing mice was also significantly lower than the water-drinking, tumor-bearing animals (Fig. 6D). These results suggest that overall, chronic alcohol consumption impairs proliferation of $CD8^+$ T cells in melanoma-bearing mice in response to anti-CD3 and anti-CD28 stimulation. Similar effects were observed in the proliferation of The $CD4^+$ T cells (data not shown).

Discussion

In the present study we demonstrated differential effects of chronic alcohol consumption on $CD8^+$ T cells in mice not inoculated with melanoma and in melanoma-bearing mice. Without exogenous stimulation chronic alcohol consumption activates T cells as evidenced by the increase in $CD8^+$ T cells expressing the memory phenotype and in the percentage of those cells producing IFN-γ (Fig. 1) and [42]. However, in the presence of continued melanoma growth in vivo, memory $CD8^+$ T cell expansion is inhibited and IFN-γ producing $CD8^+$ T cells disproportionately decrease in the alcohol consuming mice.

It is well documented that tumors cause T cell anergy by producing tumor derived factors and by inducing immune suppressor cells such as MDSC, TAM and Treg cells

[27]. In some cancers tumor derived factors increase Treg cells, which in turn induce T cell anergy [33]; however, we did not find any changes in the percentage of Treg cells in the alcohol consuming tumor-bearing mice in the present study. Therefore, it is unlikely that these cells are involved in the T cell dysfunction induced by alcohol consumption in tumor-bearing mice. We did, however, show that MDSC are disproportionately increased in the alcohol consuming, melanoma-bearing mice compared to their respective water drinking controls. MDSC are induced by tumor-derived factors such as IL-6, vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) etc. [20]. It is well documented that chronic alcohol consumption elevates the level of IL-6 in the blood and increases the production of GM-CSF [12, 36]. Results from in vivo and in vitro tumor models indicate that low doses of alcohol up regulate VEGF expression [11, 38]. Whether this is also the case with chronic high dose alcohol consumption is not known. These studies indicate that chronic alcohol consumption has the potential to increase the cytokines and growth factors that are required for the induction of MDSC in the tumor-bearing mice.

Once MDSC are induced, these cells must be activated to suppress T cell function. It is known that IFN-γ produced by T cells plays an important role in the initiation of MDSC activation [10]. Thus the expression of IFN-γ in T cells and CD124 (IL-13 receptor) in MDSC are crucial to MDSC activation. Alcohol consumption increases IFN-γ producing T cells in non-tumor-bearing mice [42] and also during the early stages of tumor growth (Fig. 3). It also increases the percentage of $CD124^{+}$ expressing $CD11b^{+}Gr\text{-}1^{int}$ MDSC (Fig. 4). This is further support for the hypothesis that chronic alcohol consumption enhances the generation and activation of MDSC. Activated MDSC

produce IFN-γ and IL-13 to maintain self-activation [10]; however, how alcohol consumption further affects the production of IFN- γ and IL-13 as well as the diverse activities of these cells remains to be elucidated.

One of the important features of MDSC is the simultaneous activation of arginase I and inducible nitric oxide synthase [10]. Both enzymes use arginine as their substrate. With the increase and activation of MDSC, the availability of arginine is significantly decreased in the tumor bearing host [28]. This arginine deficit arrests the cell-cycle at the G_0-G_1 stage in T lymphocytes due to a failure to up-regulate the expression of cyclin D3 and cyclin-dependent kinase 4 [29]. We previously found that chronic alcohol consumption significantly decreases the concentration of arginine in the plasma of B16BL6 melanoma-bearing mice compared to their water drinking, tumor-bearing counterpart [22], and this finding underscores the significance of the associated increase of MDSC and dysfunction of T cells observed in the present study.

It is well recognized that nitric oxide is involved in the progression of melanoma as well as other tumors. Specifically, the metastatic B16BL6 melanoma cells used in this study express significantly higher levels of inducible nitric oxide synthase than the parent B16 cell line and the released nitric oxide is known to decrease the cytotoxicity of B16BL6-specific T cells [44]. Inducible nitric oxide synthase, which is upregulated by alcohol consumption [2, 15], also catalyzes the production of peroxinitrites through an arginine-dependent mechanism. Peroxinitrites inhibit the production of IFN-γ in T cells [21], and could be involved in the decreased IFN-γ that we observed as a function of late stage tumor growth (Fig.3). Thus, the increase in MDSC and compromised expansion of memory T cells and tumor specific T cells in the alcohol consuming tumor-bearing mice

could be associated with and ultimately be linked to availability of arginine; however, this requires further investigation.

For most types of cancers, the precise mechanism underlying the cause of death is still unknown. It is currently known that multiple factors, such as altered metabolism, cancer-induced cachexia, and impaired immune functions contribute to the cancer-related death. Interaction among these factors is not well known. However, a recent report indicates that certain $CD4^+$ T cells might protect against tumor-induced cachexia [40]. We previously found that chronic alcohol consumption in mice bearing subcutaneous B16BL6 melanoma leads to a major loss in body fat which is reflected in a 2-fold increase in body weight loss at necropsy compared to melanoma-bearing mice drinking water [22]. This element of the cachectic response could be one factor that contributes to reduced survival in the alcohol consuming mice. In the present study we found that chronic alcohol consumption impairs tumor specific $CD8^+$ T cell expansion and IFN-γ production, which are factors that critically affect host survival in tumor immunotherapy [16, 30]. Thus, the factors leading to decreased survival of melanoma-bearing mice chronically consuming alcohol are multifactorial.

In summary, chronic alcohol consumption inhibits tumor induced memory T cell and tumor-specific T cell expansion, and accelerates the decay of IFN-γ producing T cells in B16BL6 melanoma-bearing mice. The dysfunction of T cells is consistent with the increase of MDSC in the tumor-bearing, alcohol consuming mice.

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Figure Legends

Fig. 1. Effects of chronic alcohol consumption on $CD44^{hi}$ $CD8^{+}$ T cells. A) Dot plot showing the gated $CD8^{+}$ T cells in splenocytes. B) Histogram showing the $CD44^{hi}$ cells in the gated splenic $CD8^{+}$ T cells of melanoma-bearing mice. C) Percentage of $CD8^{+}CD44^{hi}$ cells in $CD8^{+}$ splenocytes from non-tumor injected mice (Cont) and melanoma-bearing mice at the indicated time points after tumor inoculation. Circle: alcohol-consuming mice. Square: Water-drinking mice. Alcohol group different from Water group, *: $P < 0.05$. ** $P < 0.001$. Each group contained 10 mice and the results are representative to two separate experiments.

Fig. 2. Chronic alcohol consumption decreases B16BL6 melanoma-associated gp100 specific $CD8^{+}$ T cells. A) Dot plot of the gp100/H-$2D^{b}$ tetramer (3700) positive $CD8^{+}$ cells in the gated splenic $CD8^{+}$ T cell population from melanoma-bearing mice after three weeks. B) Percentage of gp100 specific T cells in the splenic $CD8^{+}$ T cell population of melanoma-bearing mice at the indicated time points after tumor-inoculation. Circle: alcohol-consuming mice. Square: Water-drinking mice. ETOH group different from Water group, * $P<0.05$. C) Number of gp100 specific $CD8^{+}$ T cells in the spleen of melanoma-bearing mice three weeks after tumor inoculation. ETOH group different from Water group, ** $P<0.001$. Each group contained 10 mice and the results are representative to two separate experiments. ETOH, alcohol consuming group. Water, water drinking group.

Fig. 3. Chronic alcohol consumption accelerates the decay of IFN-γ producing cells within the splenic $CD8^+$ T cell population in the melanoma-bearing mice. Closed diamonds: water drinking mice. Closed squares: alcohol consuming mice. Alcohol consuming group different from water consuming group, * $P<0.05$ ** $P<0.001$ Each group contained 10 mice and the results are representative to two separate experiments.

Fig. 4. Chronic alcohol consumption increases the percentages of MDSC in the PBL and of $CD124^+$ cells in MDSC and $CD11b^+$ cells. MDSC were determined one week after tumor inoculation. A) Dot plot of $CD11b^+Gr\text{-}1^{int}$ MDSC in the PBL of water drinking mice. B) Dot plot of $CD11b^+Gr\text{-}1^{int}$ MDSC in the PBL of alcohol consuming mice, respectively. C) Percentage of $CD11b^+Gr\text{-}1^{int}$ MDSC in the PBL. D) Percentage of $CD124^+$ cells in $CD11b^+Gr\text{-}1^{int}$ MDSC cells. ETOH group significantly different from Water group, *$P<0.05$ ** $P<0.001$. ETOH, alcohol consuming group. Water, water drinking group. Each group contained 10 mice and the results are representative to two separate experiments.

Fig. 5. Alcohol consumption does not alter $Foxp3^+CD4^+CD25^+$ Treg cells. Treg cells were determined two weeks after tumor inoculation. A) Dot plot of Treg cells (upper right quadrant) in cells from the inguinal lymph nodes (LN). B) Percentage of $Foxp3^+CD4^+CD25^+$ Treg cells in the spleen, PBL and LN of melanoma-bearing mice.

Fig. 6. Effects of chronic alcohol consumption on T cell proliferation *in vitro*. Splenocytes were isolated from mice consuming alcohol for three months and inoculated

with $2x10^5$ B16BL6 sc for two weeks, labeled with CFSE and stimulated with anti-CD3 and anti-CD28 monoclonal antibodies for 90 hr. A) and B) Representative histograms indicating the MFI of CFSE in $CD8^+$ T cells from water drinking tumor-bearing mice and alcohol consuming tumor-bearing mice, respectively. The marker (bar region) M1 stands for proliferating cells. C) Histogram of the MFI of CFSE in $CD8^+$ T cells, ETOH different from Water, * $P<0.05$. D) Histogram of the percentage of proliferating cells in $CD8^+$ T cells, ETOH different from Water, ** $P<0.001$. Each group contained 7 mice. ETOH, alcohol consuming group. Water, water drinking group. The results are representative of two separate experiments.

Fig.1

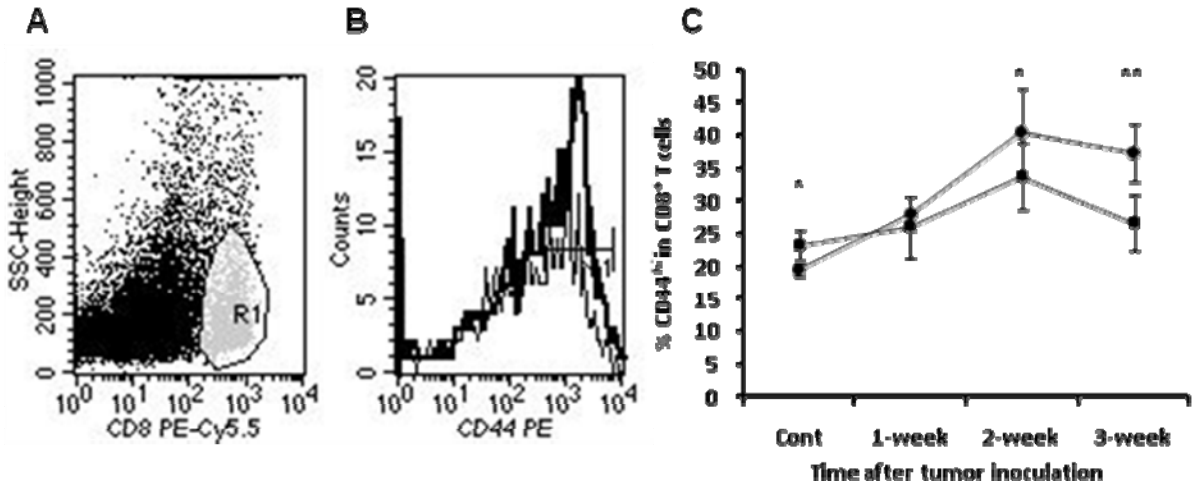

A
B
C
SSC-Height
R1
CD8 PE-Cy5.5
Counts
CD44 PE
% CD44hi in CD8+ T cells
Cont
1-week
2-week
3-week
Time after tumor inoculation

Fig. 2

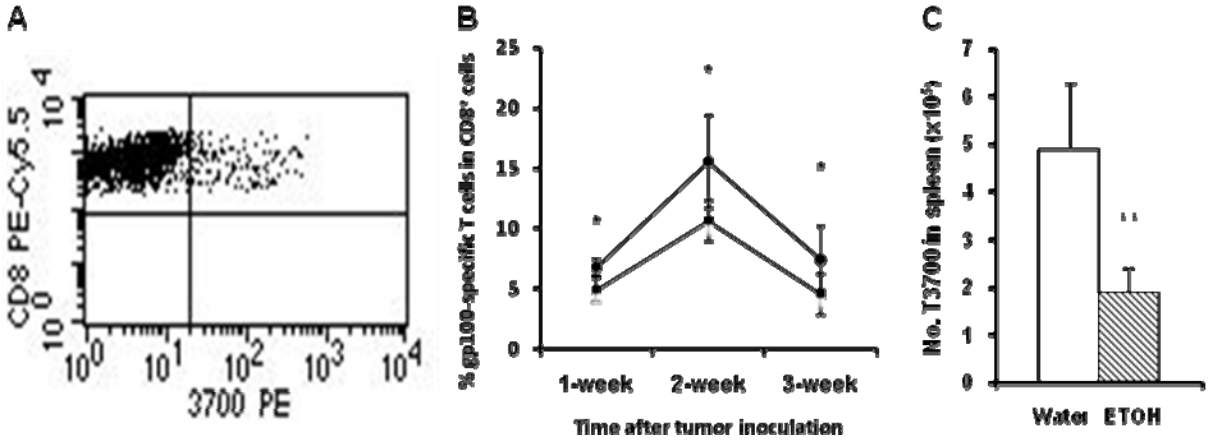

A
CD8 PE-Cy5.5
3700 PE
B
% gp100-specific T cells in CD8+ cells
1-week
2-week
3-week
Time after tumor inoculation
C
No. T3700 in spleen (x10⁵)
Water
ETOH

Fig. 3

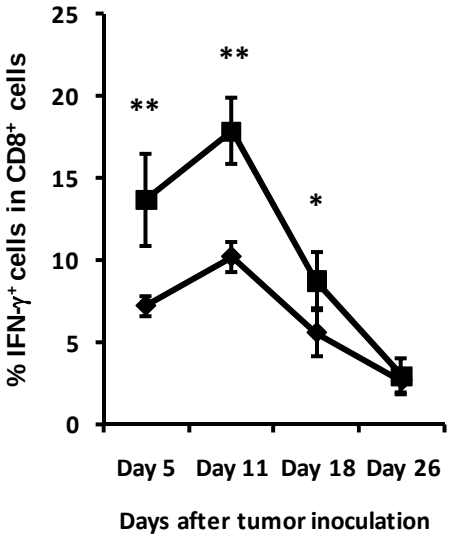

25
20
15
10
5
0
% IFN-γ+ cells in CD8+ cells
**
**
*
Day 5
Day 11
Day 18
Day 26
Days after tumor inoculation

Fig. 4

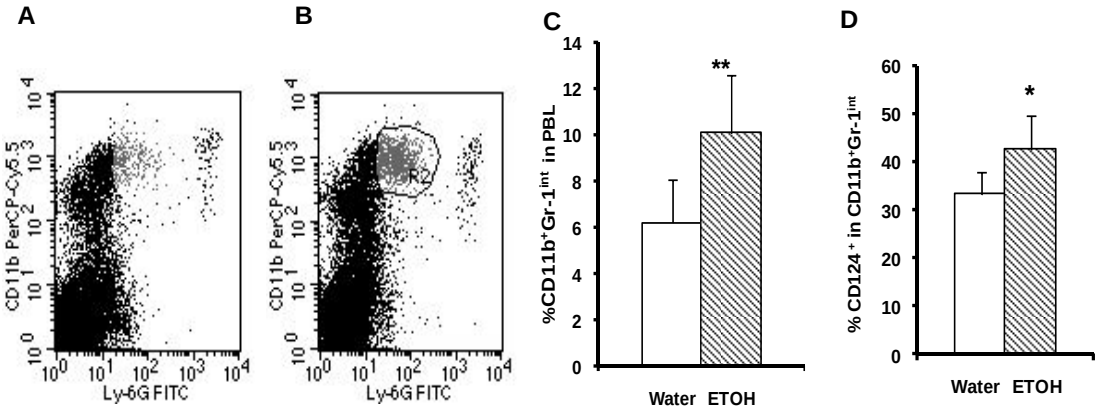

A
B
C
D
CD11b PerCP-Cy5.5
Ly-6G FITC
R2
%CD11b+Gr-1int in PBL
**
Water ETOH
% CD124+ in CD11b+Gr-1int
*
Water ETOH

Fig. 5

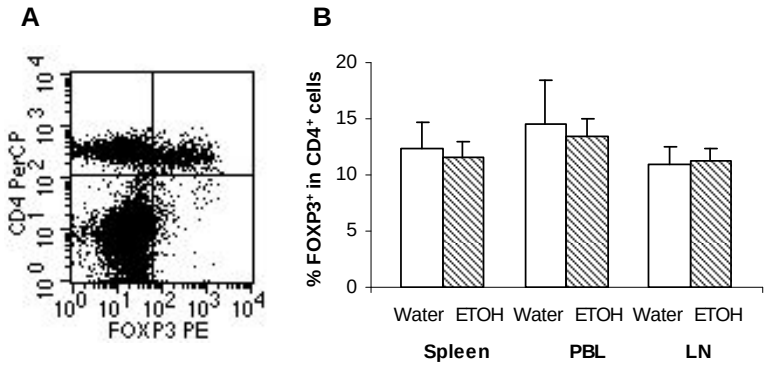
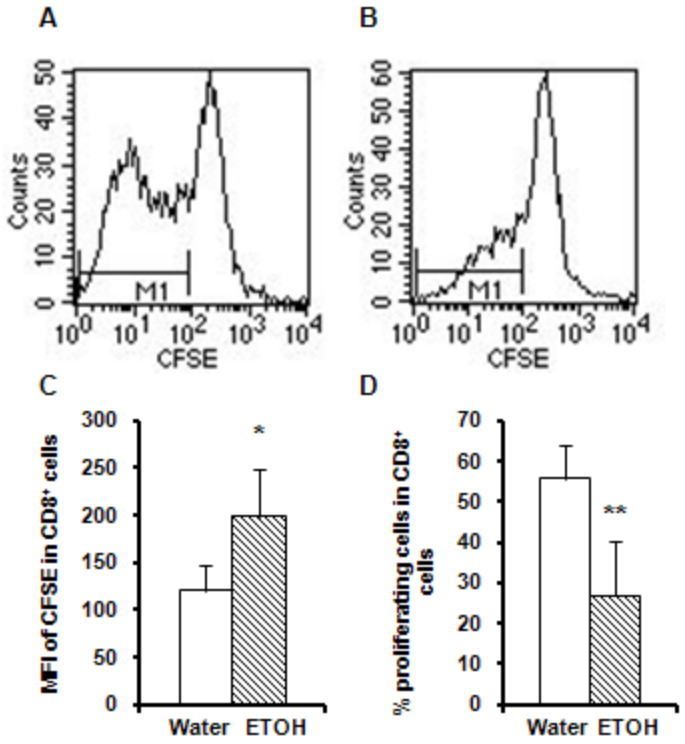

A
B
CD4 PerCP
FOXP3 PE
% FOXP3+ in CD4+ cells
20
15
10
5
0
Water ETOH Water ETOH Water ETOH
Spleen
PBL
LN

Fig. 6


A
B
Counts
M1
CFSE
C
D
MFI of CFSE in CD8+ cells
% proliferating cells in CD8+ cells
Water
ETOH
*
**