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ORIGINAL ARTICLE

The immunomodulatory, antitumor and antimetastatic responses of melanoma-bearing normal and alcoholic mice to sunitinib and ALT-803: a combinatorial treatment approach

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Abstract ALT-803, a novel IL-15/IL-15 receptor alpha complex, and the tyrosine kinase inhibitor, sunitinib, were examined for their single and combined effects on the growth of subcutaneous B16BL6 melanoma and on lymph node and lung metastasis. The study was conducted in immunocompetent C57BL/6 mice drinking water (Water mice) and in mice that chronically consumed alcohol (Alcohol mice), which are deficient in $CD8^+$ T cells. Sunitinib inhibited melanoma growth and was more effective in Alcohol mice. ALT-803 did not alter tumor growth or survival in Water or Alcohol mice. Combined ALT-803 and sunitinib inhibited melanoma growth and increased survival, and these effects were greater than sunitinib alone in Water mice. ALT-803 and alcohol independently suppressed lymph node and lung metastasis, whereas sunitinib alone or in combination with ALT-803 increased lymph node and lung metastasis in Water and Alcohol mice. Initially, ALT-803 increased IFN-γ-producing $CD8^+CD44^{hi}$ memory T cells and $CD8^+CD44^{hi}CD62L^{lo}$ effector memory T cells and sunitinib decreased immunosuppressive MDSC and T regulatory cells (Treg). However, the impact of these treatments diminished with time. Subcutaneous tumors from Water mice showed increased numbers of $CD8^+$ T cells, $CD8^+CD44^{hi}$ T cells, NK cells, and MDSC cells and decreased Treg cells after ALT-803 treatment.

Keywords IL-15 · Sunitinib · Melanoma · Metastasis · Immunotherapy

Abbreviations

Cy5.5	Cyanine 5.5
IL-15Rα	IL-15 receptor alpha
Treg	Regulatory T cells

Introduction

The incidence of melanoma has been rapidly rising for several years and shows no sign of abating. While melanoma is curable if detected early, it has a propensity to metastasize and then is virtually incurable. However, recent advances in immunotherapy offer new hope for increased survival of patients with distant metastasis.

IL-15 is one of the most promising cytokines for use in cancer immunotherapy because of its low toxicity and efficacy in promoting proliferation and survival of memory $CD8^+$ T cells as well as NK cells and for not inducing

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T cell activation-induced cell death [1]. ALT-803 is an IL-15 superagonist consisting of IL-15 complexed to the IL-15 receptor alpha, developed by Altor BioScience, Miramar, FL. It has significant antitumor activity against several murine tumors including 5T33P and MOPC-315P multiple myeloma [2], and non-muscle-invasive bladder cancer (NMIBC) [3]. ALT-803 enhanced proliferation of IFN-γ-producing $CD8^+CD44^{hi}$ memory T cells in murine myeloma, and this was responsible for its antitumor activity [2]. $CD8^+CD44^{hi}$ memory T cells and IFN-γ also are key factors that influence metastasis and host survival of melanoma [4–9]. Established s.c. B16F10 melanoma tumors were approximately fourfold smaller in mice administered two daily doses of IL-15 complexed to IL-15 receptor alpha than the tumors in untreated mice when measured 3 days later [10]. The initial antitumor effect was attributed to promotion of proliferation and effector capacity of resident $CD8^+$ T cells [10]. Dubois et al. [11] also showed effectiveness of an IL-15/IL15 receptor alpha complex in mice bearing i.v. B16 melanoma and from in vitro culture experiments attributed the antitumor effect to $CD8^+CD44^{hi}$ memory T cells. Thus, we hypothesized that ALT-803 would effectively inhibit s.c. growth and increase survival of mice inoculated with the more invasive and metastatic B16BL6 melanoma. This study is of additional significance since ALT-803 is currently being evaluated in a phase I clinical trial in patients with various surgically incurable advanced solid tumors including melanoma (ClinicalTrials.gov Identifier: NCT01946789).

In melanoma, MDSC are increased and correlate with tumor progression, metastasis, resistance to therapy, and decreased survival [12–14]. Several therapeutic approaches to targeting MDSC have been proposed to block tumor-induced immune tolerance and improve cancer immunotherapy [15–17]. Moreover, inhibiting MDSC as well as regulatory T cells (Treg) in a variety of different cancers, including melanoma, can enhance immune-based therapies [18–23]. Sunitinib is an orally effective, small molecule that inhibits multiple receptor tyrosine kinases [24] and can suppress MDSC [17] and Treg [17]. Conversely, IL-15 is a potent inducer of Treg [25]. Thus, an additional objective of the present investigation was to examine the potential additive effect of sunitinib and ALT-803 to inhibit growth and metastasis of melanoma and increase host survival in the B16BL6 murine model.

Alcohol abuse is a worldwide problem, and a recent meta-analysis confirms a positive association between alcohol consumption and melanoma risk [26]. Chronic alcohol consumption compromises the immune system, and this could impact the effectiveness of immunotherapy. We found previously that chronic alcohol intake decreases lymphocytes and in particular $CD8^+$ T cells in the spleen of non-melanoma-inoculated mice and stimulates homeostatic proliferation resulting in an increase in $CD8^+CD44^{hi}$ memory T cells and IFN-γ-producing $CD8^+$ T cells [27]. Although these cells are higher in chronic alcohol drinking mice (hereafter referred to as Alcohol mice) than in mice drinking water (hereafter referred to as Water mice) in the steady state, $CD8^+CD44^{hi}$ memory T cells fail to proliferate and IFN-γ-producing $CD8^+$ T cells as well as antigen-specific $CD8^+$ T cells decrease rapidly after s.c B16BL6 melanoma inoculation [17, 28]. This could be due to the fact that Alcohol mice exhibit an IL-15 deficiency, which can impair $CD8^+$ T cell and $CD8^+CD44^{hi}$ memory T cell proliferation and survival [29]. MDSC are elevated in the peripheral blood within 1 week after inoculation of B16BL6 melanoma in Alcohol mice, and this also could suppress T cell responses [28]. Thus, the goals of the present investigation were to (1) determine the potential additive effect of sunitinib and ALT-803 to inhibit growth and metastasis of B16BL6 melanoma and increase host survival in Water and Alcohol mice, (2) assess the relationship among these effects and the ability of the treatments to inhibit MDSC and Treg, and to increase $CD8^+$ T memory cells and their ability to produce IFN-γ and (3) determine uptake and retention of leukocytes in s.c. tumors.

Materials and methods

Animals, alcohol administration, and diet

Female C57BL/6 mice aged 6–7 weeks were purchased from Charles River Laboratories (Wilmington, MA) and housed in specific pathogen-free housing conditions within the vivarium at Washington State University. Mice were acclimated to the vivarium for at least 1 week before incorporating them into experiments. Mice in experiments were single housed in filter topped, polycarbonate cages and distributed to different treatment groups as described below. The starting body weight among the experimental groups was not different. Experimental protocols were approved by the Institutional Animal Care and Use Committee. The vivarium facilities are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Mice received autoclaved double distilled, deionized water or 0.2 μm filtered 20 % w/v alcohol consisting of double distilled, deionized water and Everclear (Luxco, St. Louis, MO) as their sole drinking source throughout the experimental period. All mice received Purina 5001 Laboratory chow ad libitum. The alcohol model has been previously validated [30].

Primary tumor inoculation, measurement and assessment of metastasis

B16BL6 melanoma was originally obtained from Dr. Menashe Bar-Eli, Department of Cancer Biology, The University of Texas MD Anderson Cancer Center, Houston, TX. It was cultured in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10 % heat-inactivated fetal bovine serum (Sigma-Aldrich) in a humidified incubator with 5 % CO_2 at 37 °C. Cells were harvested with 0.25 % trypsin-EDTA (Sigma Aldrich) when they reached 50–70 % confluence. Cells were then resuspended in sterile calcium-free, magnesium-free phosphate-buffered saline and used for injection within 5 in vitro passages. For assessment of treatment effects on primary tumors, mice were inoculated s.c. into the dorsal back near the right hip with 2×10^5 melanoma cells. Mice receiving alcohol were inoculated 12 weeks after alcohol consumption was initiated. Tumors became palpable approximately 7 days after inoculation and were then measured with calipers every 2 days thereafter. Tumor volume was calculated using the equation, $V = \pi/6 \times 1.58 \times (a \times b)^{3/2}$, where V is tumor volume, a is tumor length, and b is tumor width [31]. Metastasis to the right axillary lymph nodes and the lung as well as other potential organs was examined at necropsy. Lungs were fixed in Bouin's solution (Ricca Chem. Co.) for later enumeration of superficial tumor colony number using a dissecting microscope.

Drugs and treatment groups

The independent and combined effects of sunitinib and ALT-803 (a gift from Dr. Hing C. Wong, Altor BioScience Corp., Miramar, FL) to reduce s.c. melanoma growth, inhibit metastasis, and increase host survival were evaluated in the B16BL6 melanoma model. ALT-803 was diluted in 0.9 % sterile saline. Sunitinib malate, >99 % pure (LC Laboratories), was dissolved in 10 % DMSO. Experimental groups consisted of Water mice and/or Alcohol mice as follows: (1) a Control group that received the vehicles used to solubilize the drugs, (2) a sunitinib only treatment group, (3) an ALT-803 only treatment group and (4), and a Combination group treated with sunitinib and ALT-803.

Unless otherwise indicated, mice treated with ALT-803 received 5 weekly i.v. injections of 0.2 mg/kg ALT-803 starting at Day 7 after tumor inoculation. This dosage is equivalent to that used in another study that showed in vivo antitumor activity in murine multiple myeloma models [2]. Unless otherwise indicated, mice treated with sunitinib received daily i.p. injections of 40 mg/kg starting at Day 5 after tumor inoculation for 38 days. The continuous dosing schedule of sunitinib as well as the daily dose of 40 mg/kg is known to inhibit MDSC as well as Treg [18, 19, 23].

Assessment of metastasis after i.v. melanoma inoculation

A separate assessment of metastasis was conducted in Water mice inoculated with 2×10^5 B16BL6 melanoma cells into the lateral tail vein according to a previously established protocol [32]. Mice were treated with sunitinib and/or ALT-803 on day 2 after tumor injection. Daily i.p. sunitinib injections were continued until mice were euthanized on Day 21. ALT-803 was injected again on Day 11 and Day 18 after tumor injection. The lungs were removed, fixed in Bouin's solution, and visible lung tumor colonies were enumerated using a dissecting microscope.

Immunophenotyping in splenocytes and PBL

Immunophenotyping was conducted on the splenocytes and PBL obtained from the above-treated mice bearing s.c. melanoma tumors at various time points in a separate experiment. Splenocytes and PBL were isolated according to previously published procedures [28, 33]. Intracellular staining of splenocytes for IFN-γ was performed in splenocytes as previously described [27]. Staining for FOXP3 in $CD4^+CD25^+$ T cells in splenocytes was performed as previously described [28]. Samples were analyzed on a Becton–Dickinson FACScan flow cytometer, and data were analyzed using CellQuest Pro, version 5.1.1 software. For this experiment, Water and Alcohol mice were divided into four groups (Control, sunitinib, ALT-803, and Combination) each containing 9 mice per group and inoculated with B16BL6 melanoma as described above. The ALT-803 group received ALT-803 on day 9, 16, 23, and 30 after tumor inoculation. Sunitinib treatment began on day 7 after tumor inoculation and continued through day 32.

Treatment protocol and immunophenotyping of leukocytes in s.c. B16BL6 melanoma tumors

A separate cohort of Water mice were divided into three groups: Control, ALT-803, and Combination. The Control group received the vehicles used to solubilize the drugs. The ALT-803 group received ALT-803 (1.5 mg/kg i.v.) on day 7 and day 14 after tumor inoculation. The Combination group received sunitinib (40 mg/kg i.p) on day 7 through day 20 as well as ALT-803 (1.5 mg/kg i.v.) on day 7 and day 14. On day 21, mice were euthanized, the tumors were weighed, and the leukocytes isolated according to previously published procedures [28, 33] and then analyzed by flow cytometry as indicated above.

Antibodies used for immunophenotyping

PE-, FITC-, PerCP- and PerCP/Cyanine 5.5 (Cy5.5)-conjugated anti-mouse antibodies (clones) were purchased from the following suppliers. BioLegend: CD4 (RM4-5)-FITC, CD4 (RM4-5)-PerCP, CD8a (53-6.7)-Per/Cy5, CD3 (145-2C11)-PerCP, F4/80 (GM8)-PE, CD25 (PC61)-FITC, CD11b (M1/70)-PerCP/Cy5.5, Ly-6G/Ly-6C [Gr-1](RB6-8C5)-FITC, and IFN-γ (XMG1.2)-PE; BD Biosciences: CD44 (IM7)-FITC, CD62L (MEL-14)-PE; and eBioscience: FOXP3 (150D/E4)-PE.

Statistical analyses

Repeated measures ANOVA was used to determine differences in tumor growth over time with treatment. Kaplan–Meier survival analysis and the Gehan–Breslow–Wilcoxon test were used for determining overall survival. Two-way ANOVA was used to determine differences between Water and Alcohol mice. Differences in lung metastasis between treatment groups were analyzed by one-way ANOVA or one-way ANOVA on ranks as appropriate. Two-way ANOVA on ranks was used to determine differences between Water and Alcohol mice since the data were not uniformly distributed. Fisher's exact test was used to determine statistical significance of axillary lymph node metastases. Immunophenotyping data in organs were analyzed by one-way ANOVA followed by the Holm–Sidak method for multiple comparisons analysis between two analyses within a treatment group. For leukocytes in the tumor, normally distributed data were analyzed by one-way ANOVA followed by the Holm–Sidak method for multiple comparisons analysis between two analyses within a treatment group. Nonparametric data were analyzed by Kruskal–Wallis one-way ANOVA on ranks followed by the Tukey test or Dunn's method (NK cells) for pairwise multiple comparisons. Statistical significance was set at 95 % confidence limits. GraphPad Prism 5 and SigmaPlot 12.5 software were used for analyses.

Results

Sunitinib alone and in combination with ALT-803 plays a major role in inhibiting primary s.c. tumor growth and increasing host survival

The effects of sunitinib, ALT-803, and Combination treatments on tumor growth and survival are shown in Fig. 1. Tumors became palpable approximately 7 days after melanoma inoculation. Sunitinib significantly decreased the rate of tumor growth in Water mice ($p < 0.05$) (Fig. 1a) and Alcohol mice ($p < 0.05$) (Fig. 1b) compared to Controls, and the decrease in growth rate was greater in the Alcohol

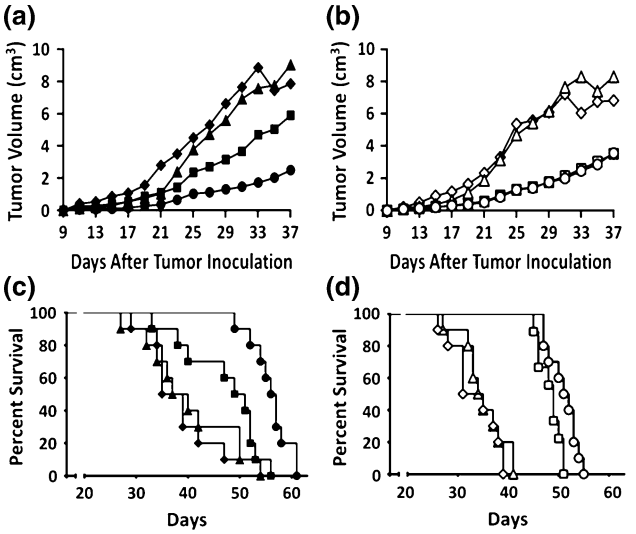

Fig. 1 Effect of treatment on primary s.c. B16BL6 melanoma tumor growth and survival. **a** Mean tumor volume as a function of treatment and time after tumor implantation in Water mice. **b** Mean tumor volume ± SEM as a function of treatment and time after tumor implantation in Alcohol mice. **c** Kaplan–Meier survival plot as a function of treatment in Water mice. **d** Kaplan–Meier survival plot as a function of treatment in Alcohol mice. *Closed symbols* Water mice, *Open symbols* Alcohol mice. *Diamonds* Control, *Squares* sunitinib, *Triangles* ALT-803, *Circles* Combination. Each group contained 10 mice. Repeated measures ANOVA was used to determine differences in tumor growth over time with treatment. The Gehan–Breslow–Wilcoxon test was used for determining overall survival in the Kaplan–Meier survival analysis

mice as compared to the Water mice treated with sunitinib ($p < 0.05$). ALT-803 treatment delayed tumor growth during the first 21 days after tumor inoculation in the Water mice (Fig. 1a) as compared to the Control group but not thereafter. Although ALT-803 also delayed tumor growth during the first 21 days after tumor inoculation in Alcohol mice (Fig. 1b), the effect was overshadowed by the pronounced effect that alcohol had on tumor growth during this time period. Overall, the combination treatment decreased the tumor growth rate when compared to Control and ALT-803 groups in Water and Alcohol mice ($p < 0.05$). In Water mice, the Combination group showed a trend toward decreased tumor growth compared to the sunitinib group ($p = 0.05$). In Alcohol mice, the overall tumor growth rate after Day 21 in the Combination group was identical to the sunitinib group indicating a lack of interaction between ALT-803 and sunitinib on tumor growth in these mice.

Median survival increased 43 % in the sunitinib group ($p < 0.05$) and 66 % in the Combination group ($p < 0.05$) compared to Control in the Water mice (Fig. 1c). ALT-803, which did not alter tumor growth overall likewise had no effect on survival in the Water mice. Survival in the Combination group was significantly different from the

sunitinib group ($p < 0.05$), indicating a beneficial interaction between sunitinib and ALT-803 to increase survival.

In Alcohol mice, sunitinib increased median survival by 53 % ($p < 0.05$) and Combination treatment increased survival by 63 % ($p < 0.05$, Fig. 1d). ALT-803 alone did not alter survival compared to Control in the Alcohol mice; however, survival in the Combination group was statistically different ($p < 0.05$) from the sunitinib group indicating a modest effect attributable to ALT-803.

Sunitinib increases and alcohol and ALT-803 decrease lymph node and lung metastasis in primary tumor-bearing mice

Metastasis to the right inguinal and axillary lymph nodes and to the lung was evaluated at necropsy in mice inoculated s.c. with B16BL6 melanoma. All mice exhibited metastasis to the right inguinal lymph node, which is the draining node closest to the primary tumor inoculation site.

Alcohol profoundly decreased the percentage of mice with right axillary lymph node involvement compared to Water Control ($p < 0.05$, Fig. 2a). The percentage of mice with visible metastasis in the right axillary lymph node was elevated in mice in the sunitinib and Combination groups in both Water and Alcohol mice compared to their respective Controls. A significantly lower percentage of mice with metastasis was observed in the Water ALT-803 group than in the Water Combination group ($p < 0.05$). The percentage of mice with right axillary lymph node metastasis treated with ALT-803 was identical to the Control in the Alcohol mice and the percentage of mice exhibiting metastasis in both these groups was significantly lower than sunitinib and Combination groups in Alcohol mice ($p < 0.05$).

The number of lung metastases determined at necropsy was lower ($p < 0.05$) in the ALT-803 groups compared to their respective Water (Fig. 2b) and Alcohol (Fig. 2c) Control, sunitinib, and Combination groups. The sunitinib and Combination groups exhibited higher levels of lung tumor colonies compared with respective Water and Alcohol Controls ($p < 0.05$). Metastasis was significantly decreased in the Alcohol Control relative to the Water Control group ($p < 0.05$). Metastasis in the sunitinib groups also was lower in Alcohol mice compared to Water mice ($p < 0.05$). While alcohol had a mitigating effect on metastasis in mice treated with sunitinib alone, this was not the case in the Combination group, where lung metastasis was significantly higher than the sunitinib group in the Alcohol mice ($p < 0.05$).

Sunitinib and ALT-803 similarly inhibit lung metastasis after i.v. tumor inoculation

Injection of tumor cells i.v. bypasses the early steps in metastasis associated with interactions between the primary

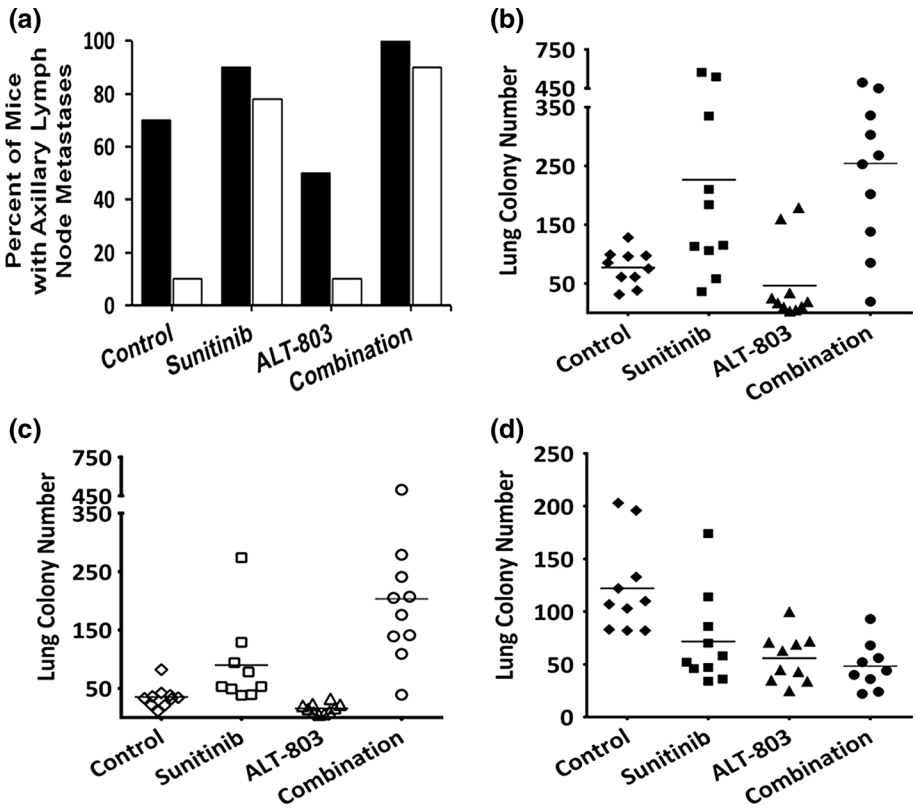

Fig. 2 Effect of treatment on metastasis of B16BL6 melanoma. **a** Percentage of mice at necropsy that exhibit right axillary lymph node metastasis after s.c. melanoma inoculation. *Black bars* Water mice, *White bars* Alcohol mice. **b** Tumor lung colony number at necropsy in Water mice after s.c. melanoma inoculation. **c** Tumor lung colony number in Alcohol mice after s.c. melanoma inoculation. **d** Tumor lung colony number after i.v. melanoma inoculation. **b–d** *Closed symbols* Water mice, *Open symbols* Alcohol mice. *Diamonds* Control, *Squares* sunitinib, *Triangles* ALT-803, *Circles*, Combination, *Bar* median number of lung colony tumors. Each group contained 10 mice. Fisher's exact test was used to determine statistical significance of axillary lymph node and lung metastases

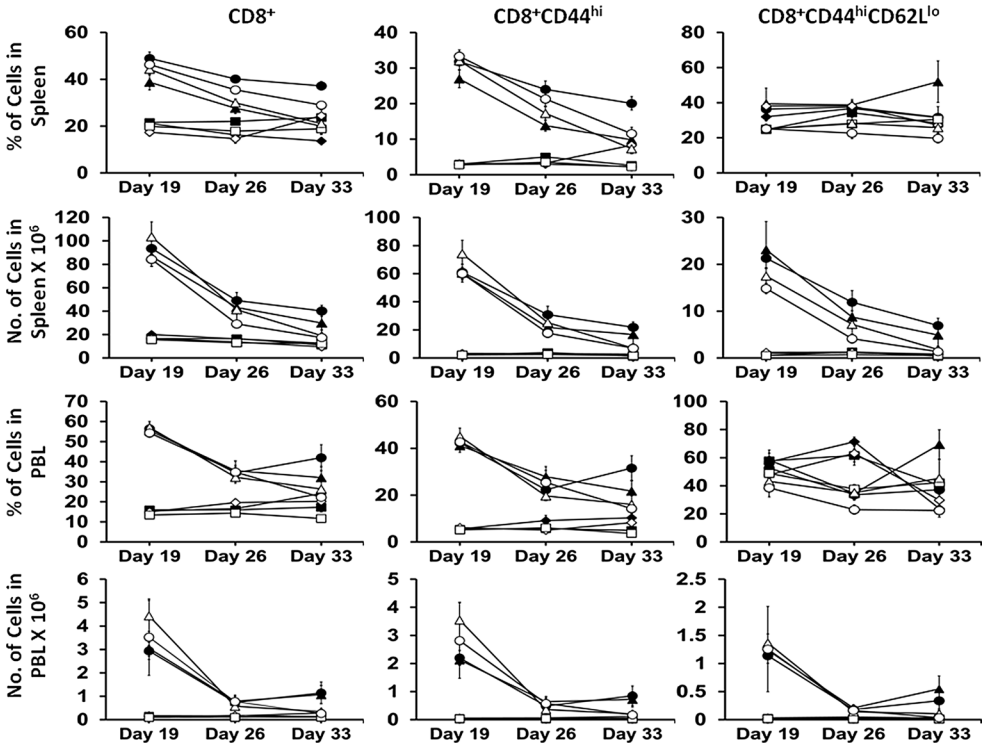

Fig. 3 Effect of treatment on $CD8^+$ T cells, $CD8^+CD44^{hi}$ memory T cells, and $CD8^+CD44^{hi}CD61L^{lo}$ effector memory T cells in the spleen and PBL of s.c. B16BL6 melanoma-bearing Water and Alcohol mice at various time periods after tumor inoculation. Values are mean for $N = 9$ mice per group. *Closed symbols* Water mice, *Open symbols* Alcohol mice. *Diamonds* Control, *Squares* sunitinib, *Triangles* ALT-803, *Circles* Combination. Data were analyzed by one-way ANOVA followed by the Holm–Sidak method for multiple comparisons analysis between two analyses within a treatment group

tumor and the microenvironment. Thus, this method of introduction of tumor cells examines more directly the effects of treatment on tumor colony development within the lung, which is a major metastatic site of melanoma. The effect of sunitinib and ALT-803 on metastasis after i.v. injection of melanoma was examined in Water mice. The results in Fig. 2d show that the lung colony number is decreased ~40–50 % in all three treatment groups compared to Control ($p < 0.05$).

ALT-803 alone and in combination treatment increase total $CD8^+$ T cells, $CD8^+CD44^{hi}$ memory T cells, and especially $CD8^+CD44^{hi}CD62L^{lo}$ effector memory T cells

Based on the results of other tumor systems, the major effect of ALT-803 on the immune response in melanoma-bearing mice was expected to be an increase in the number of $CD8^+$ T cells and $CD8^+CD44^{hi}$ memory T cells. Herein, we also examined the effect of ALT-803 on the $CD8^+CD44^{hi}CD62L^{lo}$ effector memory T cells, which are antigen-experienced T cells that upon activation secrete large amounts of IFN-γ and are able to rapidly lyse antigen-expressing tumor cells [34–36].

As expected, ALT-803 alone and in combination with sunitinib greatly increased ($p < 0.05$) $CD8^+$ T cells, $CD8^+CD44^{hi}$ memory T cells, and $CD8^+CD44^{hi}CD62L^{lo}$ effector memory T cells in the spleen and PBL in both Water and Alcohol mice on Day 19. The degree of increase on Day 19 was greater in the Water as compared to the Alcohol mice ($p < 0.05$) in the spleen but was not different in the PBL (Fig. 3). Sunitinib alone did not affect the percentage or number of $CD8^+$ T cells or $CD8^+CD44^{hi}CD62L^{lo}$ effector memory T cells in the spleen and PBL as compared to respective Control groups in Water and Alcohol mice. The response to ALT-803 was not sustained over time, and the numbers of these cells were decreased on Day 26 and Day 33 in the ALT-803 and Combination of Water and Alcohol mice. In the Alcohol mice, the numbers of these cells in the ALT-803 and Combination groups on Day 33 decreased to Control values while in the Water mice they were still somewhat elevated ($p < 0.05$) (Fig. 3). The response of $CD8^+CD44^{hi}$ memory T cells to ALT-803 in the Alcohol mice on Day 19 was significantly higher than in Water mice as compared to the Control group in the spleen and PBL; however, the decrease in these cells on Day 26 was also more precipitous ($p < 0.05$). Thus, ALT-803 was initially effective in increasing $CD8^+$ T cells, $CD8^+CD44^{hi}$ memory T cells, and $CD8^+CD44^{hi}CD62L^{lo}$ effector memory T cells; however, continued treatment was not effective in sustaining the levels of these cells over time.

ALT-803 increases IFN-γ-expressing $CD8^+$ T cells and $CD8^+CD44^{hi}$ memory T cells

The major antitumor effect of $CD8^+$ T cells and particularly $CD8^+CD44^{hi}$ memory T cells is associated with their ability to produce IFN-γ [6–9], and the goal herein

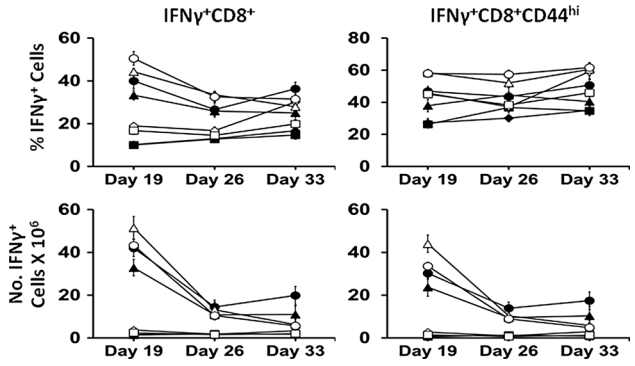

Fig. 4 Effect of treatment on IFN-γ-producing $CD8^+$ T cells and $CD8^+CD44^{hi}$ memory T cells in the spleen of s.c. B16BL6 melanoma-bearing Water and Alcohol mice at various days after tumor inoculation. Values are mean ± SEM for $N = 9$ mice per group. *Closed symbols* Water mice, *Open symbols* Alcohol mice. *Diamonds* Control, *Squares* sunitinib, *Triangles* ALT-803, *Circles* Combination. Data were analyzed by one-way ANOVA followed by the Holm–Sidak method for multiple comparisons analysis between two analyses within a treatment group

was to achieve this via administration of ALT-803. IFN-γ-producing $CD8^+CD44^{hi}$ memory T cells are responsible for the greater part of the antitumor activity against many other types of tumors [2, 37]. The numbers of IFN-γ-producing $CD8^+$ T cells and $CD8^+CD44^{hi}$ memory T cells in the spleen were dramatically elevated in the ALT-803 and Combination groups on Day 19 as compared to Control of both Water and Alcohol mice ($p < 0.05$, Fig. 4). In the Water mice these cells remained elevated in the ALT-803 and Combination groups compared to Control through Day 33 ($p < 0.05$) even though they decreased with time. This decrease was more pronounced in the Alcohol mice, where on Day 33, the numbers were not different from the Control group. The percentage and number of IFN-γ-producing $CD8^+$ T cells and $CD8^+CD44^{hi}$ memory T cells were higher on Day 19 in the ALT-803 Alcohol mice as compared to Water mice ($p < 0.05$). In addition, the numbers of these cells in the Alcohol mice were higher in the ALT-803 group compared to the Combination group on Day 19 ($p < 0.05$). Sunitinib had no effect on the number of IFN-γ-producing $CD8^+$ T cells or $CD8^+CD44^{hi}$ memory T cells in the spleen. While ALT-803 was initially effective in facilitating these IFN-γ producing cells, the effect was not long lasting.

Sunitinib alone and in combination decreases MDSC, but the effect is short lived

MDSC have strong immunosuppressive activities. They can influence tumor progression and survival as well as play an important role in the development of immune tolerance and interfere with immunotherapy in melanoma as well as other cancers [12, 14, 15, 17]. There is strong evidence that sunitinib suppresses MDSC [17, 24]. Since chronic alcohol consumption can increase MDSC in the PBL [28], we investigated whether sunitinib could mollify this effect in Alcohol mice.

The percentage of $CD11b^+GR\text{-}1^{int}$ MDSC increased in the spleen and PBL of Control groups with time in both Water and Alcohol mice ($p < 0.05$, Fig. 5). The numbers of MDSC ($CD11b^+GR\text{-}1^{int}$ cells) in the spleen of Alcohol mice did not change over time. However, in Water mice the numbers of these cells increased in the Control and ALT-803 groups over time ($p < 0.05$). Sunitinib and Combination treatment held steady the numbers of MDSC in the spleen of Water mice over time, and they were lower than Control and ALT-803 groups on Day 33 ($p < 0.05$). The

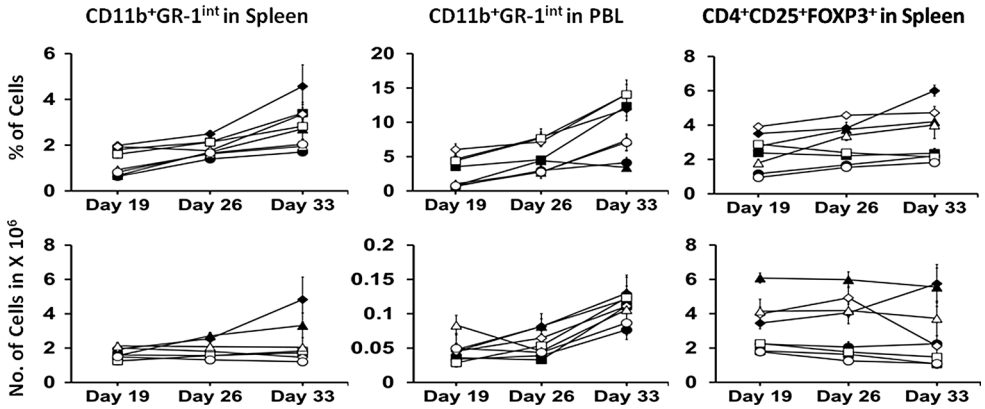

Fig. 5 Effect of treatment on $Gr\text{-}1^{int}$ cells in the spleen and PBL and $CD4^+FOXP3^+$ T cells in the spleen of s.c. B16BL6 melanoma-bearing Water and Alcohol mice at various days after tumor inoculation. Values are mean ± SEM for $N = 9$ mice per group. *Closed symbols* Water mice, *Open symbols* Alcohol mice. *Diamonds* Control, *Squares* sunitinib, *Triangles* ALT-803, *Circles* Combination. Data were analyzed by one-way ANOVA followed by the Holm–Sidak method for multiple comparisons analysis between two analyses within a treatment group

numbers of MDSC in PBL of Water mice on Day 26 were lower in the sunitinib and Combination groups as compared to Control and ALT-803 groups ($p < 0.05$). However, by Day 33, MDSC levels increased in Control and sunitinib groups as compared to Day 19 ($p < 0.05$) and increased in the ALT-803 group as compared to Day 26 ($p < 0.05$). In Alcohol mice, sunitinib did not effectively suppress MDSC in the PBL, and the numbers increased over time in Control and sunitinib groups as compared to Day 19 ($p < 0.05$) and on Day 33 were higher in the ALT-803 group as compared to Day 26 ($p < 0.05$). In both the Water and Alcohol Combination treatment groups, the numbers of MDSC in the PBL did not change significantly over time. Overall, the results indicate that sunitinib initially inhibited expansion of MDSC in melanoma-bearing Water mice; however, the effect in PBL was not sustainable.

Sunitinib prevents Treg cell expansion and their increase in response to ALT-803

Treg also are associated with inhibition of host antitumor immunity, and depletion of these cells favors immune-mediated tumor rejection. Since IL-15 is a potent inducer of Treg [25], we hypothesized that sunitinib, which is purported to decrease Treg [17], would restrict the induction of these cells after ALT-803 therapy. The numbers of Treg in the ALT-803 group were higher than Control, sunitinib, and Combination groups in Water and Alcohol mice on Day 19, Day 26, and Day 33 ($p < 0.05$), with the exception that Control was not different from ALT-803 in Alcohol mice on Day 33 (Fig. 5). Treg were lower in the sunitinib and Combination groups in Water and Alcohol mice compared to Control on Day 19 and Day 26 ($p < 0.05$), and they did not increase over time. Treg clearly increased in Water mice over time ($p < 0.05$). The overall findings were that ALT-803 resulted in elevated Treg and that sunitinib counteracted this effect.

ALT-803 increases $CD8^+$ T cells, $CD8^+CD44^{hi}$ memory T cells, and MDSC and decreases Treg cells in primary melanoma tumors in Water mice

At necropsy, the mean tumor weight was 2.96 g ± 0.99 in the Control group, 0.76 g ± 0.19 in the ALT-803 group, and 0.36 g ± 0.06 in the Combination group. Tumor weights of three groups were different from each other ($p < 0.05$) indicating that ALT-803 decreased growth and that the combination treatment was more effective than ALT-803 alone. The uptake and retention of leukocytes in the tumor is shown in Fig. 6. $CD8^+$ T cells and primarily those exhibiting the $CD8^+CD44^{hi}$ memory phenotype were the major type of leukocyte comprising almost 90 % of the total cells. ALT-803 treatment increased the numbers of these cells per 100 mg tumor by threefold compared to Control; however, combined treatment with sunitinib dampened this effect. NK cells increased 11- to 13-fold in the ALT-803 and Combination groups. Treg decreased about sevenfold in the ALT-803 group and fourfold in the Combination group.

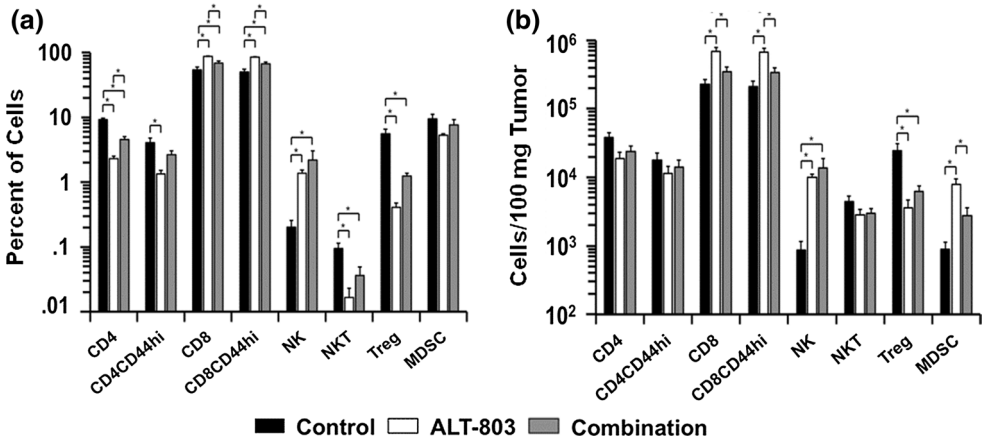

Fig. 6 Leukocytes in s.c. B16BL6 melanoma tumors. **a** Percentage of leukocytes in s.c. B16BL6 melanoma tumors expressed on a log scale. **b** Number of leukocytes per 100 mg of B16BL6 melanoma tumors expressed on a log scale. The percentage and numbers of leukocytes were determined in Water mice on day 21 after s.c. B16BL6 melanoma tumor inoculation. The Control group (*Black bars*) received vehicles as indicated in the Materials and Methods. The ALT-803 group (*White bars*) received l.5 mg/kg i.v. into the lateral tail vein on day 7 and 14 after tumor inoculation. The Combination group (*Gray bars*) received 40 mg/kg sunitinib i.p. from day 7 through day 20 after tumor inoculation and 1.5 mg/kg ALT-803 i.v. on day 7 and day 14 after tumor inoculation. Mice were euthanized on day 21 and the s.c. tumors analyzed for content of various leukocytes as indicated in the figure. Values are mean ± SEM for $N = 6$ mice per group. $^*p < 0.05$. Normally distributed data were analyzed by one-way ANOVA followed by the Holm–Sidak method for multiple comparisons analysis between two analyses within a treatment group. Nonparametric data were analyzed by Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's method for pairwise multiple comparisons (NK cells)

MDSC increased about ninefold in the ALT-803 group and only threefold in the Combination group indicating an inhibitory effect of sunitinib on MDSC accumulation in the tumor along with ALT-803 treatment.

Discussion

There are few effective treatment options for patients with melanoma. Recent clinical and preclinical studies suggest that immune-enhancing agents provide a promising approach to treatment; however, their effectiveness can be limited by development of resistance. IL-15 and particularly IL-15 combined with its receptor alpha chain including ALT-803 show great potential in the immunotherapy of cancer. The tyrosine kinase inhibitor, sunitinib, which inhibits immunosuppressive MDSC and Treg cells, can synergize with cancer immunotherapy [38]. The objective of this study was not only to examine the effectiveness of ALT-803 to inhibit primary B16BL6 melanoma growth and metastasis, and to increase survival, but also to determine the ability of sunitinib to enhance the affect of ALT-803 in mice that are immunocompetent and those that are immunodeficient due to chronic alcohol consumption.

Sunitinib decreased tumor growth throughout the experiment in both Water and Alcohol mice; however, the effect was more pronounced in the Alcohol mice (Fig. 1). ALT-803 inhibited tumor growth in Water and Alcohol mice during the first 21 days after tumor implantation but was ineffective thereafter (Fig. 1). ALT-803 in combination with sunitinib more effectively inhibited tumor growth throughout the experiment in Water mice, and these mice had the greatest survival. In Alcohol mice, ALT-803 in combination with sunitinib became ineffective in controlling growth after 21 days as compared to sunitinib treatment alone, and this was also reflected in only a modest increase in survival compared to sunitinib treatment alone. The initial growth inhibitory effect of ALT-803 and Combination therapy in Water and Alcohol mice correlated strongly with high levels of $CD8^+$ T cells and their memory phenotype in the spleen and PBL (Fig. 3) and with IFN-γ-producing $CD8^+$ T cells and $CD8^+CD44^{hi}$ memory T cells in the spleen (Fig. 4). The numbers of these cells were higher in the Alcohol mice than in the Water mice on Day 19 ($p < 0.05$); however, the decrease observed in Alcohol and Water mice on Day 26 was more precipitous in Alcohol mice. Tumor levels of $CD8^+$ T cells, $CD8^+CD44^{hi}$ T cells, and NK cells also were greatly increased in primary tumors when examined in Water mice on day 21 (Fig. 6).

The increase in tumor growth in the ALT-803 treated Water and Alcohol mice on day 21 correlated with increased MDSC and Treg (Fig. 5), decreased $CD8^+$ T cells, $CD8^+CD44^{hi}$ memory T cells, and $CD8^+CD44^{hi}CD62L^{lo}$ effector memory T cells (Fig. 3) in the spleen and PBL and decreased IFN-γ-producing $CD8^+$ T cells and $CD8^+CD44^{hi}$ memory T cells in the spleen (Fig. 4). While Treg were greatly elevated in the spleen of ALT-803-treated mice compared to Control (Fig. 5), they were greatly decreased in the primary tumors from Water mice on day 21 (Fig. 6). In contrast, MDSC were greatly elevated in the tumor by ALT-803 treatment. All together these data strongly suggest that B16BL6 melanoma cells developed resistance to ALT-803 and Combination therapy associated with elevated MDSC and decreased memory T cells. IL-15 internalization is necessary for proliferation and survival of responding cells after trans-presentation of IL-15 complexed with IL-15Rα followed by recycling of the receptor to the cell surface [39]. Thus, it is possible that the ability of the ALT-803 complex to be cleaved over time is impaired in melanoma-bearing mice. Another possibility is that various other immunosuppressive factors that foster resistance of $CD8^+$ T cells to IL-15 lead to the eventual decrease in number and function of these cells as tumor growth continues.

Sunitinib decreased tumor growth and increased survival in the Water and Alcohol mice independent of increased $CD8^+$ T cells, $CD8^+CD44^{hi}$ memory T cells, $CD8^+CD44^{hi}CD62L^{lo}$ effector memory T cells, and IFN-γ-producing $CD8^+$ T cells and $CD8^+CD44^{hi}$ memory T cells since the numbers of these cells remained unchanged over time in this treatment group compared to Controls (Fig. 3). The decrease in the number of these cells associated with continued combination treatment was generally greater in the Alcohol mice. In addition, the numbers of IFN-γ-producing $CD8^+$ T cells and $CD8^+CD44^{hi}$ memory T cells were lower in the Alcohol mice on Day 33 compared to Water mice, suggesting that maintenance of survival and/or proliferation of these cells in response to ALT-803 was inhibited by alcohol. This study further confirms our previous findings that while chronic alcohol consumption itself facilitates an enhanced immune response, the microenvironment associated with an ever-present alcohol/melanoma interaction is strongly immunosuppressive.

It is likely that the antiangiogenesis activity of sunitinib contributed to the overall antitumor response in this treatment group, since it is known to inhibit B16 melanoma neovascularization [40]. However, sunitinib in another study did not inhibit s.c. growth of B16 melanoma tumors in immunodeficient mice [41] indicating that an intact immune system is essential for its ability to inhibit melanoma growth. Thus, additional studies are required to determine the mechanism associated with the effect of sunitinib on melanoma growth and survival.

Combination therapy with ALT-803 and sunitinib in Water mice was the most effective therapy in reducing primary tumor growth and increasing survival adding support

for the original hypothesis that sunitinib by virtue of its suppressive effect on MDSC and Treg facilitated $CD8^+$ T cell-mediated antitumor immune cells. Further supporting this hypothesis is the fact that the lowest numbers of MDSC in the spleen and PBL were observed in the Combination group (Fig. 5) and in the tumors from Water mice (Fig. 6).

ALT-803 and alcohol alone and in combination had a strong inhibitory effect on spontaneous metastasis of s.c-inoculated B16BL6 melanoma. The effect of alcohol on metastasis was previously shown to be IFN-γ dependent [5]. While the mechanism underlying the antimetastatic effect of ALT-803 is not known, it is likely that the effect is related to increased levels of IFN-γ.

Sunitinib increased metastasis and overcame the antimetastatic effect of ALT-803. Similar findings have been described for sunitinib treatment in B16 melanoma and other murine and human tumor lines implanted orthotopically [42, 43]. The increase in metastasis after s.c. tumor implantation might be related to increased local tumor cell invasion [42], adaptation of the tumor cell microenvironment triggered by changes in the tumor vasculature [43], augmented intratumoral heterogeneity [44], or ensuing hypoxia induced by sunitinib antiangiogenesis therapy creating an immunosuppressive microenvironment, which fosters metastasis [45]. Oxygen deprivation clearly increases B16F10 melanoma metastasis to the lung [46, 47]. Timing and duration of treatment is critical to increasing survival in response to sunitinib treatment after i.v. tumor inoculation [42]. Survival of mice inoculated i.v. with B16 or human MeWo melanoma was decreased if sunitinib was given prior to tumor inoculation or for a short 7-day treatment after tumor injection. Moreover, sustained treatment such as that used in the present study increased survival. While we did not examine survival after i.v. inoculation of melanoma, we did observe increased survival in mice continuously administered sunitinib i.p., and we observed decreased metastasis in mice inoculated i.v. and treated continuously with sunitinib. Ebos et al. [48] examined the pre- and postsurgical effects of antiangiogenic therapy found in established breast, kidney, and melanoma models and found that sunitinib did not predict postsurgical recurrence of metastatic disease. While there is strong evidence that antiangiogenic therapy increases metastasis in animal models of cancer, the risk associated with human cancer patients is still unresolved.

Immunotherapy is a major focus and a promising area for treatment of melanoma. While the present study indicates a beneficial effect of ALT-803 to augment the immune response in s.c. B16BL6 melanoma and of sunitinib to enhance the efficacy of ALT-803, we witnessed a common problem that often arises with immunotherapy—the development of resistance to treatment over time. Moreover, the development of resistance appeared to be greater in the immune-compromised, chronic alcohol drinking mice, which could reflect additional complexity to successfully treating human alcoholic cancer patients. Further studies are warranted to assess the role of ALT-803 in combination with other immune-enhancing therapies for the treatment of melanoma as well as the role of preexisting immune deficiency in the efficacy of these therapeutic approaches.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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