Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2022**; 27(7): 215
https://doi.org/10.31083/j.fbl2707215

Original Research

IL-2 and Zoledronic Acid Therapy Restores the *In Vivo* Anti-Leukemic Activity of Human Lymphocytes Pre-Exposed to Simulated Microgravity

Preteesh L. Mylabathula[1,2], Douglass M. Diak[1,2], Forrest L. Baker[1,2], Grace M. Niemiro[2], Melissa M. Markofski[3], Brian E. Crucian[4], Emmanuel Katsanis[2,5,6], Richard J. Simpson[1,2,5,6,*]

[1]School of Nutritional Sciences and Wellness, The University of Arizona, Tucson, AZ 85721, USA
[2]Department of Pediatrics, The University of Arizona, Tucson, AZ 85721, USA
[3]Laboratory of Integrated Physiology, Department of Health and Human Performance, University of Houston, Houston, TX 77004, USA
[4]NASA Johnson Space Center, Houston, TX 77058, USA
[5]Department of Immunobiology, The University of Arizona, Tucson, AZ 85721, USA
[6]The University of Arizona Cancer Center, Tucson, AZ, 85721, USA
*Correspondence: rjsimpson@arizona.edu (Richard J. Simpson)
Academic Editor: Josef Jampílek
Submitted: 16 March 2022 Revised: 29 April 2022 Accepted: 28 June 2022 Published: 8 July 2022

Abstract

Background: We have previously shown that the anti-tumor activity of human lymphocytes is diminished *in vitro* after 12-hours pre-exposure to simulated microgravity (SMG). Here we used an immunocompromised mouse model to determine if this loss of function would extend *in vivo*, and to also test the efficacy of IL-2 and zoledronic acid (ZOL) therapy as a potential countermeasure against SMG-induced immune dysfunction. We adoptively transferred human lymphocytes that were exposed to either SMG or 1G-control into NSG-Tg (Hu-IL15) mice 1-week after they were injected with a luciferase-tagged human chronic myeloid leukemia (K562) cell line. Tumor growth was monitored 2x weekly with bioluminescence imaging (BLI) for up to 6-weeks. **Results**: Mice that received lymphocytes exposed to SMG showed greater tumor burden compared to those receiving lymphocytes exposed to 1G (week 6 BLI: $1.8e^{10} \pm 8.07e^{9}$ versus $2.22e^{8} \pm 1.39e^{8}$ photons/second; $p < 0.0001$). Peak BLI was also higher in the SMG group compared to 1G-control ($2.34e^{10} \pm 1.23e^{10}$ versus $3.75e^{8} \pm 1.56e^{8}$ photons/second; $p = 0.0062$). Exposure to SMG did not affect the ability of human lymphocytes to engraft or evoke xeno-graft-versus-host disease in the mice. Additionally, we injected the mice with IL-2 and zoledronic acid (ZOL) to expand and activate the anti-tumor activity of NK cells and $\gamma\delta$-T cells, respectively. This treatment was found to revive the loss of anti-leukemic function observed *in vivo* when lymphocytes were pre-exposed to SMG. **Conclusions**: Microgravity plays a contributory role in loss of tumor control *in vivo*. Immuno-stimulating agents like ZOL+IL-2 may offer an important countermeasure for immune dysregulation during prolonged spaceflight.

Keywords: spaceflight; immunology; rotary cell culture system; natural killer cells; humanized mice; cancer

1. Introduction

Immune dysregulation has been reported in astronauts following both short (14–17 days) [1–4] and long (5–6 months) [5,6] duration spaceflight. This includes diminished cell mediated immunity with a Th-2 cytokine shift, with latent viral reactivation being a frequent manifestation of immune dysregulation in astronauts. Many factors including microgravity, space radiation, isolation and confinement stress, altered circadian rhythms, nutritional deficiencies, and the gut microbiome are believed to play a role in altering astronaut immunity during extended space voyages [7].

A particular concern for astronauts is that space travel might impair anti-tumor immune surveillance, increasing cancer risk in future exploration class mission crew. We showed previously that the anti-tumor activity of natural killer (NK) cells was diminished in crewmembers onboard the International Space Station [8]. We further demonstrated that pre-exposing human lymphocytes to 12 h of simulated microgravity (SMG), impaired NK cell killing against a range of hematological cancer cell lines *in vitro* when returned to the 1G environment [9]. The SMG exposed NK cells expressed lower levels of the cytolytic granules perforin and granzyme b, and were less capable of degranulation (CD107a+) and secreting effector cytokines (e.g., TNF-α and IFN-γ) when co-cultured with tumor target cells after being pre-exposed to SMG. Exposing K562 chronic myeloid leukemia (CML) cells to SMG did not alter their susceptibility to NK cell killing, indicating that cancer cells might be more resistant to the effects of SMG compared to primary immune cells. This might tip the balance in the favor of tumor transformation during a long duration mission, particularly against a backdrop of continuous

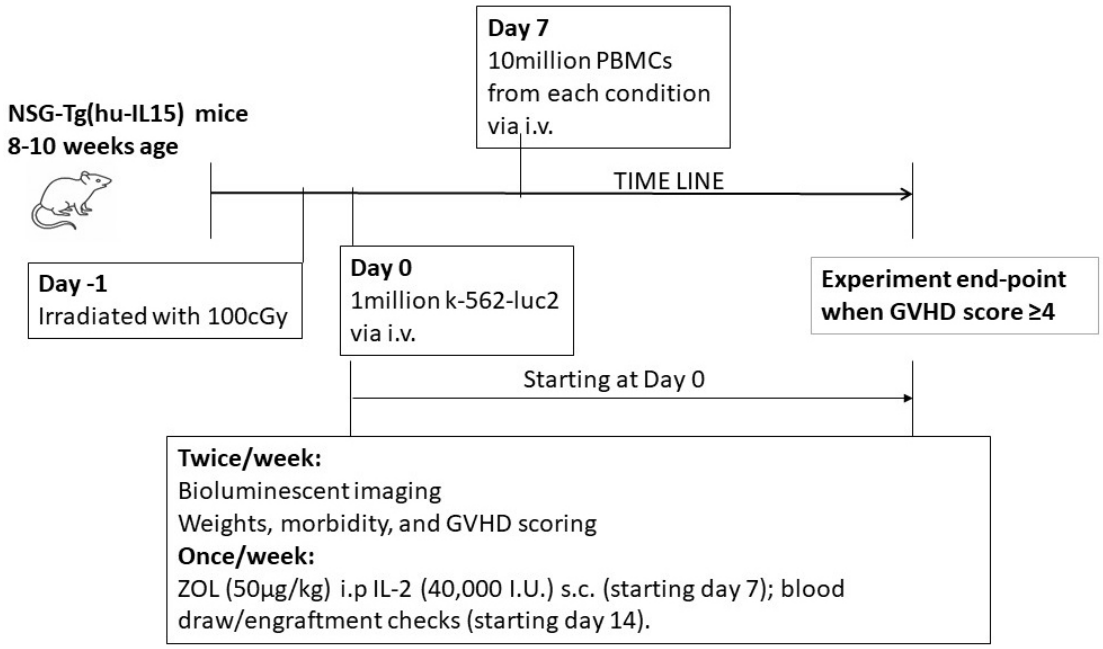

Fig. 1. Experimental design.

radiation exposure and further immune suppression due to latent viral reactivation.

A limitation of our previous work was the reliance on *in vitro* assays to assess the anti-tumor activity of human lymphocytes collected from astronauts in space or following exposure to SMG [8,9]. Unfortunately, these assays fail to capture other key elements of the anti-tumor response unique to *in vivo* setting including extravasation, homing, tumor infiltration, and killing. Humanized mouse models offer an excellent platform to determine the anti-tumor activity of human immune cells *in vivo*. NSG-Tg (Hu-IL15) animals harbor Prkdc^{scid} [10] and IL2rg^{tm1Wjl} [11–13] mutations and an insertion of humanized IL-15 sequence (Tg(IL15)1Sz/SzJ) that allows improved engraftment of primary human cells [11] and increased survival of NK cells [14]. This mouse model also allows us to test the effectiveness of certain pharmacological countermeasures that have been proposed to mitigate spaceflight associated immune dysregulation [15].

Administration of subcutaneous interleukin-2 (IL-2) has shown to improve anti-tumor activity of NK cells in preclinical mouse models [16,17] and in various clinical trials [18–20]. Zoledronic acid is an amino-bisphosphonate with clinical therapeutic applications including improving bone mineral density [21,22] and anti-tumor immunity through suppression of T_{regs} [23,24]. Zoledronic acid has also been shown to expand $\gamma\delta$-T cells *in vivo* [25] and sensitize tumors to $\gamma\delta$-T cell killing [26,27]. As such, it is possible that ZOL+IL-2 could restore the anti-leukemic activity of human effector lymphocytes pre-exposed to SMG.

The aim of this study was to determine if pre-exposure to SMG would impair the anti-leukemic function of human lymphocytes *in vivo* using the NSG-Tg (Hu-IL15) mouse model (Fig. 1, Ref. [28,29]). K562 leukemia bearing mice were engrafted with human immune cells exposed to either SMG or 1G and monitored for tumor growth over 6-weeks via bioluminescent imaging. Additionally, we tested if ZOL+IL-2 administered *in vivo* could revive SMG-induced loss of anti-leukemic function.

2. Materials and Methods

2.1 Participants and Blood Collection

Healthy adults between the age of 18–49 years volunteered for the study. Each participant provided written informed consent and had a single blood sample drawn by standard phlebotomy. Blood was collected in acid citrate dextrose (ACD) tubes and peripheral blood mononuclear cells (PBMCs) were isolated using density gradient separation method with Ficoll (Sigma-Aldrich, St. Louis, MO, USA) and processed immediately. All study procedures were approved by the Institutional Review Board (IRB) at University of Arizona.

2.2 Rotary Cell Culture System

We exposed human PBMCs to simulated microgravity (SMG) or 1-G control as previously described [9]. Briefly, a rotary cell culture system (RCCS) (Synthecon, Houston, TX, USA) was used to simulate microgravity. PBMCs were resuspended in 10% FBS+RPMI media in 10ml high-aspect ratio vessels (HARVs) and exposed to simulated microgravity for 12-hours while rotating on a horizontal axis at 10RPM. A 1G-control HARV was rotated on a vertical axis exposing PBMCs to similar shear stress while experiencing 1G gravitational force.

2.3 NSG-Tg (Hu-IL15) Mouse Model

All mouse experiments were done in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines at the University of Arizona under an approved protocol. NSG-Tg (Hu-IL15) animals were purchased from Jackson laboratory (Bar Harbor, ME, USA) and breeding colonies were maintained at the University Animal Care facility. Animals were housed for the duration of the experiments at the University Animal Care facility.

K562 (chronic myeloid leukemia) cell line was used to induce a wide-spread tumor to evaluate systemic immune control of tumor growth and luciferase-tagged k562 made tumor growth monitoring viable. K562 cells also lack MHC1 molecules on their surface making them susceptible to NK-cell killing. Animals were irradiated with 100 cGy in a Cesium^{137} irradiator (Atomic Energy of Canada Limited, Chalk River, Canada) a day before they were injected with 1 million luciferase-tagged-k562 (chronic myeloid leukemia) cells (Fig. 1). One week later, they were injected with 10 million PBMCs from SMG or 1G control conditions. 'Tumor only' control group received only 1 million K562 cells with placebo (PBS) on the day of immune cells injection. Bioluminescent intensity, weight, morbidity, and GVHD were monitored twice a week. Engraftment data was also collected from another group of mice that received only immune cells without tumor cells to examine differences in engraftment in the absence of tumor. Blood draw was performed from the sub-mandibular vein once a week to examine engraftment dynamics. Animals were sacrificed when they reached sacrificial criteria or at the end of the experiment (6 weeks). Since we propose to study the effect SMG on *in vivo* anti-leukemic activity of human PBMCs, which is largely driven by NK cells, we used this NSG-Tg (Hu-IL15) mouse model, which enhances *in vivo* survival of human NK cells. Our experiments suggested that acute GVHD signs occur around 14 days after PBMC injection and severe GVHD occurs around day 28. Therefore, we chose to evaluate graft-versus-tumor effect before severe GVHD starts setting in to prevent GVHD associated immune modulation from eclipsing the graft-versus-tumor effect of human PBMCs. Acute GVHD scoring was based on 6 parameters in accordance with previously published grading criteria [30,31]. All animals were terminated if they presented with a GVHD score of 4 or more or had lost more than 20% of their initial body weight.

2.4 Administration of Zoledronic Acid and Interleukin-2

Subgroups of mice received an intraperitoneal injection of zoledronic acid (50 μg/kg) with a subcutaneous injection of IL-2 (40,000 I.U) 1x weekly starting at day 7 (i.e., the day of PBMC transfer) of the experiment (Fig. 1).

2.5 Bioluminescent Imaging

K-562-luc2 (ATCC® CCL-243-LUC2™) (American Type Culture Collection, Manassas, VA, USA), which is a K562 chronic myeloid leukemia cell line that has been transfected with luciferase, was used for the *in vivo* experiments [28,29]. Tumor burden was measured immediately after D-luciferin (GoldBio, St Louis, MO) was injected intraperitoneally. A LagoX spectral imager (Spectral Instruments Imaging, Tucson, AZ) was used to quantify bioluminescent intensity (BLI) scores.

2.6 Flow Cytometry

Antibodies (CD8 Vioblue, CD3 Viogreen, vγ9 vδ2 FITC, CD4 PE, CD45 Per-CP, vγ9 vδ1 APC, CD56 APC-Vio770, CD16 Vioblue, NKG2D PE, PD-1 Per-CP, CD158b PE-Vio770, NKG2A APC, TCR-$\gamma\delta$ APC-Vio770, NKp30 PE) (Miltenyi Biotec, San Diego, CA) were added to 25 μL of whole blood at the proper titrated volumes and allowed to incubate for 30 minutes at room temperature in the dark. Blood samples were lysed with 1X Red Blood Cell Lysis solution (Miltenyi Biotec, San Diego, CA), washed twice in phosphate-buffered saline (PBS), and resuspended in 200 μL PBS.

2.7 Statistical Analysis

Statistical analysis was performed on Graphpad Prism 8.4.3 software (Graphpad, San Diego, CA, USA). For tumor progression, bioluminescent intensity data was log-transformed for normality as previously indicated [32]. A mixed effects model was used to analyze differences between tumor growth with either 3 (e.g., SMG, 1G, vehicle) or 5 (e.g., SMG, SMG+ZOL+IL-2, 1G, vehicle, vehicle+ZOL+IL-2) 'condition' levels included in the model, along with a main effect for 'time' and 'condition*time' included as an interaction effect. When the interaction effect was significant (indicating that tumor growth differed between conditions as the experiment progressed), systematic pairwise comparisons were conducted with Bonferroni correction to identify which conditions were significantly different from each other over time. Peak BLI was analyzed using Friedman test for paired comparisons. GVHD scores and engraftment data were analyzed using a linear mixed model or a two-way RM ANOVA. A log-rank (Mantel-Cox) test was used for survival analysis. Statistical significance was set at $p < 0.05$.

3. Results

3.1 Exposure to SMG Impaired the Anti-Leukemic Activity of Human Immune Cells in Vivo

To evaluate the effect of SMG on anti-leukemia activity of human immune cells *in vivo* we compared tumor growth between mice that were injected with PBMCs exposed to SMG (TUMOR+SMG PBMCs) or 1G-control (TUMOR+1G PBMCs). TUMOR+vehicle (PBS) was used as a control reference for unrestrained tumor growth. Representative BLI images showing tumor dynamics *in vivo* are shown in Fig. 2. For tumor growth, statistically significant effects were found for time, condition, and time*condition

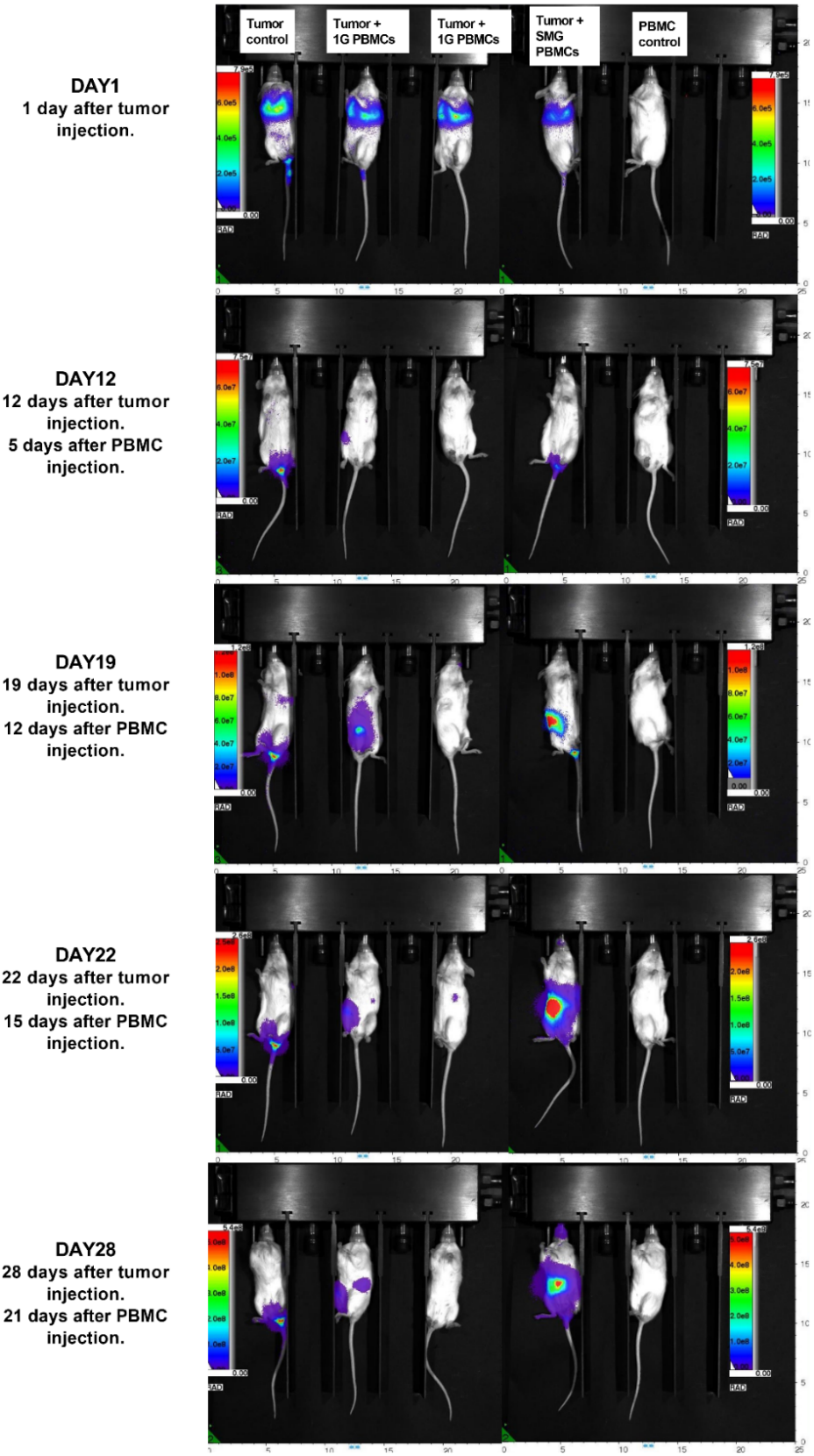

Fig. 2. Bioluminescent intensity (BLI) images. Days 1, 12, 19, 22 and 28 of the experiment. Lane 1: tumor control, lanes 3, 5: tumor+ 1G PBMCs, lane 6: tumor+ SMG PBMCs, lane 8: PBMC control.

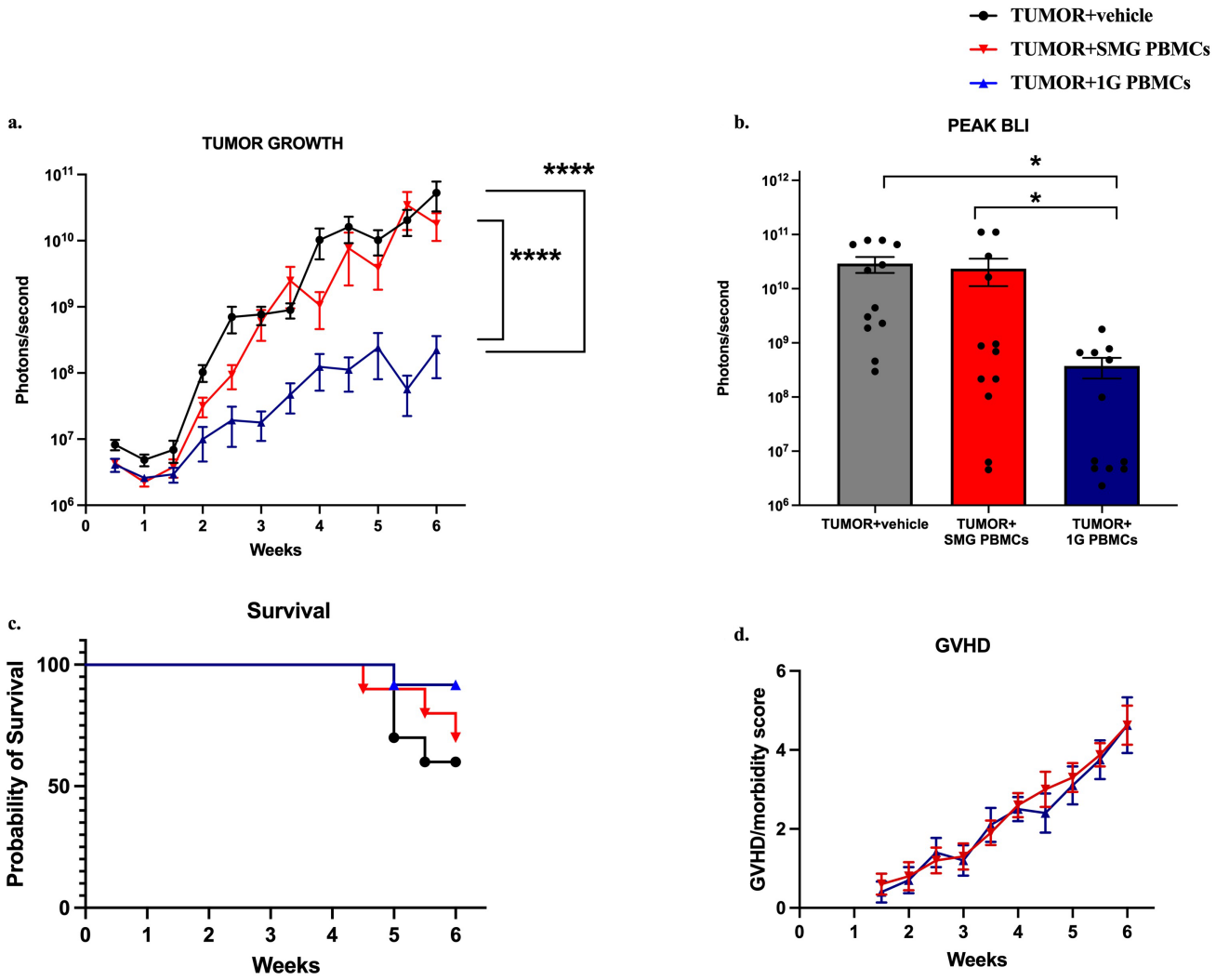

Fig. 3. Effect of SMG on effector immune cell function *in vivo*. (a) shows effect of SMG exposure of immune cells on tumor growth *in vivo*. (b) shows peak BLI scores during the experiment. (c) and (d) show survival and GVHD incidence. N = 12, MEAN±SEM.

($p < 0.0001$) (Fig. 3a). Post-hoc analyses revealed these effects to be driven by a lower tumor burden in the mice transferred with 1G exposed PBMCs compared to mice transferred with SMG exposed PBMCs and vehicle. Tumor growth kinetics were similar between SMG exposed PBMCs and vehicle control ($p > 0.05$).

Peak tumor growth (as determined by highest BLI score) was significantly higher in the mice receiving SMG exposed PBMCs and vehicle compared to mice receiving 1G exposed PBMCs.

There were no significant differences for peak tumor growth between the SMG exposed PBMC and vehicle conditions ($p > 0.05$). Overall survival up to 6-weeks did not differ significantly across the conditions ($\chi^2 = 2.934$, $p = 0.231$) (Fig. 3c). For GvHD/morbidity score, an expected significant effect was seen for time ($p < 0.0001$), but not 'condition' ($p = 0.764$) or 'condition*time' ($p = 0.981$) (Fig. 3d).

3.2 Exposure to SMG did not Affect Human Immune Cell Engraftment In Vivo

To verify that exposure to SMG did not impair the PBMCs' ability to engraft and expand in a xenograft; mice were injected with PBMCs only and not tumor. Engraftment was measured as a proportion of CD45human+ (CD45h+) cells in total immune cells in mice blood collected at weekly intervals from week 2–5. The engraftment dynamics of PBMCs in the mice were not statistically different between SMG and 1G conditions (Fig. 4). This was true for total PBMCs as defined by human CD45 expression as a percentage of the total human + mouse CD45 expressing cells (% $p = 0.175$; cells/uL, $p = 0.628$, Fig. 4a,c) and for NK-cells (CD3-/CD56+) expressed as a percentage of CD45h+ cells (% $p = 0.219$; cells/μL, $p = 0.478$, Fig. 4b,d). Similar human immune cell engraftment dynamics were observed in mice with tumor (Fig. 5).

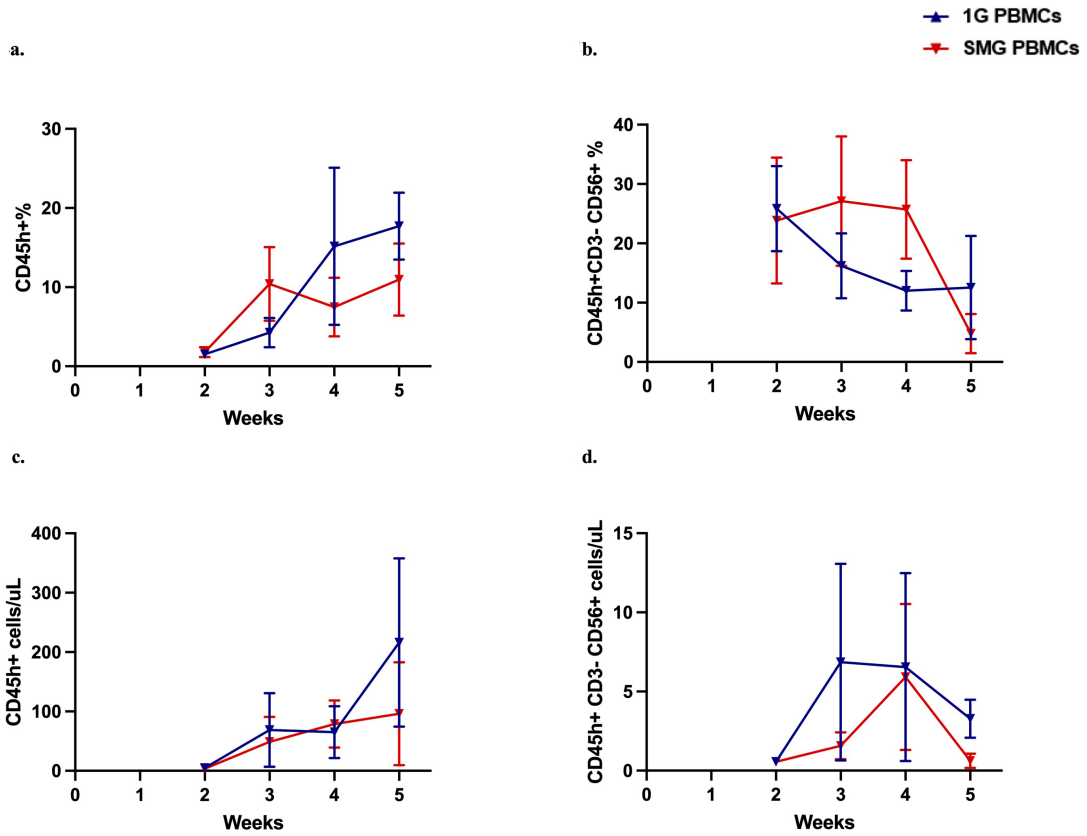

Fig. 4. Effect of exposure to SMG on human cell engraftment dynamics *in vivo* in the absence of tumor. (a) and (c) show total PBMC engraftment. (b) and (d) show NK cells engraftment. N = 10, MEAN±SEM.

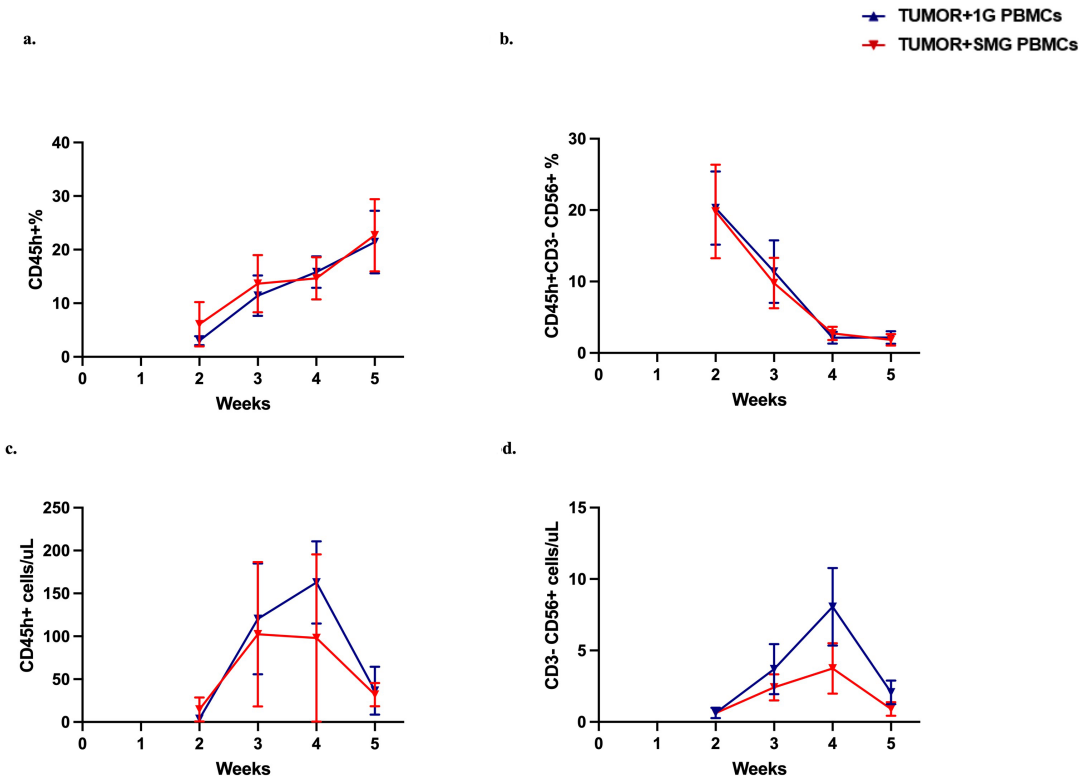

Fig. 5. Effect of SMG on human cells engraftment *in vivo* in the presence of tumor. (a) and (c) show total PBMC engraftment. (b) and (d) show NK cells engraftment. N = 5, MEAN±SEM.

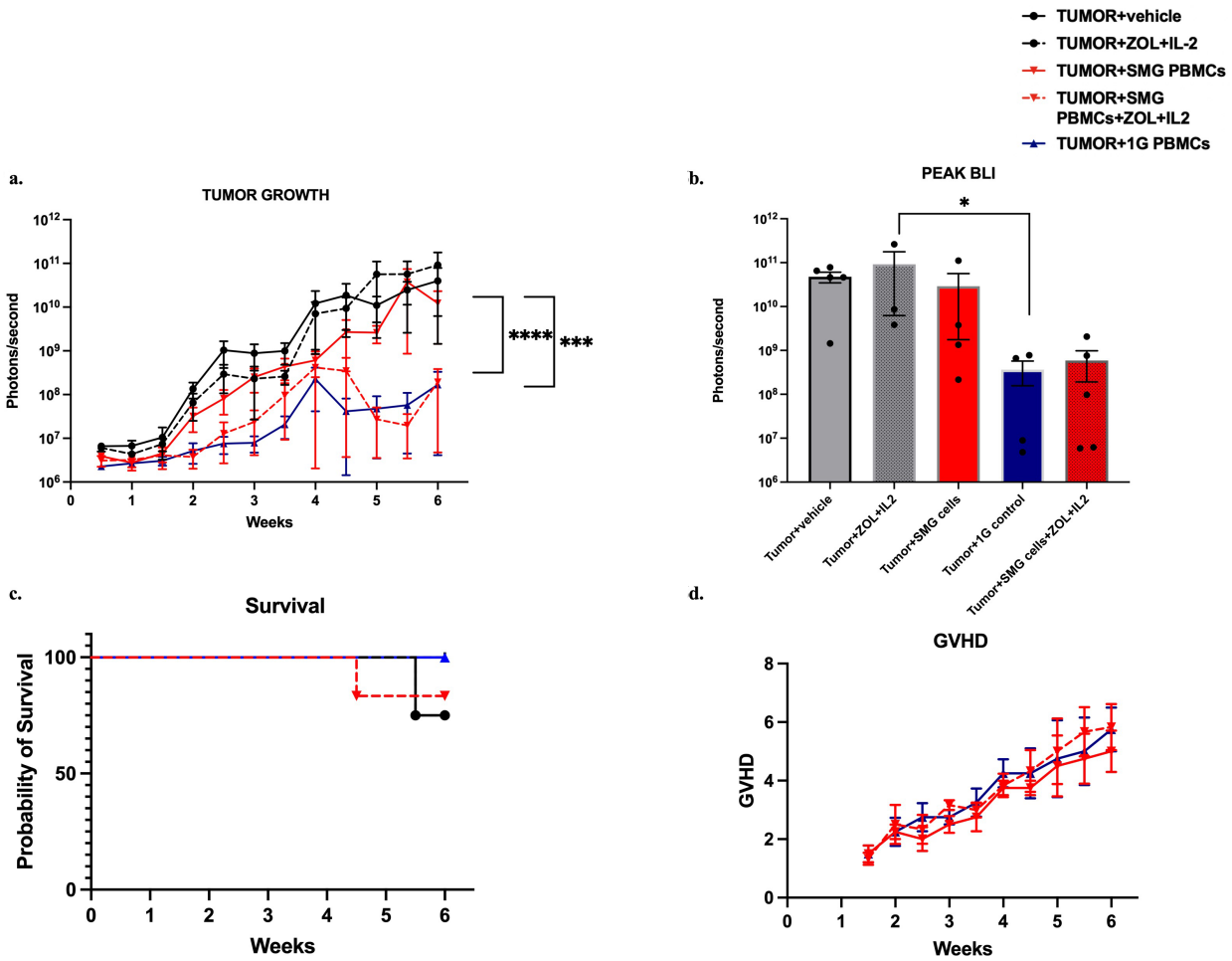

Fig. 6. Effect of zoledronic acid+IL2 (ZOL+IL2) therapy on SMG induced suppression of *in vivo* anti-leukemia activity. (a) shows effect of ZOL+IL2 therapy on tumor growth *in vivo*. (b) shows peak BLI scores during the experiment. (c) and (d) show survival and GVHD incidence. N = 5, MEAN±SEM.

3.3 Zoledronic Acid and IL-2 Therapy Improved Anti-Leukemia Activity of Human Immune Cells in Vivo

In an attempt to 'rescue' the anti-leukemic effects of PBMCs pre-exposed to SMG prior to adoptive transfer, we injected mice with ZOL+IL-2 on the day of PBMC transfer and once weekly thereafter. For tumor growth, statistically significant effects were found for time, condition, and time*condition ($p < 0.0001$) (Fig. 6a). As observed in the previous experiment, 1G exposed PBMCs significantly inhibited tumor growth compared to SMG exposed PBMCs. Post-hoc analysis revealed a significant interaction effect ($p = 0.0004$) between TUMOR+SMG PBMCs and TUMOR+SMG PBMCs+ZOL+IL2, while no interaction effects were found for TUMOR+SMG PBMCs+ZOL+IL2 versus TUMOR+1G PBMCs ($p = 0.567$). There were also no significant differences for condition ($p = 0.947$) or time ($p = 0.221$) between these groups. This revealed that ZOL+IL2 administration enabled SMG-exposed PBMCs to control tumor growth rate as efficiently as 1G-exposed PBMCs. As expected, ZOL+IL-2 had no effect on tumor growth dynamics in the absence of PBMCs ($p > 0.05$). Peak tumor growth (as determined by highest BLI score) was significantly higher in the mice receiving SMG exposed PBMCs and vehicle (with or without ZOL+IL-2) compared to mice receiving 1G exposed PBMCs and SMG exposed PBMCs+ZOL+IL-2. There were no significant differences for peak tumor growth between the 1G exposed PBMCs and the SMG exposed PBMCs+ZOL+IL-2 ($p > 0.05$), again indicating that the administration of ZOL+IL-2 rescued the anti-leukemic effect of PBMCs pre-exposed to SMG. Overall survival up to 6-weeks did not differ significantly across the conditions ($\chi^2 = 2.429$, $p = 0.6575$) (Fig. 6c). Administration of ZOL+IL2 therapy did not affect GVHD/morbidity progression ($p > 0.999$) (Fig. 6d).

3.4 Zoledronic Acid and IL-2 Therapy did not Alter the Number of NK and γδ-T Cells in Blood

To determine if ZOL+IL2 therapy increased the number and proportion of NK cells and γδ-T cells in peripheral blood, weekly human leukocyte engraftment checks were performed after adoptive transfer (Fig. 7). No significant interaction*time effects were found for total CD45h+ (%

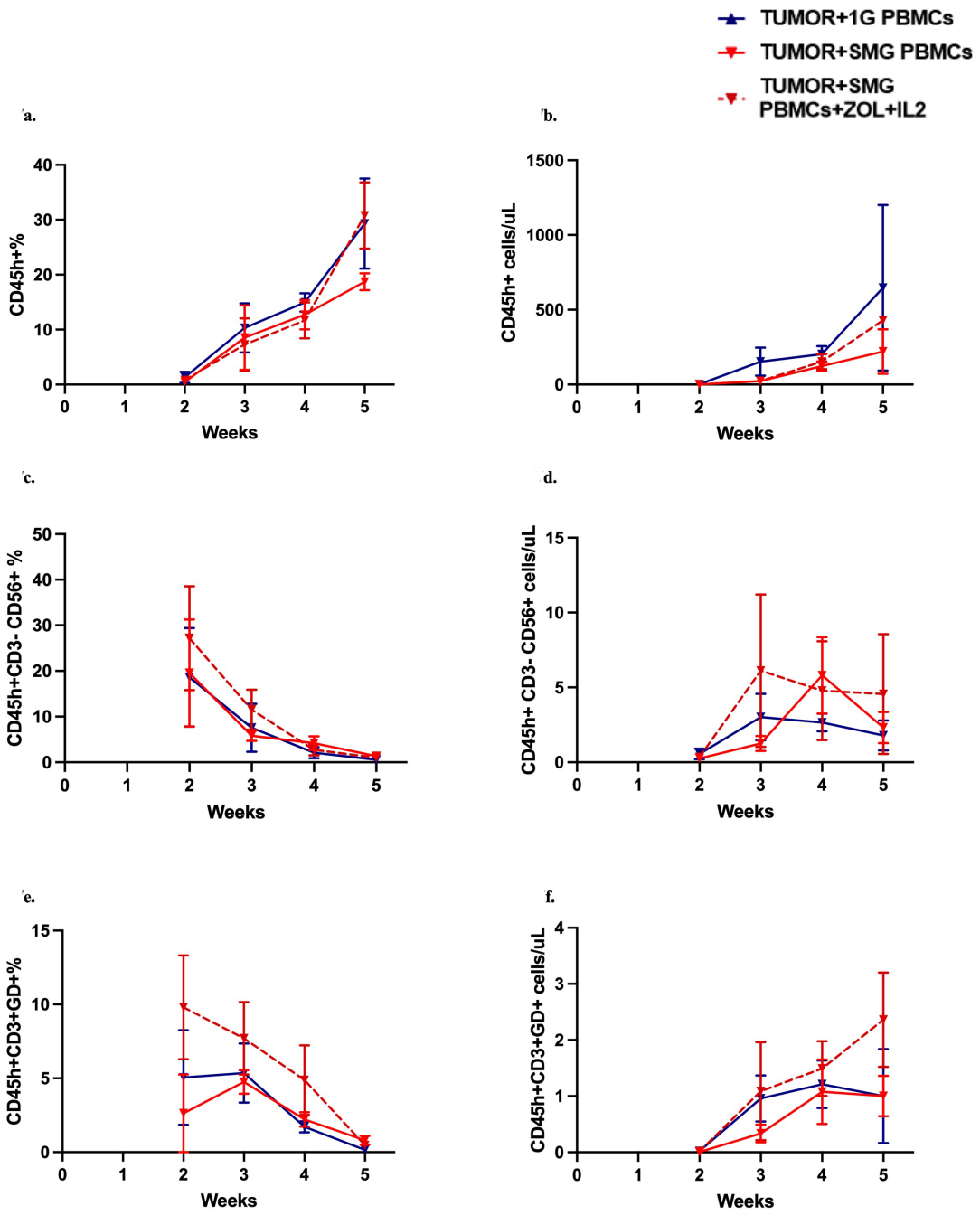

Fig. 7. Effect of SMG and ZOL+IL2 therapy on human cells engraftment *in vivo* in the presence of tumor. (a) and (b) show total PBMC. (c) and (d) show NK cell. (e) and (f) show $\gamma\delta$-T cell engraftment dynamics. N = 5 MEAN±SEM.

p = 0.713; cells/uL, p = 0.419, Fig. 7a,b), NK cells (% p = 0.99; cells/uL, p = 0.941, Fig. 7c,d), or $\gamma\delta$-T cells (% p = 0.915; cells/uL, p = 0.40.937, Fig. 7e,f), indicating that ZOL+IL-2, despite increasing tumor control, did not expand these leukocyte populations in peripheral blood.

4. Discussion

The overarching aims of this study were to determine if the loss of NK-cell function against a leukemic cell line *in vitro* due to exposure to simulated microgravity (SMG) would extend to an *in vivo* model, and to also test the efficacy of zoledronic acid and IL-2 (ZOL+IL-2) therapy as a countermeasure to SMG-induced immune dysfunction.

Using a human K562 tumor bearing immunocompromised mouse model, we found that pre-exposing human PBMCs to SMG for 12 h inhibits their ability to control leukemic growth *in vivo* after adoptive transfer, but systemic administration of ZOL+IL-2 'rescued' this SMG induced impairment of anti-leukemic activity. These findings indicate that microgravity likely plays a contributory role to immune system impairment during long duration space travel. Further, ZOL+IL-2 (a treatment that is currently

used to expand and increase the anti-leukemic activity of NK-cells and $\gamma\delta$-T cells in cancer patients [19,20,33]) could serve as an effective countermeasure to mitigate deficits in anti-tumor immunity due to prolonged exposure to microgravity during extended space missions.

This is the first study, to our knowledge, to show that pre-exposing primary human lymphocytes to SMG inhibits their ability to control leukemic growth *in vivo*. Not only were the SMG exposed PBMCs less effective at controlling leukemic growth in comparison to their 1G exposed counterparts, tumor growth kinetics were identical between the SMG exposed and vehicle control conditions indicating that SMG completely incapacitates the anti-leukemic activity of human lymphocytes. This corroborates our previous findings that pre-exposing human lymphocytes to SMG impairs their ability to kill various hematologic cancer cells lines *in vitro* [34]. While the mechanisms underpinning this response are not fully known, our prior *in vitro* studies showed that SMG impairs several anti-tumor properties of NK-cells, including their ability to deliver 'lethal hits' through cytotoxic degranulation and secretion of effector cytokines [34]. Although it is possible that SMG could have impaired human cell engraftment in the mouse, we somewhat alleviated this potential confounder by showing similar levels of human leukocyte engraftment between the SMG and 1G conditions. While blood levels of immune cells might not be reflective of engraftment levels in tissues or in mice with tumor, this showed that human immune cells (including NK cells) thrived similarly in mice after exposure to 1G and SMG. *In vivo* expansion of (CD45h+CD3-CD56+) human NK cells in the initial weeks was to be expected in an NSG-Tg (Hu-IL15) mouse model. NSG-Tg (Hu-IL15) mice sustain IL-15 blood levels of 7.1 +/- 0.3 pg/mL [35]. These levels are considerably higher than the usual undetectable levels in human blood ($\approx$ 1 pg/mL) [36]. This mouse model was precisely chosen to sustain and expand NK cells, since a loss of NK cell function was deemed the underpinning reason why SMG evokes impaired anti-tumor responses of human lymphocytes. NK cell levels in blood fell in the later weeks when the differences in BLI between the groups reached discernible levels. It remains unclear if NK cells became exhausted in the later weeks (due to sustained IL-15 stimulation) or if they simply traffic away from the peripheral blood and are more abundant in the tissues and areas of sustained tumor growth.

Hematological tumor lysis in a 4-hour *in vitro* killing assay using mixed PBMCs is majorly attributed to the anti-tumor activity of NK cells; however, the ability to control tumor growth *in vivo* over a 6-week period might be a composite effect of all effector lymphocytes. While other effector lymphocytes like CD8+ T cells could have played a role, we deem this unlikely as GvHD scores (a manifestation of the human CD3+ $\alpha\beta$ T-cells attacking xenogeneic tissue in the mouse) were not different between the SMG and 1G exposed PBMC conditions indicating that SMG did not affect the alloreactivity of CD8+ $\alpha\beta$ T-cells. Past evidence shows *in vitro* T-cell activity is suppressed after exposure to SMG [37]. However, in our *in vivo* model, there might be a recovery of the lost function over the duration of the experiment, particularly because the immunocompromised mice we used have a human IL-15 knock in that could have restored any detriments in $\alpha\beta$ T-cell function due to SMG. Since clinical symptoms of GvHD did not manifest until 3–4 weeks into the experiment, this might reflect similar GVHD onset patterns between the groups. While similar recovery is possible in NK cells too, failure to control tumor growth in the initial weeks might be contributing to tumor burden reaching an uncontrollable level before NK cells regain their activity. The more efficient 1G-exposed PBMCs could be more adept at killing K562 tumor cells *in vivo* during the initial weeks, which might have resulted in stunted tumor growth in the later weeks.

There is a critical need to identify effective countermeasures that can mitigate immune dysregulation during long duration spaceflight. Subcutaneous IL-2 injections are commonly used as an immunotherapeutic strategy to enhance graft-versus-leukemia effects in bone marrow transplant recipients by accelerating NK-cell reconstitution [38]. Additionally, zoledronic acid, an aminobisphosphonate that is used to prevent osteoporosis [21] and currently being considered as a countermeasure to prevent decreases in bone mineral density due to weightlessness [39], has also been shown to expand $\gamma\delta$-T cells *in vivo* and increase their anti-tumor activity in graft recipients [40]. We show here for the first time that that systemic administration of ZOL+IL2 'rescued' the anti-leukemia activity of SMG-exposed PBMCs *in vivo*. Indeed, while SMG exposed PBMCs were rendered completely ineffective against K562 leukemic growth *in vivo*, the tumor growth kinetics in mice that received SMG exposed PBMCs were identical to those that received 1G exposed PBMCs when ZOL+IL-2 was administered. This indicates that ZOL+IL-2 has the potential to completely reverse the negative impact of SMG on the anti-leukemic activity of human lymphocytes. It should be noted, however, that all PBMCs were returned to the 1G environment after adoptive transfer and we do not currently know if ZOL+IL-2 is capable of maintaining anti-leukemic activity during periods of microgravity. Individual effects of IL2 [41], zoledronic acid [42–44] and their combination [45] have been shown to improve and/or accelerate immune reconstitution in transplant patients as well as have anti-leukemic effect. Ongoing clinical trials are also examining the effect of ZOL+IL-2 in cancer patients (NCT01404702, NCT00582790). Since we wanted to examine the translational potential of this drug combination that is currently used clinically into a preventative measure in long duration spaceflight, we tested them together. We do acknowledge, however, that it will be important to determine the relative contribution of ZOL and IL-2 to the restoration effects we observed *in vivo*. Nevertheless, these results indicate that

ZOL+IL-2 could at least be an effective strategy to restore human lymphocyte function in conditions of 1 g (e.g., on return to Earth after a mission) or partial (e.g., on arrival at the Martian surface) gravity following prolonged periods of weightlessness.

5. Conclusions

In summary, we have demonstrated that exposure to SMG impairs the anti-leukemia activity of human effector immune cells *in vivo* as well as *in vitro*, and that ZOL+IL2 therapy improved the anti-leukemia activity of human effector immune cells after exposure to SMG. Limitations of the present study include the short-term exposure to microgravity, not identifying the lymphocyte subtypes affected by SMG, and the fact that all functional experiments were conducted in 1G. We conclude that microgravity plays a contributory role in the loss of tumor control and should be considered a risk factor for impaired anti-tumor immune responses during prolonged space voyages. Immunostimulating agents like ZOL+IL-2 may help mitigate clinical risks associated with immune dysregulation during prolonged spaceflight and its effectiveness as an immune system countermeasure in humans warrants investigation.

Abbreviations

SMG, simulated microgravity; PBMC, peripheral blood mononuclear cells; NK cell, Natural Killer cell; MHC, major histocompatibility complex; GVHD, graft-versus-host-disease; ZOL, zoledronic acid; IL-2, interleukin-2; IL-15, interleukin-15; TNFα, tumor necrosis factor- alpha; IFNγ, interferon-gamma; BLI, bioluminescent intensity.

Author Contributions

RJS, PLM and EK designed the research. PLM, FLB, DMD and GMN performed the research. PLM, RJS, EK, MMM and BEC analyzed and/or interpreted the data. PLM and RJS wrote the manuscript. All co-authors edited the manuscript and approved its submission.

Ethics Approval and Consent to Participate

All study procedures were approved by the Institutional Review Board (IRB) at University of Arizona. All mouse experiments were done in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines at the University of Arizona under an approved protocol.

Acknowledgment

The authors thank Emely Hoffman and Jessica Stokes for assistance with the animal work.

Funding

These studies were supported by NASA grants NNX16AB29G to RJS and 80NSSC19K1059 to RJS, EK and PLM.

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Crucian B, Stowe R, Mehta S, Uchakin P, Quiriarte H, Pierson D, *et al*. Immune System Dysregulation Occurs during Short Duration Spaceflight on Board the Space Shuttle. Journal of Clinical Immunology. 2013; 33: 456–465.

[2] Crucian B, Stowe R, Quiriarte H, Pierson D, Sams C. Monocyte phenotype and cytokine production profiles are dysregulated by short-duration spaceflight. Aviation, Space, and Environmental Medicine. 2011; 82: 857–862.

[3] Crucian BE, Stowe RP, Pierson DL, Sams CF. Immune system dysregulation following short- vs long-duration spaceflight. Aviation, Space, and Environmental Medicine. 2008; 79: 835–843.

[4] Mehta SK, Laudenslager ML, Stowe RP, Crucian BE, Sams CF, Pierson DL. Multiple latent viruses reactivate in astronauts during Space Shuttle missions. Brain, Behavior, and Immunity. 2014; 41: 210–217.

[5] Crucian B, Stowe RP, Mehta S, Quiriarte H, Pierson D, Sams C. Alterations in adaptive immunity persist during long-duration spaceflight. Npj Microgravity. 2015; 1: 15013.

[6] Mehta SK, Laudenslager ML, Stowe RP, Crucian BE, Feiveson AH, Sams CF, *et al*. Latent virus reactivation in astronauts on the international space station. Npj Microgravity. 2017; 3: 11.

[7] Rooney BV, Crucian BE, Pierson DL, Laudenslager ML, Mehta SK. Herpes Virus Reactivation in Astronauts During Spaceflight and Its Application on Earth. Frontiers in Microbiology. 2019; 10: 16.

[8] Bigley AB, Agha NH, Baker FL, Spielmann G, Kunz HE, Mylabathula PL, *et al*. NK cell function is impaired during long-duration spaceflight. Journal of Applied Physiology. 2019; 126: 842–853.

[9] Mylabathula PL, Li L, Bigley AB, Markofski MM, Crucian BE, Mehta SK, *et al*. Simulated microgravity disarms human NK-cells and inhibits anti-tumor cytotoxicity in vitro. Acta Astronautica. 2020; 174: 32–40.

[10] Bosma GC, Custer RP, Bosma MJ. A severe combined immunodeficiency mutation in the mouse. Nature. 1983; 301: 527–530.

[11] Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, *et al*. Human Lymphoid and Myeloid Cell Development in NOD/LtSz-scid IL2R null Mice Engrafted with Mobilized Human Hemopoietic Stem Cells. Journal of Immunology. 2005; 174: 6477–6489.

[12] Cao X, Shores EW, Hu-Li J, Anver MR, Kelsall BL, Russell SM, *et al*. Defective lymphoid development in mice lacking expression of the common cytokine receptor γ chain. Immunity. 1995; 2: 223–238.

[13] Shultz LD, Ishikawa F, Greiner DL. Humanized mice in translational biomedical research. Nature Reviews Immunology. 2007; 7: 118–130.

[14] Cooper MA, Bush JE, Fehniger TA, VanDeusen JB, Waite RE, Liu Y, *et al*. In vivo evidence for a dependence on interleukin 15 for survival of natural killer cells. Blood. 2002; 100: 3633–3638.

[15] Crucian BE, Choukèr A, Simpson RJ, Mehta S, Marshall G, Smith SM, *et al*. Immune system dysregulation during spaceflight: Potential countermeasures for deep space exploration missions. Frontiers in Immunology. 2018; 9: 1437.

[16] Cheever MA, Greenberg PD, Fefer A, Gillis S. Augmentation of the anti-tumor therapeutic efficacy of long-term cultured T lymphocytes by in vivo administration of purified interleukin 2. Journal of Experimental Medicine. 1982; 155: 968–980.
[17] Mazumder A, Rosenberg SA. Successful immunotherapy of natural killer-resistant established pulmonary melanoma metastases by the intravenous adoptive transfer of syngeneic lymphocytes activated in vitro by interleukin 2. Journal of Experimental Medicine. 1984; 159: 495–507.
[18] Rosenberg SA. IL-2: the first effective immunotherapy for human cancer. Journal of Immunology. 2014; 192: 5451–5458.
[19] Muntasell A, Ochoa MC, Cordeiro L, Berraondo P, López-Díaz de Cerio A, Cabo M, *et al*. Targeting NK-cell checkpoints for cancer immunotherapy. Current Opinion in Immunology. 2017; 45: 73–81.
[20] Kimpo MS, Oh B, Lee S. The Role of Natural Killer Cells as a Platform for Immunotherapy in Pediatric Cancers. Current Oncology Reports. 2019; 21: 93.
[21] Fobelo Lozano MJ, Sánchez-Fidalgo S. Adherence and preference of intravenous zoledronic acid for osteoporosis versus other bisphosphonates. European Journal of Hospital Pharmacy. 2019; 26: 4–9.
[22] Mei M, Xiang Z, Yang J, Xiang R. Efficacy of zoledronic acid for prevention of bone loss in early-stage breast cancer patients receiving adjuvant therapy: a meta-analysis of 13 randomized controlled trials. Current Problems in Cancer. 2020; 44: 100507.
[23] Liu H, Wang SH, Chen SC, Chen CY, Lo JL, Lin TM. Immune modulation of CD4(+) CD25(+) regulatory T cells by zoledronic acid. BMC Immunology. 2016; 17: 45.
[24] Salaroglio IC, Campia I, Kopecka J, Gazzano E, Orecchia S, Ghigo D, *et al*. Zoledronic acid overcomes chemoresistance and immunosuppression of malignant mesothelioma. Oncotarget. 2015; 6: 1128–1142.
[25] Mönkkönen H, Ottewell PD, Kuokkanen J, Mönkkönen J, Auriola S, Holen I. Zoledronic acid-induced IPP/ApppI production in vivo. Life Sciences. 2007; 81: 1066–1070.
[26] Caccamo N, Meraviglia S, Scarpa F, La Mendola C, Santini D, Bonanno CT, *et al*. Aminobisphosphonate-activated gammadelta T cells in immunotherapy of cancer: doubts no more. Expert Opinion on Biological Therapy. 2008; 8: 875–883.
[27] Hamilton E, Clay TM, Blackwell KL. New perspectives on zoledronic acid in breast cancer: potential augmentation of anticancer immune response. Cancer Investigation. 2011; 29: 533–541.
[28] Paley MA, Prescher JA. Bioluminescence: a versatile technique for imaging cellular and molecular features. MedChemComm. 2014; 5: 255–267.
[29] Duda J, Karimi M, Negrin RS, Contag CH. Methods for imaging cell fates in hematopoiesis. Methods in Molecular Medicine. 2007; 134: 17–34.
[30] Lai H, Chou T, Tzeng C, Lee OK. Cytokine Profiles in Various Graft-Versus-Host Disease Target Organs following Hematopoietic Stem Cell Transplantation. Cell Transplantation. 2012; 21: 2033–2045.
[31] Naserian S, Leclerc M, Thiolat A, Pilon C, Le Bret C, Belkacemi Y, *et al*. Simple, reproducible, and efficient clinical grading system for murine models of acute graft-versus-host disease. Frontiers in Immunology. 2018; 9: 10.
[32] Stokes J, Hoffman EA, Zeng Y, Larmonier N, Katsanis E. Post-transplant bendamustine reduces GvHD while preserving GvL in experimental haploidentical bone marrow transplantation. British Journal of Haematology. 2016; 174: 102–116.
[33] Fisher JP, Heuijerjans J, Yan M, Gustafsson K, Anderson J. Γδ T cells for cancer immunotherapy: A systematic review of clinical trials. OncoImmunology. 2014; 3: e27572.
[34] Tam YK, Klingemann H. Antileukemic effect of interleukin-2-transduced murine bone marrow after autologous transplantation. Biology of Blood and Marrow Transplantation. 1999; 5: 231–242.
[35] Weiss L, Reich S, Slavin S. Use of recombinant human interleukin-2 in conjunction with bone marrow transplantation as a model for control of minimal residual disease in malignant hematological disorders: I. Treatment of murine leukemia in conjunction with allogeneic bone marrow transplantation and IL-2-activated cell-mediated immunotherapy. Cancer Investigation. 1992; 10: 19–26.
[36] Yamasaki A, Onishi H, Morisaki T, Katano M. Induction of cytotoxic T lymphocytes by CEA peptide-pulsed γδ T-cells isolated from patients with advanced cancer. Anticancer Research. 2011; 31: 2419–2424.
[37] Nada MH, Wang H, Workalemahu G, Tanaka Y, Morita CT. Enhancing adoptive cancer immunotherapy with Vγ2Vδ2 T cells through pulse zoledronate stimulation. Journal for ImmunoTherapy of Cancer. 2017; 5: 9.
[38] Cui Q, Shibata H, Oda A, Amou H, Nakano A, Yata K, *et al*. Targeting myeloma–osteoclast interaction with Vγ9Vδ2 T cells. International Journal of Hematology. 2011; 94: 63–70.
[39] Galley HF, Lowes DA, Thompson K, Wilson ND, Wallace CA, Webster NR. Characterisation of gamma delta (γδ) T cell populations in patients with sepsis. Cell Biology International. 2015; 39: 210–216.
[40] Zoine JT, Knight KA, Fleischer LC, Sutton KS, Goldsmith KC, Doering CB, *et al*. Ex vivo expanded patient-derived γδ T-cell immunotherapy enhances neuroblastoma tumor regression in a murine model. Oncoimmunology. 2019; 8: 1593804.
[41] Bergamaschi C, Bear J, Rosati M, Beach RK, Alicea C, Sowder R, *et al*. Circulating IL-15 exists as heterodimeric complex with soluble IL-15Rα in human and mouse serum. Blood. 2012; 120: e1–e8.
[42] Hauschild S, Tauber S, Lauber B, Thiel CS, Layer LE, Ullrich O. T cell regulation in microgravity – the current knowledge from in vitro experiments conducted in space, parabolic flights and ground-based facilities. Acta Astronautica. 2014; 104: 365–377.
[43] Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, *et al*. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood. 2005; 105: 3051–3057.
[44] Cavanagh PR, Licata AA, Rice AJ. Exercise and pharmacological countermeasures for bone loss during long-duration space flight. Gravitational and Space Biology Bulletin. 2005; 18: 39–58.
[45] Bertaina A, Zorzoli A, Petretto A, Barbarito G, Inglese E, Merli P, *et al*. Zoledronic acid boosts γδ T-cell activity in children receiving αβ + T and CD19 + cell-depleted grafts from an HLA-haplo-identical donor. Oncoimmunology. 2016; 6: e1216291.