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Comparing thermal stress reduction strategies that influence MDSC accumulation in tumor bearing mice.

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Abstract

Myeloid derived suppressor cells (MDSCs) are a diverse collection of immune cells that suppress anti-tumor immune responses. Decreasing MDSCs accumulation in the tumor microenvironment could improve the anti-tumor immune response and improve immunotherapy. Here, we examine the impact of physiologically relevant thermal treatments on the accumulation of MDSCs in tumors in mice. We found that different temperature-based protocols, including 1) weekly whole-body hyperthermia, 2) housing mice at their thermoneutral temperature (TT, ~30°C), and 3) housing mice at a subthermoneutral temperature (ST, ~22°C) while providing a localized heat source, each resulted in a reduction in MDSC accumulation and improved tumor growth control compared to control mice housed at ST, which is the standard, mandated housing temperature for laboratory mice. Additionally, we found that low dose β -adrenergic receptor blocker (propranolol) therapy reduced MDSC accumulation and improved tumor growth control to a similar degree as the models that relieved cold stress. These results show that thermal treatments can decrease MDSC accumulation and tumor growth comparable to propranolol therapy.

Keywords

Myeloid derived suppressor cells; thermal stress; hyperthermia; β -adrenergic signaling; β -adrenergic receptor blockers

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1. Introduction

Myeloid Derived Suppressor Cells (MDSCs) are a collection of immune cells that play a key role in suppressing immune responses [1, 2]. This is potentially beneficial during tissue remodeling, wound healing, and prevention of auto-immunity [3-5]. However, these cells are also recruited to the tumor microenvironment when malignancies develop [6, 7]. In this context, the immunosuppressive effects of MDSCs lead to impairment of anti-tumor immunity, contributing to cancer progression and treatment resistance. Although many avenues of therapy are being investigated as methods to mitigate the negative impact of MDSCs [8], past work from our laboratory and others has shown limiting exposure to chronic stress could be one such mechanism [9, 10].

The human neuroendocrine response to chronic stress is partially mediated by catecholamines from the sympathetic nervous system signaling through beta-adrenergic receptors (β -AR) [11]. Interestingly, mice and other mammals that possess large amounts of brown adipose tissue use the sympathetic nervous system and the same neuroendocrine mediators, such as norepinephrine, to stimulate heat generation and prevent hypothermia when exposed to cold stress [12]. Our lab observed that increased production of norepinephrine occurs (needed for thermogenesis) not only during acute cold stress, but also when mice are housed at mildly subthermoneutral temperatures [13], which is approximately 22°C [14]. Because the IACUC mandated standard temperature (ST) for housing mice is typically 22-23°C, mice housed under these temperatures experience low levels of chronic cold stress. Past work from our laboratory and that of others has revealed that this mild chronic cold stress, which increases adrenergic receptor signaling throughout the body, can have effects on many immune cells, including effector T cells [15-18], regulatory T cells [19, 20], dendritic cells [21, 22], natural killer cells [23], and MDSCs [9]. Taken together, the impact of cold stress on these immune cells culminates in more rapid tumor growth; however, the relative contributions of changes in each of these cell types is not clear.

We have previously determined that the increase in systemic adrenergic signaling mediated by cold stress leads to an increase in MDSC accumulation and immunosuppressive function, which could be mitigated by housing mice at TT or blocking β -AR signaling with clinically available β -blockers [24]. The goal of this study was to investigate whether other methods of manipulating caging temperature and/or adrenergic signaling could be implemented to limit MDSC activity. We observed that allowing mice access to a simple cage heating system with a localized heat source [25], or weekly whole-body hyperthermia (WBH) treatments [26], also limited MDSC accumulation in tumors and tumor growth. This inhibition of MDSC accumulation was similar to that achieved using even very low doses of the pan β -adrenergic receptor antagonist, propranolol, in mice housed at ST. Together these data support the notion that the modulation of ambient temperature, and/or the mitigation of adrenergic stress signaling, are potential methods for mitigating MDSC mediated immune suppression.

2. Methods

Animals.

BALB/c (H-2d) mice were purchased from Charles River. All mice were maintained under standard housing conditions unless otherwise specified, and were approximately 8 weeks of age when tumor implantation occurred. All experiments were performed in accordance with the animal care guidelines at Roswell Park Comprehensive Cancer Center, and all protocols used were approved by the institutional animal care and use committee (IACUC).

Tumor models.

4T1 mammary carcinoma tumor cells were purchased from ATCC (ATCC, catalog CRL-2539), and are tested for mycoplasma yearly with the Mycoplasma Plus PCR Primer Set (Agilent Technologies, catalog 302008). Cells were cultured in RPMI 1640 (Corning), with 10% FBS, 1% l-glutamine, 1% penicillin/streptomycin, and 5% ambient CO₂ with 95% air. 10⁵ cells in 100 μL of PBS were orthotopically injected into the 4th mammary fat pad, and were always passed twice in culture after thawing before use. Tumor size was measured by the same individual using the same set of calipers throughout the experiments, and tumor volume was calculated by the equation: $2S \times L / 2$, where S is the small dimension and L is the large dimension.

Ambient temperature manipulation.

As described previously [27], rat cages with a false floor were used to house mice in groups of 5 for all standard temperature (ST, ~22°C), thermoneutral temperature (TT, ~30°C), and local heat source (~22°C with hand warmer) experiments. For the local heat source treatment group, cages were kept at ST, and newly opened hand warmers were exchanged daily at approximately 8 am. For mice at ST and TT conditions, cages were placed in rooms maintained to their appropriate temperatures, and these cages were also manipulated daily in the same way that local heat source treatment cages were. Briefly, the hand warmers produce heat as they undergo an exothermic reaction converting iron to iron oxide in the presence of oxygen in the air [24]. In both local heat and TT conditions the core body temperature did not change compared to the baseline.

Propranolol treatments (β-blocker).

For studies in which the pan-β-adrenergic receptor antagonist, propranolol, was used to assess the impact of adrenergic signaling on tumor growth and MDSC accumulation, 4T1 tumor-bearing mice were housed at ST. Daily treatment with propranolol (P0884, Sigma-Aldrich) began 4 days prior to tumor implantation and continued throughout the course of the experiment. Mice received a dose of 10.0, 1.0, or 0.1 mg/kg (200.0, 20.0, or 2.0 μg respectively in 200 μL of PBS) by intraperitoneal injection.

Whole body hyperthermia treatments.

Similar to as was described previously [28], mice received whole body hyperthermia treatments weekly for the duration of the tumor growth experiments. Once mice were received from Charles River, a temperature probe/transponder from the Electronic

Laboratory Animal Monitoring System from Biomedic Data Systems (Maywood, NJ) was implanted subcutaneously into the dorsal thoracic region of one mouse from each cage, and mice were given at least 2 weeks to heal/acclimate to their housing conditions. Immediately prior to treatment, mice received 1.0 mL of PBS via an intraperitoneal injection for hydration. WBH mice were then transferred to a cage that was pre-heated to 38.5°C and placed within the environmental chamber (Mammert model BE500; Mammert, East Troy, WI). Core body temperatures were measured every 30 minutes, and the temperature of the chamber was increased by 1°C every half hour until the core body temperature of WBH mice reached 39.0°C. That temperature was maintained, and an average core temperature was held constant between 39.0°C and 40.0°C for 6 hours. Control mice were subjected to the same conditions and temperature readings as well. It is worth noting that in the hyperthermia protocol core body temperature raised above baseline but the core body temperature did not change in local heat or TT conditions.

Flow cytometry.

For spleens, after dissection, mechanical disruption and filtration through a 70 µm filter (Corning) was done to create single cell suspensions. AKC lysis buffer (Gibco) was used to lyse red blood cells prior to staining. For tumors, after dissection and removal, a scalpel was used to mechanically break down the tissue. Collagenase/hyaluronidase (Stem Cell Technologies, 07912) was then used following the manufacturer's instructions, and samples were filtered through 70 µm filters prior to staining. 10⁶ live cells per spleen and tumor samples were used for staining. Cells were first washed in flow running buffer (0.1% BSA in PBS) and incubated with anti-CD16/32 (Fc receptor blocker, 1:200) at 4°C for 10 minutes. Live/dead aqua (ThermoFisher Scientific) was used to gate out dead cells, and the following antibodies were used to identify MDSC populations: CD45 (clone 30F11), CD11b (clone M1/70), Ly6G (clone 1A8), Ly6C (clone HK1.4). All flow cytometry data were collected on the LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo v7 software (Tree Star, Inc.). Absolute numbers of cells in tissues were determined by multiplying percentage of live CD45⁺ CD11b⁺ Ly6G⁺ Ly6C⁻ (PMN-MDSC) and live CD45⁺ CD11b⁺ Ly6C⁺ Ly6G⁻ (M-MDSC) by the cell numbers of the sample, divided by the sample mass in mg.

Statistics.

The Student's t test was used to compare data between 2 groups, and 2-way ANOVA with Tukey's post hoc analysis was used to generate tumor growth statistics using GraphPad Prism. All tumor growth data are presented as mean ± SEM, and all other data are presented as median ± minimum to maximum.

Study approval.

Generation of the mice and all mice studies were reviewed and approved by the Roswell Park Comprehensive Cancer Center IACUC (protocol numbers 757M and 1038M).

3. Results:

3.1. Self-selected temperature modulation decreases tumor growth and MDSC accumulation.

Previous data has shown that housing mice at TT (30°C) results in a decrease in MDSC accumulation compared to that seen in mice housed under the subthermoneutral temperatures required for laboratory mice [9]. We implemented a new murine housing cage system that allows for the insertion of exothermic heating packets, colloquially known as hand warmers, which can heat specific regions of a cage [27]. This allows mice to select warmer regions of the cage whenever they desire. This model is referred to here as the local heat model.

Using this system, and our previously described ST and TT models [9] in which mice are housed in rooms kept at either 22°C or 30°C, mice were allowed to acclimate to their particular housing condition for 2 weeks. Then, 4T1 tumor cells were orthotopically implanted, housing conditions were maintained, and tumor growth was monitored. We determined that TT housing conditions allowed for the greatest control of tumor growth, while our local heat source model also significantly decreased tumor growth when compared to ST housing conditions (Fig. 1A). Importantly, using flow cytometry (Fig. 1B), we determined that the local heat source housing condition suppressed the accumulation of MDSCs in tumors (Fig. 1C & D) and spleens (Fig. 1E & F) to a similar degree as TT housing. Importantly, it is possible that the difference in tumor volumes at the endpoint of these experiments was due to effects of local heat and TT on other cell types, and that the decrease in MDSC accumulation is actually a reflection of a change in tumor volume. To determine whether this was the case, analysis of tumors from mice at ST and TT was done at day 15 when tumor volumes were equivalent and at day 25. We found that MDSC populations were decreased in tumors from mice housed in TT conditions at both time points (Sup. Fig. 1A-D). Additionally, we have previously shown that mice housed at ST and TT have equivalent numbers of MDSCs prior to tumor implantation [9]. Taken together, these data indicate that cold stress by itself does not increase MDSC accumulation in healthy mice, but it does increase accumulation in tumor bearing mice.

3.2. Limiting adrenergic stress with a low dose β -blocker decreases tumor growth and MDSC accumulation.

Previous results using several murine tumor models indicate that daily treatment with propranolol at a dose of 10mg/kg (in PBS) is another intervention capable of minimizing the effects of ST housing induced cold stress, limiting MDSC activity, and improving tumor control. Here, we utilized BALB/c mice bearing orthotopically implanted 4T1 mammary carcinoma tumors, and found that even a 100-fold decrease in propranolol dose (daily i.p. injections of 0.1mg/kg) was capable of limiting tumor growth rates (Fig. 2A). Additionally, single cell suspensions of splenic tissue were prepared and stained for flow cytometry to assess levels of both major MDSC subtypes, M-MDSC and PMN-MDSC. We found that all 3 doses of propranolol: 10.0, 1.0, and 0.1 mg/kg suppressed M-MDSC and PMN-MDSC numbers to a similar degree (Fig. 2B). These data suggest that blocking stress mediated

adrenergic signaling delays tumor growth, partially by decreasing MDSC accumulation. We compared this effect to that achieved by thermal manipulation of housing temperature.

3.3. Weekly whole-body hyperthermia decreases tumor growth and MDSC accumulation.

In the experiments conducted above, body temperature remains stable. To test the impact of actually increasing the core body temperature of mice on MDSC accumulation, whole body hyperthermia (WBH) experiments were carried out. BALB/c mice were orthotopically implanted with 4T1 tumor cells, and all mice were housed at ST. Once a week, one group of mice received a WBH treatment during which they were temporarily housed at temperatures between 38.5°C and 39.5°C, which allows their core temperatures to increase [28]. The WBH treatment concluded once the core temperature of a surrogate mouse reached an average of 39.0°C or greater for six hours. We found that tumor growth was delayed significantly in WBH treated mice compared to control ST mice (Fig 3A). Additionally, flow cytometry of tumors (Fig 3B & C) and spleens (Fig 3D & E) indicated that levels of both subtypes of MDSCs were decreased as well. These effects were similar in magnitude to that achieved by using propranolol or TT housing, although the mechanism(s) of action driving WBH mediated changes may be unique because this is the only model in which the core temperature of treated mice is increased. Taken together, these data support the notion that changes in housing conditions and temperature-based therapies can significantly alter the immunosuppressive environment within the tumor by decreasing MDSC accumulation.

4. Discussion

Without a potent co-incident anti-tumor immune response, cancer therapies are less effective [29]. MDSC populations are capable of shifting the delicate balance of this immune response from anti-tumor to pro-tumor [2], and it is likely that decreasing MDSC accumulation allows for improved therapeutic efficacy along with enhanced immune cell killing of tumor cells [30, 31]. MDSCs suppress the anti-tumor immune response by inhibiting T cell and natural killer cell activity, promoting a premetastatic niche, and contributing to resistance to immunotherapy [32]. We have previously shown that housing temperature induced cold stress, and the adrenergic signaling induced by this cold stress, significantly increases MDSC accumulation and immunosuppressive functions through β 2-AR signaling [9]. Here, we have compared the effects of three temperature-based protocols on tumor growth and MDSC accumulation, including 1) weekly whole-body hyperthermia (WBH), 2) housing mice at TT (~30°C), and 3) housing mice at ST (~22°C) while providing them with access to a local heat source. We also compared these protocols to mice housed under ST conditions, but given daily treatments of different doses of propranolol. These treatment protocols exert their effects through unique mechanisms as described below.

By permanently changing housing temperatures to TT, or by using our local heat source model, mice do not require adaptive thermogenesis to maintain their core body temperature (approximately 37°C) [33]. As a result, systemic levels of norepinephrine are lower; thus β 2-AR signaling in MDSCs is reduced. In propranolol studies, all mice are housed at ST and experience cold stress, which drives an increase in systemic norepinephrine levels to drive thermogenesis in order to maintain normal core body temperature. However, propranolol

treatments inhibit signaling through β -ARs expressed by many cells throughout the body, including MDSCs, and mitigate the effects of elevated norepinephrine levels. Interestingly, our β -AR antagonist studies show that even a low dose of propranolol (0.1mg/kg) is sufficient to reduce MDSC accumulation, compared to the previously used dose of 10.0mg/kg [9]. This emphasizes how responsive this immunosuppressive pathway is to β -AR signaling.

In comparison, mice in our hyperthermia studies experience chronic cold stress throughout the week, as they are all housed at ST. Then, the hyperthermia treatment they receive every seven days temporarily increases their core body temperature from $\sim 37^{\circ}\text{C}$ to above 39°C for six hours and briefly interrupts the cold stress they are experiencing. Although we have shown that these weekly hyperthermia treatments reduce MDSC accumulation and tumor growth, it is unknown whether this is due to reduced levels of cold stress, or whether this treatment reduces MDSC accumulation through some other mechanism. It is possible that the decrease in MDSC accumulation due to hyperthermia occurred as a result of direct changes in MDSC biology, such as a decrease in MDSC survival in the tumor microenvironment or a decrease in MDSC expansion at the level of the bone marrow. Although these cold stress limiting treatments are different, it is evident that the processes driving MDSC accumulation are sensitive to each of these models.

It is important to appreciate that many cell types throughout the body, including other immune cells, are likely affected by each of the treatments in this study, in addition to MDSCs. We know that immune cells, including T cells [19], dendritic cells [21], and natural killer cells [23] express functional β -ARs, and blocking β -adrenergic signaling likely directly affects them, independent of changes in MDSC mediated immunosuppression. For example, in the past we have shown that cold stress and β -AR signaling impairs CD8^{+} T cell function and anti-PD-1 immunotherapy [15-17, 34], increases regulatory T cell activity in models of graft vs. host disease to limit severity and mortality [19], and limits effector T cell responses to radiation therapy [34, 35]. We have also shown that hyperthermia can have effects on Langerhans cells and lymphocytes [28]. Although the effects of cold stress, adrenergic signaling, and hyperthermia on these cell types are important to consider, these current studies specifically focus on how these experimental models affect MDSC accumulation. Still, even within this focused scope of investigation, there are many other potential mechanisms that could be responsible for the observed changes in MDSCs accumulation.

For example, tumor associated factors released into the tumor microenvironment are thought to generally be responsible for the expansion of MDSC populations [36], and it is possible that the responses to therapy we observed could in part be due to effects on other cell types, such as T cells [37, 38] or tumor cells, which could alter the tumor microenvironment and lead to the production of less tumor associated factors. However, this was in part controlled for because 4T1 tumor cells have been shown to lack functional $\beta 2$ -ARs [39], so changes in adrenergic signaling directly on tumor cells is unlikely to be responsible for the observed phenotype.

Additionally, the thermogenic process that occurs in brown adipose tissue as a response to cold stress is driven by β 3-AR signaling [40]. This signaling triggers the conversion of the chemical energy trapped within lipids into heat, rather than adenosine triphosphate molecules [41]. As a consequence, thermogenesis requires a great deal of metabolic energy, and interestingly, mice housed at even colder temperatures, such as 4°C, can maintain their core body temperature if they are able to consume significantly more dietary calories to use for thermogenesis [42, 43]. Considering that a non-trivial portion of the body's energy consumption is typically allocated to the immune system [44], it is possible that this decrease in available energy could also contribute to impairment of the anti-tumor immune response. However, we have shown that when ST and TT tumor growth experiments have been carried out in β 2-AR knockout mice and SCID mice, there is no change in tumor growth [16]. This suggests that while mice experiencing cold stress may burn more calories and consume more food, these changes do not significantly affect tumor growth, which is primarily driven by β 2-AR signaling in immune cells in these models.

When pharmacologically inhibiting β -adrenergic signaling, it is important to note that because propranolol is a pan- β -antagonist, β 1-ARs and γ 3-ARs are antagonized under this treatment, in addition to γ 2-ARs. In this case, it is possible that other systemic metabolic changes, such as an increase in plasma fatty acids, glycerol, and/or insulin, and a reduction in blood glucose driven by β 3-AR inhibition [45] could lead to changes in MDSC accumulation or other relevant immune populations. In addition, the cardiovascular effects of propranolol mediated by β 1-AR inhibition have been thoroughly described, and these effects could potentially result in changes in tumor growth or production of systemic tumor associated factors that expand MDSC populations by some undiscovered mechanism.

In a previous study, we determined that as tumor volumes increase, mice seek warmer environments. When allowed to move freely between cages at different temperatures, tumor bearing mice spend more time at temperatures as warm as 38°C [17]. In this study, the hand warmers heat the floor above where they are placed to ~35°C, but this heat begins to taper off after 6 hours and eventually drops to ~24°C by the time they are replaced the following morning [27]. This is still warmer than ST (~22°C), but it is possible that a more constant and/or warmer local heat source could provide even greater tumor control. This could provide insight into why our local heat model was not as effective at reducing tumor growth as constant TT housing (~30°C) was. Although we do not have data describing how often the tumor bearing mice in this local heat model are physically on the heat source, it has been shown that non-tumor bearing mice spend significantly more time on the false floor when it is heated rather than at ST [46].

This desire for tumor bearing mice to seek warmth also supports the notion of using WBH treatments to increase core body temperatures and relieve this cold stress. In these experiments, WBH was administered once a week, however more regular treatments may provide an even greater decrease in tumor growth rates. Although these findings in mouse models are important, the most vital aspect of these studies is their potential implications for human patients that could receive hyperthermia treatments for breast cancer or other malignancies. We know that breast cancer patients commonly report feeling cold [47, 48], yet little research has been done to determine how this symptom of feeling cold affects

outcomes, or whether it impacts important processes such as MDSC accumulation, macrophage accumulation, or T cell function. Further, it is unknown whether a temperature-based therapy, such as hyperthermia, might relieve these symptoms of feeling cold, skew the tumor microenvironment to a more anti-tumor state, and improve clinical survival rates.

In summary, we showed that by implementing several different methods of limiting chronic cold stress in mice, tumor growth rates and MDSC accumulation could be reduced. This study provides rationale to re-think experimental design, and consider how the environment, especially housing temperature, affects *in vivo* experiments. This also gives credence to the importance of the nervous system's role in modulating the anti-tumor immune response, indicating that more investigations of the central nervous system-immune response axis are necessary. Finally, we hope that these provocative findings will encourage the investigation of potential clinical treatment regimens that utilize the human body's response to changes in temperature to improve patient outcomes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

1. MDSC accumulation in tumors is highly sensitive to various manipulations of housing temperature, which can affect their physiological response to adrenergic stress.
2. Providing a local heat source within the cage of tumor bearing mice limits cold stress, is associated with diminished intra-tumoral MDSC accumulation, and improves tumor growth control.
3. Intermittent whole body hyperthermia treatments given to mice housed under standard cool temperatures is also associated with diminished intratumoral MDSC accumulation and improved tumor growth control.
4. Local heating, housing at thermoneutrality, and intermittent whole body hyperthermia provide similar tumor growth control and MDSC reduction as that seen with various doses of the pan β -adrenergic receptor antagonist, propranolol.

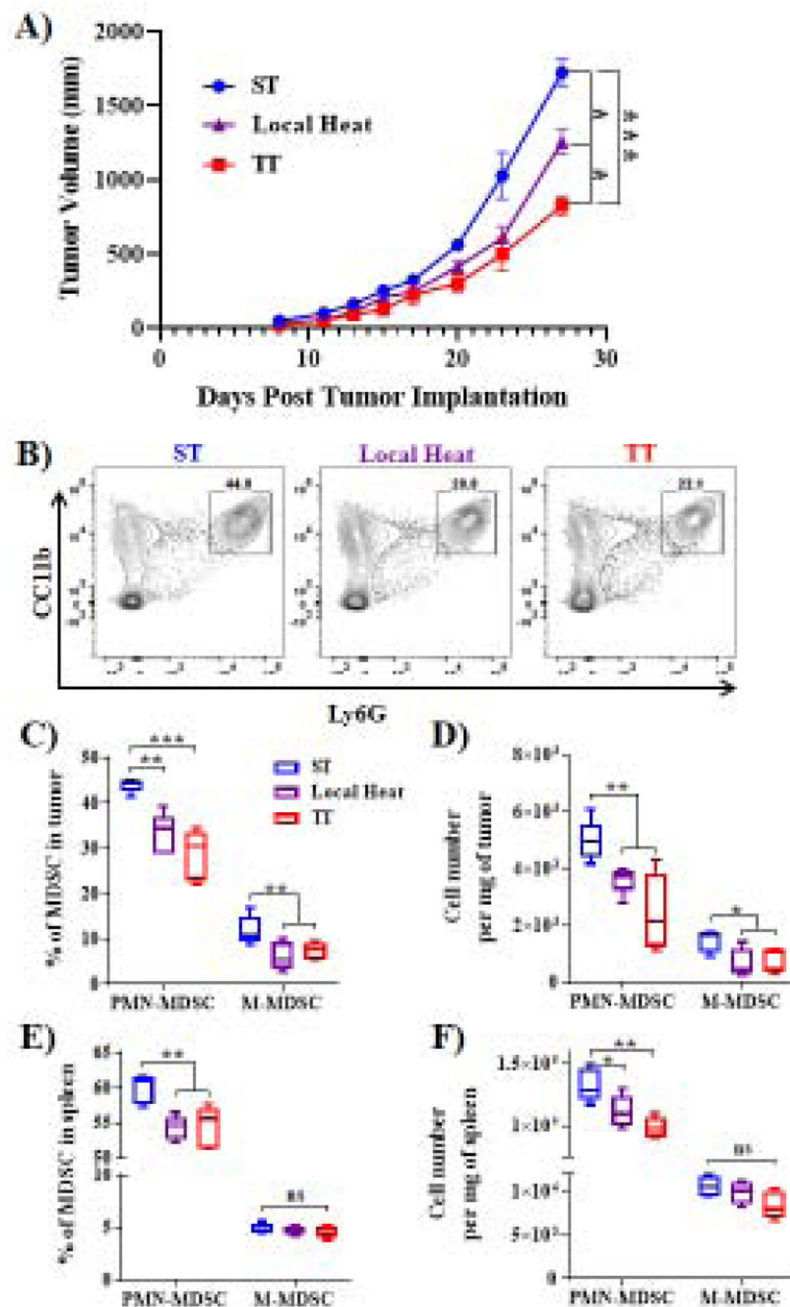


Figure 1). Housing temperature can decrease tumor growth and MDSC accumulation. (A). 4T1 tumor growth kinetics in WT BALB/c mice housed under ST (22°C), TT (30°C), or local heat source conditions (n=5 mice per group). (B) Representative flow cytometry data assessing intratumoral MDSC populations. (C and D) Percentage of PMN-MDSC and M-MDSC subpopulations, as well as absolute numbers per mg of tumor. (E and F) Percentage of PMN-MDSC and M-MDSC subpopulations, as well as absolute numbers per mg of spleen. Tumor growth curves are presented as the mean ± SEM, and MDSC data are presented as median ± minimum to maximum. Two-way ANOVA was used to analyze statistical significance among tumor growth in different groups, and One-way ANOVA was

used to analyze statistical significance among MDSC populations. In all panels, *P < 0.05, **P < 0.01., and ***P < 0.01. A P value less than 0.05 was considered significant.

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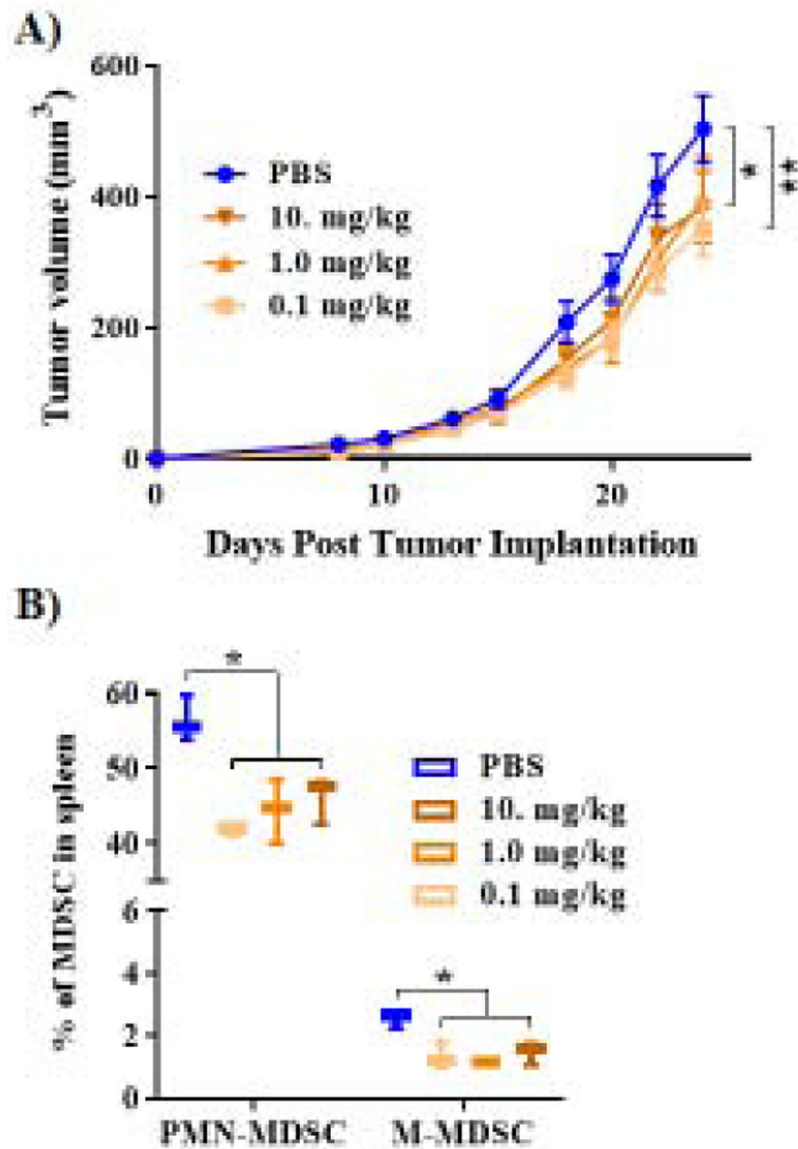


Figure 2). Low dose β -blocker treatment decreases tumor growth and MDSC accumulation. (A). 4T1 tumor growth kinetics in WT BALB/c mice treated with propranolol at doses of 10.0, 1.0, or 0.1 mg/kg (n=6 mice per group), housed at ST (22°C). (B) MDSC populations measured by flow cytometry in splenic tissue of mice bearing 4T1 tumors, treated with propranolol at doses of 10.0, 1.0, or 0.1 mg/kg (n=6 mice per group). Tumor growth curves are presented as the mean \pm SEM, and MDSC data are presented as median \pm minimum to maximum. Two-way ANOVA was used to analyze statistical significance among tumor growth in different groups, and One-way ANOVA was used to analyze statistical significance among MDSC populations. In all panels, *P < 0.05 and **P < 0.01. A P value less than 0.05 was considered significant.

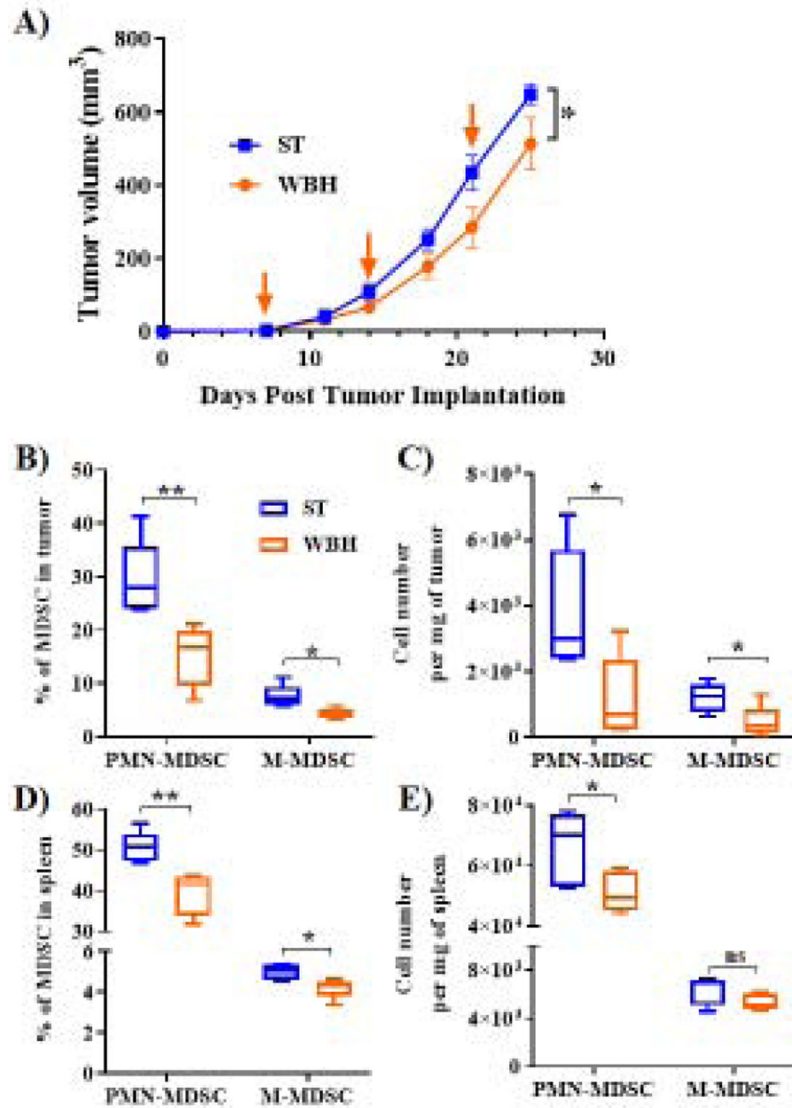


Figure 3). Whole body hyperthermia decreases tumor growth and MDSC accumulation. (A). 4T1 tumor growth kinetics in WT BALB/c mice housed at ST (22°C) and treated with whole body hyperthermia (WBH), or control ST treatment (n=5 mice per group). Arrows indicate days on which WBH or control treatments were administered. (B and C) Percentage of PMN-MDSC and M-MDSC subpopulations, as well as absolute numbers per mg of tumor. (D and E) Percentage of PMN-MDSC and M-MDSC subpopulations, as well as absolute numbers per mg of spleen. Tumor growth curves are presented as the mean \pm SEM, and MDSC data are presented as median \pm minimum to maximum. Two-way ANOVA was used to analyze statistical significance among tumor growth in different groups, and One-way ANOVA was used to analyze statistical significance among MDSC populations. In all panels, *P < 0.05 and **P < 0.01. A P value less than 0.05 was considered significant.