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Mild cold-stress depresses immune responses: Implications for cancer models involving laboratory mice

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Abstract

Physiologically accurate mouse models of cancer are critical in the pre-clinical development of novel cancer therapies. However, current standardized animal-housing temperatures elicit chronic cold-associated stress in mice, which is further increased in the presence of tumor. This cold-stress significantly impacts experimental outcomes. Data from our lab and others suggests standard housing fundamentally alters murine physiology, and this can produce altered immune baselines in tumor and other disease models. Researchers may thus underestimate the efficacy of therapies that are benefitted by immune responses. A potential mediator, norepinephrine, also underlies stress pathways common in mice and humans. Therefore, research into mechanisms connecting cold-stress and norepinephrine signaling with immune-depression in mice could highlight new combination therapies for humans to simultaneously target stress while stimulating anti-tumor immunity.

Keywords

cancer; immunology; murine model; norepinephrine; stress; T cells; thermoregulation

Introduction

Cancer has posed a burden on human society since ancient times. Over the past century, cancer therapies have evolved through medical and scientific advancement to include surgery, radiation, chemotherapy, as well as specialized biological inhibitors and other treatments that target genetic facets of cancer. For both old and new interventions, efficacy varies greatly, depending on tumor type and individual patient characteristics. It is now clear that a number of these therapeutic strategies are greatly benefitted by anti-tumor immune responses generated as side-effects of therapy [1, 2]. This highlights the value of the immune system in protecting patients from aggressive disease and cancer recurrence and also prompts the search for ways to enhance immune responses in conjunction with other therapies. Indeed, cancer immunotherapies were heralded as the major breakthrough of 2013 [3].

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Much of our pre-clinical data evaluating potential new therapeutic combinations relies on the use of animal models. Mice in particular are highly popular in the field of cancer immunology, offering a number of benefits to the researcher. Mice are generally less expensive, easier to maintain, and have relatively short generation times compared to other mammals [4, 5]. In addition to a panoply of murine specific antibodies and reagents for analysis, a large number of mouse strains have been generated as important tools in cancer immunology [6]. These include transgenic expression of cancer oncogenes, selective knock-out of tumor suppressors, as well as specific knock-out of a number of immunologically relevant receptors and ligands for study. Despite their wide use as surrogates for the study of human cancer development, significant differences exist between mice and humans, particularly in the area of metabolism [7] and thermoregulation [4]. These differences often do not receive sufficient attention from researchers using murine models to study tumor growth and anti-tumor immunity.

This review addresses current problems and paradigms in the area of cancer immunology research, with a focus on the striking role ambient temperature plays in shaping experimental outcomes in our pre-clinical mouse models. Emerging data demonstrate that ambient temperature has significant effects on murine biology and specifically on anti-cancer immune responses by mice. We propose that greater awareness of these effects will contribute to enhanced design of animal models for cancer. These observations can also be co-opted as excellent models for understanding the role of stress in tumor development.

Mammals use multiple approaches to adapt to ambient temperature

Mammals are defined as endotherms, or animals that maintain a particular metabolically favorable temperature. For humans and mice, this internal temperature is approximately 37°C. In order to maintain this temperature in a range of external environments, they have evolved a number of thermo-regulatory mechanisms [8]. These mechanisms can be divided into two main categories: conscious behaviors and non-conscious physiological responses. Warming behaviors include burrowing, huddling, increasing activity, and, for humans, adding additional layers of clothes. Involuntary, physiological methods of conserving heat include constricting blood flow (vasoconstriction), raising the hair on the skin surface to form a more insulating area (piloerection), as well as adopting a hunched posture to reduce the amount of exposed surface area [9]. In the face of continued cold, mammals have the ability to increase their own heat production, or thermogenesis. Thermogenesis occurs via two major pathways, shivering and nonshivering. Both represent increases in the mammalian basal metabolic rate and are controlled by a complicated network of signaling events. Generally, shivering thermogenesis is a short-term response and is rapidly turned off once the mammal is returned to a warm environment. Non-shivering thermogenesis, associated with altered metabolism, hormonal signaling, and activity in the brown fat pads, is considered to have more long term consequences but is more difficult to measure [9].

Thermoneutrality is defined as the temperature at which mammals need to exert the least amount of energy to maintain their normal body temperature (Figure 1). This temperature depends on the rate at which heat dissipates from the animal, controlled by a variety of factors including insulation (fur, skin thickness, and clothes), body composition, as well as

the ratio of surface area to volume. Mouse bodies have a higher surface to volume ratio than humans resulting in greater opportunity for heat loss to the external environment [10]. The Guide for the Care and Use of Laboratory Animals recognizes that mice exhibit a preference for temperatures above 26°C [11], and this figure is in line with several calculations for the expected thermoneutral range of mice [11–16]. However, the Guide's mandates on housing temperatures do not reflect this preference, as it requires that mice be maintained between 20 and 26°C [11]. Housing temperatures may be even lower in international research facilities.

Mice maintained at standard temperatures have the same core body temperature as mice in thermoneutral environments; however, they display several outward signs of 'feeling cold.' The options mice have for dealing with this cold are limited. Mice cannot add layers of clothes, but when given additional insulating materials, standard temperature mice build more elaborate nests [13, 14]. The shoebox housing commonly used for mouse research also greatly limits the space for mice to increase warming physical activity [7]. Thus, mice at standard housing spend more time huddling in groups compared to their thermoneutral counterparts [5, 13, 14]. Mice in standard housing do have unlimited access to food; it has been observed that mice in cold temperatures consume significantly more calories than mice at thermoneutrality [17, 18]. These observations underline the fact that standard temperature-housed mice experience chronic cold-induced stress. Additionally, we have observed increased levels of the hormone norepinephrine (NE) in these animals which is even further increased in tumor bearing animals (Eng et al., manuscript in preparation). NE is classically associated with inducing thermogenesis [9]; it has also been implicated in a number of human stress pathways and is a key measure of stress in these animals.

Cold stress significantly impacts murine physiology

As mentioned above, ambient temperature determines the level of metabolism in mammals that is required to maintain core body temperatures. Adaptations to chronic cold temperatures can have a profound effect on rodent physiology. By comparing mice housed at standard temperatures (20–26°C, ST) to mice housed at their thermoneutral temperature (28–32°C, TT), we and others have identified a number of significant differences that impact experimental models. Examples include basic differences in growth and development [19–22], altered sleep and activity patterns [23–27], altered hormonal balance [28–30], as well as altered metabolism and susceptibility to metabolic syndrome [31–35]. Indeed, an excellent example of the profound effect of chronic cold stress on murine physiology and the potential impact on experimental outcomes has been in the field of metabolism. UCP1, a gene involved in thermoregulation and implicated in metabolic pathways underlying human obesity [36, 37], was knocked out in mice, but this resulted in no overt obese phenotype under standard housing. It was not until mice were housed at thermoneutrality that the obesogenic nature of this deletion was revealed [31]. Thus, the means mice rely upon to cope with chronically reduced ambient temperatures in housing facilities fundamentally alters their biology. Other biological processes affected by ambient temperature are summarized in Table 1.

Not only does ambient temperature affect homeostatic properties of murine development, it also alters the ability of mice to respond to additional stressors. Though similar at baseline,

our lab has observed that mice housed at ST and TT experience differential expression of heat shock factor-1 (HSF-1) in numerous organs upon short term exposure to hyperthermic temperatures well above their thermoneutral zone (resulting in mild whole body hyperthermia) (Reed et al, manuscript pending revision). Concurrently, expression of the heat shock proteins HSP70, HSP90, and HSP110 differed significantly between ST and TT mice following this therapy. In addition to their role in temperature responses, heat shock proteins maintain protein stability and fundamental cellular biology in the face of hypoglycemia, hypoxia, and other cellular stressors. Such striking differences in heat shock protein expression suggests that mice housed at different ambient temperatures would have differential susceptibilities to a number of additional stressful interventions, including surgery, radiation, and chemotherapy, as well as models of ischemia/reperfusion injury and other conditions.

In addition to the differences in basic biology discussed above, differences in immune phenomena between ST and TT housed animals have been observed. Karp has published an excellent review of the effects of cool ambient temperatures on several facets of murine immune responses to infectious disease and other pathologies with immune components such as atherosclerosis and cystic fibrosis [5]. Generally, mice maintained under standard housing temperatures have altered susceptibilities to infectious diseases and often do not develop pathological symptoms associated with immune function in human patients. These findings have should be of concern to researchers who study immunological problems in murine models. Given the current appreciation of immunotherapy as a great advance in cancer treatment for patients [3], the effects of cold stress in murine cancer models deserves more study.

Chronic cold stress profoundly impacts cancer models

Despite the profound effects of chronic cold stress on murine physiology, data for the cancer setting have only recently been published. Kokolus et al demonstrated that mice under chronic cold stress due to standard temperature housing (22°C, ST) are more susceptible to tumor growth compared to their non-stressed thermoneutral counterparts (30°C, TT) [15]. Transplantable, orthotopic tumors grow with faster kinetics in ST hosts and also produce more metastatic lesions. Additionally, exposing mice to the carcinogen methylcholanthrene (MCA) resulted in tumor development in 100% of mice at ST but only 20% of mice at TT after 140 days. Thus, it is clear that standard housed, cold-stressed mice, used routinely for cancer studies, suffer from increased tumor burdens compared to warmer animals.

Tumor growth is dependent on a wide array of variables that could be impacted by ambient temperature as described above. Interestingly, Kokolus et al. identified endogenous immune responses as an especially strong determining factor for the observed difference in tumor growth rates. Challenging immune-compromised mice with tumors resulted in similar tumor growth rates between the two temperature conditions. Additionally, depletion of immune effector T cells, specifically CD8⁺ T cells, abolished the delay in tumor growth observed at TT. Thus, despite other potential factors in the cold-stressed mice that could contribute to tumor growth, an intact immune response plays a major role in reducing tumor burdens in non-stressed animals.

Intriguingly, gross immune populations from naïve (i.e. non-tumor bearing) mice at ST versus TT are comparable [15], suggesting that homeostatic immune development is not affected by temperature. However, under the added stress of tumor burden, the predominant immune populations are strikingly different between the two temperatures. ST mice have higher numbers of suppressive regulatory T cells (T regs) within the tumor, while TT mice have a large number of infiltrating CD8 T cells that appear to be functionally active. The size of the spleen increases drastically in tumor-bearing mice, but the size is larger for ST mice compared to TT mice [15, 38], and the difference is largely due to increased numbers of myeloid cells. These cells include macrophages, dendritic cells, and myeloid-derived suppressor cells. Comparing these populations from mice at TT, myeloid cells from ST mice are less mature and exhibit an overall decreased ability to activate T cells [38]. Immature, suppressive myeloid cells have been strongly implicated in promoting tumor growth in a number of cancer settings [39, 40]. Mechanisms underlying this difference in myeloid populations between ST and TT mice deserve more research as a major target for cancer therapies.

Adrenergic signaling likely contributes to stress-mediated immune dysfunction in cancer models

We propose here a model to highlight the systemic effects of stress on immune responses, with a focus on the cancer setting (Figure 2). As discussed above, norepinephrine (NE) is the primary signaling hormone associated with adaptive thermogenesis. NE has also been strongly associated with cancer development [41–45]. The primary source of systemic NE, the sympathetic nervous system (SNS), innervates vital immune organs including primary lymphoid tissues such as the bone marrow and secondary, peripheral lymphoid tissues such as the spleen and lymph nodes [46]. Thus, NE signaling via the SNS in response to ambient temperature potentially modulates anti-tumor immune responses at all stages, from progenitor development in the bone marrow, inhibition or suppression of antigen presentation and T cell priming in the lymphoid organs, as well as responsiveness of lymphocytes at the tumor site itself and in other peripheral tissues.

Bone marrow progenitor cells express adrenergic receptors [47], rendering them capable of responding to NE signaling [46]. Social stress and thermal injury have been associated with alterations in lympho- and myelopoiesis which could be blocked by antagonists of adrenergic signaling [48–50]. However, administration of NE alone was not enough to elicit these changes. Thus, NE, in the presence of other factors such as tumor derived G-CSF or GM-CSF, potentially contributes to granulocytic skewing of the myeloid compartment giving rise to suppressive cell types. The exact mechanism by which NE signaling contributes to lineage commitment remains to be elucidated. In addition to effects on cellular development, NE differentially promotes myeloid or lymphoid egress from bone marrow into the periphery [51–53]. Again, the exact signaling pathways behind these observations remain to be explored, and this phenomenon appears to also be dependent on other factors in the bone marrow. Taken together, the production of NE by sympathetic nerves in response to stress potentially targets immune cells directly at their site of

manufacture to alter lineage skewing and deployment to peripheral tissues. This is likely modulated by tumor derived factors.

The secondary lymphoid organs, comprised of the lymph nodes and spleen, are sites of training, or 'priming,' naive immune cells to develop effective immune responses against detected threats. It has long been known that these organs are heavily innervated by sympathetic neurons [46]. NE signaling through β -adrenergic receptors on antigen presenting cells can dramatically alter their cytokine production, suppressing inflammatory cytokines such as IL-12 and TNF α while promoting production of anti-inflammatory cytokines such as IL-10 [46, 54]. Cytokine milieu at the site of priming determines the type of cellular immunity elicited; overall, NE appears to promote a 'Th2' phenotype more typically associated with anti-helminthic or allergic responses, as opposed to 'Th1' responses which are considered to be more protective in the cancer setting. Such cytokine imbalances may also contribute to enhanced regulatory T cell responses. Thus, NE at the site of immune priming may skew potential effector cells away from an effective anti-tumor phenotype toward pro-tumor immune responses.

It is clear that NE within the tumor microenvironment also contributes to disease progression [44, 55]. Numerous sources of intratumoral NE have been identified and may be tumor specific; some tumors have been shown to trigger neural invasion for direct interaction with the SNS [56]. Additionally, tumor-associated macrophages [57–60], lymphocytes [61], or other stromal components [62] can also contribute to local NE production. It is also clear that inflammatory cytokines form a feedback loop with the SNS to regulate catecholamine production [63–65]. Regardless of source, NE signaling through β -adrenergic receptors at the tumor site can have diverse immunologically relevant effects: 1) T regulatory cells [66, 67], myeloid derived suppressor cells, or macrophages [68] experience enhanced suppressor function, 2) effector T cells experience reduced proliferation and production of survival cytokine IL-2 and effector cytokines IFN γ and TNF α [66,69,70], 3) signaling on tumor cells can directly decrease the expression of immune-stimulatory molecules [71]. These effects are primarily mediated by downstream increases in intracellular cyclic AMP and activation of PKA. It is important to note that NE can also stimulate adrenergic receptor positive tumor cells directly to promote tumor growth and metastasis via immune-independent pathways [41, 71]. Thus, targeting of NE signaling can have a global benefit on boosting immune responses, but can also be developed as a targeted therapy for patients with tumors that are adrenergic receptor positive.

Comparing animals from varied housing conditions could improve disease models

There is considerable debate in the field regarding whether the warm housing environment more or less accurately represents the human condition. As others have pointed out, humans routinely live in environments below their thermoneutral temperature [72]. Humans, however, have developed a number of tools to manipulate their environment, reducing the need for purely physiological adaptations to reduce cold- (or hot-) stress. This level of environmental control is unavailable to laboratory mice. Whether ambient temperature impacts human cancer incidence or mortality has not been determined. This is due to

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confounding factors such as infection rates, UV exposure, developmental status, and other regional variations that make such investigations difficult. However, we do not believe the observations discussed above are limited to only cold-stress. Human cancer patients face a variety of other environmental and psychological stressors over the course of pre-clinical tumor development, clinical diagnosis, and therapy. Importantly, in both mice and humans, such stressors are also largely associated with increased NE production including: social isolation [55, 73, 74], social disruption [75, 76], depression [77, 78], burn injury [50, 79] and others [80]. Standard housing may thus be an excellent model for certain patients that are experiencing long-term chronic stress or have otherwise elevated levels of NE. Indeed, epidemiological evidence has already shown that patients on antagonists of NE signaling at the time of their chemotherapy had improved disease outcomes for breast cancer as well as melanoma [81]. Such antagonists, termed ‘beta-blockers’ for their specific effects of inhibiting NE signaling through beta adrenergic receptors, are commonly prescribed to patients for treatment of high blood pressure. There is a great need for prospective studies and/or randomized trials to investigate the potential benefits to combining cancer immunotherapy with such beta-blockers or other pharmaceuticals that intervene in stress response pathways.

Interestingly, tumor growth itself represents an additional metabolic stress. This is reflected in the observation that tumor bearing mice prefer to be housed at temperatures even warmer than 30°C [15], and tumor burden increases NE levels in mice under both ST and TT housing (Eng et al., manuscript in preparation). Immune responses are considered to be metabolically expensive events [82, 83]. Under conditions of metabolic stress, immune function experiences trade-offs to preserve other physiologic processes [84]. Thus, hormones such as NE may serve as regulators of immune responses to preserve metabolic function for survival in the face of environmental stress. Intriguingly, similar to tumor-bearing mice, cancer patients frequently report feeling cold or experiencing other temperature-related symptoms [85]. Thus, changes in metabolism and thermoregulation can also be associated with cancer development in humans.

Assessment across a number of infectious disease models has shown that mice at thermoneutrality tend to more closely recapitulate human pathologies associated with active immune responses [5]. Cancer models that rely solely on mice in standard housing may not fully reflect immune effects over the course of tumor development. Non-stressed TT mice have highly functioning immune responses, but still only suppress, rather than outright reject, most tumors [15]. The tumors that grow out in mice at TT may offer insight into novel immune escape mechanisms to be targeted in human patients. Also, since TT tumors grow at a slower rate and are under greater selective pressure, they may better reflect human tumors which have also undergone strong selective pressure before becoming clinically detectable. It will be very important to determine whether these tumors are more or less refractory to common cancer therapeutics, which may help explain how a number of drugs that were promising at suppressing tumor growth in mice have been less than successful in the clinic.

We suggest that mice should be evaluated under more than one ambient temperature to more fully understand the range of their ability to respond to challenges that model human

disease. Several strategies are available for this effort, including the use of environmentally controlled incubators for maintaining mice at various ambient temperatures, including thermoneutrality, as described in the studies reported here. However, this may not always be practical. Simply introducing more bedding material to allow mice greater control over their own thermal comfort greatly reduces stress experienced by mice in standard housing [17,86]. Modeling diseases with or without environmental manipulation requires very little additional intervention and will aid the assessment of stress signaling in these disease pathways.

Conclusions and outlook

Chronic stress in murine models has limited our ability to achieve a complete understanding of potential therapeutic outcomes. Given that positive pre-clinical data are necessary to justify the expense of seeing a new therapy through clinical trials, this may have resulted in a number of missed opportunities for immune-associated therapies that might have been effective if tested in non-stressed animals with highly functioning immune systems. Appreciation of the role of patients' endogenous immune responses to cancer therapies is a developing phenomenon, and there remain a number of questions regarding the best way to stimulate this protection. Future research into novel cancer therapeutics would be benefitted by simple interventions that reduce stress in laboratory mice to allow for more robust immune outcomes. It will also be important to consider ambient temperature in development of new spontaneous cancer models; by comparing chronic mild cold-stress to reduced stress animals, investigators will be able to identify differences between positive and negative stress states, immune responses, and other processes active during disease progression that may more or less reflect human outcomes. This research should be significantly motivated by the centrality of norepinephrine signaling in multiple human stress pathways and the potential impact of NE on anti-tumor responses. Indeed, human epidemiologic data has already shown that blocking NE signaling can protect patients from tumor recurrence and metastasis. Surely the use of these and other drugs that target stress responses in combination with immunogenic therapies deserves further study.

Abbreviations

NE	norepinephrine
SNS	sympathetic nervous system
ST	standard temperature housing (approx. 22°C)
TT	thermoneutral temperature housing (approx. 30°C)

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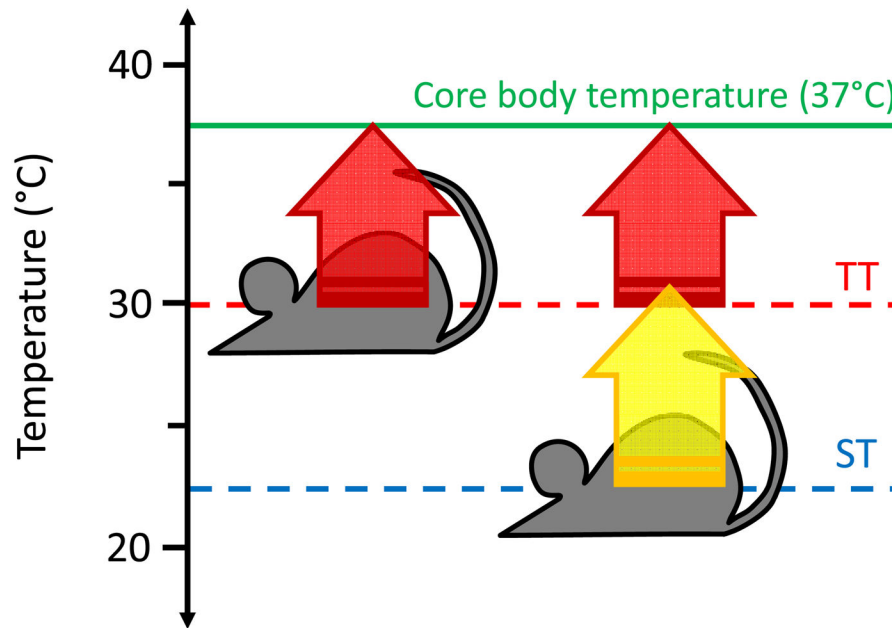


Figure 1.

Mice at lower ambient temperatures require increased rates of metabolism and thermogenesis. The normal core body temperature of mice is approximately 37°C (green line), as for most mammals. Mice housed at their thermoneutral temperature (~30°C, TT, red dashed line) are able to meet this temperature using only their basal metabolic rate (BMR) for heat production (red arrow). Mice housed under standard temperature conditions (~22°C, ST, red dashed line) cannot rely solely on their BMR, and thus a number of adaptive thermogenic pathways are turned on to maintain normal body temperature (yellow arrow).

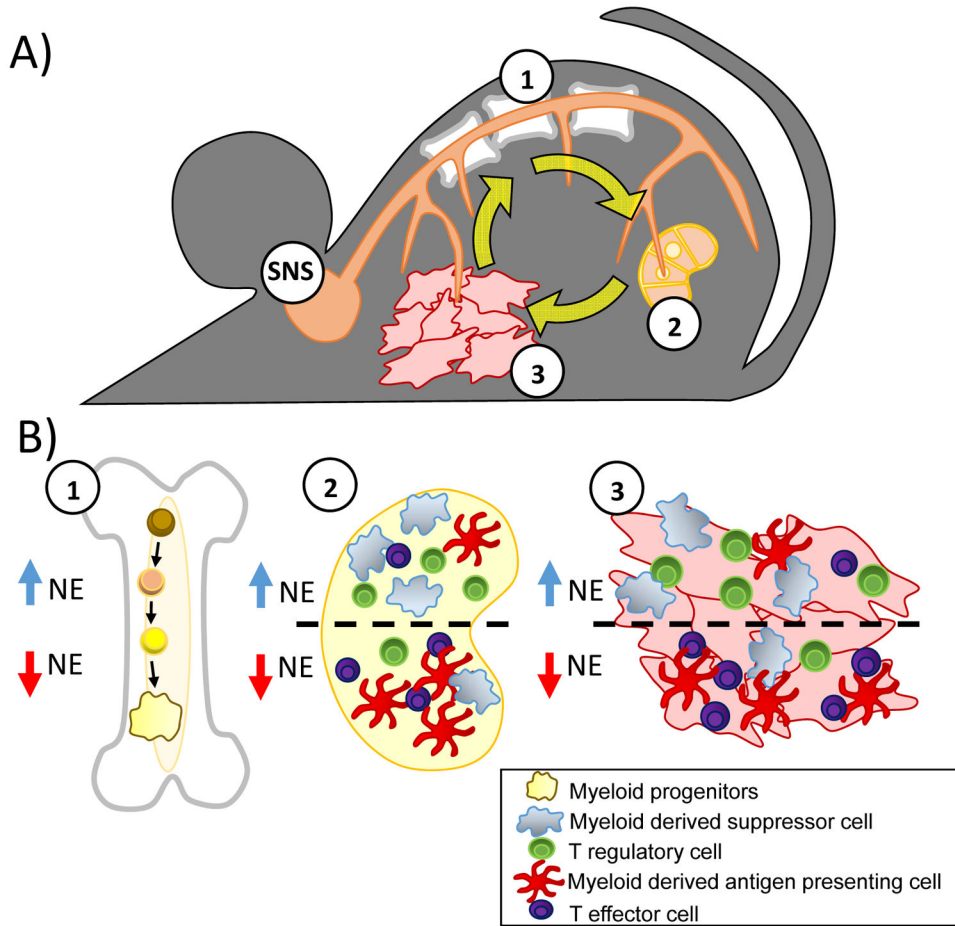


Figure 2. Chronic stress-associated norepinephrine systemically alters immune responses in the tumor bearing mouse. **A:** Stress activates the sympathetic nervous system (SN) to produce norepinephrine (NE). NE potentially targets three major organs of interest in cancer research: (1) primary lymphoid tissue - bone marrow, (2) secondary/peripheral lymphoid tissues - lymph nodes, spleen, and (3) the tumor microenvironment. **B:** Potential cellular targets at each of these sites are highlighted: (1) hematopoietic progenitor cells in the bone marrow, (2) myeloid cells and lymphocytes in peripheral lymphoid tissues, (3) tumor cells as well as tumor-associated myeloid cells and lymphocytes. With increasing NE, the balance of suppressive to effector cells within the tumor, spleen, and lymph nodes shifts toward pro-tumorigenic populations. NE signaling during both myeloid and lymphoid development and upon fully differentiated cells likely contributes to this altered immune environment.

Table 1

Differences between animals housed at Standard Temperature (ST) and Thermoneutral Temperature (TT)

	ST	TT
Core body temperature [4]	=	=
Metabolism [87]	+	-
Heart rate [28]	+	-
Food intake [17, 18]	+	-
Weight [4, 15]	+	-
Serum lipids [88]	+	-
Norepinephrine production [88]	+	-

Relative expression of the indicated measures is shown as = (equivalent), + (increased), or - (decreased) between ST and TT conditions.

Table 2

Alterations in immune responses between animals housed at Standard Temperature (ST) and Thermoneutral Temperature (TT)

	ST	TT
% CD3+ T cells [15]	-	+
% CD8+ T cells [15]	-	+
% Treg cells [15]	+	-
# Myeloid cells [15, 38]	+	-
# Myeloid-Derived Suppressor Cells [15, 38]	+	-
# Dendritic Cells (DC) [15, 38]	+	-
% MHC II+CD86+ DCs [38]	=	=
DC-T cell Activation [38]	-	+
Alternatively activated macrophages [60]	+	-
Norepinephrine production by macrophages [60]	+	-

Relative expression of the indicated measures is shown as = (equivalent), + (increased), or - (decreased) between ST and TT conditions.