β-Adrenergic Signaling in Mice Housed at Standard Temperatures Suppresses an Effector Phenotype in CD8\(^+\) T Cells and Undermines Checkpoint Inhibitor Therapy

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Abstract

The immune context of tumors has significant prognostic value and is predictive of responsiveness to several forms of therapy, including immunotherapy. We report here that CD8\(^+\) T-cell frequency and functional orientation within the tumor microenvironment is regulated by β\(_2\)-adrenergic receptor (β\(_2\)-AR) signaling in host immune cells. We used three strategies—physiologic (manipulation of ambient thermal environment), pharmacologic (β-blockers), and genetic (β\(_2\)-AR knockout mice) to reduce adrenergic stress signaling in two widely studied preclinical mouse tumor models. Reducing β\(_2\)-AR signaling facilitated conversion of tumors to an immunologically active tumor microenvironment with increased intratumoral frequency of CD8\(^+\) T cells with an effector phenotype and decreased expression of programmed death receptor-1 (PD-1), in addition to an elevated effector CD8\(^+\) T-cell to CD4\(^+\) regulatory T-cell ratio (IFN\(_γ\)/CD8\(^+\)/Treg). Moreover, this conversion significantly increased the efficacy of anti-PD-1 checkpoint blockade. These data highlight the potential of adrenergic stress and norepinephrine-driven β-AR signaling to regulate the immune status of the tumor microenvironment and support the strategic use of clinically available β-blockers in patients to improve responses to immunotherapy.

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Introduction

Immune checkpoint blockade is becoming first-line therapy for patients with melanoma and several other types of cancer (1, 2). However, despite remarkable and durable responses, long-term control of tumors is achieved in only a fraction of patients. Extensive preclinical and clinical studies to identify the differences between responders and nonresponders demonstrate a lack of antitumor T-cell activity within the tumor microenvironment (TME) of nonresponders (3–6). Multiple lines of evidence indicate that checkpoint inhibitors are more effective in combination with treatments that generate a robust CD8\(^+\) T-cell infiltrate, including radiation (7), chemotherapy (8), and immune activating agents (9). In addition, clinical studies indicate that the immunoscore, a measurement of the functional orientation, density, and location of specific immune cell subsets in the TME, has significant prognostic value (5, 10, 11). However, understanding underlying mechanisms that could be targeted to improve T-cell infiltration remains a challenge (3, 6).

We recently reported that ambient housing temperature plays an unexpected role in regulating the antitumor immune response in murine tumor models. We found that the mild, chronic cold stress caused by the required subthermoneural housing temperatures (~22°C) mandated by the Institutional Animal Care and Use Committee (12, 13) suppresses the antitumor immune response and can be reversed by housing mice at thermoneural temperatures (~30°C; ref. 14). Housing mice at 30°C increases the frequency of intratumoral effector CD8\(^+\) T cells, correlating with significantly improved control of tumor growth (14). However, the underlying mechanisms were not identified in this study.

Cold exposure causes activation of the sympathetic nervous system (SNS) and norepinephrine (NE)-mediated adaptive thermogenesis to maintain a normal core body temperature (~37°C). Previously, we demonstrated that the mild cold stress experienced by laboratory mice housed at 22°C is, in fact, sufficient to cause elevated NE in comparison with mice...
In this study, we sought to determine whether adrenergic signaling was the mechanism mediating suppression of the antitumor immune response in mice housed at 22°C compared with 30°C.

Release of neurotrophic factors that stimulate outgrowth of fibers from sympathetic ganglia was first observed in vitro in a landmark study by Cohen and colleagues (18). Recently, Magnon and colleagues (19) demonstrated that sympathetic input to tumors is required for the initiation and growth of primary tumors in a model of prostate cancer, thus demonstrating that neurogenesis of autonomic fibers plays a significant role in tumor growth and progression. Cumulatively, these and many other studies have made it clear that the release of catecholamines, primarily NE, in response to a variety of stressors facilitates tumor initiation, growth, and progression (20–22).

In nontumor settings, adrenergic signaling clearly inhibits CD8+ T-cell responses. Grebe and colleagues (23) have shown that anti-influenza CD8+ T-cell responses in vivo are limited by adrenergic signaling, and Estrada and colleagues (24) demonstrate suppression of effector function by β2-AR signaling in both human and mouse CD8+ T cells. These studies support the idea that adrenergic signaling could suppress antitumor immunity; however, the impact of adrenergic stress on the development of antitumor immunity, the immune contexture of tumors, or the role that β2-AR signaling may have in dictating the sensitivity or resistance of tumors to checkpoint inhibitor therapy has received virtually no attention.

Overall, these inhibitory effects of adrenergic signaling on CD8+ T-cell responses, taken together with our previous work on the effects of ambient housing temperature on NE levels, tumor growth, and the antitumor immune response, suggest that increased adrenergic signaling is a critical mechanism underlying suppression of the antitumor immune response. Here, using the pan-β-AR blocker propranolol, as well as β2-AR receptor knockout mice (Adrb2−/−) in which the immune cells are nonresponsive to adrenergic signaling (24), we report for the first time that the mechanism mediating differences in tumor growth and the antitumor immune response observed between mice housed at 22°C and 30°C are dependent upon β2-AR signaling. Furthermore, reducing β2-AR signaling either by housing mice at 30°C or by treating mice at 22°C with propranolol, we discovered that we could "reset" the baseline immune response in murine tumor models and significantly improve responses to anti-programmed death receptor-1 (PD-1) checkpoint blockade therapy.

These data highlight factors that directly affect interpretation of data obtained in murine tumor models as nearly all preclinical mouse work is conducted at 22°C. In addition, they also reveal, for the first time, the critical role that adrenergic stress plays in shaping the phenotype of CD8+ T cells in the TME and its role in constraining the efficacy of checkpoint inhibitors. Taken together, these data provide the first evidence that using FDA-approved β-blockers is a viable strategy to improve checkpoint inhibitor efficacy.

Materials and Methods

Mice
Female BALB/cAnNcr (BALB/c) and C57BL/6Ncr (C57BL/6) were purchased from Charles River and C.B. Igh-1b Icr Tac Prkdc scid (SCID) mice from the Laboratory Animal Resource at RPCI. BALB/c mice globally deficient in β2-ARs (Adrb2−/−) were provided by Dr. David Farrar (University of Texas Southwestern Medical Center, Dallas, TX; ref. 24). OT-1 (B6.129S7-Rag1tm1-Mom Tg[Ifnar1c2b]1,100Mjb) mice were provided by Dr. Minhyung Kim (RPCI, Buffalo, NY). Mice were 8–12 weeks old and were maintained in specific pathogen-free facilities. All studies followed approved protocols and were performed under the guidelines established by the Institutional Animal Care and Use Committee at RPCI.

Cell culture and tumor models
4T1 tumor cells were purchased from and authenticated by ATCC in 2014. B16-OVA tumor cells were provided by Dr. Protul Shrikant in 2012 and have not been genetically authenticated by our laboratory. Cell lines were confirmed to be mycoplasma negative using the Mycoplasma Plus PCR Primer Set (Aligent Technologies, 302008). Both cell lines were cultured in RPMI1640 (Gibco) supplemented with 10% FBS, 1% γ-glutamine, and 1% penicillin/streptomycin. Once thawed, cells were passed twice prior to use. A total of 1 × 10⁴ 4T1 cells in 100 μL PBS were injected into four mammary fat-pad. A total of 2 × 10⁵ B16-OVA cells in 100 μL PBS were injected subcutaneously into the lower left abdomen. Tumor growth was monitored throughout experiments; perpendicular diameters (width/length) were measured 2 to 3 times/week and tumor volume calculated = WL/2 mm³.

Ambient temperature manipulation
Mice were housed five/cage in Precision Refrigerated Plant-Growth Incubators (Thermo Fisher Scientific) maintained at either standard temperature (~22°C) or thermoneutral temperature (~30°C) as described previously (14–16). Humidity was controlled using a Top Fin Air Pump AIR 1000 with Top Fin tubing. Mice were acclimated to the assigned temperature for at least 2 weeks.

Propranolol (β-blocker) studies
For studies in which propranolol was used to assess the impact of adrenergic signaling on the endogenous antitumor immune response, mice were acclimated to 22°C or 30°C housing temperature for 2 weeks; propranolol treatment in mice at 22°C and 30°C was initiated 4 days prior to tumor cell implantation and given daily until the experimental endpoint. Mice received 200 µg propranolol (P0884, Sigma-Aldrich) in 100 μL of PBS by intraperitoneal injection; control mice received 100-μL PBS. Tumor cells were implanted and treatment continued for the duration of the experiment.

Immunotherapy and propranolol combination studies
Anti-mouse PD-1 antibody (RMP1-14) and rat IgG2a isotype control antibody (2A3) were purchased from BioXCell. The experimental design detailed above was modified for these studies in that treatment was not initiated until tumors were detectable to ensure that tumors in all groups were the same size when treatment was started. This experimental strategy was...
also designed to mimic clinical implementation of combining β-blockers with checkpoint inhibitors in patients who present to clinic with a tumor. For combination immunotherapy ± propranolol studies, tumor cells were implanted into mice housed at 22°C prior to starting treatment and mice were monitored daily for tumor formation. On a rolling basis beginning the day after tumors became detectable, mice were randomized to receive either PBS (100 µl) + isotype antibody (200 µg), propranolol (200 µg) + isotype antibody (200 µg), PBS (100 µl) + anti-PD-1 (200 µg), or propranolol (200 µg) + anti-PD-1 (200 µg). Mice received 5–6 injections of antibody spaced 2–3 days apart whereas propranolol and PBS were given daily.

**CD8⁺ and CD4⁺ T-cell depletion**
Mice received weekly intraperitoneal injections (400 µg in 100 µl PBS) of CD8⁺ T-cell-depleting antibody (53-6.72, BioXCell) or CD4⁺ T-cell-depleting antibody (LTF-2, BioXCell) beginning 4 days prior to tumor implantation. Control animals received either rat IgG2a (2A3, BioXCell) or rat IgG2b (LTF-2, BioXCell) isotype controls for CD8⁺ and CD4⁺ T-cell depletion studies, respectively. Depletion was confirmed using flow cytometry.

**Flow cytometry**
Single-cell suspensions were created by excising and cutting mouse tumors into 2–3 mm pieces. 4T1 tumors were dissociated with collagenase/hyaluronidase (STEMCELL Technologies, 07912) and B16-OVA tumors with a Murine Tumor Dissociation Kit (Miltenyi Biotec, 130-096-730) following the manufacturers’ protocols prior to passing samples through a 70-µm nylon cell strainer (Corning). Spleens were mechanically disrupted and directly passed through a 70-µm nylon cell strainer (Corning). Red blood cells were lysed using ACK buffer (Gibco). Cells were then washed with flow running buffer (0.1% BSA in PBS) and incubated with anti-CD16/32 (Fc receptor blocker, 1:200) at 4°C for 15 minutes.

Cells were then stained with the following extracellular antibodies: anti-CD8a BV439 (53-6.7), anti-CD8β PE (H35-17.2), anti-CD4 BV876 or BV395 (GK1.5), anti-CD3 BV876, or APC-Cy7 (X45-2C11), anti-CD45 FITC or BUV395 (30-F11), anti-CD4 BV786 or BUV395 (GK1.5), anti-CD3 BV786, or APC-Cy7 (X45-2C11), anti-CD45 FITC or BV395 (30-F11), anti-CD-1 BV605 (J43), anti-CD11b BV395 (M1/70), and anti-Gr-1 BV605 (RB6-8C5) all purchased from BD Biosciences. For intracellular staining, cells were fixed and permeabilized using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) as per the manufacturer’s protocol. Cells were then stained with anti-FoxP3 Alexa 488 (MA1-12.17.3), anti-IFNγ APC (XMG1.2) from BD Biosciences, anti-IFNγ APC (XMG1.2) from eBioscience, or anti-granzyme B (GzmB) Alexa 647 (GB11) from Biolegend. All data were collected on a LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo v10 software (Tree Star, Inc.).

**ELISA**
Noradrenaline Research ELISA Kits were purchased from Rocky Mountain Diagnostics (BA E-5200). Blood was collected at the experimental endpoint by retro-orbital bleeding and NE was determined in serum.

**Statistical analysis**
Student t test was used to compare data between two groups and tumor growth statistics were calculated using two-way ANOVA with Tukey analysis using GraphPad Prism. One-way ANOVA with Tukey post hoc tests was used to compare data between four groups using SAS v9.4. All data are depicted as mean ± SEM.

**Results**
Tumor growth in mice housed at ST is promoted by β-adrenergic signaling in non-tumor host cells and can be inhibited by propranolol treatment.

We previously showed that several syngeneic tumors and a carcinogen-induced tumor grow more slowly when mice were housed at thermoneutrality (~30°C) than at standard temperatures (~22°C) and this is dependent on the adaptive immune system (14). However, the mechanism(s) mediating immune suppression at 22°C was not identified. We later showed that pancreatic tumor-bearing mice housed at 22°C have significantly higher NE levels than those housed at 30°C (16).

In this study, NE levels were also found to be significantly elevated in 4T1 tumor-bearing mice at 22°C compared to 30°C, indicating that this is not a tumor-type dependent phenomenon (Fig. 1A). To test the hypothesis that increased β-AR signaling at 22°C caused accelerated tumor growth when compared to 30°C, we acclimated mice to 22°C or 30°C, began treatment with propranolol (a pan-β-AR antagonist) or PBS 4 days prior to tumor implantation, then implanted tumor cells and monitored tumor formation and growth. A detailed experimental design is depicted in Fig. 1B. Pharmacologic blockade of adrenergic signaling by propranolol significantly slowed both B16-OVA (Fig. 1C) and 4T1 (Fig. 1D) tumor growth in mice at 22°C to a degree comparable with that achieved by housing mice at 30°C. Furthermore, addition of propranolol to mice at 30°C did not improve tumor growth control, which was likely because NE levels were already significantly lower.

We next utilized a β2-AR global receptor knockout BALB/c mouse (Adhrb2⁻/⁻) to assess the role of β2-AR in host cells by comparing tumor growth between Adhrb2⁻/⁻ and wild-type (WT) BALB/c mice (8). First, we physiologically manipulated adrenergic stress by housing mice at 22°C or 30°C. Then we implanted 4T1 tumor cells, which we and others have shown to lack functional β-ARs (data not shown; ref. 25), and monitored tumor growth. Housing temperature had no impact on tumor growth in Adhrb2⁻/⁻ mice and tumors grew at a rate similar to that observed in WT mice housed at 30°C. However, tumors in WT mice at 22°C grew significantly faster than the other three groups (Fig. 1E), indicating that accelerated tumor growth at 22°C is dependent on functional β2-ARs on host cells.

Finally, we housed mice at 22°C and reduced adrenergic signaling pharmacologically with propranolol. As shown in Fig. 1F, propranolol treatment significantly slowed tumor growth in WT mice but had minimal effect in Adhrb2⁻/⁻ mice, further supporting the conclusion that signaling through β2-ARs in host cells drives immune suppression at 22°C. Unlike housing at 30°C, however, there was a small, but significant, reduction in tumor growth in Adhrb2⁻/⁻ mice treated with a pan-β-AR
antagonist, suggesting a minor role for other β-AR isoforms not found on immune cells (i.e., β1-AR and β2-AR). These data suggest that increased signaling through β2-ARs on host cells causes tumors to grow faster in mice housed at 22°C compared with those housed at 30°C.

Reduced tumor growth following β-AR blockade results from enhanced CD8⁺ T-cell–mediated antitumor immune response

NE signaling through β2-ARs on lymphocytes has recently been shown to suppress immune cell function in nonnecologic settings (17, 23, 24); therefore, we asked whether this signaling pathway mediates immune suppression in mice housed at 22°C. We repeated the experiments shown above (Fig. 1C and D) in SCID mice and found that neither physiologic (30°C) nor pharmacologic (propranolol) reductions in adrenergic signaling had any effect on tumor growth (Fig. 2A and B), indicating that the pharmacologic (propranolol) reductions in adrenergic signaling (Fig. 2C and D) did not affect tumor growth in wild-type BALB/c or Adrb2⁻/⁻ mice housed at 22°C or 30°C (E and F), further demonstrating that propranolol was slowing tumor growth in mice housed at 22°C in a CD8⁺ T-cell–dependent manner.

As T-cell killing is dependent upon contact with tumor cells in the TME, we examined the functional orientation of tumor-infiltrating lymphocytes (TIL; refs. 5, 10, 11). We evaluated the frequency and relative number (# cells / mg tumor) of CD8⁺ T cells from propranolol-treated mice bearing 4T1 or B16-OVA tumors compared with controls (Supplementary Fig. S1), indicating that propranolol was slowing tumor growth in mice housed at 22°C in a CD8⁺ T-cell–dependent manner.

To determine which immune cell(s) were responsible for the improved tumor growth control following β-blockade, we selectively depleted either CD8⁺ or CD4⁺ T cells from WT mice. Depletion of CD8⁺ T cells abrogated the effect of propranolol on both B16-OVA and 4T1 tumor growth (Fig. 2C and D), indicating dependence on CD8⁺ T cells. Furthermore, depletion of CD8⁺ T cells in Adrb2⁻/⁻ mice also accelerated tumor growth rates similar to that seen in WT mice following CD8⁺ T-cell depletion (Fig. 2E). In contrast to CD8⁺ T-cell depletion, the benefit of β-blockade was not lost following CD4⁺ T-cell depletion (Supplementary Fig. S1), indicating that propranolol was slowing tumor growth in mice housed at 22°C in a CD8⁺ T-cell–dependent manner.

As T-cell killing is dependent upon contact with tumor cells in the TME, we examined the functional orientation of tumor-infiltrating lymphocytes (TIL; refs. 5, 10, 11). We evaluated the frequency and relative number (# cells / mg tumor) of CD8⁺ T cells in tumors treated with or without propranolol. E and F, 4T1 tumor growth in wild-type BALB/c and Adrb2⁻/⁻ mice housed at 22°C or 30°C (E) or housed at 22°C, treated with or without propranolol (F). Data are presented as mean ± SEM. Comparison of norepinephrine levels by Student t-test. N = 4 to 5 per group. Tumor growth statistics analyzed using two-way ANOVA with Tukey analysis. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. N = 4 to 8 per group.
The frequency of effector CD8\(^+\) TILs expressing T-bet was significantly increased in propranolol-treated mice compared with controls. Similar observations were also made regarding the frequency and relative number of CD8\(^+\) TILs expressing IFN\(\gamma\) (Fig. 3G–I; 4T1 data in Supplementary Figs. S3D–S3F). We did not observe differences in the frequency or relative number of CD8\(^+\) TILs that expressed GzmB (Fig. 3G–I). However, further analysis of the GzmB\(^+\) fraction of CD8\(^+\) TILs revealed that the GzmB MFI was significantly increased in the CD8\(^+\) T-cell population of propranolol-treated mice (Fig. 3J and K). We also observed a significant reduction in the frequency and absolute number of MDSCs in the spleens of 4T1 tumor-bearing mice housed at 22°C treated with propranolol compared with PBS controls. Thus far, these data indicate that \(\beta\)-AR blockade reduces tumor growth in a CD8\(^+\) T-cell–dependent manner and increases the frequency of effector CD8\(^+\) TILs in both the 4T1 and B16-OVA tumor models.

\(\beta\)-AR blockade significantly increases the ratio of effector CD8\(^+\) T cells to Tregs in B16-OVA tumors and decreases the accumulation of MDSCs in the spleens of 4T1 tumor-bearing mice

Tumor infiltration by effector CD8\(^+\) T cells is a favorable prognostic indicator in many tumor types (5, 10, 11). However, the presence of CD4\(^+\) regulatory T cells (Tregs; CD4\(^+\) FoxP3\(^+\)), as well as a low CD8\(^+\) T-cell to Treg ratio, is often a negative prognostic indicator (5, 27, 28). We therefore asked whether propranolol treatment decreased numbers of Tregs and, if so, did this favorably alter the CD8\(^+\)/Treg ratio. At endpoint, we found that the frequency of CD4\(^+\) T cells in B16-OVA tumors with a Treg phenotype was significantly reduced in propranolol-treated mice (Fig. 4A and B). We next evaluated the ratio of total CD8\(^+\) T cells to Tregs, but did not observe a difference between groups (Fig. 4C). However, we did observe a significant increase in the ratio of effector (IFN\(\gamma\)\(^+\)) CD8\(^+\) T cells to Tregs in propranolol-treated mice compared with controls (Fig. 4D).

In 4T1, we assessed the frequency of another potent suppressor cell type, myeloid-derived suppressor cells (MDSC; CD11b\(^+\) Gr-1\(^+\)), because they accumulate in the spleens of mice bearing 4T1 and other mammary tumors (29). We observed that the mass of the spleens in propranolol-treated mice was significantly less than that of untreated mice (Supplementary Fig. S4A). We also observed a significant reduction in the frequency and absolute number of MDSCs in the spleens of 4T1 tumor-bearing mice housed at 22°C treated with propranolol compared with PBS controls (Supplementary Figs. S4B and S4C).

Because the MDSC frequency correlates with tumor burden (29), we next asked whether adrenergic signaling played a role in the accumulation of MDSCs in addition to tumor burden. Here, we evaluated spleens of both non-tumor–bearing and 4T1 tumor–bearing mice treated with propranolol or PBS following the experimental outline detailed in Supplementary Fig. S5A. In the absence of a tumor, propranolol treatment did not alter MDSC frequency. However, analysis of spleens at three different time-points post 4T1 tumor implantation (days 7, 12, and 20) revealed that the frequency and absolute number of MDSCs were decreased in the propranolol treated cohorts despite the fact that tumor volumes were starting to separate but were not yet significantly different (Supplementary Figs. S5B–S5F).
Figure 3.

Frequencies of intratumoral effector T cells are increased in β-blocker-treated mice. Single-cell suspensions were made from B16-OVA tumors from mice housed at 22°C, treated with PBS or propranolol, and analyzed by flow cytometry. A, Representative flow plots of T-bet expression in CD8⁺ TILs. B and C, Frequency (B) and number (C) of T-bet⁺ CD8⁺ T cells per mg of tumor. D, Representative flow plots of IFNγ expression in CD8⁺ TILs. E and F, Frequency (E) and number (F) of IFNγ⁺ CD8⁺ T cells per mg of tumor. G, Representative flow plots of GzmB expression in CD8⁺ TILs. H and I, Frequency (H) and number (I) of GzmB⁺ CD8⁺ T cells per mg of tumor. J, Representative histogram of GzmB expression in CD8⁺ TILs. K, Quantification of the GzmB MFI in the GzmB⁺ CD8⁺ TIL populations. All data are presented as mean ± SEM. Statistics analyzed by Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.005. N = 5 to 7 per group.
Overall, these data indicate that β-blockade reduces the frequency of Tregs and increases the ratio of IFN-γ+ CD8 TILs to Tregs while also decreasing the accumulation of MDSCs in the spleens of 4T1 tumor-bearing mice.

Fewer effector CD8+ TILs express PD-1 in β-blocker-treated mice compared with controls

Because we found that β-blockade reversed immunosuppression at 22°C, we next examined whether this would also change the expression of immune checkpoint molecules, which naturally suppress the antitumor immune response, specifically PD-1. Recent work indicates that tumors, as well as suppressive cells in the TME, can express the ligand PD-L1, which can bind to PD-1 and suppress antitumor immune responses. Moreover, one recent study concluded that the percentage of PD-1-expressing CD8+ T cells in murine tumors is one factor that dictates the responsiveness of tumors to anti-PD-1 (30). Specifically, responsive tumors had a low frequency of PD-1+ CD8+ TILs, whereas nonresponsive tumors had a high frequency of PD-1+ CD8+ TILs.

Therefore, prior to testing anti-PD-1 checkpoint blockade in our stress reducing murine tumor models, we first investigated whether β-AR signaling had any impact on PD-1 expression. Following treatment of mice housed at 22°C with propranolol, we observed that the percentage of CD8+ TILs expressing PD-1 was decreased in both 4T1 (Fig. 5A and B) and B16-OVA tumors (Fig. 5C and D). Further analysis of the PD-1 MFI on the PD-1+ CD8+ TIL population from 4T1 tumors revealed that the expression of PD-1 was significantly decreased when mice were treated with propranolol compared with controls (Fig. 5E and F). In contrast, the PD-1 MFI was increased on PD-1+ CD8+ TILs isolated from B16-OVA tumors in mice treated with propranolol compared with control animals (Fig. 5G and H).

To determine whether the cells that expressed PD-1 had an effector phenotype, as evidenced by expression of T-bet and IFNγ, we analyzed CD8+ TILs from 4T1 tumors that coexpressed effector molecules and PD-1. Here, we observed that the frequency of CD8+ TILs coexpressing PD-1 and either T-bet+ (Fig. 5I) or IFNγ+ (Fig. 5J) was decreased in propranolol-treated mice compared with PBS controls.

Taken together, these data indicate that the frequency of CD8+ TILs isolated from 4T1 and B16-OVA tumors, which express PD-1, is decreased in mice housed at 22°C and treated with propranolol. In addition, fewer effector CD8+ TILs from 4T1 tumors express PD-1 in β-blocker–treated mice, suggesting that not only does adrenergic signaling suppress the function of CD8+ TILs, but these effector cells are also more susceptible to suppression via PD-1/PD-L1 signaling.

β-Adrenergic stress signaling impairs the efficacy of anti-PD-1 checkpoint blockade in murine tumor models

After determining that β-AR blockade facilitates development of endogenous antitumor immunity in murine tumor models, which was associated with a decreased percentage of PD-1–expressing CD8+ TILs, we next evaluated whether anti-PD-1 checkpoint blockade would have greater efficacy when adrenergic signaling was reduced. Because all previously reported murine studies assessing checkpoint inhibitor efficacy have been conducted at 22°C, we first used a physiologic approach to test whether housing temperature induced adrenergic signaling influenced anti-PD-1 efficacy in mice bearing B16-OVA and 4T1 tumors. Here, we acclimated mice to 22 or 30°C, implanted tumor cells prior to initiation of treatment to ensure that tumors in all groups were established and of comparable size, and subsequently began treatment with anti-PD-1 or isotype antibodies as tumors became detectable. The treatment protocol is depicted in Supplementary Fig. S6A.

As shown in Supplementary Fig. S6B, although anti-PD-1 was modestly effective in slowing B16-OVA tumor growth in mice housed at 22°C, its efficacy was significantly improved in mice that had been acclimated to 30°C housing prior to tumor implantation. In the 4T1 model, anti-PD-1 had no impact on tumor growth in mice housed at 22°C (Supplementary Fig. S6C), whereas significantly improved efficacy of anti-PD-1 was observed in mice housed at 30°C.
Expression of PD-1 by effector CD8^+ TILs is reduced by propranolol treatment. Single-cell suspensions were made from B16-OVA tumors from mice housed at 22°C, treated with PBS or propranolol, and analyzed by flow cytometry. Representative flow plots of PD-1 expression on CD8^+ TILs isolated from 4T1 (A) and B16-OVA (C) tumors. Quantification of CD8^+ TILs expressing PD-1 isolated from 4T1 (B) and B16-OVA (D) tumors. Representative histograms of PD-1 expression on CD8^+ TILs isolated from 4T1 (E) and B16-OVA (G) tumors. Quantification of PD-1 MFI on CD8^+ TILs isolated from 4T1 (F) and B16-OVA (H) tumors. The percentage of effector CD8^+ TILs isolated from 4T1 tumors that express both PD-1 and T-bet (I) or IFNγ (J). All data are presented as mean ± SEM. Statistics analyzed by Student t test: ***, P < 0.005. B16-OVA studies, N = 5 per group. 4T1 studies, N = 8 to 10 per group.
We next tested the hypothesis that treating mice housed at 22°C with propranolol should recapitulate the beneficial effects on anti-PD-1 efficacy observed when housing mice at 30°C. To ensure that the tumors in each group were of comparable size, tumors were again implanted prior to treatment. In this clinically relevant therapeutic model, we began propranolol treatment after tumors became detectable. Mice treated with propranolol or PBS were then randomized to receive anti-PD-1 or isotype antibodies as detailed in Fig. 6A (Supplementary Fig. S7A).

In the B16-OVA tumor model, we again observed that anti-PD-1 alone had limited efficacy (Supplementary Fig. S7B) at 22°C. However, the addition of propranolol significantly improved

Figure 6.
β-blockade increases the efficacy of anti-PD-1 immunotherapy in the 4T1 murine tumor model. A, Experimental design for combination propranolol and anti-PD-1 studies at 22°C. Mice at 22°C were challenged with 4T1 tumors. Mice were randomized to receive anti-PD-1 or isotype antibody (200 μg), with or without daily propranolol treatments (200 μg) beginning the day after tumors became detectable. Mice received five doses of anti-PD-1 or isotype antibody on days 0, 3, 6, 9, and 12. B, 4T1 tumor growth. Data are presented as mean ± SEM. Tumor growth statistics analyzed using two-way ANOVA with Tukey analysis. ***, P < 0.0001. N = 6 to 7 per group.

Figure 7.
Anti-PD-1 and β-blocker therapy synergize to increase the frequency of IFNγ expressing intratumoral effector CD8+ T cells in 4T1 tumors. Single-cell suspensions were made from 4T1 tumors harvested from mice housed at 22°C, treated with anti-PD-1 or isotype antibody with or without propranolol. IFNγ expression was determined in intratumoral CD8+ T cells using flow cytometry. A, Representative flow plots of IFNγ expression in CD8+ TILs. B, Quantification of IFNγ expressing CD8+ T cells in all treatment groups. C, The number of IFNγ+ CD8+ T cells per mg of tumor. All data are presented as mean ± SEM. Log-transformed data analyzed using one-way ANOVA with Tukey-adjusted post hoc tests. In B and C, there was a significant overall association with treatment group (P = 0.002 and 0.001, respectively). *, P < 0.05; **, P < 0.01. N = 6 to 7 per group.
responses to anti-PD-1 (Supplementary Fig. S7B). In 4T1 tumor-bearing mice, treatment with anti-PD-1 alone had no impact on tumor growth (Fig 6B). In contrast, combining anti-PD-1 with propranolol significantly slowed 4T1 tumor growth (Fig 6B). Note that propranolol treatment alone had little effect on the ability of the immune response to control these established tumors in contrast to the beneficial effect seen when propranolol was given prior to tumor implantation.

Next, we sought to determine whether the improved efficacy of anti-PD-1 and propranolol in 4T1 tumor-bearing mice at 22°C was associated with changes in the immune contexture of the TME. We analyzed the effector phenotype of CD8+ TILs in tumor tissue taken from the experiment in Fig 6B. We observed that propranolol, anti-PD-1, or a combination of the two had no influence on the frequency of total CD8+ T cells (Supplementary Fig S8). However, the frequency and relative number of effector CD8+ TILs (IFNγ+) in tumors from mice receiving combination therapy was significantly higher than all other groups (Fig 7A–C).

Altogether, these data show that combining propranolol and anti-PD-1 in mice housed at 22°C significantly changes the immune contexture of tumors to a more inflamed TME as evidenced by an overall increase in the frequency and relative number of effector CD8+ TILs. Importantly, these changes correlate with a significant improvement in the efficacy of anti-PD-1 therapy.

Discussion

In this study, we exploited a physiologic model of mild cold stress to investigate the role of chronic adrenergic signaling in the development of antitumor immunity, the immune contexture of the TME, and the efficacy of anti-PD-1 checkpoint blockade. We report here, for the first time, that increased adrenergic signaling at 22°C, by suppressing CD8+ T-cell–mediated antitumor immunity, is the underlying mechanism by which cool housing temperatures suppress the antitumor immune response. Notably, this suppression can be reversed by treating mice with the pan-ß-AR blocker propranolol, and the improved immune contexture of treated tumors correlates with increased anti-PD-1 efficacy.

Although many studies have shown that β-blockade slows tumor growth, these previous studies have either focused on how tumor cell–intrinsic adrenergic signaling affects growth and metastasis or other protumorigenic processes such as increased angiogenesis (16, 19–21, 31). In contrast, our study investigated the effects of propranolol on the antitumor immune response. Importantly, we demonstrated that the benefit of propranolol is lost in the absence of CD8+ T cells in these models. Moreover, propranolol treatment improved the functional orientation of CD8+ TILs as evidenced by increased expression of markers of effector function (T-bet, IFNγ, and GzmB). We also found a significantly increased ratio of IFNγ+ CD8+ Treg cells, which is indicative of an inflammatory TME (28). Furthermore, the tumor promoting effects of cool housing were lost in mice lacking β2-ARs (Adrb2−/−) as was the immune enhancing role of propranolol, pointing to a specific role for the β2-AR.

We also found that effector CD8+ TILs isolated from the tumors of mice treated with propranolol expressed lower levels of PD-1 and thus are likely less susceptible to PD-L1–mediated suppression. Furthermore, we showed that reducing adrenergic signaling by either housing mice at 30°C or treating mice housed at 22°C with propranolol, significantly improved responses to anti-PD-1 in both the B16-OVA and 4T1 tumor models. It should be noted that in these clinically relevant studies, we started treatments only after tumors were established (detectable) because if we pre-treated the mice with propranolol before implanting tumors, tumors in the propranolol groups would already be significantly smaller when the anti-PD-1 was begun. Consistent with previously published work by Ngiow and colleagues (30), we observed that a decreased frequency of PD-1+ CD8+ TILs corresponded with an increased efficacy of anti-PD-1 therapy. However, to date, definitive data regarding PD-1 expression on CD8+ TILs and how this impacts responses to anti-PD-1 is debatable (6). Moreover, our data regarding PD-1 expression on CD8+ TILs is observational and warrants further investigation to more definitively evaluate if low PD-1 expression on CD8+ TILs in our model is directly linked to increased anti-PD-1 efficacy.

Analysis of 4T1 tumors from mice treated with both propranolol and anti-PD-1 showed a significant increase in the frequency of IFNγ-producing CD8+ T cells compared with those receiving anti-PD-1 or propranolol alone. These findings have broad implications related to the baseline immunosuppression of tumor-bearing mice housed at 22°C (15, 16, 32) and could help to explain why the remarkable responses seen in some patients receiving a single checkpoint inhibitor in a therapeutic setting are generally not observed in mice (33, 34).

To date, most of the investigations regarding β-AR signaling and T-cell behavior have been conducted in nononcologic models and have focused on CD4+ T cells. For example, Bellinger and Lorton have recently reviewed mechanisms by which the SNS facilitates the immune response, focusing on both sympathetic innervation to lymphoid organs and β-AR expression on immune cells (17). As they point out, NE signaling through β-ARs on CD4+ lymphocytes induces production of the Th1 cytokines IL6 and IL10 suppressing production of the Th1 cytokines IL12, TNFα, and INFγ thereby reducing the generation of cytotoxic CD8+ T cells. Recently, Estrada and colleagues investigated adrenergic signaling specifically in CD8+ T cells and found that β2-AR activation decreased IFNγ, TNFα, and IL2 production and suppressed the ability of CD8+ T cells to kill virally infected cells (24).

Several questions and future studies emerge from this work. One is the need to identify the mechanisms by which adrenergic signaling ultimately shapes the immune contexture of tumors. One possibility is that β-AR signaling undermines production of inflammatory cytokines and chemokines within the TME that may regulate the recruitment of both antitumor and suppressive immune cells to the tumor. Recent studies assessing lymph node trafficking and T-cell activation indicate that adrenergic signaling is an important mediator of egress from lymph nodes, which can significantly impact responses to vaccination and the severity of autoimmunity (35, 36). In addition, other work has shown that circadian oscillations in NE regulate chemokines and adhesion molecules on endothelial cells that are essential for T-cell trafficking (37).

Another important goal will be to improve our understanding of the degree to which adrenergic signaling shapes antitumor immune responses in more naturally developing tumors. We have previously reported that the incidence of a carcinogen-induced tumor model (3-methylcholanthrene, MCA) is reduced in mice housed at 22°C compared with 30°C (14), suggesting that adrenergic signaling likely plays a role in immune surveillance and immune editing. Using a transgenic rat model of melanoma, Wrobel and colleagues (38) found that propranolol...
also decreased tumor incidence, reduced the number of myeloid cells, and increased the presence of GzmB+ CD8+ T cells in tumors at endpoint. Future work must also address the mechanisms by which adrenergic signaling may impact the suppressive functions of myeloid cells. Relatively little is known about the importance of β-AR signaling in MDSCs, but recent work by lin and colleagues (39) showed that psychologic stress increases MDSC frequency in non-tumor–bearing mice (29). In our study, β-blockade reduced MDSC accumulation in spleens at both early and late time-points post 4T1 tumor implantation. Future work should assess whether adrenergic signaling alters the suppressive functionality of MDSCs.

In addition to MDSCs, M2 macrophages should also be evaluated as they suppress antitumor immunity, drive metastasis, and can produce NE (40). Previously, it was shown that cold stress skews macrophages in brown fat towards an M2 phenotype, which then produces NE for heat generation (41). However, a recent conflicting study by Fischer and colleagues concluded that M2 macrophages do not participate in adaptive thermogenesis in brown fat (42). Although we did not observe differences in the frequency of phenotypic M2 macrophages in our models (data not shown), future work should determine whether M2 macrophages are making NE locally in the TME.

Future studies should also assess whether housing at 30°C or β-blockade at 22°C affects the efficacy of other immunotherapies, such as anti–CTLA-4, or combinations of immunotherapies. The potential of combining β-AR blockade with anti–CTLA-4 is of particular interest because β2-AR activation in Tregs increases suppressive functions by upregulating expression of CTLA-4 (43). In addition, it will be important to investigate whether reducing adrenergic signaling increases the incidence and severity of autoimmunity often observed in patients receiving but was not predicted by mouse models. Although we did not observe any obvious increases in toxicity in our models, our ongoing studies will assess the impact of combination therapies. Because all preclinical studies that tested checkpoint inhibitors in mice were conducted at 22°C, it is possible that the general immune suppression we have demonstrated under these housing conditions also suppresses the function of autoreactive T cells, an observation we previously reported in models of GVHD (15).

Although the work presented here focused specifically on ambient housing temperature, other concurrent forms of stress also likely contributed to stress in mice and may influence data interpretation. These include but are not limited to the presence of an "aggressor" mouse in the cage as well as the frequency of handling by investigators (44). Also, as baseline levels of catecholamines vary between male and female mice (45), these studies should be repeated in male mice.

From a clinical perspective, our findings complement several retrospective studies suggesting that patients taking nonspecific β-blockers for indications other than cancer have better outcomes than those administered specific β1-β-blockers and nonusers. (46–48). Because β-blockers are generally prescribed to treat a variety of cardiovascular conditions, as well as performance anxiety, it would be interesting to see whether patients taking β-blockers have improved responses to immunotherapies and/or an increased incidence or severity of autoimmunity, both of which would be predicted by our study.

In addition to assessing retrospective β-blocker use in the context of immunotherapies, analysis of patients taking psychotropic drugs that modulate adrenergic signaling like anti-depressants and anti-psychotics should also be examined. On the basis of our data, we would predict that patients taking anti-depressants that increase NE levels, such as TCAs, SNRIs, and DNRIs would have poorer responses to checkpoint inhibitors than those taking SSRIs, which specifically modulate serotonin. These data could inform prescribing physicians as to which anti-depressants to prescribe or to avoid in cancer patients who will receive immunotherapy.

Moreover, it would be of value to determine the percentage of patients who have not responded to checkpoint inhibitors among those who have faced extended periods of stress throughout their lifetime. One study by Mundy-Bosse and colleagues (49) determined that patients who reported high levels of chronic, but not acute, stress had a significantly increased frequency of MDSCs in circulation. These findings are likely important to checkpoint inhibitor responses as recent work by Waight and colleagues showed that a high frequency of MDSCs decreases responses to checkpoint inhibitors while also serving as a negative prognostic indicator in breast cancer patients (50).

In summary, our data reveal a fundamental role for adrenergic signaling in regulating the antitumor immune response. Our findings support the idea that the effects of catecholamines and β-AR signaling are a significant underlying mechanism that causes suppression of antitumor immunity. In addition, the work presented here suggests that in some patients, strategic combinations with β-blockers should enhance the efficacy of checkpoint blockade.

Disrupting the NE/β-AR axis provides an attractive target for clinicians because there is already an extensive list of FDA-approved β-blockers with a history of safety and efficacy that have been used in patients over many decades. The availability of these β-blockers and the potential to rapidly repurpose this class of drugs for use with cancer treatments make the possibility of new combination trials especially feasible.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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β-Adrenergic Signaling in Mice Housed at Standard Temperatures Suppresses an Effector Phenotype in CD8+ T Cells and Undermines Checkpoint Inhibitor Therapy

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