ABSTRACT

Metabolic dysfunction and exhaustion in tumor-infiltrating T cells have been linked to ineffectual antitumor immunity and the failure of immune checkpoint inhibitor therapy. We report here that chronic stress plays a previously unrecognized role in regulating the state of T cells in the tumor microenvironment (TME). Using two mouse tumor models, we found that blocking chronic adrenergic stress signaling using the pan β-blocker propranolol or by using mice lacking the β2-adrenergic receptor (β2-AR) results in reduced tumor growth rates with significantly fewer tumor-infiltrating T cells that express markers of exhaustion, with a concomitant increase in progenitor exhausted T cells. We also report that blocking β-AR signaling in mice increases glycolysis and oxidative phosphorylation in tumor-infiltrating lymphocytes (TIL), which associated with increased expression of the costimulatory molecule CD28 and increased antitumor effector functions, including increased cytokine production. Using T cells from Nurr77-GFP reporter mice to monitor T-cell activation, we observed that stress-induced β-AR signaling suppresses T-cell receptor (TCR) signaling. Together, these data suggest that chronic stress–induced adrenergic receptor signaling serves as a “checkpoint” of immune responses and contributes to immunosuppression in the TME by promoting T-cell metabolic dysfunction and exhaustion. These results also support the possibility that chronic stress, which unfortunately is increased in many patients with cancer following their diagnoses, could be exerting a major negative influence on the outcome of therapies that depend upon the status of TILs and support the use of strategies to reduce stress or β-AR signaling in combination with immunotherapy.

Introduction

For decades, the relationships between stressful events and immunity have been studied by researchers in the field of psychoneuroimmunology (PNI). A new spotlight has now been placed on this field with more recent revelations that tumor growth itself may depend upon neurogenesis and sympathetic nervous system (SNS) activity (1–4). Nerves of the SNS have been found to infiltrate the tumor microenvironment (TME; refs. 5–7), providing a direct conduit by which stress, nervous stimulation, and release of catecholamines, such as norepinephrine (NE), could influence the TME. Studies have also shown that sympathetic nerves innervate primary and secondary lymphoid organs (8), and there is evidence in nononcologic settings that β-AR signaling can directly suppress CD8+ T-cell activation and effector function (9) or can act indirectly through dendritic cells (DC), reducing their ability to prime T cells (10). Overall, these studies generate concern related to how stress and the nervous system might be affecting the immunologic balance within the TME; in particular, how does stress signaling influence the development and strength of antitumor immune responses and efficacy of immunotherapy?

This is important because many patients with cancer experience an increase in chronic stress (11), which could influence the outcome of treatment, including immunotherapies. We have previously shown that adrenergic stress results in immunosuppression in graft-versus-host disease (GVHD) and cancer, both of which depend on a complex balance between immunostimulatory and immunosuppressive activities (12–14). Our previous observations show that mice housed at their standard [Institutional Animal Care and Use Committee (IACUC) mandated] housing temperature (ST, 22–23°C) experience mild, but chronic, adrenergic stress that is mediated by the SNS in response to the need for increased metabolic heat production (a process dependent on the adrenergic stress response and NE production; refs. 15–17). Housing mice at thermoneutrality (TT, 30–31°C), blocking β2-adrenergic receptor (β2-AR) signaling with β blockers in mice housed under standard cool conditions, or using mice lacking β2-AR (14, 15, 18) provide very convenient and physiologically relevant opportunities to study the impact of adrenergic stress on various aspects of the immune system. We previously found that reducing thermal stress or blocking β-ARs in stressed mice enhances antitumor immune responses by reducing the suppressive function of myeloid-derived suppressor cells (MDSC) and increasing the frequency and function of intratumoral CD8+ T cells (12, 14, 15). The improved tumor control is lost when CD8+ T cells are depleted. We also found that reducing cold stress or blocking β-AR signaling significantly improves the efficacy of immune checkpoint immunotherapy, chemotherapy, and radiation (15, 17, 19). Although these data suggest the potential for stress to inhibit antitumor immunity, the mechanisms involved are not known.
Our prior work showed that chronic adrenergic stress increases programmed cell death-1 (PD-1) expression on tumor-infiltrating lymphocytes (TIL; ref. 15). In a subsequent in vitro analysis, β-AR signaling was observed to suppress metabolic reprogramming during CD8+ T-cell activation (18). Here, we report that chronic stress in mice has the potent ability to shape both the metabolic profile and the status of exhaustion in T cells in vivo within the TME. Most prior research on T-cell exhaustion has focused on identifying the subsets of cells involved, their differentiation, the role of chronic T-cell receptor (TCR) stimulation, and the role of T-cell exhaustion in the overall response to checkpoint inhibitor therapies (20, 21). In this study, we asked whether physiologic chronic stress drives T-cell dysfunction or exhaustion, characterized by reduced effector function, limited cytokine secretion, and expression of inhibitory receptors such as PD-1 in the TME. We observed that blocking β-AR signaling in mice experiencing mild chronic stress reduced the percentage of exhausted T cells, while simultaneously increasing progenitor exhausted T cells, a condition known to result in better tumor control and response to anti–PD-1 therapy (22). We also found that blocking β-AR signaling increased the metabolic activity and function of TILs, whereas conversely, both in vitro and in vivo data showed that increased β-AR signaling decreased T-cell activation by inhibiting TCR signaling. Together, these data provide mechanistic support for the idea that the chronic stress alters the immune contexture of tumors and suppresses the activity of TILs, most likely through local secretion of NE by nerve endings in the TME. These findings also suggest that chronic stress experienced by patients with cancer (11, 23, 24) has the potential to limit response to therapies, particularly immunotherapies, that depend on antitumor immune responses.

Materials and Methods
Mice
Female C57BL/6Ncr (C57BL/6) and Balb/cAnNcr (BALB/c) were purchased from Charles River. Female β2-AR−/− mice on a BALB/c background were originally provided by Dr. David Farrar (University of Texas Southwestern Medical Center, Dallas, TX) and subsequently bred at Roswell Park Comprehensive Cancer Center (RPCCC; Buffalo, NY). Female β2-AR−/− mice on a C57BL/6 background were made by CRISPR technique in RPCCC. Guide RNA was designed, validated, and synthesized by IVT for the Adbr2 gene in C57BL/6 background by the Washington University of St. Louis CRISPR/Cas9 core facility. Briefly, algorithms predicting guide cut sites in Adbr2 were analyzed for off-target effects as well as the double-stranded DNA break leading to a nonsense-mediated decay of the DNA, which resulted in indels and thus knockouts in repair via non-homologous end joining repair pathway (NHEJ). Guide RNA and Cas9 mRNA were synthesized and sent to the RPCCC’s Gene Targeting and Transgenic Shared Resource for injection. The guide plus Cas9 mRNA were injected into the pronuclei of C57BL/6 fertilized one-cell embryos (final concentrations were as follows: IVT gRNA + Cas9 mRNA = 5 ng/μL Cas9, 2.5 ng/μL gRNA). Guide sequence was directed at exon 1: 5’ TCTGGCGCTCGGTCCGTT NGG 3’. The injected embryos were transferred into pseudopregnant CD-1 foster mothers and pups were subsequently born. At weaning, tail biopsies were taken and screened by WUSTL by targeted next-generation sequencing (NGS). It was found that 11 of the animals harbored deletions in their genomes which resulted in homozygous knockout of the Adbr2 gene in these animals. Primers were acquired from WUSTL to maintain genotyping and breeding at Roswell Park. Three independent “founder” lines were chosen that harbored deletions of 5 bp, 102 bp, and 71 bp. These lines were bred with C57BL/6Ncr counterparts in order to breed out any off-target effects that may still be present in the early generations of mice, and this was maintained as stock. Once several generations of breeding were done, intercrossing to re-create the homozygous knock-out was performed and phenotype confirmed. Female Nur77-GFP reporter mice on a C57BL/6 background were originally purchased from the Jackson Laboratory and maintained in-house. All mice used were at 8 to 12 weeks old. All mice were maintained in specific pathogen–free facilities, and all studies were conducted following protocols approved by the IACUC at RPCCC (Buffalo, NY).

Manipulation of ambient temperature and stress
Mice were housed at either standard (IACUC required) temperature (ST; 22°C–23°C) or thermoneutral temperature (TT; 30°C–31°C) as described previously (12, 13). In this model, the control, untreated groups all experience chronic physiologic cold stress, which activates the SNS to regulate generation of heat through adaptive thermogenesis (16).

β-adrenergic receptor blocker treatment
To block β-adrenergic signaling, stressed mice (housed at ST) received daily, intraperitoneal injections of 200 μg propranolol (P0884, Sigma-Aldrich) in 100 μL PBS, beginning 7 days prior to, or 7 days after, tumor cell implantation (described below). Treatments continued until end of the experiment. Mice in the control (stressed) group received 100 μL PBS injections only.

Cell culture and tumor models
The B16-OVA melanoma cell line was provided by Dr. Protul Shrikant (University of Arizona College of Medicine, Tucson, AZ) and has been genetically authenticated by our laboratory. Cell lines were confirmed to be Mycoplasma negative (Mycoplasma Plus PCR Primer Set, Aligent Technologies, 302008). The CT26.CL25 colon cancer cell line was purchased from and authenticated by ATCC in 2017. These cells were cultured as described previously (15, 19). Both cell lines were passaged twice before tumor implantation after being thawed. A total of 2 × 10⁶ B16-OVA cells were injected subcutaneously into the lower left abdominal of C57BL/6, C57BL/6 β2-AR−/−, and Nur77GFP mice; 2 × 10⁶ CT26.CL25 cells were injected subcutaneously into the lower left abdominal of BALB/c, BALB/c β2-AR−/− mice. Both cell lines were in 50 μL PBS. In each animal experiment, mice were randomly assigned to each group. Tumor monitoring started 5 days after implantation, and perpendicular diameters (width/length) were measured every 2–3 days until the end of the experiment. Tumor volume was calculated by the formula: Volume (mm³) = W² × H/2. Tumor growth in the CT26 model was blindly measured by a member of the laboratory staff (Ms. Li Feng, Department of Immunology, RPCCC, Buffalo, NY) who is not an author on this study. Experiments were terminated by days 17 to 19 after tumor implantation, except for the kinetic study (days 11 and 15). Tumors, spleens, and draining lymph nodes were collected at the endpoint.

CD8+ T-cell isolation and culture
Eight- to 12-week-old BALB/c or Nur77-GFP reporter mice were sacrificed, and spleen and inguinal and axillary lymph nodes were collected. Spleen and lymph nodes were crushed and filtered through a 70-μm nylon cell strainer (Corning). Red blood cells were lysed by ACK lysing buffer (A1049201, Thermo Fisher Scientific). CD8+ T cells were isolated using a negative CD8+ T Cell Isolation Kit (catalog no. 130–104–075, Miltenyi Biotec) and then cultured at 1 × 10⁶/mL in the presence of plate-coated anti-CD3/anti-CD28 cross-linking antibodies.
antibodies (anti-CD3 2 μg/mL; anti-CD28 2 μg/mL; Supplementary Table S1) in the presence or absence of 10 μmol/L isoproterenol (Sigma) during activation as described previously (18). For immunoblotting (described below), CD8⁺ T cells were cultured at 2 × 10⁶/mL in the presence of 10 μL anti-CD3/anti-CD28 activation beads (catalog no. 11452D, Thermo Fisher Scientific) and harvested at the indicated time points.

Flow cytometry
At the experimental endpoint, tumors were removed and cut into 2 to 3 mm pieces, then transferred into a gentle MACS C tube (Miltenyi Biotec). Enzymes from Murine Tumor Dissociation Kit (catalog no. 130–996–730, Miltenyi Biotec) were added and processed through a gentleMACS Dissociator (130–093–235). Single-cell suspensions from tumors or tumor-draining lymph nodes (TDLNs) were obtained by passing dissociated tumors through 70-μm nylon cell strainers (Thermo Fisher Scientific). Cells were washed with flow running buffer (0.1% BSA in PBS, Thermo Scientific) and incubated with anti-CD16/332 (Fc receptor blocker, 1:200, BD) at 4°C for 10 minutes.

For cell surface staining, cells were stained with following antibodies (Supplementary Table S1): anti-CD45 conjugated to BV605, anti-CD3 conjugated to APC-Cy7, anti-CD8ε conjugated to BV395, anti-CD4 conjugated to BV786, anti-LAG3 conjugated to BV421, anti-CD28 conjugated to A647, anti-FD-1 conjugated to BV711, anti-Tim3 conjugated to PE-Cy7, anti-Fasl conjugated to PE-Cy7, and anti–β2-adrenergic receptor conjugated to FITC. Live/Dead Fixable violet or aqua (Thermo Fisher Scientific) were used to gate out dead cells. For intracellular staining, cells were permeabilized using the FoxP3/Transcription Factor Staining Buffer Set (catalog no. 00–5523–00, Thermo Fisher Scientific) following the manufacturer’s protocol. Cells were then stained with anti-IFNy conjugated to BV421, anti-TNFα conjugated to PerCP-Cy5.5, anti-IL2 conjugated to BV711, and anti-TCF1 conjugated to PE (Supplementary Table S1). For staining with mitochondrial dye, cells were first stained with cell surface markers and Live/Dead Fixable dyes, then incubated with 30 mmol/L MitoTracker Green FM (mitochondrial mass; catalog no. M7514, Thermo Fisher Scientific) in RPMI1640 at 37°C for 30 minutes. For in vitro studies, CD8⁺ T cells (see “CD8⁺ T-cell isolation and culture”) were harvested at either 4 hours or 24 hours after activation and washed twice with flow running buffer. Cells were stained with cell surface markers anti-CD69 conjugated to PerCP-Cy5.5 and Live/Dead Fixable dyes. GLUT-1 (conjugated to A647) intracellular staining was performed following the protocol described above (Supplementary Table S1). All flow data were collected using an LSR Fortessa flow cytometry (BD Biosciences) and analyzed with FlowJo (RRID: SCR_008520) software v10.

Western blotting
CD8⁺ T cells (see “CD8⁺ T-cell isolation and culture”) were harvested at different time points (5, 10, and 20 minutes), washed with PBS twice, and stored at −80°C. Protein was extracted using lysis buffer consisting of RIPA Buffer (Pierce, catalog no. 89900), protease and phosphatase inhibitor mini tablets (Pierce, A32961), and 0.1 mol/L phenylmethylsulfonylfluoride (Thermo Fisher Scientific). Protein concentration was determined using the BCA Protein Assay Kit (Pierce). Protein resolution was achieved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore), and blocked with 5% nonfat milk or 5% BSA (Thermo Fisher Scientific) in Tris-buffered saline (Bio-Rad) with 0.1% Tween 20 (Bio-Rad). Mem-
temperatures (Supplementary Fig. S1A and S1B). Our previous study in the 4T1 murine breast cancer model shows that the effect of chronic adrenergic stress is through β2-AR signaling and that CD8\(^{+}\) T cells are the major cell type involved in antitumor immunity (15). We next compared tumor growth rates in wild-type (WT) mice and β2-AR\(^{-/-}\) mice, both housed under standard housing temperature (ST). We observed that in both tumor models, tumor growth was slower in β2-AR\(^{-/-}\) mice than in WT mice (Fig. 1A and B), confirming the role for host β2-AR in mediating the tumor-enhancing effect of chronic stress. We examined expression of the β2-AR on CD8\(^{+}\) T cells from TDLNs and the TME of untreated mice housed at ST by flow cytometry (gating strategy, Supplementary Fig. S1C). Tumor-infiltrating CD8\(^{+}\) T cells had higher expression of β2-AR than CD8\(^{+}\) T cells from the TDLNs (Fig. 1C-F). A higher frequency of β2-AR\(^{-}\)CD4\(^{+}\) T cells in the TME compared with the TDLNs was also found (Supplementary Fig. S1D and S1E). These data together showed that reducing β2-AR signaling in the host decreases tumor growth rate and that a higher frequency of TILs express β2-AR than T cells from the TDLNs, suggesting the possibility that TILs could be more responsive to adrenergic stress–induced signals than those in other normal tissues.

β2-AR blockade in stressed mice reduces checkpoint receptors and increases CD28 on TILs

Next, we used a pharmacologic method of blocking β2-AR signaling and compared stressed mice with those treated with the pan-β2-AR antagonist propranolol to further assess the impact of adrenergic stress signaling on T cells in the TME. Consistent with previous findings, tumor growth was slowed (Fig. 2A and B). Previous work from our lab shows that blockade of β2-AR signaling improves the efficacy of immune checkpoint inhibitors (15). T-cell resistance to immune checkpoint blockade occurs by upregulation of multiple alternative immune checkpoint molecules, a state characteristic of T-cell exhaustion (25-28). We sought to determine whether the expression of molecular markers of dysfunctional, exhausted T cells was influenced by chronic stress. In the B16 model, blocking β2-AR signaling in stressed mice associated with a decreased expression of PD-1 (15), LAG3, and Tim3 on CD8\(^{+}\) T cells (Fig. 2C and D). The frequency of triple-positive PD-1\(^{+}\)Tim3\(^{+}\)LAG3\(^{+}\) CD8\(^{+}\) T cells was also decreased by β2-AR blockade (Fig. 2E and F). This data demonstrates that blockade of β2-AR signaling counteracts the influence of chronic stress on expression of exhaustion markers on CD8\(^{+}\) TILs. This result was confirmed by comparing CD8\(^{+}\) TILs from WT and β2-AR\(^{-/-}\) mice, where we found fewer exhausted CD8\(^{+}\) T cells in the TME of β2-AR\(^{-/-}\) mice (Supplementary Fig. S2A–S2C). CD4\(^{+}\) TILs showed a similar phenotypic change when β2-AR signaling was blocked using propranolol or in β2-AR\(^{-/-}\) mice (Supplementary Fig. S2D and S2E). The reduction in frequency of exhausted T cells in the TME when β2-AR signaling was blocked in mice housed at ST was also observed in CD8\(^{+}\) T cells within CT26 tumors (Supplementary Fig. S2F–S2H). Studies show that progenitor exhausted cells retain polyfunctionality and are better able to control tumor growth than terminally exhausted T cells in response to anti–PD-1 therapy (22).

We used the transcription factor, T-cell factor 1 (TCF1), to distinguish progenitor and terminally exhausted T cells (gating strategy, Supplementary Fig. S3A). Blocking β2-AR signaling increased the frequency of progenitor exhausted T cells and decreased terminally exhausted T cells in the two tumor models (Fig. 2G; Supplementary Fig. S3B). We also performed a kinetic analysis of exhausted T cells in the B16-OVA model and found that blocking β2-AR signaling increased progenitor exhausted T cells at three different time points, even when tumors were small (Fig. 2H). Because we observed the heterogeneity of β2-AR expression on TILs, we next compared the frequency of progenitor and terminally exhausted T cells in β2-AR\(^{-}\) and β2-AR\(^{-/-}\) TILs. A high frequency of terminally exhausted T cells in β2-AR\(^{-/-}\) TILs was seen (Supplementary Fig. S3C), again indicating that adrenergic receptor signaling is associated with the exhaustion status of TILs. In these experiments, we initiated the blockade of β2-AR signaling before tumor implantation in our tumor models. Therefore, we also analyzed the phenotype of exhausted T cells when β2-AR signaling blockade began after tumor establishment, which is more clinically relevant. Although no significant difference in tumor growth control (Supplementary Fig. S3D) or frequency of progenitor exhausted T cells (Supplementary Fig. S3E) was seen, the absolute number of progenitor exhausted T cells/mg of tumor was increased by β2-AR signaling blockade (Fig. 2I). These data together suggest that β2-AR signaling blockade decreases exhausted T cells in the TME, however, an increase in progenitor exhausted T cells is also induced by blockade of β2-AR signaling and could be the reason that β2-AR signaling blockade increases anti–PD-1 therapy efficacy.

Next, we compared the gene expression of other inhibitory molecules in CD8\(^{+}\) TILs from PBS-treated (stressed) and propranolol-treated B16-OVA tumor–bearing mice by NanoString analysis. Blockade of β2-AR signaling resulted in a decrease in expression of checkpoint genes (Btha, Cd274, Tigit, Ceacam1, and CD160) and T-cell activation and function suppression genes (Lair, Klrav7, and Pteger4) in CD8\(^{+}\) TILs in the propranolol-treated compared with PBS-treated tumor–bearing mice (Fig. 3A), which correlates with the flow cytometry data showing reduced expression of other checkpoint markers when adrenergic signaling was blocked. Together, these data suggest that β2-AR signaling increases expression of multiple checkpoint inhibitory molecules in CD8\(^{+}\) TILs and promotes a T-cell–exhausted phenotype in the TME.

It has been reported that CD28 expression is required for reversing CD8\(^{+}\) T-cell exhaustion by anti–PD-1 therapy (29, 30). Therefore, we quantified the expression of CD28 on TILs from stressed mice treated with either PBS or propranolol and found that in both melanoma (Fig. 3B and C) and colon cancer (Supplementary Fig. S4A) models, blockade of β2-AR signaling increased CD28 expression on CD8\(^{+}\) TILs. Similar results were also observed in CD4\(^{+}\) TILs in melanoma (Fig. 3D) and colon cancer (Supplementary Fig. S4B) models. Consistent with our findings that the costimulatory molecule CD28 was upregulated, gene expression data showed that other costimulatory molecules (Cd226, Icos, and Tnfrf9) were also upregulated in CD8\(^{+}\) T cells when β2-AR signaling was blocked (Fig. 3A).

β2-AR blockade in stressed mice increases antitumor cytokines and proteins in TILs

Thus far, we have shown that blockade of β2-AR signaling in stressed mice decreased the percentage of exhausted TILs. Next, we sought to determine whether blockade of β2-AR signaling could improve effector functions of TILs. We isolated CD8\(^{+}\) TILs from PBS- or propranolol-treated B16 melanoma tumor–bearing mice (housed at ST) and analyzed selected immune function–related gene expression by NanoString. Expression of several cytokine genes was significantly altered by blocking β2-AR signaling, with an increased expression of effector molecules Ifng, Gzem, and Il12a and a decreased expression of proinflammatory cytokines Il1b, Il4, Il6, and Il10 (Fig. 4A). The cytokine expression by TILs from B16 tumors was also measured by flow cytometry and showed that the frequencies of IFNγ\(^^{+}\), TNFα\(^^{+}\), and double-positive IFNγ\(^^{+}\)TNFα\(^{+}\) CD8\(^{+}\) T cells were enhanced by
blocking β-AR signaling. IL2 expression by CD8+ TILs were also elevated in the propranolol group (Fig. 4B–E). In the CT26.CL25 model, the frequencies of IFNγ+ (Supplementary Fig. S5A) and FasL+ CD8+ T cells (Supplementary Fig. S5B) were also increased in the TME. CD4+ TILs also showed a similar alteration of effector phenotype in the melanoma (Fig. 4F and G) and colon cancer models (Supplementary Fig. S5C–S5E). These data together suggest that blocking β-AR signaling in stressed mice increases the expression of antitumor cytokines and cytotoxic proteins in TILs.

**β-AR blockade in stressed mice alleviates mitochondrial dysfunction in CD8+ TILs**

The data indicate that reducing stress signaling by blocking β-AR signaling in stressed mice led to a more activated and less exhausted phenotype of TILs. T-cell exhaustion is associated with mitochondrial dysfunction (31–33, 34–36), and our previous work shows that β-AR signaling impairs mitochondrial function (i.e., impairs upregulation of glycolysis and oxidative phosphorylation) during activation *in vitro* (18). Therefore, we next asked whether β-AR signaling also
Figure 2.
Blockade of adrenergic signaling in stressed mice reduces immune checkpoint receptor expression on TILs. A and B, $2 \times 10^5$ B16-OVA (A) or CT26.CL25 (B) cells were injected into C57BL/6 mice or Balb/c mice, respectively. Mice were treated with either PBS or propranolol (Prop; 200 mg) daily starting 5 days before tumor implantation until the end of experiment, and tumor growth was monitored every 2 to 3 days. n = 5–7/group; data are presented as mean ± SD; one of two independent experiments. Tumor growth was compared using two-way ANOVA with Tukey analysis. C–I, Single-cell suspensions were made from B16-OVA tumors of mice treated with PBS or Prop, and TILs were analyzed by flow cytometry. C and D, Frequency and mean fluorescence intensity (MFI) of Tim3$^+$ (C) and LAG3$^+$ (D) CD8$^+$ TILs. E, Representative flow plots of PD1$^+$ Tim3$^+$ LAG3$^+$ CD8$^+$ TILs. F, Frequency of PD1$^+$ Tim3$^+$ LAG3$^+$ CD8$^+$ TILs; n = 5–6/group; data are presented as mean ± SD; one of two independent experiments. G, Frequency of progenitor (gated by CD8$^+$ PD-1$^+$ CD44$^+$ TCF1$^+$ Tim3$^+$) and terminally exhausted CD8$^+$ TILs (gated by CD8$^+$PD-1$^+$CD44$^+$TCF1$^+$Tim3$^+$) from B16-OVA tumors. H, Frequency of progenitor exhausted CD8$^+$ TILs from B16-OVA tumors at days 11, 15, and 19 after tumor implantation. I, Absolute number of progenitor exhausted CD8$^+$ TILs from mice treated with PBS or Prop 7 days after tumor implantation. n = 4–6/group; data are presented as mean ± SD. Data were analyzed using unpaired Student t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
impaired metabolism of CD8\(^+\) T cells in vivo. First, we assessed mitochondrial mass by staining TILs with MitoTracer Green FM. Consistent with a previous report (35), CD4\(^+\) and CD8\(^+\) T cells from the TME had lower mitochondrial mass than those from the TDLNs (Supplementary Fig. S6A and S6B; ref. 35). However, the mitochondrial mass of CD8\(^+\) TILs from propranolol-treated mice was increased compared with CD8\(^+\) TILs from PBS-treated stressed mice, indicating that blocking \(\beta\)-AR signaling in stressed mice can partially rescue the mitochondrial loss in CD8\(^+\) TILs (Fig. 5A and B). This phenotype was also observed in the CT26 model (Supplementary Fig. S6C and S6D). In contrast, propranolol treatment of mice did not increase mitochondrial mass in CD8\(^+\) T cells from TDLNs, which expressed only low levels of \(\beta_2\)-AR (Supplementary Fig. S6E). The increase in mitochondrial mass with \(\beta\)-AR blockade was also seen in CD4\(^+\) TILs (Fig. 5C; Supplementary Fig. S6F). Because loss of mitochondrial mass is associated with mitochondrial dysfunction (37), we next isolated CD8\(^+\) T cells from spleens, TDLNs, or tumors (Fig. 5D; Supplementary Fig. S7A and S7B) of PBS-treated and propranolol-treated stressed mice. We used the Seahorse Extracellular Flux Analyzer to assess oxidative phosphorylation (oxygen consumption rate, OCR). There was no significant difference in baseline OCR or spare respiratory capacity of CD8\(^+\) T cells from spleens or TDLNs (Supplementary Fig. S7A and S7B), which was consistent with the similar mitochondrial mass present in these cells. However, we did see a significant increase in baseline OCR (Fig. 5E), maximum OCR (Fig. 5F), and spare respiratory capacity (Fig. 5G) of CD8\(^+\) TILs from propranolol-treated tumor-bearing mice (Fig. 5D). This phenotype was also found in the CT26 model (Supplementary Fig. S7C–S7F). Together, these data suggest that blocking \(\beta\)-AR signaling in mice experiencing chronic stress results in increased mitochondrial mass and improved metabolic function of TILs, which may lead to an increased functional phenotype.

Figure 3.
Blockade of adrenergic signaling in stressed mice reduces immune checkpoint receptor expression and increases CD28 expression on TILs. Single-cell suspensions were made from B16-OVA tumors harvested from mice treated with PBS or propranolol (Prop). A, Single-cell suspensions of B16-OVA tumors from PBS- or Prop-treated mice (CD8\(^+\) T cells were pooled from 5 mice/group) were prepared, and tumor-infiltrating CD8\(^+\) T cells were sorted and lysed, and RNA was extracted for NanoString nCounter microarray analysis. Graph shows costimulatory and inhibitor molecules by log2 (fold change of PBS/Prop). Blue shows high in the PBS group; green shows high in the Prop group. B, Representative flow plots of CD28 expression on CD8\(^+\) T cells from B16-OVA tumors. C, Frequency and mean fluorescence intensity (MFI) of CD28 expression on CD8\(^+\) T cells from B16-OVA tumors. n = 5/group. B–D, Data are presented as mean ± SD; one of two independent experiments; data were analyzed using unpaired Student t test. ***, P < 0.001; ****, P < 0.0001.
Blocking β-AR signaling in stressed mice increases glycolysis in CD8⁺ T cells in the TME

TCR-induced activation drives T-cell metabolic reprogramming to meet the cellular energetic and biosynthetic demands of activation, differentiation, and effector function. In the TME, the low-nutrient and high-acidity conditions induce metabolic competition between immune cells and tumor cells. In the TME, impaired glycolytic function of CD8⁺ T cells associates with low IFNγ expression (38), and the insufficiency of glycolytic metabolites leads to defects in TCR-induced Ca²⁺ and activation (39). β-AR signaling reduces glucose transporter-1 (GLUT1) expression on CD8⁺ TILs and decreases glycolysis in CD8⁺ T cells during activation in vitro (18). Therefore, we speculated that the glycolysis in CD8⁺ TILs was also impaired by β-adrenergic signaling. To test this idea, we isolated CD8⁺ T cells from spleens, TDLNs, and tumors from stressed mice treated with PBS or propranolol and used the Seahorse Extracellular Flux Analyzer to analyze glycolysis via measuring the extracellular acidification rate (ECAR; Fig. 6A; Supplementary Fig. S7G and S7H). Because CD8⁺ T cells from spleens or TDLNs have a very low level of glycolysis, no significant difference in ECAR of CD8⁺ T cells from spleens or TDLNs was observed between the two groups (Supplementary Fig. S7C and S7D). However, we observed a significant increase in baseline ECAR (Fig. 6B) and maximal ECAR (Fig. 6C) of CD8⁺ T cells from B16-OVA tumors. B and G, Data are presented as mean ± SD; one of two independent experiments; data were analyzed using unpaired Student t test. *, P < 0.05.
function (40), and glycolysis during early activation of CD8\(^+\) T cells in vitro is inhibited by the \(\beta\)-AR agonist isoproterenol (18). Therefore, we speculated that signaling through the \(\beta\)-AR may impair early TCR signaling. Here, we tested this by isolating CD8\(^+\) T cells from naïve mice and activating them ex vivo in the presence of isoproterenol (\(\beta\)-AR agonist), added either at the beginning of activation or 2 hours after activation. Treatment with isoproterenol during activation led to impaired T-cell activation (CD69 expression) and GLUT-1 expression compared with controls, but this suppressive effect was lost when isoproterenol was added 2 hours after activation (Fig. 7A and B). This indicates that \(\beta\)-AR signaling inhibits CD8\(^+\) T-cell activation at an early stage, likely via the TCR signaling pathway. We collected cells from these two groups at different time points after activation (5, 10, and 20 minutes) and used Western blots to assess the phosphorylation of ZAP-70, the kinase immediately downstream of TCR engagement. We showed that by activating \(\beta\)-AR signaling, the phosphorylation of ZAP-70 was significantly reduced, indicating an impairment of TCR signaling (Fig. 7C).

Figure 5.
Blocking \(\beta\)-AR signaling in stressed mice alleviates mitochondrial dysfunction of CD8\(^+\) T cells in the TME. Single-cell suspensions were made from B16-OVA tumors harvested from mice treated with PBS or propranolol (Prop), CD8\(^+\) T cells (pooled from 5 mice/group) were isolated, and oxygen consumption rate (OCR) was measured in 1 x 10\(^5\) CD8\(^+\) T cells using the Seahorse Extracellular Flux Analyzer. A, Representative flow plots of mitochondrial mass of CD8\(^+\) TILs from B16-OVA tumors. B and C, Frequency and mean fluorescence intensity (MFI) of mitochondrial mass of CD8\(^+\) (B) and CD4\(^+\) (C) TILs. D, OCR of CD8\(^+\) T cells from B16-OVA tumors. Addition of reagents indicated by arrows: (1) oligomycin, (2) FCCP, and (3) rotenone and antimycin A (R&A). E, Basal OCR of CD8\(^+\) TILs. F, Maximum OCR of CD8\(^+\) TILs. G, Spare respiratory capacity of CD8\(^+\) TILs. n = 3–5. Data are presented as mean ± SD; one of two independent experiments. Data were analyzed using unpaired Student t test. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\).
In these cells, GFP expression correlates with the strength of TCR signaling. By activating CD8\(^+\) T cells isolated from Nurr77-GFP mice in vitro, with or without isoproterenol, we again observed that adrenergic signaling impaired TCR signaling (Fig. 7D). In the B16 melanoma model, Nurr77-GFP reporter mice treated with \(\beta\)-AR blockade had increased TCR signaling in CD8\(^+\) TILs in vivo (Fig. 7E). Together, these data demonstrate that \(\beta\)-AR signaling inhibits metabolic reprogramming of CD8\(^+\) T-cell activation through impairment of TCR signaling.

**Discussion**

The equilibrium established among immune cells within tumors is known to be sensitive to physiologic factors, such as hypoxia (41) or nutrient availability, that influence metabolic changes in T cells (38, 42) and responses to immune checkpoint therapies (43). Tumors are sites that induce neurogenesis and serve as targets of stress-driven signals via these nerves (4, 44, 45). Thus, a stressful TME drives the recruitment of immune effector cells (38, 42) and responses to immune checkpoint therapies (43). Hence, we hypothesize that \(\beta\)-AR signaling is a target of anti–PD-1 therapy.

In two independent experiments. Data were analyzed using unpaired Student t test. 

![Figure 6](image_url)

**Figure 6.** Blocking \(\beta\)-AR signaling in stressed mice increases glycolysis in CD8\(^+\) T cells in the TME. Single-cell suspensions were made from B16-OVA tumors from mice treated with PBS or propranolol (Prop). CD8\(^+\) T cells (pooled from 5 mice/group) were isolated, and ECAR was measured after adding 1 \(\times\) 10\(^5\) CD8\(^+\) T cells to a Seahorse Extracellular Flux Analyzer. A, ECAR results from CD8\(^+\) TILs from B16-OVA tumors. Addition of reagents indicated by arrows: (1) glucose, (2) oligomycin, and (3) 2-DG. B, Basal ECAR of CD8\(^+\) TILs from B16-OVA tumors. C, Maximum ECAR of CD8\(^+\) TILs from B16-OVA tumors. \(n = 3\)–5. Data are presented as mean ± SD; one of two independent experiments. Data were analyzed using unpaired Student t test. ****, \(P < 0.0001\)

Our data suggests that CD28 expression on TILs is also increased by blockade of \(\beta\)-AR signaling in both tumor models. Several studies show that the CD28 signaling pathway is the target of PD-1–recruited Shp2 phosphatase and is required in reversing CD8\(^+\) T cells exhaustion by anti–PD-1 therapy (33, 47). However, it is still not clear whether there is a relationship between reducing expression of exhaustion markers and increasing expression of CD28 on TILs by blocking \(\beta\)-AR signaling. It is also not known how CD28 is regulated by \(\beta\)-AR signaling and...
β-AR signaling impairs TCR signaling. A and B, CD8⁺ T cells from BALB/c mice were isolated and purified from lymph nodes and spleens of nontumor–bearing mice, activated with anti-CD3/CD28 antibodies, and treated with or without isoproterenol (ISO; 10 μmol/L). CD69 (A) and GLUT-1 (B) were measured by flow cytometry 24 hours after activation.

C, CD8⁺ T cells activated and treated with or without ISO were collected and lysed. Total ZAP-70, phosphorylated ZAP-70, and GAPDH protein expression were assessed by Western blot analysis at the indicated times.

D, CD8⁺ T cells from Nur77-GFP reporter mice were isolated and purified from lymph nodes and spleens of nontumor–bearing mice and activated with anti-CD3/CD28 antibodies with or without ISO. Nur77, represented by GFP, was measured by flow cytometry 4 hours after activation. MFI, mean fluorescence intensity.

E, 2 x 10⁵ B16-OVA cells were injected into C57BL/6 mice. Mice were treated daily with either PBS or propranolol (Prop; 200 μg) starting 5 days before tumor implantation and continuing through the end of experiment. Single-cell suspensions were made from B16-OVA tumors. Nur77 was represented by GFP and measured by flow cytometry. n = 3/group; data are presented as mean ± SD; one of two independent experiments. Data were analyzed using Student t test. n.s., not significant; *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
whether the increase of CD28 expression leads to an increase in CD28 signaling. The role of stress in the biology of CD28 expression is, therefore, a major topic that must be addressed in future studies. We previously showed (18) that β-adrenergic signaling inhibits metabolic reprogramming in CD8+ T cells during activation in vitro. It is known that CD28 regulates T-cell metabolism; however, we still cannot conclude that β-AR inhibits metabolic reprogramming in a CD28-dependent manner in vivo. This needs to be studied in greater detail in future work.

In this study, we observed that propranolol blockade in mice experiencing chronic adrenergic stress increases both glycolysis and oxidative phosphorylation in CD8+ TILs. Previous studies exploring T-cell metabolism have revealed that the functional status of T cells associates with metabolic reprogramming, with naïve and memory T cells primarily undergoing oxidative phosphorylation and effector T cells increasing glycolysis (42). Our previous work shows that increasing β-AR signaling during CD8+ T-cell activation in vitro (using the agonist isoproterenol) impairs metabolic reprogramming, and together these data suggest that β-AR signaling is a significant factor regulating the metabolic status of CD8+ T cells in the TME. Exhausted T cells undergo metabolic insufficiency, with decreased effector function and poor responsiveness to immunotherapies. It has been shown that that PD-1 ligation decreases T-cell glycolysis and promotes lipolysis and fatty acid oxidation (48). The coexpression of immune checkpoint receptors has also been associated with mitochondrial dysfunction (35), and loss of mitochondrial function can result in increased production of reactive oxygen species (ROS), which promotes T-cell exhaustion (36). Therefore, targeting stress may be a good strategy to reverse T-cell exhaustion and improve immunotherapy. However, the specific relationships between T-cell exhaustion, T-cell metabolism, and β-AR signaling remain to be clarified. More study is needed on the impact of stress on altered T-cell metabolism during tumor development, and how this might contribute to suppressed antitumor immunity.

The new data presented here show that β-AR signaling suppresses TCR signaling during CD8+ T-cell activation. By using T cells from Nur77-GFP transgenic mice, in which the expression of Nur77 (downstream of TCR signaling) is indicated by GFP fluorescence, we observed that blocking β-AR signaling increased the expression of Nur77-GFP in CD8+ T cells from the TME, with no significant difference in frequency or relative number of antigen-specific CD8+ TILs (15), which indicates that this inhibition of TCR signaling by β-AR signaling is also involved in regulation of antitumor immunity. Studies have shown that β-AR signaling suppresses CD8+ T-cell cytokine production and cytolytic function in response to TCR activation (9). We speculate that the suppressive effect of β-AR signaling on TCR signaling occurs indirectly through GLUT-1 downregulation. It has been shown that GLUT-1 expression, and the subsequent metabolic increase in glycolysis, play a key role in T-cell activation and function. Studies show that suppression of GLUT-1 and glucose metabolism (by decreased Akt/mTORC1 signaling) drives T-cell impairment (49) and exhaustion (50). We previously published that β2-AR signaling decreases GLUT-1 expression by T cells in vitro and in vivo (18). Therefore, we propose that the β-AR signaling–induced decrease in GLUT-1 expression inhibits TCR signaling and drives T-cell exhaustion, but this must be studied in more detail. It is also important to investigate whether the same phenotype change can be observed in humans, which could explain, in part, why some patients do not respond well to immunotherapy.

The specific effect of adrenergic signaling on CD8+ T cells in tumor models needs to be clarified more completely by generating CD8+ T-cell–specific β2-AR knockout mice to further investigate the precise mechanisms of how β2-AR signaling affects CD8+ T-cell phenotype and metabolism. Although here we demonstrated that adrenergic signaling in CD8+ T cells increases exhaustion and prevents metabolic reprogramming and acquisition of effector function, our previous studies have shown that adrenergic signaling also enhances the suppressive function of MDSCs (14). How these effects, and those on other immune cells such as DCs and CD4+ T cells are integrated to regulate the antitumor immune response needs to be addressed in future studies.

These findings add to a growing appreciation of the importance of nervous stimulation and chronic stress in shaping tumor-immune contexture. Given the wide effects of adrenergic signaling on immune cells, especially suppression on T cells, we have previously suggested that the β-AR could be another immune checkpoint that can be targeted for improving antitumor immune responses (45). In this regard, β-AR antagonists, including propranolol, could be repurposed as a checkpoint inhibitor for immunotherapy. A retrospective study reveals that β-blocker usage for nononcologic purposes, which included β2 but not specific β1-blockers, can significantly improve the outcome of immunotherapy in patients with melanoma (51). A phase II biomarker clinical trial shows that when patients with breast cancer are given propranolol 7 days prior to surgery, prometastatic, and proinflammatory gene expression are downregulated, with evidence of increased tumor infiltration of CD68+ macrophages and CD8+ T cells (52). Our group has completed a phase I trial prospectively combining propranolol with pembrolizumab (anti–PD-1) and show encouraging responses in patients with advanced melanoma (53). The benefit seen in these epidemiologic and therapeutic evaluations, combined with the mechanistic data provided here, provide a compelling rationale for combining immunotherapies with stress-reducing strategies or use of pharmacologic β-AR blockade.

**Authors’ Disclosures**

No disclosures were reported.

**Authors’ Contributions**

G. Qiao: Formal analysis, validation, investigation, methodology, writing–original draft, writing–review and editing. M. Chen: Investigation, methodology, writing–review and editing. H. Mohammadpour: Investigation, methodology, writing–review and editing. C.R. MacDonald: Investigation, methodology, writing–review and editing. M.J. Bucek: Investigation, methodology, writing–review and editing. B.L. Hylander: Formal analysis, supervision, investigation, methodology, writing–original draft, writing–review and editing. J.J. Barbik: Resources, methodology, writing–review and editing. E.A. Repasky: Conceptualization, resources, data curation, software, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.

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Chronic Adrenergic Stress Contributes to Metabolic Dysfunction and an Exhausted Phenotype in T Cells in the Tumor Microenvironment

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