β2 adrenergic receptor–mediated signaling regulates the
immunosuppressive potential of myeloid-derived suppressor
cells

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Catecholamines released by sympathetic nerves can activate adrenergic receptors present on nearly every cell type, including myeloid-derived suppressor cells (MDSCs). Using in vitro systems, murine tumor models in wild-type and genetically modified (β2-AR<sup>−/−</sup>) mice, and adoptive transfer approaches, we found that the degree of β2-AR signaling significantly influences MDSC frequency and survival in tumors and other tissues. It also modulates their expression of immunosuppressive molecules such as arginase-I and PD-L1 and alters their ability to suppress the proliferation of T cells. The regulatory functions of β2-AR signaling in MDSCs were also found to be dependent upon STAT3 phosphorylation. Moreover, we observed that the β2-AR–mediated increase in MDSC survival is dependent upon Fas-FasL interactions, and this is consistent with gene expression analyses, which reveal a greater expression of apoptosis-related genes in β2-AR<sup>−/−</sup> MDSCs. Our data reveal the potential of β2-AR signaling to increase the generation of MDSCs from both murine and human peripheral blood cells and that the immunosuppressive function of MDSCs can be mitigated by treatment with β-AR antagonists, or enhanced by β-AR agonists. This strongly supports the possibility that reducing stress-induced activation of β2-ARs could help to overcome immune suppression and enhance the efficacy of immunotherapy and other cancer therapies.

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Introduction

A major hallmark of cancer cells is their ability to avoid immune detection and destruction. One mechanism of tumor immune escape is through the accumulation of several immune cell populations, including myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and regulatory T cells (Tregs), which exhibit potent immune suppressive activities (1–4). MDSCs are immature myeloid cells that share some characteristics with monocytes and neutrophils but have distinct functional differences. These include their ability to release soluble immunosuppressive factors and promote angiogenesis and metastasis (5). There are 2 distinct subsets of MDSCs: monocytic MDSCs (M-MDSCs; CD11b+Ly6G−Ly6Chi) and granulocytic or polymorphonuclear (PMN) MDSCs (PMN-MDSCs; CD11b+Ly6G+Ly6Clo), which differ somewhat in their ability to suppress immune responses (6–9). Although the protumor, immunosuppressive potential of MDSCs is well-recognized, the mechanisms through which they acquire their inhibitory functions, especially under physiological conditions, remain incompletely understood.

Several studies in mice and humans reinforce the growing recognition of a negative role for various forms of chronic stress and their activation of the sympathetic nervous system (SNS) stress response in cancer progression, metastasis, and drug resistance (10–15). Nerve fibers present in and around most tissues and organs, as well as tumors (13, 16, 17), release neurotransmitters and other neuropeptides locally and systemically. The release of catecholamines (norepinephrine [NE] and epinephrine) by ubiquitously distributed sympathetic nerves, and by some special cells such as tyrosine hydroxylase–positive cells in the spleen, can directly stimulate cells bearing adrenergic receptors (ARs) (18). ARs belong to the guanine nucleotide–binding G protein–coupled receptor (GPCR) superfamily (19). Two classes of ARs have been identified: α-ARs and β-ARs. The α1-AR is primarily expressed on endothelial cells of blood vessels, whereas the α2-AR is more ubiquitously expressed. β-ARs comprise 3 receptors, including β1, β2, and β3. β1 and β3 receptors are primarily expressed in heart and adipose tissues respectively, whereas β2-AR is expressed by most cells, including immune cells (20–22).

Many effects of adrenergic signaling in immune cells have been reported in previous studies. For example, β2-AR activation in T cells was seen to suppress their ability to secrete interferon-γ (IFN-γ) in response to infection with vesicular stomatitis virus (23) and impair metabolic reprogramming during T cell activation (11). High levels of NE also impair dendritic cell (DC) maturation (24, 25) and increase MDSC recruitment into the tumor microenvironment (TME) (26). Murine studies from our lab showed that chronic β2-AR signaling suppresses antitumor CD8+ T cell function and increases populations of MDSCs and Tregs in the spleen and tumor microenvironment, respectively (27, 28). However, the role of β2-AR in major aspects of MDSC functions associated with suppressing the

Catecholamines released by sympathetic nerves can activate adrenergic receptors present on nearly every cell type, including myeloid-derived suppressor cells (MDSCs). Using in vitro systems, murine tumor models in wild-type and genetically modified (β2-AR−/−) mice, and adoptive transfer approaches, we found that the degree of β2-AR signaling significantly influences MDSC frequency and survival in tumors and other tissues. It also modulates their expression of immunosuppressive molecules such as arginase-1 and PD-L1 and alters their ability to suppress the proliferation of T cells. The regulatory functions of β2-AR signaling in MDSCs were also found to be dependent upon STAT3 phosphorylation. Moreover, we observed that the β2-AR–mediated increase in MDSC survival is dependent upon Fas–FasL interactions, and this is consistent with gene expression analyses, which reveal a greater expression of apoptosis-related genes in β2-AR−/− MDSCs. Our data reveal the potential of β2-AR signaling to increase the generation of MDSCs from both murine and human peripheral blood cells and that the immunosuppressive function of MDSCs can be mitigated by treatment with β-AR antagonists, or enhanced by β-AR agonists. This strongly supports the possibility that reducing stress-induced activation of β2-ARs could help to overcome immune suppression and enhance the efficacy of immunotherapy and other cancer therapies.
antitumor immune response, including their generation and accumulation, immune-regulatory function, and survival, have not been addressed. The fact that stress-induced catecholamines are rapidly released systemically, indicates the potential for physiological mechanisms to influence the overall balance of immune factors dictating tumor progression and highlights a critical need to understand how MDSCs are regulated by neural activity.

Here we tested whether β2-AR signaling plays a major role in dictating the immunosuppressive function of MDSCs in the TME and in other tissues including the spleen and blood. Using in vitro and in vivo strategies, including use of β2-AR-deficient mice (referred to as β2-AR−/−) and adoptive transfer models, we examined the impact of adrenergic stress signaling through β2-ARs on MDSC frequency in tumors and other tissues, whether the β2-AR expression in MDSCs is influenced by expression of cytokines including GM-CSF, and how β2-AR signaling modulates the expression of immunosuppressive molecules such as arginase-1 and PD-L1 in MDSCs. We also examined the impact of β2-AR signaling on the immune regulatory functions of MDSCs on T cells, the survival of MDSCs in tumor and peripheral tissues, and the generation of MDSCs from human and mouse models cells. Our data reveal a major impact of β2-AR signaling on the immune suppressive potential of MDSCs and suggest that reducing stress-induced activation of β2-AR could help to overcome immune suppression and enhance the efficacy of immunotherapy and other cancer therapies.

Results

Chronic stress-mediated β2 adrenergic signaling increases MDSC dependent tumor growth. Our laboratory has relied on several in vivo models (28, 29) to investigate the effects of adrenergic stress on cancer progression. Here, we sought to determine whether the immune suppressive activity of MDSCs plays a key role in driving the increased tumor growth rates we have observed in these and other models. To this end, we first set up several models to obtain material for the analyses shown in subsequent data. We used a physiological model of adrenergic stress (29) in which NE levels can be manipulated by housing mice at either the standard subthermo-neutral housing temperature (ST; -22°C), or a thermoneutral housing temperature (TT; +30°C). When housed at ST, the sympathetic nervous system is activated, and NE production is increased to drive thermogenesis (30). Conversely, thermogenesis is not needed at TT, adrenergic stress is reduced, and NE levels are decreased (12, 28). As observed in our earlier studies, (27) we found that mice housed under TT conditions showed delayed tumor growth (Figure 1A, A and B) and decreased tumor weights (Supplemental Figure 2A; supplemental material available online with this article; https://doi.org/10.1172/JCI129502DS1). Here, we also report that at TT conditions there are reduced levels of circulating protumor cytokines (Supplemental Figure 1, A and B) compared with mice housed at ST conditions. As the β2-AR is the most prominent AR expressed by immune cells (31), we compared tumor growth in BALB/c WT mice and β2-AR−/− mice. As we previously observed (28), 4T1 tumors grew at a decreased rate in β2-AR−/− mice (Figure 1C and Supplemental Figure 2B). Here, we also found decreased levels of several protumor cytokines in the plasma (Supplemental Figure 1C) and, together with the data in Supplemental Figure 1, A and B, these results suggest a role for the β2-AR pathway in regulating the overall cytokine milieu in tumor-bearing mice. Consistent with these data, we found that the lungs of β2-AR−/− mice had fewer metastatic nodules (Supplemental Figure 2C).

We next made bone marrow chimeras, using the BALB/c WT and β2-AR−/− models defined below, to test whether the impact of β2-AR signaling on tumor growth was dependent upon cells of hematological origin or stromal cells of the tumor. Lethally irradiated BALB/c WT mice and β2-AR−/− mice were reconstituted with BM cells isolated from either β2-AR−/− mice or WT controls. We found that the growth of 4T1 tumors was significantly slower in mice reconstituted with β2-AR−/− BM than in mice reconstituted with WT BM (Figure 1D), suggesting that β2-AR signaling in a cell type derived from the bone marrow plays a key role in tumor growth promotion.

In investigating which specific type(s) of hematopoietic cells are most important in this process, we focused on MDSCs, as they are a relevant population of hematopoietic cells known to be associated with immune suppression and cancer progression. To test whether β2-AR−/− deficient MDSCs lose their protumorigenic properties, we depleted MDSCs in both WT and β2-AR−/− mice using an anti-Gr-1 antibody (31). MDSC depletion significantly delayed 4T1 tumor growth in WT mice, but led only to a small, nonsignificant decrease tumor growth rate in β2-AR−/− mice (Figure 1E). These data confirm that MDSCs from WT mice promote tumor growth, while tumor growth in β2-AR−/− mice is not affected by β2-AR−/− MDSCs.

So far, we have demonstrated that the impact of adrenergic stress on tumor growth is largely dependent on MDSCs, but the precise role adrenergic signaling in MDSCs plays in altering tumor growth rates has not yet been determined. To this end, we first visualized the expression of β2-ARs on MDSCs from 4T1 tumor-bearing WT and β2-AR−/− mice via ImageStream. After confirming β2-AR expression in WT but not β2-AR−/− MDSCs (Figure 1F), we sought to further determine whether the presence of a tumor altered the level of β2-AR expression in WT MDSCs. When comparing MDSCs from the spleens of tumor-bearing mice to those that were isolated from the spleens of healthy mice, we observed a significant increase in β2-AR expression in MDSCs from the spleens of tumor-bearing mice (Figure 1I).

When considering this variability in β2-AR expression in conjunction with the observed changes in cytokine levels in earlier experiments (Supplemental Figure 1, A-C), we sought to investigate whether increased cytokine levels originating from the TME might be involved in locally increasing the expression of β2-AR in intratumoral MDSCs. To address this question, we cultured MDSCs sorted from the BM of non-tumor bearing mice with either IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), or lipopolysaccharide (LPS) as a standard activator of MDSCs. We found that GM-CSF and LPS treatments were associated with an increase in β2-AR expression, whereas treatment with IL-6 was not (Figure 1, G and H), suggesting that β2-AR expression in MDSCs is differentially responsive to various cytokines. The ability of GM-CSF, which is found at high levels in the plasma of tumor-bearing mice (32), to induce expression of β2-ARs in MDSCs correlates with our finding that a higher percentage of the splenic MDSCs from tumor-bearing mice express β2-ARs compared with those from non-tumor bearing mice (Figure 1I).}


these data demonstrate that there is a tight association between tumor-promoting cytokines, β2-AR expression on MDSCs, and MDSC-dependent tumor growth such that the whole response may be orchestrated by sympathetic nervous system activity.

**β2-AR activation during chronic stress increases MDSC accumulation and tumor vascularization.** We next tested the role of β2-AR in MDSC accumulation in the spleen, TME, and other tissues. 4T1 cells were injected into WT or β2-AR−/− mice and on day 25, MDSC accumulation in blood, lymph node, lung, spleen, and tumor was quantified by flow cytometry. We found that the percentage of CD11b+ myeloid cells within the live CD45+ cells of the TME was significantly elevated in WT mice compared with β2-AR−/− mice. These data demonstrate that there is a tight association between tumor-promoting cytokines, β2-AR expression on MDSCs, and MDSC-dependent tumor growth such that the whole response may be orchestrated by sympathetic nervous system activity.

**Figure 1. β2-AR activation increases tumor growth in a MDSC-dependent manner.** (A and B) Tumor growth in mice bearing 4T1 and AT-3 tumor cells, housed at ST (22°C) or TT (30°C). (C) Tumor growth kinetics in WT and β2-AR−/− mice bearing 4T1 tumor cells. (D) Lethally irradiated WT mice received bone marrow transplants from WT (blue circle) or β2-AR−/− (red square) mice. Lethally irradiated β2-AR−/− mice received bone marrow transplants from WT (purple triangle) or β2-AR−/− (brown triangle) mice. Eight weeks after transplantation, chimeric mice were injected with 4T1 tumor cells and tumor growth was monitored. (E) 4T1 tumor-bearing WT or β2-AR−/− mice were injected with isotype or anti–Gr-1 antibodies (200 μg per mouse, i.p., every 4 days), and tumor growth was monitored. (F) β2-AR expression in MDSCs sorted from spleen of 4T1 tumor-bearing mice 25 days after tumor injection using Image Stream. (G and H) β2-AR expression in MDSCs sorted from bone marrow of non-tumor-bearing mice after culture with IL-6, G-CSF, and LPS (data from 3 independent replicates). (I) The levels of β2-AR in splenic MDSCs from healthy or 4T1 tumor-bearing mice using flow cytometry. Two-way ANOVA was used to analyze statistical significance among tumor growth in different groups. These data are presented as mean ± SEM of 5 mice per group from at least 2 replicate experiments. Other data are presented as median ± minimum to maximum. One-way ANOVA was used to analyze statistical significance among 4 groups, and the Student’s t test was used to analyze statistical significance between 2 groups. In all panels, **P < 0.01, ***P < 0.001 and ****P < 0.0001. A P value less than 0.05 was considered significant.
Figure 2. β2-AR activation during chronic stress increases MDSC accumulation in the spleen and tumor. (A) Representative flow cytometry analysis of PMN-MDSCs and CD14+ MDSCs populations, as well as absolute number of CD14+ MDSCs in tumors and spleen of 4T1 tumor-bearing mice on day 25 after tumor injection. The data presented are from groups of 10 mice from 2 replicate studies. (B) Absolute number of G-MDSCs and M-MDSCs in tumor and spleen of healthy or tumor-bearing mice (4T1 or AT-3) at day 25 after tumor injection housed in ST or TT. The data presented are from groups of 8 mice from 2 replicate studies. (C) Both representative immunohistochemistry analysis and absolute number of Gr-1+ (×20 magnification, scale bars = 100 μm), CD31+ (×4 magnification, scale bars: 500 μm) and VEGF-α-positive (×10 magnification, scale bars: 200 μm) cells in 4T1 tumors at day 25 after tumor injection. The data are presented as median ± minimum to maximum from groups of 6 mice from 2 replicate studies. The Student’s t test was used to analyze statistical significance between 2 groups. In all panels, *P < 0.05, **P < 0.01, and ***P < 0.001. A P value less than 0.05 was considered significant.
β2-AR expression plays an important role in MDSC turnover and survival. Based on our Nanostring data from WT and β2-AR–/– MDSCs sorted from 4T1 tumor-bearing mice, we next asked whether adrenergic stress signaling affected the expression of survival factors in MDSCs themselves. Expression of proapoptotic genes such as Fas, Casp8, and Casp3 was higher in β2-AR–/– MDSCs (Figure 3C), whereas the expression of the antiapoptotic gene Bcl2 was higher in WT MDSCs (Figure 3C). It has been shown that Fas-FasL interactions play an important role in regulating MDSC populations in different tissues (34). Therefore, we hypothesized that deletion of β2-AR increased susceptibility of MDSCs to apoptosis through Fas-FasL interactions.

We quantified the expression of Fas on WT or β2-AR–/– MDSCs and FasL expression on CD8+ T cells (one of the FasL-expressing cells in the TME) in WT and β2-AR–/– 4T1 tumor-bearing mice. The data showed that β2-AR–/– MDSCs expressed Fas at a significantly higher level than WT MDSCs (Figure 4A). Interestingly, β2-AR–/– CD8+ T cells expressed more FasL compared with WT CD8+ T cells (Figure 4A), implicating a likely source of the cognate ligand for Fas engagement. Additionally, higher levels of FasL on β2-AR–/– CD8+ T cells suggest a higher degree of activation, as FasL is upregulated in response antigenic challenges. Furthermore, we observed that WT MDSCs expressed a significantly higher level of the protein B cell lymphoma 2 (BCL-2) compared with β2-AR–/– MDSCs (Figure 4B), suggesting that WT MDSCs are less sensitive to apoptosis compared with β2-AR–/– MDSCs.

To test whether the differential expression of pro- and antiapoptotic molecules by WT and β2-AR–/– MDSCs can influence the survival of MDSCs in the TME, apoptosis of WT or β2-AR–/– MDSCs in 4T1 tumor-bearing mice was investigated. At day 25 after tumor implantation, the level of apoptosis in WT and β2-AR–/– MDSCs was measured in the tumor and spleen tissues. We found that the frequency of apoptotic cells in β2-AR–/– MDSCs was significantly higher, compared with WT MDSCs (Figure 4C) in both tumor and spleen. We also observed a higher level of apoptosis in MDSCs isolated from tumor-bearing mice housed under TT conditions (reduced NE levels) compared with MDSCs isolated from tumor-bearing mice housed under ST conditions (Figure 4C).

To further investigate the importance of β2-AR in MDSC apoptosis, we took advantage of different congenic strains of mice (CD45.1 vs. CD45.2). AT-3 tumor cells, a mammary carcinoma cell line syngeneic to C57BL/6 mice, were orthotopically injected into WT (CD45.1) or β2-AR–/– (CD45.2) mice. On day 25 after tumor injection, we isolated WT (CD45.1) or β2-AR–/– (CD45.2) MDSCs from the tumor-bearing mice, mixed them 1:1, and injected them into fresh groups of AT-3 tumor-bearing GFP-positive mice (Figure 4D). We found that the percentage of WT MDSCs was significantly higher compared with β2-AR–/– MDSCs (GFP CD45.2+) in the spleen on days 3 and 7 after injection, suggesting that WT MDSCs could survive longer compared with β2-AR–/– MDSCs (Figure 4E). These data highlight that β2-AR signaling increases the survival of MDSCs in TME at least partially through the Fas-FasL pathway.

β2-AR stimulation activates STAT3 phosphorylation. STAT3 activation in myeloid cells regulates multiple aspects of MDSC biology, including their immunosuppressive function and expansion (35). We hypothesized that ligands of the β2-AR, such as NE and ISO, can activate STAT3 in MDSCs. To test this, MDSCs were isolated from tumor-bearing mice. We then treated WT or β2-AR–/– MDSCs with ISO for different periods of time. Western blot results indicate that ISO induced STAT3 phosphorylation in WT MDSCs after 20 minutes, but not in β2-AR–/– MDSCs (Figure 5A). Moreover, we investigated the in vivo level of phospho-STAT3 (p-STAT3) in MDSCs of 4T1 tumor-bearing mice. These data show that the level of p-STAT3 was significantly higher in WT MDSCs compared with β2-AR–/– MDSCs in both the tumor tissue and spleen. A similar trend was seen in MDSCs isolated from tumor-bearing mice housed at ST compared with TT (Figure 5B), consistent with the notion that physiological chronic stress increases STAT3 activation in MDSCs. To confirm the role of STAT3 activation in these MDSCs, we inhibited STAT3 phosphorylation in 4T1 tumor-bearing mice using the STAT3 inhibitor JSI-124 (36) (Figure 5C). A significant delay in tumor growth was observed in mice receiving the STAT3 inhibitor compared with mice receiving vehicle control in WT but not β2-AR–/– tumor-bearing mice, again supporting a role for β2-AR in STAT3 phosphorylation. Twenty-five days after tumor injection, tumor tissue and spleen were collected. Inhibition of p-STAT3 significantly decreased the number of MDSCs in both tumor tissue and spleen in WT tumor-bearing mice (Figure 5D). These data indicate that the mechanism by which β2-AR signaling enhances accumulation and/or survival of MDSCs occurs through STAT3 phosphorylation, which may lead to increased expression of prosurvival and immunosuppressive genes such as Bcl-2 and arginase-1, respectively, in MDSCs.

β2-AR blockade slows tumor growth and diminishes frequency of MDSCs whereas β-AR agonists accelerate tumor growth and enhance MDSC frequency in the TME. To address the question of whether β2-AR blockade, which slows tumor growth, also reduces MDSC accumulation in the TME, we investigated the effects of proprano-
Figure 4. β2-AR prolongs MDSC survival. (A) Fas and FasL expression by MDSCs and T cells from WT or β2-AR−/− mice from tumor and spleen, respectively (n = 5). (B) Expression of Bcl-2 in intratumoral MDSCs from WT or β2-AR−/− 4T1 tumor–bearing mice (n = 5). (C) Levels of apoptosis in MDSCs from tumor and spleen of WT or β2-AR−/− tumor–bearing mice or WT tumor–bearing mice housed at ST or TT. (D) Schematic diagram of experimental design to compare the survival capability of WT or β2-AR−/− MDSCs. (E) WT (CD45.1) or β2-AR−/− (CD45.2) MDSCs were sorted from bone marrow of AT-3 tumor–bearing mice, mixed in 1:1 ratio, and injected into GFP-positive AT-3 tumor–bearing mice. The percentage of WT (CD45.1) or β2-AR−/− (CD45.2) MDSCs in the live, GFP-negative, CD11b+, and Gr-1+ population on day 3 and day 7 after coinjection were analyzed (4 mice per end point). Data are presented as median ± minimum to maximum. The Student’s t test was used to analyze statistical significance between 2 groups. In all panels, *P < 0.05, **P < 0.01, and ***P < 0.001. A P value less than 0.05 was considered significant.
we tested the effects of the β2-AR–specific agonist (salbutamol) on tumor growth and MDSC accumulation. We found that salbutamol increased both tumor growth (Supplemental Figure 5A) and MDSC accumulation in the spleen (Supplemental Figure 5B) and tumor tissue (Supplemental Figure 5C) in mice housed under ST conditions. To rule out the possibility of indirect effects of propranolol on tumor growth and MDSC accumulation, we used 6-hydroxydopamine (6-OHDA) to deplete nerve-derived NE. We found that treatment of WT mice housed at ST with 6-OHDA significantly decreased MDSC accumulation in both the spleen and tumor (a pan β-AR blocker) in our murine tumor models. As we previously reported (28), propranolol significantly slows tumor growth in WT mice but not β2-AR–/– mice (Figure 6A). In addition, the numbers of MDSCs in tumor tissue and spleen of WT mice were decreased compared with WT mice receiving the vehicle control (Figure 6B). We then performed immunohistochemistry (IHC) on tumor tissue, and found a decreased number of myeloid cells and a decreased expression of angiogenic markers (CD31 and VEGF-α) in the tumors of mice treated with propranolol compared with tumors of mice from the control group (Figure 6C). Next, we tested the effects of the β2-AR–specific agonist (salbutamol) on tumor growth and MDSC accumulation. We found that salbutamol increased both tumor growth (Supplemental Figure 5A) and MDSC accumulation in the spleen (Supplemental Figure 5B) and tumor tissue (Supplemental Figure 5C) in mice housed under ST conditions. To rule out the possibility of indirect effects of propranolol on tumor growth and MDSC accumulation, we used 6-hydroxydopamine (6-OHDA) to deplete nerve-derived NE. We found that treatment of WT mice housed at ST with 6-OHDA significantly decreased MDSC accumulation in both the spleen and tumor.
tumor tissue, but it was less efficient than propranolol, suggesting that nerves are not the only source of NE (Figure 6, D and E). These results demonstrate that the protumor effects of chronic stress mediated by β2-AR signaling in MDSCs can be regulated by commonly used β-blocker drugs.

β2-AR activation increases MDSC generation from human PBMCs. We tested whether the presence of neurotransmitters released into the vasculature (which would happen under physiological conditions) could influence the generation of MDSCs in human blood. We isolated human peripheral blood mononuclear cells (PBMCs) from healthy volunteers, and cultured them to generate MDSCs in the presence or absence of ISO as described (37). We found that PBMC-derived MDSCs express β2-AR on their surface (Figure 7A) and that addition of ISO, which provides stimulation of these receptors, significantly increased the generation of MDSCs (CD14+CD33+) 7 days after culture (Figure 7B). We also found that adding ISO into MDSC culture media increased the expression of arginase-1, PD-L1, and p-STAT3, thus replicating in human cells the effects that β2-AR activation has in mouse MDSCs (Figure 7C). Then, to investigate the immunosuppressive potency of human cells derived in culture with or without ISO treatment, we isolated these MDSCs from culture and cocultured them with human CD3+ CD8+ T cells stimulated with anti-CD3 and anti-CD28 beads. ISO-treated cells suppressed proliferation and IFN-γ production of both CD4+ and CD8+ T cells at a higher level compared with that seen using cells cultured without ISO (Figure 7D). These data highlight the potential for increased chronic stress and production of catecholamines in humans to enhance the generation and immunosuppressive function of MDSCs.

Discussion

The immune response can potentially control tumor growth, but its development requires a critical balance between the functions of immune suppressive cells such as MDSCs and immune effector cells such as CD8+ T cells. Previous studies, including those from our lab, have shown that stress (including physical or psychological stress) promotes tumor growth and metastasis and suppresses CD8+ T cell–dependent antitumor immunity (29, 38–40). The data presented here reveal that adrenergic stress signaling also increases the frequency and suppressive function of MDSCs in the tumor microenvironment (TME), spleen, and blood. Using a physiological model of chronic stress (i.e., housing mice in a mild but chronically cool housing temperature), a genetic model (i.e., deletion of β2-AR), and multiple pharmacological interventions, we identified a major role for β2-AR signaling in promoting MDSC survival and protumorigenic function. Overall, the fact that β-AR signaling increases the immune suppressive functions of murine MDSCs in the TME as well as those of human MDSC-like cells generated from PBMCs, points to a mechanism by which chronic stress could tilt the immunological balance toward suppression of the antitumor immune response. Additionally, we found that β2-AR signaling in vivo drives MDSC survival through STAT3 activation. These findings that chronic adrenergic stress promotes the immunosuppressive functions of MDSCs to constrain the antitumor immune response are clinically relevant since cancer patients often report increased symptoms of chronic stress (anxiety, pain, or depression), which are also mediated through the sympathetic nervous system and adrenergic signaling (14, 41).

Mechanistically, physiological and/or psychological stressors provide the stimulus that drives activation of the SNS, leading to increased release of NE from sympathetic nerve endings found systemically and, in particular, in the TME (42). Our work suggests that this elevated adrenergic tone is responsible for observed changes in immune cell populations, and overall promotes a protumor milieu in the TME. Recent data reported by others show that tumors recruit and are innervated by SNS fibers (13, 43), thus providing a conduit for chronic stress to provide signals to the tumor microenvironment. Our data reveal that MDSCs are highly sensitive to these signals and could mediate tumor progression in response to even mild, but chronic, stress such as the thermal stress model used in this study. It is noteworthy that some of our data, while statistically significant, show a relatively modest impact of adrenergic receptor signaling (e.g., Supplemental Figure 1, A–C). It is important to remember that chronic stress is a physiological perturbation and therefore not likely to result in major immunological changes. Nevertheless, a daily suboptimal immune control of tumor progression, over long periods of time, could result in a significant increase in tumor progression and/or metastases. This point is reinforced by recent epidemiological data coming from several different cancer settings, supporting the idea that daily use of β-blockers, for indications unrelated to cancer, is associated with improved response to therapy and increased overall survival (44).

A positive correlation between chronic stress and increased numbers of MDSCs has been shown in several settings other than cancer. A recent report by McKim et al. (45) has shown that psychological stress (exposure to an aggressor mouse) increases hematopoietic stem progenitor cell (HSPC) trafficking from bone marrow to spleen, and promotes differentiation into several types of immunosuppressive cells, including MDSCs. Another report has shown that NE increases proliferation of granulocyte-macrophage progenitors (GMPs) in the spleen and that severing splenic SNS nerves diminishes GMP proliferation and MDSC development (18).

In further support of our findings, Ben-Shaanan et al. showed that positive emotions decrease NE levels in the bone marrow, diminish the generation of MDSCs, and reduce the inhibitory effects of MDSCs on T cell proliferation and effector phenotype in mouse tumor models (46). These data support our results indicating that decreasing NE-mediated β2-AR signaling in MDSCs reduces the capacity of MDSCs to suppress T cell proliferation along with reducing expression of components of major inhibitory pathways.

We also found that a higher percentage of MDSCs in the spleens of tumor-bearing mice express β2-AR compared with those of tumor-free mice, and that soluble factors (e.g., inflammatory cytokines including GM-CSF) derived from tumor cells, stromal cells, or immune cells promote β2-AR upregulation. In agreement with our results, a recent report confirmed that the expression level of β2-AR on MDSCs residing in various tissues is different (46), suggesting that the effects of chronic stress on MDSCs in those tissues could be regulated by this differential expression level of β2-AR.

The pivotal role of STAT3 in MDSC expansion and MDSC-mediated immunosuppression has been widely reported (35). STAT3 activity is also the predominant signaling molecule in TAMs
Figure 6. Propranolol suppresses tumor growth and decreases MDSC accumulation in the spleen and tumor tissue. (A) Tumor growth kinetics in WT or β2-AR−/− mice orthotopically injected with 4T1 tumor cells receiving PBS or propranolol (i.p. daily injection) (n = 10). (B) Absolute number of MDSCs in spleen and tumor of WT mice treated with PBS or propranolol. (C) Tumor tissue was collected in WT 4T1 tumor-bearing mice at day 25 and stained for Gr-1 (×20 magnification), CD31 (×4 magnification), and VEGF-α (×10 magnification) (n = 5). (D) Representative flow cytometry plot of MDSCs in WT or β2-AR−/− 4T1 tumor-bearing mice receiving saline or 6-OHDA (50 mg/kg, i.p., weekly injection) (n = 6–10 mice from 2 replicates). (E) Percentage and absolute number of MDSCs in tumor and spleen of 4T1 tumor-bearing mice receiving saline or 6-OHDA (50 mg/kg, i.p., weekly injection) (n = 5). Two-way ANOVA was used to analyze statistical significance among tumor growth in different groups. These data are presented as mean ± SEM. Other data are presented as median ± minimum to maximum, and the Student’s t test was used to analyze statistical significance between 2 groups. In all panels, *P < 0.05, **P < 0.01, and ****P < 0.0001. A P value less than 0.05 was considered significant.
Figure 7. Isoproterenol increases MDSC generation from human PBMCs. (A) Analysis of β2-AR expression on MDSC surface analyzed by flow cytometry after culturing PBMCs with IL-6 and GM-CSF with or without ISO for 7 days. (B) Analysis of MDSC generation analyzed by flow cytometry after culturing PBMCs with IL-6 and GM-CSF with or without ISO for 7 days. (C) The expression of p-STAT3, PDL-1, and arginase-1 after culturing PBMCs with IL-6 and GM-CSF with or without ISO for 7 days. (D) Effects of in vitro differentiated MDSCs in the presence or absence of ISO on allogenic CD4+ or CD8+ T cell proliferation and IFN-γ production. One histogram example corresponding to CD8+ proliferation analyzed by ef670 dilution dye in a ratio of 1:4 is shown. These data are presented as median ± minimum to maximum from 3 biological replicates in all graphs, and the Student’s t test was used to analyze statistical significance between 2 groups. In all panels, *P < 0.05, **P < 0.01, and ***P < 0.001. A P value less than 0.05 was considered significant.
of different cancers, including glioblastoma (47). It has been shown that STAT3 activation plays crucial roles in myelopoiesis, and constitutive phosphorylation of STAT3 in myeloid cells increases the accumulation of MDSCs in spleen and tumor tissues (48). Here, we showed that β2-AR activation in MDSCs activates STAT3 phosphorylation, suggesting that the importance of STAT3 activity in MDSC biology, and possibly TAM biology, is mediated at least in part by β2-AR signaling. This upregulation of STAT3 activity is a likely mechanism underlying the increased MDSC survival and changes in immune-inhibitory function that we observed.

It is well-known that the Fas and FasL interaction plays an important regulatory role in lymphocyte homeostasis (49). Apoptosis mediated by the expression of Fas receptor on MDSCs and FasL on T cells, in particular CD8+ T cells, plays a key role in MDSC survival and turnover (50), and it has been shown that this process is impaired in tumor-bearing mice, resulting in increased MDSC accumulation (34, 50, 51). Immunotherapy using either IL-12/αCD40 (52) or irradiation plus anti–PD-1 activates cytotoxic T cells and promotes MDSC apoptosis (53). In our study, we showed that β2-AR activation in MDSCs increases the resistance of MDSCs to apoptosis induced by Fas/FasL. We found that the expression of the Fas receptor and the level of apoptosis were higher in MDSCs lacking β2-ARs. We also confirmed, by in vivo competition assays, that the level of apoptosis is increased in β2-AR–deficient MDSCs. Although the precise mechanisms by which β2-ARs regulate the expression of Fas require further investigation, these data provide new evidence about the important role of β2-AR in MDSC resistance to apoptosis. In addition, these data provide insight into the potential application of β2-AR blockers in combination with various treatments like immunotherapies (including anti–PD-1) and irradiation to improve the antitumor immune response through increased susceptibility of MDSCs to cell death.

In summary, our work demonstrates that chronic stress, acting through the β2-AR, significantly promotes proliferation, suppressive function, and survival of MDSCs and therefore has the potential to significantly suppress the antitumor immune response. Inhibiting β2-AR signaling by β-AR blockade, inhibiting NE releasing nerves, or β2-AR deletion can decrease the accumulation of MDSCs, reduce their immunosuppressive functions, and is associated with the increased efficacy of the antitumor immune response and inhibition of tumor growth that we have previously seen. Understanding the mechanisms by which the expression of β2-AR can be regulated in immune cells in different organs warrants further investigation.

Our data also provide justification for further investigation of the therapeutic potential of blocking chronic stress–mediated β2-AR signaling. Interventions focusing on this strategy have the potential to significantly improve cancer treatment outcomes, with the additional benefit of having minimal toxicity compared with other cancer therapies. Although additional research on drugs that specifically block β2-ARs is needed to increase the precision of this therapy, we have shown that pharmacologic agents like propranolol, which are currently clinically available and FDA-approved, could be a potentially efficacious approach at the present time.

Methods

Animals and tumor cells. BALB/c (H-2b), C57BL/6 (H-2b, CD45.1), and C57BL/6 (H-2b, CD45.2) mice were purchased from Charles River. β2-AR knockout (β2-AR−/−) mice on a BALB/c background were of the gift of David Farrar (UT Southwestern Medical Center). β2-AR knockout mice on C57BL/6 were developed at Roswell Park. GFP-positive mice on C57BL/6 background were gifted by Michael Nemeth (Roswell Park). All mice were maintained in SPF housing, all experiments were performed in accordance with the animal care guidelines at Roswell Park Comprehensive Cancer Center, and all protocols used were approved by the animal studies committee. 6-OHDA (162957), propranolol (P0884), salbutamol (S8260), and isoproterenol (16504) were purchased from MilliporeSigma. Cucurbitacin 1 (JSI-124) was purchased from TOCRIS, R&D Systems.

Cell culture and tumor models. 4T1 tumor cells were purchased from ATCC (ATCC, catalog CRL-2539). AT-3 tumor cells were provided by Scott Abrams (Roswell Park). Cell lines were confirmed to be mycoplasma-negative yearly using the Mycoplasma Plus PCR Primer Set (Agilent Technologies, catalog 302008). 4T1 cell lines were cultured in RPMI 1640 (Corning Cellgro) supplemented with 10% FBS, 1% l-glutamine, and 1% penicillin/streptomycin. AT-3 tumor cells cultured in DMEM (Corning Cellgro) supplemented with 10% FBS, 1% l-glutamine, and 1% penicillin/streptomycin and 7% CO2. Once thawed, cells were passed twice prior to use. 4T1 cells (1 × 10^6) in 100 μL PBS and 5 × 10^5 AT-3 cells in 100 μL PBS were subcutaneously injected into the fourth mammary fat-pad of female BALB/c and C57BL/6 mice, respectively. Tumor growth was monitored in a blinded manner throughout experiments, and perpendicular diameters (width/length) were measured every 2 days. Tumor volume was calculated using the following equation: tumor volume = (2W × L)/2 mm³, where W is the small dimension and L is the large dimension.

Ambient temperature manipulation. Mice were housed 5 per cage in Precision Refrigerated Plant-Growth Incubators (ThermoFisher Scientific) and maintained at either standard temperature (~22°C) or thermoneutral temperature (~30°C) as previously described (27, 54). 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 prior to tumor injection.

Reagents and antibodies. Antibodies including anti-mouse CD3 (clone 17A2), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD45 (clone 30F11), Gr-1 (clone RB6-8C5), CD45.1 (clone A20), CD45.2 (clone 104), CD11b (clone M1/70), LD-L (clone MIH5) CD206 (clone C068C2), F4/80 (clone BM8), Ly6C (clone HK1.4), Ly6G (clone IA8), Arginase-1 (clone 1C5868F, R&D Systems), B2-2 (clone BCL/10C4), p-STAT3 (clone 13A3-1), Fas (clone 15A7), FasL (clone MFL-3), and Caspase-8 (clone FITC-IETD-FMK) were purchased from Biolegend, BD Bioscience, and ebioscience, except as noted. Antibodies including anti-human CD33 (clone P67.6), CD14 (clone M5E2), CD4 (clone A161A1), CD8 (clone RPA-T6), Arginase-1 (clone 14D2C43), p-STAT3 (clone 13A3-1), β2-AR (clone R1IE1), and PD-L1 (clone 29E2A3) were purchased from Biolegend. Mouse MDSC isolation kit was purchased from Miltenyi Biotec. EasySep Human T Cell Isolation Kit and EasySep HLA chimerism whole-blood CD33 positive selection kit were obtained from Stem Cell Technologies.

eFluor 670 dilution. Single-cell suspensions of sorted pan T cells were suspended in 5 mL of 37°C PBS. An equal volume of 10 μM eFluor 670 (ebioscience, ThermoFisher Scientific) in 37°C PBS was added.
to the T cell suspension and incubated for 10 minutes at 37°C. After incubation, 5 mL RPMI 1640 containing 10% FBS was added, and cells were washed.

**Coculture of MDSCs and T cells.** MDSCs were sorted from 4T1 tumor-bearing mice or PBMC-derived MDSCs using mouse or human MDSC isolation kit ( Stem Cell Technologies). CD4+ and CD8+ cells were harvested from BALB/c mice or human PBMCs by using Pan T Cell Isolation Kit II (Miltenyi Biotec) and EasySep Human T Cell Isolation Kit ( Stem Cells Technologies) respectively. MDSCs were cocultured with 2 × 10^5 CD3+ T cells in RPMI 1640 culture media supplemented with 1-glutamine, penicillin/streptomycin, and 10% heat-inactivated FBS. After 72 hours, cells were collected and eFluor 670 dilutions were calculated by gating from live CD4+ or CD8+ T cells using flow cytometry. To activate mouse T cells, CD3 and CD28 antibodies (both from BioXCell) were added to a coculture of T cells and MDSCs. To stimulate human T cells, CD3 and CD28 beads (Invitrogen) were used. Cytokine production by T cells in coculture was detected by adding Brefeldin A (Invitrogen) 4 hours before staining.

**Luminex assay.** Plasma was collected by retro-orbital bleeding on the indicated days following transplant. Blood samples were placed on ice until all samples had been collected. Once the final sample was collected, all samples were incubated at room temperature for 20 minutes to allow for coagulation to occur. After incubation, vials were centrifuged at 4°C for 10 minutes at 2000 g. Serum plasma was collected and frozen at –80°C. Mouse cytokine and chemokine 11plex was performed by Flow and Image Cytometry, Luminex Division at Roswell Park, as per the manufacturer's instructions.

**Histopathology scoring.** WT and β2-AR–/– mice were injected with 1 × 10^4 4T1 cells in 100 μL PBS; mice were sacrificed 25 days after injection. Lungs were removed, fixed with formalin, sectioned, and stained with H&E.

**Bone marrow chimera.** Chimeras were generated between BALB/c WT and β2-AR–/– hosts. These mice were lethally irradiated with 8.5 Gy of total body irradiation (Cesium 137 source). One day after irradiation, BM was reconstituted with the intravenous (tail vein) injection of 5 × 10^6 BM cells and 5 × 10^6 splenocytes which were isolated from healthy β2-AR–/– mice or WT controls. After 8 weeks, tumor growth experiments were conducted and mice were injected with 1 × 10^5 4T1 cells in 100 μL PBS.

**Propranolol (β-blocker), salbutamol (β-agonist), and isoproterenol (β-agonist) studies.** For studies in which propranolol was used to assess the impact of adrenergic signaling on tumor growth and MDSC accumulation, tumor-bearing mice were housed at ST (22°C) or TT (~30°C). Propranolol treatment was initiated 4 days prior to tumor cell implantation and daily treatment continued until the experimental endpoint. Mice received 200 μg propranolol (clone P0884, Sigma-Aldrich) in 10 mg/kg by i.p. injection; control mice received 200 μL PBS. Salbutamol (clone S8260, Sigma-Aldrich) was injected 1 mg/kg daily after tumor implantation. For in vitro studies, ISO (clone 07912, Stem Cell Technologies) was used at 10 μM and 100 μM concentrations and propranolol (clone P0884, Sigma-Aldrich) was used at 10 μM.

**MDSC depletion.** Anti-mouse Gr-1 antibody (clone RB6-8C5) and IgG2a isotype control antibody (clone LTF-2) were purchased from BioXCell. WT and β2-AR–/– mice were randomized to receive treatment with either anti-Gr-1 antibody (200 μg) or an isotype antibody (200 μg). Treatment was initiated on a rolling basis beginning one day after tumors became detectable. Mice received 5 injections of antibody spaced 4 days apart.

**Flow cytometry.** Single-cell suspensions were created by excising and cutting mouse tumors into 2- to 3-mm pieces. 4T1 tumors were dissociated with collagenase/hyaluronidase (clone 07912, Stem Cell Technologies) following the manufacturer’s protocol prior to passage 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 receptors blocker, 1:200) at 4°C for 10 minutes. Cells were then stained with the different antibodies. Live/dead aqua or yellow dye (ThermoFisher Scientific) were used to gate out dead cells. For intracellular staining, cells were first surface-stained as above, fixed and permeabilized using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) as per the manufacturer’s protocol, then stained with antibodies to intracellular antigens. All data were collected on a LSR Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo v7 software (Tree Star, Inc.). Absolute number of cells in both spleen and tumor tissues was calculated by multiplying percentage of live CD45+ CD11b+ Ly6G+ (PMN-MDSC) and live CD45+ CD11b+ Ly6G+ (M-MDSC) by the cell numbers of the sample, divided by milligram weight.

**Cell sorting.** MDSCs were harvested from WT and β2-AR–/– 4T1 tumor-bearing mice for Nanostring analysis. MDSCs were sorted from single-cell suspensions of tumors excised 25 days after 4T1 tumor implantation into WT and β2-AR–/– mice. Cell sorting was performed using a BD FACSAria (BD Biosciences).

**Western blot.** MDSCs were sorted from bone marrow of 4T1 tumor-bearing WT and β2-AR–/– mice using a murine MDSC isolation kit. MDSCs were suspended in 4 mL RPMI 1640 culture media supplemented with 1-glutamine, penicillin/streptomycin, and 10% heat inactivated FBS in 2 cm^2, 24-well plates (Costar, catalog 3524). Cells were incubated at 37°C and treated with 100 μM ISO in PBS for 20, 60, or 120 minutes. Control cells were treated with PBS. After treatment time, cells were washed with PBS and frozen at –80°C. A lysis buffer consisting of RIPA Buffer (Pierce, catalog 89900), protease and phosphatase inhibitor mini tablets (Pierce, catalog A28961), and 0.1M PMSF (ThermoFisher Scientific, catalog 36978) was used to extract protein from MDSC samples. BCA assays were carried out using a clear, flat-bottom, 96-well plate (Costar, catalog 9018), the BCA Protein Assay Kit (Pierce, catalog 32225), and a plate reader (Synergy H1) to determine the concentration of protein in each sample. Protein resolution was achieved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore, catalog IPVH00010), and blocked with 5% nonfat milk or 5% BSA (ThermoFisher Scientific, catalog 10857) in Tris buffered saline (Bio-Rad, catalog 173-6435) with Tween 20 (Bio-Rad, catalog 170-6531) per primary antibody incubation specifications. Membranes were probed overnight at a concentration of 1:1000 for phospho-STAT3 (Cell Signaling, catalog 9145), STAT3 (Cell Signaling, catalog 9139), and GAPDH (Cell Signaling, catalog 5174). Anti-rabbit (Cell Signaling, catalog 7074) and anti-mouse (Cell Signaling, catalog 7076) horseradish peroxidase-conjugate secondary antibodies were used at a concentration of 1:3000. Membranes were developed with ECL-substrate (Bio-Rad, catalog 170-5060) and images were captured using the LI-COR Odyssey Fc (catalog OGF-0756).

**Generation of human MDSCs from PBMCs.** Human PBMCs were isolated from healthy volunteer donors by venipuncture and subsequent
differential density gradient separation (Ficoll Hypaque, MilliporeSigma). PBMCs were cultured in T-25 flasks at 1 × 10⁶ cells/mL in complete medium (RPMI 1640, Corning Cellgro) supplemented with the cytokines IL-6 (20 ng/mL, MilliporeSigma) and GM-CSF (20 ng/mL, R&D Systems) for 7 days, in the presence or absence of ISO (10 μM). Cultures were run in duplicate, and medium and cytokines were refreshed every 2–3 days. After 1 week, all cells were collected from PBMC cultures. Adherent cells were removed using non–protease cell detachment solution Detaching (Genelantis). At day 7, MDSC populations were characterized using CD14 and CD33 markers by flow cytometry. CD33+ cells were isolated from each culture using EasySep HLA Chimerism CD33 Whole Blood Positive Selection Kit (STEMCELL Technologies) per manufacturer’s instructions. The purity of isolated cell populations was determined to be greater than 90% by flow cytometry. 

**Nanostring.** Sorted MDSCs (CD11b+ Gr-1+) from WT or β2-AR−/− mice bearing 4T1 tumors were prepared for Nanostring analysis. In brief, RNA was isolated from sorted cells using the RNeasy Plus Mini kit (Qiagen). Nanostring analysis was performed with the nCounter Analysis System at NanoString Technologies. The nCounter Mouse Immunology Kit, which includes 561 immunology-related mouse genes, was used.

**Statistics.** The Student’s t test was used to compare data between 2 groups, 2-way ANOVA with Tukey’s post hoc analysis was used to generate tumor growth statistics using GraphPad Prism, and 1-way ANOVA with Tukey’s post hoc analysis was used to compare data between 3 groups or more using GraphPad Prism. All tumor growth data are presented as mean ± SEM, and all other data are presented as median ± minimum to maximum.

**Study approval.** The Roswell Park Comprehensive Cancer Center IRB approved human subject studies (NHR 009510). Generation of the mice and all mice studies were reviewed and approved by the Roswell Park Comprehensive Cancer Center IACUC (protocol numbers 757M and 1038M).

**Author contributions**

HM initiated the study. HM, BLH, SIA, and EAR designed the study; HM performed the experiments with assistance from CRM, GQ, BD, and MC. HM, BLH, PLM, SIA, and EAR analyzed and interpreted the data and wrote the paper.

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