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# 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 and murine tumor models, in wild-type mice and genetically modified ( $\beta$ 2-AR<sup>-/-</sup>) mice, as well adoptive transfer approaches, we found that the degree of  $\beta$ 2-AR signaling significantly influences MDSC frequency and survival in tumors and other tissues, modulates their expression of immunosuppressive molecules such as arginase-I and PDL-1 and alters their ability to suppress the proliferation of T cells. The regulatory functions of  $\beta$ -AR signaling in MDSCs were found to be dependent upon STAT3 phosphorylation. Moreover, we observed that the  $\beta$ 2-AR-mediated increase in survival of MDSCs is dependent upon Fas-FasL interactions, and this is consistent with gene expression analyses which reveal a greater expression of apoptosis-related genes in  $\beta$ 2-AR<sup>-/-</sup> MDSCs. Our data reveals the potential of  $\beta$ 2-AR signaling to increase the generation of MDSCs from both murine and human peripheral blood cells and that the immunosuppressive function of MDSCs could be mitigated by treatment with  $\beta$ -AR antagonists, or enhanced by  $\beta$ -AR agonists, strongly supporting the possibility that reducing stress-induced activation of  $\beta$ 2-ARs could help to overcome immune suppression and enhance the efficacy of immunotherapy and other cancer therapies.



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

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#### Summary

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 ( $\beta$ 2-AR<sup>-/-</sup>) mice, as well as adoptive transfer approaches, we found that the degree of  $\beta$ 2-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 PDL-1 and alters their ability to suppress the proliferation of T cells. The regulatory functions of  $\beta$ 2-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  $\beta$ 2-AR<sup>-/-</sup> MDSCs. Our data reveal the potential of  $\beta$ 2-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  $\beta$ -AR antagonists, or enhanced by  $\beta$ -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.

Keywords: β2 adrenergic receptor, MDSC, Chronic Stress, Tumor, STAT3

#### 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 (1, 5). There are two distinct subsets of MDSCs, monocytic MDSCs (M-MDSCs; CDllb<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>lo</sup>) 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:  $\alpha$ ARs and  $\beta$ -ARs. The  $\alpha$ 1-AR is primarily expressed on endothelial cells of blood vessels, while the  $\alpha$ 2-AR is more ubiquitously expressed.  $\beta$ -ARs comprise three receptors including  $\beta$ 1,  $\beta$ 2 and  $\beta$ 3.  $\beta$ 1 and  $\beta$ 3 receptors are primarily expressed in heart and adipose tissues respectively, while  $\beta$ 2-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,  $\beta$ 2-AR activation in T cells was seen to suppress their ability to secrete interferon- $\gamma$  (IFN- $\gamma$ ) 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  $\beta$ 2-AR signaling suppresses anti-tumor CD8<sup>+</sup> T cell function and increases populations of MDSCs and Tregs in the spleen and tumor microenvironment respectively (27, 28). However, the role of  $\beta$ 2-AR in major aspects of MDSC functions associated with suppressing the anti-tumor 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  $\beta$ 2-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  $\beta$ 2-AR-deficient mice (referred to as  $\beta$ 2-AR<sup>-/-</sup>) and adoptive transfer models, we examined the impact of adrenergic stress signaling through  $\beta$ 2-ARs on MDSC frequency in tumors and other tissues, whether the  $\beta$ 2-AR expression in MDSCs is

influenced by expression of cytokines including GM-CSF and how  $\beta$ 2-AR signaling modulates the expression of immunosuppressive molecules such as arginase-I and PDL-1 in MDSCs. We also examined the impact of  $\beta$ 2-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 peripheral blood cells. Our data reveal a major impact of  $\beta$ 2-AR signaling on the immune suppressive potential of MDSCs and suggest that reducing stressinduced activation of  $\beta$ 2-AR could help to overcome immune suppression and enhance the efficacy of immunotherapy and other cancer therapies.

#### Results

Chronic stress mediated  $\beta_2$  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 subthermoneutral housing temperature (ST;  $\sim 22^{\circ}$  C), or a thermoneutral housing temperature (TT;  $\sim$ 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, B) and decreased tumor weights (Figure S2A). Here, we also report that at TT there are reduced levels of circulating pro-tumor cytokines (Figure S1A, B) compared to mice housed at ST. As the  $\beta$ 2-AR is the most prominent AR expressed by immune cells (31), we compared tumor growth in BALB/c wild type (WT) mice and  $\beta$ 2-AR<sup>-/-</sup> mice. As we previously observed (28), 4T1 tumors grew at a decreased rate in  $\beta$ 2-AR<sup>-/-</sup> mice (Figure 1C, S2B). Here, we also found decreased levels of several pro-tumor cytokines in the plasma (Figure S1C) and, together with the data in Figure S1A and S1B, these results suggest a role for the  $\beta$ 2-AR pathway in regulating the overall cytokine milieu in tumor bearing mice. Consistent with this data, we found that the lungs of  $\beta$ 2-AR<sup>-/-</sup> mice had fewer metastatic nodules (Figure S2C).

We next made bone marrow chimeras, using the BALB/c WT and  $\beta$ 2-AR<sup>-/-</sup> models defined below, to test whether the impact of  $\beta$ 2-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  $\beta$ 2-AR<sup>-/-</sup> mice were reconstituted with BM cells isolated from either  $\beta$ 2-AR<sup>-/-</sup> mice or WT controls. We found that the growth of 4T1 tumors was significantly slower in mice reconstituted with  $\beta$ 2-AR<sup>-/-</sup> BM than in mice reconstituted with WT BM (Figure 1D), suggesting that  $\beta$ 2-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  $\beta$ 2-AR<sup>-/-</sup> deficient MDSCs lose their pro-tumorogenic properties, we depleted MDSCs in both WT and B2-AR<sup>-/-</sup> mice using an anti-Gr-1 antibody (31). MDSC depletion significantly delayed 4T1 tumor growth in WT mice, but led only to a small, non-significant decrease tumor growth rate in  $\beta$ 2-AR<sup>-/-</sup> mice (Figure 1E). These data confirm that MDSCs from WT mice promote tumor growth, while tumor growth in  $\beta$ 2-AR<sup>-/-</sup> mice is not affected by  $\beta$ 2-AR<sup>-/-</sup> 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  $\beta$ 2-ARs on MDSCs from 4T1 tumor-bearing WT and  $\beta$ 2-AR<sup>-/-</sup> mice via ImageStream. After confirming  $\beta$ 2-AR expression in WT, but not  $\beta$ 2-AR<sup>-/-</sup> MDSCs (Fig 1F), we sought to further determine whether the presence of a tumor altered the level of  $\beta$ 2-AR expression in WT MDSCs. When comparing MDSCs from the spleen of tumor bearing mice to those that were isolated from

the spleen of healthy mice, we observed a significant increase in  $\beta$ 2-AR expression in MDSCs from the spleens of tumor bearing mice (Figure 1I).

When considering this variability in  $\beta$ 2-AR expression in conjunction with the observed changes in cytokine levels in earlier experiments (Fig S1A, B, C), we sought to investigate whether increased cytokine levels originating from the TME might be involved in locally increasing the expression of  $\beta$ 2-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, while treatment with IL-6 was not (Figure 1G, H), suggesting that  $\beta$ 2-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  $\beta$ 2-ARs in MDSCs correlates with our finding that a higher percentage of the splenic MDSCs from tumorbearing mice express  $\beta$ 2-ARs compared to those from non-tumor bearing mice (Figure 1I). Altogether, these data demonstrate that there is a tight association between tumor promoting cytokines, B2-AR expression on MDSCs, and MDSC-dependent tumor growth such that the whole response may be orchestrated by sympathetic nervous system activity.

 $\beta$ 2-AR activation during chronic stress increases MDSC accumulation and tumor vascularization. We next tested the role of  $\beta$ 2-AR in MDSC accumulation in the spleen, TME, and other tissues. 4T1 cells were injected into WT or  $\beta$ 2-AR<sup>-/-</sup> 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<sup>+</sup> myeloid cells within the live CD45<sup>+</sup> cells of the TME was significantly elevated in WT mice compared to  $\beta$ 2-AR<sup>-/-</sup> mice (Figure 2A). In  $\beta$ 2-AR<sup>-/-</sup> mice,

we observed significantly fewer PMN-MDSCs and M-MDSCs, suggesting that the expression of β2-ARs on MDSCs increases the accumulation of both subsets in tumor-bearing mice. The absolute number of MDSCs was also higher in both the spleen and tumor tissue of WT mice compared to β2-AR<sup>-/-</sup> mice (Figure 2A). In addition, we found that housing mice under TT conditions significantly decreased MDSC accumulation in the spleen and TME in both tumor models, but we found no significant differences in phenotypically similar populations in tumorfree mice (Figure 2B). We also found that the accumulation of MDSCs significantly increased in both blood and lymph node tissues of 4T1 tumor bearing mice housed at ST compared to TT (Figure S3A, S3B). We also assessed the accumulation of MDSCs in lung tissue of WT and B2-AR-/- mice bearing either 4T1 tumors, which are metastatic, or AT3 tumors, which are nonmetastatic. We found that the lungs of  $\beta$ 2-AR<sup>-/-</sup> mice had significantly fewer numbers of MDSCs compared to WT mice in the 4T1 tumor model, but this was not observed in the AT-3 tumor model (Figure S3C). Thus, our results show that tumor growth and metastasis is diminished in mice that lack B2-ARs. To extend our flow cytometry findings, we performed immunohistochemistry to analyze myeloid cell accumulation, labeled by Gr-1, and angiogenesis, labeled by CD31 and VEGF- $\alpha$  in the TME. We observed an increase in the proportion of Gr-1 and CD31-positive cells in WT mice compared to  $\beta$ 2-AR<sup>-/-</sup> mice (Figure 2C). We also found that the VEGF- $\alpha$  positive area was significantly higher in WT mice compared with  $\beta$ 2-AR<sup>-/-</sup> mice (Figure 2C). Taken together, these data suggest that chronic stress, signaling through  $\beta$ 2-AR, increases the accumulation of myeloid cells which in turn, could be enhancing tumor growth by vascularization through  $\beta$ 2-AR dependent signaling and/or other mechanisms.

 $\beta$ 2-AR activation increases the immune-inhibitory activity of MDSCs. So far, we have demonstrated that chronic stress mediated  $\beta$ 2-AR activation increases the accumulation of

MDSCs in tumors, enhances tumor vascularization, and augments the level of tumor promoting cytokines in the plasma of tumor bearing mice. We have also shown that pro-tumor effects of  $\beta$ 2-AR are associated with the expression of B2-AR on hematopoietic cells rather than stromal cells. We next tested the impact of  $\beta$ 2-AR activation on the function of MDSCs *in vitro*. To this end, we isolated total BM cells and generated MDSCs using a GM-CSF and IL-6 cytokine cocktail, as described (33). The addition of the  $\beta$ -AR agonist isoproterenol (ISO) to the culture significantly increased the expression of well-known immunosuppressive molecules, such as arginase I and programmed death-ligand 1 (PD-L1), in MDSCs compared to the control group, and this increase was inhibited by the addition of propranolol (a β-AR blocker; Figure 3A). To test the functional activity of ISO-treated MDSCs, we performed a T cell proliferation assay. After coculture of WT control MDSCs or ISO-treated MDSCs, we observed that ISO-treated MDSCs were significantly more immunosuppressive against both CD8<sup>+</sup> and CD4<sup>+</sup> T cell proliferation (Figure 3B) compared to the controls. Furthermore, we used flow cytometry to assess levels of arginase I and PDL-1 in MDSCs isolated from 4T1 tumors, and found that intratumoral WT MDSCs have a significantly higher level of arginase I and PDL-1, compared to β2-AR<sup>-/-</sup> MDSCs (Figure S4A). Then, we sorted WT and  $\beta$ 2-AR<sup>-/-</sup>MDSCs from tumor tissue and co-cultured them with CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Inhibition of T cell proliferation was significantly greater with WT MDSCs compared to  $\beta$ 2-AR<sup>-/-</sup> MDSCs (Figure S4B). These data demonstrate that  $\beta$ 2-AR signaling in MDSCs increases the immunosuppressive function of MDSCs.

In line with this reduced immunosuppressive ability, we hypothesized that  $\beta$ 2-AR<sup>-/-</sup> MDSCs would have a less immunosuppressive phenotype. Therefore, we compared immune-related gene expression patterns of WT and  $\beta$ 2-AR<sup>-/-</sup> MDSCs by comparing sorted WT and  $\beta$ 2-AR<sup>-/-</sup> MDSCs from tumors by "Nanostring" analysis. Nanostring data showed that WT MDSCs expressed

higher levels of the immunosuppressive molecules (*Il11ra, Ahr, Cd209, Dpp4, Xcr1, Gpr44*) and cytokines (*Il4, Il2, Il5, Il33, Il-21*) than did  $\beta$ 2-AR<sup>-/-</sup> MDSCs (Figure 3C). Conversely, the expression of the costimulatory markers (*Cd86, Cd40, Cd83, Cd27, Cd6 and Tlr8*) and antitumor cytokines (*Ifng, Il12a, Il12b*) was higher in  $\beta$ 2-AR<sup>-/-</sup> MDSCs (Figure 3C). Furthermore, to confirm the different phenotypes, MDSCs were isolated from the BM of WT or  $\beta$ 2-AR<sup>-/-</sup> 4T1 tumor bearing mice, activated with LPS for 18 hours, and the levels of pro-tumor and anti-tumor cytokines were measured. Significant increases in pro-tumor cytokine production were observed in WT MDSCs, compared to  $\beta$ 2-AR<sup>-/-</sup> MDSCs. Conversely, we observed that the secretion of IFN- $\gamma$  by  $\beta$ 2-AR<sup>-/-</sup> MDSCs was significantly increased compared to WT MDSCs (Figure 3D).

To examine the importance of  $\beta$ 2-AR expression for MDSC pro-tumorigenic function *in vivo*, 4T1 cells were mixed 1:1 with MDSCs isolated from WT or  $\beta$ 2-AR<sup>-/-</sup> 4T1 tumor bearing mice, and injected orthotopically into fresh groups of WT mice. Co-injection of 4T1 with  $\beta$ 2-AR<sup>-/-</sup> MDSCs into WT mice resulted in significantly delayed tumor growth compared to co-injection with WT MDSCs (Figure 3E) suggesting that the expression of  $\beta$ 2-AR on MDSCs plays an important role in inducing MDSC-mediated pro-tumor mechanisms. We next evaluated the immunosuppressive capabilities of WT or  $\beta$ 2-AR<sup>-/-</sup> MDSCs by an adoptive transfer approach. The adoptive transfer of WT MDSCs into  $\beta$ 2-AR<sup>-/-</sup> mice increased tumor growth, whereas the adoptive transfer of  $\beta$ 2-AR<sup>-/-</sup> MDSCs 3 or 6 days post 4T1 implantation, delayed tumor growth (Figure 3F). These data show that  $\beta$ 2-AR expression and activation in MDSCs is necessary for the immunosuppressive function of MDSCs and promotion of tumor growth.

 $\beta$ 2-AR expression plays an important role in MDSC turnover and survival. Based on our Nanostring data from WT and  $\beta$ 2-AR<sup>-/-</sup> 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 pro-apoptotic genes such as *Fas*, *Casp8*, and *Casp3* was higher in  $\beta^{-/-}$  MDSCs (Figure 3C), while the expression of the anti-apoptotic 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  $\beta^{2}$ -AR increased susceptibility of MDSCs to apoptosis through Fas/FasL interactions.

We quantified the expression of Fas on WT or  $\beta$ 2-AR<sup>-/-</sup> MDSCs and FasL expression on CD8<sup>+</sup> T cells (one of the FasL-expressing cells in the TME) in WT and  $\beta$ 2-AR<sup>-/-</sup> 4T1 tumor-bearing mice. The data showed that  $\beta$ 2-AR<sup>-/-</sup> MDSCs expressed Fas at a significantly higher level than WT MDSCs (Figure 4A). Interestingly,  $\beta$ 2-AR<sup>-/-</sup> CD8<sup>+</sup> T cells expressed more FasL compared to WT CD8<sup>+</sup> T cells (Figure 4A), implicating a likely source of the cognate ligand for Fas engagement. Additionally, higher levels of FasL on  $\beta$ 2-AR<sup>-/-</sup> CD8<sup>+</sup> 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 to  $\beta$ 2-AR<sup>-/-</sup> MDSCs.

To test whether the differential expression of *pro-* and *anti-*apoptotic molecules by WT and  $\beta$ 2-AR<sup>-/-</sup> MDSCs can influence the survival of MDSCs in the TME, apoptosis of WT or  $\beta$ 2-AR<sup>-/-</sup> MDSCs in 4T1 tumor-bearing mice was investigated. At day 25 after tumor implantation, the level of apoptosis in WT and  $\beta$ 2-AR<sup>-/-</sup> MDSCs was measured in the tumor and spleen tissues. We found that the frequency of apoptotic cells in  $\beta$ 2-AR<sup>-/-</sup> MDSCs was significantly higher, compared to 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 to MDSCs isolated from tumor-bearing mice housed under ST conditions (Figure 4C).

To further investigate the importance of  $\beta$ 2-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  $\beta$ 2-AR<sup>-/-</sup> (CD45.2) mice. On day 25 after tumor injection, we isolated WT (CD45.1) or  $\beta$ 2-AR<sup>-/-</sup> (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 (GFP<sup>-</sup> CD45.1<sup>+</sup>) in the spleen was significantly higher compared to  $\beta$ 2-AR<sup>-/-</sup> MDSC (GFP<sup>-</sup> CD45.2<sup>+</sup>) at days 3 and 7 after co-injection, suggesting that WT MDSCs could survive longer compared to  $\beta$ 2-AR<sup>-/-</sup> MDSCs (Figure 4E). These data highlight that  $\beta$ 2-AR signaling increases the survival of MDSCs in TME at least partially through the Fas-FasL pathway.

 $\beta$ 2-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  $\beta$ 2-AR, such as NE and ISO, can activate STAT3 in MDSCs. To test this, MDSCs were isolated from the bone marrow of WT or  $\beta$ 2-AR<sup>-/-</sup> MDSCs from tumor-bearing mice. We then treated WT or  $\beta$ 2-AR<sup>-/-</sup> 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  $\beta$ 2-AR<sup>-/-</sup> 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 to  $\beta$ 2-AR<sup>-/-</sup> MDSCs in both the tumor tissue and spleen. A similar trend was seen in MDSCs isolated from tumor-bearing mice housed at ST compared to 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 to mice receiving vehicle control in WT but not  $\beta$ 2-AR<sup>-/-</sup> tumor bearing mice, again supporting a role for  $\beta$ 2-AR in STAT3 phosphorylation. 25 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  $\beta$ 2-AR signaling enhances accumulation and/or survival of MDSCs occurs through STAT3 phosphorylation which may lead to increased expression of pro-survival and immunosuppressive genes such as Bcl-2 and arginase-I respectively in MDSCs.

β2-AR blockade slows tumor growth and diminishes frequency of MDSCs while 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 propranolol (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<sup>-/-</sup> mice (Figure 6A). In addition, the numbers of MDSCs in tumor tissue and spleen of WT mice were decreased compared to 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 to 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 (Figure S4A) and MDSC accumulation in the spleen (Figure S5B) and tumor tissue (Figure S5C) 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 tissue, but it was less efficient than propranolol, suggesting that nerves are not the only source of NE (Figure 6D). These results demonstrate that the pro-tumor effects of chronic stress mediated by  $\beta$ 2-AR signaling in MDSCs can be regulated by commonly used  $\beta$ -blocker drugs.

 $\beta$ 2-AR activation increases MDSC generation from human PBMC. To test 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 MDSC in the presence or absence of ISO as described (37). We found that PBMCderived MDSCs express  $\beta$ 2-AR on their surface (Figure 7A) and that addition of ISO, which provides stimulation of these receptors, significantly increased the generation of MDSCs (CD14<sup>+</sup>CD33<sup>+</sup>) seven days post culture (Figure 7B). We also found that adding ISO into MDSC culture media increased the expression of arginase-I, PDL-1 and p-STAT3, thus replicating in human cells the effects that  $\beta$ 2-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 co-cultured them with human CD3<sup>+</sup> T cells stimulated with anti-CD3 and anti-CD28 beads. ISO-treated cells suppressed proliferation and IFN- $\gamma$  production of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells at a higher level compared to 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 anti-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<sup>+</sup> 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<sup>+</sup> T cell-dependent anti-tumor 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 mild, but chronically cool housing temperature), a genetic model (i.e., deletion of  $\beta$ 2-AR), and multiple pharmacological interventions, we identified a major role for  $\beta$ 2-AR signaling in promoting MDSC survival and pro-tumorigenic function. Overall, the fact that  $\beta$ -AR signaling increases the immune suppressive functions of murine MDSCs in the TME as well as those of human MDSC-like cells generated from PBMC, points to a mechanism by which chronic stress could tilt the immunological balance toward suppression of the anti-tumor immune response. Additionally, we found that  $\beta$ 2-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 anti-tumor 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 which 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 pro-tumor milieu in the TME. Recent data reported by others shows 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 reveals 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. Indeed, some of our data, while statistically significant, shows a relatively modest impact of adrenergic receptor signaling (e.g., Figure S1A-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 sub-optimal immune control of tumor progression, over long periods of time, could result in a significant difference 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  $\beta$ -blockers, for reasons 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 non-oncologic settings. 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-monocyte progenitors (GMPs) in the spleen and that severing splenic SNS nerves diminished 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  $\beta$ 2-AR signaling in MDSCs alleviates 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  $\beta$ 2-AR compared to 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  $\beta$ 2-AR upregulation. In agreement with our results, a recent report confirmed that the expression level of  $\beta$ 2-AR on MDSCs residing in different tissues is different (46), suggesting that the effects of chronic stress on MDSCs in different tissues could be regulated by this differential expression level of  $\beta$ 2-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 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  $\beta$ 2-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  $\beta$ 2-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<sup>+</sup> 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/ $\alpha$ CD40 (52) or irradiation plus anti-PD-1 activates cytotoxic T cells and promotes MDSC apoptosis (53). In our study, we showed that  $\beta$ 2-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  $\beta$ 2-ARs. We also confirmed, by *in vivo* competition assays, that the level of apoptosis is increased in  $\beta$ 2-AR-deficient MDSCs. Although the precise mechanisms by which  $\beta$ 2-ARs regulate the expression of Fas require further investigation, these data provide new evidence about the important role of  $\beta$ 2-AR in MDSC resistance to apoptosis. In addition, these data provide insight into the potential application of  $\beta$ 2-AR blockers in combination with various treatments like immunotherapies (including anti-PD-1) and irradiation to improve the anti-tumor immune response through increased susceptibility of MDSCs to cell death.

In summary, our work demonstrates that chronic stress, acting through the  $\beta$ 2-AR, significantly promotes proliferation, suppressive function, and survival of MDSC and therefore has the potential to significantly suppress the anti-tumor immune response. Inhibiting  $\beta$ 2-AR signaling by  $\beta$ -AR blockade, inhibiting NE releasing nerves, or  $\beta$ 2-AR deletion can decrease the accumulation of MDSCs, reduce their immunosuppressive functions, and is associated with the increased efficacy of the anti-tumor immune response and inhibition of tumor growth that we have previously seen. Understanding the mechanisms by which the expression of  $\beta$ 2-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  $\beta$ 2-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 to other cancer therapies. Although additional research on drugs that specifically block  $\beta$ 2-ARs is needed to increase the precision of this therapy, we have shown that pharmacologic agents like propranolol which are currently clinically available and Food and Drug Administration (FDA)-approved could be a potentially efficacious approach at the present time.

#### Material and methods

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

*Cell culture and tumor models.* 4T1 tumor cells were purchased from ATCC (ATCC, CRL-2539). AT-3 tumor cells were provided by Dr. Scott Abrams (Roswell Park). Cell lines were confirmed to be Mycoplasma negative yearly using the Mycoplasma Plus PCR Primer Set (Aligent Technologies, 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% CO<sub>2</sub>. Once thawed, cells were passed twice prior to use.  $1 \times 10^5$  4T1 cells in 100µl PBS and  $5 \times 10^5$  AT-3 cells in 100µl PBS were subcutaneously injected into 4th mammary fat-pad of C57BL/6 mice. Tumor growth was monitored in a blinded manner throughout experiments, and perpendicular diameters (width/length) were measured every two days. Tumor volume was calculated using the equation: *Tumor Volume*  $= \frac{2W \times L}{2}$  mm3, where W is the small dimension a L is the large.

*Ambient temperature manipulation.* Mice were housed five/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 (17A2), CD4 (GK1.2), CD8 (536.7), CD45 (30F11), Gr-1 (RB6-8C5), CD45.1 (A20), CD45.2 (104), CD11b (M1/70), PDL-1 (MIH5) CD206 (C068C2), F4/80 (BM8), Ly6C (HK1.4), Ly6G (1A8), Arginase-I (IC5868F, R&D), Bcl-2 (BCL/10C4), p-STAT3(13A3-1), Fas (15A7), FasL (MFL-3), and Caspase-8 (FITC-IETD-FMK) were purchased from Biolegend, BD bioscience, and ebioscience. Antibodies including anti-human CD33 (P67.6), CD14 (M5E2), CD4 (A161A1), CD8 (RPA-T6), Arginase-I (14D2C43), p-STAT3 (13A3-1),  $\beta$ 2-AR (R11E1), and PDL-1 (29E2A3) were purchased from Biolegend. Mouse MDSC isolation kit was purchased from Miltenyi Biotec. EasySep<sup>TM</sup> Human T Cell Isolation Kit and EasySep<sup>TM</sup> HLA chimerism whole blood CD33 positive selection kit were obtained from Stem Cells Company. Mouse and human IL-6, GM-CSF and G-CSF were purchased from Biolegend Company. Apoptosis level were measured using apoptosis kit (ebioscience, ThermoFisher) by flow cytometry based on manufacture's protocol.

*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) in 37°C PBS

was added to the T cell suspension and incubated for 10 min at 37°C. After incubation, 5 ml of RPMI 1640 containing 10% FBS was added, and cells were washed.

*Co-culture of MDSCs and T cells.* MDSCs were sorted from 4T1 tumor bearing mice or PBMCs derived MDSCs using mouse or human MDSC isolation kit (Stem Cells 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<sup>TM</sup> Human T Cell Isolation Kit (Stem Cells Technologies) respectively. MDSCs were co-cultured with  $2\times10^5$  CD3<sup>+</sup> T cells in RPMI1640 culture media supplemented with L-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<sup>+</sup> or CD8<sup>+</sup> T cells using flow cytometry. To activate mouse T cells, CD3 and CD28 antibodies (both from Bioxcell) were added to a co-culture of T cells and MDSCs. To stimulate human T cells, CD3 and CD28 beads (Invitrogen) were used. Cytokine production by T cells in co-culture 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 min to allow for coagulation to occur. After incubation, vials were centrifuged at 4°C for 10 min at 2000  $\times$  g. Serum plasma was collected and frozen at -80°C. Mouse cytokine and chemokine 11-plex was performed by the Flow and Image Cytometry, Luminex Division at Roswell Park, as per the manufacturer's instructions.

*Histopathology scoring.* WT and  $\beta$ 2-AR<sup>-/-</sup> mice were injected with 1×10<sup>5</sup> 4T1 cells in 100µl 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  $\beta$ 2-AR<sup>-/-</sup> hosts. These mice were lethally irradiated with 8.5Gy of total body irradiation (Cesium 137 source). One day after irradiation, BM was reconstituted with the intravenous (tail vein) injection of  $5 \times 10^6$  BM cells and  $5 \times 10^6$  splenocytes isolated from healthy  $\beta$ 2-AR<sup>-/-</sup> mice or WT controls. After eight weeks, tumor growth experiments were conducted, and mice were injected with  $1 \times 10^5$  4T1 cells in 100µl PBS.

*Propranolol (β-blocker), salbutamol (β2-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 house at standard temperature (ST, 22°C) or thermoneutral temperature (TT, ~30°C). Propranolol treatment was initiated 4 days prior to tumor cell implantation, and daily treatment continued until the experimental endpoint. Mice received 200µg propranolol (P0884, Sigma-Aldrich) in 10mg/kg by i.p. injection; control mice received 200µl PBS. Salbutamol (S8260, Sigma-Aldrich) was injected 1mg/kg daily after tumor implantation. For *in vitro* studies, isoproterenol (ISO) (16504, Sigma-Aldrich) was used at 10µM and 100µM concentrations and propranolol (Prop) (P0884, Sigma-Aldrich) was used at 10µM.

*MDSC depletion*. Anti-mouse Gr-1 antibody (RB6-8C5) and IgG2a isotype control antibody (LTF-2) were purchased from BioXCell. WT and  $\beta$ 2-AR<sup>-/-</sup> mice were randomized to receive treatment with either anti-Gr-1 antibody (200µg) or an isotype antibody (200µg). 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 2-3mm pieces. 4T1 tumors were dissociated with collagenase/hyaluronidase (STEMCELL Technologies, 07912) following the manufacturer's protocol prior to passage through a 70µm nylon cell strainer (Corning). Spleens were mechanically disrupted and directly passed through a 70µm 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) were used to gate out dead cells. For intracellular staining, cells were first surface-stained as above, then fixed and permeabilized using the FoxP3/Transcription Factor Staining Buffer Set (eBiosciences) 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 percent live CD45<sup>+</sup> CD11b<sup>+</sup> Ly6G<sup>+</sup> (G-MDSC) and live CD45<sup>+</sup>  $CD11b^+$  Ly6C<sup>+</sup> (M-MDSC) by the cell numbers of the sample, divided by mg weight.

*Cell sorting.* MDSCs were harvested from WT and  $\beta$ 2-AR<sup>-/-</sup> 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  $\beta$ 2-AR<sup>-/-</sup> mice. Cell sorting was performed using a BD FACSAria (BD Bioscience).

Western blot. MDSCs were sorted from bone marrow of 4T1 tumor bearing WT and β2-AR<sup>-/-</sup> mice using a murine MDSC isolation kit. MDSCs were suspended in 4 mL of RPMI1640 culture media supplemented with L-glutamine, penicillin-streptomycin, and 10% heat inactivated FBS in 2 cm<sup>2</sup>, 24 well plates (Costar. 3524). Cells were incubated at 37°C, and treated with 100µM 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, 89900), protease and phosphatase inhibitor mini tablets (Pierce, A32961), and 0.1 M PMSF (ThermoFisher, 36978) was used to extract protein from MDSC samples. BCA assays were carried out using a clear, flat bottom, 96 well plate (Costar, 9018), the BCA Protein Assay Kit (Pierce, 23225), 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, IPVH00010), and blocked with 5% non-fat milk or 5% BSA (ThermoFisher, 10857) in Tris buffered saline (Bio-Rad, 173-6435) with tween 20 (Bio-Rad, 170-6531) per primary antibody incubation specifications. Membranes were probed overnight at a concentration of 1:1000 for phospho-STAT3 (Cell signaling, 9145), STAT3 (Cell signaling, 9139), and GAPDH (Cell signaling, 5174). Anti-rabbit (Cell signaling, 7074) and anti-mouse (Cell signaling, 7076) horseradish peroxidase conjugate secondary antibodies were used at a concentration of 1:3000.

Membranes were developed with ECL-substrate (Bio-Rad, 170-5060) and images were captured using the LI-COR Odyssey Fc (OFC-0756).

Generation of human MDSCs from PBMCs. Human Peripheral Blood Mononuclear Cells (PBMCs) were isolated from healthy volunteer donors by venipuncture, and subsequent differential density gradient separation (Ficoll Hypaque, Sigma, St. Louis, MO). PBMCs were cultured in T-25 flasks at 1×10<sup>6</sup> cells/mL in complete medium (RPMI 1640, Corning Cellgro) supplemented with the cytokines IL-6 (20ng/mL, Sigma) and GM-CSF (20ng/mL, R&D) for seven days, in the presence or absence of ISO (10µM). Cultures were run in duplicate, and medium and cytokines were refreshed every two-three days. After one week, all cells were collected from PBMC cultures. Adherent cells were removed using non-protease cell detachment solution Detaching (Genlantis, San Diego, CA). At day 7, MDSC populations were characterized using CD14 and CD33 markers by flowcytometry. CD33<sup>+</sup> cells were isolated from each culture using EasySep<sup>TM</sup> 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<sup>+</sup> Gr-1<sup>+</sup>) from WT or  $\beta$ 2-AR<sup>-/-</sup> 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 Students t-test was used to compare data between 2 groups, two-way ANOVA with Tukey post-hoc analysis was used to generate tumor growth statistics using GraphPad Prism, and one-way ANOVA with Tukey post-hoc analysis was used to compare data between 3 groups or more using GraphPad Prism. All tumor growth data are presented as mean  $\pm$  SEM, and all other data are presented as median  $\pm$  minimum to maximum.

#### **Study Approval**

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

#### **Author Contribution**

H.M. initiated and H.M., B.H., S.A, and E.R. designed the study; H.M. performed the experiments with assistance from C.M., G.Q., B.D., and M.C. H.M., B.H., P.M., S.A., and E.R. analyzed and interpreted the data and wrote the paper.

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#### **Declaration of Interests**

The authors have declared that no conflict of interest exists.

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Figure 1



**Figure 1: β2-AR activation increase tumor growth in a MDSC-dependent manner.** (A, B) Tumor growth in mice housed at ST (22°C) or TT (30°C) bearing 4T1 und AT-3 tum *r* cells. (C) Tumor growth kinetics in wild-type and  $\beta$ 2- AR<sup>-/-</sup> mice bearing 4T1 tumor cells. (D) Lethally irradiated WT mice rec ived bone marrow transplants from wild-type (blue circle) or  $\beta$ 2-AR<sup>-/-</sup> (red square) mice. Lethally irradiated  $\beta$ 2-AR<sup>-/-</sup> mice received bone marrow transplants from WT (purple triangle) or  $\beta$ 2-AR<sup>-/-</sup> (brown triang e) mice. Eight weeks after transplant, chimeric mice were injected with 4T1 tumor cells and tumor growth was monitored. (E) 4T1 bearing WT or  $\beta$ 2-AR<sup>-/-</sup> mice were injected with isot pe or anti-Gr-1 antibodies (i.p. 200µg per mouse every 4 days), and tumor growth was monitored. (F)  $\beta$ 2-AR expression in MDSCs sorted by IDSC isolation kit from spleen of 4T1 tumor bearing mice 25 days after tumor injection using Image Stream. (G)  $\beta$ 2-AR expression in MDSCs worted from bone marrow of non-tumor bearing mice after culture with IL-6, G-CSF, and LPS (data from three independent replicates). (H) The levels of  $\beta$ 2-AR in splenic MDSCs from hea thy or 4T1 tumor bearing mice using flow cytometry. T wo-way ANOVA was used to analyze stat stical significance between tu nor growth in lifferent groups. These data are presented as mean ± SE  $\Lambda$  of 5 mice per group from at least two replicate experiments. Other data are presented as median ± min to max. One-way ANOVA was used to analyze statistical significance betwee 1 four groups, and the students T test was used to analyze statistical significance betwee 1 two 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

A



**Figure 2:**  $\beta$ 2-AR activation during chronic stress increases MDSC accumulation in the spleen and tumor. (A) Representative flow cytometry analysis of G-MDSC and M-MDSC subpopulations, as well as absolute number of -MDSCs and M-MDSCs in tumor and spleen of 4T1 tumor bearing mice on day 25 fter tumor injection. The data presented are from groups of 10 mice from two 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 standard temperature (ST) or thermoneutral temperature (TT). The data presented are from groups of 8 mice fron 1 two replicate studies. (C) Both representative immunohistochemistry analysis an 1 absolute n mber of Gr-1(20x magnification), CD31 (4x magnification) and VE  $\beta$ F- $\alpha$  (10x magnification) positive cells in 4T1 tumors at day 25 after tumor injection. These data are presented as median  $\pm$  min to max fr m groups of 6 mice from two replicate studies. The students T test was used to analyze statistical significance between two groups. In all panels \* P<0.05, \*\* P<0.01 and \*\*\* P<0.001. A P value less than 0.05 was considered significant.

## Figure 3



**Figure 3:**  $\beta$ 2-AR deletion decreases immune suppressive activity of MDSCs. (A) Representative flow cytometry data of the expression of arginase I and PDL-1 plus the percentige of arginase I and PDL-1 in MDSCs derived from bone marrow in the presence of IL-6 and GM-CSF (WT), IL-6, GM-CSF and ISO (WT + ISO) or IL-6, GM-CSF, ISO and Prop (W ? + ISO + Pro )) for 6 days. (B) T cells co-cultured with VT or WT + ISO MDSCs in various ratios (1=3). (C) Nanostring nCounter microarray analysis of WT or  $\beta$ 2-AR<sup>-/-</sup> MDSCs sorted by flow cytometry from 4T1 tumors of WT or  $\beta$ 2-AR<sup>-/-</sup> mice 25 days after tumor injection (WT or  $\beta$ 2-AR<sup>-/-</sup> MDS 's were pooled from 5 mice per each group). (D) WT and  $\beta$ 2-AR<sup>-/-</sup> MDSCs were sorted from bone marrow of 4T1 tumor bearing mice, cultured with LPS for 24 hours, and cytokines levels were analyzed in culture media using multiplex (n=3). (E) Tumor growth kinetics in WT mice orthot pically injected with 4T1 cells (black square) or co-injected with 4T1 cells and WT M DSCs (blue circle) or 4T1 cells and  $\beta$ 2-AR<sup>-/-</sup> MDSCs (red square). MDSCs were sorted from the BM of tumor bearing mice using an MDSC is blation kit. (F) Tumor growth kinetics in T or  $\beta$ 2-AR<sup>-/-</sup> mice receiving i.v. transfer (3×10<sup>6</sup> on days 3 and 6 after 4T1 injection) of MDSCs sorted the BM of tumor bearing WT or  $\beta$ 2-AR<sup>-/-</sup> mice. Two-way ANOVA was used to analyze statistical significance between tu nor growth in different groups. These data are presented as mean  $\pm$  SEM. Other data are presented as median  $\pm$  min to max. One-way ANOV . was used to analyze statistical significance between three groups, and the students T test was used to analyze stat stical significance between two groups. In all panels \* *P*<0.05, \*\* *P*<0.01 \*\*\* *P*<0.001 and \*\*\*\* *P*<0.001. A P value less than 0.05 was considered significant.

Figure 4



**Figure 4:**  $\beta$ 2-**AR** prolo igs **MDSC** survival. (A) Fas and FasL expression by DSCs and T cells from WT or  $\beta$ 2-**A**R<sup>-/-</sup> mice from tumor and spleen respectively (n=5). (B) Expression of Bcl-2 in intratumoral MDSCs from WT or  $\beta$ 2-A t<sup>-/-</sup> 4T1 tumor bearing mice (n=5). (C) Levels of apoptosis in MDSCs from tumor and spleen of WT or  $\beta$ 2-AR<sup>-/-</sup> tumor bearing mice or WT tumor bearing mice housed at ST or T <sup>'</sup>. (D) Schematic diagram of experimental lesign to compare the survival capability of WT or  $\beta$ ?-AR<sup>-/-</sup> MDSCs. (E) WT (CD45.1) or  $\beta$ 2-AR<sup>-/-</sup> (CD45.2) MDSCs were sorted from b one marrow of AT-3 tumor bearing mice, nixed in 1:1 ratio, and injected into GFP positive AT-3 tumor bearing mice. The percentage of WT (CD45.1) or  $\beta$ 2-AR<sup>-/-</sup> (CD45.2) MDSCs in the live, GFP negative, CD11b<sup>+</sup>, and Gr-1<sup>+</sup> population on day 3 and day 7 after co-injection were analyzed (4 mice per each end point). Data are presented as median ± min to max. The students T test was used to analyze statistic il significance between two groups. In all panels \* *P*<0.35, \*\* *P*<0.01 and \*\*\**P*<0.001. A P value less than 0.05 was considered significant.







**Figure 5:**  $\beta$ 2-AR stimulation in MDSCs activates STAT3 signaling. (A) Repr sentative blot of bone marrow MDSCs sorted from 4T1 tumor bearing mice were treated with or without ISO and the level p-STAT3 was analyzed by western blot. (B) p-STAT3 expression in tumor MDSCs in WT or  $\beta$ 2-AR<sup>-/-</sup> tumor bearing mic: using flowcytometry (n=10, two replicates). (C) Tumor growth kinetics in WT or  $\beta$ 2-AR<sup>-/-</sup> 4T1 tumor bearing mice receiving DMSO or JSI-124 (1mg/kg, i.p. daily injection) (n=6-10 mice from wo replicates). (D) MDSC absolute number in spleen or tumor of W ' 4T1 tumor bearing mice receiving DMSO or JSI-124 (n=5). Two-way ANOVA was used to analyze stat stical significance between tu nor growth in different groups. These data are presented as mean ± SEM. Other data are presented as median ± min to max, and the students T test was used to analyze statistical significance between two groups. In all panels \* *P*<0.05, \*\* *P*<0.01, \*\*\* *P*<0.001 and \*\*\*\* *P*<0.0001. A P value less than 0.05 was considered significant.

в

Figure 6



**Figure 6:** Propranolol suppresses tumor growth and decreases MDSC accu nulation in t te spleen and tumor tissue. (A) Tumor growth kinetics in WT or  $\beta 2$ -AR<sup>-/-</sup> mice orth topically injected with 4T1 tumor cells receiving PBS or propranolol (i.p. daily injection) (n=10). (B) Absolute number of M DSCs in splee 1 and tumor of WT mice treated with PB 3 or proprano ol. (C) Tumor tissue was collected in WT 4T1 tumor bearing mice at day 25 and stailed for Gr-1(20x magnification), CD31 (4x magnification), and VEGF- $\alpha$  (10x magnification) (N=5). (D) Representative flowcytometry plot of MDSCs in WT or  $\beta 2$ -AR<sup>-/-</sup> 4T1 tumor bearing mice receiving saline or 6-OHDA (50mg/kg, i.p weekly injection) (n=6-10 mice from two replicates). (E) Percent and absolute number of MDSCs in tumor and spleen of 4T1 tumor bearing mice receiving saline or 6-OHDA (50mg/kg, i.p weekly injection) (n=5). Two-way ANOVA was used to analyze statistical significance between tumor growth in different groups. The is data are presented as mean ± SEM. Other data are presented as median ± min to max, and the students T test was used to analyze statistical significance between two 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



**Figure 7: Isoproterenol increases 1DSC generation from human PBMCs.** (A) Analysis of  $\beta$ 2-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 with ut ISO for 7 days. (C) The expression of p-STAT3, PDL-1 and arginase-I 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-I 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-I after culturing PBMCs with IL-6 and GM-CSF with or without ISO for 7 days. ( )) Effects of *in vitro* differentiated MDSCs in the presence or absence of ISO on allogenic CD4<sup>+</sup> or CD8<sup>+</sup> T cell proliferation and IFN- $\gamma$  production. One histogram example corresponding to CD8<sup>+</sup> proliferation an lyzed by ef670 dilution dye in a ratio of 1:4 is shown. These data are presented as median  $\pm$  min to max fro n three biological replicates i i all graphs, and the students T test was used to analyze statistical significance between two groups. In all panels \* P<0.05, \*\* P<0.01 and \*\*\*P<0.001. A P value less than 0.05 was considered significant.



Supplementary Figure 1:  $\beta$ 2-AR activation increases inflammatory cytokine levels. (A and B) The levels of inflammatory cytokines in the plasma of mice housed at ST (22°C) or TT (30°C) bearing 4T1 or AT-3 tumors respectively, as determined by multiplex. (C) Inflammatory cytokine levels in WT and  $\beta$ 2-AR<sup>-/-</sup> mice bearing 4T1 tumor cells. Data are presented as mean ± SEM from 5 mice per group from two independent replicates, and the students T test was used to analyze statistical significance between two groups. In all panels \* *P*<0.05, \*\* *P*<0.01 and \*\*\**P*<0.001. A P value less than 0.05 was considered significant.



Supplementary Figure 2:  $\beta$ 2-AR activation increases tumor growth and metastasis. (A) Tumor mass of mice housed at ST (22°C) or TT (30°C) bearing 4T1 and AT-3 tumors. Tumor mass is presented as mean ± SEM from 5 mice per group from two replicates. (B) Tumor mass in WT and  $\beta$ 2-AR<sup>-/-</sup> mice bearing 4T1 tumors. Tumor mass is presented as mean ± SEM from groups of 5 mice from three replicate studies. (C) WT and  $\beta$ 2-AR<sup>-/-</sup> mice were injected with 4T1 cells. On day 25, lung tissue was collected, stained with hematoxylin and eosin, and tumor metastatic nodules were counted. These data are presented as median ± min to max from 5 mice per group from two replicates, and the students T test was used to analyze statistical significance between two groups. In all panels \* *P*<0.05. A P value less than 0.05 was considered significant.



Supplementary Figure 3: β2-AR activation during chronic stress increases the accumulation of MDSCs in blood, lymph node and metastatic lung. (A and B) Percentage of G-MDSCs and M-MDSCs in blood (A) and lymph node (B) of healthy or tumor bearing mice (4T1 or AT-3) day 15 after tumor injection housed in standard temperature (ST) or thermoneutral temperature (TT). (C) On day 25, lung tissue was collected, crushed, disassociated with a tumor disassociation kit, and single cell suspensions were prepared. CD11b<sup>+</sup> Gr-1<sup>+</sup> cells were gated from live CD45<sup>+</sup> cells. These data are presented as median ± min to max from 5 mice per group from two replicates, and the students T test was used to analyze statistical significance between two groups. In all panels \* P<0.05 and \*\* P<0.01. A P value less than 0.05 was considered significant.



Supplementary Figure 4:  $\beta$ 2-AR activation increases the immunosuppressive function of MDSCs. (A) Arginase I and PDL-1 expression by intratumoral MDSCs of 4T1 tumor bearing mice measured by flow cytometry 25 days after tumor injection (n=5). (B) T cells co-cultured with MDSCs sorted by MDSCs isolation kit from 4T1 tumors of WT or  $\beta$ 2-AR<sup>-/-</sup> mice (n=3). These data are presented as median ± min to max, and the students T test was used to analyze statistical significance between two groups. In all panels \* p<0.05 and \*\* p<0.01. A P value less than 0.05 was considered significant.



Supplementary Figure 5: Direct  $\beta$ 2-AR activation by salbutamol increases tumor growth and accumulation of MDSC in the spleen and tumor tissue. (A) Tumor growth kinetics of 4T1 tumors orthotopically injected into WT mice. Mice were treated with PBS or salbutamol (i.p. daily injection) (n=10). (B and C) Absolute number of MDSCs in spleen (B) and tumor (C) of WT mice treated with PBS or salbutamol (n=5). Two-way ANOVA was used to analyze statistical significance between tumor growth in different groups. These data are presented as mean ± SEM. Other data are presented as median ± min to max, and the students T test was used to analyze statistical significance between two groups. In all panels \* P<0.05 and \*\*\*\* P<0.0001. A P value less than 0.05 was considered significant.