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β3-adrenoceptor as a potential immuno-suppressor agent in melanoma

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Abstract

Background and purpose. Stress-related catecholamines have a role in cancer and β-adrenoceptors, specifically β2-adrenoceptors have been identified as new targets in treating melanoma. Recently, β3-adrenoceptors have shown a pleiotropic effect on melanoma microenvironment leading to cancer progression. However, the mechanisms by which β3-adrenoceptors promote this progression remain poorly understood. Catecholamines affect the immune system by modulating several factors that can alter immune cell sub-population homeostasis. Understanding the mechanisms of cancer immune-tolerance is one of the most intriguing challenges in modern research. This study investigates the potential role of β3-adrenoceptors in immune-tolerance regulation.

Experimental Approach. A mouse model of melanoma in which syngeneic B16-F10 cells were injected in C57BL-6 mice was used to evaluate the effect of β-adrenoceptor blockade on the number and activity of immune cell sub-populations (Treg, NK, CD8, MDSC, macrophages and neutrophils). Pharmacological and molecular approaches with β-blockers (propranolol and SR59230A) and specific β-adrenoceptor siRNAs targeting β2- or β3-adrenoceptors were used.

Key Results β3- only, not β2-adrenoceptors, were upregulated under hypoxia in peripheral
blood mononuclear cells and selectively expressed in immune cell sub-populations including Treg, MDSC and NK. SR59230A and β3-adrenoceptor siRNAs increased NK and CD8 number and cytotoxicity, while abrogated Treg and MDSC sub-populations in tumor mass, blood, and spleen. SR59230A and β3-adrenoceptor siRNAs increased M1/M2 macrophages ratio and N1 granulocytes.

**Conclusion and implication.** Our data suggest an involvement of β3-adrenoceptors in immune-tolerance, opening the way to new strategic therapies to overcome melanoma growth.

**Introduction**

Several studies demonstrate that tumor neurogenesis or stress-related catecholamines, norepinephrine (NE) and epinephrine (E), accelerate cancer progression and reduce the overall survival of patients (Cole and Sood, 2012; Magnon et al., 2013). The increased secretion of catecholamines usually promotes favourable environment for tumor cells to grow and metastasize predominantly by acting at β-adrenoceptors (β-ARs) (Entschladen et al., 2004). Signaling activated by β-ARs regulates tumor growth, progression and metastasis by influencing a number of cellular and molecular processes (Armaiz-Pena et al., 2013; Cheng et al., 2018).

There is evidence that stress-related catecholamines enhance tumor growth mainly through β2-ARs, and that non-selective β-AR blockers (acting at β1- and β2-ARs) provide protection against different types of cancer (Childers et al., 2015; Yazawa et al., 2016). Melanoma, like other tumors, shows a surprisingly positive response to propranolol, a β-AR blocker targeting β1- and β2-ARs (Glasner et al., 2010; Barbieri et al., 2012) although the role of β1-ARs in stimulating melanoma growth, and tumor growth in general, seems to be questionable (Armaiz-Pena et al., 2013; Dal Monte et al., 2013; Thaker et al., 2006). In addition, propranolol reduces cell proliferation in human and murine melanoma cell lines (Yang et al.,...
The positive impact of β1- and/or β2-AR blockade in the overall survival of melanoma patients (De Giorgi et al., 2011; Lemeshow et al., 2011; De Giorgi et al., 2017; Kokolus et al., 2017), although these findings have been recently called into question (Livingstone et al., 2013; McCourt et al., 2014).

A role for β3-ARs in melanoma has been proposed and recently reviewed (Dal Monte et al., 2018). In fact, the use of two different β3-AR blockers, SR59230A and L-748337, is effective in reducing tumor growth in a mouse model of melanoma (Dal Monte et al., 2013; Sereni et al., 2015). In addition, SR59230A and L-748337 as well as selective β3-AR siRNAs reduce the proliferation and induce apoptosis of human and mouse melanoma cells (Dal Monte et al., 2013; Dal Monte et al., 2014; Calvani et al., 2015), while β3-AR agonism stimulates melanoma cell proliferation and reduces apoptosis (Dal Monte et al., 2014). However, the mechanisms by which β3-ARs may promote melanoma growth are not fully elucidated yet.

There is evidence that mechanisms of immune-tolerance, which are known to prevent autoimmune diseases may be used by tumors to bypass the development of an effective immune response (King et al., 2018). Immune cells in cancer exhibit functional plasticity and undergo a dramatic phenotypic change, leading to an alternative activation promoting tumor progression by inducing immune-tolerance (Granot and Fridlender, 2015). Currently, the mechanisms of cancer immune-tolerance are not yet completely clarified. Established tumors with higher mutation rates use various escape mechanisms to bypass immune-surveillance including a decrease of cytotoxic immune cells as natural killer cells (NK) and CD8 T cells (CD8) and/or an increase of immune-suppressive cells as the so-called myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) (Vinay et al., 2015). In addition, the phenotype of myeloid cells (macrophages and neutrophils), which plays a key role within the
immunosuppressive network, may be altered by the tumor microenvironment: an environment rich in M2 macrophages and N2 neutrophils enhances immune-escape and supports tumor growth (Schouppe et al., 2012).

The β-adrenergic system has been identified as one of the major players in the regulation of the immune system. In this context, catecholamines bind to specific receptors, in particular β2-ARs (Nance and Sanders, 2007), on white blood cells and have diverse regulatory effects on the distribution and function of these cells that mainly result in immunosuppression. For instance, the stimulation of β2-ARs inhibits lymphocyte responses, NK cytotoxicity and dendritic cell functions (Marino and Cosentino, 2013). In addition, β-AR signaling significantly suppresses the proliferation and the cytolytic killing ability of cytotoxic CD8 as well as their capability to produce interferon γ (Nissen et al., 2018).

Moreover, MDSC are increased in patients with breast cancer characterized by high levels of stress (Mundy-Bosse et al., 2011). Finally, studies using rodent models of different tumors have shown that catecholamines or stress, suppress NK activity leading to tumor metastasis, likely through β2-AR stimulation (Inbar et al., 2011).

Melanoma is one of the most immunogenic tumors and it is highly sensitive to immune therapeutic agents (Tawbi et al., 2018). Of note, melanoma microenvironment is enriched in tumor-associated M2 macrophages, Treg and MDSC, which promote the defective cytotoxicity of T-cells (Fujimura et al., 2012). In addition, melanoma cells inhibit CD8 and NK activity through the production of negative modulators such as VEGF, IL-8, and IL-10 (Passarelli et al., 2017). Little is known about a role of β-ARs in regulating the immune environment in melanoma, being the only evidence limited to the findings that β-adrenergic stimulation recruits and polarizes macrophages, thus promoting tumour progression (Cole et al., 2015), and that β2-AR blockade improves the anti-tumor efficacy of immunotherapy (Kokolus et al., 2017).
This study evaluates the potential role of β3-ARs in the regulation of melanoma immune-tolerance by investigating the effects of its antagonism in cytotoxic and suppressive immune cell sub-populations. In addition, the regulatory role of β3-ARs was compared with that of β2-ARs with the use of pharmacological and molecular approaches. This research suggests the possibility that β3-AR antagonism could reduce melanoma growth in vivo by increasing the number of NK and CD8 as well as their cytotoxicity, and by abrogating Treg and MDSC sub-populations in tumor microenvironment. A shift in macrophage and neutrophil phenotypes from both M2 to M1 and N2 to N1 was also observed after β3-AR blockade.

Methods

Cell cultures.
Murine B16-F10 melanoma cell lines were obtained from American Type Culture Collection (ATCC, Cat# CRL-6475, RRID:CVCL_0159). Cells were maintained in DMEM containing 10% fetal calf serum (FCS) (Euroclone, Milan, Italy), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO2. The cell lines have been mycoplasma tested (Euroclone, Milan, Italy).

In vivo transfection assay.
SiRNA β2 (SASI_Mm01_00154297) and siRNAβ3 (SASI_Mm01_00145466) were complexed with 200 μl of Invivofectamine reagent-plasmid duplex (reagent for in vivo plasmid delivery, Invitrogen, Carlsbad, CA, USA) and were injected into the vein tail when a palpable tumor was formed (as described below). In vitro transfection assay with β2- and β3-siRNA was performed by using Polyplus INTERFERin siRNA Transfection Reagent. The efficiency of β2- and β3-siRNA was assessed by cytofluorometric analysis of proteins
Western Blot.

β-ARs expression was evaluated in murine lymphocytes, isolated from mice and cultured in normoxia (24h at 21% O₂), hypoxia (24h at 1% O₂) and 1h of normoxic re-exposure after 24h of hypoxia. Cell lysates were prepared in an appropriate volume of RIPA lysis buffer. After protein quantification (Bradford, Biorad, Hercules, CA, USA), 20 μg of proteins from total lysates were subjected to SDS-PAGE and Western blot analysis as reported in previous work. Rabbit polyclonal antibodies directed to β2-ARs (Santa Cruz Biotechnology Cat# sc-569, RRID:AB_630926) and β3-ARs (Santa Cruz Biotechnology Cat# sc-50436, RRID:AB_781613) have been recently validated (Sereni et al., 2015).

Co-cultures and MTT assay.

Tumor cells were seeded in MW24 and pre-treated with propranolol or SR59230A (10μM) for 24h. Subsequently, the tumor cells were washed with PBS and co-cultured with peripheral blood mononuclear cells (PBMC) (pre-treated or not with propranolol or SR59230A (10μM) for 24h) in a seeding ratio of 1:3 for the consecutive 24h. At the end of the total 48h, PBMC were withdrew from the medium of the co-culture and analyzed by FACS, while tumor cells were tested for viability assay. Viability of tumor cells, in all conditions, was evaluated using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) assay (Sigma Aldrich, Saint Louis, MO, USA) following manufacturer’s instructions. The intensity of the absorbance at 550nm was evaluated using a spectrophotometer (FlexStation3, Molecular Devices). The same experiment was performed in each tumor cell line silenced or not with Scr-siRNA, β2-siRNA, and β3-siRNA.
Mice

In vivo experiments and tissue collection were carried out according to the European Union (EU) guidelines for animal care procedures and the Italian legislation (DLgs 26/2014) application of the EU Directive 2010/63/EU. Studies were conducted under University of Florence and Italian Health Minister research permits n° 401/2015-PR. C57BL/6 mice (male, 20-25 g, 5-6 weeks; IMSR Cat# JAX:000664, RRID:IMSR_JAX:000664) were used. Animals were housed in a temperature and humidity-controlled vivarium (12h dark/light cycle, free access to food and water, maximum 10 animals per cage). All the experiments were performed in a quiet, temperature-controlled room (20-22°C). Animals were euthanized with inhaled CO₂ plus 10-50% O₂.

Mouse B16-F10 syngeneic model and treatments

B16-F10 were implanted in C57BL/6 recipient mice by injecting 1x10⁶ cells in 200 µl phosphate buffered saline (PBS) subcutaneously (s.c.) in the right flank of the mice. Mice were monitored daily. After 8-10 days, when B16-F10 cells formed a palpable tumor, the treatment was started. The treatments were administered twice a day with a window time of 4-6 hours between each treatment. Diluted DMSO (vehicle), propranolol (20 mg/kg/day, Saint Louis, MO, USA), and SR59230A, CAS: 174689-39-5 (20 mg/kg/day, Sigma Aldrich, Saint Louis, MO, USA) were injected intraperitoneally (i.p.). Mice were sacrificed at day 7 and 14 of treatment; peripheral blood was collected, tumor and spleen were weighed and measured. B16-F10-GFP cell lines (Creative Biogene Biotechnology Cat# CSC-RR0109, RRID:CVCL_QZ86) were injected in C57BL/6 mice to precisely discriminate the tumor cells from tumor stroma.
**In vivo magnetic resonance imaging**

For the *in vivo* MRI imaging, C57BL/6 mice were fully anesthetized for the acquisition procedure with Avertin (2,2,2-tribromoethanol; 0.3 mg/g of body weight). T2-weighted scans of the mice were performed using 7T Bruker PharmaScan MRI scanner. T2-weighted images were acquired by spin-echo sequences with TR = 1642 ms, TE = 25 ms, FOV = 4.0 × 4.0 cm, matrix size = 256 × 256, 15 slices and slice thickness of 1 mm.

**Isolation of tumor cells and preparation of spleen and blood cells**

Mouse tumor tissues were minced with scissors and incubated in C-Tubes (Miltenyi Biotec, Gladbach, Germany) with a storage tissue solution (Miltenyi Biotec, Gladbach, Germany). Tumor samples were then homogenized using a Tumor Dissociation Kit (Miltenyi Biotec, Gladbach, Germany) and the heating function of the gentle MACS Octo Dissociator (Miltenyi Biotec, Gladbach, Germany) with appropriate heaters run program. After homogenization, the samples were filtered with pre-separation filters, to remove cell aggregates or large particles. The lymphocyte cells were then separated by tumor sample, using anti-CD45 beads (Miltenyi Biotec, Gladbach, Germany) and AutoMACS separator Pro (Miltenyi Biotec, Gladbach, Germany), according to the manufacturer’s instructions. Mouse spleens were homogenized in PBS using gentleMACS Octo Dissociator and then filtered with pre-separation filters. Mouse blood was diluted in Red Blood Cell Lysis Solution 10X (Miltenyi Biotec, Gladbach, Germany), for optimal lysis of erythrocytes, according to the manufacturer’s instructions.

**Flow cytometry and morphologic analysis**

Cells isolated from mouse tumors, spleens and blood were incubated and stained with appropriate dilutions of various combinations of the following fluorochrome-conjugated
antibodies: anti-CD 45-VioBlue (Miltenyi Biotec Cat# 130-092-880, RRID:AB_1103220) or VioGreen (Miltenyi Biotec Cat# 130-096-906, RRID:AB_2660419), anti-NKp46-FITC (Miltenyi Biotec Cat# 130-102-300, RRID:AB_2661345), anti-CD8a-APC Vio 770 (Miltenyi Biotec Cat# 130-102-305, RRID:AB_2659897), anti-CD3e (17A2)-PE Vio 770 (Miltenyi Biotec Cat# 130-105-461, RRID:AB_2657921), anti-CD107-PE (Miltenyi Biotec Cat# 130-111-318, RRID:AB_2654464), anti-CD161(NK1.1)-PerCP Vio700 (Miltenyi Biotec Cat# 130-117-773, RRID:AB_2728038), anti-CD25-PE (Miltenyi Biotec Cat# 130-102-593, RRID:AB_2659902), anti-CD127-APC (Miltenyi Biotec Cat# 130-110-274, RRID:AB_2654842), anti-CD11b-PerCP Vio700 (Miltenyi Biotec Cat# 130-109-289, RRID:AB_2654659), anti-Gr1-PE (Miltenyi Biotec Cat# 130-102-426, RRID:AB_2659861), anti-CD95 (FAS)-APC (Miltenyi Biotec Cat# 130-106-907, RRID:AB_2659651), anti-F4/80-PerCP Vio700 (Miltenyi Biotec Cat# 130-102-161, RRID:AB_2651711), anti-CD16/32-VioBright FITC (Miltenyi Biotec Cat# 130-108-364, RRID:AB_2660221), anti-CD11c-APC Vio770 (Miltenyi Biotec Cat# 130-107-461, RRID:AB_2660162), anti-IL10-APC (Miltenyi Biotec Cat# 130-102-349, RRID:AB_2660626), anti-Integrin alpha 7-APC (Miltenyi Biotec Cat# 130-102-717, RRID:AB_2652466), anti-INOS-APC (Santa Cruz Biotechnology Cat# sc-7271, RRID:AB_627810), anti-Arg1-FITC (R and D Systems Cat# IC5868F, RRID:AB_10718118), anti-beta2-FITC (Biorbyt Cat# orb15065, RRID:AB_10735676), anti-beta3-PE (Biorbyt Cat# orb124479, RRID:AB_2783863) or PerCP Vio700 (Biorbyt Cat# orb123003, RRID:AB_2783864). For intracellular staining, the cells were further permeabilized using Inside Stain Kit (Miltenyi Biotec, Gladbach, Germany), and then stained for iNOS, IL10, β2- and β3-ARs. The stained cells were acquired on MACSQuant Analyzer 10 Flow Cytometer (Miltenyi Biotec, Gladbach, Germany) and the data were processed using Flowlogic software (Miltenyi Biotec, Gladbach, Germany).
**Statistical Analysis**

Statistical analysis was performed using the SAS 9.2 Software. Values are presented as mean ± SD. Differences with \( P < 0.05 \) were considered significant.

For a t-test with Bonferroni correction for multiple comparison, an expected tumor growing difference of 2.5 cm\(^3\) between groups (8, 5.5 and 3 cm\(^3\) in vehicle, propranolol and SR group respectively), a standard deviation for each group of 2 cm\(^3\) (Serien et al. 2015) and a first type error set to 1.7%, 6 mice for group were needed to guarantee a power of 80%. Allocation concealment was performed using a randomization procedure (http://www.randomizer.org/).

To assess normal distribution and homoscedasticity for each quantitative outcome in each group Kolmogorov-Smirnov’s test and Bartlett’s Test was used respectively.

In order to evaluate difference in quantitative outcomes between groups, according to normality and homoscedasticity tests results, ANOVA and posthoc t-test with Bonferroni correction for multiple comparison or Welch ANOVA and posthoc Satterthwait t-test with Bonferroni correction for multiple comparison or Kruscal-Wallis and Dwass, Steel, Critchlow-Fligner method for multiple comparison were used. Posthoc test was performed only if ANOVA, Welch ANOVA or Kruskal-Wallis analysis were statistically significant.

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018).

**Nomenclature of targets and ligands**

Key protein targets and ligands in this article are hyperlinked to corresponding entries in [http://www.guidetopharmacology.org](http://www.guidetopharmacology.org), the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017a,b,c).
Results

β-ARs expressed in immune cells influence melanoma cell viability through an effect on immune cells

We first analyzed the expression of both receptors in PBMC and in immune cell sub-populations to assess whether β2- and β3-ARs could be implicated in regulating melanoma cell viability by affecting the immune system. Previous studies demonstrated β2-ARs expression in immune cell sub-populations such as NK (Maisel et al., 1989), CD8 (Estrada et al., 2016), Treg (Guereschi et al., 2013), without exploring the presence of β3-ARs. In this study, we have observed that both β2- and β3-ARs were expressed in mouse PBMC, but only β3-ARs were up-regulated under hypoxic conditions, used to mimic the tumor microenvironment, and fast down-regulated after oxygen re-exposure (Figure 1A). We have then performed a cytofluorimetric analysis to evaluate the expression of β2- and β3-ARs in different PBMC sub-populations isolated from either blood or tumor tissue, including NK, Treg, and MDSC. The expression of β2-ARs in NK, Treg and MDSC infiltrating the tumor did not differ from that in circulating cells, while β3-ARs were up-regulated in the immune cell sub-populations infiltrating the tumor (Figure 1B).

We evaluated the hypothesis that β3-ARs expressed in PBMC, and in particular in tumor infiltrating lymphocytes (TILs), could affect tumor cell viability. We co-cultured under hypoxic conditions B16-F10 cells with mouse PBMC pre-exposed to SR59230A, propranolol or selective siRNAs targeting β2- or β3-ARs to assess this hypothesis. The results have shown that co-culturing B16-F10 cells with PBMC pretreated with SR59230A induced an increase in cell death compared to melanoma cells treated with SR59230A only, or cultured exclusively with PBMC. Pretreatment of PBMC with propranolol had no significative effects on cell viability. The silencing approach substantially confirmed that the effects obtained
with SR59230A were due to the blockade of β3-ARs (Figure 1C and 1D). The enhanced efficacy of SR59230A pre-treatment in PBMC suggests that β3-AR antagonism, by acting on immune cells, might pilot cancer cell death.

**Targeting β2- or β3-ARs reduces tumor growth in a mouse model of melanoma.**

In line with previous findings (Dal Monte et al., 2013), additional investigation performed in this work allows to confirm that propranolol or SR59230A administered for 14 days significantly reduced tumor volume (Figure 2A), with a major effect of β3- over β2-AR blockade, as also confirmed by the greater efficacy of SR59230A and β3-AR silencing approach (Figure 2B). Moreover, the administration of the β2-AR agonist terbutaline to mice treated with β3-AR siRNA showed no growth rebound, thus confirming the predominant role of β3-AR subtype in controlling tumor growth. At the same time, the administration of the β3-AR agonist BRL37344 in β3-AR-silenced mice did not affect tumor growth compared to siRNA-β3-AR condition alone (Figure Supplementary 2). The efficacy of propranolol and SR59230A in reducing the tumor volume was also demonstrated here by nuclear magnetic resonance (Figure 2C). The rate of tumor cell death in the tumor mass was analyzed by cytofluorimetric analysis, injecting into C57BL/6 mice a stable B16-F10 cell line expressing the green fluorescent protein (B16-F10-GFP), to precisely discriminate the tumor cells from tumor stroma. Both propranolol and SR59230A induced an increased early apoptotic rate after 7 days of treatment, with an effect of SR59230A which was significantly higher than that of propranolol (Figure 2D). In addition, in the tumor mass both propranolol and SR59230A, with a major effect of SR59230A, increased the apoptotic marker FAS (Figure 2E), which is known to bind its ligand in NK or neutrophils, thus leading to apoptosis of tumor cells (Abrahams et al., 2003). The early apoptotic rate after SR59230A treatment decreased after 14 days in favor of an extensive cell death in the tumor mass, as indicated by
large areas of tissue necrosis (Figure 2F).

**Targeting β3-ARs induces immune competent sub-populations in the tumor microenvironment**

The possible role of immune cells expressing β-ARs in counteracting melanoma growth was also investigated in the *in vivo* model treated with propranolol or SR59230A, or with β2- or β3-AR siRNA. As shown in Figure 3A, the spleen weight, used as an indirect indicator of the immune system activation, was significantly higher in SR59230A-treated than in vehicle-treated mice. On the contrary, propranolol did not affect the spleen weight. The effect of SR59230A was maximally detected after 7 days of treatment. As shown in Figure 3B and 3C, the cytofluorimetric analysis of cells from the tumor mass of SR59230A-treated mice showed an increased number of NK and CD8, which was already detectable after 7 days of treatment. SR59230A also led to the activation of NK and CD8 cytotoxic activity as evidenced by the increased expression of the activation markers perforin (van den Broek and Hengartner, 2000) and CD107A (Aktas et al., 2009), respectively (Figure 3D and 3E). Propranolol did not affect the number of NK or CD8 nor induced perforin or CD107A expression. The silencing approach confirmed the prevalent role played by β3-ARs (Figure 3F and 3G).

**Prevalent role of β3-ARs in reducing immune-suppressive sub-populations in the tumor microenvironment**

We have also analyzed the number of Treg and MDSC sub-populations in excised tumor mass, as shown in Figure 4A and 4B. Treatments with either propranolol or SR59230A reduced Treg and MDSC immune-suppressive sub-populations with an effect of SR59230A which was significantly higher than that of propranolol. In addition, the CD8/Treg ratio was
significantly increased by SR59230A, while it was not affected by propranolol (Figure 4C). Furthermore, the expression of the inducible form of nitric oxide synthase (iNOS), a landmark of the immune-suppressed phenotype in MDSC (Mazzoni et al., 2002), was reduced by both propranolol and, more effectively, SR59230A (Figure 4D). The silencing approach substantially confirmed the immune-suppressive action of β3-ARs (Figure 4E and 4F).

**Prevailing role of β3-ARs in inducing macrophage M1 and neutrophil N1 phenotypes in the tumor microenvironment**

An immune-suppressive microenvironment is characterized by high density of M2 macrophages and N2 neutrophils while an immune-competent microenvironment is characterized by the presence of M1 macrophages and N1 neutrophils (Mantovani and Locati, 2013; Fridlender et al., 2009). We have observed that SR59230A, but not propranolol, increased the M1/M2 ratio in the tumor microenvironment (Figure 5A), likely by reducing the M2 sub-population, as evidenced by the reduced levels of IL-10, a marker of M2 macrophages (Figure 5B). In addition, SR59230A, but not propranolol, increased the number of N1 cells and decreased the expression of arginase-1, a marker of immune suppression in immune cells (Munder, 2009), in neutrophils (Figure 5C and 5D). The silencing approach substantially confirmed that the neutrophils’ and macrophages’ polarization was influenced by both β2- and β3-ARs, but with a β3-ARs stronger activity (Figure 5E and 5F).

**Targeting β2- or β3-ARs affects immune sub-populations in the spleen and the blood**

Results from the analysis of immune-competent NK and CD8 sub-populations in the spleen and the blood from mice treated with propranolol, SR59230A or siRNAs were in line
with those obtained in tumor infiltrating cells and demonstrated a major effect of β3-AR blockade (Figure 6A-6D). On the other hand, propranolol and SR59230A resulted equally effective in reducing the immune-suppressive Treg and MDSC sub-populations in the spleen and the blood (Figure 7A and 7B). The silencing approach confirmed similar effects of β2- or β3-AR antagonism in Treg reduction. On the contrary, silencing β3-ARs was more effective than silencing β2-ARs in reducing the MDSC sub-population (Figure 7C and 7D).

Discussion

The fine control of immune cell sub-populations is completely reverted in cancer, favoring an immune-tolerant phenotype. Understanding the mechanisms promoting this immunologic shift is currently one of the most important challenges in oncologic research.

Although conflicting results have been reported on the regulation of the immune system by catecholamines in humans, the majority of reports agree that E and NE act as immunosuppressive factors. In early studies, elevated NK activity has been reported after E infusion, open-heart surgery or physical exercise (Pedersen et al., 1988). However, subsequent findings have suggested that the observed increase in NK activity is due to a marked, but transitory, increase in the number of circulating NK, rather than to an increase in activity per NK cell (Palmø et al., 1995). This increase in the number of circulating NK occurs during the time of elevated catecholamine levels, and dissipates shortly after catecholamine decline (Benschop et al., 1996). The additional fact that NE impairs the cytotoxicity of NK and that β2-AR activation suppresses CD8 cytotoxicity further supports the immune-suppressive role of the β-adrenergic system (Gan et al., 2002). While the role of β2-ARs on immune cell has been widely studied, both the expression and the possible role of β3-ARs has not been clarified yet. Here, we provide some evidences suggesting a new possible role of β3-ARs in the regulation of melanoma immune-tolerance in tumor bearing
mice.

In this study, propranolol has been used as non-selective β1-/β2-AR antagonist, and SR59230A has been chosen as a β3-AR antagonist and preferred to a different antagonist, L-748,337, because of its higher affinity for β3-ARs in rodents (Candelore et al., 1999). However, even though SR592390A has been previously indicated as a selective β3-AR antagonist, a similar affinity has been demonstrated for all the three subtypes (Hoffmann et al., 2004; Baker, 2005; Niclauss et al., 2006). A second issue is related to the fact that SR59230A can act as a partial agonist, with the degree of partial agonism strongly depending on the model system. In addition, in some systems, SR59230A acts as a full agonist (Vrydag and Michel, 2007; Sato et al., 2007). In this context, the siRNA molecular approach represents a useful tool to clarify the real mechanism of action of SR59230A and, as previously observed, the silencing approach has demonstrated that in B16-F10 cells SR59230A actually act at β3-ARs (Dal Monte et al., 2013). According to this study, the in vivo use of selective β3-AR siRNAs provides results overlapping with those obtained with SR59230A, thus confirming the role of SR59230A as β3-ARs antagonist in this scenario. Moreover, the administration of the β2-AR agonist terbutaline to pre-silenced β3-AR melanoma bearing mice, showed no rebound in tumor growth demonstrating the predominant role of β3-AR subtype in controlling tumor evolution (Supplementary Figure 2). In addition, the difference between SR59230A and propranolol treatment observed in the present study could depend on the β3-AR component, since both drugs are saturating the receptors, as we can extrapolate from previous studies in the same mouse model used here and from literature data on the relative affinity of SR59230A and propranolol for β-ARs (Hoffmann et al., 2004; Dal Monte et al., 2013). In addition, the fact that, based on the difference of molecular weight between SR59230A and propranolol, the molar doses of propranolol used in the present study are higher than those of SR59230A can be also considered. Assuming these premises, this
study supports the hypothesis that the blockade of β2- or β3-ARs may promote immune-competence in TILs of melanoma bearing mice, and that β3-ARs could play a major role over β2-ARs in melanoma immune-escape.

The observation that the expression of β3-ARs, but not β2-ARs, in mouse lymphocytes was up-regulated in a hypoxic environment and down regulated after oxygen re-exposure, suggests a fine post-translational regulation of β3-AR protein under oxygen control. This is intriguing, considering that hypoxia is indicated as one of the most important regulators of cancer immune-tolerance (Facciabene et al., 2011) and supports the hypothesis that β3-ARs might participate, in a hypoxic environment, to the acquisition of an immune-tolerant phenotype.

T-lymphocytes are known to express both β2- and β3-ARs, of which β3-ARs were mostly up-regulated in response to stress (Laukova et al., 2012). In addition, the present finding that β3-ARs are localized to immune cell sub-populations involved in both immune-suppression (Treg and MDSC) and immune-toxicity (CD8 and NK) suggests a possible role of β3-ARs in immune response. As shown by our results, both SR59230A and propranolol counteract melanoma growth in vivo and their effect is concomitant with a significant increase in NK and CD8 and a strong reduction in Treg and MDSC within the tumor mass. More precisely, SR59230A appears to act with greater effectiveness when compared with propranolol. The additional finding that the effects of SR59230A are mimicked by β3-AR silencing supports a role for β3-ARs in mediating the switch from an immunosuppressive to an immunocompetent tumor microenvironment. This shift is better documented at T7 than at T14, suggesting that after 14 days of treatment the immune reactivity is in regression and mouse melanoma in resolution, as demonstrated here by the high rate of necrosis at T14. Although such suggestions on the role of β3-ARs on the immune system, one of the main limitations of the present study is the inability to precisely discriminate if the effect observed
in immune phenotype is directly related to β3-AR blockade in immune cells or is a consequence of the tumor growth reduction. This study, indeed, did not evaluate the cause-effect relationships between tumor cell death and immune modulation in vivo, which might be evaluated in future by selective direct manipulations of immune cell sub-populations. However, the results obtained with pre-treatment of PBMC with SR59230A or selective β3-AR siRNAs suggest a direct effect of β3-AR blockade on immune cells, independent from an action on tumor cells.

The loss of the cytotoxicity of tumor-infiltrating M1 macrophages and N1 neutrophils represents a substantial barrier to immune clearance of solid tumors (Jaiswal et al., 2010; Nicolás-Ávila et al., 2017). As shown here, SR59230A and β3-AR siRNAs, but not propranolol or β2-AR siRNAs, induce a strong increase in M1 macrophage and N1 neutrophil populations within the tumor microenvironment, suggesting that the phenotypic shift in macrophages and neutrophils is mainly mediated by β3-ARs. These finding is consistent with the recent description that β3-AR agonism inhibits the pro-inflammatory (M1) activity of macrophages (Hadi et al., 2017) and with previous studies demonstrating that there is a phenotypic plasticity of macrophages and neutrophils in the tumor microenvironment (Schouppe et al., 2012).

In conclusion, this study supports the hypothesis that β3-ARs might play a role in the promotion of immune-tolerance of melanoma. If future experiments will confirm the causative effect of β3-AR blockade on the immune system editing and tumor resolution, β3-AR blockade could represent a new strategy to overcome cancer immune-editing and an effective therapy against melanoma. Unfortunately, the poor pharmacologic profile of the currently available β3-AR blockers may limit the development of future therapies. On the other hand, the fact that the results from the siRNA approach fit well with those from the pharmacological study supports the possibility that selective β3-AR antagonists (when
available) could be metamorphosed from experimental tools into therapeutic drugs.

Authors Contributions

LF and MC developed the concept and experiment and wrote the manuscript. GB, FF, and LC performed and analyzed the animal model and functional assays. MC, AC, and MB performed flow cytometry experiments. RN and FD performed immunohistochemical analysis. FB and AP performed RMI analysis. MDM, GF, GlM, LC, PB, PC, CA, PG, and CF revised the experiments and the manuscript.

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Conflicts of Interest

None of the authors has competing interests or potential conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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Figure 1. A) Representative WB of β3-ARs in murine lymphocytes after 24h of normoxic (N) or 24h hypoxic conditions (H) and 1h of normoxic re-exposure (R) and relative densitometric quantification (n=6). Results are reported as mean ± SD of relative expression.
normalized to β-actin. (**) P<0.01, (***) P<0.001 hypoxic (H) and re-exposure conditions compared with normoxic (N). B) FACS quantification of β2- and β3-ARs expression in blood and tumor infiltrating NK, Treg and MDSC. (**) P<0.01, (****) P<0.0001 β3-AR in tumor compared with β3-AR in blood (n=6) C) MTT cell viability assay in B16-F10 cells untreated or treated with Prop or SR and co-cultured for 48h under hypoxic condition with PBMC untreated or pre-treated with Prop or SR. Dot plots show the changes of each treatment compared with Ctrl (n=6). (ns= not significative, * P<0.05 PBMC/SR or SR+(PBMC/SR) compared with SR) D) MTT cell viability assay in B16-F10 cells silenced with siRNA-Ctrl, siRNA-β2 or siRNA-β3 and co-cultured for 48h under hypoxic condition with PBMC untreated or pre-treated with Ctrl-siRNA, siRNA-β2 or siRNA-β3. Dot plots show the changes of each treatment compared with Ctrl (n=6). (ns= not significative, *P<0.05 PBMC/siRNA-β3 or siRNA-β3+(PBMC/siRNA-β3) compared with siRNA-β3).
Figure 2. A) Tumor growth rate in control- (Crl), vehicle- (Veh), propranolol- (Prop) and SR59230A- (SR) treated mice (n=6). B) Tumor growth rate in siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6). C) MR images of mouse ventral section in control- (Crl),
vehicle- (Veh), propranolol- (Prop) and SR59230A- (SR) treated mice (n=6). D) FACS analysis of AnnexinV positive cells in control- (Crl), vehicle- (Veh), propranolol- (Prop) and SR59230A- (SR) treated mice (n=6). E) FACS analysis of Fas marker expression in tumors of control- (Crl), vehicle- (Veh), propranolol- (Prop) and SR59230A- (SR) treated mice (n=6). F) Representative fields of hematoxylin-eosin (H&E) staining at T14 in control- (Crl), vehicle- (Veh), propranolol- (Prop) and SR59230A- (SR) treated mice (n=6).

(**** P<0.0001, $$$ P<0.001, ** P<0.01, # P<0.05) Prop- (or siRNA-β2) compared with Veh-, # # P<0.01, # # # P<0.001, # # # # P<0.0001 SR- (or siRNA-β3) compared with Veh-, $ P<0.05, $ $ P<0.01, $ $$ P<0.001, $ $$$ P<0.0001 SR- (or siRNA-β3) compared with Prop- (or siRNA-β2))
Figure 3. A) Representative images of mouse spleens at T7 (n=6) (left) and mean weight of mouse spleens (right). B) FACS analysis and quantification at T7 and T14 of NK (NKp46+/NK1.1+ gated on CD3-/CD45+). C) FACS analysis and quantification at T7 and T14...
of CD8⁺ (gated on CD45⁺). D) FACS analysis and quantification at T7 and T14 of perforin
to NKp46⁺/NK1.1⁺ cells. Two-way Anova analysis was performed. E) FACS
expression on NKp46⁺/NK1.1⁺ cells. Two-way Anova analysis was performed. E) FACS
analysis and quantification at T7 and T14 of CD8⁺ cytotoxic (CD107⁺ gated on CD8⁺). F)
FACS analysis and quantification at T14 of NK (NKp46⁺/NK1.1⁺ gated on CD3⁻/CD45⁺)
cells in siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6). G) FACS analysis and
quantification at T14 of CD8⁺ (gated on CD45⁺) cells in siRNA-CTRL, siRNA-β2 and
siRNA-β3 treated mice (n=6).

(* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 Prop- (or siRNA-β2) compared with
Veh-, # P<0.05, ## P<0.01 ### P<0.001, #### P<0.0001 SR- (or siRNA-β3) compared with
Veh-, $ P<0.05, $$ P<0.01, $$$ P<0.001, $$$$ P<0.0001 SR- (or siRNA-β3) compared with
Prop- (or siRNA-β2))
Figure 4. A) FACS analysis and quantification at T7 and T14 of Treg (CD25⁺/CD127⁻ gated on CD45⁺/CD4⁺). B) FACS analysis and quantification at T7 and T14 of MDSC (in CD11b⁺, GR1⁺ gated on CD45⁺). C) FACS analysis and quantification at T7 and T14 of CD8⁺/Treg.
ratio. D) iNOS expression in MDSC. E) FACS analysis and quantification at T14 of Treg (CD25+/CD127- gated on CD45+/CD4+) in siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6). F) FACS analysis and quantification at T14 of MDSC (in CD11b+, GR1+ gated on CD45+) in siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6).

(* P<0.05, ** P<0.01 Prop- (or siRNA-β2) compared with Veh-, ## P<0.01, ### P<0.001, #### P<0.0001 SR- (or siRNA-β3) compared with Veh-, $ P<0.05, $$ P<0.01, $$$ P<0.001 SR- (or siRNA-β3) compared with Prop- (or siRNA-β2))
**Figure 5.** A) FACS analysis and quantification at T7 and T14 of M1/M2 ratio on CD45$^+$ cells. B) IL-10 expression in M2 macrophages C) FACS analysis and quantification at T7 and T14 of N1 granulocytes (CD54$^+$, CD95$^+$, CD11b$^+$). D) Arg1 expression in N1
granulocytes. E) FACS analysis and quantification at T14 of M1/M2 ratio on CD45+ cells in siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6). F) FACS analysis and quantification at T14 of N1 granulocytes (CD54+, CD95+, CD11b+) in siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6).

(* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 Prop- (or siRNA-β2) compared with Veh-, # P<0.05, ## P<0.01, #### P<0.0001 SR- (or siRNA-β3) compared with Veh-, $ P<0.05,$$$$ P<0.0001 SR- (or siRNA-β3) compared with Prop- (or siRNA-β2))
Figure 6. A) FACS analysis and quantification at T7 and T14 of NK (NKp46+/NK1.1+ gated on CD3+/CD45+) cells (left) and CD8+ (gated on CD45+) cells (right) in spleen of control- (Crl), vehicle- (Veh), propranolol- (Prop) and SR59230A- (SR) treated mice (n=6). B) FACS
analysis and quantification at T7 and T14 of NK (NKp46⁺/NK1.1⁺ gated on CD3⁻/CD45⁺) cells (left) and CD8⁺ (gated on CD45⁺) cells (right) in blood of control- (Crl), vehicle- (Veh), propranolol- (Prop) and SR59230A- (SR) treated mice (n=6). C) FACS analysis and quantification at T7 and T14 of NK (NKp46⁺/NK1.1⁺ gated on CD3⁻/CD45⁺) cells (left) and CD8⁺ (gated on CD45⁺) cells (right) in spleen of siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6). D) FACS analysis and quantification at T7 and T14 of NK (NKp46⁺/NK1.1⁺ gated on CD3⁻/CD45⁺) cells (left) and CD8⁺ (gated on CD45⁺) cells (right) in blood of siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6).

(* P<0.05, ** P<0.01 Prop- (or siRNA-β2) compared with Veh-, # P<0.05, ## P<0.01, ### P<0.001, #### P<0.0001 SR- (or siRNA-β3) compared with Veh-, $ P<0.05, $$ P<0.01, $$$ P<0.001 SR- (or siRNA-β3) compared with Prop- (or siRNA-β2))
Figure 7. A) FACS analysis and quantification at T7 and T14 of Treg (CD25⁺/CD127⁻ gated on CD45⁺/CD4⁺) cells (left) and MDSC (in CD11b⁺, GR1⁺ gated on CD45⁺) cells (right) in spleen of control- (Crl), vehicle- (Veh), propranolol- (Prop) and SR59230A- (SR) treated...
mice (n=6). B) FACS analysis and quantification at T7 and T14 of Treg (CD25+/CD127− gated on CD45+/CD4+) cells (left) and MDSC (in CD11b+, GR1+ gated on CD45+) cells (right) in blood of control- (Crl), vehicle- (Veh), propranolol- (Prop) and SR59230A- (SR) treated mice (n=6). C) FACS analysis and quantification at T7 and T14 of Treg (CD25+/CD127− gated on CD45+/CD4+) cells (left) and MDSC (in CD11b+, GR1+ gated on CD45+) cells (right) in spleen of siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6). D) FACS analysis and quantification at T7 and T14 of Treg (CD25+/CD127− gated on CD45+/CD4+) cells (left) and MDSC (in CD11b+, GR1+ gated on CD45+) cells (right) in blood of siRNA-CTRL, siRNA-β2 and siRNA-β3 treated mice (n=6).

(* P<0.05, ** P<0.01, *** P<0.001 Prop- (or siRNA-β2) compared with Veh-, ## P<0.01, ### P<0.001, #### P<0.0001 SR- (or siRNA-β3) compared with Veh-, $ P<0.05, $$$ P<0.001, $$$$ P<0.0001 SR- (or siRNA-β3) compared with Prop- (or siRNA-β2))