




## RESEARCH PAPER

# $\beta_3$ -Adrenoceptor as a potential immuno-suppressor agent in melanoma

Maura Calvani<sup>1</sup> | Gennaro Bruno<sup>2</sup> | Massimo Dal Monte<sup>3</sup>  | Romina Nassini<sup>2</sup>  |  
 Filippo Fontani<sup>2</sup> | Arianna Casini<sup>4</sup> | Lorenzo Cavallini<sup>5</sup> | Matteo Becatti<sup>6</sup> |  
 Francesca Bianchini<sup>6</sup> | Francesco De Logu<sup>2</sup> | Giulia Forni<sup>7</sup> | Giancarlo la Marca<sup>7</sup> |  
 Lido Calorini<sup>6</sup> | Paola Bagnoli<sup>3</sup> | Paola Chiarugi<sup>6</sup> | Alberto Pupi<sup>6</sup> | Chiara Azzari<sup>2</sup> |  
 Pierangelo Geppetti<sup>2</sup> | Claudio Favre<sup>1</sup> | Luca Filippi<sup>8</sup> 

<sup>1</sup>Oncohematology Unit, Department of Pediatric Oncology, Meyer University Children's University Hospital, Florence, Italy

<sup>2</sup>Department of Health Sciences, University of Florence, Florence, Italy

<sup>3</sup>Department of Biology, Unit of General Physiology, University of Pisa, Pisa, Italy

<sup>4</sup>Division of Immunology, Section of Pediatrics, Meyer University Children's Hospital, Florence, Italy

<sup>5</sup>Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

<sup>6</sup>Department of Experimental and Clinical Biomedical Sciences, University of Florence, Florence, Italy

<sup>7</sup>Metabolic and Newborn Screening Clinical Unit, Department of Neurosciences, Meyer University Children's University Hospital, Florence, Italy

<sup>8</sup>Neonatal Intensive Care Unit, Medical Surgical Feto-Neonatal Department, Meyer University Children's Hospital, Florence, Italy

## Correspondence

Luca Filippi, Neonatal Intensive Care Unit, Medical Surgical Feto-Neonatal Department, A. Meyer Children's University Hospital, Viale Pieraccini, 24 I-50139 Florence, Italy.  
 Email: l.filippi@meyer.it

**Background and Purpose:** Stress-related catecholamines have a role in cancer and  $\beta$ -adrenoceptors; specifically,  $\beta_2$ -adrenoceptors have been identified as new targets in treating melanoma. Recently,  $\beta_3$ -adrenoceptors have shown a pleiotropic effect on melanoma micro-environment leading to cancer progression. However, the mechanisms by which  $\beta_3$ -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  $\beta_3$ -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  $\beta$ -adrenoceptor blockade on the number and activity of immune cell sub-populations (Treg, NK, CD8, MDSC, macrophages, and neutrophils). Pharmacological and molecular approaches with  $\beta$ -blockers (propranolol and SR59230A) and specific  $\beta$ -adrenoceptor siRNAs targeting  $\beta_2$ - or  $\beta_3$ -adrenoceptors were used.

**Key Results:** Only  $\beta_3$ -, but not  $\beta_2$ -adrenoceptors, were up-regulated under hypoxia in peripheral blood mononuclear cells and selectively expressed in immune cell sub-populations including Treg, MDSC, and NK. SR59230A and  $\beta_3$ -adrenoceptor siRNAs increased NK and CD8 number and cytotoxicity, while they attenuated Treg and MDSC sub-populations in the tumour mass, blood, and spleen. SR59230A and  $\beta_3$ -adrenoceptor siRNAs increased the ratio of M1/M2 macrophages and N1 granulocytes.

**Abbreviations:** CD8, CD8 T cells; Ad, adrenaline; MDSC, myeloid-derived suppressor cells; NA, noradrenaline; PBMC, peripheral blood mononuclear cells; Treg, regulatory T cells

Maura Calvani and Gennaro Bruno are first name equal contributors to the work.  
 Claudio Favre and Luca Filippi are last name equal contributors to the work.

**Funding information**

Meyer Foundation, A. Meyer Children's University Hospital, Grant/Award Number: not applicable; Italian Ministry of Health, Grant/Award Number: RF-2011-02351158

**Conclusions and Implications:** Our data suggest that  $\beta_3$ -adrenoceptors are involved in immune-tolerance, which opens the way for new strategic therapies to overcome melanoma growth.

**LINKED ARTICLES:** This article is part of a themed section on Adrenoceptors—New Roles for Old Players. To view the other articles in this section visit <http://onlinelibrary.wiley.com/doi/10.1111/bph.v176.14/issuetoc>

## 1 | INTRODUCTION

Several studies demonstrate that tumour neurogenesis or stress-related catecholamines, **noradrenaline** (NA) and **adrenaline** (Ad), accelerate cancer progression and reduce the overall survival of patients (Cole & Sood, 2012; Magnon et al., 2013). The increased secretion of catecholamines usually promotes favourable environment for tumour cells to grow and metastasize predominantly by acting at  **$\beta$ -adrenoceptors** (Entschladen, Drell, Lang, Joseph, & Zaenker, 2004). Signalling activated by  $\beta$ -adrenoceptors regulates tumour 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 tumour growth mainly through  **$\beta_2$ -adrenoceptors** and that non-selective  $\beta$ -adrenoceptor blockers (acting at  $\beta_1$ - and  $\beta_2$ -adrenoceptors) provide protection against different types of cancer (Childers, Hollenbeak, & Cheriya, 2015; Yazawa et al., 2016). Melanoma, like other tumours, shows a surprisingly positive response to **propranolol**, a  $\beta$ -adrenoceptor blocker targeting  $\beta_1$ - and  $\beta_2$ -adrenoceptors (Barbieri et al., 2012; Glasner et al., 2010), although the role of  $\beta_1$ -adrenoceptors in stimulating melanoma growth, and tumour 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 (Moretti et al., 2013; Wrobel & Le Gal, 2015; Yang et al., 2009). Finally, clinical studies demonstrate the positive impact of  $\beta_1$ - and/or  $\beta_2$ -adrenoceptor blockade in the overall survival of melanoma patients (De Giorgi et al., 2011; De Giorgi et al., 2017; Kokolus et al., 2017; Lemeshow et al., 2011), although these findings have been recently called into question (Livingstone et al., 2013; McCourt et al., 2014).

A role for  **$\beta_3$ -adrenoceptors** in melanoma has been proposed and recently reviewed (Dal Monte et al., 2018). In fact, the use of two different  $\beta_3$ -adrenoceptor blockers, **SR59230A** and **L-748337**, is effective in reducing tumour growth in a mouse model of melanoma (Dal Monte et al., 2013; Sereni, Dal Monte, Filippi, & Bagnoli, 2015). In addition, SR59230A and L-748337 as well as selective  $\beta_3$ -adrenoceptor siRNAs reduce the proliferation and induce apoptosis of human and mouse melanoma cells (Calvani et al., 2015; Dal Monte et al., 2013; Dal Monte et al., 2014), while  $\beta_3$ -adrenoceptor agonism stimulates melanoma cell proliferation and reduces apoptosis (Dal Monte et al., 2014). However, the mechanisms by which  $\beta_3$ -adrenoceptors promote melanoma growth are not yet fully elucidated.

### What is already known about this subject

- $\beta$ -adrenoceptors have been identified as new targets in treating melanoma.
- $\beta$ -adrenergic system is one of the major players in the regulation of the immune system.

### What this study adds

- $\beta_3$ -adrenoceptors are involved in mediating the switch from an immunocompetent to an immunosuppressive tumor microenvironment.
- $\beta_3$ -adrenoceptors blockade reduces the growth of melanoma inducing a reversion of immune-tolerance.

### What is the clinical significance

- $\beta_3$ -adrenoceptors blockade could represent a new strategy to overcome melanoma immune-editing.

There is evidence that mechanisms of immune-tolerance, which are known to prevent autoimmune diseases, may be used by tumours to bypass the development of an effective immune response (King, Sharma, Davis, & Jimeno, 2018). Immune cells in cancer exhibit functional plasticity and undergo a dramatic phenotypic change, leading to an alternative activation promoting tumour progression by inducing immune-tolerance (Granot & Fridlender, 2015). Currently, the mechanisms of cancer immune-tolerance have not been completely clarified. Established tumours with higher mutation rates use various escape mechanisms to bypass immune-surveillance including decreasing the number of cytotoxic immune cells such as NK and CD8 T cells (CD8) and/or increasing immune-suppressive cells, 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 tumour microenvironment: an environment rich in M2 macrophages and N2 neutrophils enhances immune-escape and supports tumour growth (Schoupe, De Baetselier, Van Ginderachter, & Sarukhan, 2012).

The  $\beta$ -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  $\beta_2$ -adrenoceptors (Nance & 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  $\beta_2$ -adrenoceptors inhibits lymphocyte responses, NK cytotoxicity, and dendritic cell functions (Marino & Cosentino, 2013). In addition,  $\beta$ -adrenoceptor signalling significantly suppresses the proliferation and the cytolytic killing ability of cytotoxic CD8 cells as well as their capability to produce IFN- $\gamma$  (Nissen, Sloan, & Mattarollo, 2018). Moreover, MDSC are increased in patients with breast cancer characterized by high levels of stress (Mundy-Bosse, Thornton, Yang, Andersen, & Carson, 2011). Finally, studies using rodent models of different tumours have shown that catecholamines or stress suppress NK cell activity leading to tumour metastasis, likely through  $\beta_2$ -adrenoceptor stimulation (Inbar et al., 2011).

Melanoma is one of the most immunogenic tumours, and it is highly sensitive to immune therapeutic agents (Tawbi et al., 2018). Of note, the melanoma micro-environment is enriched in tumour-associated M2 macrophages, Treg and MDSC, which promote the defective cytotoxicity of T cells (Fujimura, Kambayashi, & Aiba, 2012). In addition, melanoma cells inhibit the activity of CD8 and NK cells through the production of negative modulators such as VEGF, IL-8, and IL-10 (Passarelli, Mannavola, Stucci, Tucci, & Silvestris, 2017). Little is known about a role of  $\beta$ -adrenoceptors in regulating the immune environment in melanoma, the only evidence being limited to the findings that  $\beta$ -adrenergic stimulation recruits and polarizes macrophages, thus promoting tumour progression (Cole, Nagaraja, Lutgendorf, Green, & Sood, 2015) and that  $\beta_2$ -adrenoceptor blockade improves the anti-tumour efficacy of immunotherapy (Kokolus et al., 2017).

This study evaluates the potential role of  $\beta_3$ -adrenoceptors in the regulation of melanoma immune-tolerance by investigating the effects of its antagonism on cytotoxic and suppressive immune cell sub-populations. In addition, the regulatory role of  $\beta_3$ -adrenoceptors was compared with that of  $\beta_2$ -adrenoceptors by the use of pharmacological and molecular approaches. This research suggests the possibility that  $\beta_3$ -adrenoceptor antagonism could reduce melanoma growth in vivo by increasing the number of NK and CD8 cells as well as their cytotoxicity and by attenuating Treg and MDSC sub-populations in the tumour micro-environment. A shift in macrophage and neutrophil phenotypes from both M2 to M1 and N2 to N1 was also observed after  $\beta_3$ -adrenoceptor blockade.

## 2 | METHODS

### 2.1 | 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 (Euroclone, Milan, Italy), 2 mM L-glutamine, 100 U·ml<sup>-1</sup> penicillin and 100  $\mu$ g·ml<sup>-1</sup> streptomycin at 37°C in 5% CO<sub>2</sub>. The cell lines have been mycoplasma tested (Euroclone, Milan, Italy).

### 2.2 | In vivo transfection assay

$\beta_2$ -siRNA (SASI\_Mm01\_00154297) and  $\beta_3$ -siRNA (SASI\_Mm01\_00145466) were complexed with 200  $\mu$ l of InvivoFectamine reagent-plasmid duplex (reagent for in vivo plasmid delivery, Invitrogen, Carlsbad, CA, USA) and were injected into the tail vein when a palpable

tumour was formed (as described below). In vitro transfection assay with  $\beta_2$ - and  $\beta_3$ -siRNA was performed by using Polyplus INTERFERin siRNA Transfection Reagent. The efficiency of  $\beta_2$ - and  $\beta_3$ -siRNA was assessed by cytofluorimetric analysis of protein expression (Figure S1).

### 2.3 | Western blot

The expression of  $\beta$ -adrenoceptors was evaluated in murine lymphocytes, isolated from mice and cultured in normoxia (24 hr at 21% O<sub>2</sub>), hypoxia (24 hr at 1% O<sub>2</sub>), and 1 hr of normoxic re-exposure after 24 hr of hypoxia. Cell lysates were prepared in an appropriate volume of RIPA. After protein quantification (Bradford, Biorad, Hercules, CA, USA), 20  $\mu$ g of proteins from total lysates were subjected to SDS-PAGE and western blot analysis as reported in previous work. Rabbit polyclonal antibodies directed to  $\beta_2$ -adrenoceptors (Santa Cruz Biotechnology Cat# sc-569, RRID:AB\_630926) and  $\beta_3$ -adrenoceptors (Santa Cruz Biotechnology Cat# sc-50436, RRID:AB\_781613) have been recently validated (Sereni et al., 2015).

The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology*.

### 2.4 | Co-cultures and MTT assay

Tumour cells were seeded in MW24 and pretreated with propranolol or SR59230A (10  $\mu$ M) for 24 hr. Subsequently, the tumour cells were washed with PBS and co-cultured with peripheral blood mononuclear cells (PBMC; pretreated or not with propranolol or SR59230A [10  $\mu$ M] for 24 hr) in a seeding ratio of 1:3 for the next 24 hr. At the end of the total incubation period of 48 hr, PBMC were withdrawn from the medium of the co-culture and analysed by FACS, while tumour cells were tested for viability. Viability of tumour 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 the manufacturer's instructions. The intensity of the absorbance at 550 nm was evaluated using a spectrophotometer (FlexStation3, Molecular Devices). The same experiment was performed in each tumour cell line silenced or not with Scr-siRNA,  $\beta_2$ -siRNA, and  $\beta_3$ -siRNA.

### 2.5 | 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. Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny et al., 2010) and with the recommendations made by the *British Journal of Pharmacology*. Studies were conducted under University of Florence and Italian Health Minister research permits No. 401/2015-PR. C57BL/6 mice (male, 20–25 g, 5–6 weeks-old; IMSR Cat# JAX:000664, RRID:IMSR\_JAX:000664) were used. Animals were housed in a temperature and humidity-controlled vivarium (12 hr 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 killed by the use of inhaled CO<sub>2</sub> plus 10–50% O<sub>2</sub>.

## 2.6 | Mouse B16-F10 syngeneic model and treatments

B16-F10 were implanted in C57BL/6 recipient mice by injecting  $1 \times 10^6$  cells in 200  $\mu$ l PBS s.c. in the right flank of the mice. Mice were monitored daily. After 8–10 days, when B16-F10 cells formed a palpable tumour, the treatment was started. The treatments were administered twice a day with a window time of 4–6 hr between each treatment. Diluted DMSO (vehicle), propranolol (20 mg·kg<sup>-1</sup>·day<sup>-1</sup>, Saint Louis, MO, USA), and SR59230A, CAS: 174689–39-5 (20 mg·kg<sup>-1</sup>·day<sup>-1</sup>, Sigma Aldrich, Saint Louis, MO, USA) were injected i.p. Mice were killed on Days 7 and 14 of treatment; peripheral blood was collected; tumour 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 tumour cells from tumour stroma.

## 2.7 | In vivo MRI

For the in vivo MRI, C57BL/6 mice were fully anaesthetized with Avertin (2,2,2-tribromoethanol; 0.3 mg·g<sup>-1</sup> of body weight) intraperitoneal somministration, for the acquisition procedure. Anesthetic depth in each group was assessed with noxious stimuli using atraumatic forceps. T<sub>2</sub>-weighted scans of the mice were performed using 7 T Bruker PharmaScan MRI scanner. T<sub>2</sub>-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.

## 2.8 | Isolation of tumour cells and preparation of spleen and blood cells

Mouse tumour tissues were minced with scissors and incubated in C-Tubes (Miltenyi Biotec, Gladbach, Germany) containing a storage tissue solution (Miltenyi Biotec, Gladbach, Germany). Tumour samples were then homogenized using a Tumour Dissociation Kit (Miltenyi Biotec, Gladbach, Germany) and the heating function of the gentle MACS Octo Dissociator (Miltenyi Biotec, Gladbach, Germany) with an appropriate heater programme. After homogenization, the samples were filtered with pre-separation filters to remove cell aggregates or large particles. The lymphocyte cells were then separated by tumour 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 10× (Miltenyi Biotec, Gladbach, Germany), for optimal lysis of erythrocytes, according to the manufacturer's instructions.

## 2.9 | Flow cytometry and morphological analysis

Cells isolated from mouse tumours, 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 (17A<sub>2</sub>)-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\_2660259), anti-CD4-FITC (Miltenyi Biotec Cat# 130-102-541, 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** (integrin,  $\alpha$  X sub-unit)-APC Vio770 (Miltenyi Biotec Cat# 130-107-461, RRID:AB\_2660162), anti-**IL-10**-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- $\beta_2$ -FITC (Biorbyt Cat# orb15065, RRID:AB\_10735676), anti- $\beta_3$ -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, IL-10,  $\beta_2$ -adrenoceptors, and  $\beta_3$ -adrenoceptors. The stained cells were acquired on a MACSQuant Analyzer 10 Flow Cytometer (Miltenyi Biotec, Gladbach, Germany), and the data were processed using Flowlogic software (Miltenyi Biotec, Gladbach, Germany).

## 2.10 | Statistical analysis

Statistical analysis was performed using the SAS 9.2 software. Values are presented as mean  $\pm$  SD. Differences with  $P < 0.05$  were considered significant. For a *t* test with Bonferroni correction for multiple comparison, an expected tumour growing difference of 2.5 cm<sup>3</sup> between groups (8, 5.5, and 3 cm<sup>3</sup> in vehicle, propranolol, and SR group, respectively), an SD for each group of 2 cm<sup>3</sup> (Sereni et al., 2015), and a first type error set to 1.7%, six mice per 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 were used, respectively. In order to evaluate the difference in quantitative outcomes between groups, according to normality and homoscedasticity tests results, ANOVA and post hoc *t* test with Bonferroni correction for multiple comparison or Welch ANOVA and post hoc Satterthwait *t* test with Bonferroni correction for multiple

comparison or Kruskal–Wallis and Dwass, Steel, Critchlow–Fligner method for multiple comparison were used. A post hoc 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).

## 2.11 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in <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, Fabbro et al., 2017a,b; Alexander, Christopoulos, et al., 2017; Alexander, Kelly et al., 2017).

## 3 | RESULTS

### 3.1 | $\beta$ -adrenoceptors expressed in immune cells influence melanoma cell viability through an effect on immune cells

We first analysed the expression of both receptors in PBMC and in immune cell sub-populations to assess whether  $\beta_2$ - and  $\beta_3$ -adrenoceptors could be implicated in regulating melanoma cell viability by affecting the immune system. Previous studies demonstrated  $\beta_2$ -adrenoceptor expression in immune cell sub-populations such as NK (Maisel, Fowler, Rearden, Motulsky, & Michel, 1989), CD8 (Estrada, Ağaç, & Farrar, 2016), and Treg (Guereschi et al., 2013) cells, without exploring the presence of  $\beta_3$ -adrenoceptors. In this study, we have observed that both  $\beta_2$ - and  $\beta_3$ -adrenoceptors were expressed in mouse PBMC, but only  $\beta_3$ -adrenoceptors were up-regulated under hypoxic conditions, used to mimic the tumour micro-environment, and fast down-regulated after oxygen re-exposure (Figure 1a). We then performed a cytofluorimetric analysis to evaluate the expression of  $\beta_2$ - and  $\beta_3$ -adrenoceptors in different PBMC sub-populations isolated from either blood or tumour tissue, including NK, Treg cells, and MDSC. The expression of  $\beta_2$ -adrenoceptors in NK, Treg cells, and MDSC infiltrating the tumour did not differ from that in circulating cells, while  $\beta_3$ -adrenoceptors were up-regulated in the immune cell sub-populations infiltrating the tumour (Figure 1b).

We evaluated the hypothesis that  $\beta_3$ -adrenoceptors expressed in PBMC, and in particular in tumour infiltrating lymphocytes, could affect tumour cell viability. We co-cultured under hypoxic conditions B16-F10 cells with mouse PBMC pre-exposed to SR59230A, propranolol, or selective siRNAs targeting  $\beta_2$ - or  $\beta_3$ -adrenoceptors to assess this hypothesis. The results show 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 significant effects on cell viability. The silencing approach substantially confirmed that the effects obtained with SR59230A were due to

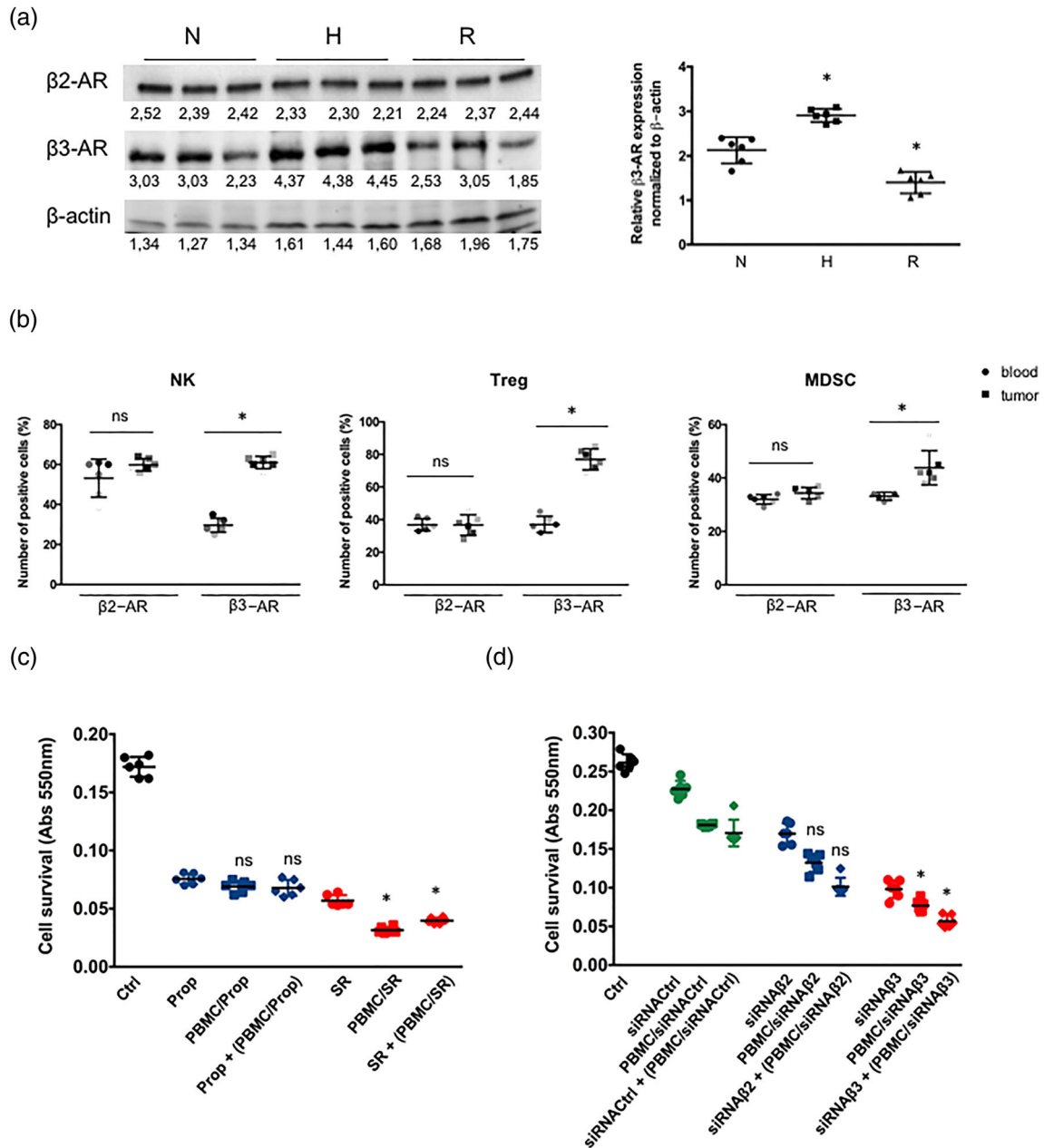
the blockade of  $\beta_3$ -adrenoceptors (Figure 1c,d). The enhanced efficacy of SR59230A pretreatment in PBMC suggests that  $\beta_3$ -adrenoceptor antagonism, by acting on immune cells, might pilot cancer cell death.

### 3.2 | Targeting $\beta_2$ - or $\beta_3$ -adrenoceptors reduces tumour growth in a mouse model of melanoma

In line with previous findings (Dal Monte et al., 2013), additional investigation performed in this study allowed us to confirm that propranolol or SR59230A administered for 14 days significantly reduced tumour volume (Figure 2a), with a major effect of  $\beta_3$ - over  $\beta_2$ -adrenoceptor blockade, as also confirmed by the greater efficacy of SR59230A and  $\beta_3$ -adrenoceptor silencing approach (Figure 2b). Moreover, the administration of the  $\beta_2$ -adrenoceptor agonist **terbutaline** to mice treated with  $\beta_3$ -adrenoceptor siRNA showed no growth rebound, thus confirming the predominant role of  $\beta_3$ -adrenoceptor subtype in controlling tumour growth. At the same time, the administration of the  $\beta_3$ -adrenoceptor agonist BRL37344 in  $\beta_3$ -adrenoceptor-silenced mice did not affect tumour growth compared to siRNA- $\beta_3$ -adrenoceptor condition alone (Figure S2). The efficacy of propranolol and SR59230A in reducing the tumour volume was also demonstrated here by NMR (Figure 2c). The rate of tumour cell death in the tumour mass was analysed by cytofluorimetric analysis, injecting into C57BL/6 mice a stable B16-F10 cell line expressing the GFP (B16-F10-GFP), to precisely discriminate the tumour cells from tumour stroma. Both propranolol and SR59230A induced an increased early apoptotic rate after 7 days of treatment, with an effect of SR59230A that was significantly higher than that of propranolol (Figure 2d). In addition, in the tumour mass, both propranolol and SR59230A, with a major effect of SR59230A, increased the apoptotic marker FAS (Figure 2e), which is known to bind to its ligand in NK cells or neutrophils, thus leading to apoptosis of tumour cells (Abrahams, Kamsteeg, & Mor, 2003). The early apoptotic rate after SR59230A treatment decreased after 14 days in favour of an extensive cell death in the tumour mass, as indicated by large areas of tissue necrosis (Figure 2f).

### 3.3 | Targeting $\beta_3$ -adrenoceptors induces immune-competent sub-populations in the tumour micro-environment

The possible role of immune cells expressing  $\beta$ -adrenoceptors in counteracting melanoma growth was also investigated in the *in vivo* model treated with propranolol or SR59230A or with  $\beta_2$ - or  $\beta_3$ -adrenoceptor siRNA. As shown in Figure 3a, the spleen weight, used as an indirect indicator of activation of the immune system, was significantly higher in SR59230A-treated than in vehicle-treated mice. In contrast, propranolol did not affect the spleen weight. The maximal effect of SR59230A was detected after 7 days of treatment. As shown in Figure 3b,c, the cytofluorimetric analysis of cells from the tumour mass of SR59230A-treated mice showed an increased number of NK and CD8 cells, which was already detectable after 7 days of treatment. SR59230A also led to the activation of NK and CD8 cytotoxic activity

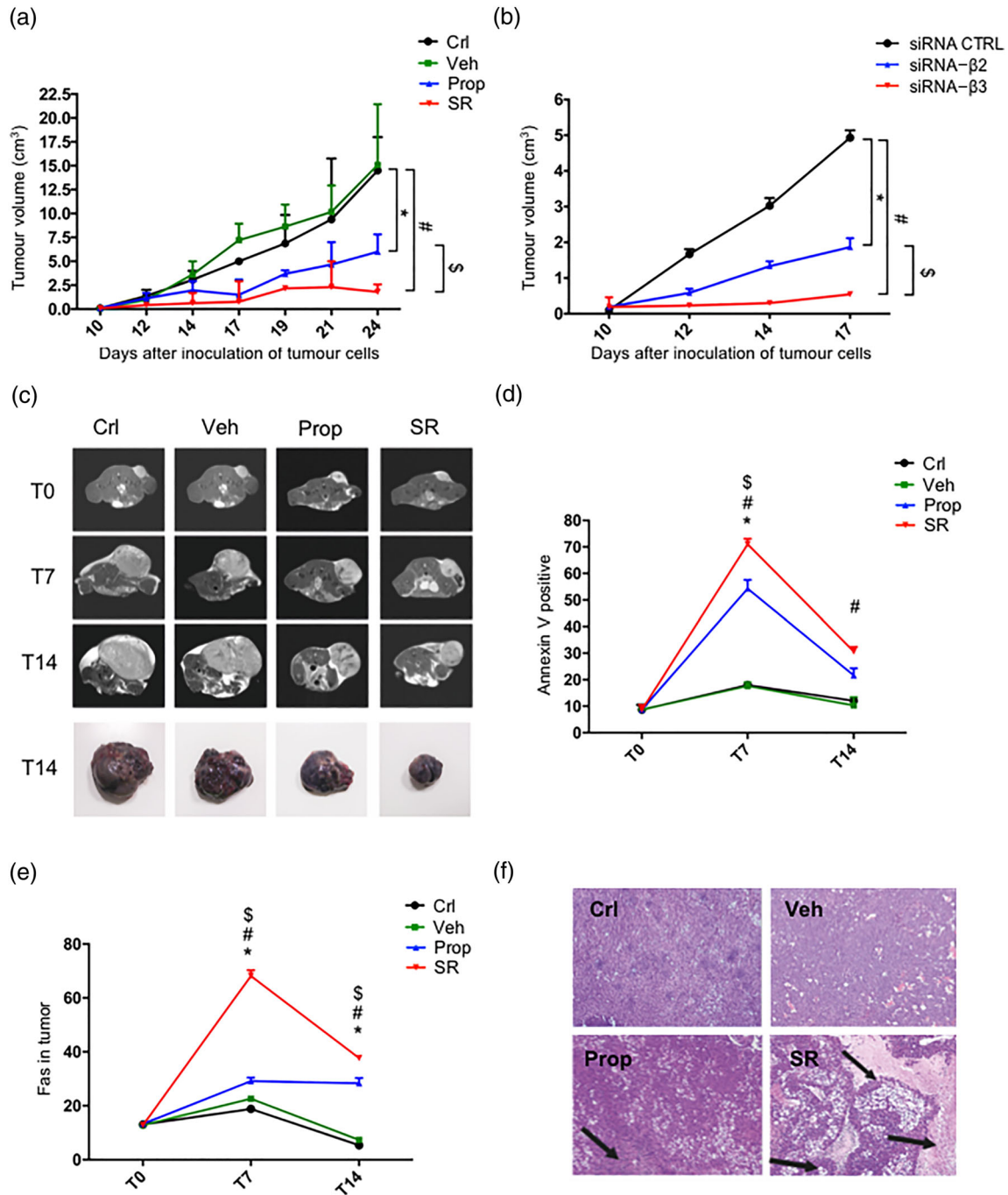


**FIGURE 1** (a) Representative WB of  $\beta_3$ -adrenoceptors in murine lymphocytes after 24 hr of normoxic (N) or 24 hr hypoxic conditions (H) and 1 hr of normoxic re-exposure (R) and relative densitometric quantification ( $n = 6$ ). Results are reported as mean  $\pm$  SD of relative expression normalized to  $\beta$ -actin. \* $P < 0.05$  hypoxic (H) and re-exposure conditions compared with normoxic (N). (b) FACS quantification of  $\beta_2$ - and  $\beta_3$ -adrenoceptors expression in blood and tumour infiltrating NK and Treg cells, and MDSC. \* $P < 0.05$   $\beta_3$ -adrenoceptor in tumour compared with  $\beta_3$ -adrenoceptor in blood ( $n = 6$ ). (c) MTT cell viability assay in B16-F10 cells untreated or treated with propranolol (Prop) or SR59230A (SR) and co-cultured for 48 hr under hypoxic conditions with PBMC untreated or pretreated with Prop or SR. Dot plots show the changes of each treatment compared with Ctrl ( $n = 6$ ). ns: not significant, \* $P < 0.05$  PBMC/SR or SR + (PBMC/SR) compared with SR. (d) MTT cell viability assay in B16-F10 cells silenced with siRNA-Ctrl, siRNA- $\beta_2$ , or siRNA- $\beta_3$  and co-cultured for 48 hr under hypoxic conditions with PBMC untreated or pretreated with Ctrl-siRNA, siRNA- $\beta_2$ , or siRNA- $\beta_3$ . Dot plots show the changes of each treatment compared with Ctrl ( $n = 6$ ). ns: not significant, \* $P < 0.05$  PBMC/siRNA- $\beta_3$  or siRNA- $\beta_3$  + (PBMC/siRNA- $\beta_3$ ) compared with siRNA- $\beta_3$

as evidenced by the increased expression of the activation markers perforin (van den Broek & Hengartner, 2000) and CD107A (Aktas, Kucuksezer, Bilgic, Erten, & Deniz, 2009) respectively (Figure 3d,e). Propranolol did not affect the number of NK or CD8 cells or induce the expression of perforin or CD107A. The silencing approach confirmed the prevalent role played by  $\beta_3$ -adrenoceptors (Figure 3f,g).

### 3.4 | Prevalent role of $\beta_3$ -adrenoceptors in reducing immune-suppressive sub-populations in the tumour micro-environment

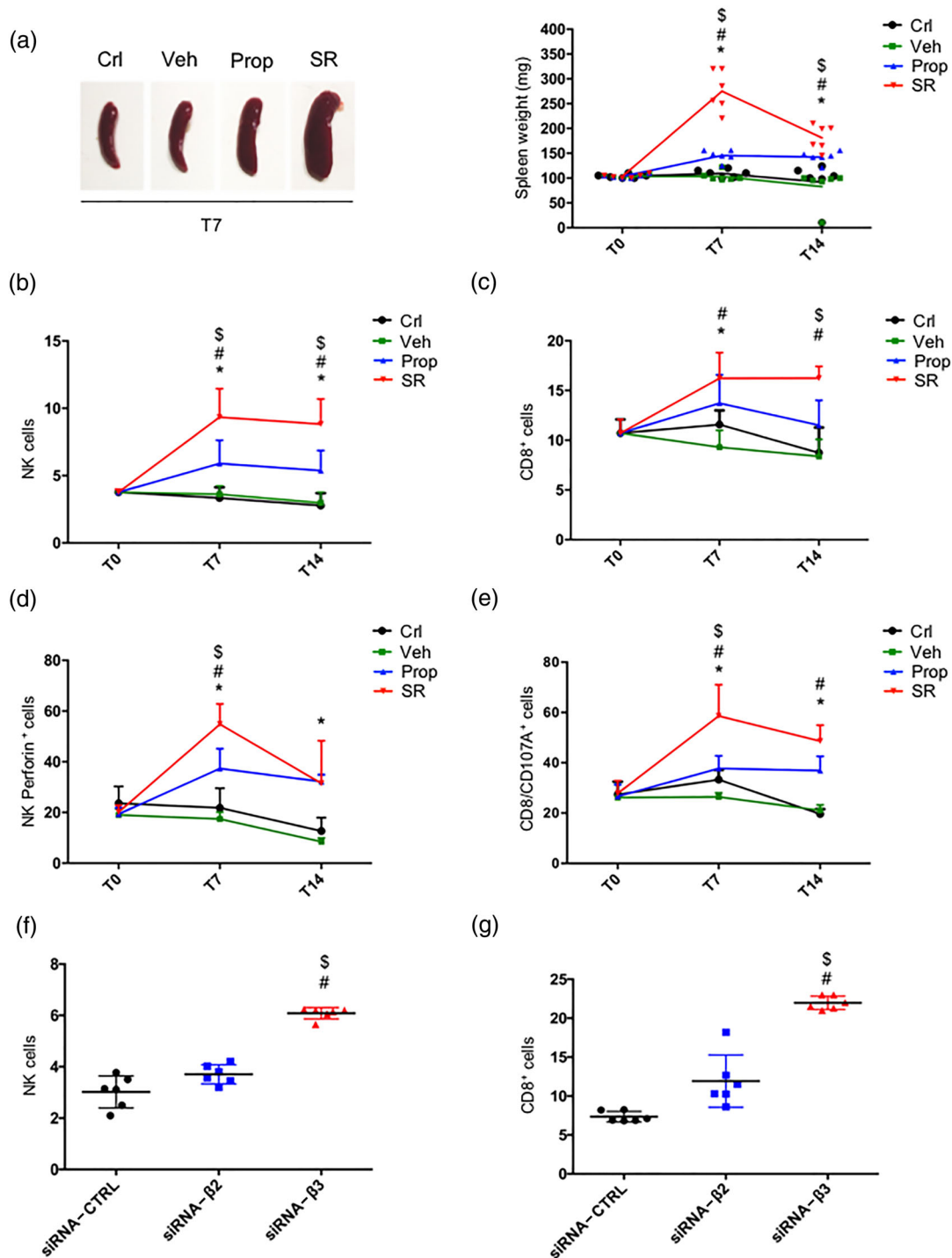
We also analysed the number of Treg and MDSC sub-populations in excised tumour mass, as shown in Figure 4a,b. Treatments with either



**FIGURE 2** (a) Tumour growth rate in control- (Crl), vehicle- (Veh), propranolol (Prop)-, and SR59230A (SR)- treated mice (n = 6). (b) Tumour growth rate in siRNA-CTRL, siRNA-β<sub>2</sub>, and siRNA-β<sub>3</sub> treated mice (n = 6). (c) MR images of mouse ventral section in control, vehicle-, propranolol-, and SR59230A-treated mice (n = 6). (d) FACS analysis of Annexin V positive cells in control, vehicle-, propranolol-, and SR59230A-treated mice (n = 6). (e) FACS analysis of Fas marker expression in tumours of control, vehicle-, propranolol-, and SR59230A-treated mice (n = 6). (f) Representative fields of haematoxylin-eosin (H&E) staining at T14 in control, vehicle-, propranolol-, and SR59230A-treated mice (n = 6). \*P < 0.05 Prop- (or siRNA-β<sub>2</sub>) compared with Veh-; #P < 0.05 SR- (or siRNA-β<sub>3</sub>) compared with Veh-; §P < 0.05 SR- (or siRNA-β<sub>3</sub>) compared with Prop- (or siRNA-β<sub>2</sub>)

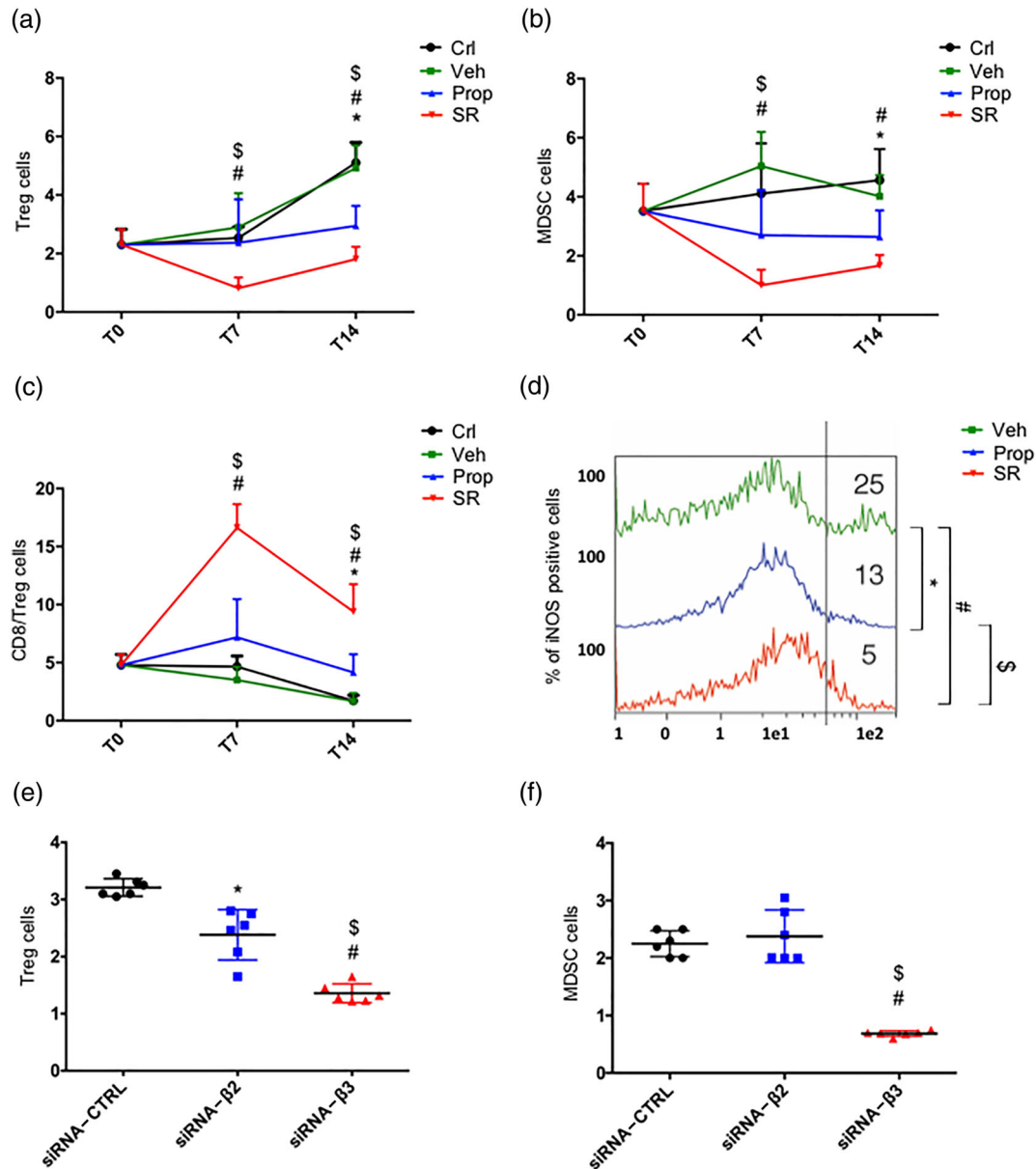
propranolol or SR59230A reduced Treg and MDSC immune-suppressive sub-populations with the effect of SR59230A being significantly higher than that of propranolol. In addition, the CD8/Treg ratio was significantly increased by SR59230A, while it was not affected by propranolol (Figure 4c). Furthermore, the

expression of the inducible form of NOS (iNOS), a landmark of the immune-suppressed phenotype in MDSC (Mazzoni et al., 2002), was reduced by both propranolol and, more effectively, by SR59230A (Figure 4d). The silencing approach substantially confirmed the immune-suppressive action of β<sub>3</sub>-adrenoceptors (Figure 4e,f).



**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<sup>+</sup>/NK1.1<sup>+</sup> gated on CD3<sup>-</sup>/CD45<sup>+</sup>). (c) FACS analysis and quantification at T7 and T14 of CD8<sup>+</sup> (gated on CD45<sup>+</sup>). (d) FACS analysis and quantification at T7 and T14 of perforin expression on NKp46<sup>+</sup>/NK1.1<sup>+</sup> cells. Two-way ANOVA analysis was performed. (e) FACS analysis and quantification at T7 and T14 of CD8<sup>+</sup> cytotoxic (CD107A<sup>+</sup> gated on CD8<sup>+</sup>). (f) FACS analysis and quantification at T14 of NK (NKp46<sup>+</sup>/NK1.1<sup>+</sup> gated on CD3<sup>-</sup>/CD45<sup>+</sup>) cells in siRNA-CTRL, siRNA-β<sub>2</sub>, and siRNA-β<sub>3</sub> treated mice (n = 6). (g) FACS analysis and quantification at T14 of CD8<sup>+</sup> (gated on CD45<sup>+</sup>) cells in siRNA-CTRL, siRNA-β<sub>2</sub>, and siRNA-β<sub>3</sub> treated mice (n = 6). \*P < 0.05 Prop- (or siRNA-β<sub>2</sub>) compared with Veh-; #P < 0.05 SR- (or siRNA-β<sub>3</sub>) compared with Veh-; \$P < 0.05 SR- (or siRNA-β<sub>3</sub>) compared with Prop- (or siRNA-β<sub>2</sub>)



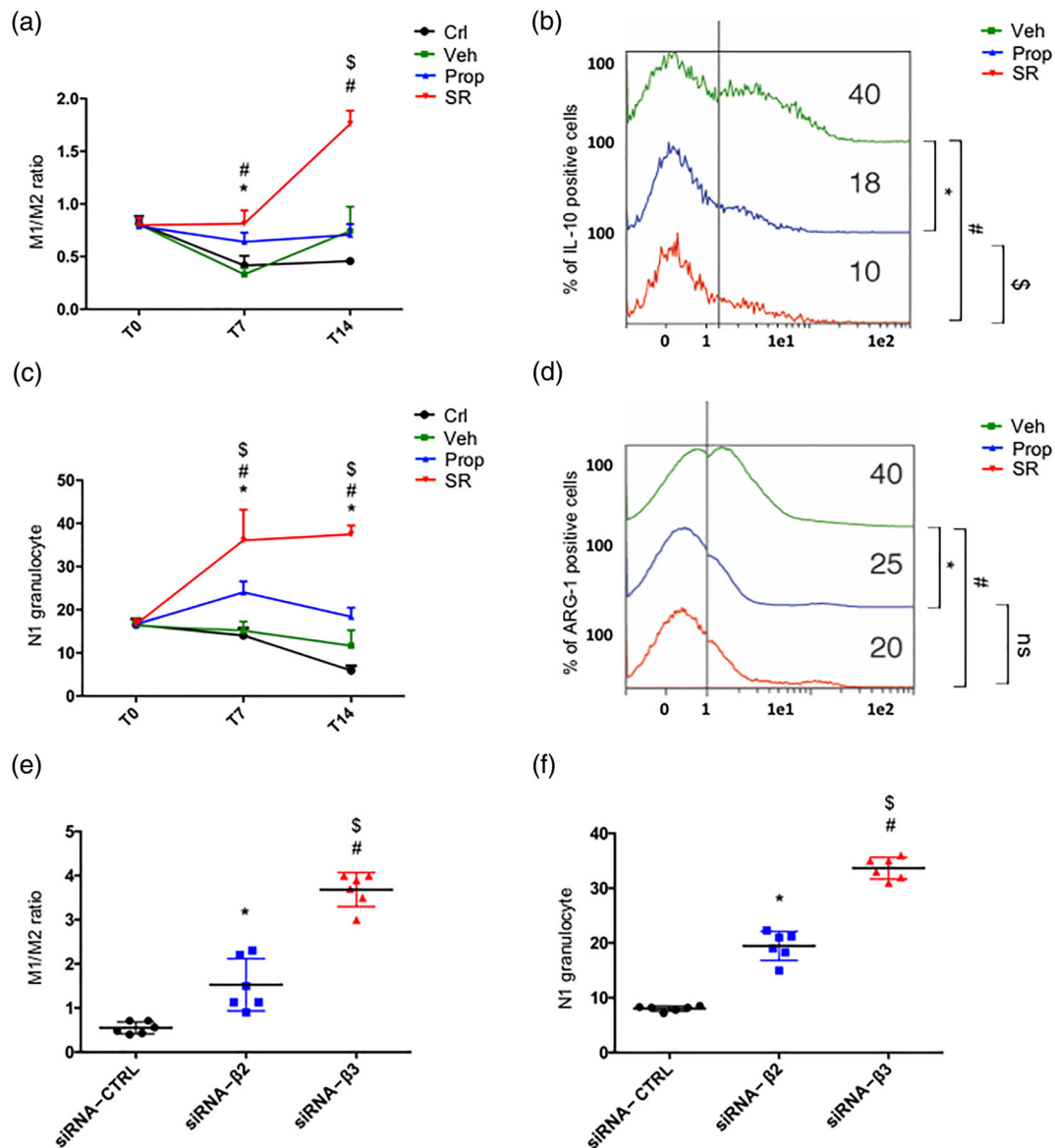


**FIGURE 4** (a) FACS analysis and quantification at T7 and T14 of Treg ( $CD25^+/CD127^-$  gated on  $CD45^+/CD4^+$ ). (b) FACS analysis and quantification at T7 and T14 of MDSC (in  $CD11b^+$ ,  $GR1^+$  gated on  $CD45^+$ ). (c) FACS analysis and quantification at T7 and T14 of  $CD8^+/Treg$  ratio. (d) iNOS expression in MDSC. (e) FACS analysis and quantification at T14 of Treg ( $CD25^+/CD127^-$  gated on  $CD45^+/CD4^+$ ) in siRNA-CTRL, siRNA- $\beta_2$ , and siRNA- $\beta_3$  treated mice ( $n = 6$ ). (f) FACS analysis and quantification at T14 of MDSC (in  $CD11b^+$ ,  $GR1^+$  gated on  $CD45^+$ ) in siRNA-CTRL, siRNA- $\beta_2$ , and siRNA- $\beta_3$  treated mice ( $n = 6$ ). \* $P < 0.05$  propranolol (Prop)- (or siRNA- $\beta_2$ ) compared with Veh-; # $P < 0.05$  SR59230A (SR)- (or siRNA- $\beta_3$ ) compared with Veh-; \$ $P < 0.05$  SR- (or siRNA- $\beta_3$ ) compared with Prop- (or siRNA- $\beta_2$ )

### 3.5 | Prevalent role of $\beta_3$ -adrenoceptors in inducing macrophage M1 and neutrophil N1 phenotypes in the tumour micro-environment

An immune-suppressive micro-environment is characterized by a high density of M2 macrophages and N2 neutrophils, while an immune-competent micro-environment is characterized by the presence of M1 macrophages and N1 neutrophils (Fridlender et al., 2009; Mantovani & Locati, 2013). We observed that SR59230A, but not propranolol, increased the M1/M2 ratio in the tumour

micro-environment (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,d). The silencing approach substantially confirmed that the polarization of neutrophils and macrophages was influenced by both  $\beta_2$ - and  $\beta_3$ -adrenoceptors but with  $\beta_3$ -adrenoceptors having a greater effect (Figure 5e,f).



**FIGURE 5** (a) FACS analysis and quantification at T7 and T14 of M1/M2 ratio on CD45<sup>+</sup> cells. (b) IL-10 expression in M2 macrophages. (c) FACS analysis and quantification at T7 and T14 of N1 granulocytes (CD54<sup>+</sup>, CD95<sup>+</sup>, and CD11b<sup>+</sup>). (d) Arg1 expression in N1 granulocytes. (e) FACS analysis and quantification at T14 of M1/M2 ratio on CD45<sup>+</sup> cells in siRNA-CTRL, siRNA-β<sub>2</sub>, and siRNA-β<sub>3</sub> treated mice (*n* = 6). (f) FACS analysis and quantification at T14 of N1 granulocytes (CD54<sup>+</sup>, CD95<sup>+</sup>, and CD11b<sup>+</sup>) in siRNA-CTRL, siRNA-β<sub>2</sub>, and siRNA-β<sub>3</sub> treated mice (*n* = 6). \**P* < 0.05 propranolol (Prop)- (or siRNA-β<sub>2</sub>) compared with Veh-; #*P* < 0.05 SR59230A (SR)- (or siRNA-β<sub>3</sub>) compared with Veh-; \$*P* < 0.05 SR- (or siRNA-β<sub>3</sub>) compared with Prop- (or siRNA-β<sub>2</sub>)

### 3.6 | Targeting β<sub>2</sub>- or β<sub>3</sub>-adrenoceptors affects immune sub-populations in the spleen and the blood

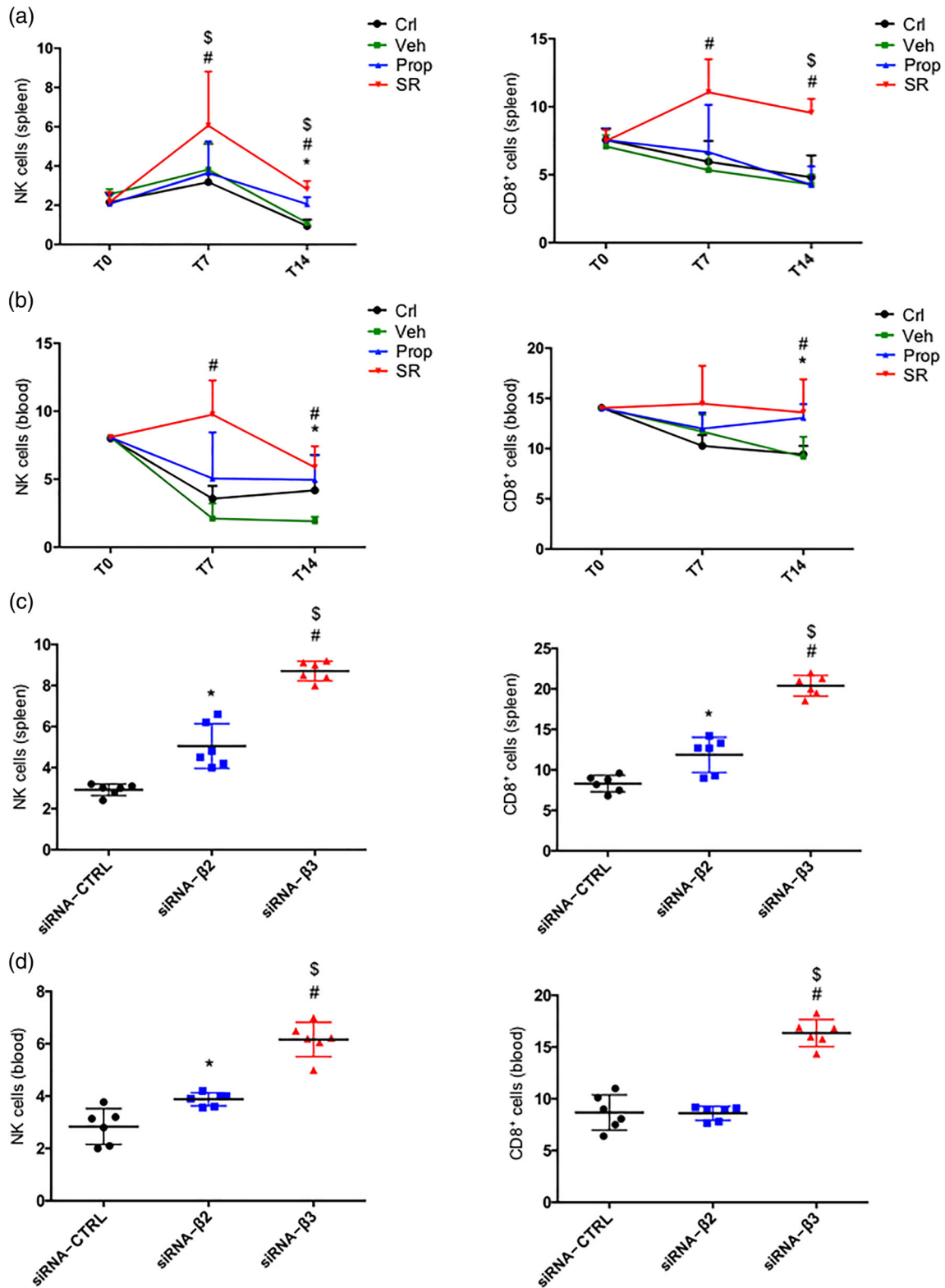
Results from the analysis of immune-competent NK and CD8 sub-populations in the spleen and the blood from mice treated with propranolol, SR59230A, or siRNAs were in line with those obtained in tumour infiltrating cells and demonstrated a major effect of β<sub>3</sub>-adrenoceptor blockade (Figure 6a-d). However, propranolol and SR59230A were equally effective at reducing the immune-suppressive Treg and MDSC sub-populations in the spleen and the blood (Figure 7a,b). The silencing approach confirmed these similar effects of β<sub>2</sub>- or β<sub>3</sub>-adrenoceptor antagonism on Treg reduction. In contrast,

the silencing of β<sub>3</sub>-adrenoceptors was more effective than the silencing of β<sub>2</sub>-adrenoceptors at reducing the MDSC sub-population (Figure 7c,d).

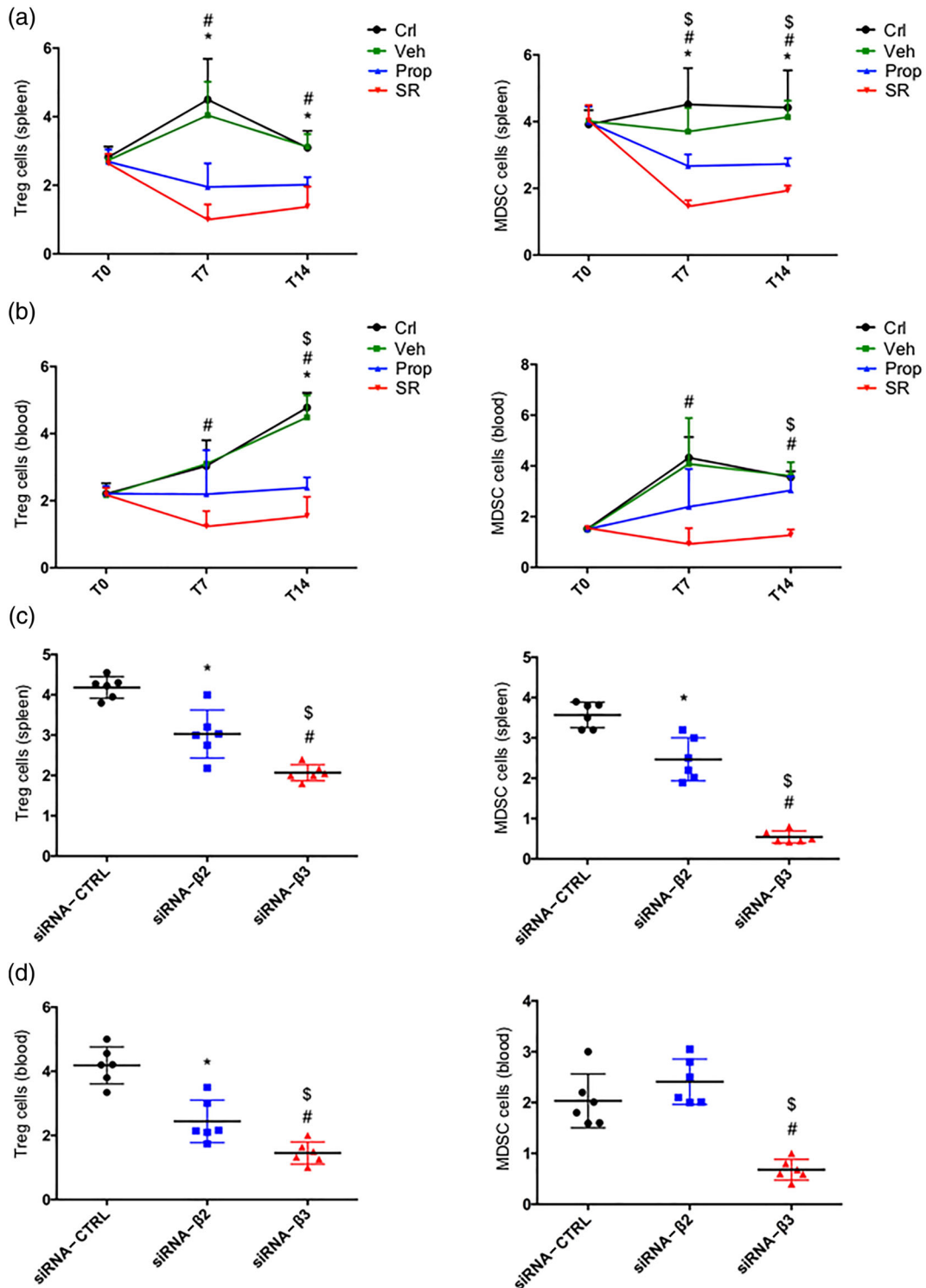
## 4 | DISCUSSION

The fine control of immune cell sub-populations is completely reversed in cancer, favouring an immune-tolerant phenotype. Understanding the mechanisms promoting this immunological shift is currently one of the most important challenges in oncological research.

Although conflicting results have been reported on the regulation of the immune system by catecholamines in humans, the majority of



**FIGURE 6** (a) FACS analysis and quantification at T7 and T14 of NK (NKp46<sup>+</sup>/NK1.1<sup>+</sup> gated on CD3<sup>-</sup>/CD45<sup>+</sup>) cells (left) and CD8<sup>+</sup> (gated on CD45<sup>+</sup>) cells (right) in spleen of control (Cri), vehicle (Veh)-, propranolol (Prop)-, and SR59230A (SR)-treated mice (n = 6). (b) FACS analysis and quantification at T7 and T14 of NK (NKp46<sup>+</sup>/NK1.1<sup>+</sup> gated on CD3<sup>-</sup>/CD45<sup>+</sup>) cells (left) and CD8<sup>+</sup> (gated on CD45<sup>+</sup>) cells (right) in blood of control, vehicle-, propranolol-, and SR59230A-treated mice (n = 6). (c) FACS analysis and quantification at T7 and T14 of NK (NKp46<sup>+</sup>/NK1.1<sup>+</sup> gated on CD3<sup>-</sup>/CD45<sup>+</sup>) cells (left) and CD8<sup>+</sup> (gated on CD45<sup>+</sup>) cells (right) in spleen of siRNA-CTRL, siRNA-β<sub>2</sub>, and siRNA-β<sub>3</sub> treated mice (n = 6). (d) FACS analysis and quantification at T7 and T14 of NK (NKp46<sup>+</sup>/NK1.1<sup>+</sup> gated on CD3<sup>-</sup>/CD45<sup>+</sup>) cells (left) and CD8<sup>+</sup> (gated on CD45<sup>+</sup>) cells (right) in blood of siRNA-CTRL, siRNA-β<sub>2</sub>, and siRNA-β<sub>3</sub> treated mice (n = 6). \*P < 0.05 Prop- (or siRNA-β<sub>2</sub>) compared with Veh-; #P < 0.05 SR- (or siRNA-β<sub>3</sub>) compared with Veh-; \$P < 0.05 SR- (or siRNA-β<sub>3</sub>) compared with Prop- (or siRNA-β<sub>2</sub>)



**FIGURE 7** (a) FACS analysis and quantification at T7 and T14 of Treg (CD25<sup>+</sup>/CD127<sup>-</sup> gated on CD45<sup>+</sup>/CD4<sup>+</sup>) cells (left) and MDSC (in CD11b<sup>+</sup>, GR1<sup>+</sup> gated on CD45<sup>+</sup>) (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<sup>+</sup>/CD127<sup>-</sup> gated on CD45<sup>+</sup>/CD4<sup>+</sup>) cells (left) and MDSC (in CD11b<sup>+</sup>, GR1<sup>+</sup> gated on CD45<sup>+</sup>) cells (right) in blood of control, vehicle-, propranolol-, and SR59230A-treated mice ( $n = 6$ ). (c) FACS analysis and quantification at T7 and T14 of Treg (CD25<sup>+</sup>/CD127<sup>-</sup> gated on CD45<sup>+</sup>/CD4<sup>+</sup>) cells (left) and MDSC (in CD11b<sup>+</sup>, GR1<sup>+</sup> gated on CD45<sup>+</sup>) (right) in spleen of siRNA-CTRL, siRNA-β<sub>2</sub>, and siRNA-β<sub>3</sub> treated mice ( $n = 6$ ). (d) FACS analysis and quantification at T7 and T14 of Treg (CD25<sup>+</sup>/CD127<sup>-</sup> gated on CD45<sup>+</sup>/CD4<sup>+</sup>) cells (left) and MDSC (in CD11b<sup>+</sup>, GR1<sup>+</sup> gated on CD45<sup>+</sup>) (right) in blood of siRNA-CTRL, siRNA-β<sub>2</sub>, and siRNA-β<sub>3</sub> treated mice ( $n = 6$ ). \* $P < 0.05$  Prop- (or siRNA-β<sub>2</sub>) compared with Veh-; # $P < 0.05$  SR- (or siRNA-β<sub>3</sub>) compared with Veh-; \$ $P < 0.05$  SR- (or siRNA-β<sub>3</sub>) compared with Prop- (or siRNA-β<sub>2</sub>)

reports agree that Ad and NA act as immunosuppressants. In early studies, elevated NK cell activity was observed after Ad infusion, open-heart surgery, or physical exercise (Pedersen et al., 1988). However, subsequent findings have suggested that the observed increase in NK cell activity is due to a marked, but transitory, increase in the number of circulating NK cells, rather than to an increase in activity per NK cell (Palmø et al., 1995). This increase in the number of circulating NK cells occurs during the time of elevated catecholamine levels and dissipates shortly after catecholamines decline (Benschop, Rodriguez-Feuerhahn, & Schedlowski, 1996). The additional finding that NA impairs the cytotoxicity of NK cells and that  $\beta_2$ -adrenoceptor activation suppresses CD8 cytotoxicity further supports the immune-suppressive role of the  $\beta$ -adrenergic system (Gan, Zhang, Solomon, & Bonavida, 2002). While the role of  $\beta_2$ -adrenoceptors on immune cell function has been widely studied, both the expression and the possible role of  $\beta_3$ -adrenoceptors have not yet been clarified. Here, we provide some evidence for a possible new role for  $\beta_3$ -adrenoceptors in the regulation of melanoma immune-tolerance in tumour-bearing mice.

In this study, propranolol has been used as non-selective  $\beta_1$ -/ $\beta_2$ -adrenoceptor antagonist, and SR59230A has been chosen as a  $\beta_3$ -adrenoceptor antagonist and preferred to a different antagonist, L-748,337, because of its higher affinity for  $\beta_3$ -adrenoceptors in rodents (Candelore et al., 1999). However, even though SR59230A has been previously indicated as a selective  $\beta_3$ -adrenoceptor antagonist, a similar affinity has been demonstrated for all the three subtypes (Baker, 2005; Hoffmann, Leitz, Oberdorf-Maass, Lohse, & Klotz, 2004; Niclauss, Michel-Reher, Alewijnse, & Michel, 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 (Sato, Horinouchi, Hutchinson, Evans, & Summers, 2007; Vrydag & Michel, 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 acts at  $\beta_3$ -adrenoceptors (Dal Monte et al., 2013). According to this study, the *in vivo* use of selective  $\beta_3$ -adrenoceptor siRNAs provides results overlapping with those obtained with SR59230A, thus confirming the role of SR59230A as a  $\beta_3$ -adrenoceptor antagonist in this scenario. Moreover, the administration of the  $\beta_2$ -adrenoceptor agonist terbutaline to pre-silenced  $\beta_3$ -adrenoceptor melanoma-bearing mice showed no rebound in tumour growth demonstrating the predominant role of the  $\beta_3$ -adrenoceptor subtype in controlling tumour development (Figure S2). In addition, the difference between the effects of SR59230A and propranolol treatment observed in the present study could depend on the  $\beta_3$ -adrenoceptor component, because both drugs saturate the receptors, as we can extrapolate from previous studies in the same mouse model used here and from previous data on the relative affinity of SR59230A and propranolol for  $\beta$ -adrenoceptors (Dal Monte et al., 2013; Hoffmann et al., 2004). In addition, the fact that, based on the difference in MW between SR59230A and propranolol, the molar doses of propranolol used in the present study are higher

than those of SR59230A should also be considered. Assuming these premises, this study supports the hypothesis that the blockade of  $\beta_2$ - or  $\beta_3$ -adrenoceptors may promote immune-competence in tumour infiltrating lymphocytes of melanoma-bearing mice and that  $\beta_3$ -adrenoceptors rather than  $\beta_2$ -adrenoceptors play a major role in the ability of melanoma to evade the immune system.

The observation that the expression of  $\beta_3$ -adrenoceptors, but not  $\beta_2$ -adrenoceptors, in mouse lymphocytes was up-regulated in a hypoxic environment and down-regulated after oxygen re-exposure suggests a fine post-translational regulation of  $\beta_3$ -adrenoceptor protein under the control of oxygen. 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  $\beta_3$ -adrenoceptors might participate, in a hypoxic environment, in the acquisition of an immune-tolerant phenotype.

T-lymphocytes are known to express both  $\beta_2$ - and  $\beta_3$ -adrenoceptors, of which  $\beta_3$ -adrenoceptors were primarily up-regulated in response to stress (Laukova et al., 2012). In addition, the present finding that  $\beta_3$ -adrenoceptors are localized to the sub-populations of immune cells involved in both immune-suppression (Treg and MDSC) and immune-toxicity (CD8 and NK) suggests a possible role for  $\beta_3$ -adrenoceptors in the immune response. As shown by our results, both SR59230A and propranolol counteract melanoma growth *in vivo*, and their effect is associated with a significant increase in NK and CD8 cells and a strong reduction in Treg cells and MDSC within the tumour 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  $\beta_3$ -adrenoceptor silencing supports a role for  $\beta_3$ -adrenoceptors in mediating the switch from an immunosuppressive to an immunocompetent tumour micro-environment. 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 our data suggest that  $\beta_3$ -adrenoceptors have a significant role in regulating the immune system, one of the main limitations of the present study is the inability to precisely discriminate if the effect observed on immune phenotype is directly related to  $\beta_3$ -adrenoceptor blockade in immune cells or is a consequence of the reduction in tumour growth. This study, indeed, did not evaluate the cause-effect relationships between tumour cell death and immune modulation *in vivo*, which should be investigated in the future by selective direct manipulations of immune cell sub-populations. However, the results obtained with pretreatment of PBMC with SR59230A or selective  $\beta_3$ -adrenoceptor siRNAs suggest a direct effect of  $\beta_3$ -adrenoceptor blockade on immune cells, independent of an action on tumour cells.

The loss of the cytotoxicity of tumour-infiltrating M1 macrophages and N1 neutrophils represents a substantial barrier to immune clearance of solid tumours (Jaiswal, Chao, Majeti, & Weissman, 2010; Nicolás-Ávila, Adrover, & Hidalgo, 2017). As shown here, SR59230A and  $\beta_3$ -adrenoceptor siRNAs, but not propranolol or  $\beta_2$ -adrenoceptor

siRNAs, induce a strong increase in M1 macrophage and N1 neutrophil populations within the tumour micro-environment, suggesting that the phenotypic shift in macrophages and neutrophils is mainly mediated by  $\beta_3$ -adrenoceptors. This finding is consistent with the recent revelation that  $\beta_3$ -adrenoceptor 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 tumour micro-environment (Schoupe et al., 2012).

In conclusion, this study supports the hypothesis that  $\beta_3$ -adrenoceptors play a role in the promotion of immune-tolerance of melanoma. If future experiments confirm this beneficial effect of  $\beta_3$ -adrenoceptor blockade on immune system editing and tumour resolution,  $\beta_3$ -adrenoceptor blockade could represent a new strategy to overcome cancer immune-editing and an effective therapy against melanoma. Unfortunately, the poor pharmacological profile of the  $\beta_3$ -adrenoceptor blockers currently available may limit the development of future therapies. Nevertheless, the fact that the results from the siRNA approach fit well with those from the pharmacological study supports the possibility that selective  $\beta_3$ -adrenoceptor antagonists (when available) could be metamorphosed from experimental tools into therapeutic drugs.

## ACKNOWLEDGEMENTS

This work was supported in part by a grant from the Italian Ministry of Health (RF-2011-02351158). This study was supported by Meyer Foundation, A. Meyer Children's University Hospital, Florence, Italy. We would like to thank Prof. Antonino Morabito for the kind revision of the manuscript.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

L.F. and M.C. developed the concept and experiment and wrote the manuscript. G.B., F.F., and L.C. performed and analysed the animal model and functional assays. M.C., A.C., and M.B. performed flow cytometry experiments. R.N. and F.D. performed immunohistochemical analysis. F.B. and A.P. performed RMI analysis. M.D.M., G.F., G.I.M., L.C., P.B., P.C., C.A., P.G., and C.F. revised the experiments and the manuscript.

## 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.

## ORCID

Massimo Dal Monte  <https://orcid.org/0000-0002-5181-4456>

Romina Nassini  <https://orcid.org/0000-0002-9223-8395>

Luca Filippi  <https://orcid.org/0000-0001-5310-9147>

## REFERENCES

- Abrahams, V. M., Kamsteeg, M., & Mor, G. (2003). The Fas/Fas ligand system and cancer: Immune privilege and apoptosis. *Molecular Biotechnology*, 25, 19–30. <https://doi.org/10.1385/MB:25:1:19>
- Aktas, E., Kucuksezer, U. C., Bilgic, S., Erten, G., & Deniz, G. (2009). Relationship between CD107a expression and cytotoxic activity. *Cellular Immunology*, 254, 149–154. <https://doi.org/10.1016/j.cellimm.2008.08.007>
- Alexander, S. P., Christopoulos, A., Davenport, A. P., Kelly, E., Marrion, N. V., Peters, J. A., ... CGTP Collaborators (2017). The concise guide to pharmacology 2017/18: G protein-coupled receptors. *British Journal of Pharmacology*, 174, S17–S129. <https://doi.org/10.1111/bph.13878>
- Alexander, S. P. H., Fabbro, D., Kelly, E., Marrion, N. V., Peters, J. A., Faccenda, E., ... CGTP Collaborators. (2017a). The concise guide to pharmacology 2017/18: Enzymes. *Br J Pharmacol*, 174, S272–S359.
- Alexander, S. P. H., Fabbro, D., Kelly, E., Marrion, N. V., Peters, J. A., Faccenda, E., ... CGTP Collaborators. (2017b). The Concise Guide to PHARMACOLOGY 2017/18: Catalytic receptors. *British Journal of Pharmacology*, 174(S1), S225–S271. <https://doi.org/10.1111/bph.13876>
- Alexander, S. P. H., Kelly, E., Marrion, N. V., Peters, J. A., Faccenda, E., Harding, S. D., ... CGTP Collaborators. (2017). The Concise Guide to PHARMACOLOGY 2017/18: Overview. *British Journal of Pharmacology*, 174, S1–S16. <https://doi.org/10.1111/bph.13882>
- Armaiz-Pena, G. N., Allen, J. K., Cruz, A., Stone, R. L., Nick, A. M., Lin, Y. G., ... Sood, A. K. (2013). Src activation by  $\beta$ -adrenoreceptors is a key switch for tumour metastasis. *Nature Communications*, 4, 1403. <https://doi.org/10.1038/ncomms2413>
- Baker, J. G. (2005). The selectivity of  $\beta$ -adrenoceptor antagonists at the human  $\beta_1$ ,  $\beta_2$  and  $\beta_3$  adrenoceptors. *British Journal of Pharmacology*, 144, 317–322. <https://doi.org/10.1038/sj.bjp.0706048>
- Barbieri, A., Palma, G., Rosati, A., Giudice, A., Falco, A., Petrillo, A., ... Arra, C. (2012). Role of endothelial nitric oxide synthase (eNOS) in chronic stress-promoted tumour growth. *Journal of Cellular and Molecular Medicine*, 16, 920–926. <https://doi.org/10.1111/j.1582-4934.2011.01375.x>
- Benschop, R. J., Rodriguez-Feuerhahn, M., & Schedlowski, M. (1996). Catecholamine-induced leukocytosis: early observations, current research, and future directions. *Brain, Behavior, and Immunity*, 10, 77–91. <https://doi.org/10.1006/brbi.1996.0009>
- Calvani, M., Pelon, F., Comito, G., Taddei, M. L., Moretti, S., Innocenti, S., ... Chiarugi, P. (2015). Norepinephrine promotes tumor microenvironment reactivity through  $\beta_3$ -adrenoreceptors during melanoma progression. *Oncotarget*, 6, 4615–4632. <https://doi.org/10.18632/oncotarget.2652>
- Candelore, M. R., Deng, L., Tota, L., Guan, X. M., Amend, A., Liu, Y., ... Weber, A. E. (1999). Potent and selective human  $\beta(3)$ -adrenergic receptor antagonists. *The Journal of Pharmacology and Experimental Therapeutics*, 290, 649–655.
- Cheng, Y., Gao, X. H., Li, X. J., Cao, Q. H., Zhao, D. D., Zhou, J. R., ... Yang, Y. (2018). Depression promotes prostate cancer invasion and metastasis via a sympathetic-cAMP-FAK signaling pathway. *Oncogene*, 37, 2953–2966. <https://doi.org/10.1038/s41388-018-0177-4>
- Childers, W. K., Hollenbeck, C. S., & Cheriya, P. (2015).  $\beta$ -Blockers reduce breast cancer recurrence and breast cancer death: A meta-analysis. *Clinical Breast Cancer*, 15, 426–431. <https://doi.org/10.1016/j.clbc.2015.07.001>

- Cole, S. W., Nagaraja, A. S., Lutgendorf, S. K., Green, P. A., & Sood, A. K. (2015). Sympathetic nervous system regulation of the tumour microenvironment. *Nature Reviews. Cancer*, *15*, 563–572. <https://doi.org/10.1038/nrc3978>
- Cole, S. W., & Sood, A. K. (2012). Molecular pathways:  $\beta$ -Adrenergic signaling in cancer. *Clinical Cancer Research*, *18*, 1201–1206. <https://doi.org/10.1158/1078-0432.CCR-11-0641>
- Curtis, M. J., Alexander, S., Cirino, G., Docherty, J. R., George, C. H., Giembycz, M. A., ... Ahluwalia, A. (2018). Experimental design and analysis and their reporting II: Updated and simplified guidance for authors and peer reviewers. *British Journal of Pharmacology*, *175*, 987–993. <https://doi.org/10.1111/bph.14153>
- Dal Monte, M., Calvani, M., Cammalleri, M., Favre, C., Filippi, L., & Bagnoli, P. (2018).  $\beta$ -Adrenoceptors as drug targets in melanoma: Novel preclinical evidence for a role of  $\beta_3$ -adrenoceptors. *British Journal of Pharmacology*. <https://doi.org/10.1111/bph.14552>
- Dal Monte, M., Casini, G., Filippi, L., Nicchia, G. P., Svelto, M., & Bagnoli, P. (2013). Functional involvement of  $\beta_3$ -adrenergic receptors in melanoma growth and vascularization. *J Mol Med (Berl)*, *91*, 1407–1419. <https://doi.org/10.1007/s00109-013-1073-6>
- Dal Monte, M., Fornaciari, I., Nicchia, G. P., Svelto, M., Casini, G., & Bagnoli, P. (2014).  $\beta_3$ -Adrenergic receptor activity modulates melanoma cell proliferation and survival through nitric oxide signaling. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *387*, 533–543. <https://doi.org/10.1007/s00210-014-0969-1>
- De Giorgi, V., Grazzini, M., Benemei, S., Marchionni, N., Geppetti, P., & Gandini, S. (2017).  $\beta$ -Blocker use and reduced disease progression in patients with thick melanoma: 8 years of follow-up. *Melanoma Research*, *27*, 268–270. <https://doi.org/10.1097/CMR.0000000000000317>
- De Giorgi, V., Grazzini, M., Gandini, S., Benemei, S., Lotti, T., Marchionni, N., & Geppetti, P. (2011). Treatment with  $\beta$ -blockers and reduced disease progression in patients with thick melanoma. *Archives of Internal Medicine*, *171*, 779–781.
- Entschladen, F., Drell, T. L., Lang, K., Joseph, J., & Zaenker, K. S. (2004). Tumour-cell migration, invasion, and metastasis: Navigation by neurotransmitters. *The Lancet Oncology*, *5*, 254–258. [https://doi.org/10.1016/S1470-2045\(04\)01431-7](https://doi.org/10.1016/S1470-2045(04)01431-7)
- Estrada, L. D., Açaç, D., & Farrar, J. D. (2016). Sympathetic neural signaling via the  $\beta_2$ -adrenergic receptor suppresses T-cell receptor-mediated human and mouse CD8<sup>+</sup> T-cell effector function. *European Journal of Immunology*, *46*, 1948–1958. <https://doi.org/10.1002/eji.201646395>
- Facciabene, A., Peng, X., Hagemann, I. S., Balint, K., Barchetti, A., Wang, L. P., ... Coukos, G. (2011). Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T (reg) cells. *Nature*, *475*, 226–230. <https://doi.org/10.1038/nature10169>
- Fridlender, Z. G., Sun, J., Kim, S., Kapoor, V., Cheng, G., Ling, L., ... Albelda, S. M. (2009). Polarization of tumor-associated neutrophil phenotype by TGF- $\beta$ : “N1” versus “N2” TAN. *Cancer Cell*, *16*, 183–194. <https://doi.org/10.1016/j.ccr.2009.06.017>
- Fujimura, T., Kambayashi, Y., & Aiba, S. (2012). Crosstalk between regulatory T cells (Tregs) and myeloid derived suppressor cells (MDSCs) during melanoma growth. *Oncoimmunology*, *1*, 1433–1434. <https://doi.org/10.4161/onci.21176>
- Gan, X., Zhang, L., Solomon, G. F., & Bonavida, B. (2002). Mechanism of norepinephrine-mediated inhibition of human NK cytotoxic functions: Inhibition of cytokine secretion, target binding, and programming for cytotoxicity. *Brain, Behavior, and Immunity*, *16*, 227–246. <https://doi.org/10.1006/brbi.2000.0615>
- Glasner, A., Avraham, R., Rosenne, E., Benish, M., Zmora, O., Shemer, S., ... Ben-Eliyahu, S. (2010). Improving survival rates in two models of spontaneous postoperative metastasis in mice by combined administration of a  $\beta$ -adrenergic antagonist and a cyclooxygenase-2 inhibitor. *Journal of Immunology*, *184*, 2449–2457. <https://doi.org/10.4049/jimmunol.0903301>
- Granot, Z., & Fridlender, Z. G. (2015). Plasticity beyond cancer cells and the “immunosuppressive switch”. *Cancer Research*, *75*, 4441–4445. <https://doi.org/10.1158/0008-5472.CAN-15-1502>
- Guereschi, M. G., Araujo, L. P., Maricato, J. T., Takenaka, M. C., Nascimento, V. M., Vivanco, B. C., ... Basso, A. S. (2013).  $\beta_2$ -adrenergic receptor signaling in CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells enhances their suppressive function in a PKA-dependent manner. *European Journal of Immunology*, *43*, 1001–1012. <https://doi.org/10.1002/eji.201243005>
- Hadi, T., Douhard, R., Dias, A. M., Wendremaire, M., Pezzè, M., Bardou, M., ... Lirussi, F. (2017).  $\beta_3$  adrenergic receptor stimulation in human macrophages inhibits NADPH oxidase activity and induces catalase expression via PPAR $\gamma$  activation. *Biochimica et Biophysica Acta*, *1864*, 1769–1784. <https://doi.org/10.1016/j.bbamcr.2017.07.003>
- Harding, S. D., Sharman, J. L., Faccenda, E., Southan, C., Pawson, A. J., Ireland, S., ... NC-IUPHAR. (2018). The IUPHAR/BPS guide to pharmacology in 2018: Updates and expansion to encompass the new guide to immunopharmacology. *Nucl Acids Res*, *46*(D1), D1091–D1106. <https://doi.org/10.1093/nar/gkx1121>
- Hoffmann, C., Leitz, M. R., Oberdorf-Maass, S., Lohse, M. J., & Klotz, K. N. (2004). Comparative pharmacology of human  $\beta$ -adrenergic receptor subtypes—Characterization of stably transfected receptors in CHO cells. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *369*, 151–159. <https://doi.org/10.1007/s00210-003-0860-y>
- Inbar, S., Neeman, E., Avraham, R., Benish, M., Rosenne, E., & Ben-Eliyahu, S. (2011). Do stress responses promote leukemia progression? An animal study suggesting a role for epinephrine and prostaglandin-E2 through reduced NK activity. *PLoS ONE*, *6*, e19246. <https://doi.org/10.1371/journal.pone.0019246>
- Jaiswal, S., Chao, M. P., Majeti, R., & Weissman, I. L. (2010). Macrophages as mediators of tumor immunosurveillance. *Trends in Immunology*, *31*, 212–219. <https://doi.org/10.1016/j.it.2010.04.001>
- Kilkenny, C., Browne, W., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Animal research: Reporting *in vivo* experiments: The ARRIVE guidelines. *British Journal of Pharmacology*, *160*, 1577–1579.
- King, G. T., Sharma, P., Davis, S. L., & Jimeno, A. (2018). Immune and autoimmune-related adverse events associated with immune checkpoint inhibitors in cancer therapy. *Drugs Today (Barc)*, *54*, 103–122. <https://doi.org/10.1358/dot.2018.54.2.2776626>
- Kokolus, K. M., Zhang, Y., Sivik, J. M., Schmeck, C., Zhu, J., Repasky, E. A., ... Schell, T. D. (2017).  $\beta$  blocker use correlates with better overall survival in metastatic melanoma patients and improves the efficacy of immunotherapies in mice. *Oncoimmunology*, *7*, e1405205.
- Laukova, M., Vargovic, P., Csaderova, L., Chovanova, L., Vlcek, M., Imrich, R., ... Kvetnansky, R. (2012). Acute stress differently modulates  $\beta_1$ ,  $\beta_2$  and  $\beta_3$  adrenoceptors in T cells, but not in B cells, from the rat spleen. *Neuroimmunomodulation*, *19*, 69–78. <https://doi.org/10.1159/000329002>
- Lemeshow, S., Sørensen, H. T., Phillips, G., Yang, E. V., Antonsen, S., Riis, A. H., ... Glaser, R. (2011).  $\beta$ -Blockers and survival among Danish patients with malignant melanoma: A population-based cohort study. *Cancer Epidemiology, Biomarkers & Prevention*, *20*, 2273–2279. <https://doi.org/10.1158/1055-9965.EPI-11-0249>
- Livingstone, E., Hollestein, L. M., van Herk-Sukel, M. P., van de Poll-Franse, L., Nijsten, T., Schadendorf, D., & de Vries, E. (2013).  $\beta$ -Blocker use and all-cause mortality of melanoma patients: Results from a population-based Dutch cohort study. *European Journal of Cancer*, *49*, 3863–3871. <https://doi.org/10.1016/j.ejca.2013.07.141>
- Magnon, C., Hall, S. J., Lin, J., Xue, X., Gerber, L., Freedland, S. J., & Frenette, P. S. (2013). Autonomic nerve development contributes to

- prostate cancer progression. *Science*, 341, 1236361. <https://doi.org/10.1126/science.1236361>
- Maisel, A. S., Fowler, P., Rearden, A., Motulsky, H. J., & Michel, M. C. (1989). A new method for isolation of human lymphocyte subsets reveals differential regulation of  $\beta$ -adrenergic receptors by terbutaline treatment. *Clinical Pharmacology and Therapeutics*, 46, 429–439. <https://doi.org/10.1038/clpt.1989.161>
- Mantovani, A., & Locati, M. (2013). Tumor-associated macrophages as a paradigm of macrophage plasticity, diversity, and polarization: Lessons and open questions. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 33, 1478–1483. <https://doi.org/10.1161/ATVBAHA.113.300168>
- Marino, F., & Cosentino, M. (2013). Adrenergic modulation of immune cells: An update. *Amino Acids*, 45, 55–71. <https://doi.org/10.1007/s00726-011-1186-6>
- Mazzoni, A., Bronte, V., Visintin, A., Spitzer, J. H., Apolloni, E., Serafini, P., ... Segal, D. M. (2002). Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *Journal of Immunology*, 168, 689–695. <https://doi.org/10.4049/jimmunol.168.2.689>
- McCourt, C., Coleman, H. G., Murray, L. J., Cantwell, M. M., Dolan, O., Powe, D. G., & Cardwell, C. R. (2014).  $\beta$ -Blocker usage after malignant melanoma diagnosis and survival: A population-based nested case-control study. *The British Journal of Dermatology*, 170, 930–938. <https://doi.org/10.1111/bjd.12894>
- Moretti, S., Massi, D., Farini, V., Baroni, G., Parri, M., Innocenti, S., ... Chiarugi, P. (2013).  $\beta$ -Adrenoceptors are upregulated in human melanoma and their activation releases pro-tumorigenic cytokines and metalloproteases in melanoma cell lines. *Laboratory Investigation*, 93, 279–290. <https://doi.org/10.1038/labinvest.2012.175>
- Munder, M. (2009). Arginase: An emerging key player in the mammalian immune system. *British Journal of Pharmacology*, 158, 638–651. <https://doi.org/10.1111/j.1476-5381.2009.00291.x>
- Mundy-Bosse, B. L., Thornton, L. M., Yang, H. C., Andersen, B. L., & Carson, W. E. (2011). Psychological stress is associated with altered levels of myeloid-derived suppressor cells in breast cancer patients. *Cellular Immunology*, 270, 80–87. <https://doi.org/10.1016/j.cellimm.2011.04.003>
- Nance, D. M., & Sanders, V. M. (2007). Autonomic innervation and regulation of the immune system (1987–2007). *Brain, Behavior, and Immunity*, 21, 736–745. <https://doi.org/10.1016/j.bbi.2007.03.008>
- Niclauss, N., Michel-Reher, M. B., Alewijnse, A. E., & Michel, M. C. (2006). Comparison of three radioligands for the labelling of human  $\beta$ -adrenoceptor subtypes. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 374, 99–105. <https://doi.org/10.1007/s00210-006-0104-z>
- Nicolás-Ávila, J. Á., Adrover, J. M., & Hidalgo, A. (2017). Neutrophils in homeostasis, immunity, and cancer. *Immunity*, 46, 15–28. <https://doi.org/10.1016/j.immuni.2016.12.012>
- Nissen, M. D., Sloan, E. K., & Mattarollo, S. R. (2018).  $\beta$ -Adrenergic signaling impairs antitumor CD8<sup>+</sup> T-cell responses to B-cell lymphoma immunotherapy. *Cancer Immunology Research*, 6, 98–109. <https://doi.org/10.1158/2326-6066.CIR-17-0401>
- Palmø, J., Asp, S., Daugaard, J. R., Richter, E. A., Klokke, M., & Pedersen, B. K. (1995). Effect of eccentric exercise on natural killer cell activity. *Journal of Applied Physiology* (1985), 78, 1442–1446.
- Passarelli, A., Mannavola, F., Stucci, L. S., Tucci, M., & Silvestris, F. (2017). Immune system and melanoma biology: A balance between immunosurveillance and immune escape. *Oncotarget*, 8, 106132–106142.
- Pedersen, B. K., Tvede, N., Hansen, F. R., Andersen, V., Bendix, T., Bendixen, G., ... Klarlund, K. (1988). Modulation of natural killer cell activity in peripheral blood by physical exercise. *Scandinavian Journal of Immunology*, 27, 673–678. <https://doi.org/10.1111/j.1365-3083.1988.tb02400.x>
- Sato, M., Horinouchi, T., Hutchinson, D. S., Evans, B. A., & Summers, R. J. (2007). Ligand-directed signaling at the  $\beta$ 3-adrenoceptor produced by SR59230A relative to receptor agonists. *Molecular Pharmacology*, 74, 1359–1368.
- Schoupe, E., De Baetselier, P., Van Ginderachter, J. A., & Sarukhan, A. (2012). Instruction of myeloid cells by the tumor microenvironment: Open questions on the dynamics and plasticity of different tumor-associated myeloid cell populations. *Oncotarget*, 1, 1135–1145. <https://doi.org/10.4161/onci.21566>
- Sereni, F., Dal Monte, M., Filippi, L., & Bagnoli, P. (2015). Role of host  $\beta$ 1- and  $\beta$ 2-adrenergic receptors in a murine model of B16 melanoma: Functional involvement of  $\beta$ 3-adrenergic receptors. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 388, 1317–1331. <https://doi.org/10.1007/s00210-015-1165-7>
- Tawbi, H. A., Forsyth, P. A., Algazi, A., Hamid, O., Hodi, F. S., Moschos, S. J., ... Margolin, K. (2018). Combined nivolumab and ipilimumab in melanoma metastatic to the brain. *The New England Journal of Medicine*, 379, 722–730. <https://doi.org/10.1056/NEJMoa1805453>
- Thaker, P. H., Han, L. Y., Kamat, A. A., Arevalo, J. M., Takahashi, R., Lu, C., ... Sood, A. K. (2006). Chronic stress promotes tumor growth and angiogenesis in a mouse model of ovarian carcinoma. *Nature Medicine*, 12, 939–944. <https://doi.org/10.1038/nm1447>
- van den Broek, M. F., & Hengartner, H. (2000). The role of perforin in infections and tumour surveillance. *Experimental Physiology*, 85, 681–685. <https://doi.org/10.1017/S0958067000020972>
- Vinay, D. S., Ryan, E. P., Pawelec, G., Talib, W. H., Stagg, J., Elkord, E., ... Kwon, B. S. (2015). Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Seminars in Cancer Biology*, 35(Suppl), S185–S198. <https://doi.org/10.1016/j.semcancer.2015.03.004>
- Vrydag, W., & Michel, M. C. (2007). Tools to study  $\beta$ 3-adrenoceptors. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 374, 385–398. <https://doi.org/10.1007/s00210-006-0127-5>
- Wrobel, L. J., & Le Gal, F. A. (2015). Inhibition of human melanoma growth by a non-cardioselective  $\beta$ -blocker. *The Journal of Investigative Dermatology*, 135, 525–531. <https://doi.org/10.1038/jid.2014.373>
- Yang, E. V., Kim, S. J., Donovan, E. L., Chen, M., Gross, A. C., Webster Marketon, J. I., ... Glaser, R. (2009). Norepinephrine upregulates VEGF, IL-8, and IL-6 expression in human melanoma tumor cell lines: Implications for stress-related enhancement of tumor progression. *Brain, Behavior, and Immunity*, 23, 267–275. <https://doi.org/10.1016/j.bbi.2008.10.005>
- Yazawa, T., Kaira, K., Shimizu, K., Shimizu, A., Mori, K., Nagashima, T., ... Kuwano, H. (2016). Prognostic significance of  $\beta$ 2-adrenergic receptor expression in non-small cell lung cancer. *American Journal of Translational Research*, 8, 5059–5070.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Calvani M, Bruno G, Dal Monte M, et al.  $\beta$ 3-Adrenoceptor as a potential immuno-suppressor agent in melanoma. *Br J Pharmacol*. 2019;176:2509–2524. <https://doi.org/10.1111/bph.14660>