

Benefits of exercise and immunotherapy in a murine model of human non–small-cell lung carcinoma

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ABSTRACT

Background: Lung cancer has the highest incidence and mortality rate in the world. One of the most promising new cancer therapies in recent years is immunotherapy, which is based on the blockade of immune checkpoints such as programmed cell death protein 1 (PD-1). Exercise training is beneficial to maintain and improve the quality of life of cancer patients, and it might also modulate the anti-tumoral efficiency of some chemotherapeutic agents. However, the potential of exercise combined with immunotherapy as a cancer therapy remains to be elucidated. Here, we examined the effects of exercise on tumor growth and its possible adjuvant effects when combined with anti-PD-1 immunotherapy (nivolumab) in a patient-derived xenograft (PDX) model of non–small-cell lung carcinoma (NSCLC).

Methods: We generated a PDX model using NOD-SCID gamma mice with subcutaneous grafts from tumor tissue of a patient with NSCLC. Animals were randomly assigned to one of four groups: non-exercise + isotype control (n=5), exercise + isotype control (n=5), non-exercise + nivolumab (n=6) or exercise + nivolumab (n=6). The animals undertook an 8-

week moderate-intensity training regimen (treadmill aerobic exercise and strength training). Immunotherapy (nivolumab) or an isotype control was administered 2 days/week, for 6 weeks. Several tumor growth and microenvironment parameters were measured after the intervention.

Results: Improvements in aerobic capacity and muscle strength ($p=0.027$ and $p=0.005$) were noted in exercised animals. Exercise alone reduced the tumor growth rate with respect to non-exercised mice ($p=0.050$). The double intervention (exercise + nivolumab) increased tumor necrosis and reduced apoptosis with respect to controls ($p=0.026$; $p=0.030$). All interventions achieved a reduction in proliferation compared with the control group ($p=0.015$, $p=0.011$, and $p=0.011$). Exercise alone increased myeloid tumor infiltrates (mostly neutrophils) with respect to the nivolumab only group ($p=0.018$). Finally, Vegf-a expression was higher in the nivolumab groups (in combination or not with exercise) than in exercise + isotype control group ($p=0.045$ and $p=0.047$, respectively). No other significant effects were found.

Conclusions: Our results would suggest that aerobic and strength training should be studied as an adjuvant to cancer immunotherapy treatment.

Keywords: exercise, training, lung cancer, patient-derived xenografts, cancer immunotherapy, immune checkpoints.

INTRODUCTION

Lung cancer is the most common cancer worldwide, and accounts for more than 2.1 million new cases each year and about 1.8 million deaths (10). Non–small-cell lung carcinoma (NSCLC) is a major sub-type of lung cancer with a particularly poor 5-year relative survival rate of 10–13% (20), which is partly attributable to inefficient methods for early detection and lack of curative treatments for advanced disease (10). While surgical resection and chemotherapy remain the cornerstone of treatment in NSCLC (9), checkpoint inhibitor-based immunotherapy is quickly emerging as a possible new treatment modality with the potential to revolutionize cancer care for patients (25).

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On the other hand, the study of the relationship between cancer and exercise continues to evolve. A healthy lifestyle that includes regular exercise and physical activity is epidemiologically associated with a lower risk of cancer – including lung tumors – and cancer mortality (54). Exercise also improves the general physical condition of patients undergoing anti-neoplastic treatment or surgery, and helps mitigate treatment side effects and other complications, and for these reasons people living with and beyond cancer should be advised to be as physically active as possible (56). Regarding lung cancer specifically, growing evidence supports the safety and efficacy of exercise interventions (4). For instance, preoperative exercise considerably reduces the postoperative complication rate (61), with a recent meta-analysis of randomized controlled trials in patients with NSCLC showing improvements in walking endurance, peak exercise capacity, dyspnoea, risk of hospitalization, and postoperative pulmonary complications (53). However, the molecular underpinnings of the potential exercise benefits on lung cancer outcomes remain to be elucidated (4). Some mechanisms have been postulated, such as p53-induced apoptosis in a murine model of lung adenocarcinoma (26), or reduced proliferation and survival of NSCLC cells (41). In addition, the potential anti-tumorigenic effects of exercise on lung cancer –and in fact in most tumors –appear to be related to immunomodulation, as explained below.

The best studied mechanisms are those associated with moderate-intensity aerobic exercise, which involve the improvement of immune function (48). Specifically, innate immunity can be regulated by exercise through reprogramming the tumor microenvironment (39), and by polarizing the relative abundance of different myeloid cell types towards a more anti-tumorigenic phenotype (1). The evidence is particularly strong for natural killer (NK) lymphocyte mobilization and redistribution to the tumor, pointing to a direct anti-tumor effect of exercise, although changes have also been reported in adaptive immunity, with exercise enhancing the mobilization, redistribution and activation of T lymphocytes (principally, cytotoxic CD8⁺ T cells) (40), increasing their infiltration into the tumor stroma (39, 49). With regard to lung cancer, Pedersen et al. (49) found that wheel running in mice inoculated with Lewis lung cancer cells significantly reduced tumor volume, with an upregulation of the proinflammatory cytokine interleukin (IL)1 α , as well as of inducible nitric oxide synthase [iNOS] and markers for NK and T-lymphocyte activity. In patients with NSCLC, tai chi training may promote proliferation of peripheral blood mononuclear cells – with an improvement in their cytotoxicity – and increase circulating NK cell percentage, natural killer T, and dendritic CD11 cells (43), and could also preserve a stable interferon (IFN) γ -producing CD3⁺ T lymphocytes (T1) to IL4-producing CD3⁺ T lymphocytes (T2) ratio (70).

Several preclinical models have been developed to study the effects of exercise in cancer (54), with some studies suggesting a greater therapeutic benefit of certain drugs (*e.g.*, low-dose doxorubicin (62)) when used in combination with exercise. The use of the aforementioned models is crucially important for testing the biological plausibility, establishing the therapeutic window and the effective “dose” (intensity, volume and duration of the exercise), and identifying possible predictors of exercise response (33). However, the potential anti-tumor

effects of exercise in combination with checkpoint inhibitor-based immunotherapy remain to be elucidated using preclinical cancer models. Although this treatment approach has resulted in unprecedented improvements in survival for patients with lung cancer, not all patients benefit equally and many issues remain unresolved, including the mechanisms of action and the possible effector function of immune cells from non-lymphoid lineages. In this regard, the clinical implementation of antibodies targeting the immune checkpoint inhibitor programmed cell death protein 1 (PD-1), for example nivolumab, can relaunch anti-tumor responses by stimulating the action of infiltrating cytotoxic T lymphocytes in the tumor microenvironment (59, 65). Yet, recent preliminary evidence from our group using a murine patient-derived xenograft (PDX) model of squamous NSCLC devoid of host lymphoid cells provided mechanistic support for an additional immunotherapy mechanism mediated by myeloid cells – specifically neutrophils –, which act as PD-1 inhibitor effector cells responsible for tumor regression by necrotic extension (44). Thus, it would be of interest to use the aforementioned model to investigate whether exercise acts as an adjuvant to anti-PD-1 immunotherapy on malignant tumor cells through mechanisms beyond those related to T lymphocyte involvement.

The aim of the present study was therefore to determine the effect of a physical exercise intervention (specifically, an 8-week combined protocol including aerobic and strength training) during immunotherapy treatment with the anti-PD-1 monoclonal antibody nivolumab in a PDX murine model of squamous cell lung carcinoma – a histologic type of NSCLC. Our main hypothesis was that the exercise intervention would induce significant beneficial effects in terms of physical capacity in PDX mice, and also that the combined effect of physical exercise with nivolumab therapy would enhance the anti-tumor response, with exercise acting as a potential adjuvant treatment.

MATERIALS AND METHODS

Ethical approval for the collection and the use of patient tumor tissue was granted by the Ethics Committee of the *Hospital Universitario Puerta de Hierro* (Madrid, Spain; approval number: PI/144-14). The study was conducted following the Declaration of Helsinki guidelines for the treatment of data and tumor tissue samples from donor patients, collected from May 2015 to October 2018. Eligibility criteria for tissue collection included: patient’s informed consent, a new diagnosis of a primary lung tumor (specifically a NSCLC), not having received previous therapy until surgery and, provision of a sufficient quantity of tumor volume to donate a section for research purposes.

All animal experimental protocols were also approved by the Institutional Ethics Committee of the *Hospital Universitario Puerta de Hierro* (approval number: PROEX 163/14) and were conducted in accordance with European (European Convention ETS 123) and Spanish (32/2007 and R.D. 1201/2005) laws on animal protection in scientific research.

Donor patient

The donor patient was a 79-year-old male who underwent a lobectomy for a nodule in the upper lobe of the right lung.

According to World Health Organization criteria for histological classification and staging (11, 66), the patient’s tumor was a basaloid infiltrating and poorly-differentiated squamous cell lung carcinoma, in clinical stage IIA (pT2a N1 L1 M0).

Animals

Female 8-week-old NOD-SCID gamma mice (NSG, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) purchased from Charles River Breeding Laboratories (Chatillon-sur-Chalaronne, France) were used for the establishment of a human squamous NSCLC PDX model. Mice were housed in the animal facility of Hospital Universitario Puerta de Hierro on a 12-hour light/12-hour dark cycle under controlled conditions of temperature (22 ± 1 °C) and humidity (45 ± 10%). Mice were accommodated in pairs in ventilated racks under specific pathogen-free conditions, with environmental enrichment (*nestlets*), and *ad libitum* access to food and water. Mice were handled in laminar airflow cabinets during all the experimental protocols.

Study design

Establishment of a human squamous NSCLC PDX model

The tumor sample was surgically obtained from the donor patient and sliced into fragments (~3 mm × 2 mm) under sterile conditions. Two specimen pieces were implanted subcutaneously into the bilateral flanks of mice (bilateral grafts) under inhalation anesthesia (sevoflurane in oxygen, Sevovane). This first transplant directly performed from the donor patient to the host mouse was referred to as passage 0 or p0. Whenever palpable, tumor volume was measured with a

caliper (AA846R, Aesculap AG, Tuttlingen, Germany) twice weekly using the following formula: $(4\pi/3) \times (w/2)^2 \times (l/2)$, where w = width and l = length. When the volume reached ~1 cm³, a piece of the tumor was harvested and regrafted into new host mice, in order to maintain the PDX line *in vivo* during subsequent passages; these serial transplants were called p1, p2, and p3, etc. Another portion of the harvested tumor was used for phenotype and molecular analyses to verify that the xenograft model was stable without histopathological changes along passages. When the PDX p2 tumors reached the appropriate size (~100 mm³), they were regrafted subcutaneously in the bilateral flanks of 22 female mice (p3) (Figure 1A). Finally, transplanted p3 mice were monitored and their bilateral tumor volumes were measured as described above. When the tumors reached ~100 mm³, the mice were included in the intervention.

Pharmacological and exercise intervention

The pharmacological intervention for the NSCLC PDX model was based on our previous study, which describes an immunotherapy mechanism based on neutrophils (44).

Transplanted p3 mice were paired-matched based on their aerobic capacity (see below), and each pair was randomly assigned to the following experimental groups: non-exercise + isotype control (n=5); exercise + isotype control (n=5); non-exercise + nivolumab (n=6); exercise + nivolumab (n=6). The study design and the experimental groups are shown in Figure 1.

Regarding the pharmacological intervention, isotype control or nivolumab was administrated intraperitoneally (i.p.) twice weekly for six consecutive weeks, beginning at day 0

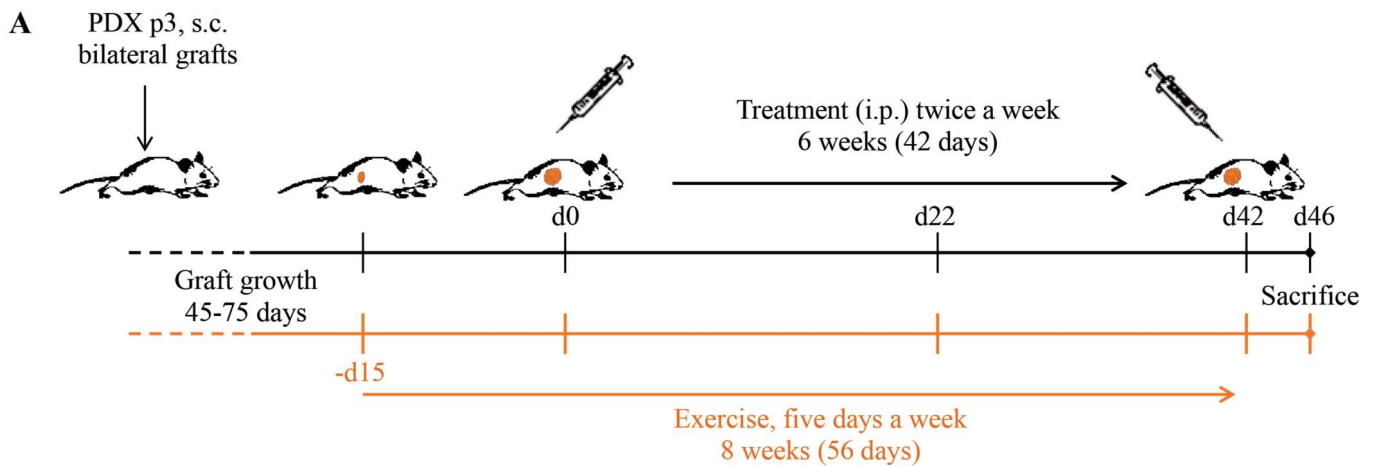


Figure 1. Study design. (A) Schematic representation of the study protocol. Tumor sensitivity to exercise and immunotherapy in a murine PDX model of squamous non-small-cell lung carcinoma (NSCLC). (B) Table showing treatment regimens. Abbreviation: PDX, patient-derived xenograft.

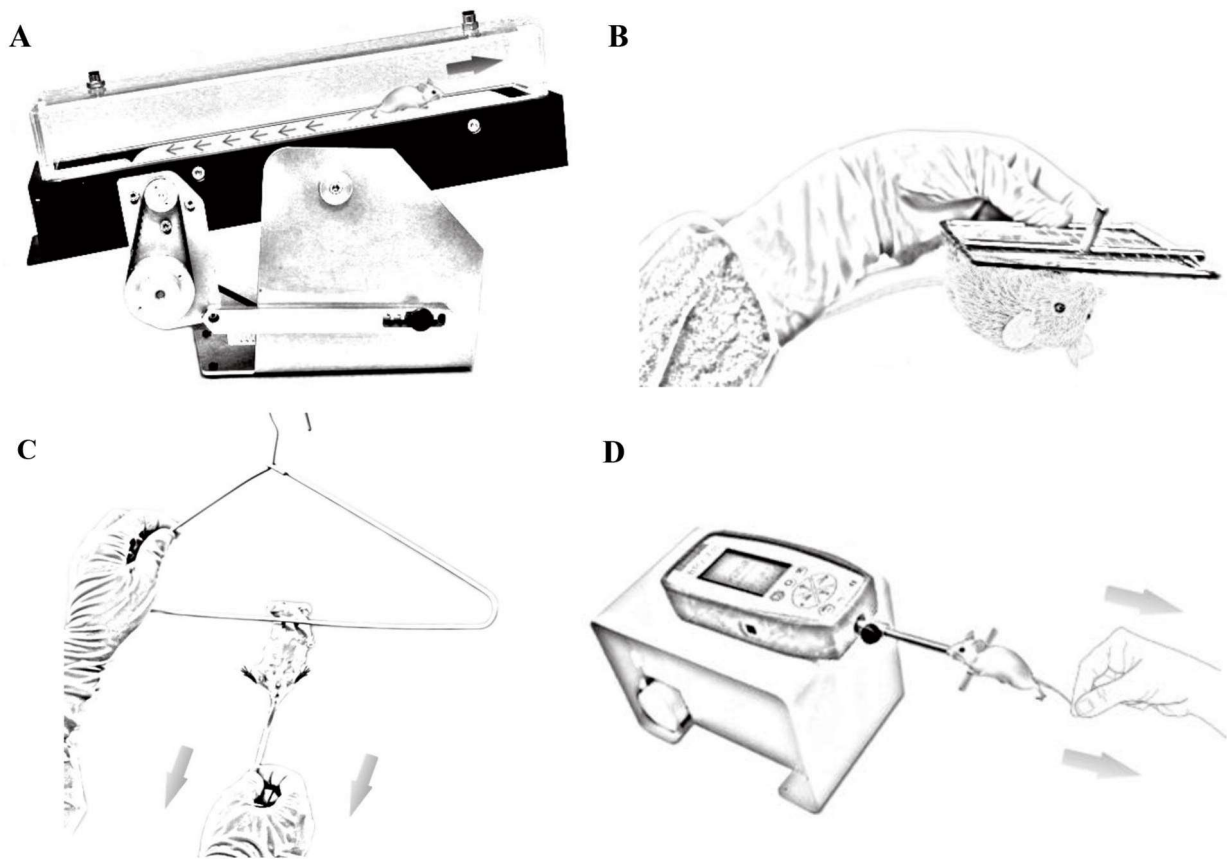


Figure 2. Diagrams of physical exercise equipment. Experimental apparatuses used in the exercise study: (A) Treadmill. (B) Grid. (C) Hanger. (D) Grip strength meter.

(d0) until the end of the experiment (d42). The treatment regimens and doses used in this study are shown in Figure 1B.

The exercise intervention was based on our previous experience (17, 18) and was adapted to the features of the PDX model. The exercise program combined both aerobic and resistance training during five days per week (Monday–Friday; session duration: 40–60 minutes) for a total of eight weeks (from day minus 15 [d-15] to d42). The *aerobic training* regimen included five weekly sessions (session duration: 30–40 minutes) during which the exercise duration, treadmill speed and inclination were gradually increased (Figure 2A). Accordingly, mice started with very low workloads (d-15, 20 minutes at 40% of the maximal velocity obtained during the aerobic performance test that is described below and 0% gradient) and ended with 40 minutes at 80% of maximal velocity and 15% gradient in the last sessions (d42). Electrical stimulation was applied (0.2 mA, 1 Hz) during the first aerobic sessions, but later only gentle tail touching was used as a stimulus to provoke the mouse to move. The *strength training* was performed after the aerobic training, twice weekly (Tuesday and Friday), and included two exercises as recently described (18): horizontal screen exercise (Tuesday; Figure 2B) and hanging exercise with two limbs (Friday; Figure 2C). For the first exercise, the mouse was placed on top of an inverted screen (BIO-GRIPGS model, Bioseb; Chaville, France) and had to climb back over to the top; when this was achieved, the mouse was placed again in the initial position. The number of repetitions was gradually increased (from two to six with a duration of one minute each) with a constant 1-minute rest

period between repetitions. For the second exercise, mice were picked up by the tail and placed on a metal cloth hanger taped to a shelf and maintained at 40 cm above a layer of bedding to cushion any fall. Mice were allowed to grasp the wire only using the two forepaws for as long as they could during one set of two-to-six repetitions, with a 2-minute rest period between repetitions. Non-exercise groups could freely move in their cages, but did not perform the exercise intervention.

Sample collection

Peripheral blood samples from the caudal vein were drawn into EDTA tubes at the end of the intervention (d46, 48 hours after the last exercise tests) to study immune cell populations by flow cytometry. Mice were sacrificed after the last blood collection (d46) by cervical dislocation. The bilateral tumors (n=44) were removed and weighed, and their volumes were measured as described earlier. Then, each tumor was divided into several portions for the following analyses: *i*) one piece was fixed in formalin and paraffin-embedded prior to histopathological and immunohistochemical studies; *ii*) a second piece was cryopreserved in OCT (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan) to determine proliferation and apoptosis by immunofluorescence; and, *iii*) a third piece was homogenized in Hank's Balanced Salt Solution (HBSS, Gibco Life Technologies, Gergy-Pontoise, France) to examine for intratumoral immune cell infiltrates using flow cytometry and real-time quantitative polymerase chain reaction (qPCR). To this end, tumors were mechanically homogenized and then digested by gentle agitation in RPMI medium

(Gibco Life Technologies) with D-collagenase (1 mg/ml; Roche Diagnostics, Mannheim, Germany) in a 3:10 proportion for 24 hours at room temperature. When the enzymatic digestion was completed, the suspension was passed through a 40 µm pore size nylon mesh (BD Biosciences, Franklin Lakes, NJ) and centrifuged for 15 minutes at 1500 rpm. After washing twice with HBSS, a part of the resuspended pellet was directly used for leukocyte population identification by flow cytometry and another part was preserved at -80 °C to analyze immune system-related gene expression.

Outcomes

Aerobic capacity

All mice had two familiarization sessions on a treadmill (Harvard Apparatus; Panlab, Barcelona, Spain; Figure 2A) before the aerobic capacity tests were performed at the beginning and the end of the study. The familiarization sessions consisted of a gradual increase in running duration (5–10 minutes), treadmill speed (0–8 cm/s), and inclination (0–15 degrees), starting with no electrical stimulation and ending with 0.2 mA (1 Hz, 200 ms). Once mice were weighed, they performed a 10-minute warm-up period at 5–10 cm/s (treadmill inclination: 15 degrees) followed by the incremental treadmill test, which started at 5 cm/s with successive speed increments of 2 cm/s every 2 minutes until exhaustion (constant treadmill inclination at 15 degrees), which was defined as the mouse spending more than five continuous seconds on the electric grid and unable to continue running at the next speed. The maximal aerobic capacity achieved was expressed as the total distance run by the mice during each incremental aerobic test considering the weight of the animal [total distance (meters)/ weight (grams)] (adapted from (30, 42)). We also registered maximal velocity achieved in order to determine training intensities for the exercised groups (exercise + isotype control; exercise + nivolumab). To assess aerobic adaptations, we compared the maximal aerobic capacity before and after the training period.

Forelimb grip strength

The maximum forelimb grip strength was measured – as the maximum force (grams) exerted by the mouse before losing grip – using an isometric force transducer (Harvard Apparatus; Panlab, Barcelona, Spain; Figure 2D) on the days following the two aerobic capacity tests. Each mouse took the test three times with a 5-minute rest period between them, and the best reading was recorded as the maximal grip strength in grams. To measure strength adaptations, we compared the maximal grip strength before and after the training period. All animals were weighed at the start and the end of the test.

Tumor volume and tumor growth rate

Tumor volume was measured twice weekly throughout the trial using a caliper, as described above. The response to treatment therapy and/or exercise intervention was quantified in terms of tumor growth rate expressed as a percentage of the total tumor growth with respect to the volume at the beginning of the intervention. The formula $(TV_{dx} / TV_{d0}) \times 100$ was applied, where TV_{dx} refers to the tumor volume measured on a specific day and TV_{d0} as the tumor volume at the beginning of the pharmacological intervention (set at 100%).

Necrotic index

The percentage of necrotic areas as a measure of tumor regression in response to therapies (pharmacological treatment and/or exercise) was quantitatively analyzed using CaseViewer software (3DHISTECH Ltd., Budapest, Hungary). The average percentage of necrotic areas was evaluated in four paraffin-embedded sections (5 µm, 4 tumors per study group) stained with hematoxylin and eosin. The area for each section was calculated according to the formula: % necrosis area = $(\Sigma \text{ necrosis area} / \text{total tumoral mass area}) \times 100$.

Cell proliferation

The expression of Ki67 as a proliferation marker (19) was evaluated in 4-µm sections of OCT-embedded tumor tissue by immunofluorescence using a specific proliferative cell antibody (anti-Ki67 human antibody, clone SP6, reference MAD-000310QD, ready to use; Master Diagnostica, Granada, Spain). Antigen unmasking was performed by immersing the slides into a specific buffer (EnVision™ FLEX Target Retrieval Solution, Low pH, 50×; Agilent Technologies Inc., Santa Clara, CA) during 20 minutes at 97°C. Tumor samples were then fixed in 100% methanol (-20°C) for 10 minutes at room temperature followed by incubation with 5% bovine serum albumin for 60 minutes to block the nonspecific protein binding. Samples were stained with undiluted anti-Ki67 mouse antibody, overnight at 4°C. After three 5-minute washes with phosphate buffered saline (PBS), samples were incubated with the secondary anti-rabbit IgG Alexa 488 antibody diluted 1:200 (Invitrogen Life Technologies, Waltham, MA). After three additional 5-minute washes with PBS, samples were stained with TO-PRO-3 (reference T3605; Invitrogen Life Technologies) diluted 1:500 in PBS for 20 minutes, followed by washing and assembling the samples in PBS/glycerol. Finally, the cell proliferation index was determined by confocal microscopy (20×, numeral aperture of 0.4; TCS-SP5-AOBS-UV; Leica-Microsystems) quantifying the positive nuclear staining (green colour; Arg laser, 500–540 nm) in 8 areas of each slide (~100 cells). The contrast with TO-PRO-3 was displayed in blue colour (He/Ne laser, 633 nm, 645–750 nm). The percentage of Ki67 positive cells (% Ki67) was calculated against the total number of nuclei per field and the average value of the eight images per sample was also calculated.

Apoptosis

End-stage apoptotic cells were investigated in 4-µm sections of OCT-embedded tumor tissue using terminal deoxynucleotidyl transferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL assay) based on DNA fragment detection (DeadEnd™ Fluorometric TUNEL System; Promega, Madison, WI). To eliminate possible autofluorescence, slides were washed with PBS and incubated with NH_4Cl before the TUNEL assay. Subsequently, samples were incubated with TO-PRO-3 (Invitrogen Life Technologies) diluted 1:1000 in PBS for 20 minutes at room temperature. After three 5-minute washes with PBS, samples were incubated with the secondary anti-rabbit IgG Alexa 488 antibody diluted 1:200 (Invitrogen Life Technologies), washed and assembled in PBS/glycerol. The fluorescent dye-conjugated dUTP-labeled DNA (displayed in green) and TO-PRO-3 (in blue) were visu-

Table 1. Antibodies per tube and per tumor homogenate sample used for flow cytometry analysis

	FITC	PE	PerCP-Cy7	PE-Cy7	APC	APC-Cy7	PB	AC
peripheral blood Tube 1	hCD33	mCD45.1	hCD4	hCD56	hCD19	hCD8	hCD3	hCD45
Tumor Tube 1	hCD4	mCD45.1	7AAD	hCD56	hCD19	hCD8	hCD3	hCD45
Tumor Tube 2		mLy6G	7AAD	mNK1.1	mCD11B	mCD3		mCD45

Abbreviations: FITC (Fluorescein isothiocyanate); PE (Phycoerythrin); PerCP-Cy7 (Peridinin chlorophyll protein); PE-Cy7 (Phycoerythrin cyanin-7); APC (Allophycocyanin); APC-Cy7 (Allophycocyanin cyanin-7); PB (Pacific blue); AC (AmCyan).

Table 2. List of antibodies used for flow cytometry analysis

Protein	Clone	Brand
hCD33	HIM3-4	BD Pharmigen
hCD4	SK3	BD Pharmigen
hCD56	NCAM16,2	BD Pharmigen
hCD19	HIB19	BD Pharmigen
hCD8	SK1	BioLegend
hCD3	UCHT1	BD Horizon
hCD45	2D1	BD Pharmigen
7AAD	-	BioLegend
mCD45.1	A20	BioLegend
mLy6G	1A8	BioLegend
mNK1.1	PK136	BioLegend
mCD11B	M1/70	BioLegend
mCD3	145-2C11	BioLegend
mCD45	30-F11	BioLegend

alized using a confocal laser scanning microscope (100×, TCS-SP5-AOBS-UV) and images were captured (5 images per preparation; 1024 × 1024 pixels) with Leica Confocal Software (both from Leica-Microsystems). The apoptotic index was determined using the following formula: (number of TUNEL-positive staining cells) / (total number of nuclei) × 100.

Identification of leukocyte populations in tumor stroma (ex vivo)

As described earlier, fresh tumor tissue was homogenized and digested in RPMI media with D-collagenase (1 mg/ml). This was then filtered and prepared for immune cell analysis by flow cytometry (FACSCanto II and FACSDiva software v6.1.2; BD Biosciences) using the specific human and mouse antibodies shown in Table 1 and Table 2.

Identification of gene expression associated with the tumor microenvironment

Total RNA extracted from digested tumor tissues using the RNeasy Plus Mini Kit (Qiagen Inc., Hilden, Germany) was reverse-transcribed into cDNA using SuperScript VILO MasterMix (Invitrogen Life Technologies) in a Mastercycler EP thermocycler (Eppendorf, Hamburg, Germany). All reactions were performed using the same protocol consisting of an incubation step at 25°C for 10 minutes, followed by one cycle at

42°C for 120 minutes, another cycle at 85°C for 5 minutes, and a cool down cycle at 4°C.

qPCR was carried out using the TaqMan Gene expression and the TaqMan Gex Master Mix (both from Applied Biosystems, Foster City, CA). Each sample was analyzed independently in duplicate for every determination with the 7500 Fast Real-Time PCR System using 7500 software v2.0.6 (Applied Biosystems). Oligonucleotide primers and probes for the amplification reactions were purchased from Applied Biosystems and are shown in Table 3. The expression of the following human and murine genes was studied, all related to immune system and tumor microenvironment: *a*) Human genes: cytotoxic T lymphocyte antigen 4 (*CTLA-4*), *PD-1*, programmed death ligand 1 (*PD-L1*), *IL4* and *IFN γ* ; and, *b*) Murine genes: arginase-1 (*Arg1*), vascular endothelial growth factor a (*Vegf-a*), inducible nitric oxide synthase (*iNos*), *Pd-1* and *Pd-11* (Table 3). Relative mRNA abundance was normal-

Table 3. List of primers used in real-time quantitative PCR analysis

Species	Gene	Primers
Human	<i>CTLA-4</i>	Hs03044418_m1
	<i>PD-1</i>	Hs01550088_m1
	<i>PD-L1</i>	Hs00204257_m1
	<i>IL-4</i>	Hs00174122_m1
	<i>IFNγ</i>	Hs00989291_m1
	<i>GADPH</i>	Hs02758991_g1
Mouse	<i>Arg1</i>	Mm00475988_m1
	<i>Pd-11</i>	Mm00452054_m1
	<i>iNos</i>	Mm00440502_m1
	<i>Vegf-a</i>	Mm01281449_m1
	<i>Pd-1</i>	Mm01285676_m1
	<i>Gadph</i>	Mm99999915_g1

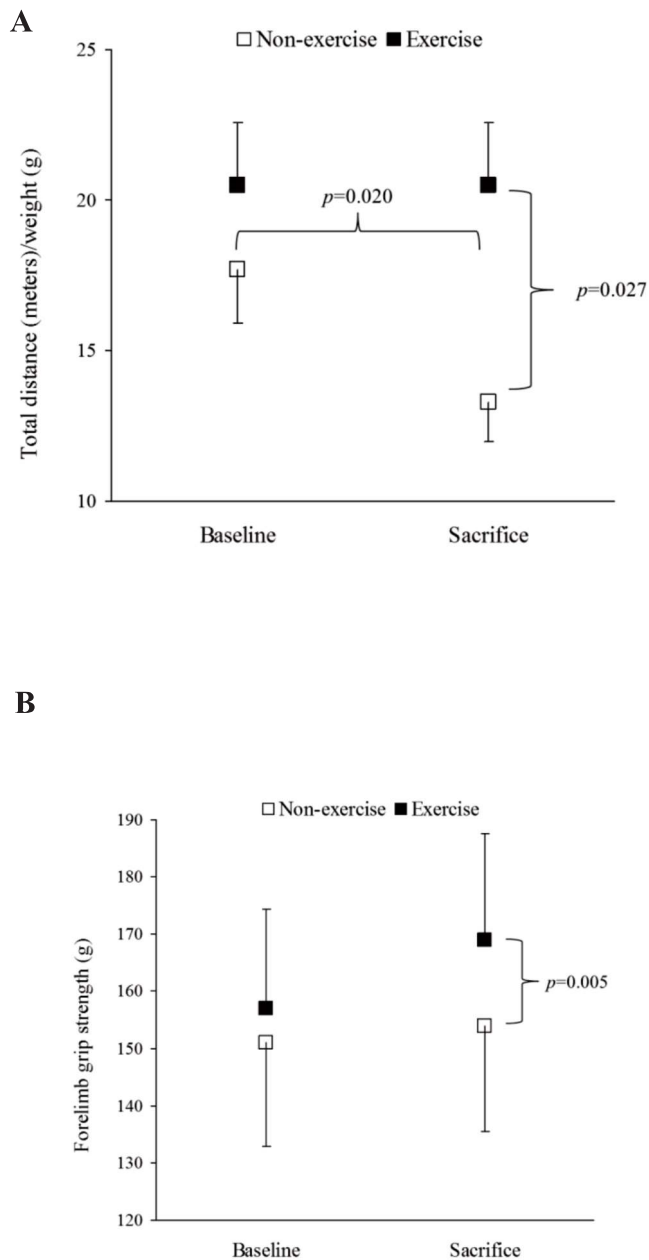


Figure 3. Effect of physical exercise on aerobic capacity. Results consider together the sedentary (non-exercise, white square) and the trained (exercise, black square) groups, before and after the eight weeks of training (to sacrifice). (A) Aerobic performance (distance covered [meters] normalized to weight [g]) before and at the end of the experiment in the control and exercised groups. (B) Muscle strength (forelimb grip strength [g]) before and at the end of the experiment in the control and exercised groups. Values represent mean \pm SEM. Data were analyzed using non-parametric tests for within and between-group comparisons (Wilcoxon signed-rank test and Mann-Whitney *U* test, respectively) and significant *p*-values are shown.

ized to the internal standard, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) for human and murine genes, using the relative quantification method. Each sample was analyzed as an independent duplicate for each set of primers and probes. Changes (Δ) in cycle threshold (*Ct*) values were calculated using the formula: $\Delta Ct = \text{mean } Ct \text{ for target gene} - \text{mean } Ct \text{ for housekeeping gene } (GAPDH)$. Relative gene expression levels were calculated using $\Delta\Delta Ct$ analysis, where $\Delta\Delta Ct = \Delta Ct \text{ of sample} - \Delta Ct \text{ of } GAPDH$. The formula $2^{(-\Delta\Delta Ct)}$ was used to calculate the relative expression ratio in treatment and control groups.

Statistical analysis

Between-group differences were analyzed with the non-parametric Kruskal-Wallis one-way ANOVA test (with post hoc pairwise comparisons done using the Mann-Whitney *U* test). The Wilcoxon signed-rank test was used for within-group comparisons over time. Since tumors were bilateral, the sample size per group for all tumor-related variables was the total number of tumors we studied per group. Statistical analyses were performed using IBM SPSS 22.0 package (SPSS, Inc., Chicago, IL) setting the significance level at $p=0.05$. All graphics were made with GraphPad Prism 6, version 6.01 software (GraphPad Software, San Diego, CA).

RESULTS

Body weight, aerobic capacity and forelimb grip strength

An increase in body weight over time was detected in all mice (1.9 ± 0.4 g on average, $p < 0.05$ for all within-group comparisons). These changes can be attributed, at least in part, to the tumor development process. Between-group analyses showed that the aerobic capacity ($p=0.027$, Figure 3A) and forelimb grip strength ($p=0.005$, Figure 3B) (both corrected by body weight) were significantly higher in the exercised mice (exercise + nivolumab or isotype groups) after the 8-week combined training intervention than in their non-exercised peers (non-exercise + nivolumab or isotype groups). Within-group analysis showed that aerobic capacity declined significantly in the non-exercised groups at the end of the study ($p=0.020$), but was maintained in the exercised groups ($p > 0.05$).

Tumor volume and tumor growth rate

When we compared tumor growth rates between groups, we found a delay in tumor growth in the exercise + isotype control group compared with the non-exercise + isotype control group ($p=0.050$) (Figure 4A), and also a smaller tumor volume at sacrifice (Figure 4B). By contrast, no differences in tumor growth were observed between the exercise + nivolumab group and the non-exercise + nivolumab group. Macroscopically, we observed that the final tumor volume in the double intervention (exercise + nivolumab) group tended to be larger than that in the other study groups, but this difference was not statistical significant ($p > 0.05$).

Necrotic index

Tumor regression was analyzed by evaluating the percentage of necrotic areas as an index of the tumor response to the different interventions at the study end. Between-group comparisons showed that the necrotic index was significantly higher in the double intervention group than in the non-exercise + isotype control group ($p=0.026$) (Figure 5).

Cell proliferation

Cell proliferation was assessed by immunostaining for Ki67. As shown in Figure 6A and B, the non-exercise + isotype control group had the highest cell proliferation between the four study groups (40% of Ki67-positive proliferating cells). Significant lower Ki67 immunostaining was found in the exercise + isotype control, non-exercise + nivolumab and exercise + nivolumab groups when compared with the non-exercise + isotype control group ($p=0.015$, $p=0.011$ and $p=0.011$, respectively; Figure 6B).

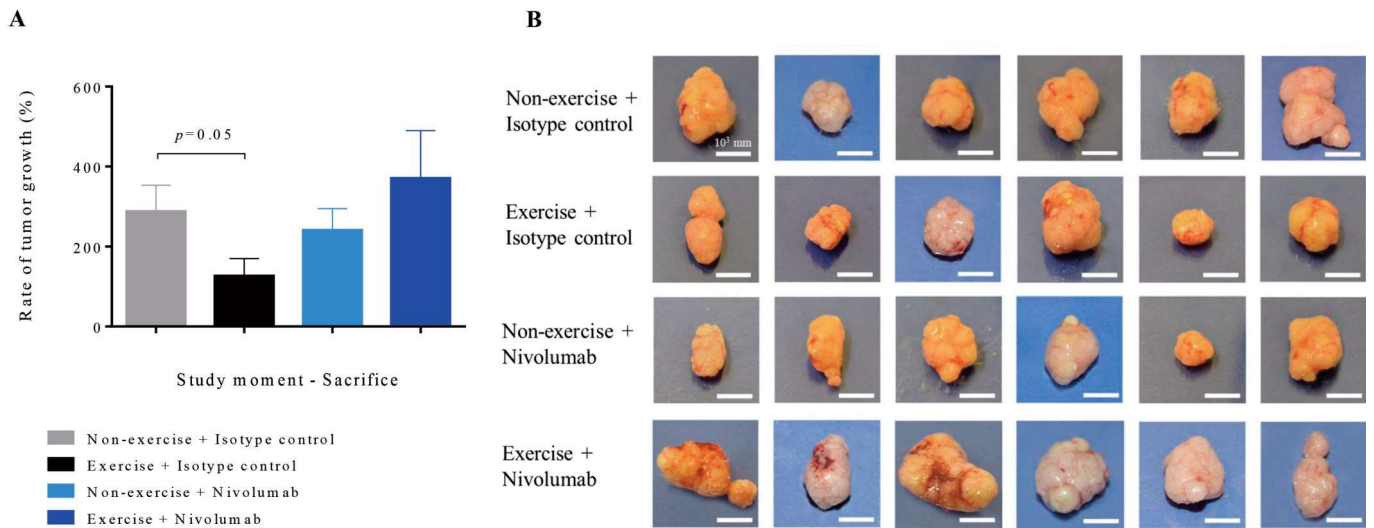


Figure 4. Tumor response to exercise and anti-PD-1 immunotherapy. (A) Tumor growth rate at sacrifice of the different groups. Tumor growth rate was expressed as a percentage of the change of tumor volume with respect to initial tumor volume, which was considered 100%. Data are expressed as mean \pm SEM and the only significant pairwise difference is shown (Kruskal-Wallis p-value for group effect = 0.173). (B) Panel with representative ex vivo images of the tumor sizes at the end of the experiment (at sacrifice moment). Scale bars, 10 mm.

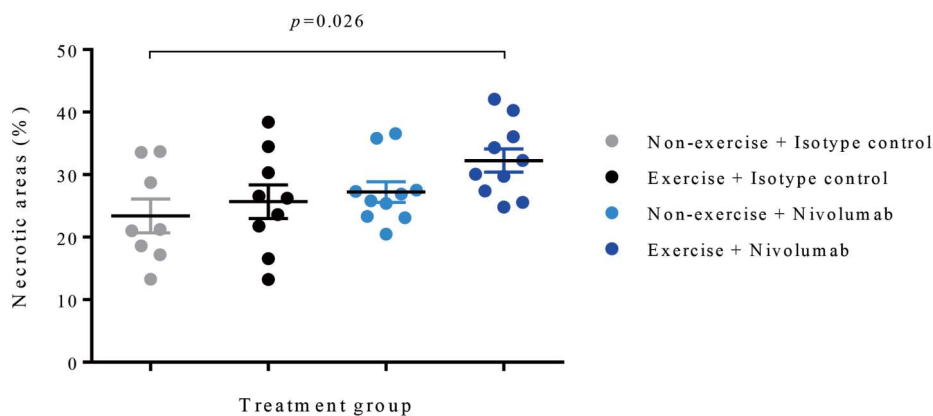


Figure 5. Analysis of tumor necrosis in the experimental groups. The necrosis index is expressed as a percentage of necrotic areas. Values represent the percentage (mean \pm SEM) and the only significant pairwise difference is shown (Kruskal-Wallis p-value for group effect = 0.074).

Apoptosis

We noted very little apparent apoptosis in any of the four groups of mice examined, and TUNEL-positive cells were not strictly located to the necrotic areas (Figure 7A). The non-exercise + isotype control group contained the largest number of TUNEL-positive cells ($1.65 \pm 0.59\%$), which was significantly greater than in the exercise + nivolumab group ($p=0.030$) (Figure 7B).

Identification of leukocyte populations in peripheral blood and in tumor stroma

We used flow cytometry to analyze several human and mouse immune cell populations in peripheral blood and in tumors in the four study groups (see Tables 1 and 2). We failed to detect human leukocytes (hCD45⁺) in both blood and tumor samples, and the immune component was 100% murine (mCD45.1⁺). Similarly, human T and B lymphocytes and NK

cells were also absent, so we concluded that the lineage present in the samples was myeloid, from the murine host. Identical results were found for all study groups.

Focusing on tumor homogenates, we found a significantly higher tumor infiltration of leukocytes in the exercise + isotype control group than in the non-exercise + nivolumab group ($p=0.018$; Figure 8A). The percentage of neutrophils, monocytes and eosinophils were very similar in all study groups (Figure 8B), although there was a quasi-significant trend for an increase in tumor-infiltrating neutrophils in the exercise + nivolumab group when compared with the non-exercise + isotype control group ($p=0.060$).

Gene expression associated with the tumor microenvironment

We next used qPCR to test for the expression of human genes related to the immune system and the tumor microenvironment in RNA extracted from tumor tissues (*i.e.*, *CTLA-4*, *PD-1*, *PD-L1*, *IL-4* and *IFN γ*), and found that their expression was undetectable in all the study mice (results not shown). In an analysis of murine genes related to the tumor microenvironment, we found that *Vegf-a* expression was higher in the nivolumab groups (in combination or not with exercise) than in exercise + isotype control group ($p=0.045$ and $p=0.047$, respectively) (Figure 9). We also noted a trend for lower *Arg1* expression when exercise was applied as the only intervention compared with the exercise + nivolumab group ($p=0.068$). The remaining genes (*iNos*, *Pd-1* and *Pd-1l*) showed similar expression levels in all experimental groups.

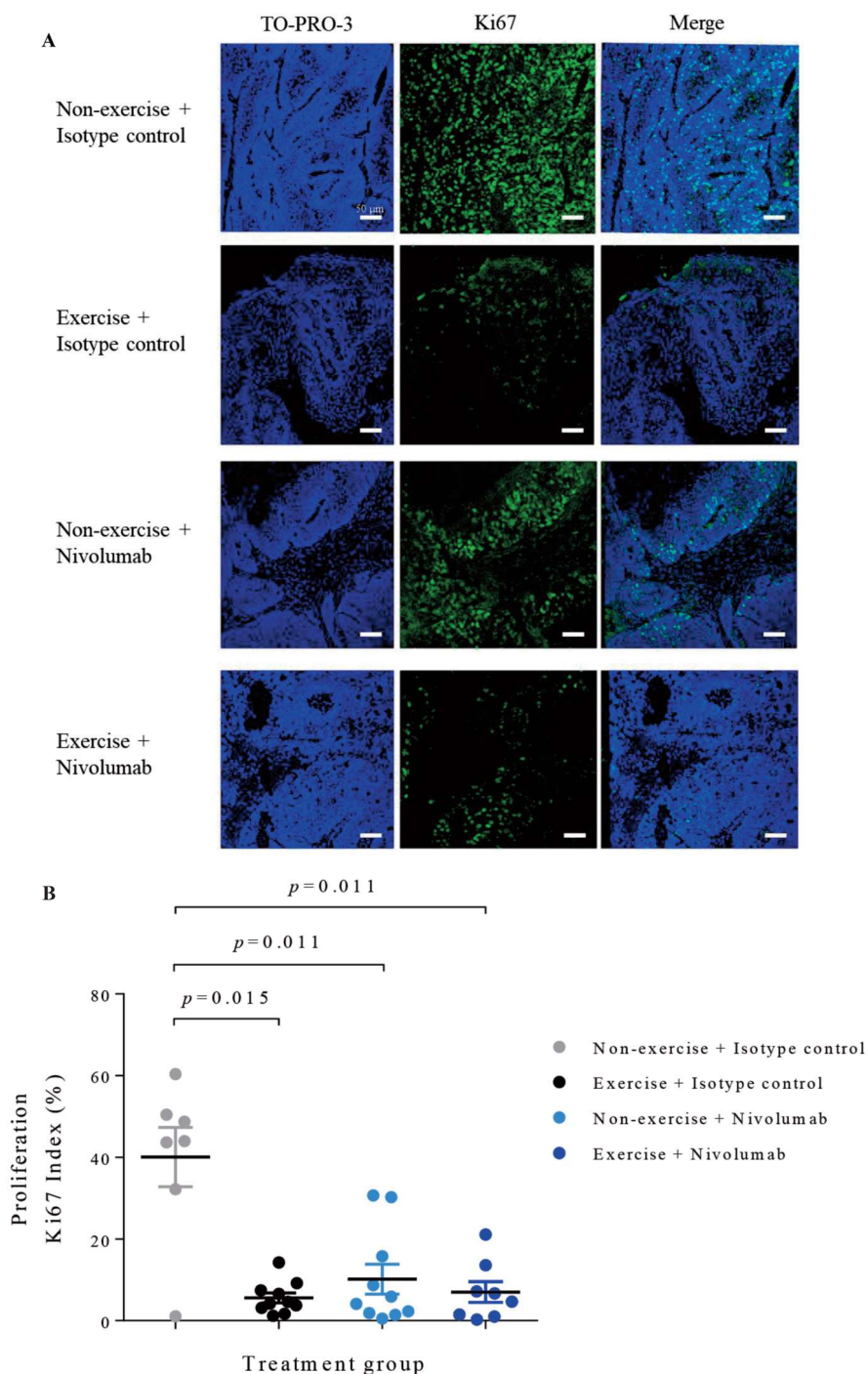


Figure 6. Effect of exercise and anti-PD-1 treatment on cell proliferation. (A) Panel of representative images of Ki67 immunofluorescence staining (green). Nuclei were stained with TO-PRO-3 (blue). Right panels show merged images. Scale bars, 50 μ m. (B) Scatter plot, with the individual percentages of Ki67-positive cells in tumors in each group. The lines in the dot plots represents the mean \pm SEM of each group and significant pairwise differences are shown (Kruskal-Wallis p-value for group effect = 0.027).

DISCUSSION

Our main findings were that: (i) exercise *per se* – and also in combination with nivolumab – reduced cell proliferation in tumors compared to non-exercise and exercise alone also increased myeloid tumor infiltrates (mostly neutrophils) with respect to the nivolumab only group; and (ii) the combined intervention (exercise + nivolumab) increased tumor necrosis with respect to the non-intervention control group. To the best of our knowledge, this is the first study to analyze the effect of

a physical exercise intervention (applying an 8-week combined protocol including aerobic and strength training) as a potential adjuvant therapy for anti-PD-1 treatment using a murine PDX model of NSCLC. The fact that this model is devoid of host lymphoid cells allows to gain mechanistic insight into the effects of exercise – combined or not with immunotherapy – on tumor development, particularly with regard to the role played by neutrophils, which have been much less studied than lymphocytes.

On the other hand, we found that an exercise intervention combining aerobic and resistance training, and based on public health recommendations for adults (24), conferred significant benefits on aerobic capacity and forelimb grip strength in mice irrespective of immunotherapy treatment. These results are in line with previous studies performed in patients with NSCLC (53) and in preclinical murine models of lung cancer (50) and other tumors (*e.g.*, slowing prevention of muscle wasting (6, 29, 52)).

Studies on physical exercise as an adjuvant therapy in patients with cancer generally focus on increasing their general health status and tolerance to treatments, which might contribute to improve their prognosis and survival. Although immunological adaptations to exercise in cancer have been studied previously (see 54 for a review), there is no *direct* biological evidence in humans for the potential beneficial effects of exercise *per se*, on tumor regression or on delaying tumor progression. However, previous studies have demonstrated how exercise can reduce the tumor growth rate in preclinical models (*e.g.*, 26, 49, 51, 73), but because of the broad heterogeneity with respect to type of cancer, murine models and exercise interventions used in these studies, it is difficult to draw clear conclusions. In our murine PDX model, physical exercise by itself was found to delay tumor growth and to result in a smaller tumor volume at the end of the study compared with non-exercised mice, irrespective of immunotherapy. However, the final tumor volume as a result of the double intervention (exercise + nivolumab) tended to be larger than that observed in the remainder of the study groups. In this regard, we recently showed in the current mouse PDX model that anti-PD-1 therapy, alone or sequentially combined with cis-

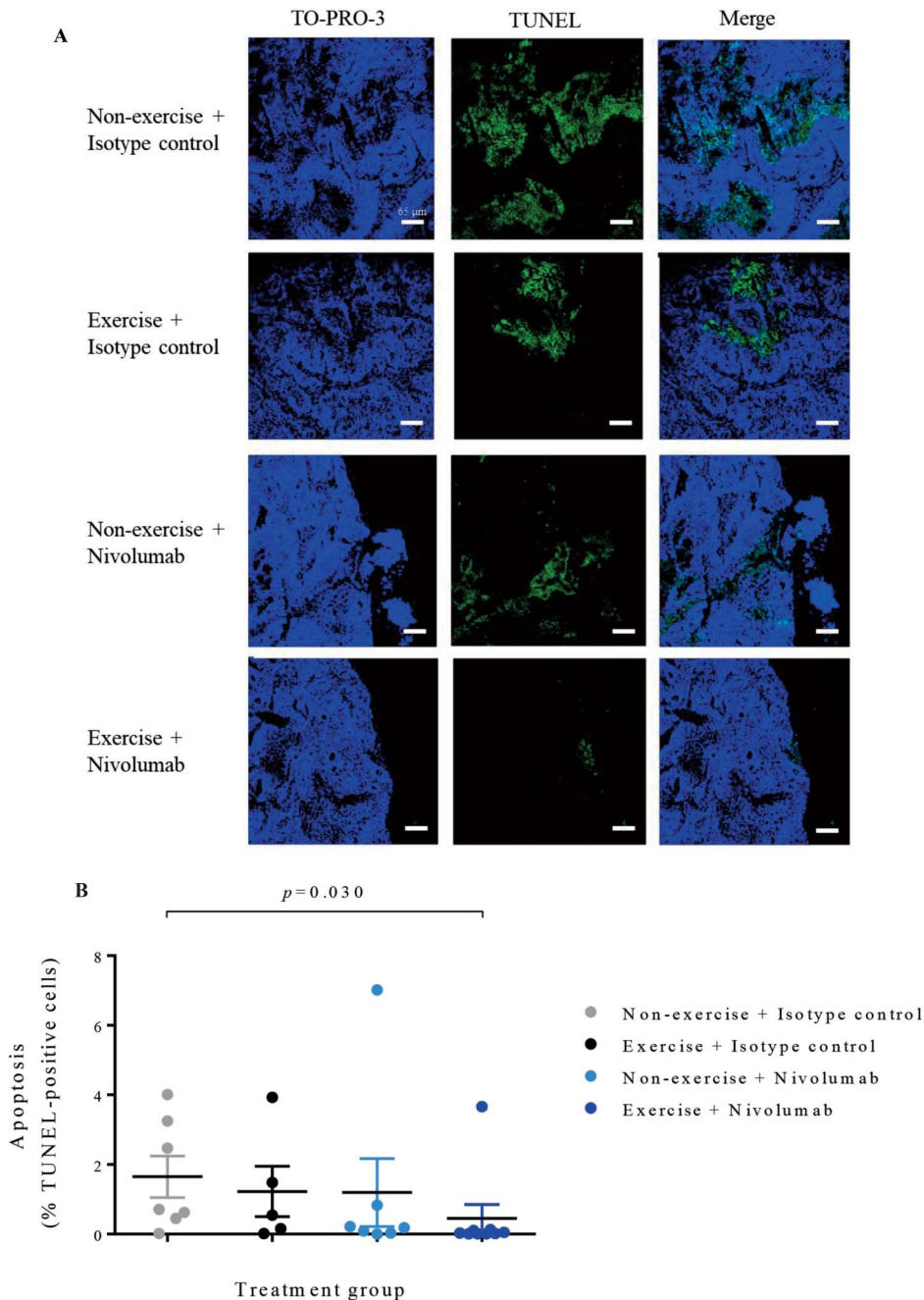


Figure 7. Effect of exercise and anti-PD-1 treatment on tumor apoptosis evaluated by TUNEL assay. (A) Panel of representative images of TUNEL immunofluorescence staining (green). Nuclei were stained with TO-PRO-3 (blue). Right panels show merged images. Scale bars, 65 μ m. (B) Scatter plot, with the individual percentages of TUNEL-positive cells (apoptotic) of the tumors in each group. The lines in the dot plots represents the mean \pm SEM of each group and the only significant pairwise difference is shown (Kruskal-Wallis p-value for group effect = 0.089).

platin, led to a paradoxical response, that is, to an increase in tumor growth rate (in the anti-PD-1 phase) with large and friable tumors in some cases, which were associated with exudates containing inflammatory polymorphonuclear neutrophils (PMNs) from areas of reactive necrosis (44). This phenomenon is reminiscent of unconventional responses of checkpoint inhibitor-based immunotherapy, such as pseudo-progression, which can be observed in patients' solid tumors (including NSCLC) treated with this type of immunotherapy (12, 13, 37). These 'paradoxical' or 'unconventional' responses are associated both with immune cell – PMNs and lymphocytes – recruitment and with the intratumoral inflammatory environment triggered by those cells (12).

together with stimulation of proliferation and survival through inhibition of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (41). However, some controversy exists because other authors associate exercise with an increase in tumor cell proliferation (71).

Exercise modulates the tumor microenvironment by acting on the innate and adaptive immune systems (28, 39), increasing the peripheral blood levels of T lymphocytes and NK cells, their mobilization to the tumor stroma (31, 49), or their cytotoxicity against tumor cells (28, 39). However, in the PDX model used in this study, flow cytometry analysis revealed the absence of T lymphocytes and NK cells in the tumor grafts and also in peripheral blood, which is common in

In murine cancer models, aerobic exercise training at moderate intensity has been shown to induce apoptosis in tumors, essentially through caspase activation and a reduction in *Bcl-2* expression (3, 7, 26, 27, 73). By contrast, our results suggest that apoptosis is probably not the mechanism of cell death that results in tumor regression in response to nivolumab, exercise or their combination, in this model. Indeed, the exercise + nivolumab combination produced the lowest levels of apoptosis, although the percentage of positive TUNEL staining was low (<2% on average) in all study groups. Regarding the results of cell proliferation in our study (measured as Ki67-positive cells), exercise and nivolumab (alone or in combination) significantly reduced cell proliferation with respect to the non-exercise + isotype control group (i.e., 6–10% versus 40% in the control group), but no differences were observed between the treatment groups. Thus, we consider that exercise per se could be responsible for the decrease in proliferation found in the tumors from this group. In this regard, exercise can inhibit cell proliferation by a variety of mechanisms. These include decreases in circulating growth factors such as insulin-like growth factor 1 (54), activation of AMP-activated protein kinase (AMPK), and down-regulation of protein kinase B, which collectively down-regulate the activity of the mammalian target of rapamycin (mTOR) (74). Direct and indirect (through Akt inhibition) downregulation of mTOR, as well as of mTOR downstream signalling (ribosomal protein S6 kinase beta-1 [p70S6K]), has also been shown in NSCLC cells exposed to post-exercise serum,

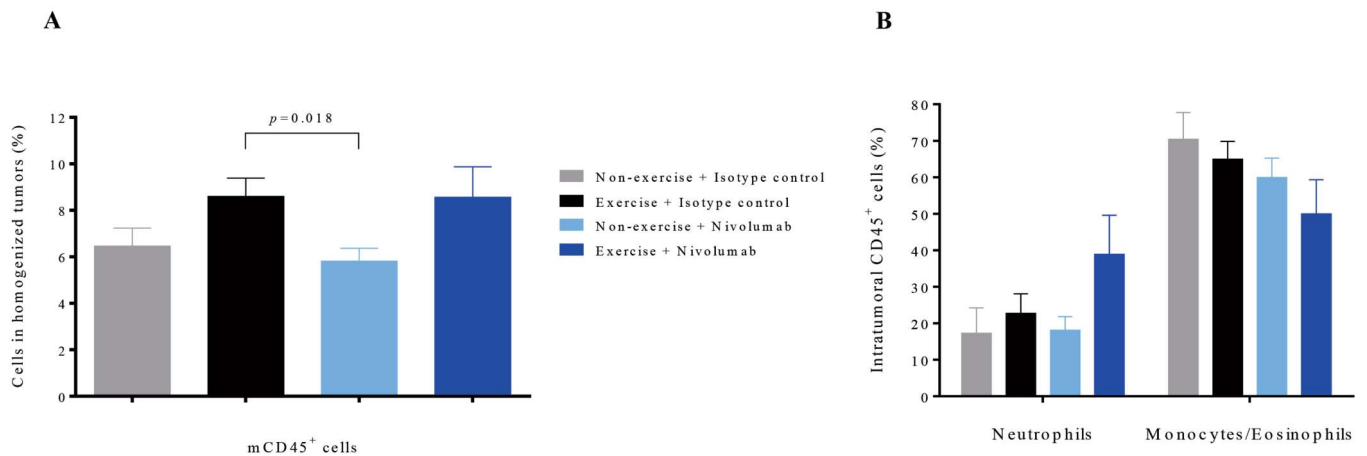


Figure 8. Leukocyte populations in PDX tumor homogenates. (A) Analysis of murine leukocytes in homogenized tumors. Results represent the percentage of mCD45.1 positive cells with respect to the total amount of cells that are detected as events in the flow cytometer. (B) Analysis of populations of murine myeloid cells in homogenized tumors. Populations of neutrophils and monocytes/eosinophils are shown, with respect to the total numbers of cells that are detected as events in the flow cytometer. The data (A and B) are expressed as the mean \pm SEM and the only significant pairwise difference is shown (Kruskal-Wallis p-value for group effect = 0.056 for mCD45.1+ leukocytes, 0.310 for neutrophils, and 0.293 for monocytes/eosinophils). Neutrophil phenotype: mCD45.1+ mCD11b+ mLy6G+; monocyte/eosinophil phenotype: mCD45.1+ mCD11b- mLy6G-.

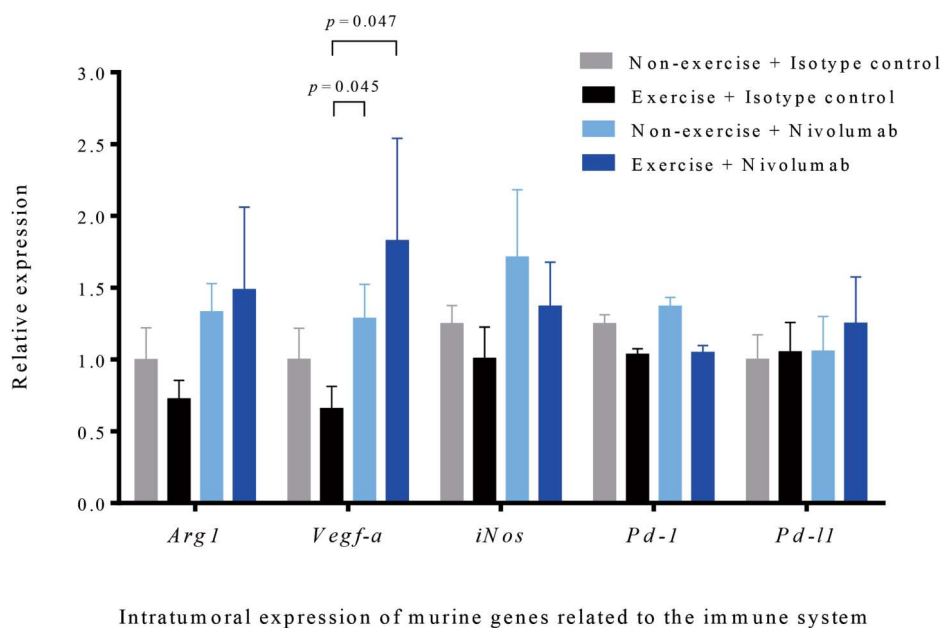


Figure 9. Analysis of murine gene expression related to immune system and microenvironment. Relative expression of arginase-1 (*Arg1*), vascular endothelial growth factor-a (*Vegf-a*), inducible nitric oxide synthase (*iNos*), programmed cell death protein 1 (*Pd-1*) and programmed death ligand 1 (*Pd-11*) by qPCR in homogenized tumors. Represented are the mRNA intratumoral relative levels, which are expressed as mean \pm SEM. Significant pairwise differences are shown ($p=0.161$ for Kruskal-Wallis group effect in *Vegf-a*).

this type of model (60). Animal and human studies show that exercise can also impact the innate immune component by increasing the levels of myeloid cells such as macrophages, monocytes and neutrophils, both in peripheral blood and in tissue infiltrates (64, 69). In addition, exercise can regulate the reprogramming of the tumor microenvironment, by promoting the polarization of myeloid cells towards a more anti-tumorigenic phenotype (1, 22, 23). As expected for PDX models of solid tumors (72), including NSCLC (46), we found that the leukocyte-infiltrated tumor component was completely replaced by a murine infiltrate. We cannot confirm whether

some human cells were preserved in host mice, but they were below the limit of detection in tumor homogenates. Accordingly, in the absence of murine NK cells and lymphocytes, myeloid cells were considered as the infiltrated cells present in the stroma. We found an increase in the number of total leukocytes in tumor infiltrates from exercised mice treated with the isotype control, which is in line with previous research on exercise-induced changes to myeloid cell abundance (64, 69). We also found a trend towards an increase ($p=0.06$) in the proportion of neutrophils in the tumor stroma of the exercise + nivolumab group (38%) versus the non-exercise + isotype control group (17%), which is an important finding as neutrophils dominate the immune cell composition in NSCLC (35). Moreover, there was a decrease in the number of monocytes/eosinophils in the tumors of exercised mice that received

nivolumab, which was concomitant with an increase in neutrophils. In turn, leukocyte populations in tumor stroma appeared to be balanced in the pharmacological (nivolumab) and non-pharmacological (physical exercise) groups, which might create an environment that can lead to potential beneficial effects of both therapies, since the tumor microenvironment is characterized by a loss of homeostasis in immune cell populations (47, 63).

Immunotherapy treatment can modify the cellular component of the tumor microenvironment and, consequently, the gene expression profile related to the immune system of the

intratumor environment (21, 57). Physical exercise also produces this effect, as it is known to mobilize myokines (mainly proteins and small peptides [*i.e.*, cytokines]) and immune cells at the systemic level (1, 39, 54). Although the expression of all murine genes evaluated was detected in the tumors of all experimental groups, a significant increase in *Vegf-a* expression was observed 8 weeks after nivolumab administration (combined or not with exercise) compared with the exercise + isotype control group, which showed the lowest expression of this gene. *Vegf-a* is involved in angiogenesis in the tumor microenvironment (5). The effect of exercise on *Vegf-a* is controversial because both a decrease (in the vicinity of Dalton's lymphoma (68) or in breast tumor tissue from female mice (58)) and an increase in its expression has been reported (67). Tsai et al. showed an increase in serum *Vegf-a* levels in tumor-bearing mice inoculated with Lewis lung carcinoma (LLC) compared with baseline, but without significant differences in terms of survival rate or tumor growth compared with the control group [67]. In turn, Alves et al. observed 2.5-fold higher *Vegf-a* mRNA levels in an LLC mice model undergoing daily high-intensity interval training after tumor cell injection compared with non-exercised mice, with a significant reduction of tumor mass and an increase in survival (2). On the other hand, the finding that exercise might result in enhanced tumor perfusion (through increases in *Vegf-a*) had led some authors to hypothesize that exercise may enhance the delivery of drugs (45) and thus exercise emerges as a potential co-adjuvant intervention when combined with drug therapy, improving the efficacy of the latter (8). Thus, the fact that *Vegf-a* expression was increased with nivolumab treatment, especially when combined with exercise, does not necessarily reflect a deleterious effect. On the contrary, this finding can be interpreted as a mechanism by which anti-PD-1 immunotherapy promotes PDX tumor perfusion, implying vascular remodeling, better drug distribution and myeloid cell – mainly neutrophils – mobilization with the potential to exert an anti-tumor (cytotoxic or 'necrotizing') effect. In effect, the treatment that tended to yield the highest necrotic index (Figure 5) as well as the highest *Vegf-a* tumoral levels was the double intervention exercise + nivolumab group. Moreover, neutrophils participate actively in the remodeling and (neo)angiogenesis processes of the tumor and its extracellular matrix as a result of VEGF- α production and activation (14, 15).

Our study has several limitations and strengths. Firstly, the sample size was low and the study was done in only one immunotherapy-responder line of PDX mice. On the other hand, the NOD-SCID gamma mice used as the experimental model lack T cells, B cells and mature NK cells, so it was not possible to study the interaction of those immune cells with both the tumor microenvironment and the interventions applied in this study. However, the fact that we studied PDX mice devoid of host lymphoid cells might be actually viewed as a potential strength as they represent a unique animal model to study the effects of exercise on tumor development through mechanisms other than the classically advocated T lymphocyte or NK cell involvement. Therefore, we were able to study the effects of the anti-PD-1 (nivolumab) immunotherapy, the exercise training program, and their combination by mechanisms independent of the adaptive immune system, specifically through myeloid cells such as neutrophils. One of the strengths of this study was the application of a training modality combining aerobic and resistance exercise, with the latter

been applied here for the first time in the field of exercise in preclinical cancer models, and with the knowledge on the potential benefits of exercise against cancer been confined up to date to aerobic exercise only. Interestingly, preliminary evidence suggests that chemotherapy-treated patients with lung cancer who joined exercise sessions using resistance bands managed to maintain white blood cell levels during treatment compared with a control group (36). Our study is also the first to combine exercise with immunotherapy, specifically an immune checkpoint inhibitor that is receiving growing attention in oncology for its potential to improve treatment responses compared to traditional treatments. In addition, the study was focused on the analysis of the tumor response to exercise and/or to the anti-PD-1 therapy, assessing several molecular markers of growth, progression and cell death and also the anti-tumoral transcriptome, with particular emphasis on the infiltrated innate immune system and the production of *Vegf-a*.

CONCLUSION

The combination of an aerobic and strength exercise training program in a murine PDX model of NSCLC improved aerobic capacity and maximal strength in mice. Exercise per se was also able to reduce cell proliferation in tumors from these mice, as reflected in a reduction in Ki67 staining and in tumor volume. When exercise was combined with nivolumab, both therapies influenced tumor regression through an increase in necrosis and a decrease in the proliferative index. An increment in *Vegf-a* expression was also found in the exercise + nivolumab group, although future research should examine the biological relevance of this result. In conclusion, we consider that despite the need for a deeper analysis of the effect of exercise in clinical and pre-clinical studies, its application should be taken into consideration as a co-adjuvant therapy to pharmacological treatment. Furthermore, PDX models as the one we used here might allow other researchers to gain deeper insight into the role of non-lymphoid immune cells, particularly neutrophils, against tumor development, with these cells being scarcely studied to date compared with lymphocytes.

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