**REDUCED LUNG CANCER BURDEN BY SELECTIVE IMMUNOMODULATORS ELICITS IMPROVEMENTS IN MUSCLE PROTEOLYSIS AND STRENGTH IN CACHECTIC MICE**

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<th>Journal:</th>
<th>Journal of Cellular Physiology</th>
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<td>Manuscript ID:</td>
<td>JCP-18-1217.R1</td>
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<tr>
<td>Wiley - Manuscript type:</td>
<td>Original Research Article</td>
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<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
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<td>Complete List of Authors:</td>
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<td>Key Words:</td>
<td>lung tumor cells, immunotherapy, cancer-induced cachexia, muscle function and physical activity, muscle proteolysis and apoptosis</td>
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REDUCED LUNG CANCER BURDEN BY SELECTIVE IMMUNOMODULATORS ELICITS IMPROVEMENTS IN MUSCLE PROTEOLYSIS AND STRENGTH IN CACHECTIC MICE

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Word count: 4,929

Running head: Tumor burden implications in cancer cachectic muscles

KEY WORDS: lung tumor cells; immunotherapy; cancer-induced cachexia; muscle function and physical activity; muscle proteolysis and apoptosis

Number of text figures: 5

Number of tables: 4

Grant information: Contract grant sponsor: Instituto de Salud Carlos-III, contract grant numbers, CIBERES, FIS 14/00713 (FEDER), FIS 18/00075 (FEDER).

Contract grant sponsor: Spanish Respiratory Society (SEPAR), contract grant numbers, SEPAR 2016 and 2018.
Contract grant sponsor: Catalan Foundation of Pulmonology (FUCAP), contract grant numbers, FUCAP 2016.

Contract grant sponsor: Unrestricted grant from Menarini SA 2018.
Identification of to what extent tumor burden influences muscle mass independently of specific treatments for cancer-cachexia remains to be elucidated. We hypothesized that reduced tumor burden by selective treatment of tumor with immunomodulators may exert beneficial effects on muscle wasting and function in mice. Body and muscle weight, grip strength, physical activity, muscle morphometry, apoptotic nuclei, troponin-I systemic levels, interleukin-6, proteolytic markers and tyrosine release, and apoptosis markers were determined in diaphragm and gastrocnemius muscles of lung cancer (LP07 adenocarcinoma cells) mice (BALB/c) treated with monoclonal antibodies (mAbs), against immune checkpoints and pathways (CD-137, CTLA-4, PD-1, and CD-19) (N=10/group). Non-treated lung cancer cachectic mice were the controls. T and B cell numbers and macrophages were counted in tumors of both mouse groups. Compared to non-treated cachectic mice, in the mAbs-treated animals, T cells increased, no differences in B cells or macrophages, the variables final body weight, body weight and grip strength gains significantly improved. In diaphragm and gastrocnemius of mAbs-treated cachectic mice, number of apoptotic nuclei, tyrosine release, proteolysis and apoptosis markers significantly decreased compared to non-treated cachectic mice. Systemic levels of troponin-I significantly decreased in treated cachectic mice compared to non-treated animals. We conclude that reduced tumor burden as a result of selective treatment of the lung cancer cells with immunomodulators elicits per se beneficial effects on muscle mass loss through attenuation of several biological mechanisms that lead to increased protein breakdown and apoptosis, which translated into significant improvements in limb muscle strength but not in physical activity parameters. Word count: 250
INTRODUCTION

Muscle wasting and cachexia are common in patients with chronic conditions including cancer and in those with critical illness. Sarcopenia defined as the loss of muscle function is also characteristic of patients with chronic diseases and in the elderly, and it is commonly associated with muscle wasting. Importantly, muscle weakness has consistently been shown to negatively influence the patients’ prognosis and survival for the same degree of the airway obstruction in patients with chronic obstructive pulmonary disease (COPD) (Barreiro et al, 2015; Barreiro, 2017; Maltais et al, 2014; Seymour et al, 2010; Vogelmeier et al, 2017; Alvarez et al, 2016; Villar et al, 2016; Gea, 2018) and in those with chronic heart failure (von and Anker, 2010; von and Anker, 2014).

In patients with cancer cachexia, important metabolic alterations take place at different levels such as increased protein breakdown and fat and carbohydrate metabolic derangements despite a correct nutritional support. The prevalence of cachexia can be as high as 20% in patients with advanced tumors (Evans et al, 2008; Fearon et al, 2011). Nonetheless, it has also been reported (Inui, 2002; MacDonald et al, 2003; Saini et al, 2006) that patients with tumors from either the lungs or the upper gastrointestinal tract already exhibit weight loss even at the time of diagnosis. Moreover, biochemical and metabolic alterations characteristic of oncologic cachexia were also demonstrated at the time of diagnosis even if weight loss was not yet clearly established in the patients (Bossola et al, 2003; Rossi et al, 1995). The gradual loss of muscle mass is the most relevant feature in patients with cancer cachexia as it leads to major functional impairment and is also associated with poor clinical outcomes (Vigano et al, 2000). Thus, cachexia and muscle wasting should be evaluated in all the patients upon diagnosis of the tumor.

In the etiology of cancer cachexia and progressive muscle loss, several clinical factors and biological mechanisms have been demonstrated to be involved in the muscles of
patients and animal models in different investigations (Barreiro et al, 2015; Barreiro, 2017; Barreiro and Jaitovich, 2018; Barreiro et al, 2018; Jaitovich and Barreiro, 2018; Maltais et al, 2014; Alvarez et al, 2016; Villar et al, 2016; Aversa et al, 2017). Metabolic derangements, enhanced protein breakdown, increased oxidative stress, autophagy, structural abnormalities, and apoptosis are counted among the most relevant biological mechanisms leading to muscle protein loss and subsequent cachexia in patients and animal models of cancer cachexia (Barreiro et al, 2015; Barreiro, 2017; Barreiro and Jaitovich, 2018; Barreiro et al, 2018; Jaitovich and Barreiro, 2018; Maltais et al, 2014; Alvarez et al, 2016; Villar et al, 2016; Penna et al, 2018). Increased catabolism as a result of the tumor burden may also contribute to enhanced protein breakdown in the skeletal muscles of the patients as well as in animal models (Saini et al, 2006). In this regard, several factors such as cytokines (e.g. tumor necrosis factor, TNF)-alpha, and anabolic factors (insulin-like growth factor, IGF)-I and –II may act as systemic mediators leading to muscle mass loss (Saini et al, 2006). In fact, an improvement in skeletal muscle mass and cachexia was seen in patients with colon cancer that underwent surgical resection of their tumor, despite the potential deleterious effects of surgery in these subjects (Williams et al, 2012). Whether medical treatment with agents that reduce tumor burden may also favor muscle mass preservation independently of specific treatments for cachexia remain to be elucidated. Immune therapy has shown promising results in the treatment of lung adenocarcinoma in patients (Toi et al, 2018). Thus, identification of the effects of immunomodulators on muscles in an experimental model of cancer cachexia would be of interest as it may result in potential clinical applicability.

In the present investigation we sought to demonstrate that treatment of tumor burden with a therapeutic strategy to specifically target lung adenocarcinoma cells improves body and muscle weights using a very well-validated mouse model of cancer cachexia (Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2015; Chacon-Cabrera et al, 2017; Fermoselle et al,
As no surgical intervention was made in this animal model, all the effects could be attributed to the medical treatment with the immunomodulators of the tumor. On this basis, we hypothesized that selective treatment with a combination of several immunomodulators of lung adenocarcinoma cells may improve cachexia and muscle wasting through the attenuation of several biochemical mechanisms in the muscles. This treatment preferentially targets the immune microenvironment of the cancer cells (Dai et al, 2013; Dai et al, 2015). Accordingly, the study objectives were that in cachectic mice bearing the LP07 adenocarcinoma tumors that received treatment with a combination of monoclonal antibodies compared to non-treated cachectic animals, to explore: 1) body and muscle weights, food intake, and tumor growth, 2) limb muscle strength and physical activity, 3) markers of specific muscle proteolysis in blood and muscle compartments, 4) muscle fiber sizes, and 5) markers of muscle apoptosis. The number of T and B cells, and macrophages was also counted in the tumors of both experimental groups of mice. In this study, the biological markers were analyzed in both diaphragm and gastrocnemius muscles in order to assess whether any differences may take place in respiratory and limb muscles of similar fiber type composition as previously shown by our group (Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2015; Chacon-Cabrera et al, 2017; Fermoselle et al, 2013; Salazar-Degracia et al, 2016; Salazar-Degracia et al, 2017; Salazar-Degracia et al, 2018).

MATERIALS AND METHODS

(See detailed methodologies in the online supporting information)

Animal experiments

Tumor. LP07 is a cell line derived from the transplantable P07 lung tumor that appeared spontaneously in the lung of a BALB/c mouse (Diament et al, 1998). After successive passages of a P07 primary culture, the LP07 cell line was previously obtained in vitro...
LP07 cell line shares identical characteristics regarding lung tumor incidence and histology \textit{well-differentiated adenocarcinoma} and cachexia with its parental P07 tumor \cite{Diament1998, Diament2006, Urtreger2001, Mateu-Jimenez2016}. One month after inoculation of the cells, all animals developed lung metastasis, spleen enlargement, and severe cachexia without affecting other organs as previously shown \cite{Chacon-Cabrera2014, Chacon-Cabrera2015, Chacon-Cabrera2017, Diament1998, Diament2006, Urtreger2001, Fermoselle2013}. Mortality rate was 5\% attributable to the severe cachectic conditions of the mice, especially during the last week of the study protocol.

**Experimental design.** Figure 1 illustrates the experimental procedures conducted in the mice. BALB/c female mice (10 weeks old, weight \textasciitilde 20 g) were obtained from Harlan Interfauna Iberica SL (Barcelona, Spain). Mice were kept under pathogen-free conditions in the animal facilities at Barcelona Biomedical Research Park (PRBB), with a 12:12 h light: dark cycle. Mice in both groups did not develop infections throughout the study period. In all experimental groups, LP07 viable cells \((4 \cdot 10^5)\) resuspended in 0.2 mL minimal essential medium (MEM) were subcutaneously inoculated in the left flank of female BALB/c mice. After tumor cell inoculation on day 0, mice were studied for a period of 30 days. Mice were randomly divided into two independent groups \((N=10 \text{ mice/group})\) as follows: 1) cancer-cachexia mice, inoculation of LP07 cells on day 0 and 2) cancer-cachexia mice that were treated with a cocktail of monoclonal antibodies (cancer-cachexia+mAbs) (Table 1). The monoclonal antibodies were selective inhibitors of immune check-points and pathways that reduce tumor lung burden as previously demonstrated \cite{Chen2013, Forsthuber2018, Jin2011, Selby2013, Yonezawa2015}: 1) anti-programmed cell death-1 antibody \(\text{(anti-PD-1 antibody; RMP1-14, Cat. \#BE0146, BioXCell, New Hampshire, USA)}\), 2) anti-cytotoxic T-lymphocyte associated protein-4 antibody \(\text{(anti-CTLA-4 antibody;)}\)
9D9, Cat. #BE0164, BioXCell), 3) anti-TNF receptor superfamily member 9 antibody (anti-CD-137 antibody; LOB12.3; Cat. #BE0169, BioXCell), and 4) anti-B-lymphocyte antigen antibody (anti-CD19 antibody; 1D3, Cat. #BE0150, BioXCell) (Table 1). The cocktail of all four monoclonal antibodies was administered intraperitoneally for 15 days [starting on day 15 up until the end of the study period on day 30 (5×10^{-3} mg of each antibody/kg body weight/72 hours, dissolved in phosphate-buffered saline (PBS)] (Figure 1). The dosage and time of administration of the monoclonal antibodies were chosen on the basis of previous studies, in which a time-frame of 72h was estimated to be sufficient for the purpose of the study (Dai et al, 2013; Dai et al, 2015).

Ethics. All animal experiments were conducted at Parc de Recerca Biomèdica de Barcelona (PRBB). This was a controlled study designed in accordance with both the ethical standards on animal experimentation in our institution (EU 2010/63 CEE and Real Decreto 53/2013 BOE 34, Spain) and the Helsinki convention for the use and care of animals. All experiments were approved by the Animal Research Committee at PRBB. Ethical approval was obtained by the Animal Research Committee (Animal Welfare Department in Catalonia, Spain, EBP-15-1704).

In vivo measurements conducted on the animals. Food and water were supplied ad libitum for the entire duration of the study. In all mice, body weight and food intake were measured daily. In addition, tumor size was also measured daily with a caliper from day 15 up until the end of the study period. Metabolic parameters: oxygen consumption (VO_2), carbon dioxide production (VCO_2), respiratory exchange ratio (RER), energy expenditure (EE), the amount of food and water and physical activity (stereotyped and locomotor movements) were determined on day 0 and right at the end of the study period (day 30) in all the animals. Limb strength was determined from the four limbs together in each mouse for all the study animals on day 0 and right at the end of the study period as previously described (Chacon-Cabrera et
al, 2014; Chacon-Cabrera et al, 2015; Chacon-Cabrera et al, 2016b; Chacon-Cabrera et al, 2016a; Chacon-Cabrera et al, 2017). Additionally, measurements of the strength of the forelimbs were also conducted and are described below. Metabolic and physical activity parameters were measured using Oxylet system (Panlab, Barcelona, Spain) and limb strength was measured using a specific grip meter (Bioseb, Vitrolles Cedex, France). Body weight and limb strength gain variables were calculated as the percentage of the measurement performed at the end of the study period with respect to the same measurements obtained on day 0 as previously described (Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2015; Chacon-Cabrera et al, 2016b; Chacon-Cabrera et al, 2016a; Chacon-Cabrera et al, 2017). Total physical activity was calculated as a result of the addition of both stereotype and locomotor movements.

**Sacrifice and sample collection.** Mice from all the experimental groups were sacrificed on day 30 post-inoculation of LP07 cells. Each mouse was inoculated intraperitoneally with 0.1 mL sodium pentobarbital (60 mg/kg). In all animals, the pedal and blink reflexes were evaluated in order to verify total anesthesia depth. The diaphragm and gastrocnemius muscles and the subcutaneous tumor were obtained from all the mice. In all samples, a fragment of the muscle specimens was immediately frozen in liquid nitrogen and subsequently stored at -80°C, while the remaining specimen was immersed in an alcohol-formol bath to be thereafter embedded in paraffin until further use. Frozen tissues were used to assess the expression of the molecular analyses, whereas paraffin-embedded tissues were used for the histological studies.

**Biological analyses**

**Inflammatory cells in tumors.** T and B cells and macrophages were identified in tumor sections using specific antibodies against membrane antigens: anti-CD3, anti-CD8, and anti-CD4 (T cells), anti-CD20 (B cells), and anti-CD68 (macrophages) and immunohistochemical

Enzyme-linked immunosorbent assay (ELISA) plasma skeletal muscle troponin-I levels.

Skeletal muscle troponin-I levels were quantified in plasma of all study groups of mice obtained at day 0, 15 and 30 time-points using a specific sandwich ELISA kit (Life Diagnostics Inc., West Chester, PA, USA) as previously described (Chacon-Cabrera et al, 2016b; Vassallo et al, 2009).

ELISA measurements of IL-6. Protein levels of the interleukin (IL-6) were quantified in the diaphragm and gastrocnemius muscles of both study groups using specific sandwich ELISA kits (RayBiotech, Norcross, GA, USA), following the manufacturer's instructions and previous studies (Salazar-Degracia et al, 2016).

Protein catabolism. In the gastrocnemius muscle (whole intact muscle is required), protein degradation was explored on the basis of the rate of production of free tyrosine from tissue proteins as previously described (Barreiro et al, 2016; Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2016b).

Muscle morphometry. Muscle fibers of diaphragm and gastrocnemius were identified on three micrometer paraffin-embedded sections of all groups of mice which had been previously stained with hematoxylin-eosin. As muscle fiber phenotype and morphometry have already been described in previous studies using this animal model (Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2015; Chacon-Cabrera et al, 2017), only cross-sectional areas of the study muscles has been measured in this investigation.

Terminal deoxynucleotidyl transferase-mediated uridine 5'-triphosphate (UTP) nick-end labeling (TUNEL) assay. In muscle paraffin-embedded sections, apoptotic nuclei were identified using the TUNEL assay (ApopTag® Peroxidase In Situ Apoptosis Detection Kit, Meck-Millipore, Darmstadt, Germany) in both diaphragm and gastrocnemius muscles from
all study groups following the manufacturer’s instructions and previously published studies 
(Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2016b; Chacon-Cabrera et al, 

**Immunoblotting.** Protein levels of the different molecular markers analyzed in the study were 
explored by means of immunoblotting procedures as previously described (Chacon-Cabrera et 
al, 2014; Chacon-Cabrera et al, 2015; Chacon-Cabrera et al, 2016b; Chacon-Cabrera et al, 
2016a; Chacon-Cabrera et al, 2017; Puig-Vilanova et al, 2014; Puig-Vilanova et al, 
2015; Salazar-Degracia et al, 2016; Salazar-Degracia et al, 2018).

Study markers were identified using the following specific primary antibodies: anti-20S 
proteasome subunit C8 antibody (1:5000, BML-PW8110-0100) from Biomol (Plymouth 
Meeting, PA, USA), anti-protein ubiquitination antibody (1:5000, A-100) from Boston 
Biochem (Cambridge, MA, USA), anti-muscle ring finger protein-1 antibody (MuRF-1; 
1:2000, sc-27642), anti-atrogin-1 antibody (1:1000, sc-166806), anti-tripartite motif-
containing protein 32 antibody (TRIM32; 1:500, sc-49265), anti-B-Cell CLL/Lymphoma 2 
antibody (BCL-2; 1:1000, sc-7382), anti-BCL2 associated X protein antibody (BAX; 1:1000, 
sc-526), and anti-glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH; 1:2000, sc-
25778) from Santa Cruz (Santa Cruz, CA, USA). Antigens from all samples were detected 
using horseradish peroxidase (HRP)–conjugated secondary antibodies (Jackson 
ImmunoResearch Inc, West Grove, PA, USA) and a chemiluminescence kit (Thermo 
Scientific, Rockford, IL, USA). The specificity of the different antibodies was confirmed by 
the omission of the primary antibody and incubation of the membranes only with secondary 
antibodies. Positive controls were used when available such as MuRF-1 and atrogin-1 
proteins. PVDF membranes were scanned with the Molecular Imager Chemidoc XRS System 
(Bio-Rad Laboratories, Hercules, CA, USA) using the software Quantity One version 4.6.5 
(Bio-Rad Laboratories).
Statistical Analysis

Statistical power was calculated using specific software (StudySize 2.0, CreoStat HB, Frolunda, Sweden). Normality of the study variables was checked using Shapiro-Wilk test. Body weight gain and limb strength gain were both selected as the target variables on the basis of the T-test to estimate the statistical power between the two experimental groups: cancer-cachexia mice and cancer-cachexia mice treated with monoclonal antibodies (cancer-cachexia+mAbs). On the basis of a standard power statistics established at a minimum of 80% and assuming an alpha error of 0.05, the statistical power was sufficiently high to detect a minimum difference percentage of 15 and 38 points respectively between groups in the sample size (minimum N=10 and N=6, respectively for body weight and limb strength gain variables) and standard deviation. Results are represented as mean (standard deviation) and the comparisons between study groups were analyzed using the Student’s *T*-test. A level of significance of \( P \leq 0.05 \) was established. Statistical analyses were performed using the Statistical Package for the Social Sciences (Portable SPSS, PASW statistics 18.0 version for Windows, SPSS Inc., Chicago, IL, USA).

RESULTS

**Tumor and physiologic characteristics of the study animals**

The subcutaneous tumor area significantly decreased in cancer-cachexia+mAbs animals compared to cancer-cachexia mice at the end of the study period (day 30, Figure 2A). The number of CD3+ and CD8+ T cells was significantly greater in the tumors of the cancer-cachexia+mAbs group than in cancer-cachexia mice, while a significant rise was not seen in CD4+ cells in the former group (Table 2). Levels of B cells (CD20+) or macrophages (CD68+) in the tumors did not significantly differ between the two study groups (Table 2). Final body weight and body weight gain variables significantly increased in the cancer-
cachexia+mAbs group compared to cancer-cachexia mice (Table 3 and Figure 2B). A tendency towards a significant increase in muscle weight (p=0.080) was observed only in the gastrocnemius, while no significant differences were seen in the diaphragm of the cachectic mice treated with the monoclonal antibodies compared to the non-treated control animals (Table 3). Importantly, no significant differences were observed in food intake between the two study groups of mice (Figure 2C). Total physical activity, metabolic parameters, and the amount of food and water consumed daily by the mice did not significantly differ between the two study groups (Table 4). At the end of the study period, limb strength gain variables significantly increased in cancer-cachexia+mAbs mice compared to the cancer-cachexia control rodents (Table 4).

Effects of treatment of the tumor with mAbs on injury and muscle structure

At the end of the study period, systemic troponin-I levels were lower in cancer-cachexia+mAbs animals than in the non-treated control mice (Figure 3A). Moreover, in the gastrocnemius muscle, tyrosine release levels significantly decreased in cancer-cachexia+mAbs mice compared to the cancer-cachexia controls (Figure 3B). No significant differences were observed in interleukin (IL)-6 levels in either diaphragm or gastrocnemius muscles between the study groups (Figure 3C). The fiber cross-sectional areas significantly increased in both diaphragm (21% improvement) and gastrocnemius (38% improvement) muscles in cancer-cachexia+mAbs mice compared to non-treated cachectic controls (Figures 3D and S2). Compared to non-treated cachectic mice, the percentage of TUNEL positively-stained nuclei significantly decreased in diaphragm and gastrocnemius muscles of cancer-cachexia+mAbs rodents (Figures 3E and S3).

Effects of treatment of the tumor with mAbs on apoptosis in muscles

Protein levels of BAX significantly decreased in the diaphragm and limb muscles of cancer-cachexia+mAbs mice compared to the non-treated cachectic controls (Figures 4A and S4).
both respiratory and gastrocnemius, BCL-2 protein levels significantly increased in cancer-cachexia+mAbs mice compared to the cancer-cachexia controls (Figures 4B and S4). Levels of active caspase-3 in diaphragm and gastrocnemius significantly decreased in the treated cachectic mice compared to the non-treated controls (Figures 4C and S4).

**Effects of treatment of the tumor with mAbs on proteolytic markers in muscles**

Protein levels of MuRF-1 and atrogin-1 were significantly lower only in the gatrocnemius of cancer-cachexia+mAbs mice than in the non-treated cachectic controls (Figures 5A, 5B and S5), while no differences were seen in TRIM32 protein levels in any study muscle (Figures 5A-5C, and S5 respectively). In both diaphragm and gastrocnemius muscles, total protein ubiquitination levels significantly decreased in cancer-cachexia+mAbs mice compared to the non-treated cachectic controls, while no differences were observed in C8 proteasome subunit protein levels between the two study groups (Figures 5D and 5E, respectively, and S5).

**DISCUSSION**

In the current study, the hypothesis has been confirmed to a great extent. Interestingly, the number of T cells was increased in the tumors of the mice treated with the cocktail of the monoclonal antibodies, whereas no differences were observed in the counts of B cells or macrophages. These results are in line with previous investigations (Dai et al, 2013;Dai et al, 2015), in which intratumoral injection of the same cocktail of antibodies elicited a regression of the lung tumors through reverting a Th2 to a Th1 immune response. A significant decrease in the tumor area (almost 75% reduction) was observed in response to treatment with the immunomodulators, which implied that the treatment was effective as it induced a substantial decline in the tumor burden of the animals at the end of the study period on day 30.

At the end of the study period, the reduction in body weight and limb strength was significantly attenuated in the cancer cachectic mice treated with the monoclonal antibodies
compared to the non-treated animals. In fact, the parameters body weight and limb strength gains, but not physical activity, were also significantly greater in the treated tumor-bearing mice than in the non-treated cachectic rodents, while food intake was similar in both study groups. The weight of the diaphragm did not significantly increase in the cachectic mice treated with the monoclonal antibodies, whereas the gastrocnemius weight showed a tendency to be greater in these animals compared to the non-treated controls. It should be emphasized that food intake did not vary between the two study groups of animals thus, suggesting that the improvements seen in total body weight were not attributable to nutritional support, but to biological processes leading to a better metabolic status in the animals as discussed below. In this regard, it is likely that adipose tissue might have also contributed to the improvement in body weight gain seen in the animals treated with the monoclonal antibodies. Indeed, the implications of adipose tissue in cancer cachexia are of increasing interest (De Lerma, 2015; Penna et al, 2018). This compartment should be thoroughly explored in this experimental model in future studies.

The results demonstrated herein are original and shed light into the potential relationships between tumor burden and total body and muscle weights in cancer cachectic mice. In patients with colon cancer cachexia, the resection of the tumor induced a favorable metabolic response characterized by an attenuation of muscle protein breakdown and increased muscle protein anabolism (Williams et al, 2012), despite the potential deleterious effects derived from the surgical procedures. The findings reported in that study suggested that the reduction in tumor burden was a notable factor in the preservation of muscle mass and body weight as well as in the maintenance of protein anabolism in response to nutrition (Williams et al, 2012). Interestingly, differences in the levels of inflammatory mediators (IL-10, TNF-alpha, and C-reactive protein) were not detected in the patients with colon cancer cachexia before and after the surgical resection of the tumor (Williams et al, 2012). The
authors concluded that these molecules were probably not driving the increased muscle protein catabolism observed in the cancer cachectic patients (Williams et al, 2012) as plasma levels of those cytokines remained invariable before and after surgery. In keeping with, levels of the cytokine IL-6 in either diaphragm or gastrocnemius muscles, did not significantly differ between the two experimental groups. This suggests that this cytokine did not play a major role in muscle protein breakdown in this model of cancer-induced cachexia. Indeed, levels of this cytokine were undetectable in the blood of the mice (data not shown). Whether these inflammatory mediators may play a significant role at different time-points (e.g. from day 22 on, time at which body weight initiated its decline) remains an open question, which should be addressed in future studies.

In the current investigation, we selected this animal experimental model since it has been well-validated in our group (Chacon-Cabrera et al, 2014;Chacon-Cabrera et al, 2015;Chacon-Cabrera et al, 2017;Fermoselle et al, 2013), while a medical rather than a surgical treatment was applied to the tumor-bearing mice in order to avoid any additional stress derived from the surgery. Furthermore, a combination of several immunomodulators that targeted lung cancer cells through different immune pathways was used in the investigation (Dai et al, 2013;Dai et al, 2015). With this approach, we aimed to avoid any potential interference of the use of other drugs and conditions that may exert effects simultaneously on both tumor cells and skeletal muscle fibers (Chacon-Cabrera et al, 2014;Chacon-Cabrera et al, 2015;Chacon-Cabrera et al, 2017;Fermoselle et al, 2013). Indeed, the combination of monoclonal antibodies used in the current study was already shown to elicit a complete regression of the cancer in the mice with syngeneic tumors (melanoma and lung cancer) (Dai et al, 2015). We reasoned that targeting different mechanisms of action (PD-1, CTLA-4, CD-137, and CD-19) that prompted a shift from Th2 to Th1 type immunity as in the cocktail of monoclonal antibodies used in the present study, the therapeutic efficacy
could be significantly improved as previously demonstrated in other experimental models of cancer including mice with lung cancer (Dai et al, 2013; Dai et al, 2015; Wei et al, 2013).

Additionally, to our knowledge, the monoclonal antibodies used in this work do not exert any significant direct effects on skeletal muscles (Dai et al, 2013; Dai et al, 2015; Wei et al, 2013). This was a major concern when designing the study as our main goal was to determine whether treatment of the tumor with specific therapies may have induced a beneficial effect on the skeletal muscle resulting from a decline in tumor burden.

Importantly, a significant attenuation of markers of muscle damage and proteolysis was observed in the cancer cachectic mice treated with the monoclonal antibodies. These are very relevant findings that clearly demonstrate that the documented greater muscle protein catabolism consistently seen in the cachectic mice (Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2015; Chacon-Cabrera et al, 2017; Fermoselle et al, 2013; Salazar-Degracia et al, 2016) was attenuated in response to immunotherapy of the tumor in this model. Specifically, plasma levels of troponin-I were significantly lower in the cancer-cachexia+mAbs mice than in the non-treated cachectic animals. Moreover, levels or tyrosine release within the gastrocnemius were also significantly reduced in the treated mice compared to the non-treated control rodents. Interestingly, signs of muscle damage (membrane leakiness) were not detected in the histological preparations that were stained for IgG in both experimental groups of mice (data not shown). In view of these results, it is possible to conclude that enhanced muscle proteolysis was notably attenuated in response to the blockade of cancer cells induced by the immunomodulators in the study mice, and this may account for the lower levels of troponin I detected in these animals.

In keeping with this, the cross-sectional area of the myofibers in both diaphragm and gastrocnemius muscles were significantly greater in the mice treated with the monoclonal antibodies than in the non-treated animals. Taken together, these findings suggest that
attenuation of cachexia-induced protein breakdown led to larger muscle fibers in both respiratory and limb muscles of the treated animals despite that only a tendency to increased muscle weight was seen in the gastrocnemius of the cachectic mice treated with the monoclonal antibodies. It could be argued that the duration of the model (30 days) and/or the magnitude of the increase in the size of the myofibers, especially in the diaphragm (21% improvement), may have not been sufficiently high to induce a statistically significant rise in the muscle weights of the cachectic mice treated with the monoclonal antibodies.

In the study, several biological mechanisms that are known to participate in the loss of muscle mass and proteolysis have been explored. As such, protein levels of the pro-apoptotic BAX and levels of TUNEL-positive nuclei significantly decreased in both respiratory and limb muscles of the cancer cachectic mice treated with the monoclonal antibodies, whereas protein levels of the anti-apoptotic BCL-2 increased. Collectively, these findings imply that attenuation of apoptosis within the skeletal muscle fibers most likely played a significant role in the improvement of the sizes of the myofibers. In fact, apoptosis was already shown to mediate muscle wasting in the respiratory and limb muscles of cachectic mice with lung tumors (carcinogenesis model) (Salazar-Degracia et al, 2016).

Markers of the ubiquitin-proteasome system have also been explored in the study. In this regard, a significant decline in protein levels of the E3 ligases MuRF-1 and atrogin-1 was detected only in the limb muscle of the cachectic mice treated with the monoclonal antibodies compared to the non-treated cachectic animals. Interestingly, expression levels of the E3 ligase TRIM32 and the marker 20S proteasome did not significantly differ in any study muscle between the two experimental groups. Nonetheless, levels of total protein ubiquitination were, indeed, significantly reduced in both respiratory and limb muscles of the cachectic mice treated with the monoclonal antibodies. These findings are consistent with the decline in the levels of the E3 ligases MuRF-1 and atrogin-1 along with those of total muscle
proteolysis as indicated by the tyrosine release assay. Collectively, these findings are also consistent especially in the limb muscle with previously published results in our group using this experimental model, in which levels of the study proteolytic markers were significantly increased in the diaphragm and limb muscles of the cancer cachectic mice. Differences in the activity of each muscle type may account for the differential expression levels of the E3 ligases seen in response to immunotherapy in the cachectic mice in this model. (Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2017).

Finally, it should be mentioned that limb muscle strength rather than physical activity, as measured using several parameters, significantly improved in the cachectic mice treated with the monoclonal antibodies compared to the non-treated cachectic animals. These results are also in agreement with previous reports in which the cachexia-induced muscle weakness (reduced strength) ameliorated in response to several pharmacological therapeutic strategies (Chacon-Cabrera et al, 2014; Chacon-Cabrera et al, 2017) in the animals. The relevance of these findings rely on the fact that a decline in tumor burden elicited an attenuation of muscle wasting through the blockade of muscle proteolysis and apoptosis that translated into an improvement in both the cross-sectional area of the myofibers and the strength of the limb muscles. Levels of physical activity, however, did not significantly differ in the animals between the two study groups. As physical activity is a more complex process that depends on other factors, potential differences between the two groups may have not been of sufficient magnitude in the cancer-cachexia model used in the study (one month).

Conclusions

We conclude that reduced tumor burden as a result of selective treatment of the lung cancer cells with immunomodulators elicits beneficial effects on body weight and muscle mass loss through attenuation of several biological mechanisms involved in increased protein catabolism and apoptosis, which effectively translated into a significant improvement in limb
muscle strength but not in physical activity. Hence, muscle strength rather than physical
activity may be a better surrogate to monitor improvement in muscle wasting conditions and 
oncologic cachexia.
LIST OF ABBREVIATIONS

CD: cluster of differentiation
COPD: chronic obstructive pulmonary disease
CTLA: cytotoxic T-lymphocyte associated protein-4
ELISA: enzyme-linked immunosorbent assay
HRP: horseradish peroxidase
IGF: insulin-like growth factor
IL: interleukin
MEM: minimal essential medium
PD-1: programmed cell death-1
PRBB: Barcelona Biomedical Research Park
PVDF: polyvinylidene difluoride
TdT: terminal deoxynucleotidyl transferase
TUNEL: terminal deoxynucleotidyl transferase-mediated uridine 5’-triphosphate (UTP) nick-end labeling (TUNEL) assay
TMB: tetramethylbenzidine
TNF: tumor necrosis factor
ACKNOWLEDGEMENTS

The authors are very grateful to Mr. Xavier Duran for his continuous statistical advice and analyses of the study results, to Dr. José Yélamos (immunologist) for providing us with the antibodies to identify the immune cells in the tumors, and to Dr. Lara Pijuan (pathologist) and Judith Prat-Duran (Master student) for their help with the identification and counting of T and B cells, and macrophages in the lung histological preparations.

Authors’ conflicts of interest in relation to the study: None to declare.

Editorial support: None to declare.

Authors' contributions: Study conception and design: EB, ASD; Animal experiments and sample collection: ASD; Molecular biology analyses: ASD, PGM, AMS, JT, APC; Statistical analyses and data interpretation: ASD, EB; manuscript drafting and intellectual input: EB, ASD; manuscript writing final version: EB.


FIGURE LEGENDS

Figure 1: Graphical time-line representation of cancer-cachexia and cancer-cachexia+mAbs mice. Definition of abbreviations: mAbs, monoclonal antibodies; mg, milligram; kg, kilogram.

Figure 2: Mean values of the progression of (A) subcutaneous tumor area (mm²), (B) body weight (grams), and (C) food intake (grams) in cancer-cachexia mice (grey diamonds) and cancer-cachexia+mAbs mice (black squares) during the study protocol. Definition of abbreviations: mAbs, monoclonal antibodies. Statistical significance: * p ≤ 0.05 and ** p ≤ 0.01 between cancer-cachexia mice and cancer-cachexia+mAbs animals.

Figure 3: (A) Mean values and standard deviation of fast troponin-I plasma levels (ng/ml) on day 0, 15 and 30 in cancer-cachexia mice (grey diamonds) and cancer-cachexia+mAbs animals (black squares). (B) Mean values and standard deviation of tyrosine release (nmol/mg/2h) in the gastrocnemius of cancer-cachexia mice (grey bars) and cancer-cachexia+mAbs animals (black bars). (C) Mean values and standard deviation of interleukin (IL)-6 protein levels in the diaphragm (white bars) and gastrocnemius (black bars) muscles of cancer-cachexia and cancer-cachexia+mAbs mice. (D) Mean values and standard deviation of cross-sectional areas in the diaphragm (white bars) and gastrocnemius (black bars) muscles of cancer-cachexia mice and cancer-cachexia+mAbs animals. (E) Mean values and standard deviation of the percentage of positively stained nuclei for the TUNEL assay in the diaphragm (white bars) and gastrocnemius (black bars) muscles of cancer-cachexia mice and cancer-cachexia+mAbs animals. Definition of abbreviations: mAbs, monoclonal antibodies; ng, nanogram; ml, milliliter; nmol, nanomol; mg, milligram; h, hour; µm, micrometer; IL, interleukin. Statistical significance: * p ≤ 0.05, ** p ≤ 0.01 and *** p ≤ 0.001 between cancer-cachexia mice and cancer-cachexia+mAbs animals.
Figure 4: Mean values and standard deviation of the following markers: (A) BAX, (B) BCL-2 and (C) active caspase-3 levels in diaphragm (white bars) and gastrocnemius (black bars) muscles as measured by optical densities in arbitrary units (OD, a.u.). Definition of abbreviations: mAbs, monoclonal antibodies; BAX, BCL2 associated X protein; BCL-2, B-Cell CLL/Lymphoma 2. Statistical significance: * \( p \leq 0.05 \) and *** \( p \leq 0.001 \) between cancer-cachexia mice and cancer-cachexia+mAbs animals.

Figure 5: Mean values and standard deviation of the following markers: (A) MuRF-1, (B) atrogin-1, (C) TRIM32, (D) total protein ubiquitination levels, and (E) 20S proteasome C8 protein content in diaphragm (white bars) and gastrocnemius (black bars) muscles as measured by optical densities in arbitrary units (OD, a.u.). Definition of abbreviations: mAbs, monoclonal antibodies; MuRF-1, muscle ring finger protein-1; TRIM32, tripartite motif-containing protein 32. Statistical significance: * \( p \leq 0.05 \) between cancer-cachexia mice and cancer-cachexia+mAbs animals.
Table 1. Cocktail of antibodies used to reduce tumor burden

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Target</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-PD-1</td>
<td>PD-1</td>
<td>PD-1 receptor is expressed in activated T cells and induces immune tolerance by repressing T cell effector function (1-2).</td>
</tr>
<tr>
<td>Anti-CTLA-4</td>
<td>CTLA-4</td>
<td>CTLA-4 receptor is expressed in T cells and induces immune tolerance by repressing antigen presentation (3-4).</td>
</tr>
<tr>
<td>Anti-CD137</td>
<td>CD-137</td>
<td>CD-137 receptor activates CD8+ T and NK cells while it also inhibits Treg cell function (5).</td>
</tr>
<tr>
<td>Anti-CD19</td>
<td>CD-19</td>
<td>CD-19 activates B cells (6).</td>
</tr>
</tbody>
</table>

Definition of abbreviations: PD-1, programmed cell death-1; CTLA-4, cytotoxic T-lymphocyte associated protein-4; CD-137, TNF receptor superfamily member 9; CD-19, B-lymphocyte antigen; NK, natural killer.

(Chen and Mellman, 2013; Forsthuber et al, 2018; Jin et al, 2011; Selby et al, 2013; Yonezawa et al, 2015)

Literature Cited


Table 2. Immune cells in the tumors of the study groups of mice

<table>
<thead>
<tr>
<th></th>
<th>Cancer-cachexia</th>
<th>Cancer-cachexia+ mAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>T cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3+ cells (%)</td>
<td>8.83 (0.92)</td>
<td>11.20 (0.77)***</td>
</tr>
<tr>
<td>CD8 + cells (%)</td>
<td>4.95 (1.41)</td>
<td>7.70 (1.08)***</td>
</tr>
<tr>
<td>CD4+(cells/µm²)</td>
<td>2.28×10⁻⁶ (0.84×10⁻⁶)</td>
<td>3.04×10⁻⁶ (1.67×10⁻⁶)p=0.289</td>
</tr>
<tr>
<td><strong>B cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD20+, Hscore 0 (%)</td>
<td>0.18 (0.28)</td>
<td>0.16 (0.35) p=0.912</td>
</tr>
<tr>
<td>CD20+, Hscore 1 (%)</td>
<td>5.94 (6.17)</td>
<td>8.96 (9.53) p=0.455</td>
</tr>
<tr>
<td>CD20+, Hscore 2 (%)</td>
<td>52.86 (12.63)</td>
<td>57.17 (10.01) p=0.473</td>
</tr>
<tr>
<td>CD20+, Hscore 3 (%)</td>
<td>40.51 (17.43)</td>
<td>32.68 (12.32) p=0.331</td>
</tr>
<tr>
<td>CD20+, Hscore 4 (%)</td>
<td>0.46 (0.54)</td>
<td>0.98 (0.73) p=0.126</td>
</tr>
<tr>
<td><strong>Macrophages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD68+ cells (%)</td>
<td>21.3 (7.4)</td>
<td>20.1 (8.3)p=0.768</td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation). **Definition of abbreviations:** mAbs, monoclonal antibodies. **Statistical significance:** *p ≤ 0.05 and **p ≤ 0.01 between cancer-cachexia mice and cancer-cachexia+mAbs animals.
Table 3. Physiological characteristics of the mice in both study groups

<table>
<thead>
<tr>
<th></th>
<th>Cancer-cachexia</th>
<th>Cancer-cachexia+ mAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>20.32 (1.03)</td>
<td>20.13 (1.92)</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>16.75 (1.74)</td>
<td>19.46 (2.05)**</td>
</tr>
<tr>
<td>Body weight gain (%)</td>
<td>-17.48 (8.47)</td>
<td>-2.90 (10.19)**</td>
</tr>
<tr>
<td>Diaphragm weight (g)</td>
<td>0.066 (0.016)</td>
<td>0.071 (0.011)</td>
</tr>
<tr>
<td>Gastrocnemius weight (g)</td>
<td>0.087 (0.013)</td>
<td>0.097 (0.012), p=0.080</td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation). Definition of abbreviations: mAbs, monoclonal antibodies; g, gram. Statistical significance: **p ≤ 0.01 between cancer-cachexia mice and cancer-cachexia+mAbs animals.
### Table 4. Metabolic, physical activity, and grip strength variables in the study groups of mice

<table>
<thead>
<tr>
<th></th>
<th>Cancer-cachexia</th>
<th>Cancer-cachexia+ mAbs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metabolic parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VO$_2$ (mL/min)</td>
<td>1.18 (0.24)</td>
<td>1.32 (0.05)</td>
</tr>
<tr>
<td>VCO$_2$ (mL/min)</td>
<td>1.11 (0.21)</td>
<td>1.29 (0.09)</td>
</tr>
<tr>
<td>RER</td>
<td>0.94 (0.03)</td>
<td>0.97 (0.04)</td>
</tr>
<tr>
<td>EE (kcal/day)</td>
<td>8.46 (1.74)</td>
<td>9.55 (0.46)</td>
</tr>
<tr>
<td>Food (g)</td>
<td>3.77 (3.61)</td>
<td>3.64 (1.05)</td>
</tr>
<tr>
<td>Water (ml)</td>
<td>1.37 (0.58)</td>
<td>1.87 (0.61)</td>
</tr>
<tr>
<td><strong>Physical activity 24h</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total physical activity</td>
<td>2784 (1326)</td>
<td>3193 (1363)</td>
</tr>
<tr>
<td>Stereotyped movements</td>
<td>2721 (1283)</td>
<td>3136 (1346)</td>
</tr>
<tr>
<td>Locomotor movements</td>
<td>63 (43)</td>
<td>58 (34)</td>
</tr>
<tr>
<td><strong>Grip strength</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forelimb strength gain (%)</td>
<td>-20.76 (24.74)</td>
<td>+16.81 (26.35)*</td>
</tr>
<tr>
<td>Four-limb strength gain (%)</td>
<td>-24.93 (22.32)</td>
<td>-4.15 (14.84)**</td>
</tr>
</tbody>
</table>

Values are expressed as mean (standard deviation). **Definition of abbreviations:** mAbs, monoclonal antibodies; mL, milliliter; min, minute; VO$_2$, oxygen consumption; VCO$_2$, carbon dioxide production; RER, respiratory exchange ratio; EE, energy expenditure; kcal, kilocalorie; g, gram. **Statistical significance:** *p ≤ 0.05 and **p ≤ 0.01 between cancer-cachexia mice and cancer-cachexia+mAbs animals.
Figure 1

Cancer-cachexia mice

Day 0

Subcutaneous inoculation of $4 \times 10^6$ LP07 adenocarcinoma cells

Day 15

Phosphate-saline buffered (PBS) intraperitoneal 72 hours

Day 30

Sacrifice

Cancer-cachexia + mAbs

Day 0

Subcutaneous inoculation of $4 \times 10^6$ LP07 adenocarcinoma cells

Day 15

Cocktail of monoclonal antibodies ($5 \times 10^3$ mg/kg) intraperitoneal 72 hours

Day 30

Sacrifice

Figure 1

288x195mm (300 x 300 DPI)
Figure 2A-2C

268x201mm (300 x 300 DPI)
Figure 3A-3C

274x211mm (300 x 300 DPI)
Figure 3D-3E

280x106mm (300 x 300 DPI)
Figure 4A-4C

259x199mm (300 x 300 DPI)
Figure 5A-5C

260x196mm (300 x 300 DPI)
Figure 5D-5E

260x106mm (300 x 300 DPI)