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THE INFLUENCE OF PHYSICAL ACTIVITY IN THE ANTI-TUMOR IMMUNE RESPONSE IN EXPERIMENTAL BREAST TUMOR

Thiago M. Bianco^{*a}, Douglas R. Abdalla^{*a}, Chamberttan S. Desidério^a, Sofie Thys^b, Cindy Simoens^b, John-Paul Bogers^b, Eddie F. C. Murta^c, Márcia A. Michelin^d

** Equal contribution for datas*

¹*Oncology Research Institute (Instituto de Pesquisa em Oncologia-IPON), Federal University of the Triângulo Mineiro (UFTM);* ²*Laboratory of Cell Biology and Histology, University of Antwerp, Belgium;* ³*Titular Professor, Oncology Research Institute (IPON)/ Discipline of Gynecology and Obstetrics, UFTM,* ⁴*Associate Professor, Oncology Research Institute (IPON)/Discipline of Immunology, UFTM,. Uberaba, Minas Gerais, Brazil.*

Corresponding Author: Márcia Antoniazi Michelin, PhD, Oncology Research Institute (IPON)/Discipline of Immunology, Federal University of the Triângulo Mineiro (UFTM), Avenida Guilherme Ferreira n° 1940 Uberaba (MG), Brazil, CEP 38100-000, Bairro São Benedito. Tel: (+55 34) 3318-5500; Fax:(+55 34) 3318-5342. E-mail: michelinimuno@icbn.uftm.edu.br

Highlights

The physical influence the innate immunity in experimental breast cancer by interfering in process of maturation of DCs both in tumor and systemically, that by its turn promote a modification in acquired immune cells, representing by T helper to induce an important alteration transcription factors that are responsible to maintain a suppressive microenviroment, and thereby, allowing the latter cells can thus activate antitumor immune response. The PA was able improve the Th1 systemic response by enhance to Tbet gene expression, promote a slightly increased of Th1-type cytokines and decrease Gata3 and Foxp3 gene expression in which can inhibit the Th1 immune response.

ABSTRACT

This study aimed to investigate the influence of physical activity in innate immunity to conduce to an effective antitumoral immune response analyzing the phenotype and activation status of infiltrating cells. We analyzed the intracellular cytokines and the transcription factors of tumor infiltrating lymphocytes (TILS) and spleen leukocytes. The Nos2 gene expression was evaluated in spleen cells and futhermore the ROS production was measured and spleen cells; another cell evaluated was dendritic cells (TIDCs), their cytokines expression and membrane molecules; finally to understood the results obtained, we analysed the dendritic cells obtained from bone marrow. Were used female Balb/c mice divided into 4 groups: two

controls without tumor, sedentary (GI) and trained (GII) and two groups with tumor, sedentary (GIII) or trained (GIV). The physical activity (PA) was realized according swimming protocol. Tumor was induced by injection of 4T1 cells. All experiments were performed in biological triplicate. After the experimental period, the tumor was removed and the cells were identified by flow cytometry with labeling to CD4, CD8, CD11c, CD11b, CD80, CD86 and Ia, and intracellular staining IL-10, IL-12, TNF- α , IFN- γ , IL-17, Tbet, GATA3, ROR γ t and FoxP3. The bone marrow of the animals was obtained to analyse the derived DCs by flow cytometry and culture cells to obtain the supernatant to measure the cytokines. Our results demonstrated that the PA inhibit the tumoral growth although not to change the number of TILS, but reduced expression of GATA-3, ROR- γ T, related with poor prognosis, and TNF- α intracellular; however occur one significantly reduction in TIDCS, but these cells expressed more co-stimulatory and presentation molecules. Furthermore, we observed that the induced PA stimulated the gene expression of Tbet and the production of inflammatory cytokines suggesting an increase of Th1 systemic response. The results evaluating the systemic influence in DCs showed that the PA improve significantly the number of those cells in bone marrow as well the number of co-stimulatory molecules. Therefore, we could conclude that PA influence the innate immunity by interfering to promote in process of maturation of DCs both in tumor and systemically, that by its turn promote a modification in acquired immune cells, representing by T helper to induce an important alteration transcription factors that are responsible to maintain a suppressive microenvironment, and thereby, allowing the latter cells can thus activate antitumor immune response. The PA was able improve the Th1 systemic response by enhance to Tbet gene expression, promote a slightly increased of Th1-type cytokines and decrease Gata3 and Foxp3 gene expression in which can inhibit the Th1 immune response.

Key Words: dendritic cells; transcription factors; helper lymphocytes; antitumoral immune response; physical activity.

INTRODUCTION

About female cancers that can be diagnosed, the World Health Organization (WHO) estimated around of 1,500,000 new cases of breast cancer in the world, also estimating a total 500,000 deaths[1,2]. Breast cancer is not a single pathology but a diversified group of diseases characterized by heterogeneity in

histology, genomic aberrations, and protein expression that influence treatment response and patient outcome [3]. In this sense, this diverse set of factors could change the immune system and it is necessary to investigate the relationship between tumor and immune response, what profiles are acting in tumor progression as well as in other hand what work to regression and/or elimination of this tumor.

Tumor microenvironments include, among the extracellular matrix and cells of stroma, innate immune cells (macrophages, neutrophils, myeloid derived suppressor cells, natural killer cells, and dendritic cells) and adaptive immune cells (B and T lymphocytes). The intercellular interactions, cytokine pathways and transcription factors polarize the tumor microenvironment, being tumor-promoting, profiles of T lymphocytes helper (Th)1, Th 2, Th17 and Treg that could directly influence the immune response[4,5]. On the other hand, immune response patterns are controlled by different proteins, in which, are responsible by drive the activity of lymphocytes and other cells of immunological system. The Tbet is the chief transcription factor involved in the differentiation of TCD4⁺ cells in Th1 cells. The Th1 cytokines, for example IL12, are capable of inhibiting angiogenesis, metastasis and decreasing tumor growth. Gata3 is responsible for inducing the differentiation in Th2 cells. Th2 immune response can inhibit the Th1 profile and has been associated with tumor progression. Bahria- Sediki (2016)[11], showed that the expression of Tbet and Gata3 was resulted an increase survival of carcinoma bladder patients, but the mechanisms remain unclear. The expression of Foxp3 in the immunological system is essential for the development of T CD4⁺CD25⁺ helper cells (Treg cells) and lead a tolerant state. However, this protein in cancer might have a dual role. Although the expression of Foxp3 by T cell result in an immunosuppressive microenvironment, its expression in tumor cells has been associated with a good prognosis [6-11].

On the other hand, it is known that cells of the immune system work synergistically and coordinated manner, being of fundamental importance the correct interaction between cells of the innate and acquired immunity. Briefly, the main cell of innate immunity is the DCs that are fundamental to correctly activate the helper lymphocytes, which in turn are the central cell of acquired immunity and responsible by coordinate all the immune cell. Several works with human and experimental models described that the helper lymphocytes could control the regression or progression of different tumors [4, 5, 12, 13, 14].

In this context, immune cells tumor infiltration, the presence of DCs into primary tumor has been associated with significantly prolonged patient survival and a reduced incidence of metastasis (e.g. oral, head and neck tumors, nasopharyngeal tumors, lung, bladder, esophageal, and gastric carcinomas)[12]. Likewise, Langerhans cell (LC) infiltration has been linked to regression of primary cutaneous melanomas [13,14]. However, in breast cancer, tumor-infiltrating dendritic cells (TIDCs) were found to drive inflammatory Th2 (iTh2) cells and pro-tumor inflammation [15]. Therefore, the behavior of the immune cells inside the tumoral microenvironment can promote immunological escape, through the decreased or absent expression of MHC molecules, co-stimulatory molecules, or via secretion of immunosuppressive factors by tumors[16-19].

Despite the many complex functions of the human immune system is the regulation of susceptibility to cancer as highlighted in the present study. Therefore, the established beneficial effect of exercise on the immune system implies that it can also reduce the risk of cancer. In addition, there is a growing interest in alternative options in order to achieve a better quality of life. A thorough understanding of the effects of exercise and physical activity on the mortality/morbidity rates of breast cancer patients would have an important clinical impact [20].

Several studies about cancer prospects have demonstrated that physical exercise after cancer diagnosis is associated with a reduced risk of cancer recurrence and improves overall mortality rates in multiple cancer survivor groups, including breast, ovarian, prostate and colorectal cancer[21-24].

Previous studies by our group have revealed that moderate physical activity is able to potentiate a systemic polarization profiles of T helper lymphocytes and macrophages to antitumor patterns, i.e., Th1 and M1, respectively, in chemical carcinogenesis [25,26], in other hand still not clear if physical activity is capable of modifying the phenotype of dendritic cells and T cells into the tumor. Thus, this study aimed to investigate the influence of physical activity on the systemic immune response, phenotype of dendritic cells intratumoral and systemic, and explain how this change could influence the antitumor immune response in animals with breast cancer.

MATERIALS AND METHODS

Animals

A total of 40 adult female Balb/c mice (8 wks old, mean of body weight $23,1 \pm 0,7$ g) from the Research Institute of Oncology, Uberaba, Minas Gerais, were used; the animals were divided into 4 experimental groups (described below) and group-housed in sufficiently large plastic cages and maintained on a 12-hour light/dark cycle in a temperature-controlled (21 ± 3 °C) vivarium, with *ad libitum* access to food and water. After the experimental period, the animals were euthanized with an overdose of ketamine (75 mg/kg) and xylazine (15 mg/kg). This study was approved by the ethics committee on the use of animals by the Federal University of Triangulo Mineiro (registration no. 317). Were used the ethical principles for the use of animals contained in Brazilian Guiding Principles for Biomedical Research Involving Animals. All experiments were performed in biological triplicate.

Experimental groups

The animals were divided into four groups (n = 10 per group): no tumor/non-trained (GI), non tumor/trained (GII), tumor/non-trained (GIII), and tumor/trained (GIV). In the tumor groups, 4T1 mouse breast tumor cells (2×10^5), were injected into the mammary gland of host mice. The tumoral volume was measured every two-three days (for calculation of the volume the major and minor diameters were collected) and then used in the Steel [27] formula:

$$\frac{[\text{major diameter} \times (\text{minor diameter})^2]}{2}$$

2

The figure 1a represent the study design. The animals were given swim training 5 days/week for 4 weeks, in separate circular line (15cm of diameter) inside an insulated box. The first week was used for adaptation to water and afterwards the mice were submitted to a progressive protocol (one week of 30 min of training and two final weeks 45 min of training). The water was replaced daily and temperature was maintained in the range of 30–35 °C. This protocol was adapted from previous studies developed by our group and literature reports [22,23,25,26].

The animals who did not show physical fitness to follow the training protocol were withdrawn from the study, as were those animals that show any signs of discomfort when performing some of the procedures.

Collection of tumor and bone marrow cells

Animals were euthanized (as described above), had their tumor removed, and were necropsied. Each tumor was submitted to mechanical disruption and the cells were homogenized immediately in saline solution and washed three times by centrifugation at $290 \times g$ for 10 min at 4°C in physiologic solution. Following centrifugation, the cells were counted and resuspended at a concentration of 1×10^6 cells for use in flow cytometry label protocol.

The bone marrow from femur and tibiae from each animal were removed, homogenized immediately in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma-Aldrich®, St Louis, MO, USA), and washed three times by centrifugation at $290 \times g$ for 10 min at 4°C in IMDM. Following centrifugation, the cells were counted and resuspended in complete IMDM supplemented with fetal bovine serum, this protocols was standardized in our laboratory.

Cells were distributed in flat-bottom, 6-well plates at a concentration of 5×10^6 cells/well in a 4.0-mL medium. Two days later, they were stimulated with 10 ng/ml of GM-CSF and IL-4 (both BD Pharmingen™, BD Biosciences, San Diego, CA, USA), and after five days they were stimulated with 10 ng/ml of TNF- α (BD Pharmingen™) and tumor lysate. Half of the plates were stimulated with lipopolysaccharide (LPS) (Sigma-Aldrich®) (10 $\mu\text{g/ml}$). After 48 incubation at 37°C in a humidified 5% CO_2 atmosphere, supernatant samples were obtained, divided into six aliquots, and stored at -80°C . The adherent cells were collected for the flow cytometry label protocol.

RNA Extraction

RNA extraction was done in two steps. The first one using the Trizol method. Later, in the second step for RNA extraction, the SV RNA Isolation (PROMEGA®) kit was used, both of them were performed following manufacturer's recommendations.

Reverse Transcriptase (RT)

After RNA extraction, quantification, and RNA/protein analysis, the Reverse Transcriptase assay was performed with the Reverse Transcriptase (Invitrogen®) kit. 50ng-1µg total RNA was used for this technique, following the protocol recommended by the manufacturer. For the reaction in the first step, the conditions were: one cycle at 65°C for 5 min. In the second step, the conditions were: one 5 min cycle at 25°C, 50°C for 60 min and 70°C for 15 min.

Real-Time Polymerase Chain Reaction (q-PCR)

For the q-PCR specific primers were used, with their sequences and concentrations displayed in the following table.

The q-PCR was performed with the GoTaq® qPCR Master Mix (PROMEGA®) kit, following the protocol suggested by the manufacturer. The reaction conditions are one cycle at 95°C for 2 min, 45 cycles at 95°C for 25 seconds, and 1 minute at 60°C. The calculations of gene expression were based on the $\Delta\Delta C_t$.

Flow cytometry Protocol

After the spleen collection, cells were isolated and cultured with lysis buffer (FACS Lysing Solution; BD Biosciences, San Diego California, USA) for erythrocyte exclusion. After this step, the sample was centrifuged to remove lysed cells. The next stage was comprised of two washes followed by centrifugation. Then, the labelling was performed with the following antibodies.

Isolated tumor cells were placed in lysing solution (BD Biosciences - FACS™ Lysing Solution) at a proportion of 1 mL sample in 20 mL lysing solution. After a 5-min incubation at room temperature, cell lysis solution was removed by centrifugation for 10 min at a temperature of 4°C, at $290 \times g$, and the cells were washed three times in phosphate buffered saline solution (PBS). The tumor cells obtained from these protocol and the bone marrow cells werethen combined with 1 mL of PBS, supplemented with 2 µL of protein transport inhibitor (BD Golgistor™) per 3 mL of cell solution, and then incubated for at least 20 min at 4°C. The cells were then washed with PBS by centrifugation, as described above, to remove excess protein.

After centrifugation, the cells were resuspended, counted, and submitted to extracellular immunolabeling. Cells were incubated with each antibody at 4 °C for 30 min in the dark, and then washed

with PBS to remove excess antibodies. Permeabilization and fixation were performed (BD Cytofix/Cytoperm™ solution) at 4°C for 20 min in the dark. Cells were similarly submitted to intracellular immunolabeling, some tubes were reserved for control isotypes. After intracellular labeling, the cells were again incubated at 4°C for 30 min in the dark and washed in buffer solution (BD Perm/Wash™ Buffer) to remove excess labeling molecules. Finally, cell aliquots were resuspended in 500 µL of PBS for flow cytometry analysis in a BD FACS Calibur™ cytometer.

Tumor and bone marrow cells were marked for total myeloid innate cells (anti-CD11b PerCP-Cy5.5 conjugated antibody), mature dendritic cells (anti-CD11c APC conjugated antibody) and tumor-associated macrophage (CD14 FITC conjugated antibody), as well as for the co-stimulatory molecules (anti-80 FITC, anti-CD86 APC and Ia PE-conjugated antibody). For TILs, the following labeling with conjugated-antibody was used to reveal total T (anti-CD3 FITC), helper T lymphocytes (anti-CD4 Pe-Cy), cytotoxic T lymphocytes (anti-CD8 Alexa 647 associated with the cells type were labeling also the transcription factors: Th1 (anti-Tbet Alexa488), Th2 (anti-GATA3 PE), Th17 (anti-ROR-γT) and Treg (anti-FoxP3 Alexa488). Furthermore, immunolabeling of the expression of intracellular cytokines by T cell was performed: anti-IL-12 PE, anti-TNF-α PE, anti-IFN-γ FITC, anti-TNF-α PE, and anti-IL-10 FITC. All antibodies used in flow cytometry protocol were from BD Biosciences, San Diego, CA, USA.

Cytokine levels

The presence of cytokines (IFN-γ, IL-4, IL-12, IL-10 and TGF-β) in the supernatant samples obtained from dendritic cells culture was measured by enzyme linked immunosorbent assay (ELISA) using pairs of monoclonal antibodies purchased from BD OptEIA™ (BD Biosciences, San Diego, CA, USA) The procedure was performed in accordance with the manufacturer's protocol. All cytokines were measured in pg/ml.

Statistical analysis

The data for each variable were tested whether they were normally distributed. For variables for which the distribution was found to be normal, parametric tests were performed. For comparisons of normally distributed data, Student's t-tests were used for two-group comparisons and analyses of variance

(ANOVAs) and Post-hoc Bonferroni tests were used for comparisons among three or more groups. The results are expressed as means $\pm$ standard error of the mean (SEM). For comparisons of abnormally distributed data, Mann-Whitney tests were used for two-group comparisons, and Kruskal-Wallis and Post-hoc Dunn's tests were used for comparisons among three or more groups. The results are expressed as median $\pm$ ranges. Proportions were compared using the chi-square (χ^2) test. The observed differences were considered significant if probability (p) value less than 0.05 (5%).

RESULTS

Tumor volume in the 4T1 cell-injected groups.

Each group in this study was composed of ten animals, no death cases registered during the experimental period, and only one animal of group II was changed for another animal, due to lack of adaptation to the aquatic environment. In accordance with the study design (figure 1a), the animals of groups III and IV were injected with 4T1 cells on day 0 and the animals of groups II and IV were gradually adapted to the water environment for 15 min/day during the first week, 30 min/day during the second week and 45 min/day up to the end of the experimental period.

In the tumor (GIII) and tumor/trained (GIV) groups, the tumors, once present, were measured every 2-3 days from 7th days after injection of the 4T1 cells onwards (see figure 1b). The group submitted to physical activity showed significantly smaller tumors according to measurements between day 21 and day 25 ($p < 0.05$). The slope of the tumor growth rate in the tumor/trained group (GIV: 12.9 ± 2.6) was significantly lower than that in the tumor/non-trained group (GIII: 37.1 ± 3.9) and we observed that the tumor volume was lower in tumor/trained group than tumor/non-trained mice (fig 1c).

The physical activity enhanced the Th1 immune response in spleen of tumor mice

The Tbet is a transcriptional factor that lead Th1 activation. The Th1 pattern driver the expression of No2 gene and promote the anti-tumor immune response (27, 28). First, we evaluated the expression of Tbet and others transcriptional factor that can inhibit tbet in the spleen of mice. The results showed a

significant increase of *tbet* gene expression ($p < 0.0003$) and decrease of *Gata3* gene expression in tumor/trained group, whereas the tumor/non-trained group presented lower levels of expression of these genes. Although the levels of gene expression of *Tbet* and *Foxp3* were increased in the mice subjected to physical activity, the level of *Tbet* gene expression was 1.5 higher than *Foxp3* in tumor/trained group (fig2a). Then, we evaluated the balance Th1/Th2 response and we observed the Th1 balance was significantly increased in tumor/trained group than tumor/untrained group (fig2b). After that, we were evaluated another marker associated with Th1 pattern and the results showed a reduction of *Nos2* gene expression in tumor/untrained group and an increase of positive samples that express the *Nos2* gene in tumor/trained group (fig2c) where we can observe in this group, that the increase of *Nos2* gene expression was close to the control group (fig2d). The *in vitro* assay showed that the splenic cells in tumor/trained group presented an increase of nitrite production, suggesting an enhanced of iNOS enzyme activity in the mice subjected to physical activity (fig2d). Furthermore, we observed an increase of $CD4^+ CD25^+$ lymphocytes (Tregs cells) in the spleen of tumor group ($p < 0.05$) while the mice that were submitted the physical activity in both groups (trained and tumor/trained group) presented a decrease of $CD4^+ CD25^+$ cells (Treg cells) (fig3a). We observed an increase of pro-inflammatory cytokines produced by $CD4^+$ cells, like IL-12 and IFN- γ ($p < 0.05$), but not the TNF- α in mice with breast cancer and submitted the swimming, (Fig 3b, 3c and 3d). The results showed that the lymphocytes reduced the production of IL-10, which is a Th2-like cytokine, in the tumor/trained group ($p < 0.05$) (Fig 3e). Taken together, the results demonstrated an increase of Th1 markers, like *Tbet* and *Nos2* gene expression as well as the Th1-like cytokines and a reduction Th2 profile in tumor/trained group. The data shown that the physical activity was able improve the Th1 immune response patterns of systematic way.

Tumor-Infiltrating Lymphocytes (TILs), Tumor-Infiltrating Dendritic Cells (TIDCs) And Tumor-Associated Macrophages (TAMs)

We investigated the behavior of tumor-infiltrating immune cells, in this sense was examined whether training could modify the cells inside the tumor by labeling the lymphocytes and dendritic cells inside the tumor.

Figure 4A show the representation the lymphocytes (CD3+) distribution in two subtypes, T Helper (CD4+) and T cytotoxic (CD8+), in groups submitted to Tumor induction and physical activity protocol. Revealed no difference in the proportions of CD4+ T-cells within the TIL population between the tumor/non-trained and the tumor/trained group. Analysis of the CD8+-positive T-cells indicates that physical activity tends to increase the infiltration of cytotoxic T-cells (figure 4B).

Subsequently, the expression of transcription factor and cytokines by CD4+ T cells was assessed inside the tumor. The figure 4C show the double expression of CD4+ T cells and transcription factor, physical training promoted the reduction of MFI (mean of fluorescence intensity) for ROR γ T+ and GATA3 ($p<0.05$) and no change the expression of Tbet and FoxP3.

Taken together, Figure 4D, depicts the expression of cytokines. The mean fluorescence intensity of Th1 cytokines (IFN- γ and IL-12) was higher in the tumor/trained group (GIV), while the expression of the IL-17, the Th17 profile, decreased substantially in this group. The same pattern was seen for TNF- α ($p<0.05$), suggestive of inflammation reduction inside the tumor. On the other hand, physical activity did not cause any change in the expression of the suppressive cytokine, IL-10.

Figure 5A show the representation the dendritic cells (CD11b+ and CD11c+) in groups submitted to breast tumor and/or physical activity, and also the expression of costimulatory molecules (CD80+, CD86+ and I-A). Our results show that physical training reduce the number of Dendritic cells inside the tumor, figure 5B. Regarding the expression of costimulatory molecules in the dendritic cells, training promoted an increase in the expression of CD80+, CD86+ and I-A, when compared group GIV with GIII. Figure 5D shows the results of the synthesis of cytokines by DCs: physical activity, represented by GIV (tumor/trained), tend to increase the mean of fluorescence intensity for IL-12, TNF- α , IL-10, an in particular IL-12, an important cytokine to adopt the antitumor patterns.

Regarding the infiltration of TAM (Figure 6A represent the gating strategy), the physical activity tend to increase the number of macrophage into the tumor, and also tend to increase the expression of CD86+, in other hand reduce significantly the expression of MHC class II (figure 6B and 6C).

Bone Marrow-Derived Dendritic Cell Phenotype By The Expression Of Co-stimulatory Molecules

In order to verify to which extent dendritic cells can be differentiated from hematopoietic progenitors, which could serve as a tool for future immunotherapeutic approaches, this study assessed whether the differentiation into DCs and the maturation process are influenced by tumor presence, physical activity, as well as the association tumor-training. To this end, bone marrow was removed from the femur and tibia and the differentiation culture was started.

After the bone marrow cells had been differentiated from dendritic cells by stimulation with GM-CSF and IL-4, and matured with TNF- α and tumor lysate, the adherent cells were resuspended and labeled to assess the number of cells by CD11b+ and CD11c+ expression. Additionally, the maturation was checked with co-stimulatory molecules (CD80+ and CD86+). Figure 7A shows a reduction in the number of double stainings (CD11b+ and CD11c+) in the non-tumor/trained group (GII), comparable to the control group (GI), as well as an increase in DC numbers in the tumor/trained group (just like in the non-tumor/non-trained group (GI)), compared to the former two groups.

Surprisingly, the expression of co-stimulatory molecules was higher in the non-tumor/trained group than in control and tumor/non-trained groups. High expression of co-stimulatory molecules was found in tumor/trained group; when comparing this group (GIV) to GI and GIII, a significantly higher expression of CD80+ and CD86+ similar to that in the tumor/trained group (GIV), was measured (figure 7B).

Synthesis Of Cytokines By Bone Marrow-Derived Dendritic Cells

Figure 7C, 7D, 7E, 7F and 7G provides an overview of the expression of cytokines (IL-12, IFN- γ , IL-4, IL-10 and TGF- β) by bone marrow-derived DCs, The concentration of IFN- γ was found to be significantly higher in the non-tumor/trained group than in the control group. However, tumor presence significantly ($p < 0.05$) decreased the synthesis of IFN- γ (fig7C). Analysis of IL-12 synthesis (figure 7D)

showed that tumor induction decreased the expression of IL-12, while physical activity tends to increase the synthesis of IL-12 (albeit not significantly).

IL-4 synthesis by dendritic cells (figure 7E) disclosed an opposite behavior compared to IL-12 and IFN- γ , with the tumor groups (GIII and GIV) having higher levels than the other groups ($p < 0.05$). Physical activity in tandem with tumor presence (group GIV) tends to reduce the expression of IL-4, but it is not significantly.

Tumor induction does not influence the concentration of IL-10 produced by bone marrow-derived dendritic cells, when compared GI and GIII. However, the expression of IL-10 tended to be lower in both trained groups, especially in the tumor/trained group (GIV), where it was significantly ($p < 0.05$) reduced compared to the other groups (figure 7F).

Finally, both tumor groups showed a significantly reduced expression of TGF- β compared to GI and GII (figure 7G). Also the tumor/non-trained group displayed reduced TGF- β synthesis compared to GI ($p < 0.05$). Our cytokine synthesis results suggest that physical activity increases the expression of antitumor cytokines, as evidenced from the increased levels of IFN- γ and IL-12, and also the protumor cytokines (IL-4 and IL-10) reduced the levels.

DISCUSSION

In this study, we measured tumor sizes and provided evidence that physical activity reduced tumor growth, probably decrease the proliferation of tumor cells. In a similar study by Lane and cols (1991)[27], who used a DMBA (7,12- Dimetil-benzantraceno) induced tumor model, animals that performed physical activity and received DMBA showed decreased tumor frequency. Another study using Walker's tumor injection in different regions showed that the groups submitted to exercise had decreased tumor sizes [30].

In a meta-analysis conducted by Wu et al. (2013) [31], combining the results of 31 studies about physical activity and breast cancer, found that physically active women had lower risks of

developing breast cancer compared to the inactive women. Two hours/week of moderate to vigorous activity, decreases the risk of breast cancer by 5%. Also, after the menopause, this risk is reduced by 10%, regardless of a woman's physical fitness, weight or waist circumference [32].

What could explain the reduction of tumor size in our study and the lower risk of breast cancer in physically active women is the fact that physical activity influence the immune system. For an effective anti-tumor response to occur, the components of this response need to work synergistically. The most distinguished cells in the innate immune response are macrophages, NK cells, and dendritic cells, in the acquired immune response, principally involving T and B lymphocytes [4,5].

Our study aimed to evaluate the systemic immune response through the Th1 response pattern that have play a critical role in cancer by drive an effective anti-tumor immune response [28, 29] and the phenotypes of DCs in tumor (TIDCs) and systemic (bone marrow-derived DCs), given the fact that the maturation stage of these cells is directly linked to the activation process of the cells of the adaptive immune response, CD4+ and CD8+ T lymphocytes [33]. These are meaningful findings in that Fields et al. (1998)[34] and Asavaroengchai et al. (2002)[35], described that mature DCs are able to induce a potent and specific anti-tumor response by T cell stimulation.

We observed in tumor/untrained group a reduction of pro-inflammatory cytokines production and increase of IL10. On the other hand, the enhanced Th1 response was stronger in tumor/trained group, in which was increased Th1-type cytokines production, like IFN gamma and IL12. It has been demonstrated in mice with DMBA-induced breast cancer and subjected to physical activity an enhance of Th1 response pattern [24, 25]. In our molecular approach we observed low level of Tbet gene expression in the tumor/untrained group. However we show in tumor/trained group a significant increase of Tbet gene expression in splenic cells. Furthermore, Nos2 gene expression was higher in mice of tumor /trained group than tumor /untrained group, in which it was observed in this group, that the number of mice expressing Nos2 was similar to the control group. Besides the increase of iNOS and Tbet gene expression, it was observed an increase of iNOS molecule. A study demonstrated in healthy mice that the physical activity lead an enhanced Th1 response and NO production [36]. Several studies showed that peritoneal macrophages in breast cancer model have a reduction of Nos2 gene expression and the production of oxide

nitric. These studies demonstrated physical activity is able to reestablish of Nos2 expression as well as the production of nitric oxide [37-39].

The Gata3 is a negative regulator of Th1-type genes, and it has been proposed the Th2-type inflammation is associated with tumor growth [40,41,10]. The results showed a decrease of Th2 response by reduce Gata3 gene expression and IL-10 production in the splenic cells of tumor/trained mice, in which, that reduction corroborated with Gholamnezhad et al (2014) [42], which showed in healthy rat that moderate exercise might reduce Th2-type inflammation, while the overtraining was able to increase the Th2-type cytokines and reduce IFN gamma.

Our data demonstrated a slight increase of Foxp3 gene expression in mice subjected to physical activity. A study showed in peripheral blood of octogenarian walkers an increase of leukocytosis and differentiation of Treg cells [43]. Nevertheless, the Tbet gene was more expressed than Foxp3 in the tumor/trained group. Recently, a study demonstrated that Tbet is a critical modulator of Foxp3 gene in autoimmunity disease. In this study, the authors showed the *tbx21*^{-/-} mice presented an increase of Treg response, while the expression of Tbet leads a reduction of Treg activity [44].

The exercise improved iNOS and Tbet gene expression and the enhanced of iNOS activity indicating an improved of Th1 response. and Beside, was observed a reduction of Gata3 genes expression in mice subjected to physical activity, in which, lead us to believed the exercise may improve the systemic antitumor immune response in spleen of mice by increase the Th1-response markers.

We demonstrated that physical activity increased the number of cells labeled with CD11c, i.e., differentiated bone marrow-derived DCs. This increase is higher than that observed in the non-trained groups, regardless of tumor presence or absence. The expression of co-stimulatory molecules was also higher in the animals submitted to physical activity. These results are in accordance with those by Chiang et al. (2007)[45] and Liao et al. (2006)[46], who observed in rodents an increase in the number of DCs, together with increased expression of class II MHC, CD80 and CD86 in DCs after training. On the other hand, Ru and Peijie (2009)[47] showed that overtraining could induce immunosuppression whereby the number of DCs and co-stimulatory molecules expression are reduced. Regarding cytokine production by DCs, the present study showed that bone marrow-derived DCs in conditions of physical activity were able

to produce more IFN- γ and IL-12, but less IL-4 and IL-10, while the expression of TGF- β did not change. Chiang et al (2007)[45] reported that the group of animal submitted to physical activity displayed higher cytokine levels than the groups that were not submitted to exercise. In our study, the tumor/trained group exhibited increased IL-12 expression and reduced expression of suppressive cytokines (IL-4 and IL-10). No data are available in the literature to compare these results.

Despite the presence of tumor-infiltrating DC (TIDC), tumor cell outgrowth often occurs, demonstrated by the data of animals with tumour and not trained, indicating that immunity against tumor cells is either improperly induced or bypassed by the tumor. This raises questions regarding the status of TIDC. Studies have shown that tumor cells produce molecules that inhibit DC maturation such as IL-10, vascular endothelial growth factor, PGE2, and TGF- β [48-52]. Therefore, in the majority of solid tumors, more TIDCs are present in well-differentiated and less-invasive tumors compared to less-differentiated and high-invasive tumors, which proves that TIDC density inversely correlates with tumor pathologic grade and stage and positively correlates with favourable prognostic features [52]. In the case of breast tumors, TIDCs drive inflammatory Th2 (Th2) cells and pro-tumor inflammation [15]. In our results, the group submitted to physical activity increase the absolute number of CD11c+, and the phenotype was more mature (higher expression of CD80 and CD86) than the group tumor/non-trained, and also synthesized more IFN- γ , TNF- α and IL-12 than the group tumor/non-trained. Indicating that the physical activity could influence the innate immune response to induce maturation of DCs. Such as the maturation of innate immune cells are fundamental to induce a correct acquired immune response, another aspect investigated in this study was the tumor-infiltrating lymphocytes, once that some studies showed a high number of TIL is usually associated with a better prognosis (e.g., melanoma, colorectal, and ovarian cancer) [53-58]. About breast cancer, some studies report that higher TIL scores were associated with a better prognosis [56-58]. Our results showed that physical activity are not able to alter the numbers of helper T cells inside the tumor, neither cytotoxic lymphocytes, which may kill the tumor cells. But found another interesting data, demonstrating that exercise reduce the expression of transcription factors that polarize the pro-tumor patterns (Th2 and Th17), as evidenced by decreased GATA3 and ROR γ t levels, which are known to have a worse effect on the the anti-tumor response. Some data from literature has been shown that, ascites Th17-derived IL-17 production lead to myeloid cell recruitment in the tumor environment and accelerated tumor growth [59]; higher pretreatment Th17 numbers correlated with faster disease progression [60-61].

Regarding Th2/GATA3, some authors reported that GATA3 is within the networks that govern breast cancer progression. Changes in genomic targets and regulatory activity may control tumor-associated mechanisms [62]. When lymphocytes expression GATA3 adopt a Th2 profile and these cells secrete anti-inflammatory cytokines and can be pro-tumorigenic [63].

Thus, the immune system's defense against tumors is mediated by the function of DCs, macrophages, T cells, and IL-12 and IFN- γ expression contributes to the differentiation of T cells into Th1 cells. Therefore, the initial production of IFN- γ , IL-2, IL-12 and TNF- α is important for the generation of adaptive immunity, as well as for innate defense against tumors. Moreover, the production of IL-4 and IL-10 (Th2 cytokines) and TGF- β (Treg cytokine), promotes a shift of Th2 and Treg patterns, suppressing the antitumor resistance. Our results suggesting that the low production of IL-4 and IL-10, by dendritic cells from trained mice, may contribute to the Th1 immune response against tumors, which was confirmed by increase expression of Tbet in CD4⁺ T cells, and even lower expression of GATA3, ROR γ t and FoxP3.

Our results indicate that regular physical activity may be highly beneficial to cancer patients, as an adjuvant therapy to conventional therapies, such as chemotherapy, radiotherapy and surgery, as well as new therapies as immunotherapies. The physical activity not only reduces the tumor growth but also polarizes the immune response pattern by reduce the Th2 and Th17 profile, since the cytokines of this profile were overexpressed in the trained groups, and the actions of the competent immune cells were potentiated in conditions of physical activity.

Nevertheless, more studies with different types of tumors and more correlations between immune and neuroendocrine factors are needed to verify the extent to which physical activity is beneficial not only in breast cancer.

Taken together, our results allow us to concluded that physical activity could improve the systemic antitumoral immune response by increase the Tbet gene expression, Th1-type cytokines and decrease markers of Th2 and Treg response. Besides, the physical activity improve the antitumoral immune response by alter the maturation of dendritic cells, that are an important cell of innate

response, and responsible by activate correctly the antitumoral acquired immune cells represented by the essential helper lymphocytes.

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Conflict of Interest

There is no conflict of interest to report.

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Figure 1 – Evaluation of tumor growth in experimental groups. Ten animals were used in each group (a) Representation of study design, showing the induction of tumors with 4T1 cell line and training schedule. (b) Representation of tumor volume of the tumor/non-trained group and the tumor/trained group. The values were expressed by mean $\pm$ SEM, Student's t-test was used and * $p < 0.05$ vs GIV. (c) Representation of tumor volume of each mouse in the tumor/non-trained group and the tumor/trained group. The values were expressed by mean $\pm$ SEM, Student's t-test was used GIII vs GIV.

Figure 2: Influence of physical activity in systemic immune response patterns in spleen of mice. (a) RTq-PCR of transcriptional factors in splenic cells of control group, tumor group and tumor/trained group. The statistical test used was 2way ANOVA; * $p < 0.03$, *** $p < 0.0003$. (b) Representation of fold change of Tbet and Gata3 gene expression in splee cells of tumor group and tumor/trained group. The statistical test used was Student T test; * $p < 0.0001$. (c) RT-PCR of splenic cells of control group, trained group, tumor group and tumor/trained group. (d) Representation of fold change of Nos2 gene expression in spleen cells of tumor group and tumor trained group. Red dashed line represent control group. The statistical test used was Student T test * $p < 0.0001$. (e) The supernatant of splenic cells culture obtained of 12 hours, 24 hours and 48 hours were evaluated by griess method. The statistical test used was two way ANOVA, showed with SEM.

Figure 3: Evaluation of influence of PA in T cells phenotype by flow cytometry in spleen cells of mice of the all groups. (a) Percentage of Treg cells (CD4⁺CD25⁺) in splenic cells in mice of groups. (b) Production of IL12 by T cells (CD3+CD4⁺) in spleen in mice of groups. (c) Production of INF-gamma by T cells (CD3+CD4⁺) in spleen in mice of groups. (d) Productions of TNF-alpha by T cell (CD3+CD4⁺). (e) Production of IL10 by T cells (CD3+CD4⁺) in the spleen. The statistical test used was one way ANOVA test and the values were showed by median. $p < 0.05^*$

Figure 4 - Tumor-infiltrating immune cells (A) TILs in dot plot representation for select the gate R1 and confirmed the presence of total lymphocytes and double labeling using Gate R3 (CD3+CD4+ and CD3+CD8+ in groups tumor and tumor/trained); (B) the absolute value of CD3+CD4+ and CD3+CD8+; (C) the percentage of gate (CD3+CD4+ and CD3+CD8+); (D) mean of fluorescence intensity of transcription factors and (E) cytokines expressed by T helper lymphocytes. Note: Tumor (GIII) - tumor/non-trained and Tumor Trained (GIV) - tumor/trained. (D) ANOVA with Bonferroni's posttest vs GIII for GATA3 and RORγt * p<0,05; (E) ANOVA with Post-hoc Bonferroni vs GIII for TNF-α *p<0,05.

Figure 5 - Tumor-infiltrating immune cells (A) TIDCs in dot plot representation for select the gate R2 and confirmed the presence of dendritic cells and double labeling using Gate R3 (CD11b+CD11c+) and also double labeling for CD80+/CD86+ and CD80+/MHC-II in groups tumor and tumor/trained; (B) percentage of gate and absolute value of CD11b+ and CD11c+ (C) percentage of gate double labeling for CD80+ and CD86+, percentage of gate double labeling for CD80+ and MHC class II; (D) mean of fluorescence intensity of cytokines. Note: Tumor (GIII) - tumor/non-trained and Tumor Trained (GIV) - tumor/trained. * p<0,05 and ** p<0,01.

Figure 6 - Tumor-infiltrating immune cells (A) Tumor-Associated Macrophage (TAM) in dot plot representation for select the gate R2; (B) percentage of gate of CD14+ and (C) Mean of Fluorescence

Intensity of double labeling for CD14⁺ and CD86⁺, and double labeling for CD14⁺ and MHC class II;
 Note: Tumor (GIII) - tumor/non-trained and Tumor Trained (GIV) - tumor/trained. * p<0,05.

Figure 7 - Representation by box plot of markings for bone marrow-derived dendritic cells; (a) percentage of gate of CD11b+ and CD11c+ (b) percentage of gate double labeling for CD80+ and CD86+. Note: GI – control group; GII - no tumor/trained; GIII - tumor/non-trained and GIV - tumor/trained. Kruskal-Wallis and Post-hoc Dunns tests were used and the symbols represent the statistical significance: # p<0,05 vs GI and * p<0,05 vs GII and ** p<0,05 vs GIII. Cytokine synthesis by dendritic cells, (c) IFN- γ , (d) IL-12, (e) IL-4 (f) IL-10 and (g) TGF- β . Note: the bars represent the mean $\pm$ SEM; GI – control group; GII – non-tumor/trained group; GIII - tumor/non-trained group and GIV - tumor/trained group. Newman-Keuls test was used and the symbols represent the statistical significance: # p<0.05 vs GI and * p<0.05 vs GII and ** p<0.05 vs GIII.

Table 1: Primer sequences used for the qPCR

Gene	Primers
Tbet FORWARD	5'-TCAACCAGCACCAGACAGAG-3'
Tbet REVERSE	5'-AAACATCCTGTAATGGCTTGTG-3'
Gata3 FORWARD	5'-TTATCAAGCCCAAGCGAAG-3'
Gata3 REVERSE	5'-TGGTGGTGGTCTGACAGTTC-3'
Foxp3 FORWARD	5'-ACTGCTGGCAAATGGAGTCT-3'
Foxp3 REVERSE	5'-AAGTAGGCGAACATGCGAGT-3'
Nos2 FORWARD	5'-CACCTTGGAGTTCACCCAGT-3'
Nos2 REVERSE	5'-ACCACTCGTACTTGGGATGC-3'
Nos3 FORWARD	5'-CATGGAAATGTCAGGCCCG-3'
Nos3 REVERSE	5'-TTCCACAGAGAGGATTGTAGC-3'
β -actin FORWARD	5'-TGTGATGGTGGGAATGGGTCAG-3'
β -actin REVERSE	5'-TTTGATGTCACGCACGATTTC-3'