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Different roles of helper T lymphocytes during dendritic cells vaccine in experimental breast cancer

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Breast cancer is the most common women's tumor. Treatments though effective, are often aggressive and leave the patients vulnerable to developing opportunistic infections. There is a need for less aggressive more effective new therapies. In this study, we have made an attempt to evaluate DCs immunotherapy by flow cytometry, analyzing the presence of infiltrating cells, cytokines and transcription factors in the spleens, lymph nodes and tumors of breast cancer mice undergoing immunotherapy. The 4T1 cell transfection was used to induce breast cancer in Balb/c mice aged 6 to 8 weeks. Mice were treated with 3 doses of DCs vaccine. After the treatment, animals were euthanized, the spleen, lymphnodes and tumors were removed and used to perform flow cytometry. Results have shown higher $CD4^+$ T lymphocytes that produce IL-12 in the spleen of group treated with DCs vaccine (149,8-271,3) 172,4. Further, there was an increase in T-bet (976,1-1075) 1022 in the lymph nodes of tumor group treated with DCs and less FOXP3 (795,7 - 895) 832. Tumoral volume and the FOXP3 were decreased in the treated group. There was an increase in the transcription factor of Th1 profile, and of cytotoxic T lymphocytes, inferring a good immune response. With these observations, it can be concluded that the DC vaccine is effective in combating the development of tumors.

Keywords: Cytokines, Immunotherapy, Transcription factors, Tumor infiltrate lynfocytes

The IL-12 secretion stimulates and activates the immune response by T cells of the Th1 profile. This profile is ideal in an antitumoral response[3]. At low IL-12 level, the balance between Th1/Th2 induces secretion of Th2 profile cytokines[4]. IL-12 acts on T lymphocytes as well in natural killer cells (NK), prompting the production of IFN-γ[5]. Banchereau & Palucka report in the same paper that cytotoxic T lymphocytes (CD8+) stimulated by IL-12 have a higher amount of CD40L, which increases its cytotoxicity.

The Dendritic Cells (DCs) play a fundamental role in the polarization of the T lymphocytes profiles, in which we have Th1, Th2, Th17, and Treg. The DC participation in the activation of these T lymphocytes profiles happens with the activation process of the T lymphocyte, whereby the specific cytokine secretion for each profile the DCs induct this polarization[6].

The polarization of T cells in Th1 cells also stimulates the secretion of IFNs among them IFN-γ, a cytokine, produced mainly by NK and Th1 lymphocytes[7]. Tumor produces immunosuppressor cytokines capable of polarizing immune cells to an absence of antitumoral response or even modulate an active T cell in an immunosuppressor profile[8]. Treg is an immune-suppressor profile characterized by the presence of the surface markers CD25, IL-10R, and the presence of the transcription factor FOXP3[9].

For many years, numerous studies have tried to classify helper T lymphocytes according to cytokine synthesis and secretion. However, one of the difficulties is that the expression of these mediators is carried out by various profiles, such as IL-10[10]. Therefore, one of the most accurate tools for classifying the profile of T lymphocytes is the expression of transcription factors[11]. Thereby: T-bet is essential for Th1 polarization[10], GATA3 for the Th2 polarization[13], FOXP3 for Treg polarization[12], and RORγt is necessary for the Th17 polarization[15].

The reversion of immunosuppression induced by tumor escape mechanisms is essential for an effective treatment and a good prognosis. Therefore, in this study, we intended to verify if the dendritic cell vaccination could alter the main profile of helper

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T lymphocytes both at the systemic level (lymph nodes and spleen) and in the tumor microenvironment using an experimental model of cell line-induced breast tumor 4T1.

Materials and Methods

Experimental models

About 180 mice were used in four independent experiments, all Balb/c (*Mus musculus*) females aged 6-8 weeks, acquired at the biological sciences institute of the Federal University of Minas Gerais - UFMG and kept in plastic cages in the vivarium of the Oncology Research Institute - IPON, from the Federal University of Triângulo Mineiro - UFTM, with controlled dark/light cycle and temperature maintained at 23°C, with food and water. The animals were euthanized with ketamine hydrochloride (50 mg/kg) and xylazine (15 mg/kg). All procedures performed in this study were approved by the Ethics Committee on Animal Use - CEUA of the Federal University of Triangulo Mineiro under registration no. 327.

Experimental groups

The experimental groups were divided as follows: Gr. I, control group, without breast cancer induction and there was no vaccination with DCs; Gr. II, Tumor group, breast cancer was induced by transplantation of 4T1 cells, but not treated with DCs; and Gr. III, tumor group treated with DC vaccine.

DC vaccine

For each dose of DC vaccine, two Balb/c mice were euthanized with an excessive dose of anesthetic ketamine and xylazine to obtain bone marrow cells and differentiate them in DCs to make the DC vaccine. The cells were removed with a 4.5 × 13 mm^3 syringe containing 0,9% saline and placed in a conical tube. Then, they were centrifuged @ 290×g for 10 min at 4°C and added to Iscove's Modified Dulbecco's Medium - Thermo Fisher Scientific® (IMDM) culture medium complete with 0.1 mM vitamins, 2 mM L-glutamine, 100 mg/mL of gentamicin, 1 mM sodium pyruvate and 5% fetal bovine serum (Sigma) bone marrow cells[1].

The cells were then transferred to a culture bottle with a final volume of 7.5 mL of IMDM 32×10^6 of cells. The day after the cells were plated in the bottles, granulocyte colony stimulating factor (GM-CSF, 13 µL; 10 ng/µL) and interleukin 4 (IL-4, 13µL; 10 ng/µL) were added. The cultures were kept in a CO_2 chamber with 5% humidity at 37°C for 5 days[2]. On the 7th day, the cells received tumor necrosis factor (TNF-α, 23 µL; 10 ng/µL), followed by the tumor antigen (10µg/ml), obtained by the lysis of 4T1 cells. On the 9th day, the cells were washed and resuspended in 0.9% saline. During the four weeks in the experimental period, 3 doses of DCs vaccine were applied @ 2×10^6 cells per animal, with one dose per week applied to the tumor group treated with DC vaccine after application of the last dose one week was saved for euthanasia of animals.

Flow cytometry

On the 21st day after tumor induction, the animals were euthanized, and it was removed, the spleen, the lymph node, and the tumor of animals from three groups. Flow cytometry was performed on the spleen, lymph node, and tumor cells, but spleen cells were subjected to osmotic lysis with a BD Bioscience® solution - FACSTM (lysis solution) in a 1:20 ratio. After incubation at room temperature (23°C) for 20 min, the tube was removed and centrifuged for 10 min @290×g at 4°C. Later, for cells of all organs (spleen, lymph node and tumor), the inhibitory protein transporter (BD GOLGI STOP TM) was added in a proportion of 2 µL for each 2 mL of cell solution, then it was incubated for 20 min and washed with 30 mL of PBS. Subsequently, the cell counting was performed by adding 180 µL of Turk's solution and 20 µL of cells ins a separate conical tube and counted in a Neubauer chamber.

About 1.0×10^6 cells were added to each flow cytometry tube. The first staining was performed with surface marker antibodies (BD PharmingenTM®) with a volume of 0.5 µL for each 2.0×10^6 cells. The antibodies used were: CD3, CD4, CD19, CD25, CD11C, CD80, CD86, IA, incubated for 30 min at room temperature. After extracellular staining, the cells were washed, and 100 µL of BD CitofixTM® was added to each tube to permeabilize the cells. The cells were then labeled with intracellular antibodies (BD PharmingenTM®): T-bet, GATA3, RORγt, FOXP3, TNF-α, IL-12 IFN-γ, IL-10 and IL-17. The cells were rewashed. During the incubation of extracellular antibodies, the wash buffer (BD PERM/WASHTM Buffer) was diluted. The wash buffer was diluted 1X, and after incubation, the samples were washed twice with it and centrifuged for 10 min @290×g, 4°C, and. After being washed with the washing solution, approximately 50 µL of BD PERM/WASH was left in each tube, and 0.5 µL of intracellular antibodies added. It was incubated for 30 min at 4°C in the dark and washed twice with BD PERM/WASH washing buffer. The cells were

submitted to the BD FACS Calibur Biosciences cytometer, Franklin Lakes, New Jersey, USA.

Statistical analysis

The data were analyzed and when the distribution was considered normal, a parametric test was performed to compare the groups. When the distribution was not considered normal or Gaussian, nonparametric tests were performed between groups such as the Mann-Whitney test. The results were expressed as medians and values of P <0.05 were considered statistically significant. For data analysis we used the GraphPad Prism 5 software.

Results

Tumoral volume

Regarding the development of the tumor volume, in the first two weeks of the experiment, we noticed that in the tumor group and the tumor group treated with DCs, the tumor volume did not change significantly. However, in the subsequent week, we observed a higher volume in the tumor group compared to the tumor group treated with DCs. In the fourth week of experimental observation, the tumor group had a tumor volume twice as large as the tumor group treated with DCs, as shown in Fig,. 1.

Presence of helper t lymphocytes (CD4$^+$) and cytotoxic t lymphocytes (CD8$^+$) in the spleen, tumor and lymph node

When analyzing the presence of TCD4+ and TCD8+ lymphocytes in spleens, lymph nodes and tumors of the mice in the experimental groups, we observed, through the flow cytometry technique, the presence in a more significant quantity of CD4+ T cells in lymph nodes in the tumor group. In the spleen, we obtained a higher CD4+ MFI in the tumor group treated with DCs when compared to the other groups (P <0.001) Fig, 2. About T CD8+ cells, we noticed that the control group had a higher amount of these cells both in the spleen and in the lymph node of this group when compared to the other groups (P <0.001). We can infer that these cells may be in

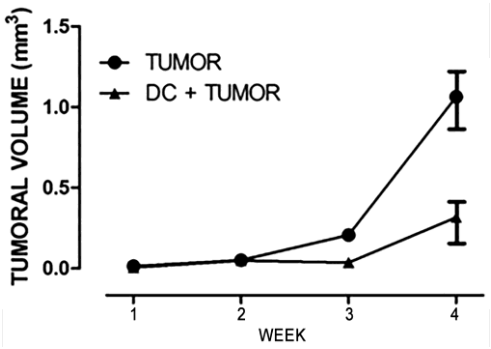

Fig. 1 — Measurement of tumor volume against immunotherapy with DCs. [It represents in mm3 the tumor volume of the experimental groups induced to develop a tumor with 2×10^5 of 4T1 cells over 4 weeks of the experiment. The groups in question are: the tumor group represented by • was induced to develop a tumor, but was not treated with the DCs vaccine. The second group represented by ▲ was induced to develop breast tumor in 4T1 cells and was treated with 3 doses of the DCs vaccine.]

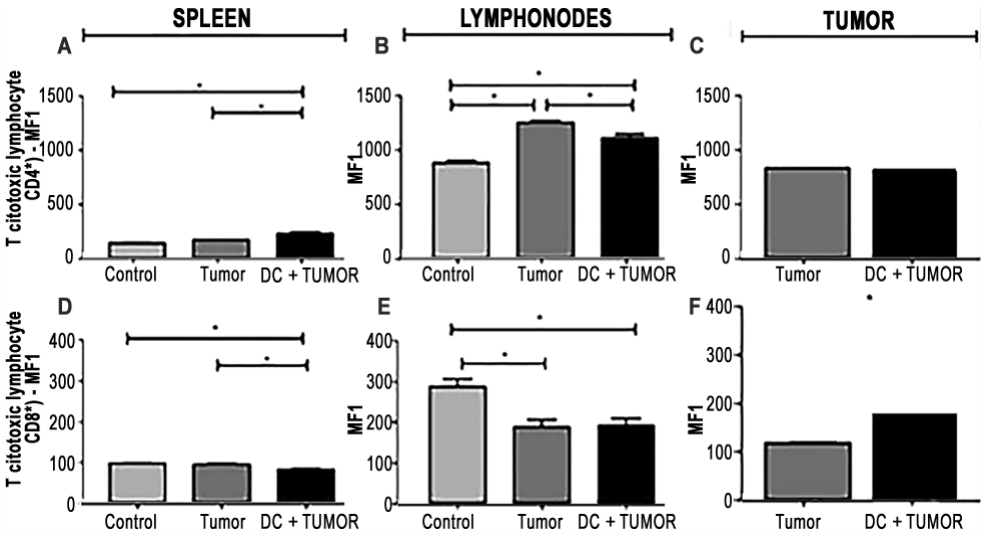

Fig. 2 — Analysis of the presence of T helper (CD4$^+$) and cytotoxic (CD8$^+$) T lymphocytes in the spleen, lymph node and tumors of tumor-induced mice treated with DCs immunotherapy (DCs). Represents the mean fluorescence intensity (MFI) obtained by flow cytometry of the CD4$^+$ and CD8$^+$ clusters of differentiation (CD's) present in total T lymphocytes (CD3$^+$) in the spleen, lymph node and tumor in the mice of the three experimental groups: control group, tumor group and tumor group treated with DCs vaccine. (A-C) MFI of CD4$^+$; and (D-F) MFI of CD8$^+$ in the spleen, lymph nodes and the tumors of the experimental groups, respectively. [*P <0.0001 in the turkey statistical test]

these organs because they don't need to perform an activity possibly. In the tumor, we noticed a higher MFI of CD8+ cells in the tumor group treated with DCs.

Synthesis of cytokines and transcription factors in the spleen, tumor and lymph node of helper t cells (*CD4*$^{+}$)

To verify which profile of activated CD4+ T cells found in these lymphoid organs, we analyzed by flow cytometry the main transcription factors present, and their respective produced cytokines that could characterize a given profile. Regarding the Th1 cell profile, our results showed that there is a higher mean fluorescence intensity of the transcription factor T-bet in the lymph nodes of the tumor group treated with DCs when compared to the other groups and the spleen and tumor Fig, 3.

Regarding cytokines IL-12, IFN-γ and IL-10, we noticed that the tumor group treated with DCs vaccine presented a higher IL-12 MFI in the tumor group treated with DCs vaccine in the lymph nodes (P <0.001) and in the tumor when compared to the other groups, of IFN-γ the tumor group treated with DCs vaccine showed a higher MFI in the spleen (P <0.001) and in the tumor when compared to the other groups, IL-10 had a higher MFI in the spleen tumor group (P <0.001) and lymph nodes (P <0.001) when compared to the tumor group treated with DC vaccine

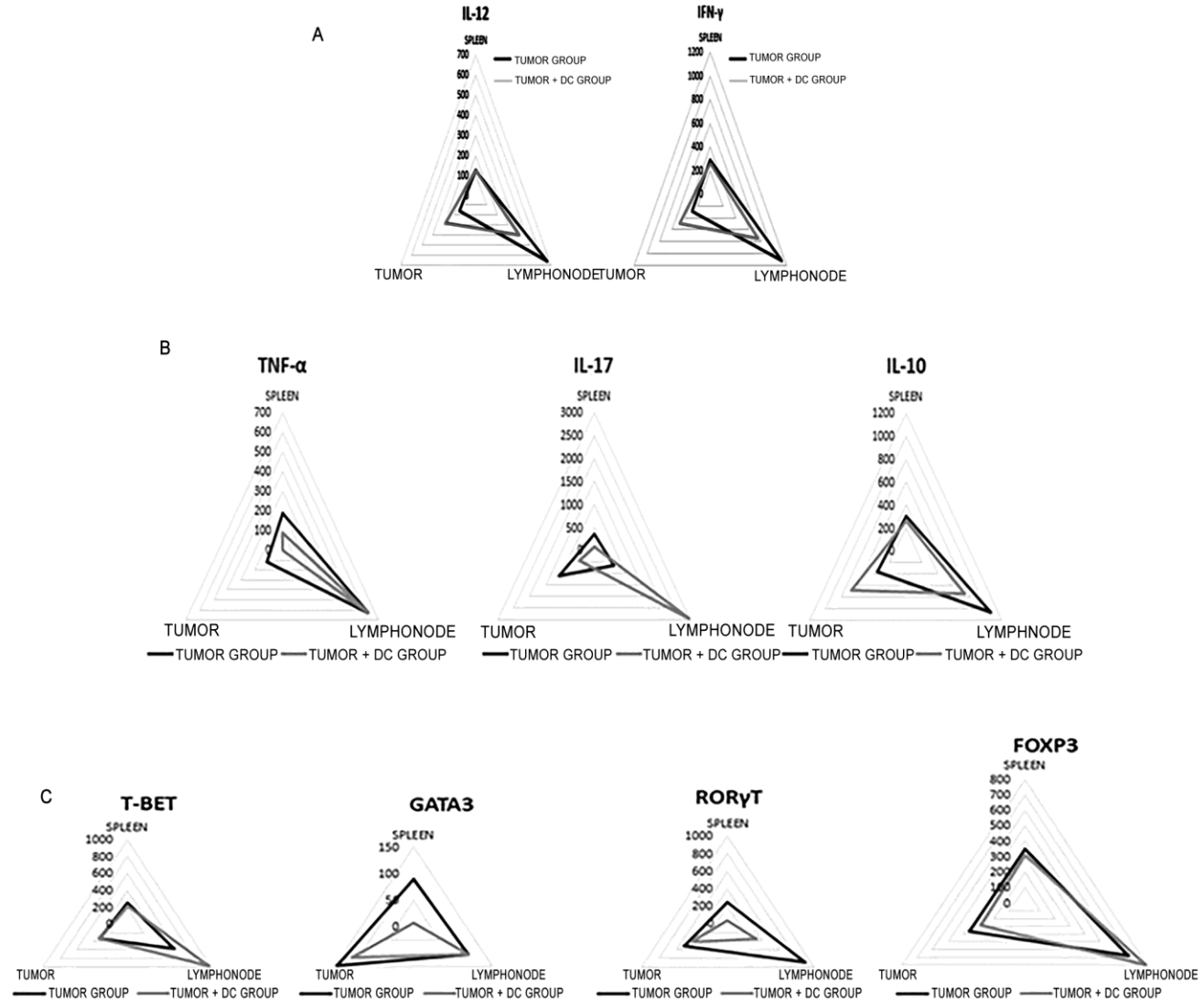

Fig. 3 — Satellite graphs showing the relationship in the distribution of mean fluorescence intensity (MFI) between 3 analyzed organs, spleen, lymph node and tumor (A) IL-12 and IFN-γ labeling; (B) TNF-α, IL-17 and IL-10 labeling; and (C) T-bet, GATA3, RORγt and FOXP3 marking. [The spleen, lymph node and tumor of animals from the tumor group and the tumor group treated with DCs were removed, mechanically grounded, placed in suspension and analyzed by flow cytometry technique]

Fig, 3. IL-12 was found with a higher MFI in the lymph nodes of the tumor group treated with DCs when compared to the tumor group. Concerning the tumor, this cytokine is increased in the treated tumor group DC when compared to the tumor group.

The IFN-γ cytokine, in the group treated with DCs, showed an increase in the MFI in the group that received immunotherapy with DCs observed the spleen. In lymph nodes, the increase of this cytokine in the tumor group was relevant in comparison to the tumor group treated with DCs. However, when we observe the tumor of the animals, the tumor group treated with DCs, we noticed a higher MFI of the studied cytokine, when compared to the tumor group.

Studying the cytokine IL-10, we noticed that the MFI in the spleens and lymph nodes and tumors analyzed was higher in the tumor group when compared to the tumor group treated with DCs Fig, 3. The presence of CD4+ T cells producing IL-17, one of the critical cytokines of the Th17 pattern, was more significant in the analyzed lymph nodes when compared to the spleen and tumor. In the lymph nodes, the highest MFI of the cytokine IL-17 was in charge of the tumor group treated with DCs when compared to the other groups (P <0.001) Fig, 2, whereas in the tumors and spleens analyzed, the highest MFI of IL- 17 17 was in charge of the tumor group Fig, 2.

We found a higher MFI of IL-17 in the lymph nodes of the tumor group treated with DCs immune therapies when compared to the others (P <0.001), and subsequently, we found a higher MFI of TNF-α in tumors in the same group. When spleen and lymph nodes were analyzed, we found a higher MFI of TNF-α in the tumor group when compared to the tumor group treated with DC immunotherapies (P <0.001) Fig, 3. TNF-α, on the other hand, found a higher MFI in the spleens and lymph nodes analyzed in the tumor group when compared to the tumor group treated with DCs. In tumors, we observed that the average MFI was higher in the tumor group treated with DCs immunotherapy when compared to the tumor group.

For Th2 profile, we only performed flow cytometry for its main transcription factor, GATA3, and we noticed that there is a higher MFI of GATA3 in the lymph nodes when compared to the spleen and the tumor. In lymph nodes, the MFI of GATA3 is similar in the tumor group and the tumor group treated with DCs vaccine. In the spleen, there is a higher MFI in the tumor group when compared to the tumor group treated with DCs vaccine (P <0.001) Fig, 4. In lymph nodes, the highest MFI of FOXP3 was in charge of the tumor group treated with DCs immunotherapy when compared to the other groups (P <0.001), whereas in the spleen, the highest average intensity of FOXP3 fluorescence was in charge of the tumor group when compared to the other groups, the opposite result when the tumor was analyzed. RORγt, the main transcription factor of the Th17 cell profile, showed a higher MFI in the lymph node of the tumor group when compared to the other groups (P <0.001) Fig, 4. In tumors and spleens, the transcription factor also had a higher MFI in the tumor group when compared to the tumor treated with the DCs vaccine.

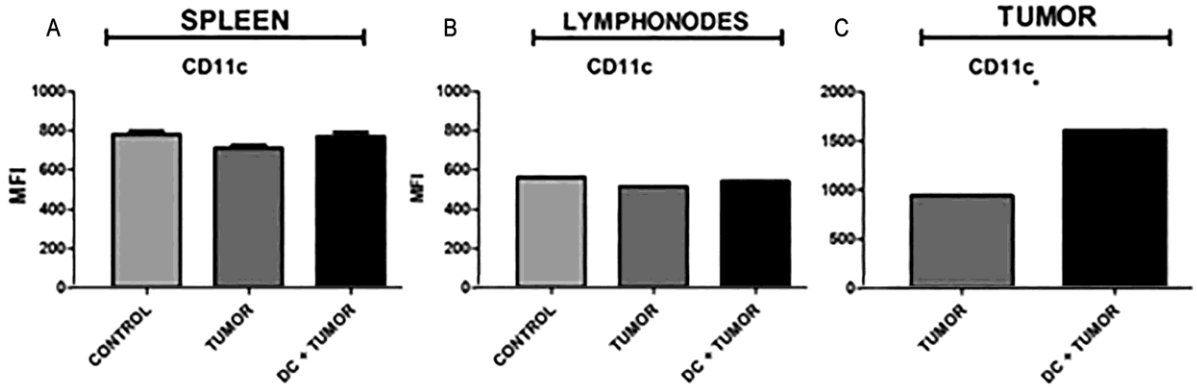

Fig. 4 — Presence of CD11c in the spleen, lymph node and tumors of tumor-induced mice treated with dendritic cells immunotherapy (DCs). It represents the average fluorescence intensity by flow cytometry of the cluster of differentiation (CD11c$^+$) in CD11b$^+$ cells in the spleens, lymph nodes and tumors of the mice in the experimental groups. The control group was not subjected to tumor induction by 4T1 cells or treatment with DCs vaccine, the tumor group was subjected to breast tumor induction with 4T1 cells, but was not treated with DCs vaccine and the tumor group treated with DCs vaccine was subjected to breast tumor induction by 4T1 cells and treated with 3 doses of DCs vaccine. [*statistically significant values (P <0.0001) in the turkey test]

Presence of co-stimulatory molecules in the spleen, tumor and lymph node

Flow cytometry for CD11c was performed to verify whether the DCs exercise their effector function and the location where the highest concentration of functional myeloid DCs is present and which had the highest MFI of co-stimulatory molecules such as CD80, CD86 and the MHCII (IA) Fig, 5.

Analyzing the antigen presentation molecules, our results showed that the presence of IA was more significant in the lymph nodes and tumors analyzed. In the lymph nodes, when the tumor group and treated tumor were compared, the highest MFI of the IA was in charge of the treated tumor group, a result similar to that found in the analyzed tumors Fig, 6. Of the co-stimulatory molecules analyzed, CD80 had its most significant expression in the spleens, and lymph nodes analyzed, in the spleens, the highest MFI of CD80 was in charge of the treated tumor group when compared to the untreated tumor group, a result similar to that found in lymph nodes and tumors

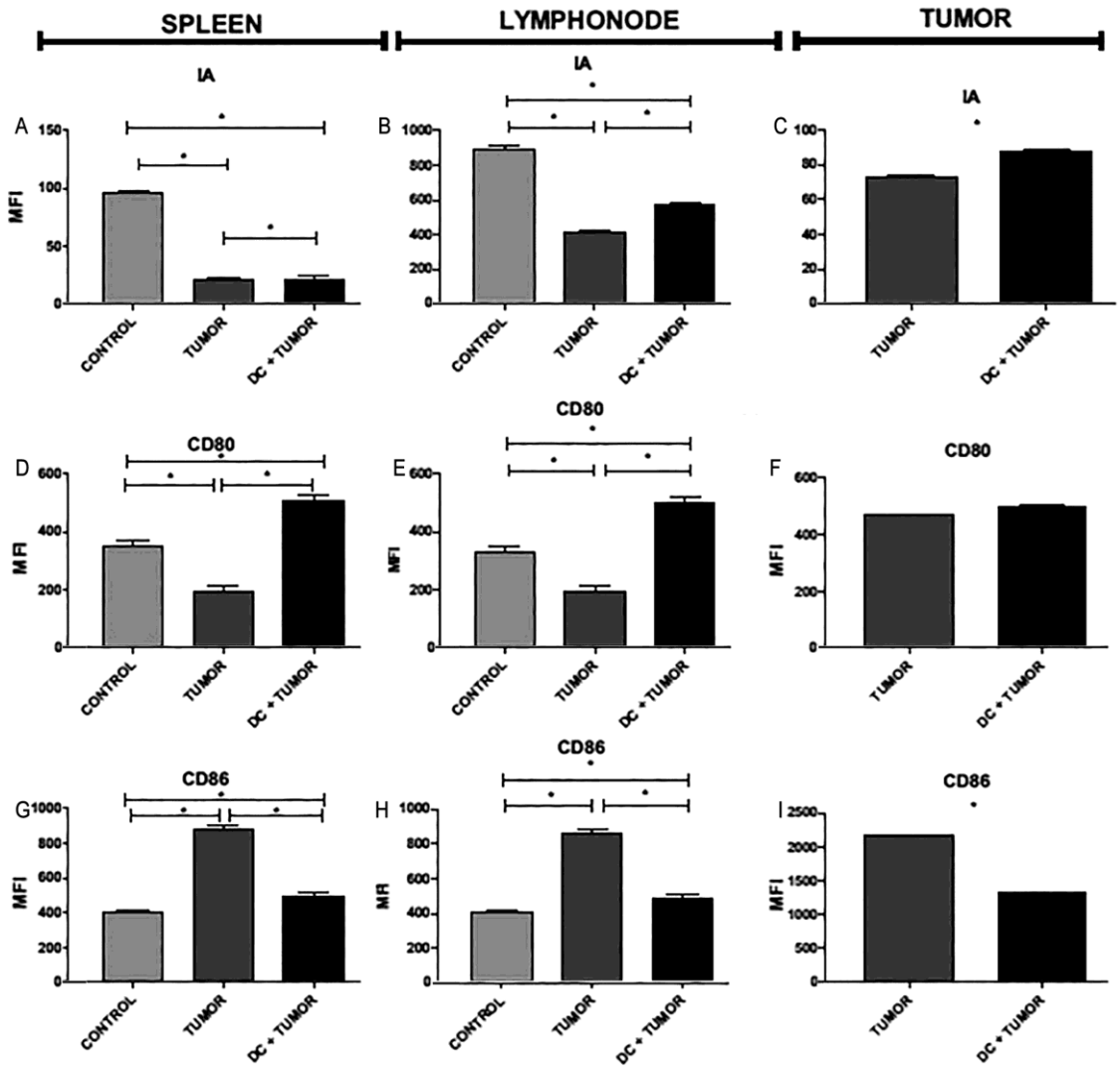

Fig. 5 — Presence of co-stimulatory molecules and MHCII in CD11c$^+$ cells in the spleen, lymph node and tumors of tumor-induced mice treated with dendritic cells immunotherapy (DCs). It represents the average fluorescence intensity by flow cytometry of co-stimulatory molecules (CD80 and CD86) and MHCII (IA) of CD11b$^+$ and CD11c$^+$ cells in the spleens, lymph nodes and tumors of the mice in the experimental groups. The control group was not subjected to tumor induction by 4T1 cells or treatment with a DCs vaccine, the tumor group was subjected to breast tumor induction with 4T1 cells, but was not treated with DCs vaccine and the tumor group treated with DCs vaccine was subjected to breast tumor induction by 4T1 cells and treated with 3 doses of DCs vaccine. (A-C) IA MFI; and (D-F) MFI of CD80; and (G-I) MFI of CD86 in the spleens, lymph nodes and the tumors of the experimental groups, respectively. [*statistically significant values (P <0.0001) in the turkey test]

analyzed. The co-stimulatory molecule CD86 had its highest MFI in the analyzed tumors, where the tumor group had a more significant presence when compared to the treated tumor group, similar results were found in the analyzed spleens and lymph nodes.

On transcription factors, our data demonstrated that the highest MFI was found in the analyzed lymph nodes. Comparing the tumor and tumor groups treated with DCs vaccine in the lymph nodes, the transcription factor that had the highest MFI was RORγT in the tumor group followed by FOXP3, T-bet, and GATA3. In the tumor group treated with DCs vaccine, the largest MFI was in charge of the T-bet, FOXP3 RORγT, and GATA3, respectively Fig, 7. In the spleens analyzed, the largest MFI in the tumor group was in charge of FOXP3, RORγT, T-BET, and GATA3, respectively. In the tumor group treated with DCs vaccine, the largest MFI was in charge of FOXP3, T-BET, RORγT, and GATA3, respectively. In the analyzed tumors, we observed the same pattern both in the tumor group and in the tumor group treated with DCs vaccine, where the largest MFI was in charge of RORγT, FOXP3, T-BET, and GATA3 respectively Fig, 7.

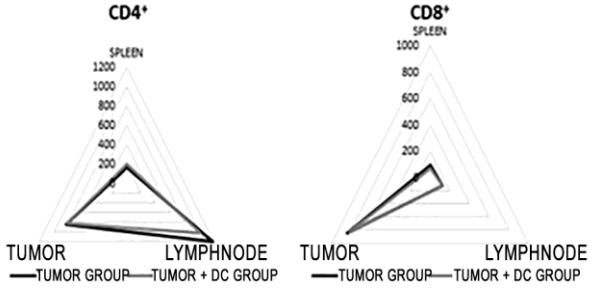

Fig. 6 — Satellite graph showing the relationship in the distribution of the mean fluorescence intensity (MFI) between 3 organs analyzed, spleen, lymph node and tumor. CD4$^+$ and CD8+ marking. The spleen, lymph node and tumor of animals from the tumor group and the tumor group treated with DCs were removed, mechanically grounded, placed in suspension and analyzed by flow cytometry technique.

Discussion

The effectiveness of the various immunotherapies in combating tumor development is known. Several works in the literature demonstrate its effectiveness. Coca *et al*.[16] have reported the development of colorectal tumors with dimethylhydrazine in Wistar rats induced and treated with intraperitoneal injections of IL-12 and this cytokine in combination with IL-2. It was found that combined treatment was more effective when compared to IL-12 injections only. It has suggested that the efficacy of immunotherapies is related to the development of tumors, but to understand how immunotherapy with DCs influences this decrease in tumor volume, other experiments were carried out. It is known that CD4+ T cells after maturation migrate to lymphoid organs to end the process and exercise its effector function[17]. We can relate this function to the more significant presence of CD4 + T cells in the lymph nodes both in the tumor group and in the tumor group treated with DCs immunotherapy since we know that the first place where the dendritic cells migrate to exercise their effector function and end their maturation process is the lymph nodes[6]. After the activation of CD4+ T cells, its effector function is the production of cytokines to help in the combat or tumor development[18].

In our study, the presence of CD4+ T cells in the tumors had no difference when compared to the tumor group to the tumor group treated with DCs immunotherapy. However, the profile of cytokine production and transcription factors showed a difference. It is known that lymphoid organs have the function of ending the maturation process of T cells and that in the same CD4 T cells, they perform their

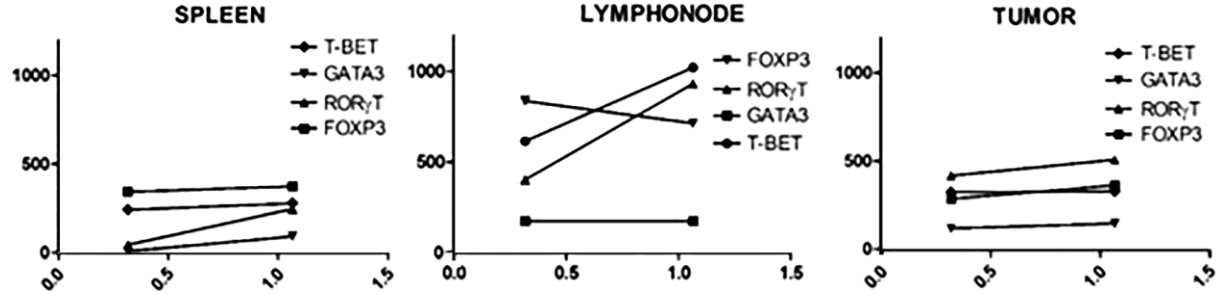

Fig. 7 — Representative line graph showing the transcription factors with the highest mean fluorescence intensity (MFI) in the spleens, lymph nodes and tumors analyzed from the tumor and tumor groups treated with DCs vaccine. Spleen, lymph node and tumor of animals from the tumor group and the tumor group treated with DCs were removed, mechanically grounded, placed in suspension and analyzed by flow cytometry technique.

effector function[18]. Our results showed that among the three organs analyzed, the most significant amount of CD4 T lymphocytes was in charge of the lymph nodes both in the tumor group and in the treated tumor group, confirming that in the lymph nodes, as a lymphoid organ, the activation, maturation and effector function of the lymphocytes occurs. The density of lymphoid organs is associated with a more significant presence of lymphocytes of the Th1 profile[17], our results showed that there is an attempt by the immune system to fight the tumor since there is a higher production of cytokines than profile Th1 as IL-12 and IFN-γ in the lymph nodes of the tumor group and tumor treated with DCs.

However, some indications circulating T lymphocytes with a Treg profile induced by the tumor environment can induce a change in the class of cells that are not in the lymphoid organs[20], our results indicate the significant presence of lymphocytes with a regulatory profile. By the significant presence of IL-10 and FOXP3 in the lymphoid organs, both in the spleen and in the lymph nodes, as well as the high presence of the transcription factor of the Th1 profile, T-bet in the lymph nodes, showing that there is a balance between the attempt of the immune response to tumor and the influence of the tumor environment.

It is known that CD8+ T cells are responsible for destroying tumor cells releasing their granular content, and it is also known that the density of these cells present in the tumor in immunotherapy is related to a better prognosis[21]. Our results showed a lower mean fluorescence intensity of CD8+ T cells in the spleen and lymph nodes in all groups when compared to the tumor. In the tumor, the tumor group treated with DCs immunotherapy showed a higher mean intensity of CD8+ T cell fluorescence when compared to the tumor group. As previously mentioned, several studies demonstrate that a more significant infiltration of CD8+ T cells is associated with a better prognosis[9,22] a result that goes in agreement with results obtained when analyzing the tumor volume. The presence of a CD8 T lymphocyte tumor infiltrate gives the patient a good prognosis concerning cancer treatment, since the lymphocytes have the function of destroying cells that present the antigens to which they were presented in the lymphoid organs[24]. Our results demonstrate that there is a large amount of tumor infiltrate both in the tumor group and in the treated tumor group.

What should be analyzed is under what influence these CD8 T lymphocytes are activated, there is evidence that there are cells in the CD8 T lymphocyte tumor infiltrate that also express FOXP3 and have the ability to suppress the function of other lymphocytes such as IFN-γ production by CD4 T lymphocytes[9]. Our results demonstrate that among the 3 organs analyzed, the highest MFI of T-bet is in the lymph nodes, and when compared to the transcription factors of the CD4 T lymphocyte profiles, the largest MFI in the lymph nodes is T-bet followed by FOXP3 in the treated tumor group. With DCs and then in the tumor group without treatment.

The coexpression of T-bet and FOXP3 is related to the development of tumor volume. This cell initially expresses T-bet can start to express the only FOXP3 at a specific moment of the immune response to the tumor, or even express the two transcription factors when the cells express the two transcription factors the tendency is to develop an immunosuppressive profile[12]. The prognosis for patients who express FOXP3 in tumor infiltrates in several is not favorable[25].

Naive CD4 T cells can present after activating one of the four known T cell subtypes: Th1, Th2, Th17 or Treg. It is known that the Th1 profile is present in responses to intracellular pathogens, and its main transcription factor is the T-bet and among its signature cytokines IFN-γ and IL-12[4]. The profile of Th2 cells is present in immune responses against parasites and allergens, and this profile has GATA3 as its main transcription factor and as IL-4 signature cytokines[4]. Th17 profile cells are present in inflammatory responses, and their main transcription factor is RORγt and the central signature cytokines IL-17, TNF-α and IL-23. Finally, the immunosuppressive cell profile, also called Treg, has FOXP3 as its main transcription factor and TGF-β and IL-10 as the central signature cytokines[26]. Our results also demonstrate that there are large amounts in the lymph nodes of the tumor group treated with DCs of the transcription factors of the profiles Th1 and Th17, T-bet and RORγt, respectively, as well as their signature cytokines IL-12, IFN-γ, IL-17 and TNF antitumor immune response with the Th17 profile is beneficial for the patient, but if the Th17 response extends the Th17 profile can further accelerate tumor development as well as all adverse developments induced by the disease, such for example, cachexia induced by high TNF-α[27–29].

Similar results are described in the literature, in a study published in 2016, a higher level of gene expression of GATA3 was found when compared to T-bet in low and medium grade bladder cancer[30]. FOXP3, the main transcription factor of regulatory CD4+ T cells, has a higher mean intensity of fluorescence in the lymph nodes when compared to the spleen and tumor[31].

The presence of CD4+ T cells producing IL-17, one of the critical cytokines of the Th17 pattern, was more significant in the analyzed lymph nodes when compared to the spleen and tumor. In the lymph nodes, the highest mean intensity of IL-17 fluorescence was in charge of the tumor group treated with DCs immunotherapy, whereas in the tumors and spleens analyzed, the highest mean intensity of IL-17 fluorescence was in charge of the tumor group. There is a correlation with the profile of Th17 cells and their cytokines with the production of TNF-α, as demonstrated by Lin *et al*.[32], where the treatment of patients with an antibody against Th17 cells (adalimumab) reduced the presence of TNF-α in the serum of these patients.

Myeloid DCs are the primary antigen-presenting cells, and among its main markers, we can mention the integrin CD11c[31], among other co-stimulatory molecules. During activation of the specific immune response to combat a particular antigen, activation and signaling of three signals must occur. The first being the link between MHC to the T cell receptor (TCR), the second the interaction of co-stimulatory molecules such as CD80 (B7.1), CD86 (B7.2) of the CD with the CD28 or CD152 (CD152) of the lymphocytes and finally, the production of cytokines that enhance or inhibit the immune response[35]. In the absence of the co-stimulatory molecules CD80 and CD86, although there is the first signal from the MHC binding to the T cell receptor, there is no progression in the activation of the immune system[33]. Our results showed a higher MFI of the co-stimulatory molecule CD80 in the spleens, lymph nodes, and tumors of the tumor group treated with DCs and CD86 in the spleens, lymph nodes and tumors of the tumor group. This inversion in the CD80 and CD86 values can be explained by the works published earlier where it was verified that in DCs at rest the presence of CD80[37] is slightly higher when compared to the presence of CD86[35]. CD80 and CD86 are known to correlate with better prognosis in some types of cancer[36]. The importance of CD80 and CD86 in the induction of essential cytokines for the immune response, such as IFN-γ, is also noteworthy[39].

For both the CD11c+ cell population, cytokine production was verified of these cells present in the spleens, lymph nodes and tumors. Regarding the cytokines that work together with the profile of Th1, IL-12, and IFN-γ cells, we noticed that both in the spleen and in the lymph nodes, there is a higher average intensity of fluorescence in the tumor group treated with DCs immunotherapy when compared to the tumor group.

In the analyzed tumors, we noticed that the average fluorescence intensity of IL-12 is higher in the tumor group compared to the treated tumor group, the average fluorescence intensity of IFN-γ is slightly higher in the tumor group treated with dendritic cell immunotherapies when compared to the tumor group. As cited by Galina et al. in a review published in 2013, the cytokines produced in the tumor microenvironment can polarize the DCs to a protumor profile as well[33].

We note that there is a balance between the profile of pro-tumor cells and those that aim to combat tumor development. Even so, we realized that DCs immunotherapy effectively activates the immune system, but the tumor environment unbalances this response, inducing the transformation of different activated cells into cells of an immunosuppressive profile. It is necessary to understand the dynamics of the immune response to tumors and to verify when this change occurs so that there is an effective intervention.

Conclusion

The observations from the above study suggest that the dendritic cell vaccine is effective in combating tumor development because the treated group has less tumor volume. It is indicated by the increase in infiltration of cytotoxic T lymphocytes in the tumors of the treated group. We can also infer that in the treated tumor group there is a greater activation of lymphocytes of the Th1 profile in the lymph nodes, which are essential in combating tumor development by increasing the signature cytokine of this profile, IL-12. Another factor by which we can affirm that immunotherapy of dendritic cells is effective in combating tumor development is the smaller tumor volume present in the treated tumor group when compared to the untreated tumor group. With regard

to combating tumor development, the role of lymphocytes of the Th17 profile should be further investigated, as well as of the cytokines of this profile.

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Conflicts of interest

Authors declare no competing interests.

References

1 Michelin MA, Abdalla DR, Aleixo AAR, Murta EFC. Peripheral Helper Lymphocytes Produce Interleukin 12 in Cancer Patients. *Clinical Medicine Insights: Oncology*. January 2013. doi:10.4137/CMO.S11292.

2 Abdalla DR, Gomes BBM, Murta EFC & Michelin MA, Bone marrow-derived dendritic cells under influence of experimental breast cancer and physical activity. *Oncol Lett*, 13 (2017) 1406. doi:10.3892/ol.2017.5589.

3 Tugues S, Burkhard SH, Ohs I, Vrohlings M, Nussbaum K, Vom Berg J, Kulig P & Becher B, New insights into IL-12-mediated tumor suppression. *Cell Death Differ*, 22 (2015) 237.

4 Hirahara K & Nakayama T, CD4+ T-cell subsets in inflammatory diseases: Beyond the Th1/Th2 paradigm. Int Immunol, 28 (2016) 163.

5 Mirlekar B & Gupta YP, IL-12 family cytokines in cancer and immunotherapy. *Cancers* (Basel),13 (2021) 167.

6 Luckheeram RV, Zhou R, Verma AD & Xia B, CD4 +T cells: Differentiation and functions. Clin Dev Immunol, 2012 (2012) 925135. doi: 10.1155/2012/925135.

7 Cosmi L, Maggi L, Santarlasci V, Liotta F & Annunziato F, T helper cells plasticity in inflammation. *Cytometry A*, 85 (2014) 36. doi: 10.1002/cyto.a.22348.

8 Lisiecka U & Kostro K, Mechanisms of tumour escape from immune surveillance. *J Vet Res*, 60 (2016) 453. DOI: 10.1515/jvetres-2016-0068.

9 Ward-Hartstonge KA & Kemp RA, Regulatory T-cell heterogeneity and the cancer immune response. *Clin Transl Immunol* 6 (2017) e154. doi.org/10.1038/cti.2017.43.

10 García-martínez E, Gil GL, Benito AC, González-billalabeitia E, Angeles M, Conesa V, García TG, Garre EG, Vicente V & Peña FA, Tumor Infiltrating immune cell profiles & changes after neoadjuvant chemo predict response. *Breast Cancer Res*, 16 (2014) 488. doi: 10.1186/s13058-014-0488-5.

11 Yamane H & Paul WE, Memory CD4+ T Cells: Fate Determination, Positive Feedback and Plasticity. *Cell Mol Life Sci*, 69 (2013) 1577. doi: 10.1007/s00018-012-0966-9.

12 Kachler K, Holzinger C, Trufa DI, Sirbu H & Finotto S, The role of Foxp3 and Tbet co- expressing Treg cells in lung carcinoma. *Oncoimmunology*, 8 (2018) e1456612. doi.org/10.1080/2162402X.2018.1456612.

13 Gimferrer I, Hu T, Simmons A, Wang C, Souabni A, Busslinger M, Bender TP, Hoyos GH & Ila JA, Regulation of GATA-3 expression during CD4 lineage differentiation. *J Immunol*, 186 (2011) 3892. doi: 10.4049/jimmunol.1003505.

14 Linehan DC, Goedegebuure PS. CD25 + CD4 + Regulatory T-Cells in Cancer. *Immunol Res*, 32 (2005) 155.

15 Wu L, Luo LH, Zhang YX, Li Q, Xu B, Zhou GX, Luan HB & Liu YS, Alteration of Th17 and Treg cells in patients with unexplained recurrent spontaneous abortion before and after lymphocyte immunization therapy. *Reprod Biol Endocrinol*, 12 (2014) 74. doi: 10.1186/1477-7827-12-74.

16 Coca S, Enrech S, Moreno García V, Sáez MA, Gutiérrez C, Colmenarejo A, Hernández JM & Piqueras JP, Estudio de la respuesta antitumoral de la interleucina-12 en cáncer de colon inducido mediante 1,2-dimetilhidracina (DMH). *Rev Esp Enferm Dig*, 97 (2005) 619.

17 Castell SD, Harman MF, Morón G, Maletto BA & Pistoresi-Palencia MC, Neutrophils which migrate to lymph nodes modulate CD4+ T cell response by a PD-L1 dependent mechanism. *Front Immunol*, 10 (2019) 105. doi: 10.3389/fimmu.2019.00105.

18 Tay RE, Richardson EK & Toh HC, Revisiting the role of CD4+ T cells in cancer immunotherapy—new insights into old paradigms. *Cancer Gene Ther*, 28 (2021) 5. http://doi.org/10.1038/s41417-020-0183-x.

19 Hiraoka N, Ino Y & Yamazaki-Itoh R, Tertiary lymphoid organs in cancer tissues. *Front Immunol*, 7 (2016) 244. doi: 10.3389/fimmu.2016.00244.

20 Chemin K, Gerstner C & Malmström V, Effector functions of CD4+ T cells at the site of local autoimmune inflammation-lessons from rheumatoid arthritis. *Front Immunol*, 10 (2019) 353. doi: 10.3389/fimmu.2019.00353.

21 Durgeau A, Virk Y, Corgnac S, Mami-Chouaib F. Recent Advances in Targeting CD8 T-Cell Immunity for More Effective Cancer Immunotherapy. *Front Immunol*, 9 (2018) 14. doi:10.3389/fimmu.2018.00014.

22 Bruno A, Pagani A, Pulze L, Albini A, Dallaglio K, Noonan DM & Mortara L, Orchestration of angiogenesis by immune cells. *Front Oncol*, 4 (2014) 131.

23 Redondo-Muñoz J, García-Pardo A & Teixidó J, Molecular players in hematologic tumor cell trafficking. *Front Immunol*, 10 (2019) 156. doi: 10.3389/fimmu.2019.00156.

24 van der Leun AM, Thommen DS, Schumacher TN. $CD8^+$ T cell states in human cancer: insights from single-cell analysis. *Nat Rev Cancer*, 20 (2020) 218. doi:10.1038/s 41568-019-0235-4.

25 Shang, B, Liu, Y, Jiang Sj & Liu Y, Prognostic value of tumor-infiltrating $FoxP3^+$ regulatory T cells in cancers: a systematic review and meta-analysis. *Sci Rep* 5, 15179 (2015). https://doi.org/10.1038/srep15179

26 Alves JJP, Fernandes TAA de M, De Araújo JMG, Cobucci RNO, Lanza DCF, Bezerra FL, Andrade VS & Fernandes JV, Th17 response in patients with cervical cancer. *Oncol Lett*, 16 (2018) 6215.

27 Carvalho DFG, Zanetti BR, Miranda L, Hassumi-Fukasawa MK, Miranda-Camargo F, Crispim JCO & Soares EG, High IL-17 expression is associated with an unfavorable prognosis in thyroid cancer. *Oncol Lett*, 13 (2017) 1925.

28 Lee MH, Chang JTC, Liao CT, Chen YS, Kuo ML & Shen CR, Interleukin 17 and peripheral IL-17-Expressing T cells are negatively correlated with the overall survival of head and neck cancer patients. *Oncotarget*, 9 (2018) 9825.

29 Bahria-Sediki I Ben, Yousfi N, Paul C, Chebil M, Cherif M, Zermani R, Gaaied ABAE & Bettaieb A, Clinical significance of T-bet, GATA-3, and Bcl-6 transcription factor expression in bladder carcinoma. *J Transl Med*, 14 (2016) 144.

30 Sato T, Terai M, Tamura Y, Alexeev V, Mastrangelo MJ & Selvan SR, Interleukin 10 in the tumor microenvironment: A target for anticancer immunotherapy. *Immunol Res*, 51 (2011) 170.

31 Lin YC, Lin YC, Wu CC, Huang MY, Tsai WC, Hung CH & Kuo PL, The immunomodulatory effects of TNF-α inhibitors on human Th17 cells via RORγt histone acetylation. *Oncotarget*, 8 (2017) 7559.

32 Ma Y, Shurin GV, Peiyuan Z, Shurin MR. Dendritic Cells in the Cancer Microenvironment. *J Cancer*, 4 (2013) 36. doi:10.7150/jca.5046.

33 Collin M & Bigley V, Human dendritic cell subsets: an update. *Immunology*, 154 (2018) 3.

34 Yang X, Lv T, Qiang J, Li X, Zheng C, Ding J, Lim Z, Pang L, Du W, Zhu P, Ba Y, Zheng Y & Wu Z, Expression and significance of CD28, CTLA-4, CD80 AND CD86 in gastric cancer. *Biomed Res*, 28 (2017) 10159.

35 Ma Y, Shurin GV, Peiyuan Z & Shurin MR, Dendritic cells in the cancer microenvironment. *J Cancer*, 4 (2013) 36.

36 Mir MA & Agrewala JN, Signaling through CD80: An approach for treating lymphomas. *Expert Opin Ther Targets*, 12 (2008) 969.

37 McLellan AD, Starling GC, Williams LA, Hock BD & Hart DNJ, Activation of human peripheral blood dendritic cells induces the CD86 co- stimulatory molecule. *Eur J Immunol*, 25 (1995) 2064.

38 Chang CS, Chang JH, Hsu NC, Lin HY & Chung CY, Expression of CD80 and CD86 costimulatory molecules are potential markers for better survival in nasopharyngeal carcinoma. *BMC Cancer*, 7 (2007) 88.

39 Bak SP, Barnkob MS, Bai A, Higham EM, Wittrup KD & Chen J, Differential Requirement for CD70 and CD80/CD86 in Dendritic Cell-Mediated Activation of Tumor-Tolerized CD8 T Cells. *J Immunol*,189 (2012) 1708.