Dynamic analysis of the immunological response of Balb/c mice with experimental breast cancer submitted to immunotherapy treatment of dendritic cell

Análise dinâmica da resposta imunológica de camundongos Balb/c com câncer de mama experimental submetido a imunoterapia de células dendríticas

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Marcia Antoniazi Michelin
Dra. Oncology Research Institute, Federal University of Triangulo Mineiro
Avenida Guilherme Ferreira n°1940, CEP 38022-200, São Benedito, Uberaba, Minas Gerais, Brazil
E-mail: marcia.michelin@uftm.edu.br

Eddie Fernando Cândido Murta
Dr. Oncology Research Institute, Federal University of Triangulo Mineiro
Avenida Guilherme Ferreira n°1940, CEP 38022-200, São Benedito, Uberaba, Minas Gerais, Brazil
E-mail: eddiemurta@mednet.com.br

Saulo Fernando Moreira da Silva
MSc. Oncology Research Institute, Federal University of Triangulo Mineiro
Avenida Guilherme Ferreira n°1940, CEP 38022-200, São Benedito, Uberaba, Minas Gerais, Brazil
E-mail: saulo.fernando@yahoo.com.br

ABSTRACT

INTRODUCTION: Cancer is a complex disease because it is capable of inhibiting the immune response through tumor escape mechanisms. OBJECTIVE: analyze the dynamics of the immune system during cancer treatment with dendritic cell immunotherapy. We evaluated the presence of tumor infiltrate of CD8 T lymphocytes, transcription factors of CD4 T lymphocytes in the spleen of these animals, the development of tumor volume and the behavior of coestimulatory molecules. METHODS: 70 female Balb/c mice were divided into experimental and control groups, they were evaluated on the 7th e 14th days after tumor challenge and dendritic cell immunotherapy. RESULTS: Molecules such as T-bet, showed an increased expression in treated tumor group. Our results also demonstrated the higher MFI of CD8 + T lymphocytes infiltrate in the treated groups. Thus, there is a greater MFI of protumoral co-stimulatory molecules such as CTLA4 in the untreated groups. CONCLUSION: the immune system is able to modulate an immune response against tumor within 14 days if the organism is being treated with dendritic cell immunotherapy.

Keywords: dendritic cell immunotherapy, breast cancer, active immunotherapy.

RESUMO
INTRODUÇÃO: O câncer é uma doença complexa, pois é capaz de inibir a resposta imune por meio de mecanismos de escape tumoral. OBJETIVO: analisar a dinâmica do sistema imunológico durante o tratamento do câncer com imunoterapia de células dendríticas. Avaliamos a presença de infiltrado tumoral de linfócitos T CD8, fatores de transcrição de linfócitos T CD4 no baço desses animais, o desenvolvimento do volume tumoral e o comportamento de moléculas coestimulatórias. MÉTODOS: 70 camundongos Balb/c fêmeas foram divididos em grupos experimental e controle, avaliados no 7º e 14º dias após o desafio tumoral e imunoterapia com células dendríticas. RESULTADOS: Moléculas como a T-bet, mostraram uma expressão aumentada no grupo de tumor tratado. Nossos resultados também demonstraram a maior IMF de infiltrado de linfócitos T CD8 + nos grupos tratados. Assim, há uma maior MFI de moléculas coestimulatórias protumorais, como CTLA4, nos grupos não tratados. CONCLUSÃO: o sistema imune é capaz de modular uma resposta imune contra o tumor em 14 dias se o organismo estiver sendo tratado com imunoterapia com células dendríticas.

Palavras-chave: imunoterapia com células dendríticas, câncer de mama, imunoterapia ativa.

1 INTRODUCTION

Immunotherapies are therapies that have the function of activating the immune system against an infection, and in a case of cancer, immunotherapy aims to activate the immune system previously inhibited by tumor escape mechanisms [1]. Immunotherapies are being highlighted in research in recent years precisely because they are as effective as conventional therapies and have minimal side effects when compared to conventional therapies [1–3] . Among a plenty of immunotherapies, there are passive immunotherapies such as the immune checkpoint blockade [4] and active immunotherapies such as the use of adoptive T cell therapy [5], dendritic cell vaccination [6], and others.

The immune surveillance theory postulates that the body's own cells verify the body looking for infections that can be combated [7]. Once the tumor formation is an inflammatory process, the concept of Immunoediting divides immune response to tumors into three phases: recognition, where the cells of the immune system recognize tumor development and destroy tumor cells [7]. The equilibrium phase, where a group of cells with potential to become cancer are in anergy state, so they do not induce immune response and do not present cancer characteristics [8,9]. Finally the escape phase, where the anergic state cells begin an unregulated division, thus presenting high tumorigenic potential, also called tumor escape mechanisms. These mechanisms are different ways that the tumor can circumvent the immune response in order to keep growing [7–9].

In an ideal immune response against cancer, tumor antigens are processed in small peptides into antigen presenting cells and presented via MHC molecule to T lymphocytes [10]. T lymphocytes are divided in two classes, CD8+ T lymphocytes and CD4+ T lymphocytes. CD8+ T lymphocytes, also known as cytotoxic T lymphocytes, have the function of first recognizing the antigen presented via MHCI and then destroying the cell that presented that antigen [11]. CD4+ T lymphocytes recognize the antigen presented via MHCII and, when activated, the cell modulates the immune response by producing cytokines that will lead the immune response to fight against the presented antigen [12].

Cancer is seen as a complex disease because it inhibits the immune response through tumor escape mechanisms. Among these mechanisms, it is possible to cite the tumor ability of the producing soluble mediators that contribute tumor development such as immunosuppressive cytokines, metalloproteinases, and vascular growth factors [13]. Another tumor escape mechanism is the suppression of the immune response through the suppression of the MHCI, thus preventing tumor cells from being destroyed by CD8+ T lymphocytes [14]. Dendritic cells are used in active immunotherapies because they are also antigen-presenting cells [15,16]. They are able to process and present antigens both via MHCI and MHCII without being destroyed by CD8 + T lymphocytes, which is a mechanism called cross-presentation [17].

It is extremely important to understand the mechanisms of action of dendritic cells in immunotherapies and the mechanisms triggered by it, so researchers will be able to develop new and better protocols for differentiation and maturation of dendritic cells *in vitro* in order to improve dendritic cell immunotherapy quality [18].

2 OBJECTIVE

Our aim is to evaluate the immune response against breast cancer in mice submitted to dendritic cells immunotherapy and also understand how long it takes to a modulation of the immune response towards tumor control and reduction of tumor volume by performing techniques such as immunofluorescence and qPCR .

In order to do this, immunofluorescence will evaluate the presence of tumor infiltrate and also the presence of co-stimulatory molecules in lymphoid organs. In addition, CD8+ will be stained in tumors while CD3+, CD152 (CTLA4), and IA (MHCI) will be stained in lymph nodes. Furthermore, it is going to be evaluated if dendritic cells immunotherapy plays a role on the differentiation of auxiliary T lymphocytes to Th1,

Th2, Th17 and Treg profiles through qPCR expression assessment of transcription factors T-bet, GATA3, RORγt, and FOXP3, respectively.

3 METHODOLOGY

EXPERIMENTAL DESIGN

The experiment used 88 female Balb/c mice, aged 6 to 8 weeks, from the sectoral vivarium of IPON - Oncology Research Institute. The animals were housed in cages (dimensions - 305x198x133mm), grouped in no more than 5 animals per cage, separated into different experimental groups, being subjected to environments with light/dark cycle (12h), controlled temperature around 22 ° C, +/- 2 ° C, with food and water *ad libitum*. Of these 88 mice, 70 were divided into the experimental groups described below:

GI- Control group consisting of 10 animals. This group was not induced to tumor development by the 4T1 breast tumor cell line and was also not treated with a dendritic cell vaccine;

GII - Dendritic Cell Vaccine Group, consisting of 20 animals that were not induced to tumor development by the 4T1 cell line, but were submitted to the dendritic cell vaccination protocol;

GIII - Tumor Group without treatment with dendritic cell vaccine, this group consists of 20 animals that were induced to tumor development by the 4T1 cell line, but were not submitted to the dendritic cell vaccination protocol.

GIV - Tumor Group Treated with Dendritic Cell Vaccine, consisting of 20 animals that were induced to tumor development by the 4T1 cell line and also submitted to a dendritic cell vaccination protocol.

The 4T1 cells were maintained in complete RPMI media in a CO2 incubator with 5% humidity at 37°C. For transplants, cells were removed from the incubator, washed twice with saline and centrifuged for 10 min. at 290g at 4°C. Then they were injected with a 13x4.5 syringe in the left breast of each animal with a transplant of 2.0 x 10^5 4T1 cells in 50 µL of solution. From day 7 the tumor of animals in groups submitted to developing a breast tumor was measured at least every three days. The tumor volume was determined using the following formula:

$$[\text{largest diameter} \times (\text{smallest diameter})^2]$$

The remaining 18 animals were used to make dendritic cell vaccines. For each 1 euthanized animal, 4 doses of dendritic cell vaccine were made. In this way the 18 animals produced sufficient doses of dendritic cell vaccine to vaccinate the 40 animals in groups GII and GIV. For the production of vaccines applied in GII (Dendritic Cell Vaccine Group) and GIV (Tumor Group treated with Dendritic Cell Vaccine) cells were removed from the bone marrow of femurs and tibiae.

The removed cells were then placed in 25cm^2 flasks (volume 7.5 ml) in an amount of 32.5x10^6 per flask, with supplemented IMDM culture medium. Under stimuli of GM-CSF (13µL; 10ng / µL), IL-4 (13µL; 10ng / µL), TNF-α (23 µL; 10ng / µL) and tumor antigen of 4T1 cells (23µL), the cells were differentiated into dendritic cells.

Subsequently, the groups were subdivided into other groups in which animals were euthanized seven days after the first dose of dendritic cell vaccine and other groups where the animals were euthanized 14 days after the first dose of dendritic cell vaccine. In this way, the total amount of groups increased from 4 to 7 groups: GI (control group), GII (control group dendritic cells 7 days), GII (tumor group 7 days), GIV (tumor group dendritic cells 7 days), GV (control group dendritic cells 14 days) , GVI (tumor group 14 days) and GVII (tumor group dendritic cells 14 days).

Therefore, on the 7th day of the experimental period, animals related to the groups GI, GII, GIII and GIV were euthanized, the other animals related to the groups GV, GVI and GVII were euthanized on the 14th day of the experimental period. The results obtained were verified and analyzed by the GraphPad Prism 5.0 software.

IMMUNOFLUORESCENCE

The following protocol was used for immunofluorescence staining of tumors and lymph nodes. The sample previously frozen in tissue-tek is taken to the immunofluorescence sector where it is thawed and placed in a mold, it is filled with TISSUE TEK and the sample to be frozen is placed. This mold with TISSUE TEK and the fragment to be analyzed are placed in liquid nitrogen for 4 minutes. Once frozen, the sample must not be defrosted.

The TISSUE TEK block frozen with the sample was taken to the cryostat. The block was placed in the holder using TISSUE TEK. The cryostat was at a temperature of -24°C, if it reached -20°C the block would start to thaw, impairing its handling and cutting.

Due to the temperature difference, the cut (5µm) adheres to the blade at room temperature. After cutting, we used a 30% methanol solution to fix the cut on the blade for 10 minutes. We washed the slide 10 times with PBS previously prepared with monobasic potassium phosphate, bi-basic sodium phosphate, sodium chloride. We then followed the staining that was done in a dark vat in order to preserve the antibodies from direct light exposure, which can impair the reading of the slide. We placed the antibody (variable volume) and incubated for 120 minutes. We washed 10 times with PBS. To cover the coverslips, 3% glycerol was used. The markings performed were as follows: CD11a PE, CD49d PE, CD102 PE and CD54 PE.

qPCR

Part of the spleen was stored in trizol and RNA was subsequently extracted. The cDNA obtained from the RNA using the GoScript ™ Reverse Transcription System kit, according to the manufacturer's protocol, was subjected to a qPCR reaction to check the gene expression of the following transcription factors: T-bet, GATA-3, FOXP3 and RORγt. For the same samples, the endogenous control used was β-actin. The pre-assembled primers are:

TABLE 1 – Genes used for qPCR assay. Nucleotide sequence and annealing temperature for each primer.

GENE	PRIMERS	ANNEALING TEMPERATURE (CELSIUS)
T-BET FORWARD	5'-TCAACCAGCACCAGACAGAG-3'	
T-BET REVERSE	5'-AAACATCCTGTAATGGCTTGTG-3'	65
GATA3 FORWARD	5'-TTATCAAGCCCAAGCGAAG-3'	
GATA3 REVERSE	5'-TGGTGGTGGTCTGACAGTTC-3'	65
RORγt FORWARD	5'-ATGGACAGGGCCCCACACAGAGA-3'	
RORγt REVERSE	5'CAAGTTCAGGACGCCTGGTTTCCTC-3'	58
Foxp3 FORWARD	5'-ACTGCTGGCAAATGGAGTCT-3'	
Foxp3 REVERSE	5'-AAGTAGGCGAACATGCGAGT-3'	61
β-ACTIN FORWARD	5´TGTGATGGTGGGAATGGGTCAG 3´	
β-ACTIN REVERSE	5´-TTTGATGTCACGCACGATTTCC- 3´	65

STATISTICAL ANALYSIS

Data obtained were analyzed using the GraphPad Prism 5 software. Kolmogorov-smirnov and Shapiro-wilk normality tests were carried out, if the results passed the

normality tests, ANOVA and T test parametric tests were performed, if they did not pass the normality tests, non-parametric Kruskal-Wallis and Mann-Whitney tests were chosen. Tests with a result of p <0.0001 were considered statistically significant. For the correlations, normality tests and Pearson or Spearman correlation were performed.

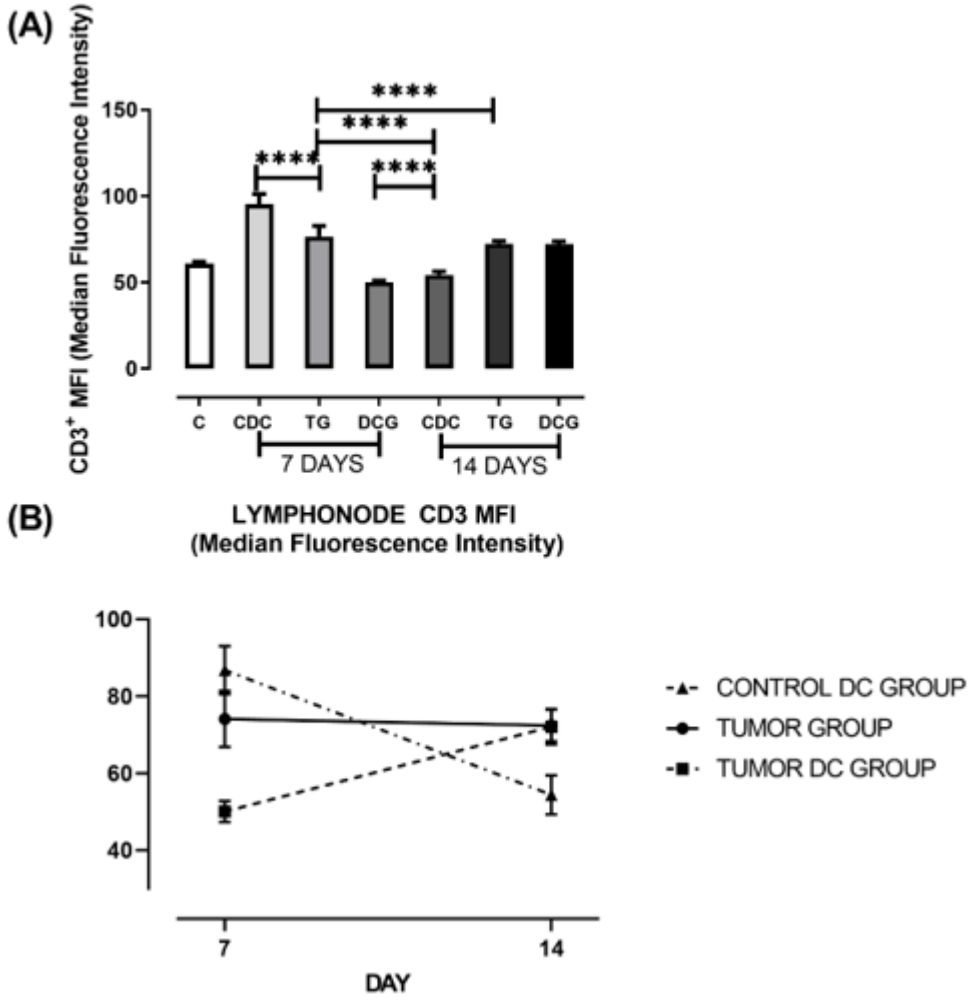
4 RESULTS

Dendritic cell vaccine in the activation of T lymphocytes

Our results demonstrated that the mean fluorescence intensity of cells with CD3 in the lymph nodes among all experimental groups is the highest in the control group DC 7 days (76.49 - 116) 92.8, followed by the tumor group 7 days (55.63 - 98.73) 74.45 with a statistical difference of p <0.05 between the two groups. When comparing the groups Tumor 14 days (68.25 - 78.92) 71.99 and Tumor Treated 14 days (67.66 - 78.36) 71.27, we did not notice any statistically significant difference. When comparing CD3 + in the Treated Tumor and Tumor groups, we noticed that in 7 days the MFI is higher in the Tumor group (55.63 - 98.73) 74.45 when compared to the Treated Tumor group (47.20 - 53.20) 49 , 33. In 14 days, we noticed that MFI levels are close when we compare the Tumor group (68.25 - 78.92) 71.99 to the Treated Tumor group (67.66 - 78.36) 71.27.

In terms of the CD8+ cells in the tumor, we noticed the highest MFI in the Tumor Treated 14 days (740.6 - 1347) 1123 when compared to the other groups. When comparing the group Tumor 14 days (544.9 - 1873) 544.9, there is a statistically significant difference with a value of p <0.0001. When comparing the CD8+ MFI kinetics in the tumors from treated and untreated groups, we noticed first that the Treated Tumor group had a higher MFI when compared to the Tumor group in 7 days and second, the Treated Tumor group had a higher MFI when compared to the Tumor group in 14 days.

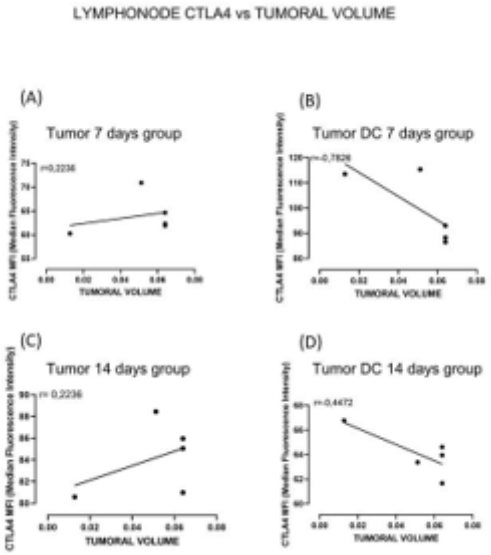
FIGURE 1 – Mean Fluorescence Intensity (MFI) of CD3+ cells in lymph nodes with immunofluorescence technique. (A) represents MFI of CD3+ cells in experimental groups. C= control group, CDC= control dendritic cell group, DCG= dendritic cell group. (B) representation of the kinetics involving MFI of CD3 + cells in the tumor and tumor DC groups from 7 to 14 days after the first dose of dendritic cell immunotherapy. **** represents the value of p <0.0001.



Activation of co-stimulatory molecules

CTLA-4 or C152 is known as a co-stimulatory molecule holding an inhibitory role. It was performed a correlation between the tumor volume and the presence of this molecule in the lymph nodes. Our results demonstrate that the treated groups show a negative correlation, wherein the Tumor group DC 7 days (r = -0.7826) and the Tumor group DC 14 days (r = -0.4472). This means that the greater the presence of CTLA4, the lower the tumor volume in both groups. In groups without any treatment, for both 7 and 14 days, there is a positive correlation for the Tumor group 7 days(r = 0.2236) and the Tumor group 14 days (r = 0.2236), which means that the greater the tumor volume, the greater the presence of co-stimulatory molecules. The presence of dendritic cell immunotherapy can unbalance this correlation.

FIGURE 2 - Spearman's correlation showing the correlation between tumor development and the presence of CTLA4 in the lymph nodes of the experimental groups. (A) represents the correlation between the tumor volume and the presence of CTLA4 in the lymph nodes of the tumor group 7 days. (B) represents the correlation between the tumor volume and the presence of CTLA4 in the lymph nodes of the DC tumor group 7 days. (C) represents the correlation between the tumor volume and the presence of CTLA4 in the lymph nodes of the tumor group 14 days. (D) represents the correlation between the tumor volume and the presence of CTLA4 in the lymph nodes of the DC tumor group 14 days.



In order to evaluate an essential molecule in the activation of cells such as CD8+ T lymphocytes, we performed a correlation between the MFI of MHCI in lymph nodes and the tumor infiltrate of CD8+ T lymphocytes. The treated tumor groups, for both 7 and 14 days, present an r=1,000 and therefore a strong positive correlation. It indicates that the greater the presence of tumor infiltrate of CD8 + T lymphocytes, the greater the presence of MHC molecules in the lymph nodes. In the untreated tumor groups, we noticed an r = 1,000 and a strong positive correlation in 7 days , which indicates that the greater the presence of CD8+ T lymphocyte infiltrate in the tumor, the greater the presence of MHCI in the lymph nodes. This strong positive correlation turns into a negative correlation with a r = -0.02000 in 14 days, thus suggesting that the immune system needs this time to modulate its immune response against many pro-tumor stimuli.

FIGURE 3 - Mean Fluorescence Intensity (MFI) of CD8 + cells in tumors using the immunofluorescence technique. (A) represents the MFI of CD8 + cells in the tumors of the tumor groups 7 days, tumor treated 7 days, tumor 14 days and tumor treated 14 days, respectively. (B) kinetic representation involving the MFI of CD8 + cells in the tumor and tumor DC groups from 7 to 14 days after the first dose of dendritic cell immunotherapy. **** represents the value of p <0.0001**.**

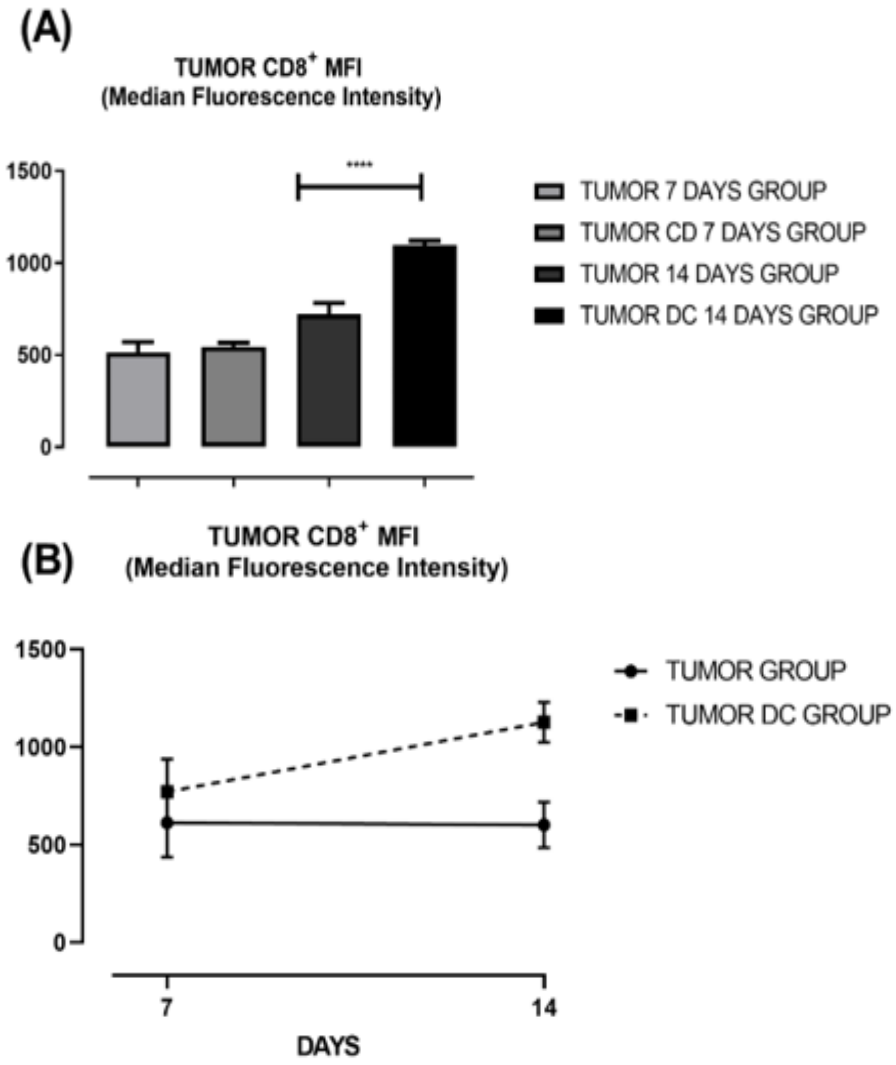
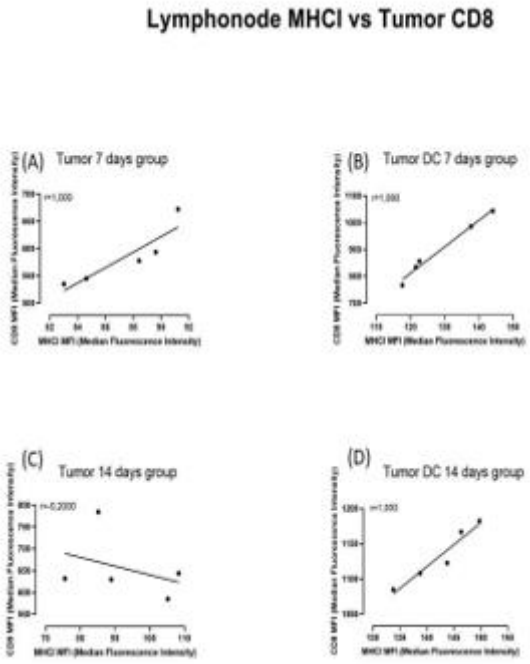


FIGURE 4 - Spearman correlation showing the correlation between tumor development and the presence of CD8 + in the tumors of the experimental groups. (A) represents the correlation between the tumor volume and the presence of CD8 + in the tumors of the tumor group 7 days. (B) represents the correlation between the tumor volume and the presence of CD8 + in the tumors of the DC tumor group 7 days. (C) represents the correlation between the tumor volume and the presence of CD8 + in the tumors of the tumor group 14 days. (D) represents the correlation between the tumor volume and the presence of CD8 + in the tumors of the DC tumor group 14 days.



The presence of transcription factors of cd4+ t lymphocyte and tumor volume

T-bet, the main transcription factor responsible for CD4+ T lymphocytes differentiation into lymphocytes of Th1 profile, demonstrated its highest expression in spleens from the Control group dendritic cells, where it is 6 times more expressed in the Control group dendritic cell when compared to Control group. In a comparison of T-bet in the Tumor group and Treated Tumor group, we noticed a higher final T-bet expression in the Tumor group CD compared to the Tumor group.

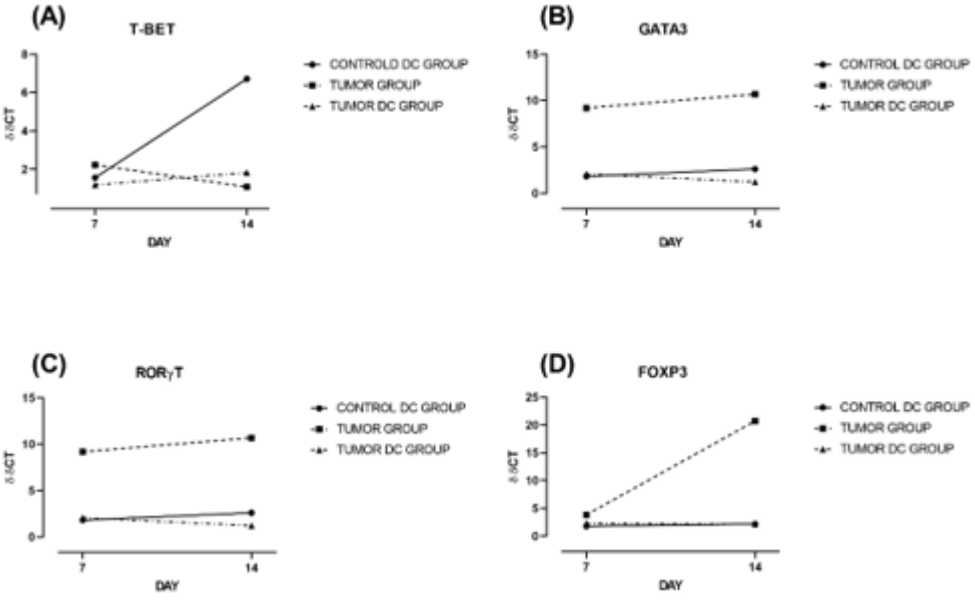
GATA3, the transcription factor responsible for the differentiation of CD4 lymphocytes into Th2 profile cells, is about 10 times more expressed in the tumor group without treatment in 14 days while the same transcription factor is about 8 times more expressed in the tumor group 7 days . We note that the greatest expression of GATA3 is found in tumor groups without treatment.

RORγt is the main transcription factor responsible for the differentiation of CD4 + T lymphocytes into Th17 profile lymphocytes. Our results showed that the greatest expression of this transcription factor was also found in the tumor groups without treatment both in 7 and 14 days. In the tumor group 7 days the transcription factor is about 8 times more expressed when compared to the tumor group and in the tumor group 14

days the same transcription factor is about 10 times more expressed when compared to the control group.

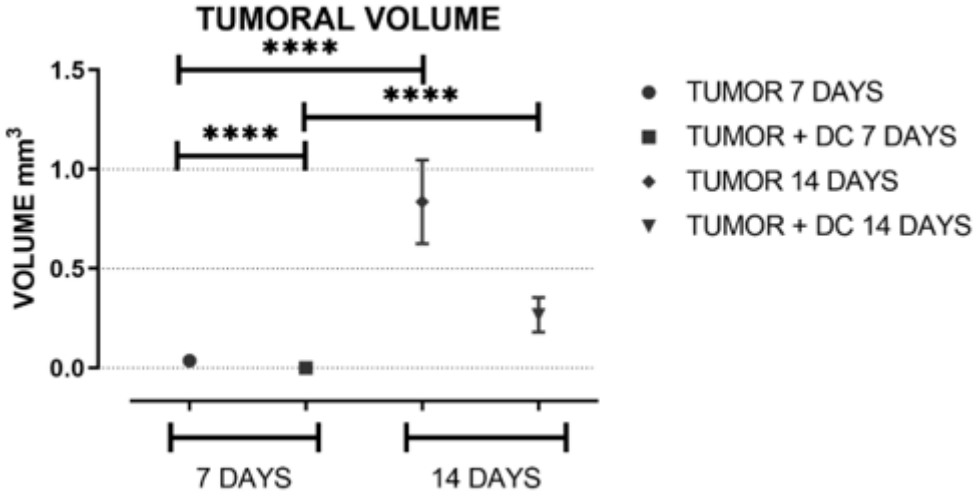
FOXP3, an essential transcription factor for the differentiation of T lymphocytes into Treg lymphocytes, is about 20 times more expressed in the tumor group 14 days when compared to the tumor group. In the 7-day tumor group, the same transcription factor is about 5 times more expressed when compared to the control group. When we analyze the kinetics of FOXP3 in the groups at 7 and 14 days we noticed that the greatest increase in the expression of this transcription factor is in the tumor group, going from about 5 times more expressed in the tum or group in 7 days to about 20 times more expressed in the tumor group in 14 days.

FIGURE 5 - graphical representation of the kinetics involving the main transcription factors of T lymphocytes from 7 to 14 days after the first dose of immunotherapy with dendritic cells. (A) graphical representation of the δδCT of the transcription factor T-bet in the control groups DC, tumor and tumor DC from 7 to 14 days. (B) graphical representation of the δδCT of the transcription factor GATA3 in the control groups DC, tumor and tumor DC from 7 to 14 days. (C) graphical representation of the δδCT of the transcription factor RORγt in the control groups DC, tumor and tumor DC from 7 to 14 days. (D) graphical representation of the δδCT of the FOXP3 transcription factor in the DC, tumor and DC tumor groups from 7 to 14 days.



Given the aforementioned molecular data relating immunotherapy to a better anti-tumor immune response, we also measured the tumors of animals in the experimental groups. Our results show that in 7 days there is no difference between the tumor volume of the tumor group and the tumor volume of the tumor group treated with DC. At 14 days there is a great difference between the tumor volume of the two groups analyzed. The tumor volume is higher on average in the tumor group when compared to the tumor group treated with DC with a value of $p < 0.0001$, a statistically significant result.

FIGURE 6 - Tumor development in experimental groups induced to develop breast tumor with 4T1 cells in 7 and 14 days. Balb/c mice were induced to develop breast tumor from 4T1 cells. Part of these animals was treated with dendritic cell immunotherapy, the so-called DC tumor group and another group received no treatment at all. The graph shows the development of tumors in these groups 7 days after the first dose of dendritic cell immunotherapy and 14 days after the first dose of dendritic cell immunotherapy. **** represents the value of p <0.0001.



5 DISCUSSION

In lymphocyte activation, it is known that CD3 associates to TCR and aids the activation of T lymphocytes. Our results showed that in lymph nodes, the lymphoid organ activates lymphocytes, there is a higher MFI of CD3 in the Tumor group when compared to the Treated Tumor group 7 days. In 14 days, MFI is similar in the Tumor group and Treated Tumor group, thus demonstrating there are CD4 or CD8 T lymphocytes differentiation in lymph nodes of the Treated group in 14 day. In a prospective analysis, it was found that nonresponsive patients to CD3 in the lymph nodes had a greater recurrence of head and neck tumors when compared to the group of responsive patients [18], thus demonstrating the importance of the molecule in the immune response to tumors.

CD8 is associated with cytotoxic T lymphocytes, therefore showing cytotoxic function against tumor cells [19]. It is known that the presence of tumor infiltrate of cytotoxic T lymphocytes is a sign of good prognosis in the course of several types of cancers [1,20,21]. In the analyzed tumors, our results showed the highest MFI of CD8 T lymphocyte in the Treated Tumor group 14 days when compared to other groups. By analysing the tumors in 7 days, we noticed that the infiltrate of CD8 T lymphocytes is similar in both the Tumor group and in the Treated Tumor group with dendritic cell vaccine. It demonstrates that 14 days are sufficient for CD8 T lymphocytes differentiation and activation by dendritic cell immunotherapy.

CTLA4 is associated with a poor prognosis in the development of cancer. It is known that CTLA4 is a co-stimulatory molecule and has an immunosuppressive role through several mechanisms, such as: competition with CD28 during the lymphocyte activation process [22]. Checkpoint blockers are already well described and even used as a treatment for cancer, such as ipilimumab, which is an antibody that blocks the binding site of CTLA4, thus preventing it from exerting its immunosuppressive function [23]. A study demonstrated that CTLA4 blockade combined with immunotherapy prolonged CD8 T lymphocyte cell activity without the need to stimulate proliferation with IL-2 [24]. Our results demonstrated that in an analysis of tumor development versus CTLA4 MFI in lymph nodes, there is a positive correlation in the Tumor group 14 days, which means that the greater the tumor volume, the greater the MFI of CTLA4. However, in the Treated Tumor group 14 days there is a negative correlation negative, which means the greater the tumor volume, the lower the MFI of CTLA4 in the lymph nodes. As previously shown, the dendritic cell vaccine induces a fight against the tumor by decreasing its volume in the Treated groups.

Due to its immunosuppressive role, it is known that CTLA-4 is associated with a poor prognosis in several types of cancers [25]. CTLA-4 blockade is already used as immunotherapy, so it was suspected that blocking this molecule could also potentiate the immune response. Suspicion confirmed in a work published in 2019 [26]. This result is in agreement with the work published in 2020 [27], and we can correlate these data with our results which demonstrate that the dendritic cell vaccine has the potential to inhibit CTLA-4 MFI due to the fact that the tumor group treated with dendritic cells present higher CTLA-4 MFI in 7 days and in 14 days MFI has decreased, thus increasing the potential for tumor destruction by dendritic cells.

It is well described that lymphocytes are activated in the secondary lymphoid organs by dendritic cells. In the case of CD8 T lymphocytes, after interaction of the MHCI of the antigen presenting cells with their TCR, they migrate to the infection site to execute their effector function, which is to destroy cells that have known antigen in their MHCI [28]. CD8 T lymphocytes are part of the adaptive immune response, therefore more specific, one of the reasons why this cell type is essential in the immune response against cancer [29].

Several studies demonstrate that the tumor infiltrate of CD8 T lymphocytes and the presence of this cell type is a sign of a good prognosis for the cancer patient [30,31]. Our results demonstrated in the analyzed lymph nodes of the tumor group in 14 days that the

lower the MFI of MHCI in the lymph nodes, the lower the MFI of CD8 lymphocytes in the tumor with a negative correlation demonstrating the inability of the immune system to fight the tumor without stimulation. When we analyzed the tumor group treated with dendritic cell immunotherapy, we obtained a strong correlation with r = 1 when we analyzed the CD8 MFI in tumors vs MHCI MFI in lymph nodes, demonstrating that immunotherapy positively stimulated T lymphocyte activation CD8 in 14 days. Result that is repeated in the tumor group treated when MFI analysis of TCR in lymph nodes vs MFI of CD8 in tumors.

Among the transcription factors that act during an immune response to cancer, we can mention the T-bet, which is the main transcription factor related to the cell profile Th1, a profile responsible for an ideal immune response against tumor cells[32]. Among the transcription factors described in the early 2000s [33], it is now known that the expression of t-bet by helper T lymphocytes induces the production of cytokines such as IFN-γ and IL-12. IFN- γ induces greater differentiation in T lymphocytes in Th1 lymphocytes and inhibition of Th2 profile, in macrophages it induces differentiation in M1, increases the maturation of dendritic cells and the expression of costimulatory molecules and inhibits the differentiation of regulatory T lymphocytes [34]. Our results demonstrated that the behavior of T-bet in immunotherapy with dendritic cells increased from 7 to 14 days in the treated tumor group and in the tumor group the opposite behavior, suggesting that from 14 days on we can start to notice improvements in the behavior of the immune system in combating tumor cells. Complementing these results, we note what is well described in the literature, that T-bet has an opposite behavior or an equilibrium behavior with GATA3[35] : from 7 to 14 days T-bet increases in the treated tumor group and GATA3 decreases in the same group and in the tumor group, T-bet decreases and GATA3 increases from 7 to 14 days.

It has been shown that for the development of regulatory T cells, the activation of the FOXP3 transcription factor is sufficient in mice [36]. It is known that for the activation of the proinflammatory Th17 profile, the main transcription factor is RORγT [37]. The profile of Th17 cells plays a controversial role in the immune response to tumors, sometimes fighting tumor development, and other times contributing to tumor development [38]. However, the role of the regulatory T profile has been well described and it is known that cells of this profile have a pro-tumor role in the immune response to cancer, with its markers being successful targets in immunotherapies against cancer [23,39]. Our results demonstrated that in 14 days of dendritic cell immunotherapy the gene

expression of FOXP3 did not change in the treated tumor group, but in the tumor group without treatment there was a great increase in the genetic expression of FOXP3. Considering RORγT, our results showed that in 14 days in the treated tumor group there was a decrease in the genetic expression of this factor, but in the tumor group without treatment there was, as well as FOXP3, a great increase.

6 CONCLUSION

Our results demonstrate that 14 days of immunotherapies with dendritic cells are sufficient for modulation of the immune system. The levels of great prognostic markers such as T-bet and infiltrate of CD8 T lymphocytes increase when compared to bad prognostic markers such as FOXP3, CTLA4 among other studied markers. In practice, we note these results by assessing the tumor volume where we notice a significant difference in tumor volume between the tumor group and the treated tumor group. In general, immunotherapy of dendritic cells gives the treated mice a good prognosis in the fight against cancer and this immunotherapy should be studied more thoroughly, so that in the near future it can be used as a base treatment in most diverse cancers.

REFERENCES

1. Zhang Y, Zhang Z. The history and advances in cancer immunotherapy: understanding the characteristics of tumor-infiltrating immune cells and their therapeutic implications. Cell Mol Immunol [Internet]. 2020;8:807–21. Available from: http://dx.doi.org/10.1038/s41423-020-0488-6

2. Kaufman HL, Atkins MB, Subedi P, Wu J, Chambers J, Joseph Mattingly T, et al. The promise of Immuno-oncology: Implications for defining the value of cancer treatment. J Immunother Cancer. 2019;7:1–11.

3. Riley RS, June CH, Langer R, Mitchell MJ. Delivery technologies for cancer immunotherapy. Nat Rev Drug Discov. 2019;18:175–96.

4. Lee JY, Lee HT, Shin W, Chae J, Choi J, Kim SH, et al. Structural basis of checkpoint blockade by monoclonal antibodies in cancer immunotherapy. Nat Commun [Internet]. 2016;7:1–10. Available from: http://dx.doi.org/10.1038/ncomms13354

5. Perica K, Varela JC, Oelke M, Schneck J. Adoptive T Cell Immunotherapy For Cancer. Rambam Maimonides Med J. 2015;6:4.

6. Calmeiro J, Carrascal MA, Tavares AR, Ferreira DA, Gomes C, Falcão A, et al. Dendritic cell vaccines for cancer immunotherapy: The role of human conventional type 1 dendritic cells. Pharmaceutics. 2020;12:1–20.

7. Ribatti D. The concept of immune surveillance against tumors. The first theories. Oncotarget. 2017;8:7175–80.

8. O'Donnell JS, Teng MWL, Smyth MJ. Cancer immunoediting and resistance to T cell-based immunotherapy. Nat Rev Clin Oncol. 2019;16:151–67.

9. Swann JB, Smyth MJ. Review series Immune surveillance of tumors. J Clin Invest. 2007;117:1137–46.

10. Comber JD, Philip R. MHC class I antigen presentation and implications for developing a new generation of therapeutic vaccines. Ther Adv Vaccines. 2014;2:77–89.

11. Andersen MH, Schrama D, Thor Straten P, Becker JC. Cytotoxic T cells. J Invest Dermatol [Internet]. 2006;126:32–41. Available from: http://dx.doi.org/10.1038/sj.jid.5700001

12. Goadsby PJ, Kurth T, Pressman A. Present Yourself! By MHC Class I and MHC Class II Molecules. Trends Immunol. 2016;35:1252–60.

13. Landskron G, De La Fuente M, Thuwajit P, Thuwajit C, Hermoso MA. Chronic inflammation and cytokines in the tumor microenvironment. J Immunol Res. 2014;2014.

14. Messerschmidt JL, Prendergast GC, Messerschmidt GL. How Cancers Escape Immune Destruction and Mechanisms of Action for the New Significantly Active

Immune Therapies: Helping Nonimmunologists Decipher Recent Advances. Oncologist. 2016;21:233–43.

15. Butterfield LH. Dendritic cells in cancer immunotherapy clinical trials: Are we making progress? Front Immunol. 2013;4:3–9.

16. Geijtenbeek TBH, Gringhuis SI. Dendritic Cell Immunotherapy, the Next Step in Cancer Treatment. Multidiscip Cancer Investig. 2017;1:465–79.

17. Breckpot K, Escors D. Dendritic Cells for Active Anti-Cancer Immunotherapy: Targeting Activation Pathways Through Genetic Modification. Endocrine, Metab Immune Disord - Drug Targets. 2012;9:328–43.

18. Shibuya TY, Nugyen N, McLaren CE, Li KT, Wei WZ, Kim S, et al. Clinical significance of poor CD3 response in head and neck cancer. Clin Cancer Res. 2002;8:745–51.

19. Tian CT, Lu SX, Fan QX, Zhang WJ, Jiao SC, Zhao X, et al. Prognostic significance of tumor-infiltrating CD8+ or CD3+ T lymphocytes and interleukin-2 expression in radically resected non-small cell lung cancer. Chin Med J (Engl). 2015;128:105–10.

20. Al-Saleh K, El-Aziz NA, Ali A, Abozeed W, El-Warith AA, Ibraheem A, et al. Predictive and prognostic significance of CD8+ tumor-infiltrating lymphocytes in patients with luminal B/HER 2 negative breast cancer treated with neoadjuvant chemotherapy. Oncol Lett. 2017;14:337–44.

21. Shimizu S, Hiratsuka H, Koike K, Tsuchihashi K, Sonoda T, Ogi K, et al. Tumor-infiltrating CD8+ T-cell density is an independent prognostic marker for oral squamous cell carcinoma. Cancer Med. 2019;8:80–93.

22. Zhao Y, Yang W, Huang Y, Cui R, Li X, Li B. Evolving Roles for Targeting CTLA-4 in Cancer Immunotherapy. Cell Physiol Biochem. 2018;47:721–34.

23. Callahan MK, Postow MA, Wolchok JD. CTLA-4 and PD-1 Pathway Blockade: Combinations in the Clinic. Front Oncol. 2015;4:1–6.

24. Sckisel GD, Mirsoian A, Bouchlaka MN, Tietze JK, Chen M, Blazar BR, et al. Late administration of murine CTLA-4 blockade prolongs CD8-mediated anti-tumor effects following stimulatory cancer immunotherapy. Cancer Immunol Immunother. 2015;64:1541–52.

25. Santoni G, Amantini C, Morelli MB, Tomassoni D, Santoni M, Marinelli O, et al. High CTLA-4 expression correlates with poor prognosis in thymoma patients. Oncotarget. 2018;9:16665–77.

26. Neeha Zaidi, Sergio A. Quezada, Janelle M.Y. Kuroiwa1, Li Zhang, Elizabeth M. Jaffee , Ralph M. Steinman and BW. Anti–CTLA-4 synergizes with dendritic cell–targeted vaccine to promote IL-3–dependent CD4+ effector T cell infiltration into murine pancreatic tumors. 2019;1445:62–73.

27. Esmaily M, Masjedi A, Hallaj S, Nabi Afjadi M, Malakotikhah F, Ghani S, et al. Blockade of CTLA-4 increases anti-tumor response inducing potential of dendritic cell vaccine. J Control Release [Internet]. 2020;326:63–74. Available from: https://doi.org/10.1016/j.jconrel.2020.06.017

28. Mondino A, Khoruts A, Jenkins MK. The anatomy of T-cell activation and tolerance. Proc Natl Acad Sci U S A. 1996;93:2245–52.

29. Farhood B, Najafi M, Mortezaee K. CD8+ cytotoxic T lymphocytes in cancer immunotherapy: A review. J Cell Physiol. 2019;234:8509–21.

30. Oshi M, Asaoka M, Tokumaru Y, Yan L, Matsuyama R, Ishikawa T, et al. CD8 T cell score as a prognostic biomarker for triple negative breast cancer. Int J Mol Sci. 2020;21:1–16.

31. Kim SH, Go S Il, Song DH, Park SW, Kim HR, Jang I, et al. Prognostic impact of CD8 and programmed death-ligand 1 expression in patients with resectable non-small cell lung cancer. Br J Cancer. 2019;120:547–54.

32. Mori H, Kubo M, Kai M, Yamada M, Kurata K, Kawaji H, et al. T-bet+ lymphocytes infiltration as an independent better prognostic indicator for triple-negative breast cancer. Breast Cancer Res Treat [Internet]. 2019;176:569–77. Available from: https://doi.org/10.1007/s10549-019-05256-2

33. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH, et al. A Novel Transcription Factor , T-bet , Directs Th1 Lineage Commitment. 2000;100:655–69.

34. Jorgovanovic D, Song M, Wang L, Zhang Y. Roles of IFN-γin tumor progression and regression: A review. Biomark Res. 2020;8:1–16.

35. Ylikoski E, Lund R, Kyläniemi M, Filén S, Kilpeläinen M, Savolainen J, et al. IL-12 up-regulates T-bet independently of IFN-γ in human CD4+ T cells. Eur J Immunol. 2005;35:3297–306.

36. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. J Immunol. 2003;198:986–92.

37. Chen Z, Lin F, Gao Y, Li Z, Zhang J, Xing Y, et al. FOXP3 and RORγt: Transcriptional regulation of Treg and Th17. Int Immunopharmacol. 2011;11:536–42.

38. Dahal LN. The dichotomy of T helper 17 cells in cancer. Nat Rev Immunol [Internet]. 2017;17:592. Available from: http://dx.doi.org/10.1038/nri.2017.93

39. Ohue Y, Nishikawa H. Regulatory T (Treg) cells in cancer: Can Treg cells be a new therapeutic target? Cancer Sci. 2019;110:2080–9.