

# Phenotypic differences of teidual T<sub>DCs</sub> obtained from breast cancer mice

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## Summary

**Objective:** To evaluate T<sub>DC</sub> expression by flow cytometry for surface markers (CD4, CD8 and CD86), transcription factors (Tbet, Foxp3, Gata3 and Ror $\gamma$ t), and cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-10, IL-12 and IL-17) in spleen, liver, lymph node, bone marrow and tumor of 4T1 induced and healthy mice. **Results:** T<sub>DC</sub> are more frequent in lymph nodes in the control and tumor groups, compared to the other environments studied ( $p < 0.0001$ ). When we compare the expression of surface markers between control and 4T1 induced groups we noted decreased CD4 T<sub>DC</sub> expression in liver ( $p = 0.0001$ ), and the same with CD8 T<sub>DC</sub> expression in spleen ( $p = 0.0012$ ) and liver ( $p = 0.0028$ ), as well as the expression of CD86 T<sub>DC</sub> in spleen and liver ( $p = 0.0337$ ), in the 4T1-induced tumor group. When comparing transcription factors, there was a decrease T<sub>DC</sub> Tbet and T<sub>DC</sub> Foxp3 in spleen and liver ( $p = 0.0001$ ); and the same with T<sub>DC</sub> Gata3 in liver ( $p = 0.0028$ ), and increase in T<sub>DC</sub> Ror $\gamma$ t in bone marrow in the tumor group ( $p < 0.0001$ ). Regarding cytokines, we found decreased IFN- $\gamma$  T<sub>DC</sub> in spleen ( $p < 0.0001$ ) and bone marrow ( $p = 0.0002$ ), and the same with TNF- $\alpha$  T<sub>DC</sub> in spleen and liver ( $p < 0.0001$ ), as well as the expression of IL-10 T<sub>DC</sub> in spleen ( $p < 0.0001$ ), liver ( $p < 0.0001$ ) and bone marrow ( $p < 0.001$ ), of IL-12 T<sub>DC</sub> in spleen and bone marrow ( $p < 0.001$ ), and IL-17 T<sub>DC</sub> in spleen and liver ( $p < 0.001$ ) in the 4T1-induced tumor group in all comparisons. Phenotypic changes may be driven by the tissue microenvironment in the presence of the tumor. Directions are needed to understand the functionality associated with possible antitumor immunotherapy.

**Key words:** T<sub>DC</sub> cells; Breast cancer; Tissue microenvironment; Antitumor immune response.

## Introduction

T<sub>DC</sub> cells represent a rare and newly discovered subset of hematopoietic cells [1]. Phenotypic characteristics refer to the nomenclature of this cell type, as they have the T-cell receptor (TCR $\alpha\beta$ ) marker as well as conventional dendritic cells (CD11c and MHC II) [2].

T lymphocytes, functionally important for the immune system, have in their structure the TCR receptor, consisting of an alpha and beta chain or a gamma and delta chain. This receptor recognizes major histocompatibility complex (MHC) molecules present in almost all nucleated cells, thus enabling T cell activation leading to a multitude of immune responses [3].

CD11c and MHC II surface markers are expressed by dendritic cells (DCs), ie professional antigen presenting cells, derived from pluripotent hematopoietic progenitors in the bone marrow [4-5].

DCs constitute about 1% of mononuclear leukocytes present in peripheral blood, and their localization in other tissues provides the function of immune system sentinels, continuously monitoring antigens [6].

In healthy mice 7% of splenic DCs express the TCR $\alpha\beta$  receptor, a different characteristic from conventional DCs.

These cells have similar characteristics in their origin and development to conventional DCs and T cells, being called T<sub>DC</sub> cells. In addition, they are self-sufficient for antigen presentation and, when stimulated with lipopolysaccharides (LPS), are capable of producing IL-12 cytokine and exhibiting cytotoxic gene expression [1].

Understanding and exploring the phenotypic characteristics of T<sub>DC</sub> cells regarding the expression of surface markers, transcription factors and cytokines in the spleen, liver, lymph node, bone marrow and tumor in healthy conditions and in the presence of tumor cells in mice is of utmost importance.

However, few studies directed to this characterization have been performed. Thus, the objectives of the present study were to verify the frequency of T<sub>DC</sub> cell expression in the tissues of the 4T1 cell-induced control and tumor groups, as well as to compare such cell frequency between the tissues of both groups, by evaluating the phenotypic characteristics of related T<sub>DC</sub> cells. surface markers (CD4, CD8 and CD86), transcription factors (Tbet, Foxp3, Gata3 and Ror $\gamma$ t), and cytokine expression (INF- $\gamma$ , TNF- $\alpha$ , IL-10, IL-12 and IL-17) in spleens, livers, sentinel lymph nodes, bone marrow and tumors obtained from healthy mice and 4T1-induced breast cancer.

Table 1. — *Antibody reagentes for flow cytometry*

Reagent	Supplier	Cat. No/lot no.	Isotype	Clone
FITC-anti-mouse TCR $\beta$	BD Biosciences	553170	Hamster IgG2	H57-597
PE-Cy 5-anti-mouse CD4	BD Biosciences	553050	Rat (DA) IgG2a, $\kappa$	RM4-5
PerCP-Cy 5.5-anti-mouse CD8a	BD Biosciences	551162	Rat (LOU) IgG2a, $\kappa$	53-6.7
APC-anti-mouse CD11c	BD Biosciences	550261	Hamster IgG1, $\lambda$ 2	HL3
APC-anti-mouse CD86	BD Biosciences	553768	Rat (LOU) IgG2a, $\kappa$	GL1
PE-anti-mouse MHC Class II (I-A)	BD Biosciences	553548	Mouse (SJL) IgG2b, $\kappa$	MAS-32.1
ALEXA FLUOR®488-anti-mouse Tbet	BD Biosciences	561266	Mouse IgG1, $\kappa$	O4-46
ALEXA FLUOR®488-anti-mouse Foxp3	BD Biosciences	560407	Rat IgG2b	MF23
PE-anti-mouse Gata3	BD Biosciences	560074	Mouse (BALB/c) IgG1, $\kappa$	L50-823
PE-anti-mouse Ror $\gamma$ t	BD Biosciences	562607	Mouse IgG2a, $\kappa$	Q31-378
FITC-anti-mouse IFN- $\gamma$	BD Biosciences	554411	Rat IgG1, $\kappa$	XMG1.2
PE-anti-mouse TNF- $\alpha$	BD Biosciences	554419	Rat IgG1	MP6-XT22
FITC-anti-mouse IL-10	BD Biosciences	554466	Rat IgG2b	JES5-16E3
PE-anti-mouse IL-12	BD Biosciences	554479	Rat IgG1	C15.6

## Materials and Methods

### *Animals and experimental groups*

For this study, 30 8-week-old female Balb/c mice were obtained from the Oncology Research Institute (IPON) of the Federal University of Triângulo Mineiro in Uberaba, Minas Gerais. The animals were divided into two groups: control (n = 15), without tumor cell inoculation, and 4T1-induced tumor group (n = 15), which were inoculated with breast cancer tumor cells of the 4T1 cell line. During the experimental phase, mice were kept in plastic cages under a 12 h light / dark cycle at  $21 \pm 3$  °C, with food and water available ad libitum. After 28 days they were euthanized by an overdose of 50mg/kg ketamine and 15mg/kg xylazine. This study was approved by the Animal Use Ethics Committee (CEUA) of the Federal University of Triângulo Mineiro, under number 317.

### *Tumor induction*

The 4T1 cells were cultured in RPMI medium in a humidified greenhouse (Water Jacket Incubator 3110, Thermo Fisher Scientific, Marietta, OH) at 37 °C and 5% CO<sub>2</sub>. For tumor induction, cells were washed with 0.9% saline and centrifuged at 290 xg at 4 °C for 10 minutes. Subsequently, when removing the supernatant, the cells were counted and  $2 \times 10^5$  cells were injected into the left mammary gland of the last pair of breasts of the 4T1 mouse group (Figure 1).

### *T<sub>DC</sub> cell characterization*

T<sub>DC</sub> cells were characterized according to immunophenotypic analysis by flow cytometry, performed by Kuka *et al.* (2012) by simultaneous expression of TCR $\alpha\beta$  (T lymphocyte receptor), CD11c (adhesion molecule) and MHC II (Molecule Histocompatibility Complex) [1].

To characterize T<sub>DC</sub> cells in different tissues, we performed the phenotypic analysis with surface markers with the same methodology as the authors cited above, that is, using the same gate strategy, revealing differences between the control and 4T1-induced tumor groups. Surface molecules represented by CD4 (helper T lymphocytes),

CD8 (cytotoxic T lymphocytes) and CD86/B7-2 (costimulatory molecules) were analyzed, as were helper T lymphocytes, transcription factors (Tbet, Gata3, Ror $\gamma$ t and Foxp3) and cytokine expression (INF- $\gamma$ , TNF- $\alpha$ , IL-10, IL-12 and IL-17).

### *Flow cytometry*

After 28 days of the experimental period, the 4T1-induced Control and Tumor mice were euthanized and the spleens, livers, sentinel lymph nodes and tumors were removed and subjected to a mechanical rupture process to obtain suspended cells. Bone marrow cells were extracted from the femur and tibia of the mice of the studied groups as well. The suspended cells were homogenized and washed with 0.9% saline and centrifuged at 290xg for 10 minutes at 4 °C. The supernatant was collected and the cell pellet counted and distributed at  $1 \times 10^6$  cells in each cytometry tube.

The obtained tissue cells were incubated for 30 minutes at 4 °C in the dark with extracellular monoclonal antibodies listed in Table 1. They were then washed with PBS (phosphate buffered saline) in order to remove unattached antibodies. After this procedure, cells in the tubes that would receive the intracellular labeling were permeabilized, fixed and incubated for 20 min with permeabilizing solution (BD Cytotfix / Cytoperm™) and then washed with buffered solution (BD Perm / Wash™ Buffer) and intracellularly labeled.

For intracellular labeling we used monoclonal antibodies (Table 1) for transcription factors and cytokines, which were incubated for 30 min according to the pre-established protocol and then washed with Perm/Wash Buffer solution (BD Perm / Wash™) to remove excess antibodies. Subsequently, they were resuspended in 50  $\mu$ L of PBS for reading using a BD FACSCalibur™ cytometer.

The collected data were analyzed and the average fluorescence intensities were determined by Flowing Software.

The gating strategy used was primarily size and granularity delimitation (FSCxSSC). In lymphocytes, positive

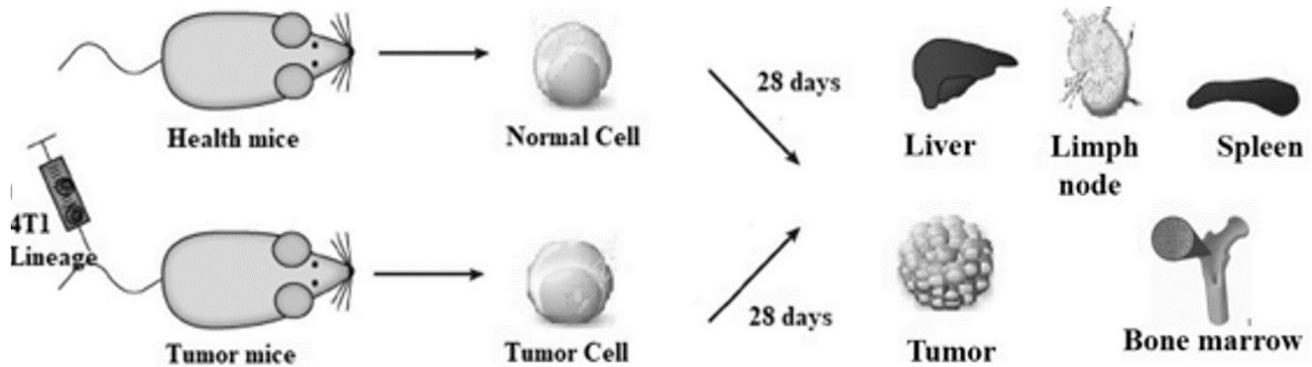


Figure 1. — Representation of tumor induction with 4t1 cell line in balb/c mice.

populations were selected simultaneously for  $CD3^+$  and  $IA^+$  (MHCII). An FSCx  $TCR\alpha\beta$  graph was plotted from this selection. Within each of these populations, a dot plot was used to delineate  $T_{DC}$  cells and thus average fluorescence intensity by histograms for surface markers, transcription factors and cytokines of interest.

#### Statistical analysis

For statistical data analysis we used the GraphPad Prism 6.0 software (GraphPad Software). The Mann-Whitney test was used to compare the 4T1-induced tumor and control groups and the Kruskal-Wallis and Dunn's post hoc tests to compare  $T_{DC}$  cell expression in tissue microenvironments: spleens, livers, sentinel lymph nodes, bone marrow and tumors. Results were expressed as medians (minimum and maximum values). Differences were considered statistically significant when  $p$  was less than 5% ( $p < 0.05$ )

## Results

### $T_{DC}$ cells predominate in healthy and tumor-induced 4T1 mouse lymph nodes

The representative cytometric cell flow profile revealed a predominant  $T_{DC}$  cell infiltrate ( $TCR\alpha\beta^+CD11c^+MHCII^+$ ) in the lymph nodes (Figure 2) of healthy mice (control) and 4T1 cell-induced breast cancer animals.

The frequency of  $T_{DC}$  cells was significantly higher in the sentinel lymph nodes of the control group 70.05 (52.78-87.32) and 4T1-induced tumor group 67.02 (67.02 - 97.88) ( $p < 0.0001$ ) compared to the other environments studied: lymph node versus spleen ( $*p < 0.05$ ), lymph node versus liver ( $**p < 0.001$ ), lymph node versus bone marrow ( $***p < 0.0001$ ) of the control group; and lymph node versus spleen ( $*p < 0.05$ ), lymph node versus liver ( $***p < 0.0001$ ), lymph node versus bone marrow ( $*p < 0.05$ ), lymph nodes versus tumor ( $**p < 0.0001$ ) of the 4T1-induced tumor group.

### The tissue microenvironment determines the immunosuppressive phenotype of $T_{DC}$ cells in the 4T1 cell-induced tumor group.

We evaluated surface markers (CD4, CD8 and CD86) and found in healthy mice (control group) increased expression of the CD4 molecule (helper T lymphocytes) in

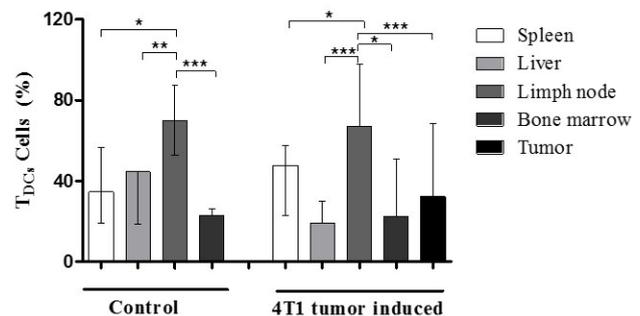


Figure 2. — Expression of  $T_{DC}$  cells in tissues of control and 4T1 tumor induced. (a) Representative graphs of two independent experiments ( $n = 15$ ) of flow cytometric analyses to identify  $TCR\alpha\beta^+ CD11c^+ MHC II^+ T_{DC}$ s cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced. (b) Percentual  $TCR\alpha\beta^+ CD11c^+ MHC II^+ T_{DC}$ s cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced. The results were analyzed by Kruskal-Wallis and Dunn's post hoc tests to compare the percentage of  $T_{DC}$ s cells in organs (represent by median with range). Differences were considered statistically significant at  $p < 0.05$  (5%).  $*p < 0.05$ ;  $**p < 0.001$ ;  $***p < 0.0001$ .

$T_{DC}$  cells in the liver compared to the other organs analyzed with median of 1662 (1551-1662) ( $p = 0.0001$ ) (Figure 3a). When looking at the 4T1-induced Tumor group, we noted that the tumor has the highest  $CD4^+ T_{DC}$  expression in tumor, with median of 2621 (1243 - 4962) ( $p = 0.0001$ ) (Figure 3a). When comparing groups, we observed in the 4T1-induced tumor group there was a significant decrease in  $CD4^+ T_{DC}$  compared to the control group with median of 1662 (1551 - 1662) ( $p = 0.0001$ ) in the livers of the mice studied (Figure 3a).

Liver-resident  $T_{DC}$  express significantly high levels of the  $CD8^+$  marker in the control group, with median of 3593 (2372 - 3593) ( $p = 0.0089$ ) (Figures 3b). In the 4T1-Induced Tumor group we found high  $CD8^+ T_{DC}$  in lymph node, with median of 2911 (1181 - 4342), but reduced in bone marrow 488.5 (488.5 - 2678) ( $p < 0.0001$ ) (Figures 3b). Comparing the control and 4T1-induced tumor groups, we

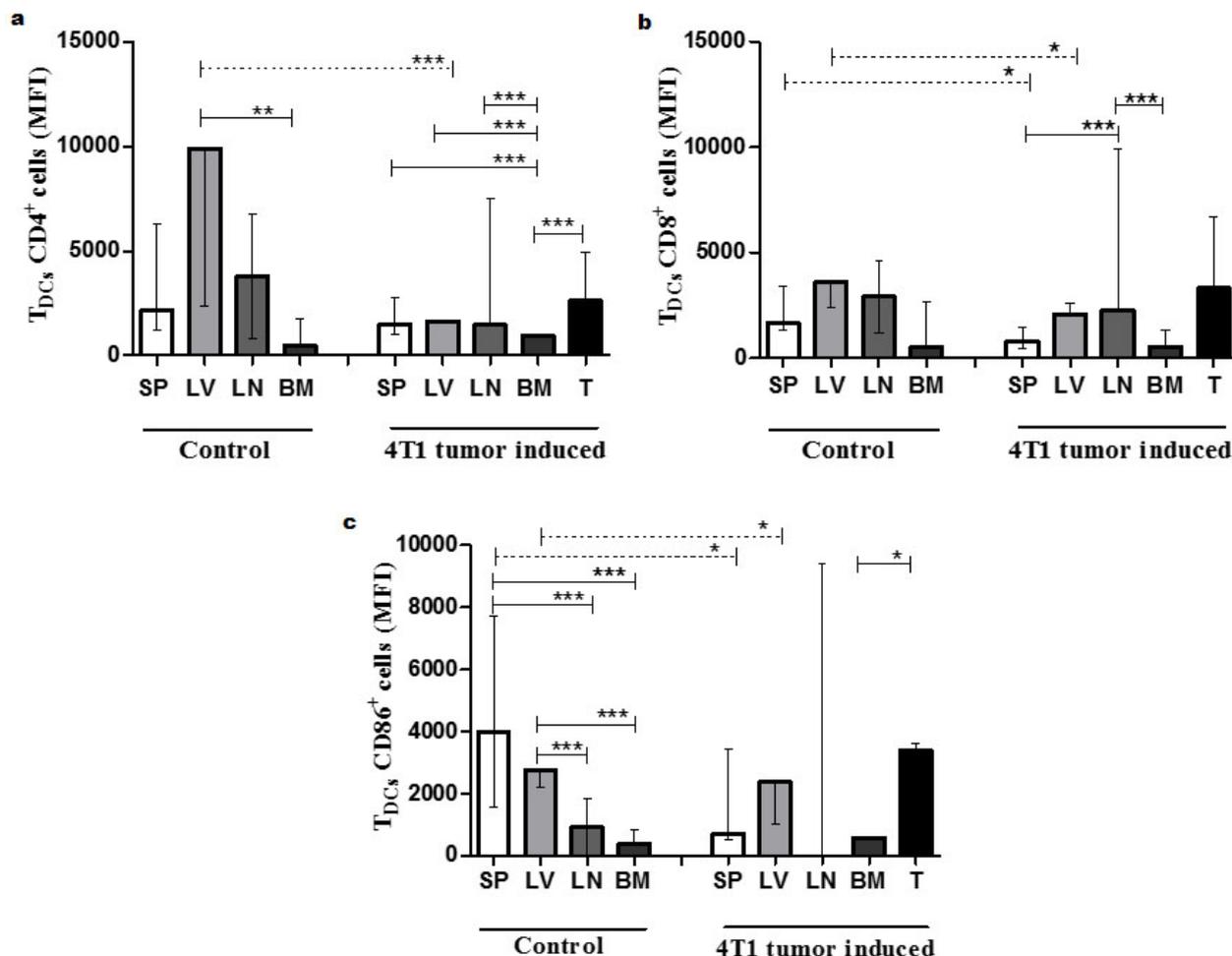


Figure 3. — The tissue microenvironment determines the  $T_{DC}$  cells phenotype positive for CD4, CD8 and CD86 control mice and 4T1 tumor induced. (a, b) Flow cytometric analyses to identify  $T_{DCs}$  CD4<sup>+</sup>, CD8<sup>+</sup> and CD86<sup>+</sup> cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced. Representative graphs of two independent experiments,  $n = 15$  each (median with range). (c) Mean fluorescence intensity of  $T_{DC}$  CD4<sup>+</sup> cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (d) Mean fluorescence intensity of  $T_{DC}$  CD8<sup>+</sup> cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. (e) Mean fluorescence intensity of  $T_{DC}$  CD86<sup>+</sup> cells in spleen, liver, lymph node, bone marrow and tumor of control mice and 4T1 tumor induced. The results were analyzed by and Kruskal-Wallis and Dunn's post hoc tests to compare the mean fluorescence intensity of  $T_{DCs}$  cells in organs statistical differences represented by solid line) and Mann-Whitney test to compare control and 4T1 tumor induced groups (statistical differences represented by dashed line). Differences were considered statistically significant at  $p < 0.05$  (5%). \* $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ . BM: Bone Marrow, LN: Lymph node, LV: Liver, MFI: Mean fluorescence intensity, T: Tumor.

noted low levels of CD8<sup>+</sup>  $T_{DC}$  cells in spleen, with median of 764.7 (485.8 - 1467) and liver with 2078 (2078-2602) ( $p = 0.0012$  and  $p = 0.0028$ , respectively) in the 4T1-induced tumor group. compared to the control group (Figure 3b).

In the analysis of the organs studied, we observed that in the control group, the spleen showed higher expression of CD86<sup>+</sup>, with median of 3997 (1550-7700)  $T_{DC}$  cells ( $p < 0.0001$ ) and bone marrow showed lower expression of these cells, with 398.0 (398.0 - 829.9) ( $p < 0.0001$ ) (Figures 3c). In the 4T1-induced Tumor group we found higher CD86<sup>+</sup>  $T_{DC}$  expression in tumor, with median of 3395 (1757-3604) ( $p = 0.0021$ ) (Figure 3c). By analyzing the

relationship of CD86<sup>+</sup>  $T_{DC}$  cells between control and 4T1-induced Tumor we identified low levels in spleen, with median of 3997 (1550-7700) and liver with 2742 (2197-2742) of the 4T1-induced tumor group ( $p = 0.0012$  and  $p = 0.0337$ , respectively) (Figure 3c).

#### *Different tissue microenvironments induce the positivity of transcription factors in $T_{DC}$ cells*

We investigated Tbet expression levels in  $T_{DC}$  cells in the various tissues studied (spleen, liver, lymph node, bone marrow and tumor). We observed that in the control group high levels of  $T_{DC}$  Tbet<sup>+</sup> levels were found in the sentinel lymph nodes, with a median of 6425 (6153 - 6697) ( $p <$

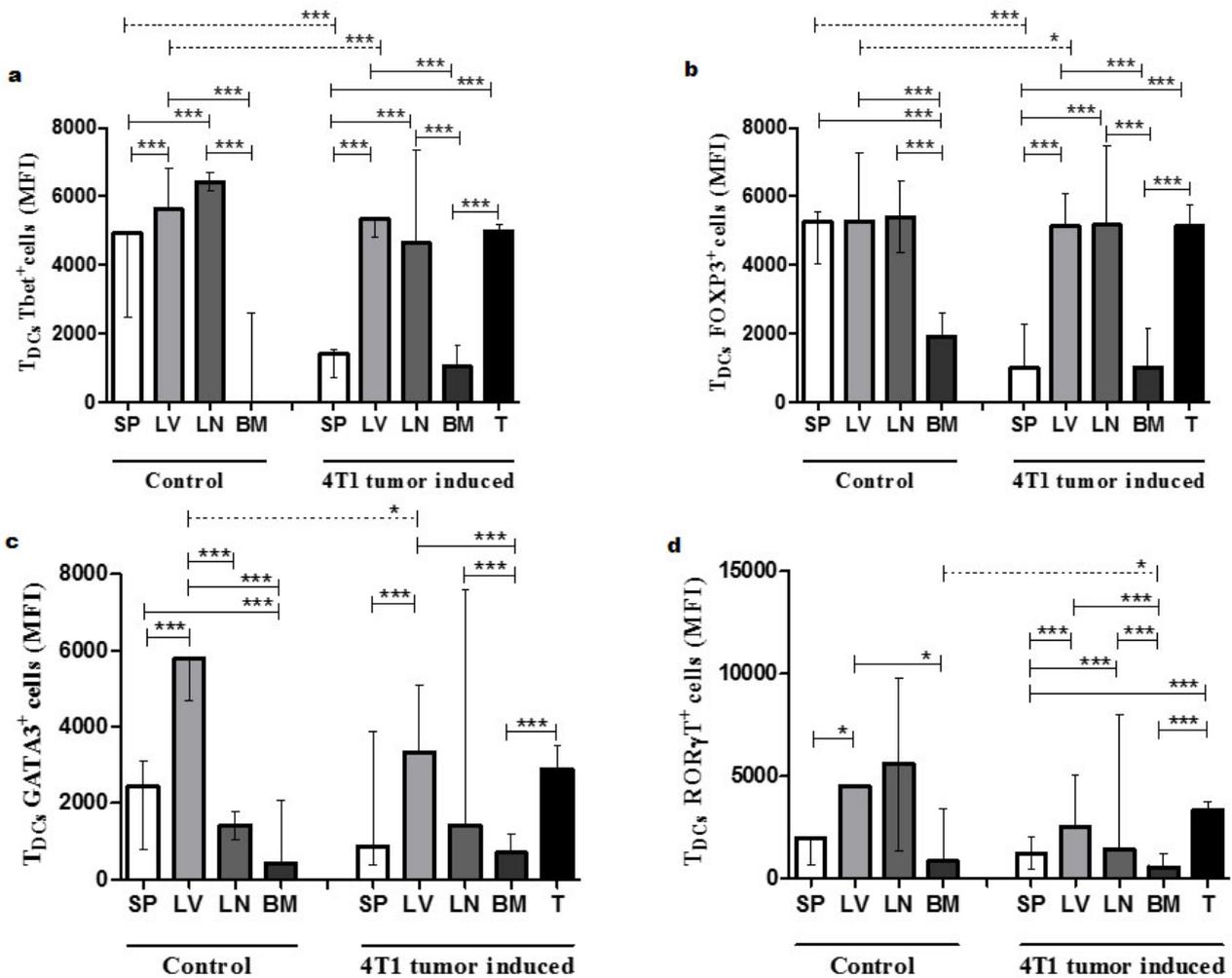


Figure 4. — Different tissue microenvironments induce positivity of transcription factors in  $T_{DC}$  cells Tbet<sup>+</sup>, Foxp3<sup>+</sup>, Gata3<sup>+</sup> and RorγT<sup>+</sup> cells in control and 4T1 tumor induced mice. Flow cytometric analyses to identify  $T_{DC}$  Tbet<sup>+</sup>, Foxp3<sup>+</sup>, Gata3<sup>+</sup> and RorγT<sup>+</sup> cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced mice. Representative graphs of two independent experiments,  $n = 15$  each. (a, b, c and d). Graphs represent mean fluorescence intensity data from two independent experiments,  $n = 15$  each (median with range); (e) Representation by heat map about mean fluorescence intensity of Tbet<sup>+</sup>, Foxp3<sup>+</sup>, Gata3<sup>+</sup> and RorγT<sup>+</sup>  $T_{DC}$ s, respectively cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced mice. The results were analyzed by Kruskal-Wallis and Dunn's post hoc tests to (statistical differences represented by solid line) compare the mean fluorescence intensity of  $T_{DC}$ s cells in organs in A, B, C. and D. The Mann-Whitney test to compare control and 4T1 tumor induced mice groups in A, B and C (statistical differences represented by dashed line). Differences were considered statistically significant at  $p < 0.05$  (5%). \* $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ . BM: Bone Marrow, LN: Lymph node, LV: Liver, MFI: Mean fluorescence intensity, T: Tumor.

0.0001), while in the 4T1-induced tumor group, we found greater expression of this transcription factor in liver, with a median of 5338 (4804 - 5338) ( $p < 0.0001$ ). Comparing control and 4T1-induced tumor groups, we found that the presence of  $T_{DC}$  Tbet<sup>+</sup> in 4T1-induced breast cancer animals were decreased in spleen, with a median of 1427 (704.1 - 1517) ( $p < 0.0001$ ) and liver with a median of 5338 (4804 - 5338) ( $p = 0.0001$ ) compared to the control group (Figure 4a).

Analyzing the organs, we found that the presence of  $T_{DC}$  cells positive for the transcription factor Foxp3,

showed higher expression in the sentinel lymph nodes of the control group, with a median of 5404 (4371 - 6436) ( $p < 0.0001$ ) and also of the 4T1 induced tumor group, with a median of 5165 (5165 - 7473) ( $p < 0.0001$ ). Evaluating between the 4T1-induced tumor and control groups, we noted a decrease in Foxp3<sup>+</sup>  $T_{DC}$  in the 4T1-induced tumor group in spleen, with a median of 1016 (962.9 - 2265) ( $p < 0.0001$ ) and in liver with a median of 5133 (5133 - 6077) ( $p = 0.0337$ ) (Figure 4b).

Gata3<sup>+</sup>  $T_{DC}$  cells showed significantly higher levels in the liver of the control mice, with a median of 5788 (4700-

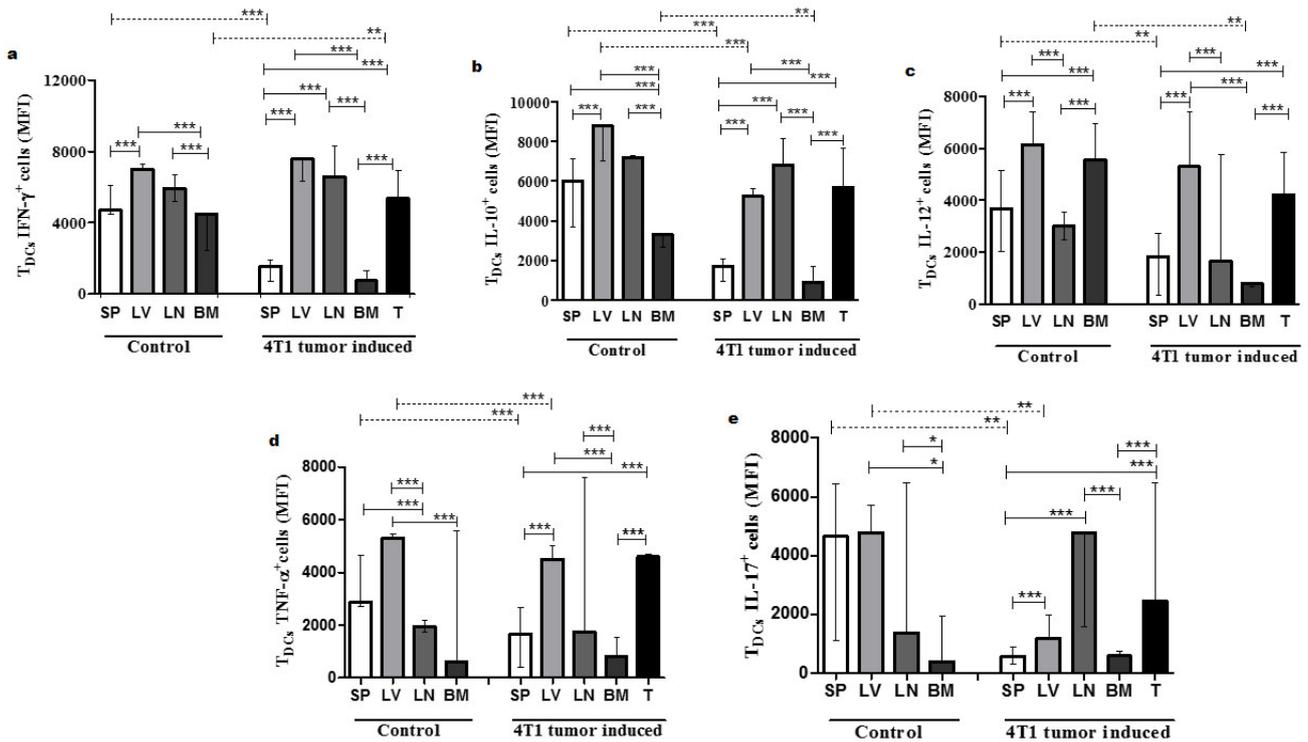


Figure 5. — Increased proinflammatory cytokine levels in  $T_{DC}s$   $IFN-\gamma^+$ ,  $TNF-\alpha$ ,  $IL-10^+$ ,  $IL-12^+$  and  $IL-17^+$  from different tissue microenvironments from control and 4T1 tumor induced mice. Flow cytometric analyses to identify  $T_{DC}s$   $IFN-\gamma^+$ ,  $TNF-\alpha$ ,  $IL-10^+$ ,  $IL-12^+$  and  $IL-17^+$  cells in spleen, liver, lymph node, bone marrow and tumor of control and 4T1 tumor induced mice. Representative graphs of two independent experiments,  $n = 15$  each. (a, b, c, d, e, f, g, h, i and j) mean fluorescence intensity of  $T_{DC}s$   $IFN-\gamma^+$ ,  $TNF-\alpha$ ,  $IL-10^+$ ,  $IL-12^+$  and  $IL-17^+$ , respectively cells in spleen, liver, lymph node, bone marrow and control group 4T1 tumor induced mice. Graphs represent Mean fluorescence intensity data from two independent experiments,  $n = 15$  each (median with range). The results were analyzed by Kruskal-Wallis and Dunn's post hoc tests to (statistical differences represented by solid line) compare the mean fluorescence intensity of  $T_{DC}s$  cells in organs and Mann-Whitney test to compare control and 4T1 tumor induced mice groups (statistical differences represented by dashed line). Differences were considered statistically significant at  $p < 0.05$  (5%). \* $p < 0.05$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ . BM: Bone Marrow, LN: Lymph node, LV: Liver, MFI: Mean fluorescence intensity, T: Tumor.

5788) ( $p < 0.0001$ ) and the 4T1-induced tumor group, with a median of 3346 (3346 - 5101) ( $p < 0.0001$ ), compared to the spleen, lymph node, bone marrow and tumor. However, comparing the control group with the 4T1-induced tumor group we found a lower expression of these cells in the liver of the 4T1-induced tumor group, with a median of 3346 (3346 - 5101) compared to the 5788 (4700- 5788) control group ( $p = 0, 0028$ ) (Figure 4c).

Comparing organs, we found in the lymph node of the control group a higher level of  $T_{DC}$  expression  $Ror\gamma^+$ , with a median of 5589 (1373 - 9805) ( $p = 0.0010$ ) and in the group of mice induced by 4T1, the tumor has a higher expression, with a median of 3340 (3223 - 3722) ( $p < 0.0001$ ) (Figure 4d).

#### Increased levels of proinflammatory cytokines in $T_{DC}s$ from different tissue microenvironments

The positive  $IFN-\gamma$  antiinflammatory cytokine in  $T_{DC}$  was higher in the liver tissue microenvironment of control mice, with a median of 7010 (7010 - 7293), data also found in the 4T-induced tumor group, with a median of 7590

(6335 - 7590) ( $p < 0.0001$ ). In the comparison between the two groups studied, we found that in the 4T1-induced tumor group, the expression of  $IFN-\gamma^+$   $T_{DC}$  significantly decreased in the spleen, with a median of 1554 (705.3 - 1885) ( $p < 0.0001$ ) and bone marrow, 733.1 (733.1 - 1307) ( $p = 0.0002$ ) (Figure 5a).

We evaluated the expression of cytokine  $IL-10$  in  $T_{DC}$  in the tissues studied, and higher expression was found in the control mouse liver with a median of 8777 (6999 - 8777) ( $p < 0.0001$ ) and in the 4T1-induced tumor group lymph node with 6791 (6791 - 8162) ( $p < 0.0001$ ). The results found showed that when tumor cells were involved,  $IL-10^+$   $T_{DC}$  expression decreased significantly in spleen, with a median of 1689 (914.3 - 2049) ( $p < 0.0001$ ), 5219 (5219 - 5619) ( $p < 0.0001$ ) in liver, and 894.0 (894.0 - 1712) ( $p = 0.0002$ ) in bone marrow (Figure 5b).

The  $IL-12^+$   $T_{DC}$  evaluated were more evident in the liver of both study groups, control the median was 6153 (6153 - 7415) ( $p = 0.0022$ ) and 4T1-induced tumor the median was 5300 (5300-7415) ( $p < 0.0001$ ). Again, we note that the presence of tumor cells induces a significant de-

crease in tumor cell expression, particularly in the spleen with a median of 1841 (360.7-2728) and bone marrow with a median of 791.0 (688.8 - 791.0) of 4T1-induced breast cancer mice ( $p = 0.0002$ ) (figure 5c).

Represented by Figure 5d, tumor necrosis factor (TNF- $\alpha$ ) expressed in  $T_{DC}$  was found to be highest in the liver of both groups, with control with a median of 5303 (5303 - 5454) ( $p = 0.0001$ ) and 4T1-induced tumor with a median of 4494 (4494 - 5032), respectively. Findings observed in the comparison between both groups, we found significant decrease in TNF- $\alpha^+$   $T_{DC}$  levels in spleen, with a median of 1655 (403.8 - 2673) and bone marrow 819.2 (819.2 - 1521) ( $p < 0.0001$ ).

We also analyzed the expression of IL-17 +  $T_{DC}$  and observed elevated levels in the liver of the control group, with a median of 4793 (4783 - 5717) mice ( $p = 0.0026$ ) and the 4T1-induced tumor lymph node with a median of 4776 (1590 - 4776) ( $p < 0.0001$ ). We found that in the presence of tumor cells this immunosuppressive cytokine was decreased in the spleen, with a median of 578.5 (326.3 - 873.8) ( $p < 0.0001$ ) and liver cancer-induced mice with 1200 (1200 - 1988) ( $p = 0.0001$ ) (Figure 5e).

## Discussion

Kuka *et al.* (2012), pioneers in the discovery and characterization of  $T_{DC}$  cells, reported that approximately 7% of mouse DCs expressed on their surface the TCR  $\alpha\beta$  receptor, with 0.04% present in the spleen, a differentiated characteristic related to conventional  $\alpha\beta$  T cells [1].

Recent studies have shown that some subpopulations of macrophages have the expression combinatorial receptor TCR $\alpha\beta$  [7]. This immune receptor is present in macrophages in autoimmune diseases [8], in diseases such as atherosclerosis [9] and in the tumor microenvironment playing an anti-inflammatory role [10]. A study carried out with human neoplasms (colon cancer, esophageal cancer, liver carcinoma, melanoma) demonstrated that 40% of macrophages express TCR $\alpha\beta$  in the tumor microenvironment and more than 30% in experimental breast cancer induced in mice with adenocarcinoma cells breast [9].

In our studies, we used a 4T1 murine mammary adenocarcinoma cell line. This experimental model is widely used in the evaluation and better understanding of tumor biology. It is a highly tumorigenic and invasive cell line, that is, spontaneous metastases are observed in various organs [11]. According to the literature, research uses this line because it allows to evaluate the immune response to malignant neoplasms effectively [11-15].

The mouse mammary carcinoma 4T1 was originally isolated as subpopulation 410.4 derived from a spontaneously arising mammary tumour in BALB/c fC3H mice [16-17]. The 6-thioguanine-resistant 4T1 tumour metastasizes via the haematogenous route to liver, lungs, bone and brain, making it a good model of human metastatic breast cancer [18]. 4T1 grows progressively and causes a uniformly lethal disease, even after excision of the primary tumour

[19].

According to the experimental design used, the parameters related to the animals' weight were not evaluated. The mice used in our study were born, raised and fed under the same conditions, according to international standards for the management of experimental animals [20]. Thus, the animals showed no obvious signs of obesity or malnutrition. The time required for the study was not enough for the malignancy to result in weight changes due to obesity or cachexia.

In lymph nodes, this same characterization study found that 0.06% of lymph node DCs had the TCR  $\alpha\beta$  marker [1]. In view of this, this study verified the presence of these cells in different tissue microenvironments such as the spleen, liver, lymph node, bone marrow and tumor in healthy conditions and in the presence of breast cancer in mice induced by the 4T1 cell line.

Lymph nodes are frameworks that specialize in interception between innate and adaptive immunity. Professional antigen presenting cells (APCs) such as dendritic cells (DCs) and lymphocytes are brought to this organ for antigens [21].  $T_{DC}$  are believed to perform the same mechanism [1].

As a result, we found that  $T_{DC}$  are present in larger amounts in lymph nodes when studying the expression of these cells in the other environments studied ( $p < 0.0001$ ). Regarding the comparison between healthy and breast cancer-induced mice, no significant differences were observed related to the expression of these cells.

Taken together, these results suggest that  $T_{DC}$  cells play a previously unknown role in the presentation or recognition of antigens on lymph nodes. In addition, we also demonstrate that the presence of neoplastic cells has no influence on the frequency of  $T_{DC}$  cells.

Following the phenotypic characterization line of  $T_{DC}$  in different tissue microenvironments, we analyzed molecules that characterize helper T lymphocytes (CD4) and cytotoxic T lymphocytes (CD8) and dendritic cells with the presence of the B7-2 costimulatory molecule (CD86).

In healthy mice we found helper  $T_{DC}$  (CD4<sup>+</sup>  $T_{DC}$ ) and cytotoxic  $T_{DC}$  (CD8<sup>+</sup>  $T_{DC}$ ) cells in greater quantity in the liver compared to the other organs ( $p < 0.0001$ ). In contrast to animals with breast cancer, these markers were more evident in the tumor and sentinel lymph nodes, so we can infer from the regulatory cells, ie, suppressed by the tumor conditions present there ( $p < 0.0001$ ).

Our results corroborate the study by Rad *et al.* (2015) which indicated that the ratio of helper and cytotoxic T lymphocytes was somewhat different in peripheral blood, tumor microenvironment and lymph nodes, resulting in lymph node as tissue. with the largest amount of these lymphocytes followed by peripheral blood and tumor tissues [6-22].

Another hypothesis would be that these cells may be in a stage of lymphocyte or dendritic cell maturation, thus not being able to migrate to the tumor microenvironment, thus

inferring the characterization of a greater presence of these cells in the lymph node compartments. Studies with mouse models and human samples have shown that metabolic dysregulation caused by hepatic tumor precursor non-alcoholic fatty liver disease (DHGNA) causes selective loss of intrahepatic T-lymphocytes, but this change was not found with regard to hepatic cytotoxic T lymphocytes in the presence of tumor cells, which consequently accelerate the processes of hepatocarcinogenesis [23].

The liver is considered an immunological organ formed by a complex histological structure composed of 70% hepatocytes, 16 to 22% intrahepatic lymphocytes and 30% non-parenchymal cells [24] of which the hepatic macrophages correspond 80 to 90% [24-25].

Recent studies have demonstrated the presence of several types of lymphocytes residing in the liver. We find in the microenvironment of this organ CD8 memory lymphocytes, invariant natural killer cells (iNKT), mucosa associated T cells (MAIT) and  $\gamma\delta$ T cells, in addition to innate lymphoid cells (ILCs) and NK that can remodel phenotypic and functional regulatory characteristics transcriptional [25].

Functionally, these lymphocytes recognize a wide variety of harmful signals, playing the role of sentinels contributing to immune surveillance in the face of infectious and non-infectious responses in the liver [25].

Regarding  $T_{DC}$  cells positive for the B7-2 molecule ( $T_{DC}$  CD86<sup>+</sup>), we observed the presence of significant spleen in healthy mice and breast cancer-induced tumor ( $p < 0.0001$ ) ( $p < 0,0001$ ).

The CD86 costimulatory molecule is related to the B7/CD28 family and the tumor necrosis factor (TNF) family, which are involved in triggering the cell-mediated immune response, and in later stages, by activating T-receptor-bound T lymphocytes (TCR) [26]. Thus, the  $T_{DC}$  were characterized and studied because they have a functional TCR and are self-sufficient for antigen presentation. (1). Our results indicate that the B7-2 molecule may be present in splenic  $T_{DC}$  and favor antigen presentation by  $T_{DC}$ . The immune system cells present in the spleen include various subsets of T cells, B and DCs, and other cell types [27].

Studies by Slits and colleagues (2016) analyzed subsets of myeloid cell-derived suppressor cells (MDSC) in the hepatic parenchyma of mice with hepatocellular carcinoma, which may cause phenotypic changes in efficient defensive cells called Kupffer cells, and found that these cells expressed less CD86 and MHCII costimulatory molecule in the livers of these mice [28].

In order to verify the  $T_{DC}$  cell profile, we evaluated the transcription factors Tbet, Gata3, Ror $\gamma$ t and Foxp3. Changes in these transcription factors were found in TDCs from different tissues in both groups.

Our results demonstrated that Tbet transcription factor in TDCs is present more in lymph nodes of healthy mice and liver of mice with breast cancer ( $p < 0.0001$ ). Tbet is considered a chief regulator of Th1 lymphocytes [29]. Kach-

ler and colleagues (2018) observed increased expression of Tbet and Foxp3 transcription factors in pulmonary helper T lymphocytes (TCD4<sup>+</sup>) of lung cancer mice, accompanied by increased production of TGF- $\beta$ , immunosuppressive cytokine [31]. In our study, we observed an increase in Foxp3 expression in  $T_{DC}$  in sentinel lymph nodes ( $p < 0.0001$ ) and a decrease in spleen and liver in mice with breast cancer ( $p < 0,0001$ ).

In Gata3  $T_{DC}$ , we found a marked presence in the liver ( $p < 0.0001$ ) in both healthy and breast cancer mice. Gata3 transcription factor is a major determinant of Th2 lymphocyte polarization [31] and studies suggest that expression of this marker in breast cancer is highly related to luminal transcription of this cancer [32], in part because it causes an inflammatory process and favors tumor growth [33].

Wei *et al.* (2017) reported that Gata3 expression was negatively regulated in the presence of gastric cancer mainly in humans, and was still associated with tumor size, stage in which it was present and episodes of metastasis [34]. In our study, we also identified a remarkable expression of  $T_{DC}$  Ror $\gamma$ t cells in the tumors of mice with experimental breast cancer ( $p < 0.0001$ ), thus assuming that the high levels of this transcription factor are related to a Th17 profile that can influence the tumor microenvironment in its promotion and also regulate the activity of neighboring stromal cells [35]. The results are similar to those observed in the study by Wang *et al.* (2016), who reported in their studies that the transcription factor Ror $\gamma$ t was strongly expressed in human prostate cancer tumors [36].

With these results we can establish that the expression of transcription factors in TDCs cells varies according to the tissue in which they are found, and this variation can often be due to the escape mechanism that tumor cells acquire for an ineffective immune response. .

By evaluating the phenotypic and functional characteristics of TDCs cells related to expression of INF- $\gamma$ , TNF- $\alpha$ , IL-10, IL-12 and IL-17 cytokines in tissues obtained from healthy mice and 4T1-induced breast cancer, we have seen that IFN- $\gamma$  expression in TDC cells was suppressed in lymph nodes obtained from mice with experimental breast cancer ( $p < 0,0001$ ).

IFN- $\gamma$  cytokine plays an important role in immune response to infectious agents and tumors [37]. Studies by Tanner and colleagues (2016) have suggested that immune system failures responsible for carcinogenesis may contribute to tumor development and progression; furthermore, mice with colorectal cancer demonstrated that helper T lymphocytes produced less IFN- $\gamma$  due to mutations caused by tumor cells to immune cells [38].

We evaluated the expression of tumor necrosis factor alpha (TNF- $\alpha$ ) in  $T_{DC}$  cells in the tissues studied and found a higher amount of this cytokine in the liver of healthy and breast cancer mice ( $p < 0,0001$ ). TNF- $\alpha$  is a cytokine with proinflammatory effect comprised in inflammatory circumstances [39], but also present in advanced tumors, responsible for cachexia and even involved in escape mechanisms

[40]. Studies by Kastl *et al.* (2014) have shown that hepatocellular carcinoma (HCC) is enriched by high levels of TNF- $\alpha$  responsible for NF- $\kappa$ B activation, favoring the migration of immune cells to the site [41].

We found that T<sub>DC</sub> cells constitute a cellular repertoire, which is IL-10 producing, and in mice with experimental breast cancer, IL-10<sup>+</sup> T<sub>DC</sub> were reduced in all studied organs, being more evident in sentinel lymph nodes ( $p < 0,0001$ ).

Studies related to IL-10 cytokine have shown that many cells have the ability to produce it, including immune system cells such as monocytes/macrophages, dendritic cells, B lymphocytes, regulatory T cells, CD4 T lymphocytes, CD8 T and NK cells [42].

In addition to IL-12, we also studied the production of IL-17 by T<sub>DC</sub> in different tissue microenvironments. Our results consisted of the decrease in IL-17<sup>+</sup> T<sub>DC</sub> cells in the lymph node, spleen and liver obtained from mice with experimental breast cancer ( $p < 0.0001$ ). IL-17 is a cytokine produced by Th17 profile T helper cells which is associated with down regulation of immune cells and tumor promotion to various immune responses [43].

Studies of mice with cervical cancer by Yang *et al.* (2016) showed that lymphocyte expression of this cytokine may reduce the size of tumors in transplanted mice, and association with tumor cells may increase lymphocytic infiltration into the tumor tissue microenvironment [44].

Overall, these data show that although there were differences in tissue microenvironment, the phenotype of cytokine expression in T<sub>DC</sub> showed detectable differences in the presence of tumor cells, thus inferring the action of the tumor evasion mechanism provided by the presence of tumor cells.

Among the limitations of this study, it should be mentioned that the methodology did not include performing cell sorting or cDNA microarray analysis in order to provide a better evaluation of gene expression of T<sub>DC</sub> cells, which would have allowed us to achieve more.

The main objectives have been elucidated, allowing a further explanation of the importance and functionality of these cells. This study is unprecedented in the analysis of T<sub>DC</sub> cells, with respect to the surface markers (CD4, CD8 and CD86), transcription factors (Tbet, Foxp3, Gata3 and Ror $\gamma$ t) and cytokine expression (INF- $\gamma$ , TNF- $\alpha$ , IL-10, IL-12 and IL-17 in different mouse organs (spleen, liver, lymph node, bone marrow and tumor) under the influence of breast cancer tumor cells.

## Conclusions

T<sub>DC</sub> cells showed a decrease in several surface markers, transcription factors and cytokines in the studied tissue microenvironments obtained from animals with 4T1 cell-induced cancer. In the lymph nodes the presence of T<sub>DC</sub> cells was quite evident, but it is in the liver that we found that these cells can play an immunoactivating role, as evidenced by positive T lymphocyte markers characteristic of

the Th1 profile, by the expression of Tbet and also the production of cytokines favorable to effective antitumor immune response such as TNF- $\alpha$ , INF- $\gamma$  and IL-12.

Taken together, these results reveal that the tissue microenvironment provides strong indications for a phenotypic adequacy of T<sub>DC</sub>, raising the question of whether phenotypic alterations can be driven by the tissue microenvironment in the presence of the tumor and thus result in functional differences in relation to the tumor expression of these markers against an immune response.

Future research is needed to address crucial issues related to T<sub>DC</sub> cell immaturity, which will certainly provide additional information on the expression process and functionality related to possible cancer immunotherapy.

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## Conflict of Interest

The authors declare no conflicts of interest.

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