Original Article

Dendritic Cell-Based Immunity: Screening of Dendritic Cell Subsets in Breast Cancer-Bearing Mice

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Abstract

Background: Breast cancer (BC) is the most devastating disease, particularly the lethal invasive form. It is the most underlying cause of death among women worldwide. The expansion of BC is controlled by a variety of alterations in the tumor cells themselves, in addition to the state of the immune system, which has a direct influence on the tumor microenvironment. Numerous receptors expressed by T-cells interact with ligands on antigen-presenting cells to provide activation signals results in mounting effector anti-tumor T-cell responses. On the other hand, there is a dearth of information about the actual interactions and reactions of T-cells and dendritic cells (DCs) all through the progression of tumor development. **Aim:** Immune system response against BC was investigated through tumor induction in mice. The size and volume of the tumor were calculated. Moreover, the phenotypical profile of T-cells and DCs from lymph nodes (LN) and spleens of BC-bearing mice was investigated. In addition, the levels of Transforming growth factor-β, Interferon-gamma (IFN-γ), Interleukin IL-2, IL-10, IL-4, IL-12, and tumor necrosis factor (TNF)-α were determined. **Materials and Methods:** MDA231 cells were utilized to induce BC in 30 white BALB/C mice, whereas the other 30 mice acted as healthy controls and were not treated with any cancer-causing agents. The impact of malignancy was evaluated using flow cytometry based on the marking surface molecules, as well as the titer of specific cytokines of the mice's LN culture using the ELISA method. These cytokines included transforming growth factor-β (TGF-β), IFN-γ, IL-2, IL -10, IL -4, IL -12, and TNF-α. **Results:** The findings showed that the maturation of DCs was inhibited, followed by an accumulation of immature DCs. These immature DCs increase the release of TGF-β and cytokines like IL-10 and inhibit the release of IFN-γ and IL-12 in the culture supernatant of nodal lymph and spleen suspension of BC-bearing mice compared to control. In addition, there was a low expression of CD80 and CD86 on DCs, which indicates a low maturation process. **Conclusion:** According to the findings, the tumor microenvironment may have been responsible for preventing the maturation of DCs. This, in turn, weakened the immune response and facilitated the ability of the tumor to proliferate. Furthermore, the tumor microenvironment increased the number of immature DCs by inhibiting their stimulation by overexpression of TGF-β-produced by regulatory T lymphocytes and stimulation of tumor cells. In addition, the tumor microenvironment stimulated the secretion of cytokines such as IL-10, and CD4 and decreased the secretion of IFN-γ-and IL-12 in tumor-induced mice cultured LN and spleen.

Keywords: And transforming growth factor-beta, breast cancer, dendritic cells, interferon-gamma, interleukin-10, interleukin-12, interleukin-2, interleukin-4, T lymphocytes, tumor necrosis factor-α

Introduction

Dendritic cells (DCs) are the masters of the antigen-presenting cells (APCs) because they display the tumor antigen (capture and process) on their surface for T-cells as an exogenous antigen for T helper (CD4+) and endogenous antigen for cytotoxic (CD8+) cells.[1] They are often discovered in an immature form in peripheral tissue, where they function to capture and process antigens that have been collected through the use of complement and immunoglobulins.[2] Following this, DCs move to secondary lymphoid tissue as a result of inflammatory circumstances and the release of cytokines. This causes immature DCs to develop. At this stage, DCs are able to activate naive T-lymphocytes through the process of

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cytokine secretion.[3,4] MHC molecules are expressed on the surface of DCs at this time.

Breast cancer (BC) is a form of terminal cancer with the highest mortality rate.[5] The progression of the tumor is caused by the formation of a vascular link between tumor cells, which then attracts lymphatic cells and immune cells through the influx of cytokines and chemokines, which in turn suppresses the immune system's reaction to advanced cancer.[1] On the other hand, the immune system either inhibits the growth of the tumor by eradicating tumor cells or promotes tumor growth.[6] This occurs because of a cooperative action between T lymphocytes and DCs in lymph nodes (LN), which are essential for the activation of anti-cancer immune reactions. Through the T-cell receptor (TCR) that is located on the DCs receptor that expresses MHC, T-cells are able to recognize tumor antigen.[7] Following the activation of T-cells and the production of cytokines, rapid proliferation takes place.[8] Using molecules that have a stimulatory action and adhere to the surface of DCs, several researchers have pointed out the critical role that DCs play in the activation, growth, maturation, and tolerance of T-cells.[9-11] These researchers have used molecules that have the ability to stimulate DCs. One of the primary goals of advanced malignancies is to suppress DCs by decreasing the number of mature DCs capable of doing their jobs effectively and increasing the number of immature DCs unable to activate T-cells or induce regulatory T-cells.[12]

The immune response is inhibited by BC, which leads to increased numbers of $CD25^+$ (regulatory T-cells) and $CD4^+$ cells.[13,14] In addition, levels of cytokines that inhibit the immune response, such as interleukin (IL-10) and Transforming growth factor-β (TGF-β), are increased. For instance, tumor-induced models, such as mice, have been utilized to demonstrate the mechanism of tumor progression. In certain tumors, mice are regarded as the perfect models in which the interaction between clone DCs subsets and T-cells.[15] This is because rats are more susceptible to developing certain types of tumors. Unfortunately, specific answers are lacking owing to discrepancies between the immune systems of humans and the models used in laboratories that use animals.[16]

cDC1 (myeloid conventional DCs) are responsible for the process of cross-presenting antigens to MHC class I and initiating cytotoxic immune responses of type I. Innate lymphoid cells 2 (ILC2) and TH2 cells are activated and induced to respond by cDC2, along with ILC3 and TH17 immunological responses. In most tumor models, the effector function of $CD8^+$ must be supported by $CD4^+$ cells.[17-19] The researchers have not yet determined why cDC2 cannot transmit tumor antigen to LN in advanced tumors which is still an open question. In other words, the quantity of $CD11b^+$ cDC2 within LN is equivalent to the number of $CD103^+$ cDC1, according to.[20] CD11c and MHC-II are present in mice. In addition, it has been shown that the endocytic receptors CD36, which are accountable for the detection of apoptotic cells, are expressed in reduced amounts.[21] Dead cells boost the expression of CD40, CD80, CD86, and MHC-II on DCs, in addition to the release of cytokines that contribute to an inflammatory state, such as IL-1, IL-12, IL-6, and TNF-α.[16] It is essential to provide evidence of macrophages (CD115, CD14, and CD64), $CD11b^+$ cDCs, and cDC markers (CD26 and CD24).[22] Marked expression of PD-1, as well as PD-L1 and PD-L2, is caused by hypoxia, which is caused by a hypoxic tumor microenvironment. CD152 (CTLA-4) also shows a high level of expression.[23]

Research that focuses on the effect of DCs on the induced tumors can provide a source of speculation on upcoming vaccines, particularly immunotherapeutic vaccinations that rely on inducing cytotoxic $CD8^+$ and NK cells as well as Interferon (IFN)-gamma-producing lymphocytes. These vaccinations are necessary for the body to mount an effective anti-tumor immune response. As a result, the purpose of this study was to investigate the role that DCs play in the evolution of BC tumors in mice that were induced by MDA231 cells. Initially, flow cytometry was used to measure the immune response to generate BC. The evaluation was based on markers that were found on the surface the immune cells. In addition, the supernatant of the culture of mice LN and spleen was evaluated using ELISA to measure the levels of IL-2, IL-4, IL-10, IL-12, IFN-gamma, TGF-β, and TNF-alpha.

Materials and Methods

Cells

The Immunology Unit of King Fahd Medical Research Center (KFMRC), King Abdulaziz University (KAU), located in Jeddah, Saudi Arabia, provided the MDA231 cells used in this study. DMEM, which stands for Dulbecco's Modified Eagle's Medium, was used to cultivate the cells (Thermo Fisher, USA). The media were improved by adding 1 mmol/mL of L-glutamine, 10% heat-inactivated fetal bovine serum (Gibco, USA), and 1% penicillin/streptomycin solution comprising 10,000 units/mL penicillin and 10,000 g/mL streptomycin (HyClone, South Logan, USA).

Animals and *In vivo* Protocol

All of the experiments on animals were conducted following procedures approved by the Animal Care and Use Committee at KFMRC, KAU reference number 07-CEGMAR-BIOETH 2021. These approvals were given based on the bio-ethical guidelines for animal studies that were adopted by The National Bioethical Committee of Saudi Arabia (NACSA), which was registered at KACST.

Female BALB/c mice that were between 6 and 8 weeks old were obtained from the KFMRC's Animal House and then split into two groups of 30 mice each. The first group, which included a total of 30 participants, served as a healthy control and was given simply saline. The second group, which included 30 mice, was a tumor-induced group (TG) that was given 1.5×10^7 MDA231 cells suspended in 0.1 ml of phosphate-buffered saline (PBS). The experiment lasted for a total of 45 days, at the end of which time every mouse was euthanized.

Induction of MDA231 cell Breast Cancer in mice

Collecting MDA231 cells that had reached a 60% confluent state, washing them once in serum-free plane media, and then re-suspending them in PBS at a concentration of 1.5×10^7 cells/0.1 mL PBS were the subsequent steps. In one group of female BALB/c mice, 0.1 mL of the cell suspension was injected subcutaneously into the abdomen mammary fat, whereas in the other group, 0.1 mL of PBS was injected in the same manner. Both groups were given the injections at the same time. The following equation was used to determine the size of the tumor:

$$V = 0.5236\ d1\ d2$$

It was used in the process of calculating the volume of the tumor, with d1 representing the most minor diameter and d2 representing the maximum diameter. At the end of the experiment, on day 45, all of the mice were put to sleep (also known as "euthanasia").[24]

Examination of tumor

The spleens, LN, and tumors of mice that had been subjected to MDA231 cell-induced BC, in addition to mice that served as normal standard control, were removed. In addition, tumor size was measured and compared to the negative control group (CG) injected with saline.

In order to digest the tissue and generate cell suspensions, the samples of both LN and spleen were first diced and then subjected to a treatment with 500 units/mL of collagenase IV (Sigma) for 1 h at 37°C with constant stirring. Part of the cell suspension was processed (as described in section 1.5) for flow cytometry and the supernatant was collected for ELISA (section 1.6).

Single-cell suspensions were diluted with 10% fetal calf serum (FCS) and RPMI 1640 and then added to the mixture (Gibco, England). Cells were allowed to grow in the presence of GM-CSF and IL-4 at concentrations of 20 and 10 ng/mL, respectively, for a total of 6 days. On days 3 and 5, cells were refed by replacing half of the media with fresh new media. This process was repeated. After 7 days, the morphology of DCs was obtained by treating immature DCs with 1000 ng/mL of lipopolysaccharide (LPS), while the latter was left untreated for negative control.[25] The cells were examined daily under an inverted microscope to evaluate the morphological character of DCs.

Flow cytometry analysis

In order to obtain single cell suspensions, tumor-draining LN and spleen cells were first subjected to collagenase IV treatment for 1 h, as was previously described. Flow cytometry was then used to evaluate the cells' characteristics (FACSCalibur cytometer). Cytometry procedures were carried out per the guidelines provided by the manufacturer, with specific monoclonal antibodies being used for the intended purpose. In short, the cells were first re-suspended in PBS. For extracellular identification, we employed the antibodies of CD80 fluorescein isothiocyanate (FITC), CD86 phycoerythrin (PE), and T-cell markers TCR-αβ FITC, CD4 FITC, CD8 PE, CD11a FITC, and TCR-δλ PE (BD-Bioscience, USA), and the antibodies of CD80 FITC and CD86 PE (eBioscience, USA) and antibodies identified DCs. Following a 30-min incubation at 4°C, the cells were centrifuged to remove the PBS. Each staining operation was carried out in a final volume of 100 μL on ice. The staining was done separately in different tubes as 2 colors only were used. Flow data were analyzed with the help of the Flow Jo v7.6.2 software, while the data themselves were acquired with the help of a FACS Caliber flow cytometer.

Enzyme-linked immunosorbent assay and analysis

The levels of cytokines (IL-2, IL-4, IL-10, IL-12, TNF-α, IFN-γ, and TGF-β) were measured using ELISA (RandD Systems, USA) with monoclonal antibodies in accordance with the manufacturer's guidance. In conclusion, 96-well plates were coated with 100 μL of chosen antibodies that had been diluted in a coating buffer and then left at 4°C overnight. After rinsing the plate with washing solution (0.05% Tween-PBS), a blocking buffer was added. The plate was then incubated. After that, 1 h was spent adding 50 L of the diluent, which was then followed by the washing and rinsing steps. In accordance with the concentrations at the outset, 100 μL of a specified standard positive (recombinant cytokine) was injected into each well of the first row. The volume of 100 μL of LN and spleen supernatant was added to each well in the other rows. After an incubation period of 2 h at room temperature and three washes with washing buffer, the plates were prepared for the examination. After that, conjugated detection enzyme antibodies were added to each well in a volume of 100 μL. After incubation for an hour at room temperature, the plates were given a final washing in PBS-Tween before being stored. After that, the substrate solution was put into each well at a volume of 100 μL and left there for half an hour. Each well received an additional 50 μL of the Stop Solution, which consisted of phosphoric acid at a concentration of 1 M. For the purpose of reading the ELISA plate, an automated ELISA reader was used. The findings show that there is a significant discrepancy between the absorbance that was measured at 450 nm and the correction that was performed at 570 nm. The concentration of each cytokine in the blood was compared to a standard curve concurrently produced, and the resultant value, stated in pg/mL, was the serum concentration.

Statistical analysis

The results were analyzed using SPSS version 21 (IBM, NY, USA) through the Kruskal–Wallis nonparametric test and compared between the two groups. In addition, the results were summarized as median, maximum, and minimum values. By using the "Dun," test.

Results

In the current work, we examined the profile of DCs and T lymphocytes in the blood, serum, spleen, tumor tissue, and LN of female BALB/C mice between the ages of 6 and 8 weeks old that had been given an MDA231 cell to cause BC. The cell suspension

was injected subcutaneously into the abdominal mammary fat of each mouse that was part of the first group, while each mouse that was part of the second group received an injection of saline through the same route and location as the first group.

Proliferation of MDA231 cell to induce breast cancer

The tumor volume has reached that of the group serving as the negative control [Figure 1d]. In a similar vein, the weight and size of the tumors were considerably enhanced in the MDA231 cell BC-induced group compared to the CG ($P < 0.001$) [Figure 1a-c]. It was found that there were no significant differences in body weights between the two groups. All tumor-induced animals passed away between 45 and 60 days after being inoculated with MDA231 cells.

Morphological date of dendritic cells generated from spleen and lymph node homogenate

The spleen and LN samples were chopped and digested with 500 U/mL collagenase IV with agitation to create cell suspensions. Both spleen and LN cell suspension were cultured in the presence of IL-4 and GM-CSF cytokines and induced by LPS on their last day of culture. The morphology of the cultured cells was presented in the following photos [Photos 1-9]. It was found that the immature DCs of LN and spleen suspension taken from mice of the CG proliferated, and mature DCs were found on day 7 after the addition of LPS [Photos 7 and 8], while no maturation occurred for DCs in the suspension of LN and spleen taken from tumor-induced mice group [Photos 5 and 9].

Flow cytometry analysis

After obtaining single-cell suspensions, cells were placed in RPMI 1640 and were followed by the addition of FCS. In this investigation, we explored the characteristics of the DCs and T-cells in the LN tumor draining, and the spleen, of BALB/C mice. Flow cytometry was used to examine the surface molecules [Figure 2], and the following T-cell markers were evaluated: TCR-αβ FITC, CD4 FITC, CD8 PE, CD3 FITC, CD11a FITC, and TCR-δλ PE [Figure 2]. Markers known as CD80 FITC and CD86 PE were used to determine the existence of DCs surface molecules [Figure 2]. The data obtained from the female mouse tumor BALB/C's draining LNs and spleen are displayed as MGP (the median gate percentage which is number of positive cells for each marker) or MFI (the median fluorescence intensity which is the amount of protein, regardless of the number of positive cells).

In this study, the surface molecules ($CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$, respectively) were evaluated using positive gate percentage values to determine which cells were cytotoxic and which were helper cells. In contrast to the healthy control negative group, which had median values of 31.48 for $CD3^+$ $CD4^+$ cells, the TG had median values of 61.68 for these cells [Figure 2a]. Although tumor-induced lymphocytes had higher gate percentage values (57.27), there were no significant differences between the tumor-induced lymphocytes and the other research group [Figure 2a].

Antigen-specific receptors, referred to as TCR, are found on the surface of T-cells. It was determined, with the

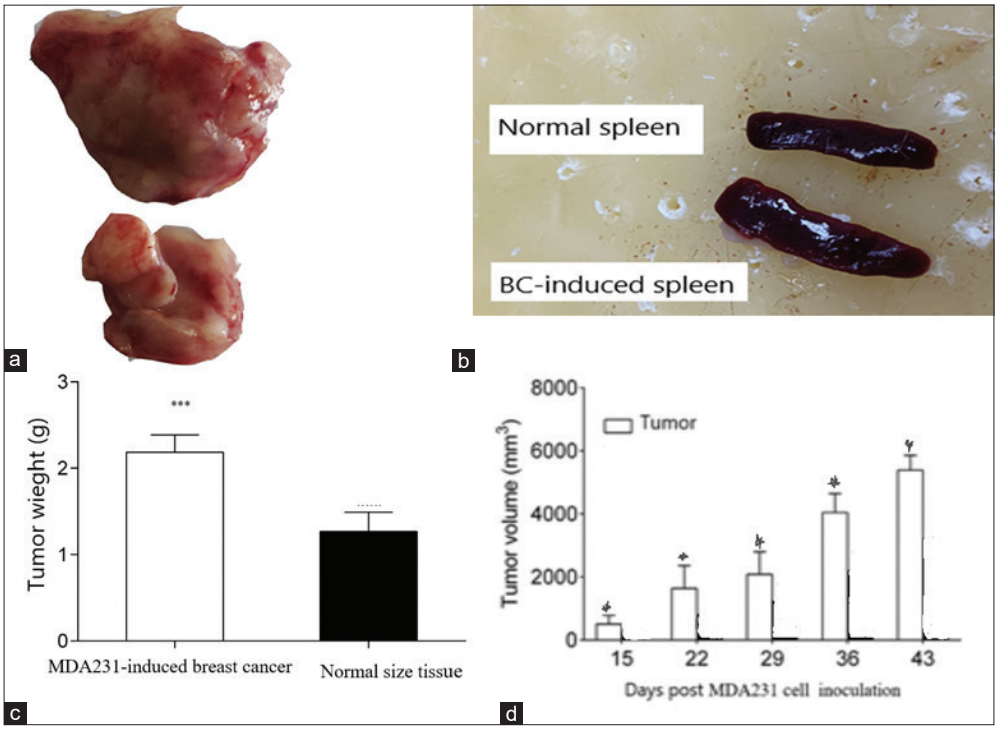

Figure 1: MDA231 cell inoculated in mice to MDA231 induce breast cancer, Tumors were removed from mice, and both the size and weight of tumors were recorded a, c, and d. Where $**P < 0.01$ and $***P < 0.001$ compared to the control group. b. The size of the spleen markedly increased in tumor-induced mice

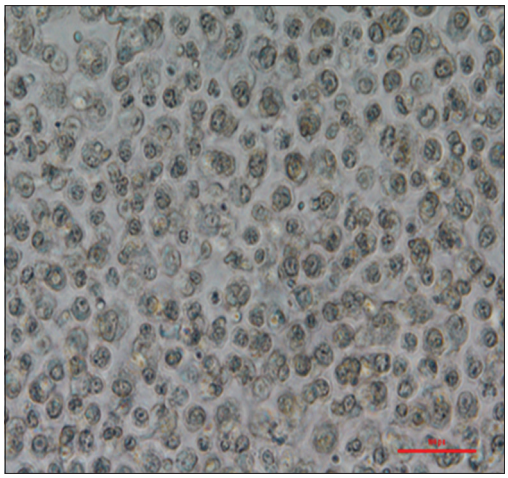

Photo 1: Morphological analysis of mature and immature DCs cultured from spleen and lymph nodes of normal mice as well as BC-induced tumor in mic where the experiment was repeated three times. Spleen tissue. DC: Dendritic cell, BC: Breast cancer

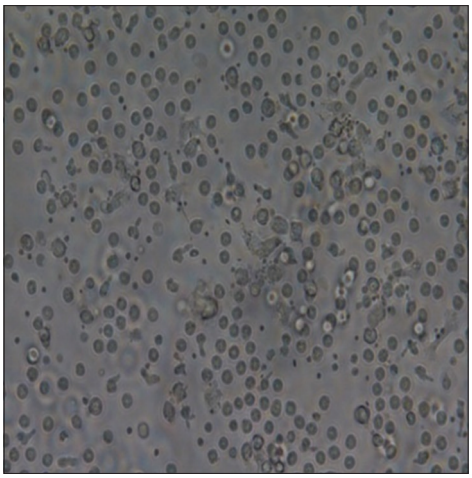

Photo 2: Morphological analysis of mature and immature DCs cultured from spleen and lymph nodes of normal mice as well as BC-induced tumor in mic where the experiment was repeated three times. Day 1 of isolated monocytes. DC: Dendritic cell, BC: Breast cancer

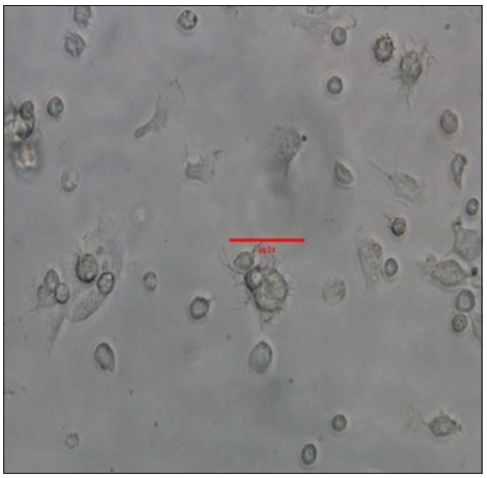

Photo 3: Morphological analysis of mature and immature DCs cultured from spleen and lymph nodes of normal mice as well as BC-induced tumor in mic where the experiment was repeated three times. Day 1 after washing. DC: Dendritic cell, BC: Breast cancer

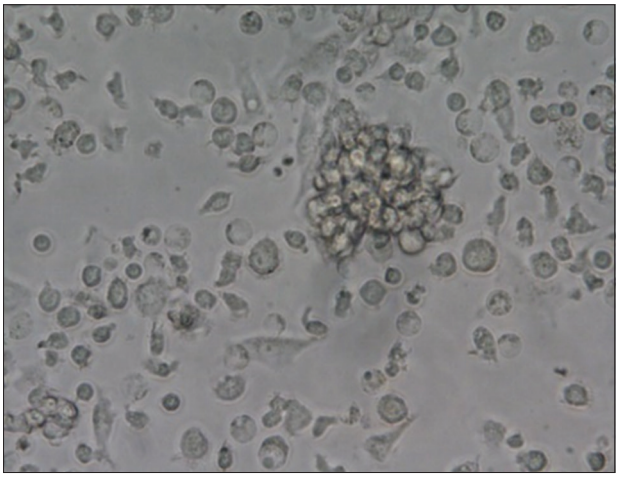

Photo 4: Morphological analysis of mature and immature DCs cultured from spleen and lymph nodes of normal mice as well as BC-induced tumor in mic where the experiment was repeated three times. Day 4 showing aggregation of monocytes. DC: Dendritic cell, BC: Breast cancer, BC: Breast cancer

use of gate percentage, whether or not T helper cells and cytotoxic cells carried TCR chains (αβ) and (δλ). There were no statistically significant changes detected between the two groups in TCD3$^+$ CD4$^+$ TCR-αβ$^+$ cells [Figure 2a]. Analysis of the TCR-αβ median positive value gate % in TCD3$^+$ TCD8$^+$ TCR-αβ $^+$ cells indicated a substantial difference between the TGs (32.97), on the one hand, and the healthy control negative group (41.22), on the other hand [Figure 2a]. Another difference is shown in Figure 2a in which the values for T CD3$^+$ CD4$^+$ TCR-δλ$^+$ were statistically more significant in the TG (4.80) and the healthy control negative group compared to the TG (median). This was the case when comparing the TG to the healthy negative CG (4.98).

According to the data, the levels of CD3$^+$ CD8$^+$ TCR-αβ$^+$ cells value gate percentage median were significantly lower in the groups that had been tumor-induced (32.95), as compared to the healthy control negative group (41.49) [Figure 2b]. Similar statistical values were found for CD3$^+$ CD8$^+$ TCR-δλ$^+$ in the control negative group (4.97) when compared with TG (4.5) [Figure 2b].

When compared to the control negative group (9.21), the myeloid DCs with the CD11c$^+$ marker exhibit significantly higher gate percentage values for the TG (13.77) [Figure 2c]. The mean fluorescence intensity (MFI) of CD80$^+$ and CD86$^+$ was used in an investigation of the maturation of DC. When CD80$^+$ molecules are analyzed, an increase in MFI values can be shown in TG (4282) in compared to the control negative group (2406); in addition, there is a statistically significant difference between the two groups. Moreover, the tumor-induced (1120) and control negative (1459) groups, the

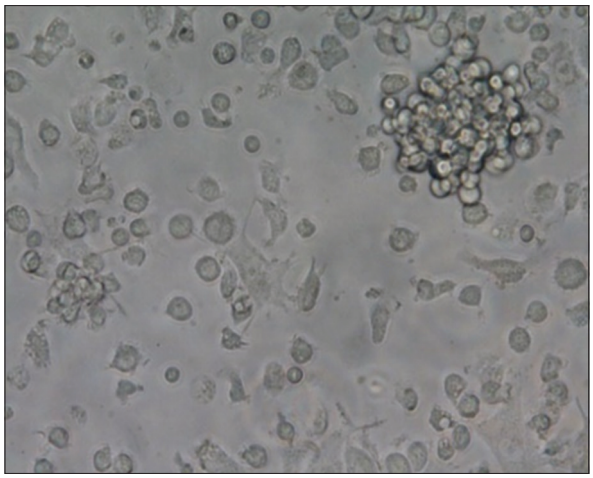

Photo 5: Morphological analysis of mature and immature DCs cultured from spleen and lymph nodes of normal mice as well as BC-induced tumor in mic where the experiment was repeated three times. Day 5 immature DC with IL-4 and LPS. DC: Dendritic cell, BC: Breast cancer

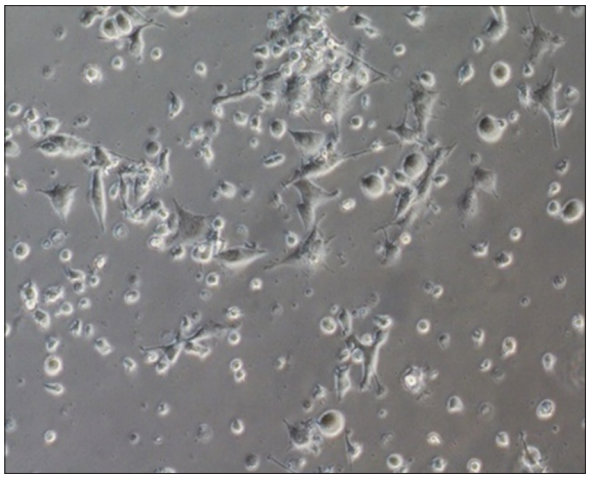

Photo 6: Morphological analysis of mature and immature DCs cultured from spleen and lymph nodes of normal mice as well as BC-induced tumor in mic where the experiment was repeated three times. Day 5 immature DC with IL-4 and LPS. DC: Dendritic cell, BC: Breast cancer

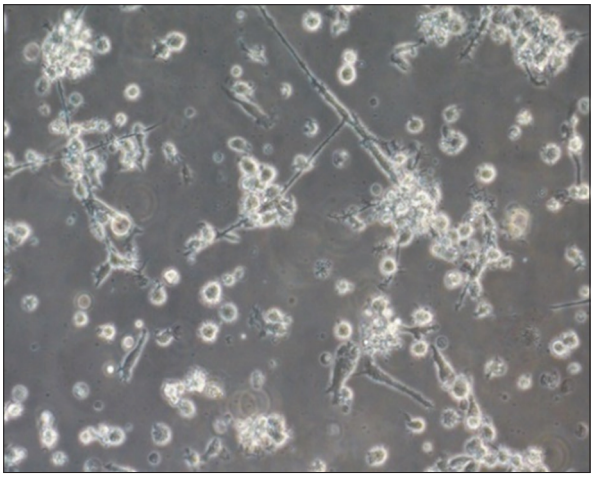

Photo 7: Morphological analysis of mature and immature DCs cultured from spleen and lymph nodes of normal mice as well as BC-induced tumor in mic where the experiment was repeated three times. Day 7 mature DC from the normal control group. DC: Dendritic cell, BC: Breast cancer

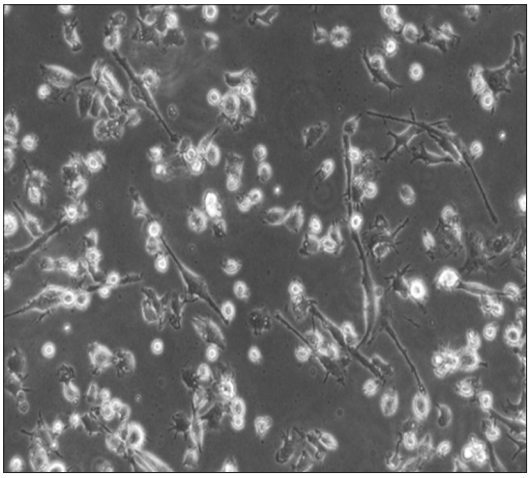

Photo 8: Morphological analysis of mature and immature DCs cultured from spleen and lymph nodes of normal mice as well as BC-induced tumor in mic where the experiment was repeated three times. Another photo showing Day 7, mature DC from normal control group. DC: Dendritic cell, BC: Breast cancer

MFI values for $CD86^+$ molecules were significant, according to the statistical analysis [Figure 2c].

Analysis of data obtained by ELISA

Cytokines, which are considered to be inflammatory mediators, are responsible for inducing a wide variety of cellular processes, including cell proliferation, chemotaxis, apoptosis, and differentiation.[26] The production of cytokines by effector cells is closely connected to the development of an appropriate immune response to tumor induction as well as the establishment of an immune-competent milieu. The following cytokines were measured using ELISA in the LN infiltration supernatant to supplement the findings for the immune response brought on by tumor induction: IL-2, IL-4, IL-10, IL-12, TNF-α, IFN-γ, and TGF-β. This was done to complement the findings for the immune response brought on by tumor induction. The outcomes are shown in Figures 3 and 4. After culturing the cell suspension, the quantities of cytokines were measured between 12 and 48 h after the stimulation with LPS. The distinctive kinetic fluctuations of the cytokine may be caused by a vast number of processes, including those that control its function and secretion. As a result, a preliminary literature review was conducted to identify cytokine production peaks that occurred simultaneously with the activation of responses to LPS. According to the findings, the release peaks of the cytokines IL-4, IL-10, and TNF-α were found to occur between 12 and 24 h [Figures 3 and 4].

On the other hand, the levels of the TGF-β cytokine fluctuated statistically throughout the period, with the release

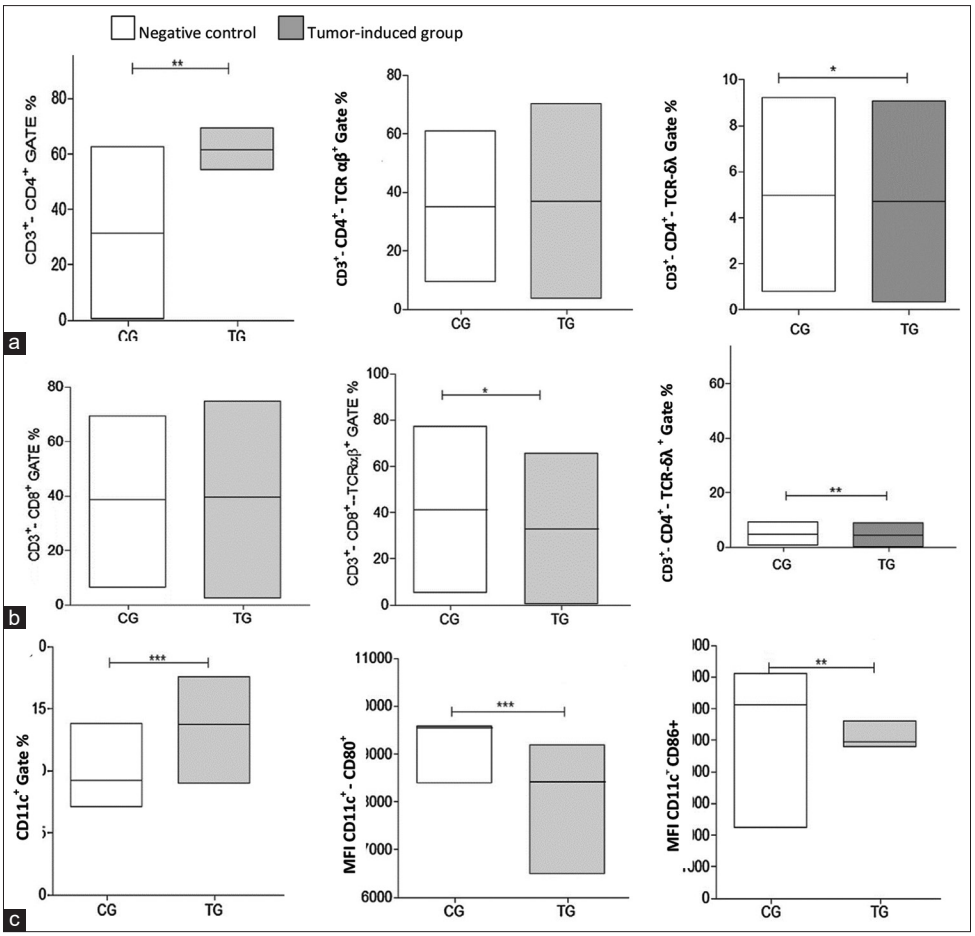

Figure 2: The statistical findings of flow cytometry as the median, maximum, and lowest gate percentages of cells that are Th $CD4^+$ (a) while T cytotoxic $CD8^+$ (b) that are +ve for markers $CD4^+$ $CD3^+$; $CD8^+$ $CD3^+$; TCR-$\alpha\beta^+$) and; and TCR-$\gamma\lambda^+$ respectively (c) that are positive for $CD11c^+$, $CD80^+$ and $CD86^+$, respectively. The results were implemented using SPSS program including Kruskal–Wallis test and the "Dun" test. It was determined that the observed differences were statistically significant when the probability of rejecting the null data was less than 0.05 (5%), *P 0.05, **P 0.0006, and ***P 0.0001, respectively

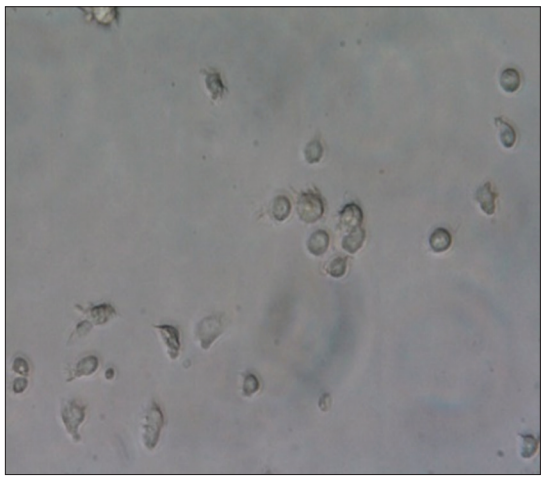

Photo 9: Day 7 immature DCs from tumor-induced group. DC: Dendritic cell

peaks happening between 24 and 48 h. IFN-γ and IL-12 cytokine production reached a statistically significant peak after 48 h of culture with LPS. This peak was measured in pg/mL.

There was no discernible statistical significance between the two experimental groups or the culture time with LPS in terms of the levels of IL-2 that were produced, and the levels increased 12 h after stimulation reaching its peaks 24 h then decreased below the negative CG [Figure 3]. In summary, the data lend credence to the release kinetics peaks reported in the literature, as mentioned earlier.[27-29]

It was found that IL-4 in TG had higher release [pg/ml] at 12 h (1.456) in a statistically significant average, as compared to the values discovered for the same group after 24 h (0.357). Furthermore, the analysis of the groups shows that there is a statistically significant difference between the tumor-induced (0.357) and healthy control negative (1.456) groups after 24 h of stimulation [Figure 3].

The maximum quantity of the IL IL-10 might be released between 12 and 24 h. After 12 h of LPS stimulation, there was no statistical difference between the TG and the healthy control negative group; however, the TG had larger statistical values, on average, compared to the healthy control negative

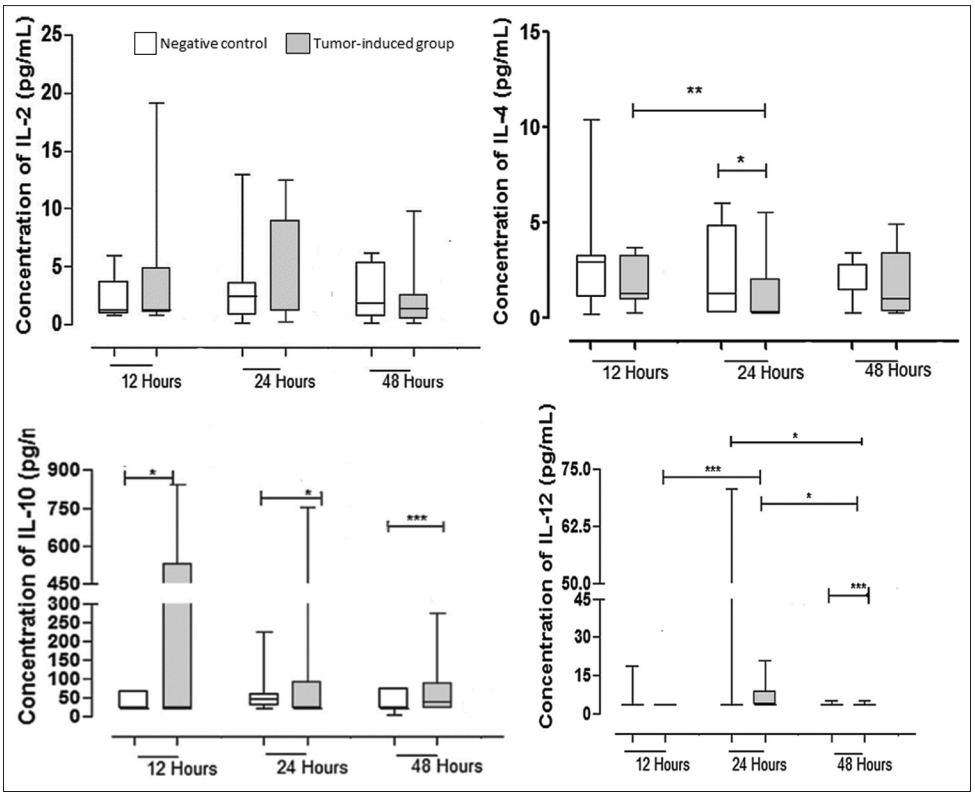

Figure 3: The amounts of cytokines that may be found in the supernatant of lymph nodal infiltration and spleen tissue after culturing during 12–48 h after the addition of LPS. The concentrations (in pg/mL) of the cytokines IL-10, IL-12, IL-4, and IL-2, across the different groups. 30 mice were used for the negative Control Group, and 30 animals were used for the Untreated Tumor-induced Group. The data were analyzed by using Kruskal-Wallis test known as the "Dun" test in SPSS program. The findings were presented with their median, maximum, and lowest values. It was determined that the differences were statistically significant when *P* was less than 0.05, where **P* 0.05***P* 0.0006 and ****P* 0.0001. IL: Interleukin

group. There was a statistically significant increase in cytokine production after 48 h for the healthy CG (46.23) compared to the TG (21.12) in the IL secretion [pg/ml]. This was the case despite the fact that very high levels were discovered for the TG 12 h after LPS addition [Figure 3].

Concerning the IL IL-12, a release peak was seen after 48 h, when the concentration (pg/ml) was more significant. While lower levels were observed after 12 and 24 h of LPS addition. In addition, there was an increase in IL-12 production in the TG at 24 h (3.65), but there was no significant difference between them at 12 h after stimulation with LPS [Figure 3].

The quantity of TGF-β, measured in pg/ml, changes greatly over the course of the 48 h; nonetheless, the highest level of cytokine production was 24–48 h after stimulation. A significant difference was found between the tumor-induced (1057) and the healthy control negative group (962.2) was recorded 12 h after LPS addition, where the cytokine concentration was higher. This difference was due to the fact that the TG had been exposed to a higher amount of LPS. After 24 and 48 h, a change takes place in the profile, and both the healthy control negative group and the TG reach their maximum cytokine production. Within 48 h of LPS stimulation, the TG (2567) demonstrates significantly higher cytokine levels (pg/ml) compared to the negative CG. This finding is statistically significant (2304) [Figure 4].

After the first 12 h of LPS stimulation, the median concentration of TNF-α in the LN infiltration culture reaches its highest point [pg/ml], and it then starts to rise again over the next 48 h. After 12 h, the values shown by the TG (122.8) are significantly larger than those exhibited by the control negative group (105.7). After 24 h, there is a statistically significant difference in the rate at which the concentration drops in the tumor-induced (37.91) group compared to the control negative (8.89) group. TNF-α a production rose considerably in both study groups after 48 h of stimulation, with increases being found in both the control negative (201) and tumor-induced (203) groups [Figure 4].

After 12 h of LPS stimulation, there was a difference that could be considered statistically significant in the production of IFN-γ between the negative CG (3498), which served as a control, and the TG (0.096). After 24 h, the median values of IFN-γ production in culture dropped in both groups, despite the fact that there were no noticeable differences between them. 48 h after stimulation, there was a statistically significant increase in the production of IFN-γ in both the TG (0.09) and the negative CG (0.678) [Figure 4].

Discussion

Even with the advances that have been made in therapy, BC remains the primary cause of mortality among women.

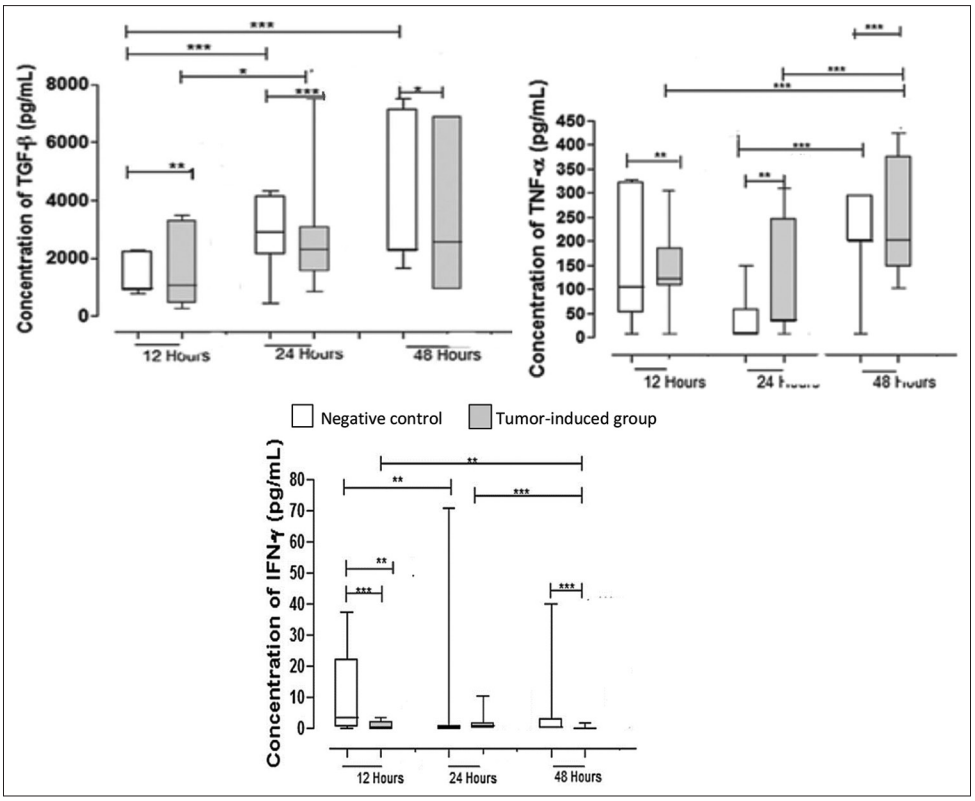

Figure 4: Shows the amounts of cytokines that may be found in the supernatant of lymph nodal infiltration anywhere between 12 and 48 h after stimulation with lipopolysaccharide (LPS). Comparison of the TNF-α, IFN-γ, and TGF-β concentrations (in pg/mL) across the different groups. 30 mice were used for the Control negative Group (GC), and 30 animals were used for the Untreated TG. The data were evaluated using Kruskal–Wallis test known as the "Dun" test. The findings were presented in three different ways: the median, the maximum, and the least values. It was determined that the differences found were statistically significant when *P* was less than 0.05, where **P* 0.05***P* 0.0006 and ****P* 0.0001. LPS: Lipopolysaccharide, TNF-α: Tumor necrosis factor alpha, IFN-γ: Interferon-gamma, TGF-β: Transforming growth factor-β, TG: Tumor-induced Group

There are a variety of treatments available for BC; however, some of these treatments are quite hazardous and may lead to medication resistance in the patient. As a result, there is a pressing need for the development of brand-new medicines that can selectively attack cancer cells without triggering any adverse effects. Animals that serve as models, such as the BALB/C mice, have been used to produce tumors and evaluate the effects of medications.[30] However, oncologists are continually concerned by the recurring topic of whether or not the immune response of mice to tumors and antitumor medicine would be the same pattern as the immunological response of humans. Therefore, most research focused on the anticancer effect on laboratory animals with artificially produced tumors. The current study aims to evaluate the role of DCs and T lymphocytes in mice that have been manipulated to develop tumors and to demonstrate that BC progresses more rapidly when the immune response is suppressed.

Commonly, an effective immune response against cancer should include the costimulatory molecules linked to the membrane, such as co-stimulatory soluble molecules, for example, CD80 and CD86, IL-12, which is considered as pro-inflammatory cytokine, and the T-cell, which presents tumor antigen as peptide epitopes on MHC molecules. This is because of the known association between these molecules and the immune system's cancer-fighting ability. For example, the co-stimulation of T-cells that are present in CD28 and its ligands CD80 or CD86) (which are called B7-1 and B7-2, respectively) on the surface of DCs amplifies the contact between DCs and T-cells.[11,31,32] On the other hand, immature DCs in our study may induce tolerance through the unwanted proliferation or energy of antigen-specific $CD4^+$ and $CD8^+$ T-cells, the production of Treg cells that stifle immune responses by secreting IL-10 and TGF-β, or any combination of these mechanisms depending on the stage of malignancy. These mechanisms may also work in conjunction with one another.

In addition to playing a part in the formation and development of the mammary epithelium, TGF-β also promotes the spread of cancer by inducing cancer cells to go through the epithelial-to-mesenchymal transition.[33,34] TGF-β has been shown to suppress tumor growth initially; nevertheless, TGF-β constitutive activation enhances tumor growth.[35] This finding is consistent with a growing number of other research findings. Previous biomedical research has found a connection between excessive levels of TGF-α in BC and metastases to the bone and local LN.[36] According to the findings of this research, the levels of TGF-β protein in the LN of mice that developed tumors were much higher than those discovered in the control-negative group.

Conjugates can be stabilized owing to specific MHC-peptide complexes developed on mature DCs, which also facilitate the development of mature immunological synapses and the efficient maturation of T-cells. In contrast, proliferated DCs create high-affinity stable conjugates, mature immunological synapses, and successful T-cell activation.[37] Immature DCs make many short connections with a low affinity and do not adequately cluster TCRs. The amount of TCR protein (αβ) and (δλ) in $CD4^+$ and CD8+ cells was measured by gate percentage, and the results showed that the amount of TCR protein in the TG was statistically lower when compared to the negative CG in this test. T-cell development is characterized by several characteristics, including the need for specific positive selection, TCR (αβ) binding onto foreign peptides coupled to MHC, and subsequent specific proliferation, activation, and effector function of T-cells against tumors.[38] Specific positive selection, TCR (δλ) binding onto these foreign peptides coupled to MHC, and subsequent specific activation, proliferation, and effector function of T lymphocytes, which are fully mature in the absence of MHC and appear to respond to molecules that signal possible danger or cellular stress,[39,40] play a role in tumor immunosurveillance and immunoregulation. This is the case because T lymphocytes are utterly mature without MHC.

When contrasting different polarizer signals to either Th1 or Th2, the contribution made by the cytokine microenvironment is thought to be an important factor.[41] The microenvironment of the tumor may affect the immune response, perhaps making it possible for the immune system to be used by the tumor as a means of evasion. A recent study[42] found that the tumor microenvironment in BC patients with BC is immunosuppressive. This is because it promotes T-cells to transform into regulatory T-cells. IFN-γ, tumor necrosis factor (TNF-α)), IL-2, and IL 12 are all examples of cytokines that belong to the Th1 profile. These cytokines have immune competence responses (tumor suppressors) that primarily induce cell-mediated immunity. On the other hand, Th2 profile cytokines (IL-4, IL-10, and regulatory T (TGF-β) are immune inhibitory cell-mediated responses.[43]

Research indicates that the IL IL-12 is the primary agent responsible for the differentiation of $CD4^+$ T-cells into Th1 cells from virgin progenitors.[44,45] This differentiation into Th1 is facilitated further by IFN-γ, which works by promoting the synthesis of IL-12 receptors while simultaneously suppressing the expansion of Th2 cells.[46] The production of IFN-γ is dependent on the generation of cytokines such as IL-12, the upregulation of CD80 and CD86 DCs, several TNF-α receptors, and other factors.[47,48] On the other hand, the production of TGF-β and IL-10 by regulatory T-cells suppress them on a molecular level.[49,50] In addition, IFN-γ is the factor responsible for regulating the levels of checkpoint inhibitors such as PDL-1 and indoleamine 2, 3-dioxygenase.[51] The combination of these findings reduced the amount of antigen that was presented to T-cells in people and animals.[52] This reduction was related to MHC-II and other molecules found on the surface of DCs.[53]

Conclusion

The current study investigated BC in mice (white female BALB/C). T-cells and DCs profile was obtained from cultured LN and spleen through flow cytometry, and the cytokines were detected by ELISA. MDA231 cells reacted with the immune system to progress BC inside the body of mice. In this assay, we directly analyzed the role changes occurred DCs and T-cells during the progression of tumor in mice. As a result, the tumor microenvironment was able to increase the number of immature DCs by inhibiting their stimulation by overexpression of TGF-β produced by regulatory T lymphocytes and stimulation of tumor cells and stimulate the secretion of cytokines such as IL-10 as well as CD4+ and to decrease the secretion of IFN-γ and IL-12 in tumor-induced mice LN. It is hoped that the data may provide light on the development of anti-cancer treatment tools as well as future vaccines that are based on the interactions between immune cells.

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Conflicts of interest

There are no conflicts of interest.

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