Th-17 CELLS AS NOVEL PARTICIPANTS IN IMMUNITY TO BREAST CANCER

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TH-17 LIMFOCITI, NOVI UČESNIK U IMUNSKOM ODGOVORU NA TUMOR DOJKE

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ABSTRACT

*Breast cancer is a leading cause of cancer-related deaths among women worldwide. Tumour surveillance constitutes a process of recognising and modifying tumour development and involves both innate and adaptive immune systems. During the progression of malignancy, the immune response is dynamically changed. In our breast cancer model, we used 4T1 mouse mammary tumour cell lines with the capacity to metastasise efficiently to sites affected by human breast cancer. This model was used to evaluate anti-tumour immunity and tested **in vivo** whether tumour progression affected anti-tumour immunity. Female BALB/c mice were injected with 5 x 10^4 4T1 tumour cells into 4-th mammary fat-pad. Tumour size was evaluated daily and the number and size of tumour metastases was determined on day 36. Serum levels of pro-inflammatory cytokines, leukocyte cytotoxicity and cellular make up of the draining lymph nodes were tested in animals on day 13 after tumour inoculation. On day 36, metastases were found in the lungs and livers of the mice. IL-17 levels were higher in tumour bearing mice compared to healthy animals, while TNF-α serum levels showed no significant differences during tumour progression. Total cellularity of the draining lymph nodes was higher in tumour bearing mice. There were no differences in the total number of CD8+ and CD4+ cells; however, significant increases in CD19+ cells were found on the 13th day after tumour inoculation. Finally, MTT tests indicated higher cytotoxic activity levels in the draining lymph node cells of tumour bearing mice. We provide evidence suggesting that tumour induction may enhance immune responses most likely via the enhancement of Th-17 cells and the attenuation of CD4+Foxp3+ Treg cells.*

Key words: *mouse breast cancer, 4T1, metastasis, Th-17, Treg*

SAŽETAK

Tumor dojke je vodeći uzrok smrti kod žena širom sveta. Imunski nadzor predstavlja proces prepoznavanja i eliminacije malignih ćelija, koji uključuje i urođenu i stečenu imunost. Tokom progresije tumora, imunski sistem trpi dinamične promene. U ovom eksperimentu koristili smo 4T1 ćelijsku liniju mišjeg tumora dojke kao model tumora koji daje metastaze u organima zahvaćenim kod humanog karcinoma dojke. Cilj našeg istraživanja bio je ispitati efekte progresije tumora na anti-tumorsku imunost kod eksperimentalnog modela karcinoma dojke na BALB/C miševima. BALB/C miševima ženskog pola ubrizgano je 5 x 10^4 4T1 tumorskih ćelija direktno u masno jastuče mlečne žlezde broj 4. Veličina primarnog tumora merena je svakodnevno, a broj i veličina metastatskih kolonija 36-og dana erksperimenta. Trinaestog dana od ubrizgavanja tumorskih ćelija merili smo serumske nivoe pro-inflamatornih citokina, citotoksičnost leukocita i ćelijski sastav drenirajućih limfnih čvorova. Tridesetšestog dana od indukcije tumora, nadjene su metastaske kolonije na plućima i jetri. Izmeren je znatno viši serumski nivo IL-17 u miševima sa tumorima, a nije nadjena značajna promena u nivou TNF-α tokom progresije bolesti. Ukupna celularnost drenirajućih limfnih čvorova je povećana, 13-og dana nakon indukcije tumora. Nije pronadjena razlika u ukupnom broju CD8+ i CD4+ limfocita, ali je registrovano značajno povećanje ukupnog broja CD19+ limfocita. Ukupan broj CD4+Foxp3+ limfocita je značajno smanjen istog dana eksperimenta. Konačno, izmerena je veća citotoksična aktivnost tumor drenirajućih limfocita. Na osnovu navedenih rezultata, mi smatramo da indukcija tumora može da facilitira imunski odgovor, najverovatnije kroz aktivaciju Th-17 limfocita i smanjenje broja CD4+Foxp3+ T regulatornih limfocita (Treg).

Ključne reči: *mišji tumor dojke, 4T1, metastaze, Th-17, Treg*

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INTRODUCTION

Breast cancer is a leading cause of cancer-related deaths among women worldwide. Breast cancer genesis is caused, in part, by a combination of oncogenic mutations that promote genetic instability and accelerated cellular proliferation (1). The major cause of mortality from breast cancer is due to metastasis to distant organs, such as the lungs, bones, liver and brain (2). Breast cancer does not induce potent and effective immune responses (3). However, tumour surveillance constitutes a process of recognising and modifying tumour development and involves both innate and adaptive immune systems (4).

Detection of T lymphocytes in carcinoma tissue has revealed that they are associated with tumour development. The important role of T cells as effectors in anti-tumour immunity was first shown in numerous experimental models. For instance, UV light-induced tumours have been shown to grow progressively in the absence of T cells and are normally rejected by normal mice (5-7). The mature T-cell population is composed of 1) αβ T cells expressing CD4 or CD8 and 2) CD4-/CD8- γδ T-cell receptor (TCR)-expressing cells.

Most tumours are positive for MHC class I and negative for MHC class II, and CD8+ T cells are able to induce tumour killing upon direct recognition of peptide antigens, which are presented by the tumour's MHC class I molecules (8).

CD4+ T cells (T-helper lymphocytes, Th) can also recognise tumour antigens either directly or via cross-presentation by host antigen presenting cells (8). Th cells, as an integral part of adaptive immunity, have a bipolar role in mounting anti-tumour responses. The CD4+ T cell population can be divided into two subpopulations based on types of cytokine secretion (9). Type 1 Th cells characteristically secrete IFN-g, whereas type 2 T cells secrete IL-4, IL-5, IL-10, and IL-13. The commitment of CD4+ T cells to either a type 1 or type 2 pathway is influenced by many factors, including the nature of antigen (10), costimulatory molecules (11), the type of antigen-presenting cells and the cytokine environment (12, 13). Th1 and Th2 cells play important immunoregulatory roles in cancer development (14). There have been many reports suggesting that the Th1-type anti-tumour immune response provides a greater therapeutic impact. The role of Th1 cells in anti-tumour response is often to aid in the activation of CD8+ T cells. On the other hand, Th2-type cytokines usually downregulate anti-tumour immunity, although they can promote the recruitment of tumouricidal eosinophils and macrophages into the tumour microenvironment (15-18).

B lymphocites present contributors to the anti-cancer immune response via the secretion of antigen-specific immunoglobulins. They likewise facilitate the recruitment of innate leukocytes and the targeted destruction of neoplastic cells (19). Besides the role of B cells in tumour regression through immunoglobulin-mediated mechanisms, recent data are also pointing to a potential role in tumour development.

Interleukin-17 (IL-17), T cell-derived cytokine, was originally described as cytotoxic T lymphocyte (CTL)-associated antigen 8 (20). Interleukin 17 is predominantly produced by activated CD4 T-cells, but some studies in humans have demonstrated that CD8 T-cells can also produce IL-17 (21). It is considered to be a proinflammatory cytokine because it increases IL-6 and IL-8 production by macrophages, fibroblasts, keratinocytes, and synovial cells (22-26) and also induces the secretion of IL-1b and TNF-α by human macrophages and endothelial cells (24, 27). TNF-α was originally identified for its capacity to induce hemorrhagic necrosis of solid tumours (29). Its anti-tumour effects work both through direct cytotoxicity against tumour cells, but also through the activation of macrophages, cytotoxic lymphocites and neutrophils (30, 31), as well as specific damage to tumour blood vessels (32-34). IL-17 and TNF-α represent pleiotropic cytokines that are critical to multiple biological processes and exert a great influence on the development, progression and immune surveillance of tumours.

Regulatory T cells (Treg) represent a subset of CD4+T cells that function to modulate immune responses through the ability to suppress T-cell proliferation and cytokine production (35). The majority of Treg lymphocytes express high levels of interleukin-2 (IL-2) receptor α chain (CD25) and transcription factor Foxp3. These cells constitute 2-3% of CD4+ human blood T cells. Tregs have considerable influence on the regulation of immune response in autoimmunity but also play an important role in cancer development.

In the current study, we developed a breast cancer model using a 4T1 mouse mammary tumour cell line with the capacity to metastasise efficiently to sites affected in human breast cancer to evaluate the role of Th-17 cells in a particular tumour model.

MATERIALS AND METHODS

Animals

Female BALB/c mice (obtained from the Military Medical Academy), aged 8 to 9 weeks, were used in the experiments. Mice were housed under standard conditions. The experiments were approved by the ethics board of the Medical Faculty of Kragujevac.

Tumour cells

The weakly immunogenic mouse breast tumour cell line 4T1, which is singenic to the BALB/c background, was purchased from the American Type Culture Collection (Manassas, USA). The tumour cell line was derived from a single spontaneously arising mammary tumour from a BALB/C mouse (36). The rapid and efficient metastasis to organs affected in human breast cancer makes the 4T1 model an excellent mouse model for the study of the progression of breast cancer in humans. 4T1 cells were maintained in DMEM supplemented with 10% FBS, 2 mmol/l L-glutamine, 1 mmol/l penicillin-streptomycin and 1

mmol/l mixed nonessential amino acids (PAA Laboratories GmbH), a complete growth medium. Subconfluent monolayers in log growth phase were harvested by brief trypsin treatment, using 0,25% trypsin and 0,02% EDTA in PBS (PAA Laboratories GmbH) and washed three times in serum-free PBS before use in all in vitro and in vivo experiments. The number of viable tumour cells was determined by the trypan blue, and only those cell suspensions with more than 95% viable cells were used.

Induction of tumour

Syngenic female BALB/c mice were injected with 50 µl of a single-cell suspension containing 5×10^4 4T1 mammary carcinoma cells, orthotopically into the fourth mammary fat-pad of mice (direct injection). The size of the primary tumour in diameter was daily assessed morphometrically using electronic callipers and is presented as the mean ± SEM. Mice were sacrificed on the 13th and 36th days after tumour cell injection, and the primary tumours were surgically removed. Blood (from the mice's abdominal aortas), and samples of lungs, liver, brain and sentinel lymph nodes were collected. For the purposes of the study, the sentinel lymph node was defined as the primary draining lymph node for the primary cancer (37). Specimens of lungs, liver and brain were routinely embedded in paraffin, stained with hematoxylin and eosin (H&E) and reviewed to confirm the presence of metastatic colonies. Tumour cells appeared heterogeneous in size but were easily differentiated from non-tumour cells as predominately larger cells with an elevated nuclear to cytoplasm ratio. To avoid missing micrometastases, 4 µm H&E-stained sections from at least three different levels were examined for the presence of metastases. The number and size of metastatic colonies were examined with light microscopy by an independent observer.

Measurement of cytokines

Sera from animals were collected by a single needle stick and were stored at –20 °C until thawed for assay. Serum levels of IL-17 and TNFα were measured in one sample with highly sensitive enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Minneapolis, MN) specifically receptive to the mouse cytokines. In brief, premixed standards were reconstituted in PBS (pH 7.2), generating a stock concentration of 2000 pg/mL for TNF-α and 1000 pg/mL for IL-17. The standard stocks were serially diluted in Reagent Diluent to generate 7 points for the standard curves. Diluted Capture Antibody was added in a 96-well, flat-bottomed, polystyrene microtiter plate (MTP), with a final volume of 100µl. The plates were sealed and incubated overnight at room temperature and then washed with Wash Buffer (autowasher). The samples were diluted 1:4 in the Reagent Diluent. Premixed standards or diluted samples (100 µl) were added to each well containing washed beads, and then were covered with an adhesive strip and incubated for 2 hours at room temperature. After incubation and washing, 100 µL of the premixed Detection Antibody was added to each well, and then the wells were covered with a new adhesive strip and incubated for 2 hours at room temperature. After incubation and washing, Streptavidin-HRP was added to each well (100 µL). The incubation was terminated after 20 min. at room temperature (avoiding placement of the plates in direct light). After washing, the beads were then re-suspended in 100 µl of Substrate Solution. Then, 50 µL of Stop Solution were added to each well, and optical density of each well was immediately determined using a microplate reader set to 450 nm.

Cell preparation

Thirteen days after injection with the tumour cells, the mice were sacrificed, and their sentinel (inguinal) lymph nodes were isolated. Further, single-cell suspensions from the sentinel lymph nodes were obtained by mechanical dispersion through steel and nylon mesh screens in complete growth medium. After three washes, the cells were re-suspended in complete growth medium.

Cytotoxicity assay

To examine cytotoxic activity, we divided the mice into two groups: mice injected with tumour cells and healthy mice. Cells isolated from sentinel lymph nodes were used as effector cells in this assay. 4T1 mouse breast tumour cells were used as targets. The target cells were plated in 96-well flat bottom plates at a density of $1x10^4$ cells/well (V= 100 µl) in growth medium, in triplicate. After culture at 37°C for 24 h, effector cells were added at $4x10^4$ cells/well (V= 100 µl) to yield a target:effector (T:E) ratio of 1:4. After co-culture at 37°C for 24 h, methylthiazolyldiphenyl-tetrazolium (MTT; Sigma Chemical, St. Louis, MO) was added to each well for a final concentration of 5 mg/ml. Four hours later, the plates were centrifuged at 1000 rpm for 5 min, the medium was gently removed, MTT crystals were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma Chemical, St. Louis, MO), and the optical density was read on a spectrophotometer (OD570). The percentage of cytotoxity was calculated as: cytotoxity (%) = [1 - (experimental group (OD)/control group (OD))] x 100 (38). Data are expressed as the mean of triplicate wells ± SEM.

Flow cytometry

To investigate whether the administration of breast cancer cells could affect the number of lymphocytes derived from draining lymph nodes, the number of lymphocytes was measured using flow cytometric analysis scan (FACS). Single-cell suspensions of sentinel lymph nodes were obtained from mice on day 13 after the tumour cells were injected. Cells (5 x 105/ml) were washed three times and re-suspended in cold PBS containing 0.1% sodium azide (Sigma) and 10% mouse serum. Subsequently, they were incubated with FITC- or PE-labelled mAbs specific for mouse CD4, CD8, CD25, CD19 and F4/80 (BD Pharmingen, USA) or isotype-matched controls (5 mg/ml), for 30 min at 4°C in PBS.

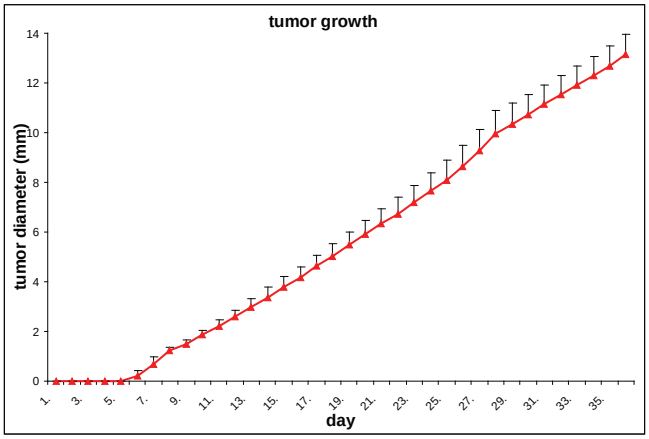
For the analysis of regular T cells, we used double staining. After labelling surface marker CD4, we conducted an intracellular staining technique for detecting Foxp3. CD4-labelled cells were washed in cold PBS. Cell pellets were then re-suspended using pulse vortex in 1 ml of freshly prepared fixation/permeability working solution and incubated for 2 hours in the dark. They were washed once by adding 2 ml of permeabilisation buffer followed by centrifugation and decanting of the supernatant. The washing procedure was then repeated. Then, the cells were incubated in the dark in 100 µl of Fc block in permeabilisation buffer for 30 min at 4°C. After blocking and without washing, PE-labelled anti-Foxp3 antibody (BD Pharmingen, USA) was added to the suspension, and the mixture incubated in the dark for 30 min at 4°C. As a control, we used an isotype control in the permeabilisation buffer instead of fluorochrome. The cells were then washed in 2 ml of permeabilisation buffer and centrifuged before the supernatant was decanted.

Stained cells were analysed by FACS calibre flow cytometry (Becton Dickinson, Mountain View, CA, USA) and CellQuest software (Becton Dickinson). Dead cells were excluded by gating out propidium iodide-positive cells.

number of mice with metastases/ total number of mice		
lungs	liver	brain
6/7	4/7	0/7

Table 1.
The incidence of metastases in mice inoculated with 4T1 tumour cells

1A)



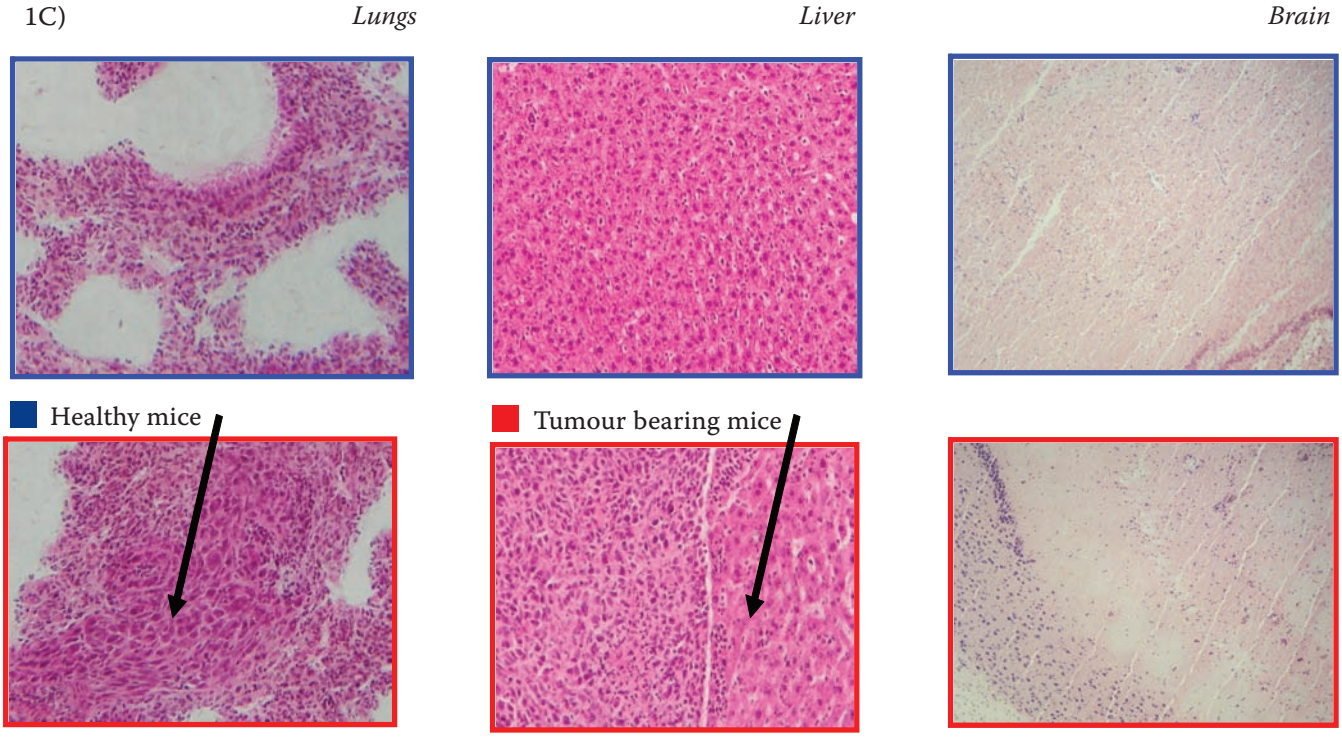
1B)



Figure 1:

A. Mean values of tumour diameters in BALB/C mice at 36 days after inoculation of 5x104 4T1 cells per mouse. On the 36th day of the experiment, the mean value of primary tumour diameters was 13,16 ± 0,79 mm.
B. Picture of surgically removed primary tumour.
C. Light-microscopic pictures of sections through pulmonary, liver and brain tissue (arrows are pointing on metastatic colonies).

1C)



Statistical analysis

For statistical analysis, the two-tailed Student's t-test or nonparametric Mann–Whitney Rank Sum test was used. The data were analysed using the SPSS statistical package, version 13.

RESULTS

1. Detection of tumour growth and metastasis

The primary tumour was established in the BALB/c mice by a unilateral subcutaneous injection of 5×10^4 4T1 mammary carcinoma cells, orthotopically into the fourth mammary fat-pad. Tumour growth was measured daily, using callipers, as described in the materials and methods sections. The results pertaining to tumour growth and metastasis are shown in **Figure 1** and **Table 1**. Systemic tumour involvement was determined by microscopic assessment. Specimens of lungs, liver and brain were investigated for the presence of metastatic colonies. Metastasis became apparent 5 to 6 weeks after tumour inoculation, although metastasising cells had probably seeded these sites earlier (77,78). Six out of seven BALB/C mice (86%) developed numerous lung metastatic colonies, while four out of seven (57%) developed lung metastases, as shown in Table 1. No brain metastases were detected.

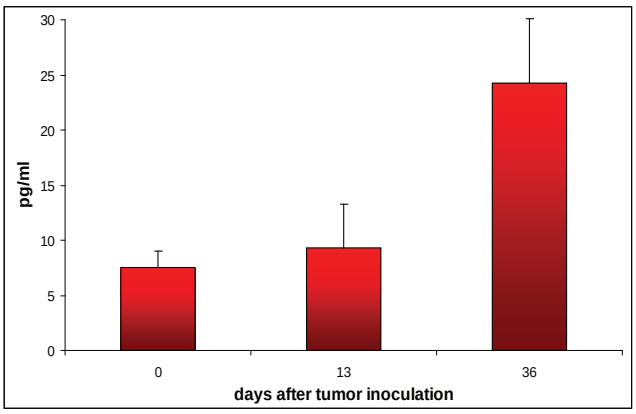
2. Serum levels of proinflammatory cytokines after tumour inoculation

To assess the anti-tumour immune response, we investigated the systemic production of proinflammatory cytokines. The measurements were performed before and on days 13 and 36 after tumour inoculation. After tumour inoculation, we noticed an increase in IL-17, and on the 36th day of the experiment, increases became significant when compared to baseline (24,21 ± 5,89 vs. 7,54 ± 1,45), as shown in **Figure 2A** (p=0.047). At the same time, we found the opposite trend in TNF-α serum levels. That is, TNF-α levels showed evident, but not significant, decreases during tumour progression, as shown in **Figure 2B.**

3. Anti-tumour cytotoxicity

To investigate the anti-tumour immune response, we analysed the cytotoxicity of sentinel lymph node cells. BALB/C mice were inoculated subcutaneously with 5×10^4 4T1 breast tumour cells orthotopically into the fourth mammary fat-pad. On day 13, 4T1-treated and equivalent untreated mice were killed, and their sentinel (inguinal) lymph nodes were removed. Lymph node cell suspensions were prepared and $4x10^4$ cells were plated into 96-well flat bottom plates and pre-incubated with $1x10^4$ 4T1 cells to yield a target:effector (T:E) ratio of 1:4. The percentage of cytotoxicity was determined after 24 hours of culture. Cells from tumour bearing mice manifested significantly higher cytotoxic activity compared with untreated BALB/C mice (57,13 ± 1,11 vs. 39,83 ± 1,47 %; p=0.027), as shown in **Figure 3.**

2A)



2B)

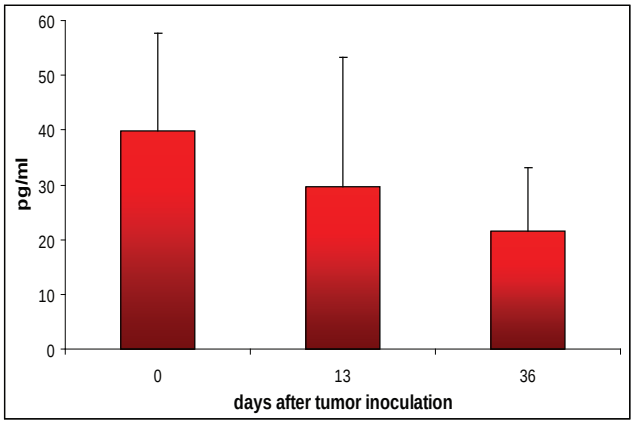


Figure 2:

A. Serum levels of IL-17 in BALB/C mice at 0, 13 and 36 days after inoculation of 5x104 4T1 cells per mouse. Serum levels of IL-17 were higher in tumour bearing mice 36 days after inoculation when compared with healthy mice (24,21 ± 5,89 vs. 7,54 ± 1,45; p=0.047).

B. Serum level of TNF-α in BALB/C mice at 0, 13 and 36 days after inoculation of 5x104 4T1 cells per mouse. Serum levels of TNF-α were lower in tumour bearing mice 36 days after inoculation when compared with the healthy animals (21,52 ± 11,69 vs. 39,93 ± 17,77).

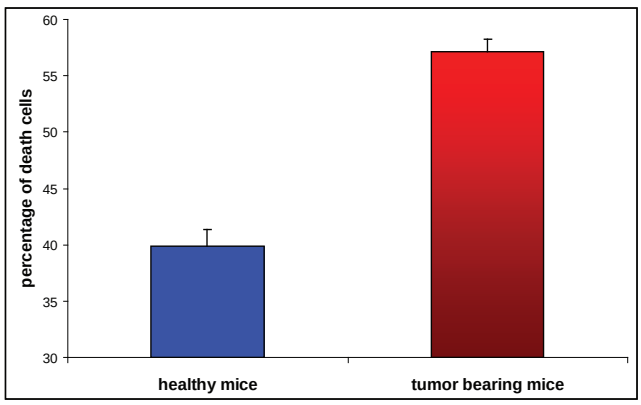
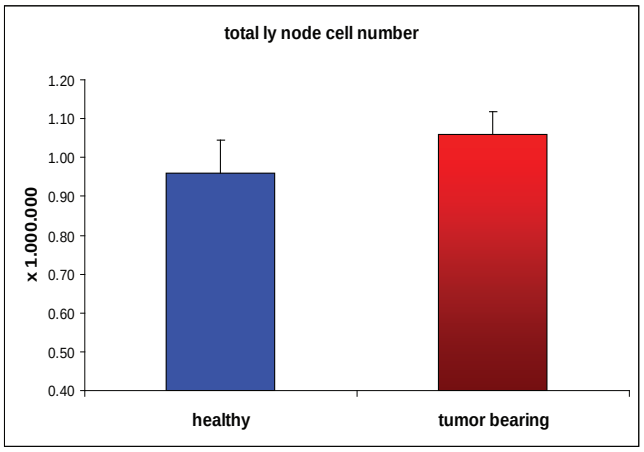
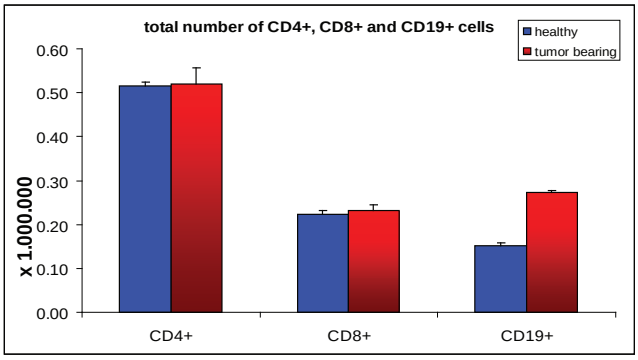


Figure 3. Cytotoxity of leukocytes derived from tumour draining lymph nodes 13 days after the injection of 5x104 4T1 tumour cells and from the inguinal nodes from healthy animals. When compared with the healthy mice (39,83 ± 1,47), the percentage of cytotoxity of leukocytes from tumour bearing mice was higher (57,13 ± 1,11; p=0.027).

4A)



4B)



4C)

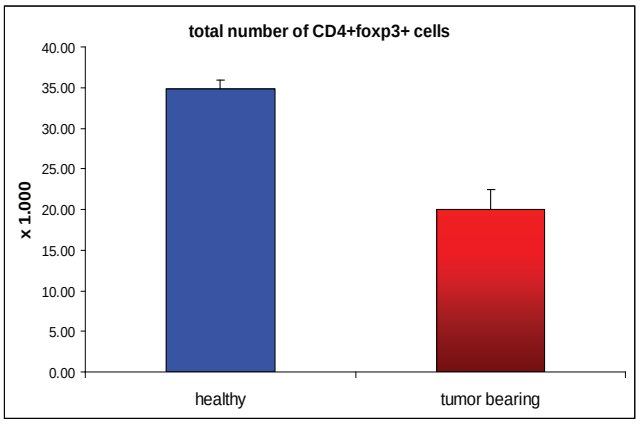


Figure 4. FACS analysis of lymph node derived leukocytes in BALB/C mice, before and after tumour inoculation:

A. The total lymph node cell number. Results showed no significant increases after tumour injection (0,96 ± 0,08 x 106 vs. 1,06 ± 0,06 x 106 cells).

B. Total number of different T and B cell populations. After tumour inoculation, no significant changes were found in the number of CD+ cells (515.225 ± 8.351 vs. 519.448 ± 37.866) or CD8+ cells (223.887 ± 7.647 vs. 232.290 ± 12.742). However, the number of B lymphocytes significantly increased after the tumour injection (150.890 ± 6.170 vs. 273.820 ± 2.380).

C. Total number of CD4+Foxp3+ cells in the draining lymph nodes. The number of CD4+Foxp3+ cells decreased after tumour inoculation (34.830 ± 1.040 vs. 20.040 ± 2.470).

4. Cellular composition of lymphoid cells in sentinel nodes (day 13)

To assess and characterise the cellular make up of sentinel nodes and their possible correlations with disease progression, sentinel lymph nodes were extirpated on 13th day after tumour inoculation, and lymphocyte populations were enumerated by multicolour flow cytometric analysis. As shown in **Figure 4A**, the results suggest that there is an slight increase in the number of total cells in the draining lymph nodes after tumour inoculation (0,96 ± 0,08 x 106 vs. 1,06 ± 0,06 x 106 cells; p>0.05). The same trend was evaluated in CD4+ (515.225 ± 8.351 vs. 519.448 ± 37.866) and CD8+ T cell populations (223.887 ± 7.647 vs. 232.290 ± 12.742). The number of CD19+ cells (B- lymphocytes) derived from inguinal lymph nodes showed significant increases after tumour injection (150.890 ± 6.170 vs. 273.820 ± 2.380), as shown in **Figures 4B** and 5E-F (p=0.001). Furthermore, it appears that the number of CD4+Foxp3+ cells was decreased during tumour progression (34.830 ± 1.040 vs. 20.040 ± 2.470), as shown in **Figures 4C** and 5G-H (p=0.054).

DISCUSSION

The 4T1 mammary carcinoma cell line was originally isolated by Fred Miller and his colleagues at the Karmanos Cancer Institute (39). We introduced this weakly immunogenic mouse breast tumour orthotopically into the mammary fat pad of the animals. The tumours grew rapidly at the primary site and formed metastases in the lungs, liver, bone and brain over a period of 3-6 weeks. Its use has increased in recent years because of its high propensity to metastasise to bone and other sites (40). Because this model is syngenic in BALB/c mice, we are using it to study the role of the immune system in tumour progression. In the current study, we showed rapid tumour growth, reflected through primary tumour diameters. On day 36 after the inoculation of tumour cells, 4T1 tumour cells had spread into different anatomical locations. The lungs, liver and brain from mice killed at day 36 were recovered. Visible metastases were found in the lungs and liver.

During the progression of malignancy, immune responses changed dynamically. When studying the inflammatory responses against tumours, we discovered a higher expression of the pro-inflammatory cytokine IL-17 on the 36th day after tumour inoculation as compared to baseline levels. This hints at a role of IL-17 in the inflammatory response to breast cancer progression. Interleukin 17 is predominantly produced by activated CD4 T-cells (41). CD4+ T cells can be classified into T-helper (Th) 1 cells, which secrete interferon (IFN) γ, IL-2, and tumour necrosis factor (TNF), and β and Th2 cells, which produce IL-4, IL-5, IL-6, IL-10, and IL-13, Additionally, there are also Th0 cells, a common precursor with the ability to release both IFNg and IL-4 (42). Thirty percent of Th0/Th1 clones have been shown to produce IL-17, whereas Th2 clones never express IL-17 (41). However, there is consensus now that IL-17 and IL-22 producing cells represent separate Th-

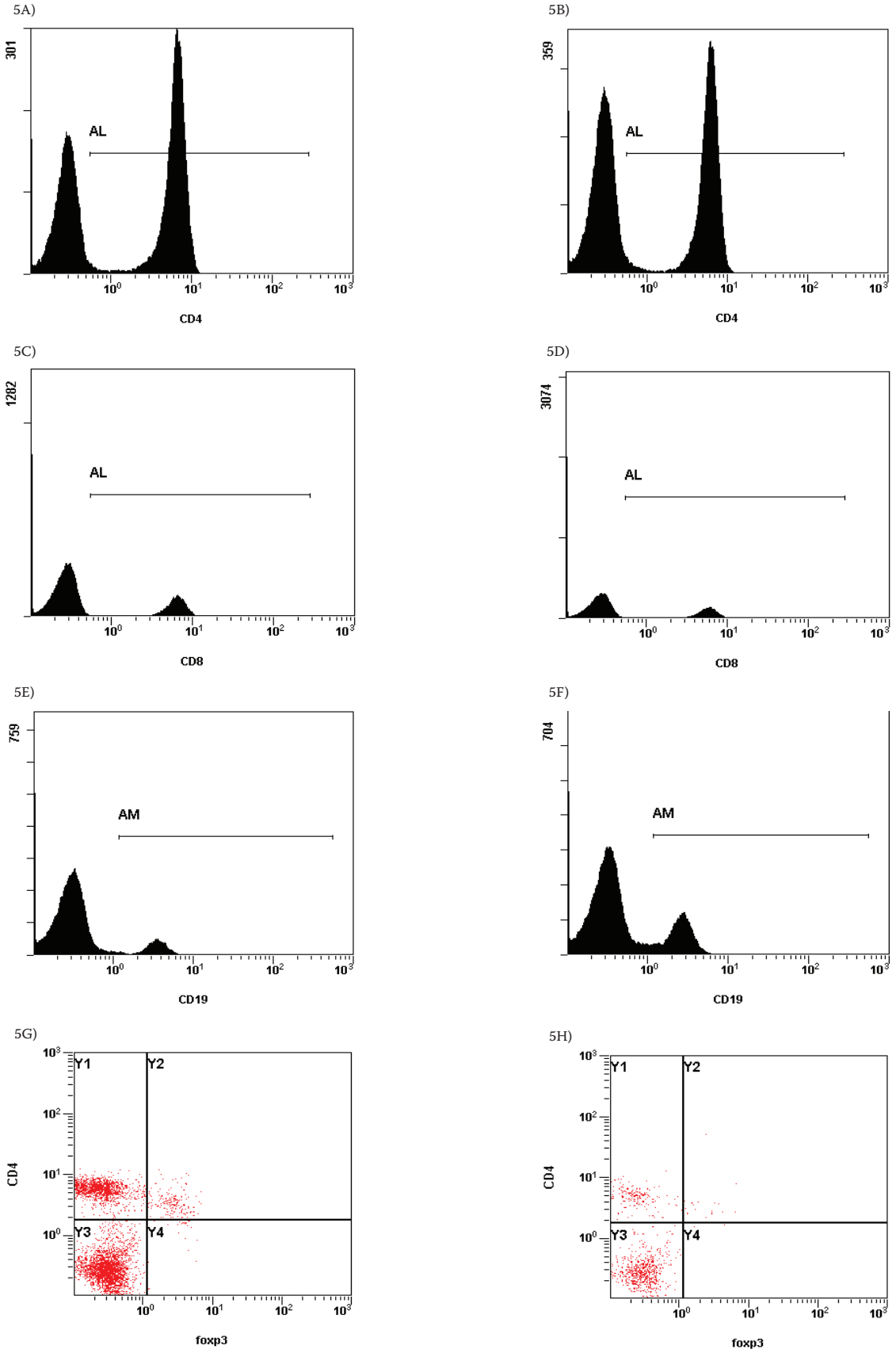


Figure 5. FACS analysis of lymph node derived leukocytes in BALB/C mice, before and after tumour inoculation:
A. CD4+ cells in healthy mice; B. CD4+ cells in tumour bearing mice; C. CD8+ cells in healthy mice; D. CD8+ cells in tumour bearing mice; E. CD19+ cells in healthy mice; F. CD19+ cells in tumour bearing mice; G. CD4+foxp3+ cells in healthy mice; H. CD4+foxp3 cells in tumour bearing mice.

17 cell populations. IL-17 is a pro-inflammatory cytokine because it increases IL-6, IL-8, IL-1b and TNF-α by many different cell populations (22-27). IL-17 is upregulated in breast cancer (28) and can influence tumour progression in a dual manner. IL-17 can inhibit the growth rate of tumours through the enhancement of tumour-specific T-cell activity (42). It has been shown that IL-17 increases the production of IL-6 by different cells (43), which is associated with the induction of tumour-specific CTLs (44, 45). It is also known (24) that IL-17 stimulates the secretion of IL-12 by macrophages, promoting Th1 immunity, and leads to the activation of CTLs (46). Additionally, IL-17 promotes breast cancer invasion (47), through upregulation of the metalloproteinases MMP-2 and MMP-9 (48), which indicates a pro-tumour effect of inflammation.

It was understood that TNF-α had a critical role in chronic inflammatory diseases such as rheumatoid arthritis (49), but it appears that it also plays a role in tumour progression. For many years, TNF-α was thought to have only anti-tumour effects (29, 30-34), but recent studies are demonstrating its tumour-promoting role (50-55). We found no significant changes in TNF-α serum levels during tumour progression.

The purpose of the next study phase was to characterise and quantify cells that are involved in the anti-tumour immune response in sentinel lymph nodes 13 days after tumour induction as well as compare them with control lymph nodes from healthy mice. This was felt to be very important because immune response against tumours initially occurs in sentinel nodes. We showed that the number of total cells was slightly increased in the SNs of tumour bearing mice, as compared to the healthy controls. There were no significant differences in the numbers of CD4+ or CD8+ cells in SNs before and after tumour inoculation. However, the number of B cells significantly increased after tumour injection, which explains the slight increase in the number of total SN cells. Most recently, it has been reported that IL-17 from CD4+ cells plays an important role in B-cell development (47, 56). The B cells' increase may be a consequence of Th determination. Th1 cells activate a cellular immunological response through increased IFN–γ and IL-2 production (57, 58), while Th2 cells suppress cellular immunological responses and promote mainly humoral immunity through increased IL-4, IL-5 IL-10 and IL-13 production (59, 58, 60).

In addition, we also investigated, in vitro, the cytotoxicity of sentinel lymph node cells. The cytotoxic capacity of SN cells was tested 13 days after tumour inoculation. We have shown higher cytotoxic activity in cells from tumour bearing mice as compared to untreated mice (57,13 vs. 39,83%). We believe that the difference in tumour-induced and spontaneous cytotoxicity is due to adaptive immunity. Th1-polarised cells secrete IFN-γ, TNF-α and IL-2 (61), which enhance the cytotoxic function of CD8+ cells (57) and macrophages (58). In general, we found no differences in the number of CD4+ and CD8+ cells in SNs after tumour inoculation, but the cytotoxic capacity of the aforementioned cells was significantly increased.

Regulatory T cells (Treg) are important in the control of the immune response (62). The majority of Treg lymphocytes express high levels of interleukin-2 (IL-2) receptor α chain (CD25) and transcription factor FoxP3 (critical for the development and function). Further, they constitute 2-3% of CD4+ human blood T cells (63). Treg lymphocytes express CTLA-4 and membrane bound TGF-β, which inhibit cytokine production and the responses of effector lymphocytes (35). They also secrete immunosuppressive cytokines such as IL-10 and TGF-β. Treg cells are a key contributor to the maintenance of immune tolerance and regulate immune responses in autoimmune diseases, graft-versus-host diseases, allograft rejections and allergies (64).

In addition, Tregs have an important role in cancer development. Cancer cells can modulate host anti-tumour immune responses indirectly, through the activation of Treg lymphocytes. Tumours promote the accumulation of immunosuppressive Treg lymphocytes in the tumour bed or in the blood. Patients with breast (65), liver (66), gastric and esophageal cancer (67) have higher numbers of Tregs in peripheral blood as compared to healthy controls. Furthermore, increased numbers of tumour-infiltrating Tregs have been demonstrated in hepatocellular (66), lung (68), ovarian (69), gastric, esophageal (67), and, more recently breast cancer (70).

Recent studies showed that Tregs play an important role in tumour growth by suppressing anti-tumour T-cell immunity (69, 71). The accumulation of Tregs within the tumour microenvironment effectively prevents tumour destruction (72) via the inhibition of CD8+ T cell function (73). The loss of regulatory function from the depletion of tumour-induced Treg lymphocytes may enhance the effector anti-tumour response, thereby resulting in tumour rejection (73-75). In our study, we showed that CD4+Foxp3+ cells numbers were decreased during tumour progression, which is thought to facilitate anti-tumour immunity.

We provide evidence suggesting that tumour progression may enhance the anti-tumour response in a model of primary breast tumours as well as pulmonary and liver metastases. This was reflected through the production of proinflammatory cytokines, the cellular composition of draining lymph node cells and cytotoxic activity. It remains to be formally shown whether Th-17 cells play a protective role in anti-tumour immunity in mammary carcinoma.

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