

SUPPRESSED INNATE IMMUNE RESPONSE AGAINST MAMMARY CARCINOMA IN BALB/C MICE

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SUPRIMIRANI URODGENI IMUNSKI ODGOVOR TUMORA DOJKE KOD BALB/C MIŠEVA

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ABSTRACT

Breast carcinoma is one of the leading causes of deaths among women worldwide. The immune response in breast cancer is mediated by innate and adaptive immune cells, including natural killer (NK) cells, dendritic cells (DCs) and T lymphocytes. The 4T1 mammary carcinoma line derived from BALB/c mice shares many characteristics with naturally occurring human breast cancer. We aimed to investigate the mechanisms of anti-tumour immunity using the experimental 4T1 breast cancer model in syngeneic BALB/c mice. After 12 days of tumour inoculation, mammary carcinoma-bearing mice had significantly decreased numbers of NKp46⁺ NK cells compared with healthy mice and lower cytotoxic activity of total splenocytes and NK cells *in vitro*. Additionally, significantly higher numbers of CD11c⁺ DCs were detected in the spleens of tumour-bearing mice, but the number of activated CD80⁺CD86⁺ dendritic cells was entwithsimilar to that in healthy mice, indicating an increased number of immature DCs in tumour-bearing mice. The data indicate that 4T1 mammary carcinoma progression in BALB/c mice is associated with suppressed innate anti-tumour immunity.

Keywords: 4T1 mammary carcinoma, BALB/c mice, NK cells, dendritic cells.

APSTRAKT

Rak dojke je jedan od najčešćih uzroka smrti žena, širom sveta. Imunski odgovor na tumor dojke posredovan je ćelijama urođene i stečene imunosti, uključujući ćelije ubice (NK), dendritske ćelije (DCs) i T limfocite. 4T1 mišji karcinom dojke, dobijen iz BALB/C miša, deli mnoge karakteristike sa spontano nastalim humanim karcinomom dojke. Cilj istraživanja je bio ispitati mehanizme anti-tumorske imunosti koristeći 4T1 eksperimentalni model tumora dojke singen sa BALB/c miševima. Dvanaest dana nakon inokulacije tumora, miševi sa tumorom imali su značajno manji broj NKp46⁺ NK ćelija, u poređenju sa zdravim miševima kao i manju citotoksičnost ukupnih splenocita i NK ćelija, *in vitro*. Takođe, detektovan je značajno veći broj CD11c⁺ dendritskih ćelija u slezini miševa sa tumorom, dok se broj aktiviranih CD80⁺CD86⁺ dendritskih ćelija nije značajno razlikovao u poređenju sa zdravim miševima, ukazujući na povećan broj nezrelih dendritskih ćelija u miševa sa tumorom. Rezultati ukazuju da je progresija 4T1 karcinoma dojke povezana sa suprimiranim urođenim anti-tumorskim odgovorom.

Ključne reči: 4T1 karcinom dojke, BALB/c miševi, NK ćelije, dendritske ćelije.

INTRODUCTION

Breast cancer is characterised by the development of metastasis in distant organs, such as the lungs, bones, liver and brain, and it is one of the leading causes of cancer deaths among women (1, 2). The role of innate immunity in breast cancer growth and progression remains unknown, but the role of the specific immune response has been extensively studied (3-4). The role of NK cells in immune surveillance as a first line of antitumor defence is well established (5-8). NK cell activity is variable during tumour progression and is related to clinical stage and disease outcome (4, 8-11). T cells are important effector cells against tumours, according to many studies on tumour models

in mice (12-16). Cytotoxic CD8⁺ T cells kill tumour cells, while the anti-tumour immune response of CD4⁺ T cells can be polarised towards Th1, Th2 or Th17 type. The type-1 immune response is characterised by the secretion of interferon-gamma (IFN-γ), which contributes to tumour rejection by stimulating the cytotoxic activity of CD8⁺ T and NK cells (17-20). In contrast, in the type-2 anti-tumour immune response, *interleukin-4* (IL-4), *interleukin-5* (IL-5) and *interleukin-10* (IL-10) suppress cellular immunity and therefore facilitate tumour growth and metastases (21-22). The role of the type-17 anti-tumour immune response has not been clarified. Interleukin-17 (IL-17), a hallmark Th17

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cell cytokine, is a potent mediator of inflammation in autoimmune diseases (23-24), but it has dual roles in antitumor immunity: not only can it promote tumour growth and metastasis by stimulating neoangiogenesis (25-27), IL-17 can also induce the cellular immunity responsible for tumour rejection (28-30). Dendritic cells (DCs) are part of the innate immune system involved in the activation and proliferation of tumour-specific T cells (31-33) and in enhancing the tumoricidal activity of NK cells (34-35). Immature DCs induce immunosuppressive CD4⁺ T cells (36-39), and tumour cells produce factors that could prevent DC maturation (40-41). Immature DCs are characterised by the weak expression of MHC II and co-stimulatory molecules with low IL-12 production and therefore have limited capacities to stimulate T cells (36-39). B cells contribute to antitumor immunity by secreting tumour-specific antibodies that facilitate the killing of tumour cells (42), but they can also induce a pro-angiogenic and pro-tumorigenic microenvironment that supports tumour growth (42).

In this study, using the 4T1 metastatic breast cancer model in BALB/c mice, we aimed to investigate anti-tumour innate immune mechanisms during the progression of primary tumours.

MATERIALS AND METHODS

Mice

In all of the experiments, we used female BALB/c mice that were 10-11 weeks old. The experiments were approved by the Animal Ethics Board of the Faculty of Medicine, University of Kragujevac, Serbia.

4T1 tumour cells

The mouse breast cancer cell line 4T1, which is syngeneic to the BALB/c background, was purchased from the American Type Culture Collection (ATCC, USA). 4T1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum, 2 mmol/l L-glutamine, 1 mmol/l penicillin-streptomycin, and 1 mmol/l mixed nonessential amino acids (Sigma, USA) (complete growth medium) and cultured under standard conditions as previously described (43). The number of viable tumour cells was determined by trypan blue exclusion, and only cell suspensions with $\geq 95\%$ viable cells were used. Mice were orthotopically injected with 5×10^4 4T1 cells in the fourth mammary fat pad, as previously described (44).

Cellular analysis of the spleen

Mice were sacrificed on day 12 after tumour inoculation, and their spleens were removed. Single-cell suspensions were obtained from the spleens by mechanical dispersion through a cell strainer (BD Pharmingen, USA) in complete growth medium. Additionally, erythrocytes were removed from the splenocyte cell suspension by lysing solution (BD Pharmingen). After three washes, cells were resuspended in complete growth medium.

Cell stimulation

For analysis of CD107a expression, splenocytes were activated with phorbol 12-myristate 13-acetate (PMA, Sigma) (50 ng/ml) and ionomycin (500 ng/ml, Sigma) with GolgiS-top (BD Pharmingen) as previously described (43, 45).

Flow cytometry

Single-cell suspensions from spleens were incubated with mAbs specific for mouse CD3, CD4, CD8, CD19, NKp46, CD107a, CD11c, CD80, and CD86 or isotype-matched controls (BD Pharmingen/BioLegend) and analysed with a FACSCalibur flow cytometer (BD). The gate used for FACS analysis was the mononuclear cell region in FSC/SSC plots (20000 events were acquired). Data were analysed using CELLQUEST (BD) and FlowJo (Tristar) software.

NK cell separation

NK cells were isolated from the spleen by magnetic cell sorting using FlowCompTM Mouse CD49b antibody (Invitrogen, USA), as previously described (43).

CD8⁺ T cell separation

CD8⁺ T cells were isolated from spleens by depleting non-CD8⁺ T cells (CD4⁺ T cells, B cells, monocytes/macrophages, NK cells, dendritic cells, erythrocytes and granulocytes) using a mixture of monoclonal antibodies against non-CD8⁺ T cells (Invitrogen), as previously described (43). Isolated cells were highly enriched mouse CD8⁺ T cells (purity >90%).

Cytotoxicity assay

The cytotoxic activity of splenocytes, enriched NK cells and enriched CD8⁺ T cells was measured using a 4 h MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay at various target-effector (T:E) ratios, as previously described (45). 4T1 tumour cells were used as targets. Data were expressed as the mean of triplicate wells \pm SEM. Cytotoxic activity was also presented by lytic units ($LU_{20}/10^7$ cells), which were calculated from the means of triplicate percentages of killing obtained at four different T:E ratios (46).

Statistical analysis

The data were analysed using the SPSS statistical package, version 13. The two-tailed Student's t test was used. The normality of distribution was tested by the Kolmogorov-Smirnov test. The results were considered significantly different when $p < 0.05$ and highly significantly different when $p < 0.01$.

RESULTS

Twelve days after tumour inoculation, the percentage and total number of CD19⁺ B cells were increased while the frequency and number of NKp46⁺ cells were decreased

We assessed the frequencies and the numbers of major lymphocyte populations in the spleens of naive and tumour-bearing mice at day 12 following tumour chal-

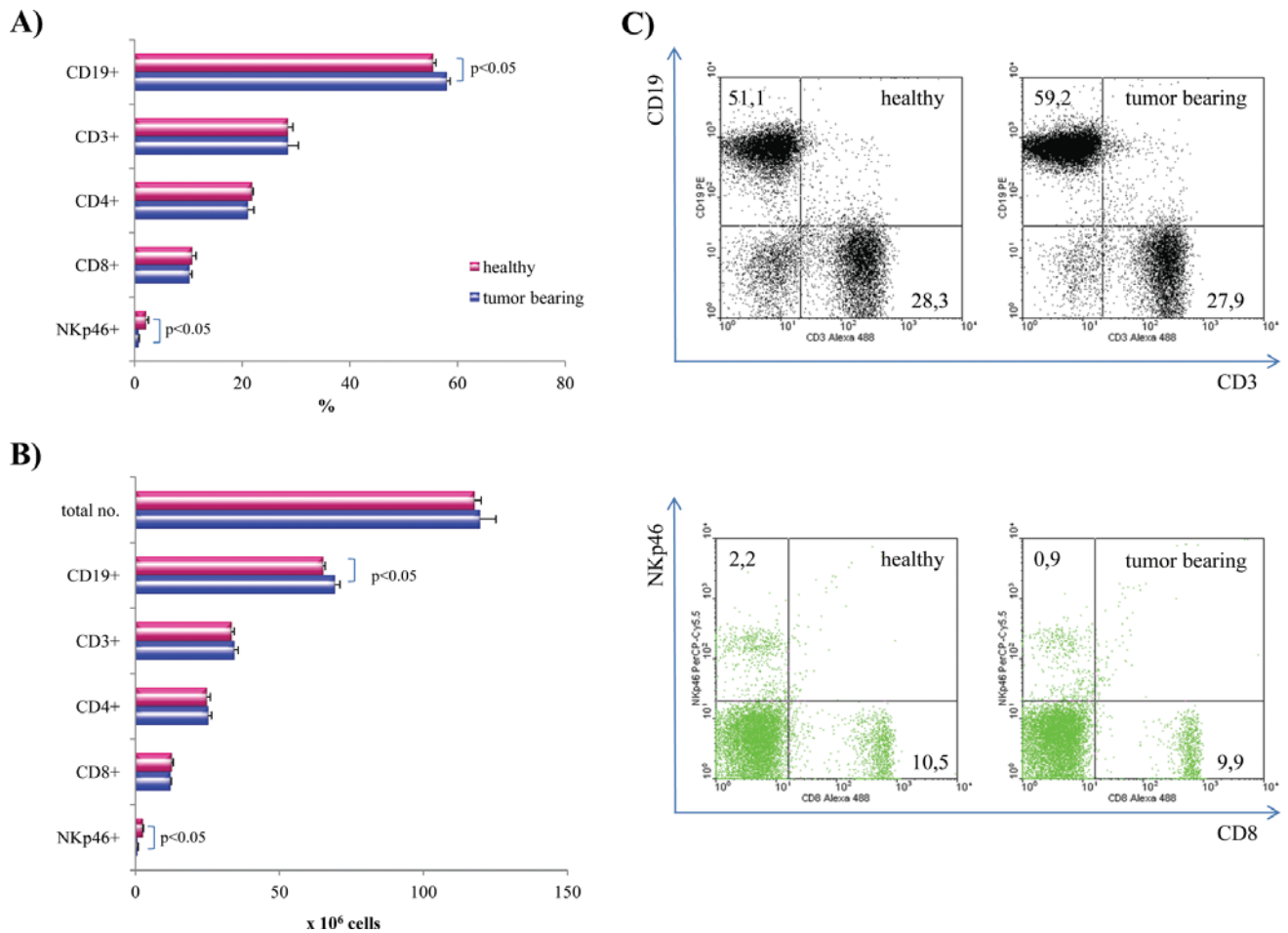


Figure 1. FACS analysis of splenocytes from tumour-bearing versus tumour-naïve mice

A-B) The total cell number of splenocytes was determined in healthy and tumour-bearing mice on day 12 after tumour inoculation. Percentages and total numbers of CD19⁺, CD3⁺, CD4⁺, CD8⁺ and NKp46⁺ cells were determined by staining splenocytes with fluorochrome-labelled mAbs and analysing them with a FACSCalibur flow cytometer.

C) Representative flow cytometry dot plots show percentages of CD19⁺, CD3⁺, CD8⁺ and NKp46⁺ cells in spleens from healthy and tumour-bearing mice. The gate used for analysis was the mononuclear cell region in FSC/SSC plots. Data are presented as the mean ± SEM of two separate experiments, each carried out with four mice per group. Statistical significance was tested by Student's t-test.

length. The total number of splenocytes was not significantly changed in tumour-bearing mice compared with healthy mice (Fig. 1B). The frequency and total number of splenic CD19⁺ B cells were significantly increased after tumour inoculation (p < 0.05). There was no difference in the percentage or total number of CD3⁺, CD4⁺ or CD8⁺ cells (Fig. 1A and 1B) between tumour-bearing and naïve mice. 4T1 tumour administration markedly reduced the percentages and numbers of NKp46⁺ cells (p < 0.05), as shown in Figs. 1A and 1B.

The cytotoxic activity of NK cells but not CD8⁺ T cells was suppressed after tumour inoculation

On day 12 of tumour progression, the *in vitro* cytotoxic activity of total splenocytes was decreased (Fig. 2). We subsequently isolated NK cells (CD49b⁺) and CD8⁺ T cells from spleens and assessed their antitumor cytotoxic-

ity. The cytotoxic activity of CD8⁺ T cells was higher after tumour inoculation (p < 0.05; Fig. 2), while the cytotoxicity of NK cells derived from spleens of tumour-bearing mice was significantly lower compared with healthy animals. In addition, activated CD107a⁺ NK cells were less frequent in tumour-bearing mice, while the percentage of CD107a⁺CD8⁺ T cells was higher at the same time point (both p < 0.05; Fig. 2).

The frequency and total number of CD11c⁺ dendritic cells were increased after tumour induction

After 12 days of 4T1 tumour inoculation, the percentage and number of CD11c⁺ DCs were higher in spleens from tumour-bearing mice (both p < 0.05; Fig. 3). Analyses of the activation status of these cells revealed no significant difference in frequencies or numbers of activated CD80⁺CD86⁺ dendritic cells between healthy and tumour-bearing mice (Fig. 3).

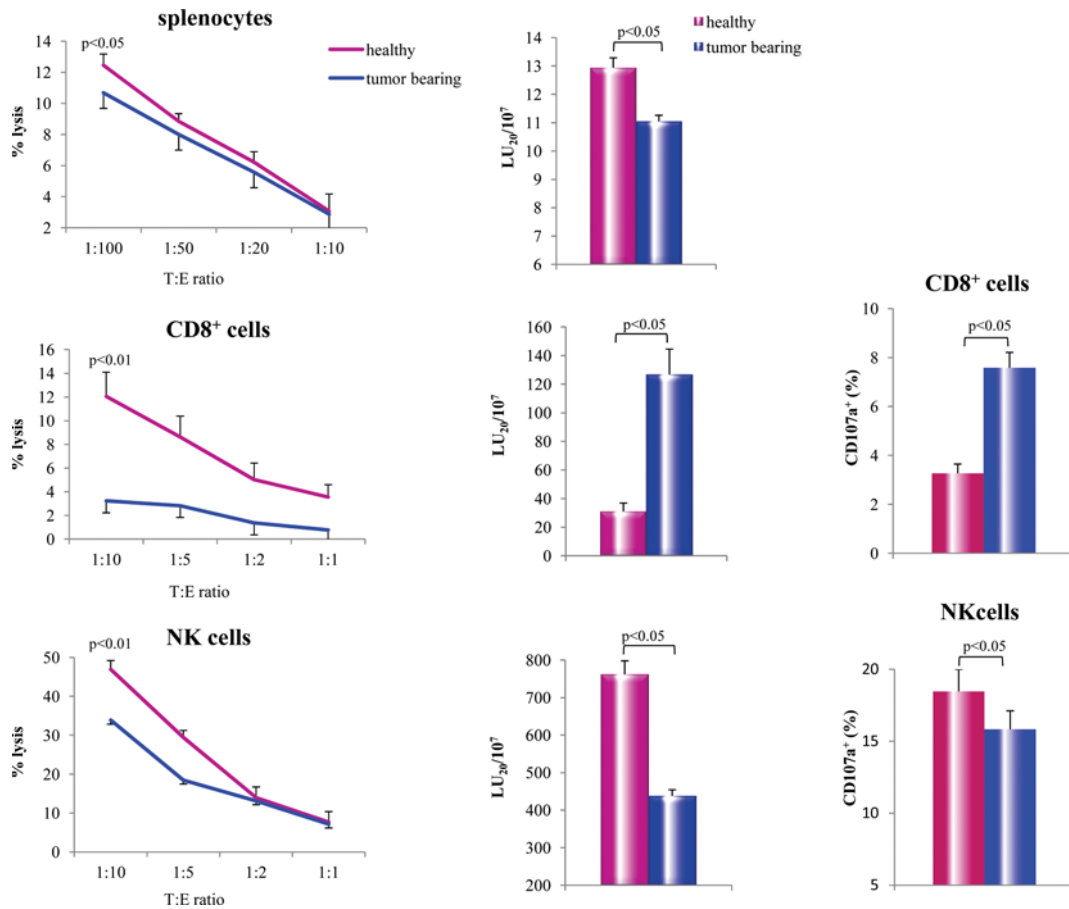


Figure 2. Cytotoxic activity of total splenocytes, CD8+ T cells and NK cells

The cytotoxicity of splenocytes, isolated CD8+ T cells and NK cells was tested with a 4-h MTT assay against 4T1 cell targets at day 12 after tumour inoculation. The data are presented as the mean percentages of specific cytotoxicity and as LU20/107 effector cells, which was calculated on the basis of mean percentages of killing at four different E:T ratios and percentages of effector cells found in the spleen. The cytotoxic capacity of NK and CD8+ T cells was also determined by flow cytometric analysis of CD107a expression on NKp46+ and CD8+ cells. The gate used for analysis was the mononuclear cell region in FSC/SSC plots. Data are means \pm SEM of two individual experiments, each carried out with four mice per group. Statistical significance was tested by Student's t-test.

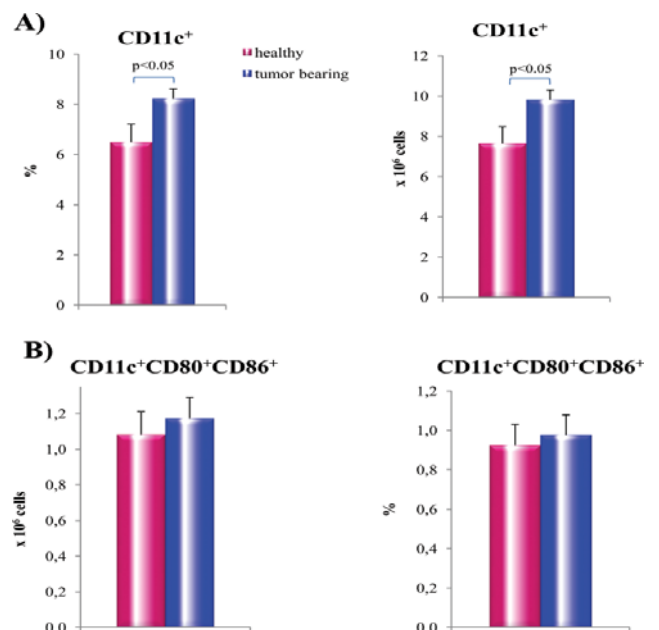


Figure 3. Frequency, number and functional phenotype of CD11c+ dendritic cells derived from the spleen

A) The percentage and total number of CD11c+ dendritic cells were determined with a FACSCalibur flow cytometer before and at day 12 following 4T1 tumour challenge. The results are presented as the mean \pm SEM of 8 mice per group. B) The percentage and total number of CD80+CD86+ dendritic cells were also analysed by flow cytometry. The results are presented as the mean \pm SEM of 4 mice per group. Statistical significance was determined by Student's t-test.



DISCUSSION

In this study, we report that 12 days after the inoculation of 4T1 mammary carcinoma cells, the percentage and number of CD19⁺ B cells in spleens were significantly increased, while the number and cytotoxic activity of NK cells were decreased (Fig. 1). Additionally, the frequency and number of CD11c⁺ dendritic cells were higher in spleens from tumour-bearing mice compared with naïve mice, but the frequency of activated dendritic cells in both groups was not significantly different (Fig. 3).

The increased frequency and number of CD19⁺ B cells in spleens of tumour-bearing BALB/c mice could be the result of Th2 polarisation, which implicates the predominance of the type-2 antitumor immune response (22). However, there is evidence that the type-17 response can also induce B cell proliferation and the formation of germinal centres (47). Several studies have shown that B cell proliferation in lymph organs correlates with tumour progression (48-49). Furthermore, we found no difference in the percentage or number of splenic T cells and CD4⁺ and CD8⁺ subpopulations, indicating weak or no activity of adaptive cellular immunity, on day 12 after tumour challenge (Fig 1).

We also detected the decreased cytotoxicity of total splenocytes isolated from tumour-bearing mice (Fig. 2). To determine which cell population was responsible for this phenomenon, we isolated NK and CD8⁺ T cells and tested their antitumor cytotoxic activity *in vitro* at day 12. We found enhanced cytotoxicity of CD8⁺ T cells (Fig. 2). However, the cytotoxic activity of NK cells was significantly decreased at the same time point, which contributed to the lower cytotoxic activity of total splenocytes (Fig. 2). During the cytotoxic killing of tumour cells, NK cells and CD8⁺ T cells rapidly release granules containing perforin and granzymes into the immunological synapse, thereby inducing the death of target cells (50). Lysosomal-associated membrane protein-1 (LAMP-1), also known as CD107a, is a marker of cytotoxic degranulation, as it lines the membrane of these granules (51). CD107a can be used as an indirect indicator of the cytotoxic capacity of NK and CD8⁺ T cells (52). In line with these findings, we found a higher frequency of activated CD8⁺CD107a⁺ T cells while activated NKp46⁺CD107a⁺ NK cells were less frequent in spleens from tumour-bearing mice (Fig. 2). Several studies have revealed lower cytotoxic activity of NK cells in patients with breast cancer compared with healthy controls, with a negative correlation of NK cell lytic activity with lymph node progression of disease (9).

Functional maturation and the activity of NK and T cells appear to be dependent on the functional phenotype of DCs (53). We showed that tumour inoculation led to an increase in the frequencies and numbers of CD11c⁺ DCs in spleens (Fig. 3). During maturation/activation, DCs express more MHC class II and CD80 and CD86 co-stimulatory molecules, all of which have T-cell stimulatory capacity. However, there was no difference in the percentage

or total number of fully functional CD80⁺CD86⁺ DCs in tumour-bearing mice compared with healthy animals (Fig. 3). Thus, it could be assumed that 12 days after tumour inoculation, the functional maturation of dendritic cells was absent. The interaction of mature DCs with NK and T cells is essential for the tumoricidal activity of these effector cells, while immature DCs can induce immunosuppressive activity of the same effector cells (34-35).

Taken together, we demonstrate that 12 days after inoculation with 4T1 mammary breast carcinoma, NK cell cytotoxicity was markedly reduced, and DCs were not activated after tumour challenge. These findings suggest a suppressed innate anti-tumour immune response in mammary carcinoma-bearing BALB/c mice.

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