Inhibitory effects of 4T1 breast tumor transplantation on mouse peripheral blood immune cell populations

Abstract

Aims: One of serious threats to women's health is mammary cancer whose occurrence, development, and treatment are related to the body's immunological circumstances. In addition, the cancer also imposes the some effects on the body's immune system. However, the body's response is very diverse because it varies from type to type of cancer. This paper reported that the effects of 4T1 cell transplantation on immune cells and spleen in mice.

Methods: Twenty female BALB/C mice were randomly divided into a control group and transplantation group. 4T1 cells were injected into the forth mammary fat pad to construct an animal model of breast cancer metastasis. The lymphocytes from mouse peripheral blood after transplantation and were analyzed by flow cytometry.

Results: The transplantation of 4T1 cells rapidly and continuously decreased the percentages of total T cells, total B cells, cytotoxic T cells and helper T cells in peripheral blood during experimental period (28 days). In addition, memory T cells in the transplantation group were increased at 28 days after transplantation. Only the natural killer (NK) cell percentage was significantly increased at 14 days after transplantation.

Conclusions: 4T1 cell transplantation exerted distinctive effects on the different types of immune cells in mouse peripheral blood: the transplantation of 4T1 cells decreased the levels of total T cells, total B cells, cytotoxic T cells, helper T cells and memory T cells, and the natural killer (NK) cell was increased transiently than in control group.

Key words: T cell; B cell; NK cell; Breast cancer; BALB/C mice.

1. Introduction

Breast cancer remains a serious problem to human health worldwide and is associated with high mortality rates [1]. The poor prognosis of breast cancer patients is generally due to the high rates of recurrence and metastasis of tumors [2]. In addition, the age of onset has gradually become younger, and increasing numbers of younger patients are being diagnosed with breast cancer [3]. Although a large number of studies have helped elucidate the mechanisms of breast cancer development and metastasis, there are still many problems around the clinical treatment and prevention of cancer [4]. Increasing evidence has shown that immune function is critical to restrain the initiation, development and metastasis of cancers [5, 6]. Therefore, improving the body's own anti-cancer defense potential is the most important direction to prevent and treat malignant tumors [7]. However, successful immune therapy and prevention for cancers requires understanding of the relationship between the development of cancers and changes in the immune system.

The immune system is a very complex defense system that protects the body against exogenous pathogens and autologous diseases and consists of multiple types of organs, tissues, cells and biomolecules [8]. Lymphocytes, including B cells, T cells and NK (natural killer) cells [9], are major constituents of this system and carry out multiple functions in the body. T cells directly destroy pathogens and tumor cells and also help antigens to stimulate B cells to produce antibodies [10]. For instance, the costimulatory molecule B7 expressed by melanoma activates CD8+ T cells to directly destroy melanoma cells, and then inhibited the development of melanoma in mice [11, 12]. CD4+ T cells and CD8+ T cells generated from the mouse immune system previously stimulated by human pathogen chlamydia trachomatis could directly kill the pathogens [13]. NK cells also function to kill target pathogens and tumor cells directly after activation by cell stimulatory factors [14]. NK cells were expressed in the highest activity by C57BL/6 mice during the 2-6 days after infection with rickettsia [15], and their synergy with IFN-γ could stimulate the mice early anti-rickettsia immune response [15, 16]. NK

cells isolated from malignant pleural effusions in mice had high antitumor activity *in vitro* after activated by IL-2 and IL-15 [17]. Thus, the defense of the human body against foreign pathogens requires close synergistic effects among T, B, and NK cells and other immune cells [14, 18, 19].

There is a considerable diversity in the type of the immune cells. B lymphocytes are classified into two categories, B1 and B2, according to their functions [20]. T lymphocytes are divided into helper T cells, cytotoxic T cells, regulatory T cells and memory T cells [21]. NK cells consist of activated and anergic cells [21]. Within each of these immune cell types, a number of subsets have been identified and more novel sorts of immune cells are continually being uncovered [22]. This diversity of the immune cells aids in defending the body against the infection of various exogenous pathogens and the development of autologous tumors.

The relationship between the immune system and tumor development is extremely complicated. The immune system is capable of recognizing and eradicating sporadic neoplasm cells in the body, but a certain proportion of primary tumor cells still manage to escape from the immune surveillance to develop and metastasize [23]. Loss of HLA class I antigen expression on the surface of tumor cells would lead to colorectal cancer, lung cancer and other tumors to escape from T cell-mediated immune surveillance and metastasize [24-26]. The interaction between CD147 on hepatoma cell surface and its natural ligand cyclophilin A activated the ERK1/2 signaling pathway to help hepatoma cells to evade the immune surveillance of T cells and promote their proliferation [27]. Tumors can also avoid immune attack by suppressing the body's immune system or recruiting the immune cells [28]. The myeloid derived suppressor cells (MDSCs) derived from immature myeloid cells suppress the body immune system. The levels of MDSCs were increased by cancer development in hepatic carcinoma, lung cancer and acute myeloid leukemia [29-31]. In leukocyte infiltrating tumors such as breast cancer and colorectal cancer, MDSCs reduced immune attack on tumors [32-34]. Although studies have shown that tumor development is associated with changes in the immune system, the understanding of the

mechanisms by which tumors interact with the immune system remains rather limited.

For an effective immune response to tumors, the body's immune defense system should be able to discriminatively respond to the different types of tumors and tumor cells, as cell types are diverse within a single type of tumor and tumors also show great diversity [35, 36]. The types of cells within a single tumor vary as cancers develop, and this variation then imposes a continuously changing effect on the immune system [37]. Therefore, each tumor has its specific relationship with the immune system and immune cells show exceptional variation patterns in response to a given cancer. The understanding of the exact effects of specific types of tumor on immune cells will be helpful to study the anti-tumor mechanisms of the immune system and provide valuable references for medical treatments of cancers and health care practice.

In this study, to examine the relationship between the development of breast tumors and the response of immune cells, we used a 4T1 breast cancer cell transplantation mouse model. The processes of growth and metastasis of 4T1 breast cancer cells in mouse are similar to those observed in human breast cancer. After transplantation into BALB/C mice, 4T1 cells form orthotopic breast tumors at the transplantation site and metastasize, spontaneously and rapidly, to lung, liver, lymph nodes and brain [38]. Here we examined B cells, T cells and NK cells from peripheral blood in mice transplanted with 4T1 breast cancer cells to detect changes in the immune system in response to the development of breast tumors.

2. Materials and methods

2.1. Animals

BALB/C female mice 4–6 weeks of age were purchased from Liaoning Changsheng Biological Technology Company in China. Twenty mice were randomly divided into the control group and the 4T1 breast cancer cell transplantation group, and all mice were kept in SPF (Specific pathogen free) conditions. Mice were allowed

for a 1-week acclimatization period at room temperature with a 12 h light/dark cycle before treatment. The animals were fed with normal rodent chow and allowed free access to drinking water.

2.2. Cell culture and transplantation

The mouse breast cancer cell line 4T1 cells were cultured in RPMI 1640 media supplemented with 10% FBS (Fetal bovine serum), 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37ºC. We collected 4T1 cells in the logarithmic phase and mice in the transplantation group were injected with 2×10^5 cells into the fourth breast fat pad.

2.3. Peripheral blood collection and immune cell isolation

Mouse peripheral blood was collected from the mouse facial vein vascular bundle at four time points: day 0, 7, 14 and 28 (Fig.1). Lymphocytes were isolated with lymphocyte separation medium (MP Biomedicals, CA, US).

2.4. Lymphocyte labeling

The peripheral blood immune cells were washed twice with washing buffer (0.15 M PBS (Phosphate buffered solution), 0.5% BSA (Bovine serum albumin）, 0.1% NaN3 (Sodium azide)). The cells were then re-suspended in 100 μL washing buffer and incubated with optimized amount of fluorochrome conjugated mAbs (Monoclonal antibodies) for 30 min at 4ºC in the dark. The total B cells were labeled by CD19-PerCP/Cy5.5 (Biolegend, San Diago, California, US. Catalog#115533); the total T cells were labeled by CD3e-FITC (Miltenyi Catalog#130-102-496), helper T cells by CD4-FITC (Biolegend Catalog#100405), cytotoxic T cells by CD8a-PE (Biolegend Catalog#100707) and memory T cells by CD127-PE (Biolegend Catalog#135009). NK cells were labeled by CD49b-PE (Miltenyi Catalog#130-108-174). The labeled cells were then washed twice with washing buffer and reserved for flow cytometry analysis.

2.5. Flow cytometry analysis

Flow cytometry was conducted using a FACSCalibur flow cytometer (BD Biosciences) and the data were analyzed with FlowJo software.

2.6. Anatomic observation

Mice were sacrificed and dissected to observe visceral organ morphology at day 29 (Fig. 1). The spleen, lung and liver were harvested for observation.

2.7. Statistical analysis

All data are presented as the mean ± S.E.M. Statistical significance between more than two groups was tested using one way ANOVA. P values < 0.05 and < 0.01 were considered statistically significant and extremely statistically significant, respectively.

3. Results

3.1. Effects of 4T1 cell transplantation on total T cells and total B cells

We examined changes in total T cells and total B cells in mouse peripheral blood in the 4T1 cell transplantation and control mice (Fig. 2) and detected significant variations in both cell populations in the 4T1 cell transplantation mice compared with control mice. At day 7, we observed a significant decrease in the total T cells to 36.67 ± 1.91% in mice transplanted with 4T1 cells compared with controls (45.27 ± 1.62%) ($P < 0.05$), with further decreases to 26.2 ± 2.8% at day 14 compared with controls (44.37 ± 4.05%) ($P < 0.01$) and 13.47 ± 3.11% at day 28 compared with controls (44.57 ± 3.16%) ($P < 0.01$). This result showed that the transplantation of 4T1 cells had a rapid inhibitory effect on the total T cell level in mouse peripheral blood.

On examining the total B cells in peripheral blood, a very significant decline ($P < 0.01$) was observed at day 7 in the 4T1 cell transplantation group compared with controls (22.8 ± 1.44% versus 29.37 ± 2.3%, respectively). The total B cells further decreased to 10.27 ± 1.7% at day 14 compared with controls (29.57 ± 1.32%) ($P < 0.01$).

At day 28, the total B cells in the 4T1 cell transplantation group were still significantly lower than that in control mice (5.05 ± 1.52% versus 27.93 ± 3.11%, respectively) ($P < 0.01$). These results showed that the transplantation of 4T1 cells had a strong inhibitory effect on the total B cell level in mouse peripheral blood.

3.2. Effects of 4T1 cell transplantation on helper T cells and cytotoxic T cells

We also examined the helper T cells and cytotoxic T cells in mouse peripheral blood in the 4T1 cell transplantation and control groups (Fig. 3). On examining helper T cells, a significant decrease was detected in the 4T1 cell transplantation group at day 7 compared with the control (22.4 ± 1.65% vs. 27.2 ± 4.19%, respectively) ($P < 0.05$). The helper T cells in the 4T1 cell transplantation group further decreased to 16 ± 1.67% at day 14, which was a very significant difference ($P < 0.01$) compared with the control (26.33 ± 4.04%). At day 28, the helper T cells in the 4T1 cell transplantation further decreased to 7.6 ± 2.51%, which also was very significantly different compared with the control (27.2 ± 3.62%) ($P < 0.01$). This result showed that the transplantation of 4T1 cells had a strong inhibition effect on the helper T cell level in mouse peripheral blood.

We also detected a significant alteration in the cytotoxic T cell population after transplanting 4T1 cells. At day 7, we observed a remarkable decrease in cytotoxic T cells in the 4T1 cell transplantation group compared with the control (9.69 ± 1.15% and 11.07 ±1.78%, respectively) ($P < 0.05$). At day 14, the cytotoxic T cells in the 4T1 cell transplantation group continued to decrease compared with controls (7.92 ± 1.67% and 12.13 ± 1.1%, respectively) ($P < 0.01$). At day 28, the cytotoxic T cells in the 4T1 cell transplantation group were even more significantly lower than that in controls (3.75 ± 1.09% and 11.57 ± 1.63%) ($P < 0.01$). This result showed that the transplantation of 4T1 breast cancer cells had an inhibitory effect on the cytotoxic T cell level in mouse peripheral blood.

3.3. Effects of 4T1 cell transplantation on memory T cells

To explore whether transplantation of 4T1 cells has a long-lasting effect on the immune system, we examined memory T cells by monitoring the surface marker CD127 with flow cytometry. As shown in Fig. 4, at day 28, the level of memory T cells in the 4T1 cell transplantation group was 3.55 ± 2.14%, which was extremely significantly reduced compared with the control (35.77 ± 1.66%) ($P < 0.01$). This result suggested that the transplantation of 4T1 cells could suppress memory T cells and exert a long-lasting inhibition effect on the immune system in mice.

3.4. Effects of 4T1 cell transplantation on NK cells

As shown in Fig. 5, we did not detect a significant change between the treatment and control groups after 4T1 cell transplantation at day 7. However, at day 14, the NK cells increased to 36.93 ± 1.83% in the 4T1 cell transplantation group, which was significantly higher compared with controls (20.67 ± 1.46%) ($P < 0.01$). At day 28, the NK cells in the 4T1 cell transplantation group fell back to 26.33 ± 4.93%, which was similar to that detected in the control (22.23 ± 2.81%). This result showed that the transplantation of 4T1 cells had a lagged and transient promotion effect on the NK cell level in mouse peripheral blood.

3.5. Effects of 4T1 cell transplantation on mouse visceral organs

To observe whether transplantation of 4T1 breast cancer cells has a direct influence on the mouse visceral organs, we sacrificed and dissected mice on day 29. As shown in Fig. 6, the spleen in the 4T1 cell transplantation group was larger than that of control mice. However, we did not observe any significant difference in lung and liver between the transplantation and control mice. This result showed that the transplantation of 4T1 cells had an effect on the spleen development in mouse.

4. Discussion and Conclusion

Our investigation showed that the transplantation of 4T1 cells in mice remarkably reduced the amount of B cells and T cells, including cytotoxic T cells, helper T cells and memory T cells, in peripheral blood and induced NK cells to transiently increase and then decrease. These results indicate that B cells and T cells in the peripheral blood of mice were vulnerable and susceptible to 4T1 breast cancer development. In other words, the development of breast cancer can strongly inhibit the proliferation of immune cells or destroy immune cells. The similar results to this experiment were also observed in some studies. The contents of CD4+ T and CD8+ T cells in peripheral blood in breast cancer patients was found significantly lower than in healthy controls [39, 40]. T lymphocytes are the main tumor-infiltrating immune cells with antitumor effects in breast cancer [41]. The elevated ratio of infiltrating lymphocytes CD4 / CD8 indicated the better prognosis of breast cancer patients after surgery and chemotherapy [42]. The content of infiltrating B lymphocytes increased in tumor tissue of breast cancer patients [43], and the infiltrating B lymphocytes in malignant breast cancer tissue was significantly higher than in benign tumors [44]. However, the relationship between the changes of B lymphocyte content and the development of tumor in peripheral blood of breast cancer patients is rarely studied. Memory T cells in bone marrow of breast cancer patients was significantly higher than healthy individuals [45]. The percentage of NK cells was decreased in peripheral blood of mice after transplantation of 4T1 breast cancer cells, while it was significantly increased after tumor resection [46]. Although increasing evidence suggests that tumor-infiltrating leukocytes may promote angiogenesis, growth and invasion of the tumors [47, 48], the decline of leukocyte levels in the peripheral blood likely has an adverse effect on the body's defense against solid tumors, as the vascular system is an important pathway for leukocyte transport and a pivotal defense line to block solid tumor metastasis through vascular system.

The function of B cells is to carry out humoral immunity *in vivo* [49]. After stimulation by antigens, B lymphocytes differentiate and proliferate into plasmocytes, which synthesize and release antibodies to defend the body against the infection of various pathogens [50]. The mice, in which the effecter subset of B cells was deficient or depleted, displayed a slower tumor growth compared with control mice [48, 51-53], however, in our experiment, B cell level was decreased in peripheral blood of 4T1 transplantation mice. This suggests that the decrease of B cell level is adverse to the defense of body to cancer. The effecter subset of B cells was also shown to directly kill cancer cells via the Fas/FasL pathway [54]. A higher density of cancer-filtrating CD20+ B cells significantly correlated with an improved overall survival in colorectal cancer patients [55]. Moreover, B cells provide co-stimulatory signals and serve as antigen-presenting cells to activate T cells, contributing to cellular immunity [10]. Antigen-presenting B cells were shown to activate tumor-specific T cell cytotoxicity [56] and stimulate NK cells [51].

The function of T cells is to implement cellular immunity in the body [8]. Cellular immunity is critical in preventing diseases such as HIV and in targeting pre-cancerous and cancerous cells [57]. After initial stimulation by antigens, T lymphocytes differentiate and proliferate into effecter T cells and memory T cells, and the effecter T cells then destroy target cells upon secondary exposure to the same antigen [58]. cytotoxic T cells belong to the effecter T cells. Helper T cells not only participate in cellular immunity, but are also involved in humoral immunity by assisting antigens to stimulate B cells to synthesize and release antibodies [59]. Immunological memory is one of the pivotal features of the immune response and is key in resisting repeated pathogen invasion and elimination of malignant cells [60]. NK cells kill target pathogens or cells directly after activation by antibodies. Recent studies have suggested that the activated NK cells play an important role in tumor defense [61]. Together these data support the idea that the reduced numbers of populations of immune cells in the peripheral blood of mice will have an adverse effect on anti-cancer responses.

Despite our current understanding of some of the functions of immune cells in cancer response, the mechanism of inhibition of immune cells by breast cancer development in peripheral blood is still elusive. The reduction of leukocyte levels in mouse peripheral blood may be attributed to the suppression from the tumor-recruited immune cells.

Regulatory B cell subpopulations produce cytokines and/or immune regulatory ligands such as IL-10, TGF-β and PD-L1 in murine autoimmune models [51, 62]. IL-10 is also produced by monocytes, type 2 T helper cells (Th2), mast cells, regulatory T cells and certain subset of activated T cells [63]. However, the variations of IL-10 level in cancer patient serum compared with healthy controls were not shown a consistent patterns, that is, IL-10 level increases in some patients and decreases in others. IL-10 enhances B cell antibody production, proliferation and survival, and also down regulates the expression of co-stimulatory molecules on macrophages, Th1 cytokines and MHC class II antigens [64]. PD-L1 represses T cell and/or NK cell reactions [65]. TGF-β signaling has been demonstrated to suppress memory T cell development [66] and supports the maintenance of regulatory function and homeostasis in peripheral regulatory T cells [67]. Regulatory T cells are critical to maintain the homeostasis of the immune system via negative regulation of other types of immune cells. Adaptive regulatory T cells can be induced and recruited by cancers [68]. The supernatants from cultured follicular dendritic cells also inhibit human B-lymphocyte proliferation [69]. In the tumor environment, dendritic cells can be transformed into immunosuppressive regulatory dendritic cells [70, 71].

The function of immune system depends on the coordination among diverse immune cells. Some of the T cells regulate cellular immunity by helping antigens to stimulate B cells to synthesize and release antibodies [59]. Some of the antibodies activate NK cells to destroy target cells and pathogens directly [72]. Our observations have shown that 4T1 breast cancer cell transplantation treatment severely inhibited the immune system in the mouse. Although the 4T1 cell transplantation induced a transient increase of NK cell levels in the peripheral

blood, this increase may not be sufficient to inhibit tumor development as helper T cells, cytotoxic T cells, B cells and memory T cells were severely suppressed.

In summary, our results show that 4T1 cell transplantation exerts inhibitory effects on the body's immune system. Therefore, further research is required to investigate the mechanisms of 4T1 breast cancer cell transplantation effects on the immune cells in peripheral blood.

Conflict of interest: We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence the manuscript entitled, "Inhibitory effects of 4T1 breast tumor transplantation on mouse peripheral blood immune cell populations".

Ethical Approval:

"All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee"

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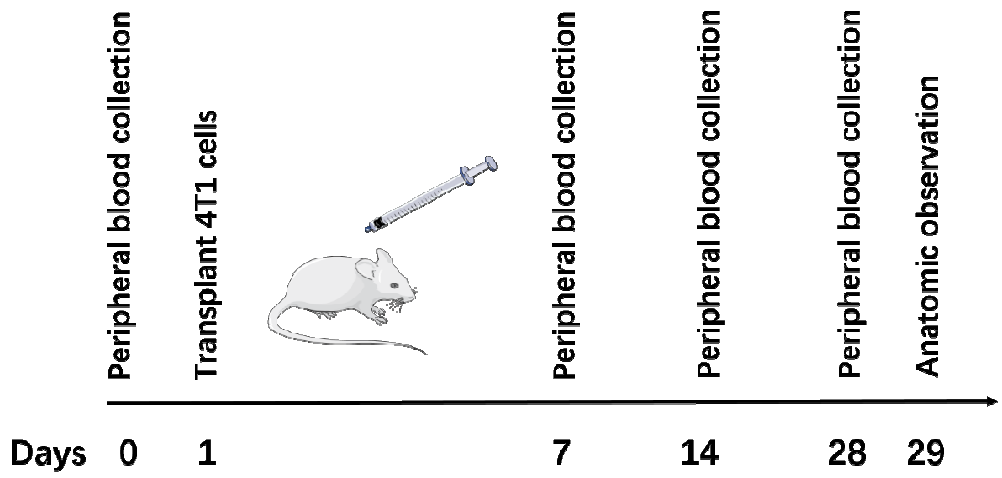
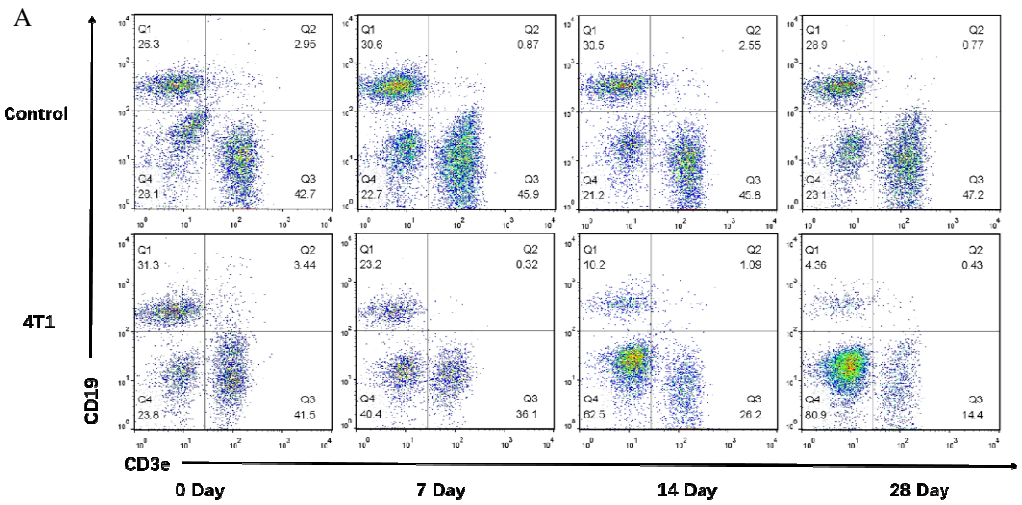


Fig. 1. Experimental strategy for 4T1 cell transplantation and peripheral blood collection



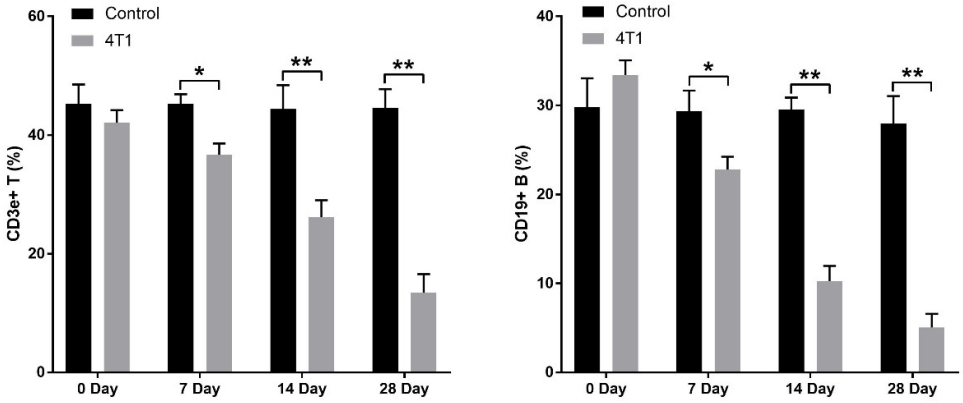
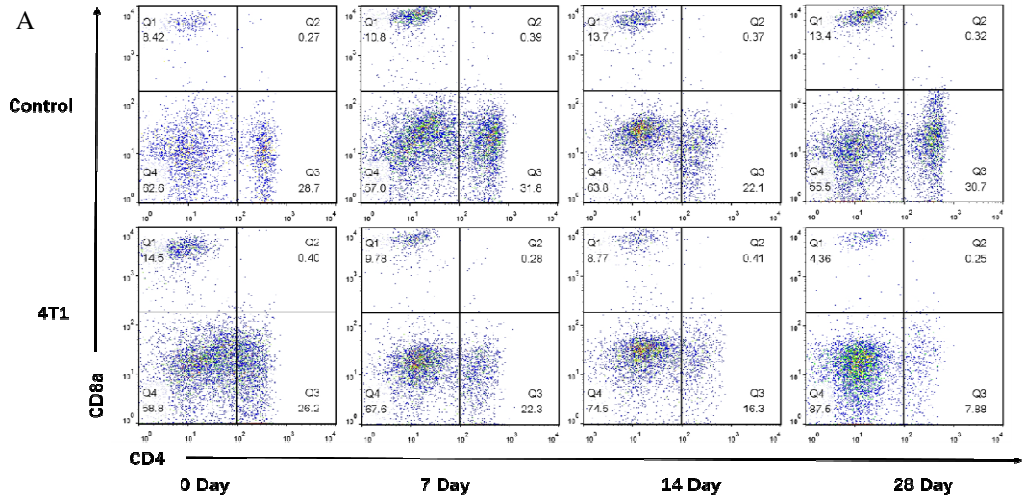
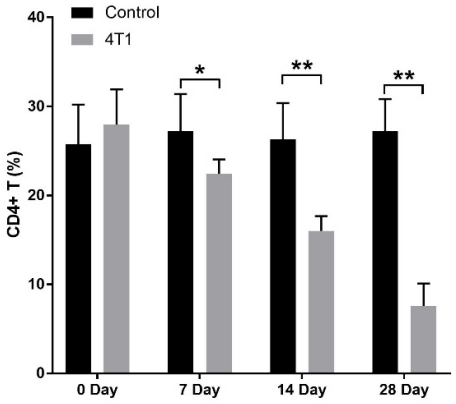


Fig. 2. Effects of 4T1 cell transplantation on total T cells and total B cells in mouse peripheral blood. A. Flow cytometry plots of total T cells and total B cells. B. Comparison of total T cells between the transplantation and control mice. C. Comparison of total B cells between the transplantation and control mice. T cells were labeled by CD3e-FITC and B cells were labeled by CD19-PerCP/Cy5.5. N = 10. *$P < 0.05$, **$P < 0.01$.





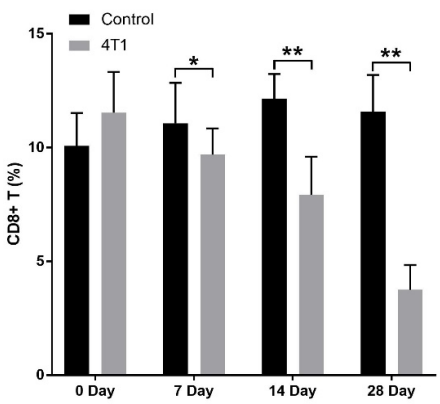


Fig. 3. Effects of 4T1 cell transplantation on helper T cells and cytotoxic T cells in mouse peripheral blood. A. Flow cytometry plots of helper T cells and cytotoxic T cells. B. Comparison of helper T cells between the transplantation and control groups. C. Comparison of cytotoxic T cells between the transplantation and control groups. T cells were labeled by CD3e-FITC and B cells were labeled by CD19-PerCP/Cy5.5. N = 10. $*P < 0.05$, $**P < 0.01$.

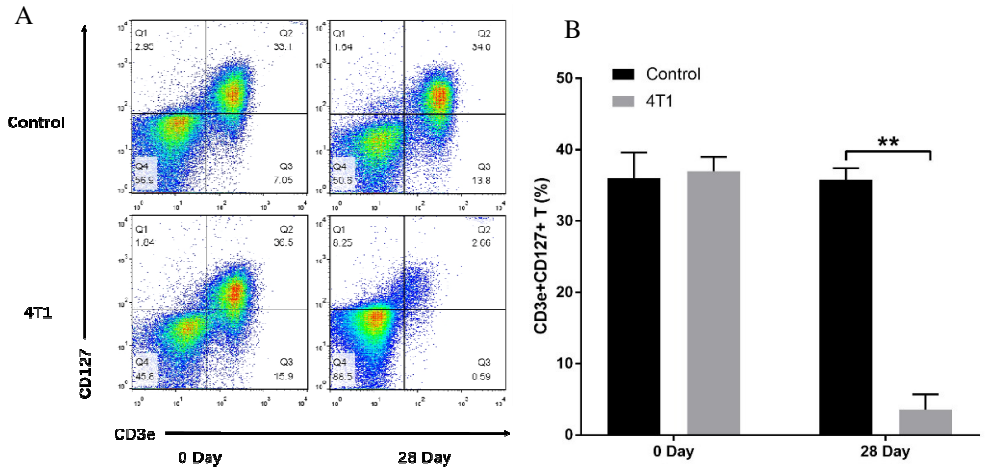
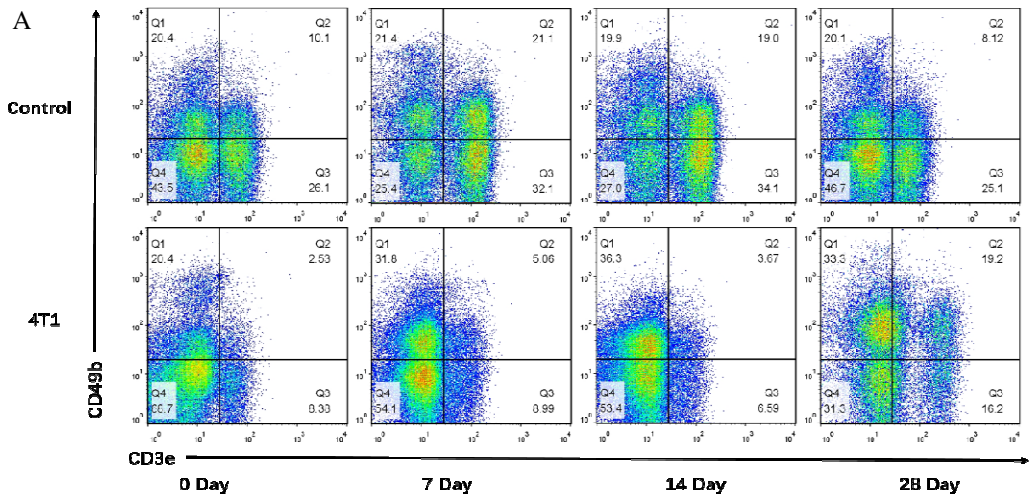


Fig. 4. Effects of 4T1 cell transplantation on memory T cells in mouse peripheral blood. A. Flow cytometry plots of memory T cells. B. Comparison of memory T cells between the transplantation and control. Memory T cells were labeled by CD127-PE. N = 10. $^{**}P < 0.01$.



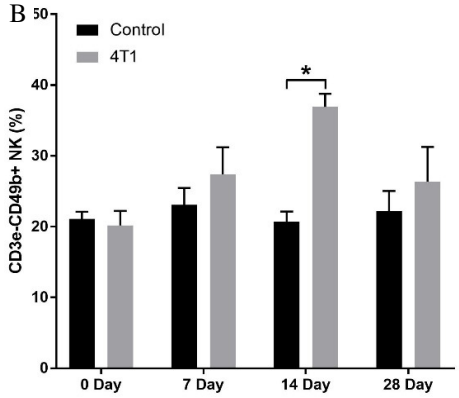


Fig. 5. Effects of 4T1 cell transplantation on NK cells in mouse peripheral blood. A. Flow cytometry plots of NK cells. B. Comparison of NK cells between the transplantation and control groups. NK cells were labeled by CD49b-PE. N = 10. *$P < 0.05$.

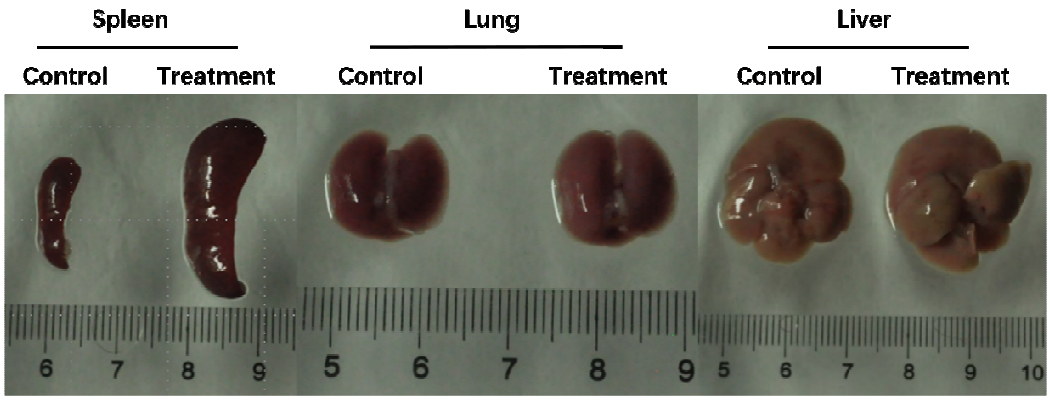


Fig. 6. Effects of 4T1 cell transplantation on mouse visceral organs.