original article

In vitro-activated tumor-bearing host T cells and the effectiveness of tumor vaccine immunotherapy

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BACKGROUND AND OBJECTIVE: Vaccination during periods of lymphopenia may facilitate immune responses to weak self-antigens and enhance antitumor immunity. The objective of this study was to determine the effectiveness of tumor vaccine immunotherapy combined with immune reconstruction using tumor-bearing host immune cells in lymphopenia, and to investigate the role of tumor-bearing host T cells activated in vitro during immunotherapy.

DESIGN AND SETTING: Animal study conducted in the First Affiliated Hospital of Xi'an Jiaotong University from January 2009 to January 2010.

PATIENTS AND METHODS: Lymphopenia was induced by cyclophosphamide. A reconstituted immune system with different syngeneic lymphocytes was employed, including lymphocytes from naïve rats (unsensitized group), tumor-bearing rats (tumor-bearing group), and tumor-bearing rats activated in vitro (activated group). All rats were immunized with granulocyte-macrophage colony-stimulating factor (GM-CSF)-modified NuTu-19 ovarian cancer (GM-CSF/NuTu-19) cells. Tumor vaccine-draining lymph nodes (TVDLNs) were harvested, and then stimulated to induce effector T cells (T_E). T_E were then adoptively transferred to rats bearing a 3-day pre-established abdominal tumor (NuTu-19), and the survival rate was calculated.

RESULTS: Compared with the unsensitized group, the levels of interleukin-2 (IL-2) were significantly lower in the tumor-bearing group, whereas that of IL-4 were significantly higher ($P<.05$). The number of CD4+ T cells secreting interferon-γ and the specific cytotoxicity of CD8+ cytotoxic T lymphocytes were significantly lower ($P<.05$). The survival was significantly higher in the activated group compared with the other groups.

CONCLUSIONS: Lymphocytes from tumor-bearing rats activated in vitro can effectively reverse the immunosuppressive effects of tumor-bearing hosts.

A major impediment to the development of effective cancer immunotherapy is the lack of strong tumor rejection antigens. Most of the tumor-associated antigens that have been identified are weak self-antigens.[1] Recently, it has been suggested that vaccination during periods of lymphopenia may facilitate immune responses to weak self-antigens and enhance antitumor immunity.[2,3] These observations provide a strong impetus to exploit the critical period of immune reconstitution in lymphopenic mice for active tumor vaccination. Immune reconstitution in clinical application has involved mainly autogenous peripheral blood mononuclear cells (PBMNCs) transfusion. However, the immune status of a tumor-bearing host could be inhibited by TGF-β, IL-10, VEGF, and other cytokines as well as the Fas/FasL system secreted by autogenous immune system.[4] Therefore, the immune reconstituted effect with lymphocytes of tumor-bearing hosts still requires further investigation.

We reconstituted the immune systems of tumor-bearing rats with lymphocytes to simulate the reconstitution of the immune systems of clinical tumor patients with PBMNC transfusion. At the same time, the rat hosts were given tumor vaccine immunotherapy. We studied the effectiveness of this therapy and unraveled the mechanism of immune suppression under a tumor-bearing state, and explored the feasi-

bility of tumor-bearing rat lymphocytes activated in vitro in a reconstituted immune system for reversing T-cell inhibition.

PATIENTS AND METHODS

Reagents

Anti-CD3 and fluorescein isothiocyanate (FITC)-anti-CD4 were from USA eBioscience (San Diego, CA, USA). Recombinant interleukin 2 (rIL-2) was from Shenyang Sansheng Pharmaceutical Limited Company (Shenyang, China). Rat IL-2 ELISA Kit and rat IL-4 ELISA Kit were from Shanghai Xitang Biological Technology Limited Company (Shenyang, China). BD Cytofix/Cytoperm Plus fixed rupture of membrane kit and PE-anti-interferon-γ monoclonal antibody (PE-anti-IFN-γ mAbs) were from BD Company (Franklin Lakes, USA). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Sigma-Aldrich, St. Louis, NO, USA. 7-Amino-actinomycin (7-AAD) cells Detect Liquid was purchased from Beijing Jing Mei Limited Company (Beijing, China).

Animals and cells

Female F344 rats between the ages of 8-10 weeks from the Slac Laboratory Animal Ltd. (Shanghai, China) were used for experiments. Standard principles of laboratory animal care and handling were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996). NuTu-19 is a cell line derived from a poorly differentiated adenocarcinoma formed in a F344 female athymic rat (a gift from Dr. He Wang, Huaxi Medical University, Sichuan, China). Granulocyte-macrophage colony-stimulating factor-modified NuTu-19 ovarian cancer (GM-CSF/NuTu-19) cell lines were produced by transducing NuTu-19 with recombinant retrovirus vector PLGM-CSFSN as described previously.[5] Cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum, 0.017% penicillin G, and 0.01% gentamycin in a humidified incubator at 37°C and 5% CO_2.

Animal studies

The animals were divided into three groups with 10 rats in each group. Group 1 was defined as the unsensitized group: Chemotherapy–immune reconstitution with lymphocytes from naïve syngeneic rats-carcinovaccine immunity. Group 2 was defined as the tumor-bearing group: Chemotherapy-immune reconstitution with tumor-bearing host lymphocytes–carcinovaccine immunity. Group 3 was defined as the activated group: Chemotherapy-immune reconstitution with tumor-bearing host lymphocytes activated in vitro–carcinovaccine immunity.

Preparation of tumor-bearing host lymphocytes

NuTu-19 cells (1×10^7 cells/rat) were injected into the abdominal cavity of the rats. The spleen cells were harvested 7 days after injection. The tumor-bearing host lymphocytes in suspension were then used to reconstitute immune systems in group 2 or were activated with anti-CD3 (5 μg/mL) for 2 days and rIL-2 (60 IU/mL) for 3 additional days to be used in group 3.

Reconstitution of immune system and carcinovaccine immunity

Cyclophosphamide (20 mg/kg) was injected into the abdominal cavity of F344 rats once per day for 2 days to induce acute lymphocytopenia; 48 hours after the second injection, the naïve splenocytes (2×10^8 cells/rat) from syngeneic rats were injected into vena caudalis of rats from group 1 for the reconstituted immune system. The lymphocytes from tumor-bearing rats were injected into rats in group 2. The activated lymphocytes in vitro were used in group 3. The live GM-CSF/NuTu-19 carcinovaccine was injected 24 hours after immune reconstitution by subcutaneous inoculation in 4 spots on both sides of the auxiliary fossa and inguina (3×10^6 cells/spot). Nine to 10 days after carcinovaccine immunization, tumor vaccine–draining lymph nodes (TVDLNs) from the rats were harvested. TVDLN cells were cultured in 24-well plates and activated by anti-CD3 (5 μg/mL, eBioscience) for 2 days. The active cells were harvested in the culture flask and treated with rIL-2 (60 IU/mL) to induce proliferation for 3 days to obtain effector T cells (T_E).

Quantitative detection of the IL-2 and IL-4

T_E cells were distributed into the 96-well plates at a concentration of 1×10^5 cells/well. Anti-CD3 mAbs (1 μg/well, eBioscience) and rIL-2 (60 IU/mL) were added and cultured for 3 days at 37°C in a 5% CO_2 incubator. NuTu-19 cells (1.5×10^3) were then added into each well with specific stimulation after being cultured under the same conditions for 3 days. The supernatant of each well was harvested and tested by an enzyme-linked immunosorbent assay (ELISA) kit (Westang Biotech, Shanghai, China) for additional 3 days.

Cytokines staining of interferon-γ

The prepared T_E cells were evenly distributed onto 24-well plates (4×10^6 cells/well). NuTu-19 cells (2×10^5 cells/well) were added into each well for spe-

cific stimulation. Brefeldin A (BFA) (1 μL/mL, BD Biosciences, San Diego, CA, USA) was added into each well to prevent the cytokine secretion at the same time. The cells were collected after being cultured for 10 hours. The cells were stained with FITC-anti-CD4 mAbs (eBioscience), fixed and permeabilized with BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit (BD Biosciences). Then the cells were stained with PE-anti-IFN-γ mAb (BD PharMingen, San Diego, CA, USA), and the FACS assay was performed within 0.5 hours.

Cytotoxicity test

For measuring the cell-mediated cytotoxicity, NuTu-19 target cells (1×10^6 cells/mL) were prelabeled with CFSE (Sigma) at a final concentration of 2.5 μmol/mL. After centrifugation, the labeled target cells were collected and cultured in a 5% CO_2 incubator at 37°C. T_E cells in a nonspecific stimulation group were obtained after being activated by anti-CD3 (1 μg/well) and rIL-2 (60 IU/mL) for 3 days. T_E cells in a specific stimulation group were obtained after being activated by NuTu-19 cells (1.5×10^3 cells/well) for 3 days. Then the effector cells were added at the ratio of 25:1 (effector:target cells). Five hours later, the cocultured cell mixture was harvested and stained with 7-AAD. The cytotoxicity response of immune target cells to effector cells was quantified by fluorescence-activated cell sorting (FACS). According to the assay, the CFSE+7-AAD+ cells were dead target cells, while CFSE+7-AAD cells represented live target cells. The cytotoxicity was expressed as the death rate of the target cells and calculated using the following calculation: Specific cytotoxic rate = [Dead target cells in experiment groups (%) – natural dead target cells (%)]/[100 – natural dead target cells (%)] × 100.

Adoptive immunotherapy

T_E cells were centrifuged, resuspended with phosphate-buffered saline, and injected through the tail vein into the F344 rats carrying tumor cells in their abdominal cavity for 3 days. rIL-2 (125 000 IU/rat) was injected into the abdominal cavity on that day and the following 3 days. The rats were monitored 7 to 18 weeks for body weight, abdominal circumference, ascites, and survival. The control rats were injected only with rIL-2.

Statistical analysis

The statistical analysis was performed by SPSS software (release 13.0, IBM Corp., Armonk, NY USA) All data were expressed as mean (standard deviation). The results of ELISA, Intracellular cytokine staining, cytotoxicity test, and adoptive immunotherapy were analyzed by the least significant difference t tests. The survival curves were analyzed by the Kaplan-Meier method. $P<.05$ was considered significant.

RESULTS

IL-2 and IL-4 measurements in the supernatant of the culture medium by ELISA

After T_E cells in the tumor-bearing group were exposed to the nonspecific (anti-CD3, rIL-2) and the specific stimuli (NuTu-19), the secreted IL-2 was found to be 33.2 (8.3) and 40.6 (0.9) pg/mL, respectively (**Table 1**). IL-2 values were 57.0 (2.4) and 65.7 (4.0) pg/mL in the unsensitized group 1, significantly higher than that in the tumor-bearing group 2 (both $P<.001$). IL-2 levels were 44.7 (2.4) and 60.4 (6.7) pg/mL in the activated group 3, significantly higher than that in tumor-bearing group ($P=.002$, $P<.001$, respectively).

After T_E cells in the tumor-bearing group were exposed to the nonspecific and the specific stimuli (NuTu-19), the mean (SD) secreted IL-4 levels were 192.3 (24.1) and 313.7 (36.8 pg/mL, respectively. No significant difference was observed compared with those in the unsensitized group ($P=.336$, .061) or with those in the activated group under nonspecific stimulation ($P=.089$), but a significant difference ($P<.001$) was observed compared with those in the activated group under specific stimulation (**Table 1**).

Intracellular cytokine staining

The rate of CD4+ T cells secreting IFN-γ in the tumor-bearing group 5% (0.9%) was lower than that in the unsensitized group 13.0% (2.1%) and activated group 11.3% (1.1%) (both $P<.001$) (**Figure 1**).

T_E cells were added into a 24-well plate, and mixed with NuTu-19 cells. BFA was added into each well to inhibit cytokine secretion. They were cocultured for 10 days. The cells were collected and stained by FITC-anti-CD4 and PE-anti-IFN-γ mAbs. The number in the upper right region in each figure represents the killing rate of target cells.

Cytotoxicity test

As shown in **Figures 2a**, the natural mortality of target cells in 5 hours was less than 5%. The death rate of target cells in the unsensitized group was significantly higher than that in the tumor-bearing group and the activated group (86.5% (1.1%) vs. 80.3% (0.69%) and 83.2 (0.7%), both $P<.001$) (**Figures 2b**). A pre-gating graph is shown in **Figure 3**.

Table 1. IL-2 and IL-4 in the culture medium.

Group	Number of samples	IL-2 (pg/mL)	IL-4 (pg/mL)
Group 1 nonspecific stimulation	6	57.0 (2.4)[a]	211.2 (21.8)[b]
Group 1 specific stimulation	6	65.7 (4.0)[a]	277.4 (48.9)[b]
Group 2 nonspecific stimulation	6	33.2 (8.3)	192.3 (24.1)
Group 2 specific stimulation	6	40.6 (0.9)	313.7 (36.8)
Group 3 nonspecific stimulation	6	44.7 (2.4)[a]	160.2 (15.7)[b]
Group 3 specific stimulation	6	60.4 (6.7)[a]	195.7 (14.2)[a]

IL: Interleukin. [a]*P*<.05 compared with group 2; [b]*P*>.05, compared with group 2. Values are mean (standard deviation).

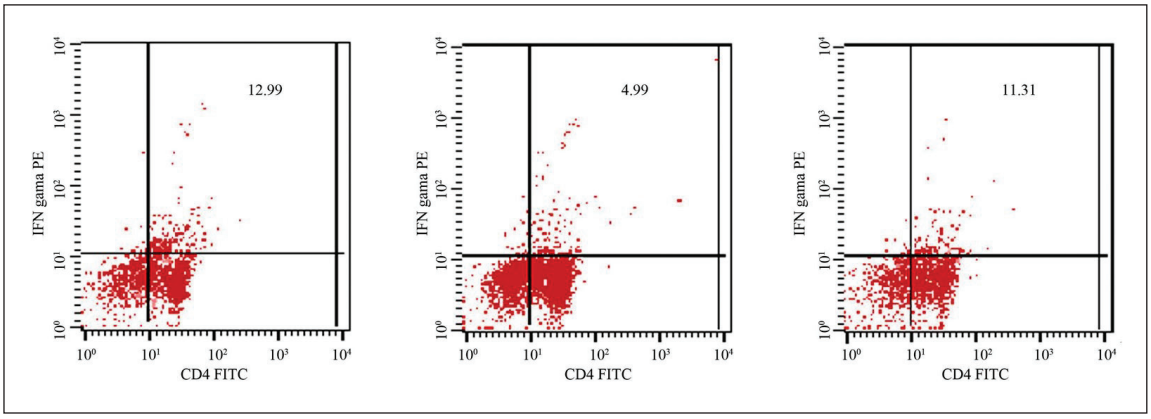

Figure 1. The rate of CD4+ T cells secreting IFN-γ. From left to right: unsensitized group; tumor-bearing group; activated group.

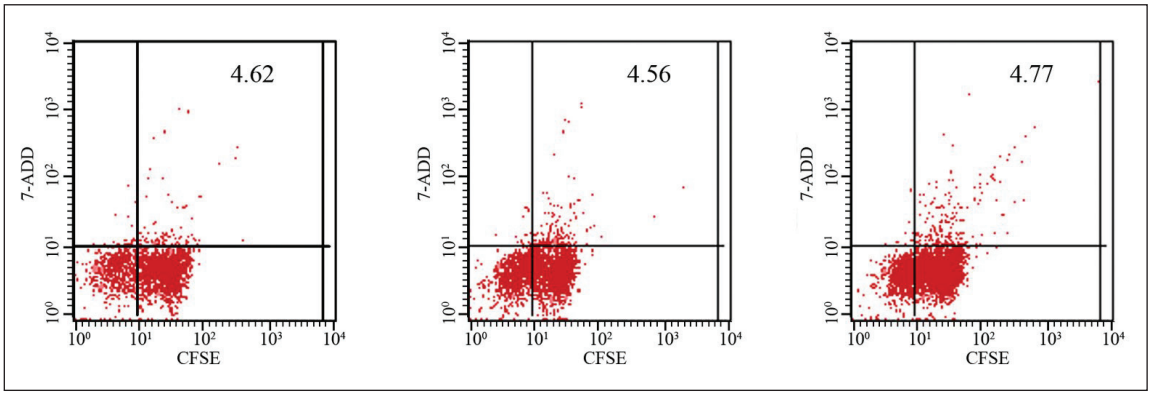

Figure 2a. The death rate of the target cells in control groups.

Antitumor activity of T_E cells

In the tumor-bearing group, bloody ascites was observed on the sixth week and became more severe during the eighth week (80–110 mL) with pale skin apparent in every rat. Death occurred because of extreme exhaustion in the 10th week. The mean survival time was approximately 73 (10) days. From the autopsy, we found disseminated miliary-like nodules in the peritoneum and on the surface of nearly all the organs in the abdominal cavity, and the nodules in the omenta had integrated with the surrounding tissues to form large areas of mass. In the unsensitized group, death occurred on the 12th week, and the mean (SD) survival time was 110 (16) days. Fifty percent of the rats were still alive on the 14th week. In the activated group, the mean (SD) survival time was 100 (19) days. However,

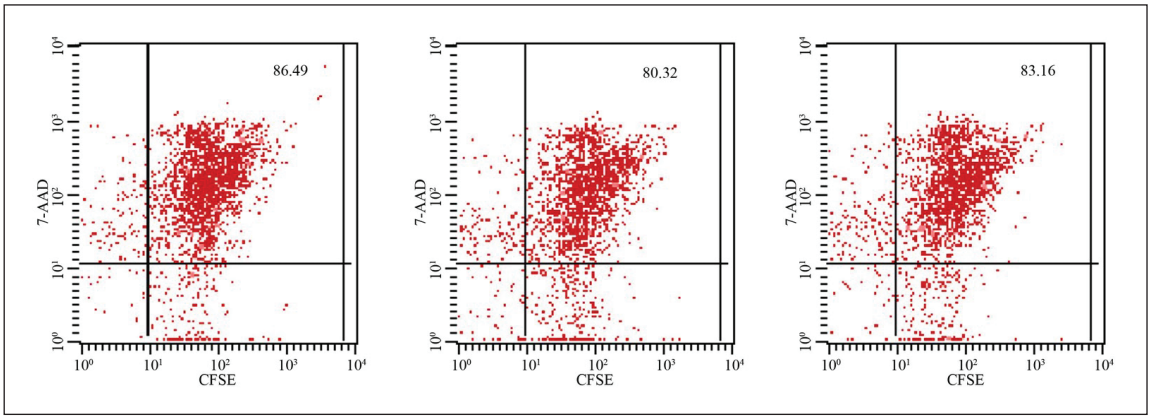

Figure 2b. The death rate of the target cells.

still 33.3% rats were alive on the 14th week. The tumor-specific protective immunity was decreased significantly, and disease progressed in the tumor-bearing group. Compared with other groups, the difference in survival time was significant ($P<.001$, $P=.006$). The antitumor activity of T_E cells increased significantly in the activated group (**Figure 4**). T_E cells were injected into the tail veins of F344 rats, which had peritoneal tumor for 3 days. rIL-2 was injected via abdominal cavity. The survival period of each group was observed in 7 to 18 weeks. Curves in the figure showed the survival rates with the development of tumor-bearing time.

DISCUSSION

When lymphocytopenia is transfused with homologous unsensitized lymphocytes in mice, lymphocytopenia commonly induces T lymphocyte proliferation.[1-3] Exogenous immune reconstitution may induce an effective antitumor immune reaction when lymphocytopenia is combined with immunotherapy. In a previous study, we found that immune reconstitution can induce effective antitumor effects.[2]

In this study, we measured the capability of stimulated tumor-specific T_E to secrete Th1 and Th2 cytokines in both special and nonspecial conditions. We also measured the frequency and function of the special tumor T_E. Data showed that the deflection of Th1 to Th2 happened in TVDLNs of the tumor-bearing group, resulting in reduced Th1-type cytokines (IL-2, IFN-γ) and increased Th2-type cytokines (IL-4), which suggested the inhibition of cell-mediated immunity. In cytotoxicity experiments, CD8+ killer T cells play major roles—as cytotoxic T lymphocytes (CTL), which can directly kill specific target cells. The purpose of this experiment was to examine whether immune function was inhibited in the tumor-bearing state by comparing the unsensitized

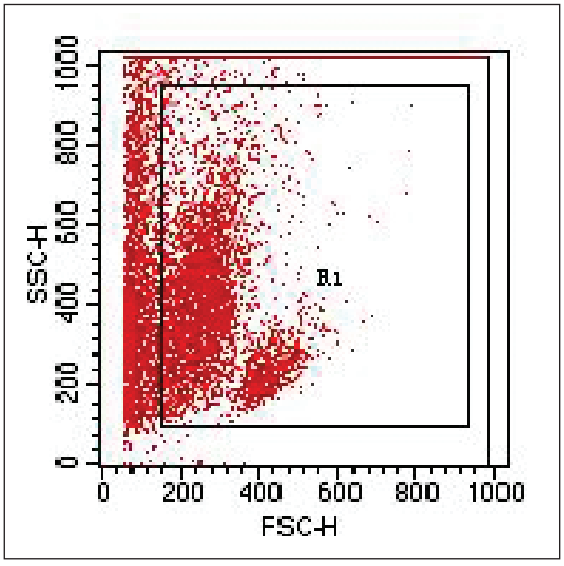

Figure 3. Pre-gating.

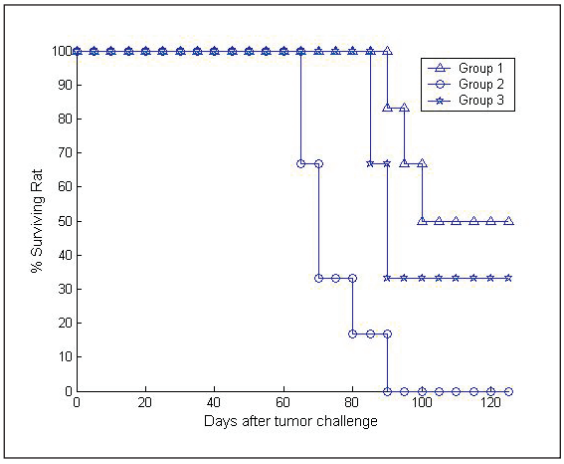

Figure 4. The survival rates of tumor-bearing rats.

group with the tumor-bearing group. The tumor-bearing host lymphocytes were much less effective IN the immune reconstitution of naïve T cells.

Since adoptively transferred antigen-specific CTLs are highly dependent on exogenous cytokines for their continued growth and survival,[3] systemic administration of IL-2 has been used to enhance their in vivo expansion and persistence.[4] However, the prolonged administration of IL-2 is often associated with serious side effects, limiting the amount and duration of cytokine administration.[4] Moreover, the effects of systemically administered cytokines are nonselective. IL-2 may favor the expansion of unwanted cell subsets, such as regulatory T cells,[5] that constitutively express the IL-2 receptor and adversely affect the function of antitumor CTLs.[6-9] In this study, we presumed that with the decrease of IL-2, unwanted regulatory T cells may decrease, which would improve the function of antitumor CTLs.

Recently, numerous studies have shown that tumor-bearing hosts inhibit the immune status from multiple aspects.[10-13] In the tumor-bearing state, TGF-β and CD8(+) CD28(−) T cells, which regulate T lymphocytes, can inhibit the T-cell proliferation and CTL. The tumor-derived TGF-β plays an important role in transforming CD4(+) CD25(−) T cells to CD4(+) CD25(+) regulation T lymphocytes (T-reg cells). T-reg cells with CD8(+) CD28(−) T cells can inhibit the immune activity of tumor-infiltrating auxiliary T cells (Th).[14-18] B7-H1 can stimulate the resting T cells to produce IL-10 and subsequently induce T cells apoptosis, leading to immune suppression.[19,20] Research has also shown that tumor cells express surface PDLs, which can damage the antitumor immune function in the body and lead to immune escape of tumor cells. PID-L1 on tumor cells directly inhibits CD8(+) T cells, which have antitumor effects.[21] Dendritic cells (DCs) are closely associated with the antitumor T-cell response. However, in the tumor-bearing state, high VEGF expression levels reduce the quantity and function of the DCs, and rebalance Th1/Th2, which reduces immunosuppression.[10,22]

We activated T cells in vitro to change the inhibitory state. The T-cell activation requires that TCR/CD3 identify the first signal provided by MHC–peptide complexes on the surface of antigen-presenting cells (APCs), as well as the secondary signal provided by the interaction of costimulatory molecules CD28/B7, LFA-1/ICAM-1, and others. Anti-CD3 specifically binds to CD-3 molecules on the T-cell surface, and activates T cells proliferation by activating the TCR-CD3 complex combined with MHC-II type molecule–antigenic peptides on the APC surface. After in vitro activation by the CD3 antibody, the T lymphocytes are transfused back to the patients with an infection or a tumor caused by the immune decline.[23] It was reported that T cells, sensitized by tumor cells and given an appropriate activation stimulus in vitro, not only can effectively restore the antitumor function of T cells, but also enhance the proliferation capacity of T cells in local tumor tissues.[24] In addition, some studies also indicated that T cells activated appropriately can remarkably change the tumor environment (immune inhibition, immune tolerance, apoptosis, for example). This study compared the effect of immunotherapy of the tumor-bearing group with that of the activated group. The results showed that immune cells activated in vitro can significantly reverse the immune system suppression of the tumor-bearing state. These activated T cells used for immune reconstitution can significantly increase the effect of the combined immunotherapy.

We found that the therapeutic effect was significantly lower in the condition of immune reconstitution with unactivated lymphocytes of tumor-bearing hosts while combined with the tumor vaccine. It is known that many factors may cause microenvironment at changes in the immune system in the tumor-bearing state, such as immunosuppression, immune tolerance, apoptosis, and others. The in vitro tumor-bearing host T cells can reverse such changes after being activated appropriately in vivo, and induce a tumor-specific immune response.

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