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Immune microenvironment profiles of tumor immune equilibrium and immune escape states of mouse sarcoma

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ABSTRACT

Cancer immunoediting consists of three distinct phases: elimination, equilibrium and escape. Here, for the first time, we investigated the immune microenvironment profiles of tumor immune equilibrium and immune escape states in 3′-methylcholanthrene-induced murine sarcoma model. Our study indicates the relative balance of monocytic MDSCs and antitumor immunity cells (especially CTLs, NK cells and γδT cells) may involve in maintaining tumor cells in a state of immune-mediated dormancy. In addition, high percentages of Treg cells and PMN-MDSCs are associated with the tumor immune escape state – mice with progressing sarcomas. In summary, the relative balance of immune effector cells and suppressive populations in the tumor microenvironment may involve in determining the fate of tumors.

1. Introduction

Cancer immunoediting comprises three distinct phases: elimination, equilibrium and escape. In the elimination phase, the innate and adaptive immune responses collaborate to detect the presence of a developing tumor and destroy the tumor before it becomes clinically apparent. Few tumor cell variants can survive the elimination phase and enter the equilibrium phase, in which the immune system maintains residual, latent tumor cells in a functional state of dormancy for a considerable period of time. If the tumor cell population changes in response to the immune system's editing functions and/or an immunosuppressive state is established within the tumor microenvironment, the tumor cells may then enter the escape phase, progressively growing into visible neoplasms [1–3]. There have been numerous studies on the elimination and escape phases. Koebel et al. demonstrated the existence of an immunologically mediated equilibrium phase using a low-dose methylcholanthrene (MCA)-induced mouse sarcoma model [3]. The researchers further showed that equilibrium was maintained by components of adaptive immunity, and especially $CD4^+$ and $CD8^+$ T cells, IFN-γ and IL-12p40 [3]. However, very little is known about the immune environment and the effector mechanisms underlying the equilibrium phase.

The immune microenvironment, comprising various immune cell subsets and the cells products, plays a critical role in promoting and inhibiting tumor development and/or progression. Tumor-infiltrating lymphocytes (TILs) include several different lymphocyte subsets: $CD4^+$ cells, which include T helper 1 (Th1), Th2, Th17 and T regulatory (Treg) cells, and $CD8^+$ CTLs, γδT cells, natural killer T (NKT) cells and natural killer (NK) cells [4–7]. Extensive evidence has revealed that not only $CD8^+$ and/or $CD4^+$ αβT cells but also other lymphocyte subsets, including γδT cells, NK cells and NKT cells, infiltrate tumor tissues diversely and dynamically and play a critical role in antitumor immunity [7–11]. However, Treg cells and myeloid-derived suppressor cells (MDSCs) promote tumor progress by inhibiting other immune cell [12–15]. These studies were performed on tumor tissue in the escape phase. In contrast, due to the difficulties to obtain the available samples, the immune milieu profile of the dormant tumor in the equilibrium phase remain unclear.

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In this study, we used a low-dose MCA-induced mouse sarcoma model and analyzed the percentages of various immune cell subsets among the TILs from the dormant sarcomas, which displayed small, stable masses, and the apparent sarcomas, which exhibited progressive growth. The spleen is the main peripheral lymphoid organ that plays a key role in antitumor immunity and possesses unique biological properties. Hence, we also detected the percentages of various immune cell subsets among the splenic lymphocytes of mice with dormant sarcomas, sarcoma-positive mice and wild-type mice. Our further analysis showed that high proportions of $CD3^+$ T cells, CTLs, NK cells, γδT cells, splenic memory CTL ($CTLs_M$) and memory γδT ($γδT_M$) cells; low proportions of NKT cells, $Foxp3^+$ Treg cells, splenic polymorphonuclear granulocytic myeloid-derived suppressor cells (PMN-MDSCs), infiltrating memory NKT (NKT_M) cells, $γδT_M$ cells and effector memory Th (Th_{EM}) cells; and a normal proportion of splenic Th_{EM} cells is associated with maintaining transformed cells in an immune-mediated equilibrium state. The relative balance of immunosuppressive cells (especially mononuclear myeloid-derived suppressor cells, MO-MDSCs) and antitumor immunity cells (especially effector or memory CTLs, NK cells and γδT cells) in the immune microenvironment is associated with maintaining tumor cells in a state of immune-mediated dormancy. In addition, high $CD8^+/CD4^+$ and $CD8^+$/Treg cell ratios are associated with a maintained equilibrium state and improved mouse survival.

Taken together, the relative balance of immune effector cells and suppressive immune cell populations in the tumor microenvironment is associated with determining the fate of a tumor.

2. Materials and methods

2.1. Mice

Female wild-type C57BL/6 mice at 6–8 weeks of age were purchased from the Academy of Military Medical Science (Beijing, China). The animals were housed and fed in a specific pathogen-free animal facility at the Experimental Animal Center of Tianjin Medical University. The experiments were in accordance with guidelines for animal care and were approved by the Animal Ethics Committee of Tianjin Medical University.

2.2. MCA tumor induction

Groups of C57BL/6 mice were subcutaneously injected in the hind flank with 25 μg of MCA emulsified in corn oil by heating in boiling water [16]. The mice were monitored every 7 days for tumor development for 200 days, beginning 50 days after MCA treatment. Tumors of >0.5 cm^2 in area and demonstrating progressive growth were recorded as sarcomas. Small, stable masses were identified as stable masses/dormant sarcomas, meaning that the mice were in the equilibrium phrase.

2.3. Isolation of peripheral mononuclear cells and TILs

Splenic and tumor tissue samples were collected from the tumor-bearing mice. Single-cell suspensions derived from the splenic tissues (splenocytes) were prepared by mechanical disruption and filtered through a 40-μm cell strainer (BD, USA), and then the red blood cells were lysed.

The tumor tissues were minced into small pieces and were digested with 0.05 mg/ml each of type-IV collagenase, hyaluronidase and DNase I (Sigma, USA) for 30 min at 37 °C. Single-cell suspensions were obtained by grinding the digested tissues and filtering the ground tissues through a 40-μm cell strainer (BD, USA). TILs were isolated using Ficoll density gradient centrifugation.

2.4. Flow cytometry analysis of the immune microenvironment

Splenocytes and TILs were blocked with 2% rat serum and 10% bovine serum albumin prior to surface marker immunostaining. Next, the cells were stained for surface markers using rat anti-mouse CD3-PE, CD4-APC, CD8-APC, γδ-APC, NK1.1-APC, CD11b-FITC, Ly6C-PE and Ly6G-PE-Cy7. For the Treg cell assay, the cells were first stained for surface markers using rat anti-mouse CD3-FITC, CD4-APC and CD25-PE. Next, intracellular staining was performed using rat anti-mouse Foxp3-PE-Cy5.5 (eBioscience, CA). For intracellular cytokine staining, the cells were fixed and permeabilized with Cytofix/Cytoperm buffers (BD Biosciences, USA) for 20 min at 4 °C and then washed with permeabilization wash buffer (BD Biosciences, USA). The Fc receptors were blocked with 2% rat serum and 10% bovine serum albumin prior to intracellular cytokine staining. Next, the cells were stained with rat anti-mouse Foxp3-PE-Cy5.5 (eBioscience, CA, USA).

Finally, the cells were analyzed using a FACSCalibur. The controls for nonspecific staining consisted of isotype-matched mAbs, and nonspecific staining was always subtracted from the specific staining results. The acquired data were analyzed using CellQuest and WinMDI 2.9 software (BD Biosciences, USA).

2.5. Statistical analysis

All of the results were derived from at least three independent experiments. The data represent the mean values ± the standard deviation (SD). Comparisons between the groups were performed using a Student's unpaired *t*-test, and *p*-values of <0.05 were considered statistically significant.

3. Results

3.1. An equilibrium state occurred in primary MCA-induced murine sarcomas

To obtain mice in the equilibrium state, groups of sex- and age-matched wild-type C57BL/6 mice were subcutaneously injected with a single low dose of the chemical carcinogen MCA and monitored for tumor development for the following 200 days. We observed that 8/30 (26.7%) mice developed progressively growing sarcomas and that 7/30 (23.3%) mice displayed small, stable masses at the site of the MCA inoculation (Fig. 1). In addition, there were 15/30 (50.0%) mice that were tumor-free (data not shown).

3.2. The percentages of T cells in the tumor tissue and spleen

To understand the differences in the immune microenvironment between the three distinct phases of immunoediting, we first detected the percentages of T cells among the TILs of progressing sarcomas and dormant sarcomas (Fig. 2B and D). Our results indicated that the percentage of T cells (49.93%) in the TILs of mice with dormant sarcomas was significantly higher than in mice with progressing sarcomas (33.60%, $p < 0.01$). We also detected the percentages of T cells in the splenetic mononuclear cells of normal mice, mice with dormant sarcomas and mice with progressing sarcomas (Fig. 2A and C). Our data show that among the splenocytes of mice with dormant sarcomas, the percentage of T cells was the highest (40.33%) compared with the percentage in mice with progressing sarcomas (12.76%, $p < 0.01$) or in normal mice (35.69%, $p < 0.05$) (Fig. 2A and C). Moreover, the percentage of splenetic T cells in mice with progressing sarcomas was significantly decreased compared with the percentage in normal mice ($p < 0.01$). These results demonstrate that the infiltration of T cells into the tumor tissue and spleen of the mice at an equilibrium induced by MCA was significantly increased, suggesting that T cells might be conducive to maintain the equilibrium state.

3.3. The percentages of CTLs, NK cells and γδT cells increased and the percentage of NKT cells decreased among the TILs and splenetic mononuclear cells in the equilibrium state

We further analyzed the percentages of T cell subsets (Th cells, CTLs, NKT cells and γδT cells) and NK cells (Fig. 3). In mice with dormant sarcomas (Fig. 3A and C), the percentages of tumor-infiltrating CTLs (36.43% vs. 23.52%, $p < 0.05$), γδT cells (18.97% vs. 8.44%, $p < 0.05$), Th cells (51.60% vs. 39.10%, $p < 0.05$) and NK cells (4.42% vs. 1.87%, $p < 0.01$) were significantly increased compared with the percentages in mice with progressing sarcomas. In contrast, the percentage of tumor-infiltrating NKT cells (3.89% vs. 22.33%, $p < 0.01$) in mice with dormant sarcomas was significantly decreased compared with the percentage in mice with progressing sarcomas (Fig. 3A and C). Moreover, the percentages of splenic γδT cells (5.18% vs. 3.59%, $p < 0.05$) and NK cells (2.13% vs. 1.54%,