International Immunopharmacology 17 (2013) 245–253

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Hypothermic microenvironment plays a key role in tumor immune subversion

Gangjun Du *[,1], Yinghui Liu [1], Jiahuan Li, Weijie Liu, Yingying Wang, Hong Li

Institute of Pharmacy, Pharmaceutical College of Henan University, Jinming District, Kaifeng, Henan 475001, China

ARTICLE INFO

Article history:
Received 29 March 2013
Received in revised form 8 June 2013
Accepted 14 June 2013
Available online 3 July 2013

Keywords:
Hypothermia
Immunosuppressive microenvironment
Tumorigenesis
Hyperthermia

ABSTRACT

There are diverse immunosuppressive factors in the tumor microenvironment, but the main reason for these factors is unclear. Because hypothermia induces micronuclei in mouse bone marrow cells and because alterations in body temperature may influence immune system function, we aimed to investigate whether the hypothermic microenvironment is associated with the presence of diverse immunosuppressive factors. We found that hypothermic culture (34 °C) decreased lymphocyte proliferation, cytotoxic CD8 + T cell function and Th1 cell expression of IFN-γ and IL-2 in vitro, with a concomitant increase in Th2 and Treg cell populations. Whole-body hypothermia at a temperature less than 34 °C produced an immunosuppressive microenvironment, resulting in an increase in splenic Treg and Th2 cell populations as well as increased serum IL-4 and IL-10 levels in vivo. As a result of whole-body hypothermia, less than one of two cells in a conventional syngeneic tumor model was capable of tumor formation. In contrast, febrile-range hyperthermia (39–40 °C) promoted a T cell-mediated immune response, resulting in an increase in splenic Th1 and Tc1 cell populations, which caused more than a two-fold increase in the number of nontumorigenic cells in a conventional syngeneic tumor model. Similarly, local hypothermia induced by microcirculatory dysfunction or the application of a cold compress increased intratumor Treg cells and TGF-β1 levels, which promote lung metastasis, whereas local hyperthermia induced by capsaicin stimulation or a hot compress had the opposite effect. These results confirmed the hypothesis that tumor hypothermia determines the immunosuppressive microenvironment and suggested a novel treatment strategy for solid tumors.

1. Introduction

The main cause of cancer mortality is disseminated disease, not the primary tumor. Tumor metastasis and recurrence by residual tumor cells affect the prognosis of cancer patients after radical surgery [1]. Conventional treatments, such as surgery, radiotherapy and chemotherapy, can eradicate the primary tumor but have little effect on tumor metastasis and recurrence [2], whereas immunotherapy is particularly well suited to eliminate residual tumor cells [3]. However, the clinical results of immune-based strategies for treating human cancer have been disappointing [4]. This limited success is largely attributed to the immune tolerance observed in cancer patients [5]. Thus far, most immunotherapies for metastasis and recurrence have focused on promoting peripheral immune responses, ignoring the negative impact that the immunosuppressive tumor microenvironment has on the trafficking of cancer-specific T cells. This immunosuppressive microenvironment results in the inability to elicit an anti-tumor immune response during the metastatic and recurrent period [6]. For this reason, various strategies to enhance tumor immunity have been attempted as supportive anti-cancer therapies, but the optimal treatment of metastasis and recurrence continues to pose a major challenge in cancer therapy.

A variety of human and murine cancers have been proven to be antigenic and recognizable by T cells [7], and it is also believed that immunotherapy will potentially elicit tumor-reactive T cells that can seek and destroy disseminated tumor Ag-positive cancer cells while sparing the surrounding healthy tissues [8]. However, there is growing awareness that tumor cells build up a "self-advantageous" microenvironment that reduces the effectiveness of the anti-tumor immune response [9]. Unfortunately, while tumor immunotherapy progresses at a rapid pace, we lack an understanding of the causes and progression of the tumor immunosuppressive microenvironment [10]. During progression, tumors become colder than the external environment due to disordered vascularization and ischemia [11]. It is not yet known whether the hypothermic microenvironment is associated with the presence of diverse immunosuppressive factors. In the current study, we explored the relationship between hypothermia and the tumor immunosuppressive microenvironment. We

Abbreviations: FBS, fetal bovine serum; FCM, flow cytometry; IL-, interleukin-; IFN-γ, interferon-gamma; ELISA, enzyme-linked immunosorbent assay; LLC, Lewis lung cancer; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide; PMA, phorbol myristate acetate; CFSE, carboxyfluorescein succinimidyl ester; Treg, regulatory T cell; TGF-β1, transforming growth factor-β1.

* Corresponding author. Tel.: +86 378 3880680.
E-mail address: 724200@henu.edu.cn (G. Du).

[1] Gangjun Du and Yinghui Liu contributed equally to this work.

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http://dx.doi.org/10.1016/j.intimp.2013.06.018

found that the hypothermic microenvironment plays a key role in tumor immune subversion.

2. Materials and methods

2.1. Materials

RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco Invitrogen, Carlsbad, CA) and 2 mM L-glutamine was used throughout the experiments. Fluorescence-conjugated Abs specific for CD4, CD8, CD25, FOXP3, IL-2, IL-4, IFN-γ and TGF-β1 were obtained from BD Pharmingen. Calcein-AM and CFSE staining kits were obtained from Molecular Probes. ConA, MTT, mitomycin C, saponin, phorbol myristate acetate (PMA) and ionomycin were obtained from Sigma Chemical Co. (St. Louis, MO, USA). IL-2, IL-4, IFN-γ and TGF-β1 ELISA kits were purchased from R&D Systems (Minneapolis, MN). Anti-CD + 3 and anti-CD28 + T cell expansion beads were purchased from Invitrogen. The MACS separation system was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). All other chemicals were of the highest purity available and were purchased from Sigma (St. Louis, MO).

2.2. Mice and cell line

Five- to 6-week-old C57BL/6 mice were obtained from Beijing Weitong Lihua Animal Co. The mice were maintained under specific pathogen-free conditions. All procedures involving animals were approved by the Henan University Institutional Animal Care and Use Committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals. Mouse Lewis lung cancer cells (LLC) were purchased from the Cell Bank of the Chinese Academy of Sciences in Shanghai and grown in RPMI 1640 supplemented with 10% FBS.

2.3. Splenic lymphocyte proliferation assay

Spleens were aseptically collected from female C57BL/6J mice, crushed gently and separated into single cells by squeezing in 10 ml D-Hank's solution. After lysing the erythrocytes with 10 ml of sterile 0.01 mol/l Tris and 0.83% NH4Cl, the single cell suspension was washed twice with D-Hank's and resuspended in RPMI 1640 medium supplemented with 10% FBS. Cells were plated in 96-well plates at a density of 5×10^5 cells/well and stimulated with Con A (5 μg/ml) for 48 h at 37 °C, 34 °C and 31 °C. During the final 4 h of the 48 h incubation, the supernatants were discarded, and 100 μl of MTT (0.5 mg/ml) was added to each well. After 4 h, the MTT was discarded, and 100 μl of DMSO was added to each well. Optical density (OD) was determined with a microplate reader (BIO-TEK) at 570 nm. The results of the cell proliferation assay are presented as the percent of control cells inoculated at 37 °C ($n = 5$).

2.4. T cell proliferation analysis by CFSE

T cells were isolated from the spleen using a MACS separation system (Miltenyi Biotec, Bergisch Gladbach, Germany) and stained with 0.5 μM CellTrace carboxyfluorescein succinimidyl ester (CFSE) for 15 min at 37 °C according to the manufacturer's instructions. Cells were plated in a 12-well plate at a density of 5×10^6 cells/well and then stimulated with anti-CD3/anti-CD28 T cell expansion beads (50 μl/well) for 48 h at 37 °C, 34 °C or 31 °C. T cell proliferation was analyzed by flow cytometry ($n = 5$).

2.5. T cell type analysis

Isolated T cells were cultured and stimulated as described above. After 48 h, cells were harvested and stained with fluorescence-conjugated monoclonal antibodies against CD4 and CD8. The cells were fixed, permeabilized, and intracellularly stained with fluorescence-conjugated monoclonal antibodies against IL-2, IFN-γ, IL-4 and IL-10. T cell populations were analyzed by flow cytometry ($n = 5$).

2.6. CTL assay

The cytotoxic CD8 + CTL activity was measured using a calcein-release assay, as described previously [12]. Briefly, CD8 + CTLs were isolated from C57BL/6J mice using a MACS anti-PE magnetic separation kit and anti-CD8 PE antibodies. CTLs were cultured in 6-well plates and stimulated with PMA (10 ng/ml) and ionomycin (0.1 μg/ml) at 37 °C. After 24 h in culture, the cells were washed and harvested. Mitomycin C-treated LLC target cells were labeled with 10 μM calcein-AM for 30 min at 37 °C and placed into a 96-well plate with CTLs at 20:1, 10:1, and 5:1 (CTL:LLC) ratios for 6 h at 37 °C, 34 °C and 31 °C, respectively. After incubation, the supernatants were transferred from each well to another 96-well plate. The fluorescence of each supernatant was monitored at 490 nm excitation and 520 nm emission wavelengths using a Synergy2 multi-mode microplate reader (BIO-TEK). Maximum release was obtained from detergent-released target cells, and spontaneous release was obtained from target cells incubated in the absence of effector cells ($n = 5$). Cytotoxicity was determined as follows: The cytotoxicity = (experimental release − spontaneous release) / (maximum release − spontaneous release) × 100%.

2.7. Tumor challenge models

LLC cells were maintained in 10% FBS RPMI 1640 medium and cultured until the cells reached 80% confluence. A total of 5×10^6 irradiated LLC cells were injected subcutaneously into the left axilla of C57BL/6J mice to induce tumor immunization. Seven days after tumor immunization, the mice were inoculated subcutaneously with 10^5–2.5×10^6 living LLC cells into the right axilla. Control mice that were not immunized were also challenged with living LLC cells. Twenty-four hours after tumor challenge, the challenged mice were either treated or not treated with reserpine (50 mg/kg, i.p.) or 2,4-dinitrophenol (DNP, 15 mg/kg, s.c.) once daily for one week. The mice were housed at 30 °C or 24 °C and monitored for tumor size twice weekly ($n = 5$). Twenty-nine days after tumor challenge, sera from challenged mice were isolated for cytokine ELISA (IL-2, IFN-γ, IL-4 and IL-10) according to the manufacturer's instructions ($n = 5$). Spleens were also removed for T cell type analysis as described above, and tumors were removed and weighed ($n = 5$).

The tumor volume (mm^3) was calculated according to the following equation: volume = $width^2 \times length / 2$.

2.8. Local hypothermia and tumor immunity

Local hypothermia was induced by 2 mg of epinephrine injected subcutaneously once into the backs of C57BL/6J mice. Three days after the epinephrine injection, 5×10^5 LLC cells were inoculated subcutaneously into the site of the epinephrine injection. Control mice that had not been injected with epinephrine were also inoculated with LLC cells following the same schedule ($n = 10$). One day after tumor inoculation, 50 μg of capsaicin was dropped once daily on the skin at the site of tumor inoculation to produce local hypothermia. In addition, a hot compress was used to antagonize microcirculatory dysfunction, and a cold compress was used to antagonize vasodilation. The skin temperature and central tumor temperature were measured by an infrared thermometer (American Omega) and a probe thermometer (British ETI), respectively. The mice were