International Immunopharmacology 9 (2009) 900–909

Contents lists available at ScienceDirect

International Immunopharmacology

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

Gemcitabine directly inhibits myeloid derived suppressor cells in BALB/c mice bearing 4T1 mammary carcinoma and augments expansion of T cells from tumor-bearing mice

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ARTICLE INFO

Article history:
Received 30 October 2008
Received in revised form 22 March 2009
Accepted 23 March 2009

Keywords:
Myeloid derived suppressor cells
Gemcitabine
4T1
T cell
Immunosuppression
Mammary carcinoma

ABSTRACT

Myeloid derived suppressor cells (MDSCs) accumulate in 4T1 mammary carcinoma bearing mice and present a barrier to the success of adoptive immunotherapy (AIT) by suppressing T cell immunity. In this study, we investigated the inhibition of MDSCs by gemcitabine (GEM), a chemotherapy agent that may have favorable immunologic effects. BALB/c mice were inoculated with 4T1 mammary carcinoma cells and treated with GEM either once a week starting 5 days after tumor inoculation (EARLY GEM) or as a single dose at days 20–25 (LATE GEM). Splenic mononuclear cells were isolated, activated in vitro, expanded, and stimulated with tumor antigen. T cells were then used for AIT to treat tumor-bearing mice. EARLY GEM treatment of 4T1 tumor-bearing mice significantly inhibited tumor growth, reduced splenomegaly, and significantly decreased MDSC proportion in the spleen. Support for a direct effect was demonstrated through suppression of MDSCs in spleens, bone marrow, and blood harvested 24 and 48 h after LATE GEM treatment, despite no significant decrease in tumor burden. Interestingly, treatment of tumor-bearing mice with GEM augmented in vitro expansion of splenic T cells and boosted IFN-γ secretion in response to stimulation by tumor antigen. However, despite GEM-mediated inhibition of MDSC suppression, splenic T cells from mice with advanced tumors were ineffective in vivo against established tumors. This study provides support for direct inhibition of MDSCs and direct reduction of tumor burden by GEM in 4T1 tumor-bearing mice. GEM treatment of mice with advanced tumors improves T cell function and growth in vitro.

1. Introduction

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of undifferentiated cells that express markers of monocytes (CD11b) and neutrophils (Gr1) and cause T cell dysfunction in tumor-bearing mice and humans [1–7]. They reduce antigen specific CD8+ T cell proliferation, increase T cell death by apoptosis, foster T cell tolerance, and change the profile of cytokines secreted by activated T lymphocytes [3,5,8–10]. MDSCs exert their immunosuppressive effects primarily through the production of arginase-I, nitric oxide synthase, and reactive oxygen species as well as by cell contact dependent cross talk with macrophages that results in decreased IL-12 production by macrophages and increased IL-10 production by CD11b+Gr-1+ MDSCs [5,9,11,12,33,34]. There may also be direct contact dependent inhibition of T cell proliferation [9,21,35]. MDSCs additionally inhibit T cell function by inducing the development of CD4+CD25+FoxP3+ T regulatory cells in vitro and in vivo [13].

These CD11b+ Gr-1+ cells accumulate in the bone marrow, blood, spleen, lymph nodes, and at the tumor site in tumor-bearing hosts [3,14,36]. Accumulation of MDSCs is caused by tumor-derived soluble factors, like VEGF, GM-CSF, and M-CSF, that stimulate myelopoiesis and maintain the immature state of myeloid cells [3,20]. MDSCs are important in murine as well as human cancer progression. They have been shown to increase 5-fold in the spleens of mice bearing large subcutaneous bronchoalveolar carcinoma, TC-1 tumors, Lewis-lung carcinoma, or mesothelioma [14,15,17]. In patients with renal cell carcinoma (RCC), there is an upregulation of CD11b+CD14− MDSCs compared to healthy individuals (from ~10% in peripheral blood mononuclear cells to ~40% in RCC patients) [5]. Importantly, increased levels of circulating MDSCs have recently been correlated with disease stage and extensive metastatic tumor burden in patients with breast cancer [16].

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doi:10.1016/j.intimp.2009.03.015

MDSCs present a barrier to cellular immunity and abrogation of their influence may increase the likelihood of successful immunotherapy. Gemcitabine (GEM), a pyrimidine antimetabolite that has been used clinically to treat patients, was suggested to have immunological properties and was used against MDSCs by Suzuki et al. [17,18,32]. GEM is believed to be tumoricidal, inducing apoptosis caused by early termination of DNA synthesis. In mice, GEM has a plasma half life of 0.28 h after a dose of 20 mg/kg given intravenously and 86.3% is excreted in urine 24 h after administration [19]. The drug is quickly distributed in the spleen, thymus, testicles, kidney, femur, small intestines, and lymph nodes, correlating to sites of MDSC accumulation.

GEM given to animals bearing large TC-1 lung epithelial tumors (1000 mm^3) reduced splenic MDSCs from 28.5% in control mice to 9.3% in treated mice while leaving important CD4+ T cell, CD8+ T cell, and B cell populations unperturbed [17]. CD8+ T cells given in conjunction with splenocytes from GEM-treated animals and TC-1 tumor cells significantly inhibited tumor development compared to combination with splenocytes from untreated tumor-bearing mice. GEM has also been shown to slow the growth of primary tumors and decrease MDSC proportion in the blood of tumor-bearing mice [11]. These results prompted the question: does GEM act directly on MDSCs or does its anti-tumor effect decrease tumor load and thereby inhibit MDSCs indirectly?

Although GEM has been associated with myelosuppression, it has not been applied clinically as an agent to inhibit the immunosuppression caused by MDSCs. The purpose of the following studies was to elucidate the mechanism by which GEM exerts its inhibitory effect on MDSCs in a mouse mammary carcinoma model. Our goal was to determine whether the observed suppression of MDSCs is a direct effect of the drug or an indirect result of cytotoxicity to tumor cells, which will help clarify whether GEM is useful in preventing the immunosuppression of T cell responses caused by tumor-induced MDSCs.

2. Materials and methods

2.1. Mice

Virus-free female BALB/c and athymic nude mice (National Cancer Institute) were used between 8 and 12 weeks of age. Animals were caged in groups of 6 or fewer and provided food and water ad libitum. All guidelines of the Virginia Commonwealth University Institutional Animal Care and Use Committee, which conform to the American Association for Accreditation of Laboratory Animal Care and the U.S. Department of Agriculture recommendations for the care and humane experimental use of animals, were followed.

2.2. Tumor cell lines

4T1 mammary tumor cells were kindly provided by Dr. Jane Tsai at the Michigan Cancer Foundation, Detroit, Michigan. Cells were passaged in complete Dulbecco's Modified Essential Medium (DMEM) with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 1 mM sodium pyruvate (Mediatech, Inc, Herndon, VA), 100 U/ml penicillin (Invitrogen, Grand Island, NY), 100 mg/ml streptomycin, 0.075% sodium bicarbonate, and 10 mM HEPES buffer. MethA sarcoma, an unrelated syngeneic tumor cell line (ATCC, Rockville, MD) was maintained in complete RPMI 1640 with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.075% sodium bicarbonate, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES buffer, and 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). All cells were incubated in 250 ml T-flasks (PGC, Gaithersburg, MD) at 37 °C in humidified air with 5% CO_2. Tumor cells were harvested for inoculation of mice with 0.05% trypsin–EDTA (Invitrogen), washed twice with 1× PBS and resuspended in 1× PBS.

2.3. Tumor inoculation

BALB/c and nude mice were inoculated subcutaneously (S.C.) in the flank with 50,000 4T1 mammary carcinoma cells. Tumor growth was monitored with bidirectional tumor measurements using calipers every 2–3 days. The product of the two perpendicular measurements measured was recorded as the tumor area in mm^2. Results are reported as the mean tumor area ± standard error (SE) with 3–6 mice per group.

2.4. Cyclophosphamide and gemcitabine treatments

Cyclophosphamide (CYP) was given at a dose of 100 mg/kg via intraperitoneal (I.P.) injection on day 5 after tumor inoculation (Bristol-Myers Squibb Company, Princeton, NJ). Gemcitabine-HC1 (GEM) was given at 60 mg/kg via I.P. injection (Eli Lilly and Company, Indianapolis, IN). Two GEM treatment regimens were employed: 1) EARLY GEM: GEM was given on day 5 after 4T1 tumor inoculation and repeated once weekly and 2) LATE GEM: GEM was given in a single dose on days 20–25.

2.5. Spleen harvest and expansion of splenocytes

Spleens were harvested 24–48 h after GEM treatment in complete RPMI, weighed, and crushed through a cell strainer. Splenocytes were resuspended in 1× ammonium chloride solution to lyse red blood cells. Splenocytes were stained with 0.04% trypan blue to exclude dead cells and viable cell numbers were counted under a light microscope using a Neubauer type hemacytometer. Splenocytes that were expanded were first subjected to Ficoll density gradient centrifugation to isolate splenic mononuclear cells. These cells were then washed and brought to a concentration of 1×10^6 cells/ml and activated by incubation with 5 nM bryostatin-1 (provided by the National Cancer Institute, Bethesda, MD), 1 mM ionomycin (Calbiochem, San Diego, CA) (B/I), and 80 U/ml of rIL-2 (Chiron, Emeryville, CA) in 50 ml conical polystyrene tubes at 37 °C, 5% CO_2, for 18 h. Cells were washed three times with warm complete RPMI and resuspended at 1×10^6 cells/ml. Splenocytes were expanded in complete RPMI supplemented with an additional 5% heat-inactivated fetal calf serum with an alternating regimen, with IL-2 (40 U/ml) alone, or with IL-7 + IL-15 (10 ng/ml each, Peprotech Inc, Rocky Hill, NJ). In the alternating regimen protocol, B/I activated splenocytes were incubated for 24 h in IL-7 + IL-15 (10 ng/ml each), exposed to IL-2 (40 U/ml) for 24 h and re-cultured in IL-7 + IL-15 for the remainder of the expansion period. The cells were allowed to proliferate in culture until peak growth was reached and were split every 2–3 days to 1×10^6 cells/ml.

2.6. Separation of MDSC from splenocytes and T cell expansion

BALB/c mice were injected in the flank with 50,000 4T1 carcinoma cells. Twenty-one days later, splenocytes were harvested and prepared as described above. Some splenocytes were set aside as a control group while the remaining cells were sorted to positively select out the myeloid derived suppressor cells using an Easy Sep PE Selection Kit (StemCell Technologies, Vancouver, BC, Canada). Splenocytes were treated with FcR blocking antibody (BD Biosciences Pharmingen, San Diego, CA) and PE-conjugated anti-mouse Ly-6G/Ly-6C (Gr-1) (Biolegend, San Diego, CA). The positive MDSC cells adhered to the magnetic beads while the MDSC-depleted splenic lymphocytes were collected separately. Both the MDSC positive and depleted cell groups were counted using a Neubauer type hemacytometer, and the percentages of MDSC originally present were calculated. Using the total number of cells for the control groups as the starting point, the number of cells in the MDSC positive group and MDSC-depleted groups were adjusted by the calculated MDSC percentage to ensure that the same number of lymphocytes was plated in each well. In