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Differential effects of low-dose fludarabine or 5-fluorouracil on the tumor growth and myeloid derived immunosuppression status of tumor-bearing mice

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ABSTRACT

Myeloid-derived suppressor cells (MDSCs) accumulate in tumor-bearing hosts and play a key role in the suppression of the innate and adaptive immunity. Chemotherapeutic strategies have been developed to deplete or deactivate MDSCs in different tumor models. The pyrimidine analog, 5-fluorouracil (5-FU) is found to reduce the tumor size by depleting MDSCs. Here, we asked whether the purine analog, fludarabine (Flu), could exert similar effects. Employing a lymphoma model, we demonstrated that in mice with advanced tumors (where MDSC-induced suppression was present), treatment with a single low-dose Flu (25, 50, 100 mg/kg) elevated the numbers of splenic MDSCs and serum arginase activity, and simultaneously, increased the tumor growth (only the highest dose). On the other hand, in mice with palpable tumors (where the MDSC-induced suppression was in progress), treatment with Flu had no significant effects on the tumor growth or the number of splenic MDSCs. In contrast to Flu, treatment with low-dose 5-FU, irrespective of tumor stage, caused tumor regression which coincided with significant reductions in the numbers of splenic MDSCs and blood neutrophils, but increases in the ratios of splenic $CD4^+$ T and $CD8^+$ T cells to suppressive MDSCs. Finally, in healthy mice (where MDSC-induced immuosuppression did not exist), 5-FU, but not Flu induced significant decreases in the number of myeloid cells in the bone marrow, naturally occurring splenic MDSCs and thymocytes. In conclusion, Flu exacerbates MDSC-induced immunosuppression in a tumor stage-dependent manner, whereas 5-FU alleviates the suppressive effects of MDSC at all stages of tumor development.

1. Introduction

It is now well known that during tumor development and progression, malignant cells gain certain properties (e.g., expressing membrane molecules or releasing soluble factors) that enable them to alter hematopoiesis, in particular myelopoiesis [22,36]. This tumor-induced aberrant myelopoiesis leads to an abundant expansion of myeloid cell populations that are generally termed as myeloid derived suppressor cells (MDSCs) [12,35,36]. Studies have shown that MDSCs are heterogenous immature myeloid cells that have lost their ability to differentiate into cells such as granulocytes, macrophages, and DCs [12,35]. In mouse, these cells are characterized by the expression of cell surface markers CD11b and Gr-1 and subdivided into two major subpopulations: granulocytic MDSCs (G-MDSCs) and monocytic MDSCs (M-MDSCs). In this species, G-MDSCs predominantly express CD11b and Ly6G markers along with low levels of Ly6C ($CD11b^+ Ly6G^{high} Ly6C^{low}$), whereas, M-MDSCs express CD11b together with high levels of Ly6C ($CD11b^+ Ly6G^- Ly6C^{high}$) [12,35]. G-MDSCs have neutrophilic morphology and are predominantly found in tumor and spleen of nearly all tumor models [24,41], whereas M-MDSCs have typical monocytic features and expand only in a few tumor models [41].

It has been proposed that MDSCs play two major roles in the development and progression of tumors [36]. Firstly, these cells can promote tumor angiogenesis, metastasis and tumor cell stemness by producing vascular endothelial growth factor (VEGF), matrix metallopeptidase 9 (MMP9), versican, transforming growth factor beta (TGF-β)

Abbreviations: 5-FU, 5-fluorouracil; Flu, Fludarabine; G-MDSCs, Granulocytic MDSCs; M-MDSCs, Monocytic MDSCs; MDSCs, Myeloid-derived suppressor cells; SGR, Specific growth rate; Tregs, T regulatory cells; WBC, white blood cells

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and interleukin-1 receptor antagonist (IL-1RA) [36]. Second, MDSCs can effectively suppress anti-tumor immunity through diverse mechanisms that alter specific adaptive immune responses [10,11,24,36]. These mechanisms mainly involve the production of high levels of soluble factors including: arginase I, reactive oxygen/nitrogen species (ROS/RNS), VEGF and TGF-β [10,11,24] and induction of regulatory T cells (Tregs) [18,40]. Among the soluble factors, arginase may play a major role in induction of immunosuppression i.e., this enzyme converts arginine into urea and ornithine in a cell-to-cell contact independent manner [19,29]. Subsequently, arginine deprivation inhibits the proliferation and function of T cells through G0/G1 arrest of the cell cycle [10,11,34].

In addition to MDSCs, Tregs also emerges as a key player in the development of immunosuppression which favors tumor growth, angiogenesis and likely metastasis [13]. These cells are defined as a subset of $CD4^+$ $CD25^+$ T cells that co-express the transcription factor, forkhead box P3 (Foxp3) [2,6,25]. Although, the suppressive mechanisms of Tregs have been clearly addressed in the context of immunological tolerance and regulation of immune and autoimmune responses [21,30], they have not been exclusively investigated in tumor settings. However, it has been suggested that during tumor development, these cells suppress tumor-specific immune responses via multiple mechanisms that include cell-cell contact, the production of immunosuppressive and pro-angiogenic cytokines (e.g., TGF-β, interleukin-10 (IL-10) and VEGF) and induction of production of tolerance-inducing enzyme, indoleamine 2,3-dioxygenase (IDO) in antigen-presenting cells [42].

On the basis of information provided above, it is rational that elimination of MDSCs and Treg cells might lead to the restoration of anti-tumor immunity, thereby increasing therapeutic effects of anticancer drugs. In this connection, we and others have shown that a single low-dose of the antimetabolite pyrimidine analogs such as 5-fluorouracil (5-FU) and gemcitabine are able to reduce the tumor size as well as the numbers of circulatory, splenic and tumor MDSCs, without exerting significant effects on Tregs [1,3,4,33,38]. On the other hand, studies have demonstrated that in patients with chronic lymphocytic leukemia, chemotherapeutic doses of the antimetabolite purine analog, fludarabine (Flu) are able to activate anti-tumor T cells by reducing the frequencies and/or functions of Tregs [5,14]. This information led us to ask whether low doses of pyrimidine and purine analogs share similar properties in suppressing the tumor growth by targeting MDSC-induced immunosuppression. To answer this question, in the current study, we investigated the effects of 5-FU and Flu on MDSCs in the murine lymphoma model EL4-luc2 at two different stages (advanced and palpable) of tumor development. Our results demonstrated that 5-FU and Flu exert different and even opposing effects on the tumor growth as well as immunosuppression caused by MDSCs.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's phosphate buffer saline (DPBS), horse serum (HS), sodium pyruvate, L-glutamine, 2-mercaptoethanol, penicillin and streptomycin were obtained from Invitrogen Life Technologies (Invitrogen AB, Stockholm, Sweden). 5-fluorouracil (5-FU) and fludarabine (Flu) were purchased from Sigma (Sigma–Aldrich, Stockholm, Sweden) and Actavis (Hafnarfjördur, Iceland), respectively.

2.2. Cell culture

Murine EL4 lymphoma cell line (referred to hereafter as EL4-luc2 cells) was obtained from American Type Culture Collection (Manassas, VA, USA) and engineered to express the firefly luciferase gene, luc2 (PerkinElmer, MA, USA) enabling in vivo bioluminescent detection for monitoring tumor status and treatment follow-up. Phenotypic and tumorigenic characteristics of EL4-luc2 cells were proven to be similar to those of their parental EL4 cells as declared by the producer (PerkinElmer, MA, USA). The cells were cultured in DMEM supplemented with 10% HS, 1 mM sodium pyruvate, 2 mM L-glutamine, 50 μM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. This cell line was routinely passaged every 3–4 days.

2.3. Mice

Female C57BL/6 N ($H\text{-}2^b$) mice (8–10 weeks old at the beginning of each experiment) were obtained from Charles River (Charles River Laboratories, Germany). These animals were housed in our animal facility (Karolinska University Hospital, NOVUM) at 22 (± 2) °C with a 12-h light/12-h dark cycle, 50% humidity and access to the standard mouse diet and tap water ad libitum. The mice were allowed to acclimatize for 7 days before inoculation with tumor cells. All experiments in this study were pre-approved by the Stockholm Southern Ethics Committee for Animal Research and were conducted in accordance with the animal wellfare law, the Animal Protection Regulation and the Regulation of the Swedish National Board for Laboratory Animals (approval number: S67-14).

2.4. Tumor induction

EL4-luc2 cells were harvested from culture flasks, washed in DPBS and adjusted to a concentration of 5×10^6 cells/ml in DPBS, prior to inoculation. For tumor induction, mice were injected subcutaneously (s.c.) in the loose skin of the neck with 5×10^5 EL4-luc2 tumor cells in 100 μl DPBS. Tumor volumes were evaluated daily by measuring two perpendicular diameters with a caliper. Tumor volume (V) was calculated using the following equation: $V = (W^2 \times L)/2$, where W is the width of the tumor (small diameter), and L the length (large diameter), both in centimeters.

2.5. Characterization of immune status in mice with palpable or advanced tumor prior to administration with 5-fluorouracil (5-FU) or fludarabine (Flu)

The tumor development was monitored in tumor-bearing mice until the tumor reached the palpable (approximately 0.3 cm^3) or advanced (approximately 1.3 cm^3) size. For each tumor stage (palpable or advanced), a group of healthy mice (n = 3–5) was used as the control group. For the assessment of immune status tumor-bearing and control mice were bled by cardiac puncture under isoflurane anesthesia and thereafter euthanized by cervical dislocation. The spleens were removed for preparation of splenocytes.

2.6. 5-Fluorouracil (5-FU) or fludarabine (Flu) administration in mice with advanced tumor

Mice with tumor size of approximately 1cm^3 were randomly divided into six groups (n = 5). Mice in group 1 were used as the control group for tumor growth and injected intraperitoneally (i.p.) with vehicle (DPBS, 100 μl). Animals in groups 2, 3 and 4 were injected (i.p.) with a single dose of Flu at concentrations of 25, 50 and 100 mg/kg body weight, respectively (the maximum dose of Flu used in this study was found not to be toxic to mice [20]. Mice in group 5 were injected i.p. with a single dose of 5-FU at the concentration of 50 mg/kg body weight. This dose was selected because it is non-toxic and has optimal diminishing effects on MDSCs [1]. For combination therapy with 5-FU and Flu, mice in group 6 were administered (i.p.) with a single dose of 5-FU (50 mg/kg) in combination with Flu (100 mg/kg). A group of mice (n = 3–5) was used as untreated non-tumor-bearing control group (for weight and health control). In all cases, the tumor development was further monitored for four days after which the animals were bled, euthanized by cervical dislocation and the spleens were removed as