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Research Paper

Inhibition of IL-18-mediated myeloid derived suppressor cell accumulation enhances anti-PD1 efficacy against osteosarcoma cancer

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ABSTRACT

Myeloid derived suppressor cells (MDSC) are very important in tumor immune evasion and they dramatically increased in peripheral blood of patients with osteosarcoma cancer. The association between MDSC and various cytokines has been studied in the peripheral blood. However, little is known about the mechanism drawing MDSC into tumor parenchyma. This study was to analyze the correlation between MDSC subsets and interleukin 18 (IL-18) level in osteosarcoma tumor model and its effect on the immunotherapy. MDSC were isolated from the blood and parenchyma and analyzed in the osteosarcoma tumor model. IL-18 levels were detected by enzyme-linked immunosorbent assay (ELISA) assay, real-time PCR, western blot and flow cytometry. Moreover, combination treatment with IL-18 inhibition and anti-PD1 was conducted to assess the therapeutic effects of IL-18 blockade. Results showed MDSC levels had a positive correlation with IL-18, suggesting IL-18 may attract MDSC into the parenchyma. IL-18 gene and protein expression significantly increased in blood and tumor lysates of tumor-bearing mice. Anti-IL-18 treatment significantly decreased G-MDSC and M-MDSC in the peripheral blood and tumor. Furthermore, combination therapy decreased the tumor burden and increased $CD4^+$ and $CD8^+$ T cell infiltration, as well as the production of interferon gamma (IFNγ) and granzyme B. Our study revealed a possible correlation between MDSC subsets and IL-18 inducing MDSC migration into the tumor tissue, in addition to provide the potential target to enhance the efficacy of immunotherapy in patients with osteosarcoma.

1. Introduction

As the most common type of malignant bone tumor in children and young adults, osteosarcoma is characterized by the proliferation of tumor cells producing osteoid or immature bone matrix. Most osteosarcomas are detected at the end of long bone in adolescence when they are rapidly growing [1,2]. Despite the advances in multimodality treatment consisting of chemotherapy and radiation, the survival rate remained low in the past two decades because pulmonary metastasis occurred in approximately 40–50% of patients with osteosarcoma as a major cause of fatal outcome. The effectiveness of conventional therapies for osteosarcoma has remained unchanged with a dismal five-year survival rate of less than 20% [3–5]. Therefore, it is important to search for other effective option for treating osteosarcoma.

Overwhelming evidences have proved that immunotherapy shows to be a promising treatment modality, however, most anti-tumor immune responses are rendered ineffective by tumor-mediated immunosuppression and immune evasion contributing to decreased clinical efficacy [6,7]. There are various types of cells involving in tumor mediated immune suppression, such as regulatory T cells (Treg), tumor associated macrophages (TAMs), and myeloid derived suppressor cells (MDSC) [8,9]. Among these types of cells, MDSC have become the focus of intense study in recent years because they play a pivotal role in the tumor-associated immune suppression [10].

Arising from myeloid progenitor cells and losing differentiation into mature dendritic cells, granulocytes or macrophages, MDSC possess the capacity to suppress T cell and natural killer cell through down-regulation of CD8 T cell homing to lymph nodes, and induction of $FoxP3^+$ Treg cells, etc. [11,12]. In peripheral blood of patients with cancer, accumulation of MDSC have been observed, and increased MDSC levels are associated with a poor prognosis of the tumor-bearing host. Tumor-infiltrated MDSC promoted tumor cell proliferation and facilitated tumor cell dissemination from the primary site [13]. MDSC-induced 'metastatic gene signature' derived from murine syngeneic model predicts poor patient survival in the majority of solid tumors [14]. According to the content of immunosuppressive substances and nuclear morphology, murine MDSC were characterized as granulocytic MDSC (G-MDSC) and monocytic MDSC (M-MDSC).

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Tumor-derived factors and inflammatory cytokines are essential to the differentiation and expansion of MDSC, and associations have been confirmed between MDSC and various cytokines in the peripheral blood [8]. However, little is known about the mechanism drawing MDSC into the tumor parenchyma. Studies showed that interleukin 1β (IL-1β) activated the generation and function of MDSC and has been shown to induce the MDSC accumulation [15,16]. Because IL-1β and interleukin 18 (IL-18) are related closely, we questioned whether IL-18 influences MDSC accumulation [17]. Moreover, the association of this cytokine production with MDSC accumulation in osteosarcoma cancer has not been fully investigated.

In this study, we analyzed the relationship between peripheral or intratumoral levels of MDSC and IL-18 expression in the osteosarcoma tumor model, which would elucidate some of the factors that might promote MDSC accumulation to result in tumor immunosuppression. Our findings suggest that blockade of IL-18 may be a potential strategy to target immune inhibitory MDSCs for combination treatments as methods of enhancing the efficacy of immunotherapy in patients with osteosarcoma.

2. Materials and methods

2.1. Mouse tumor model and treatment

Six-week old female Balb/cJ mice were purchased from Vital River Lab (Beijing, China) and maintained under specific pathogen-free conditions. Mice had free access to food and water during the whole experimental period. All animal experiments were performed in compliance with the Chinese legislation on the use and care of laboratory animals and approved by the Ethical Committee on Animal Care and Use of Harbin Medical University. Anti-PD1 antibody was purchased from Bioexcell. IL-18 binding protein (IL-18BP, 122-BP-100) was purchased from R&D Systems.

The osteosarcoma carcinoma K7M2 cell line was purchased from Shanghai Cell Bank (Shanghai, China) and 5×10^5 cells were injected into Balb/cJ mice subcutaneously [2]. 28 days after cell injection, mice were sacrificed to collect blood and tumor for analysis, normal mice as the control (five mice per group). In the treatment studies, 7 days after cell injection, tumor-bearing mice were treated with IL-18BP (5 mg/kg, once daily I.P.), anti-PD1 antibody (5 mg/kg, once a week I.P.) or the combination. Mice treated with IgG were used as the negative control (five mice per group).

2.2. MDSC subsets from tumor analysis

M-MDSC and G-MDSC were phenotyped using flow cytometry. Tumor was cut into small fragments followed by digestion with tumor disassociation kit for 30 min (Miltenyi Biotec, USA), and then filtered by 70 μm cell strainers. Mononuclear cells was enriched by subjecting the single cell suspension to percoll gradient. Briefly, 70% percoll (4 ml) was added to the centrifuge tube, 30% percoll (4 ml) was carefully layered, and then tumor suspension (4 ml) was carefully layered on the top. After centrifuging at 1500 rpm for 15 mins, the buffy coat layer in the middle was collected and washed with PBS, followed by staining with anti-CD4 FITC, anti-CD8 Percp, anti-Ly6G AF700, anti-Ly6C APC, anti-CD11b APC-Cy7, anti-IFNγ PE-CF594, and anti-granzyme B PE antibodies, along with appropriate isotype controls (all from BD) for flow cytometry analysis (BD FACSCalibur). M-MDSC were defined as $CD11b^+Ly6C^+Ly6G^-$ and G-MDSC were defined as $CD11b^+Ly6G^+Ly6C^-$.

2.3. Analysis of MDSC subsets from peripheral blood

Peripheral blood was obtained from tumor-bearing mice and normal mice. Blood was stained with the same antibodies as the tumor for flow cytometry analysis.

2.4. Cytokine levels from peripheral blood and tumor

IL-18 levels were quantitated by enzyme-linked immunosorbent assay (ELISA) assay in plasma and tumor lysates according to the manufacturer's instructions (R&D systems). Protein was quantitated by bicinchoninic acid (BCA) assay to ensure equal amounts of protein were aliquoted in each well.

2.5. Quantitative real-time RT-PCR

The total RNA was extracted with 1000 μl TRIzol reagent. Complementary DNA was generated by adding 0.5 μg total RNA to SuperScript master mix and performing reverse transcription. Quantitative PCR was performed using SYBR green supermix (Bio-Rad, CA). Comparative C_t value method was used to quantify the expression of genes of interest in different samples. The mRNA levels were normalized to the housekeeping gene Gapdh. The gene-specific primer sequences are the following. For Il-18, forward: aatcacttcctcttggccca; reverse: gttgtacagtgaagtcggcc, NCBI Reference: NM_008360.1. For Gapdh, forward: aacgacccctcattgacct; reverse: atgttagtggggtctcgctc, NCBI Reference: NM_001289726.1.

2.6. Western blot

After boiling, equal amounts of protein (40 μg) were subjected to electrophoresis on a 12% (v/v) SDS-polyacrylamide gel. Protein was then electroblotted to a polyvinylidene difluoride (PVDF) membrane from gel. The membrane was blocked with phosphate buffered saline (PBS) containing 5% non-fat milk at room temperature for 1 h, and incubated with indicated primary antibodies (anti-IL-18 rabbit pAb, ab71495, 1:1000; anti-iNOS rabbit pAb, ab15323, 1:250; anti-Arg-1 rabbit pAb, PA5-29645, 1:1000; anti-GAPDH rabbit pAb, SC-25778, 1:3000) at 4 °C overnight, followed by incubating with the goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h. Membrane was washed three times, and visualized by the enhanced chemiluminescence system.

2.7. Statistical analysis

Data were expressed as mean ± SD and analyzed by one-way ANOVA with SAS 9.1 software (SAS Institute, USA). The comparison between each two groups is by post hoc analysis. $p < 0.05$ was considered as significant difference.

3. Results

3.1. MDSC subset levels increased in tumor-bearing mice

5×10^5 K7M2 cells were injected into Balb/cJ mice subcutaneously. 28 days after cell injection, mice were sacrificed to collect blood and tumor for flow cytometry analysis, normal mice as the control (five mice per group). MDSC subsets were analyzed based on staining for CD11b, Ly6G and Ly6C. Comparing to normal mice, the amount of G-MDSC and M-MDSC significantly increased in tumor-bearing mice ($p < 0.01$) (Fig. 1).

3.2. IL-18 increased in tumor-bearing mice

5×10^5 K7M2 cells were injected into Balb/cJ mice subcutaneously. 28 days after cell injection, mice were sacrificed to collect blood and tumor for analysis, normal mice as the control (five mice per group). IL-18 levels in blood and tumor were detected by ELISA assay, real-time PCR and western blot. Results showed that IL-18 levels significantly increased in tumor-bearing mice comparing to normal mice. IL-18 surface expression was detected by flow cytometry and results showed that expression of IL-18 significantly increased on MDSC