



Research paper

Blockade of CCL2 enhances immunotherapeutic effect of anti-PD1 in lung cancer

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ABSTRACT

Myeloid derived suppressor cells (MDSC) play a pivotal role in tumor immune evasion and MDSC levels increased in patients with cancer. Studies confirmed the associations between MDSC and various cytokines in the peripheral blood. However, little is known about the association between parenchymal MDSC subsets and cytokines, or the mechanism drawing MDSC into tumor parenchyma. This study was to analyze the correlation between MDSC subsets and CCL2 level in lung cancer model. G-MDSC and M-MDSC from the blood and parenchyma were analyzed by flow cytometry and western blot in the lung tumor model. CCL2 was detected by ELISA assay, real-time PCR, western blot and flow cytometry. Furthermore, the therapeutic effects of combination treatment combining CCL2 antagonist and anti-PD1 antibody were assessed. Results showed that MDSC subsets had a positive correlation with CCL2, suggesting CCL2 may attract MDSC into the parenchyma. Gene and protein expression of CCL2, as well as the CCL2 surface expression significantly increased in blood and tumor of tumor-bearing mice. Anti-CCL2 treatment decreased G-MDSC and M-MDSC in the periphery and tumor through inhibiting the protein expression of arginase 1 and iNOS. In addition, combination therapy enhanced CD4⁺ and CD8⁺ T cell infiltration, as well as the production of interferon gamma (IFN γ), and increased the survival time of tumor-bearing mice. Our study provided potential new target to enhance the efficacy of immunotherapy in patients with lung cancer, in addition to elucidate a possible association between MDSC subsets and the cytokine drawing MDSC migration into the tumor tissue.

1. Introduction

Lung cancer is characterized by the proliferation of tumor cells. Although the advances in multimodality treatment leading to a relative good prognosis, the 5-year survival rate drops with metastatic disease [1–4]. To improve survival, it is imperative to search for new effective strategies.

In spite of overwhelming evidences that immunotherapy has been shown to be a promising treatment modality, most anti-tumor immune responses are rendered ineffective by tumor-mediated immunosuppression and immune evasion contributing to decreased clinical efficacy [5]. Various types of cells are involved in tumor-mediated immune suppression, such as tumor associated macrophages (TAMs), regulatory T cells (Treg), and myeloid derived suppressor cells (MDSC) [6,7].

As a heterogeneous cell population, MDSC have become the focus of intense study in recent years [8]. MDSC arise from myeloid progenitor cells and lose differentiation into mature dendritic cells, macrophages

or granulocytes, but possess the capacity to suppress T cell and natural killer cell through induction of FoxP3⁺ Treg cells, depletion of L-arginine to arrest T cells in mitosis, and down-regulation of CD4 and CD8 T cell homing to lymph nodes, etc. [9,10].

MDSC levels significantly increased in peripheral blood in patients with cancer, and correlate with clinical metastatic burden and stage [11]. The content of immunosuppressive substances and nuclear morphology have been used to characterize murine MDSC as granulocytic MDSC (G-MDSC) and monocytic MDSC (M-MDSC). Tumor-infiltrated MDSC facilitated tumor cell dissemination from the primary site and promoted tumor cell proliferation. MDSC-induced 'metastatic gene signature' derived from murine syngeneic model predicts poor patient survival in the majority of human solid tumors [12].

While associations have been confirmed between MDSC and various cytokines in the peripheral blood, little is known about the mechanism drawing MDSC into the tumor parenchyma [13]. Studies showed that inflammatory cytokines such as CCL2 was related to the accumulation of MDSC and suppression of T cells [14,15]. However, the association of

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this cytokine production with accumulation of MDSC in lung cancer has not been fully investigated.

In this study, we analyzed the relationship between peripheral and intratumoral levels of MDSC and CCL2 expression in the lung tumor model. This study would elucidate some of the factors that might promote MDSC accumulation to result in tumor immunosuppression.

2. Materials and methods

2.1. Mouse experiments

Four-week old female C57 mice were purchased from Charles River (Beijing, China) and maintained under pathogen-free conditions. Mice were free access to food and water during the 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. CCL2 antagonist *N*-[2-[(3*R*)–1-[(4-chlorophenyl)methyl]–3-pyrrolidinyl]amino]–2-oxoethyl]–3-(trifluoromethyl)benzamide hydrochloride (BHC) was purchased from Tocris Bioscience. Anti-PD1 antibody was purchased from Bioexcell.

The lung carcinoma cell line was purchased from Cell Bank (Beijing, China) and 6×10^5 cells were injected into C57 mice by tail vein. 25 days after cell injection, mice were sacrificed to collect blood and tumor for analysis, normal mice as the control. In the therapeutic experiments, 14 days after cell injection, tumor-bearing mice were treated with CCL2 antagonist BHC (40 $\mu\text{g}/\text{mouse}$, once daily I.P.), anti-PD1 antibody (100 $\mu\text{g}/\text{mouse}$, once per week I.P.) or the combination. Mice treated with IgG were used as the negative control.

2.2. Phenotype analysis of tumor lysate

G-MDSC and M-MDSC were phenotyped using flow cytometry. Tumor was cut into small fragments followed by digestion for 30 min with tumor disassociation kit (Miltenyi Biotec, USA), and then filtered by 70 μm cell strainers. The percoll gradient was used to enrich mononuclear cells. After washing with PBS, cells were stimulated with PMA/ionomycin in the presence of Golgi stop and Monesin (eBioscience, San Diego, CA, USA) for 4 h. Cells were washed with PBS and then stained with Live/Dead dye, anti-CD11b PE-Cy7, anti-Ly6G AF700, anti-Ly6C APC, anti-CD4 FITC and anti-CD8 Percp antibodies (all from BD). After fixation-permeabilization, cells were stained with anti-IFN γ PE-CF594 antibody, along with appropriate isotype controls (all from BD) for flow cytometry analysis (BD FACSCalibur). G-MDSC was defined as CD11b⁺Ly6G⁺Ly6C⁻ and M-MDSC was defined as CD11b⁺Ly6C⁺Ly6G⁻. MDSC subsets were sorted with corresponding beads from the tumor lysates for western blot assay.

2.3. Phenotype analysis of blood

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

2.4. Cytokine levels from peripheral blood and tumor

CCL2 levels were quantified by ELISA assay in tumor lysates and plasma according to the manufacturer's instructions (R&D systems). Protein was quantified by BCA assay (Thermo Scientific) to ensure equal amounts of protein were aliquoted in each well.

2.5. Quantitative real-time PCR

Samples were collected and placed in 1000 μl TRIzol reagent to extract the total RNA. Complementary DNA was generated by adding 1 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 Ccl2, forward: aggtgtcccaagaagctgt; reverse: acagaagtgtctgagtggt, NCBI Reference: NM_011333.3. For Gapdh, forward: cccactctccaccttgcag; reverse: ctgtctcagtccttctgt, NCBI Reference: NM_001289726.1.

2.6. Western blot

After boiling, equal amounts of protein (50 μg) were subjected to electrophoresis on a 12% (v/v) SDS-polyacrylamide gel. Protein was then electroblotted to a PVDF membrane from gel. The membrane was blocked with PBS containing 5% non-fat milk at room temperature for 1 h, and incubated with indicated primary antibodies (anti-CCL2 rabbit pAb ab25124, 1:2000; anti-GAPDH rabbit pAb SC-25778, 1:2000) at 4 °C overnight, followed by incubating with the goat-anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h. After incubation, membrane was washed three times, and visualized by the enhanced chemiluminescence system.

2.7. Statistical analysis

Data were expressed as mean \pm SD and analyzed by one-way ANOVA. Kaplan-Meier survival analysis was performed to compare differences among the groups and *p* values were calculated with the log-rank test. The comparison between each two groups is by post hoc analysis. All data analysis was used with SAS 9.1 software (SAS Institute, USA). *p* < 0.05 was considered as significant difference.

3. Results

3.1. MDSC subset levels increased in tumor-bearing mice

Analysis of MDSC subsets was based on staining for CD11b, Ly6G and Ly6C. Compared to normal mice, G-MDSC and M-MDSC levels significantly increased in tumor-bearing mice (*p* < 0.01) (Fig. 1).

3.2. CCL2 increased in tumor-bearing mice

CCL2 levels in blood and tumor were detected by ELISA assay, real-time PCR and western blot. Surface expression of CCL2 on MDSC subsets was detected by flow cytometry. Results showed that CCL2 levels significantly increased in tumor-bearing mice comparing to normal mice. CCL2 surface expression also significantly increased on MDSC subsets of tumor-bearing mice (*p* < 0.01) (Fig. 2).

3.3. CCL2 blockade reduced MDSC recruitment

Lung tumor model was used to test whether blocking CCL2 activity would impact MDSC subset levels. Anti-CCL2 treatment significantly reduced CCL2 levels that corresponded to the MDSC reduction, both in the blood and tumor (*p* < 0.05) (Fig. 3).

3.4. CCL2 blockade enhanced the efficacy of anti-PD1 treatment

The effects of combination treatment using the CCL2 antagonist with anti-PD1 antibody were explored in this study. Treatment of tumor-bearing mice with anti-PD1 antibody afforded modest effect. Treatment with the CCL2 antagonist alone also had modest impact on animal survival time. However, the combination treatment improved the efficacy of the immune checkpoint blockade by increasing the infiltration of both CD4⁺ and CD8⁺ T cells, as well as the production of IFN γ , and the survival time of tumor-bearing mice (*p* < 0.05) (Fig. 4).

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