Increased circulating myeloid-derived suppressor cells correlate with cancer stages, interleukin-8 and -6 in prostate cancer

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Abstract: Aim: Myeloid-derived suppressor cells (MDSCs) are a population of cells which negatively regulate immune response during tumor progression. In this study, we assessed the accumulation of MDSCs (CD33+CD11b+HLA-DR−CD14−) in patients with prostate cancer and its clinical relevance. Methods: We tested the frequency of MDSCs in the peripheral blood of patients with prostate cancer or benign prostate hyperplasia and healthy donors. Serum interleukin-8, -6 and -10 were analyzed. Effects of MDSCs on the T cell response were determined. Results: MDSCs increased in cancer patients, and there was an association between MDSCs and cancer stages or overall survival. Elevated serum interleukin-8 and -6 in cancer patients correlated with MDSCs. Moreover, accumulation of MDSCs was associated with defective T cell function. Conclusion: Our study showed an increased population of MDSCs in patients with prostate cancer. Interleukin-8 and -6 in serum may play a new important role companied with MDSCs in prostate cancer.

Keywords: IL-6, IL-8, MDSCs, prostate cancer

Introduction

Recent research has established that many cancers are characterized by the overproduction of a range of immature myeloid immunosuppressive cells [1, 2], and these cells are defined as myeloid-derived suppressor cells (MDSCs). MDSCs represent a heterogeneous population comprised of progenitors and precursors of myeloid cells and its induction is an important immune-evading mechanism used by tumors [3]. In preclinical models, the phenotype of MDSCs consists of co-expression of the myeloid lineage differentiation antigens Gr-1 (Ly6G) and CD11b (CR3, Mac-1), including monocytic (Ly6G Ly6C+++) and granulocytic (Ly6G-Ly6C−) cells [4]. In cancer patients, MDSCs express either or both of the common myeloid markers CD33 or CD11b [5], and are LIN and/or HLA-DR [6]. Thus, human MDSCs were initially defined as HLA-DRCD33+ or CD14 CD11b+ cells [7], with both phenotypes identifying cell populations with T cell suppressive activity. They are also further classified as subset of the CD11b+CD14+ polymorphonuclear granulocyte morphology distinct from the mononuclear CD11b+CD14+ monocytes. Previous work also established the presence of granulocytic CD15+ MDSCs in the circulation of human cancers including renal, lung cancer, breast, colon, and pancreatic cancers [8]. Even so, more and more markers have been associated with MDSCs function in recent year.

MDSCs play a pivotal role in cancer progression by suppressing immune response [9]. The increasing frequency and phenotype of circulating MDSCs in peripheral blood have been reported in many types of cancers, both in the preclinical models and human patients [10]. Several reports also have shown increased infiltration of MDSCs in cancers including breast cancer, lung cancer, and multiple myeloma, both in the primary tumor and metastatic sites [11-13]. However, due to their heterogeneity, the frequency, phenotype and suppressive
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Table 1. Clinicopathological parameters of patients

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>80 (100)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>≤70 y</td>
<td>35 (43.8)</td>
</tr>
<tr>
<td>&gt;70 y</td>
<td>45 (56.2)</td>
</tr>
<tr>
<td>Preoperative PSA</td>
<td></td>
</tr>
<tr>
<td>&lt;4 ng/mL</td>
<td>33 (41.3)</td>
</tr>
<tr>
<td>4-10 ng/mL</td>
<td>25 (31.2)</td>
</tr>
<tr>
<td>&gt;10 ng/mL</td>
<td>22 (27.5)</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
</tr>
<tr>
<td>4-6</td>
<td>32 (40.0)</td>
</tr>
<tr>
<td>7</td>
<td>27 (33.8)</td>
</tr>
<tr>
<td>8-10</td>
<td>21 (26.2)</td>
</tr>
<tr>
<td>TNM classification</td>
<td></td>
</tr>
<tr>
<td>I-II</td>
<td>20 (25.0)</td>
</tr>
<tr>
<td>III</td>
<td>29 (36.3)</td>
</tr>
<tr>
<td>IV</td>
<td>31 (38.8)</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>10 (12.5)</td>
</tr>
<tr>
<td>T2</td>
<td>12 (15.0)</td>
</tr>
<tr>
<td>T3</td>
<td>27 (33.8)</td>
</tr>
<tr>
<td>T4</td>
<td>31 (38.8)</td>
</tr>
<tr>
<td>Nodal status</td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>30 (37.5)</td>
</tr>
<tr>
<td>N1</td>
<td>50 (62.5)</td>
</tr>
<tr>
<td>Distant metastasis</td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>43 (53.8)</td>
</tr>
<tr>
<td>M1</td>
<td>37 (46.2)</td>
</tr>
</tbody>
</table>

In the present study, we investigated the clinical characteristics of circulating MDSCs in patients with prostate cancer. We isolated peripheral blood mononuclear cells (PBMCs) and measured percentages of MDSCs to determine the relationship between MDSCs levels and clinical cancer stages. We detected three subtypes of MDSCs, including granulocytic CD33^+CD11b^+HLA-DR CD14^-, monocytic CD33^+CD11b^+HLA-DR CD14^+, and granulocytic CD33^+CD11b^+HLA-DR CD15^+ MDSCs. Among these phenotypes, the major one is CD33^+CD11b^+HLA-DR CD14^+, so we defined the MDSCs as CD33^+CD11b^+HLA-DR CD14^+ in this study and further explore their correlation with T progression, nodal status and metastasis of prostate cancer. Levels of cytokine including Interleukin (IL)-8, -6 and -10 were also determined to explore their relationship with MDSCs.

Patients and methods

Peripheral blood specimens were collected from 80 patients with prostate cancer (mean age 67.8; range 38-84) with newly diagnosed and histologically confirmed tumors: stages I-II (n=20); stage III (n=29); stage IV (n=31) in accordance with the TNM classification of the American Joint Committee on Cancer Criteria. All the patients were hospitalized at Department of Urology, Inner Mongolia People’s Hospital, from 2009 to 2013. The study was carried out in accordance with the institutional ethical guidelines and the use of human tissues was approved by the Medical Ethics Committee of Inner Mongolia Medical University (IMMP study ID 013-332115). Every patient involved in the study was asked to sign a piece of written informed consent which has been approved by the ethics committee of Inner Mongolia Medical University, and all the consents were saved by the ethics committee. The study was conducted according to the principles expressed in the Declaration of Helsinki. Patient characteristics are detailed in Table 1. Twenty age-matched normal healthy volunteers served as normal controls. Additional twenty patients with benign prostatic hyperplasia (BPH) were included. Clinical follow-up data were available for 62 patients, while 18 patients were excluded for lack of information.

PBMCs isolation and flow cytometry analysis

Five milliliters of venous blood was collected into EDTA-coated evacuated tubes and processed within 2 hours. Blood collected from patients was obtained prior to surgery, radiation or any systemic chemotherapy. Blood was diluted with an equal volume of plain RPMI and then layered over Ficoll-Paque Plus. The solution was centrifuged at 1100 x g for 20 minute by density centrifugation. PBMCs were isolated from the interface and washed in RPMI. Platelets were removed by an additional density centrifugation over cold PBS. Cells were counted and stored at liquid N2 for subsequent analysis.
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Figure 1. MDSCs FACS and scatter plots of cancer patients and healthy controls. CD33⁺CD11b⁺HLA-DR⁻CD14⁻ MDSCs FACS gating of PBMC of a patient with prostate cancer (A) and an age-matched normal control (B). Dot plots represent live gated events. The forward and side scatter gate was analyzed for CD33⁺HLA-DR⁻ cells. Then the CD33⁺HLA-DR⁻ gate was analyzed for cells expressing CD11b and CD14. MDSCs were calculated as a percentage of live cells in PBMCs. Markers analyzed are indicated in the axis of each FACS plot.
For flow cytometry, PBMCs were incubated with specific antibodies including CD33-PE, CD11b-FITC, CD14-PerCP-Cy5.5, HLA-DR-APC and CD15-PE-Cy7 (BioLegend, CA, USA). PBMCs were also labeled with the appropriate isotype control antibodies as negative controls. After staining, cells were resuspended in 500 μL of FACS buffer and evaluated by multicolor flow cytometry in BD FACSCanto II flow cytometer (BD Bioscience). Data were analyzed with Flowjo software. MDSCs (CD33+CD11b−HLA-DR−CD14−) were calculated as a percentage of total live PBMCs.

**Serum isolation and measurement of cytokines**

Three milliliters of venous blood was collected into promoting coagulating tubes and centrifuged within 1 hour. The serum obtained was stored frozen in aliquots at -80°C for subsequent analysis. Serum inflammatory cytokines were analyzed by enzyme-linked immunosorbent assay in accordance to the manufacturer's instructions.

**Determination of T cell function**

T cell function was determined as previously described [5]. PBMCs were incubated in plates with anti-CD3/CD28-coated beads at 37°C for 3 days, followed by determination of total viable cell number using a hemacytometer. Cell culture supernatant was assayed for IL-2 and IFN-γ concentration using the Searchlight™ multiplex assay system. Briefly, 50 μL supernatant was added to a 96-well plate pre-spotted with either IL-12 or IFN-γ antibodies. After several washes, biotinylated antibodies with different specificity within the same cytokines were added. Streptavidin conjugated to horse radish peroxidase and SuperSignal ELISA Femto Chemiluminescent Substrate was added successively to generate luminescent signal. The amount of signal was proportional to the amount of cytokine.

PBMCs were labeled with carboxyfluorescein diacetate succinimidy ester (CFSE, 10 μM) and plated in 96-well plates. After 5 days, cells were harvested and stained for CD4 and CD8 markers; the data were acquired and detected by flow cytometry. CD33+HLA-DR myeloid cells and CD3+ T cells were purified using the Rosettesep™ kit according to the manufacturer’s instructions. Increasing ratios of CD33+HLA-DR cells were added to T cells. For T cell activation, anti-CD3 and anti-CD28-coated beads were added to each well at a bead to T cell ratio of 1:1. Plates were cultured for 4 days, then pulsed with 1.0 μCi of (H)-TdR (NEN, MA, USA) for 8 hours and lysed with distilled water. Thymidine incorporation was determined by detecting the amount of radioactivity using a β-counter.

**Statistical analysis**

Results are expressed as mean ± SD and processed using SPSS 13.0 statistical software. The statistical significance of differences between groups was determined by one-way ANOVA or Mann-Whitney U test. Spearman’s correlation analysis was used to analyze the relationship between MDSCs and cytokines. Survival curves were plotted according to the KaplanMeier method and compared using the Log-rank test. P<0.05 was considered to be statistically significant.

**Results**

**Circulating CD33+CD11b−HLA-DR−CD14− cells are major subtype of MDSCs in prostate cancer patients which are elevated and associated with clinical stages**

PBMCs from patients with newly diagnosed prostate cancer were analyzed for MDSCs and compared with healthy, age-matched normal controls and patients with BPH. Three subtypes of MDSCs, including granulocytic CD33+CD11b+HLA-DR−CD14+, monocytic CD33+CD11b+HLA-DR−CD14+, and granulocytic CD33+CD11b+HLA-DR−CD14− MDSCs were detected. Figure 1 shows the representative dot plots for one of the patients and normal controls included in the study to illustrate the gating strategy. Acquired cells were first gated based on their expression of CD33 and HLA-DR and then cells expressing the markers CD11b and CD14 were determined. The level of all the three subtypes of MDSCs were significantly elevated in patients with prostate cancer compared with both normal controls and patients with BPH (P=0.001, Figure 2A-C). Moreover, among these phenotypes, the major one is CD33+CD11b+HLA-DR−CD14−, so we defined the MDSCs as CD33+CD11b+HLA-DR−CD14− in this study (Figure 2D). As shown in Figure 3, CD33+CD11b+HLA-DR−CD14− MDSCs levels increased along with the clinical stages (Figure 3A), including progres-
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Cytokines including IL-8, -6 and -10 are higher in patients with prostate cancer and correlate with clinical stages

Levels of inflammatory cytokines important for MDSCs expansion and function were evaluated in this cohort of patients with prostate cancer [14-16]. Serum levels of IL-8, -6 and -10 were all elevated in cancer patients compared with normal controls and patients with BPH (P<0.01, Figure 4, left lanes). While serum levels of IL-1b and TNF-α were up-regulated in only 13 and 7 patients respectively (data not shown). Besides, levels of IL-8, -6 and -10 were also correlated to the clinical stages of cancer (Figure 4, right lanes).

MDSCs correlate with serum IL-8 and -6 in patients with prostate cancer

Analysis of MDSCs and cytokines revealed strong correlation between the percentage of MDSCs and IL-8 (Figure 5A, r=0.7149, P<0.0001) or IL-6 (Figure 5B, r=0.6392, P<0.0001), but not IL-10 (r=0.267, P=0.233).

MDSCs are associated with the poor prognosis of patients with prostate cancer

To investigate the prognostic value of MDSCs, its association with an overall survival was evaluated using Kaplan-Meier survival curves with the log-rank test. Sixty-two patients were enrolled for this analysis. The follow-up time ranged from 1 to 60 months. The median survival time of the group with higher level of MDSCs was 19.158 months, and the cumula-
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**Figure 3.** CD33+/CD11b+/HLA-DR−/CD14− MDSCs correlated with clinical stages. Correlation between percentage of MDSCs and clinical stages (A), T classification (B), N classification (C) and distant metastasis (D) in patients with prostate cancer and normal controls (*P<0.05, **P<0.01).

**Immunosuppressive effect of tumor-derived MDSCs**

To determine if circulating MDSCs negatively impact the activity of T cells, PBMCs from three normal controls and three patients with stage IV cancer were cultured with anti-CD3/CD28-coated beads. As shown in **Figure 6A-C**, T cell activation in patients with advanced cancer was impaired compared to normal controls, as determined by decreased cell proliferation and secretion of IL-2 and IFN-γ. The proliferation of CD4+ T cells and CD8+ T cells were also significantly decreased (**Figure 6D**). To better understand the impact of MDSCs on T cell activity, increasing ratios of CD33+/HLA-DR− cells were co-cultured with equal numbers of purified autologous T cells. After enrichment, purity of the CD33+/HLA-DR− fraction was determined by flow cytometry. Approximately 80% of the cells showed positive CD33+ and negative HLA-DR (**Figure 6E**). Purified autologous T cells were activated in the presence of increasing ratios of CD33+/HLA-DR− cells. Increasing numbers of CD33+/HLA-DR− cells isolated from normal controls had a minimal impact on the proliferation of their autologous T cells. By contrast, a significant decrease in T cell proliferation was observed when CD33+/HLA-DR− cells derived from cancer patients were added (**Figure 6F**).

**Discussion**

Prostate cancer is one of the major public health problems causing cancer-related deaths...
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throughout the world with very poor prognosis and high possibilities of tumor invasion and migration [17]. It has been evident that prostate cancer is commonly infiltrated by a high number of immune cells, including T and B lymphocytes, macrophages, natural killer cells, dendritic cells and mast cell. All of these cells are irregularly scattered within the tumor and loaded with an assorted array of cytokines, chemokines, and inflammatory mediators [18]. Quite recently, studies on tumor and immune cells revealed the critical role of MDSCs, a novel and heterogeneous population of myeloid cells with specific inhibitory activity, in the pro-

Figure 4. IL-8, -6 and -10 are significantly higher expressed in patients with prostate cancer and correlated with stages of cancer. Serum cytokines levels including IL-8 (A), IL-6 (B) and IL-10 (C) in age-matched normal controls and patients with prostate cancer were measured using ELISA assays. Serum samples from patients had a significantly higher level of IL-8, 6 and 10 than control samples (left lanes) and the levels increased with clinical stages (right lanes) (*P<0.05, **P<0.01).
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cess of tumorigenesis and metastasis [19]. MDSCs were significantly elevated in pancreatic, esophageal and gastric cancer compared with controls, and increasing percentage of MDSCs was associated with increased risk of death, and was an independent prognostic factor for survival [20]. MDSCs were also elevated in patients with metastatic renal cell carcinoma and patients with a relatively low proportion of MDSCs exhibited prolonged survival [21]. The high percentage of MDSCs in patients with advanced-stage melanoma correlated with disease progression and decrease overall survival partially by inhibiting the T cell activation [22]. Based on these findings, we speculated MDSCs might be correlated with prostate cancer development and progression. In this study, we demonstrated elevation of circulating MDSCs in patients with prostate cancer (defined as CD33^+CD11b^+HLA-DR^-CD14^-). Increasing MDSCs percentage was associated clinical stages and a prognostic factor for survival. Patients with prostate cancer also presented with increased serum levels of pro-inflammatory IL-8 and -6, which may be partly responsible for MDSCs accumulation.

MDSCs may exert their immunosuppressive effects against tumor partially through some cytokines [16, 23]. In preclinical models, inflammatory cytokines including IL-8, CCL2 and CCL5 recruit MDSCs with pro-cancerous activities from the blood stream into the tumor site. These cytokines have potent angiogenic activities, promoting the motility of endothelial cells within tumor, sprouting and branching [24]. In parallel, TNF-α, which up-regulate expression of these cytokines, increases the release of matrix metalloproteinases and directly induces epithelial-to-mesenchymal transition and motility processes in cancer cells [16]. We found significant increases in serum levels of IL-8, -6 and -10 in patients with prostate cancer and their correlation with circulating MDSCs percentage.
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A

B

C

D

E

F

CD4

CD8

CD33+HLA-DR- T cell=0

CD33+HLA-DR- T cell=0.2

These data complement and provide clinical evidences for previously published data on intratumoral recruitment and differentiation of MDSCs and IL-8 production [25, 26]. And it is also consistent with previous study that IL-6 was the most potent generator of MDSCs-like suppressor cells from normal donor PBMCs, and therefore a significant inducer of MDSCs [27].

Recently, MDSCs-centered therapeutic approach, characterized by inhibiting immune response against tumor in cancer patients and tumor-bearing mice, has drawn people’s attention. In patients with metastatic renal cell carcinoma, sunitinib significantly reduced accumulation of MDSCs in peripheral blood and reversed T cell suppression, thus provided a rationale for combining sunitinib with immunotherapy for treatment of certain tumors [28]. In murine models of lung cancer, depletion of MDSCs inhibited tumor growth, enhanced tumor cell apoptosis, reduced migration of tumors from the primary site to lung and enhanced therapeutic vaccination responses [29]. Treatment with IL-12 plus cyclophosphamide eliminated MDSCs in patients with colorectal cancer, which was essential to facilitate T cell infiltration and subsequent tumor elimination [30]. Our results showed that the elevated MDSCs in prostate patients were associated with clinical characteristics of patients and increased level of both IL-8 and -6. Moreover, higher level of MDSCs associated with poor prognosis of patients. Taken together, these results suggested MDSCs plus IL-8 or -6 may be a new potential prognostic factor and target of prostate cancer. Further investigation will be required to elucidate this question.

It is appreciated that MDSCs expressing arginase I deplete L-arginine and profoundly inhibit T cell function [5, 22]. Inhibition of arginase I restores T cell function in vitro and induces an antitumor response in vivo [31]. Increased numbers of MDSCs in the peripheral blood of renal cancer patients correlated with a profound T cell dysfunction [7]. Our results, consistent with the previous study, demonstrated that increased circulating MDSCs were associated with decreased T cell activation in terms of proliferation and secretion of IL-2 and IFN-γ. Moreover, increasing numbers of MDSCs in direct contact with T cells was associated with greater inhibition of T cell proliferation. Therefore, MDSCs is a potential important mechanism of cancer-related T cell immunosuppression.

Conclusion

We demonstrated a marked increase in circulating CD33+CD11b+HLA-DR-CD14+ MDSCs in patients with prostate cancer. Importantly, we demonstrated the elevation of MDSCs increased with stages of cancer and correlated with IL-8/IL-6 significantly. Abnormal accumulation of MDSCs is an important mechanism of T cell unresponsiveness in cancer patients. These data not only add to our understanding of the immunobiology of prostate cancer but also may be of importance in informing studies incorporating MDSCs inhibition strategies and IL-8/IL-6 directed therapies.

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Disclosure of conflict of interest

None.
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Abbreviations

BPH, benign prostatic hyperplasia; IL, Interleukin; MDSCs, Myeloid-derived suppressor cells; PBMC, peripheral blood mononuclear cells.

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