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Subsets of myeloid-derived suppressor cells in hepatocellular carcinoma express chemokines and chemokine receptors differentially☆,☆☆,★

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ABSTRACT

Tumors induce the recruitment and expansion of myeloid-derived suppressor cells (MDSCs), a heterogeneous population of cells that can be further sub-divided into polymorphonuclear Ly6G$^+$ PMN-MDSCs and monocytic Ly6G$^-$ Mo-MDSCs. To identify chemokines and chemokine-related genes that are differentially expressed within the tumor microenvironment in these two MDSC subsets, we established an orthotopic hepatocellular carcinoma model in immunocompetent mice. Splenic PMN-MDSCs and Mo-MDSCs were isolated to >95% homogeneity by flow cytometry. Using a real-time PCR array, we investigated the expression of 84 genes encoding chemokines and cytokines, chemokine receptors, and related signaling molecules involved with chemotaxis. Clustering analysis suggested that a core set of chemokine-related genes is expressed in both PMN-MDSC and Mo-MDSC populations, but that the expression profile is broader for Mo-MDSCs. Furthermore, 11 genes are more highly expressed in PMN-MDSCs and 12 genes are more highly expressed in Mo-MDSCs. Among these, PMN-MDSCs express Cxcr1, Cxcr2 and Il1b at 33.03- to 109.76-fold higher levels than in Mo-MDSCs, and Mo-MDSCs express eight genes (Ccr2, Ccr5, Cmklr1, Cx3cr1, Ccr3, Ccl9, Cmtm3 and Cxcl16) at 30.2 to 515.5-fold higher levels than in PMN-MDSCs. These results suggest that the profile of chemokines and chemokine-related genes is more expansive for Mo-MDSCs than for PMN-MDSCs. The differential expression of chemokines and chemokine-associated genes may regulate the presence and activity of PMN-MDSCs and Mo-MDSCs in the tumor microenvironment.

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1. Introduction

Tumor escape from immune elimination is a hallmark of cancer [1]. Multiple mechanisms of cancer immune evasion have been demonstrated. It is well established that tumors can secrete multiple cytokines and chemokines, and that these soluble factors recruit or expand immunosuppressive cells, such as regulatory T lymphocytes (Tregs) and myeloid-derived suppressor cells (MDSCs), into the tumor microenvironment [2,3]. MDSCs represent a heterogeneous population of myeloid progenitor cells and other myeloid cells, including immature myeloid cells, immature macrophages, granulocytes, and dendritic cells [DCs]. MDSCs contribute to cancer immune evasion via their suppressive effects on both the innate immune response by NK cells and the adaptive immune response by T cells via direct cell-cell contact [4,5]. A growing body of evidence from the clinic and experimental animal models has demonstrated that MDSCs accumulate in the bone marrow, spleen, peripheral blood, and lymph nodes, as well as at the tumor sites [4,6,7]. In mice, MDSCs are characterized by the dual expression of CD11b and Gr-1 [7]. According to the preferential expression of Ly6G on granulocytes and Ly6C on monocytes, MDSCs can be divided into two groups: CD11b$^+$Ly6G$^+$Ly6C$^{int/low}$ PMN-MDSCs (with polymorphonuclear morphology) and CD11b$^+$Ly6G$^-$Ly6C$^+$ Mo-MDSCs (with monocytic morphology) [8–10].

Diverse chemokine/chemokine receptor axes participate in the regulation of MDSC mobilization. The CCL2/CCR2 pathway is established to play a pivotal role in MDSC migration in several mouse cancer models [11]. Furthermore, the dynamics of MDSCs in tumor-bearing hosts are mediated by CCR2, and CCR2 deficiency causes striking conversion of infiltrating cellular dominance from CD11b$^+$Gr-1$^{int/dull}$Ly-6C^{hi} macrophages to CD11b$^+$Gr-1^{hi}Ly-6C^{int} neutrophils without affecting tumor growth [12]. CXCR2 is required for homing of granulocytic MDSCs from the circulatory system to the colonic inflamed mucosa and the tumor in rhabdomyosarcoma [13], and CCL5, a member of the CC chemokine family involved in the response to stress signals, promotes mammary tumor progression by generating MDSCs in the bone marrow in cooperation with tumor-derived colony-stimulating factors [14].

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Though it is known that differential expression of chemokines and chemokine receptors plays a crucial role in determining tissue-specific trafficking and infiltration of different leukocyte subsets, how MDSCs are recruited to local inflamed tissues and the tumor micro environment is unclear. The tumor microenvironment contains a vast array of pro- and anti-inflammatory cytokines that result in the infiltration of different MDSC subsets depending on their chemokine receptor expression. Additionally, there is some evidence that chemokine receptors are differentially expressed by PMN-MDSCs and Mo-MDSCs, which provides a potential explanation for their differential recruitment [15].

In this study, we found that PMN-MDSCs and Mo-MDSCs display different mobility towards conditioned medium form hepatic stellate cells (HSC-CM), which make us seek to perform a comprehensive examination of the chemokine and chemokine receptor expression profiles of PMN-MDSCs and Mo-MDSCs. To this end, we established an orthotopic hepatocellular carcinoma model in immunocompetent mice, and sorted PMN-MDSCs and Mo-MDSCs in the spleen of tumor-bearing mice via flow cytometry.These findings help to establish patterns of chemokines and chemokine receptors that may regulate differential recruitment of PMN-MDSCs and Mo-MDSCs.

2. Materials and methods

2.1. In situ hepatic cancer model

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Xiamen University. To establish an orthotopic hepatocellular carcinoma model in immuno-competent mice, H22 cells (5×10^5 in 20 μl PBS) were injected intrahepatically into BALB/c mice. After 2 weeks, the mice were humanely sacrificed and spleens were aseptically removed.

2.2. Isolation of PMN-MDSCs and Mo-MDSCs by flow cytometry

Aseptically removed spleens were mechanically dispersed in cold RPMI-1640. The cell suspension was filtered, depleted of erythrocytes using RBC lysis buffer (BD Pharmingen), washed twice in RPMI-1640 medium containing 10%FBS, and adjusted to a concentration of 1×10^7 cells/ml in PBS containing 1%BSA. PMN-MDSCs and Mo-MDSCs were isolated using an MDSC isolation kit (MiltenyiBiotec, Germany) according to the manufacturer's instructions.

2.3. The migration of PMN-MDSCs and Mo-MDSCs

For the migration of PMN-MDSCs and Mo-MDSCs, the sorted MDSCs were cultured in a transwell (Costar, Cambridge, MA). The up chamber were added 5×10^5/100 μl MDSCs, the lower chamber were added 800 μl RPMI-1640 medium or HSC-CM contained 5% FBS. After 8 h, the cells in the lower chamber were collected for flow analysis and counting.

2.4. RNA extraction and real-time PCR profiling

RNA was extracted from Mo-MDSCs and PMN-MDSCs using Trizol (Invitrogen) and then cleaned using the RNA easy MinElute Cleanup kit (Qiagen, USA). Subsequently, total RNA was reverse transcribed using Super Script III reverse transcriptase (Invitrogen, USA), and cDNA was amplified by PCR using 2 × Super Array PCR master mix (SA Biosciences, USA). Real-time PCR was then performed on each sample using the Mouse Chemokines and Receptors RT^2 Profiler™ PCR Array (SA Biosciences, USA). The genes targeted are shown in Table 1. Reactions were conducted using the ABI PRISM 7900 with the following reaction profile: cDNA pre-denaturation for 10 min at 95 °C; PCR amplification for 40 cycles with 15 s at 95 °C and 60 s at 60 °C. This was followed by a melting curve analysis to determine the reaction specificity.

Table 1
mRNAs tested by PCR array.

Category	Sub-category	mRNAs
Chemokines	Beta chemokines	CCL1–9, 11, 12, 17, 19, 20, 22, 24–26, and 28
	Alpha chemokines	CXCL1–3, 5, and 9–16
	XC chemokine	XCL1
	Delta chemokine	CXC3L1
Cytokines with potential chemotactic activity		Cmtm2a,Cmtm3, Cmtm4, Cmtm5, Cmtm6, Ifng, Il16, Il1b, Il4, Il6, Pf4, Ppbp, Tgfb1, and Tnf
Typical chemokines receptors	CXC receptors	CXCR1–6
	CC receptors	CCR1–11
	CX3C receptor	CX3CR1
	XC receptor	XCR1
Atypical chemokine receptors		ACKR1 (DARC), ACKR2 (D6/Ccbp2), ACKR3 (CXCR7), ACKR4 (Ccrl1), Ccrl2
Cytokine receptors		C5ar1 (Gpr77), Cmklr1, Fpr1, Gpr17
Other important gene involved in chemotaxis		Hif1a, Itgam, Itgb2, Mapk1, Mapk14, Slit2, Tlr2, Tlr4, Tymp

Agarose gel electrophoresis was performed for confirmation of the size of each PCR product.

For gene expression quantification, we used the comparative Ct method. First, gene expression levels for each sample were normalized to the expression level of the housekeeping gene (Actb and B2m). The expression of target genes was classified into four groups according to the value of Ct: 0 = undetected/absent or >20 Ct, representing negative expression (−); 1 = 15–20 Ct, representing weak expression (+); 2 = 10–15 Ct, representing moderate expression (++); and 3 = 1–10 Ct, representing strong expression (+++). The relative expression of each gene was calculated by determining $10^6 \times$ Log2 (− Ct), and the expression difference between PMN-MDSCs and Mo-MDSCs was determined by calculating the Log2 (− Ct). Statistical differences ($p < 0.05$) were calculated by the *T*-test using SPSS 16.0 software. Clustering was done using MEV v4.9 software (Clustering type: hierarchical clustering, distance metric: Pearson correlation).

3. Results

3.1. Purity and mobility of PMN-MDSCs and Mo-MDSCs from spleens of tumor-bearing mice

To detect the gene expression profiles of PMN-MDSCs and Mo-MDSCs, an orthotopic hepatocellular carcinoma model was established in BALB/C mice (n = 9 mice). Splenocytes were isolated and approximately 94.5% of splenocytes was CD45 positive (data not shown). The percentage of MDSCs in splenocytes from tumor-bearing mice was 10.1%, and the percentage of PMN-MDSCs and Mo-MDSCs in CD11b$^+$ cells was 64.7% and 24.5% (Fig 1A). After isolation, the purity of PMN-MDSCs and Mo-MDSCs was 96.2% and 95%, respectively (Fig. 1B). Next, we investigated the mobility of these two subsets toward HSC-CM. As shown in Fig. 1C, the ratio of PMN-MDSCs and Mo-MDSCs varied from 220:1 in control to 18:1 in HSC-CM. The results showed that the two cell subsets had different mobilities toward HSC-CM, which confirms their differences in functionality.

3.2. Gene profiling using real-time PCR chips

The mobilization of MDSC subsets was related to their expression of chemokine receptors to a great degree [13,16]. To investigate the chemokine receptors expression of PMN-MDSCs and Mo-MDSCs, a real-time RT-PCR array was used to investigate the mRNA expression levels of 84 genes that encode chemokines, chemokine receptors and associated signaling molecules (Fig. 2A). To date over 50 chemokines

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