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Transmembrane tumor necrosis factor-α promotes the recruitment of MDSCs to tumor tissue by upregulating CXCR4 expression via TNFR2

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ABSTRACT

Myeloid-derived suppressor cells (MDSCs) accumulated in tumor sites promote immune evasion. We found that TNFR deficiency-induced rejection of transplanted tumor was accompanied with markedly decreased accumulation of MDSCs. However, the mechanism(s) behind this phenomenon is not completely understood. Here, we demonstrated that TNFR deficiency did not affect the amount of MDSCs in bone marrow (BM), but decreased accumulation of Gr-1$^+$CD11b$^+$ MDSCs in the spleen and tumor tissues. The chemotaxis of T*nfr*$^{-/-}$ MDSCs was prominently decreased in response to both tumor cell culture supernatants and tumor tissue homogenates from T*nfr*$^{-/-}$ and wild-type mice, indicating an effect of TNFR signaling on chemokine receptor expression in MDSCs. We used real-time PCR to detect gene expression for several chemokine receptors in MDSCs from BM and found that CXCR4 was the most affected molecule at the transcriptional level in T*nfr*$^{-/-}$ MDSCs. Neutralizing CXCR4 in wild-type MDSCs by a specific antibody blocked their chemotactic migration. Interestingly, it was tmTNF-α, but not sTNF-α, that induced CXCR4 expression in MDSCs. This effect of tmTNF-α was totally blocked in TNFR2$^{-/-}$ but not in TNFR1$^{-/-}$ MDSCs, and partially inhibited by PDTC or SB203580, an inhibitor of NF-κB or p38 MAPK pathway, respectively. Adoptive transfer of wild-type MDSCs restored MDSCs accumulation in tumors of T*nfr*$^{-/-}$ mice, but this could be partially blocked by treatment with a CXCR4 inhibitor AMD3100. Our data suggest that tmTNF-α upregulates CXCR4 expression that promotes chemotaxis of MDSCs to tumor, and give a new insight into a novel mechanism by which tmTNF-α facilitates tumor immune evasion.

1. Introduction

It has been well known that chronic inflammation is closely associated with the development and progression of cancer, although the mechanisms by which this relationship can be regulated are poorly understood. Recently, increasing evidence demonstrates that myeloid-derived suppressor cell (MDSC) is one of links between chronic inflammation and tumor [1]. MDSCs are a heterogeneous population of immature myeloid cells including immature precursors of macrophages, granulocytes, and dendritic cells. The population is widely regarded as Gr1$^+$CD11b$^+$ cells in mouse [2] and HLA-DR$^-$CD11b$^+$CD33$^+$ cells in human being [3,4], and it has a remarkable ability to suppress innate and adaptive immune response in vitro and in vivo [1,5–9]. These cells are found not only in tumor microenvironment but also in inflamed tissue [10,11], but the mechanism behind directional migration of MDSCs from bone marrow to the site of tumor or infection has not been fully understood.

Previous studies showed that the inflammatory mediators interleukin-1β (IL-1β) [12], IL-6 [13], prostaglandin E2 (PEG_2) [14] produced by tumor cells induce the differentiation of Gr-1$^+$CD11b$^+$ MDSCs from bone marrow stem cells in tumor-bearing mice, and their accumulation in tumor tissue, which facilitates tumor progression by preventing the activation of T lymphocytes. MDSCs in tumor-bearing mice also synthesize and secrete proinflammatory mediators, such as S100A8/A9 that acts as an autocrine feedback loop and promotes the migration of MDSCs to tumor tissues. In addition, PGE_2-induced production of CXCL12 and CXCR4 regulates the accumulation of MDSCs in the microenvironment of ovarian cancer [15]. However, MDSCs can be accumulated in the absence of elevated levels of IL-1β, IL-6, PGE2, and S100A8/A9, indicating additional possible inflammatory factors responsible for MDSC accumulation.

Tumor necrosis factor-α (TNF-α) is an important pro-inflammatory cytokine, and plays a versatile role in chronic inflammatory diseases and tumors [16,17], although it was originally regarded as a serum factor inducing hemorrhagic necrosis of solid tumors in mice. TNF-α exists in

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two biologically active forms, transmembrane TNF-α (tmTNF-α) and soluble TNF-α (sTNF-α). It is first produced as a 26-kDa transmembrane protein that can be cleft by the TNF-α–converting enzyme, releasing a 17-kDa soluble molecule. Both forms of TNF-α exert biological activities via two types of receptor, TNF receptor-1 (TNFR1) and TNFR2. TNFR1 is expressed universally on almost all cell types, while TNFR2 expression is restricted to immune cells [18]. TNFR2-mediated signal pathway has been reported to be involved in MDSCs survival [19] and activity [20]. TNF-α is able to affect the leukocyte movement by virtue of regulation of chemokine expression [21,22]. For example, TNF-α acts as an autocrine or paracrine regulator of functional CXCR4 expression on ovarian cancer cells in a NF-κB-dependent manner and promotes cancer cell invasion and metastasis [23,24]. We hypothesized that TNF-α may promote the migration of MDSCs by regulating the expression of related chemokine receptors in these cells. In the present study, we demonstrated that deficiency of TNFR1 and TNFR2 reduced MDSC accumulation in tumor site, which was due to down-regulation of CXCR4 expression in MDSCs. In addition, we found that tmTNF-α but not sTNF-α, stimulated MDSCs to express CXCR4 via TNFR2 through activation of both NF-κB and p38 MAPK pathways.

2. Materials and methods

2.1. Animals and cell lines

T*nfr*1$^{-/-}$, T*nfr*2$^{-/-}$ and T*nfr*$^{-/-}$ (T*nfr*1 and T*nfr*2 double knockout) mice are on a BALB/c background and were kindly provided by Prof. Zhihai Qin (National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China). Wild-type BALB/c mice were obtained from the Center of Medical Experimental Animals of Hubei Province (Wuhan, China). All the mice were bred and housed in the specific pathogen-free barrier facility and allowed ad libitum access to food and water. The animal study was approved by the Animal Care and Use Committee of Huazhong University of Science and Technology. Mice aged 6–8 week(s) and weighing about 15–18 g were used.

A murine hepatic carcinoma cell line H22, murine breast cancer cell line 4T1 and murine macrophage RAW264.7 cells were cultured in RPMI 1640 medium (Gibico) containing 10% FBS (Sijiqing, Hangzhou, China), 100 U/ml penicillin, and 100 mg/ml streptomycin.

2.2. Tumor model and MDSC isolation

1×10^6 H22 cells were injected intraperitoneally into wild-type BALB/c mice, after one week, the cells in the ascites were harvested and washed for 3 times with phosphate buffer saline (PBS) before inoculation into wild-type and T*nfr*$^{-/-}$ mice. 1×10^6 H22 or 4T1 cells were subcutaneously inoculated into the right flank or right mammary fat pads, respectively. The tumor size was measured every 3 days with slide calliper rule. Mean tumor size was calculated by a formula of width^2 × length × 0.52 [25], in the meantime, the survival of tumor-bearing animals was recorded.

A part of animals was sacrificed at the day 18 or 30 after inoculation of H22 or 4T1, respectively. MDSCs were isolated and purified as previously described [20]. In brief, the cells were isolated from bone marrow and fractionated by Percoll density gradient centrifugation, and cells between the gradient interfaces 50% and 60% were harvested. Gr-1$^+$ cells were further purified by BD IMag Anti-Mouse Ly-6G and Ly-6C Particles-DM (BD Biosciences). The purity of the Gr-1$^+$CD11b$^+$ MDSC population was >90% as evaluated by flow cytometry.

For the stimulation, MDSCs as target cells were cocultured for 16 h with sTNF-α (Peprotech, Rocky Hill, NJ) or tmTNF-α expressed by RAW264.7 cells as effector cells at an effector/target (E/T) ratio of 10:1. tmTNF-α originated from 4 h LPS (1 μg/ml) stimulation of RAW264.7 cells followed by fixation with 4% paraformaldehyde for 30 min at room temperature (RT). For identification of tmTNF-α specific action, fixed Raw264.7 cells were pretreated with an anti-TNF-α antibody (BD Pharmingen, San Jose, CA) for 30 min to neutralize tmTNF-α prior to addition to the target cells.

2.3. Adoptive transfer of MDSCs

MDSCs were isolated from wild-type and T*nfr*$^{-/-}$ mice-bearing H22 tumor on day 12 after inoculation. 1×10^6 purified wild-type or T*nfr*$^{-/-}$ MDSCs were transferred through tail vein into T*nfr*$^{-/-}$ mice on day 4 after H22 inoculation, respectively. One day later, AMD3100 (5 mg/kg) was administered i.p. every day in a volume of 100 μl of PBS [26]. Recipients were sacrificed at the day 18 after tumor inoculation.

2.4. Flow cytometry

Single cell suspensions were prepared directly from bone marrow, peripheral blood, spleen and tumor tissue. The cells were stained with the following mouse-specific mAbs, including FITC-anti-Gr1, PE-anti-CD11b, APC-anti-CXCR4 (BioLegend, San Diego, CA), PE-anti-ly6C and ly6G, APC-cy7-anti-CD11b, APC-anti-ly6C, PE-cy7-anti-ly6G (BD, San Jose, CA). Expression of the cell surface molecules was analyzed on an LSR II flow cytometer (Becton Dickinson, San Jose, CA) using BD FACS Diva software.

2.5. Immunohistochemistry

A rat anti-mouse Ly-6G and Ly-6C (BD, San Jose, CA) was used to examine accumulation of MDSCs in the tumor tissue by the avidin/biotin complex method described previously [20]. In tumor tissue, the number of Gr-1$^+$ cells was counted in 10 fields per tumor section, two sections per mouse and five mice per group and the percentage of Gr-1$^+$ cells was calculated.

2.6. Chemotaxis assay

Chemotaxis assays were performed in 24 well plates with an 8 μm pore size transwell polycarbonate filter (BD PharMingen). The lower compartment was filled with 500 μl of culture supernatant from H22 or 4T1 cells or 10% homogenate of tumor tissue, while 200 μl of 5×10^5 purified Gr-1$^+$CD11b$^+$ MDSCs was added to the upper compartment. For neutralization, Gr-1$^+$CD11b$^+$ MDSCs were pretreated for 30 min with anti-CXCR4 antibody (2 μg/ml; Abcam) prior to addition to the upper compartment. The plates were incubated at 37 °C for 3 h. After removing cells stayed on the upper surface of the membrane using cotton swabs, the membrane with cells migrated to its lower surface was fixed in 95% ethanol at RT for 10 min, and subsequently stained with 0.1% crystal violet for 30 min. The number of migrated cells was counted in 10 randomly selected fields (×200 magnifications) per membrane. The chemotactic index was calculated as the following formula: Number of migrated MDSCs to tumor tissue homogenate or to conditioned media / number of migrated MDSCs to control media.

2.7. Quantitative real-time PCR

Total RNA was extracted from MDSCs of bone marrow and tumor tissues using Trizol (Invitrogen, China). Then 2 μg of RNA was reverse-transcribed to cDNA by the EasyScript First-Strand cDNA Synthesis SuperMix kit (Transgene, China). cDNA was amplified by real-time PCR system (Mx3000P, Stratagene) using 2 × Super Array PCR Master Mix (Transgene, China). The sequences of primer pairs for real-time PCR were as follows: CXCR4, 5′-ACCTCTACAGCAGCGTTCTCATC-3′ (forward) and 5′-GGATCCAGACGCCCACATAG-3′ (reverse); CCR2, 5′-TCCTGCCTCCACTCTACTCCCT-3′ (forward) and 5′-TGCAGCATAGTGAGCCCAGAAT-3′ (reverse); CCR5, 5′-CTGCTGCCTAAACCCTGTCAT-3′ (forward) and 5′-TGCAAAAGCGTTTGACCATGT-3′ (reverse); CCR6, 5′-GCTCCAGAACACTGACGCA-3′ (forward) and 5′-