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Granulocytic subset of myeloid derived suppressor cells in rats with mammary carcinoma

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ABSTRACT

Limited knowledge is available on myeloid derived suppressor cells (MDSCs) of rat origin. We examined the myeloid cells from peripheral blood, bone marrow and spleens of healthy and mammary tumor bearing rats employing a novel immunophenotyping strategy with CD172a, HIS48, and Rp-1 antibodies. We addressed rat granulocytes by Rp-1 positivity and used HIS48 in discrimination of two mononuclear cell subsets. An expansion of granulocyte numbers was detected in peripheral blood and spleens of mammary tumor-bearing animals. The purified granulocytes were able to impair antigen-specific helper T-cell proliferation, and therefore nominated as granulocytic MDSCs of this rat tumor model. HIS48$^+$ mononuclear cell numbers were also increased in the blood and spleens of mammary tumor bearing rats with a lower MHC class II positivity. Despite the lack of an antigen specific suppression of CD4$^+$ T cells, HIS48$^+$ monocytes resemble monocytic MDSCs with their inflammatory phenotype. Together, these results provide evidence for the existence and phenotypic characterization of a granulocytic MDSC subset in a rat model of mammary carcinoma.

1. Introduction

Myeloid derived suppressor cells (MDSCs) are activated immature cells of myeloid origin that pile up in various pathological conditions including cancer [1]. Most of the MDSC accumulation in tumor-bearing mice has been found in the spleen, peripheral blood and bone marrow tissues. In addition, peripheral blood of cancer patients were shown to contain increased numbers of MDSCs in correlation with the tumor burden [2]. One of MDSCs' main impact against anti-tumor immunity is the suppression of tumor antigen specific T cell responses.

MDSCs were previously characterized in tumor-bearing mice as Gr-1$^+$ CD11b$^+$ cells, addressing both monocytic and granulocytic subsets together [3]. The application of Ly6G and Ly6C antibodies further enabled the discrimination of different subsets of MDSCs in the course of time [4]. Even though there are no markers yet available to distinguish MDSCs from non-suppressive granulocyte or monocytes in any species, Ly6G$^+$ Ly6C^{low} subset is widely used to address the granulocytic or polymorphonuclear MDSCs (G-MDSC) of tumor bearing mice [4]. Furthermore, diverse functions were associated with these MDSC subtypes such as the high reactive oxygen species (ROS) production of G-MDSCs and high nitric oxide (NO^-) or more proliferative capacity of monocytic MDSCs [4,5]. MDSCs present in the tumor-bearing mice are mostly comprised of the G-MDSCs subset, and M-MDSCs were reported to differentiate towards G-MDSCs in tumor-bearing mice and cancer patients [6,7].

MDSCs of rat origin were first described as CD11b/c$^+$ and HIS48$^+$ cells in a T9 glioma model [8]. CD172a (SIRPα), TGF-β, iNOS or arginase expressions were also reported for rat and mice MDSCs, and NO^- was found to be responsible for the suppressive activity of glioma infiltrating rat MDSCs [9–12]. Here we employed Rp-1 antibody discrimination of granulocytic cells from monocytic ones and introduced a new classification for rat monocytes depending on HIS48 positivity. This phenotyping strategy enabled us to address an enhanced accumulation of granulocytes at mammary tumor-bearing rat spleens and blood, which are functionally suppressive representing granulocytic-MDSCs.

Abbreviations: MDSC, myeloid-derived suppressor cells; SPL, splenocytes; CIR, conalbumin immunized rat.

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2. Materials and methods

2.1. Animals, establishment of mammary tumors and conalbumin immunization

Female Sprague–Dawley rats were obtained from the Hacettepe University Experimental Animals' Breeding Unit. All animals were housed under the conventional laboratory conditions, food and water ad libitum. The Institutional Ethics Committee on the Care and Use of Laboratory Animals, Hacettepe University, Ankara, Turkey approved all protocols and the project before commencement of the studies (Approval No. 2007/68-5). All procedures were performed in accordance with the ethical standards described in the declaration of Helsinki.

N-nitroso-N-methyl urea (NMU) (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in sterile filtered physiological saline (0.9%, w/v; pH: 5.00) before injections [13]. The NMU solution was administrated intraperitoneally to 21 days old rats at a dose of 50 mg/kg and repeated three times with weekly intervals [14]. In order to generate antigen-specific T cells, 100 μg Conalbumin in Complete Freund's Adjuvant (CFA) (Sigma–Aldrich, St. Louis, MO, USA) was injected at footpads of healthy rats and boosted one 7 days in advance.

2.2. Cell isolations and cell culture

Peripheral blood (PB) was drawn with cardiac puncture during the terminal anesthesia; bone marrow cells (BM) were obtained from the femurs and single-cell suspensions of splenocytes (SPL) were acquired by passing the tissue through a 70 μm cell strainer. Erythrocytes were depleted by 1/1 ACK buffer lysis. For functional assays, cells were layered on 1.119 g/ml and 1.077 g/ml Histopaque (Sigma–Aldrich, USA) double density gradient and spinned down at 700×*g* for 30 min as indicated in product sheet. 1.119 g/ml fraction was further sorted with FACS. In vitro assays were performed for 96 h in RPMI-1640 (Lonza, Walkersville, MD, USA) supplemented with 10% FBS, 2.1 mM ultra-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-Mercaptoethanol with or without 25 μg/ml conalbumin in 37 °C humidified 5% CO_2 incubator.

2.3. Antibodies, flow cytometry, cell sorting and Giemsa staining

FITC labeled mouse anti-rat CD11b/c (OX-42); PE-labeled mouse anti-rat Granulocytes (RP-1); PerCP labeled mouse anti-rat MHC class II (OX-6); PE-labeled mouse anti-rat macrophages (HIS-36) were obtained from BD Biosciences. APC labeled mouse anti-rat CD3 (G4.18) and biotin labeled mouse anti-rat Granulocytes (HIS48) were obtained from E-bioscience. APC/Cy7 labeled Streptavidin; PE-labeled mouse anti-rat CD172a (OX-41); PE-labeled mouse anti-rat CD4 (OX-35); APC labeled anti-rat CD161 (3.2.3) were obtained from Biolegend. APC labeled mouse anti-rat CD80 (3H5) were obtained from Invitrogen.

1×10^6 cells were incubated with the antibodies for 30 min in PBS +1% FBS, then washed with PBS. Non-specific isotype-matched antibodies were included as control. Single stained samples were incorporated in automated compensation matrices. Kaluza and Flowjo software were used for analysis. Cells isolated from a total spleen, both femurs or 5 ml peripheral blood of an animal was subjected to FACS purification by Aria II flow cytometer (BD Biosciences, San Jose, CA, USA). The purity of sorted cells was over 95% as determined by post sort acquisitions. FACS purified samples were transferred to microscope slides, air-dried, and incubated with Giemsa stain for 15 min at RT.

2.4. Ag-specific proliferation

Freeze/Thawed conalbumin immunized rat (CIR) splenocytes were used as responder cells and were stained with 5 μM CFSE for 8 min at 0.1% BSA/PBS. Co-cultures of CIR-splenocytes and tumor-bearing (TB) or control (Ctr) rat myeloid cell subsets were performed at $1/2(5 \times 10^4/10^5)$ effector/responder cell ratios in the presence of 25 μg/ml conalbumin. Freshly isolated TB and Ctr myeloid subsets were included in each experiment. Some wells with only CIR splenocytes were incubated with conalbumin to get a maximum proliferation for each experiment. Proliferated $CD3^+CD4^+$ T cell percentages were determined by CFSE intensity. Proliferation ratios were obtained as the ratio of (% proliferation of co-culture samples/% proliferation of only CIR-SPL samples) to minimize inter-assay variance (Supp. Fig. 3-b). No allogeneic proliferation was observed at Conalbumin free wells with both CIR + Ctr or CIR + TB cells as negative controls in any of the experiments.

2.5. Analysis of NO^- production and cytokine levels

96-h co-culture supernatants were analyzed by colorimetric Griess reaction for NO^- levels. 100-μl sample was mixed with 100-μl Greiss reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-1-naphthylethylenediaminedihydrochloride in dH_2O). After 30-min incubation at room temperature, the absorbance was measured at 535 nm by a microplate reader (SpectraMax Plus, Molecular Devices, CA, USA). A standard curve generated by serial dilutions of 100 mM sodium nitrite was used to calculate nitrite concentrations (Softmaxpro 2.6.1, Molecular Devices, CA, USA). Rat TNF-α, IL-6, IL-10 and TGF-β1 ready to use sandwich ELISA kits (Platinum ELISA, E-bioscience, San Diego, CA, USA) were used to detect corresponding cytokines at serum samples.

2.6. Phagocytosis and reactive oxygen species (ROS) assay

Fluorescent red labeled, carboxylate-modified, polystyrene latex beads with 2 μm diameter (Sigma–Aldrich, St. Louis, MO, USA) were opsonized with normal rat serum and incubated at 37 °C for 1 h. 1×10^5 myeloid cells were mixed with 3 μl of bead/serum suspension and incubated at 37 °C for 2 h. After washing, cells were analyzed by flow cytometry for red fluorescence. Sorted myeloid cells were incubated with 3 μM Carboxy-H_2-DCF-DA (85703, Anaspec, CA, USA) for 1 h at 37 °C in serum-free RPMI media, immediately transferred on ice and fluorescence intensities were recorded by flow cytometer.

2.7. Statistical analyses

Statistical analyzes were performed with SPSS18 (IBM, Armonk, NY, USA) and Graph Pad Prism 6 (GraphPad Software, La Jolla, CA, USA). Means of control or tumor-bearing groups were compared in either parametric unpaired *T*-tests or non-parametric Mann-Whitney *U* tests depending upon the normality. Relationships were analyzed by Pearson Correlation tests.

3. Results

3.1. CD172a, HIS48, and Rp-1 staining discriminate rat granulocytes and monocyte subsets

In order to define myeloid cell subsets, all cells were initially plotted for CD11b/c and CD172a (Fig. 1A) where $CD161^+$ NK cells were found to be also positive for CD11b/c, (Supp. Fig. 1). Therefore, CD172a positivity was preferred to gate all myeloid