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Brief communication

Inhibition of lymphocyte proliferation: An ability shared by murine mesenchymal stem cells, dermal fibroblasts and chondrocytes

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ABSTRACT

It has been demonstrated that mesenchymal stem cells (MSCs) have potent immunosuppressive capacities. But it is controversial whether differentiated mature stromal cells (SCs) share the immunosuppressive capacities. A previous study examined the ability of SCs from different human tissue sites to inhibit the proliferation of lymphocytes. The results are all positive but the mechanism isn't clear, and few mouse data have been published on this topic. Using an efficient mixed cell culture assay, our in vitro data show that the anti-proliferative ability of murine MSCs on lymphocytes is shared by mature murine SCs, i.e. chondrocytes and fibroblasts. Though conflicting results have been published, our results suggest that nitric oxide and IFN-γ are critical to the immunosuppressive effect. We also demonstrate that murine MSCs cultivated in chondrogenic differentiation medium still possess the anti-proliferative capacities on lymphocytes in vitro.

1. Introduction

Besides their well-known multi-potent differentiation potential, mesenchymal stem cells (MSCs) are capable to suppress the proliferation of activated lymphocytes [1]. MSCs have been investigated as immunosuppressive agents to treat graft versus host disease (GVHD) [1,2]. They ameliorated autoimmune conditions such as experimental autoimmune encephalomyelitis (EAE) and autoimmune hepatitis in animal models [3,4]. And further, the injection of MSCs prolonged allogeneic solid organ transplantations in animal models, e.g. allogeneic islet engrafts, allogeneic hearts and allogeneic corneal grafts [5–8].

Stromal cells (SCs) are often referred to as fibroblasts, and have different functions related to their anatomic site [9]. Is this immunosuppressive effect specifically endowed to MSCs or a universal effect shared by all mature SCs across the body? Data are contradictory [9–13]. It has been reported that the chondrogenic differentiation increases antidonor immune responses to allogeneic mesenchymal stem cell transplantation [11,12]. A previous study of Jones et al., 2007 on human cells showed the anti-proliferative effect of human MSCs on lymphocytes is a fundamental property shared by all human stromal cells, but in the article there is not much mechanism study [9]. In addition, there is no equivalent mouse study article on that topic. As a result, the current research aims to investigate whether the immunosuppressive effect and the associated mechanisms are shared by MSCs and mature SCs.

In a great number of studies that investigated the mechanisms of the immunosuppressive effect of MSCs, interferon (IFN) -γ was indicated as playing an important role in its mediation. When murine MSCs were mixed with lymphocytes derived from IFN-$\gamma^{-/-}$ mice, the immunosuppressive effect was largely abrogated [14,15]. Many studies suggested that the mechanisms involve a negative feedback loop. The proliferation of lymphocytes produces a large amount of pro-inflammatory cytokines, which activate MSCs. After the activation, MSCs express immunosuppressive molecules including programmed cell death ligand 1 (PD-L1), prostaglandin E2 (PGE2), indoleamine-2,3-dioxygenase (IDO) and nitric oxide (NO), which inhibit the proliferation of lymphocytes. PD-L1, PGE2 and IDO were reported to be associated with human MSCs [13,16–18]. PD-L1, IDO, and NO were associated with murine MSCs [14,19–22]. Species difference of the mechanisms was also reported between human and murine MSCs [22].

The current study aims to investigate whether the immunosuppressive effect of murine MSCs is shared by primary SCs and SCs differentiated from murine MSCs, and to clarify the mechanisms of the immunosuppressive effect.

Abbreviations: imCCs, immature costal chondrocytes; MBP, myelin basic protein; PαS cells, PDGFRα$^+$Sca-1$^+$ cells; SCs, stromal cells

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2. Materials and methods

2.1. Animals

Tg4 T cell receptor (TCR) transgenic mice expressing Vβ8.2 TCR specific for peptide myelin basic protein (MBP) Ac1-9 [4K] (AcASQKRPSQR) in the context of I−A^u, which were described previously, were bred in-house [23,24]. C57BL/6 (H-2^b) mice were purchased from Harlan Laboratories. The animal experiments comply with Guangdong Laboratory Animal Regulations. The experiment protocol was approved by the Animal Welfare and Ethics Committee of Guanhao Biotech Co., Ltd. The mice were sacrificed by cervical dislocation before any tissues or cells were obtained from them, with the exception that the newborn mice were sacrificed by decapitation under general anesthesia with isoflurane.

2.2. Cell isolation and culture

Platelet derived growth factor receptor α (PDGFRα) and stem cell antigen 1 (Sca-1) have recently been identified as selective markers of murine MSC. Murine MSCs isolated in this way is named as PDGFRα$^+$Sca-1$^+$ (PαS) cells [25]. Mouse femurs and tibias from 6 to 8 C57BL/6 mice of around 5 weeks age were dissected, crushed and minced. The minced bone fragments were washed once in HBSS (Gibco) supplemented with 2% FBS (Invitrogen), 10 mM HEPES buffer, and 1% P/S (Penicillin/Streptomycin) to remove the hematopoietic cells, then incubated for 1 h at 37 °C in 0.2% collagenase I (Wako) in DMEM (Sigma) containing 10 mM HEPES and 1% P/S (100 μg/ml, Invitrogen). The derived cell suspension was filtered through a cell strainer (Falcon) and collected by centrifugation. The cell pellet was resuspended for 5 s in 1 ml water to burst red blood cells, after which 1 ml of 2 × PBS containing 4% FBS was added. The cells were washed and resuspended in HBSS$^+$. Ter-119-PE (eBioscience, 12-5921-81, use 1:200), CD45-PE (eBioscience, 12–0451-81, use 1:200), Sca-1-FITC (eBioscience, 11-5981-81, use 1:200) and PDGFRα-APC (eBioscience, 17–1401-81, use 1:125) were used for staining and sorting. Freshly prepared propidium iodide (PI, 2.5 μg/ml final concentration) was added as a live/dead marker. A BD Influx machine was used for cell sorting. According to the isotype control signal, live cells positive for PDGFRα and Sca-1 but negative for CD45 and TER119 were sorted and collected (Fig. 1). Sorted cells were initially seeded at 2000 cells/cm^2 and maintained in αMEM (Gibco) containing 10% FBS, 1% P/S, 10 mM HEPE, in an atmosphere of 5% (v/v) CO_2 in air. Medium was changed twice a week. When 80% confluent, PαS cells were detached with 0.05% trypsin-EDTA (Invitrogen Life Technologies) at 37 °C for 5 min and passaged at a 1 to 2 ratio. PαS cells from passage 4–5 were used for analyses. Cells were routinely tested for their differentiation capacities.

Immature costal chondrocytes (imCCs) were isolated as previously described [26]. Briefly, rib cages dissected from a litter (4–9) of newborn C57BL/6 mice (5–7 days old) were incubated for one hour in 0.3% collagenase IV (Lorne Laboratories) in DMEM and then shaken in PBS to remove the soft tissues, followed by digestion in 0.05% collagenase solution overnight in an incubator. The derived imCC suspension was collected and seeded at 6 × 10^4/cm^2 in 6 well plates. imCCs from passage 2 were used for analyses. imCCs were characterized by their expression of collagen type II (in the supplement data).

Skin fibroblasts were isolated as previously described [27]. Briefly, the mouse skin from the abdomen and lower back was cut and removed from C57BL/6 mice of approximately 5 weeks of age, placed into dispase solution (1 mg/ml Stem Cell Technology, 07923) with dermis side touching the solution and incubated overnight at 4 °C. The dermis was then separated and incubated in collagenase I (Sigma, C0130) solution (7.5 mg/ml, 75 mg collagenase I in 10 ml DMEM medium without FBS) for 1 h at 37 °C in an atmosphere of 5% (v/v) CO_2 in air. The derived cell suspension was collected, washed and filtered through a 70 μm sterile filter (BD Falcon, 352,350). Around 500,000 cells were derived from each mouse. Cells from one mouse were seeded into one well of a plate. All SCs were maintained and cultured as outlined above for PαS cells.

L cells (NCTC clone 929) were purchased from ATCC and cultivated for at least one passage and stored over liquid nitrogen at 1 million cells/ml in culture medium until use. L cells were recovered and cultivated for at least one passage for analyses. The maintenance and culture condition was the same as SCs.

2.3. Surface marker analysis

Surface markers were analyzed using an MSC Marker Antibody Panel (R&D systems, SC018), containing rat anti-mouse CD29, CD73, CD105, CD106, Sca-1, CD11b, CD45, CD44, IgG2A isotype control and IgG2B isotype control antibodies. Cells were prepared and stained following the instructions provided in the kit. Samples were processed using a FACSCalibur flow cytometer (BD Bioscience) and analyzed with FlowJo Software (Treestar).

2.4. Adipogenic differentiation and osteogenic differentiation

Adipogenic differentiation and osteogenic differentiation capacities of cells were tested as described [28].

2.5. Immunosuppression assay (mixed cell culture)

Splenocytes were freshly isolated from the spleens of Tg4 mice. Briefly, the spleen was placed on and mashed through a cell strainer. Red blood cells were lysed by incubating in lysis buffer (R7757, Sigma) for 4 min. The rest of the cells were washed and resuspended in RPMI complete medium consisting of RPMI 1640 (Lonza) supplemented with 10% (v/v) FBS, 1% (v/v) Glutamax (1 ×; Invitrogen), 1% P/S, 20 mM HEPES buffer and 50 μM 2mercapto-ethanol (Gibco). Every 10^7 splenocytes were resuspended in 1 ml PBS containing 1 μM carboxyfluorescein-succinimidyl-ester (CFSE) (eBioscience) for 8 min at 37 °C in an atmosphere of 5% (v/v) CO_2 in air, followed by a further incubation period of 10 min with the addition of 1 ml FBS to quench the reaction. Splenocytes were washed and resuspended at 10^6 cells/ml and put on ice. Peptide MBP Ac1-9 [4K] (AcASQKRPSQR) were custom-synthesized by GL Biochem Shanghai, and diluted to 20 μg/ml for use. Effector cells (PαS cells, fibroblasts, imCCs or L cells) were trypsinized and collected from culture, washed and resuspended in RPMI complete medium at 2 × 10^5 cells/ml. In each culture well of a 96 well plate, 50 μl peptide (20 μg/ml), 100 μl CFSE stained splenocytes (10^6 cells/ml) and 50 μl effector cells (2 × 10^5 cells/ml) were added and cultivated for three days at a 1:10 (effector cells: responder cells) ratio. The cell concentration was adjusted for the test of other ratios as indicated in the results. The related inhibitors, the neutralizing antibodies and the isotype control antibodies were suspended in the peptide solution as indicated, which includes L-NMMA (Sigma M7033), anti-IFN-γ (Bioxcell, clone XMG1.2), rat IgG1 isotype control (Bioxcell, clone HPRN). After three days in culture, cells were collected and then stained with anti-mouse CD4-APC (eBioscience 17-0041-81) in FACS buffer for 30 min at 4 °C. Freshly prepared PI was added as a live/dead marker (2.5 μg/ml final concentration). The fluorescence of cells was then measured using a FACS-Calibur flow cytometer (BD) and analyzed using FlowJo (Tree Star) software.

2.6. Greiss assay

NO production was measured as nitrite in the supernatant using a Greiss reagent G4410 (Sigma) and a nitrite standard as described [29].

2.7. Chondrogenic differentiation culture

For the differentiation in monolayer culture, 5 × 10^5 PαS cells were