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# The research on the immuno-modulatory defect of Mesenchymal Stem Cell from Chronic Myeloid Leukemia patients

Zhu Xishan<sup>\*</sup>, An Guangyu, Song Yuguang and Zhang Hongmei

## Abstract

Overwhelming evidence from leukemia research has shown that the clonal population of neoplastic cells exhibits marked heterogeneity with respect to proliferation and differentiation. There are rare stem cells within the leukemic population that possess extensive proliferation and self-renewal capacity not found in the majority of the leukemic cells. These leukemic stem cells are necessary and sufficient to maintain the leukemia. While the hematopoietic stem cell (HSC) origin of CML was first suggested over 30 years ago, recently CML-initiating cells beyond HSCs are also being investigated. We have previously isolated fetal liver kinase-1-positive (Flk1<sup>+</sup>) cells carrying the BCR/ABL fusion gene from the bone marrow of Philadelphia chromosome-positive (Ph<sup>+</sup>) patients with hemangioblast property. Here, we showed that CML patient-derived Flk1<sup>+</sup>CD31<sup>-</sup>CD34<sup>-</sup>MSCs had normal morphology, phenotype and karyotype but appeared impaired in immuno-modulatory function. The capacity of patient Flk1<sup>+</sup>CD31<sup>-</sup>CD34<sup>-</sup>MSCs to inhibit T lymphocyte activation and proliferation was impaired in vitro. CML patient-derived MSCs have impaired immuno-modulatory functions, suggesting that the dysregulation of hematopoiesis and immune response may originate from MSCs rather than HSCs. MSCs might be a potential target for developing efficacious cures for CML.

## Introduction

Chronic Myeloid Leukemia (CML) is a malignant myelo-proliferative disorder originating from a pluripotent stem cell that expresses the BCR/ABL oncogene and is characterized by abnormal release of the expanded, malignant stem cell clone from the bone marrow into the circulation[1,2]. The discovery of the Philadelphia chromosome followed by identification of its BCR/ABL fusion gene product and the resultant constitutively active P210 BCR/ABL tyrosine kinase prompted the unravelling of the molecular pathogenesis of CML. However, regardless of greatly reduced mortality rates with BCR/ABL targeted therapy, most patients harbor quiescent CML stem cells that may be a reservoir for disease progression to blast crisis. Under steady-state conditions, these cancer stem cells are localized in a microenvironment known as the stem cell “niche”, where they are maintained in an undifferentiated and

quiescent state. These niches are critical for regulating the self-renewal and cell fate decisions, yet why and how these cells are recruited to affect leukemia progression are not well known.

Local secretion of proteases has been implicated in this tumor-stroma crosstalk. Matrix metalloproteinase-9 (MMP-9) is one of the proteases that has the preferential ability to degrade denatured collagens (gelatin) and collagen type IV, the 2 main components of basement membranes and therefore plays a critical role in tumor progression and metastasis[3,4]. Previous studies have demonstrated localization of MMP-9 on the plasma membrane of various tumor cells[5-7] and recently, the role of MMP-9 in CML pathogenesis has become a focus of attention[8-11]. But the research is mainly focusing on the MMP-9 inducing molecules[12-14] or the effect of MMP-9 inhibitors[15]. However, it has become clear that the role of MMP-9 in CML is not limited to simple extracellular matrix (ECM) degradation[16]. The regulation of MMP-9 is found to be involved in multiple pathways induced by different kinds of cytokines in different cell types and illness[17,18].

\* Correspondence: mountain.red@163.com  
Institute of Medical Oncology, Beijing Shijitan Hospital, Capital Medical University, Beijing, 100038, P.R. China

Therefore, it is necessary to verify a specific MMP-9 induced pathway in a given cell type.

Recent research[6,10,4] showed that T lymphocytes isolated from CML patients suppressed the forming of CFU-GM (colony forming unit-granulocyte and macrophage) and CFU-E (colony forming unit-erythroid) and furthermore this kind of inhibition could be blocked by CsA(cyclosporine A)[19,20];besides, the rate of the forming of the HSCs (hematopoietic stem cells) increased with the removal of T lymphocytes. Therefore, immunological inhibitors like CsA. and ATG (anti-human thymocyte globulin) was helpful for CML patients and was widely used in clinic therapy[21-23]. All these evidence indicated there might existed immunological abnormalities, that is, the T lymphocytes in CML might existed in a unusually activated state leading to self injury.

Besides HSCs, there also existed another kind of stem cells called MSCs (Mesenchymal Stem Cells), they could differentiated into stroma cells and acted as the “niche” in the micro-environment[24]. MSCs also had the immunological regulation ability and were believed to be the “immune protection site” in the cells environment. So, we believed that MSCs might play important role in the pathogenesis of CML, but there was no article examined the immunological function of MSCs.

Previous studies[19,21] from our laboratory have identified Flk1<sup>+</sup> (fetal liver kinase-1 positive) CD31<sup>-</sup>CD34<sup>-</sup> cells carrying the BCR/ABL fusion gene from the bone marrow of Philadelphia chromosome positive (Ph<sup>+</sup>) patients with CML and found that these cells could differentiate into malignant blood cells and phenotypically defined endothelial cells at the single-cell level, suggesting these cells have the properties of hemangioblasts.

The main purpose of our article was to examine the immune characteristics of Flk1<sup>+</sup>CD31<sup>-</sup>CD34<sup>-</sup> MSC in CML and analyse if there existed abnormalities comparing with the healthy donors.

## Patients, materials, and methods

### Patient samples

20 patients with newly diagnosed CML (12 male and 8 female, aged 17-63 years) were recruited in this study (table 1). All were Ph<sup>+</sup> patients with CML in chronic phase as revealed by bone marrow histology and cytogenetic analysis. The immunophenotypes of thawed cells were quite variable. None was treated with hydroxyurea or interferon before. The control samples were from 20 healthy donors (12 male and 8 female, aged 21-60 years). Bone marrow samples were collected after obtaining informed consent according to procedures approved by the Ethics Committee at the 309<sup>th</sup> Hospital of Peoples Liberation Army.

### Cell preparations and culture

Isolation and culture of bone marrow-derived CML hemangioblasts were performed as described previously with some modifications[19,21]. Briefly, mononuclear cells were separated by a Ficoll-Paque gradient centrifugation (specific gravity 1.077 g/mL; Nycomed Pharma AS, Oslo, Norway) and the sorted cells were plated at concentration of 1 cell/well by limiting dilution in a total of 96 × 10 wells coated with fibronectin (Sigma, St Louis, MO) and collagen (Sigma) for each patient. Culture medium was Dulbecco modified Eagle medium and Ham F12 medium (DF12) containing 40% MCDB-201 medium complete with trace elements (MCDB) (Sigma), 2% fetal calf serum (FCS; Gibco Life Technologies,

**Table 1 The general conditions of the patients**

Patient	Age	Sex	Diagonosis	Diagnosis time	Ph chromosome	Immunosuppressive therapy
1	84	F	CML	Aug-04	positive	yes
2	54	M	CML	Jun-87	positive	yes
3	56	M	CML	May-99	positive	yes
4	49	M	CML	Feb-87	positive	yes
5	66	M	CML	Aug-04	positive	yes
6	40	F	CML	Feb-05	positive	No
7	50	F	CML	Sep-04	positive	No
8	76	F	CML	Aug-04	positive	No
9	64	F	CML	Dec-05	positive	No
10	55	M	CML	Apr-00	positive	yes
11	49	M	CML	Feb-05	positive	No
12	51	M	CML	Jun-01	positive	yes
13	40	F	CML	Dec-05	positive	No
14	43	F	CML	Dec-05	positive	No
15	60	M	CML	Nov-05	positive	No

M: male; F:female;

Paisley, United Kingdom), 1 × insulin transferrin selenium (Gibco Life Technologies), 10<sup>-9</sup> M dexamethasone (Sigma), 10<sup>-4</sup> M ascorbic acid 2-phosphate (Sigma), 20 ng/mL interleukin-6 (Sigma), 10 ng/mL epidermal growth factor (Sigma), 10 ng/mL platelet-derived growth factor BB (Sigma), 50 ng/mL fetal liver tyrosine kinase 3 (Flt-3) ligand (Sigma), 30 ng/mL bone morphogenetic protein-4 (Sigma), 100 U/mL penicillin and 100 ug/mL streptomycin (Gibco Life Technologies) at 37°C and a 5% CO<sub>2</sub> humidified atmosphere. Culture media were changed every 4 to 6 days.

#### **FISH analysis**

We cultured BCR/ABL<sup>+</sup> hemangioblasts from male CML patients (n = 12) and Y chromosome was detected using a probe (CEP Y Spectrum Red; Vysis, Downers Grove, IL) according to the manufacturer's instructions. Normal cells showed 2 red abl signals and 2 green bcr signals. BCR/ABL<sup>+</sup> hemangioblasts showed a single red and a single green signal representing normal abl and bcr genes and the yellow signal representing fusion of abl and bcr genes.

#### **Fluorescence activated cell sorting (FACS)**

For immunophenotype analysis, expanded clonal cells were stained with antibodies against Flk1, CD29, CD31, CD34, CD44, CD45, CD105, (all from Becton Dickinson Immunocytometry Systems, Mountain View, CA). For intracellular antigen detection, cells were first fixed in 2% paraformaldehyde (Sigma) for 15 minutes at 4°C and permeabilized with 0.1% saponin (Sigma) for 1 hour at room temperature. Cells were washed and labeled with fluorescein isothiocyanate (FITC) conjugated secondary goat antimouse, goat antirabbit, or sheep antigoat antibodies (Sigma), then washed and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

#### **Mitogen proliferative assays**

In mitogen proliferative assays, triplicate wells containing responder 1 × 10<sup>5</sup> MNCs were cultured with 50 g/ml PHA (Roche, USA) in a total volume of 0.1 ml medium at 37°C in 5% CO<sub>2</sub>, and Flk1+CD31-CD34- MSCs were added on day 0. Irradiated Flk1+CD31-CD34- MSCs (30 Gy) were cocultured with the MNCs at different ratios (MSCs to MNCs = 1:2, 1:10, 1:100). Control wells contained only MNCs. Cultures were pulsed with 1 Ci/well [3H]-TdR (Shanghai Nucleus Research Institute, China) on day 2, and harvested 18 h later with a Tomtec (Wallac Inc., Gaithersburg, MD) automated harvester. Thymidine uptake was quantified using a liquid scintillation and luminescence counter (Wallac TRILUX).

#### **Mixed lymphocyte reaction assays (MLR)**

Blood mononuclear cells (MNCs) were prepared from normal volunteers' peripheral blood by Ficoll-Paque

density gradient centrifugation and suspended in RPMI 1640 medium supplemented with 10% (vol/vol) FCS, 2 mM l-glutamine, 0.1 mM nonessential amino acids (Life Technologies, Grand Island, NY), 1 mM sodium pyruvate, 100 U/mL penicillin,

#### **Effect of MSCs on T cell cycle**

MSCs and MNCs were prepared as described before. T cells, stimulated with PHA (50 g/ml, final concentration) stimulation for 3 days, were cultured alone or cocultured with MSCs (derived from normal and MDS patient) or 3T3 cell line, then harvested and quantified. One million T cells were fixed with 70% cold ethanol at 4°C for 30 min, washed with PBS twice, and stained with 50 g/ml PI (Sigma, USA) at room temperature for 5 min. Data were analyzed with Mod-FIT software.

#### **Effect of MSCs on T cell activation**

MSCs and MNCs were prepared as described before, respectively. T cells were cultured alone or cocultured with prepared MSCs and stimulated with PHA (50 g/ml final concentration). The expression of CD25 (BD, USA) and CD69 (BD, USA) was detected by flow cytometry at 24 h, and CD44 (BD, USA) was detected at 72 h.

#### **Effect of MSCs on T cell apoptosis**

MSCs and MNCs were prepared as described before. T cells were cultured alone or cocultured with MSCs with PHA (50 g/ml final concentration) stimulation for 3 days, then harvested and quantified, stained with Annexin-V kit (BD, USA), and analyzed by flow cytometry (FACS Vantage).

#### **RNA-i experiments**

The si-RNA sequence targeting human MMP-9 (from mRNA sequence; Invitrogen online) corresponds to the coding region 377-403 relative to the first nucleotide of the start codon (target = 5'-AAC ATC ACC TAT TGG ATC CAA ACT AC-3'). Computer analysis using the software developed by Ambion Inc. confirmed this sequence to be a good target. si-RNAs were 21 nucleotides long with symmetric 2-nucleotide 3'overhangs composed of 2'-deoxythymidine to enhance nuclease resistance. The si-RNAs were synthesized chemically and high pressure liquid chromatography purified (Gen-set, Paris, France). Sense si-RNA sequence was 5'-CAU CAC CUA UUG GAU CCA AdT dT-3'. Antisense si-RNA was 5'-UUG GAU CCA AUA GGU GAU GdT dT-3'. For annealing of si-RNAs, mixture of complementary single stranded RNAs (at equimolar concentration) was incubated in annealing buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, and 10 mM MgCl<sub>2</sub>) for 2 minutes at 95°C followed by a slow cooling to room temperature (at least 25°C) and then proceeded to

storage temperature of 4°C. Before transfection, cells cultured at 50% confluence in 6-well plates (10 cm<sup>2</sup>) were washed two times with OPTIMEM 1 (Invitrogen) without FCS and incubated in 1.5 ml of this medium without FCS for 1 hour. Then, cells were transfected with MMP-9-RNA duplex formulated into Mirus *TransIT*-TKO transfection reagent (Mirus Corp, Interchim, France) according to the manufacturer's instructions. Unless otherwise described, transfection used 20 nM RNA duplex in 0.5 ml of transfection medium OPTIMEM 1 without FCS per 5 × 10<sup>5</sup> cells for 6 hours and then the medium volume was adjusted to 1.5 ml per well with RPMI 2% FCS. Silencer<sup>TM</sup> negative control 1 si-RNA (Ambion Inc.) was used as negative control under similar conditions (20 nM). The efficiency of silencing is 80% in our assay.

#### Enzyme-linked Immunosorbent Assays

This was carried out according to the manufacturer's recommendations (Oncogene Research Products). Results were compared with those obtained with serially diluted solutions of commercially purified controls. Anti-human cytokine antibodies (R&D Systems, Minneapolis, MN) was added at 0.4 µg/ml in 0.05 M bicarbonate buffer (pH 9.3) to 96-well, U-bottom, polyvinyl microplates (Becton Dickinson and Co., Oxnard, CA) and the cell number was 1 × 10<sup>5</sup>/100 µl. After incubation overnight at 4°C, the plates were washed and blocked with 1% gelatin for 1 hour. Samples (50 µl) or standard protein diluted in 0.5% gelatin were added to the wells. After incubation for 1 hour at 37°C, the plates were washed again, and 50 ng/ml biotinylated antimouse antibody (R&D Systems) was added for 1 hour at 37°C. The plates were then washed and incubated with streptavidin-HRP for 1 hour at 37°C. After washing, 0.2 mM ABTS (Sigma Chemical Co.) was added to the wells, and after 10 minutes, the colorimetric reaction was measured at 405 nm with an ELISA reader VERSAmax (Molecular Devices, Sunnyvale, CA).

#### Western blot

CML hemangioblasts were harvested at specific times after treatment with regents as indicated in each experiment. Cells were mixed with loading buffer and subject to electrophoresis. After electrophoresis, proteins were transferred to polyvinyl difluoride membranes (Pall Filtron) using a semidry blotting apparatus (Pharmacia) and probed with mouse mAbs, followed by incubation with peroxidase-labeled secondary antibodies. Detection was performed by the use of a chemiluminescence system (Amersham) according to the manufacturer's instructions. Then membrane was striped with elution buffer and re-probed with antibodies against the nonphosphorylated protein as a measure of loading control.

Controls for the immunoprecipitation used the same procedure, except agarose beads contained only mouse IgG.

#### Statistics

Statistical analysis was performed with the statistical SPSS 13.0 software. The paired-sample t-test was used to test the probability of significant differences between samples. Statistical significance was defined as  $p < 0.05$ .

#### Results

##### The biological characteristics of CML hemangioblasts

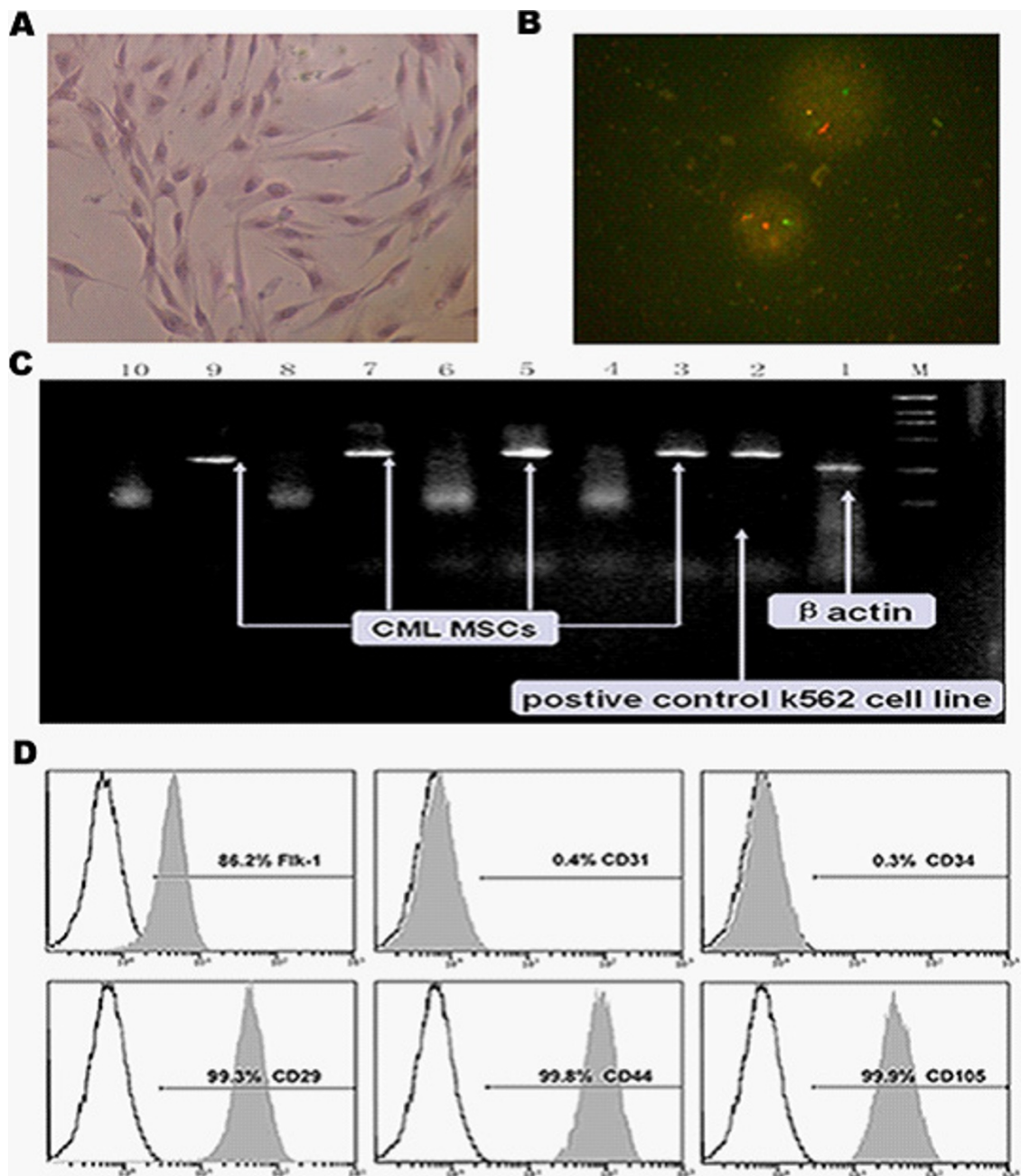
To establish the characteristics of CML hemangioblasts, we first examined the morphology, phenotype and growth patterns of them respectively. Results showed that they persistently displayed fibroblast-like morphology (Figure 1A) and CML specific BCR/ABL oncogene was observed by FISH analysis (Figure 1B) and PCR (Figure 1C) in CML hemangioblasts. Isotype analysis indicated they were all persistently negative for CD34 and CD31 but positive for Flk1, CD29, CD44 and CD105 (Figure 1D).

##### Immunomodulatory decrease on T cell proliferation

To analyse immunomodulatory effects on T cell proliferation, irradiated MSCs were added to mitogen-stimulated T cell proliferation reactions and mixed lymphocyte reactions (MLR). A previous study showed that MSCs from healthy volunteers could obviously inhibit the proliferation of T cells not only stimulated with mitogen but also in MLR. Additionally, this inhibitory effect occurred in a dose-dependent manner. In mitogen-stimulated T cell proliferation assays, the proliferation of T cells at 1:2 ratio (MSCs to MNCs) was significantly inhibited to about 1% with normal MSCs, but proliferation at the same ratio was inhibited only to about 37% with CML-derived MSCs (compared with co-culture system of normal MSCs,  $p < 0.05$ ). Similarly, inhibitory rates were impaired at 1:10 ratio (MSCs to MNCs) in CML-derived MSCs (compared with co-culture system of normal MSCs,  $p < 0.05$ ). Also the inhibitory effect was dose dependent in CML-derived MSCs. (Figure 2A). In MLR, a similar impaired inhibitory effect with MDS-derived MSCs was observed. (Figure 2B)

##### Immunomodulatory attenuation of MSCs on T cell cycle

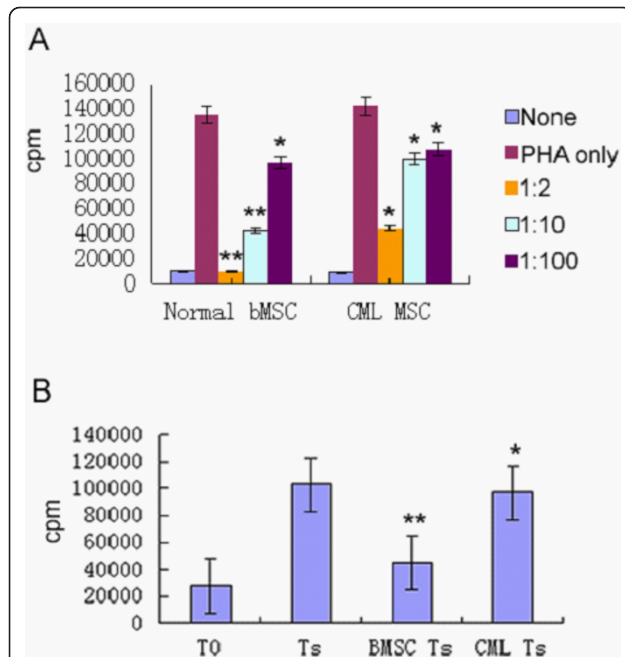
A previous study showed that MSCs could silence T cells in G<sub>0</sub>/G<sub>1</sub> phase, which might be one of the possible mechanisms of MSC's inhibitory effect on T cells. When the inhibitory effect of CML-derived MSC on T cell proliferation was impaired, the related inhibitory effect on cell cycle was analyzed. In a PHA-stimulating system without MSC co-culture, there were 67.3 ± 3.7% and 28.4 ± 2.9% T cells in G<sub>0</sub>/G<sub>1</sub> phase and S phase, respectively. When normal MSCs were present in co-



**Figure 1 Biological characteristics of the CML MSCs.** (A) The morphology of hemangioblasts from CML (Magnification  $\times 200$ ). (B) BCR/ABL fusion gene was detected by FISH (yellow signal is the positive one) in CML hemangioblasts from male patients. (C) BCR/ABL fusion gene was detected by RT-PCR (line 4, 6, 8, 10 correspond to non-special amplification). (D) Isotype analysis showed they were all persistently negative for CD34 and CD31 but positive for Flk1, CD29, CD44 and CD105.

culture, the percentages of T cells in G0/G1 phase and S phase were  $94.0 \pm 1.9\%$  and  $3.1 \pm 1.9\%$ , respectively (compared with PHA stimulated T cells,  $p < 0.05$ ). MSCs from healthy volunteers could have most of their T cells in G0/G1 phase with fewer cells entering S phase. However, T cells in G0/G1 phase and S phase remained  $74.5 \pm 1.2\%$  and  $22.1 \pm 2.4\%$  in the co-culture

system of CML-derived MSCs (compared with co-culture system of normal MSCs,  $p < 0.05$ ). This result was confirmed by five independent tests (Figure 3). The 3T3 cell line was used as a control, and no effects on cell cycle were observed ( $70.3 \pm 3.1\%$  in G0/G1 and  $27.3 \pm 5.1\%$  in S, respectively (compared with PHA stimulated T cells,  $p > 0.05$ ). These results suggested that the



**Figure 2 The effects of Flk-1+CD31-CD34- MSCs on T lymphocyte proliferation.** (A) The effects of Flk-1+CD31-CD34- MSCs on T lymphocyte proliferation in mitogen proliferative assays. There are three groups, including nonstimulated T cells (none), PHA-stimulated T cells (Ts) and PHA-stimulated T cells cocultured with MSC at different ratios (MSC to T cell = 1:2, 1:10, :100). Data are shown as means  $\pm$  S.D. of three independent experiments (\* $p < 0.05$ ,\*\* $p < 0.005$  vs. Ts). (B) The effects of Flk-1+CD31-CD34- MSCs on T lymphocyte proliferation in MLR. Flk-1+CD31-CD34- MSCs at 1:10 ratios (irradiated MSCs to T cells); there are four groups, including nonstimulated responder T cells (T0), irradiated stimulator cells plus responder T cells; normalMSC plusMLR (BMSC Ts), CML-derived MSC plus MLR (CML Ts). Data are shown as means  $\pm$  S.D. of three independent experiments (\* $p \geq 0.05$ ,\*\* $p = 0.001$  vs. Ts)

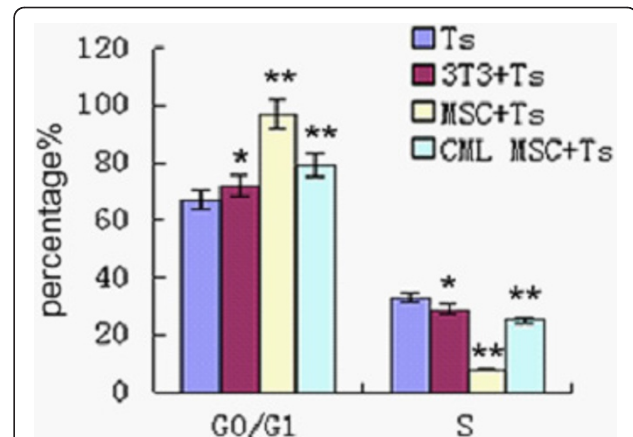
inhibitory effect of CML-derived MSCs on cell cycle arrest was also impaired.

#### Impaired effects of MSCs on T cell activation

MSCs from CML patients could significantly inhibit activation of T cells. The percentage of CD25, CD69 and CD44 in PHA induced T lymphocyte was  $12.3 \pm 3.5\%$ ,  $34.5 \pm 5.9\%$  and  $29.4 \pm 7.0\%$  respectively. But they were  $3.1 \pm 2.3\%$ ,  $6.4 \pm 3.2\%$  and  $2.1 \pm 1.7\%$  when co-cultured with normal hemangioblasts and, when co-cultured with CML hemangioblasts, they were  $5.4 \pm 2.3\%$ ,  $31.5 \pm 6.8\%$  and  $24.5 \pm 3.6\%$  respectively. All data presented here were confirmed by repeated tests (Figure 4). These results also indicated that MSCs from CML patients were impaired in their immuno-modulatory function.

#### Dampening effect of MSCs on T cell apoptosis

In apoptosis tests, we have observed that MSCs from healthy volunteers could significantly dampen the effect

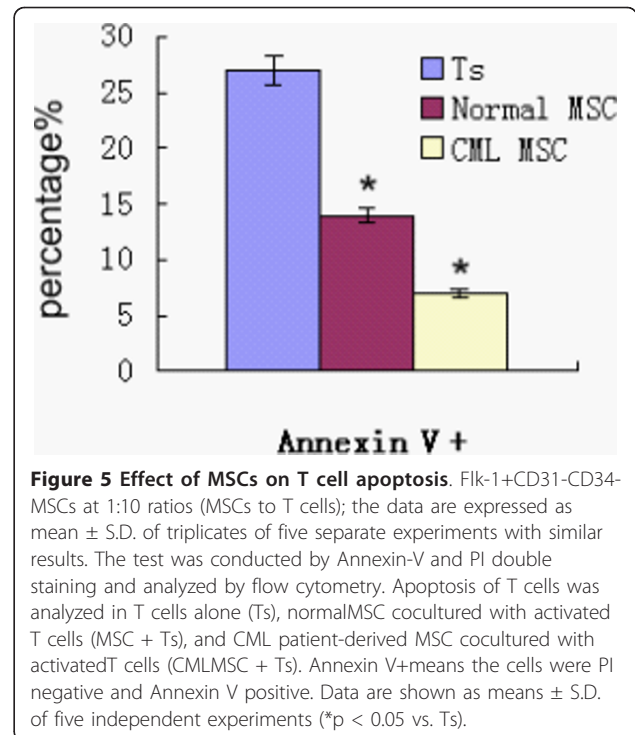
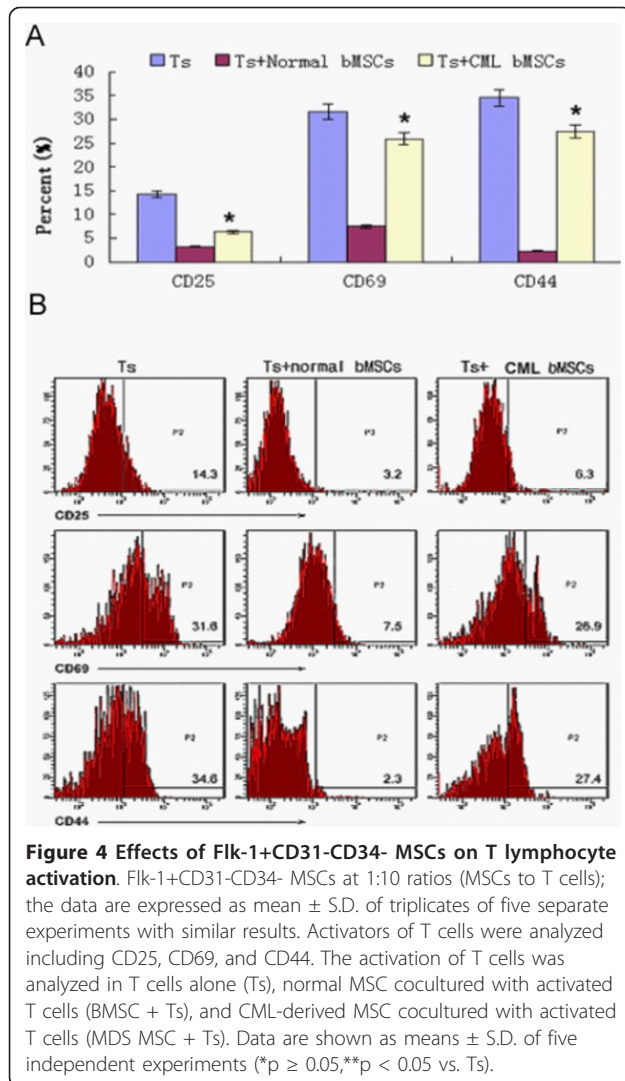


**Figure 3 Effects of MSCs on T cell cycle.** Flk-1+CD31-CD34- MSCs or 3T3 at 1:10 ratios (MSCs to T cells); the data are expressed as mean  $\pm$  S.D. Of triplicates of five separate experiments with similar results. Cell cycles of PHA-stimulated T cells were analyzed in T cells alone (Ts), cocultured with MSCs (MSC + Ts) group and MSCs derived from CML patient group (CML MSC + Ts). 3T3 cell line was used as control (3T3 + Ts). Data are shown as means  $\pm$  S.D. of five independent experiments (\* $p \geq 0.05$ ,\*\* $p < 0.05$  vs. Ts)

of activation-induced apoptosis of T cells. Following stimulation with PHA for 3 days, the rate of apoptosis of T cells was  $23.37 \pm 2.71\%$ . When PHA-stimulated T cells were cocultured with MSCs obtained from healthy volunteers, the percentage of apoptotic T cells decreased to  $14.1 \pm 0.65\%$  (compared with PHA stimulated T cells,  $p < 0.05$ ). In the same condition, the apoptosis percentage of T cells co-cultured with MDS-derived MSCs further decreased to  $8.36 \pm 1.31\%$  (compared with co-culture system of normal MSCs,  $p < 0.05$ ). We repeated the experiment five times to confirm this result (Figure 5). These results suggested the dampening effect of CML-derived MSCs on activation-induced T apoptosis seemed to be enhanced.

#### Efficient extinction of MMP-9 expression in HT1080 cells by RNAi strategy and the concomitantly upregulation of s-ICAM-1

We used an RNAi method to target MMP-9 in the CML MSC and the constructs we designed encoded an RNA that targets the MMP-9 mRNA. The target sequence had no homology with other members of the MMP family. The ds-RNA and Silencer negative control si-RNA (snc) were each tested for their ability to suppress MMP-9 specifically. We first assessed whether RNAi was dose and time-dependent. A MMP-9 dependent ds-RNA-mediated inhibition was observed in a dose and time dependent manner (Figure 6A). The time-course assay performed with 20 nM ds-RNA-transfected CML MSC showed that the induced MMP-9 silencing could be maintained for at least 3 days (Figure



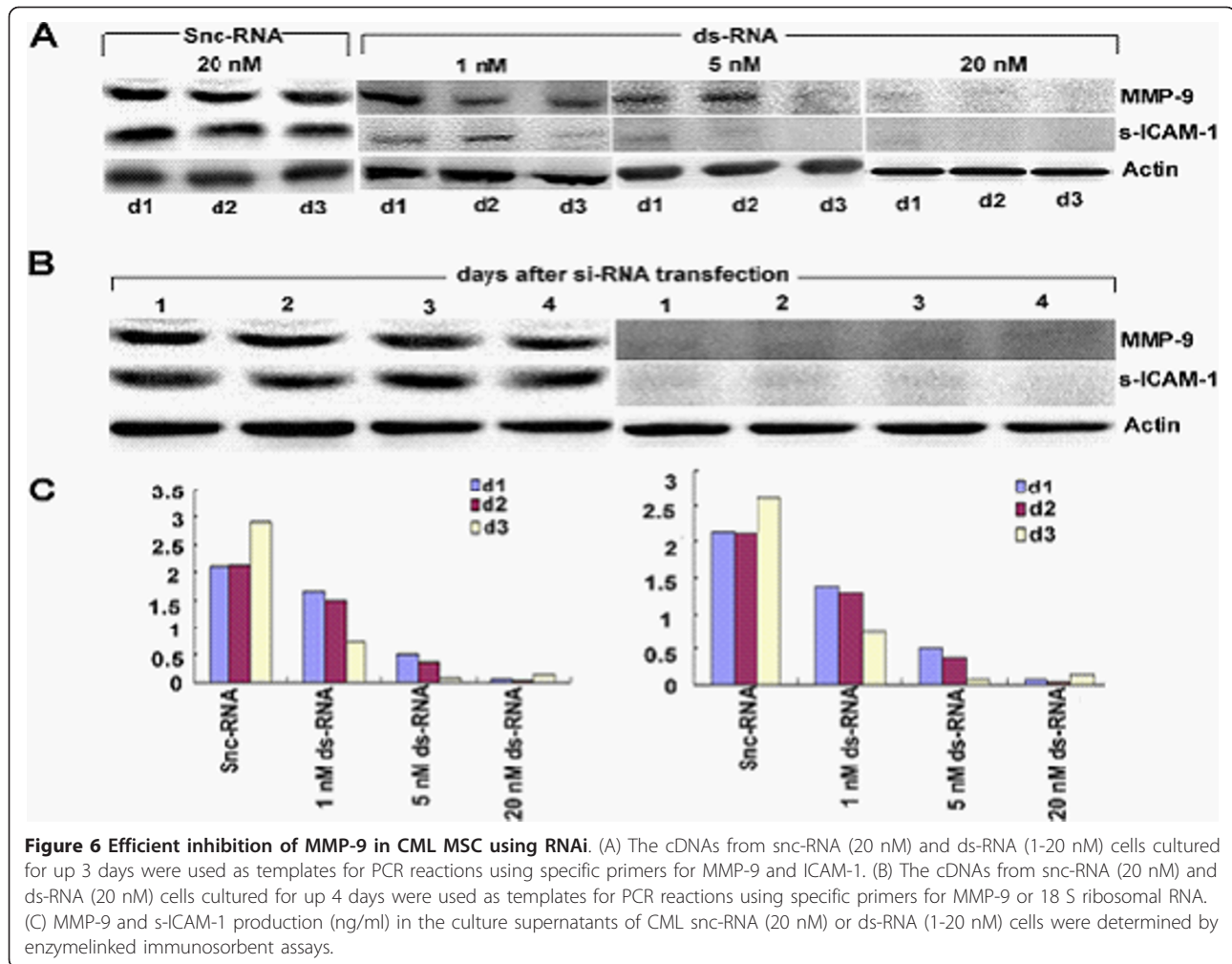
6B). Besides, serum ICAM-1 was concomitantly changing with MMP-9. The Western blotting results were confirmed by enzyme-linked immunoadsorbent assay. CML snc-RNA-transfected cells cultured up to 3 days spontaneously released high amount of MMP-9 into the culture conditioned medium whereas ds-RNA-transfected cells showed a marked time- and dose- dependent inhibition in MMP-9 protein levels. Importantly, levels of s-ICAM-1 were also affected with ds-RNA transfection (Figure 6C).

## Discussion

MSC isolated from different tissues had immune regulation ability not only in vivo but in vitro and it might consist the “immune protection site” in human body [25,26]. Considering their richness in source, availability for expansion, and most importantly, their robust immuno-modulatory activity, MSCs appear to be a

primary candidate for cellular therapy in immune disorders [12,16,27]. In normal physiological conditions, MSCs are very scarce (one MSC per 10,000-100,000MNC), therefore, normal immune responses against foreign antigens are not affected. This is consistent with in vitro results showing that immuno-suppressive function was abolished when the ratio of MSC to T cells was less than 1:100. However, once a large number of MSCs were infused for immune therapy, influx of MSC in the circulation and bone marrow could bring the hypersensitive immune response to normal. Moreover, MSC infusion could not only modulate immune responses but enhance the hematopoietic microenvironment. Transplantation of MSCs offers bright prospects in developing new therapies for blood diseases caused by an abnormal immune system and impaired hematopoietic microenvironment. To date, MSCs have been used to treat GVHD, which is a disorder of hyper-immunoresponse, and shown to be effective clinically [28,29].

Chronic myeloid leukemia is a clonal hematopoietic stem cell disorder characterized by the t(9;22) chromosome translocation and resultant production of the constitutively activated *BCR/ABL* tyrosine kinase [30]. Interestingly, this *BCR/ABL* fusion gene, was also detected in the endothelial cells of patients with CML, suggesting that CML might originate from hemangioblastic progenitor cells that can give rise to both blood cells and endothelial cells. Although Interferon- $\alpha$ ,



Intimab(a *BCR/ABL* tyrosine kinase inhibitor) and stem cell transplantations are the standard therapeutic options, transplant-related morbidity from graft-versus-host disease and mortality rates of 10% to 20% have greatly reduced the allogeneic hematopoietic cell transplantation in clinics[31], while interferon- $\alpha$  is only effective in some patients to some degree and chemotherapeutic intervention does not result in prolonged overall survival[32,33] and the reason is possibly due to some unknown biology of the CML immune regulation[34].

We conducted this study of CML patient-derived MSCs to evaluate the safety and effectiveness of autologous MSCs in treating CML. We tested the karyotype and genetic changes of in vitro-expanded MSCs for safety evaluation. The immuno-modulatory function of MSCs was also examined. The investigation of CML patient-derived MSCs could help to further elucidate etiology and pathology of CML. Specifically, the answers to questions of whether gene aberrations exist in MSCs

and whether the functions of MSCs are impaired are crucial for understanding of CML development and finding effective treatments.

We utilised Flk1+CD31-CD34- MSCs from CML patients for 4-6 passages, and there were chromosomal abnormalities, indicating that mutation of CML happened at the hematoangioblast level[35]. We thereby hypothesized that malignant mutation existed in stem cells more primordial than HSCs. Data from functional tests proved that CML-derived MSCs had abnormal immuno-modulatory function, although their MSCs showed normal karyotype. An inhibitory effect on T cell proliferation is an important characteristic of MSC in immuno-modulatory action. A previous study, in accordance with another report, suggested that the inhibitory effect on T cell proliferation might be through cell cycle arrest. MSCs from healthy volunteers could obviously block T cells in G0/G1 phase. In this study, inhibitory effects of MDS-derived MSCs on T cell proliferation were obviously impaired. Moreover, no significant cell



cycle arrest was observed in PHA-stimulated T cells cocultured with CML-derived MSCs. In addition, an inhibitory effect on T cell activation is another key point of immuno-modulatory function for MSCs, although there are still disputes[21,22]. CD25, CD69 and CD44 are candidates for T cell activation in different phases. In our study, MSCs from healthy volunteers showed significant inhibitory effects on expression of T cell activation markers, but MSCs from CML patients showed very limited inhibitory effects. These results suggested that CML-derived MSCs have immunologic abnormalities and their application in immuno-modulation might be limited.

Normally, the invasion and metastasis by malignant tumor cells consists of three major steps: the receptor-mediated adhesion of tumor cells to the extracellular matrix, the degradation of the extracellular matrix by the proteinase secreted by the tumor cells, and the transfer and proliferation of tumor cells[36]. So, the loose of ECM and secreted cytokines are important for the metastasis of the tumor cells from the primary tumor[37]. Pathological conditions will change the tumor cell fate leading to invasion and metastasis[38], Local secretion of proteases have been implicated in this tumor-stroma crosstalk. Matrix Metalloproteinase-9 (MMP-9) is one of them which has the preferential ability to degrade denatured collagens (gelatin) and collagen type IV, the 2 main components of basement membranes and therefore plays a critical role in tumour progression and metastasis[39]. Moreover, its expression increases with the increased or greater proliferation of tumor cells.

We used a ds-RNA to interfere with the expression of MMP-9 gene in CML MSC and our findings support the conclusion that MMP-9 constitutes a trigger for the switch between adhesive and invasive states in CML MSC by changing the ICAM-1 from membrane-anchored state to solvable one leading to tumor cell immune evasion and metastasis.

In conclusion, the immune function of CML patient-derived MSCs showed that their immuno-modulatory ability, compared to MSCs from healthy volunteers, was impaired, which might be a cause for an abnormal hematopoietic environment. This indicates that autologous MSCs transplantation might be futile. Instead, allogenic MSCs transplantation might be a better choice to ameliorate CML.

#### Acknowledgements

Supported by grants from the "863 Projects" of Ministry of Science and Technology of PR China (No. 2006AA02A109, 2006AA02A115); National Natural Science Foundation of China (No.30570771; Beijing Ministry of Science and Technology (No. D07050701350701) and Cheung Kong Scholars programme.

#### Authors' contributions

ZH carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscript. AG carried out the immunoassays. SY participated in the design of the study and performed the statistical analysis. All authors read and approved the final manuscript.

#### Competing interests

The authors declare that they have no competing interests.

Received: 23 September 2010 Accepted: 2 May 2011

Published: 2 May 2011

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doi:10.1186/1756-9966-30-47

Cite this article as: Xishan et al.: The research on the immunomodulatory defect of Mesenchymal Stem Cell from Chronic Myeloid Leukemia patients. *Journal of Experimental & Clinical Cancer Research* 2011 **30**:47.

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