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Mesenchymal Stem Cells Enhance Chemotaxis of Activated T Cells through the CCL2-CCR2 Axis *In Vitro*

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Activation and migration of donor T cells to the host target organs are critical mechanisms in the pathogenesis of graft-versus-host disease (GVHD). The role of monocyte chemoattractant protein-1 (MCP-1/CCL2) and its receptor CCR2 in the recruitment of T cells during immune or inflammatory response is also well known. For elucidation of the mechanism of the therapeutic effect of human bone marrow derived-mesenchymal stem cells (MSC) in GVHD, we studied the effect of these cells on migration of activated donor T cells through the CCL2-CCR2 axis *in vitro*. MSC were expanded from donors' bone marrow mononuclear cells. After co-culturing of IL-2-activated T cells with allogeneic MSC at different ratios, the levels of CCL2 in supernatants were measured by ELISA, and CCR2 expression in $CD4^+$/$CD8^+$ T cells subsets were detected by flow cytometry. The effect of MSC on the migration of activated T cells in the Transwell system was studied in the absence or presence of CCL2. Our results show that CCL2 levels in supernatants of co-cultures were significantly higher than in MSC monoculture and this increase depended on the number of MSC. MSC inhibited proliferation of T cells, but did not change the percentages of $CD4^+$ and $CD8^+$ T cells subsets. MSC can up-regulate the CCR2 expression in $CD8^+$ subsets rather than in $CD4^+$ subsets; MSC enhanced migration of IL-2-activated T cells to CCL2 by increasing the expression of CCR2. The data demonstrate that MSC can enhance chemotaxis of cytokine-activated T cells through the CCL2-CCR2 axis *in vitro*.

Key Words: *mesenchymal stem cells; T lymphocytes; CCL2 protein; CCR2 receptor; GVHD*

Graft-versus-host disease (GVHD) is the main complication of allogeneic transplantation of hematopoietic stem cells and a crucial risk factor affecting the survival and outcomes of patients [12]. Activation, proliferation, differentiation, and migration of donor T cells to the host target organs are very critical elements in the pathogenesis of GVHD. Various immunosuppressive agents, such as steroids, methotrexate, and mycophenolate mofetil are now extensively used for the prevention and treatment of GVHD [3,22]. Immunosuppressants effectively inhibit the proliferation of donor T cells and reduce the rate of GVHD; however, treatment with these preparations is associated with increased risk of infectious complications, sometimes life-threatening for the recipients [17].

Mesenchymal stem cells (MSC) are a self-renewing population of multipotent non-hematopoietic progenitor cells capable of differentiating into a variety of mesenchymal lineage cells [13]. MSC have potent immunosuppressive and immune-modulating effects on various immune cells *in vitro* and *in vivo* [1]. Several studies have demonstrated that infused MSC clearly suppress GVHD and promote engraftment of transplanted allogeneic hematopoietic stem cells [11,21]. Although the underlying mechanisms are not completely understood, MSC are considered as a promising approach for GVHD due to their ability to modulate immune reactions and repair damaged tissue [2,10].

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Monocytic chemoattractant protein-1 (CCL2) was first cloned as a member of the CC chemokine subfamily. This protein and its receptor (CCR2) constitute the CCL2-CCR2 axis that is activated in many inflammatory and immune diseases [6]. In experimental GVHD, some chemokines CCL2-CCL5, CXCL2, and CXCL9 are overexpressed and enhance migration of effectors to the target organs [14,23]. A potent role of the CCL2-CCR2 axis in GVHD has also been demonstrated in genetically modified mice [9]. However, very little is known about the interaction of MSC with the CCL2-CCR2 axis.

In this study, we measured CCL2 levels in supernatants and CCR2 expressions in activated T cells after co-culturing with bone marrow MSC and the effect of MSC on chemotaxis of activated T cells to find out whether MSC can inhibit migration of activated T cells to the target organs and exhibit therapeutic effect on GVHD *in vivo*.

MATERIALS AND METHODS

Isolation, culturing, and identification of human bone marrow MSC. The study protocol was approved by the Research Ethics Committee at the Second Hospital of Hebei Medical University. All adult healthy donors signed informed consent form. MSC were isolated and expanded from bone marrow of healthy donors as described previously [4]. After three passages, adherent cells were harvested and stained with fluorescein-conjugated monoclonal antibodies against CD13, CD14, CD31, CD34, CD45, HLA-DR, CD29, CD105, CD71, CD106, HLA-ABC and then analyzed by flow cytometry. For evaluation of osteogenic and adipogenic differentiation of MSC, passage 5 cells were treated with NH AdipoDiff or NH OsteoDiff induction medium according to the manufacturer's protocol. Osteogenesis and adipogenesis were assessed by staining with Oil Red O and staining for alkaline phosphatase (AP), respectively.

Isolation, culturing, and identification cytokine-activated T cells. Peripheral blood mononuclear cells were isolated from healthy blood donors by using density-gradient centrifugation. The isolated cells were washed 3 times with PBS and cultured in 25 cm^2 plastic flasks at a density of 2×10^6 cells/ml in RPMI-1640 culture medium with 10% fetal calf serum, penicillin/streptomycin, and L-glutamine. In 6 h, adherent cells were removed and nonadherent cells were continuously cultured in complete medium with stimulation with recombinant human IL-2 (2000 U/ml). The medium was changed every 2 days and supplemented with recombinant human IL-2 (2000 U/ml). Seven days later, the cytokine-activated T cells were evaluated by flow cytometry using ECD-conjugated CD3 antibodies.

Co-culturing of MSC with cytokine-activated T cells. Passage 3 confluent MSC cultures were used as the feeder layer. MSC were seeded in a 6-well plate (2×10^4, 4×10^4, 10^5, and 2×10^5 per well) with complete medium and incubated at 37°C for 24 h until adhesion. Then, 2×10^6 T cells activated with IL-2 were plated in each well at 100:1, 50:1, 20:1, and 10:1 T cell/MSC ratios. These co-cultures were grown in triplicates in a humidified atmosphere at 37°C and 5% CO_2. In wells of another 6-well plate, the same numbers of MSC were cultured without cytokine-activated T cells (control). In addition, IL-2 activated T cells (2×10^6 per well) were solely seeded as negative control. In 48 h, the levels of CCL2 in supernatants were measured by ELISA according to the manufacturer's instructions. CCR2 expression on the surface of IL-2 activated $CD3^+$ T cells and $CD3^+CD4^+$/$CD3^+CD8^+$ subsets were determined using PE-conjugated CCR2 antibodies, FITC-conjugated CD4 antibodies, and PC5-conjugated CD8 antibodies. Background fluorescence was measured using the corresponding PE-, FITC-, and PC5-conjugated isotypic antibodies.

Transwell analysis of chemotaxis of cytokine-activated T cells. Chemotaxis was assayed using 5-μm pore-size 24-well Transwell plates. IL-2-activated T cells cultured alone or co-cultured with MSC at different ratios (T cell/MSC 100:1, 50:1, 20:1 and 10:1, respectively) for 48 h were harvested, washed 3 times with PBS, and resuspended in serum-free RPMI-1640 medium; then, 200 μl cell suspension were seeded at a density of 2×10^6 cells/ml into the upper chamber. Serum-free RPMI-1640 medium alone (500 μl) or with recombinant human CCL2 (1000 pg/ml) was added as the chemoattractant to the lower chamber and incubated for 4 h at 37°C and 5% CO_2 in humidified atmosphere. All variants were incubated in triplicates. At the end of the chemotaxis assay, 100 μl cell suspensions that had migrated to the lower chamber and 100 μl cell suspensions in upper chamber were counted in a FACScan flow cytometer (BD) for 30 sec under identical flow conditions. The chemotaxis index (CI) was calculated by the formula: CI=(cell number in the lower chamber/cell number in the upper chamber)×100%. Each assay was repeated 3 times.

Statistical analysis. All data were analyzed using GraphPad Prism 5.0. For continuous variables with normal distribution, the analysis of treatment effects in different groups was performed using one-way ANOVA at $p<0.05$. The results are expressed as the $M\pm SD$.

RESULTS

Morphological features, immunophenotype, and multilineage differentiation of bone marrow MSC.

The spindle-shaped cells adherent to plastic were successfully isolated and expanded from the bone marrow over ~7-8 days. After three passages, homogeneous MSC cultures were obtained (Fig. 1, *a*, *b*). Flow cytometry results showed that passage 3 MSC were characterized by intensive expression of CD13, CD29, CD105, CD71, CD106, and HLA-ABC and did not express CD14, CD31, CD34, CD45, and HLA-DR. Passage 5 MSC could successfully differentiate into osteoblasts and adipocytes under specific culturing conditions, which was confirmed by staining with Oil Red O (Fig. 1, *c*, *d*) and staining for AP (Fig. 1, *e*, *f*). Thus, adherent cells obtained from the bone marrow fulfill the recommended criteria of human MSC which

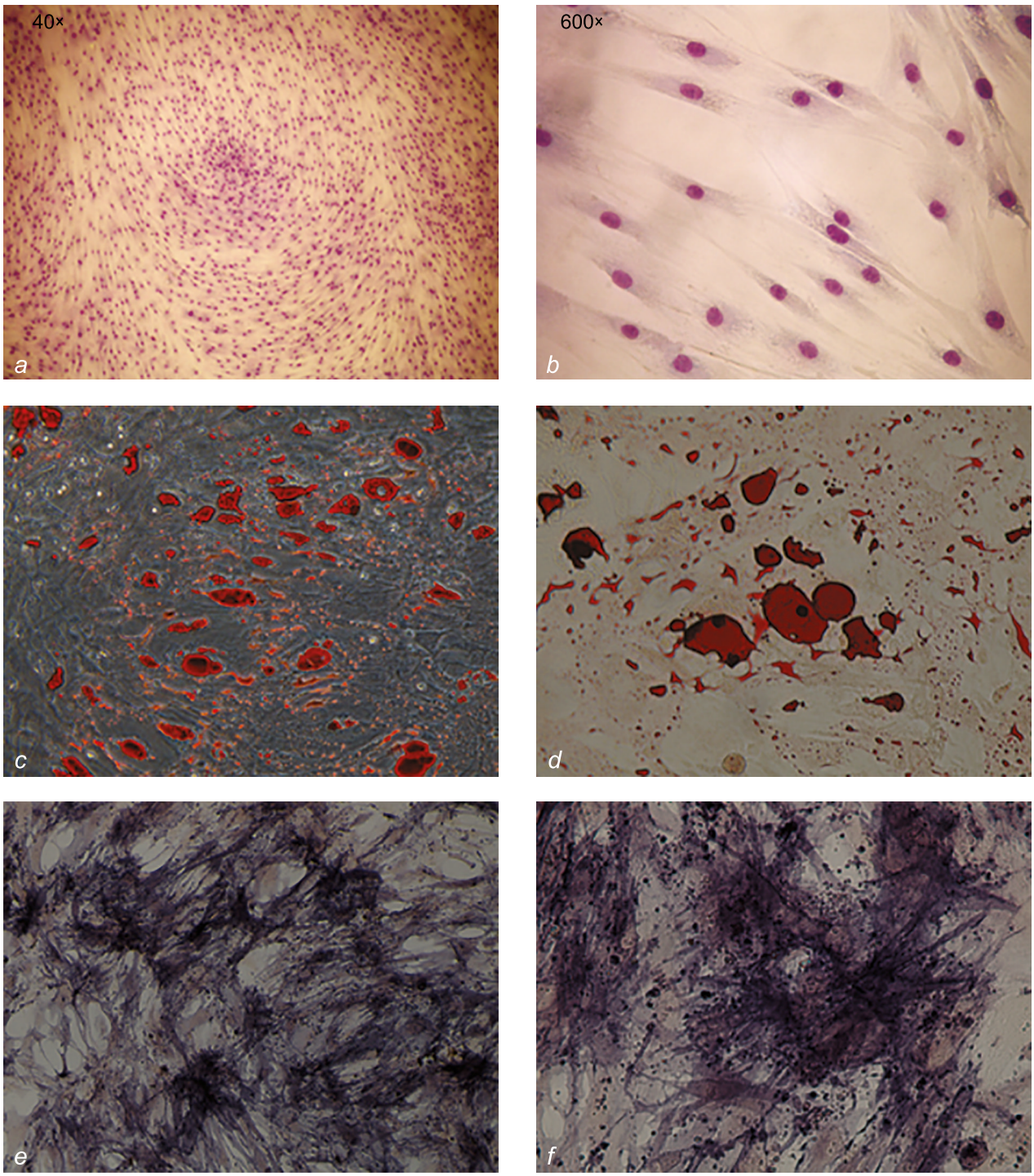

Fig. 1. Fibroblast-like morphology of passage 3 MSC (*a*, *b*) and their adipogenic (*c*, *d*) and osteogenic (*e*, *f*) differentiations. Wright—Giemsa staining, ×40 (*a*), ×600 (*b*); Oil Red O staining, ×200 (*c*), ×400 (*d*); staining for AP, ×200 (*e*), ×400 (*f*).

include adhesion to plastic, expression of certain surface markers, and multi-differentiation ability.

Morphology and identification of cytokine-activated T cells colony. T-cell colonies were generated by culturing isolated 5×10^5 cells mononuclear cells in the presence of recombinant human IL-2 (2000 U/ml) for 7 days. The percentage of $CD3^+$ cells among activated nuclear cells was about 93-99%.

The levels of CCL2 in MSC increased after co-culturing with IL-2-activated T cells. The results from 5 experiments showed that IL-2-activated T cells did not solely secrete CCL2, whereas MSC did, and the CCL2 levels secreted by MSC were dose-dependent. Furthermore, CCL2 levels (pg/ml) significantly increased in all co-cultures with different T cell/MSC ratios (except for 100:1) in comparison with pure MSC cultures; the effects depended on the proportion of MSC. For T cell/MSC ratios of 10:1, 20:1, 50:1, and 100:1, the CCL2 levels in the supernatants was 5005.14±494.94 pg/ml (*vs* 1954.35±196.35 pg/ml in the control), 3374.46±591.83 pg/ml (*vs* 630.95±137.50 pg/ml in the control), 1645.94±552.30 pg/ml (*vs* 330.87±109.55 pg/ml in the control), and 418.44±275.65 pg/ml (*vs* 143.86±30.82 pg/ml in the control), respectively. These data also indicated that CCL2 levels in supernatants significantly increased in MSC dose-dependent manner, whether MSC were cultured alone or co-cultured with IL2-activated T cells at different ratios ($p<0.05$; Fig. 2, *a*).

MSC increased CCR2 expressions on $CD8^+$ T cells subsets, but did not change the percentages of $CD4^+/CD8^+$ subsets in IL-2-activated T cell. Flow cytometry results showed that the percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells did not differ significantly in co-cultures with different T cell/MSC ratios and in activated T cells cultured alone ($p=0.8720$ and $p=0.975$; respectively). Furthermore, the ratio of $CD4^+$ to $CD8^+$ cells was also unchanged (Fig. 2, *b*). In addition, we found that CCR2 was not expressed by $CD4^+$ T cell subsets, but could be expressed on $CD8^+$ T cell subsets at a low level. CCR2 expressions on $CD8^+$ T subsets were parallelly up-regulated with the MSC increasing, although significant differences were not found. The expression of CCR2 on $CD3^+$ and $CD3^+CD8^+$ T cells is shown in Figure 2, *c*.

MSC increased migration of IL-2 activated T cells to CCL2 in co-culture. The cell chemotaxis was assayed during co-culturing in the Transwell system (Fig. 3, *a*). In the presence of CCL2 in the bottom chamber, percentage of activated T cells in this chamber after co-culturing with MSC was higher than in culture containing T cells alone. Chemotaxis index (CI) in all co-cultures was significantly higher than in pure T-cell cultures, and this effect depended on MSC dose. For T cell/MSC ratios of 100:1, 50:1, 20:1, and 10:1, the CI values significantly differed from those in T-cell monocultures ($p=0.049$, $p=0.035$, $p=0.002$, and $p=0.000$; respectively) (Fig. 3, *b*). In the absence

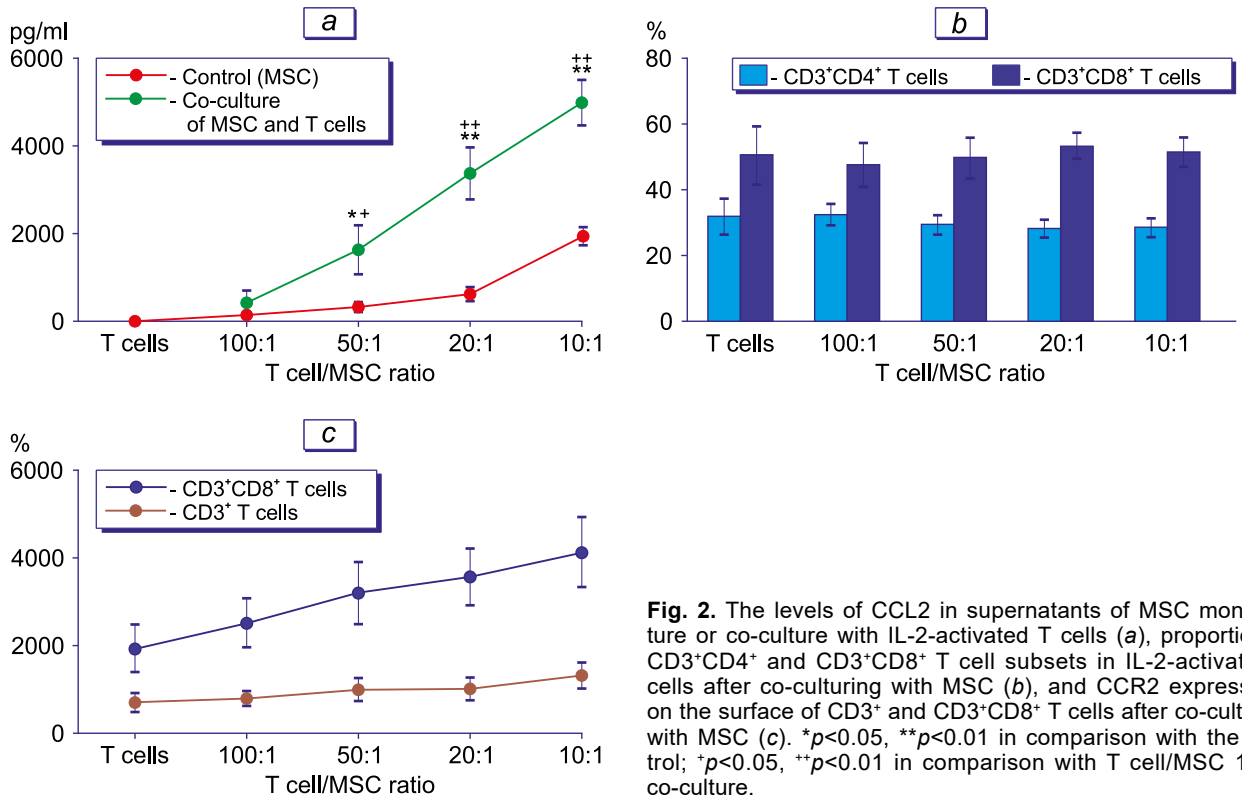

Fig. 2. The levels of CCL2 in supernatants of MSC monoculture or co-culture with IL-2-activated T cells (*a*), proportion of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell subsets in IL-2-activated T cells after co-culturing with MSC (*b*), and CCR2 expressions on the surface of $CD3^+$ and $CD3^+CD8^+$ T cells after co-culturing with MSC (*c*). $^*p<0.05$, $^{**}p<0.01$ in comparison with the control; $^+p<0.05$, $^{++}p<0.01$ in comparison with T cell/MSC 100:1 co-culture.

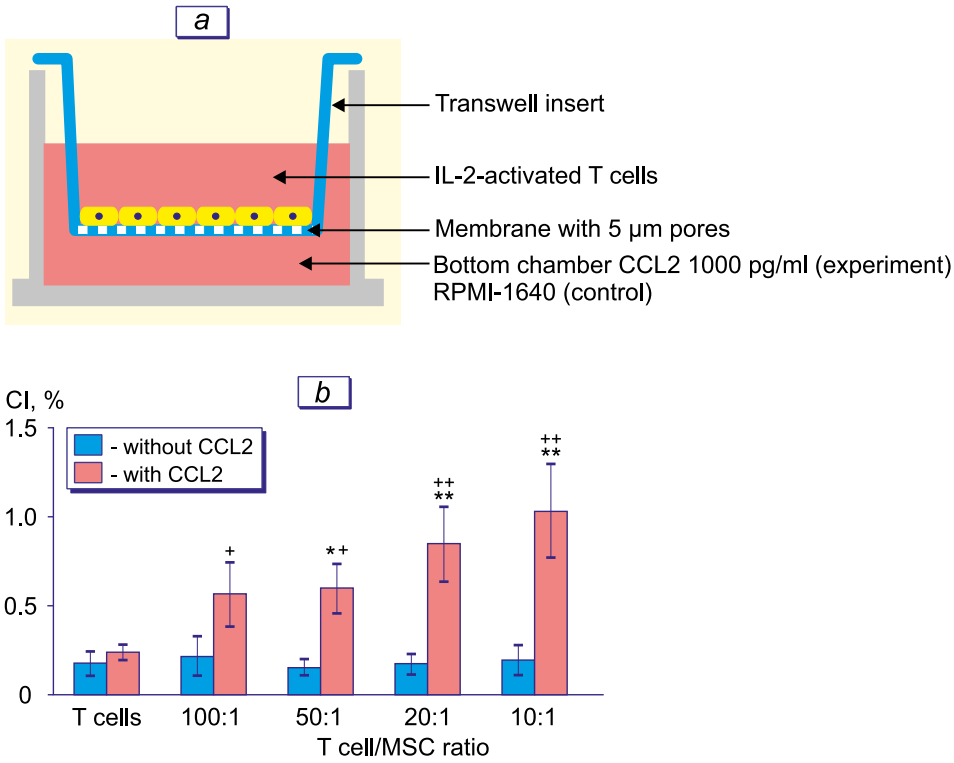

Fig. 3. Chemotaxis assay in Transwell system. *a*) Scheme of the experiment; *b*) effect of MSC on migration of activated T cells in co-culture with different T cell/MSC proportions. **p*<0.05, ***p*<0.01 in comparison with culturing without CCL2; ^{+}p<0.05, ^{++}p<0.01 in comparison with T cell monoculture.

of CCL2 in the bottom chamber, no significant differences in CI were observed. Furthermore, CI were significantly increased in all co-cultures (except the culture with T cell/MSC ratio of 100:1) in the presence of CCL2 in comparison with cultures without CCL2 (t=2.355, p>0.05; t=2.961, p<0.05; t=.4.509, p<0.01; t=5.653, p<0.01 for 100:1, 50:1, 20:1, and 10:1 ratios, respectively). These results suggest that MSC contact enhance migration capacity of IL-2-activated T cells to CCL2 by improving CCR2 expressions on the surface of $CD8^+$ T cells.

It is known that chemokines direct migration of donor T cells to the target organs in which they may cause the GVHD. CCL2 protein plays the major role in selective recruiting of monocytes and T lymphocytes through binding to its receptor CCR2 [15]. It was previously reported that recipients of $CCR2^{-/-}CD8^+$ T cells presented less damage of gut and liver compared to recipients of wild-type $CD8^+$ T cells, which determined lower overall GVHD-related morbidity and mortality in murine GVHD models [20]. Importantly, graft-versus-tumor (GVT) effect mediated by $CCR2^{-/-}CD8^+$ T cells was preserved. It suggests that knockout of *CCR2* gene in $CD8^+$ T cells may contribute to separate GVHD from GVL [20].

Previous studies reported that CCL2 can be produced by many cell types, including monocyte, endothelial, fibroblasts and smooth muscle, *etc*. [5]. However, the data on the secretion of CCL2 by T cells are still controversial. It was reported that CCL2, MIP-1α, MIP-β, and RANTES mRNA can be detected in mouse Th1 cells by using RNase protection assay [4]. However, there are reports that T cells do not secrete CCL2 [7]. Our data show that CCL2 levels in supernatants of T cells cultured alone were negligibly low. This can be due to the following aspects. First, $CD4^+$ T subsets accounted for a low proportion of all T cells resulting in a fewer number of Th1 cells. Thus, the level of CCL2 protein production is below the minimum ELISA detection threshold. Second, we only detected the CLL2 protein, which is easy degraded *in vitro*, rather than mRNA. Therefore, we speculate that IL-2-activated T cells may not secrete CCL2 *in vitro*. In addition, our results showed that CCL2 can be secreted by MSC, which was consistent with previous reports [16]. CCL2 levels in supernatants of co-culture of MSC with T cells are significantly higher than in MSC monoculture, and the effect positively correlated with the number of MSC. It is known that some proinflammatory cytokines secreted by activated T cells such as IFNγ and TNFα can provoke intensive expression of some chemokines by MSC [18]. The levels of CCL2 mRNA increase by 3600 times in MSC co-cultured with activated T cells [18]. Therefore, we

presumed that proinflammatory cytokines secreted by activated T cells in co-culture can stimulate CCL2 secretion by MSC.

Previous studies have demonstrated that MSC can suppress the proliferation of T cell and cytokine release by these cells through NO production, which inhibits STAT5 phosphorylation [19]. In our study, we also found that MSC can suppress proliferation of T cells, but do not change the proportion of $CD4^+$/$CD8^+$ T cell subsets. As NO is a gaseous bioactive unstable molecule, MSC must be close enough to the immune effector cells to exert the inhibitory effect. Our data show that $CD8^+$ T cells can express low levels of CCR2, whereas $CD4^+$ T cells do not express this receptor. In co-culture with activated T cells, MSC can up-regulate the CCR2 expressions on the surface of $CD8^+$ subsets rather than $CD4^+$ subsets. However, the difference was insignificant due to low number of experiments.

Our findings suggest that MSC can not only secrete high levels of CCL2, but also up-regulate the CCR2 expression on $CD8^+$ T cells. So, it was assumed that MSC can promote migration of activated T cells by increasing activity of the CCL2-CCR2 axis. Chemotaxis assay showed that chemotaxis index CI was significantly increased in all co-cultures in comparison with pure T cell cultures when CCL2 was added in the bottom chamber. Based on these results, we put forward a hypothesis that infused MSC can attract *in vivo* alloreactive donor $CD8^+$ T by secreting high levels of CCL2 and up-regulating CCR2 expressions on donor $CD8^+$ T cells, thereby preventing recruitment of donor $CD8^+$ T to the target organ of GVHD. It was reported that a transgenic mouse line in which MCP-1 expression is controlled by the MMTV-LTR was characterized by high levels of CCL2 expression in multiple organs and high serum levels of CCL2, which can eliminate the concentration gradient of this factor between the inflammation sites and blood circulation, thereby preventing the migration of effector cells to the inflammation sites [8]. These results to a certain extent support our hypothesis. The mechanism responsible for the immunomodulatory effects of MSC are very complex and have not been completely understood. We believe that regulation of chemokines and their receptors by MSC can be a very important aspect in numerous immunomodulatory mechanisms.

In conclusion, our study shows that MSC enhance the chemotaxis of cytokine-activated T cells through the CCL2-CCR2 axis *in vitro*. Therefore, it was presumed that infused MSC might attract alloreactive donor $CD8^+$ T cells in vivo by eliminating the CCL2 concentration gradient between the circulating blood and the target organ by increasing CCR2 expression on alloreactive donor T cells, especially $CD8^+$ T cells. Thus, MSC not only reduce recruitment of donor activated T cells to the target organ, but also exert local inhibitory effect on the donor activated T cells through direct cell—cell interactions or secretion of soluble molecules. Future experiments on animal models and in vivo tests will help to verify our hypothesis.

Y. L. Zhang was responsible for experimental design and performed research, analysis, and interpretation of the results; drafted the manuscript; and provided final approval of the version to be published. S. K. Qiao participated in the design of the study and provided general support. All other authors helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

Conflicts of interest. The authors declare no potential conflicts of interest.

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