Models and Technologies

Human Umbilical Cord Blood–Derived Mesenchymal Stem Cells Producing IL15 Eradicate Established Pancreatic Tumor in Syngeneic Mice

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Abstract
Mesenchymal stem cells (MSC) represent a new tool for delivery of therapeutic agents to cancer sites because of their strong tropism toward tumors. IL15 has demonstrated a potent antitumor activity in various animal models as well as clinical trials. However, because of its short half-life, effective therapeutic effects usually require a high dose, which often results in undesired side effects; thus, new strategies for overcoming this disadvantage are needed. In this study, human MSCs were isolated from umbilical cord blood as delivery vehicles and transduced with lentivirus vector expressing murine IL15 (MSC-IL15). In vitro assays of lymphocyte activation and proliferation demonstrated that IL15 produced by MSCs was biofunctional. In syngeneic mice bearing Pan02 pancreatic tumors, systemic administration of MSC-IL15 significantly inhibited tumor growth and prolonged the survival of tumor-bearing mice, which were associated with tumor cell apoptosis, and natural killer (NK)– and T-cell accumulation. Furthermore, we confirmed that MSC-IL15 could migrate toward tumor and secreted IL15 in tumor-specific sites. Depletion of NK and CD8+ T cells abolished the antitumor activity of MSC-IL15, suggesting that NK and CD8+ T cells play a key role for MSC-IL15–mediated effect. Interestingly, cured mice after MSC-IL15 treatment were resistant to Pan02 pancreatic tumor rechallenge, and adoptive transfer of lymphocytes from cured mice also could cause rejection of Pan02 tumor inoculation in naïve mice, indicating that MSC-IL15 induced tumor-specific T-cell immune memory response. Overall, these data support that MSCs producing IL15 might represent an innovative strategy for therapy of pancreatic tumor. Mol Cancer Ther; 13(8); 2127–37. ©2014 AACR.

Introduction
The administration of cytokines to augment immunosurveillance has been proven to have efficacy in the treatment of several cancers (1). IL15 is a member of the common chain family of cytokines and is functionally similar to IL2 (2–4). Besides, IL15 does not promote acti-

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including type I IFN, TNF-related apoptosis-inducing ligand (TRAIL), LIGHT, and IL12 (16–21). MSCs differ depending on their sources, such as bone marrow, umbilical cord blood (UCB), or adipose tissue (22). UCB-derived MSCs have clinical advantages because of the immaturity of newborns compared with adult cells, large ex vivo expansion capacity, low risk of viral infection, lack of donor attrition, and less pronounced immune response (23, 24). These advantages may result in the replacement of other adult stem cells with UCB–MSCs as the vehicle of choice in gene therapy against tumor.

Pancreatic adenocarcinoma (PDAC) is among the most fatal cancers, with a mortality rate approximates its incidence rate. The all-stage 5-year survival, which is about 5%, has remained unchanged over the last 25 years (25, 26). In contrast to other malignancies, PDAC is highly resistant to chemotherapy and targeted therapy. Therefore, novel therapies such as immunotherapy, which could improve the prognosis of patients with advanced PDAC, are urgently needed (27). Until now, limited information was available about antitumor role of IL15 in pancreatic cancer. Thus, in this study, we investigated whether UCB–MSCs transfected with a lentivirus vector expressing murine IL15 (MSC-IL15) would represent an efficient IL15 delivery system, and further evaluated the antitumor activity and long-term protective immunity of IL15-expressing MSCs in a syngeneic mouse model of Pan02 pancreatic tumors.

Materials and Methods

Isolation, culture, and identification of MSCs from human UCB

The UCB samples were obtained from the umbilical vein immediately after delivery. Informed consent was received from the mothers, and this study was approved by the Institutional Review Board of the Changhai and PLA 301 Hospital. Isolation and culture of MSCs were conducted as previously reported (28). Briefly, the UCB samples were mixed with Hetasep solution (StemCell Technologies) at a ratio of 5:1, and then incubated at room temperature to deplete erythrocyte counts. The supernantant was collected carefully, and mononuclear cells were obtained using Ficoll (GE Healthcare Life Sciences) density gradient centrifugation at 2,500 rpm for 20 minutes. Cells were washed twice in PBS. Cells were then seeded at a density of $2 \times 10^5$ to $2 \times 10^6$ cells/cm² in growth media that consisted of D-media (formula 78-5470EF; Gibco BRL) containing EGM-2 SingleQuot and 10% FBS (Gibco BRL). After 3 days, nonadherent cells were removed. The adherent cells formed colonies and grew rapidly, showing spindle-shaped morphology. The adherent cells at the third to fifth passages were trypsinized and centrifuged, then fixed in neutralized 2% paraformaldehyde solution for 30 minutes. The fixed cells were washed twice and resuspended in PBS, incubated with FITC-labeled anti-human CD34, CD44, CD29, HLA-DR, CD38, and HLA-1 (all from biolegend) for 30 minutes. Positive cells were counted by flow cytometry with FACs.

To ascertain the in vitro differentiation ability, the third passages of MSCs were cultured in osteogenic, adipogenic, and chondrogenic differentiation medium, respectively (Cyagen Biosciences Inc.). To observe the calcium deposition, the cultured cells were fixed with 4% paraformaldehyde, and stained with Alizarin Red S. To determine the adipogenic differentiation, the cultured cells were stained with the Oil Red O solution. To determine the chondrogenic differentiation, the cultured cells were stained with alcian Blue.

Lentivirus vector expressing IL15 construction and transduction

Replication defective lentivirus encoding the complete IL15 open reading frame (LV-15) and a lentivirus vector encoding green fluorescent protein (GFP; LV-GFP), which was used as the control, were constructed by Invitrogen. The third to fifth passages of MSCs were transduced with lentivirus at a multiplicity of infection (MOI) of 50 pfu per cell in 2% FBS medium with 10 μg/mL polybrene (Sigma-Aldrich). At 24 hours after transfection, 1 × 10^6 cells were harvested and determined for IL15 mRNA expression using RT-PCR. The supernatants were collected and tested for IL15 protein secretion using the ELISA method in accordance with the manufacturer’s instructions (R&D Systems). One week later, cells were harvested by trypsinization and resuspended in PBS for injection.

Determination of bioactivity of IL15 secreted by MSCs

To confirm the bioactivity of secreted IL15, splenocytes (2 × 10^6/ well) were seeded in 96-well plates and tested in triplicates for their proliferative response to culture supernatants collected from IL15-expressing MSCs. Viability and proliferation of cells were analyzed in 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) metabolism assays. Furthermore, to analyze splenocyte activation, phosphorylation of STAT5 and STAT3 were determined by FACS analysis. IFNγ production by splenocytes after 3 days of treatment was detected by ELISA.

Tumor therapy experiments

Mouse pancreatic cancer cell line Pan02 was purchased from Cell Resource Center of the Chinese Academy of Sciences. Pan02 cells were grown as monolayer in RPMI 1640 (Invitrogen) supplemented with 5% FBS (Invitrogen) and 2 mmol/L L-glutamine (Invitrogen). Cells were seeded at a density of $2 \times 10^5$ cells/cm² and incubated at 37°C with 5% CO₂ and 95% humidity. Six-week-old specific pathogen-free male C57BL/6 mice were purchased from Second Military Medical University (Shanghai, China). All animals in this study were housed under pathogen-free conditions and were maintained in accordance with guidelines of the Committee on Animals of Second Military Medical University.
To assess the efficacy of MSC-IL15 in the prophylactic protocol, mice were injected with various doses of MSC-IL15 via tail vein. The next day, mice were challenged with 1 × 10^6 Pan02 cells subcutaneously in the flank. To study the therapeutic effects of MSC-IL15, mice were challenged with 1 × 10^6 Pan02 cells in the flank; 7 days after tumor inoculation, the mice received 1 × 10^6 MSC-IL15 via i.v. injection. Control mice were injected with PBS or MSC-GFP. The tumors were measured every 2 days with vernier calipers, and the diameters were recorded. Tumor volume was calculated by the formula: \( V = \frac{a \times b^2}{2} \), where \( a \) and \( b \) are the 2 maximum diameters. If mice were in a moribund state or the diameter of the tumors reached 15 mm, the mice were sacrificed and the date of death was recorded for survival analysis.

**Histology**

Tumor tissues were collected at the indicated time, fixed in 10% neutral formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was performed according to the protocol of the manufacturer. For NK- and T-cell staining, tumor tissues were embedded in optimal cutting temperature compound and frozen at -70°C. Frozen sections were stained with PE-conjugated antibodies to CD3 and to CD8 (clone 2.43; BD-pharMingen), and biotinylated rat anti-mouse-IFNγ (clone XMG1.2; BD-PharMingen). Pan02 cells were added as APC over the spleen cells. The ratio of responded cells to APC was 10:1. After 48 hours of incubation, cells were removed and biotinylated rat anti-mouse-IFNγ (clone XM1G2; BD-PharMingen) was added to incubate for 12 hours at 4°C. Then avidin–horseradish peroxidase (BD-PharMingen) was added and incubated at 20°C for 2 hours. The substrates AEC was then added. Five minutes later, the plates were washed and the spots were enumerated.

**ELISPOT assay**

ELISPOT assay was performed as described previously (20). Briefly, 4 × 10^5 spleen cells (responder cells) were added to each well precoated with anti-mouse IFNγ (clone R4-6A2; BD-pharMingen). Pan02 cells were added as APC over the spleen cells. The ratio of responded cells to APC was 10:1. After 48 hours of incubation, cells were removed and biotinylated rat anti-mouse-IFNγ (clone XM1G2; BD-PharMingen) was added to incubate for 12 hours at 4°C. Then avidin–horseradish peroxidase (BD-PharMingen) was added and incubated at 20°C for 2 hours. The substrate AEC was then added. Five minutes later, the plates were washed and the spots were enumerated.

**ELISA**

Cell-free supernatants and tumor tissue homogenates were harvested and analyzed for the production of IL15 and IFNγ by using ELISA Kits (R&D Systems).

**Statistical analysis**

Data were compiled as mean ± SE in qualitative experiments. Two-way ANOVA was conducted. Statistical significance was determined at the \( P < 0.05 \) level. Survival estimates were determined using Kaplan and Meier method.
Results

Isolation and identification of UCB–MSCs

According to previous reports (22, 23), we identified the characteristics of isolated UCB–MSCs, including fibroblastic-like morphology, immunophenotype, and differentiation potentials. As shown in Fig. 1A, UCB–MSCs presented a homogeneous population of spindle fibroblast-like cells. CD29, CD44, and HLA-I were positively expressed and CD34, CD38, and HLA-DR were negatively expressed as demonstrated by flow cytometry (Fig. 1B). The purity of MSC is >95%. The differentiation property of UCB–MSCs was further determined. Numerous lipid droplets were observed with Oil Red O staining in UCB–MSCs after incubation with the adipogenic supplementation for 21 days. Positive staining of Alizarin Red S was shown after osteogenic induction. Also blue staining indicated synthesis of proteoglycans by chondrocytes after chondrogenic induction (Fig. 1C). Collectively, these data demonstrated that we successfully obtained abundant MSCs from human umbilical cord blood.

To determine whether immunocompetent cell contamination might exist in MSC culture, we performed a more extended phenotypic characterization of MSCs by analyzing the expression of CD14, CD11b, and CD16. As shown in Supplementary Fig. S1, the monocytic marker CD14, macrophage marker CD11b, and NK marker CD16 were not detected in the MSCs.

UCB–MSCs can be successfully transduced to release functional IL15

UCB–MSCs were transduced with lentivirus encoding GFP (MSC-GFP) together with mouse IL15 (MSC-15). More than 90% cells were successfully transduced, as shown by GFP expression (Fig. 2A). No apparent change in morphology and proliferation rate between MSC-GFP and MSC-IL15 was observed (data not shown). IL15 mRNA expression was verified by semiquantitative RT-PCR analysis (Fig. 2B). To examine the IL15-secreting level of MSC-IL15, we quantified IL15 level in the supernatant of MSCs cultured at 1 × 10^6 cells per mL for 24 hours (Fig. 2C). ELISA assay showed that there was about 5 ng/mL of IL15 in the supernatant of MSC-IL15, whereas no IL15 was detected in the supernatant of MSC-GFP cultured under the same condition. In addition, because IL15 also exists as a membrane-bound form, we determined whether MSC-IL15 expressed membrane-bound IL15. FACS analysis showed that membrane-bound IL15 was not detected (Fig. 2D).

We further determined whether IL15 produced by MSC-IL15 was biofunctional. First, splenocytes were cultured in the culture supernatants of MSC-IL15, and proliferation was followed by MTT metabolization assays (Fig. 2E). The results showed that splenocytes could continue to proliferate compared with the group treated with the culture supernatants of MSC-GFP. To investigate whether IL15 produced by MSC-IL15 was able to activate lymphocyte cells, we analyzed phosphorylation of STAT5 and STAT3 (10, 28), which act as signal transducers downstream of IL2/IL15 receptors (Fig. 2F). As expected, splenocytes treated with the culture supernatants of MSC-IL15 resulted in high and consistent levels of phospho-STAT5 and phospho-STAT3, compared with the culture supernatants of MSC-GFP. Next, we investigated whether the IL15 produced by MSC-IL15 activated cytotoxicity. The IFNγ contents of the medium were measured by ELISA. The culture supernatants of MSC-IL15 exhibited much higher IFNγ-inducing activity than the controls.
Moreover, addition of anti-IL15 mAb into the culture neutralized the effect mediated by MSC-IL15 (Fig. 2G). Taken together, these data clearly demonstrated that MSC-IL15 produced biologically functional IL15.

Recently, it was reported that cord blood–derived MSCs were as efficient as bone marrow–derived MSCs in the inhibition of NK cells and other immune-competent cells (29, 30). Thus, we further assessed the effect of the MSCs on both human and murine NK cells activation through analyzing the expression of CD69 and CD107a. As shown in Supplementary Fig. S2, expressions of CD69 and CD107a in both human and murine NK cells were significantly increased after recombinant human and murine IL15 stimulation, respectively. However, CD69 and CD107a expressions in activated murine NK cells were modestly decreased after coculture with MSCs, whereas MSCs showed more effective inhibition on their expressions in activated human NK cells. Furthermore, MSC-IL15 could also significantly increase CD69 and CD107a expressions in murine NK cells. These data suggested that although MSC itself seemed to exert immune-regulatory properties on NK cells activation, IL15 could completely abolish the effect of MSCs.

The antitumor activity of MSC-IL15 in a syngeneic mouse model of pancreatic cancer

To investigate the effect of MSC-IL15 on tumor growth in vivo, mouse Pan02 pancreatic tumor model was used. In this system, all cells and mice were in the C57BL/6 background. We first tested whether MSC-IL15 could prevent tumor growth when mice were challenged with 1 x 10^6 Pan02 cells, and various doses of MSC-IL15 were simultaneously administered by systemic injection. Tumors were removed and weighed 20 days later. Strikingly, 1 x 10^6 MSC-IL15 almost completely halted tumor growth. Moreover, Pan02 pancreatic cancer showed reduced growth in a dose-dependent manner (Fig. 3A). This effect was found to be specific to the cytokine and not attributable to vector transduction or MSCs because there was no significant therapeutic effect observed in mice treated with MSC-GFP (Fig. 3A). We then analyzed the therapeutic potential of MSC-IL15. The mice were challenged with 1 x 10^6 Pan02 cells. Seven days later, 1 x 10^6 MSC-IL15 were administered by i.v. injection. In contrast to control groups, MSC-IL15 could also effectively repress tumor-aggressive growth (Fig. 3B). Moreover, all control mice inoculated with tumor cells alone died within 30 days, whereas 40% of mice treated with MSC-IL15...
survived more than 100 days (Fig. 3C). To exclude the possibility that high number of cells might interfere with tumor stroma and shape the tumor microenvironment and subsequently affect the magnitude of antitumor response and tumor growth, we further compared effects of injection of different number of cells. As shown in Supplementary Fig. S3, the antitumor effect of MSC-IL15 was mediated via a dose-dependent manner. Moreover, we found that administration of \( \frac{1}{10^5} \) MSC-IL15 also demonstrated a significant antitumor property.

To compare the antitumor capacity of MSC-IL15 with recombinant IL15, we treated mice with 5 \( \mu \)g recombinant IL15 or \( \frac{1}{10^6} \) MSC-IL15 after Pan02 cell inoculation. Based on our in vitro assay, we estimated that the \( \frac{1}{10^6} \) injected MSC-IL15 cells only produced 5 ng of IL15 daily. This is far below the 5 \( \mu \)g recombinant IL15 injected. Importantly, even with this low amount of IL15 produced by MSC-IL15, MSC-IL15 had much more potent antitumor effect than recombinant IL15 (Fig. 3D). Furthermore, when tumors were examined histologically, MSC-IL15 increased tumor cell apoptosis, as shown by TUNEL assay (Fig. 3E). Massive lymphocyte infiltration, including NK and T cells, was detected in the MSC-IL15 group compared with MSC-GFP treatment (Fig. 3F). IL15 was also able to stimulate expansion and survival of \( \gamma \delta \) T cells. As expected, the intratumor infiltration of \( \gamma \delta \) T cells was also increased after MSC-IL15 administration (Supplementary Fig. S4).

In addition, MSC was demonstrated to be able to induce regulatory T cells expansion. Thus, we further determined whether MSC-IL15 could affect the regulatory T cells population in this study, and the intratumoral infiltration of CD4+ FoxP3+ cells was examined by flow cytometry. The administration of MSC-GFP led to increased localization of regulatory T cells in the tumor site. Moreover, the mRNA level of FoxP3 was also increased by MSC-GFP. However, MSC-IL15 significantly inhibited the infiltration of regulatory T cells (Supplementary Fig. S5A and S5B). Furthermore, we performed an additional experiment in which MSC-GFP with tumor cells were coinjected, and 7 days later, MSC-IL15 was administered. As shown in Supplementary Fig. S5C, MSC-GFP coinjection with tumor cells modestly promoted tumor growth. However, MSC-IL15 was still able to abolish tumor development, suggesting that although MSC-GFP might possess immunoregulatory properties, IL15 steadily produced by MSC-IL15 secured the protumor effect of MSCs. Overall, these data clearly demonstrated that IL15-secreting MSCs possess highly potent antitumor activity in vivo.
MSC-IL15 can home to tumors and produce IL15 in vivo

It is well known that MSCs have a strong tropism property toward tumors (14,15). To test whether this property existed in our genetically modified MSCs, in vitro migration assays using Transwell plates were performed. We found that only a few cells migrated toward medium, whereas the migration of genetically modified MSCs was significantly increased by the conditional medium of Pan02 cells. Moreover, we confirmed that migration of unmodified MSCs toward the conditional medium was similar to MSC-IL15, suggesting that IL15 transfection did not affect the ability of MSC migration (Fig. 4A).

To further investigate whether genetically modified MSCs migrate toward Pan02 tumor in vivo, MSC-IL15 was labeled using the cell tracker dye PKH-26 (red). Labeled MSC-IL15 was intravenously administered to mice bearing Pan02 tumors. Three days after the administration of MSC-IL15, the animals were sacrificed and tumor tissues were harvested. We found that these cells accumulated into the tumor bed. To better investigate the intratumoral persistence and survival of MSC cells, we further detected the bioluminescent imaging of the profile of tumor at 7, 15, and 21 days after MSC-15 injection. Interestingly, we found that initially, MSC-IL15 was expanded in tumor site, and persistence lasted for more than 21 days with gradual decrease (Fig. 4B). In addition, PKH-26-positive cells were not observed in the lung, liver, and spleen tissue (data not shown).

We further tested the level of systemical IL15 and intratumoral IL15 following MSC-IL15 therapy. Results indicated that the mice treated with MSC-IL15 showed a dramatically increase in IL15 concentrations of tumor tissue, compared with controls. There was merely modest increase of serum IL15 level after MSC-IL15 therapy (Fig. 4C). Taken together, these data showed that MSC-IL15 was able to target tumor tissue, and effectively produced intratumoral IL15.

CD8$^+$ T and NK cells were required for MSC-IL15-mediated antitumor efficacy

To investigate possible involvement of NK or CD8$^+$ T cells as effectors in MSC-IL15-mediated antitumor activity, we treated Pan02 tumor-bearing mice with anti-asialo-GM1 or anti-mouse CD8 antibody to eliminate NK or CD8$^+$ T cells. Compared with the group receiving...
MSC-IL15 therapy alone, depletion of CD8\(^+\) T cells significantly but not completely eliminated the antitumor efficacy. Administration of anti-asialo-GM1 also reversed the tumor inhibition. Simultaneous depletion of both CD8\(^+\) and NK cells abrogated antitumor efficacy mediated by the MSC-IL15 therapy (Fig. 5A). Animal survival experiments also showed that depletion of CD8\(^+\) or NK cells decreased the survival of mice bearing Pan02 tumors, compared with the group receiving MSC-IL15 therapy alone (Fig. 5B). Thus, these studies supported that CD8\(^+\) T cells played a major role in the regimen-mediated antitumor efficacy whereas NK cells also contributed to the therapeutic efficacy.

**Induction of tumor-specific long-term memory response by MSC-IL15**

One of the beneficial characteristics of T-cell–mediated immunity is the development of an effective memory response. To determine whether a tumor-specific memory response was established in animals that long-term survived after MSC-IL15 treatment, effector cells were generated from the splenocytes obtained from the Pan02 tumor-bearing mice 10 days after MSC-IL15 treatment by culture with the irradiated Pan02 cells. Only cells from MSC-IL15–treated mice exhibited specific lysis activity toward Pan02 target cells (Fig. 6A), and CTL activity was not observed against syngeneic B16 cells (Fig. 6B), indicating that MSC-IL15–induced CTLs are tumor specific. Furthermore, we compared the tumor-specific CTL activity between MSC-IL15 and IL15 treatment groups. The results showed that the specific lysis activity of Pan02 cells in MSC-IL15 group was significantly higher than that of IL15 group (Fig. 6C). In addition, we also performed ELISPOT assays to determine whether MSC-IL15 could activate tumor-specific T cells. Our data clearly showed that MSC-IL15 could effectively promote tumor-specific T-cell–secreting IFN\(\gamma\), and had a much stronger activity than IL15 treatment (Fig. 6D).

To test whether immunologic memory was induced by MSC-IL15, the animals survived for 100 days were subsequently rechallenged with the same tumor cells Pan02. The syngeneic tumor B16 cells were used as controls. Mice cured of Pan02 tumors rejected subsequent challenges with the same tumor, and remained tumor free until the end of the experiment, whereas the mice could not reject syngeneic unrelated B16 tumor in control group (Fig. 6E), suggesting that mice developed a long-lasting memory against the same tumors, which was consistent with results of CTL assays. Moreover, all mice rechallenged with Pan02 cells showed a long-time survival, and control mice died of progressive tumor growth within 40 days (Fig. 6F).

In additions, adoptive transfer experiment was performed to determine whether this immunologic memory could be transferred into naïve mice. Recipient mice (C57BL/6) were challenged with Pan02 tumor cells 1 day after the adoptive transfer of total splenocytes (bulk cells) from cured mice. We observed tumor protection in 100% of these mice, and these mice remained tumor free until the end of the experiment. Adoptive transfer with purified CD4\(^+\) or CD8\(^+\) T cells resulted in partial protection over the control group. However, tumor developed rapidly in control group of mice, adoptively transferred with spleenocytes from naïve mice. All control mice died within 40 days of tumor challenge. Adoptive transfer with bulk cells protected mice (Fig. 6G and H). Thus, these results suggested that MSC-IL15 treatment induced tumor-specific memory T cells and had a long-term antitumor effect. This also implied that this therapeutic strategy could potentially elicit protection for patients with pancreatic tumor from cancer relapse or metastasis, as well as to eliminate the primary tumor.

**Discussion**

Although in several studies IL15 was an effective cytokine that could induce eradication of experimental tumors...
To investigate whether IL15-producing MSCs also induce antitumor effect of MSC-IL15 was mediated by NK cells. Recently, some studies demonstrated that MSCs had immune-regulation capacity, and thus might contribute to the growth of tumor (34). Moreover, indeed, we found that MSCs were able to reduce the activation of NK cells by IL15 stimulation in vitro. We also observed that MSC-GFP treatment increased mild accumulation of Treg in tumor site. However, compared with PBS treatment, we did not observe significant protumor growth effect of MSC-GFP treatment group in vivo. Li and colleagues demonstrated that MSCs might be a double-edged sword in regulating immune responses. MSCs were also able to enhance immune responses upon stimulation of inadequate inflammatory cytokines (34). In addition, we postulated whether part of MSCs could differentiate into other tissue immune cells in vivo, including immunocompetent cells, leading to reduced immuno-regulatory properties. Moreover, we observed that the Treg accumulation was significantly inhibited by MSC-IL15, suggesting that IL15 was able to reverse MSC-induced Treg. Thus, although the underlying mechanism needs to further clarified, MSC delivery system might not affect the antitumor activity of IL15 in vivo.

NK and T cells are known to play crucial roles in killing tumor cells. Several reports have described that infiltration of NK and T cells into tumor could be enhanced by IL15 therapy (8, 10). Consistent with previous reports, in our study NK- and T-cell populations in Pan02 tumor site were significantly accumulated by MSC-IL15 therapy. Through NK cells depletion in vivo, we found that the antitumor effect of MSC-IL15 was mediated by NK cells. To investigate whether IL15-producing MSCs also induce

Figure 6. Induction of tumor-specific long-term immunity by MSC-IL15 in Pan02 pancreatic tumor model. A and B, induction of tumor-specific CTLs after MSC-IL15 injection. Ten days after MSC-IL15 treatment, the splenocytes were isolated and restimulated in vitro for 5 days with the irradiated Pan02 cells and assayed for CTL activity against 51Cr-labeled Pan02 (A) and B16 cell targets (B). C, tumor-specific CTL activity of IL15-treated mice was also determined by CTL assay. D, ELISPOT assay. After 10 days of MSC-L or IL15 treatment, spleen cells were isolated and stimulated by Pan02 cells. The number of IFNγ+ cells was determined by ELISPOT assay. All experiments were repeated in triplicate. Columns, mean; bars, SE. E and F, rechallenge experiments. Tumor-free mice were rechallenged either with Pan02 or unrelated syngeneic B16 tumor cells. Tumor growth and survival were recorded over time. G and H, adoptive transfer experiments. Antigen-specific lymphocytes were generated from tumor-free mice of 100 days of posttumor challenge, and CD4+ and CD8+ T cells were purified by magnetic bead cell sorting. Stimulated cells were infused i.v. into naïve mice. On the next day, mice were challenged with Pan02 tumor cells. Control mice were infused with splenocytes from naïve mice. Tumor growth and survival were recorded over time. * P < 0.05; ** P < 0.01, versus control groups.
adaptive immune responses, we performed the depletion of CD8+ T cells in vivo. Indeed, our results supported that CD8+ T cells also contribute to the effect of MSC-IL15. Moreover, when compared with NK cells, CD8+ T cells might play a major role in the antitumor activity of MSC-IL15. We should also note that in addition to NK and CD8+ T cells, other immune cells may also mediate therapeutic effects of IL15. Di Carlo and colleagues demonstrated that IL15 gene transfer induced tumor rejection without T and NK cell involvement, which could be mediated by anti-tumor-specific antibodies, due mainly to B cells and the cross-talk between innate immunity and B cells (35). IL15 could also enhance antitumor response of DC vaccine, through increasing the capacity of DC to prime specific CD8 T cells (36, 37). Recently, Yu and colleagues found that combination cyclophosphamide and IL15 combination mediated antitumor effect depends not only on NK cells, but also γδ T cells (38). In fact, we have observed that γδT cells were also accumulated in tumor site, and Treg expansion was decreased after MSC-IL15 treatment. Thus, in this study, we could not exclude the possibility that there are other mechanisms for the antitumor effect of MSC-IL15.

The most interesting finding of this study is that application of MSC to deliver IL15 could elicit a strong tumor-specific memory T-cell response. In vitro CTL assay showed that the CTL responses were directed to Pan02 tumor cells, not other tumors. ELISPOT results further indicated that tumor memory CD8+ T cells were induced and expanded by MSC-IL15. Accumulative evidence demonstrated that the main nonredundant role of IL15 seemed to be in the maintenance of long-lasting T-cell immunity by supporting the proliferation and survival of memory CD8+ T cells (5). In fact, the tumor-free mice cured of Pan02 tumor were able to reject subsequent rechallenges with the identical tumor cells. The ability to transfer immunity by the infusion of CTLs indicated the presence of tumor-specific memory T cells in tumor-protected mice. Moreover, we found that both CD4+ and CD8+ T cells seemed to be required for achieving tumor protection. Thus, CD4 effector memory T cells might also be induced by MSC-IL15 in this setting. In addition, interestingly, although IL15 is well known to be able to induce memory CD8+ T expansion, no significant tumor-specific CTL response was observed in IL15 single injection, compared with MSC-IL15 treatment group, as demonstrated by in vitro CTL assay and ELISASPOT. These data suggested that tumor-specific memory CD8+ T expansion might need persistent IL15 stimulation, particularly in tumor site. However, because of rapid renal clearance (8), single injection of IL15 could not reach effective concentration for induction of tumor-specific CTL response. In contrast, MSC-IL15 was able to target the tumor tissue, and constitutively secreted IL15, leading to development of long-term tumor-specific memory CTL response.

It is well known that recombinant adeno-associated virus serotype 2 (rAAV2) has been used in human clinical trials because of low immunogenicity and long-term gene expression. Recently, Yang and colleagues also demonstrated that rAAV2-hIL15 was effective for human glioblastoma treatment (39). However, compared with rAAV2, lentivirus was easy to manipulate and its gene expression was more stable. Moreover, our aim was to prove the concept that the MSC carried IL15 has a strong antitumor effect. Thus, lentivirus was chosen to deliver IL15 in our study. rAAV2-IL15 transfected MSC will be further constructed and the anti-tumor property between them will be evaluated to better characterize whether lentivirus-carried IL15 has a superior effect than the rAAV2 carried.

Toxic effects related to IL15 have been described in several reports that high systemic level of IL15 could lead to NK or T leukemias (40, 12). In this study, there was an absence of high systemic level of IL15, suggesting that MSC-IL15 may be able to reduce undesired side effects. Moreover, we also did not observe intravenously administered MSC-IL15 homing in other tissues, which is consistent with previous reports (19, 20). This property adds advantage toward using MSC to deliver IL15 in avoiding organ toxicity. Furthermore, because of IL15 gene modified MSCs constitutively secrete IL15 in tumor location, repeated injection of recombinant IL15 would be avoided. Moreover, the low immunogenicity of MSCs made them a prime candidate for prolonged gene expression in allogeneic recipients, or in the xenogeneic setting (41). Taken together, preferential homing of MSCs to tumor sites and the antitumor effects of genetically modified MSCs provided the possibility that this new method could potentially be translated in clinic for tumor therapy in future. Further studies investigating the fate of the modified MSCs and its long-term effects in vivo are warranted to better characterize this strategy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Hu, K. Fan, G. Jin
Development of methodology: W. Jing, X. Zhou, Y. Zhou, K. Fan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Jing, Y. Chen, L. Lu, K. Fan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Chen, L. Lu, C. Shao, Y. Zhang, L. Wu, R. Liu, K. Fan, G. Jin
Writing, review, and/or revision of the manuscript: L. Wu, K. Fan, G. Jin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Zhang, X. Zhou, Y. Zhou, K. Fan
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