LIGHT Delivery to Tumors by Mesenchymal Stem Cells Mobilizes an Effective Antitumor Immune Response

Weibin Zou¹, Huilin Zheng¹, Tong-Chuan He², Jinjia Chang¹, Yang-Xin Fu¹,³, Weimin Fan¹, ⁴

¹Program of Innovative Cancer Therapeutics, The First affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China; ²Department of Surgery and ³Pathology and Committee on Immunology, The University of Chicago, Chicago, Illinois, USA. ⁴Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina, USA;

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Addresses to Correspondence to: Weimin Fan, The First affiliated Hospital of Zhejiang University School of Medicine, Hangzhou, China, or Department of Pathology and Laboratory Medicine, Medical University of South Carolina, SC 28425, USA. Phone: 843-860-6816; Fax: 843-793-0368; E-mail: fanw@zju.edu.cn or fanw@musc.edu

Yang-Xin Fu, Department of Pathology and Committee on Immunology, The University of Chicago, Chicago, Illinois 60637, USA. Phone: (773) 702-0929; Fax: (773) 834-8940; E-mail: yfu@midway.uchicago.edu

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Abstract

Bone marrow-derived mesenchymal stem cells (MSCs) have been shown to home to tumor tissues, where they promote tumor growth and suppress immune rejection. In this study, we tested whether MSCs engineered to express the immune stimulating factor LIGHT, a member of the tumor-necrosis factor superfamily, could induce tumor regression. Using in vitro and in vivo migration assays, we found that LIGHT-expressing MSCs (MSC-L) displayed a strong tropism for tumor tissues. MSC-L treatment activated the LIGHT signaling pathway, effectively organizing a potent anti-tumor immune response that stimulated an influx of T cells and inhibited tumor growth in vivo. CD4 T cells were found to play a role in the induction phase of the immune response, and CD8 T cells were shown to be essential for the effector phase. Together, our findings indicate that MSCs can effectively home to and deliver immune stimulating molecules to tumor tissues, thereby reversing the immune-suppressive environment, promoting anti-tumor immunity, and inhibiting tumor growth.
Introduction

Tumor tissues consist of cancer cells and stromal cells. These non-tumor cells may provide suitable niche for tumor progression, and act as barrier to block anti-tumor agents into tumor tissues (1). Recent studies have suggested that MSCs were potential sources of tumor related stroma (2-6). MSCs mainly reside within the bone marrow. They are characteristically able to differentiate into osteoblasts, chondrocytes, adipocytes and other cells (7-9). Moreover, these cells can migrate into tumor and participate in tumor development (2-5, 10-12).

It is increasingly recognized that MSCs can exert immune suppression against host eradication of tumor. T cell anergy can be induced by the lack of costimulatory molecules CD40, B7-1 and B7-2 on MSCs (10, 11). In addition, MSCs can induce immune tolerance via secretion of some soluble inhibitory factor (13). On the other hand, owing to their potent tropism to tumor tissues, MSCs could be ideal vehicles for targeted delivery of anti-tumor agents to tumor cells. It has been reported that the approaches could mediate tumor regression to certain extents (14-16). However, there were some limitations with these previous studies. For example, some of research just only mixed with modified MSCs with tumors (15) or used xenograft animal model (16). Furthermore, there were rare reports investigating how to reverse tumor suppressive microenvironments when MSCs used as vehicle.

LIGHT, an acronym for homologous to lymphotoxin, shows inducible expression, and competes with herpes simplex virus glycoprotein D for HVEM, a receptor expressed by T lymphocytes, is a member of the TNF family. LIGHT has a potent, CD28-independent, costimulatory role in T cell activation (17). LIGHT can interact with lymphotoxin-β receptor (LTβR) and HVEM expressed on stromal cell and T cell respectively (18). Constitutive
expression of LIGHT can enhance the extravasation and homing of naïve T cells (19, 20). Moreover, recent data further confirmed the role of LIGHT in priming potent anti-tumor immunity (17, 21-25). For instance, we have showed that forced expression of LIGHT in the tumor could induce a massive infiltration of naïve T cells into tumor tissues. Activation of these T cells subsequently led to the rejection of established, highly progressive tumors at local and distal sites (22). Therefore, LIGHT is an excellent candidate molecule to target tumors by MSCs.

In this study, we investigated whether a stable expression of LIGHT in MSCs (MSC-L) can target delivering LIGHT into tumor tissues, and thereby reverse tumor immunosuppressive environment via LIGHT priming immunity against tumors. Our data showed that MSCs efficiently delivered LIGHT into tumor tissues. We observed that MSC-L enhanced T cell infiltrating into tumors and reversed the local suppressive environment, thereby efficiently control tumor in LIGHT dependent adaptive immunity.
Materials and Methods

Mice and cell lines. Female aged 6-8 weeks of BALB/c mice were from Shanghai SLAC animal facility. All animal care and experiments were conducted according to Zhejiang University Animal Care Committee guidelines. The murine breast tumor cell line TUBO and human embryonic kidney derived cell line AD293 were cultured in DMEM with 10% FCS.

Isolation, culture and retroviral transduction of marrow MSCs. A female 6-week-old BALB/c mouse was sacrificed, and whole bone marrow was retrieved by flushing the bones with DMEM. All of the bone marrow cells were then cultured in DMEM with 15% FCS for 3 days. The cultured supernatant was harvested as MSCs conditional medium and the nonadherent hematopoietic cells were discarded (7, 9). The adherent cells were then washed three times with PBS, added 10ml of DMEM with 10% FCS. When the cells grew to 60% confluence, the cells were transfected with LIGHT expressing retrovirus vector.

For packaging LIGHT expressing retrovirus, AD293 cells were plated with cell density about 50%. After 3-5 hours, the retrovirus was produced by transfecting AD293 with 250 μl Opti-MEM plus 37.5 μl of a solution made by mixing 15 μl LipofectAMINE (Invitrogen) with 7.5 μl of the packaging plasmid pAmpho and 15 μl of pSEB-LIGHT (22). 3-5 hours later, the medium was discarded and replaced with 4 ml fresh complete DMEM. The retrovirus supernatants were collected at 36, 60 and 84 hours respectively. The generated virus were kept at 4°C, or directly used to infect MSCs.

LIGHT gene-modified MSCs were generated by transduction of MSCs twice per day for 2 consecutive days; for more efficient transduction, before 6 hours of adding the retrovirus supernatant, the medium was replaced with fresh MSCs conditional medium to promote MSCs
proliferation. 5.0 μg/ml of Blasticidin S (Sigma-Aldrich) selection started at the end of the second round infection. The expression of LIGHT on MSCs was determined by flow cytometry with LTβR-Ig (26).

**In vitro determination of MSC-L differentiation potential.** To determine *in vitro* differentiation potential of MSC-L, the medium was replaced with osteogenic and adipogenic medium respectively when MSC-L were about 70% confluence. For osteogenic differentiation, MSC-L was cultured for 4 weeks in osteogenic medium containing 10 nM dexamethasone, 50 μg/ml ascorbic 2-phosphate and 10 mM β-glycerol phosphate. To observe calcium deposition, the cultured cells were fixed with 4% paraformaldehyde, and stained with Alizarin Red S. To induce adipogenic differentiation, MSC-L was cultured in adipogenic medium containing 100 nM dexamethasone and 50 μg/ml indomethacine. After fixed with 4% paraformaldehyde, the cells were stained with Oil Red O solution (7, 27).

**In vitro and in vivo migration assay.** *In vitro* migration assay was performed according to the protocol previously described (6, 28). 2×10^4 MSC-L with 300ul medium was placed on the top well of the transwell plate with 8 mm pores (Millipore). 1×10^5 TUBO cells were transferred to the bottom wells in 800 μl of the same medium. The migration of MSC-L was assessed 12 h later. The migrated cells were then fixed, stained, and enumerated.

For *in vivo* migration assay, BALB/c mice were challenged with 4×10^5 TUBO cells in 200ul of PBS at the left flank of the animals. Ten days later when tumors were amount to 3×3 mm, 1×10^6 MSC-L labeled with PKH26 (Sigma Aldrich) were injected subcutaneously into the right flank. After two weeks, the migration was determined by bioluminescent imaging of the profile of tumor, and through frozen section (5-10 μm) of tumor, the images were acquired via
fluorescent microscope to determine the location of MSC-L in tumor tissues (28).

**Inoculation of MSC-L and tumor challenge.** To assess the efficacy of MSC-L in the prophylactic protocol, BALB/c mice were injected with $10^6$ MSC-L via s.c. in the left flank. About two weeks later, mice were challenged with $4 \times 10^5$ TUBO cells s.c. in the right flank. To study the therapeutic effects of MSC-L, mice were challenged with $4 \times 10^5$ TUBO cells in the right flank, about 7 days after tumor inoculation, the mice were received with $10^6$ MSC-L s.c. in the contralateral flank. Controls included mice injected with nontransduced MSCs or MSC-mock (only with vector).

**Cell deletion and in vivo blockade of LIGHT activity.** For *in vivo* cell deletion, mice were received the following mAbs via *i.p.* injection: anti-CD4 (clone GK1.5) 500 μg/injection, and anti-CD8 (clone 2.43) 500 μg/injection for consecutive 3 days respectively. Deletions were confirmed by analysis of blood samples using flow cytometry. To block LIGHT activity in mice, LTβR-Ig (100 μg per injection) was given intraperitoneally at the indicated time as described previously (22).

**Measurement of γ-IFN secreting T cells by ELISPOT assay.** The performance of ELISPOT was done as described previously (29). Briefly, $4 \times 10^5$ spleen cells (responder cells) were added to each well, which had been precoated with 2.5 μg/ml rat anti-mouse γ-IFN (clone R4-6A2; BD-pharMingen). TUBO 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 100 μl of 2 μg/ml biotinylated rat anti-mouse-IFN-γ (clone XMG1.2; BD-PharMingen) were added to incubate for another 12 hours at 4°C, then unbound antibody were removed. 100 μl of 0.9 μg/ml avidin-horseradish peroxidase (BD-PharMingen) were added and incubated at 20°C for 2
hours. The substrate AEC was added. 3-5 minutes later, the plates were washed and the spots were enumerated.

**ELISA.** After about 2 weeks of MSC-L treatment, tumor and spleen were collected and homogenized, the debris were spun down and supernatant were used for detection of IFN-\(\gamma\) ((BD optEIA\textsuperscript{TM}), IL-6 (BD optEIA\textsuperscript{TM}), IL-10 (BD optEIA\textsuperscript{TM}), TGF-\(\beta\) (abcam) with ELISA kit respectively.

**Histology.** Tumor tissues were collected at the indicated time, fixed in 10% neutral formalin, embedded in paraffin, and stained with hematoxylin and eosin, mAb to Ki67, or isotype Ab. TdT-mediated dUPT nick end labeling (TUNEL) staining was performed as protocol of the manufacturer. For CD31, CD4, CD8 staining, tumor tissues were embedded in OCT compound and frozen at -70°C. Frozen sections (5-7 \(\mu\)m) were stained with PE-conjugated antibodies to CD4 and to CD8 (BD pharmingen), and biotinylated goat antibodies to CD31 (ABcom), respectively.
Results

**MSC-L has multi-differentiation potential and strong tropism to tumor tissues.** MSCs have multi-differentiation capability and could home to tumor tissues (2, 28). To assess whether MSC-L still possess the above property, MSCs from six-week BALB/c mice were stably transduced with retrovirus expressing LIGHT. LIGHT was readily detected by LTβR-Ig on the surface by flow cytometric analysis (Fig. 1A). After transduction with LIGHT, MSC-L adapted similar mesenchymal stromal cell-like morphology (Fig. 1B). Further, mineral deposition was readily detected when MSC-L were stimulated with osteogenic medium, indicating that MSC-L have osteogenic potential (Fig. 1B). Meanwhile, the oil droplet was found in adipogenic medium, suggesting that MSC-L may differentiate into adipocytes (Fig. 1B). These results showed that MSC-L retained the MSC’s multi-differentiation potential.

We next determined whether MSC-L exhibited any tropism to tumors. With *in vitro* transwell experiments, we found that MSC-L possessed the potential of migration into tumors (Fig. 1C). For *in vivo* migration assay, we found that MSC-L may specifically migrate into tumor and mainly locate in tumor periphery (Fig. 1D, Fig. 1E and Suppl. Fig. 1B). In addition, the infiltration pattern of MSC-L in tumor was similar with MSC or MSC-mock (Suppl. Fig. 2). Further, LIGHT could be readily detected in tumor tissues after 2 weeks of MSC-L injection (Suppl. Fig. 1A). These findings suggest MSC-L still can home and thereby deliver LIGHT into tumor tissues.

**MSC-L controls tumor growth efficiently.** To determine whether MSC-L can help host resistant against tumor, we first tested whether MSC-L could prevent tumor formation when mice were challenged with lethal dosage of TUBO cells at right flank after two weeks of $1 \times 10^6$
MSC-L inoculation at the left flank. We observed that MSC-L could efficiently provide protection against tumor (Fig. 2A and Suppl. Fig. 3A). We then analyzed the therapeutic potential of MSC-L. The mice were challenged with $4 \times 10^5$ of TUBO cells. Seven days later, MSC-L were inoculated into the other flank. In contrast to control groups, we found that MSC-L can effectively repress tumor aggressive growth (Fig. 2B and Suppl. Fig. 3B).

**Tumor inhibition by MSC-L is not mainly due to tumor apoptosis or inhibition of angiogenesis and proliferation.** Tumor inhibition often ensues when cancer cells are counteracted by mechanisms such as growth arrest, impaired tumor vascularization, apoptosis, and immunosurveillance (30-33). To determine which mechanisms involve in MSC-L mediated-tumor control, tumor histology was assessed after about 2 and 4 weeks of tumor inoculation respectively. Ki67 was used to detect tumor cell proliferation. There were no difference of cell proliferation between MSC-L treated group and control group (Fig. 3A and Suppl. Fig. 5A), which indicated that tumor cell growth arrest was not mainly involved in tumor control in this model.

To determine whether apoptosis may contribute to tumor inhibition in our experiments, TUNEL were performed after about 2 and 4 weeks of tumor inoculation respectively. Only marginal apoptosis was observed for different groups (Fig. 3B and Suppl. Fig. 5B). Therefore, it is unlikely that tumor control in our experiments is mainly due to the enhanced tumor apoptosis.

Recently, reports showed that MSCs could contribute to the tumor vasculature formation (15, 34). To test whether tumor inhibition was due to the MSC-L reversion of the pro-angiogenic effect of MSC, we detected CD31 expression in tumor tissues after about 2 and 4 weeks of
tumor inoculation. The vessel density of MSC-L treated group is the same as control groups (Fig. 3C and Suppl. Fig. 5C), suggesting that tumor angiogenic inhibition might not be the main mechanism for MSC-L mediated tumor inhibition.

**Immune activation by MSC-L contributes to tumor regression.** Our previous data have shown that LIGHT could prime potent anti-tumor immunity (22, 24, 25). To determine whether expression of LIGHT on MSCs still possesses potent anti-tumor immunity, we first performed the experiments with nude mice. Unlike in the model of immune competent BALB/c mice, MSC-L could not provide host any protection against tumor challenge (Fig. 4A and Fig. 4B), which suggests that T cells might involve in the MSC-L induced tumor control.

Next, we examined the mechanism underlying MSC-L mediated-tumor control. Tumor tissues were assessed after 3 weeks of tumor inoculation. In contrast to MSCs treatment, MSC-L treated tumors showed profound of CD4+, CD8+ T cells infiltration (Fig. 4C), indicating that MSC-L may promote the infiltration of T cells into tumor tissues. Further, we tested whether MSC-L could activate tumor specific T cells by ELISPOT assays using tumor cells as antigen presenting cell (APC). Our data clearly showed that MSC-L could effectively promote tumor specific T cell secreting γ-IFN (Fig. 5A), which might in turn contribute to T cell mediated-tumor control.

Moreover, we observed that MSC-L may induce pro-inflammatory environment in local tumor tissues. After about 2 weeks of MSC-L injection, some cytokines in tumor tissues were detected with ELISA assay in therapeutic setting. As shown in Suppl. Fig. 4, the level of IFN-γ, IL-6 was largely increased, while IL-10, TGF-β decreased for MSC-L treatment. In addition, we found that in spleen, although IFN-γ level was enhanced to some extents for MSC-L treated
mice, but its level was still lower than in tumor tissues, which indicated that after migration into tumors, MSC-L may largely enhance local inflammation reaction.

Further, we analyzed whether CD4 and CD8 T cells were essential in maintaining tumor control. Either in prophylactic protocol or therapeutic protocol, we observed that, in the induction stage before tumor challenge, CD4 T cells played an important role in tumor control. At this stage, deletion of CD4 T cells but not CD8 T cells could completely abort the MSC-L induced anti-tumor immunity (Fig. 5B and Fig. 5C). Whereas in the effector stage, CD8 T cells were essential for tumor control, since deletion of CD8 T cells largely promoted tumor growth after short time of the continued inhibition (Fig. 5D and Fig. 5E); nevertheless, with CD4 deletion in effector stage, the host could still repress tumor growth (Fig. 5D and 5E).

Additionally, for the tumor-free or dormant tumor-bearing mice (surgical removal of occult tumors before tumor re-challenge) treated with MSC-L, when re-challenged with $1 \times 10^6$ TUBO, they still resisted against tumor growth (Table 1 and Suppl. Table1), which suggests the presence of immune memory for MSC-L treated mice.

**Priming immunity of MSC-L was mainly dependent on LIGHT.** LIGHT has potent capability of priming T cell activation independent of CD28 signaling (17). Our previous studies have shown that expression of LIGHT in the tumor can greatly enhance host resistance to tumor (22, 24). MSCs might have many molecules that are regulated after transfection of LIGHT. To investigate whether LIGHT is essential for MSC-L mediated- anti-tumor immunity, we first injected LTβR-Ig intraperitoneally to block LIGHT activity *in vivo* before MSC-L injection. Interestingly, in contrast to the mice without blockade of LIGHT, via consecutive 3 days injection of LTβR-Ig, the tumor posed with aggressive growth tendency (Fig. 6A and 6B).
Next, we tested whether injection LTβR-Ig after MSC-L inoculation can still abolish MSC-L protection against tumor. At initial duration, for the mice receiving LTβR-Ig after about 1 week and 3 weeks of MSC-L inoculation respectively, the host could continue to resist tumor growth for about 2-3 weeks, but after that time point, the tumors grew aggressively again (Fig. 6C and 6D), suggesting blockade of LIGHT activity could completely abolish the anti-tumor effect of MSC-L. We then tested whether the blockade of LIGHT activity could delete MSC-L priming T cells activation. Through ELISpot assays for γ-IFN secretion of T cells, we observed that blockade of LIGHT nearly completely inhibited the T cells producing γ-IFN (Fig. 6E). Clearly, these data present strong evidence that LIGHT signal pathway is essential for MSC-L priming anti-tumor immunity.
Discussion

Tumor micro-environment is immunosuppressive (35, 36), and tumor stroma is often referred as barrier blocking anti-tumor agents into tumors. Since MSCs can home to tumor, MSCs have been used as vehicle for tumor therapy (14, 16). However, owing to the immunosuppression of MSCs (3, 10, 11, 13, 37-39), the efficacy of the treatments would be largely compromised. How to circumvent its immunosuppression remains illusive when MSCs used as vehicle. Herein, we have shown that expression of LIGHT on MSCs can break tumor barrier and prime anti-tumor immunity to control tumor growth. First, MSC-L can migrate and thereby target delivery of LIGHT into tumor; Second, the tumor control of MSC-L was not due to induction of tumor apoptosis, or inhibition of tumor angiogenesis and proliferation; Third, the infiltration of T cells and pro-inflammatory factor level were increased in tumor tissues with MSC-L treatment. Furthermore, we observed that CD4 T played a role in induction phase, while CD8 T was essential for effector phase. In addition, γ-IFN producing T cells were increased in lymphoid tissues and immune memory existed with MSC-L treatment. Fourth, LIGHT is essential for MSC-L priming anti-tumor immunity, suggesting the direct effect of LIGHT-expressing MSCs on tumor control. These findings indicate that MSC-L can circumvent the tumor immunosuppressive environment and may be used as vehicle for tumor treatment.

The ability of priming T cell activation in tumors by MSC-L is an intriguing finding. MSCs have been broadly recognized as immunosuppressors (3, 10, 11, 13, 37-39). Nevertheless, recent studies suggested that, in specific conditions, MSCs might be able to behave as conditional APC to prime immune reactions. For instance, MSCs can cross-present soluble
exogenous antigens to activate CD8 T cells (40). MSCs may also act as APC to prime CD4+ T cell proliferation (41, 42). More recently, report showed that MSCs expressing ErbB-2/neu elicited full protective anti-tumor immunity *in vivo* (43). In line with the above findings, we observed that MSC-L can prime anti-tumor immunity to control tumor. Since tumor targeted LIGHT not only can recruit DC and T cells into tumors, but also has a potent, CD28-independent, costimulatory role in priming T cell activation (17); whereas MSCs are usually lack of costimulatory molecules CD40, B7-1 and B7-2, which may induce T cell anergy when interact with T cells (13, 37). In this regard, it remains interesting to investigate whether MSC-L, after migration into tumor, may directly act as APC via LIGHT as costimulatory molecule to prime immunity against tumor, so as to distinct from the role of LIGHT itself directly priming potent anti-tumor immunity.

Tumor stromal cells are critical for tumor growth and immune monitoring. Reports showed that destruction of tumor stromal cell was essential for eradication of established tumors (1, 44-47). However, the source of tumor stromal cells has not been well defined. MSCs have been suggested as one source of tumor stroma. Some reports showed that, after migration into tumor, MSCs can differentiate into carcinoma-associated fibroblasts (CAFs) and pericytes (2, 3, 5, 6, 15). It has been observed that at least 20% of CAFs derived from MSCs (5). Recently, another report even showed that epithelial-mesenchymal transition-derived (EMT) cells might exhibit multilineage differentiation potential similar to MSCs (48). It has been increasingly recognized that tumor cells may experience EMT transition, especially in the case of metastasis. The current study suggests that MSC-L likely survive for more than 14 days before tumor inoculation and could prime T cells infiltrating into tumor tissues, and thereby reverse
suppressive environment leading to tumor regression. Meanwhile, since LIGHT has potent priming-immunity activity, overexpression of LIGHT may incur auto-immunity (17, 21); Accordingly, it remains to be determined whether expression of LIGHT on MSCs can prime anti-MSCs immunity and subsequently lead to destruction of MSCs, and even to destruction of tumor stroma as well as tumor cells when they experienced EMT transition.

Some studies have shown that MSCs can inhibit Kaposi sarcoma (KS) and some breast tumors. It was believed that MSCs inhibition of KS cells is mediated by a contact-dependent inhibition of Akt-signaling (49), while MSCs inhibition of mammary carcinoma is via stimulating apoptosis by cleavage of PARP-1 and caspase-3 (50). In the current study, our results support that MSC-L mediated-tumor control is not due to MSC-L direct contact inhibition of tumor cells. There were no apparently apoptosis or arrested cells in tumor tissues with MSC-L treatment. Instead, there were large number of T cell infiltration into tumor tissues, and deletion T cell may abolish MSC-L’ anti-tumor effect. Further, in the experiments with nude mice, treatment with MSC-L, MSCs or PBS has no different effect on tumor growth. Presently, the complex interaction of MSCs and tumors is poorly defined. Whether MSCs inhibit tumor growth may depend on specific conditions, which vary widely as a function of the source of MSCs and the tumor model used. Accordingly, how to decipher the mechanism of the interaction between MSCs and tumor cells will largely benefit to MSCs as the vehicle for tumor treatment.

We previously tested that LIGHT, when expressed in tumor tissues, can prime potent anti-tumor immunity leading to the clearance of well established tumor at local and distal sites (22). More recently, we further observed that targeting the primary tumor with Ad-LIGHT can
generate CTL to effectively eradicate the metastasis tumor (24). In the present experiments, although we observed large numbers of T cell infiltration into tumor tissues and T cells activation by MSC-L, we do not know why MSC-L can not reject the established tumor, but only repress tumor aggressive growth and maintain host-tumor in an equilibrium state. Some previous works have supported this notion that, when MSCs in the milieus of inflammation, MSCs might increase the production of large arrays of chemokines and immunosuppressive factors (13, 37). This indicates that MSCs may respond to inflammatory cytokines by attracting immune cells via chemokine secretion and thereby suppress these immune cells. Accordingly, although MSCs have been exhibited to act as APC to prime anti-tumor immunity in specific conditions, it remains unclear in our model whether MSC-L, when engraft in tumor tissues, still possesses some kind of immunosuppression, which would partly compromise the LIGHT-primed immunity.

Overall, our data showed that MSC-L could specifically migrate into tumor and control aggressive tumor growth by priming potent anti-tumor immunity. This study has addressed one efficient way to deliver immune stimulating factor LIGHT into tumors by MSCs and an approach of circumventing the immunosuppression of tumor environment via LIGHT. Since MSCs, as tumor stroma, play a pivotal role in tumor development and may compromise clinic treatment, this strategy has potential for translation to clinical application.
Acknowledgments:

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References


Table 1

MSC-L prevents tumor growth and induces anti-tumor immune memory

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>First TUBO challenge</th>
<th>Incidence of tumor growth</th>
<th>Surgical removal(^a) and tumor re-challenge</th>
<th>Incidence of tumor growth</th>
<th>percent</th>
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<tbody>
<tr>
<td>1</td>
<td>PBS</td>
<td>4x10(^5)</td>
<td>8/8</td>
<td>1x10(^6)</td>
<td>6/6</td>
<td>100%</td>
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<tr>
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<td>MSCs</td>
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<td>8/8</td>
<td>1x10(^6)</td>
<td>8/8</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>MSC-L</td>
<td>4x10(^5)</td>
<td>2/8(^b)</td>
<td>1x10(^6)</td>
<td>0/8</td>
<td>0</td>
</tr>
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<td>None</td>
<td>None</td>
<td></td>
<td>1x10(^6)</td>
<td>6/6</td>
<td>100%</td>
</tr>
</tbody>
</table>

\(^a\) Tumor free mice were directly re-challenged with 1x10\(^6\) TUBO cells; Mice bearing tumors were re-challenged with 1x10\(^6\) TUBO cells after surgical removal of the tumor. \(^b\) Of the 8 mice treated with MSC-L, the tumors of 2 mice maintained in dormancy.
Legends

Figure 1 MSC-L has multi-differentiation potential and can migrate into tumor tissues.
(A) LIGHT expression on MSCs, as determined with LTβR-Ig by flow cytometry. (B) The morphology of MSC-L, the differentiation of MSC-L to adipocytes (Oil-Red-O staining) and osteoblasts (Alizarin Red S staining; magnification, x20). (C) in vitro transwell migration assays. All experiments were repeated in triplicate, with five fields of view counted on each membrane. Columns, mean; bars, SE. (D) in vivo MSC-L migration assays, bioluminescent imaging of the profile of tumor to detect MSC-L labeled with PKH26 in tumor tissues. (E) Sections of tumor tissues (5-um thick) were counterstained with DAPI (blue) to visualize nuclei and PKH26-labelled MSC-L (red), showing that MSC-L incorporated into tumor periphery.

Figure 2 MSC-L controls tumor aggressive growth. (A) For prophylactic protocol, BALB/c mice (eight per group) were injected with 1x10^6 MSC-L s.c. in the left flank and 13 days later, the mice were challenged with 4x10^5 TUBO in the contralateral flank. P values were determined by one-way ANOVA test and log-rank test respectively; P<0.001. (B) For therapeutic protocol, BALB/c mice (eight per group) were challenged with 4x10^5 TUBO in right flanks 7 days before 1x10^6 of MSC-L inoculation in the left flanks, MSCs or PBS as controls. Points, mean for each group of mice; bars, SD. P value was calculated using one-way ANOVA test and log-rank tests, respectively; P<0.001.

Figure 3 MSC-L-mediated tumor regression is mainly not associated with tumor proliferation, tumor apoptosis and angiogenesis. Mice tumor model were set up in the
prophylactic and therapeutic protocol respectively, as described in Figure 2. At 4 weeks of TUBO challenge, tumor tissues were collected and immunohistochemistry was performed with Ki67 staining (A), TUNEL assay (B), and CD31 detection (C).

**Figure 4** MSC-L protects against tumor by priming potent immune reactions. (A and B) for nude mice of animal model in the prophylactic protocol (A) and therapeutic protocol (B), the tumor growth in MSC-L group has no statistic difference with the control groups. (C) Immune competent BALB/c mice were treated with MSC-L or MSCs in the prophylactic and therapeutic protocol respectively as described aforementioned, and tumor tissues were collected at 3 weeks after tumor challenge. Sections were stained with H&E (a, d, g, j) or with PE-conjugated anti-CD4 (b, e, h, k) or anti-CD8 (c, f, i, l). Images are representative of three to five groups of MSC-L or MSCs treated mice in the prophylactic and therapeutic protocol respectively.

**Figure 5** CD4+ cells are essential in induction phase while CD8+ cells in effector phase for MSC-L mediated-tumor control. (A) Mice were treated with MSC-L in the prophylactic and therapeutic protocol respectively. After 7 days of MSC-L inoculation, spleen cells were isolated and used for ELISPOT assay. All experiments were repeated in triplicate, Columns, mean; bars, SE. (B and C) either in the prophylactic protocol (B) or in the therapeutic protocol (C), deletion of CD4 with GK1.5 before 3 days of tumor challenge (induction phase) abolished the anti-tumor effect of MSC-L. (D and E) In effector phase after about 12 days of tumor challenge, either in prophylactic protocol (D) or therapeutic protocol (E), deletion of CD8, but not deletion of CD4 promoted tumor growth after short time of continued-repression.
Figure 6 The anti-tumor immunity of MSC-L is mainly dependent on LIGHT. Mice were treated with MSC-L or MSCs in the prophylactic and therapeutic protocol respectively. (A and B) Either in prophylactic protocol (A) or therapeutic protocol (B), blocking LIGHT with LTβR-Ig for consecutive 3 days before MSC-L inoculation can abort MSC-L protection against tumor. (C and D) Blockage of LIGHT after MSC-L inoculation also abolished MSC-L protection against tumor. In prophylactic protocol (C) or therapeutic protocol (D), blocking LIGHT with LTβR-Ig after about 1 week and 3 weeks of MSC-L inoculation respectively, abrogated the protection of MSC-L against tumor growth. (E) Mice were treated with MSC-L in the prophylactic and therapeutic protocol respectively. With 100ug LTβR-Ig blockage of LIGHT signaling, 3 days later, spleen cells were isolated and used for ELISPOT assay. All experiments were repeated in triplicate, Columns, mean; bars, SE.
Figure 2

A

MSC-L or MSC
inoculation

D-13

D0

TUBO challenge

Tumor volume (mm³)

Days after tumor inoculation

Percent survival

Days after tumor inoculation

PBS
MSC
MSC-L

B

D0

D7

TUBO challenge

MSC-L or MSC
inoculation

Tumor volume (mm³)

Days after tumor inoculation

Percent survival

Days after tumor inoculation

PBS
MSC
MSC-L
Figure 3

A  Prevention  Therapeutics

Ki67 detection

B  

Tunel assay

C  

CD31 detection

MSC  MSC-L  MSC  MSC-L
Figure 4

A

B

C

Prevention

Therapeutics

H&E

Anti-CD4

Anti-CD8

MSC

MSC-L

MSC

MSC-L
Figure 5

A

![Graph showing IFNγ+ cell count with PBS, MSC, and MSC-L treatments with P<0.001 difference between Prevention and Therapeutics](image)

B

<table>
<thead>
<tr>
<th>D-13</th>
<th>D-3</th>
<th>D0</th>
</tr>
</thead>
<tbody>
<tr>
<td>MSC</td>
<td>MSC-L</td>
<td>TUBO</td>
</tr>
</tbody>
</table>

Tumor volume (mm³)

![Graph showing tumor volume over days after tumor inoculation](image)

C

<table>
<thead>
<tr>
<th>D-3</th>
<th>D0</th>
<th>D7</th>
</tr>
</thead>
<tbody>
<tr>
<td>GK1.5 or 2.43</td>
<td>TUBO</td>
<td>MSC or MSC-L</td>
</tr>
</tbody>
</table>

Tumor volume (mm³)

![Graph showing tumor volume over days after tumor inoculation](image)

D

<table>
<thead>
<tr>
<th>D-13</th>
<th>D0</th>
<th>D12</th>
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</thead>
<tbody>
<tr>
<td>MSC</td>
<td>MSC-L</td>
<td>TUBO</td>
</tr>
</tbody>
</table>

Tumor volume (mm³)

![Graph showing tumor volume over days after tumor inoculation](image)

E

<table>
<thead>
<tr>
<th>D0</th>
<th>D7</th>
<th>D13</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUBO</td>
<td>MSC or MSC-L</td>
<td>GK1.5 or 2.43</td>
</tr>
</tbody>
</table>

Tumor volume (mm³)

![Graph showing tumor volume over days after tumor inoculation](image)
Figure 6

A

B

C

D

E

Prevention

Therapeutics

P<0.001

MSC-L+LTβR-Ig

MSC-L

MSC

IFN-γ+cell/2x10^5
LIGHT Delivery to Tumors by Mesenchymal Stem Cells Mobilizes an Effective Antitumor Immune Response

Weibin Zou, Huilin Zheng, Tong-Chuan He, et al.

Cancer Res  Published OnlineFirst April 17, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-11-4216

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2012/04/17/0008-5472.CAN-11-4216.DC1

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