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14. ABSTRACT

Bone metastasis remains an incurable condition in breast cancer patients and new therapeutic methods are urgently needed. In this study, bone marrow-derived mesenchymal stem cells (BM-MSCs), with intrinsically homing capability towards ``tumor wounds'', are selected as a natural vehicle to deliver anti-tumor agents into the bone lesions. However, due to MSCs' dual roles in pre-clinical models, MSC-delivered targeted therapy could be potentially impaired by their intrinsic tumor-promoting effects. Therefore we will try to ablate the major tumor-promoting mechanisms in MSCs to build a more effective cell-based tumoricidal vehicle in treating metastatic breast cancer. During the reporting period, in Aim 1 we have measured the engraftment efficiency and safety of the genetically engineered BM-MSCs in vivo, and then we optimized the administration routes and timing of these engineered MSCs for better therapeutic effects. In Aim 2, we found that the adoptive transfer of engineered BM-MSCs potentially accelerated the bone local T cell expansion, and the immune checkpoint inhibitors were able to improve the therapeutic effect of BM-MSC treatment in vivo. In the last Aim, we have constructed engineered human BM-MSCs and will apply them to treat bone metastases of human breast cancer cells in humanized mice model.

15. SUBJECT TERMS

Mesenchymal stem cells; cell engineering; bone metastases; breast cancer; cell-based cancer therapy; interferon-alpha; immunotherapy; humanized mice

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1. INTRODUCTION:

Mesenchymal stem cells (MSCs) are a type of adult stem cells with intrinsic capability to home to the wound sites (1, 2). As tumors function as wounds that do not heal (3, 4), recent studies have revealed that endogenous or exogenous MSCs could favorably and precisely migrate into the malignant lesions in various pre-clinical cancer models (5, 6). Such a mechanism has been utilized for genetic engineering of anti-tumor cytokines in MSCs for a targeted therapy (7-9). This is particularly critical for treating metastases which are difficult to detect and monitor by current medical technologies. In MSC-based therapeutic settings, MSCs exert dual roles: targeted delivery of anti-tumor agents, and their intrinsic tumor-promoting abilities (5, 10, 11). Therefore, when MSCs were given at middle to late stages of breast tumor progression, the therapeutic effects became minimal. In this study, we try to build the next generation of MSC-based cancer therapeutic by genetic ablation of tumor-promoting factors in MSCs in both syngeneic and humanized mouse models. To our knowledge, this is the first study to explore MSC-based delivery of anti-tumor agents in treating bone metastases of breast cancer. Our study will directly lead to clinically applicable new strategy in treating the incurable bone metastases of breast cancer.

2. KEYWORDS:

Mesenchymal stem cells; cell engineering; bone metastases; breast cancer; cell-based cancer therapy; interferon-alpha; immunotherapy; humanized mice

3. ACCOMPLISHMENTS:

1) What were the major goals of the project?

- Specific Aim 1: Optimize MSC constructs with genetic deletion of tumor-promoting genes in $IFN\alpha^{OE}$-MSCs.
- Specific Aim 2: Reveal mechanisms underlying the anti-tumor effects of engineered MSCs.
- Specific Aim 3: Adapt our mouse findings to treating bone metastases of human breast cancer with engineered human MSCs in the humanized NSG™-SGM3 mouse model.

2) What was accomplished under these goals?

- **Specific Aim 1: Optimize MSC constructs with genetic deletion of tumor-promoting genes in $IFN\alpha^{OE}$-MSCs.**

Major Task 1: Optimize the MSC administration protocol to treat bone metastases in mouse syngeneic model

➢ Subtask 1: Submit documents for ACURO approvals

Before the award was officially started (04/01/2018), we have submitted the animal protocol to ACURO and obtained the ACURO approval.

➢ Subtask 2: Construct stable *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} MSC lines and measure the engraftment efficiency and evaluate the safety of these MSCs in vivo

Under this subtask, we recently generated over 10 stable *iNOS*$^{-/-}$ bone marrow-derived MSCs (BM-MSCs) with *CCL-2* knockdown (KD) and *IFNα* overexpression (OE) using the lentiviral transduction system. The resultant *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} BM-MSC lines were further constructed to express luciferase and GFP dual-reporters to facilitate non-invasive detection and purification by bioluminescent imaging (BLI) and flow cytometry.

These MSC lines were administered into tumor-free Balb/c mice or orthotopically inoculated 4T1.2 tumor-bearing mice by intracardiac (i.c.) injection, and their engraftment and persistence were monitored by BLI. As shown in **Figure 1A**, the implanted BM-MSCs were found to preferably home to the bone among the tested organs at the early time point after their inoculation. Moreover, a higher level of engraftment of BM-MSCs was detected in the tumor-bearing mice compared to the tumor-free mice, confirming the capability of MSCs homing to the tumor-associated "wounds" (12).

The implanted BM-MSCs can persist in the recipient mice for no more than 3 weeks in both tumor-free and tumor-bearing conditions (**Figure 1B**). Further, the tumor-free mice had been monitored over 6 months and did not show irregular symptoms compared to the untreated mice. When administered into tumor-bearing mice, the genetically engineered BM-MSCs did not enhance the tumor progression. These data indicated that the engineered MSC lines will not persist in the recipients for a long term and they also do not directly form or promote malignancy development within the observation period. This supports the notion that MSCs are largely safe in vivo in treating cancer, degenerative diseases and autoimmune disorders as shown in a diversity of pre-clinical studies and most completed clinical trials (13). We plan to further measure the safety of MSC treatment by necropsies at month 9 following injections. The pathological conditions will be determined by a board certified pathologist in our histopathology sciences core at The Jackson Laboratory. This information will be critical for the clinical translation of this novel cell-based therapeutic.

➢ Subtask 3: Validate the therapeutic efficacy of *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} MSC in treating bone metastases in vivo; and Subtask 4 Optimize the mode and timing of genetically engineered *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} MSC administration

Under these two subtasks, we recently tested different inoculation routes and timing schedules for administrating *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} BM-MSC in order to find an optimal MSC-based treatment

protocol to achieve the best therapeutic effect. In a pilot experiment, we utilized a spontaneous 4T1.2 bone metastases model in the Balb/c mice (14). With this 4T1.2 model, we found a single i.c. injection of the engineered BM-MSCs had a comparable therapeutic effect in controlling the bone metastasis (**Figure 2A**) compared to the multiple injections as shown in our preliminary study. We therefore applied the single injection rather than multiple injections in the following studies to avoid any complications and stresses on the experimental animals.

Next we compared the intrafemoral (i.f.) injection route with i.c. injection using the same dose of $2X10^5$ engineered BM-MSC cells in the 4T1.2 spontaneous bone metastasis model. As shown in **Figure 2A**, the i.f. route was significantly superior to i.c. route in repressing the local bone metastatic progression of 4T1.2 cells. Furthermore, we determined the influence of MSC cell inoculation timing on their therapeutic effects. Using the same 4T1.2 model, MSC treatment at relatively early time points (bone BLI signal $<10^5$) showed a better therapeutic efficacy than those receiving treatment at later time points (bone BLI signal $> 5X10^5$) (**Figure 2B**).

Based on above results, in the following mechanism studies the i.f. inoculation of engineered BM-MSCs with a single dose of $2X10^5$ cells at the relatively early metastasis development stages, was adopted.

- **Specific Aim 2: Reveal mechanisms underlying the anti-tumor effects of engineered MSCs.**

Major Task 2: Identify the functional contribution of host immunity to the therapeutic effects by engineered MSCs

➢ Subtask 1: Determine the role of $CD8^+$ T cells in the therapeutic effects of *CCL-2*^{KD} *iNOS*$^{-/-}$ *IFNα*^{OE} MSCs

To determine how engineered BM-MSCs modulate the local bone metastatic niche, particularly the immune microenvironment, we recently developed comprehensive immune cell testing panels based on the newest flow cytometry system- BD ACSymphony A5. From these settings (**Figures 3A and 3B**), distinct myeloid and lymphoid cell subsets were able to be compared between the untreated and MSC-treated groups. Particularly we pay close attention to the effector and exhausted T-cell subsets using the markers CD44, CD62L, PD-1 and Tim3.
As shown in **Figure 4A**, three days later after *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} MSC i.f. treatment in the 4T1.2 tumor model, the BM-infiltrating $CD8^+$ and $CD4^+$ T cells were found to be significantly increased in comparison to the sham group. However, the MSC treatment did not change the ratio of the effector to exhausted T cells (**Figure 4B**). This suggested the introduction of engineered BM-MSCs into the local bone metastatic areas may mainly serve to recruit or expand T cells, but not function to modulate the T cell effector statuses. For other immune cell subsets tested, MSC treatment led to a mild decrease of BM-infiltrating $Ly6G^+Ly6C^-$ neutrophils and natural killer (NK) cells, with other population unchanged (**Figure 4C**).

In the next period of studies, we will further identify the functions of expanded T cells in the MSC-treated groups by in vivo T cell depletion, ex vivo T cell cytotoxicity assay and T cell effector molecules (granzyme B and perforin), cytokine and transcriptional factor measurement.

- Subtask 2: Test if immune checkpoint inhibitors enhance the effects of *CCL-2*^{KD}*iNOS*$^{-/-}$ *IFNα*^{OE} MSCs

Immunotherapy, particularly blockage of the immune checkpoints such as CTLA-4 and PD-1/PDL-1, is very promising in treating many solid tumors, including breast cancer in clinic (15, 16). Our above results indicated that adoptive transfer of genetically engineered BM-MSCs potentially accelerated the expansion of local T cell populations but did not change the T cell effector status. We were therefore wondering whether the immune checkpoint inhibitors would improve the therapeutic effect of engineered BM-MSCs via enhancing the T cell effector functions.

To do so, anti-PD1 (clone J43, BioXCell) and anti-CTLA4 (clone 9D9, BioXCell) were injected at a dose of 10 mg/kg by intraperitoneal injection three times a week after the *CCL-2*^{KD}*iNOS*$^{-/-}$ *IFNα*^{OE} BM-MSC i.f. administration in the above 4T1.2 model. By BLI monitoring, the metastatic progression of 4T1.2 cells was more effectively controlled in the combination treatment groups, when compared to either MSC or checkpoint inhibitor treatment alone (**Figure 4D**). As inspired by these preliminary results, we will optimize the treatment regimens and also determine how the combinational treatment modulates the bone microenvironment at cellular and functional levels in the next period of study.

- **Specific Aim 3: Adapt our mouse findings to treating bone metastases of human breast cancer with engineered human MSCs in the humanized NSG™-SGM3 mouse model.**

Major Task 3: Build the humanized mice platform for human MSC-based therapeutic in treating bone metastases of human breast cancer

- Subtask 1: Construct *IDO*^{KO}*CCL-2*^{KO}*IFNα*^{OE} human BM-MSCs

Under this subtask, we have recently constructed over 5 stable human BM-MSCs with IDO knockdown, CCL-2 knockdown and IFNα overexpression using the lentiviral transduction system, as did for mouse cells. These cells are now undergoing in vitro identification for their surface marker expression, differentiation capabilities and immunoregulatory function, as well as in vivo engraftment efficiency and safety assay. We will determine the therapeutic effects of these engineered human BM-MSC lines in treatment of bone metastases of human breast cancer in humanized mice in the next period of studies.

- Subtask 2: Establish the breast cancer-bone metastasis model in humanized NSG™-SGM3 mice

We are now in the middle of working with our collaborators Dr. Lenny Shultz and Dr. Bora Lim to establish the human breast cancer bone metastasis models (SCP2 and SCP28) in humanized mice. We will apply above genetically engineered human BM-MSC cells in these bone metastasis models in the upcoming year.

3) What opportunities for training and professional development has the project provided?

During this period of awarding time, the postdoctoral fellow (Dr. Pei-shan Li) received rigorous training from the PI, Dr. Guangwen Ren for both research and career development. Dr. Li significantly increased his knowledge in breast cancer research, molecular biology and immunology. He presented his research in the Integrated Translational Science Center (ITSC) Workshop, Bar Harbor in July 2018 and also attended the AACR Special Conference on Metabolism and Cancer, New York in September 2018. Dr. Li also published a first-authored review article in Pharmacology & Therapeutics, and a book chapter in Methods in Molecular Biology as a co-author.

4) How were the results disseminated to communities of interest?

Nothing to Report.

5) What do you plan to do during the next reporting period to accomplish the goals?

During the next reporting period, we will mainly focus on Aim 2 and Aim 3. In particular, we will determine the role of $CD8^+$ T cells in the therapeutic effects of genetically engineered BM-MSCs and test how immune checkpoint inhibitors enhance the effects of these engineered BM-MSCs in breast cancer bone metastases model in Aim 2. In Aim 3, we will test the effect of $\textit{CCL-2}^{KO}\textit{IDO}^{KO}\textit{IFN}\alpha^{OE}$ human BM-MSCs on human breast cancer bone metastases in the humanized mouse model.

4. IMPACT:

1) What was the impact on the development of the principal discipline(s) of the project?

During the reporting period, we have optimized the treatment protocol using genetically engineered BM-MSCs in treating pre-existing bone metastases in syngeneic mouse breast cancer models. Furthermore, we have found the adoptive transfer of engineered BM-MSCs accelerated the local T cell expansion, and immune checkpoint inhibitors can enhance the therapeutic efficacy of MSC treatment. Therefore, our results will facilitate us to design combined immunotherapy in treating bone macrometastases of breast cancer.

2) What was the impact on other disciplines?

Nothing to Report.

3) What was the impact on technology transfer?

Nothing to Report.

4) What was the impact on society beyond science and technology?

Nothing to Report.

5. CHANGES/PROBLEMS:

Nothing to Report.

6. PRODUCTS:

1) Publications, conference papers, and presentations

- **Journal publications.**

Mesenchymal Stem Cells: from Regeneration to Cancer. Li P, Gong Z, Shultz LD, Ren G. *Pharmacol Ther*. 2019 Apr 15. pii: S0163-7258(19)30062-2. doi: 10.1016/j.pharmthera.2019.04.005. [Epub ahead of print] Review. PMID: 30998940. In press; acknowledgement of federal support (yes).

- **Books or other non-periodical, one-time publications.**

  Nothing to Report.

- **Other publications, conference papers, and presentations.**

  Nothing to Report.

2) Website(s) or other Internet site(s)

Nothing to Report.

3) Technologies or techniques

Nothing to Report.

4) Inventions, patent applications, and/or licenses

Nothing to Report.

5) Other Products

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

1) What individuals have worked on the project?

Name:	*Guangwen Ren*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Ren has overall responsibility for this project*
Funding Support:	*The Jackson Laboratory Startup funds; NIH-R00 CA188093; this award*

Name:	*Pei-Shan Li*
Project Role:	*Postdoctoral Associate*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*12*
Contribution to Project:	*He is responsible for the experimental work in this project.*
Funding Support:	*This award.*

Name:	*Jiayuan Shi*
Project Role:	*Research Assistant*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4*
Contribution to Project:	*He is responsible for the experimental work in this project.*
Funding Support:	*This award.*

2) Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Other Support

**Entries in italics represent changes from the last time other support information was provided for this project.*

Ren, Guangwen

ACTIVE

W81XWH-18-1-0013 (Ren) 4/1/2018 - 3/31/2021 3.00 Calendar
Department of Defense $124,420
Mesenchymal Stem Cell-based Therapy for Treatment of Bone Metastases in Breast Cancer
The goal of this project is to develop mesenchymal stem cells (MSCs) as biological vehicles to deliver anti-tumor agents into the bone by exploiting the natural behavior of MSCs to home towards sites of damaged tissue.

5 R00 CA188093-05 (Ren) 2/15/2017 - 1/31/2020 7.80 Calendar
NIH/NCI $142,285
Mesenchymal Stromal Cells and Stromal Fibroblasts in Radiotherapy Resistance
In this study, we will elucidate how mesenchymal stem cells (MSCs) and their derivative stromal fibroblasts functionally contribute to cancer resistance and early recurrence after radiotherapy.

OVERLAP
None

3) What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report.

9. APPENDICES:

Supporting Data

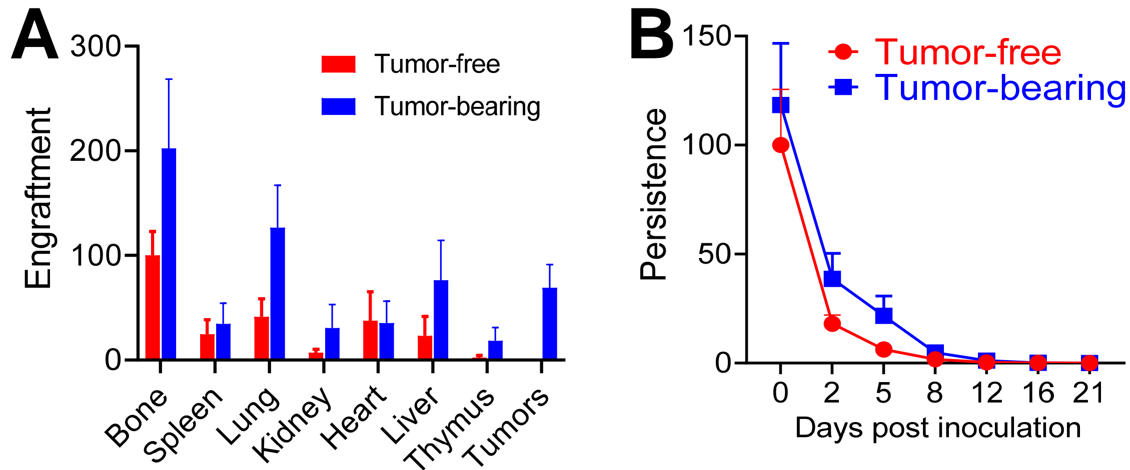

Figure 1. Measurement of the engraftment efficiency and safety of implanted genetically engineered BM-MSCs. (**A**) Tumor-free or 4T1.2 orthotopic tumor-bearing mice received 2X10^5 *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} BM-MSC cells which were labeled with luciferase. Two days later, the mice were sacrificed and the engraftment of BM-MSCs was detected by ex vivo BLI. The average value of BLI signals in the femur and tibia of tumor-free mice was set as 100. n=4; (**B**) In the same experimental setting as in (**A**), the persistence of implanted BM-MSC cells in the recipient mice was monitored by BLI within 3 weeks post MSC inoculation. The average values of BLI signals in the whole body of mice on day 0 (just after MSC injection) were set as 100. n=4. These results represent 3 independent experiments. Data are presented as mean ± SEM.

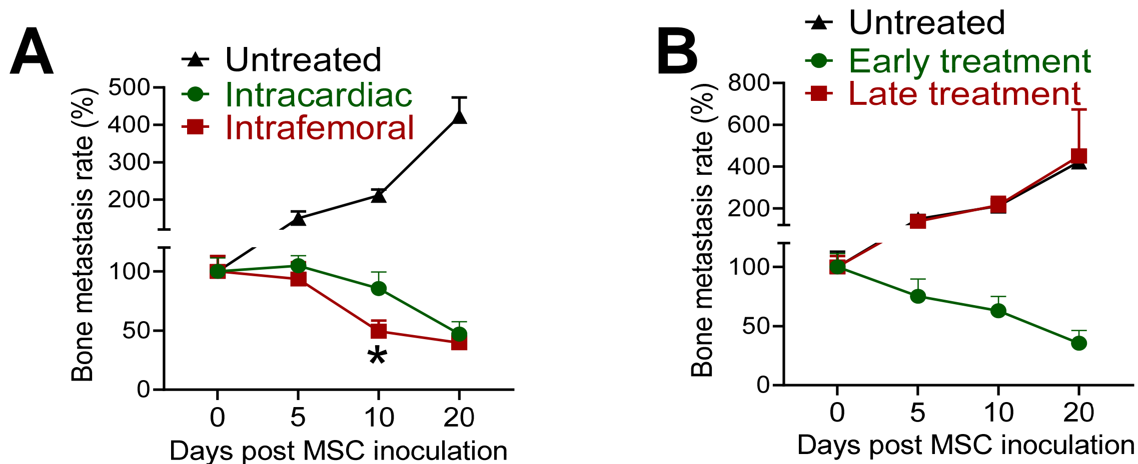

Figure 2. Optimization of the administration routes and timing of engineered BM-MSCs. (**A**) In a spontaneous 4T1.2 (luciferase labeled) bone metastasis model, when bone metastases (femur and tibia areas) were able to be detected by BLI ($<10^5$), a single dose of $2X10^5$ *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} BM-MSC cells were implanted by either intracardiac (i.c.) or intrafemoral (i.f.) routes and the bone metastasis progression was then monitored by BLI. The average values of BLI signals before the MSC treatment were set at 100 and the following BLI signals were then normalized. n=10. Data are presented as mean ± SEM; *$p<0.05$. The comparison was made between i.c. and i.f. routes on day 10 post MSC inoculation; (**B**) In the same experimental setting as in (**A**), a single dose of $2X10^5$ *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} BM-MSC cells were given at early bone metastasis progression stages ($<10^5$) or later stages ($> 5X10^5$) by i.f. injection. The therapeutic effects were then compared by BLI monitoring. Again, the average values of BLI signals before the MSC treatment were set at 100 and the following BLI signals were then normalized. n=10. These results represent 2 independent experiments. Data are presented as mean ± SEM.

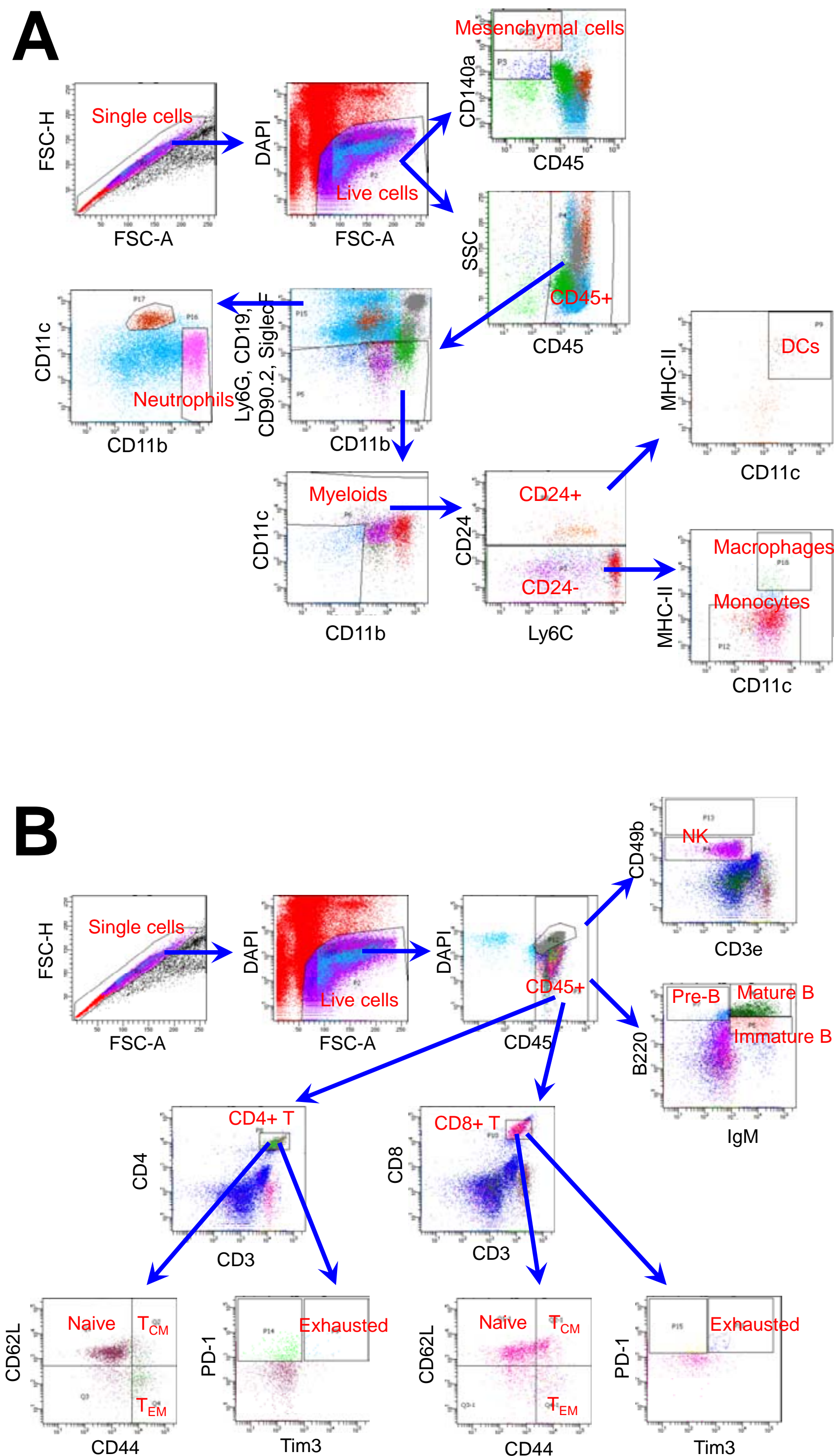

Figure 3. The myeloid cell (A) and lymphoid cell (B) profiles of mouse immune cells tested by flow cytometry. With these settings, the major myeloid cell and lymphoid cell subsets can be determined using the BD ACSymphony A5.

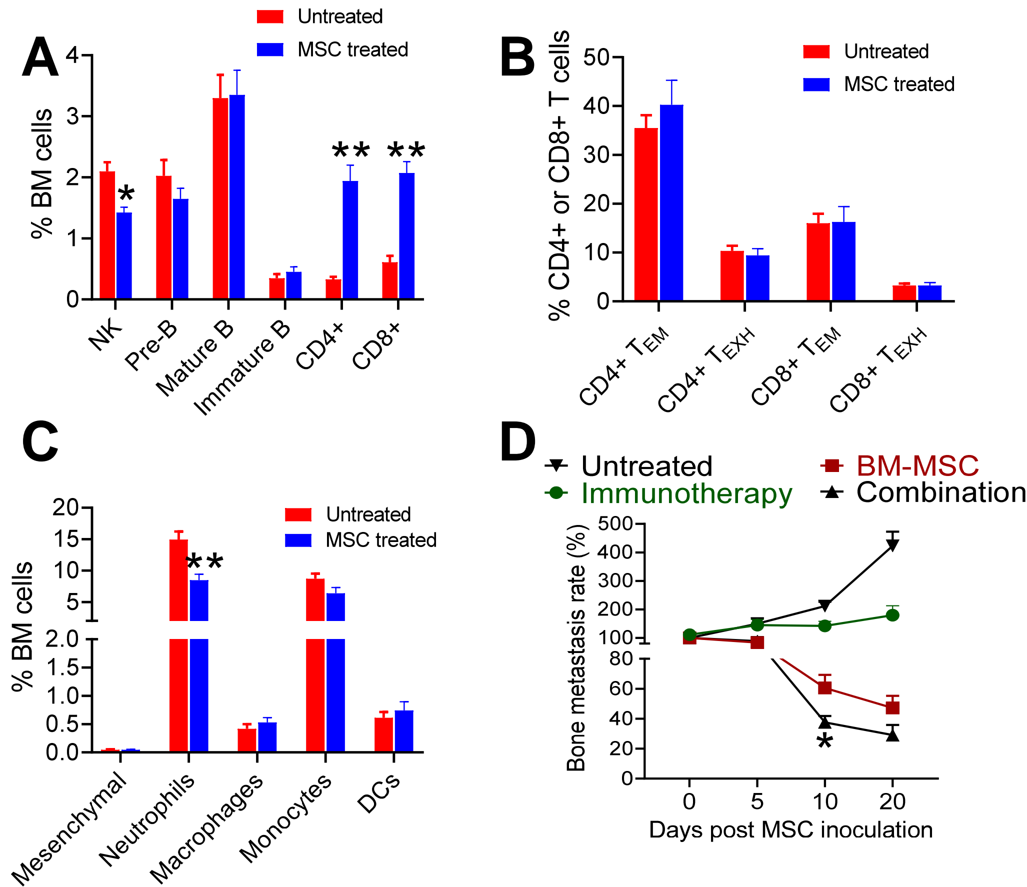

Figure 4. Immune checkpoint inhibition enhanced the therapeutic effect of engineered BM-MSCs in treating bone metastases of 4T1.2 tumors. (**A-C**) in a spontaneous 4T1.2 bone metastasis model, when bone metastases (femur and tibia areas) were able to be detected by BLI, a single dose of 2X10^5 *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} BM-MSC cells were implanted by intrafemoral route. Three days later after MSC cell inoculation, the mice were sacrificed and the lymphoid cells (**A**), T cell subsets including T effector cells (T_{EM}) and exhausted T cells (T_{EXH}) (**B**), and myeloid cell subsets (**C**), as indicated in **Figure 3**, were measured and compared with the untreated group by flow cytometry; (**D**) in a same experimental setting as in (**A-C**), a single dose of 2X10^5 *CCL-2*^{KD}*iNOS*$^{-/-}$*IFNα*^{OE} BM-MSC cells were given at early bone metastasis progression stages (<10^5). To determine whether immune checkpoint inhibitors can improve the therapeutic effect of BM-MSC treatment, anti-PD1 and anti-CTLA4 were injected at a dose of 10 mg/kg by intraperitoneal injection three times a week after MSC inoculation (combination group). The following bone metastases were monitored by BLI. n=4 for (**A-C**) and n=10 for (**D**). The average values of BLI signals before the MSC treatment were set at 100 and the following BLI signals were then normalized. These results represent 2 independent experiments. Data are presented as mean ± SEM; *p<0.05; **p<0.01. The significance comparison in (**D**) was made between BM-MSC and combination groups.

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