# Response of mouse thymic cells to radiation after transfusion of mesenchymal stem cells

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## Abstract

Thymic lymphoma is a highly invasive and even metastatic cancer. This study investigated the effects of mesenchymal stem cells (MSCs) transfusion on cell cycle, cell proliferation, CD3 expression, mutation frequency of T cell receptor using mouse model of thymic lymphoma.

C57BL/6J young mouse models of thymoma were injected with MSCs. Six months later, the thymus was taken for pathological examination and flow cytometry studies. The cells were labeled with anti-CD4, CD8, CD3, propidium iodide, or CFDA-SE, cell cycle, proliferation kinetics, and mutation frequency of T cell receptor, respectively.

Pathologic results showed that control had clear corticomedular structure with regularly shaped lymphocytes. After radiation, the thymus structure was completely destroyed, with lymphoid tumor cells diffusely distributed and heavily stained, and large nuclei. Transfusion of MSCs resulted in normal thymus structure. Cytometry studies showed that there were more CD4-/CD8- T cells in the thymus of irradiated mice than in control; transfusion of MSCs led to reduced CD4-/CD8- T cells. In irradiated mice, there were less CD4+/CD8+ T cells than in control and MSCs transfusion groups. It was observed that there were more cells arrested in G1 phase in the thymus cells and CD4-/CD8- T cells in irradiated mice than in other 2 groups, whereas there were more cells arrested in S phase in CD4+/CD8+ and CD4+/CD8- T cells in irradiated mice than in the other mice. In the thymus cells, and CD4+/CD8+ and CD4+/CD8- T cells, irradiated mice group had significantly less parent, G2, G3, and G4 cells, and more cells at higher generations, and also higher proliferation index. In CD4-/CD8- T cells, irradiated mice had significantly more parent, G2, and G3 cells, and less G4, G5, G6, and propidium iodide, as compared with the other 2 groups. The expression of CD3 in CD4/CD8 T cells was significantly higher than in control. MSCs transfusion improved CD3 expression, but was still less than the control. Irradiation resulted in very high mutation frequency of T cell receptor, which was barely affected by MSCs transfusion.

Mesenchymal stem cell transfusion is able to restore the cell cycle and cell proliferation, but not CD3 expression and mutation frequency of T cell receptor in irradiated mice to control level.

**Abbreviations:** CD = cluster of differentiation, CFDA-SE = carboxyfluorescein diacetate succinimidyl ester, DNA = deoxyribonucleic acid, EDTA = ethylene diamine tetraacetic acid, FCM = flow cytometry, FITC = fluorescein isothiocyanate, H&E = hematoxylin and eosin, L-DMEM = low glucose Dulbecco modified eagle medium, Mf = mutation frequency, MSCs = mesenchymal stem cells, PBS = phosphate buffer saline, PE = phycoerythrin, PE-CF594 = phycoerythrin-cyanine-based fluorescent dyes594, PE-Cy5 = phycoerythrin-cyanine 5, PI = propidium iodide, RNase = ribonuclease RNAse, rpm = revolutions per minute.

**Keywords:** cycle cell, MSCs, mutation, proliferation, radiation, T cell receptor, thymic lymphoma

## 1. Introduction

Radiotherapy is 1 of the irreplaceable means for cancer therapy; however, there are increasing reports of induced secondary malignant tumors, such as thymic lymphoma, leukemia, bladder cancer, and osteosarcoma.[1–4] Thymic lymphoma induced by radiotherapy is a highly invasive and even metastatic cancer[5]

with poor prognosis.[6] Since the establishment of mouse models of radiation-induced thymoma in 1967 by Kaplan,[7] many studies have been conducted regarding the induction of thymoma by radiation. For example, Mao et al[8] used Hipk2 in combination with the p53 gene to treat radiation-induced thymic lymphoma; Zhao et al[9] found that hydrogen protects mice from radiation-induced lymphoma. However, no effective therapy is available to treat thymic lymphoma.

Bone marrow-derived mesenchymal stem cells (MSCs) are originated in stromal cells, and can proliferate and differentiate into hematopoietic stem cells,[10,11] or migrate to the injured tissue for differentiation.[12] The MSCs are shown to enhance the survival of transplanted hematopoietic stem cells or serve as the precursor for tissue reconstruction.[13] Many in vitro studies show that MSCs play in immunomodulation role and injury repair by regulating the differentiation and maturation of dendritic cells,[14–16] B cells,[17–19] natural killer (NK) cells,[19] and T cells,[20–22] and secretion of cytokines, probably as a result of generation of soluble factors.[20,23,24]

Since 2010, we have been using MSCs in therapy of radiation-induced thymoma, and found that MSCs reduce the incidence of thymoma.[25] To better understand the mechanism underlying the formation of thymoma and role of MSCs in thymoma therapy, we investigated the effect of MSCs transfusion on cell cycle, proliferation, CD3 expression, and mutation frequency (Mf) of T cell receptor. The findings would provide new insights for treatment of the secondary malignancies.

## 2. Materials and methods

### 2.1. MSCs culture

All protocols were approved by ethics review board of Second Hospital of Jilin University.

Neonatal C57BL/6J mice were sacrificed by cervical dislocation, and the medullary cavity was rinsed with low glucose Dulbecco modified eagle medium (L-DMEM) medium to obtain cell suspension. The cells were inoculated in 25 cm$^2$ culture bottle at density of $2 \times 10^5$ cells/mL, and cultured at 37°C and 5% CO2 in incubator with saturated humidity. The half amount of medium was first refreshed after 48 hours and then after every 3 days. The cells at 90% confluence were digested with 0.25% trypsin (containing 0.02% ethylene diamine tetraacetic acid [EDTA]) for 2 minutes, and passaged at the ratio of 1:2 for 3 generations before use.

### 2.2. Thymoma models

Fifty-four female C57BL/6J mice (weighting $13 \pm 2$ g) were randomly divided into 3 groups to receive no treatment (control), radiation, and MSCs transfusion. Radiation (6X MV) was given as described[7] using Varian 23 EX linear accelerator (Varian Medical Systems, Inc.) at 1.75 Gy/time at dose rate of 300 Mu/min once a week for 4 weeks. On the day of last irradiation and 1 week after the irradiation, the irradiated mice were injected with 0.2 mL MSCs ($2 \times 10^6$ cells/mL) via tail veins. Six months later, the mice were sacrificed and the thymus isolated. Half of the thymus was fixed in formalin for pathological examination and the other half was grinded into single cells for flow cytometry studies.

### 2.3. Pathological examination

The fixed thymus was embedded in paraffin, sliced, stained with hematoxylin and eosin (H&E) and examined under microscope.

### 2.4. Flow cytometry

Thymus cell suspensions were filtered through 200-mesh screen and centrifuged at 1500 rpm for 5 minutes. The pellet was resuspended in 5 mL red cell lysis buffer at room temperature for 5 minutes. The lysate was centrifuged at 1500 rpm for 5 minutes and the pellet was washed twice with phosphate buffer saline (PBS). Prechilled 2 mL 70% ethanol was added to $(0.2–1) \times 10^7$ cells/mL cell suspension and fixed at 4°C overnight. The fixed cells were centrifuged at 1000 rpm for 5 minutes, washed twice with PBS, and then resuspended in 400 µL PBS, added with 50 µL of ribonuclease RNAse (RNAse) (1 mg/mL) and propidium iodide (PI, 400 µg/mL), and incubated at 37°C for 30 minutes. The cells were then labeled with anti-CD4-PE-CF594, anti-CD8-FITC antibodies (Becton, Dickinson and Company) at room temperature for 30 minutes in the darkness and analyzed using Beckman flow cytometry (Epics XL, Beckman Coulter Inc.) at the excitation wavelength of 488 nm according the manufacturer's instructions. The Cellquest analysis software was used to determine the DNA content and cell cycle distribution.

For analysis of cell proliferation, the cells were suspended in diluted carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) solution, incubated at 37°C for 10 minutes, and added with complete cell culture medium DMEM with 10% fetal bovine serum (HyClone). The cells were centrifuged at 1000 rpm for 3 minutes, washed once with complete culture medium, and incubated in complete culture medium for 5 minutes to allow unreacted CFDA-SE to release into the medium. The cells were collected by centrifugation and labeled with anti-CD4-PE-CF594 and anti-CD8-PE antibodies (eBioscience). Unlabeled cells were used as control. The fluorescence intensity was measured using Beckman flow cytometer and analyzed using the Modfit software.

For analysis of CD3, $2 \times 10^6$ to $1 \times 10^7$ cells were incubated with anti-CD3-PE-Cy5 (eBioscience), anti-CD4-PE-CF594, and anti-CD8-PECF594 antibodies at room temperature and darkness for 30 minutes. The cells were subjected to flow cytometry analysis, and CD3 expression was analyzed using FCS Express 4. Mf of T cell receptor was calculated as follows[26]:

$$Mf = \text{count of CD3}-/\text{CD4} + \text{T cells/count of CD3}-/\text{CD4}+$$
$$\text{and CD3}+/\text{CD4} + \text{T cells,}$$

where CD3-/CD4+ cells were T cell receptor mutants and CD3+/CD4+ cells were normal cells.

### 2.5. Statistical analysis

Each experiment was repeated at least 3 times. Data were shown as mean ± SD and analyzed using SPSS 18.0. Variance analysis was used to compare the means and differences between groups, and values with $P < 0.05$ or $<0.01$ were considered statistically significant or highly significant.

## 3. Results

### 3.1. Differentiation thymocytes into CD4/CD8 cells

As shown in Table 1, T cells isolated were mainly CD4-/CD8-, CD4+/CD8+, or CD4+/CD8-; the proportion of CD4-/CD8- subset was significantly higher in the irradiated mice than that in the control, and was reduced to normal levels in the MSCs transfusion group; the proportion of CD4+/CD8+ T cells in the irradiated mice was 50.68%, significantly lower than that in the control (80.82%) and MSCs transfusion group (72.85%); and

**Table 1**

**Percentages of CD4/CD8 T cells in the thymus of mice irradiated with or without transfusion of MSCs.**

| Group | No. sample | CD4−/CD8− | CD4+/CD8+ | CD4+/CD8− | CD4−/CD8+ |
|---|---|---|---|---|---|
| Control | 3 | 6.36±1.53 | 80.82±1.39 | 12.62±0.72 | 0.19±0.05 |
| Irradiation | 3 | 40.29±10.33[**] | 50.68±9.06[**] | 8.94±1.39 | 0.10±0.03 |
| MSCs transfusion | 3 | 9.36±6.36[##] | 72.85±10.24[#] | 17.47±6.38[#] | 0.32±0.19 |

The symbols * and ** denote significant or highly significant difference versus control ($P < 0.05$ or $P < 0.01$).
The symbols # and ## denote significant or highly significant difference versus radiation group ($P < 0.05$ or $P < 0.01$).

**Table 2**

**Percentages of cells arrested at different cell phases after irradiation and MSCs transfusion.**

| Group | No. sample | G1 | S | G2 |
|---|---|---|---|---|
| Control | 3 | 69.98±4.60 | 22.62±3.69 | 7.40±1.38 |
| Irradiation | 6 | 90.53±3.83[**] | 5.77±3.17[*] | 3.70±1.12[*] |
| MSCs transfusion | 3 | 64.24±10.43[##] | 21.43±9.00[#] | 14.33±1.42[**##] |

The symbols * and ** denote significant or highly significant difference versus control ($P < 0.05$ or $P < 0.01$).
The symbols # and ## denote significant or highly significant difference versus radiation group ($P < 0.05$ or $P < 0.01$).

the percentage of CD4+/CD8− T cells in the transfusion group was significantly higher than that in the radiation group. There were very few CD4−/CD8+ T cells in all groups.

### 3.2. Effect of MSCs on the cell cycle

Cytometry analysis showed that there were similar proportions of G1 cells in MSCs transfusion and control mice (64.24% vs 69.98%; Table 2), whereas irradiated mice had significantly more G1 cells than the other 2 groups (90.53% vs 64.24% and 69.98%). There were less cells arrested in S phase in the radiation group compared with that in the control group (5.77% vs 22.62%), but the difference disappeared after MSCs transfusion (21.43%; Table 2).

### 3.3. Effect of MSCs on the cell cycle in CD4/CD8 subsets

We further analyzed the impact of MSCs on the cell cycle in different CD4/CD8 subsets. The results showed that in CD4−/CD8− T cells, there were significantly more G1 cells in radiation group than in control group (68.44% vs 35.73%; Table 3), and MSCs transfusion restored G1 cells to the level in control. On the contrary, significantly less numbers of cells

(26.03%) were arrested at S phase in the radiation group than that in the control (57.26%) and MSCs transfusion (47.35%) groups. For CD4+/CD8+ T cells, there were significantly more cells at S phase in the radiation group than in the control group, and MSCs transfusion resulted in normal numbers of these cells (Table 3). The numbers of cells at G2 or M phase were similar among the 3 groups. When looked at CD4+/CD8− T cells, less numbers of cells were arrested at S phase in radiation group than in control group, and the number increased insignificantly after MSCs transfusion (Table 3). Furthermore, the numbers of cells at G2 phase were similar among the 3 groups (Table 3).

### 3.4. Effect of MSCs on the proliferation of thymocytes

Compared with control, radiation produced less cells in lower generations (<sixth generation) and more in higher generations (>sixth generation; Table 4, Fig. 1). MSCs transfusion reversed the radiation-induced effect, although not completely, resulting in cell proliferation profiles similar to the control, with similar numbers of PI-stained cells (Table 4). PI in radiation group (94.12%) was much higher than that in control group (7.27%), and MSCs transfusion greatly reduced the PI to the level (6.41%) that is even below the control (Table 4).

**Table 3**

**Percentages of different CD4/CD8 T cells after irradiation and MSCs transfusion.**

| CD4/CD8 subset | Group | No. sample | G1 | S | G2 | G2/M |
|---|---|---|---|---|---|---|
| CD4−/CD8− | Control | 3 | 35.73±6.45 | 57.26±12.34 | 6.54±11.27 | |
| | Irradiation | 6 | 68.44±9.83[**] | 26.03±4.91[**] | 5.53±6.37 | |
| | MSCs transfusion | 3 | 36.50±18.25[##] | 47.35±4.60[#] | 16.71±23.63 | |
| CD4+/CD8+ | Control | 3 | 98.62±0.32 | 1.38±0.32 | | 0±0 |
| | Irradiation | 6 | 79.68±13.34[*] | 16.29±7.56[**] | | 4.04±5.77 |
| | MSCs transfusion | 3 | 88.70±4.46 | 4.34±1.86[##] | | 6.94±3.23 |
| CD4+/CD8- | Control | 3 | 95.98±3.47 | 0.17±0.30 | 3.86±3.56 | |
| | Irradiation | 6 | 81.90±9.25[*] | 12.97±4.58[**] | 5.14±5.85 | |
| | MSCs transfusion | 3 | 88.75±6.41 | 2.27±3.93[##] | 8.98±6.67 | |

The symbols * and ** denote significant or highly significant difference versus control ($P < 0.05$ or $P < 0.01$).
The symbols # and ## denote significant or highly significant difference versus radiation group ($P < 0.05$ or $P < 0.01$).

**Table 4**

**The percentages of CFDA-SE-labeled mouse thymocytes in different generations after irradiation and MSCs transfusion.**

| Group | No. sample | Parent | G2 | G3 | G4 | G5 | G6 |
|---|---|---|---|---|---|---|---|
| Control | 3 | 0.14±0.13 | 2.23±0.98 | 24.97±9.92 | 47.14±6.05 | 17.03±6.48 | 4.76±1.14 |
| Irradiation | 3 | 0.19±0.21* | 0.27±0.27* | 0.46±0.33 | 0.85±0.46* | 2.09±1.30* | 3.08±1.80 |
| MSCs transfusion | 3 | 1.47±0.71* | 0±0* | 32.01±26.87 | 32.25±20.65# | 0±0* | 8.61±4.57 |

| | G7 | G8 | G9 | G10 | PI |
|---|---|---|---|---|---|
| Control | 2.41±0.74 | 0.28±0.12 | 0.11±0.09 | 0.94±0.52 | 7.27±0.53 |
| Irradiation | 7.75±6.24 | 16.50±6.36** | 30.90±9.26** | 37.92±16.47** | 94.12±44.21** |
| MSCs transfusion | 0±0# | 6.49±3.68# | 0±0## | 10.89±7.13# | 6.41±2.81## |

The symbols * and ** denote significant or highly significant difference versus control ($P < 0.05$ or $P < 0.01$).
The symbols # and ## denote significant or highly significant difference versus radiation group ($P < 0.05$ or $P < 0.01$).
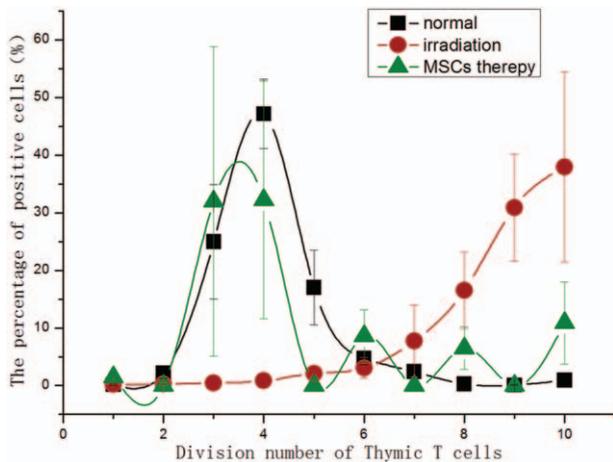


**Figure 1.** Proliferation kinetics of mouse thymocytes after irradiation and MSCs transfusion. Mouse thymocytes were stained with CFDA-SE and analyzed using flow cytometry and the Modfit software.

### 3.5. Effect of MSCs on the proliferation of different CD4/CD8 cells

For CD4-/CD8- T cells, there were more cells in the third and fourth generations, and less cells in the fifth and sixth generations in control and MSCs transfusion groups than in radiation group (Table 5, Fig. 2A). After MSCs transfusion, the mice had significantly more PI-stained cells (6.24%) as compared with the radiation group (2.88%).

For CD4+/CD8+ cells, analysis showed that there were more cells in the third and fourth generations, and less in the fifth and sixth generations in control and MSCs transfusion groups as compared with radiation group (Table 5, Fig. 2B), which had significantly higher PI (12.92%) than the former 2 groups (6.23% and 5.78%, respectively; Table 5).

For CD4+/CD8- cells, there were more second and third generation, and less fifth and sixth generation cells in control and MSCs transfusion groups than in radiation group (Table 5, Fig. 2C), which also had significantly higher PI (10.01%) than the former 2 groups (2.95 and 3.92%, respectively; Table 5).

### 3.6. Expression of CD3 in different CD4/CD8 cells

As shown in Table 6, 16.48% of the cells in the CD4-/CD8- subset were positive for CD3 in control, and no CD3+ T cells were detected after radiation and MSCs transfusion. The percentages of CD3+ T cells were nearly 100% in CD4+/CD8+ and CD4+/CD8- subsets in control (Table 6), and were significantly lower in radiation and MSCs transfusion groups. MSCs transfusion increased the percentages in CD4+/CD8+ and CD4+/CD8- subsets from 1.05% and 1.29% in radiation group to 7.89% and 13.23%, respectively, but was still much less than control (98.92% and 99.36%).

### 3.7. T cell receptor mutation

The profiles of CD3/CD4 expression are shown in Fig. 3, and analysis showed that mutation frequencies of the T cell receptor was very low in control (0.008%), dramatically increased in radiation group (0.996%), and MSCs transfusion slightly but

**Table 5**

**The percentages of CFDA-SE-labeled mouse CD4-/CD8- thymocytes in different generations after irradiation and MSCs transfusion.**

| CD4/CD8 subset | Group | No. sample | Parent | G2 | G3 | G4 | G5 | G6 | PI |
|---|---|---|---|---|---|---|---|---|---|
| CD4−/CD8− | Control | 3 | 2.65±2.97 | 13.84±7.42 | 19.29±18.48 | 37.54±9.15 | 10.85±9.40 | 11.45±1.65 | 5.53±0.37 |
| | Irradiation | 3 | 0.03±0.05 | 47.39±14.71* | 49.34±14.99 | 0.66±0.97** | 0.99±0.46 | 1.59±0.35** | 2.88±0.21 |
| | MSCs transfusion | 3 | 2.27±3.21 | 12.37±5.72# | 19.09±15.86 | 24.60±5.19## | 28.83±16.16# | 12.81±3.47## | 6.24±2.35# |
| CD4+/CD8+ | Control | 3 | 0.12±0.02 | 3.97±0.59 | 28.32±2.69 | 46.99±1.25 | 14.10±3.15 | 4.45±0.89 | 6.23±0.27 |
| | Irradiation | 3 | 0.32±0.34 | 0.66±0.70* | 4.66±5.15** | 19.77±7.98** | 37.77±0.72** | 36.82±2.91** | 12.92±1.36** |
| | MSCs transfusion | 3 | 0.16±0.04 | 1.58±2.58 | 38.27±11.24## | 54.22±10.79## | 4.27±3.25**## | 0.65±0.09**## | 5.78±0.78## |
| CD4+/CD8- | Control | 3 | 18.67±11.83 | 15.93±17.14 | 40.47±1.84 | 20.76±1.23 | 0±0 | 4.18±5.91 | 2.95±0.21 |
| | Irradiation | 3 | 0.87±1.23* | 4.05±4.27 | 9.04±7.17** | 16.71±3.33 | 27.18±12.63* | 35.74±14.81* | 10.01±1.79** |
| | MSCs transfusion | 3 | 1.89±2.04* | 21.76±1.52 | 21.76±1.52## | 32.08±5.42*# | 2.75±4.77# | 0±0## | 3.92±0.35## |

The symbols * and ** denote significant or highly significant difference versus control ($P < 0.05$ or $P < 0.01$).
The symbols # and ## denote significant or highly significant difference versus radiation group ($P < 0.05$ or $P < 0.01$).
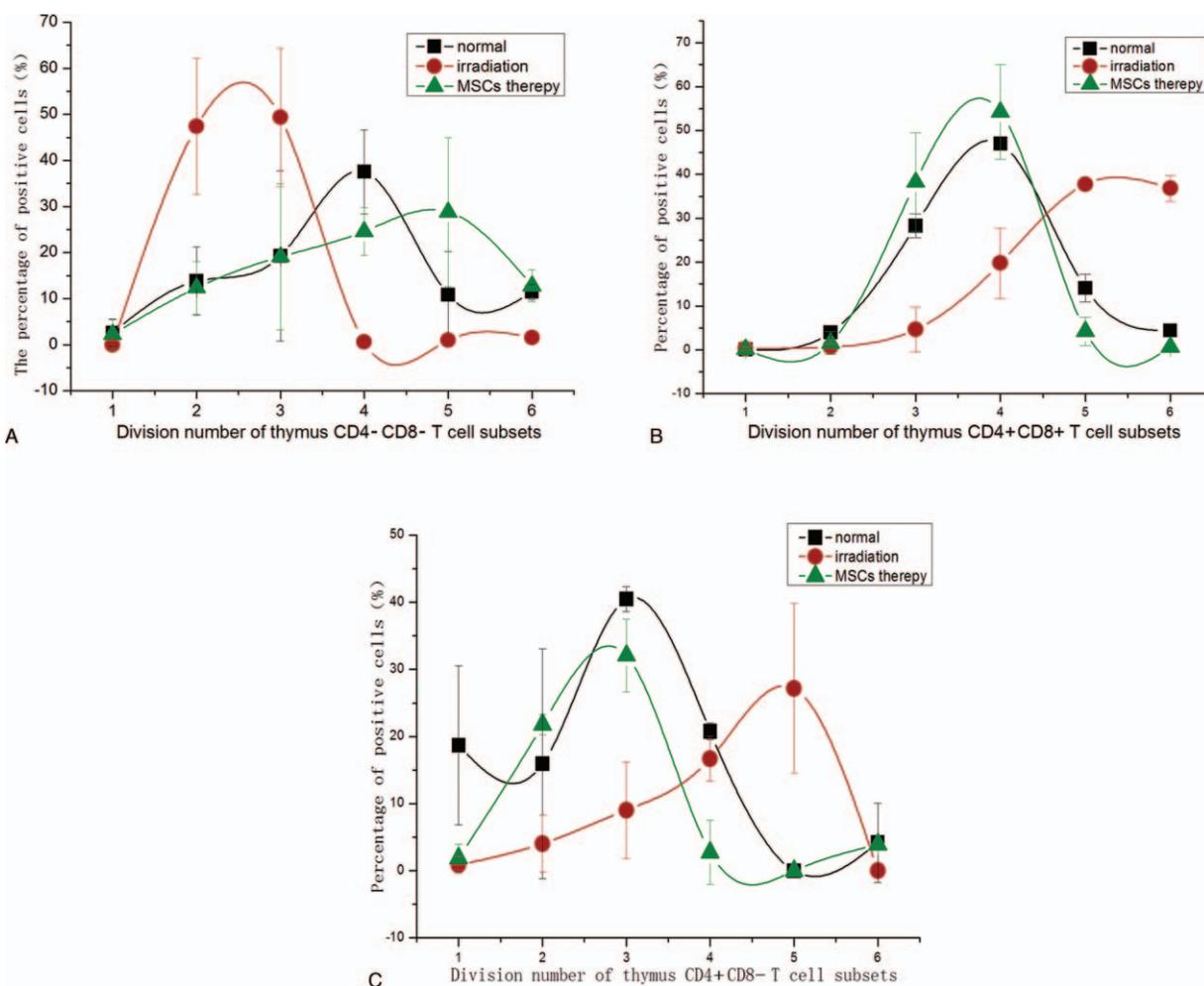
**Figure 2.** Proliferation kinetics of mouse thymocytes after irradiation and MSCs transfusion. Mouse thymocytes were labeled with CD-specific antibodies and stained with CFDA-SE, and analyzed using flow cytometry and the Modfit software. A, CD4-/CD8- T cells; B, CD4+/CD8+ T cells; and C, CD4+/CD8- T cells.

<div style="border:1px solid #888;padding:2px 8px;display:inline-block;background:#888;color:#fff;">Table 6</div>

**Percentages of CD3+ cells in different CD4/CD8 subsets after irradiation and MSCs transfusion.**

| | | CD3+ cells in | | |
|---|---|---|---|---|
| Group | n | CD4-/CD8- | CD4+/CD8+ | CD4+/CD8- |
| Control | 3 | $16.48 \pm 3.18$ | $98.92 \pm 0.20$ | $99.36 \pm 0.07$ |
| Irradiation | 6 | $0 \pm 0$ ** | $1.05 \pm 0.37$ ** | $1.29 \pm 1.08$ ** |
| MSCs transfusion | 3 | $0 \pm 0$ ** | $7.89 \pm 8.59$ **# | $13.23 \pm 6.63$ **## |

The symbols * and ** denote significant or highly significant difference versus control ($P < 0.05$ or $P < 0.01$).
The symbols # and ## denote significant or highly significant difference versus radiation group ($P < 0.05$ or $P < 0.01$).

insignificantly reduced the frequency as compared with the radiation group (Table 7).

## 4. Discussion

It has been shown that mouse thymus T cells are derived from the bone marrow,[27] they migrate in different parts of the thymus gland to differentiate and mature, and are released into the blood for immune response. Most studies suggest that immature thymus T cells normally develop into T cell lymphoma continuously, when CD4-/CD8- T cells are differentiated into CD4+/CD8- T cells and CD4-/CD8+ T cells from CD4+/CD8+ T

cells.[28] Some differences in the percentages of CD4/CD8 subsets have been noticed in different mouse strains and at different differentiation and maturation stages; however, the maturation process is consistent. Our study showed a significant increase in CD4-/CD8- T cells after radiation. This is different from results in an earlier work, where CD4-/CD8- cells were reduced and CD4+/CD8- or CD4-/CD8+ cells were increased after adult mice were irradiated with x-rays.[29] The difference may result from the differences in radiation dose applied, mouse genotype used, and detection times postirradiation. In our study, we analyzed the samples 6 months after irradiation, whereas in the early study, analysis was conducted 32 days after irradiation.[29] Study also
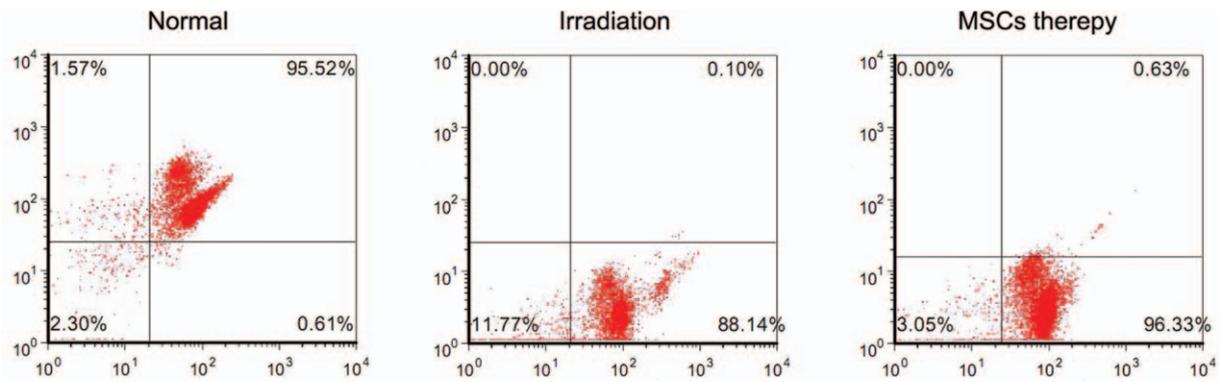
**Figure 3.** Flow cytometry analysis of CD3/CD4 expression in mouse thymocytes after irradiation and MSCs transfusion.

showed that there are some differences in the percentages of CD4/CD8 subsets in different mouse strains and at different differentiation and maturation stage, although the maturation process is consistent.[30] To elucidate the inhibitory effect of MSCs on thymoma, we analyzed the effect of MSCs on cell cycle and proliferation. It is found that in the irradiated mice, the cell arrest was mainly at G1 phase. This is consistent with early results that x-ray irradiation results in arrest of thymic cells at G1 phase in mice.[31] This may be due to the irradiation-induced DNA damage or repair error in the thymus tissue, which triggers cells to response by activating the cell cycle arrest mechanism that arrests the cells at G1 phase to initiate DNA repair mechanism to repair DNA damage. Similarly, CD4-/CD8- T cells were also mainly arrested at G1 phase after radiation. This is probably because the immature CD4-/CD8- T cells in the thymus are very sensitive and vulnerable to radiation. Arrest at G1phase would prevent the errors from being transmitted to next generation. On contrast, for the relatively mature CD4+/CD8+ and CD4+/CD8- T cells, radiation mainly increased the cells arrested at S phase, suggesting that radiation simulates the advance and proliferation of cells as compared with control and MSCs transfusion. This also implies that high proliferation of tumor is mainly due to the increased proliferation of mature cells.

The proliferation kinetics analysis showed that in the irradiated mice, thymocytes became more proliferative, which is particularly remarkable for CD4+/CD8+ and CD4+/CD8- subsets. Early study showed that 24 hours after x-ray irradiation, there were less CD4+/CD8+, and more CD4-/CD8+ and CD4+/CD8- cells in mice.[32] MSCs transfusion reduced the proliferation to near control level. On the contrary, proliferation of the CD4-/CD8- T cells in the irradiated mice were less than those in control and MSCs transfusion groups, suggesting that the CD4-/CD8- T cells in the irradiated mice are immature cells

released from the bone marrow after irradiation and they are less proliferative.

We also analyzed the percentage of CD3+ cells in different subsets of CD4/CD8 cells as a measure of cell maturity. The results showed that 16.48% of cells in the CD4-/CD8- subset were mature in control, but none of them was found in the irradiated or MSCs-transfused mice; for CD4+/CD8+ and CD4+/CD8- subsets, the mature cells were close to 100%, whereas they were only about 1% after irradiation. Su et al[32] found that the number of CD3+ thymocytes is highly correlated to the total number of CD4+ and CD8+ cells. MSCs transfusion increased the percentages of CD3+ cells, but the percentages were till much lower than those in control, suggesting that the cell population after fractionated irradiation is mainly composed of immature cells, and is mainly mature cells after single low-dose irradiation, as a result of cell supplement from bone marrow,[33] and such situation is not able to be restored completely by the MSCs transfusion used in the study. Our work demonstrated that radiation increases the Mf of T-cell receptor.[34] As expected, dramatic increase in the mutation frequency was observed after irradiation in our experiments. These radiation-induced mutations were barely affected by MSCs transfusion, suggesting they are irreversible.

Taken together, our results show that MSCs transfusion to irradiated mice restores the cell cycle and proliferation kinetics to normal levels; however, it has limited impact of CD3 expression and no effect on the mutation frequency of T cell receptor. These findings suggest that MSCs may adjust the cellular microenvironment to restore normal cell growth after radiation and demonstrate the cellular roles of MSCs in the prevention and treatment of thymoma.

## References

[1] Goldstein MG, Siegel R. Thymoma developing 30 years after mantle radiation for Hodgkin's lymphoma. Conn Med 2009;73:521–3.
[2] Meulepas JM, Ronckers CM, Smets AM, et al. Leukemia and brain tumors among children after radiation exposure from CT scans: design and methodological opportunities of the Dutch Pediatric CT Study. Eur J Epidemiol 2014;29:293–301.
[3] Kansara M, Leong HS, Lin DM, et al. Immune response to B1-regulated senescence limits radiation-induced osteosarcoma formation. J Clin Invest 2013;123:5351–60.
[4] Kadota Y, Utsumi T, Inoue M, et al. Radiation-induced osteosarcoma 17 years after mediastinal irradiation following surgical removal of thymoma. Gen Thorac Cardiovasc Surg 2010;58:651–3.

**Mutation frequency of TRC genes in mouse thymocytes after irradiation and MSCs transfusion.**

| Group | No. sample | Mutation frequency (%) |
|---|---|---|
| Control | 3 | $0.008 \pm 0.001$ |
| Irradiation | 6 | $0.996 \pm 0.003$[**] |
| MSCs transfusion | 3 | $0.989 \pm 0.001$[**,#] |

[**] Denote significant or highly significant difference versus control ($P < 0.01$).
[#] Denotes significant or highly significant difference versus radiation group ($P < 0.05$).

[5] Patel S, Macdonald OK, Nagda S, et al. Evaluation of the role of radiation therapy in the management of malignant thymoma. Int J Radiat Oncol Biol Phys 2012;82:1797–801.

[6] Nagamata M, Okuma Y, Yamada Y, et al. Crucial role of treatment with palliative intent for a patient with advanced thymic carcinoma. Oncol Lett 2014;8:513–6.

[7] Kaplan HS. On the natural history of the murine leukemias: presidential address. Cancer Res 1967;27:1325–40.

[8] Mao JH, Wu D, Kim IJ, et al. Hipk2 cooperates with p53 to suppress (-ray radiation-induced mouse thymic lymphoma. Oncogene 2012;31:1176–80.

[9] Zhao L, Zhou C, Zhang J, et al. Hydrogen protects mice from radiation induced thymic lymphoma in BALB/c mice. Int J Biol Sci 2011;7:297–300.

[10] Ball LM, Bernardo ME, Roelofs H, et al. Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. Blood 2007;110:2764–7.

[11] Muguruma Y, Yahata T, Miyatake H, et al. Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. Blood 2006;107:1878–87.

[12] Devine SM, Cobbs C, Jennings M, et al. Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. Blood 2003;101:2999–3001.

[13] Li N, Feugier P, Serrurrier B, et al. Human mesenchymal stem cells improve ex vivo expansion of adult human CD34 +peripheral blood progenitor cells and decrease their allostimulatory capacity. Exp Hematol 2007;35:507–15.

[14] Jiang XX, Zhang Y, Liu B, et al. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. Blood 2005;105:4120–6.

[15] Beyth S, Borovsky Z, Mevorach D, et al. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T cell unresponsiveness. Blood 2005;105:2214–9.

[16] Nauta AJ, Kruisselbrink AB, Lurvink E, et al. Mesenchymal stem cells inhibit generation and function of both CD34+-derived and monocyte-derived dendritic cells. J Immunol 2006;177:2080–7.

[17] Corcione A, Benvenuto F, Ferretti E, et al. Human mesenchymal stem cells modulate B-cell functions. Blood 2006;107:367–72.

[18] Ame-Thomas P, Maby-El Hajjami H, Monvoisin C, et al. Human mesenchymal stem cells isolated from bone marrow and lymphoid organs support tumor B-cell growth: role of stromal cells in follicular lymphoma pathogenesis. Blood 2007;109:693–702.

[19] Spaggiari GM, Capobianco A, Becchetti S, et al. Mesenchymal stem cell-natural killer cell interactions: Evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood 2006;107:1484–90.

[20] Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood 2005;105:1815–22.

[21] Glennie S, Soeiro I, Dyson PJ, et al. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. Blood 2005;105:2821–7.

[22] Krampera M, Glennie S, Dyson J, et al. Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen specific T cells to their cognate peptide. Blood 2003;101:3722–9.

[23] Le Blanc K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. Cytotherapy 2003;5:485–9.

[24] Meisel R, Zibert A, Laryea M, et al. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indole amine 2,3-dioxygenase-mediated tryptophan degradation. Blood 2004;103:4619–21.

[25] Chen YB, Wang HY, Liu LP, et al. Inhibitory effect of mesenchymal stem cells on thymoma induced by ionizing radiation in mice. Chin J Biol 2010;23:36–8.

[26] Ya MA, Dian-Jun HOU, Wei LIU, et al. Estimation value of TCR gene mutation analytical technique to the biological dose of radiotherapy patients. Chin J Cancer Prev Treat 2011;18:1541–3.

[27] Shushimita S, de Bruijn MJ, de Bruin RW, et al. Dietary restriction and fasting arrest B and T cell development and increase mature B and T cell numbers in bone marrow. Plos One 2014;9:e87772.

[28] Siggs OM, Yates AL, Schlenner S, et al. A ZAP-70 kinase domain variant prevents thymocyte-positive selection despite signalling CD69 induction. Immunology 2014;141:587–95.

[29] Muto M, Kubo E, Kamisaku H, et al. Phenotypic characterization of thymic prelymphoma cells of B10 mice treated with split-dose irradiation. J Immunol 1990;144:849–53.

[30] Jine Y, Lis M, Szczypka M, et al. Influence of betulinic acid on lymphocyte subsets and humoral immune response in mice. Pol J Vet Sci 2012;15:305–13.

[31] Ju GZ, Fu HQ, Fu SB, et al. G1 arrest and relative protein expressions in mouse thymocytes induced by whole body X-ray irradiation. Biomed Environ Sci 2001;14:278–82.

[32] Su X, Zhang YC, Liu SZ. Radiosensitivity of thymocytes in different stages of their differentiation. J Radiat Res Radiat Proces 1998;16:38–41.

[33] Sado T, Kamisaku H, Kubo E. Bone marrow–thymus interactions during thymic lymphomagenesis induced by fractionated radiation exposure in B10 mice: analysis using bone marrow transplantation between Thy 1 congenic mice. J Radiat Res 1991;32:168–80.

[34] Taooka Y, Takeichi N, Noso Y, et al. Increased T-cell receptor mutation frequency in radiation-exposed residents living near the Semipalatinsk nuclear test site. J Radiat Res 2006;47(suppl A):A179–81.