Cooperation of CD4+ T cells and CD8+ T cells and release of IFN-γ are critical for antileukemia responses of recipient mice treated by microtransplantation

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Abstract. Previous studies have demonstrated that infusion of allogeneic matched and haploidentical peripheral blood stem cells with minimal conditioning (microtransplantation) achieved durable responses in patients with refractory leukemia/lymphoma in the absence of engraftment. The mechanisms underlying this response have not been thoroughly elucidated, while host-versus-graft reactions are likely to have an important role. The present study established a mismatched microtransplantation mouse model of leukemia to study the roles of CD4+ T cells and CD8+ T cells in changes of interferon (IFN)-γ and interleukin (IL)-4 release to explore the mechanisms of the effects of microtransplantation. It was demonstrated that IFN-γ is critical to the antileukemia response in a mouse model of microtransplantation. The therapeutic efficacy was associated with the number of CD4+ T cells (Pearson's r=0.722). In addition, CD8+ T cells increased the release of IFN-γ with assistance from CD4+ T cells. IL-2 augmented IFN-γ release, partly by increasing CD4+ T cells (42.8 vs. 35.6%; P<0.05). The present study suggested that the release of IFN-γ via cooperation of CD4+ T cells and CD8+ T cells represents a crucial mechanism in the antileukemia responses of recipient leukemic mice treated by microtransplantation. During this process, the cooperation of CD4+ T cells and CD8+ T cells was demonstrated to have a major role in the antileukemia effect. IL-2 may be developed into an agent used for improving the efficacy of microtransplantation by increasing CD4+ T cells.

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Introduction

As with allogeneic stem cell transplantation (allo-SCT), much of the antileukemia effect is derived from graft vs. leukemia (GVL) responses that are dependent on donor cell engraftment (1). To attain engraftment, large doses of chemotherapy or irradiation are administered to suppress the host's immune system. Toxicities associated with allo-SCT are derived from these chemotherapy or irradiation doses, causing myelosuppression. In addition, the resultant bleeding and infection risk that comes with myelosuppression, as well as the threat of engrafted donor immune cells recognizing normal cells instead of leukemia cells, results in graft vs. host disease (GVHD), rather than GVL. As the incidence of acute myeloid leukemia (AML) increases with age, the majority of AML patients have numerous types of comorbidities and are less tolerant to high-intensity consolidation and allo-SCT, leading to a significantly worse prognosis.

Microtransplantation, also known as human leucocyte antigen (HLA)-partially matched donor leucocyte infusion (DLI), is a fractional infusion of small amounts of allogeneic hematopoietic stem cells and has rapidly developed in recent years (2-6). In 2011, Guo *et al* (7) as well as Mackinnon and Chakraverty (8) first reported that the 2-year overall survival (OS) rate in elderly patients with AML increased from 11 to 39% with microtransplantation following chemotherapy. Microtransplantation was reported to have antileukemic effects and promoted hematopoietic recovery. For AML patients aged <65, the 6-year OS rate for low- and medium-risk patients reached 89.5 and 65.2%, respectively, when microtransplantation was added to chemotherapy (9). These results indicated that microtransplantation utilized as a postremission therapy may improve outcomes and prevent GVHD in patients with AML at any age.

The mechanisms of the beneficial effect of microtransplantation have remained largely elusive. However, it is thought that the rejection response is important in mediating antileukemia effects. Indeed, animal experiments in which the process of rejection of donor hematopoietic cells was deliberately simulated have demonstrated that enhancement of specific antitumor responses involved interferon-γ (IFN-γ) and host immune cells (e.g., CD4+ T cells, CD8+ T cells and invariant

natural killer T cells), with IFN-γ being primarily produced by $CD8^+$ T cells (10-14). Hence, the present study hypothesized that the underlying mechanism of action of microtransplantation possibly comprises the generation of a host vs. leukemia response, which results in the release of IFN-γ, interleukin-4 (IL-4) or other cytokines, primarily by $CD4^+$ T cells and $CD8^+$ cytotoxic T cells.

The purpose of the present study was to establish an H-2 completely mismatched microtransplantation mouse model of leukemia. Using this model system, the present study sought to demonstrate the mechanism of the antileukemia efficacy associated with $CD4^+$ T cells, $CD8^+$ T cells and the release of several cytokines, including IFN-γ and IL-4.

Materials and methods

Animals. A total of 64 female BALB/c ($H\text{-}2K^{d/d}$) mice (8-12 weeks) served as recipients, while 64 male C57BL/6J ($H\text{-}2K^{b/b}$) mice (6-8 weeks) were used as donors in the present study. They were purchased from the Shanghai Experimental Animal Research Center (Shanghai, China). All mice were housed in autoclaved, specified pathogen-free microisolator environments, and all manipulations were performed in a laminar flow hood. The use of mice in this study was approved by the Medical Ethics Committee of Wuhan University Zhongnan Hospital and the Cancer Research Center (permission no. ZN2015021; Wuhan, China).

Cell lines. WEHI-3 is a cell line of myelomonocytic cell leukemia that was purchased from the Cell Bank of Sun Yat-sen University (Guangzhou, China). The WEHI-3 cell line was cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (both from Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin), at 37˚C in a humidified air atmosphere with 5% CO_2. WEHI-3 cells grown in the exponential phase were selected and observed under an inverted microscope to ensure good activity, and were subsequently digested and the cell concentration was adjusted to ~$5x10^6$ cells/ml.

Microtransplantation. Male C57BL/6J ($H\text{-}2K^{b/b}$) mice were used as recipients, while female BALB/c ($H\text{-}2K^{d/d}$) mice served as donors. WEHI-3 cells were administered through the tail vein on day 5 prior to transplantation ($1x10^6$/mouse). The recipients were treated with a chemotherapy regimen including mitoxantrone (MA; 4 mg/kg) on day 4 and cytarabine (200 mg/kg) (both from Pfizer, Inc., New York, NY, USA) on days 3-1. Mobilization of hematopoietic stem cells from donors began on day 5 by subcutaneously injecting granulocyte colony-stimulating factor (G-CSF; 100 μg/kg, q 12 h x 5 days; Qilu Pharmaceutical Co., Ltd., Jinan, China). IL-2 was added to donors through tail intravenous injection on days 4-2 at a dose of 3 MU/day. The experiment included 8 groups: Group A, MA + cytarabine + normal saline; group B, MA + spleen mononuclear cells (sMNC) without mobilization; group C, MA + mobilized sMNC; group D, MA + mobilized sMNC + IL-2; group E, MA + sMNC without mobilization + $CD4^+$ T-cell depletion; group F, MA + mobilized sMNC + $CD4^+$ T-cell depletion; group G, MA + mobilized sMNC + $CD8^+$ T-cell depletion; group H, MA + mobilized sMNC + $CD4^+$ T-cell depletion + $CD8^+$ T-cell depletion. At 12 h after the last injection, donor mice were sacrificed by cervical dislocation. Donor spleens were separated, ground, filtered and centrifuged to obtain a splenic mononuclear cell (sMNC) suspension. Red blood cells were removed using red blood cell lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and washed with Iscove's modified Dulbecco's medium (270 x g, 5 min, at 4˚C (Gibco; Thermo Fisher Scientific, Inc.) prior to and after the above procedure. Afterwards, the cells were counted and the cell concentration was adjusted. Donor-derived sMNC ($6x10^6$/mouse) were infused into recipients on day 0 within 8 h after the last dose of chemotherapy. The control group received the same volume of saline solution.

General characteristics and GVHD. General characteristics of the recipient mice, such as mental condition, appetite, activity, diarrhea, skin, weight and incidence of early death, were observed and recorded. The incidence of GVHD was assessed once daily after transplantation. The degree of GVHD was scored based on five indexes, namely weight loss, posture, activity, hair texture and skin integrity (15). Three recipient mice in every group were sacrificed under ether anesthesia by cervical dislocation. Specimens of liver, spleen and small intestine were surgically dissected on day +3 after transplantation. Next, the specimens were fixed with 10% formaldehyde solution, conventionally paraffin-embedded, sectioned, hematoxylin and eosin stained and observed under a light microscope.

Observation of leukemia load. Five recipient mice in every group were sacrificed 3 weeks after inoculation with WEHI-3. Bilateral femurs were separated, muscles were removed, and bone marrow was flushed out of the bone by PBS and stained using the method of Wright-Giemsa (Leagene Biotechnology, Co., Ltd, Beijing, China) at room temperature after centrifugation (280 x g, 5 min, 4˚C). A total of 200 nucleated cells were counted under a microscope and the ratio of blasts was calculated.

Flow cytometric analysis of T-cell subset. T-cell depletion was performed using αCD4 monoclonal antibody (mAb) (1:1,000 dilution, cat. no. SC-19643; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and αCD8 mAb (1:800 dilution, cat. no. AB22374; Abcam, Cambridge, UK) prior to transplantation. Donor sMNCs were added to the corresponding test tube with anti-mouse monoclonal antibodies, including fluorescein isothiocyanate (FITC) anti-mouse CD4 antibody (1:200, cat. no. 100509), phycoerythrin (PE)-CD8 anti-mouse CD8 antibody (1:200, cat. no. 118006) (both from Biolegend Inc., San Diego, CA, USA). The two groups with FITC-immunoglobulin G (IgG)/PE-IgG and without adding any antibodies were used as controls. Cells were incubated with the antibodies for 30 min at 4˚C under exclusion of light and washed twice with PBS buffer. The supernatant was discarded, and the pellet was resuspended in PBS and tested using fluorescence-activated cell sorting.

ELISA for the determination of serum IFN-γ and IL-4 levels. At 7 days after transplantation, blood of mice from different groups was collected from the tail vein. IFN-γ and IL-4 concentrations were measured using mouse IFN-γ (cat. no. MIF00)

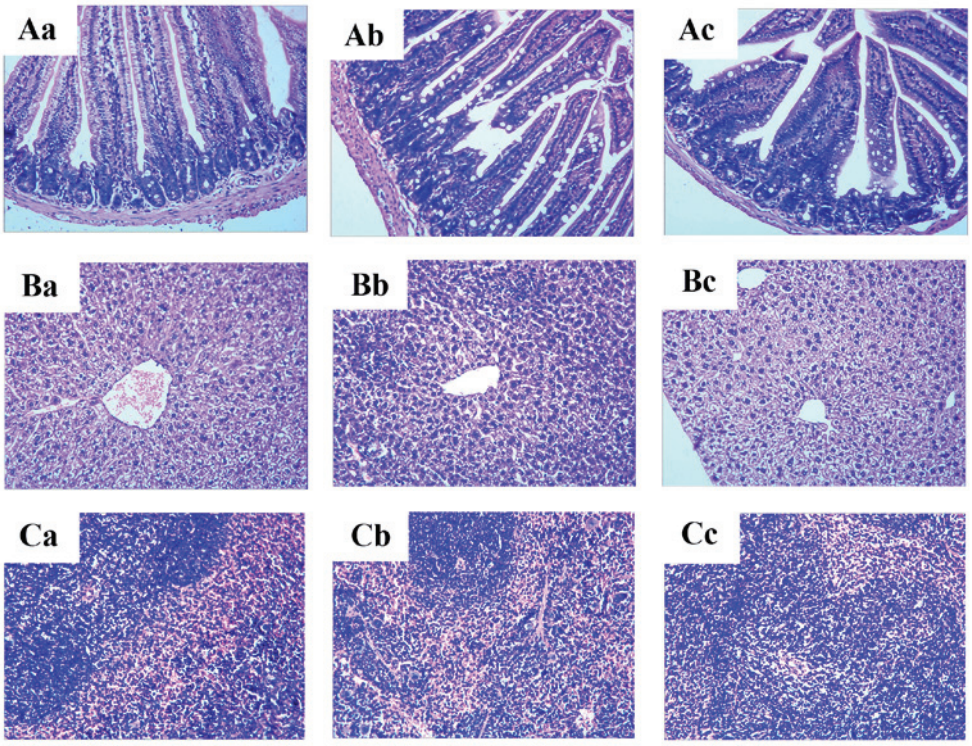

Figure 1. Morphological changes in tissues of mice from different groups. (A) Chemotherapy, (B) chemotherapy + mobilized sMNC and (C) chemotherapy + mobilized sMNC + interleukin-2 groups (hematoxylin and eosin staining; magnification, x200). a, intestine; b, liver; and c, spleen; sMNC, spleen mononuclear cells.

and mouse IL-4 ELISA kits (cat. no. M4000B; both R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis. Data were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Continuous variables were expressed as the mean ± standard deviation. Categorical variables were expressed as a percentage. Statistical comparisons were performed by one-way analysis of variance and Dunnett's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference. Correlations between $CD4^+$ T cells and IFN-γ were analyzed using Pearson's correlation analysis.

Results

General characteristics and GVHD. No early death was observed until the end-point of the experiments at +3 days, which was set according to international guidelines (16). Regarding weight, mental condition, appetite, activity, diarrhea, skin and hair, no significant differences were present among the groups. No symptoms or signs including rash or skin ulcers typically associated with acute GVHD were observed. Furthermore, no significant histopathological differences in tissue biopsy specimens of intestine, liver and spleen were detected. The histopathological results for groups A-C are displayed in Fig. 1. After microtransplantation, the following slight changes were observed: Reduction of intestinal mucosal glands, scattered necrosis of epithelial cells, apoptotic bodies, crypt formation and cell proliferation. The sinusoidal structure of the liver was integrated and a small amount of lymphocytic infiltration was observed. The structure of the spleen had a normal appearance, as the cortex and medulla were clearly distributed, and the spleen was rich in lymphocytes.

Microtransplantation effectively reduces the leukemic load mediated by $CD4^+$ and $CD8^+$ T cells. To assess the antileukemic effect of microtransplantation and determine the importance of $CD4^+$ and $CD8^+$ T cells, the change in leukemia cells caused by infusion of sMNC was observed, as presented in Fig. 2. In the group that received chemotherapy with mobilized sMNC (group C), the percentage of leukemic cells was significantly decreased (8.9 vs. 26.1% in group A; $P<0.05$), and the effectiveness of the microtransplantation was thereby confirmed. When IL-2 was added (group D), the percentage of leukemic cells was further reduced. Even when the infused MNCs were not mobilized (group B), they still had a certain effect. There were no obvious differences in the percentage of leukemic cells in groups E, F and H, which all had depleted $CD4^+$ T cells and as a result had more leukemic cells. These results demonstrate that $CD4^+$ T cells are essential for the antileukemia effect of microtransplantation. In addition, the percentage of leukemic cells in group G (with $CD8^+$ T cells solely depleted) was higher than that in group C (13.8 vs. 8.9%; $P<0.05$). This result demonstrated that $CD8^+$ T cells are also accountable for the antileukemia effects of microtransplantation, while their effect was lower than that of $CD4^+$ T cells.

$CD4^+$ and $CD8^+$ T cells exert their antileukemia effects through releasing IFN-γ after microtransplantation. The serum levels of IFN-γ and IL-4 were measured at 7 days after transplantation (Figs. 3 and 4). The results indicated that

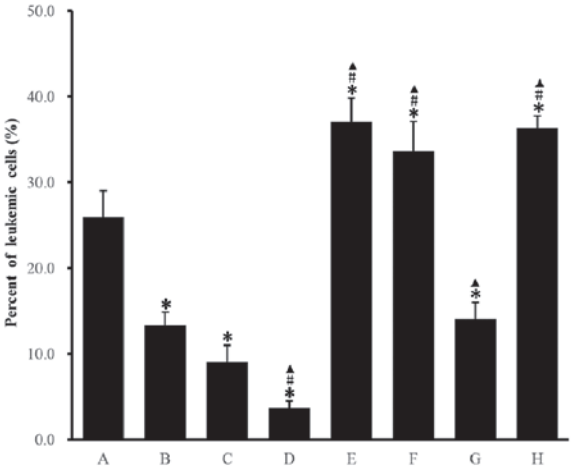

Figure 2. Leukemia load in each group. Blasts in the bone marrow were stained using the method of Wright-Giemsa and counted under a microscope. Values are expressed as the mean ± standard deviation of results from at least three independent experiments. *P<0.05 vs. group A; #P<0.05 vs. group B; ▲P<0.05 vs. group C. Groups: A, MA + cytarabine + normal saline; B, MA + sMNC without mobilization; C, MA + mobilized sMNC; D, MA + mobilized sMNC + interleukin-2; E, MA + sMNC without mobilization + $CD4^+$ T-cell depletion; F, MA + mobilized sMNC + $CD4^+$ T-cell depletion; G, MA + mobilized sMNC + $CD8^+$ T-cell depletion; H, MA + mobilized sMNC + $CD4^+$ T-cell depletion + $CD8^+$ T-cell depletion; MA, mitoxantrone; sMNC, spleen mononuclear cells.

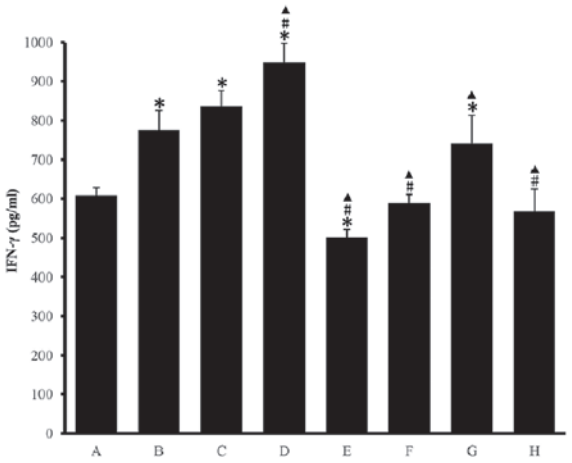

Figure 3. IFN-γ levels in all the groups. IFN-γ concentrations were measured using ELISA. Values are expressed as the mean ± standard deviation of results from at least three independent experiments. *P<0.05 vs. group A; #P<0.05 vs. group B; ▲P<0.05 vs. group C. Groups: A, MA + cytarabine + normal saline; B, MA + sMNC without mobilization; C, MA + mobilized sMNC; D, MA + mobilized sMNC + interleukin-2; E, MA + sMNC without mobilization + $CD4^+$ T-cell depletion; F, MA + mobilized sMNC + $CD4^+$ T-cell depletion; G, MA + mobilized sMNC + $CD8^+$ T-cell depletion; H, MA + mobilized sMNC + $CD4^+$ T-cell depletion + $CD8^+$ T-cell depletion; MA, mitoxantrone; sMNC, spleen mononuclear cells; IFN, interferon.

the serum IFN-γ levels in groups B-D and G were significantly increased compared with those in group A (P<0.01). It was therefore indicated that microtransplantation results in elevated IFN-γ levels, in particular in the groups with mobilized MNCs, and IFN-γ was further increased upon addition of IL-2. Furthermore, it was observed that the levels of IFN-γ in groups F-H were significantly decreased compared with those in group C (P<0.01). In addition, in group F with

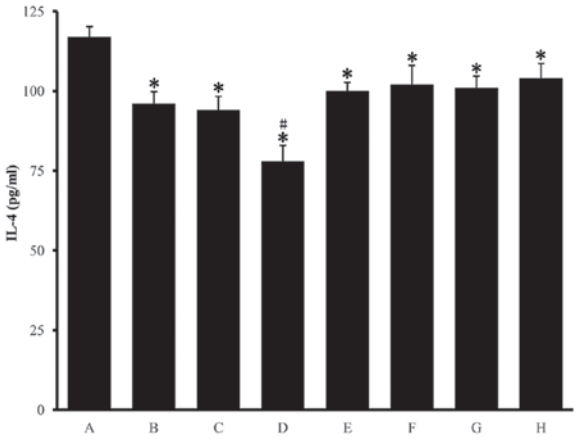

Figure 4. IL-4 levels in all groups. IL-4 concentrations were measured using the method of ELISA. Values are expressed as the mean ± standard deviation of results from at least three independent experiments. *P<0.05 vs. group A; #P<0.05 vs. group B. Groups: A, MA + cytarabine + normal saline; B, MA + sMNC without mobilization; C, MA + mobilized sMNC; D, MA + mobilized sMNC + IL-2; E, MA + sMNC without mobilization + $CD4^+$ T-cell depletion; F, MA + mobilized sMNC + $CD4^+$ T-cell depletion; G, MA + mobilized sMNC + $CD8^+$ T-cell depletion; H, MA + mobilized sMNC + $CD4^+$ T-cell depletion + $CD8^+$ T-cell depletion; MA, mitoxantrone; sMNC, spleen mononuclear cells; IL, interleukin.

depleted $CD4^+$ T cells, IFN-γ was significantly decreased compared with that in group G with depleted $CD8^+$ T cells. These results indicate that $CD4^+$ T cells have a more important role in promoting the release of IFN-γ than $CD8^+$ T cells. In fact, $CD4^+$ T cells may enhance the effect of $CD8^+$ T cells during the process. The trend of IFN-γ levels was synchronous with the change in the leukemic load. Therefore, it may be inferred that the antileukemia effect of microtransplantation was induced by IFN-γ release, which was highly correlated with $CD4^+$ T cells rather than $CD8^+$ T cells. Of note, IL-2 may further strengthen the release of IFN-γ and reduce the leukemic load after microtransplantation, while $CD4^+$ T cells increased from 35.6 to 42.8% (P<0.05).

Th2 cells have antitumor activities that may be mediated through IL-4 and the recruitment of innate immune cells (17). Of note, in the present study, a change in IL-4 levels was detected following microtransplantation. In addition, the levels of IL-4 were decreased in every experimental group, particularly in group D. Regarding IL-2, no significant difference was present among the groups, except for group D. Hence, it was concluded that IL-4 did not have a major role in the antileukemic effects of microtransplantation.

Changes of IFN-γ are correlated with the extent of $CD4^+$ but not $CD8^+$ T cells. As the abovementioned results indicated that the release of IFN-γ is mainly performed or facilitated by $CD4^+$ T cells, the correlation between IFN-γ levels and the amount of $CD4^+$ T cells was then investigated. Indeed, the results indicated that the release of IFN-γ is correlated with the amount of $CD4^+$ T cells (Fig. 5). The Pearson's correlation coefficient of $CD4^+$ T cells and IFN-γ was 0.722, indicating that these two parameters were strongly correlated. These results confirmed that $CD4^+$ T cells were essential for the release of IFN-γ and the antileukemic effects of microtransplantation.

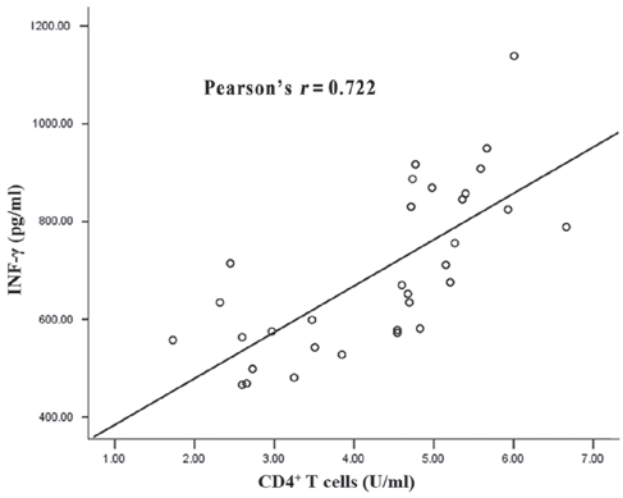

Figure 5. Correlation of $CD4^+$ T cells and IFN-γ. Correlations between $CD4^+$ T cells and IFN-γ were analyzed using Pearson's correlation coefficients. $CD4^+$ T cells and IFN-γ were identified to be correlated, as the correlation coefficient was 0.722. IFN, interferon.

Discussion

To determine the mechanism of the antitumor effect of microtransplantation, a microtransplantation mouse model of leukemia was used and the effectiveness of microtransplantation combined with chemotherapy was further demonstrated. It was demonstrated that amplification of the effects was associated with elevated levels of IFN-γ. By depleting donor-derived $CD4^+$ and/or $CD8^+$ T-cells prior to microtransplantation, it was determined that the release of IFN-γ in recipients was positively correlated with the number of $CD4^+$ but not $CD8^+$ T cells. MNCs without mobilization were less effective, while IL-2 further enhanced the effect of microtransplantation. Therefore, it was inferred that IL-2 may strengthen this effect by increasing the number and function of $CD4^+$ and $CD8^+$ T cells.

In 2011 and 2012, Guo *et al* (7,9) reported the largest clinical trial with high efficacy of HLA haploidentical peripheral blood stem cell infusions in combination with chemotherapy for patients with AML and myelodysplastic syndrome. The failure to observe T-cell engraftment makes it unlikely that the increased responses observed in the above study were associated with a 'classical' GVL response. Rejection of donor hematopoietic cells has been linked to reduced rates of relapse, and it is suggested that the rejection response itself is important in mediating antileukemia effects (18). Kraus *et al* (19) demonstrated that an increase in $CD8^+$ T cells and IL-2-producing T-helper cells occurs in association with graft rejection. Certain patients who lost chimerism following nonmyeloablative hematopoietic cell transplantation still achieved sustained tumor remission (20,21). Rubio *et al* (10) confirmed this in a murine model and revealed that recipient-derived IFN-γ had an important role. Further studies indicated that $CD8^+$ T cells were the major source of IFN-γ. Invariant/constant natural killer T (NK) T-cells, dendritic cells and NK cells, and the interaction among these different immune cells also participate in the antitumor process (11,22). The above course may be classified as host vs. graft (HVG) effects associated with massive systemic cytokine release.

The present study also confirmed several of these points; however, donor-derived $CD4^+$ T cells rather than $CD8^+$ T cells were positively associated with the antileukemia response, which was different from the results obtained by Rubio *et al* (11). It was speculated that this discrepancy may be attributed to different methods of modeling. While Rubio *et al* used receptor lymphocyte infusions, microtransplantation in the present study was performed using donor MNC infusions. Conversely, $CD4^+$ T cells acted upstream of $CD8^+$ T cells, and the production of chemokines, including IFN-γ, IL-6 and IL-10, was measured. Patients with leukemia have a cadre of 'anergic' leukemia-specific T cells that are awakened to become cytotoxic antileukemia effector cells by donor-derived alloreactive $CD4^+$ T cells, which provide support by activating antigen-presenting cells (APCs) (23,24). These same APCs, which are activated by T cells recognizing donor alloantigens via an indirect pathway may also process and present tumor antigens in the context of the host's major histocompatibility molecules to T cells. APC activation by alloreactive T cells may induce an effective tumor antigen-specific response (10). The present study demonstrated that alloreactive $CD4^+$ T cells were critical in promoting IFN-γ production in a microtransplantation model. It was also indicated that $CD4^+$ T cells may prevent $CD8^+$ T-cell exhaustion and mediate a graft vs. leukemia response. Furthermore, $CD4^+$ T cells may optimize tumor eradication by infiltrating $CD8^+$ T cells by producing IL-2. This result is supported by the previous observation that the absence of IL-2 and IFN-γ resulted in completely abrogated $CD4^+$ T cell responses and a lack of tumor killing (25). Based on the present findings, the antileukemia effect was enhanced when IL-2 was added to a traditional microtransplantation model.

Symons *et al* (26) had demonstrated that donor $CD4^+$ T cells cooperate with and effectively awaken the host's $CD8^+$ T cells to induce tumor regression in hematologic and solid malignancies. In particular, they demonstrated that treatment of mice with cyclophosphamide (Cy) followed by infusion of $CD8^+$ T cell-depleted allogeneic donor lymphocytes (Cy + $CD8^-$ DLI) induced the regression of established tumors with minimal toxicity in models of hematologic and solid cancers, even though the donor cells are eventually rejected by the host immune system. In addition, they proved that the antitumor activity involves at least two distinct mechanisms: i) A direct graft vs. tumor effect that requires $CD4^+$ T cells in the DLI and alloantigen expression by the tumor itself; and ii) an indirect antitumor effect mediated by host $CD8^+$ T cells and requiring a GVH reaction against non-malignant host tissue. The experimental outcomes of the present study are consistent with the above results. Therefore, it is implied that the cooperation of $CD4^+$ and $CD8^+$ T cells may have a major role in the antileukemia activity of microtransplantation. In addition, a GVH but not HVG reaction through 'allogeneic effects' may awaken dormant antileukemia immunity by activating host APCs and leukemia-specific host $CD8^+$ T cells.

Chimeric antigen receptor-modified T cells therapy was effectively applied in leukemia, particularly in acute lymphoid leukemia (27). One important mechanism behind this was the release of IFN-γ, IL-6 and IL-10 (28,29). As most studies hypothesized that the mechanism of antitumor effect of microtransplantation is associated with the release of chemokines (4,30,31), this context should be further explored.

In conclusion, the present study provided a pivotal mechanism of the anti-leukemia effects of microtransplantation. To illustrate the mechanism, it was demonstrated that donor-derived $CD4^+$ T cells have an important role in anti-leukemic processes. $CD4^+$ T cells may function directly or via activating host APCs and leukemia-specific host $CD8^+$ T cells to amplify the release of IFN-γ and other cytokines, which eventually facilitates host antitumor immunity. These results may have important implications for the design and implementation of microtransplantation for the treatment of malignant hematological diseases, highlighting methods designed to elevate donor $CD4^+$ T cells, including the addition of IL-2.

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