



## Effects of CD<sub>3</sub>McAb and rhIL-2 activated bone marrow on the killing and purging of leukemia cells

X.C. Wei, D.D. Yang, X.R. Han, Y.A. Zhao, Y.C. Li, L.J. Zhang and J.J. Wang

Institute of hematological research, Shaanxi Provincial People's Hospital, Xi'an, China

Corresponding author: X.C. Wei  
E-mail: weixc22015@163.com

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**ABSTRACT.** We investigated the roles of CD<sub>3</sub>McAb and rhIL-2 activated bone marrow in the killing and purging of leukemia cells. Cytotoxicity of activated bone marrow was detected with MTT assay. CFU-GM level in activated bone marrow and the destruction of leukemia cells were measured using the semi-solid cell culture. Immune activation markers in activated bone marrow were detected by indirect immunofluorescence assay. Bone marrow activated by CD<sub>3</sub>McAb and rhIL-2 displayed significantly upregulated the killing and purging abilities on the leukemia cell line K562 and HL-60. Such effects were superior to that of bone marrow activated by rhIL-2 or CD<sub>3</sub>McAb alone ( $P < 0.05$ ,  $P < 0.01$ ). Activation by rhIL-2 and (or) CD<sub>3</sub>McAb exerted no obvious influence on CFU-GM level in bone marrow. Compared with bone marrow activated by rhIL-2 or CD<sub>3</sub>McAb alone, the synergistic effect of both CD<sub>3</sub>McAb+ and rhIL-2 caused significant increase of CD3<sup>+</sup>, CD8<sup>+</sup>, CD19<sup>+</sup>, CD25<sup>+</sup>, CD38<sup>+</sup>, and CD56<sup>+</sup> levels. Our study indicates that CD<sub>3</sub>McAb enhanced the killing and purging effects of rhIL-2 activated bone marrow on leukemia cells.

**Key words:** CD<sub>3</sub>McAb; rhIL-2; Activated bone marrow; Killing effect; Bone marrow purification

## INTRODUCTION

Autologous bone marrow transplant (auto-BMT) generally has a higher recurrence rate than allogeneic bone marrow transplant (allo-BMT). It is known that autologous bone marrow confers no anti-leukemia effect, and contains residual leukemia cells. *In vitro* purification of bone marrow contaminated by residual leukemia cells is a key technique for improving the efficacy of auto-BMT. Recent studies show that bone marrow activated (ABM) by IL-2 preserves the activity of hematopoietic progenitor, and IFN- $\alpha$  and TNF- $\alpha$  can act synergistically with IL-2 to enhance activity of ABM (Charak et al., 1991; Li et al., 1997; Dong et al., 1997). In the present study, we aim to determine the effect of CD<sub>3</sub>McAb and rhIL-2 activated bone marrow CD<sub>3</sub>McAb and rhIL-2 in killing and purifying leukemia cells.

## MATERIAL AND METHODS

### Specimens

Discarded ribs from 13 patients receiving operations for non-hematological diseases at the Department of Thoracic Surgery were used to harvest bone marrows. Bone marrows obtained were determined to be normal in both morphological and pathological tests. The patients recruited included 6 males and 7 females ranging between 16 to 60 years in age. The leukemia cell lines K562 and HL-60 were donated by the Immunology Teaching and Research Section of the Fourth Military Medical University.

### Reagents

RhIL-2 and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma Corporation (USA); GM-CSF and CD<sub>3</sub>McAb from Jingmei Biological Engineering Co., Ltd (Shenzhen); rat anti-human CD monoclonal antibodies (mAbs) CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, CD19<sup>+</sup>, CD25<sup>+</sup>, CD34<sup>+</sup>, CD38<sup>+</sup>, and CD56<sup>+</sup> were obtained from the Immunology Teaching and Research Section of Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences.

### *In vitro* culture of ABM

Bone marrow was harvested from the discarded ribs under sterilized conditions, and was stored with heparin to prevent coagulation. Hypaque-Ficoll density gradient centrifugation was performed to isolate bone marrow mononuclear cells (BMMNCs). Cells were counted after washing with RPMI 1640 medium three times. BMMNC ( $1 \times 10^6$ /mL) was added into RPMI 1640 medium containing 20% (volume fraction) FCS, 100 U/mL rhIL-2 and (or) 10 ng/mL CD<sub>3</sub>McAb. Cells were cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator. Killing activity and immune phenotypes were determined at day 1, 3, 5, and 7.

### Cytotoxicity detection

Killing activity was detected by MTT assay (Zhu et al., 1998) with an effector to target ratio of 20:1.

## Phenotypic analysis

Immune activation markers were detected by indirect immunofluorescence assay.

## Purification

BMMNC ( $1 \times 10^6$ ) was added into 1 mL RPMI 1640 complete medium with or without CD<sub>3</sub>McAb (10 ng/mL) and (or) rhIL-2 (100 U/mL). K562 or HL-60 ( $5 \times 10^3$ ) proliferating cells were inoculated and cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator for 1-3 days. For the control group, no leukemia cells were inoculated to determine the effect of cytokines on CFU-GM levels.

## CFU-GM assay

BMMNC ( $2 \times 10^5$ /mL) and 20 ng/mL GM-CS were added into RPMI 1640 medium containing 20% FCS, and were thoroughly mixed. Equal volume of 0.3% agar solution was added to the mixture. Cells were dispersed by gentle shaking, and were seeded in 35 mm culture dishes and cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator.

## Leukemia cell cultures

K562 and HL-60 cells were seeded for 1-3 days prior to bone marrow purification. Using 1 mL complete RPMI 1640 medium supplemented with 20% FCS and 0.3% agar solution, 1000 leukemia cells were inoculated and cultured at 37°C in a humidified 5% CO<sub>2</sub> incubator for 7 days. Colonies containing more than 20 cells were counted under the inverted microscope.

## Statistical analysis

Data were expressed as mean  $\pm$  SEM. Intergroup comparisons were performed by Student *t*-test, and  $P < 0.05$  was considered statistically significant.

## RESULTS

### Killing activity of ABM on K562 and HL-60 cells

Killing activity of bone marrow activated by rhIL-2 and (or) CD<sub>3</sub>McAb was significantly higher compared to control samples not supplemented with cytokines ( $P < 0.01$ ). Moreover, the killing activity of bone marrow activated by rhIL-2 and CD<sub>3</sub>McAb was superior to that activated by rhIL-2 or CD<sub>3</sub>McAb alone ( $P < 0.05$ ,  $P < 0.01$ ). Killing activity was initially increased in the first 5 days in culture and declined as time progressed (Table 1).

### Phenotypic analysis

After culture with rhIL-2 and (or) CD<sub>3</sub>McAb, BMMNCs showed significant changes in the level of surface markers. CD3<sup>+</sup>, CD8<sup>+</sup>, CD19<sup>+</sup>, CD25<sup>+</sup>, CD38<sup>+</sup> and CD56<sup>+</sup> cells were increased to varying extent after activation, but CD34<sup>+</sup> cells did not vary significantly in quantity (Table 2).

**Table 1.** Killing activity of bone marrow activated by CD3McAb and (or) rhIL-2 on leukemia cells ( $\pm$  SE, %).

Group	K562				HL-60			
	1 day	3 days	5 days	7 days	1 day	3 days	5 days	7 days
Control group	3.5 $\pm$ 0.9	5.2 $\pm$ 1.2	4.3 $\pm$ 1.0	3.1 $\pm$ 0.7	2.1 $\pm$ 1.0	3.5 $\pm$ 1.1	5.0 $\pm$ 1.7	4.1 $\pm$ 1.2
CD3McAb	10.4 $\pm$ 2.1	14.3 $\pm$ 1.3	16.7 $\pm$ 1.8	13.2 $\pm$ 1.3	9.3 $\pm$ 1.7	15.7 $\pm$ 3.1	17.7 $\pm$ 4.6	14.2 $\pm$ 1.8
CD3McAb + rhIL-2	27.2 $\pm$ 6.1	48.0 $\pm$ 5.7	50.1 $\pm$ 6.2	40.2 $\pm$ 5.0	26.8 $\pm$ 6.0	52.3 $\pm$ 8.0	53.6 $\pm$ 4.0	45.0 $\pm$ 6.9

**Table 2.** Variations of surface markers (percentage of positive cells) before and after activation (means  $\pm$  SE).

Group	n	CD3	CD4	CD8	CD19	CD25	CD38	CD56	CD34
Before activation	9	8.2 $\pm$ 4.1	2.6 $\pm$ 2.1	6.2 $\pm$ 1.2	4.0 $\pm$ 2.0	2.1 $\pm$ 0.4	3.8 $\pm$ 2.0	3.3 $\pm$ 1.4	2.2 $\pm$ 1.1
CD3McAb	9	11.4 $\pm$ 3.1*	3.2 $\pm$ 1.5	8.3 $\pm$ 3.0	4.3 $\pm$ 1.8	4.0 $\pm$ 2.1	4.9 $\pm$ 2.4	5.0 $\pm$ 1.8	2.0 $\pm$ 0.9
rhIL-2	9	14.7 $\pm$ 3.1*	4.0 $\pm$ 1.7	11.4 $\pm$ 2.2*	12.5 $\pm$ 5.7*	9.0 $\pm$ 4.1*	10.2 $\pm$ 4.7*	13.3 $\pm$ 6.0 <sup>a</sup>	3.1 $\pm$ 1.5
CD3McAb+rhIL-2	9	19.7 $\pm$ 4.5 <sup>ab</sup>	4.3 $\pm$ 3.1	15.7 $\pm$ 3.7 <sup>ab</sup>	14.3 $\pm$ 6.8*	11.0 $\pm$ 5.2*	21.3 $\pm$ 7.0 <sup>ab</sup>	15.5 $\pm$ 4.0 <sup>a</sup>	4.0 $\pm$ 2.0

\*P < 0.05 and <sup>a</sup>P < 0.01 compared with that before activation; <sup>ab</sup>P < 0.05 compared with activation by rhIL-2 alone.

### CFU-GM assay for bone marrow activated by CD<sub>3</sub>McAb and (or) rhIL-2

CFU-GM colonies decreased as the culture proceeded regardless of the presence of cytokines. However, no significant differences were found between the non-activation groups and control group ( $P > 0.05$ ). CFU-GM colonies of the groups supplemented or not supplemented with cytokines did not show significant differences on either day 1 or day 3 ( $P > 0.05$ ) (Table 3).

**Table 3.** CFU-GM yield of ABM (means  $\pm$  SE, colonies/1x10<sup>5</sup>% MNC).

Group	Numbers of specimen	Control group	CD3McAb	rhIL-2	CD3McAb-rhIL-2
0 days	6	72.5 $\pm$ 30.1			
1 day	6	70.2 $\pm$ 40.2	71.3 $\pm$ 20.1	69.8 $\pm$ 25.1	74.1 $\pm$ 29.2
3 days	6	62.3 $\pm$ 10.4	67.4 $\pm$ 18.3	66.4 $\pm$ 23.1	70.8 $\pm$ 20.4

### Purifying effect of ABM on leukemia cells

BMMNCs were cultured with 0.5% K562 or HL-60 cells in the presence of CD<sub>3</sub>McAb or rhIL-2 or rhIL-2 plus CD<sub>3</sub>McAb for 1-3 days. Leukemia cells were cultured, and the purifying effect of ABM on leukemia cells was observed. The results showed that ABM had varying purifying effect on leukemia cells; the purifying effect on day 3 was superior to that of day 1. ABM in rhIL-2 plus CD<sub>3</sub>McAb group exhibited enhanced purifying effect as compared with the rhIL-2 or CD<sub>3</sub>McAb group ( $P < 0.05$ ,  $P < 0.01$ ) (Table 4).

**Table 4.** Purifying effect of ABM on leukemia cells (means  $\pm$  SE, %).

Group	K562		HL-60	
	1 day	3 days	1 day	3 days
CD3McAb	23.4 $\pm$ 10.2	56.2 $\pm$ 12.3	25.1 $\pm$ 9.3	60.2 $\pm$ 7.3
rhIL-2	37.5 $\pm$ 8.3	90.5 $\pm$ 7.7	34.7 $\pm$ 9.1	89.3 $\pm$ 6.7
CD3McAb + rhIL-2	42.2 $\pm$ 6.5	99.9 $\pm$ 0.4	47.3 $\pm$ 6.2	99.7 $\pm$ 0.2

## DISCUSSION

ABMT is an important therapy for leukemia treatment despite the high recurrence rate due to residual leukemia cells and the lack of anti-leukemia effects. Thus *in vitro* purification of bone marrow before transplant is crucial. IL-2 is a growth factor secreted by T cells via autocrine/paracrine pathways. Previous experiments showed that ABM produced by co-incubation of IL-2 with bone marrow cells not only preserved activities of the hematopoietic progenitor, but also displayed anti-tumor effects *in vitro* and *in vivo* (Dong et al., 1997; Agab et al., 1989). CD3 is an important leukocyte differentiation antigen expressed on the surface of nearly all T cells. CD3/TCR complex is formed by binding of CD3 to the T-cell receptor (TCR), which can activate nearly all T cells if bound to anti-CD3 monoclonal antibodies. This then enhances non-specific killing effect and proliferation of T cells. Anti-CD3 monoclonal antibodies and

IL-2 can act synergistically to promote non-specific killing activity and proliferation of T cells (Yun et al., 1989). We found that ABM induced by CD<sub>3</sub>McAb and rhIL-2 had a prominent killing effect on K562 or HL-60 cells *in vitro*; the difference was of statistical significance compared with ABM induced by CD<sub>3</sub>McAb or rhIL-2 ( $P < 0.01$ ,  $P < 0.05$ ). The combined use of CD<sub>3</sub>McAb and rhIL-2 greatly improved the killing effect of ABM on leukemia cells without the need to increase rhIL-2 concentration.

Many techniques have been invented for purification of bone marrow before ABMT, but nearly all of them have shortcomings. ABM activated by IL-2 may lead to the dissolution of several human and mouse cell lines and fresh tumor cells. An *in vitro* purification experiment showed that co-incubation of normal ABM and 1-10% leukemia cell line CEM for 2 weeks caused significant inhibition of colonies. In contrast, normal cell colonies proliferated rapidly. This suggested that ABM could clear low-level leukemia cells (Charak et al., 1990). In our purification experiment, ABM induced by rhIL-2 and (or) CD<sub>3</sub>McAb exhibited a clearing effect on K562 or HL-60 cells, and the purifying effect on day 3 was better than that on day 1. After a 3-day culture, ABM induced by rhIL-2 and CD<sub>3</sub>McAb cleared almost all leukemia cells. However, a few number of leukemia cells were still present in the groups activated by rhIL-2 or CD<sub>3</sub>McAb alone. It was concluded that rhIL-2 and CD<sub>3</sub>McAb activated bone marrow achieved a better purifying effect.

Phenotypic changes were also observed before and after bone marrow activation. In our experiment, CD3<sup>+</sup>, CD8<sup>+</sup>, CD19<sup>+</sup>, CD25<sup>+</sup>, CD38<sup>+</sup> and CD56<sup>+</sup> cells in ABM induced by CD<sub>3</sub>McAb + rhIL-2 were significantly increased, and their numbers were greater than those in groups activated by rhIL-2 or CD<sub>3</sub>McAb alone. It was indicated that CD<sub>3</sub>McAb + rhIL-2 induction played an important role in the activation of T cells, B cells and NK cells in bone marrow. The high killing and purifying activity in the CD<sub>3</sub>McAb + rhIL-2 group may be related to the increased level of immunocyte subgroups. Hematopoietic activity was not compromised by anti-tumor effect as evidenced by no obvious reduction of CD34<sup>+</sup> cells. ABM produced by this method can confer anti-leukemia effect.

To conclude, bone marrow activated by CD<sub>3</sub>McAb and (or) rhIL-2 displayed killing and purifying effect on leukemia cell K562 and HL-60, especially when activated by both CD<sub>3</sub>McAb and rhIL-2. Moreover, hematopoietic activity was not obviously inhibited. The findings confirm the feasibility of rhIL-2 and CD<sub>3</sub>McAb activated bone marrow *in vitro* to eradicate leukemia cells before ABMT.

## Conflicts of interest

The authors declare no conflicts of interest.

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