Detailed Analysis of Bone Marrow From Patients With Ischemic Heart Disease and Left Ventricular Dysfunction
BM CD34, CD11b, and Clonogenic Capacity as Biomarkers for Clinical Outcomes


Rationale: Bone marrow (BM) cell therapy for ischemic heart disease (IHD) has shown mixed results. Before the full potency of BM cell therapy can be realized, it is essential to understand the BM niche after acute myocardial infarction (AMI).

Objective: To study the BM composition in patients with IHD and severe left ventricular (LV) dysfunction.

Methods and Results: BM from 280 patients with IHD and LV dysfunction were analyzed for cell subsets by flow cytometry and colony assays. BM CD34+ cell percentage was decreased 7 days after AMI (mean of 1.9% versus 2.3%–2.7% in other cohorts; P<0.05). BM-derived endothelial colonies were significantly decreased (P<0.05). Increased BM CD11b+ cells associated with worse LV ejection fraction (LVEF) after AMI (P<0.05). Increased BM CD34+ percentage associated with greater improvement in LVEF (+9.9% versus +2.3%; P=0.0355). BM-derived endothelial colonies were significantly decreased (2.3%–2.7% in other cohorts; P<0.05). Increased BM CD34+ cell nadir 7 days after AMI, a negative correlation between CD11b percentage and postinfarct LVEF, and positive correlation of CD34 percentage with change in LVEF after cell therapy. These results serve as a possible basis for the small clinical improvement seen in autologous BM cell therapy trials and support selection of potent cell subsets and reversal of comorbid BM impairment.

Conclusions: In this study, we show a heterogeneous mixture of BM cell subsets, decreased endothelial colony capacity, a CD34+ cell nadir 7 days after AMI, a negative correlation between CD11b percentage and postinfarct LVEF, and positive correlation of CD34 percentage with change in LVEF after cell therapy. These results serve as a possible basis for the small clinical improvement seen in autologous BM cell therapy trials and support selection of potent cell subsets and reversal of comorbid BM impairment.


Key Words: angiogenesis effect ▪ blood cells ▪ bone marrow ▪ myocardial infarction ▪ stem cells
clinical studies have shown increased percentage of endothelial progenitor cells (EPCs) in the peripheral blood (PB) after AMI and correlation with improved LV systolic function after AMI. It is possible that the BM is a source of circulating EPCs after infarction; however, a paucity of information is available that describes the BM niche in patients with IHD.

Editorial, see p 814

Of particular interest after AMI are CD34+ cells in the BM. These stem and progenitor cells, when injected in the ischemic or infarcted myocardium, reduce the number of angina pectoris episodes in a dose-dependent manner and improve myocardial perfusion after AMI. In the absence of understanding the BM niche after AMI, some have raised the possibility that earlier BMC therapy trials showing improved LV ejection fraction (LVEF) were either red herrings or that later studies because of cell processing procedures vitiated BMC potency. Others have called for abandoning BMCs, altogether. To improve on these early clinical investigations, we must understand the composition and functional status of the delivery agent (BM).

Therefore, we chose to study the BM of a large cohort of patients with IHD and severe LV dysfunction (LVD) with particular respect to BM surface expression and vasculogenic colony capacity. The goal of this report is to describe the detailed composition of the BM obtained at different times after AMI.

Methods

Study Populations and Sources of Cells

BM and PB were obtained from consenting patients enrolled in the Cardiovascular Cell Therapy Research Network (CCTRN), Transplantation in Myocardial Infarction Evaluation (TIME), LateTIME, and First Mononuclear Cells injected in the United States conducted by the CCTRN (FOCUS) trials. The TIME trial randomized 120 patients with AMI and severe LVD to intracoronary injection of BM mononuclear cells (MNCs) versus placebo at 3 versus 7 days after AMI. The LateTIME trial randomized 87 patients with AMI to intracoronary injection of BM MNCs versus placebo. The FOCUS trial randomized 92 patients with chronic IHD and severe LVD not amenable to surgical revascularization to intramyocardial injection of BM MNCs versus placebo.

Among the 3 studies, 299 study participants were recruited at 5 clinical centers and their satellites under institutional review board approvals. Of the 299 subjects, 291 consented to donate to the biorepository. Because of insufficient volume in 11 samples, the final evaluable data set consisted of samples from 280 patients. An automated closed-system density gradient centrifugation separation protocol using Ficoll was used to separate BMCs from whole BM (Sepax device; Biosafe Group, Eysins, Switzerland). Within 12 hours of the BM harvest, a prescribed number of autologous BMCs were administered in the hearts of subjects after MI. Extra aliquots of BMCs were shipped overnight to a central biorepository for rapid assessment of cell phenotype, evaluation of cell function, and cryopreservation. Immediately on receipt in the central biorepository, BMCs were separated by Ficoll and density gradient centrifugation.

Cell Phenotyping and Flow Cytometry

BMC phenotyping was performed by immunostaining (BD Biosciences) and flow cytometry (BD LSRII) using antibody-fluorochrome conjugates (BD Biosciences) for 30 minutes on ice. Appropriate isotype controls were also used (BD Biosciences). Stained cells were washed, resuspended in Dulbecco’s phosphate-buffered saline plus 2% fetal bovine serum containing Via-Probe (BD Biosciences), and analyzed using a Becton Dickinson LSRII flow cytometer. International Society of Hemotherapy and Graft Engineering (ISHAGE) protocols were used for enumerating CD34+ and CD133+ cells. FlowJo software (TreeStar, Inc, Ashland, OR) was used to analyze the flow cytometry data. Confocal imaging of fluorescently labeled BM cells was performed to confirm labeling (Online Figure I).

Progenitor Cell Analyses

BMCs were evaluated for clonogenic capacity by assays for hematopoietic and EPC activity, as previously described. Colony-forming cell (CFC) assay (Methodult; Stem Cell Technologies) was performed at all 5 study sites to evaluate hematopoietic progenitor cell activity. Endothelial colony formation assays were performed in the centralized biorepository core laboratory using methods previously described to evaluate for vasculogenic and proangiogenic progenitor cell activity. In brief, BMCs were plated in Endocult (Stem Cell Technologies) or endothelial growth media-2 (Stem Cell Technologies) according to manufacturer guidelines and incubated at 37°C in a fully humidified atmosphere with 5% CO₂. Colony formations were enumerated weekly for 4 weeks, and the maximum number of colonies per plate were used for analyses. BM and PB from healthy individuals (Lanza, Walkersville, MD) were used to demonstrate viable progenitor cell assays. BM and PB from healthy individuals were processed using the same MNC preparation (ie, overnight shipment; Sepax MNC separation) as the patients with IHD.

Statistical Analysis

Summary statistics are tabulated as percentages for discrete variables for TIME, LateTIME, and FOCUS. Summarizations of baseline characteristics are compared across studies, with differences between continuous variables assessed using the general linear model, whereas differences between dichotomous variables were evaluated using χ² testing. Therapy groups were combined because of the absence of differences for the Table 1 baseline characteristics across therapy groups in each of the studies. BM and PB characteristics were assessed for congruency with Pearson correlation coefficients.

Results

Patient Characteristics

Between July 8, 2008, and November 15, 2011, BM from 280 patients with acute and chronic IHD and LVD (LVEF≤45%) were collected. The majority of subjects were older, obese white men with a history of smoking, hypertension, and hyperlipidemia (Table 1). After multiplicity correction, P values of <0.003 were deemed as statistically significant differences.
among the proportions of patients. As expected, there was a greater proportion of patients with chronic IHD that also had cardiovascular disease–relevant comorbidities (ie, diabetes mellitus, hypertension, and hyperlipidemia) and angina pectoris. BM from 9 healthy volunteers aged 20 to 40 years (median, 36 years) were recruited during this same time period, and their BM was processed using the same MNC isolation methods as the patients with IHD.

Heterogeneous BMC Phenotypes With Quantitative Variation in Patients With IHD
The BM from patients with IHD was predominately (>50%) composed of CD45+ and CD11b+ cells (Figure 1A–1D). To a lesser extent (5%–20%), the BM contained cells expressing CD3+, CD14+, and CXCR4+. In addition, the BM contained minor populations (<5%) of cells expressing CD19+, CD133+, CD34+, CD31+CD45−, and VEGFR2+.

Decreased Colony Formations Generated From BM of Patients With IHD and LVD
BM from all patients with IHD and healthy controls showed hematopoietic progenitor activity by generating CPC colonies in Methocult media (Table 2). However, shortly after AMI, the number of individuals whose BM showed proangiogenic and vasculogenic activity by colony-forming unit Hill (CFU-Hill) assay (Endocult) and endothelial CFC (ECFC) assay was significantly reduced (CFU-Hill 55% versus 100%; P<0.001 and ECFC 43% versus 100%; P<0.001). Even in the subacute period (2–3 weeks) after AMI significantly fewer patients generated CFU-Hill (74% versus 100%; P<0.0001) and ECFC colonies (78% versus 100%; P<0.0001). Although fewer Late TIME patients grew colonies, the number of CFU-Hill colonies was not reduced in BM where colonies grew (Figure 2A). However, ECFC colony number was significantly decreased in the patients at 2 to 3 weeks after AMI (Figure 2B).

Interestingly, in patients with heart failure from the FOCUS group, which had higher proportion of patients with comorbid factors, such as diabetes mellitus, hyperlipidemia, and hypertension, BM was more likely to generate CFU-Hill and ECFC colonies than BM from the TIME group.

Decreased BM CD34+ Cells 7 Days After AMI
Of the 10 BMC subsets enumerated (Figure 1), only CD34+ cells differed according to time from MI, with study participants 7 days from AMI showing the lowest percentage of CD34+ cells (1.9%) when compared with subjects 3 days after AMI (2.3%; P=0.05), 14 to 21 days from AMI (2.6%; P<0.05), and patients with chronic IHD (2.7%; P<0.05; Figure 3).

Postinfarct Heart Function and BM Composition
To compare BM composition with postinfarct heart function, regression analyses were performed on the 10 BMC subsets (Figure 1) and 2 endothelial assays (CFU-Hill and ECFC) when compared with LVEF. Only CD11b+ cell (monocyte...
and macrophage) percentage significantly (and inversely) associated with postinfarct LVEF (P < 0.05): for every 1% greater in CD11b+, LVEF was lesser by 0.22%. These results support previous reports of increased innate immune cell activity after AMI and their importance in mediating myocardial remodeling.18–21

BM CD34+ Cells as a Biomarker for Clinical Outcome After Cell Therapy for IHD

Given the importance of CD34+ stem/progenitor cells in various tissue repair processes, we scrutinized the BM CD34+ cell percentage of patients with IHD in this study and found a distinct cohort of patients with elevated CD34+ cell percentage. Nine AMI patients showed a >2 SD increase in BM CD34+ cell percentage (mean, 5.7%) when compared with the rest of the patients with IHD (mean, 2.2%). Normally, in a resting state, human BM CD34+ cell percentage lies below 5%. Therefore, we hypothesized that increased CD34+ stem/progenitor cell percentage correlated with improvement in LVEF at 6-month follow-up. In fact, patients presenting with a high BM CD34+ percentage after AMI achieved greater increase in LVEF at 6 months when compared with others (+9.9% absolute increase in LVEF versus +2.32%; P = 0.03; Figure 4A). When applying this same analysis in patients with chronic IHD and severe LVD, 3 subjects with >2 SD increase in BM CD34+ also showed a greater increase in LVEF when compared with others (+6.6% versus −0.02%; P = 0.021; Figure 4B).

In complementary fashion, we hypothesized that lower BM CD34+ cell percentage after AMI indicated a suppression or lack of response in the BM resulting in a decrement in LVEF after AMI. To test this hypothesis, the 10 patients with IHD and the lowest BM CD34+ percentages immediately after AMI were compared with the others. These individuals had no significant change in LVEF at 6-month follow-up (mean change in LVEF, +2.55% versus +2.68%; P = 0.9671). However, the 10 chronic patients with LVD and the lowest BM CD34+ percentage demonstrated a significant decline in their LVEF at 6-month follow-up when compared with others (mean change LVEF, −2.93% versus +0.69%; P = 0.0355). Together, these results suggest that BM CD34+ stem/progenitor cell percentage may be a biomarker for response after AMI.

### Table 2. Frequency of BM-Derived Progenitor Cell Colony Outgrowth Among Patients With IHD Compared With Healthy Controls

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<th>Healthy, %</th>
<th>TIME, %</th>
<th>Comparison With Control</th>
<th>LateTIME, %</th>
<th>Comparison With Control</th>
<th>FOCUS, %</th>
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<td>78</td>
<td>P &lt; 0.0001</td>
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<td>P &lt; 0.0001</td>
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BM mononuclear cell from healthy control subjects (n=9), TIME, LateTIME, and FOCUS patients were grown in Methocult (CFC assay), Endocult (CFU-Hill assay), and endothelial growth media-2 (endothelial CFC assay). Outgrowth of hematopoietic progenitor cell colonies (CFC) was observed in all patients. However, BM from patients with IHD enrolled in TIME, LateTIME, and FOCUS trials were less likely to generate endothelial cell colonies (CFU-Hill and ECFC) when compared with healthy individuals. BM indicates bone marrow; CFC, colony-forming cell; and IHD, ischemic heart disease.
No Correlation Between PB and BMC Subsets and Progenitor Activities

Given the minimal risk in obtaining PB and the higher risk of sampling BM, we examined whether PB measurements of cell subsets and progenitor activities correlated with BM. Nine cell lineages and progenitor outgrowth results were selected based on previous reports of repairing ischemic/infarcted myocardium (Online Table I). None of the 9 subsets and progenitor activities showed strong correlation between PB and BM. Of the 9, the strongest correlation between PB and BM was the CD34+CD133+ percentage, but its strength of correlation was weak (0.55; 95% confidence interval, 0.40–0.67).

Discussion

In this detailed analysis of BM from patients with IHD, we show a heterogeneous mixture of cell subsets, decreased endothelial colony capacity, a CD34+ cell nadir 7 days after AMI, inverse relation between CD11b percentage and LVEF immediately after AMI, and positive correlation of CD34 percentage with change in LVEF 6 months after autologous BMC therapy.

Because this is the first presentation of major and minor BMC subsets from patients with IHD, no comparisons can be made to other studies. However, the proportions of major cell lineages (ie, CD45+, CD3+) are consistent with the proportions observed in the standard clinical practice of BM transplant for patients with hematologic malignancies.

In terms of BMC function, the data show that BM from patients with IHD and severe LVD contains hematopoietic progenitor cell activity (as measured by the CFC assay) but has reduced ability to form EPC colonies (as measured by ECFC and CFU-Hill assays). This study confirms a previous report showing no change in hematopoietic progenitor activity after MI but extends previous knowledge by revealing impairment in BM-derived vascular precursors after AMI. When
Figure 4. Bone marrow (BM) CD34+ cell percentage and change in left ventricular ejection fraction (LVEF) at 6 months. BM aspirations were performed in patients presenting with acute myocardial infarction (AMI) and chronic LV dysfunction (LVD). CD34+ percentage was quantified by ISHAGE criteria and LVEF was examined at baseline and 6 months later. A, Patients with AMI who presented with high BM CD34+ percentage (>2 SD from the mean) showed significantly greater increase in LVEF at 6-month follow-up. B, Likewise, patients with chronic LVD and high BM CD34+ percentage (>2 SD from the mean) also showed significantly greater increase in LVEF at 6-month follow-up.

considering that the BM can be a source for circulating EPCs and that number of circulating EPCs correlates with LVEF function after AMI, the results suggest that the BM from patients with IHD is a plentiful resource for hematopoiesis but potentially a finite reservoir of vasculogenic precursors. One consideration to make when evaluating these data is that the healthy control study participants were younger in age and without known cardiac disease. Although their BMCs were processed exactly like the IHD study patients, it is possible that age may have been a determinant in BM cell-derived endothelial colony formation. In a nonhuman primate model of age-related changes, decreased number and function of thymus mononuclear circulating ECFCs were found in aged primates. Whether age affects BM-derived ECFC in humans has yet to be determined.

The only BMC subset that significantly differed according to time from AMI was the CD34+ cell fraction. Although a minor population in the BM, the percentage of BM CD34+ stem and progenitor cells was decreased in patients with AMI 7 days after percutaneous intervention, suggesting a temporary depression of CD34+ cells in the BM. In the setting of MI, possible instigators of this depression could include proinflammatory cytokines, angiogenic factors, and sympathetic nervous system signaling. In an experimental model of BMC therapy for AMI, regional MI led to systemic inflammation that triggered proliferation of activated myeloid cells in the BM. However, the BMCs showed a time-dependent depression in regenerative capacity with a nadir of activity at 3 days after AMI in mice. This relationship between the injured heart and BM mirrors what we observed in the current human study with particular respect to BM CD11b+ and CD34+ percentages. After AMI in experimental models, systemic cytokines that alter BM composition and depress regenerative function include interleukin (IL)-1α, IL-1β, IL-6, and GM-CSF (granulocyte macrophage colony-stimulating factor), in addition to others, providing a possible explanation for the decrease in BM CD34+ percentage and impaired clonogenic capacity in patients with IHD. Interestingly, in rodents, the cardiac regenerative capacity of BMCs can be recovered after treating BMCs ex vivo with immune suppression. Follow-up translational studies will include evaluations of BM and PB plasma for inflammatory cytokines and attempted recovery of BM function by inflammation antagonists.

Although our data are supported by experimental evidence, there are a few differences in relation with previous clinical reports from other groups. Only one other cell therapy group has reported CD34+ values over multiple time points after AMI. In contrast to our results, data from Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) subjects showed a small increase in BM CD34+ percentage at days 6 to 8 when compared with days 2 to 6 after AMI with mean BM CD34+ increasing from 1.4% to 1.9%. Although the direction of change differed in our study, the absolute value seen at day 7 was similar (1.9%). When comparing our BM CD34+ cell percentages with the previous report, absolute differences are ≤0.5%, which calls into question whether the differences are biologically significant. Moreover, differences in study populations between the CCTRN trials and the REPAIR-AMI trial could account for the differences in CD34 percentages. This fine point may bear further examination.

Although CD34+ stem and progenitor cells are a minor subset within the BM, they have multipotent potential and are closely enumerated in the standard practice of hematology, oncology, and BM transplantation. In patients with IHD, we found that BM CD34+ cell percentage after AMI correlates with change in LVEF at 6 months. Patients with AMI and severe LVD who had increased BM CD34+ cell percentage showed markedly improved LVEF (+10% absolute increase) at 6 months. These individuals represent an interesting cohort of enhanced responders. Whether their early LV improvement is sustained long term remains to be determined. Another question of interest is the biological significance of increased BM CD34+ cells. Because 4 of 9 (44%) CD34+ enhanced responders were randomized to active cell therapy, it is possible that the high level of CD34+ cells may have served a direct role in heart regeneration. However, it is also possible that the high level of BM CD34+ cells is a biomarker for some other process, such as inflammation, angiogenesis, and catecholaminergic signaling instigated by an infarcted myocardium.

Data from this study show BM impairments in patients with IHD and severe LVD as a potential explanation for the mixed trial results in autologous BMC therapy trials. Rather than abandon BMCs as a therapeutic source, more investigation should occur into selecting potent cell subsets or reversing cell impairments before clinical use. First, these data confirm the importance of CD34+ cells in cardiac cell therapy. Leading up to this report, Losordo et al. and Wang et al. demonstrated reduced frequency of angina pectoris and improved exercise tolerance in patients with IHD who received intramyocardial and intracoronary injections of autologous CD34+ cells when compared with placebo. In patients with nonischemic dilated cardiomyopathy, transendocardial injection of autologous CD34+ cells was associated with higher myocardial retention.
rates and greater improvements in ventricular function when compared with intracoronary route.27 Our data confirm the importance of CD34 number in BMC-mediated repair after AMI but go on to show that even when cell number is intact, progenitor cell function can be decreased in these patients—reinforcing the concept that reversal of loss of function may be equally as important as improving cell number.

Although the mechanisms by which BMCs improve myocardial function are still unclear, the CD11b and CD34 data from this report suggest an important relationship between inflammatory cues and BMC response. In the ischemic/infarcted myocardial microenvironment, BMCs most likely act as paracrine regulators, mitigating toxic inflammation, and triggering capillary regrowth, thereby preventing cardiomyocyte apoptosis or stimulating resident cardiac stem cells.1,28 Therefore, selection of potent BMC subsets may best be defined in terms of homing (eg, CXCR4 expression) and controlling inflammation and angiogenesis. If so, the optimal BMCs for cardiac regeneration after MI may require upregulation of chemokine receptors, such as IL-1Rs or CXCR4. More simply, it may be possible to treat BMCs ex vivo before patient administration with agents that reverse BMC impairment(s). For example, given the upregulation of the proinflammatory cytokine, IL-1, in the BM and hearts of patients with IHD, pretreating BMCs with IL-1R antagonists, such as anakinra, could reverse BMC impairment and improve cardiac outcomes.29 Ultimately, merging data about the milieu of the postinfarct myocardial microenvironment with data from reports, such as this one, will be necessary to select and engineer the most potent BMC subtype in the future.

Conclusions

In this study, we show a heterogeneous mixture of BMC subsets, decreased endothelial colony capacity, a CD34+ cell nadir 7 days after AMI, a negative correlation between CD11b percentage and postinfarct LVEF, and positive correlation of CD34 percentage with change in LVEF after cell therapy. These results serve as a possible basis for the small clinical improvement seen in autologous BMC therapy trials and support selection of potent cell subsets and reversal of comorbid BM impairment.

Sources of Funding

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Disclosures

None.

References


What Is Known?

- Bone marrow (BM) contains stem and progenitor cells capable of generating blood vessels in response to ischemia and inflammation.
- After acute myocardial infarction (AMI), BM cell (BMC) therapy improves left ventricular (LV) function in experimental models, but effects in patients with AMI are minimal.
- Limited information is available about the BM niche and links with LV function in patients with acute and chronic ischemic heart disease (IHD).

What New Information Does This Article Contribute?

- A heterogeneous mixture of cell subsets is present in the BM of patients with IHD.
- Post-AMI endothelial colony capacity is decreased with a nadir in CD34+ cell number at 7 days and a negative correlation between CD11b percentage and LV function.
- There is a positive correlation between CD34+ percentage and change in LV function after BMC therapy.

Experimental studies that document BMC therapy improves LV function in AMI models. However, only minimal LV functional improvement has been reported after BMC therapy in patients with AMI or those with chronic IHD. We found that the BM niche of patients with LV dysfunction because of acute or chronic IHD, analyzed for cell subsets by flow cytometry and colony assays, contains a heterogeneous mixture of cell subsets. Both the cell numbers and the colony growth characteristics vary over time after AMI. The CD34+ cell percentage is significantly decreased 7 days after AMI when compared with BM from patients with less acute or chronic IHD. Also BM-derived endothelial colonies are significantly decreased. In patients with chronic IHD, a decreased CD34+ percentage is associated with greater improvement in LV function among the patients with AMI and patients with chronic IHD, correlated with the decrease in LV function observed 6 months after study treatment. These findings may explain, in part, the only minimal and variable LV functional improvement observed in autologous BMC therapy trials and support selection of more potent cell types and attempts to reverse comorbid BMC impairment.

Novelty and Significance
Detailed Analysis of Bone Marrow From Patients With Ischemic Heart Disease and Left Ventricular Dysfunction: BM CD34, CD11b, and Clonogenic Capacity as Biomarkers for Clinical Outcomes


for the Cardiovascular Cell Therapy Research Network (CCTRN)

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Supplemental Material

Online Figure I. Multiple Bone Marrow Cell Types in Ischemic Heart Disease Patients. Representative confocal images of fluorescently labeled bone marrow cells from IHD patients. Hematopoietic cells were most abundant and included CD45+ (green), CD11b+ (red), CD3+ (red), and CD14+ (red) cells. Nuclear staining with DAPI (blue). Magnification 40X.
## Supplemental Table I

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Abbreviations: CI, confidence interval; LB, lower bound; UB, upper bound; CD, cluster of differentiation; ECFC, endothelial colony-forming cell; CFU-Hill, colony forming unit Hill; BM, bone marrow; PB, peripheral blood; MSC, multipotent mesenchymal stromal cell