High expression of heme oxygenase-1 in target organs may attenuate acute graft-versus-host disease through regulation of immune balance of TH17/Treg

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Abstract

The high incidence of acute graft-versus-host disease (aGVHD) is a serious complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT). Grades III and IV aGVHD are the leading causes of death in allo-HSCT recipients. Heme oxygenase-1(HO-1) has anti-inflammatory and immune-regulatory functions. In this study, we evaluated the none GVHD and grade I-IV patients samples which were collected at the first re-examination after successful allo-HSCT, we found that expressions of HO-1 mRNA in the bone marrow and peripheral blood mononuclear cells of allo-HSCT recipients who had subsequent non-GVHD and grade I aGVHD were significantly higher than those in patients with Grade III-IV aGVHD. We then demonstrated that enhanced expression of HO-1 in target organs by infusing HO-1-gene-modified Mesenchymal stem cells (MSCs) alleviated the clinical and histopathological severity of aGVHD in experimental mice. Flow cytometry revealed a higher expression of Treg cells and a lower expression of TH17 cells in splenic and lymph node tissues of mice with enhanced HO-1 expression, as compared to that in the aGVHD mice. This was further substantiated by lower expression levels of ROR- γ t and IL-17A mRNA, and higher levels of Foxp3 mRNA in the splenic tissue of mice with enhanced HO-1 expression. Our results indicate that high expression of HO-1 may reduce the severity of aGVHD by regulation of the TH17/Treg balance.

Keywords: Heme oxygenase-1; Acute graft-versus-host disease; Mesenchymal stem cell; TH17; Treg

1. Introduction

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) is among the most effective therapies for a variety of hematological malignancies and autoimmune diseases (1, 2). However, aGVHD is the main complication of allo-HSCT and causes significant morbidity and mortality (3,4). 30%-60% of patients which receive an

allo-HSCT from an HLA-identical sibling donor subsequently develop grade II-gradeIV aGVHD (5). aGVHD remains a significant obstacle to more widespread use of allo-HSCT (6,7). Moreover, the advances in molecular biology notwithstanding, the molecular mechanisms that mediate GVHD are poorly understood. MSCs are good carrier cells (8), they have been widely used in gene therapy, and can migrate to areas of inflamed tissue and tissue damaged by GVHD, after which, they assist in repairing the damaged tissue (9,10). The inducible form of heme oxidase (HO-1) plays a pivotal role in anti-inflammation and immunosuppression (11,12). Several recent studies have shown the protective effect of HO-1 gene against rejection of transplanted organs (13,14). Some investigators have used COPP to induce high levels of endogenous HO-1 expression in prevention of GVHD following experimental allogeneic bone marrow transplantation and high levels of HO-1 expression were shown to be associated with improved survival of transplant recipients (15-19). However, whether a causal association exists between HO-1 expression levels and aGVHD in allo-HSCT recipients is not known.

In this study, we aim to explore a correlation between expression levels of HO-1 mRNA at the first re-examination after allo-HSCT and severity of subsequent aGVHD in allo-HSCT recipients. We further hypothesized that enhanced expression of HO-1 by lentiviral gene transfer alleviated GVHD-induced target organ damage in experimental mice. We also investigated that HO-1 mediates aGVHD by its affect on the Th17/Treg immune balance.

2. Materials and methods

2.1 Patients

Bone marrow mononuclear cells of 62 allo-HSCT recipients and 30 healthy bone marrow donors were examined. All patients were admitted in a hospital Affiliated to Guizhou Medical University (Guizhou, China) in the period between January 2013 and August 2015. aGVHD was evaluated (grade1-4) according to the overall severity (20,21). Clinical characteristics of the patients are summarized in Table 1. Written informed consent was obtained from all patients as well as the healthy donors prior to

their enrolment in the study. The study protocol was approved by the Ethics Committee at the Hospital affiliated to Guizhou Medical University.

2.2 Experimental Animals

Eight-to10-week-old male C57BL/6 (H-2b) and female BALB/c (H-2d) mice were purchased from the Chinese Academy of Medical Sciences (Beijing, China) and the Third Military Medical University (Chongqing, China), respectively. We made slight modifications to the protocols for induction of aGVHD reported elsewhere (22-24). Briefly, On the day of transplantation, C57BL/6 (donor) were sacrificed by cervical dislocation. Their femora were removed aseptically, and bone marrow (BM) was removed from the femoral shaft by insertion of a 1 ml syringe needle at the proximal end. A single cell suspension of spleen cells was obtained by passage of minced spleen through 200 mesh nylon filter and resuspended in RPMI 1640 medium. Female BALB/c (recipient) mice received a single dose of 5.8 Gy x-ray total body irradiation, and were infused intravenously with 2×10^7 bone marrow cells from male C57BL/6 donor mice four hours later. 3×10^7 donor splenocytes (as a source of allogeneic T cells) were co-infused with bone marrow cells to induce aGVHD. The day of transplantation was designated as day zero.

C57BL/6 mouse BMSCs (Cyagen Biosciences Inc., Guangzhou, China) were transduced with murine *HO-1*-expressing lentiviruses (rLV-mHO-1-ZsGreen) or control vector (rLV-ZsGreen) (Viral therapy Technologies, Wuhan, China) in the presence of 6 µg/mL polybrene (Sigma). 5×10^5 of lentivirus-transduced BMSCs (MSC-ZsGreen or MSC-HO-1-ZsGreen) were injected into the recipient mice 24 hours after HSC transplantation.

All mice were maintained in pathogen-free animal facilities. All animal experiment protocols were approved by the Animal Ethics Committee at the Guizhou Medical University.

2.3 Immunohistochemistry

Two-step detection of tissue antigen expression was performed with the PV6001 two-Step immunohistochemistry (IHC) Detection Reagent. Blocks of paraffin-embedded tissue were cut into 3 μm thick serial sections. The sections were then deparaffinized, hydrated, and incubated in 3% hydrogen peroxide at room temperature (RT) for 10 min to eliminate endogenous peroxidase activity. In the next step, the sections were washed 3 times with PBS (5 min per wash) and subjected to antigen retrieval using a heated high pressure technique. Briefly, the sections were immersed in a solution containing 0.001 mol ethylenediamine tetraacetic acid disodium salt (EDTA 2Na), at pH 8.0, and heated for two minutes; after which, they were cooled at RT. The primary antibody (HO-1) (Proteintech, Chicago, IL, USA) was diluted at ratios of 1:150 and 1:130. Each tissue section was incubated with 50 μL of primary antibodies, while samples of control tissue were incubated with PBS. Following incubation, all tissue samples were stored overnight at 4°C. Each section was subsequently washed 3 times with PBS (5 min per wash), and then incubated with 50 μL of goat anti-rabbit or mouse IgG antibody (HRP multimers) at 37°C for 30 min. The tissue sections were then washed 3 times with PBS (5 min per wash) and incubated with 100 μL of DAB (Zhongshan Xinqiao Company; Beijing, China) staining solution for color development. When microscopic examination revealed the presence of brownish yellow color, the sections were immediately rinsed with tap water to terminate the reaction. In the next step, the sections were washed with tap water, re-stained with hematoxylin, dehydrated, transparentized, and mounted on neutral gum.

2.4 Western Blot Analysis

Splenic tissue of mouse were washed twice in ice-cold PBS, the cells were lysed by sonication in RIPA buffer containing 1 mM Phenylmethanesulfonyl fluoride (Solarbio Science & Technology; Beijing, China) for 20 min. The lysate was then centrifuged at 12,000 $\times g$ for 10 min at 4°C. The supernatant was collected and mixed with loading buffer. The final solution was boiled for 10 min. An equal amount of protein (100-150 μg) was loaded and run on a 10% SDS-PAGE gel and transferred onto PVDF membranes (Millipore Corporation, Milford, MA, USA), which were then blocked in

5% non-fat milk in TBST buffer at 4°C overnight. The membranes were treated with corresponding primary antibodies at a dilution of 1:500 (HO-1 33 kDa) (Proteintech, Chicago, IL, USA) and (β -actin 42 kDa) (Beyotime Institute of Biotechnology, Shanghai, China) for 2h while shaking at RT. Subsequently, the membranes were washed in TBST 3 times (10 min each) and incubated with the corresponding conjugated secondary antibody at a dilution of 1:1,000 [horseradish peroxidase-labeled (HRP) goat anti-rabbit IgG (H+L), HRP-labeled goat anti-mouse IgG (H+L)] (Beyotime Institute of Biotechnology, Shanghai, China) for 2 h at RT. After washing in TBST three times (10 min each), protein bands were visualized on film by enhanced chemiluminescence (7sea Biotech, Shanghai, China) as per the manufacturer's instructions. The expression levels of the target genes were normalized to that of β -actin.

2.5 Real-time PCR

Total RNA were extracted from mononuclear cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Real-time PCR using SYBR-Green PCR Master Mix (TianGen Biotech, Beijing, China) and the ABI Prism 7500 Sequence Detection System (Applied Biosystems Life Technologies, Foster City, CA, USA). Melting curve analysis was applied to ascertain the specificity of amplification. Relative levels of target mRNA were compared with β -actin using the $1/2^{(C_{T\text{target}}-C_{T\text{control}})}$ method.

Sequences were as follows:

Murine RoRYt:5'- TGAACCTGGGGAACCAGAACAG -3'(sense),5'-TAGAAGG TCCTCCAGTCGCAG-3'(antisense);IL-17A:5'-CCCTAAGAAACCCCCACG-3' (sense),5'-AAGTCCACAGAAAAACAAACACG-3'(antisense);Foxp3:5'-CTCTAG CAGTCCACTTCACCAA-3'(sense),5'-CACCCACCCTCAATACCTCTCT-3'(antisense), β -actin:5'-GGGAAATCGTGCGTGAC-3'(sense),5'-AGGCTGGAAAAGAGCC T-3'(antisense).HumanHO-1:5'-ACCCATGACACCAAGGACCAGA-3'(sense),5'-GT GTAAGGACCCATCGGAGAAGC-3'(antisense), β -actin,5'-TCACCCACACTGTGCC ATCTACGA-3'(sense),5'-CAGCGGAACCGCTCATTGCCAATGG-3' (antisense).

cDNA samples were mixed with primers and SYBR-Master Mix to a total volume of 20 μ L. The thermal cycling conditions used in the protocol were: 1 minute at 94°C, followed by 40 cycles of 10 sec at 94°C and 15 seconds at 60°C for 15 cycles.

2.6 Assessment of acute GVHD in mice

For clinical GVHD scoring, five parameters were monitored in mice: weight loss, posture, activity, fur texture, and skin integrity; the assessment was based on consensus criteria (25). Liver, lung, small intestine and spleen were fixed in 4% paraformaldehyde, embedded in paraffin blocks, and 5 μ m thick H&E-stained sections prepared. Pathological scores were calculated following a mice GVHD histopathology scoring system (26).

2.7 Flow cytometric analysis

The spleens and LNs were isolated to prepare single-cell suspensions, then we exact lymphocytes by Lymphocyte Separation Medium (Solarbio Science & Technology; Beijing, China). Cells were immunostained with several combinations of fluorescence-conjugated antibodies against CD4, IL-17A and Foxp3. The antibodies were purchased from BD Biosciences Pharmingen (San Jose, CA) and Biolegend (San Diego, CA). Briefly, before intracellular staining, cells were stimulated for 5h with 50 μ g/L phorbol myristate acetate (PMA) and 1mg/mL ionomycin (eBioscience, San Diego, CA, USA). Intracellular staining was conducted using an intracellular staining kit (eBioscience, San Diego, CA, USA), according to the manufacturer's instructions. Gates were determined by isotype staining performed on the same cells and time point, and analyzed on a BD FACS can using Cell Quest software (BD Biosciences, San Jose, CA).

2.8 Statistical analysis

Data analyses were performed using GraphPad Prism 5.0 (GraphPad Software. San Diego, CA, USA). Mann-Whitney non-parametric test was used to assess between-group differences; two-tailed *P* values < 0.05 were considered indicative of a

statistically significant difference. Data are presented as mean $\pm$ standard error of the mean. Log-rank test was used for mouse survival analysis.

3. Results

3.1 Correlation of aGVHD severity with *HO-1* gene expression in bone marrow and peripheral blood mononuclear cells of allo-HSCT patients.

Out of the 62 patients who participated in the study, 20 did not have GVHD, 16 showed evidence of grade I, 14 had grade II, and 12 had grade III-IV GVHD (Table 1). *HO-1* gene expression (mRNA) levels in bone marrow and peripheral blood mononuclear cells of the patients were assessed. We evaluated the none GVHD and grade I-IV patients samples which were collected at the first re-examination after successful allo-HSCT (i.e. before the onset of acute GVHD), we showed that *HO-1* mRNA expression in bone marrow of patients with none GVHD and those with grade I aGVHD were comparable (0.0011 ± 0.00012 vs. 0.0012 ± 0.00023 , $P > 0.05$) which, nonetheless, were significantly higher than those in the patients with severe aGVHD (grade III-IV) ($P < 0.05$) (Fig. 1A and Table 2). Equally, *HO-1* mRNA expression in peripheral blood of patients with none GVHD and those with grade I aGVHD were comparable (0.0010 ± 0.00020 vs. 0.0010 ± 0.00017 , $P > 0.05$) which, nonetheless, were significantly higher than those in the patients with severe aGVHD (grade III-IV) ($P < 0.05$) (Fig. 1B and Table 2).

Interestingly, the *HO-1* levels in the cohort of healthy donors were significantly lower than those in the none GVHD and grade I-II aGVHD patients ($P < 0.05$) (Fig. 1A-B). These results indicate a correlation between *HO-1* expression levels and the severity of aGVHD.

3.2 Enforced overexpression of *HO-1* by lentiviral gene transfer alleviated inflammatory injury to target organs in aGVHD and improved survival.

Efficient lentiviral transduction of mouse MSCs was indicated by the positive green

fluorescent protein signals on fluorescence microscopy (Figure. 2A, 72 hours post-transduction). Western blot further validated the over-expression of HO-1 protein in the lentivirus-transduced mouse MSC (MSC-HO-1-ZsGreen) (Figure. 2B). At 14 days after transplantation, mice in the MSC-ZsGreen and MSC-HO-1-ZsGreen groups were sacrificed, and tissue samples were examined by immunohistochemical staining (Figure. 3A). HO-1 expressions in damaged target organs of mice that received HO-1-gene-modified MSCs (MSC-HO-1-ZsGreen) were considerably higher than those in the ZsGreen-infected control group (MSC-ZsGreen) (Figure. 3B). MSCs with HO-1 overexpression significantly enhanced the survival of recipients, reduced weight loss, and attenuated clinical and pathological scores of mice (Figure. 4A-B). Gene transfer-mediated HO-1 overexpression evidently reduced GVHD-related tissue damage and inflammation in multiple organs (liver, lung, small intestine, and spleen) (Figure. 4C).

3.3 HO-1 over-expression increased Treg cell number and reduced TH17 cell number in aGVHD mice.

Splenocytes and lymph nodes were collected after euthanizing all experimental mice on day 14 post-transplantation. We added the without GVHD (BMT) mice as control. On flow cytometry, TH17 cell numbers and Th17/Treg cell ratio in both lymph nodes and spleens in the MSC-HO-1-ZsGreen group were lower, while Treg cell numbers were higher than that in the aGVHD and MSC-ZsGreen groups. However, there were not statistically significant between without GVHD (BMT) group and MSC-HO-1-ZsGreen group (Figure. 5A-C). On Real time PCR, ROR- γ t and IL-17A mRNA levels in the HO-1 overexpression (MSC-HO-1-ZsGreen) mice were lower while the FoxP3 expression was higher than that in the aGVHD and MSC-ZsGreen mice, and there were not statistically significant between without GVHD (BMT) mice and MSC-HO-1-ZsGreen mice (Figure. 5D).

4. Discussion

In this study, we observed a correlation between HO-1 mRNA levels and aGVHD severity in allo-HSCT recipients. Based on this, we hypothesized that high expression of HO-1 may ameliorate aGVHD. To the best of our knowledge, this is the first report of correlation between HO-1 and allo-HSCT aGVHD, although some controversial findings of the correlation between HO-1 expression and solid organ transplant rejection, e.g. heart and lungs, have been reported (27-29). However, the small sample size in our study is a notable limitation, which mandates more comprehensive study in a larger sample of patients. We are on course to recruit more patients to assess the impact of other variables (e.g. patient and donor age, degree of HLA-identity, use of ATG and other immunosuppressants) on the observed correlation in more complicated patients.

We then proved the hypothesis by showing that enforced overexpression of HO-1 in target organs by gene transfer can significantly alleviate aGVHD in experimental HSCT/aGVHD mice. Our results represent a further progress from a report in which high expression of endogenous HO-1 in mice induced by COPP was shown to prevent the occurrence of GVHD, and prolong survival of allogeneic transplant recipient mice (19). However, the induced HO-1 expression in that study may have been transient, in addition to the potential inherent COPP toxicity. MSCs has the characteristics of low toxicity and long-term expression of exogenous genes (30-32). The gene transfer-mediated HO-1 over-expression in our study provided persistent protection against GVHD.

High HO-1 expression induced by hemin in mice was shown to alleviate mice neutrophilic airway inflammation and acute murine colitis via regulation of TH17 cells and Treg cells, the suppressive effect of HO-1 on Th17/Treg-related inflammation may be helpful for developing novel therapeutic approaches in managing these diseases (33,34). Recent studies have also demonstrated the ability of TH17 cells to initiate and promote GVHD. For example, Kappel found that Th17 cells and their secretion (IL-17) further aggravates early inflammation following a transplant, and promote the occurrence of aGVHD (35-37). The retinoic acid-related orphan receptor

Yt (ROR- γ t) is a key transcription factor that mediates the differentiation of Th17 cells (38). Yu *et al* reported alleviation of aGVHD after blockage of ROR- γ t (39). IL-17 is the characteristic cytokine secreted by Th17 cells, which is known to have a biological effect (40). The transcription factor Foxp3 is a key molecule that regulates the differentiation and development of Treg cells (41). Treg cell expansion has been shown to be capable of reducing the severity of acute GVHD in murine acute GVHD models(42,43). Further, in our study, we found that HO-1 over-expression may alleviated inflammatory injury of aGVHD mice by modulation of TH17/Treg ratio. Similarly, investigators also indicated that change of Treg/Th17 cell ratio was shown to induce a series of inflammatory reactions, which further aggravated aGVHD in mice (44,45). Our results strongly suggest that HO-1 expression mediates aGVHD severity via regulation of TH17/Treg balance.

5. Conclusions

Our results demonstrate that higher levels of HO-1 expression ameliorate aGVHD in allo-HSCT recipients via immune regulation of TH17/Treg cell balance.

Acknowledgments

This study was supported by the National Science Foundation of China (No. 81070444) and International Cooperation Project of Guizhou Province (No. 2012-7043). The authors would like to thank Dr. Ying Chen (Department of Hematology, Affiliated Hospital of Guizhou Medical University) for her advice.

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Figure 1. Comparison of HO-1 mRNA expression in healthy donors, none GVHD and aGVHD patients at the first re-examination after allo-HSCT. Total RNA was extracted from the mononuclear cells of bone marrow(A) and peripheral blood (B), and relative expression levels of HO-1 mRNA determined by Realtime PCR. *P < 0.05, **P < 0.01.

Figure2. MSCs of C57BL/6 mouse were transfected with lentivirus to express HO-1. A. MSCs were transfected by lentivirus vectors, the expression of HO-1 was upregulated by rLV-mHO-1-ZsGreen (MSC-HO-1-ZsGreen), and rLV-ZsGreen (MSC-ZsGreen) was control. The expression of ZsGreen were examined by fluorescent microscope. B. The expression of HO-1 protein in each group was analysed by western blot. This experiment was performed once using five mice per group. *** $P < 0.001$.

A

B

Figure3. Fourteen days after transplantation, the expression of HO-1 protein in target organs of the MSC-ZsGreen and MSC-HO-1-ZsGreen groups. A. Representative models of HO-1 immunohistochemical staining in liver, lung, small intestine and spleen tissue from MSC-ZsGreen and MSC-HO-1-ZsGreen mouse. Positive immunoreactivity appears as a brown color and is counterstained with hematoxylin as blue color. original magnification $\times 400$. B. The protein expression of HO-1 in each group's splenocytes was analyzed by western blot. *P < 0.01.

A

B

C

Figure4. Infusion of HO-1-overexpressing MSCs can attenuates aGVHD in mouse. A. The typical model respectively represent the three groups of mice. B. Mouse were monitored for proportion surviving, weight loss, clinical GVHD score and GVHD histological grade. C. Fourteen days after transplantation. Histopathologic assessment of the liver, spleen (original magnification, $400\times$), Lung and small intestine(original magnification, $200\times$). This specimens was stained with H&E. Results are shown as mean $\pm$ SEM of five mice. $**P < 0.01$, versus aGVHD group. ► denotes roach back, ➡ denotes messy hair, ➤ denotes tail skin ulceration and perianal abscess, ● yellow color was picric acid for signing, ➡ denotes coagulation necrosis foci, ► denotes proliferation of fibroblasts and fibrocytes.

Figure 5. HO-1 over-expression increased Treg cell number and reduced TH17 cell number. A. Representative data for IL-17A and Foxp3 expressions on CD4⁺ T cells from splenocytes and lymph nodes are shown. B and C. Data were presented as the ratio of Th17/Treg among CD4⁺T-cells. D. Fourteen days after transplantation, total RNA was extracted from the spleen tissues, and relative expression levels of ROR- γ t, Foxp3 and IL-17A were determined by realtime PCR. Data in the graphs represent mean $\pm$ SD, data represent three independent experiments. BMT, bone marrow transplant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table1. Summary of characteristics about aGVHD patients and non-GVHD patients after receiving allo-HSCT.

Characteristics	patients. n(%)
Total number of patients	62
aGVHD/non- GVHD	42/20 (68/32)
Median patient age, years (range)	27 (6-56)
Patients gender, male/female	35/27 (56/44)
Donor relationship to patient	
Sibling/Parent/Offspring/Other	48/10/3/1 (77/16/5/2)
Donor-patient gender	
Female-male/ Male-female	17/15 (27/24)
Female-female/Male-male	12/18 (19/29)
HLA-pattern	
partially matched/matched	35/27 (56/44)
Conditioning regimen	
BU/CY	25 (40)
BU/CY+ATG	35 (57)
Flu+CTX+ATG	2 (3)
Source of HSC: PBSC+BM/PBSC	61/1 (98/2)
Type of diagnosis	
AML/ALL/CML	26/21/8 (42/34/13)
MDS/NHL/SAA	3/1/3 (5/2/5)
Involved organs	
Skin/GI tract/Liver/Multiorgans	42/22/12/8 (68/35/19/13)
Severity classification of aGVHD	
GradeI/GradeII/GradeIII/GradeIV	16/14/4/8 (26/23/6/13)

Abbreviations: allo-HSCT, allogeneic hematopoietic stem cell transplantation; aGVHD, acute graft-versus-host disease; HLA, human leukocyte antigen; BU, Busulfan; CY, Cyclophosphamide; ATG, antithymocyte globulins; Flu, Fludarabine; HSC, hematopoietic stem cells; PBSC, peripheral blood stem cells; BM, bone marrow; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic syndrome; CML, chronic myelogenous leukemia; NHL, Non-Hodgkin's lymphoma; SAA, severe aplastic anemia; GI tract , Gastrointestinal tract. partially matched: Degree of HLA-identity between donor and recipient include 3/6, 4/6 or 5/6.

Table 2. Comparison of bone marrow and peripheral blood mononuclear cells HO-1 mRNA levels in healthy donors, none-GVHD and grade I-IV aGVHD patients during the first re-examination after successful allo-HSCT.

Groups	No.patients	Bone marrow	Peripheral blood
		Mean±SEM	Mean±SEM
Healthy donors	30	0.0006±0.00008	0.0004±0.00007
None GVHD	20	0.0011±0.00012	0.0010±0.00020
Grade I aGVHD	16	0.0012±0.00023	0.0010±0.00017
Grade II aGVHD	14	0.0001±0.00012	0.0008±0.00019
Grade III-IV aGVHD	12	0.0007±0.00013	0.0005±0.00019

Abbreviations: HO-1=Heme oxygenase-1; aGVHD=acute graft-versus-host disease; allo-HSCT=allogeneic hematopoietic stem cell transplantation.