RESEARCH ARTICLE

Immune Reconstitution of CD4+T Cells after Allogeneic Hematopoietic Stem Cell Transplantation and its Correlation with Invasive Fungal Infection in Patients with Hematological Malignancies

Xin-Guo Peng*, Yan Dong, Ting-Ting Zhang, Kai Wang, Yin-Jian Ma

Abstract

Objective: To explore the immune reconstitution of CD4+T cells after allogeneic hematopoietic stem cell transplantation (Allo-HSCT) and its relationship with invasive fungal infection (IFI) in patients with hematological malignancies. Materials and Methods: Forty-seven patients with hematological malignancies undergoing Allo-HSCT in Binzhou Medical University Hospital from February, 2010 to October, 2014 were selected. At 1, 2 and 3 months after transplantation, the immune subpopulations and concentration of cytokines were assessed respectively using flow cytometry (FCM) and enzyme linked immunosorbent assay (ELISA). The incidence of IFI after transplantation and its correlation with immune reconstitution of CD4+T cells were investigated. Results: The number of CD4+T cells and immune subpopulations increased progressively after transplantation as time went on, but the subpopulation cell count 3 months after transplantation was still significantly lower than in the control group ($p<0.01$). In comparison to the control group, the levels of interleukin-6 (IL-6) and IL-10 after transplantation rose evidently ($p<0.01$), while that of transforming growth factor-β (TGF-β) was decreased ($p<0.01$). There was no statistically significant difference level of interferon-γ (IFN-γ) ($p>0.05$). The incidence of IFI was 19.2% (9/47), and multivariate logistic regression revealed that IFI might be related to Th17 cell count ($p<0.05$), instead of Th1, Th2 and Treg cell counts as well as IL-6, IL-10, TGF-β and IFN-γ levels ($p>0.05$). Conclusions: After Allo-HSCT, the immune reconstitution of CD4+T cells is delayed and Th17 cell count decreases obviously, which may be related to occurrence of IFI.

Keywords: Hematological malignancy - allogeneic hematopoietic stem cell transplantation - immune reconstitution

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Introduction

Allogeneic hematopoietic stem cell transplantation (Allo-HSCT) has been an effective way to treat solid tumors, hematological malignancy and immunodeficiency disease (Mohty et al., 2011; Yetisyigit et al., 2014). Hematopoietic reconstitution is possible after Allo-HSCT, and most of patients could immediately obtain granulocytes and platelet reconstitution 2~3 weeks after transplantation, but the process of immune reconstitution is delayed (Mensen et al., 2014). Immune reconstitution after Allo-HSCT is divided into reconstitution of inherent immune system and reconstitution of specific immune system. The former including granulocytes and mononuclear macrophages usually occurs 2~3 weeks after transplantation, whereas the latter including T-lymphocytes and B-lymphocytes is very slow after transplantation, especially for CD4+T cells (Perlingeiro Beltrame et al., 2014).

Studies have shown that the immune reconstitution of patients with Allo-HSCT plays important roles in the prevention of disease recurrence and decrease of mortality, and delayed immune reconstitution is closely associated with infection and secondary tumors (Perales et al., 2012; Qin et al., 2013; Holtick et al., 2014). Invasive fungal infection (IFI) is one of the major reasons for patients' deaths after Allo-HSCT. Specific immune response mediated by CD4+T cells plays a key role in the body antifungal action mechanisms (Liu et al., 2013).

Under induction of different cytokines, initial CD4+T cells in peripheral blood can differentiate into different subpopulations of effector T cells. These subpopulations of cells then secret different cytokines on their own and perform different functions in immune response. In this study, the relevant indexes of different subpopulations of effector T cells were detected at the levels of cells and cytokines so as to further explore immune reconstitution characteristics of CD4+T cells after Allo-HSCT and its relationship with IFI in patients with hematological malignancy.

*Laboratory Department, Binzhou Medical University Hospital, Binzhou, China *For correspondence: bzyfxxyl@126.com*

Materials and Methods

General data

A total of 47 patients with hematological malignancy and undergoing Allo-HSCT in Binzhou Medical University Hospital from February, 2010 to October, 2014 were selected as research objects, in which males and females were respectively 33 and 14 cases. They were 16~55 years old, with the median age of 37.5 years. There were 34, 8 and 5 patients respectively with acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL) and chronic myeloid leukemia (CML). The transplantation of 38 cases was supplied by compatriot donors and 9 by unrelated donors. There were 2 and 45 donors respectively consistent and completely consistent with beyond site HLA 9/10, 17 and 30 cases respectively given myeloablative preconditioning regimen with/without antithymocyte globulin (ATG). Preventive regimens for acute graft-versus-host disease (aGVHD): 30 cases given tacrolimus (FK506)+rapamycin (MTX)+mycophenolate mofetil (MMF) and 17 receiving ciclosporin +MTX+MMF. Additionally, 40 healthy people who had physical examination in Binzhou Medical University Hospital were set as control group, in which males and females were respectively 28 and 12 cases. They were 18~56 years old, with the median age of 38 years.

The research was conducted in accordance with the ethical principles of human body medical research in Declaration of Helsinki at Institute of Jinan Stem Cell Regeneration and Translational Medicine. Experimental protocols were approved by Ethics Committee of Binzhou Medical University Hospital. All subjects had been told detailed experimental contents by researchers and signed informed consent form before enrollment.

Methods

Detection of number of immune subpopulation after transplantation by flow cytometry (FCM): 5 mL peripheral blood was drawn from fasting subjects in the morning, and heparin was used as anticoagulation. Centrifugation was conducted for 5 min in 1 500/min. Single nuclear cell layer was separated using density gradient centrifugation after leukocytic membrane was taken. A single karyocyte was resuspended with appropriate RPMI 1640 medium+10% fetal bovine serum (FBS)+100 U/mL mycillin and counted. Amount of medium was regulated to make cell concentration be 2×10^6/mL approximately. Cell suspension was subpackaged into No. 1~6 EP tubes, 250 μL per tube. No. 1 and 2 tubes were placed in a 5% CO_2 incubator for incubating 4~6 h at 37°C, and then the supernatant was discarded and FCM was performed after centrifugation for 5 min in 1 500/min. BD Diva software was applied to analyze the proportions of CD4+cells in lymphocytes and Treg in CD4+cells, and the absolute number of Treg and CD4+cells was counted by combining absolute lymphocyte count in peripheral blood revealed by blood routine examination. Both 1 μL of phorbol ester/ionomycin suspension and 1 μL of brefeldin A/monensin suspension were added into No. 3~6 EP tubes to make them reach 50 ng/mL, 1 μg/mL, 3 μg/mL and 1.4 μg/mL of concentrations, respectively. After that, EP tubes were placed in a 5% CO_2 incubator for incubating 4~6 h at 37°C, and then the supernatant was taken and FCM was performed after centrifugation for 5 min in 1 500/min. BD Diva software was applied to analyze the respective proportion of Th17, Th1 and Th2 in CD4+cells, and the absolute number of each subpopulation was counted according to absolute lymphocyte count in peripheral blood.

Detection of cytokine concentration by enzyme linked immunosorbent assay (ELISA): 5 mL peripheral blood was drawn from fasting subjects in the morning, and EDTA was used as anticoagulation. The supernate was taken after centrifugation for 5 min in 1 500/min. The concentrations of interlukin-6 (IL-6), IL-10, interferon-γ (IFN-γ) and transforming growth factor-β (TGF-β) were respectively detected using ELISA. Additionally, a microplate reader imark made in Bio-Rad, USA was used for measurement. All kits were purchased from BioLegend, USA, and all operations were strictly operated according to kit instructions.

Observation indexes

Immune reconstitution characteristics of $CD4^+$T cells after Allo-HSCT were observed. $CD4^+$T cell count and number of Th17, Th1, Th2 and Treg cells were compared between control group and the patients with hematological malignancy 1, 2 and 3 months after transplantation, and dynamic changes of cytokines in those who were continuously detected for 3 months were observed and compared with control group. Besides, the incidence of IFI was also observed in the patients after transplantation, and its correlation with immune reconstitution of $CD4^+$T cells was analyzed.

Statistical data analysis

SAS 9.3 software package was used for statistical analysis. Measurement data was given normality test, and those in accordance with normal distribution were expressed with (mean±standard deviation). An independent-sample t test was used for comparison between two groups, while a pair-sample t test for comparison of each index in one patient at different time points. Those unmatched with normal distribution were expressed with the median, and an independent-sample Mann-Whitney U test was used. Multivariate Logistic regression analysis was used to analyze the correlation between indexes related to immune reconstitution of $CD4^+$T cells and IFI. All statistical tests were adopted two-sided tests, with $\alpha=0.05$ as an inspection level.

Results

Number of immune subpopulation at different time points after Allo-HSCT

After transplantation, the number of $CD4^+$T cells and its immune subpopulation was markedly lower than control group ($p<0.01$), and that of $CD4^+$T, Th1, Th2 and Treg cells increased progressively as time went on. 2 and 3 months after transplantation, each index was significantly higher than that 1 month after transplantation ($p<0.05$ or $p<0.01$). Besides, Th17 cell count was also on

Table 1. Number of Immune Subpopulation at Different Time Points after Allo-HSCT(x±s) ×10⁶/L

	n	CD4+T	Th17	Th1	Th2	Treg
Control group	40	976.34±319.62	14.82±6.93	207.14±71.44	5.45±4.26	15.03±6.87
1 month after transplantation	47	311.13±175.46**	2.81±1.51**	51.23±27.20**	0.67±0.34**	5.19±2.07**
2 month after transplantation	38	453.29±337.24**##	3.90±2.05**	103.45±54.14**##	2.28±1.98**##	8.14±3.18**##
3 month after transplantation	41	488.19±211.74**##	5.18±2.32**##	120.44±51.36**##	3.11±2.30**##	8.92±4.32**##

*Compared with control group, **p<0.01; Compared with 1 month after transplantation, ##p<0.01

Table 2. Dynamic Changes of Cytokine Levels at Different Time Points after Allo-HSCT pg/mL

	n	IL-6	IL-10	IFN-γ	TGF-β
Control group	40	1.58±0.95	1.34±0.85	3.26(0~131.25)	1509.26±641.06
1 month after transplantation	36	5.69±4.71**	12.59±6.27**	6.07(0~172.98)	502.63±225.18**
2 month after transplantation	36	19.26±7.32**##	11.17±4.51**	4.16(0~53.44)	535.74±241.35**
3 month after transplantation	36	11.53±5.91**##△△	14.66±9.79**	1.49(0~230.17)	481.95±163.24**

*Compared with control group, **p<0.01; Compared with 1 month after transplantation, ##p<0.01; Compared with 2 month after transplantation, △△p<0.01

a progressive increase, but no significant difference was presented by comparison at adjacent time points (p>0.05) (Table 1).

Dynamic changes of cytokine levels at different time points after Allo-HSCT

After transplantation, IL-6 level went up gradually, reached the peak at 2 months and then went down, but it was still higher than in control group 3 months after transplantation (p<0.01). Compared with control group, IL-10 level after transplantation increased notably (p<0.01), while TGF-β level down (TGF-β). There was no statistical significance by comparison to IL-10 and TGF-β levels at different time points after transplantation (p>0.05), and there was also no statistical significance by comparison to IFN-γ level among each group (p>0.05) (Table 2).

Correlation between incidence of IFI and immune reconstitution of CD4+T cells

In this study, the incidence of IFI was 19.15% (9/47), in which candida and aspergilli respectively accounted for 55.56% (5/9) and 44.44% (4/9). Infection parts: the lung, oropharynx and intestinal tract respectively occupied 55.56% (5/9), 33.33% (3/9) and 22.22% (2/9) (1 patient simultaneously suffered from 2 parts of infection).

Multivariate Logistic regression analysis was conducted with presence or absence of IFI as a dependent variable, 4 subpopulation cell counts of CD4+T cells, IL-6, IL-10, IFN-γ and TGF-β as independent variables. The results revealed that incidence of IFI might be related to Th17 cell count (p<0.05) instead of Th1, Th2 and Treg cell count as well as IL-6, IL-10, TGF-β and IFN-γ levels (p>0.05) (Table 3).

Table 3. Correlation between Incidence of IFI and Immune Reconstitution of CD4+T Cells

	P value	OR	95%CI
Th17	0.025	0.174	0.051~0.596
Th1	0.817	0.996	0.975~1.028
Th2	0.209	0.137	0.009~3.001
Treg	0.098	0.621	0.401~0.979
IL-6	0.799	1.000	0.991~1.024
IL-10	0.257	1.024	0.986~1.041
TGF-β	0.812	1.003	0.998~1.006
IFN-γ	0.057	1.017	1.002~1.043

Discussion

Because application of a large dose of cytotoxic drugs and long-term immunosuppressive drugs in transplantation regimen affects immune reconstitution after Allo-HSCT, the patients after transplantation will suffer from a longer immunodeficiency so as to increase the susceptibility to pathogenic microorganism (Toubert et al., 2012; Lehrnbecher et al., 2013; Gu et al., 2014; Dong et al., 2014). IFI, a very common severe complication after Allo-HSCT, still has high incidence and mortality in the patients undergoing Allo-HSCT (Omer et al., 2013; Tacke et al., 2014), but not all patients will encounter IFI, suggesting that the sensitivity of patients undergoing Allo-HSCT to IFI exists individual difference due to some unknown factors. Hence, it has great significance and guidance value for further improvement of transplantation efficacy and formulation of preventive and therapeutic strategies in clinic to understand whether there is some correlation between IFI and immune reconstitution of CD4+T cells or not.

T-cell reconstitution includes two pathways after Allo-HSCT. One is thymus-dependent pathway, namely pre-T cells from donors develop in the thymus; the other is thymus-independent pathway, namely mature T cells augment to the periphery. As the main pathway of T cells after transplantation, the former generates initial T cells and initiates slowly; under the stimulation of antigens and cytokines, the latter that mainly includes memory T cells can transform into effector cells fast and perform immune responses, which is an important alternative pathway of dramatic decrease of lymphocytes at an early stage and unrecovered thymic function after transplantation. It also plays a key role in the early anti-infectious mechanism after transplantation (Wils et al., 2011). Regarding the immune reconstitution of each subpopulation of CD4+T cells, the research results in this study revealed that the cell count of each subpopulation 3 months after transplantation was still lower than in control group, which might be related to insufficiency of initial CD4+T cells in peripheral blood. The cells of each subpopulation are not

activated normally under the circumstance of insufficient initial CD4+T cells even though there exists sufficient induction of antigens or cytokines (Bastien et al., 2012). Additionally, polymorphism deficiency of T-cell receptor bank in the patients after transplantation may also weaken the response ability to antigens, consequently leading to weakened transforming capability to effector T cells and delayed reconstitution of CD4+T cell subpopulation.

In this study, IFN-γ, IL-6, IL-10 and TGF-β levels were also detected. The research results demonstrated that within 3 months after transplantation, TGF-β level was continuously lower than in control group, while IL-6 and IL-10 levels persistently higher than in control group, and no significant difference was presented by comparison to IFN-γ level. These might be associated with the cytokines above originating from CD4+T cells and secretion of rapidly-recovered mononuclear macrophages and granulocytes after transplantation. Activation of these cells can maintain, even increase the level of some cytokines.

The study on correlation between each subpopulation of CD4+T cells and cytokines and IFI revealed that Th17 was a protective factor for IFI occurrence, suggesting that for the patients after transplantation, timely detection of Th17 may predict the incidence of IFI. However, these research results still need to be further confirmed by larger samples due to limited cases in this study.

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