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Original Article

The variance of peripheral blood lymphocyte subsets of streptozotocin-induced diabetic mice after bone marrow transplantation

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Abstract: Type 1 diabetes (T1D) results from the host immune disorder, which elicits the selective destruction of insulin-producing s in the pancreatic islets. Bone marrow transplantation (BMT) has been reported to treat T1D in numerous studies, and has been proved to be effective in treating T1D based on immune ablation and regeneration. In this study, we aimed to evaluate the curative effect of syngeneic bone marrow transplantation (syn-BMT) and to analyze peripheral blood lymphocyte phenotypes of streptozotocin (STZ)-induced diabetic mice after syn-BMT, and further to reveal possible mechanisms of syn-BMT involved in normalization of blood glucose. After multiple injections of low-dose STZ, most male C57BL/6J inbred mice got hyperglycemia, and then underwent syn-BMT. Fasting blood glucose was detected every 10 days after syn-BMT. The hemocytes count was evaluated every 3 days after syn-BMT in mice. Before syn-BMT, and on days 30, 60, and 90 after syn-BMT, we examined proportion of peripheral blood T lymphocytes, $CD19^+$ B lymphocytes, and NK cells by flow cytometry. Our data showed that hyperglycemia could be reversed and normal blood glucose level could be maintained in the whole observation period after syn-BMT. The peripheral blood elevated $CD4^+/CD8^+$ T lymphocyte ratio, $CD19^+$ B lymphocyte proportion and NK cell proportion in diabetic mice significantly decreased after syn-BMT. This study indicated that syn-BMT could reverse hyperglycemia and revealed immune ablation and immune system regeneration might be a possible mechanism of syn-BMT involved in normalization of blood glucose.

Keywords: Bone marrow transplantation, lymphocytes, stem cell, T lymphocyte reconstitution, type 1 diabetes

Introduction

The most important factor in the development of type 1 diabetes (T1D) is the host immune system, which elicits selective destruction of insulin-producing β cells in the pancreatic islets. Bone marrow transplantation (BMT) is a novel therapy for autoimmune disease which is based on immune ablation and immune regeneration. There have been many animal studies on bone marrow transplantation for T1D [1-4], and showed significant effect of BMT for T1D. Besides, in one study, ninety-three percent of patients achieved different degrees of insulin independence after autologous hematopoietic stem cell transplantation (AHSCT) [5]. Previous clinical research have found that AHSCT modulated lymphocytes and preserved β-cell function in Chinese patients experiencing new-onset T1D and diabetic ketoacidosis [6].

In humans, T1D is multi-factorial disease which is influenced by both genetic factors (multiple susceptible alleles) and environmental factors. Studies on human beings from the same ethnic group but located in different geographic areas (e.g., Finland vs. Estonia) have shown different incidence rates of diabetes [7]. In humans, T1D may be initiated by environmental exposure [8]. The identification of such environmental factors has been proved to be frustratingly difficult. Certain studies have suggested an association between T1D and certain types of enteroviral infections [7]. The repeated injections of low-dose streptozotocin (STZ) to an otherwise non-diabetic mice of C57BL/6J strain, provides a useful T1D model induced by environmental exposure [7].

In our previous study, $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells were implicated in the mechanism

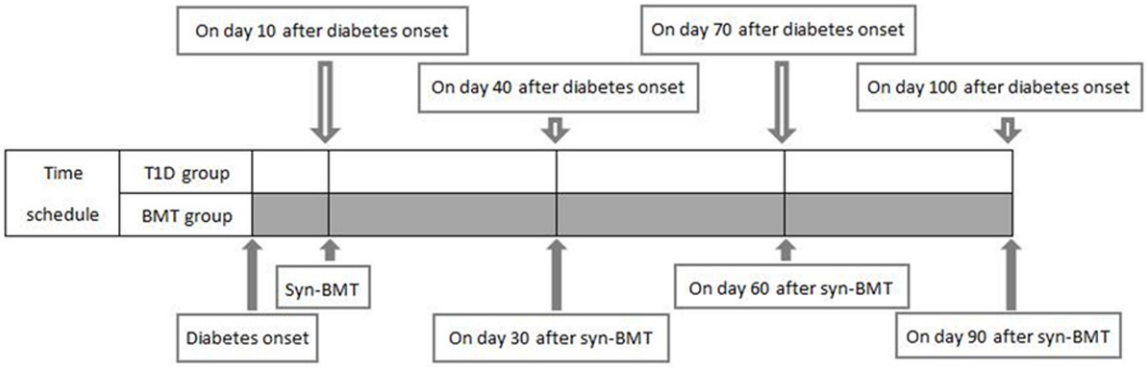

Figure 1. Time schedule and grouping of experimental animal. BMT, bone marrow transplantation; syn-BMT, syngeneic bone marrow transplantation; T1D, type 1 diabetes.

for remission from diabetes after syngeneic-BMT (syn-BMT), which reversed high blood glucose in STZ-induced diabetic mice [3]. However, there have been no data on other lymphocyte subpopulations of T1D recipients undergoing syn-BMT. The aim of this study was to evaluate the curative effect of syn-BMT, and to analyze the dynamic variance of lymphocyte phenotypes in STZ-induced diabetic mice after syn-BMT, and further to reveal possible mechanisms of syn-BMT involved in normalization of blood glucose.

Methods

STZ-induced diabetic mice

C57BL/6J inbred male mice, 6 weeks old and weighing between 18 and 21 g, were purchased from the Model Animal Research Center of Nanjing University. All the mice were housed under specific pathogen-free conditions with ad libitum access to food and water. The animal use protocols complied with the Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1995). The mice were injected intraperitoneally with streptozotocin (STZ, 40 mg/kg body weight; Sigma-Aldrich, USA) daily for 5 consecutive days. STZ was solubilized in 0.1 ml of chilled citrate buffer (0.1 mol/L trisodium citrate and 0.1 mol/L citric acid, pH 4.5) and injected within 15 minutes after preparation. Fasting blood glucose was measured twice weekly with a glucometer (Roche Diagnostic, Frankfurt, Germany). The mice were considered to be overtly diabetic if fasting blood glucose was > 13.9 mmol/L (250 mg/dL) for 2 consecutive days [3].

Grouping

Random overtly diabetic mice underwent syn-BMT (BMT, n = 18), and comprised three subgroups killed on day 30 (30d-BMT, n = 6), day 60 (60d-BMT, n = 6), and day 90 (90d-BMT, n = 6) after syn-BMT. The other overtly diabetic mice (n = 24) as T1D group constituted four subgroups killed on day 10 (10d-T1D, n = 6), day 40 (40d-T1D, n = 6), day 70 (70d-T1D, n = 6), and day 100 (100d-T1D, n = 6) after the onset of diabetes. Inbred mice of a similar age (n = 6) served as normal control group (NC). To be noted, diabetic mice underwent syn-BMT on day 10 after diabetes onset, hence mice on day 30 after syn-BMT were at the similar age to mice on day 40 after diabetes onset (**Figure 1**).

Bone marrow transplantation

Donor normal inbred mice were euthanized by CO_2 narcosis, and both femurs and tibias were collected in cold phosphate buffer saline (PBS). Bone marrow from femurs and tibias were flushed into cold RPMI 1640 medium, and the erythrocytes were removed using a lysis buffer (0.15 mol/L NH_4Cl, 1 mmol/L $KHCO_3$, and 0.1 mmol/L Na_2-EDTA, pH 7.4). The remained bone marrow mononuclear cells were washed in RPMI 1640 medium and collected by centrifugation. Cell viability after isolation was determined using the Typan blue (Sigma, St. Louis, USA) dye exclusion method. Recipient mice on day 10 after diabetes onset were irradiated (800 cGy from a 60Co source, 1 Gy/min), and then transplanted with approximately 5×10^6 bone marrow cells intravenously through the tail vein within 6 h of irradiation, as we reported previously [3].

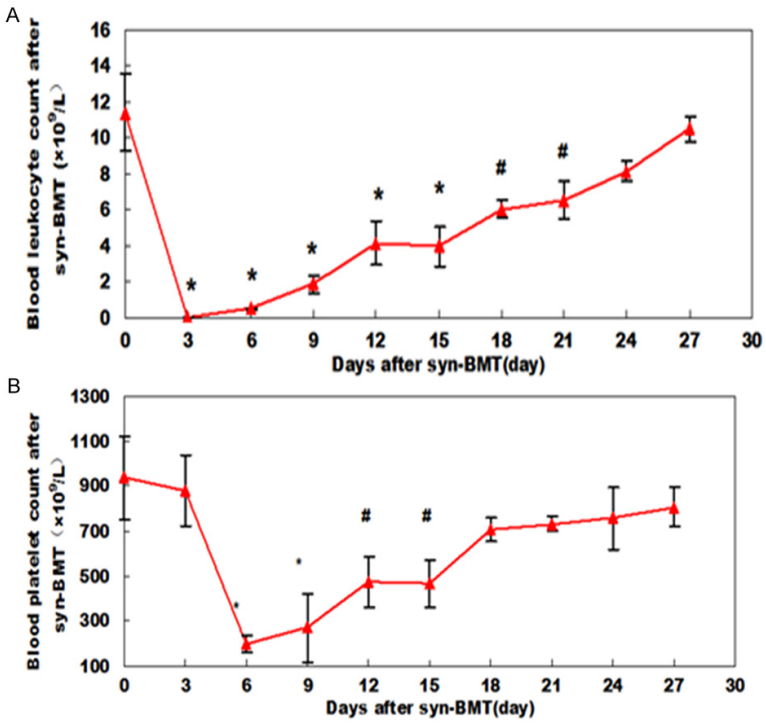

Figure 2. The peripheral blood leukocyte and platelet count of BMT group mice after syn-BMT. A. The peripheral blood leukocyte count of mice decreased to minimum on day 3 after syn-BMT, and then gradually increased to nearly normal on day 24 after syn-BMT. B. The peripheral blood platelet count of mice decreased to minimums on day 6 after syn-BMT, and gradually increased to nearly normal on day 18 after syn-BMT. BMT, bone marrow transplantation; syn-BMT, syngeneic bone marrow transplantation. $^{*}P < 0.01$ compared with other groups. $^{\#}P < 0.05$ compared with other groups.

Blood glucose monitoring

We measured fasting blood glucose of NC, DC, and BMT mice every 10 days with a glucometer (Roche Diagnostic, Frankfurt, Germany). The blood leukocyte and platelet counts of BMT mice were detected every 3 days after syn-BMT with an automatic hemocyte analyzer (Sysmex, Kobe, Japan).

Flow cytometry analysis of peripheral blood immunocytes

The mice (NC mice, DC mice, 30d-BMT mice, 60d-BMT mice, 90d-BMT mice, 10d-T1D mice, 40d-T1D mice, 70d-T1D mice, 100d-T1D mice) were euthanized by CO_2 narcosis and then were killed by bloodletting to obtain peripheral blood. Flow cytometry analysis of the cells was performed with combinations of monoclonal antibodies directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-Cy5 (PE-Cy5), or allophycocyanin (APC). Monoclonal antibodies were added to 50 μL of peripheral blood suspended in RPMI 1640 supplemented with 2% newborn calf serum and then incubated in the dark for 30 min at 4°C. Erythrocytes were removed with Flow Cytometry Lysing Solution (Multisciences, Nanjing, China). The remained nucleated cells were rinsed twice in PBS, sedimented by centrifugation at 1500 rpm for 5 min, resuspended with 0.25 ml of PBS, stored at 4°C and analyzed within 24 h. The following antibodies (eBioscience, San Diego, USA) were used for cell phenotyping: anti-CD45-APC, anti-CD3-PC5, anti-CD8-FITC, anti-CD4-PE, and anti-CD19-FITC, anti-pan-NK-PE and fluorochrome-conjugated isotype-matched non-specific mAbs as negative controls. Flow cytometry analysis was performed with a BD FACScanto™ Flow Cytometer running CellQuest software (Becton-Dickinson, San Jose, California, USA), and a minimum of 8,000 events were required. T and B lymphocytes were identified as small cells with high CD45-APC expression.

Statistics

One-way ANOVA was used for comparisons between the groups. *P* values less than 0.05 were considered statistically significant. All the statistical analyses were performed using SPSS 13.0 software. The graphical data are presented as the mean ± standard deviation (S.D.). The experiment was approved by the Animal Ethics Committee of Nanjing University.

Results

Blood glucose monitoring

Before syn-BMT, fasting blood glucose of diabetic mice was 21.06 ± 3.732 mmol/L, which was higher than that of NC mice (7.22 ± 0.397) ($P < 0.01$). Without syn-BMT, the diabetic mice remained hyperglycemic during the subsequent

observation period. After the diabetic mice underwent syn-BMT, the fasting blood glucose level gradually decreased to nearly normal, as previously reported [3].

Hematopoietic reconstitution

The peripheral blood leukocyte and platelet counts decreased to minimums on day 3 and day 6, respectively, and then gradually increased to nearly normal on day 24 and day 18, respectively after syn-BMT (**Figure 2**).

Dynamic variance of peripheral blood $CD4^+$/$CD8^+$ T lymphocyte ratio

The peripheral blood $CD4^+$/$CD8^+$ T lymphocyte ratio was significantly elevated in the new-onset diabetic mice compared with that of NC mice. In the 40d-T1D, 70d-T1D, and 100d-T1D groups, peripheral blood $CD4^+$/$CD8^+$ T lymphocyte ratio decreased but remained higher than that in the NC group. In the 30d-BMT, 60d-BMT, and 90d-BMT groups, peripheral blood $CD4^+$/$CD8^+$ T lymphocyte ratio was significantly decreased compared with that in the NC group ($P < 0.01$; **Figure 3A**; **Table 1**).

Dynamic variance of peripheral blood $CD19^+$ B lymphocytes

Peripheral blood $CD19^+$ B lymphocytes significantly increased in the new-onset diabetic mice compared with that in the NC mice ($P < 0.01$). However, they remained significantly increased in the 40d-T1D group, while there were no significant differences among the 70d-T1D, 100d-T1D groups and NC group. In the 30d-BMT group, peripheral blood $CD19^+$ B lymphocytes were significantly decreased ($P < 0.05$), but became nearly normal in the 60d-BMT and 90d-BMT groups (**Figure 3B**; **Table 1**).

Dynamic variance of peripheral blood NK cells

The proportion of peripheral blood NK cells were significantly increased in the new-onset diabetic mice compared with that in the NC mice ($P < 0.01$). They remained more abundant over the subsequent observation period. After syn-BMT, the proportion of peripheral blood NK cells decreased significantly compared with that in the NC group ($P < 0.01$; **Figure 3C**; **Table 1**).

Discussion

Our previous study indicated that syn-BMT, when performed in the mice with new-onset (within 10 days) T1D, was safe and could reverse the diabetes status [3]. Our clinical research also found that AHSCT modulated lymphocytes and preserved β-cell function in Chinese patients experiencing new-onset type 1 diabetes and diabetic ketoacidosis [6]. However, the influence of syn-BMT on immune system in T1D is not well understood. T cell tolerance was achieved after immune reconstitution by syn-BMT. The achievement of immunological balance might play an important role as an effector of AHSCT therapy.

T1D in humans represents end-stage insulitis, and it has been hypothesized that insulitis is characterized by the infiltration of mononuclear cells into the islets. In humans, MHC genetic variation may lead to immune diseases, including type 1 diabetes, partly by deregulating peripheral blood $CD4^+$/$CD8^+$ T lymphocyte homeostasis [9]. The peripheral blood $CD4^+$/$CD8^+$ T lymphocyte ratio was higher in T1D patients [10]. We found that the peripheral blood $CD4^+$/$CD8^+$ T lymphocyte ratio was significantly increased in the new-onset diabetic mice compared with that in the NC mice, and decreased after syn-BMT. Decreased peripheral blood $CD4^+$ T and increased peripheral blood $CD8^+$ T lymphocytes proportion altered the $CD4^+$/$CD8^+$ T lymphocyte ratio in diabetic mice after syn-BMT, which may involving in improvement of diabetes status.

The immune response is normally regulated by various mechanisms that control hyperactivity and prevent self-destruction. Our research showed that compared with that of NC mice, peripheral blood $CD19^+$ B lymphocytes proportion of diabetic mice increased significantly but became nearly normal at a later time point in the observation period after syn-BMT. In some studies, the number of $CD5^+$ $CD19^+$ B lymphocytes was higher in type 1 diabetic children with a very recent onset disease compared with that of patients on insulin therapy for more than 30 days and normal controls [11]. In our study, after syn-BMT, peripheral blood $CD19^+$ B lymphocyte proportion decreased significantly, but were nearly normalized at later time points. Based on these results, we hypothesized that B

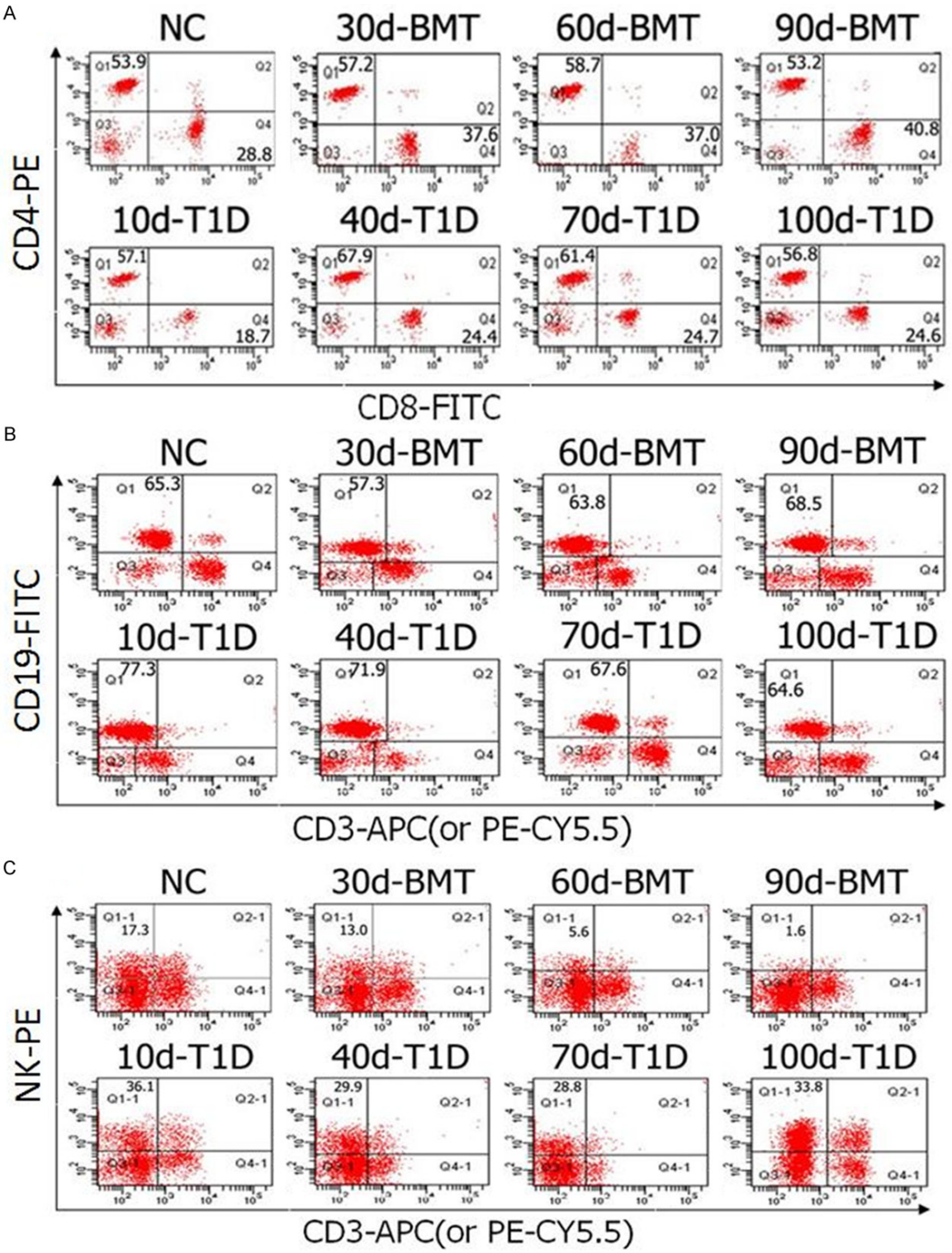

Figure 3. Peripheral blood lymphocytes changes in new-onset diabetes after syn-BMT. A. The peripheral blood $CD4^+$/$CD8^+$ T lymphocyte ratio change in new-onset diabetes after syn-BMT. B. The peripheral blood $CD19^+$ B lymphocytes proportion change in new-onset diabetes after syn-BMT. C. The peripheral blood NK cells proportion change in new-onset diabetes after syn-BMT. BMT, bone marrow transplantation; syn-BMT, syngeneic bone marrow transplantation; T1D, type 1 diabetes; NC, normal control. $^*P < 0.01$ compared with other groups. $^\#P < 0.05$ compared with other groups.

Table 1. Peripheral blood lymphocyte subsets changes in mice

	Peripheral blood $CD4^+/CD8^+$ T lymphocyte ratio (%)	Peripheral blood $CD19^+$ B lymphocytes (%)	Peripheral blood NK cells (%)
NC	1.94 ± 0.24	64.46 ± 3.94	16.50 ± 2.37
10d-T1D	2.95 ± 0.40*	77.77 ± 4.31*	36.66 ± 3.68*
40d-T1D	2.80 ± 0.39*	72.37 ± 3.91*	29.67 ± 4.53*
70d-T1D	2.41 ± 0.18*	66.73 ± 1.52	28.05 ± 6.13*
100d-T1D	2.28 ± 0.14#	65.17 ± 2.85	33.51 ± 3.91*
30d-BMT	1.52 ± 0.18*	57.46 ± 3.41#	11.73 ± 3.50*
60d-BMT	1.53 ± 0.11*	63.61 ± 7.42	5.56 ± 2.59*
90d-BMT	1.10 ± 0.17*	67.86 ± 5.19	1.46 ± 1.04*

Values are presented as mean ± standard deviation. BMT, bone marrow transplantation; T1D, type 1 diabetes. *$P < 0.01$ compared with other groups. #$P < 0.05$ compared with other groups.

lymphocytes were involved in the recent onset diabetes, which might be reversed by syn-BMT.

NK cells are programmed to kill target cells and to interact with antigen-presenting cells and T cells. NK cells are a major source of γ-interferon, a key proinflammatory cytokine that modulates the aggressiveness of the immune attack in diabetes and the rate of progression from insulitis to overt diabetes. In a model of coxsackievirus-induced autoimmunity, NK cells were important to disease progression [12], and in a T-cell receptor transgenic model, an increased number of NK cells was observed under conditions of aggressive insulitis [13]. A reduction in the NK cells frequency was observed in patients with new-onset diabetes compared with that of patients with long-term diabetes [14]. In contrast, in our study, the peripheral blood NK cells proportion increased with disease progression, and a lower peripheral blood NK cells proportion in BMT group after syn-BMT was observed. Diminished NK cell proportion may correlate with the mechanism of immune regulation, and further improve the diabetes status.

In conclusion, the data confirmed the curative effect of syn-BMT in new-onset diabetic mice, and revealed diverse immune cells played important roles in disease progression and maintaining immune tolerance to self during the early stage after syn-BMT. The immune system dysregulation may correlate with the maintenance of euglycemia after syn-BMT, which induces immune ablation and subsequent regeneration, and suggested that the dynamic variance of lymphocyte phenotypes in STZ-induced diabetic mice after syn-BMT might be possible mechanisms of syn-BMT involved in normalization of blood glucose.

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Disclosure of conflict of interest

None.

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References

[1] Banerjee M, Kumar A, Bhonde RR. Reversal of experimental diabetes by multiple bone marrow transplantation. Biochem Biophys Res Commun 2005; 328: 318-325.

[2] Gao X, Song L, Shen K, Wang H, Niu W, Qin X. Transplantation of bone marrow derived cells promotes pancreatic islet repair in diabetic mice. Biochem Biophys Res Commun 2008; 371: 132-137.

[3] Wen Y, Ouyang J, Yang R, Chen J, Liu Y, Zhou X, Burt RK. Reversal of new-onset type 1 diabetes in mice by syngeneic bone marrow transplantation. Biochem Biophys Res Commun 2008; 374: 282-287.

[4] Lv C, Wang J, Xie T, Ouyang J. Bone marrow transplantation reverses new-onset immunoinflammatory diabetes in a mouse model. Int J Clin Exp Pathol 2014; 7: 5327-5336.

[5] Voltarelli JC, Couri CE, Stracieri AB, Oliveira MC, Moraes DA, Pieroni F, Coutinho M, Malmegrim KC, Foss-Freitas MC, Simões BP, Foss MC, Squiers E, Burt RK. Autologous nonmyeloablative hematopoietic stem cell transplantation in newly diagnosed type 1 diabetes mellitus. JAMA 2007; 297: 1568-1576.

[6] Li L, Shen S, Ouyang J, Hu Y, Hu L, Cui W, Zhang N, Zhuge YZ, Chen B, Xu J, Zhu D. Autologous hematopoietic stem cell transplantation modulates immunocompetent cells and improves β-cell function in Chinese patients with new onset of type 1 diabetes. J Clin Endocrinol Metab 2012; 97: 1729-1736.

[7] Acharjee S, Ghosh B, Al-Dhubiab BE, Nair AB. Understanding type 1 diabetes: etiology and models. Can J Diabetes 2013; 37: 269-276.

[8] de Kleer I, Vastert B, Klein M, Teklenburg G, Arkesteijn G, Yung GP, Albani S, Kuis W, Wulffraat N, Prakken B. Autologous stem cell transplantationfor autoimmunity induces immunologic self-tolerance by reprogramming-autoreactive T cells and restoring the CD4+ CD25+ immune regulatorynetwork. Blood 2006; 107: 1696-1702.

[9] Ferreira MA, Mangino M, Brumme CJ, Zhao ZZ, Medland SE, Wright MJ, Nyholt DR, Gordon S, Campbell M, McEvoy BP, Henders A, Evans DM, Lanchbury JS, Pereyra F, Walker BD. Quantitative trait loci for CD4: CD8 lymphocyte ratio are associated with risk of type 1 diabetes and HIV-1 immune control. Am J Hum Genet 2010; 86: 88-92.

[10] Kaaba SA , Al-Harbi SA. Abnormal lymphocyte subsets in Kuwaiti patients with type-1 insulin-dependent diabetes mellitus and their first-degree relatives. Immunol Lett 1995; 47: 209-213.

[11] De Filippo G, Pozzi N, Cosentini E, Cavalcanti M, Carel JC, Tamasi S, Franzese A, Pignata C. Increased CD5+ CD19+ B lymphocytes at the onset of type 1 diabetes in children. Acta Diabetol 1997; 34: 271-274.

[12] Flodstrom M, Maday A, Balakrishna D, Cleary MM, Yoshimura A, Sarvetnick N. Target cell defense prevents the development of diabetes after viral infection. Nat Immunol 2002; 3: 373-382.

[13] Poirot L, Benoist C, Mathis D. Natural killer cells distinguish innocuous and destructive forms of pancreatic islet autoimmunity. Proc Natl Acad Sci U S A 2004; 101: 8102-8107.

[14] Rodacki M, Svoren B, Butty V, Besse W, Laffel L, Benoist C, Mathis D. Altered Natural Killer Cells in Type 1 Diabetic Patients. Diabetes 2007; 56: 177-185.