Biochemical and Biophysical Research Communications 374 (2008) 282–287

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Reversal of new-onset type 1 diabetes in mice by syngeneic bone marrow transplantation

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ARTICLE INFO

Article history:
Received 30 June 2008
Available online 14 July 2008

Keywords:
Syngeneic bone marrow transplantation
Type 1 diabetes
CD4+CD25+FoxP3+ regulatory T cells
Immunologic tolerance

ABSTRACT

Autologous hematopoietic stem cell transplantation (HSCT) has recently been performed as a novel strategy to treat patients with new-onset type 1 diabetes (T1D). However, the mechanism of autologous HSCT-induced remission of diabetes remains unknown. In order to help clarify the mechanism of remission-induction following autologous HSCT in patients with T1D, mice treated with multiple low doses of streptozotocin to induce diabetes were used as both donors ($n = 20$) and recipients ($n = 20$). Compared to streptozocin-treated mice not receiving transplantation, syngeneic bone marrow transplantation (syn-BMT) from a streptozocin-treated diabetic donor, if applied during new-onset T1D (day 10 after diabetes onset), can reverse hyperglycemia without relapse ($P < 0.001$), maintain normal blood insulin levels ($P < 0.001$), and preserve islet cell mass. Compared to diabetic mice not undergoing HSCT, syn-BMT, results in restoration of Tregs in spleens ($P < 0.01$), increased Foxp3 mRNA expression ($P < 0.01$) and increased Foxp3 protein expression ($P < 0.05$). This diabetic-remission-inducing effect occurred in mice receiving bone marrow from either streptozocin-treated diabetic or non-diabetic normal donors. We conclude that autologous HSCT remission of diabetes is more than transient immune suppression, and is capable of prolonged remission-induction via regeneration of CD4+CD25+FoxP3+ Tregs.

Type 1 diabetes (T1D) is a prototypical organ-specific autoimmune disease, induced mainly by T helper 1 and CD8+ T lymphocytes, characterized by progressive autoimmune destruction of the insulin-producing cells of the islets of Langerhans of the pancreas [1]. Non-obese diabetic (NOD) mice are commonly used as a spontaneous-onset genetically pre-ordained model of T1D in which disease occurs independent of any known environmental exposure. For NOD mice, an allogeneic HSCT is required to re-establish islet cell tolerance [2,3]. Environmental exposure to streptozotocin in non-diabetic prone strains can induce T1D by repeated injection of multiple low doses of streptozotocin [4,5].

Autologous HSCT is a novel therapy based on immune ablation and regeneration of a new immune system [6,7] that has been recently performed in patients with new-onset T1D [8]. After HSCT, patients with new-onset T1D remain off all immune suppressive medications and the majority remain insulin-free with normal blood sugar and glycated hemoglobin. The duration of this medication-free remission remains unknown but some have remained insulin-/treatment-free for more than 3 years [8]. Since autologous hematopoietic stem cells (HSC), rather than allogeneic HSC, are re-infused, genetic predispostion to disease is unchanged and the mechanism of autologous HSCT-induced remission may be transient immune suppression. Alternatively, changes in peripheral immune regulation that maintain peripheral tolerance may be re-established by the transplant procedure [9]. In order to better clarify the mechanism of autologous HSCT-induced remission, we analyzed Treg cell regeneration following syn-BMT in an environmentally-induced animal model of T1D.

Materials and methods

Animals. Male C57BL/6J inbred mice weighing 19–23 g were obtained from the Model Animal Research Center of Nanjing University, Nanjing, China. Mice were kept under specific pathogen-free (SPF) conditions. The protocols of animal use complied with the Principles of Laboratory Animal Care (NIH Publication 85-23, revised 1995).

Induction of experimental diabetes by mld-SZ. C57BL/6J mice were injected intraperitoneally with 40 mg/kg streptozotocin (Sigma, St. Louis, MO) prepared in 0.1 ml chilled citrate buffer (0.1 mol/L trisodium citrate, 0.1 mol/L citric acid, pH 4.5) for 5 consecutive days to

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doi:10.1016/j.bbrc.2008.07.016

induce T1D or with 0.1 ml citrate buffer as control. Diabetic status was confirmed by measurement of blood glucose (Accu-Chek blood glucose meter, Roche Diagnostic, Germany) from day 8 after the first injection. Mice were classified diabetic when fasting blood glucose (FBG) was >13.9 mM (250 mg/dl) for 2 consecutive days. Mice were euthanized by CO_2 narcosis on day 10 after T1D onset or on day 120 after syn-BMT. Untreated SZ-diabetic mice were euthanized on day 120 of T1D as control. After syn-BMT, FBG and urine glucose were monitored biweekly and weekly, respectively, for 120 days.

Bone marrow cell isolation and transplantation. Donor mice (either normal ($n = 13$) or streptozocin-treated diabetic ($n = 7$)) were euthanized by CO_2 narcosis, and both femurs and tibias were collected. Bone marrow (BM) was flushed into ice-cold RPMI 1640 medium (GIBCO) and erythrocytes were removed by lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM Na_2–EDTA, pH 7.4). Viability of the cells was checked by Trypan blue (Sigma, St. Louis, MO) dye exclusion method. Recipient diabetic mice were sub-lethally irradiated (800 cGy from a ^{60}Co source, 0.95 Gy/min) and were transplanted intravenously through tail vein approximately 10^7 BM cells (in about 0. 8 ml) within 6 h of irradiation. Recipients were provided with antibiotic supplemented water (neomycin sulfate, 2 mg/ml) for 3 weeks after BMT.

Glucose tolerance test. Streptozocin-treated mice 120 days after onset of T1D ($n = 8$), and 120 days after either normal donor syn-BMT ($n = 7$) or diabetic donor syn-BMT ($n = 13$) were injected intraperitoneal with 2 g/kg body weight of glucose after fasting 8 h. Blood glucose was measured at 0, 30, 60, and 120 min, respectively, after the injection.

Insulin quantification in sera. Upon euthanasia whole blood was collected from retro-orbital vein, and sera were isolated by centrifugation. Insulin levels were measured using a murine insulin ELISA kit (Linco Research, MO, USA) according to the manufacturers' instructions. Each sample was analyzed in duplicate.

Pancreata histological and morphometry analysis. Pancreata were fixed in 10% formalin and processed for paraffin embedding. Semi-serial sections of 5 μm were cut 3 μm apart from the paraffin-embedded blocks and stained with hematoxylin and eosin (HE) to assess the pancreatic islet morphology.

Flow cytometry analysis (FCAS) of CD4+CD25+FoxP3+ cells percentage. Mononuclear cells (MNCs) were collected from spleens and resuspended in RPMI 1640. Fluorescein isothiocyanate (FITC) conjugated anti-mouse CD4, phycoerythrin (PE) conjugated anti-mouse CD25, PE-Cy5 anti-mouse/rat Foxp3 and appropriate isotype controls were purchased from eBioscience (San Diego, USA). Flow cytometry was performed on a BD FACScaanto™ Flow

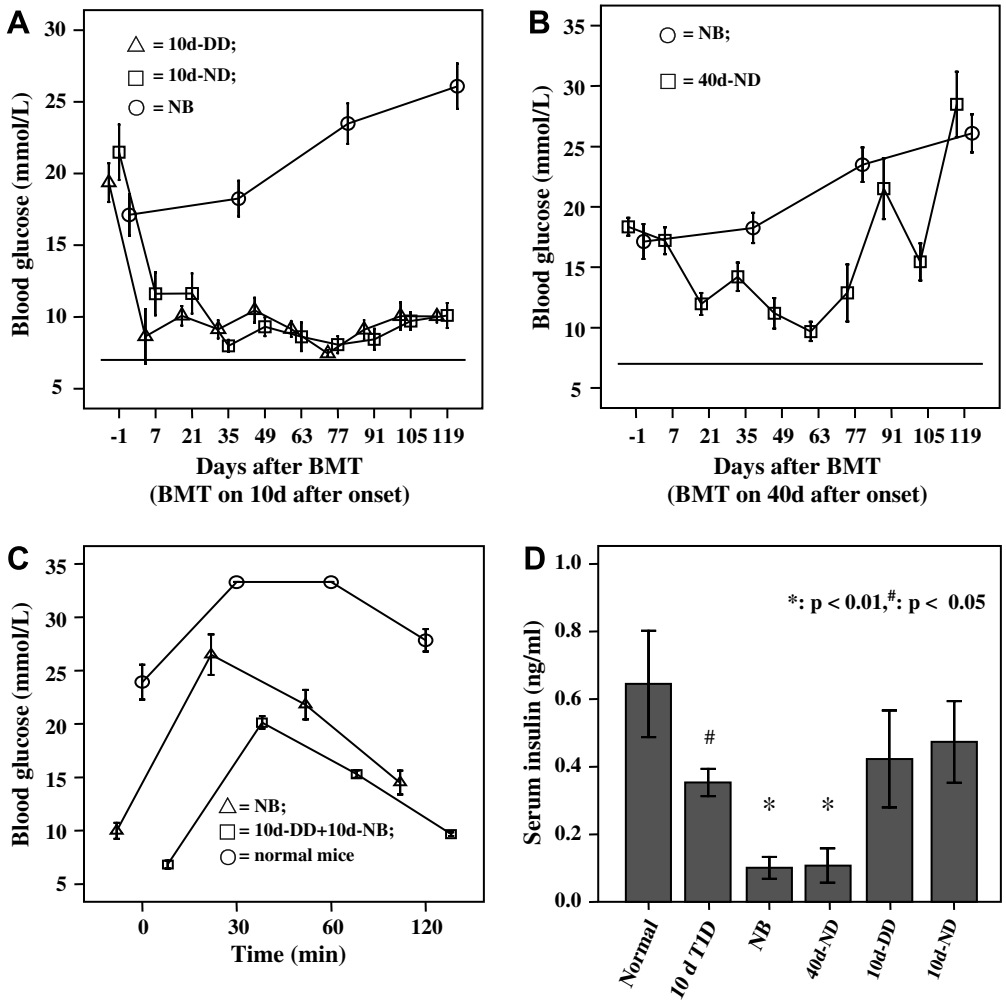

Fig. 1. (A) Fasting blood glucose (FBG) measured after syn-BMT performed on day 10 after T1D onset. The line at the bottom stands for the mean FBG of normal mice. (B) FBG measured after syn-BMT performed on day 40 after T1D onset. (C) Glucose tolerance test on day 120 after syn-BMT. The Accu-Chek blood glucose meter has a top limit, so the cases of FBG > 33.3 mmol/L was replaced with 33.3 mmol/L. (D) Serum insulin test. Normal = normal mice. 10 d T1D = mice 10 days after the onset of T1D. Groups 10d-DD and 10d-ND showed restored blood insulin, while group 40d-ND did not demonstrate significant improvement. *Difference was significant compared to normal controls ($P < 0.01$). #Difference was significant compared to normal controls ($P < 0.05$).

Cytometer running with Cellquest software (Becton Dickinson). Cells were gated on the CD4+ lymphocyte population. Data represent 50,000 events.

Western blot analysis of FoxP3 protein. Splenocytes were lysed and analyzed with 12% SDS–polyacrylamide gel electrophoresis (PAGE) gels. Rabbit anti-mouse FoxP3 antibody (1:800) (Santa Cruz Biotechnology, CA, USA), and goat anti-rabbit IgG HRP-conjugated antibody (1:3000) (Mulityscience, USA) were used for the detection of FoxP3 protein. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was detected with mouse anti-GAPDH antibody (1:4000) (Chemicon, Temecula, CA) and goat anti-mouse IgG HRP-conjugated antibody (1:2000) (Mulityscience, USA) as an internal control.

Quantitative real-time RT-PCR assessment of FoxP3 mRNA expression. Total RNA of MNCs was prepared using the RNAiso Reagent (Takara, Japan). The samples were treated with DNase I. RT-PCR

Table 1
FBG checked on day 120 after syn-BMT performed on day 10 or day 40 after T1D onset, respectively

Days after T1D onset when receiving BMT	Donor	Group	No.	Mean FBG (mmol/L)	Lowest FBG (mmol/L)	Highest FBG (mmol/L)
10	Diabetic	10d-DD	7	10.04*	8.7	11.4
10	Normal	10d-ND	6	10.1*	7.5	13.7
No BMT	—	NB	8	26.08	18.3	28.3
40	Normal	40d-ND	7	>24.88	13.4	>33.3

* Difference was significant compared to group NB ($P < 0.001$).

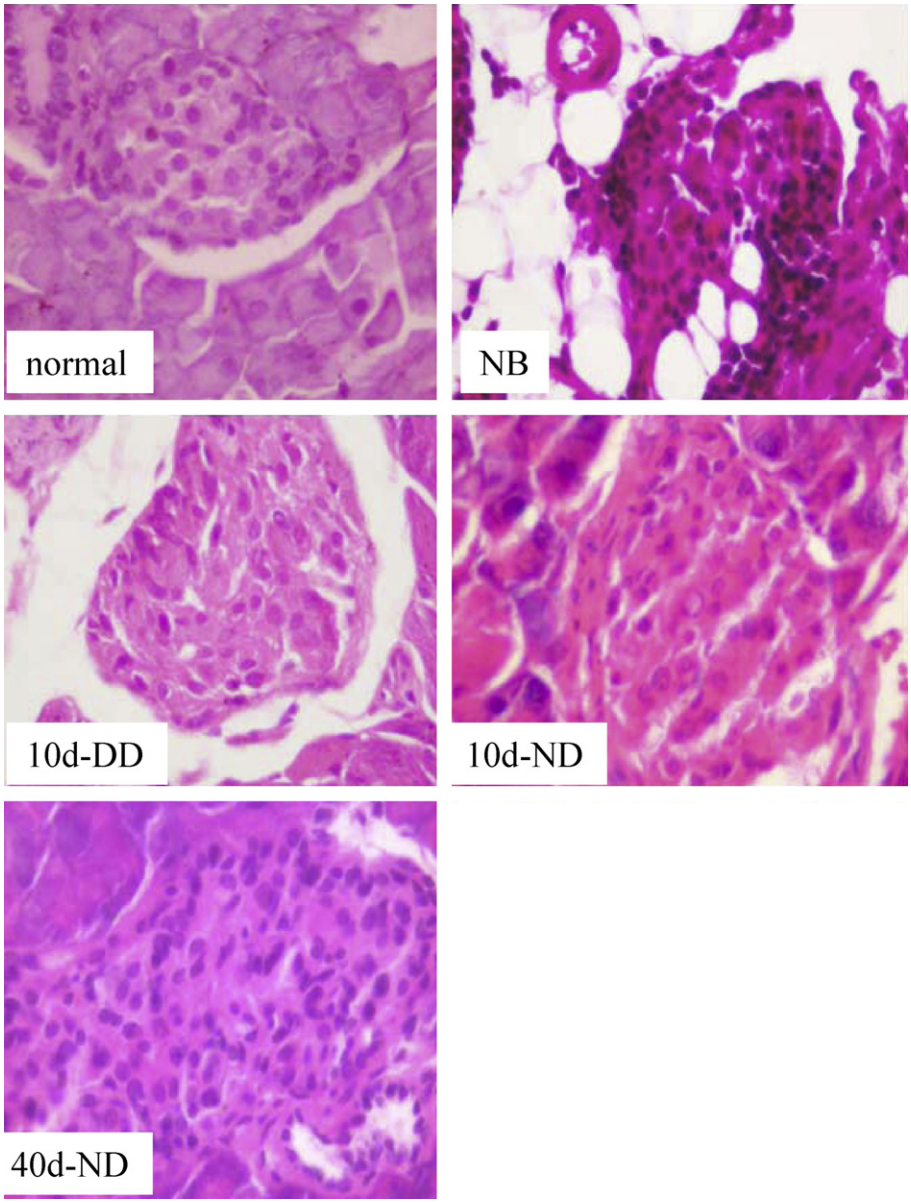

Fig. 2. Pancreata histological analysis. Normal = islet from a normal mouse. Group NB showed fewer islets, massive destruction of morphology. Groups 10d-DD and 10d-ND showed no obvious lesions. Group 40d-ND showed destruction of morphology (400×).

was performed using ExScriptTM RT-PCR kit (Takara). Amplification was performed on a DNA Engine Opticon™ 2 Continuous Fluorescence Detector (MJ Research) using a TaqMan-MGB protocol. The primer sequences were as follows: Foxp3: forward, TGGCCTGGTTGTGAGAAGGT; reverse, TCTGCTTGGCAGTGCTTGAG, Foxp3-MGB-Probe: FAGGAGCCAGAAGAGTTP. GAPDH as housekeeping gene: forward: GCACAGTCAAGGCCGAGAA, reverse: CCTCACCCCATTTGATGTTAGTG, GAPDH-MGB-Probe: FTCTTCCAG GAGCGAGACP. Cycling parameters: 95 °C × 10 s; 95 °C × 5 s → 59 °C × 35 s for 40 cycles. Each sample was analyzed in triplicate. The relative amount of FoxP3 was expressed as $2^{\Delta\Delta C_T}$ values.

Statistical analysis. One-way ANOVA was used for comparisons between the groups. *P* values less than or equal to 0.05 were considered to be significant difference. Statistical analyses were performed with SPSS 13.0 software. Error bars in the graphs represent means ± standard error of the mean (SEM).

Results

Effects of syn-BMT on diabetic mice survival

To evaluate the effect of syn-BMT on diabetic mice, three groups were analyzed in new-onset (10 days) SZ-diabetic mice: group 10d-DD ($n = 7$) received BM from the fraternal inbred SZ-diabetic mice, group 10d-ND ($n = 6$) received BM from normal mice, and group NB ($n = 13$) received no treatment. Group 40d-ND ($n = 7$) received syn-BMT from normal mice on day 40 after T1D onset. Group normal ($n = 7$) were normal mice (shown in Supplementary Fig. 1 and Table 1). In the experimental period (120 days after BMT), no death was observed in groups 10d-DD, 10d-ND, and normal, whereas group NB had a diminished survival rate of 61.5% (8/13).

Effects of fasting blood glucose after syn-BMT

While the FBG of group NB maintained at a gradually higher level during the whole experimental period, FBG in groups 10d-DD and 10d-ND dropped significantly on day 7 after receiving syn-BMT and then decreased gradually (Fig. 1A). FBG in groups 10d-DD and 10d-ND remained close to normoglycemia for up to 120 days after BMT. The difference of FBG between groups NB and 10d-DD or 10d-ND was statistically significant ($P < 0.001$). Interestingly, the FBG in groups 10d-DD (syn-BMT from diabetic donors) and 10d-ND (syn-BMT from normal donors) had no significant difference (Fig. 1A and Table 1). However, in group 40d-ND, when syn-BMT was delayed until day 40 after diabetes onset, although there was a temporary drop of blood glucose from day 21, it relapsed to hyperglycemia on day 77, and showed no statistical difference with group NB on day 120 (Fig. 1B and Table 1). The glucose tolerance test showed that compared with group NB, groups 10d-DD and 10d-ND had improved glucose tolerance, which had a curve similar to the normal mice (Fig. 1C).

Effects of syn-BMT on insulin

Insulin had dropped from day 10 after diabetes onset. While mice in groups NB and 40d-ND demonstrated decreased of blood insulin compared to normal controls ($P < 0.01$, Fig. 1D), normalization of blood glucose in groups 10d-DD and 10d-ND correlated with restoration of blood insulin.

Effects of syn-BMT on pancreas histology

Histological examination on day 120 after BMT of pancreata in the mice of groups 10d-DD and 10d-ND had relatively intact islets similar to in normal mice, while those in group NB developed massive loss of islet cells, and those in mice of group 40d-ND have partial loss of islets morphology. Fig. 2 shows the representative results from one mouse of each group.

Effects of syn-BMT on the CD4+FoxP3+ Tregs

We measured the percentage of cells expressing both CD25 and FoxP3 in total CD4+ T cells in spleens by FCAS (shown in Fig. 3A and Supplementary Fig. 2). The percentages were significantly higher in group 10d-DD (12.90% ± 1.64%) or 10d-ND (13.43% ± 0.59%) compared with normal mice (8.43% ± 0.30%, $P < 0.001$), group NB (8.10% ± 0.96%) or group 40d-ND (3.01% ± 0.30%, $P < 0.001$), or with mice on day 10 after T1D onset (10.16% ± 0.80%, $n = 6$, $P < 0.01$). The percentage of Tregs revealed no statistic difference between groups 10d-DD and 10d-ND. In mice on day 10 after T1D onset, Tregs increased compared to the normal controls ($P = 0.041$), but showed no statistical changes from group NB. Interestingly, the percentage of Tregs in group 40d-ND were statistical decreased compared with either normal mice or group NB ($P < 0.001$) (Fig. 3A). In groups of mice undergoing BMT or not, CD4+ cell compartment showed no differences (Fig. 3B).

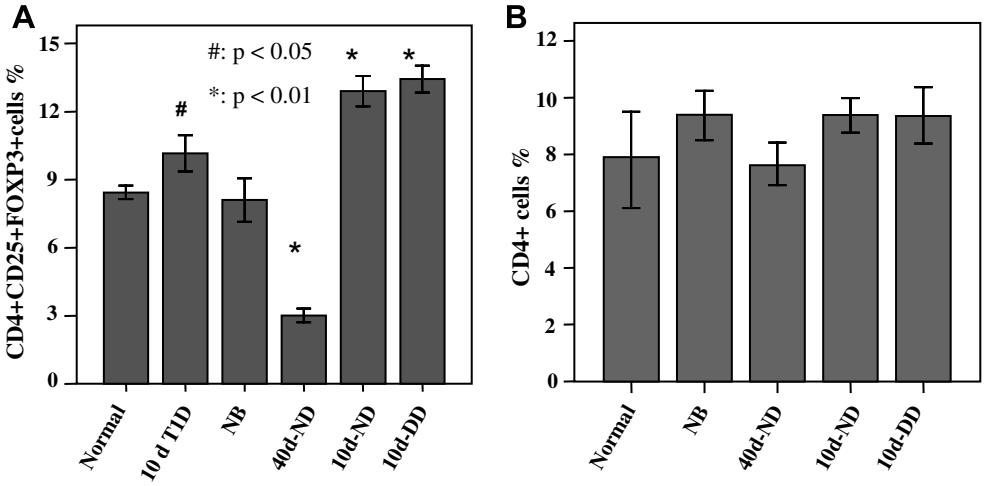

Fig. 3. (A) Percentage of CD25+FoxP3+ cells in CD4+ lymphocytes in spleens analyzed by FACS. Normal = normal mice. 10 d T1D = mice 10 days after the onset of T1D. *Difference was significant compared to normal controls ($P < 0.01$). #Difference was significant compared to normal controls ($P < 0.05$). (B) Percentage of CD4+ lymphocytes in MNCs in spleen analyzed by FACS. The CD4+ cell compartment showed no differences in mice undergoing BMT when compared with normal mice or with group NB.

Effects of syn-BMT on the expression of the FoxP3 protein

FoxP3 protein expression was higher in either group 10d-DD and 10d-ND compared with group NB or 40d-ND ($P < 0.01$), or with normal mice ($P < 0.05$ and $P < 0.01$, respectively), or with mice on day 10 after T1D onset ($P < 0.05$ and $P < 0.01$, respectively). FoxP3 protein expression showed no statistic changes within groups 10d-DD and 10d-ND, or among group NB, group 40d-ND and mice on day 10 after T1D onset compared to normal controls (Fig. 4A).

Effects of syn-BMT on the transcription factor FoxP3 mRNA expression

Foxp3 mRNA increased in the either group 10d-DD or 10d-ND compared with group NB ($P < 0.001$), with group 40d-ND ($P < 0.05$), with mice on day 10 after T1D onset ($P < 0.001$), or with normal controls ($P < 0.001$). However, in group 40d-ND, an increase of FoxP3 mRNA was revealed compared to group NB ($P = 0.012$), or to normal mice ($P < 0.001$). No statistical changes were seen between groups 10d-DD and 10d-ND, or among group NB, normal controls, and mice on day 10 after T1D onset (Fig. 4B).

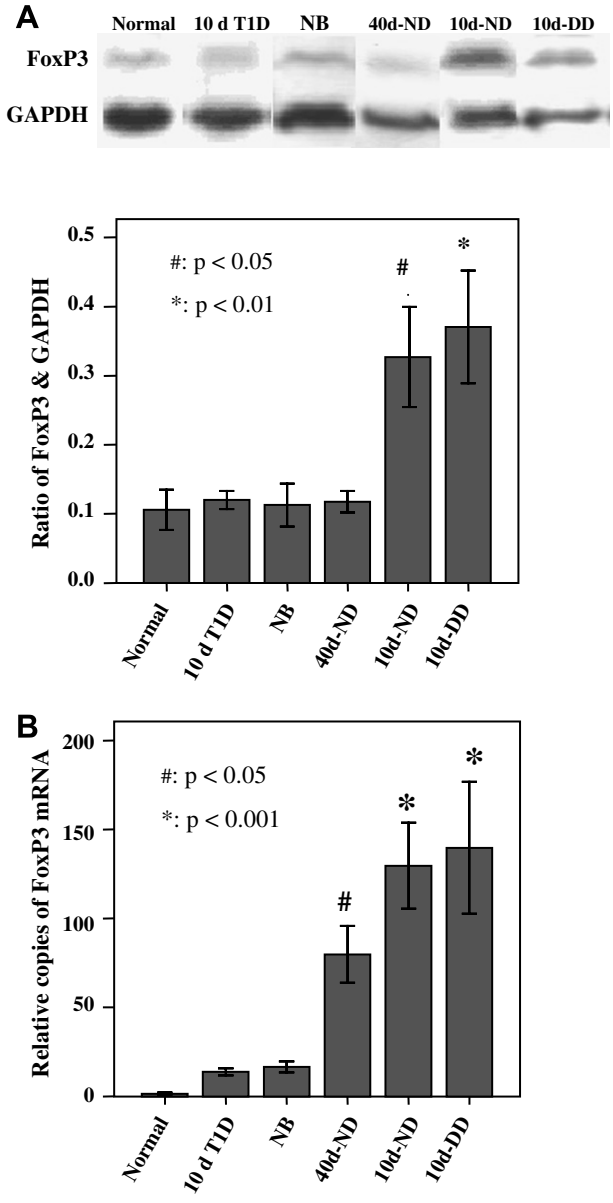

Fig. 4. (A) Western blot analysis for FoxP3 protein compared with GAPDH. Normal = normal mice. 10 d T1D = mice 10 days after the onset of T1D. Figures are representative blots of six separate experiments. Densitometry shows fold induction of FoxP3: GAPDH ratio of each mice groups. *Significant difference in ratios ($P < 0.01$); #$P < 0.05$. (B) FoxP3 mRNA measured by means of quantitative real-time PCR. Normal = normal mice. 10 d T1D = mice 10 days after the onset of T1D. *Difference was significant compared with normal controls ($P < 0.001$). #Significant difference in ratios compared with normal controls ($P < 0.05$).

Discussion

Since Voltarelli et al. reported their pioneering results of autologous HSCT in patients with T1D [8], autologous HSCT has become an exciting potential therapy for patients with new-onset T1D. The main question we wanted to address was whether the favorable effects of autologous HSCT could be replicated in a murine model in order to decipher the mechanism(s) of this remission. It has already been established that diabetes may be prevented in NOD mice by allogeneic bone marrow transplantation [2,3,10–12]. In NOD mice, diabetes is a spontaneous-onset disease that is genetically pre-ordained occurring without known environmental triggers. As a result, in NOD mice, an allogeneic BMT from a diabetic-resistant donor is necessary for cure [10,13].

In humans, T1D is multi-factorial disease in which both genetic factors consisting of multiple susceptible alleles and environmental factors contribute to disease. The environmental importance of diabetes in humans is demonstrated in monozygotic twin studies in which the majority of identical twins are discordant for T1D, although the number of diabetes susceptible genes shared between twins may correlate with concordance [14]. This suggests that T1D in humans may be initiated by environmental exposure [15]. We, therefore, chose an animal model of diabetes using an otherwise non-diabetic strain of mice, C57BL/6J, in which T1D is induced by environmental exposure to multiple low doses of streptozocin.

We demonstrated that BMT, performed when T1D was new-onset (10 days) or even at a latter stage (40 days), can reverse hyperglycemia. If performed on new-onset (10 days) T1D, BMT ameliorated blood glucose to near normoglycemia, maintained euglycemia for at least 120 days of subsequent follow-up and improved blood insulin and glucose tolerance. Even when performed later at 40 days after disease onset, syn-BMT reduced blood glucose for about 77 days compared to non-transplant diabetic controls. These results indicate that BMT, if performed early after disease onset, is a promising therapy for T1D. The euglycemic remission-inducing effect of syn-BMT occurred independent of whether the bone marrow was from a normal or diabetic mouse. Since the donor marrow was not lymphocyte depleted, remission was maintained despite infusion of potential diabetes-inducing immune cells from a SZ-diabetic donor, and suggests an active tolerance-inducing effect from BMT.

Recent publications suggested that in T1D animal models, self-tolerance in autoimmune diabetes depends upon the induction of CD4+CD25+ T regulatory lymphocytes (Tregs) [16,17]. T-cell tolerance is achieved, in part, by the action of Tregs, which are powerful inhibitors of T-cell activation both in vivo and in vitro [18]. Tregs can suppress not only T effector cells but also the cytokines secreted by them. They play an important role in the maintenance of immune tolerance to self, and their antigen-specific population expansion can establish transplantation tolerance [19,20]. We, therefore, determined if Treg expansion can contribute to tolerance following BMT for T1D. Our data demonstrated that although the percentage of total CD4+ cells remained unchanged, an expansion of the CD4+CD25+Foxp3+ Treg population occurred in the spleen after syn-BMT when it was performed when T1D was new-onset (10 days), whereas no increase occurred when it was performed

at a latter stage (40 days after diabetes onset). This change correlated with the maintenance of euglycemia.

Foxp3 is a key regulatory gene for the development of Tregs [21,22], and acts as a T regulatory cell lineage specification factor and mediator of the genetic mechanisms for active tolerance [23,24]. Both FoxP3 mRNA and protein increased after syn-BMT performed early after onset of T1D (10 days). Meanwhile, if BMT was performed later after disease onset (40 days), there was an increase in FoxP3 mRNA, but not in FoxP3 protein. The expansion of FoxP3 mRNA and protein is consistent with increase of functional Tregs. In the group receiving BMT at later stage of disease, our data suggest a blockage in translation of FoxP3 mRNA, which may have contributed to the risk of disease relapse.

Tregs have been previously implicated as a mechanism for BMT-induced autoimmune disease remission in juvenile idiopathic arthritis [25], autoimmune arthritis [26], and multiple sclerosis [7]. We have now extended this observation to SZ-diabetic T1D. In addition, we demonstrate the importance of analyzing both Foxp3 mRNA and protein levels, since, at least in streptozocin-induced T1D, BMT performed later in disease course, after more extensive islet cell destruction, may lead to a less robust regeneration of functional Tregs.

Taken together, this study indicates that syn-BMT, performed when T1D was new-onset (10 days), is safe and is able to reverse the diabetic status. Whether syn-BMT directly induces CD4+CD25+FoxP3+ Tregs in vivo and then the Tregs induce the amelioration of hyperglycemia remains to be determined. Despite its preliminary character, these animal strials provide a rationale to treat human new-onset T1D with autologous HSC. The expansion of Tregs may be one of the mechanisms of BMT-induced T1D remission. Further studies are required to investigate the curative period of syn-BMT for T1D mice and to better understand the mechanisms that underlie these observations.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.07.016.

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