Treatment of Type I Diabetes in the NOD Mouse with Syngeneic Cord Blood Stem Cells

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Abstract: Human type 1 diabetes mellitus occurs due to chronic inflammation with lymphocytic infiltration and (usually) the presence of antibodies against insulin and characteristic islet cell proteins (e.g., GAD). This autoimmune process destroys the islets of Langerhans (including the beta cells) leading to insulin insufficiency. In several studies to date diabetes in the non-obese diabetic (NOD) mouse model of human type 1 diabetes has either been prevented or reversed by allogeneic transplantation of stem cells derived from mouse bone marrow or spleen. Immunosuppressive regimens have been ablative in some studies, but non-ablative regimes have been used in others. With non-ablative regimens, recipient mice develop chimeric immune systems. In addition, two recent clinical trials using stem cells have shown similar benefit for human patients with type 1 diabetes.

We investigated whether stem cells isolated from genetically identical, but non-diabetic neonatal mouse pups (i.e., the mouse equivalent of human cord blood stem cells) would reverse diabetes when transfused into NOD mouse recipients which have already developed hyperglycemia. It was observed that cord blood stem cell infusion after diabetic conversion resulted in reversal of hyperglycemia whether given with or without the immune ablative drug, cytoxan. However, cytoxan alone had no beneficial effect**.** Stem cell-treated mice also lived longer than untreated mice. In these studies it was not possible to demonstrate islet cell regeneration resulting from the infused stem cells. These experiments provide evidence that cord blood stem cells may be suitable for improving or eliminating the autoimmune process in human type 1 diabetes patients, and thereby modulating their disease. However, additional studies are needed to clarify the mechanisms behind these observations.

Keywords: Cord blood, stem cells, diabetes.

INTRODUCTION

Type 1 Diabetes (T1D) is expected to affect 1 in every 300 births in the United States. Approximately 5-10% of all diabetics will display the type 1 diabetic phenotype (i.e., immune mediated). That is, approximately 2-million individuals in the United States currently have type 1 diabetes. Estimated annual costs related to of both types of diabetes mellitus (DM) and their complications in the U.S. is 132 billion dollars (www.diabetes.org and www.jdrf.org). Thus, the economic impact of type 1 DM can be estimated at 16 billion dollars annually in the U.S. Type I diabetes results from destruction by the immune system of the beta cells in the pancreatic islets responsible for insulin production. The end result is uncontrolled blood glucose levels. Diabetic complications include renal failure, retinal hemorrhage and blindness, cataracts, cardiomyopathy, coronary artery disease, peripheral vascular disease and peripheral neuropathies.

The ideal treatment for type 1 DM would be the permanent restoration of functional islet cells, which secrete insulin in response to glucose (glucoregulate), in sufficient numbers to keep blood sugar within the physiologic range (euglycemia). One strategy for T1D, which has shown promise, is immunosuppression targeted against the population(s) of T-cells that mediate islet cell destruction. Immunosuppressive drugs, such as cyclosporin have successfully prolonged the "honeymoon" phase of new-onset type 1 DM, during which no, or only low, doses of insulin may be required, but have been considered too toxic for general application [1]. Recently short-term treatment of new-onset type 1 DM patients ages 12-39 with an anti-lymphocyte antibody targeted against specific T-cell surface receptors (CD3) has been reported to preserve C-peptide secretion and reduce insulin requirements for up to 18 months [2]. However, long term immune compromise makes this approach potentially dangerous.

In an effort to treat type I diabetes, surgical procedures have been developed to transplant islets across histocompatability barriers with limited success due to immune rejection and the lack of cadaver donors. Even efficient usage of the US donor pool would provide, at most, a few thousand donor pancreata per year. However, annual incidence of type 1 DM is approximately 30,000. Thus, there is not enough donor material even to treat all the new patients, let alone the "backlog" of approximately 1.6 million established patients. Moreover, success of transplantation depends on a good match of donor and recipient HLA tissue compatibility antigens, which is difficult to achieve when prospective donors are genetically unrelated to recipients (as

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cadaver donors nearly always are). Investigators have tried to address the issue of T1D through the use of stem cells and regenerative medicine [3]. Currently, autologous CB mononuclear (stem) cells are being evaluated in a clinical trial to treat type 1 diabetes in children [4]. To date, 23 children have been treated, and the first child treated under the study protocol showed significant improvement in glucose control and was able to produce insulin much longer than children with a similar prognosis [5]. The protocol for the clinical trial was established in studies which showed that in animals with T1D, those treated with xenogeneic (human) CB stem cells had lower blood glucose levels, reduced insulitis, and increased lifespan compared to control diabetic animals [6-8]. Similar stem cell trials are being proposed at other centers as well [4]. Although the mechanism(s) of action behind CB stem cell therapy for T1D are not known, it is postulated that once *in vivo* the infused CB stem cells differentiate into new islet cells and mediate an immune tolerance to the new derived islet cells [5]. In fact, recent results have indicated that *in vitro* CB stem cells can indeed be driven to become insulin secreting islet cells as indicated by the production of C-peptide, an offshoot of the de novo secretion of insulin [9, 10]. In both instances, the islet cell differentiation was attributed to the presence of the ES-like stem cells found in cord blood.

Thus, the main obstacles to restoring and maintaining functional islet mass are the ongoing autoimmune reaction which destroys islets, probably due to the action of infiltrating T-lymphocytes and; the probability that, in long-standing type 1 DM, the pancreatic stem/progenitor cell population has been depleted by a process of attrition. However, treatment of T1D patients shortly after diabetogenic conversion might be able to reverse the pathogenic process and preserve sufficient numbers of islet cells to mediate long term benefits. In the current study we utilized the non-obese, diabetic (NOD) mouse, a model of type 1 DM [11], to analyze the effects of cord blood stem cell infusion on T1D. The congenic but non-diabetic C57Bl/6 mouse strain was utilized as a normal control and as the cord blood stem cell donor. It was observed that cord blood stem cell infusion after diabetic conversion resulted in reversal of hyperglycemia whether given with or without the immune ablative drug, cytoxan. However, cytoxan alone had no long-term beneficial effect.

MATERIALS AND METHODS

Mice and Diabetes Screening

Pregnant C57BL/6xBalb.C (B6: H-2^{bxd}) mice and congenic NOD (8-10 weeks old, females, H-2^{g7}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were selected to be mismatched at CD45 for stem cell tracking and immune chimerism (CD45.1 vs. CD45.2 alleles). NOD mice were monitored for hyperglycemia daily

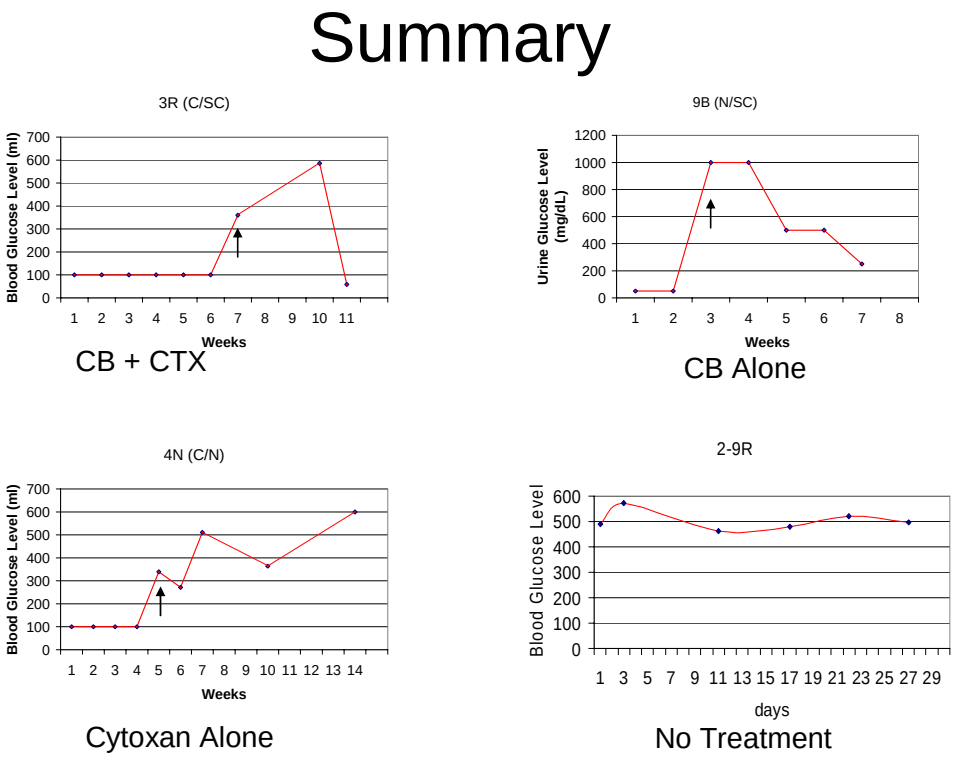

Fig. (1). Representative outcomes for diabetic therapies.

Mice were treated as shown with combinations of cytoxan (CTX) or cord blood stem cells (CB) after onset of diabetes. Blood glucose was monitored weekly, except for the no treatment groups which was monitored daily.

by a urine "dip-stick" screening (Diastix, Bayer) and by weekly sampling of blood glucose by sub-mandibular venipuncture (Basic One Touch glucometer from LifeScan). The mice were monitored for up to 6 months for changes in blood or urine glucose levels. During this time approximately 60% of the mice converted to become hyperglycemic (i.e., diabetic), as measured by urine glucose level over 200 mg/dL for three consecutive days and confirmed by blood glucose over 200 mg/dL. After diabetic onset the mice were treated with daily insulin injections (1 unit, s.c.) until therapy (Humalin, Eli Lilly).

The animal protocols used in these studies were approved by the IACUC committee of the University of Arizona. IACUC approval implies that in order for the project to be approved that the proposed research is based on sound scientific principles, that the minimum number of animals to complete the project has been requested, that the animal model proposed is appropriate for the work, that use of animals in the work is justified and necessary, that manipulations of the animals (e.g., anesthetics) is appropriate, that the research is not duplicative and unnecessary, and that the personnel involved on the project are trained and certified to perform such work. The University of Arizona is approved by AAALAC for research employing the use of animals.

Stem Cells

Timed pregnant mice were sacrificed at d20 of gestation (1 day before parturition), pups were removed, and fetal blood obtained by exsanguination (heparinized), cells counted, immunophenotyped (CD3, CD34, Thy1, Sca1), and separated into mononuclear cells (MNC) by centrifugation over Lmypholyte-M (Cedarlane Labs, Burlington, NC). The MNC were utilized as the murine version of cord blood stem cells [12].

Diabetes Treatment and Analyses

Four weeks after onset of hyperglycemia, mice were treated with a non-ablative immunosuppressive regimen. That is, 2 days prior to stem cell infusion, half of the mice received 200 mg/kg Cytoxan i.p. (CTX). Conditioned diabetic NOD mice received either $1.0x10^7$ neonatal mononuclear cells or the cell suspension medium as a control intravenously via a tail vein. Blood glucose and body weight were monitored weekly and mice from each group were sacrificed periodically after transplantation for analysis. Insulin was withheld for 36 hr before sacrifice. For each mouse blood glucose was measured and blood cells were subjected to FACS analysis to estimate degree of lymphocyte chimerism. Pancreata were analyzed by quantitative histologic and immuohistochemical analysis for islet inflammation, glomerular hypertrophy, tubular dilatation and numbers of insulin containing cells by the small animal pathology laboratory of the University of Arizona. The treatment groups were: (1) no cells, no CTX; (2) no cells, CTX alone; (3) stem cells, no CTX; or (4) stem cells and CTX.

Data Analysis

Statistical methods used to validate the significance of the numeric data collected in each set of experiments included the Student's *t* test and the unpaired Student's **t** test (performed by Microsoft® Excel), while the animal survival data was validated for significance using a Wilcoxon test. Significance was set at $p<0.05$.

RESULTS

Stem Cell Infusion Results in Reversal of Diabetic Hyperglycemia

Diabetic mice were treated as described in Materials and Methods with combinations of cord blood stem cells and cytoxan. As shown in Fig. (**1**), treatment with cytoxan alone resulted in temporary reductions in blood glucose but no long term remissions of hyperglycemia. However, mice treated with a combination of CTX and cord blood stem cells became euglycemic within 4-6 weeks of therapy. Interestingly, mice infused with cord blood stem cells alone also rapidly displayed normal blood glucose levels after therapy. No untreated NOD mice survived long enough for complete analysis.

All diabetic NOD mice were monitored before and after therapy for blood glucose levels. As shown in Table **1**, untreated NOD mice rapidly became hyperglycemic and died within 2 weeks. Only one mouse of 10 survived long enough to obtain multiple glucose measures. No spontaneous cures were observed. Mice treated with cytoxan alone to ablate the peripheral immune system displayed elevated blood glucose levels that persisted in the hyperglycemic range. One mouse (of five) however, did display some long term benefit (decrease in blood glucose levels from 590 mg/dL to 350 mg/dL) from the therapy. The overall blood glucose levels for the group averaged 690 mg/dL. However, all mice treated with a combination of cytoxan plus an infusion of cord blood stem cells after diabetic conversion either remained constant in their glucose levels or exhibited reduced blood glucose measurements. Seven of 15 animals significantly benefited from the therapy in terms of reduced blood glucose levels of 50% or more. One of 15 mice (#2) appeared to be "cured" of diabetes, although overall blood glucose levels for the group averaged 598 mg/dL. This treatment group was the largest in number of animals in that it was the treatment group expected to show the most benefit based on published literature (see below). Interestingly, mice treated solely with cord blood stem cell infusions seemed to benefit similarly, with all mice showing reduced blood glucose levels and all mice appearing to benefit from the therapy. The overall blood glucose levels for this group averaged 290 mg/dL although no single mouse appeared to be cured of diabetes (as evidenced by blood glucose levels of 100 mg/dl or below). It should be noted that almost one-third of the animals in this group displayed no overall change in blood glucose levels (from their highest levels before treatment to their final measures before sacrifice), although day to day and even week to week variations were seen. The reason(s) for this observations is unknown.

Mechanisms of Stem Cell Therapy

Efforts were made to ascertain possible mechanisms of the beneficial effects of the stem cell therapies. Mice were sacrificed at various time points and harvested for blood, spleen, thymus, bone marrow and pancreas. Tissues were

Table 1. Summary of the Effects of Therapy on Diabetes

Therapy	Mouse (N)	Low	Blood Glucose High	Final	Avg	Number that Benefited
None	1	150	500	500	500	0/1
Cytoxan	1	100	600	600		
	2	80	580	500		
	3	50	1000	1000	690	1/5
	4	50	2000	1000		
	5*	50	590	350		
CB + Ctx	1*	100	520	220		
	2*	100	600	80		
	3	100	600	450		
	4	100	340	350		
	5*	220	500	220		
	6	420	550	600		
	7	50	2000	1000		
	8*	50	2000	500	598	7/15
	9	50	2000	2000		
	10	50	1000	1000		
	11	50	500	500		
	12	200	1000	1000		
	13*	380	600	380		
	14*	360	600	400		
	15*	280	520	270		
CB	1*	100	600	380		
	2*	50	1000	210	290	4/4
	3*	80	400	190		
	4*	250	600	380		

NOD mice were allowed to develop diabetes and then treated as described in Materials and Methods. Urine glucose readings were used to follow mice until a level of 300 mg/dL was attained, after which blood glucose levels were measured. Mice were followed until the time of death or sacrifice. Data is shown as the lowest blood glucose reading prior to diabetic conversion and therapy, the highest blood glucose reading after diabetic conversion, and the final blood glucose reading after conversion and therapy (where appropriate). (*) indicates mice achieving a statistically significant therapeutic benefit from their therapy (i.e., significantly lowered blood glucose levels). All surviving mice were sacrificed at the same time point (d60).

measured for donor cell engraftment and restored insulin producing capacity by flow cytometry and immunohisto-chemistry. At various time points mice in each group were sacrificed and analyzed for indications of islet regeneration and stem cell engraftment (Table **2**). It was not possible to determine if any chimerism had occurred based on CD45 allele expression and no significant islet regeneration from donor stem cells could be determined. Islets were maintained in the stem cell treated groups but it was not possible to measure increased C-peptide production due to assay sensitivity. Anti-insulin IHC staining of pancreas tissue was performed for several mice in each group. No significant differences were detected in the treated groups. Although it did not appear that many, if any, of the treated mice were cured of their diabetes (i.e., blood glucose levels below 300 mg/dL) the type of treatment received did impact overall survival (see Fig. (**2**)). That is, stem cell treated mice (CTX plus stem cells or stem cells alone) survived significantly longer (median survival of 50 and 80 days, respectively) than untreated animals (27 days). As animals treated with cytoxan alone had not shown benefit in earlier experiments (see Fig. (**1**) and (Table **1**)), this group was not examined in the survival experiment presented in Fig. (**2**).

Table 2. Other Data

Islets	No consistent or significant islet regeneration was observed in any group although analyses were performed late in the time course.
C-peptide	ELISA measurements of mouse C-peptide did not detect differences between groups, but sensitivity of the assay was low.
Chimerism	No significant chimerism was detected by FACS at the time of autopsy/death.
Survival	The untreated group died quickest; while the stem cell treated groups lived longest

DISCUSSION

Diabetes mellitus is defined as an insufficiency of insulin action, leading to an inability to metabolize glucose, and hence hyperglycemia (high blood sugar) and the complications thereof. Type 1 DM, which usually appears in childhood or adolescence, is caused by autoimmune destruction of islet cells. It is characterized by infiltration of the islets with lymphocytes and most patients have serum autoantibodies against insulin and other islet cell proteins [13]. Blood levels of insulin and C-peptide are extremely low or undetectable in untreated type 1 DM, although a few patients with established Type 1 DM maintain residual islet cell function [14]. Type 1 DM can only be treated successfully with exogenous insulin. Blood glucose levels in insulin-dependent diabetics tend to be labile and unpredictable, despite best efforts at control, and patients with frequent hypoglycemic reactions and hyperglycemia, in the short term may experience life-threatening ketoacidosis and in the long term experience renal failure, blindness, painful neuropathy, and premature death. DM is also associated with accelerated atherosclerosis and both coronary disease and peripheral circulatory insufficiency leading to amputations. Experimental evidence suggests that "tight control" of blood glucose, as indicated by low circulating levels (<7.0 mg/dl) of hemoglobin A1c (glycohemoglobin) can reduce the incidence of both microvascular [15, 16] and macrovascular [17] complications.

Successful treatment of type 1 DM has been achieved by transplantation of whole pancreas [18] and more recently by transplanting isolated islets from human cadaver donors. However, transplantation is limited by the availability of suitable donors [19-21]. This lack of available islets for transplantation has led investigators to experiment with *in vitro* production of glucoregulating, insulin-secreting cells [22-29], with limited success. Stem cell therapy provides a potential solution to the afore-described problems. That is, appropriate stem cell infusions might provide a source of pancreatic islet beta cell replacement via regenerative medicine. For example, although bone marrow stem cells are recruited to sites of pancreatic beta cell injury [30] evidence is mixed as to whether the cells differentiate into functional islets [31-35].

In the last few years, blood drained from the placenta after delivery of an infant has been found to be a rich source of pluripotent stem cells [36-39] that could be used for regenerative medicine. In one study [40] multiple markers for islet cell differentiation were expressed in cultured human cord blood mononuclear cells. In another experiment, intravenous human umbilical cord blood administration to prediabetic nonobese diabetic (NOD) mice significantly reduced blood glucose levels and insulitis and increased lifespan, compared with untreated mice. Prolonged lifespan appeared to be related to better control of blood glucose levels [41]. In this same model cord blood transfusion also normalized renal glomerular hypertrophy and tubular dilatation [42]. These results resemble the reported "cures" of diabetes in NOD mice achieved with mouse bone marrow cells and splenic lymphocytes. Finally, in another study, 2 months after T cell-depleted mononuclear cells from human cord blood were given intravenously to NOD, immunodeficient, beta2-microglobulin null mice, pancreata were positive for human insulin by immunofluorescence staining and PCR analysis. *In situ* hybridization analysis indicated that human insulin-producing cells were present at a frequency of 0.65 %, with about half the insulin-positive cells showing evidence of fusion [43]. The authors concluded that human cord blood progenitor cells can generate insulin-producing cells in recipient pancreas *in vivo* by fusion-dependent and fusion-independent mechanisms.

Three recent reports demonstrate "cure" of diabetes in the NOD mouse model of autoimmune diabetes using stem cell transplantation. The first study employed a combination of whole body irradiation and bone marrow cells [44]; in the second study [45] mice received donor splenocytes partially matched for major histocompatibility complex (MHC) class I antigens and Freund's adjuvant without immunosuppression. In both studies, mice became euglycemic without exogenous insulin, and evidence of endogenous insulin secretion was found. In the most recent such report [46] in spontaneously diabetic NOD mice, nonmyeloablative conditioning followed by bone marrow stem cell transplantation achieved mixed hematopoietic chimerism across MHC barriers. This regimen preserved both alloreactive and autoreactive diabetogenic host NOD T-cells, but, when mixed chimerism was established, diabetic NOD mice accepted donor-type allogeneic islet grafts and were cured of diabetes, demonstrating reversal of autoimmunity, despite continued presence of significant numbers of recipient T-cells. However, the mechanism(s) behind these

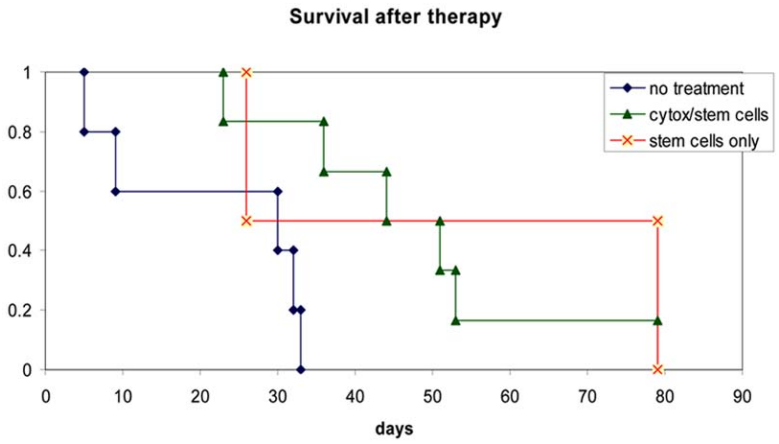

Fig. (2). Overall survival of stem cell treated diabetic mice.

Mice were treated upon diabetic conversion as described. Mice were then followed until death or 80 days post-treatment. The overall median survival for each group was 27 days for untreated mice, 50 days for mice treated with cytoxan plus stem cells, and 80 days for mice treated with stem cells alone. Both the Cytoxan + Stem Cells and the Stem cells alone groups were statistically significant from the No treatment group ($p<0.05$). N = 10 for each group.

observations have been difficult to ascertain and the findings difficult to reproduce.

Interestingly, a recent clinical trial using bone marrow-derived stem cells has produced similar findings in newly diagnosed juvenile diabetic patients [47]. A total of 15 patients received cytoxan-mobilized stem cells intravenously with cytoxan pre-conditioning. Significantly, 14 of the patients became insulin-free after treatment for periods of time ranging from 1 to 35 months. Further, it appeared that insulin production was either maintained or increased as measured by C-peptide production indicative of maintained or regained islet function. Thus, high dose immunosuppression in combination with autologous stem cell infusions appeared to be beneficial in most of the subjects in terms of either prolonging islet function or regenerating islet mass. A caveat is the potential toxicity that can be associated with the high dose immunosuppression (200 mg/kg cyclophosphamide and ATG), albeit not myeloablative. Our study produced similar findings in the diabetic NOD mice.

Also consistent with this hypothesis are the initial results obtained in a clinical trial utilizing autologous cord blood stem cells to treat newly diagnosed type I diabetics. In contrast to the above trial, no immunosuppression was used [5]. A total of 15 patients have been studied and a total of 23 patients are scheduled to be treated. It appeared that simple cord blood stem cell infusion (similar to our own observations in the study) was sufficient to prolong islet function in the patients, possibly via the generation of immune regulatory cells and/or regeneration of islet mass.

Our study was designed to simulate the above clinical trials with the hope of elucidating the mechanisms of any beneficial effects. We found that treatment of diabetic mice with stem cells shortly after diabetic conversion resulted in an overall reduction in blood glucose levels and a prolongation of overall survival. Approximately half of all mice treated with a combination of CTX and stem cells appeared to show benefit from the therapy, while all mice treated with stem cells alone displayed significant benefit. The reason for this difference could not be ascertained. Mice treated with CTX alone showed little long term benefit, although there were temporary reductions in blood glucose. Untreated mice rapidly succumbed to hyperglycemia. It is not clear whether the therapy resulted in any true cures. That is, stem cell infusions did not appear to result in chimerism or any significant islet cell regeneration of donor origin as assessed by IHC or C-peptide production. However, it did appear that stem cell infusions resulted in lowered blood glucose levels and a longer "honeymoon" period in the treated mice. Whether multiple stem cell infusions and/or larger stem cell doses would be more beneficial are topics that should be investigated further.

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ABBREVIATIONS

1D = Type I diabetes

CB = Cord blood

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