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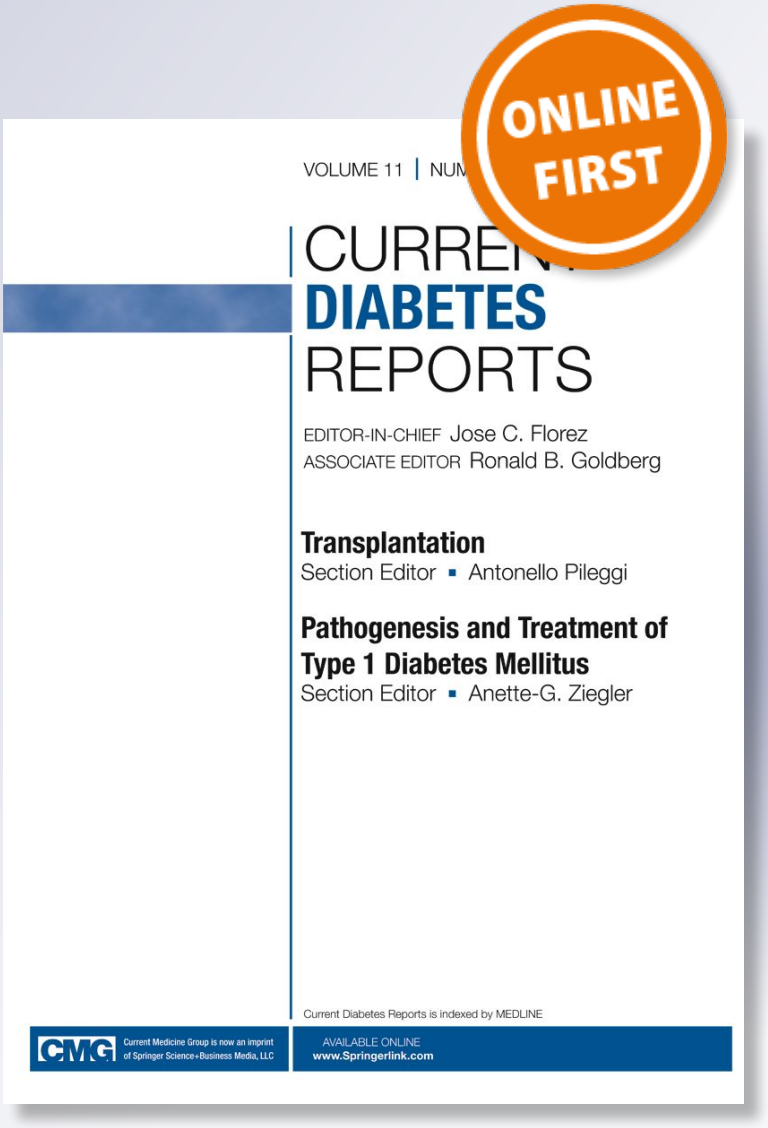

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Stem Cell Educator Therapy and Induction of Immune Balance

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Abstract Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease that causes the deficit of pancreatic islet β cells. A true cure has proven elusive despite intensive research pressure by using conventional approaches over the past 25 years. The situation highlights the challenges we face in conquering this disease. Alternative approaches are needed. Increasing evidence demonstrates that stem cells possess the function of immune modulation. We established the Stem Cell Educator therapy by using cord blood-derived multipotent stem cells (CB-SCs). A closed-loop system that circulates a patient's blood through a blood cell separator, briefly co-cultures the patient's lymphocytes with adherent CB-SCs in vitro, and returns the educated lymphocytes (but not the CB-SCs) to the patient's circulation. Our clinical trial reveals that a single treatment with the Stem Cell Educator provides lasting reversal of autoimmunity that allows regeneration of islet β cells and improvement of metabolic control in subjects with long-standing T1D.

Keywords Stem cell educator therapy · Immune balance · Immune modulation · Cord blood-derived multipotent stem cells · Mesenchymal stem cells · Hematopoietic stem cells · Autoimmune regulator (Aire) · Type 1 diabetes

Abbreviations

Aire	autoimmune regulator
CB-SCs	cord blood-derived multipotent stem cells
DCs	dendritic cells
HLA	human leukocyte antigen
HSCs	hematopoietic stem cells
MSCs	mesenchymal stem cells
NK	natural killer cells
NO	nitric oxide
PB-IPC	peripheral blood-derived insulin-producing cells
PD-L1	programmed death ligand 1
TGF-β1	transforming growth factor-β1
T1D	type 1 diabetes
Tregs	regulatory T cells
TLR 3	Toll-like receptor 3
TLR 4	Toll-like receptor 4

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Introduction

Type 1 diabetes (T1D) is an autoimmune disease caused by a T cell-mediated destruction of pancreatic islet β cells and thereby limits insulin production and glucose homeostasis. Millions of individuals worldwide have T1D, and the number of individuals with T1D is increasing annually among different populations. While daily insulin injections offer some control over blood sugar levels and may delay the onset of chronic complications due to dysglycemia, insulin injection is not a cure. Ideally, therapeutic approaches to treating or curing T1D should address multiple or all of the underlying causes of autoimmunity in T1D. Unfortunately, the etiology of T1D remains largely unknown in humans. Possible triggers for autoimmunity in T1D include genetic, epigenetic, physical, social, and environmental factors. These factors may act independently or jointly to initiate or potentiate the development of autoimmunity [1••, 2]. As is expected in conditions with multiple contributing factors, T1D-related dysfunction in the immune system has been traced to dysfunctions in multiple cell types and targets including T cells, B cells, regulatory T cells (Tregs), monocytes/macrophages, dendritic cells (DCs), natural killer (NK) cells, and natural killer T (NKT) cells [3]. Due to the polyclonal nature of T1D-related autoimmune responses

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and the global challenges of immune regulation in T1D patients, therapies and trials that only target one or a few components of the autoimmune response are likely to fail just as recent trials involving anti-CD3 antibody for T cells and GAD 65 vaccination have failed [4–6]. Successful therapies will likely restore immune balance and peripheral tolerance by addressing changes in multiple targets within the immune system. This review will highlight the recent progress in the development of stem cell-based immune therapy for treating T1D.

CB-SC and Stem Cell Educator Therapy

Certain adult stem cells possess the capacity to modulate immune responses. We recently developed a novel therapy designated Stem Cell Educator therapy [7••], based on our results in non-obese diabetic (NOD) mice and other pre-clinical evidence that CB-SCs can control autoimmune responses by altering Tregs and human islet β cell-specific T cell clones [1••, 8•, 9]. Briefly, a 16-gauge IV needle is placed in the median cubital vein to isolate lymphocytes from the patient's blood by using a Blood Cell Separator. The collected lymphocytes are transferred into the device for exposure to CB-SCs, and other blood components are automatically returned to the patient [7••]. The Stem Cell Educator functions as part of a closed-loop system that circulates a patient's blood through a blood cell separator, briefly co-cultures the patient's lymphocytes with CB-SCs in vitro, and returns the educated lymphocytes to the patient's circulation [7••, 10]. CB-SCs tightly attached to interior surfaces in the device, and only the CB-SC-educated autologous lymphocytes are returned to the subjects. The Stem Cell Educator therapy requires only two venipunctures with minimal pain, and does not introduce stem cells or reagents into patients in comparison with other stem cell-based therapies (eg, mesenchymal stem cells [MSCs] and hematopoietic stem cells [HSCs]) [7••]. In addition, CB-SCs display very low immunogenicity, eliminating the need for human leukocyte antigen (HLA) matching prior to treatment [1••, 7••, 11, 12]. Thus, these advantages of Stem Cell Educator therapy may provide CB-SC-mediated immune modulation therapy while mitigating the safety and ethical concerns associated with other stem cell-based approaches and conventional immune therapies.

CB-SCs are a unique type of stem cells identified from human cord blood [1••, 11], which are different from other types of stem cells including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs) (Table 1), endothelial progenitor cells (EPC), and monocyte-derived stem cells [13]. Phenotypic characterization demonstrates that CB-SCs display embryonic cell markers (eg, transcription factors OCT-4 and Nanog, stage-specific embryonic antigen (SSEA)-3, and SSEA-4) and leukocyte common antigen CD45, but they are negative for blood cell lineage markers (eg, CD1a, CD3, CD4, CD8, CD11b, CD11c, CD13, CD14, CD19, CD20, CD34, CD41a, CD41b, CD83, CD90, CD105, and CD133). Furthermore, CB-SC displayed very low immunogenicity as indicated by the expression of a very low level of major histocompatibility complex (MHC) antigens and failure to stimulate the proliferation of allogeneic lymphocytes [11, 12]. They can give rise to 3 embryonic layer-derived cells in the presence of different inducers [1••]. More specifically, CB-SCs tightly adhere to culture dishes with a large rounded morphology and are resistant to common detaching methods (trypsin/EDTA),

Table 1 Phenotypic comparison between CB-SCs and MSCs

	CB-SCs	MSCs
Major source	Human cord blood	Human bone marrow, adipose tissue, placenta, umbilical cord
Morphology	Round	Spindle or fibroblast-like
Attaching surface	Hydrophobic surface	Hydrophilic surface
Cell detachment	Tightly adhered, resistant to EDTA/trypsin detachment	Sensitive to the EDTA/ trypsin detachment
Cell surface markers:		
Hematopoietic stem cell marker CD34	negative	negative
Leukocyte common antigen CD45	Strongly positive	Clearly negative
Thy-1 antigen CD90	negative	positive
Endoglin CD105	negative	positive
Immunogenicity:		
Class I: HLA-ABC	Very low	High
Class II: HLA-DR, DQ	negative	negative

making it easy to collect suspended lymphocytes and separate with CB-SCs after *ex-vivo* co-culture [8•, 11, 12]. Thus, during the Stem Cell Educator therapy, only the CB-SC-educated autologous lymphocytes are returned to the subjects [7••].

Safety and Clinical Efficacy of Stem Cell Educator Therapy Compared to Other Stem Cell-Based Immune Therapies

Safety

Our published data [7••] and unpublished data demonstrated that Stem Cell Educator therapy was well tolerated in all participants with minimal pain from 2 venipunctures. Most patients experienced mild discomfort during venipuncture and some soreness of the arm during aphaeresis, but discomfort and soreness resolved quickly following the conclusion of the procedure [7••]. There were no participants that experienced any significant adverse events during the course of treatment, and no adverse events during 1 year follow-up studies. In comparison with the application of MSCs, autologous bone marrow-derived MSC has been limited for clinical applications due to the painful operations and potential infections in the procedure for harvesting bone marrow. To this end, placenta or umbilical cords are easy to access and represent valuable sources for provision of allogeneic MSCs. However, patients usually showed medium or high fever following transplant of allogeneic MSCs through interventional therapy such as intravenous delivery or direct infusion into pancreatic islets via transfemoral cannulation under angiography.

Efficacy

Findings from our clinical trials provide powerful evidence that a single treatment with the Stem Cell Educator provides lasting reversal of autoimmunity that allows regeneration of islet β cells and improvement of metabolic control in individuals with long-standing T1D [7••]. In an open-label, phase1/phase 2 study, 15 patients with T1D received 1 treatment with the Stem Cell Educator. Their median age was 29 years (range, 15 to 41), and median diabetic history was 8 years (range, 1 to 21). Stem Cell Educator therapy can markedly improve C-peptide levels, reduce the median glycated hemoglobin A_1C (HbA_1C) values, and decrease the median daily dose of insulin in patients with some residual β cell function (n=6) and patients with no residual pancreatic islet β cell function (n=6). Treatment also produced an increase in basal and glucose-stimulated C-peptide levels through 40 weeks. However, participants in the Control Group (n=3) did not exhibit significant change at any follow-up [7••]. Notably, a single treatment could improve islet β function that lasts a year (Fig. 1). Individuals who received Stem Cell Educator therapy exhibited increased expression of costimulating molecules (specifically, CD28 and ICOS), increases in the number of $CD4^+CD25^+Foxp3^+$ Tregs, and restoration of Th1/Th2/Th3 cytokine balance [7••]. Thus, findings from these trials indicate that CB-SC-mediated reversal of autoimmunity results from modulation of the immune response in multiple immune cell types, thereby meeting the expectation that successful therapies

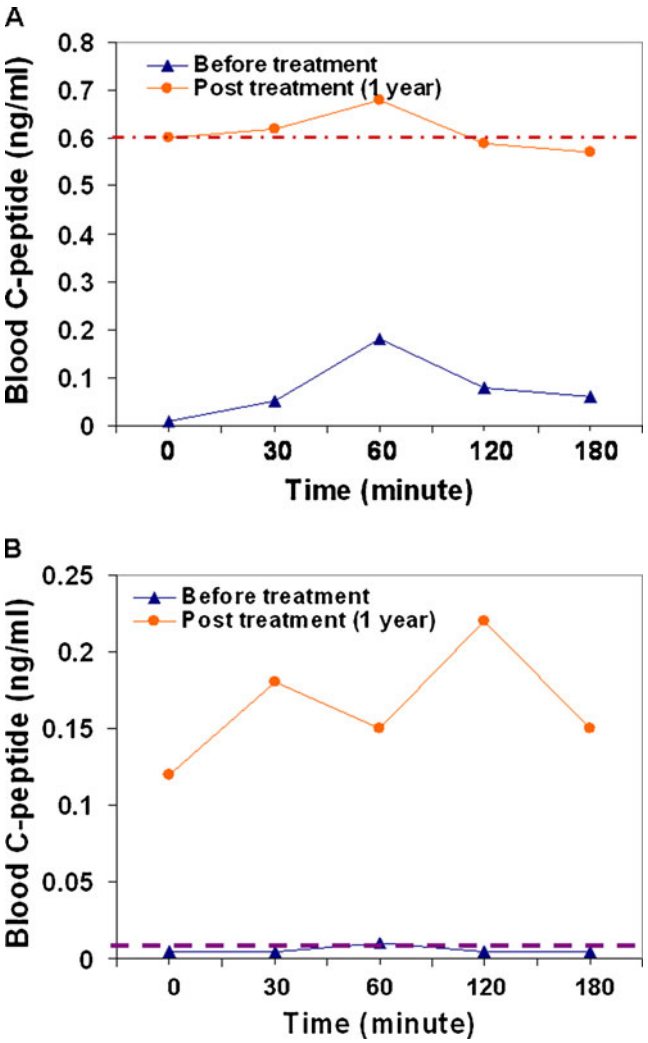

Fig. 1 Improvement of β-cell function by Stem Cell Educator therapy. **a** C-peptide response following a 75-g oral glucose tolerance test (OGTT). A T1D subject (female, 15-year old, 5-year diabetic duration) received 1 treatment with Stem Cell Educator therapy, with one-year follow-up. **b** C-peptide response following a 75-g OGTT from another severe T1D subject (male, 40-year-old, 17-year diabetic duration) received 1 treatment with Stem Cell Educator therapy, with 1-year follow-up. The *dashed red line* indicates the lower limit for normal C-peptide levels in Chinese populations. The level of 0.01 ng/ml is the minimum detectable level (sensitivity, the *dashed purple line*) of C-peptide by radioimmunoassay (RIA). To convert C-peptide value to nmol/L, multiply the ng/ml by 0.331

will likely address different arms of the autoimmune response and balance the immune system through the systemic and local modulations [7••, 8•].

Increasing evidence demonstrates that HSCs display immune modulations and therapeutic potential in autoimmune diseases [14]. Voltarelli and colleagues reported the use of autologous hematopoietic stem cell transplantation (HSCT) for the treatment of new onset T1D, in combination with high doses of immunosuppressive drugs [15]. This protocol achieved insulin independence in some patients with adequate residual β cell mass. However, the response could not be maintained; reoccurrence of the disease is the major challenge for HSCT protocol to treat T1D [16], in addition to the application of chemotherapy. In addition, a new pilot study from the same group showed that bone marrow-derived mesenchymal stromal cells failed to improve the metabolic controls and C-peptide levels in all new onset T1D subjects (total n=5, <6 weeks after T1D diagnosis) after the 7th intravenous infusion of mesenchymal stromal cells. Flow cytometry did not reveal significant differences in the immune markers after transplantation of mesenchymal stromal cells [17]. Thus, a large scale of trial with different routes of administrations is required to clarify whether bone marrow-derived MSCs (or mesenchymal stromal cells) are beneficial for the treatment of T1D.

Additional studies in humans have documented that both autologous and allogeneic hMSCs have met safety endpoints; however, efficacy has not been demonstrated in recent clinical trials [18]. Therefore, it is vital for human MSC-based therapy to optimize the whole protocol from the isolation of MSCs, to in vitro culture and expansion of MSCs, and to the clinical administration of MSCs. To improve the clinical efficacy, most importantly, the function and properties of MSCs should be maintained after *ex-vivo* expansion in cell culture medium [19]. Otherwise, an infusion of functionless MSCs at a large cell dose will not be favorable for clinical achievement. Due to the heterogeneity and scarcity of MSCs populations in the primary starting materials, the processing protocols for isolation and cultivation should be standardized and safe to maintain chromosome stability [20] and reduce the tumorigenicity after ex vivo expansion. Development of novel cell-tracking techniques in humans will provide direct evidence for the cellular fate and function of systemically infused MSCs and might improve clinical application [18, 21].

Molecular Mechanisms Underlying the Immune Modulation of Stem Cell Educator Therapy and Other Stem Cell-Based Therapies

Preclinical studies and clinical data demonstrated the immune modulation of CB-SCs and therapeutic efficacy of Stem Cell Educator therapy in T1D. The immune modulation of CB-SCs can be achieved by a variety of molecular and cellular mechanisms, which include: (1) Expression of autoimmune regulator (Aire) in CB-SCs plays an essential role. Using human Aire-specific small interfering RNAs (siRNA) to knock down Aire expression in CB-SCs, the data indicate that Aire is involved in immune modulation and induction of immune tolerance following Stem Cell Educator therapy [7••]. (2) Increase the percentage of regulatory T cells (Tregs) following Stem Cell Educator therapy [7••]. (3) Correct the functional defects of Tregs [8•]. (4) Directly suppress the islet β cell-specific T cell clones [1••]. (5) Act through the cell-cell contacting mechanism via the surface molecule programmed death ligand 1 (PD-L1) on CB-SCs [12]. 6) Act through the soluble factors released by CB-SCs (eg, nitric oxide, TGF-β1) [12]. During the ex vivo co-culture, T1D-derived effector T cells and/or Tregs can be educated by the favorable microenvironment created by CB-SCs through cell to cell contact and soluble factors [8•, 12, 22]. Quantitative real time PCR array indicated that in vitro co-culture with CB-SCs causes substantial modifications of gene expressions in Tregs, specifically for function-related cytokine and chemokine genes along with signaling pathway molecules and transcription factors [8•].

Specifically, our clinical trial provides evidence that CB-SCs in the device educate effector T cells and/or Tregs, resulting in lasting changes in the expression of costimulating molecules, increasing the population of Tregs, and restoring Th1/Th2/Th3 cytokine balance, each of which is expected to improve control of autoimmunity of T1D [8•, 23]. Therapy also increases production of TGF-β1 in plasma of T1D subjects, one of the best-characterized cytokines contributing the induction of peripheral immune tolerance [24]. Results from a non-obese diabetic (NOD) mouse study [8•], demonstrated that increased plasma TGF-β1 may contribute to the formation of a "TGF-β1 ring" around pancreatic islets that protects β cells against infiltrating lymphocytes, providing a safe environment for promotion of β cell regeneration [8•, 9].

MSCs are rare cell populations physiologically located in the connective tissues (eg, bone marrow and adipose tissue), with self-renewal and potential of differentiation. However, MSCs derived from different tissues display diverse properties [21]. Although given the morphological similarity under in vitro cultures, it is hard to discriminate the MSCs from the populations of bone marrow-derived mesenchymal stromal cells (BMSCs) and tissue-derived fibroblasts [25]. Generally, MSCs are positive for CD73, CD90, and/or CD105 and negative for leukocyte common antigen CD45 and other blood lineage markers [1••]. Thus, it is difficult to generate a pure population of MSCs for basic research and clinical applications, specifically in human MSCs. To date, in most clinical trials, different tissue-derived MSCs are

expanded through in vivo culture with 5–6 passages, collected, and administered through interventional therapy [10].

Recently, in vivo studies reveal that MSCs have limited differentiation potential to regenerate the damaged tissues [21, 26]. Instead, MSCs appear to be more important to regulate the immune responses in clinical settings such as tissue injuries, organ transplantations, and autoimmune diseases [21, 26]. Mounting evidence from animal studies and clinical trials demonstrates that MSCs possess the immune modulation properties through targeting various types of immunocytes including $CD4^+$ T cells, $CD8^+$ T cells, B cells, monocytes/macrophages, DCs, and NK cells [21, 25, 27]. Using in vitro cultured MSCs, mechanistic studies showed that nitric oxide (NO) plays a key role in the mouse MSCs' immune modulation; while indoleamine 2,3- dioxygenase (IDO) is predominantly involved in the immune modulation of human MSCs on T cell suppression [28, 29]. Different from human MSCs, NO plays a key role in mediating the immune modulation of human CB-SCs [12]. Importantly, toll-like receptors (TLR3 and TLR4) are abundantly expressed on human bone marrow-derived MSCs [30, 31]. Waterman et al. reported that specific TLR-activation polarizes hMSCs into 2 distinct sub-populations (MSC1 and MSC2) that display an opposite function in immune modulation. Sub-population MSC1 induced by TLR4 primarily releases proinflammatory factors and activate lymphocytes, whereas MSC2 primed by TLR3 produce predominantly immunosuppressive effects [32]. Importantly, MSCs may function as antigen-presenting cells (APCs) in the presence of interferon-γ (IFN-γ) [33, 34] to evoke the immune system. Thus, the dual effects of MSCs in immune modulation challenge the clinical application of MSCs to find out appropriate time points, cell doses, route of administration, and personal conditions of subjects.

Cellular Mechanisms Underlying the Regeneration of Islet β Cells of Stem Cell Educator Therapy

Pancreatic islets in long-standing T1D are completely destroyed by autoimmune cells [8•]. Abrogation of autoimmunity without an adequate residual β-cell mass will not restore normoglycemia and improve metabolic control. Promotion of β-cell neogenesis must be part of any therapy aimed at T1D treatment. Notably, our clinical data provide powerful evidence that reversal of autoimmunity by Stem Cell Educator therapy leads to regeneration of islet β cells and improvement of metabolic control in long-standing T1D subjects [7••]. CB-SCs from the device are not likely to be the source of this regeneration because they are not transferred to the patient during therapy [7••]. As demonstrated in other studies, the regenerated cells may be derived from multiple endogenous resources such as transdifferentiations of duct cells or α cells [35, 36], and peripheral blood-derived insulin-producing cells [37].

Using the ultrasensitive assay, Wang and colleagues revealed that C-peptide production persists for decades after onset of T1D [38]. Probably, the function of these residual β cells in long-standing T1D subjects may be recovered to replicate after treatment with the Stem Cell Educator therapy. Additionally, the formation of a "TGF-β1 ring" around pancreatic islets [8•] may provide a safe environment to promote the regeneration of these residual β cells and protect them against autoimmune re-attacking [8•, 9].

Our previous work identified a cell population from adult human blood displaying high potential for producing insulin (designated peripheral blood-derived insulin-producing cells, PB-IPC) by using a similar approach by attaching to a plastic surface, without any genetic manipulation and any induction of differentiation [37]. In vitro characterization demonstrates that PB-IPC display the characteristics of islet β-cell progenitors, including the expression of β cell-specific insulin gene transcription factors (eg, MafA, Nkx6.1, and PDX-1), prohormone convertases (PC1 and PC2), the production of insulin and its by-product C-peptide. In vivo transplantation demonstrated that PB-IPC can give rise to functional insulin-producing cells after administering into the chemical streptozotocin (STZ)-induced diabetic NOD-scid mice, as indicated by the production of human C-peptide in mouse plasma and reduction of hyperglycemia [37]. The data imply that PB-IPC may be a potential resource contributing the neogenesis of β cells after the Stem Cell Educator therapy.

Recently, insulin-producing cells have been generated from bone marrow-derived mesenchymal cells under in vitro conditions. Yet they remain controversial. Most bone marrow-derived stem cells originate from mesenchymal cells, such as very small embryonic-like (VSEL) characterized by Ratajczak and colleagues [39], and the marrow-isolated adult multilineage inducible (MIAMI) cells characterized by Schiller and colleagues [40]. Furthermore,, mesenchymal stem cells from adult human islet-derived precursor cells (hIPCs), and from Wharton's Jelly of the human umbilical cord can also give rise to insulin-expressing cells [41]. Due to lack of the hallmark leukocyte common antigen CD45, they are different from our reported PB-IPC.

Islet transplantation, drug-mediated promotion of β-cell regeneration, and stem cell transplantation have been proposed and tested as likely approaches for treating T1D. However, the continued presence of autoreactive effector T cells and B cells in the circulation may destroy insulin-producing cells generated through these approaches, thereby minimizing their therapeutic potential. An alternative approach using ex vivo co-culture of immune cells through

Stem Cell Educator therapy holds promise for addressing both persistent autoimmunity and the regeneration of insulin-producing β cells.

Conclusions

Immune dysfunction of T1D is complicated not only in localizing in pancreatic islets, but also appearing outside of pancreata. There are different compartments of immune system (eg, T cells, Tregs, B cells, DCs, Mo/Mϕ, iNKT) contributing to the autoimmune responses. Therefore, the comprehensive immune modulations via local and systematic approaches are needed to simultaneously address these multiple dysfunctions in clinics. Stem Cell Educator therapy functions as "an artificial thymus" that circulates a patient's blood through a blood cell separator, briefly co-cultures the patient's lymphocytes with CB-SCs in vitro, induces the immune tolerance through the action of Aire, returns the educated lymphocytes to the patient's circulation, and restores the immune balance and homeostasis. During this procedure, both peripheral and pancreatic infiltrated lymphocytes can be isolated by a blood cell separator and treated by CB-SCs. This treatment leads to the global immune modulations and immune balance as demonstrated by clinical data and animal studies [1••, 7••, 8•, 12]. Finally, due to the limitation obtaining pancreatic tissues from human subjects and the limited approaches for tracing the β cell neogenesis, further mechanistic studies in humanized immune-caused T1D mouse model [42, 43] may provide additional insight into the β cell regeneration and the source of regenerated cells in long-standing T1D subjects.

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Disclosure Conflicts of interest: Y. Zhao: is employed by, is the Chairman of the Board for, and has stock/stock options for Tianhe Stem Cell Biotechnologies Inc.; is the inventor for Stem Cell Educator therapy and related technologies at the University of Illinois at Chicago;

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