Hackensack Meridian *Health*

A Pilot Study of the Therapeutic Potential of Stem Cell Educator Therapy in Type 1 Diabetes

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Study Product: Stem Cell Educator

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The Detailed Table of Contents for Clinical Protocol

Study Summary

Title	A Pilot Study of Therapeutic Potential of Stem Cell Educator Therapy in Type 1 Diabetes
Short Title	SCE therapy in type 1 diabetes
Protocol Number	N/A
Phase	Pilot study
Methodology	Single arm, open-label
Study Duration	2 year
Study Center(s)	Hackensack University Medical Center
Objectives	**Primary Objective:** 1) To assess the safety of SCE therapy in subjects with T1D. **Secondary Objectives:** 1) To assess the feasibility of SCE therapy in patients with T1D 2) To evaluate preliminary efficacy of SCE therapy for improving β cell function in patients with T1D through 12 months. 3) To evaluate markers of immune function in T1D patients after SCE therapy.
Endpoints	**Primary Endpoint** 1) The occurrence of treatment-related adverse events **Secondary Endpoints** 1) Feasibility as determined by: • The number of patients who were unable to complete SCE Therapy • The number of patients who are lost to follow-up prior to the 12-month follow-up visit. 2) Preliminary efficacy as measured by • Area under the C-peptide curve (AUC) over the first 2 hours of a 3-hour mixed meal tolerance test (MMTT) • Peak C-peptide levels over a 3 MMTT. Basal C-peptide levels Daily insulin requirements • Change in HbA1C levels over time • Daily insulin requirements • Changes in auto-antibody levels over time 3) Measurements of immune markers at baseline, 1, 3, 6, 9, 12, and 24 months
Number of Subjects	10 subjects projected for the entire study
Diagnosis and Main Inclusion Criteria	Patients with T1D who meet the 2015 diagnosis standards of the American Diabetes Association and a blood test confirmed the presence of at least one autoantibody to pancreatic islet β cells.
Study Product, Dose, Route, Regimen	SCE-treated MNCs, single treatment, infused intravenously, one time infusion.
Duration of administration	3 – 5 hours
Statistical Methodology	All study data will be entered into a Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA) and imported into SAS 9.2. The database will be validated in SAS to ensure any out range values are queried with the multiple sources and all data issues are resolved. Any P-value < 0.05 will be considered statistically significant. All data analysis will be performed using SAS 9.2 (SAS Institute Inc, Cary, NC).

6 Background

6.1 Introduction

In Type 1 diabetes (T1D), autoimmune destruction of pancreatic islet β cells reduces an individual's ability to regulate blood glucose, ultimately resulting in poor blood circulation, heart disease, stroke, infection, renal failure, and often premature death. Each day, millions of patients with T1D receive insulin injections to survive, but these injections do nothing to address the underlying T cell-mediated autoimmune dysfunction. Ideally, therapeutic approaches for treating or curing T1D should address multiple or all of the underlying causes of autoimmunity in the disease. Unfortunately, the etiology of T1D in humans remains largely unknown. Possible contributors to the initiation or potentiation of autoimmunity in T1D include genetic, epigenetic, physical, social, and environmental factors [9,12]. As is expected in conditions with multiple contributing factors, T1D-related immune system dysfunction has been traced to dysfunctions in multiple cell types and targets including T cells, B cells, regulatory T cells (Tregs), monocytes/macrophages (Mo/Mϕs), dendritic cells (DCs), natural killer (NK) cells, and natural killer T (NKT) cells [13]. Due to the polyclonal nature of T1D-related autoimmune responses and the global challenges of immune regulation in T1D patients, therapies and trials that target only one or a few components of the autoimmune response are likely to fail. A true cure has proven elusive despite intensive research pressure using conventional approaches over the past 30 years [14]. Several recent clinical trials [15,16] highlight the challenges in conquering T1D, but their failures provide some valuable lessons about the limitations of conventional immune therapy and the future direction of the quest. Specifically, they point to the need for an approach that produces comprehensive immune modulation at both the local pancreatic and systemic levels rather than targeting the pancreatic effects of one or a few components of the immune system. Stem Cell Educator (SCE) therapy takes this broader approach [1,5,10], which was developed by Dr.Yong Zhao.

While traditional stem cell therapy is not likely to be effective for long-term treatment of T1D, recent studies suggest alternative approaches using stem cells may overcome the autoimmune component of the disease. Human cord blood-derived multipotent stem cells (CB-SCs) [1,2] and mesenchymal stem cells (MSCs) [3-5] have been shown to modulate immune activity *in vitro*. Subsequent studies have demonstrated that CB-SCs can be used to alter immune function and improve markers of T1D in nonobese diabetic (NOD) mice [6], and they can modulate the immune function of T1D patient-derived islet β cell-specific pathogenic T cell clones in co-culture [9]. Studies in an animal model also suggest that CB-SC treatment may allow the patient to regenerate the native population of islet β cells without stem cell transplantation [6,9,24].

To translate these findings into a clinically feasible therapy, Dr.Zhao (currently working at Hackensack University Medical Center) developed the procedure of "SCE" therapy in which a patient's blood was circulated through a closed-loop system that separated lymphocytes from the whole blood and briefly co-cultured them with adherent CB-SCs before returning them to the patient's circulation [1]. In an open-label, phase1/phase 2 study conducted in China, patients (n = 15) with T1D received one treatment with the SCE. Findings from this study demonstrated the feasibility and safety of SCE therapy and demonstrated that T1D patients could achieve improved metabolic control and reduced autoimmunity that lasted months following a single treatment. Notably, our initial clinical data provided powerful evidence that reversal of autoimmunity could lead to regeneration of islet β cells and improvement of metabolic control in long-standing T1D subjects [1].

The SCE device is essentially a stack of specially-designed Petri dishes with tightly adherent CB-SCs cultured to confluency. In the closed-loop system, the patient's lymphocytes slowly pass through the device at a constant flow rate and can interact with the CB-SCs *in vitro*, and the cells returned to the patients are thus autologous lymphocytes that have been treated (or educated) by the CB-SCs. The SCE therapy required only two venipunctures, carried a lower risk of infection than a typical blood transfusion, and did not introduce stem cells or reagents into the patients. In addition, CB-SCs have very low immunogenicity, and the CB-SCs cultured in the device are a highly restricted population and contain no CD3+ T cells or other lymphocyte subsets, eliminating the need for human leukocyte antigen (HLA) matching prior to treatment [8,9,11]. This innovative approach has the potential to provide CB-SC-mediated immune modulation therapy for multiple autoimmune diseases while mitigating the safety and ethical concerns associated with other approaches [2,3,5]. The relative simplicity of the approach may also provide cost and time savings relative to other approaches.

Results from this initial Chinese trial confirmed prior studies indicating that the adherence of CB-SCs could be exploited to develop therapies that do not introduce the CB-SCs into the patient [1,6,9]. The improvement of islet β cell function in T1D patients with residual islet β cells is impressive, but the recovery of islet β cell function in T1D patients without evident β cell function prior to treatment suggested that SCE therapy could address the underlying challenge of autoimmunity and could control the immune response sufficiently to allow regeneration of the native β cell population. Thus, this trial provided initial evidence that exposing a patient's lymphocytes to CB-

SCs can achieve the two essential outcomes required to cure T1D: reversal of autoimmunity and regeneration of islet β cells [1,5].

6.2 SCE Device

The SCE is made of a hydrophobic material from FDA-approved (USP Class VI) Petri dishes that tightly binds CB-SCs without interfering with their immune modulating capability [1]. We originally designed a chamber for co-culture of lymphocytes and CB-SCs that included nine discs of the material with a flow pathway and adherent CB-SCs sandwiched between a top cover plate and a bottom collecting plate. In this trial, we are going to use the 12-layer SCE device (**Figure 3**). The device was manufactured in a Class 100K clean room and gamma- irradiated prior to introducing CB-SCs [8]. The SCE device has been registered as a Class I medical device with the FDA (Registered Establishment Number 3010031995). This innovative device and related technology have been patented (United States Patent and Trademark Office Patent # 13/514, 694).

In the initial SCE Chinese trial, lymphocytes separated from a patient's blood were slowly passed through the stacked discs of material with adherent CB-SCs, and lymphocytes finally collected through a hole in the bottom plate were returned to the patient. The materials used to produce the device are approved for *in vivo* use per the United States Pharmacopeia (i.e., Grade Class VI Plastic).

Figure 3. Overview of SCE Device. CB-SCs are initially cultured inside the SCE device for 10-20 days at a cGMP facility. One allogeneic cord blood unit generates one Educator for one-patient treatment. Lymphocytes are isolated from the T1D participant by a Blood Cell Separator and transferred into the device, where they come in contact with CB-SCs attached to the interior surfaces of the device. Educated lymphocytes are collected from the bottom chamber and infused intravenously back to the patient.

6.3 Preclinical Data

6.3.1 Introduction of cord blood-derived multipotent stem cells (CB-SCs)

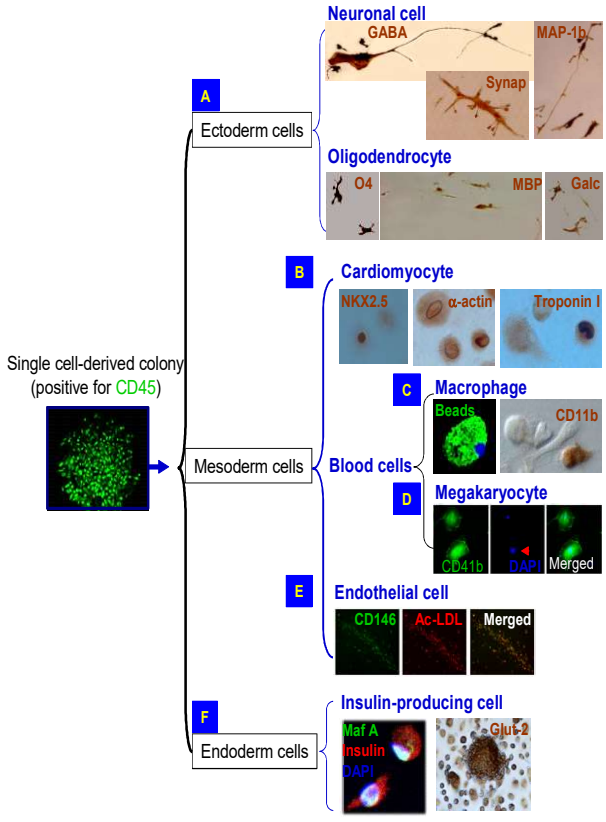
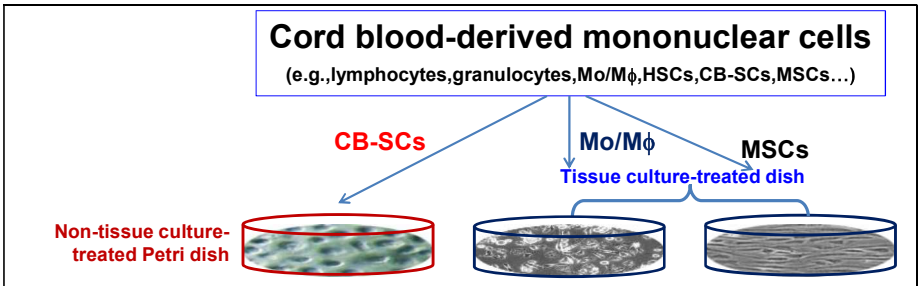


Figure 13. CB-SCs give rise to multiple lineages in response to different physiological growth factors and inducers. CB-SCs are the only cells that tightly adhere to hydrophobic plastic (non-tissue culture treated Petri dishes) and after lymphocytes and monocytes die off, the CB-SCs form a single cell-derived colony positive for leukocyte common antigen CD45 (left panel) and expressing embryonic markers SSEA-3 and SSEA-4. These CB-SCs were capable of: **(A)** differentiation to neuronal cell and oligodendrocyte when treated with 100 ng/ml NGF for 10–14days and characterized with lineage markers including γ-aminobutyric acid (GABA), microtubule associated protein (MAP)1B, Synaptophysin (Synap), sulfatide O4, myelin basic protein (MBP), galactocerebroside(Galc); **(B)** differentiation to cardiomyocytes when treated with a chemical 3 μM 5-aza-2′ Deoxycytidine for 24h, followed by testing with cardiomyocyte markers including nuclear transcription factor Nkx2.5, cardiomyocyte specific α-actin and troponin I; **(C)** differentiation to macrophages when treated with5 0 ng/ml M-CSF for 7–10 days and then characterized with phagocytosis of fluorescence beads and surface marker CD11b/Mac-1; **(D)** differentiation to megakaryocytes when treated with 10 ng/ml TPO for 10–14 days and then characterized with specific marker CD41b and polyploidy nuclear (red arrow); **(E)** differentiation to endothelial cells when treated with 50 ng/ml VEGF for 10–14 days, and then characterized with specific marker CD146 and incorporation of the acetylated low density lipoprotein(Ac-LDL); and **(F)** differentiation to insulin-producing cells when treated with10 nM exendin-4+25 mM glucose for 5–8days and characterized with markers for β cell marker insulin and Glut2. Isotype-matched IgG served as negative controls for every experiment.

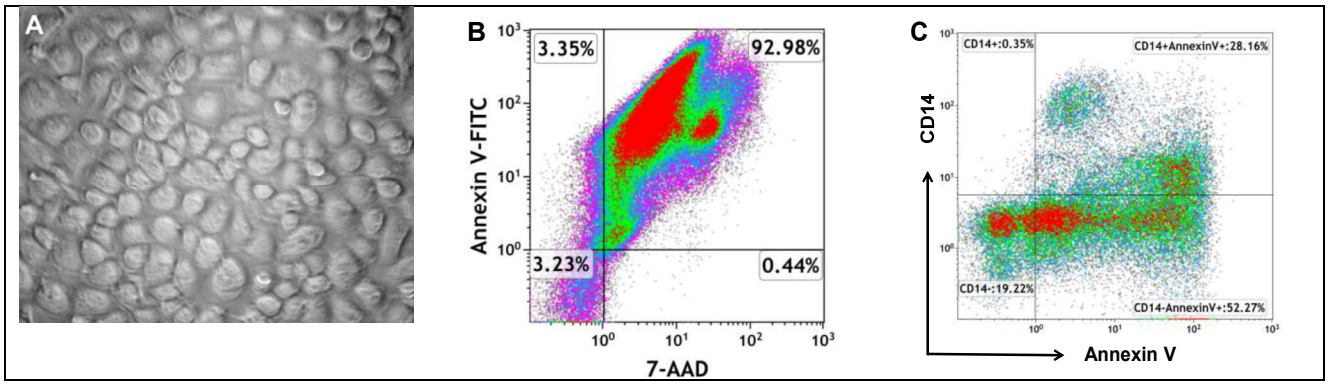
Human cord blood contains several types of stem cells including hematopoietic stem cells (HSCs), multipotent stem cells that have been designated cord blood-derived stem cells (CB-SCs) [8], mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), and monocyte-derived stem cells [25]. Here, we focus on the novel type of stem cells--CB-SCs (**Figure 13**). We identified a unique type of stem cell CB-SC by virtue of their capability to attach to a hydrophobic plastic surface of non-tissue culture-treated Petri dishes [6,8] (**Figure 14A**); other mononuclear cells such as lymphocytes and monocytes were apoptotic and necrotic (**Figure 14 B and C**).



Cellular markers	CB multipotent stem cells CB-SCs	Monocytes/macrophages Mo/Mϕ	Mesenchymal stem cells MSCs
Leukocyte common antigen CD45	Strongly positive 100%	Strongly positive 100%	Negative
Mo/Mϕ markers CD11b and CD14	Negative	Positive	Negative
MSC markers CD90 and CD105	Negative	Negative	Positive
MHC class II antigens HLA-DR, DQ	Negative	Positive	Negative
MHC class I antigens HLA-ABC	Very low	High	High
Embryonic stem (ES) markers: Oct3/4, Nanog, Sox2	Positive	Negative	Negative

Table 5. The phenotype of CB-SCs is different from that of Mo/Mϕ and MSCs.

Phenotypic characterization of CB-SCs indicated that they are different from mesenchymal stem cells (MSCs) and monocytes/ macrophages (Mo/Mϕ) (**Table 2**). CB-SCs display embryonic cell markers (e.g., transcription factors OCT3/4 and Nanog, stage-specific embryonic antigen (SSEA-3, and SSEA-4) and leukocyte common antigen CD45, but are negative for blood cell lineage markers (e.g., CD1a, CD3, CD4, CD8, CD11b, CD13, CD14, CD19, CD20, CD34, CD41a, CD41b, CD83, CD105, CD131, and CD133). Flow cytometry revealed the expression of HVEM (herpesvirus-entry mediator, CD270) and PD-L1 (programmed death ligand 1) molecules on their surface, which may contribute to their immune modulation capacity [3,11] (**Figure 14 D and E**). Additionally, CB-SCs displayed very low immunogenicity as indicated by expression of a very low level of MHC antigens and failure to stimulate the proliferation of allogeneic lymphocytes [8,11]. They can give rise to three embryonic layer-derived cells in the presence of different inducers (**Figure 13**). In comparison with using bone marrow-derived mesenchymal stem cells (MSCs), application of CB-SCs possess several unique advantages including large resources of cord blood worldwide, no risk to the donor, ease to culture and expand *in vitro*, and possession of embryonic characteristics [8]. More specifically, CB-SCs tightly adhere to culture dishes with a large rounded morphology and are resistant to regular detaching methods (trypsin/EDTA), making it easy to collect only the lymphocytes in suspension after co-culture [6,8,11]. Next, we summarize our recent findings in preclinical models that shed light on the therapeutic potential of these cord blood stem cells in T1D.



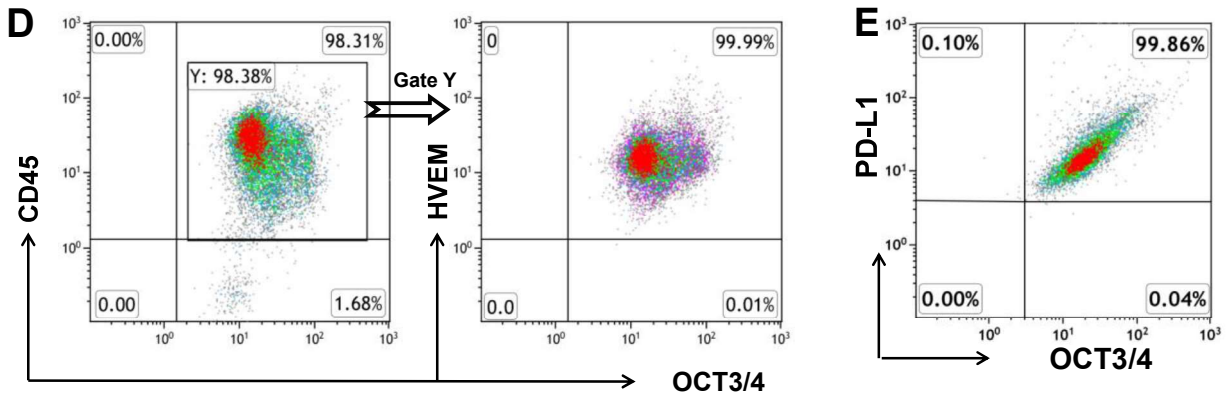


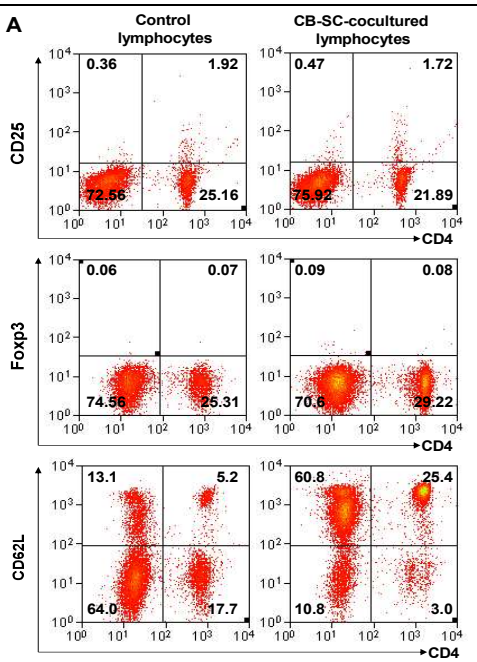
Figure 14. Characterization of CB-SC culture by flow cytometry. (A) Morphology of CB-SC culture under phase-contrast microscope. All floating cells and debris were washed away. **(B)** The apoptosis and necrosis of floating cells in the Petri dish. Human cord blood-derived mononuclear cells were plated in the Bioreactor in serum-free X-Vivo 15 culture medium (Lonza, Walkersville, MD) and incubated at 37°C, in 8% CO_2. After overnight culture (17 hours), flow cytometry demonstrated that 92.98% of floating cells became Annexin V^+7-ADD^+ double positive cells, with only 3.23% of viable cells. The results represented from seven independent experiments with the similar results. **(C**) The apoptosis of monocytes. Human cord blood-derived mononuclear cells were planted in non-treated Petri Dishes in regular cell culture medium. (B) After 20 hours, flow cytometry demonstrated that 99% of $CD14^+$ monocytes became Annexin V-positive apoptotic cells. The data confirmed the apoptosis of monocytes; monocytes could not grow on the surface of non-treated Petri dishes. The results represented from five independent experiments with the similar results. **(D)** Expression of HVEM on the gated $CD45^+OCT3/4^+$ CB-SCs. CD45, a leukocyte common antigen; OCT3/4, a transcription factor marker of embryonic stem (ES) stem cells. **(E)** The gated $CD45^+$ $Oct\text{-}4^+$ CB-SCs display PD-L1 molecule. The isotype-matched IgGs served as controls for all flow cytometry.

6.3.2 Human Cord Blood Stem Cell-Modulated Regulatory T Lymphocytes Reverse the Autoimmune-Caused Type 1 Diabetes in Nonobese Diabetic (NOD) Mice

We reported that treatment of established autoimmune-induced diabetes in NOD mice with purified autologous $CD4^+CD62L^+$ Tregs co-cultured with human CB-SCs can eliminate hyperglycemia, promote islet β-cell regeneration to increase β-cell mass and insulin production, and reconstitute islet architecture. Correspondingly, treatment with CB-SCs modulated $CD4^+CD62L^+$ Tregs (mCD4CD62L Tregs), resulted in a marked reduction of insulitis, restored Th1/Th2 cytokine balance in blood, and induced apoptosis of infiltrated leukocytes in pancreatic islets. These data demonstrated that treatment with mCD4CD62L Tregs can reverse overt diabetes, providing a novel strategy for the treatment of type 1 diabetes as well as other autoimmune diseases.

6.3.2.1 CB-SC modulation of NOD mouse regulatory T lymphocytes.

To investigate the therapeutic potential of Tregs in T1D, we employed an experimental nonobese diabetic (NOD) mouse model. Initially, we tested the co-culture of CB-SC and NOD mouse spleen-derived lymphocytes and found that co-culture with CB-SC did not significantly stimulate the proliferation of mouse lymphocytes at different ratios of CB-SC : lymphocytes (1:5, 1:10, and 1:20) (p = 0.25, p = 0.15, p = 0.16 respectively), which is similar to the co-culture of CB-SC and human lymphocytes [11]. Next, we analyzed co-cultures of CB-SC and mouse lymphocytes for the presence of Tregs including conventional $CD4^+CD25^+$ Treg and $CD4^+Foxp3^+$ Treg, and the $CD4^+CD62L^+$ Treg. We found no significant differences in $CD4^+CD25^+$ Treg and $CD4^+Foxp3^+$ Treg in total mouse spleen lymphocytes that were either cultured alone or with CB-SC. In contrast, the percentage of $CD4^+CD62L^+$ Treg was increased about 5-fold after co-culture with CB-SC (**Figure 9A**). Further flow cytometry revealed that only a very small proportion of these $CD4^+CD62L^+$ Tregs was $CD4^+CD25^+CD62L^+Foxp3^+$ positive (**Figure 9B**), and this percentage was not different between lymphocytes co-cultured with or without CB-SC (0.11 ± 0.04 % vs 0.10 ± 0.03 %, P = 0.44). We subsequently focused on $CD4^+CD62L^+$ Tregs, which were primarily affected by co-culture with CB-SC (designated CB-SC-modulated $CD4^+CD62L^+$ Tregs, mCD4CD62L Tregs).



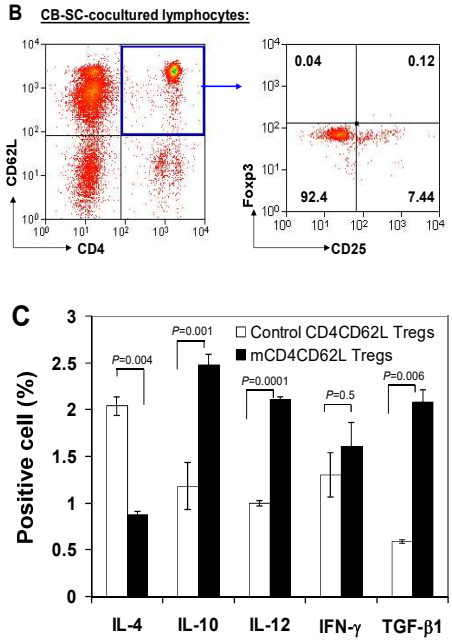


Figure 9. Flow analysis of CD4$^+$CD62L$^+$ Tregs following *in vitro* co-culture with human cord blood stem cells CB-SC. Mouse spleen lymphocytes were isolated from female NOD mice (aged 6-8 weeks) and then co-cultured with CB-SC for 2-4 days at a ratio 1:10 of CB-SC: lymphocytes. Subsequently, the total lymphocytes were harvested for flow analyses. Lymphocytes cultured in absence of CB-SC served as control. **(A)** Percentage of CD4$^+$CD25$^+$ Treg, CD4$^+$Foxp3$^+$ Treg, and CD4$^+$CD62L$^+$ Treg after *in vitro* co-culture with CB-SC. **(B)** Flow analysis of CD25 and Foxp3 expressions in CD4$^+$CD62L$^+$ Tregs after *in vitro* co-culture with CB-SC. **(C)** Flow analysis on CD4$^+$CD62L$^+$ Tregs after intra-cellular cytokine staining. Isotype-matched IgG served as control. Data were representative of three to five experiments.

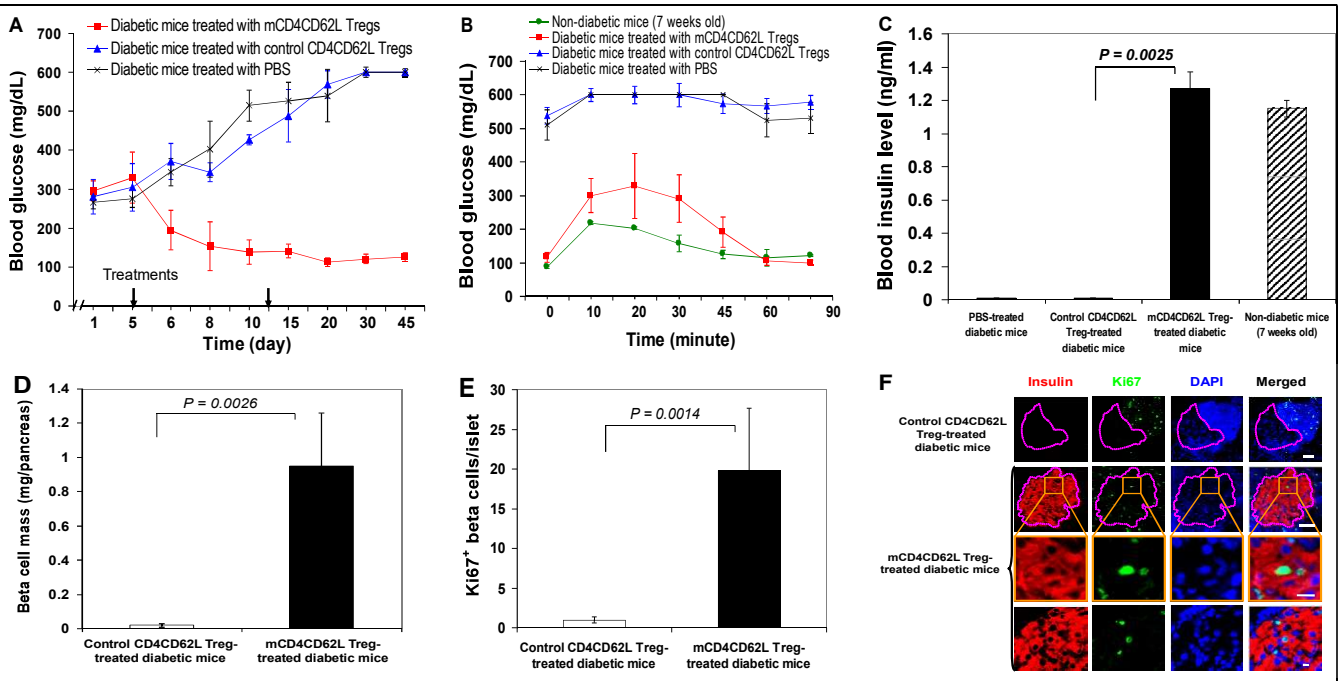


Figure 10 CB-SC-modulated CD4$^+$CD62L$^+$ Tregs (mCD4CD62L Tregs) reverse hyperglycemia in overt diabetic NOD mice. (**A**) The mCD4CD62L Tregs correct hyperglycemia in diabetic NOD mice. Overt diabetic NOD mice were treated with mCD4CD62L Tregs (total 5 million cells/mouse, i.p., red line; representative data are from 6 diabetic mice sensitive to mCD4CD62L Treg treatment with euglycemia, n = 8 mice). Purified control CD4CD62L Tregs served as control (total 5 million cells/mouse, i.p., blue line, n = 5 mice). PBS served as an additional control (black line, n = 5 mice). (**B**) Intraperitoneal glucose tolerance testing (IPGTT) 3 weeks following the 1st treatment with mCD4CD62L Tregs. Seven-week old NOD mice served as normal control. (**C**) Determination of blood insulin levels by ELISA. (**D**) Morphometric analysis of pancreatic β-cell mass. Pancreatic β-cell mass was determined by point-counting morphometry on insulin-positive islet β cells followed by immunostaining with guinea pig anti-insulin Ab (Dako) and counter-staining with hematoxylin. (**E**) Quantification of Ki67-positive cells in pancreatic islets after double immunostaining with Ki67 and insulin Abs. Isotype-matched rabbit IgG served as control for rabbit anti-Ki67 mAb. (**F**) Confocal microscopy shows double-immunostaining for insulin (red) and a cell proliferation nuclear marker Ki67 (green) (scale bar, 50 μm), with a high magnification (two bottom rows, scale bar 10 μm). Control

CD4CD62L Treg-treated diabetic mice (top panels) showed the Ki67-positive cells distributed in the infiltrated inflammatory cells (blue, with high density), not in β-cell area (dashed pink circle), with almost complete disappearance of β cells (red).

To document modulation of $CD4^+CD62L^+$ Tregs by CB-SC after *in vitro* co-culture, intracellular cytokines related to helper T (Th)1 and Th2 immune responses were measured using flow analysis (**Figure 9C**). Results demonstrated that the IL-4 level was significantly down-regulated ($p = 0.004$), whereas IL-10, IL-12 and TGF-β1 levels were up-regulated in mCD4CD62L Tregs compared with control CD4CD62L Tregs ($p = 0.001$, $p = 0.0001$, and $p = 0.006$ respectively). In contrast, the IFN-γ expression did not change following co-culture with CB-SC (**Figure 9C,** $p = 0.5$). Next, we investigated expression of Th1-Th2-Th3 cell-related genes by using quantitative real time PCR array in the purified $CD4^+CD62L^+$ Tregs following co-culture with CB-SC. Results demonstrated that mCD4CD62L Tregs displayed marked down-regulation of Th cell-related genes including multiple cytokines and their receptors, chemokines and their receptors, cell surface molecules, along with signaling pathway molecules and transcription factors [6]. These data clearly indicate that *in vitro* co-culture with CB-SC causes substantial modifications of gene expression in mouse $CD4^+CD62L^+$ Tregs, specifically for function-related cytokine and chemokine genes.

6.3.2.2 CB-SC-modulated CD4+CD62L+ Tregs (mCD4CD62L Tregs) correct hyperglycemia in overt diabetic NOD mice.

Next, overt diabetic NOD mice (female, at 24-28 weeks of age) were treated with mCD4CD62L Tregs (total 5 million cells/mouse, i.p., n = 8 mice) for 5-20 days after the diagnosis of T1D to determine their therapeutic potential. The control CD4CD62L Tregs at the same cell amount (i.p., n = 5 mice) and vehicle PBS (total 200 μl/mouse, i.p., n = 5 mice) served as controls. Notably, we found that treatment with mCD4CD62L Tregs restored euglycemia in these overt diabetic mice (6/8 mice) (**Figure 10A**). However, treatment with control CD4CD62L Tregs or PBS failed to reduce hyperglycemia in diabetic mice (5/5, 5/5 mice respectively) (**Figure 10A**). Diabetic mice that had been rendered euglycemic after treatment with mCD4CD62L Tregs also showed an improved glucose tolerance test (IPGTT), similar to that of non-diabetic NOD mice at 7 weeks (**Figure 5B**). However, diabetic mice treated with PBS or control CD4CD62L Tregs maintained high glucose levels (>500 mg/dL) without any observable down-regulation (**Figure 10B**). Moreover, we monitored blood insulin levels 6 weeks after treatment with mCD4CD62L Tregs. Results showed that insulin in diabetic mice treated with control CD4CD62L Tregs or PBS vehicle was undetectable by ELISA (0.019 ng/ml sensitivity for the ELISA kit, **Figure 10C**). These mice had to be sacrificed because of severe hyperglycemia (BG> 600 mg/dL) and loss of body weight (>20%) according to the protocol approved by the Animal Care Committee. In contrast, blood insulin levels in diabetic NOD mice treated with mCD4CD62L Tregs were significantly increased (**Figure 10C,** $p = 0.0025$).

At 45 days after treatment, we subjected pancreata to histological analysis and evaluated total β-cell mass followed by immunostaining with insulin Ab on serial pancreatic sections. Morphometric analysis demonstrated that treatment with mCD4CD62L Tregs significantly increased total β-cell mass (**Figure 10E,** $p = 0.0026$). In contrast, β-cell mass was markedly lower after vehicle PBS treatment or control CD4CD62L Treg treatment (**Figure 10E**). To understand the mechanism of the increase in total β-cell mass, we determined the expression of a cell proliferation nuclear marker Ki67 [18] in pancreatic islets. Double immunostaining with insulin and Ki67 Abs revealed that 20 ± 8 β cells/islet expressed Ki67 in pancreatic islets of mCD4CD62L Treg-treated mice (**Figure 10E** and **F**), which was much higher than that in pancreatic islets of mice treated with control CD4CD62L Tregs (1 ± 0.4) ($p = 0.0014$). It suggests that *de novo* proliferation of β cells accounts for the noted increase in total β cell mass. Moreover, double immunostaining with β-cell-marker insulin and α-cell-marker glucagon revealed that pancreatic islets in diabetic mice treated with mCD4CD62L Tregs displayed a similar pattern of α- and β-cell distribution as that noted in normal islets of non-diabetic NOD mice. However, islet architecture was completely destroyed with almost complete disappearance of β cells in the diabetic mice treated with control CD4CD62L Tregs [6]. Thus, treatment with mCD4CD62L Tregs can correct hyperglycemia of T1D mice by promoting β-cell regeneration and reconstitution of islet cell architecture.

6.3.2.3 Treatment with mCD4CD62L Tregs reverses insulitis and immune dysfunction in NOD mice.

To establish whether mCD4CD62L Tregs exert an immunosuppressive influence on autoreactive effector T cells, we performed pancreatic histological analysis and scored insulitis at 45 days after treatment. Histological evaluations showed that approximately 80% of islet β cells (profound insulitis) were destroyed in diabetic NOD mice prior to treatment. Six weeks post treatment, we found that in diabetic mice receiving mCD4CD62L Tregs, 36% of islets had no or few signs of infiltration of inflammatory cells; 20% of islets displayed mild insulitis; 15% of islets exhibited moderate insulitis; 18% of islets had severe insulitis and only 11% of islets showed profound insulitis (**Figure 11A** and **B**). The insulitis-free islets were of smaller size and positive for the proliferation marker Ki67 (data not shown), suggesting that these islets may have been newly generated. In contrast, all pancreatic

islets in diabetic mice receiving control CD4CD62L Tregs showed massive infiltration of inflammatory cells and severe destruction of pancreatic architecture (**Figure 11A**), and had few or no insulin-positive cells present. Similarly, pancreatic histological examination demonstrated that those two mice (2/8 mice) that were resistant to mCD4CD62L Tregs treatment also displayed profound insulitis (data not shown) after 45 days observation.

To understand the molecular mechanism underlying reduction of insulitis, we measured plasma Th1/Th2 cytokine levels by ELISA. We found that Th1 cytokine IFN-γ and Th2 cytokine IL-4 were considerably reduced in the plasma of mCD4CD62L Treg-treated diabetic mice relative to control CD4CD62L Treg-treated diabetic mice (P = 0.017, **Figure 11C;** P = 0.018, **Figure 11D** respectively). In contrast, diabetic mice receiving mCD4CD62L Tregs showed a marked increase in plasma IL-10 level compared with those treated with control CD4CD62L Tregs (P = 0.016; **Figure 11E**) and non-diabetic NOD mice at age of 6 weeks (P = 0.014; **Figure 11E**). Additionally, plasma TGF-β1 level was significantly elevated in mCD4CD62L Treg-treated diabetic mice compared with control CD4CD62L Treg-treated diabetic mice (P = 0.041; **Figure 11F**). These data suggest that both IL-10 and TGF-β1 may contribute to an induction of immune tolerance after treatment with mCD4CD62L Tregs [19-21]. These data demonstrate that exposure to CB-SC induced profound changes in mCD4CD62L Tregs that helped restore "normal" islet architecture and β-cell function resulting in the suppression of diabetes.

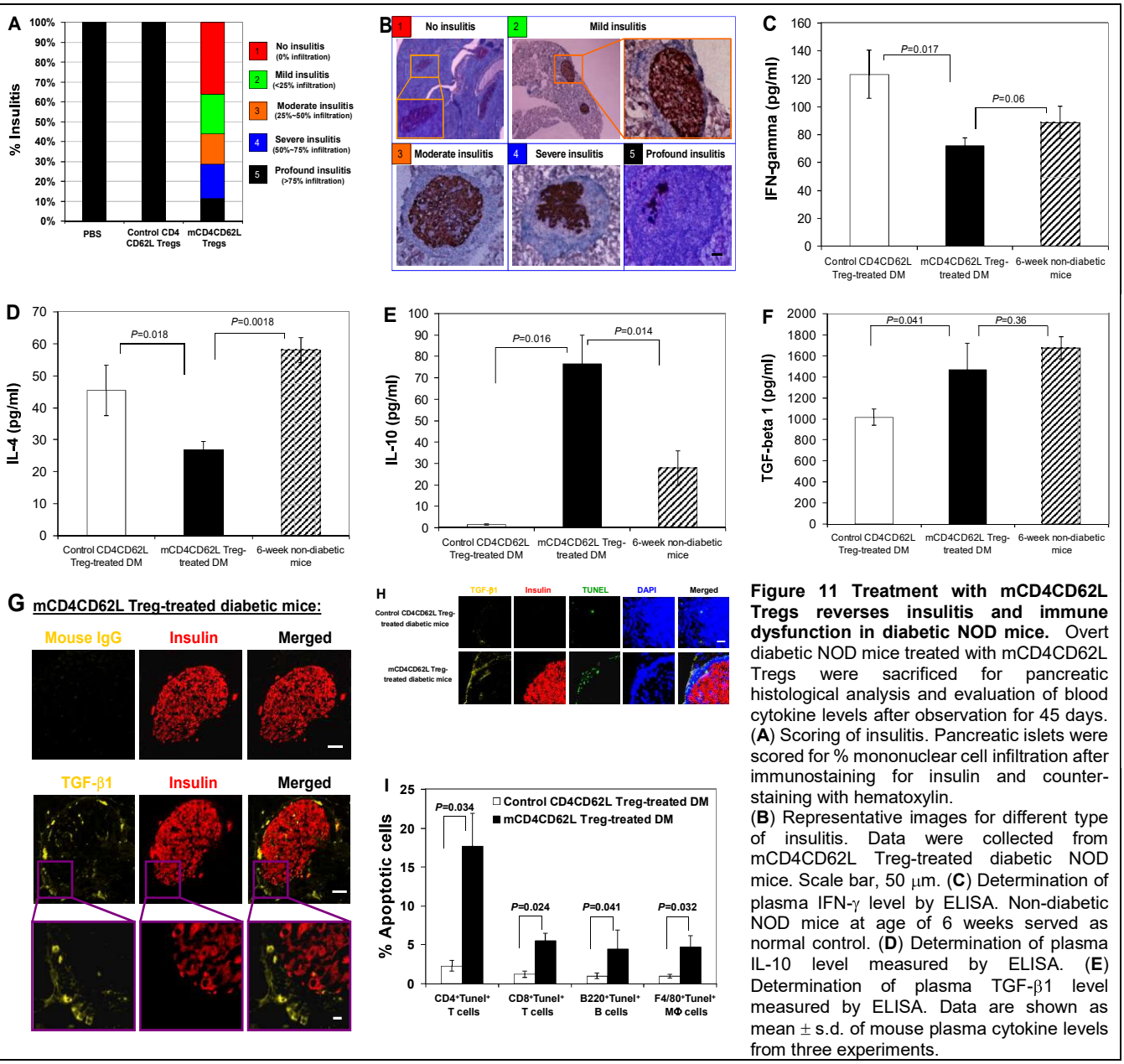


Figure 11 Treatment with mCD4CD62L Tregs reverses insulitis and immune dysfunction in diabetic NOD mice. Overt diabetic NOD mice treated with mCD4CD62L Tregs were sacrificed for pancreatic histological analysis and evaluation of blood cytokine levels after observation for 45 days. (**A**) Scoring of insulitis. Pancreatic islets were scored for % mononuclear cell infiltration after immunostaining for insulin and counter-staining with hematoxylin. (**B**) Representative images for different type of insulitis. Data were collected from mCD4CD62L Treg-treated diabetic NOD mice. Scale bar, 50 μm. (**C**) Determination of plasma IFN-γ level by ELISA. Non-diabetic NOD mice at age of 6 weeks served as normal control. (**D**) Determination of plasma IL-10 level measured by ELISA. (**E**) Determination of plasma TGF-β1 level measured by ELISA. Data are shown as mean ± s.d. of mouse plasma cytokine levels from three experiments.

TGF-β1 is one of the best characterized cytokines contributing to the induction of immune suppression and maintaining of self-tolerance [19]. To elucidate *de novo* molecular mechanism underlying the protection of islet β cells following treatment with mCD4CD62L Tregs, we determined TGF-β1 expression in pancreatic islets by immunohistochemistry in addition to plasma TGF-β1 measurement (**Figure 11F**). Results demonstrated that TGF-β1 was presented at higher level in pancreatic islets of mCD4CD62L Treg-treated diabetic mice compared with control CD4CD62L Treg-treated diabetic mice (**Figure 11G** and **H**). Staining of TGF-β1-positive cells showed two patterns: one was distributed among islet β cells, with average positive cell number of 14 ± 9 cells/islet, and another was located around islet β cells. Importantly, we found that these surrounding TGF-β1-positive cells (negative for macrophage marker F4/80, but positive for dendritic cell marker CD11c [22], data not shown), along with their released TGF-β1 in the matrix (faint staining), formed a ring surrounding pancreatic islets (**Figure 11G**). This ring may protect newly-generated islets against attack by inducing apoptosis of auto-aggressive effector lymphocytes, as determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (**Figure 11H**, 58 ± 23 $TUNEL^+$ infiltrated leukocytes in mCD4CD62L Treg-treated group vs. 9 ± 3 $TUNEL^+$ infiltrated leukocytes in control CD4CD62L Treg-treated group, $p = 0.02$). To clarify which cell type became apoptotic, we performed double staining with different cell markers including CD4 for $CD4^+$ T cells, CD8 for $CD8^+$ T cells, B220 for B cells, and F4/80 for macrophages respectively in combination TUNEL staining. We found that treatment with mCD4CD62L Tregs increased the apoptosis of infiltrated T cells, B cells, and macrophages compared with control CD4CD62L Treg treatment ($p = 0.0034$, $p = 0.024$, $p = 0.041$, and $p = 0.032$ respectively). In comparison with the other three cell types however, $CD4^+$ T cells showed a much higher percentage of apoptotic cells (**Figure 11I**). Thus, these data suggest that treatment with mCD4CD62L Tregs enhances expression of TGF-β1 in pancreatic islets that may contribute to local protection of newly-generated pancreatic islets from the re-destruction of autoreactive immune cells.

6.3.3 Safety of using CB-SCs—No teratoma formation.

To demonstrate the safety of application of CB-SCs, CB-SCs were inoculated into the muscles of NOD-scid mice (n = 8, the muscle of single rear leg) at 2×10^7 cells/mouse to test the tumor formation [23]. Mice were monitored for tumor formation twice a week for 12 weeks. After injection for 3 months, histological examinations failed to show evidence of tumor (or teratoma) formations in the muscles, lungs, livers, and kidneys (**Figure 12**). The data prove that application of CB-SCs is safe.

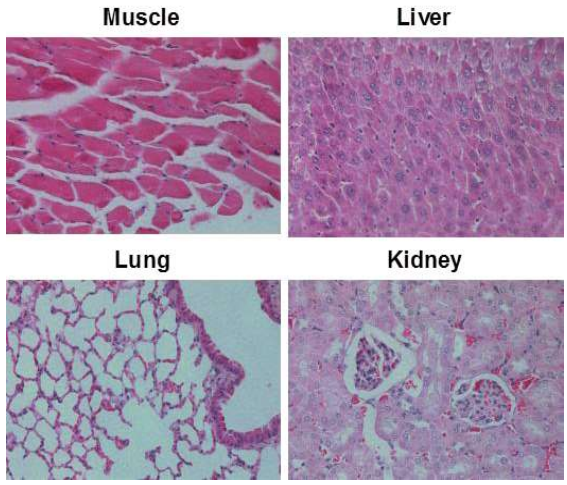


Figure 12. Histological examination of teratoma formation. CB-SCs were inoculated into the muscles of single rear leg of NOD-scid mice at 2×10^7 cells/mouse in 100 μl PBS. After injection for 3 months, mice were sacrificed for testing tumor formation by histological analysis. The isolated tissues were fixed in 10% formaldehyde at room temperature for 10 minutes, paraffinized, and processed for hematoxylin and eosin (H&E) staining. Original magnification, ×400.

6.4 Clinical Data to Date

We recently developed a novel therapy designated "SCE" therapy (**Figure 15**), based on our results in the NOD mouse model and other preclinical evidence that CB-SCs can control autoimmune responses by altering Tregs and human islet β cell-specific T cell clones [6,9,24]. To date, several international clinical trials have confirmed the safety and therapeutic efficacy of SCE therapy for the treatment of: T1D in adults (in China and Spain, respectively) (NCT01350219) [1,5]; T1D in children (NCT01996228); type 2 diabetes (NCT01415726) [2]; and alopecia areata (NCT01673789) [3]. Briefly, a 16-gauge IV needle was placed in the median cubital vein to isolate lymphocytes from the patient's blood by using a Blood Cell Separator. The collected lymphocytes were transferred into the device for exposure to CB-SCs, and other blood components were automatically returned to the patient [1]. In these trials, the SCE functioned as part of a closed-loop system that circulated a patient's blood through the blood cell separator, briefly co-cultured the patient's lymphocytes with CB-SCs *in vitro as they slowly flowed through the Educator*, and returned the "educated" lymphocytes to the patient's circulation [1,26]. CB-SCs tightly attached to interior surfaces in the device, and only the CB-SC-educated autologous lymphocytes were returned

to the subjects. The SCE therapy required only two venipunctures with minimal pain, and did not introduce stem cells or reagents into the patients in comparison with other stem cell-based therapies (e.g., MSCs and HSCs) [1]. Additionally, CB-SCs displayed very low immunogenicity and no presence of CD3+ T cells or other lymphocyte subsets derived from the cord blood, eliminating the need for human leukocyte antigen (HLA) matching prior to treatment [1,8,9,11]. Thus, SCE therapy proved to be advantageous in providing CB-SC-mediated immune modulation therapy while mitigating the safety and ethical concerns associated with other stem cell-based approaches and conventional immune therapies.

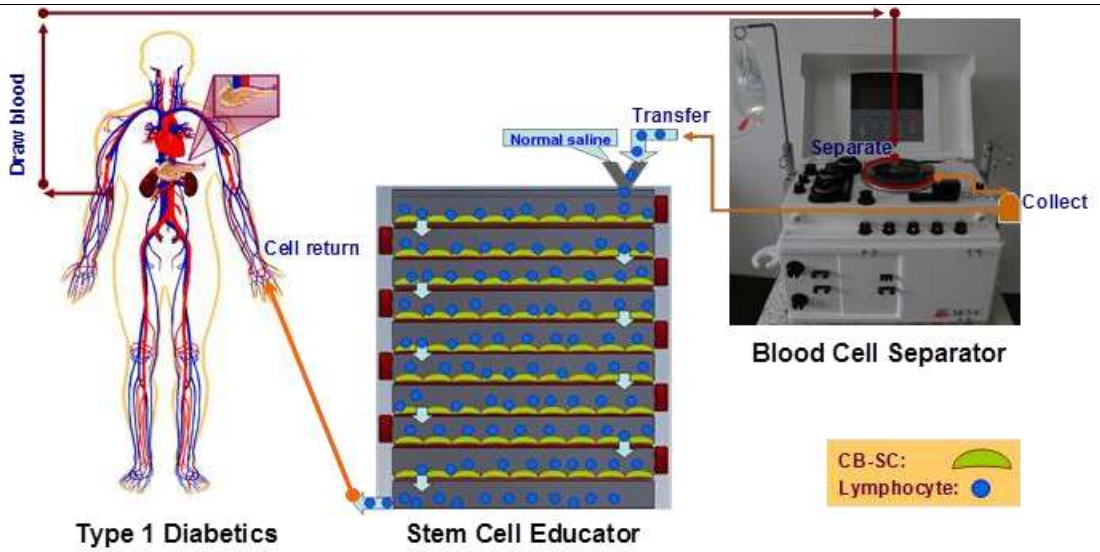


Figure 15. Scheme of the SCE Therapy Approach. CB-SCs are initially cultured inside the SCE device for 10-14 days at cGMP facility and shipped to clinical sites for bedside treatment. One allogeneic cord blood unit generates one Educator for one-patient treatment. Briefly, a patient's blood is passed through a Blood Cell Separator that isolates the lymphocytes from peripheral blood, the collected lymphocytes are transferred into the SCE device where they encounter CB-SCs as they slowly transit through the chambers from the top to bottom layers; after that, **only the "educated" autologous cells** will return back to the patient via a dorsal vein in the hand (note: CB-SCs remain tightly attached inside the Educator). This is a closed-loop system and blood is continually processed for a total of about 8-9 hrs.

6.4.1 SCE Therapy for the treatment of type 1 diabetes in a Chinese Population

6.4.1.1 Feasibility and Safety of SCE Therapy

Fifteen T1D patients were enrolled (baseline characteristics presented in **Table 6**). Median age was 29 years (range, 15 to 41), and median diabetic history was 8 years (range, 1 to 21). Participants were randomly assigned to receive SCE therapy (n=12) or sham therapy (n=3). Each participant received one treatment. Based on fasting C-peptide levels (a by-product of insulin biosynthesis, as an indicator for islet β cell function), participants in the treatment group were characterized as having moderate T1D with some residual β cell function (n = 6, Group A) or severe T1D with no residual pancreatic islet β cell function (n = 6, Group B) (**Table 6**). All Control Group participants had moderate T1D.

No participants experienced any significant adverse events during the course of treatment. 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. Twenty-four hours post treatment, no significant difference was noted in white blood cell counts relative to baseline (Total white blood cell count: 6.95×10^9/L ± 1.98 vs 6.39×10^9/L ± 1.72, $p = 0.38$; Granulocytes: 3.79×10^9/L ± 1.43 vs 3.66×10^9/L ± 1.05, $p = 0.77$; Lymphocytes: 2.31×10^9/L ± 0.9 vs 2.08×10^9/L ± 0.67, $p = 0.40$; Monocytes: 0.49×10^9/L ± 0.13 vs 0.46×10^9/L ± 0.10, $p = 0.48$). Participants' body temperatures were not significantly changed during the two-day post-treatment observation (36.44 °C ± 0.24 vs 36.5 °C ± 0.22, n = 15, $p = 0.35$). No changes were observed in blood cell count or temperature at the 12-week follow-up.

CB-SCs are tightly adherent [8,9] and not expected to escape the device. To confirm CB-SCs are completely retained in the Educator and not transferred to the patient, we examined cells leaving the device to check for stage specific embryonic antigen 3 (SSEA-3), a CB-SC-specific marker. Flow cytometry confirmed the absence of SSEA-3 in cells leaving the Educator [1]. These data indicate that the cells returned to the patients are autologous. Additionally, HLA matching is not required prior to SCE therapy because CB-SCs are not transferred to the patient and because CB-SCs have very low immunogenicity [8,9,11]. Thus, SCE therapy is a very safe approach.

Table 6. Characteristics of the T1D subjects before treatment.

Patient No.	Age	Gender	Marriage	History (year)	Height (cm)	Body weight (kg)	C-peptide (ng/ml)*	Auto-Antibodies IA-2A	GAD	ICA	IAA	HbA1C (%)	Insulin dose (unit/day)
Group A: Long-standing patients having some residual islet β cell function, received Stem Cell Educator therapy with CB-SC													
1	17	F	No	5	145	35	0.30	-	+	-	-	12.3	52
2	23	F	No	5	167	59	0.56	-	+	-	-	6.6	50
3	38	F	Yes	11	160	60	0.12	-	+	-	-	7.3	23
4	39	F	Yes	1	156	49	0.636	+	+	+	-	11.3	22
5	31	M	Yes	14	170	70	0.18	+	+	-	-	6.5	30
6	30	M	Yes	2	182	70	0.18	-	+	-	-	8.4	40
Mean	**30**			**6**	**163**	**57**	**0.33**					**8.7**	**36.2**
(SD)	**(9)**			**(5)**	**(12.7)**	**(13.4)**	**(0.22)**					**(2.5)**	**(13.2)**
Group B: Long-standing severe patients with no residual islet β cell function, received Stem Cell Educator therapy with CB-SC													
7	40	M	Yes	17	170	72.5	0.01	-	+	-	-	8.8	48
8	15	F	No	5	163	65	0.01	-	+	-	-	15.5	50
9	21	F	No	4	168	65	0.01	+	-	-	-	9.9	46
10	23	F	No	12	162	80	0.01	+	+	+	-	16.5	60
11	40	F	Yes	21	160	67	0.01	+	-	-	-	8.6	37
12	21	F	No	5	157	56	0.01	-	+	-	-	13.6	50
Mean	**27**			**11**	**163**	**67.6**	**0.01**					**12.2**	**48.5**
(SD)	**(11)**			**(7)**	**(4.9)**	**(8.1)**	**(0)**					**(3.5)**	**(7.4)**
Control group: Long-standing patients having some residual islet β cell function, received sham therapy													
13	35	M	Yes	1	178	73.5	0.37	+	-	+	-	9.8	30
14	41	M	Yes	14	165	65	0.55	-	+	-	-	6.4	48
15	24	M	No	3	175	58	0.3	-	+	-	-	10.7	48
Mean	**33**			**6**	**173**	**65.5**	**0.41**					**9.0**	**42**
(SD)	**(9)**			**(7)**	**(6.8)**	**(7.8)**	**(0.13)**					**(2.3)**	**(10.4)**

***** The level of 0.01 ng/ml is the minimum detectable level (sensitivity) of C-peptide by radioimmunoassay (RIA). To convert C-peptide value to nmol/L, multiply the ng/ml by 0.331.

6.4.1.2 Clinical Efficacy Outcomes in Improving Islet Beta Cell Function [1]

Participants in Group A (i.e., those with moderate T1D and some residual β cell function) exhibited improved fasting C-peptide levels at 12 and 24 weeks post treatment (**Figure 16A and B**), and participants in Group B (i.e., those with severe T1D and no residual pancreatic islet β cell function) exhibited successive improvement in fasting C-peptide levels at each follow-up (**Figure 16A and 9C**). C-peptide response following a 75-g oral glucose tolerance test (OGTT) improved among Group A participants at 4 and 12 weeks (**Figure 16B**). Notably, participants in Group B exhibited essentially no C-peptide production following glucose challenge at baseline (i.e., < the minimum sensitivity of 0.01 ng/ml at all time points) but demonstrated marked improvement at 12 weeks (**Figure 16C**). Improvement was maintained through the final follow-up (i.e., 40 weeks post treatment; $p = 0.026$) **(Figure 16C).** Participants in the Control Group did not exhibit significant change at any follow-up (**Figure 16A**).

Consistent with improved β cell function, the median daily dose of insulin was reduced 38% at 12 weeks post treatment in Group A (36 ± 13.2 units/day at baseline vs. 22 ± 1.8 units/day 12 weeks post treatment) and 25% in Group B (48 ± 7.4 units/day at baseline vs. 36 ± 4.4 units/day 12 weeks post treatment), but no change was observed in the Control Group. The reduced daily dose of insulin in Group A and B was maintained through the last follow-up for this measure (24 weeks). The median glycated hemoglobin (HbA_1C) in Group A was significantly lowered from 8.73% ± 2.49 at baseline to 7.67% ± 1.03 at 4 weeks post treatment ($p = 0.036$), and to 6.82% ± 0.49 at 12 weeks post treatment ($p = 0.019$). The median HbA_1C in Group B was reduced 1.68% ± 0.42 at 12 weeks post treatment, but no change was observed in the Control Group (9.0% ± 2.3 at baseline vs. 8.7% ± 1.9 at 12 weeks post treatment, $p = 0.86$). Thus, the *ex vivo* immune education by exposure to CB-SCs leads to regeneration of islet β cells and improvement of β cell function in long-standing T1D subjects.

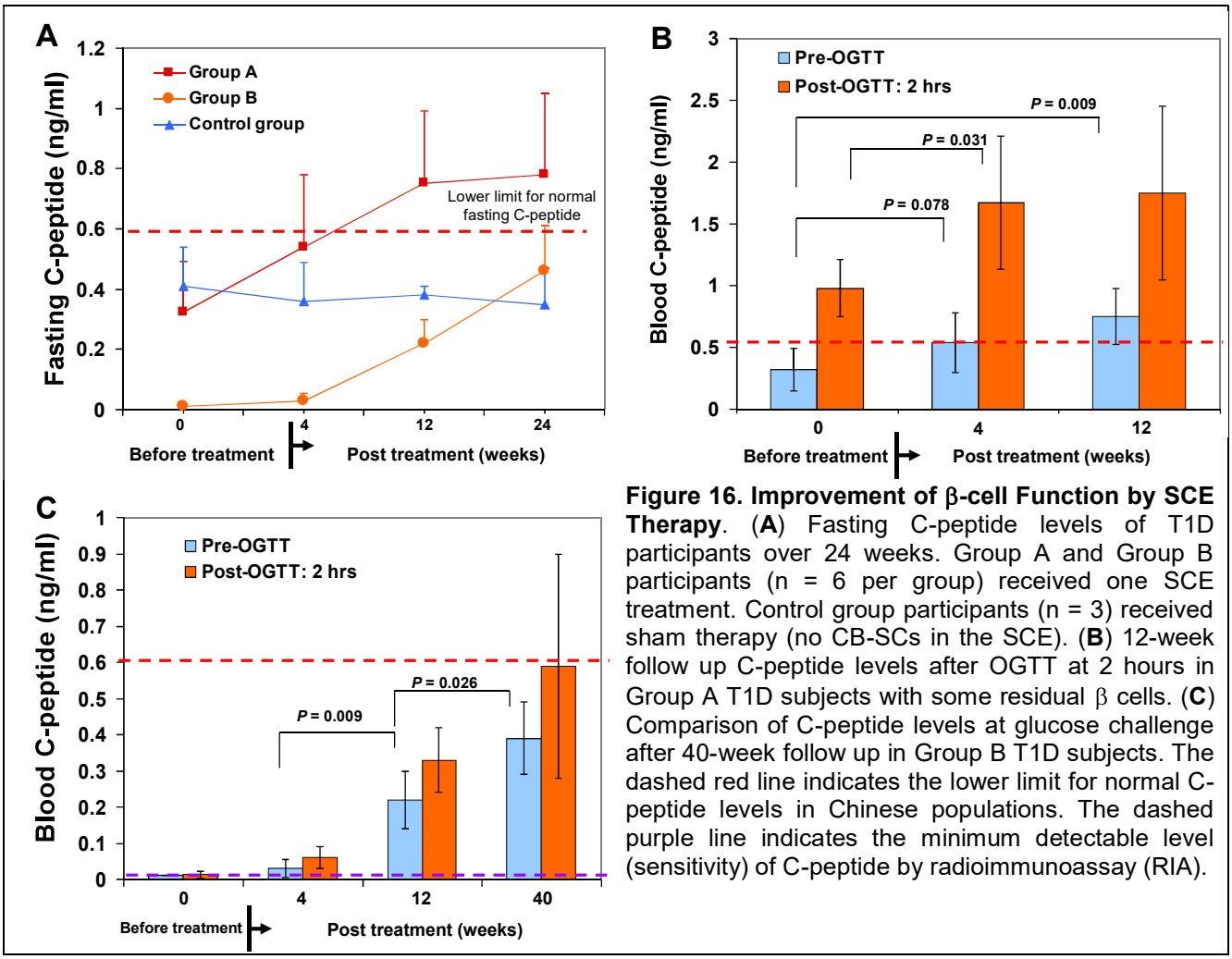


Figure 16. Improvement of β-cell Function by SCE Therapy. (**A**) Fasting C-peptide levels of T1D participants over 24 weeks. Group A and Group B participants (n = 6 per group) received one SCE treatment. Control group participants (n = 3) received sham therapy (no CB-SCs in the SCE). (**B**) 12-week follow up C-peptide levels after OGTT at 2 hours in Group A T1D subjects with some residual β cells. (**C**) Comparison of C-peptide levels at glucose challenge after 40-week follow up in Group B T1D subjects. The dashed red line indicates the lower limit for normal C-peptide levels in Chinese populations. The dashed purple line indicates the minimum detectable level (sensitivity) of C-peptide by radioimmunoassay (RIA).

6.4.1.3 Efficacy Outcomes in Autoimmune Control [1]

Next, we explored mechanisms underlying CB-SC-mediated immune modulation. Regulatory T lymphocytes (Tregs) play a crucial role in maintaining homeostasis and self-tolerance by inhibiting the action of autoreactive effector T cells [6,27,28], but previous attempts to manipulate Tregs for clinical applications have been problematic [29]. We measured changes in the percentage of $CD4^+CD25^+Foxp3^+$ Tregs in peripheral blood of participants following SCE treatment. The percentage of Tregs in peripheral blood of participants was significantly increased 4 weeks after SCE therapy (**Figure 17A)**, whereas the percentage of Tregs in peripheral blood of participants receiving sham therapy was unchanged from baseline (**Figure 17A**). TGF-β1 has also been implicated in Treg-mediated immune suppression [30] as well as in maintenance of self-tolerance in T1D animal models subjected to stem cell-mediated immune modulation [9,19,24]. We examined TGF-β1 and IL-10 expression to explore whether these pathways are activated following SCE therapy. Participants in the treatment group exhibited significant increases in plasma level of TGF-β1 at the 4-week follow-up (P = 0.001, **Figure 17B**) but did not exhibit changes in the plasma level of IL-10 (P = 0.44, **Figure 17B**). Both TGF-β1 and IL-10 failed to show changes in the control group.

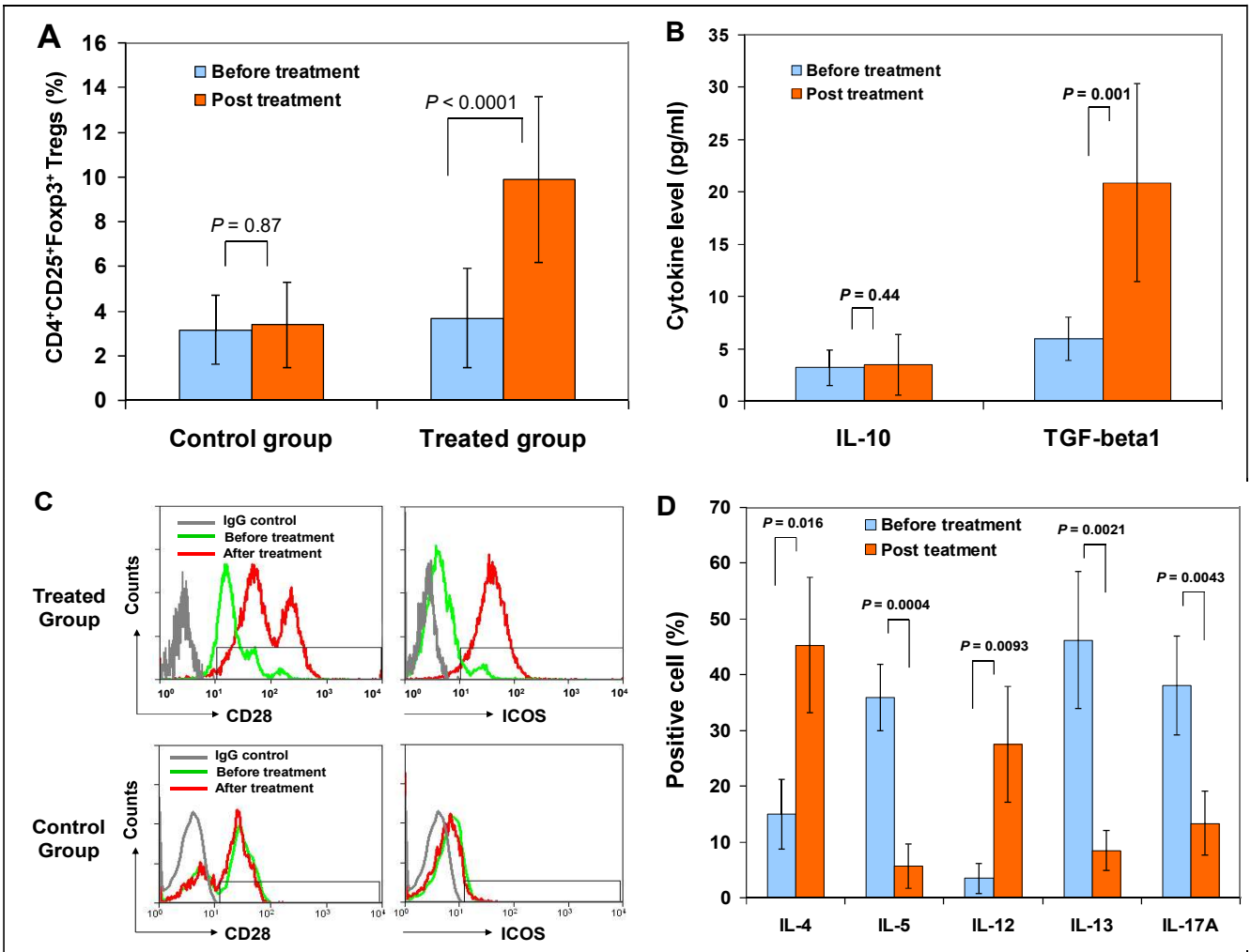


Figure 17. Markers of immune function in T1D patients after SCE therapy. Patient lymphocytes were isolated from peripheral blood by Ficoll-Hypaque (γ = 1.077) for flow cytometry analyses in T1D patients at baseline and 4 weeks after SCE therapy. Isotype-matched IgG served as control. **(A)** Flow Analysis of $CD4^+CD25^+Foxp3^+$ Tregs demonstrating an increase in the percentage of Tregs at 4 weeks post treatment. **(B)** Cytokine ELISAs demonstrating an increase in TGF-β1 but not IL-10 at 4 weeks post treatment. **(C)** Flow cytometry on co-stimulating molecules indicating increases in CD28 and ICOS at 4 weeks post treatment with SCE therapy (top panels). Control group failed to show increases (bottom panels). **(D)** Flow analysis of intra-cellular cytokines demonstrating differential effects on key interleukins at 4 weeks post treatment. Data are representative of preparations from all T1D participants (n = 12) that received SCE therapy.

We also examined levels of CD28 [31-35] and inducible costimulator (ICOS) [36,37], which are essential for the establishment, maintenance, and efficacy of Tregs [31-39]. Flow cytometry revealed an increase in CD28 and ICOS in lymphocytes 4 weeks after SCE therapy (**Figure 17C**), but levels of both molecules were unchanged in participants receiving sham therapy [1]. We also noted other changes at the 4-week follow-up consistent with improved helper T cell 1 (Th1) and Th2-mediated immune function (**Figure 17D**). Expression of IL-4 and IL-12 was significantly increased (p = 0.016 and p = 0.0093, respectively), and expression of IL-5 and IL-13 was decreased (p = 0.00039 and p = 0.00206, respectively). The production of pro-inflammatory IL-17A was also decreased 4 weeks after treatment (**Figure 17D**, p = 0.0043). No changes were observed in levels of these cytokines in participants who received sham therapy [1].

6.4.2 SCE Therapy for the treatment of type 1 diabetes in a Caucasian Population

6.4.2.1 Safety profile and feasibility of two treatments with SCE therapy in Caucasian T1D subjects

In previous clinical trials, all subjects received one treatment with the SCE therapy [1-3]. Due to the likelihood that significant numbers of pathogenic autoimmune cells may have remained in lymph nodes and other tissues, failing to enter into the bloodstream during the procedure, and thus may have escaped the exposure to CB-SCs, we added a second treatment three months following the initial session in these T1D subjects (n = 15) (**Figure 18A**). No participants experienced any significant adverse events during the course of the two treatments with SCE

therapy or during 56-week follow-up. During the procedure, only mild discomfort at the site of venipuncture (the median cubital vein) and some soreness of the arm were noted for some participants. No fever or rejection was noted during follow-up studies.

Additionally, our ongoing children (NCT01996228) also confirmed the safety in Caucasian children (**Figure 18 B and C**). The procedure of treatment was well tolerated in all children without any significant adverse events.

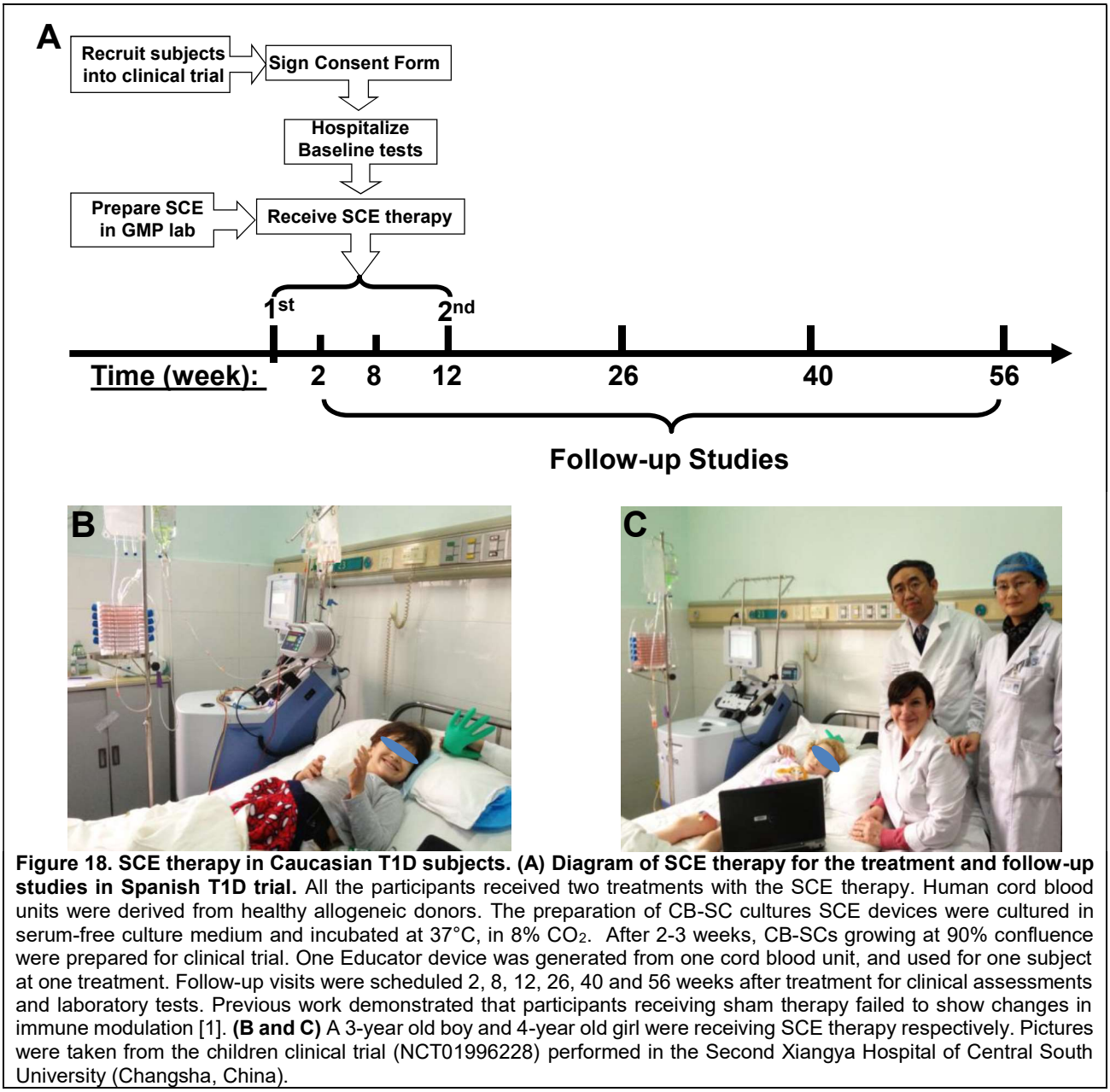


Figure 18. SCE therapy in Caucasian T1D subjects. (A) Diagram of SCE therapy for the treatment and follow-up studies in Spanish T1D trial. All the participants received two treatments with the SCE therapy. Human cord blood units were derived from healthy allogeneic donors. The preparation of CB-SC cultures SCE devices were cultured in serum-free culture medium and incubated at 37°C, in 8% CO_2. After 2-3 weeks, CB-SCs growing at 90% confluence were prepared for clinical trial. One Educator device was generated from one cord blood unit, and used for one subject at one treatment. Follow-up visits were scheduled 2, 8, 12, 26, 40 and 56 weeks after treatment for clinical assessments and laboratory tests. Previous work demonstrated that participants receiving sham therapy failed to show changes in immune modulation [1]. **(B and C)** A 3-year old boy and 4-year old girl were receiving SCE therapy respectively. Pictures were taken from the children clinical trial (NCT01996228) performed in the Second Xiangya Hospital of Central South University (Changsha, China).

6.4.2.2 Clinical efficacy of SCE therapy in the modulation of memory T cell compartment of Caucasian subjects [10]

To evaluate the immune modulating effects of the SCE therapy, we used flow cytometry to examine immune markers in 15 participants following SCE therapy. Clinical data indicated no changes in total cell numbers of each cell population during one-year follow-up, including leukocyte common antigen $CD45^+$ nuclearized cells, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells, $CD56^+$ NK cells, and $CD20^+$ B cells (**Figure 19A**). Quantification of the percentages of total CD4+ and CD8+ T cells in peripheral blood remained very stable over a year (**Figure 19B**). Notably, the percentage of naïve $CD4^+$ T ($CD45RA^+$ $CCR7^+$) cells was significantly increased at 26 weeks after the treatment with SCE therapy (P = 0.0042), and maintained through the final follow-up (at 56 weeks post-treatment, P = 0.0021) (**Figure 19C**). The percentage of naïve $CD8^+$ T cells did not exhibit significant changes at

any follow-ups (**Figure 19C**). These findings suggested that the SCE therapy restored the regeneration of naïve CD4⁺ T cells, an essential part of normal immune capacity.

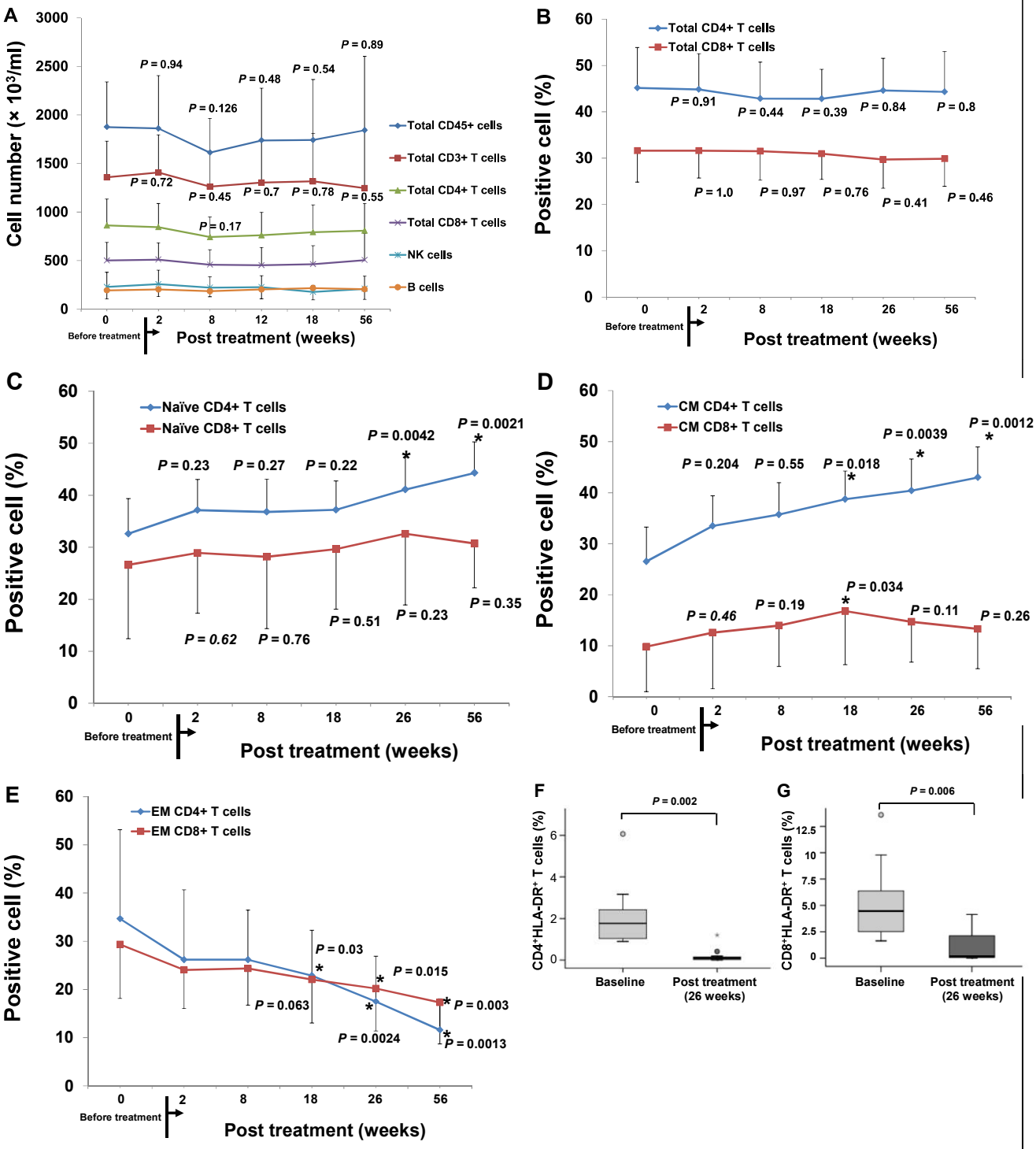


Figure 19. Changes in immune markers in Caucasian T1D patients after SCE therapy. All subjects received two treatments with SCE therapy. After 3 months, all subjects received a 2nd treatment with SCE therapy. Follow-up visits were scheduled 2, 8, 12, 26, 40 and 56 weeks after treatment for clinical assessments and laboratory tests. Patient lymphocytes were isolated from peripheral blood by Ficoll-Hypaque (γ = 1.077) for flow cytometry analyses in T1D patients at baseline and different time points after SCE therapy. Isotype-matched IgG served as control. **(A)** Immune cell quantification in peripheral blood. **(B)** Percentage of CD4⁺ and CD8⁺ T cells in peripheral blood. **(C)** Flow Analysis of naïve CD4⁺ and CD8⁺ T cells in peripheral blood, demonstrating an increase in the percentage of naïve CD4⁺ T cells at 26 weeks post treatment. **(D)** Flow Analysis of CD4⁺ T_{CM} and CD8⁺ T_{CM} cells in peripheral blood, demonstrating an increase in the percentage of CD4⁺ T_{CM} cells at 18 weeks post treatment. **(E)** Flow Analysis of CD4⁺ T_{EM} and CD8⁺ T_{EM} cells in peripheral blood, demonstrating a decline in the percentage of CD4⁺ T_{EM} and CD8⁺ T_{EM} cells at 18 weeks and 26 weeks respectively post

treatment. **(F)** Flow Analysis of $CD4^+HLA\text{-}DR^+$ in peripheral blood, demonstrating a decline in their percentages at 26 weeks post treatment. **(G)** Flow Analysis of $CD8^+HLA\text{-}DR^+$ T cells in peripheral blood, demonstrating a decline in their percentages at 26 weeks post treatment. Data are shown as mean ± SD for all statistical analyses (A-E), pAIREd Student's t test (A-F).

Naive T cells constantly recirculate between secondary lymphoid tissue (SLTs) using the blood and lymph as conduits. Naive T cells become activated only after recognizing cognate antigens presented by antigen-presenting cells (APCs) within lymphoid organs. After the initial immune response, 90-95% of antigen-specific T cells undergo apoptosis; the remaining cells mainly give rise to long-term memory T cells, including central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) [40,41]. These memory T cells are meant to protect the human body from the re-exposure to pathogens by quickly and efficiently responding to the challenge. However, these memory T cells, specifically the $T_{EM,}$ may also play a critical role in the persistence of disease in autoimmune conditions [42]. To explore the effects of the SCE therapy on these memory T cells, T_{CM} and T_{EM} were examined by flow cytometry. Overall analysis in these subjects demonstrated that the percentage of $CD4^+$ T_{CM} ($CD45RA^-$ $CCR7^+$) cells was markedly and constantly increased after receiving SCE therapy at 18 weeks (P = 0.018) (**Figure 19D**). In contrast, the percentage of $CD8^+$ T_{CM} cells was only temporarily improved at 18 weeks (P = 0.034), but return to baseline levels during continued follow-ups (**Figure 19D**). In comparison with Group B subjects (4/9, 44%), Group A subjects (4/6, 67%) were more efficiently increasing the percentage of $CD4^+$ T_{CM} cells over 30% of positive cells at 18 weeks follow-up (data not shown). Notably, overall analysis of T_{EM} ($CD45RA^+$ $CCR7^-$) cells revealed that both $CD4^+$ T_{EM} cells and $CD8^+$ T_{EM} cells were considerably decreased at 18 weeks (P = 0.03) and 26 weeks (P = 0.0024) respectively (**Figure 19E**). Group A subjects (6/6, 100%) were more efficiently decreasing the percentage of $CD8^+$ T_{EM} cells over 15% of positive cells than that in Group B subjects (7/9, 78%) at 26 weeks follow-up; 5/6 (83%) of Group A subjects vs 7/9 (78%) of Group B subjects for the reduction of $CD4^+$ T_{EM} cells at 26 weeks follow-up (data not shown).

In addition, using HLA-DR as an activation marker for T cells [43], clinical data demonstrated that the percentage of $CD4^+HLA\text{-}DR^+$ T cells and $CD8^+HLA\text{-}DR^+$ T cells were markedly declined at 26 weeks follow-up relative to the baseline levels (P = 0.002 and P = 0.006 respectively) (**Figure 19F and G**).

6.4.2.3 Up-regulation of CCR7 expression on T cells after receiving SCE therapy in Caucasian T1D subjects [10]

C-C chemokine receptor 7 (CCR7) plays important roles in lymph-node homing of T cells via high endothelial venules and mediating the T-cell homeostasis [44,45]. To further explore the modulation of SCE therapy, we analyzed the level of CCR7 expression on naïve T, T_{CM}, and T_{EM} cells by flow cytometry. Clinical data revealed that both Group A and B subjects significantly increased the expression of CCR7 on Naïve $CD4^+$ T cells (**Figure 20A**), naïve $CD8^+$ T cells (**Figure 20B**), and $CD4^+$ T_{CM} cells (**Figure 20C**). The marked responses of Naïve $CD4^+$ T cells in Group A subjects happened as early as at 8 weeks post SCE therapy, comparable to that of delayed responses in Group B subjects at 26 weeks (**Figure 20A**). The up-regulation of CCR7 expression on naïve $CD8^+$ T cells was shown simultaneously at 8 weeks follow-up in both Group A and B subjects (**Figure 20B**). The expression of CCR7 on $CD8^+$ T_{CM} cells in Group A subjects was also improved and started at 18 weeks follow-up (**Figure 20D**), but with a postponed response in Group B subjects at 56 weeks follow-up (P = 0.046, **Figure 20D**). The levels of CCR7 expression on both $CD4^+$ T_{EM} and $CD8^+$ T_{EM} cells were markedly enhanced at 56 weeks follow-up after receiving SCE therapy in both groups (**Figure 20E**). The data suggest that the up-regulation of CCR7 expression on $CD4^+$ and $CD8^+$ T cells may lead to the re-distribution of T cells in T1D subjects after the treatment with SCE therapy.

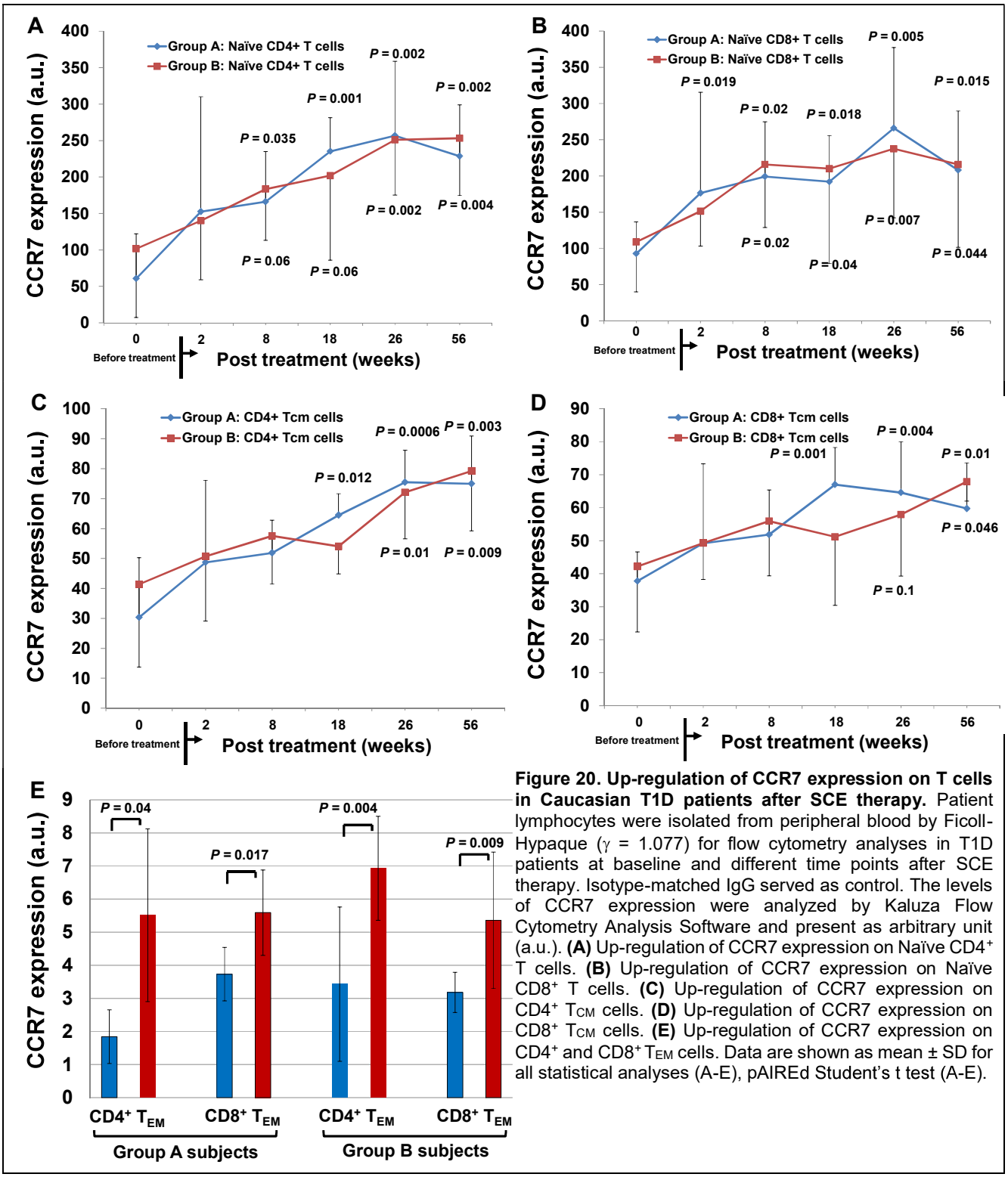


Figure 20. Up-regulation of CCR7 expression on T cells in Caucasian T1D patients after SCE therapy. Patient lymphocytes were isolated from peripheral blood by Ficoll-Hypaque (γ = 1.077) for flow cytometry analyses in T1D patients at baseline and different time points after SCE therapy. Isotype-matched IgG served as control. The levels of CCR7 expression were analyzed by Kaluza Flow Cytometry Analysis Software and present as arbitrary unit (a.u.). **(A)** Up-regulation of CCR7 expression on Naïve $CD4^+$ T cells. **(B)** Up-regulation of CCR7 expression on Naïve $CD8^+$ T cells. **(C)** Up-regulation of CCR7 expression on $CD4^+$ T_{CM} cells. **(D)** Up-regulation of CCR7 expression on $CD8^+$ T_{CM} cells. **(E)** Up-regulation of CCR7 expression on $CD4^+$ and $CD8^+$ T_{EM} cells. Data are shown as mean ± SD for all statistical analyses (A-E), pAIREd Student's t test (A-E).

6.4.2.4 Clinical efficacy of SCE therapy in the improvement of pancreatic islet β cell function of Caucasian subjects [10]

The deficit of pancreatic islet β cells is another key issue for the treatment of T1D subjects. Previous work demonstrated that treatment with SCE therapy provided lasting reversal of autoimmunity that allowed the regeneration of islet β cells and improved metabolic control in Chinese subjects with long-standing T1D [1,5]. To test the therapeutic potential of SCE therapy in the metabolic control of Caucasian T1D subjects, islet β-cell function was examined through the measurement of fasting plasma C-peptide and glucagon-stimulated C-peptide

levels. In Group A subjects, clinical results demonstrated up-regulation of both fasting and glucagon-stimulated C-peptide levels at 12 weeks in two recent-onset T1D subjects (i.e., those most likely to have residual β cell populations) (**Figure 21A and B**). Recovered fasting and glucagon-stimulated C-peptide levels were retained in subject 1 through the final follow-up at 56 weeks post-treatments (**Figure 21A**). Glucagon-stimulated C-peptide levels in subject 2 were stable during one-year follow-up, while fasting C-peptide levels declined slightly (**Figure 21B**). Subject 3 who had T1D 10 years at the time of study, still achieved modest improvements including an increase in fasting C-peptide from 0.25 ng/ml at basal to 0.36 ng/ml at 56 weeks and an increase in glucagon-stimulated C-peptide level from 0.4 ng/ml at basal to 0.52 ng/ml at 26 weeks (**Figure 21C**). Subject 4 who had T1D 3 years at the time of the study, retained normal β-cell function with no significant change over time in fasting C-peptide levels from 1.05 ng/ml at baseline to 0.88 ng/ml at 40 weeks and in glucagon-stimulated C-peptide levels from 2.18 ng/ml at baseline to 2.01 ng/ml at 40 weeks (**Figure 21D**).

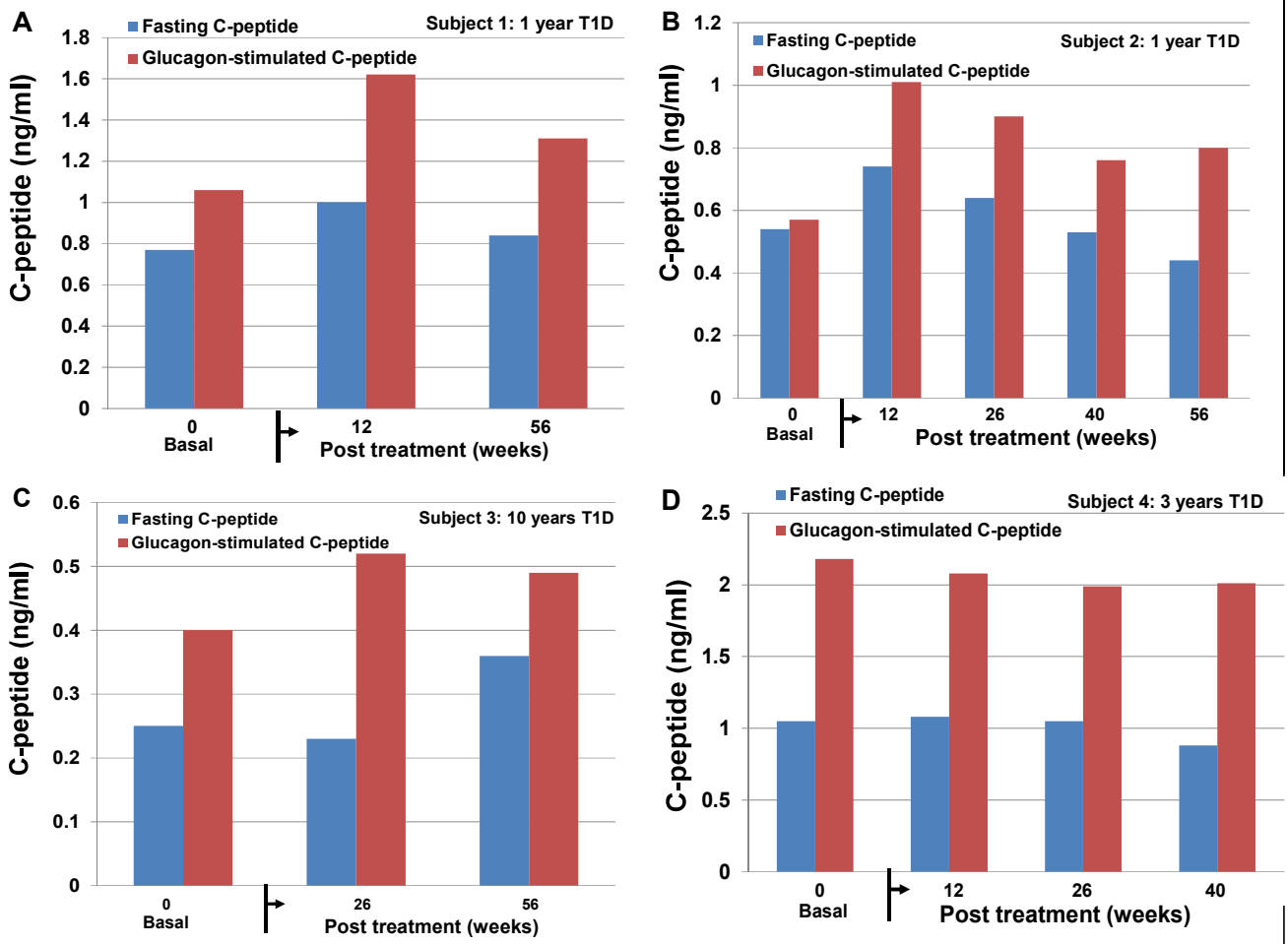


Figure 21. Effects of SCE therapy on β-cell function in Caucasian T1D subjects. All subjects received two treatments with SCE therapy. T1D subjects received two treatments with SCE therapy at the beginning and 3rd month respectively. Fasting (blue) and glucagon-stimulated C-peptide levels (brown) were examined at different time points according to the protocol. For glucagon-stimulated C-peptide production, glucagon (1mg, i.v.) was administrated within 30 seconds, and six minutes later, plasma samples were collected for the C-peptide test by Ultrasensitive C-peptide ELISA kit. These data were from six T1D subjects with some residual islet β-cell function (Group A). **(A-D)** Recovered fasting and glucagon-stimulated C-peptide levels were retained in subject 1 through the final follow-up at 56 weeks post-treatments in subject 1 -4 respectively.

Additionally, no changes were observed in fasting C-peptide levels of severe long-standing Group B patients with no residual pancreatic islet β cell function after receiving two SCE therapies. Their responses to SCE therapy were strikingly different from that reported in long-standing severe Chinese T1D subjects [1,5]. The potential mechanisms underlying this difference need to be explored.

6.4.3 SCE Therapy for the treatment of type 2 diabetes

The prevalence of type 2 diabetes (T2D) is increasing worldwide and creating a significant burden on health systems, highlighting the need for the development of innovative therapeutic approaches to overcome immune

dysfunction, which is likely a key factor in the development of insulin resistance in T2D. It suggests that immune modulation may be a useful tool in treating the disease. In an open-label, phase1/phase 2 study, patients (N = 36) with long-standing T2D were divided into three groups (Group A, oral medications, n = 18; Group B, oral medications + insulin injections, n = 11; Group C having impaired β-cell function with oral medications + insulin injections, n = 7). All patients received one treatment with the SCE therapy in which a patient's blood is circulated through a closed-loop system that separates mononuclear cells from the whole blood, briefly co-cultures them with adherent CB-SCs, and returns the educated autologous cells to the patient's circulation.

Clinical findings indicate that T2D patients achieve improved metabolic control and reduced inflammation markers after receiving SCE therapy. Median HbA_1C in Group A and B was significantly reduced from 8.61% ± 1.12 at baseline to 7.25% ± 0.58 at 12 weeks (p = 2.62E-06), and 7.33% ± 1.02 at one year post treatment (p = 0.0002). Homeostasis model assessment (HOMA) of insulin resistance (HOMA-IR) demonstrated that insulin sensitivity was improved post treatment. Notably, the islet beta-cell function in Group C subjects was markedly recovered, as demonstrated by the restoration of C-peptide levels. Mechanistic studies revealed that SCE therapy reverses immune dysfunctions through immune modulation on monocytes and balancing Th1/Th2/Th3 cytokine production. Clinical data from current phase 1/2 study demonstrate that SCE therapy is a safe approach that produces lasting improvement in metabolic control for individuals with moderate or severe T2D who receive a single treatment. In addition, this approach does not appear to have the safety and ethical concerns associated with conventional stem cell-based approaches.

6.4.3.1 Feasibility and safety of SCE therapy in T2D

Baseline characteristics of participants with T2D are provided in the **Table [2]**. Thirty six patients with T2D have received SCE therapy in a safety study, and their results are similar to the safety evaluation with T1D participants [1]. No participants experienced any significant adverse events during the course of treatment and post treatment for over a year. Patient complaints were limited to mild discomfort during venipunctures at the site of median cubital vein and some soreness of the arm that resolved quickly following aphaeresis.

6.4.3.2 Efficacy outcomes in improving metabolic control

After receiving SCE therapy and being discharged from the hospital, patients continued their regular medications. Follow-up studies demonstrated that the median glycated hemoglobin (HbA_1C) in Group A (n = 18) and Group B (n = 11) was significantly lowered from 8.61% ± 1.12 at baseline to 7.9% ± 1.22 at 4 weeks post treatment (p = 0.026), 7.25% ± 0.58 at 12 weeks post treatment (p = 2.62E-06) (**Figure 22A**), and 7.33% ± 1.02 at one-year post treatment (p = 0.0002). According to the A1C goal (<7%) recommended by the American Diabetes Association (ADA) for the treatment of adult diabetics, 28% (5/18) of subjects in Group A, 36% (4/11) of subjects in Group B, and 29% (2/7) of subjects in Group C achieved this goal at 12 weeks post treatment. More than 31% of total subjects achieved and maintained the <7% standard for over a year. Additionally, based on the efficacy criteria, 11 of 18 (61.1%) subjects in Group A, 8 of 11 (72.7%) subjects in Group B, and 4 of 7 (57.1%) subjects in Group C with reduction of A1C value (> 0.5%) at 4 weeks post treatment. Thirteen of 18 (72.2%) subjects in Group A, 9 of 11 (81.8%) subjects in Group B, and 6 of 7 (85.7%) subjects in Group C with reduction of A1C value (> 0.5%). Twenty eight of 36 (78%) of total subjects reduced by 1.28 ± 0.66 at 12 weeks post treatment. The data demonstrate that glycemic control was improved in T2D patients after SCE therapy.

To explore the change in insulin sensitivity, we analyzed homeostasis model assessment (HOMA) of insulin resistance (HOMA-IR) by the product of fasting plasma glucose and C-peptide (instead of insulin due to subjects receiving insulin injections) in Group A and B. The data revealed that levels of HOMA-IR c-pep were markedly reduced at 4 weeks follow-up (**Figure 22B**). It suggests that insulin sensitivity has been improved post treatment. Consistent with their improved β cell function, the median daily dose of metformin was reduced 33% ~ 67%, and insulin was reduced 35% at 12 weeks post treatment.

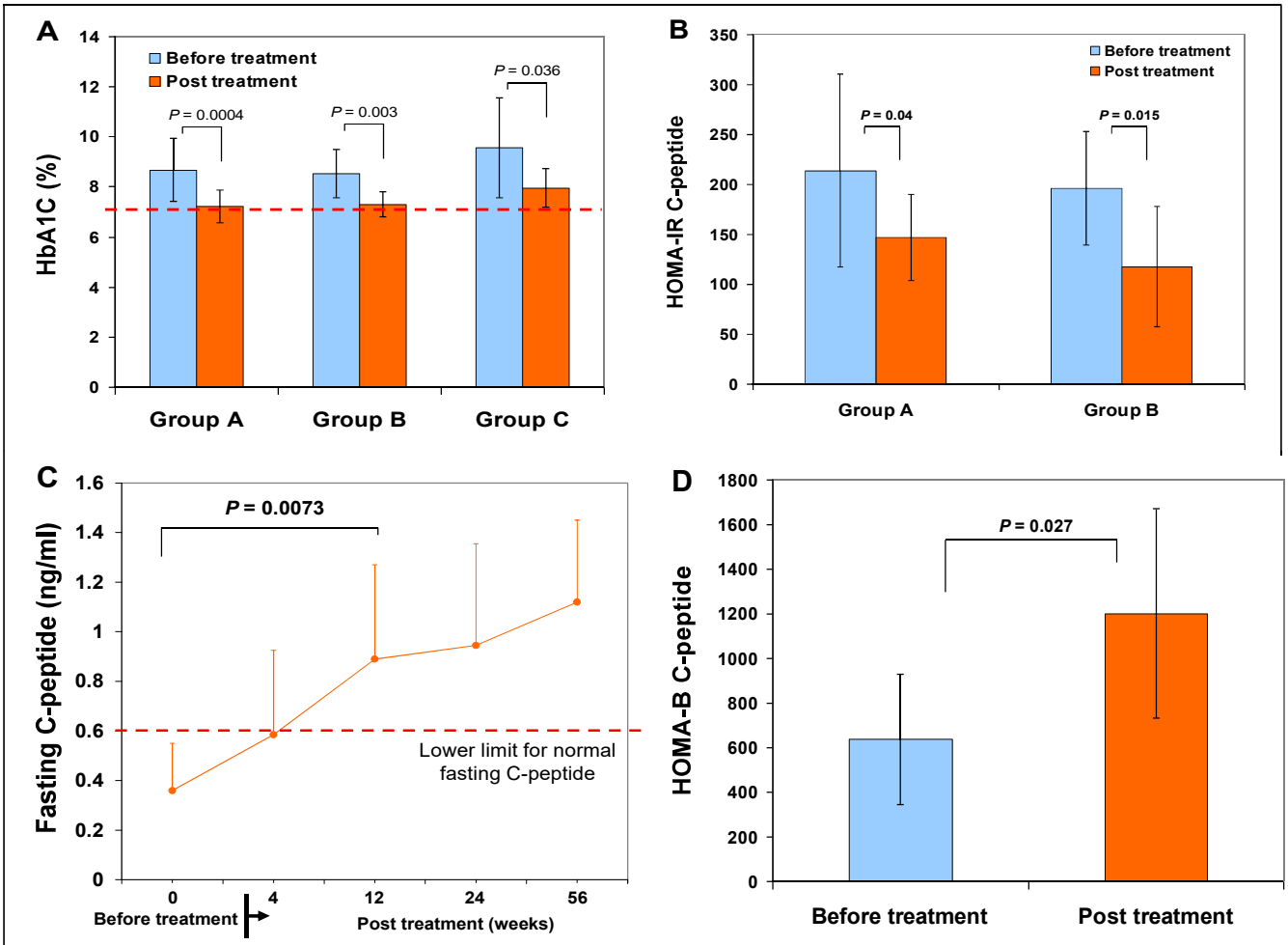


Figure 22. Improvement of metabolic control by SCE therapy. (**A**) 12-week follow-up HbA1C levels in T2D subjects. (**B**) Analysis of insulin sensitivity by HOMA-IR C-peptide at 4 weeks post treatment with SCE therapy. (**C**) 56-week follow-up C-peptide levels in Group C T2D subjects with impaired islet β cell function. (**D**) Analysis of islet β cell function by HOMA-B C-peptide at 12-week follow-up post treatment with SCE therapy.

Notably, we found that levels of fasting C-peptide were markedly increased in the long-standing T2D subjects with impaired islet β cell function (Group C, diabetic duration 14 ± 6 years, n = 7, $P = 0.0073$) (**Figure 22C**). Twelve weeks after receiving the SCE therapy, fasting C-peptide levels reached normal physiological levels and were maintained through the last follow-up for this measure (56 weeks) (0.36 ± 0.19 ng/ml at baseline vs 1.12 ± 0.33 ng/ml at one year post treatment, $p = 0.00045$, **Figure 22C**). The β-cell functional analysis by using HOMA-B C-peptide demonstrates that the function of islet β cells was markedly enhanced in group C subjects after receiving SCE therapy (**Figure 22D**). The data suggest that the restoration of C-peptide may be associated with the regeneration of islet β cells as we demonstrated in our previous work in T1D [1,6].

6.4.3.3 Efficacy outcomes in correcting the immune dysfunction

To determine the molecular and cellular mechanisms underlying the improvement of metabolic control, we examined the effects of anti-inflammation and immune modulation of SCE therapy in T2D. We used ELISA to examine proinflammatory cytokines IL-1, IL-6, and TNFα in the plasma, which are primarily involved in the insulin resistance and T2D [46,47]. We found that IL-1, IL-6, and TNFα were all at background levels in these long-standing T2D subjects and failed to show changes after SCE therapy ($p = 0.557$, $p = 0.316$, $p = 0.603$ respectively), probably because metabolic inflammation is a chronic sub-degree inflammation [47] and the serum samples which were directly collected from the blood of T2D patients, not from the lipopolysaccharide (LPS)-activated monocytes of T2D subjects [48]. Importantly, we found that anti-inflammatory and immune suppressive cytokine TGF-β1 was markedly increased in the plasma of T2D subjects post treatment at 4 weeks relative to the baseline levels (**Figure 23A**). However, IL-10 was unchanged in all participants ($p = 0.497$). These findings suggest up-regulation of TGF-β1 may be one of potential mechanisms contributing to the reversal of insulin resistance by SCE therapy.

Next, using a more sensitive intra-cellular flow cytometry analysis, we examined interleukin-17 (IL-17**,** also known as IL-17A) and Th1/Th2 immune response-associated cytokines in the peripheral blood of T2D subjects. IL-17A is a well-known proinflammatory cytokine involved in the autoimmune diseases. Importantly, mounting evidence collected over the past decade indicates that the etiology of T2D includes an autoimmune component that initiates an inflammation affecting pancreatic islet β cells [47,49-53], which provides new insight into the mechanism and potential treatment of insulin resistance through immune modulation. Recent clinical studies showed the increasing of circulating Th17 cells and IL-17 production in T2D patients [54] and obese patients [55]. Additionally, recent studies showed that the level of Th1-associated cytokine IL-12 is increased in T2D subjects [56,57]. We found that the production of IL-17, IL-12, and Th2-associated cytokine IL-4 and IL-5 were all markedly decreased after SCE therapy (**Figure 23B**).

To explore the cellular mechanism underlying the modulation on the Th1/Th2 immune responses, we focused on the changes of co-stimulating molecules CD80/CD86 expressed on the monocytes/macrophages, the professional antigen-presenting cells that play a key role in the onset of chronic inflammation and obese-associated insulin resistance of T2D [58-62]. Flow results demonstrated that the percentage of $CD86^+CD14^+$ monocytes was markedly decreased 4 weeks after treatment (**Figure 23C,** $P = 0.0212$). There was no significant change in the level of $CD80^+CD14^+$ monocytes ($P = 0.13$). The ratio of $CD86^+CD14^+$ monocytes / $CD80^+CD14^+$ monocytes was reduced from 3.86 ± 2.56 to 1.22 ± 0.48 ($P = 0.01$). Further flow analysis of the ligands of CD80/CD86, CD28/CTLA-4 expressed on lymphocytes revealed that the expression of CTLA-4 was markedly increased 4 weeks after receiving SCE therapy (0.51% ± 0.5 before treatment vs 1.98% ± 0.51 post treatment, P = 9.02E-05). However, flow analysis failed to show differences in the expression of co-stimulating molecule CD28 (69.98% ± 14.17 before treatment vs 61.5% ± 10.89 post treatment, $P = 0.225$). Additionally, we examined changes in the $CD4^+CD25^+Foxp3^+$ Tregs population after receiving SCE therapy. Flow analysis did not identify any differences between baseline and 4- or 12-weeks post treatment (**Figure 23D,** $P = 0.689$). Therefore, these data suggest that SCE therapy may modulate the Th1/Th2 immune responses through the action of antigen-presenting cells monocytes rather than Tregs.

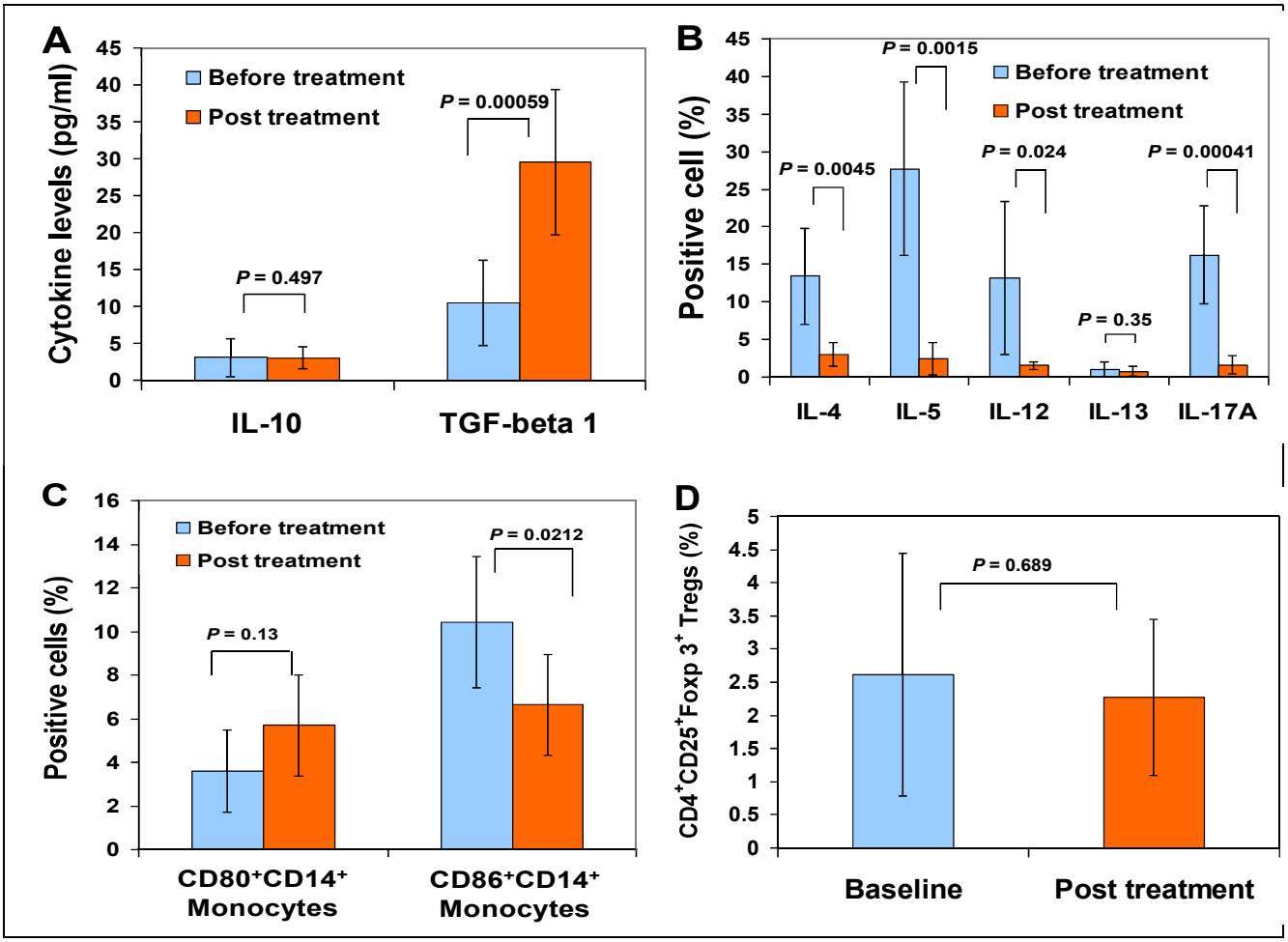


Figure 23. Anti-inflammatory effects of SCE therapy. (A) Up-regulation of plasma level of TGF-β1 in T2D patients at baseline and 4 weeks after SCE therapy. **(B)** Flow analysis of intra-cellular cytokines demonstrating differential effects on key interleukins at 4 weeks post treatment. **(C)** Down-regulation percentage of $CD86^+CD14^+$monocytes in T2D patients at baseline and 4 weeks after SCE therapy. **(D)** Flow Analysis of $CD4^+CD25^+Foxp3^+$ Tregs demonstrating no change in the percentage of Tregs at 4 weeks post treatment.

6.4.3.4 *In vitro* mechanistic studies of the immune modulation of CB-SCs on monocytes.

To better understand the immune modulation of CB-SC on monocytes, we performed *in vitro* co-culture experiments by using $CD14^+$ monocytes purified from human peripheral blood. The purified $CD14^+$ monocytes were co-cultured with CB-SCs at different ratios. We found that there were strong reactions after adding the $CD14^+$ monocytes to CB-SCs (**Figure 24A, bottom left panel**). Flow analysis demonstrated that co-culture with CB-SCs for 18 hrs resulted in the significant apoptosis of monocytes at the ratio 1 : 5 of CB-SC : monocytes (**Figure 24B**). Correspondingly, both the cell viability and attachment of CB-SCs were also affected in the presence of apoptotic monocytes (**Figure 24A, bottom left panel**). The cellular processes of CB-SCs were reduced in length, but most were still attached to the bottom (**Figure 24A, bottom left panel**). Interestingly, these impaired CB-SCs were restored after co-culture for 2-3 days; they continually expanded and became 90 ~ 100% confluence after 7-10 days (**Figure 24A, bottom right panel**). Mechanistic studies revealed that CB-SCs displayed the cellular inhibitor of apoptosis protein (cIAP) 1 [63] that protects CB-SCs against the cytotoxic effects of monocytes, allowing them to survive and proliferate (**Figure 24C**). To further explore the molecular mechanisms underlying the cytotoxic effects of monocytes on CB-SCs, we found that CB-SCs expressed tumor necrosis factor receptor II (TNF R II) but not TNF R I (**Figure 24D**). Recombinant TNF showed cytotoxicity to CB-SCs at different doses (**Figure 24E**). Notably, CB-SCs pre-treated with TNF RII mAb (20 μg/ml) at ratio of 1 : 10 could markedly block the toxic action of monocytes and protect 50% of CB-SCs with good cell viability and morphology.

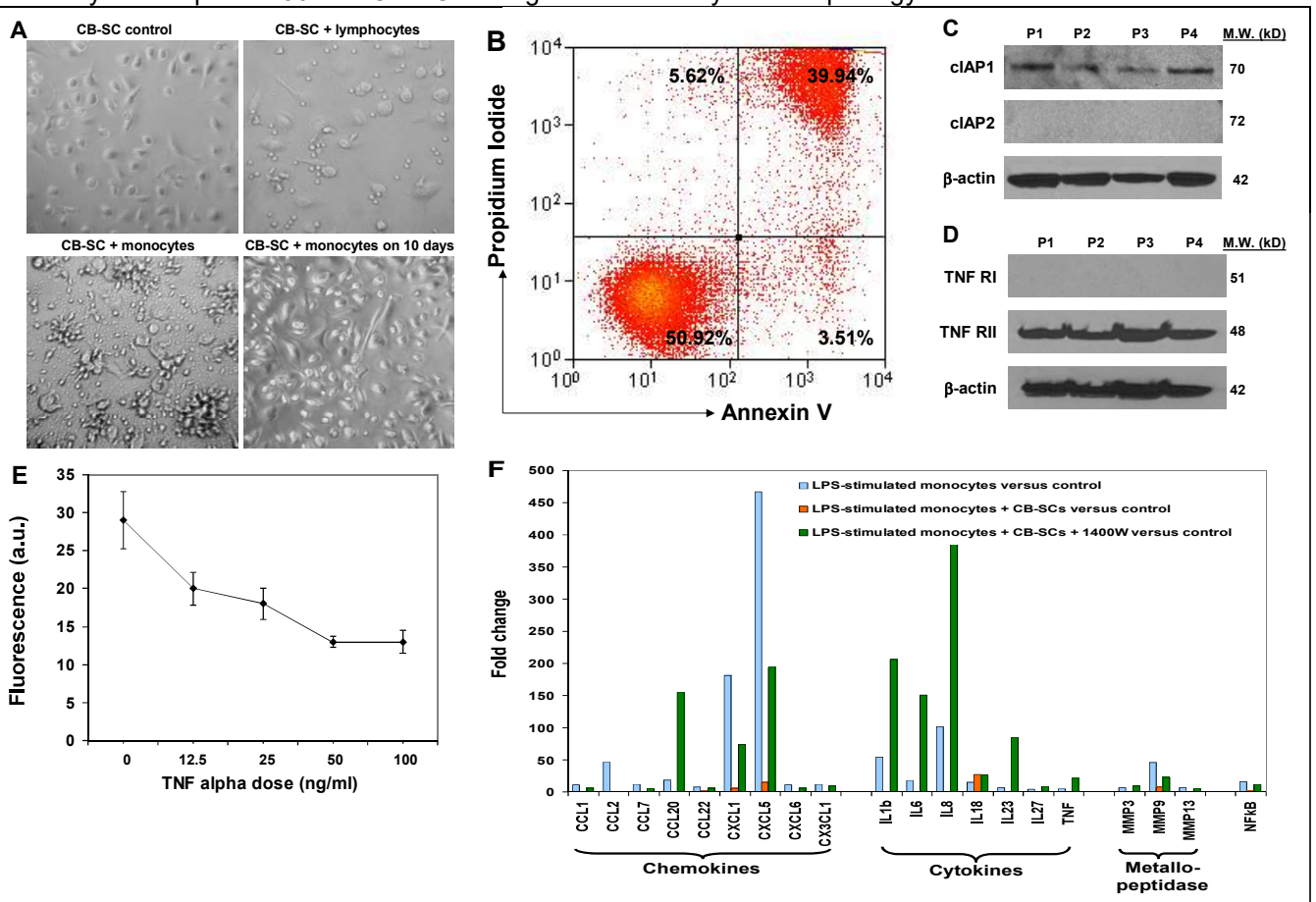


Figure 24. *In vitro* study of the immune modulation of CB-SCs on monocytes. (A) Phase contrast microscopy shows the co-culture of CB-SC with monocytes (bottom left panel) for 18 hrs. CB-SCs co-culture with lymphocytes (top right panel) served as control. The impaired CB-SCs after co-culture with monocytes were restored to expansion and became 90 ~ 100% confluence after 7-10 days (bottom right). Original magnification, × 100. **(B)** Apoptotic analysis of floating cells from the co-culture of CB-SCs with monocytes for 18 hrs. **(C)** Western blotting show the expression of the cellular inhibitor of apoptosis protein (cIAP) 1, not cIAP2, in four preparations of CB-SCs. **(D)** Western blotting show the expression of tumor necrosis factor receptor II (TNF R II), not TNF-RI, in four preparations of CB-SCs. **(E)** TNFα suppress the proliferation of CB-SCs in a dose-

response manner. Cell proliferation was evaluated using CyQUANTR Cell Proliferation Assay Kit (Millipore, OR) [25]. **(F)** Blocking experiment with iNOS inhibitor 1400W demonstrates that CB-SC-derived nitric oxide (NO) contributes to the immune modulation of CB-SCs on monocytes. Monocytes were initially stimulated with lipopolysaccharide (LPS, 10 μg/ml) for 8 hrs, and then co-cultured with CB-SCs at ratio 1 : 5 of CB-SCs : monocytes for 48 hrs in the presence or absence of 1400W (100nM), followed by real time PCR analysis by using Human Th17 for Autoimmunity & Inflammation PCR Array kit (SABiosciences, Valencia, CA).

To further explore the immune modulation of CB-SCs on monocytes, lipopolyssacharide (LPS)-stimulated purified $CD14^+$ monocytes were co-cultured with CB-SCs. Real time PCR array showed that co-culture with CB-SC could significantly down-regulate numbers of LPS-stimulated, inflammation-related genes including chemokines, multiple cytokines, and matrix metallopeptidase, along with signaling pathway molecule NF-κB (**Figure 24F**). These data clearly indicate that *in vitro* co-culture with CB-SCs causes substantial down-regulation of inflammation-associated gene expressions in monocytes. Previous work showed that CB-SCs function as immune modulators on lymphocytes via nitric oxide (NO) production [11]. To confirm the action of NO involved in the immune modulation of CB-SCs on monocytes, the specific inducible nitric oxide synthase (iNOS) inhibitor 1400W was applied to the co-culture system. The data demonstrated that the inhibitory effects of CB-SC on LPS-stimulated monocytes could be significantly reversed in the presence of iNOS inhibitor 1400W (**Figure 24F**). Interestingly, we found that blocking NO production in CB-SCs could markedly increase the expressions of chemokine CCL20 and cytokines (e.g., IL-1β, IL-6, IL-8, IL-23, and TNFα) in monocytes. Thus, it indicates that CB-SC-derived NO plays an essential role in the immune modulating and anti-inflammatory effects of CB-SCs on monocytes.

6.4.4 SCE Therapy for the treatment of Alopecia Areata

Alopecia areata (AA) is one of the most common autoimmune diseases and targets the hair follicles, with high impact on the quality of life and self-esteem of patients due to hair loss. Clinical management and outcomes are challenged by current limited immunosuppressive and immunomodulating regimens. In an open-label, phase 1/phase 2 study, patients (N = 9) with severe AA received one treatment with the SCE therapy. The median age was 20 years (median alopecic duration, 5 years). Clinical data demonstrated that patients with severe AA achieved improved hair regrowth and quality of life after receiving SCE therapy. Flow cytometry revealed the up-regulation of Th2 cytokines and restoration of balancing Th1/Th2/Th3 cytokine production in the peripheral blood of AA subjects. Immunohistochemistry indicated the formation of a "ring of transforming growth factor beta 1 (TGF-β1)" around the hair follicles, leading to the restoration of immune privilege of hair follicles and the protection of newly generated hair follicles against autoimmune destruction. Mechanistic studies revealed that co-culture with CB-SC may up-regulate the expression of coinhibitory molecules B and T lymphocyte attenuator (BTLA) and programmed death-1 receptor (PD-1) on $CD8\beta^+NKG2D^+$ effector T cells and suppress their proliferation via herpesvirus entry mediator (HVEM) ligands and programmed death-1 ligand (PD-L1) on CB-SCs. Current clinical data demonstrated the safety and efficacy of the SCE therapy for the treatment of AA. This innovative approach produced lasting improvement in hair regrowth in subjects with moderate or severe AA.

6.4.4.1 **Suppressed Proliferation of Antigen-Specific T cells by co-culture with CB-SC**

The expansion of antigen-specific autoreactive T cells is the critical step leading to the destruction of tissues in autoimmune diseases. Recently, mouse and human data have demonstrated that $CD8^+NKG2D^+$ effector T cells function as a key mediator in the pathogenesis of AA [27]. To explore the therapeutic potential of CB-SC in AA, $CD8^+NKG2D^+$ effector T cells from human peripheral blood mononuclear cells (PBMC) were activated and expanded with Dynabeads coupled with anti-CD3, anti-CD28, and anti-CD137 mAb in the presence of IL-2 and IL-7. After *ex vivo* expansion with this mAb combination for 5 days, there were large numbers of cell clusters with different sizes floating in the supernatant (**Figure 25A**, left panel), suggestive of significant cell proliferation. However, this phenomenon was not evident in the presence of CB-SCs (Figure 2A, right panel). Flow cytometry revealed that 52% of lymphocytes proliferated in response to costimulation with this combination of mAb molecules and growth factors (**Figure 25B**, middle panel). By contrast, there were only 13% of lymphocytes proliferating after co-culture with CB-SCs (**Figure 25B**, right panel). Triple color staining demonstrated that 25% of $CD8^+NKG2D^+$ T cells were proliferated upon costimulation in the absence of CB-SCs. Notably, the percentage of proliferating $CD8^+NKG2D^+$ T cells was reduced to 5% following co-culture with CB-SCs. Further multi-color flow cytometry indicated that the percentage of $CD8^+NKG2D^+$ T cells was decreased from 25.6% ± 0.43% to 13.87% ± 3.43% in the presence of CB-SCs (Figure 2C, left panels) ($P = 0.04$). Additionally, we examined the expression of coinhibitory molecules on $CD8^+NKG2D^+$ T cells, such as BTLA (B and T lymphocyte attenuator) and PD-1 (programmed death-1 receptor). Results confirmed that co-culture with CB-SCs increased the percentage of $CD8^+NKG2D^+BTLA^+PD\text{-}1^+$ T cells from 69% to 91%. Their mean fluorescence intensity (MFI) also increased after co-culture with CB-SCs (**Figure 25C**, right panels). These data demonstrated that CB-SCs could markedly

suppress the proliferation of $CD8^+NKG2D^+$ T cells and up-regulate the expression of coinhibitory molecules on those cells. This finding supports the clinical-translational potential of CB-SCs in AA subjects.

6.4.4.2 Hair Regrowth in Alopecia Areata Subjects

Nine AA subjects (the median age was 20 years; median alopecic duration was 5 years) received one treatment with SCE therapy and completed the study. All patients tolerated the procedure well, without any significant adverse events during the course of treatment. Their baseline clinical characteristics are described in Table 1. No participants experienced any significant adverse events during the course of treatment and the 2-year follow-up period. At 4 weeks post-treatment with SCE therapy, there was hair regrowth in subjects with patchy AA and Alopecia totalis (**Figure 22**). There were short vellus hairs found over the scalp of AA universalis patients (3/4) at the 12-week follow-up. Two participants (one having Alopecia totalis and another having multiple patches of AA) achieved complete hair regrowth at 12 weeks and 16 weeks, respectively, post-treatment, and remained completely recovered with no relapse after 2 years (**Figure 22**). Patients (3/4) with Alopecia universalis exhibited regrowth of eyebrows and eyelashes at the 12-week follow-up. Notably, the regrowth of eyebrows and a mustache were found in a 17-year-old boy affected by severe Alopecia universalis since he was 1-year-old. Additionally, one of nine subjects with nail pitting was also improved, as indicated by the reduction of the number and the cavity of nail pitting at 4 weeks after receiving SCE therapy. All of these improvements were maintained throughout the final follow-up at 2 years. Of nine AA subjects, only one participant with Alopecia universalis failed to show a response to the SCE therapy, possibly due to the previous long-term therapy with oral prednisone. Overall, the proof-of-concept data demonstrated the therapeutic potential of SCE therapy for the treatment of AA subjects.

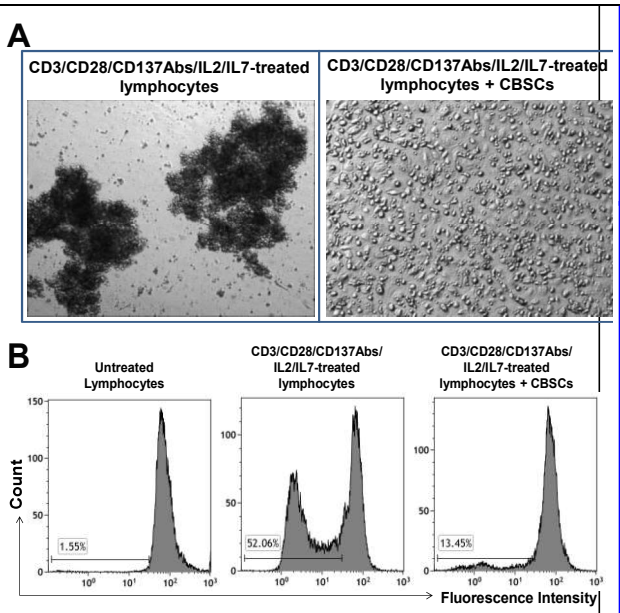


Figure 25. Ex vivo studies of the immune modulation of CB-SCs on T cells. (A) Phase contrast microscopy shows the formation of cell clusters in human peripheral blood-derived lymphocytes that were activated with Dynabeads coupled with anti-CD3, anti-CD28 and anti-CD137 antibodies, 50 U/ml rIL-2 and 5 ng/ml rIL-7 for 5 days, in absence (left panel) and presence (right panel) of CB-SCs. co-culture with lymphocytes (top right panel) served as control. Original magnification, × 100. **(B)** Cell proliferation was analyzed with CellTrace™ CFSE Cell Proliferation kit. Untreated lymphocytes (left panel) served as control. Flow cytometry dot plots and the percentage of each population were representative of three independent experiments with similar results.

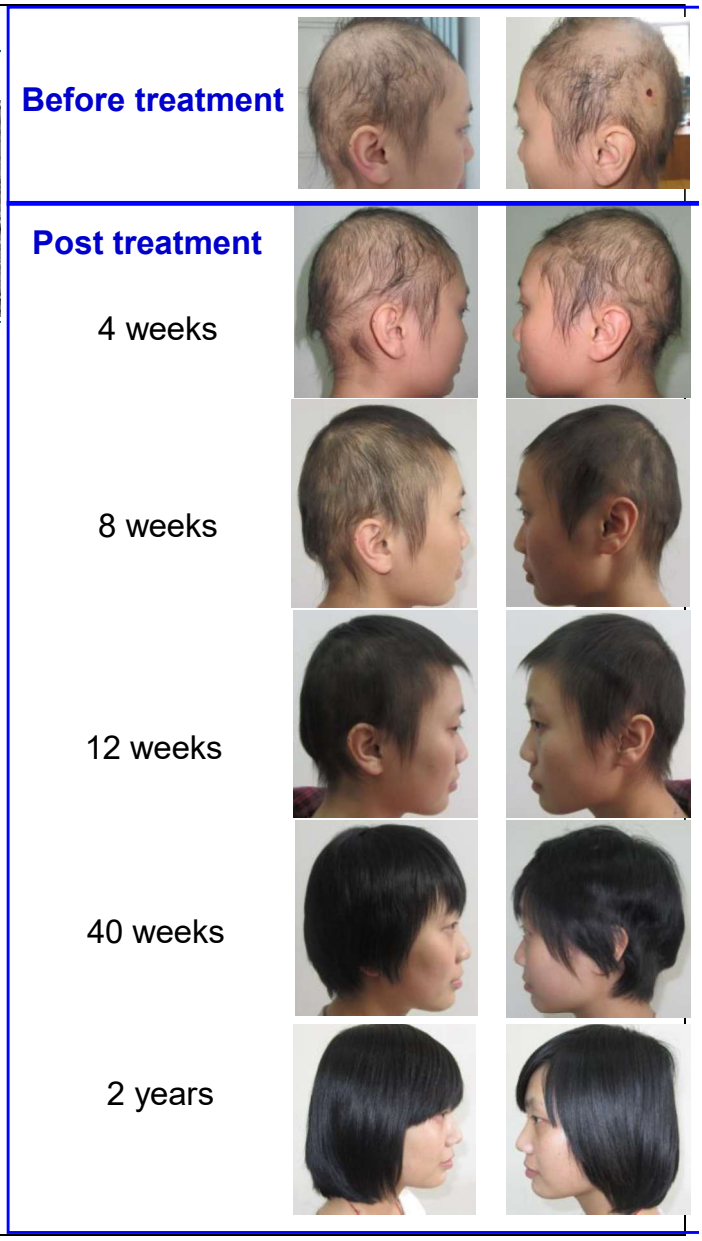


Figure 22. Regrowth of hair following SCE therapy. A patient with alopecia totalis received SCE therapy and followed up for two years, without relapse.

6.4.4.3 Systematic immune modulation after receiving SCE therapy To explore the immune modulation of SCE therapy in AA subjects, we examined changes in the percentage of regulatory T cells (Treg) in their peripheral blood by using the specific intracellular marker, FoxP3. Flow cytometry revealed that the percentage of $FoxP3^+$ Treg at 4 weeks was unchanged from the baseline (1.69% ± 1.02 versus 1.38% ± 0.85, $P = 0.49$). This result suggested that the immune modulation via SCE therapy in AA subjects may act through a different mechanism than induction of Treg. TGF-β1, one of the best-characterized cytokines contributing to the induction of peripheral immune tolerance [29], also plays a crucial role in modulating the normal cycling of hair follicles [30]. Flow cytometry demonstrated a marked increase in TGF-β1 expression by blood mononuclear cells 4 weeks after receiving SCE therapy ($P = 0.015$, **Figure 23A**). Additionally, participants exhibited significant up-regulation of Th2 cytokines IL-4 and IL-5 expression at the 4-week follow-up ($P = 0.012$ and $P = 0.022$, respectively, Figure 5A). Expression of IL-13 was also significantly increased in 6/9 participants (3.69 ± 3.27 versus 15.55 ± 7.48, $P = 0.005$). No changes were observed in the level of Th1 cytokine IL-12 ($P = 0.24$, Figure 5A). Thus, these data suggested that the up-regulation of Th2 cell responses may suppress the Th1 cell-mediated autoimmune

response in AA subjects [1,4,31] via associated cytokines [32]. The SCE therapy may shift the balance towards Th2-mediated immune responses, leading to the clinical efficacy in AA subjects.

The costimulatory molecule, CD28, functions as a key signal leading to Th2 cell differentiation and activation [33-36]. To determine changes in expression of costimulatory molecules, we examined lymphocytes for their levels of CD28 and inducible costimulator (ICOS). Flow cytometry demonstrated that the expression of CD28 was markedly increased in 8/9 participants 4 weeks after SCE therapy (***P*** = 0.013, **Figure 23B**), but levels of ICOS were unchanged in all participants (***P*** = 0.84, **Figure 23B**). Therefore, the up-regulation of CD28 expression, together with the increase of IL-4 production in AA subjects, can provide critical signals that shift the differentiation of human $CD4^+$ T cells into Th2 cells [36] and attenuate Th1 cell responses.

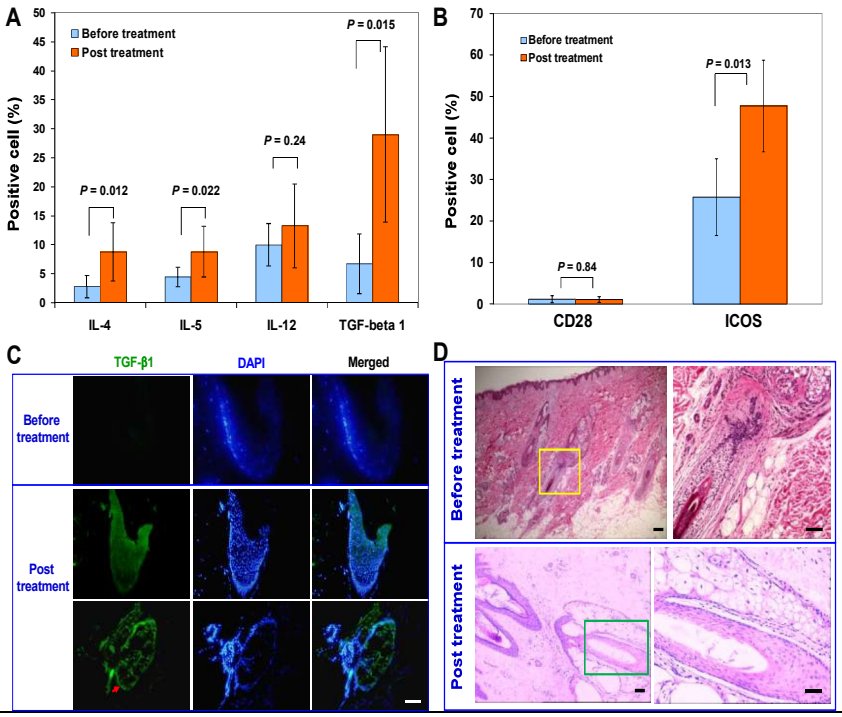


Figure 23. Immune modulation of SCE therapy. Patient lymphocytes were isolated from peripheral blood by Ficoll-Hypaque technique (γ = 1.077) for flow cytometric analyses in AA patients at baseline and 4 weeks after SCE therapy. Isotype-matched IgG served as control. **Histologic examination of alopecic skin (C and D). (A)** Flow analysis of intracellular cytokines demonstrating differential effects on key interleukins at 4 weeks post-treatment. **(B)** Flow analysis of costimulating molecules demonstrating an increase of CD28 expression at 4 weeks post-treatment. Data are representative of preparations from all AA participants (n = 9) who received SCE therapy. **(C)** Fresh skin tissues were collected from the scalps via biopsy for immunohistochemistry testing in participants with alopecia totalis before treatment and 12 weeks after receiving SCE therapy. TGF-β1 staining surrounds a hair follicle of AA participants after receiving SCE therapy, with vertical section of hair follicle (top panels) and horizontal section of hair follicle. Isotype-matched mouse IgG_1 served as a negative control. Representative images were obtained from five experiments. Scale bar, 25 μm. **(D)** H&E staining of scalp tissues. Scale bar, 25 μm.

6.4.4.4 Formation of a "ring of TGF-β1" leading to local immune modulation and restoration of immune privilege of hair follicles after SCE therapy To clarify the molecular and cellular mechanism underlying the regrowth of hairs and the immune modulation, we performed immunohistochemistry on fresh tissues via the biopsy of alopecic lesions from subjects after receiving SCE therapy. The histology of the alopecic lesions demonstrated a dense, perifollicular lymphocytic infiltration around anagen hair follicles. Twelve weeks after receiving SCE therapy, histological examination revealed the restoration of hair follicle architecture and the disappearance of the substantial perifollicular infiltration of lymphocytes. Additionally, horizontal sections confirmed an increase in the density of total hair follicles in these participants with alopecia totalis.

TGF-β1 is a pleiotropic growth factor that plays a key role not only in the induction of local immune tolerance [29], but also in the regulation of cycling of hair follicles via the inhibition of keratinocyte proliferation and the induction of apoptosis [30,37,38]. Previous works demonstrated that TGF-β1 contributed to the therapeutic efficacy of SCE therapy in both autoimmune-caused diabetic NOD mice [23] and diabetic patients [24,25]. Notably, in addition to the up-regulation of TGF-β1 expression in the peripheral blood mononuclear cells (**Figure 15A**), immunohistochemistry demonstrated that the level of TGF-β1 expression post-treatment (21.78 ± 0.27) was much higher than that of baseline before treatment (5.14 ± 0.01, *P* = 3.14337E-14), specifically at the proximal anagen hair follicle (**Figure 15C**, middle panel). The expression of TGF-β1 formed a cycle around the hair follicles (**Figure 15C**, bottom panel), similar to the "ring of TGF-β1" that we observed in a previous study [23]. It suggested that this unique feature of TGF-β1 distribution may contribute to reconstitution of the immune privilege of hair follicles and protect the regenerated hair follicles against further destruction by autoimmune cells. HE staining further demonstrated the reduction of immune cell infiltration and restoration of normal architecture of the hair follicles (**Figure 15D**).

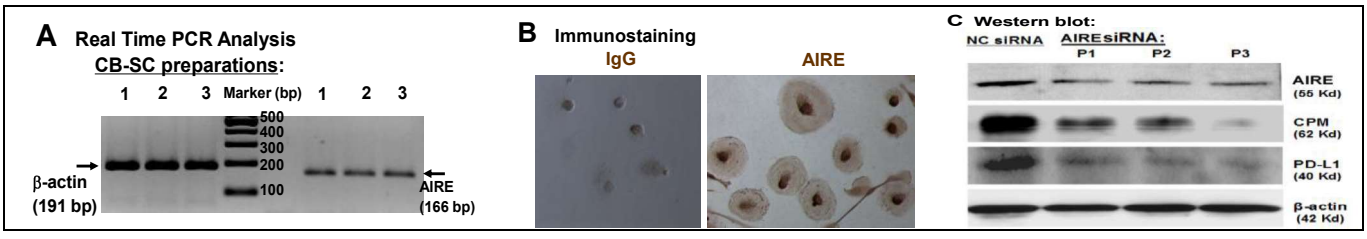
To elucidate the major origin of the TGF-β1 production involved in the formation of the "ring of TGF-β1," we performed double immunostaining in biopsied tissues by using the markers of monocyte/macrophage (CD14 and CD11b), Langerhans/dendritic cells (CD83 and DEC205), myeloid dendritic cells (CD1c), and Treg FoxP3, respectively. There were a few $CD14^+$ monocytes/$CD11b^+$ macrophages, Langerhans/dendritic cells, and $FoxP3^+$

Treg cells distributed in the dermal tissues. Double immunostaining showed weak staining for TGF-β1 in these immune cells, indicating that these immune cells were not the major source of TGF-β1. Notably, we found that there was a strong expression of TGF-β1 in the proximal root sheath of hair follicles of participants after receiving SCE therapy (**Figure 15C**, bottom panels) relative to the baseline level (**Figure 15C**, top panel). These data suggested that keratinocytes were recovered after receiving SCE therapy and became the major TGF-β1-producing cells leading to the restoration of immune privilege and the induction of immune tolerance in hair follicles. The molecular mechanisms underlying the up-regulation of TGF-β1 in keratinocytes need to be clarified in current studies.

6.4.5 Molecular Mechanisms underlying the SCE Therapy

In comparison with conventional immune therapies (e.g., monoclonal antibodies, vaccines, Treg therapy, and dendritic cell therapy) for T1D, the ex vivo immune modulation by CB-SCs inside a device can be controlled and monitored during the treatment. The lymphocytes (including Naïve T cells, TCM, and TEM) purified by apheresis can be intensively educated by directly contact with CB-SCs, with minimum interference from red blood cells, granulocytes, and other blood components. This approach also reduces side effects associated with conventional immune therapies. Based on our combined preclinical [1,5,6] and clinical studies [8,12] to date, immune modulation by CB-SCs seems to be mediated by a variety of molecular and cellular mechanisms including: 1) Expression of autoimmune regulator (AIRE) in CB-SCs plays an essential role (**Figure 24A-C**) [8]; 2) Functioning via cell-cell contact mechanisms involving the surface molecule programmed death ligand 1 (PD-L1) [5] and CD270 on CB-SCs, and their ligands PD-1 and BTLA on variety of immune cells (e.g., T cells, B cells, monocytes, dendritic cells, and granulocytes (**Figure 24D and E**) [10]; 3) Acting through soluble factors released by CB-SCs (e.g., nitric oxide, TGF-β1) (**Figure 24F**) [5]; and 4) Adjusting the cell-cell interaction between antigen-presenting cells monocytes/macrophages and T cells through co-stimulating molecules and their ligands [12]. Thus, during the ex-vivo brief exposure to CB-SCs, T1D-derived TCM and TEM can be "educated" by the favorable microenvironment created by CB-SCs through cell to cell contact and soluble factors. Additionally, previous work demonstrated that the proliferation of the T cell clone stimulated with antigen-presenting cells (APC) and different doses of GAD peptide were markedly and specifically decreased in the presence of CB-SCs compared to control group in the absence of CB-SCs [1]. Thus, it indicates that CB-SCs have a potential to eliminate the pathogenic T cells.

Human CB-SCs mediate immune modulation through the release of soluble factors (for example, nitric oxide and TGF-β1) and the expression of surface molecules such as PD-L1 (programmed death-1 ligand) [19,20,28]. To investigate additional potential molecular mechanisms underlying the immune modulation, we also found that CB-SCs strongly displayed the surface molecule HVEM (**Figure 24D**), the ligand of BTLA. Triple color staining confirmed the co-expression of HVEM on CB-SCs positive with molecular markers of leukocyte common antigen CD45+ and embryonic transcription factor OCT3/4+ (**Figure 24D**).To further substantiate that HVEM may be a mechanism of immune modulation by CB-SCs, flow cytometry also demonstrated the expression of BTLA and PD-1 on most immune cells, including CD4+ and CD8β+ T cells, CD19+ B cells, CD14+ monocytes, CD11c+ myeloid dendritic cells (mDCs), CD123+ plasmacytoid dendritic cells (pDCs), and CD66b+ granulocytes (**Figure 24E**). There were about 40-80% BTLA+PD-1+ cells in each subpopulation of immune cells [10]. These data suggest that CB-SCs may display a broad spectrum of modulatory capacity on immune cells via HVEM/BTLA and PD-L1/PD-1 signaling pathways.



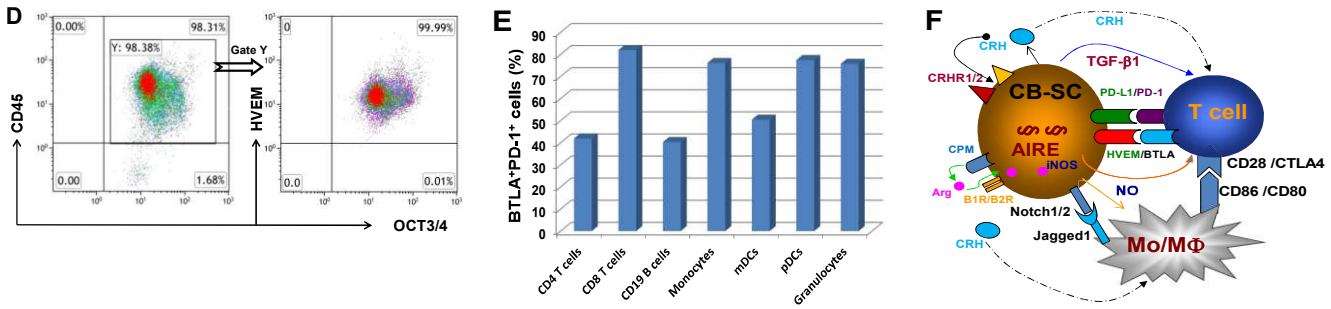


Figure 24. Characterization of AIRE and molecular mechanisms in CB-SC. (**A**) Expression of AIRE mRNA in CB-SCs. Real time PCR analysis for AIRE mRNA expression followed by electrophoresis in 2% agarose gel. Data are representative of three CB-SC preparations. **(B)** Immunocytochemistry for AIRE. Isotype- matched IgG served as control (left) for AIRE staining (right) with magnification ×200. **(C)** Western blot showed the dose-dependent knockdown response of AIRE following AIRE siRNA treatment, and decreased expression of PD-L1 and carboxypeptidase M (CPM) in CB-SCs. **(D)** Expression of HVEM on the gated $CD45^{+}OCT3/4^{+}$ CB-SCs. CD45, a leukocyte common antigen; OCT3/4, a transcription factor marker of embryonic stem (ES) stem cells. **(E)** Expression of BTLA and PD-1 on peripheral blood-derived immune cells. The percentage of $BTLA^{+}$ $PD\text{-}1^{+}$ cells in each subpopulation that was gated using the specific surface markers. **(F)** Outline of the molecular mechanisms for the immune modulation by CB-SC. AIRE, autoimmune regulator; Arg：arginine; B1R, kinin B1 receptor; B2R, kinin B2 receptor; BTLA, B and T lymphocyte attenuator; CRH, corticotropin-releasing hormone; CRHR, CRH receptor; CTLA4, cytotoxic T lymphocyte-associated molecule-4; iNOS, inducible nitric oxide synthase; NO, nitric oxide; PD-L1, programmed death ligand 1; PD-1, programmed death 1; TGF-β1, transforming growth factor.

6.5 Dose Rationale and Risk/Benefits

To date, a true cure for T1D has proven elusive despite intensive research pressure using conventional approaches over the past 30 years [64]. The situation highlights the challenges that we face in conquering this disease and the need for novel alternative approaches for the treatment of T1D. Our previous work demonstrated that CB-SCs are a unique type of stem cell identified from human cord blood [8,9], distinct from other stem cell types including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs) [25]. We characterized these stem cells and harnessed some of their unique properties with SCE therapy by using CB-SCs in 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 (**Figure 15**) [1,2,5,6,8,9,11,24,65]. This treatment leads to global immune modulations and immune balance as demonstrated by clinical data and animal studies [1,3,6,9,11]. The SCE therapy may revolutionize the clinical treatment of diabetes and other immune-related diseases through CB-SCs' immune education and induction of immune balance, without the safety and ethical concerns associated with conventional stem cell-based approaches.

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. 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 [13]. Due to the polyclonal nature of T1D-related autoimmune responses 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 Ab for T cells, anti-CD19 Ab for B cells, and GAD 65 vaccination have failed [15,16,66]. Successful therapies will likely restore immune balance and peripheral tolerance by addressing changes in multiple targets within the immune system. Specifically, they point to the need for an approach that produces comprehensive immune modulation at both the local pancreatic and systematic levels rather than targeting the pancreatic effects of one or a few components of the immune system. Our SCE therapy takes this broader approach.

In the initial use of this therapy, a novel device (the SCE) functioned as part of a closed-loop system that circulated a patient's blood through a blood cell separator, briefly co-cultured the patient's lymphocytes with adherent CB-SCs *in vitro*, and returned the educated lymphocytes (but not the CB-SCs) to the patient's circulation. It takes 8 -9 hours for the whole process. CB-SCs attached to interior surfaces in the device present secreted and cell-surface signaling molecules to passing lymphocytes, and only the autologous lymphocytes are returned to the subjects. Findings from ongoing clinical trials provide powerful evidence that a single treatment with the SCE provides lasting reversal of autoimmunity that allows regeneration of islet β cells and improvement of metabolic

control in individuals with long-standing T1D [1,5]. Findings from these trials indicated 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 will likely address multiple sources of the autoimmune response.

Additionally, the clinical data from the Spanish trial (NCT01350219) demonstrated that SCE therapy was well tolerated in all Caucasian subjects. The percentage of naïve $CD4^+$ T cells was significantly increased at 26 weeks and maintained through the final follow-up at 56 weeks. The percentage of $CD4^+$ central memory T cells (T_{CM}) was markedly and constantly increased at 18 weeks. Both $CD4^+$ effector memory T cells (T_{EM}) and $CD8^+$ T_{EM} cells were considerably decreased at 18 weeks and 26 weeks respectively. Additional clinical data demonstrated the up-regulation of C-C chemokine receptor 7 (CCR7) expression on naïve T, T_{CM}, and T_{EM} cells. Following two treatments with SCE therapy, islet β-cell function was improved and maintained in individuals with residual β-cell function. Thus, SCE therapy provided lasting reversal of autoimmune memory that could improve islet β-cell function in Caucasian subjects. These findings provide a much-needed boost for T1D therapeutic research. If further shown to be safe and effective in subjects, immune modulation by CB-SCs has the potential to address T1D and other autoimmune diseases [9].

7 Study Objectives

7.1 Primary Objective

To assess the safety of SCE therapy in patients with T1D.

7.2 Secondary Objectives

7.2.1 To assess the feasibility of SCE therapy in patients with T1D

7.2.2 To evaluate preliminary efficacy of SCE therapy for improving islet β cell function in patients with T1D through 12 months.

7.2.3 To evaluate markers of immune function in T1D patients after SCE therapy.

8 Study Design

8.1 General Design

This is a prospective, single arm, open-label, single-center pilot study to assess the safety, feasibility, and efficacy of SCE therapy for the treatment of patients with T1D. Up to 10 patients meeting eligibility criteria will be enrolled. Patients with T1D will be evaluated by the study principal investigator or co-investigators. Informed consent will be obtained at the initial screening visit (Visit 1). The initial screening visit will occur within 30 days of initiation of SCE therapy (**Table 3**). The second screening visit (Visit 2) will occur within 7 days of therapy. Subjects who meet all criteria will be scheduled for treatment. All enrolled subjects will receive treatment with the SCE system consisting of a single session of MNC collection by apheresis where 10 L (expected collection of ~10^{10} mononuclear cells) of blood will be processed on day -1. The MNC product will then be exposed over a 17-hour period of time to the SCE and on day 0 the product will be infused intravenously back to the patient. The infusion will take place in the Outpatient Transplant Facility at John Theurer Cancer Center (JTCC) or Inpatient at Hackensack University Medical Center (HUMC) on the stem cell transplantation or cellular therapy unit. The infusion will be performed according to the standard operating procedure, titled "Name of Procedure: Infusion of Non-Frozen Hematopoietic Stem Cell (HSC) Products and Cytotoxic T Lymphocytes". After finishing the infusion, the patients will be hospitalized at the main hospital of Hackensack University Medical Center for a 24-hours post-infusion observation. If the patients show the stable blood glucose, blood pressure, temperature, and normal vital signs, they can be discharged from the hospital after 24 hours. Subjects will be treated sequentially, with a minimum of one week between subjects for the first three subjects. . All treated subjects will attend a safety visit on Day +7 ± 1 day. Follow-up visits will occur on 1st, 3rd, 6th, 9th, 12th, and 24th month (± 5 days) post-therapy. Subjects will be instructed to record daily insulin doses, sugar levels, and physical activities. After receiving the SCE therapy, patients' daily insulin doses will be monitored and adjusted their own endocrinologist.

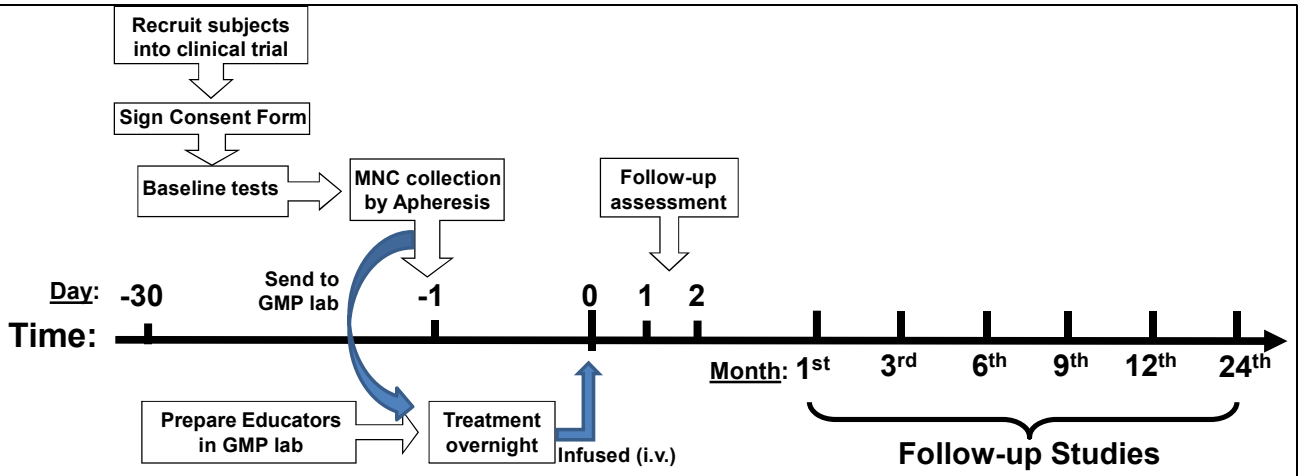


Figure 25. Outline the SCE therapy and follow-up. Ten participants will receive a single treatment with the SCE and follow-up studies, as described in above diagram. The preparation of CB-SC cultures and SCEs will be performed as previously described [1]. Briefly, human cord blood units derived from healthy allogeneic donors will be obtained from FDA-registered CORD : USE Cord Blood Bank (Orlando, FL). All cord blood samples will be screened for HIV ½, HBsAg, HBcAg, HCV, HIVNAT, STS, HBVNAT, HCVNAT, AbScr, HTLV I/II, West Nile, Chagas, and CMV, and only pathogen-free cord blood units will be used for clinical treatment. Human CB-SC will be generated as previously described [6,8]. Cord blood mononuclear cells will be plated in serum-free culture medium (Lonza, Walkersville, MD) and incubated at 37°C, in 8% CO_2. After 2-3 weeks, CB-SC growing at 80% confluence will be prepared for clinical trial. One Educator device will be generated from one cord blood unit, and used for one subject. After overnight treatment through the co-culture of MNC product with CB-SCs, the SCE (SCE)-treated MNC will be collected and infused intravenously (i.v.) back to the subject in 3 - 5 hours.

8.2 Primary Study Endpoint

The primary study endpoint will be the occurrence of treatment-related adverse effects. Adverse events that occur during therapy (especially those that necessitate temporary or permanent discontinuation of therapy) and over the 12-month follow-up period will be assessed.

8.3 Secondary Study Endpoints

8.3.1 Feasibility endpoints:

8.3.1.1 The number of patients who were unable to complete SCE Therapy. Any non-adverse event related reasons for discontinuation of therapy will be recorded (eg: technical problems with the SCE system).

8.3.1.2 The number of patients who are lost to follow-up prior to the 12-month follow-up visit.

8.3.2 Efficacy endpoints

8.3.2.1 Area under the C-peptide curve (AUC) over the first 2 hours of a 3-hour mixed meal tolerance test (MMTT). The AUC at 12-months and changes over time will be assessed.

8.3.2.2 Peak C-peptide levels over a 3-hour MMTT. The peak C-peptide level at 12-months and changes over time will be assessed.

8.3.2.3 Basal C-peptide levels at 12-months and changes over time will be assessed.

8.3.2.4 Daily insulin requirements

8.3.2.5 Change in HbA1C levels over time

8.3.2.6 Changes in auto-antibody levels over time

8.3.3 Exploratory endpoints

8.3.3.1 Measurements of regular immune cell markers at baseline, 1, 3, 6, 9, 12, and 24 months

8.3.3.2 Flow cytometry of memory T cell markers at baseline, 1, 3, 6, 9, 12, and 24 months

Table 3. Schedule of Events

PROCEDURE	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7	Visit 8	Visit 9	Visit 10	Visit 11	Visit 12
	Within 30 Days of Cord Blood Order	Day -7 to -1	Day -7 to -1	Day 0	Day +1 to +2	Day +7 ±1 day	Month 1 ± 5 days	Month 3 ± 5 days	Month 6 ± 5 days	Month 9 ± 5 days	Month 12 ± 5 days	Month 24 ± 5 days
Informed Consent	x											
Verify Inclusion/Exclusion Criteria	x											
Vital Signs (Ht/Wt/Temp/BP/HR/RR)	x			x		x		x	x	x	x	x
Medical History	x			x		x		x	x	x	x	x
Physical Exam	x			x		x		x	x	x	x	x
Venous assessment for apheresis	x											
LABS												
CBC	x			x		x	x	x	x	x	x	x
CMP	x						x	x	x	x	x	x
ABO Blood Typing Test	x											
Infectious disease testing (HIV, HTLV, Hep B, cAB, Hep C ab.	x											
HbA1C	x						x	x	x	x	x	x
Serum Pregnancy Test	x	x					x	x	x			
Auto-antibodies (IAA, IA2, GAD, ZnT8)	x						x	x	x	x	x	x
Fasting C-peptide level	x	x	x	x	x	x	x	x	x	x	x	x
Fasting Blood Glucose Level	x	x	x	x	x	x	x	x	x	x	x	x
Fasting plasma pro-insulin level		x					x	x	x	x	x	x
Insulin dose recording	x						x	x	x	x	x	x
EKG	x											
METABOLIC STATUS												
Mixed meal tolerance test		x						x	x	x	x	x
Pro-Insulin level		x						x	x	x	x	x
Glucagon levels		x						x	x	x	x	x
Daily Insulin Doses: Patient Diary		**Continuous**										
Daily Glucose Levels: Patient Diary		**Continuous**										
IMMUNE MARKERS												
$CD4^+CD25^+CD127^{low/-}$-Tregs		x					x	x	x	x	x	x
$CD4^+CD45RO^+CCR7^+$ T_{CM} cells		x					x	x	x	x	x	x

Table 3. Schedule of Events

PROCEDURE	Visit 1 Within 30 Days of Cord Blood Order	Visit 2 Day -7 to -1	Visit 3 Day -7 to -1	Visit 4 Day 0	Visit 5 Day +1 to +2	Visit 6 Day +7 ±1 day	Visit 7 Month 1 ± 5 days	Visit 8 Month 3 ± 5 days	Visit 9 Month 6 ± 5 days	Visit 10 Month 9 ± 5 days	Visit 11 Month 12 ± 5 days	Visit 12 Month 24 ± 5 days
$CD4^+ICOS^+$ and $CD8^+ICOS^+$ T cells		x					x	x	x	x	x	x
IL-1, TNFα, IL-17		x					x	x	x	x	x	x
IL-10 and TGF – β1		x					x	x	x	x	x	x
Th1/Th2 cytokines (IL4, IL5, IL12, IL13)		x					x	x	x	x	x	x
PLATELET MARKERS												
Platelet function testing		x					x	x	x	x	x	x
$CD41^+CD42^+CD270^+CD274^+Galectin9^+$		x					x	x	x	x	x	x
TREATMENT												
Apheresis			day -1 x	**cell infusion**								
SAFETY ASSESSMENTS												
Phone call for safety follow-up					x	x						
Concomitant med monitoring				**Continuous**								
Adverse Event monitoring				**Continuous**								

9 Subject Selection and Withdrawal

This study will enroll a total of 10 subjects who meet the following eligibility criteria.

9.1 Inclusion Criteria

1) Adult patients (≥ 18 years)
2) Must have a diagnosis of type 1 diabetes mellitus based on the 2015 American Diabetes Association criteria for the Clarification and Diagnosis of diabetes.
3) Must have a blood test confirming the presence of at least one autoantibody to pancreatic islet β Cells (IAA, IA2, GAD 65, ZnT8).
4) Fasting C-peptide level ≥ 0.3 ng/ml
5) HbA1C < 10% at enrollment
6) Recent diagnosis (within two years of enrollment)
7) Adequate venous access for apheresis
8) Must be equipped with a continuous glucose monitoring system (CGMS)
9) Ability to provide informed consent
10) For female patients only, willingness to use FDA-recommended birth control (http://www.fda.gov/downloads/ForConsumers/ByAudience/ForWomen/FreePublications/UCM356451.pdf) until 6 months post treatment.
11) Must agree to comply with all study requirements and be willing to complete all study visits

9.2 Exclusion Criteria

Potential subjects meeting any of the following criteria will be excluded from participation:

1) AST or ALT 2 ≥ x upper limit of normal.
2) Abnormal bilirubin (total bilirubin > 1.2 mg/dL, direct bilirubin > 0.4 mg/dL)
3) Creatinine ≥ 2.0 mg/dl.
4) Known coronary artery disease or EKG suggestive of coronary artery disease unless cardiac clearance for apheresis is obtained from a cardiologist.
5) Known active infection such as Hepatitis B, Hepatitis C, or Human Immunodeficiency Virus (HIV)
6) Pregnancy assessed by a positive serum pregnancy test or breastfeeding mothers
7) Use of immunosuppressive medication within one month of enrollment including but not limited to prednisone, cyclosporine, tacrolimus, sirolimus, and chemotherapy.
8) Presence of any other autoimmune diseases (lupus, rheumatoid arthritis, scleroderma, etc.)
9) Anticoagulation other than ASA.
10) Hemoglobin < 10 g/dl or platelets < 100 k/ml
11) Is unable or unwilling to provide informed consent
12) Presence of any other physical or psychological medical condition that, in the opinion of the investigator, would preclude participation

9.3 Subject Recruitment and Screening

Subjects will be recruited for the study by any of the investigators, co-investigators, endocrinologists, other medical professionals who treat Type 1 Diabetes. Patients may also be self-referred. If subjects consent to participate in the trial, diagnostic testing necessary to meet above inclusion or exclusion criteria will be performed. The list of screening requirements will include a regular blood count analysis, a blood test to confirm the presence of at least one autoantibody to pancreatic islet β cells, and other associated testing to exclude pregnancy, clinically significant liver, kidney, or heart disease. Patients will be referred to the Principal Investigator, Dr. Donato, for the apheresis and re-infusion therapy portion of the protocol only. There will be a follow-up with a safety phone call on Day 1 or 2 after re-infusion and on Day 7 $\pm$1. This will be performed by the research nurses.

Copies medical records from all visits to the JTCC will be sent to the treating physicians.

The rescreening of subjects who previously failed eligibility is allowable.

9.4 Early Withdrawal of Subjects

9.4.1 When and How to Withdraw Subjects

Based on our current published [16] and unpublished clinical data, there were no subjects who needed to be withdrawn from the study prior to completion. The risks to the patients are expected to be minimal and akin to a standard apheresis procedure. The cells returned to the patients are autologous cells that will be treated (or educated) by CB-SCs.

In this study a patient may be withdrawn from this protocol at any points:

1) If they suffer some type of allergic or hypersensitivity reaction to the SCE treated cells precluding the infusion of the product.
2) If technical difficulties preclude the completion of apheresis.
3) If they do not want to complete the required post treatment follow-up testing.

Participation in this trial is VOLUNTARY for all subjects. If they choose not to participate, this will not affect their relationships with Hackensack University Medical Center or their rights to health care or other services to which they are otherwise entitled. If they decide to participate, patients are free to withdraw their consents and discontinue participation at any time without affecting their future care at Hackensack University Medical Center. In the event that a patient is withdrawn for above or any other unexpected reasons they will be allowed to continue to receive medical care for treatment associated problems.

9.4.2 Data Collection and Follow-up for Withdrawn Subjects

Even though subjects may be withdrawn prematurely from the study, it is imperative to collect toxicity data for 12 weeks after. We will use all possible methods (e.g. number of phone calls to subject, phone calls to health care proxy, if possible, certified letters, etc.) to confirm that the subject is truly lost for follow-up. If a patient withdraws from the study prior to their 12 month follow-up, enrollment of a replacement patient will occur.

10 Study Device

10.1 Description

To build upon previous clinical trials abroad [1-3] using a closed-loop system, we will investigate the use of the SCE as a static device, which will allow increased interaction time (14-17 hours) between CB-SCs and patient lymphocytes/monocytes under a controlled good manufacturing process (GMP) environment before reintroducing the lymphocytes back into the patient. The longer interaction between CB-SCs and lymphocytes/monocytes will improve treatment efficacy using this optimized discontinuous system. Through secreted and cell-surface signaling molecules, the CB-SCs educate the lymphocytes [1,5]. The cells returned to the patients are autologous cells that have been treated (or educated) by CB-SCs. CB-SCs remain inside of the device due to their unique attaching ability [1-3,5]. Additionally, in this clinical trial, we design a chamber (160×160×200 cm) for co-culture of lymphocytes and CB-SCs that includes twelve discs of the material with adherent CB-SCs sandwiched between a top cover plate and a bottom

collecting plate (**Figure 1**). The device is manufactured, assembled and packaged in a Class 100K clean room. After being sterilized by gamma-irradiation (Cesium-137), the devices are stored at the room temperature in dark cabinets of Class 100K clean room, which is FDA-approved facility for cell isolation and cultures. The materials used to produce the device are FDA-approved for *in vivo* use per the United States Pharmacopeia (i.e., Grade Class VI Plastic). The sterilized device is a single use, which CB-SCs are generated from one cord blood unit for one subject application.

10.2 Treatment Regimen

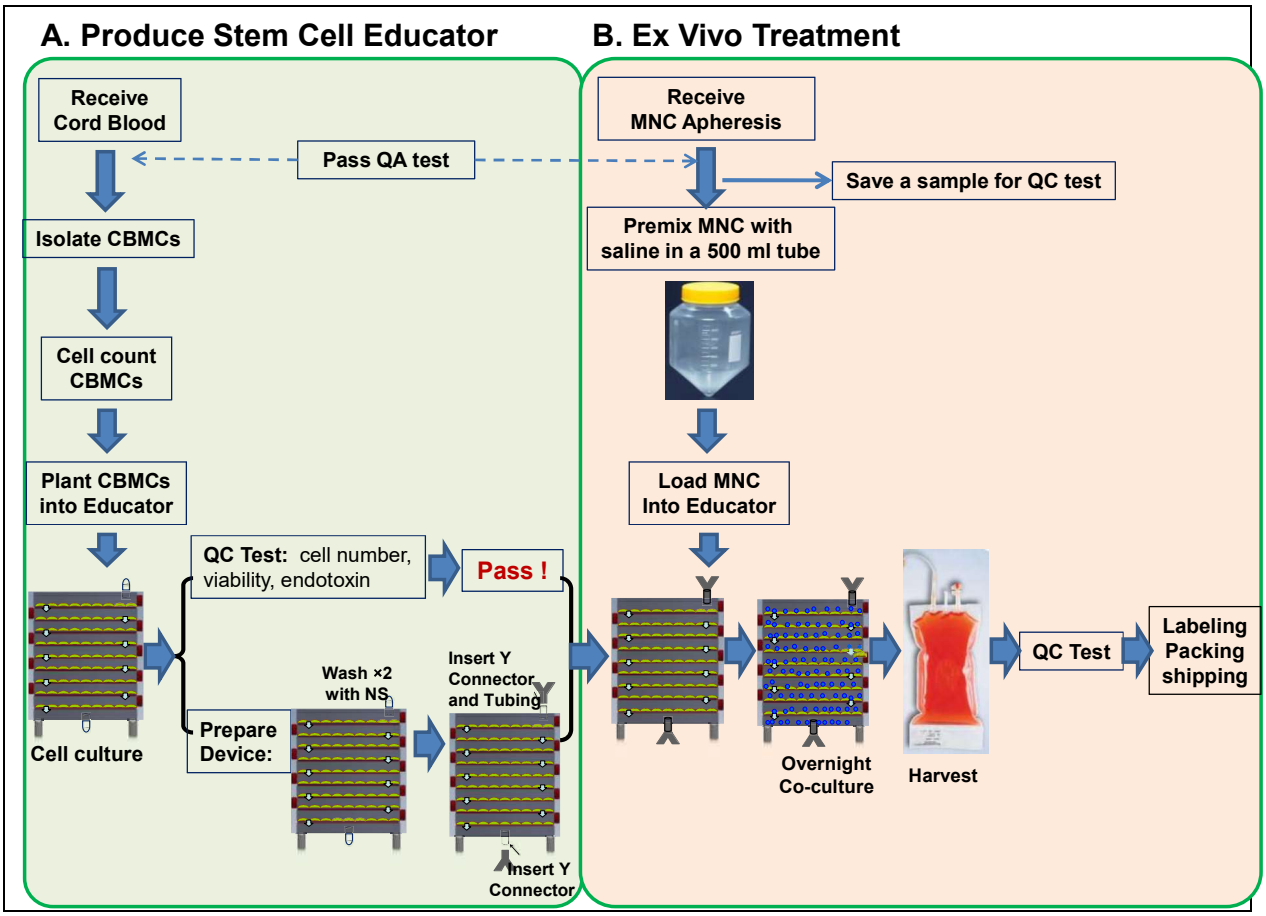
Subjects who meet all criteria will be scheduled for treatment. All enrolled subjects will receive treatment with the SCE system consisting of a single session of MNC collection by apheresis approximately 10 L (~10^{10} mononuclear cells) of blood will be processed on day -1. The MNC product will then be exposed over a 17-hour period of time to the SCE and on day 0 the product will be infused intravenously back to the patient. All treated subjects will receive a telephone follow-up assessment on Day +1 to monitor for acute adverse events. All treated subjects will attend a safety visit on Day +7 ± 1 day. Follow-up visits will occur on 1st, 3rd, 6th, 9th, 12th, and 24th month (± 5 days) post-therapy. Subjects will be instructed to record daily insulin doses, sugar levels, and physical activities. After receiving the SCE therapy, patients' daily insulin doses will be monitored and adjusted by their own physician.

10.3 Method for Assigning Subjects to Treatment Group

There is only one treatment group. Based on fasting C-peptide levels (a by-product of insulin biosynthesis, with normal reference range 0.8 – 3.1 ng/mL), as an indicator for islet β cell function, all participants will be characterized and assigned as one group having moderate T1D with some residual β cell function (fasting C-peptide level $\geq$ 0.3 ng/mL, n = 10). Each participant will receive one treatment.

10.4 Preparation and Use of Study Device

To build upon previous clinical trials abroad [1-3] using a closed-loop system, we will investigate the use of the SCE as a static device, which will allow increased interaction time (at least 17 hours) between CB-SCs and patient lymphocytes/monocytes under a controlled good manufacturing process (GMP) environment before reintroducing the lymphocytes back into the patient. The longer interaction between CB-SCs and lymphocytes/monocytes will improve treatment efficacy using this optimized discontinuous system (**Figures 13 A and B**). Please see the Appendix 4 Master Batch Record (MBR) for the detailed information on the Preparation and Use of Study Device. Briefly, the whole procedure is outlined in the diagram (**Figure 4C**): 1) Preparation of SCE: A fresh donated human umbilical cord blood unit is received, and mononuclear cells are isolated using a Ficoll gradient separation followed by removal of remaining Red Blood Cells (RBCs) using lysis buffer. The entire mononuclear cell suspension is seeded in the SCE, and cultured for 10-20 days. Once the culture is $\geq$ 80% confluent, the SCE is ready to use. 2) Preparation of SCE-treated MNC: The day after the culture is $\geq$ 80% confluent, the recipient's MNC Apheresis is collected on day -1 and shipped to the GMP facility; the SCE is washed and the CB-SCs in the SCE are cocultured overnight with the recipient's MNC Apheresis product, which contains lymphocytes and monocytes. After incubation, the patient cells are drained into a product collection bag. After final QC testing and product release, the product is sent to the recipient for administration.


A. Produce Stem Cell Educator
B. Ex Vivo Treatment
Receive Cord Blood
Pass QA test
Isolate CBMCs
Cell count CBMCs
Plant CBMCs into Educator
QC Test: cell number, viability, endotoxin
Pass !
Cell culture
Prepare Device:
Wash ×2 with NS
Insert Y Connector and Tubing
Insert Y Connector
Receive MNC Apheresis
Save a sample for QC test
Premix MNC with saline in a 500 ml tube
Load MNC Into Educator
Overnight Co-culture
Harvest
QC Test
Labeling Packing shipping

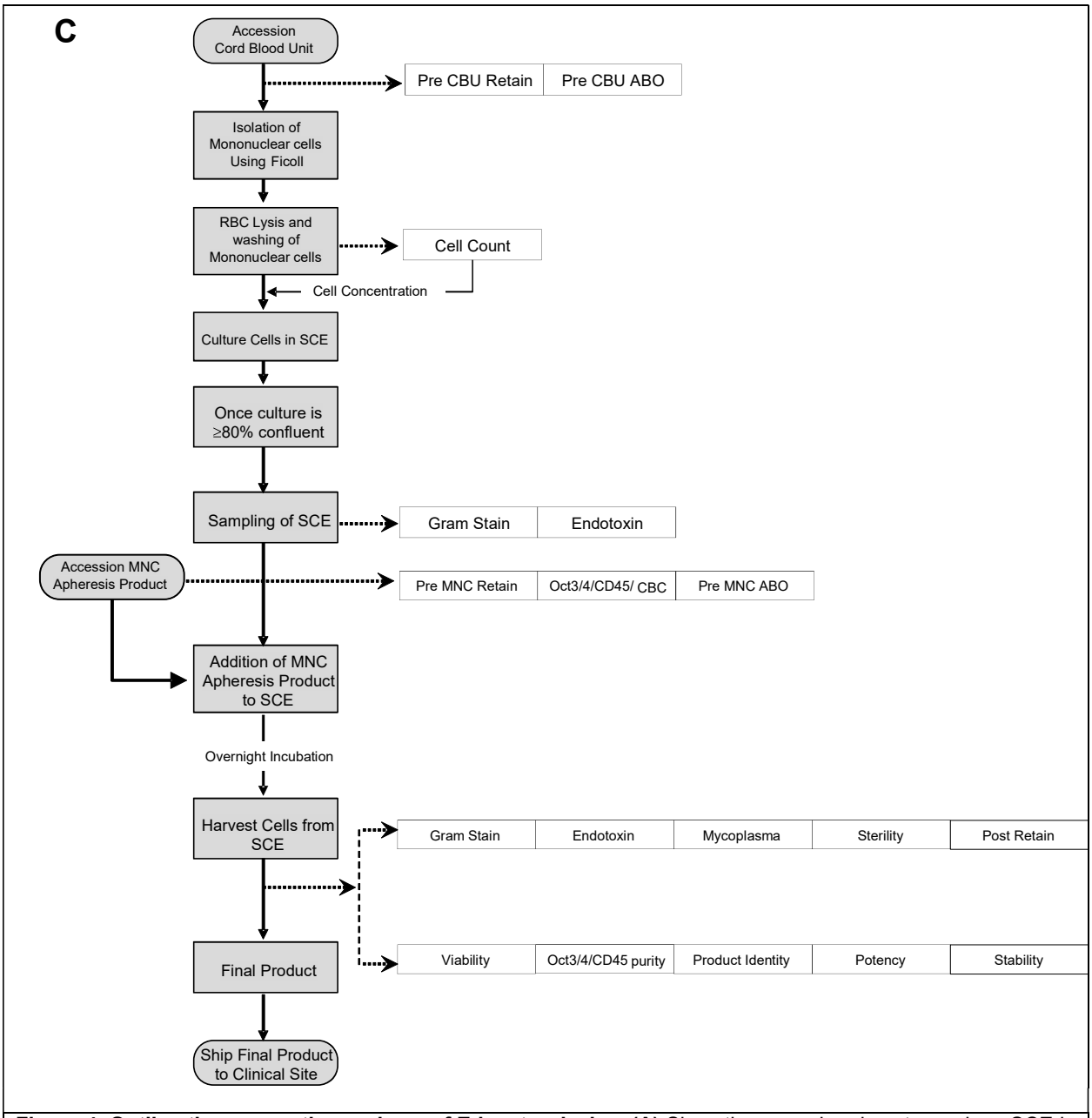


Figure 4. Outline the preparation and use of Educator device. (A) Show the procedure how to produce SCE in GMP facility. **(B)** Display the protocols on ex vivo treatment of MNC by overnight co-culture with CB-SCs in GMP facility. **(C)** The detailed process overview.

Following steps will be applied for the Preparation and Use of Study Device. The production of clinical-grade SCEs and associated protocol have been mirrored by two Performance Qualification (PQ) runs at the FDA-registered cGMP facility, as demonstrated by PQ Reports from NYBC (see Appendix 2).

Step 1: Collection of human cord blood units.
Human cord blood units derived from healthy donors will be provided by FDA-registered CORD:USE Cord Blood Bank Inc. All cord blood samples are screened for communicable disease as requested by regulatory agencies.

Step 2: Preparation of CB-SC in the device.
Prior to introducing CB-SCs into the device, cord blood mononuclear cells are isolated from fresh cord blood unit (at least 100 ml/unit) according to the following protocol:

1) Take 50ml tubes and put in holders: normally use 4 tubes;
2) Add HISTOPAQUE®-1077 to each tube at 20ml/tube to isolate the mononuclear cells;
3) Plant mononuclear cells in the SCE Device (Tianhe Stem Cell Biotechnologis Inc.) at 1×10^6cells/ml, 25-30ml/dish in serum-free culture medium; if > $20x10^9$ CD3 positive cells are collected by the apheresis procedure, the collection may be divided between two SCE Devices for one treatment.
4) Incubate cells at 37°C, 8% CO_2 conditions for 10~20 days.
5) Cell observation: CB-SC are round and attach on the bottom of dishes. If cell density reaches the 80% of confluence, CB-SC can be prepared for clinical application.

Step 3: Test the CB-SC culture for endotoxin and Gram stain.

1) **Testing for endotoxin.** The supernatant from the culture of CB-SCs will be collected into 1.8 ml sterilized tubes. Endotoxin will be tested by using the Endosafe-PTS Portable Test System (Charles River, Charleston, SC) and Endosafe-Licensed PTS Endotoxin Cartridge (0.005 EU/mL sensitivity, Catalogue #: PTS20005F, Fisher Scientific). The standard endotoxin level will be < 0.5 EU/mL. Only the Educator apparatus that meets this standard can be used for the clinical trial.
2) **Gram stain.** The supernatant from the culture of CB-SCs will be collected into 1.8 ml sterilized tubes. Gram staining will be performed by using the Gram Stain kit (BD Diagnostic Systems, Sparks, MD). Only the Educator apparatus that is negatively stained can be used for the clinical trial.

Step 4: Preparation of SCE.

1) Sterilized the hood in a GMP facility for preparation of SCE;
2) Clean out side of device with 70% ethanol gauze and carefully remove all caps;
3) Discard all supernatants inside of device;
4) Add physiological saline to each layer at 20ml/layler, washing each layer and removing all floating cells and debris;
5) Additional wash with physiological saline (20ml/layler);
6) Remove the caps from the top and bottom, replace with Plastisol Horseshoe Y Connectors;

Step 5: Treatment of MNC with SCE therapy.

1) On day -1 patients with undergo a steady state MNC collection by apheresis, with an optimal goal of processing approximately 10 L (~1010 mononuclear cells) of blood. Apheresis may be stopped before the goal is reached based on tolerability and venous access. A femoral apheresis catheter may be placed for the duration of the procedure if venous access is found to be insufficient on the day of collection, despite the initial venous assessment. The femoral apheresis catheter will be removed approximately 1 hour after the collection as per institutional SOP. A minimum of 6 L processed will be required to continue on to the next phase. Plasma will be added to the collection bag at the end of apheresis to target a product volume of 250 mL. Labeling of the product will be done prior to disconnecting the product from the donor in accordance with the Blood and Marrow Transplant program labelling policy.
2) The product will be packaged, stored and picked up by New York Blood Center (NYBC) in accordance with the Blood and Marrow Transplant program SOPs.
3) Testing for stem cell markers CD45 and OCT3/4 by flow cytometry on the cellular product. To exclude the contamination of CB-SCs in the Final Product, we will perform the flow cytometry by double-staining with CB-SCs' markers CD45 and OCT3/4 (Figure 3D).
4) Testing for identity markers $CD4^+CD25^+CD127^{Low/-}$ Tregs by flow cytometry on the cellular product. The isotype-matched IgG antibodies will be served as negative controls. The Fc Blocker (BD Pharmingen) will be applied to block non-specific staining.
5) Testing for potency markers $CD4^+CD45RO^+CCR7^+$ central memory T (T_{CM}) cells, $CD4^+ICOS^+$,$CD8^+ICOS^+$ T cells by flow cytometry on the cellular product. The isotype-matched IgG

antibodies will be served as negative controls. The Fc Blocker (BD Pharmingen) will be applied to block non-specific staining.

6) Once at NYBC, the cellular product will be processed and treated by CB-SCs according to the SOPs in Master Batch Record (Appendix 4).
7) On day 0, the educated cellular product will be infused to the patient in accordance with the Blood and Marrow Transplant program cellular infusion SOP.

Step 6: Test the Final Product for cell viability, endotoxin, Gram stain, mycoplasma, and stem cell markers.

1) **Cell viability.** The sample from the CB-SC-treated MNCs will be collected into 1.8 ml sterilized tubes. The cell viability will be tested by the trypan blue–based exclusion assay. The mononuclear nuclear cell number will be tested by using the Automated Complete Blood Counts (CBC) Using the Beckman Coulter® AC-T DIFF ™.
2) **Testing for endotoxin.** The sample from the CB-SC-treated MNCs will be collected into 1.8 ml sterilized tubes. Endotoxin will be tested by using the Endosafe-PTS Portable Test System (Charles River, Charleston, SC) and Endosafe-Licensed PTS Endotoxin Cartridge (0.005 EU/mL sensitivity, Fisher Scientific). The standard endotoxin level for the final product will be ≤ 5 EU/kg/hr according to FDA guidelines. Only the Final Product that meets this standard can be used for the clinical infusion.
3) **Gram stain.** The sample from the CB-SC-treated MNCs will be collected into 1.8 ml sterilized tubes. Gram staining will be performed by using the Gram Stain kit (BD Diagnostic Systems, Sparks, MD). Only the Educator apparatus that is negatively stained can be used for the clinical trial.
4) **Testing for mycoplasma and sterility.** The Sterility and contamination of mycoplasma in the sample from the CB-SC-treated MNCs can be tested and excluded by following methods: 1). Regular cell culture: Cell viability can be monitored under phase-contrast microscope to exclude the contamination of bacteria. 2). Real time PCR: The sample from the CB-SC-treated MNCs will be collected into 1.8 ml sterilized tubes for mycoplasma and and 50 mL tubes (total 120 mL) for sterility tests by Eurofins (Lancaster, PA) in compliance with the requirements of the US FDA Good Laboratory Practice Regulations (21 CFR 58). 3). The Sterility Testing will be performed by using a Direct Inoculation Method at Eurofins Company.
5) **Testing for stem cell markers CD45 and OCT3/4 by flow cytometry**. To exclude the contamination of CB-SCs in the Final Product, we will perform the flow cytometry by double-staining with CB-SCs' markers CD45 and OCT3/4 (**Figure 3D**).
6) **Testing for stem cell markers CD45 and OCT3/4 by flow cytometry**. To exclude the contamination of CB-SCs in the Final Product, we will perform the flow cytometry by double-staining with CB-SCs' markers CD45 and OCT3/4 (**Figure 3D**). The isotype-matched IgG antibodies will be served as negative controls. The Fc Blocker (BD Pharmingen) will be applied to block non-specific staining.
7) **Testing for identity markers $CD4^+CD25^+CD127^{Low/-}$** Tregs **by flow cytometry**. The isotype-matched IgG antibodies will be served as negative controls. The Fc Blocker (BD Pharmingen) will be applied to block non-specific staining.
8) **Testing for potency markers $CD4^+CD45RO^+CCR7^+$ central memory T (T_{CM}) cells, $CD4^+ICOS^+$,$CD8^+ICOS^+$ T cells by flow cytometry**. The isotype-matched IgG antibodies will be served as negative controls. The Fc Blocker (BD Pharmingen) will be applied to block non-specific staining.

10.5 Subject Compliance Monitoring

Subjects will receive one treatment with the SCE.

Follow-up visits will be scheduled 1, 3, 6, 9, 12, and 24 months (± 5 days) after treatment for clinical assessments and laboratory tests as described in Section 4.2. A telephone follow-up will be performed the day after the cell infusion.

10.6 Prior and Concomitant Therapy

All T1D subjects receive their daily insulin injections prior to or during the study. Their daily insulin doses will be adjusted after receiving the SCE therapy by their own physicians, not the study PI. There will be no other new diabetes medicines or therapies will be permitted during this study.

10.7 Packaging

The SCEs will be packaged in box (4 devices/box) and shipped to the GMP facility NYBC, with the Good Manufacturing Practices (GMP) Labeling System.
After treatment with the SCE, the Final Product will be shipped to clinical site at Hackensack University Medical Center in a sterilized cooler.

10.8 Blinding

In this single-arm study, all subjects will receive open label treatment with the SCE.

10.9 Receiving, Storage, Dispensing and Return

10.9.1 Receipt of Devices

Upon receipt of the study treatment supplies, an inventory must be performed and a device receipt log filled out and signed by the person accepting the shipment. It is important that the designated study staff counts and verifies that the shipment contains all the items noted in the shipment inventory. Any damaged or unusable study device in a given shipment will be documented in the study files. The investigator must notify study sponsor of any damaged or unusable study treatments that were supplied to the investigator's site.

10.9.2 Storage

The devices are stored at the room temperature in dark cabinets of Class 100K clean room (or GMP facility for cell cultures). There is no need for protection from light. Storage considerations include avoiding dropping of the devices or placing heavy weight on top of the devices.

10.9.3 Dispensing of Study Device

All empty devices (without stem cells) are in the same quality. Any single device will be assigned to any single subject and dispensed. The only standard will be the quality of CB-SCs cultured in the device. If cell density reaches the ≥80% of confluence, endotoxin level <0.5 EU/mL, the device with CB-SCs can be assigned to subject and prepared for clinical application.

Regular study device reconciliation will be performed to document device assignments, devices used, devices remaining, and inadvertently damaged devices. This reconciliation will be logged on the device accountability form, and signed and dated by the study team.

10.9.4 Return or Destruction of Study Device

Devices are discarded at the conclusion of the treatment as per institutional biohazard waste disposal SOP. At the completion of the study, there will be a final reconciliation of devices shipped, devices consumed, and devices remaining. This reconciliation will be logged on the device reconciliation form, signed and dated. Any discrepancies noted will be investigated, resolved, and documented prior to return or destruction of unused study devices. Any devices destroyed on site will be documented in the study files.

11 Study Procedures

11.1 **Visit 1**

Visits to the John Theurer Cancer Center will be as outlined in this study. The investigators will not be adjusting the participants' insulin or other diabetic medications, with the following exceptions:

1) If medically necessary while the participant is physically present at the JTCC during a scheduled visit.

2) On the day of apheresis

3) On the day of re-infusion.

4) If one of the co-investigators is the primary endocrinologist for one of the participants.

5) In case of emergency or if the participant is unable to contact their primary treating physician.

Screen for Enrollment: First, all consented subjects will be screened for enrollment in accordance with the Inclusion Criteria and Exclusion Criteria of SCE therapy. Patients will be qualified for enrollment if they meet the 2015 diagnosis standards of the American Diabetes Association and a blood test confirm the presence of at least one autoantibody to pancreatic islet beta cells. Depending on age, either the adult endocrinologist (Dr. Van Hoven) or pediatric endocrinologist (Dr.Ghanny, for patients aged from 18 to 22 years old) will review pre-procedure blood sugar logs. Respective MD would be accessible by medical team for any questions during visit regarding blood sugars. Patients will be provided additional blood sugar logs for the duration of the study. We will ask the patient to check their blood sugars at least 4 times daily and twice a week overnight. We will also give option to obtain a cloud-based glucometer (e.g., Accucheck Aviva Connect). This meter is a cloud-based meter and has a physician portal so MD can review blood sugars remotely as weekly. We can also recommend that the patient obtain a CGMS (continuous glucose monitoring system) unit to help monitor blood sugars more closely. The visit must be completed within 30 days prior to the cord blood order from cord blood bank.

1) CBC with different immune cell markers

2) Complete chemistry

3) EKG

4) Venous assessment done by an apheresis nurse. Patients without sufficient venous access will be deferred. Patients with acceptable venous access at the time of screening but who fail to collect peripherally

on the day of apheresis may have an apheresis catheter placed femorally by a surgeon for the purpose of the collection.

5) Autoantibody testing (see Table 3).

6) Infectious disease testing (HIV, HTLV, Hep B, cAB, Hep C ab.)

11.2 Visit 2

Women of childbearing potential will require a serum pregnancy test between day -7 and day -1.

The Mixed Meal Tolerance Test (MMTT): MMTT testing should be done in the morning while the subject is fasting. Subjects will be nothing by mouth (NPO) after midnight except water. Liquids that contain only simple sugars can be used to treat or prevent hypoglycemia during the night and morning prior to study. The target glucose level at the start of the test should be between 70 and 200 mg/dL for subjects with T1DM.

Insulin Application: Subjects with T1DM should take their usual dose of insulin on the evening before the test. Subjects on Lantus or Levemir should take their usual injection the evening before and/or on the morning of the test, as per their normal routine. Subjects on CSII should continue with their usual basal settings on night prior to test. Usual doses of rapid-acting insulin analog will be used until midnight the evening before the test. Small correction doses of rapid-acting insulin analog may also be used up to 4 hours before the test if needed to achieve a blood glucose level <150 mg/dl at the start of the study. Usual doses of NPH insulin and rapid-acting insulin analogs are not given on the morning of the test.

Blood Sampling: Blood will be obtained for measurement of plasma glucose, c-peptide and glucagon at -10, 0, 15, 30, 60, 90, 120, 150 and 180 minutes. A split-duplicate sample for quality control will also be collected. IV lines will be inserted in one arm for blood sampling.

Boost Dose: The mixed meal used will be Boost High Protein Gluten Free Drink with 20g of sugar per bottle. The MMTT should take 180 minutes to perform. The dose of Boost High Protein Gluten Free Drink with 20g of sugar per bottle, given at time 0 minutes. The dose should be consumed in no more than 5 minutes.

11.3 Visit 3

Patients will undergo leukapheresis. Adult endocrinologist or Pediatric Endocrinologist to be on-call for questions.

11.4 Visit 4

Infusion of the cellular product after exposure to the SCE. Adult endocrinologist or Pediatric Endocrinologist to be on-call for questions.

The cellular product will be released based on the results of a Gram stain and endotoxin test, with results of sterility available after the 14 day sterility test. If the tests reveal a sterility failure and there are any signs (e.g., fever, vomiting, and increased CBC count) of infection in subjects after infusion, the management and handling will be performed according to our standard SOPs at HackensackUMC, “Name of Procedure: Management and Handling of Cellular Products with Positive Microbial Cultures”. Antibiotics need to be administered in the event that microbial contamination is present in the product that the subject already received. The route (oral or i.v.) and dosage of administering antibiotics will be determined according to the type of infection. To identify the microorganism(s), anti-microbial agent sensitivity testing needs to be performed during the sterility test. If the subject experiences any serious and unexpected adverse reaction that could be from administration of a non-sterile cellular product, then the endocrinologist must report this information to the FDA in an IND safety report no more than 15 calendar days after the initial receipt of the information. The endocrinologist must also report the sterility failure, results of investigation of cause and any corrective actions, in an information amendment submitted to the IND within 30 calendar days after

initial receipt of the positive culture test result. All these reports will be handled according to our SOPs at HackensackUMC, “Name of Procedure: Biological Product Deviation” and “Name of Procedure: Incidents, Errors, Accidents, Deviations, Variances and Complaints Reporting”.

To determine which step caused contamination and to prevent sterility lapses in the future, all procedures will be fully examined from the collection of the cord blood unit, all steps of Operations, the isolation of mononuclear cells (MNCs) by apheresis, and to the packing and shipping of final product between the clinical site and the GMP facility. If the reagents and materials are contaminated, they will be discarded. New reagents and materials will be used for the production of Stem Cell Educators (SCE) and SCE-treated MNCs.

11.5 Visit 5

Safety Phone Call Day + 1 to + 2 after the re-infusion.

11.6 Visit 6

Safety visit 7 days ± 1 day after the re-infusion.

11.7 Visit 7

Follow-up visit 1: It is scheduled 1 month (± 5 days) after treatment for clinical assessments and laboratory tests as per Section 3.2, Table 3. To be reviewed by respective Endocrinologist.

11.8 Visit 8

Follow-up visit 2: It is scheduled 3 months (± 5 days) after treatment for clinical assessment (e.g., fasting blood glucose, C-peptide, and HbA1C) and laboratory tests as per Section 3.2, Table 3. To be reviewed by respective Endocrinologist.

11.9 Visit 9

Follow-up visit 3: It is scheduled 6 months (± 5 days) after treatment for clinical assessment (e.g., fasting blood glucose, C-peptide, and HbA1C) and laboratory tests as per Section 3.2, Table 3. To be reviewed by respective Endocrinologist.

11.10 Visit 10

Follow-up visit 4: It is scheduled 9 months (± 5 days) after treatment for clinical assessment (e.g., fasting blood glucose, C-peptide, and HbA1C) and laboratory tests as per Section 3.2, Table 3. To be reviewed by respective Endocrinologist.

11.11 Visit 11

Follow-up visit 5: It is scheduled 12 months (± 5 days) after treatment for clinical assessment (e.g., fasting blood glucose, C-peptide, and HbA1C) and laboratory tests as per Section 3.2, Table 3. To be reviewed by respective Endocrinologist.

11.12 Visit 12

Follow-up visit 6: It is scheduled 24 months (± 5 days) after treatment for clinical assessment (e.g., fasting blood glucose, C-peptide, and HbA1C) and laboratory tests as per Section 3.2, Table 3. To be reviewed by respective Endocrinologist.

12 Statistical Plan

12.1 Statistical Methods

This is a prospective, single-arm, open-label, single-center study to assess the safety, feasibility, and efficacy of SCE therapy for the treatment of patients with T1D. Ten patients meeting eligibility criteria will be enrolled for this study, with an effect size of 0.8 ng/mL at a 0.025 significance level and 80% power. All patients will be included in the safety analyses. The primary safety analysis will be performed in the safety population. The occurrences of treatment-related adverse events will be evaluated and presented as counts (percentages) and binomial 95% exact binomial confidence intervals (CI).

The feasibility of the SCE therapy will assessing by analyzing the number of the patients unable to complete the therapy and the number of patients who are lost to follow-up prior to the 12-month visit. Both of these parameters will be analyzed as counts (percentages) and corresponding 95% CI as described in the safety analysis section above.

Statistical analyses of data will be performed by *t*-test using Power and Precision software (www.**power-analysis**.com). The paired *t* test will be used to study the significance between baseline and follow-ups. Using the fasting blood glucose levels and the plasma C-peptide, as well as their levels following OGTT or MMTT as the parameters, we want to detect the difference after the SCE therapy with an effect size of 0.8 ng/mL at 2.5% significance level, 80% power, and one side. According to our calculations, we will need 10 subjects in order to find this difference.

Area under the C-peptide curve will be calculated with C-peptide level measured at 0, 30, 60, 90, 120, 150 and 180 minutes of the MMTT using the trapezoid rule. The AUC for C-peptide over 120 minutes of the MMTT will be evaluated and summarized using descriptive stats mean (SD) or median (IQR), as appropriate. This calculation will be obtained for each of the time points, baseline, 3, 6, 9 12, and 24 months and longitudinal analysis will be performed to evaluate the changes over time. This analysis of changes over the time points will be achieved using mixed effect models for repeated measures (MMRM) implemented using PROC MIXED in SAS version 9.4. As this is a secondary analysis no adjustment for multiple test will be employed for this analysis.

Mean AUC for C-peptide over 3 hour MMTT, which is defined as the AUC divided by the duration of the test i.e., 180 minutes, will be calculated at baseline, and all subsequent post-baseline points, and changes over time will be analyzed using MMRM method as defined in the AUC for C-peptide subsection above.

HbA1C levels assessed at baseline, 3, 6, 9, 12, and 24 months will be summarized using descriptive statistics at each time point. To evaluate changes in HbA1C levels assessed at baseline and post-baseline time points, the MMRM analysis defined in AUC for C-peptide will be performed.

All study data will be entered into a Microsoft Office Excel 2013 (Microsoft Corporation, Redmond, WA) and imported into SAS 9.4. The database will be validated in SAS to ensure any out of range values are queried for all multiple source until all data issues are resolved. Any $p < 0.05$ will be considered statistically significant. All data analysis will be performed using SAS version 9.4 (SAS Institute Inc. Cary, NC).

Stopping rule: If a grade 3 adverse event, as per the Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0, related to the study procedure occurs in 2 patients, the study will be terminated.

12.2 Subject Population(s) for Analysis

All subjects who received treatment will be included in the safety analyses. The primary efficacy end point will be the change in C-peptide secretion between baseline and follow-up.

13 Safety and Adverse Events

13.1 Definitions

13.1.1 Unanticipated Problems Involving Risk to Subjects or Others

Any incident, experience, or outcome that meets all of the following criteria:

- Unexpected in nature, severity, or frequency (i.e. not described in study-related documents such as the IRB-approved protocol or consent form, the investigators brochure, etc)
- Related or possibly related to participation in the research (i.e. possibly related means there is a reasonable possibility that the incident experience, or outcome may have been caused by the procedures involved in the research,
- Apheresis-related side effects such as citrate toxicity (symptomatic hypocalcemia), hypotension, vasovagal reaction, and access site thrombophlebitis. If central venous catheter (CVC) is used, additional adverse events include bleeding at the cvc site, deep-vein thrombosis (DVT), thrombophlebitis, and central line infections.
- Serious (as defined below) "***Serious" is different than "severe" as reported in the CTC criteria that applies a grade to the AE.***

13.1.2 Adverse Event

An ***adverse event*** (AE) is any symptom, sign, illness or experience that develops or worsens in severity during the course of the study. Concurrent illnesses or injuries should be regarded as adverse events. Abnormal results of diagnostic procedures are considered to be adverse events if the abnormality:

- results in study withdrawal
- is associated with a serious adverse event
- is associated with clinical signs or symptoms
- leads to additional treatment or to further diagnostic tests
- is considered by the investigator to be of clinical significance

13.1.3 Serious Adverse Event

Adverse events are classified as serious or non-serious. A ***serious adverse event*** is any AE that is:

- fatal
- life-threatening
- requires or prolongs hospital stay
- results in persistent or significant disability or incapacity
- a congenital anomaly or birth defect
- an important medical event

Important medical events are those that may not be immediately life threatening, but are clearly of major clinical significance. They may jeopardize the subject, and may require intervention to prevent one of the Patient logs in in-patient hospitalization, or intensive treatment of bronchospasm in an emergency department would typically be considered serious.

All adverse events that do not meet any of the criteria for serious should be regarded as ***non-serious adverse events***.

13.1.4 Adverse Event Reporting Period

The study period during which adverse events must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up.

For this study, the study treatment follow-up is defined as 30 days following the administration of study treatment. Serious adverse events should be reported to the local IRB and FDA within 7-10 days from the time of the event. Minor events can be reported in an annual report

13.1.5 Preexisting Condition

A preexisting condition is one that is present at the start of the study. A preexisting condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

13.1.6 General Physical Examination Findings

At screening, any clinically significant abnormality should be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event must also be recorded and documented as an adverse event.

13.1.7 Post-study Adverse Event

All unresolved adverse events should be followed by the investigator until the events are resolved, the subject is lost to follow-up, or the adverse event is otherwise explained. At the last scheduled visit, the investigator should instruct each subject to report any subsequent event(s) that the subject, or the subject's personal physician, believes might reasonably be related to participation in this study. The investigator should notify the study sponsor of any death or adverse event occurring at any time (during the 6-month follow-up) after a subject has discontinued or terminated study participation that may reasonably be related to this study. The sponsor should also be notified if the investigator should become aware of the development of cancer or of a congenital anomaly in a subsequently conceived offspring of a subject that has participated in this study.

13.1.8 Abnormal Laboratory Values

A clinical laboratory abnormality should be documented as an adverse event if <u>any one of the following</u> conditions is met:

- The laboratory abnormality is not otherwise refuted by a repeat test to confirm the abnormality
- The abnormality suggests a disease and/or organ toxicity
- The abnormality is of a degree that requires active management; e.g. change of dose, discontinuation of the drug, more frequent follow-up assessments, further diagnostic investigation, etc.

13.1.9 Hospitalization, Prolonged Hospitalization or Surgery

Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as a serious adverse event unless specifically instructed otherwise in this protocol. Any condition responsible for surgery should be documented as an adverse event if the condition meets the criteria for and adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an adverse event in the following circumstances:

- Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition. Surgery should ***not*** be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.
- Hospitalization or prolonged hospitalization required to allow efficacy measurement for the study.
- Hospitalization or prolonged hospitalization for therapy of the target disease of the study, unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

13.2 Recording of Adverse Events

Based on our current clinical data in 200 subjects including Chinese and Caucasian patients, we did not see any significant adverse events during the treatment with SCE therapy, and follow-up studies after treatment for 4 years. No participants experienced any significant adverse events during the course of treatment. 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. No tumor formation and other safety concerns in all subjects after receiving SCE for 4 years.

At each contact with the subject, the investigator must seek information on adverse events by specific questioning and, as appropriate, by examination. Information on all adverse events should be recorded immediately in the source document, and also in the appropriate adverse event module of the case report form (CRF). All clearly related signs, symptoms, and abnormal diagnostic procedures results should recorded in the source document, though should be grouped under one diagnosis.

All adverse events occurring during the study period must be recorded. The clinical course of each event should be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. Serious adverse events that are still ongoing at the end of the study period must be followed up to determine the final outcome. Any serious adverse event that occurs after the study period and is considered to be possibly related to the study treatment or study participation should be recorded and reported immediately.

13.3 Reporting of Serious Adverse Events and Unanticipated Problems

Investigators and the protocol sponsor must conform to the adverse event reporting timelines, formats and requirements of the various entities to which they are responsible, but at a minimum those events that must be reported are those that are:

- related to study participation,
- unexpected, and
- serious or involve risks to subjects or others (see definitions, section 8.1).

If the report is supplied as a narrative, the minimum necessary information to be provided at the time of the initial report includes:

- Study identifier
- Study Center
- Subject number
- A description of the event
- Date of onset
- Current status
- Whether study treatment was discontinued
- The reason why the event is classified as serious
- Investigator assessment of the association between the event and study treatment

13.3.1 Investigator reporting: notifying the study sponsor

Any study-related unanticipated problem posing risk of harm to subjects or others, and any type of serious adverse event, must be reported to the study sponsor by telephone within 24 hours of the event. To report such events, a Serious Adverse Event (SAE) form must be completed by the investigator and faxed to the study sponsor within 24 hours. The investigator will keep a copy of this SAE form on file at the study site. Report serious adverse events by phone and facsimile to:

Dr.Michele Donato, 551 996 5900 (Phone)
Dr.Steven Ghanny, 551 996 5329 (phone)
Dr. Anne Van Hoven, 551 996 3500 (Phone)

Within the following 48 hours, the investigator must provide further information on the serious adverse event or the unanticipated problem in the form of a written narrative. This should include a copy of the completed Serious Adverse Event form, and any other diagnostic information that will assist the understanding of the

event. Significant new information on ongoing serious adverse events should be provided promptly to the study sponsor

13.3.2 Investigator reporting:

For reportable deaths, the initial submission to the Hackensack UMC IRB may be made by contacting the IRB Director or Associate Director. The AE/Unanticipated Problem Form is required as a follow up to the initial submission.

Other Reportable events:
For clinical drug trials, the following events are also reportable to the HackensackUMC's IRB:

- Any adverse experience that, even without detailed analysis, represents a serious unexpected adverse event that is rare in the absence of drug exposure (such as agranulocytosis, hepatic necrosis, Stevens-Johnson syndrome).
- Any adverse event that would cause the sponsor to modify the investigators brochure, protocol or informed consent form, or would prompt other action by the IRB to assure protection of human subjects.
- Information that indicates a change to the risks or potential benefits of the research, in terms of severity or frequency. For example:
  - An interim analysis indicates that participants have a lower rate of response to treatment than initially expected.
  - Safety monitoring indicates that a particular side effect is more severe, or more frequent than initially expected.
  - A paper is published from another study that shows that an arm of your research study is of no therapeutic value.
- Change in FDA safety labeling or withdrawal from marketing of a drug, device, or biologic used in a research protocol.
- Breach of confidentiality
- Change to the protocol taken without prior IRB review to eliminate apparent immediate hazard to a research participant.
- Incarceration of a participant when the research was not previously approved under Subpart C and the investigator believes it is in the best interest of the subject to remain on the study.
- Complaint of a participant when the complaint indicates unexpected risks or the complaint cannot be resolved by the research team.
- Protocol violation (meaning an accidental or unintentional deviation from the IRB approved protocol) that in the opinion of the investigator placed one or more participants at increased risk, or affects the rights or welfare of subjects.

13.3.3 Sponsor reporting: Notifying the FDA

If this protocol is being conducted under an FDA IND, it is the responsibility of the study regulatory sponsor (Hackensack University Medical Center./Dr.Michele Donato responsible for the initiation, management, of the clinical trial --- i.e. the IND holder) to report certain adverse events or unanticipated problems to the FDA.

The study sponsor is required to report certain study events in an expedited fashion to the FDA. These written notifications of adverse events are referred to as IND safety reports. The following describes the safety reporting requirements by timeline for reporting and associated type of event:

- ***Within 7 calendar days***
  Any study event that is:
  - associated with the use of the study drug
  - unexpected,
  - fatal or life-threatening, and

- ***Within 15 calendar days***
  Any study event that is:
  - associated with the use of the study drug,
  - unexpected, and
  - serious, but not fatal or life-threatening
    -or-
  - a previous adverse event that was not initially deemed reportable but is later found to fit the criteria for reporting (reporting within 15 calendar days from when event was deemed reportable).

  Any finding from tests in laboratory animals that:
  - suggest a significant risk for human subjects including reports of mutagenicity, teratogenicity, or carcinogenicity.

Additional reporting requirements
Sponsors are also required to identify in IND safety reports all previous reports concerning similar adverse events and to analyze the significance of the current event in light of the previous reports.

Reporting Process
Adverse events may be submitted on FDA Form 3500A or in a narrative format. If supplied as in a narrative format, the minimum information to be supplied is noted above at the beginning of section 8.3. The contact information for submitting IND safety reports is noted below:

FDA Emergency Operations Branch
Office of Regional Operations, HFC-162
Phone: 1-866-300-4374 or 301-796-8240
FAX: 301-443-3757

13.3.4 Sponsor reporting: Notifying participating investigators

It is the responsibility of the study sponsor (Hackensack University Medical Center) to notify all participating investigators, in a written IND safety report, of any adverse event associated with the use of the drug that is both serious and unexpected, as well as any finding from tests in laboratory animals that suggest a significant risk for human subjects. Additionally, sponsors are also required to identify in IND safety reports all previous reports concerning similar adverse events and to analyze the significance of the current event in light of the previous reports.

13.4 Stopping Rules

This single-arm study, in which all patients will receive the same experimental treatment at one dose, assess treatment safety and efficacy. The conduct of this trial may thus be stopped by a stopping rule setting out the circumstances under which the trial will end and the action that will then be taken, if the primary safety endpoint (Section 3.3.1) and efficacy endpoints (Section 3.3.2) have been achieved, and/or the confirmation of the treatment is safe and efficacious.
If a grade 3 adverse event, as per the Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0, related to the study procedure occurs in 2 patients, the study will be terminated. This is defined in the statistical section of the protocol (Section 7.1).

13.5 Medical Monitoring

Michele Donato, MD, FACP, Division of Stem Cell Transplantation, HUMC is assigned as the medical monitor and will review all unanticipated problems involving risks to subjects or others, serious adverse events associated with the protocol.
It is the responsibility of the study sponsor (Hackensack University Medical Center) to notify all participating investigators, in a written IND safety report of any adverse event associated with the use of the drug that is both serious and unexpected, as well as any finding from tests in laboratory animals that suggest a significant risk for human subjects. Additionally, sponsors are also required to identify in IND safety reports

all previous reports concerning similar adverse events and to analyze the significant of the current event in light of the previous reports.

13.5.1 Internal Data and Safety Monitoring Board or DSMP

Hackensack University Medical Center (HUMC) places the highest priority on ensuring the safety of patients participating in clinical trials. The initial review of a new protocol lies with the Institutional Review Board (IRB) in reviewing new trials to ensure each trial, regardless of sponsorship or support, contains adequate plans for data and safety monitoring. Our Data and Safety Monitoring Board (DSMB) is responsible for monitoring all investigator-initiated trials (IITs) authored by HUMC investigators, regardless of federal, institutional or industry support. This includes single site IITs, as well as multicenter IITs coordinated by HUMC investigators with data management and site coordination by the principal investigator and includes all phases of clinical trials conducted at the HUMC. The members of DSMB at HUMC have clinicians, biostatisticians, bioethicists, and research scientists. Where appropriate, the use of an external/independent DSMB for a trial (e.g., high risk, multicenter) will be recommended.

Once a trial is determined as appropriate for monitoring by the internal DSMB, the DSMB has the responsibility for continuing review and monitoring of the study. Our DSMB's review and oversight are written in to the IRB-approved protocol for such trials. The DSMB provides oversight of study progress and safety by review of the following information by un-blinded analyses: 1) Rates of accrual and accrual retention. 2) Frequency and severity of adverse events (AEs) and serious adverse events (SAEs). 3) Response rates, where appropriate. 4) New information related to the trial, i.e., published scientific reports or other developments that may affect subject safety or ethical concerns. 5) Any changes to the anticipated risk/benefit ratio of the study that would affect its continuation. 6) Protocol deviations and violations. 7) Matters that pertain to serious errors or potential misconduct by any of the investigators or research staff, i.e., breaches in confidentiality, research fraud. 8) Subject complaints. 9) Conflict of interest.

The timeline for review of trials by the DSMB is determined at the outset of the study with approval by the IRB. The frequency of DSMB review required for a protocol (i.e., 6 month, yearly) is recorded by the PRMS Administrator and tracked in order that the appropriate DSMB submission documentation is requested from the study team in advance of a scheduled DSMB meeting. The PI and clinical research coordinator (CRC) are provided forms for submission of required documentation to the DSMB.

14 Data Handling and Record Keeping

14.1 Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Those regulations require a signed subject authorization informing the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (i.e. that the subject is alive) at the end of their scheduled study period.

14.2 Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in source documents. Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays,

subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

14.3 Case Report Forms

The study case report form (CRF) is the primary data collection instrument for the study. All data requested on the CRF must be recorded. All missing data must be explained. If a space on the CRF is left blank because the procedure was not done or the question was not asked, write "N/D". If the item is not applicable to the individual case, write "N/A". All entries should be printed legibly in black ink. If any entry error has been made, to correct such an error, draw a single straight line through the incorrect entry and enter the correct data above it. All such changes must be initialed and dated. DO NOT ERASE OR WHITE OUT ERRORS. For clarification of illegible or uncertain entries, print the clarification above the item, then initial and date it.

14.4 Records Retention

It is the investigator's responsibility to retain study essential documents for at least 2 years after the last approval of a marketing application in their country and until there are no pending or contemplated marketing applications in their country or at least 2 years have elapsed since the formal discontinuation of clinical development of the investigational product. These documents should be retained for a longer period if required by an agreement with the sponsor. In such an instance, it is the responsibility of the sponsor to inform the investigator/institution as to when these documents no longer need to be retained.

15 Study Monitoring, Auditing, and Inspecting

15.1 Study Monitoring Plan

This study will be monitored according to the monitoring plan. The investigator will allocate adequate time for such monitoring activities. The Investigator will also ensure that the monitor or other compliance or quality assurance reviewer is given access to all the above noted study-related documents and study related facilities (e.g. pharmacy, diagnostic laboratory, etc.), and has adequate space to conduct the monitoring visit. The following issues will be addressed at this visit: all CRFs have been completed and appropriately filed, a copy of the monitoring Patient Log is obtained, maintenance and retention of study records.

The close-out visit will occur after the last subject's case report forms have been completed, study has been closed with reviewing IRB/IEC and all regulatory issues have been addressed.

In summary the Monitor will serve an important role in the successful conduct of the study. The relationship between the Monitor and the site staff is strengthened by open effective communication with the Monitor providing training and support to ensure participants' rights and safety as well as data quality and compliance with all applicable regulations of the regulatory authorities.

15.2 Auditing and Inspecting

The investigator will permit study-related monitoring, audits, and inspections by the IRB, the sponsor, government regulatory bodies, and University compliance and quality assurance groups of all study related documents (e.g. source documents, regulatory documents, data collection instruments, study data etc.). The investigator will ensure the capability for inspections of applicable study-related facilities (e.g. pharmacy, diagnostic laboratory, etc.).

Participation as an investigator in this study implies acceptance of potential inspection by government regulatory authorities and applicable University compliance and quality assurance offices.

16 Ethical Considerations

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted independent Ethics Committee (EC) or Institutional Review Board (IRB), in agreement with local legal prescriptions, for formal approval of the study conduct. The decision of the EC/IRB concerning the conduct of the study will be made in writing to the investigator and a copy of this decision will be provided to the sponsor before commencement of this study. The investigator should provide a list of EC/IRB members and their affiliate to the sponsor.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. This consent form will be submitted with the protocol for review and approval by the EC/IRB for the study. The formal consent of a subject, using the EC/IRB-approved consent form, must be obtained before that subject undergoes any study procedure. The consent form must be signed by the subject or legally acceptable surrogate, and the investigator-designated research professional obtaining the consent.

17 Study Finances

17.1 Funding Source

This study is financed through a funding from Hackensack University Medical Center Foundation.

17.2 Conflict of Interest

No investigators have conflict of interests that may be relevant to the submitted clinical trial. All Hackensack UMC investigators will follow the HUMC conflict of interest policy.

18 Publication Plan

Neither the complete nor any part of the results of the study carried out under this protocol, nor any of the information provided by the sponsor for the purposes of performing the study, will be published or passed on to any third party without the consent of the study sponsor. Any investigator involved with this study is obligated to provide the sponsor with complete test results and all data derived from the study.

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