FEATURED NEW INVESTIGATOR

New type of human blood stem cell: a double-edged sword for the treatment of type 1 diabetes

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Type 1 diabetes (T1D) is an autoimmune disease caused by an autoimmune destruction of pancreatic islet insulin-producing cells. Autoimmunity and shortage of insulin-producing cells are 2 key issues for the treatment of T1D. To cure T1D in a comprehensive manner, both issues need to be addressed simultaneously. Not only must the islet cells be replaced, the patient's immune system also must be dealt with. Regulatory T cells (Tregs) play a crucial role in maintaining homeostasis and self-tolerance through their inhibitory impacts on autoreactive effector T cells. We identified a novel type of stem cells from human umbilical cord blood, designated cord blood stem cells (CB-SC), which may be able to address immune modulation of the autoimmune process and allow for β-cell replacement. We are the first group using CB-SC to correct functional defects of $CD4^+CD62L^+$ Tregs, leading to a reversal of overt diabetes in an autoimmune-caused diabetic NOD mouse model. Notably, treatment with CB-SC-modulated $CD4^+CD62L^+$ Tregs (mCD4CD62L Tregs) simultaneously can overcome the autoimmunity via systemic and local immune modulations and the shortage of insulin-producing cells via stimulating the β-cell regeneration. These new stem cells will offer a promising avenue for the development of powerful autologous therapeutic products for prevention and reversal of T1D. (Translational Research 2010;155:211–216)

Abbreviations: CB-SC = cord blood stem cells; ES = embryonic stem; IL-10 = interleukin-10; NO = nitric oxide; NOD = non-obese diabetic; PB-IPC = peripheral blood-derived insulin-producing cells; SSEA = stage-specific embryonic antigen; T1D = type 1 diabetes; TGF-β 1 = transforming growth factor-β 1; Tregs = regulatory T cells

Type 1 diabetes (T1D) is a T-cell-mediated autoimmune disease that leads to a major loss of pancreatic insulin-producing β cells. To stay alive, people with T1D must take multiple insulin injections daily or continually infuse insulin through a pump. However, pump therapy is not a cure; it does not halt the persistent autoimmune response. Nor can it reliably prevent devastating complications such as neuronal and cardiovascular diseases, blindness, and kidney failure. Thus, T1D has created serious burdens on families and society. This compelling need brings a sense of urgency to finding a cure for T1D that cannot only overcome the shortage of insulin-producing β-cells but also halt the progression of autoimmunity.[1-3] Treatment with β-cell surrogates (eg, insulin and islet transplantation) or immunosuppressive agents (eg, anti-CD3 mAb,

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Submitted for publication November 1, 2009; revision submitted January 12, 2010; accepted for publication January 13, 2010.

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1931-5244/$ – see front matter

doi:10.1016/j.trsl.2010.01.003

anti-CD20 mAb, GAD 65 vaccination, and cyclosporine) cannot address efficiently both key issues to cure T1D. Recently, *in vitro* co-culture with stem cells, including human cord blood stem cells (CB-SCs)[3,4] or mesenchymal stem cells,[5] has been shown to modulate lymphocytes.

Notably, we have developed a novel approach that can address both issues simutaneously, leading to reversal of overt T1D.[3] This review will focus on the recent progress of CB-SCs.

CHARACTERIZATION OF NEW TYPE OF BLOOD STEM CELLS

We have identified and characterized a unique type of stem cell from human CB-SC by virtue of their capability to attach to a plastic surface of nontissue culture-treated petri dishes.[3,6] The surface of petri dishes is hydrophobic with a positive charge; however, the regularly used tissue culture dishes or plates treated with vacuum gas plasma by manufacturers become hydrophilic with a negative charge, which monocytes/macrophages are used for adhering.[6,7] CB-SC displayed embryonic-stem-cell-specific (ES) molecular markers, which included transcription factors OCT-4 and Nanog along with stage-specific embryonic antigen (SSEA)-3 and SSEA-4. Interestingly, CB-SC also expressed leukocyte common antigens CD45 but was negative for CD34. CB-SC displayed very low immunogenicity as indicated by an expression of a very low level of major histocompatibility complex antigens and a failure to stimulate the proliferation of allogeneic lymphocytes.[4,6] Notably, CB-SC could give rise to cells with 3 embryonic layer-derived cells, which include endothelial cells, neuronal cells, and functional insulin-producing cells.[6]

CB-SC AND AUTOIMMUNITY OF T1D

T1D is a complex and chronic autoimmune disease. Despite the possible involvement of viral infection, environmental factors, and genetic predisposition that can increase the risk of T1D, the specific factor or factors that trigger autoimmunity remain elusive. A major challenge for the treatment of T1D is to identify therapeutic approaches that fundamentally modulate autoimmune responses. Tregs play a critical role in controlling the immune balance and maintaining self-tolerance through their inhibitory impacts on autoreactive effector T cells, such as releasing immunosuppressive cytokines interleukin-10 (IL-10) and/or transforming growth factor-β 1 (TGF-β1). Compelling evidence demonstrates that abnormalities of Tregs, either in cell number or in function, are associated with the initiation and the progression of T1D, both in diabetic patients and in animal models.[8,9] It becomes an attractive approach via the manipulation of Tregs for prevention and treatment of T1D. We found that CB-SC displayed potential in correcting the intrinsic functional defects of Tregs and held promise for the prevention and treatment of T1D.[3,10]

CB-SC-modulated Tregs prevent T1D onset. The development of T1D is a chronic disease process. It is important to preserve β-cell function and to prevent the pathogenesis of T1D via the manipulation of autoimmune destruction of islet β cells in subjects at high risk. A tremendous amount has been learned from T1D animal models. Makino et al first generated the nonobese diabetic (NOD) mouse in 1980 that spontaneously developed T1D, which resulted from autoimmune destruction of pancreatic islet β cells[11] and shared many similarities with those observed in T1D patients. Therefore, the NOD mouse model has provided a valuable tool for T1D study during the past 30 years.[12,13] Increasing evidence demonstrates that intrinsic defects of Tregs were involved in the initiation and progression of islet β-cell autoimmunity in NOD mice in a fashion similar to T1D in patients.[9,14-19] To determine the therapeutic potential of CB-SC-modulated Tregs for the prevention of diabetes onset, we have performed the following experiments at the prediabetic stage (8–9 week, n = 10 for each group) of NOD mice. First, the mice treated with CB-SC-modulated $CD4^+CD62L^+$ Tregs (mCD4CD62L Tregs, 2×10^6 cells/mouse, i.p.) significantly delayed and prevented diabetes onset in the NOD mice (Fig 1, *A*, red line) in comparison with the control mice treated with freshly-isolated $CD4^+CD62L^+$ Tregs. After 30 weeks, only 20% of mCD4CD62L Treg-treated NOD mice developed overt diabetes in contrast to 75% of the mice treated with control CD4CD62L Tregs (Fig 1, *A*, blue line). Second, we performed glucose tolerance testing. Results showed no significant difference between the group of mCD4CD62L Treg-treated NOD mice (Fig 1, *B*, red line) and the 7-week-nondiabetic NOD mice (Fig 1, *B*, green line). However, control CD4CD62L Treg-treated mice with diabetes had much higher blood glucose levels after the glucose challenge (Fig 1, *B*, dark blue line). Even the control CD4CD62L Treg-treated mice with euglycemia after 30 weeks (around 25% of the mice in the control group) displayed an impaired glucose tolerance test and maintained high levels of blood glucose after 60 min following glucose challenge (Fig 1, *B*, bright blue line, $p < 0.05$). Thus, these data demonstrate that treatment with mCD4CD62L Tregs at the prediabetic stage significantly can delay and prevent diabetes onset in an NOD mouse model.

Reversal of established T1D by CB-SC-modulated Tregs. T1D is a T-cell-mediated autoimmune disease

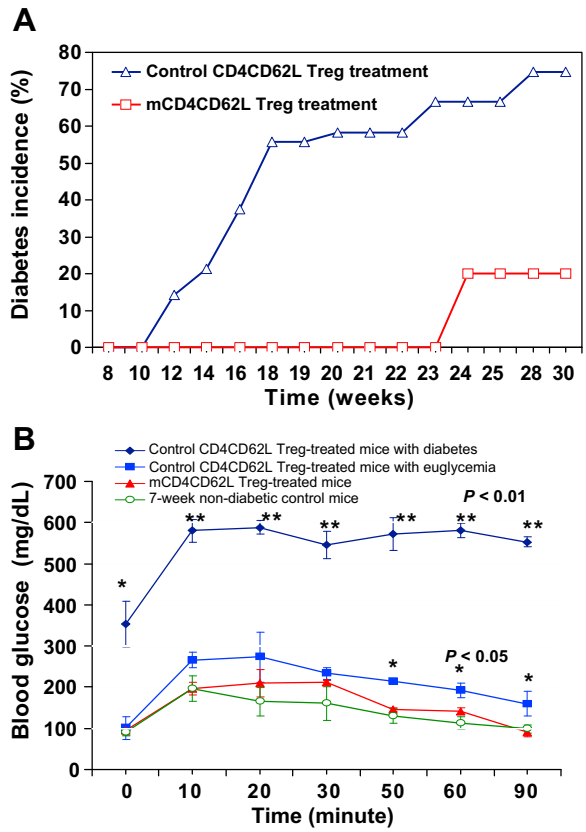

Fig 1. CB-SC-modulated mouse CD4CD62L Tregs prevent diabetes onset. Mouse lymphocytes were isolated from female NOD mice (aged 6 to 7 weeks) and then cocultured with CB-SC for 2 to 4 days at a ratio 1:10 of CB-SC: lymphocytes. Subsequently, the floating lymphocytes were harvested for cell sorting. The purified $CD4^+CD62L^+$ Tregs were administered into NOD mice (female, 8 to 9 weeks old, 2 million cells/mouse, i.p., n = 10). The $CD4^+CD62L^+$ Tregs sorted from freshly isolated NOD mouse spleen mononuclear cells without coculture with CB-SC served as the control. (**A**) Diabetes incidence following the administering of CB-SC-modulated CD4CD62L Tregs (mCD4CD62L Tregs, red line). The mice treated with CD4CD62L Tregs without coculture with CB-SC serve as control (blue line). (**B**) Intraperitoneal glucose tolerance testing 25 weeks after treatment with mCD4CD62L Tregs (red line, n = 5). Mice treated with control CD4CD62L Tregs with diabetes (dark blue line, n = 5), and mice treated with euglycemia (bright blue line, n = 3) after 30 weeks served as controls. Nondiabetic NOD mice at 7 weeks served as the normal control. Mice were challenged with high glucose (2 mg/kg of body weight, i.p.). Data represent mean ± s.d.

with both $CD4^+$ and $CD8^+$ T cells essentially involved in the pathogenesis of T1D. Tregs have emerged as key players in controlling immune responses and maintaining the peripheral tolerance via multiple mechanisms, including the deletion and anergy of autoreactive T cells and the secretion of suppressive cytokines.[20] Functional defects of Tregs are essential for the development of T1D.[8,9] We found that the frequency of Tregs such as $CD4^+CD25^+$ Treg, $CD4^+CD25^+$ Foxp3 Treg, and $CD4^+CD62L^+$ Treg failed to show significant differences at different stages of diabetes in comparison with prediabetic and/or nondiabetic NOD mice.[3] These data suggest the intrinsic functional defects of Tregs in NOD mice that are consistent with previous reports.[8,9] To determine further the therapeutic potential of CB-SC-modulated Tregs in T1D, we treated the established diabetic NOD mice with CB-SC-modulated $CD4^+CD62L^+$ Tregs. Notably, using CB-SC-modulated $CD4^+CD62L^+$ Tregs can correct their functional defects, leading to a reversal of overt diabetes in an autoimmune-caused diabetic NOD mouse model.[3] In contrast, treatment with control CD4CD62L Tregs could not reverse diabetes onset.

Mechanistic studies demonstrated that control of diabetes was correlated with systemic immune alterations, including the restoration of Th1/Th2 cytokine balance in blood as well as local regulations in pancreatic islets through a unique distributional pattern of TGF-β1 that may protect islet β cells against the infiltrated lymphocytes.[3] IL-10 and TGF-β1 are representative cytokines contributing the induction of immune tolerance. Treatment with mCD4CD62L Tregs in diabetic NOD mice markedly increased plasma levels of IL-10 and TGF-β1. These suppressor cytokines can help create a tolerogenic environment after treatment with mCD4CD62L Tregs. Specifically, TGF-β1 represents one of the best-characterized cytokines contributing to the induction of immune suppression and the maintaining of self-tolerance.[21] To elucidate *de novo* the molecular mechanism underlying the protection of newly generated islet β cells after treatment with mCD4CD62L Tregs, importantly, we have found that the TGF-β1-positive cells along with their released TGF-β1 in the matrix formed a ''TGF-β1-ring'' surrounding pancreatic islets. This ring may protect newly generated islets against reattack by inducing apoptosis of autoaggressive effector lymphocytes.[3]

Molecular mechanisms and immune modulation of CB-SC. We found that CB-SC displayed very low immunogenicity and functioned as immune regulators.[3,4] Mechanistic studies revealed that programmed death ligand 1 expressed on a CB-SC membrane together with a soluble factor nitric oxide (NO) released by CB-SC mainly contributed to the regulation of human T cells.[4] CB-SC could modulate mouse $CD4^+CD62L^+$ Tregs via the NO signaling pathway because its action can cross species. In comparison with using bone marrow-derived mesenchymal stem cells for immune modulation, the application of CB-SC possess several unique advantages, which include large resources of cord blood worldwide, no risk to the donor, easy to culture and expand *in vitro*, as well as its possession of

embryonic characteristics. More specifically, CB-SC tightly adheres to culture dishes with a large, rounded morphology and are resistant to regular detaching methods (trypsin/EDTA), which makes it easy to collect suspended Tregs for treatment.

CB-SC AND SHORTAGE OF INSULIN-PRODUCING CELLS IN T1D

The shortage of insulin-producing cells is another key issue for T1D. Our current work reveals that treatment with mCD4CD62L Tregs not only diminished the autoimmunity but also overcame the shortage of insulin-producing cells to eliminate hyperglycemia.[3] In mCD4CD62L Tregs-treated diabetic NOD mice, morphometric analysis demonstrated that treatment with mCD4CD62L Tregs significantly increased total β-cell mass. In contrast, β-cell mass was markedly lower after vehicle phosphate-buffered solution treatment or control CD4CD62L Treg treatment. The mechanistic studies have revealed that an increase in the total β-cell mass and a reconstitution of islet architecture via a marked increase in the residual β-cell proliferation both play a key role in the restoration of euglycemia after treatment with mCD4CD62L Tregs.[3]

TGF-β signaling may contribute to β-cell regeneration and restore islet architecture after treatment with mCD4CD62L Tregs in overt diabetic NOD mice. TGF-β1 is a multifunctional factor known to regulate cell growth, differentiation, and function in developing mammalian systems. An expression of TGF-β1 has been found in all embryonic stages and normal human pancreatic islet cells, acinar cells, and ductal cells.[22] Altering TGF-β1 expression in the developing pancreas has been shown to affect both exocrine and endocrine development, which suggests that it is an important regulator of pancreatic organogenesis. Sanvito et al reported that pancreatic buds cultured for 8–10 days in the presence of TGF-β1 (1 ng/mL) were characterized by the absence of acinar cells and by an abundance of epithelial buds containing all types of endocrine cells with a preponderance of cells containing insulin. The shape and arrangement of stromal cells also was modified by TGF-β1. Thus, TGF-β1 plays a key role in directing β-cell differentiation.[22,23] To explore TGF-β1 action in β-cell regeneration, we performed immunohistochemistry. Results showed a higher percentage of cell proliferation marker Ki67-positive islet β cells in the mCD4CD62L Treg-treated mice than that in the control CD4CD62L Treg-treated mice (Fig 2). However, the Ki67-positive cells in the pancreatic islets of the control CD4CD62L Treg-treated mice were infiltrated with inflammatory cells positive for T-cell marker CD3. Notably, most of Ki67-positive islet β cells were distributed around the edge of pancreatic islets, similar in pattern as the ''ring of TGF-β1'' (Fig 2). Triple immunostaining revealed that the Ki67-positive islet β cells were close to the TGF-β1-producing cells (Fig 2, bottom panels). Furthermore, an expression of Sma- and Mad-related protein 4—a primary intracellular mediator downstream in the TGF-β1 signaling pathway—was up-regulated in pancreatic islets and in the Ki67-positive islet β cells (data not shown) of the mCD4CD62L Treg-treated diabetic mice relative to the control CD4CD62L Treg-treated diabetic mice. Thus, TGF-β1 may play a key role in the restoration of total β-cell mass and islet architecture after treatment with mCD4CD62L Tregs.

APPLICATION OF BLOOD STEM CELL-DERIVED INSULIN-PRODUCING CELLS

For late-stage T1D, it will be necessary to combine mCD4CD62L Tregs with stem cell-derived insulin-producing cells and/or other β-cell surrogates because of the almost complete disappearance of β cells in advanced T1D.[3] *In vitro* characterization demonstrates that CB-SC display similar characteristics of islet β-cell progenitors as adult peripheral blood-derived insulin-producing cells (PB-IPC[7]), which include the expression of β cell-specific insulin gene transcription factors (eg, MafA, Nkx6.1, and PDX-1), prohormone convertases (PC1 and PC2), the production of insulin, and its by-product, C-peptide. *In vivo* transplantation demonstrated that CB-SC can give rise to functional insulin-producing cells after administering into the chemical streptozotocin-induced diabetic NOD-scid mice, as indicated by the production of human C-peptide in mouse plasma and by the reduction of hyperglycemia.[6] Thus, CB-SC can be combined with mCD4CD62L Tregs to treat T1D patients at late stage. Additionally, we identified a cell population from adult human blood that displayed a high potential for producing insulin (PB-IPC) by using a similar approach by attaching to a plastic surface without any genetic manipulation and any induction of differentiation.[7]

Our findings provide a novel approach for the generation of autologous insulin-producing cells from patients themselves to treat diabetes. In comparison with the generation of insulin-producing cells from ES cells, this technology efficiently can generate insulin-producing cells without any ethical issues or the hazards of immune rejection. Voltarelli et al performed small clinical trials in new-onset T1D by autologous nonmyeloablative hematopoietic stem cell transplantation and achieved insulin independence in most trial participants.[24] Our findings[3,4,6,7,25] bring new hope for the treatment of diabetic patients by using autologous cells without immune rejection and ethical issues.

Recently, insulin-producing cells have been generated from bone marrow-derived cells under *in vitro*

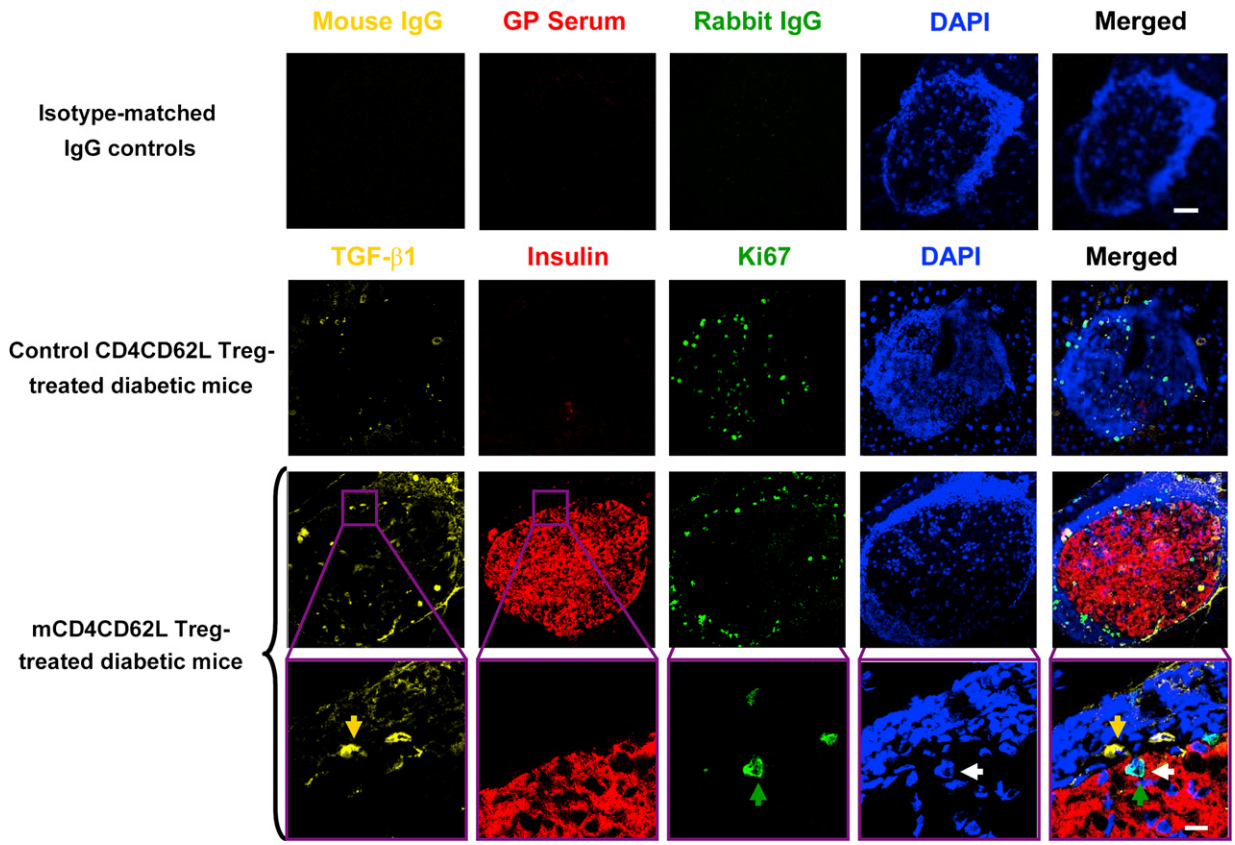

Fig 2. Pancreatic immunohistochemistry. Pancreata from mCD4CD62L Treg-treated diabetic mice and a control group were used for triple immunostaining: TGF-β1 (yellow) in combination with the cell proliferation marker Ki67 (green) and islet β-cell staining (red). Isotype-matched Abs served as the control (top row).

conditions. Yet they remain controversial. Most bone marrow-derived stem cells originate from mesenchymal cells, such as very small and embryonic-like, characterized by Ratajczak et al,[26] and the marrow-isolated adult multilineage inducible cells, characterized by Schiller et al.[27] Additionally, mesenchymal stem sells from adult human islet-derived precursor cells, and from Wharton's Jelly of the human umbilical cord also can give rise to insulin-expressing cells.[28] Because of the lack of the hallmark leukocyte common antigen CD45, they are different from our reported PB-IPC.

CONCLUING REMARKS

Pancreatic islets in T1D are destroyed completely by autoimmune cells.[3] Abrogation of autoimmunity without an adequate residual β-cell mass will not restore normoglycemia. The promotion of β-cell neogenesis must be part of any therapy aimed at T1D treatment. Additionally, it is essential to restore islet architecture for the restoration of normal metabolic control by returning not only endogenous insulin secretion but also the secretion of other important islet hormones like glucagon. These new blood stem cells naturally exist in human blood circulation. They are easy to isolate, culture, and expand, *in vitro*.[3,6] During coculture, CD4CD62L Tregs can be "educated" by the favorable microenvironment created by CB-SC through cell to cell contact and soluble mediators.[3,4] After coculture with CB-SC, only floating lymphocytes were collected for purification of the mCD4CD62L Tregs. Thus, these mCD4CD62L Tregs are the autologous therapeutic product without concern for immune rejection in comparison with the current trial with heterologous Tregs. They show promise in dealing with the many facets of T1D treatment and overcome both key issues (autoimmunity and shortage of insulin-producing cells) simultaneously. We have demonstrated that treatment with mCD4CD62L Tregs results in both restoration of islet architecture and in normoglycemia in an NOD mouse model.[3] Ongoing studies have demonstrated the similar immune modulation of CB-SC on T1D patient-derived lymphocytes, including Tregs and islet β cell-specific pathogenic T cells. Because studies in nonhuman primates are costly, of limited availability, and because of a lack of an

autoimmune model for T1D, establishment of a humanized autoimmune mouse model is a crucial goal for translational research.

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