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Bone marrow- and cord blood-derived stem cell transplantation for diabetes therapy

E. Cantarelli, S. Pellegrini, A. Citro, V. Sordi, L. Piemonti

Diabetes Research Institute, IRCCS San Raffaele Scientific Institute, Milan, Italy

Corresponding Author: Lorenzo Piemonti, MD; e-mail: piemonti.lorenzo@hsr.it

Abbreviations

T1D: Type 1 Diabetes, T2D: Type 2 Diabetes, BM: Bone Marrow, HSC: Hematopoietic Stem Cells, EPC: Endothelial Precursors Cells, MSC: Mesenchymal Stem Cells, UCB: Umbilical Cord Blood, Tx: Transplantation, HSCT: Hematopoietic Stem Cell Transplantation, GVHD: Graft-Versus-Host Disease, Treg: regulatory T cells, APC: Antigen Presenting Cells, NOD: Non-Obese Diabetic, VEGF: Vascular Endothelial Growth Factor, TNC: Total Nucleated Cells.

Abstract

In the last years, the widely consolidated clinical experience in the field of hematology has encouraged the use of bone marrow (BM)- and cord blood (CB)-derived stem cells in non-haematological disease. In the field of diabetes, a huge amount of clinical trials for the cure of type 1 and type 2 diabetes, involving BM-derived HSC and both BM- and CB-derived MSC got underway, thanks also to the availability of simple protocols for collection, culture and storage of these stem cells. Many groups have investigated their potential role in tolerance induction and/or restoration, in pancreatic tissue remodelling as "feeder" cells and in direct differentiation into insulin-producing cells, with the shared final goal to preserve β cell function. This review recapitulates the historical use of BM- and CB-derived stem cells in diabetes therapy, alone or in combination with islet transplantation, and focuses on the most relevant information on preclinical experimental data and provides an update on the most recent clinical trials.

Keywords: Bone marrow, Mesenchymal stem cell, Umbilical cord blood, Diabetes, Immunomodulation, Tolerance, Transplantation, Islet.

Introduction

Diabetes affects 382 million people throughout the world and this number will rise to 592 millions by 2035 (http://www.idf.org/diabetesatlas/introduction). Both type 1 (T1D) and type 2 diabetes (T2D) share a deficit in β cell mass, although due to different pathogenic events: autoimmunity and insulin resistance, respectively[1,2]. Exogenous administration of insulin is routinely used to control both types of diabetes, but it does not sufficiently replace β cells and the adverse short- and long-term effects of the disease remain. Therefore, the cure for diabetes lies in the possibility to replace the lost β cell mass with a new endocrine component capable of assessing blood sugar levels and secreting appropriate levels of insulin in the vascular bed. β cell replacement, through whole pancreas or islet transplantation, is the only treatment capable of establishing long-term euglycemia in T1D patients[3]. Unfortunately these procedures, despite advances in recent years[4], are hindered by the need of immunosuppression, the use of many donors for a single recipient and the short life of the grafts. Accordingly, new approaches aimed to overcome these limits are strongly required. An attractive possibility to treat diseases like diabetes could be represented by stem cell therapy. In the last years, the use of stem cells in clinical protocols is over and over increasing. The remarkable plasticity of different cell subsets obtained from human embryonic and adult tissues from different sources (including bone marrow, adipose tissue, umbilical cord and amniotic fluid) has been the focus of many efforts in research, also in the field of diabetes[5]. Among stem cells, those derived from bone marrow (BM), which mostly comprise hematopoietic stem cells (HSC), endothelial precursors cells (EPC) and mesenchymal stem cells (MSC), can be easily recovered and cultured and have been studied for a long time (Fig. 1). In this review we will report the most relevant preclinical and clinical applications of total BM, isolated BM cells subpopulations and cord blood (CB) cells for the treatment of both T1D and T2D, alone and in combination with pancreatic islet transplantation (tx) (Table 1).

Total BM transplantation for β cell replacement

At first, the possibility for BM cells to differentiate into β cells following signals of tissue remodelling, was reported; in fact, some studies suggested that undifferentiated BM cells transplanted *in vivo* could become glucose-responsive insulin producing cells[6-8]. Using transgenic BM cells that express the green fluorescent protein (GFP) under the guidance of insulin promoter, Ianus and colleagues were the first to demonstrate the ability of BM cells to transdifferentiate into insulin producing cells within the pancreatic islets[7]. These data have resulted in conflicting reports because several other groups have not been able to confirm these findings. In fact, although the infusion of BM cells into diabetic mice lowered the blood glucose and increased β cell mass, their ability to transdifferentiate into β cells was not supported[9-13]. In this direction it was reported that the uptake of exogenous insulin by differentiated cells could lead to false conclusions about the ability of BM-derived cells to differentiate in β cells[14]. Therefore, the concept of *in vivo* transdifferentiation of BM cells into insulin producing cells still remains elusive. Hess et al gave a new dimension to BM tx for diabetes therapy demonstrating that the transplanted cells can initiate endogenous pancreatic regeneration by β cell rapid proliferation and neogenesis[15]. The experience with unpurified BM-derived cells in clinical to treat diabetes is very limited. Few years ago, a Spanish study evaluated the impact of the infusion into the pancreatic artery of autologous, unfractionated BM-derived mononuclear cells obtained after mobilization with G-CSF from the iliac crest of long-standing T1D patients. The pilot clinical study showed no effects in terms of C-peptide serum levels, both basal and stimulated, and no changes in insulin requirement or metabolic control after tx. Due to the lack of efficacy this study, initially aimed at enrolling 10 subjects, was stopped after the third patient by the local research ethic committee[16]. Possible criticisms on this trial included the lack of immune interventions aimed at favouring self tolerance restoration and the selection of a subpopulation of T1D patients with undetectable C-peptide levels before tx.

Hematopoietic stem cell transplantation for T1D and T2D therapy: toward tolerance restoration?

The use of HSC, instead, has aroused much more interest and success. HSC transplantation (HSCT) is now widely recognized as a curative therapy for

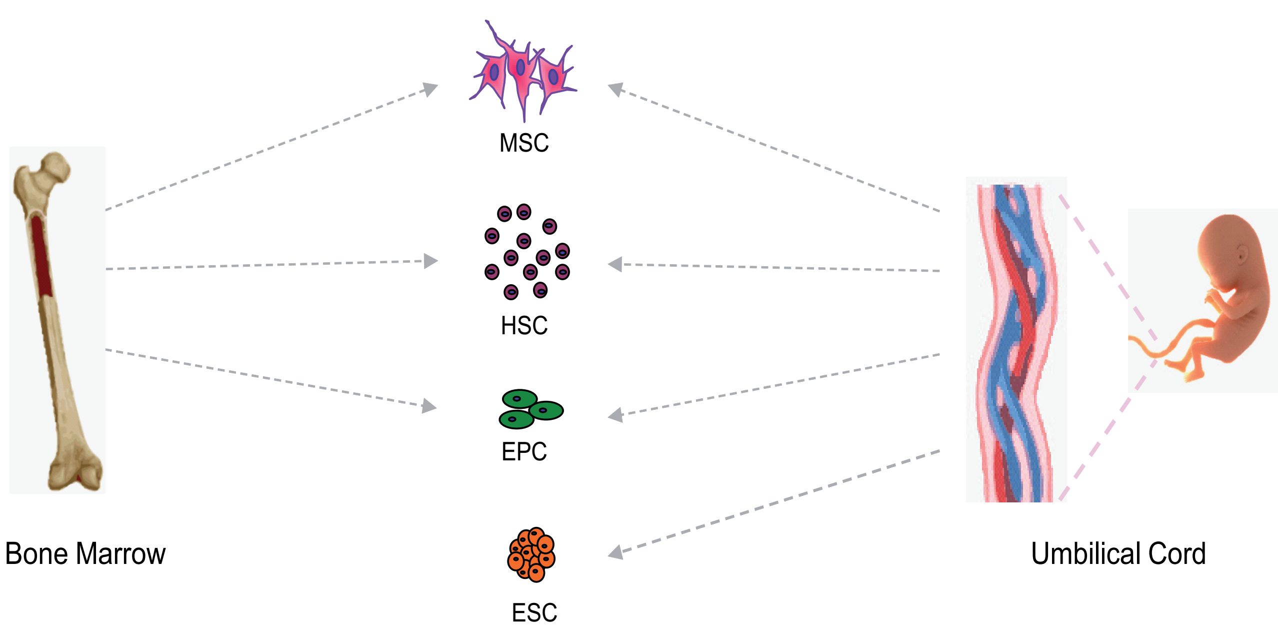

Figure 1. Schematic representation of the different kind of stem cells that can be obtained from bone marrow or umbilical cord. MSC: Mesenchymal Stem Cells, HSC: Hematopoietic Stem Cells, EPC: Endothelial Precursor Cells, ESC: Embryonic Stem Cells.

Table 1. Clinical trials on BM- and UCB-derived HSC and MSC therapy. References or ClinicalTrilas.gov Identifier were reported for completed or still active clinical studies, respectively.

	T1D	T2D	In association with Islet Tx
BM-HSC	[36-37, 38, 39, 40, 41, 42, 43, 44, 45] NCT01121029 NCT01285934	[47, 48, 49, 50, 51] NCT00767260 NCT00465478	-
BM-MSC	[86] NCT02057211 NCT00690066	NCT01576328	[158] NCT00646724
UCB	[114-115, 116, 117] NCT01996228	[118, 121-122]	-

many high-risk hematological diseases. Over the last two decades, it has also been extensively investigated as a therapeutic opportunity for patients affected by severe autoimmune diseases considered refractory to conventional therapies[17]. The idea that a strong relationship between HSC and the organ target of the autoimmune response exists, was further supported by the evidence that either susceptibility or resistance to autoimmunity could be transferred by HSCT, as widely confirmed in animal models for many autoimmune diseases[18,19] including systemic lupus erythematosus, experimental autoimmune encephalomyelitis, adjuvant arthritis, antiphospholipid syndrome and T1D[20]. In particular, the definitive proof-of-principle that HSCT may represent a promising therapeutic opportunity for T1D patients was borne accidentally from finding a patient who, after undergoing HSCT for hematological indications, developed the autoimmune disease[21]. The rationale of HSCT for the cure of autoimmune diseases is the substitution of the defective immune system by an healthy one that can start from scratch and regenerate undergoing tolerization to self antigens, hopefully in the absence of the supposed accidental environmental circumstances that have led to the initial autoimmune response.

HSCT consists in the administration of HSC which are self-renewing cells identified as $CD34^+$ $CD59^+$ $Thy1^+$ $CD38^{low/-}$ $c\text{-}Kit^{-/low}$ and Lin^- in humans ($CD34^{low/-}$ $Sca\text{-}1^+$ $Thy1^{+/low}$ $CD38^+$ $c\text{-}Kit^+$ and Lin^- in mice) able to give rise to all mature hematopoietic cells and possibly to some non-hematopoietic cells. In the clinical routine, recipients undergoing HSCT are pre-conditioned with a potent immunosuppressive therapy before autologous (auto-HSCT; cells harvested from the recipient before pre-conditioning) or allogeneic HSCT (allo-HSCT; cells harvested from donor other than the recipient). In both instances, HSC are mobilized from the BM to the peripheral blood before conditioning by using different protocols, many of which involve granulocyte colony-stimulating factor (G-CSF) and/or cyclophosphamide, a myelosuppressive drug that leads to a 'rebound' mobilization of these cells. The first choice between allo-HSCT and auto-HSCT in the clinical practice is influenced by the balance between the risk to develop graft-versus-host-disease (GVHD) and the effectiveness to initiate an hopeful graft-versus-autoimmunity (GVA) response. GVHD arises from the attack of donor allogeneic T cells on recipient antigens, while GVA is the result of the immune-mediated destruction of residual recipient's memory T and B cells by host's T cells, a mechanism applicable to allo-HSCT only.

Despite the well-documented clinical success of HSCT in correcting autoimmune diseases[22], an accurate explanation of the mechanisms of action of this treatment is still tricky. Clearly, HSCT relies on an extensive debulking of the recipient's immune system by potent immunosuppressive conditioning protocols such as total body irradiation (TBI), cyclophosphamide, anti-CD2 antibodies, anti-CD52 antibodies, fludarabine and anti-thymocyte globulin (ATG), which leads to profound long-lasting lymphopenia and persistently reduced levels of long-living autoantibody-producing plasma cells[23]. It was demonstrated both in animal models and clinical trials that the use of these lymphoablative therapies alone (without HSCT) for the conditioning regimen can halt or slow *per se* the progression of autoimmune diseases[24]. Aside this quite non-specific immunosuppression, there are growing evidences that auto-HSCT not only has a role in shortening aplasia, but also holds the potential to re-establish the immunological tolerance thanks to: (1) an increase in the number of $CD4^+$ $FoxP3^+$ regulatory T cells (Treg) that are crucial for tolerance preservation[25]; (2) the reactivation of thymic function re-establishing T cell receptor heterogenicity as showed by the presence of recent thymic emigrating cells (TREC) and CD31 expression[26,27]. However, autoimmunity relapse, mainly due to the persistence of autoreactive cells such as surviving memory T cells, memory B cells and long-lived plasma cells in genetically predisposed recipients, may occur. Further studies are strongly required for the evaluation of the optimal condition regimen in relation to the duration and stability of the induced remission. Mild conditioning treatments that do not completely ablate donor's HSC followed by allo-HSCT leads to a condition called "mixed hematopoietic chimerism" in which donor and recipient HSC, and therefore multi-lineage hematopoietic populations, co-exist. Thus in the recipients, a life-long source of donor antigen presenting cell (APC) pool that most effectively presents antigens to T cells positively selected in the recipient thymus is present. In this context, T cells with high affinity for self peptide-MHC complexes are deleted, ensuring tolerance towards donor and recipient antigens (Figure 2). Mixed chimerism induction after allo-HSCT prevents the development of autoimmune diseases with greater efficacy than auto-HSCT does, as demonstrated in animal models[28]. Kaminitz A et al compared auto- and allo-HSCT using the NOD mouse model and explored the degree of donor hematopoietic chimerism required to prevent T1D development. This study demonstrated that: (1) low levels of allogeneic hematopoietic chimerism were sufficient to suppress the autoimmune response and to lead to the resolution of the inflammatory insulitis and (2) transplantation of syngeneic BM cells was largely not effective in insulitis prevention. In order to facilitate allo-HSC engraftment overcoming a potential recipient's T cells response to donor's antigens in the periphery (i.e. GVHD), costimulatory blockade of the CD40-CD154 and CD28-CD80/CD86 pathways has been recently introduced in pre-conditioning non-myeloablative protocols[29]. This strategy, together with allo-HSCT,

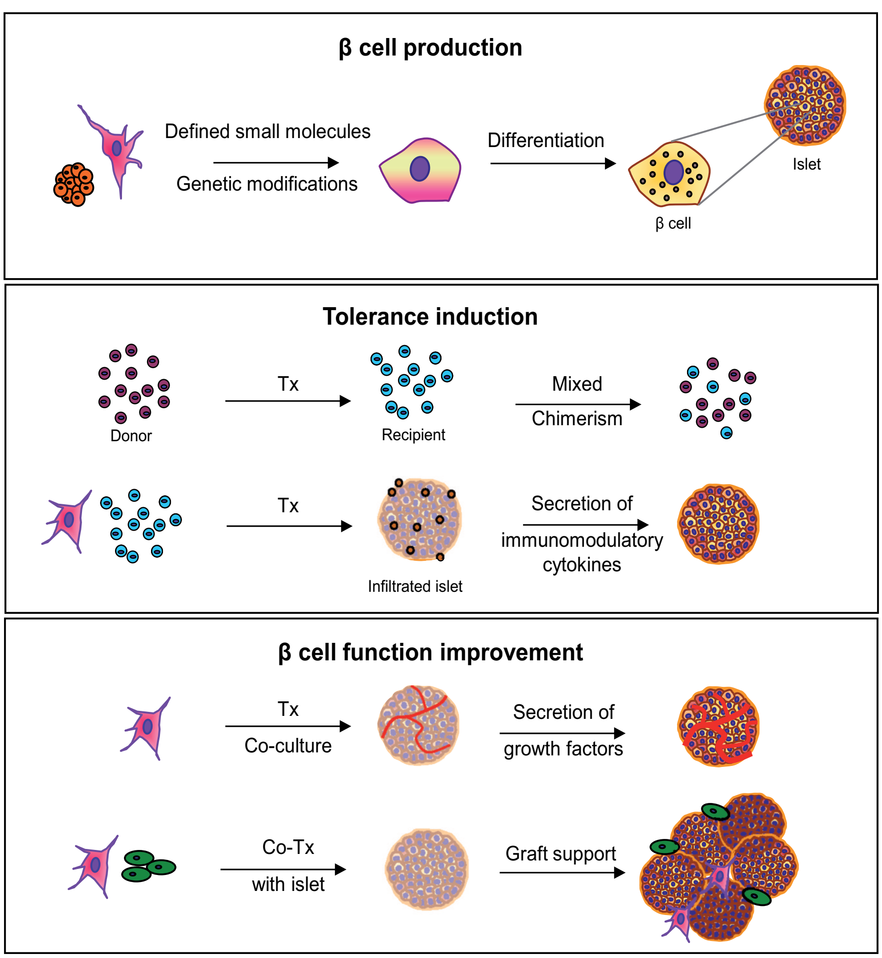

Figure 2. Potential therapeutic application of HSC, MSC, EPC and ESC for the treatment of diabetes. (1) β cell production: MSC and CB-ESC differentiate in vitro into insulin producing β-like cells; (2) Tolerance induction: transplantation of donor HSC induces mixed chimerism in recipient, eliminating autoreactive T cells. Transplantation of MSC or recipient HSC blocks immune attack against islets by cytokine secretion; (3) β cell function improvement: MSC and EPC, alone or in combination with pancreatic islets, improve islet survival and function by secreting cytokines and growth factors able to stimulate vascularization and protect β cells.

is able to lead to tolerance toward existing alloreactive CD4$^+$ T cells in the periphery through anergy (followed by deletion) of these cells, due to the presentation of donor antigens on APCs in the absence of an activation signal. Focusing our attention on T1D among all the autoimmune diseases, the availability of murine models of spontaneous T1D such as Biobreeding (BB) rat[30] and Non-Obese Diabetic (NOD) mouse[31] has allowed to investigate the potential of HSCT in this context. The potential use of BM to alter the course of T1D pathogenesis was first proposed in 1985 in NOD mice through allogeneic BM transplantation[32]. In the last years, allo-HSCT and the induction of mixed hematopoietic chimerism received greatest attention for T1D therapy. Numerous studies have demonstrated that allo-HSCT resulted effective for diabetes prevention and remission in NOD mice[33-35]. Despite the promising results obtained in NOD mice by using allo-HSCT, in the clinical practice the auto-HSCT procedure has been preferred over allo-HSCT because of the lower risk of severe toxicity. Firsts clinical trials were designed to demonstrate that auto-HSCT is safe and feasible for the achievement of a stable normoglycemic state in T1D patients with sufficient residual β cell mass. The first attempt to determine safety and efficacy of a non-myeloablative immunosuppression regimen followed by auto-HSCT in early onset T1D patients comes from a Brazilian phase I/II clinical trial by Voltarelli and colleagues (ClinicalTrials.gov Identifier: NCT00315133). In this study 23 new onset patients (aged 13-31 years) within 6 weeks from T1D diagnosis underwent HSC mobilization with cyclophosphamide and daily G-CSF administration, followed by collection and cryopreservation. Before the reinfusion of autologous HSC, patients received an intensive immunosuppressive conditioning therapy with ATG and cyclophosphamide. During a 7- to 58-month follow-up (mean 29.8 months), 20 out of the 23 patients became insulin independent. Twelve patients maintained this status for 31 months (range 14-52 months) and 8 patients relapsed and resumed insulin use albeit at low dose (0.1-0.3 IU/kg). There was no treatment-related mortality, although two patients developed bilateral nosocomial pneumonia, three late endocrine dysfunction and nine of them oligospermia[36,37]. In 2009 Snarsky at al reported the safety and feasibility of auto-HSCT in a 28-years-old patient with a 4-week history of T1D. Insulin independence was achieved 3 weeks after the transplant, thus confirming the results obtained in the Brazilian group[38]. Subsequently, a Polish group applied the same protocol for HSC mobilization, recipient pre-conditioning and auto-HSCT to a larger number of subjects with T1D diagnosis no longer than 6 weeks. All eight transplanted patients reached insulin independence and achieved a good glycemic control with average HbA1c levels decreasing from 12.3% at T1D diagnosis to 6.2% at 6 months after auto-HSCT. During the follow-up, only one patient resumed low-dose insulin 7 months after transplant[39]. Li et al reported in a cohort of Chinese T1D patients diagnosed within the previous 12 months, that intravenous administration of autologous HSC resulted in: (i) a significant reduction in insulin requirement for an adequate glycemic control in 11 out of 13 patients; (ii) insulin independence in 3 out of 11 patients maintained for 7 months, more than 3 or more 4 years; (iii) normal HbA1c levels for 2 years in 7 out of 8 patients (ClinicalTrials.gov Identifier: NCT01341899)[40]. Using a similar treatment, the same group published a case report demonstrating that insulin independence can be achieved after auto-HSCT in a patient with new onset T1D and concomitant diabetic ktoacidosis (DKA)[41]. Although this successful case report, Gu et al showed in a prospective phase II clinical trial on 28 patients with T1D that auto-HSCT can be an effective long-term treatment to reach insulin independence, but that it's possible to achieve greater efficacy in subjects without DKA at diagnosis[42]. The same group performed a phase II clinical trial (ClinicalTrials.gov Identifier: NCT00807651) in 9 patients diagnosed with T1D within the previous 6 months trying to specifically evaluate whether auto-HSCT was safe when chemotherapy and immunotherapy were combined together. Six of the 9 patients became insulin free, while the remaining three still required insulin injection, although with reduced dosage. Immuno-monitoring of these patients during the 6 months follow-up revealed that: (i) there was no significant differences in immune cell populations (CD4$^+$ and CD8$^+$ T, B and NK cells) despite insulin independence achievement; (ii) T cells differentiated toward Th1 subset after auto-HSCT; (iii) the pro-inflammatory IFNγ signalling pathway was the most significantly modified pathway in patients that remained insulin-dependent[43]. Although the application of auto-HSCT has shown increasing potential for the cure of T1D in adult patients, the above mentioned clinical studies did not contain data from children with T1D. To address this point, a Chinese group designed a clinical study to determine the safety and efficacy of immune intervention combined with auto-HSCT and conventional insulin therapy in the treatment of 42 children (aged 1.5-12.5 years) with newly diagnosed T1D. The study included a case group of 14 patients undergoing auto-HSCT within the first 3 months after T1D diagnosis and a control group of 28 patients with newly diagnosed T1D enrolled in the same period. During the 3-5 years follow-up, the auto-HSCT lead to: (i) a stop of the insulin therapy in 3 out of 14 patients for 2, 3 and 11 months respectively; (ii) no DKA in all the patients that have received auto-HSCT; (iii) significant lower HbA1c levels in control in comparison to the transplanted group and (iv) no significant differences in insulin requirement and serum C-peptide levels between the two groups[44]. The results of a multicenter clinical study involving two Chinese and one Polish centers in 65 individuals with new onset T1D was published in the last months, with the aim to determine the safety and the efficacy of autologous non-myeloablative HSCT. Insulin independence was achieved in 59% of the patients within the first 6 months after the pre-conditioning therapy with ATG and cyclophosphamide and a single infusion of auto-HSCT and maintained in 32% of individuals at the last time point of their follow-up. In all treated patients HbA1c levels were decreased and serum C-peptide levels increased. Despite the encouraging results on the possibility of T1D remission by combining auto-HSCT and immunosuppression, 52% of treated subjects experienced adverse events including one death, suggesting that safer HSC-based therapies are still required and strongly encouraged[45]. Beyond the above mentioned studies, other clinical trials are still active due to ongoing patients recruitment or waiting for a longer follow-up, or they are completed but results have not yet been published. Among them, in Mexico a phase I/II clinical trial tested the efficacy of non-myeloablative auto-HSCT in 15 T1D patients (aged 2-35 years) (ClinicalTrials.gov Identifier: NCT01121029) in order to determine whether it can induce prolonged and significant increases in C-peptide levels and/or absence or reduction of daily insulin injections. Patients enrolled in this clinical protocol received a combination of filgrastim and cyclophosphamide to mobilize HSC and were then pre-conditioned with cyclophosphamide and fludarabine before auto-HSCT. The study was completed, but results have not yet been published. Another phase I/II clinical trial is ongoing in Brazil and will be finished in December 2017 (ClinicalTrials.gov Identifier: NCT01285934). The protocol design include an experimental group undergoing auto-HSCT and a control group treated with intensive insulin injections. Despite numerous clinical studies have been performed, the majority of them did not include in their design a randomized control group that either received no intervention or received only immunosuppression or immunomodulation. Furthermore, only long-term monitoring of β cell function over the coming months and years could finally established how long the achieved clinical results could be maintained and then prove whether the cost/benefit ratio of this approach can support the procedure. Although these clinical data collectively suggested that auto-HSCT could be beneficial for pancreatic β cell function preservation and/or improvement in T1D patients, the question whether this is due to β cell regeneration or to the blockade of the autoimmune destruction of the residual β cells, or both, remains open.

As BM tx has been demonstrated to improve β cell function and/or mass increasing C-peptide levels and potentially leading to insulin independence achievement, thus opening new perspectives in the management of T1D, similarly its potential has been investigated for the treatment of T2D, where β cell loss is due to metabolic exhaustion. The rationale for the use of HSC in T2D included: (i) the secretion of different growth factors such as hepatocyte growth factor (HGF) and vascular endothelial growth factors (VEGF) by HSC resulting in angiogenesis and stimulation of growth, differentiation and survival of the β cells; (ii) trans-differentiation of HSC into β cell and (iii) islet regeneration due to pancreatic stem cells around the pancreatic ducts[46]. Taking advantage from these potential mechanisms of action, HSC were directly injected into the pancreas through the dorsal pancreatic artery. Twenty-five patients with T2D enrolled between March 2004 and October 2006 at the Stem Cells Argentina Medical Center of Buenos Aires, received a combination therapy of intra-pancreatic auto-HSCT along with peri-infusion hyperbaric oxygen treatment. The results of this prospective phase I study were recently published: all metabolic variables tested (fasting glucose, HbAlc, fasting C-peptide, C-peptide/glucose ratio and insulin requirements) showed significant improvement over a period of one-year follow-up when compared to the baseline[47]. Improvement in glucose control and decrease in insulin requirement and oral hypoglycemic agents were reported also in 31 patients with T2D enrolled at the Central Hospital of Wuhan in China[48]. In a recently published study, Hu et al demonstrated the long-term (3 years follow-up) efficacy and safety of autologous BM mononuclear cells infusion in comparison to intensive insulin therapy in 118 patients with T2D. The transplanted group achieved significantly lower HbA1c levels with reduction in oral hypoglycemic drugs and insulin requirement in comparison to the control group. One of the critical points for this clinical study is that it is not conducted in double-blind but patients were allowed to choose among the different treatment, thus potentially leading to wrong conclusions[49]. Intrapancreatic autologous stem cell infusion was also reported as a safe and effective treatment to improve β cell function in 10 patients with T2D at Postgraduate Institute of Medical Education and Research in India[50]. The results of the phase II clinical trial (ClinicalTrials.gov Identifier: NCT00644241) performed to test safety and efficacy of auto-HSCT for the cure of T2D in the same center were recently published. Patients enrolled in this study received a super-selective injection of HSC under fluoroscopic guidance through the superior pancreaticoduodenal artery which is feeding the head and the part of the body of the pancreas composed by a relatively higher density of β cells. Six out of 10 patients showed a reduction in insulin requirement by 74% as compared to the baseline and one patients achieved and maintained insulin independence till the end of the study (15 months follow-up) without any adverse events. Responder patients showed a reduction in HbA1c levels and a significant improvement in glucagon-stimulated C-peptide levels and Quality Of Life scores. However, non-responder patients did not show any significant changes in these parameters[51]. Further randomized controlled clinical trials will be required to confirm these findings. Phase I/II clinical trials of intra-arterial pancreatic infusion of total autologous BM and/or BM derived stem cell are currently underway in China for the treatment of T2D at Fuzhou General Hospital (in combination with hyperbaric oxygen therapy; ClinicalTrials.gov Identifier: NCT00767260) and at Shandong University (ClinicalTrials.gov Identifier: NCT00465478).

Altogether these up-to-date clinical trials involving auto-HSCT and, although to a small extent, allo-HSCT to cure T1D and T2D supported the increasing evidences on the crosstalk between BM-derived cells and pancreatic islets. However, both higher number of transplanted patients and longer duration of follow-up are required to substantiate these observations. Future studies should also evaluate and clarify the effect of HSCT on prevention and cure of diabetes by unravelling the mechanisms involved, allowing the identification of new molecular pathways and the development of new pharmacological strategies to improve both safety and efficacy.

Mesenchymal stem cell transplantation for T1D and T2D therapy: differentiation into insulin producing β cells and/or immunomodulation?

MSC constitute another cellular component of the BM and are an essential HSC niche component. Together with HSC, MSC have been the object of extensive research for decades. More than thirty thousands papers regarding MSC have been published in indexed journals and their capacity to differentiate into multiple lineages, to support hemopoiesis, to exert immunoregulation and secrete growth factors/cytokines have been described. This field of study has gone widening in the last 20 years as new features of these cells were discovered[52-54]. In fact at the beginning MSC were isolated only from BM and classified as the postnatal, self-renewing, and multipotent stem cells for the mesenchymal lineage (bone, fat, cartilage) and as a key player in maintaining HSC in their niche[55,56]. A panel of minimal criteria to define an MSC was then reported and is still greatly in use: ability to adhere to plastic surfaces when cultured under standard conditions, expression of a defined panel of phenotypic markers ($CD73^+$ $CD90^+$ $CD105^+$ $CD45^-$ $CD14^-$ $CD11b^-$$CD19^-$ HLA-DR^- $CD34^-$) and capacity to differentiate into osteogenic, chondrogenic and adipogenic lineages when cultured in specific inducing media[57]. Afterwards, in a second period, MSC have started to be isolated from virtually all post natal tissues (adipose tissue, Wharton's jelly, dental pulp, pancreas, amniotic fluid, liver) and their capacity to differentiate also along ectodermic and endodermic lineages has been reported. As a matter of fact, some studies suggested that MSC might differentiate into nerve cells, heart muscle cells, liver cells and endothelial cells[58], although controversial[59]. In the third and most recent phase, the interest for MSC has shifted from their plasticity to their ability to modulate the function of host tissues, also thanks to the deeper experience acquired with the *in vivo* use of these cells. In fact, a large number of studies reported that MSC hold immunomodulatory and feeder cell functions which are exerted by direct cell-to-cell contacts, secretion of cytokines and/or by a combination of both mechanisms[60] (Fig. 2). The discovery that MSC contribute to tissue regeneration by modulating inflammation ushered in a new interest in MSC as a promising therapeutic tool to suppress inflammation and down-regulate pathogenic immune responses in GVHD, Chron's disease and autoimmune disorders such as diabetes, multiple sclerosis and rheumatoid arthritis.

Some key points about MSC immunomodulatory potential has been established by now, and excellently reviewed recently by Wang and colleagues[60]. Briefly:

Migration. When MSC are exogenously administered by intravenous infusion a large number of cells remains trapped in the lungs, but some MSC migrate to damaged tissue sites such as infarcted myocardium, traumatic brain injury, fibrotic liver and chemically damaged lungs, where they participate in tissue repair[61].

Engraftment. The rate of MSC engraftment *in vivo* is poor, and engrafted MSC tend to be short-lived, which suggest a "hit-and-run" effect of MSC on target tissue[54].

Cytokine release. In response to inflammatory mediators, MSC produce a large number of cytokines, growth factors and cell-mobilization factors able to regulate inflammation and tissue. Among the factors produced there are TNF-α, IL-1, IL-6, IFN-γ, transforming growth factor-β (TGF-β), HGF, epidermal growth factor (EGF), insulin growth factor (IGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), keratinocyte growth factor (KGF), angiopoietin-1 (Ang-1), prostaglandin E2 (PGE_2), VEGF, stromal cell-derived factor-1 (SDF-1), tryptophan-catabolic enzyme IDO, nitric oxide (NO) and inducible nitric oxide synthase (iNOS)[62].

Anti/pro inflammatory action. MSC have the capacity to modulate immune response both as suppressor and as enhancer, depending on the type and on the intensity of the signals they receive from the microenvironment.

Effect on immune cells. MSC exert an effect on cells of the innate and adaptive immune systems and in particular they are able to suppress the function of T and B cells, NK cells, dendritic cells, macrophages and neutrophils[63].

Feeder cell action. In the process of tissue repair, MSC are thought to exert an action also on endogenous cells of damaged tissue, for instance protecting cells from apoptosis or stimulating cell proliferation[64].

This path of knowledge described until here about MSC and their use, from multipotent cells to cells that secrete key factors for the immune response and tissue remodelling, was likewise followed in the field of diabetes. In fact the first efforts have been focused on *in vitro* transdifferentiation of MSC into insulin producing cells, with the aim to provide a tissue source for autologous cell tx.

In vitro differentiation into β cells

Many attempts have been made to differentiate isolated MSC *in vitro* into insulin producing cells (Fig. 2). Several studies reported the appearance of insulin mRNA in cultures of MSC treated with defined combinations of growth factors[65-67]. To give an example, also very recently a study was published about the differentiation of MSC into β cells: a protocol of 18 days of differentiation with the addition of FGF-β, EGF, activinA and β-cellulin. Differentiated cells formed cell clusters some of which resembled pancreatic islet, stained positive with dithizone and were able to produce C-peptide[68]. The limits of this and of many studies published before is that, at a deeper look, none of these differentiated cells exhibit the necessary conditions to be defined as β cells: insulin secretion in response to glucose stimuli and capacity to normalize glycemia in diabetic animal models. Moreover, safety is an issue when stem cells are forcedly converted in another cell type. For instance, in a recent study, MSC were induced to differentiate into islet-like clusters: newly formed islet-like cells expressed multiple genes related to islet development and β cell function, produced insulin, demonstrated time-dependent glucose-stimulated insulin release, and the ability to ameliorate hyperglycemia in chemically-induced diabetic mice, but, when transplanted in diabetic immunocompromised mice, differentiated cells became tumorigenic[69]. So far, although knowing that the risk of neoplastic transformation may be even greater, the most convincing data of MSC reprogramming to functional β cells involve the use of genetic modifications. To this purpose, pancreatic transcriptional factors are the mostly used candidates[70]. This approach is mainly based on the forced expression of pancreatic duodenal homeobox-1 (Pdx1) and/or Ngn3 in MSC, as reported for MSC derived from BM[71-74] and from CB[75-78]. Pdx1 gene is crucial for the transdifferentiation to pancreatic endocrine cells: in fact, it can shuttle to the nucleoplasm of MSC under high glucose stimulus, then initiate the expression of Ngn3 and recruit other proteins, resulting in transactivation of relevant genes (including insulin) and generating β cell phenotype. For example, MSC transfected with Pdx1 cDNA were shown to secrete insulin in response to glucose stimulus and the formed islet-like structure resulted positive to dithizone staining[79]. Besides, in a recent paper by Guo and colleagues a procedure for induction of insulin-producing cells from murine BM-MSC based on the transfection and expression in these cells of a combination of the pancreatic transcription factors Pdx-1, NeuroD1 (neurogenic differentiation-1), and MafA (V-maf musculoaponeurotic fibrosarcoma oncogene homolog A) genes was reported. With this procedure insulin biosynthesis and secretion were induced in MSC, and transplantation of the transfected cells into mice with streptozotocin-induced diabetes resulted in the reversal of the glucose challenge[80]. This strategy of MSC transdifferentiation through genetic manipulations still needs improvements to increase the efficacy in order to generate a good candidate for β cell replacement, although it is obviously anyhow limited by the risk of tumorigenesis. This year a new interesting contribution came with the use of Laminin 411 for the induction of MSC differentiation into β cells: in fact Laminin 411 strongly promoted the expression of the genes Foxa2 and Sox17 which leads to up-regulation of insulin transcription and translation. Besides, treatment with Laminin 411 was able to induce the expression of Pdx1 and Ngn3 and the insulin producing cells obtained with this treatment were able to normalize glycemia and improve the survival of diabetic rats[76].

MSC exert immunomodulatory and feeder cell activity in vivo

The immunomodulatory capacities displayed by MSC have been tested as beneficial agents for autoimmune diseases and in particular for prevention and treatment of T1D. Several studies in preclinical models have shed lights on different aspects of MSC effect. First, the role of MSC as feeder cells for endogenous pancreatic cells (Fig. 2). Lee and colleagues reported that MSC home to and promote repair of pancreatic islets and renal glomeruli in diabetic mice[81]. In this paper human MSC were delivered via multiple intracardiac infusions in hyperglycemic NOD/scid mice. MSC infusion was able to lower blood glucose levels in diabetic mice and mouse insulin measurement was higher in the MSC-treated compared with untreated group, but the presence of human insulin in the serum was not detected. Rare islets containing human cells that co-labelled for human insulin or Pdx-1 were found into mouse pancreases, but most of the β cells within the islets were cells that expressed mouse insulin, demonstrating that MSC effect was mainly exerted on recipient pancreatic cells[82]. A single intravenous injection of MSC in diabetic mice was subsequently tested in order to study the recovery of pancreatic and renal function and structure. One week after tx, only MSC-treated diabetic mice exhibited significant reduction in their blood glucose levels, reaching nearly euglycemic values a month later. Reversion of hyperglycemia and glycosuria remained for 2 months at least. An increase in the number of morphologically normal pancreatic islets was observed only in MSC-treated diabetic mice. Thus, MSC administration resulted in pancreatic islets regeneration and prevented also renal damage in diabetic animals. In an attempt to enhance the effect of MSC, BM cells were administered together with syngeneic or allogeneic MSC into sublethally irradiated diabetic mice[83]. Blood glucose and serum insulin concentrations rapidly returned to normal levels, accompanied by efficient tissue regeneration after a single injection of a mixture of BM cells and MSC. Successful treatment of diabetic animals was not due to the reconstitution of the damaged islet cells, since no donor-derived β cells were found in the recovered animals, indicating a graft-initiated endogenous repair process. Moreover, MSC injection caused the disappearance of β cell-specific T cells from diabetic pancreas. These evidences suggest that BM cells and MSC were able to induce the regeneration of recipient-derived pancreatic insulin-secreting cells and that MSC inhibited T-cell-mediated immune responses against newly formed β cells. As reported above, MSC exert an effect on many types of immune cells and indeed they were shown to protect NOD mice from diabetes by inducing Treg cells[84]; in this paper, MSC were able to suppress *in vitro* both allogeneic and insulin-specific proliferative responses and this suppressive effect was associated with the induction of IL10-secreting $FoxP3^+$ T cells. Moreover, MSC infusion reduced the capacity of diabetogenic T cells to infiltrate pancreatic islets *in vivo* and to transfer diabetes. Finally, MSC co-transfer inhibited the decrease in levels of Treg induced by injection of diabetogenic T cells. The effect of MSC on immune cells is mainly sustained by cytokine secretion. In one study diabetic mice transplanted with intravenous injection of syngeneic MSC reverted their hyperglycemia state even if presented no donor-derived insulin-producing cells. In contrast, 7 and 65 days post-tx, MSC were engrafted into secondary lymphoid organs. This correlated with a systemic and local reduction in the abundance of autoreactive T cells together with an increase in Treg cell number. Additionally, in the pancreas of mice treated with MSC, a cytokine profile shift from pro-inflammatory to anti-inflammatory was observed. Besides, EGF circulating levels were found increased in MSC transplanted mice. This study underlined the capacity of MSC to restore the balance between Th1 and Th2 immunological responses and to modify the pancreatic microenvironment[12].

The experiences *in vitro* and in animal models of diabetes, together with the increasing number of data regarding clinical applications of MSC in other diseases[85], has led to the development of trials also in diabetes field. Among these clinical trials, until today only one has been completed and data have been published[86]. This study (ClinicalTrials.gov Identifier: NCT01068951) was performed at the University of Uppsala (Sweden) and was aimed to evaluate the safety and efficacy of BM derived autologous MSC tx in patients with recent onset of T1D. The starting hypothesis was that an increased number of circulating MSC would provide immunomodulation, and thereby stop the immune process causing progressive β-cell death in islets. Twenty patients were randomized in MSC or control group. Safety of treatment was proved, since autologous treatment with MSC was well tolerated and no side effects were observed. Changes during the first year in C-peptide response to a Mixed Meal Tolerance Test (MMTT) were evaluated as primary efficacy end point. In response to MMTT, patients in the control arm had an expected decrease in both C-peptide peak values and C-peptide when calculated as Area Under Curve (AUC) during the first year; in contrast, these responses were preserved in MSC-treated patients. These encouraging results opened the way to a larger, randomized, and double-blinded study, with a longer follow-up, to validate the findings obtained. This new study (ClinicalTrials.gov Identifier: NCT02057211) is now recruiting participants and the estimated completion date is May 2017. Another important clinical trial was performed by Mesoblast International Srl in partnership with Juvenile Diabetes Research Foundation. This study (ClinicalTrials.gov Identifier: NCT00690066) was a phase II, multicenter, randomized, double-blind, placebo-controlled study aimed to test safety and efficacy of Prochymal®, a human BM-derived MSC line, in recently diagnosed T1D patients. The *interim* assessment at one year showed that systemic infusions of Prochymal® were well-tolerated and there were no differences in adverse event rates between the Prochymal® and the placebo groups. At that early time point no significant differences in disease progression, as measured by stimulated C-peptide levels, have been observed; however, there was a trend towards fewer hypoglycemic events for patients Prochymal-treated compared to controls. This study is now concluded and a complete analysis of the data is expected. A new study (ClinicalTrials.gov Identifier: NCT01157403) is ongoing at the Third Military Medical University of Chongqing, in China and it is at the moment recruiting patients. In this case the aim of the trial is to test the effect of autologous tx of BM-MSC administered intravenously in recently diagnosed T1D patients.

The potential of MSC to ameliorate hyperglycemia in diabetic animals by the release of trophic factors have pushed the research on the use of MSC also in T2D. In fact it was recently reported that multiple intravenous MSC infusions may reverse hyperglycemia in T2D rats[87]. Briefly, allogeneic MSC were administrated to T2D rats intravenously once every 2 weeks; hyperglycemia decreased only transiently after a single infusion in early-phase (1 week) T2D rats, but normoglycemia was achieved after at least three infusions and maintained for at least 9 weeks. Serum concentrations of both insulin and C-peptide were dramatically increased after serial MSC infusions. Oral glucose tolerance tests revealed that glucose metabolism was significantly improved. In another paper the hypothesis that MSC might also contribute to amelioration of the insulin resistance was tested[88]. MSC infusion was performed during an early (7 days) or a late phase (21 days) after diabetes induction to test their therapeutic effects. Infusion of MSC during the early phase not only promoted β cell function, but also ameliorated insulin resistance, whereas infusion in the late phase had a mild positive effect. The therapeutic potential of MSC infusion was investigated also through infusion into the pancreatic artery of diabetic macaques[89]. Six weeks after BM-MSC tx, blood glucose and lipid levels were significantly lower in the treated compared to the control group. Additionally, the serum C-peptide levels were significantly increased and an intravenous glucose tolerance test and C-peptide release test showed significant changes to the AUC. Si et al proposed that MSC can enhance β cell function by elevating phosphorylation of insulin receptor substrate 1 (IRS-1) and Akt (protein kinase B) in insulin target tissues thereby reducing hyperglycemia[88]. Collectively, these reports suggested that the secreted trophic factors or the MSC themselves had a positive effect on T2D outcome by either protecting the remaining β cells or stimulating the generation of endogenous β cells from resident stem cells, or by reducing the peripheral insulin resistance. Also clinical trials of MSC therapies for the treatment of T2D have been approved, but the final results have not yet been published. Currently, two clinical trials are ongoing: the first is a study by Mesoblast (ClinicalTrials.gov Identifier: NCT01576328), the same company that was testing Prochymal®, which is conducting a study of mesenchymal precursor cells transplantation in T2D. It is a randomized, placebo-controlled, dose-escalation study with the aim to assess safety and tolerability of a single intravenous infusion of allogeneic MSC in patients sub-optimally controlled on metformin. The other is a Chinese study (ClinicalTrials.gov Identifier: NCT01954147) which is testing a combined therapy of umbilical cord derived MSC tx and Liraglutide in T2D patients. The investigators hypothesized that this combined treatment will allow stem cells differentiation into insulin producing cells, improve their survival, protect the residual β cells and improve insulin secreting function, so as to achieve a favourable glucose homeostasis. Another study (ClinicalTrials.gov Identifier: NCT01759823) focused on efficacy and safety of autologous MSC tx is now recruiting patients in India. The hypothesis was that intra-pancreatic MSC infusion in T2D patients may lead to increased angiogenesis, secretion of various cytokines and VEGF, upregulation of pancreatic transcription factors and contribute to create a microenvironment which supports β cell survival and resident stem cell activation. Other clinical trials, aimed to establish safety and efficacy of MSC infusion in T2D patients, are still recruiting patients: in Florida (ClinicalTrials.gov Identifier: NCT01453751) with autologous adipose-derived MSC, which will be intravenously implanted; in China (ClinicalTrials.gov Identifier: NCT02302599) with allogeneic UCB-MSC; in India (ClinicalTrials.gov Identifier: NCT01759823) with autologous BM-derived MSC.

Umbilical cord as a source of stem cells

Another source of stem cells with differentiation potential and immunomodulatory capacities comparable to BM-derived stem cells is umbilical cord blood (UCB), which consists in the blood left over in the placenta and in the umbilical cord (UC) after childbirth. In humans, UC normally contains two umbilical arteries and one vein, included within the surrounding connective tissue called Wharton's jelly[90]. Following the first UCB tx in 1988 for the treatment of Fanconi's anemia[91], the past decades have led to increased use of UCB as a source of cells for tx to treat many hematological and non-hematological diseases[92]. In fact, compared to other stem cells, UCB-derived cells can be easily collected, cryopreserved and stored for years without significant loss of viability[93,94]. In the last decades, because of the increased demand of UCB storage, public and private banking of UCB became more widespread in many parts of the world[95]. The umbilical cord contains about 60-200 ml CB[96] and harvesting UCB can yield an average of $10x10^6$ total nucleated cells (TNC) per ml of tissue collected[97]. UCB is composed of red blood cells, white blood cells, plasma, platelets and is also rich of cord blood stem cells (CB-SC) that are self-renewable multipotent/pluripotent progenitor with the potential to differentiate into various lineages[98]. In contrast to adult BM-derived HSC, CB-SC display many advantages, including an eightfold greater proliferative potential, a higher cell-cycle rate and a relatively longer telomere length[99]. Moreover, because of the immunological immaturity of this tissue, unrelated UCB tx tolerates greater HLA disparity between the donor and the recipient and may result in reduced severe acute GVHD[100,101]. UCB has been reported to be a source of many different kinds of stem cells, including embryonic stem cells, EPC, MSC and HSC[98,99] (Fig. 1). CB embryonic stem cells are a recently discovered cell population characterized by cells with very small size and low density[102] that express the embryonic markers Oct4, Nanog and SSEA-4[103] and are considered to be virtually totipotent. CB-derived EPC are $CD133^+$ $CD34^+$ $VEGFR2^+$ cells and are considered as the most promising source of stem cells for integration into vascular structures with the goal of regenerating vascularization processes[104]. MSC are identified as $CD44^+$ $CD73^+$ $CD90^+$ $CD105^+$ cells with the potential to differentiate into various lineages such as chondrogenic, adipogenic and osteogenic. These cells can be easily collected from UCB and Wharton's jelly[90] and utilized for both differentiation studies or *in vivo* tx as discussed before.

Related and unrelated CB-derived HSC are now considered the most appropriate cells for tx procedures for the treatment of hematological and non hematological diseases[105] for the majority of patients who are unable to identify a fully matched donor[92].

During the past years, CB cells tx for the regulation of immune imbalance in various autoimmune diseases has gained great interest[106-108]. In particular, the application of UCB-derived cells for the treatment of diabetes has a high therapeutic potential due to the variety of stem cells available in this tissue; in fact, all the key issues of this disease can be addressed such as control of autoimmunity through induction of hematopoietic chimerism and immune tolerance restoration or overcoming the shortage of insulin-producing cells through differentiation processes. Indeed, it has been demonstrated that CB-SC can be driven *in vitro* to become insulin secreting cells, as confirmed by the production of insulin and C-peptide, but their engraftment and survival *in vivo* has not been tested[109,110]. The presence of human insulin$^+$ cells was also reported within pancreatic tissue after *in vivo* differentiation of CB-SC transplanted into immunodeficient normoglycemic mice[111] albeit with a very low efficiency (<1%). In another study the efficiency of *in vivo* differentiation of UCB-derived cells into insulin producing cells was incremented when mice underwent pancreatectomy two weeks after tx, but these cells were not glucose-responsive[112].

Despite these promising works, the greatest interest regarding the use of UCB-SC for diabetes treatment still remains related to their potential role in restoring immune regulation. The fact that UCB contains a large population of immature unprimed highly functional subpopulation of CD4$^+$ T cells, the CD4$^+$ CD25$^+$ FoxP3$^+$ Treg, has become the base of the first clinical trial for UCB tx in patients with T1D[113]. CB Treg cells in fact have the potential to decrease the inflammatory cytokine response and anergize the effector T cells that play a key role in cellular-mediated autoimmune processes[114] restoring immune tolerance. In the first pilot study 15 children (mean age of 5.5 years) with recently diagnosed T1D (mean time of 4.1 months since diagnosis) have been infused with autologous UCB and monitored for immunologic and metabolic assessment every 3 to 6 months. At 6 months, an increased Treg population in the peripheral blood, a slowing of the loss of endogenous insulin production and no significant adverse events associated with UCB infusion, were observed[113]. One year post-infusion however, no changes were observed in insulin requirement, C-peptide measurement, autoantibody titers or Treg cell numbers, indicating that the procedure is feasible and safe, but has yet to demonstrate efficacy[115]. The same results were observed at the end of the study (2 years follow-up), concluding that a single infusion of minimally manipulated autologous UCB in young children with T1D fails to preserve C-peptide[116], neither when infusion was followed by 1 year of supplementation with immunomodulatory agents such as vitamin D and docosahexaenoic acid[117]. One reason for the failure of these trials could be that an insufficient number of cells carrying regenerative or immunoregulatory capacity may have been transferred into patients. In fact, in a recently published work in which seven children with newly diagnosed T1D underwent a single autologous UCB infusion, Giannopoulou et al demonstrated that patients who received more TNC per kg showed better preservation of residual β cell function, as assessed by C-peptide measurement after stimulus[118]. To address this issue, strenuous efforts are ongoing to isolate and expand specific cell populations within UCB in order to increase their therapeutic potential[117]. In another study the efficacy of UCB tx has been tested also in T2D patients. UCB cells were infused by micro-catheter into the dorsal pancreatic artery in 3 subjects with different diabetic histories. The most important observations of the study were that after UCB tx (i) C-peptide levels increased in all patients by the third month and (ii) the requirement for insulin and oral hypoglycemic agents was reduced. The positive outcome of this study compared to those performed in T1D patients was possibly due to a less serious immune injury, a better microenvironment surrounding transplanted cells in T2D patients and a different method of UCB perfusion[119].

A different approach has been designed by Zhao et al, who discovered that UCB-SC displayed immunomodulatory effects *in vitro* on human allogeneic T lymphocytes[120]. Recently, the same group demonstrated that co-culture of human UCB-SC with purified NOD mouse spleen cells was able to induce a subpopulation of Treg CD4$^+$CD62L$^+$ but CD25$^-$ that reversed established diabetes in NOD mice. The treatment with these autologous unconventional subset of Tregs was able to eliminate hyperglycemia promoting islet β cell regeneration, reducing insulitis and inducing apoptosis of infiltrated leukocytes in pancreatic islets[121]. The same strategy, called "Stem Cell Educator therapy", has been then translated to human: 15 subjects (median age of 29 years old, range 15-41) with a median diabetic history of 8 years (range 1-21) were infused with autologous blood-derived T lymphocytes "re-educated" through the exposure to allogeneic CB-SC. Stem Cell Educator therapy markedly improved C-peptide levels, reduced the median HbA1C values, and decreased the median daily dose of insulin in both patients with residual (n = 6) or no evident (n = 6) β cell function, indicating that this therapy is able to control the immune response sufficiently to allow regeneration of the native β cell population. Moreover, patients who received educated cells exhibited an increase in the number of Treg cells and in the production of the immunoregulatory cytokine TGF-β1 four weeks after infusion. The therapy was well-tolerated and no adverse effects were reported[122]. An open-label, phase I/II Stem Cell Educator therapy study has been performed also in 36 patients with long-standing T2D. Clinical findings one year after infusion of autologous educated cells indicated that treated patients achieved improved metabolic control (significantly reduced median HbA1C and increased insulin sensitivity) and reversed immune dysfunctions through immune modulation of monocytes/macrophages and balance of Th1/Th2 cytokine production[123]. The efficacy and safety of this innovative approach is currently being tested in a phase I/II clinical trial in children with T1D (ClinicalTrials.gov Identifier: NCT01996228).

In conclusion, among the broad array of potential cell-based therapies, the use of autologous UCB as a source of immunomodulatory cells or exposing a patient's lymphocytes to CB-SC represent two promising strategies for the treatment not only of diabetes, but also of other autoimmune diseases.

BM-derived stem cells in combination with pancreatic islet transplantation

The physical replacement of the β cell mass constitutes the rationale for islet tx. Allogeneic pancreatic islet tx is a minimally invasive and safe option for patients with T1D able to induce restoration of physiological glucose sensing and insulin delivery. Sustained graft survival is achieved in the majority of islet tx recipients, but the rate of insulin independence may progressively decline after tx reaching about 10-50% at 5 years[124]. Several factors contribute to the progressive islet graft failure observed over time and limit the widespread application of this procedure: (i) the generation of nonspecific inflammation early after tx, which leads to loss of a substantial mass of the implanted islets, (ii) the increase of hypoxia, due to a delayed revascularization, (iii) the activation of allo- and auto-reactive T cells, (iv) the need for life-long immunosuppressive therapy[125]. Co-tx of islet with stem cells is a promising option to improve their survival and function, overcoming the current challenges of islet tx. Among BM-derived stem cells, the best candidate for a protective therapy in diabetes are EPC, MSC and HSC.

Stem cells and islet tx: re-vascularization of the graft

BM-EPC are one of the main experimental tools aimed at improving revascularization (Fig. 2). A recent report by Quaranta et al. suggested that vascularization is a crucial step to achieve stable normoglycemia. Syngeneic islets and GFP+ EPC were co-transplanted in diabetic rats; recipients co-tx with islets and EPC exhibited a better glycemic control than the control group transplanted with islets alone, thus highlighting the importance of a newly formed viable vascular network to obtain a functional graft[126]. Recently also another group emphasized the relevance of BM-EPC infusion in a preclinical model of islet tx; indeed, BM-EPC co-transplanted with islets improved the outcome of marginal mass islet transplantation by promoting revascularization and preserving islet morphology[127].

Also MSC have been investigated for their possible action on islet revascularization after tx. The capacity of MSC, by secretion of a large number of cytokines, chemokines and other factors, to produce repair and functional improvement in injured tissues is well known and was detailed before in this review. Park et al reported that MSC secreted numerous trophic molecules such as IL6, IL8, HGF, insulin like growth factor binding protein 4 (IGFBP4), VEGFA, Von Willebrand factor and TGF-β[128]. Other studies in animal models described the ability of MSC co-transplanted with islets to enhance graft function and survival by increasing islet revascularization[128-130]. *In vitro* co-culture of MSC with islets before tx increase their ability to reverse hyperglycemia *in vivo*, thus suggesting that pre-conditioning could exert a positive effect on islet tx outcome[131]. Figliuzzi et al demonstrated that MSC co-tx with the islets under the kidney capsule improved graft function and revascularization by secreting VEGF[132]. Accordingly, we also demonstrated that co-localization of

MSC and islets in a marginal mass islet tx promoted graft function and vascularisation[133]. The same observations were reported also when syngeneic islet and MSC were co-tx into the liver of diabetic rats. One week after infusion the histological analysis revealed a well-preserved and vascularized graft only in the rats co-tx with MSC and islets[130]. Similarly, in a preclinical monkey model of allogeneic islet tx, Berman et al demonstrated that the co-tx with MSC enhanced the engraftment providing an increase in the revascularization process[132]. Other mechanisms of action behind the ability of MSC to sustain islet function after tx was recently reported by Remuzzi et al. The authors described a "double" effect exerted by MSC: the release of trophic factors increased islet survival, while the expression of Pdx1, induced by direct contact of MSC with islets, resulted in their differentiation into insulin releasing cells[134]. An innovative strategy adopted in order to overcome the limitation of current islet tx strategy has been the tx of co-encapsulated islets and MSC under the kidney capsule. Results demonstrated the ability of MSC to improve graft revascularization and insulin content and secretion both *in vitro* and *in vivo*[135]. Pancreatic islets were also co-cultured with MSC on a silk-based scaffold incorporating ECM proteins (Laminin and Collagen IV) able to improve insulin secretion and gene expression of functional genes such as insulin I, insulin II, glucagon, somatostatin and Pdx1[136]. Further development of this system may become a suitable platform for *in vivo* islet delivery. EPC and MSC were also combined together with human islets on a composite structure to promote islet revascularization before the infusion in immune-deficient animal model, in order to enhance neo angiogenesis and islet survival[137].

Overall, the local and systemic effects of multiple infusions of stem cells could provide new perspectives in islet tx, whereby these cells support pancreatic β cell replacement providing them an adequate supply of survival and trophic factors and inducing revascularization.

Stem cells and islet tx: immunomodulation of the graft site

One of the principal goal to be addressed in islet tx is the optimization of immunosuppressive therapies thus limiting the undesired side effects. In this way a relevant aspect of stem cell therapy application in islet tx is the possibility to modulate the immune response against graft antigens. As previously described, BM-derived MSC and HSC could exert immunomodulatory activity. In particular, recent studies revealed that MSC affect several mechanisms of different cellular components of both innate and adaptive immunity (Fig. 2). In this context, it has been demonstrated that MSC strongly act on T cells by (i) efficiently suppressing the proliferation of $CD4^+$ and $CD8^+$ T cells[138-140]; (ii) reducing IFN-γ production by $CD4^+$ Th1 cells and IL-17 release by $CD4^+$ Th17 cells, whereas increasing IL-4 secretion by $CD4^+$ Th2 cells[141-143]; (iii) impairing the cytolytic potential of Cytotoxic T lymphocyte (CTL)[144]; (iv) markedly promoting the expansion and the inhibitory capacity of regulatory T cells[145]. Other studies have also shown that MSC have the capacity to modulate DC by (i) impairing the differentiation of human blood monocytes into immature DC as well as DC maturation[146-148]; (ii) inhibiting endocytosis and IL-12 production by DC[148]; (iii) suppressing the capacity of DC to stimulate T cell proliferation, reducing DC-mediated polarization of naïve $CD4^+$ T lymphocytes into pro-inflammatory Th1 cells and promoting the induction of Th2 cell responses. In few studies was also investigated the impact of MSC on macrophages[149] and NK cells[141,144,150]. In 2009 a pioneer study demonstrated for the first time in a preclinical model of marginal mass islet tx, the efficacy of MSC in prolonging graft function and survival with a low dose of immunosuppressive drugs[151]. Ding et al then dissected the molecular mechanism at the basis of immunomodulation when MSC were combined with allogeneic islet tx. They clearly described that MSC are responsible of the modulation of T cell response by reducing CD25 expression through the secretion of matrix metalloproteinases (MMP)-2 and 9. *In vitro* the abrogation of MMP-2 and 9 completely abolished MSC-induced suppression of T cell proliferation and restored CD25 expression in T cells and their sensibility to IL-2[152].

Yeung et al recently demonstrated that MSC were able to protect islets from cytokines-induced damage. Human islets co-cultured with BM- and pancreas-derived MSC and exposed to IFN-γ, TNF-α and IL-1β, were protected from inflammatory-induced damage and apoptosis thanks to the release of the cytoprotective factors HGF and MMP-2 and 9 by MSC[153]. In a preclinical mouse model, MSC co-transplanted with islets under the kidney capsule were able to delay graft rejection by inhibiting the proliferation and the development of alloreactive effector T cells and potently enhancing the induction of regulatory T cells[154]. In order to further improve the outcome of human islet and MSC co-tx, Mundra et al genetically modified MSC by inducing the expression of hIL-Ra and VEGF. Islet co-transplanted with these modified MSC into diabetic immunodeficient mice showed improved glycemic control and better islet viability after cytokines stimuli[155]. In 2010 Berman et al published a promising study about the co-tx of islets, MSC and BM in a cynomolgus monkey model. Allogeneic MSC were intra-portally infused simultaneously with islet and a HSC suspension was injected intravenously at days 5 and 11. They observed that MSC significantly improved islet engraftment and function 30 days after tx. Moreover, the additional infusion of HSC determined a reversion of the rejection episodes and prolonged islet function in a small number of monkeys. Immunophenotype analysis of T cells in recipients with stable graft function showed an increase in the total number of Treg in peripheral blood[156].

To test the real immunosuppressive ability of stem cells in islet tx setting, is crucial to study their effect in a model of autoimmune diabetes. The fascinating hypothesis that donor cell chimerism is necessary to obtain a central tolerant state and prevent autoimmune response, triggered different protocol to combine HSC or MSC prior or with islet infusion. In a NOD mouse model of islet tx Kang et al. reported that infusion of unfractionated BM before the onset of T1D was able to prevent the disease in all treated mice for one year after tx, while the same treatment performed at 2 weeks after the onset was unsuccessful. Moreover, in order to test whether tolerance to islets was achieved, islets from the same allogeneic donor strain as the BM cells were transplanted two weeks after BM infusion in four recipients: two of them showed graft acceptance and reversion of the disease[157]. Another similar experience was reported by Itakura and colleagues: diabetic rats were co-transplanted with islets, allogeneic BM cells and MSC after a pre-conditioning total body irradiation. Although all the recipients rejected the islets, half of them developed a stable mixed chimerism and donor-specific immune tolerance, as shown by the engraftment of a second islet transplant[158].

Altogether the evidences obtained studying the preclinical models promoted the development of several clinical trials. Many of them showed an increase in allograft islet survival and a reduction of adverse events by using high doses of donor allo-HSC. Diabetes Research Group in Miami was one of the first to co-tx stem cells and pancreatic islets into diabetic patients; since 1994 to 2007 they started to combine islet tx to the use of BM stem cells with different clinical indications. The unfractionated BM or HSC used in these studies were obtained from the vertebral bone of the same allogeneic pancreas donors. The primary end points in all the trials were tolerance induction against islet graft and hematopoietic chimerism. The first trial started in 1994 and enrolled eight patients; seven of them received simultaneously islets and kidney (SIK) and only one islet after kidney (IAK). In this report, chimerism was achieved and maintained for 12 months, but the loss of islet function was observed within in the first 6 months after tx. From 1998 to the latest trial in 2007 Miami group collected three different experiences of islet alone tx (IAT) co-infused with HSC. In the latest clinical trial the five patients enrolled received a single islet infusion on day 0 with an Edmonton-like immunosuppression therapy and two intravenous injection of donor HSC at day 5 and 11. Clinical primary endpoint in this study was the acceptance of islets after weaning of immunosuppression thanks to the induction of hematopoietic chimerism. Unfortunately, the co-tx did not lead to a solid chimerism and the islet function was prematurely lost during the follow up or after the suspension of immunosuppressive regimen[159]. Another clinical trial was started in 2008 in China, at Fuzhou General hospital. This trial, with an estimate study completion in 2014, is aimed to the evaluation of the safety and the efficacy of co-tx of islets and MSC in T1D patients. The rational of the trial is based on the hypothesis that MSC infusion could improve engraftment in the transplant site and protect the graft from inflammatory damage and allo- and auto-immune reaction (ClinicalTrial.gov Identifier: NTC00646724). Although the preclinical experience highlighted the pivotal role of stem cells in improving islet tx during engraftment of the islets and immune reaction, clinical experience is limited to a reduced number of cases. In these reports the failure of the expected clinical outcomes is probably related to some limitations in these studies, including the use of conventional immunosuppression and the lack of myeloablative strategies. Since preclinical experience suggested the use of MSC as paracrine cells able to modulate the transplant microenvironment, probably the co-localization of these cells and islets within the tx site could improve the efficacy also in the clinical practice. More clinical trials are probably needed in order to better display the stem cell potency also from the clinical point of view.

Conclusions

BM transplantation, either autologous or allogeneic, is successfully used to treat hematopoietic diseases. BM transplantation was firstly used to treat leukemia in 1978 and recent research has suggested its efficacy also in non-hematological diseases such as autoimmune diseases, aging-related disorders and malignant tumors. In diabetes field, a huge amount of clinical trials for the cure of T1D and T2D, involving BM-derived HSC and both BM- and CB-derived MSC are ongoing, thanks also to the availability of simple protocols for collection, culture and storage of these stem cells. Despite this, consistent and reproducible results are still lacking and only a small subset of patients with early-onset T1D could benefit from this kind of approach. To date, the results obtained with MSC in preclinical and in particular in the first clinical experiences are only preliminary, and require higher numbers and longer follow up. Instead, pioneering studies using HSC have assessed the efficacy of autologous BM reconstitution following immunosuppressive therapy, especially in new onset T1D patients. Overall, these studies, based on the harvest by aphaeresis of mobilized BM progenitors under the coverage of cyclophosphamide to prevent add back of effector lymphocytes, suggest that immunosuppressive therapy and auto-HSCT decrease exogenous insulin requirement in approximately 60% and 40% of the patients for one and two years, respectively. Despite this, the short-term outcome of these clinical transplants is similar to the predictions drawn from NOD mice: the debulking of diabetogenic cells by immunosuppressive drugs is ineffective, and resetting of immune homeostasis does not restrain autoimmunity. Finally, all the described cell therapy retain the concern for potential adverse effects. In the case of auto-HSCT for example, even if there are not enough data from T1D patients, this procedure has been used to treat other autoimmune diseases in children or adults for more than 15 years. Instead, over time we learned that allogeneic stem cells transplantation is associated with significant morbidity and mortality, and is therefore not yet considered the standard of care for non hematological diseases. In the early post-HSCT phase, bacterial or fungal infections occur and therapy-associated lymphopenia sets patients at risk for reactivation of endogenous viruses and other opportunistic infections. During re-activation of lymphopoiesis after transplant, *de novo* autoimmunity may develop through loss of central or peripheral control mechanisms. Late effects of auto-HSCT, like a potentially increased frequency of secondary malignancies, are also of concern. In the case of transplantation of other stem cells, such as MSC, the knowledge of these kind of short- and long-term complications is even more limited by the reduced number of clinical studies ongoing.

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Conflict of Interest

The authors declare no conflict of interest.

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