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Therapy of experimental type 1 diabetes by isolated Sertoli cell xenografts alone

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Abstract

Type 1 diabetes mellitus is caused by autoimmune destruction of pancreatic β cells, and effective treatment of the disease might require rescuing β cell function in a context of reinstalled immune tolerance. Sertoli cells (SCs) are found in the testes, where their main task is to provide local immunological protection and nourishment to developing germ cells. SCs engraft, self-protect, and coprotect allogeneic and xenogeneic grafts from immune destruction in different experimental settings. SCs have also been successfully implanted into the central nervous system to create a regulatory environment to the surrounding tissue which is trophic and counter-inflammatory. We report that isolated neonatal porcine SC, administered alone in highly biocompatible microcapsules, led to diabetes prevention and reversion in the respective 88 and 81% of overtly diabetic (nonobese diabetic [NOD]) mice, with no need for additional β cell or insulin therapy. The effect was associated with restoration of systemic immune tolerance and detection of functional pancreatic islets that consisted of glucose-responsive and insulin-secreting cells. Curative effects by SC were strictly dependent on efficient tryptophan metabolism in the xenografts, leading to TGF- β -dependent emergence of autoantigen-specific regulatory T cells and recovery of β cell function in the diabetic recipients.

A cure for type 1 diabetes in humans will probably require the provision or elicitation of new pancreatic islet β cells in conjunction with reestablishment of immunological tolerance. Sertoli cells (SCs) are normally found in the testes, where they couple trophic effects with prevention of immune damage to developing germ cells. SCs engraft and self-protect when transplanted into allogeneic and xenogeneic environments ([Emerich et al., 2003](#)). In different settings, SCs manifest an ability to provide local immunoprotection to cografted tissues, including those from xenogeneic donors ([Shamekh et al., 2006](#)). In murine type 1 diabetes, SCs protect cografted allogeneic and xenogeneic islets from immune destruction ([Suarez-Pinzon et al., 2000](#); [Yang et al., 2002](#)). Thus, SC might confer immunoprotection for transplanted islets in humans, representing a possible means of overcoming the major obstacle associated with cell therapy. In addition, porcine SC culture supernatants mediate the homologous transdifferentiation in vitro of neonatal pancreatic duct cells into endocrine cells ([Basta et al., 2004](#)).

SCs can be implanted into the central nervous system to locally deliver molecules with trophic and antiinflammatory effects on the surrounding tissue (Sanberg et al., 1996). When SCs are grafted into an experimental model of Huntington's disease, the beneficial effects are quite similar to those obtained by systemic treatment with nonsteroidal antiinflammatory drugs (Emerich, 2004). SCs also provide protection in experimental models of Parkinson's disease and amyotrophic lateral sclerosis (Sanberg et al., 1997; Luca et al., 2007). Although these data do not clarify the underlying mechanisms, they do suggest that the graft of SCs alone may exert beneficial effects on specific tissues through their ability to provide trophic effects and/or modulation of pathogenic inflammation.

The nonobese diabetic (NOD) strain of mice has become a prototypic model of autoimmune disease (Delovitch and Singh, 1997; Atkinson and Leiter, 1999). A large proportion of female mice generally dies of type 1 diabetes, reflecting the onset of severe insulinitis at ~4 wk of age and the T cell-mediated destruction of pancreatic β cells. The predisposition of NOD mice to develop autoimmunity is the result of defects in both peripheral and central tolerance mechanisms (Kishimoto and Sprent, 2001). Several abnormalities have been described in those mice, including aberrant APC function (Serreze et al., 1993) and impaired activity of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO; Grohmann et al., 2003a; Fallarino et al., 2004), whose effects are typically linked to the generation and function of regulatory T (T reg) cells in the periphery (Puccetti and Grohmann, 2007).

In different experimental settings, immunological reversal of autoimmune diabetes does not require externally provided β cell precursors, suggesting that control of the autoimmune disease at a crucial time in diabetogenesis can result in recovery of β cell function (reversion rates of 30–41%; Chong et al., 2006; Suri et al., 2006). In one such study, the recovered islets from long-term survivors were, indeed, all of host origin, indicating that the diabetic NOD mice actually retain substantial β cell mass, which can be reconditioned to reverse disease upon adjuvant-dependent dampening of autoimmunity (Kodama et al., 2003; Nishio et al., 2006).

In the present study, we aimed at revitalizing pancreatic islet β cells in NOD mice by means of the trophic and immunoregulatory effects of neonatal porcine SC administered alone in highly biocompatible alginate microcapsules. In these conditions, the grafted SCs remain viable and functional for remarkably long periods of times (Luca et al., 2007). We obtained evidence that IDO-expressing xenogeneic SC could represent a novel form of cell therapy in NOD mice, combining control of autoimmunity and emergence of T reg cell responses with pancreas regeneration from adult multipotent progenitor cells.

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RESULTS

Prevention and reversion of diabetes by SC xenografts

Severely diabetic NOD mice (blood glucose levels of 350–400 mg/dl for at least 10 d) were transplanted on day 0 with empty capsules (ECs; $n = 18$) or encapsulated SC ($n = 21$). All recipients of EC developed progressive disease, with survival times of 28–62 d. Four recipients of SC died within 60–90 d of transplantation, although 17 of 21 (81%) became long-term survivors (>120 d), and 10 of those became normoglycemic (Fig. 1, A and B; Table I). The percentage of disease-free animals rose to 88% (22 out of 25) when the SCs were administered to prediabetic mice that were 8–10 wk of age, with glucose levels <200 mg/dl (Fig. S1 depicts a Kaplan-Meier plot for normoglycemia, in which $P < 0.001$ for comparison between EC and SC treatment groups).

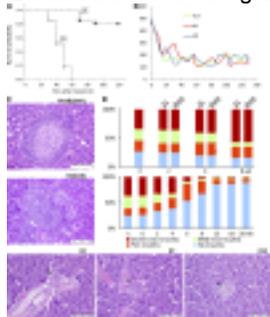


Figure 1.

Effects of treatment with encapsulated SC on disease progression and pancreatic histology in diabetic NOD mice. (A) Kaplan-Meier plot for survival. Diabetic NOD females were treated with an

implant of EC or SC, and survival probability was plotted over ...



Table I.

Effect of SC xenografts on course of disease in overtly diabetic mice

The diabetic mice on SC therapy that developed a slower but progressive disease exhibited the occurrence of insulin-producing pancreatic islet cells without invasive insulinitis (that is, autoreactive cells within the islets, as seen in the EC recipients) but with pronounced peri-insulinitis (circumferential lymphoid cells that did not progress, for the most part, to invasion). In contrast, in the pancreata of mice on successful SC therapy, reappearance of islets occurred without invasive insulinitis and with minimal or no peri-insulinitis that decreased over time (Fig. 1, C and D).

18 wk after SC treatment, the mean number of islets in the pancreas was 20.7 ± 3.6 (mean \pm SD; $n = 5$), a number not significantly different from that in prediabetic controls (25.8 ± 4.5 ; $P = 0.081$). The mean area of individual islets from successfully treated mice was $2,326 \pm 470 \mu\text{m}^2$ (of which $\sim 55\%$ stained positive for insulin), which represents roughly one-third of the corresponding value in a prediabetic control. 3 of the 10 mice that became normoglycemic after SC therapy were subjected to intraperitoneal glucose testing, displaying nearly normal responses (Fig. S2).

Cure by SC xenografts is associated with the emergence of a regulatory environment

In sorted CD4^+ cells from pancreatic LNs (PLNs) of mice receiving SC therapy, flow cytometry analysis revealed significantly increased expression of Foxp3 (T reg cell specification factor) relative to controls (Fig. 2 A). This was reflected in the spleen by changes in the expression of the T reg markers CTLA-4 and glucocorticoid-inducible TNF receptor (GITR; Fig. 2 A). Upon immunofluorescence analysis, CD4^+ Foxp3 $^+$ cells characterized the still insulinitic pancreata of mice treated with SC for 2–3 wk relative to controls (Fig. 2 B). In sorted PLN CD3^+ T cells from diabetic mice, SC therapy caused a >100 -fold reduction in message expression of *Rorc* (encoding the Th17 transcription factor ROR- γ t), as well as strong induction (>50 -fold) of *Foxp3* (Fig. 3).

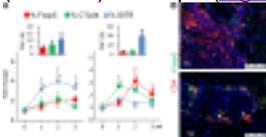


Figure 2.

Induction of a regulatory response by xenogeneic SC in diabetic mice. (A) Cytofluorometric analyses of Foxp3, CTLA-4, and GITR expression in CD4^+ cells from spleens (left) or PLN (right) of mice ($n = 3$) treated with EC or SC for 1–3 wk. Isotype ...

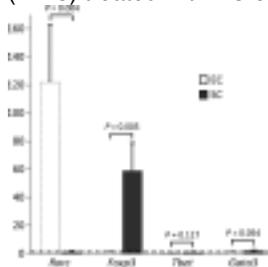


Figure 3.

In diabetic NOD mice, SC therapy causes reduced expression of the ROR- γ t–encoding gene and augmentation of Foxp3, with negligible effects on Tbet and Gata3.

Rorc, *Foxp3*, *Tbet*, and *Gata3* transcripts were evaluated in CD3^+ T cells from PLN ...

The antigen specificity of the T reg activity induced by SC graft was examined in terms of T cell response to fusion proteins encompassing proinsulin-related P3UmPI, glutamic acid decarboxylase (GAD)–related P3UmG, or IA-2–related P3UhIA domains (Fig. S3, A and B). Proliferative responses were measured in pancreatic $\text{CD4}^+\text{CD25}^+$ and $\text{CD4}^+\text{CD25}^-$ cells from SC-treated NOD mice, stimulated with individual fusion

proteins. In addition, inhibition of CD4⁺CD25⁻ cells from diabetic NOD mice by antigen-specific CD4⁺CD25⁺ cells from SC-treated mice was also investigated. The results showed that effector T cells from EC-treated diabetic mice proliferated vigorously in response to each autoantigen, and so did the regulatory cells from SC-treated mice (P3UmG ≥ P3UmPI > P3UhIA). However, these in vitro–expanded T reg cells of single antigen specificities were poorly capable of inhibiting the polyclonal anti-CD3–stimulated effector population.

Effective SC therapy alters the local pancreatic as well as systemic cytokine milieu

Th17 cells promote autoimmunity in mice and have been implicated in the pathogenesis of human inflammatory diseases (Laurence and O'Shea, 2007). Differentiation of proinflammatory Th17 cells requires IL-23. In vitro, however, Th17 differentiation is independent of IL-23 and is induced by TGF-β plus IL-6 or IL-21, with IL-10 having a controversial role in regulating pathogenetic Th17 cells (Jankovic and Trinchieri, 2007). In NOD mice, IFN-γ induced by adjuvant-free antigen restores normoglycemia through inhibition of IL-17 (Jain et al., 2008). Both in vitro and in vivo, differentiation of the Th17 lineage requires up-regulation of ROR-γt (Yang et al., 2008), and evidence indicates that TGF-β–induced Foxp3 inhibits Th17 cell differentiation by antagonizing ROR-γt function (Zhou et al., 2008).

We measured IL-17, IL-23, and TGF-β in the splenic CD4⁺ and/or pancreatic leukocyte fractions of diabetic mice treated with SC or EC for 6 wk, together with IL-6, IL-10, IFN-γ, and IL-4 in the spleen and/or pancreas (Fig. 4 A). SC therapy strongly opposed IL-23 and IL-17 productions both in the spleen and pancreas of diabetic mice. Reduced production was also observed for IL-6, IL-10, and IFN-γ, mostly in the pancreas. Although present in control animals on EC, TGF-β was greatly increased by SC therapy. When challenged with the proinsulin-related fusion protein P3UmPI, CD4⁺CD25⁺ (but not CD25⁻) cells in PLNs from SC-treated mice produced noticeable amounts of TGF-β, as detected by intracellular staining. In EC-treated mice, preponderant production of IL-17A was observed in parallel, mostly by the CD4⁺CD25⁻ fraction (Fig. 4 B).

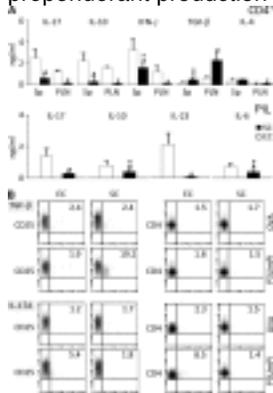


Figure 4.

Effects of SC therapy on cytokine secretion profile of Th cells from diabetic mice. (A) Sorted CD4⁺ T cells from spleens (Sp) or PLNs, as well as unfractionated pancreatic-infiltrating leukocytes (PIL), were assayed for cytokine release in response to ...

The response initiated by SC requires TGF-β and can be adoptively transferred

Clonotypic T cell NOD mice expressing the BDC2.5–TCR-αβ transgene (BDC2.5/NOD) are characterized by a prevalent population of pathogenetic CD4⁺ T cells that recognize an islet-related 12-mer peptide of GAD 65 (Katz et al., 1993; Judkowski et al., 2001; Quinn et al., 2001). Most BDC2.5/NOD mice do not develop spontaneous diabetes but do develop insulinitis accompanied by the appearance of activated islet-specific T cells. Evidence suggests that these cells are actively prevented from causing disease by a CTLA-4–dependent immunoregulatory mechanism and by the action of CD4⁺CD25⁺CD69⁻ T reg cells (Herman et al., 2004). At variance with BDC2.5/NOD mice, BDC2.5 TCR transgenic mice backcrossed into the NOD-SCID background show fulminant diabetes (Kurrer et al., 1997). Rapid diabetes also develops in NOD-SCID mice adoptively transferred with spleen cells or purified CD4⁺ T cells from BDC2.5/NOD donors (You et al., 2004). In this setting, however, onset of disease is prevented by the cotransfer of CD4⁺CD25⁺CD62L⁺ cells from prediabetic NOD mice (You et al., 2004) or of T reg cells generated in vitro by tryptophan catabolites (Fallarino et al., 2006).

Using this cotransfer model, we obtained evidence that pancreatic CD4⁺ cells from SC-treated mice were

capable of protecting NOD-SCID mice from the induction of diabetes by BDC2.5 transgenic T cells (Fig. 5 A). Protection from diabetes was also demonstrable on transfer of splenocytes from diabetic NOD/Mrk mice injected in combination with CD4⁺ cells from SC-treated mice (Fig. 5 B). Similar to previous results in other models (Suarez-Pinzon et al., 2000; Belghith et al., 2003), TGF- β had an indispensable role in the induction of the autoimmune-preventive cell-transferable regulatory response initiated by SC in NOD mice ($P < 0.001$; Fig. 5 C). No significant effect was instead afforded by the in vivo administration of anti-IL-10, anti-GITR, or anti-CTLA-4, though a trend toward worsening of the disease was associated with CTLA-4 neutralization ($P = 0.522$; Fig. 5 C). Neither TGF- β nor CTLA-4 was required for diabetes reversal in the recipients of an adoptively transferred protective CD4⁺ T cell response (Fig. 5, A and B). Depleting CD4⁺ cells of the CD25⁺ fraction in both models abrogated transfer of protection (unpublished data).

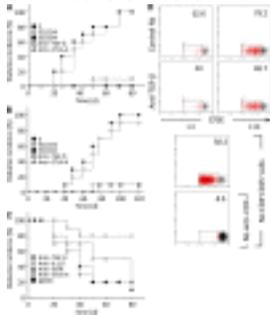


Figure 5.

Induction but not effector phase of protection by SC xenografts requires TGF- β and is cell transferable to NOD-SCID recipients. (A) 10^6 8–12-wk-old NOD/BDC2.5 splenocytes were injected intravenously alone (control [C]) or in combination ...

In contrast, purified CD4⁺CD25⁺ cells cultured with dendritic cells presenting multiple autoantigen-related fusion protein epitopes greatly suppressed the proliferation of CD4⁺CD25⁻ from diabetic donors in a TGF- β -independent fashion (Fig. 5 D; $P = 0.543$). Thus, TGF- β was required for the generation and/or expansion, but not effector function, of T reg cells in SC-treated NOD mice.

IDO expression by SC is instrumental in their therapeutic efficacy

IDO is an immune regulatory enzyme that fosters the generation or activation of IL-10-producing T reg cells (Mellor and Munn, 2004; Puccetti and Grohmann, 2007). We investigated IDO expression by SC and the possible contribution of IDO-dependent immune regulation by SCs to their therapeutic activity in diabetic mice. In contrast to freshly harvested cells, SC differentiating in vitro progressively acquired messenger RNA (mRNA) predictably encoding IDO (Fig. 6 A and Fig. S4). Detection of IDO protein by cross-reactive rabbit anti-mouse and mouse anti-human IDO reagents did indeed occur on immunoblot analysis at 3–7 d of culture, as well as in SC recovered from successfully treated mice (Fig. 6 B). Functional IDO expression in those cells was demonstrable in vitro, in terms of tryptophan conversion to kynurenine, and this was negated either by adding a synthetic substrate competitor (i.e., 1-methyl-tryptophan) or by stably silencing IDO-encoding transcript expression by small interfering (si) mRNA technology (Fig. 6 C). In contrast to control SC, cells with silenced IDO expression failed to protect hosts from diabetes development (Fig. 6 D; Fig. S5 provides data on silencing efficiency by immunoblot and immunofluorescence analyses).

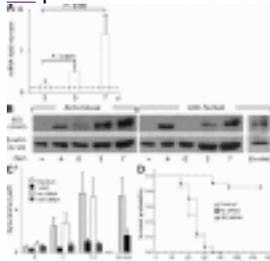


Figure 6.

IDO expression by SC is instrumental in their therapeutic efficacy. (A) In vitro differentiation of SC progressively increases mRNA putatively encoding porcine IDO. SCs were cultured for 3, 5, or 7 d, and IDO-encoding mRNA was analyzed by real-time PCR ...

Gene expression pattern and insulin production in islets from SC-treated mice

In different diabetes models, both β cell proliferation and neogenesis contribute to restoration of β cell function. Early proliferation of host residual β cells did not appear to occur to a significant extent in NOD recipients of SC (Fig. S6). In pancreas regeneration, multipotent precursor cells proliferate and differentiate to form pancreatic islets. Among the numerous genes that orchestrate their expression, *Pdx1* is detected early (Hardikar, 2004). Using an experimental injury model of the pancreas, Xu et al. (2008) reported the involvement of Ngn3 (neurogenin 3), a marker for embryonic-type endocrine progenitor cells, in the formation of new insulin-producing β cells. These Ngn3⁺ facultative endocrine progenitor cells in the adult pancreas could be of potential value in the provision or elicitation of new pancreatic islet β cells once the underlying immune disease has been eliminated.

After the implant of EC or SC in diabetic mice, we measured pancreatic *Pdx1*, *Ngn3*, and *c-kit* transcript expression (all considered to be markers of progenitor cells) together with transcripts specific for *Neurod1*, *Nkx6.1*, *Pax4*, and *Pax6*, which are known to encode transcription factors downstream of Ngn3 in the differentiation cascade and which result in predominant β cell, δ cell (*Pax4*), or α cell (*Pax6*) development (Fig. 7 A). With the exception of *Nkx6.1*, *Pax4*, and *Pax6*, whose expression was marginally or not affected at all, onset of diabetes increased pancreatic gene expression by itself (>10-fold increase relative to prediabetic mice [in which fold change = 1], perhaps reflecting a self-repair attempt by the damaged islets), most notably, *Ngn3*. Grafting diabetic mice with SC resulted, however, in fold increases ranging from 8–15 (*Pax4*, *Pax6*, and *Nkx6.1*) to 30–60 (*c-kit*, *Pdx1*, *Ngn3*, and *Neurod1*).

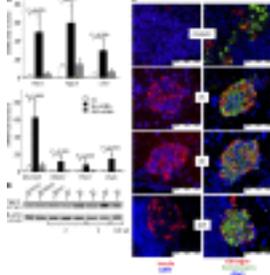


Figure 7.

Gene expression pattern and insulin production in islets from successfully treated mice. (A) Real-time PCR analysis of pancreatic gene expression in diabetic mice treated with EC or SC for 2 wk. SCs were treated with either IDO siRNA or negative control ...

Immunoblot analysis of Ngn3 expression in diabetic mice grafted with EC or SC for different times revealed progressively increasing expressions of the transcriptional regulator in mice cured by SC therapy, with peak levels at 2–4 wk of treatment (Fig. 7 B). In contrast, in control mice on EC treatment, detection of the protein at diabetes onset was accompanied by persistent yet low levels. Serum insulin levels at 6–8 wk of treatment suggested recovery of β cell function (Fig. S7). Immunofluorescence analysis revealed that the increased Ngn3 expression induced by effective therapy was accompanied by an ability of the islets to produce insulin, glucagon, and somatostatin (Fig. 7 C). Notably, the occurrence of glucagon and somatostatin double-stained cells would apparently recapitulate the process of embryonic stem cell development and, thus, be indicative of authentic regeneration.

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DISCUSSION

Cell therapy is a potentially powerful tool in the treatment of many grave disorders including leukemia, immune deficiencies, autoimmune diseases, and diabetes. Bone marrow mesenchymal stem cells have potent immunosuppressive properties and have been advocated for IDO-dependent control of inflammatory diseases in humans (Matysiak et al., 2008). In different experimental settings, mesenchymal stem cells do, indeed, exploit the IDO mechanism for tolerogenesis (Haniiffa et al., 2007; Jones et al., 2007; Matysiak et al., 2008). Like bone marrow-derived mesenchymal stem cells, which are multipotent adult stem cells, SCs are of mesodermal origin, and mRNA expression and protein secretion of mesenchymal markers have been found in rat SCs in addition to stem cell factor (c-kit ligand) and other products, which contribute to maintaining the spermatogonial stem cell niche (Konrad et al., 2005).

Among the latter, immature pig SCs secrete IGF-1 (insulin-like growth factor 1), which is crucially involved in regulating β cell growth (Luca et al., 2007). Porcine c-kit ligand and IGF-1 have 82% and >80% protein

identity with their respective mouse counterparts. Our unpublished observations with cultured SC demonstrated IGF-1 production by RIA testing and c-kit ligand expression by PCR analysis. Encapsulated cells maintained both expressions. Although FasL has also been reported to be expressed by SC ([Yao et al., 2007](#)), cultured SCs do not secrete soluble forms of FasL ([Riccioli et al., 2000](#)).

Administration of SCs across xenogeneic barriers induces transplantation tolerance without altering systemic immune competence, and SC could be used as a novel and potentially powerful tool in cell transplantation therapy and for protection of transplanted adult or embryonic stem cells ([Shamekh et al., 2006](#)). In this study, we tested the hypothesis that a peculiar combination of self-protective, immunoregulatory, and trophic properties might confer a unique potential for type 1 diabetes therapy on SC, with no absolute need for the cotransfer of allogeneic or surrogate stem cell-derived β cells or insulin therapy.

We obtained evidence that (a) porcine SCs are competent at eliciting functional pancreatic islets in severely diabetic NOD mice, reversing disease in a remarkably high percentage of recipient hosts; (b) the immunoregulatory component of SC activity is dependent on the tolerogenic effects of tryptophan catabolism, initiated in the host by SC xenografts, such that restoration of the kynurenine pathway ([Puccetti and Grohmann, 2007](#); [Romani et al., 2008a](#)) by SC could represent a new form of immunotherapy in autoimmune diabetes; and (c) SC might effectively combine the advantages of mesenchymal cells (which are also IDO-positive and immunoregulatory yet lack trophic effects) with those of maneuvers that foster β cell regeneration but lack substantial impact on the underlying autoimmune condition, linking IL-17 antagonism ([Jain et al., 2008](#)) and emergence of T reg responses ([Zhou et al., 2008](#)) with pancreas regeneration from adult multipotent progenitor cells ([Xu et al., 2008](#)).

In fact, we obtained data showing that autoimmunity as a whole is suppressed in NOD recipients of SC, including onset of thyroiditis (unpublished data). Similarly, we attempted to determine whether encapsulated SCs are protective in other experimental models of autoimmunity. In experiments parallel to those in NOD mice, we have obtained preliminary evidence that SC can protect against experimental autoimmune encephalomyelitis, in a model system which we have previously described in detail and which makes use of susceptible strains of mice immunized with the encephalitogenic MOG peptide ([Orabona et al., 2005](#)). In this setting, IDO-dependent mechanisms are, indeed, highly protective ([Orabona et al., 2006](#); [Matysiak et al., 2008](#)).

Human islets may produce IDO themselves, and it has been suggested that in the short term, IDO activation could physiologically protect islets from cytotoxic damage ([Sarkar et al., 2007](#)). This is consistent with previous observations on the protective role of IDO ([Grohmann et al., 2003a](#)) and IDO-inducing maneuvers in NOD mice ([Alexander et al., 2002](#); [Fallarino et al., 2004](#); [Ueno et al., 2007](#)), and with resistance of pancreatic islets to the direct effects of the IDO-induced general control nonderepressible 2 kinase stress pathway ([Jalili et al., 2009](#)). We became interested in evaluating whether the administration of natural ([Romani et al., 2008a](#)) or synthetic tryptophan catabolites ([Platten et al., 2005](#)) to NOD mice would be as protective as SC therapy. Although a detailed account of this study will be the subject of a subsequent paper, our preliminary data demonstrate that, consistent with the concept that the therapeutic efficacy of SC results from a combination of immunoregulatory and trophic effects, supplemental kynurenines have only partial effects, i.e., those related to immunoregulation. Optimal exploitation of supplemental kynurenine therapy requires synergic effects by additional maneuvers, at least in severely diabetic mice.

Accumulating evidence points to the β cell itself as the most promising source for generating new β cells, and different maneuvers may stimulate islet precursor cells to undergo neogenesis or to induce replication of existing β cells, emphasizing the importance of pancreas-resident stem or progenitor cells in islet regeneration ([Xu et al., 2008](#)). Expansion of β cell mass from endogenous sources, either in vivo or in vitro, represents an area of increasing interest. In vitro, one potential source of islet progenitors is the islet proper via the dedifferentiation, proliferation, and redifferentiation of facultative progenitors residing within the islet. Recently, short-term treatment with a peptide fragment of INGAP (islet neogenesis-associated protein) was found to induce adult human pancreatic islet-derived proliferative duct-like components to reform islet-like structures, which resembled freshly isolated islets with respect to the frequency and distribution of the four endocrine cell types, islet gene expression and hormone production, insulin content, and glucose-responsive insulin secretion ([Hanley and Rosenberg, 2009](#)). These studies emphasize how the plasticity of adult human islets may have significant implications for islet regeneration. Additionally, neogenesis or the budding of new islet cells from pancreatic ducts has been reported, although the existence and identity of a progenitor cell have been debated ([Inada et al., 2008](#)).

In vivo, residual β cells that are present soon after onset of diabetes may respond to experimental regeneration ([Reddy et al., 2008](#)). In addition, combination therapy with glucagon-like peptide 1 and gastrin restores normoglycemia in overtly diabetic NOD mice by increasing the pancreatic β cell mass and down-regulating the autoimmune response ([Suarez-Pinzon et al., 2008](#)). [Suarez-Pinzon et al. \(2000\)](#) had previously found that SC production of TGF- β 1, not Fas ligand, protects islet β cells from autoimmune

destruction and TGF- β 1 diverts islet-infiltrating cells from a β cell-destructive (IFN- γ ⁺) phenotype to a nondestructive (IL-4⁺) phenotype. We show in this paper that not only are xenogeneic SCs capable of sustained suppression of autoimmunity through TGF- β induction of T reg cells and down-regulation of IL-17-dependent responses but they are also competent at eliciting functional pancreatic islet cells in the severely diabetic host, and they might do so by inducing neogenesis in periductal areas. The process does not require substantial replication of residual β cells and is instead associated with transcriptional activation of genes that are markers of progenitor cells and encode transcription factors downstream of Ngn3 in the differentiation cascade of the four endocrine cell types (O'Neill et al., 2008). Islet gene expression correlated with hormone production, insulin content, and glucose-responsive insulin secretion, according to developmental kinetics apparently reminiscent of the process of embryonic stem cell differentiation. Castrated mice grafted with porcine immature SC manifest an endocrine status and hormonal profiles suggestive of an intense cross-talk between the mouse hypothalamo-pituitary axis, and the grafted porcine tissue, which reaches maximum amplification at ~60 d after grafting (Kaneko et al., 2008). Testicular SC dysfunction has recently been described in male patients with systemic lupus erythematosus, suggesting a possible bidirectional link between SC function and onset of systemic autoimmunity (Suehiro et al., 2008). Antiandrogen exposure in utero disrupts expression of insulin-like factor 3 in the developing fetal rat testis (Brokken et al., 2009). Finally, a stable SC line lacks the immunoprotective properties associated with primary SC in a model system of allogeneic islet transplantation (Dufour et al., 2008). It is therefore possible that multiple mechanisms participate in the SC-induced generation of an optimally trophic environment fostering β cell regeneration once autoimmunity has been dampened in NOD female mice by the suppressive properties of encapsulated SC. Although cultured SCs are competent at initiating transdifferentiation in vitro of neonatal porcine pancreatic duct cells into endocrine cells (Basta et al., 2004), further studies are needed to clarify this issue.

Recent evidence indicates that Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells (Martin-Orozco et al., 2009). NOD-SCID mice, recipient of BDC2.5 transgenic CD4⁺ cells matured to a Th17 phenotype, developed rapid onset of diabetes with extensive insulinitic lesions, which progressed to overt disease in concurrence with the appearance of a strong IFN- γ response. However, at the prediabetic stage, IFN- γ induced by an adjuvant-free antigen restores normoglycemia, most likely by localized bystander suppression of pathogenic IL-17-producing cells (Jain et al., 2008). We found that SC therapy was associated with reduced production of IL-17A by CD4⁺CD25⁻ cells in PLNs, which was concomitant with enhanced production of TGF- β by the CD4⁺CD25⁺ counterpart.

IL-17 and IFN- γ show bidirectional influences with IDO regulation and function, and they influence each other through the IDO mechanism. Both IFN- γ (Grohmann et al., 2003a) and TGF- β (Belladonna et al., 2008) are transcriptional activators of the gene encoding IDO, which is instead subject to IL-6-driven regulatory proteolysis (Orabona et al., 2008). The IDO mechanism initiates the peripheral TGF- β -dependent generation of autoimmune-preventive T reg cells in NOD mice (Fallarino et al., 2006). Both T reg cell activity and kynurenine products of tryptophan degradation oppose Th17 responses through down-regulation of *Rorc* transcription (Romani et al., 2008a,b). Thus, the immunoactive component of SC therapy in our model system may call for a staged response, which combines an early counter-inflammatory action of tryptophan catabolism (Grohmann et al., 2003b) with the generation of CTLA-4⁺ GITR⁺ T reg cells (Fallarino et al., 2006), which could then use the IDO mechanism as an effector system in the homeostatic regulation of peripheral immunity (Fallarino et al., 2003; Grohmann et al., 2007; Puccetti and Grohmann, 2007). The early IDO-dependent tryptophan catabolizing activity of the grafted SC appears to be indispensable for therapeutic activity. Analogous to several experimental settings of IDO-associated "infectious tolerance" (Belladonna et al., 2009), our data provide evidence that TGF- β is required for the generation, but not the effector function, of T reg cells in SC-treated NOD mice.

In conclusion, IDO, a "metabolic" enzyme conserved through the last 600 million years of evolution, suppresses T cell responses and promotes tolerance in mammalian pregnancy (Munn et al., 1998), as well as in autoimmune (Grohmann et al., 2003a) and allergic (Grohmann et al., 2007) inflammation. Its regulation (Orabona et al., 2008), as well as the mechanisms of its action as an immune regulator, are composite (Belladonna et al., 2009) and include an arrest in T cell proliferation, induction of Th cell apoptosis, reversible impairment of T cell activity through down-regulation of T cell receptor ζ -chain, and the generation or activation of T reg cells (Mellor and Munn, 2004; Puccetti and Grohmann, 2007). Our current study provides evidence for effective therapy of experimental autoimmune diabetes by implants of IDO-expressing encapsulated SC, which likely act through multiple mechanisms, including dampening of autoimmunity, protection and functional recovery of β cells, and neogenesis, as proven by up-regulation of specific transcription factors, and/or transdifferentiation. This may pave the way to the implementation of new forms of cell therapy for human diabetes, namely immunotherapy with cell types of nonhuman origin that would

combine the advantages of mesenchymal cells with those of maneuvers that promote β cell regeneration but lack impact on the underlying autoimmune condition.

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MATERIALS AND METHODS

Mice, pancreatic cell isolation, and histopathology.

Female NOD/Mrk mice, 4 and 12 wk of age, were purchased from Taconic. The mice were housed and fed under specific pathogen-free conditions. Autoimmune diabetes develops in ~80% of NOD/Mrk female mice by 24 wk of age. Glycemia of <200 mg/dl (in the absence of glycosuria) and of 350–400 mg/dl (stably for at least 1 wk) in the presence of glycosuria defined prediabetes (5–6 wk) and diabetes (>15 wk), respectively, at the time of SC transplantation (day 0). The NOD mice that were kept for 10 d with blood glucose levels of 350–400 mg/dl did change their weight significantly, and no insulin pellets were necessary for survival. Transplanted mice were housed individually and blood glucose concentrations were monitored once a week up to day 120. All in vivo studies were in compliance with national (Italian Approved Animal Welfare Assurance A-3143-01) and Perugia University Animal Care and Use Committee guidelines.

Pancreatic cell purification involved treating the organ with Complete Mini Protease inhibitors (Roche) followed by digestion with collagenase type IV (Sigma-Aldrich) in the presence of bovine pancreatic DNase (Sigma-Aldrich) for 30–45 min at 37°C. The digested pancreata were further disrupted by gently pushing the tissue through a nylon screen, and pancreatic-infiltrating leukocytes were separated on a percoll gradient (Sigma-Aldrich). Purification of CD3⁺ or CD4⁺ T cell subsets from the spleen or LNs was conducted as previously described ([Grohmann et al., 2007](#); [Romani et al., 2008a](#)).

For histopathology, 3–4 μ m of paraffin-embedded sections of pancreata (five per organ) were stained with hematoxylin and eosin and analyzed by light microscopy. Insulinitis scoring was according to the following criteria: severe insulinitis, 50% or higher of the islet area is infiltrated; mild insulinitis, <50% of the islet area is infiltrated; peri-insulinitis, infiltration is restricted to the periphery of islets; and no insulinitis, absence of cell infiltration.

Alginate-based microencapsulation of SC.

Neonatal prepubertal large white pigs, 7–15 d of age, were used as SC donors. SCs were isolated, cultured, and alginate microcapsules were prepared by a dripping method, as previously described ([Luca et al., 2007](#)), resulting in the production of microcapsules measuring 400–500 μ m in equatorial diameter, with no loss of SC functional and morphological properties, either in vitro or in vivo, for extraordinarily long periods of time. Once implanted in the peritoneal cavity of NOD mice, the SC-containing capsules appear to be freely floating in the peritoneal cavity and morphologically intact, with the majority of them remaining free of fibrotic tissue overgrowth at 4 and 8 mo after transplant. When the capsules were explanted at 8 mo of grafting, the embodied SCs were extraordinarily viable and appeared to be organized in tubule-like structures. Freshly harvested SCs, with the typical morphological and secretory features of somatic testicular cells from immature donors ([Chatelain et al., 1987](#); [Suarez-Pinzon et al., 2000](#); [Yang et al., 2002](#); [Mancuso et al., 2006](#)), were differentiated in vitro for 5–7 d, and each mouse received a single intraperitoneal implant of encapsulated neonatal SC (2×10^7) through a small abdominal incision, as previously described ([Luca et al., 2007](#)). SCs were culture maintained in HAMF12 (EuroClone), supplemented with 0.166 nM retinoic acid (Sigma-Aldrich) and 5/500 ml of insulin-transforming selenium (BD) in 95% air–CO₂ at 37°C. Cells were extensively washed before use. In selected in vitro experiments, encapsulated hepatocytes or peripheral blood leukocytes from the same donors were used as a control to test for nonspecific effects of cell therapy, and none were found. Approximately 120×10^6 cells are retrieved from neonatal pig testis pairs, and 20×10^6 cells are required for reversing diabetes in a NOD mouse weighing 25 g.

IDO expression and functional analysis.

IDO induction was investigated by immunoblotting with rabbit polyclonal anti–murine IDO antibody ([Grohmann et al., 2007](#)) or a monoclonal mouse anti–human IDO reagent (Millipore). Anti-actin AC-40 antibody (Sigma-Aldrich) was used as a normalizer in all assays. IDO functional activity was measured in vitro in terms of the ability to metabolize tryptophan to L-kynurenine, whose concentrations were measured by high-performance liquid chromatography ([Romani et al., 2008a](#)).

Vector construction and siRNA transfection.

For silencing porcine IDO-encoding mRNA expression, siRNA was cloned into pSUPER-puro (Oligoengine), and a construct was prepared, as previously described ([Brummelkamp et al., 2002](#)). Among several potential target sequences, a 19-nt gene-specific sequence was selected spanning nucleotides 344–363. After BLAST analysis to ensure lack of significant homology with other known *Sus scrofa* (pig) genes, the sequence was inserted into a BglII–HindIII-cut pSUPER-puro vector to generate the pSUPER-Ssc_IDOi vector. Freshly isolated SCs were transfected with negative control pSUPER or pSUPER-Ssc_IDOi to obtain cells stably expressing siRNA, and stable integrants were selected by puromycin, starting 24 h after transfection. Negative control (null) pSUPER consisted of SC transfected with a sequence proven by BLAST analysis not to match any known *S. scrofa* sequence.

In brief, SCs were transfected with pSUPER or pSUPER-Ssc_IDOi vector by DOTAP liposomes (Roche). The plasmids were combined with DOTAP to form complexes to be used to transfect SC. 3×10^6 SCs were silenced with 5 μg of plasmid DNA diluted to a concentration of 0.1 mg/ml in HBS buffer (Hepes-buffered saline: 20 mM HEPES, pH 7.4, containing 150 mM NaCl; final volume, 50 μl) in a sterile tube. In a separate sterile tube, 30 μl DOTAP (1 mg/ml) was mixed with HBS buffer to a final volume of 100 μl . 50 μl of the DNA solution was transferred to the reaction tube already containing 100 μl DOTAP in HBS buffer and carefully mixed. The transfection (DOTAP–DNA) mixture was incubated for 30 min at room temperature. The mixture was gently added to 5 ml of SC cultures. The cells were then cultured for 48 or 72 h and inserted into the capsules to be administered in vivo. In parallel, the efficiency of IDO silencing was determined by immunoblotting at the same time points.

PCR analyses.

Real-time PCR (for *Rorc*, *Tbet*, *Foxp3*, *Gata3*, *Pdx1*, *Ngn3*, *c-kit*, *Neurod1*, *Nkx6.1*, *Pax4*, and *Pax6*) analyses were conducted as previously described ([Romani et al., 2008a](#)), using the primers listed in [Table S1](#). Also shown in the table are primers designed to amplify *S. scrofa* mRNA predicted to encode porcine IDO, as revealed by multiple sequence alignment analysis (Fig. S4). Primers were designed according to the putative coding region.

Flow cytometry.

In all FACS analyses, cells were treated with rat anti-CD16/32 (2.4G2) for 30 min at 4°C for blockade of Fc receptors before assaying on an EPICS flow cytometer using EXPO 32 ADC software (Beckman Coulter). GITR and total CTLA-4 expressions were analyzed as previously described ([Fallarino et al., 2003](#); [Grohmann et al., 2007](#)). For intracellular Foxp3, cells from PLNs were stained with anti-CD4 (GK1.5)-PE (BD), fixed, permeabilized, and stained with FITC-labeled anti-Foxp3 (FJK-16s; eBioscience) or isotype control rat IgG2a-FITC. Intracellular TGF- β and IL-17A staining in sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ from the spleen was conducted as described upon overnight activation with 5 $\mu\text{g}/\text{ml}$ of plate-bound anti-CD3 ([Romani et al., 2008a](#)). For CFSE (Invitrogen) labeling, the fluorescent dye in the form of 5 mM of stock solution was added to 2×10^6 CD4⁺CD25⁻ T cells/ml to a final concentration of 2 μM . The cells were incubated at room temperature for 10 min and washed twice with an excess of FCS-enriched culture medium.

Fusion proteins.

The following fusion proteins were used: murine proinsulin-related P3UmPI, murine GAD-related P3UmG, and human IA-2-related P3UHIA, with recombinant OVA being the control protein. Purified CD4⁺CD25⁺ and CD4⁺CD25⁻ cells were cultured for 3–5 d with fusion protein-pulsed splenic CD11c⁺ cells as antigen-presenting cells (5 $\mu\text{g}/\text{ml}$ of fusion protein; overnight, in the presence of 5 $\mu\text{g}/\text{ml}$ of purified mouse IgG). The recovered T cells were assayed for intracellular cytokine production, proliferation, and suppressive function.

Immunohistochemistry.

In immunostaining for insulin, glucagon, and somatostatin, 4- μm sections were cut from paraffin blocks and captured on electrically charged slides (Sigma-Aldrich). Sections were dewaxed in xylene and stained with primary guinea pig anti-mouse insulin antibodies (Dako), FITC-labeled mouse anti-mouse glucagon monoclonal (Sigma-Aldrich), and Alexa Fluor 555-labeled rabbit anti-mouse somatostatin antibody (Millipore) for 1 h at room temperature, washed in PBS, and incubated with a goat anti-guinea pig TRITC (tetramethyl rhodamine isothiocyanate) conjugate for 45 min (for insulin staining). Nuclei were counterstained with DAPI and mounted in DABCO (all from Sigma-Aldrich). In double immunostaining for Foxp3 and CD4, after dewaxing the sections, antigen retrieval was accomplished by microwave pressure cooking for 4 min at full pressure in 50 mM TRIS and 2 mM EDTA, pH 9. Slides were incubated for 1 h with primary unconjugated rat anti-mouse CD4 (Millipore) and Alexa Fluor 488-conjugated anti-mouse Foxp3 (150D; BioLegend). Sections were washed with PBS and incubated with goat anti-rat TRITC conjugate for

45 min (Millipore). Nuclei were counterstained with DAPI and mounted in DABCO. All slides were examined using a BX 41 apparatus in conjunction with F-View software (both from Olympus).

Statistical analysis.

In the in vivo experiments, survival and glycemia data were analyzed by Kaplan-Meier plots. Paired data were evaluated by Student's *t* test, and a one-way analysis of variance was used for multiple comparisons. All in vitro determinations are means \pm SD from at least three independent experiments, unless otherwise indicated. All *n* values were computed by power analysis, so as to yield a power of at least 80% with an α -level of 0.05.

Online supplemental material.

Fig. S1 demonstrates that the effect of SC therapy is even more dramatic when SCs are administered to prediabetic mice. Fig. S2 shows that diabetic mice rendered normoglycemic by SC therapy display nearly normal responses on intraperitoneal glucose testing. Fig. S3 deals with antigen specificity patterns of T reg cells induced by SC grafts in terms of proliferative responses to fusion proteins of proinsulin-related P3UmPI, GAD-related P3UmG, and IA-2-related P3UhIA. Fig. S4 depicts multiple amino acid sequence alignment of *S. scrofa*, *Bos taurus*, *Homo sapiens*, and *Mus musculus* IDOs. Fig. S5 provides data on IDO silencing efficiency in SC by immunoblot and immunofluorescence analyses. Fig. S6 demonstrates that early proliferation of host residual β cells does not occur to a significant extent in NOD recipients of SC. Fig. S7 displays serum insulin levels at 6–8 wk of SC treatment, further confirming recovery of β cell function. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20090134/DC1>.

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Footnotes

Abbreviations used:

EC empty capsule

GADglutamic acid decarboxylase

GITRglucocorticoid-inducible TNF receptor

IDO indoleamine 2,3-dioxygenase

mRNA messenger RNA

NOD nonobese diabetic

PLN pancreatic LN

SC Sertoli cell

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