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# Is Facilitating Pancreatic Beta Cell Regeneration a Valid Option for Clinical Therapy?

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Type 1 diabetes (T1D) is an autoimmune disease in which the clinical onset most frequently presents in adolescents who are genetically predisposed. There is accumulating evidence that the endocrine pancreas has regenerative properties, that hematopoietic chimerism can abrogate destruction of beta cells in autoimmune diabetes, and that, in this manner, physiologically sufficient endogenous insulin production can be restored in clinically diabetic NOD mice. Recapitulating what also has been seen sporadically in humans, we set out to test reliable and clinically translatable alternatives able to achieve these same goals. Recently, Tian and colleagues demonstrated that T1D can be prevented in genetically susceptible mice by substituting a "diabetes-susceptible" class II MHC beta chain with a "diabetes-resistant" allelic transgene on their hematopoietic stem cells through gene supplantation. The expression of the newly formed diabetes-resistant molecule in the reinfused hematopoietic cells was sufficient to prevent T1D onset even in the presence of the native, diabetogenic molecule. If this approach to obtain autoimmunity abrogation could facilitate a possible recovery of autologous insulin production in diabetic patients, safe induction of an autoimmunity-free status might become a new promising therapy for T1D.

Key words: Autoimmunity; Type 1 diabetes; Beta cell regeneration; Tolerization; Beta cell precursors

## INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease the clinical onset of which most frequently presents in adolescents who are genetically predisposed (4,7). The disease onset frequently is a totally unexpected, abrupt, frightening event involving families with no previous history of diabetes. From that moment on the patients can only control glycemia with daily injections of recombinant human insulin. The control of glycemia via repetitive insulin administrations, even guided by the most sophisticated schedule, is far from the delicate and fine-tuned control offered by the physiologic secretion of the hormone. The suboptimal control of glycemia seems to be the determining factor, which predisposes to diabetes complications (11,12). Transplantation of whole pancreas or isolated islets offers a therapeutic solution to T1D in which insulin production and secretion are much closer to physiologic conditions (15,33,34,37). However, these two therapeutic approaches suffer from all the drawbacks associated with any allotransplant. In particular, the immunosuppressive regimens necessary for organ or islet acceptance are extremely difficult to bear for the recipient (35), toxic not only for the beta cells (5,31) but, in the long run, also quite toxic for the kidney, the function of which may eventually become totally impaired (5,31).

## BETA CELL REGENERATION AND AUTOIMMUNITY

The case of a 13-year-old Caucasian boy who, after suffering with a conventional T1D onset (i.e., after a history of polyuria, polydipsia, and weight loss, the boy presented with serum glucose up to ~500 mg/dl, glucosuria, and ketonuria), needed less and less insulin doses over time, to ultimately have insulin treatment completely stopped after 11 months of treatment, was recently published by a group from Ulm, Germany (18). The authors also reported that: "Without further treatment, $HbA_{1c}$, and fasting glucose levels remained normal throughout the entire follow up of currently 4.5 years," and that serum autoantibodies to GAD65, IA-2, insulin, and ICA "were initially positive but showed a progressive decline or loss during follow-up." A similar case was recently reported by David Harlan's group working at the National Institute for Health (35).

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It is therefore not surprising to fine that in the nonobese diabetic (NOD) mouse the abrogation of autoimmunity is sufficient to promote regeneration or rescue of the insulin-producing beta cells in the host endocrine pancreas, even after the onset of the disease (16,36,48). NOD mice spontaneously develop T1D with etiopathogenetic characteristics very similar to the disease in humans (3).

If the approach to obtain autoimmunity abrogation is safe, it could facilitate a possible recovery of autologous insulin production in already-diabetic patients, and might become the basis for new, less invasive, promising therapies for T1D.

Precursors of a perhaps unconventional type, located both in close proximity and/or inside the endocrine tissue, seem to exist and be activated by increased metabolic demand or by still unknown secreted factors, normally able to accelerate the process that guarantees homeostasis of islets of Langerhans under normal conditions (43). The physiologic equilibrium between lost and newly generated cells is dramatically altered in T1D by the action of beta cell-specific, autoreactive T-cell clones, in instances in which autoimmunity develops (46). Once T-cell-mediated destructive activity overcomes the regenerative compensatory activity of the organ, the number of functional beta cells progressively decreases until they become too few for maintaining homeostasis of the glucose in the body. The time of transition over this metabolic threshold becomes immediately evident with the presentation of the characteristic signs of the clinical onset of T1D. During the disease, even if the regenerative properties of the pancreas remain functional, the continued presence of diabetogenic, autoreactive T cells consistently nullifies the reparative effort (43,46). The fact that these autoreactive T cells remain present in the body of the diabetic patient for a long time is proven by experiments in which healthy islet cells transplanted into syngeneic, long-term diabetic mice or humans were quickly killed by these same autoreactive T cells (38).

The autoimmune response can be successfully averted in the NOD mouse either by directly eliminating the majority of the autoreactive T cells with anti-T cell antibodies (26) or by substituting all or part of the immunocompetent cell repertoire with bone marrow (BM) cells obtained from diabetic-resistant donors (49). We and others (16,21,48) have shown that the successful induction of a mixed allogeneic chimerism obtained after transplanting BM from a diabetes-resistant donor into a diabetic recipient, following a sublethal dose of irradiation, is sufficient to block and eventually also revert the systematic invasion into the islets of autoreactive T cells. This invasion results in insulitis (i.e., the presence of beta cell-reactive T cells in the pancreatic islets themselves) (Fig. 1). Within the endocrine pancreas, once the insult of autoimmunity is abrogated, the physiologic process of regeneration can continue efficiently, eventually replenishing the population of insulin-producing cells to a number sufficient to maintain euglycemia, thus curing the diabetic recipient (Fig. 2) (16,43,48). However, while this slow process takes place, the recipient's glycemia must be controlled by additional, independent measures. The most commonly used technique is to transplant islets from the same BM donor under the kidney capsule of the recipient (6,16,48). However, the successful engraftment of the transplanted bone marrow, or the establishment of a steady hematopoietic chimerism, would have to be maintained without the use of immunosuppressive agents (43). These potent drugs may kill not only the still present autoreactive T cells of the recipient, but also the beta cells themselves, thereby defeating the purpose of the transplant (5,31,39). The use of immunosuppressive agents may also interfere with the observed rise of regulatory T cells, a possible explanation for the long-lasting immunoregulatory cell-dominant condition observed in cured animals (9,32,43).

A subject of ongoing debate is whether either or both the transplanted BM and the cotransplanted islets are necessary for promoting an efficient regenerative process, independent of their ability to block autoimmunity or preserve euglycemia, respectively. Strong evidence suggests that the hematopoietic precursors present in the BM cell population do not directly participate in the reparative process of the insulin-producing cell population (Fig. 2) (16,43,48); they might, for example, secrete factors such as glucagon-like peptides, which are useful in order to sustain an efficient regenerative process (13,26, 28). In the cured recipient, insulin-producing cells that are genetically marked to indicate they are of donor origin are extremely rare, occurring in no more than 2 out of more than 100,000 beta cells. These cells may actually be the result of sporadic cell fusion processes (19).

## SUCCESSFUL APPROACHES TO ABROGATE AUTOIMMUNITY

In order to reduce the complications associated to the radioablation of the BM of the recipient—a procedure difficult to transfer to clinical trials because of all its inherent severe contraindications (e.g., GVHD)—we originally focused on the generation of hematopoietic chimeras in sublethally irradiated recipients as a possibly less dangerous yet still efficient approach to abrogate autoimmunity (49). Donor BM cell number required for the establishment of the chimeric status in the NOD mice was titrated to define a dose-based correlation with the level of the chimerism achieved. NOD mice exhibited not only a higher resistance to allogeneic BM engraftment but also a greater inconsistency in the level of chimerism, induced within one group when

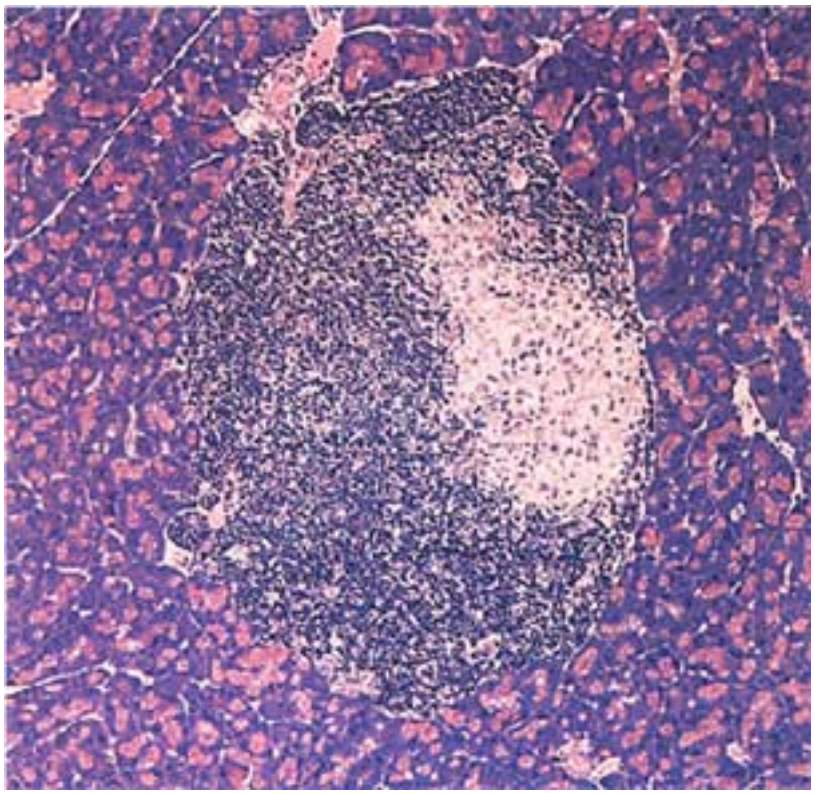

**Figure 1.** Insulitis: the presence of autoreactive T cells in the islet of pancreas.

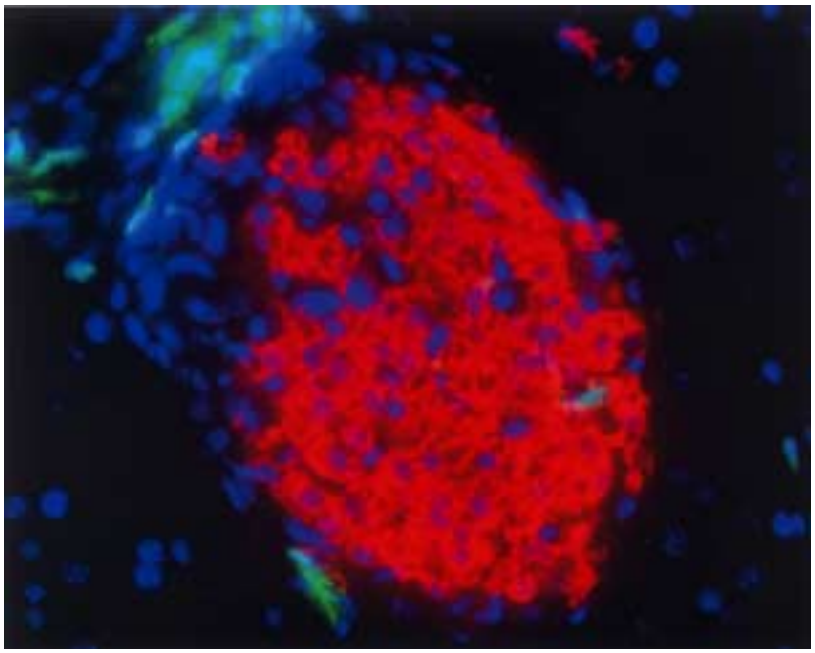

**Figure 2.** Regeneration of the endogenous pancreas. Using a GFP transgenic mouse donor, it is possible to observe how the transplanted BM cells (green) do not directly participate in the regeneration of the insulin-producing beta cells (red). There are not double-positive (orange) cells in the newly formed islets (17). (Reprinted with permission from *Journal of Clinical Investigation*.)

compared with other autoimmunity-free mouse strains studied (49). Another interesting and distinct feature of the NOD chimerae from other, non-autoimmunity-prone strains of mice that underwent allogeneic BM transplantation (BMT), is the increase of the level of chimerism with time when compared with the initial numbers determined at 4 weeks after BMT (49).

The recovery of the endocrine pancreas was reproducibly observed also in NOD mice treated just after the clinical onset of the disease (48). Diabetic mice that received islet grafts after 3 days of glycemia of >300 mg/dl became euglycemic within 24 h following the transplantation procedure and remained so for the length of observation. After surgical removal of islet graft-bearing kidney, performed at 17–26 weeks after islet transplantation, all mice remained euglycemic for up to 18 days (length of observation). Direct assessment of the insulin content in the endogenous islets was performed by histological evaluation of pancreata harvested from euthanized animals upon termination of the experiments (48,49). Insulin-positive beta cells in these pancreata collected from treated mice about 5 months after the clinical onset of the disease were found in quantities and morphologies similar to those of the normal mouse pancreas (Fig. 3). Allochimerism was multilineage and reached levels over 90% in all animals (49). None of these animals developed clinical signs of GVHD (49). When we used a green fluorescent protein (GFP) transgenic mouse (27) as the BM donor, GFP-positive cells were detected in the pancreas, but these cells did not look as directly involved with the restoration of the endocrine pancreas because both insulin- and GFP-positive cells were not detected (27,48) (Fig. 2). The GFP-positive cells were considered more probably circulating, mature blood cells rather than BM hematopoietic stem cells (HSCs) able to transdifferentiate into insulin-producing beta cells (43).

## ALTERNATIVE APPROACHES TO ABROGATE AUTOIMMUNITY

To avoid the possible complications associated with a conventional BMT, we then decided to use the strategy recently proposed by Tian et al. (40), in which T1D can be prevented by substituting a "diabetes-susceptible" class II MHC beta chain with a "diabetes-resistant" allelic transgene on hematopoietic stem cells of genetically susceptible NOD mice through gene supplantation (44).

In the late 1980s, in collaboration with Dr. Hugh McDevitt, Stanford University, we were able to map and identify the most influential single hereditary susceptibility factor in T1D: a single amino acid of the beta chain of the HLA-DQ histocompatibility molecule (25,41). Although T1D is recognized to be a multigenic disease (10), in humans the principal genetic susceptibility component was proposed to be any allelic form of the HLA-DQ molecule that lacks a charged amino acid at position 57 of its beta chain. Conversely, resistance to disease is associated with the inheritance of HLA-DQ alleles containing a charged amino acid such as aspartic acid, at the same position (Asp-57). Physical explanation of the unusual importance of this particular single amino acid location for the development of the autoimmune characteristics of T1D came with the elucidation of the crystal structure of the HLA-DQ8 molecule, a non-Asp-57 molecule that confers the highest susceptibility to the disease (20). The most important structural features of the susceptibility HLA-DQ8 molecule relevant to diabetes immunology are identical to the homologous I-$Ag^7$ molecule present in the diabetes-prone NOD mouse (1). The peptide binding site of the majority of human HLA-DQ and murine I-A molecules have an Asp-57 that points into the groove (24,45). In these allelic forms, Asp-57 forms an electrostatic salt bridge with the arginine in juxtaposition (i.e., in position 76) of the alpha chain of the molecule (Arg-76), which also points into the groove. Both HLA-DQ8 and I-$Ag^7$ molecules lack Asp-57 and this variation disrupts the electrostatic interaction, leaving the Arg-76 free to interact with the aqueous environment and with any peptide able to lodge inside the binding groove of the molecule. The absence of Asp-57 allows the binding of peptides that may not find appropriate lodging inside other Asp-$57^+$ molecule grooves, and may jeopardize an efficient presentation by the histocompatibility molecule to T cells because of incorrectly positioned self-peptides. The eventually created susceptibility status can be correlated, in immunological terms, with impaired peptide lodging, impaired peptide presentation to T cells, with consequent reduced efficiency on negative selection of self-reactive T-cell clones and positive selection of regulatory T cells (24,44,45). Indirect evidence supporting these hypotheses derives from transgenic NOD mice that express class II genes other than I-$Ag^7$, which do not develop diabetes (14,22), and from the fact that transplantation of allogeneic BM from strains that do not spontaneously develop diabetes also prevents the occurrence of diabetes in NOD mice (16,21,48,49).

Instead of performing an alloreactive bone marrow transplant, Tian's approach (40) consisted of the transfection ex vivo of the gene encoding a resistant, Asp-57-positive beta chain into the BM cells isolated from the diabetes-prone NOD mouse. The expression of the newly formed diabetes-resistant molecule in the reinfused hematopoietic cells was sufficient to prevent T1D onset in the NOD recipient, even in the presence of the native, diabetogenic, non-Asp-57, $Ag^7$ molecule (Fig. 4) (40,44). Mechanistically, the authors suggest a model in

which a subset of the engineered BM cells migrate, populate the thymus, and become efficient antigen-presenting cells involved in the negative selection of thymocytes that would otherwise mature into autoreactive T cells (44). In fact, diabetes-free NOD mice exhibited no emergence into the blood stream of T cells capable of responding to putative autoantigens, nor insulitis. The mice remained diabetes free even after cyclophosphamide treatment, a maneuver that tests the robustness of a prophylactic antidiabetic therapy (2). Similar experiments performed by Leiter's group (14) in which the T cells from the cured mice were used to transfer diabetes into NOD scid recipient mice seem to indicate, however, that central tolerance was not completely restored because some autoreactive T-cell clones were not deleted; rather, they were still present in the successfully treated recipient. The possible complementary involvement of activated CD4+CD25+ T regulatory (T reg) cells, able to alter the balance of the circulating cytokines and consequently mount an efficient peripheral tolerance, seems to be deserving equal consideration.

If this approach to obtain autoimmunity abrogation could also facilitate a possible recovery of autologous insulin production in the already-diabetic individual, safe induction of an autoimmunity-free status might become a new promising therapy for T1D.

## IMMEDIATE GOALS

With this working hypothesis we intend to test whether: a) the targeting of a diabetes-resistant beta chain gene transfection specifically on enriched precursors cells isolated from BM may improve the procedure outcome; b) nonradiation-based preconditioning approaches would be sufficient to promote engraftment and repopulation capabilities of these engineered cells; c) this approach can enhance thymic repopulation with engineered cells to promote an efficient negative selection of autoreactive T-cell clones and an improved positive selection of T reg cells, to consequently abrogate autoimmunity; d) the abrogation of autoimmunity will allow the rescue of endogenous insulin production even in newly onset diabetic NOD mice.

We have already been able to reproduce the results of Tian and collaborators as far as retroviral vector preparation, transfection, and BM reconstitution in radioablated recipients are concerned. We are now in the process of comparing the experimental protocol proposed by the Harvard group (40) with the new one we intend

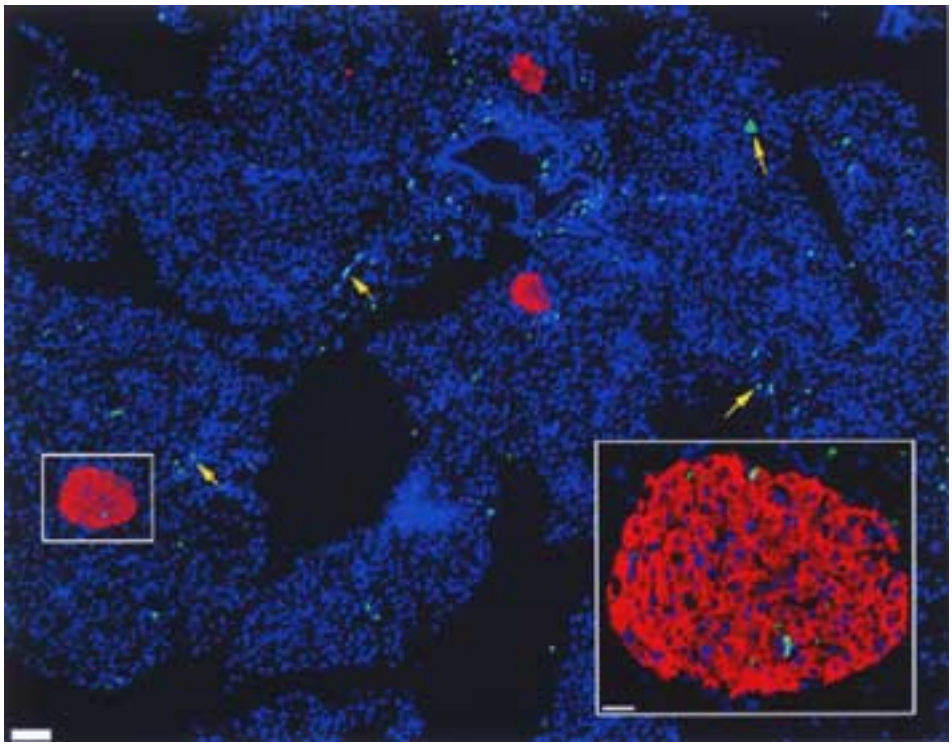

**Figure 3.** Regeneration (or rescue) of the endogenous pancreas. After 4 months from obliteration of the autoimmune process, via allogeneic bone marrow transplant, we have fond clusters of insulin-producing cells (red) resembling pancreatic islets (48). (Reprinted with permission from *Stem Cells*.)

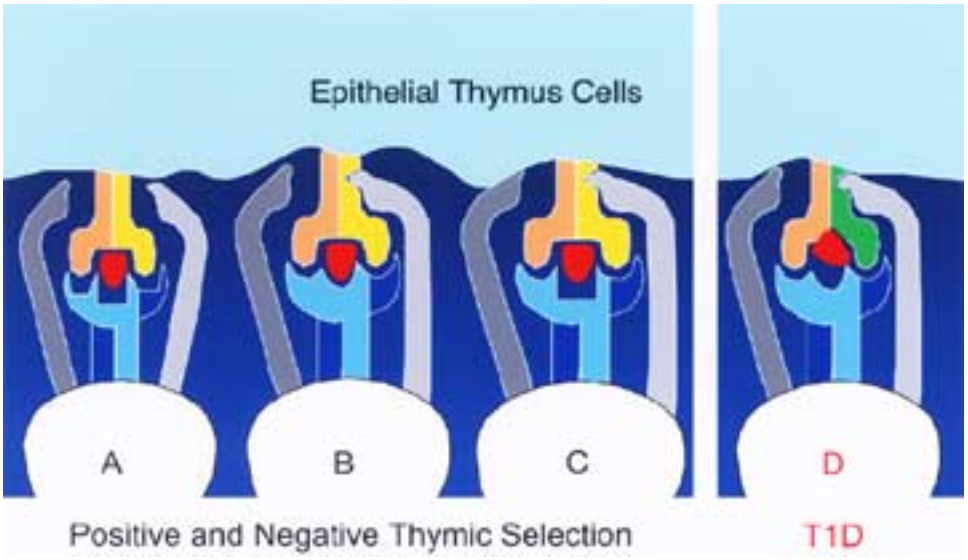


**Figure 4.** Theoretical basis of Tian's et al.'s (40) approach for autoimmunity abrogation in the NOD mouse. In a healthy individual, the maturation of the T cells, coming from precursors present in the bone marrow, is taking place in the thymus, where they undergo a positive and a negative selection. In the thymus, peptides (in red) from antigens of self-tissues are presented to the various immature double-positive (CD8, in gray, and CD4, lighter gray) T cells (A, B, C) via the MHC molecule. MHC class II molecules are heterodimers composed of an alpha (in orange) and a beta (in yellow) chain that form their antigen combining site. When, like for the A cell, the T-cell receptor (TCR: in blue, alpha chain; in azure, beta chain) has for the MHC molecule/self-peptide complex a very low affinity (in the figure contours of the MHC molecule/self-peptide complex do not fit with the contours of the TCR molecule), the developing T cell does not receive the necessary positive signal to survive and exit the thymus for release into the periphery. However, if the affinity between the MHC molecule/self-peptide complex and the TCR is too high, like for the B cell (in the figure the contours of the MHC molecule/self-peptide complex fit precisely into the contours of the TCR molecule), the T cell undergoes negative selection and dies inside the thymus. The T cell shown in C receives instead a positive survival signal because of the high-affinity interactions between its TCR with the MHC molecule; an affinity, however, that is not further enhanced by the presence of a self-peptide in its groove, so that the negative selection does not take place. This T cell matures and goes in the circulation to protect the body from foreign (non-self) invaders, with which it is able to efficiently interact. The immunological basis of type 1 diabetes is schematically described in T1D. Here the D cell binds to an MHC molecule (orange and green chains) conferring susceptibility to diabetes (like the HLA-DQ8 in humans and the I-$Ag^7$ in the mouse), because it does not present the self-peptide properly. The T cell, then, even if potentially autoreactive (D has the same TCR as B), is not subjected to negative selection and is free to leave the thymus to circulate in the blood. T cells that are potentially reactive to self-antigens but failed to be deleted inside the thymus are able to attack tissues of the body expressing these same antigens, generating autoimmunity. The approach taken by Tian and colleagues (40) can be illustrated imagining that the diabetogenic I-$Ag^7$ molecule, carrying a non-Asp-57 beta chain (in green like in D), was supplemented, in the hematopoietic cells of the NOD mouse, with a nondiabetogenic MHC molecule, like the one interacting with A, B, or C. The ex vivo transfection of a gene encoding an Asp-$57^+$ beta chain (in yellow) into the bone marrow stem cells allowed the reconstruction of an efficient MHC molecule (orange and yellow chains) that, once the cells were returned into the donor, allowed the restoration of an efficient negative selection in the thymus (like for B), sufficient per se to delete autoreactive T cells and consequently to prevent diabetes (44). (Reprinted with permission from *Gene Therapy*.)

to use instead. It must also be proven that the enriched precursors are able to generate new, more efficient APC and in sufficient numbers to efficiently repopulate the thymus and eventually favor peripheral tolerance.

Our first goal certainly is proving that regeneration (or rescue) of beta cell mass, in the absence of autoimmunity, is a function of the endogenous pancreas we may exploit for possible clinical applications, and that Tian et al.'s approach is useful to this aim. However, the proposition of considering the use of this same ap-

proach for a possible clinical trial of tomorrow may be complicated by the presence in humans of more than one susceptibility molecule [i.e., not only the H2-IA (equivalent to HLA-DQ) like in the NOD mouse, but also the H2-IE (HLA-DR)]. However, the efficiency of Tian et al.'s gene-based treatment, even in the presence of the native diabetogenic molecule, may offer solutions also to the problem of dealing with more than one susceptibility molecule. In fact, as opposed to the HLA-DQ molecule that is composed of a polymorphic alpha chain and polymorphic beta chain, the HLA-DR molecule is formed of a nonpolymorphic alpha chain combined with a polymorphic beta chain (30). The substitution of any HLA-DR beta chain will then be sufficient to create a new DR molecule. The protective molecule may act in an epistatic or dominant manner over the susceptibility molecule also in the case of the DR alleles.

Our second goal will be to systematically substitute for irradiation different antibody-based, immunoreductive conditioning protocols. We selected three approaches that seem to be giving the most reliable results, with the aim to see whether they may be able to promote engraftment and repopulation capabilities of our engineered BM precursor cells and consequently the abrogation of autoimmunity. The first is the protocol originally described by Lucienne Chatenoud (9), in which the anti-CD3 antibody was successfully used initially to delete the onset of the disease in prediabetic NOD mice. Further expanding the goals of her experiments, Chatenoud was able to reverse recent-onset disease by restoring the

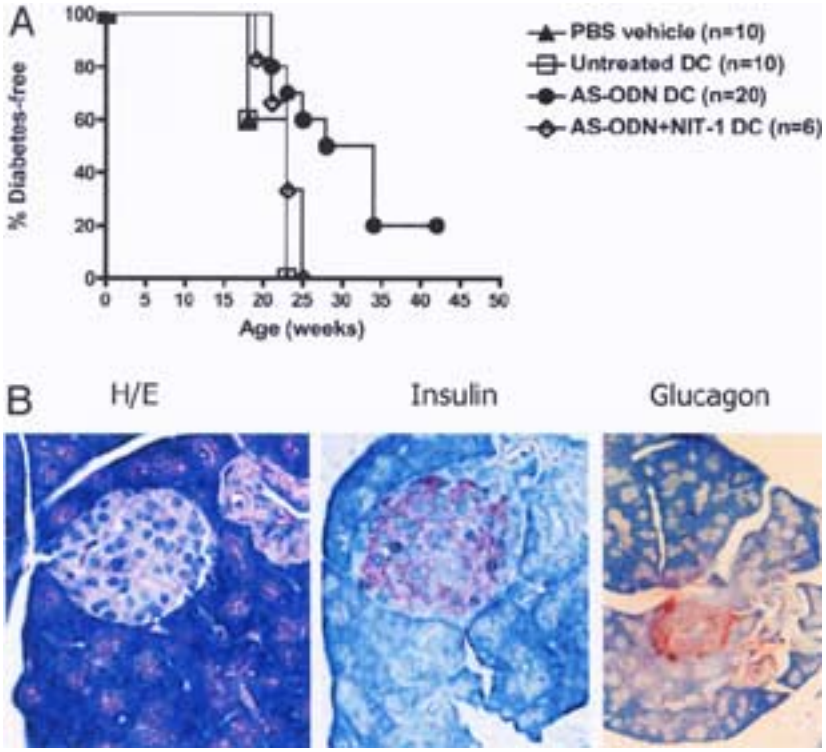


**Figure 5.** Diabetes incidence in NOD recipients of DC. (A) DC from bone marrow progenitors of 6–8-week-old female NOD mice were propagated in GM-CSF/IL-4 and further treated with AS-ODN with or without NIT-1 lysate as described. PBS-resuspended cells (in 200 µl; $2 \times 10^6$) were injected IP into syngeneic age- and sex-matched NOD recipients. Blood glucose was measured by electronic sampling of tail vein blood beginning at 15 weeks of age. AS-ODN DC: DC treated with a mixture of CD40, CD80, CD86 AS-ODN, each oligo at 3.3 µM; AS-ODN + NIT1 DC: DC cotreated with the AS-ODN mixture and NIT-1 lysate. $p = 0.012$, AS-ODN DC recipients vs. AS-ODN NIT-1 DC recipients, Kaplan-Meier log rank test. (B) Immunohistochemistry of pancreata from diabetes-free recipients of AS-ODN DC. For immunohistochemistry, the sections were fixed in paraffin, treated to block peroxidase, and incubated with nonfat dried milk. The slides were then incubated with anti-insulin or anti-glucagon Ab followed by an isotype-reactive biotinylated secondary Ab. Avidin-HRP was then added followed by diaminobenzidine after which a brown color could be observed. No evidence of insulitis was observed with insulin and glucagon readily detectable (23). (Reprinted with permission from *Journal of Immunology*.)

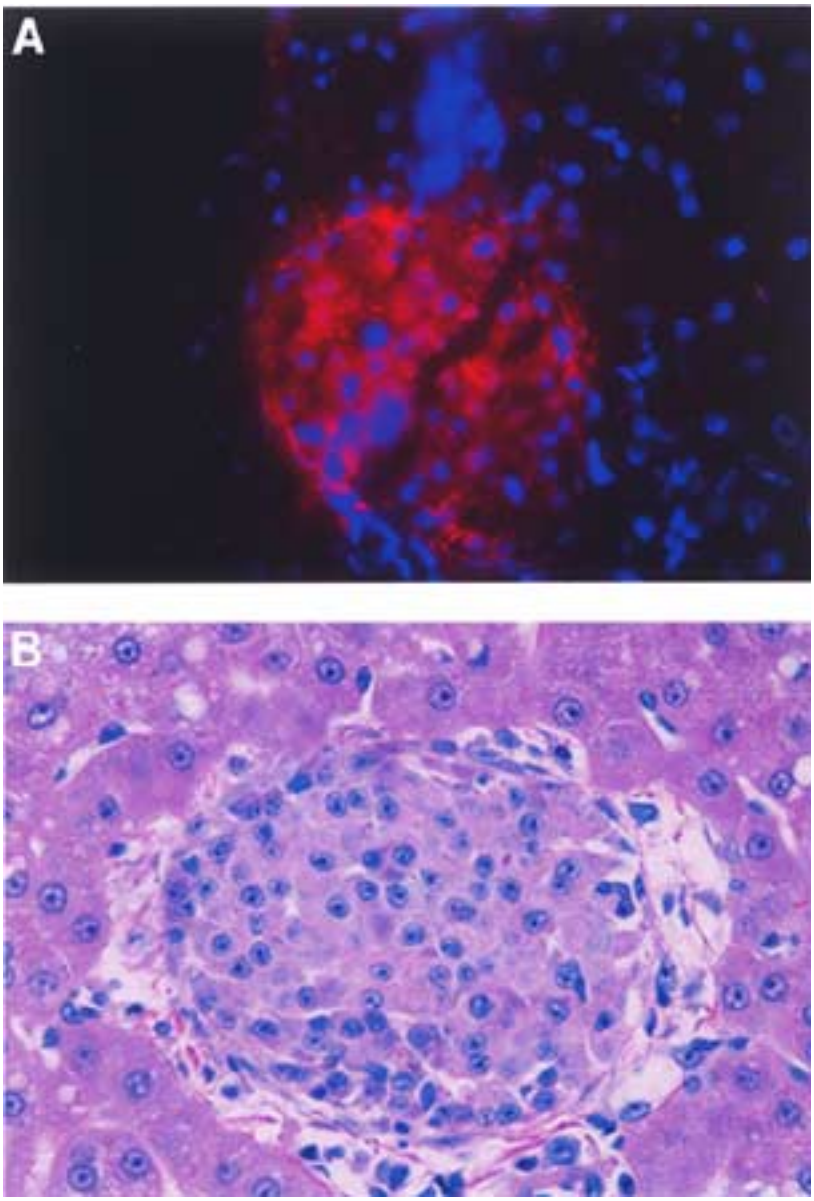


**Figure 6.** Adult pig islet transplanted into the liver of nonhuman primates. (A) Insulin immunofluorescent staining (red). Nuclear staining with Hoescht (blue). (B) Hematoxilin and eosin staining (40×).

lost self-tolerance to beta cell antigens in NOD mice. The treatment involved five consecutive doses of anti-CD3 antibody administered within 2–4 weeks (9). The second approach, proposed by Dale Greiner's group in Worcester (17), uses instead the anti-CD154 antibody. This protocol considers one donor-specific transfusion administered before transplant plus a brief course (e.g., four doses of 0.5 mg/mouse, on days −7, −4, 0, and +4 of transplant) of anti-CD154. The third is Megan Sykes's proposed protocol (42) in which anti-CD4 and anit-CD8 monoclonal antibodies are administered on days −6 and −1 of transplant followed by a 3-Gy total body irradiation and 7-Gy thymic irradiation on day 0. The possible involvement of CD4+CD25+ T cells in the preservation of tolerance, postulated on the basis of results obtained in cured mice (9,44), is also proposed by Zheng and collaborators (47) to explain the effect of a treatment that involves the administration of rapamycin, agonistic IL-2–Ig fusion protein, and a mutant, antagonist-type IL-15-related cytolytic Ig fusion protein to obtain long-term engraftment and tolerance of allogeneic islets transplanted into overtly diabetic NOD mice. The

CD4+CD25+ T-cell population is also increased once CD40, CD80, and CD86 cell surface molecules are specifically downregulated by ex vivo treatment of NOD mice dendritic cells (DCs) with a mixture of antisense oligonucleotides targeting CD40, CD80, and CD86 primary transcripts (23). The incidence of diabetes is significantly delayed by a single injection of the engineered NOD mouse-derived DCs into syngeneic recipients (Fig. 5) (23). Different quantities of antibodies and injection time schedules will be compared to find optimal conditions for acceptance of transfected, enriched BM precursor cells.

Although we do not have a proven record of our ability to properly use in the mouse model these conditioning protocols, we are obtaining extremely encouraging results (i.e., more than 2 months of pig C-peptide production) (Fig. 6) using the humanized anti-CD154 antibody from Novartis, to immunologically suppress diabetic (i.e., streptozotocin-treated) monkeys, recipients of intrahepatically transplanted, double knock-out (DKO) pig islets that are lacking the α1-3 galactosyltransferase enzyme (8,29). The dangerously thrombogenic characteristics of the anti-CD154 antibody may not be too worrisome if the treatment can be limited in time. The success of a limited in time, antibody-mediated conditioning will suggest approaches more transferable to humans than lethal irradiation.

## CONCLUDING REMARKS

Given that clinical solutions, like whole pancreas or pancreatic islet allotransplantation, are plagued by the paucity of pancreas donors and the toxicity of the immunosuppressive drugs that precludes their implementation in young recipients, relatively simple and possibly safe gene therapy-based approaches may become extremely useful in facilitating new types of clinical interventions.

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