Toward a cure for type 1 diabetes mellitus: diabetes-suppressive dendritic cells and beyond


Abstract: Insulin has been the gold standard therapy for diabetes since its discovery and commercial availability. It remains the only pharmacologic therapy for type 1 diabetes (T1D), an autoimmune disease in which autoreactive T cells specifically kill the insulin-producing beta cells. Nevertheless, not even molecularly produced insulin administered four or five times per day can provide a physiologic regulation able to prevent the complications that account for the morbidity and mortality of diabetic patients. Also, insulin does not eliminate the T1D hallmark: beta-cell-specific autoimmunity. In other words, insulin is not a ’cure’. A successful cure must meet the following criteria: (i) it must either replace or maintain the functional integrity of the natural, insulin-producing tissue, the endocrine islets of Langerhans’ and, more specifically, the insulin-producing beta cells; (ii) it must, at least, control the autoimmunity or eliminate it altogether; and (iii) it must be easy to apply to a large number of patients. Criterion 1 has been partially realized by allogeneic islet transplantation. Criterion 2 has been partially realized using monoclonal antibodies specific for T-cell surface proteins. Criterion 3 has yet to be realized, given that most of the novel therapies are currently quasi-patient-specific. Herein, we outline the current status of non-insulin-based therapies for T1D, with a focus on cell-based immunomodulation which we propose can achieve all three criteria illustrated above.

Type 1 diabetes mellitus: nature of the autoimmunity

Type 1 diabetes (T1D) is an autoimmune disorder that culminates in uncontrollable hyperglycemia because of the destruction of the insulin-producing beta cells of the pancreatic islets of Langerhans. The major effectors of beta-cell destruction are T cells reactive to beta-cell-specific antigens. A strong genetic predisposition is a conditio sine qua non of T1D and a large body of studies support that key genetic susceptibility loci affect the genesis, function and survival of immune cell subsets including T cells (effectors and putative regulatory T cells) and dendritic cells (DC) (1–4).

To understand the critical role played by the genetic predisposition in T1D, it is necessary to consider the processes that shape the immune system. A randomized pool of immature cells continuously generated in the bone marrow (BM) eventually travel across the thymus. Once in the thymus, these immature cells, individually expressing unique receptors, undergo positive and negative selection through receptor interaction with fragments of proteins present in our bodies (self-peptides) presented by antigen-presenting cells (APC) once properly inserted in the peptide-binding groove of major histocompatibility complex (MHC) molecules. Indeed, the epithelial thymus is now known to express a wide array of self-antigens including insulin, thyroperoxidase, thyroglobulin, and myelin basic protein, all of which are normally produced by cells targeted in a number of autoimmune disorders including T1D, Hashimoto’s thyroiditis and multiple sclerosis. Human leukocyte antigens (HLA), the human MHC molecules, anchored in the cell membrane of thymic epithelial and other APC display HLA/self-peptide complexes for T-cell receptor (TCR) interaction. A cell that interacts strongly with the HLA/self-peptide complex dies in
the thymus and is thus eliminated, i.e., negatively selected. On the contrary, cells that interact poorly with the complex do not proliferate sufficiently or become unable to function (i.e., anergic) and are eventually lost. The cells between these two extremes proliferate modestly, survive (positive selection), and emerge from the thymus to circulate in the periphery. Once in the periphery, the cells that matured in the thymus (T cells) can be engaged by circulating APC. DC are extremely powerful APC that collect foreign or ‘ignored’ (i.e., not previously exposed to the immune system) material, to present it as ‘new’ antigens to T cells through their HLA molecule. These T cells interact with the new antigens more strongly than with self-peptides, which enabled their positive selection and consequently the establishment of a T-cell-based protective immune response (5–7). The epitope spreading phenomenon (i.e., the expansion of newly recognized antigens) (8) observed in the islet inflammation is due to both islet-reactive T cells that were generated in the thymus early in ontogeny along with the generation and survival of T cells activated in the periphery by these new antigens.

The pathologic vicious circle of continuous presentation of old and new antigens, collected by the DC from the newly destroyed beta cells, to naive T cells in the pancreatic lymph nodes that eventually go back to the pancreas to kill other beta cells, is illustrated in Fig. 1.

The genetic predisposing background of autoimmune diseases, like T1D, is mainly constituted of specific HLA alleles (9–12). Allelic forms of the HLA-DQ molecule that lack a charged amino acid at position 57 of its beta chain were shown to be strongly correlated with the development of T1D. Conversely, resistance to the disease was found to be associated with the inheritance of an HLA-DQ allelic form with an aspartic residue at the same position (Asp57). The importance of this amino acid change has to do with the physical structure of the non-Asp57 alleles constituting class II molecules with a suboptimal functional groove. In fact, the molecular interactions that normally drive positive and negative selection are altered by the disease-associated HLA molecules so that even strongly self-reactive T-cell clones are allowed to escape to the periphery. HLA structural variations between alleles with Asp 57 and those lacking

![Image](Type 1 diabetes onset the pancreatic environment)

Fig. 1. The autoimmune vicious circle favoring the anti-beta-cell epitope spreading. Activated by an environmental stimulus, the autoreactive T cells that escaped thymic censorship, leave the lymph nodes and move into the tissues where eventually they find the self-peptide with which they were originally set up to react. Once the first beta cells are damaged, dendritic cells (DC) come to clean up the scene. Debris from dead cells are brought back to the lymph nodes in which even cytoplasmic markers – thus far not exposed to the immune system – are presented by DC to naive T cells. T cells that were so far ‘ignorant’ of their existence, recognize these self-antigens as foreign and react against them once back into the islet of Langerhans, killing new beta cells. This constitutes a vicious circle that does not allow the recovery of the insulin-secreting cells, even when the physiologic homeostasis process tries to substitute the lost cells with new cells. APC, antigen-presenting cells.
a charged amino acid at this position (non-Asp57) provide the foundation for HLA-associated diabetic susceptibility and resistance. Susceptibility is closely related to an impaired negative selection of self-reactive T cells. Another not necessarily mutually exclusive consequence is that T-regulatory cells are not as effectively selected and, in their reduced abundance, peripheral reactions to self-peptides are not held in check as well as would occur in a normal immune system.

The importance of the MHC alleles and the thymic antigen-presenting environment was confirmed in studies in which autoimmunity was prevented in non-obese diabetic (NOD) mice by transplanting BM cells derived from diabetes-resistant (Asp57) strains (13, 14). Instead of relying on allogeneic BM transplantation, Tian et al. (15) successfully prevented diabetes by reconstituting sublethally irradiated non-Asp57 NOD mice with their own BM genetically engineered ex vivo to express a resistance (Asp57) MHC class II molecule. The reconstituted mice, carrying BM-derived cells that coexpressed both their own diabetogenic (non-Asp57) and the transfected Asp57 beta chain, were diabetes-free. The thymus, repopulated by the engineered BM cells, which differentiated into APC, had restored negative selection and consequently the ability to delete T cells potentially autoreactive to pancreatic beta cells. Autoreactive T-cell clones, which were not found in the treated animals, were eliminated because of the stronger affinity of their TCR for the self-peptide now properly presented by the newly expressed MHC molecule.

Immediately, it became clear to us that once this approach had obtained autoimmunity abrogation also in already diabetic individuals, it could possibly facilitate the recovery of autologous insulin production. Safe induction of an autoimmunity-free status might become a new promising therapy for T1D.

We are working on this aim using a modification of Tian’s approach that may be transferable to clinical trials in the near future (16, 17). A reason to believe it comes from the study of Voltarelli et al. in which autologous transplantation of hematopoietic stem cell-enriched BM was used to treat T1D patients (18). The risk of exposing the patient to a non-myeloablative yet quite powerful preconditioning was not totally justified, however, by the results obtained. The effects were limited to simple postponement of diabetes recurrence, i.e., just delayed by the time necessary for the transplanted BM to reorganize itself and to reestablish all of its immunocompetent cell subpopulations. The autologous BM did not change the patient’s genetic characteristics under which tolerance for the insulin-producing beta cells was not achieved in the first place; in this context, autoimmunity easily recurred. Our approach should safely change the patient’s diabetogenic characteristics (16, 17).

New types of intervention are becoming available everyday, which may allow a successful ‘take’ of the transplanted BM without the need for a deleterious type of preconditioning (19). Furthermore, new gene therapy approaches that do not involve vector integration at potentially transformative gene loci are continuously discovered (20–22). A protocol that takes into account the choice of the gene transfection vector, any form of safer preconditioning of the patient, and the genetic background of the transplanted BM will significantly improve the one proposed by Voltarelli et al. because efficient negative selection will be reestablished along with central (and possibly also peripheral) tolerance.

The prevalent belief that beta-cell mass is fixed by adulthood and that all adult beta cells are fully differentiated is now being reexamined in light of recent studies showing a regenerative capacity, albeit low, of pancreatic islets of Langerhans during T1D progression. These studies suggest that, although the physiological state of islet cells tends towards a fully differentiated phenotype, the lack of autoimmune aggression, together with the ‘danger’ signals generated by massive beta-cell destruction may trigger processes inside progenitors (whether islet-resident or ductal epithelium-resident) that result in some degree of islet cell regeneration (14, 23–26).

**Immunomodulation: current state-of-the-art in the clinic**

In general, immunomodulation aims at reestablishing central and/or peripheral tolerance to self. The reestablishment of tolerance can include the deletion of autoreactive immune cells, the attenuation of the activity of autoreactive immune cells (T-cell anergy), the generation/augmentation in vivo of immunosuppressive cells that can be antigen-specific (T-regulatory cells). Many experimental immunomodulatory interventions have been carried out preclinically in the NOD mouse model and almost all involve treatment of young mice ‘prior’ to the clinical onset of hyperglycemia. An insightful article by Atkinson and Leiter was instrumental in illustrating the plethora of specific (and sometimes even quite unorthodox) approaches by which diabetes onset was delayed or prevented in this model (27).

As of yet, only one of these methods has shown any significant clinical efficacy. This is the use of a humanized anti-CD3 antibody [TRX-4 (28); and hOKT3g (Ala-Ala) (29)], which can reverse new-onset disease, although for a limited amount of time (30–32).

The manipulation of T-cell responses by autoantigen-derived peptides has been another approach used to attenuate autoimmunity with demonstrated efficacy in rodent models of T1D including the NOD mouse (33–36). The majority of pathogenic CD8+ T-cell clones isolated from pancreata of diabetic NOD mice react specifically with the 9–23 peptide of the insulin B
The first clinically adapted immunoregulatory cell therapeutic: diabetes-suppressive autologous DC

DC are the body’s sentinels largely responsible for host surveillance against microenvironmental anomalies including pathogen invasion, infection, and damaged tissue architecture, while coordinating the mechanisms of self-tolerance (70–74). DC continuously traffic throughout all body tissues’ sampling molecules from their surroundings, where it is believed they maintain potentially autoreactive immune cells in quiescence either directly or via indirect regulatory immune cell networks (75–82). When DC encounter local disruption of tissue architecture and elevated proinflammatory signals from infected cells, DC undergo ‘maturation’ through a series of internal changes. While conceptually thought of as a series of discrete checkpoint-like events, maturation is rapid and often nonlinear (63, 83, 84). Concurrent with maturation, DC migrate away from the site of ‘danger’ and into the anatomically closest lymph nodes. Within the lymph node, the DC, as a powerful APC, initially interacts – using its class I or class II MHC/peptide complex – with the TCR present on a naïve T cell. This will constitute the so-called ‘first signal’ for T-cell activation. To bring a T cell to full activation, however, a subsequent contact between co-receptors is necessary. Co-stimulatory molecules are so-called because they are present on the APC (e.g., CD80/CD86 or CD40), with their counterparts on the T cell (i.e., CD28 and CD40 ligand, respectively), that, by interacting, further stabilize the signal for activation between the two cells, thus providing the ‘second signal’.

Absence of co-stimulatory molecule binding and consequently lack of secondary signal generation has been shown to lead to impaired activation of the responding T cell, eventually bringing it to functional anergy or apoptosis. This is indeed the outcome of many immunosuppressive strategies aimed at co-stimulation blockade (85–90).

Many lines of investigation support the concept that DC in a functionally immature state (characterized by low to absent co-stimulation) are powerful agents of immune hyporesponsiveness (80, 82, 91–95). Exogenous administration of functionally immature DC achieves long-term and stable allograft survival in a variety of mouse and rat models and prevents a number of autoimmune diseases (96–103). Mechanistically, functionally immature DC act by inducing anergy either via direct cell contact and/or cytokines (104–106) and, as described more recently, by up-regulating the number and function of regulatory immune cell subsets, especially CD4+ CD25+ Foxp3+ T cells (Treg) and a class of CD8+ immunosuppressive T cells (106–114).

We have shown that in vitro administration of Nuclear Factor-KappaB (NFκB) decoys to DC as well as direct targeting of CD40, CD80, and CD86 with antisense oligodeoxynucleotides (AS-ODN), reduce co-stimulatory molecule levels producing functionally immature DC capable of preventing or reversing new-onset diabetes in the NOD mouse (115–117). This was accomplished while maintaining T-cell responsiveness to alloantigens in animals that received repeated injections of modified DC. Co-stimulatory-depleted DC also augmented the number of Treg that were CD4+ CD25+ Foxp3+ through short-range interleukin-7 signaling (115).

Numerous clinical trials have safely used DC-based treatments for cancer therapy providing the basis for...
clinical adaptation of DC administration for T1D treatment. A National Institutes of Health-funded protocol approved by the Food and Drug Administration (FDA) is currently underway in phase I clinical trial with an adult (18 yr or older) cohort documented with insulin-requiring T1D of at least 5-yr duration. Leukocytes are obtained from the patient by apheresis and DC are generated in vitro and engineered in Good Manufacturing Practice (GMP) facilities with the addition of AS-ODN. These DC, which express low levels of CD40, 80 and CD86 are injected into the patient by intradermal administration at an anatomical site proximal to the pancreas (Fig. 2) (118). DC will migrate to the nearest lymph nodes where they will start to interrupt the vicious circle that maintains islet-specific inflammation, i.e., insulinitis. This therapeutic approach should be more successful when DC injections start close to the clinical onset of the disease. In the pancreas, DC acquire beta-cell-specific antigens from apoptotic cells, leading to the eventual display of these antigens to T cells in the pancreas-draining lymph nodes. The lack of co-stimulatory molecules will result in an anergizing signal to the T cells, induce regulatory immune cells (like Foxp3<sup>1</sup>Treg), and interrupt the T-cell-mediated anti-beta-cell epitope spreading phenomenon. The abrogation of the autoimmune diabetogenic insult should be sufficient to promote rescue of still present insulin-producing beta cells and/or neogenesis of other insulin-producing cells in the host endocrine pancreas, even after the onset of the disease. This trial is underway at the time of this writing and once safety has been demonstrated, a phase II efficacy trial will start, involving new-onset diabetic patients.

**Beyond autologous DC: a diabetes-suppressive microsphere vaccine**

In spite of the promise of this study, we have encountered cumbersome logistical requirements to generate these diabetes-suppressive DC, which may limit the future enrollment of new-onset diabetic children in the efficacy phase of the trial. Leukopheresis takes 2 or 3 h to provide sufficient precursor cells to generate the number of DC necessary for six to eight injections. The obtained DC should be exposed to AS-ODN in GMP facilities in which the laboratory practices are frequently difficult to reproduce. GMP facilities are frequently located far away from the clinic where the patients are treated. Many DC are lost during the freezing/thawing procedures.

In an effort to avoid these steps, we have been concurrently pursuing an alternative method to stabilize DC immaturity directly in vivo using microparticle carriers of immunomodulating agents like AS-ODN.

Many studies confirm that microparticle carriers can direct DC to the administration site and once phagocytosed, the contents can shape the DC functional phenotype (119, 120). We have incorporated the AS-ODN directed against CD40, CD80 and CD86 into Baxter Healthcare’s PROMAXXX<sup>®</sup> microsphere delivery system. The inert PROMAXXX microsphere technology has been shown to be safe and effective in human trials (121). More importantly, when administered in vivo, this technology is neutral with respect to DC maturation state compared with the known immunostimulatory properties of other microsphere
formulations. In other words, other polyplex formulations have an inherent capacity to induce the upregulation of co-stimulatory proteins at the DC surface (possibly via Toll-like receptors), whereas the PROMAXX technology does not. This neutrality on DC maturation is a critical criterion in adapting microsphere chemistry for immunosuppressive objectives where DC are involved as mediators. Our very recently developed PROMAXX antisense-formulated vaccine rendered DC diabetes suppressive and newer data show that it can prevent and reverse new-onset autoimmune diabetes in the NOD mouse model (122). This recent study was aimed at ascertaining the efficacy of AS-ODN-formulated PROMAXX microspheres to prevent T1D and to reverse new-onset disease. Microspheres carrying AS-ODN to CD40, CD80 and CD86 were delivered into NOD mice. Glycemia was monitored to determine disease prevention and reversal. In recipients that remained and/or became diabetes free, spleen and lymph node T cells were enriched to determine the prevalence of Foxp3+ putative T-regulatory cells. Splenocytes from diabetes-free microsphere-treated recipients were adoptively cotransferred with splenocytes from diabetic NOD mice into NOD-SCID recipients. To rule out non-specific systemic immunosuppression, splenocytes from successfully treated recipients were pulsed with beta-cell antigen, ovalbumin or cocultured with allogeneic splenocytes. The microspheres prevented T1D and, most importantly, exhibited a capacity to reverse clinical hyperglycemia, suggesting reversal of new-onset disease. The microspheres augmented Foxp3+ T-regulatory cells, induced hyporesponsiveness to NOD-derived pancreatic beta-cell antigen, without compromising global immune response to alloantigens and nominal antigens. T cells from successfully treated mice suppressed adoptive transfer of disease by diabetogenic splenocytes into secondary immunodeficient NOD-SCID recipients. Finally, microspheres accumulated within the pancreas and the spleen. Live animal in vivo imaging measured the microsphere accumulation pattern (Fig. 3). DC from the spleen of the microsphere-treated mice exhibit decreased cell surface CD40, CD80, and CD86. This novel microsphere formulation represents the first diabetes-suppressive and reversing nucleic acid vaccine that confers an immunoregulatory phenotype to endogenous DC (122). We predict that once all preclinical studies are completed, a phase I/II trial can be initiated (Fig. 4). The microspheres are simple to manufacture to clinical grade on a large scale and do not involve the cumbersome logistics outlined earlier that are necessary for the DC-based therapy. Although we have focused on autoimmune diabetes as a disease target throughout our studies, our microsphere technology can be readily and rapidly applied to producing other immunosuppressive vaccines for other autoimmune conditions in which nucleotides along with disease-specific antigens can be formulated to target the transcripts of other critical molecules involved in immunoregulation inside endogenous DC without affecting their maturation status in vivo.

**Fig. 3.** In vivo accumulation of antisense-oligonucleotide-formulated microspheres (AS-MSP). (A) Alive non-obese diabetic (NOD) mice received a subcutaneous injection containing sterile phosphate buffered saline (control, CN) or fluorescent microspheres with 50 μg of AS-MSP (AS). Three hours postinjection, the spheres accumulated in the area of the pancreas and spleen. (B) Pancreas and spleen removed at 3, 24, and 48 h postinjection are shown to contain the fluorescently labeled microspheres [used by permission of Diabetes, Phillips et al. (122)].
Conclusion

In the past 20 years, benchside research has made many promises to ‘cure’ T1D. Only recently has it been possible to clinically implement a limited number of benchside successes. This has been primarily because of the reluctance of clinicians to intervene in a disease where a therapeutic ‘gold standard’ in the form of insulin replacement is considered by many to be sufficient to guarantee an almost normal life for many diabetics. The persistent presence of complications in almost all type 1 diabetics, despite insulin replacement, forced us to conclude that insulin is not a real cure. With this knowledge, it has become easier to consider immunotherapies aimed at preventing and perhaps reversing T1D. Also, data from clinical trials from other antibody- and cell-based therapies for other diseases (e.g., cancer and rheumatoid arthritis) have paved the way for cell-based immunotherapy to enter routine clinical practice. For T1D, these other trials have uncovered critical reference points (e.g., biochemical, physiologic and immunologic profiles) for the clinician to monitor safety and immune activity in vivo. Such key reference points and safety measurements have encouraged us to adapt autologous DC therapy to reverse new-onset T1D. In the near future, we envision trials with the microsphere vaccine. Whether the autologous diabetes-suppressive DC or the microsphere vaccine will prove to be ‘cures’ awaits demonstration of safety, proven lowering of insulin requirements with evidence of C-peptide level amelioration, considered to be physiologic and pharmacologic markers of preservation of residual beta-cell mass and/or possible regeneration. At the same time, as basic research identifies novel molecular pathways of immunoregulation, more such cell- and particle-based therapies will become acceptable for clinical consideration.

References

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Fig. 4. Antisense-oligonucleotide-formulated microsphere-based clinical trial. Once compared with the procedures involved in the current clinical trial (see Fig. 2), it is evident how the use of the AS-MSP simplifies the logistics while guaranteeing the same immunologic result.
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