Chapter III.6

In Vivo Delivery of Nucleic Acid-Formulated Microparticles as a Potential Tolerogenic Vaccine for Type 1 Diabetes

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Abstract

Originally conceived as a method to silence transcription/translation of nascent RNA, nucleic acids aimed at downregulating gene expression have been shown to act at multiple levels. Some of the intriguing features of these gene-silencing nucleic acids include activation of molecular signals in immune cells which confer tolerogenic properties. We have discovered a method to induce stable tolerogenic ability to dendritic cells in vivo using a mixture of phosphorothioate-modified antisense DNA targeting the primary transcripts of CD40, CD80 and CD86. Autologous human dendritic cells generated in the presence of these oligonucleotides prevent and reverse type 1 diabetes (T1D) in the non-obese diabetic (NOD) strain mouse model of the human disease, and have been shown to be safe in established diabetic human patients. Even though this in vivo approach is clinically feasible, we have gone beyond a cell therapy approach to develop a “population-targeting” microparticle formulation of the three antisense oligonucleotides. Effectively, such a product could constitute an “off-the-shelf” vaccine. In this paper, we describe the progress made in developing this approach, as well as providing some insight into potential molecular mechanisms of action.

Keywords: type 1 diabetes · immune tolerance · vaccination · autoimmunity · beta-cell · dendritic cell · antisense oligonucleotide · microparticles · regeneration

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease caused by the loss of insulin-producing β-cells below a critical mass necessary to maintain proper glucose metabolism consequent to chronic impairment. A combination of multilocus genetic susceptibility and exposure to environmental triggers facilitates a T-cell-driven process of islet inflammation, impairment, and eradication [1]. Studies conducted in non-obese diabetic (NOD) mice, a strain which spontaneously develops diabetes of an autoimmune nature, have highlighted the critical role of the adaptive immune response in the pathogenesis of the disease [2]. β-cell death is first observed at 2-3 weeks of age, possibly as part of a natural remodeling and/or metabolic reprogramming of β-cells. Alternatively, or in addition, β-cell death could occur as a consequence of host response to viral infection [3].

Irrespective of the actual initiating trigger, which is now considered to be insulin itself [4], the role of dendritic cells (DC) is critical. β-cell death leads to activation of islet-resident and pancreas-accumulating DCs in response to an as yet unidentified inflammatory signal deriving from the islets. Both islet-resident DCs and those that respond to an islet “damage” signal contribute to β-cell autoreactivity. They take up β-cell-derived material, which includes the antigens that drive the cell-specific inflammation before migrating to the pancreas-draining lymph-nodes, where they initiate
the expansion of specific β-cell autoreactive T cells that have escaped negative thymic selection [5]. This cascade of events initiates a chronic inflammation and creates a vicious circle that eventually results in sufficient β-cell mass destruction to require exogenous insulin replacement, while peripheral mechanisms attempt to prevent autobody, e.g. through Foxp3 T regulatory cells (Figure 1). Even if insulin replacement can maintain normal glucose homeostasis, it is unable to provide the physiologic control necessary to prevent serious diabetic complications such as cardiovascular diseases, nephropathy and neuropathy, which decrease quality of life and result in significant morbidity and mortality. Thus, insulin cannot cure T1D, but only mitigate the symptoms. An effective cure would need to preserve an adequate amount of the patients’ residual β-cell mass to maintain physiologic glucoregulation, while eliminating or stably suppressing the underlying autoimmunity.

There has been some success in achieving preservation of a residual β-cell mass which is able to regulate glucose homeostasis physiologically by using immunosuppressive drugs such as cyclosporin A and anti-CD3 antibody [6, 7]. However, the significant adverse events associated with these approaches require ongoing research activities to discover new and safer methods, including biologic or cell-related therapies aimed at mobilizing tolerogenic cell networks in vivo.

Immunotherapy to halt or reverse autoimmune diabetes needs to establish and maintain stable peripheral T cell tolerance. Given the important role of DCs in the initiation of immune responses, DC-based strategies seem to be attractive options to induce functional self-tolerance in T1D [8]. Studies involving DCs in therapeutic applications focus on the exogenous generation of tolerogenic DCs for administration as a cellular vaccine [9, 10]. An attractive alternative to ex vivo DC manipulation can be in vivo targeting of DCs with biologic or chemical drugs which stabilize the cells into tolerogenic states. Given the labile nature of biologics and most of the immunosuppressive chemicals, as well as their potential for systemic spread, biodegradable microparticles have evolved which have been shown to mitigate these unwanted effects. Additionally, microparticles can be formulated in a manner that can result in the co-delivery of immunosuppressive agents along with disease-relevant antigens [11]. Furthermore, microparticles can be engineered to be multifunctional and modular. For example, they can be coated with chemoattractants (e.g. CCL19, CCL20, CCL21) that specifically stimulate DC accumulation to the area of administration in vivo [12, 13]. Once taken up, the microparticles can release their tolerogenic payload with or without the provision of antigens. Finally, microparticles can also be programmed to release their various contents at different times after in vivo injection.

### 2. Dendritic cells as immunoregulators

The interest in the use of DCs as cellular therapy began with the identification of their role as the most potent antigen-presenting cells. This led to more than 50 clinical trials worldwide using DCs as adjuvant immunotherapy for many malignancies [10, 14-16]. The common characteristic of
these DCs is their high expression of costimulation surface proteins like CD86, CD40, and OX40L, conferred during the generation process by the addition of immunostimulatory cytokines ex vivo or triggered after the generation process by the addition of nucleic acids. Intensive costimulation inside the lymph nodes draining the site of DC administration results in very robust Th1 type reactions and activation of naive and memory T cells. Following ex vivo generation, immunostimulatory DCs are poorly phagocytic and exhibit lower thresholds for TLR stimulation. They consequently express high levels of NF-κB, and much of this transcription factor is found in the nucleus compared to non-stimulatory DCs where NF-κB is mainly cytosolic. Immunostimulatory DCs produce significant levels of IL-12, IL-6, and TNF-α. Through these cytokines the DCs manage and maintain a proinflammatory cell loop at the lymph nodes that drain their site of administration. This loop includes the activation of naive and memory T cells, macrophages, NK cells, and B cells. Most immunostimulatory DC protocols involve the provision of antigens derived from the cells or tissues to which an immune response should be targeted. For example, in prostate cancer trials, DCs are of-
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Tolerogenic DCs can be generated in vitro from bone marrow precursors of rodents, or blood monocytes from non-human primates and humans, by controlling their exposure to cytokines, growth factors, pharmacological agents, or by genetic engineering [25]. It is therefore reasonable to exploit these effects to formulate microparticles that could target DCs in vivo. Different studies have shown that a downregulated costimulation capacity in DCs can lead to a tolerogenic therapeutic outcome [26, 27]. Indeed, in states of impaired costimulation, functionally immature DCs can achieve long-term and stable allograft survival, and can also prevent autoimmune disease in animal models [28, 29]. Mechanistically, functionally immature DCs, with low to absent costimulatory molecule expression, have been shown to mediate peripheral tolerance by inducing T cell anergy. This was observed in the context of cell-cell contact and/or cytokine secretion, and promoted the function and the expansion of regulatory cell subsets such as Foxp3+CD25+CD4+ Treg and novel B regulatory cell populations [10, 30, 31].

There are different mutually non-exclusive mechanisms which explain how tolerogenic DCs (i.e. functionally immature cells) maintain an overall state of immune hyporesponsiveness, of which the induction of anergy is the most clearly understood. In the absence of costimulation, T cells that interact with antigen through class II MHC on costimulation-deficient DCs (e.g. low surface levels of CD86 and OX40L) cannot enter the cell cycle. Those T cells that enter the cell cycle, exhibit aborted G1-S phase transition.

Costimulation can be thought of as a means to increase the time of interaction between the T cell receptor complex and the antigen-MHC on the DC. The lower the costimulatory ability the shorter is the interaction between DC and T cell, and the less time the TCR has to interact with the MHC-peptide. Increasing evidence demonstrates that a critical threshold exists for the time that a TCR is engaged with the MHC to stabilize the TCR signaling complex and to initiate phosphorylation of TCR epsilon-associated proteins. The latter amplifies the initial phosphorylation cascade. This culminates in the activation of cyclin production, cyclin-dependent kinase activation, and transition into G1-S. When the time of TCR-MHC engagement does not meet the critical threshold (e.g. consequent to impaired costimulation), cell cycle entry is aborted, which results in anergy in the T cell. Anergic T cells obtain an antigen-specific immunosuppressive ability. Through mechanisms which are still unclear, they silence or impair bystander

DCs with immunosuppressive roles occur naturally in vivo and have been generated ex vivo to induce negative immunomodulation. The naturally occurring DCs have been characterized as CD11c+, CD11b+, CD8 alpha+, CD45RB+, BDCA4+, CD123+, CCR7+, and CCR9+. Other DCs of this kind have been characterized as CD83+, CD1a+, ILT2+, ILT3+, and ILT4+, and yet others exhibit CD200R3+ and CD49+. Some reports indicate that expression of indoleamine 2,3-dioxygenase (IDO) is characteristic of immunosuppressive DCs. However, a respectable body of data suggests that IDO expression is limited to very specific and limited populations which are possibly in a metastable developmental stage in vivo [23, 24]. Although DCs have a natural ability to switch between activating immunostimulatory and suppressive tolerogenic states in vivo, the microenvironment determines their phenotype and the stability of the phenotype. Understanding the microenvironmental signals that enforce this modulation is the key to acquiring insight into DC-related biological mechanisms and to discovering approaches to achieve stable tolerogenic characteristics in ex vivo generated DCs.

Ten pulsed with tumor-derived antigens in the form of the specific patient's tumor lysate. This increases the probability that T cells reactive to tumor antigens in vivo will be preferentially expanded by the DCs as the tumor antigen is presented on class II MHC.

Eventually, it was appreciated that DCs play a substantial role as regulators of immunity by providing activation and maintenance signals for a variety of immunosuppressive cells [17, 18]. Largely acting on T cell populations (CD4, CD8), DC-related regulation of immunity also involves the stimulation of novel immune cells which are not well characterized (NKT cells, gamma-delta T cells, T-follicular helper cells, and T-follicular regulatory cells) [19, 20]. General key features of DCs that activate and maintain immunosuppressive states include:

- Low antigen-presentation capacity (low levels of class I and class II MHC expression on the cell surface)
- Low-to-absent co-stimulation ability
- Poor or absent allostimulatory ability to induce T cell proliferation in allogeneic mixed leukocyte culture or in antigen-specific recall responses
- Production of Th2 type cytokines and retinoic acid [21, 22]

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immune cells to enter into a proinflammatory state.

Functionally immature and tolerogenic DCs also increase the frequency of regulatory T and B cells. Although some studies suggest that DC-derived anti-inflammatory cytokines (e.g., IL-10 and TGF-β) and/or DC surface proteins (e.g., GITR) expand pre-existing Foxp3+ Tregs, the biological processes are still unclear. Interestingly, oligonucleotides, including RNAi and antisense DNA, have an intrinsic capacity to trigger tolerogenicity in DCs, in a manner that is independent of their expected molecular effects (e.g., RNase H-dependent cleavage of the primary transcript). It is highly probable that they act through alternative activation of oligonucleotide-sensing receptors such as TLR2, 3, 7, and 9, and RIG-1 and MDA-5 [32]. While TLR signaling in DCs frequently results in an immunostimulatory state, in some instances, TLR signaling can confer immunosuppressive abilities. We speculate that this is not a TLR-dependent phenomenon per se, but depends on the higher order structure of the oligonucleotides which determine how TLRs and which TLRs will transmit a “higher order”-dependent signal. Such a possibility would involve differential TLR signaling dependent on the topology of its ligand.

3. Tolerogenic dendritic cells as cell therapy for type 1 diabetes

Promising preclinical data were obtained from studies in NOD mice, showing that DCs generated in vitro in the presence of a mixture of CD40-, CD80-, and CD86-targeting antisense DNA can prevent and reverse the disease. This success led to a phase I clinical trial using autologous ex vivo engineered DCs from established diabetic patients (clinicaltrials.gov identifier NCT00445913), conducted at the University of Pittsburgh Medical Center (UPMC) [10]. The trial included adult volunteers, aged from 18 to 60 years, with documented evidence of insulin-requiring T1D of at least 5 years’ duration. Leukocytes were obtained from the patients by apheresis. DCs were generated in the presence of antisense DNA, targeting the primary transcripts of CD40, CD80, and CD86 costimulatory genes under good manufacturing practices (GMP). These DCs expressed low levels of CD80, CD86, and CD40, and were then injected into the patients by intradermal/subcutaneous administration at an anatomical site proximal to the pancreas. The choice of this anatomic region for DC delivery was based on the location of the lymphatic conduits (microvessels) which drain the injection site and favor migration of the DCs into the pancreatic lymph nodes. Once inside the pancreatic lymph nodes, the tolerogenic DCs could interact from the injection site with either soluble β-cell-derived antigens from the inflamed islets. The DCs can also acquire β-cell antigens through cross-presenting islet-resident DCs that moved into the pancreatic lymph nodes subsequent to β-cell destruction [33]. Having acquired the β-cell antigens, the tolerogenic DCs can then suppress autoreactive T cells and stimulate suppressive immune cells inside the pancreatic lymph nodes, which may stop the vicious circle maintaining the islet-specific inflammation in the pancreas.

Our study, the first ever to use tolerogenic DCs in humans, provided some interesting observations in addition to demonstrating its complete safety. First, treatment of diabetic patients with non-manipulated DCs or DCs generated in the presence of antisense DNA resulted in a C-peptide level that became detectable in some patients. Whereas, previous to and at enrolment of the patients, the C-peptide level was not detectable. Moreover, DC administration was associated with an increased frequency of B220+CD11c B cells that we discovered to be comprised of a potentially regulatory B cell population (Breg) [10].

Further characterization of this B cell population confirmed their immunosuppressive activity in vitro [10]. In contrast to other reported Breg cells [34, 35], this immunosuppressive ability is independent of IL-10 secretion [10]. Although Bregs have been characterized by various phenotypes (reviewed in [36]), much attention has recently focused on a rare splenic B lymphocyte population, CD19+CD122+CD5+, which is able to suppress experimental contact hypersensitivity (CHS) in an antigen-restricted and IL-10-dependent manner [35, 37, 38]. These cells represent about 1% of the total splenic B cells. Adoptive transfer of these B lymphocytes effectively reduces inflammation in recipient mice sensitized with the same, but not with a different chemicals (in a CHS model in vivo), indicating that the suppressive function was antigen-specific. These cells require IL-10 for their suppressive effect [35, 37, 38]. Immature B lymphocytes, presenting what is very likely a transitional B220+CD21+CD23+ phenotype, have been shown to suppress the adoptive transfer of diabetes into immunodeficient NOD mice with diabetogenic immune cells [39]. B lymphocytes stimulated with LPS are suppressive in NOD and prevent diabetes. This effect is largely
mediated by TGF-β [40]. B-lymphocyte-receptor-stimulated B cells from NOD mice delay T1D in syngeneic prediabetic recipients in an IL-10-dependent manner [41]. Our recent data suggest that the production of retinoic acid by DCs could be the possible link that mechanistically connects tolerogenic DCs and Bregs. Retinoic acid increases the survival of Bregs in vitro, but not that of non-suppressive CD19^+ B220^+ B cells. Therefore, Bregs involve a selective survival advantage induced by tolerogenic DCs, which could maintain and/or amplify a suppressive milieu that would also include Foxp3^+ Tregs (Di Caro et al., manuscript submitted).

After demonstrating that tolerogenic DCs are safe and well tolerated in patients with established T1D, an imminent phase II clinical trial in new onset T1D patients is scheduled. In addition to increasing the frequency and function of immunoregulatory cell populations in new-onset T1D patients, this trial is intended to test the efficacy of the tolerogenic DCs in improving functional residual β-cell mass. Stopping the inflammation process that promotes the T-cell-mediated β-cell destruction at such a stage could be sufficient to promote the rescue or regeneration of the remaining insulin-producing β-cell mass in the pancreas.

4. Dendritic cell-targeting microparticles

Despite the encouraging outcome of the phase I clinical trial, and the optimistic expectation for phase II, there are also limiting aspects for possible application in new onset diabetic children. Firstly, the leukapheresis process is invasive. It usually takes 2 or 3 hours to obtain precursor cells for the generation of a sufficiently large number of DCs. Moreover, tolerogenic DCs need to be generated in a well-equipped GMP facility for the collection, generation, storage, and distribution of a clinical-grade cell product. To overcome these issues we have been concurrently pursuing an alternative method for stable generation of immature DCs directly in vivo using microspheres as delivery vehicles of the antisense DNA [36].
In the NOD mouse model, we have shown that these microspheres are phagocytosed by DCs, and confer a diabetes-suppressive phenotype on them. When injected, these microspheres mobilized endogenous DCs to the injection site, and within 3 hours the loaded DCs moved to the closest lymph nodes. Microsphere-administered mice remained diabetes-free when injected prior to disease, and at least 40% exhibited reversal of new onset disease. Diabetes-free mice exhibited an augmented Foxp3+/CD25+/CD4+ Treg cell frequency and hyporesponsiveness to β-cell antigens, without constraints on systematical immune responses to alloanitgens. Additionally, T cells from successfully treated mice suppressed adoptive transfer of disease by diabetogenic splenocytes into secondary immunodeficient NOD-SCID recipients. Finally, a fraction of the microspheres was found to be accumulated within the pancreas and the spleen, indicating uptake and migration by phagocytes. In vivo imaging measured the microsphere accumulation pattern (Figure 2). Based on these observations, we suggest that our “first-generation” microsphere formulation can be considered to be the first diabetes-suppressive vaccine to confer an immunoregulatory phenotype on endogenous DCs [42].

Other types of microparticle-based approaches have been explored as possible vaccines for T1D. Administration of T-cell-targeting nanoparticles coated with disease-relevant peptide/major histocompatibility complexes (pMHC-NPs) has been demonstrated to prevent T1D in prediabetic mice, and to restore normoglycemia in diabetic animals. Treatments of NOD mice, with specific pMHC-NPs, expand memory-like autoregulatory CD8+ T cells, and suppress local presentation of autoantigens [43]. Compared to ours, this approach is based on a different concept. While our approach envisages direct targeting of DCs to make them tolerogenic, pMHC-NPs trigger a pool of specific memory-like CD8+ T cells to modulate the autoimmune response. This includes suppression of the antigen-presenting function of DCs in an IFN-γ- and IDO-associated manner, and direct elimination of the antigen-loaded DCs via perforin [43]. Even though there is no obvious overlap in the biological mechanisms between the approaches, it is worth noting that the congruence of actions relates to the DCs.

Concerning the physical characteristics of micro-/nanoparticles, the most critical aspect is that the polymer carrier and chemical backbone is not immunogenic on its own, i.e. it does not stimulate immune cells through specific/non-specific processes. Most micro-/nanosphere formulations are comprised of three portions:

1. A hydrophilic polymer
2. A linker molecule
3. The biologically active compound

The properties of the polymer can be modified to affect the rate of drug clearance, solubility, immune responsiveness, and in vivo stability, facilitating decreased drug dosage and frequency of administration. Polyethylene glycol (PEG) is a non-toxic polymer which is a constituent of many clinical and nutritional products. Currently, two PEG conjugates have undergone clinical trials for the treatment of autoimmune diseases. In 1996, the interferon beta-1a drug Avonex was approved by the FDA for the treatment of multiple sclerosis. Phase III clinical trials with a PEGylated form of the drug designated BIIB017 are currently in progress. Another bioconjugate of PEG is an anti-tumor necrosis factor alpha (anti-TNFα) antibody, initially tested for the treatment of rheumatoid arthritis and later extended to the treatment of Crohn’s disease. The conjugate, termed Certolizumab pegol, was FDA-approved for the treatment of Crohn’s disease in 2008 and rheumatoid arthritis in 2009. Despite the widespread effectiveness of PEG drug compounds, recent studies have found that their use can lead to the development of anti-PEG antibodies which may inhibit drug effectiveness. It needs to be clarified in future investigations whether this is a common problem not yet realized or specific only to certain PEG-compound formulations.

Apart from non-specific effects of formulation chemistry, the size of the particle decides whether DCs take it up by phagocytosis. Microspheres less than 200 microns in diameter are readily phagocytosed by macrophages and DCs. Interestingly, nanoparticles less than 50 microns in diameter are ignored by these cells. Thus, if the intention of the therapist is to take advantage of phagocytosis to deliver molecules inside DCs, then the critical diameter must be taken into account. If the intention is to modulate cell surface processes, the choice of the formulation size must exceed or be below the critical diameter (for example, using nanoparticles to target activation of surface receptors). Specific targeting of naturally occurring tolerogenic DCs in vivo may be required to avoid systemic immunosuppression. Even though there is no cell surface protein that selectively defines a
5. Conclusion and perspective

Since T1D is a disease which largely affects children and adolescents, any therapy aimed at modifying the immune system towards re-establishment of physiologic gluco-regulation would have to be minimally invasive, free of significant adverse events, and stably effective for at least a few months prior to a need to re-administer the agent(s). Considering the available clinical trial data, it seems that our approach of tolerogenic DCs is the safest with a promising potential to reverse autoimmune diabetes. We anticipate that adaptation of microparticle delivery will make the approach more appealing to patients, and will achieve the same biologic outcome with minimal logistical outlays and time from clinical confirmation of disease to initial therapy. Today, microspheres exist in numerous formulations that are inactive to the immune system (i.e., the particle chemistries do not induce non-specific immune activation). These very recent developments in polymer chemistry offer a variety of useful vehicles to target DCs in vivo.

As we approach the onset of a phase II clinical trial using the DCs, we are equally enthusiastic about the possibilities of a microparticle formulation for antisense DNA targeting CD40, CD80, and CD86, and for RNAi targeting of other costimulation pathways. The technology for verification of minimal off-target effects exists, and a careful selection of the most effective and specific oligonucleotide(s) coupled with disease-relevant antigen(s) could provide the elusive and long-sought T1D vaccine. By understanding how DCs process synthetic polymers, and how they respond to oligonucleotides, we are confident that this approach represents one of the safest ways of creating an off-the-shelf product. It is simple to manufacture to clinical grade on a large scale. The easy delivery to patients would make it possible to repeat the treatment by multiple administrations to achieve the final goal of reaching a complete tolerance against β-cell-specific antigens in the pancreas. It is also possible that these microparticles could represent a means for early intervention in first-degree relatives of T1D patients who exhibit signs of underlying disease (e.g., impaired first-phase insulin response to a mixed meal challenge along with high titers of T1D-related autoantibodies).

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