# World Journal of

# *Diabetes*

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# Current advances in using tolerogenic dendritic cells as a therapeutic alternative in the treatment of type 1 diabetes

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## Abstract

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin-producing β-cells of the pancreatic islets by autoreactive T cells, leading to high blood glucose levels and severe long-term complications. The typical treatment indicated in T1D is exogenous insulin administration, which controls glucose levels; however, it does not stop the autoimmune process. Various strategies have been implemented aimed at stopping β-cell destruction, such as cellular therapy. Dendritic cells (DCs) as an alternative in cellular therapy have gained great interest for autoimmune disease therapy due to their plasticity to acquire immunoregulatory properties both *in vivo* and *in vitro*, performing functions such as anti-inflammatory cytokine secretion and suppression of autoreactive lymphocytes, which are dependent of their tolerogenic phenotype, displayed by features such as semimature phenotype, low surface expression of stimulatory molecules to prime T cells, as well as the elevated expression of inhibitory markers. DCs may be obtained and propagated easily in optimal amounts from peripheral blood or bone marrow precursors, such as monocytes or hematopoietic stem cells, respectively; therefore, various protocols have been established for tolerogenic (tol)DCs manufacturing for therapeutic research in the treatment of T1D. In this review, we address the current advances in the use of tolDCs for T1D therapy, encompassing protocols for their manufacturing, the data obtained from preclinical studies carried out, and the status of clinical research evaluating the safety, feasibility, and effectiveness of tolDCs.

**Key Words:** Type 1 diabetes; Dendritic cells; Autoimmunity; Immune tolerance; Cell therapy; Immunotherapy

**Core Tip:** Autoimmunity in type 1 diabetes (T1D) is severe and leads to pancreatic dysfunction; therefore, therapies that can lessen this process are required. Cell therapy with tolerogenic dendritic cells (tolDCs) is a promising strategy. Various protocols have been implemented for tolDC generation, using stimuli such as cytokines, growth factors, and drugs. These cells are also subjected to treatments with antisense oligonucleotides, liposomes, toll-like receptor ligands, and peptides of the pancreatic islets, for optimization as T1D immunotherapy. Preclinical and clinical trials have demonstrated effectiveness of tolDC-based therapy. This review aims to give a detailed understanding of current advances in tolDC-based T1D treatment.

## INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the dysfunction and destruction of insulin-producing β-cells in the pancreatic islets of Langerhans[1,2]. Genetic susceptibility contributes to the loss tolerance of β-cells antigens, such as insulin, glutamic acid decarboxylase 65 (GAD65), insulinoma-associated-2 autoantibodies, and ZnT8 by autoreactive CD4+ and CD8+ T cells, leading to islet destruction, insulin deficiency, and elevated blood glucose levels[3-7].

Some current therapeutic strategies for T1D treatment include the exogenous insulin replacement therapy and the use of immunosuppressive drugs, leading to the amelioration of several aspects inside the pathology, but not the causal factors[8], as well as other different conditions[9]. Furthermore, serious side effects, like chronic infections or malignant transformation, may be driven by the use of immunosuppressive drugs. Therefore, alternative therapeutic strategies are necessary to reach the maintenance, restoration, or induction of autoantigen-specific immunological tolerance. In this line, cellular immunotherapy is emerging as a promising approach for the treatment of a T1D, inside which the use of tolerogenic dendritic cells (tolDCs) has attracted special attention[10].

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) specialized in the initiation of both immunogenic and tolerogenic response[11]. For immunogenic activities, DCs mature in response to inflammatory stimuli. During this maturation process, DCs markedly increase the expression of major histocompatibility complex (MHC)-peptide complexes and costimulatory molecules, secrete a wide variety of pro-inflammatory [tumor necrosis factor alpha (TNF-α), interleukin (IL)-1α, and IL-6] and immunomodulatory [interferon (IFN)-α, -β and -γ, and IL-12] cytokines, augmenting their ability to prime T cells[12,13]. On the other hand, for tolerogenic functions, both in central and peripheral tolerance[13], DCs acquire tolerogenic properties, named as "tolerogenic DCs or tolDC" with the capacity to regulate potential harmful adaptive responses[14,15]. Such tolerogenic properties result from their low capability to stimulate T cells, their high secretion of immunoregulatory factors such as anti-inflammatory cytokines [IL-10 and transforming growth factor (TGF) -β], indolamine 2,3-dioxygenase, and the expression of surface inhibitors like programmed death-ligand 1 (PD-L1)[15-17].

In addition, T1D-associated genetic factors are expressed in DCs affecting their tolerogenic properties *in vivo*[18], and the hyperglycemic state, as well as the control degree of patients, may affect the optimal regulatory functions of tolDCs[19]; by this reason, the immunoregulatory characteristics of tolDCs have made them potential tools for therapeutic research for T1D[20]. In this line, several protocols have harnessed DC plasticity to respond to external immunomodulatory agents modifying their phenotype, cytokine profile, and stimulatory ability, with the aim of developing manufacturing of tolDCs as a method to control T cell-mediated immunopathologic processes occurring in T1D and, simultaneously, as a replacement mechanism that might lead to the restoration of innate tolerance control. However, owing to several aspects concerning the efficacy, safety, and feasibility, essentially about the per-

formance of the protocols to obtain tolDC with stable tolerogenic phenotype, the therapeutic use of tolDCs requires further analysis. This review approaches advances in the investigation of protocols for tolDC manufacturing, as well as the underlying tolerogenic mechanisms described from their pre-clinical and clinical use in T1D.

## STRATEGIES FOR TOLDCS MANUFACTURING: ALTERNATIVELY AND *EX VIVO* GENERATED

DCs endowed with tolerogenic properties have been widely characterized for presenting a semimature state accompanied with high antigen uptake ability, a reduced antigen presentation capability owing to an attenuated antigen processing, and a reduced expression of MHC-II/MHC-I and costimulatory molecules, which in turn limits their competence to stimulate naïve or effector/memory T cells. Additionally, tolDCs produce reduced o null levels of pro-inflammatory cytokines such as IL-12p70; in contrast, they secrete a high level of anti-inflammatory cytokine like IL-10[21]. Nevertheless, the expression of some markers may be variable depending on the protocols used for tolDC generation. Concerning their functionality, tolDCs avoid the activation of autoreactive T cells by inducing various tolerance mechanisms, such as apoptosis, skewing phenotype, anergy, and expansion or induction of regulatory T cells (Tregs)[13,21]; a general view of tolerogenic features of tolDCs is shown in Figure 1.

Several protocols have been established for the differentiation and propagation of tolDCs. In humans, tolDCs are generated from peripheral blood monocytes (alternatively generated tolDC) and in murine models from bone marrow progenitors (*ex vivo* generated tolDC)[22,23]. The manufacturing of tolDC is carried out by exposing the cells to growth factors like granulocyte-macrophage colony-stimulating factor (GM-CSF) and cytokines like IL-4, which induce differentiation to immature DC, and the simultaneous use of immunomodulatory agents such as anti-inflammatory cytokines (IL-10 and/or TGF-β) or pharmacological agents (dexamethasone, rapamy-cin, and vitamin D3) that allow obtainment of tolerogenic properties[24-27] (Table 1 and Figure 2).

At first, typical protocols for alternative and *ex vivo* tolDC generation are carried out in the presence of GM-CSF alone or plus IL-4 (GM-CSF/IL-4). GM-CSF is important in the functional regulation of DCs; studies in a murine model revealed that generation of bone marrow-derived DCs with low concentrations of GM-CSF possess an immature phenotype, resistant to maturation and restore T cell tolerance *in vivo* and *in vitro*[28]. It has been reported that GM-CSF provides protection against diabetes in non-obese diabetic (NOD) mice. DCs of these GM-CSF-protected mice express low levels of MHC-II, CD80, and CD86, produce IL-10, and are less effective in stimulating diabetogenic CD8+ T cells[29]. Additionally, DCs that are treated to express IL-4 can delay or prevent the onset of autoimmune diabetes in NOD mice, maintaining stable glucose levels for a long time[30]. Strikingly, it has been documented that GM-CSF/IL-4 combination synergistically improved the regulatory roles of DCs, demonstrating optimal prevention of diabetes in NOD mice[22,31].

Besides using GM-CSF/IL-4, tolDCs have been generated *in vitro* by adding immunomodulatory agents during the process of differentiation. Our research group demonstrated that tolDC inducing antigen-specific tolerance may be generated when they are alternatively differentiated in the presence of cytokines such as IL-10/TGF-β1 together, displaying enhanced efficiency to generate anergy and Tregs[24]. These tolDC displayed lower expression of CD40, enhanced endocytic ability, increased secretion of IL-10 and prostaglandin E, and lowered secretion of IL-12 and IL-23. On the other hand, tolDCs have also been generated by only using a suppressive modulator like IL-10[24,32] or TGF-β[33]; such cells show increased secretion of IL-10 and IL-6, reduced IL-12p70 production, and a semi-mature phenotype demonstrated by intermediate expression of CD80, CD86, CD40, CD83, and MCH-II. Another protocol for tolDC generation is GM-CSF/IL-10. TolDC obtained by this route modulate the autoimmunity in a specific form when they are differentiated in the presence of autologous serum[34]. It has also been demonstrated that tolDC (GM-CSF/IL-10) in animal models of T1D suppress insulitis and spontaneous diabetes in NOD mice. These results suggest that IL-10-treated DC acquire tolerogenic characteristics and induce tolerance in pancreatic islets in a non-antigen-specific way[35]. Likewise, DCs induced by TGF-β display tolerogenic phenotype and func-tions. The addition of these TGF-β-treated tolDC to grafted islets led to graft survival in autoimmune diabetic recipient mice[33].

**Table 1 Tolerogenic dendritic cells manufacturing for type 1 diabetes therapy**

| Protocol | Treatment | DC phenotype | Therapeutic effects in T1D | Ref. |
|---|---|---|---|---|
| GM-CSF | Apoptotic bodies-loaded | ↓ Costimulatory molecules (CD40, CD86); ↓ IL-6; ↓ TNF-α | Reduces disease incidence in NOD mice. Reduces insulitis | Marin-Gallen *et al*[62], 2010 |
| | Liposomes-loaded | ↑ TIM4, CD36; ↓ MHC-II; ↓ Costimulatory molecules (CD40, CD86); ↑ CCR7, CCR2; ↑ DC-SING; ↓ IL-6; ↑ Anti-inflammatory cytokines (IL-10, TGF-β1) | Decreases CD8+ T cell proliferation. Reduces disease incidence in NOD mice. Reduces insulitis | Pujol-Autonell *et al*[64], 2015 |
| GM-CSF/IL-4 | Antisense oligonucleotides | ↓ Costimulatory molecules (CD40, CD80, CD86); ↓ NO; ↓ TNF-α, IL-12p70 | Prevents diabetes in NOD mice. Reduces insulitis. Promotes Tregs. Increases B cells. Suppresses T cells proliferation: Clinicaltrials.gov identifier: NCT00445913; Clinicaltrials.gov identifier: NCT02354911 | Machen *et al*[50], 2004 |
| | | | | Di Caro *et al*[51], 2014 |
| | | | | Di Caro *et al*[52], 2012 |
| | | | | Phillips *et al*[53], 2008 |
| | | | | Giannoukakis *et al* [54], 2011 |
| | | | | NIH[68], 2007 |
| | | | | NIH[69], 2015 |
| | Antigen-loaded: Proinsulin | Tolerogenic phenotype (not specifically described) | Delays or halts progressive destruction of β-cell and loss function. -Clinicaltrials.gov identifier: NCT04590872 | Nikolic *et al*[70], 2020 |
| | | | | FACT[71] |
| | Liposomes-loaded | ↓ Costimulatory molecules (CD40, CD86); ↑ PDL1 expression; ↑ VEGF secretion | Arrests autoimmunity in the model of experimental diabetes | Rodriguez-Fernandez *et al*[61], 2019 |
| | | | | Rodriguez-Fernandez *et al*[63], 2018 |
| | TLR´s ligand: 1Z1 | ↑ PD-L1; ↑ IRAK-M; Minimum increases of MHC-II, CD40, CD80, CD83, CD86 | Suppresses T cell activation and proliferation. Delays insulitis in NOD mice | Kim *et al*[67], 2012 |
| GM-CSF/IL-10 | | ↓ Costimulatory molecules; ↓ IL-12, IL-23, IL-6; ↑ IL-10 | Reduces insulitis. Prevents spontaneous diabetes in murine models. Induces Tregs. Induces hyporesponsiveness of T cells. Inhibits T cells proliferation | Haase *et al*[34], 2005 |
| | | | | Tai *et al*[35], 2011 |
| GM-CSF/IL-4 + IL-10 or TGF-β | | Intermediate expression of MHC-II, CD40, CD80, CD86, CD83; ↓ IL-12p70, IL-23, TNF-α; ↑ IL-10; ↑ IL-6; ↑ PD-L1 | Decreases T cells infiltration. Reduces T cells proliferation. Induces Tregs. Prolongs the survival of syngeneic Islet graft in NOD mice | Torres-Aguilar *et al*[24], 2010 |
| | | | | Boks *et al*[32], 2012 |
| | | | | Thomas *et al*[33], 2013 |
| GM-CSF/IL-4 + IL-10/TGF-β | Antigen-loaded: Insulin; GAD65 | ↑ CD1a; ↓ Costimulatory molecules (CD40, CD86); ↓ CD83; ↓ MHC-II; ↓ IL-12; ↓ IL-23; ↑ PGE | Suppresses effector/memory T cells. Induces T cells anergy. Induces Tregs. Induces IL-10 production by T cells. Suppresses T cells proliferation. Induces hyporesponsiveness of T cells | Torres-Aguilar *et al*[44], 2010 |
| | | | | Segovia-Gamboa *et al*[58], 2014 |
| GM-CSF/IL-4 + Vitamin D/Dexamethasone | Antigen-loaded: -Proinsulin | ↓ MHC-II; ↓ IFN-γ; ↓ CD86; ↑ IL-10; ↑ PD-L1 | Controls autoimmunity. Induces Tregs. Inhibits effector T cells. Eliminates CD8+ T cells | Suwandi *et al*[55], 2020 |
| | | | | Gibson *et al*[56], 2015 |
| | -GAD65 | ↓ Costimulatory molecules (CD40, CD86); ↓ CD83; ↓ MHC-II; ↑ CD14; ↑ TLR-2; ↑ PD-L1; ↑ IL-10; ↓ IL-6, TNF-, IL-23, IL-12p70 | Decreases Th1/Th17 responses. Suppresses antigen-specific T cell activation and proliferation. Prevents onset diabetes in NOD-SCID mice. Decreases IFN-γ production by T cells | Phillips *et al*[20], 2017 |
| | | | | Funda *et al*[57], 2018 |
| GM-CSF/IL-4 + Rapamycin | | ↓ Costimulatory molecules (CD40, CD80); ↓ IL-6, IL-23; ↑ PD-L1 | Induces Tregs. Inhibits T cell proliferation. Reduces Th17 cells | Boks *et al*[32], 2012 |
| | | | | Navarro-Barriuso |

T1D: Type 1 diabetes; IL: Interleukin; TNF-α: Tumor necrosis factor alpha; NOD: Non-obese diabetic; GM-CSF: Granulocyte-macrophage colony-stimulating factor; MHC: Major histocompatibility complex; TGF-β1: Transforming growth factor-β1; VEGF: Vascular endothelial-derived growth factor; TLR: Toll-like receptor; PD-L1: Programmed death-ligand 1; IRAK-M: IL-1 receptor-associated kinase M; PGE: Prostaglandin E; IFN-γ: Interferon-γ; DC: Dendritic cell; Treg: Regulatory T cell; GAD65: Glutamic acid decarboxylase 65.
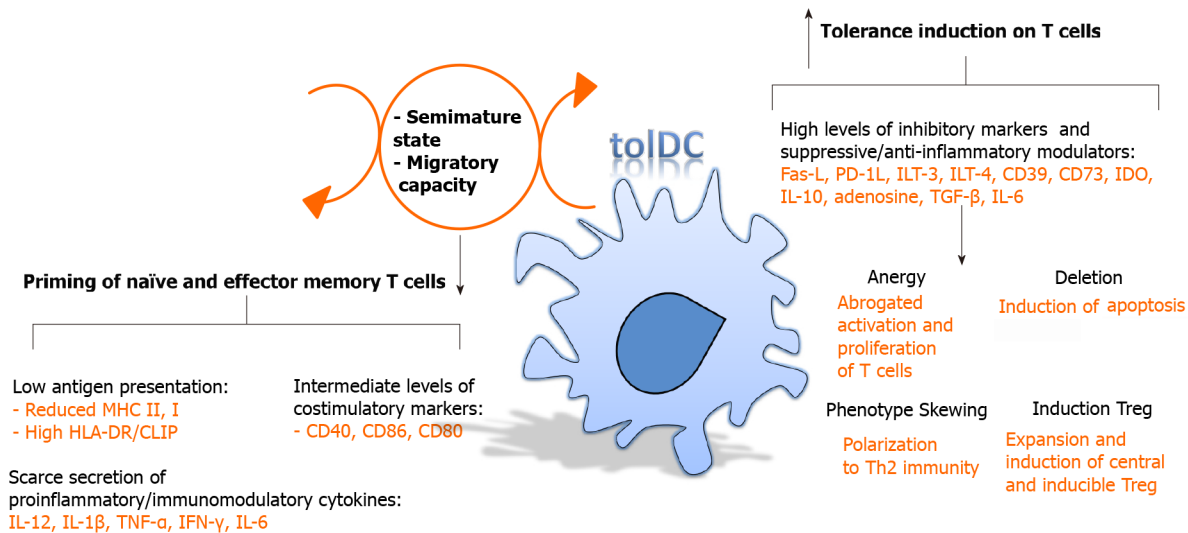


**Figure 1 Phenotypic and functional hallmarks describing the immunobiology of the tolerogenic state of dendritic cells.** Tolerogenic dendritic cells (tolDCs) display a semimature state with high antigen uptake capability and bear low/intermediate surface levels of factors essential for T cell priming. In contrast, tolDCs bear high surface levels of inhibitory markers, allowing them to inhibit autoreactive T cells. Further, tolDCs display reduced secretion of inflammatory/immunomodulatory agents accompanied by the high secretion of anti-inflammatory/suppressive modulators. All those features are essential for inducing specific tolerance for self, microbiome, and environmental derived antigens by mechanisms such as anergy, deletion, phenotype skewing, and/or expansion of regulatory T cells. Additionally, tolDCs display optimal migratory capability, which has been documented to be essential to inducing periphery tolerance *in vivo*. HLA: Human leukocyte antigen; IFN: Interferon; IL: Interleukin; TGF: Transforming growth factor; tolDC: Tolerogenic dendritic cells; TNF: Tumor necrosis factor; MHC: Major histocompatibility complex; PD-L1: Programmed death-ligand 1.

Additionally, one of the major drugs used to induce tolDC differentiation *in vitro* is the biologically active form of vitamin D, 1,25-dihydroxyvitamin D3. These tolDC display different morphological features than immunogenic DCs, showing lower expression of costimulatory molecules and high CD11c and DC-SIGN expression, confirming their semi-mature phenotype, with an increased expression of IL-10 and inhibitory molecules like PD-L1[36-39]. Vitamin D3 and dexamethasone combination generate tolDC characterized by a low expression of MHC-II, the costimulatory molecules CD40 and CD86, and the maturation marker CD83, as well as low levels of IL-12p70[40]. Nevertheless, a systematic comparative analysis of tolDC generated with vitamin D3, IL-10, dexamethasone, TGF-β, or rapamycin showed that IL-10-generated tolDCs are optimal for functional Treg induction, which display strong suppression activity[32]. Likewise, several agents of different nature have been used for tolDC generation; such agents encompass tissue-derived factors, cytokines, some pathogen-derived antigens, and pharmacological molecules[41,42].

One significant aspect taking special attention is the notion that tolDCs might not be advisable for clinical research, because the *in vivo* permanence of their phenotype and tolerogenic functions might not be guaranteed, especially when they reach tissues with chronic inflammation in conditions such as T1D[43]. In this line, several investigations have addressed protocols that allow obtaining functionally stable tolDC to keep their regulatory properties under pro-inflammatory environments. These protocols include addition of maturation stimuli, such as lipopolysaccharide, TNF-α, prostaglandin $E_2$, CD40-L, or IL-6 between others[43-45], during or after the tolerogenic stimuli. Although the rising idea about the maturation state is not necessarily a fully distinguishing feature of immunogenic activity on DCs, a mature state is not opposed to their tolerogenic activity either[46]. Besides the stable tolerogenic phenotype, a mature state of tolDC may optimize some features for optimal regulatory mechanisms *in vivo*, such as their migratory capability, which is required to promote T cells into regulatory control. Moreover, such migratory capability has been considered as a pivotal
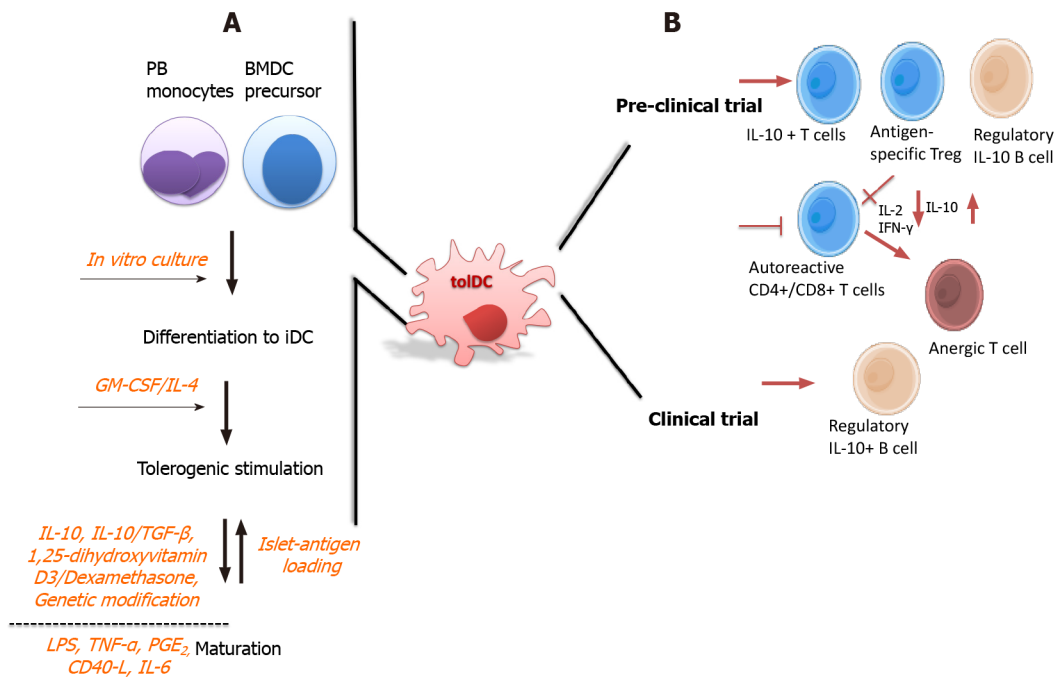
**Figure 2 Tolerogenic dendritic cells in type 1 diabetes therapy: manufacturing and tolerogenic mechanisms described in preclinical and clinical trials.** A: Tolerogenic dendritic cells (tolDCs) are alternatively generated from peripheral blood monocytes, or bone marrow precursors, which are subjected in culture with sequentially stimulation processes. Immature DC differentiation is firstly generated with growth factors, which in turn, owing to their plasticity, are subjected to tolerogenic stimulation with immunomodulatory agents to obtain tolDCs. Besides, some protocols perform the manufacturing with additional maturation stimuli, such as lipopolysaccharide or tumor necrosis factor-α previous to or after the tolerogenic stimulus to obtain stable tolDCs; B: According to their regulatory mechanism, tolDCs may induce an increased frequency of interleukin (IL)-10-expressing T cells and expand the antigen-specific regulatory T cell population, which show optimal suppressive activity; further, tolDCs reduce the activation and proliferation of autoreactive naïve and memory CD4+ and CD8+ T cells, otherwise becoming anergic. Additionally, the regulatory roles of tolDCs also reach B cells, since a high level of regulatory B cells expressing IL-10 are expanded by tolDCs, which are associated to a protective role in type 1 diabetes, being the only described immunoregulatory mechanism obtained from a clinical trial. BMDC: Bone marrow-derived dendritic cell; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IFN: Interferon; PGE$_2$: Prostaglandin E$_2$; PB: Peripheral blood; IL: Interleukin; tolDC: Tolerogenic dendritic cell; TGF: Transforming growth factor; LPS: Lipopolysaccharide.

tolerogenic feature of DCs[32,45].

*Preclinical assays: In vitro and in vivo studies evaluating the promising tolDC application for T1D therapy*

Several studies have revealed a diversity of regulatory mechanisms employed by tolDCs both *in vitro* and in animal models. Such mechanisms differ according to the type of generated tolDC. Hence, this evidence has prompted their use for further clinical research (Figure 2).

According to *in vitro* analysis, IL-10-generated tolDCs have been documented to be optimal to induce Tregs with strong suppressive activity[32,45]. Such tolDCs are resistant to inflammatory conditions and exhibit strong migratory capacity toward the secondary lymphoid organ chemokine CCL21, allowing an optimal migratory capability to induce T cell regulatory actions. In line with their regulatory activity on autoreactive T cells, *ex vivo* 1,25-dihydroxyvitamin D3-generated tolDCs derived from diabetes-prone (NOD) mice decrease the proliferation and activation of autoreactive CD4+ T cells *in vitro*. Further, these tolDCs are optimal to induce increased IL-10 expression in T cells and may expand the CD25+ Foxp3+ T cell population[47]. Regarding CD8+ T cells' activity as an independent risk factor governing the detrimental destruction of insulin-producing β-cells by their cytotoxic role[48], vitamin D3/dexamethasone-modulated DCs (Combi-DCs) loaded with human leukocyte antigen class I epitopes were described with the capability to impede priming of autoreactive naïve CD8+ T cells and to reduce memory CD8+ T cells[36].

Other studies have displayed the effective regulatory role of tolDCs on T cells, but data illustrating further tolerogenic mechanisms are scarce. Additionally, some studies have shown that inhibitory roles of tolDC are not limited to T cells, since such effects may even reach B cells by increasing their frequency. *Ex vivo* generated tolDCs appear to be capable of inducing the expansion of regulatory B cells, displaying an IL-10-dependent suppressive effect on T cells through the proliferation of preexisting IL-10-

expressing B cells as well as by differentiation of their precursors. This mechanism performed by tolDCs is mediated in a retinoic acid-dependent manner, favoring the FoxP3^{+} Treg differentiation[49]. These findings describe a novel mechanism of tolDCs exerting their regulatory mechanism on other cellular entities different than Tregs.

Regarding the *in vivo* analysis, the NOD mouse model is a well-established approach extensively used to investigate several aspects of the molecular and cellular mechanisms underlying T1D as well as to evaluate therapeutic agents. TolDC have been shown to prevent and reverse T1D in the NOD mouse model. *Ex vivo* generated tolDCs with impaired costimulatory capability, delay or revert new-onset hyperglycemia for the long-term, increasing the expansion of Tregs[50,51]. Additionally, an increased number of regulatory B cells (Breg) expressing higher levels of IL-10 has been obtained from NOD mice. Such Bregs resulted from the conversion of precursor B cells into IL-10-expressing cells, being, in this way, involved in the mechanism of tolerogenic reversal of T1D by tolDC[51]. Moreover, *ex vivo* 1,25-dihydroxyvitamin D3-generated tolDCs transferred into NOD severe combined immunodeficiency mice exhibited the capability to dampen autoreactive T cell proliferation in pancreatic draining lymph nodes. This action probably might be an effect of the functional migratory capability of tolDC, since these tolDCs exhibited optimal homing to the pancreas in adult NOD-SCID mice[47].

Nowadays, there is a diversity of preclinical trial protocols for tolDC aimed at T1D immunotherapy. During or after differentiation and propagation of tolDC, different strategies have been implemented in order to improve the functional capability of tolDC. Within those protocols, there is the use of antisense oligonucleotides targeting the expression of costimulatory molecules, tolDCs pulsed or loaded with antigens for a specific antigen immune response, liposomes or apoptotic bodies, and the use of Toll-like receptor (TLR) ligands.

**Antisense oligonucleotides:** TolDCs may be obtained by genetic modification, including transference or silencing of selected genes through several approaches, with the aim to modulate their maturation. In T1D immunopathogenesis there are active DCs favoring the increase of costimulatory molecules to realize immunogenic functions; for this reason, a protocol was implemented to negatively regulate their expression through *ex vivo* treatment of immature DCs from NOD mice with a mixture of antisense oligonucleotides targeting the CD40, CD80, and CD86 transcripts[50].

The single administration of these tolDCs promotes a higher prevalence of Tregs, conferring protection against T1D[50]. These phosphorothioate-modified antisense oligonucleotides confer tolerogenic properties to cells and prevent T1D in NOD mice, therefore, some research groups proposed developing microspheres "DC populations targeting" of the three antisense oligonucleotides, and this broadens the perspective towards a possible vaccine of treated tolDCs[52,53]. This method of antisense oligonucleotides has allowed launching of the first phase I study of autologous tolDC administration in T1D therapeutics[54].

**Antigen-loaded tolDCs:** Immunotherapies with tolDCs can be performed with antigen-loaded or -unloaded cells; both methods have shown results preventing T1D. However, the antigen-loaded methods promise being more feasible, owing to these, should specifically inhibit the action of autoreactive T cells, thereby allowing a tolerance restoration to self-antigens and avoiding general immunosuppression.

Proinsulin, insulin, and GAD65 are some target autoantigens involved in T1D development and, hence, utilized to load tolDCs. Vitamin D3/dexamethasone-generated and proinsulin-loaded tolDCs induce antigen-specific Tregs with various phenotypes *in vitro*, expressing regulatory markers, such as Lag-3, CD161, and inducible co-stimulator, and effectively suppress effector CD8^{+} and CD4^{+} T cells[55]. Likewise, in a humanized mouse model of proinsulin autoimmunity, the administration of proinsulin in vitamin D3-generated tolDCs may control the autoimmunity *via* IL-10 production[56]. Controversially, in another study, the authors tested the efficacy of vitamin D2/dexamethasone-generated GAD65-loaded tolDCs to prevent the adoptive transfer of diabetes by diabetogenic splenocytes to NOD-SCID receptor mice. However, in this study the GAD65-loaded tolDCs decrease the protective effect against disease in T1D, compared to tolDCs without antigen-loading[57].

The evidence that the metabolic control of T1D individuals affects the functionality of tolDCs takes special relevance in tolDC-based strategies. Alternatively generated tolDCs modulated with vitamin D2/dexamethasone were loaded with the antigen GAD65 from well- and deficient-controlled T1D patients. Results showed that, in both groups, tolDCs induced Tregs *in vitro*. However, only the tolDCs derived from well-controlled T1D patients decreased the T helper (Th)1/Th17 responses and suppressed

the activation of antigen-specific T cells, unlike the tolDCs derived from patients with a deficient metabolic control. Additionally, the functionality of these tolDCs was evaluated in an adoptive transfer model of NOD-SCID mice, resulting in a delay in the onset of the disease[20].

The relevance of the activation state of each T1D patient in the functionality of tolDC strategies is strengthened due to the evidence obtained with human cells by our research group. Our results showed that alternatively IL-10/TGF-β1-generated tolDCs effectively induce insulin-specific tolerance in autologous effector/memory CD4+ T cells derived from T1D individuals, without affecting the proliferative response to an unrelated antigen. TolDC-stimulated T cells reproducibly displayed a decrease in activation molecules and pro-inflammatory cytokines (IL-2, IFN-γ), with high levels of the anti-inflammatory cytokine IL-10 and exhibition of an anergic state. Nevertheless, the degree of tolerance induction was dependent on the initial T cell activation state of each patient[44]. These results agreed with another study with IL-10/TGF-β-gene-rated, insulin, or GAD65-loaded tolDCs from T1D patients, which similarly showed antigen-specific autoreactive cell hypo-responses, lower IL-2 and IFN-γ secretion, and higher IL-10 production by T cells[58]. These studies demonstrate the ability of *in vitro*-generated tolDCs to induce antigen-specific tolerance in T cells.

**Liposomes or apoptotic bodies:** Apoptosis is an effective mechanism to induce tolerance. The capture of apoptotic bodies by APCs (macrophages and DCs), also called efferocytosis, is due to a specific recognition and phagocytosis through phosphatidylserine (PS)[59]. In T1D, the increase in apoptotic pancreatic β-cells or defects in efferocytosis contributes to the loss of tolerance[60]. Nevertheless, it has been shown that DCs from T1D patients may acquire defective apoptotic bodies' clearance. In a child population with T1D, the tolerogenic functionality of DCs derived from monocytes was evaluated using liposomes with PS (PS-liposomes), demonstrating that the DCs of pediatric patients with T1D phagocyte PS-liposomes function in a less efficient way than the controls, which inversely correlated with the evolution of the disease. However, the tolerogenic profile in DCs was consistent after efferocytosis[61].

DCs acquire a tolerogenic phenotype and functionality after ingestion of apoptotic β-cells and prevent T1D when transferred to NOD mice, significantly decreasing its incidence and correlating positively with insulitis reduction[62]. However, the limitation of a large source of apoptotic autologous β-cells for immunotherapeutic application is the wide outlook for a biomimicry alternative consisting of PS-liposomes containing β-cell autoantigens (insulin). However, owing to the limitation of having a large source of apoptotic autologous β-cells for their immunotherapeutic application, the need arises to extend the outlook toward a biomimicry alternative consisting of PS-liposomes containing β-cell autoantigens (insulin). Liposomes that mimic apoptotic β-cells have been shown to arrest autoimmunity and prevent T1D through the generation of tolDCs. These DCs exposed to PS-liposomes decrease the proliferation of autologous T cells, deregulate genes associated with antigen presentation, and increase tolerogenic genes as well as anti-inflammatory pathways[63]. A similar study establishes that insulin-loaded PS-liposomes also reduce the severity of insulitis and that the administration of PS-free liposomes demonstrates the importance of PS in modulating the expansion of antigen-specific CD4+ T cells[64]. Immunotherapy based on the use of liposomes constitutes a promising strategy for autoimmune diseases, including T1D.

**TLR ligand:** Hayashi *et al*[65] proposed an innate immune response modulator generated by conjugating a TLR-7 Ligand to six subunits of polyethylene glycol, "PEGylated TLR-7 Ligand", or 1Z1. DCs treated *ex vivo* with 1Z1 and injected into NOD mice delay the appearance of insulitis, suggesting that 1Z1-treated DCs are functionally tolerogenic since these cells suppress the proliferation of antigen-specific T cells. Besides, these tolDCs do not promote an inflammatory response *in vitro* or *in vivo* and show an increase of the expression of PD-L1 and IL-1 receptor-associated kinase M.

TLR-2 involvement in T1D development has been shown by the late apoptotic β-cells ability to stimulate APCs through this receptor, contributing to activate diabetogenic T cells. Hence, as a T1D therapeutic alternative, TLR-2 blocking or tolerization is proposed. TLR-2 tolerization was carried out with a prolonged treatment of the agonist (Pam₃CSK4). This treatment attenuates T cell activation mediated by DCs[66]. Furthermore, the combination of this therapy with the inhibitor (DA-1229) of dipeptidyl peptidase 4, which increases the mass of β-cells, can reverse the appearance of diabetes in NOD mice[67].

*Perspective obtained from clinical trials with the use of tolDCs for T1D therapy*

**Clinical trials with good progress, but limitations and barriers:** The first tolDC-based clinical trial for T1D treatment supports their safety administration (Clinicaltrials.gov identifier: NCT00445913)[68]. In this protocol, alternatively generated tolDCs were developed with antisense phosphorothioate-modified oligonucleotides targeting the transcripts of the costimulatory molecules CD40, CD80, and CD86. Available data show that the administration of these tolDCs upregulate the frequency of a B-lymphocyte subpopulation that was later discovered to possess immunosuppressive capability[49,51]. In this study, the procedures, equipment, and facilities comply with recommendations and are approved by the Food and Drug Administration (FDA), and no toxicity or adverse effects associated with the tolDC therapy were reported. Hence with FDA and Institutional Review Board approval, a new phase 2 study was started. This clinical trial in phase 2 (Clinicaltrials.gov identifier: NCT02354911)[69], aims to assess the capability of these tolDCs to disrupt the autoimmune process leading to β-cell destruction in individuals with T1D. To evaluate the expected effect, indirect studies will be carried out with C-peptide measurement, glycosylated hemoglobin A1c, and basal and postprandial glucose. The investigators will mainly evaluate the number of potentially tolerogenic/Tregs, B cells, and DCs and also aim to identify molecular signatures of these cell populations and correlate them with the clinical response. However, the status of this phase 2 clinical trial is unknown. It is worth mentioning that it has been reported that these tolDCs generated with antisense oligonucleotide induce T cell anergy[40].

On other hand, as have been reviewed in preclinical section, several preclinical studies have documented the ability of tolDCs loaded with antigen to induce antigen-specific tolerance. In this line, the intradermal administration of proinsulin peptide-pulsed tolDC, showed no signs of systemic immune suppression, no induction of allergy to insulin, no interference with insulin therapy, and no accelerated loss in β-cell function in patients with the remaining C-peptide level, assuming the tolDC therapy appears to be feasible and safe[70]. Yet, this study shows that the residual β-cells' function assessed by C-peptide detection did not change after tolDC administration. However, it´s important to highlight that this study was carried out with long-standing T1D patients, and this aspect get special attention owing to preclinical data point out that the efficacy of tolDC to promote optimal tolerance to specific antigen might be useful just in certain subsets of T1D patients, since the extent grade of disease (metabolic indicator as uncontrolled glycemia, uncompensated patients, activate state of T cells), being a barrier to get optimal effectiveness[20,44,51].

It is worth mentioning that the clinical trials described are carried out according to the standards for effector immune cells regulated by the foundation for the accreditation of cellular therapy[71].

**Perspectives for TID therapy:** Despite the existence of data showing optimal regulatory properties of tolDC, which might encourage the rising of additional clinical trial studies, important aspect must be considered; for instance, given the uncertainty of DCs' plasticity under inflammatory microenvironment prolonged, the road for the safe use of tolDC vaccines in T1D patients has been taken into account. In addition, owing to the grade of the disease may reflect distinctive efficacy, spotlighting the interest in testing patients with a shorter clinical diagnosis, where tolDC might delay the progressive autoimmune process.

In line with the aforementioned, one phase 1 study evaluating the use of tolDC as immunotherapy vaccine for the treatment of patients with T1D who use insulin and don't have any other diabetes-related health complications, is currently driven (Clinicaltrials.gov identifier: NCT04590872)[72]. Here, the safety and viability of autologous tolDCs loaded with proinsulin peptide "C19-A3" (PIpepTolDC) in new-onset T1D patients is evaluated, being C19-A3, a pharmaceutical product regulated by FDA. The PIpepTolDC vaccine aims to protect β-cells to lead an efficient insulin production to control blood glucose levels and reduce T1D-related complications by reducing the autoimmune process. The clinical effect in this study is evaluated by measuring levels of glucose, C-peptide, and hemoglobin A1c, and the effect on the autoimmune process by analyzing changes in autoantibody of pancreatic islets, T cell responsiveness, CD4+ T cells producing IFN-γ and IL-10, the number of autoreactive CD8+ T cells, as well as the immune phenotype. Thus, as perspective, the further research should encompass the viability of tolDC useful according to the clinical status of the disease.

## CONCLUSION

Based on the fact that there is no specific treatment against the autoimmune process underlying T1D, which in the long-term may imply disease complications. The use of tolDC as alternative immunotherapy arises as a promising approach for T1D therapy. tolDCs have been shown to ameliorate the disease, owing to their capability to downregulate several immune cells' hyperactivity in a specific manner. Furthermore, this particular focus of tolDCs as T1D therapy is also due to the feasibility for their obtainment, since several protocols have been established, which have harnessed the DC plasticity to respond to external immunomodulatory agents, modifying in this way their phenotype, cytokine profile, and stimulatory ability, endowing them with immunoregulatory properties. Hence, various preclinical trials have demonstrated their effectiveness. Current clinical trials evaluate the safety and efficacy of tolDC administration in patients with T1D, continuing to be a viable and promising alternative therapy to reduce the autoimmune process of this disease.

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