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Recent nanotechnological approaches for immunotherapy in type 1 diabetes

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Monografia orientada pela Doutora Liane Isabel Ferreira Moura, Categoria Investigadora Júnior e coorientada pela Professora Doutora Helena Isabel Fialho Florindo Roque Ferreira, Categoria Professora Associada com Agregação.

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Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Resumo

A diabetes tipo 1, também conhecida como diabetes autoimune ou diabetes insulinodependente, é uma doença crónica que afeta maioritariamente crianças e jovens. Esta patologia é marcada por uma destruição maciça das células produtoras de insulina, as células beta. Trata-se de uma doença heterogénea, em que está envolvido um mecanismo de destruição mediado pelas células T, devido a uma perda de imunotolerância, que conduz à não produção de insulina e, consequentemente, a elevados níveis de açúcar no sangue. Se a hiperglicemia não for adequadamente controlada a longo prazo, podem surgir complicações graves em vários órgãos.

Atualmente, esta doença metabólica não tem cura. A utilização de injeções ou bombas de insulina para controlo dos sintomas são os únicos tratamentos disponíveis. No entanto, mesmo quando essas estratégias são rigorosamente seguidas, os doentes continuam a desenvolver complicações e a viver com menor qualidade de vida face ao esperado.

Com o intuito de ultrapassar essas limitações, foram desenvolvidas novas terapias com foco na prevenção e tratamento da doença. Entre elas, a imunoterapia é a que tem ganho particular destaque. O recurso à modulação e reprogramação da resposta imune que se encontra exacerbada melhora os níveis de insulina em resposta à hiperglicemia e atrasa o aparecimento da doença. Alguns exemplos são as terapias antigénio-específicas, comumente designadas de imunoterapias antigénio-específicas, que incluem as vacinas tolerogénicas. Através da combinação da imunoterapia com a nanotecnologia, observou-se um aumento significativo na eficácia destas vacinas. A presença de nanopartículas para a entrega de imunomoduladores, insulina ou vacinas modificadas para células imunes endógenas conferiu vantagens, nomeadamente um restabelecimento parcial da imunotolerância anteriormente perdida, por anergia e/ou deleção de células T diabetogénicas, e diferenciação e/ou expansão de células T reguladoras, levando assim a uma melhoria no desequilíbrio observado entre essas células T. Embora sejam necessários esforços para aprimorar as terapias já existentes ou desenvolver novas abordagens, é inegável que é na nanotecnologia que reside o futuro da prevenção e tratamento da diabetes tipo 1.

Palavras-chave: células T reguladoras; diabetes tipo 1; doenças autoimunes; imunoterapia; nanotecnologia

Abstract

Type 1 diabetes, also known as autoimmune diabetes or insulin-dependent diabetes, is a chronic disease that mostly affects children and young people. This pathology is marked by a massive destruction of insulin-producing cells, the beta cells. It is a heterogeneous disease, in which a mechanism of destruction mediated by T cells is involved, due to a loss of immunotolerance, which leads to the non-production of insulin and, consequently, to high blood sugar levels. If hyperglycemia is not properly managed over the long term, serious complications can arise in multiple organs.

Currently, this metabolic disease has no cure. The use of insulin injections or pumps to control the symptoms are the only treatments available. However, even when these treatments are strictly followed, patients continue to develop complications and live with a lower quality of life than expected.

To overcome these limitations, novel therapies have been developed focusing on disease prevention and treatment. Among them, immunotherapy has gained prominence. The use of modulation and reprogramming of the exacerbated immune response improves insulin levels in response to hyperglycemia and delays the onset of the disease. Some examples are antigen-specific therapies, commonly referred to as antigen-specific immunotherapies, which include tolerogenic vaccines. Through the combination of immunotherapy with nanotechnology, a significant increase in the effectiveness of these vaccines was observed. The presence of nanoparticles for the delivery of immunomodulators, insulin, or engineered vaccines to endogenous immune cells conferred advantages namely a partially restoration of the immunotolerance previously lost, by anergy and/or deletion of diabetogenic T cells and differentiation and/or expansion of regulatory T cells, thus leading to an improvement in the observed imbalance between these T cells. While efforts are needed to improve existing therapies or to develop novel approaches, it is undeniably that the future of type 1 diabetes prevention and treatment lies in nanotechnology.

Keywords: autoimmune diseases; immunotherapy; nanotechnology; regulatory T cells; type 1 diabetes

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Abbreviations

AhR	Aryl hydrocarbon receptor
APC	Antigen-presenting cells
aTregs	Adaptive Treg cells
ASI	Antigen-specific immunotherapy
AuNP	Gold nanoparticles
ATG	Antithymocyte globulin
CD	Cluster of differentiation
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
DCs	Dendritic cells
DZB	Daclizumab
FOXP3	Forkhead box P3 transcription factor
GAD	Glutamic acid decarboxylase
GLP-1	Glucagon-like-peptide-1 agonists
GM-CSF	Granulocyte-macrophage colony-stimulating factor
Hb1Ac	Glycated haemoglobin
HLA	Human leukocyte antigen
Hsp	Heat shock protein
IA-2	Insulin antigen 2
IAA	Insulin autoantibodies
ID	Intradermal
iDCs	Immature dendritic cells
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IL-2R	Interleukin-2 receptor
IFN	Interferon

IM	Intramuscular
iNKT	Invariant NKT cells
iTreg	Induced Treg cells
IV	Intravenous
LD	Low dose
mAb	Monoclonal antibody
MMF	Mycophenolate mofetil
MP	Microparticles
MΦ	Macrophages
MHC	Major histocompatibility complex
NC	Nanocarriers
NK	Natural killer
NP	Nanoparticles
NOD	Non-obese diabetic
nTregs	Natural Treg cells
PD-L1	Death-ligand 1
PEG	Polyethylene glycol
PLGA	Poly(lactic-co-glycolic) acid
pLN	Pancreatic lymph nodes
pMHC	Peptide-MHC complexes
PS	Phosphatidylserine
ROS	Reactive oxygen species
SC	Subcutaneous
SCFAs	Short-chain fatty acids
TCR	T cell receptors
Teff	Effector T cells

TolDCs	Tolerogenic dendritic cells
tTreg	Thymic Treg cells
Tregs	Regulatory T cells
TNF	Tumor necrosis factor
T1D	Type 1 diabetes
T2D	Type 2 diabetes
Th	Helper T-cells
ZnT8	Zinc transporter 8

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1. Introduction

Type 1 Diabetes (T1D) is a highly prevalent autoimmune disorder, in which pancreas does not produce insulin, the hormone responsible for controlling blood glucose levels (1). In the absence of insulin, the body is unable to utilize glucose as an energy source, since its entry into cells is compromised, leading to hyperglycemia (1, 2). The destruction process of insulin-producing β-cells occurs progressively and may last months or even years before any symptoms appear, which makes it difficult to detect the disease in an early stage. As a result, T1D is a silent but potentially fatal disease since severe complications can arise without the proper glycemic control. To date, there is no cure for T1D, only treatments that help to control and relieve its symptoms, representing a huge financial burden for the state. High costs, as well as serious complications resulting from a late detection and inexistence of a cure, are factors that require a change in the focus of present therapies: efforts must be made to ensure T1D prevention or delay its onset, enabling fewer costs and greater security for individuals at risk of developing the disease (2, 3).

Immunotherapy has a great potential for the treatment and prevention of T1D, being one of the most studied strategies in clinical trials. This type of therapy can modulate immune responses using a variety of materials, such as immunosuppressors, cytokines or even antibodies, resulting either in the activation or suppression of the immune system (4). Since T1D is an autoimmune disease, where the immune system does not work properly, mistakenly attacking the insulin-producing cells in the pancreas, the use of immunotherapy could attack its immune problem directly. Basically, as immune cells recognize self-antigens and destroy β-cells, it is necessary to reprogram the immune system, suppressing it. According to various studies, the combination of immunotherapy and nanotechnology could potentiate the efficacy of these therapeutics (4, 5). Nanoparticles (NP) are often used in medicine, helping in the diagnosis, prevention, and treatment of diseases. In the last decade, nanotechnology has gained significant relevancy in the treatment and prevention of T1D. Thus, using NP as autoantigens nanocarriers (NC), it could be possible to achieve a targeted drug delivery, in a sustained and controlled manner, and with fewer adverse effects, enabling the modulation of immune system in a safer and more effective way (6, 7).

Here, the role that nanotechnology, associated with promising immunotherapeutic strategies, has in the development of novel and effective approaches for the prevention and treatment of T1D will be explored.

2. Type 1 diabetes

Diabetes Mellitus is a metabolic chronic disease, characterized by high glucose levels in blood (8). This long-term condition can occur due to innumerous factors, in particular by a deficiency in the production and/or function of insulin (9). Insulin, a peptide hormone produced by pancreatic β-cells (10), is responsible for the input of glucose in the cells (8), and affects protein, lipid and mineral metabolism (11), whereby an insulin deficit leads to diverse symptoms such as polyuria, polydipsia and weight loss (9). Without appropriate glycemic control, it can cause life-threatening complications, including retinopathy, nephropathy, neuropathy, vasculopathy, cardiovascular diseases, diabetic ketoacidosis, etc (12).

T1D, also called insulin-dependent diabetes, is an autoimmune disorder in which T cell-mediated destruction of pancreatic β-cells is present (10), due to a loss of immunological tolerance (13). In T1D, pancreas does not produce and release any insulin to the bloodstream, differing from type 2 diabetes (T2D), in which reduced secretion of insulin by the β-cells and insulin resistance play a significant role (1).

T1D is a complex disorder, where multiple genetic and environmental factors are involved. However, the triggering event for the development of this disease in not yet fully understood (12). Antibodies against specific β-cell proteins have been identified in most of the people diagnosed with T1D. During months or even years, these important biomarkers of presymptomatic disease, can progress to symptomatic disease (8).

Thus, T1D is subdivided into three stages (figure 1): stage 1 (presence of autoantibodies and absence of dysglycaemia) and stage 2 (presence of antoantibodies and dysglycaemia), that comprise the presymptomatic stage, where a decline in β-cell mass is observed; and stage 3, that comprises the symptomatic phase. The risk of progression to the final stage is associated with age of seroconversion and the number of autoantibodies detected (1).

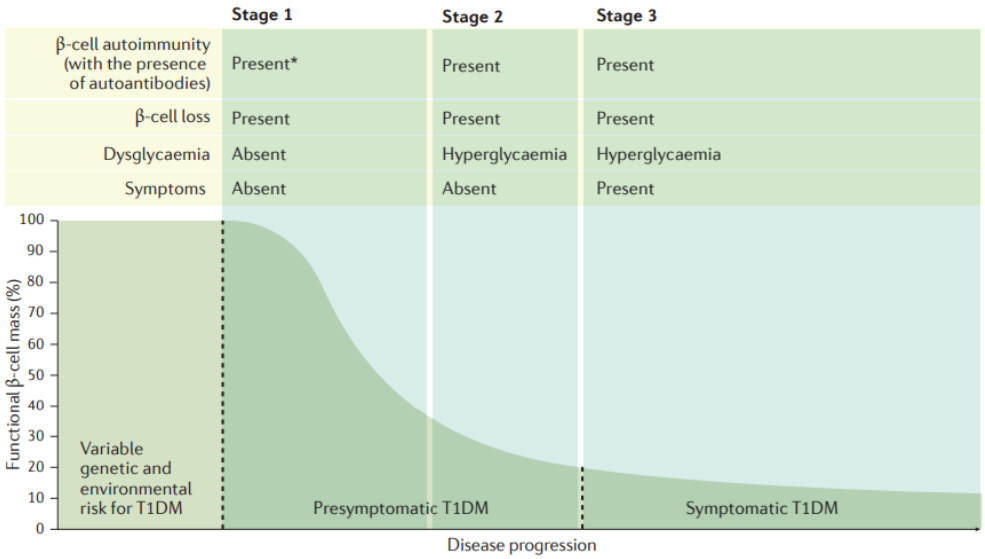

Figure 1. Stages of T1D. Adapted from (1).

According to the International Diabetes Federation, T1D is one of the most prevalent diseases in the world, where an estimated 537 million adults aged 20-79 years (10,5% of all adults in that age gap) have diabetes and it is believed that an increase of 46% will be seen by 2030. This disease is generally more prevalent in men than in women, with children and adolescents being affected as well. According to the data available, the prevalence and incidence of T1D are increasing all over the world (8).

2.1. Pathophysiology

T1D is known to be a heterogeneous disease. For T1D development, an immune response to β-cells antigens must first be triggered. Then, a strong pro-inflammatory response followed by. An ineffective regulatory control of the autoimmune response originates a chronic and deadly response to pancreatic β-cells. Thus, autoimmunity is recognized as the major factor in the pathophysiology of T1D (14).

Pancreatic islet inflammation, termed insulitis, is characterized by the infiltration of immune effectors including macrophages (MΦ), dendritic cells (DCs), $CD8^+$ and $CD4^+$ T cells and B cells (Figure 2). This inflammation progresses over time, promoting hyperglycemic blood levels when a substantial quantity of β-cell mass has been destroyed or converted to nonfunctional (12).

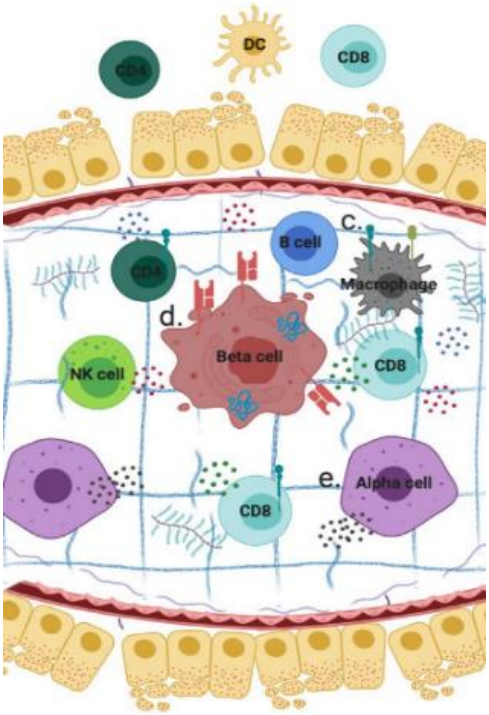

Figure 2. T1D islet microenvironment. Adapted from (15).

Despite the exact pathogenesis remains unknown, it is believed that the susceptibility to the development of T1D and, therefore, the process of autoimmune destruction, is determined by a combination of genetic and environmental factors (14). The key genes that predispose to T1D are found on chromosome 6, within the major histocompatibility complex (MHC) region, usually designated human leucite antigen (HLA) (16), the family of genes that is responsible for controlling antigen presentation in the immune system (10). Environmental factors can also trigger T1D namely viruses, especially enteroviruses such as coxsackievirus B1, toxins and some nutrients present in cow´s milk and cereals (16). Recent studies indicated that gut microbiota is involved in T1D pathogenesis, due to its potential to modulate peripheral immune tolerance, and to induce a regulatory effect on β-cell autoimmunity (17). It is important to mention that the most frequent theory for T1D pathogenesis include microbial/viral infections of β-cells, causing or worsening islet inflammation, since pathogens can cause direct cytolysis and/or local inflammation (18).

3. Immune system in pathogenesis of Type 1 diabetes

To understand the pathogenesis of T1D, it is important to understand the complex interaction between pancreatic β-cells and the immune system, resulting in pancreatic islets damage, mainly towards β-cells, and uncontrollable glucose homeostasis (19).

3.1. Innate and adaptive immune systems – overview

The immune system recognizes and protect the organism against pathogens. This complex system needs a strict regulation to prevent autoimmune complications, being one of them the loss of immunotolerance seen in T1D (20).

This function is achieved through communication between the immune system's two fundamental lines of defense: innate immune system and adaptive immune system. The first line of defense, that acts in a non-specific, antigen-independent, and immediate manner, is provided by innate immune system, which do not have immunologic memory (21). It is responsible for an inflammatory response by secreting pro-inflammatory cytokines. Otherwise, adaptive immune system is antigen-specific, and can differentiate between self and non-self-antigens, having the capacity of immunologic memory and production of specific antibodies. These properties allow a faster and more efficient response when a second exposure to the same antigen occurs. These immune systems have a synergistic and complementary action, whereby a dysfunction in either of them can result in an autoimmune disorder (20, 22).

3.2. Immune cell crosstalk between pancreatic beta cells and innate and adaptive systems

Several studies have shown that innumerous cell types are involved in β-cell destruction, including autoreactive $CD4^+$ and $CD8^+$ T cells, responsible for cell-mediated immunity, and B cells, involved in the adaptive immune response; and MΦ, DCs and natural killer (NK) cells, involved in the innate immune response (Figure 3) (21, 22). T1D occurs due to a loss of immunological tolerance, where autoreactive T cells, T cells that escaped thymic negative selection, plays an important role in the development and progression of the disease, due to its capacity to recognize autoantigens, mainly insulin, proinsulin, islet antigen 2 (IA-2) glutamic acid decarboxylase 65 (GAD65), zinc transporter 8 (ZnT8) and heat shock protein 60 (Hsp60) (23, 24).

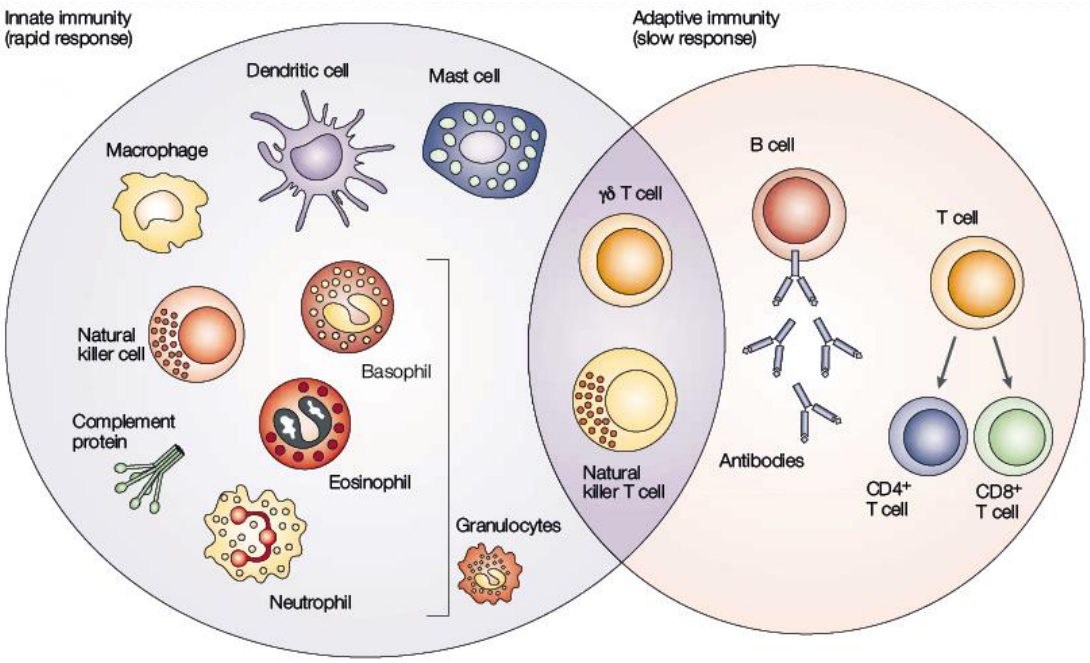

Figure 3. Immune cells responsible for innate and adaptive responses. Adapted from (25).

Even though the exact triggering event remains unclear, T1D pathogenesis could be related to a direct viral attack against β cells. In a normal condition, autoantigens are produced by apoptotic cells, being after removed by antigen-presenting cells (APC) through a process known as efferocytosis. After this mechanism, anti-inflammatory mediators are released by APC, mainly DCs, whilst the release of inflammatory cytokines (TNF, IFN-γ, IL-12, IL-2) is inhibited, preventing DCs maturation and contributing to the maintenance of immune tolerance to self, rather than evolving to autoimmunity (26). Nevertheless, if part of this process fails, due to efferocytosis abnormality or an increase in the rate of β-cells apoptosis, β-cells will become necrotic, favoring inflammation, activation of DCs, insulitis and autoimmunity. For example, when a defective clearance of apoptotic β-cells is present, there is an increase of apoptotic bodies, translating into higher self-antigens release and, therefore, a higher involvement of resident MΦ and mature DCs, both capable of endocytosis (18). When an effective efferocytosis occurs, DCs do not undergo the maturation process, remaining immature, and, therefore, being unable to activate naïve T cells (26). At this stage, immature dendritic cells (iDCs) sustain the peripheral self-tolerance, through a specific phenotype named tolerogenic. These tolerogenic dendritic cells (TolDCs) express low levels of co-stimulatory molecules, such as CD80 and CD86, and low levels of MHCI and MHCII, secreting minor quantities of pro-inflammatory cytokines. TolDCs are also qualified to secrete immunosuppressive agents, such as TGF-β, contributing to an expansion of regulatory T cells (Tregs), and expression of inhibitory co-receptors, namely death-ligand 1(PD-L1), contributing to T cell anergy. Thus, the presence of this phenotype is important to prevent the development of T1D (Figure 4) (27).

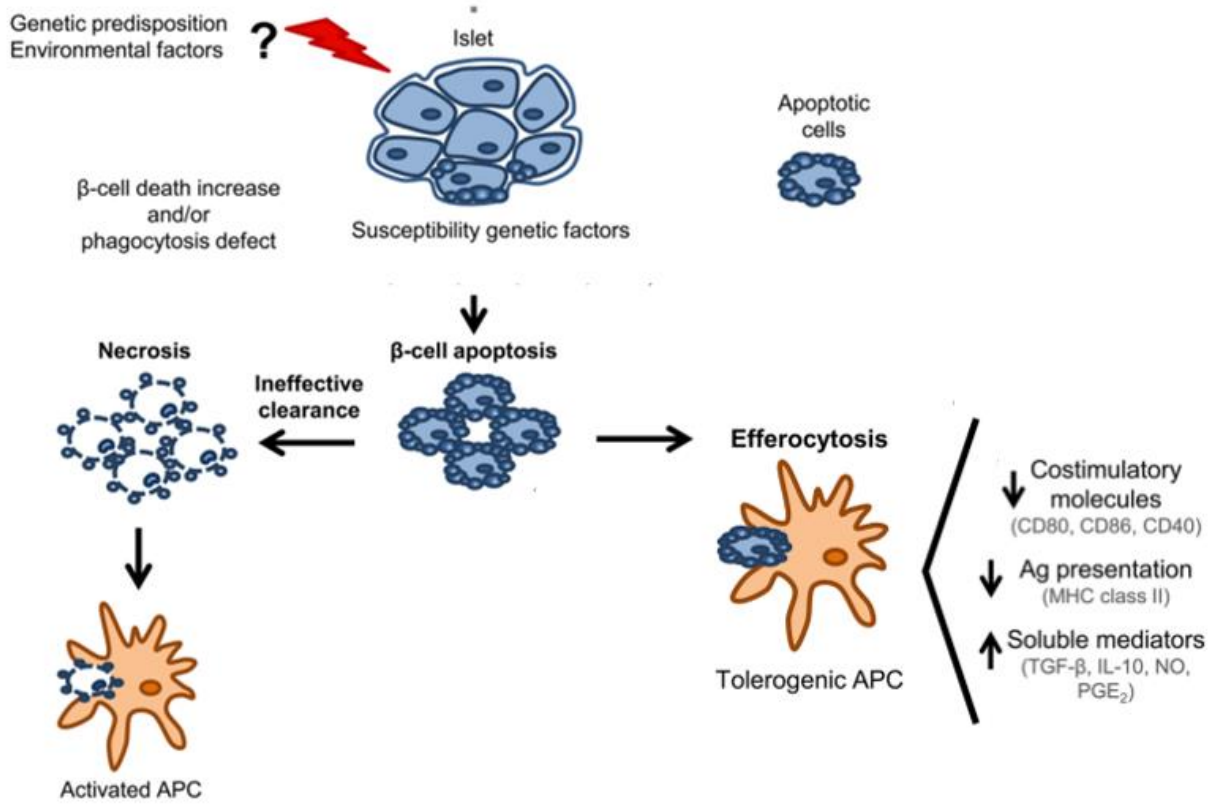

Figure 4. Schematic representation of T1D initiation. Adapted from (26).

Activated DCs, i.e., mature DCs can produce high levels of pro-inflammatory chemokines and cytokines, including IL-15 and IL-12, that contribute to autoimmunity against the pancreatic tissue and exacerbation of inflammation (21), being also responsible for the capture of the released self-antigens, presenting them to specific naïve T cells, that escaped the peripheral tolerance mechanisms, in draining pancreatic lymph nodes (pLN), where the diabetogenic response start (28). This process leads to T cell priming, followed by the activation of islet antigen specific $CD4^+/CD8^+$ T cells, turning them into effector T cells (Teff), also known as diabetogenic. An expansion, recruitment, and migration of these autoreactive $CD4^+/CD8^+$ T cells to pancreatic islet takes place after their activation. Therefore, Teff can promote β-cell destruction and progression of T1D (18).

Through the presentation of self-antigens by mature DCs, is possible to achieve Teff cells activation. For this to happen, a set of signals must be present. Other than the existence of MHC molecules on DCs, with the capacity to bind to T cell receptors (TCR), providing a "signal 1", other three signals are required for DCs to act as APCs. This antigen specificity "signal 1", needs to be paired to with a co-stimulatory "signal 2", possible by the presence of clusters of differentiation, receptors of the B7 family, specifically CD80 and CD86. The co-stimulation occurs when CD80/CD86 binds to CD28, the main co-stimulatory receptor in T cells (29). It is also required a growth "signal 3", provided by cytokines and a subset polarization "signal 4", that determines the subtype of cells that will be present after the process of priming, relying on cytokines and differentiation factors (30).

Destruction and loss of β-cell mass requires both $CD4^+$ and $CD8^+$ cells, being $CD4^+$ cells activated through MHC class II-mediated antigen presentation, and $CD8^+$ cells activated through MHC class I-mediated antigen presentation (Figure 5).

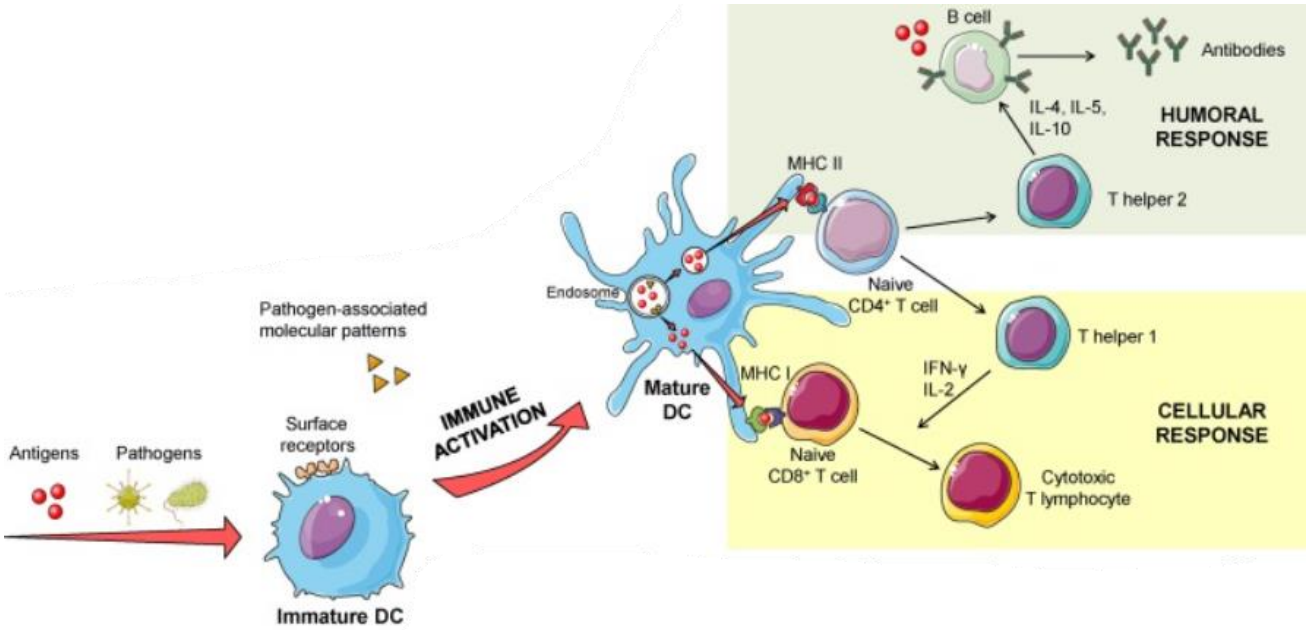

Figure 5. Mechanism of immune activation of $CD4^+$ and $CD8^+$ T cells. Adapted from (31).

The main functions of these adaptive immune cells are mentioned below:

CD4$^+$T cells: known as helper T-cells (Th), they present two subsets, T helper cells 1 (Th1) and T helper cells 2 (Th2). Th1 phenotype is accountable to produce IL-2 and IFN-γ, a pro-inflammatory cytokine that causes an induction of MHC-I and MHC-II expression in β-cells, increasing the inflammatory response and the destruction of the pancreatic cells. CD4$^+$ T cells also exert a harmful effect via activation of DCs, maturation of CD8$^+$ T cells (21) and activation of MΦ, through a set of important interactions with surface markers, such as TLRs, and pro-inflammatory cytokines, such as IFN-γ (14). This cytokine stimulates the production of TNF-α and IL-1β by MΦ and, since the IL-1 receptor is highly expressed on pancreatic cells, the IL-1β induced apoptosis is favored (28). Additionally, IFN-γ promotes a migration of T, B and innate cells into the islets and induces the production of reactive oxygen species (ROS) (18). Contrarily, Th2 phenotype produces a protective effect, mainly through the release of IL-5, IL-6, and IL-4 (14).

CD8$^+$T cells: known as cytotoxic T cells, were shown to be dominant in peripheral blood of T1D patients, in relation to CD4$^+$ T cells, proving the CD8$^+$ T cells important contribution to T1D development (19). CD8$^+$ T cells recognize self-antigens presented by MHC class I molecules, onto β-cell surface, leading to their activation and differentiation into Teff cells (32). Cytotoxicity results from a contact-dependent manner, involving perforin and granzyme B, or from a FAS-FAS ligand (FASL) pathway (14). When some inflammation appears, the pancreatic β-cells become more susceptible to destruction, due to an enhance expression of FAS. This susceptibility is the result of IFN-γ activity released from CD8$^+$ T cells, being also potentiated by other pro-inflammatory cytokines, such as IL-1β and TNF-α (33). Alternatively, perforin and granzyme B release play a crucial role to achieve apoptosis, with CD8$^+$ T cells secreting these membrane-disrupting proteins, able to induce death signaling pathways, directly on target cells (34).

Even though T cells could exert a negative effect, contributing for the T1D onset, they are also capable of a regulatory mechanism, preventing the loss of β-cell mass. This beneficial outcome is possible due to the existence of Treg cells (35).

Tregs are a subpopulation of T cells, mainly a subset of CD4$^+$T cells, considered immunosuppressive (27). Responsible for maintaining self-tolerance, these cells are distinguished by the presence on the surface not only of CD4, but also high levels of interleukin-2 receptor, IL-2R, known as CD25, and expression of the forkhead box P3 transcription factor, FOXP3, being, for this reason, commonly mentioned as CD4$^+$CD25$^+$FOXP3$^+$ Treg cells (36). These cells play a fundamental role in the control of autoimmunity, through the suppression of Teff cells responses, by contact-dependent mechanisms, involving APCs, and by anti-inflammatory cytokine release, especially IL-10 and TGF-β (12). Treg cells are classified into two groups based on their development origin: thymic Treg cells (tTregs), formed in the thymus, being known as natural Treg cells (nTregs), or induced Treg cells (iTreg), formed in the peripheral blood from the induction of CD4$^+$T cells after antigen presentation, being known as adaptive Treg cells (aTregs) (36, 37). Having the ability to migrate to the periphery, tTregs are usually found in peripheral tissues when a local inflammatory process is occurring. This type of regulatory cells presents CTLA-4 (cytotoxic T lymphocyte-associated antigen 4) or PD-L1, inhibitory molecules expressed on its surface (11), high levels of the transcription factor

FOXP3$^+$, low levels of CD127 and TCR with high affinity for self-antigens (38). FOXP3$^+$ is the marker of Tregs, having an important role in the upregulation of IL-2R and CTLA-4 genes (16, 39). Responsible for the immunosuppressive properties of this T phenotype, FOXP3$^+$ is necessary for its development and transcription, controlling and regulating immune homeostasis and reactivity to self (18, 39). The presence of aTregs in peripheral blood is not only dependent on antigen presentation, but also dependent on the presence of specific cytokines, particularly IL-2 and TGF-β. For example, IL-2 is considered the most critical cytokine for Treg cells, since its presence is mandatory for Treg function, survival, expansion, and homeostasis (18, 36). In this manner, when some perturbation takes place in the Treg suppressive network, the possibility of an autoimmune disease emerging needs to be considered.

B lymphocytes also have a pathogenic role in the development of T1D. Studies in non-obese diabetic (NOD) mice, using a depletion of B cells with anti-IgM antibodies, led to an impairment of disease progression (21). Even though some of the exact functions of these cells in T1D are not yet fully known, two activities were confirmed: B cells are producers of autoantibodies, working as APC to diabetogenic CD4$^+$ and CD8$^+$ T cells (Figure 6). The production of autoantibodies against islet cell proteins in an earlier stage of the disease helps to predict the onset of T1D, as they function as diagnostic biomarkers (40). In an advanced stage of T1D, these cells work as APC, stimulating the activation of effector cells and, therefore, the destruction of β-cells (18). Due to its involvement in T1D progression, targeting B cells is a promising strategy.

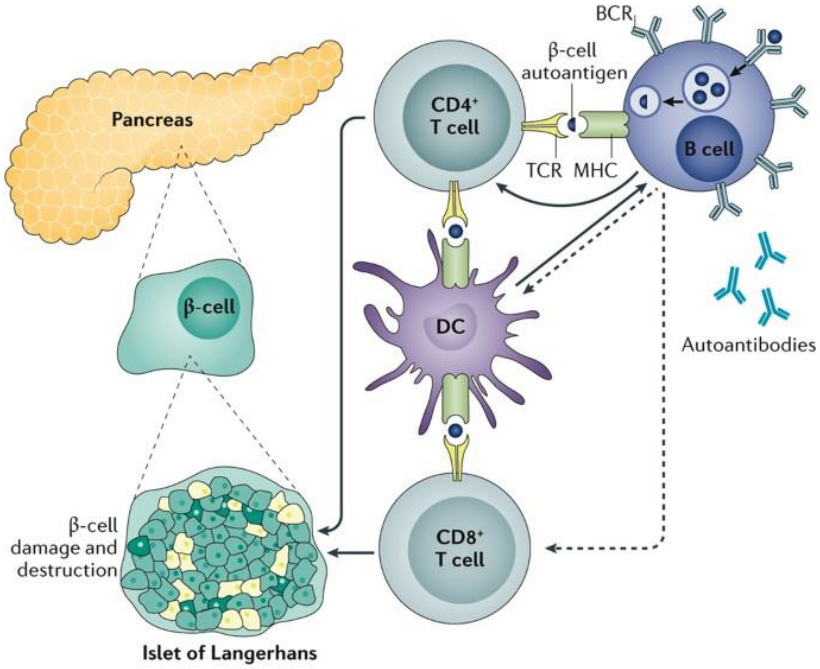

Figure 6. Interaction between activated B cell with DCs, CD4$^+$ and CD8$^+$ T cells. Adapted from (1).

Neutrophils, mast cells and NK cells are also involved in T1D pathogenesis. Being the most abundant leukocyte in blood, neutrophils are characterized by their phagocytic properties, in order to fight bacterial infections (22). Apoptotic β-cells can activate these short-lived cells, which can cause a long-lasting inflammation by secreting pro-inflammatory cytokines. Besides this function, infiltrated neutrophils in the pancreatic region can stimulate MΦ and DCs,

favoring the inflammatory process (21). Studies have demonstrated that neutrophil infiltration and neutrophil extracellular traps (NETs) formation are involved in the initiation of T1D (41).

As neutrophils, mast cells also play an effector role in this autoimmune disease. These long-lived tissue-resident cells are activated in response to allergic diseases and parasites, releasing histamine, lipid mediator, such as leukotrienes, and most importantly cytokines, such as TNF-α and IL-6, both pro-inflammatory, contributing to the destruction of β-cells and progression of the disease (12). NK cells are known for having a dual effect, since they are responsible not only for a pathogenic role, but also for a protective effect. Once these lymphoid cells are activated, they release perforin and granzymes, but also cytokines, namely TNF-α in high amount (22, 28). These agents contribute to the destruction of target cells, like β-cells (21). Through these mechanisms NK cells exert its pathogenic effect on T1D development. Several studies showed that depletion of NK cells leads to a delay in the progression of the disease, showing a positive correlation between the number of activated NK cells with the damage on pancreatic islets. However, some data suggest that NK cells exhibit a protective role. Here, the presence of a specific NK subtype, named invariant NKT cells (iNKT) reduces T1D incidence in NOD mice, whose protection is associated with the induction of Th2 phenotype, responsible for the production of anti-inflammatory cytokines (28).

MΦ are a lineage of white blood cells with a significant role in T1D development, since they secrete pro-inflammatory cytokines, including TNF, IL-12, IL-1β, and ROS (21). TNF, IL-1β and ROS are involved in apoptosis, increasing β-cell destruction and death. IL-12 is implicated in the differentiation of $CD8^{+}$T cells, a type of diabetogenic T cells that produce harmful effects (28). MΦ are related to the initial and destructive stages of T1D, having been reported the presence of pro-inflammatory MΦ in the islets of newly diagnosed patients (14). The immune cell crosstalk between pancreatic β-cells and innate and adaptive immune systems are schematized in Figure 7.

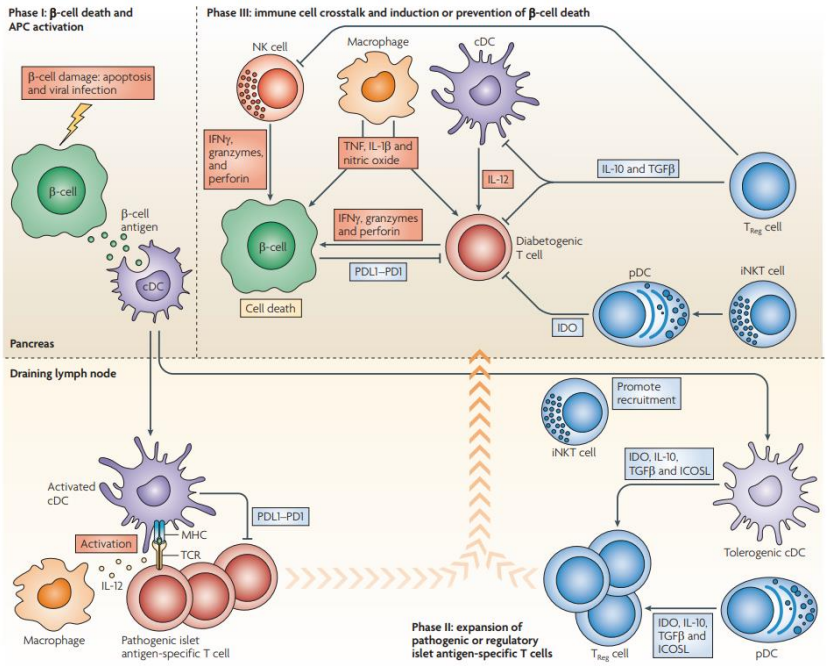

Figure 7. Summary of immune cell crosstalk between pancreatic β-cells and innate and adaptive immune systems leading or preventing T1D. Adapted from (28).

Many of the cells mentioned function as APC, recognizing autoantigens from pancreatic islet, presenting them to T cells, promoting their activation and, thus, their effector phenotype. For example, $CD8^+$T cells can recognize epitopes derived from GAD65, ZnT8 and insulin (40). Moreover, another type of peptides can be recognized by the immune system, named neoantigens, implicated in T1D development (42). Neoantigen production needs a chronic exposure to pro-inflammatory cytokines, inducing β-cell stress and, consequently, β-cell destruction (32). These peptides are a result of mutations, frameshifts, post-translational, post-transcriptional and proteasomal modifications, resulting in hybrid peptides or a combination of amino acid sequences (43). Since they are considered non-self-antigens, a stronger immune response can be triggered, being important to develop treatments that targets these peptides, aiming the achievement of immunotolerance.

Thus, many adaptive immune cells and innate immune cells can have a dual role in T1D pathogenesis, being able to activate the specific immune response or act as regulatory cells, preventing the autoimmune disease.

3.3. Mechanisms of immunotolerance and regulatory T cell dysfunction

To prevent the onset of autoimmune disorders, it is crucial to maintain the immune tolerance. When an immune response occurs against self-antigens, it means that a loss of immune tolerance is present (14). Immune tolerance involves specific processes that leads to a state of immune unresponsiveness or to a state of a low response to autoantigens (27), while detecting non-self-antigens and maintaining an effective response to these particles viewed as potentially dangerous to the human body (44).

This complex system involves two mechanisms: central and peripheral tolerance. Together, they avoid modifications in immunological tolerance and, consequently, a harmful response directed to islet cells.

The central tolerance involves the development of T and B lymphocytes in the primary lymphoid organs, namely T lymphocytes in the thymus, and B lymphocytes in the bone narrow (42).Occurring in the first years of life, central tolerance implies a selection process where immature T cells and immature B cells are exposed to a high concentration of self-antigens (38). Lymphocytes with specific receptors for self-antigens, able to recognize MHC I and MHC II molecules with high affinity, undergo a negative selection (16). Thus, self-reactive cells are directed to programmed cell death, known as apoptosis, where lymphocytes do not receive survival signals (Figure 8). This process allows the identification of self-reactive T and B lymphocytes, with the same being eliminated before they escape from the primary lymphoid organs and develop into fully immunocompetent cells. Otherwise, lymphocytes that recognize MHC I and MHC II molecules with low affinity undergo a positive selection, receiving survival signals and, therefore, surviving and proceeding to secondary lymphoid organs. This first type of immune tolerance can prevent autoimmunity, as its discriminate self to non-self, leaving in circulation mature T cells capable of recognize peptides presented by HLA, but not from MHC complexes (41, 42, 44).

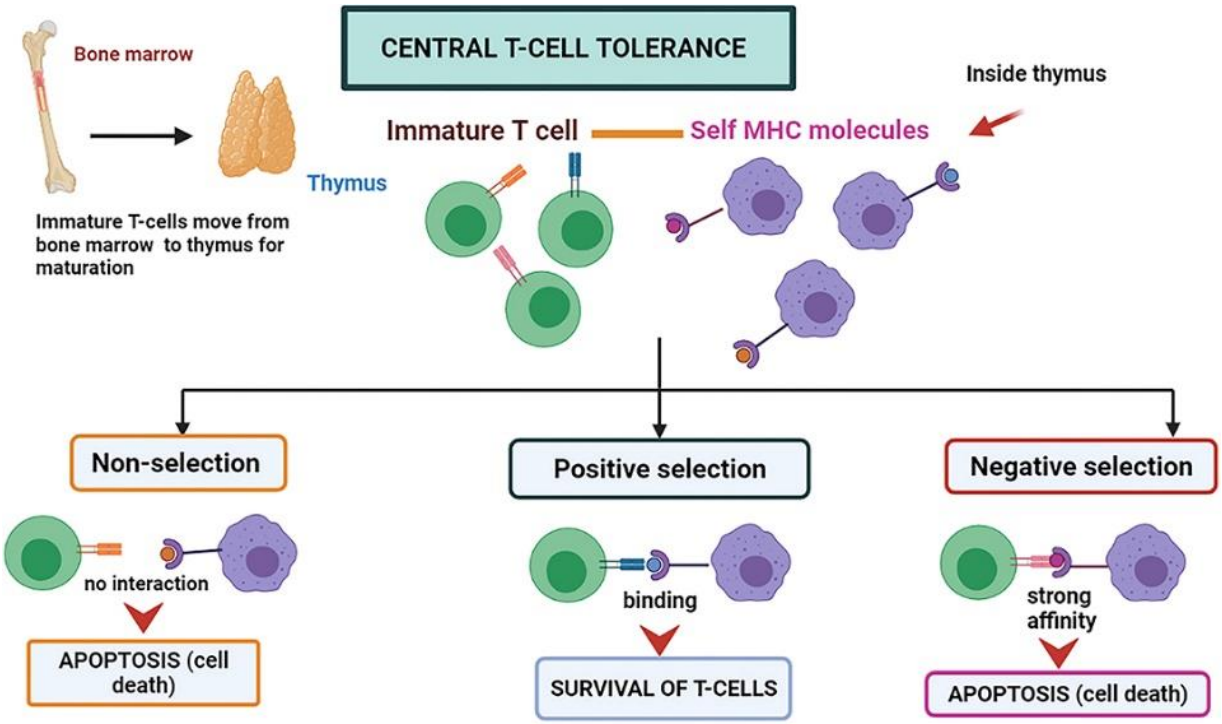

Figure 8. Possible outcomes of central tolerance mechanism. Adapted from (44).

Despite the central tolerance process**,** self-reactive T cells can still be detected in the peripheral blood, showing that this mechanism alone is generally not sufficient to obtain the high protection needed against self-peptides (42). This information confirms that central tolerance is not a completely effective mechanism, needing an additional method, named peripheral tolerance. This second type of immune tolerance occurs in the peripheral tissues and secondary lymph organs (lymph nodes, spleen, etc.) and its responsible to regulate self-reactive clones, that escaped into the bloodstream, mainly through tolDCs and Tregs functions (27, 31). T cells that escaped thymic deletion are present not only in ill individuals, but also in healthy ones, which means that, in a patient with genetic predisposition and in the presence of favorable environmental factors, a failure in the peripheral protective mechanisms may be sufficient to lead to disease development (31). Self-reactive T lymphocytes can become activated when they first encounter their autoantigen outside the thymus and, therefore, becoming harmful and diabetogenic (45). To control tolerance of lymphocytes, peripheral tolerance operates by one of four mechanisms (Figure 9): promotion of T cell anergy; deletion of T cells via apoptosis; Tregs induction and proliferation, and T cell ignorance (40).

T cell ignorance: reactive T cells do not come across their self-antigen in the periphery (40). Some autoantigens are ignored by T cells, since it is believed that these cells can be present in immune privileged areas or because they can be less immunogenic, not triggering the bond with their autoantigen and, therefore, preventing the initiation of harmful immune responses (46).

Anergy: known as an unresponsiveness state, this process is possible due to a specific DCs phenotype, tolDCs (27). These semi-immature DCs present autoantigens to autoreactive T clones, but not provide sufficient or efficient co-stimulatory signals to promote Teff activation, since this regulatory phenotype expresses low levels of MHCI/MHCII molecules and co-stimulatory molecules CD49, CD80 and CD86, high levels of inhibitory co-receptors,

such as PD-L1, and produces low amounts of pro-inflammatory cytokines. Thus, after autoantigen presentation and without proper signals 2 and 3, tolDCs are not capable to activate T cells, turning them anergic to that self-antigen (26, 27, 45). This process occurs mainly due to a high avidity interaction between CTLA-4-CD80/CD86. This link promotes a negative signal and, consequently, decreases the activation of immune cells and promotes the regulation of the immune response.

Deletion of autoreactive T cell clones: tolDCs are also able induce T cell depletion via apoptosis. As no co-stimulation signal is observed, in the long term, could lead to death in inflammatory conditions (27, 44). Apoptotic pathways mediated by FAS appear to be crucial for self-reactive lymphocytes deletion in the periphery, namely after they suffer several stimulations by their autoantigen *in vivo* (45). This apoptotic mechanism occurs via the caspase pathway, culminating in the end of the immune response.

Induction and development of FOXP3+Tregs: An immune regulation is achieved by "induced" Tregs expressing the transcription factor FOXP3^{+}, responsible for Tregs stability and their immunosuppressive properties, to control reactivity to self. The presence of these cells is crucial to inhibit pathogenic effects, since they compete with reactive T cells for the same antigen presentation by APC and, consequently, being able to block their activation into Teff cells. Also, tolDCs are involved in this mechanism. Their presence is important for iTregs development and induction, since they secrete immunosuppressive molecules, such as TGF-β, and express inhibitory co-receptors, such as PD-L1 (26, 40). In turn, pTregs have similar protective functions as nTregs, maintaining immune homeostasis.

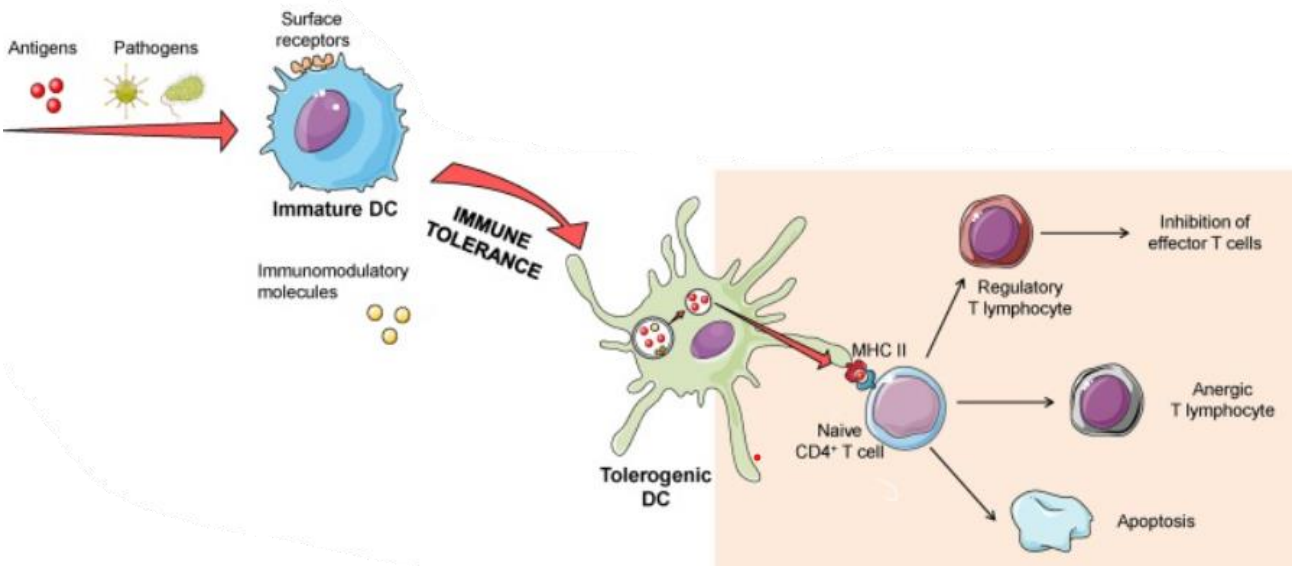

Figure 9. Different mechanisms of peripheral tolerance. Adapted from (31).

Treg cells are essential to maintain the peripheral tolerance to autoimmune responses, exhibiting their suppressive functions by cell-contact mechanisms and by secretion of anti-inflammatory cytokines and other immunosuppressive factors (18). These $CD4^+FOXP3^+$T cells express CTLA-4, an inhibitory co-stimulatory molecule responsible for a cell-to-cell contact (36). Even though the exact mechanism by which this receptor operates remains unclear, it is believed to compete with CD28, present in T cells, for the linkage to CD80 and CD86, present in APC. Expressed at high levels on Tregs, binds to the CD80 and CD86 molecules, preventing binding to CD28 from happening and, consequently, inhibiting Teff activation and proliferation, since antigen presentation is blocked (18, 30). Thus, CTLA-4 is considered a key molecule to prevent the onset of T1D. For immune hemostasis to remain undisturbed, the production of some cytokines and soluble factors also needs to occur (47). IL-10, IL-35 and TGF-β are immunosuppressive cytokines produced by activated Tregs involved in their contact-independent mechanisms, directly suppressing the effector properties of T cells, inducing the same to differentiate into the regulatory phenotype (27). Tregs also benefit from their high expression of CD25, favoring the link between IL-2-CD25. Competing with Teff, they deprive these cells from their growth factor and, therefore, block their activation, expansion, and differentiation (36).

Susceptibility to an autoimmune disorder increases when some of the suppressive functions are disturbed, broking the self-immunotolerance established. Many authors argue that this default occurs due to a combination of uncontrolled/defected Teff activity/function and a change in the frequency of Tregs. Both processes are believed to lead to an imbalance between regulatory and effector cells and therefore leading to a loss of tolerance (24, 37). However, there is still no consensus, mainly about the frequency of Treg cells. There are studies that suggest a decrease in frequency of Tregs in the peripheral blood of diabetic patients, while others argue that the number of these cells are very similar between healthy and diabetic patients (36).

$FOXP3^+$ is crucial for the development, homeostasis, and suppressor functions of Tregs cells, so it is extremely important to maintain $FOXP3^+$ expression, even in inflammatory environments, to maintain Tregs stability. However, there is a set of mechanisms responsible for the opposite, leading to its instability (figure 10). The decrease of function can be caused not only by genetic deficiencies of $FOXP3^+$, but also from a failure in thymic development of $FOXP3^+$ Tregs due to, for example, an irregularity in signaling through IL-2/IL-2Rα or IL-2/IL-2Rβ (24, 27). When this cytokine is present in low amounts, the induction of $FOXP3^+$ expression, differentiation of $FOXP3^+$Tregs and the achievement of their immunosuppressive capabilities become impaired, resulting in a loss of $FOXP3^+$ expression and in a gain of effector properties, leading to a downregulation of regulatory T cells (11, 39). Besides those mentioned, other processes are accountable for a defective Tregs function, such as disturbances in CTLA-4 pathway and in signal transducer and activator of transcription 5 (STAT5) signaling, this last one resulting in a decrease expression of CD25 and $FOXP3^+$ (48). This leads to an increase of pathogenic activity of the dysregulated Tregs, where a production of pro-inflammatory cytokines, such as IFN-γ and IL-17, is observed (24, 36). For these cells demonstrating stability means being able to express $FOXP3^+$ and to produce anti-inflammatory cytokines, such as IL-10 and IL-35. However, due to some complication, they can alter their phenotype, becoming "ex-Tregs," also known as destabilized Tregs, where $FOXP3^+$ is lost and, hence, acquiring effector properties. In these situations, the production of anti-inflammatory cytokines is replaced with the production of pro-inflammatory ones, causing tissue damage (47).

Diabetic patients had shown a combination of less-stable Tregs, due to a decrease in stability of FOXP3 expression, resulting in a lower number of stable Tregs capable to exert the protection needed against Teff; and a higher number of pathogenic Tregs, which produce harmful cytokines, contributing to the inflammatory environment. Moreover, a resistance by Teff to Tregs suppression is also detected, not only because of the Treg cells that changed their suppressive phenotype to an effector one, but also due to the specific T cells that were activated by autoantigen presentation through APC, allowing their conversion to Teff. Together, these anomalies create the ideal environment for T1D onset, with an imbalance between Tregs and Teff cells, with a higher number of Teff cells present, but also with Tregs function compromised (36, 48).

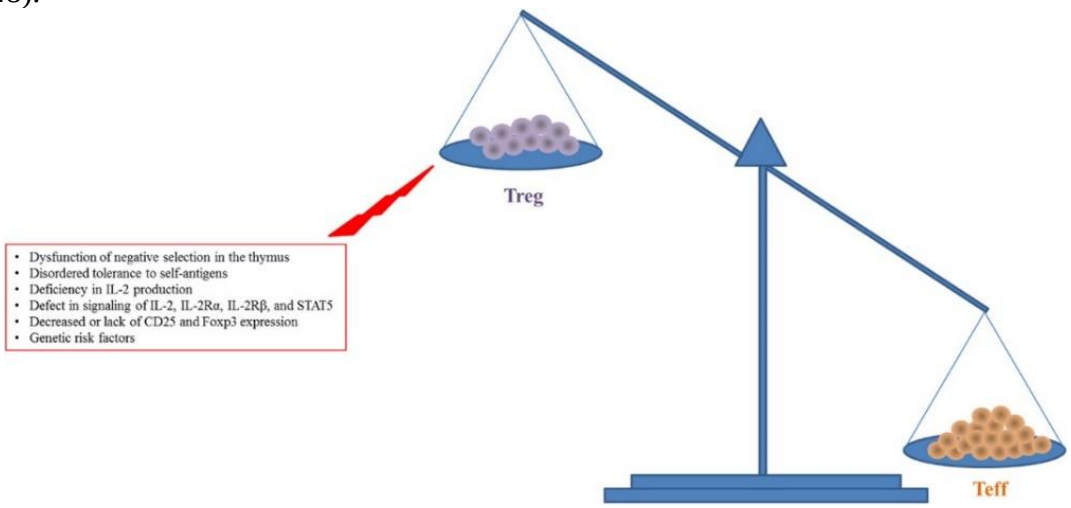

Figure 10. Mechanisms responsible for Tregs instability. Adapted from (24).

4. Alternative immunotherapeutic strategies for the treatment of type 1 diabetes

T1D is a heterogeneous disease, where genetic predisposition and environment factors are involved. These effects result in a breakdown of immunotolerance, namely an imbalance between diabetogenic T cells and Tregs. As a result of this heterogeneity, it is important to develop novel target therapies for T1D treatment. Nowadays, most of the therapies approved can control the disease, by correcting the hyperglycemia and, therefore, the symptoms associated. This type of therapy is immunomodulatory and uses essentially small molecules and antibodies.

4.1. Antigen-independent immunotherapies

In T1D both innate and adaptive immune systems are implicated in the interaction with β-cells, causing their death. During the last years, immunotherapy has emerged as a treatment. It has the potential to preserve insulin production and prevent the development of the disease. Some of the therapies consists in antigen-independent strategies, that is, a non-antigen specific intervention.

4.1.1. Antibody- and cytokine-based immunotherapies

One of the approaches that has been mostly studied to manage the symptoms or to prevent the development of autoimmune diseases are antibody-based therapies, which consists in a cell-directed therapy. As T1D is also characterized by an imbalance in cytokines, where pro-inflammatory ones are dominant, a cytokine-based therapy is also expected (Figure 11).

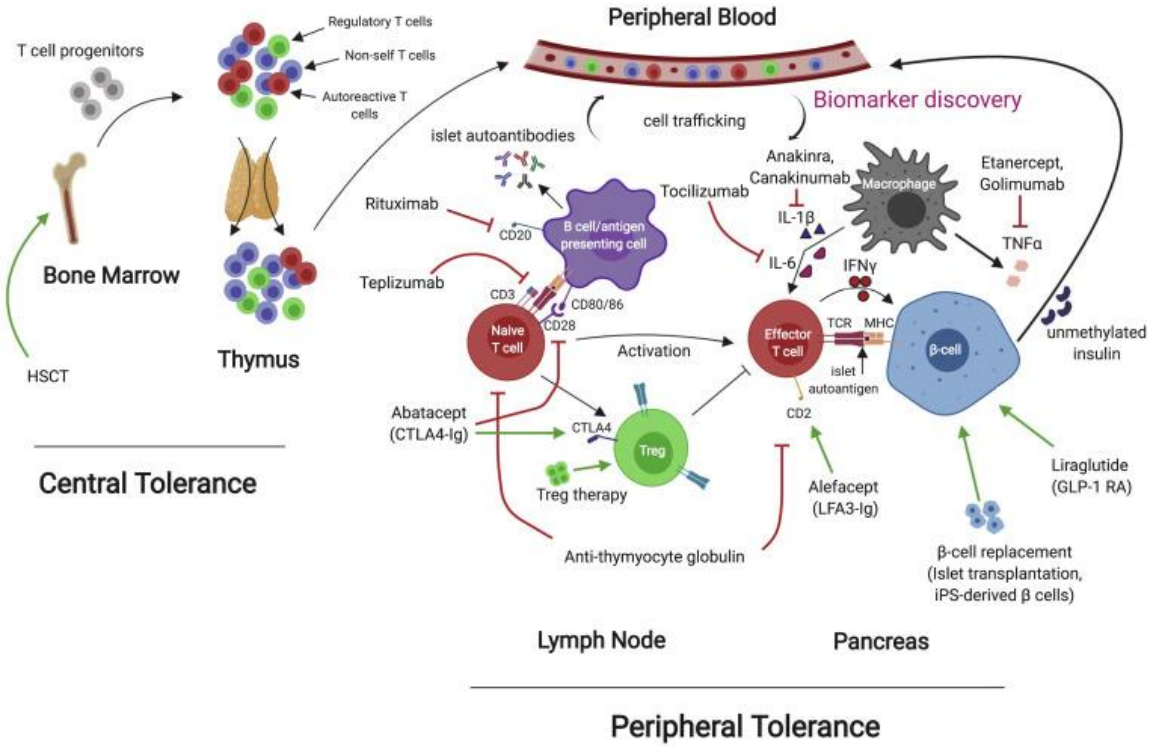

Figure 11. Schematization of antigen-independent immunotherapies in T1D. Adapted from (4).

4.1.1.1. Antibody-based therapies

Even though antibody treatments are not antigen-specific, they manifest specificity to either T cells or B cells. In both, the goal is the same: restore the previously lost immunotolerance, via target cell depletion or by blocking cell receptors (49).

Modulation of T cells: targeting and modulating T cells is one of the most well studied strategies, because these therapies are responsible for inhibit T-cell activation, decreasing the activity or the number of pathogenic T cells, or contributing to the expansion of Tregs (50). CD3 is an essential element of TCR complex, necessary for T cell maturation and activation. This protein complex is expressed in Teff cells, along with CD4 or CD8 (51). Several clinal trials tested anti-CD3 agents, including Teplizumab and Otelixizumab. These anti-CD3 monoclonal antibodies have the ability to bind to CD3, preventing the development of the CD3/TCR/MHC complex. Through this mechanism, the monoclonal antibody (mAb) prevents the effector cells to produce an immune and pathogenic response to the autoantigens, as GAD65 or insulin, since the activation of naïve T cells are compromised (30). This pathway provides a depletion and an exhaustion of Teff cells, leading T cells to an anergic state to its autoantigens, playing a role in the protective effect (50). This sort of treatment targets the antigen specificity "signal 1" of lymphocyte activation, where TCR signaling should take place (30). Since TCR

signaling is compromised, maintaining Teff function becomes a difficult task, since "signal 1" is required for that purpose. For example, a phase II clinical trial in newly diagnosed diabetic patients receiving a 14-day treatment with teplizumab showed a transient preservation in C-peptide levels, sustaining the insulin production, lasting appreciably 24 months, when compared with placebo-treated groups (52, 53). Otelixizumab had also showed positive results in a phase II clinical trial, where a preservation of β-cell function were noticed (30).

Targeting co-stimulatory "signal 2" is also a way to modulate the progression of T1D, via binding and blocking cell receptors, mainly using fusion proteins (30). When a T cell recognizes an antigen through MHC, and the CD3/TCR/MHC complex is formed, a link between B7 ligands, including CD80 and CD86 on APC, its either possible with the main co-stimulatory protein, CD28, in naïve T cells or with co-inhibitory CTLA-4, also known as CD152, present in Tregs (53). The differentiation and activation of Teff cells is modulated by the connection between CD80/CD86 and CD28, with CTLA-4 competing with the co-stimulatory receptor, so that this connection does not occur (30). Abatacept, an CTLA4-IgG fusion protein, showed promising results in clinical trials, with the decline of C-peptide levels in newly diagnosed diabetic patients, the maintenance of glycated haemoglobin (Hb1Ac) values and the delay in the loss of β-cell function (54, 55). Exhibiting a protective function, Abatacept is capable to prevent the linkage between CD80/CD86 and T cell co-stimulatory molecule CD28, blocking the co-stimulatory signal required to Teff activation, promoting an anergic state of naïve T-cells and impairing the activation of these type of cells (54). Belatacept is another CTLA4-IgG fusion protein used to strongly block CD86-CD28 interaction. Other fusion protein that has been used is Alefacept, a recombinant LFA3-Ig fusion protein, that binds with CD2, a surface adhesin molecule expressed in T and NK cells, to block T cell activation and proliferation and, consequently, leading to an increased Treg/Teff ratio (4). Thus, depletion of target cells, mainly Teff cells, has been obtained using mAb, promoting the reestablishment of immunotolerance.

Modulation of B cells: although T cells are the most involved in the autoimmunity process in T1D, B cells also have an important role related to its immunopathogenesis.

Anti-CD20 therapy is one strategy used to modulate B cells. CD20 is a B cell protein marker, that appear on the surface of these cells in the earlier stages of life cycle, namely in the pre-B cell stage (53). Studies using Rituximab, a mAb that targets CD20, showed a delay in the decline of C-peptide levels in the first 8 months after treatment, which indicated some type of preservation regarding β-cell function (30).

4.1.1.2. Cytokine-based therapies

One main strategy for T1D treatment is to target β-cell inflammation, manifested in the form of insulitis, which plays an important role in the pathogenesis of the disease. The imbalance between pro-inflammatory and anti-inflammatory cytokines is one of the main causes that lead to the destruction of β-cells and, as a result, progression and worsening of the disease (Figure 12) (41). Thus, therapies to neutralize the inflammatory environment, inhibiting the expression of Th1-secreted cytokines are necessary (10).

Tumor Necrosis Factor-α (TNF-α):

TNF-α, produced by DCs and MΦ, is elevated in patients with T1D, presenting an important role for the activation of specific naïve T cells, and to promote apoptosis of pancreatic cells (41, 56).

Studies with Etanercept, a recombinant soluble TNF receptor fusion protein, showed a decrease in Hb1Ac levels and an elevation of C-peptide levels, helping to preserve β-cell function, particularly in newly diagnosed children (4, 50). Adalimumab, an anti-TNF mAb, trigger Tregs expansion, by inducing a conformational change in TNF-α receptors (53). Another TNF-α blocker named Golimumab had shown a positive outcome in clinical trials, preserving C-peptide levels after one year of treatment. All the approaches are capable of blocking or antagonizing TNF-α, preventing its activity and leading to a better control of T1D (57).

Interleukin-12/23 (IL-12/23):

Targeting IL-12 and IL-23 is an alternative therapeutic approach, since these cytokines are involved in several immune pathways. Regarding the development of T1D, IL-12 is important in the differentiation of naïve $CD4^+$ T cells into Th1 cells linage, a phenotype responsible for IFN-γ production and stimulation of pathogenic Teff functions. Likewise, IL-23 is committed to support effector functions of some innate immune cells, such as NKT cells and Th17, a subtype of T cells that play a key role in the normal function of the immune system, by providing a pro-inflammatory response (58). Ustekinumab, an anti-IL12/23 mAb, was tested and no conclusive results were obtained (58).

Interleukin-6 (IL-6):

In T1D, an overexpression of IL-6 is related to insulitis (56). Additionally, IL-6 is involved in the development of Th17 effector cells and inhibition of Tregs. A recent clinical trial using Tocilizumab, an anti-IL-6 receptor mAb, revealed that blocking IL-6R had no significant changes in the loss of β-cell function in newly-onset T1D patients during the first-year post-treatment (59).

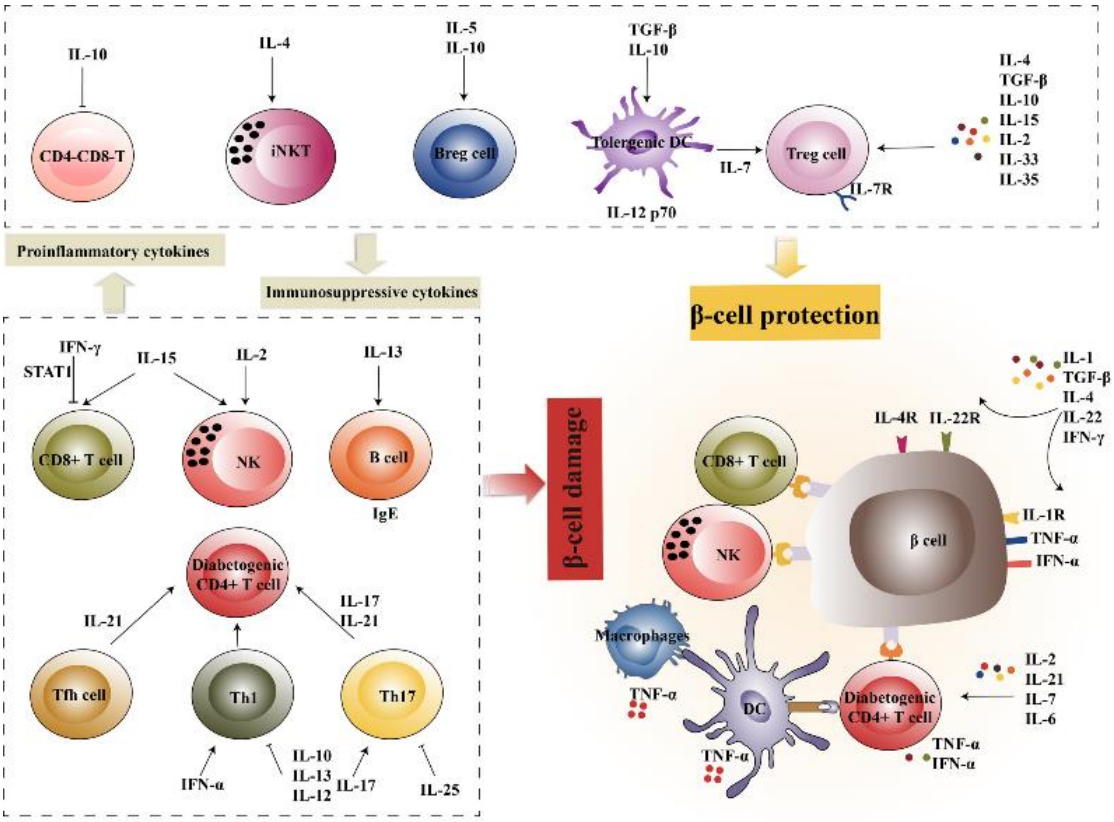

Figure 12. Cytokines involved in β-cell protection and β-cell destruction. Adapted from (56).

Other pro-inflammatory cytokines are related to T1D onset, such as IFN-α, IFN-βγ, IL-21 and IL-7. Targeting these cytokines and, consequently, blocking their functions would be a great approach to consider (40).

4.1.2. Treg mediated therapies

In T1D there is an imbalance between Treg and Teff cells, responsible for the loss of immunotolerance, and, therefore, autoimmunity (39). Due to the role of $CD4^+FOXP3^+$Tregs on peripheral immune regulation, new strategies emerged to promote and restore functional Tregs, in order to reverse the mechanism behind the onset of the disease (36).

Induction of Tregs by IL-2 mediated therapy: IL-2 is a cytokine that either can exert anti-inflammatory or pro-inflammatory functions, depending on the dose at which its presented (50). IL-2, secreted by $CD4^+$ and $CD8^+$ T cells, is one of the most important cytokines to maintain immunotolerance, because it plays a critical role in Tregs development, particularly in its differentiation, function, expansion, and maintenance (12, 27, 36). For this protective function to work properly, an IL2-IL2R (CD25) interaction needs to occur. $CD25^+$ expressed at a higher level on regulatory cells allows them to work as a "IL-2 sink", meaning that a low dose (LD) of IL-2 will lead to a preferential expansion of Tregs, depriving Teff cells from this cytokine that, as for Tregs, is important for their differentiation, proliferation, and activity (36, 49, 56).

Various clinical trials used a low-dose IL-2 treatment in NOD-mice and results showed that administration of LD of IL-2 alone selectively promote Treg cells development and function, and suppression of pathogenic responses (27, 36). They also verified that IL-2 is also effective to prevent differentiation of naïve $CD4^+$T cells into Teff cells (39). This type of

strategy targets growth and differentiation "signal 3", a signal that combined with "signal 1" and "signal 2" is responsible for lymphocyte activation and differentiation (30). Although LD IL-2 administration may be helpful enhancing the protection in T1D patients, due to an expanded pool of Tregs, it is still unclear whether this therapy is effective, as there is a lack of studies in newly diagnosed patients with this disease (56).

Treg-cell transplantation: One of the goals of T1D treatment is to restore Tregs, mainly due to their deficiency in function, but also deficiency in number. Therefore, the manipulation of FOXP3^{+}Treg population *in vivo* by transferring expanded autologous Tregs *in vitro* is a strategy (12).

Adoptive transfer therapy, a cell-based method, increase Tregs number, leading to an improvement in the loss of β-cell mass and to a possible attempt to reverse the autoimmunity (60). As the transfer cannot only be performed by translocating Treg cells into the human body, isolation of polyclonal FOXP3^{+}Treg from peripheral blood, under good manufacturing practice-compliant protocols, is required (53). Afterwards, the expansion of Treg cells *in vitro* occurs, resulting in many polyclonal Treg cells ready to be reinfused into the donor (39). It is noteworthy that one single Treg cell is sufficient to expand the number of them by billions and, consequently, it is possible to achieve a safe therapy capable of suppressive effects, due to the high expression levels of CTLA4, CD25 and FOXP3^{+} (36). Bluestone and colleagues have studied this approach, conducting clinical trials demonstrating the safety and viability of the Treg-cell transplantation, and showing that, one year post transfer, some of the patients needed less exogenous insulin to control the disease, without significant changes in C-peptide or Hb1Ac levels (50, 60, 61).

4.1.3. Gut microbiome manipulation

The gut microbiota consists of a set of microorganisms, such as viruses and bacteria, living in our organism, mainly in our gastrointestinal tract (16). Studies conducted in both animals and humans have demonstrated that defects in the intestinal microbiota are correlated with the onset of T1D (55). These studies also demonstrated that patients with T1D have differences in their intestinal microbiota, with a higher ratio of *Bacteroidetes* to *Firmicutes* (43). Thus, the microbiota is responsible for maintaining the normal function of the immune system, through modulation of the migration and differentiation of immune cells (12). When an imbalance of gut microbes occurs, a processed called dysbiosis, the development of T1D became susceptible (Figure 13). A compromised intestinal permeability and an immune dysfunction had also been reported to the development of T1D (61).

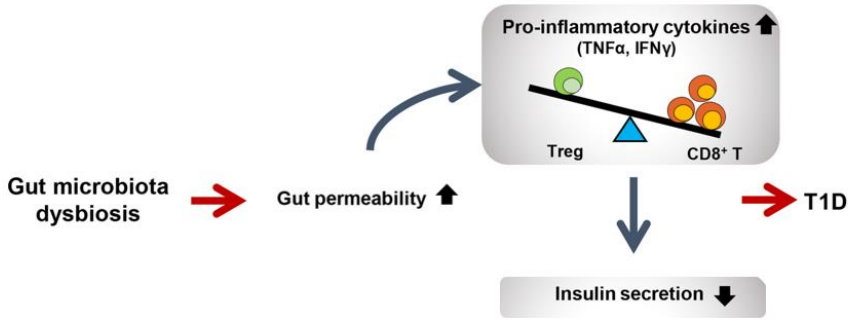

Figure 13. Contribution of dysbiosis for T1D development. Adapted from (55)

Microbiome plays an important role for glucose homeostasis because some of the metabolites generated by the process of fermentation, such as short-chain fatty acids (SCFAs), induce the production of glucagon-like peptide 1 (GLP-1) and, consequently, more insulin. T cells are directly impacted by SCFAs, including acetate, propionate, and butyrate. Diets that stimulate the production of these metabolites by the gut microbiota may manifest a protective effect. For example, acetate reduces the β cell-specific Teff that are present in the pLN (55). Also, presence of butyrate enhances the number of Tregs, also promoting the production of anti-inflammatory cytokine IL-10 and the display of FOXP3 (43).

Thus, it is also important to fully understand the crosstalk between the gut microbiota and the immune system via mechanisms involved in TLR-mediated signaling (55). Several studies using knock out NOD mice for toll-like receptor adaptor protein MyD88 have shown a protective effect, regarding the development of T1D, in pathogen-free conditions. However, this effect is no longer observed in germ-free conditions meaning that the susceptibility for T1D in NOD mice is conditioned by the environment, where microbial colonization has a protective effect (12, 62).

The gut microbiome manipulation could be an alternative and/or an adjuvant therapy, to ensure that immunotolerance is not jeopardized and β-cells function is not completely lost. Alterations in diet and nutrition, but also in personal hygiene, antibiotic use, and probiotic or prebiotic use are important for those effects (43).

Prebiotics are non-digestible carbohydrates which stimulates the growth and activity of bacteria, being independent of the process of colonization. Probiotics are commensal microorganisms that provide a health benefit to host. Several studies have shown that manipulation of gut microbiome is generally beneficial to T1D prevention and to enhance immune responses and insulin production (63). Some probiotics used to modulate the incidence of T1D are shown in Table 1.

Table 1. Summary of some probiotics used in gut microbiota manipulation.

Probiotics	Effects	Reference
Lactobacillus johnsonni N62	This bacterium helped the immune responses because inhibits indoleamine 2,3-dioxygenase (IDO), allowing tryptophan to be more available to DCs, inducing T cell activation (Tregs).	(63, 64)
Lactobacillus rhamnosus* and *Bifidobacterium lactis	This bacterium has an anti-inflammatory effect, inducing regulation of T cells. However, without protective effect since it did not maintain pancreatic β-cell function.	(65)
VSL#3® (includes eight different bacteria, all of them lactic acid producers)	It has a protective and preventive mechanism in mice, due to the increase of IL-10, an anti-inflammatory cytokine that is extremely important for Tregs development.	(62)

4.2. Combinatorial strategies to improve treatment of autoimmune diabetes

Over the years, most of the clinical trials studied one single agent on newly diagnosed patients. Of the clinical trials carried out, a significant number failed to achieve the intended outcomes, namely the induction of long-lasting full remission (66). To address this, and given the better understanding of the disease, studies were conducted to evaluate the combination of immunotherapies and, hence, verifying their efficacy in restoring immunotolerance (4, 50). Thus, a synergism with the beneficial effects of the therapies when used alone is expected (57). Various combinations were tested, including combinations using anti-inflammatory agents, immunomodulatory agents, and even antigen-specific therapies (66). Some combinatorial approaches and their clinical outcomes are presented in Table 2.

Table 2. Summary of combinatorial immunotherapy studied in T1D.

Combinatorial Immunotherapy	Clinical Trial and Outcomes	References
LD ATG and GCSF	LD of antithymocyte globulin (ATG) is responsible for Teff cells depletion, through lysis and apoptosis. LD ATG alone showed positive outcomes in diabetic patients, with preservation of C-peptide levels one year after treatment. Granulocyte colony stimulating factor (GCSF) has an upregulation function, supporting leukocyte recovery and increasing the production of anti-inflammatory cytokines, such as IL-4 and IL-10. A clinical trial conducted on patients with establish T1D (stage 3) with LD ATG/GCSF showed to be safe and presenting some C-peptide preservation. When conducted on patients with newly diagnosed diabetes, no discernible preservation of C-peptide AUC was observed, in comparison to the placebo group. This effect demonstrated that no synergistic effect was obtained from this combination, over the use of LD ATG in monotherapy, that can reduce the level of Hb1Ac and preserve β-cell function.	(50, 60, 67)
Rapamycin and IL-2	IL-2 is a crucial cytokine in T1D, since in lower doses is responsible for promoting Tregs activity, function, maintenance, and expansion, increasing the expression of $FOXP3^+$. All this functions leaded to a preservation of hyperglycemia in a safe way. Rapamycin, in lower doses, can promote Tregs function. When these two strategies were combined in newly diagnosed patients an increased level of $FOXP3^+$Treg cells in blood was detected, similar to those found in IL-2 treatment alone.	(12, 60)
MMF and DZB	Mycophenolate mofetil (MMF) is an immunosuppressant agent used in the prophylaxis of acute transplant rejection. Its inhibition leads to an impairment of T and B cells. Daclizumab (DZD) is an anti-CD25 mAb, acting as an antagonist of the IL-2 receptor expressed on activated T cells. The	(54, 60)

	combination of these two agents was studied. After 2 years, MMF/DZB combination did not prevented loss of C-peptide, believing being the result of DZB's activity, reducing the levels of $CD4^+CD25^+$Tregs.	
IL-21 and GLP-1 agonist (liraglutide)	Il-21 has an important role in T1D progression. This cytokine is able to support the function of Th cells and acts on DCs, due to the presence of its receptor, IL-21R, promoting the production of IL-6. It is also involved in the migration of $CD8^+$ T cells to pancreatic islets. GLP-1 stimulates insulin secretion, suppresses β-cell stress and apoptosis and, therefore, lead to a better glycemic control. A combinatory therapy using a mAb anti-IL-21 and liraglutide, a GLP-1 agonist, revealed a preservation of insulin secretion, by sustaining C-peptide levels.	(56, 68)

5. Nanoimmunotherapeutic approaches for the prevention and treatment of T1D

5.1. Autoantigen-specific therapies

T1D is still a disease without a cure. Despite the continuous optimization of insulin therapy regimens, the administration of this hormone is only effective in the treatment of symptoms, having no effect on the pathology and progression of the disease (69). The daily injection of recombinant human insulin often remains the most accessible therapeutic alternative for patients (70).

Due to the limitations found in the treatment of the disease, it was necessary to change approaches, shifting the focus to prevention. These prevention approaches focus on high-risk individuals, identified by the presence of autoantibodies to the autoantigens in considerable amounts in the serum (69, 71). Through a therapeutic intervention applied in the initial phase of the disease, it may be possible to maintain the function of endogenous β-cells, preserving their residual reservoir from the autoimmune attack. Thus, individuals with high risk to develop T1D are the perfect targets, since β-cells mass is still preserved (69).

All these conditions can be targeted through antigen-specific therapy or commonly named antigen-specific immunotherapy (ASI) (70). This therapeutic strategy targets the presymptomatic stage of T1D (69). This form of therapy consists of β-cell autoantigens administration, which may correspond to the whole antigen or to natural peptide sequences from these antigens (72). Thus, the identification of the main autoantigens driving T1D is extremely important, since knowing them enables the selection of the appropriate and most relevant autoantigen(s) for the construction of antigen-dependent immunotherapies. This parameter gains even more relevance, considering that there are numerous autoantigens present in the serum of diabetic patients (73).

Because the main goal is to achieve selective immunotolerance, ASI adopts "inverse vaccination" instead of conventional vaccination (70, 74). Vaccination is an easy, secure and reliable method of preventing harmful diseases and infections by exposing the immune system

to a dead or weakened form of microorganisms, such as viruses and bacteria, boosting the body's natural defenses and immune system against those particular infectious agents. Inverse vaccination has an opposite objective where, through presentation of β-cell autoantigens, an inhibition of certain immune responses is expected, leading to an induction of immunological tolerance (70).

This technology could be extremely beneficial in the management of autoimmune diseases (73). ASI has been proven particularly interesting, with potential of offering full prevention of onset or progression of T1D (75). Additionally, this strategy shows to be safer than other forms of treatment based on modulation or suppression of the immune system, named systemic immunomodulating therapies, such as anti-CD3 antibodies (e.g., teplizumab), immunosuppressive drugs (e.g., cyclosporine) or anti-CD20 (e.g., rituximab), that may require repeated administrations and may lead to significant side effects (70, 73).

Therefore, ASI is a more specific immunosuppressive therapy, with a lower risk to change acquired immunity and aiming to induce peripheral immunological tolerance, while avoiding off-target effects spotted in antigen-independent therapies (75). Although its use may have a disadvantage compared to traditional immunosuppressive strategies, a lower potency, remains one of the most studied and sought-after strategies (41).

Based on the administration of β-cell autoantigens, this therapy should be able to restore immune homeostasis and, therefore, immunotolerance by DCs mediated mechanisms (70, 71), including: induction or augmentation/expansion of autoantigen-specific Tregs, improving immunological response by an active tolerance mechanism; deletion, anergy and/or exhaustion of pathogenic T cells, removing harmful islet-specific effector reactions by a passive tolerance mechanism; and differentiation of naïve β cell-specific T cell into aTregs, namely for the anti-inflammatory phenotype Th2 (12, 41, 70).

Thus, a balance of T cell population between Treg cells and Teff cells is expected to be achieved striving to selectively tolerize the pathogenic pool and to expand the regulatory pool. This method centers essentially around the enhanced expression of self-antigen-specific aTregs, where a "bystander suppression" is present (12, 57). A "bystander suppression" implies an unusually intensive suppression of cells exceeding the normal mechanisms of downregulation, such as Teff, or a cellular inhibition by direct cell-to-cell contact or soluble products, such as short-range cytokines (76). The release of IL-10 and TGF-β by $CD4^+$aTreg cells leads to a disruption of diabetogenic T cells function or development, downregulating the activity of pro-inflammatory APC (4, 57). On this wise, it is fundamental that Tregs have the ability to inactivate self-reactive T cells in the periphery that escaped negative selection in the thymus, competing with them for APC, and to induce an anti-inflammatory environment. Besides, it is noteworthy that administration of different autoantigens can lead to different effects (73).

The use of "inverse vaccination" was initially based on the administration of whole autoantigens or multiples peptides from the same autoantigen, as they correspond to the most immunogenic portions (70, 73).

Insulin is an autoantigen largely used in clinical trials for the prevention of T1D (70). Nonetheless, other single peptide vaccines were formulated based on GAD and Hsp60. The effect of the combination of multiple peptides from two different autoantigens is being studied

and investigated in phase one clinical trials, consisting of a different and novel approach that needs to be further explored (73).

5.1.1. Antigen identification and selection

The autoantigen identification and selection, or portion of it, is one of the most crucial factors to ensure the efficacy of the usage of "inverse vaccination," even though there are other factors that also affect the outcome of this approach, such as dose, frequency, and route of administration (12, 77).

This step has become a huge challenge not only because there is a difficulty to identify all autoantigens during the disease progression, but also due to the presence of neoepitopes which can vary between patients (73, 78). Vaccines can be formulated with an antigen as a whole or with portions of it thought to be more immunogenic and, therefore, responsible for the disease onset. To date, insulin autoantibodies (IAA), GAD, ZnT8, IA-2, and Hsp60 are some examples of antigens used in clinical trials to determine their safety and effectiveness in preventing T1D (4). The antigen selection is not random, being necessary a check-up of some criteria, such as their involvement in T1D pathogenesis and whether it is relevant or not; how the autoantigen is presented to the immune system, by their specific MHC/HLA complex, and how strongly the immune system manifestly responds to that self-antigen (73). Thus, it is essential to identify the specific self-antigens to what T lymphocytes responds with greater affinity so that, through an effective intervention by choosing the right antigen, the autoimmune response against pancreatic β-cells can be stopped (79). The main vaccine-based strategies using peptide immunotherapy to modulate the pathogenic responses are summarized in Table 3.

Table 3. Main non-nanoparticle-based vaccines studied in clinical trials and important discoveries.

Antigen	Clinical Trials Description	Findings and major outcomes	References
Insulin Main β-cell antigen, with specific-autoantibodies found in newly diabetic patients. Pointed as the initiating autoantigen in TID and considered the major autoantigen, since it is the hormone responsible for the glucose homeostasis.	**NCT00004984** Prevention Trial-Type 1 diabetes (DTP-1) consists in a randomized trial, where oral and parenteral insulin were studied. Participants received insulin capsules daily or subcutaneous (SC) injections of insulin twice a day.	After a four-year follow-up, no protective effect was verified. No significant differences in insulin C-peptide levels were shown. Only in a specific subgroup with high levels of anti-insulin autoantibodies, a positive outcome was observed.	(69, 70, 80)
	NCT02547519 The Pre-POINT randomized clinical trial	The treatment seemed to induce insulin and pro-insulin responsive-Tregs cells and IL-21-expressing	(80, 81)

	studied the effects of high doses of oral insulin in children at high risk for T1D.	$CD4^+$ T cells. This cytokine stimulates B cell responses to autoantigens.	
Most studied natural peptide: derived from proinsulin peptide, C19-A3, and insulin B chain 9-23 epitope. **C19-A3 Proinsulin peptide** Proinsulin is the precursor of insulin, and it is detected in considerable amounts in diabetic patients.	An initial phase I study was conducted to determine the effect of intradermal (ID) C19-A3 injections in newly diagnosed patients.	The treatment was safe and well tolerated. It was detected an increase of $FOXP3^+$ expression, no accelerated declined of C-peptide levels and a higher IL-10 production. Therefore, these can be encouraging results for the development of more studies regarding peptide immunotherapy.	(81-83)
Insulin B chain 9-23 epitope	**NCT00873561** In this study, the outcome from the SC administration of altered peptide ligand B9-23 was evaluated in adolescents and adults.	The initial results were promising, since a conversion of the pathogenic response, predominantly IFN-γ mediated, to a regulatory response was seen (Th2 phenotype detected). However, there was no clinical benefit associated with the preservation of β-cell function.	(12, 81, 84)
GAD65 GAD enzyme converts glutamate into GABA, a neurotransmitter not only involved in the central nervous system, but also in the regulation of pancreatic hormone release, through the GAD65 isoform.	**NCT01122446** The effect of GAD65-alum (Diamyd) SC administration in children with multiple islet cell autoantibodies was studied for five years. Aluminum hydroxide is used as adjuvant, inducing IL-4-secreting Th2 cells.	An increase in GAD-specific Treg cells and a decreased in GAD-specific Teff cells were detected. The effectiveness of the treatment depends on the duration of T1D at the time the vaccine is given, showing that C-peptide levels were better preserved in patients treated within 6 months after diagnosis, than those treated after 30 months.	(12, 41, 85)
Hsp	**NCT00615264** Efficacy of DiaPep277 SC	Treatment showed Th1 to Th2 phenotype conversion and, consequently, a	(41, 86)

Hsp are chaperones that get triggered under stress conditions, in order to protect proteins from potential damaging. Peptide p277 was identified as the most strongly immunogenic epitope.	administration in newly diagnosed T1D patients were reported in this study. DiaPep277 is a stabilized version of p277.	preservation of C-peptide levels. However, these effect does not occur in young patients, where a decline of C-peptide levels is present.	

5.2. Nanoparticle-based vaccines for T1D

Over time, it became essential to find alternatives that would address some of the issues and challenges that ASI had raised, including concerns about the progression of T1D, induction of hypersensitivity and "off-target" autoimmunity (77). To this extent, nanotechnology, and the use of NP as antigen-carriers started being used due to its higher and improved efficacy (87, 88). NP, particles whose size range from 1 to 100 nm, have some chemical and physical properties as strong immunomodulatory agents, described in Table 4 (89). Microparticles (MP), whose size range from 1 to 1000 μm, also function as NC (90).

Table 4. Features promoting nanoparticles as drug delivery systems.

NP properties		**References**
Easy manipulation of particle size and surface properties	Increased stability – protection against enzymatic degradation	(91)
Versatile control release properties – precise cell targeting and delivery, increasing drug's half-life and a lower frequency of administration	Increased solubility – carriers to poorly soluble drugs	
Controlled release and distribution of drugs – higher efficiency and fewer adverse effects	Different routes of administration – oral, parenteral, nasal, etc	

Vaccines for T1D can work through several mechanisms, but the focus is always either to prevent the onset of T1D or to restore the previously lost immunotolerance, without affecting others immune system processes (31, 92).

These vaccines' mechanisms generally entail switching from a harmful (Th1) to a benign and unharmful (Th2) immune response, modifying effector T cell function to a more antigen-specific regulatory phenotype or by deleting/tolerizing Teff cells or limiting immune cell contact, for example through MHC-TCR interaction (93). The autoantigens used in these nanovaccines are the identical ones responsible for T1D development. However, when these autoantigens are delivered under specific circumstances, such as non-inflammatory conditions, they might have the reverse effect, encouraging the regulation of Teff (7).

Traditional vaccines present one or more antigens, which usually are proteins or peptides poorly immunogenic, and an adjuvant, to induce an adequate immune response (94). In this case "inverse" vaccines, unlike the traditional ones, resort to a nanoscale strategy where autoantigens are delivered in nano-sized formulations, based on different biomaterials such as

polymers, lipids, and inorganic metals (7). The precise *in vivo* delivery of autoantigens should be able to activate alternative signals 1, 2 or 3 that inhibit or inactivates immune cells. Such tolerogenic vaccines often consist in customized-antigen delivered, without any adjuvants or stimulatory signals associated, which can target innate immunity, such as DCs, or adaptive immunity, including B cells and T cells (94, 95). The focus on the delivery of autoantigens to DCs via NP is related with the peripheral tolerance mechanisms naturally mediated by this cell type and the attempt to fight malignant self-reactive T cells (31). It is also possible to alter adaptive immune responses, by anergy or deletion of Teff or by Tregs differentiation and expansion (Figure 14) (95, 96). The mindset should focus on reducing the effector and diabetogenic characteristics of immune cells and/or inducing a more tolerogenic and benign phenotype, preferably ensuring that NP are biodegradable and that nanovaccines are both safe and affordable, for patients to have access to these treatment innovations.

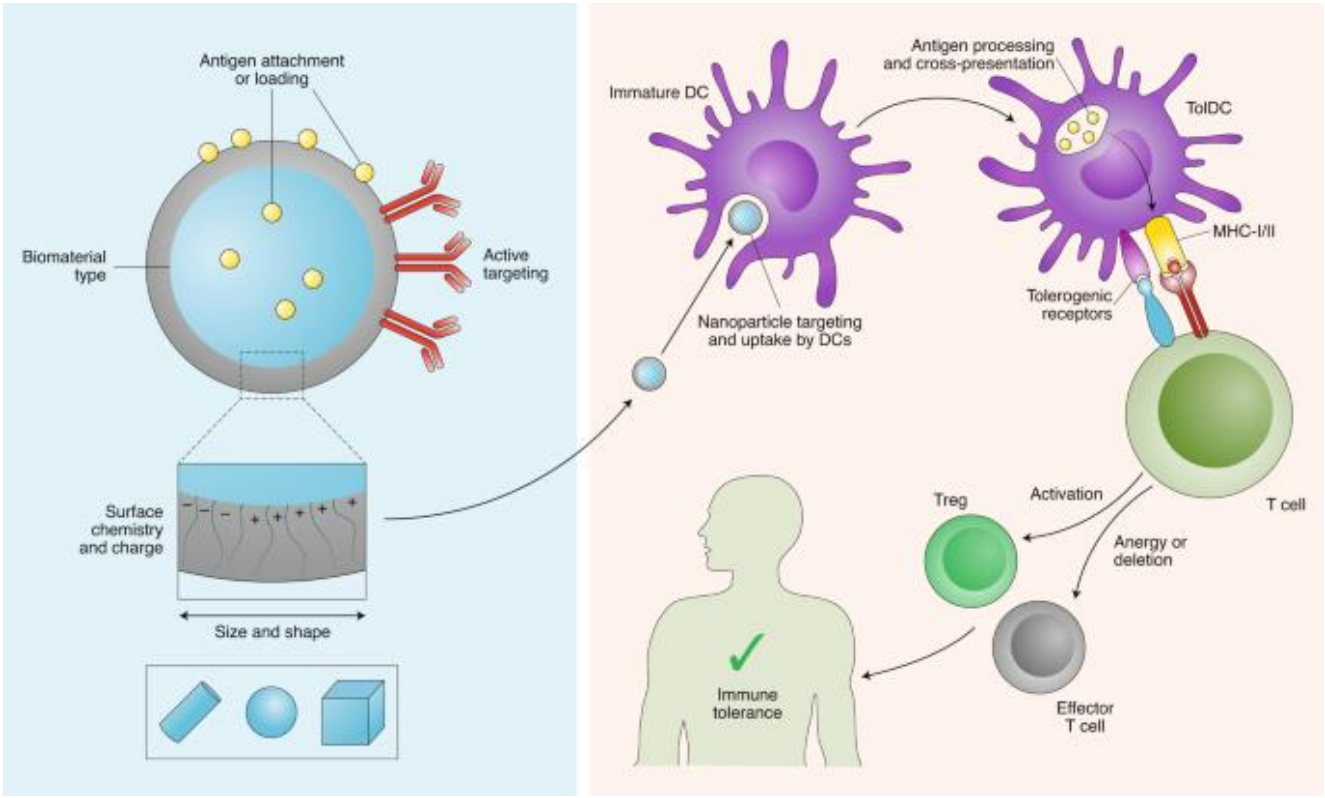

Figure 14. Immunological outcomes of inverse vaccination: anergy/deletion of autoreactive T cells or development of Treg phenotype. Adapted from (97).

5.2.1. Design of engineering nanoparticles to overcome autoimmunity and induce T cell tolerance in T1D

5.2.1.1. Biomaterial based-nanosystems

Nanomedicine has been used to overcome the drawback of existing T1D therapy, such as insulin administration as conventional vaccines (98). Nano- and microparticles have been described as a great alternative to restore and promote immunotolerance against autoimmune diseases (78). The selection of the biomaterials used is judicious, and it is necessary to ensure three important requirements: biocompatibility, non-toxicity, and the ability to easily manipulate NP physical and chemical properties (97).

Nanoformulations can offer the possibility to co-deliver multiple autoantigens, as a vehicle, while providing a better controlled release kinetics and protection of the cargo against enzymatic and pH degradation, also increasing cargo stability and solubility (94, 95, 99). Furthermore, NP can have their surface modified, with specific receptors ligands, for targeting specificity and, consequently, attenuation of adverse systemic effects (78, 90). Moreover, the control of their physical size and shape and surface charge, allows different interactions between immune cells and different organs and tissues distribution (95, 100).

These nanosystems can be based of organic materials, including lipid-based and polymeric NP, and inorganic materials, such as metal and metal oxide NP (96, 99). All biomaterials have distinct properties. Some of them have tolerogenic characteristics and, therefore, are beneficial for inducing tolerance (101). Thus, the selection of the right biomaterial is fundamental for the development of inverse vaccines.

Lipid-based nanovaccines: Lipid-based nanovaccines mainly include liposomal nanovaccines. Liposomes are small spherical vesicles, composed of cholesterol and phospholipids bilayers with hydrophilic and hydrophobic character (102, 103). Liposomes are one of the most used NC, not only because they can encapsulate both hydrophobic and hydrophilic cargoes within the membrane and aqueous core respectively, but also due to their biocompatibility, non-toxicity, biodegradability, and lower immunogenicity (98, 99, 104). Through encapsulation, liposomes can protect one or more autoantigens from degradation, increasing the cargo stability as well as cargo solubility (105).

One major advantage of these lipid NP is the flexibility of formulation. Thus, lipid NP surface charge, size, lipid composition and fluidity, and surface targeting ligands can be modulated to obtain an active targeting, with less toxic systemic effects and overall better outcomes (98, 103). However, liposomes also present some disadvantages that need to be considered, such as poor stability, since they can go through conglomeration, fusion sedimentation, oxidation, and phospholipid hydrolysis-like reaction, also presenting vulnerability to leakage of loaded autoantigens and high production and sterilization costs (102, 105).

Polymeric nanovaccines: Polymeric nanovaccines can consist of either natural, e.g., chitosan, or synthetic polymers e.g., polyethylene glycol (PEG) and poly(lactic-co-glycolic) acid (PLGA) (94). With a size range from 10 to 1000 nm, these colloidal systems are often non-toxic, biodegradable, and non-immunogenic (96, 103). Similarly to liposomes, it is possible to modulate their structure and function (104). They also present other properties that allow them

to be frequently employed as NC, including the ease of preparation, controlled release kinetics by polymer degradation, ease of surface functionalization and increased stability in biological fluids (98, 106).

Autoantigens are usually encapsulated in nanocapsules or nanospheres, which allows cargo protection against degradation and an improvement in cargo bioavailability (99, 105). There are numerous polymers available for the development of nanovaccines, PLGA being the most popular, due to its biodegradability, biocompatibility, ability to encapsulate various molecules and immunosuppressive degradation products (96, 97). Polymeric NP presents high production costs and a low shelf-life (105). Moreover, they present an unpredictable stability, which can cause some degree of toxicity (104).

Inorganic nanovaccines: Other biomaterial nanosystems are metal and nonmetal inorganic NP. These types of particles gained particular interest when they began to be used in diagnosis and therapies, namely for bioimaging and drug delivery. Inorganic particles are biocompatible, hydrophilic, and present a very high safety profile (107). Similar to other NP, these nanosystems exhibit an ease surface functionalization, for fine-tune cellular responses (97). Variation in the size and shape of the particles can lead to alterations in the same cellular responses. Silica, carbon, gold, and iron oxide are some inorganic NP examples. Of these, gold nanoparticles (AuNP) are most involved in T1D nanovaccines development (98). AuNP exhibit immunosuppressive properties, being able to diminish hyperglycemia in animal models without systemic toxic side effects (105). It has been also reported that AuNP may act as size- and shape-dependent adjuvants, potentially stimulating the immune system and improving antigen recognition (104). Despite having several favorable characteristics for application in nanotechnology, AuNP face a significant obstacle: non-biodegradability, which can result in toxicity (96).

5.2.1.2. Strategies to enhance efficacy of nanovaccines in T1D

While engineering nanovaccines, it is crucial to consider intrinsic features of NP, since these properties determine the recognition and uptake of vaccines by APC, affecting their interaction with the immune system and, therefore, the outcomes reached (92). These inherent properties include size, shape, and composition of NC, charge, hydrophobicity, particle coating, and even NP stiffness and fluidity. The route of administration is another parameter that must be considered (Figure 15) (96).

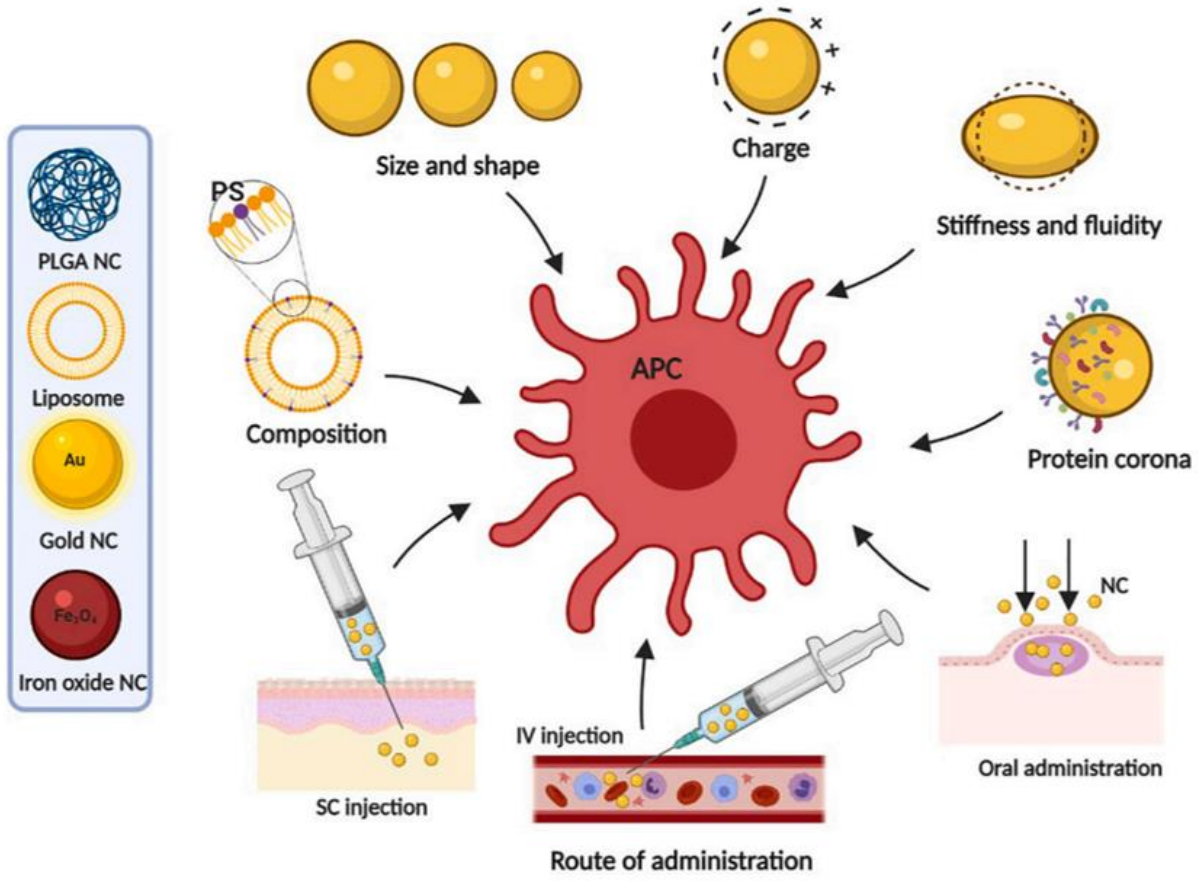

Figure 15. NP properties affecting recognition and uptake of vaccines by APC. Adapted from (96).

5.2.1.2.1. Modification of Physicochemical properties of NP

Size: size is one of the most critical features of NP, as it influences their bioavailability and cellular absorption process (96). For a faster and enhanced immune response, NP size should enable a direct delivery of autoantigens to the APC, preferably to lymph-node-resident DCs (97). When a modulation in the NP size occurs, a modulation in autoantigen trafficking kinetics could happen. It was found that small NP (diameter <100-200nm), especially the ultra-small NP (diameter ≤25nm) drain directly and more rapidly to lymph nodes, presenting autoantigens to lymph-node-resident DCs within hours of ID administration. Contrarily, larger NP (diameter >200 nm) present a higher difficulty to reach the lymphatic system (92, 94). These particles either remain at the injection site or are phagocytosed and transported to lymph nodes by DCs, presumable in the blood, liver, spleen, or injection site (figure 16) (97). Hence, it is

verified that DCs carrying smaller NP can activate T cells easier and more rapidly, generally leading to a more potent immune response compared to the one obtained with DCs carrying larger NP. NP size also affects their distribution, site of accumulation and the cargo loaded (12, 92, 96). Larger particles can, as expected, carry a higher amount of cargo, which may translate into a more effective restoration of immunotolerance (97). Distribution within the spleen and liver was noted for NP <50-100nm, which can be directly drained to lymph nodes whereas the larger ones usually stay within extracellular matrix before being carried to lymph nodes (78, 94). Smaller NP (<15nm) suffer an accumulation in the kidneys and lungs (94).

Shape: alongside with size, NP shape can equally modify the interaction with APC, since it can influence how the autoantigens are displayed to immune cells (92). Different studies have attained different conclusions, with no consensus having been reached on the ideal shape that NP should present. The impact of the NP shape on the immunological response was, in fact, well evidenced in a study carried out by Niikura and other investigators, where spherical, rod-shape and cubic AuNP, owning the same surface characteristics, were explored. By the end of the study, different profiles of cytokine production were observed, with rod-shape being the one that most induced a Th1 immune response (97). This indicated the possibility of DCs being able to differentiate NP based on their different shapes, possibly displaying the autoantigens differently from shape to shape. There are numerous shapes that NP can adopt, such as cylindrical, cubical, spherical, and ellipsoidal (108). Some authors defend that, to activate APC easier, by direct autoantigen presentation through NP, a spherical shape is preferable rather than a rod-shape (96). Other studies have pointed out spherical NP as being more likely to be phagocytized by MΦ, when compared to ellipsoidal NP and, therefore, the spherical shape being the most suitable to promote a favorable immune response. However, contradictory outcomes were obtained while studying ellipsoidal and spherical PLGA NP (99). This property is considered responsible for initiating the phagocytosis process since, when changing NP shape, a change usually occurs in the autoantigen presentation to APC (96).

Composition: it is important to design NP capable of being recognized by APC. This can be achieved by changing the composition of the NP, using apoptotic signal molecules, also consider immunomodulatory molecules (31). One of the molecules used is phosphatidylserine (PS), that has the capability to enhance the NP uptake by APC, thus granting a tolerogenic autoantigen presentation (96). It is also possible to alter NP cargo, introducing adjuvants (98). Similarly to PS, adjuvants are immunomodulators that improve, accelerate, and extend the specific immune response to vaccine autoantigens, thereby enhancing vaccines potency through several mechanisms. Other benefits are expected, such as an improvement of the immunogenicity by weaker autoantigens and less autoantigen required for a successful immunization. Aluminum hydroxide was one of the adjuvants used in T1D approaches, in combination with GAD65. The therapy was safe and showed some positive results.

5.2.1.2.2. Modification of NP surface and charge

Charge: in addiction to size, shape and composition, charge of NP is an important factor to determine their distribution, clearance, and interaction with the immune system, mainly affecting their uptake by APC (96, 104). Regarding NP charge and their influence on endocytosis process, its noteworthy that no clear and exact conclusion was yet reached (100). Most of studies showed that cationic NP are more effective in inducing a Th1 response, being more extensively taken by APC, when in comparison with neutral and negative NC (92). Conversely, other studies have demonstrated that the anionic charge is the charge preferable for an efficient uptake by APC (100). The Th1 response achieved while using cationic NP is a result of their extended and preferable cellular internalization by DCs and their innate propensity to regulate positive costimulatory ligands (96). These positively charge NP are also highly immunogenic, exhibiting toxic properties (92, 97). For the anionic NP it was observed a lower rate of endocytosis, meaning that these particles are less prone to suffer that process (figure 16). Surface charge also influences NP distribution, with negative particles being easily drained to the lymph nodes within negatively charged extracellular matrix, where repulsion acts as driving force. Contrastingly, positive particles reveal sedimentation characteristics, forming a depot that is removed either by a direct but slow drainage to lymph nodes or by APC in circulation (31). Precise and careful NP distribution to specific organs and tissues is only achievable when a balance between charge, size, and other inherent features of the NP is present.

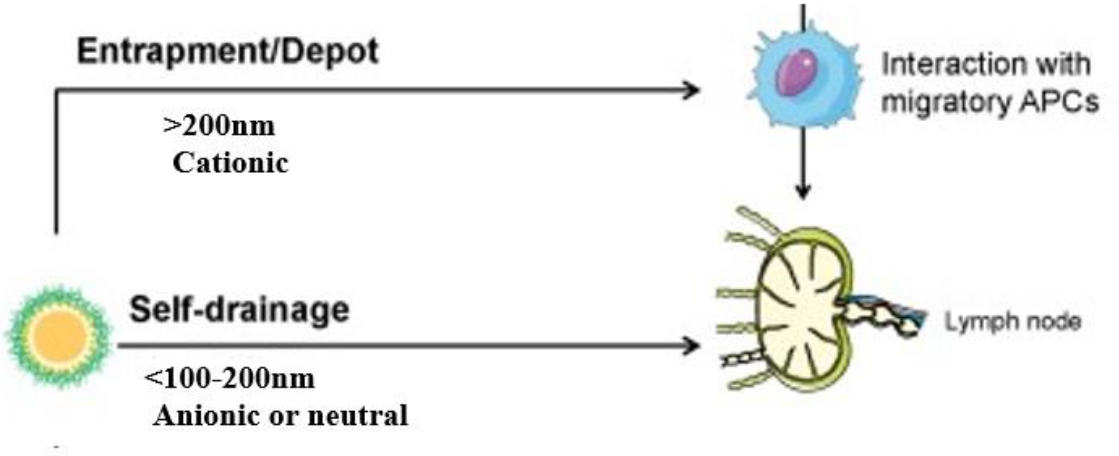

Figure 16. Influence of size and surface charge of NP on their distribution after administration. Adapted from (31).

NP surface ligands: the surface of NP is commonly functionalized by attaching specific ligands or functional groups, to improve targeting towards tissues, cells or even organs (95). This is possible, considering a key feature of NP: the easy manipulation of surface properties. It has been determined that the major target of T1D nanovaccines is DCs and to achieve a better and more effective response of nanotherapy, it is essential to understand and recognize the surface markers of different DCs phenotypes (97). Knowing these receptors enable the understanding and knowledge of DCs specific targeting ligands, becoming possible to decorate NP with them. This leads to a higher uptake of autoantigens by DCs, since NP have their surface functionalized to target these specific cells and their receptors (98). One of the strategies include

the decoration of NP surface with antibodies against specific DCs receptors, including anti-CD11c, anti-DEC-205 antibodies, and mannose (78, 94). The mannose receptor is not only expressed on the surface of DCs, but is also present on MΦ surface, suggesting that these cells can also be targeted (100). An investigation was carried out to study the effect of anti-DEC-205 Ab conjugated with autoantigens. Positive results were obtained, including the induction of T immunotolerance, by the conversion of $CD4^+$Teff cells into Treg cells (5). Another strategy that is generally used is to coat NP with T1D-relevant peptide-MHC class I/II complex, since MHC molecules are responsible for autoantigen presentation to T cells via TCR (78). Iron oxide NP decorated with autoantigen-MHC complexes function as a tolerogenic artificial APC, targeting not DCs, as the therapies previously mentioned, but T cells, ending up promoting differentiation of $CD4^+$Teff cells into Tr1-cells, a regulatory T cell subset (12, 94). Another alternative is to coat NP with anti-$CD4^+$ and anti-$CD8^+$ antibodies, responsible for recognizing surface T receptors (94).

5.2.1.2.3. Impact of different routes for administration of nanovaccines

The route of administration affects not only the distribution of the NP, but also the efficacy of nanovaccines (90). To successfully modify the immune system responses, nanovaccines must target and reach specific organs, such as lymph nodes, liver, and spleen, which are crucial for the development of tolerance to self-antigens (96). Thus, the success of nanovaccines is conditioned by the ease of access to immune cells, which is increased when using mucosal routes, including oral, nasal, and vaginal administration. Through these pathways, NC can activate both mucosal and systemic immune systems, since they are able to reach mucosal-associated lymphoid tissues (MALT), but also lymph nodes, where draining DCs carrying the NP could modulate T cells and suppress malignant immune responses (31). Despite the direct access to lymphoid tissues conceived by mucosal administration, parental delivery continues to be preferred in clinical practice, including ID, intramuscular (IM), and SC injections (92). The path taken by NP to reach the lymphatic system can differ depending on the physicochemical features of the NC, particularly their size and charge. Succeeding SC, IM, or ID administration, smaller NP can drain directly to the nearest lymph node, without any help from the APC. Conversely, larger ones generally remain at the injection site, requiring uptake and transportation by APC to reach the lymphatic system (Figure 17) (31).

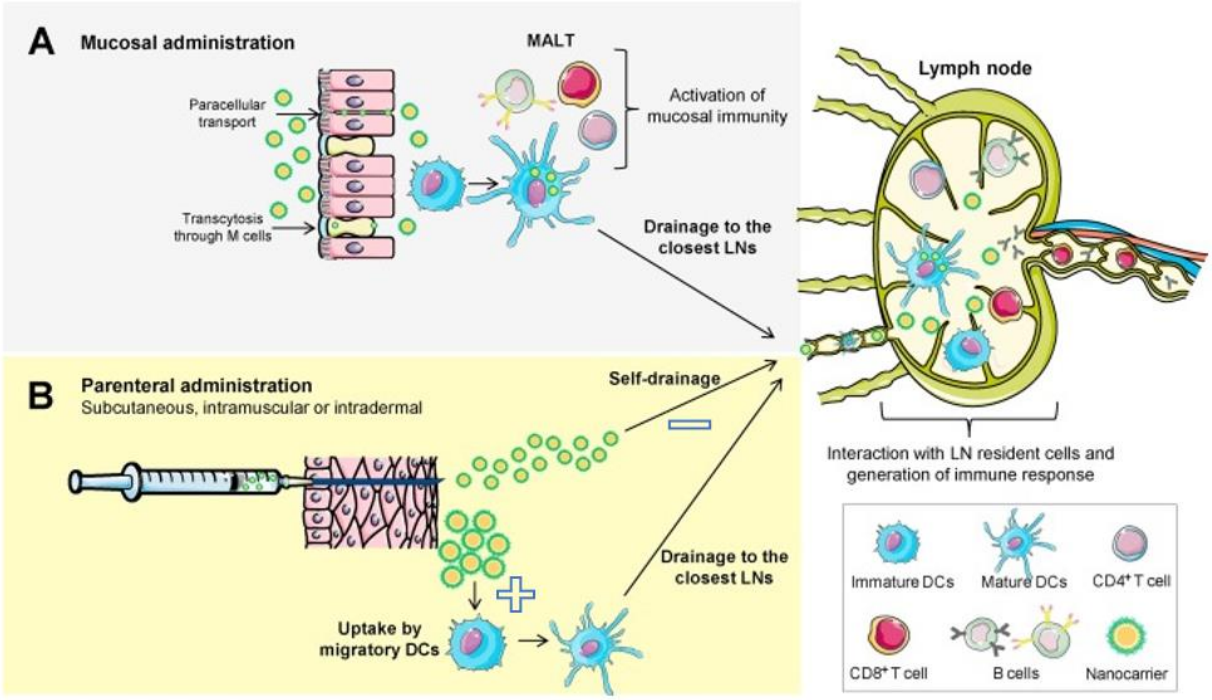

Figure 17. Different paths taken by NP depending on the vaccination route. Adapted from (31).

Intravenous (IV) administration resulted in NP accumulation, also depending on the NC physicochemical features (92). Larger NP are more retained in the spleen and liver, while smaller ones are mainly accumulated in the kidneys and lungs (94). A study demonstrated that after IV administration, if NP are accumulated in the liver and subsequently captured by Kupffer cells, a tolerogenic effect can be achieved, since this type of MΦ express higher levels of PD-L1 at their surfaces, which function as a "protective signal" against autoimmune attacks by binding with PD-1 present on T cells (31).

5.2.2. Pre-clinical nanovaccines for T1D treatment and prevention

pMHC-coated iron oxide nanoparticles: The use of NP coupled with disease-related peptides bound to major histocompatibility complex is an approach that has been researched over the years (80). These peptide-MHC complexes (pMHC) are extremely relevant, since naïve T cells require a MHC-TCR interaction to become active (87). In a first approach Tsai and other researchers studied the effect of IV mono-specific pMHC class I-coated iron oxide NP administration into prediabetic NOD mice, where a considerable expansion of autoregulatory $CD8^+$T cells was observed (80). This outcome reflected positive effects, including the restoration of normoglycemia in newly diagnosed diabetic animals, while also preventing the onset of the disease in prediabetic mice, considering that pMHC-coated iron oxide NP expanded $CD8^+$T cells were able to suppress antigen presentation to other autoreactive T cells, by APC-killing mechanisms, including IFN-γ, IDO and perforin (87, 96). In another study, developed by Santamaria's research team, islet-specific peptide glucose-6-phosphatase catalytic subunit-related protein (IGRP) MHC class II-coated iron oxide NP systemic delivery were investigated (100). It was reported a proliferation of disease-specific regulatory $CD4^+$T type 1 (Tr1)-like cells, a distinct subset of Tregs that secretes high amounts of IL-10 and TGF- β, while lacking $FOXP3^+$ expression, which allowed to restore normal blood

sugar levels in spontaneously diabetic mice (5, 109). These cells exert an immunomodulatory role, by downregulating the expression of co-stimulatory molecules and pro-inflammatory cytokines by APC, being also responsible for the expansion of regulatory B cells, capable of reducing the production of pro-inflammatory cytokine and suppressing Teff proliferation in an IL-10-dependent manner. This leads to a reversal of diabetes in NOD mice (Figure 18) (4, 110).

In this therapy, nanovaccine acts as an artificial APC, binding directly to the TCR without APC interaction. These artificial APC act in the absence of co-stimulation signals, known as signals 2, promoting T1D prevention (94).

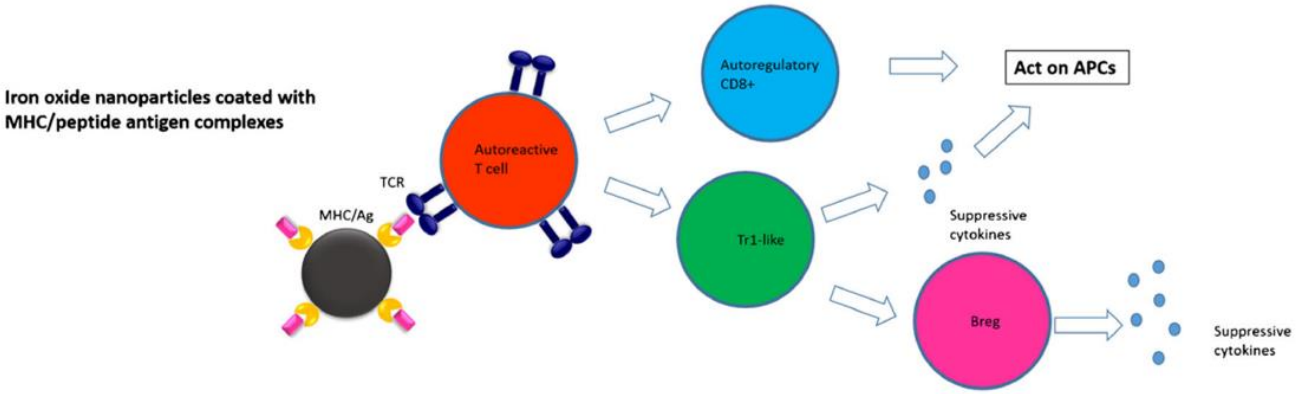

Figure 18. Iron oxide NP acting as tolerogenic artificial APC. Adapted from (92).

PEG-coated gold nanoparticles: Other studies were carried out in NOD mouse investigating the effect of an IV or intraperitoneal gold-based nanovaccine administration (96). These gold particles co-delivered a tolerogenic molecule, an aryl hydrocarbon receptor (AhR) ligand 2-(1´H-indole-3´-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE), and a disease-specific autoantigen, proinsulin, both attached to NP surface (88). Additionally, NP were coated with PEG in an attempt to improve their stability and solubility and, consequently, increase the effectiveness of the treatment (87). As a result of weekly intraperitoneal injections into 8-week-old NOD mice, a differentiation of DCs into a tolerogenic phenotype, due to AhR activation, was observed, with lower levels of surface molecules (CD40, CD80 and MHCII) and pro-inflammatory cytokines, but a higher secretion of anti-inflammatory ones (27, 94). The change in DC phenotype promoted $FOXP3^+$ gene expression and, therefore, $FOXP3^+CD4^+$Tregs differentiation and expansion, while decreasing the number of Th1 and Th17 effector cells activated in pLN, allowing T1D prevention (88, 96). These alterations were observed, since an internalization of the NP by the major APC, DCs, were distinguish (Figure 19). Holding anti-inflammatory properties, AuNP are commonly used due to their easy surface functionalization and size manipulation (7, 88). More recently, Dual et al, conducted a study where gold NP coated with proinsulin $P_{IC19\text{-}A3}$ were administered by a microneedle delivery system, into human skin (87). *In vitro* studies showed that DCs "affected" by this nanovaccine had a lower capacity to activate naïve T cells and, therefore, a greater protective capacity (88).

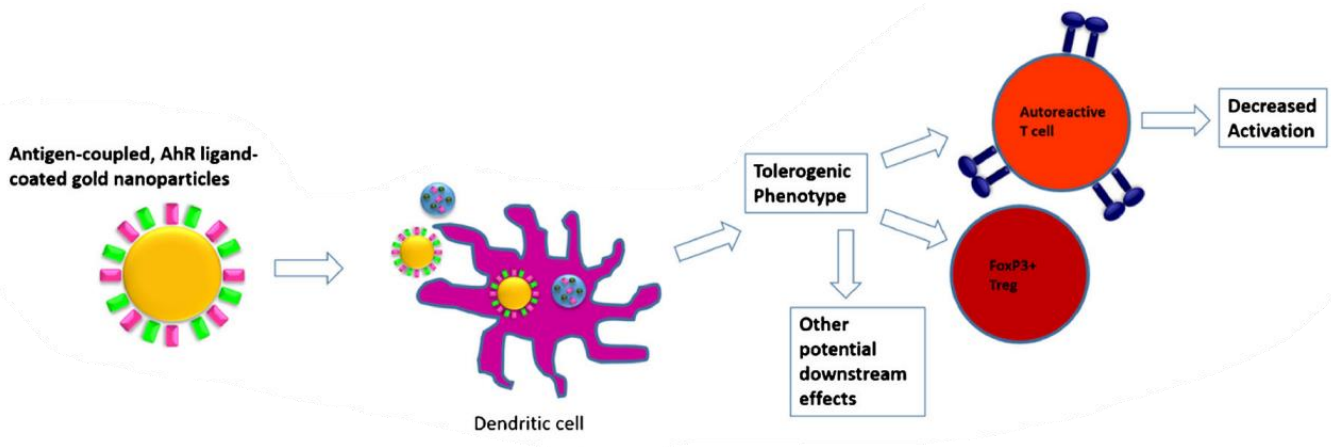

Figure 19. Protective mechanism of PEG-coated AuNP: induction of tolerogenic DCs. Adapted from (92).

PLGA-nanoparticles: Another alternative treatment is based on the use of biodegradable polymeric NP PLGA. One of the approaches consisted of SC injection of a two-sized PLGA MP formulation. Delivered in large non-phagocytosable MP, which presented 30 µm in diameter, were anti-inflammatory cytokines, including TGF-β and granulocyte-macrophage colony-stimulating factor (GM-CSF) (7, 31, 97). The presence of these chemokines at the injection site turned the microenvironment tolerogenic which, in turn, enabled the recruitment of DCs and differentiation into protective phenotypes. At the same time, phagocytosable MP loaded with vitamin D3 (calcitriol) and autoantigen insulin B9-23, whose size range from 0,5 µm to 2,5 µm, were also delivered (31, 94). Since the non-phagocytosable MP attracts APC to the site, the phagocytosis of the smaller MP becomes possible. Once in the DCs, the autoantigen is presented in a tolerogenic manner to the lymphocytes, resulting in a prevention and temporary reversal effect of T1D in NOD mice (94). However, these positive outcomes were not seen when the administration occurred individually. It is also important to highlight Vitamin D3, that is fundamental to the success of this nanotherapy. Being a protolerogenic agent, Vitamin D3 can differentiate DCs into tolDCs, a phenotype that holds suppressive properties, considering their production of anti-inflammatory cytokines and low expression of co-stimulatory molecules (111).

A different approach used PLGA NP formulations with BDC2.5 mimotope 1040-31 (p31) and/or NY8.3 mimotope (NRP-A7), known diabetogenic peptides. Coupled to or encapsulated within PLGA NP, these formulations showed positives results, such as induction of Treg cells and downstream regulation of Teff cells, with an inhibition of autoreactive $CD8^+$ T cells (112). This strategy significantly improved APC uptake and increased the level of anti-inflammatory cytokines and the expression of negative co-stimulatory molecules, such as PD-1 and CTLA-4 in Tregs, leading to an increased immunotolerance. Thus, the restoration of immunotolerance occurs in a PD-1 and CTLA-4 dependent manner, with an expansion of Tregs able to overcome the effect of autoreactive T cells (78).

Keselowsky and other researchers used a peptide hydrogel that co-delivered GM-CSF, oligodeoxynucleotides CpG and denatured insulin within 1,5 µm PLGA MP (87). A delay in the onset of the disease, from 12 to 19 weeks, was observed after administering this vaccine in

SC dosages to NOD mice. Additionally, a rise in the survival rate of 40% was noted, as a preventive effect was attained by an enhanced IL-10 production (5).

Liposomes: PS liposomes loaded with insulin A and B peptides were given intraperitoneally. PS, usually present in apoptotic cells, works as an immunosuppressive signal, promoting liposomes phagocytosis by DCs (97, 113, 114). Thus, by mimicking apoptotic β-cells, it is possible to induce tolerogenic DCs in a safe way (87, 105). This tolerogenic behavior indicate that DCs may operate to silence potential autoreactive T cells and, consequently, reduce the damage caused by these cells (113).

In another study, related to nanoparticle-based vaccines development, rapamycin, an immunomodulator, and p31 were encapsulated within 0.7 μm acetalated dextran MP. Results showed a preventive effect marked by a decline in $CD4^+$ T cell proliferative ability and an increase in $FOXP3^+$/IFN-γ T cell ratio (94). Moreover, an oral vaccine composed by chitosan particles loaded with heat shock protein 65–6xP227 was developed (96). Results showed a preventive effect, with expansion of Treg cells and a decrease of Th1 cells in the pLN (90).

Even though it is not yet commonly employed, nanoparticle-based gene therapy is an alternative to consider. In this strategy, NP are use as NC to plasmids encoding T1D-related genes. Investigators developed a nanoscale system where poly-(amino-butylated)-1-glycolic acid (PAGA) NP were loaded with chimeric plasmids encoding to anti-inflammatory cytokines, dallying inflammation of the islets of Langerhans (87). The administration of immunomodulatory genetic material, such as antisense oligonucleotides against CD40, CD80 and CD86, encapsulated in PLGA NP, is also an alternative therapeutic strategy for T1D (94). During this treatment, DCs acquired a protective phenotype, with decreased CD40, CD80 and CD86 expression, enabling expansion of Treg cells (115). This approach does not use autoantigens, whereby not belonging to “inverse” vaccines group.

6. Ongoing clinical trials of nano and immunotherapeutic approaches in T1D

Clinical trials are studies conducted in humans that provide the most robust scientific information about a particular drug, device, or treatment. Trial results can guide investigators in the right path even when studies do not deliver the outcomes anticipated (70). For this reason, the development of more clinical trials is necessary, not only to better understand the mechanisms of T1D pathogenesis, but also for a better comprehension of the existing therapies, including nanovaccines and immunotherapeutic approaches, and to discover and develop new ones.

Regarding antigen-independent immunotherapies, there are some clinical trials that remain active and the results on hold. For example, a clinical trial studying the impact of ATG, Adalimumab (anti-TNF mAb) and LD IL-2 administration is now ongoing (116). The main objective of the study is to evaluate C-peptide levels, which translate endogenous insulin secretion, and Tregs blood levels during 1 year treatment. Over a period of 52 weeks, newly diagnosed diabetic patients with a range age from 18 to 35 years and undergoing insulin therapy receive ATG, Adalimumab and Aldesleukin (IL-2) subcutaneously, all in different doses and frequency. Study results are expected in 2026.

Another randomized clinical trial is being studied, to prevent the onset of T1D in at-risk individuals (117). In this study, participants receive Abatacept (CTL4-Ig) given as 30-minute IV infusion for 1 year, to determine whether using this drug will prevent or delay the onset abnormal glucose tolerance. To date, the results have not yet been released.

There is another study involving Abatacept, in which T1D at-risk individuals, aged 8 years and older, receive Rituximab (anti-CD20 mAb) given by IV infusion over a 3-8hour period for 3 months, followed by Abatacept, given subcutaneously for 2 years (118). The primary purpose is to prevent T1D. To verify that this is achieved, several parameters are evaluated throughout the study, including C-peptide levels, insulin production and glucose tolerance status. Results are expected by 2026.

Related to nanotechnology, there is an ongoing clinical trial, where the formulation of Proinsulin C19-A3 AuNP is being evaluated (118). Proinsulin C19-A3 AuNPs is administered intradermally every 28 days for 8 weeks. Participants are diabetic patients, on insulin treatment and aged between 18 and 40 years old. To determine whether the treatment is having any effect on modulating T cell responses and restoring immunotolerance, an assessment of IFN-γ blood levels after nanovaccine administration is required.

One promising therapy, non-nanotechnology related, involves NNC0361-0041 drug, which consists of recombinant plasmid encoding four proteins: TGF-β, IL-10, IL-2, and pre-proinsulin (119). TGF-β and IL-2 are necessary in the formation of Tregs. TGF- β also acts, along with IL-10, as an anti-inflammatory cytokine. The ongoing clinical trial was developed, since an efficacy improvement was seen, measured by an almost total hyperglycemia prevention, when co-expressing these immune modulators in NOD mice models (120). Diabetic participants, aged 18 to 45 years, receive NNC0361-0041 subcutaneously, in an effort to treat the disease. Throughout the study, C-peptide concentration-time curve needs to be traced, allowing to verify if the therapy is being effective in reaching its goal. Other ongoing clinical trial study the effect of an experimental drug, IMCY-0098, a modified peptide developed to induce immune cells, such as $CD4^+$ T cells, to target and attack specifically other immune cells involved in β-cell destruction (121). Diabetic patients, aged 18 to 44 years, receive either a low or high dose of IMCY-0098 subcutaneously, evaluating C-peptide response and hoping that it will translate into the treatment of T1D, the main purpose of this study. Results are expected by 2024.

7. Conclusion

T1D is a common disease that still does not have a cure. At the time of diagnosis, it is typically too late to reverse the pathology and the damage caused, since about 80% of β-cell mass is already dysfunctional or destroyed. Thus, the identification of early-stage immunological biomarkers has become crucial, as it allows an early intervention at a preclinical stage and, consequently, the possible reversal of the disease and prevention of its progression. Most of the therapies studied aim to restore immunotolerance, through the expansion of Treg cells and/or exhaustion/anergy of Teff cells. Due to this dysregulation of immune cells, immunotherapy has emerged as the most promising strategy for a preventive therapy. Clinical trials focused on antigen-independent immunotherapies, demonstrated not to achieve the expected goals, despite some positive results regarding, for example, C-peptide levels. To overcome this limitation, autoantigen-specific therapies began to emerge, resorting to nanotechnology. The use of NP along with autoantigens has shown great potential, since by

altering their properties, such as size, charge, shape and/or composition, it is possible to modulate the generated immune response, making it more specific and with fewer adverse effects. Nonetheless, the advancement in the development of these tolerogenic vaccines has been slow and difficult, mainly because the mechanism of action that leads to immunotolerance restoration is not yet fully known and understood. Thus, there is still a long way to go. There is also a variety of factors that need to be considered, such as production on industrial scale, requirement of investment and large expenditures in health, balance between the risk/benefit ratio, and knowledge about the biomarkers that allow the measurement of therapies effectiveness that, until now, are still poorly understood.

Despite limitations, the impact of nanotechnology on T1D therapies has increased, verifying that NP are, in fact, the most suitable platforms for the construction of inverse vaccines and the best strategy to overcome immunotolerance loss. Thus, combining nanotechnology with immunotherapy for T1D prevention and treatment has great potential.

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