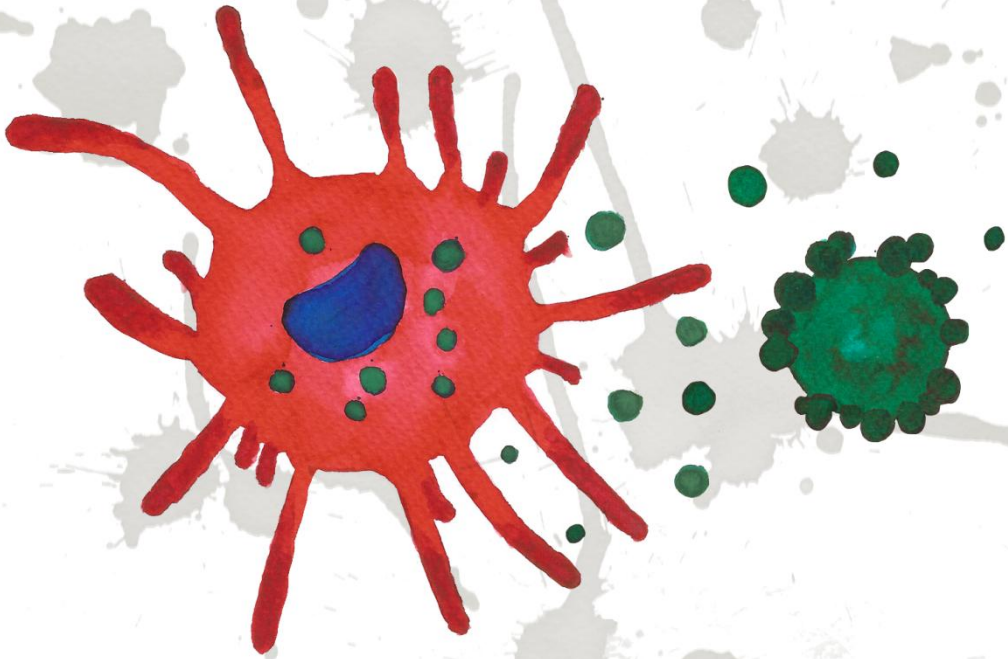
APOPTOSIS-BASED IMMUNOTHERAPY FOR TYPE 1 DIABETES:

FROM DENDRITIC CELLS TO ANTIGEN-SPECIFIC LIPOSOMES



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APOPTOSIS-BASED IMMUNOTHERAPY FOR TYPE 1 DIABETES:

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Certifica:

Que el treball experimental i la redacció de la memoria de la Tesi Doctoral 'Apoptosis-based immunotherapy for type 1 diabetes: from dendritic cells to antigen-specific liposomes' han estat realitzats per Irma Pujol Autonell sota la seva direcció i considera que és apta per ser presentada per optar al grau de Doctor en Immunologia per la Universitat Autònoma de Barcelona.

I per tal que en quedi constancia, signa aquest document a Badalona, el 28 de maig del 2014.

Dra. Marta Vives Pi

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Als meus pares,
Manel i Roser

ABBREVIATIONS

AIRE	autoimmune regulator
ALS	anti-lymphocyte serum
APCs	antigen presenting cells
Apo	apoptotic
ATG	anti-thymocyte globulin
BB	bio-breeding
BM	bone marrow
CBA	cytometric bead array
CD	cluster differentiation
cDCs	conventional dendritic cells
CFSE	carboxyfluorescein succinimidyl ester
CH	cholesterol
CsA	cyclosporin A
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated protein 4
DAMPs	damage associated molecular patterns
DCs	dendritic cells
DNA	deoxyribonucleic acid
DZB	daclizumab
EAE	experimental autoimmune encephalomyelitis
eTACs	extrathymic Aire-expressing cells
GAD	glutamic acid decarboxylase
GLUT2	glucose transporter 2
GM-CSF	granulocyte macrophage colony-stimulating factor
HLA	human leukocyte antigen
Hsp60	heat shock protein 60
IA-2	associated protein 2
IAA	insulin autoantibodies
ICAM	intercellular adhesion molecule
IDO	indolamine 2,3 dioxygenase

IFN	Interferon
IGRP	islet-specific glucose-6-phosphatase
IL	interleukin
IPA	ingenuity pathway analysis
INS	insulin
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
MSCs	mesenchymal stem cells
mTECs	medullary thymic epithelial cells
NITApo-DCs	dendritic cells pulsed with NIT-1 apoptotic cells
NKs	natural killer cells
NKTs	natural killer T cells
NOD	non-obese diabetic
PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffer saline
PC	phosphatidylcholine
pDCs	plasmacytoid dendritic cells
PEG	poly ethylene glycol
PGE_2	prostaglandin E_2
PRRs	pattern recognition receptors
PS	phosphatidylserine
PTA	peripheral tissue antigens
RIP	rat inulin promoter
RA	retinoic acid
RNA	ribonucleic acid
SPF	specific pathogen-free
STZ	streptozotocin
SVApo-DCs	dendritic cells pulsed with SV-T2 apoptotic cells
T1D	type 1 diabetes
TCR	T cell receptor
TGF	transforming growth factor
Th	T helper

TLRs	toll-like receptors
TNF	tumour necrosis factor
tolDC	tolerogenic DCs
Tregs	regulatory T cells
TSLP	thymic stromal lymphopoietin
VNTR	variable number of tandem repeat

INDEX

SUMMARY

Type 1 diabetes (T1D) is a metabolic disease that results from the autoimmune attack against insulin-producing β-cells in the pancreatic islets of Langerhans. Currently, there is no treatment to restore endogenous insulin secretion in patients with T1D, so the development of new therapies to induce long-term tolerance has been an important medical health challenge. An ideal immunotherapy should inhibit the autoimmune attack, avoiding systemic side effects and allowing islet regeneration to improve β-cell function. There are physiological mechanisms that contribute to the maintenance of immune tolerance. On one hand, apoptotic cells are a source of autoantigens that induce tolerance after its removal by antigen presenting cells through a process called efferocytosis. On the other, dendritic cells (DCs) are powerful antigen presenting cells capable of maintaining immunological tolerance. Based on these two traits of the immune system, we hypothesized that tolerogenic features acquired by antigen presenting cells after efferocytosis can help to restore tolerance lost in autoimmunity. The main goal of this study was to generate an antigen-specific immunotherapy based on the inherent tolerogenic properties of apoptosis to reestablish tolerance to β-cells in T1D.

To that aim, we generated a cell-based immunotherapy with DCs loaded with apoptotic β-cells. After the uptake of apoptotic β-cells, DCs displayed a molecular switch to a tolerogenic phenotype, low secretion of proinflammatory cytokine and impaired ability to stimulate autologous T cell proliferation even after proinflammatory stimuli, thus demonstrating their stability. Moreover, efferocytosis promoted suppressive ability in DCs, mediated at least in part by prostaglandin E_2. The administration of the DC-based immunotherapy to the gold standard animal model for T1D, the non-obese diabetic (NOD) mouse, impaired the progress of insulitis, resulting in the prevention of the disease. However, it was not able to reverse the disease in overtly diabetic in mice.

Due to the difficulties in obtaining and standardizing β-cell apoptotic cells, we designed a synthetic strategy, based on liposomal microparticles. Liposomes are

vesicles composed of a lipid bilayer with a hydrophilic center. A central event on the surface of apoptotic cells is the exposure of phosphatidylserine (PS), which provide the main signal for efferocytosis. Therefore, PS-liposomes loaded with insulin peptides -as autoantigens- were generated to simulate the PS recognition of apoptotic cells by antigen presenting cells. These liposomes were phagocyted by DCs resulting in tolerance induction and impairing autoreactive T cell proliferation, in a similar way than the DC-based immunotherapy. In addition, this synthetic immunotherapy arrested the autoimmune aggression, reduced the severity of insulitis and prevented T1D in NOD mice.

The fact that efferocytosis contributes to the maintenance of self-tolerance led us to design a cell therapy and later, to encapsulate β-cell autoantigens in liposomes to induce specific immunosuppression. Apoptotic mimicry using liposomes can offer a solution to the complexity of cell-therapy with design benefits like low-cost and easy to standardize, large-scale production and customization. Although further research is needed, the clinical relevance of the herein proposed immunotherapies could prove to be very important, as it has translational potential in situations that require the reestablishment of immunological tolerance, such as autoimmune diseases.

RESUM

La diabetis tipus 1 (DT1) és una malaltia metabòlica causada per la destrucció selectiva de les cèl·lules β pancreàtiques productores d'insulina per part del sistema immunitari. Actualment no hi ha cura ni prevenció per restaurar la secreció endògena d'insulina als pacients amb DT1. Per tant, un important repte biomèdic és el desenvolupament de noves teràpies per recuperar la tolerància immunològica. Una immunoteràpia ideal hauria d'inhibir la resposta autoimmunitària, sense efectes sistèmics adversos, i permetre la regeneració de les cèl·lules insulars per tal de recuperar la massa β. Hi ha mecanismes fisiològics que contribueixen a mantenir la tolerància immunològica. D'una banda, les cèl·lules apoptòtiques són una important font d'autoantígens que indueixen tolerància després de ser fagocitades per les cèl·lules presentadores d'antigen a través d'un procés anomenat eferocitosi. D'altra banda, les cèl·lules dendrítiques (CDs) són potents presentadores d'antigen amb capacitat de mantenir la tolerància immunològica. Basant-nos en aquestes dues característiques de la resposta immunitària, la hipòtesi del present treball és que les característiques tolerogèniques adquirides per les cèl·lules presentadores d'antigen després de l'eferocitosi poden ajudar a recuperar la tolerància perduda durant l'autoimmunitat. El principal objectiu ha estat generar una immunoteràpia antigen-específica basada en les propietats tolerogèniques inherents a l'apoptosi, per tal de restablir la tolerància a les cèl·lules β en la diabetis autoimmunitària.

Amb aquesta finalitat, hem generat una immunoteràpia cel·lular amb CDs que havien capturat cèl·lules β apoptòtiques. Després de l'eferocitosi, les CDs van mostrar un canvi de patró molecular cap a un fenotip tolerogènic i una baixa producció de citocines proinflamatòries, mostrant també una disminució de la seva capacitat d'estimular la proliferació de cèl·lules T autòlogues. Això es va observar fins i tot després d'un estímul proinflamatori, fet que recolza l'estabilitat d'aquestes CDs tolerogèniques. A més, l'eferocitosi va promoure funció supresora en les CDs, un fenomen mediat -al menys en part- per la prostaglandina E_2. L'administració d'aquesta immunoteràpia al model més utilitzat de DT1, el ratolí

no-obès diabètic (NOD), va aturar la progressió de la insulitis i va prevenir la diabetis. Malgrat això, no va ser possible revertir la malaltia en ratolins diabètics.

Degut a la dificultat en obtenir i estandarditzar les cèl·lules β apoptòtiques, vam dissenyar una estratègia sintètica, basada en les micropartícules liposomals. Els liposomes són vesícules formades per una bicapa lipídica amb un nucli hidrofílic. Una característica clau en el reconeixement de les cèl·lules apoptòtiques és l'exposició a la membrana de la molècula fosfatidilserina (FS), que genera el principal senyal de reconeixement per l'eferocitosi. Per tant, vam generar FS-liposomes amb pèptids d'insulina a l'interior -com a autoantígens-, per tal de simular el reconeixement de les cèl·lules apoptòtiques per part de les cèl·lules presentadores d'antigen. Aquests liposomes van ser fagocitats per les CDs, resultant en la inducció de tolerància i disminuint la capacitat d'induir proliferació en els limfòcits T autoreactius, de manera similar a la teràpia cel·lular de CDs. A més, aquesta immunoteràpia sintètica va ser capaç d'aturar l'atac autoimmunitari, reduint la severitat del infiltrat insular i prevenint la DT1 en ratolins NOD.

Les característiques tolerogèniques de l'apoptosi ens van portar a dissenyar una teràpia cel·lular i posteriorment, a encapsular autoantígens en liposomes per induir una immunosupressió específica. El mimetisme de l'apoptosi utilitzant liposomes pot oferir una solució a la complexitat de la teràpia cel·lular, amb avantatges en quant a baix cost i la possibilitat de producció a gran escala, estandardització i personalització. Malgrat cal aprofundir molt en aquest camp, l'aportació al camp clínic de les immunoteràpies presentades en aquest treball pot ser molt important, donat el seu potencial translacional en situacions que requereixen el restabliment de la tolerància immunològica, com ara les malalties autoimmunitàries.

INTRODUCTION

INTRODUCTION

1. TYPE 1 DIABETES

Type 1 diabetes (T1D) is a metabolic disease that results from the autoimmune attack against insulin-producing β-cells in the islets of Langerhans of the pancreas. The pancreas is a glandular organ situated in the upper left abdomen, below the stomach and surrounded by the duodenum. Adult pancreas is composed by two main tissues: exocrine and endocrine. Exocrine tissue is arranged in clusters called acini that produce bicarbonate ions and digestive enzymes. Endocrine tissue is organized in small clusters of cells called islets of Langerhans. The islets, taken together, can be thought of as a single organ occupying 1% of the pancreas weight [1]. Each islet contains on average a total of 1,000 cells [2] that secrete hormones related to the glucose homeostasis: β-cells (65–80%) produce insulin and amylin. α-cells (15–20% of total islet cells) secrete the hormone glucagon in response to low glucose levels. δ-cells (3–10%) secrete the hormone somatostatin, which inhibits insulin and glucagon secretion. PP-cells (3–5%) produce pancreatic polypeptide, which appears to inhibit exocrine secretions. ε-cells (<1%) secrete ghrelin, which seems to inhibit insulin release [3]. There are two major forms of T1D: type 1A results from an autoimmune attack on β-cells, whereas type 1B, named idiopathic, has an unknown cause [4]. From this point onward, type 1A diabetes will be referred to as T1D, as most of the literature does.

T1D etiology is unidentified, but it is known that disease appears in genetically susceptible individuals after environmental triggers, which affect β-cell autoimmunity. The onset of the disease is preceded by a long asymptomatic period named prediabetes. During this prediabetic stage, islet reactive autoantibodies arise in the sera and autoreactive T cells start to destroy β-cells, resulting in a progressive loss in insulin secretory function. Clinical onset of T1D occurs when 80% - 90% of the β-cells have been destroyed **(Figure 1)**.

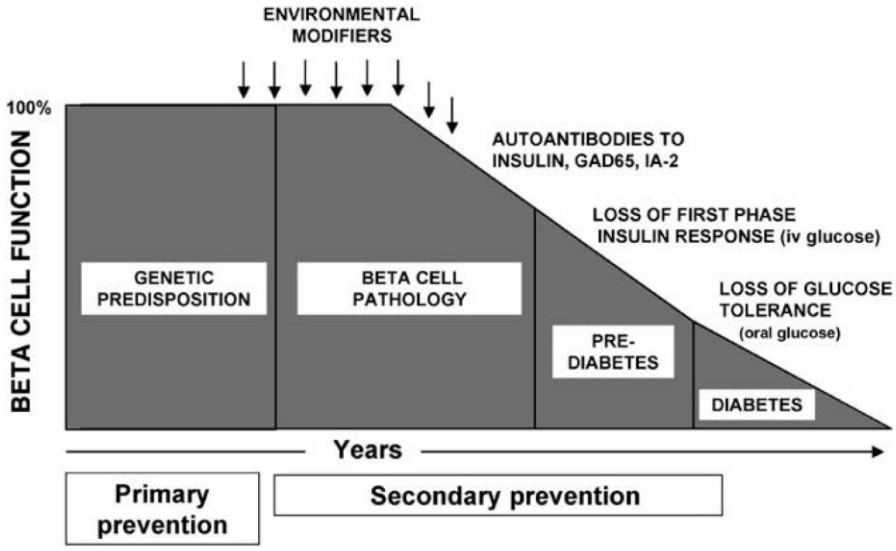

Figure 1. Natural history of T1D. Adapted from LC Harrison, 2008, Immunol and Cell Biol [5].

Both the American Diabetes Association, as well as the World Health Organization defined the criteria for the diagnosis of T1D [6, 7]. Clinical symptoms of T1D are polyuria, polydipsia, polyphagia, weight loss, along with hyperglycemia, requiring the immediate need for exogenous insulin administration. Recurrent infections and impairment of growth may also accompany hyperglycemia. However, not all those symptoms are always present, complicating the diagnosis of this disease, especially in infants. At present, there is no cure or prevention for T1D. Since the discovery of insulin at the beginning of the 20th century, treatment of T1D is based on lifelong insulin therapy to keep blood glucose levels as normal as possible and to prevent health problems developing later in life, leading to vascular damage associated with kidney failure, heart diseases, retinopathy and neuropathy. Pancreas or pancreatic islets transplantation were performed in very few T1D patients to restore endogenous insulin secretion [8, 9]. However, there are many limiting factors for the general implementation of islet transplantation: the need for immunosuppressive drugs after transplant, limited availability of donor pancreases for islet isolation, high costs and complicated islet isolation procedures.

1.1. Epidemiology

T1D is one of the most common metabolic diseases in childhood and its incidence is increasing in developing countries. T1D incidence has a marked geographic variability, being higher in some Central and Eastern European and North America countries and limited or almost non-existent in sub-Saharan Africa [10]. Within Europe, the highest rates of childhood diabetes are found in north-west Europe, with Finland the highest (56.7 new cases per 100,000 inhabitants per year), with a clear north-south gradient [11]. T1D incidence in Spain is about 20.6 new cases per 100.000 inhabitants per year. In contrast, India and China have relatively low incidence rates for diabetes (0.1 per 100,000 inhabitants and year). In addition, T1D has been increasing throughout the world at 3% per year in the last decades [12]. The peak age for diagnosis of T1D is at 12-14 years, but the average age of T1D onset has decreased in the past two decades and second peak at 4-6 years has been observed [13]. However, T1D can also be diagnosed in adults [14]. In general terms, there is not a gender bias in human T1D, although it remains controversial [15, 16].

1.2. Etiology

T1D is a multifactorial disease with an unknown etiology. However, it is known that genetic and environmental factors influence the disease. Concordance between identical twins is close to 40%, while concordance between siblings with same HLA is about 15%. The rate of diabetes among siblings is 6%, compared to 0.5% in the general population. Moreover, 15% of T1D patients have relatives with diabetes [17]. Nevertheless, T1D concordance in monozygotic twins is less than 50%, providing strong evidence that environmental factors also influence susceptibility to the disease.

1.2.1. Genetic factors

T1D is a polygenic disease. More than 50 loci of susceptibility to T1D have been identified in humans, defined as IDDM, and in mice, defined as *Idd*. New T1D

susceptibility regions can be continuously identified through association studies (T1Dbase, www.t1dbase.org), but the most established susceptibility locus are to be mentioned next. Current genetic data indicate that highest contribution (~50%) of the genetic predisposition in humans is due to polymorphisms in genes of the human leukocyte antigen (**HLA**) **class II** region (IDDM1) in chromosome 6p21 [18]. HLA class II genes encode molecules related to antigen presentation; among them HLA-DRB1, HLA-DQA1 and HLA-DQB1 genes have been associated to T1D. The haplotype DR3-DQ2 (DRB1*03-DQA1-DQB1*0201) and DR4-DQ8 (DRB1*04-DQB1*0302) confers susceptibility to T1D, while the haplotype HLA-DR2 (DRB1*1501-DQB1*0602) confers protection to T1D. The **HLA class I** region is also associated to T1D, even if it is less prominent. The HLA-B*39 allele was found to be a risk factor associated with a lower age at diagnosis of T1D. Additionally, HLA-A*02 increases the risk in individuals possessing the high-risk class II DR3/4-DQ8 haplotype [19]. Another locus associated to the disease is the **insulin** (ISN) gene region (IDDM2) in chromosome 11p15.5, which susceptibility resides in a variable number of tandem repeat (VNTR) pholymorphisms in the promoter region. It controls the expression of the insulin gene in the thymus by affecting the transcription factor AIRE (autoimmune regulator) binding to its promoter region [20]. Other immune-related genes associated to T1D include **PTPN22** gene, in chromosome 1p13, which encodes the lymphoid protein tyrosine phosphatise (LYP), the gene encoding cytotoxic T lymphocyte-associated protein 4 (**CTLA-4**) in the 2q33 region (IDDM12), the allelic variation in the interleukin (IL)-2 receptor-α gene (**IL2RA**) in the 10p15 region, the gene encoding the interferon-induced helicase 1 (**IFIH1**) in the 2q24.2 region (IDDM19) and the gene **CYP27B1** on 12q13, which encodes for the 1a-hydroxylase, which is involved in the production of active vitamin D are other immune-related genes associated to T1D [18]. Currently, new T1D susceptibility regions can be identified through association studies (T1Dbase, www.t1dbase.org). In addition, environmental factors may alter gene expression via epigenetic mechanisms. This is pertinent to T1D in which DNA methylation and histone modifications have been associated with altered gene expression [21]. Epigenetic mechanisms can influence not only the immune system, but also beta cell physiology, homeostasis, and regeneration [22].

1.2.2. Environmental factors

Epidemiologic studies have provided strong evidences to involve environmental factors in the etiology of T1D. The incidence of T1D is increasing so sharply in developed countries that cannot be explained by genetic changes among generations and has to be attributed to environmental changes [23, 24]. Moreover, seasonal changes influence the incidence of the disease, which is higher in autumn and winter and lower in summer [25]. In this sense, it was observed that families from Pakistan, an area of low T1D incidence, when migrating to the United Kingdom, developed T1D that reached similar incidence as people in their host country [23]. Numerous environmental factors have been associated with this disease; however, no single one has been confirmed as the cause.

Viral infections have been associated to the etiology of T1D [26]. Despite there is no direct evidence for a particular viral strain being causative, enteroviruses, and more specifically coxsackieviruses, are viral candidates to trigger T1D [27]. In this sense, it has been observed that enteroviral infections are frequent in children positive for antibodies anti-islet [28]. Moreover, specific IgM response against coxsackie B virus was found in recently diagnosed T1D children from England and Austria [29]. Another evidence for the viral infection hypothesis is the presence of type I IFNs -antiviral cytokines- in pancreas from diabetic patients [30, 31]. Among the suggested mechanisms, molecular mimicry is one of those. It has been described crossreactivity to autoantigen glutamic acid decarboxylase (GAD) and viruses such as coxsackievirus [32], cytomegalovirus [33] or rubella virus [34]. Other proposed mechanisms are the epitope spreading after virus infection, the "bystander" damage caused by proinflammatory cytokines released during viral infection [35] or the "fertile field hypothesis", which postulates that the inflammation induced by viral infection creates the optimum conditions to develop T1D only in susceptible individuals [36].

Diet is another environmental factor that can be related to the etiology of T1D [19]. **Cow's milk** has been proposed as a possible contributor to T1D, due to cross-

reactivity between the albumin component of the milk and ICA-1 (p69), a β-cell surface protein [37]. Also, early introduction of gluten-containing foods (before 3 months of age) can be a risk factor for the development of T1D [38]. In fact, antibodies anti-gliadin were reported at the onset of T1D in children [39]. However, a recent study showed that breastfeeding at the time of wheat or barley introduction is protective against T1D [40].

Vitamin D has been related to T1D with a protective role. On one hand, higher T1D incidence inversely correlated with the ultraviolet irradiance in hours of sunshine [41]. On the other, low levels of vitamin D were detected in T1D patients [42] and other autoimmune diseases. The proposed mechanism is that the biologically active form of vitamin D (1,25-dihydroxyvitamin, $1.25(OH)_2D3$) is a potent modulator of the immune system [43].

Data from animal and human studies link alterations in the **intestinal microbiome** with T1D. Microbiome studies suggest that an aberrant intestinal microbiota, as well as an increased permeability of the intestinal barrier and altered intestinal immune responsiveness form the "perfect storm" for the development of T1D [44]. In the most used spontaneous model of T1D, the non-obese diabetic (NOD) mice, the interaction of the intestinal microbiota with the innate immune system is critical in T1D predisposition. The knock out NOD mice for MyD88 protein -i.e. an adaptor for multiple innate immune receptors that recognize microbial structures- did not develop T1D in specific pathogen-free (SPF) conditions, but it did in germ-free conditions [45].

In the last decades, infectious diseases have been reduced in industrialized countries by the use of antibiotics and vaccines, but also by the increased hygiene and better socioeconomic conditions. The "**hygiene hypothesis**" proposes that a lack of early childhood exposure to infectious agents increases susceptibility to allergic and autoimmune diseases, by suppressing the natural development and maturation of immune system, and leading to defects in the establishment of immune tolerance [46]. Epidemiological comparisons between Finland and the

neighboring Karelian Republic of Russia support the “hygiene hypothesis” [47]. These neighboring populations are genetically similar, but live in different socioeconomic circumstances. After collecting samples of children from both countries, the results showed a higher incidence of autoimmune and allergic diseases (T1D, celiac disease, autoimmune thyroid disease and IgE-mediated allergic sensitization) in Finland than in Russian Karelia, and inverse correlation with microbial infections. In addition, studies in animal models support the “hygiene hypothesis”. The incidence of T1D in NOD mice is increased in a specific pathogen-free (SPF) environment [48].

1.3. Experimental models of type 1 diabetes

Experimental models for T1D offer the great advantage of allowing interventions rarely possible in humans, while improving the understanding of genetics, etiology and pathogenesis. Moreover, experimental therapies in animal models open the door to test the administration pathway and dose, to evaluate their safety and efficacy and to understand the molecular mechanisms of the therapy. However, many therapies that could prevent or cure T1D have failed in human disease, so it is important to understand the discrepancies in the immune system between mice and humans for productive research using experimental models [49].

1.3.1. Induced

T1D can be induced in rodent strains that do not have genetic predisposition to T1D through surgical methods, by partial or total pancreatectomy, or by chemical compounds that induce β-cell death. The main induced models of T1D are detailed below.

Surgical models were initially used in pancreas transplantation studies, in the case of total pancreatectomy, or regeneration studies in the partial duct obstruction model [50] or partial pancreatectomy, where an increase of the β-cell mass after the extirpation was observed [51, 52].

Pharmacological induced models employ the injection of chemicals compounds that destroy β-cells (β-cytotoxic agents) to induce T1D in rodents. Three main compounds have been used:

- Alloxan (2,4,5,6-pyrimidinetetrone) is an oxygenated pyrimidine derivative that acts a toxic glucose analogue which preferentially accumulates in pancreatic β-cells via the glucose transporter 2 (GLUT2). It generates superoxide radicals that are responsible for the death of the β-cells, which have a particularly low antioxidative defence capacity. The result is an insulin-dependent, but not autoimmune, 'alloxan diabetes' [53]. Moreover, as a thiol reagent, alloxan also selectively inhibits glucose-induced insulin secretion through its ability to inhibit the β-cell glucose sensor glucokinase.

- Streptozotocin (STZ) (N-(Methylnitrosocarbamoyl)-α-D-glucosamine) is an antimicrobial agent that has been used as a chemotherapeutic agent. It is a glucose-conjugated nitrosourea that accumulates in pancreatic β-cells via the GLUT2 glucose transporter. It acts as toxic glucose analog by inducing DNA damage through the generation of free radicals (alkylating agent). STZ is also able to inhibit glucose-induced insulin secretion [53]. STZ dosage defines two different models: a single and high dose of STZ induces massive β-cell necrosis and an acute hyperglycaemia in 24 hours; however, multiple low doses produce gradual hyperglycaemia which is associated with insulitis [54].

- Cyclophosphamide (CY) is a nitrogen mustard compound that has been used as an immunomodulatory agent, as well as a chemotherapeutic agent in the treatment of chronic lymphocytic leukemias, lymphomas, and solid tumors. Administration of high-dose CY to prediabetic NOD mice induces T1D [55], possibly by the breakdown of regulatory networks [56].

1.3.2. Spontaneous

Over time and the generation of different animal models, some of them emerged hyperglycemic and susceptible to T1D. These animals have been the founders of strains that develop spontaneous and autoimmune diabetes. The main spontaneous murine models of T1D are the NOD mouse and the BB rat.

The NOD mouse is the most widely used mouse model of T1D, because of its similarities with human autoimmune diabetes [57]. The NOD strain was developed in 1980 by Makino and colleagues at Shionogi Research Laboratories in Aburahi, Japan by selecting cataract-prone mice strains [58]. Spontaneous autoimmune diabetes in NOD starts at 3-4 weeks of age with an initial insulitis formed by an initial leukocytic aggregation at the perimeter of the islets and followed by an infiltration into the pancreatic islets. $CD4^+$ T is the main subset of the infiltrate in NOD mice, but $CD8^+$ T, natural killer (NK) cells, B cells, dendritic cells (DCs), and macrophages are also present in the lesions [59]. Infiltration starts with antigen presenting cells (APCs), such as macrophages, DCs and B cells, which present self-antigens to the autoreactive $CD4^+$ T cells that drive the disease [60, 61]. In fact, DCs and macrophages are the first immune cells detectable at 3 - 4 weeks of age in the islets of NOD mice [62]. The effector attack on the insulin-producing β-cells in the islets is primarily mediated by $CD8^+$ T cells [63]. Diabetes onset occurs from 12 -14 weeks of age and the cumulative incidence of the disease at 30 weeks of age is 60-80% in females and 20-30% in males [58]. The specific diabetes incidence of NOD colony also depends on the "cleanliness" status of the colony, as well as other environmental factors [48]. NOD mice are also prone to develop other autoimmune syndromes, such as autoimmune sialitis, autoimmune thyroiditis, autoimmune peripheral polyneuropathy and prostatitis [59].

Autoimmune diabetes in NOD mice shares genetic characteristics with human T1D [59], presenting genetic regions that confer susceptibility to disease, designated as insulin-dependent diabetes (Idd) loci. The dominant genetic contributor to disease predisposition in NOD mice is the unique major histocompatibility complex (MHC)

class II haplotype (*Idd1*), denominated H-2^{g7}. This MHC haplotype does not express the I-E molecule due to a defective Eα locus. Moreover, the unique I-A molecule lacks the usual aspartic residue at position 57 of the β chain (I-A^{g7}), constituting the major genetic trait for diabetic susceptibility in NOD [64], since that alters the repertoire of MHC binding peptides. In addition, other non-MHC *Idd* regions also mediate T1D risk [65]. *Ctla4* gene (*Idd5.1*) is one of the first risk locus found in NOD mice that shows correspondence to humans. Another genetic variation with T1D susceptibility is in IL-2 gene (*Idd3*), altering the IL-2 signaling pathway. Finally, *Ptpn8* gene, the mouse ortholog of PTPN22, has been described to influence disease in NOD [19]. In contrast, the insulin gene in NOD mice does not have a regulatory variation analogous to human insulin gene. Unlike humans, mice have two insulin genes *Ins1* and *Ins2*, located in two different chromosomes [66]. They are both expressed in pancreatic β-cells, but *Ins2* is the one that is predominantly, if not almost exclusively, expressed in the thymus.

It has been reported that a number of immune defects in NOD mice that may contribute to their pathogenesis. Autoreactive T cells recognize autoantigens (Pro/insulin, GAD65/67, insulinoma-associated protein 2 (IA-2)/IA-2β, heat shock protein 60 (Hsp60) and IGRP, among others), which are produced in pancreatic islets [67]. However, islet-reactive T cells may also target other cells than β-cells, such as the nonmyelinated Schwann cells that surround the islets [68]. B cells produce autoantibodies against many autoantigens. Anti-insulin antibodies are first detectable at 6 weeks of age and between 8 and 16 weeks [69]. Although high titers of anti-insulin and anti-GAD autoantibodies are found in prediabetic NOD mice, a non pathogenic role has been confirmed to them [70]. However, it is suggested that B cells have an important role in the development of autoreactivity in NOD mice through the secretion of pro-inflammatory cytokines, as well as APCs [71]. Other proposed alterations are a defective macrophage maturation and function [72], low levels of NK cell activity [73] and defects in natural killer T (NKT) cells [74], among others.

The NOD RIP-IFNβ mouse is a transgenic model of accelerated T1D. Genetic engineering techniques have allowed the development of numerous genetically modified mouse models. In transgenic mice, an exogenous gene -i.e. transgene- is introduced into the DNA of fertilized oocyte, which will be transmitted to the offspring. In this study, in addition to the wild type NOD model, we have used the transgenic model NOD RIP-IFN-β [NOD rat insulin promoter-human interferon-β (RIP-IFN-β)] generated in our laboratory by backcrossing the CD1 RIP-HuIFN-β transgenic mice [75] to NOD mice [76].

NOD RIP-IFN-β mice express human interferon (IFN)-β under the control of the insulin promoter. This model was generated to study the role of the antiviral cytokine IFN-β in T1D, since viruses have been proposed as environmental trigger events in this disease. IFN-β is secreted by many cell types including fibroblasts, plasmacytoid DCs (pDCs), NK cells and T cells, among others. Transgenic NOD RIP-IFN-β mice were created with human IFN-β to avoid the sterility observed in mice expressing high levels of mouse IFN-β [77]. Human IFN-β is active in mouse cells, although its efficacy is 1000 times lower than that of mouse IFN-β [75]. NOD RIP-IFN-β mouse model suggests that the initial cell damage may induce the production of IFN-β by β-cells, thus triggering inflammation and cell-mediated autoimmunity. NOD RIP-IFN-β mice develop early autoimmune diabetes at 3-4 weeks of age with similar incidence in males and females **(Figure 2)** [76], resembling the T1D presented in very young humans.

The insular expression of human IFN-β is involved in the development of autoimmune diabetes: Islets from transgenic mice display a higher degree of insulitis than the wild type NOD mice and hyperexpress MHC class I molecules as a consequence of human IFN-β. It was demonstrated that the transfer of lymphocytes from a diabetic NOD RIP-IFN-β animal caused the disease in NOD-SCID immunodeficient recipient mice and accelerated the onset of diabetes in prediabetic NOD recipient mice. In addition, human IFN-β expression did not altered the expression of pancreatic hormones (prepoinsulin and glucagon) or β_2m in the thymus, thus supporting the idea that the disease could be caused by a local

effect of human IFN-β, strong enough to break the peripheral tolerance to β-cells rather than by a change in central tolerance [76]. In this sense, it was shown that NK cells had a crucial role in the induction of accelerated diabetes in this model, since the accelerated onset was characterized by an increase of NK cells and their depletion completely abolished the acceleration of T1D [78].

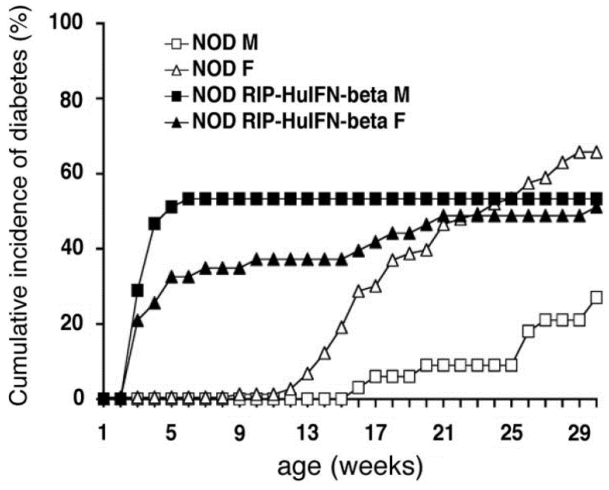

Figure 2. The transgenic expression of human IFN-β in islet β-cells accelerates the onset of the disease in NOD transgenic mice. From Alba A, *et al.*, 2005, J Immunol [76]. Cumulative incidence of diabetes (%) in RIP-HuIFNβ transgenic mice during 30 wk of follow-up (n>40). NOD RIP-HuIFNβ mice developed early diabetes, after 3 wk of age, when compared with NOD wild-type mice. The incidence of the disease in transgenic NOD mice was similar in males and females; as expected, wild-type NOD mice showed a higher incidence of the disease in females than in males.

The BioBreeding rat is the most extensively studied rat model of T1D is the BioBreeding (BB) rat. BB rat is derived from a Canadian colony of outbred Wistar rats, in which spontaneous hyperglycemia and ketoacidosis took place in the 1970s. Affected animals were the founders for two colonies that were later used to establish all subsequent BB rat colonies. One colony established in Worcester, Massachusetts (BB/Wor), were inbred and a second colony in Ottawa, Canada, "BBdp" rats were outbreed [79]. Diabetes in BB rats is also a polygenic disease, presenting genetic susceptibility in MHC class II genes (*RT1u* allele, Iddm1) and GTPase immunity-associated protein family member 5 (*Gimap5*) gene (Iddm2),

among others [80]. BB rats of both sexes develop diabetes between 50 and 90 days of age [81] with insulitis mediated mainly by Th1-type lymphocytes. However, all diabetic BB rats develop a profound T cell lymphopenia characterized by a severe reduction of $CD4^+$ T cells and an almost complete absence of $CD8^+$ T cells [82], differing from T1D pathology of either NOD mice and humans.

2. IMMUNOLOGY OF TYPE 1 DIABETES

The **immune system** is one of the most complex network of the body. Its main role is to recognize and protect the organism from the invading pathogens and neoplastic cells, while maintaining tolerance to self. The first and immediate line of defense to protect the organism from infection is provided by the **innate immune response**, which triggers an inflammatory response and initiates the response of the adaptive immune system. The **adaptive immune response** is capable to distinguish among self/nonself antigens, being highly specific to each particular pathogen or dangerous antigen (antigenic specificity). A key feature of the adaptive immune system is memory, which confers life-long immunity to those previously encountered pathogens. However, this complex network can fail in certain individuals or life stages, thus allowing the immune system to attack own constituent parts of the body. This disorder is **autoimmunity**, which can be demonstrated by the presence of autoantibodies and autorecative T lymphocytes capable to transfer the disease [83]. Autoimmunity is the cause of a broad spectrum of human illnesses, known as autoimmune diseases, such as T1D.

2.1. Immunopathogenesis

T1D involves a complex cross-talk between pancreatic β-cells and cells from the innate and adaptive immune system. T1D involves a complex inflammatory process that progresses in spite of the regulatory and regenerative mechanism **(Figure 3)**. The contribution from several immune mechanisms is detailed below.

2.1.1. Innate immune cells

Although is known that T1D results from a T cell-mediated destruction of β-cells, the development of the disease involves a deregulation of the innate immune system. The innate immune response is the first line of defense of the organism. Cells from innate immunity are able to recognize conserved and exclusive structures expressed in microbes named pathogen-associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) that are released by stressed tissues when cells die by immunologic not-silent cell death such as necrosis through pattern recognition receptors (PRRs), among them, toll-like receptors (TLRs). Specialized cells like neutrophils, macrophages and DCs are activated to rapidly engulf and destroy extracellular microbes through phagocytosis. After the encounter with the pathogen, activated innate immune cells produce a variety of antimicrobial proteins that help to kill pathogens, as well as cytokine and chemokine proteins that recruit cells, molecules, and fluid to the site of infection, leading to swelling and other symptoms collectively known as inflammation.

In T1D, **inflammation** contributes to the induction and amplification of the immune attack against pancreatic β-cells; and at later stages, to the stabilization and maintenance of insulitis [84]. The insulitic infiltrate, mainly composed by T and B cells, but also macrophages, DCs, NKs and NKTs, release proinflammatory cytokines and chemokines. IL-1β, TNF-α, IFN-α, IFN-β, IFN-γ, IL-6 [30, 85, 86], as well as CXCL-10, CCL5, CCL8, CXCL9, and a few CX3CL1 [87] have been found in pancreas from T1D patients. Those molecules may contribute to suppress β-cell function and induce cell death, and also to the recruitment of macrophages, inflammatory monocytes, DCs and T cells into the pancreatic islets. Moreover, TNF-α and IFN-γ can induce nitric oxide secretion by β-cells, promoting their apoptosis [88]. In addition, β-cells express TLRs [89], which can contribute to autoimmune response, as it has been reported in experimental models [90]. The transcriptome from four T1D patients who died at different stages of the disease

revealed that the expression of genes of innate immunity was maintained in the long-standing cases contributing to the chronification of the disease [91].

Natural killer cells are cytotoxic lymphocytes that constitute the host's first line of defense against infecting viruses, by producing cytokines, mainly IFN-γ, and directly destroying virus infected cells [92]. In NOD mice, the lack of NK cells has been associated to protective insulitis [93]. Moreover, NK cells are required for accelerated T1D driven by IFN-β in NOD mice [78]. NK cells express the activating receptor NKp46, which recognizes mouse and human ligands on pancreatic β-cells. It was shown that NKp46 engagement by β-cells led to degranulation of NK cells, contributing to the development of T1D in NOD mice [94]. In humans, NK cells have been found in infiltrates of pancreas from T1D patients [95] and abnormalities in NK cells of diabetic patients have been also described. Other studies showed a reduction of the frequency of NK cells in peripheral blood [96] and described functional abnormalities [97].

2.1.2. Antigen presenting cells: a bridge among innate and adaptive immunity

APCs, which are DCs, macrophages and B cells, play the key role in the initiation of adaptive immune responses. Activated APCs migrate through lymphatic vessels to nearby lymph nodes, where they present antigen-derived peptides on their MHC proteins to T cells, and drive T cell clonal expansion and differentiation to effector and memory cells. Antigen-activated T cells, then, initiate adaptive immune responses against the pathogen or dangerous antigen. APCs exhibit remarkable plasticity and can activate the specific immune response or act as regulatory cells. Their pathogenic role in T1D is detailed below.

Conventional DCs (**cDCs**) are professional APCs and have a unique capacity for priming $CD4^+$ and $CD8^+$ T cells, and an essential role in the presentation of β-cell-derived antigens during the initial stages of T1D, in the islet of Langerhans, as well as in the lymph nodes (LN) that drain the pancreas. An early study showed that

DCs were one of the main populations of cells infiltrating the islets at early stage of insulitis in NOD mice [62]. It has been described that DCs are found infiltrating pancreatic islets in close association with the blood vessels [98]. Those intra-islet DCs are divided into two subsets: the main population (85% of the $CD11c^+$) express CD11b, F4/80, CX_3CR1 and SIRPα and the minority (15%) express CD103. After inflammation, the number of DCs in the islets increases, and phenotype changes can be observed, increasing the expression of B7-2, as well as CD40, CD11b and the intercellular adhesion molecule 1 (ICAM-1) [99, 100]. It has been proposed that islet DCs take up antigens, migrate to the peri-islet stroma, and from there, into the draining lymph nodes, to recruit and activate autoreactive T cells [99]. Activated diabetogenic T cells enter directly from blood into islets, perhaps helped by islet DC dendrites exposed inside the blood vessels, through the MHC class II and ICAM-1 interaction. The entry of those diabetogenic T cells may trigger inflammatory signals and gene changes in the islet (upregulation of chemokines and adhesion molecules) to promote the subsequent entry of non-specific T cells [98].

Plasmacytoid DCs have a high capacity to produce type I IFN in response to nucleic acids [101]. In NOD mice, a progressive infiltration of pDC within the islets was observed [102]. Moreover, pDCs are the main producers of type I IFNs, which are associated to T1D as a viral infection footprint [26]. In addition, an abnormal expression of type I IFNs has been reported in the sera of patients affected with autoimmune diseases [103] and type I IFNs have been found present in pancreata of T1D patients [30, 31].

Macrophages have an effective phagocytic activity, increased ability to kill ingested microbes and high capacity to secrete proinflammatory and cytotoxic mediators when activated, but also can function as APCs to activate T cells. In NOD mice, the block of macrophage adhesion prevents insulitis [104]. In humans, macrophages are the second cell subset among the infiltrating mononuclear cells in the islets of newly diagnosed T1D patients [105]. Macrophages may promote apoptosis of β-cells by the production of proinflammatory cytokines [85].

Moreover, a deficiency in the clearance of apoptotic cells by macrophages was described in NOD mice [106, 107], where the accumulation of apoptotic cells could result in necrosis, thus contributing to the autoimmune process.

2.1.3. Adaptive immunity

The adaptive immune response is capable to distinguish among self/nonself antigens, being highly specific to each antigen. The principal cell players of the adaptive immune response are B and T cells, which express high specific receptors to each antigen. The presentation of the antigen to naive lymphocytes induces them to proliferate and differentiate into effector cells in the primary response. Most of effector cells die upon resolution of infection, but a few remain as memory cells. All subsequent encounters with the same antigen are referred to as the secondary response, which is greater in magnitude, peaks in less time and is more antigen specific than the primary response. Adaptive immune response can proceed through two classes of responses, the cell-mediated immunity or the humoral immunity (or antibody-mediated immunity).

T cells are the main players of the cell-mediated immunity. They express a unique antigen-binding receptor, called T-cell receptor (TCR), which reacts against dangerous antigens presented by major histocompatibility complex (MHC) molecules. There are two classes of MHC molecules (HLA in humans): class I MHC molecules are expressed by nearly all nucleated cells of vertebrate species, and class II MHC molecules are expressed by antigen presenting (APCs) cells, like DCs, but also macrophages and B cells. T cells are divided into two major cell types, T helper (Th) cells or T cytotoxic (CTL) cells, that can be distinguished from each other by the presence of either CD4 or CD8 membrane glycoproteins on their surfaces. Naive T cells need three signals provided by DCs, as powerful APCs, to be activated to effector or memory cells. The signal 1 is the TCR/MHC-peptide interaction, together with CD4 and CD8 coreceptors and adhesion molecules. The signal 2 is the costimulation by other membrane molecules, including CD28. The

signal 3 is the secretion of transcription factors and cytokines, which is initiated after signals 1 and 2.

$CD4^+$ T cells recognize antigens presented by MHC class II molecules and differentiate into effector T cell subsets, depending on the received signals. Those subsets are defined by the secretion of a concrete cytokine profile. Th type 1 (Th1) cells produce IFN-γ and IL-2 and promote a cell-mediated immunity through the activation of the bactericidal activities of macrophages and the cytotoxic T lymphocytes. Th type 2 (Th2) cells release IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, and they lead to a humoral response to regulate B cell activity and differentiation [108]. Th type 17 (Th 17) cells, which secrete IL-17, play an important role in cell-mediated immunity and their original function is the defense against bacteria and fungi [109]. $CD8^+$ T cells recognize antigens presented by MHC class I molecules. After the encounter their MHC-peptide complex naive $CD8^+$ T cells differentiate into effector cytotoxic T lymphocytes (CTLs). Once activated, CTL have a vital function in fighting against virus-infected cells and tumor cells [110]. Activation of CTL is done by the three signals: TCR signalling, costimulation and cytokines, but also seems to require help from mature $CD4^+$ T cells [111].

T cells have an important role in the autoimmunity against β-cells. Several studies in NOD mice confirmed the pathogenic role of $CD4^+$ and $CD8^+$ T cells after showing their capacity to transfer the disease [112]. Both $CD4^+$ and $CD8^+$ T cells are found infiltrating the islets in T1D patients, but $CD8^+$ T cells are the most abundant in the insulitis [30, 113]. More than 150 $CD4^+$ T cell epitopes have been described from pro/insulin, GAD65, IA-2, HSP60, HSP70, IGRP and ICA69 autoantigens and more than 20 $CD8^+$ T cell epitopes have been identified from pro/insulin, GAD65, IA-2, IGRP and IAPP autoantigens in T1D patients [67]. Autoreactive T cells that recognize islet autoantigens have been identified in diabetic patients [114-116].

It has been postulated that the activation of autoreactive T cells starts after β-cell antigenic presentation by DCs in the draining pancreatic lymph nodes [98, 117]. However, a pathogenic population of $CD4^+$ T cells has been described which

recognize an unstable peptide of insulin, that are directly recruited into islets of Langerhan, bypassing the initial priming stage in the pancreatic lymph node and being activated by intra-islet DCs [118]. It has also been proposed that some autoreactive $CD8^+$ T cells can be directly activated in pancreatic islets [119] by direct recognition of islet β-cell antigens presented by MHC calls I on β-cells [120]. Once activated, diabetogenic T cells contribute to recruit and activate inflammatory cells, such as macrophages, and amplify the cellular response through the production of cytokines, contributing to β-cell death. In fact, there is a direct association of islet-autoreactive $CD8^+$ T cells with β-cell destruction in human pancreatic islets in T1D patients [121] and NOD mice [122]. Moreover, activated cytotoxic $CD8^+$ T cells play an important role as effectors in T1D, by killing β-cell via Fas pathway and through the secretion of perforin and serine proteases (granzymes) [123].

B cells are the main players of the humoral immunity. They express the immunoglobulin receptor, named B cell receptor, which is specific for each antigenic epitope. Naive B cell can be activated to secrete antibodies after the encounter with the pathogenic antigen through a T cell-dependent or independent mechanism, based on the nature of the antigen. Moreover, B cells have an important role as APCs. It has long been established that T1D is a T cell-mediated autoimmune disease. However, there is an important role of B cells in the T1D pathogenesis. It was demonstrated that B cell depletion resulted in a resistance to T1D in NOD mice [124]. B cells have been found in insulitis of T1D patients, being present in only small numbers in early insulitis, but also being recruited to islets when β-cell death progresses [113]. During prediabetes, B cells produce autoantibodies against islet cell proteins. Insulin, GAD, IA-2 and IA-2β are the β-cell antigens most frequently targeted by autoantibodies in T1D susceptible subjects [125]. Those have been utilized as effective biomarkers to identify subjects at risk and predict the onset of disease, but their contribution in disease development seems to be unlikely [126]. However, as was shown in NOD mice, B cells seem to have an important role in diabetes related to their efficient presentation of self-antigens to autoreactive $CD4^+$ T cells [70] and to their capacity to cross-present

islet-derived autoantigens to self-reactive $CD8^+$ T cells in the pancreatic lymph nodes [127].

Natural killer T cells are lymphoid cells that express a semi-invariant TCR that recognize specific lipids and glycolipids presented by MHC class I-like CD1 molecules [128]. Once activated, NKT cells release cytotoxic granules that kill target cells, but also large amounts of cytokines and chemokines [129]. Despite NKT cells having a potential pathogenic role in T1D, some studies suggest them as regulatory cells. Increasing the frequency of NKT cells reduces T1D incidence in NOD mice [130], whose protection is associated to a switch from Th1 to Th2 response to islet autoantigens [131]. In human T1D, there is controversial data on NKT cell frequency and function; some authors reported a low frequency of NKT cells in T1D patients [132] and others reported a high frequency of NKT cells in recent-onset T1D patients [133].

2.1.4. Central tolerance

During T cell development in the thymus, lymphocytes go through two check points: positive selection chooses those thymocytes bearing receptors capable of binding self-MHC molecules, and negative selection deletes self-reactive thymocytes bearing high-affinity TCR for self-MHC/peptide complexes, eliminating the autoreactive T cells [134]. This mechanism is called central tolerance and is the primary barrier that autoreactive T cells must overcome prior to carry out an aberrant response in the periphery, triggering autoimmunity in T1D.

In the thymus there are medullary thymic epithelial cells (mTECs), which express the transcription factor AIRE, that allows those cells to express, process and present proteins that are only found in specific organs [135], such as insulin, among other T1D autoantigens. To explain the contribution of central tolerance to T1D, the VNTR minisatellite at the 5' of the insulin gene (IDDM2) was found to correlate with the level of insulin transcription in the thymus from T1D patients [136]. Other results point to the nature of the T cell epitopes and the generation of

the peptide-MHC complexes. It was reported that disease-associated MHC II alleles form low avidity interactions with the peptides that they present, with a deficient presentation of the autoantigens in thymus, thus allowing autoreactive T cells to escape from central tolerance in NOD mice [137]. Another explanation is that the self peptides presented in the periphery have undergone post-translational changes, creating unique epitopes that are not presented in the thymus [138]. A recent study proposed a novel pathway of presentation by MHC class II molecules involving peptides or denatured proteins important in autoimmunity. It identified a set of pathogenic $CD4^+$ T cells that can only recognize soluble B:9–23 insulin peptide, but not the insulin protein when processed by APCs in NOD mice. This situation allows those self-reactive $CD4^+$ T cells to escape thymic control. Moreover, the peptide that activate those autoreactive $CD4^+$ T cells is normally derived from the insulin secretory granules and can be loaded on MHC class II in the intra-islet DCs, activating those autoreactive $CD4^+$ T cells to induce T1D [118, 139].

2.1.5. Peripheral tolerance

Despite central mechanisms of tolerance, autoreactive T cells can be found in most healthy individuals [140], indicating that additional mechanisms to maintain peripheral tolerance exist [141]. Those mechanisms could fail in subjects with susceptibility to the disease, contributing to the activation of autoreactive cells.

Costimulatory signals: After its maturation in the thymus, naïve T cells circulate throughout the body in order to find the antigen that, presented by MHC molecules, will induce their proliferation and differentiation into effector cells. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1) molecules are negative regulators [142] selectively expressed on activated T cells to shut down immune activation and promote T cell homeostasis and peripheral tolerance. CTLA-4 and PD-1 both are important to control T1D, maintaining a certain peripheral tolerance in the NOD strain [143, 144]. Potential defects in the CTLA-4 and/or PD-1 pathways in T1D patients were suggested by the association of

polymorphisms in both genes with susceptibility to disease [145, 146]. Additionally, decreased expression of PD-1 gene was found in peripheral blood-derived $CD4^+$ T cells from a small group of T1D patients [147].

Depletion of autoreactive T cells: It has been postulated that in secondary lymphoid organs there is an additional checkpoint to delete autoreactive T cells that escape negative selection in the thymus. Lymph node stromal cells [148] and extrathymic Aire-expressing cells (eTACs) [149] are found in secondary lymphoid tissues and are able to express peripheral tissue antigens (PTA) peptides. These peptides are presented by MHC molecules to promote deletional tolerance to autoreactive PTA-specific T cells. In addition, it was reported that the expression of PTAs in lymph node stromal cells was also regulated by another transcriptional regulator named deformed epidermal autoregulatory factor 1 (Deaf1). In T1D patients and NOD mice, a Deaf1 variant isoform that suppresses PTA expression in secondary lymphoid tissues was increased, thus contributing to the pathogenesis T1D [150].

Regulatory T cells: Another mechanism involved in the maintenance of pheripheral tolerance is the action of regulatory T cells (Tregs), which can produce IL-10 and TGF-β to inhibit immune response. Regulatory $CD4^+$ T cells are classified in natural Tregs (nTregs), which arise during maturation in the thymus from autoreactive cells or adaptive/induced Tregs (iTregs), which can be induced at the site of an immune response in an antigen-dependent manner [151]. Tregs cells are important in maintaining the peripheral tolerance in the host, which helps to suppress autoreactive responses that have not been avoided via other mechanisms. However, there is not a clear consensus on this subject in human T1D. Some studies have shown reduced Treg cell frequency [132] or suppressive function in T1D patients [152]. However, others have shown no changes when comparing diabetic subjects to healthy individuals [153, 154]. In another sense, it was reported that effector T cells of diabetic subjects are more resistant to regulation by $CD4^+$ $Foxp3^+$ Treg cells than in healthy individuals [155]. Regulatory $CD8^+$ T cells have also been described [156]. In NOD mice, low-avidity autoreactive

CD8 T cells can convert to into memory-like autoregulatory CD8 T cells after chronic-antigen exposition, thus acting as a negative feed-back regulatory loop in the disease [157].

Cytokines: The cytokine production from activated T cells by self-antigen may be important for the outcome of the immune response. There is a general consensus that Th1 cells (producing IFN-γ) tend to be pathogenic whereas Th2 cells (producing IL-4) can protect from diabetes [158, 159]. However, NOD mice genetically deficient for IFN-γ [160] or for IL-4 [161] showed no difference in diabetes incidence, suggesting that cytokine deficiency, per se, is not sufficient to alter disease progression, being more an outcome that the cause for diabetes [162]. Furthermore, proinflammatory Th17 cells, which have been implicated in many autoimmune diseases [109], also appear to have a role in T1D. It was reported that peripheral blood autoreactive $CD4^+$ T cells from patients with new-onset T1D secreted IL-17 in response to β-cell autoantigens, contributing to β-cell destruction [163].

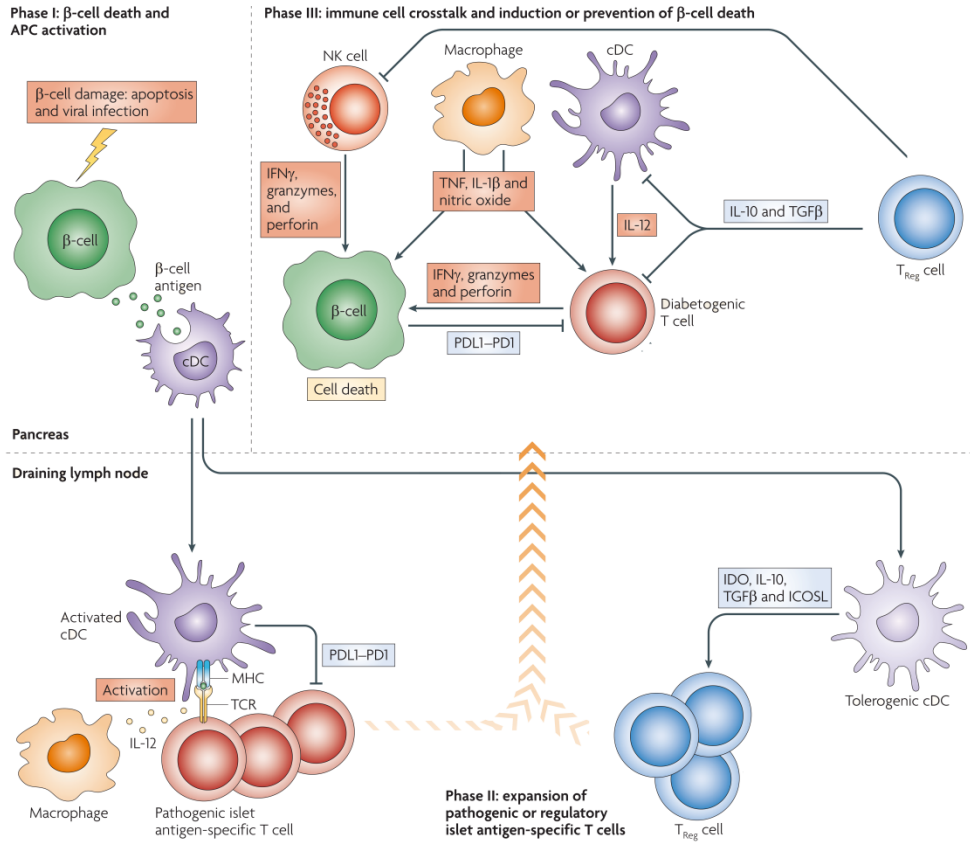

Figure 3. Cellular and molecular mechanisms in the development or prevention of type 1 diabetes. Adapted from Lehuen A, 2010, Nat Immunol Rev [164].

2.2. Role of β-cells in autoimmunity

It has been postulated that the initiation of the disease occurs in the islets of the pancreas, where under undefined pathogenic conditions, β-cell antigens are released [165]. The immunological alterations in the pancreatic islets in at T1D onset have been studied in a small number of observations from autopsies [91, 166] and few pancreatic biopsies of T1D patients performed in Japan [167]. As expected, there is a clear decrease of β-cell mass in the islets of T1D patients and the presence of a leukocyte infiltrate mainly composed by $CD8^+$ T cells, but also macrophages and in less extent, $CD4^+$ T cells, B cells and DCs [91]. The pathogenic insulitis goes together with molecular alterations in the islet cells. Hyperexpression

of MHC class I molecules, increase of the expression of adhesion molecules and other molecules involved in antigen presentation [168, 169] and ectopic expression of MHC class II molecules were described in the islet cells [170]. Moreover, the increased Fas expression was identified in β-cells of inflamed human islets, which can induce apoptosis after binding to its receptor [171]. Immunoglobulin and complement deposits were also found in the islets [30].

Moreover, physiological apoptosis of β-cells also contributes to autoimmunity in T1D. A peak of physiological β-cell death is observed in mice 2 weeks after birth [172], precipitating the arrival of $CD11c^+$ $CD11b^+$ $CD8\alpha^-$ DCs and the priming of islet antigen-specific $CD4^+$ T cells in NOD model [173]. Interestingly, a similar peak of β-cell death has been described in humans, at perinatal period, when the process of islet remodeling has been seen [174]. Therefore, it is postulated that, due to either too much loss of cells with little regenerative capacity or by too little apoptotic cell removal, the wave of physiological β-cell death is crucial in the initiation of T1D [120]. If these dying β-cells are not efficiently removed, they release danger signals that can activate DCs and contribute to insulitis progression and diabetes onset in NOD mice [175].

In addition, a low but constant capacity of regeneration of β-cells in adult T1D patients was described [176], leading to propose a relapsing-remitting nature of T1D [177]. In this sense, β-cell regeneration could have a dual role in T1D. It was described that Reg proteins, involved in islet regeneration, may contribute to accelerate the autoimmune process leading to diabetes in a vicious cycle [178].

2.3. Autoantigens

An autoantigen is an endogenous body constituent that is the target of a cell-mediated or humoral immune response. In T1D disease, the immune response can be against pancreatic islets autoantigens, such as insulin or IGRP, neuroendocrine antigens, such as GAD, IA-2 or ubiquitous, such as HSP [179]. The main autoantigens targeted by T and B cells are detailed in **Table 1**. It is important to

identify the autoantigens of T1D in order to understand its pathogenesis and design new immunetherapies to prevent or treat the disease.

Table 1. Major autoantigens in T1D

Autoantigen	Expression	Function	Specie	Immune response
Insulin/Proinsulin	β-cells	Glucose homeostasis	Hu, Mu	Cellular, Humoral
GAD65	Islet cells (mainly β-cells), neuronal cells	Synthesis of GABA	Hu, Mu	Cellular, Humoral
GAD67	Islet cells (mainly β-cells), neuronal cells	Synthesis of GABA	Mu	Cellular, Humoral
IA-2	Neuroendocrine cells	Maintenance of hormone content	Hu, Mu	Cellular, Humoral
IA-2β	Neuroendocrine cells	Maintenance of hormone content	Hu, Mu	Cellular, Humoral
IGRP	β-cells	Glucose metabolism	Hu, Mu	Cellular
ICA69	Islet cells (mainly β-cells), neuronal cells	Regulator of secretion	Hu	Cellular, Humoral
Carboxypeptidase H	Neuroendocrine cells	Insulin secretion	Hu	Humoral
ZnT8	Islet cells (mainly β-cells)	Zinc transporter	Hu	Humoral
HSP60	Ubiquitous	Mitochondrial chaperonin	Hu, Mu	Cellular, Humoral
HSP70	Ubiquitous	Chaperones	Mu	Cellular, Humoral
Peripherin	Neuroendocrine cells	Intermediate filament	Mu	Cellular, Humoral
REG1A	Islet cells, acinar cells	Cell proliferation	Hu	Humoral
Chromogranin A	Neuroendocrine cells	neuroendocrine secretory protein	Mu	Cellular
IAPP	β-cells	Glycemic regulation	Hu, Mu	Cellular

GAD65, glutamic acid decarboxylase 65; IA-2, insulinoma antigen-2; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; ICA69, Islet cell antigen-69; ZnT8, Zinc transporter-8; HSP60, Heat shock protein; REG1A, regenerating islet-derived 1 alpha, IAPP, islet amyloid polypeptide

3. IMMUNOTHERAPIES FOR TYPE 1 DIABETES

Currently, T1D patients require lifelong administration of exogenous insulin, which is associated with adverse effects, such as the risk of hypoglycemia and side effects. Trying to prevent autoimmunity in T1D is a real medical health challenge. Many strategies have been developed in order to prevent or reverse the disease and halt the autoimmune attack against pancreatic β-cells. Some have proven effective in preventing spontaneous T1D in mice and a few have reported a reversal of the disease. No therapy has yet reversed T1D in humans even though some were able to maintain residual endogenous insulin production. Immunotherapies are classified as systemic, which are directed to suppress the immune response to some extent and antigen-specific, which are directed to restrict the immune response against the specific target of the disease, without compromising the host's defense against infections and tumors.

3.1. Systemic immunotherapies

In the 1980s, immunosuppressive drugs were used in T1D resulting from the clear evidence of the autoimmune component of the disease. The main antigen-specific strategies to arrest β-cell destruction will now be detailed.

The first and most extensively used drug was **cyclosporin A** (**CsA**) [180]. CsA is a peptide of fungal origin with a strong immunosuppressive capacity. It is a calcineurin inhibitor that interferes with TCR-mediated signal transduction, thereby inhibiting T cell activation and the production of IL-2 by T cells, limiting the amplification of immune responses [181]. CsA provided the first proof of concept that the inhibition of the immune system at the onset of T1D could prolong residual β-cell function and insulin production. Treatment of diabetic T1D patients with CsA was able to achieve insulin independence [180, 182]; however, the effect was not sustained after the discontinuation of the treatment and potential renal toxicity was observed [183]. Eventually, CsA trials in T1D were stopped, given that the risks outweighed the benefits.

Another immunosuppressive drug, **Mycophenolate mofetil** (**MMF**), which has cytostatic effects on B and T lymphocytes [184], was used to treat newly diagnosed patients with T1D in combination with **Daclizumab** (**DZB**), a monoclonal antibody specific for CD25, the α-chain of the IL-2 receptor [185]. Despite its efficacy in organ transplantation, MMF alone or with DZB was not able to preserve β-cell function in new onset T1D patients [186].

Because T cells are the logical targets to prevent β-cell destruction, the polyclonal **anti-lymphocyte serum** (**ALS**) (or anti-thymocyte globulin, ATG) has been used as systemic immunotherapy for T1D. ALS or ATG is a mixture of immunoglobulins directed at various T and B lymphocyte receptors. In NOD mice, ALS restored normoglycemia in diabetic mice lifelong [187]. ATG made in horses, in combination with prednisone [188], or ATG made in rabbit [189] were used in two trials to treat recent onset T1D patients. Complete diabetes remission occurred in only a few treated patients, but significant side effects were observed. However, a third study did not preserve β-cell function [190].

In the 1990's, anti-CD3 monoclonal antibodies were developed as a new treatment for T1D. Their administration to diabetic NOD mice resulted in a complete remission of the disease [191]. **Teplizumab** and **otelixizumab** are humanized versions of anti-CD3 monoclonal antibodies. These two humanized anti-CD3 mAbs display mutations in the Fc portion (the constant region of the immunoglobulin) of the human IgG1, to avoid the binding by Fc receptor. Modified anti-CD3 antibodies showed a reduction capacity to induce *in vitro* T cell proliferation and cytokine release by human peripheral blood mononuclear cells. They have been tested in over 1500 patients with new and recently diagnosed T1D, with encouraging results in preserving β-cell function. However, adverse effects of the treatment have to be taken into consideration [192]. The suggested mechanism of action of anti-CD3 antibodies involve the depletion of activated T cells and the induction of regulatory cells, but further investigations needed as they remain unclear [193].

As the contribution of B cells as APCs in the pathogenesis of T1D gained evidence, anti-CD20 antibodies have been administered to T1D patients. In NOD mice, those antibodies reduced B cell population, and prevented and reversed diabetes [194]. **Rituximab** is a chimeric murine/human monoclonal antibody against the CD20 antigen containing human IgG1,κ constant region sequences and murine light-and heavy-chain variable region sequences. Rituximab was administered to newly diagnosed T1D patients and achieved to preserve β-cell function, but with some adverse effects [195].

Because functional interactions between T and B lymphocytes are necessary for optimal activation of an immune response, **cytotoxic T lymphocyte antigen 4** (**CTLA4**)**-Ig** (**Abatacept**) has been tested in T1D patients. Abatacept is a fusion protein composed of an Fc portion of IgG1 fused to the extracellular domain of CTLA4, a T cell-negative costimulation receptor that binds the B7 complex (CD80/CD86) on APCs. Administration of Abatacept to recent onset T1D patients slowed the decline of β-cell function [196]. Unfortunately, the effect was short-lived despite prolonged dosing. The drug is now being investigated in a prevention trial in relatives at-risk for T1D (ClinicalTrials.gov NCT01773707).

In addition to cells from adaptive immunity, inflammation is another pathogenic component of T1D [84]. Anti-inflammatory agents have been used with good effect in several autoimmune diseases such as rheumatoid arthritis and psoriasis [197, 198], and were also tested in T1D. **Etanercept** is a recombinant soluble tumor necrosis factor (TNF)-α receptor fusion protein that binds to TNF-α, thereby blocking its activity. Etanercept was administered in a small pilot trial to children with new onset T1D and preserved β-cell function [199]. **Anakinra**, the human IL-1 receptor antagonist and **canakinumab**, the human anti-IL-1β (IgG1) monoclonal antibody were administered to recent onset T1D patients. Although both treatments were well tolerated, they failed to prevent or ameliorate the disease [200].

The outcome of the immunomodulatory effects of the active form of **vitamin D3** has lead to study its effects in T1D. $1.25(OH)_2D3$ can block DCs maturation and IL-12 secretion [201] and inhibit lymphocyte proliferation [202]. Observational studies point to a protective role in T1D development for Vitamin D supplementation in infancy [203, 204]. In NOD mice, short treatment with $1.25(OH)_2D3$ reduced T1D incidence [205], but only when it was administered at an early stage [206]. In the clinic, recent-onset T1D patients were treated [207, 208], providing less encouraging results: treatment was safe, but it was not able to reduce loss of β-cell function.

3.2. Antigen-specific immunotherapies

The use of immunosuppressive drugs in clinical trials has shown that it is possible to arrest the progression of T1D once hyperglycemia is recently established. However, the general depression of immune responses and the required chronic drug administration lead to potential side effects that are unacceptable. In this sense, antigen-based therapies are aimed to "reprogram" the immune system towards inducing antigen-specific tolerance, by targeting the regulatory immune responses or by eliminating antigen-reactive T cells without affecting the immune response homeostasis. The main antigen-specific strategies to arrest β-cell destruction are detailed below.

3.2.1. Protein

Insulin is a major autoantigen in the NOD model and an essential target in T1D [209], and therefore it represents an obvious target for antigen-specific interventions. Administration of insulin through different routes, being oral, nasal or subcutaneous, resulted in disease prevention in animal models [57]. However, in humans, results have not been as encouraging. The Diabetes Prevention Trial (DPT-1) tested oral and subcutaneous insulin treatments in individuals at high risk for T1D without achieving diabetes prevention [210, 211]. Nasal administration also failed to prevent T1D [212, 213]. More efforts have been done in treating

recent onset T1D patients with insulin, but oral insulin administration failed again [214-216]. Nonetheless, the Pre-POINT (Primary Oral/intranasal INsulin Trial) proposed to identify optimal dose and route of administration of insulin in genetically at risk children before the appearance of islet autoantibodies [217].

The T1D associated β-cell autoantigen **glutamic acid decarboxylase** (**GAD**), is an enzyme that catalyzes the decarboxylation of glutamate to the inhibitory neurotransmitter γ-aminobutyric acid (GABA) [218]. At least two isoforms of GAD exist in mammals, with 65 kDa (GAD65) and 67 kDa (GAD67) of molecular weight. Mice β-cells express mainly GAD67 while human ones express only GAD65 [219]. Various approaches in tolerize NOD mice against GAD have proved successful [220, 221]. A dose escalation study in adults with T1D confirmed that GAD65 administration with aluminum hydroxide (alum) was safe and revealed an optimal dose for β-cell function preservation [222]. Three trials in recently diagnosed T1D patients were conducted, reporting a delayed loss of C-peptide in one [223], but not in the others [224, 225].

Another putative T1D autoantigen is **heat shock protein 60** (**HSP60**) [226]. Its major T cell epitope, the peptide p277, conferred T1D protection in NOD mice [227]. The compound tested in clinical trials, **DiaPep277** (TEVA Pharmaceuticals), contains two amino acid substitutions in the native p277 sequence to stabilize the peptide. Clinical trials showed a significant preservation of β-cell function in adult T1D patients, but not children. However, daily insulin was always required [228-230]. Very recently, a large trial treated newly diagnosed T1D patients, showing improved β-cell function [231].

3.2.2. DNA “vaccines”

DNA “vaccines” constitute a new approach used to induce antigen-specific tolerance in autoimmune diseases. These vaccines are plasmids encoding for relevant autoantigens of the disease. In NOD mice, diabetes was prevented by the administration of plasmids encoding insulin B chain [232] or a mimotope peptide

(2.5mi) [233], and reversed by preproinsulin-encoding plasmids [234]. In the clinic, the administration of plasmids encoding for human proinsulin (BHT-3201) to recent-onset T1D patients reduced the frequency of $CD8^+$ T cells reactive to proinsulin and preserved β-cell function in treated patients [235].

3.3. Cell-based immunotherapies

In the last few years, some cell types with immunomodulatory capacities have been isolated from individuals to modulate or expand them *ex vivo* and reinfuse them with the objective to treat autoimmunity. Importantly, all those therapies need the use of good manufacturing practice (GMP).

3.3.1. Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that have the potential to differentiate to multiple cell lineages [236], with immunosuppressive effect [237]. In the last few years, MSC have been used in various experimental disease models with promising results and consequently they have been transferred to the clinical setting to evaluate their safety and efficacy in cancer, graft-versus-host disease, among others [238]. In NOD mice, it was demonstrated that MSC were able to prevent diabetes [239] and reverse hyperglycemia [240]. In the clinic, an ongoing trial treated newly diagnosed T1D patients with a formulation of allogenic MSCs derived from bone marrow (BM) of adult donors (Prochymal) to establish the safety and efficacy of that product (Clinicaltrials.gov, NCT00690066). However, therapeutic applications of MSC raise questions about their safety for human use.

3.3.2. Regulatory T cells

Tregs play an important role in maintaining peripheral tolerance to self-antigens and in the suppression of excessive responses. Therefore many efforts have been carried out to isolate them from patients, expand them and increase their suppressive function *ex vivo*, to reininfuse them to treat autoimmune diseases. In

animal models, the adoptive transfer of antigen-specific Tregs has been successful in suppressing T1D [241, 242]. In T1D patients, the expansion of Treg cells with suppressive activity is technically possible [243]. In the clinics, *ex vivo* expanded Treg cells have been administered to recent-onset T1D children, without adverse reactions and improved β-cell function [244]. Another ongoing clinical trial is recruiting adult T1D patients, but results are not yet available (Clinicaltrials.gov, NCT01210664). Since Treg treatment for T1D remains promising, it is essential to have robust purity controls to guarantee therapy safety and optimized expansion methods to guarantee their efficacy.

3.3.3. Dendritic cells

Since tolerogenic/suppressive DCs are effector DCs capable of inducing tolerance, they have been used to treat autoimmune diseases. Experimental data in NOD mice have demonstrated the prevention and reversal of T1D with the autologous administration of DCs capable to induce peripheral tolerance. It was reported that DCs isolated from pancreatic lymph nodes prevented diabetes in NOD mice [245]. In addition, different approximations have been used to generate tolerogenic DCs in vitro.

On one hand, DCs have been generated with biologic cytokines and growth factors, acquiring tolerogenic capabilities. As example, DCs derived from BM precursors through *in vitro* culture with GM-CSF and IL-4 culture were capable to prevent diabetes in NOD mice [246]. The proposed mechanism by which these DCs induce tolerance is a shift towards a Th2 response, restoring the balance between pathogenic Th1 cells and protective Th2 cells [247]. The presence of IL-10 and GM-CSF in the culture media also provided the combination to generate suppressive DCs, capable to promote T cell tolerance and prevent experimental T1D [248]. Other approximation used thymic stromal lymphopoietin (TSLP), a member of the IL-7 cytokine family expressed by thymic epithelial cells among others, to condition DCs. TSLP conditioned DCs induced differentiation of Tregs and protected NOD mice from diabetes development [249].

On the other, DCs have also been genetically modified to promote tolerogenic function. DCs deficient in the nuclear transcription factor-κB activity did not mature, secreted low amount of cytokines, showed decreased allostimulatory activity and prevented T1D in NOD mice [250]. Another approach used DCs transduced to express IL-4 to prevent [251-253] and treat [254] T1D in NOD mice. Tolerogenic DCs deficient in costimulatory molecules were also used. These DCs were modified *ex vivo* with antisense DNA, targeting the primary transcripts of CD40, CD80 and CD86 costimulatory molecules. Their administration prevented [255] and reversed T1D in NOD mice [256].

These results have been the basis for a phase I trial to assess tolerogenic DC safety in the clinic [257]. Established T1D patients were treated with autologous DCs unmanipulated or engineered ex vivo with anti-sense oligonucleotides towards a costimulation-deficient state. DC treatment was safe and well tolerated. In addition, engineered DCs increased the frequency of a potentially beneficial $B220^+$ $CD11c^-$ B cell population in T1D autoimmunity. Importantly, this is the first time that autologous DC therapy achieves clinical use for T1D patients, confirming its safety and opening doors to other DC-based interventions.

3.4. Nanotherapies

In the last years, innovative experimental approaches based on nanotechnology have been developed to modulate immune response, and some of them have been designed to induce tolerance in T1D. Nanotechnology uses the properties of objects that function as a unit within the overall size range of 1–1,000 nanometres as immunomodulatory agents. The administration of **peptide-MHC complexes** has been explored as an approach to induce antigen-specific tolerance in experimental models of T1D. As an example, the administration of iron oxide nanoparticles coated with MHC class I molecules loaded with peptides relevant for T1D prevented and reversed diabetes in NOD mice, by expanding a regulatory $CD8^+$ T population that naturally occurs in the progression of autoimmunity [157].

Another approximation used **microspheres** loaded with antisense oligonucleotides to CD40, CD80 and CD86 costimulatory genes, and prevented and reversed T1D in NOD mice. Treatment decreased costimulatory molecule expression on DCs and generated Foxp3$^+$ Treg cells [258].

In the end, more efforts are required in the search for new immunotherapies, which ideally should be antigen-specific, safe and stable to avoid unwanted side effects, customizable, easy to produce and economically reasonable.

4. EFFEROCYTOSIS: THE PHAGOCYTOSIS OF APOPTOTIC β-CELLS BY DENDRITIC CELLS

Efferocytosis is a crucial process by which apoptotic cells are cleared by phagocytes, maintaining immune tolerance to self in the absence of inflammation [259]. Apoptosis is the physiological and programmed cell death that occurs throughout life in essentially all tissues and has a key role in maintaining tissue homeostasis. Despite the constant turnover of cells through apoptosis, apoptotic cells are rarely seen under physiological conditions because of a high rate of apoptotic cell clearance by phagocytes. Phagocytes can be classified into tissue-resident professional phagocytes (such as neutrophils, monocytes, macrophages and DCs) or by neighbouring non-professional phagocytes (such as fibroblasts, epithelial cells and endothelial cells) [260]. When a cell dies through apoptosis, it maintains their membrane integrity, without the release of their content DAMPs. Under physiological circumstances, the phagocytosis of apoptotic cells by DCs results in an immunological silent event, contributing to induce specific tolerance rather that autoimmunity [261].

Evidence that links apoptosis and tolerance has been reflected in several studies where the administration of specific apoptotic cells made *ex vivo* demonstrate tolerance induction in T1D [262], inflammatory arthritis [263], in induced lung inflammation [264, 265] and sepsis [266]. In addition, strategies that induce

apoptotic cells in situ in models of inflammation have shown potential benefits [267, 268].

4.1. Apoptotic cell clearance

Over the last years, new insights into the engulfment process of apoptotic cells by phagocytes have been reported [260]. *In vivo* cell clearance is performed through four steps: the sensing of corpses, their recognition, the engulfment, the digestion and the final response of the phagocyte that keeps cell clearance "immunologically silent" **(Figure 4)**.

First step: The apoptotic cells send "find me" signals to recruit phagocytes, in combination with "keep out" signals, to inhibit the recruitment of inflammatory cells as neutrophils [269] and maintain the apoptotic clearance process as a non-inflammatory process. Nucleotides, such as ATP and UTP [270], the intercellular adhesion molecule 3 (ICAM-3) [271] and CX_3CL1 [272] have been described as "find me" signals.

Second step: Apoptotic cells express "eat me" signals to trigger phagocytic uptake. Many of them have been described, but the most studied and universally detected is the phospholipid PS [273-275]. Viable cells kept PS on the inner leaflet of the cell membrane and only after apoptosis induction, PS is exposed outer leaflet of the cell membrane, by increasing more than 280-fold within only a few hours after induction of apoptosis [276]. Many receptors that recognize PS on apoptotic cells have been described on the surface of phagocyte cells, such as CD36, CD14, CD68 and αVβ3 integrin [275], as well as the recently described as brain-specific angiogenesis inhibitor 1 (BAI1) [277], members of the T cell immunoglobulin mucin domain (TIM) protein family including TIM-1 and TIM-4 [278, 279] the Stabilin-2 [280] and the receptor for advanced glycation end products (RAGE) [281] among others. In addition to "eat me" signals, "don't eat me" signals on the surface of living cells help phagocytes to distinguish between live and dead cells. The interaction between phagocytes and apoptotic can also be influenced by the

presence of opsonins, which are serum derived proteins that can bind to the cell surface of the apoptotic cell to enhance phagocytosis. Complement proteins, such as C1q and iC3b have been associated to enhanced phagocytosis and induction of tolerance by DCs [282, 283]. Antibodies, collectins, pentraxins and anticoagulant proteins can also influence the apoptotic cell clearance process [284].

Third step: The engulfment process requires signaling pathways leading to the rearrangement of the phagocyte cytoskeleton necessary for corpse internalization.

Fourth step: The apoptotic cell is digested through the phagosome maturation, which becomes increasingly acidic, ultimately fusing with lysosomes, which contain the digestive enzymes [285]. Finally, the processed apoptotic cells peptides are presented by MHC class II molecules, but also cross-presented by MHC class I molecules, contributing to maintain self-tolerance [286, 287]. Normally, antigens from exogenous proteins phagocytosed by APCs are presented in the context of MHC class II molecules to $CD4^+$ T cells and antigens from endogenous proteins are presented through MHC class II molecules to $CD8^+$ T cells. However, exogenous proteins can also be presented through MHC class I molecules in a process named cross-presentation. In this sense, it was proposed that $CD8^+$ DCs, which are the ones specialized for uptake dying cells, are much better than $CD8^-$ DCs for cross-presentation on MHC class I [288].

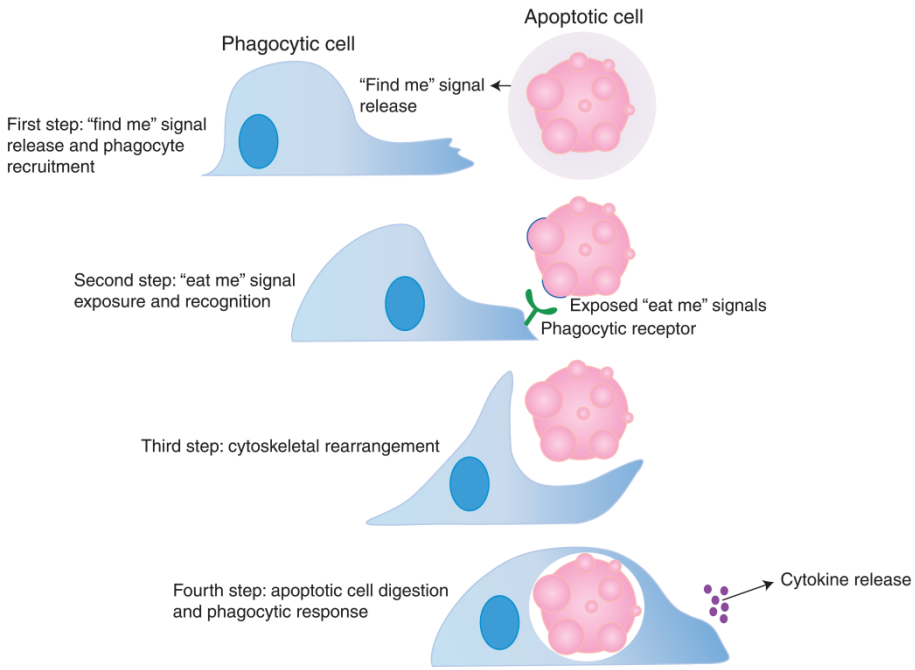

Figure 4. The steps of apoptotic cell clearance. Adapted from A Hochreiter-Hufford et al., 2013, Cold Spring Harb Perspect Biol [289].

There are potential variables that occur with the cell death that are important in the subsequent immune modulation [260]: 1) The "quality" of apoptotic cells, which includes the cell type, the cause of cell death and the activation status of the dying cells. It determines which type of "eat me" signals is being exposed to the phagocytes. 2) The ratio of apoptotic cells, which can determine the magnitude of resulting immune response. 3) The apoptotic cell microenvironment that determines which phagocyte is in charge to mediate clearance and regulate the immune response. 4) The timing of cell death and duration of apoptotic cell-derived signals. Therefore, the specific conditions of the cell death can determine if apoptotic cells promote immunity or induce tolerance.

4.2. Role of dendritic cells in self tolerance

As it has previously mentioned, DCs are innate immune cells that play a dual role in the immune system: DCs play a primordial role in the initiation of the immune

response by presenting antigenic peptides when activated, but in absence of inflammation, immature DCs are essential to maintain tolerance to self. Comprehension of the DCs role in the induction of T cell tolerance will be briefly described to establish the basis for DCs further use as an immunomodulatory therapy.

cDCs are professional APCs. They have a high phagocytic activity as immature cells, with low expression of MHC class II and costimulatory (CD80, CD86) molecules, and a broad range of PRRs, which recognize a wide range of microbial structures. Once activated, mature DCs decrease endocytosis capacity and increase the expression of MHC class II and costimulatory molecules to enhance antigen presentation, together with production of cytokines [290]. Mature DCs also upregulate CCR7 chemokine receptor that enables DCs in peripheral tissues to migrate to the draining lymph nodes [291]. In mice, cDCs express $CD11c^+$ and can be divided in **lymphoid DCs** ($CD8\alpha^+$ cDCs or $CD8\alpha^-$ cDCs) found in thymus, spleen and lymph nodes. $CD8\alpha^+$ DCs play an important role in cross-presentation to cytotoxic $CD8^+$ T cells, whereas $CD8\alpha^-$ DCs are most potent at stimulating $CD4^+$ T cells. **Migratory (or non-lymphoid) DCs** ($CD103^+$ cDCs, $CD11b^+$ cDCs, interstitial cDCs and langerhans cells) reside in peripheral non-lymphoid tissues and migrate to draining lymph nodes. $CD103^+$ cDCs, similar to lymphoid-tissue $CD8^+$ cDCs, are efficient cross-presenting antigens to $CD8^+$ T cells [292, 293].

pDCs are specialized to respond to viral infection through type I interferons (IFNs). They also can act as APCs, but less effectively; pDCs are unable to capture antigens by phagocytosis or macropinocytosis, but instead, they internalize antigens by receptor mediated endocytosis. pDCs also express a narrow range of PRRs (TLR7 and TLR9) and low levels of MHC class II and costimulatory molecules in steady a state. In mice, pDCs express intermediate levels of CD11c and they are positive for B220 (CD45RA) and LY6C and they selectively express BST2 (CD317) and SIGLEC-H (PDCA-1) [293, 294].

4.2.1. Dendritic cells in central tolerance

DCs are crucial in central tolerance in the thymus. A subset of thymic-resident DCs, which are differentiated from thymic lymphoid precursor cells were described [295] and defined by the expression of CD8α and Sirpα molecules [296]. Thymic DCs capture peripheral tissue antigens from mTECs and cross-present them to delete autoreactive thymocytes [297]. It was proposed that thymic DCs acquire PTA by phagocytosing apoptotic mTECs [298, 299]. Moreover, cytokine environment in the thymus, such as TSLP produced by Hassall's corpuscles in the thymic medulla instruct DCs to induce Tregs [300]. In addition, it was proposed that peripheral DCs can also migrate into the thymus and contribute in negative selection and Tregs generation [301, 302].

4.2.2. Dendritic cells in peripheral tolerance

It is well known that some autoreactive T cells escape from selection in the thymus. Therefore, additional mechanisms are crucial in maintaining peripheral tolerance to self [303]. DCs have a key role on this process. Understanding these mechanisms is a crucial to develop new strategies to recover tolerance to self, lost in autoimmune diseases.

The induction of T cell response depends on the maturation stage of the DCs. DCs are, in absence of inflammation, in an immature stage, capable to deliver only signal 1 in antigenic presentation to T cells, thereby tolerizing to the presented antigen. This can leads to a state of hiporesponsiveness (anergy) of the autoreactive T cells, characterized by an active repression of the TCR signalling and the IL-2 production [304]. However, this bimodal model is too simple and tolerance induction in the periphery is a complex process that involves several molecular mechanisms to arrest autoreactive T cells. The balance among Th cell subsets (immune deviation) producing different patterns of cytokines is also relevant in the maintenance of tolerance. While Th1/Th17 phenotype has been associated to pathogenic immune response, Th2 phenotype can promote anti-

inflammatory cytokines and counteract autoimmunity [305]. Elimination (deletion) of autoreactive T cells [306] and the induction of Tregs play important roles in tolerance induction [307].

Tolerogenic/suppressor DCs -reffered as tolDCs- are effector DCs capable of inducing peripheral tolerance. The identity of tolerogenic DCs has been a matter of debate in the last years and much effort is being done to define specific biomarkers for tolDCs for clinical use. At present, tolDCs can be characterized by the low expression of costimulatory molecules (CD40, CD80, CD86), reduced expression of pro-inflammatory cytokines (IL-12, IL-1, IL-6, TNF) and low capability to induce T cell proliferation [294]. However, it is a simplified vision and sometimes controversial.

The molecular mechanisms of tolDCs to induce tolerance in the periphery include an antigenic presentation with inadequate costimulation, inducing anergy or apoptosis to autoreactive T cells. However, tolDCs are not immature DCs, since they have an active role in the tolerance induction. TolDCs have been associated with the secretion of anti-inflammatory molecules (IL-10, TGFβ) involved in the differentiation of Tregs [308], the production of tryptophan metabolites through the action of indoleamine 2,3-dioxygenase (IDO), which can suppress T cell response and induce Tregs [309, 310], the synthesis of retinoic acid, a metabolite of vitamin A, related to Treg induction [311, 312] and the secretion of prostaglandin E_2 (PGE_2), a lipid mediator derivate of arachidonic acid, involved the regulation of the immune response [313] among others.

Moreover, tolDCs can also express several membrane receptors that may contribute to peripheral tolerance induction. Among them, the programmed death-1 ligands (PD-L1 and PD-L2) negatively regulate T cell activation through engagement of the immunoinhibitory PD-1 [314, 315] and might contribute to generate Tregs [316], the inhibitory immunoglobulin-like transcript (ILT) receptors (ILT3, ILT4), which inhibit T cell response [317] and can stimulate the

differentiation of Treg [318], as well as the expression of FasL (CD95L) that can trigger T-cell death [319].

In the last decades, many efforts have been carried out to simulate biological conditions to generate tolDCs *in vitro*, to understand their biology and test them in experimental immunotherapies. Anti-inflammatory biologicals that are physiologically present in tolerogenic situations have been used as strategy. For example, IL-10, TGF-β, the active vitamin D3 metabolite, as well as, estrogen, TSLP, GM-CSF, PGE_2 and TNF-α, resulted effective in promoting tolDCs to induce Treg [307]. Pharmacologic agents can also affect DC immunogenicity by intervening in their maturation. Glucocorticoids (GCs) used as immunosuppressants in clinical practice, such as prednisolone or dexamethasone condition tolDCs cells to promote Tregs [320]. Cellular metabolism also plays an important role in DC immunogenicity. The inhibition of the serine/threonine protein kinase named mammalian target of rapamycin (mTOR) by rapamycin exerts immunosuppressive effects and promotes tolDCs [321]. Finally, genetic modifications have been attained to force expression of tolerogenic factors in DCs by transducing the expression immunoregulatory molecules, such as IL-4, IL-10 and TGF-β among others, or silencing immunogenic molecules by antisense oligonucleotides (ODNs) and small interfering RNA (siRNA) against costimulatory or proinflammatory genes, among others [322].

4.2.3. Dendritic cells, apoptotic cells and tolerance

A physiological mechanism involved in tolDCs generation is the uptake of apoptotic cells [261, 323], which has been proposed to suppress DC immunogenicity **(figure 5)**. Other studies have confirmed that the capture of apoptotic cells by immature DCs turn them into tolerogenic DCs [324]. It was shown that apoptotic-loaded DCs expressed low levels of MHC class II molecules and costimulatory molecules CD40, CD80 and CD86, even after the exposure to some proinflammatory stimulus [325], secreted low amounts of proinflammatory cytokines IL-1α, IL-1β, IL-6, IL-12p70 and TNF-α [326], decreased their ability to

stimulate T cell proliferation [327] and induced Tregs [328]. Moreover, it was reported that efferocytosis can induce DCs to secrete immunosuppressive mediators such as TGF-β, which triggers the differentiation of $Foxp3^+$ Tregs [329], IL-10 [330], nitric oxide [331, 332] or IDO [333].

It was also reported that the tolerogenic effect acquired by DCs after efferocytosis resulted in tolerance induction in vivo. The uptake of apoptotic DCs by viable DCs suppressed their maturation and promoted tolerance through the secretion of TGF-β and induction of Tregs [334]. In the context of transplant immunology, the administration of donor apoptotic cells prolonged cardiac allograft survival in mice [335] and improved chronic allograft vasculopathy [336] through the interaction with recipient DCs. In addition, recipient DCs loaded with donor-derived apoptotic cells restrained allorecognition in cardiac allograft transplant [336, 337].

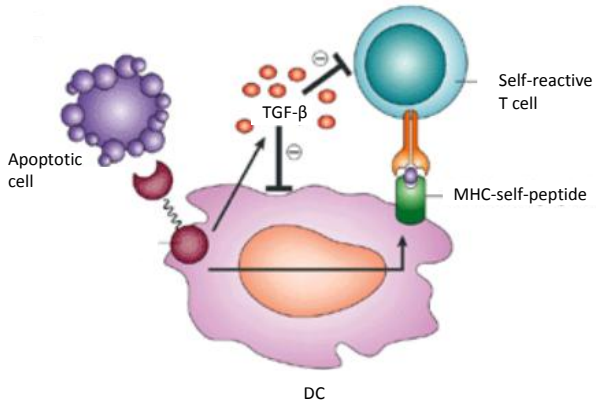

Figure 5. Tolerogenic DCs and and apoptotic cells. Adapted from L Gorelik and RA Flavell, 2002, Nat Rev Immunol [338].

4.3. Liposomes as synthetic approach to apoptotic cells

Liposomes are vesicles composed of a lipid bilayer with a hydrophilic center. They have been broadly used as drug delivers, mainly applied in cancer [339], but also as in immunologic adjuvants in vaccines [340] or as immunosuppressive vehicles

[341]. Currently, liposomes formulations are clinically approved for cancer treatment or fungal infections [342].

4.3.1. Liposomes and immune response

Liposomes have the advantages of being easy to prepare and administer, and of constituting a low-cost strategy compared to other current immunotherapies. Moreover, liposomes protect encapsulated antigens from degradation by proteases in the plasma. In general, nanoparticles can be engineered specifically to avoid, inhibit or enhance the immune responses. Particle size, surface charge, hydrophobicity/hydrophilicity, and the effects of particle coating can determine its effects in the immune system **(Figure 6)** [343]. It has been proposed that particle size affects particle immunogenicity [344]. As an example, small polystyrene particles (<100 nm) were more prone to promote induce CD8 and CD4 T cell responses than larger ones (>0.5 μm), although the latter could induce better antibody responses [344]. In addition, particle size is essential to trigger phagocytosis and avoid cell fusion. It has been shown that larger particles are phagocytosed more efficiently than smaller particles [345]. In addition, cationic or anionic particles are more attractive to phagocyte than neutral particles of the same size [346]. Surface charge of the particles not only influences in their uptake by phagocytes, but also in their effect on the immune system. It has been reported that cationic liposomes are much more potent in generating immune response than anionic or neutral liposomes [347]. Polymers such as poly(ethylene glycol) (PEG) have been attached to liposomes to provide an hydrophilic environment, hiding them from immune recognition and allowing them to circulate for longer periods of time in the blood circulation after intravenous administration, which is important in the use of liposomes as drug delivery vehicles [348, 349]. The advantage of liposomes is that they can be customized for a specific purpose and can be combined with specific ligands, such as antibodies, peptides, and glycoprotein, to target their deliver to APCs, thus improving the immune response [350-352].

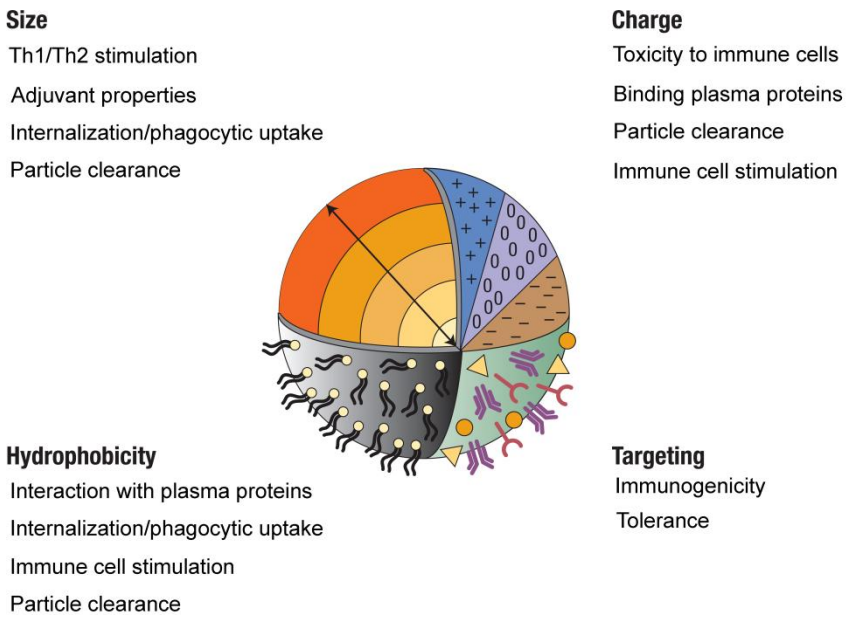

Figure 6. Liposomes properties: size, charge, hydrophobicity and targeting determine their interaction with the immune system. Adapted from Dobrovolskaia M, 2007, Nat Nanotechnol [353].

When liposomes are administered to an organism and enter into the bloodstream, they immediately interact with a complex environment of plasma proteins and immune cells. Liposomes uptake may occur by phagocytes in the circulation or in tissues. It can be facilitated by the adsorption of opsonins, such as immunoglobulins and components of the complement system, into the surface of the particle [354]. Particles whose surfaces were not modified to prevent adsorption of opsonins were removed from the bloodstream within seconds by phagocytes [355]. When liposomes are quickly cleared from the blood, interaction with their components is minimized; however, hemolysis, thrombogenicity and complement activation are conditions that could influence their biodistribution and has to be taken into consideration [354].

4.3.2. Phosphatidylserine-liposomes in autoimmunity

There are many possible lipid formulations suitable for liposome manufacturing. The choice of lipids for liposome prepatration is entirely dependent on the system to be studied. It is well known that phosphatidylserine (PS) exposition outer leaflet of the cell membrane of apoptotic cells triggers phagocytosis, induces an anti-inflammatory milieu and contributes to maintain self-tolerance [274]. Therefore, anionic PS-containing liposomes have been used to mimic apoptotic cells in a very basic way.

Several groups have demonstrated that the use of PS-liposomes may inhibit immune responses through downmodulation of macrophage and DC activation and the production of anti-inflamatory mediators. It was shown that PS-liposomes inhibited *in vitro* maturation of human DCs and impaired the capability to stimulate allogeneic T cell proliferation and to activate IFN-γ production by $CD4^+$ T cells [356, 357]. Other studies have found that PS-liposomes induce the release of TGF-β1 and PGE_2 by macrophages, microglia and osteoclast precursors [358-362]. The anti-inflammatory effect of PS-liposomes was demonstrated: PS-liposomes were able to reduce carrageenan-induced mouse paw oedema, in a partly dependent way on PPAR activation [363] and improve infarct repair in mouse modulating macrophages to an anti-inflammatory state [364]. Since antigen specific PS-liposomes resemble apoptotic cells in inhibiting maturation and immunostimulatory function of DCs, these liposomal microparticles could be optimum vehicles to restore tolerance to autoantigens thus preventing the progression of autoimmunity.

HYPOTHESIS AND OBJECTIVES

HYPOTHESIS

There is no cure or prevention for type 1 diabetes, so patients will need exogenous insulin administration for the rest of their lives. An important medical goal is the development of immunotherapies capable to arrest the autoimmune attack by recovering tolerance to autoantigens, thus allowing β-cell recovery. Physiological mechanisms contributing to the maintenance of tolerance to self have been characterized. Among them, apoptosis that results from cell turnover and the ability of antigen presenting cells not only to activate lymphocytes, but also to tolerize T cells to self-antigens are key factors contributing to minimizing autoimmune reactions.

Based on these two traits of the immune system, the **hypothesis** of this work is that tolerogenic features of dendritic cells acquired after apoptotic cell clearance -efferocytosis-, will arrest autoimmune mechanisms in type 1 diabetes and will restore tolerance to β-cells.

OBJECTIVES

The **main aim** of this study was to generate an antigen-specific immunotherapy based on the inherent tolerogenic features of apoptosis to reestablish tolerance to β-cells in type 1 diabetes.

To accomplish the main aim, **specific objectives** were arranged in two groups.

Cell-based immunotherapy

1. To generate a cell-based immunotherapy by efferocytosis with dendritic cells loaded with apoptotic β-cells for type 1 diabetes.
2. To elucidate the tolerogenic effects of efferocytosis in dendritic cells in terms of phenotype, cytokine secretion, T cell proliferation induction and stability.
3. To determine the transcriptome of dendritic cells after the capture of apoptotic β-cells, in order to define molecular mechanisms and biomarkers of tolerogenicity.
4. To assess the biodistribution and the effects of dendritic cells loaded with apoptotic β-cells in the prevention and treatment of type 1 diabetes.

Liposome-based immunotherapy

5. To optimize cell-immunotherapy by developing a synthetic strategy based on liposomes that mimic apoptotic β-cells in terms of membrane composition, uptake and antigenic content.
6. To characterize the tolerogenic effects of liposomes after their phagocytosis by dendritic cells in terms of phenotype, cytokine secretion, T cell proliferation induction and stability.
7. To determine the liposome biodistribution and to assess its preventive effect in type 1 diabetes.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. MICE

The NOD mouse colony was established by breeding NOD/LtJ mice obtained from the Jackson Laboratory (Bar Harbor, ME, USA). NOD mice develop spontaneous diabetes after 12 weeks of age with higher incidence in females than males. Transgenic NOD RIP-IFNβ mice that express IFN-β in their β-cells (RIP-IFNβ) were generated by our group by back-crossing CD-1 RIP-IFNβ (kindly provided by Dra. Fàtima Bosch, CBATEG, Autonomous University of Barcelona [75]) on to the NOD background [76]. NOD RIP-IFNβ mice develop spontaneous accelerated autoimmune diabetes after 3 weeks of age, with similar incidence in males and females. Mice were kept under specific pathogen-free (SPF) conditions. Temperature and humidity were maintained between 19-23ºC and 40-60% respectively, and light cycle was maintained at 12-hour light on / 12-hours dark. Mice were fed irradiated teklad global 18% protein rodent diet (Harlan, Indianapolis, IN, USA) *ad libitum* and acidic water at pH 5. The Catalan Government's guidelines for the use and care of laboratory animals were followed and protocols were approved by our Institutional Animal Care and Use Committee.

2. REAGENTS

Cells were cultured in RPMI-1640 (PAA: The Cell Culture Company, Pasching, Austria). Complete RPMI medium was supplemented with 10% of fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin (Normon SA, Madrid, Spain), 100 µg/ml streptomycin (Laboratorio Reig Jofre, Sant Joan Despi, Spain), 2 mmol/l glutamine (Sigma, Saint Louis, MO, USA), 1 mmol/l sodium pyruvate (Gibco) and 25 µmol/l β-mercaptoethanol (Sigma). Hypotonic buffer is 140 mM NH_4Cl (Panreac, Barcelona, Spain) and 16.8 mM Tris (Sigma). TE buffer is 10 mM Tris-HCl (Sigma) and 1 mM EDTA (Sigma). Recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF) (Prospec, Rehovot, Israel) was added at 1000 U/ml to all DCs cultures for the differentiation of BM progenitor

cells into DCs. Carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes, Invitrogen, Carlsbad, CA) was used at final concentration of 0.625 μM to label NIT-1 cells or SV-T2, previously to co-culture by DCs, and T cells, in T cell proliferation assays. Proinflammatory stimuli lipopolysaccharide (LPS) (Sigma), poly I:C (P(I:C)) (InvivoGen, San Diego, CA, USA) or zymosan (Zy) (InvivoGen) were added at 100 ng/ml, 0.5 μg/ml and 1 μg/mL respectively, for 22-24h, in the indicated cell cultures. Mitogen stimuli poly(methyl acrylate) (PMA, 25ng/ml, Sigma) and ionomycin (IO, 250ng/ml, Sigma) were used in T cell proliferation assays. The COX-2 inhibitor (NS-398; Sigma) was added at 10 μM to indicated suppression assays. Purified PGE_2 (Cayman Chemical, Ann Arbor, MI) was added at 50 pg/ml, 250pg/ml, 50 μg/ml and 250 μ/ml to indicated suppression assays. Insulin peptides were obtained from Genosphere Biotechnologies (Paris, France) and were >95% pure, with trifluoroacetic acid removed. Peptide A is from insulin A chain (21 AAs, GIVDQCCTSICSLYQLENYCN) and peptide B is from insulin B chain (30 AAs, FVKQHLCGSHLVEALYLVCGERGFFYTPMS). 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS) and 1,2-didodecanoyl-sn-glycero-3-phosphocholine (DLPC) were purchased from Lipoid (Steinhausen, Switzerland). Cholesterol (CH) was purchased from Sigma Aldrich. Lipid-conjugated fluorescent dye Oregon Green 488 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE) was purchased from Invitrogen (Carlsbad, CA, USA). Alexa Fluor 750 was obtained from Invitrogen in its succinimidyl ester form and was conjugated with the lipid 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) supplied by Avanti Polar Lipids (Alabaster, AL, USA). Rapamycin (Rapamune, Pfizer Inc, Bedminster, NJ, USA) was orally administered to NOD mice at the clinical onset of diabetes at a dose of 2.5 mg/kg by gavage (gauge 20G, Fine Science Tools, Foster City, CA, USA).

3. CELL CULTURE

3.1. Cell lines

The NIT-1 cell line, derived from and insulinoma from NOD/Lt mice, was chosen because of its expression of β-cell-specific autoantigens [365]. NIT-1 is a pancreatic

β-cell line established from NOD/Lt mice harboring a hybrid rat insulin-promoter/SV40 large T-antigen gene, which spontaneously develop β-cell adenomas. Immunocytochemical staining of NIT-1 cells showed most contained insulin, with less than 5% containing glucagon. In addition, sera from diabetic NOD mice stained NIT-1 cells and $CD8^+$ T cells from NOD mice were able to recognize and destroy NIT-1 cells *in vitro* [366]. The SV-T2 cell line was obtained from embryonic fibroblasts from BALB/c mice [367] and used as control (ATCC). Both cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in complete RPMI medium, as indicated by the suppliers.

3.2. Dendritic cell generation

DCs were propagated from BM progenitors of 10- to 14-week-old female NOD mice. BM cells were obtained by flushing the tibias and femurs from euthanized mice. After this, erythrocytes were depleted by hypotonic buffer for 5 minutes at room temperature (RT). Recovered cells were washed in phosphate buffer saline (PBS) (1ml PBS, 400xg 5 min) and counted using Perfect Count microspheres (Cytognos SL, Salamanca, Spain). BM cells were cultured at $1x10^6$ cell/ml in complete RPMI medium supplemented with 1000 U/ml GM-CSF for 8 days. Cells were fed every two days by replenishment of half the volume of fresh medium and cytokine. Immature DCs (iDCs) were obtained from DCs cultured in basal conditions and mature DCs (mDCs) were obtained from DCs stimulated with 100 ng/mL of LPS for 22-24 hours. Purity and viability were assessed by flow cytometry, after PeCy7-CD11c staining and PE-Annexin V plus 7-Amino-actinomycin D labelling (7aad) (BD Biosciences), respectively.

3.3. Apoptosis induction and monitoring

NIT-1 or SV-T2 cells were plated at $1.2x10^6$ cell/ml and $0.15x10^6$ cell/cm^2 in RPMI medium and apoptosis was induced by UVB irradiation (10 mJ/m^2) for 45 minutes or 1 hour, followed by overnight (o/n) culture at 37°C, when indicated. Apoptosis

was confirmed by flow cytometry after staining with PE-Annexin V and 7aad. To purify DCs loaded with apoptotic cells by cell sorting, NIT-1 or SV-T2 cells were previously labelled with CFSE (Molecular Probes, Invitrogen, Carlsbad, CA). Briefly, cells were previously washed (1 ml PBS, 400xg 5 min), resuspended in PBS at 5×10^6 cells/ml and incubated with an equal volume of 1.25 µM CFSE (Molecular Probes Europe, Leiden, The Netherlands) for 10 minutes at room temperature and protected from light. Then unbound dye was quenched with an equal volume of complete RPMI medium for 15 minutes at 37ºC. $CFSE^+$ cells were cultured in complete RPMI medium o/n before inducing them apoptosis.

3.4. Efferocytosis and purification of DCs after the uptake of apoptotic cells

Efferocytosis, a process by which apoptotic cells are cleared by phagocytes, was performed by co-culturing DCs with apoptotic NIT-1 cells (NITApo) at 1:3 ratio and $1.2x10^6$ cell/ml, for 2 hours in complete RPMI medium **(Figure 7)**. SV-T2 apoptotic cells were used as antigen-irrelevant source, instead of NIT-1 cell line. DCs that captured $CFSE^+$-NITApo cells ($CD11c^+$ and $CFSE^+$), henceforth denominated NITApo-DCs, were purified by fluorescence-activated cell sorting (FACSAria II, BD Biosciences, San Jose, CA, USA) in all *in vitro* assays. Purity was assessed by CD11c and CFSE positive staining. Viability was assessed by PE-Annexin V and 7aad labelling.

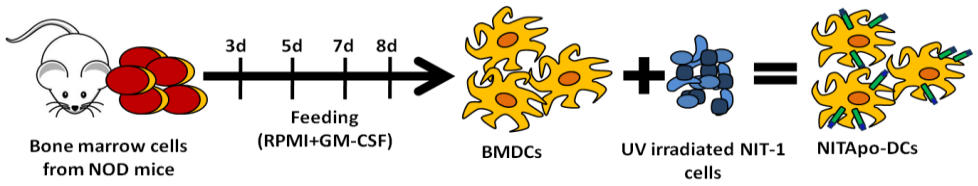

Figure 7. Schematic representation of the generation of DCs and uptake of apoptotic cells (efferocytosis). DCs were differentiated from $1x10^6$ BM cells in complete RPMI medium supplemented with 1000 U/ml GM-CSF for 8 days. Cells were fed every two days by replenishment of half the volume of fresh medium and cytokine. DCs were co-cultured with apoptotic NIT-1 cells (NITApo) at 1:3 ratio for 2 hours in complete RPMI medium and purified by cell sorting (FACSAria II, BD Biosciences).

3.5. T cell obtaining

T lymphocytes were obtained after mechanical disruption of the spleen from NOD female mice. Erythrocytes were depleted by hypotonic buffer for 5 minutes at RT. Cells were washed in PBS (1ml PBS, 400xg 5 min) and filtered at 70 µm with cell strainers (Biologix, Jinan, China). Splenocytes were stained with antibodies to CD19, CD16/32, CD11c, CD11b and Ly-6G(Gr-1) and the non-stained cell population (T cells) was purified by sorting with FACSAria II (BD). Purity was assessed by CD3, CD8 and CD4 staining and viability was assessed by PE-annexin V and 7aad.

4. LIPOSOMES

4.1. Liposome generation

Due to the difficulty in obtaining and standardizing apoptotic cells, liposomes were designed to simulate apoptotic bodies' membrane composition and loaded with insulin peptides, to mimic the effects of apoptotic β-cells in phagocytes. Liposomes were prepared using the thin film hydratation method from a lipid mixture of PS, phosphatidylcholine (PC) and cholesterol (CH) at 1:1:1.33 molar ratio respectively [364] under sterile conditions at Catalan Institute of Nanoscience and Nanotechnology (ICN2, Bellaterra, Spain). The final amount of total lipid was 30 mM. Lipids and lipid-conjugated fluorescent dyes were dissolved in chloroform and the solvent was removed by evaporation under vacuum and nitrogen. The lipids were hydrated with the appropriate buffer (PBS, 0.5mg/mL solution of peptide A or B) and the liposomes thus obtained were homogenized to 1 µm by means of an extruder (Lipex Biomembranes, Vancouver, Canada). The non-encapsulated peptide was removed from the liposome formulation by centrifugation at 110000g for 30 min at 10ºC. Encapsulation efficiencies (EE) were calculated according to the equation $EE(\%)=[(C_{peptide,total}-C_{peptide,out})/ C_{peptide,total}] \times 100$, where $C_{peptide,total}$ is the initial peptide A or B concentration and $C_{peptide,out}$ is the concentration of non-encapsulated peptide. Peptide A or peptide B concentration was quantified by

Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., IL, USA), with some modifications to the manufacturer's instructions. BCA kit combines the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a reagent containing bicinchoninic acid (BCA) in a working range (20-2000µg/mL). Standard curve was performed with stock insulin peptide A or B, for each quantification, and the assay was developed in a NUNC Maxisorp 96 well ELISA plate with 25µl of Standard/Sample and 200µl of BCA working reagent for 1h at 37ºC. The absorbance was measured at 562nm in a spectrophotometer (Varioskan®, Thermo Fisher Scientific). PS-liposomes loaded with insulin peptides A or B were referred to as PSA-liposomes or PSB-liposomes, respectively. Empty liposomes were referred to as PS-liposomes. Particle size distributions and stability of liposomes, expressed as zeta potential (ζ), were measured by dynamic light scattering (DLS) using Malvern Zetasizer, (Malvern Instruments, UK) in undiluted samples. The morphology and lamellarity of liposomes were examined using cryogenic transmission electron microscopy (cryo-TEM) in a JEOL-JEM 1400 microscope (JEOL USA, Inc., Peabody, MA, USA).

4.2. Liposome uptake by DCs

iDCs were cultured in 24-well plates as previously described (see "3.2. Dendritic cell generation") at a final concentration of $1x10^6$ cell/ml with complete medium plus 1000 U/ml GM-CSF. PS- or PSAB-liposomes (empty or loaded with insulin peptides) were added at 100 µM, 300 µM and 1000 µM doses, for 24h, to obtain PS- or PSAB-iDCs. To mature iDCs, LPS was added 2 hours after adding liposomes at 100 ng/ml for the next 22h. After 24h, DCs were extensively washed (1 ml of PBS, 400xg 5 min) and DCs phenotype was assessed or T cell proliferation assays were performed.

5. FLOW CYTOMETRY

Flow cytometry is a technology that simultaneously measures and then analyzes multiple physical characteristics of single particles, usually cells, as they flow in a fluid stream through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence.

5.1. Viability and cell counting

To asses cell viability, cells were washed (1 ml of PBS, 400xg 5 min), resuspended in 50 µl of annexin V binding buffer (BD Biosciences) with 2 µL of PE-annexin V plus 2 µL of 7-Amino-actinomycin D labelling (7aad) (BD Biosciences) and incubated for 20 minutes at RT. Cells were acquired by FACSCanto II flow cytometer (BD Biosciences) using the Standard FACSDiva software (BD Biosciences) and subsequent analyses were performed using FlowJo software, 6.4 version (Tree Star, Ashland, OR, USA) or FACSDiva software (BD Biosciences).

Cell count was performed using Perfect Count microspheres (Cytognos SL, Salamanca, Spain) according to the manufacturer's instructions. Cell count was combined with viability staining, to exclude dead cells, or with a specific membrane staining, to target cells of interest.

5.2. Immunophenotype

Monoclonal antibodies used to phenotype DCs or T cells by flow cytometry are detailed in **Table 2**.

Table 2. Monoclonal antibodies used to phenotype DCs and T cells by flow cytometry.

Antigen	Isotype	Clone	Fluorochrome	Use	Staining	Brand
CD107a	Rat IgG2b, κ	1DB4	Non	2 µg/ml	20' 4ºC	eBioscience
CD11b	Rat IgG2b	M1/70.15	PE	10 µg/ml	20' 4ºC	Immunotools
CD11c	Hamster IgG1, λ2	HL3	PECy7	1 µg/ml	20' 4ºC	BD
CD16/32	Rat IgG2b, κ	2.4G2	PE	2 µg/ml	20' 4ºC	BD
CD19	Rat IgG2a, κ	1D3	PE	2 µg/ml	20' 4ºC	BD
CD25	Rat IgG1, λ	PC61.5	PE	2 µg/ml	20' 4ºC	eBioscience
CD3	Hamster	500A2	V450	2 µg/ml	20' 4ºC	BD
CD4	Rat IgG2b, κ	GK1.5	APC Cy7	2 µg/ml	20' 4ºC	BD
CD40	Rat IgG2a, κ	3/23	APC	2 µg/ml	20' 4ºC	BD
CD8a	Rat IgG2a, κ	53-6.7	PerCP Cy5.5	2 µg/ml	20' 4ºC	BD
CD80	Hamster IgG	16-10A1	FITC	2 µg/ml	20' 4ºC	eBioscience
CD86	Rat IgG2a, κ	GL1	PE	0.2 µg/ml	20' 4ºC	eBioscience
Foxp3	Rat IgG2a, κ	FJK-16s	APC	2 µg/ml	30' 4ºC	eBioscience
Ly-6G	Rat IgG2b, κ	RB6-8C5	eFluor660	2 µg/ml	20' 4ºC	eBioscience
MHC I	Mouse IgG2a	SF1-1.1.1	eFluor 450	2 µg/ml	20' 4ºC	eBioscience
MHC-II	Rat IgG2b, κ	AMS-32.1	APC	2 µg/ml	20' 4ºC	eBioscience

5.2.1. Dendritic cell phenotype

DCs ($2.5x10^5$ – $5x10^5$ cells) were cultured in 24-well plates at a final concentration of $1x10^6$ cell/ml with complete medium plus 1000 U/ml GM-CSF for 24h, with or without specified stimulus. Membrane expression of CD11c, costimulatory molecules CD40, CD86 and major histocompatibility complex (MHC) class II was assessed **(Table 2)**. Fluorescence minus one (FMO), which contains all fluorochromes in a panel except the one that is being measured, was used as a control. Membrane staining protocol proceeds as follows. Cells were washed with 1 ml of PBS, centrifuged at 400xg for 5 minutes and supernatant was decanted. After, cells were resuspended in 50 µl of PBS with monoclonal antibodies and incubated for 20 minutes at 4ºC. Finally, cells were washed (1 ml PBS, 400xg, 5 min) and resuspended in 100 µl of PBS. Stained cells were acquired by FACSCanto II flow cytometer (BD Biosciences) using the Standard FacsDiva software (BD Biosciences). Subsequent analyses were performed using FlowJo software 6.4

(Tree Star Inc., Ashland, OR, USA). Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris.

5.2.2. T cell phenotype

Purified T cells (10^5) were CFSE labelled and co-cultured with autologous DCs at 10:1 ratio in the presence of insulin to evaluate the generation of Tregs (see "6.1. Autologous T cell proliferation assays"). After 7 days, percentages of Tregs were assessed by flow cytometry after membrane staining of CD3, CD8, CD4, CD25, and intracellular staining of FoxP3 **(Table 2)**. Fluorescence minus one (FMO) was used as control. Intracellular staining protocol proceeds as follows. After membrane staining (20 min at 4ºC), cells were washed (1 ml PBS, 400xg, 5 min), and fixed and permeabilized with 500 µl of freshly prepared Fixation/Permeabilization working solution (Fix/Perm, eBioscience) for 1h at 4ºC. Afterwards, cells were washed with 1 ml of 1X Permeabilization buffer (PermBuff, eBioscience), centrifuged (400xg 5 min) and supernatant was decanted. Cells were blocked with 1.25 µl of anti-mouse CD16/32 (Fc Block, eBioscience) in 50 µl of Perm Buffer for 15 minutes at 4ºC. Without washing after blocking step, cells were stained with 1 µl of anti-mouse FoxP3 in 50 µL of Perm Buffer for 30 minutes at 4ºC. Finally, cells were washed (1 ml PermBuff, 400xg 5 min) and resuspended in 150 µl of PermBuff. Stained cells were immediately acquired by FACSCanto II flow cytometer (BD Biosciences) using the Standard FacsDiva software (BD Biosciences). Subsequent analyses were performed using FlowJo software 6.4 (Tree Star Inc.). Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris.

5.3. Phagocytosis assays of liposome capture by DCs

In order to determine whether liposome uptake by DCs is through phagocytosis, *in vitro* assays were performed by co-culturing DCs at $1x10^6$ cell/ml with 100 µM fluorescence labelled with Oregon green 488 DHPE (Invitrogen) PS-liposomes. Capture was assessed by flow cytometry (FACSCanto II, BD) at different times, for

5 min to 4 hours, at 37º and at 4ºC after extensively washing in PBS to remove the liposomes attached to the cell membrane.

6. T CELL PROLIFERATION ASSAYS

6.1. Autologous T cell proliferation assays

To determine the ability of DCs to induce T cell proliferation after the capture of apoptotic cells or liposomes, purified splenic T lymphocytes from NOD mice (10^5) were co-cultured with autologous DCs (10^4 cells). DCs were loaded with 20 µg/ml of insulin (Sigma, St Louis, MO, USA), added in all DCs cultures and left for 24h. To determine the stability of tolerogenic function of DCs, LPS (100ng/ml, Sigma) was used to mature DCs after the capture of NIT-1 apoptotic cells **(Figure 8A)** or after PS- or PSAB-liposomes addition **(Figure 8B)** for the last 22h. After extensive washes, DCs were co-cultured with T cells from NOD mice female spleen at 1:10 ratio in complete RPMI medium. As a control, T lymphocytes were cultured in basal conditions or with mitogen stimuli Poly(methyl acrylate) (PMA, 25ng/ml, Sigma) and Ionomycin (IO, 250ng/ml, Sigma). After 5-6 days, cells were pulsed with 1 µCi of (3H)-thymidine (Perkin Elmer, Waltham, MA, USA) for 16 h. Cells were harvested (Harvester 96, Tomtec Inc., Hamden, CT, USA) and analysed using a scintillation counter (1450 Microbeta, TriluxWallac, Turku, Finland). T cell proliferation was expressed as counts per minute (c.p.m). In another set of experiments, purified T cells (10^5) were previously labelled with CFSE and were co-cultured with autologous DCs (10^4 cells). T cell proliferation was analysed at day 7 by flow cytometry (FACSCanto II, BD). Briefly, T cells were previously washed (1 ml PBS, 400xg 5 min) and resuspended in PBS at 5×10^6 cells/ml. Next, T cells were incubated with an equal volume of 1.25 µM of CFSE for 10 minutes at RT and protected from light. Then, unbound dye was quenched with an equal volume of complete RPMI media for 15 minutes at 37ºC. Finally, $CFSE^+$ cells were washed twice (1ml PBS, 400xg 5 min). Cells were acquired by FACSCanto II flow cytometer using the Standard FacsDiva software and analysed by FlowJo software 6.4.

Samples were gated using forward (FSC) and side (SSC) scatter to exclude dead cells and debris.

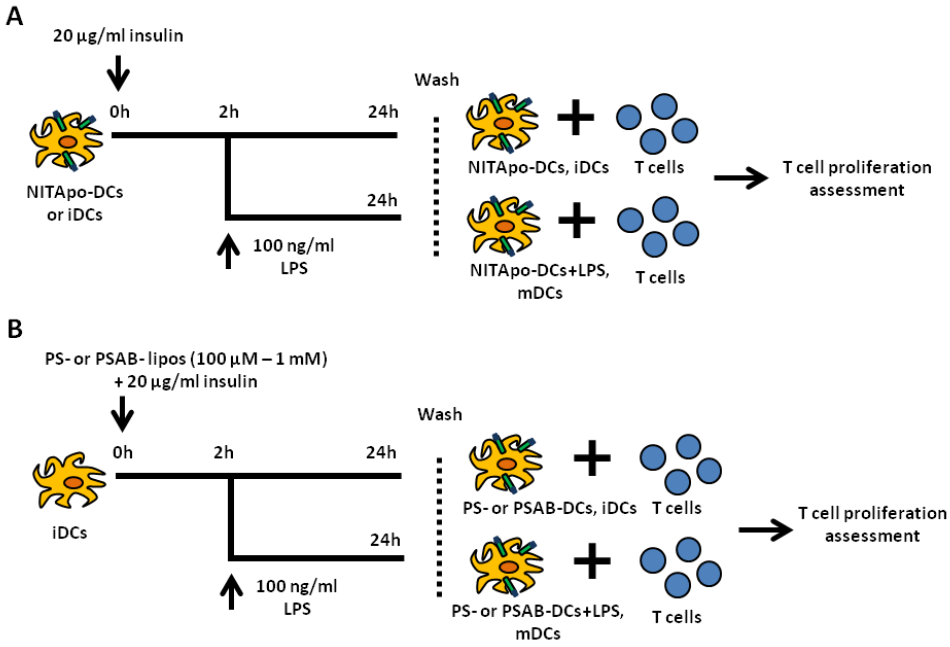

Figure 8. Schematic representation of autologous T cell proliferation assays. (A) Purified NITApo-DCs cells or control DCs were cultured with insulin (20 µg/ml, sigma). After 2h, some DCs were stimulated with LPS (100ng/ml, Sigma) for additional 22h. Finally, DCs were extensively washed in PBS, counted and cultured with T lymphocytes at 1:10 ratio (DCs:Ts) for 6 days. **(A)** iDCs cells cultured with insulin (20 µg/ml, sigma) and co-cultured with PS- or PSAB-liposomes (100 µM – 1mM). After 2h, LPS (100ng/ml, Sigma) was added in some DCs cultures for another 22h. Finally, DCs were extensively washed in PBS, counted and cultured with T lymphocytes at 1:10 ratio (DCs:Ts) for 5 days.

6.2. Stability assays

To determine the stability of the tolerogenic function of DCs acquired after efferocytosis, three maturation stimuli were used in the assays. LPS (100 ng/ml; Sigma), Poly I:C (0.5 µg/ml; InvivoGen, San Diego, CA, USA), or Zymosan (1 µg/ml; InvivoGen) were added to cultures to mature DCs after the capture of NIT-1 apoptotic cells for 22h. Afterwards, cells were washed, counted and proliferation assays were performed as described above (see "6.1. Autologous T cell

proliferation assays"). Results were represented as T cell proliferation index (T cell proliferation of each condition divided by T cell proliferation induced by iDCs).

6.3. Suppression assays

To assess the immunosuppressive ability of DCs after efferocytosis, purified splenic T lymphocytes from NOD mcie (10^5) were co-cultured with autologous mDCs (10^4) in the presence of iDCs or NITApo-DCs at the following ratios 1:2, 1:1, 1:0.5 and 1:0.25 (mDCs: iDCs or NITApo-DCs) for 7 days. All DCs were loaded with 20 μg/ml of insulin (Sigma). After 6 days, cells were pulsed with (3H)-thymidine, harvested and counted as described above (see "6.1. Autologous T cell proliferation assays"). A specific-COX2 inhibitor (NS-398, Sigma) that inhibits PGE_2 production was used to confirm PGE_2 role in suppressive function of NITApo-DCs. NS-398 was added to suppression assays at 10μM. To determine if the mechanism depends on cell-cell contact, supernatants from NITApo-DCs cultures, in which PGE_2 concentration was previously measured by ELISA (PGE_2 EIA Kit-Monoclonal; Cayman Chemical) were added to suppression assays instead of NITApo-DCs in a final concentration of 50 pg/ml and 250pg/ml. When applied, purified PGE_2 (Cayman Chemical) was added at final concentration of 50 pg/ml, 250pg/ml, 50 μg/ml and 250 μ/ml to suppression assays instead of NITApo-DCs.

6.4. Classical Tregs assessment

The percentage of CD4+, CD25+, FoxP3+ Tregs was assessed in cell co-cultures after T cell proliferation experiments (see "6.1. Autologous T cell proliferation assays" and "5.2.2. T cell phenotype"). Briefly, 10^5 purified T cells were co-cultured with autologous iDCs, mDCs and NITApo-DCs with insulin (20 μg/ml) at 10:1 ratio (Ts:DCs). After 7 days, percentages of Tregs were assessed by flow cytometry after membrane staining (CD3, CD8, CD4 and CD25), permeabilization/fixation with Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience) and intracellular staining (FoxP3).

7. IMMUNOMODULATORY FACTORS QUANTIFICATION

The immune system acts through many soluble molecules to immunomodulate immune responses, such as cytokines or lipid mediators like PGE_2. Cytokines are low-molecular-weight (<30 kDa) regulatory proteins or glycoproteins secreted by white blood cells, and various other cells, that that act as mediators between cells. On the other hand, PGE_2 is a potent lipid molecule produced by many cells of the body -including fibroblasts, macrophages and DCs- that affects key aspects of immunity.

7.1. Cytokine production by dendritic cells after efferocytosis

The production of interleukin (IL)-6, IL-10, IL-12 and tumour necrosis factor (TNF)-α was quantified in DCs cultures after efferocytosis, by the bead-based immunoassay BD™ CBA Mouse Flex Set (BD Biosciences). BD™ Cytometric Bead Array (CBA) assays provide a method to detect soluble analytes with beads of known size and fluorescence using flow cytometry. Each capture bead has been conjugated with a specific antibody and present distinct fluorescence intensity for its identification. The detection reagent is a mixture of phycoerythrin (PE)–conjugated antibodies which provides a fluorescent signal in proportion to the amount of bound analyte. After acquiring samples on a flow cytometer, FCAP Array™ software was used to generate results in graphical and tabular format. In our case, four beads with distinct fluorescence intensities in APC and APC-Cy™7 channels have been coated with capture antibodies specific for each cytokine. To determine the amount of those cytokines, DCs ($2.50x10^5$ – $5x10^5$ cells) were cultured in 24-well plates at a final concentration of $1x10^6$ cell/ml with complete RPMI medium plus 1000 U/ml GM-CSF for 24h, with or without LPS stimulus. Culture supernatants were collected and frozen at -80°C until use. Lyophilized mouse cytokine standards were reconstituted, settled for 15 minutes at room temperature and gently mixed by pipette. Serial half dilutions were performed from top standard (2500 pg/ml) to 1:256 dilution (10 pg/ml). Next, all four capture beads were vigorously vortexed and pooled immediately before using them in the assay. Then, samples and

standards were mixed with the same volume of capture beads and incubated for 1 hour at room temperature, protected from light. Finally, the same volume of mixed detector reagent was added to each tube and incubated for another 1 hour at room temperature, protected from light. Finally, samples were washed (1 ml of wash buffer, 200xg, 5 min) and acquired by BD FACSCanto™ II flow cytometer (BD Bioscience). The theoretical limit of detection (pg/mL) was1.4 pg/ml for IL-6, 9.6 pg/ml for IL-10, 3.6 pg/mL for IL-12 and 2.8 pg/ml for TNF.

7.2. Cytokine production in T cell proliferation assays

The amount of **IL-2**, **IL-4**, **IL-6**, **IFN-γ**, **TNF**, **IL-17A** and **IL-10** was measured in the supernatant from T cell proliferation assays by BD™ CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences). In this kit, seven bead types with distinct fluorescence intensities resolved in a red channel of a flow cytometer have been coated with antibodies specific for each cytokine. To quantify those cytokines in T cell proliferation assays, purified T lymphocytes (10^5 cells) were co-cultured with DCs (10^4 cells) with 20 μg/ml of insulin as described previously (see "6.2. Autologous T cell proliferation assays"). At day 5 or 6 of culture, supernatants were collected and frozen at -80ºC until use. To measure cytokines, mouse cytokine standards were reconstituted and serial half dilutions were performed from top standard (5000 pg/ml) to 1:256 dilution (20 pg/ml). Next, all seven capture beads were vigorously vortexed and pooled immediately before using them in the assay. Finally, samples and standards were mixed with the same volume of capture beads and detector reagent in a 96-well polypropylene round bottom plate and incubated for 2 hours at room temperature and darkness. After cytometer set up, samples were washed twice (400 μl of wash buffer, 200xg, 5 min) and acquired by BD LSRFortessa™ flow cytometer (BD Bioscience). The theoretical limit of detection was 0.1 pg/ml for IL-1, 0.03 pg/ml for IL-4, 1.4 pg/ml for IL6, 0.5 pg/ml for IFN-γ, 0.9 pg/ml for TNF, 0.8 pg/ml for IL-17A, 16.8 pg/ml for IL-10.

Transforming growth factor (**TGF**)**-β** was measured by the "sandwich" ELISA Human/Mouse TGF-β1 Ready-SET-Go! (eBioscience). TGF-β is a pleiotropic

cytokine which exists in five isoforms, known as TGF-β1-5. TGF-β1 is the most abundant isoform and is ubiquitously expressed, being highly conserved with 99% homology between the human and murine proteins. TGF-β1 is synthesized as a long precursor polypeptide, which is cleaved to yield the mature protein and the Latency associated peptide (LAP). LAP and mature TGF-β1 remain non-covalently associated through secretion, forming homodimers known as the small latent complex (SLC). This ELISA recognizes the mature/active form of TGF-β1 without association with LAP, so samples require acid-treatment and neutralization to remove LAP from TGF-β1 prior to evaluation in this assay. Purified T lymphocytes (10^5 cells) were co-cultured with DCs (10^4 cells) with 20 µg/ml of insulin as described previously (see "6.1. Autologous T cell proliferation assays"). At day 6 of culture, culture supernatants were collected and frozen at -80ºC until use. TGF-β ELISA was performed according to the manufacturer's instructions on a NUNC Maxisorp 96 well ELISA plate. Samples were acid activated with 1N HCl for 10 minutes at room temperature and neutralized with 1N NaOH. The procedure consists in capture antibody – sample – biotin-conjugated antibody – avidin-horseradish peroxidase (HRP) complex formation. The addition of the tetramethylbenzidine (TMB) substrate solution starts the enzymatic reaction, which was stopped using 1M H_3PO_4. The plate was read at 450 nm, with 570 nm wavelength subtraction in a spetrophotometer (Varioskan®, Thermo Fisher Scientific). The standard curve range was from 1000 pg/ml to 8 pg/ml. Sensitivity was 8 pg/mL.

7.3. Prostaglandin E_2 quantification

The assessment of PGE_2 production by DCs was performed by the competitive immunoassay PGE_2 EIA Kit-Monoclonal (Cayman Chemical). PGE_2 is a primary product of arachidonic acid metabolism in many cells. Like most eicosanoids, it does not exist preformed in any cellular reservoir. When cells are activated, PGE_2 is synthesized *de novo* and released into the extracellular space. To measure PGE_2 production in DCs cultures, cells were cultured in 24-well plates at final concentration of $1x10^6$ cell/ml ($2.5x10^5$ – $5x10^5$ cells/well) with complete RPMI

medium plus 1000 U/ml GM-CSF after the capture of NIT-1 apoptotic cells or after PS- or PSAB-liposomes addition (300 µM and 1000 µM) for 24h after. Culture supernatants were collected and frozen at -80°C until use. PGE_2 quantification was based on the competition between PGE_2 and PGE_2-acetylcholinesterase (AChE) conjugate (PGE_2 Tracer) for a limited amount of PGE_2 monoclonal antibody. Therefore, when the same amount of samples or standard and PGE_2 Tracer was added to each well, the antibody-PGE_2 complex binds to goat polyclonal anti-mouse IgG that has been previously attached to the well. The enzymatic reaction was started when the Ellman's reagent is added to the well and the product of this enzymatic reaction has distinct yellow colour that absorbs strongly at 412 nm (Varioskan®, Thermo Fisher Scientific). All controls: non-specific binding, maximum binding, blank and total activity were performed according to the manufacturer's instructions. The PGE_2 EIA Kit standard curve range from 1000 pg/ml to 7.8 pg/ml and the detection limit (80% B/B_0) was 15 pg/ml. Results were expressed as pg of $PGE_2/10^6$ cells.

8. GENE EXPRESSION PROFILE

Gene expression profile is the measurement of the expression of thousands of genes at once, to create a global picture of cellular function, called transcriptome. Microarrays, a concept and methodology that started to grow after 1995, has became a new tool widely used to provide simultaneous measurement of tens of thousands of messenger RNA (mRNA) transcripts for gene expression analysis.

8.1. RNA isolation and cDNA synthesis and labelling

RNA was obtained from NITApo-DCs and sorted iDCs using RNeasy Micro (QIAGEN, Hilden, Germany). This kit combines the selective binding properties of a silica-based membrane with the speed of microspin technology to purify purifies RNA from small amounts of tissues or cells ($<5x10^5$ cells). Cells from four different mice were used in four paired experiments. RNA was also obtained from NIT-1 apoptotic cells. RNA extraction was performed according to the manufacturer's

instructions, with few modifications. After harvesting, cells were immediately lysed in 350µl lysis buffer (RLT) to prevent unwanted changes in the gene expression profile, vortex and frozen at -70ºC until use. The guanidine-thiocyanate-containing lysis buffer and ethanol create conditions that promote selective binding of RNA to the RNeasy MinElute membrane. Next, lysate was homogenized passing through a 20-gauge needle attached to a sterile plastic syringe 10 times. β-mercaptoetanol, carrier (5 µl of a 4 ng/µl solution) and one volume of 70% ethanol were added to the lysate. It was well mixed by pipetting and transferred into the spin column to allow the RNA binding to the silica membrane. Traces of DNA that may copurify are removed by DNase treatment. After, DNase and any contaminants were washed away, and high-quality total RNA was eluted in 12µl of RNase-free water. With this procedure, all RNA molecules longer than 200 nucleotides are purified, so the procedure enriches for mRNA, since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together make up 15-20% of total RNA) are selectively excluded. RNA integrity, purity and concentration were assessed using the Agilent 2100 bioanalyzer (Agilent Technologies Inc., CA, USA). High-quality RNA sample was observed in the electropherogram **(Figure 9)**. Two well-defined peaks corresponding to the 18S (pink) and 28S (green) ribosomal RNAs were observed, similar to results obtained with a denaturing agarose gel, showing an optimal RNA integrity (RIN) in all samples for microarray experiments.

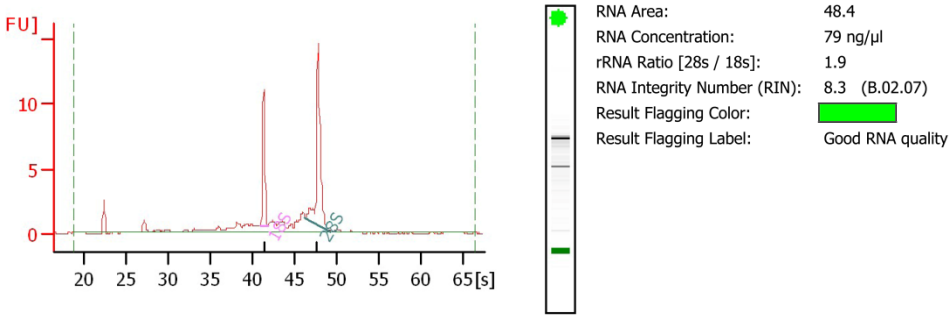

Figure 9. Example of a representative electropherogram of a RNA sample from NITApo-DCs (Agilent 2100 bioanalyzer). [FU]=Fluorescence, [s]= Seconds.

cDNA was synthesized with 50-100ng of total RNA using the WT expression kit (Ambion, Applied Biosystems, CA, USA), according to the manufacturer's instructions. This protocol requires as little as 50 ng sample input and selectively reverse-transcribes non-ribosomal RNA for complete and unbiased coverage of the transcriptome. The kit employs an engineered set of primers that exclude sequences that match ribosomal RNA (rRNA) in the first-strand cDNA synthesis from total RNA. Next, during the second-cycle, first-strand reverse transcription reaction, dUTP is incorporated in the DNA in order to reproducibly fragment the single-stranded DNA. This single stranded cDNA sample is then treated with a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) that specifically recognizes the unnatural dUTP residues and breaks the DNA strand. cDNA is labeled by terminal deoxynucleotidyl transferase (TdT) with the DNA Labeling Reagent that is covalently linked to biotin with the Terminal labelling kit (Affymetrix Inc. Santa Clara, CA). After, cDNA is purified (GeneChipH Sample Cleanup Module, Affymetrix Inc.) and fragmented and checked to verify the integrity with Agilent 2100 bioanalyzer (Agilent Technologies).

8.2. Microarrays hybridization and analysis

Labelled and fragmented cDNA was added to hybridization cocktail (Affymetrix Inc.) containing control oligonucleotide B2 (50 pM) and eukaryotic hybridization controls pre-mixed biotin-labelled bioB, bioC, bioD and cre (1.5, 5, 25 and 100 pM, respectively) from the GeneChip Hybridization Control Kit (Affymetrix Inc.). Eukaryotic hybridizations were heated to 65°C for 5 minutes to completely resuspend the cRNA before aliquoting and hybridization was performed at 99°C for 5 minutes and cooled to 45°C for 5 minutes. Mouse GeneChip 1.0 ST Arrays was equilibrated to room temperature immediately before use and samples were injected into the array and incubated at 45°C, at 60 rpm for 17 hours ± 1 hour. Mouse Gene 1.0 ST Arrays were hybridized and scanned by an Affymetrix G3000 GeneArray Scanner. The Mouse Gene 1.0 ST Array interrogates 28,853 well-annotated genes with 770,317 distinct probes. The design of the array was based

on the February 2006 mouse genome sequence (UCSC mm8, NCBI build 36) and it has 100 percent coverage of NM sequences present in the April 3, 2007, RefSeq database.

Raw expression values obtained from CEL files were pre-processed using the Robust Multiarray Averaging method [368]. These normalized values were used for all subsequent analyses. Experimental data have been uploaded into ArrayExpress for the European Bioinformatics Institute (EBI, http://www.ebi.ac.uk/arrayexpress; E-MEXP-3374). Data were subjected to non-specific filtering to remove low signal and low variability genes. Conservative (low) thresholds were used to reduce possible false negative results. The selection of differentially expressed genes was based on a linear model analysis with empirical Bayes modification for the variance estimates, as described [369]. This method is similar to using a 't-test' with an improved estimate of the variance. To account for the multiple testing probability effects arising when many tests (one per gene) are performed simultaneously, p-values were adjusted to obtain a strong control over the false discovery rate using the Benjamini-Hochberg method [370]. Genes with a p-value ≤ 0.002, adjusted p-value ≤ 0.08 and fold change (FC) ≥ 1.38 were considered upregulated, whereas genes with FC ≤ -1.37 were considered downregulated. The Ingenuity Pathway Analysis (IPA) (Ingenuity Systems H) was used to identify the canonical pathways from the IPA library that were most significant to the data sets.

8.3. Validation of microarray results by qRT-PCR

Total RNA from each sample was reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) **(Tables 3 and 4)**. Quantitative RT-PCR validation was performed with the same microarray RNA samples (<100 ng) and other new RNA samples (>1 μg).

Table 3. List of compounds for the reverse transcription reaction

Component	µl/reaction
10X RT Buffer	2.0
25X dNTPs (100mM)	0.8
10X RT Random Primers	2.0
MultiScribe Reverse Transcriptase (50 U/µl)	1.0
20X Rnase Inhibitor	1.0
Nuclease-free H2O	3.2
RNA	10.0
Total volume	20.0

Table 4. Thermal cycling conditions for the reverse transcription reaction

	Step 1	Step 2	Step 3	Step 4
Temperature	25ºC	37ºC	85ºC	4ºCº
Lenght	10 min	120 min	5 min	∞

In small samples (< 100ng of RNA), a preamplification step was included. cDNA was preamplified using TaqMan PreAmp Master Mix Kit (Applied Biosystems), for each gene-specific target using a pool of TaqMan Gene Expression Assays (Applied Biosystems) as a source of primers **(Tables 5 and 6)**. This preamplification reaction generated approximately a 1.000- to 16.000-fold increase without inducing any bias.

Table 5. List of compounds for the preamplification reaction

Component	µl/reaction
2X TaqMan PreAmp Master Mix	25.0
0.2X Pooled assay mix	12.5
1-250 ng cDNA sample + nuclease free water	12.5
Total volume	50.0

Table 6. Thermal cycling conditions for the preamplification reaction

	Enzyme activation	Preamplification PCR (12 cycles)	
	hold	denature	anneal/extend
Temperature	95ºC	95ºC	60ºC
Length	10 min	15 sec	4 min

The resulting preamplified material was diluted (1:7 in TE buffer) and used as the starting material for the subsequent qRT-PCR reaction, performed on a LightCyclerH 480 (Roche, Mannheim, Germany). Quantitative RT-PCRs were performed under TaqMan universal assay conditions **(Tables 7 and 8)** and using the following TaqMan Assays: Ccr7 (Mm01301785_m1), Ccl5 (Mm01302428_m1), Cd74 (Mm00658576_m1), Cd83 (Mm00486868_m1), Il2ra (Mm01340213_m1), Tnfrsf9 (Mm00441899_m1), Ins2 (Mm00731595_gH) and Iapp (Mm00439403_m1). Ptgs1 (Mm00477214_m1), Ptgs2 (Mm00478374-m1), Alox15 (Mm00507789_m1) and Ltc4s (Mm00521864_m1). The relative quantification was determined by normalizing the expression for each gene of interest to the housekeeping gene Gapdh (Mm99999915_g1), as described in the $2^{-\Delta Ct}$ method [371].

Table 7. List of compounds for the PCR reaction

Component	µl/reaction
20X TaqMan Gene Expresión Assay	1.0
1:7 diluted preamplified cDNA product	4.0
2X TaqMan Gene Expression Master Mix	10.0
Nuclease-free water	5.0
Total volume	20.0

Table 8. Thermal cycling conditions for the PCR reaction

	UDG Activation	AmpliTaq Gold Enzyme Activation	PCR (40 cycles)	
	hold	Hold	denature	anneal/extend
Temperature	50ºC	95ºC	95ºC	60ºC
Time	2 min	10 min	15 sec	1 min

9. BIODISTRIBUTION BY FLUORESCENCE IMAGING

Fluorescence imaging is a technique that allows *in vivo* imaging and dynamically tracking of native, unaltered cells in the animal.

9.1. Bioimage analysis of DCs

To determine if DCs reached the lymphoid organs and sites of autoimmune attack, in vivo and ex vivo near-infrared fluorescence imaging of treated mice was performed using the Pearl Impulse imaging system (LI-COR, Lincoln, NE, USA). Prediabetic NOD mice (8-10 wks) were i.p. treated with 0.5-1x10^6 NIR815 near-infrared fluorescent labelled NITApo-DCs or DCs, as control in 200 µL of saline solution. A Sham-treated mouse was used as control to set background signal. CellVue NIR815 fluorescent cell labeling kit (LI-COR Biosciences, NE, USA) uses proprietary membrane labeling technology to stably incorporate a fluorescent dye with long aliphatic tails (CellVue NIR815) into lipid regions of the cell membrane. CellVue NIR815 excitation and emission spectra is 786 nm and 814 nm, respectively. Briefly, DCs were resuspended in a final concentration of 2x10^7 cells/ml and mixed rapidly with by pipetting with the same volume of 8x10^{-6}M NIR815 dye for 5 minutes. Unbound dye was quenched with an equal volume of complete RPMI medium and after, extensively washed (1 ml PBS, 400xg 5 min). Prior to imaging, mice were anesthetized with ketamine/xylazine at 50 and 5 mg/kg body weight respectively. In vivo imaging was performed at 24, 48 and 72 hours after treatment. At 24 hours after injection, kidney, spleen, pancreas, pancreatic lymph nodes, mesenteric lymph nodes, liver, mediastinal lymph nodes and thymus from a group of mice were harvested, washed in PBS, and imaged ex vivo using the Pearl Impulse imaging system (LI-COR). Fluorescent signal intensity was semi-quantitatively assessed by comparing treated mice to the signal intensity from sham untreated mice.

9.2. Bioimage analysis of liposomes

To determine if liposomes reached the lymphoid organs and sites of autoimmune attack, in vivo and ex vivo near-infrared fluorescence imaging of treated mice was performed using the LiCor Pearl imaging system. Prediabetic NOD mice (8-10 wks) were anesthetized with ketamine/xylazine at 50 and 5 mg/kg body weight respectively, prior to imaging. Mice were given a single intraperitoneal dose of 3.5

mg of A750 fluorescent labelled PS-liposomes in 200 µl saline solution. In vivo imaging was performed at 6 hours, 24 hours and 48 hours after i.p. injection of fluorescence labeled PS-liposomes (Alexa Fluor 750). At 24 hours after injection, perigonadal adipose tissue, kidney, spleen, pancreas, pancreatic lymph nodes, mesenteric lymph nodes, liver, mediastinal lymph nodes and thymus from a group of mice were harvested washed in PBS, and imaged ex vivo using the Pearl Impulse imaging system (LI-COR). Fluorescent signal intensity was semi-quantitatively assessed by comparing treated mice to the signal intensity from untreated mice.

10. IMMUNOTHERAPY FOR T1D

With the aim of prevent and treat T1D, different immunotherapies were use in wild type NOD and transgenic NOD RIP-IFNβ mice **(Table 9)**. The autoimmunity in NOD mice started at 3-4 weeks of age with an initial insulitis with a lymphocytic infiltration into the pancreatic islets. This prediabetic stage was chosen for preventive assays, where the autoimmunity was started and an initial insulitis with a lymphocytic infiltration into the pancreatic islets was found. In treatment assays, mice were treated after the T1D diagnose. Immunotherapy assays with NITApo-DCs were firstly performed in transgenic NOD RIP-IFNβ mice and validated after in wild type NOD mice. Prevention assays with PS-liposomes filled with insulin peptides were performed in wild type NOD mice.

Table 9. Immunotherapies in NOD mice models.

Mouse model	Therapy	Rationale	Prevention	Treatment
NOD RIP-IFNβ	Tol-DCs	TolDCs loaded with NIT-1 apoptotic cells (NITApo-DCs)	YES	YES
NOD	Tol-DCs	TolDCs loaded with NIT-1 apoptotic cells (NITApo-DCs)	YES	YES
NOD	PS-liposomes	PS-containing liposomes with insulin peptides (PSAB-lipo)	YES	NP

NP means not performed.

10.1. Immunotherapy for the prevention of T1D

In preventive assays, tolerogenic DCs after capturing apoptotic cells, or liposomes, and control DCs were extensively washed (1 ml saline solution, 400xg, 5 min) and resuspended in saline solution before injection. A sham control group that received only saline solution was always included. Three preventive assays were performed.

1st preventive assay: Pre-diabetic NOD RIP-IFNβ mice (12–14 days old) were given a single intraperitoneal (i.p.) dose of (1) 10^6 DCs cultured with NIT-1 apoptotic cells (NITApo-DCs), (2) 10^6 non-pulsed iDCs (DCs), (3) 10^6 DCs cultured with SV-T2 apoptotic cells (SVApo-DCs) and (4) saline solution (Sham), all in 150 µl. A total of 11-26 animals, 50% males and 50% females, per group were used.

2nd preventive assay: Pre-diabetic NOD mice (12- to 14-days old) were given a single i.p. dose of (1) 10^6 DCs cultured with NIT-1 apoptotic cells (NITApo-DCs), (2) 10^6 control iDCs (DCs) and (3) saline solution (Sham), all in 150 µl saline solution. A total of 8-11 females per group were used.

3rd preventive assay: Pre-diabetic NOD mice (8 weeks old) were given a single i.p. dose of (1) 3.5 mg of peptide-filled PSAB-liposomes (2) 3.5 mg of empty PS-liposomes empty and (3) PBS, all in 200 µl PBS. A total of 12-26 females per group were used.

10.2. Immunotherapy for the treatment of T1D

Diabetic mice were diagnosed and maintained with daily subcutaneously (s.c.) injected insulin (1U, Insulatard Flex-Pen, Novo-Nordisk, Bagsvaerd, Denmark) unless if normoglycaemia was achieved. Two treatment assays were performed.

1st treatment assay: Diabetic RIP-IFNβ mice received a single i.p. dose of (1) purified 10^6 NITApo-DCs in 150 µl saline and (2) saline solution (Sham), 7 days after

the onset of the disease (time to generate NITApoDCs *in vitro*). A total of 3–4 animals, 50% males and 50% females, were included in each group.

2nd treatment assay: Mice received a single i.p. dose of (1) purified 10^6 NITApo-DCs in 150 µl saline solution 7 days after the onset of the disease. To restrain autoimmunity after the onset until the obtaining of NITApo-DCs, NITApo-DCs immunotherapy was combined with the immunosuppressant rapamycin (Rapamune, Pfizer Inc, Bedminster, NJ), an endogenous inhibitor of mTOR activity. (2) Rapa + NITApo-DCs group was orally treated with rapamycin at the clinical onset of diabetes at a dose of 2.5mg/kg by gavage (gauge 20G, Fine Science Tools, Foster City, CA) for 7 days, once day, after the diabetes onset, and 3-4 weeks, each two days, after cell treatment. (3) Rapa-control group only received rapamune and (4) Sham group was treated with saline solution. A total of 3–6 females were included in each group.

10.3. Metabolic follow up

Mice were monitored daily for urine glucose using Glucocard strips (Menarini, Barcelona, Spain) during a 30-week period. Animals with glucosuria were confirmed diabetic when successive blood glucose levels were higher than 200 mg/dL or when a measure was higher than 360 mg/dL. For treatment protocols, blood glucose levels were monitored weekly (AccuCheck, Roche Diagnostics, Indianapolis, IN). All blood glucose levels were performed after fasting for 2 hours.

10.4. Insulitis score

The degree of islet infiltration by leucocytes (insulitis) was determined in mice at the indicated times. Briefly, pancreata from three to six animals for each group were snap-frozen in an isopentane/ cold acetone bath. Cryosections of 5 µm were obtained at five non-overlapping levels. The sections were stained with haematoxylin and eosin (H&E), coded and analysed by two independent observers who were blinded to the experimental conditions. Each observer assessed a

minimum of 40 islets per animal. Insulitis was scored as described elsewhere [76]: 0, no insulitis; 1, peri-insular; 2, mild insulitis (<25% of the infiltrated islet); 3, 25–75% of the islet infiltrated; 4, >75% islet infiltration.

10.5. C-peptide measurement

The recovery of β-cell function was assessed by measuring C-peptide levels in sera of mice at the end of the study by RayBio® Human/Mouse/Rat C-Peptide Enzyme Immunoassay Kit (Raybiotech, Norcross, GA). C-peptide is the middle segment of proinsulin (31 amino acids) that is made when proinsulin is split into insulin and C-peptide. They split before proinsulin is released from endocytic vesicles within the pancreas and there is one C-peptide for each insulin molecule. Due to the fact that C-peptide has a 2-to-5-time longer half-life than insulin, there are higher concentrations of C-peptide than insulin in the peripheral circulation. Therefore, plasma C-peptide concentrations may reflect pancreatic insulin secretion more reliably than the level of insulin itself. Moreover, c-peptide distinguishes endogenous beta cell secretion from exogenously administered insulin. At the end of follow-up (30 days), after fasting for 2 hours, diabetic mice were sacrificed by cervical dislocation and whole blood was obtained through cardiac puncture. After leaving the blood undisturbed at room temperature to allow the blood to coagulate (15-30 minutes), the clot was removed by centrifugation (600xg, 5 min and 2.300xg, 5 min) and the serum was immediately stored at -80ºC. The C-peptide was quantified by the principle of competitive enzyme immunoassay. The microplate was pre-coated with anti-rabbit secondary antibody. After a blocking step and incubation of the plate with anti-C-peptide antibody, both biotinylated C-peptide and C-peptides from samples or standards interacts competitively with the C-peptide antibody. Uncompeted (bound) biotinylated C-peptide then interacts with Streptavidin-horseradish peroxidase (SA-HRP) which catalyzes a colour development reaction. The intensity of colorimetric signal is directly proportional to the amount of biotinylated C-peptide-SA-HRP complex and inversely proportional to the amount of C-peptide in the standard or samples. This is due to the competitive binding to C-peptide antibody between biotinylated C-peptide and

peptides in standard or samples. Sensitivity of the assay is 772 pg/mL and the reproducibility Intra-Assay: CV < 10% and Inter-Assay: CV < 15%. This ELISA shows no cross-reactivity with any of the cytokines tested: ghrelin, nesfatin, angiotensin II, NPY, and APC. Results were expressed as pg/ml.

11. STATISTICAL ANALISIS

Statistics was performed using the Prism 5.0 software (GraphPad software Inc., San Diego, CA), SPSS Statistics 17.0 software (SPSS Inc. Chicago, IL) and "R" software (www.r-project.org). The analysis of variance (ANOVA) was used for comparisons among multiple groups. For comparisons between two groups, non-parametric Wilcoxon test was performed for paired data. Otherwise, Mann Whitney test was used. Unpaired T test or paired T test were used when specified for unpaired and paired data, respectively. Kaplan-Meier log-rank analysis was used for comparing incidence of diabetes between different groups. A p-value < 0.05 was considered significant.

RESULTS

RESULTS

SECTION I: DENDRITIC CELLS PULSED WITH APOPTOTIC β-CELLS PREVENT EXPERIMENTAL TYPE 1 DIABETES

The first part of this work was focused on the development of an antigen-specific cell-based immunotherapy based on DCs and the tolerogenic potential of apoptotic cells. In order to generate the therapy, DCs were derived from BM of NOD mice and pulsed with apoptotic cells from the β-cell line NIT-1 by efferocytosis; this is a process by which apoptotic cells are cleared by phagocytes. NITApo-DCs were generated and their phenotype, cytokine secretion ability, autologous T cell stimulatory capacity and suppressive function were assessed. Due to the translational potential of NITApo-DCs, LPS was used as inflammatory stimulus to determine the stability of NITApo-DCs in terms of phenotype, cytokine production and autologous T cell stimulatory ability. Gene expression profile of DCs after the capture of apoptotic cells was determined, as well as the role of PGE_2 pathway. Finally, NITApo-DCs were administered to NOD mice and a transgenic variant of accelerated T1D, the NOD RIP-IFN-β mice, in order to determine their effects in diabetes prevention and treatment.

1. Efferocytosis of apoptotic β-cells by DCs

DCs were generated *in vitro* from BM progenitors by culture in GM-CSF for 7 days. DCs obtained using GM-CSF were immature, as previously described [372]. Mature DCs were generated after LPS stimulus. iDCs and mDCs had similar cell size (FSC) and granularity (SSC) and no differences were found in terms of DC differentiation yield, based on staining for the DC marker CD11c **(Figure 10)**. iDCs and mDCs used in this study were >80% pure. Viability was determined by annexin V and 7aad negative staining. iDCs and mDCs used in this study were always >85% viable. iDCs and mDCs differ in phenotype and functionality, as will be shown throughout this work.

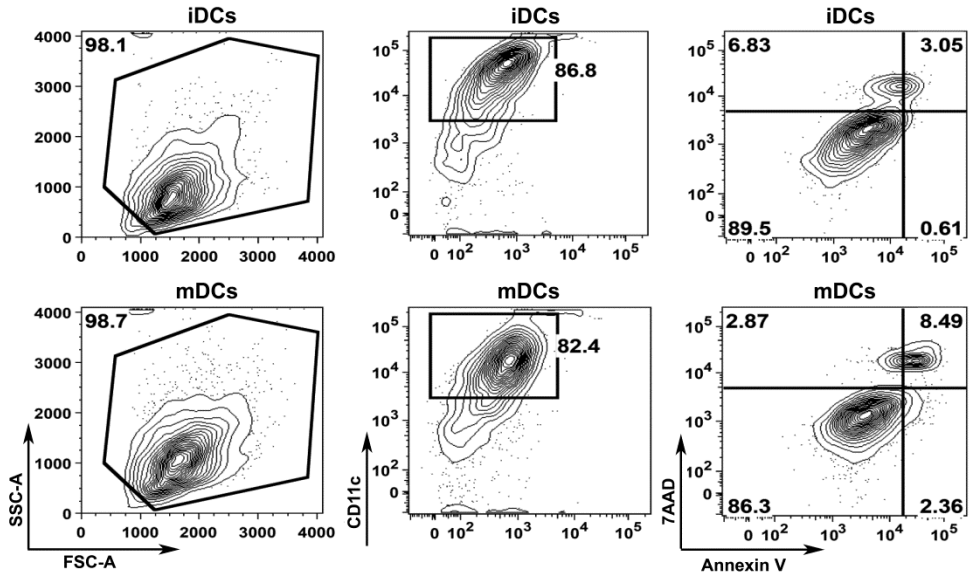

Figure 10. Cell size and granularity, purity and viability of BM derived DCs. Flow cytometry contour plots show cell size (forward scattered light, FSC) and granularity (side scattered light, SSC), purity ($CD11c^+$) and viability (annexin V^-, $7aad^-$) of iDCs and mDCs. Contour plots are from one representative experiment.

The NIT-1 cell line (NOD β-cell line) was used as a source of T1D autoantigen and the SV-T2 cell line (BALB/c fibroblast-cell line) was used as control, as an antigen-irrelevant source. After UVB irradiation, cells showed typical morphological features of apoptosis, since >70% of cells were annexin V positive **(Figure 11A)**. Monitoring of apoptotic cells was performed by labelling NIT-1 cells with CFSE **(Figure 11B)**, previously to apoptosis induction.

iDCs were co-cultured with apoptotic NIT-1 cells to promote phagocytosis. The percentage of DCs that captured apoptotic cells was determined by flow cytometry as CD11c and CFSE positive **(Figure 12A)**. DCs loaded with apoptotic NIT-1 cells were isolated by cell sorting, reaching >95% purity and >90% viability **(Figure 12B)**.

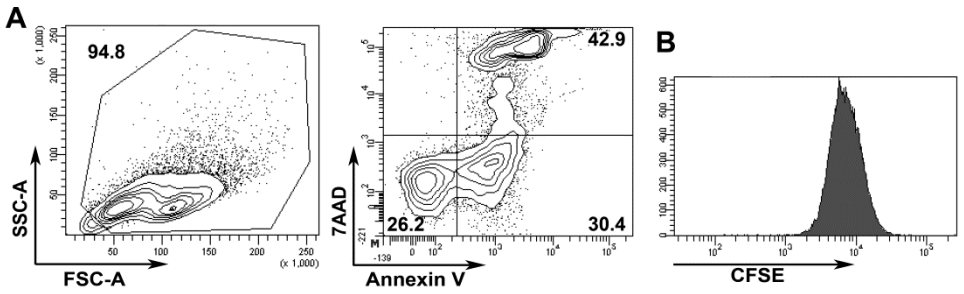

Figure 11. Cell size and granularity, viability and CFSE staining of apoptotic NIT-1 cells. **(A)** Flow cytometry contour plot of cell size and granularity (FSC and SSC) and percentage of apoptotic cells (annexin V^+). **(B)** Flow cytometry histogram of CFSE staining ($CFSE^+$) of NIT-1. Contour plots and histogram are from one representative experiment.

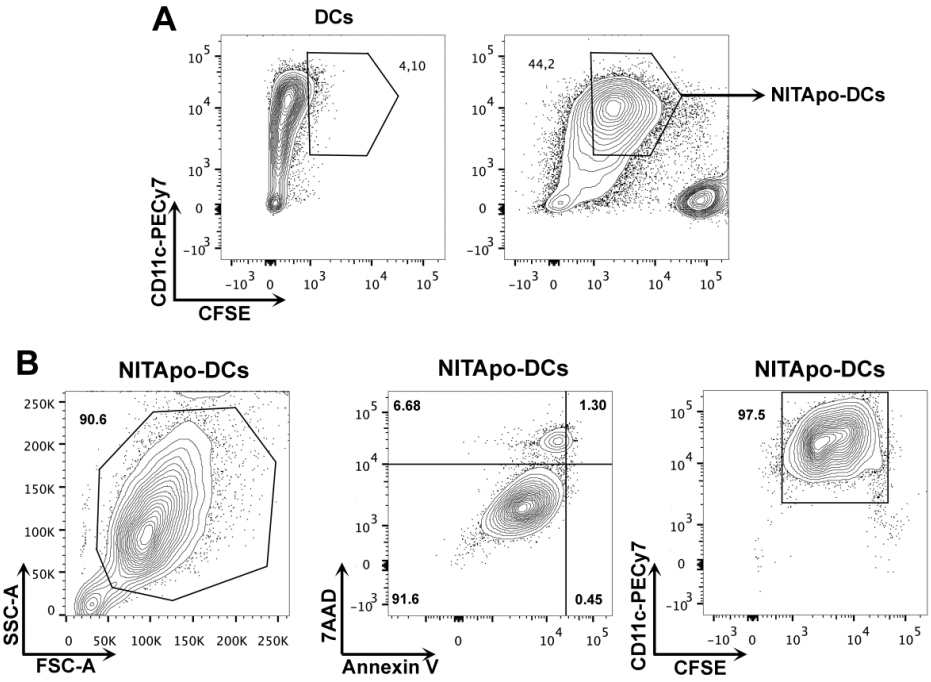

Figure 12. Cell size and granularity, viability and purity of NITApo-DCs. **(A)** Left**:** Flow cytometry contour plot of DCs ($CD11c^+$) before co-culture with apoptotic cells. Right: Flow cytometry contour plot of DCs ($CD11c^+$) after 2 hours of co-culture with apoptotic NIT-1 cells ($CFSE^+$) at 37ºC. **(B)** Flow cytometry contour plot of cell size and granularity (FSC and SSC), viability (annexin V^-, $7aad^-$) and purity ($CD11c^+$ and $CFSE^+$) of NITApo-DCs purified by sorting. Contour plots are from one representative experiment.

2. DCs display a low costimulatory phenotype after the capture of apoptotic cells, even after LPS activation

The membrane expression of costimulatory molecules CD40 and CD86, and major histocompatibility complex (MHC) class II was assessed on NITApo-DCs and compared to iDCs **(Figure 13A)**. NITApo-DCs showed similar expression of CD40 and CD86 molecules in comparison to iDCs **(Figure 13B)**. Therefore, NITApo-DCs had an immature phenotype, expressing low levels of costimulatory molecules after the capture of apoptotic NIT-1 cells.

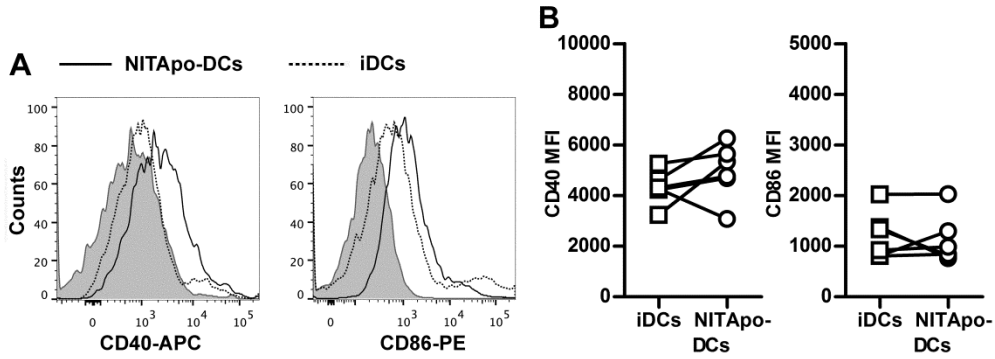

Figure 13. CD40 and CD86 membrane expression on NITApo-DCs. (A) Flow cytometry histograms of CD40 and CD86 membrane expression on iDCs (discontinuous line) and NITApo-DCs (continuous line) in basal conditions. Grey areas are FMOs controls. One representative experiment of six performed is shown. **(B)** MFI of CD40 and CD86 membrane expression on iDCs (white squares) and NITApo-DCs (white circles) in basal conditions from six independent experiments.

LPS was used as inflammatory stimulus to determine the maturation ability of DCs after efferocytosis. The results showed that CD40 and CD86 expression was lower in NITApo-DCs after LPS exposure when compared to mDCs **(Figure 14A)**. Therefore, after LPS exposure, NITApo-DCs were significantly more resistant to increase the expression of costimulatory molecules than iDCs in proinflamatory conditions ($p<0.05$) **(Figure 14B)**. As expected, no differences were found for MHC class II expression due to the previously described feature of BM-derived DCs of NOD mice [373].

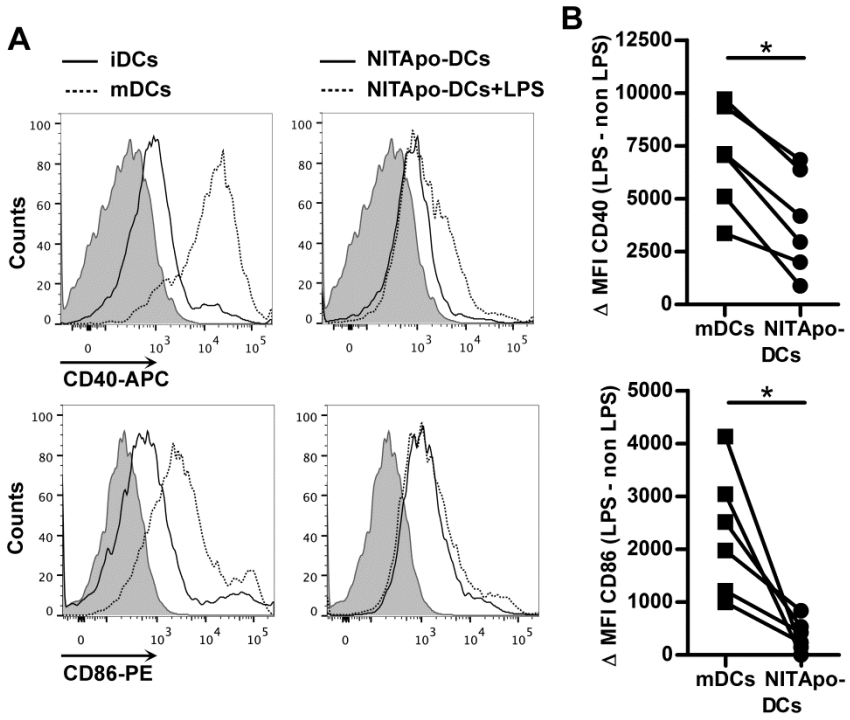

Figure 14. Effect of LPS on CD40 and CD86 membrane expression on NITApo-DCs. (A) Flow cytometry histograms of CD40 and CD86 membrane expression on control DCs (left), NITApo-DCs (right), in basal conditions (continuous line) and after LPS stimulus (discontinuous line). Grey areas are FMOs controls. One representative experiment of six performed is shown. **(B)** MFI of CD40 and CD86 membrane expression on mDCs (black square) and NITApo-DCs (black circles) after 24h of LPS (100 ng/ml) stimulation. Results are expressed as the subtraction between median fluorescence intensity (MFI) of LPS stimulated cells and the MFI of unstimulated cells (ΔMFI). Data are from six independent experiments (*p<0.05, Wilcoxon test).

3. DCs show reduced proinflammatory cytokines profile after efferocytosis, even after LPS stimulus

The secretion of proinflammatory cytokines IL-6 and TNF-α was determined in the supernatants from NITApo-DCs cultures and compared to iDCs. The results showed similar amounts of IL-6 and TNF-α secretion by NITApo-DCs when compared to iDCs **(Figure 15A)**. Very little IL-12 secretion was found, as has been described in BM-derived DCs of NOD mice [373]. A proinflammatory stimulus was used to

determine the maturation ability of DCs after efferocytosis. Results show that after a LPS stimulus, NITApo-DCs were significantly more resistant to increase IL-6 and TNF-α secretion than iDCs ($p<0.05$) **(Figure 15B)**.

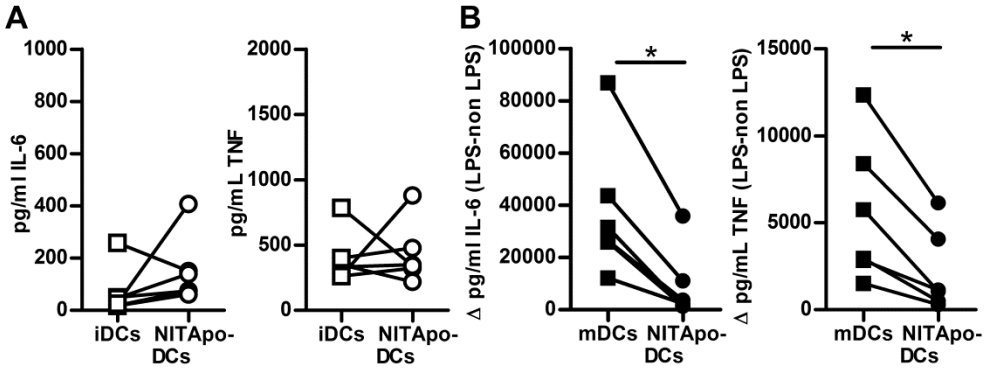

Figure 15. Cytokine secretion from NITApo-DCs in basal conditions and after LPS stimulus. (A) IL-6 and TNF-α secretion (pg/ml) by iDCs (white square) and NITApo-DCs (white circles) during 24h in basal conditions from six independent experiments. **(B)** IL-6 and TNF-α secretion (pg/ml) by mDCs (black squares) and NITApo-DCs (black circles) 24h after LPS (100 ng/ml) stimulation. Results are expressed as the subtraction between cytokine secretion of LPS stimulated and unstimulated cells (Δ pg/ml). Data are from six independent experiments (*$p< 0.05$, Wilcoxon test).

4. Efferocytosis impairs DCs ability to stimulate autologous T cell proliferation, even after a proinflamatory stimulus

A key feature of tolDCs is their low capacity for priming T cells. Autologous T cell proliferation assays were performed to assess the ability of efferocytosis to generate tolDCs. T cell purity and viability were always over 85% and 90% respectively **(Figure 16)**. We observed that the capture of apoptotic cells by DCs significantly impaired autologous T cell proliferation when compared with iDCs ($p<0.05$) **(Figure 17A)**. These results were obtained using T cells from prediabetic NOD mice. After LPS stimulus, NITApo-DCs induced a similar T cell proliferation to that of non-stimulated NITApo-DCs and statistically different to T cell proliferation induced by mDCs previously activated with LPS ($p<0.05$) **(Figure 17A)**. Similar results were obtained in T cell proliferation assays using T cells from recent onset diabetic NOD mice ($p<0.01$) **(Figure 17B)**. Two additional proinflammatory stimuli

were used to validate these results, so the effect of Poly I:C and Zymosan was determined, which stimulate viral infection and inflammation, respectively **(Figure 17C)**. The results indicated that the proliferation of T cells induced by NITApo-DCs did not increase, not even after the effect of these proinflammatory stimuli.

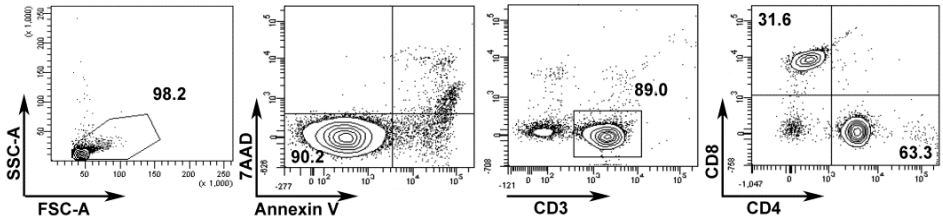

Figure 16. Cell viability and purity of T cells used in T cell proliferation assays. Flow cytometry contour plots of cell size and granularity (FSC and SSC), viability (annexin V^-, $7aad^-$), purity ($CD3^+$), and T cell populations ($CD4^+$ and $CD8^+$ expression, gated on $CD3^+$ T cells) of negative selected T cells from NOD mice spleen.

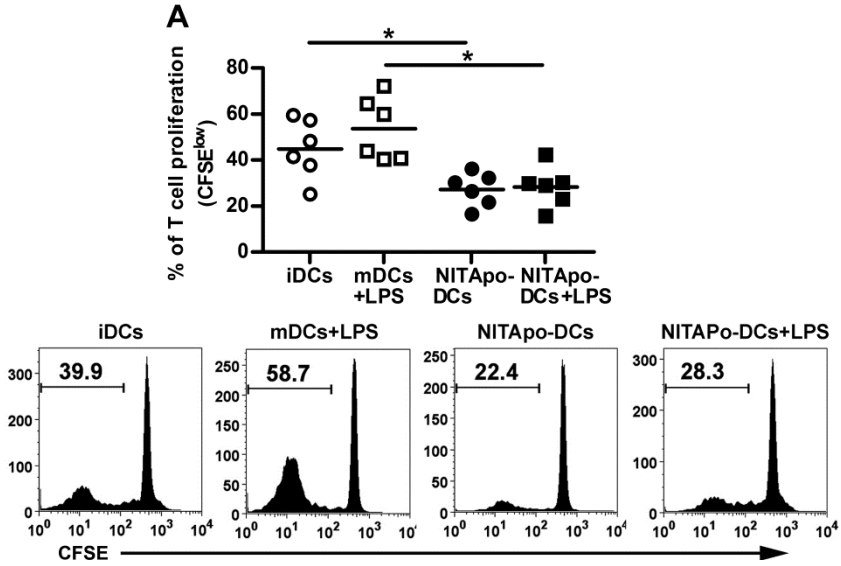

Figure 17A.

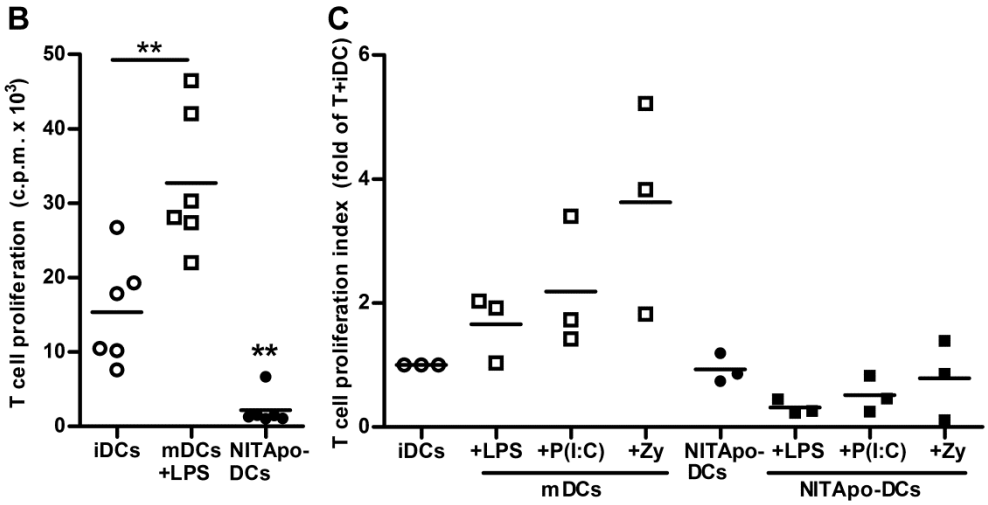

Figure 17. Impaired ability of DCs to stimulate autologous T cell proliferation after the capture of apoptotic cells, even after proinflammatory stimuli. (A) Top: Autologous T cell proliferation (% $CFSE^{low}$ proliferating T cells) after stimulation induced by iDCs (white circles) and by NITApo-DCs (black circles) with insulin (20 µg/ml) at a ratio of 1:10 for 7 days. mDCs and NITApo-DCs+LPS were previously activated with LPS (+LPS, 100 ng/ml, squares) during 24 hours. Lines show the mean of six independent experiments. (*p<0.05, Wilcoxon test) (**Bottom:** One representative flow cytometry histogram of autologous T cell proliferation is shown. **(B)** Autologous T cell proliferation from diabetic NOD mice (c.p.m. for (3H)-thymidine assay) after stimulation induced by iDCs (white circles), NITApo-DCs (black circles) and mDCs (white squares) previously loaded with insulin (20 µg/ml) at a ratio of 1:10 for 6 days. One representative experiment out of three is shown. (**p<0.01, Mann Whitney test) **(C)** Autologous T cell proliferation (c.p.m. for (3H)-thymidine assay) induced by iDCs and mDCs (white circles and squares) and NITApo-DCs (black circles and squares) with insulin (20 µg/ml) at a ratio of 1:10 for 7 days. mDCs and NITApo-DCs were previously activated with LPS (+LPS, 100 ng/ml), Poly I:C (+P(I:C), 0.5 µg/ml) and Zymosan (+Zy, 1 µg/ml) during 24 hours. Lines show the mean of three independent experiments represented as T cell proliferation index (T cell proliferation of each condition divided by T cell proliferation induced by iDCs, mean).

5. Cytokine profile in T cell proliferation assays with DCs after efferocytosis is similar to iDCs and stable after LPS stimulus

To determine the cytokine profile in supernatants from autologous T cell proliferation assays, CBA analysis was performed. Results showed that NITApo-DCs promoted a cytokine profile (IFN-γ, IL-17A, IL-10, IL-6 and TNF) similar to iDCs

(Figure 18). The production of TGF-β was higher in iDCs than in NITApo-DCs, in which TGF-β concentration was below the limit of detection. IL-2 and IL-4 were not detected in any condition of the assay. This cytokine profile induced by NITApo-DCs was stable after proinflammatory stimulus, while T cells co-cultured with mDCs displayed a biological, although non significant, increase of IL-17, IFN-γ, TNF, IL-6 and IL-10. As expected, the production of TGF-β was inhibited after LPS stimulation in mDCs.

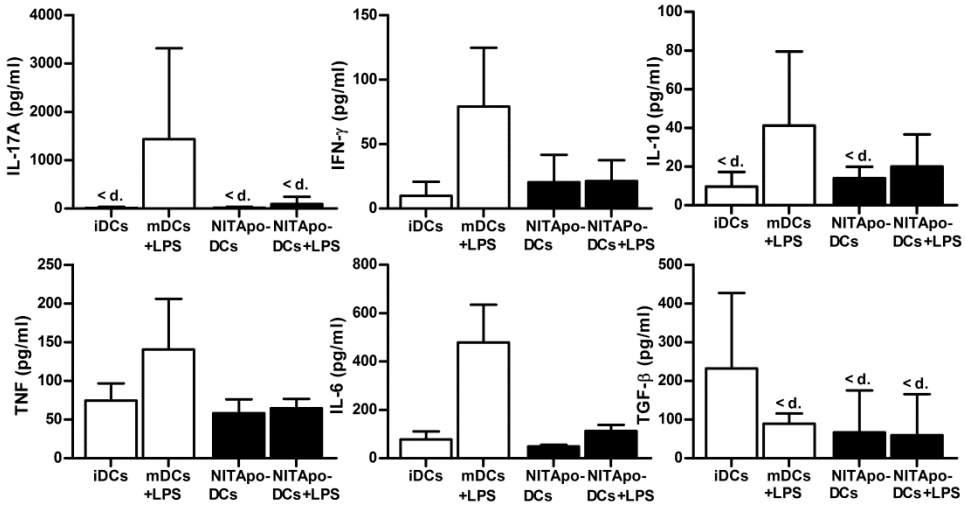

Figure 18. Cytokine production in T cell proliferation experiments. Levels of IL-17A, IL-10, IFN-γ, TNF and IL-6 were measured in supernatants from autologous T cell proliferation experiments induced by iDCs and mDCs (white bars) or by NITApo-DCs (black bars) in basal conditions or previously activated with LPS, at day 6 of culture. Results are expressed as mean+SEM from four independent experiments. <d. means values below the standard.

6. $CD4^+$ $CD25^+$ $FoxP3^+$ classical regulatory T cell subset is not increased by DCs after efferocytosis

The induction of classical $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs by NITApo-DCs was assessed as a possible mechanism of tolerance induction. *In vitro* proliferation assays showed that the percentage of proliferating Tregs induced by NITApo-DCs was not higher than those induced by iDCs or mDCs. The result indicated that T cell

hyporesponsiveness induced by NITApo-DCs was not due to an increased number of classical Tregs **(Figure 19)**.

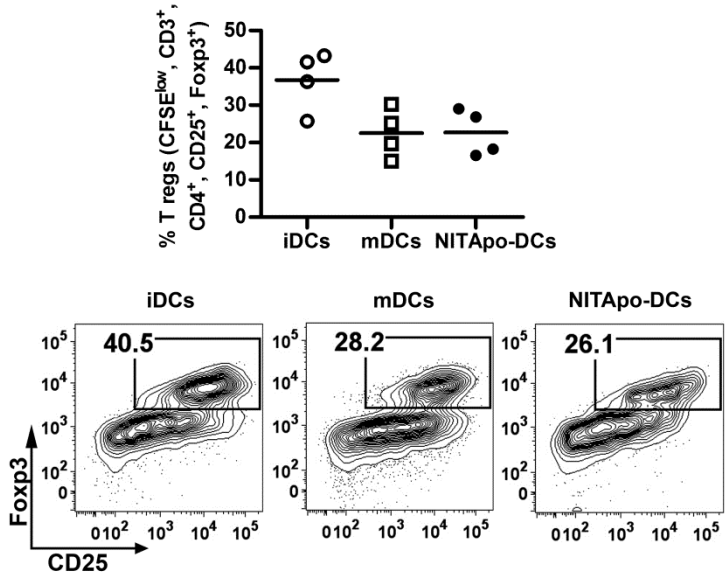

Figure 19. CD4$^+$ CD25$^+$ FoxP3$^+$ classical Treg cell subset is not increased by DCs after efferocytosis. Top: Percentage of proliferating CFSE^{low}, CD3$^+$, CD4$^+$,CD25$^+$, FoxP3$^+$ Tregs in autologous T cell proliferation assays with iDCs (white circles), mDCs (white squares) and NITApo-DCs (black circles), all DCs loaded with insulin (20 µg/ml) at a ratio of 1:10 for 7 days. Plots show the mean (line) of four independent experiments. **Bottom:** Representative flow cytometry contour pots showing CD25$^+$ FoxP3$^+$ Tregs gated on CFSE^{low} CD3$^+$ CD4$^+$.

7. Efferocytosis promotes suppressive function in DCs

The suppressive effect of NITApo-DCs on T cell proliferation induced by mDCs was determined. The percentage of proliferating T cells stimulated with mDCs was not altered by iDCs at different ratios (mDCs:iDCs, from 1:2 to 1:0.25). In contrast, when NITApo-DCs were added to T cells cultured with mDC (mDCs:NITApo-DCs, from 1:2 to 1:0.25), we observed a reduction in T cell proliferation **(Figure 20)** in a dose-dependent manner, up to a 79.6% reduction at a ratio of 1:2, when compared to T cell proliferation induced by mDCs ($p<0.05$). In this condition, the

suppressive effect of NITApo-DCs was dose-dependent, showing significant differences at 1:2 and 1:1 ratios.

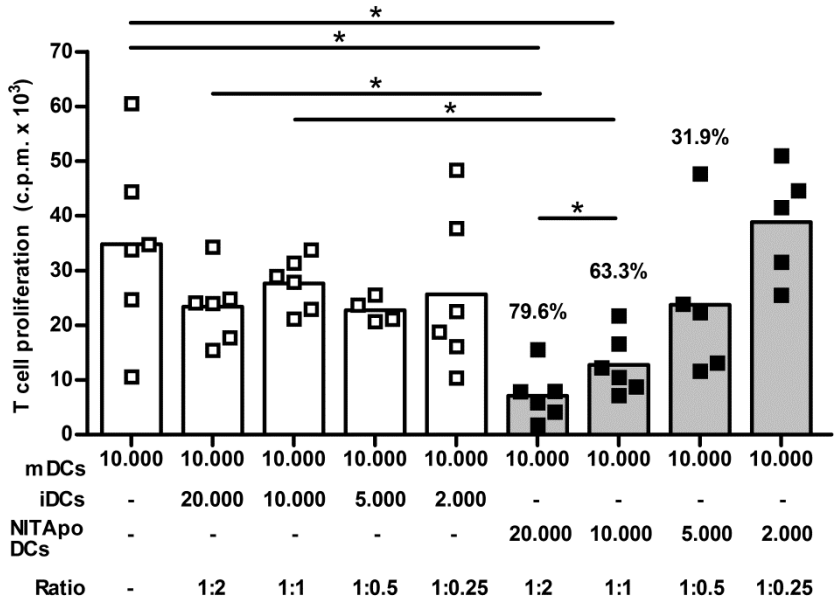

Figure 20. Suppressive effects of NITApo-DCs impaires T cell proliferation. Autologous T cell proliferation (c.p.m. for 3H thymidine assay) induced by mDCs (white bar) in the presence of iDC (white bars) or NITApo-DCs (grey bars) with insulin (20 µg/ml) at different ratios (from 1:2 to 1:0.25) for 7 days. Percentage of inhibition is given on top of histogram bars. Bars and squares represent means from six independent experiments and individual experiments, respectively. (*p<0.05, Wilcoxon test).

8. Gene expression profile of DCs after efferocytosis

To determine gene expression changes in DCs after efferocytosis, microarray analysis was performed by comparing transcriptome of sorted NITApo-DCs and iDCs **(Table 10)**. All RNA samples had an integrity value >7.6, a sufficient quality for microarray experiments. Bioinformatic analysis was performed, taking into account genes that showed a p value < 0.002 and an adjusted p value < 0.083, considering *Cd86* gene expression as a cut off. A total of 278 genes out of the 28,853 mouse genes represented in the gene chip were differentially expressed in

NITApo-DCs when compared to iDCs. In addition, 177 (64%) out of these 278 genes were downregulated, and the remaining 101 (36%) were upregulated.

Table 10. Features of NITApo-DCs and iDCs and RNA quality in microarray experiment

Experiment	Cell type	Num. of cells	ng of RNA	RIN	28s/18s
1	NITApo-DCs	270,000	492	8.2	1.7
	DCs	188.500	324	7.8	1.9
2	NITApo-DCs	205,000	336	7.6	1.4
	DCs	333,000	984	7.8	1.5
3	NITApo-DCs	277,000	648	8.6	1.9
	DCs	510,000	1,800	8.8	1.5
4	NITApo-DCs	370,000	948	8.3	1.9
	DCs	455,500	1,212	8.2	1.9

To define altered processes in DCs after capturing apoptotic cells, information of each gene was collected through genomic and biomedical databases (GeneCards, Entrez Gene, Gene Ontology, Pubmed). Differentially expressed genes in DCs after efferocytosis **(Annex)** were classified in several functional categories related to immune tolerance of DCs to diabetogenic autoantigens **(Table 11)**.

1) Genes involved in antigen processing and presentation (*H2-Ab1, H2-Eb1, H2-DMb2, Cd74, Il4i1*) and costimulation (*Cd80, Cd83, Cd86, Tnfsf4, Tnfsf8, Tnfrsf9, Tnfrsf18*) were downregulated. Only the MHC class Ib gene *H2-M2* was upregulated.

2) Chemokine related genes involved in T cell recruitment (*Ccl5, Ccl17, Ccl22, Cx3cl1*) were downregulated as well as chemokine receptor genes (*Ccr2, Ccr7, Cxcr2*). By contrast, chemokine genes involved in the recruitment of leucocytes were upregulated (*Ccl2, Ccl3, Ccl4, Ccl7, Ccl12, Cxcl1, Cxcl5* and *Ppbp*). Moreover, genes related to adhesion and cell migration were downmodulated (Cd34, Cldn1)

3) Cytokine related genes were also differentially expressed. *Il2ra* gene -corresponding to Cd25, a DC maturation marker- was downregulated. By contrast, *Il1a* and *Tnf* genes were upregulated.

4) The expression of genes with immunoregulatory function was also altered. Overexpressed genes were serpin peptidase inhibitor genes *Serpinb2* and

Serpinb8. Genes for immunomodulatory receptors of the signaling lymphocyte activation molecule (SLAM) family were downmodulated (*Ly9, Salmf6*).

5) Specific transcripts for islet cells were found in DCs after the engulfment of apoptotic bodies, some of them being T1D autoantigens. Among all 101 genes upregulated in NITApo-loaded DCs when compared to DCs, only 14 were selective for NIT-1 cells (genes overexpressed in NITApo cells but not in DCs). Six transcripts of these came from islet specific genes in the pancreas (*Cpe, Iapp, Ins1, Ins2, Sst, Tspan7*).

6) Metabolism-related genes showed changes in expression after the uptake of apoptotic cells. The most relevant changes were found in functional categories of actin-based motility, solute transporters, cell cycle and metabolism. Two genes also described as DC maturation markers were downregulated: an actin-based motility gene -*Fscn1*- and a voltage-dependent Ca^{2+} channel regulatory subunit -*Cacnb3*- [374]. Interestingly, the eicosanoids biosynthesis from the arachidonc acid pathway was altered. Two genes involved in prostaglandin synthesis pathway were upregulated: cyclooxygenase enzyme called prostaglandin-endoperoxide synthase 2, *Ptgs2* (Cox-2), and prostaglandin E synthase, *Ptges*. Furthermore, genes involved in the leukotriene synthesis from arachidonic acid, such as *Alox15* and *Ltc4s*, were downmodulated.

7) The natural immunity category was also altered, showing differential expression in PRR related genes. Mannose receptor gene *Mrc1*, encoding for an endocytic receptor, was upregulated, as well as the scavenger receptor gene *Marco*, the long pentraxine gene *Ptx3* and the TLR homolog gene *CD180*. By contrast, c-type lectin receptor genes *Cd209a, Clec10a* and *Mgl2* were downregulated, as well as the activating receptor gene *Cd300e*.

8) The signaling category displayed downregulated expression of *Jak2, Mapk13,* and *Pik3cg* kinases genes. Ingenuity analysis indicated alterations in the canonical pathway PPAR signaling, p-Value = 0.00005 (upregulated: *Pdgfb, Pdgfa, Ptgs2, Il1a, Tnf*; downregulated: *Map3k14, Il1r2, Il18rap*).

9) Some transcription factors involved in DC maturation were downregulated: *Stat4* - involved in Th1 polarization-, *Nr4a3* -related to apoptosis in mDCs-, *Irf4* -

important in the regulation of interferons- and *Xbp1* – involved in the regulation of MHC class II genes-.

Microarray data was uploaded into the public repository ArrayExpress for the European Bioinformatics Institute (EBI, www.ebi.ac.uk/aerep/ experiment: E-MEXP-3374).

Table 11. Summary of differentially expressed genes related to immune response and diabetogenic autoantigens.

Categories	P val	Genes
Adhesion	<0.000001	*Cd34, Cldn1*
Antigen Presentation	<0.000757	*Cd74, Il4i1, H2-Ab1, H2-DMb2, H2-Eb1, H2-M2*
Chemokines	<0.001271	*Ccl12, Ccl17, Ccl2, Ccl22, Ccl3, Ccl4, Ccl5, Ccl7, Ccr2, Ccr7, Cx3cl1, Cxcl1, Cxcl5, Cxcr2, Ppbp*
Costimulation	<0.001957	*Cd80, Cd83, Cd86, Tnfsf4, Tnfsf8, Tnfrsf9, Tnfrsf18*
Cytokines	<0.000001	*Il1a, Il2ra, Tnf*
Immunoregulation	<0.000215	*Ly9, Serpinb2, Serpinb8, Slamf6*
Islet cells	<0.000190	*Cpe, Iapp, Ins1, Ins2, Sst, Tspan7*
Metabolism	<0.001269	*Alox15, Cacnb3, Fscn1, Ltc4s, Ptges, Ptgs2*
Natural Immunity	<0.001838	*Cd209a, Cd300e, Marco, Mrc1, Ptx3, Clec10a, Mgl2*
Signalling	<0.000975	*Jak2, Mapk13, Pik3cg*
Transcription Factor	<0.000022	*Irf4, Nr4a3, Stat4, Xbp1*

Validation of microarray results was performed by qRT-PCR assay, an alternative technique that has a higher sensitivity. The eight selected genes (*Ccr7*, *Ccl5*, *Cd74*, *Cd83*, *Il2ra*, *Tnfrsf9*, *Ins2*, *Iapp*) confirmed the microarray findings **(Figure 21)**. As expected, Ins2 and Iapp expression was not found in iDCs before efferocytosis of apoptotic islet cells.

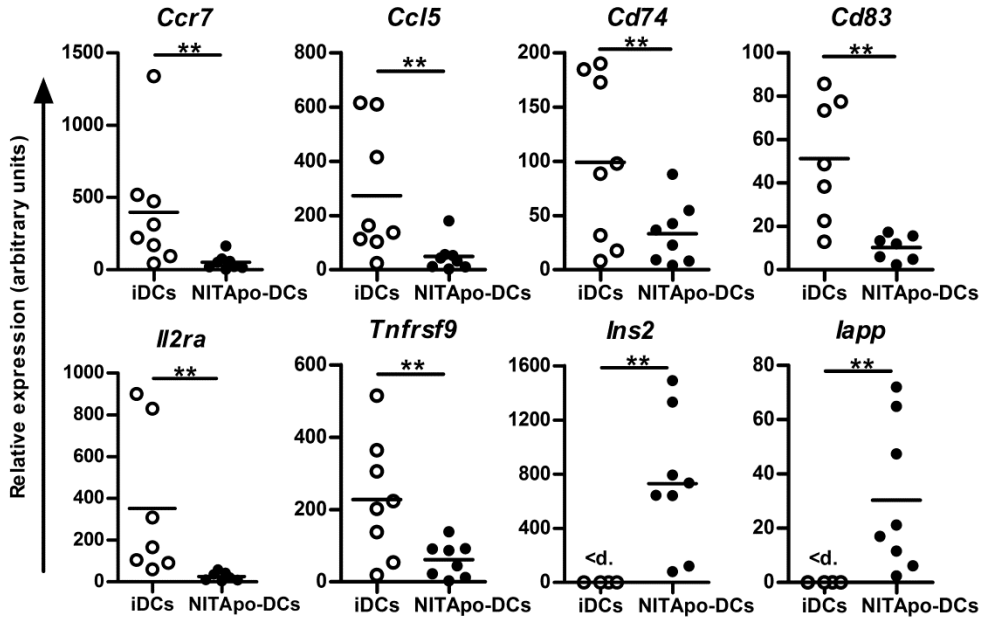

Figure 21. Validation of microarray results by quantitative RT-PCR. Histograms represent quantitative RT-PCR results for the selected genes in iDCs (white dots) and in NITApo-DCs (black dots). Gene expression signals were normalized to *Gapdh*. Plots show the mean (line) of eight independent experiments. (**p<0.01, One-sided Wilcoxon test). <d. means values below limit of detection.

9. Suppressive effects of DCs after efferocytosis involve prostaglandin E_2 production

Based on microarray results, the production of PGE_2 by NITApo-DCs was determined. PGE_2 is a lipid messenger synthesized from arachidonic acid that has been attributed to possess ambivalent immunological functions, having a role in immune suppression [313]. Interestingly, the concentration of PGE_2 was significantly increased in the supernatant of NITApo-DCs cultures when compared to iDCs **(Figure 22A)**. As expected, the concentration of PGE_2 in the supernatant of NIT-1 cells after the induction of apoptosis was very low, thus ruling out the possibility that the increase in PGE_2 production would come from these cells. These results agree with the microarray data and were validated by qRT-PCR **(Figure 22B)**. The expression of *Ptgs2* gene (encoding for COX-2) was significantly higher in DCs after efferocytosis. Furthermore, the expression of *Ptgs1*, *Alox15* and

Ltc4s genes -encoding for COX-1, Arachidonate 15-lipoxygenase and Leukotriene C4 synthase respectively- was significantly lower in DCs after the capture of apoptotic cells.

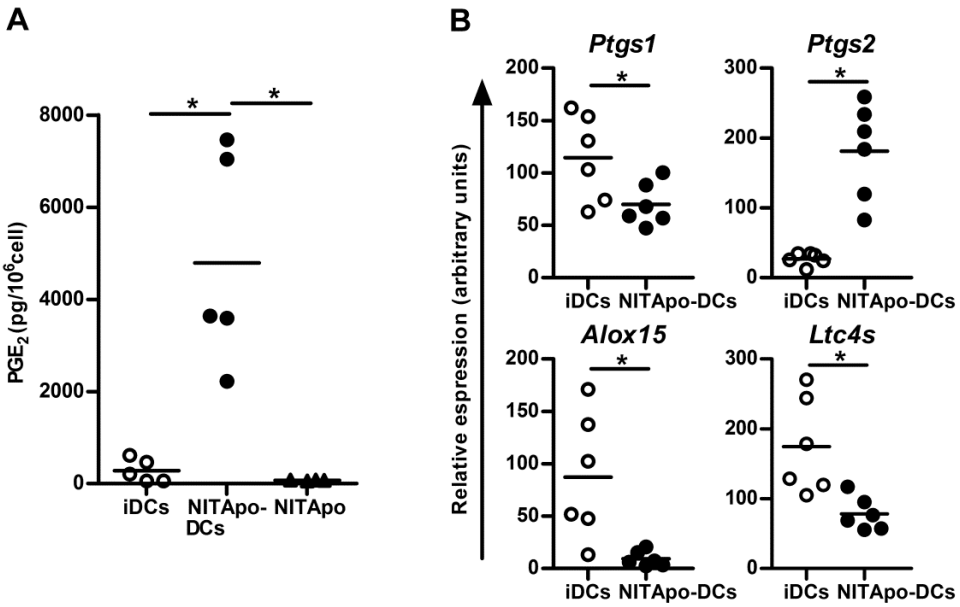

Figure 22. PGE_2 production by DCs after efferocytosis. (A) Quantification of the PGE_2 by ELISA in culture supernatants of iDCs (white circles), NITApo-DCs (black circles) and apoptotic NIT-1 cells (NITApo, black triangles). ELISA data are represented as pg/10^6 cells. Plots show the mean (line) of five independent experiments. (*p<0.05, One-sided Wilcoxon test). **(B)** Quantitative RT-PCR results for *Ptgs1*, *Ptgs2*, *Alox15* and *Ltc4s* genes in iDCs (white circles) and in NITApo-DCs (black circles). Gene expression signals were normalized to *Gapdh*. Plots show the mean (line) of six independent experiments. (*p<0.05, One-sided Wilcoxon test).

To determine the role of PGE_2 in the suppressive function of NITApo-DCs previously shown **(Figure 20)**, T cell proliferation experiments were performed using a specific-COX-2 inhibitor (NS-398) that inhibits PGE_2 production. First, we confirmed that NS-398 inhibited the production of PGE_2 **(Figure 23A)**. Interestingly, by inhibiting the activity of COX-2, the effect of efferocytosis on DCs in T cell proliferation was reverted **(Figure 23B)**, reaching similar levels of T cell proliferation induced by mDCs. NS-398 had no effect on T cell proliferation

induced by mDCs. These results indicated that PGE_2 was involved in the suppression ability of NITApo-DCs. Finally, we tested the effect of the supernatant of NITApo-DCs cultures in the suppression of T cell proliferation induced by mDCs. The addition of supernatant from NITApo-DCs (50 and 250 pg/ml of PGE_2) to T cell proliferation cultures significantly decreased T cell proliferation **(Figure 23C)**, reaching levels of suppression similar to those induced by NITApo-DCs with similar PGE_2 concentration (34.5%, 50.1% and 60.6% respectively). Moreover, the addition of high concentrations of pure PGE_2 to the cultures (50 ng/ml) resulted in an inhibition of >90.9% **(Figure 23D)**. These data confirmed the role of PGE_2 released by DCs in the suppressive effects of efferocytosis in a cell-cell contact independent way.

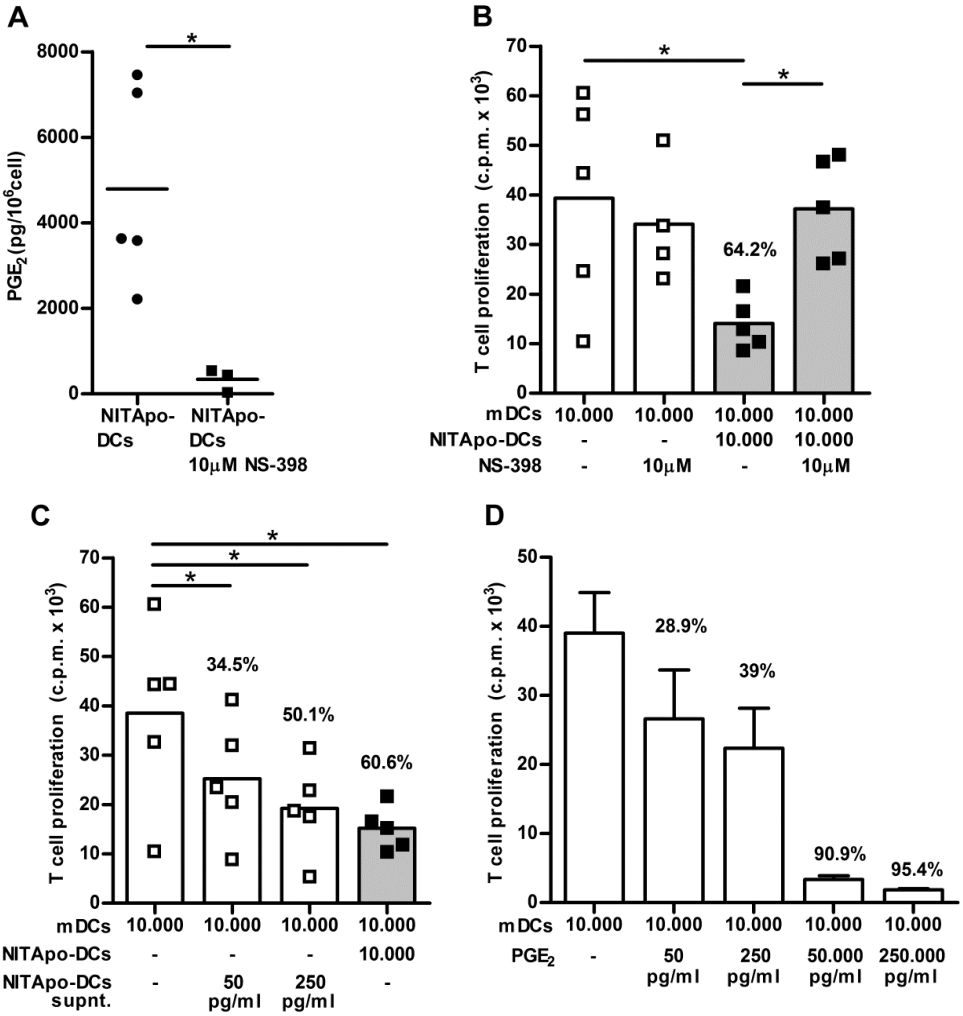

Figure 23. Suppressive effects of NITApo-DCs impaires T cell proliferation through PGE_2 production.

Figure 23. Suppressive effects of NITApo-DCs impaires T cell proliferation through PGE_2 production. (A) Quantification of the PGE_2 by ELISA in culture supernatants of NITApo-DCs (black circles) or cultured with NS-398, specific-COX2 inhibitor (NITApo+DCs 10µM NS-398, black squares). ELISA data are represented as pg/10^6 cells. Plots show the mean (line) of at least three independent experiments. (*p<0.05, Mann Whitney test) **(B)** Autologous T cell proliferation (c.p.m. for 3H thymidine assay) induced by mDCs (white bars) in the presence of NITApo-DC (grey bars) with insulin (20 µg/ml) at a ratio of 1:1 for 7 days. NS-398 (10µM) was added to block PGE_2 production and reverse the suppressive effect of NITApo-DCs. Percentage of inhibition is given on top of histogram bar. Results from five independent experiments are shown. Bars and symbols represent mean and individual experiments, respectively. (*p<0.05, One-sided Wilcoxon test). **(C)** Autologous T cell proliferation (c.p.m. for 3H thymidine assay) induced by mDCs (white bar) in the presence of NITApo-DC (grey bar) with insulin (20 µg/ml) at a ratio of 1:1 for 7 days. To determine if the mechanism depends on cell-cell contact, supernatants from NITApo-DCs cultures, (white bars, 50 and 250 pg/ml of PGE_2) were added to suppression assays instead of NITApo-DCs. Percentage of inhibition is given on top of histogram bars. Results from five independent experiments are shown. Bars and symbols represent mean and individual experiments, respectively. (*p<0.05, One-sided Wilcoxon test). **(D)** Autologous T cell proliferation (c.p.m. for 3H thymidine assay) induced by mDCs (white bar) loaded with insulin (20 µg/ml) at a ratio of 1:1 for 7 days. PGE_2 (white bars, 50 pg/ml, 250 pg/ml, 50 ng/ml and 250 ng/ml) was added to suppression assays instead of NITApo-DCs. Percentage of inhibition is given on top of histogram bars. Results from three independent experiments are shown (mean±SD).

10. Monitoring DCs after i.p. administration.

Bioimaging allows *in vivo* monitoring and tracking of cells in animal models. To evaluate the *in vivo* biodistribution of the immunotherapy, NIR815-labeled NITApo-DCs or DCs were i.p. injected into prediabetic mice. The *in vivo* analysis showed that cell signal was detected 3 days after the injection, mainly in the peritoneal area **(Figure 24)**. The *ex vivo* analysis of the organs showed that at 24 hours post administration, NITApo-DCs signal took place primarily in pancreatic lymph nodes, pancreas and mediastinal lymph nodes **(Figure 25)**. Traces of signal were detected in the spleen, mesenteric lymph nodes, kidney and liver.

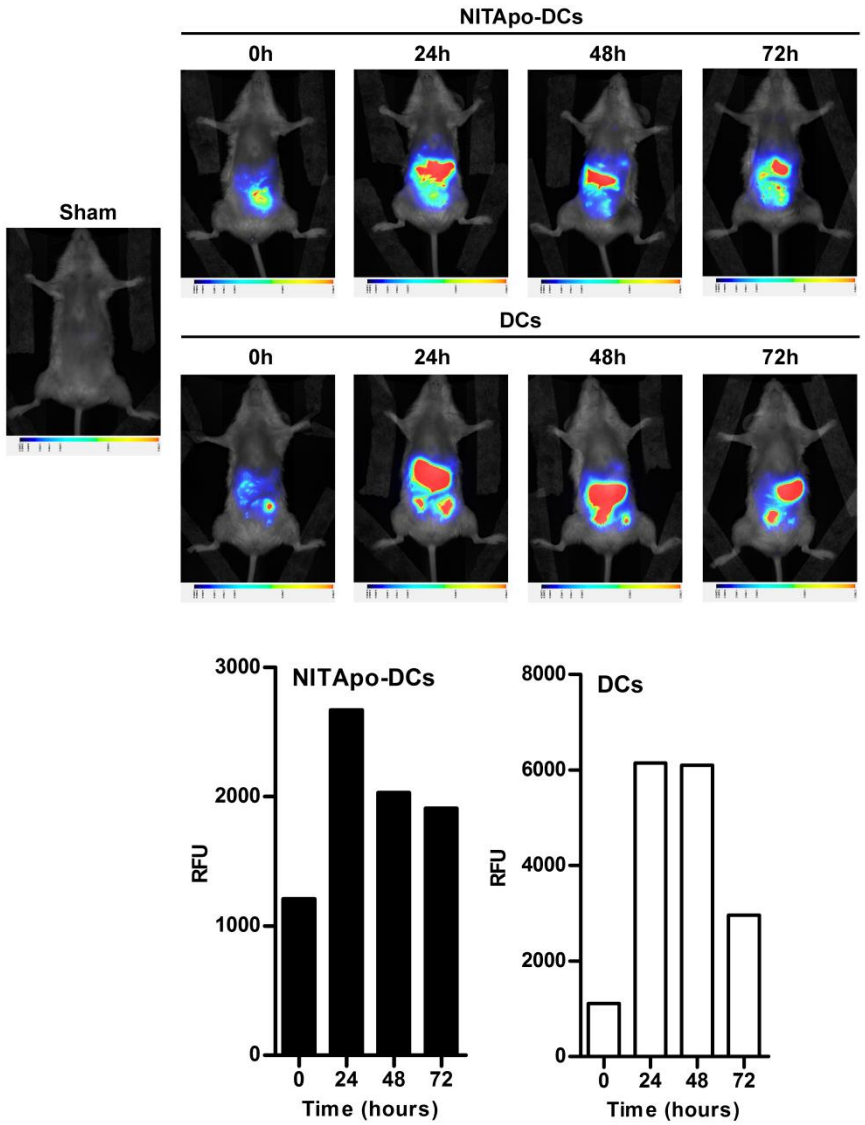

Figure 24. ***In vivo*** **analysis of NIR815-labeled DCs in prediabetic NOD mice. Top:** In vivo imaging (Pearl Imager, LI-COR) of NIR815 stained NITApo-DCs or DCs at 24, 48 and 72 hours after i.p. injection in 8-10wk old NOD mice. Sham mouse shows background signal. **Bottom:** *In vivo* histogram of fluorescence signal (RFU, Relative Flourescence Units) of DCs (white bars) and NITApo-DCs (black bars) from peritoneal area. Results of one representative experiment from two performed.

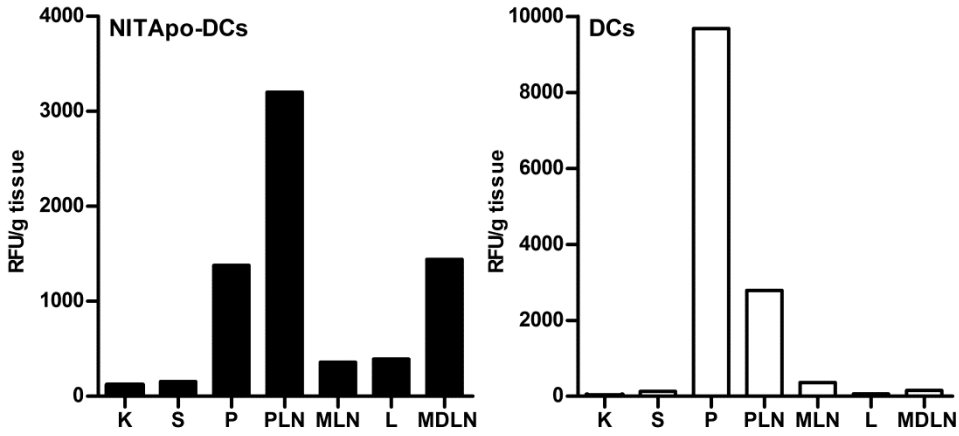

Figure 25. Biodistribution of NIR815-labeled DCs in prediabetic NOD mice 24h after i.p. injection. Histograms of fluorescent signal (RFU, Relative Flourescence Units) relative to grams of tissue of each organ from NOD mice treated with NIR815 stained NITApo-DCs (black bars) or DCs (white bars) after 24 hours. K, kidney; S, spleen; P, pancreas; PLN, pancreatic lymph nodes; MLN, mesenteric lymph nodes; L, liver; MDLN, mediastinal lymph nodes. Results of one representative experiment from two performed.

11. Administration of DCs pulsed with NIT-1 apoptotic cells prevents experimental T1D

To assess the efficacy of DCs pulsed with NIT-1 apoptotic cells in T1D prevention, mice were treated at the pre-diabetic period. Two T1D experimental models were chosen: NOD RIP-IFN-β mice, which express interferon (IFN)-β under the control of the insulin promoter, and wild type NOD mice. NOD RIP-IFN-β mice develop accelerated autoimmune diabetes after 3 weeks of age, with similar incidence (~60%) in males and females, and with aggressive insulitis. Wild type NOD mice develop autoimmune diabetes after 12 weeks of age with an incidence of 60-80% in females and 20-30% in males. We used these two models because on one hand, the NOD RIP-IFN-β mice present an aggressive form of T1D, which represents a big challenge for NITApo-DCs in arresting the autoimmune attack. On the other, we wanted to validate the results in wild type NOD mice, the widely used model to study human T1D.

11.1. RIP-IFN-β NOD mice

NOD RIP-IFN-β mice were treated at 12–14 days of age with DCs loaded with apoptotic NIT-1 cells. iDCs and DCs pulsed with SV-T2 apoptotic cells were used as controls. SV-T2 cell line is derived from BALB/c fibroblasts and was chosen as an antigen-irrelevant source to study the antigen specificity of the immunotherapy. A control group that received saline solution was included. At this early age, which was chosen for preventive immunotherapy prior to diabetes onset, pups are still unweaned, but the treatments were safe and well tolerated, as the mice exhibited the appearance and behaviour similar to that of untreated mice. Mice from the sham control group developed diabetes after 3 weeks of age with an incidence of 65.4% **(Figure 26)**. The administration of NITApo-DCs resulted in a significant reduction of diabetes incidence when compared to the sham, DCs and SVApo-DCs groups, that did not cause any preventive effect (21.05% of diabetes incidence in comparison to 65.38%, 57.14% and 54.55% diabetes incidence, respectively; $p<0.05$).

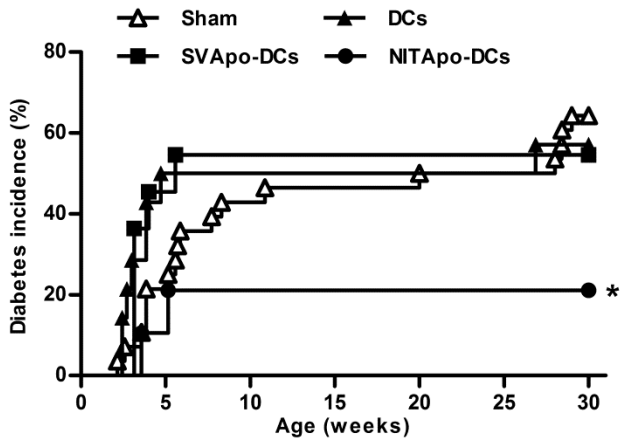

Figure 26. Immunotherapy using NITApo-DCs decreases T1D incidence in NOD-RIP-IFN-β mice. Cumulative incidence (%) of diabetes in NOD-RIP-IFN-β mice treated with 10^6 NITApo-DCs (black circles, n = 19), 10^6 SV-T2 apoptotic cells (SVApo)-DCs (black squares, n = 11), 10^6 iDCs (black triangles, n = 14) and in the control group that received saline solution (sham) (white triangles, n = 26) (*$p<0.05$, Kaplan–Meier log-rank analysis).

Insulitis is the degree of lymphocytic infiltration in the islets of Langerhans. Insulitis was scored in the pancreas of non-diabetic animals from each group at the end of the follow-up period (30 weeks of age). As expected, mice in the sham group showed a higher insulitis score, as previously reported [76] **(Figure 27A)**. Cell therapy with NITApo-DCs reduced insulitis score significantly ($p<0.05$) when compared to the sham group, to the DCs group and to the SVApo-DCs group. Insulitis was also scored at the cell administration check point (12 days of age), showing a weak peri-insulitis. Mice treated with NITApo-DCs slightly increased the degree of insulitis in relation to mice at 12 days of age (1.95-fold), although the differences were lower than control groups (3.43-fold). Moreover, analysis of the percentage of islets classified in each of the five infiltration categories showed that in mice treated with NITApo-DCs, 66% of the islets remained free of insulitis or with peri-insulitis, whereas 23%, 35% and 27% of the islets were non-destructed in the SVApo-DCs, DCs and sham groups, respectively, scored with a severe degree of insulitis **(Figure 27B)**. Thus, the insulitis degree observed after antigen-specific immunotherapy correlated well with the prevention of the disease **(Figure 27C)**.

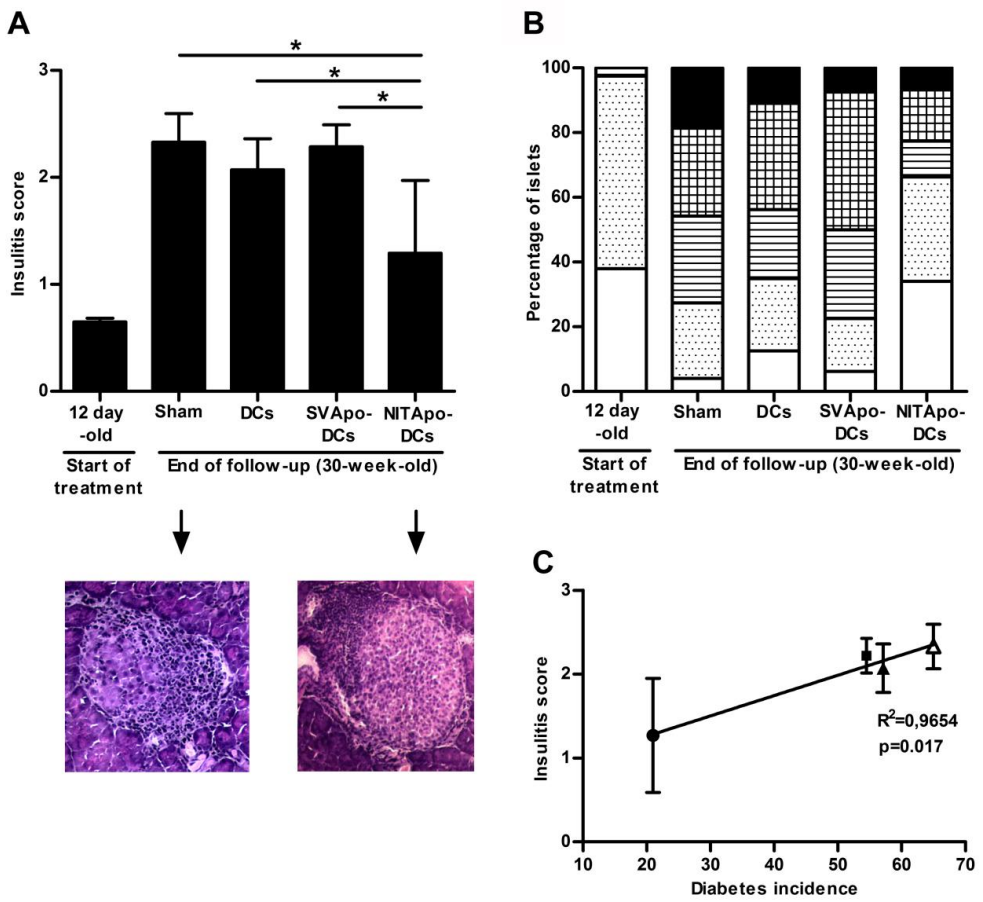

Figure 27. Effect of the treatment with NITApo-DCs on insulitis in NOD-RIP-IFN-β mice. (A) Upper panel: Insulitis score from non diabetic animals at the beginning of the treatment (12-day-old) and at the end of the follow-up (30-week-old) in different treatment groups. Pancreata from 5-7 animals/group were analysed (mean±SD) (*p<0.05, Mann Whitney test). **Lower panel:** Representative images of islets from haematoxylin and eosin-pancreatic cryostat sections from sham and NITApo-DC treated mice. **(B)** Percentage of islets classified in each of the five infiltration categories at the beginning of the treatment (12-day-old) and at the end of the follow-up (30-week-old). White= 0, no insulitis; Dotted = 1, peri-insulitis; Striped = 2, mild insulitis (<25% of the infiltrated islet); Squared = 3, severe insulitis (25-75% of the islet infiltrated); Black = 4, destructive insulitis (>75% islet infiltration). **(C)** Significant correlation between diabetes incidence (%) and insulitis score (mean±SD) in different treatment groups (R^2 = 0.9654, *p<0.05, Pearson test).

11.2. NOD mice

NOD RIP-IFN-β is a model of spontaneous accelerated and aggressive autoimmune T1D. To confirm the efficacy of NITApo-DCs for preventing T1D in the wild type mouse model, immunotherapy was administered to NOD mice at also the pre-diabetic stage (12- to 14-days old). iDCs (DCs) and saline solution (sham) were used as controls. The treatment with NITApo-DCs showed a tendency to reduce diabetes incidence when compared to sham and DCs groups (37.5% of diabetes incidence in comparison to 63.64% and 62.5% diabetes incidence, respectively) **(Figure 28A)**. Moreover, NITApo-DCS therapy showed a delay of the disease (20 weeks of age versus 12-14 weeks of age in control and sham groups, respectively). Despite the difference to control groups was not statistically significant and the number treated animals was small, there was a clear biological effect of the immunotherapy in preventing T1D. Insulitis was scored for non-diabetic animals from treated and control group at the end of the follow-up period. Results showed that cell therapy with NITApo-DCs reduced insulitis score when compared to sham group and to DCs group **(Figure 28B)**, although the difference was not statistically significant. Analysis of the percentage of islets classified in each of the five infiltration categories showed that in mice treated with NITApo-DCs, 56% of the islets remained free of insulitis or with peri-insulitis, whereas 48% and 37% of the islets were non-destructed in the DCs and sham groups **(Figure 28C)**.

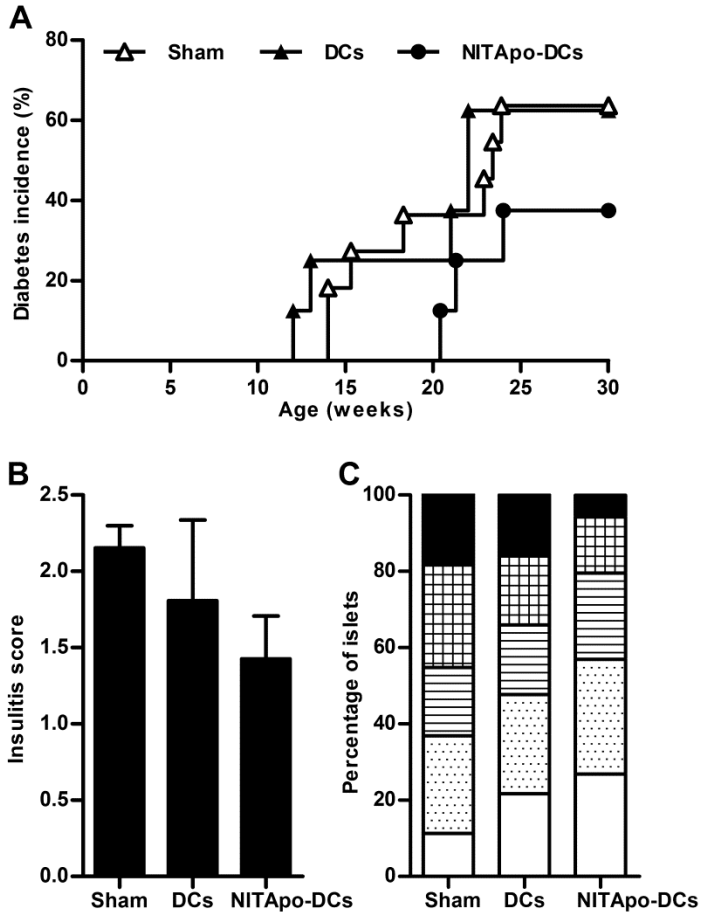

Figure 28. Effect of the treatment with NITApo-DCs on diabetes incidence and insulitis in NOD mice. (A) Cumulative incidence (%) of diabetes in NOD mice treated with 10^6 NITApo-DCs (black circles, n = 8), 10^6 iDCs (black triangles, n = 8) and in the control group that received saline solution (sham) (white triangles, n = 11). **(B)** Insulitis score from non diabetic animals at the end of the follow-up (30-week-old) in different treatment groups. Pancreata from 3-5 animals/group were analysed (mean±SD). **(C)** Percentage of islets classified in each of the five infiltration categories at the end of the follow-up as follows: White= 0, no insulitis; Dotted = 1, peri-insulitis; Striped = 2, mild insulitis (<25% of the infiltrated islet); Squared = 3, severe insulitis (25-75% of the islet infiltrated); Black = 4, destructive insulitis (>75% islet infiltration).

12. Treatment with DCs pulsed with NIT-1 apoptotic cells does not reverse experimental T1D

After confirming the preventive effect in T1D of DCs pulsed with NIT-1 apoptotic cells, the immunotherapy was administered to recent-onset diabetic NOD mice with the aim to reverse the disease. NOD RIP-IFN-β mice and wild type NOD mice were treated once with NITApo-DCs and compared to a control group that received saline solution. Mice were always treated with daily subcutaneous injections of insulin

12.1. NOD-RIP-IFN-β mice

Mice were spontaneously rendered diabetic after >3 weeks of age showing elevated fasting blood glucose concentration (345.5±39.6 mg/dl, mean ± SD). To assess the efficacy of NITApo-DCs in the reversal of T1D, diabetic NOD RIP-IFN-β mice were treated at day 7 after the onset of the disease, the required time to obtain NITApo-DCs, and compared to the sham group. Fasting blood glucose levels showed that all diabetic NOD mice maintained hyperglycemia with glucose concentrations >300 mg/dl at the end of the follow-up **(Figure 29A)**. To determine the recovery of β-cell function, C-peptide concentrations were measured in the sera of NOD-RIP-IFN-β at the end of the study. No significant differences were observed between mice treated with NITApo-DCs and those from the control group **(Figure 29B).** Insulitis was scored 30 days after T1D onset. Animals from the treated and sham group showed similar insulitis scores **(Figure 29C)**. Analysis of the percentage of islets classified in each of the five infiltration categories showed the severity of insulitis in both groups **(Figure 29D)**.

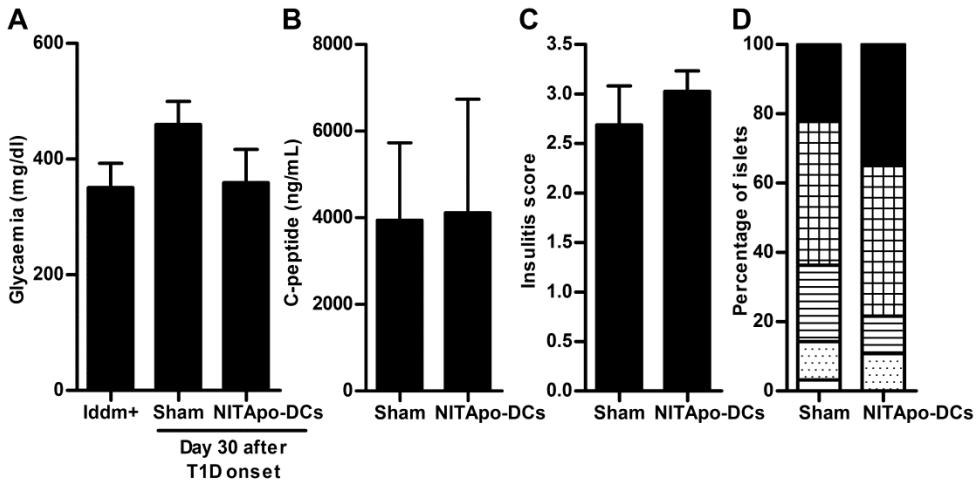

Figure 29. Effect of NITApo-DCs administration on glycaemia, endogenous insulin secretion and insulitis in diabetic NOD-RIP-IFN-β mice. (A) Glucose levels in diabetic mice at the onset of the disease (Iddm+) and at the end of the study (30 days after T1D onset) in mice treated with 10^6 NITApo-DCs and the control group that received saline solution (sham). **(B)** Serum C-peptide levels were determined at the end of the follow-up (30 days) by ELISA. **(C)** Insulitis score at the end of the follow-up (30 days after T1D onset) in NITApo-DCs treated group and sham control group. **(D)** The percentage of classified islets in each of the five infiltration categories in different groups was as follows: White= 0, no insulitis; Dotted = 1, peri-insulitis; Striped = 2, mild insulitis (<25% of the infiltrated islet); Squared = 3, severe insulitis (25-75% of the islet infiltrated); Black = 4, destructive insulitis (>75% islet infiltration). Results are means±SD from 3-4 mice per group.

12.2. NOD mice

The efficacy of the immunotherapy in T1D reversal was assessed in NOD mice, which showed a less accelerated and aggressive disease than NOD RIP-IFN-β mice. NOD mice were spontaneously rendered diabetic after 12 weeks of age showing elevated fasting blood glucose concentration (363.5±109.6 mg/dl of blood glucose, mean±SD). NITApo-DCs were administered to diabetic wild type NOD mice at day 7 after the onset of the disease, the required time to obtain the corresponding NITApo-DCs.

To restrain autoimmunity before the immunotherapy administration, a second group of diabetic mice was treated daily with rapamycin until the end of follow up.

A third group was treated only with rapamycin and a fourth group received saline solution (sham) as control. Mice were treated with daily subcutaneous injections of insulin. At the end of the study, fasting blood glucose levels were not significantly different between mice treated with NITApo-DCs immunotherapy and the sham group **(Figure 30A)**. The administration of rapamycin did not improve glycaemia when compared to the sham group. Moreover, no effect in glucose levels was observed with the group that received the combination of NITApo-DCs and rapamycin. Among the three treated groups, the lowest glycaemia was achieved by mice treated with NITApo-DCs, showing significant differences when compared to mice treated with rapamycin alone or in combination with NITApo-DCs ($p<0.01$ and 0.05, respectively). All diabetic NOD mice sustained hyperglycaemia at the end of the study. To determine the recovery of β-cell function, C-peptide was measured in sera at the end of the study **(Figure 30B)**. No significant differences were observed when comparing NITApo-DCs treated mice with those from the sham group. Only the treatment with rapamycin alone significantly increases C-peptide concentration in NOD mice when compared to those in the sham group ($p<0.05$). However, C-peptide levels in mice treated with NITApo-DCs or simultaneously treated with NITApo-DCs and rapamycin were similar to those in the sham group, and significantly lower than those in mice treated with rapamycin. Insulitis was scored in diabetic mice 4 weeks after treatment. No significant differences were found between the four groups of diabetic mice **(Figure 30C)**. Insulitis score in mice treated with NITApo-DCs was similar to that in the sham group. The insulitis score in mice treated with rapamycin in combination of NITApo-DCs was similar to that in mice treated with rapamycin alone. The percentage of islets classified in each of the five infiltration categories in different groups showed that the severity of insulitis fits well with the percentage of islets with destructive insulitis and severe insulitis **(Figure 30D)**.

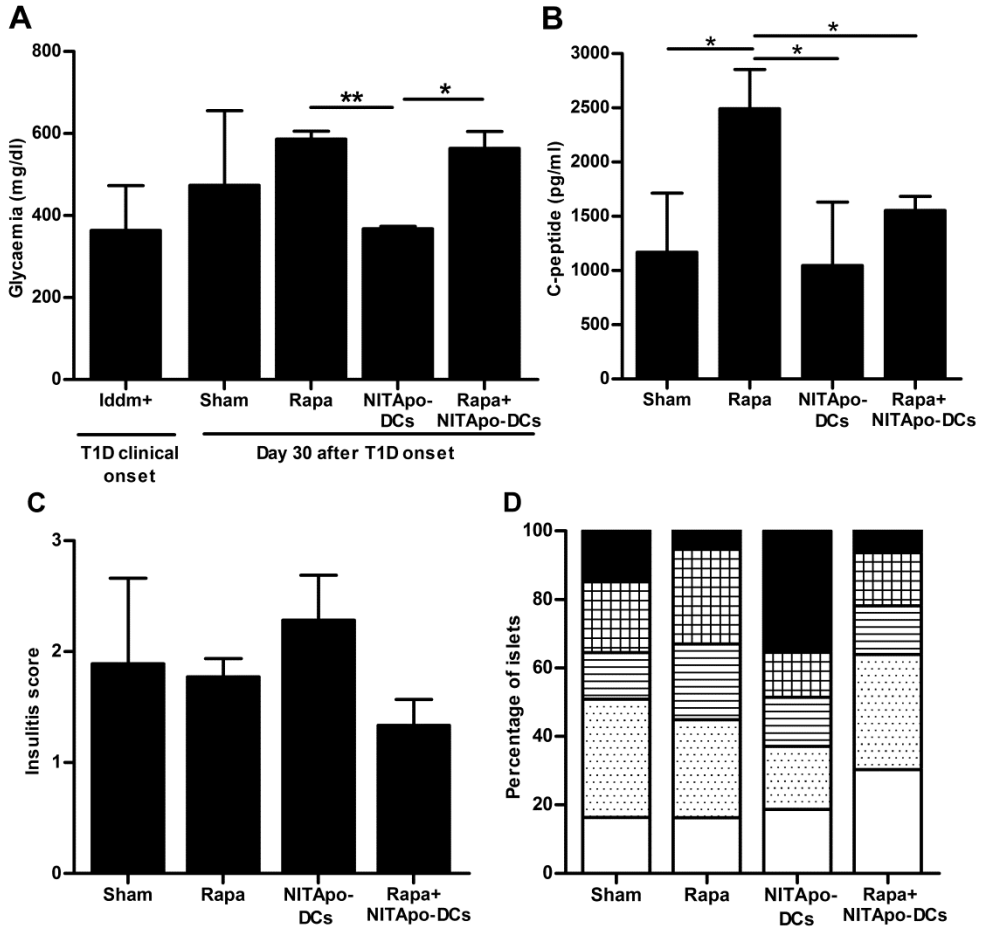

Figure 30: Administration of NITApo-DCs do not reverse glycaemia, do not improve insulin secretion and do not reduce insulitis in diabetic NOD mice. (A) Glucose levels in diabetic mice at the onset of the disease and at the end of the study (day 30) in different treatment groups with rapamycin (Rapa), NITApo-DCs and rapamycin (Rapa+NITApo-DCs), NITApo-DCs and the control group that received saline solution (sham). Results are means ± SD from 3-6 mice per group (*$p < 0.05$ and **$p < 0.01$, Unpaired t-test). **(B)** Serum C-peptide levels at the end of the follow-up (30 days) determined by ELISA. Data from 3-6 animals/group are expressed as means ± SD. (*$p<0.05$, Unpaired t-test). **(C)** Insulitis score at the end of follow up (30 days after T1D onset) in sham, Rapa, NITApo-DCs and Rapa+NITApo-DCs treated groups. The pancreas from 3 animals/group were analyzed. Results are means±SD. **(D)** The percentage of classified islets in each of the five infiltration categories in different groups was as follows: White= 0, no insulitis; Dotted = 1, peri-insulitis; Striped = 2, mild insulitis (<25% of the infiltrated islet); Squared = 3, severe insulitis (25-75% of the islet infiltrated); Black = 4, destructive insulitis (>75% islet infiltration).

SECTION II: ENCAPSULATION OF β-CELL AUTOANTIGENS INTO PHOSPHATIDYLSERINE-LIPOSOMES ARRESTS AUTOIMMUNITY AND PREVENTS TYPE 1 DIABETES

Apoptotic cells are a key factor in the maintenance of immunological homeostasis. The phagocytosis of apoptotic cells prevents maturation of DCs, and this process could induce specific tolerance rather than autoimmunity. In fact, in the first part of "Results" section, we have demonstrated that apoptotic β-cells captured by autologous DCs prevent T1D in mice. Due to the difficulty in obtaining and standardizing β-cell apoptotic bodies, the aim of this second part was to evaluate a synthetic strategy to arrest autoimmune diabetes, based on nanomedicine and liposomal microparticles that mimic apoptotic β-cells in membrane recognition and antigenic content. Apoptotic cells exhibit surface changes, especially exposure of the plasma membrane inner leaflet phospholipid PS that distinguish them from viable cells and allow recognition by efferocytic receptors. Therefore, PS-liposomes loaded with insulin peptides were generated, to simulate the PS recognition of apoptotic bodies by APCs. These liposomes, when administered to NOD mice, down-regulated autoimmunity and prevented T1D by inducing tol DCs and impairing autologous T cell proliferation after being captured by DCs. We believe that this innovative immunotherapy constitutes a novel and simple strategy for human T1D and to restrain unwanted autoimmune reactions.

13. PS-presenting liposomes filled with insulin peptides are captured by DCs

PS-liposomes were prepared with DOPS/DLPC/CH at 1:1:1.33 molar ratio to present the 'death signal' PS on their surface. Empty liposomes presented a mean particle size of 997 nm, a size >500 nm to promote phagocytosis [375], with a polydispersity index (PdI) of 0.31. Additionally, zeta potential measurements revealed a net surface charge of -29.26 mV on PS-liposomes. When PS-liposomes were loaded with peptide A or B from mouse Insulin2, the mean diameter was 1051 nm (PdI=0.31) and 968 nm (PdI=0.27), respectively. Regarding the zeta potencial, both encapsulated liposomes had a negative surface charge of -30.8 mV

for peptide A and -29.4 mV for peptide B. The mean of % of encapsulation achieved was 41.07% for peptide A and 87.44% for peptide B. **Table 12** summarizes the characteristics of liposomes used in the study.

Table 12. Liposome characteristics

	Particle size (nm)	Polydispersity index (PdI)	Zeta potential (mV)	Encapsulation efficiency (%)
PS-liposomes	996.71 ± 89.42	0.31 ± 0.05	-29.26 ± 2.82	-
PSA-liposomes	1051.43 ± 45.15	0.31 ± 0.06	-30.79 ± 2.35	41.07 ± 23.58
PSB-liposomes	996.71 ± 86.32	0.27 ± 0.08	-29.44 ± 1.48	87.44 ± 4.54

Data are expressed as mean ± SD.

Cryo-TEM analysis revealed that most liposomes (empty or peptide encapsulated) showed a multivesicular vesicle morphology **(Figure 31A)**. To characterize the kinetics of the capture by DCs, time course analysis was performed. To that end, PS-liposomes were marked with Oregon green 488 (OG488) fluorescent PS-liposomes (PS-lipo-OG488). The experiments confirmed that after 5 min of co-culture at 37ºC, 75.20±6.38% phagocytosis was achieved **(Figure 31B)**. As expected, OG488 fluorescent signal was significantly higher ($p<0.01$) in each checkpoint when compared to cocultures at 4ºC, confirming the capture of liposomes by phagocytosis at 37ºC.

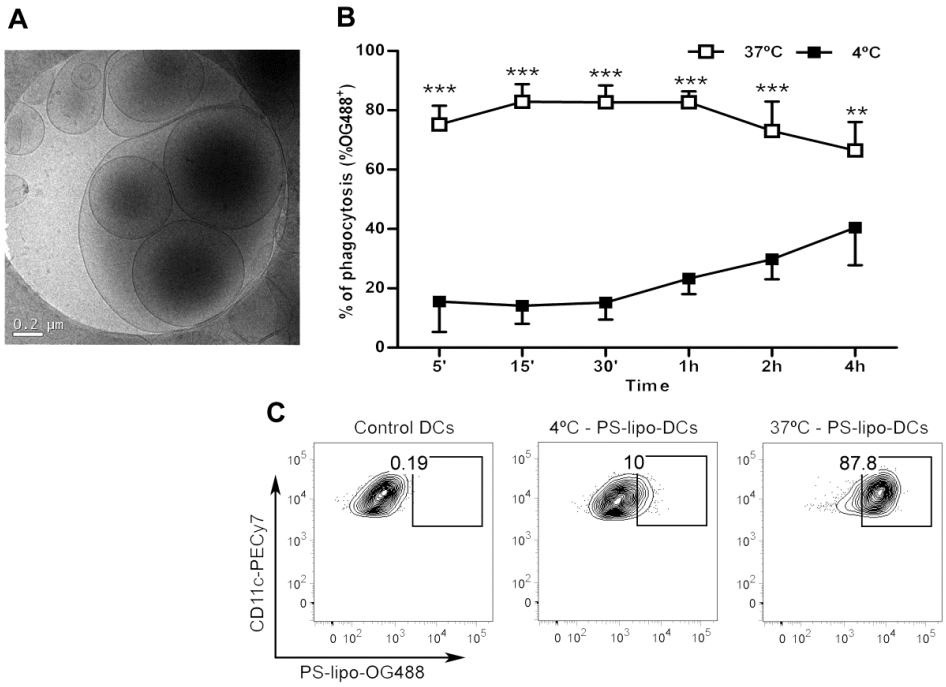

Figure 31. Liposome features. (A) Cryogenic transmission electron microscopy (cryo-TEM, JEOL-JEM 1400 microscope) images of PSAB-liposomes. Bar=0.2µm. **(B) Top:** Time course analysis of the capture of 100 TM Oregon green 488 DHPE labelled PS-liposomes (OG488 PS-liposome) by DCs at 37ºC (white squares) and at 4ºC (black squares) (***p<0.001, **p<0.01, Two-way ANOVA). **Bottom:** Dot plot image of -from left to right control DCs, DCs co-cultured for 30 minutes with OG488 PS-liposome at 4ºC and at 37ºC. One representative experiment of three is shown.

The effect of PS- and PSAB-liposomes on DCs viability was studied. After the capture of PS- or PSAB-liposomes, DCs gained cell granularity **(Figure 32A)**. Viability of DCs after co-culture with PS- or PSAB- liposomes was always >80%, similar to control DCs, even after a proinflammatory stimulus, regardless of the dose (range 100-1000 µM, only the highest dose was shown) **(Figure 32B and 32C)**.

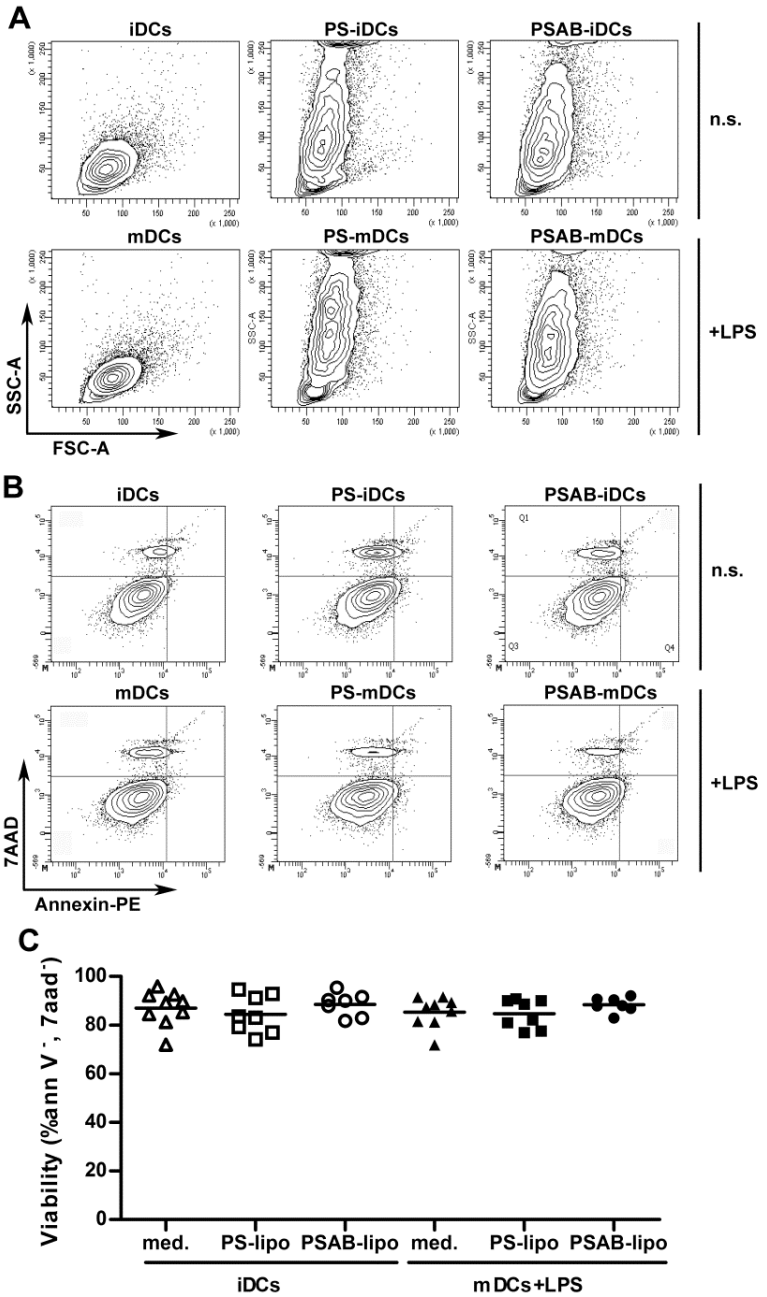

Figure 32. DCs morphology and viability after the capture of PS- or PSAB-liposomes. (A) Flow cytometry contour plot of DCs size and granularity (FSC and SSC), 24h after capturing PS- or PSAB-liposomes, in basal conditions (med.) or with LPS (+LPS). One representative experiment of at least eight independent experiments is shown. **(B)** Flow cytometry contour plot of DCs viability (annexin V^- and $7aad^-$, gated on $CD11c^+$ cells), 24h after

capturing PS- or PSAB-liposomes, in basal conditions (med.) or with LPS (+LPS). One representative experiment of at least eight independent experiments is shown. **(C)** DCs viability (% annexin V^- and $7aad^-$). White symbols represent iDCs, before (triangles) and after the capture of PS-liposomes (squares) or PSAB-liposomes loaded with insulin peptides (circles), 24 hours after culture. Black symbols represent viability of mDCs before (triangles) and after the capture of PS-liposomes (squares) or PSAB-liposomes loaded with insulin peptides (circles) after proinflamatory stimulus (LPS). Lines show the mean of at least eight independent experiments.

14. Decreased expression of costimulatory molecules by DCs after the capture of PS-liposomes

The expression of costimulatory molecules was assessed on the surface of DCs. CD40 and CD86 membrane expression did not increase in DCs after liposome capture, maintaining low expression levels **(Figure 33A, B).** After LPS exposure, control mDCs significantly increased CD40 and CD86 membrane expression in comparison to iDCs ($p<0.001$) **(Figure 33C)**. DCs loaded with PS-liposomes significantly increased CD86 ($p<0.001$) after LPS stimulus, but not CD40 expression. In contrast, PSAB-liposomes did not significantly increase CD40 or CD86 membrane expression.

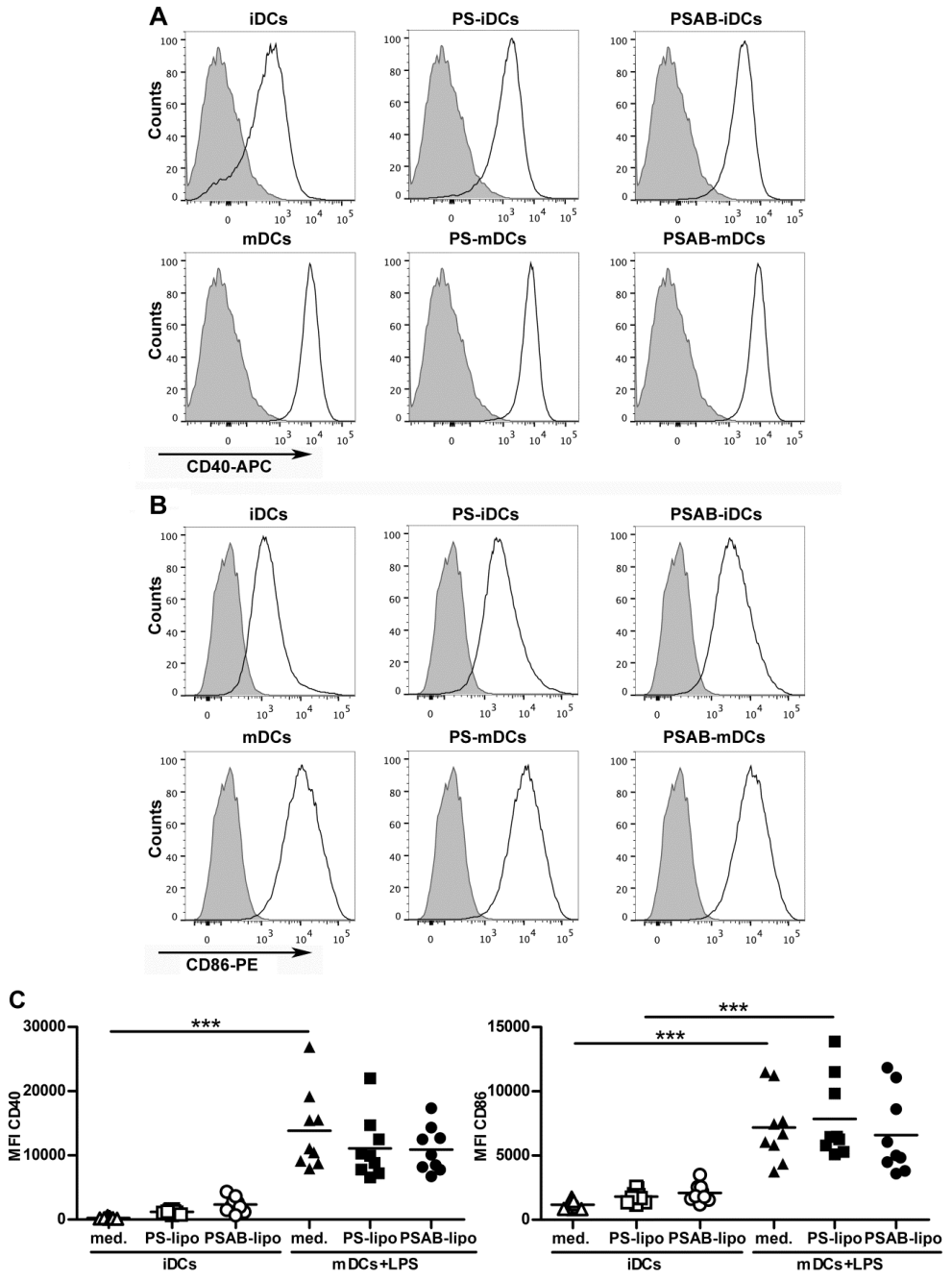

Figure 33. CD40 and CD80 expression on DCs membrane after the capture of PS-liposomes. (A) Histograms of CD40 expression on DCs, PS-DCs or PSAB-DCs at 24h after culture in basal conditions or with LPS stimulus. One representative experiment of nine independent experiments is shown. **(B)** Histograms of CD86 expression on DCs, PS-DCs or

PSAB-DCs at 24h after culture in basal conditions or with LPS stimulus. Grey areas are FMOs controls. One representative experiment of nine independent experiments is shown. **(C)** Median of fluorescence intensity (MFI) for CD40 (up) and CD86 (down) membrane expression on iDCs (white triangles), PS-iDCs (white squares), PSAB-iDCs (white circles) and mDCs (black triangles), PS-mDCs (black squares), PSAB-mDCs (black circles) after 24h in culture media or with LPS stimulus (100 ng/ml), respectively. Lines show the mean of nine independent experiments (each symbol represents an individual experiment). Comparisons between groups showed significant differences (***p<0.001, One-way ANOVA).

15. Increased production of PGE_2 by DCs after the capture of PS-liposomes

Based on our previous results **(Figure 34)**, the production of PGE_2 by DCs after PS-liposomes uptake was measured. The concentration of PGE_2 was significantly increased in the supernatant of DCs co-cultured with PS-liposomes ($p<0.01$) or PSAB-liposomes ($p<0.05$) when compared to iDCs, in a dose dependent manner **(Figure 34)**. As expected, PSAB-liposomes alone did not give a positive signal in the ELISA for PGE_2.

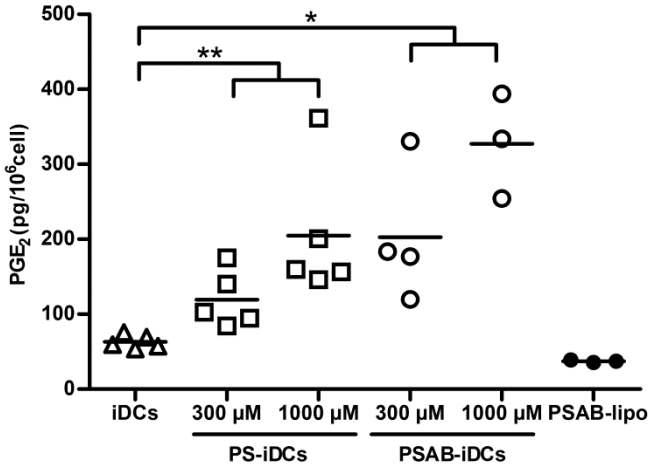

Figure 34. PGE_2 production by DCs after the capture of PS-liposomes. Quantification of the PGE_2 production by iDCs in basal conditions (white triangles), loaded with empty PS-liposomes (PS-iDCs, white squares) or loaded with PSAB-liposomes containing autoantigen (PSAB-iDCs, white circles) after 24 hours of culture. Two doses of PS- or PSAB-liposomes were used: 300 µM and 1000 µM. Data from DCs are represented as $pg/10^6$ cells. 1000 µM of PSAB-liposomes alone (PSAB-lipo, black circles) were used as control. Plots show the mean (line) of a minimum of three independent experiments. Comparisons between groups showed significant differences (*p<0.05 and **p<0.01, Mann Whitney test).

16. Impairment of DCs to stimulate autologous T cell proliferation after PS-liposome capture

Autologous T cell proliferation assays were performed to assess the ability of PS- and PSAB-liposomes to generate tolDCs with low capability to induce autoreactive T cell proliferation. T cell purity and viability were always over 85% and 90% respectively, as previously shown in **Figure 16**. T cell proliferation assays showed that the capture of PS- or PSAB-liposomes by iDCs did not increase autologous T cell proliferation when compared to iDCs alone, regardless of the dose **(Figure 35)**. As expected, T cell proliferation induced by mDCs (matured with LPS) was higher than proliferation induced by iDCs ($p<0.001$). Interestingly, T cell proliferation induced by mDCs loaded with the highest doses of PS- or PSAB-liposomes was significantly lower than proliferation induced by mDCs ($p<0.05$), even after the effect of proinflammatory stimulus. Moreover, results showed a clear dose-effect between PS- or PSAB-liposomes and mDCs capability to induce T-cell hyporesponsiveness. Therefore, the capture of PS- and PSAB-liposomes by DCs conferred them stable tolerogenic function.

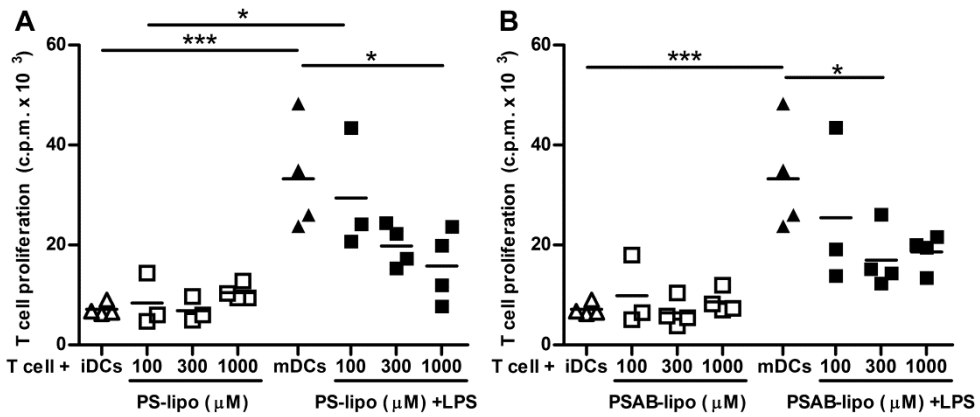

Figure 35. Impaired ability of DCs to stimulate autologous T cell proliferation after the capture of PS- and PS-liposomes, and cytokine secretion. (A) Autologous proliferation of T cells (c.p.m. for 3H thymidine assay) after stimulation induced by iDCs (white triangles), PS-iDCs (white squares), mDCs (black triangles) and PS-mDCs (black squares) with insulin (20 μg/ml) at ratio of 1:10 (DCs:Ts) for 6 days. Three doses of PS- or PSAB-liposomes were used: 100 μM, 300 μM and 1000 μM. Lines show the mean of four independent experiments (*$p<0.05$ and ***$p<0.001$, ANOVA for a randomized complete block design).

(B) Autologous proliferation of T cells (c.p.m. for 3H thymidine assay) after stimulation induced by iDCs (white triangles), PSAB-iDCs (white squares), mDCs (black triangles) and PSAB-mDCs (black squares) with insulin (20 µg/ml) at ratio of 1:10 (DCs:Ts) for 6 days. Three doses of PS- or PSAB-liposomes were used: 100 µM, 300 µM and 1000 µM. Lines show the mean of four independent experiments (*p<0.05 and ***p<0.001, ANOVA for a randomized complete block design).

17. T cell cytokine profile induced by DCs after PSAB-liposome uptake is similar to that induced by iDCs

CBA analysis showed that T cells co-cultured with DCs loaded with liposomes displayed a cytokine profile (IFN-γ, IL-17A) similar to iDCs. IL-2, IL-10 and IL-4 were not detected in any condition of the assay. After LPS stimulus, T cells co-cultured with mDCs and DCs loaded with PS-liposomes significantly increased IFN-γ and IL-17A production **(Figure 36)** ($p<0.01$ and $p<0.05$ respectively). In contrast, T cell proliferation assays induced by DCs loaded with PSAB-liposomes did not significantly increase the production of IFN-γ and IL-17A. No differences were observed in IL-6 and TNF secretion due to the uptake of PS- or PSAB-liposomes.

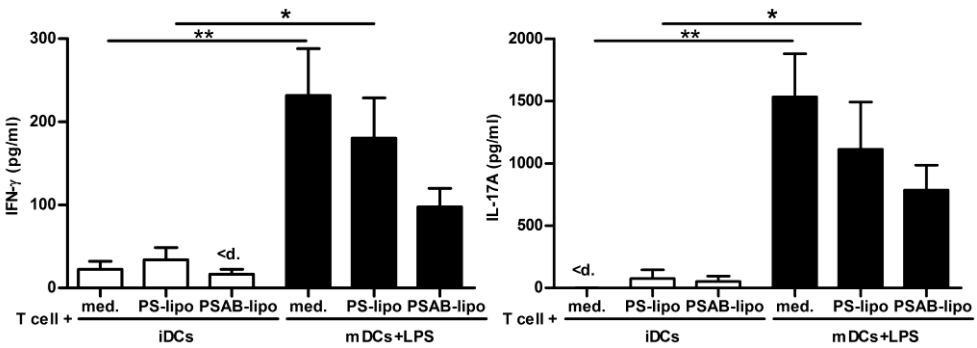

Figure 36. IFN-g and IL17 production in T cell proliferation experiments. Levels of IFN-γ and IL-17A were measured in supernatants from autologous T cell proliferation experiments induced by DCs, DCs loaded with empty liposomes (PS-lipo) and DCs loaded with liposomes filled with insulin peptides in basal conditions (white bars) or previously activated with 100 ng/ml of LPS (black bars), with insulin (20 µg/ml), at ratio of 1:10 (DCs:Ts) for 5 days. Results are expressed as mean+SEM from four independent experiments (*p≤0.05 and **p<0.01, ANOVA for a randomized complete block design). <d means values below the standard.

18. Monitoring liposomes after i.p. administration

Pre-diabetic mice were i.p. injected with a single dose of fluorescence labeled PS-liposomes in order to evaluate the *in vivo* biodistribution of this immunotherapy. At different times post-injection, mice were sacrificed after being anesthetized and their main organs removed. The *in vivo* analysis showed that PS-liposomes signal was detected up to 24 hours after the injection, mainly in the peritoneal area **(Figure 37A, B)**. The *ex vivo* analysis of the organs showed that, at 24 hours post administration, liposome signal took place primarily in perigonadal adipose tissue, mediastinal lymph nodes, pancreatic lymph nodes, pancreas, spleen, mesenteric lymph nodes and liver **(Figure 37C)**. Traces of signal were detected in the kidneys and thymus.

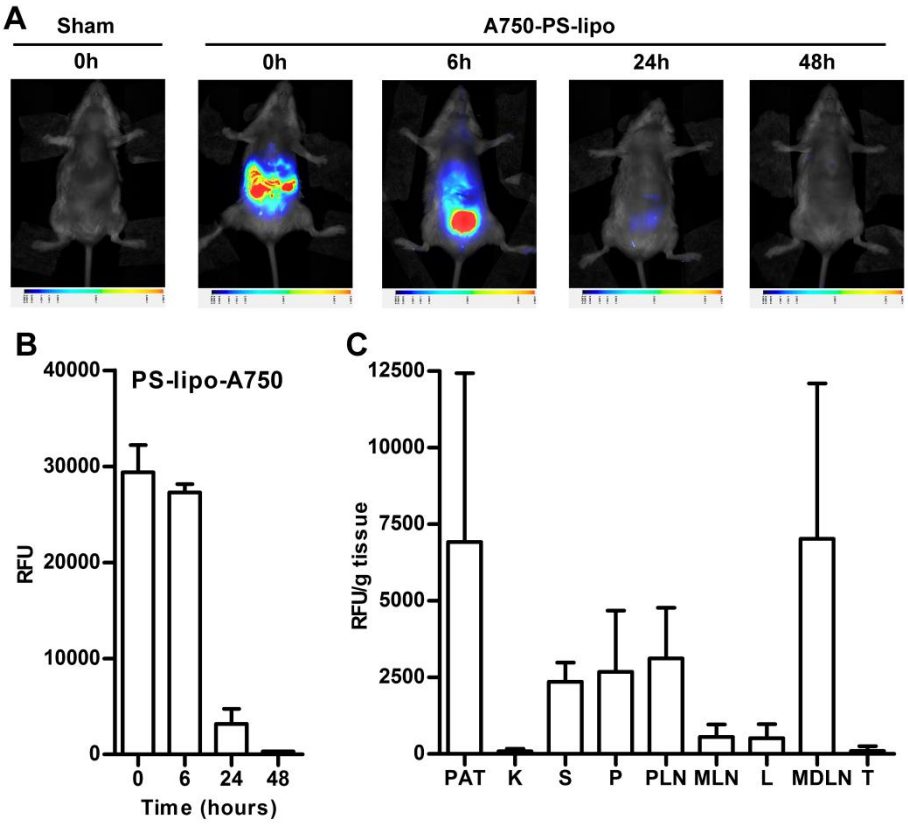

Figure 37. Tracking of A750-labeled PS-liposomes in prediabetic NOD mice.

Figure 37. Tracking of A750-labeled PS-liposomes in prediabetic NOD mice. (A) *In vivo* imaging (Pearl Imager, LI-COR) of 3.5 mg fluorescent A750-labeled PS-liposomes (Alexa Fluor 750) at 6, 24 and 48 hours after i.p. injection in 8-10wk old NOD mice. sham mouse shows background signal. **(B)** Histogram of *in vivo* fluorescence signal (RFU, Relative Flourescence Units) of A750-labelled PS-liposomes from peritoneal area. **(C)** Histogram of *ex vivo* fluorescent signal relative to grams of tissue (RFU/g tissue) of several organs from NOD mice i.p. injected with fluorescence labeled PS-liposomes (Alexa Fluor 750) after 24 hours. PAT, perigonadal adipose tissue; K, kidney; S, spleen; P, pancreas; PLN, pancreatic lymph nodes; MLN, mesenteric lymph nodes; L, liver; MDLN, mediastinal lymph nodes; T, thymus. Results are the mean of three independent experiments.

19. Insulin peptide-filled PSAB-liposomes decrease diabetes incidence in NOD mice

To assess the efficacy of liposomes for preventing T1D, NOD mice were treated with a single dose of PSAB-liposomes during the pre-diabetic period (8 weeks old). As expected, animals from the sham-control group developed diabetes from the age of 11 weeks and with a final incidence of 84.6% **(Figure 38A)**. The treatment with empty PS-liposomes resulted in a disease incidence of 83.3% starting the disease at 15 weeks of age. Interestingly, mice treated with PSAB-liposomes containing β-cell autoantigens showed a T1D incidence of 50%, significantly lower than sham group ($p \leq 0.05$) and starting at 16 weeks of age. No significant differences were found in body weight in mice treated with PSAB-liposomes loaded with insulin peptides, empty PS-liposomes, and control group treated with saline solution **(Figure 38B)**.

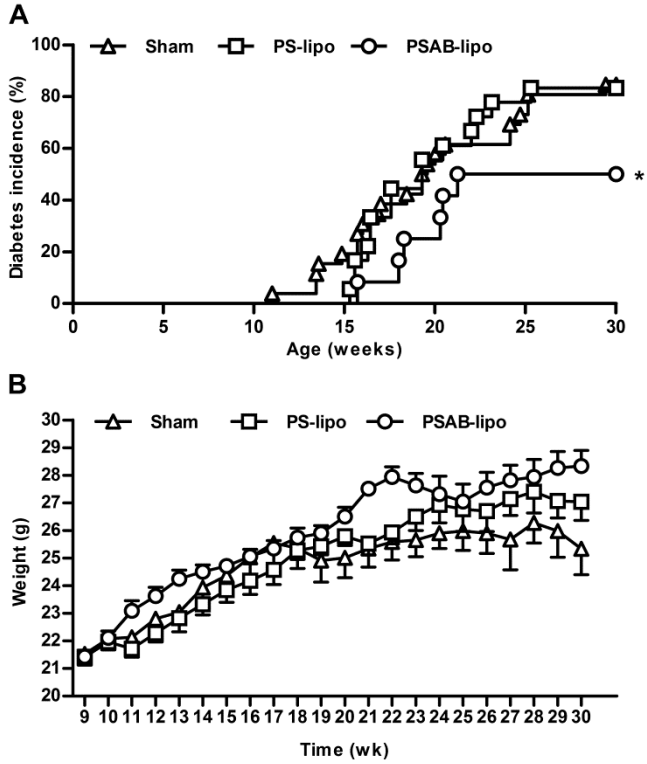

Figure 38. Immunotherapy using PS-liposomes filled with insulin peptides decreases T1D incidence. (A) Cumulative incidence (%) of diabetes in NOD mice treated with PSAB-liposomes loaded with insulin peptides (PSAB-lipo, circles, n=12), empty PS-liposomes (PS-lipo, squares, n=18), and in control group that received saline solution (sham, triangles, n=26). Significant differences were found when compared group treated with PSAB-liposomes versus sham group (*p≤0.05, Kaplan-Meier log-rank analysis). **(B)** Weight (g) of NOD mice treated with PSAB-liposomes (circles), empty PS-liposomes (squares) and sham group (triangles).

20. Insulin peptide-filled PSAB-liposomes reduce insulitis in NOD mice

To determine whether the insulin peptide-filled PSAB-Liposomes may prevent leukocyte recruitment to islets, insulitis score in treated mice was determined at the end of the follow-up period **(Figure 39A)**. As expected, upon completion of the follow up, non diabetic mice in the sham group showed high insulitis score. Mice treated with PS-liposomes showed a similar insulitis degree than sham group. Interestingly, immunotherapy with insulin PSAB-liposomes displayed a significant

reduction of insulitis score when compared to sham group ($p<0.05$). Moreover, the analysis of the islets classified in each of the five infiltration categories showed that 52% of the islets remained non-destructed -free of insulitis or with peri-insulitis- in mice treated with PSAB-liposomes, whereas 35% and 32% of the islets were non-destructed in the sham and the PS-liposomes groups, respectively **(Figure 39B)**.

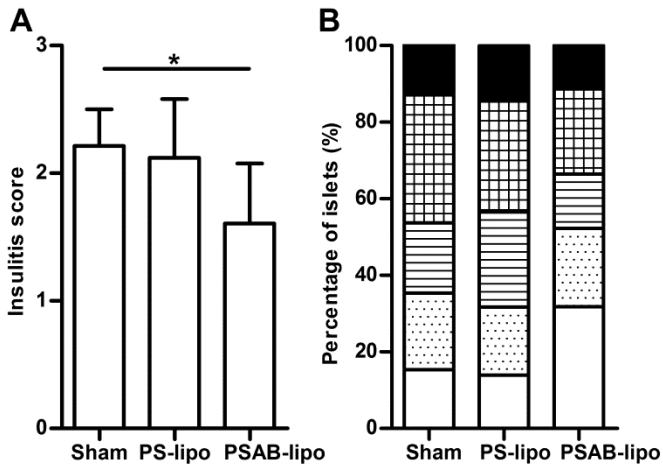

Figure 39. Insulitis score in mice treated with PSAB-liposomes. (A) Insulitis score for different groups, sham, mice treated with empty PS-liposomes (PS-lipo) and mice treated with PSAB-liposomes containing autoantigens (PSAB-lipo). Pancreata from 3-6 non diabetic mice/group were analyzed at the end of the study period (30 weeks). Results are means ± SD (*$p\leq0.05$, Mann Whitney test). **(B)** Percentage of islets classified in each of the five infiltration categories in different groups: White= 0, no insulitis; Dotted = 1, peri-insulitis; Striped = 2, mild insulitis (<25% of the infiltrated islet); Squared = 3, severe insulitis (25-75% of the islet infiltrated); Black = 4, destructive insulitis (>75% islet infiltration).

As a consequence of Results in section II, European patent application has been sent (patent number: 14151629.4-1460)

DISCUSSION

DISCUSSION

1. How apoptotic β-cells can induce tolerance through dendritic cells and prevent experimental type 1 diabetes

During the last years, considerabe efforts have been invested in generating new immunotherapies for T1D. However, no therapy has yet been able to prevent or cure human T1D. Ideally, a T1D immunotherapy should inhibit autoimmune attack against β-cells, avoiding systemic side effects and permitting the regeneration of the damaged tissue. We took advantage of the inherent immunomodulatory properties of apoptotic cells to design an immunotherapy to arrest the immune response against β-cells in experimental models of T1D. When a cell dies, the immune system is alerted by several mechanisms, the processes of which are relevant to host defense and disease pathogenesis [376]. Apoptotic cells serve a dual purpose: they prevent the spreading of cellular debris into the extracellular milieu, and when engulfed by APCs, such as DCs, they contribute to the maintenance of self-tolerance through the presentation of autoantigens in an active suppressive process that constitutes a silencer event [377]. The physiological uptake of apoptotic β-cells by DCs promulgates a preventive effect in T1D [378]. In fact, transfusion of apoptotic NIT-1 cells decreased diabetes incidence in NOD mice [262], supporting the suppressor role of apoptotic cells. The strong evidence linking the removal of apoptotic cells with tolerance to self is the association of autoimmune diseases and defective apoptotic cell clearance [379, 380]. DCs are critical for the initiation of immune responses, as well as for the maintenance of tolerance to self [176]. In fact, various authors have been generated tolerogenic DCs capable to modulate autoimmunity in experimental models [381]. In the clinical setting, a phase I trial has assessed tolerogenic DCs safety in T1D patients with good results [257].

In the present study we combined the immunomodulatory abilities of DCs with the immunosuppressive properties of apoptotic cells to generate an antigen-specific immunotherapy for T1D. Results showed that DCs, when loaded with apoptotic β-

cells, acquire potent immunomodulatory capacities. A link between innate and adaptive immunity via DCs depends upon costimulation during the maturation process [382], which also affects the decision between either tolerance or autoimmunity. We demonstrated that DCs that engulf apoptotic β-cells express low costimulatory molecules and secrete small amounts of proinflammatory cytokines, key features of tolerogenic DCs [294]. This could at least partially explain the re-establishment of peripheral tolerance in our models. This protective function of DCs may be due to antigenic presentation in a tolerogenic manner, since costimulation is necessary for complete T cell activation [383] and antigen presentation with deficient costimulation can induce anergy in autoreactive T cells [304]. In other studies, the low expression of costimulatory molecules in DCs was associated with a reduced incidence of experimental diabetes [250, 255]. Therefore, our results indicated that efferocytosis promote DCs with tolerogenic phenotype. Furthermore, an important requirement of tolerogenic DCs to constitute a safe immunotherapy is their stability, which implies that tolerogenic DCs should be unable to revert into immunogenic DCs in a proinflammatory situation. We showed that efferocytosis conferred DCs resistance to increase costimulation and proinflammatory cytokines secretion after LPS stimulation, in agreement with other results [325].

A key feature of tolerogenic DCs is a low capacity for priming T cells. Therefore, autologous T cell proliferation assays were performed to confirm the ability of efferocytosis to generate functionally tolerogenic DCs. The whole T cell response was analyzed because DCs can present exogenous antigens to $CD4^+$ T cell subset and efficiently cross-present exogenous antigens to $CD8^+$ T cells, a mechanism enhanced in efferocytosis *in vivo* [287]. The results showed the impairment of autologous T cell response after efferocytosis, in concordance with previous data [327], demonstrating that the observed tolerogenic phenotype in DCs resulted in a tolerogenic function. Emphasizing the DC stability, results confirmed that tolerogenic function acquired by DCs after efferocytosis was stable and resistant to maturation; this is a very important feature to take into account for future therapy design. However, we are well aware of the low T cell proliferation index in basal

conditions, probably due to experimental design which uses NOD mice, and the whole T cell repertoire instead of islet specific systems [384]. In terms of cytokine secretion, a low IL-6, TNF-α, IFN-γ and IL-17 phenotype partially mediates the effect in re-establishing peripheral tolerance, through the impairment of the pathogenic Th1/Th17 phenotype progression. IL-17 is very important in autoimmune diseases, since the blockade of IL-17 was shown to prevent diabetes in NOD mice [385]. Interestingly, IL-17 production was found increased in circulating autoreactive $CD4^+$ T cells from T1D patients and related to β-cell death [163]. Nonetheless, we were not able to detect IL-4 production, which is a typical Th2 cytokine [108], suggesting that even the pathogenic Th1/Th17 profile is reduced by DCs after efferocytosis, the mechanism of peripheral tolerance induction should be different from the immune deviation towards Th2 phenotype. Surprisingly, our results showed a lack of IL-10 and TGF-β secretion, which are immunosupressor mediators related to the generation of Tregs [307]. In this sense, DCs did not induce $CD4^+$ $CD25^+$ $FoxP3^+$ Treg generation after efferocytosis, thus suggesting that the induction of tolerance is not dependent on the increase in these regulatory cells, as reported [386]. However, the changes may be qualitative and not quantitative. Because Tregs constitute a heterogeneous cell population, without specific markers to define some of them, other types of Tregs, including $CD8^+$ T cells [156], could be involved in the tolerance reeducation observed after the administration of our immunotherapy.

The reported suppressive function of DCs acquired after efferocytosis is a key feature in this study. After the uptake of apoptotic cells, DCs suppressed T cell proliferation induced by mDCs in a dose dependent manner, thus indicating the existence of an active suppressor mechanism. Microarray data supported this concept and showed overexpression of two genes related to the pathway of PGE_2 synthesis (*Ptgs2* and *Ptges*). In this sense, it was reported that the removal of apoptotic cells by macrophages, which is crucial for the active suppression of inflammation, involved PGE_2 production [387]. PGE_2 is a lipid mediator produced by the liberation of arachidonic acid from cell membrane phospholipids by the action of phospholipase A2 and the subsequent action of cyclooxygenases (COX;

constitutively active cyclooxygenase COX1 and inducible COX2) and prostaglandin synthase E enzymes (cPGES, mPGES-1 and mPGES-2) [388, 389]. PGE_2 has been generally designed to modulate inflammation, promoting local vasodilatation and local attraction and activation of innate immune cells at early stages of inflammation [390, 391]. However, PGE_2 has also a suppressive function that limits inflammation [392]. In agreement with this, it has been proposed that PGE_2 is released during tissue injury and inflammation to protect against immunity to self antigens [393]. In fact, PGE_2 levels were found significantly higher in T1D patients [394], maybe as a response to the inflammation in an immunoregulatory attempt. The possibility of heterogeneous effects described for PGE_2 can partially be explained by the existence of several receptors (EP1, EP2, EP3 and EP4) with different sensitivity and different signaling pathways [395] expressed by the major cells of the immune system, such as DCs, macrophages, T and B cells, among others [396]. In addition, many cells of the body, such as fibroblasts, macrophages, DCs and some types of malignant cells are able to produce PGE_2.

Overall, we confirmed that DCs produce PGE_2 after efferocytosis and we observed that PGE_2 mediated the mentioned suppressive effect in DCs, decreasing T cell proliferation induced by mDCs. The blockade of PGE_2 production, by a COX-2 specific inhibitor, had no effect on T cell proliferation induced by mDCs, but avoided the suppressive function of DCs acquired efferocytosis, thus confirming that PGE_2 was responsible for this effect. In addition, the suppressive effect was also achieved by the supernatants of DCs after efferocytosis, confirming that the suppression was a cell-cell contact independent mechanism. A direct effect of PGE_2 in modulating the T cell response through shifting the Th1 response toward to Th2 has been reported [397, 398] promoting the development of Tregs [399]. In this sense, a study reported PGE_2 production in a shift from Th1 to Th2 response, contributing to insulitis suppression in NOD mice [400]. However, we were unable to either detect IL-4 production in T cell proliferation assays or quantify an increase of classical Tregs *in vitro*. Other studies related PGE_2 suppressive effects in the activation and expansion of naive T cells with the inhibition of IL-2 production [401] and the expression of IL-2 receptor [402, 403]. Therefore, we believe that

PGE_2 could induce a state of anergy in the autoreactive T cells, a well described mechanism of peripheral tolerance induction that depends on IL-2 [304], impairing T cell proliferation even after a complete presentation through mDCs. In addition, PGE_2 can also modulate antigen-specific immune responses by interacting with DCs. The role of PGE_2 in DCs maturation has been broadly studied, given that it has been used in *in vitro* maturation cocktails with proinflammatory cytokines. PGE_2 matured DCs present enhanced ability to activate naive T cells [404, 405]. Nevertheless, an increased production of suppressive factors, such as IL-10 [406] and IDO [407] has also been reported, as well as the development to an exhausted phenotype, manifested by its impaired ability to induce the Th1-, CTL-, and NK cell-mediated type 1 immunity, while promoting Th2 responses [408-410]. Therefore, we cannot rule out an autocrine or paracrine effect of PGE_2 on the DCs, which could inhibit DCs activation after efferocytosis, as well as silence other activated mDCs in the milieu, resulting in the observed suppression of T cell proliferation. In this sense, prostaglandins activate the peroxisome proliferator-activated receptor (PPAR) γ-dependent pathway [411], which is responsive to the lipid status of the cell and has a determinant role in the engulfment of apoptotic cells, negatively regulating DC maturation and avoiding the autoimmune attack against dying cells [412]. Ingenuity analysis of microarray data showed that the canonical pathway of the PPAR is altered in DCs after efferocytosis, suggesting a PGE_2 role in the tolerogenic potential of these DCs. Furthermore, the genes involved in the synthesis of leukotriene from arachidonic acid, such as *Alox15* and *Ltc4s*, were downmodulated after efferocytosis. Leukotrienes are mediators of inflammation and their excessive production has been associated with inflammatory and autoimmune disorders [413].

The use of DC-based immunotherapies to induce tolerance in autoimmune diseases and transplantation has emerged as a promising strategy. However, it has been shown that phenotypic determination of maturation markers in DCs does not guarantee a tolerogenic function [414]. For that reason, the need to define tolerogenic biomarkers has become evident. Gene expression profiles of DCs rendered tolerogenic by different approaches have been previously reported [415-

417]. Until now, the transcriptome of tolDCs after efferocytosis remains unknown. In this study we revealed new altered suppression pathways, such as the previously mentioned PGE_2 pathway one, that support DCs suppressive function after efferocytosis. In addition, the decrease in the expression of antigen processing, presentation and costimulation related genes was confirmed. Surprisingly, the only MHC molecule found upregulated was a non-classical MHC class I (MHC Ib) molecule; whose function is unknown [418], but could have a role in the tolerance induction [419]. The DCs gene expression profile suggests that after efferocytosis, DCs remain immature, but not inactive. Endocytosis-related genes were found upregulated, a feature of tolerogenic DCs [420]. Moreover, a reduced ability of DCs to attract T cells can be suggested in terms of chemokine genes downregulation. The decreased expression of CCL5 gene, an imprint of DC maturation, can be a regulatory effect of PGE_2 as previously described [421]. This fact, together with the decreased expression of CCL17 and CCL22 genes both related to Th2 cells recruitment [422], may alter the lymphocyte subsets recruited by tolerogenic DCs. The expression of chemokine receptor genes related to DCs migratory function [423] were downregulated, in apparent contrast to the previously described upregulation of CCR7 after efferocytosis [261] and through the PGE_2 effect [424]. Our results could be explained by the reported dissociation between CCR7 membrane expression and mRNA amounts [425]. Inflammatory chemokine genes were also upregulated after efferocytosis, involving an active role for DCs in the recruitment of innate immune cells, such as APCs and phagocytes, among others [426]. Surprisingly, proinflammatory TNF and IL-1α genes were upregulated, contrary to low secretion of TNF-α previously quantified in vitro, possibliy due to the low sensitivity of the arrays to detect cytokines [427]. Interestingly, specific transcripts for islet cells, whose expression was confirmed in apoptotic NIT-1 cells microarray, were found in DCs after efferocytosis, some of them defined as T1D autoantigens [179]. The presence of DNA and mRNA molecules, structurally and functionally preserved, within apoptotic bodies was previously described [428, 429]. Other studies have provided evidence that apoptotic bodies that are released from many cell types can transfer microRNAs (miRNAs) to neighboring cells modulating cell functions [430], pointing to a

possible mechanism of epigenetic regulation of gene expression. One study reported that the simple injection of naked mRNA resulted in local uptake and translation [431]. In fact, it has been demonstrated that mRNA transfection of DCs delivers encoded antigen to MHC class I [432, 433]. We believe that after efferocytosis, DCs could translate those mRNA molecules and may present the peptides derived from the neotranslated antigen to $CD8^+$ T cells in a tolerogenic way. In this sense, it was shown that T1D autoantigenic peptides presented in the context of MHC class I suppressed T1D in diabetic NOD mice by expanding a memory-like autoregulatory $CD8^+$ T cells [157].

After confirming the tolerogenic potential of DCs after the uptake of apoptotic β-cells, those DCs were administered to NOD mice, in order to evaluate their safety and capacity to restore tolerance in T1D. The immunotherapy biodistribution is a key feature in tolerance re-establishment, so we expected that DCs should enter the afferent lymph nodes and inhibit autoreactive T cells. Since fluorescent signal from DCs was observed in the target organ or draining lymph nodes, we hypothesized that DCs contributed to an effective local and systemic reestablishment of tolerance after tolerogenic antigen presentation. This fits well with previous results in NOD mice that described DCs signal up to one week after intraperitoneal injection and preferential migration to pancreatic lymph nodes [434]. Surprisingly, signal of DCs loaded with apoptotic NIT-1 was also detected in the mediastinal or parathymic lymph nodes situated at the posterior face of the two lobes of the thymus. Because cortical thymocytopoiesis can be enhanced in the segment adjacent to parathymic lymph nodes after i.p. injection of stimulants [435], we believe that DCs may translocate to the thymus, thus contributing to the maintenance of central tolerance to β-cell antigens.

The transgenic variant of NOD mice, the NOD RIP-IFN-β mouse [76] was used in this study due to its resemblance with T1D in children and young adults in terms of age at onset and similar incidence in males and females. Furthermore, the success of this treatment in NOD RIP-IFN-β mice, a rigorous model to test immunotherapies due to the intrinsic inflammatory environment of the islets, may

encourage the use of tolerogenic DCs to achieve tolerance. The present work reports prevention of T1D in both NOD RIP-IFN-β and wild type NOD mice. T1D prevention correlated with a significant reduction of insulitis, and fits well with the tolerogenic features of DCs after efferocytosis determined *in vitro*. The prevention of T1D was achieved using DCs loaded with apoptotic β-cells, but not with apoptotic cells from an irrelevant source of antigen or unloaded iDCs, suggesting that the antigenic specificity of the apoptotic bodies is a key factor in the process of selective tolerance induction. Among other β-cell autoantigens, NIT-1 cells express insulin and glutamic acid decarboxylase (GAD) [365], which could be processed and presented by DCs in a tolerogenic manner, thus silencing autoreactive T cells. According to these results, NOD mice treated with immature BM-derived DCs pulsed with a mixture of three islet antigen-derived peptides from hsp60 and GAD65 showed a decreased T1D incidence [246], however these were iDCs that could revert to immunogenic DCs in a proinflammatory milieu. Therefore, the administration of apoptotic cells to DCs rather than peptides highlights selectivity for the re-establishment of immunological tolerance and conferred tolerogenic stability, ensuring that DCs, after efferocytosis, remain immature once administered *in vivo*. The synergy between DCs and specific-apoptotic bodies was reported to be a promising strategy in the prevention of chronic rejection [336, 337] by inducing allograft tolerance. Recently, it was reported their capacity to arrest autoimmunity, thus preventing experimental autoimmune encephalomyelitis (EAE) by blocking Th17 cell activity [436]. We are well aware that T1D prevention is not complete and believe that to define the optimal dose and timing of immunotherapy administration would substantially improve the observed preventive effect. In fact, many treatments have used several doses to induce tolerance in NOD mice [437]. In addition, the timing of treatment administration is also important [57]. We treated NOD mice before the initiation of autoimmunity [59], which might be too early and less effective than when administered at the prediabetic stage.

After confirming the prevention of T1D we attempted the reversal of the disease in diabetic mice. Since no effects were observed using DCs loaded with apoptotic

NIT-1 in NOD RIP-IFN-β mice, we used the less aggressive NOD wild type model and included a group of mice simultaneously treated with DCs and rapamycin. Rapamycin, the noncalcineurin-based inhibitor of the serine/threonine protein kinase mammalian target of rapamycin (mTOR), that has been used as an immunosuppressant to prevent acute graft rejection [438]. Rapamycin inhibits cell proliferation and induces anergy in T cells [439]. In addition, it has been used as a pharmacological grade agent to induce tolerogenic DCs *in vitro* [321]. In NOD mice, rapamycin treatment prevented T1D; although no reversal has been reported [440, 441]. Interestingly, ramapycin increased suppressive capability of Tregs from T1D patients [442]. Since our DC-based immunotherapy takes one week to be generated, we thought that rapamycin would contribute to arrest autoimmunity until its administration in diabetic NOD mice, and we predicted a positive synergism among rapamycin and cell therapy. Once more, there was a lack of effect of the DCs loaded with apoptotic β-cells in ameliorating endogenous insulin secretion, even in combination with the rapamycin treatment. In fact, the effect of rapamycin was counteracted, thus indicating that the combination of immunosuppressants with tolerogenic DCs did not increase the ability of our DCs to recover insulin secretion. Our results are according with reported data, where rapamycin combined with other experimental immunotherapies that successfully prevented and cured T1D (e.g., anti-CD3 antibodies) [443] exerted a detrimental effect on disease outcome [444]. Recently, rapamycin/IL-2 combination therapy was used to treat T1D patients, resulting in transient β-cell dysfunction despite an increase in Tregs [445]. This is consistent with the reported impairment of human β-cell function and survival by immunosuppressants [446].

We are well aware that prevention in NOD mice is easier that reversal, since many immune interventions have been described to prevent diabetes in NOD mice [57, 437], but a few have restored insulin secretion. Only two studies reported T1D reversal with tolerogenic/suppressive DCs treatment, showing that T1D reversion with tolerogenic DCs is possible. One study achieved T1D reversal with DCs transfected to secrete IL-4 [254] and the other used costimulation-defficient DCs, but only achieved T1D reversion after several injections in mice with low levels of

non-fasting glucose [256]. Therefore, we think that T1D reversion with tolerogenic DCs generated after efferocytosis of apoptotic β-cells could be possible, and that the results reported here could be due to several factors. On one hand, the stage of insulitis at the time of immunotherapy administration was very severe, thus hindering the recovery of β-cell mass, which is considerably low. On the other hand, the amount of DCs and the number of doses could be too to recover endogenous insulin secretion. Finally, although it has been demonstrated that β-cells can regenerate throughout life [447], the cure of T1D will could require both, the reestablishment of immunological tolerance and the regeneration of a functional β-cell mass in T1D patients.

In summary, the tolerogenic behavior of DCs after the uptake of apoptotic β-cells suggests a mechanism of silencing potential autoreactive T cells in the microenvironment of autoimmunity. This mechanism is mediated, at least in part, through PGE_2 production. We propose that the presentation of autoantigens through deficient costimulation, together with the immunosuppressive milieu created by PGE_2 production could induce anergy of autoreactive T lymphocytes or the expansion of a regulatory cell population, thus reeducating lost tolerance to β-cells and preventing the disease in antigen-specific way. Even if we did not achieve T1D reversal in diabetic mice, we believe that the presented immunotherapy has the potential to do so, and we are exerting our greatest efforts to optimize the immunotherapy to attain it.

2. A new synthetic strategy, based on phosphatidylserine-liposomes loaded with insulin peptides, arrests autoimmunity in type 1 diabetes

After confirming the tolerogenic potential conferred to DCs after efferocytosis and considering its broad clinical implications, a main question arise as to: how can we obtain apoptotic β-cells for the clinical use? We ruled out the procurement of islets cells from organ donors, since the enzymatic digestion and the long process to purify pancreatic islets can be detrimental to the subsequent obtaining of apoptotic bodies [448]. Moreover, the complex procedure to obtain and isolate

islet cells induces inflammatory markers [449] and will add further complication in the generation of the immunotherapy. We also ruled out the obtaining of β-cells derived from adult stem cells [450]. Although many efforts have been carried out in this field [451], at present the yield is very low and costs unaffordable. Therefore, we changed the question as to: How can we mimic apoptotic β-cells for therapeutic purposes? On one hand, a central event on the surface of apoptotic cells is the loss of phospholipid arrangement and the exposure of PS component, which is present in the inner layer of the cell membrane and will provides the main signal for efferocytosis [289]. On the other, antigenic content is very important, since we reported that DCs loaded with antigenic irrelevant apoptotic cells did not prevent T1D and that he presence of autoantigenic transcripts in DCs after the uptake of apoptotic β-cells seems to be crucial. Therefore, we designed a synthetic approach and we generated PS-liposomes filled with insulin peptides to simulate the PS recognition of apoptotic β-cells by APCs.

Liposomes herein presented have an optimal size for an efficient uptake by phagocytic cells [452, 453], were anionic and display MVV (multivesicular vesicles) morphology, often observed in large liposomes with advantages in terms of antigen encapsulation [454] and immunological activity [375]. In addition, the reported anti-inflammatory effects of PS-liposomes [361, 363, 364, 455] are mediated by PS [356], an “eat me” signal of apoptotic cells with anti-inflammatory effects [264]. Additional components of the liposomes are PC, which is a neutral liposomal constituent, and CH, which is incorporated for stability purposes [456]. In fact, efferocytosis in most cases is considered a beneficial event that keeps cell clearance immunologically silent [457]. It has been shown that anionic liposomes, composed of PS, PC, and CH, interact with DCs promoting phagocytosis of antigen-containing liposomes [458]. In addition, PS-liposomes could be optimum vehicles of autoantigens from β-cells, by simulating apoptotic cell recognition and inhibiting maturation and immunostimulatory function of DCs [356, 357].

Many T1D autoantigens are shared between humans and NOD mice. Among them, insulin is an obvious target for antigen specific interventions. Insulin

administration by various routes has proved effective in preventing T1D in NOD mice [459, 460]. However, since human results with insulin have been disappointing [57], we believe that an added benefit of using liposome approach is delivering insulin peptides along with PS, mimicking apoptosis. Therefore, the combination of PS-liposome with T1D autoantigens can contribute to a stable and long-term antigen-specific tolerance reestablishment. To that end, two peptides from A and B insulin chains were chosen from the Insulin2 sequence, the predominant form of insulin expressed in murine β-cells, because they were known target epitopes in autoimmune diabetes [461]. Peptide A showed a 50% reduction in the encapsulation when compared to peptide B. This difference was attributed to aminoacid composition and peptide solubility. However, it has been suggested that the insulin B chain bears major T1D-associated epitopes of significance for disease in humans and NOD mice [462]. Nevertheless, we think that this immunotherapy could be improved by evaluating other candidate T1D peptides for liposome encapsulation in terms of solubility, size and antigenicity [463]. After liposome engulfment by DCs, preselected autoantigens could be presented to $CD4^+$ T lymphocytes through MHC class II molecules and to $CD8^+$ T lymphocytes through MHC class I molecules by cross presentation [464] in tolerogenic forms [156, 157], thus preventing the progression of autoimmune destruction.

In vitro experiments revealed important features of liposomes. First, PS- and PSAB-liposomes, empty and loaded with insulin peptides, respectively, were phagocytosed by DCs and were nontoxic. Moreover, liposomes do not degenerate into toxic side products, such as necrotic bodies. Second, after PS- or PSAB-liposomes uptake, DCs maintained low membrane levels of costimulatory molecules CD40 and CD86, and displayed a low capacity for priming T cells, similar to those observed in iDCs and significantly lower than those observed in mDCs. In mice, levels of CD40 are used as a marker to distinguish between inactivated and activated DCs [465]. Regarding to the stability of DCs after capturing the liposomes, the presence of LPS induced a semi-mature phenotype of DCs. However, PS- and PSAB-liposomes conferred mature-resistant functionality to DCs

in a dose dependent manner, maintaining their low capability to stimulate autologous T cell proliferation. In this sense, it has been reported that phenotipically iDCs and semi-mature DCs can expand antigen-specific Tregs [466, 467]. For this reason, some authors suggest to define tolerogenic DCs based on their effector functions in T cells rather than on their phenotype [468, 469]. Third, the uptake of PS- or PSAB-liposomes induced PGE_2 secretion by DCs in a dose dependent manner, a previously described tolerogenic mediator produced by APCs [387, 400] and fitting well with our previous results of DC loaded with apoptotic cells. We noticed that the PGE_2 amount produced by DCs after the capture of liposomes was lower than that produced after the capture of apoptotic β-cells. Since PGE_2 production derives from cell membrane phospholipids [388] and is enhanced by phosphatydilserine presence [359], we believe that liposome membrane composition could be optimized to increase its production and improve tolerance induction. Finally, given the role of IL-17 and IFN-γ in β-cell apoptosis and T1D [163], we showed that IL-17A and IFN-γ production was not increased in T cell proliferation assays induced by DCs after PSAB-liposome uptake, suggesting that PSAB-liposomes contribute to tolerance reestablishment, even after a proinflammatory stimulus, by antigen specific memory cells. Taken together, these results suggest that PSAB-liposomes may induce tolerogenic DCs to promote hyporesponsivness in autoreactive T cells and trigger tolerance, in a similar way than apoptotic β-cells.

Another important issue for this immunotherapy is liposomes biodistribution. After i.p. administration, fluorescent signal from liposomes was detected for at least 24 hours in the pancreatic lymph nodes, spleen, pancreas, and again, in the mediastinal or parathymic lymph nodes, a similar pattern to that observed with DC loaded with apoptotic β-cells. PS-liposomes biodistribution to the target organ and relevant draining lymph nodes might contribute to an effective local and systemic reestablishment of tolerance after tolerogenic antigen presentation. Again, the signal in the mediastinal or parathymic lymph nodes suggests that liposomes may translocate to the thymus, thus contributing to the maintenance of central tolerance to β-cell antigens, as described after i.p. administration of stimulants

[435]. The high amount of signal in the perigonadal adipose tissue was unexpected and it can be due to liposome affinity for fat. However, adipose tissue resident immune cells, such as adipose tissue macrophages and B-2 cells [470] have a key role in the immunometabolism [471], and could contribute to tolerance induction.

In vivo experiments demonstrated that PSAB-liposomes loaded with insulin peptides decreased T1D incidence and slightly delayed the onset of the disease. However, no effect was observed in T1D incidence when NOD mice received empty PS-liposomes, hence demonstrating the antigen specificity of the therapy. Empty liposomes have anti-inflammatory effects [364] that fit well with our *in vitro* results in terms of costimulatory molecule expression, anti-inflammatory mediators and T cell proliferation. However, empty liposomes had no effect in the prevention of T1D *in vivo*, highlighting the role of insulin peptides in the specific arrest of the autoimmune progression and generating antigen specific memory cells capable to induce long-term tolerance. This antigen specificity is well supported by results of previous studies using apoptotic cells with irrelevant antigenic content for T1D. Nevertheless, the assessment of PS-liposomes loaded with irrelevant peptides in T1D prevention should be performed in order to confirm the antigenic specificity of this new immunotherapy. As expected, the prevention of T1D by PSAB- liposomes were in line with a significant reduction of insulitis. The percentage of islets classified in each of the five infiltration categories indicated that immunotherapy with PSAB-liposomes prevented destructive insulitis thus contributing to β-cell preservation.

We are conscious that liposome design should be improved to better simulate apoptotic cells. We chose two insulin peptides as autoantigen sources because insulin is essential for initiation of T1D in NOD mice [209]. However, autorreactivity to other autoantigens occurs during the progression of autoimmunity [472], therefore PS-liposomes could be customized encapsulating peptides from other relevant molecules (GAD, IA-2 and IGRP, among others), thus trying to inactivate the whole β-cell autoreactive T cell repertoire. This can be also prove valuable in a future clinical use, as it would easily allow personalizing each patient's treatment,

according to the presence of islet autoantibodies in sera [473]. Moreover, liposomes could be loaded with mRNAs instead of peptides to increase tolerogenic presentation through MHC class I, or siRNAs or miRNAs to silent the expression of genes involved in antigen presentation and induce tolerogenic DCs *in vivo* [258]. Besides the antigenic content, PS-liposomes could be engineered in membrane composition to target liposomes to DCs and improve efferocytosis. As example, liposomes may be coupled with antibody fragments (Fab) against endocytic receptors expressed in iDCs, such as DEC-205 (CD205) [474], which interestingly recognizes apoptotic cells [475].

Overall and with regards to a future clinical use, there are many considerations to be contemplated. First of all, we have to take into account the administration route. We contend that i.p. administration provides advantages in terms of biodistribution in treating T1D due to the proximity of the pancreas. Chemotherapy drugs have been i.p. administered for ovarian cancer [476]. Second, the dose of the immunotherapy is critical in the tolerance induction and not always easy to calibrate from experimental models to humans. As an example, T1D was prevented in NOD mice with 1 mg of orally insulin, but not with lower doses (0.01-0.1 mg) or higher ones (5 mg) [57]. Third, the number of doses of the immunotherapy is also important, since many treatments need several doses to achieve T1D tolerance [437]. For example, three weekly transfusions of apoptotic NIT-1 cells were required to prevent experimental T1D [262]. Fourth, the optimal check point of the autoimmune process for immunotherapy administration that is still an open question regarding its future clinical application. Immunotherapy use in prediabetic individuals could be possible since the presence of multiple autoantibodies has been a useful value to predict T1D in subjects at genetic risk [473]. Although we have not been able to reverse T1D in already diabetic mice with DCs loaded with apoptotic β-cells, we are hopeful to accomplish it with PS-liposomes loaded with insulin peptides. Finally, we need to define all the molecular mechanisms involved in the tolerance induction to establish biomarkers for the immunotherapy monitoring.

In consequence and after confirming the safety and the tolerogenic potential of PSAB-liposomes loaded with insulin peptides, we believe that the administration of PS-liposomes filled with autoantigens represents a novel and simple approach to down-regulate autoimmunity in T1D. However, we accept that it is important to optimize liposome membrane composition and antigenic content, as well as determine the critical dosing window and optimal pattern of administration in order to improve the presented preventive effect of T1D and achieve T1D reversal in a future treatment to approach.

3. Final considerations: looking for an ideal immunotherapy

We are fully conscious that the NOD mouse strain has some immunological differences compared to human disease [477]. However, experimental models constitute an important tool for dissecting tolerance mechanisms and to test experimental immunotherapies. Obviously, one must be very cautious when translating results from animal models to a clinical setting. First, diabetic patients do not share all the immune defects existing in the NOD mouse. Second, human β-cells are less able to regenerate than rodent β-cells. Third, NOD mice, unlike humans, are genetically identical. An important pre-clinical step will be the assessment of the in vitro effects of the immunotherapies reported here in human cells DCs from healthy subjects and diabetic patients. In addition, immunotherapies will benefit from the use of novel humanized MHC class II mouse models [478]. In any case, efforts should be put into guiding translational research to successful clinical therapy for T1D.

The potential advantages of the treatment with DCs loaded with apoptotic cells presented here are: 1) treatment with autologous cells that would not require immunosuppressants; 2) the possibility to obtain tolerogenic DCs in the laboratory; 3) the possibility of several doses, if long-term tolerance is not achieved with a single course of DCs and 4) the safety of the treatment. However, our DC-based immunotherapy still has limitations for the clinical practice: 1) the obtaining procurement of apoptotic β-cells; 2) the quality and safety controls to

ensure tolerogenic function of DCs and 3) the requirements to obtain autologous DCs in terms of costs and patient discomfort.

The use of liposomes rich in phosphatidylserine filled with autoantigens to mimic apoptotic cells shares the antigen specificity and the safety of DC-based immunotherapy and overcomes the main limitation of apoptotic β-cells obtention. In addition, it includes the following advantages: 1) the facility of manufacture and standardization; 2) the relative low-cost of production; 3) the facility of administrate several doses; 4) the potential to personalize the treatment. However, many concerted efforts should be done to optimize its tolerogenic potential and to study the molecular mechanisms involved.

Our study proposes an experimental antigen-specific immunotherapy that could be of paramount importance for the design of future treatments in human T1D. We believe that the optimization of this immunotherapy that has proven capable to reestablish peripheral immunological tolerance, in combination with agents able to dampen inflammation and help β-cell regeneration, will result in a treatment for T1D.

CONCLUSIONS

CONCLUSIONS

1. We have generated a cellular immunotherapy based on autologous DCs loaded with apoptotic β-cells for the treatment of autoimmunity in experimental T1D.

2. DCs acquire tolerogenic features after the uptake of apoptotic cells. Low costimulatory molecules expression, decreased proinflammatory cytokine secretion, impaired autologous T cells proliferation, reduced Th1/Th17 polarization and suppressive function explain the reestablishment of peripheral tolerance. Moreover, efferocytosis induces stable tolerogenic DCs.

3. PGE_2 is responsible for the immunosuppressive function of DCs after the capture of apoptotic cells.

4. DCs loaded with apoptotic β-cells prevent experimental T1D through the reestablishment of antigen-specific tolerance, although disease reversal has not been achieved. Biodistribution of DCs in the target organ and lymphoid tissues concurs with immunological effects of the therapy.

5. To overcome limitations of apoptotic β-cell source, we have generated a synthetic therapy based on liposomes rich in PS loaded with insulin peptides.

6. PS-liposomes are efficiently phagocytosed by DCs resulting in a tolerogenic phenotype, impaired autologous T cell proliferation, reduced Th1/Th17 polarization and increased PGE_2 secretion, pointing to them as an alternative to the cell therapy.

7. PS-liposomes loaded with insulin peptides prevent experimental T1D. Their biodistribution contributes to the tolerance reestablishment.

8. Apoptotic mimicry provided by liposomes can offer a solution to the complexity of cell-based therapies with many benefits, such as being low-cost and easy to standardize, large-scale production and customization.

ARTICLES

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Publications

- **Pujol-Autonell I,** Planas R, Ampudia R, Marín-Gallén S, Sanchez A, Carrascal J, , Marin A, Puig-Domingo M, Pujol-Borrell R, Verdaguer J, Vives-Pi M. Efferocytosis promotes suppresive effects in dendritic cells through prostaglandin E2 production in the context of autoimmunity. *PLOS ONE* 8:e63296, 2013. PMID: 23691013

- **Pujol-Autonell I**, Ampudia RM, Monge P, Lucas AM, Carrascal J, Verdaguer J, Vives-Pi M. Immunotherapy with tolerogenic dendritic cells alone or in combination with rapamycin does not reverse diabetes in NOD mice. *ISRN Endocrinology* 2013:346987, 2013. PMID:23555060

- Ampudia RM, Alba A, Planas R, **Pujol-Autonell I**, Mora C, Verdaguer J, Vives-Pi M. PCR-based microsatellite analysis to accelerate diabetogenic genetic background acquisition in transgenic mice. *Inmunología* 30:2-7, 2011.

- Vives-Pi M, **Pujol-Autonell I**. Inmunointervención en diabetes tipo 1: vacunación preventiva y terapéutica. En: *Nuevas Fronteras en Diabetes*. Vol 2, pp 27-42. Ed. Elsevier España, Barcelona, 2011.

- Marín-Gallén S, Clemente-Casares X, Planas R, Carrascal J, **Pujol-Autonell I**, Carrillo J, Ampudia R, Pujol-Borrell R, Verdaguer J, Borràs FE, Vives-Pi M. Prevention of Type 1 Diabetes by Immunotherapy with Syngeneic Dendritic Cells Pulsed with Antigen-Specific Apoptotic Bodies. *Clin exp Immunol* 160:207-214, 2010. PMID: 20030670

Patent

- European patent application number: 14151629.4-1460. Autoantigen-containing liposomes for the treatment of autoimmune diseases. IGTP-ICN2

Publications from scientific collaborations

- Vives-Pi M. Takasawa S, Pujol-Autonell I, Planas R, Cabre E, Ojanguren I, Montraveta M, Santos AL, Ruiz-Ortiz E. Biomarkers for diagnosis and monitoring of celiac disease. *J Clin Gastroenterol*, 47:308-313, 2013. PMID: 23388848

- Ruiz-Ortiz E, Santos AL, **Pujol-Autonell I**, Planas R, Montraveta M, Pintos G, Dolade M, Cabre E, Vives-Pi M. Urinary levels of regenerating gene Ia do not differentiate celiac patients and healthy subjects. *Biomarkers* 18: 178-80, 2013. PMID: 23312007

- Pedrosa E, Carretero-Iglesia L, Boada A, Colobran R, Faner R, **Pujol-Autonell I**, Palou E, Esteve A, Pujol-Borrell R, Ferrándiz C, Juan M, Carrascosa JM. CCL4L Polymorphisms and CCL4/CCL4L Serum Levels Are Associated with Psoriasis Severity. *J Invest Dermatol* 131:1830-7, 2011. PMID: 21614014.

- Planas R, **Pujol-Autonell I**, Ruiz E, Montraveta M, Cabre E, Lucas-Martín A, Pujol-Borrell R, Martínez-Caceres E, Vives-Pi M. Regenerating gene Iα is a biomarker for diagnosis and monitoring of celiac disease: A preliminary study. *Translational Research*, 158:140-5, 2011. PMID: 21867979

ANNEX

ANNEX

Table 13. Differentially expressed genes in DCs after efferocytosis of islet cells

Category	Symbol	Gene ID	Name	Log2 FC
Actin-Based Motility	**Ankrd44**	329154	ankyrin repeat domain 44	-0.52
	Enah	13800	enabled homolog	-0.53
	Fmnl1	57778	formin-like 1	-0.50
	Fscn1	14086	fascin homolog 1, actin bundling protein	-0.65
	Myo1b	17912	myosin IB	0.68
	Ssh2	237860	slingshot homolog 2	-0.85
	Tns3	319939	Tensin 3	0.59
Adhesion	**Adora3**	11542	adenosine A3 receptor	-0.72
	Amica1	270152	adhesion molecule, interacts with CXADR antigen 1	-0.88
	Ccdc80	67896	coiled-coil domain containing 80	-0.88
	Cd34	12490	CD34 antigen	-1.26
	Cd69	12515	CD69 antigen	-0.68
	Cldn1	12737	claudin 1	-1.05
	Flrt2	399558	fibronectin leucine rich transmembrane protein 2	0.50
	Flrt3	71436	fibronectin leucine rich transmembrane protein 3	0.50
	Hepacam2	101202	HEPACAM family member 2	-0.50
	Pcdh7	54216	protocadherin 7	0.66
	Pdpn	14726	podoplanin	0.48
Ag Presentation & Costimulation	**Adra2a**	11551	adrenergic receptor, alpha 2a	-0.55
	Cd74	16149	invariant polypeptide of MHC complex, class II antigen-associated	-0.93
	Cd80	12519	CD80 antigen	-0.54
	Cd83	12522	CD83 antigen	-1.59
	Cd86	12524	CD86 antigen	-0.46
	Clec2i	93675	C-type lectin domain family 2, member i	-0.89
	Fgl2	14190	fibrinogen-like protein 2	-0.76
	H2-Ab1	14961	histocompatibility 2, class II antigen A, beta 1	-1.28
	H2-DMb2	15000	histocompatibility 2, class II, locus Mb2	-0.63
	H2-Eb1	14969	histocompatibility 2, class II antigen E beta	-1.23
	H2-M2	14990	histocompatibility 2, M region locus	0.50
	Il4i1	14204	interleukin 4 induced 1	-1.42
	Klrb1f	232408	killer cell lectin-like receptor subfamily B member 1F	-1.03
	Pdcd1lg2	58205	programmed cell death 1 ligand 2	-0.99
	Rab27a	11891	RAB27A, member RAS oncogene family	-0.52
	Tmem123	71929	transmembrane protein 123	-0.49
Apoptosis	**Bmf**	171543	BCL2 modifying factor	-0.48
	Clec4e	56619	C-type lectin domain family 4, member e	0.50
	Clu	12759	clusterin	-0.94
	Gas5	14455	growth arrest specific 5	-0.46
	Gpr65	14744	G-protein coupled receptor 65	-1.07
	Rassf2	215653	Ras association (RalGDS/AF-6) domain family member 2	-0.65
	Spint2	20733	serine protease inhibitor, Kunitz type 2	-0.49
	Trp53i11	277414	transformation related protein 53 inducible protein 11	-0.53
	Tspan13	66109	tetraspanin 13	-1.11
Cell Cycle	**Ccnd2**	12444	cyclin D2	-0.55
	Ccng2	12452	cyclin G2	-0.51
	Cdc14a	229776	CDC14 cell division cycle 14 homolog A	-0.46
	Ctdsp2	52468	CTD small phosphatase 2	-0.48
	Dna2	327762	DNA replication helicase 2 homolog	-0.64
	Hemk1	69536	HemK methyltransferase family member 1	-0.64
	Ms4a6d	68774	membrane-spanning 4-domains, subfamily A, member 6D	0.54
	Plk2	20620	polo-like kinase 2	0.54
	Pten	19211	phosphatase and tensin homolog	-0.51
	Trp53inp1	60599	transformation related protein 53 inducible nuclear protein 1	-0.50
Chemokines	**Ccl12**	20293	chemokine (C-C motif) ligand 12	0.62
	Ccl17	20295	chemokine (C-C motif) ligand 17	-1.20
	Ccl2	20296	chemokine (C-C motif) ligand 2	0.83
	Ccl22	20299	chemokine (C-C motif) ligand 22	-2.18
	Ccl3	20302	chemokine (C-C motif) ligand 3	0.48
	Ccl4	20303	chemokine (C-C motif) ligand 4	0.52
	Ccl5	20304	chemokine (C-C motif) ligand 5	-2.24
	Ccl7	20306	chemokine (C-C motif) ligand 7	1.04
	Ccr2	12772	chemokine (C-C motif) receptor 2	-1.03
	Ccr7	12775	chemokine (C-C motif) receptor 7	-2.42

	Cx3cl1	20312	chemokine (C-X3-C motif) ligand 1	-0.72
	Cxcl1	14825	chemokine (C-X-C motif) ligand 1	0.74
	Cxcl5	20311	chemokine (C-X-C motif) ligand 5	1.57
	Cxcr2	12765	chemokine (C-X-C motif) receptor 2	-0.55
	Ppbp	57349	pro-platelet basic protein	0.54
Cytokines	**Il18rap**	16174	interleukin 18 receptor accessory protein	-0.80
	Il1a	16175	interleukin 1, alpha	0.84
	Il1r2	16178	interleukin 1 receptor, type II	-1.75
	Il2ra	16184	interleukin 2 receptor, alpha chain	-1.37
	Tnf	21926	tumor necrosis factor	0.78
	Tnfrsf18	21936	tumor necrosis factor receptor superfamily, member 18	-0.50
	Tnfrsf9	21942	tumor necrosis factor receptor superfamily, member 9	-1.52
	Tnfsf4	22164	tumor necrosis factor (ligand) superfamily, member 4	-1.18
	Tnfsf8	21949	tumor necrosis factor (ligand) superfamily, member 8	-0.64
Immunoregulation	**Arrdc3**	105171	arrestin domain containing 3	-0.55
	Asb2	65256	ankyrin repeat and SOCS box-containing 2	-0.72
	Cytip	227929	cytohesin 1 interacting protein	-1.33
	Dok7	231134	docking protein 7	0.54
	Fst	14313	follistatin	0.59
	Htra1	56213	HtrA serine peptidase 1	-0.89
	Klk1b11	16613	kallikrein 1-related peptidase b11	-1.00
	Klk1b21	16616	kallikrein 1-related peptidase b21	-0.52
	Klk1b9	13648	kallikrein 1-related peptidase b9	-0.53
	Lilra6	18726	leukocyte immunoglobulin-like receptor, subfamily A, member 6	-0.48
	Ly9	17085	lymphocyte antigen 9	0.56
	Mmp25	240047	matrix metallopeptidase 25	-1.75
	Pkib	18768	protein kinase inhibitor beta, cAMP dependent, testis specific	-0.51
	Rcan1	54720	regulator of calcineurin 1	0.58
	Retnla	57262	resistin like alpha	-2.09
	Rgs18	64214	regulator of G-protein signaling 18	-0.84
	S100a8	20201	S100 calcium binding protein A8	-0.61
	S100a9	20202	S100 calcium binding protein A9	-0.51
	Serpinb10	241197	serpin peptidase inhibitor, clade B (ovalbumin), member 10	-0.50
	Serpinb2	18788	serine (or cysteine) peptidase inhibitor, clade B, member 2	1.06
	Serpinb6b	20708	serine (or cysteine) peptidase inhibitor, clade B, member 6b	-0.55
	Serpinb8	20725	serpin peptidase inhibitor, clade B (ovalbumin), member 8	0.79
	Sesn2	230784	sestrin 2	-0.58
	Sh2d1b1	26904	SH2 domain protein 1B1	-0.60
	Slamf6	30925	SLAM family member 6	0.55
	Slamf7	75345	SLAM family member 7	-0.78
	Slfn1	20555	schlafen 1	-1.39
	Socs2	216233	suppressor of cytokine signaling 2	-0.56
	Stfa1	20861	stefin A1	-0.56
	Stfa3	20863	stefin A3	-0.48
	Tmem176a	66058	transmembrane protein 176A	-0.61
	Tmem176b	65963	transmembrane protein 176B	-1.32
	Trmt61a	328162	tRNA methyltransferase 61 homolog A	0.52
	Vasn	246154	vasorin	0.50
Metabolism	**Acat3**	224530	acetyl-Coenzyme A acetyltransferase 3	-0.66
	Acsl1	14081	acyl-CoA synthetase long-chain family member 1	0.78
	Akr1c18	105349	aldo-keto reductase family 1, member C18	-1.03
	Aldh1a2	19378	aldehyde dehydrogenase family 1, subfamily A2	-1.27
	Alox15	11687	arachidonate 15-lipoxygenase	-0.62
	B3galt2	26878	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2	-0.78
	Car2	12349	carbonic anhydrase 2	-0.63
	Car4	12351	carbonic anhydrase 4	-0.55
	Ch25h	12642	cholesterol 25-hydroxylase	1.20
	Cyp11a1	13070	cytochrome P450, family 11, subfamily a, polypeptide 1	-0.48
	Cyp4f18	72054	cytochrome P450, family 4, subfamily f, polypeptide 18	-0.66
	Dgka	13139	diacylglycerol kinase, alpha	-0.64
	Dio2	13371	deiodinase, iodothyronine, type II	0.70
	Ereg	13874	epiregulin	0.95
	F13a1	74145	coagulation factor XIII, A1 subunit	0.65
	F2rl2	14064	coagulation factor II (thrombin) receptor-like 2	-0.63
	F3	14066	coagulation factor III (thromboplastin, tissue factor)	0.55
	Fabp4	11770	fatty acid binding protein 4, adipocyte	0.76
	Fbp1	14121	fructose bisphosphatase 1	-0.81
	Igf1	16000	insulin-like growth factor 1 (somatomedin C)	0.83
	Kmo	98256	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	-0.84
	Lipe	16890	lipase, hormone sensitive	-0.79
	Lipn	70166	lipase, family member N	0.75
	Ltc4s	17001	leukotriene C4 synthase	-0.48

	Maoa	17161	monoamine oxidase A	0.58
	Mtmr4	170749	myotubularin related protein 4	-0.85
	Nedd4	17999	neural precursor cell expressed, developmentally down-regulated 4	-0.72
	P2ry10	78826	purinergic receptor P2Y, G-protein coupled 10	-1.37
	Pde3b	18576	phosphodiesterase 3B, cGMP-inhibited	-0.49
	Pip4k2a	18718	phosphatidylinositol-5-phosphate 4-kinase, type II, alpha	-0.51
	Ppap2b	67916	phosphatidic acid phosphatase type 2B	0.52
	Ptges	64292	prostaglandin E synthase	1.10
	Ptgs2	19225	prostaglandin-endoperoxide synthase 2	0.97
	Ramp3	56089	receptor (calcitonin) activity modifying protein 3	-2.21
	St3gal4	20443	ST3 beta-galactoside alpha-2,3-sialyltransferase 4	-0.48
	Stfa2l1	268885	stefin A2 like 1	-1.64
	Uck2	80914	uridine-cytidine kinase 2	-0.56
Natural Immunity	**Abp1**	76507	amiloride binding protein 1 (amine oxidase, copper-containing)	-0.54
	Aoah	27052	acyloxyacyl hydrolase (neutrophil)	0.55
	Apobec1	11810	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	0.63
	Cd180	17079	CD180 antigen	0.56
	Cd209a	170786	CD209a antigen	-0.46
	Cd300e	217306	CD300e antigen	-0.63
	Cfb	14962	complement factor B	0.52
	Clec10a	17312	C-type lectin domain family 10, member A	-0.61
	Colec12	140792	collectin sub-family member 12	0.50
	Epx	13861	eosinophil peroxidase	-0.54
	Ifitm6	213002	interferon induced transmembrane protein 6	-1.41
	Lcn2	16819	lipocalin 2	-1.69
	Marco	17167	macrophage receptor with collagenous structure	1.07
	Mgl2	216864	macrophage galactose N-acetyl-galactosamine specific lectin 2	-1.23
	Mrc1	17533	mannose receptor, C type 1	0.54
	Mx1	17857	myxovirus (influenza virus) resistance 1	-0.55
	Myo10	17909	myosin X	0.50
	Pglyrp1	21946	peptidoglycan recognition protein 1	-0.68
	Prg2	19074	proteoglycan 2, bone marrow	-1.06
	Ptx3	19288	pentraxin 3, long	0.54
	Saa3	20210	serum amyloid A 3	0.49
	Sp140	434484	Sp140 nuclear body protein	-0.61
	Trim13	66597	tripartite motif-containing 13	0.49
NIT-1 cells	**Blnk**	17060	B-cell linker	0.85
	Cck	12424	Cholecystokinin	1.03
	Cpe	12876	carboxypeptidase E	0.56
	Dcbld2	73379	discoidin, CUB and LCCL domain containing 2	0.72
	Iapp	15874	islet amyloid polypeptide	1.70
	Igf2	16002	insulin-like growth factor 2	0.69
	Ins1	16333	insulin I	0.55
	Ins2	16334	insulin II	1.82
	Maob	109731	monoamine oxidase B	0.51
	Mela	17276	melanoma antigen	1.99
	Nt5dc2	70021	5'-nucleotidase domain containing 2	0.53
	Plxna2	18845	plexin A2	0.48
	Sst	20604	somatostatin	2.17
	Tspan7	21912	tetraspanin 7	1.37
Other	**6330407A03Rik**	70720	RIKEN cDNA 6330407A03 gene	-0.46
	A530032D15Rik	381287	RIKEN cDNA A530032D15Rik gene	-0.52
	A530064D06Rik	328830	RIKEN cDNA A530064D06 gene	-0.51
	A930001N09Rik	77128	RIKEN cDNA A930001N09 gene	-0.61
	AB041803	232685	cDNA sequence AB041803	-0.50
	AI607873	226691	expressed sequence AI607873	0.91
	Arap2	212285	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2	-0.59
	BC018473	193217	cDNA sequence BC018473	-1.08
	D16Ertd472e	67102	DNA segment, Chr 16, ERATO Doi 472, expressed	-0.50
	Fam117b	72750	family with sequence similarity 117, member	-0.57
	Fam169b	434197	family with sequence similarity 169, member B	-0.48
	Fam198b	68659	family with sequence similarity 198, member B	0.79
	Fam20c	80752	family with sequence similarity 20, member C	0.72
	Fam49a	76820	family with sequence similarity 49, member A	-0.54
	Fam60a	56306	family with sequence similarity 60, member A	-0.48
	Fam60a	56306	family with sequence similarity 60, member A	-0.50
	Fam82a1	381110	family with sequence similarity 82, member A1	0.49
	Gapt	238875	Grb2-binding adaptor, transmembrane	-0.69
	Klrb1b	80782	killer cell lectin-like receptor subfamily B member 1B	-0.93
	Ms4a6c	73656	membrane-spanning 4-domains, subfamily A, member 6C	0.62
	Phxr4	18689	per-hexamer repeat gene 4	-0.51
	Pmp22	18858	peripheral myelin protein 22	0.63

	Rab44	442827	RAB44, member RAS oncogene family	-0.49
	Rogdi	66049	rogdi homolog	-0.47
	Tarm1	245126	T cell-interacting, activating receptor on myeloid cells 1	-0.53
	Tm4sf19	277203	transmembrane 4 L six family member 19	0.53
	Tmem149	101883	transmembrane protein 149	-0.50
	Tmtc2	278279	transmembrane and tetratricopeptide repeat containing 2	-0.71
	Tspan33	232670	tetraspanin 33	-0.49
	Tubb2a	22151	tubulin, beta 2A	0.57
Signaling	**Angptl2**	26360	angiopoietin-like 2	0.55
	Arhgap26	71302	Rho GTPase activating protein 26	-0.49
	Arhgef12	69632	Rho guanine nucleotide exchange factor (GEF) 12	-0.46
	Dusp4	319520	dual specificity phosphatase 4	0.69
	Eepd1	67484	endonuclease/exonuclease/phosphatase family domain containing 1	0.52
	Fyn	14360	Fyn proto-oncogene	-0.51
	Gpr114	382045	G protein-coupled receptor 114	-0.60
	Gpr126	215798	G protein-coupled receptor 126	-0.65
	Gpr171	229323	G protein-coupled receptor 171	-0.85
	Gpr55	227326	G protein-coupled receptor 55	-0.47
	Gpr97	54672	G protein-coupled receptor 97	-0.51
	Gprc5c	70355	G protein-coupled receptor, family C, group 5, member C	-0.52
	Jak2	16452	Janus kinase 2	-1.04
	Lphn2	99633	latrophilin 2	0.59
	Map3k14	53859	mitogen-activated protein kinase kinase kinase 14	-0.59
	Mapk13	26415	mitogen-activated protein kinase 13	-0.49
	Met	17295	met proto-oncogene	0.68
	Olfr111	545205	olfactory receptor 111	0.73
	Olfr566	258168	olfactory receptor 566	0.51
	Pdgfa	18590	platelet derived growth factor, alpha	0.60
	Pdgfb	18591	platelet-derived growth factor beta polypeptide	0.80
	Pgf	18654	Placental growth factor	0.68
	Pik3cg	30955	phosphoinositide-3-kinase, catalytic, gamma polypeptide	-0.61
	Rabgap1l	29809	RAB GTPase activating protein 1-like	-0.73
	Rasgef1b	320292	RasGEF domain family, member 1B	0.64
	Rasgrp4	233046	RAS guanyl releasing protein 4	-0.58
	Rgl1	19731	ral guanine nucleotide dissociation stimulator,-like 1	0.55
	Rhob	11852	ras homolog gene family, member B	0.54
	Rhof	23912	ras homolog gene family, member f	-0.48
	Samsn1	67742	SAM domain, SH3 domain and nuclear localization signals, 1	-0.61
	Stk39	53416	serine/threonine kinase 39, STE20/SPS1 homolog	-0.67
	Vrk1	22367	vaccinia related kinase 1	-0.47
Solute Transporter	**Aqp9**	64008	aquaporin 9	-0.79
	Atp2a3	53313	ATPase, Ca++ transporting, ubiquitous	-0.54
	Cacnb3	12297	calcium channel, voltage-dependent, beta 3 subunit	-1.30
	Lpcat1	210992	lysophosphatidylcholine acyltransferase 1	0.50
	Mcoln2	68279	mucolipin 2	0.57
	Ms4a7	109225	membrane-spanning 4-domains, subfamily A, member 7	0.57
	Nup210	54563	nucleoporin 210	-0.46
	Slc16a7	20503	solute carrier family 16 (monocarboxylic acid transporters), member 7	0.50
	Slc24a3	94249	solute carrier family 24 (sodium/potassium/calcium exchanger), member 3	-0.54
	Slc36a2	246049	solute carrier family 36 (proton/amino acid symporter), member 2	0.58
	Slc40a1	53945	solute carrier family 40 (iron-regulated transporter), member 1	0.73
	Slc7a5	20539	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	-0.69
	Slc7a8	50934	solute carrier family 7 (amino acid transporter, L-type), member 8	0.56
	Slco3a1	108116	solute carrier organic anion transporter family, member 3a1	-0.48
	Slco4a1	108115	solute carrier organic anion transporter family, member 4a1	0.64
	Snx16	74718	sorting nexin 16	0.48
	Tacstd2	56753	tumor-associated calcium signal transducer 2	-0.67
Transcription Factor	**Bhlhe41**	79362	basic helix-loop-helix family, member e41	0.77
	Btg2	12227	B-cell translocation gene 2, anti-proliferative	-0.71
	Ifi204	15951	interferon activated gene 204	0.51
	Irf4	16364	interferon regulatory factor 4	-0.80
	Irg1	16365	immunoresponsive gene 1	0.76
	Nfe2	18022	nuclear factor, erythroid derived 2	-0.51
	Nfil3	18030	nuclear factor, interleukin 3, regulated	-0.82
	Nr4a3	18124	nuclear receptor subfamily 4, group A, member 3	-1.57
	Sp140	434484	Sp140 nuclear body protein	-0.58
	Stat4	20849	signal transducer and activator of transcription 4	-1.27
	Trps1	83925	trichorhinophalangeal syndrome I	-0.69
	Vdr	22337	vitamin D (1,25- dihydroxyvitamin D3) receptor	-0.92
	Vgll4	232334	vestigial like 4	-0.54

	Xbp1	22433	X-box binding protein 1	-0.63
	Zc3h12c	244871	zinc finger CCCH type containing 12C	0.66
	Zfp217	228913	zinc finger protein 217	-0.69

REFERENCES

REFERENCES

[1] Weir GC, Bonner-Weir S. Islets of Langerhans: the puzzle of intraislet interactions and their relevance to diabetes. J Clin Invest 1990;85:983-7.

[2] Bishop AE, Polak JM. The anatomy, organisation and ultrastructure of the islets of Langerhans. Textbook of diabetes (eds. J. Pickup and G. Williams) 1997:6.1–6.16. Blackwell Science, Oxford, UK.

[3] Wierup N, Sundler F, Heller RS. The islet ghrelin cell. J Mol Endocrinol 2014;52:R35-49.

[4] Imagawa A, Hanafusa T, Miyagawa J, Matsuzawa Y. A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. Osaka IDDM Study Group. N Engl J Med 2000;342:301-7.

[5] Harrison LC. Vaccination against self to prevent autoimmune disease: the type 1 diabetes model. Immunol Cell Biol 2008;86:139-45.

[6] Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 1997;20:1183-97.

[7] Diabetes mellitus. Report of a WHO Study Group. World Health Organ Tech Rep Ser 1985;727:1-113.

[8] Robertson RP, Davis C, Larsen J, Stratta R, Sutherland DE, American Diabetes A. Pancreas and islet transplantation in type 1 diabetes. Diabetes Care 2006;29:935.

[9] Zinger A, Leibowitz G. Islet transplantation in type 1 diabetes: hype, hope and reality - a clinician's perspective. Diabetes Metab Res Rev 2014;30:83-7.

[10] International Diabetes Federation. 2013. IDF Diabetes Atlas, 6th edn., ed. International Diabetes Federation. Brussels, Belgium

[11] Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care 2000;23:1516-26.

[12] Gale EA. The rise of childhood type 1 diabetes in the 20th century. Diabetes 2002;51:3353-61.

[13] Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G, Group ES. Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study. Lancet 2009;373:2027-33.

[14] Thunander M, Petersson C, Jonzon K, Fornander J, Ossiansson B, Torn C, et al. Incidence of type 1 and type 2 diabetes in adults and children in Kronoberg, Sweden. Diabetes Res Clin Pract 2008;82:247-55.

[15] Wandell PE, Carlsson AC. Time trends and gender differences in incidence and prevalence of type 1 diabetes in Sweden. Curr Diabetes Rev 2013;9:342-9.

[16] Karvonen M, Pitkaniemi M, Pitkaniemi J, Kohtamaki K, Tajima N, Tuomilehto J. Sex difference in the incidence of insulin-dependent diabetes mellitus: an analysis of the recent epidemiological data. World Health Organization DIAMOND Project Group. Diabetes Metab Rev 1997;13:275-91.

[17] Jahromi MM, Eisenbarth GS. Genetic determinants of type 1 diabetes across populations. Ann N Y Acad Sci 2006;1079:289-99.

[18] Ounissi-Benkalha H, Polychronakos C. The molecular genetics of type 1 diabetes: new genes and emerging mechanisms. Trends Mol Med 2008;14:268-75.

[19] van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiol Rev 2011;91:79-118.

[20] Vafiadis P, Bennett ST, Todd JA, Nadeau J, Grabs R, Goodyer CG, et al. Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus. Nat Genet 1997;15:289-92.

[21] Dang MN, Buzzetti R, Pozzilli P. Epigenetics in autoimmune diseases with focus on type 1 diabetes. Diabetes Metab Res Rev 2013;29:8-18.

[22] MacFarlane AJ, Strom A, Scott FW. Epigenetics: deciphering how environmental factors may modify autoimmune type 1 diabetes. Mamm Genome 2009;20:624-32.

[23] Bodansky HJ, Staines A, Stephenson C, Haigh D, Cartwright R. Evidence for an environmental effect in the aetiology of insulin dependent diabetes in a transmigratory population. BMJ 1992;304:1020-2.

[24] Gillespie KM, Bain SC, Barnett AH, Bingley PJ, Christie MR, Gill GV, et al. The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. Lancet 2004;364:1699-700.

[25] Moltchanova EV, Schreier N, Lammi N, Karvonen M. Seasonal variation of diagnosis of Type 1 diabetes mellitus in children worldwide. Diabet Med 2009;26:673-8.

[26] Coppieters KT, Boettler T, von Herrath M. Virus infections in type 1 diabetes. Cold Spring Harb Perspect Med 2012;2:a007682.

[27] Filippi CM, von Herrath MG. Viral trigger for type 1 diabetes: pros and cons. Diabetes 2008;57:2863-71.

[28] Salminen K, Sadeharju K, Lonnrot M, Vahasalo P, Kupila A, Korhonen S, et al. Enterovirus infections are associated with the induction of beta-cell autoimmunity in a prospective birth cohort study. J Med Virol 2003;69:91-8.

[29] Banatvala JE, Bryant J, Schernthaner G, Borkenstein M, Schober E, Brown D, et al. Coxsackie B, mumps, rubella, and cytomegalovirus specific IgM responses in patients with juvenile-onset insulin-dependent diabetes mellitus in Britain, Austria, and Australia. Lancet 1985;1:1409-12.

[30] Somoza N, Vargas F, Roura-Mir C, Vives-Pi M, Fernandez-Figueras MT, Ariza A, et al. Pancreas in recent onset insulin-dependent diabetes mellitus. Changes in HLA, adhesion molecules and autoantigens, restricted T cell receptor V beta usage, and cytokine profile. J Immunol 1994;153:1360-77.

[31] Huang X, Yuang J, Goddard A, Foulis A, James RF, Lernmark A, et al. Interferon expression in the pancreases of patients with type I diabetes. Diabetes 1995;44:658-64.

[32] Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK. Cellular immunity to a determinant common to glutamate decarboxylase and coxsackie virus in insulin-dependent diabetes. J Clin Invest 1994;94:2125-9.

[33] Hiemstra HS, Schloot NC, van Veelen PA, Willemen SJ, Franken KL, van Rood JJ, et al. Cytomegalovirus in autoimmunity: T cell crossreactivity to viral antigen and autoantigen glutamic acid decarboxylase. Proc Natl Acad Sci U S A 2001;98:3988-91.

[34] Ou D, Mitchell LA, Metzger DL, Gillam S, Tingle AJ. Cross-reactive rubella virus and glutamic acid decarboxylase (65 and 67) protein determinants recognised by T cells of patients with type I diabetes mellitus. Diabetologia 2000;43:750-62.

[35] Seewaldt S, Thomas HE, Ejrnaes M, Christen U, Wolfe T, Rodrigo E, et al. Virus-induced autoimmune diabetes: most beta-cells die through inflammatory cytokines and not

perforin from autoreactive (anti-viral) cytotoxic T-lymphocytes. Diabetes 2000;49:1801-9.

[36] von Herrath MG, Fujinami RS, Whitton JL. Microorganisms and autoimmunity: making the barren field fertile? Nat Rev Microbiol 2003;1:151-7.

[37] Karjalainen J, Martin JM, Knip M, Ilonen J, Robinson BH, Savilahti E, et al. A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. N Engl J Med 1992;327:302-7.

[38] Ziegler AG, Schmid S, Huber D, Hummel M, Bonifacio E. Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies. JAMA 2003;290:1721-8.

[39] Catassi C, Guerrieri A, Bartolotta E, Coppa GV, Giorgi PL. Antigliadin antibodies at onset of diabetes in children. Lancet 1987;2:158.

[40] Frederiksen B, Kroehl M, Lamb MM, Seifert J, Barriga K, Eisenbarth GS, et al. Infant exposures and development of type 1 diabetes mellitus: The Diabetes Autoimmunity Study in the Young (DAISY). JAMA Pediatr 2013;167:808-15.

[41] Mohr SB, Garland CF, Gorham ED, Garland FC. The association between ultraviolet B irradiance, vitamin D status and incidence rates of type 1 diabetes in 51 regions worldwide. Diabetologia 2008;51:1391-8.

[42] Littorin B, Blom P, Scholin A, Arnqvist HJ, Blohme G, Bolinder J, et al. Lower levels of plasma 25-hydroxyvitamin D among young adults at diagnosis of autoimmune type 1 diabetes compared with control subjects: results from the nationwide Diabetes Incidence Study in Sweden (DISS). Diabetologia 2006;49:2847-52.

[43] Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. Curr Opin Pharmacol 2010;10:482-96.

[44] Vaarala O, Atkinson MA, Neu J. The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. Diabetes 2008;57:2555-62.

[45] Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, et al. Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 2008;455:1109-13.

[46] Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. N Engl J Med 2002;347:911-20.

[47] Kondrashova A, Seiskari T, Ilonen J, Knip M, Hyoty H. The 'Hygiene hypothesis' and the sharp gradient in the incidence of autoimmune and allergic diseases between Russian Karelia and Finland. APMIS 2013;121:478-93.

[48] Ohsugi T, Kurosawa T. Increased incidence of diabetes mellitus in specific pathogen-eliminated offspring produced by embryo transfer in NOD mice with low incidence of the disease. Lab Anim Sci 1994;44:386-8.

[49] Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. J Immunol 2004;172:2731-8.

[50] Rosenberg L. Induction of islet cell neogenesis in the adult pancreas: the partial duct obstruction model. Microsc Res Tech 1998;43:337-46.

[51] Peshavaria M, Larmie BL, Lausier J, Satish B, Habibovic A, Roskens V, et al. Regulation of pancreatic beta-cell regeneration in the normoglycemic 60% partial-pancreatectomy mouse. Diabetes 2006;55:3289-98.

[52] Brockenbrough JS, Weir GC, Bonner-Weir S. Discordance of exocrine and endocrine growth after 90% pancreatectomy in rats. Diabetes 1988;37:232-6.

[53] Lenzen S. The mechanisms of alloxan- and streptozotocin-induced diabetes. Diabetologia 2008;51:216-26.

[54] Like AA, Rossini AA. Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. Science 1976;193:415-7.

[55] Harada M, Makino S. Promotion of spontaneous diabetes in non-obese diabetes-prone mice by cyclophosphamide. Diabetologia 1984;27:604-6.

[56] Brode S, Raine T, Zaccone P, Cooke A. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4+CD25+Foxp3+ regulatory T cells. J Immunol 2006;177:6603-12.

[57] Shoda LK, Young DL, Ramanujan S, Whiting CC, Atkinson MA, Bluestone JA, et al. A comprehensive review of interventions in the NOD mouse and implications for translation. Immunity 2005;23:115-26.

[58] Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y. Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu 1980;29:1-13.

[59] Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. Annu Rev Immunol 2005;23:447-85.

[60] Hoglund P, Mintern J, Waltzinger C, Heath W, Benoist C, Mathis D. Initiation of autoimmune diabetes by developmentally regulated presentation of islet cell antigens in the pancreatic lymph nodes. J Exp Med 1999;189:331-9.

[61] Saxena V, Ondr JK, Magnusen AF, Munn DH, Katz JD. The countervailing actions of myeloid and plasmacytoid dendritic cells control autoimmune diabetes in the nonobese diabetic mouse. J Immunol 2007;179:5041-53.

[62] Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and beta-cell destruction in NOD mice. Diabetes 1994;43:667-75.

[63] Wong FS, Janeway CA, Jr. The role of CD4 and CD8 T cells in type I diabetes in the NOD mouse. Res Immunol 1997;148:327-32.

[64] Kanagawa O, Martin SM, Vaupel BA, Carrasco-Marin E, Unanue ER. Autoreactivity of T cells from nonobese diabetic mice: an I-Ag7-dependent reaction. Proc Natl Acad Sci U S A 1998;95:1721-4.

[65] Maier LM, Wicker LS. Genetic susceptibility to type 1 diabetes. Curr Opin Immunol 2005;17:601-8.

[66] Wentworth BM, Schaefer IM, Villa-Komaroff L, Chirgwin JM. Characterization of the two nonallelic genes encoding mouse preproinsulin. J Mol Evol 1986;23:305-12.

[67] Di Lorenzo TP, Peakman M, Roep BO. Translational mini-review series on type 1 diabetes: Systematic analysis of T cell epitopes in autoimmune diabetes. Clin Exp Immunol 2007;148:1-16.

[68] Winer S, Tsui H, Lau A, Song A, Li X, Cheung RK, et al. Autoimmune islet destruction in spontaneous type 1 diabetes is not beta-cell exclusive. Nat Med 2003;9:198-205.

[69] Yu L, Robles DT, Abiru N, Kaur P, Rewers M, Kelemen K, et al. Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. Proc Natl Acad Sci U S A 2000;97:1701-6.

[70] Wong FS, Wen L, Tang M, Ramanathan M, Visintin I, Daugherty J, et al. Investigation of the role of B-cells in type 1 diabetes in the NOD mouse. Diabetes 2004;53:2581-7.

[71] Hinman RM, Smith MJ, Cambier JC. B cells and type 1 diabetes ...in mice and men. Immunol Lett 2014;

[72] Serreze DV, Gaedeke JW, Leiter EH. Hematopoietic stem-cell defects underlying abnormal macrophage development and maturation in NOD/Lt mice: defective regulation of cytokine receptors and protein kinase C. Proc Natl Acad Sci U S A 1993;90:9625-9.

[73] Ogasawara K, Hamerman JA, Hsin H, Chikuma S, Bour-Jordan H, Chen T, et al. Impairment of NK cell function by NKG2D modulation in NOD mice. Immunity 2003;18:41-51.

[74] Naumov YN, Bahjat KS, Gausling R, Abraham R, Exley MA, Koezuka Y, et al. Activation of CD1d-restricted T cells protects NOD mice from developing diabetes by regulating dendritic cell subsets. Proc Natl Acad Sci U S A 2001;98:13838-43.

[75] Pelegrin M, Devedjian JC, Costa C, Visa J, Solanes G, Pujol A, et al. Evidence from transgenic mice that interferon-beta may be involved in the onset of diabetes mellitus. J Biol Chem 1998;273:12332-40.

[76] Alba A, Puertas MC, Carrillo J, Planas R, Ampudia R, Pastor X, et al. IFN beta accelerates autoimmune type 1 diabetes in nonobese diabetic mice and breaks the tolerance to beta cells in nondiabetes-prone mice. J Immunol 2004;173:6667-75.

[77] Iwakura Y, Asano M, Nishimune Y, Kawade Y. Male sterility of transgenic mice carrying exogenous mouse interferon-beta gene under the control of the metallothionein enhancer-promoter. EMBO J 1988;7:3757-62.

[78] Alba A, Planas R, Clemente X, Carrillo J, Ampudia R, Puertas MC, et al. Natural killer cells are required for accelerated type 1 diabetes driven by interferon-beta. Clin Exp Immunol 2008;151:467-75.

[79] Mordes JP, Bortell R, Blankenhorn EP, Rossini AA, Greiner DL. Rat models of type 1 diabetes: genetics, environment, and autoimmunity. ILAR J 2004;45:278-91.

[80] Wallis RH, Wang K, Marandi L, Hsieh E, Ning T, Chao GY, et al. Type 1 diabetes in the BB rat: a polygenic disease. Diabetes 2009;58:1007-17.

[81] Guberski DL, Butler L, Manzi SM, Stubbs M, Like AA. The BBZ/Wor rat: clinical characteristics of the diabetic syndrome. Diabetologia 1993;36:912-9.

[82] Jackson R, Rassi N, Crump T, Haynes B, Eisenbarth GS. The BB diabetic rat. Profound T-cell lymphocytopenia. Diabetes 1981;30:887-9.

[83] Rose NR, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). Immunol Today 1993;14:426-30.

[84] Eizirik DL, Colli ML, Ortis F. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. Nat Rev Endocrinol 2009;5:219-26.

[85] Uno S, Imagawa A, Okita K, Sayama K, Moriwaki M, Iwahashi H, et al. Macrophages and dendritic cells infiltrating islets with or without beta cells produce tumour necrosis factor-alpha in patients with recent-onset type 1 diabetes. Diabetologia 2007;50:596-601.

[86] Foulis AK, McGill M, Farquharson MA. Insulitis in type 1 (insulin-dependent) diabetes mellitus in man--macrophages, lymphocytes, and interferon-gamma containing cells. J Pathol 1991;165:97-103.

[87] Sarkar SA, Lee CE, Victorino F, Nguyen TT, Walters JA, Burrack A, et al. Expression and regulation of chemokines in murine and human type 1 diabetes. Diabetes 2012;61:436-46.

[88] Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 2005;54 Suppl 2:S97-107.

[89] Vives-Pi M, Somoza N, Fernandez-Alvarez J, Vargas F, Caro P, Alba A, et al. Evidence of expression of endotoxin receptors CD14, toll-like receptors TLR4 and TLR2 and associated molecule MD-2 and of sensitivity to endotoxin (LPS) in islet beta cells. Clin Exp Immunol 2003;133:208-18.

[90] Lang KS, Recher M, Junt T, Navarini AA, Harris NL, Freigang S, et al. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. Nat Med 2005;11:138-45.

[91] Planas R, Carrillo J, Sanchez A, de Villa MC, Nunez F, Verdaguer J, et al. Gene expression profiles for the human pancreas and purified islets in type 1 diabetes: new findings at clinical onset and in long-standing diabetes. Clin Exp Immunol 2010;159:23-44.

[92] Bach JF, Bendelac A, Brenner MB, Cantor H, De Libero G, Kronenberg M, et al. The role of innate immunity in autoimmunity. J Exp Med 2004;200:1527-31.

[93] Poirot L, Benoist C, Mathis D. Natural killer cells distinguish innocuous and destructive forms of pancreatic islet autoimmunity. Proc Natl Acad Sci U S A 2004;101:8102-7.

[94] Gur C, Porgador A, Elboim M, Gazit R, Mizrahi S, Stern-Ginossar N, et al. The activating receptor NKp46 is essential for the development of type 1 diabetes. Nat Immunol 2010;11:121-8.

[95] Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S, et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci U S A 2007;104:5115-20.

[96] Herold KC, Huen A, Gould L, Traisman H, Rubenstein AH. Alterations in lymphocyte subpopulations in type 1 (insulin-dependent) diabetes mellitus: exploration of possible mechanisms and relationships to autoimmune phenomena. Diabetologia 1984;27 Suppl:102-5.

[97] Rodacki M, Svoren B, Butty V, Besse W, Laffel L, Benoist C, et al. Altered natural killer cells in type 1 diabetic patients. Diabetes 2007;56:177-85.

[98] Calderon B, Unanue ER. Antigen presentation events in autoimmune diabetes. Curr Opin Immunol 2012;24:119-28.

[99] Calderon B, Suri A, Miller MJ, Unanue ER. Dendritic cells in islets of Langerhans constitutively present beta cell-derived peptides bound to their class II MHC molecules. Proc Natl Acad Sci U S A 2008;105:6121-6.

[100] Calderon B, Carrero JA, Miller MJ, Unanue ER. Entry of diabetogenic T cells into islets induces changes that lead to amplification of the cellular response. Proc Natl Acad Sci U S A 2011;108:1567-72.

[101] Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 2008;8:594-606.

[102] Welzen-Coppens JM, van Helden-Meeuwsen CG, Leenen PJ, Drexhage HA, Versnel MA. The kinetics of plasmacytoid dendritic cell accumulation in the pancreas of the NOD mouse during the early phases of insulitis. PLoS One 2013;8:e55071.

[103] Hooks JJ, Moutsopoulos HM, Geis SA, Stahl NI, Decker JL, Notkins AL. Immune interferon in the circulation of patients with autoimmune disease. N Engl J Med 1979;301:5-8.

[104] Hutchings P, Rosen H, O'Reilly L, Simpson E, Gordon S, Cooke A. Transfer of diabetes in mice prevented by blockade of adhesion-promoting receptor on macrophages. Nature 1990;348:639-42.

[105] Itoh N, Hanafusa T, Miyazaki A, Miyagawa J, Yamagata K, Yamamoto K, et al. Mononuclear

cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. J Clin Invest 1993;92:2313-22.

[106] Maree AF, Komba M, Dyck C, Labecki M, Finegood DT, Edelstein-Keshet L. Quantifying macrophage defects in type 1 diabetes. J Theor Biol 2005;233:533-51.

[107] O'Brien BA, Geng X, Orteu CH, Huang Y, Ghoreishi M, Zhang Y, et al. A deficiency in the in vivo clearance of apoptotic cells is a feature of the NOD mouse. J Autoimmun 2006;26:104-15.

[108] Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today 1996;17:138-46.

[109] Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol 2009;27:485-517.

[110] Andersen MH, Schrama D, Thor Straten P, Becker JC. Cytotoxic T cells. J Invest Dermatol 2006;126:32-41.

[111] Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. Nature 1998;393:474-8.

[112] Bendelac A, Carnaud C, Boitard C, Bach JF. Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells. J Exp Med 1987;166:823-32.

[113] Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG. Analysis of islet inflammation in human type 1 diabetes. Clin Exp Immunol 2009;155:173-81.

[114] Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shah SC, Bu DF, et al. Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. Lancet 1992;339:458-9.

[115] Naquet P, Ellis J, Tibensky D, Kenshole A, Singh B, Hodges R, et al. T cell autoreactivity to insulin in diabetic and related non-diabetic individuals. J Immunol 1988;140:2569-78.

[116] Hawkes CJ, Schloot NC, Marks J, Willemen SJ, Drijfhout JW, Mayer EK, et al. T-cell lines reactive to an immunodominant epitope of the tyrosine phosphatase-like autoantigen IA-2 in type 1 diabetes. Diabetes 2000;49:356-66.

[117] Zhang Y, O'Brien B, Trudeau J, Tan R, Santamaria P, Dutz JP. In situ beta cell death promotes priming of diabetogenic CD8 T lymphocytes. J Immunol 2002;168:1466-72.

[118] Mohan JF, Calderon B, Anderson MS, Unanue ER. Pathogenic CD4(+) T cells recognizing an unstable peptide of insulin are directly recruited into islets bypassing local lymph nodes. J Exp Med 2013;210:2403-14.

[119] Pang S, Zhang L, Wang H, Yi Z, Li L, Gao L, et al. CD8(+) T cells specific for beta cells encounter their cognate antigens in the islets of NOD mice. Eur J Immunol 2009;39:2716-24.

[120] Mathis D, Vence L, Benoist C. beta-Cell death during progression to diabetes. Nature 2001;414:792-8.

[121] Coppieters KT, Dotta F, Amirian N, Campbell PD, Kay TW, Atkinson MA, et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. J Exp Med 2012;209:51-60.

[122] Coppieters K, Amirian N, von Herrath M. Intravital imaging of CTLs killing islet cells in diabetic mice. J Clin Invest 2012;122:119-31.

[123] Santamaria P. Effector lymphocytes in autoimmunity. Curr Opin Immunol 2001;13:663-9.

[124] Noorchashm H, Noorchashm N, Kern J, Rostami SY, Barker CF, Naji A. B-cells are required for the initiation of insulitis and sialitis in nonobese diabetic mice. Diabetes 1997;46:941-6.

[125] Yu L, Eisenbarth GS. Humoral autoimmunity. Adv Exp Med Biol 2004;552:247-67.

[126] Wong FS, Hu C, Xiang Y, Wen L. To B or not to B--pathogenic and regulatory B cells in autoimmune diabetes. Curr Opin Immunol 2010;22:723-31.

[127] Marino E, Tan B, Binge L, Mackay CR, Grey ST. B-cell cross-presentation of autologous antigen precipitates diabetes. Diabetes 2012;61:2893-905.

[128] Bendelac A. CD1: presenting unusual antigens to unusual T lymphocytes. Science 1995;269:185-6.

[129] Coquet JM, Chakravarti S, Kyparissoudis K, McNab FW, Pitt LA, McKenzie BS, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. Proc Natl Acad Sci U S A 2008;105:11287-92.

[130] Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, et al. Activation of natural killer T cells by alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. Nat Med 2001;7:1057-62.

[131] Hong S, Wilson MT, Serizawa I, Wu L, Singh N, Naidenko OV, et al. The natural killer T-cell ligand alpha-galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. Nat Med 2001;7:1052-6.

[132] Kukreja A, Cost G, Marker J, Zhang C, Sun Z, Lin-Su K, et al. Multiple immuno-regulatory defects in type-1 diabetes. J Clin Invest 2002;109:131-40.

[133] Oikawa Y, Shimada A, Yamada S, Motohashi Y, Nakagawa Y, Irie J, et al. High frequency of valpha24(+) vbeta11(+) T-cells observed in type 1 diabetes. Diabetes Care 2002;25:1818-23.

[134] Anderson G, Moore NC, Owen JJ, Jenkinson EJ. Cellular interactions in thymocyte development. Annu Rev Immunol 1996;14:73-99.

[135] Anderson MS, Venanzi ES, Klein L, Chen Z, Berzins SP, Turley SJ, et al. Projection of an immunological self shadow within the thymus by the aire protein. Science 2002;298:1395-401.

[136] Pugliese A, Zeller M, Fernandez A, Jr., Zalcberg LJ, Bartlett RJ, Ricordi C, et al. The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nat Genet 1997;15:293-7.

[137] Carrasco-Marin E, Shimizu J, Kanagawa O, Unanue ER. The class II MHC I-Ag7 molecules from non-obese diabetic mice are poor peptide binders. J Immunol 1996;156:450-8.

[138] Anderton SM. Post-translational modifications of self antigens: implications for autoimmunity. Curr Opin Immunol 2004;16:753-8.

[139] Mohan JF, Levisetti MG, Calderon B, Herzog JW, Petzold SJ, Unanue ER. Unique autoreactive T cells recognize insulin peptides generated within the islets of Langerhans in autoimmune diabetes. Nat Immunol 2010;11:350-4.

[140] Danke NA, Koelle DM, Yee C, Beheray S, Kwok WW. Autoreactive T cells in healthy individuals. J Immunol 2004;172:5967-72.

[141] Walker LS, Abbas AK. The enemy within: keeping self-reactive T cells at bay in the periphery. Nat Rev Immunol 2002;2:11-9.

[142] Bour-Jordan H, Bluestone JA. Regulating the regulators: costimulatory signals control the

homeostasis and function of regulatory T cells. Immunol Rev 2009;229:41-66.

[143] Luhder F, Hoglund P, Allison JP, Benoist C, Mathis D. Cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) regulates the unfolding of autoimmune diabetes. J Exp Med 1998;187:427-32.

[144] Fife BT, Guleria I, Gubbels Bupp M, Eagar TN, Tang Q, Bour-Jordan H, et al. Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. J Exp Med 2006;203:2737-47.

[145] Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature 2003;423:506-11.

[146] Nielsen C, Hansen D, Husby S, Jacobsen BB, Lillevang ST. Association of a putative regulatory polymorphism in the PD-1 gene with susceptibility to type 1 diabetes. Tissue Antigens 2003;62:492-7.

[147] Tsutsumi Y, Jie X, Ihara K, Nomura A, Kanemitsu S, Takada H, et al. Phenotypic and genetic analyses of T-cell-mediated immunoregulation in patients with Type 1 diabetes. Diabet Med 2006;23:1145-50.

[148] Lee JW, Epardaud M, Sun J, Becker JE, Cheng AC, Yonekura AR, et al. Peripheral antigen display by lymph node stroma promotes T cell tolerance to intestinal self. Nat Immunol 2007;8:181-90.

[149] Gardner JM, Devoss JJ, Friedman RS, Wong DJ, Tan YX, Zhou X, et al. Deletional tolerance mediated by extrathymic Aire-expressing cells. Science 2008;321:843-7.

[150] Yip L, Su L, Sheng D, Chang P, Atkinson M, Czesak M, et al. Deaf1 isoforms control the expression of genes encoding peripheral tissue antigens in the pancreatic lymph nodes during type 1 diabetes. Nat Immunol 2009;10:1026-33.

[151] Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. Nat Rev Immunol 2003;3:253-7.

[152] Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes 2005;54:92-9.

[153] Brusko T, Wasserfall C, McGrail K, Schatz R, Viener HL, Schatz D, et al. No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. Diabetes 2007;56:604-12.

[154] Putnam AL, Vendrame F, Dotta F, Gottlieb PA. CD4+CD25high regulatory T cells in human autoimmune diabetes. J Autoimmun 2005;24:55-62.

[155] Schneider A, Rieck M, Sanda S, Pihoker C, Greenbaum C, Buckner JH. The Effector T Cells of Diabetic Subjects Are Resistant to Regulation via CD4+FOXP3+ Regulatory T Cells. The Journal of Immunology 2008;181:7350-5.

[156] Tsai S, Clemente-Casares X, Santamaria P. CD8(+) Tregs in autoimmunity: learning "self"-control from experience. Cell Mol Life Sci 2011;68:3781-95.

[157] Tsai S, Shameli A, Yamanouchi J, Clemente-Casares X, Wang J, Serra P, et al. Reversal of autoimmunity by boosting memory-like autoregulatory T cells. Immunity 2010;32:568-80.

[158] von Herrath MG, Oldstone MB. Interferon-gamma is essential for destruction of beta cells and development of insulin-dependent diabetes mellitus. J Exp Med 1997;185:531-9.

[159] Ploix C, Bergerot I, Fabien N, Perche S, Moulin V, Thivolet C. Protection against autoimmune diabetes with oral insulin is associated with the presence of IL-4 type 2 T-cells in the pancreas and pancreatic lymph nodes. Diabetes 1998;47:39-44.

[160] Hultgren B, Huang X, Dybdal N, Stewart TA. Genetic absence of gamma-interferon delays but does not prevent diabetes in NOD mice. Diabetes 1996;45:812-7.

[161] Wang B, Gonzalez A, Hoglund P, Katz JD, Benoist C, Mathis D. Interleukin-4 deficiency does not exacerbate disease in NOD mice. Diabetes 1998;47:1207-11.

[162] Serreze DV, Chapman HD, Post CM, Johnson EA, Suarez-Pinzon WL, Rabinovitch A. Th1 to Th2 cytokine shifts in nonobese diabetic mice: sometimes an outcome, rather than the cause, of diabetes resistance elicited by immunostimulation. J Immunol 2001;166:1352-9.

[163] Arif S, Moore F, Marks K, Bouckenooghe T, Dayan CM, Planas R, et al. Peripheral and islet interleukin-17 pathway activation characterizes human autoimmune diabetes and promotes cytokine-mediated beta-cell death. Diabetes 2011;60:2112-9.

[164] Lehuen A, Diana J, Zaccone P, Cooke A. Immune cell crosstalk in type 1 diabetes. Nat Rev Immunol 2010;10:501-13.

[165] Atkinson MA, Bluestone JA, Eisenbarth GS, Hebrok M, Herold KC, Accili D, et al. How Does Type 1 Diabetes Develop?: The Notion of Homicide or β-Cell Suicide Revisited. Diabetes 2011;60:1370-9.

[166] Foulis AK, Liddle CN, Farquharson MA, Richmond JA, Weir RS. The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. Diabetologia 1986;29:267-74.

[167] Imagawa A, Hanafusa T, Tamura S, Moriwaki M, Itoh N, Yamamoto K, et al. Pancreatic biopsy as a procedure for detecting in situ autoimmune phenomena in type 1 diabetes: close correlation between serological markers and histological evidence of cellular autoimmunity. Diabetes 2001;50:1269-73.

[168] Vives-Pi M, Armengol MP, Alcalde L, Costa M, Somoza N, Vargas F, et al. Expression of transporter associated with antigen processing-1 in the endocrine cells of human pancreatic islets: effect of cytokines and evidence of hyperexpression in IDDM. Diabetes 1996;45:779-88.

[169] Vives-Pi M, Vargas F, James RF, Trowsdale J, Costa M, Sospedra M, et al. Proteasome subunits, low-molecular-mass polypeptides 2 and 7 are hyperexpressed by target cells in autoimmune thyroid disease but not in insulin-dependent diabetes mellitus: implications for autoimmunity. Tissue Antigens 1997;50:153-63.

[170] Pujol-Borrell R, Todd I, Londei M, Foulis A, Feldmann M, Bottazzo GF. Inappropriate major histocompatibility complex class II expression by thyroid follicular cells in thyroid autoimmune disease and by pancreatic beta cells in type I diabetes. Mol Biol Med 1986;3:159-65.

[171] Moriwaki M, Itoh N, Miyagawa J, Yamamoto K, Imagawa A, Yamagata K, et al. Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset Type I diabetes mellitus. Diabetologia 1999;42:1332-40.

[172] Trudeau JD, Dutz JP, Arany E, Hill DJ, Fieldus WE, Finegood DT. Neonatal beta-cell apoptosis: a trigger for autoimmune diabetes? Diabetes 2000;49:1-7.

[173] Turley S, Poirot L, Hattori M, Benoist C, Mathis D. Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. J Exp Med 2003;198:1527-37.

[174] Kassem SA, Ariel I, Thornton PS, Scheimberg I, Glaser B. Beta-cell proliferation and apoptosis in the developing normal human pancreas and in hyperinsulinism of infancy.

Diabetes 2000;49:1325-33.

[175] Han J, Zhong J, Wei W, Wang Y, Huang Y, Yang P, et al. Extracellular high-mobility group box 1 acts as an innate immune mediator to enhance autoimmune progression and diabetes onset in NOD mice. Diabetes 2008;57:2118-27.

[176] Ohnmacht C, Pullner A, King SB, Drexler I, Meier S, Brocker T, et al. Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. J Exp Med 2009;206:549-59.

[177] von Herrath M, Sanda S, Herold K. Type 1 diabetes as a relapsing-remitting disease? Nat Rev Immunol 2007;7:988-94.

[178] Planas R, Alba A, Carrillo J, Puertas MC, Ampudia R, Pastor X, et al. Reg (regenerating) gene overexpression in islets from non-obese diabetic mice with accelerated diabetes: role of IFNbeta. Diabetologia 2006;49:2379-87.

[179] Roep BO, Peakman M. Antigen targets of type 1 diabetes autoimmunity. Cold Spring Harb Perspect Med 2012;2:a007781.

[180] Stiller CR, Dupre J, Gent M, Jenner MR, Keown PA, Laupacis A, et al. Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset. Science 1984;223:1362-7.

[181] Sigal NH, Dumont FJ. Cyclosporin A, FK-506, and rapamycin: pharmacologic probes of lymphocyte signal transduction. Annu Rev Immunol 1992;10:519-60.

[182] Feutren G, Papoz L, Assan R, Vialettes B, Karsenty G, Vexiau P, et al. Cyclosporin increases the rate and length of remissions in insulin-dependent diabetes of recent onset. Results of a multicentre double-blind trial. Lancet 1986;2:119-24.

[183] Parving HH, Tarnow L, Nielsen FS, Rossing P, Mandrup-Poulsen T, Osterby R, et al. Cyclosporine nephrotoxicity in type 1 diabetic patients. A 7-year follow-up study. Diabetes Care 1999;22:478-83.

[184] Brazelton TR, Morris RE. Molecular mechanisms of action of new xenobiotic immunosuppressive drugs: tacrolimus (FK506), sirolimus (rapamycin), mycophenolate mofetil and leflunomide. Curr Opin Immunol 1996;8:710-20.

[185] Vincenti F, Kirkman R, Light S, Bumgardner G, Pescovitz M, Halloran P, et al. Interleukin-2-receptor blockade with daclizumab to prevent acute rejection in renal transplantation. Daclizumab Triple Therapy Study Group. N Engl J Med 1998;338:161-5.

[186] Gottlieb PA, Quinlan S, Krause-Steinrauf H, Greenbaum CJ, Wilson DM, Rodriguez H, et al. Failure to preserve beta-cell function with mycophenolate mofetil and daclizumab combined therapy in patients with new- onset type 1 diabetes. Diabetes Care 2010;33:826-32.

[187] Maki T, Ichikawa T, Blanco R, Porter J. Long-term abrogation of autoimmune diabetes in nonobese diabetic mice by immunotherapy with anti-lymphocyte serum. Proc Natl Acad Sci U S A 1992;89:3434-8.

[188] Eisenbarth GS, Srikanta S, Jackson R, Rabinowe S, Dolinar R, Aoki T, et al. Anti-thymocyte globulin and prednisone immunotherapy of recent onset type 1 diabetes mellitus. Diabetes Res 1985;2:271-6.

[189] Saudek F, Havrdova T, Boucek P, Karasova L, Novota P, Skibova J. Polyclonal anti-T-cell therapy for type 1 diabetes mellitus of recent onset. Rev Diabet Stud 2004;1:80-8.

[190] Gitelman SE, Gottlieb PA, Rigby MR, Felner EI, Willi SM, Fisher LK, et al. Antithymocyte globulin treatment for patients with recent-onset type 1 diabetes: 12-month results of a

randomised, placebo-controlled, phase 2 trial. Lancet Diabetes Endocrinol 2013;1:306-16.

[191] Chatenoud L, Thervet E, Primo J, Bach JF. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci U S A 1994;91:123-7.

[192] Daifotis AG, Koenig S, Chatenoud L, Herold KC. Anti-CD3 clinical trials in type 1 diabetes mellitus. Clin Immunol 2013;149:268-78.

[193] Chatenoud L, Bluestone JA. CD3-specific antibodies: a portal to the treatment of autoimmunity. Nat Rev Immunol 2007;7:622-32.

[194] Hu CY, Rodriguez-Pinto D, Du W, Ahuja A, Henegariu O, Wong FS, et al. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest 2007;117:3857-67.

[195] Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Goland R, et al. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 2009;361:2143-52.

[196] Orban T, Bundy B, Becker DJ, DiMeglio LA, Gitelman SE, Goland R, et al. Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial. Lancet 2011;378:412-9.

[197] Ogilvie ALJ, Antoni C, Dechant C, Manger B, Kalden JR, Schuler G, et al. Treatment of psoriatic arthritis with antitumour necrosis factor-α antibody clears skin lesions of psoriasis resistant to treatment with methotrexate. British Journal of Dermatology 2001;144:587-9.

[198] Feldman SR, Gordon KB, Bala M, Evans R, Li S, Dooley LT, et al. Infliximab treatment results in significant improvement in the quality of life of patients with severe psoriasis: a double-blind placebo-controlled trial. British Journal of Dermatology 2005;152:954-60.

[199] Mastrandrea L, Yu J, Behrens T, Buchlis J, Albini C, Fourtner S, et al. Etanercept treatment in children with new-onset type 1 diabetes: pilot randomized, placebo-controlled, double-blind study. Diabetes Care 2009;32:1244-9.

[200] Moran A, Bundy B, Becker DJ, DiMeglio LA, Gitelman SE, Goland R, et al. Interleukin-1 antagonism in type 1 diabetes of recent onset: two multicentre, randomised, double-blind, placebo-controlled trials. Lancet 2013;381:1905-15.

[201] D'Ambrosio D, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, et al. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. J Clin Invest 1998;101:252-62.

[202] van Halteren AG, van Etten E, de Jong EC, Bouillon R, Roep BO, Mathieu C. Redirection of human autoreactive T-cells Upon interaction with dendritic cells modulated by TX527, an analog of 1,25 dihydroxyvitamin D(3). Diabetes 2002;51:2119-25.

[203] Vitamin D supplement in early childhood and risk for Type I (insulin-dependent) diabetes mellitus. The EURODIAB Substudy 2 Study Group. Diabetologia 1999;42:51-4.

[204] Hypponen E, Laara E, Reunanen A, Jarvelin MR, Virtanen SM. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. Lancet 2001;358:1500-3.

[205] Mathieu C, Waer M, Laureys J, Rutgeerts O, Bouillon R. Prevention of autoimmune diabetes in NOD mice by 1,25 dihydroxyvitamin D3. Diabetologia 1994;37:552-8.

[206] Gysemans CA, Cardozo AK, Callewaert H, Giulietti A, Hulshagen L, Bouillon R, et al. 1,25-Dihydroxyvitamin D3 modulates expression of chemokines and cytokines in pancreatic islets: implications for prevention of diabetes in nonobese diabetic mice. Endocrinology 2005;146:1956-64.

[207] Pitocco D, Crino A, Di Stasio E, Manfrini S, Guglielmi C, Spera S, et al. The effects of calcitriol and nicotinamide on residual pancreatic beta-cell function in patients with recent-onset Type 1 diabetes (IMDIAB XI). Diabet Med 2006;23:920-3.

[208] Walter M, Kaupper T, Adler K, Foersch J, Bonifacio E, Ziegler A-G. No Effect of the 1α,25-Dihydroxyvitamin D3 on β-Cell Residual Function and Insulin Requirement in Adults With New-Onset Type 1 Diabetes. Diabetes Care 2010;33:1443-8.

[209] Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, et al. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature 2005;435:220-3.

[210] Skyler JS, Krischer JP, Wolfsdorf J, Cowie C, Palmer JP, Greenbaum C, et al. Effects of oral insulin in relatives of patients with type 1 diabetes: The Diabetes Prevention Trial--Type 1. Diabetes Care 2005;28:1068-76.

[211] Effects of insulin in relatives of patients with type 1 diabetes mellitus. N Engl J Med 2002;346:1685-91.

[212] Harrison LC, Honeyman MC, Steele CE, Stone NL, Sarugeri E, Bonifacio E, et al. Pancreatic beta-cell function and immune responses to insulin after administration of intranasal insulin to humans at risk for type 1 diabetes. Diabetes Care 2004;27:2348-55.

[213] Nanto-Salonen K, Kupila A, Simell S, Siljander H, Salonsaari T, Hekkala A, et al. Nasal insulin to prevent type 1 diabetes in children with HLA genotypes and autoantibodies conferring increased risk of disease: a double-blind, randomised controlled trial. Lancet 2008;372:1746-55.

[214] Pozzilli P, Pitocco D, Visalli N, Cavallo MG, Buzzetti R, Crino A, et al. No effect of oral insulin on residual beta-cell function in recent-onset type I diabetes (the IMDIAB VII). IMDIAB Group. Diabetologia 2000;43:1000-4.

[215] Chaillous L, Lefevre H, Thivolet C, Boitard C, Lahlou N, Atlan-Gepner C, et al. Oral insulin administration and residual beta-cell function in recent-onset type 1 diabetes: a multicentre randomised controlled trial. Diabete Insuline Orale group. Lancet 2000;356:545-9.

[216] Ergun-Longmire B, Marker J, Zeidler A, Rapaport R, Raskin P, Bode B, et al. Oral insulin therapy to prevent progression of immune-mediated (type 1) diabetes. Ann N Y Acad Sci 2004;1029:260-77.

[217] Achenbach P, Barker J, Bonifacio E, Pre PSG. Modulating the natural history of type 1 diabetes in children at high genetic risk by mucosal insulin immunization. Curr Diab Rep 2008;8:87-93.

[218] Baekkeskov S, Aanstoot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 1990;347:151-6.

[219] Velloso LA, Kampe O, Hallberg A, Christmanson L, Betsholtz C, Karlsson FA. Demonstration of GAD-65 as the main immunogenic isoform of glutamate decarboxylase in type 1 diabetes and determination of autoantibodies using a radioligand produced by eukaryotic expression. J Clin Invest 1993;91:2084-90.

[220] Tian J, Atkinson MA, Clare-Salzler M, Herschenfeld A, Forsthuber T, Lehmann PV, et al. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. J Exp Med 1996;183:1561-7.

[221] Petersen JS, Karlsen AE, Markholst H, Worsaae A, Dyrberg T, Michelsen B. Neonatal tolerization with glutamic acid decarboxylase but not with bovine serum albumin delays the onset of diabetes in NOD mice. Diabetes 1994;43:1478-84.

[222] Agardh CD, Cilio CM, Lethagen A, Lynch K, Leslie RD, Palmer M, et al. Clinical evidence for the safety of GAD65 immunomodulation in adult-onset autoimmune diabetes. J Diabetes Complications 2005;19:238-46.

[223] Ludvigsson J, Faresjo M, Hjorth M, Axelsson S, Cheramy M, Pihl M, et al. GAD treatment and insulin secretion in recent-onset type 1 diabetes. N Engl J Med 2008;359:1909-20.

[224] Wherrett DK, Bundy B, Becker DJ, DiMeglio LA, Gitelman SE, Goland R, et al. Antigen-based therapy with glutamic acid decarboxylase (GAD) vaccine in patients with recent-onset type 1 diabetes: a randomised double-blind trial. Lancet 2011;378:319-27.

[225] Ludvigsson J, Krisky D, Casas R, Battelino T, Castano L, Greening J, et al. GAD65 antigen therapy in recently diagnosed type 1 diabetes mellitus. N Engl J Med 2012;366:433-42.

[226] Elias D, Markovits D, Reshef T, van der Zee R, Cohen IR. Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein. Proc Natl Acad Sci U S A 1990;87:1576-80.

[227] Elias D, Reshef T, Birk OS, van der Zee R, Walker MD, Cohen IR. Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein. Proc Natl Acad Sci U S A 1991;88:3088-91.

[228] Raz I, Elias D, Avron A, Tamir M, Metzger M, Cohen IR. Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial. Lancet 2001;358:1749-53.

[229] Lazar L, Ofan R, Weintrob N, Avron A, Tamir M, Elias D, et al. Heat-shock protein peptide DiaPep277 treatment in children with newly diagnosed type 1 diabetes: a randomised, double-blind phase II study. Diabetes Metab Res Rev 2007;23:286-91.

[230] Schloot NC, Meierhoff G, Lengyel C, Vandorfi G, Takacs J, Panczel P, et al. Effect of heat shock protein peptide DiaPep277 on beta-cell function in paediatric and adult patients with recent-onset diabetes mellitus type 1: two prospective, randomized, double-blind phase II trials. Diabetes Metab Res Rev 2007;23:276-85.

[231] Raz I, Ziegler AG, Linn T, Schernthaner G, Bonnici F, Distiller LA, et al. Treatment of Recent-Onset Type 1 Diabetic Patients With DiaPep277: Results of a Double-Blind, Placebo-Controlled, Randomized Phase 3 Trial. Diabetes Care 2014;37:1392-400.

[232] Bot A, Smith D, Bot S, Hughes A, Wolfe T, Wang L, et al. Plasmid vaccination with insulin B chain prevents autoimmune diabetes in nonobese diabetic mice. J Immunol 2001;167:2950-5.

[233] Rivas EI, Driver JP, Garabatos N, Presa M, Mora C, Rodriguez F, et al. Targeting of a T cell agonist peptide to lysosomes by DNA vaccination induces tolerance in the nonobese diabetic mouse. J Immunol 2011;186:4078-87.

[234] Solvason N, Lou YP, Peters W, Evans E, Martinez J, Ramirez U, et al. Improved efficacy of a tolerizing DNA vaccine for reversal of hyperglycemia through enhancement of gene expression and localization to intracellular sites. J Immunol 2008;181:8298-307.

[235] Roep BO, Solvason N, Gottlieb PA, Abreu JR, Harrison LC, Eisenbarth GS, et al. Plasmid-encoded proinsulin preserves C-peptide while specifically reducing proinsulin-specific CD8(+) T cells in type 1 diabetes. Sci Transl Med 2013;5:191ra82.

[236] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage

potential of adult human mesenchymal stem cells. Science 1999;284:143-7.
[237] Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. Blood 2007;110:3499-506.
[238] Le Blanc K, Ringden O. Immunomodulation by mesenchymal stem cells and clinical experience. J Intern Med 2007;262:509-25.
[239] Madec AM, Mallone R, Afonso G, Abou Mrad E, Mesnier A, Eljaafari A, et al. Mesenchymal stem cells protect NOD mice from diabetes by inducing regulatory T cells. Diabetologia 2009;52:1391-9.
[240] Jurewicz M, Yang S, Augello A, Godwin JG, Moore RF, Azzi J, et al. Congenic mesenchymal stem cell therapy reverses hyperglycemia in experimental type 1 diabetes. Diabetes 2010;59:3139-47.
[241] Tarbell KV, Yamazaki S, Olson K, Toy P, Steinman RM. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. J Exp Med 2004;199:1467-77.
[242] Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med 2004;199:1455-65.
[243] Putnam AL, Brusko TM, Lee MR, Liu W, Szot GL, Ghosh T, et al. Expansion of human regulatory T-cells from patients with type 1 diabetes. Diabetes 2009;58:652-62.
[244] Marek-Trzonkowska N, Mysliwiec M, Dobyszuk A, Grabowska M, Derkowska I, Juscinska J, et al. Therapy of type 1 diabetes with CD4CD25CD127-regulatory T cells prolongs survival of pancreatic islets - Results of one year follow-up. Clin Immunol 2014;153:23-30.
[245] Clare-Salzler MJ, Brooks J, Chai A, Van Herle K, Anderson C. Prevention of diabetes in nonobese diabetic mice by dendritic cell transfer. J Clin Invest 1992;90:741-8.
[246] Feili-Hariri M, Dong X, Alber SM, Watkins SC, Salter RD, Morel PA. Immunotherapy of NOD mice with bone marrow-derived dendritic cells. Diabetes 1999;48:2300-8.
[247] Feili-Hariri M, Falkner DH, Morel PA. Regulatory Th2 response induced following adoptive transfer of dendritic cells in prediabetic NOD mice. Eur J Immunol 2002;32:2021-30.
[248] Tai N, Yasuda H, Xiang Y, Zhang L, Rodriguez-Pinto D, Yokono K, et al. IL-10-conditioned dendritic cells prevent autoimmune diabetes in NOD and humanized HLA-DQ8/RIP-B7.1 mice. Clin Immunol 2011;139:336-49.
[249] Besin G, Gaudreau S, Menard M, Guindi C, Dupuis G, Amrani A. Thymic stromal lymphopoietin and thymic stromal lymphopoietin-conditioned dendritic cells induce regulatory T-cell differentiation and protection of NOD mice against diabetes. Diabetes 2008;57:2107-17.
[250] Ma L, Qian S, Liang X, Wang L, Woodward JE, Giannoukakis N, et al. Prevention of diabetes in NOD mice by administration of dendritic cells deficient in nuclear transcription factor-kappaB activity. Diabetes 2003;52:1976-85.
[251] Feili-Hariri M, Falkner DH, Gambotto A, Papworth GD, Watkins SC, Robbins PD, et al. Dendritic cells transduced to express interleukin-4 prevent diabetes in nonobese diabetic mice with advanced insulitis. Hum Gene Ther 2003;14:13-23.
[252] Creusot RJ, Yaghoubi SS, Kodama K, Dang DN, Dang VH, Breckpot K, et al. Tissue-targeted therapy of autoimmune diabetes using dendritic cells transduced to express IL-4 in NOD mice. Clin Immunol 2008;127:176-87.
[253] Ruffner MA, Robbins PD. Dendritic cells transduced to express interleukin 4 reduce diabetes onset in both normoglycemic and prediabetic nonobese diabetic mice. PLoS One

2010;5:e11848.

[254] Creusot RJ, Chang P, Healey DG, Tcherepanova IY, Nicolette CA, Fathman CG. A short pulse of IL-4 delivered by DCs electroporated with modified mRNA can both prevent and treat autoimmune diabetes in NOD mice. Mol Ther 2010;18:2112-20.

[255] Machen J, Harnaha J, Lakomy R, Styche A, Trucco M, Giannoukakis N. Antisense oligonucleotides down-regulating costimulation confer diabetes-preventive properties to nonobese diabetic mouse dendritic cells. J Immunol 2004;173:4331-41.

[256] Di Caro V, Phillips B, Engman C, Harnaha J, Trucco M, Giannoukakis N. Involvement of suppressive B-lymphocytes in the mechanism of tolerogenic dendritic cell reversal of type 1 diabetes in NOD mice. PLoS One 2014;9:e83575.

[257] Giannoukakis N, Phillips B, Finegold D, Harnaha J, Trucco M. Phase I (safety) study of autologous tolerogenic dendritic cells in type 1 diabetic patients. Diabetes Care 2011;34:2026-32.

[258] Phillips B, Nylander K, Harnaha J, Machen J, Lakomy R, Styche A, et al. A microsphere-based vaccine prevents and reverses new-onset autoimmune diabetes. Diabetes 2008;57:1544-55.

[259] Erwig LP, Henson PM. Immunological consequences of apoptotic cell phagocytosis. Am J Pathol 2007;171:2-8.

[260] Poon IK, Lucas CD, Rossi AG, Ravichandran KS. Apoptotic cell clearance: basic biology and therapeutic potential. Nat Rev Immunol 2014;14:166-80.

[261] Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med 2000;191:411-6.

[262] Xia CQ, Peng R, Qiu Y, Annamalai M, Gordon D, Clare-Salzler MJ. Transfusion of apoptotic beta-cells induces immune tolerance to beta-cell antigens and prevents type 1 diabetes in NOD mice. Diabetes 2007;56:2116-23.

[263] Perruche S, Saas P, Chen W. Apoptotic cell-mediated suppression of streptococcal cell wall-induced arthritis is associated with alteration of macrophage function and local regulatory T-cell increase: a potential cell-based therapy? Arthritis Res Ther 2009;11:R104.

[264] Huynh ML, Fadok VA, Henson PM. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-beta1 secretion and the resolution of inflammation. J Clin Invest 2002;109:41-50.

[265] Lee YJ, Moon C, Lee SH, Park HJ, Seoh JY, Cho MS, et al. Apoptotic cell instillation after bleomycin attenuates lung injury through hepatocyte growth factor induction. Eur Respir J 2012;40:424-35.

[266] Ren Y, Xie Y, Jiang G, Fan J, Yeung J, Li W, et al. Apoptotic cells protect mice against lipopolysaccharide-induced shock. J Immunol 2008;180:4978-85.

[267] Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA, et al. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. Nat Med 2006;12:1056-64.

[268] McGrath EE, Marriott HM, Lawrie A, Francis SE, Sabroe I, Renshaw SA, et al. TNF-related apoptosis-inducing ligand (TRAIL) regulates inflammatory neutrophil apoptosis and enhances resolution of inflammation. J Leukoc Biol 2011;90:855-65.

[269] Bournazou I, Pound JD, Duffin R, Bournazos S, Melville LA, Brown SB, et al. Apoptotic human cells inhibit migration of granulocytes via release of lactoferrin. J Clin Invest 2009;119:20-32.

[270] Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature 2009;461:282-6.

[271] Torr EE, Gardner DH, Thomas L, Goodall DM, Bielemeier A, Willetts R, et al. Apoptotic cell-derived ICAM-3 promotes both macrophage chemoattraction to and tethering of apoptotic cells. Cell Death Differ 2012;19:671-9.

[272] Truman LA, Ford CA, Pasikowska M, Pound JD, Wilkinson SJ, Dumitriu IE, et al. CX3CL1/fractalkine is released from apoptotic lymphocytes to stimulate macrophage chemotaxis. Blood 2008;112:5026-36.

[273] Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992;148:2207-16.

[274] Fadok VA, Bratton DL, Frasch SC, Warner ML, Henson PM. The role of phosphatidylserine in recognition of apoptotic cells by phagocytes. Cell Death Differ 1998;5:551-62.

[275] Fadok VA, Bratton DL, Henson PM. Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. J Clin Invest 2001;108:957-62.

[276] Ravichandran KS. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. J Exp Med 2010;207:1807-17.

[277] Park D, Tosello-Trampont AC, Elliott MR, Lu M, Haney LB, Ma Z, et al. BAI1 is an engulfment receptor for apoptotic cells upstream of the ELMO/Dock180/Rac module. Nature 2007;450:430-4.

[278] Kobayashi N, Karisola P, Pena-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, et al. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. Immunity 2007;27:927-40.

[279] Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S. Identification of Tim4 as a phosphatidylserine receptor. Nature 2007;450:435-9.

[280] Park SY, Jung MY, Kim HJ, Lee SJ, Kim SY, Lee BH, et al. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. Cell Death Differ 2008;15:192-201.

[281] He M, Kubo H, Morimoto K, Fujino N, Suzuki T, Takahasi T, et al. Receptor for advanced glycation end products binds to phosphatidylserine and assists in the clearance of apoptotic cells. EMBO Rep 2011;12:358-64.

[282] Mevorach D, Mascarenhas JO, Gershov D, Elkon KB. Complement-dependent clearance of apoptotic cells by human macrophages. J Exp Med 1998;188:2313-20.

[283] Verbovetski I, Bychkov H, Trahtemberg U, Shapira I, Hareuveni M, Ben-Tal O, et al. Opsonization of apoptotic cells by autologous iC3b facilitates clearance by immature dendritic cells, down-regulates DR and CD86, and up-regulates CC chemokine receptor 7. J Exp Med 2002;196:1553-61.

[284] Hart SP, Smith JR, Dransfield I. Phagocytosis of opsonized apoptotic cells: roles for 'old-fashioned' receptors for antibody and complement. Clin Exp Immunol 2004;135:181-5.

[285] Kinchen JM, Ravichandran KS. Phagosome maturation: going through the acid test. Nat Rev Mol Cell Biol 2008;9:781-95.

[286] Bellone M, Iezzi G, Rovere P, Galati G, Ronchetti A, Protti MP, et al. Processing of engulfed apoptotic bodies yields T cell epitopes. J Immunol 1997;159:5391-9.

[287] Albert ML, Pearce SF, Francisco LM, Sauter B, Roy P, Silverstein RL, et al. Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present

antigens to cytotoxic T lymphocytes. J Exp Med 1998;188:1359-68.
[288] Dudziak D, Kamphorst AO, Heidkamp GF, Buchholz VR, Trumpfheller C, Yamazaki S, et al. Differential antigen processing by dendritic cell subsets in vivo. Science 2007;315:107-11.
[289] Hochreiter-Hufford A, Ravichandran KS. Clearing the dead: apoptotic cell sensing, recognition, engulfment, and digestion. Cold Spring Harb Perspect Biol 2013;5:a008748.
[290] Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767-811.
[291] Alvarez D, Vollmann EH, von Andrian UH. Mechanisms and consequences of dendritic cell migration. Immunity 2008;29:325-42.
[292] Miller JC, Brown BD, Shay T, Gautier EL, Jojic V, Cohain A, et al. Deciphering the transcriptional network of the dendritic cell lineage. Nat Immunol 2012;13:888-99.
[293] Ganguly D, Haak S, Sisirak V, Reizis B. The role of dendritic cells in autoimmunity. Nat Rev Immunol 2013;13:566-77.
[294] Hopp AK, Rupp A, Lukacs-Kornek V. Self-Antigen Presentation by Dendritic Cells in Autoimmunity. Front Immunol 2014;5:55.
[295] Ardavin C, Wu L, Li CL, Shortman K. Thymic dendritic cells and T cells develop simultaneously in the thymus from a common precursor population. Nature 1993;362:761-3.
[296] Wu L, D'Amico A. Isolation of mouse thymic dendritic cell precursors. Methods Mol Biol 2010;595:265-80.
[297] Gallegos AM, Bevan MJ. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. J Exp Med 2004;200:1039-49.
[298] Gray D, Abramson J, Benoist C, Mathis D. Proliferative arrest and rapid turnover of thymic epithelial cells expressing Aire. J Exp Med 2007;204:2521-8.
[299] Hubert FX, Kinkel SA, Davey GM, Phipson B, Mueller SN, Liston A, et al. Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. Blood 2011;118:2462-72.
[300] Watanabe N, Wang YH, Lee HK, Ito T, Wang YH, Cao W, et al. Hassall's corpuscles instruct dendritic cells to induce CD4+CD25+ regulatory T cells in human thymus. Nature 2005;436:1181-5.
[301] Bonasio R, Scimone ML, Schaerli P, Grabie N, Lichtman AH, von Andrian UH. Clonal deletion of thymocytes by circulating dendritic cells homing to the thymus. Nat Immunol 2006;7:1092-100.
[302] Proietto AI, van Dommelen S, Zhou P, Rizzitelli A, D'Amico A, Steptoe RJ, et al. Dendritic cells in the thymus contribute to T-regulatory cell induction. Proc Natl Acad Sci U S A 2008;105:19869-74.
[303] Mueller DL. Mechanisms maintaining peripheral tolerance. Nat Immunol 2010;11:21-7.
[304] Schwartz RH. T cell anergy. Annu Rev Immunol 2003;21:305-34.
[305] Ghoreschi K, Rocken M. Immune deviation strategies in the therapy of psoriasis. Curr Drug Targets Inflamm Allergy 2004;3:193-8.
[306] Marrack P, Kappler J. Control of T cell viability. Annu Rev Immunol 2004;22:765-87.
[307] Maldonado RA, von Andrian UH. How tolerogenic dendritic cells induce regulatory T cells. Adv Immunol 2010;108:111-65.
[308] Wakkach A, Fournier N, Brun V, Breittmayer JP, Cottrez F, Groux H. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo.

Immunity 2003;18:605-17.
[309] Mellor AL, Munn DH. IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol 2004;4:762-74.
[310] Matteoli G, Mazzini E, Iliev ID, Mileti E, Fallarino F, Puccetti P, et al. Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. Gut 2010;59:595-604.
[311] Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. J Exp Med 2007;204:1775-85.
[312] Cassani B, Villablanca EJ, De Calisto J, Wang S, Mora JR. Vitamin A and immune regulation: role of retinoic acid in gut-associated dendritic cell education, immune protection and tolerance. Mol Aspects Med 2012;33:63-76.
[313] Kalinski P. Regulation of immune responses by prostaglandin E2. J Immunol 2012;188:21-8.
[314] Probst HC, McCoy K, Okazaki T, Honjo T, van den Broek M. Resting dendritic cells induce peripheral CD8+ T cell tolerance through PD-1 and CTLA-4. Nat Immunol 2005;6:280-6.
[315] Pena-Cruz V, McDonough SM, Diaz-Griffero F, Crum CP, Carrasco RD, Freeman GJ. PD-1 on immature and PD-1 ligands on migratory human Langerhans cells regulate antigen-presenting cell activity. J Invest Dermatol 2010;130:2222-30.
[316] Fukaya T, Takagi H, Sato Y, Sato K, Eizumi K, Taya H, et al. Crucial roles of B7-H1 and B7-DC expressed on mesenteric lymph node dendritic cells in the generation of antigen-specific CD4+Foxp3+ regulatory T cells in the establishment of oral tolerance. Blood 2010;116:2266-76.
[317] Chang CC, Ciubotariu R, Manavalan JS, Yuan J, Colovai AI, Piazza F, et al. Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. Nat Immunol 2002;3:237-43.
[318] Brenk M, Scheler M, Koch S, Neumann J, Takikawa O, Hacker G, et al. Tryptophan deprivation induces inhibitory receptors ILT3 and ILT4 on dendritic cells favoring the induction of human CD4+CD25+ Foxp3+ T regulatory cells. J Immunol 2009;183:145-54.
[319] Suss G, Shortman K. A subclass of dendritic cells kills CD4 T cells via Fas/Fas-ligand-induced apoptosis. J Exp Med 1996;183:1789-96.
[320] van Kooten C, Stax AS, Woltman AM, Gelderman KA. Handbook of experimental pharmacology "dendritic cells": the use of dexamethasone in the induction of tolerogenic DCs. Handb Exp Pharmacol 2009:233-49.
[321] Fischer R, Turnquist HR, Taner T, Thomson AW. Use of rapamycin in the induction of tolerogenic dendritic cells. Handb Exp Pharmacol 2009:215-32.
[322] Hilkens CM, Isaacs JD, Thomson AW. Development of dendritic cell-based immunotherapy for autoimmunity. Int Rev Immunol 2010;29:156-83.
[323] Ravichandran KS, Lorenz U. Engulfment of apoptotic cells: signals for a good meal. Nat Rev Immunol 2007;7:964-74.
[324] Morelli AE. The immune regulatory effect of apoptotic cells and exosomes on dendritic cells: its impact on transplantation. Am J Transplant 2006;6:254-61.
[325] Takahashi M, Kobayashi Y. Cytokine production in association with phagocytosis of apoptotic cells by immature dendritic cells. Cell Immunol 2003;226:105-15.
[326] Morelli AE, Larregina AT, Shufesky WJ, Zahorchak AF, Logar AJ, Papworth GD, et al.

Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. Blood 2003;101:611-20.

[327] Stuart LM, Lucas M, Simpson C, Lamb J, Savill J, Lacy-Hulbert A. Inhibitory effects of apoptotic cell ingestion upon endotoxin-driven myeloid dendritic cell maturation. J Immunol 2002;168:1627-35.

[328] da Costa TB, Sardinha LR, Larocca R, Peron JP, Rizzo LV. Allogeneic apoptotic thymocyte-stimulated dendritic cells expand functional regulatory T cells. Immunology 2011;133:123-32.

[329] Kushwah R, Wu J, Oliver JR, Jiang G, Zhang J, Siminovitch KA, et al. Uptake of apoptotic DC converts immature DC into tolerogenic DC that induce differentiation of Foxp3+ Treg. Eur J Immunol 2010;40:1022-35.

[330] Urban BC, Willcox N, Roberts DJ. A role for CD36 in the regulation of dendritic cell function. Proc Natl Acad Sci U S A 2001;98:8750-5.

[331] Zhong K, Song W, Wang Q, Wang C, Liu X, Chen D, et al. Murine myeloid dendritic cells that phagocytose apoptotic T cells inhibit the immune response via NO. PLoS One 2012;7:e49378.

[332] Ren G, Su J, Zhao X, Zhang L, Zhang J, Roberts AI, et al. Apoptotic cells induce immunosuppression through dendritic cells: critical roles of IFN-gamma and nitric oxide. J Immunol 2008;181:3277-84.

[333] Williams CA, Harry RA, McLeod JD. Apoptotic cells induce dendritic cell-mediated suppression via interferon-gamma-induced IDO. Immunology 2008;124:89-101.

[334] Kushwah R, Oliver JR, Zhang J, Siminovitch KA, Hu J. Apoptotic dendritic cells induce tolerance in mice through suppression of dendritic cell maturation and induction of antigen-specific regulatory T cells. J Immunol 2009;183:7104-18.

[335] Wang Z, Larregina AT, Shufesky WJ, Perone MJ, Montecalvo A, Zahorchak AF, et al. Use of the inhibitory effect of apoptotic cells on dendritic cells for graft survival via T-cell deletion and regulatory T cells. Am J Transplant 2006;6:1297-311.

[336] Wang Z, Shufesky WJ, Montecalvo A, Divito SJ, Larregina AT, Morelli AE. In situ-targeting of dendritic cells with donor-derived apoptotic cells restrains indirect allorecognition and ameliorates allograft vasculopathy. PLoS One 2009;4:e4940.

[337] Xu DL, Liu Y, Tan JM, Li B, Zhong CP, Zhang XH, et al. Marked prolongation of murine cardiac allograft survival using recipient immature dendritic cells loaded with donor-derived apoptotic cells. Scand J Immunol 2004;59:536-44.

[338] Gorelik L, Flavell RA. Transforming growth factor-beta in T-cell biology. Nat Rev Immunol 2002;2:46-53.

[339] Fenske DB, Cullis PR. Liposomal nanomedicines. Expert Opin Drug Deliv 2008;5:25-44.

[340] Gregoriadis G. The immunological adjuvant and vaccine carrier properties of liposomes. J Drug Target 1994;2:351-6.

[341] Look M, Stern E, Wang QA, DiPlacido LD, Kashgarian M, Craft J, et al. Nanogel-based delivery of mycophenolic acid ameliorates systemic lupus erythematosus in mice. J Clin Invest 2013;123:1741-9.

[342] Allen TM, Cullis PR. Liposomal drug delivery systems: from concept to clinical applications. Adv Drug Deliv Rev 2013;65:36-48.

[343] Zolnik BS, Gonzalez-Fernandez A, Sadrieh N, Dobrovolskaia MA. Nanoparticles and the

immune system. Endocrinology 2010;151:458-65.

[344] Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol 2004;173:3148-54.

[345] Fang C, Shi B, Pei YY, Hong MH, Wu J, Chen HZ. In vivo tumor targeting of tumor necrosis factor-alpha-loaded stealth nanoparticles: effect of MePEG molecular weight and particle size. Eur J Pharm Sci 2006;27:27-36.

[346] Zahr AS, Davis CA, Pishko MV. Macrophage uptake of core-shell nanoparticles surface modified with poly(ethylene glycol). Langmuir 2006;22:8178-85.

[347] Nakanishi T, Kunisawa J, Hayashi A, Tsutsumi Y, Kubo K, Nakagawa S, et al. Positively charged liposome functions as an efficient immunoadjuvant in inducing cell-mediated immune response to soluble proteins. J Control Release 1999;61:233-40.

[348] Allen TM. Long-circulating (sterically stabilized) liposomes for targeted drug delivery. Trends Pharmacol Sci 1994;15:215-20.

[349] Moghimi SM. Chemical camouflage of nanospheres with a poorly reactive surface: towards development of stealth and target-specific nanocarriers. Biochim Biophys Acta 2002;1590:131-9.

[350] Cui Z, Mumper RJ. Coating of cationized protein on engineered nanoparticles results in enhanced immune responses. Int J Pharm 2002;238:229-39.

[351] Kim TH, Lee SY, Cho NK, Seong HK, Choi HJ, Jung SW, et al. Dielectrophoretic alignment of gallium nitride nanowires (GaN NWs) for use in device applications. Nanotechnology 2006;17:3394-9.

[352] Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. Cell 2001;106:255-8.

[353] Dobrovolskaia MA, McNeil SE. Immunological properties of engineered nanomaterials. Nat Nanotechnol 2007;2:469-78.

[354] Dobrovolskaia MA, Aggarwal P, Hall JB, McNeil SE. Preclinical studies to understand nanoparticle interaction with the immune system and its potential effects on nanoparticle biodistribution. Mol Pharm 2008;5:487-95.

[355] Gref R, Minamitake Y, Peracchia MT, Trubetskoy V, Torchilin V, Langer R. Biodegradable long-circulating polymeric nanospheres. Science 1994;263:1600-3.

[356] Chen X, Doffek K, Sugg SL, Shilyansky J. Phosphatidylserine regulates the maturation of human dendritic cells. J Immunol 2004;173:2985-94.

[357] Shi D, Fu M, Fan P, Li W, Chen X, Li C, et al. Artificial phosphatidylserine liposome mimics apoptotic cells in inhibiting maturation and immunostimulatory function of murine myeloid dendritic cells in response to 1-chloro-2,4-dinitrobenze in vitro. Arch Dermatol Res 2007;299:327-36.

[358] Otsuka M, Tsuchiya S, Aramaki Y. Involvement of ERK, a MAP kinase, in the production of TGF-beta by macrophages treated with liposomes composed of phosphatidylserine. Biochem Biophys Res Commun 2004;324:1400-5.

[359] Zhang J, Fujii S, Wu Z, Hashioka S, Tanaka Y, Shiratsuchi A, et al. Involvement of COX-1 and up-regulated prostaglandin E synthases in phosphatidylserine liposome-induced prostaglandin E2 production by microglia. J Neuroimmunol 2006;172:112-20.

[360] Takayama F, Wu Z, Ma HM, Okada R, Hayashi Y, Nakanishi H. Possible involvement of aiPLA2 in the phosphatidylserine-containing liposomes induced production of PGE2 and

PGD2 in microglia. J Neuroimmunol 2013;262:121-4.
[361] Wu Z, Ma HM, Kukita T, Nakanishi Y, Nakanishi H. Phosphatidylserine-containing liposomes inhibit the differentiation of osteoclasts and trabecular bone loss. J Immunol 2010;184:3191-201.
[362] Wu Z, Nakanishi H. Phosphatidylserine-containing liposomes: potential pharmacological interventions against inflammatory and immune diseases through the production of prostaglandin E(2) after uptake by myeloid derived phagocytes. Arch Immunol Ther Exp (Warsz) 2011;59:195-201.
[363] Ramos GC, Fernandes D, Charao CT, Souza DG, Teixeira MM, Assreuy J. Apoptotic mimicry: phosphatidylserine liposomes reduce inflammation through activation of peroxisome proliferator-activated receptors (PPARs) in vivo. Br J Pharmacol 2007;151:844-50.
[364] Harel-Adar T, Ben Mordechai T, Amsalem Y, Feinberg MS, Leor J, Cohen S. Modulation of cardiac macrophages by phosphatidylserine-presenting liposomes improves infarct repair. Proc Natl Acad Sci U S A 2011;108:1827-32.
[365] Hamaguchi K, Gaskins HR, Leiter EH. NIT-1, a pancreatic beta-cell line established from a transgenic NOD/Lt mouse. Diabetes 1991;40:842-9.
[366] Shimizu J, Kanagawa O, Unanue ER. Presentation of beta-cell antigens to CD4+ and CD8+ T cells of non-obese diabetic mice. J Immunol 1993;151:1723-30.
[367] Aaronson SA, Todaro GJ. Development of 3T3-like lines from Balb-c mouse embryo cultures: transformation susceptibility to SV40. J Cell Physiol 1968;72:141-8.
[368] Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249-64.
[369] Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article3.
[370] Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. J R Stat Soc Ser B 1995;57:289–300.
[371] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-8.
[372] Lutz MB, Suri RM, Niimi M, Ogilvie AL, Kukutsch NA, Rossner S, et al. Immature dendritic cells generated with low doses of GM-CSF in the absence of IL-4 are maturation resistant and prolong allograft survival in vivo. Eur J Immunol 2000;30:1813-22.
[373] Strid J, Lopes L, Marcinkiewicz J, Petrovska L, Nowak B, Chain BM, et al. A defect in bone marrow derived dendritic cell maturation in the nonobesediabetic mouse. Clin Exp Immunol 2001;123:375-81.
[374] Bros M, Dexheimer N, Ross R, Trojandt S, Hohn Y, Tampe J, et al. Differential gene expression analysis identifies murine Cacnb3 as strongly upregulated in distinct dendritic cell populations upon stimulation. Gene 2011;472:18-27.
[375] Latif N, Bachhawat BK. Liposomes in immunology. J Biosci 1984;6:491-502.
[376] Kono H, Rock KL. How dying cells alert the immune system to danger. Nat Rev Immunol 2008;8:279-89.
[377] Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I. Immunosuppressive effects of apoptotic cells. Nature 1997;390:350-1.
[378] Hugues S, Mougneau E, Ferlin W, Jeske D, Hofman P, Homann D, et al. Tolerance to islet antigens and prevention from diabetes induced by limited apoptosis of pancreatic beta

cells. Immunity 2002;16:169-81.
[379] Lauber K, Blumenthal SG, Waibel M, Wesselborg S. Clearance of apoptotic cells: getting rid of the corpses. Mol Cell 2004;14:277-87.
[380] Korns D, Frasch SC, Fernandez-Boyanapalli R, Henson PM, Bratton DL. Modulation of macrophage efferocytosis in inflammation. Front Immunol 2011;2:57.
[381] Trucco M, Giannoukakis N. Immunoregulatory dendritic cells to prevent and reverse new-onset Type 1 diabetes mellitus. Expert Opin Biol Ther 2007;7:951-63.
[382] Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J Exp Med 2004;199:1607-18.
[383] Guermonprez P, Valladeau J, Zitvogel L, Thery C, Amigorena S. Antigen presentation and T cell stimulation by dendritic cells. Annu Rev Immunol 2002;20:621-67.
[384] Formby B, Miller N. Autologous CD4 T-cell responses to ectopic class II major histocompatibility complex antigen-expressing single-cell islet cells: an in vitro insight into the pathogenesis of lymphocytic insulitis in nonobese diabetic mice. Proc Natl Acad Sci U S A 1990;87:2438-42.
[385] Emamaullee JA, Davis J, Merani S, Toso C, Elliott JF, Thiesen A, et al. Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice. Diabetes 2009;58:1302-11.
[386] Keino H, Takeuchi M, Kezuka T, Hattori T, Usui M, Taguchi O, et al. Induction of eye-derived tolerance does not depend on naturally occurring CD4+CD25+ T regulatory cells. Invest Ophthalmol Vis Sci 2006;47:1047-55.
[387] Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J Clin Invest 1998;101:890-8.
[388] Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol 2011;31:986-1000.
[389] Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulators of immunity. Trends Immunol 2002;23:144-50.
[390] Weller CL, Collington SJ, Hartnell A, Conroy DM, Kaise T, Barker JE, et al. Chemotactic action of prostaglandin E2 on mouse mast cells acting via the PGE2 receptor 3. Proc Natl Acad Sci U S A 2007;104:11712-7.
[391] Wang XS, Lau HY. Prostaglandin E potentiates the immunologically stimulated histamine release from human peripheral blood-derived mast cells through EP1/EP3 receptors. Allergy 2006;61:503-6.
[392] Phipps RP, Stein SH, Roper RL. A new view of prostaglandin E regulation of the immune response. Immunol Today 1991;12:349-52.
[393] Takano M, Nishimura H, Kimura Y, Washizu J, Mokuno Y, Nimura Y, et al. Prostaglandin E2 protects against liver injury after Escherichia coli infection but hampers the resolution of the infection in mice. J Immunol 1998;161:3019-25.
[394] Izuora KE, Chase HP, Jackson WE, Coll JR, Osberg IM, Gottlieb PA, et al. Inflammatory markers and diabetic retinopathy in type 1 diabetes. Diabetes Care 2005;28:714-5.
[395] Hata AN, Breyer RM. Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation. Pharmacol Ther 2004;103:147-66.
[396] Tilley SL, Coffman TM, Koller BH. Mixed messages: modulation of inflammation and

immune responses by prostaglandins and thromboxanes. J Clin Invest 2001;108:15-23.

[397] Betz M, Fox BS. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J Immunol 1991;146:108-13.

[398] Snijdewint FG, Kalinski P, Wierenga EA, Bos JD, Kapsenberg ML. Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. J Immunol 1993;150:5321-9.

[399] Baratelli F, Lin Y, Zhu L, Yang SC, Heuze-Vourc'h N, Zeng G, et al. Prostaglandin E2 induces FOXP3 gene expression and T regulatory cell function in human CD4+ T cells. J Immunol 2005;175:1483-90.

[400] Hancock WW, Polanski M, Zhang J, Blogg N, Weiner HL. Suppression of insulitis in non-obese diabetic (NOD) mice by oral insulin administration is associated with selective expression of interleukin-4 and -10, transforming growth factor-beta, and prostaglandin-E. Am J Pathol 1995;147:1193-9.

[401] Walker C, Kristensen F, Bettens F, deWeck AL. Lymphokine regulation of activated (G1) lymphocytes. I. Prostaglandin E2-induced inhibition of interleukin 2 production. J Immunol 1983;130:1770-3.

[402] Rincon M, Tugores A, Lopez-Rivas A, Silva A, Alonso M, De Landazuri MO, et al. Prostaglandin E2 and the increase of intracellular cAMP inhibit the expression of interleukin 2 receptors in human T cells. Eur J Immunol 1988;18:1791-6.

[403] Kolenko V, Rayman P, Roy B, Cathcart MK, O'Shea J, Tubbs R, et al. Downregulation of JAK3 protein levels in T lymphocytes by prostaglandin E2 and other cyclic adenosine monophosphate-elevating agents: impact on interleukin-2 receptor signaling pathway. Blood 1999;93:2308-18.

[404] Jonuleit H, Kuhn U, Muller G, Steinbrink K, Paragnik L, Schmitt E, et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur J Immunol 1997;27:3135-42.

[405] Rieser C, Bock G, Klocker H, Bartsch G, Thurnher M. Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production. J Exp Med 1997;186:1603-8.

[406] Kalinski P, Hilkens CM, Snijders A, Snijdewint FG, Kapsenberg ML. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. J Immunol 1997;159:28-35.

[407] Braun D, Longman RS, Albert ML. A two-step induction of indoleamine 2,3 dioxygenase (IDO) activity during dendritic-cell maturation. Blood 2005;106:2375-81.

[408] Kalinski P, Schuitemaker JH, Hilkens CM, Kapsenberg ML. Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. J Immunol 1998;161:2804-9.

[409] Gustafsson K, Ingelsten M, Bergqvist L, Nystrom J, Andersson B, Karlsson-Parra A. Recruitment and activation of natural killer cells in vitro by a human dendritic cell vaccine. Cancer Res 2008;68:5965-71.

[410] Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today 1999;20:561-7.

[411] Nencioni A, Wesselborg S, Brossart P. Role of peroxisome proliferator-activated receptor gamma and its ligands in the control of immune responses. Crit Rev Immunol 2003;23:1-

13.

[412] Roszer T, Menendez-Gutierrez MP, Lefterova MI, Alameda D, Nunez V, Lazar MA, et al. Autoimmune kidney disease and impaired engulfment of apoptotic cells in mice with macrophage peroxisome proliferator-activated receptor gamma or retinoid X receptor alpha deficiency. J Immunol 2011;186:621-31.

[413] Jozefowski S, Biedron R, Bobek M, Marcinkiewicz J. Leukotrienes modulate cytokine release from dendritic cells. Immunology 2005;116:418-28.

[414] Naranjo-Gomez M, Raich-Regue D, Onate C, Grau-Lopez L, Ramo-Tello C, Pujol-Borrell R, et al. Comparative study of clinical grade human tolerogenic dendritic cells. J Transl Med 2011;9:89.

[415] Torres-Aguilar H, Aguilar-Ruiz SR, Gonzalez-Perez G, Munguia R, Bajana S, Meraz-Rios MA, et al. Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory CD4+ T cells. J Immunol 2010;184:1765-75.

[416] Suciu-Foca Cortesini N, Piazza F, Ho E, Ciubotariu R, LeMaoult J, Dalla-Favera R, et al. Distinct mRNA microarray profiles of tolerogenic dendritic cells. Hum Immunol 2001;62:1065-72.

[417] Morel PA, Srinivas M, Turner MS, Fuschiotti P, Munshi R, Bahar I, et al. Gene expression analysis of dendritic cells that prevent diabetes in NOD mice: analysis of chemokines and costimulatory molecules. J Leukoc Biol 2011;90:539-50.

[418] Moore YF, Lambracht-Washington D, Tabaczewski P, Fischer Lindahl K. Murine MHC class Ib gene, H2-M2, encodes a conserved surface-expressed glycoprotein. Immunogenetics 2004;56:1-11.

[419] Hofstetter AR, Sullivan LC, Lukacher AE, Brooks AG. Diverse roles of non-diverse molecules: MHC class Ib molecules in host defense and control of autoimmunity. Curr Opin Immunol 2011;23:104-10.

[420] Torres-Aguilar H, Blank M, Jara LJ, Shoenfeld Y. Tolerogenic dendritic cells in autoimmune diseases: crucial players in induction and prevention of autoimmunity. Autoimmun Rev 2010;10:8-17.

[421] Van Elssen CH, Vanderlocht J, Oth T, Senden-Gijsbers BL, Germeraad WT, Bos GM. Inflammation-restraining effects of prostaglandin E2 on natural killer-dendritic cell (NK-DC) interaction are imprinted during DC maturation. Blood 2011;118:2473-82.

[422] Luther SA, Cyster JG. Chemokines as regulators of T cell differentiation. Nat Immunol 2001;2:102-7.

[423] Cravens PD, Lipsky PE. Dendritic cells, chemokine receptors and autoimmune inflammatory diseases. Immunol Cell Biol 2002;80:497-505.

[424] Scandella E, Men Y, Gillessen S, Forster R, Groettrup M. Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. Blood 2002;100:1354-61.

[425] Muthuswamy R, Mueller-Berghaus J, Haberkorn U, Reinhart TA, Schadendorf D, Kalinski P. PGE(2) transiently enhances DC expression of CCR7 but inhibits the ability of DCs to produce CCL19 and attract naive T cells. Blood 2010;116:1454-9.

[426] Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. Annu Rev Immunol 2004;22:891-928.

[427] Park WD, Stegall MD. A meta-analysis of kidney microarray datasets: investigation of

cytokine gene detection and correlation with rt-PCR and detection thresholds. BMC Genomics 2007;8:88.

[428] Andollo N, Boyano MD, Andrade R, Zalduendo MM, Eguizabal C, Asumendi A, et al. Structural and functional preservation of specific sequences of DNA and mRNA in apoptotic bodies from ES cells. Apoptosis 2005;10:417-28.

[429] Hasselmann DO, Rappl G, Tilgen W, Reinhold U. Extracellular tyrosinase mRNA within apoptotic bodies is protected from degradation in human serum. Clin Chem 2001;47:1488-9.

[430] Chen X, Liang H, Zhang J, Zen K, Zhang CY. Horizontal transfer of microRNAs: molecular mechanisms and clinical applications. Protein Cell 2012;3:28-37.

[431] Probst J, Weide B, Scheel B, Pichler BJ, Hoerr I, Rammensee HG, et al. Spontaneous cellular uptake of exogenous messenger RNA in vivo is nucleic acid-specific, saturable and ion dependent. Gene Ther 2007;14:1175-80.

[432] Boczkowski D, Nair SK, Snyder D, Gilboa E. Dendritic cells pulsed with RNA are potent antigen-presenting cells in vitro and in vivo. J Exp Med 1996;184:465-72.

[433] Weissman D, Ni H, Scales D, Dude A, Capodici J, McGibney K, et al. HIV gag mRNA transfection of dendritic cells (DC) delivers encoded antigen to MHC class I and II molecules, causes DC maturation, and induces a potent human in vitro primary immune response. J Immunol 2000;165:4710-7.

[434] Creusot RJ, Yaghoubi SS, Chang P, Chia J, Contag CH, Gambhir SS, et al. Lymphoid-tissue-specific homing of bone-marrow-derived dendritic cells. Blood 2009;113:6638-47.

[435] Mueller C, Tschumper A, Tschumper JC, Hess MW, Cottier H. Parathymic lymph node: oriented proliferative response of the murine thymic cortex to intraperitoneal stimulation. Thymus 1987;9:3-12.

[436] Zhou F, Lauretti E, di Meco A, Ciric B, Gonnella P, Zhang GX, et al. Intravenous transfer of apoptotic cell-treated dendritic cells leads to immune tolerance by blocking Th17 cell activity. Immunobiology 2013;218:1069-76.

[437] Chaparro RJ, Dilorenzo TP. An update on the use of NOD mice to study autoimmune (Type 1) diabetes. Expert Rev Clin Immunol 2010;6:939-55.

[438] Ponticelli C. The pleiotropic effects of mTor inhibitors. J Nephrol 2004;17:762-8.

[439] Powell JD, Lerner CG, Schwartz RH. Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. J Immunol 1999;162:2775-84.

[440] Baeder WL, Sredy J, Sehgal SN, Chang JY, Adams LM. Rapamycin prevents the onset of insulin-dependent diabetes mellitus (IDDM) in NOD mice. Clin Exp Immunol 1992;89:174-8.

[441] Rabinovitch A, Suarez-Pinzon WL, Shapiro AM, Rajotte RV, Power R. Combination therapy with sirolimus and interleukin-2 prevents spontaneous and recurrent autoimmune diabetes in NOD mice. Diabetes 2002;51:638-45.

[442] Monti P, Scirpoli M, Maffi P, Piemonti L, Secchi A, Bonifacio E, et al. Rapamycin monotherapy in patients with type 1 diabetes modifies CD4+CD25+FOXP3+ regulatory T-cells. Diabetes 2008;57:2341-7.

[443] Belghith M, Bluestone JA, Barriot S, Megret J, Bach JF, Chatenoud L. TGF-beta-dependent mechanisms mediate restoration of self-tolerance induced by antibodies to CD3 in overt autoimmune diabetes. Nat Med 2003;9:1202-8.

[444] Valle A, Jofra T, Stabilini A, Atkinson M, Roncarolo MG, Battaglia M. Rapamycin prevents and breaks the anti-CD3-induced tolerance in NOD mice. Diabetes 2009;58:875-81.

[445] Long SA, Rieck M, Sanda S, Bollyky JB, Samuels PL, Goland R, et al. Rapamycin/IL-2 combination therapy in patients with type 1 diabetes augments Tregs yet transiently impairs beta-cell function. Diabetes 2012;61:2340-8.

[446] Johnson JD, Ao Z, Ao P, Li H, Dai LJ, He Z, et al. Different effects of FK506, rapamycin, and mycophenolate mofetil on glucose-stimulated insulin release and apoptosis in human islets. Cell Transplant 2009;18:833-45.

[447] Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC. Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia 2005;48:2221-8.

[448] Ilieva A, Yuan S, Wang RN, Agapitos D, Hill DJ, Rosenberg L. Pancreatic islet cell survival following islet isolation: the role of cellular interactions in the pancreas. J Endocrinol 1999;161:357-64.

[449] Vargas F, Vives-Pi M, Somoza N, Fernandez-Llamazares J, Pujol-Borrell R. Endotoxin activity of collagenase and human islet transplantation. Lancet 1997;350:641.

[450] Vija L, Farge D, Gautier JF, Vexiau P, Dumitrache C, Bourgarit A, et al. Mesenchymal stem cells: Stem cell therapy perspectives for type 1 diabetes. Diabetes Metab 2009;35:85-93.

[451] Pagliuca FW, Melton DA. How to make a functional beta-cell. Development 2013;140:2472-83.

[452] Ulrich AS. Biophysical aspects of using liposomes as delivery vehicles. Biosci Rep 2002;22:129-50.

[453] Smith DM, Simon JK, Baker JR, Jr. Applications of nanotechnology for immunology. Nat Rev Immunol 2013;13:592-605.

[454] Endruschat J, Henschke K. Bench scale manufacture of multilamellar liposomes using a newly developed multistage pressure filtration device. Int J Pharm 2000;196:151-3.

[455] Martin DS, Walsh M, Miller AM, Skerrett HE, Byrne P, Mandel A, et al. A novel phospholipid-based drug formulation, VP025, modulates age- and LPS-induced microglial activity in the rat. Neuroimmunomodulation 2009;16:400-10.

[456] Kersten GF, Crommelin DJ. Liposomes and ISCOMS as vaccine formulations. Biochim Biophys Acta 1995;1241:117-38.

[457] Elliott MR, Ravichandran KS. Clearance of apoptotic cells: implications in health and disease. J Cell Biol 2010;189:1059-70.

[458] Foged C, Arigita C, Sundblad A, Jiskoot W, Storm G, Frokjaer S. Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition. Vaccine 2004;22:1903-13.

[459] Daniel D, Wegmann DR. Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23). Proc Natl Acad Sci U S A 1996;93:956-60.

[460] Muir A, Peck A, Clare-Salzler M, Song YH, Cornelius J, Luchetta R, et al. Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intraislet interferon-gamma transcription. J Clin Invest 1995;95:628-34.

[461] Pugliese A. Central and peripheral autoantigen presentation in immune tolerance. Immunology 2004;111:138-46.

[462] Alleva DG, Crowe PD, Jin L, Kwok WW, Ling N, Gottschalk M, et al. A disease-associated

cellular immune response in type 1 diabetics to an immunodominant epitope of insulin. J Clin Invest 2001;107:173-80.

[463] Lutsiak ME, Kwon GS, Samuel J. Analysis of peptide and lipopeptide content in liposomes. J Pharm Pharm Sci 2002;5:279-84.

[464] Blachere NE, Darnell RB, Albert ML. Apoptotic cells deliver processed antigen to dendritic cells for cross-presentation. PLoS Biol 2005;3:e185.

[465] Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C, et al. The CD40 antigen and its ligand. Annu Rev Immunol 1994;12:881-922.

[466] Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends Immunol 2002;23:445-9.

[467] Menges M, Rossner S, Voigtlander C, Schindler H, Kukutsch NA, Bogdan C, et al. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. J Exp Med 2002;195:15-21.

[468] Rutella S, Danese S, Leone G. Tolerogenic dendritic cells: cytokine modulation comes of age. Blood 2006;108:1435-40.

[469] Reis e Sousa C. Dendritic cells in a mature age. Nat Rev Immunol 2006;6:476-83.

[470] Schipper HS, Prakken B, Kalkhoven E, Boes M. Adipose tissue-resident immune cells: key players in immunometabolism. Trends Endocrinol Metab 2012;23:407-15.

[471] Mathis D, Shoelson SE. Immunometabolism: an emerging frontier. Nat Rev Immunol 2011;11:81.

[472] Achenbach P, Bonifacio E, Koczwara K, Ziegler AG. Natural history of type 1 diabetes. Diabetes 2005;54 Suppl 2:S25-31.

[473] Pihoker C, Gilliam LK, Hampe CS, Lernmark A. Autoantibodies in diabetes. Diabetes 2005;54 Suppl 2:S52-61.

[474] Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J Exp Med 2002;196:1627-38.

[475] Shrimpton RE, Butler M, Morel AS, Eren E, Hue SS, Ritter MA. CD205 (DEC-205): a recognition receptor for apoptotic and necrotic self. Mol Immunol 2009;46:1229-39.

[476] Elit L, Oliver TK, Covens A, Kwon J, Fung MF, Hirte HW, et al. Intraperitoneal chemotherapy in the first-line treatment of women with stage III epithelial ovarian cancer: a systematic review with metaanalyses. Cancer 2007;109:692-702.

[477] Roep BO, Atkinson M, von Herrath M. Satisfaction (not) guaranteed: re-evaluating the use of animal models of type 1 diabetes. Nat Rev Immunol 2004;4:989-97.

[478] Serreze DV, Marron MP, Dilorenzo TP. "Humanized" HLA transgenic NOD mice to identify pancreatic beta cell autoantigens of potential clinical relevance to type 1 diabetes. Ann N Y Acad Sci 2007;1103:103-11.