Universitat Autònoma de Barcelona

Combined therapy for type 1 diabetes: Immunotherapy and Regenerative Strategies.

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Terapia combinada para la diabetes tipo 1: Inmunoterapia y estrategias regenerativas.

Teràpia combinada per a la diabetis tipus 1: Immunoteràpia i estratègies regeneratives.

Thesis presented by Adrián Villalba Felipe to qualify for the PhD degree in Advanced Immunology by the Universitat Autònoma de Barcelona.

The presented work has been performed in the Immunology Section, at the Germans Trias i Pujol Research Institute (IGTP), and has been directed by Dr. Marta Vives-Pi.

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Cover and back cover: Confocal microscopy pictures of murine pancreatic ducts. Insulin is in red, Pdx1 in green and nuclei in blue (magnified at 40X). The book spine is a microscopy picture of a ductal area with insulin in green and nuclei in blue (40X). Covers and thesis design by Adrián Villalba.

La Dra. Marta Vives Pi, investigadora biomèdica de la Fundació Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol i professora associada del Departament de Biologia Cel·lular, Fisiologia i Immunologia de la Universitat Autònoma de Barcelona,

Certifica:

Que el treball experimental i la redacció de la memòria de la Tesi Doctoral **"Combined therapy for type 1 diabetes: Immunotherapy and Regenerative Strategies"** han estat realitzats per Adrián Villalba Felipe sota la seva direcció i considera que és apta per ser presentada per optar al grau de Doctor en Immunologia Avançada per la Universitat Autònoma de Barcelona.

I per tal que quedi constància, signa aquest document a Badalona,

el 14 de juliol del 2020.

Dra. Marta Vives Pi.

A mis padres, Pascual y Angelita,

que no han dejado nunca de luchar.

“Las cosas podían haber sucedido de cualquier otra manera y, sin embargo, sucedieron así.”

Miguel Delibes, El Camino.

Abbreviations

aGLP1: analogue of Glucagon-like Peptide 1

BB: BioBreeding (rat)

BMI: Body Mass Index

CAR: Chimeric Antigen Receptor

cf-Ins: cell-free unmethylated insulin

DC: Dendritic Cells

dpc: days post-conception

DRiPs: Defective Ribosomal Products

EAE: Experimental Autoimmune Encephalomyelitis

ECM: Extracellular Matrix

ER: Endoplasmic Reticulum

EVs: Extracellular Vesicles

GLP1: Glucagon-like Peptide 1

GLP1R: Glucagon-like Peptide 1 Receptor

HA: Hyaluronan

Hba1c: glycated haemoglobin

hESCs: human Embryonic Stem Cells

HIP: Hybrid Insulin Peptides

HTS: High-Throughput Screenings

iDCs: immature Dendritic Cells

iPSCs: induced Pluripotent Stem Cells

LADA: Latent Autoimmune Diabetes in Adults

lncRNA: long non-coding RNA

LNSCs: lymph Node Stromal cells

mDCs: mature Dendritic Cells

miRNA: micro RNA

MODY: Mature Onset Diabetes of the Young

MOG: Myelin Oligodendrocyte Glycoprotein

mTECs: medullary Thymic Epithelial Cells

NET: Neutorophil Extracellular Trap

NGS: Next Generation Sequencing

NOD: Non-Obese Diabetic (mice strain)

NSG: NOD.SCID ILR2$\gamma^{-/-}$ (mice straing)

OPG: Osteoprotegirin

PLNs: Pancreatic lymph nodes

PR: Partial Remission

PS: phosphatidyl-serine

PTM: Post-translational modifications

siRNA: small interference RNA

SLE: Systemic Lupus Erythematosus

SPK: pancreas-kidney transplant

STZ: Streptozotocin

T1D: Type 1 diabetes

T2D: Type 2 diabetes

TCR: T cell receptor

Treg(s): regulatory T cell(s)

VNTR: Variable Number Tandem Repeat

wkc: weeks post-conception

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SUMMARY

Type 1 diabetes (T1D) is a metabolic disease caused by the **autoimmune** destruction of the insulin-producing **β-cells** in the pancreas. Currently, the only treatment available is the administration of exogenous insulin. Unfortunately, this treatment does not allow the continuous maintenance of normoglycaemia and may lead to dysregulation episodes. Also, these events are responsible for short- and long-term complications.

One alternative approach to overcome the loss of insulin-producing cells in T1D is **β-cell regeneration.** Because the design of new molecules with a therapeutic potential is a long process, **this study aimed** to identify already-existing and approved compounds with β-cell regenerative properties by drug repurposing (or repositioning). This is an *in silico* technique intended to search for novel uses of drugs available to be used in patients.

Drug repurposing of pathways targeted in β-cell regeneration resulted in the identification of liraglutide —an agonist of Glucagon-like Peptide 1— as a regenerative drug. When liraglutide was administered to diabetic and immunodeficient NSG mice there was observed a transient amelioration of hyperglycaemia throughout the treatment. It correlated well with the induction of bihormonal ($glucagon^{+}insulin^{+}$) cells and $Cytokeratin19^{+}insulin^{+}$ cells in the ductal areas. Furthermore, the administration of liraglutide combined with previously developed immunotherapy based on phosphatidyl-serine liposomes also ameliorated hyperglycaemia in autoimmune non-obese diabetic mice. Moreover, the addition of liraglutide did not alter the induction of a tolerogenic phenotype by PS-liposomes in human dendritic cells from adult subjects with T1D.

Finally, with the aim to identify optimal stages for immune intervention, several candidate biomarkers were screened in peripheral blood from paediatric subjects with T1D. The PR phase, also known as honeymoon, is an interesting stage due to partial recovery of the hyperglycaemia along with lower insulin requirements. This resulted in the identification of circulating molecules, like TGF-β and betatrophin, and regulatory immune cell subsets that arise as candidate biomarkers of early stages of paediatric T1D.

Altogether, the results presented in this PhD thesis describe the identification of liraglutide as a repurposed drug for islet β-cell regeneration and its therapeutic potential in experimental autoimmune diabetes when combined with an immunotherapeutic strategy. Indeed, the identification of candidate biomarkers of early phases of T1D points toward the selection of optimal stages for future immune intervention trials.

RESUMEN

La diabetes tipo 1 (DT1) es una enfermedad metabólica causada por la destrucción **autoinmunitaria** de las **células β** productoras de insulina. Actualmente, el único tratamiento consiste en la administración exógena de insulina. Desafortunadamente este tratamiento no permite un mantenimiento continuo de la normoglicemia y puede ocasionar episodios de disglicemia. Estos eventos son los responsables de complicaciones a corto y largo plazo.

Una alternativa para superar la pérdida de células β en la DT1 es la **regeneración de célula β**. Debido a que el diseño de moléculas con potencial terapéutico es un proceso largo, este estudio pretendía identificar compuestos ya existentes y aprobados que mostrasen potencial de regeneración de célula β mediante Reposicionamiento de Fármacos. Esta es una técnica *in silico* utilizada para buscar nuevos usos a fármacos ya aprobados para su uso en pacientes.

El Reposicionamiento de Fármacos aplicados a vías de señalización implicadas en la regeneración de célula β resultó en la identificación de liraglutide —un agonista de Glucagon-like Peptide 1— como fármaco regenerador. Con la administración de liraglutide a ratones NSG inmunodeficientes se observó una mejora transitoria de la hiperglicemia durante el tratamiento. Esta mejora correlacionó con la aparición de células bihormonales (glucagón^{+}insulina^{+}) y células Citoqueratina19+insulina+ en las áreas ductales. Además, la administración de liraglutide combinado con una inmunoterapia previamente desarrollada y basada en liposomas con fosfatidil-serina también redujo la hiperglicemia en ratones no-obesos diabéticos con patología autoinmunitaria. Asimismo, la adición de

liraglutide no alteró el fenotipo tolerogénico inducido por PS-liposomas en células dendríticas obtenidas de pacientes adultos con DT1.

Finalmente, con el objetivo de identificar etapas óptimas para la inmuno-intervención, se cribaron biomarcadores candidatos en sangre periférica de pacientes pediátricos con DT1. La fase de remisión parcial, popularmente conocida como luna de miel, es una etapa interesante debido a la mejora parcial de la hiperglicemia junto a una disminución de los requerimientos de insulina exógena. Esto llevó a la identificación de moléculas circulantes, tales como TGF-β y betatrofina, así como subpoblaciones leucocitarias reguladoras que se proponen como biomarcadores candidatos de etapas iniciales de la DT1 pediátrica.

En conjunto, los resultados presentados en esta tesis doctoral describen la identificación de liraglutide como fármaco reposicionado con efecto regenerativo en células β pancreáticas y que muestra un potencial terapéutico en la diabetes autoinmunitaria experimental cuando se combina con una inmunoterapia. En efecto, la identificación de biomarcadores candidatos de etapas tempranas de DT1 encamina la selección de fases óptimas para futuros ensayos de inmuno-intervención.

INTRODUCTION

INTRODUCTION

1. Type 1 Diabetes.

1.1. Clinical overview.

Type 1 diabetes (T1D) is a metabolic disease caused by the autoimmune destruction of the insulin-producing β-cells. These cells are located in the pancreas, a mixed gland constituted by both the exocrine tissue —involved in the synthesis and secretion of digestive enzymes— and the endocrine, also known as islets of Langerhans —clusters of cells participating in the production of different hormones—. The lack of β-cells leads to sustained hyperglycaemia, which means elevated values of blood glucose. The cause of T1D is the autoimmune reaction when the self-tolerance to β-cell is lost and the immune system promotes its destruction. It is differentiated from type 2 diabetes (T2D), a disease caused by an insulin resistance factor combined with a relative lack of β-cells. Besides the autoimmune factors causing the selective destruction of insulin-producing cells, the aetiology of T1D remains unknown.

T1D affects approximately 40 million people worldwide and represents the minority fraction of cases of diabetes (~5%) whereas there are diagnosed more than 350 million patients suffering from T2D (~95%) (International Diabetes Federation Report, IDF 2019). The incidence of T1D is progressively increasing by 3% yearly. The disease affects equally both genders and despite it can be diagnosed during adulthood, T1D shows an increased prevalence in paediatric subjects (Global Report on Diabetes, WHO 2016).

The classical symptoms are related to continuous hyperglycaemia: polyphagia —increased hunger—, polydipsia —increased thirst— and polyuria —increased urination—. Usually accompanied by weight loss and

fatigue. When the period of hyperglycaemia remains sustained enough diabetic ketoacidosis can be present due to the appearance of ketonic bodies in the blood.

Currently, there are no cure nor prevention therapies for regular clinical practice. The only treatment is based on exogenous insulin administration. Unfortunately, hormone replacement strategies based on insulin cannot mimic perfectly the endogenous insulin release and may lead to different episodes of hyper- and/or hypoglycaemia. These events are short-term complications that can promote later long-term complications, such as retinopathy, neuropathy or nephropathy. Overall, T1D is associated with a worsened quality of life and a short life-span. For that reason, novel therapies achieving normoglycaemia and embracing insulin independence are needed to cure T1D.

1.2. Islet pathology.

The pancreas is the target organ of T1D. The major pathological events are loss of β-cells and concomitant islet inflammation. Both of them are triggered mainly by autoreactive T cells, which are known as the primary mediators of specific β-cell destruction (Pugliese 2017). These key features of pancreas pathology at the disease stage can be identified by three different mechanisms: a) leukocytic islet infiltrate, named insulitis, b) a reduction in the number of insulin-positive ($insulin^+$) cells and c) alteration of the expression of cytological β-cell markers.

It is common to find lymphocyte infiltration within the islets and the surroundings named **insulitis**. This observation lays on the T-cell based autoimmunity that lies as a mediator of T1D. The first exhaustive study of this phenomenon reported the presence of insulitis in 78% of patients

within a year of disease progression (Foulis 1984). More recently, a metadata analysis comprising several case-studies concluded that the existence of insulitis, in humans, shows a differential pattern along with the progression of the disease. Intriguingly, while 73% of young patients exhibited insulitis during the first month and 60% during the first year, it was only reported in 4% of the young subjects with disease duration longer than a year. Furthermore, the percentage of insulitic observations remained lower in older patients, revealing only 29% of cases during the first month in subjects between 15 and 40 years (In't Veld 2011). Nevertheless, the fraction of islets presenting insulitis in the pancreas with T1D is moderate and differ with disease duration and age (Campbell-Thompson 2016).

The insulitis was defined by consensus as a lymphocyte infiltration of a minimum of 15 $CD45^+$ cells per islet (Gepts 1981 and Campbell-Thompson 2013). Albeit T and B lymphocytes are usually present in the infiltrate, $CD8^+$ T cells constitute the majority, being increased through disease duration. Among these cells, insulitic lesions also display macrophages, $CD4^+$ T and B lymphocytes (Somoza 1994). Within the latter, there can be found two distinct subpopulations of B lymphocytes expressing a different pattern of CD20: CD20 low and CD20 high (Leete 2016). An interesting feature of this lesion is that the vast majority of the islets from a subject show an almost identic insulitis profile but this pattern is highly variable among different individuals (Morgan 2017).

The most predominant sign in the pancreas with T1D is the **β-cell destruction**, histologically identified by a lack of insulin staining. A classical belief has been considering for decades that 90% of the β-cell mass is lost in subjects with the disease at the moment of the onset. Despite that, large cohorts studies from deceased and living subjects point to different percentages (Klinke 2008 and 2011, Krogvold 2016). Younger

patients indeed present severe destruction of β-cell number at the onset but subjects developing the disease when teenagers or at adulthood can preserve more of the 50% of the β-cell mass. That indicates the loss of β-cells is not homogenous. This observation, taken together with the highly variable profile of insulitis, can reflect an autoimmune reaction affecting not all the islets at a point, which can persist after the onset.

Finally, the less extent mechanism is the **altered expression** of many islet cell molecules. Islets in the pancreas with T1D can hyper express HLA class I molecules and perform aberrant expression of HLA II (Pujol-Borrell 1987). This fact supports the evidence of inflammation in the islets and is usually associated with insulitis (Morgan 2014). It has been proposed that β-cells hyperexpressing HLA class I molecules could present self- and neo-antigens to autoreactive $CD8^+$ T lymphocytes. The upregulation of HLA class I molecules, together with the presence of type 1 IFN (Somoza 1994), has been associated with viral infections (Krogvold 2015) but it remains unclear if autoreactive $CD8^+$ T lymphocytes show specificity for viral epitopes (Kundu 2018). The alteration in the expression of these molecules is predominantly found in islets with insulitis and can persist many years after the onset (Richardson 2016). Moreover, further alteration in the expression of molecules was found in islets of human patients, such as adhesion molecules, cytokines and even transcription factors (Planas 2010).

Besides HLA, other molecules have found aberrantly expressed. Hyaluronan (HA) —a protein of the extracellular matrix— and associated proteins have been identified in capillaries draining islets presenting insulitis in the pancreas with T1D (Bogdani 2014a). Further studies indicate that this abnormal localization of HA and extracellular matrix proteins may promote the insulitis targeting the islets (Bogdani 2014b).

Furthermore, this microenvironment directly favours effector T cells by impairing T cell differentiation into a regulatory phenotype. This mechanism has been reverted in mice through the administration of an inhibitor of HA synthesis resulting in a restored balance of regulatory/effector T cells (Nagy 2015). Apart from HA, other extracellular components such as cathepsins, collagen, ICAM-1 and laminin have been identified at insulitic lesions (Vives-Pi 1996, Korpos 2013). Several other markers point to significant β-cell stress. Supporting the hypothesis of viral infections linked to T1D (Falke 1988), viral proteins are detected in a reduced portion of infiltrated β-cells (Richardson 2016). Moreover, other observations reflect endoplasmic reticulum (ER) stress leading to impaired insulin secretion at prediabetic stages (Eizirik 2013 and 2017).

In summary, several key features reinforce a β-cell-centric view of pancreas pathology during the different stages of T1D. Notwithstanding, some observations display evidence about damaged exocrine tissue. Pancreatic exocrine insufficiency during T1D was first described in 1943 (Pollard 1943). It consists of a reduction in the production of enzymes like amylase and lipase after stimulation and these discoveries were confirmed by later publications (Vacca 1964 and Semakula 1996). This evidence was obtained from invasive techniques that could easily give rise artifacts. However, non-invasive tests like measuring faecal elastase-1 concentration and chymotrypsin activity have already confirmed previous results (Hardt 2000 and Larger 2012). Pancreatic exocrine insufficiency was reported in 51% of subjects with T1D (Hardt 2000). Also, altered expression of genes in the exocrine tissue has been found in individuals with T1D (Planas 2010).

More recently, imaging techniques like magnetic resonance and computerized tomography show altered pancreatic volume and function of

the exocrine tissue (Bilgin 2009). Intriguingly, several studies report a reduced pancreas volume not only in subjects with T1D but also in pre-diabetic ones (Altobelli 1998 and Williams 2012). Whether this diminished pancreatic volume is due to exocrine atrophy or impaired organ growth remains unclear. Some propose this may be led by the loss of insulinotropic effect on acinar cells by β-cells (Henderson 1981) but this is still controversial while others have observed acinar damage independent from β-cell loss (Löhr 1987).

Beyond the autoimmune reaction selectively focused on β-cells (and reflected as insulitis), infiltrating immune cells have been identified in large portions of exocrine tissue during T1D. Among others, T lymphocytes and dendritic cells are reported in higher numbers in pancreases from subjects with the disease when compared to healthy individuals (Rodriguez-Calvo 2014). Neutrophils can be found next to acinar cells in pancreas samples from deceased patients (Valle 2013) and exhibiting neutrophil extracellular trap (NET) morphology (Vecchio 2018). Further studies link this particular activity of neutrophils with the development of acute pancreatitis both in humans and mice (Merza 2015 and Leppkes 2016). The presence of neutrophils in pancreases with T1D is correlated with a decrease of this cell type in peripheral blood, also known as neutropenia (Valle 2013). Neutropenia can be accompanied by an IFN signature, while type I IFN is associated with the development of T1D (Newby 2017). Complement activation presence was reported through C4d too (Rowe 2013). Despite B lymphocytes have not been detected, there are autoantibodies against specific exocrine proteins (cytokeratin, anhydrase, lactoferrin, and amylase) (Kobayashi 1990, Taniguchi 2003 and Endo 2009).

Taking all these observations together, seems obvious that it needs to be addressed if the exocrine tissue is an innocent bystander of the autoimmune attack during T1D or a silent accomplice.

1.3. Autoimmune etiology.

From the last 40 years, there has been much-accumulated evidence supporting that T1D is an autoimmune disease. Despite that, the triggering factors leading to the activation of the autoimmune reaction against β-cells remain unknown. Four evidences are demonstrating that T1D is caused by an autoimmune attack: genetic risk related to HLA, autoantigens, insulitis, and leukocytic transfer of the disease.

1.3.1. Genetic risk.

The initiation of islet autoimmunity is balanced between both genetic and environmental factors, being all the attempts failed to identify a unique trigger of the disease. It seems clear that T1D lies in a genetic feature, as its incidence is significantly higher in relatives of patients (Pociot 2016 and Redondo 2018). Besides that, the genetic risk is not a fully determinant when low concordance of T1D was found between monozygotic, di-zygotic and non-twin siblings (Redondo 1999). There are currently 57 identified loci that can contribute to the development of the disease (Noble 2015).

HLA is the most relevant locus whereas the other contribute lesser. HLA is known to be a highly polymorphic locus, and there are either protective or prone variants. The haplotypes conferring the higher risk are class II HLA DR4-DQ8 and DR3-DQ2 (Schober 1981 and Noble 2015). It is thought

that HLA risk haplotypes are related to antigen presentation but its contribution to thymic selection or T cell activation is still not fully understood. On the opposite, it is well stablished that these variants are the initiators of autoimmunity while paediatric subjects carrying the above mentioned haplotypes seroconvert progressively to insulin or GAD65 autoantibodies positivity (Krischer 2017). Class I HLA loci are also involved, especially HLA A*24), driving the progression of T1D by promoting β-cell destruction (Tait 2003).

Apart from risk HLA haplotypes, other non-HLA influences seroconversion from multiple autoantibodies to clinical diagnosis (Lempainen 2012 and Bonifacio 2014). Large cohort studies assessing the influence of non-HLA loci have concluded that cumulative risk provided by those is able not only to discriminate progressors of T1D but also to discern from other types of diabetes such as Mature Onset Diabetes of the Young (MODY) (Patel 2016). For instance, some genetic variants lead to a reduced ability in response to IL2 resulting in impaired function of regulatory T cells (Tregs), a subset known to be involved in self-recognition (Long 2010). This molecular axis is mastered by variants in the non-coding region of IL2Rα, which lately modulates the expression of CD25, a key marker of Tregs. Also, variants in the IL2 gene related to a reduced expression are linked to immune dysregulation in the Non-obese Diabetic (NOD) mice model of T1D (Yamanouchi 2007). Another locus related to Tregs function in the human disease is CTLA-4 (Padma-Malini 2018). Finally, PTPN22 —among many others— is an influential locus for the development of T1D (Smyth 2004). PTPN22 is a negative regulator of B and T lymphocyte activation. Its overexpression in mice produces a hyperactivation of both lymphocyte families resulting in the spontaneous development of diabetes (Dai 2013). Oppositely, some variants of PTPN22

locus may confer protection against diabetes by reducing effector T cell differentiation (Yeh 2013).

1.3.2. Autoantigens: cell and humoral autoimmunity.

The immunological synapse is the molecular complex involved in T cell activation. An antigen-presenting cell (either dendritic cells (DC), macrophages or B lymphocytes) expresses molecules of class II HLA that can bind a peptide. The dimer of this peptide joined to the class II HLA molecules on the surface of the presenting cell can interact with a T cell receptor (TCR) located on a $CD4^+$ T cell, leading its activation. This constitutes the immunological synapse (or trimolecular complex) as it comprises an antigen-presenting cell expressing class II HLA molecules on the surface, which bind peptides from different sources and interact with a TCR located in the surface of a $CD4^+$ T lymphocyte. During the physiological homeostasis of the immune system, the MHC binds to pathogenic peptides but in the case of autoimmunity, the peptide target is a self-peptide. The presentation of self-peptides, those produced by the self-individual, leads the activation of T lymphocytes into an autoreactive phenotype.

As it has been reported in the previous section (1.3.1. Genetic risk), the variants involved in class II HLA loci have a predominant impact on the development of T1D as they are responsible to interact with self-peptides. Furthermore, defects on the other two components of the trimolecular complex have been observed during the triggering and progression of T1D.

Regarding the peptide as an intermediate participant of the immune synapse, in the case of autoimmunity, the self-peptide is considered as an autoantigen. Insulin is the major autoantigen known in T1D as it is one of

the main β-cell products. There are studies linking polymorphisms of the non-coding regions of the insulin gene with an increased risk of developing T1D. These variants differ in the number of tandem repeats (VNTR) upstream of the insulin gene and correlate with low levels of insulin expression in the thymus and consequently lower self-recognition by differentiated T cells (Pugliese 1997).

Autoimmunity against insulin also comprises its unprocessed form, the pro-insulin. Unfortunately the majority of the work relying on pro-insulin as an autoantigen comes from animal models, especially the NOD mouse. The development of diabetes in this model requires a specific sequence of aminoacids in the B chain of the insulin gene (B9:23) (Nakayama 2005). Just a mutated single aminoacid contained in this sequence impairs diabetes progression (Nakayama 2007). Further studies have demonstrated that the B9:23 sequence binds unexpectedly to murine class II HLA becoming an autoantigen for autoreactive T cells targeting insulin-producing β-cells (Crawford 2011). Reactivity against insulin epitopes in humans have been identified in islet-infiltrating autoreactive T cells (Pathiraja 2015) but also in pancreatic lymph draining nodes (Kent 2005) and peripheral blood (Yang 2014).

Autoimmunity against insulin peptides is not restricted to its native form but also to post-translational modified (PTM) insulin. For instance, the first reported PTM in T1D corresponds to an internal disulfide-bond formation in the A-chain sequence A:1-13. T cell clones against this epitope have been reported in T1D but not in healthy subjects (Mannering 2005). Additionally, insulin has shown to increase affinity to class II HLA molecules and T cell autoreactivity following the conversion of glutamine to glutamic acid within the sequence B:30-C:13 in patients (van Lummel 2014). Common PTMs such as phosphorylation, acetylation or

citrullination contributing to autoantigenicity of self-peptides have been extensively documented in several other autoimmune diseases (Doyle 2012).

Apart, several molecules present in β-cells and other cell types have been recognized as T cell autoantigens. Among others, the most relevant ones are IA2 —regulating hormone secretion—, GAD65 and GAD67 —involved in GABA synthesis—, ZnT8 —participating in Zinc uptake—, IGRP and amylin —both of them are part of the glucose metabolism— (Arvan 2012). A recent project intended to search for novel epitopes recognized by T cells in T1D has already uncovered several candidates (Babon 2016). The work is based on screening target and modified epitopes against banked T cells (both $CD4^+$ and $CD8^+$) sorted from islets of subjects with T1D. To date, they have been identified more than 30 epitopes from autoantigens able to be targeted by autoreactive T cells. Islet autoantigens, including insulin, have been detected as targets of autoantibodies both in the native conformation and with PTM (Yu 2013) but this topic is discussed more deeply later in this introduction (4. Biomarkers for T1D).

Modified epitopes can be recognized by both $CD4^+$ and $CD8^+$ T lymphocytes. Some GAD65-derived peptides are autoantigens targeted by autoreactive $CD4^+$ T cells in both unmodified and citrullinated form (McGinty 2014). However, other GAD65-derived peptides that are recognized in both unmodified and modified forms are targeted by two distinct subpopulations of autoreactive $CD4^+$ T lymphocytes (Yang 2013). Furthermore, four β-cell modified peptides (phosphorylated and citrullinated) were found to be recognized by $CD8^+$ T cells from subjects with T1D (McGinty 2015).

Classically, loss of self-tolerance in autoimmune diseases such as T1D has been related to the recognition of self-peptides as autoantigens. Far from that, it has been demonstrated that stressed β-cell that underwent autoimmune attack can be a source of a new kind of autoantigens. By transpeptidation, a mechanism of fusion of two different peptides, β-cells can create new peptides susceptible to be targeted by the immune system. This novel peptides resulting from the fusion of previous ones are also called neo-epitopes as are targets for the immune system generated *de novo*. Sorted $CD4^+$ T cells from human infiltrate with T1D were shown to react against 2 hybrid insulin peptides (HIP), resulting from the fusion of an insulin sequence with other different peptides. Moreover, these $CD4^+$ T cells were reported to detect this HIP when the presentation was restricted to HLA DQ8, the risk haplotype of T1D (Delong 2016). A source new of neo-epitopes are the defective ribosomal products (DRiPs). DRiPs can be produced from errors during the translation of pre-proinsulin or the translation of insulin mRNA from alternative open reading frames (Kracht 2017).

Finally, large cohort studies revealed the presence of autoantibodies — antibodies recognizing autoantigens— against islet proteins that may be detectable between 2 and 5 years of age (Ziegler 1999 and Bonifacio 2004). Indeed, autoantibodies against insulin can be detected even between 6 and 12 months of age (Roll 1996). These autoantibodies appear early in life (Krischer 2015) and the immune response may spread to other islets targets consecutively (Ziegler 2011). Furthermore, other autoantigens of T1D such as GAD65, IA2, and ZnT8 are targets of autoantibodies (Steck 2011). Despite the detection of autoantibodies is intended to predict the chances of developing T1D, the pathophysiology of the autoimmune reaction is far more complex. The positivity for some antibodies (i.e. anti-insulin or anti-

IA2) is age dependent whereas for others (anti-GAD65) is regardless of age (Pihoker 2005). Moreover, not all individuals with positivity for one or two autoantibodies will finally develop T1D while other subjects with the disease may relapse the positivity in long-standing stages (Sørgjerd 2019). Currently, the detection of autoantibodies is used to confirm T1D in candidate patients but it still lacks of properly assessment (von Oettingen 2016).

1.3.3. Insulitis and autoreactive lymphocytes.

There are three different stages of T cell development: naïve, effector and memory T cells. Naïve T lymphocytes (either $CD4^+$ or $CD8^+$) are those cells differentiated in the bone marrow that successfully undergone central selection in the thymus. Then naïve T lymphocytes migrate to the periphery without having encountered yet the antigen (or even if they do with low affinity in the context of HLA). Once they reach the antigen, are activated and differentiate into effector T lymphocytes and might respond to antigenic stimuli. This group includes helper $CD4^+$, cytotoxic $CD8^{+,}$ and regulatory $CD4^+$ T lymphocytes. After antigen recognition, effector T cells can differentiate into memory T lymphocytes, retaining its capacity to respond against the previous antigen that has detected after the first recognition.

In the insulitic lesions from patients with T1D, both $CD8^+$ and $CD4^+$ β-cell antigen-specific lymphocytes have been found (Foulis 1984, Campbell-Thomson 2013 and 2016, Pathiraja 2015). Despite that, high heterogeneity was found at TCR clones between different subjects with the disease (Babon 2016). The vast majority of the $CD8^+$ lymphocytes were identified

as tissue-resident T cells —a subset of memory T cells— revealing that not only cytotoxic $CD8^+$ effector T cells are present in the target tissue (Kuric 2017). Furthermore, $CD8^+$ memory T cells can be also found in benign-infiltrated islets of healthy subjects thus revealing a non-exclusively pathogenic phenotype (Radenkovic 2017). An indispensable tool to determine and characterize the properties of insulitis is the nPOD program (Campbell-Thompson 2012), which aims the development of a tissue biobank from deceased donors with T1D.

Another effector T cell players are $CD4^+$ Tregs. Classical Tregs fail to progress during physiological development in the absence of its master transcription factor Foxp3 (Fontenot 2017). Actually, both human and murine individuals carrying a mutated Foxp3 variant develop lethal autoimmunity (Brunkow 2001 and Liet 2007). Two different subsets of Tregs are classified depending on the environmental maturation. On one hand, thymic Tregs have developed in the thymus through the expression of self-antigens driven by AIRE gene expression (Jordan 2001). On the other hand, Tregs can arise in the periphery from naïve $CD4^+$ T cells when are exposed to low-dose antigens in a non-inflammatory local environment (Kretschmer 2005). Both subsets are characterized by the expression of CD25 also known as IL2Rα (Malek 2002). Development and maintenance of Tregs are dependent on IL-2 signalling (Lohr 2006) and as previously reviewed defects on this gene lead to an increased risk of developing T1D. These cells also express CTLA-4, an inhibitor of T cell activation (McCoy 1999) and its deletion leads to systemic autoimmunity (Wing 2008). Apart from classical $Foxp3^+$ Tregs, there might exist other regulatory subsets that are not expressing this marker. On the other side, there has been reported the presence of $CD8^+$ Tregs, whose defects have been linked to

autoimmunity (Tsai 2011). Notwithstanding, their origin and development is still poorly understood.

While some authors proposed defects in Tregs number and function in patients with T1D (Kukreja 2002) others failed to replicate these observations (Putnam 2005 and Battaglia 2006). This may be due to the lack of characterization of *bona fide* Tregs markers. Overall, Tregs obtained from subjects with the disease seem to be prone to apoptosis (Glisic 2009) and secretion of IFNγ (McClymont 2011). These results are consistent with findings in mice models, in which individuals with impaired Tregs function and development revealed the accelerated onset of diabetes (Salomon 2000).

Besides the balance between memory and effector (including Tregs), other factors associated with the ontogeny of the immune system may be implicated in the loss of self-tolerance. To avoid the production of autoreactive T cells in physiological conditions, a negative selection process is carried in the thymus by medullary thymic epithelial cells (mTECs). mTECs can express a wide range of peripheral tissue antigens mastered by the AIRE gene (Anderson 2002). Through this mechanism, autoreactive cells can be depleted by cell death or anergy. Additionally, self-tolerance is not only promoted at the thymus level but also in the lymph nodes and is mediated by lymph node stromal cells (LNSCs). The lymph nodes contain B, T lymphocytes and antigen-presenting cells surrounded by LNSCs that do not express the AIRE gene (Gardner 2013). The master regulator responsible for inducing self-tolerance in LNSCs is DEAF1 (Fletcher 2010). Loss of DEAF1 expression in pancreatic lymph nodes (PLNs) of both subjects with T1D and NOD mice has been linked to reduced peripheral tissue antigens expression (Yip 2013). In summary, these data may indicate that the establishment of autoimmunity and loss of

self-tolerance in T1D is a very complex condition that involves several different mechanisms at the molecular, cell and tissue levels (brief overview in Figure 1).

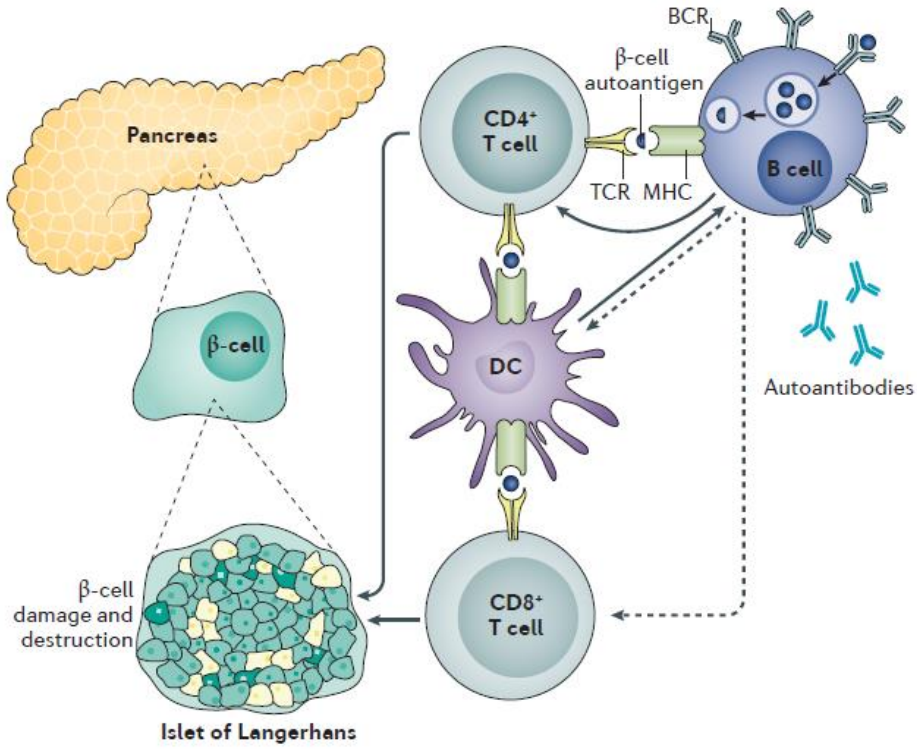

Figure 1. Main immune cell participants in autoimmune-mediated β-cell destruction (adapted from Katsarou 2017).

1.3.4. Disease transfer.

One of the scientific proofs of the autoimmune pathogeny of T1D is that the disease can be transferred from an affected to a healthy individual. Apart from the demonstration in experimental models (Serreze 1998a and De Leenheer 2016), some clinical cases reported autoimmune insulin-

dependent diabetes transfer between human subjects (Lampeter 1993 and 1998).

Autoimmune diabetes transfer is a common approach to induce the disease in immunodeficient mice. Immunocompromised mice are known to lack of B, T lymphocytes and NK cells. Hence, they cannot develop spontaneous autoimmune diabetes. However, bone marrow or splenocyte cells transfer to immunocompetent NOD mice can also promote the autoimmune disease (De Leenheer 2016).

The first report is referred to a Caucasian female, aged 29 and diagnosed with myelodysplastic syndrome. After unsuccessful therapeutic myeloablation, the patient underwent bone marrow transplantation from a male sibling with T1D. Both of the siblings were HLA identical carrying the risk alleles DR3,4. At the moment of transplantation, the recipient was negative for islet autoantibodies. Despite that, 4 years after the transplantation —following a total recovery from the myelodysplastic syndrome—, she was diagnosed with T1D. She presented positive islet cell autoantibodies and the complete chimerism of blood cells (Lampeter 1993). Further retrospective studies revealed also that T1D is transferred in some patients —but not in all of them— following bone marrow transplantation from donors with the disease (Lampeter 1998). Furthermore, this study revealed that T1D is more likely transferred from donors carrying the risk HLA than from those who not.

1.4. β-cell destruction.

Histological analysis of the pancreas from deceased donors led to a long-standing dogma that almost all β-cells were destroyed at the diagnosis of T1D. This belief is also known as the Eisenbarth model of T1D (Eisenbarth

1986). However, further histological studies revealed the presence of insulin$^+$ cells in the islets of patients with T1D for decades after diagnosis (Löhr 1987 and Lam 2017). Furthermore, some subjects still retain detectable levels of C-peptide in serum (Davis 2015 and McGee 2014).

Despite β-cells can be observed in pancreases from subjects with long-standing T1D, the β-cell mass is significantly reduced when compared with non-diabetic donors (Keenan 2010 and Rodriguez-Calvo 2018). Besides that, the β-cell mass is highly heterogeneous in both non-diabetic and subjects with the disease.

During the progression of T1D, it has been reported a sustained decrease of β-cell mass (Lam 2017) and function, matching with decreasing levels of C-peptide (Dabelea 2012) until reaching a stationary state 7 years after diagnosis (Shields 2018). Counterintuitively, studies reported more than 40 years ago that almost 15% of the subjects with T1D for 15-35 years of progression had detectable C-peptide (Madsbad 1978). Further studies using assays with improved sensitivity and specificity to detect C-peptide revealed that between 10-80% of the subjects with long-standing T1D show detectable low levels (Oram 2014, 2015 and Williams 2016). Taking all together, it suggests that marginal subpopulations of β-cells may survive to the autoimmune destruction and be functionally responsible.

Current determinations of β-cell mass are limited due to technical issues as it is limited to the staining of insulin$^+$ cells in pancreases from deceased donors. Currently, none of the *in vivo* bioimaging techniques resulted in enough sensitivity to counting β-cells or even single islets *in vivo* (Laurent 2016). On the other hand, there are few techniques to test the functionality of β-cells such as glucose tolerance tests, glucose-potentiated arginine tests and glucagon administration (McCulloch 1990 and 1991). Taken these

data, some authors tried to correlate metabolic parameters with β-cell insulin secretion and mass *in vivo*. Because this extrapolation is affected by many other factors different than insulin secretion, it has been reported a high heterogeneity among different studies (McCulloch 1991 and Greenbaum 2008). At the moment the β-cell mass picture in T1D remains blurred until novel techniques lead to clearer and deterministic results.

Given that not all the β-cells are destructed at the moment of the diagnosis nor with T1D progression, the key question that arises is: what are the features of the existing insulin$^+$ remaining cells?

One of the hypotheses living in the field is that those insulin$^+$ cells are newly formed β-cells (either by neogenesis or proliferation). Whereas both neogenesis from different sources and replication of pre-existing β-cells have been observed in murine models, the existence of these mechanisms remains unknown in T1D (Dolenšek 2015). In humans, it is known that β-cells arise through neogenesis from ductal progenitors during the embryonic developmental stage, while replication occurs in the perinatal age (Gregg 2012). However, there is no evidence that β-cells can undergo regeneration mechanisms during T1D progression, including transdifferentiation from other pancreatic cell types (Lam 2017 and Md Moin 2017).

Furthermore, there is no direct evidence of β-cell death markers in human histological studies (Meier 2006) and the only hint of apoptosis is the detection of circulating cell-free unmethylated preproinsulin DNA in long-standing patients (Fisher 2015). This reflects a limitation on the use of histological samples from the pancreases of deceased donors that may not provide enough evidence of cell rearrangement events.

Another explanatory hypothesis that needs to be challenged is that surviving β-cells in the pancreases of donors with T1D have the ability to evade the autoimmune reaction. It has been recently known that most long-standing patients can secrete residual C-peptide upon glucose stimuli. Among the subjects without C-peptide detection, it has been observed that they are able to secrete measurable proinsulin (Sims 2019). Moreover, proinsulin accumulation in the pancreas is related to hormone dysfunction and reduced insulin production (Wasserfall 2017 and Rodriguez-Calvo 2017). It may suggest that there exist subpopulations of β-cells able to synthesize hormones but not to secrete them, hiding them in histological analysis. To corroborate this hypothesis, single-cell approaches unravelled a vast heterogeneity of β-cell subpopulations (Rowe 2011 and Avrahami 2017). This fact would correlate well with a differential insulitis pattern, given that not all islets show infiltrating lymphocytes, depending especially on the age of onset (Leete 2016). For that reason, this hypothesis leads to the belief that β-cells are not just innocent targets in the disease but are the causal factor leading to the islet pathology.

Overall, all the data in human T1D come from post-mortem donors (summarised in Keenan 2010 and Lam 2017) or primary islets co-cultured with pro-inflammatory cytokines to mimic the autoimmune environment. Some groups have reported pancreas biopsy under laparoscope as a surgical method to harvest tissue without serious complications (Imagawa 2001) but the surgery failed to be commonly implemented. Due to that, it seems that technical limitations have established by far the boundaries of our current knowledge about the disease.

1.5. Experimental models of T1D.

There are two main animal models for T1D: the NOD mouse and the BioBreeding rat (BB). The BB rat was originated from Wistar strain in the decade of the 1970s (Nakhooda 1977) while the NOD mouse came from an outbred of cataract-prone Jcl:ICR mice strain during the 1980s (Makino 1980). Both models show classical symptoms of T1D —polydipsia, polyphagia and polyuria— and lymphocyte infiltrated islets similar to humans (Nakhooda 1977 and Makino 1980). Among both of them, the NOD mouse has become favoured over BB rat because of some flaws mimicking the human disease that showed the last one. The BB rat has a narrower range of autoantigens (only insulin and GAD) when compared to NOD that share most of the known autoantigens with humans. In addition, the TCR repertoire is biased when compared to NOD and human subjects. Another important limitation of the BB rat is a chronic and severe lymphopenia that may impair immunological-based studies. Apart from that, there is a unique trait in which the BB rat can be preponderated over the NOD mouse: the gender effect. Males and female BB rats show the same gender predisposition to spontaneous T1D whereas in the NOD mice the females are predominantly affected.

1.5.1. Immunopathology of T1D in the NOD mouse.

NOD mice can develop spontaneous autoimmune reactions to a wide variety of tissues, including the pancreas, salivary glands, thyroid, adrenal glands and testis (Alyanakian 2003). Further, this model is also prone to experimentally induced autoimmunity by genetic manipulation like encephalomyelitis and systemic lupus erythematosus (SLE) (Baxter 1994 and Salomon 2001). These facts may reflect that there is an underlying

autoimmune susceptibility that makes the NOD mouse a suitable model to study not only T1D but also other autoimmune-related diseases.

The immune infiltration within the islets begins as early as 3 weeks of age (despite NOD mice do not develop experimental T1D until 12 weeks of age), and at this time point, insulitis is composed of antigen-presenting cells (macrophages and DCs) and neutrophils (Jansen 1994, Bouma 2005 and Diana 2013), similarly to humans (Willcox 2009). Interestingly, when these infiltrating cells are depleted, the lymphocytic infiltration is delayed (Lee 1988). Gene manipulation experiments in the NOD mouse model revealed that both $CD4^+$ and $CD8^+$ T lymphocytes are required for the development of spontaneous T1D (Serreze 1994). These studies were further confirmed by antibody-neutralizing therapies against T lymphocyte molecules (Makhlouf 2004), and transfer experiments, reflecting that T cells are required for β-cell destruction. B lymphocytes contribute to the development and progression of T1D too. Located in the islets, some of them can secrete autoantibodies against known β-cell proteins (Pontesilli 1987) while others act as antigen-presenting cells (Diana 2013). However, their role in the pathogenesis of T1D —not only in NOD mouse but also in humans— is not fully elucidated.

Genetic susceptibility has been linked to T1D in the NOD mouse. This strain carries the $H\text{-}2^{g7}$ haplotype leading to class II MHC (the equivalent to HLA in mice) molecule $I\text{-}A^{g7}$ (McDevitt 1996). This molecule differs from the most common murine $I\text{-}A^{\beta}$ chains in only two aminoacids (Proline and Serine at positions 56-57). This conformational structure is a "promiscuous" binder that may interact with many different peptides (Carrasco-Marin 1996). Like in humans, other non-MHC molecules correlate to the progression of T1D (Wicker 1995). Among them, IL2 and CTLA4 were found to be the most relevant contributors (Podolin 2000 and

Hill 2000) exactly as previously reviewed in humans (1.3.1 Genetic risk). Defects in thymic selection allow the escape of autoreactive T cells to the periphery as seen in humans too. These defects have been related to the genetic risk conferred by non-MHC genes (Kishimoto 2001 and Lesage 2002). Because not all the NOD mice develop spontaneous diabetes, some studies have addressed the environmental factors that contribute to the disease, such as gluten (Marietta 2013), infections (Zaccone 2004) and variations in gut microbiota (Peng J 2014). Overall, the onset of diabetes in the NOD model is a complex pathophysiological process both affected by genetic and environmental triggers, as observed in humans too.

T cells are important players in the development of human T1D as well as in NOD mice. There have been described abnormalities in the activation of $CD4^+$ T cells such as increased secretion of IFNγ and decreased IL-4 (Koarada 2002). The latter defect is associated with impaired activation-induced cell death in the NOD (Arreaza 2003). On the other side of activation, both macrophages and DCs from NOD showed reduced expression of CD86 when compared with diabetes-resistant strains C57/BL6 and BALB/c (Dahlén 2000) impairing T cell activation. These observations were further demonstrated by CD86 knock-out experiments in NOD (Bour-Jordan 2004). Also, other defects have been reported in antigen-presenting cells (APC) from NOD strain, such as increased expression of IL-12 that accelerates the onset of the disease (Trembleau 2003).

Given the tolerogenic nature of Tregs, the induction of T lymphocytes able to block autoreactive T cell activity in NOD has been successfully prevented T1D (Chen 2003). In physiological conditions, NOD mice have a lower count of Tregs than other mice strains resistant to T1D (Salomon 2000). Treatments aimed to increase the number of Tregs in NOD mice

also resulted in amelioration of hyperglycaemia in diabetic individuals (Wu 2002).

Finally, despite the doubts on the role of B lymphocytes in T1D, but it is known that they are needed to develop the disease in the NOD mouse. Strains with deletion of IgM, resulting in arrested B lymphocyte development, develop diabetes with an extremely low incidence (Serreze 1996). When B lymphocyte precursors were transplanted into this model, the incidence of T1D increased to normal values (Serreze 1998b). Moreover, transient depletion of B lymphocytes reverses hyperglycaemia in some diabetic NOD mice (Hu 2007). In summary, B lymphocytes are also essential in the development of T1D.

The other players triggering autoimmunity in the islets of NOD mice are the APC, mainly tissue-resident macrophages and DCs (Unanue 2014). DCs expressing the *Batf3* transcription factor are the initiators of the autoimmune reaction in the pancreas along with effector $CD4^+$ T cells. This subset of DCs named $CD103^+$ DCs are essential to promote the disease as knock-out mice for *Batf3* cannot develop T1D (Ferris 2016). APCs orchestrate the autoimmune reaction through effector T lymphocytes by both direct interaction and transfer of granules containing autoantigens (Unanue 2014 and Ferris 2016).

1.5.2. Humanized mouse models.

There are many differences in the immune system between humans and mice, also in terms of autoimmune diabetes (Mestas 2004 and Driver 2011). For this reason, additional research may be useful before undergoing a clinical trial (Roep 2007) and mice humanization is a further step. Besides, there exist many different models of humanized mice; the main

approaches to humanize consist of transplanting human cells (either stem or mature immune cells) and/or to induce the expression of human genes.

The first trial of humanizing mice to study T1D was based on cloning the human HLA haplotype of risk (DQ8) replacing the endogenous variant in the C57/BL6 strain (Wen 1998). These mice were unable to develop spontaneous and autoimmune diabetes. They only were able to produce autoantibodies when challenged with islet peptides. This reveals that apart from the immunological background, the β-cell environment is still essential to the development of diabetes.

Then, instead of using the C57/BL6 strain lacking diabetes susceptibility, the chosen background was the NOD strain. The NOD.SCID is a strain containing the NOD background, which is prone-diabetic, but carrying a mutation in the Prkdc gene that hampers the development of B and T lymphocytes (Greiner 1995). This makes this model a valuable tool to study transplantation because of its lack to perform graft vs host disease after NK cell ablation through the anti-asialo GM1 antibody (Yoshino 2000). The first approach humanizing the NOD.SCID consisted of the co-transplantation of T cell clones isolated from human subjects with T1D and APCs from HLA-matched donors (van Halteren 2005). This resulted in the improved migration of the T lymphocytes clones from patients with diabetes to the pancreas but failed to infiltrate the islets. It reflects that not only the effector and APC cells are required but also other immune accessory cells are indispensable to perform the target recognition. Given that NOD.SCID mice cannot develop autoimmune diabetes, it is commonly used as a model of the chemically induced disease. Streptozotocin (STZ), an antimycotic compound, can be used based on its specificity targeting GLUT2 transporters (specifically expressed in β-cells) (Deeds 2011).

Alloxan can be also used as this compound is able to target the enzyme glucokinase (Lenzen 2008).

Further approaches in NOD.SCID with knocked IL2Rγ (named NSG mice: NOD SCID Gamma) —leading to ablation of NK cells too— could undergo human islet destruction *in vivo* after islet antigen-specific T cell transplantation in HLA matched animals (Unger 2012). This lead to the use of humanized NSG mice to test different immunotherapies. For instance, anti-CD3 (Teplizumab) treatment has resulted in reduced effector T cells accompanied by increased Tregs leading to islet tolerance and diabetes remission in NOD mice (Chatenoud 2010). When other authors replicated these experiments in NSG mice (Waldron-Lynch 2012), they were able to identify a subpopulation of $CCR6^+$ T cells in peripheral blood secreting IL-10 that was later discovered in subjects with T1D too. In this sense, humanized models have been useful not only to test therapeutical approaches but also to discover common mechanisms underlying the disease in both mice and humans.

The latest developed humanized models (Rongvaux 2014) are NSG mice lacking not only IL2Rγ but also Rag genes while carrying GSF, GMCS-F and TPO genes to facilitate human cell engraftment.

2. Islet transplantation and immunotherapies for T1D.

Currently, there are no cure nor prevention strategies able to treat T1D. Despite that, much effort has been carried during the last 50 years in order to develop alternatives that can reach insulin-independence. Besides none of the developed therapies is routinely used to treat patients, all of them let us gain new insights into the pathology and revealed new mechanisms and targets that may be relevant for future treatments.

2.1. Islet transplantation.

2.1.1. The procedure and current limitations.

The first approach to overcome T1D was the direct β-cell replacement through islet transplantation. In this first attempt, autologous islets were transplanted into recipient chemically-induced diabetic rats, resulting in the partial restoration of the normoglycaemia (Ballinger 1972). Later in humans, intra-portal vein islet auto-transplantation in 10 patients with surgical derived diabetes revealed temporary insulin-independence for 3 patients (two for less than a year and one for three years) (Najarian 1980). Islet allotransplantation also reported some cases of insulin-independence but under immunosuppression therapy to avoid rejection (Sharp 1990). Besides technical and surgical improvements, the state-of-the-art of islet transplantation in 1999 reported less than 10% of temporary insulin-independence success. For that reason, the Edmonton protocol was designed to establish a surgical standardized protocol leading to comparable clinical outcomes among different centres (Shapiro 2000). The Edmonton protocol demonstrated its clinical efficacy in a 5-years following study by maintaining 10% of insulin-independent receivers up to a year. Moreover, 80% of the receivers showed C-peptide secretion and HbA1c

under 7% (Ryan 2005). Currently, the Edmonton protocol is still used to perform islet transplantation even when islets are co-transplanted with other organs.

At the moment, the most common site for islet transplantation is intra-portal. It offers many advantages, for instance, patients do not need anaesthesia and the surgery is minimally invasive and safe. Despite that, intra-portal transplanted islets may become attached to sinusoidal capillaries undergoing cell death (Matsumoto 2014). Moreover, because the majority of the islet cells are destroyed within by the innate immune system after the first hours of transplantation, alternative transplantation sites have been considered. One of the proposed extra-hepatic sites for islet transplantation is the omentum (Al-Abdullah 1995), eye-chamber (Adeghate 1990), pancreas (Stagner 2007), renal subcapsular (Sakata 2008) or testis (Ferguson 1977). The main limitation for alternative transplantation sites is the lack of vascularization and hence poor oxygenation of the transplanted tissue. Vascularization is crucial not only because for the engraftment but also for islet cell homeostasis as they take up until 20% of the total pancreas blood flow (Stendahl 2009).

The immediate rejection at early transplantation is driven by the exposure of tissue factor on the islet surface (Kanak 2014). An approach to avoid innate immune response could be islet encapsulation. Macroencapsulation is the encapsulation of multiple islets (Vaithilingam 2017) while microencapsulation implies the coating of individual islets (Chang 1964). Many studies are confirming that mice but not human islets can survive into macroencapsulation systems (Scharp 2014 and Desai 2017). Microencapsulation of dispersed islets has been achieved in several materials such as alginate, polyethylene glycol or chitosan (Wang 1997).

Another approach with more clinical relevance is xenotransplantation, the procedure of transplant living tissues across different species. It has been already evaluated when fetal pig islets were transplanted (Groth 1994). The main pitfall in xenotransplantation is the high risk of zoonotic infections through endogenous species-specific viruses. Indeed, xenotransplantation did not result in clinically relevant outcomes even in the most recent trials (Matsumoto 2016).

2.1.2. Alternative sources of endogenous insulin.

Since the establishment of the Edmonton protocol, at least two donors (i.e. two fresh islets preparations) or more are needed for every recipient. That drives to a loose point in the chain of islet transplantation. These can be overcome with alternative cell sources for islet transplantation.

A promising approach is gene therapy, consisting of transfecting an insulin coding gene construction into non-insulin producing cells like hepatocytes (Bouwens 2013). This cell type was reported to secrete insulin and achieve diabetes remission in animal models (Gan 2016). Nevertheless, gene therapy still lacks of tested issues (especially *ex vivo*) and needs to take into account other glucose metabolism players, like Glut2 and Glucokinase.

For that, it has been proposed to differentiate autologous pluripotent human embryonic stem cells (hESCs) or induced pluripotent stem cells (iPSCs) due to their ability to be immune-matched with some other individuals. hESCs have been successfully differentiated into pancreatic endoderm progenitors through the expression of specific endocrine lineage transcription factors such as PDX1, NKX6.1, and MAFA in several studies (Lumelsky 2001 and Ku 2004). In addition, transplanted endocrine progenitors resulted in successful insulin production after *in vivo*

differentiation in an animal model (D'Amour 2006). Pancreatic progenitors derived from hESCs have been also further differentiated into insulin-producing cells (Zhou 2008), also named β-like cells, able to revert diabetes in mice (Pagliuca 2014 and Russ 2015). Apart from the ethical issues arising from the use of human embryonic cells another flaw concerning hESCs is that they must match receiver HLA to avoid organ rejection. For that reason, iPSCs generated from adults have been considered (Takahashi 2006). Intense research is still ongoing in order to further improve differentiation protocols, to obtain β-like cells mimicking a more real insulin-producing cell function and to avoid teratogenicity of *in vitro* cultured cells. Indeed, reduced costs and increased efficiency are needed to facilitate translation into clinical practice.

2.1.3. Post-transplant limitations.

The *Edmonton protocol* supposed a milestone in the field as it successfully standardized the procedure among the huge majority of clinical centres. Despite that, the islet transplantation still shows major restrictions. First, the scarce of organ donors impedes that future successful transplantations would be possible given the huge number of patients with T1D. Second, the need for immunosuppression makes islet transplantation not suitable for all kinds of patients. And lastly, the still reduced number of responders that can achieve insulin-independence after this complex surgical procedure. Even if future islet transplantations can reach normoglycaemia with insulin-independence, avoiding early innate immune responses and hampering graft-vs-host disease, there still would be necessary to arrest recurrent autoimmunity with the potential to selectively destroy insulin-producing β-cells. In the following sections, the most relevant immunotherapies against T1D will be reviewed.

2.2. Immunological pathways targeted in T1D.

The time-point of intervention in T1D has been classified into three distinct categories: 1) primary prevention for asymptomatic subjects with genetic risk and two or more autoantibodies, 2) secondary prevention for subjects with multiple autoantibodies and impaired glucose metabolism and 3) treatment for subjects already diagnosed of T1D (Insel 2015). Similarly, these approaches can be classified into systemic or antigen-specific depending on the targeted pathway.

Several strategies have been tested for both prevention and cure of T1D. On one hand and for primary prevention, the Pre-POINT study (Bonifacio 2015) administered high-dose oral insulin to HLA-risk subjects, resulting in anti-insulin IgA and IgG response within mucosal tissue and expanded Tregs. On the other hand, for secondary prevention insulin peptides have been used too. Their administration orally or intranasal elicited Treg generation and suppression of effector T lymphocytes (Skyler 2005). In NOD, antigen-specific insulin mimotope administration complete prevention of diabetes through Tregs induction but this strategy failed to revert hyperglycaemia in already diabetic animals (Daniel 2011). These observations reflect that targeting the immune system would be useful to achieve future prevention but not enough for the remission of established disease.

Immunotherapies for subjects with T1D have been proposed for early stages, such as recent onsets, because of the expected β-cell loss during disease progression (Table 1). None of them has currently resulted in successful total remission for all the individuals included in the following studies. One of the targeted pathways previously considered is the ablation

of T lymphocyte action. It can be achieved with general immunosuppression with cyclosporine and anti-CD3 drugs like Teplizumab (Sobel 2010 and Long 2017), resulting in T cell exhaustion. Alefacept, an anti-CD3 compound too, decreased T cell subsets for two years (Rigby 2015) without diabetes remission. Besides targeting all T cell subpopulations ($CD3^+$, not only including the effector ones), an alternative approach is to increase or promote Tregs actions. ATG + G-CSF resulting in a temporary increase in Tregs concentration correlating well with augmented C-peptide in some individuals (Haller 2016). Given the substantial role of IL-2 in maintaining Tregs identity, its administration has been explored. In combination with rapamycin resulted in Tregs expansion but side-effects were found to be deleterious on β-cells (Long 2012). Furthermore, adoptive cell therapies for increasing Tregs populations are undergoing. Apart from the immune effect from effector and regulatory players, the focus has been settled on the targeted β-cell too. For instance, compounds aiming to block pro-inflammatory cytokines could putatively overcome β-cell death (Moran 2013 and Cabrera 2016).

None of the mentioned assays performed a clear remission nor prevention of T1D. Two main lessons can be learned from these clinical trials. First, whether some individuals are responders with significant clinical outcomes and others not, it could provide novel and interesting insights into diabetes progression. Second, the timing and dosage of treatments should be considered as high heterogeneity among patient's response has been observed. These data point to a new perspective on personalized strategies to design future immunotherapies against T1D.

Table 1. Summary of clinical trials and targeted pathways for T1D. Adapted from Jacobsen 2018.

Clinical trial intervention	Presumed targeted pathway	Outcome of responders	Reference
Cyclosporin + methotrexate	Global immunosuppression	12-month lower insulin dose	Sobel 2010
Rituximab (anti-CD20)	Decreasing B cells	12-month lower insulin dose	Pescovitz 2009 and 2014
Teplizumab (anti-CD3)	Decreasing T cells	12-month lower insulin dose	Hagopian 2013, Sherry 2011
Otelixizumab (anti-CD3)	Decreasing T cells	48-month lower insulin dose	Keymeulen 2010
Thymoglobulin (ATG)	Preserving Tregs and reducing effector T cells	1 subject 24-month insulin free	Orban 2011 and 2014
ATG + G-CSF	Preservation of Tregs	12-month increased C-peptide	Haller 2018
Abatacept (CTLA-4)	Reduction of effector T cells	24-month increased C-peptide	Orban 2011 and 2014
Ex vivo expanded Tregs	Expansion of Tregs	Increased Tregs	Bluestone 2015
Autologous hematopoietic stem cell transplant	Expansion of Tregs	Short- and long-term insulin remission (10- and	Malmegrim 2017
Alpha-1 antitrypsin	Expansion of Tregs	2h increased C-peptide	Weir GC 2018, Fleixo-Lima 2014
IL-2	Expansion of Tregs	Increased Tregs	Hartemann 2013, Rosenzwajg 2015
Etanercept (anti-TNFα)	Blocking TNFα	6-months lower insulin dose	Mastrandrea 2009
Verapamil (Ca^{+2} channel blocker)	Inhibition of β-cell apoptosis	12-month increased C-peptide	Ovalle 2018

2.3. Clinical trials with Tregs therapy.

The clinical potential of Tregs arises from their known role of physiological self-tolerance induction. Taken this into account, several authors have proposed to expand the population of Tregs to prevent and treat T1D.

One of the main options to target Tregs is through specific compounds. As previously reviewed, IL-2 is required for maintaining Tregs identity. Hence, low-dose IL-2 therapy resulted in increased Tregs number and function in NOD treated mice. It resulted in successful prevention and even remission of T1D (Grinberg-Bleyer 2010 and Manirarora 2015). Intriguingly, other authors reported a dose-dependent outcome while a higher dose may accelerate diabetes onset mediated by effector T lymphocytes and NK cells (Tang 2008). In human subjects with the disease, low-dose IL-2 therapy was able to promote Treg expansion (Long 2012). Another targeted molecule is CTLA4, required for costimulation in both effector T cells and Tregs. An immunoglobulin targeting CTLA4 (CTLA4-Ig) can partially block CTLA4 impeding effector T cell development without affecting Tregs maintenance in both humans and mice (Bluestone 2008). A commercial drug named Abatacept (a CTLA4-Ig) partially impairs β-cell destruction in human subjects resulting in diabetes amelioration during 12 months (Orban 2011). Lastly, another compound named Alefacept selectively targets Tregs. Alefacept is an LFA3-Ig molecule that is able to bind CD2 on T lymphocytes, a target molecule necessary to enhance its activation upon APC contact (Miller 1993). This drug treatment resulted in effector T lymphocyte depletion while Tregs preservation (Rigby 2013) thus preserving β-cell function in subjects with T1D temporarily (Pinckney 2016).

Other strategies intended to promote Tregs function may be cell therapies. These cells can be obtained from human donors by FACS sorting based on

surface markers and expanded *ex vivo* (Putnam 2009). When administered to paediatric patients at recent-onset of the disease, increased C-peptide concentration was detected in all participants (Marek-Trzonkowka 2012 and 2014). Moreover, two patients remained insulin-independent for more than one year while a significant increase in Tregs in peripheral blood was observed.

A key point is that none of the above mentioned strategies were antigen-specific therapies. Hence, new prospective strategies may be based on this feature. Clinical trials that underwent another autoimmune disease — Chron's disease— taking into account that antigen-specific Tregs expanded ex vivo showed relative success (Desreumaux 2012). These could point to the next step in the designing and validation of immunotherapies based on Tregs pathways for T1D and other autoimmune pathologies.

Additionally, it is possible to go besides Tregs for the induction of self-tolerance through re-balancing the effector T/Tregs equilibrium. Earlier in the introduction (1.3.3. Insulitis and autoreactive lymphocytes), it was mentioned the function of DEAF1 protein in promoting self-tolerance during T lymphocyte ontogeny at the PLNs. *Deaf1* splicing can be corrected by promoting an anti-inflammatory environment in the PLNs through IL-4 overexpression in NOD mice (Kodama 2008). IL-4-derived treatments resulted in normalized gene expression in PLNs and T1D prevention in the NOD model (Feili-Hariri 2003).

Beyond the use of physiological sources of immune cells, autologous chimeric antigen receptor (CAR) T cells have been proposed to treat T1D and several other autoimmune diseases. CAR T cells were originally proposed to express a tumour antigen-specific region fused to TCR and

costimulatory domains to treat some types of cancer (Jaspers 2017). The main advantage of this therapeutic approach is that T lymphocytes can be activated without the need of APC-driven presentation.

Hence, regulatory CAR T cells for the treatment of T1D could be produced by encoding a region able to interact with βcell-specific antigens. CAR regions can be induced by gene transfer in a temporarily (Zhao 2010) or stable manner (Brusko 2010). Indeed, stable transduction of CAR sequences targeting autoantigens could potentially lead to tolerance against regular infections (Gravano 2012) but alternative dosages and time-points are under investigation for other pathologies (Getts 2012). Furthermore, novel approaches to control the expression of CAR regions against autoantigens are ongoing to deplete CAR T cells after committing its target (Jones 2014).

2.4. Experimental nanotherapies.

Nanomedicine is the development of therapeutic strategies based on systems at the supramolecular level. A major part of the field has focused on the encapsulation, transport, and delivery of biological compounds like proteins or DNA/RNA (Veiseh 2015). In the current section, it will be reviewed the state-of-the-art in nanotherapies to tackle T1D.

Despite there is no formal definition, nanoparticles are commonly considered to be between 1-1500nm of diameter (Getts 2015). Its biodistribution depends on both the size and administration route. While small nanoparticles (<200nm) can transport free, and accumulate in organs and tissues (Manolova 2008). On the other hand, the larger particles (> 200nm) need to be transported attached to APCs (Shao 2015). Within this group, DCs have preferentially uptake of the smaller and macrophages to

the larger nanoparticles (Garciafigueroa 2015). Not only the size but also the structure is relevant in the uptake of nanoparticles by APCs (Verma 2008). More relevance is even paid when these nanoparticles display conformational antigens (Wahome 2012). Despite that, caution is needed when antigens are exposed in the outer part of nanoparticles because it can lead to anaphylaxis due to exacerbated antibody recruitment (Smarr 2016).

2.4.1. Experimental tolerogenic nanoparticles.

Several strategies involving nanoparticles have been proposed to induce tolerance. This means the reestablishment of self-tolerance and the inability of the immune system to react against self-antigens.

One approach in the design and development of nanoparticles for immune-related diseases are those targeting immunomodulatory molecules or inducing immune tolerance. For instance, pegylated-nanoparticles carrying anti-sense oligonucleotides targeting costimulatory molecules —CD40, CD80 and CD86— resulted in DCs with an immunosuppressive phenotype, reversing autoimmune diabetes in animal models (Phillips 2008). This strategy also prevents and reverses diabetes in NOD.SCID mice transferred with diabetogenic T cells from NOD mice. Similarly, lipid nanovesicles carrying small interference RNA (siRNA) silencing CCR2 hampered monocyte tissue recruitment (Leuschner 2011).

Another common approach is the design of antigen-specific nanoparticles. Iron oxide nanoparticles coated with class I or II MHC presenting disease-specific autoantigens resulted in the expansion of Tregs and reduced effector and memory T lymphocytes (Singha 2017). This approach was used in an antigen-specific manner for T1D, it was able to prevent and reverse the disease in young NOD mice and humanized mice models by

expanding specific $CD8^+$ Tregs (Tsai 2010). Moreover, a similar strategy also resulted in the expansion of different immune regulatory subsets, including regulatory B cells and reversal of hyperglycaemia in NOD diabetic mice (Clemente-Casares 2016). Besides iron nanoparticles, gold particles coated with proinsulin targeting DCs also showed Tregs induction and diabetes prevention in different models of transgenic NOD mice (Yeste 2016). The main advantage of these approaches is that they are disease-specific, as they can be customized with autoantigens corresponding to other autoimmune diseases showing efficacy in other disease models such as multiple sclerosis or arthritis (Clemente-Casares 2016).

Lastly, approaches leading to the encapsulation of molecules have been developed and successfully resulted in therapeutic potential in models of T1D. Despite that, some authors claimed that displaying antigens on the nanoparticle surface may have better safety outcomes than its encapsulation (Smarr 2016). Indeed, encapsulation of the whole insulin molecule remains a challenge for some types of nanoparticles (Chopra 2017). This approach could be useful when combined with islet regenerative strategies. For instance, nanoparticle encapsulating approaches combined with rapamycin, an immunosuppressor, may increase its efficacy (Bryant 2014).

2.4.2. microRNAs.

The microRNAs (miRNAs) are small non-coding RNA molecules between 18 and 22 nucleotides (Huntzinger 2011). They are endogenous cell products of nuclear DNA transcription. Hitherto they are known to be involved in the regulation of gene expression through inhibition of protein translation by targeting specific sequences of mRNA (Bartel 2009). Among

several other functions, miRNAs play different roles in both immune system homeostasis (Bernstein 2001) and islet development (Joglekar 2007).

During the last decade, much effort has been focused on the identification of miRNAs involved in β-cell death. These studies resulted in the identification of different miRNAs molecules targeting anti-apoptotic β-cell proteins at the onset of T1D (Filios 2014). Since then, a regulatory network of miRNAs have been pointed out to participate in the pathway of programmed β-cell death triggered by cytokine release during insulitis (Roggli 2010). In summary, miRNAs constitute a new promising target to develop new therapies tackling T1D. Furthermore, specific miRNAs have been discovered to play a key role in the differentiation of iPSCs into β-like-cells (Shaer 2015) opening the door to target them not only *in vivo* but also in β-cell generation.

2.4.3. Extracellular vesicles.

Extracellular vesicles (EVs) are lipid-bound nanoparticles, ranging from 30nm to 5000nm, released by many cell types (Thery 2006). The β-cells, like other cell types, are a source of EVs. Insulinoma-derived β-cell lines can release the autoantigens insulin, IA2, ZnT8 and GAD65 encapsulated in EVs (Sheng 2011) as well as other islet-specific proteins (Cianciaruso 2017) or miRNAs (Guay 2015). These suggest that β-cells are contributing to its identity maintenance by cell-specific cargo released in EVs.

Additionally, EVs can also be released by DCs and contain both costimulatory and HLA molecules participating in antigen presentation (Segura 2005). These released EVs that also contain autoantigen cargo can undergo DC activation triggering an autoimmune response *in vitro*

(Rutman 2018). These observations correlate well with similar findings in mice models of the disease (Bashratyan 2013).

Also, customizable manufactured EVs have been explored as they can be loaded with the desired cargo. EVs loaded with vitamin D3 resulted in almost complete prevention of T1D in the NOD mice model (Lewis 2015), revealing a potential therapeutic effect *in vivo*. Overall, EVs constitute a promising tool for the design of nanotherapies for T1D and other autoimmune diseases but also for the manipulation of drug delivery to concrete histological locations. Besides, they are still very difficult to obtain from natural sources rather than *in vitro* production.

2.4.4. PS-Liposome based strategies.

Liposomes are nanovesicles composed of a lipidic bilayer that can contain an aqueous cargo. Liposomes tagged with phosphatidyl-serine (PS) in the membrane can mimic apoptotic antibodies by PS exposure. Efferocytosis is the process of self-tolerance maintenance mediated through apoptotic bodies phagocyted by DCs (Nagata 2010).

In T1D, the removal of apoptotic β-cells is slowed down so they easily become necrotic thus contributing to the release of pro-inflammatory cytokines by DCs feeding back the vicious circle of autoimmunity (Vives-Pi 2015). On the opposite, DCs contribute to maintaining self-tolerance in physiological conditions (Mbongue 2014). Normal cells regularly die through apoptosis within their environmental tissue. Hence, DCs can phagocyte them and process its intracellular content reinforcing the self-tolerance to these molecules and this process is named efferocytosis.

Taking these observations into account it is reasonable to explore the suppressive activity of DCs upon this condition. Our group developed a strategy to arrest the autoimmune attack based on an efferocytosis mechanism (Marin-Gallen 2011). This approach consisted of DCs loaded with apoptotic β-cells resulting in a preventive effect through tolDCs generation and a reduction in the incidence of diabetes. Nevertheless, this strategy is difficult to translate into the clinical, due that is not possible to obtain autologous β-cells from living patients. For this reason, liposomes mimicking apoptotic bodies were designed (Pujol-Autonell 2015). Liposomes tagged with phosphatidyl-serine (PS) in the membrane and encapsulating insulin peptides were generated (PSAB-liposomes). These nanovesicles were captured by DCs from NOD mice, inducing a tolerogenic phenotype and impairing autologous T cell proliferation *in vitro*. *In vivo*, PSAB-liposomes reduced the incidence of T1D and also decreased insulitis.

The fact that liposomes can contain a widely different cargo into their core makes them highly customizable. PS-liposomes can contain other different autoantigens and when loaded with MOG (myelin oligodendrocyte glycoprotein) peptide, they were also efficient in preventing EAE (Experimental Autoimmune Encephalomyelitis), the experimental model of multiple sclerosis (Pujol-Autonell 2017). This versatility opens the door to further explore the effect of encapsulating autoantigens in several other autoimmune diseases.

To determine liposome possibilities in human T1D, PSAB-liposomes encapsulating the human sequences of A and B chains of insulin were manufactured. The uptake of PSAB-liposomes by human DCs resulted in the induction of a tolerogenic phenotype and impaired autologous T cell proliferation, similar to the effects observed in mice (Rodriguez-Fernandez

2018). The transcriptomic analysis of DCs after PSAB-liposome capture pointed to an immunoregulatory profile by regulating specific pathways related to immunological functions. These observations were also validated in a paediatric cohort (Rodriguez-Fernandez 2019).

Further research is still needed to explore the possibilities that this new nanotechnology-based application may lead to the field of biomedicine. Because apoptotic β-cells contain other autoantigens apart from insulin, more effort is needed in order to better mimic apoptotic β-cells.

3. β-cell regeneration.

Several approaches for β-cell regeneration have been proposed based on producing β-cells *de novo*. Many efforts have been made in the past 40 years to generate β-cells *in vitro* to get transplanted in recipient subjects with T1D. Because this strategy would face similar problems as in islet transplantation if cells are not autologous —immune rejection and recurrent autoimmunity—, the paradigm in the regenerative field shifted to develop protocols triggering endogenous β-cell regeneration.

Indeed, the study of the islet and more deeply of β-cell biology arose much knowledge about pancreas structure and organogenesis, hormone secretion and cell plasticity, among several others. This knowledge, coming from basic research, has been used to understand other pathologies like T2D, pancreatitis or pancreatic cancer. Even though, tools used to decipher the genetic mechanisms underlying islet biology like lineage-tracing were previously used in other fields and have been spread to new ones.

3.1. Embryonic development: β-cell neogenesis.

To achieve proper β-cell regeneration, the β-cell generation during normal embryo development (also named β-cell neogenesis) has been historically the gold standard of the regenerative mechanisms (Shih 2013).

As mentioned, the pancreas is a mixed gland involved in both exocrine and endocrine functions related to nutrient metabolism. It is composed of a ductal-tree structure containing enzyme-producing cells, named acinar cells, and endocrine cells (Figure 2). The later are clustered into aggregates called islets of Langerhans and represent the 2% of the total pancreatic content while the acinar and ductal cells are the 98% (Steiner 2010). Islets

of Langerhans are composed of glucagon-producing α cells, insulin-producing β cells, somatostatin-producing δ cells, ghrelin-producing ε cells, and pancreatic-polypeptide γ cells (also known as PP-cells) (Baeyens 2018). These hormone-producing cells have tightly regulated secretion mechanisms since their genesis at the development and during the adult stage, being the β-cells the most common type (80%).

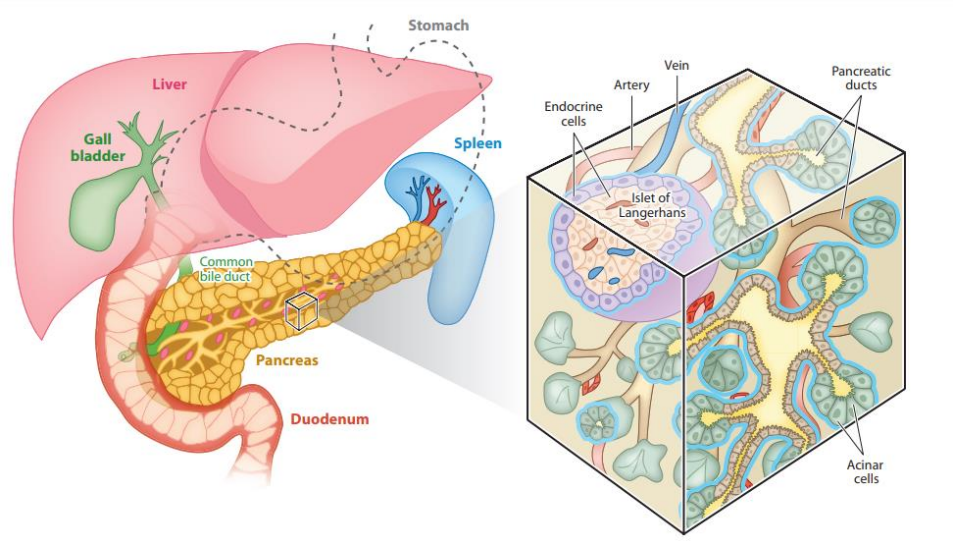

Figure 2. Anatomy and tissue distribution of the adult pancreas (adapted from Shih 2013).

Murine and human pancreas development share common features. In both, the different adult pancreatic cell types arose from a pool of common progenitor cells located in the embryo gut endoderm (Gu 2002). In mice the first morphological evidence of pancreas organogenesis relates to the budding of two (ventral and dorsal) epithelial structures from the foregut, arising at embryonic day 8.5 (E8.5) and growing by E9 (Slack 2004). Gene manipulation experiments determined that the exclusion of *Shh* and the presence of retinoic acid is required in the pre-pancreatic foregut to induce pancreatogenesis (Molotkov 2005). Furthermore, the ventral bud in the

foregut at E8.5 will give rise not only to the pancreas but also to the liver, in a developmental program tightly regulated by surrounding mesenchymal tissue secreting pro-hepatic factors such as FGF and BMP (Deutsch 2001 and Rossi 2001).

The murine pancreatic buds grow and fuse by E12.5 leading through cell proliferation leading to a branching primitive structure. This developmental process is named the primary transition (Pictet 1972). In this stage, there appeared specific transcription factors that establish the pancreatic identity —*Pdx1*, *Ptf1a*, and *Sox9*— (Guz 1995, Krapp 1998 and Kawaguchi 2002). Mice deficient in one of these transcription factors showed pancreatic hypoplasia and partial agenesis despite budding is still observed (Gittes 2009). For instance, *Ptf1a* deficient mice resulted in the differentiation of pancreatic progenitors into extra-pancreatic lineages while *Ptf1a* ectopic expression induces the differentiation of liver progenitors into pancreatic cells (Afelik 2006). Indeed, *Pt1a* expression is required to achieve another transcription factor (*Onecut-1*) expression, which simultaneously regulates *Ptf1a* and *Tcf2* —among other transcription factors— (Maestro 2003). For that reason, the deletion of an early transcription factor results in an amplified response affecting the subsequent developmental program.

Similarly in humans, the pancreatic ventral and dorsal buds emerged at 30-33 days post-conception (dpc) (Jennings 2015) —from *PDX1* progenitors (Sherwood 2009) and by *SHH* repression as well as rodents (Jennings 2013)— that later fuses around 58 dpc.

At the secondary transition (from E12.5 to birth), the pancreatic epithelium expands while differentiating into the ductal, acinar and endocrine cell types. At E12.5 the pancreas is mainly composed of multipotent progenitors that differentiate into two different lineages: tip and trunk cells

(Zhou 2007) (Figure 3). The trunk progenitors will give rise to adult ductal and endocrine cells whereas the tip domain will originate acinar cells (Solar 2009 and Kopp 2011). Opposite to murine, in humans, the tip and trunk progenitors appear before ventral and dorsal bud fusion at 47 dpc (Jennings 2013).

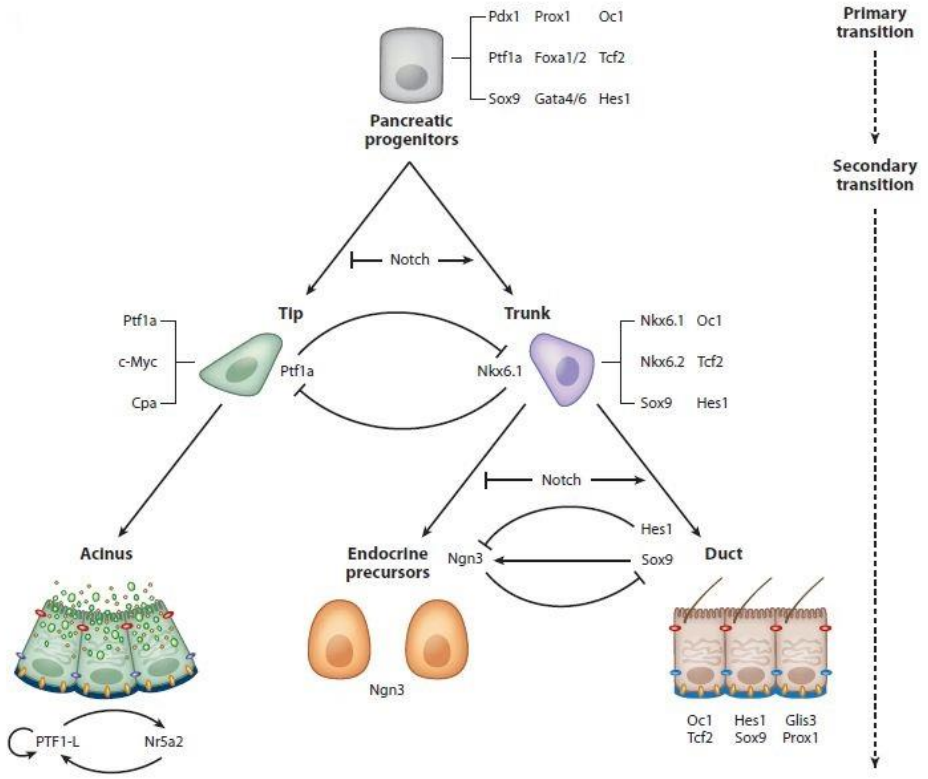

Figure 3. Tip and trunk segregation lineages during murine pancreatic development (adapted from Shih 2013).

The trunk domain is usually restricted to the edge area of the developing pancreas and marked by the expression of *Sox9*, *Nkx6.1/6.2*, *Tcf2*, *Hnf6* and *Hes1* (Jacquemin 2003 and Solar 2009). On the other side, the tip domain is characterised by the marked expression of the following transcription factors: *Ptf1a*, *Cpa*, and *c-Myc* (Villasenor 2010). Surprisingly, *Ptf1a* was shown to specify the pancreatic pattern during the primary transition and

to specify the acinar identity during the second, which reflects that transcription factors may be able to regulate many different processes upon the tissue environment (Masui 2010).

Tip progenitors underwent mature acinar cells by E15.5 and then the subsequent expansion is driven by acinar cell proliferation until the birth (Zhou 2007). Acinar cell fate specification is established by *Ptf1a* but its expression is not sufficient enough to achieve acinar differentiation (Schaffer 2010). Apart from the acinar phenotype specification, it is known that *Ptf1a* is a suppressor of subsequent acinar cell proliferation until birth (Rodolosse 2004). Hence, it reveals the need to further explore and discover the gene regulatory network involved in the last step of acinar cell specification during embryo development.

3.1.1. Endocrine cell fate specification.

While the tip progenitors originate almost exclusively in the acinar domain, trunk cells are bipotent progenitors that originate both ductal and endocrine lineages (Kopp 2011). By E12.5 (secondary transition), the pancreatic epithelial layer corresponding to the trunk pattern displays morphogenetic changes to extensively conform a tree-branched structure that will serve as a scaffold for the future ductal tree (Kesavan 2009). Most of the genes that were exclusive of the trunk lineage during tip/trunk specification were later restricted to the ductal domain, as is the case of *Sox9*, *Hes1*, *Hnf6* and *Tcf2* (Maestro 2003 and Shih 2012). Deficient mice for one of these transcription factors resulted in abnormal ductal structures (Shih 2012).

The endocrine cell fate is specified from a pool of trunk progenitors expressing *Ngn3* (Gradwohl 2000 and Gu 2002) as well as trunk cells that are not expressing this master transcription factor are limited to the ductal

fate (Beucher 2012). Indeed, the ductal transcription factor Hes1 plays an essential role in balancing the endocrine/ductal lineage by repressing Ngn3 expression (Apelqvist 1999). It has been described how Notch signalling activation prevents *Ngn3* expression too (Greenwood 2007) by promoting the expression of specific ductal transcription factors like Hes1 and Sox9 (Shih 2012). Conversely, after ductal pattern specification by the secondary transition, $Ngn3^+$ progenitors stop to arise from (Kopinke 2011), suggesting that signals from this environment are required to induce endocrine lineage. Further experiments performing macroscopic ductal injury have shown to reinitiate the endocrine neogenesis programme (Xu 2008). How some primordial trunk cells express Ngn3 and upon which specific signals it is governed still remains unexplored.

$Ngn3^+$ trunk progenitors exit the cell cycle and become post-mitotic cells (Miyatsuka 2011). Then, these progenitors have the ability to differentiate into the five major endocrine subtypes in the following temporary order —and through differentiating in previous unipotent progenitors (extensively reviewed in Desgraz 2009)—: α cells, β-cells, δ cells and γ cells (Johansson 2007). Fully differentiated hormone-producing cells still express specific transcription factors to maintain its identity and avoid spontaneous transdifferentiation. β-cells express putative markers such as *Pdx1*, *Nkx6.1*, and *Pax4* (Sosa-Pineda 1997 and Collombat 2003), which actively repress the expression of *Arx*, the determinant of α-cell identity (Collombat 2005). Gain- and loss-of-function experiments in mice with both *Arx* and *Pax4* revealed high plasticity between mature α- and β-cells (Collombat 2007 and 2009). In humans, single $insulin^+$ cells are the first to appear in the developing pancreas by 50 dpc, followed by glucagon+ and somatostatin+ cells (Riedel 2012). They arise as single, dispersed, hormone-producing cells while true islet-like structures are not detected in

the human foetal pancreas until 12 weeks post-conception (wpc) (Piper 2004 and Meier 2010).

After birth, both murine and human β-cells expand briefly by mitosis in the peri-natal stage and undergo a very slow ratio of proliferation during adulthood (Dor 2004). There is no direct demonstration of adult human β-cell neogenesis, albeit scarce observations of single insulin+ cells dispersed along with the pancreas or even in ducts (Bouwens 1996). There are technical limitations to study human β-cell proliferation, given that Ki67 assessment (self-proliferation maker) yields high variation upon tissue sample conditions such as biopsy procedure or storage (In't Veld 2010 and Sullivan 2015). Nevertheless, the highest proliferation ratio has been detected in human subjects from 0 to 3 years old, decreasing from youth to adulthood (Meier 2010) revealing a single peak before puberty (Moran 1999). Intriguingly, the highest apoptotic rates in β-cells correlate well in humans with the peri-natal age in which the proliferation is highest too (Georgia 2004), suggesting this is an important stage for endocrine cell remodelling.

Most of the work leading to the nowadays accepted model of pancreas development has been obtained from mouse models due to the ethical issues to manipulate human foetal tissue. It may lead to assumptions not strongly demonstrated in human development, limiting the progression of novel regenerative strategies. Despite that, all this work has been very useful to highlight loose-ends that opens the door to continue the exploratory analysis of the pancreatic and endocrine neogenesis programmes.

3.2. β-cell heterogeneity and accessory islet cells.

At this point, the question about if all mature β-cells are identical remains unavoidable. There is extensive and solid evidence of β-cell heterogeneity leading to different subpopulations (Johnston 2016a). This is a relevant aspect to develop preventive and therapeutic strategies.

Further studies in human β-cells from non-diabetic donors reported heterogeneity in stress-related markers (Baron 2016) and UCN3, a maturation marker (van der Meulen 2012). More studies supported the evidence that there exist β-cell heterogeneity related to ER- and age-stress related proteins (Zeng 2017 and Aguayo-Mazzucato 2017). These observations correlated well with functional heterogeneity in normal β-cells, given that the β-cells that are most sensitive to glucose are also susceptible to cytokine stress (Ling 1998). Moreover, proliferation markers are significantly decreased in cells with upregulated pro-inflammatory pathways (Janjuha 2018). It is clear that this heterogeneity does not emerge from its clonal identity —supposed to be almost identical in an individual— but from the islet architecture in which β-cells are coupled by cell to cell junctions (van der Meulen 2017). Other studies proposed that adult subpopulations with reduced capacity to uptake glucose, but active proliferation, are transcriptionally immature (van der Meulen 2017, Bader 2016 and Puri 2018). These subsets would be prone to show reduced bona fide β-cell markers such as *Pdx1* and glucokinase activity (Roma 2015), resulting in a loss of β-cell identity. That indicates a relationship between proliferation and immaturity.

Focusing on residual β-cells from subjects with T1D, normal insulin secretion can be restored when cultured ex vivo in a non-pro-inflammatory environment. It is proposed that changes affecting residual β-cell identity

in T1D may act through impairing normal islet architecture by de-coupling β-cells from each other (Farnsworth 2015).

Nevertheless, β-cells in the islets are not isolated but in tight connection and interplaying with other islet cells and with the accessory islet cells. The neurovascular tissue of the islets mainly consists of blood vessels and nerves. In spite of endocrine cells represent 2% of the pancreatic cell mass they require upon 20% of the total pancreatic blood flow (Meyer 1982 and Lifson 1985), through thin islet capillary endothelial cells (Nyqvist 2011). The vascular network of the islet is essential for hormone secretion (Jansson 2016) as when it is impaired the glucose-stimulated insulin secretion remains affected (Iwashita 2007). VEGF-A is one of the pivotal factors in the interface between the vascular and endocrine tissue to control the β-cell mass. The vascular tissue also contributes to the establishment of an extracellular matrix (ECM) by supplying proteins (Johansson 2009). It has been shown that in diabetogenic conditions, ECM may facilitate autoreactive T lymphocyte penetration within islet to perform β-cell destruction (Bogdani 2014a and 2014b).

Finally, the nervous system is also implied in the normal homeostasis of the endocrine functions as both sympathetic and parasympathetic nerves regulate enzyme release and hormone secretion (Tang 2014). Their ablation leads to islet structural abnormalities resulting in hormone production dysfunction (Borden 2013) similarly as observed in hypoglycaemic conditions (Taborsky 2012). Overall, this indicates that islet biology is a very complex process in which there are involved many cell participants, even though from extra-pancreatic tissue.

3.3. Regenerative approaches.

Since the majority but not all the β-cells are destroyed during the autoimmune attack in T1D, the most obvious strategy to increase the residual β-cell mass would be to promote its proliferation. Self-replication of pre-existing β-cells within the pancreas constituted an attempt to navigate into the pathways of β-cell division to target specific molecular participants and explore new compounds able to stimulate it. Despite it is widely accepted that human adult β-cells have a low proliferation rate, it is also known that the β-cell mass can partially expand by replication upon specific metabolic stimuli like obesity and pregnancy (Rieck 2010). In order to identify molecules capable to induce replication of pre-existing β-cells, numerous high-throughput screenings (HTS) have been performed to identify them (reviewed in Figure 4).

The WS1 was identified as a compound promoting the proliferation of a quiescent clonal cell line from rat (Shen 2013). Later, its analogue WS6 showed similar results in both human β- and α-cells (Shen 2013 and Boerner 2015). Their mechanism of action relies on the inhibition of a cell replication suppressor (EBP1). Following a similar strategy, harmine —a plant-derived alkaloid— was found able to stimulate the MYC promoter in both cultured and transplanted human β-cells (Wang 2015). Harmine is a specific inhibitor of DYRK1A. When inhibited, its target NFAT translocates from the cytosol to the nucleus acting as a mitogen (Wang 2015). HTS experiments focused on the same pathway found another DYRK1A inhibitor named 5-IT able to expand the β-cell mass in transplanted human islets (Dirice 2016 and Walpita 2012).

TGF-β inhibitors also resulted in successful human β-cell replication by recruiting TGF-β to the cytosol and aborting its associated gene expression in the nucleus (Derynck 2003). The compound SB431542 promotes β-cell

proliferation in islets by targeting this pathway (Dhawan 2016). Indeed, the combination of SB431542 and harmine increases synergistically the β-cell replication (Wang 2019). Similarly, given the previous knowledge about GSK3β and its role blocking β-cell replication, two molecules have been synthesized to inhibit GSK3β resulting in successful proliferation (Shen 2015). Notwithstanding, the systemic administration of cytokines and inhibitors may have detrimental effects on the rest of tissues, especially in the immune system.

As explained above, pregnancy is a situation of increased insulin demand that leads to a moderate β-cell expansion. This is due to the effect of prolactin and placental lactogen in the activation of FoxM1 pathway (Rieck 2010). This pathway converging in GSK3β leads to the use of osteoprotegerin (OPG) or denosumab to promote human β-cell proliferation (Kondegowda 2015). Similarly to pregnancy, obesity and insulin resistance also increases substantially β-cell mass (Shirakawa 2016). Thus, the screening of molecules involved in this pathophysiological process resulted in SerpinB1 identification (El Ouaamari 2016). SerpinB1 is a cytokine produced by the liver under insulin-resistance conditions that also blocks GSK3β (El Ouaamari 2019). Hence, the design of molecules targeting components of the pathway triggered by SerpinB1, like GW311616A, promoting human β-cell replication (El Ouaamari 2016).

Finally, the Glucagon-like Peptide 1 (GLP1) has been able to induce successfully rodent β-cell proliferation (Campbell 2013) but these observations were not identified in human cells except in transplanted human islets only in young donors (Tian 2011). Because the half-life of GLP1 is very short (about 5 minutes, Hui 2002), there have been developed many analogues of GLP1 with an increased half-life. Their administration

or pathway activation leads to the replication not only of insulin-producing cells but also rodent pancreatic progenitors (Chen 2012) and ductal cells (Bulotta 2002). Additionally, GLP1 associated pathways —targeted by incretins— also affect β-cell replication and may suggest the occurrence of neogenesis events (Cho 2011).

One of the main problems regarding target β-cell proliferation is that these small, unspecific molecules, may present off-target effects. For that reason, further studies are needed to pay special attention to safety issues. Furthermore, it is still not clear if β-cell replication would be enough to overcome latent autoimmunity in T1D.

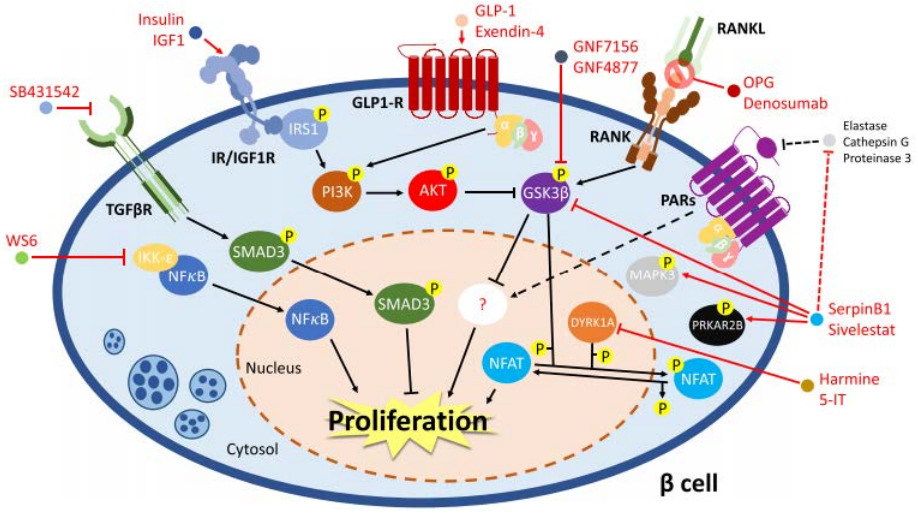

Figure 4. Pathways and compounds regulating human β-cell replication (adapted from Basile 2019).

Because favouring β-cell proliferation may not result in the expected outcome for the treatment of T1D, alternative regenerative strategies have been proposed. The most relevant paradigm is the reprogramming into insulin-expressing β-like-cells from different cell sources (reviewed in Figure 5).

Among the non-endocrine types, hepatocytes and gastrointestinal cells are suitable candidates given that both arise from common progenitors in the foregut during embryo development. Murine hepatocytes can be switched into an insulin-expressing phenotype by the controlled transduction of β–cells transcription factors like *Pdx1* (Ber 2003), *NeuroD1* (Kojima 2003), *Ngn3* (Tang 2013) or *MafA* (Nagasaki 2014), resulting in the restoration of normoglycaemia in diabetes models. Also, human hepatocytes (Sapir 2005) have been converted into insulin-expressing β-like-cells achieving glycaemia restoration upon transplantation in NOD.SCID diabetic mice. On the other side, murine enterocyte cells can be either reprogrammed by *Pdx1*, *MafA* and *Ngn3* expression (Chen 2014) or GLP1 administration (Suzuki 2003), which ultimately triggers *Ngn3* expression.

Other pancreatic cell types have been proposed as a cell source of insulin-expressing cells due to their close proximity to the islet environment. Rodent acinar cells have been successfully reprogrammed through *Pdx1*, *Ngn3* and *MafA* expression (Zhou 2008) but also by exposure to growth factors like EGF, CNTF, and BMP7 (Rooman 2004, Lemper 2016 and Klein 2015). Notwithstanding, it is difficult to translate these approaches to human acinar cells due to the fact that they tend to transdifferentiate into ductal cells when cultured (De Lisle 1990 and Hall 1992). Despite that, to transdifferentiate acinar cells may have side-effects like developing pancreatic ductal adenocarcinoma (Fukushima 2005). On the other side, rodent ductal cells can be converted in β-like-cells upon *Pdx1* expression too (Noguchi 2003). Ductal cells are the most closely related non-endocrine cells to β-cells as both arise from trunk progenitor cells during embryo development. Curiously, insulin-expressing ductal cells were found in some patients with T1D that underwent simultaneous pancreas-kidney transplant (SPK) (Martin-Pagola 2008). There are major concerns about

ductal cell transdifferentiation studies because $Ngn3^{+}$ progenitors that can differentiation into endocrine cells are also located in the ductal part and may mask the origin cell of the reprogramming observation.

Lastly, transdifferentiation from other islet cell types has been explored too. The major advantage is that switch other hormone-producing cells into insulin-expressing would maintain the islet structure thus favouring the normal islet cell crosstalk and regulation. Due to the cell identity balance between and β- and α-cells, governed by *Pax4* and *Arx* expression respectively, this mutual exclusion axis has been manipulated to induce transdifferentiation. It is widely known that glucagon-producing α-cells can be converted into β-cells by either repressing *Arx* (Courtney 2013 and Chakravarthy 2017) or overexpressing *Pax4* (Collombat 2009). Recently, and avoiding gene manipulation, compounds like GABA or artemisinins showed endogenous transdifferentiation of glucagon-producing cells into β-like-cells by restoring normoglycaemia in animal models of diabetes (Ben-Othman 2017 and Li 2017).

An additional tool that might target different regenerative purposes is drug repurposing. This is an *in silico* technique intended to search for novel uses of already approved compounds, which resulted successful in drug discovery for many other diseases (Sleire 2017 and Kumar 2017). Drug repurposing is based in articial neural networks (ANN) that screen mathematical models built with curated biological data (Jorba 2020). This data comprises a map of proteins (named edges) and their relationship (named links) retrieved from public databases such as KEGG (Kanehisa 2000), REACTOME (Croft 2014), INTACT (Orchard 2014), BIOGRID (Salwinski 2004), HPRD (Keshava 2009) and TRRUST (Han 2015). The ANN search for potential relationship between the target proteins of interest and predict potentially interactions with drugs from the DrugBank

database (Wishart 2008). This process has been previously applied for both T1D and T2D resulting in the *in silico* identification of potential repurposed drugs for both diseases (Zhang 2015) but it still lacks of *in vivo* validation.

Additional regenerative strategies have been proposed, like stem-cells-derived β-cells. However, they seem to have a limited prospective clinical outcome due to the fact they are proposed as alternative sources for islet transplantation. This would entail all the problems associated with this clinical procedure that was previously reviewed in the chapter: Immunotherapies 2.1. Islet transplantation. At the moment further research is needed to find an optimal strategy able to be translated into patients. Its main advantage is that β-cells derived from an autologous source would avoid recurrent autoimmunity targeting insulin-expressing reprogrammed cells.

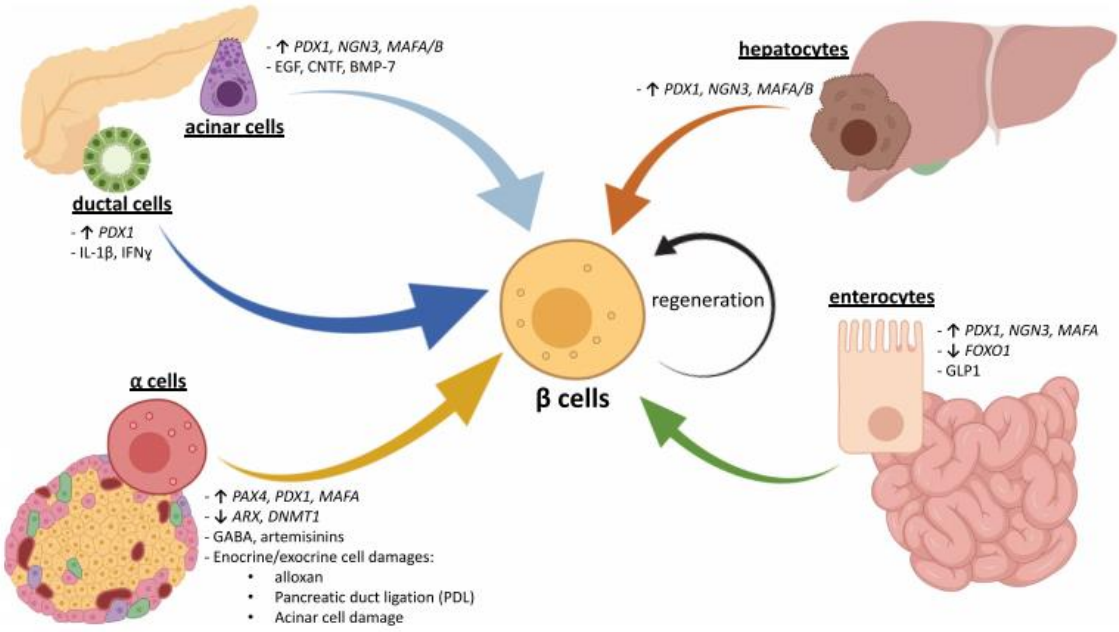

Figure 5. Main regenerative strategies aimed to reprogram other cell types into insulin-expressing β-like-cells (adapted from Basile 2019).

4. Biomarkers for T1D.

Even if there would exist a successful regenerative strategy to obtain newly formed endogenous β-like-cells and an immunotherapy to arrest autoimmunity, the time-point of administration of this immunotherapy would either be critical. For that reason is essential to deploy the different stages of disease progression and identify them with proper biomarkers. A biomarker is any measurable biological parameter that indicates a state or condition (von Herrath 2016). Currently, there is a lack of biomarkers able to reflect the pathophysiology of T1D. Individuals carrying the risk HLA haplotypes are likely to develop T1D (Lambert 2004) but as explained before, there are other unknown determinants of the disease that are independent of genetics. Moreover, not only to predict the onset of the disease is difficult and inaccurate but also to discriminate different stages of diabetes progression is still unrealistic. Hence, novel biomarkers are needed to determine the variations of the autoimmune response against β-cells. This knowledge would lead to consider novel approaches for the design of clinical trials and to allow the immune-monitoring of patients' response to interventions during clinical trials. Despite our scarce availability of biomarkers for T1D, autoantibodies and C-peptide currently help to track events related to disease development and progression.

A very interesting stage of T1D is the spontaneous and transient partial remission stage (PR), also known as honeymoon (Mortensen 2009). This phase arises after the onset, upon insulin administration and it can last few months. PR is characterized by lower exogenous insulin requirements and a decreased HbA1c. For that reason, HbA1c is the widest biomarker used for clinical issues in T1D. Taken all this data together it is reasonable to suggest that PR may be due, at least in part, to immunomodulatory mechanisms after insulin administration (Fonolleda 2017). Robust

biomarkers should fulfil the following requirements: (1) to be obtained with minimal discomfort for the patient, (2) to be able to reflect the variations of autoimmune response and (3) to display significant differences along with the progression of the disease. It is important that candidate biomarkers could monitor the disease stages.

4.1. Autoantibodies.

Given the link between islet autoantibodies and the development of the disease, they have been considered as biomarkers with a relative accuracy of the pre-diabetic phases (Insel 2015). This positivity for autoantibodies may disappear for some individuals. In a large cohort of paediatric subjects, 43% of them had no detectable autoantibodies a year after the first positivity (Krischer 2017). For that reason, autoantibody positivity is clinically used to confirm the autoimmune nature of the already diagnosed diabetes and discard other forms of immune-independent diabetes like MODY (Figure 6).

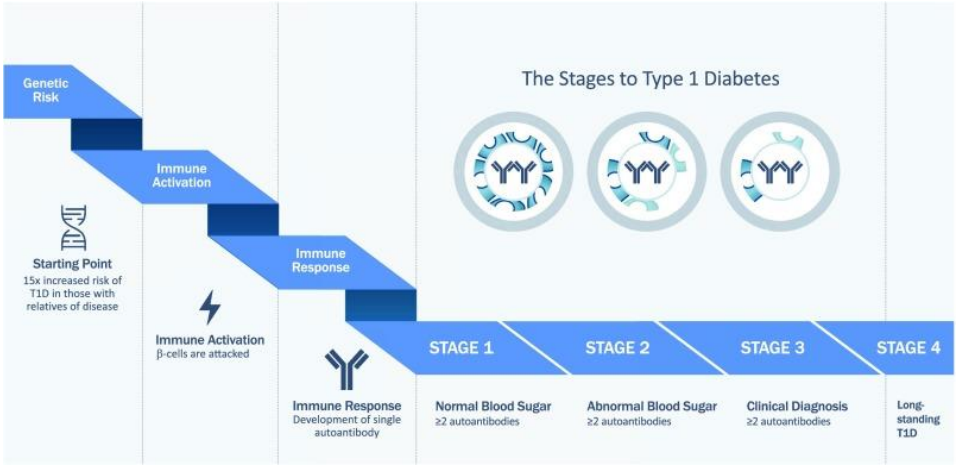

Figure 6. The stages of prediabetes and T1D based on positivity for autoantibodies (adapted from Greenbaum 2018).

4.2. Other serological biomarkers.

Apart from autoantibodies, other serological biomarkers have been proposed in T1D but their current use is limited to the experimental field. One of them is cell-free unmethylated insulin DNA (cf-Ins) (Akirav 2011 and Usmani-Brown 2014). Its origin may come hypothetically from actively destroyed β-cells (Husseiny 2014) but the short half-life of circulating cf-Ins is a drawback for its implementation as a biomarker (Olsen 2016). Further work is intended to combine cf-Ins with other specific unmethylated β-cell genes (Sklenarova 2017). Despite that, a decline in cf-Ins was observed in a clinical trial using Teplizumab (Lebastchi 2013).

On the cellular side, T lymphocytes reactive against neoepitopes have been previously identified in T1D (Skowera 2008) through next-generation sequencing (NGS). If widely implemented, this tool would provide new insights into the relationship between the autoimmune reaction and the islet microenvironment. It would be especially useful to monitor immune cell frequencies in clinical trials devoted to re-establish the balance between effector and Tregs. Currently, it still remains as a challenge to implement NGS to large cohorts in the clinical practice.

Furthermore, metabolomics tools have already been implemented to screen metabolite fluctuations at different phases of T1D. A significant reduction in a wide range of phospholipids in cord blood within a cohort of 129 high-risk children before seroconversion to positive autoantibodies (Oresic 2008) was found. Within the phospholipid group, reduced concentrations were detected, especially in phosphatidylcholine and sphingomyelin. In concordance with these observations, low levels of triglycerides were revealed as a differential parameter of T1D in a cohort of 152 individuals (LaTorre 2013). Similarly, lower methionine concentration was found in

29 high-risk children before seroconversion compared to healthy age-matched individuals (Jørgenrud 2016). These results were further observed in both prediabetic humans and transgenic mice models (Overgaard 2015 and Fahrmann 2015). It has been hypothesized that low methionine levels may be related to epigenetic reprogramming, suggesting a link to environmental factors triggering T1D (Hewagama 2009). Moreover, methionine has also an impact on the immune system development as it is necessary for both T and B cell proliferation (Chuang 1990). Notwithstanding, it is difficult to screen these biomarkers to the whole population and even found altered, most of them by themselves are unable to predict T1D.

4.3. Biomarkers of β-cell death and stress.

Most of the biomarkers studied until the moment have been detected in screening experiments intended to search for altered biological parameters and their discovery has been narrowly linked to autoimmunity. Besides that, another approach to find novel biomarkers is to look specifically for the triggered event of T1D: the β-cell death.

Insulin is synthesized as preproinsulin and converted to pro-insulin in the ER. Pro-insulin granules mature across the Golgi network resulting and the molecule is excised into insulin and C-peptide, secreted in an equimolar manner (Liu 2015). Hence, the C-peptide levels are clinically measured from blood samples in order to determine the residual β-cell sensitivity after the autoimmune destruction.

The pro-insulin/C-peptide ratio is used to detect the storage of pro-insulin. ER β-cell stress leads to the accumulation of pro-insulin in granules increasing the pro-insulin/C-peptide ratio (Tersey 2012). It can be

measured by ELISA from fresh blood samples in a minimally invasive manner, leading to monitoring T1D progression after the onset (Engin 2016).

Studies have found increased pro-insulin/C-peptide ratios in paediatric subjects 12 months prior to onset, especially in those with less than 10 years of age (Sims 2016). The ratio is increased as well in recent-onset patients (Watkins 2016). Interestingly, the ratio decreases in the PR period (Schölin 2011). The PR —also known as honeymoon phase— is a transient stage after the onset that may last more than 6 months, in which the requirements of daily insulin and glycated haemoglobin (Hba1c) are significantly reduced. A clinical trial consisting of recent onset patients treated with cyclosporine resulted in an insulin-independent PR phase with reduced proinsulin/C-peptide ratios (Snorgaard 1990). Additionally, this ratio is intrinsically related to the β-cell condition, as random analysis of this parameter in cord-blood of siblings of subjects with T1D were similar to controls (Lindgren 1993).

At present, many different sequences of non-coding RNAs have been proposed as biomarkers of β-cell death. That includes both miRNAs and long-non coding RNAs (lncRNAs), involved in the regulation of gene expression.

Due to the length of miRNAs —20-30 nucleotides— none can be considered cell-type-specific but some of them have been found enriched in both human islets and dispersed β-cells (van de Bunt 2013). One of them is miR-375, required for the balance of α and β-cell mass and glucose homeostasis in animal models (Poy 2004 and Erener 2013). Because this molecule is also upregulated in human individuals lacking circulating C-peptide (Latreille 2015) and after islet transplantation (Kanak 2015), it is

suggested that miR-375 upregulation results from β-cell death. Serum sequencing from subjects with recent-onset revealed a dozen of different upregulated miRNAs, some of them also found in β-cells but others not (Nielsen 2012). It still remains to be addressed whether these miRNAs can be used as biomarkers and which is their putative role in the pathophysiology of the disease.

The lncRNAs are molecules consisting of more than 200 nucleotides of length. lncRNAs are non-overlapping coding-sequences involved in the transcriptional, post-transcriptional and epigenetic regulation. The sequences of lncRNAs related to T1D susceptibility have arisen from genome-wide association studies (Todd 2007). Nevertheless, it is difficult to relate lncRNAs to a specific cell type with this kind of data and consequently, it would need further confirmation. Using β-cells obtained from NOD mice there were revealed 4 different lncRNAs sequences that remain upregulated upon the onset of the disease (Motterle 2015).

4.4. Leukocyte cell subsets.

Because immune cells are responsible for autoimmune diseases, they have been considered in many cases as biomarkers of different disease stages. The leukocyte cell subsets comprise a wide range of immune cells, i.e. DCs, monocytes, NK cells and the vast family of B and T lymphocytes (including naïve, effector, memory and regulatory). Different cell subsets have been proposed as biomarkers to track the disease progression of autoimmune diseases such as systemic lupus erythematosus (SLE) (Silva-Neta 2018 and Kim 2017), rheumatoid arthritis (Chen 2019), Graves' disease (Teniente-Serra 2019) and psoriasis (Polat 2017). Moreover, lymphocyte subsets have shown its usefulness as biomarkers to predict

treatment response and effect of immunotherapies in diseases like rheumatoid arthritis (Schreiber 2017). Also, immune cell subsets have been used as biomarkers during the treatment of multiple sclerosis (Teniente-Serra 2017, Quirant-Sánchez 2018 and Villarrubia 2019). In summary, given that T1D relies upon an autoimmunological cause, leukocyte cell subsets —still unexplored in this disease— may be of interest as candidate biomarkers of the different disease stages.

Overall, much more work is needed in order to screen and identify potential biomarkers for T1D. These candidates must fulfill the following criteria: to have a sensitive and reproducible detection method, to be obtained in a minimally invasive manner and to be able to reflect at least one stage of disease progression.

HYPOTHESIS AND AIMS

HYPOTHESIS AND AIMS

1. Hypothesis

At the moment, there are no therapeutic nor preventive therapies available for T1D. For that reason, patients need exogenous insulin administration to survive. However, this treatment does not allow the maintenance of normoglycaemia continuously and often may lead to events of hyper- and hypoglycaemia. Then, patients with T1D display a vast number of associated complications, worsened quality of life and a shortened life-span. The β-cell regeneration has been proposed as a promising strategy to recover endogenous insulin secretion and restore normoglycaemia. Several approaches have been very useful to unravel the cell and molecular mechanisms of reprogramming but lack of direct clinical application. Overall, there is an urgent need for novel therapies allowing the regeneration of endogenous β-cells and arresting autoimmunity.

The **hypothesis** of this work is that a novel regenerative agent can be identified by drug repurposing to promote β-cell regeneration, and thus be used to revert T1D in combination with an immunotherapeutic strategy, previously developed by our group and based in nanomedicine. Additionally, to determine the optimal stage for the administration of this and other therapies, it is also hypothesized that novel biomarkers of immunoregulation can be identified during PR and early stages of human T1D.

2. Aims.

The **main aim** of this PhD thesis is to develop a new strategy for therapeutic purposes in experimental T1D, by combining a novel regenerative agent identified by drug repurposing and an optimized antigen-specific immunotherapy to restore self-tolerance.

To accomplish the main aim, specific **objectives** are:

1. To optimize the immunotherapy based on liposomes to arrest autoimmunity, by broadening the range of β-cell autoantigens encapsulated in liposomes and by determining their effect in experimental T1D.

2. To identify and test an already existing compound able to promote endogenous β-cell regeneration, selected by drug repurposing.

3. To revert experimental T1D by combining the improved liposome-based nanotherapy with the repurposed drug.

4. To evaluate the effects of the combined therapy in human immune cells from patients with T1D.

5. To define new biomarkers of early stages of paediatric T1D aimed to identify an optimal disease stage for immune-intervention.

RESULTS

RESULTS

The results of the present PhD Thesis are reported as a compendium of three original articles already published and a manuscript. Because the data of the *Results III* corresponds to a submitted manuscript (but still not published), the whole manuscript in its current form is presented in an additional chapter named Supplementary Article prior to the Annex section.

First, the work aiming to evaluate the preclinical effect of different PS-liposomes encapsulating β-cell autoantigens in the prevention of experimental T1D is included in the ***Results I*** section, and presented as an original article. The study resulted in the identification of insulin chains A and B, among other autoantigens, as the best choice to be encapsulated in PS-liposomes (PSAB-liposomes) in order to prevent the development of the disease in the NOD mouse model. Indeed, the safety of the therapy was assessed, showing that an intensive dosage administration of PS-liposomes did not reveal toxicity nor secondary effects.

Second, we explored new strategies with the aim to combine the immunotherapy based on PS-liposomes encapsulating insulin peptides with endogenous β-cell regeneration. To that end, a drug repurposing analysis was performed to identify an already existing compound able to promote β-cell regeneration. This work is enclosed in the ***Results II*** section in an article published. The results revealed that liraglutide, an agonist of GLP1 (aGLP1), induces β-cell replacement by both α-to-β cell transdifferentiation and new insulin$^+$ cells generation from the pancreatic ductal cells. These newly formed insulin$^+$ cells may be responsible for partial and transient amelioration of hyperglycaemia in experimental T1D. Overall, this work demonstrates that a pre-existing drug identified by drug

repurposing is able to improve hyperglycaemia by β-cell regeneration in T1D.

Third, and given the potential of liraglutide in inducing β-cell regeneration, this drug was combined with the immunotherapy previously developed by the group and validated in *Results I* section, consisting of PS-liposomes encapsulating insulin peptides (PSAB-liposomes). This work is annexed as part of the ***Results III*** section in a manuscript currently under review. This combined therapy was tested in the spontaneous murine model of autoimmune diabetes, the NOD mouse. The administration of the combined therapy to NOD mice with overt diabetes resulted in the amelioration of hyperglycaemia. To move closer to the clinic, the combined therapy of liraglutide with PSAB-liposomes was also tested in DCs derived from patients with T1D and in human leukocytes by means of humanized mice. It was observed that liraglutide did not alter the tolerogenic potential induced by the immunotherapy nor have a detrimental effect on human T lymphocytes. All together suggests that the combined therapy consisting of PSAB-liposomes as immunotherapy and liraglutide as a regenerative agent has curative potential for T1D.

Fourth, and with the aim to identify an optimal stage of T1D for immune-intervention, a search for new biomarkers at early phases of the disease was performed in paediatric patients. This work, constituting the section ***Results IV,*** is enclosed as an original article. It leads to the identification of quantitative changes in soluble molecules, betatrophin and TGF- β, and regulatory immune cell subsets alterations that point to immunoregulatory attempts in the spontaneous PR stage and early diabetes. These candidate biomarkers could contribute to stratify different early phases of paediatric T1D.

Results I: Preclinical evaluation of antigen-specific nanotherapy based on phosphatidylserine-liposomes for type 1 diabetes.

Adrian Villalba, Silvia Rodriguez-Fernandez, Rosa-Maria Ampudia, Mary Cano-Sarabia, David Perna-Barrull, Cesc Bertran-Cobo, Clara Ehrenberg, Daniel Maspoch and Marta Vives-Pi.

This study is based on a previously immunotherapy developed by our group and consisting of liposomes for tolerance induction in T1D. Liposomes are nanoparticles composed by a lipid bilayer with an aqueous core, able to contain other molecules such as proteins or genes. Large liposomes rich in phosphatidylserine (PS) and encapsulating insulin peptides can prevent T1D in the NOD mice model. PS-liposomes encapsulating insulin peptides (A and B chains) act by mimicking apoptotic β-cells —exposing PS in the membrane— being phagocyted by DCs through a process called efferocytosis. This mechanism allows the induction of self-tolerance to β-cells through dendritic cells.

In this publication, the aim was to expand the range of peptide-loaded PS-liposomes considering other T1D autoantigens such as GAD65, IA2 and C-peptide or a mix of liposomes containing the different molecules (Cock-lipos). The results presented in this article showed that when administered to prediabetic NOD mice at 8 weeks of age (i.p., one dose), PS-liposomes containing insulin peptides were the best option in reducing the incidence of T1D among the other tested autoantigens (33.3% of incidence when compared to 66.6% of the sham group). Furthermore, only a mix of all the non-encapsulated peptides was able to reduce the incidence (42.8%) in

comparison to the sham group (Cock-pep). Both treatments reduced insulitis score and maintained a high percentage of islets with benign insulitis.

Finally, it was observed that PS-liposome administration did not show a toxic effect nor secondary complications after a daily dose of 3.5mg (30 mM-lipid) PS-liposomes during 14 consecutive days. Overall, this work confirms that PS-liposomes encapsulating insulin peptides constitute a safe immunotherapy to arrest the autoimmunity against β-cells in the experimental model of T1D, the NOD mice.

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Preclinical evaluation of antigen-specific nanotherapy based on phosphatidylserine-liposomes for type 1 diabetes

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ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disease caused by the destruction of insulin-producing cells. Due to the ability of apoptotic cells clearance to induce tolerance, we previously generated liposomes rich in phophatidylserine (PS) –a feature of apoptotic cells– loaded with insulin peptides to mimic apoptotic beta-cells. PS-liposomes arrested autoimmunity in experimental T1D through the induction of tolerance. The aim of this study was to investigate the potential of several peptides from different T1D autoantigens encapsulated in (PS)-liposomes for T1D prevention and to assess its safety. T1D autoantigens (Insulin, C-peptide, GAD65 and IA2) were encapsulated in PS-liposomes. Liposomes were administered to the 'gold-standard' model for the study of autoimmune T1D, the Non-Obese Diabetic mouse, that spontaneously develop the disease. Safety and toxicity of liposomes were also determined. Only PS-liposomes encapsulating insulin peptides decrease T1D incidence in the Non-Obese Diabetic mouse model. Disease prevention correlates with a decrease in the severity of the autoimmune islet destruction driven by leukocytes. PS-liposomes neither showed toxic effect nor secondary complications. Among the here referred autoantigens, insulin peptides are the best candidates to be encapsulated in liposomes, like an artificial apoptotic cell, for the arrest of autoimmunity in T1D in a safe manner.

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Nanovesicles; immunotherapy; autoimmunity

Introduction

An essential goal to both prevent and cure type 1 diabetes (T1D) is to arrest the autoimmune reaction against β-cells. Insulin is a key autoantigen in T1D and the only β-cell-specific autoantigen, as well as a major target of the autoimmune attack mediated by T lymphocytes [1]. Moreover, there is also a genetic association between polymorphisms of the insulin gene and the risk to develop T1D [2]. However, in addition to insulin, other autoantigens such as the glutamic acid decarboxylase 65 (GAD65), islet-antigen 2 (IA2) and zinc transporter 8 (ZnT8) have been described in T1D. They are known to induce autoantibodies and T cell responses. Autoantibodies against insulin or GAD isoforms are the most common in the early phases of T1D and, occasionally, multiple auto-antibodies arise during advanced stages of the pathology [3]. Although insulin is a primary autoantigen in T1D, other autoantigens are involved in the progression of the disease after epitope spreading [4].

Furthermore, in order to arrest the autoimmune reaction against β-cells, immunological tolerance to T1D autoantigens must be restored. A process known to promote tolerance is efferocytosis, or phagocytosis of apoptotic cells. These dying cells exhibit surface phosphatidylserine (PS) to be distinguished from living cells and to allow for their recognition by phagocytes [5,6]. Following these principles, an antigen-specific cell immunotherapy using apoptotic β-cells was demonstrated efficacious in re-establishing specific immunological tolerance, which was mediated by dendritic cells (DCs) rendered tolerogenic after efferocytosis [7]. Because autologous apoptotic β-cells for T1D treatment cannot be easily obtained, artificial cells (PS-liposomes) were generated to mimic tolerogenic apoptotic cell function [8]. A previous work demonstrated the therapeutic potential of PS-liposomes encapsulating insulin peptides – as a main autoantigen in T1D – to prevent T1D in an experimental model of the disease, the Non-Obese Diabetic (NOD) mouse [8]. The NOD mouse, developed in 1980 [9] is considered the 'gold-standard' model for the study of T1D [10]. These mice develop T1D spontaneously around 12 weeks of age, after the immune-mediated destruction of insulin-producing β cells, commonly called insulitis. NOD mice have been extensively utilised to study the ethiopathogenesis of the disease and to test new immunotherapies. This model mouse closely

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resembles human disease, albeit the obvious differences with human populations.

In order to better mimic the apoptotic β-cell, and to try to increase the efficacy of the nanotherapy, the present study aimed at encapsulating other peptides from several T1D autoantigens in PS-liposomes and to test its therapeutic effect in arresting autoimmunity. In addition, and in order to confirm the safety of the nanotherapy, the effects of a severe administration strategy were assessed in NOD mice.

Material and methods

Mice

Wild-type non-obese diabetic (NOD) mice were purchased from Charles River (Calco, Lecco, Italy) and kept under specific pathogen-free conditions at the Animal House facility of the Germans Trias i Pujol Research Institute. Mice were in a temperature- and humidity-controlled room with 12 h light/12h dark cycle, provided with standard chow diet (Teklad Global 14% Protein Rodent Diet, Envigo, Sant Feliu de Codines, Spain) and water *ad libitum*. To detect spontaneous development of T1D, NOD mice were monitored daily for urine glucose using Glucocard strips (Menarini, Barcelona, Spain), starting at 10 weeks of age and until 25 weeks of age. Mice with glycosuria were confirmed diabetic when the blood glucose level was >300 mg/dL. Mice were anesthetised with isofluorane inhalation to obtain serum and euthanized by cervical dislocation to harvest the pancreas at 25 weeks of age or at T1D onset. The study was approved by the Committee on the Ethics of Animal Experimentation of the Germans Trias i Pujol Research Institute and carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Generalitat de Catalunya, Catalan Government.

Peptides and liposomes

Peptides were selected because they are target epitopes in T1D [4]. Peptides from Insulin, C-peptide, GAD65 and IA2 (Table 1) were chosen (GL Biochem, Shanghai, China). Peptides were >95% pure and Trifluoroacetic acid was removed. Liposomes were composed of 1,2-dioleoyl-sn-glycero3-phospho-L-serine (sodium salt) (DOPS, Lipoid, Steinhausen, Switzerland) and 1,2-didodecanoyl-sn-glycero-3-phosphocholine (DLPC, Lipoid), and cholesterol (CH, Sigma Aldrich, MO, USA). Liposomes were prepared using the thin-film hydration method from a lipid mixture, to mimic apoptotic bodies as described, under sterile conditions and at a final concentration of 30 mM [11]. Encapsulation efficiencies (EE) were calculated according to the equation EE (%) = [(peptide, total–peptide, out)/peptide, total] × 100, where peptide, total is the initial peptide concentration and peptide, out is the concentration of non-encapsulated peptide. To measure the peptide, out, liposome suspensions were centrifuged at 110.000 × G at 10 °C for 30 min. The concentration of non-encapsulated peptide was assessed in the supernatants by PIERCE BCA protein assay kit (Thermo Fisher Scientific Inc., IL, USA). For toxicity experiments, empty PS-liposomes were generated. The distribution of the particle size and the stability – given as ζ potential – were measured by dynamic light scattering using Malvern Zetasizer (Malvern Instruments, Malvern, UK) in undiluted samples. The morphology of liposomes was examined by cryogenic transmission electron microscopy with a JEOL-JEM 1400 device (Jeol Ltd., Tokyo, Japan).

T1D prevention and assessment of islet leukocytic infiltration

Normoglycemic 8-week-old NOD mice were treated with a single dose of liposomes. Briefly, 3.5 mg of the different peptide-filled PS-liposomes in 200 μL saline solution were injected i.p. Thus, PSAB-lipo group received 200 μL of a mix of PSA and PSB liposomes, PSGAD65-lipo group received 200 μL of a mix of PSG_1 and PSG_2 liposomes, PSIA2-lipo group received 200 μL of PSI_1 and PSI_2 liposomes and PSCpep-lipo group received 200 μL of PSC liposomes. A group containing 200 μL of a mix of all the different peptide-loaded PS-liposomes (Cock-lipo) was included, in which each liposome fraction was present in an equal amount. A group containing the mix of all the different peptides (200 μL) was also included (Cock-Pep), the amount of peptide given being in accordance to encapsulation efficiency inside the liposomes. Sham group received saline solution. Each group contained 5–9 animals. Mice were monitored daily for glycosuria (Glucocard strips, Menarini, Barcelona, Spain) until 25 weeks of age or detection of T1D. To determine the destructive islet leukocytic infiltration, insulitis score was assessed at 25 weeks in all non-diabetic mice. Pancreases were snap frozen in an isopentane/cold acetone bath. Non-overlapping cryosections (5 μm) were stained with haematoxylin and eosin (H/E). A blind analysis was performed by two independent observers, analysing a minimum of 40 islets per animal. Insulitis was scored as previously described: 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 [12].

Table 1. Sequences of peptides encapsulated in PS-liposomes.

Peptide	Sequence (N′–C′)	Length Aminoacids
$C\text{-peptide}_{57-87}$	EVEDPQVAQLELGGGPGAGDLQTLALEVAQQ	31
$GAD65_{216-236}$	EYVTLKKMREIIGWPGGSGD	20
$GAD65_{290-309}$	AALGIGTDSVILIKCDERGK	20
$IA2_{816-846}$	GVKQCDRYWPDEGSSLYHVYEVNLVSEHIWC	31
$IA2_{870-898}$	FHFLSWPAEGTPASTRPLLDFRRKVNKCY	29
$Insulin_{90-110}$ (A chain)	GIVDQCCTSICSLYQLENYCN	21
$Insulin_{25-54}$ (B chain)	FVKQHLCGSHLVEALYLVCGERGFFYTPMS	30

Subindex refers to the position of the query sequence to the protein.
GAD65: glutamic acid decarboxylase 65 ; IA2: islet-antigen 2.

Table 2. Features of the liposomes used in the study.

Liposome	Encapsulated peptide	Diameter (nm)	Polydispersity index	ζ-potential (mV)	Encapsulation efficiency (%)
PSI_1	$IA2_{816-846}$	459	0.236	−30.8	98.69
PSI_2	$IA2_{870-898}$	436	0.275	−31.4	72.61
PSG_1	$GAD65_{216-236}$	618	1.000	−36.7	39.20
PSG_2	$GAD65_{290-309}$	440	0.285	−32	31.00
PSC	C $peptide_{57-87}$	437	0.126	−34	16.80
PSA	$Insulin_{90-110}$ (A chain)	712	0.364	−46.6	32.29
PSB	$Insulin_{25-54}$ (B chain)	628	0.325	−44.9	89.63
PS	Empty	645	0.638	−35.9	–

Subindex of peptides refers to the position of the query sequence to the protein.
PS: Phosphatidylserine-rich liposomes; GAD65: glutamic acid decarboxylase 65 ; IA2: islet-antigen 2.

Evaluation of the toxicity of PS-liposomes

In order to determine the safety of PS-liposomes, a severe administration strategy was tested in NOD mice. Six normoglycemic mice (8–12-week-old, 3 males and 3 females) were daily injected i.p. with 200 μL of 3.5 mg 30 mM-lipid PS-liposomes during 14 consecutive days. Simultaneously, PBS administration was used as control (sham) in 6 mice (3 males and 3 females). Mice health and welfare was monitored (Supplementary Table S1) during 21 days in terms of weight, glucosuria, physical appearance (fur, presence of secretions or bruises) and behaviour (barbering, physical activity, aggressiveness, stereotyped activities and pain). Mice were euthanised at the end of the follow-up or if the total score reached a maximum value of 3. Serum from 4 mice of each group was analysed for biochemical parameters, such as total cholesterol (Total CH), high-density lipoprotein (HDL) CH, triglycerides, creatinine, urea and alanine transferase (ALT).

Statistical analysis

The statistical analysis was performed using the Prism 7.0 software (GraphPad software Inc., CA, USA). Mantel-Cox Log-Rank was used to T1D incidence analysis. Mann–Whitney test was used for comparisons of nonparametric and unpaired data. A p-value $\leq .05$ was considered significant.

Results

PS-liposomes encapsulating insulin, but not C-peptide, GAD65 or IA2 peptides decrease T1D incidence

Liposomes were characterised in terms of diameter, polydispersity index (PdI), surface charge (ζ-potential) and efficiency of peptide encapsulation (Table 2). All liposomes had a final lipid concentration of 30 mM. NOD mice were treated with a single dose of liposomes before T1D onset (8 weeks old) (Table 3). At the end of the follow-up period, T1D incidence tended to be lower in both PSAB-lipo and Cock-Pep groups (33.3% and 42.8%, respectively) when compared with the sham group (66.6%) (Figure 1). Hence, and as expected, a 50%-fold reduction in T1D incidence was achieved in the PSAB-lipo group. The groups treated with liposomes encapsulating IA2, GAD65 or C-peptide did not show any reduction in the incidence of the disease (80%, 65% and 83.3% respectively) when compared with sham-treated animals (Figure 1). Moreover, the group treated with the mix of the peptide-filled liposomes did not show a decrease in the incidence either (80%) (Figure 1). In order to confirm that mice at the end of follow-up did not develop T1D, urine glucose concentration (glycosuria) and serum C-peptide concentration –as indicators of β-cell function– were assessed. All mice showed negative glycosuria and detectable C-peptide concentration (range 900–3000 pg/mL). No statistical differences were found between groups (data not shown).

Table 3. Amount of peptide encapsulated in a dose (200 μL) of PS-liposomes (3.5 mg lipid).

Treatment	Peptide	Peptide per dose (μg)
PSG	$GAD65_{216-236}$	39,2
	$GAD65_{290-309}$	31
PSI	$IA2_{816-846}$	98,69
	$IA2_{870-898}$	72,61
PSC	C-$peptide_{57-87}$	16,8
PSAB	Insulin (A-chain)$_{90-110}$	32,29
	Insulin (B chain)$_{25-54}$	89,63
Cock-Pep	$GAD65_{216-236}$	14,28
	$GAD65_{290-309}$	14,28
	$IA2_{816-846}$	14,28
	$IA2_{870-898}$	14,28
	C-$peptide_{57-87}$	14,28
	$Insulin_{90-110}$	14,28
	$Insulin_{25-54}$	14,28
Cock-lipo	$GAD65_{216-236}$	5,59
	$GAD65_{290-309}$	4,42
	$IA2_{816-846}$	14,09
	$IA2_{870-898}$	10,37
	C-$peptide_{57-87}$	2,40
	$Insulin_{90-110}$	4,61
	$Insulin_{25-54}$	13,26

Cock-Pep: mixture of all non-encapsulated peptides; Cock-lipo: mixture of all PS-liposomes encapsulating different peptides.

PS-liposomes encapsulating insulin peptides reduce the severity of the autoimmune islet destruction

Given the reduced incidence of T1D in both PSAB-lipo and Pep-Cock treated mice, islet leukocytic infiltration was determined at the end of the follow-up period showing a biological reduction of insulitis, although non-significant, in both treated groups (PSAB-lipo: 1.45 ± 0.57 and Cock-Pep: 1.32 ± 0.34) when compared to sham mice (1.85 ± 0.60) (Figure 2(A)). Furthermore, the analysis of the percentages of islets classified in each infiltration category revealed that in both treated groups more than 64% of the islets remained insulitis-free (type 0) or with benign insulitis (type 1); in the control group, 48% of the islets were scored with destructive insulitis (type 2, 3 and 4) (Figure 2(B,C)).

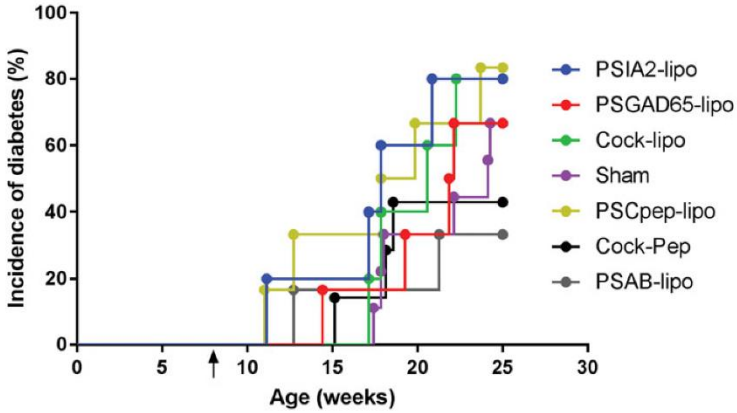

Figure 1. T1D incidence in NOD mice treated with PS-liposomes filled with different autoantigenic peptides. Cumulative incidence (percentage) of T1D in NOD mice treated with IA2 liposomes (PSIA2-lipo, blue, $n = 5$), GAD65 liposomes (PSGAD65-lipo, red, $n = 6$), C-peptide liposomes (PSCpep-lipo, yellow, $n = 6$), insulin liposomes (PSAB-lipo, grey, $n = 6$), a mixture of all liposomes (Cock-lipo, green, $n = 5$) and a mixture of all non-encapsulated peptides (Cock-Pep, black, $n = 7$). A sham group was also included (violet, $n = 9$). The arrow indicates the time-point of the single-dose administration (at 8 weeks). No significant differences in T1D incidence were found between groups (Mantel-Cox Log-Rank). PSIA2-lipo: IA2 liposomes; PSGAD65-lipo: GAD65 liposomes; Cock-lipo: a mixture of all liposomes; PSCpep-lipo: C-peptide liposomes; Cock-Pep: a mixture of all non-encapsulated peptides; PSAB-lipo: insulin liposomes.

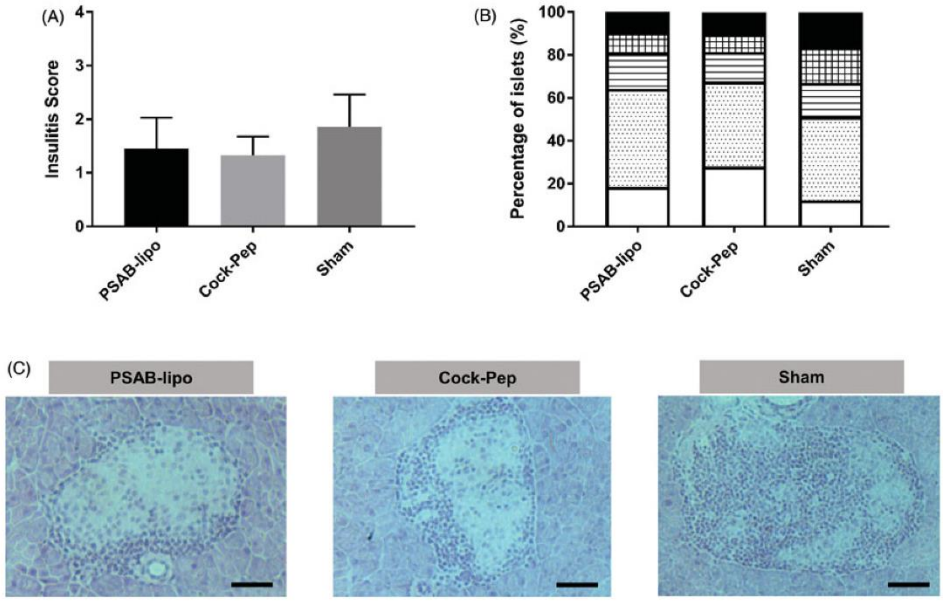

Figure 2. Islet leukocytic infiltrate (insulitis) following administration of nanotherapy. (A) Insulitis score from non-diabetic mice at the end of the follow-up period at 25 weeks, including the PSAB-lipo ($n = 3$), Cock-Pep ($n = 3$) and sham ($n = 3$) groups. Results are mean ± Standard Deviation (SD). No statistical differences were found between groups (Mann-Whitney test). (B) Percentage of islets in each of the infiltration categories: White = 0, no insulitis; Dotted = 1, peri-insular; Striped = 2, mild insulitis (<25% of the infiltrated islet); Squared = 3, 25–75% of the islet infiltrated; Black = 4, >75% islet infiltration. (C) Representative images of insulitis in islets from H/E-pancreatic cryostat sections (5 μm) from the three different groups. Scale bar represents 100 μm. PSAB-lipo: insulin PS-liposomes; Cock-Pep: mixture of all non-encapsulated peptides.

PS-liposomes treatment demonstrated optimal safety and tolerability in NOD mice

The observational study, taking into account the criteria used in welfare monitoring of mice used for toxicity and tolerability assays (Supplementary Table S1), demonstrated the safety of daily PS-liposomes administration in NOD mice. Only mild complications were observed both in daily PS-liposomes-treated and sham group (Table 4) and no animal had to be euthanised due to severe complications of the treatment. All mice were negative for glucosuria during the 14 days of treatment and 7 days after withdrawal. The analysis of the serum showed no significant differences between PS-liposomes and

sham groups at the end of the study (Figure 3). Total CH, HDL CH, triglycerides and ALT concentrations remained unaltered and within reference values for NOD mice [13]. Urea concentration in both the groups was found above reference values. Creatinine concentration was similar in both groups, but reference values were not available.

Discussion

This study shows that the administration of PS-liposomes encapsulating insulin peptides is the best strategy to reduce the incidence of T1D, spontaneously developed in NOD mice, when compared to other encapsulated autoantigen peptides (GAD65, IA2 and C-peptide). Liposomes are vesicles composed of lipid bilayers that can be used as a vehicle for drug delivery. Considering that the physicochemical features of the liposomes are homogeneous, and that empty liposomes have no effect on T1D and other autoimmune diseases [8,14],

Table 4. NOD mice daily treated with PS-liposomes (14 days) with disturbances.

	Mice with disturbances	
Parameter	Sham	PS-liposomes
Loss of 10% of body weight	1/6 (16%)	2/6 (33%)
Piloerection	0/6 (0%)	1/6 (16%)
Mild bruises on the abdomen	1/6 (16%)	0/6 (0%)
Mild bruises on the tail	1/6 (16%)	0/6 (0%)
Mild aggressive behaviour	0/6 (0%)	1/6 (16%)

Data presented as number of mice with disturbances in each group and percentage (%).

the differences here reported can be due to the biological features of the encapsulated autoantigens. The role of the different autoantigens in the stages of autoimmunity in T1D is also relevant. On the one hand, insulin is the most expressed specific autoantigen in the target β-cells on which the autoimmune reaction is focussed and, on the other hand, single positivity for autoantibodies against insulin but not for the rest of autoantigens is associated with the greatest risk to develop T1D [15]. Moreover, it has been described that insulin is an early autoantigen in T1D whereas the other autoantigens seem to be the consequence of epitope spreading, after the activation of autoimmunity [15]. IA2 expression is not exclusive of pancreatic β-cells in humans nor rodents, being found expressed also in many neuroendocrine cells [16–18]. This lack of specificity would explain the unsuccessful therapeutic use by its single encapsulation in PS-liposomes. GAD65 expression is also present in other tissues due to its role in the biosynthesis of gamma-aminobutyric acid (GABA) like GABAergic cells [19]. Furthermore, it has been recently reported that GAD65 has no capacity to prevent T1D in wild type NOD mice [20]. Finally, the role of C-peptide as autoantigen in T1D remained controversial despite recent work hints at its action as an autoantigen [21–23]. This work validates the use of PS-liposomes encapsulating insulin peptides to prevent the development of autoimmune T1D and tends to reduce the insulitis in the experimental NOD model [8].

A different encapsulation efficiency has been observed for the different peptides and this fact must be taken into

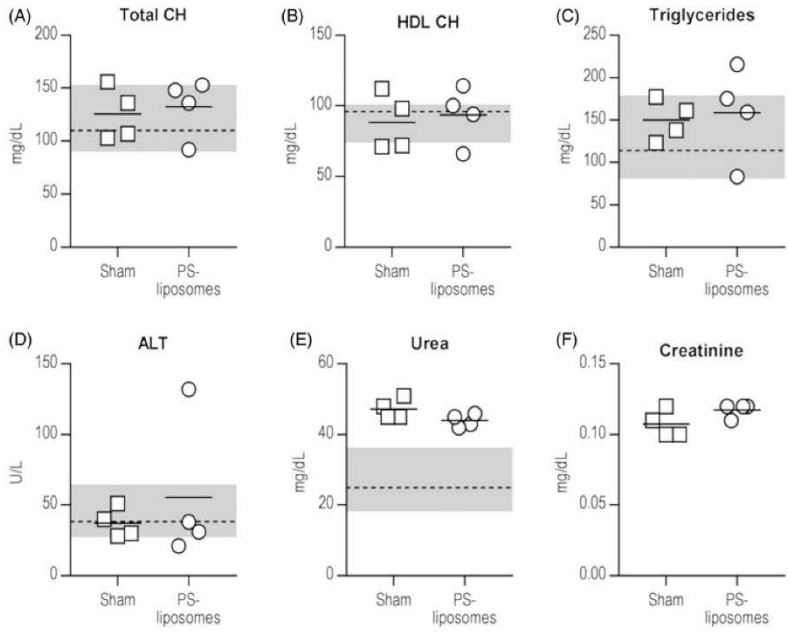

Figure 3. Effect of daily administration of PS-liposomes on blood biochemical parameters of NOD mice at the end of the study (Day 21). *In vivo* toxicity assay by measuring the levels of (A) Total CH, mg/dL, (B) HDL CH, mg/dL), (C) triglycerides (mg/dL), (D) ALT, U/L, (E) urea, mg/dL and (F) creatinine, mg/dL in serum of PBS-treated mice ($n = 4$, squares) and PS-liposomes-treated mice ($n = 4$, circles), at the end of the study (Day 21). Continuous lines represent the mean of each group. The grey area depicts normal ranges of parameters and the median (discontinuous line) of the different analytes for NOD mice. Significant differences were not found between groups (Mann-Whitney test). CH: cholesterol; ALT: Alanine transferase.

account to both understand the results in this study and to design future nanotherapies. The lowest percentage of encapsulation corresponds to liposomes encapsulating C-peptide and, as mentioned above, the role of this molecule as autoantigen remains unclear. Both facts may be the reason of the lack of effect in T1D prevention. Moreover, the mixture of liposomes (Cock-lipo) contains a 7-fold reduction of the total insulin peptide amount carried in the PSAB-lipo, resulting in a loss of therapeutic action.

Apart from the nanotherapy described here, a mixture of the selected autoantigens (Cock-Pep) was found to reduce the incidence of the disease when compared with the sham group but with a minor effect than the observed with PS-liposomes encapsulating insulin. The synergistic effect of PS in the liposomes and the encapsulated peptide has been described in previous work showing a tolerogenic effect mediated by DCs [8]. Many therapies have been tested both for prevention and treatment of T1D in the NOD mice based on peptides [24]. The here reported effect of combined peptides fits well with previous data showing a preventive effect of T1D after peptide administration of insulin, GAD65 and IA2 in NOD mice and in other autoimmune models [25,26].

This works shows that the choice of autoantigen and derived peptides is key in the design of vesicles for T1D immunotherapies. Despite that, questions still remain about the therapeutic contribution of each peptide and about the molecular mechanisms involved in their immune-recognition. Of note, the timing of administration of nanotherapies to arrest spontaneous autoimmunity in T1D plays an essential role in the success of the immunotherapy. Moreover, the best peptide option for encapsulation in nanovesicles will probably depend not only on the checkpoint of intervention but also on the immunophenotype (specific autoantibodies and autoreactive T cells) of each patient.

A vital issue for the successful implementation of the PS-liposomes immunotherapy in the clinics is its potential toxicity and tolerability. The daily injections of PS-liposomes during two weeks, revealed a well-tolerated and safe profile in NOD mice. The minor complications found among both groups are probably due to the stress of the injections the mice received daily. Also, the loss of 10% of body weight reported in mice in both the groups suggests that these mice were on the road to developing diabetes. The analysis of the serum confirmed the safety of the treatment.

We are fully aware that they are preliminary results and that a low number of mice have been analysed. Further studies are required to validate the tendencies observed in a larger group of mice and over a longer period of time, and to determine the effect of the therapy in several tissues by histological analyses. However, the effect of only one dose of PS-liposomes encapsulating autoantigens is clear and resulted in T1D prevention without side-effects, providing a simple and innovative system to recover self-tolerance.

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Disclosure statement

MVP, MCS and DM hold patents that relate to liposomes and are co-founder of Ahead Therapeutics S.L. The other authors report no conflicts of interest in this work.

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Results II: Repurposed analog of GLP-1 ameliorates hyperglycaemia in type 1 diabetic mice through pancreatic cell reprogramming.

Adrian Villalba, Silvia Rodriguez-Fernandez, David Perna-Barrull, Rosa-Maria Ampudia, Laia Gomez-Muñoz, Irma Pujol-Autonell, Eva Aguilera, Mireia Coma, Mary Cano-Sarabia, Federico Vazquez, Joan Verdaguer and Marta Vives-Pi.

Drug repurposing is an in silico technique intended to search for non-canonical uses of already-approved compounds. This work aims to identify a new commercially-available β-cell regenerative compound for its use in T1D. For that reason, a drug-repositioning analysis was performed to screen existing drugs that could putatively promote β-cell regeneration by one or more of the following mechanisms: neogenesis from ductal progenitors, transdifferentiation of glucagon-producing α -cells and/or replication of pre-existing β-cells.

The search resulted in the identification of liraglutide, an agonist of Glucagon-like Peptide 1 (aGLP1), among 6.605 registered compounds. To assess its effect in promoting β-cell recovery, liraglutide was administered for 30 days to NSG (NOD.SCID IL2Rɣ$^{-/-}$) mice rendered diabetic through STZ injection. Treated mice showed a significant amelioration of the hyperglycaemia. This phenomenon was not further observed after the withdrawal of liraglutide. Histological analysis of the pancreas of treated mice revealed that liraglutide was able to induce almost a 10% of bihormonal cells (insulin^{+}glucagon^{+}) of the total insulin^{+} cells after 48 hours of treatment, but not longer. This fact correlated well with an increase in the expression of insulin and glucagon genes in islets co-cultured with

liraglutide for 48 hours. Furthermore, it was also detected a partial and transient increase in the β-cell mass after 2 weeks of daily treatment due to a boost in the β-cell number. Moreover, neo-islets emerging from the ducts were detected both at 2 weeks of *liraglutide* administration and after its removal and were formed by $CK19^{+}insulin^{+}$ cells (and were ~ 50% of total $CK19^{+}$ ductal areas). Additionally, within these $CK19^{+}insulin^{+}$ ductal cells, a subpopulation of cells expressing PDX1 was observed. Overall, these data indicate that liraglutide can ameliorate hyperglycaemia in an experimental mice model of T1D through cell reprogramming.

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ORIGINAL RESEARCH
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Repurposed Analog of GLP-1 Ameliorates Hyperglycemia in Type 1 Diabetic Mice Through Pancreatic Cell Reprogramming

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Type 1 diabetes is an autoimmune disease caused by the destruction of the insulin-producing β-cells. An ideal immunotherapy should combine the blockade of the autoimmune response with the recovery of functional target cell mass. With the aim to develop new therapies for type 1 diabetes that could contribute to β-cell mass restoration, a drug repositioning analysis based on systems biology was performed to identify the β-cell regenerative potential of commercially available compounds. Drug repositioning is a strategy used for identifying new uses for approved drugs that are outside the scope of the medical indication. A list of 28 non-synonymous repurposed drug candidates was obtained, and 16 were selected as diabetes mellitus type 1 treatment candidates regarding pancreatic β-cell regeneration. Drugs with poor safety profile were further filtered out. Lastly, we selected liraglutide for its predictive efficacy values for neogenesis, transdifferentiation of α-cells, and/or replication of pre-existing β-cells. Liraglutide is an analog of glucagon-like peptide-1, a drug used in patients with type 2 diabetes. Liraglutide was tested in immunodeficient NOD-*Scid IL2rg*$^{-/-}$ (NSG) mice with type 1 diabetes. Liraglutide significantly improved the blood glucose levels in diabetic NSG mice. During the treatment, a significant increase in β-cell mass was observed due to a boost in β-cell number. Both parameters were reduced after withdrawal. Interestingly, islet bihormonal glucagon^{+}insulin^{+} cells and insulin^{+} ductal cells arose during treatment. *In vitro* experiments showed an increase of insulin and glucagon gene expression in islets cultured with liraglutide in normoglycemia conditions. These results point to β-cell replacement, including transdifferentiation and neogenesis, as aiding factors and support the role of liraglutide in β-cell mass restoration in type 1 diabetes. Understanding the mechanism of action of this drug could have potential clinical relevance in this autoimmune disease.

Keywords: beta cell regeneration, neogenesis, transdifferentiation, liraglutide, drug repositioning

INTRODUCTION

An essential requirement to cure type 1 diabetes is the recovery of β-cells lost after the autoimmune destruction. The pancreas can restore β-cells from different sources, after injury (1) or drug administration (2, 3) in mice, and upon pathophysiological conditions in humans (4). With the aim to develop new therapies for β-cell replacement, a repositioning analysis based on systems biology was performed to identify the regenerative potential of commercially available compounds.

Drug repositioning is a strategy for identifying new uses for approved drugs that are outside the scope of the medical indication. This approach uses a bioinformatics tool, based on networks of drugs, proteins, and diseases, which screens approved compounds that can be repurposed for other diseases (5). This has resulted in successful drug discovery for diseases (6) such as cancer (7) and Alzheimer's disease (8). Given that, we aimed to look for drugs that can induce β-cell replacement through α-cell to β-cell transdifferentiation (9), neogenesis from multipotent ductal progenitors (1), and/or replication of pre-existing β-cells (10). This resulted in the identification of liraglutide—an analog of glucagon-like peptide-1 (aGLP-1), a drug used in patients with type 2 diabetes (11), especially those with obesity (12).

GLP-1 is produced in the gut after food intake and acts by increasing insulin release. Liraglutide ameliorates insulin resistance in type 2 diabetes models (13, 14). Recently, GLP-1 has been shown to promote transdifferentiation from α-cells to β-cells (15, 16).

We report here that liraglutide ameliorates hyperglycemia in mice with type 1 diabetes by inducing insulin$^+$glucagon$^+$ cells and insulin-producing cells from the pancreatic ducts. This is the first description of an approved drug—identified by a repositioning—that promotes the generation of insulin-expressing cells from pancreatic ducts and ameliorates hyperglycemia in experimental type 1 diabetes.

MATERIALS AND METHODS

Systems Biology Analysis for Drug Discovery and Repositioning Analysis

The therapeutic performance mapping system is a top-down systems biology approach based on artificial intelligence and pattern recognition that integrates all available pharmacological knowledge to create mathematical models that simulate human pathophysiology *in silico*. The methodology employed has been previously described (17) and applied elsewhere (18, 19).

A manually curated list of proteins known to be involved in the mechanisms of β-cell regeneration—α-cell to β-cell transdifferentiation, neogenesis from ductal precursors, and β-cell replication—was obtained (**Supplementary Table 1**) and used for focusing the analysis toward β-cell regeneration in a human biological network context.

The human biological network created incorporated the available relationships (edges or links) between proteins (nodes) from a regularly updated in-house database drawn from public sources: KEGG (20, 21), REACTOME (22), INTACT (23), BIOGRID (24), HPRD (25), and TRRUST (26). All information of the key proteins defined during the molecular and the biological characterization and stored in relevant databases (drug targets, other diseases effectors, biomarkers…) was incorporated into the biological networks (27).

Artificial neural networks (ANNs) are supervised algorithms that identify relations between proteins (e.g., drug targets) and clinical elements of the network (18, 19) by inferring the probability of the existence of a specific relationship between two or more protein sets. This is based on a validation of the predictive capacity of the model toward the truth table, a selected collection of known input (drug targets)–output (indications) relationships defined through specific scientific literature search and hand-curated assignment of proteins to the conditions included in the biological effector database (17). The learning methodology used consisted of an architecture of stratified ensembles of neural networks as a model, trained with a gradient descent algorithm to approximate the values of the given truth table. The neural network model used was a multilayer perceptron (MLP) neural network classifier. The MLP gradient descent training depends on randomization initialization and, to avoid random errors, 1,000 MLPs are trained with the training subset and the best 100 MLPs are used. In order to correctly predict the effect of a drug independently of the number of targets, different ensembles of neural networks are trained for different subsets of drugs according to their number of targets (drugs with one target, two targets, three targets…). Then, the predictions for a query drug are calculated by all the ensembles and pondered according to the number of targets of the query drug (the difference between the number of targets of the query and the number of targets of the drugs used to calculate each ensemble is used to ponder the result of each ensemble). A cross-validation with the truth table information showed that the accuracy of the described ANNs to reproduce the indications compiled in DrugBank (28, 29) is 81.23% for those drugs with all targets in the human biological network. ANNs were used to screen the predicted relationship of 6,605 different drugs toward the β-cell regeneration molecular definition. The repurposed drug candidates were sorted and selected by its relationship with β-cell regeneration mechanisms.

Mice and Diabetes Induction

NOD mice, immunodeficient NOD.SCID-IL2Rγ$^{-/-}$ (NSG) mice, and C3HeB/FeJ mice were bred in our own facility. The NOD and NSG mice were kept under specific pathogen-free conditions. The NSG model was selected for its lack of an adaptive immune system, resulting in the absence of autoimmunity, and allowing to determine the raw effect of the drug. Type 1 diabetes was induced in NSG mice at 8–14 weeks of age by a single i.p. injection of streptozotocin (STZ, 150 mg/kg)

Abbreviations: ANN, artificial neural network; AUC, area under the curve; aGLP-1, analog glucagon-like peptide-1; NSG, immunodeficient NOD.SCID-IL2Rγ$^{-/-}$ mice; IPGTT, intraperitoneal glucose tolerance test; ITT, insulin tolerance test; STZ, streptozotocin.

(Sigma-Aldrich). Type 1 diabetes was confirmed at 48–72 h post-STZ injection, after either two successive 2-h fasting blood glucose levels higher than 250 mg/dl or with one higher than 300 mg/dl. The mice were euthanized by cervical dislocation. All animal studies were approved by the institutional animal ethics committee.

Treatment With aGLP-1

Immediately after type 1 diabetes diagnosis, the NSG mice (n = 12 mice/group) were treated with liraglutide (Victoza®, Novo Nordisk A/S), injected (s.c.) daily up to 30 days, following the dosage of 0.3 mg/kg at day 1, 0.6 mg/kg at day 2, and 1 mg/kg from day 3 onwards as described (30). After the withdrawal of the liraglutide treatment, the mice were maintained for 5 days. The control group (n = 6 mice) received phosphate-buffered saline (PBS). Blood glucose was determined twice weekly, after 2 h of fasting, throughout the study.

Intraperitoneal Glucose Tolerance Test and Insulin Tolerance Test

Intraperitoneal glucose tolerance test (IPGTT) was performed in fasting conditions in the three groups: (1) diabetic NSG mice responding to liraglutide after 15 days of treatment (Lira, n = 3), (2) untreated diabetic and hyperglycemic NSG mice (T1D, n = 3), and (3) healthy and normoglycemic NSG mice (sham, n = 3). At point 0, basal glucose level was determined. The mice were subsequently given an i.p. injection of 2 mg of glucose (Sigma-Aldrich) per gram of body weight and glycemia was measured after 15, 30, 60, 120, and 210 min. Insulin tolerance test (ITT) was performed in fasting conditions in 8-week-old and normoglycemic NOD mice and C3HeB/FeJ mice injected s.c. with insulin (0.5 U/kg, n = 3) or liraglutide (1 mg/kg, n = 3). Glycemia was determined after 15, 30, and 60 min.

Immunofluorescence Staining and Histometric Analysis

Immunofluorescence staining was performed to identify pancreatic insulin-producing cells in a minimum of three mice per condition. Briefly, the pancreas were harvested and snap-frozen in an isopentane/cold acetone bath. A minimum of eight cryostat sections (5 µm) from every organ were sequentially stained by indirect immunofluorescence with antibodies to insulin, glucagon, CK19 (Sigma-Aldrich), or Pdx1 (Abcam) and FITC- or TRITC-labeled secondary antibodies (Sigma-Aldrich) as described (31). The nuclei were stained with Hoechst (Invitrogen). The samples were observed in a fluorescence microscope and analyzed (ImageJ Software) (32). For histometric analysis, six mice per group were used. To determine the β-cell counts, one section every 150 µm of tissue was sampled as described (33), resulting in 12–16 sections per pancreas. The β-cell mass was calculated by multiplying the relative insulin$^+$ area per total pancreas weight, and the β-cell number as well as the insulin$^+$ aggregates were calculated by manually counting the nuclei within the insulin$^+$ area and extrapolating to the whole organ as previously described (34). The β-cell size was assessed by dividing the insulin$^+$ area per total nuclei (34). The intensity of fluorescence was measured in arbitrary units using Fiji (32).

To determine the insulin$^+$glucagon$^+$ cells, pancreas from three mice from each group were analyzed (T1D, Lira 48 h, Lira, post-Lira, and sham). Briefly, 12 non-overlapping pancreatic cryostat sections from each mouse were stained for insulin and glucagon. A minimum of 72 islets per mouse was considered and the percentage of islets that contained bihormonal cells was determined. To assess ductal insulin$^+$ cells, pancreas from four mice from each group were analyzed (T1D, sham, and Lira). Briefly, four non-overlapping pancreatic cryostat sections from each mouse were stained for CK19 and insulin. A minimum of 23 ductal areas *per section* was considered and the percentage of ducts that contained insulin$^+$ cells was determined. To prove the colocalization of insulin and glucagon in islet cells and insulin and CK19 in ductal cells, confocal microscopy was performed using an Axiobserver Z1 (Zeiss) and by analyzing 1-µm sections.

In vivo Tracking of Liraglutide

Liraglutide was conjugated to AlexaFluor750 (AF750, Invitrogen) using a standard method (Thiol-Reactive Probes, Invitrogen). *In vivo* and *ex vivo* near-infrared fluorescence imaging was performed (Pearl Impulse imaging system, LI-COR) in NOD mice anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) at 15 and 60 min after the s.c. administration of 1 mg/kg of AF750-liraglutide in 50 µl of PBS. At the end of each checkpoint, spleen, stomach, fat, heart, liver, pancreas, lungs, kidney, salivary glands, thymus, and bladder were imaged *ex vivo*. Fluorescent signal intensity was semi-quantitatively assessed: the levels were normalized by subtracting the background and represented as a relative index of fluorescence in each organ per gram of tissue.

Islets and β-Cell Line

Islets from young non-diabetic NOD mice (n = 7) were obtained from pancreas digested with collagenaseP (2.5 mg/ml in PBS; Sigma-Aldrich), after having been injected through the common bile duct, resected, and incubated at 37°C for 30 min. The pancreas was dissociated and the islets were hand-picked. Groups of 50 islets were cultured in a medium of high (10 mM, RPMI-1640, Biowest) or normal glucose concentration (6.1 mM, Ham F-10, Gibco) as described (35), both in basal conditions—culture media—and by adding liraglutide at 1,000 nM for 48 h.

The β-cell line NIT-1 (ATCC) (36) was cultured (35) with liraglutide at 10, 100, and 1,000 nM for 12, 24, and 48 h. Viability was assessed with annexin V (AnnV) PE (Immunotools) and 7-amino-actinomycin D (7aad) labeling (BD Pharmingen) and analyzed with FACS Canto II (BD Biosciences).

Gene Expression Analysis

To determine the effects of liraglutide on gene expression, quantitative RT-PCR was performed. Briefly, RNA was isolated from islets using RNeasy Micro Kit (Qiagen) and reverse-transcribed with a cDNA Reverse-Transcription Kit (ThermoFisher Scientific). cDNA synthesis was performed using random hexamers (0.5 mg/ml, BioTools) and reverse

transcriptase Moloney–murine–leukemia–virus (200 U/ml, Promega). Targeted cDNA was pre-amplified with TaqMan PreAmp MasterMix (ThermoFisher Scientific). Quantitative RT-PCR assays were performed with TaqMan universal assay (ThermoFisher Scientific) on a LightCycler®480 (Roche) using the following TaqMan assays: *Ins2* (Mm00731595_gH), *Gcg* (Mm00801714_m1), *Ki67* (Mm01278617_m1), and *Il17a* (Mm00439618_m1). The expression for each gene of interest was normalized to that of the housekeeping gene *Gapdh* (Mm99999915_g1), as described in the $2^{-\Delta Ct}$ method (37). Values from islets were normalized using their respective basal controls and represented as a ratio (relative gene expression).

Effect of Liraglutide on NIT-1 Cell Line

NIT-1 cells were stained with anti-CD44 BV786 (BD Biosciences), anti-class I major histocompatibility complex (MHC) eFluor-450 (eBioscience), anti-CD14 PE, and anti-CD49b FITC (Immunotools). Viability was assessed with AnnV PE (Immunotools) and 7aad (BD Biosciences) as detailed above. Median fluorescence intensity and viability were determined using flow cytometry (LSR Fortessa, BD Biosciences). Corresponding fluorescence minus one staining was used as control. The data were analyzed using FlowJo (Tree Star Inc).

Statistical Analysis

Prism 7.0 (GraphPad Software Inc.) was used to perform the statistical analyses. For comparisons of unpaired data, a non-parametric Mann–Whitney test was used. The statistical tests applied to each data set are specified in each figure legend.

RESULTS

Liraglutide Is a Repurposed Candidate for β-Cell Regeneration

A total of 28 non-synonymous repurposed drug candidates showed a $p < 0.05$ (predicted value $\geq$76.075%), and 16 were selected as type 1 diabetes treatment candidates in the context of pancreatic β-cell regeneration. Drugs with poor safety profile were further filtered out. Lastly, we selected liraglutide (predicted value of 96.88%) that fulfilled all the criteria and had a novel, potentially beneficial mechanism toward β-cell regeneration.

Liraglutide Improves Hyperglycemia in Diabetic Mice

We determined the effect of liraglutide in immunodeficient NSG mice because of their absence of autoimmunity and low phenotypic heterogeneity. Type 1 diabetes was induced in NSG mice by a single STZ injection. All mice showed disease symptoms at 48–72 h after administration. A total of 50% (six of 12) of the treated NSG mice were responders to liraglutide according to the improvement of blood glucose levels during treatment. The remaining six mice did not have decreased blood glucose levels in any way. No differences in terms of initial fasting glycemia (mg/dl) were found between the responder (346.8 ± 72.5, mean ± SD) and the non-responder (397.7 ± 57.71) mice. The NSG mice with diabetes partially recovered normoglycemia during liraglutide administration until day 30 (Lira responders) when compared to the non-treated mice (T1D) (**Figure 1A**). After treatment withdrawal, all mice became hyperglycemic. A statistical difference between the treated and the control groups was observed when analyzing the area under the curve (AUC) after 15 days of treatment (**Figure 1B**). The response of the NSG mice to liraglutide after an acute increase in blood glucose levels showed that the treated diabetic mice (Lira) recovered normoglycemia at 210 min after glucose injection similarly to the non-diabetic group, whereas the diabetic non-treated mice remained hyperglycemic (>400 mg/dl, T1D) (**Figure 1C**). Significant differences found between the treated and the non-treated mice demonstrate that insulin production and secretion improve by the effect of this aGLP-1. The analysis of AUC showed an intermediate response to glucose stimulation in the liraglutide-treated group (**Figure 1D**). To elucidate whether liraglutide acts in a similar manner as insulin administration, an ITT was performed in normoglycemic NOD mice. The group treated with insulin displayed a reduction in glycemia after 30 min that was maintained until 60 min. By contrast, the liraglutide-treated group displayed an increase in glycemia until 30 min, which was normalized at 60 min similarly to the insulin-treated animals (**Figure 1E**). Despite that no significant differences were observed, the opposite tendency between both groups observed in the AUC (**Figure 1F**) revealed that liraglutide and insulin do not act similarly. To determine the effect of the genetic background, the acute effect of liraglutide was also determined on the C3HeB/FeJ strain. The results showed an insulinotropic effect of liraglutide in this strain of mice (**Supplementary Figure 1**) with a tendency to differ at the endpoint (60 min) in comparison to insulin.

Liraglutide Transiently Increases the β-Cell Mass

The β-cell mass of the NSG mice after 7–15 days of treatment with liraglutide significantly increased when compared to that of the diabetic non-treated mice, although they did not reach normal levels (**Figure 2A**). This effect was lost 5 days after withdrawal. In this sense, an increase in β-cell number was detected during the treatment in comparison to T1D and post-treatment groups (**Figure 2B**). These alterations were maintained even with normalized values to body weight or pancreatic tissue (**Supplementary Table 2**). Similarly, the percentage of islets emerging from ducts was increased both during treatment and after therapy removal (**Figure 2C**). These alterations were not related to changes in either β-cell size (**Figure 2D**) or insulin fluorescence intensity (33) (**Figure 2E**).

Liraglutide Induces Endocrine Cell Rearrangement in the Pancreas

The histological analysis revealed the presence of bihormonal cells ($insulin^+glucagon^+$) in the islets of NSG mice at the beginning of the treatment with liraglutide but not after 7–15 days or withdrawal (**Figure 3**). A total of 48.14 ± 1.53% of islets contained bihormonal cells, and the

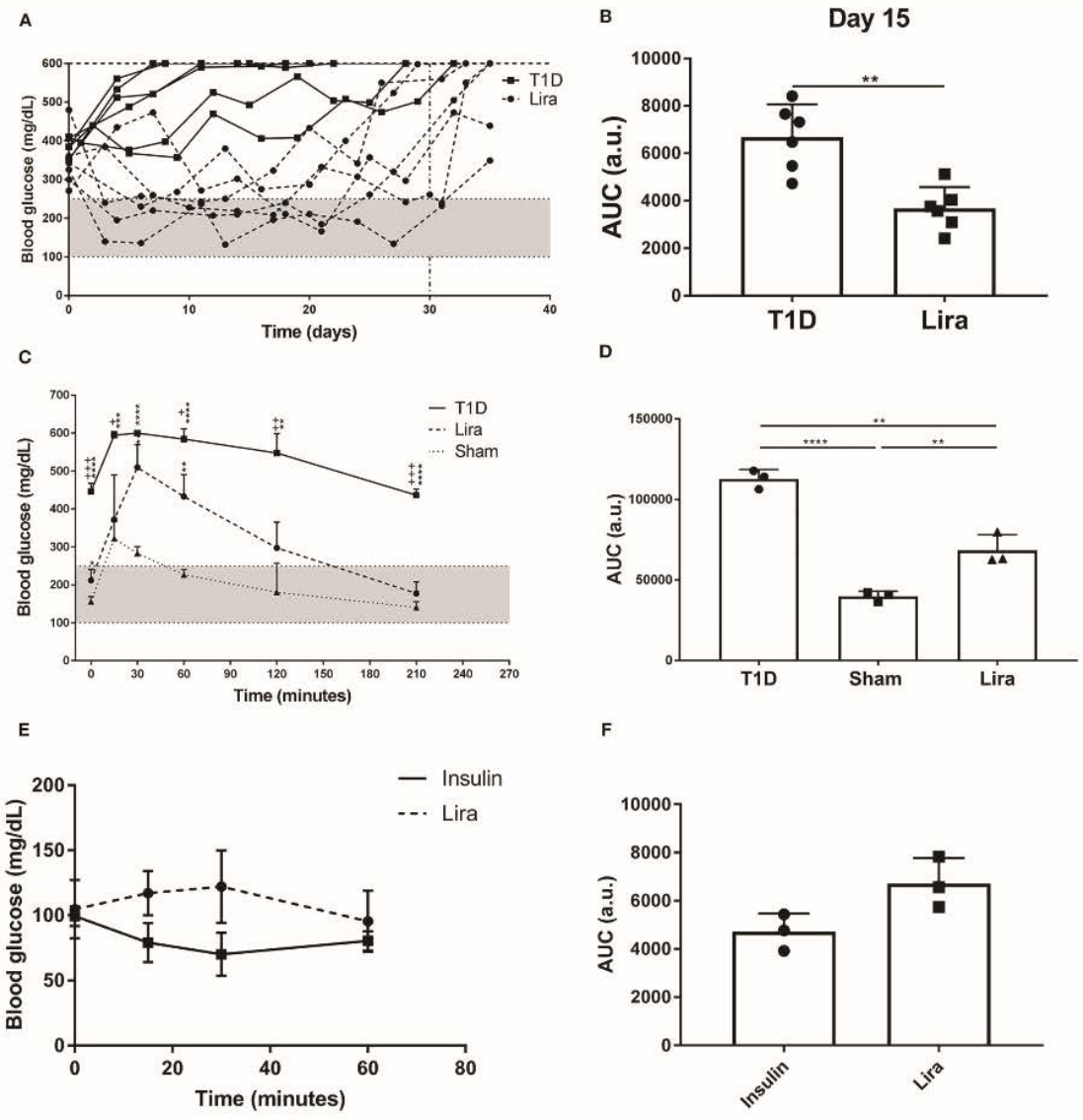

FIGURE 1 | Effect of liraglutide in diabetic immunodeficient NOD.SCID-IL2Rγ$^{-/-}$ (NSG) mice. **(A)** Two-hour fasting blood glucose levels (mg/dl) in mice rendered diabetic by a single dose of streptozotocin (150 mg/kg) and then treated with daily s.c. injections of liraglutide (Lira responders, circles) and phosphate-buffered saline (T1D, squares) from day 0 to 30 (dashed line). The filled area corresponds to normoglycemia levels in mice. **(B)** Area under the curve (AUC) of the graph in **(A)** at day 15, when all animals remain alive. Results are mean ± SD, and differences were found between groups (**$p < 0.01$, Mann–Whitney test). **(C)** Intraperitoneal glucose tolerance test in diabetic NSG mice treated with daily s.c. liraglutide for 15 days (Lira, dashed line), non-treated diabetic animals (T1D, continuous line), and normoglycemic mice (sham, dotted line). Statistical differences were found between Lira and T1D groups (+$p < 0.05$, ++$p < 0.01$, +++$p < 0.001$, Mann–Whitney test) and in both groups when compared to sham (dotted line) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, Mann–Whitney test). The filled area corresponds to normoglycemia levels in mice. **(D)** AUC of the graph in **(C)**. The results are mean ± SD, and differences were found between groups (**$p < 0.01$, ****$p < 0.0001$, Mann–Whitney test). **(E)** Insulin tolerance test performed in normoglycemic mice injected with Lira (1 mg/kg, dashed line) or insulin (0.5 U/kg, continuous line). **(F)** AUC of the graph in **(E)**. The results are mean ± SD; no statistical differences were found between groups.

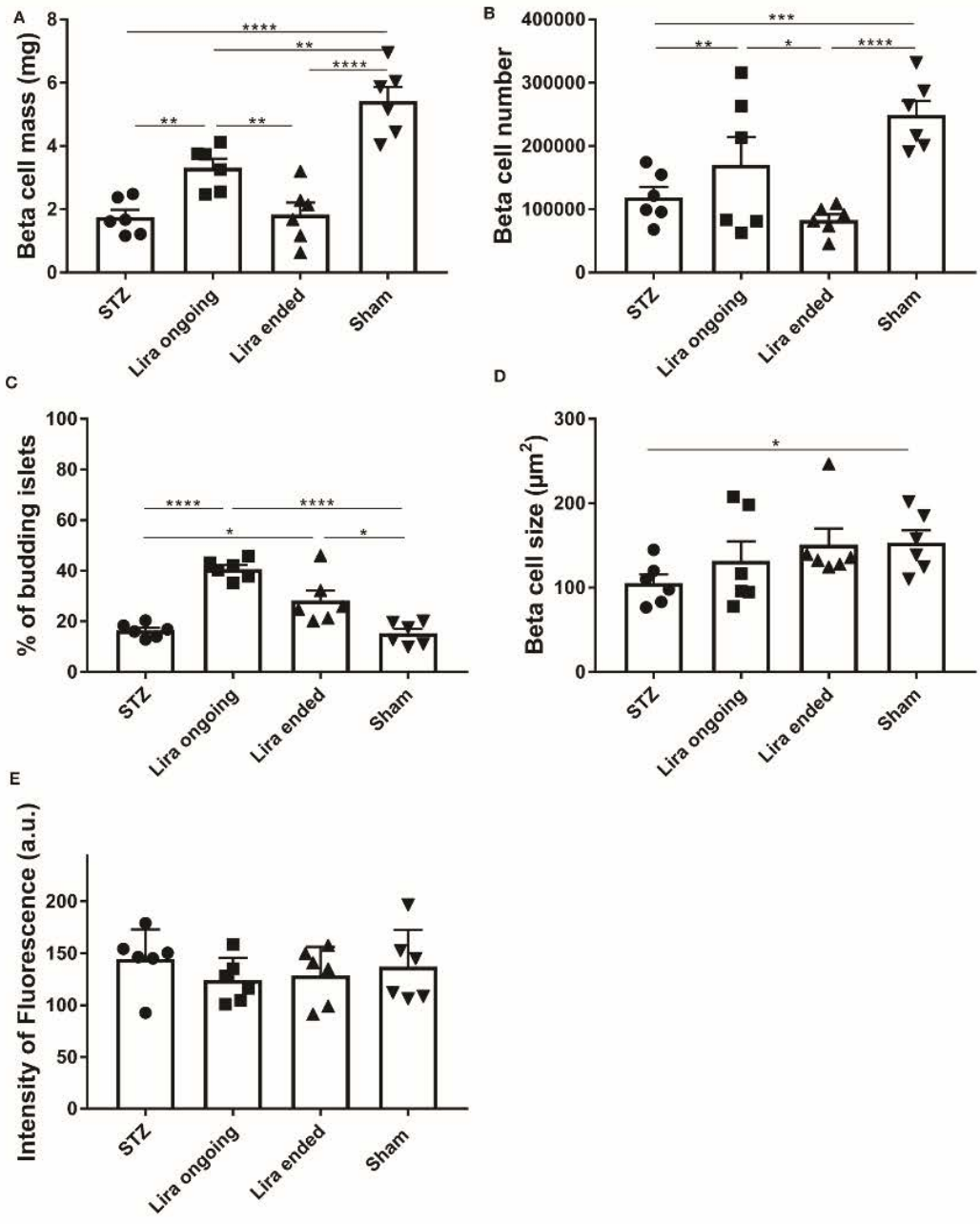

FIGURE 2 | Effect of liraglutide in the endocrine pancreas of diabetic immunodeficient NOD.SCID-IL2R$\gamma^{-/-}$ (NSG) mice. **(A)** β-cell mass (mg), **(B)** β-cell number, **(C)** percentage of budding islets (arising from ducts), and **(D)** β-cell size (μm^2) from NSG mice rendered diabetic by streptozotocin and treated with phosphate-buffered saline (T1D), with daily s.c. injections of liraglutide during 7–15 days (Lira), 5 days after the withdrawal of the liraglutide treatment (post-Lira) and normoglycemic mice (sham). The results are mean ± SD, and differences were found between groups (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, Mann–Whitney test).

(Continued)

FIGURE 2 | (E) Intensity of fluorescence of insulin staining in 5-μm cryostat pancreatic sections of NSG mice. The intensity was measured for all the islets analyzed per animal, and the mean of the intensities of all the islets is represented as a symbol. The results are mean ± SD, and no statistical differences were found between groups (Mann–Whitney test).

percentage of insulin$^+$glucagon$^+$ cells in relation to the total number of beta cells in the islets was 11.38 ± 2.97%. No bihormonal cells were detected in the other groups. Confocal microscopy images prove the colocalization of insulin and glucagon in the same cell (bihormonal cells) (**Supplementary Figure 2**).

Moreover, the pancreatic sections of the NSG mice treated for 7–15 days and after withdrawal revealed the existence of insulin$^+$ bodies emerging from ducts and without resembling the classical islet shape (**Figure 4A**). Between 23 and 47 ducts (CK19+) were analyzed in every pancreatic section. The percentage of ducts that contained insulin$^+$ cells was 50.83 ± 7.31% in mice treated with liraglutide (Lira group), whereas no ducts with insulin$^+$ cells were detected in normoglycemic mice (sham group, 0%) or untreated T1D mice (T1D, 0%). The ducts that contained insulin$^+$ cells were analyzed and, in these ductal areas, 82.85 ± 4.37% of doble-positive CK19$^+$ insulin$^+$ cells were found. Interestingly, the ductal areas positive for insulin in mice treated with liraglutide were detected from 48 h to the end of the treatment and even 5 days after the withdrawal (**Figure 4B**, white arrows). Confocal microscopy images prove the colocalization of insulin and CK19 in the same cell (**Supplementary Figure 3**). These insulin$^+$CK19$^+$ cells were glucagon-negative (**Figure 4C**). Moreover, we assessed the expression of the β-cell marker Pdx1 in ductal cells, identifying a subpopulation of Pdx1$^+$ cells in pancreatic ducts from treated animals (white arrow, **Figure 4D**).

To determine if liraglutide effects could be due to the accumulation in the pancreas, its biodistribution was assessed. The *in vivo* tracking of AF750-liraglutide showed an affinity for several organs including the pancreas that was higher at 15 min (**Figure 5A**) than at 60 min (**Figure 5B**), revealing an acute effect.

To further investigate the short-term effects of liraglutide, the expression of specific genes was assessed in islets from healthy NOD mice (three to four per group) cultured with or without liraglutide for 48 h in normal and high glucose concentrations. Previously, and in order to discard autoreactive insulitis in the islets, the expression of *Il17a* was assessed and found negative (data not shown). After exposure to liraglutide, a trend to upregulate *Gcg* and to downregulate *Ki67* was observed both at doses of 6.1 mM glucose (**Figure 5C**) and 10 mM glucose (**Figure 5D**). Finally, after exposure to liraglutide, the insulin gene (*Ins2*) appeared upregulated in normal glucose concentration conditions and slightly downregulated in high glucose concentration conditions.

Liraglutide Alters Membrane Molecule Expression in NIT-1 Cell Line

NIT-1 cell viability remained unaffected by liraglutide (10, 100, and 1,000 nM) at both 12 and 48 h (**Figure 6A**) but showed a deleterious effect on the NIT-1 cell line at 100 and 1,000 nM during 24 h (**Figure 6A**). Interestingly, liraglutide increased the expression of adhesion molecules such as CD49b, CD44 (**Figure 6B**), and CD14, a receptor of the innate immunity, and reduced the expression of MHC class I.

DISCUSSION

Drug repositioning is an attractive strategy for identifying new uses for approved drugs. The algorithm of this method was fed with target proteins known to be involved in β-cell restoration processes—(i) α-cell to β-cell transdifferentiation (9), (ii) neogenesis from multipotent ductal progenitors (1), and (iii) self-replication of pre-existing β-cells (10)—and resulted in the identification of liraglutide as a repurposed drug. As mentioned, liraglutide is an aGLP-1 used to treat type 2 diabetes (11, 12). A recent clinical trial demonstrates that liraglutide reduced HbA1c and insulin requirements in patients with long-standing type 1 diabetes (38). Moreover, it has been described that liraglutide improves β-cell function in alloxan-induced diabetic mice (39).

Our data show that liraglutide improves hyperglycemia, even reaching normoglycemia, in NSG mice. This model was selected for its lack of an adaptive immune system, resulting in immunodeficiency, and absence of autoimmunity. This fact allows us to determine the effect of the drug without autoimmunity interferences and reduced heterogeneity. The amelioration of hyperglycemia was also observed upon glucose stimuli (IPGTT) in diabetic mice treated with liraglutide, whereas diabetic non-treated mice remained hyperglycemic. By contrast, the administration of liraglutide to normoglycemic NOD mice resulted in a weak and transient increase of glycemia levels, as opposed to the effects of insulin administration. Taken together, these results indicate that liraglutide ameliorates hyperglycemia both in fasting and fed conditions but acting differently to insulin. The acute effect of liraglutide on a different mouse strain was insulinotropic as expected. These differences between NOD and C3 mice could be due, at least in part, to genetic differences in the structure and the size of the endocrine pancreas in both strains, specifically in the α- and β-cell mass (40). Another influencing factor should be the islet leukocyte infiltration, a feature of the NOD model, which promotes an inflammatory microenvironment, thus affecting insulin metabolism. The acute effect of liraglutide on a different mouse strain—C3 mice were normoglycemic and free of insulitis—was insulinotropic as expected. The NOD mice are also normoglycemic, but they display islet leukocyte infiltration despite no signs of overt diabetes having been observed at 8 weeks of age. The observation of spontaneous insulitis is restricted to mice with a diabetogenic genetic background, specifically NOD and NOR strains, and CD-1 outbred mice (31). The C3 mice display non-diabetogenic genetic background, do not develop spontaneously

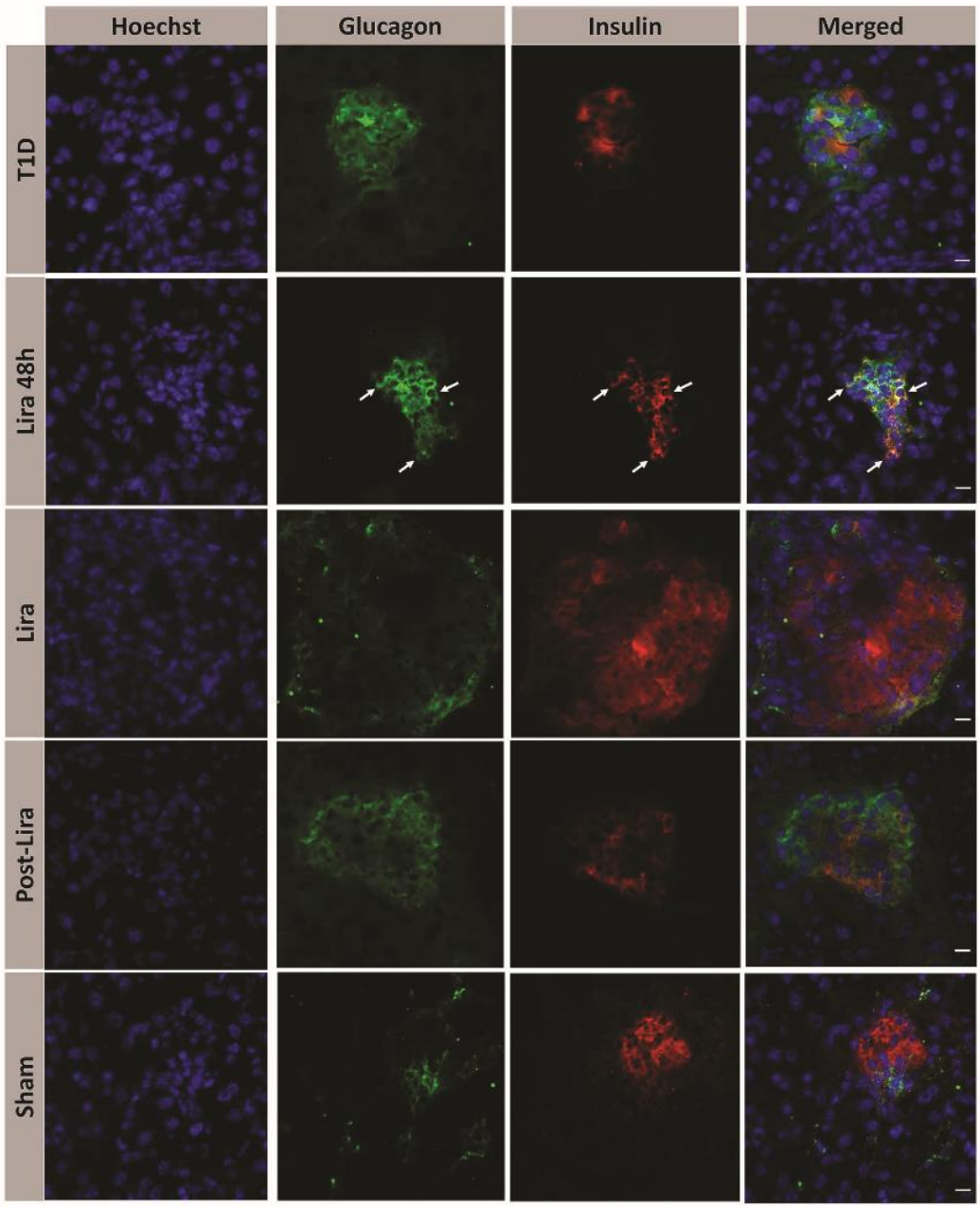

FIGURE 3 | Assessment of the effect of liraglutide in the induction of bihormonal insulin$^+$ glucagon$^+$ cells. Triple immunofluorescence staining of 5-μm cryostat pancreatic sections for α-cells (glucagon, green) and β-cells (insulin, red) in immunodeficient NOD.SCID-IL2Rγ$^{-/-}$ mice rendered diabetic by streptozotocin and treated with phosphate-buffered saline (T1D), or with liraglutide for 48 h (Lira 48 h), for 7–15 days (Lira) and after withdrawal of liraglutide at day 30 (post-Lira). Normoglycemic mice were included as control (sham). The white arrows in Lira 48 h staining depict bihormonal cells. The nuclei in all pictures were stained with Hoechst (blue). The scale bar indicates 5 μm.

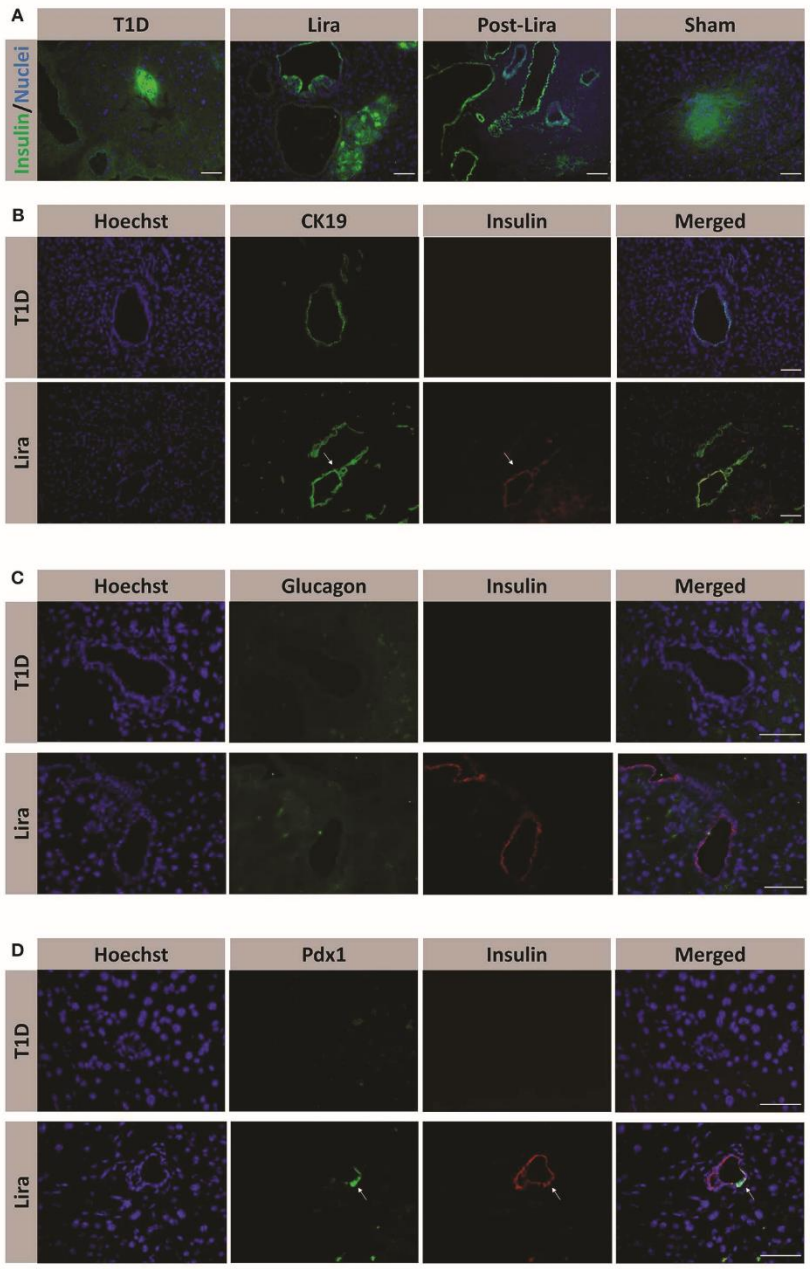

FIGURE 4 | Assessment of the effect of liraglutide in the induction of insulin-expressing ducts. **(A)** Staining for β-cells in the pancreas of immunodeficient NOD.SCID-IL2Rγ$^{-/-}$ (NSG) mice rendered diabetic by streptozotocin and treated with phosphate-buffered saline (PBS; T1D), or with liraglutide for 48 h (Lira 48 h), for 7–15 days (Lira) and after the withdrawal of liraglutide at day 30 (post-Lira). The Lira and post-Lira groups show the presence of neo-islets emerging from the ducts. Normoglycemic mice were included as control (sham). **(B)** Staining for ductal cells (CK19, green) and β-cells (insulin, red) in the same groups than in **(A)**. The white arrow depicts a CK19^{+} duct that is positive for insulin expression. **(C)** Staining for glucagon (green) and insulin (red) in ductal cells of animals treated with

(Continued)

FIGURE 4 | liraglutide for 7–15 days (Lira). **(D)** Staining for Pdx1 (green) and insulin (red) in the ductal part of diabetic NSG mice treated with PBS (T1D) or liraglutide for 7–15 days (Lira). The white arrows depict positivity for both Pdx1 and insulin in the ducts. The nuclei in all pictures are stained with Hoechst (blue). The scale bar in all pictures indicates 50 μm.

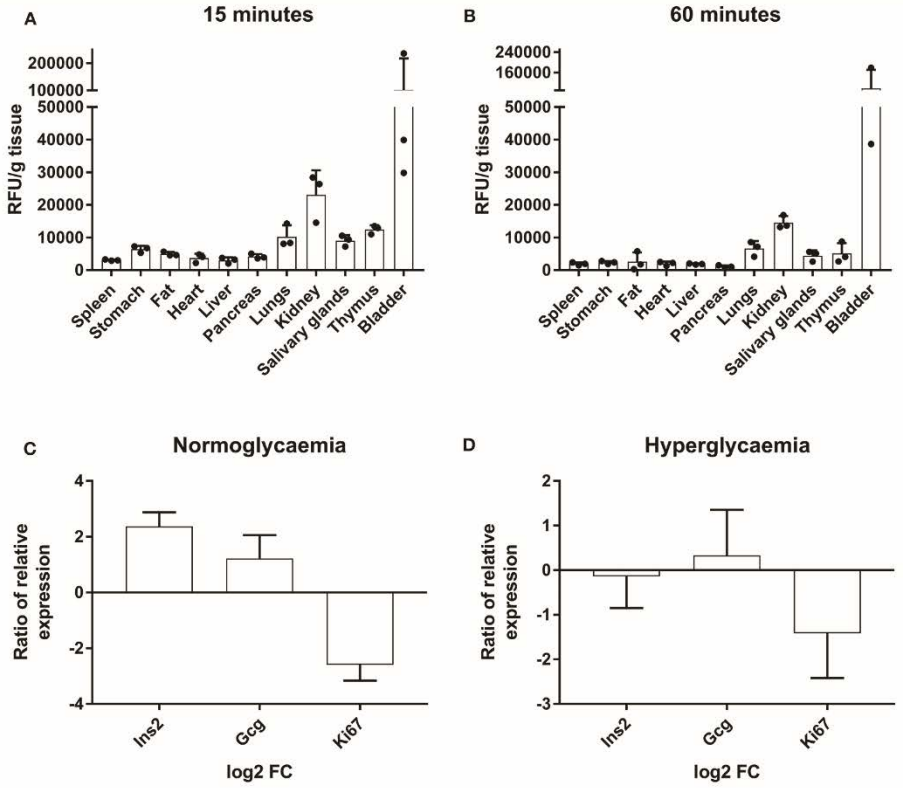

FIGURE 5 | Tracking of AF750-labeled liraglutide in prediabetic NOD mice and determination of gene expression in islets of Langerhans. **(A)** Histogram of *ex vivo* fluorescent signal relative to the grams of tissue of several organs from NOD mice at 15 min and **(B)** at 60 min after a s.c. injection of AF750-labeled liraglutide. The results are mean ± SEM of three independent experiments. **(C,D)** Ratio of relative gene expression of the selected genes in isolated islets of Langerhans after 48 h of 1,000-nM liraglutide treatment ($n \geq 3$) in relation to basal conditions analyzed by qRT-PCR in conditions of normoglycemia and hyperglycemia. Gene expression was normalized to *Gapdh*. The bars show the mean ± SD of the log2 of fold change using basal transcription as standard value.

autoimmune diabetes, and may have diabetes that can be induced by STZ treatment (41). In NOD mice, insulitis promotes an inflammatory microenvironment, thus causing β-cell stress and metabolic abnormalities (42, 43). Liraglutide administered to NOD mice may also impair insulin secretion at this stage because of the inflammatory environment in this strain.

Intriguingly, the effect was transient as previously described (30). This effect correlated with the transitory increase in the β-cell mass, confirming that insulin secretion induced by liraglutide (11) is not the only event that contributes to the restoration of normoglycemia. This increase is not due to β-cell hyperplasia but by an increase in the number of insulin$^+$ cells and the formation of neo-islets emerging from ducts. Thus, it is reasonable to speculate that the continuous presence of liraglutide is required for the maintenance of islet β-cell mass by promoting the main mechanism responsible for the improvement of blood glucose levels in treated mice, at least during the first 30 days of treatment stages. Elucidating the mechanisms of action of liraglutide in diabetic mice with diabetogenic background will contribute to the design of β-cell regenerative strategies.

To further explore the regeneration mechanism, we then searched for processes described in any of the pathways that liraglutide was predicted to act on. Suggesting transdifferentiation, transient bihormonal cells were found within the islets, a feature of α-cell to β-cell conversion detected after β-cell loss (44). Because glycemia normalization occurs

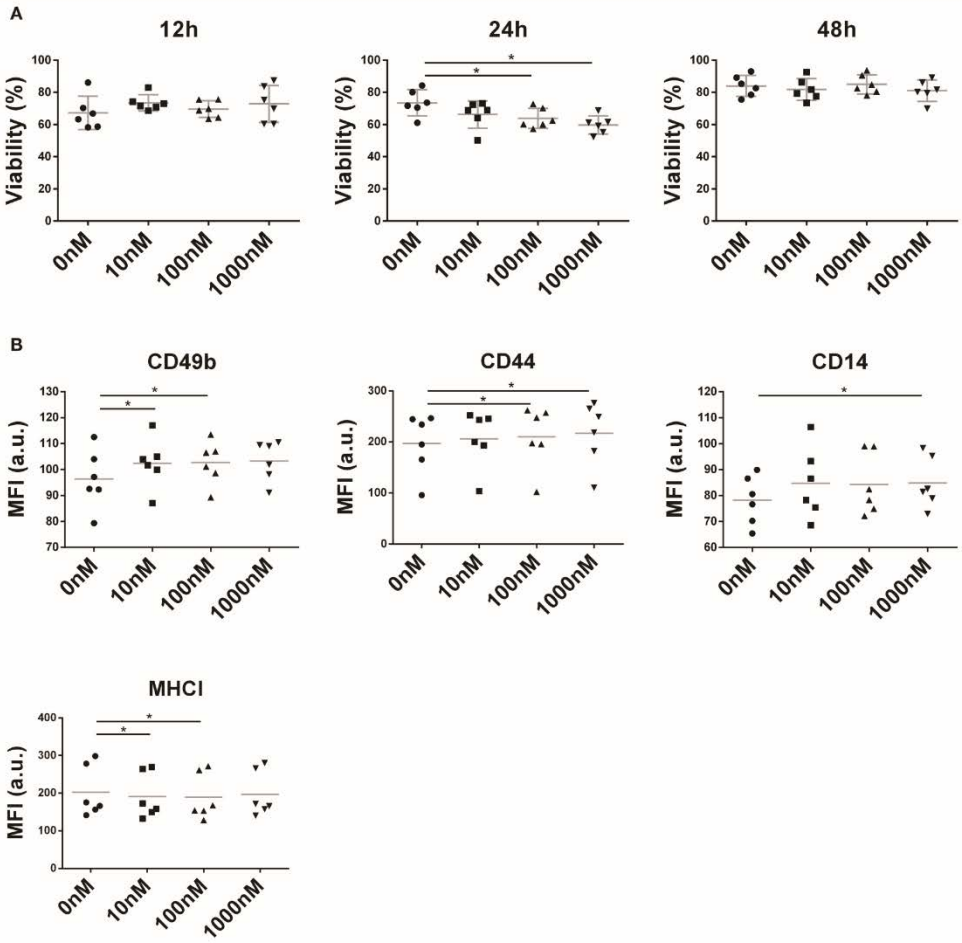

FIGURE 6 | Effect of liraglutide in the NIT-1 β-cell line. **(A)** Percentage of viable NIT-1 cells (annexin V PE^-, $7aad^-$) after 12, 24, and 48 h of co-culture with increasing liraglutide concentrations (10, 100, and 1,000 nM) and control condition (*$p < 0.05$, Mann–Whitney test). **(B)** Median of fluorescence intensity for CD49b, CD44, CD14, and MHC class I surface expression (*$p < 0.05$, Mann–Whitney test).

almost immediately after the first injections, newly formed $insulin^+$ cells with an α-cell origin could contribute to glucose homeostasis. Our results fit well with the recent demonstration that exendin-4, another aGLP-1, also induces bihormonal cells as a consequence of transdifferentiation (16). Other drugs that induce this type of transdifferentiation also cause extreme islet hyperplasia during the early stages of administration (3), but this fact has not been observed with liraglutide, thus suggesting that liraglutide might act through different pathways. Bihormonal cells detected at 48 h after administration agreed with an increase in both insulin and glucagon transcripts in islets treated *in vitro* with liraglutide. Although hormone colocalization *per se* is not a demonstration of a real conversion from α-cell to β-cell, it is logical to speculate that these cells correspond to emergent β-cells. In mice, α-cells can become insulin-expressing cells after β-cell ablation, thus promoting diabetes recovery. Recent results confirm that human α-cells are able to secrete insulin and reverse diabetes, but surprisingly, these cells express α-cell markers (45). Nevertheless, to prove that liraglutide induces a real conversion from glucagon-producing cells into insulin-producing β-cells—as reported with GLP-1 peptide (16), it would be of interest to perform lineage tracing experiments with transgenic models.

Furthermore, during the treatment and even after removal of liraglutide, $insulin^+$ bodies were found to be emerging from $CK19^+$ ductal cells that expressed insulin. Whether

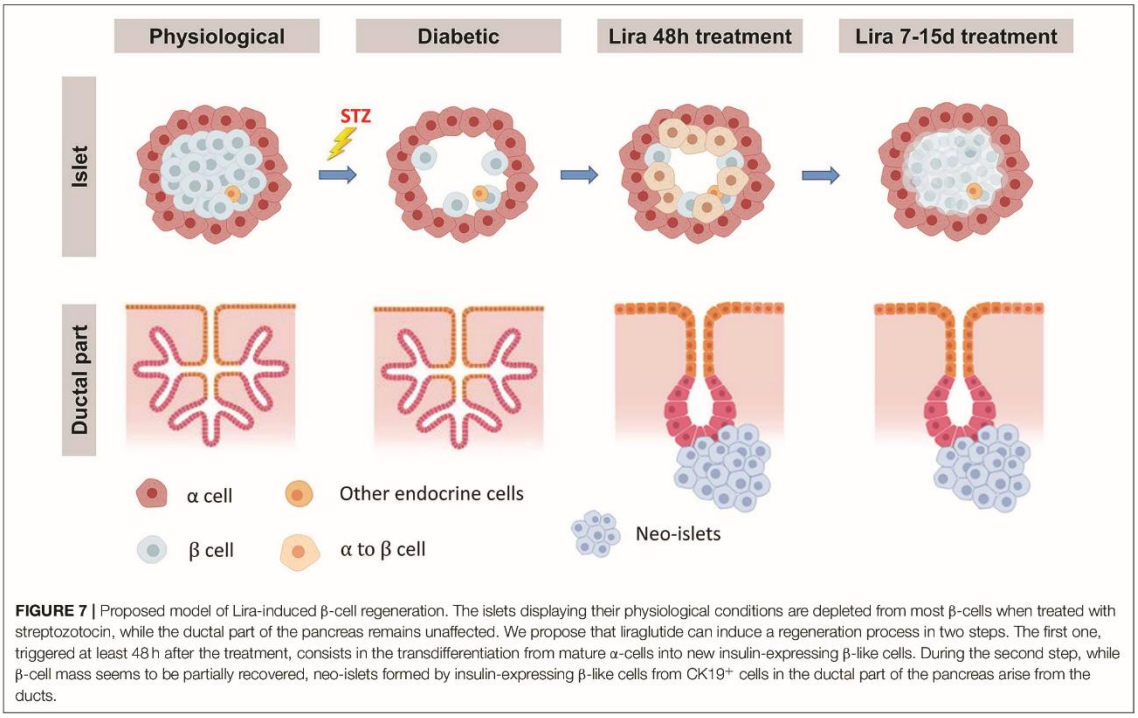

FIGURE 7 | Proposed model of Lira-induced β-cell regeneration. The islets displaying their physiological conditions are depleted from most β-cells when treated with streptozotocin, while the ductal part of the pancreas remains unaffected. We propose that liraglutide can induce a regeneration process in two steps. The first one, triggered at least 48 h after the treatment, consists in the transdifferentiation from mature α-cells into new insulin-expressing β-like cells. During the second step, while β-cell mass seems to be partially recovered, neo-islets formed by insulin-expressing β-like cells from CK19$^+$ cells in the ductal part of the pancreas arise from the ducts.

these neo-islets emerged from specific multipotent progenitors remains to be explored, but it has been proposed that early transdifferentiation promotes later neogenesis from ducts (3, 46). Further experiments are required in order to confirm this, given that the cell population identified in the ductal part are Neurogenin3$^+$ progenitors that underwent epithelial-to-mesenchymal transition to differentiate into β-like cells (9, 47). Neo-islets have also been reported in human pancreas (48), including those from patients with type 1 diabetes (49), but this is the first demonstration of insulin$^+$ cells with a ductal origin caused by liraglutide. Pdx1, a mature β-cell and endocrine progenitor marker, also depicted a ductal population. Because GLP-1 receptor is also expressed in ductal cells (50), insulin expression could be induced by liraglutide in these cells. These results suggest that the role of this drug in ameliorating hyperglycemia follows a mechanism that is non-exclusive to β-cells and has other players involved, such as islet insulin$^+$glucagon$^+$ cells and ductal insulin-expressing cells. The herein reported findings go beyond the previously reported beneficial effect of liraglutide in β-cell function, both *in vivo* (39) and *in vitro* in terms of insulin secretion (51). However, neogenesis from ducts was not reported in a previous study due to differences in dosage, disease induction, and especially lineage tracing issues (39). It is difficult to assess which insulin-producing cell types are quantitatively contributing to the improvement of hyperglycemia. First, despite the fact that the percentage of bihormonal cells at 48 h is almost 11% of total insulin$^+$ cells, these data reflect a specific stage, and it is still unknown how many insulin$^+$ cells are arising from transdifferentiation throughout the treatment. Indeed it is reasonable to speculate that ductal insulin$^+$ cells are participating in insulin secretion, but it remains to be investigated if they are glucose responsive. Finally, both a decrease in the percentage of budding islets and the fact that liraglutide is an insulinotropic agent may explain the transient effect in the glycemia after withdrawal.

Taking these observations together, we propose a two-step simultaneous mechanism of β-cell regeneration (**Figure 7**): first, an early, acute, and transient transdifferentiation mechanism from α-cell to β-cell (3, 38) and, second, an early and permanent neogenic process of insulin$^+$ cells from the ducts. After withdrawal, the first mechanism seems to be suppressed—probably after the loss of insulin$^+$ cell identity—while the second one persists.

In conclusion, liraglutide, a repurposed compound, ameliorates hyperglycemia in experimental type 1 diabetes. Our results point to β-cell replacement, including transdifferentiation and neogenesis, as aiding factor. Liraglutide could be a candidate to restore β-cell mass in combined therapies, together with an immunomodulatory strategy to arrest autoimmunity.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/ **Supplementary Material**.

ETHICS STATEMENT

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Generalitat de Catalunya, Catalan Government. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Germans Trias i Pujol Research Institute (Permit DAAM 9521) and has followed the principles outlined in the Declaration of Helsinki for animal experimental investigation.

AUTHOR CONTRIBUTIONS

AV, FV, IP-A, and MV-P designed the experiments. MC conducted the drug reposition analysis. AV, DP-B, SR-F, and R-MA performed the experiments in mice. AV, DP-B, SR-F, and LG-M carried out the *in vitro* experiments. MC-S bound the drug to the fluorochrome. JV contributed to islet isolation. AV and MV-P wrote the manuscript. SR-F, IP-A, EA, and JV contributed to the discussion. All the authors revised the manuscript and gave final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00258/full#supplementary-material

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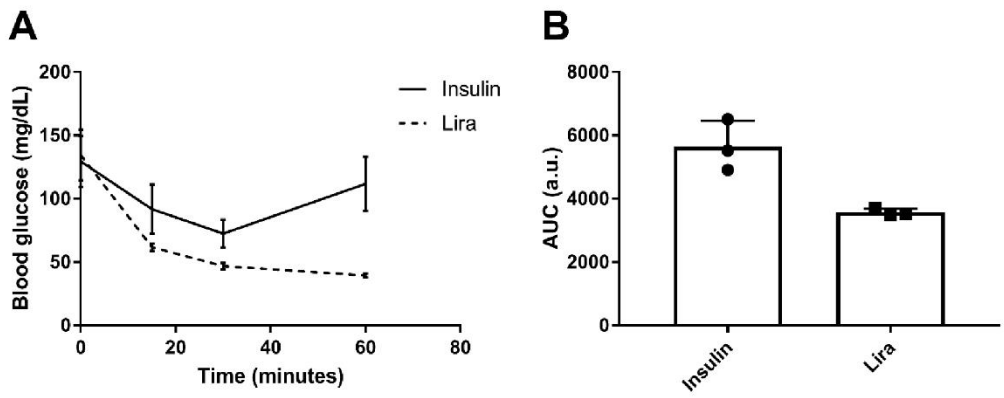
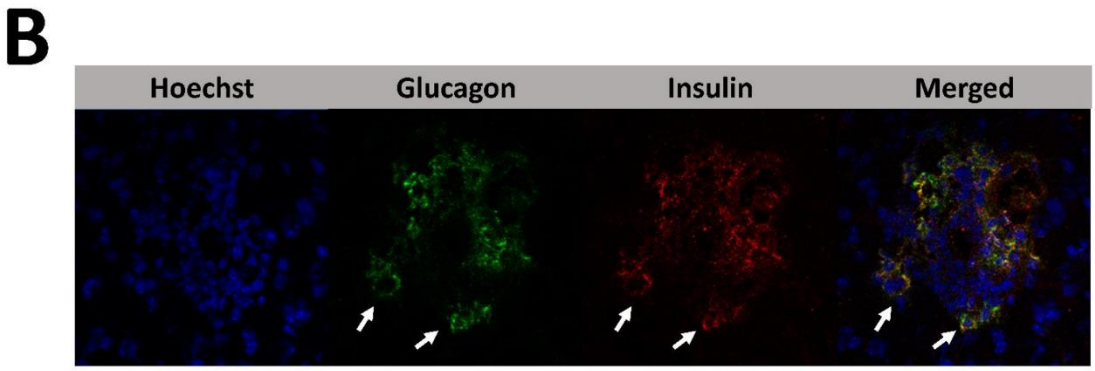
Conflict of Interest: MC-S and MV-P are co-founders of Ahead Therapeutics SL, which aims at the clinical translation of immunotherapies for the treatment of autoimmune diseases. MC was employed by the company Anaxomics Biotech SL.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

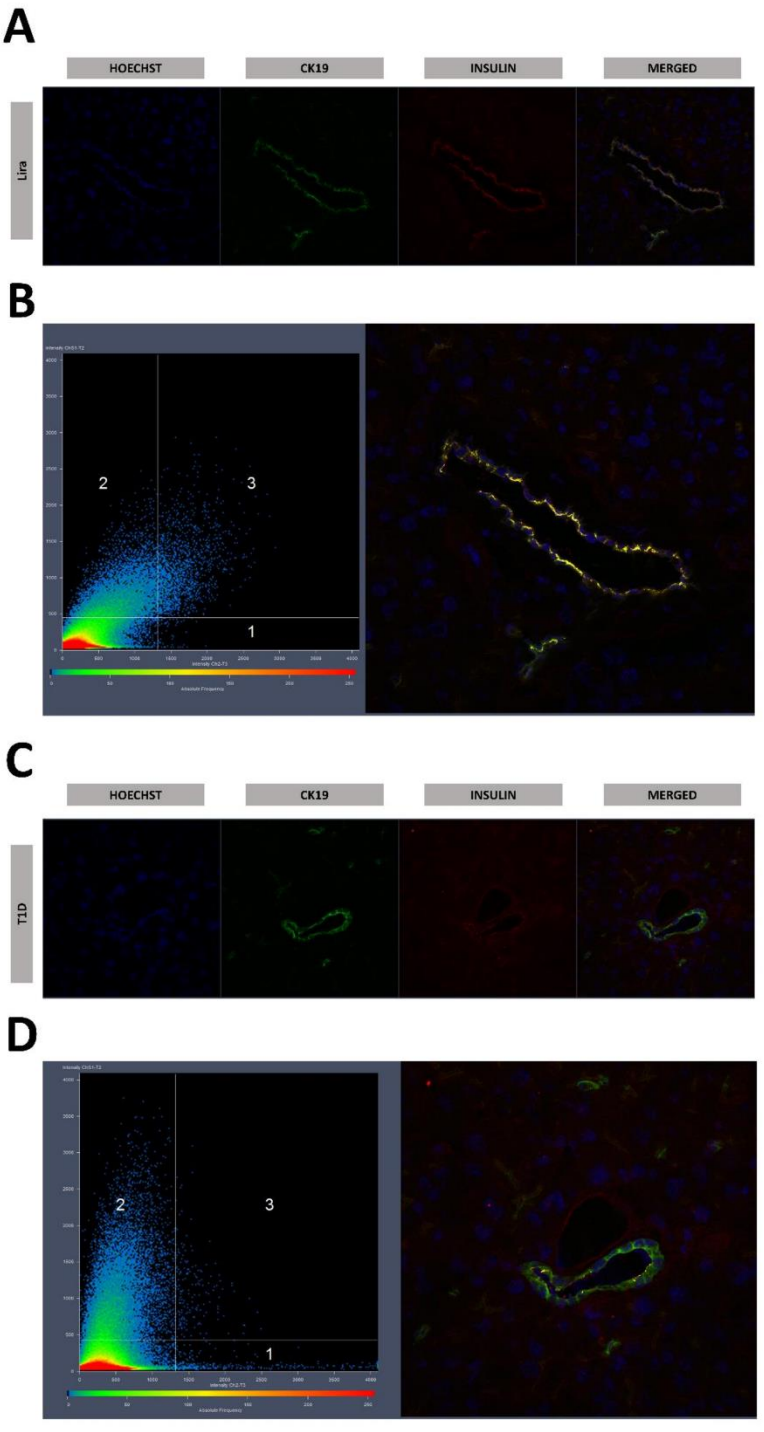


Supplementary Figure 1. Effect of liraglutide in normoglycaemic C3HeB/FeJ mice. A) Insulin Tolerance test (ITT) performed in normoglycaemic mice injected with Lira (1mg/kg, dashed line) or insulin (0.5 U/kg, continuous line). **B)** AUC of the graph in A). Results are mean ± SD, no statistical differences were found between groups.



Supplementary Figure 2. Colocalization of insulin and glucagon in bihormonal insulin$^+$glucagon$^+$ cells. Colocalization of glucagon and insulin in a pancreatic islet of a Lira 48h mouse revealed insulin$^+$glucagon$^+$ cells (white arrows). Section of 1µm thickness was recorded at 40X with a confocal microscope.

Supplementary Material



Supplementary Figure 3. Colocalization of insulin and CK19 in CK19^{+}insulin^{+} ductal cells. A) Colocalization of CK19 (green) and insulin (red) in a ductal area from a pancreas of a Lira mouse revealed insulin^{+} ductal cells. **B).** Colocalization dot plot of CK19 (y-axes) and insulin (x-axes) of the picture in A). Colocalization area highlighted in yellow pseudocolor (Zen Black, Zeiss). **C).** No colocalization of CK19 (green) and insulin (red) was observed in ductal cells from pancreases from non-treated T1D mice. **D).** Colocalization dot plot of CK19 (y-axes) and insulin (x-axes) of the picture in C) (Zen Black, Zeiss). Sections of 1µm thickness were recorded at 40X with a confocal microscope (Axiobserver Z1, Zeiss).

Supplementary table 1. Effector proteins selected for the *Drug Repositioning* analysis.

Pathway	Effector Protein	Uniprot ID
Self-replication	INGAP	Q92778
Self-replication	PTF1A	Q7RTS3
Self-replication	MYC	P01106
Self-replication	CPA1	P16085
Self-replication	NEUROG3	Q9Y4Z2
Self-replication	MKI67	P46013
Self-replication	CCND2	P30279
Self-replication	CDK4	P11802
Self-replication	E2F1	Q01094
Self-replication	AKT1	P31749
Self-replication	KMT2A	Q03164
Self-replication	SLC2A2	P11168
Self-replication	INS	P01308
Self-replication	PDX1	P52945
Self-replication	MAFA	Q8NHW3
Self-replication	NEUROD1	Q13562
Self-replication	GCK	P35557
Self-replication	ISL1	P61371
Self-replication	NOTCH1	P46531
Self-replication	REG1B	P48304
Self-replication	REG1A	P05451
Neogenesis	PDX1	P52945
Neogenesis	SOX9	P48436
Neogenesis	SLC2A2	P11168
Neogenesis	NEUROG3	Q9Y4Z2
Neogenesis	SST	P61278
Neogenesis	MSX2	P35548
Neogenesis	EGF	P01133
Neogenesis	FBXW7	Q969H0
Neogenesis	STAT3	P40763
Neogenesis	IL6	P05231
Transdifferentiation	PAX4	O43316
Transdifferentiation	GCG	P01275
Transdifferentiation	NEUROG3	Q9Y4Z2
Transdifferentiation	GLP1R	P43220
Transdifferentiation	ARX	Q96QS3
Transdifferentiation	PDX1	P52945
Transdifferentiation	NKX2-5	P52952

Transdifferentiation	FOXO1	Q12778
Transdifferentiation	CDK4	P11802
Transdifferentiation	PAX6	P26367
Transdifferentiation	NKX6-1	P78426

In order to perform the repositioning of approved drugs, selected list of proteins known to be involved in the following pathways —(i) self-replication from pre-existing β cells, (ii) neogenesis from ductal progenitors and (iii) transdifferentiation from α-cells to β-cells— was obtained.

Supplementary table 2. Histometric data from NGS mice treated or not with Lira.

Parameter	STZ	Lira ongoing	Lira ended	Sham
Body weight (g)	27.95±0.41	25.68±4.53	26.47±2.41	26.93±0.80*
Pancreas weight (mg)	122±15.83	128.8±37.41	141.2±14.48	114±20.21*
Pancreas weight/ g of body weight	4.36±0.57	5.05±1.21	5.38±0.91*	4.01±0.71
β cell % of pancreatic tissue	1.45±0.50	2.73±0.87*	1.30±0.65	4.94±1.44***
β cell number/mg of pancreatic tissue	975.3±294.5	1355±911.4	596±167.9*	2266±691.9**
β cell number/g of body weight	4254±1411	6683±4587	3106±687.3	9280±2197***
Insulin+ aggregates	2686±798.8	5138±1842*	2272±932.6	4991±1207**

Histometry from NSG mice rendered diabetic by a single-dose STZ treated with PBS (STZ), daily s.c. Liraglutide for 7-15 days (Lira ongoing), after the withdrawal (Lira ended) and a normoglycemic group (Sham). Data presented as mean ± SD. Asterisks denote statistical significance when comparing each parameter in any group with the STZ group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, Mann-Whitney test).

Results III: Antigen-specific immunotherapy combined with a regenerative drug in the treatment of experimental type 1 diabetes

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An option to revert T1D, is the suppression of the autoimmune attack combined with an autologous β-cell replacement strategy. Previous work demonstrated that liraglutide (an aGLP1) restores β-cell mass in T1D, via α-cell transdifferentiation and neogenesis. The aim of this study was to design a combined therapy consisting of PS-liposomes as immunotherapy and liraglutide as regenerative agent.

The results show that liraglutide accelerates and does not prevent T1D in the spontaneous and autoimmune model of the disease —the NOD mouse— but induced a tendency to reduce leukocytic islet infiltration. However, in combination with an immunotherapy based on liposomes, it is effective in ameliorating hyperglycaemia in NOD mice overtly diabetic. Importantly, liraglutide is not detrimental for the tolerogenic phenotype induced by PSAB-liposomes on DCs from adult subjects with T1D, referring at the terms of molecules involved in antigen presentation, immunoregulation and activation. Moreover, the *in vivo* effect of the combined therapy was tested in mice humanized with peripheral blood mononuclear cells from patients with T1D, showing no effects in leukocyte subsets.

Overall, these data —included in the Supplementary Article chapter of the present PhD thesis— demonstrate that a combined therapy consisting of PSAB-liposomes (immunotherapy) and liraglutide (regenerative agent) can ameliorate hyperglycaemia in a mice model of spontaneous autoimmune diabetes.

Results IV: Partial remission and early stages of pediatric type 1 diabetes display immunoregulatory changes. A pilot study.

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Based on clinical data from patients with T1D, it has been proposed that the disease is not steady-state but it displays a progression through relapsing and remitting stages of autoimmunity. A very interesting stage of T1D is the PR, which occurs during the first year after the onset, showing a significant reduction of insulin requirements and an amelioration of the HbA1c.

This study was aimed to identify novel biomarkers related to different stages of the pathophysiological progression and relapsing-remitting phases of T1D. A screening of immune cells subsets and immune-response related molecules was performed in 52 paediatric subjects with T1D and 30 age-related control subjects. Special attention was paid to immunological changes of PR, for its potential implication in the transient reestablishment of self-tolerance.

The results showed a decline in the concentration of circulating TGF-β at 6 and 12 months after T1D onset only in patients undergoing PR. Moreover, circulating betatrophin —a hormone with an unclear role in lipid metabolism— concentration was shown to be increased in all the stages considered of T1D when compared to controls. Regarding cell biomarkers, an increase in the amount of regulatory lymphocyte subsets (T, B and NK

regulatory cells) was found during initial stages of T1D progression, while a reduction of the numbers and percentage of monocytes in peripheral blood was detected.

These data point to novel molecular and cellular candidate biomarkers of paediatric T1D progression. Despite more studies are required, the here described altered parameters related to immunological regulatory mechanisms could be likely reflecting an attempt to restore self-tolerance at early phases of T1D.

ORIGINAL ARTICLES

Partial remission and early stages of pediatric type 1 diabetes display immunoregulatory changes. A pilot study

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Type 1 diabetes (T1D) is a chronic metabolic disease of unknown etiology that results from β-cell destruction. The onset of the disease, which arises after a long asymptomatic period of autoimmune attack, may be followed by a relapsing and remitting progression, a phenomenon that is most evident during the partial remission phase (PR). This stage lasts for a few months, shows minor requirements of exogenous insulin and could be explained by a recovery of immunological tolerance. This study aims to identify new biomarkers at early stages of pediatric T1D that reflect immunoregulatory changes. To that end, pediatric patients with T1D ($n = 52$) and age-related control subjects ($n = 30$) were recruited. Immune response-related molecules and lymphocyte subsets were determined starting at T1D onset and until the second year of progression. Results showed that circulating TGF-β levels decreased during PR, and that betatrophin concentration was increased in all the considered stages without differing among studied checkpoints. Moreover, an increase of regulatory T, B and NK subsets was found during T1D progression, probably reflecting an attempt to restore self-tolerance. By contrast, a reduction in monocyte levels was observed at the early stages of diabetes. The results reveal significant changes in immunological parameters during the different early stages of T1D in children, which could ultimately serve as potential biomarkers to characterize the progression of T1D. (Translational Research 2019; 210:8–25)

Abbreviations: aTreg = activated regulatory T; BLMS = B lymphocyte maturation stages; BMI = body mass index; Breg = regulatory B; CM = central memory; DP = double-positive;

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EM = effector memory; HbA1c = glycated hemoglobin; IDDAA1c = insulin dose-adjusted HbA1c; mTreg = memory regulatory T; NKreg = regulatory Natural Killer; PR = partial remission; PBMCs = peripheral blood mononuclear cells; Treg = regulatory T; TLMS = T lymphocyte maturation stages; T1D = type 1 diabetes

AT A GLANCE COMMENTARY
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Background

This study provides new candidate biomarkers – betatrophin, TGF-β, and regulatory T, B and NK subsets – that reflect the immunoregulatory attempts during early progression of pediatric type 1 diabetes, including partial remission stage.

Translational Significance

These findings suggest that regulatory lymphocytes as well as betatrophin and TGF-β molecules may be related to partial remission and progression of type 1 diabetes in children. Moreover, and because partial remission may be a good stage for immunointervention, these candidate biomarkers can be considered for clinical trials.

INTRODUCTION

Type 1 diabetes (T1D) is a metabolic disease caused by the autoimmune destruction of insulin-producing β-cells. After the clinical onset, and probably due to the beginning of insulin therapy, between 50% and 80% of pediatric patients with T1D undergo a partial remission (PR) phase, also known as honeymoon, starting at 6 months of disease progression and lasting up to 2 years.[1] This transient period is defined by low requirements of insulin and diminished glycated hemoglobin[2,3] (HbA1c) and could be an optimal phase for immune intervention and other therapeutic strategies. Clinical trials using immunotherapies have failed to provide long-term β-cell protection, partly due to the stage chosen for immunointervention.[4] Hence, it is necessary to identify new biomarkers which will enable the monitoring of the PR and the early stages of T1D.

A reliable biomarker has to be robust and allow for the distinction of control and patients, or in this case, patients at different stages of disease. For T1D, it should fulfil several requirements[4]: (1) To reflect the variations of the autoimmune response or β-cell regeneration[5]; (2) To be easy to obtain with minimum discomfort or risk for the patient; (3) To display variations over the progression of the disease; (4) To allow the monitoring of the patient's response to immunotherapies; (5) To have available a sensitive, specific, and highly reproducible detection method. At present, most used biomarkers in T1D are metabolic (HbA1c and C-peptide), but they do not reflect the pathophisiology of T1D. HbA1c is strongly dependent on daily glycemia and C-peptide levels are usually not detectable after the clinical onset.[6] Autoantibodies against β-cell antigens are immunological biomarkers for T1D but they do not always predict the development of the disease or the severity of secondary complications. Specific autoreactive T cells were also proposed as biomarkers but the reproducibility of experiments involving antigen-specific T cells has been questioned.[7]

Several candidate biomarker have arised in the last years. Betatrophin is one of these candidates, since its circulating levels in plasma were found increased in adult patients with long-standing T1D.[8] Another candidate was TGF-β, due to its role in immunoregulation and the alterations found in the pancreases from patients.[9] Moreover, plasma levels of TGF-β are increased in adult patients at the onset, indicating that TGF-β plays a role in chronic inflammation in T1D.[10] Other candidates are molecules with altered expression in the pancreases from patients at different disease stages,[11] and circulating leukocyte subsets,[12,13] that can be easily obtained from peripheral blood of pediatric subjects and be identified with a high resolution in an automated manner.[14] Our study aims at unravelling for the first time the characterization of the changes in these markers/subsets during the early stages of pediatric T1D.

MATERIAL AND METHODS

Participants. Pediatric patients with T1D ($n = 52$) and age-related control subjects ($n = 30$) were included in this study (Table 1). All patients fulfilled the classification criteria for T1D. Inclusion criteria were 1–18 years of age, and normal body mass index (BMI) according to the Spanish BMI pediatric cohort growth chart.[15] Exclusion criteria were being under immunosuppressive or anti-inflammatory treatment, the presence of other autoimmune diseases, type 2 diabetes, pregnancy, compromised kidney function, or liver diseases. All patients with T1D were positive for autoantibodies to GAD65 and/or IA-2 at the onset, and were negative for other autoantibodies (antithyroglobulin, anti-transglutaminase, and antithyroid peroxidase). Twenty-two out of the total 52 patients and 14 out of 30

Table 1. Clinical data for pediatric control subjects and patients with T1D at onset, and at 6, 12 and 18 months of disease progression

Variable	Control subjects ($n = 14$)	Patients at onset ($n = 22$)	Patients at 6 mo ($n = 15$)	Patients at 12 mo ($n = 14$)	Patients at 18 mo ($n = 13$)
Age (years)	8.1 ± 3.6	9.1 ± 4.5	9.6 ± 4.6	10.1 ± 4.6	10.6 ± 4.6
Gender (M/F)	3/11	11/11	8/7	8/6	9/5
BMI (kg/m^2)	19.4 ± 2.4	17.3 ± 3.4	18.3 ± 3.4	18.3 ± 3.6	17.8 ± 1.8
HbA1c (%)	ND	11.4 ± 2.5	7.2 ± 1.1	7.7± 1.04	7.6 ± 1.04
HbA1c (mmol/mol)	ND	101 ± 27.3	55 ± 11.6	61 ± 11.4	61 ± 11.4
Insulin dose (UI/kg/day)	ND	0.8 ± 0.4	0.53 ± 0.2	0.79 ± 0.7	0.92 ± 0.72
IDDAA1C (%)	ND	14.1 ± 3.4	9.4 ± 1.3	8.9 ± 3.5	10.2 ± 2.7

All values are mean ± SD. No statistical differences were found in age between groups. Statistical differences were found in: insulin dose (onset vs 6 mo: ¥, 6 mo vs 12 mo: *, 6 mo vs 18 mo: §, 12 mo vs 18 mo: #); glycated hemoglobin (HbA1c) (onset vs 6 mo: §, onset vs 12 mo: #, onset vs 18 mo: #); insulin dose-adjusted HbA1c (IDDAA1c) (onset vs 6 mo: ¥, onset vs 12 mo: ¥ and onset vs 18 mo: §). Statistics: Mann-Whitney test (*$P < 0.05$, #$P < 0.01$, §$P < 0.001$, ¥$P < 0.0001$). BMI, body mass index; HbA1c, glycated hemoglobin; IDDAA1c, insulin dose-adjusted HbA1c; ND, not determined.

control subjects were selected to be included in the ELISA experiments, whereas the remaining 30 patients and 16 control subjects were included in the leukocyte subsets' analysis. Patients were considered to be in PR when they fulfilled one or both of the two accepted criteria. PR was defined as <0.5 UI/kg/d and <7% HbA1c, or <9 insulin dose-adjusted HbA1c[3] (IDDAA1c). All the participants gave informed consent, and the study was approved by the Committee on the Ethics of Research of the Germans Trias i Pujol Research Institute and Hospital.

ELISA. ELISA method was used to determine plasmatic concentrations of TGF-β (ThermoFisher Scientific, Waltham, Massachusetts), betatrophin (Wuhan Eiaab Science, Wuhan, China), and HLA-G (sHLA-G, Enzo Life Sciences, Farmingdale, New York) in plasma samples obtained from control subjects and patients with T1D at the onset and at 6, 12, and 18 months of progression of the disease. Basic analytical characteristics of the methods were: for betatrophin, detection range: 78−5000 pg/mL and sensitivity: >0.051 pg/mL; for TGF-β, detection range: 8−1000 pg/mL and sensitivity: 8.6 pg/mL; for sHLA-G, detection range: 3.91−125 U/mL, and sensitivity: 0.6 U/mL.

Flow cytometry. For the analysis of cellular subsets, fresh venous blood samples were collected in ethylene diamine tetra acetic acid tubes from control subjects and patients with T1D at the onset and at 6, 12, and 18 months of disease progression. Samples of 1 mL of whole blood were washed and erythrocytes were lysed (Lysing Buffer, BD Biosciences, San Jose, California). A sample of 100 μL was then marked with a panel of different monoclonal antibodies (BD Biosciences) at room temperature and protected from light for 20 minutes. The panels, detailed in Table 2, were built as follows: (1) Th17 lymphocytes panel[16]: CD4 V450, CCR6 PE, CCR7 PECy7, and CCR4 AF647; (2) Treg panel[17]: CD45 FITC, CD3 V450, CD4 PerCPCy5.5, CD25 PE, CCR4 PECy7, CD127 AF647, CD45RO APCH7, HLA-DR V500; (3) T lymphocyte maturation stages panel[16]: CD3 V500, CD4 PerCPCy5.5, CD8 APCH7, CD45RA FITC, PTK7 PE, CCR7 PECy7, CD31 AF647, CD27 BV421; (4) Transitional B cell panel[16]: CD19 PerCPCy5.5, CD24 FITC, CD38 PE, CD27 APC; (5) B lymphocyte maturation stages panel[16]: CD3 V450, CD19 V500, CD27 APC, CD21 PE, IgD FITC, IgM PerCPCy5.5; (6) γδ T cell panel[16]: CD3 PerCP, γδ TCR PE, αβ TCR FITC, CD8 APCH7, CD4 V450 and (7) DC/Monocytes/NK[17]: CD45 AF700, CD3 APCH7, CD19 APCH7, CD14 V450, CD16 APC, CD11c PECy7, CD123 PerCPCy5.5, CD56 PE, HLA-DR V500, Slan FITC. At least 10,000 leukocyte events per sample were acquired using FACS Canto II and LSR Fortessa Flow Cytometers (BD Biosciences). Corresponding fluorescence minus one staining was used as control. The gating strategy to analyze specific leukocyte subsets was based on international consensus.[18] Absolute counts (cells/μL) were analyzed for all subsets using Perfect Count Microspheres (Cytognos SL, Salamanca, Spain). Data were analyzed using FACSDiva software (BD Biosciences).

Quantitative RT-PCR. RNA was isolated using RNeasy Micro Kit (QIAGEN) and reverse-transcribed with a High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). cDNA synthesis was carried out using random hexamers (0.5 mg/mL, BioTools, Valle de Tobalina, Madrid, Spain) and reverse transcriptase Moloney murine Leukemia virus (200 U/mL, Promega, Madison, Wisconsin). Quantitative RT-PCR assays were performed on a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using SYBR Green I Master mix (Roche Diagnostics) and specific primers for *HLA-E, HLA-F, TLR3, LYZ, CD36, IL-8,* and *CXCL1* (Invitrogen, Life Technologies, Gaithersburg,

Table 2. Lymphocyte subsets analyzed in pediatric patients with T1D and their markers according to international consensus[18]

Lymphocyte subset	Phenotype	Reference subset
aTreg	$CD3^+CD4^+CD127^{low}CD25^+$ $CCR4^+$ $CD45RO^+HLADR^+$	mTreg
mTreg	$CD3^+CD4^+CD127^{low}CD25^+$ $CCR4^+$ $CD45RO^+$	$CD3^+CD4^+$
Th17	$CD4^+$ $CCR7^-$ $CCR4^+$ $CCR6^+$	$CD4^+$
Breg	$CD19^+CD27^+CD24^{hi}$	$CD19^+$
NKreg	$CD3^-CD19^-CD56^+CD14^-CD56^{br}CD16^-$	NK cells
Monocytes	$CD3^-CD19^-CD14^+$	$CD3^-CD19^-$
$CD14^+CD16^-$	$CD3^-CD19^-CD14^+CD16^-$	Monocytes
Total $CD27^+$	$CD19^+CD27^+$	$CD19^+$
Total B transitional	$CD19^+CD27^-CD24^{hi}CD38^+$	$CD19^+$
B transitional high	$CD19^+CD27^-CD24^{hi}CD38^{hi}$	$CD19^+$
B transitional low	$CD19^+CD27^-CD24^{hi}CD38^{lo}$	$CD19^+$
αβ	$CD3^+$ $\alpha\beta^+$ $\gamma\delta^-$	$CD3^+$
$CD3^+$ DP	$CD3^+CD4^+CD8^+$	$CD3^+$
γδ $CD8^+$	$CD3^+$ $\alpha\beta^-$ $\gamma\delta^+CD8^+$	Total γδ
$CD3^+CD4^+$	$CD3^+CD4^+$	Total lymphocytes
Recent thymic emigrants	$CD3^+CD4^+CCR7^+CD45RA^+CD31^+PTK7^+$	$CD4^+$ Naive
$CD4^+$ CM	$CD3^+CD4^+CCR7^+CD45RA^-CD27^+$	$CD3^+CD4^+$
$CD4^+$ EM	$CD3^+CD4^+CCR7^-CD45RA^-$	$CD3^+CD4^+$
$CD4^+$ EM $CD27^+$	$CD3^+CD4^+CD27^+CCR7^-CD45RA^-$	$CD3^+CD4^+$
$CD4^+$ EMRA	$CD3^+CD4^+CCR7^-CD45RA^+$	$CD3^+CD4^+$
$CD4^+$ EMRA $CD27^+$	$CD3^+CD4^+CD27^+CCR7^-CD45RA^+$	$CD3^+CD4^+$
$CD4^+$ EMRA $CD27^-$	$CD3^+CD4^+CD27^-CCR7^-CD45RA^+$	$CD3^+CD4^+$
$CD3^+CD8^+$	$CD3^+CD8^+$	Total lymphocytes
$CD8^+$Naive	$CD3^+CD8^+CCR7^+CD45RA^+CD27^+$	$CD3^+CD8^+$
$CD8^+$ CM	$CD3^+CD8^+CCR7^+CD45RA^-CD27^+$	$CD3^+CD8^+$
$CD8^+$ EMRA	$CD3^+CD8^+CCR7^-CD45RA^+$	$CD3^+CD8^+$
$CD8^+$ EMRA $CD27^+$	$CD3^+CD8^+CD27^+CCR7^-CD45RA^+$	$CD3^+CD8^+$

aTreg, activated regulatory T cell; mTreg, memory regulatory T; Breg, regulatory B; NKreg, regulatory Natural Killer; DP, double positive; CM, central memory; EM, effector memory; EMRA, $CD45^+$ effector memory.

Maryland) (Supplementary Table 1). Relative quantification was performed by normalizing the expression of each gene of interest to that of the housekeeping *HPRT* gene following the 2-DDCt method.[19]

Statistical analysis. The statistical analysis was performed using Prism 7.0. Software (GraphPad software Inc., San Diego, California). For comparisons of paired data, a nonparametric Wilcoxon test was used, whereas a nonparametric Mann-Whitney test was used for comparisons of unpaired data. To find statistically significant correlations between parameters, Spearman's test was used. A P value of <0.05 was considered significant.

RESULTS

Clinical features of pediatric patients with T1D throughout the study. No statistical differences between groups were found in age and BMI; however, they were found in terms of insulin dose, HbA1c values and in IDDAA1c values (Table 1).

Circulating TGF-β levels decrease during PR stage. Circulating TGF-β concentration was determined in 10 control subjects and in 22 patients with T1D. Samples from these patients were obtained at 3 checkpoints: clinical onset ($n = 14$), 6 months ($n = 13$), and 12 months ($n = 13$) of progression of the disease. TGF-β concentration was significantly decreased at the checkpoint of first year when compared to the onset (Fig 1, A). Then, the possible correlation between this alteration and PR was evaluated. In fact, there is a significant decrease in circulating levels of TGF-β in patients with PR at 6 and 12 months of disease progression (Fig 1, B). In addition, patients at onset were divided in 2 groups (PR or not PR) and TGF-β levels were compared to understand whether those with PR have a less aggressive T1D onset or rather have a real different evolution of their diabetes. The results showed that both groups have similar circulating TGF-β concentration at the onset, independenlty from T1D progression. These results show a differential pattern of circulating TGF-β that discriminates between patients with or without spontaneous PR. Patients with PR do not have a less aggressive T1D onset, but a real different evolution of their diabetes.

Plasmatic concentration of betatrophin is increased in pediatric patients with T1D. Plasma betatrophin levels (pg/mL) were significantly increased in patients with T1D at

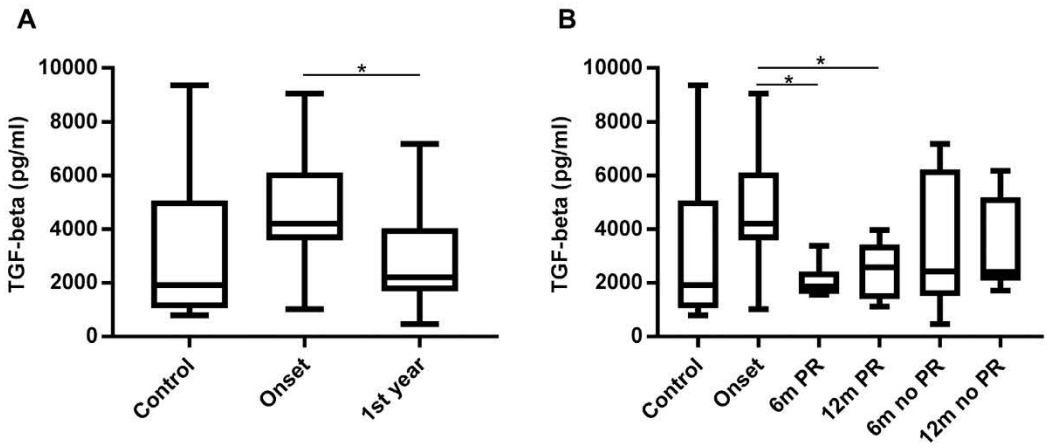

Fig 1. Plasma levels of TGF-β in pediatric patients with T1D. *A*, Concentration of TGF-β (pg/mL) in plasma of control subjects and patients with T1D at different checkpoints (onset and first year of progression). Data presented as plot box and whiskers (Tukey). (*$P < 0.05$, Mann-Whitney test). *B*, Circulating TGF-β concentration (pg/mL) in plasma of control subjects and patients with T1D at different checkpoints (onset, 6, and 12 months) grouped with and without spontaneous partial remission (PR) development at 6 months. Data presented as plot box and whiskers (Tukey). (*$P < 0.05$, Mann-Whitney test).

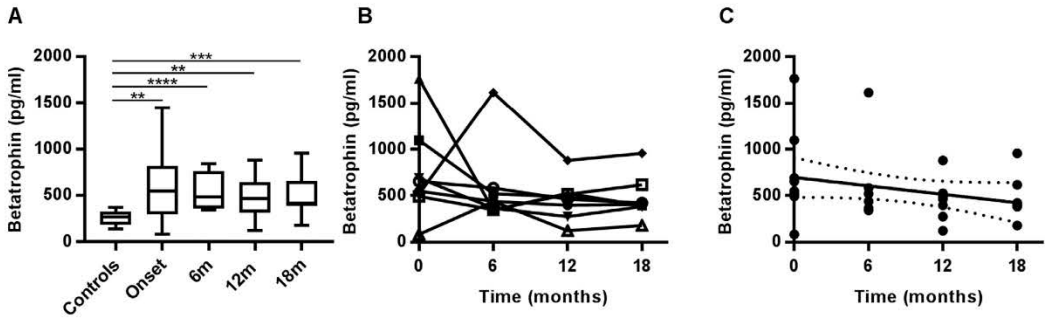

Fig 2. Plasma levels of betatrophin in pediatric patients with T1D. *A*, Circulating betatrophin concentration (pg/mL) in plasma of control subjects and patients with T1D at different checkpoints (onset, 6, 12, and 18 months of progression). Data presented as plot box and whiskers (Tukey). Statistical differences were found between patients and controls (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, Mann-Whitney test). *B*, Follow-up of the circulating plasma betatrophin concentration (pg/mL) of the patients with T1D in partial remission stage. *C*, Linear regression of the individual values of plasma betatrophin concentration (mg/dL) (Spearman $r = -0.3118$, P value = 0.0823).

the 4 different checkpoints (Fig 2, A) when compared to control subjects, whereas no statistical differences were found between patients at different disease stages. However, 12 months after the onset of the disease a trend to decrease betatrophin concentration was observed ($P = 0.057$). No statistical differences were found when grouping separately patients with and without spontaneous PR at 6 months.

To determine betatrophin levels in spontaneous PR, 8 patients with T1D showing PR criteria at 6 months were selected from the initial group of 22 patients (Table 3) and follow-up was performed during 18 months after diagnosis. No statistical differences were found in BMI between groups, although they were observed in insulin dose, HbA1c and in IDDAA1c. No statistical differences were found in circulating betatrophin levels in plasma between the 4 different checkpoints in PR patients (Fig 2, B), although a tendency for betatrophin to decrease was observed during disease progression ($P = 0.0823$) (Fig 2, C).

The correlation between circulating betatrophin levels with BMI was explored both in general T1D and

Table 3. Clinical data for pediatric patients with T1D at disease onset, and at 6, 12, and 18 mo of disease progression included in the follow-up measurements of plasma betatrophin

	Onset	6 mo	12 mo	18 mo
n	8	8	8	8
Gender (M/F)	5/3	5/3	5/3	5/3
Age (years, mean ± SD)	9.63 ± 3.78	10.13 ± 3.78	10.63 ± 3.78	11.13 ± 3.78
BMI (kg/m^2)	17.06 ± 4.59	18.27 ± 2.33	17.57 ± 1.2	17.67 ± 1.41
Insulin dose (UI/kg/day)	0.72 ± 0.58	0.43 ± 0.25	0.66 ± 0.22	0.75 ± 0.31
HbA1c (%)	10.26 ± 1.66	6.84 ± 0.90	7.86 ± 1.10	8.23 ± 1.04
HbA1c (mmol/mol)	89 ± 18.1	51 ± 9.8	62 ± 12	66 ± 11.4
IDDAA1C (%)	13.15 ± 1.93	8.15 ± 1.19	10.58 ± 1.47	11.33 ± 1.05

Data presented as mean ± SD. No statistical differences were found in age between groups. Statistical differences were found in insulin (onset vs 6 mo: #, 6 mo vs 12 mo: *, 6 mo vs 18 mo: *); glycated hemoglobin (HbA1c) (onset vs 6 mo: #, onset vs 12 mo: *, onset vs 18 mo: *, 6 mo vs 12 mo: #, 12 mo vs 18 mo: #); and in insulin dose-adjusted HbA1c (IDDAA1c) (onset vs 6 mo: §, onset vs 12 mo: * and 6 mo vs 18 mo: §). Statistical differences calculated from Mann-Whitney test (*$P < 0.05$, #$P < 0.01$, §$P < 0.001$, ¥$P < 0.0001$). BMI, body mass index; HbA1c, glycated hemoglobin; IDDAA1c, insulin dose-adjusted HbA1c.

PR patients as well as in control subjects (Fig 3). No correlation was found between BMI-betatrophin levels at the onset stage in T1D patients or in PR patients. Finally, At 18 months, a statistically significant positive correlation was found between BMI-betatrophin levels both in general and in PR patients ($P = 0.0047$ and 0.0480, respectively).

Immune response-related molecules are altered at the onset of T1D. Different molecules involved in immune response were analyzed as potential biomarkers. These molecules were selected in basis of the altered expression in the pancreases from patients with T1D.[11] One of these molecules is HLA-G, considered an immunomodulatory molecule involved in tolerance and proposed as a pancreatic regulator.[20] However, circulating sHLA-G did not reveal significant differences between control subjects and patients at different stages (Fig S1, A). To gain further knowledge within the nonclassical HLA network, the expression of these molecules was also measured in peripheral blood mononuclear cells (PBMCs) from patients with T1D. Overexpressed mRNA levels of HLA-E were found in PBMCs from patients at the onset (Fig S1, B) when compared to control subjects. In contrast, mRNA levels of HLA-F were not altered in PBMCs from patients at the onset when compared to control subjects (Fig S1, C). The expression of other altered molecules such as IL-8, TLR3, and CXCL1 was increased in PBMCs from newly diagnosed patients (Fig S2). A positive correlation was found between the expression of 2 chemokines involved in leukocyte recruitment, IL-8 and CXCL1 (Fig S3), being moderately positive correlated in control subjects ($R^2 = 0.8615$) and strongly positive correlated ($R^2 = 0.9498$) in patients at the onset of T1D. The expression of other genes, such as CD36 and LYZ, in PBMCs was not significantly different than that of control subjects (Fig S2).

Regulatory leukocyte subsets are biomarkers of T1D onset and progression stages. No statistically significant differences were found in age and BMI between groups (Table 4). Lymphocyte subsets involved in different immunoregulation strategies were studied, including regulatory T cells (Treg), both activated (aTreg) and memory (mTreg), Th17 cells (which contain a regulatory Th17 subset[21]), regulatory B cells (Breg), and regulatory Natural Killer cells (NKreg) (Fig 4). No correlation was found between these subsets (% and numbers) and the age of the subjects in the different groups, ie, controls, onset, first year, and second year.

Different percentages of the Treg subset including both aTreg and mTreg were observed at each T1D stage (Fig 4, A–D). The percentage of aTreg was increased during the first year of disease in comparison to the second year and control subjects (Fig 4, B), whereas the percentage of mTreg was decreased only at the onset when compared with control subjects (Fig 4, D). In both cases the concentration was maintained along the different groups (Fig 4, A and 4, C). The Th17 cell subset, which includes a specific and minority Th17 regulatory subset, showed an increased percentage during the first year of progression when compared to the second year (Fig 4, F). The corresponding cell concentration was maintained during T1D progression, at levels similar to those found in control subjects (Fig 4, E). The Breg cell subset was found increased both in concentration and in percentage during the first year of disease (Fig 4, G and H). Finally, the percentage of NKreg cells was increased during the second year when compared to the first year of disease (Fig 4, I and J). In summary, an increase in regulatory T, B and NK subsets was found during T1D progression, reflecting the attempts to restore tolerance to self during these early stages.

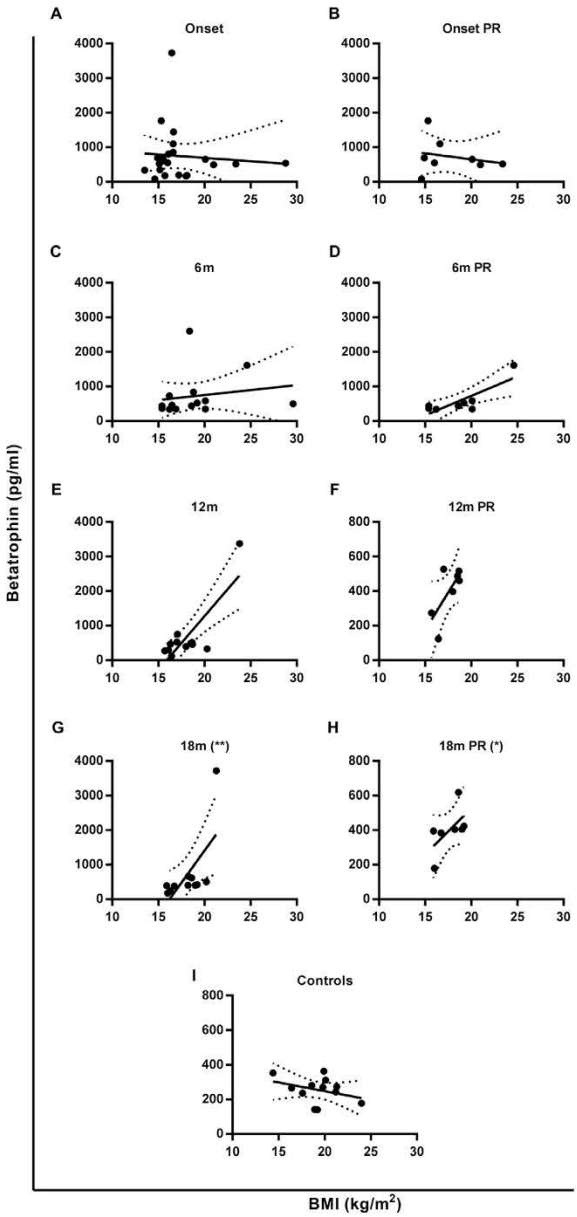

Fig 3. Correlation of betatrophin levels with BMI in pediatric patients with T1D at different stages of the disease. Linear regression of circulating plasma betatrophin concentration (pg/mL) vs BMI (kg/m^2) in patients with T1D and control subjects. *A*, Patients with T1D at the onset (Spearman $r = -0.00226$, P value = 0.9999). *B*, Patients who underwent partial remission (PR), at the onset of T1D (Spearman $r = -0.1429$, P value = 0.7520). *C* and *D*, Patients at 6 months of disease progression (Spearman $r = 0.634$, P value = 0.1705) and those who underwent PR (Spearman $r = 0.3877$, P value = 0.1009). *E* and *F*, Patients at 12 months of disease progression (Spearman $r = 0.4895$, P value = 0.1098), and those who underwent PR (Spearman $r = 0.5$, P value = 0.2667). *G* and *H*, Patients at 18 months of disease progression (Spearman $r = 0.8$, P value = 0.0047, **) and PR group (Spearman $r = 0.7857$, P value = 0.0480, *). *I*, Control group (Spearman $r = -0.07692$, P value = 0.8171).

Table 4. Clinical data for controls, and patients at onset, first and second year of T1D progression included in lymphocyte subsets analysis

	Control	Onset	1 y	2 y
N	16	11	12	7
Gender (M/F)	9/7	6/5	3/9	1/6
Age (years, mean ± SD)	8.58 ± 4.42	8.69 ± 5.16	10.20 ± 3.87	10.01 ± 2.78
BMI (kg/m^2)	19.43 ± 1.26	18.24 ± 3.10	17.53 ± 2.67	18.51 ± 2.13
Insulin dose (UI/kg/day)	ND	0.69 ± 0.27	0.62 ± 0.20	0.83 ± 0.23
HbA1c (%)	ND	8.87 ± 1.98	7.86 ± 1.17	7.82 ± 1.68
HbA1c (mmol/mol)		73 ±21.58	62 ±12.75	62 ±18.3
IDDAA1C (%)	ND	10.11 ± 13.21	8.93 ± 11.79	7.09 ± 15.25

Data presented as mean±SD. Statistical differences were found in BMI (control subjects vs patients with T1D during the first year: (*)). Statistical differences calculated using the Mann-Whitney test (*$P < 0.05$). ND, not determined.

Low circulating monocyte count is associated with T1D onset and second year of progression. Alterations in monocyte counts were found at the early stages of T1D (Fig 5). Total monocyte count showed a trend to be lower at the onset of the disease when compared to control subjects, but at the second year of T1D progression differences were significant (Fig 5, A and B). Within this monocyte family, the numbers of classical monocytes ($CD14^+CD16^-$) confirmed these results and also showed a decrease at the onset of the disease (Fig 5, C), but this was not reflected in the percentage (Fig 5, D). The nonclassical monocyte subset ($CD14^+CD16^+$) showed no statistical differences or tendencies between the different groups (data not shown). In summary, the concentration of total monocytes and its major subpopulation, the classical $CD14^+CD16^-$ monocyte subset, was diminished at the early stages of diabetes. Since monocytes are precursors of antigen-presenting cells, their decrease in periphery at the second year of disease progression suggests an active extravasation to target tissues, probably to contribute to the chronification of the autoimmune response.

B lymphocyte subsets are quantitatively altered at the onset and at early stages of T1D progression. The analysis of B lymphocytes at different maturation stages, memory/activation subsets, and B transitional lymphocytes, revealed quantitative alterations during the first 2 years of disease progression (Fig 6). The concentration of $CD27^+$ B cells was increased at the first year of disease when compared to the onset and to the second year of disease. However, it was not reflected in the percentage (Fig 6, A and B). The total B transitional subset also showed an increase in concentration during the first year of disease when compared to the second year and to control subjects, but this alteration was not observed in the percentage (Fig 6, C and D). Within the B transitional population, alterations were found both in B transitional high and low subsets (Fig 6, E–H), previously referred as T1 and T2 in the literature. A decrease in B transitional high subset counts was found at the second year when compared to control subjects and to the first year of disease (Fig 6, E), but this change was not reflected in the percentage (Fig 6, F). Regarding B transitional low subpopulation, the amount of these cells was lower at the second year of disease when compared to the first year (Fig 6, G and H). This subset includes functional regulatory B cells and despite its role is not completely understood, their increase may reflect the attempts to restore self-tolerance during T1D early stages.

T lymphocyte subsets are quantitatively altered at the onset and at early stages of diabetes progression. Because T cells are the final effector cells in T1D, both αβ and γδ T cell populations were screened (Fig 7). The concentration and percentage of αβ T cell subpopulation (Fig 7, A and B) were decreased at the second year of disease when compared to control and other disease stages. Among different αβ T cells subsets, only double positive T lymphocytes were decreased in counts at the second year when compared to the first year of disease (Fig 7, C) but not in percentage (Fig 7, D). Regarding the γδ $CD4^+$ T cell subset, no differences were found among different groups (data not shown). The $CD8^+$ γδ subset was decreased in concentration at the first year of progression when compared to the second year, and the percentage tended to decrease during disease progression (Fig 7, E and F). In summary, alterations in total αβ and γδ T cell subpopulations were observed during 2 years of T1D progression.

The different T lymphocyte maturation stages were determined and showed alterations at the onset and at the different stages of T1D (Figs 8–10). Only the total $CD3^+$ $CD4^+$ T lymphocyte population concentration was found decreased at the onset of T1D when compared to control subjects and the first year of disease (Fig 8, A and B). Within this population, the central memory (CM) $CD27^+$ subset revealed alterations both in concentration and percentage. There was an increase in concentration in the second year when compared to

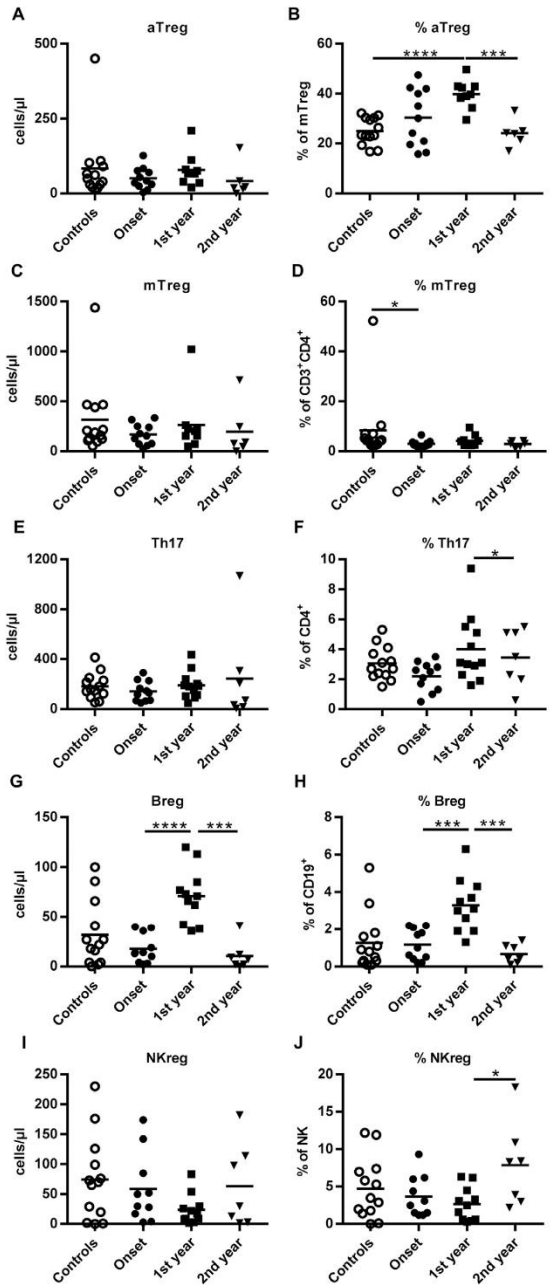

Fig 4. Concentration and percentage of regulatory lymphocyte subsets in peripheral blood from pediatric patients with T1D at different stages of the disease. Concentration (cells/µL) and percentage (%) of *A* and *B*, activated T regulatory cells (aTreg), *C* and *D*, memory T regulatory cells (mTreg), *E* and *F*, Th17 cells, *G* and *H*, B regulatory cells (Breg), and *I* and *J*, Natural Killer regulatory cells (NKreg). White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis. (*$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$, Mann-Whitney test).

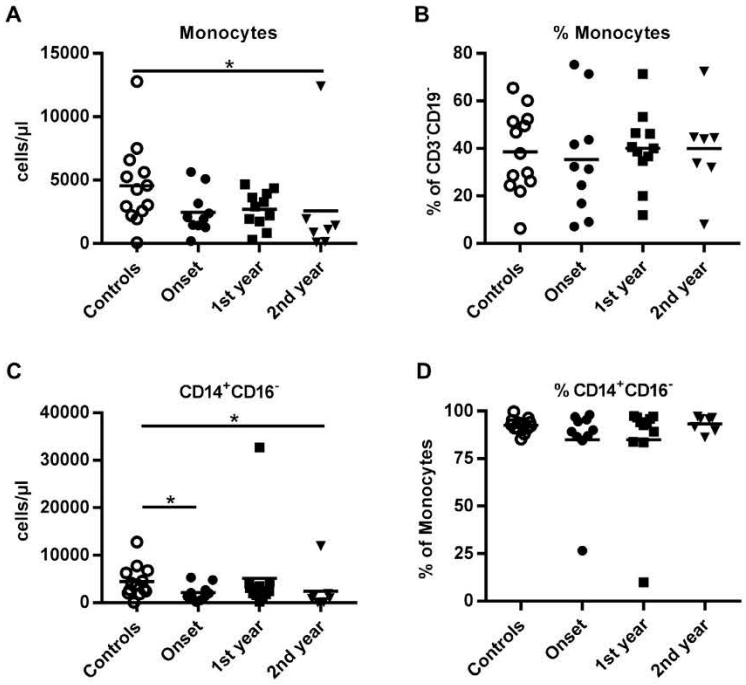

Fig 5. Concentration and percentage of monocytes in peripheral blood from pediatric patients with T1D at different stages of the disease. Concentration (cells/µL) and percentage (%) of *A* and *B*, total monocytes, and *C* and *D*, its major subset, classical monocytes. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis. (*$P < 0.05$, Mann-Whitney test).

the first year of disease (Fig 8, C). Also, there was an increase in percentage at disease onset when compared to control subjects, with a tendency to decrease with progression of diabetes until a rate similar to control group at second year (Fig 8, D). The PTK7$^+$ subpopulation, included in the CD4$^+$ naive subset, revealed an increase in percentage during the second year of disease (Fig 8, F).

The CD4$^+$ T lymphocyte effector memory subsets were analyzed (Fig 9). Total CD4$^+$ effector cells expressing the marker CD45RA (EMRA) subsets showed a remarkable decrease in both concentration (Fig 9, A) and percentage at the onset of the disease (Fig 9, B). Furthermore, this decrease in concentration was also observed in both CD4$^+$ EMRA subsets: CD27$^+$ (Fig 9, C) and CD27$^-$ (Fig 9, E), but not in their percentage (Fig 9, D and Fig 9, F, respectively). In addition, the CD4$^+$ EM cell subset revealed lower counts at disease onset when compared to the first year of disease (Fig 9, G). This is due to alterations in the CD4$^+$ EM CD27$^+$ subset (Fig 9, I). This effect was not reflected in percentage (Fig 9, H and Fig 9, J, respectively).

Besides the CD4$^+$ population, CM and EM in the CD8$^+$ subsets were also altered (Fig 10). Total counts of CD3$^+$ CD8$^+$ T cells were decreased at the onset of the disease when compared to control subjects, and normalized after diagnosis (Fig 10, A and B). Regarding the CM subset, a decrease in counts and percentage, was observed during the second year of progression (Fig 10, C and D). The analysis of the CD8$^+$ naïve cells subset showed a decrease in cell number at the onset and during the first year of disease (Fig 10, E), in agreement with the data of CD3$^+$ CD8$^+$ population (Fig 10, A). Moreover, a decrease in EMRA CD8$^+$ T cell counts (Fig 10, G) was observed, but not in percentage (Fig 10, H). This decrease in concentration was observed at T1D onset in the EMRA CD27$^+$ subset (Fig 10, I), whereas in percentage, a decrease was found at the first year of the disease (Fig 10, J). In summary, alterations in the CM and EM lineages of T lymphocytes and their maturation stages have been detected at early T1D progression. All these cell subsets are commonly found in the spleen and in the lymph nodes, and the decrease in peripheral blood

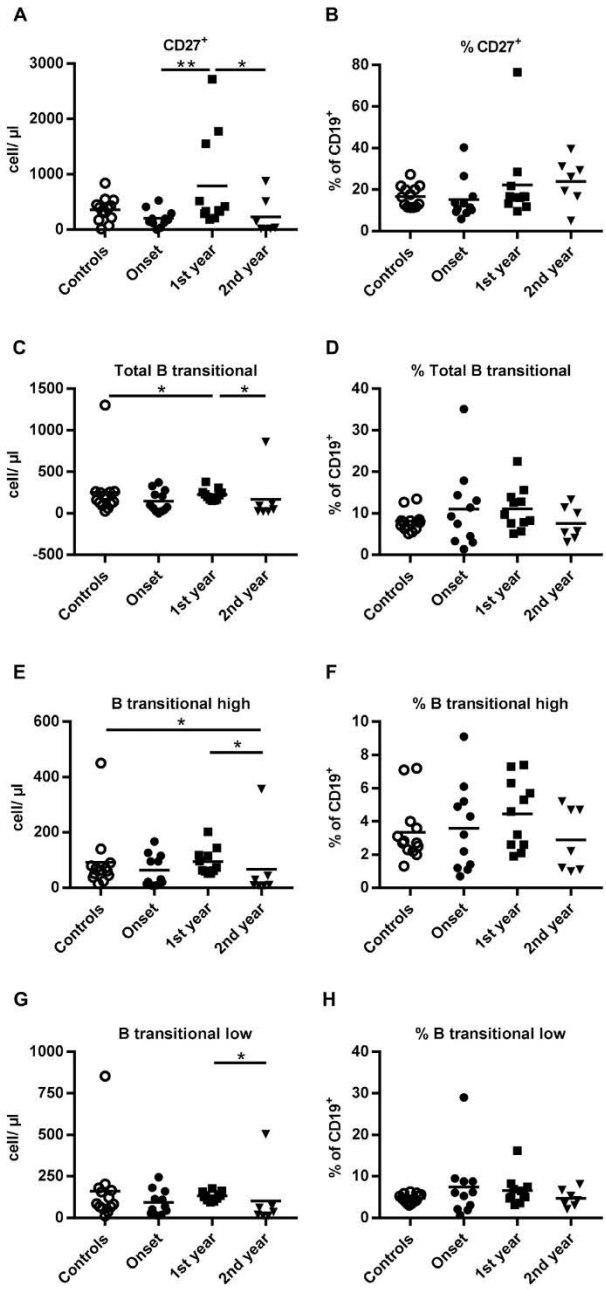

Fig 6. Concentration and percentage of B lymphocytes in peripheral blood from pediatric patients with T1D at different stages of the disease. Concentration (cells/µL) and percentage (%) of *A* and *B*, total $CD27^+$ peripheral blood mononuclear cells (PBMCs), *C* and *D*, total B transitional lymphocytes, *E* and *F*, B transitional high, and *G* and *H*, B transitional low. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis. ($^*P < 0.05$, $^{**}P < 0.01$, Mann-Whitney test).

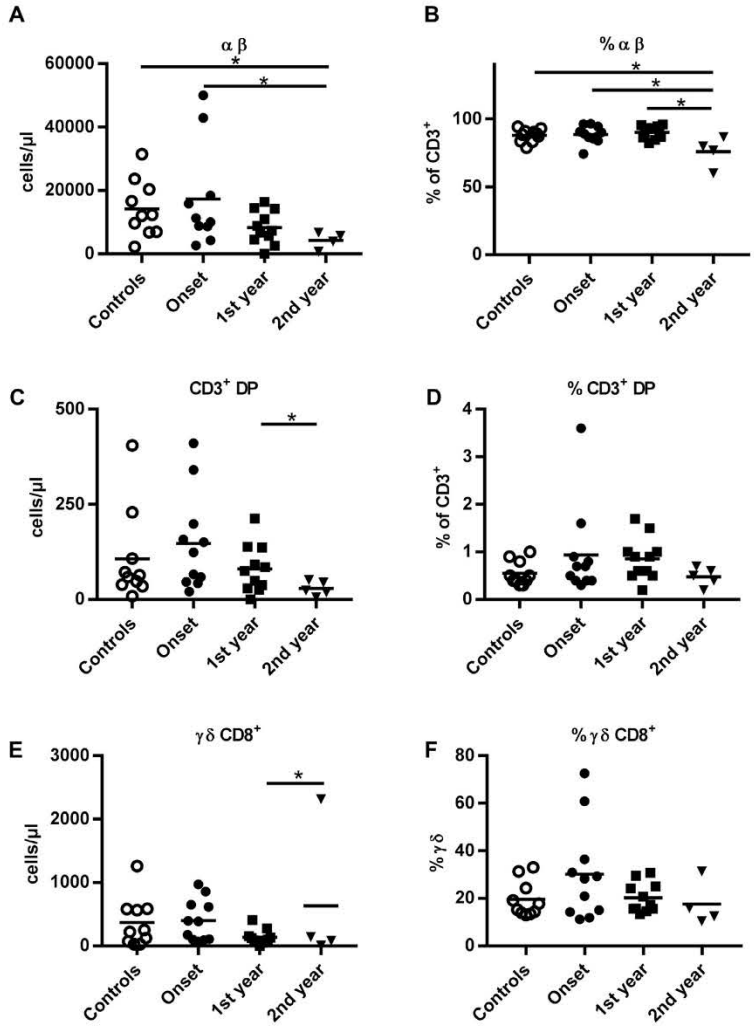

Fig 7. Concentration and percentage of αβ and γδ T lymphocytes in peripheral blood from pediatric patients with T1D at different stages of the disease. Concentration (cells/μL) and percentage (%) of *A* and *B*, total αβ, *C* and *D*, its subset $CD3^+$ double positive (DP), and *E* and *F*, γδ $CD8^+$. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis. (*$P < 0.05$, Mann-Whitney test).

of EM and EMRA T cells both in the $CD4^+$ and the $CD8^+$ T cell subsets at the onset could reflect the need for a compensatory mechanism, due to an active migration to target tissue.

DISCUSSION

It is known that the lack of biomarkers of T1D stages hinders the proper stratification patients, the prediction of secondary complications, and the discovery of optimal checkpoints for potential therapies. Metabolic parameters are currently the most reliable biomarkers, and during disease progression, C-peptide levels are considered to reflect residual insulin storage. Although this is a good biomarker for endogenous insulin production and secretion, the sensitivity of the technique prevents the assessment of low values[22] and most pediatric patients show undetectable C-peptide levels at disease onset.[6] Thus, novel biomarkers for stratifying subjects and for

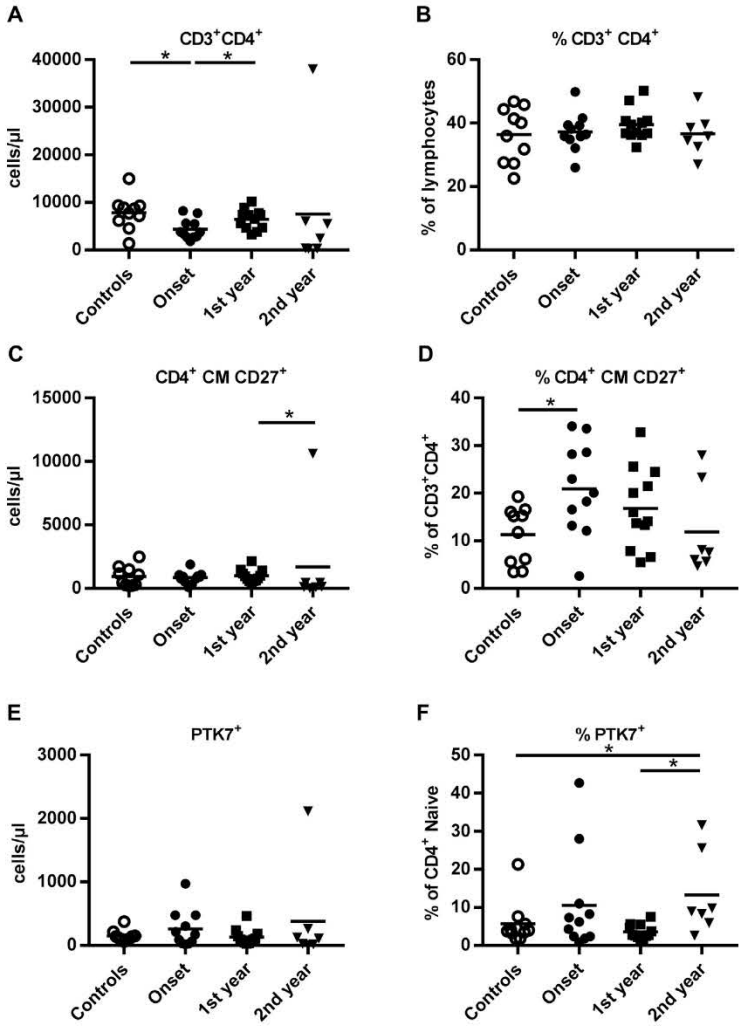

Fig 8. Concentration and percentage of $CD4^+$ T lymphocyte subsets. Concentration (cells/µL) and percentage (%) of *A* and *B*, total $CD3^+CD4^+$, *C* and *D*, $CD4^+$ CM $CD27^+$ and *E* and *F*, its major subset $PTK7^+$. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis (*$P < 0.05$, Mann-Whitney test).

immunomonitoring are needed. This article presents a screening of candidate biomarkers in pediatric patients with T1D, with special interest in the early stages.

There are many and diverse immune biomarkers of T1D.[23] In this pilot study, aimed at determining differential molecular and cell patterns in children diagnosed with T1D in our geographic area, potential candidates have been considered. Betatrophin, a promising biomarker in long-standing adult patients with T1D,[8] showed a triplicated concentration in plasma at the onset of pediatric T1D. Furthermore, those increased levels remain steady during the early progression of the disease, including the PR stage. Thus, circulating betatrophin levels in plasma do not distinguish PR stage. Our results of the correlation between a metabolic parameter such as BMI and circulating betatrophin levels are in keeping with those described in T1D[8] and T2D[24] that suggest that hyperglycemia may be the pivotal point for betatrophin upregulation in diabetes.

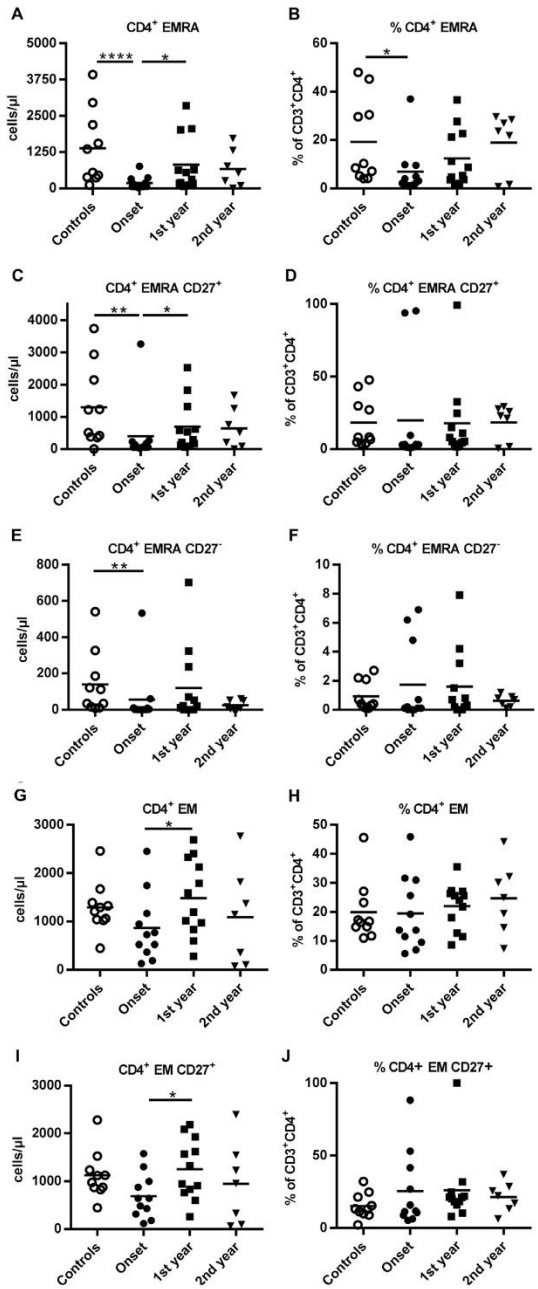

Fig 9. Concentration and percentage values of $CD4^+$ and its effector memory (EM) subset, included in the T lymphocytes maturation stages. Values in concentration (cells/µL) and percentage (%) of *A* and *B*, total EMRA $CD4^+$, *C* and *D*, $CD4^+$ EMRA $CD27^+$, *E* and *F*, $CD4^+$ EMRA $CD27^-$, *G* and *H*, total $CD4^+$ EM, and *I* and *J*, $CD4^+$ EM $CD27^+$. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis ($^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, Mann-Whitney test).

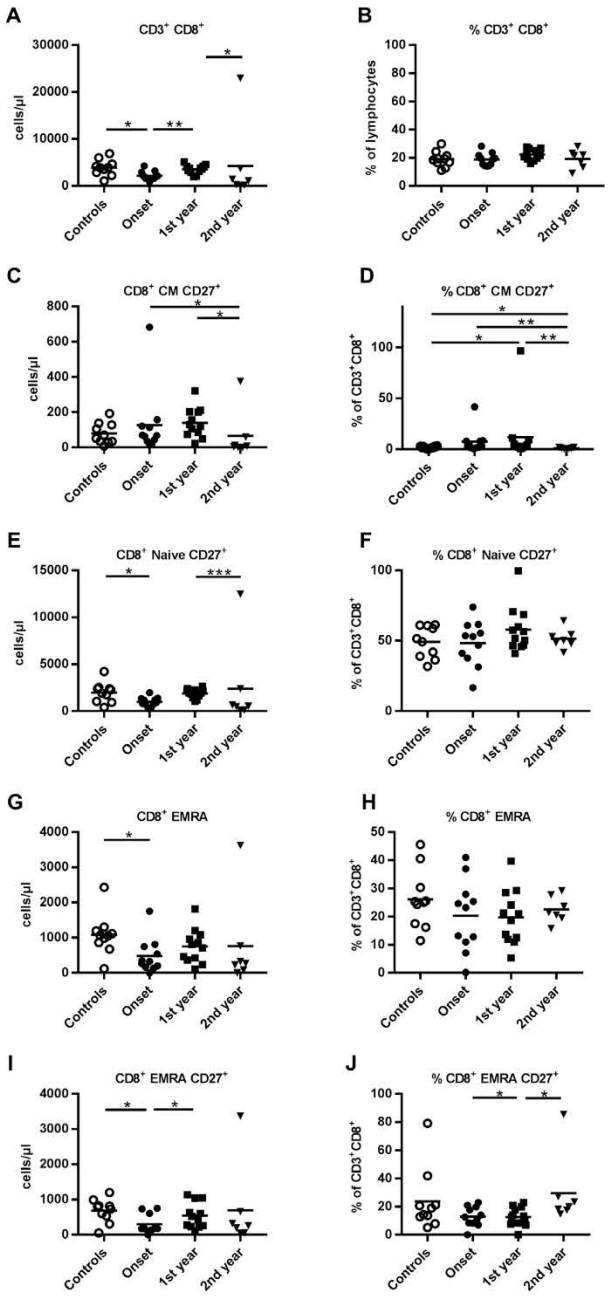

Fig 10. Concentration and percentage values of $CD8^+$ lymphocytes and their subsets, included in the T lymphocytes maturation stages. Values in concentration (cells/µL) and percentage (%) of *A* and *B*, total $CD3^+CD8^+$, *C* and *D*, $CD8^+$ CM $CD27^+$, *E* and *F*, $CD8^+$ Naïve CD27, *G* and *H*, total $CD8^+$ EMRA, and *I* and *J*, its subset $CD8^+$ EMRA $CD27^+$. White symbols represent control subjects, whereas black symbols represent patients with T1D at onset (dots), 1 year (squares) and 2 years (triangles) after diagnosis (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Mann-Whitney test).

Table 5. Summary of candidate biomarkers altered during pediatric T1D early progression

Candidate biomarker	Source	Stage	Outcome
Betatrophin	Plasma	Onset, 6, 12, and 18 mo of progression	Increased
TGF-β	Plasma	Partial remission	Decreased
aTreg (%)	Blood	First year of progression	Increased
Breg (% and counts)	Blood	First year of progression	Increased
NKreg (%)	Blood	Second year of progression	Increased

Then, the potential of other immune mediators whose expression was strongly altered in the pancreases from patients with T1D[11] was analyzed. Here we demonstrate for the first time a decrease in circulating TGF-β during PR when compared to the onset. This candidate biomarker for PR distinguishes pediatric patients until 1 year after diagnosis. Low TGF-β levels in PR could reflect the transient recovery of self-tolerance proposed at this stage.[1] In fact, TGF-β is increased in patients at the onset of T1D[10] reflecting a response to autoimmunity activation and correlating with the fact that hyperglycemia stimulates TGF-β expression.[25] Because many cells can secrete TGF-β, a deeper understanding of the mechanisms involved in the decrease of plasma concentration during PR is needed. In this stage, the remaining β-cells are still able to produce enough insulin and, in some cases, transient insulin-independence can be achieved. The identification of reliable biomarkers would allow for the identification and monitorization of PR. Because there are no differences in TGF-β levels when comparing patients at PR and non PR stages at 6 and 12 months of evolution of the disease, at present it would be useful for the monitoring of PR for each particular patient.

Additionally, lymphocyte cell subsets have been used as biomarkers for several immune mediated diseases. One of the main advantages of using them is the amount of given information due to the multiple parameters that can be analyzed in the sample, and its ease of tracking during disease progression. Lymphocyte regulatory subsets have been partially characterized in T1D. Since they are crucial in self-tolerance and show phenotypic, functional and migration alterations in autoimmunity,[26,27] this study aimed at determining the changes in regulatory leukocyte subsets during the first 2 years of T1D in children. Alterations were found in all regulatory leukocyte subsets both in concentration and percentage, reflecting a robust significance. Moreover, the alterations observed in one variable can be due to compensatory mechanisms undergone by other cell lineage subsets to accommodate the first change. Since lymphocyte subsets can only be determined in peripheral blood at a time, peripheral subsets are tightly regulated by their generation in primary lymphoid organs and by their migration to the target tissue, ie, the pancreas in T1D. The decrease of the percentage of mTreg at the onset of the disease in children is accompanied by a strong increase in aTreg at the first year. Since Tregs are crucial in self-tolerance, these data reflect the autoimmune attack at T1D onset as mTregs are reduced. The increase in aTregs during the first year of disease, which is also when the classical appearance of PR occurs, may reveal the attempts to restore self-tolerance. In this sense, a mathematical model explains that T1D onset is the consequence of an increase in effector T lymphocytes over Treg and that the PR stage may be due to an inversion in the frequencies of these subsets.[28] As for Breg and NKreg cell subsets, their role in T1D is not completely understood. This is the first report showing an increase of Breg and NKreg in early stages of pediatric T1D. Future studies may provide insight into the role of these cells in PR and T1D.

We are well aware of the limitations of this study. Despite a relatively small sample size, the *P* value of the here proposed biomarkers – betatrophin, TGF-β and regulatory T, B, and NK subsets – determines statistical differences. Furthermore, these candidates were selected from previous data and in basis of their biological effect. Future analysis of "big data" obtained from an independent cohort would be beneficial for both validation of these candidate biomarkers and for the screening of new ones. Nevertheless, the strength of the study is the identification of immunologic alterations in early stages of the disease, with special interest in the PR, an unexplored – and often undetected – phase of T1D. In our cohort PR was evident at 6 months after diabetes onset. Thus, we plan to include this time checkpoint for all patients and variables to be investigated in future studies.

Overall, the results (summarized in Table 5) reveal the characterization of the changes in these parameters during the different early stages of T1D in children – betatrophin, TGF-β and regulatory T, B and NK subsets – that could be used in clinical practice to characterize the progression of T1D. Moreover, and because PR may be an ideal stage for immunointervention, these candidate biomarkers can be considered for clinical trials. This study constitutes an open door, not only

to explore the underlying pathophysiology of the early stages of T1D, but also to stratify the patients for future therapeutic strategies.

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AUTHOR CONTRIBUTIONS

AV, MF, MM, SRF, RMA, MBR, RP, JB, and MVP designed the experiments, AV, MF, SRF, RMA, MBR, and RP performed the experiments, AV, SRF, and MVP wrote the manuscript, MM, DPB, JB reviewed the manuscript, BQS and AT contributed to the discussion. All authors revised the manuscript and gave final approval of the current version.

CONFLICT OF INTEREST STATEMENT

The authors have read the journal's policy on disclosure of potential conflicts of interest and all authors have disclosed any financial or personal relationship with organizations that could potentially be perceived as influencing the described research.

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SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2019.03.002.

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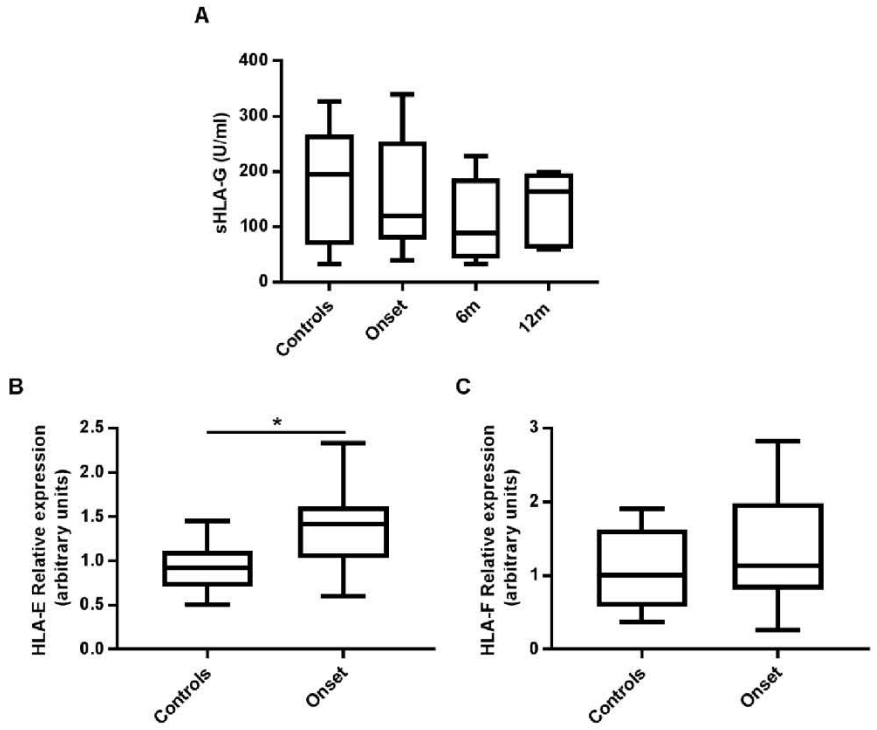

Figure S1. **Plasma levels of non-classical HLA protein and transcripts in patients at the onset of T1D.** (**A**) Plasmatic sHLA-G concentration in patients with T1D at the onset and controls. Data are represented as U/ml and plot box and whiskers (Tukey). (**B**) Relative gene expression of HLA-E in PBMCs from patients with T1D at the onset and control subjects analysed by qRT-PCR. Gene expression was normalized to *HPRT* (*$p < 0.05$, Mann-Whitney test). (**C**) Relative gene expression of HLA-F in PBMCs from patients with T1D at the onset and control subjects analysed by qRT-PCR. Gene expression was normalized to *HPRT* (Mann-Whitney test).

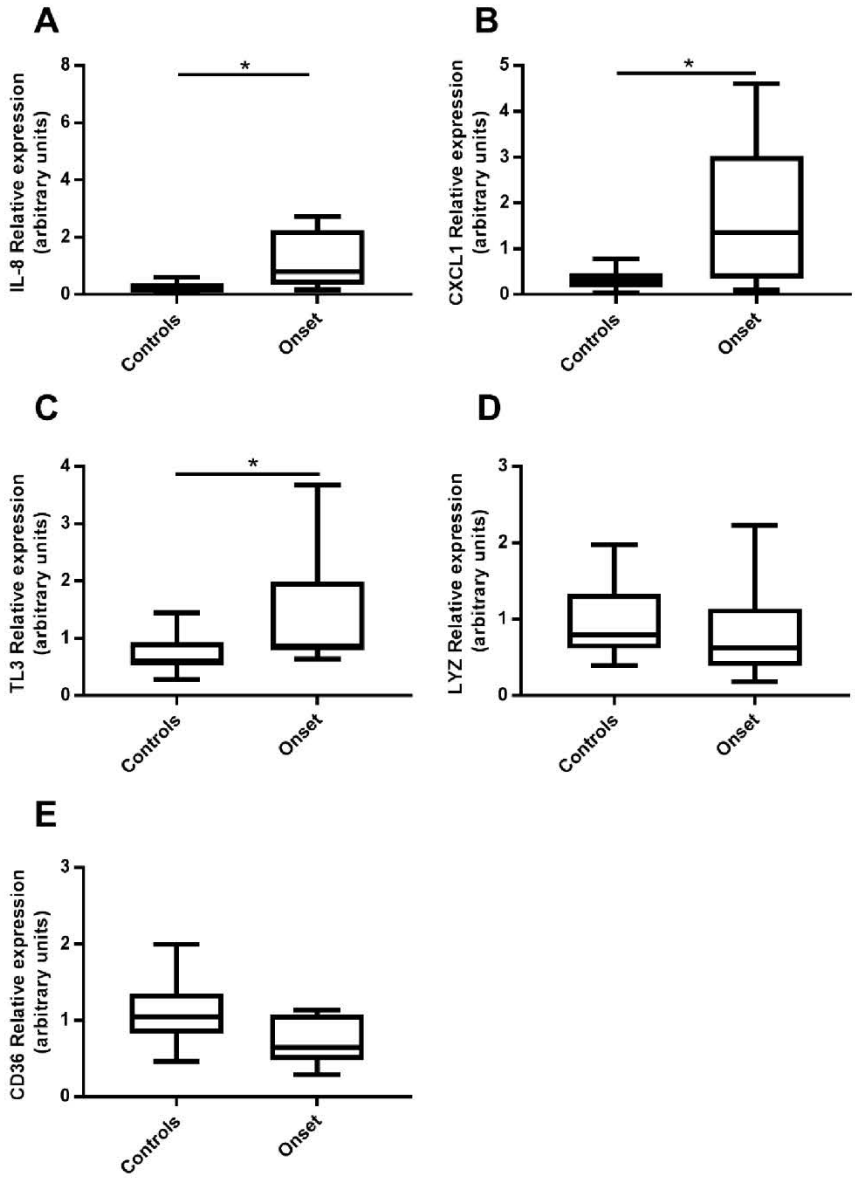

Figure S2. **Plasma levels of biomarker candidates in patients with T1D at the onset.** Relative gene expression (transcripts) of **(A)** *IL-8*, **(B)** *CXCL-1*, **(C)** *TLR3*, **(D)** *LYZ* and **(E)** *CD36* in PBMCs from patients with T1D at the onset and control subjects analysed by qRT-PCR. Gene expression was normalized to *HPRT* (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Mann-Whitney test).

Supplementary Table 1. List of primers used for quantitative reverse transcription–polymerase chain reaction (qRT-PCR).

Gene	Sense (5'-3')	Anti-sense (5'-3')	Amplimer length
HLA-E	TCTCCGAGCAAAAGTCAAA	GAGATGGGGTGGTGAGTC	160
HLA-F	AATGGGAAGGAGACGCTACA	CACAGCTCCAAGGACAACAAG	372
IL-8	TACTCCAAACCTTTCCACC	AACCCTACAACAGACCCAC	288
CXCL1	GTTAATATTTCTGAGGAGCCTGC	AAACAGTTACAAAACAGATGTGC	128
TLR3	ACACCATCTCAAAACTGGA	AAACACCCTGGAGAAAACT	397
LYZ	GGGCTTGTCCTCCTTTCTGTT	GTTGTAACCACTCTCCCATTTG	150
CD36	GACAGTTTTGGATCTTTGATG	CTTTGCTTAACTTGAATGTTG	76

DISCUSSION

DISCUSSION

Most of the work presented in this PhD thesis relies in the first part of the hypothesis: to consider that a novel regenerative agent can be identified by drug repurposing to promote β-cell regeneration, and thus be used to revert T1D in combination with a previously developed immunotherapy.

For that reason, and after our group developed an immunotherapeutic strategy based on liposomes encapsulating autoantigens, a first aim was the optimization of the immunotherapy in terms of the choice of the best autoantigen to encapsulate. This question was addressed in **Article 1:** *Preclinical evaluation of antigen-specific nanotherapy based on phosphatidylserine-liposomes for type 1 diabetes*.

Hence, the strategy consisted of encapsulating different peptides from autoantigens of T1D into PS-liposomes (Insulin, IA2, GAD65, and C-peptide). Because previously developed PSAB-liposomes consisted of a mix of two different PS-liposomes encapsulating either peptides from the A or B chain of insulin, the same system was used for the rest of autoantigens. C-peptide was the only autoantigen encapsulated as a unique peptide due to its reduced length compared to the rest of the autoantigens. In order to get insights into their preventive effect, different PS-liposomes were administered to prediabetic NOD mice and T1D incidence was assessed until 25 weeks of age. We decided this checkpoint because after 25 weeks of age, hyperglycaemia in NOD mice could be due to insulin-resistance and therefore not considered T1D.

The physicochemical properties of the resulting PS-liposomes were measured and found homogeneous in terms of diameter, polydispersity index and ζ–potential. It may indicate that the effect observed in the

incidence of T1D is due to the combination of PS-liposomes encapsulating autoantigens and not to other features related to the nanovesicle itself.

PSAB-liposomes encapsulating insulin peptides were the only that reduced T1D incidence. It would be likely due to the fact that insulin is the most expressed and specific autoantigen for β-cells (Nakayama 2011). Insulin is also the first autoantigen that elicits an autoimmune reaction during the natural progression of the disease, at a prediabetic stage (Nakayama 2011). This fact would also explain the reduction in the insulitis score levels upon PSAB-liposomes administration. Despite that, because the insulitis score is still performed in a semi-quantitative manner by a double-blind procedure, qualitative characterization of the infiltrating cells as well as bioimage analysis would help to determine the role of insulitis more precisely.

Surprisingly PSIA2- and PSGAD65- liposomes did not prevent T1D in NOD mice when compared to the sham group. One explanation would be that the IA2 molecule is not specific for β-cells (Solimena 1996, Takeyama 2009, and Mally 1996). Furthermore, other immunotherapies involving tolerogenic DCs loaded with GAD65 reduced their capability to prevent T1D in the NOD model, correlating well with our observations (Funda 2018). Finally, PSCpep-liposomes resulted also in a null reduction of diabetes incidence. The role of C-peptide as an autoantigen remains controversial. Moreover, C-peptide displayed a low encapsulation efficiency in liposomes. To determine if PSCpep- and PSIA2-liposomes have a detrimental effect on the incidence of T1D (as they showed an increased percentage of T1D individuals when compared to sham) it would be necessary to increase the number of treated mice.

Apart from the specific autoantigen containing liposomes, a mix comprising all of them was included in the experiment and named as a

cocktail of liposomes (Cock-lipo). This cocktail of liposomes contained all the above-mentioned liposomes in the same volume they were administered to other groups (200 µl). Consequently, there is a seven-fold reduction of every PS-liposome in Cock-lipo, and this dosage reduction may be an explanation for its lack of preventive effect. Thus, a new dosage and administration schedule would be required to asses if a combination of all the autoantigen containing PS-liposomes can prevent the disease.

A proper control for the Cock-lipo was included, consisting of all the peptides present in it but without being encapsulated in PS-liposomes (Cock-pep). The Cock-pep also showed a reduction in the incidence of diabetes when compared to the sham group and in the insulitis score. There has been previously described that the sole administration of autoantigens resulted in a tolerogenic effect in the NOD model (Peakman 2001). Similar outcomes were observed in the EAE model of autoimmune multiple sclerosis when the MOG peptide was administered (Pujol-Autonell 2017). It could be due to the fact that Cock-pep contains more autoantigen amount than Cock-lipo, that the first one reduces T1D incidence while no effect is observed with Cock-lipo. In addition, it would be interesting to test the effect of PS-liposomes encapsulating other autoantigens of T1D such as ZnT8. Besides, the effect of PS-liposomes encapsulating non-peptide autoantigens —like cfINS— remains to be studied.

The choice of PSAB-liposomes is a validation of previous studies (Pujol-Autonell 2015). Moreover, an additional confirmation would be desirable in other animal models, such as NSG mice transferred with PBMCs from diabetic NOD mice. It has been also observed that PSAB-liposomes administration at 8 weeks reduces the insulitis score at 25 weeks of age. Due to that, it would be of interest to evaluate this effect in a model of accelerated onset of diabetes —the NOD 8.3— upon different time-points

of administration. Additionally, fluorescent or radioisotope marked liposomes could be other approaches to track the encapsulated peptide, which could help to gain new insights into this efferocytosis mediated process. Taking this experiment into account, it would be easy to perform by co-culturing PSAB-liposomes — properly labelled on the surface and in the autoantigen— with human DCs *in vitro*.

Overall in this publication, it is shown that to broaden the range of autoantigens resulted in the choice of PSAB-liposomes as the best preventive option. Notwithstanding, PS-liposomes could be further optimized not only by means of the encapsulated peptides but also by adding novel find-me signals apart from PS. Additionally, this work supports previous data demonstrating that liposomes are safe products, even in clinical use (Bulbake 2017).

It is essential to hamper the chronic autoimmunity to avoid T1D but when the disease is well established it is also necessary to replace the damaged target tissue. For that reason, this or other immunotherapies should be combined with a regenerative strategy. The next aim was to identify the effects in β-cell regeneration of a currently used drug. That was addressed in **Article 2:** *Repurposed analog of GLP-1 ameliorates hyperglycaemia in type 1 diabetic mice through pancreatic cell reprogramming*. It is widely known that to develop from scratch a molecule targeting a pathway that may later result in a therapeutic effect is a long-time and expensive process. In order to overcome both aspects, drug repurposing was considered as a strategy to identify drugs that are clinically used and may promote β-cell regenerative properties.

Drug repurposing is an *in silico* technique intended to search for novel uses of already existing compounds that are available in the market. For instance

sildenafil, which was firstly used for heart failure was later repurposed for erectile dysfunction (Goldstein 2019). Additionally, thalidomide that was originally employed to treat nausea —especially on pregnancy— is currently available to treat leprosy (Teo 2002). The main advantage of drug repurposing is the ability to make use of an already-existing compound and hence its development and improvement are avoided. Furthermore, because the screening is performed among approved drugs it ensures the selected compound has successfully overcome the toxicity issues, being ready to use in patients. That is an important goal to perform translational medicine.

To find potential drugs that could perform β-cell regeneration, different target proteins known to be involved in 1) transdifferentiation from glucagon-producing α-cells, 2) neogenesis from ductal progenitors and 3) self-replication of pre-existing β-cells were selected. The drug repurposing screening was performed in close collaboration with Anaxomics Biotech SL., a systems biology company.

Overall, 22 approved compounds were identified (Annex 1) able to putatively promote β-cell regeneration through one or a combination of the three previously mentioned mechanisms. Among them, exenatide resulted in the highest predicted global efficacy value. Exenatide was the first designed and tested aGLP1, used to treat T2D especially if present with obesity (Aroda 2018). This drug scored a high predicted efficacy value (96.88), mainly by its contribution to transdifferentiation but scored poor values for both neogenesis and self-replication. Given that, a proof of concept experiment was performed using liraglutide, which is a recent aGLP1 widely used in the clinical practice (Jacobsen 2016).

Primarily, liraglutide was selected because of the effect in the improvement of the hyperglycaemia in a mice model but other repurposed drugs were also considered. Another option was pregabalin —used to treat neuropathic pain— because of its high predicted value for self-replication but not for neogenesis or transdifferentiation. Hence, pregabalin was predicted to act only on pre-existing β-cells without altering other cell type identities. Pregabalin was administered to NSG mice rendered diabetic and to spontaneously diabetic NOD mice following the same pattern than liraglutide alone and with PSAB-liposomes. Pregabalin alone or in combination with liposomes was unable to restore normoglycaemia even some animals delayed the development of sustained hyperglycaemia (Annex 2). Despite additional experiments are required to further demonstrate if this effect on the hyperglycaemia delay there exists, liraglutide was finally selected because of its sustained metabolic effect throughout the treatment.

The aGLP1 candidate compound selected among the drug list was tested in NSG mice rendered diabetic by STZ administration. This model allows the evaluation of the regenerative effect of the drug without interference of autoimmunity because NSG is an immunodeficient strain (lacking of T, B lymphocytes and NK cells). The effect of the drug in the amelioration of hyperglycaemia was assessed as well as the β-cell regenerative mechanisms. Notwithstanding, the effect of a given drug on the amelioration of hyperglycaemia is strongly dependent on both dosage and administration pattern. For that reason, drugs administered to NSG may have no impact depending on the dose and the administration schedule. Because repurposed drugs have been identified *in silico*, it is difficult to determine the dosage to observe an effect *in vivo*.

Taken this into account, a bottom-to-top strategy should intuitively be more useful to screen several drugs simultaneously. It would let to evaluate different increasing dosages and time-points in *in vitro* models and to select those promoting regeneration to further evaluate in the *in vivo* models. We tried to replicate the results of other authors employing this bottom-to-top strategy. Massive compound screening in engineered cell models revealed that molecules targeting the GABA A receptor in α-cells may downregulate *Arx* transcription factor thus promoting their conversion to β-cells (Li 2007 and Ben-Othman 2017). When GABA was administered to NSG mice following the same pattern and dosage as previously described (Ben-Othman 2017) it did not result in hyperglycaemia amelioration (Annex 3). Similar results were observed when GABA was administered in combination with PSAB-liposomes to NOD diabetic mice (Annex 3). Our results were replicated by other groups that did not find hyperglycaemia amelioration nor transdifferentiation in transgenic mice models (Van der Meulen 2018 and Ackerman 2018). In summary, that reveals that both strategies present their strengths and weaknesses, likely due to our lack of knowledge in human β-cell physiology and regeneration.

Finally, liraglutide was chosen because of the observed amelioration of hyperglycaemia during 30 days of continuous treatment in 50% of diabetic NSG treated mice. This amelioration resulted in both fasting conditions and upon glucose stimuli, confirming its robust effect. The factors determining which individuals are responders while the others are not remain to be elucidated. Regardless of all the mice are housed in identical conditions —thus excluding environmental factors— and treated animals are congenic —thus reducing genetic differences— that may point to some unknown factors. Whether STZ differentially affects the endogenous the β-cell pool could be a choice. Besides that, even drug administration issues could be

taken into account to explain these differences. At the biological level, the epigenetic diversity is the most evident factor that arises among different individuals. But even more importantly, it would be necessary to unravel the cross-talk signalling of the pathway triggered by the GLP1R (target of liraglutide) and its main metabolic regulators.

Due to the insulinotropic effect of liraglutide, its administration was compared with exogenous insulin in prediabetic NOD mice. Conversely to the expected, non-diabetic mice injected with liraglutide suffered from short-transient hyperglycaemia while all mice treated with insulin suffered hypoglycaemia. Since NOD mice display insulitis from 8 weeks of age (Alba 2004) it may reasonable to speculate that insulitis might impair insulin secretion. For that reason, the experiment was challenged with another strain, the C3 mice, free of insulitis and showing an increased α- and β-cell mass (Bock 2005). In this strain, the insulinotropic effect of liraglutide was demonstrated in all treated mice. Overall, the insulinotropic effect of liraglutide is maintained in physiological conditions but maybe impaired upon islet inflammation.

The next step in the characterization of liraglutide as a repurposed drug for T1D was to perform islet analysis at different time-points of the treatment. First, the β-cell mass and number were assessed during the treatment (Lira, between 7 and 15 days of liraglutide administration) and after the withdrawal of liraglutide (Post-Lira, 5 days after treatment). Both β-cell mass and number increased partially during the treatment —because the values are higher than NSG diabetic mice but did not reach the levels of non-diabetic individuals— and transient because the values remained similar to those of diabetic mice after treatment (Post-Lira). These data indicate that liraglutide may improve hyperglycaemia by β-cell replacement as there are fluctuations in β-cell numbers throughout the

treatment. Intriguingly, the percentage of neoislets —those islets emerging from ductal areas— showed an increase upon liraglutide administration (both during and after the treatment), which may suggest neogenesis events. Finally, β-cell area and insulin content did not show differences between treated and non-treated groups discarding β-cell hyperplasia. Besides the amount of newly generated β-cell, it would be of interest to understand the functionality of these cells. For instance, whether they are glucose-responsive, mature and able to secrete insulin remain to be explored. It points to a relevant aspect to understand how the different partners arising from these regeneration mechanism would contribute to hyperglycaemia amelioration during the treatment and lose their effect after the withdrawal.

At this step, the most evident question was: what is the effect of liraglutide removal on the β-cells? One option is that β-cells would undergo apoptosis after liraglutide withdrawal but further experiments employing TUNEL assays or β-cell death markers are required. Nevertheless, it is important to pay attention to the technical method of β-cell mass determination (Chintinne 2010). The assessment of the β-cell mass and number is based on the manual counting of insulin^{+} cells. While less insulin^{+} cells were found after the withdrawal of liraglutide, no alteration in islets nor nuclei (pyknosis) from insulin^{-} cells was detected. In contrast, it was detected a reduced number of insulin^{+} cells correlating with an abnormal distribution within the islet (Annex 4). Given that liraglutide is an insulinotropic agent it may be reasonable to speculate that the drug is required to maintain continuous insulin expression upon its administration and also acts as a trophic factor ensuring β-cell viability. Further electrophysiological studies focusing on Ca^{+2} oscillations of dispersed islet cells or β-cell lines may be of interest to address this question.

Once the histological data suggested β-cell replacement events, the focus was settled firstly on transdifferentiation from glucagon-producing α-cells. This event was not only the highest scored by the drug repurposing process but also demonstrated to be induced by some aGLP1 in transgenic models (Lee 2018) and supported by the expression of GLP1R detected in α-cells (Nakashima 2018). Given our lack of a transgenic reporter strain to perform lineage tracing, the evaluation of this process lied on the identification of bihormonal (glucagon^{+}insulin^{+}) cells as an intermediate stage of transdifferentiation. Bihormonal cells were found 48h after the treatment in the islets of diabetic NSG mice, but not later nor after the withdrawal (Post-Lira). Furthermore, bihormonal cells were detected in both diabetic and non-diabetic NOD mice at 6, 24 and 48h post-treatment (Annex 5). It reveals that transdifferentiation mediated by liraglutide administration is independent of the metabolic stage, in accordance with previous studies on aGLP1 (Lee 2018). Altogether, the detection of bihormonal stages before 48h but not after 7 days of treatment could indicate that only one stage of transdifferentiation is achieved throughout the treatment. One concern that arose from this speculation was if transdifferentiation could be re-challenged after the withdrawal of liraglutide. To address this aspect, prediabetic NOD mice were treated either with liraglutide or PBS for 1 week, then 1 additional week without treatment and finally they were re-challenged 48h with liraglutide or PBS. No bihormonal cells were found in any group (Annex 6), revealing that transdifferentiation probably takes place only once after the start of the treatment.

Apart from the bihormonal cells, other insulin^{+} cells were found located in the ductal areas of both treated groups (Lira and Post-Lira). Further immunostaining demonstrated that these cells were positive for CK19 —a specific marker of ductal cells—, thus suggesting that they are of ductal

origin. Some of these CK19$^+$insulin$^+$ cells seem to emerge from ducts, pointing that may evaginate while forming new pseudoislets. This fact remains to be explored, but it would be of interest to analyse the presence of epithelial-to-mesenchymal transition markers such as Vimentin and Snail (Ben-Othman 2017). Moreover, lineage-tracing experiments would be desirable to confirm the ductal origin of these newly formed insulin$^+$ cells. For instance, ductal cell reporter models have been previously generated like the HFN1b-GFP mice (Al-Hasani 2013).

These ductal insulin$^+$ cells were negative not only for glucagon but also for somatostatin (Annex 7). Interestingly, CK19$^+$insulin$^+$ cells were detected even at 48h but only in diabetic animals (Annex 8), suggesting that hyperglycaemia may play an important role in the ductal phenotype switching mediated by liraglutide. Indeed, other models of hyperglycaemia should be analysed to further prove this aspect. Additionally, it was found that these CK19$^+$insulin$^+$ cells also express the transcription factor PDX1, a marker of mature and functional β-cells. Confocal imaging revealed that PDX1 co-localizes in the cytoplasm of the ductal cells but not in the nucleus where it is usually located (Annex 9). Despite other studies correlated an unexpected location of islet transcription factors with abnormal cell function (MacFarlane 1999), additional transcriptomic approaches would be of interest to characterize the identity of these cells. Especial attention must be paid to the expression of *Glut2* and *Glucokinase*, because these proteins are involved in the glucose uptake and the associated insulin response.

It is extremely difficult to determine quantitatively the contribution of each insulin$^+$ cell type to the improvement of hyperglycaemia. At least, three different insulin$^+$ cell types can be identified: insulin$^+$ cells with a probable α-cell origin, CK19$^+$insulin$^+$ cells in the ductal areas and pre-existing β-

cells within the islets. Notwithstanding, transgenic models could help to unravel the role of each one in future experiments. First, while STZ injection usually deletes around 80% of β-cells (Deeds 2011), 99.9% ablation of insulin-producing cells may be achieved with the RIP-DTR strain after diphtheria toxin administration (Thorel 2010). Similarly, Gcg-DTR has been developed to perform almost total α-cell ablation (Pedersen 2013). These models would allow to explore the contribution of ductal insulin$^+$ cells independently from pre-existing β-cells and hampering transdifferentiation. More interestingly, these tools —together with duct isolation— could help to explore if the above-mentioned events of regeneration keep a relation between them. It is relevant to note that while bihormonal cells induction is independent of T1D, ductal insulin-expressing cells have been detected only in mice with T1D. Lastly, given the lack of tracing, it is not possible to determine by Ki67 (proliferation marker) staining whether liraglutide can promote replication of pre-existing β-cells.

Furthermore, given the increase in β-cell mass induced between 7 and 15 days of treatment with liraglutide, it was addressed if this effect is dose-dependent and subsequently, if a higher dose would favour a higher β-cell mass. This question was explored by applying an overdose administration pattern to NOD mice and evaluating the β-cell mass (Annex 10). Conversely, the higher dose resulted in a minor increase of the β-cell mass when compared with the normal dose and even when the normal schedule increased the α-cell mass, no alterations were observed in this cell type with the higher dose. That points to an effect underlined in the α-cell mass responsible of the subsequent increase of the β-cell mass that needs to be further addressed.

Finally, to validate these discoveries about the effect of liraglutide on cell rearrangement, another aGLP1 (Semaglutide) was tested. The same administration pattern —previously designed for liraglutide— resulted in insulin$^+$ cells present within the ducts but not in the gut nor stomach —as previously reported for GLP1 administration (Suzuki 2003)— in NSG mice with T1D. This experiment reveals that following this administration schedule, a common cell reprogramming phenomenon is shared along with these two aGLP1 compounds (Annex 11).

Besides the histological analysis, *in vivo* and *ex vivo* bioimaging experiments with fluorescent liraglutide were performed to determine drug biodistribution. Liraglutide was found in the pancreas —among other organs— thus indicating that the signals for cell reprogramming are at least due to local effects. Recently, it has been developed an aGLP1-derived fluorochrome named LUXendin (Ast 2020) with a cell resolution level. It would be able to reveal whether can bind to different cell types like ductal or bihormonal ones.

However, histological and imaging data could not provide us with quantitative information related to the effect of liraglutide in gene expression. To do that, expression was addressed by qPCR in purified islets from non-diabetic NOD mice, cultured in normo- (~5mM of glucose) or hyperglycaemic conditions (~10mM). Both glucagon and insulin genes were upregulated by liraglutide, consistent with *in vivo* bihormonal cells appearance. Despite glucagon remained upregulated at hyperglycaemic conditions, insulin was slightly downregulated, likely reflecting that liraglutide is unable to perform its insulinotropic action in an impaired metabolic stage. Furthermore, *Ki67* downregulation was found in both conditions, suggesting that eventual transdifferentiation from α- to β-cells may result in restricted proliferation. Because bihormonal cells were found

in the pancreas after 48h (the same time that lasts the islets culture) the balance between the expression of two transcription factors was determined: *Arx* (α-cell specific) and *Pax4* (β-cell specific). It has been previously described that transdifferentiation occurs after downregulation of *Arx* in α-cell, which actively suppresses *Pax4* transcription, triggering the subsequent upregulation of *Pax4* and promoting the switch of phenotype (Ben-Othman 2017). Unfortunately, islet transcription factors were not detected by qPCR. Due to that, further experiments of global islet transcriptomics or even single-cell RNA sequencing of islets would be of interest to decipher if bihormonal cells follow this misbalance between transcription factors and correlate them with hormone expression.

Since semaglutide resulted in $CK19^{+}insulin^{+}$ cell induction too, its effect in gene expression was determined in cultured islets from non-diabetic NOD (Annex 12). Consistent with previous findings, both insulin and glucagon genes were upregulated by liraglutide or semaglutide. Additionally, the GLP1 receptor (*Glp1r*) gene was found upregulated, probably due to positive feedback of the targeted pathway. *Pcsk1*, which is an enzyme involved in the maturation of the pre-proinsulin molecule, was also found upregulated upon both aGLP1, evidencing the insulinotropic effect of this drug family. Conversely, *Glut2* resulted in downregulation by liraglutide or semaglutide. Given the insulinotropic effect of the aGLP1 compounds, it is difficult to propose an explanation for this observation. Maybe, this coupled to electrophysiological experiments could provide an elucidation about the overall effect on glucose metabolism. Finally, the expression of a β-cell dedifferentiation marker (*Aldh1A3*, Diedisheim 2018) was strongly downregulated upon liraglutide and non-detected in islets exposed to semaglutide. This data indicates that aGLP1 drugs did not differentiate pre-

existing β-cells within the islets but previous histological results after the withdrawal may still lead to speculate this.

Besides the study with islets, because they are mainly composed of β-cells but also α-cells (and after liraglutide administration, bihormonal cells too), and other endocrine cells, a β-cell line was explored. The chosen cell line was NIT-1, obtained from a NOD insulinoma (Hamaguchi 1991). Despite the viability was not strongly altered with the addition of liraglutide, NIT-1 is not the best cell model to study the effect of aGLP1. With a clear insulinotropic effect of these drugs resulting in increased insulin secretion in primary dispersed β-cells (Xu 2018), NIT-1 cells were unable to show differences in C-peptide secretion as expected (Annex 13A). Notwithstanding, insulin expression was found altered at the transcriptomic level, along with other genes such as *Glis3* and *Ngn3* —both transcription factors implied in the maintenance and establishment of β-cells— (Annex 13B). Better models like primary and dispersed β-cells could provide stronger insights.

Finally, to gain more knowledge into the effect of liraglutide on ductal cells another cell line was used. The human PANC1 cell line has a ductal origin and was expanded from a pancreatic ductal adenocarcinoma (Lieber 1975). PANC1 cells bond liraglutide-AF750, displaying a logarithmic dynamics of bonding, with more than 80% of PANC1 cells linking liraglutide-AF750 at 180 minutes (Annex 14A). Liraglutide co-culture did not affect the viability of PANC1 cells (Annex 14B) but did over some membrane molecules expression. Increase in the expression of CD54 and class I MHC molecules could result in a more immunogenic phenotype of these cells upon liraglutide addition (Annex 14C). However, insulin expression in PANC1 cultured with or without liraglutide was negative (Annex 14D. The induction of $CK19^{+}insulin^{+}$ cells in mice treated with liraglutide may result

from a complex mechanism and cannot be discarded that this effect could not be achieved in human ductal cells by inter-species differences.

Altogether, the Results II section drives from the identification of an aGLP1 (liraglutide) as a repurposed drug with potential for the treatment of T1D through the description of specific cell type rearrangements. This section is an example of how a question that arose in the context of translational research contributed to gain knowledge in islet plasticity. Taking all these observations together we proposed a model of liraglutide-induced β-cell regeneration. Islets from NSG are depleted of the vast majority of β-cells by STZ administration, while only ~20% of them are still present. After that and until 48h after liraglutide treatment, glucagon$^+$insulin$^+$ cells appear within the islets. Because other authors have reported that GLP1 triggers β-cell transdifferentiation (Lee 2018), we propose that these bihormonal cells are the results of this ongoing process. Transdifferentiation would eventually contribute to the transient and partial restoration of islet β-cell mass between 7 and 15 days of treatment. From 48h on and simultaneously to this process, CK19$^+$insulin$^+$ cells would arise from the ductal areas. Despite these newly formed insulin$^+$ cells are maintained even after treatment withdrawal, islet β-cell mass decreased at this point for reasons that still need to be elucidated. We propose this model but being well aware of the limitations. Nevertheless, this model uncovers some loose ends that need to be addressed. For instance, the fact that bihormonal cells could be detected in both diabetogenic and non-diabetogenic conditions after the first liraglutide challenge (but not in further re-challenges) triggers the question about the α-cell source. No glucagon$^+$ cells were found around the ductal areas, as other models proposed to restore the transdifferentiated α-cell mass (Al-Hasani 2013). Replication of pre-existing glucagon$^+$ cells could be investigated through

subsequent staining with Ki67 along the treatment check-points. Another issue that needs to be unravelled is their relationship with $CK19^{+}insulin^{+}$ cells. It could be explored after α-cell depletion if the existence of these cells is related to prior transdifferentiation of α- into β-cells. Furthermore, the appearance of these ductal insulin-producing cells only in diabetogenic conditions lead to speculate the existence of hyperglycaemia signals governing this switch of phenotype. Transcriptomics analysis of the ductal cells would be necessary to decipher the origin of these cells: whether they are $Ngn3^{+}$ precursors able to express insulin or are mature ductal cells switching to a β-like-cell phenotype. A recent study employing a novel aGLP1 named puerarin (Wang 2020), also reported the existence of $CK19^{+}insulin^{+}$ cells in a mice model of induced T2D with high fat diet. These cells express not only NGN3 but also PDX1, both located in the nucleus. Hence this study confirms the existence of $CK19^{+}insulin^{+}$ induced via GLP1R signalling in diabetogenic conditions.

Besides that, it is important to keep a global perspective of the experimental plan. Here a repurposed drug intended for novel uses in human patients is employed to target the murine GLP1R, which sequence slightly differs from the human one. For that reason, it would be extremely useful to obtain future pancreatic samples from deceased donors with T2D treated with aGLP1 drugs to observe if this phenomenon happens in humans.

At this point, liraglutide was thought to be combined with the previously developed PSAB-liposomes to be tested in spontaneous autoimmune diabetes. This question was evaluated in **Article 3:** *Antigen-specific immunotherapy combined with a regenerative drug in the treatment of experimental type 1 diabetes* included in the Supplementary Publication chapter.

The first issue to check is if a regenerative agent could improve hyperglycaemia in the context of chronic autoimmunity. To do that, prediabetic NOD mice were injected with liraglutide and the incidence of diabetes was determined at 25 weeks. Although the final incidence was similar to the sham group, an acceleration of T1D onset was found in the treated mice. Conversely, the insulitis score was reduced showing an increased percentage of islets with benign insulitis. Because liraglutide can induce ductal-derived insulin$^+$ cells, the insulitis score was calculated again for neo-islets (pseudo-islets emerging from ducts) and mature islets (those displaying a physiological phenotype). In sham mice, insulitis score was higher in mature than in neo-islets, with an increased percentage of severe insulitis in the first ones. On the opposite, in liraglutide treated mice, insulitis score was higher in neo-islets when compared to sham, displaying a similar percentage of islets with severe insulitis to the mature islets. Overall, this may indicate that β-cell regeneration during autoimmune attack promotes the recruitment of leukocytes to the regenerative focus (neo- or pseudo-islets), likely due to autoantigen exposure in an inflammatory microenvironment. Moreover, leukocytic infiltrate found in the ductal areas of mice treated with liraglutide supports the above-mentioned statements. (Annex 15). These results indicate that liraglutide alone cannot be used to prevent autoimmune T1D because regeneration would trigger increased autoimmunity.

For T1D treatment, a combined therapy was designed including both PSAB-liposomes and liraglutide. The administration schedule consisted of first administration of PSAB-liposomes in three doses to arrest the autoimmunity followed by liraglutide to promote β-cell regeneration once the autoimmune reaction was hampered (4 days after first dose of immunotherapy was administered, similar to Rydén 2017). The combined

therapy resulted in the amelioration of hyperglycaemia when administered to diabetic NOD mice. Despite PSAB-liposomes alone could delay the hyperglycaemia, the addition of liraglutide was essential to decrease it. The improvement was transient as previously observed with NSG treated alone with liraglutide. The combined therapy was effective only in 50% of the treated mice. When these individuals were stratified between age and blood glucose value at the onset, it was found that the responders were those developing the disease with the lower glycaemia. This observation indicates that residual β-cell function is an important feature to be a responder to both regenerative and immunotherapeutic agents. Furthermore, novel dosages and alternative administration schedules should be tested to improve the outcomes on hyperglycaemia amelioration.

Due to the translationality of this strategy, the use of models more close to the human physiology was desired, to decipher if liraglutide affects the human immune system and thus it can interfere —either by synergistic or antagonistic effect— with the immunotherapy. For that reason, human DCs from adult subjects with T1D were co-cultured with PSAB-liposomes alone and with liraglutide to determine changes in phenotype and transcriptome (as previously described at Rodriguez-Fernandez 2018). At the phenotype level, while PSAB-liposome treatment can induce a semi-mature phenotype, considered tolerogenic, the addition of liraglutide to PSAB-liposomes did not alter this phenotype. This semi-mature phenotype was determined by the relative expression of antigen-presenting (HLA-ABC and HLA-DR), adhesion (CD54 and CD49d), activation (CD25), costimulation molecules (CD40 and CD86) as well as PS- (CD36 and TIM4), chemokine- (CCR7 and CXCR4) and pattern recognition receptors (TLR2). The suitability of the previous markers to define the semi-mature phenotype was previously determined (Rodriguez-Fernandez 2018 and

2019). Liraglutide did not show any synergistic effect when added to PSAB-liposomes but did not alter the semi-mature phenothype induced by PSAB-liposomes alone. Conversely, the effect on these specific molecules was determined by the presence or absence of PSAB-liposomes, independently from liraglutide addition.

At the transcriptome level, both PSAB-liposomes and the combined therapy shared 13 common altered (up- and downregulated) annotated genes. Intriguingly, *in vitro* culture with PSAB-liposomes resulted in the altered expression of 179 genes while the culture with only liraglutide resulted in the altered expression of 559 genes (Annex 16 and 17). Altogether, it pointed to different regulation of the gene expression between PSAB-liposomes and liraglutide with a different outcome when combined. Because the effect that PSAB-liposomes exerted in human DCs were previously reported (Rodriguez-Fernandez 2018), it would be interesting to elucidate how liraglutide alters gene expression in this cell type. It is still unknown if liraglutide shows any effect of tropism or gene regulation in immune cells, but labelling experiments with liraglutide coupled to AF750 revealed its ability binding to human DCs (Annex 18). Overall, this opens the door to further explore the putative effects of aGLP1 in the immune system and specifically in APCs.

Finally, in the road to translationality, humanized mice consisting of NSG injected with PBMCs from adult patients with T1D were treated with the combined therapy. In general terms, the combined therapy did not alter the mice health nor surveillance. T cell subsets persisted and no detrimental effect was observed in total T lymphocyte counts and percentages, suggesting no toxicity related to the combined therapy. A trend of increased lymphocytes and mTregs was found in mice treated with the combined therapy. Notwithstanding, this model showed null B lymphocyte

engraftment thus it seems not appropriate at all to evaluate the safety of this treatment in most of the immune cell subsets (Shultz 2012). An increase in sample size would be essential to appreciate more robust differences. Another limitation of this model is that it does not develop spontaneously T1D like other humanized mice do (Luce 2018).

Finally, and after the design and evaluation of a combined therapy, to identify the optimal checkpoint for future administration of this or other innovative immunotherapies seems to be a reasonable aim. Many clinical trials using immunotherapies with successful outcomes in mice have failed, in part by the inappropriate T1D stage of administration. This topic was approached in **Article 4:** *Partial remission and early stages of pediatric type 1 diabetes display immunoregulatory changes. A pilot study*.

Here, the strategy to dissect early stages of T1D consisted of the screening of candidate biomarkers (i.e. molecular and lymphocyte cell subsets) that may be related to immunological responses. The almost total absence of clinical biomarkers impedes the stratification of patients avoiding the identification of disease progression stages. T1D lacks properly characterized stages as other diseases such as multiple sclerosis (Teniente-Serra 2017 and Quirant-Sánchez 2018). Consequently, it also hinders the discovery of optimal checkpoints for immune intervention in clinical trials.

A very interesting stage of T1D is the spontaneous PR stage. This phase suddenly occurs during the first year after the onset and may last up to the second year of progression (Mortensen 2009). It is a transient period determined by lower requirements of insulin, even achieving insulin-independence for some individuals, and correlating with an improved glycaemia. The clinical determination of the PR is based in both the HbA1c and the daily insulin dose. It has also been suggested that PR may be due

to a transient restoration of self-tolerance and even successful endocrine cell regeneration (Fonolleda 2017).

Because PR and other early stages of T1D can be optimal immune intervention phases, new biomarkers should be identified. All the subjects included in this study were paediatric patients, given that the onset of T1D is clearly defined in young patients while in adult ones may induce a misdiagnosis due to Latent Autoimmune Diabetes in Adults (LADA) or other forms of diabetes (Grill 2019). Peripheral blood was the sample of choice because it means a minimal invasive method and is easy to obtain.

First, plasmatic betatrophin —a hormone involved in lipid transport— was found increased from the onset to 18 months of disease progression and did not distinguish the PR stage from other checkpoints. Betatrophin is also upregulated in other metabolic diseases like T2D, giving a clue that hyperglycaemia could be the pivotal point modulating this molecule (Espes 2014a and 2015). Previous results showed relationship between BMI (Body Mass Index) and betatrophin in T2D (Espes 2014a), similar to the correlation here showed in T1D. Betatrophin as a candidate biomarker of T1D along early stages would be useful to confirm the disease when the classical symptoms are not clear (Espes 2014b). Furthermore, while the current biomarker for pancreatic response is C-peptide, most individuals showing negativity both at basal and stimulated conditions are already positive for betatrophin (Annex 19). Hence, using betatrophin as a biomarker of sustained hyperglycaemia would be probably more representative than negativity for C-peptide in those subjects. The major flaw of measuring betatrophin is that cannot discern between different disease phases as it is increased in all the evaluated checkpoints. Besides, it would be of interested to assess the levels of betatrophin in prediabetes as a disease predictor.

It was also considered to determine immunosuppressive mediators like TGF-β, whose gene expression was previously found altered in pancreases from donors with T1D (Planas 2010). This is an important point because the pancreas is an inaccessible organ and finding biomarkers correlating at periphery would help us to predict tissue events. TGF-β is a cytokine with different functions related to cell development (from angiogenesis to lineage commitment) (Clark 1998). Plasmatic TGF-β concentration — found increased at the onset— decreased after 6 and 12 months of disease progression in paediatric subjects that underwent PR stage. Further validation in stages longer than 12 months of PR would be desirable. Despite that, because many peripheral blood cells can secrete TGF-β, extensive research is needed to unravel the underlying modulation of this candidate biomarker. Notwithstanding, because PR is a phase thought to be driven by immune tolerance or even local immune suppression it would be reasonable to relate a decrease in peripheral TGF-β as a negative feedback of immune tolerance in pancreas. TGF-β has been shown to be involved in immune tolerance (Johnston 2016b) and suppression (Dzik 2003) mainly driven by Tregs (Liu 2018) and DCs (Esebanmen 2017).

Lastly, alterations in cell subsets were also considered as biomarkers at the onset and during the first and second year of disease progression (Annex 20). Different cell subsets from peripheral blood are currently used as biomarkers because they are altered, not only to diagnose but also to assess the outcomes of clinical trials in many immune-related diseases (Teniente-Serra 2017 and Villarrubia 2019).

Despite differences in both the number and concentration of cell subsets related to natural immunity depict more robust biomarkers, changes in only one parameter can be due to compensatory mechanisms arranged by other cell types. Another aspect to be taken into account is that peripheral

lymphocytes are the result of the balance between their generation in primary lymphoid organs and migration to target organs. The determination of their levels in peripheral blood depicts a steady-state picture but cannot let us infer what is happening in other locations.

Changes in activated and memory T regulatory lymphocytes were found at the onset and during the first year of disease progression, and that may reflect an immunoregulatory attempt. Changes in percentage and number were also observed in other regulatory subsets, Bregs and NKreg cells. This report shows regulatory subsets altered in T1D but their role is still poorly understood both in physiological and pathological conditions. Overall, these altered parameters suggest that early stages of T1D display immunoregulatory changes, likely as a counter-response to the autoimmune attack.

These experiments resulted in the identification of candidate biomarkers of PR (TGF-β) and early stages of T1D (regulatory leukocytes) but further validation in an independent cohort is highly required. Indeed, it would be interesting to validate these results in a longitudinal study to avoid inter-subject perturbations. Overall and if finally validated, it would be of interest to consider the determination of these candidate biomarkers in future clinical trials.

In summary, the work comprised in the current PhD thesis encompasses the design and development of a combined therapy to treat experimental T1D focusing on the regeneration of β-cells and the arrest of autoimmunity. It resulted in the identification through drug repurposing of liraglutide, an aGLP1, that exerted β-cell regeneration from an α-cell and a ductal cell origin. The combination of liraglutide with a previously developed nano-immunotherapy elicited the choice of PSAB-liposomes (insulin containing

PS-liposomes). This combination resulted in the amelioration of hyperglycaemia in a spontaneous autoimmune model of T1D. Besides that, the further screening of immune-related molecules also identified specific candidate biomarkers of early phases of T1D. The present studies pretend to help for a future translation of the proposed models into the clinical application either for the treatment, prevention or stratification of T1D either alone or in additional combinations. Indeed, the results comprised in this PhD thesis propose a novel experimental therapy for T1D but still require extensive research. This strategy needs further validation and scalability prior to be adapted to the human disease. Even more important, these results prove that a combined therapy both targeting the autoimmune reaction and boosting the β- (or insulin-producing) cell number could be used to ameliorate hyperglycaemia in T1D. Besides that, the use of liraglutide in diabetic mice revealed the existence of ductal cells switching into an insulin-producing phenotype, hereby described for the first time. Furthermore, in order to encompass the progression and stratification of T1D stages during the natural patophisiology of the disease there has been proposed the use of candidate circulating biomarkers such as TGF-β and regulatory leukocytes.

CONCLUSIONS

CONCLUSIONS

The work comprised in the present PhD thesis allows to reach the following conclusions:

1. PS-liposomes encapsulating insulin peptides (PSAB-liposomes) are the most efficient in preventing experimental T1D when compared to PS-liposomes encapsulating other single autoantigens (GAD65, IA2, C-peptide).

2. Liraglutide, an agonist of GLP-1, has been identified by drug repositioning as a repurposed compound able to ameliorate hyperglycaemia in experimental T1D by cell reprogramming. Indeed, liraglutide improves hyperglycaemia in diabetic mice, correlating with the development of new bihormonal insulin$^+$glucagon$^+$ cells and ductal (CK19) insulin$^+$ cells.

3. The use of liraglutide as a regenerative strategy, combined with PSAB-liposomes as antigen-specific immunotherapy, ameliorates hyperglycaemia in diabetic NOD mice.

4. Liraglutide does not alter the tolerogenic phenotype induced by PSAB-liposomes in human DCs derived from patients with T1D. In addition, the *in vivo* interaction between liraglutide and PSAB-liposomes is not detrimental for the *in vivo* expansion of T lymphocyte subsets as observed in a humanized mice model.

5. TGF-β is a candidate biomarker of PR stage. Changes in the regulatory leukocyte subsets (B, T lymphocytes and NK cells) are restricted to early phases of paediatric T1D progression.

SUPPLEMENTARY ARTICLE

Antigen-specific immunotherapy combined with a regenerative drug in the treatment of experimental type 1 diabetes

Running title: Combined therapy for type 1 diabetes

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Type 1 diabetes is an autoimmune disease caused by the destruction of the insulin-producing β-cells. To revert type 1 diabetes, the suppression of the autoimmune attack should be combined with a β-cell replacement strategy. It has been previously demonstrated that liraglutide, a glucagon-like peptide-1 receptor agonist, restores β-cell mass in type 1 diabetes, via α-cell transdifferentiation and neogenesis. We report here that treatment with liraglutide does not prevent type 1 diabetes in the spontaneous non-obese diabetic (NOD) mouse model, but it tends to reduce leukocytic islet infiltration. However, in combination with an immunotherapy based on tolerogenic liposomes, it is effective in ameliorating hyperglycaemia in diabetic NOD mice. Importantly, liraglutide is not detrimental for the tolerogenic effect that liposomes exert on dendritic cells from patients with type 1 diabetes in terms of membrane expression of molecules involved in antigen presentation, immunoregulation and activation. Moreover, the *in vivo* effect of the combined therapy was tested in mice humanised with peripheral blood mononuclear cells from patients with type 1 diabetes, showing no adverse effects in leukocyte subsets. In conclusion, the combination therapy with liraglutide and a liposome-based immunotherapy is a promising candidate strategy for type 1 diabetes.

Abbreviations: Type 1 diabetes (T1D); Analog Glucagon-like peptide-1 (aGLP-1); Non-obese diabetic mouse (NOD).

INTRODUCTION

Type 1 diabetes (T1D) is a metabolic disease caused by the autoimmune destruction of the insulin-producing pancreatic β-cells. Currently, there is no cure nor prevention for the disease and subjects with T1D need exogenous insulin administration to survive. However, this treatment does not lead to continuous normoglycaemia and may often produce events of hyper- and hypoglycaemia. Patients with T1D display important associated complications —such as neuropathy, nephropathy and retinopathy— that, together with episodes of glycaemia dysregulation, worsen their quality of life and may shorten their lifespan [1].

At present, single immunotherapies that have been successful in experimental models have failed in humans [2]. On the other hand, autologous regenerative strategies, by recovering β-cell mass, are not able to ameliorate the disease due to the chronic autoimmune process. Given that, there is an urgent need for novel therapies combining the regeneration of endogenous β-cells and the arrest of the autoimmune reaction against β-cells. Combined therapies using different strategies must be designed for human use. There is a clinical trial already completed in humans [3], which consists of the administration of GAD-aluminium conjugate as immunotherapy [4] and GABA [5] as a regenerative agent (NCT02464033). This resulted in a decrease in daily insulin requirements from baseline to

12 months and the improvement of residual β-cell function by means of C-peptide secretion (a product of proinsulin processing). Additionally, a more recent combined strategy —consisting of anti-IL21 and liraglutide— resulted in the restoration of normoglycaemia in diabetic mice [6]. On the one hand, the administration of anti-IL21 achieved normoglycaemia in some diabetic mice. On the other hand, liraglutide, a commercially available drug for type 2 diabetes, contributed to the recovery of the β-cell mass. Because anti-IL21 has systemic effects in the immune system, antigen-specific immunotherapies could be safer and help to overcome the side-effect issues derived from total immunosuppression. All these data indicate that combined therapies both targeting the autoimmune attack and promoting β-cell regeneration could result in a greater beneficial outcome for the treatment of T1D.

Our previous work demonstrated the ability of apoptotic β-cells to induce self-tolerance through efferocytosis by dendritic cells (DCs), thus preventing T1D in the non-obese diabetic (NOD) mouse model [7]. For clinical application, β-cell apoptotic cells were mimicked using nanotherapeutic tools. Liposomes, lipidic vesicles with an aqueous core, were designed with phosphatidylserine (PS) in the membrane and encapsulating insulin A and B chains (PSAB-liposomes). PSAB-liposomes prevented T1D in the NOD mice [8,9] by inducing tolerogenic DCs.

Furthermore, this tolerogenic effect was also validated in human DCs from subjects with T1D [10,11]. With the aim to combine this immunotherapy with a regenerative strategy, we previously performed a Drug Repurposing analysis to search for already-existing compounds able to promote β-cell regeneration. Drug Repurposing has the advantage to propose novel uses for compounds existing in the market and overcomes the safety issues. The analysis identified liraglutide, an agonist of GLP1 (aGLP1), as a regenerative drug [12]. In fact, liraglutide administration ameliorated hyperglycaemia in immunodeficient NOD-Scid IL2rg$^{-/-}$ (NSG) mice rendered diabetic by Streptozotocin. This improvement correlated with the appearance of bihormonal insulin^{+}glucagon^{+} cells and insulin^{+} cells in the ductal areas [12]. Previous studies demonstrated the ability of aGLP1 to induce transdifferentiation of glucagon-producing α-cells into β-cells [13]. Furthermore, aGLP1 induce β-cell proliferation through the inhibition of *DYRK1A* gene expression [14]. Ongoing clinical trials aim to assess the effect of liraglutide in subjects with T1D (NCT02617654 and NCT02516657).

Taking all this information into account, an experimental combined therapy consisting of an immunotherapy based on PSAB-liposomes to arrest autoimmunity and liraglutide to promote β-cell regeneration was designed. Here we show that this strategy is able to ameliorate hyperglycaemia in the spontaneous model of autoimmune T1D, the NOD mice. Importantly, we

also show for the first time that liraglutide does not alter the tolerogenic effect of the immunotherapy in DCs from human adult patients with T1D. Additionally, the combined therapy does not result detrimental for T lymphocyte cell subsets in a humanised mouse model. Investigation of combined therapies to restore tolerance and to allow the reestablishment of β-cell mass should continue to completely reverse T1D in a clinical setting.

RESULTS

Liraglutide accelerates the onset of T1D in NOD mice. Liraglutide has a regenerative effect on β-cells, which might alter the development of T1D. To assess their impact on the onset of the disease, NOD mice (n=7) were treated with three doses of 1mg/kg of liraglutide on alternate days during the prediabetic period (8 weeks old). Sham group (n=12) was treated with PBS. liraglutide treatment significantly accelerated T1D onset when compared to sham group. Mice from the sham group developed the disease from the age of 16.4 weeks and with an incidence of 58.33% at 25 weeks of age. Treated mice developed T1D starting at 9.71 weeks of age and reaching an incidence of 57.14% at 25 weeks of age (Fig. 1A). No significant differences in terms of incidence were found between both groups at the end of the experiment.

Insulitis score was determined at the end of the follow-up period in non-diabetic mice. Mice treated with liraglutide showed a tendency to reduce insulitis, although non-significant, (1.7±0.4, mean±SD) when compared to sham group (2.2±0.07) (Fig. 1B). In mice treated with liraglutide, 62% of the islets remained non-destructed –insulitis free or with peri-insulitis–, whereas only 40% of the islets were non-destructed in the sham group (Fig. 1C).

Given that liraglutide can induce the formation of pseudoislets arising from the ducts [12], hereinafter called neoislets, insulitis score was determined for both neoislets and mature islets. Neoislets show an increased insulitis score in mice treated with liraglutide when compared to control mice, probably promoted by the β-cell regenerative effect of liraglutide in the ducts. On the opposite side, mature islets remain less affected by insulitis in liraglutide-treated mice than in controls (Supp. Fig. 1).

Liraglutide combined with liposome-based immunotherapy ameliorates hyperglycaemia in NOD mice. Our previous results on the effect of liraglutide in regenerating β-cell mass [12] led us to combine this drug with a PS-liposome based immunotherapy, which restores β-cell tolerance. NOD mice were assigned to treatment groups –sham, liraglutide, liposomes or combined– as soon as T1D was detected (Fig. 2A). Age and glycaemia at T1D onset were comparable between groups (data not shown). Liraglutide monotherapy did not ameliorate hyperglycaemia in mice with T1D, despite one mouse transiently achieved normoglycaemia at day 3 and immediately returned to hyperglycaemia. PSAB-liposome immunotherapy partially ameliorated blood glucose levels, but glycaemia was always maintained higher than 270 mg/dL. Importantly, the combined therapy resulted in a significant amelioration of glycaemia in 3 of 6 NOD

mice (only the responders are presented in the graph). Moreover, this group has a significant reduction on the glycaemia area under the curve when compared with sham and PSAB groups (Fig. 2B), correlating with an improvement in survival compared to the sham group and group treated only with PSAB-liposomes. These responder mice showed a tendency, although non-significant, to display lower blood glucose levels at T1D onset when compared to non-responders (Fig. 2C).

Liraglutide does not interfere with the tolerogenic ability of liposomes in human DCs. In order to identify possible interactions between the two parts of the combined therapy, DCs from patients with T1D were exposed to liraglutide and to PS-liposomes. As expected, iDCs acquired a tolerogenic phenotype when treated with PSAB-liposomes (Fig. 3) in terms of CD36, TIM4, CD49d, HLA Class I, HLA Class II, CD54, CD40, CD86, CD25, CCR7, PD-L1, CXCR4, and TLR2, in comparison to mDCs. Liraglutide alone was not able to modify iDC membrane expression of these molecules except for an increase in HLA Class I and CCR7. Importantly, the treatment of DCs with the combination of liraglutide and PSAB-liposomes resulted in a phenotype identical to tolerogenic treatment with PSAB-liposomes in terms of CD36, TIM4, CD49d, HLA Class I, HLA Class II, CD54, CD40, CD86, CD25, CCR7, CXCR4, and TLR2.

Interestingly, liraglutide in combination with PSAB-liposomes increased the expression of PD-L1 in DCs when compared to PSAB-liposomes monotherapy, reinforcing the tolerogenic potential of DCs.

The transcriptional changes induced in DCs from patients with T1D by the combined therapy point to a tolerogenic effect. In order to determine if liraglutide altered the previously described tolerogenic effect of PSAB-liposomes in human DCs [10], RNA-seq analysis was performed in DCs from 4 patients with T1D. Their phagocytosis capability was confirmed by flow cytometry using fluorescent liposomes. After 4h of co-culture, 72.83 ± 9.27% (mean ± SD) of DCs were positive for fluorescent signal. RNA integrity was determined for each sample, RIN 9.38 ± 0.23 (mean ± SD) being optimal for RNA-seq experiment. Bioinformatics analysis of the RNA-seq revealed 179 differentially expressed genes when comparing iDCs and PSAB-DCs (adjusted p value < 0.05 and Log2 of fold change > or < 1.2). Of these 179 genes, 142 (79.33%) were downregulated and the remaining 37 (20.67%) were upregulated, and 152 corresponded to protein-coding genes. The comparison between iDCs and combined therapy (liraglutide + PSAB-liposomes) displayed 24 differentially expressed genes (adjusted p value < 0.5). Of these 24 genes, 7 (29.16%) were downregulated and the remaining 17 (70.84%) were upregulated, and

finally 21 corresponded to protein-coding genes. In both cases, the gene expression was modulated toward a similar profile in DCs exposed to both conditions. Taking into account the genes with identical altered expression were found in both analysis (Fig. 4A), three of them were validated by qRT-PCR (Fig. 4B).

The combined therapy does not alter human leukocyte subsets in humanised mice. To further investigate the short-term effects of the combined therapy in human leukocyte subsets, NSG mice humanised with PBMCs from patients with T1D were exposed to the combined therapy and monitored for 4 weeks. No alterations in leukocyte counts or percentage were found when comparing treated mice to control group (Fig. 5A). The number and percentages of T lymphocytes (both $CD4^+$ and $CD8^+$) were not altered by the combined therapy (Fig. 5B&C). Similarly, the number and percentages of memory regulatory T cells (mTreg) cells were similar to the control group during the follow-up (Fig. 5D).

DISCUSSION

The present study shows that a combined therapy consisting of a regenerative drug and a liposome-based immunotherapy —PSAB-liposomes and liraglutide– ameliorates hyperglycaemia in the spontaneous model of autoimmune diabetes, the NOD mouse. First, the effects of liraglutide as a regenerative agent in autoimmune diabetes were assessed by its administration in prediabetic NOD mice. Liraglutide administration resulted in an acceleration of the onset but without altering the incidence of diabetes. These results fit well with a previous study showing that liraglutide did not prevent or delay the onset of the disease in NOD mice [6]. It is reasonable to speculate that the here reported acceleration of T1D onset can be due to the increase in β-cell autoantigens exposure/release induced by liraglutide in a model with active autoimmunity. This could be due to the fact that neoislets increase the availability of autoantigens –at least insulin [12]– that accelerate the autoimmune attack and actively recruit lymphocytes. Overall, these data would not only explain the acceleration in the onset of diabetes but also suggest that regenerative agents like liraglutide are not efficient in blocking an autoimmune attack and thus would need to be combined with an immunotherapy to restore β-cell tolerance in T1D. An interesting point is that previous studies with combined therapies including liraglutide did not observe β-cell

proliferation [6], but our previous results demonstrated that liraglutide is involved in β-cell replacement, mainly by induction of bihormonal (glucagon^{+}insulin^{+}) cells and from insulin-producing cells in the ducts [12].

In view of this, a therapy was designed by combining the administration of PSAB-liposomes to arrest autoimmunity [8,9] prior to liraglutide treatment to promote β–cell regeneration [12] in diabetic NOD mice. This strategy resulted in the amelioration of hyperglycaemia in 50% of the treated mice, with some even reaching normoglycaemia. The improvement in the blood glucose levels was transient and mice became hyperglycaemic again after the withdrawal of liraglutide. This phenomenon was previously observed upon liraglutide administration in diabetic immunodeficient mice [12], suggesting that this drug may be necessary for maintaining physiological β–cell function. However, it would be of interest to explore further modifications of the current approach such as dosages and administration time-points. Intriguingly, the stratification between responders and non-responders showed that those developing T1D with lower glycaemia at the onset were prone to be responders independently of the age. This may indicate that, as proposed [15], residual β–cell function is a key feature to be considered in the design of combined therapies. It would be of relevance to further expand these observations to other models of autoimmune diabetes, like the inducible RIP-B7.1 mouse model [16].

Hence, to determine if liraglutide affected the immunoregulatory effects of PSAB-liposomes, DCs from human subjects with T1D were exposed *in vitro* to both products. In summary, PSAB-liposomes can induce a tolerogenic phenotype in DCs on their own and even combined with liraglutide. This is relevant data because it demonstrates that liraglutide does not interfere with the tolerance induction mediated by PSAB-liposomes phagocytosis in DCs. As expected, liraglutide *per se* was not able to induce phenotypic alterations in iDCs. The question about liraglutide affecting the tolerogenic properties of DCs treated with PSAB-liposomes was also addressed at the gene expression level. RNA-seq experiments revealed that the main gene alterations related to tolerance were found in DCs both with and without liraglutide treatment. Further research is still needed at the transcriptomics level to find a robust and strong genetic signature by both increasing the sample size and by exploring different time-points of co-culture.

Finally, the establishment of a humanised mouse model by injecting PBMCs from adult subjects with T1D in immunocompromised NSG mice enabled us to study the effect of the combined therapy in a broader range of human immune cells. No detrimental effect of the combined therapy in the different subsets of human T lymphocytes was detected. The poor, almost null, engraftment of B lymphocytes and NK cells limited the

outcome of this experiment. It would be interesting to evaluate the combined therapy in other humanisation models, especially if they can develop diabetes, to achieve a clearer picture of the effects of the combined therapy and smooth its way for a clinical translation.

Currently, there are other combined therapies in the experimental field comprising liraglutide as a regenerative drug [6]. The main disadvantage in this approach is the use of anti-IL21 as immunotherapy, given that it is a systemic immunosuppressive agent. Other immunosuppressive agents totally ablating the immune system have proven useful in the arrest of autoimmunity in T1D and even in the reversal of the disease [17,18], but one would have to assume the side effects related to immunosuppression as well [19]. In that case, it would be much more advantageous to focus on antigen-specific therapies targeting specifically the autoimmune reaction against the insulin-producing β–cells.

Further studies are required to ameliorate and fully characterise the effect of the here presented combined therapy. New schedules of administration should be explored in order to gain insights into the amelioration of hyperglycaemia upon different dosages and time-points of treatment. Additionally, it would be of interest to evaluate the effect of the combined therapy in other murine models of the disease, such as those induced by diabetogenic T cell transfer [20].

In conclusion, the combination therapy consisting of PSAB-liposomes and liraglutide is able to ameliorate hyperglycaemia in NOD mice with overt T1D. Moreover, the regenerative effect of liraglutide does not interfere with the tolerogenic consequences of PSAB-liposomes in the human immune system. Despite additional research is still needed to explore the regenerative potential of liraglutide, the combined therapy could be useful for the treatment of T1D.

METHODS

Liposome manufacturing. Liposomes consisted of 1,2-dioleoyl-sn-glycero3-phospho-L-serine (sodium salt) (DOPS, Lipoid, Steinhausen, Switzerland) and 1,2-didodecanoyl-sn-glycero-3-phosphocholine (DLPC, Lipoid), and cholesterol (CH, Sigma Aldrich, Saint Louis, MO, USA). Liposomes were generated encapsulating the murine or human insulin A and B chains (PSAB-liposomes) (Table 1) using the thin film hydration method from the lipid mixture, at a final lipid concentration of 30mM, as described [8–10].

Mice and treatment. NOD mice were bred in our own facility and kept under specific pathogen-free conditions in a 12h dark/12h light cycle provided with food and water *ad libitum*. Mice with either successive 2h fasting blood glucose levels higher than 250 mg/dl or with a measure higher than 300 mg/dl were considered diabetic. For prevention experiments, prediabetic NOD females –8 weeks-old– were injected with 3 doses of either PBS (Sham) or liraglutide (Lira, 1 mg/kg of body weight) biweekly. For treatment experiments, NOD mice with overt diabetes –12 to 25 weeks-old– were treated daily s.c. only with liraglutide throughout the experiment (Lira, n=6), only with i.p. PSAB-liposomes at days 1, 3 and 7 (PSAB, n=6), combining i.p. PSAB-liposomes at days 1, 3 and 7 with daily s.c. liraglutide

from day 4 to 30 (combined group) (PSAB+Lira, n=6). A sham group was included by treating mice daily s.c. with PBS (Sham, n=6). Mice were monitored biweekly for fasting glucose levels. Mice were euthanised when blood glucose was higher than 600 mg/dL for ethical reasons. At the end of the follow-up, mice were euthanised by cervical dislocation and pancreases were harvested and snap-frozen in an isopentane/cold acetone bath.

Insulitis Score. Pancreases from non-diabetic animals (n=3) of the incidence study at 25 weeks of age were used to determine the insulitis score. Non-overlapping cryosections of 5 μm were obtained and stained with haematoxylin and eosin. A double-blind analysis was performed by independent observers. A minimum of 40 islets per animal was scored to determine the degree of leukocyte infiltration (insulitis), as previously described [21]: 0, no insulitis; 1, peri-insular; 2, mild insulitis (<25% of the islet infiltrated); 3, 25–75% of the islet infiltrated; 4, >75% islet infiltration.

Patients. Adult patients with T1D (n = 13, Table 2) were included in the study. All patients fulfilled the diagnosis criteria for T1D. The inclusion criteria were 18 – 45 years of age and a normal body mass index (BMI,

18.5 – 30 kg/m^2). Exclusion criteria were being under immunosuppressive or anti-inflammatory treatment, or undergoing pregnancy. All study participants gave informed consent, and the study was approved by the Committee on the Ethics of Research of the Germans Trias i Pujol Hospital.

Generation of human DCs. Peripheral blood mononuclear cells (PBMCs) were obtained from 50 ml blood samples of adult patients with T1D (n=7) after Ficoll Paque density gradient centrifugation (GE Healthcare, Marlborough, USA). Monocytes were isolated using the EasySep Human CD14 Positive Selection Kit (STEMCELL Technologies, Vancouver, Canada) and cultured with IL-4 and GM-CSF as described [10] to derive DCs. After 6 days of culture, DC differentiation yield was assessed by CD11c-APC staining (Immunotools, Friesoythe, Germany) using flow cytometry (FACS Canto II, BD Biosciences, San Jose, USA). The negatively selected fraction of PBMCs was cryopreserved in Foetal Bovine Serum (ThermoFisher Scientific, Waltham, MA, USA) with 10% dimethylsulfoxide (Sigma-Aldrich).

Effects of the combined therapy in human DCs. DCs from patients with T1D (n=7) were co-cultured with 1mM PSAB-liposomes (PSAB-DC),

1000nM Lira (Lira-DC) or combined (PSAB + Lira DC) for 24h in the presence of 20 μg/ml human insulin (Sigma-Aldrich). DCs were cultured with 20 μg/ml human insulin (Sigma-Aldrich) to obtain immature DCs (iDCs) and adding a cytokine cocktail [1000 IU/ml TNFα and 2000 IU/ml IL-1β (Immunotools) and 1 μM Prostaglandin E_2 (Cayman Chemical, Ann Arbor, USA)] to obtain mature DCs (mDC). To assess DCs phenotype, CD25-PE, CD86-FITC, HLA ABC-FITC, HLA DR-FITC, CD14-PE and CD40-APC (Immunotools), CD36-APCCy7, TIM4-APC, CD54-PECy7, TLR2-FITC, CXCR4-APCCy7, CCR2-APC, PD-L1-PECy7, ILT3-PECy7 (Biolegend, San Diego, USA) and CCR7-PECy7 (BD Biosciences) monoclonal antibodies were used to determine their membrane expression.

RNA-seq. DCs from 4 patients with T1D were cultured in basal conditions (iDCs), with 1mM PSAB-liposomes (PSAB-DC), 1000nM Lira (Lira-DC) or combined (PSAB + Lira DC) for 4h. Cells were then harvested from culture wells with Accutase (eBioscience, San Diego, CA, USA). Viability and DC percentage were assessed by flow cytometry (FACS Canto II, BD Biosciences) after staining with 7aad (BD Biosciences), annexin V-PE and CD11c-APC (Immunotools). RNA was obtained using the RNeasy Micro Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions, and RNA integrity was assessed by capillary electrophoresis

in the TapeStation 2200 instrument (Agilent Technologies Inc., Santa Clara, CA, USA). Thus, 500 ng of total RNA was used to prepare the library. Ribosomal RNA (rRNA) was depleted through oligo-dT binding (mRNA Magnetic Isolation, New England Biolabs). Strand-specific libraries were made using the NEBNext® Ultra II Directional RNA LibPrep kit (New England Biolabs), and the quality was evaluated again with TapeStation 2200 (Agilent Technologies Inc), and quantity, with KAPA Library quantification kit (Roche, Mannheim, Germany). RNA sequencing was performed using an Illumina sequencer (Illumina, San Diego, CA, USA) in a sequencing-by-synthesis protocol consisting of 2x75 cycles with 20 million reads. The differential gene expression analysis was performed using the DESeq2 algorithm [22]. Genes with an adjusted p-value <0.5 and Log2 of fold change >1.2 were considered upregulated, whereas those with Log2 of fold change <1.2 were considered downregulated. Experimental data have been uploaded into the European Nucleotide Archive (EBI, https://www.ebi.ac.uk/ena; accession number: GSE144348). Confirmation through qPCR was performed in an independent cohort of 3 patients as previously described [10,11].

Humanised mice. Normoglycaemic immunodeficient NSG mice at 8 weeks of age were humanised i.p. with 10^7 PBMCs from 3 different adult

subjects with T1D. PBMCs were obtained Ficoll Paque density gradient centrifugation (GE Healthcare) as aforementioned. All mice with more than 10% of human/murine CD45 chimerism at day 13 were injected either with PBS (n=3) or the combined therapy (n=7). The percentage and count of regulatory T cells (Tregs) was analysed once a week throughout the treatment and total T lymphocyte subsets were analysed at endpoint.

Statistical analysis. Prism 7.0 software (GraphPad software Inc., San Diego, USA) was used to perform the statistical analysis. For comparisons of unpaired data, a non-parametric Mann-Whitney test was used and for paired comparisons, a non-parametric Wilcoxon test was used.

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Duality of interest. M.C.-S., D.M., and M.V.-P. are co-founder of Ahead Therapeutics SL, which aims at the clinical translation of immunotherapies for the treatment of autoimmune diseases.

Author contributions. A.V., I.P-A., and M.V.-P. designed the experiments; A.V., D.P-B., S.R.-F. R.M.R., and R-M.A., performed the experiments in mice; F.V., and E.A. selected the patients and obtained blood samples. A.V., D.P-B., S.R.-F. and L.G-M. carried out the *in vitro* experiments; M.C-S. and D.M. generated the liposomes. A.V., S.R.-F, D.P.-B., and M.V.-P analysed the results and wrote the manuscript; S.R.-

F., I.P-A., E.A, and J.V. contributed to the discussion. All authors revised the manuscript and gave final approval of the version to be published.

Guarantor statement: M.V.-P./A.V. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Ethics statement. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Generalitat de Catalunya, Catalan Government. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Germans Trias i Pujol Research Institute (Permit DAAM 9521) and has followed the principles outlined in the Declaration of Helsinki for animal experimental investigation. Human samples were obtained after the approval and in strict accordance with the recommendations of the guidelines of Germans Trias i Pujol Ethical Committee. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

FIGURE AND FIGURE LEGENDS

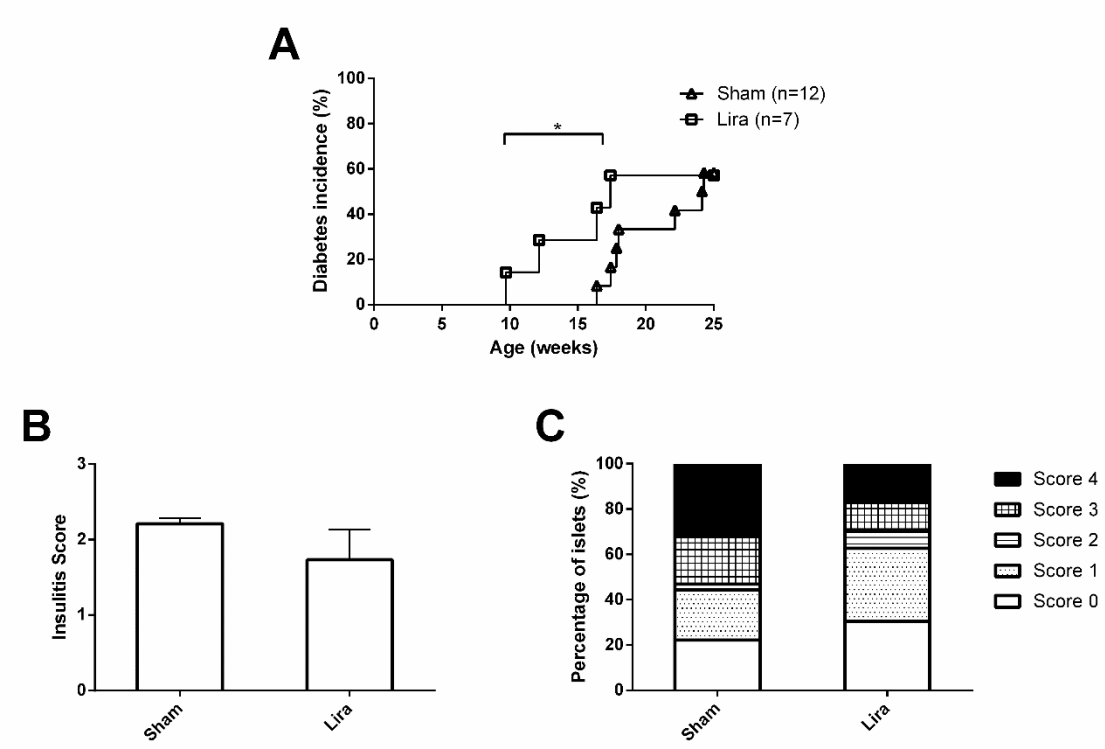


Fig 1. Effect of liraglutide in pre-diabetic NOD mice. A) Incidence of diabetes (%) in NOD mice treated with liraglutide (Lira, squares, n=7) or PBS (Sham, triangles, n=12). No significant differences in T1D incidence were found between groups (Mantel-Cox Log-Rank). Significant differences in the age of the onset were found between Lira and Sham groups (*$p < 0.05$, Mann-Whitney test). B) Islet leukocytic infiltrate (insulitis score) in sham group (n=3) and NOD mice treated with liraglutide (n=3) at the end of the follow-up. Results are mean ± Standard Deviation (SD). No statistical differences were found between groups (Mann-

Whitney test). C) Percentage of islets in each of the infiltration categories, in sham and liraglutide treated groups: White = 0, no insulitis; Dotted = 1, peri-insular; Striped = 2, mild insulitis (<25% of the infiltrated islet); Squared = 3, 25–75% of the islet infiltrated; Black = 4, >75% islet infiltration.

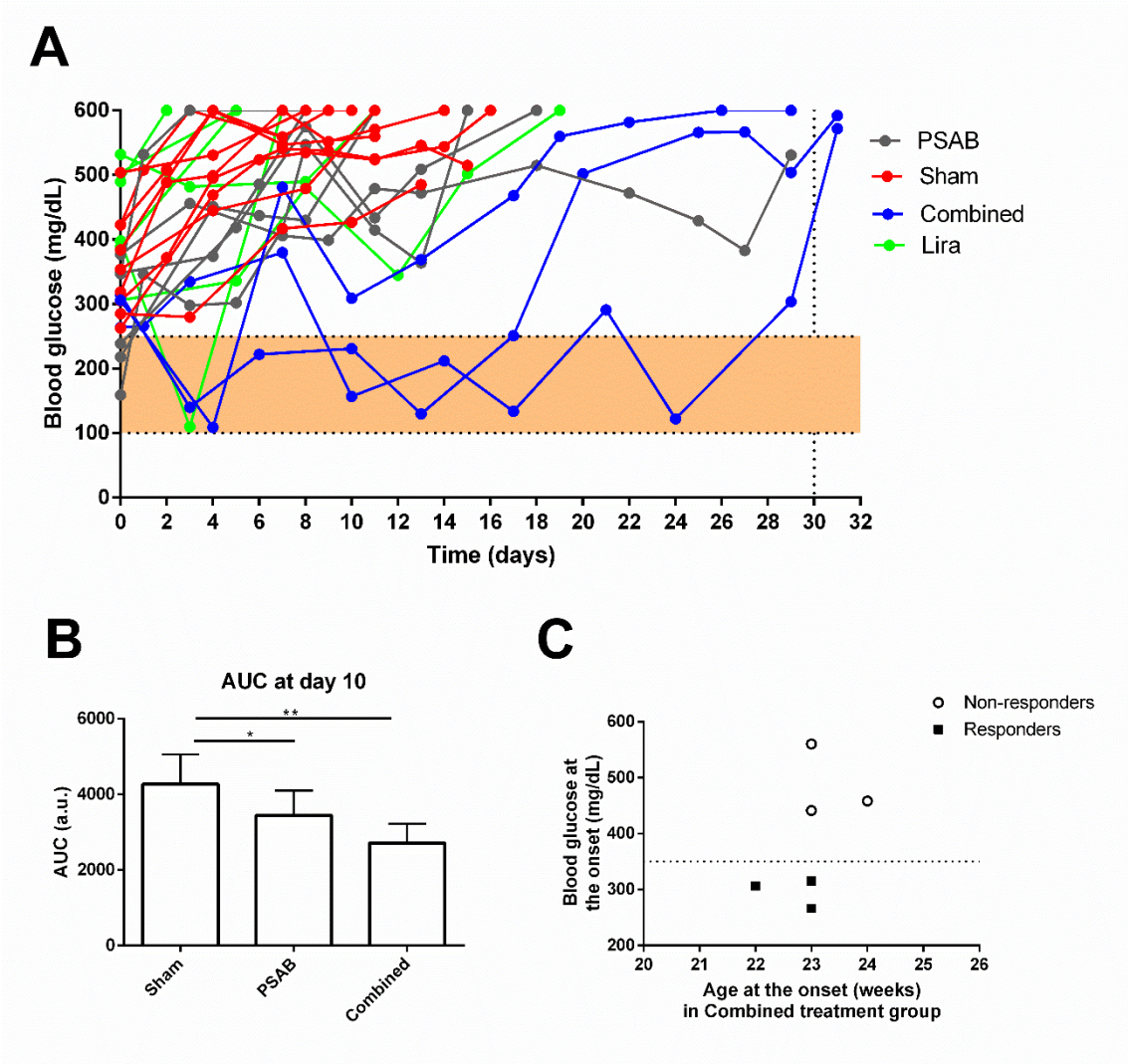


Fig 2. Effect of liraglutide in diabetic NOD mice. A) 2h fasting blood glucose levels (mg/dL) in diabetic NOD mice treated with either PBS (Sham, red, n=9), PSAB-liposomes (PSAB, grey, n=6), liraglutide (Lira, green, n=6) or combined (PSAB + Lira, blue, n=3). Filled area corresponds to normal blood glucose levels in NOD mice. B) Area Under the Curve (AUC) of the graph in A) at day 10, when all mice of Sham, PSAB, and

combined groups remain alive. Results are expressed as mean ± SD. Differences were found between Sham and PSAB (*$p < 0.05$, Mann-Whitney test) and between Sham and combined therapy (**$p < 0.01$, Mann-Whitney test). C) Stratification of responders (black squares) and non-responders (white circles) in the group treated with combined therapy regarding the age (weeks) and blood glucose levels (mg/dL) at the onset.

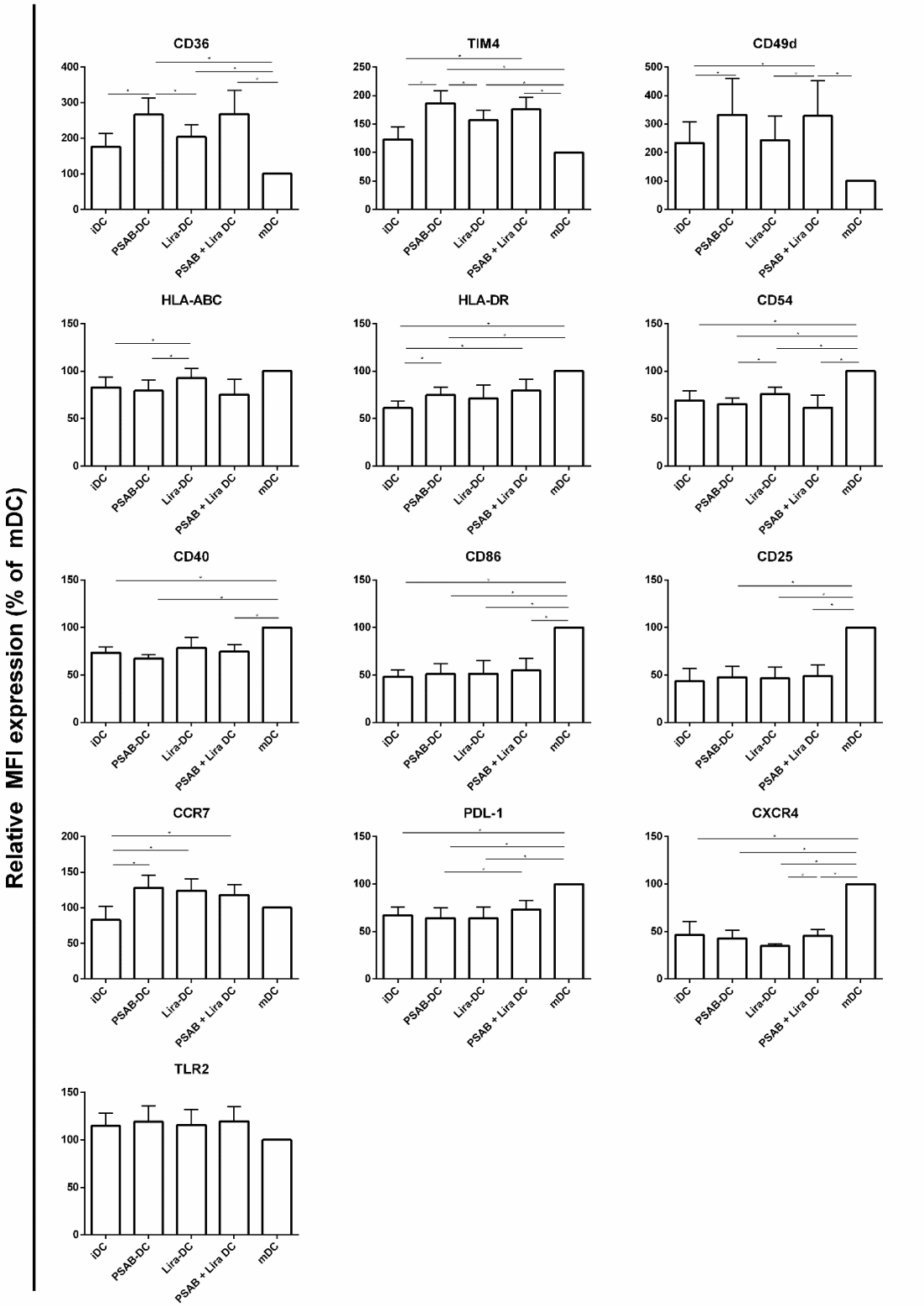

Relative MFI expression (% of mDC)
CD36
TIM4
CD49d
HLA-ABC
HLA-DR
CD54
CD40
CD86
CD25
CCR7
PDL-1
CXCR4
TLR2
iDC
PSAB-DC
Lira-DC
PSAB + Lira DC
mDC

Fig 3. Combined therapy regulates dendritic cell (DCs) phenotype. Relative CD36, TIM4, CD49d, HLA-ABC, HLA-DR, CD54, CD40, CD86, CD25, CCR7, PDL-1, CXCR4 and TLR2 membrane expression in DCs (n=7) obtained from adult subjects with T1D. Results are mean ± SD. Significant differences were found when comparing culture conditions (*p ≤ 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, Wilcoxon test).

A

GENE	Control vs Lipos		Control vs Combined	
	log2FC	adj p-value	log2FC	adj p-value
ABCA1	1,9311	$4{,}27{\cdot}10^{-7}$	1,6106	0,0084
ZNF160	1,6646	$3{,}39{\cdot}10^{-5}$	1,5138	0,0084
DMXL2	1,5948	0,0114	1,5745	0,0114
DGKA	1,6592	0,0033	1,6136	0,0147
ZFYVE16	1,5035	0,0201	1,4880	0,0178
PTPN12	1,2665	0,0138	1,2858	0,0201
SLC25A36	1,4056	0,0009	1,3345	0,0228
CD1D	-1,6655	0,0081	-1,6367	0,0267
DNPH1	-1,8277	0,0242	-1,9128	0,0282
TLR2	1,4346	0,0124	1,4297	0,0325
FOS	-1,7777	0,0233	-1,8715	0,0337
BCL6	1,8044	0,0056	1,7333	0,0366
PRC1	2,0179	0,0081	1,8453	0,0366

B

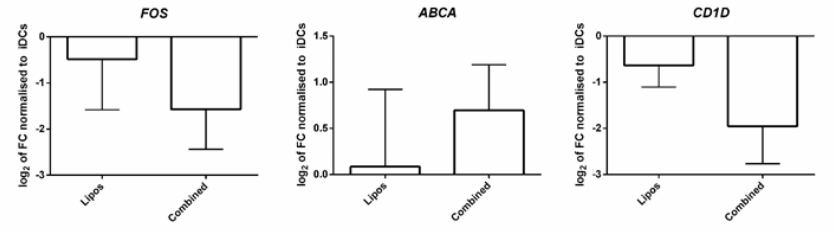


Fig 4. Gene expression analysis of human DCs co-cultured with PSAB-liposomes and combined therapy. A) List of differentially expressed

genes in both PSAB and combined groups in comparison to control conditions, showing log_2 of Fold Change and adjusted p-value. B) Relative gene expression of 3 selected genes from A) (FOS, ABCA, CD1D) analysed by qRT-PCR. Gene expression was normalized to *GAPDH*. Bars show the mean ± s.d. of the Log2 of FC using basal transcription as standard value (Wilcoxon test). Validation of the differential expression of three selected genes from A) (*FOS*, *ABCA*, *CD1D*) by quantitative RT-PCR (n=3). Results are mean ± SD.

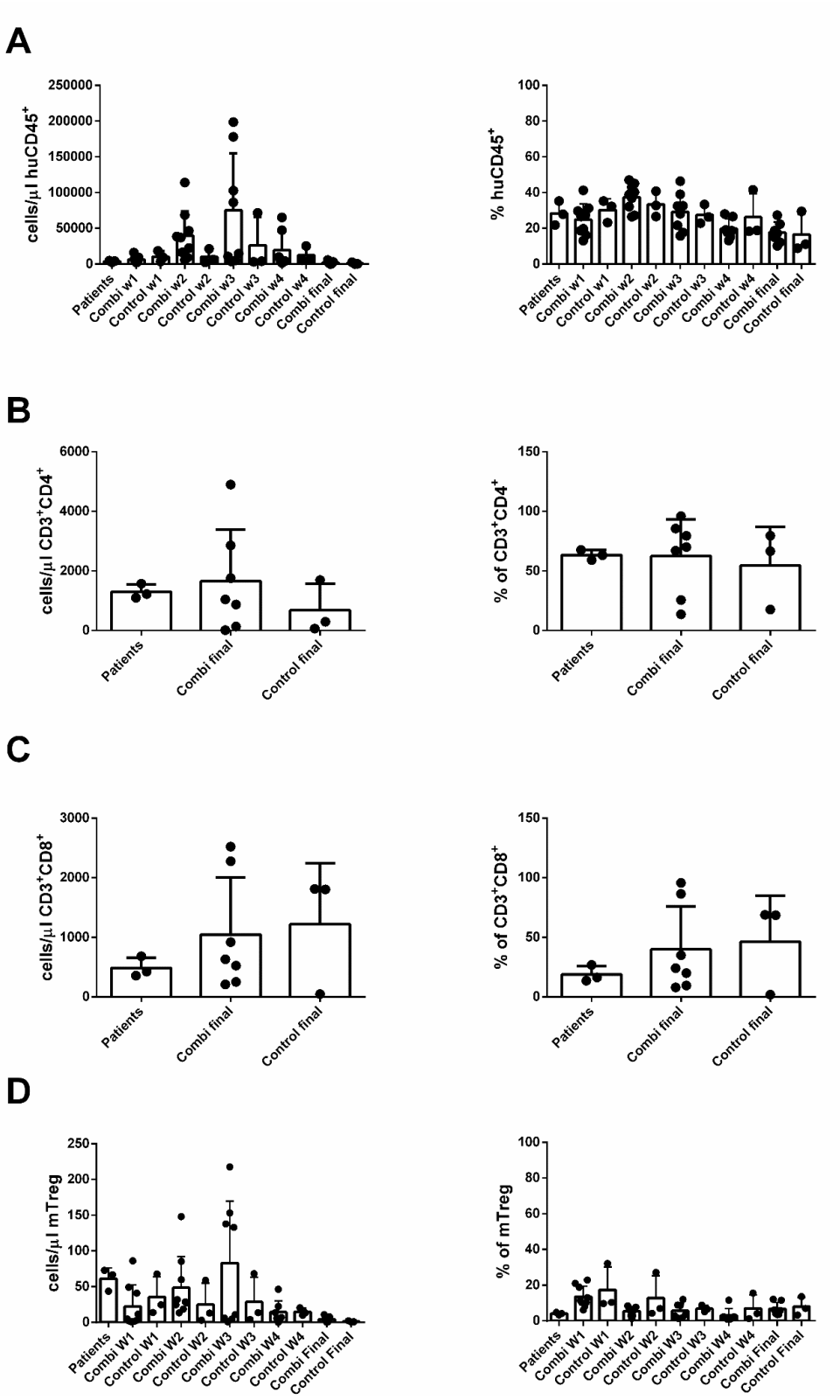
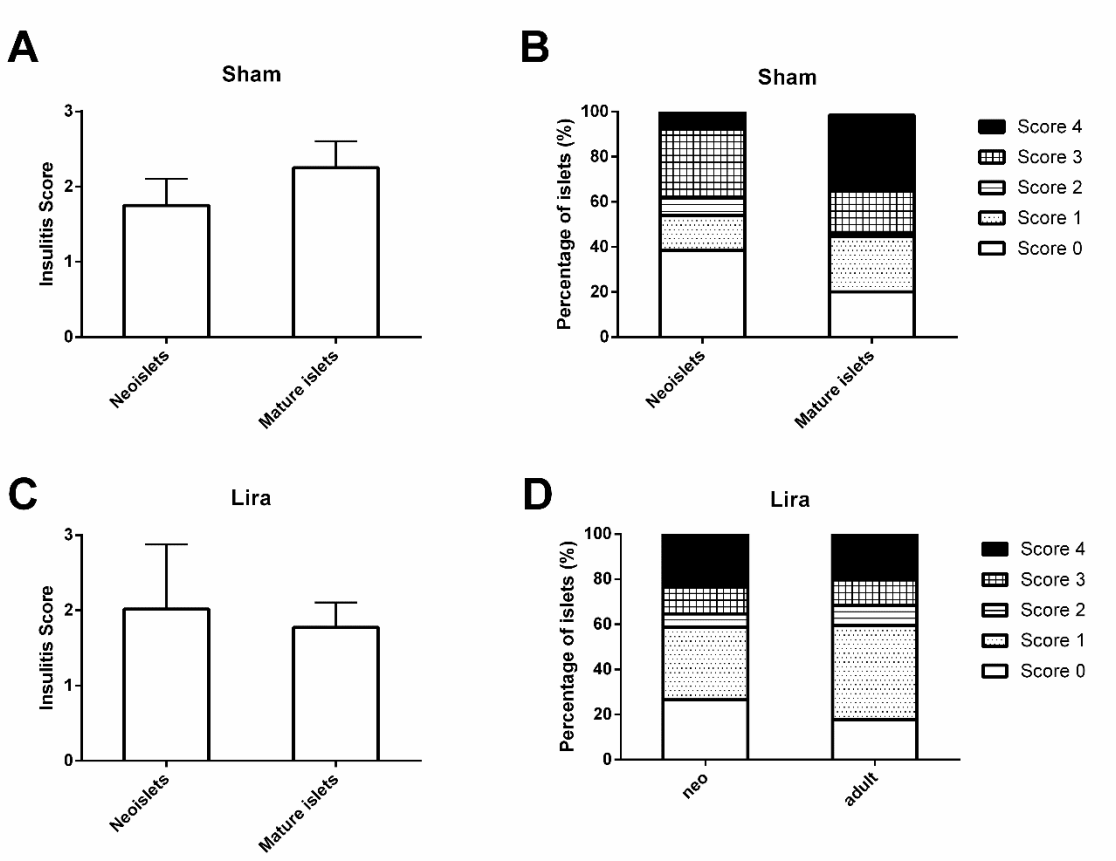

A
cells/µl huCD45+
250000
200000
150000
100000
50000
0
Patients
Combi w1
Control w1
Combi w2
Control w2
Combi w3
Control w3
Combi w4
Control w4
Combi final
Control final
% huCD45+
100
80
60
40
20
0
B
cells/µl CD3+CD4+
6000
4000
2000
0
Patients
Combi final
Control final
% of CD3+CD4+
150
100
50
C
cells/µl CD3+CD8+
3000
2000
1000
% of CD3+CD8+
D
cells/µl mTreg
250
200
150
100
50
Patients
Combi W1
Control W1
Combi W2
Control W2
Combi W3
Control W3
Combi W4
Control W4
Combi Final
Control Final
% of mTreg

Fig 5. *In vivo* effect of the combined therapy in PBMC-humanised NSG mice. Total counts (left) and percentage (right) of human cells at the starting point (in patients), in mice from week 1 (w1) to week 4 (w4) post-transplantation and at the endpoint (day 30 of treatment, final). A) Data of human $CD45^+$ cells. B) Data of human $CD3^+CD4^+$ cell subset. C) Data of human $CD3^+CD8^+$ cell subset. D) Data of human memory T regulatory cells (mTreg) subset. Data refers either to patients (n=3), combined (n=7) or control (n=3) group.



Supp Fig 1. Effect of Liraglutide administration in the insulitis score of non-diabetic NOD mice. A) Total insulitis score and B) percentage of insulitis score islets in sham mice at 25 weeks of age, revealing increased insulitis and percentage of score 3 and 4 islets in mature islets when compared to neoislets. C) Total insulitis score and D) percentage of insulitis score islets in liraglutide treated mice at 25 weeks of age, revealing an increased insulitis score in neoislets and a similar percentage of score 3 and 4 islets.

TABLES

Table 1. Features of the liposomes used in the study.

Liposome	Peptide	Specie	Diameter (nm)	Polydispersity Index (PdI)	ζ-potential (mV)	Encapsulation (%)
mPSA-lipos	$Insulin_{90-110}$ (A chain)	*Mus musculus*	712	0.364	-46.6	32.29
mPSB-lipos	$Insulin_{25-54}$ (B chain)	*Mus musculus*	628	0.325	-44.9	89.63
hPSA-lipos	Insulin* (A chain)	*Homo sapiens*	690 ± 29	0.40 ± 0.28	−38.57 ± 6.76	39.74 ± 22.10
hPSB-lipos	Insulin* (B chain)	*Homo sapiens*	788 ± 264	0.52 ± 0.42	−37.50 ± 7.16	93.19 ± 0.92

PS: Phosphatidylserine; A: Insulin A chain; B: Insulin B chain; The subindex refers to the position of the query sequence to the protein. Data are expressed as mean ± SD.

*These peptides correspond to the whole A or B chains of the insulin peptides.

Table 2. Data from the patients included in the peripheral blood collection.

N	Gender (M/F)	Age (years)	BMI	Age at onset (years)	HbA1c (%)
13	3/13	26,7 ± 4,5	22,2 ± 2,4	13,9 ± 9,6	7,3 ± 0,9

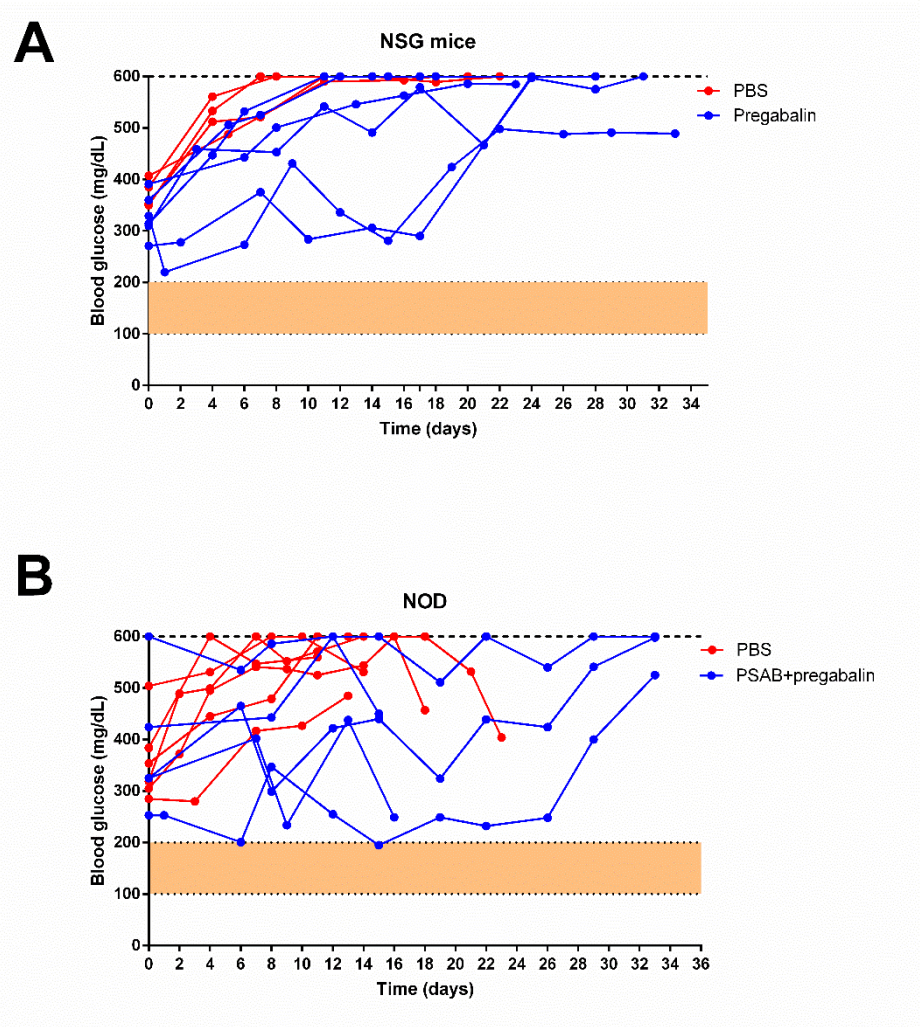
BMI: Body Mass Index. Data presented as mean ± SD.

ANNEX

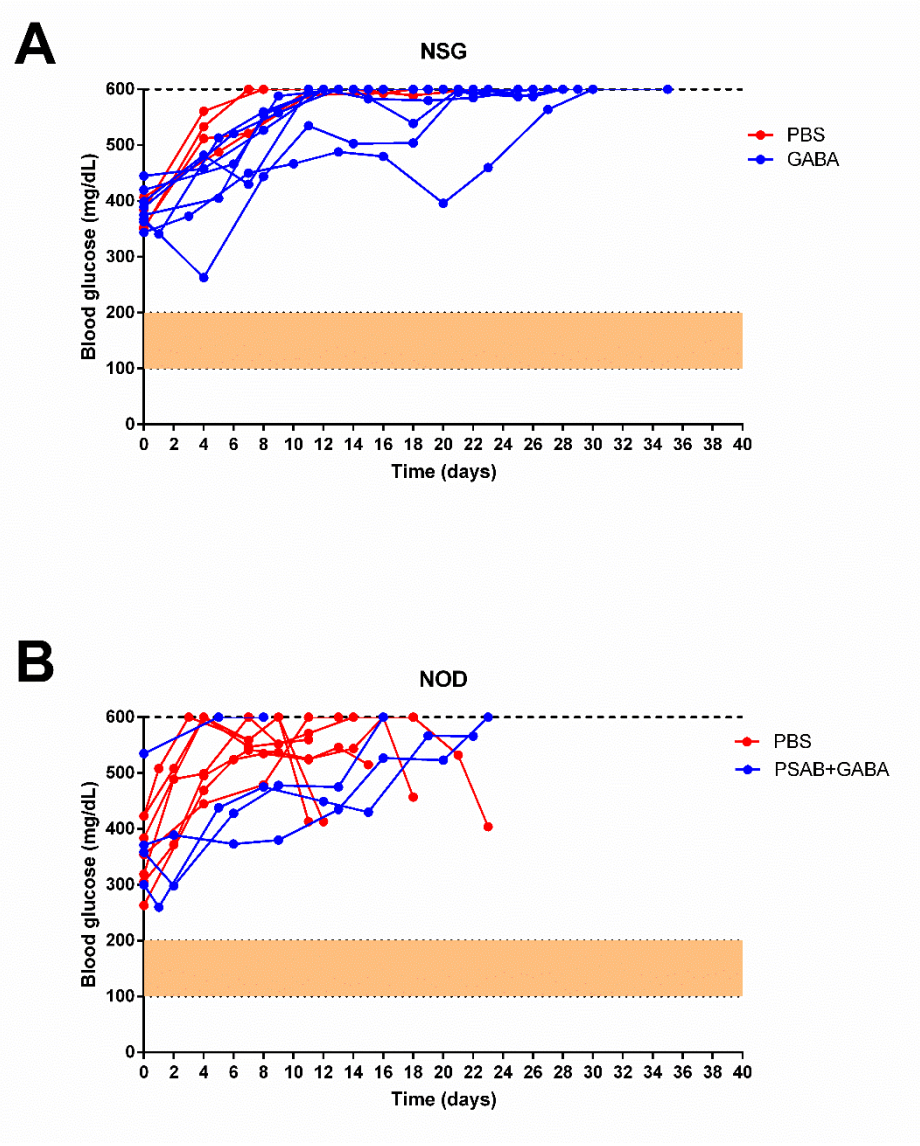
Annex 1. List of repurposed drugs with putative potential to induce β-cell regeneration. The predicted efficacy value (scored in arbitrary units from 0 to 100) is scored globally and for each regenerative pathway: transdifferentiation, neogenesis and self-replication. This list comprises only approved compounds scoring a global efficacy value higher than 60.

Drug identification		Predictive values			
Drugbank ID	Drug Name	Global	Transdifferentiation	Neogenesis	self-regeneration
DB01276	Exenatide	96,88	68,60	6,92	5,05
DB00208	Ticlopidine	73,15	5,21	63,89	5,38
DB01132	Pioglitazone	73,12	61,23	16,81	45,72
DB00230	Pregabalin	72,78	5,33	5,45	67,64
DB00412	Rosiglitazone	70,78	39,06	29,14	37,76
DB00966	Telmisartan	70,67	43,61	29,14	37,76
DB00351	Megestrol acetate	70,49	36,06	56,51	57,24
DB01029	Irbesartan	70,38	48,22	46,36	65,28
DB00731	Nateglinide	70,24	39,06	29,14	28,31
DB00183	Pentagastrin	70,15	58,09	8,65	5,41
DB00017	Salmon Calcitonin	70,08	51,87	5,33	7,87
DB00272	Betazole	68,57	50,24	12,54	5,38
DB01261	Sitagliptin	68,09	61,93	5,42	5,32
DB00227	Lovastatin	67,79	17,92	44,99	10,81
DB00615	Rifabutin	67,41	15,88	5,44	70,50
DB00331	Metformin	64,38	62,92	12,77	8,85
DB08900	Teduglutide	64,35	56,12	16,45	5,41
DB01035	Procainamide	64,22	7,07	54,12	63,47
DB00549	Zafirlukast	63,75	55,47	6,24	5,43
DB00364	Sucralfate	63,08	14,61	56,03	5,31
DB01076	Atorvastatin	61,25	37,36	19,07	4,98
DB01393	Bezafibrate	61,07	29,08	15,02	12,40

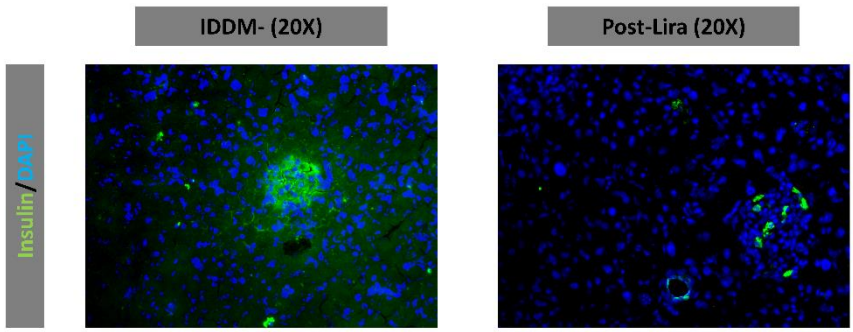
Annex 2. A) Fasting blood glucose values of NSG mice rendered diabetic through STZ administration and treated daily with Pregabalin (i.p, 25mg/kg). **B)** Fasting blood glucose values of spontaneous diabetic NOD mice treated with PSAB-liposomes (i.p, days 1, 3 and 7) and Pregabalin (i.p, 25mg/kg daily from day 4).



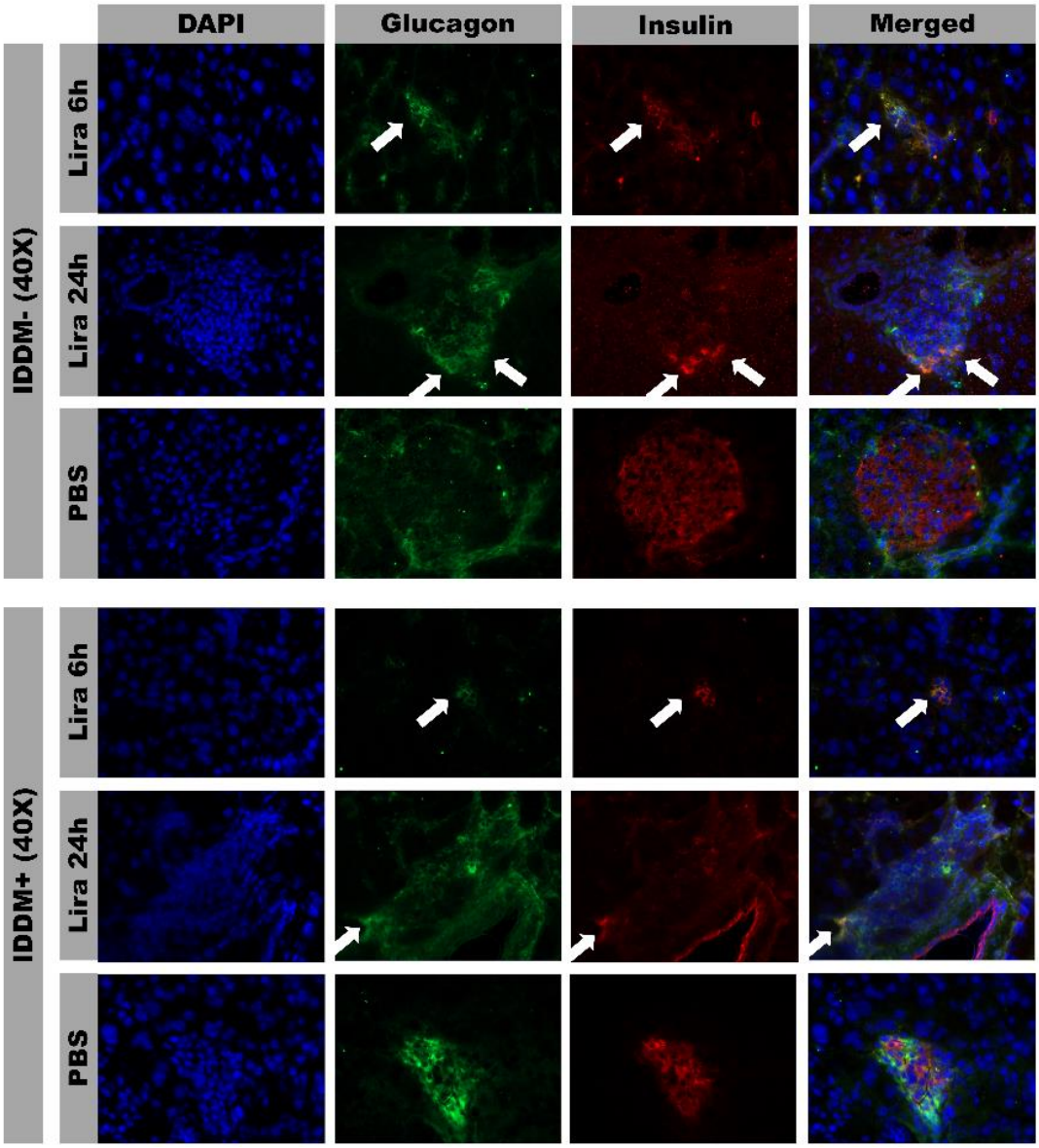
Annex 3. A) Fasting blood glucose values of NSG mice rendered diabetic through STZ administration and treated daily with GABA (i.p, 10mg/kg). **B)** Fasting blood glucose values of spontaneous diabetic NOD mice treated with PSAB-liposomes (i.p, days 1, 3 and 7) and GABA (i.p, 10mg/kg daily from day 4).



Annex 4. Representative histological staining of an islet from a non-diabetic animal (IDDM-), and the Post-Lira group (NSG diabetic mice after liraglutide withdrawal). While there is insulin positivity in the ductal area from the Post-Lira picture, the islet display an abnormal insulin distribution. Insulin is stained in green, nuclei in blue and the magnification is 20X.

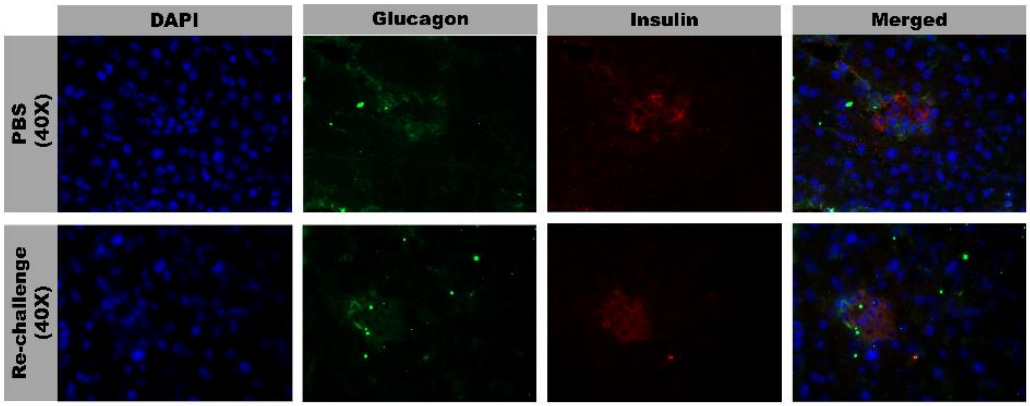


Annex 5. Representative histological images of bihormonal (glucagon^{+}insulin^{+}, white arrows) cells at 6h and 24h after Lira treatment is initiated. Bihormonal cells can be detected at these stages in both diabetic and non-diabetic animals. All images are magnified at 40X.

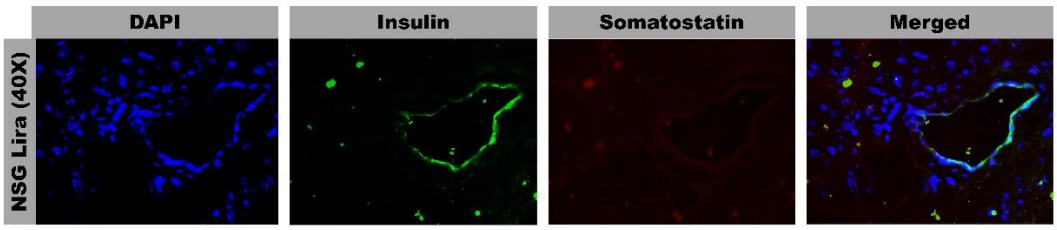


Annex 6. Schedule of liraglutide treatment re-challenge after a transient withdrawal and representative histological pictures. No bihormonal cells were found after liraglutide re-challenge. All images are magnified at 40X.

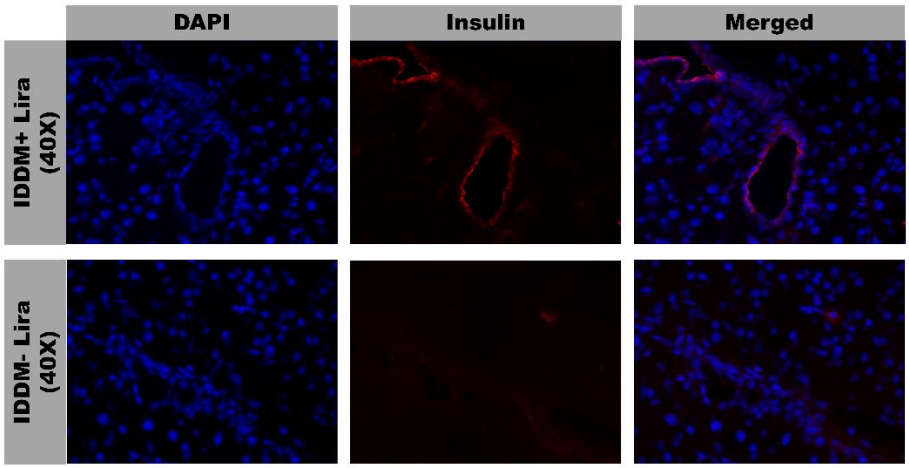
Treatment	Day 1	Day 2	Day 3	Day 4-7	Day 8-14	Day15	Day 16
Sham	PBS	PBS	PBS	PBS	-	PBS	PBS
Lira re-challenged	0.3 mg/kg	0.6 mg/kg	1 mg/kg	1 mg/kg	-	1 mg/kg	1 mg/kg



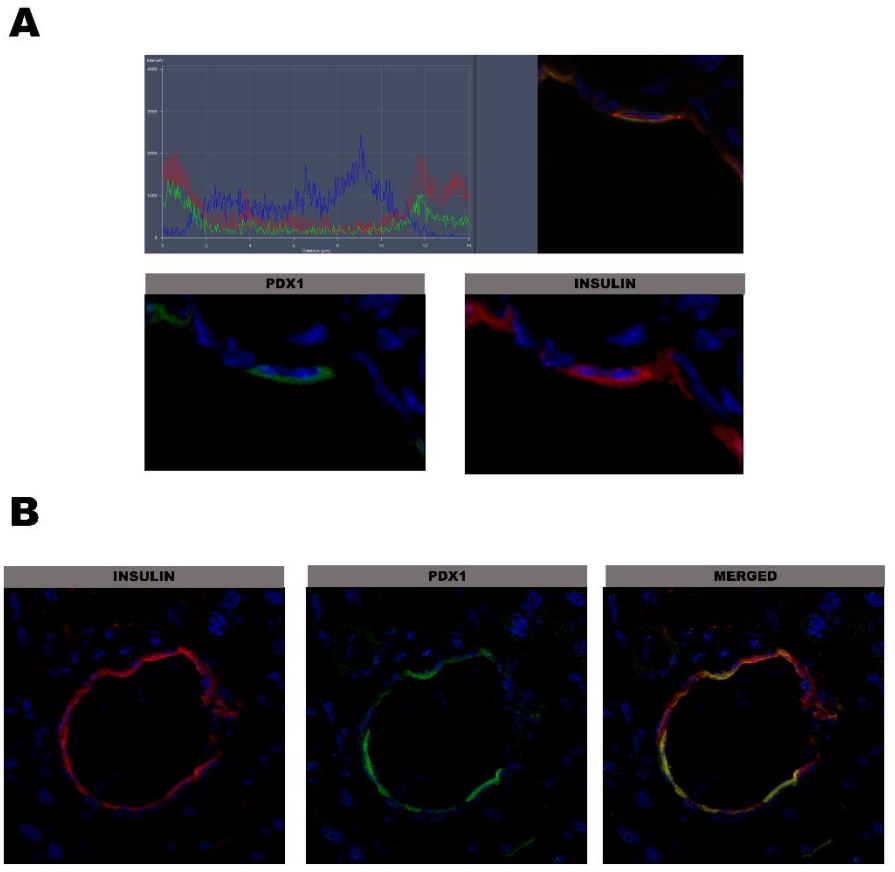
Annex 7. Representative histological image of an insulin^{+} ductal area from a NSG Lira mouse, revealing that the ducts are somatostatin^{-}. Images magnified 40X.



Annex 8. Representative images from ductal areas of NSG diabetic (IDDM+) and non-diabetic mice (IDDM-), both treated with liraglutide, revealing that only diabetic treated animals display insulin^{+} ductal cells. Images are magnified at 40X.

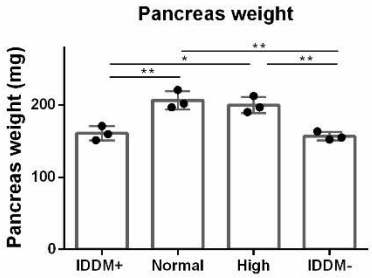


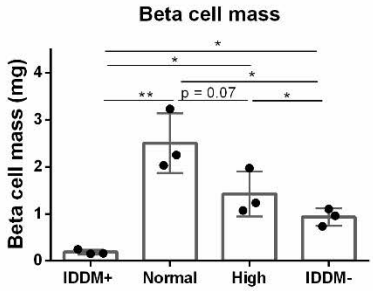
Annex 9. A-B) Confocal analysis reveals subcellular location of PDX1 in the cytoplasm rather than nucleus. Top image (A) is magnified at 40X and bottom images (B) are digitally magnified. Z-stack images of 10 sections (1 section per 1 µm) confirming a similar pattern of insulin and PDX1 co-localization. All images were magnified at 40X.

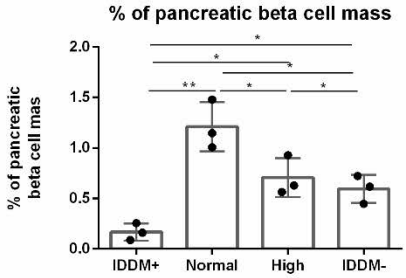


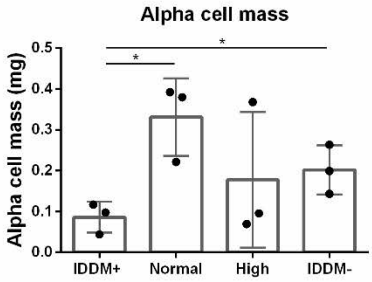
Annex 10. Schedule of liraglutide high dose administration to non-diabetic 30 weeks-old NOD mice. The higher dose results in a reduced increase of the β-cell mass while did not altered the α–cell mass.

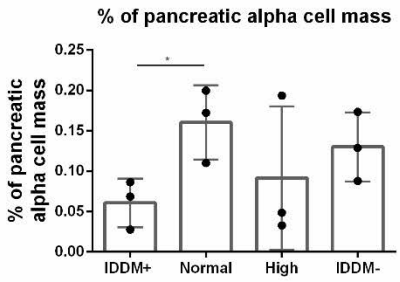
Treatment	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Normal dose	0.3 mg/kg	0.6 mg/kg	1 mg/kg	1 mg/kg	1 mg/kg	1 mg/kg	1 mg/kg
High dose	0.3 mg/kg	0.6 mg/kg	1 mg/kg	2 mg/kg	5 mg/kg	10 mg/kg	10 mg/kg



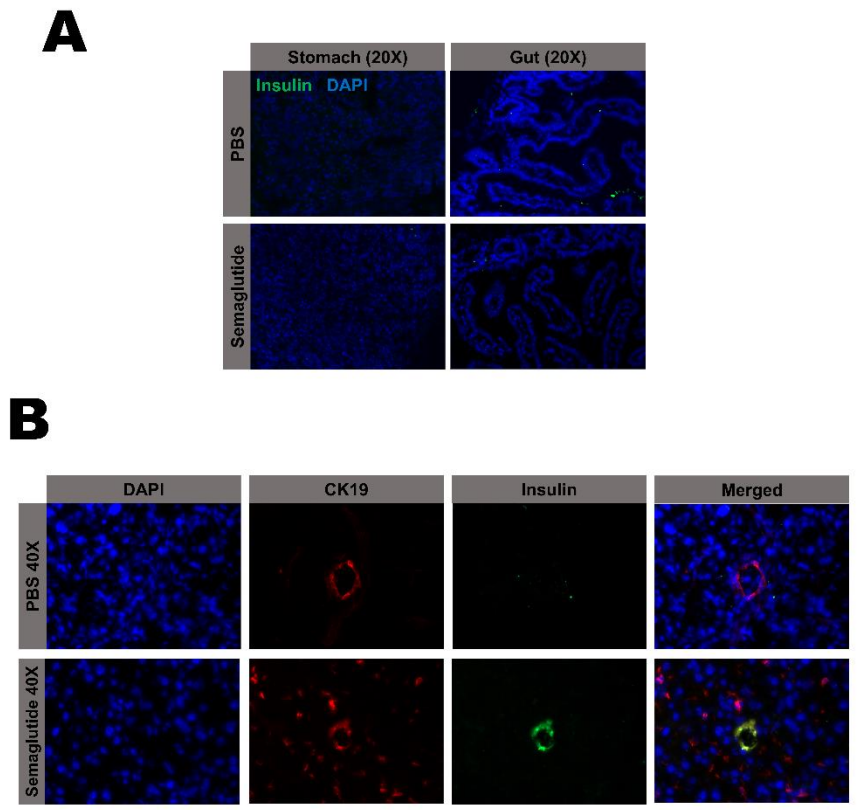




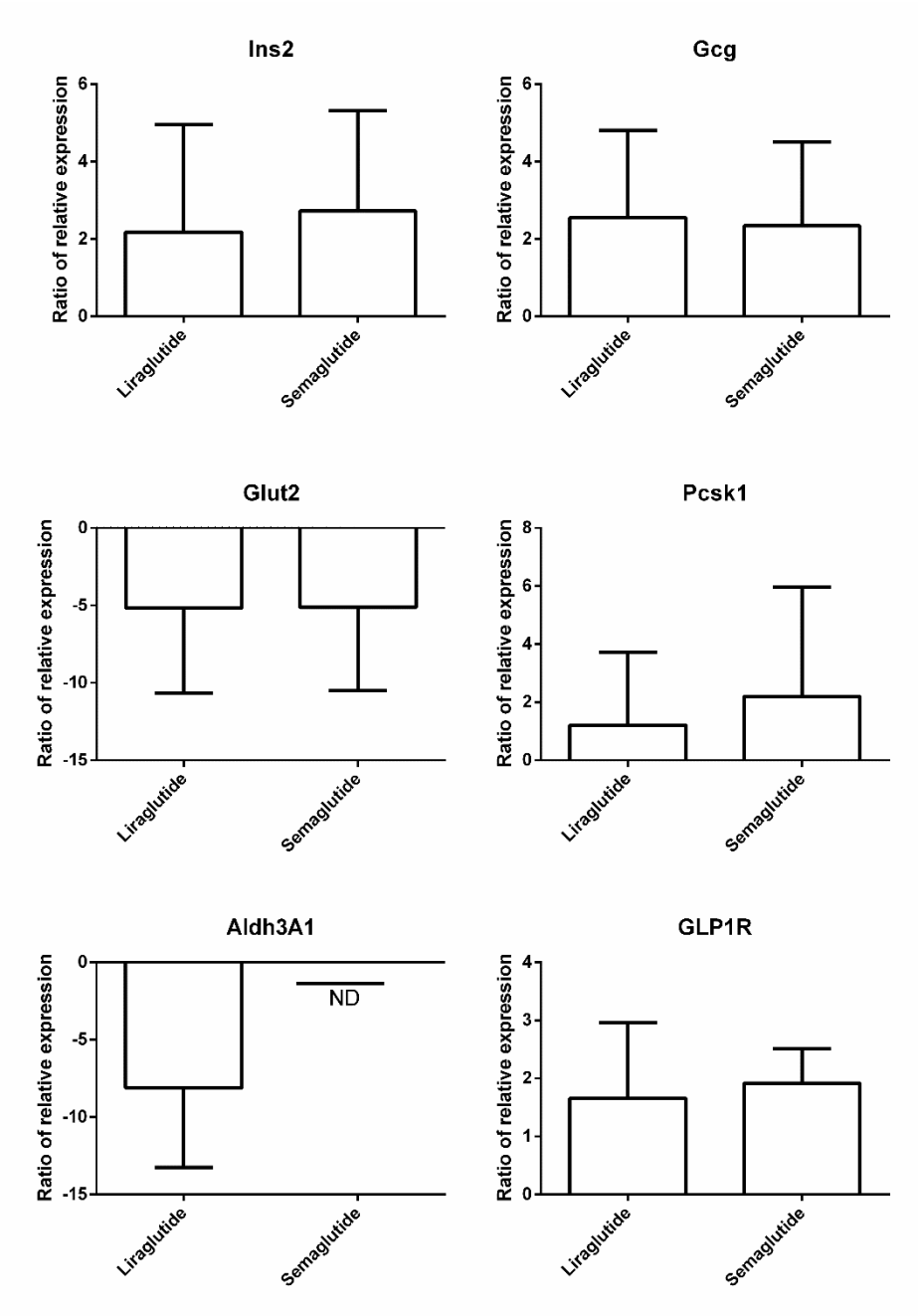




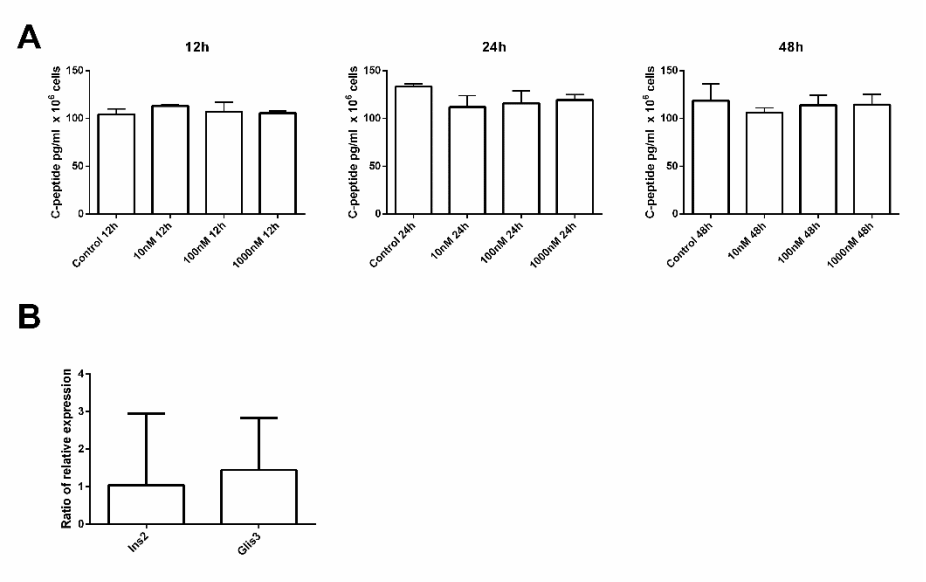
Annex 11. Representative images of diabetic NSG mice treated with semaglutide. **A)** No insulin^{+} cells were found at stomach nor gut tissues. **B)** Semaglutide can also induce CK19^{+}insulin^{+} cells within the ductal areas. Images from gut and stomach are magnified at 20X whereas pancreas images are magnified at 40X.



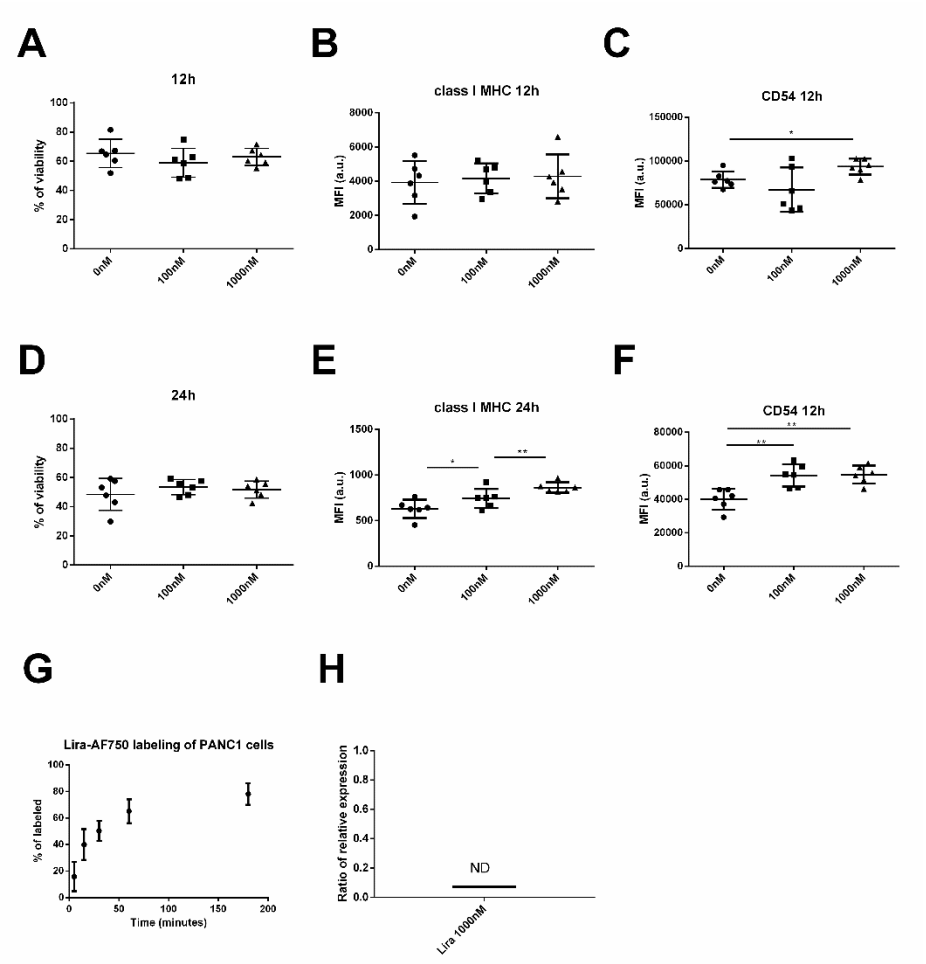
Annex 12. Ratio of relative expression of *Ins2* (insulin), *Gcg* (glucagon), *Glut2*, *Pcsk1*, *Aldh3A1* and *Glp1r* in islets from non-diabetic 8 weeks-old NOD mice.



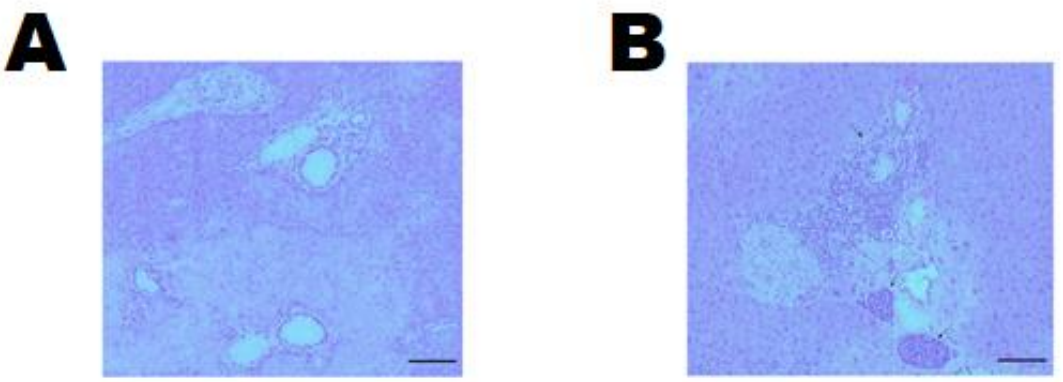
Annex 13. A) C-peptide secretion of NIT-1 cells cultured with increasing concentrations of liraglutide. **B)** Gene expression analysis of *Ins2* (insulin) and *Glis3* genes in NIT-1 cells co-cultured 48h with liraglutide 1000 nM.



Annex 14. A and D) Viability of PANC1 cells at 12h and 24h. **B, C, E and F)** Alterations of class I MHC and CD54 membrane expression at 12h and 24h. **G)** Labelling of PANC1 cells with Lira-AF750. **H)** No detectable levels of *INS* (insulin) gene expression after 48h of PANC1 cell co-culture with liraglutide 1000nM.



Annex 15. Haematoxylin/Eosin stainings from non-diabetic 8 weeks-old NOD mice non-treated **(A)** and treated with three doses of liraglutide at the prediabetic stage **(B)**. Black arrows in B indicate an abundant leukocyte infiltration targeting the ductal areas. Scale bars indicate 100 μm.



Annex 16. List of up- and downregulated genes in human DCs from adult patiens with T1D co-cultured only with PSAB-liposomes during 4h.

Gene	HGNC ID	log 2 FC	adj p-value
ENSG00000165029	ABCA1	1,93	4,2725E-07
ENSG00000170949	ZNF160	1,66	3,3958E-05
ENSG00000165175	MID1IP1	-1,51	0,0001
ENSG00000145780	FEM1C	1,59	0,0001
ENSG00000225855		2,18	0,0006
ENSG00000115008	IL1A	1,79	0,0006
ENSG00000114120	SLC25A36	1,41	0,0009
ENSG00000162461	SLC25A34	2,07	0,0023
ENSG00000006459	KDM7A	1,47	0,0032
ENSG00000065357	DGKA	1,66	0,0033
ENSG00000104093	DMXL2	1,59	0,0036
ENSG00000132952	USPL1	1,42	0,0036
ENSG00000272145		2,31	0,0037
ENSG00000197044	ZNF441	1,57	0,0044
ENSG00000039319	ZFYVE16	1,51	0,0056
ENSG00000113916	BCL6	1,84	0,0056
ENSG00000115548	KDM3A	1,54	0,0056
ENSG00000089057	SLC23A2	1,50	0,0056
ENSG00000109046	WSB1	1,61	0,0059
ENSG00000137817	PARP6	1,51	0,0062
ENSG00000145194	ECE2	-2,12	0,0064
ENSG00000165997	ARL5B	1,34	0,0070
ENSG00000177602	HASPIN	1,76	0,0070
ENSG00000196739	COL27A1	2,07	0,0070
ENSG00000197180		-1,57	0,0078
ENSG00000138688	KIAA1109	1,46	0,0078
ENSG00000244682		1,91	0,0078
ENSG00000158473	CD1D	-1,66	0,0081
ENSG00000198901	PRC1	1,92	0,0081
ENSG00000099985	OSM	-3,14	0,0081
ENSG00000173575	CHD2	1,62	0,0081
ENSG00000214021	TTLL3	1,80	0,0081
ENSG00000229644		1,94	0,0081

ENSG00000261604		3,30	0,0081
ENSG00000130749	ZC3H4	-1,57	0,0081
ENSG00000112425	EPM2A	-1,61	0,0081
ENSG00000166839	ANKDD1A	1,47	0,0083
ENSG00000169155	ZBTB43	1,60	0,0083
ENSG00000099251		2,05	0,0104
ENSG00000231064		2,35	0,0104
ENSG00000018280	SLC11A1	2,14	0,0104
ENSG00000225507		1,67	0,0104
ENSG00000112763	BTN2A1	1,33	0,0105
ENSG00000253476		2,86	0,0107
ENSG00000137462	TLR2	1,43	0,0124
ENSG00000168137	SETD5	1,52	0,0124
ENSG00000185745	IFIT1	-2,04	0,0124
ENSG00000165288	BRWD3	1,43	0,0125
ENSG00000047634	SCML1	1,60	0,0127
ENSG00000189223		1,35	0,0127
ENSG00000267040		-2,04	0,0127
ENSG00000105339	DENND3	2,10	0,0135
ENSG00000111186	WNT5B	-2,67	0,0136
ENSG00000242861		1,62	0,0136
ENSG00000127947	PTPN12	1,27	0,0138
ENSG00000163755	HPS3	1,57	0,0138
ENSG00000197608	ZNF841	1,67	0,0138
ENSG00000168061		-2,19	0,0140
ENSG00000135617	PRADC1	-1,77	0,0140
ENSG00000083097	DOP1A	1,54	0,0140
ENSG00000176055	MBLAC2	-1,90	0,0143
ENSG00000168769	TET2	1,37	0,0143
ENSG00000270231		1,51	0,0143
ENSG00000107036	RIC1	1,36	0,0143
ENSG00000197555	SIPA1L1	1,50	0,0162
ENSG00000198563	DDX39B	1,78	0,0165
ENSG00000154277	UCHL1	-2,36	0,0165
ENSG00000160961	ZNF333	1,74	0,0165
ENSG00000177483	RBM44	1,85	0,0177
ENSG00000165879	FRAT1	-1,68	0,0179
ENSG00000141068	KSR1	2,02	0,0180

ENSG00000263847		2,69	0,0184
ENSG00000172869	DMXL1	1,30	0,0187
ENSG00000121741	ZMYM2	1,63	0,0187
ENSG00000183549	ACSM5	-2,38	0,0187
ENSG00000124224		1,61	0,0187
ENSG00000102057	KCND1	2,33	0,0198
ENSG00000120709	FAM53C	1,52	0,0203
ENSG00000239779	WBP1	1,74	0,0203
ENSG00000182810	DDX28	-1,48	0,0203
ENSG00000115295	CLIP4	1,39	0,0213
ENSG00000131791	PRKAB2	1,43	0,0223
ENSG00000122035	RASL11A	-2,25	0,0224
ENSG00000165617	DACT1	-2,06	0,0227
ENSG00000176410	DJC30	-1,71	0,0232
ENSG00000278600		1,69	0,0232
ENSG00000170345	FOS	-1,79	0,0233
ENSG00000120217	CD274	1,36	0,0233
ENSG00000117000	RLF	1,41	0,0234
ENSG00000180667	YOD1	1,45	0,0235
ENSG00000112667	DNPH1	-1,80	0,0242
ENSG00000188206		1,82	0,0242
ENSG00000152518	ZFP36L2	-1,45	0,0244
ENSG00000205560	CPT1B	1,97	0,0249
ENSG00000158050	DUSP2	-2,12	0,0251
ENSG00000170322	NFRKB	1,54	0,0262
ENSG00000176170	SPHK1	-1,87	0,0285
ENSG00000151726	ACSL1	1,56	0,0285
ENSG00000164327	RICTOR	1,45	0,0285
ENSG00000185989	RASA3	1,54	0,0285
ENSG00000179041	RRS1	-1,68	0,0285
ENSG00000163872	YEATS2	1,46	0,0291
ENSG00000198862	LTN1	1,29	0,0293
ENSG00000132196	HSD17B7	1,60	0,0294
ENSG00000140455	USP3	1,29	0,0294
ENSG00000185947	ZNF267	1,48	0,0298
ENSG00000113249	HAVCR1	1,83	0,0300
ENSG00000203668	CHML	1,54	0,0303
ENSG00000125772	GPCPD1	1,94	0,0303

ENSG00000174516	PELI3	-1,49	0,0303
ENSG00000237399		1,99	0,0303
ENSG00000185432	METTL7A	-1,41	0,0303
ENSG00000116883		2,00	0,0303
ENSG00000085552	IGSF9	3,06	0,0312
ENSG00000162601	MYSM1	1,44	0,0312
ENSG00000111801	BTN3A3	1,45	0,0318
ENSG00000162923	WDR26	1,45	0,0329
ENSG00000152256	PDK1	1,41	0,0329
ENSG00000274925		1,61	0,0329
ENSG00000236304		3,69	0,0336
ENSG00000138658	ZGRF1	1,59	0,0336
ENSG00000169826	CSGALCT2	1,35	0,0336
ENSG00000124222	STX16	1,39	0,0336
ENSG00000091592	NLRP1	1,60	0,0349
ENSG00000125843	AP5S1	-1,59	0,0349
ENSG00000198590		1,90	0,0351
ENSG00000156535	CD109	1,41	0,0351
ENSG00000135912	TTLL4	1,68	0,0356
ENSG00000058063	ATP11B	1,27	0,0364
ENSG00000118620	ZNF430	1,30	0,0377
ENSG00000157890	MEGF11	2,47	0,0378
ENSG00000102606	ARHGEF7	1,43	0,0378
ENSG00000272950		1,81	0,0380
ENSG00000119686	FLVCR2	-1,30	0,0380
ENSG00000131470	PSMC3IP	1,88	0,0380
ENSG00000161800	RACGAP1	1,35	0,0380
ENSG00000131381	RBSN	1,32	0,0380
ENSG00000111674	ENO2	1,88	0,0380
ENSG00000146587	RBAK	1,28	0,0392
ENSG00000026950	BTN3A1	1,63	0,0400
ENSG00000204618	RNF39	2,39	0,0402
ENSG00000162639	HENMT1	-1,43	0,0405
ENSG00000061987	MON2	1,39	0,0405
ENSG00000125633	CCDC93	1,40	0,0405
ENSG00000170477	KRT4	3,29	0,0405
ENSG00000185669	SI3	-1,83	0,0418
ENSG00000279689		1,85	0,0418

ENSG00000225828	FAM229A	1,85	0,0421
ENSG00000114857	NKTR	1,75	0,0421
ENSG00000186866	POFUT2	1,53	0,0421
ENSG00000109920	FNBP4	1,62	0,0422
ENSG00000157617	C2CD2	1,30	0,0426
ENSG00000071189	SNX13	1,27	0,0429
ENSG00000184635	ZNF93	1,32	0,0440
ENSG00000116698	SMG7	1,27	0,0440
ENSG00000135473	PAN2	1,54	0,0440
ENSG00000160193	WDR4	-1,54	0,0443
ENSG00000104517	UBR5	1,43	0,0443
ENSG00000101596	SMCHD1	1,59	0,0443
ENSG00000236255		2,19	0,0443
ENSG00000068097	HEATR6	1,36	0,0443
ENSG00000259820		1,62	0,0443
ENSG00000156127	BATF	-1,83	0,0444
ENSG00000153317	ASAP1	1,46	0,0444
ENSG00000155463	OXA1L	1,26	0,0445
ENSG00000158636	EMSY	1,40	0,0448
ENSG00000122008	POLK	1,34	0,0448
ENSG00000170270	GON7	-1,59	0,0448
ENSG00000116985	BMP8B	1,87	0,0453
ENSG00000143847	PPFIA4	2,62	0,0461
ENSG00000175066	GK5	1,47	0,0465
ENSG00000090686	USP48	1,35	0,0465
ENSG00000074935	TUBE1	1,43	0,0469
ENSG00000136152	COG3	1,32	0,0470
ENSG00000119004	CYP20A1	-1,40	0,0480
ENSG00000161618	ALDH16A1	-1,60	0,0484
ENSG00000125735	TNFSF14	1,88	0,0487
ENSG00000154978	VOPP1	-1,29	0,0487
ENSG00000158669	GPAT4	1,37	0,0490

Annex 17. List of up- and downregulated genes in human DCs from adult patiens with T1D co-cultured only with liraglutide 1000nM during 4h.

Gene	HGNC ID	log 2 FC	adj p-value
ENSG00000165029	ABCA1	1,83	7,0348E-06
ENSG00000170949	ZNF160	1,71	7,0348E-06
ENSG00000197180		-1,80	0,0001
ENSG00000176170	SPHK1	-2,55	0,0001
ENSG00000156127	BATF	-2,61	0,0002
ENSG00000112578	BYSL	-1,71	0,0002
ENSG00000138688	KIAA1109	1,59	0,0002
ENSG00000160193	WDR4	-1,95	0,0002
ENSG00000105676	ARMC6	-2,60	0,0003
ENSG00000162881	OXER1	-2,23	0,0004
ENSG00000262919	CCNQ	-1,66	0,0004
ENSG00000225507		1,83	0,0008
ENSG00000196155	PLEKHG4	-2,15	0,0009
ENSG00000125877	ITPA	-1,79	0,0010
ENSG00000175416	CLTB	-1,81	0,0012
ENSG00000099985	OSM	-3,58	0,0013
ENSG00000235173	HGH1	-2,81	0,0013
ENSG00000177602	HASPIN	1,85	0,0014
ENSG00000187837	HIST1H1C	-2,34	0,0014
ENSG00000115548	KDM3A	1,57	0,0014
ENSG00000145194	ECE2	-2,23	0,0014
ENSG00000165916	PSMC3	-1,60	0,0014
ENSG00000135617	PRADC1	-1,94	0,0018
ENSG00000183684		-2,05	0,0018
ENSG00000128228	SDF2L1	-2,59	0,0018
ENSG00000188177	ZC3H6	1,41	0,0018
ENSG00000114120	SLC25A36	1,37	0,0018
ENSG00000185669	SI3	-2,22	0,0018
ENSG00000137331	IER3	-1,51	0,0020
ENSG00000168237	GLYCTK	-2,10	0,0023
ENSG00000138964	PARVG	-1,70	0,0025
ENSG00000006459	KDM7A	1,44	0,0026
ENSG00000132952	USPL1	1,41	0,0026

ENSG00000173868	PHOSPHO1	-2,85	0,0026
ENSG00000176945	MUC20	-2,09	0,0026
ENSG00000263528	IKBKE	-2,05	0,0026
ENSG00000168061		-2,40	0,0027
ENSG00000148300	REXO4	-1,71	0,0027
ENSG00000083097	DOP1A	1,62	0,0027
ENSG00000242028	HYPK	2,07	0,0027
ENSG00000213563	C8orf82	-2,02	0,0027
ENSG00000104093	DMXL2	1,56	0,0027
ENSG00000177700	POLR2L	-2,87	0,0031
ENSG00000165288	BRWD3	1,47	0,0034
ENSG00000174917	C19orf70	-2,49	0,0034
ENSG00000184887	BTBD6	-1,62	0,0038
ENSG00000173575	CHD2	1,64	0,0039
ENSG00000143801	PSEN2	-1,98	0,0039
ENSG00000197647	ZNF433	2,40	0,0039
ENSG00000124299	PEPD	-1,78	0,0043
ENSG00000143614	GATAD2B	1,42	0,0043
ENSG00000146826	C7orf43	-1,77	0,0044
ENSG00000197044	ZNF441	1,53	0,0046
ENSG00000244556		3,03	0,0046
ENSG00000121741	ZMYM2	1,71	0,0047
ENSG00000166166	TRMT61A	-2,21	0,0047
ENSG00000168890	TMEM150A	-1,77	0,0048
ENSG00000004809	SLC22A16	-1,84	0,0048
ENSG00000186281	GPAT2	-1,76	0,0048
ENSG00000158050	DUSP2	-2,34	0,0050
ENSG00000133812	SBF2	1,47	0,0050
ENSG00000161267	BDH1	-1,72	0,0050
ENSG00000257838		-2,23	0,0050
ENSG00000064201	TSPAN32	-2,34	0,0050
ENSG00000123685	BATF3	-1,89	0,0054
ENSG00000213339	QTRT1	-1,86	0,0054
ENSG00000012061	ERCC1	-1,70	0,0063
ENSG00000184470	TXNRD2	-1,64	0,0066
ENSG00000263847		2,85	0,0068
ENSG00000231064		2,33	0,0071
ENSG00000242861		1,64	0,0071

ENSG00000171853	TRAPPC12	-1,51	0,0071
ENSG00000100889	PCK2	-1,67	0,0072
ENSG00000167588	GPD1	-2,61	0,0072
ENSG00000261236	BOP1	-2,57	0,0072
ENSG00000169220	RGS14	-2,41	0,0072
ENSG00000126106	TMEM53	-1,58	0,0072
ENSG00000164327	RICTOR	1,51	0,0073
ENSG00000071655	MBD3	-2,00	0,0075
ENSG00000097021	ACOT7	-1,90	0,0075
ENSG00000116691	MIIP	-2,00	0,0078
ENSG00000048162	NOP16	-1,47	0,0079
ENSG00000104517	UBR5	1,51	0,0079
ENSG00000048140	TSPAN17	-1,77	0,0079
ENSG00000063241	ISOC2	-2,89	0,0081
ENSG00000173137	ADCK5	-2,48	0,0081
ENSG00000122359	ANXA11	-1,27	0,0081
ENSG00000167543	TP53I13	-1,91	0,0081
ENSG00000258818	RSE4	3,10	0,0085
ENSG00000172869	DMXL1	1,31	0,0085
ENSG00000234289	H2BFS	-3,62	0,0085
ENSG00000105699	LSR	-2,60	0,0085
ENSG00000188643	S100A16	-1,95	0,0085
ENSG00000279838		1,64	0,0088
ENSG00000154277	UCHL1	-2,39	0,0089
ENSG00000196998	WDR45	-1,57	0,0089
ENSG00000169718	DUS1L	-1,67	0,0089
ENSG00000142634	EFHD2	-1,36	0,0092
ENSG00000187796	CARD9	-2,63	0,0092
ENSG00000168769	TET2	1,37	0,0092
ENSG00000158636	EMSY	1,47	0,0092
ENSG00000132906	CASP9	-1,35	0,0092
ENSG00000185658	BRWD1	1,46	0,0092
ENSG00000072506	HSD17B10	-1,69	0,0092
ENSG00000101945	SUV39H1	-1,76	0,0092
ENSG00000099800	TIMM13	-2,33	0,0092
ENSG00000104886	PLEKHJ1	-1,98	0,0092
ENSG00000113916	BCL6	1,71	0,0095
ENSG00000162923	WDR26	1,33	0,0095

ENSG00000125843	AP5S1	-1,66	0,0095
ENSG00000128011	LRFN1	2,03	0,0095
ENSG00000159692	CTBP1	-1,36	0,0095
ENSG00000280046		2,08	0,0095
ENSG00000125753	VASP	-1,40	0,0095
ENSG00000128923	MINDY2	1,52	0,0095
ENSG00000169689	CENPX	-2,30	0,0095
ENSG00000132481	TRIM47	-1,86	0,0095
ENSG00000270231		1,50	0,0095
ENSG00000189223		1,33	0,0098
ENSG00000120709	FAM53C	1,53	0,0098
ENSG00000272950		1,91	0,0098
ENSG00000131470	PSMC3IP	2,00	0,0098
ENSG00000184205	TSPYL2	2,07	0,0098
ENSG00000277687		1,80	0,0098
ENSG00000117139	KDM5B	1,39	0,0098
ENSG00000167705	RILP	-1,75	0,0098
ENSG00000114767	RRP9	-2,01	0,0098
ENSG00000116096	SPR	-1,71	0,0102
ENSG00000139546	TARBP2	-1,80	0,0102
ENSG00000145780	FEM1C	1,38	0,0104
ENSG00000209082		2,14	0,0104
ENSG00000158483	FAM86C1	-2,01	0,0105
ENSG00000165175	MID1IP1	-1,33	0,0106
ENSG00000174516	PELI3	-1,52	0,0106
ENSG00000133597	ADCK2	-1,54	0,0106
ENSG00000040608	RTN4R	-2,66	0,0106
ENSG00000173889	PHC3	1,43	0,0106
ENSG00000241553	ARPC4	-1,53	0,0106
ENSG00000140386	SCAPER	1,50	0,0106
ENSG00000148296	SURF6	-1,49	0,0106
ENSG00000165724	ZMYND19	-1,65	0,0106
ENSG00000167114	SLC27A4	-1,96	0,0106
ENSG00000146232	NFKBIE	-1,61	0,0106
ENSG00000176058	TPRN	-2,53	0,0106
ENSG00000137877	SPTBN5	-2,73	0,0106
ENSG00000146192	FGD2	-1,45	0,0106
ENSG00000114395	CYB561D2	-1,58	0,0106

ENSG00000166986	MARS	-1,38	0,0109
ENSG00000140563	MCTP2	2,05	0,0113
ENSG00000254901	BORCS8	-1,58	0,0113
ENSG00000112667	DNPH1	-1,82	0,0113
ENSG00000168924	LETM1	-1,71	0,0114
ENSG00000260630		-1,76	0,0114
ENSG00000189042	ZNF567	1,54	0,0117
ENSG00000140365	COMMD4	-1,75	0,0117
ENSG00000104522	TSTA3	-1,59	0,0117
ENSG00000099821	POLRMT	-2,26	0,0117
ENSG00000233621		-1,54	0,0122
ENSG00000138658	ZGRF1	1,62	0,0123
ENSG00000075618	FSCN1	-2,79	0,0126
ENSG00000100294	MCAT	-1,62	0,0127
ENSG00000103995	CEP152	1,59	0,0128
ENSG00000107036	RIC1	1,34	0,0131
ENSG00000161618	ALDH16A1	-1,68	0,0131
ENSG00000164885	CDK5	-1,66	0,0131
ENSG00000071859	FAM50A	-1,41	0,0131
ENSG00000236304		3,88	0,0133
ENSG00000105447	GRWD1	-1,53	0,0134
ENSG00000120913	PDLIM2	-1,64	0,0134
ENSG00000047457	CP	2,11	0,0138
ENSG00000125772	GPCPD1	1,98	0,0138
ENSG00000061987	MON2	1,42	0,0138
ENSG00000160688	FLAD1	-1,43	0,0146
ENSG00000167700	MFSD3	-2,56	0,0147
ENSG00000168137	SETD5	1,46	0,0154
ENSG00000187688	TRPV2	-2,02	0,0156
ENSG00000108798	ABI3	-1,57	0,0158
ENSG00000119673	ACOT2	-1,44	0,0159
ENSG00000179958	DCTPP1	-1,75	0,0159
ENSG00000196705	ZNF431	1,32	0,0159
ENSG00000133265	HSPBP1	-2,35	0,0159
ENSG00000174177	CTU2	-1,89	0,0159
ENSG00000159915	ZNF233	2,06	0,0159
ENSG00000177483	RBM44	1,79	0,0161
ENSG00000104524	PYCR3	-2,67	0,0161

ENSG00000047634	SCML1	1,53	0,0162
ENSG00000236397		-2,97	0,0162
ENSG00000182154	MRPL41	-2,33	0,0163
ENSG00000125503	PPP1R12C	-1,91	0,0163
ENSG00000124224		1,57	0,0163
ENSG00000174775	HRAS	-2,06	0,0163
ENSG00000163832	ELP6	-1,57	0,0165
ENSG00000130204	TOMM40	-2,05	0,0165
ENSG00000198265	HELZ	1,33	0,0166
ENSG00000165886	UBTD1	-2,69	0,0166
ENSG00000183828	NUDT14	-2,35	0,0169
ENSG00000135763	URB2	-1,75	0,0170
ENSG00000100413	POLR3H	-1,86	0,0170
ENSG00000124357	GK	-1,34	0,0171
ENSG00000262358		2,28	0,0172
ENSG00000249992	TMEM158	-1,57	0,0172
ENSG00000185813	PCYT2	-1,81	0,0173
ENSG00000178896	EXOSC4	-2,15	0,0174
ENSG00000166839	ANKDD1A	1,40	0,0175
ENSG00000149600	COMMD7	-1,49	0,0176
ENSG00000166582	CENPV	-1,43	0,0176
ENSG00000269970		-2,12	0,0176
ENSG00000115884	SDC1	-2,71	0,0176
ENSG00000163933	RFT1	-1,29	0,0176
ENSG00000134698	AGO4	1,32	0,0177
ENSG00000115008	IL1A	1,51	0,0178
ENSG00000065357	DGKA	1,48	0,0178
ENSG00000117000	RLF	1,39	0,0178
ENSG00000047346	FAM214A	1,42	0,0178
ENSG00000196544	BORCS6	-1,63	0,0178
ENSG00000182544	MFSD5	-1,38	0,0178
ENSG00000278233		-6,48	0,0178
ENSG00000129465	RIPK3	-1,52	0,0178
ENSG00000108932	SLC16A6	1,59	0,0181
ENSG00000160961	ZNF333	1,67	0,0185
ENSG00000165997	ARL5B	1,27	0,0185
ENSG00000121417	ZNF211	1,54	0,0185
ENSG00000264343	NOTCH2NLA	2,02	0,0185

ENSG00000229097		1,75	0,0185
ENSG00000188229	TUBB4B	-1,73	0,0185
ENSG00000102970	CCL17	-2,42	0,0186
ENSG00000058063	ATP11B	1,27	0,0186
ENSG00000236255		2,25	0,0186
ENSG00000158481	CD1C	-1,64	0,0186
ENSG00000179218	CALR	-1,24	0,0186
ENSG00000172009	THOP1	-2,30	0,0186
ENSG00000007264	MATK	-2,24	0,0186
ENSG00000147548	NSD3	1,31	0,0190
ENSG00000258581		2,21	0,0190
ENSG00000144040	SFXN5	-1,96	0,0191
ENSG00000177352	CCDC71	-1,80	0,0191
ENSG00000277632	CCL3	-2,32	0,0191
ENSG00000163406	SLC15A2	1,79	0,0191
ENSG00000123933	MXD4	-1,89	0,0191
ENSG00000178691	SUZ12	1,26	0,0191
ENSG00000167850	CD300C	-1,85	0,0191
ENSG00000119004	CYP20A1	-1,42	0,0192
ENSG00000157890	MEGF11	2,47	0,0196
ENSG00000006744	ELAC2	-1,41	0,0196
ENSG00000274523	RCC1L	-1,63	0,0196
ENSG00000125912	NCLN	-2,41	0,0196
ENSG00000116191	RALGPS2	1,75	0,0198
ENSG00000033011	ALG1	-1,62	0,0198
ENSG00000113971	NPHP3	1,55	0,0198
ENSG00000048545	GUCA1A	-2,20	0,0198
ENSG00000051523	CYBA	-2,46	0,0198
ENSG00000254986	DPP3	-1,69	0,0198
ENSG00000142556	ZNF614	1,34	0,0198
ENSG00000167085	PHB	-1,47	0,0198
ENSG00000261654		2,13	0,0198
ENSG00000229644		1,74	0,0200
ENSG00000163866	SMIM12	-1,35	0,0201
ENSG00000177548	RABEP2	-1,65	0,0201
ENSG00000137817	PARP6	1,39	0,0206
ENSG00000146733	PSPH	-1,85	0,0208
ENSG00000103415	HMOX2	-1,53	0,0208

ENSG00000089057	SLC23A2	1,38	0,0208
ENSG00000161981	SNRNP25	-1,51	0,0208
ENSG00000189306	RRP7A	-1,79	0,0208
ENSG00000109046	WSB1	1,46	0,0209
ENSG00000140474	ULK3	-1,43	0,0209
ENSG00000100605	ITPK1	-1,65	0,0211
ENSG00000154222	CC2D1B	-1,36	0,0211
ENSG00000174485	DENND4A	1,30	0,0211
ENSG00000165915	SLC39A13	-2,20	0,0212
ENSG00000139410	SDSL	-2,85	0,0212
ENSG00000168566	SNRNP48	1,32	0,0215
ENSG00000106305	AIMP2	-1,59	0,0215
ENSG00000276600	RAB7B	-2,22	0,0215
ENSG00000099949	LZTR1	-1,88	0,0218
ENSG00000174744	BRMS1	-1,78	0,0218
ENSG00000183741	CBX6	-1,41	0,0219
ENSG00000273136		1,88	0,0219
ENSG00000176208	ATAD5	1,56	0,0219
ENSG00000164051	CCDC51	-1,54	0,0219
ENSG00000108256	NUFIP2	1,29	0,0221
ENSG00000132507	EIF5A	-1,51	0,0221
ENSG00000104907	TRMT1	-1,88	0,0221
ENSG00000088053	GP6	2,70	0,0227
ENSG00000183549	ACSM5	-2,17	0,0228
ENSG00000155463	OXA1L	1,26	0,0228
ENSG00000173273	TNKS	1,30	0,0228
ENSG00000176022	B3GALT6	-1,55	0,0230
ENSG00000148180	GSN	-1,74	0,0230
ENSG00000007520	TSR3	-2,15	0,0235
ENSG00000224975		1,84	0,0235
ENSG00000167797	CDK2AP2	-1,99	0,0235
ENSG00000140859	KIFC3	-2,07	0,0235
ENSG00000115307	AUP1	-1,40	0,0235
ENSG00000167130	DOLPP1	-1,42	0,0236
ENSG00000082898	XPO1	1,31	0,0236
ENSG00000100060	MFNG	-1,55	0,0236
ENSG00000278619	MRM1	-1,54	0,0238
ENSG00000197858	GPAA1	-2,41	0,0238

ENSG00000163468	CCT3	-1,42	0,0240
ENSG00000104356	POP1	-1,54	0,0240
ENSG00000156931	VPS8	1,43	0,0242
ENSG00000109501	WFS1	-1,81	0,0242
ENSG00000205220	PSMB10	-2,56	0,0242
ENSG00000169683	LRRC45	-2,21	0,0242
ENSG00000138166	DUSP5	-1,42	0,0242
ENSG00000007541	PIGQ	-2,25	0,0242
ENSG00000214021	TTLL3	1,60	0,0243
ENSG00000089094	KDM2B	-1,29	0,0243
ENSG00000130830	MPP1	1,25	0,0243
ENSG00000123159	GIPC1	-1,90	0,0243
ENSG00000105254	TBCB	-1,60	0,0243
ENSG00000146828	SLC12A9	-1,94	0,0243
ENSG00000055609	KMT2C	1,38	0,0243
ENSG00000215305	VPS16	-1,59	0,0243
ENSG00000253476		2,42	0,0247
ENSG00000175063	UBE2C	3,10	0,0247
ENSG00000142507	PSMB6	-1,53	0,0247
ENSG00000173875	ZNF791	1,28	0,0248
ENSG00000187051	RPS19BP1	-2,03	0,0248
ENSG00000198736	MSRB1	-1,98	0,0252
ENSG00000101596	SMCHD1	1,58	0,0253
ENSG00000163755	HPS3	1,47	0,0253
ENSG00000102057	KCND1	2,12	0,0253
ENSG00000279978		2,36	0,0253
ENSG00000163872	YEATS2	1,42	0,0254
ENSG00000114857	NKTR	1,73	0,0254
ENSG00000225783		1,98	0,0254
ENSG00000147874	HAUS6	1,35	0,0254
ENSG00000006015	REX1BD	-1,67	0,0254
ENSG00000119333	WDR34	-2,18	0,0254
ENSG00000152439	ZNF773	1,43	0,0254
ENSG00000115295	CLIP4	1,34	0,0256
ENSG00000147421	HMBOX1	1,38	0,0256
ENSG00000023191	RNH1	-2,17	0,0256
ENSG00000167711	SERPINF2	-1,85	0,0257
ENSG00000145982	FARS2	-1,52	0,0258

ENSG00000096717	SIRT1	1,32	0,0258
ENSG00000138621	PPCDC	-1,68	0,0258
ENSG00000114127	XRN1	1,37	0,0263
ENSG00000111652	COPS7A	-1,23	0,0263
ENSG00000095970	TREM2	-1,80	0,0263
ENSG00000141098	GFOD2	-1,48	0,0263
ENSG00000152256	PDK1	1,38	0,0264
ENSG00000167112	TRUB2	-1,47	0,0264
ENSG00000185803	SLC52A2	-2,21	0,0264
ENSG00000198862	LTN1	1,27	0,0266
ENSG00000119686	FLVCR2	-1,28	0,0266
ENSG00000164663	USP49	1,53	0,0266
ENSG00000101384	JAG1	-1,31	0,0266
ENSG00000161835	GRASP	1,78	0,0268
ENSG00000005483	KMT2E	1,37	0,0268
ENSG00000155366	RHOC	-1,92	0,0268
ENSG00000007376	RPUSD1	-3,07	0,0272
ENSG00000205534		1,51	0,0272
ENSG00000272145		1,86	0,0272
ENSG00000075624	ACTB	-1,53	0,0272
ENSG00000129255	MPDU1	-1,31	0,0273
ENSG00000204237	OXLD1	-1,98	0,0274
ENSG00000114491	UMPS	-1,35	0,0274
ENSG00000128973	CLN6	-1,85	0,0274
ENSG00000026950	BTN3A1	1,59	0,0278
ENSG00000196976	LAGE3	-2,21	0,0281
ENSG00000130311	DDA1	-1,45	0,0282
ENSG00000135250	SRPK2	1,23	0,0282
ENSG00000131791	PRKAB2	1,37	0,0283
ENSG00000226091		1,93	0,0283
ENSG00000101639	CEP192	1,51	0,0283
ENSG00000141858		-1,37	0,0283
ENSG00000071127	WDR1	-1,42	0,0283
ENSG00000143379	SETDB1	1,35	0,0283
ENSG00000102879	CORO1A	-2,04	0,0283
ENSG00000090686	USP48	1,34	0,0287
ENSG00000258890	CEP95	1,60	0,0287
ENSG00000116984	MTR	1,28	0,0287

ENSG00000149635	OCSTAMP	-2,86	0,0287
ENSG00000266338	NBPF15	1,56	0,0287
ENSG00000270392		1,97	0,0287
ENSG00000101181	MTG2	-1,76	0,0287
ENSG00000155719	OTOA	-1,73	0,0287
ENSG00000183773	AIFM3	-1,80	0,0287
ENSG00000260735		1,65	0,0287
ENSG00000250041		-2,00	0,0287
ENSG00000088827	SIGLEC1	-2,36	0,0288
ENSG00000129235	TXNDC17	-1,55	0,0289
ENSG00000100347	SAMM50	-1,33	0,0291
ENSG00000166562	SEC11C	-1,58	0,0292
ENSG00000140455	USP3	1,26	0,0292
ENSG00000136490	LIMD2	-2,24	0,0294
ENSG00000176225	RTTN	1,50	0,0294
ENSG00000147133	TAF1	1,36	0,0298
ENSG00000176410	DJC30	-1,59	0,0300
ENSG00000110107	PRPF19	-1,32	0,0300
ENSG00000118482	PHF3	1,45	0,0301
ENSG00000101265	RASSF2	-1,34	0,0305
ENSG00000130590	SAMD10	-2,19	0,0305
ENSG00000105619	TFPT	-2,45	0,0305
ENSG00000258920		-2,04	0,0305
ENSG00000063244	U2AF2	-1,56	0,0305
ENSG00000104980	TIMM44	-1,52	0,0305
ENSG00000169627	BOLA2B	-2,93	0,0305
ENSG00000177225	GATD1	-1,60	0,0305
ENSG00000197785	ATAD3A	-2,52	0,0308
ENSG00000175283	DOLK	-1,58	0,0309
ENSG00000227057	WDR46	-1,54	0,0310
ENSG00000256269	HMBS	-1,40	0,0313
ENSG00000164707	SLC13A4	1,88	0,0315
ENSG00000127947	PTPN12	1,21	0,0316
ENSG00000138002	IFT172	1,54	0,0316
ENSG00000184445	KNTC1	1,50	0,0316
ENSG00000136718	IMP4	-1,32	0,0316
ENSG00000198053	SIRPA	-1,25	0,0316
ENSG00000172531	PPP1CA	-1,58	0,0316

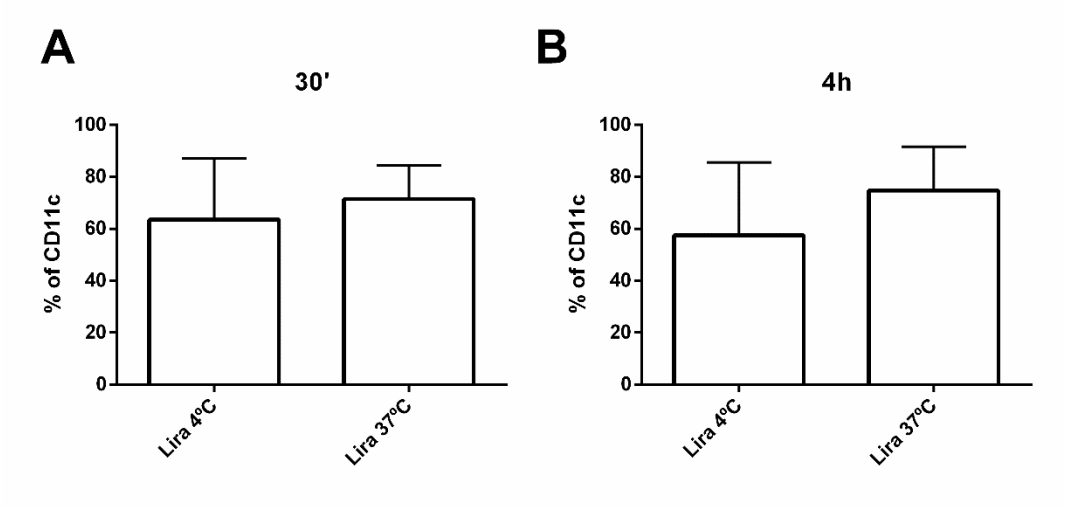
ENSG00000112425	EPM2A	-1,44	0,0318
ENSG00000244687	UBE2V1	1,59	0,0318
ENSG00000136827	TOR1A	-1,35	0,0318
ENSG00000171608	PIK3CD	-1,41	0,0318
ENSG00000111801	BTN3A3	1,40	0,0319
ENSG00000024048	UBR2	1,36	0,0319
ENSG00000164463	CREBRF	1,50	0,0319
ENSG00000122705	CLTA	-1,33	0,0322
ENSG00000137462	TLR2	1,33	0,0323
ENSG00000166189	HPS6	-1,73	0,0323
ENSG00000188994	ZNF292	1,44	0,0326
ENSG00000267731		2,25	0,0326
ENSG00000160972	PPP1R16A	-2,36	0,0326
ENSG00000176108	CHMP6	-1,46	0,0326
ENSG00000103540	CCP110	1,37	0,0326
ENSG00000093010	COMT	-1,75	0,0326
ENSG00000164081	TEX264	-1,71	0,0326
ENSG00000130749	ZC3H4	-1,41	0,0326
ENSG00000103254	FAM173A	-3,05	0,0326
ENSG00000182325	FBXL6	-2,38	0,0331
ENSG00000088682	COQ9	-1,34	0,0333
ENSG00000197608	ZNF841	1,52	0,0335
ENSG00000165097	KDM1B	1,24	0,0336
ENSG00000157778	PSMG3	-1,86	0,0336
ENSG00000246705	H2AFJ	-2,10	0,0338
ENSG00000157216	SSBP3	-1,30	0,0340
ENSG00000123505	AMD1	-1,28	0,0342
ENSG00000228300	C19orf24	-2,42	0,0342
ENSG00000197619	ZNF615	1,37	0,0342
ENSG00000103522	IL21R	-1,70	0,0342
ENSG00000127884	ECHS1	-1,28	0,0342
ENSG00000175463	TBC1D10C	-1,70	0,0342
ENSG00000087087	SRRT	-1,54	0,0342
ENSG00000006327	TNFRSF12A	-1,76	0,0342
ENSG00000179271	GADD45GIP1	-2,63	0,0343
ENSG00000160404	TOR2A	-1,87	0,0343
ENSG00000274925		1,53	0,0344
ENSG00000115129	TP53I3	-1,64	0,0344

ENSG00000184207	PGP	-1,58	0,0351
ENSG00000103274	NUBP1	-1,42	0,0351
ENSG00000074800	ENO1	-1,50	0,0351
ENSG00000131480	AOC2	2,26	0,0352
ENSG00000233038		-2,54	0,0352
ENSG00000235373		1,72	0,0352
ENSG00000239040		2,26	0,0354
ENSG00000105197	TIMM50	-1,43	0,0354
ENSG00000145495	MARCH6	1,28	0,0356
ENSG00000173638	SLC19A1	-1,96	0,0357
ENSG00000130706	ADRM1	-1,86	0,0360
ENSG00000135912	TTLL4	1,60	0,0361
ENSG00000122335	SERAC1	1,25	0,0364
ENSG00000159210	SNF8	-1,33	0,0364
ENSG00000145494	NDUFS6	-1,64	0,0366
ENSG00000162302	RPS6KA4	-1,46	0,0367
ENSG00000116285	ERRFI1	1,38	0,0369
ENSG00000172366	MCRIP2	-1,89	0,0369
ENSG00000099377	HSD3B7	-1,49	0,0369
ENSG00000121966	CXCR4	1,51	0,0376
ENSG00000131652	THOC6	-1,70	0,0376
ENSG00000277048		-6,38	0,0376
ENSG00000087076	HSD17B14	-1,79	0,0376
ENSG00000143179	UCK2	-1,30	0,0379
ENSG00000225828	FAM229A	1,75	0,0382
ENSG00000279696		2,17	0,0382
ENSG00000256087	ZNF432	1,35	0,0389
ENSG00000164896	FASTK	-1,82	0,0392
ENSG00000141994	DUS3L	-1,99	0,0392
ENSG00000167962		-2,00	0,0393
ENSG00000084070	SMAP2	1,49	0,0393
ENSG00000123992	DNPEP	-1,51	0,0393
ENSG00000247596	TWF2	-2,47	0,0396
ENSG00000148291	SURF2	-1,59	0,0397
ENSG00000118894	EEF2KMT	-1,74	0,0401
ENSG00000160789	LM	-1,61	0,0401
ENSG00000135968	GCC2	1,36	0,0402
ENSG00000122483	CCDC18	1,36	0,0403

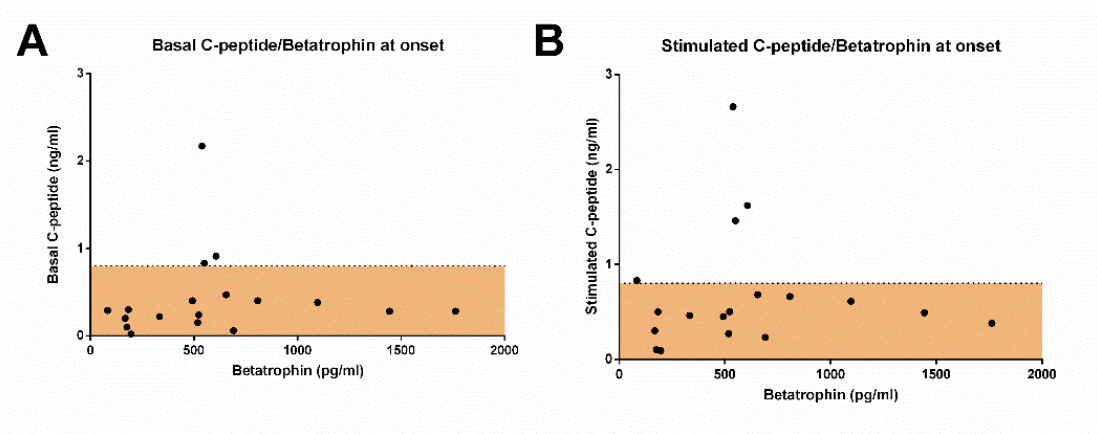
ENSG00000206082		1,62	0,0405
ENSG00000105677	TMEM147	-1,44	0,0405
ENSG00000197150	ABCB8	-1,73	0,0405
ENSG00000118412		1,26	0,0407
ENSG00000112561	TFEB	-1,81	0,0409
ENSG00000179115	FARSA	-1,93	0,0409
ENSG00000052841	TTC17	1,33	0,0409
ENSG00000101049	SGK2	2,14	0,0409
ENSG00000182307	C8orf33	-1,29	0,0413
ENSG00000258667		2,14	0,0416
ENSG00000171611	PTCRA	-2,54	0,0417
ENSG00000135365	PHF21A	1,46	0,0419
ENSG00000143847	PPFIA4	2,43	0,0423
ENSG00000179979		1,83	0,0427
ENSG00000066422	ZBTB11	1,29	0,0427
ENSG00000068079	IFI35	-1,67	0,0427
ENSG00000039650	PNKP	-1,71	0,0427
ENSG00000178860	MSC	-1,49	0,0427
ENSG00000179051	RCC2	-1,33	0,0430
ENSG00000011009	LYPLA2	-1,72	0,0432
ENSG00000001631	KRIT1	1,48	0,0438
ENSG00000244682		1,60	0,0440
ENSG00000100139	MICALL1	-1,82	0,0443
ENSG00000241860		1,54	0,0443
ENSG00000188807	TMEM201	-1,82	0,0444
ENSG00000137168	PPIL1	-1,54	0,0444
ENSG00000153165	RGPD3	1,47	0,0444
ENSG00000131381	RBSN	1,28	0,0445
ENSG00000042493	CAPG	-1,87	0,0448
ENSG00000130684	ZNF337	1,71	0,0452
ENSG00000206344		2,13	0,0454
ENSG00000153443	UBALD1	-2,11	0,0454
ENSG00000112773	TENT5A	1,64	0,0455
ENSG00000135587	SMPD2	-1,43	0,0456
ENSG00000106351	AGFG2	-1,76	0,0458
ENSG00000275582		2,36	0,0458
ENSG00000176563	CNTD1	1,83	0,0458
ENSG00000132669	RIN2	-1,27	0,0458

ENSG00000169084	DHRSX	-1,35	0,0460
ENSG00000005075	POLR2J	-1,56	0,0461
ENSG00000226950		-1,73	0,0462
ENSG00000083223	TUT7	1,30	0,0463
ENSG00000167113	COQ4	-1,45	0,0463
ENSG00000163344	PMVK	-1,56	0,0467
ENSG00000204618	RNF39	2,16	0,0469
ENSG00000136514	RTP4	-1,61	0,0469
ENSG00000138434	ITPRID2	1,25	0,0469
ENSG00000163686	ABHD6	-1,36	0,0470
ENSG00000105607	GCDH	-1,55	0,0473
ENSG00000123066	MED13L	1,29	0,0476
ENSG00000243749	TMEM35B	-2,92	0,0476
ENSG00000276045	ORAI1	-1,62	0,0476
ENSG00000068097	HEATR6	1,31	0,0479
ENSG00000101546	RBFA	-1,35	0,0479
ENSG00000145214	DGKQ	-1,82	0,0481
ENSG00000143190	POU2F1	1,35	0,0482
ENSG00000146587	RBAK	1,24	0,0483
ENSG00000238105		2,26	0,0483
ENSG00000186591	UBE2H	1,25	0,0484
ENSG00000198901	PRC1	1,62	0,0484
ENSG00000106635	BCL7B	-1,37	0,0484
ENSG00000173465	SSSCA1	-1,90	0,0486
ENSG00000173786	CNP	-1,38	0,0486
ENSG00000188206		1,63	0,0489
ENSG00000224051	CPTP	-1,77	0,0489
ENSG00000148719	DJB12	-1,39	0,0493
ENSG00000174915	PTDSS2	-1,62	0,0496
ENSG00000132005	RFX1	-1,73	0,0499
ENSG00000100258	LMF2	-2,08	0,0499
ENSG00000035141	FAM136A	-1,40	0,0499

Annex 18. Labelling of human DCs from adult subjects with T1D after 30min **(A)** or 4h **(B)** of co-culture with liraglutide-AF750.

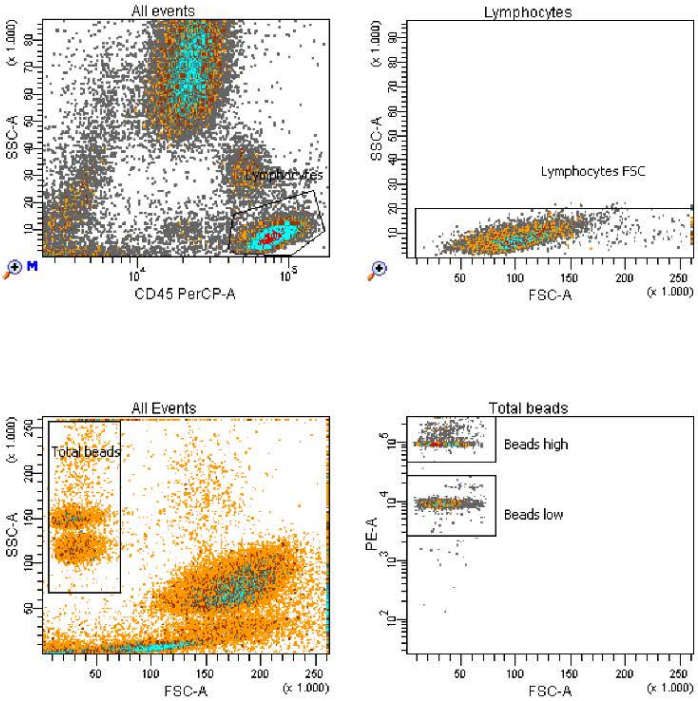


Annex 19. Stratification of betatrophin/C-peptide levels at basal **(A)** or 6min post-stimulation **(B)**. In both cases, all undetectable C-peptide values (filled area) present detectable betatrophin levels.



Annex 20. Gating strategy for the flow cytometry analysis of human leukocyte cell subsets. Including total lymphocytes, B transitional lymphocytes, DCs, monocytes, NK cells, T and B lymphocyte cell subsets, Gamma-Delta and Th17 lymphocytes.

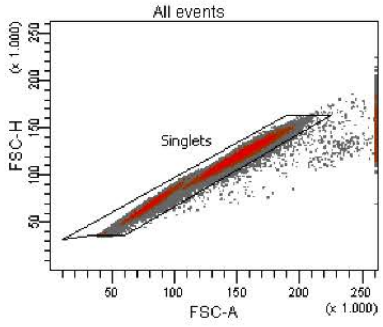
TOTAL LYMPHOCYTES

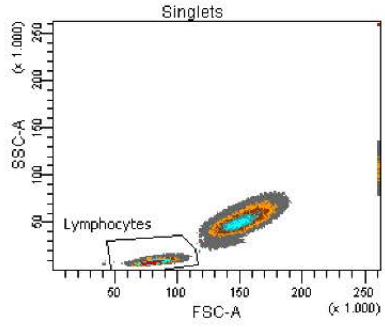


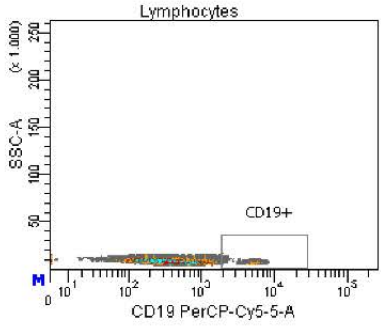
Tube: Total lymphocytes

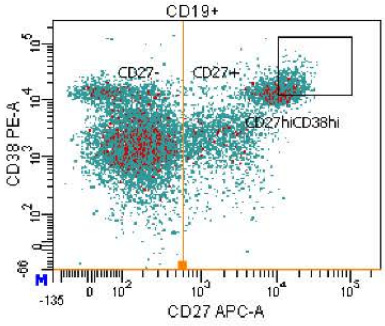
Population	#Events	%Parent	%Total
All Events	53.522	####	100,0
Total beads	4.810	9,0	9,0
Beads high	2.373	49,3	4,4
Beads low	2.419	50,3	4,5
Lymphocytes	8.808	16,5	16,5
Lymphocytes FSC	8.794	99,8	16,4

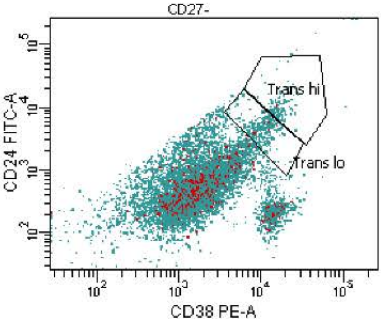
B transitional lymphocytes

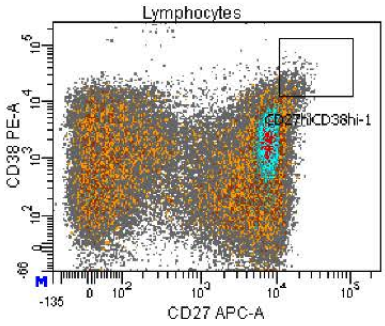


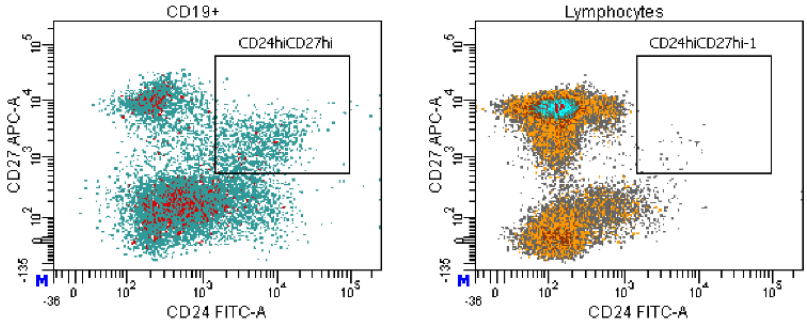








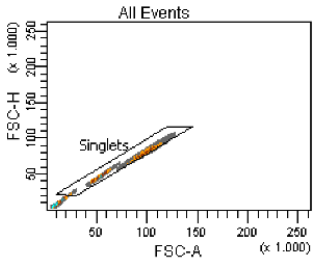


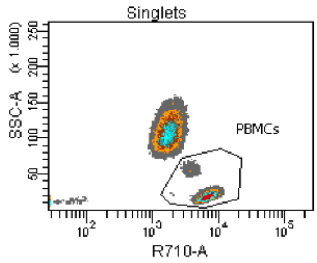


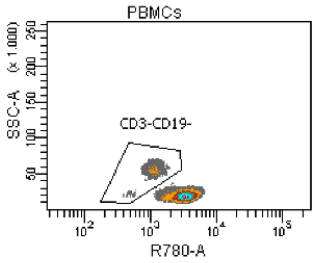
Tube: Btrans

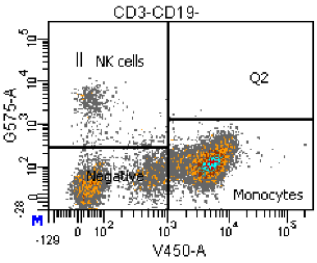
Population	#Events	%Parent	%Total
All Events	526.525	####	100,0
Singlets	515.924	98,0	98,0
Lymphocytes	129.238	25,0	24,5
CD19+	11.990	9,3	2,3
CD27-	8.439	70,4	1,6
Trans lo	318	3,8	0,1
Trans hi	144	1,7	0,0
CD27+	3.550	29,6	0,7
CD27hiCD38hi	718	20,2	0,1
Q3	1	0,0	0,0
Q4	0	0,0	0,0
CD24hiCD27hi	1.097	9,1	0,2
CD24hiCD27hi-1	1.186	0,9	0,2
CD27hiCD38hi-1	745	0,6	0,1

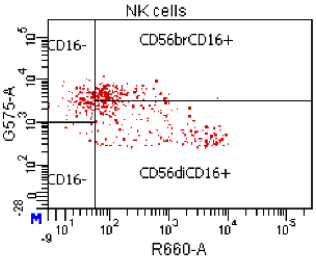
DCs, monocytes and NK cells

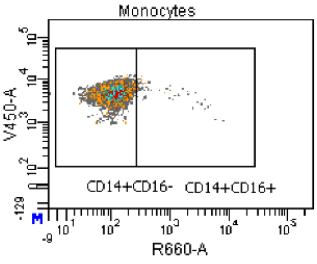


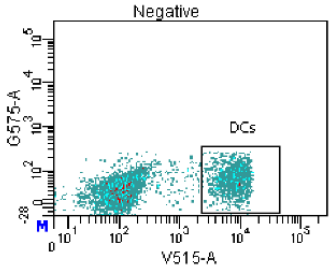


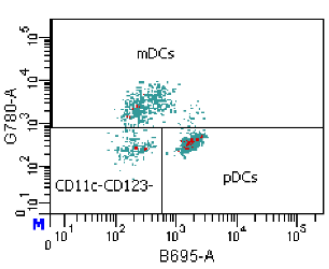


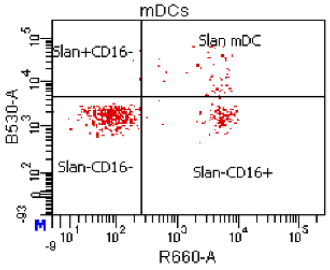








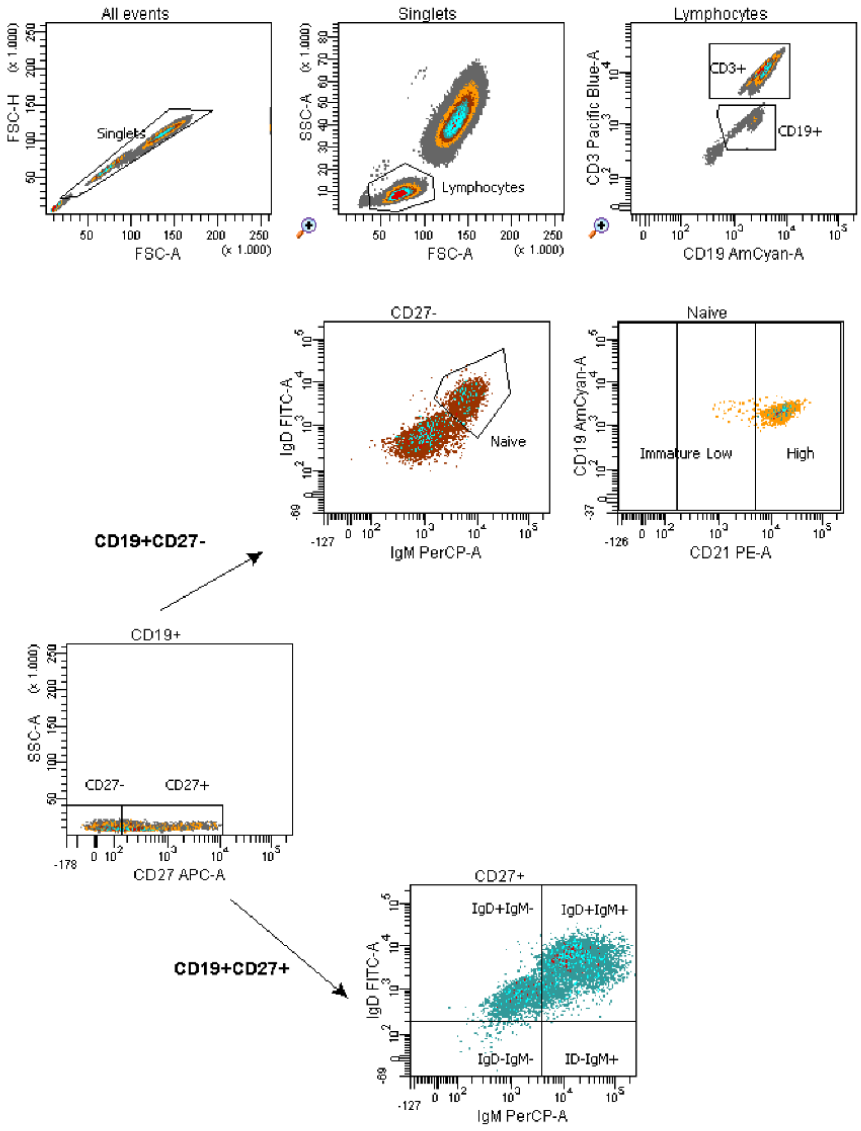




Tube: DC/MONO/NK

Population	#Events	%Parent	%Total
All Events	1.026.285	####	100,0
Singlets	544.695	53,1	53,1
PBMCs	163.152	30,0	15,9
CD3-CD19-	31.030	19,0	3,0
NK cells	456	1,5	0,0
CD56brCD16-	100	21,9	0,0
CD56brCD16+	151	33,1	0,0
CD56diCD16-	7	1,5	0,0
CD56diCD16+	198	43,4	0,0
Q2	5	0,0	0,0
Negative	5.393	17,4	0,5
DCs	1.418	26,3	0,1
Q1-1	0	0,0	0,0
mDCs	503	35,5	0,0
Slan+CD16-	3	0,6	0,0
Slan mDC	47	9,3	0,0
Slan-CD16-	356	70,8	0,0
Slan-CD16+	97	19,3	0,0
CD11c-CD123-	162	11,4	0,0
pDCs	753	53,1	0,1
Monocytes	25.176	81,1	2,5
CD14+CD16-	24.055	95,5	2,3
CD14+CD16+	1.169	4,6	0,1

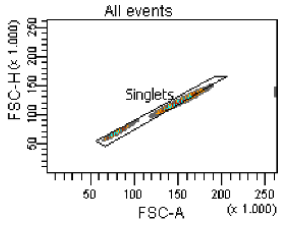
B lymphocytes subsets

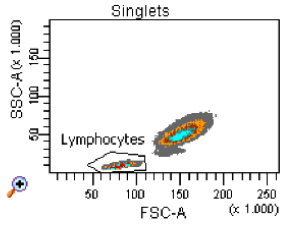


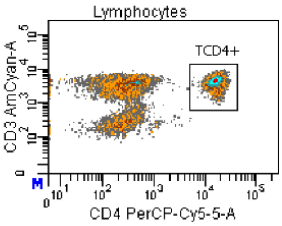
Tube: EMLB

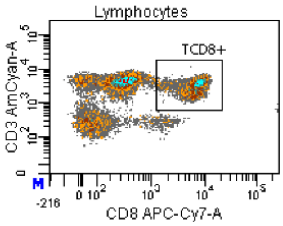
Population	#Events	%Parent	%Total
All Events	758.141	####	100,0
Singlets	602.520	79,5	79,5
Lymphocytes	149.797	24,9	19,8
CD3+	121.095	80,8	16,0
CD19+	18.710	12,5	2,5
CD27-	7.726	41,3	1,0
Naive	2.071	26,8	0,3
High	2.030	98,0	0,3
Low	44	2,1	0,0
Immature	0	0,0	0,0
CD27+	11.225	60,0	1,5
IgD+IgM-	3.120	27,8	0,4
IgD+IgM+	8.026	71,5	1,1
IgD-IgM-	79	0,7	0,0
ID-IgM+	0	0,0	0,0

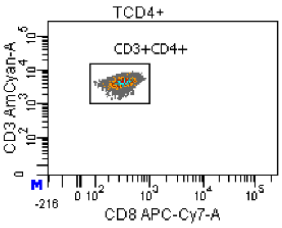
T lymphocytes subsets

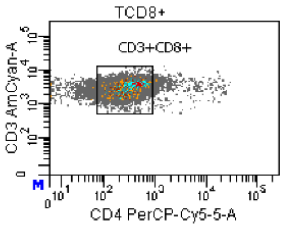


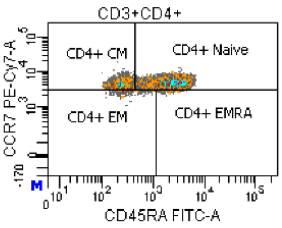


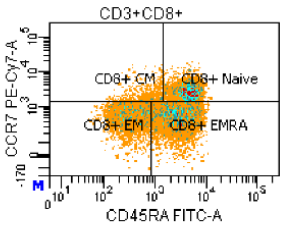


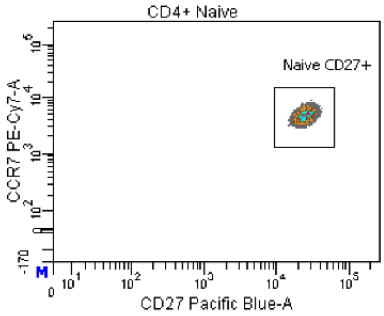








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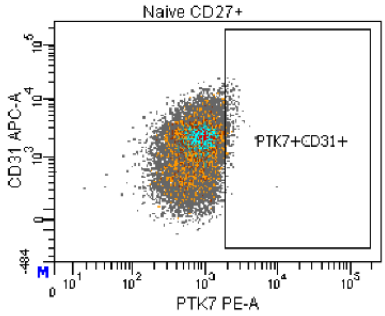
Naive CD27+
CCR7 PE-Cy7-A
CD27 Pacific Blue-A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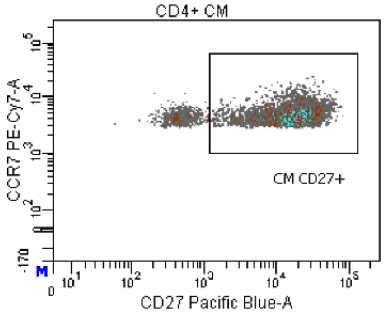
CD31 APC-A
PTK7 PE-A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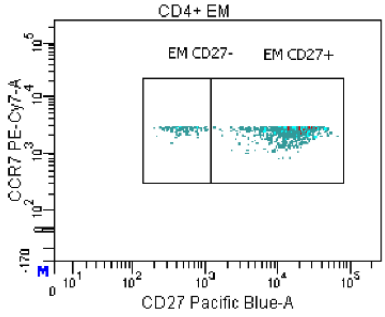
CCR7 PE-Cy7-A
CD27 Pacific Blue-A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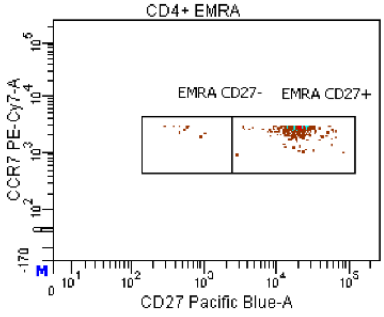
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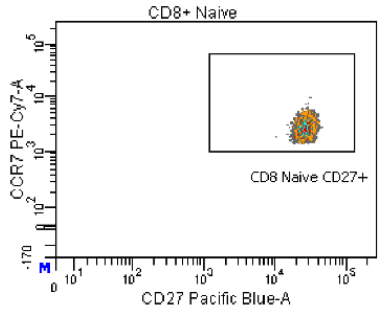
EMRA CD27-
EMRA CD27+
CCR7 PE-Cy7-A
CD27 Pacific Blue-A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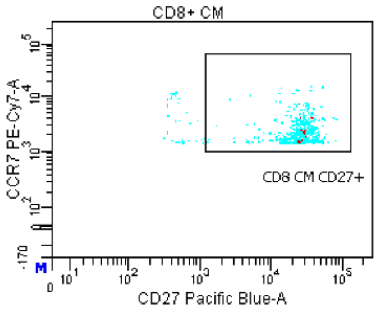
CD8 Naive CD27+
CCR7 PE-Cy7-A
CD27 Pacific Blue-A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CD8 CM CD27+
CCR7 PE-Cy7-A
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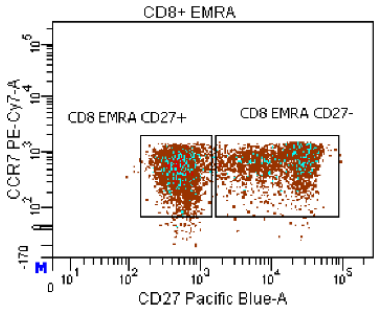
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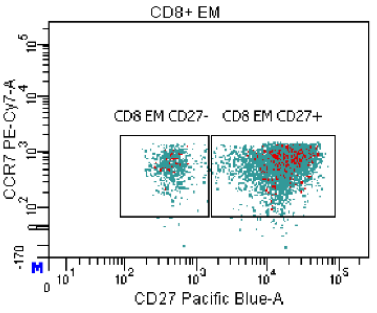
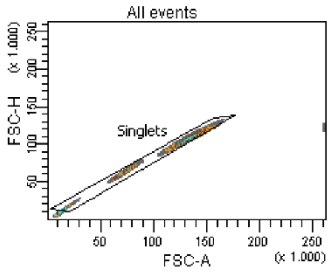
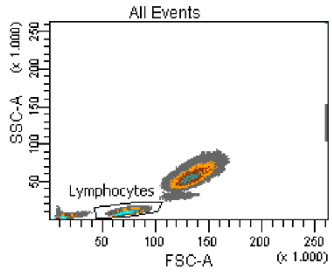

CD8+ EM
CD8 EM CD27-
CD8 EM CD27+
CCR7 PE-Cy7-A
CD27 Pacific Blue-A

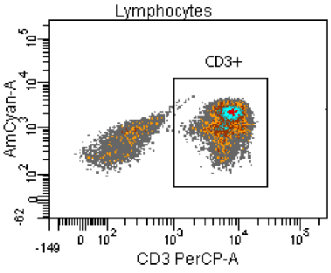
Tube: RTEs

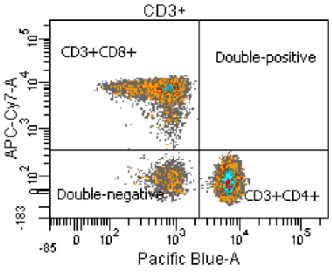
Population	#Events	%Parent	%Total
All Events	445.313	####	100,0
Singlets	400.809	90,0	90,0
Lymphocytes	82.297	20,5	18,5
TCD4+	37.673	45,8	8,5
CD3+CD4+	37.412	99,3	8,4
CD4+ CM	9.133	24,4	2,1
CM CD27+	8.383	91,8	1,9
CD4+ Naive	26.580	71,0	6,0
Naive CD27+	26.343	99,1	5,9
PTK7+CD31+	327	1,2	0,1
CD4+ EM	1.169	3,1	0,3
EM CD27+	1.061	90,8	0,2
EM CD27-	108	9,2	0,0
CD4+ EMRA	530	1,4	0,1
EMRA CD27+	521	98,3	0,1
EMRA CD27-	9	1,7	0,0
TCD8+	23.312	28,3	5,2
CD3+CD8+	21.888	93,9	4,9
CD8+ CM	510	2,3	0,1
CD8 CM CD27+	478	93,7	0,1
CD8+ Naive	8.683	39,7	1,9
CD8 Naive CD27+	8.611	99,2	1,9
CD8+ EM	4.645	21,2	1,0
CD8 EM CD27-	680	14,6	0,2
CD8 EM CD27+	3.936	84,7	0,9
CD8+ EMRA	8.050	36,8	1,8
CD8 EMRA CD27-	2.976	37,0	0,7
CD8 EMRA CD27+	4.982	61,9	1,1

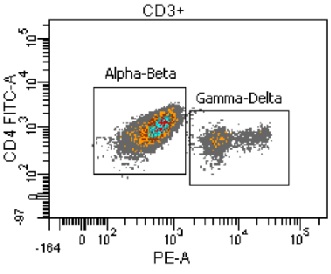
Gamma-Delta lymphocytes

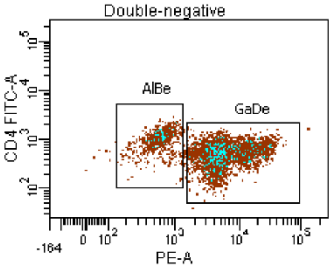


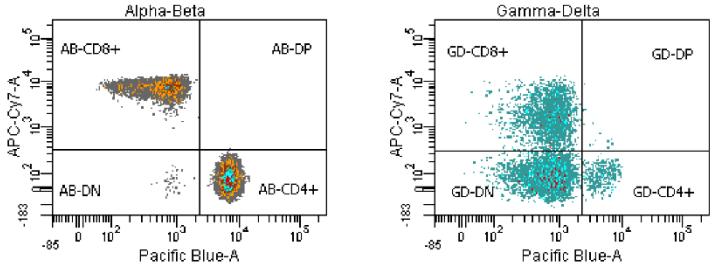








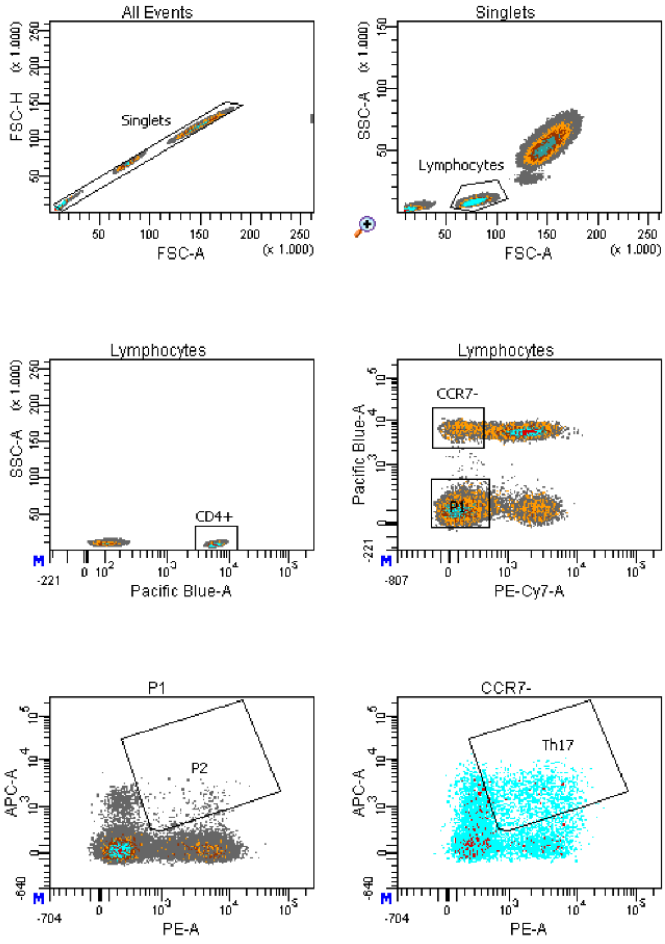




Tube: GD

Population	#Events	%Parent	%Total
All Events	479.466	####	100,0
Singlets	368.372	76,8	76,8
Lymphocytes	80.807	21,9	16,9
CD3+	67.251	83,2	14,0
CD3+CD8+	24.686	36,7	5,1
Double-positive	201	0,3	0,0
Double-negative	6.263	9,3	1,3
AlBe	775	12,4	0,2
GaDe	5.452	87,1	1,1
CD3+CD4+	36.101	53,7	7,5
Alpha-Beta	58.446	86,9	12,2
AB-CD8+	21.857	37,4	4,6
AB-DP	183	0,3	0,0
AB-DN	800	1,4	0,2
AB-CD4+	35.606	60,9	7,4
Gamma-Delta	8.474	12,6	1,8
GD-CD8+	2.746	32,4	0,6
GD-DP	17	0,2	0,0
GD-DN	5.346	63,1	1,1
GD-CD4+	365	4,3	0,1

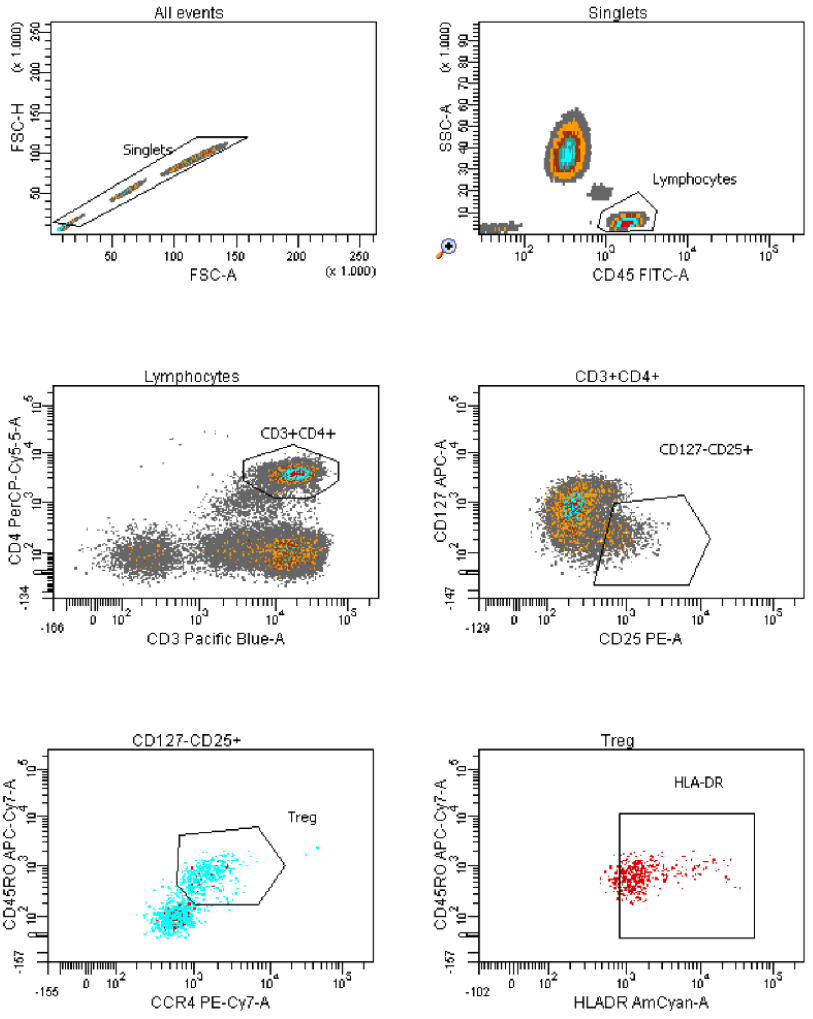
Th17 lymphocytes



Tube: TH17

Population	#Events	%Parent	%Total
All Events	589.770	####	100,0
Singlets	498.005	84,4	84,4
Lymphocytes	92.971	18,7	15,8
CD4+	42.435	45,6	7,2
CCR7-	6.654	15,7	1,1
Th17	1.096	16,5	0,2
P1	37.425	40,3	6,3
P2	160	0,4	0,0

Regulatory T cells



Tube: TREG

Population	#Events	%Parent	%Total
All Events	340.225	####	100,0
Singlets	252.032	74,1	74,1
Lymphocytes	58.644	23,3	17,2
CD3+CD4+	27.416	46,7	8,1
CD127-CD25+	1.288	4,7	0,4
Treg	448	34,8	0,1
HLA-DR	404	90,2	0,1

CONTRIBUTIONS

Publications included in this PhD thesis:

- **Partial remission and early stages of pediatric type 1 diabetes display immunoregulatory changes. A pilot study.** Adrian Villalba, Mireia Fonolleda, Marta Murillo, Silvia Rodriguez-Fernandez, Rosa-Maria Ampudia, David Perna-Barrull, Maria Belen Raina, Bibiana Quirant-Sanchez, Raquel Planas, Aina Teniente-Serra, Joan Bel, Marta Vives-Pi. **Transl Res. 2019**; 210:8-25.

- **Preclinical evaluation of antigen-specific nanotherapy based on phosphatidylserine-liposomes for type 1 diabetes.** Adrian Villalba, Silvia Rodriguez-Fernandez, Rosa-Maria Ampudia, Mary Cano-Sarabia, David Perna-Barrull, Cesc Bertran-Cobo, Clara Ehrenberg, Daniel Maspoch, Marta Vives-Pi. **Artif Cells Nanomed Biotechnol. 2020**; 48(1):77-83.

- **Repurposed analog of GLP-1 ameliorates hyperglycemia in type 1 diabetic mice through Pancreatic Cell Reprogramming.** Adrian Villalba, Silvia Rodriguez-Fernandez, David Perna-Barrull, Rosa-Maria Ampudia, Laia Gomez-Muñoz, Irma Pujol-Autonell, Eva Aguilera, Mireia Coma, Mary Cano-Sarabia, Federico Vázquez, Joan Verdaguer, Marta Vives-Pi. **Front Endocrinol. 2020**; 11:258.

- **Antigen-specific immunotherapy combined with a regenerative drug in the treatment of experimental type 1 diabetes.** Adrian Villalba, Silvia Rodriguez-Fernandez, David Perna-Barrull, Rosa-Maria Ampudia, Laia Gomez-Muñoz, Irma Pujol-Autonell, Eva Aguilera, Ruth M Risueño, Mary Cano-Sarabia, Daniel Maspoch, Federico Vázquez, Marta Vives-Pi. **Submitted.**

Publications from scientific collaborations:

- **Phosphatidylserine-liposomes promote tolerogenic features on dendritic cells in human type 1 diabetes by apoptotic mimicry.** Silvia Rodriguez-Fernandez, Irma Pujol-Autonell, Ferran Brianso, David Perna-Barrull, Mary Cano-Sarabia, Sonia Garcia-Jimeno,

Adrian Villalba, Alex Sanchez, Eva Aguilera, Federico Vazquez, Joan Verdaguer, Daniel Maspoch, Marta Vives-Pi. **Front Immunol. 2018**; 9:253.

- **Prenatal Betamethasone interferes with immune system development and alters target cells in autoimmune diabetes.** David Perna-Barrull, Silvia Rodriguez-Fernandez, Irma Pujol-Autonell, Anna Gieras, Rosa M Ampudia-Carrasco, Adrian Villalba, Laura Glau, Eva Tolosa, Marta Vives-Pi. **Sci Rep. 2019**; 9(1):1235.

- **Impaired phagocytosis in dendritic cells from pediatric patients with type 1 diabetes does not hamper their tolerogenic potential.** Silvia Rodriguez-Fernandez, Marta Murillo, Adrian Villalba, David Perna-Barrull, Mary Cano-Sarabia, Laia Gomez-Muñoz, Eva Aguilera, Daniel Maspoch, Federico Vazquez, Joan Bel, Marta Vives-Pi. **Front Immunol. 2019**; 10:2811.

Presentations in National and International meetings:

- Laura Bosch, Adrian Villalba, Marta Murillo, Mireia Fonolleda, Federico Vázquez, Silvia Rodríguez-Fernández, Joan Bel, Marta Vives Pi**. Betatrofina como nuevo biomarcador de Diabetes Mellitus tipo 1 en pediatría.** Sociedad Española de Endocrinología Pediátrica, SEEP (Bilbao, 16th-18th May 2018).

- Adrian Villalba, Rosa Ampudia, Silvia Rodriguez Fernandez, David Perna Barrull, Irma Pujol Autonell, Eva Aguilera, Daniel Maspoch, Federico Vazquez, Marta Vives Pi**. Combined therapy for type 1 diabetes based on drug-repurposing. Immunotherapy and regenerative strategies.** A Roadmap towards Diabetes Treatment: To the Beta Cell and Beyond, F-TALES: Flanders Training Network Life Sciences (Brussels, Belgium, 4th-5th Jun 2018).

- Adrian Villalba, Rosa Ampudia, Silvia Rodriguez-Fernandez, David Perna-Barrull, Irma Pujol-Autonell, Clara Ehrenberg, Eva Aguilera, Daniel Maspoch, Federico Vazquez, Marta Vives-Pi. **Combined therapy for type 1 diabetes based on drug-**

repurposing. Immunotherapy and regenerative strategies. XII Congrés de la Societat Catalana d'Immunologia (Barcelona, 15th-16th Nov 2018).

- Laura Bosch, Adrian Villalba, Marta Murillo, Mireia Fonolleda, Silvia Rodríguez-Fernández, Marta Vives-Pi, Joan Bel. **Betatrophin as a new biomarker of Type 1 Diabetes Mellitus in Paediatrics**. European Society of Paediatric Endocrinology, ESPE (Athens, 27th-29th Sep 2018).

- Adrian Villalba, Marta Murillo, Silvia Rodríguez-Fernández, David Perna-Barrull, Joan Bel, Marta Vives-Pi. **Biomarcadores inmunólogicos candidatos de etapas iniciales de diabetes mellitus tipo 1 pediátrica**. Sociedad Española de Endocrinología Pediátrica, SEEP (Madrid, 22nd-24th May 2019).

- Adrian Villalba, Silvia Rodriguez-Fernandez, David Perna-Barrull, Rosa Maria Ampudia, Federico Vazquez, Laia Gómez-Muñoz, Irma Pujol-Autonell, Eva Aguilera, Mary Cano-Sarabia, Marta Vives-Pi. **Novel combined therapy for autoimmune diabetes designed by drug repositioning.** DZD Diabetes Research School, EASD (Barcelona, 14th-16th Sep 2019).

- Adrian Villalba, Silvia Rodriguez-Fernandez, David Perna-Barrull, Rosa Maria Ampudia, Federico Vazquez, Laia Gómez-Muñoz, Eva Aguilera, Daniel Maspoch, Joan Verdaguer, Marta Vives-Pi. **Novel compound identified by drug repositioning ameliorates experimental autoimmune diabetes.** XIII Congrés de la Societat Catalana d'Immunologia. (Barcelona, 14th-15th Nov 2019).

- Adrian Villalba, Silvia Rodriguez-Fernandez, David Perna-Barrull, Rosa Maria Ampudia, Federico Vazquez, Laia Gomez-Muñoz, Eva Aguilera, Mary Cano-Sarabia, Daniel Maspoch, Joan Verdaguer, Marta Vives-Pi. **Repurposed analog of GLP-1 ameliorates hyperglycaemia in type 1 diabetic mice through pancreatic cell reprogramming.** EASD islet study group meeting (Strasbourg, 18th-20th May 2020)**.** *Note: Accepted for oral presentation. Following the Covid-19 pandemic, the workshop has been rescheduled to 20th-22nd Mar 2021.*

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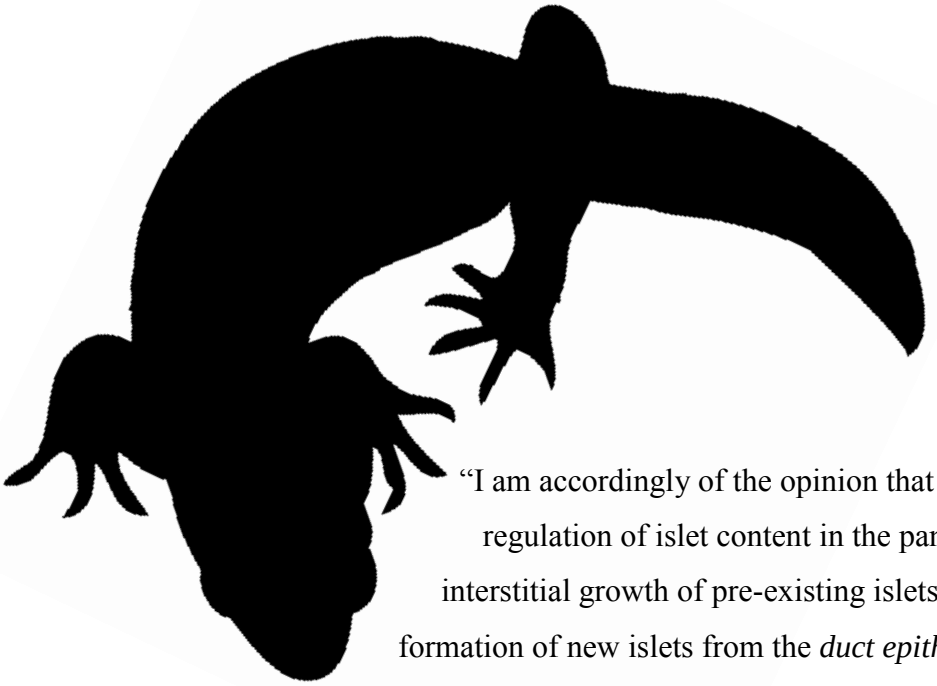

"I am accordingly of the opinion that the normal regulation of islet content in the pancreas is by interstitial growth of pre-existing islets and by the formation of new islets from the *duct epithelium*, and not at all by the formation of new islets out of acini." -

***R.R. Bensley*, 1911.**

Drawing: The quote is extracted from R.R. Bensley. *Studies on the pancreas of the guinea pig*. Am J Anatomy 1911; 12:297–388. The shape corresponds to the venomous lizard *Heloderma suspectum*, commonly known as Gila monster. Its saliva contain **Exendin-4**, a natural analogue of GLP1 that lead the design and manufacturing of **Exenatide**, the first commercial aGLP1 drug. Design by Adrián Villalba.