TWO NOVEL THERAPIES FOR THE TREATMENT OF TYPE 1 DIABETES MELLITUS

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Tesis doctoral

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Two patents (WO 2011 144725 A2 and WO 2016 156531 A1) related to BL001 have been published. The owner is Fundación Progreso y Salud and the patents have been licensed to ARIDDAD Therapeutics. Data related to BL002 and BL003 are properties of Benoit Gauthier and Fundación Progreso y Salud.

The director Benoit Gauthier is an employee of Fundación Progreso y Salud and co-founder of ARIDDAD Therapeutics. The director Miguel Garcia Toscano is an employee of Amarna Therapeutics S.L. and partner of ARIDDAD Therapeutics. The tutor Ricardo Pardal Redondo, as an employee of the Universidad de Sevilla, and Irene Herrera Gómez, as PhD student, do not declare conflict of interest.

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TABLE OF ABBREVIATIONS

AAV: adeno-associated vectors
AD: autoimmune diseases
Ag: antigen
ALT: alanine aminotransferase
AMPr: bacterial Ampicillin resistance gene
APC: antigen presenting cell
AST: antigen specific tolerance
ATP: adenosine triphosphate
BGH-pA: BGH polyadenylation signal
BrdU: 5-bromo-2'-deoxyuridine
CDA: confidential disclosure agreement
C-peptide: connecting peptide
CTL: cytotoxic T lymphocyte
CTLA4: T lymphocyte associated antigen 4
DAPI: 4',6-diamidino-2-phenylindole
DC: dendritic cell
DKA: diabetes ketoacidosis
DM: diabetes mellitus
DMEM: Dulbecco's modified eagle medium
DTT: dithiothreitol
EAD: experimental autoimmune diabetes
ER: endoplasmic reticulum
FBS: fetal bovine serum
GAD65: glutamate decarboxylase 65
GDM: gestational diabetes mellitus
GLUT2: glucose transporter 2
GMP: good manufacturing practice
HepG2: hepatoma cell line
HLA: human leukocyte antigen
IL-1β: interleukin-1β
IFNγ: interferon γ
Ins-1E: rat insulinoma cell line
iTregs: induced T regulatory cells

IVIS: *in vivo* imaging system
LCMV: lymphocytic choriomeningitis virus
LN: lymph nodes
LV: lentiviral vector
LPS: lipopolysaccharide
LRH1: liver receptor homolog 1
LTag: large T antigen
MDI: multiple daily injection therapy
MHC: major histocompatibility complex
miRNA: micro RNA
miRT: micro RNA target sequence
MODY: maturity-onset diabetes of the young
NOD: non obese diabetic
ORI: origin of bacterial replication
PAMP: pathogen associated molecular pattern
PBS: phosphate buffered saline
PPI: preproinsulin
ProI: proinsulin
RC: recombinant competent
RD: recombinant deficient
RIP: rat insulin promoter
RISC: RNA-induced silencing complex
ROS: reactive oxygen species
SHP: small heterodimer partner
STag: small T antigen
STZ: streptozotocin
SPF: specific-pathogen-free
SV40: simian virus 40
SV40 ORI: SV40 origin of replication
SV40 pA: SV40 polyadenylation signal
T1DM: type 1 diabetes mellitus
T2DM: type 2 diabetes mellitus
TCR: T cell receptor
Th: T helper cell
TNF: tumor necrosis factor
Tregs: T regulatory cells
VP: viral protein

WHO: World Health Organization

WPRE: Woodchuck hepatitis virus posttranscriptional regulatory element

ABSTRACT

Type 1 diabetes mellitus (T1DM) is caused by an autoimmune destruction of islet β cells. Current treatments are based on replacement therapy using insulin analogs but, due to the impossibility to simulate physiological glucose control, it leads to diabetes complications. Thus, novel treatments are required. As a difference of what happens with the immunosuppressor therapy, which have limited clinical efficacy, the induction of antigen specific tolerance (AST) can specifically block the activation of autoreactive T cells, preserving the survival and function of the pancreatic β cells and preventing the development of T1DM. Here, two different strategies are followed, in order to induce a tolerogenic environment. The expression of the self-Ag insulin under non-inflammatory conditions, using the SV40 background allows the restoration of the AST in the RIPB7.1 mouse model. Also, the protective role in pancreatic islets of the liver receptor homologue 1 (LRH1) against apoptosis was considered, as well as the prevention of hyperglycemia in T1DM mouse models by the induction of an anti-inflammatory environment promoted by the LRH1 agonist BL001. However, the limitations of BL001 as potential medication prompted us to develop an *in vitro* drug-screening platform that allowed the identification of two novel LRH1 agonists, BL002 and BL003. These non-toxic agonists protect mouse islets from cytokines-induced apoptosis, improving their survival. Thus, the AST induced by SV40 vector as well as the new generation of LRH1 agonists BL002 and BL003 must be considered as two promising therapies for the treatment of T1DM

I. INTRODUCTION

1. Autoimmune diseases

1.1. The immune system in autoimmune diseases

Autoimmune diseases (AD) are a group of disorders in which the immune cells react against the body's own cells leading to a severe tissue damage with subsequent harmful effects for the subject. Some examples of major ADs include rheumatoid arthritis, in which the synovial cells of the joints are attacked [1], type 1 diabetes mellitus (T1DM) in which the insulin producing β cells are destroyed [2] or multiple sclerosis, in which the immune system reacts against oligodendrocytes, the cells protecting the axons of motor neurons [3]. Although most of the mechanisms underlying tissue destruction are known, the etiology of these diseases remains unclear. Currently, the hypothesis mostly accepted establishes that patients suffering AD present a genetic predisposition that in combination with environmental factors favors the development of the autoimmune attack [4]. At the cellular and molecular level, the immune system, responsible to defend us from foreign invaders, is the effector of the AD. The immune system establishes a delicate balance between inflammation (recognition of foreign) and tolerance (recognition of own) and the break of this tolerance is what triggers the AD.

The immune response can be functionally divided into the innate and the adaptive responses. The innate response is the first line of defense. The cells of the innate immune system express cell surface receptors that directly recognize pathogens through pathogen associated molecular patterns (PAMPS) which are shared between the different groups of microorganisms (bacteria, viruses…). The strength of the innate response is the capacity to take immediate action since it is not specialized for specific pathogens. If this innate response is unsuccessful in destroying the pathogens, after 4 to 7 days the adaptive immune response sets in, targeting the pathogens more accurately [5,6]. This is based on the capacity of the adaptive response to precisely recognize specific pathogen structures known as antigens (Ags). These Ags need to be processed before being available to be recognized.

The knowledge obtained from the study of animal models of AD and the data collected from patients, suggests that among all the cells of the adaptive immune system the two main drivers of AD are the T and the B cells. Based on this, the AD can be classified in those triggered mainly by T cells and those triggered mainly by B cells, although eventually, both cell types are involved in all AD in some degree. Additionally, in animal models of AD the pathogenic symptoms of the disease can be triggered by only one of the two groups of T cells that can be distinguished by their surface markers as $CD4^+$ or $CD8^+$ T cells. In this thesis, we will focus in AD triggered by T cells.

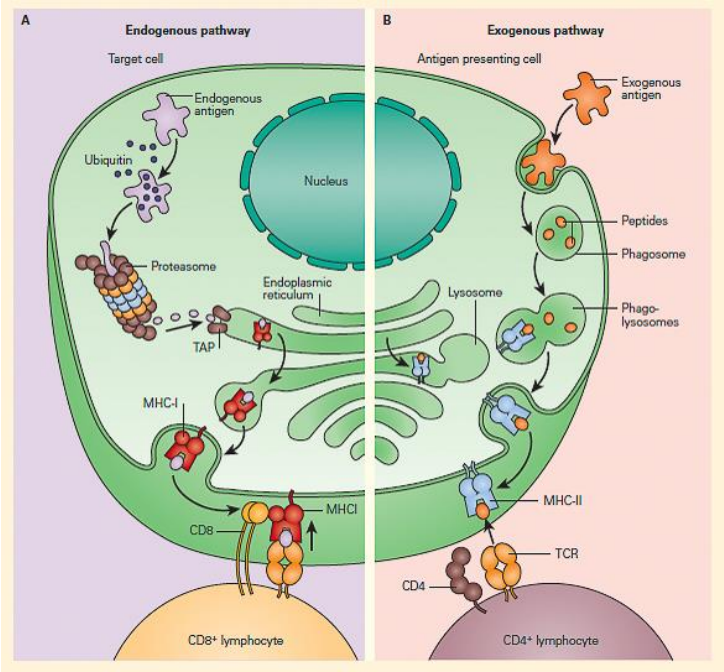

Figure 1. Antigens can be presented by MHC I or MHC II determining the class of lymphocytes activated. Schematic representation of the two modes of Ag processing. **A**. Endogenous pathway for presenting Ag from a host cell to $CD8^+$ T cell by MHC I. **B**. Exogenous pathway presents the peptide products from an endocytosed Ag by an APC to a $CD4^+$ T cell in the context of MHCII. Image taken from Bellanti, 2012 [8].

The T cells, through their surface molecule T cell receptor (TCR), can identify both foreign and self-Ags, when the Ags are loaded and presented by the major histocompatibility complex (MHC). There are two types of MHC, class I, which is expressed and present in the surface of all body cells, and class II, which is expressed only by antigen presenting cells (APCs) [7]. Cells expressing class I MHC molecules present endogenous Ags that are recognized by one or very few clones of $CD8^+$ T cytotoxic lymphocytes (CTL) (Figure 1). Thus, in an event of a viral infection, the infected cells process and express via class I MHC the viral Ags, which are recognized by the CTLs that then, are activated. These activated CTLs remove the affected cells

by releasing cytotoxic mediators. The Ags loaded in class II MHC molecules come from either pathogens or cell debris that have been phagocytosed by APCs. Mainly, dendritic cells and monocytes function as APCs. Also, the monocyte-differentiated cells (macrophages) such as Kupffer cells (located in the liver), Langerhans cells (located in the skin) or the glial cells (located in the nervous system) act as APCs. In this way, APCs are distributed all over the body. The Ags presented by class II MHC are recognized by the $CD4^+$ T helper (Th) lymphocytes, which produce cytokines and stimulate B cells to generate/secrete antibodies.

The key factor that determines a tolerance or an inflammation state is the way that Ags are presented and the maturation state of the APCs (Figure 2) [9,10]. Under inflammatory conditions (Figure 2, left), immature APCs, which have a great phagocytic capacity, collect either pathogens or cell debris (produced by the inflammation and the action of the innate immune response) and then migrate to the lymph nodes where they mature (upregulate the expression of costimulatory molecules in their cell surface and secrete pro-inflammatory cytokines). Activation of $CD4^+$ Th cells by activated APCs induces their differentiation into inflammatory T cells that migrate outside of the lymph nodes [7 11–13]. On the other hand, in the absence of inflammatory signals, immature APCs are able to capture and process Ags, and when these Ags are presented to T cells without the presence of costimulatory signals, they induce tolerance instead of inflammation (Figure 2, right).

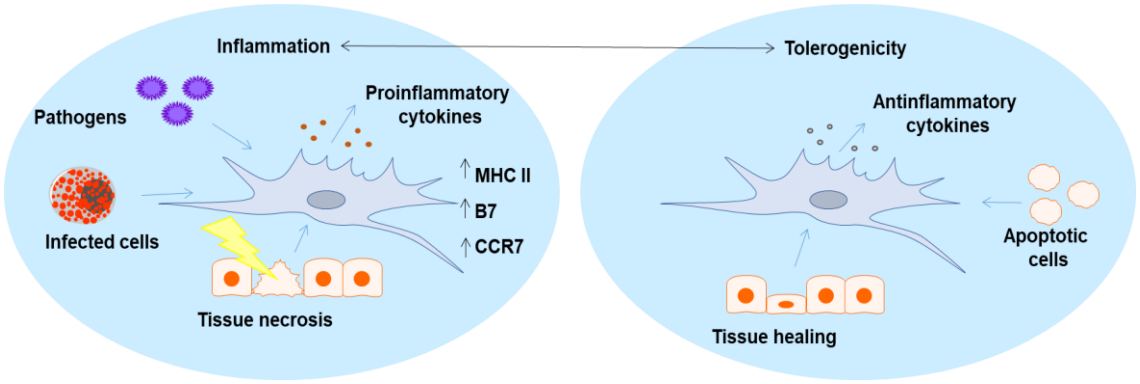

Figure 2. The maturation state of APCs induces tolerance or inflammation. The presence of pathogen or infected cells, as well as tissue necrosis favors the activation of the APCs, upregulating the expression of costimulatory surface markers and the secretion of proinflammatory cytokines. In the case of apoptotic cells or tissue healing, APCs induce a tolerogenic environment due to the lack of stimulatory signals.

There are two mechanisms that preserve the homeostasis and the tolerance. By these mechanisms, those clones of T cells that react against the self-Ags, known as

autoreactive T cells are recognized and eliminated. During development, a genetic recombination process happening in the T progenitor cells, randomly generates a plethora of T $CD4^+$ and $CD8^+$ cell clones [14]. By an exhaustive selection mechanism occurring in the thymus, known as clonal deletion, host's self-Ags are presented by the thymic stromal cells and most of the T cell clones that recognize them with high affinity are depleted [15]. This phenomenon is known as "central tolerance" [15–19]. A secondary check point, mediated by APCs and known as "peripheral tolerance", removes those autoreactive T cell clones that escaped from the central tolerance (Figure 3) [20]. Three different ways account for peripheral tolerance: a) by anergy, in which the $CD4^+$ cells becomes unresponsive after Ag presentation [11,12,21,22]; b) by depletion, which consists in the elimination of autoreactive T cells by apoptosis after persistently encounter self-Ags [23]; and c) by suppression that is mediated by regulatory T cells (Tregs), which are specialized T cells that block the activation of the autoreactive T cells [24,25].

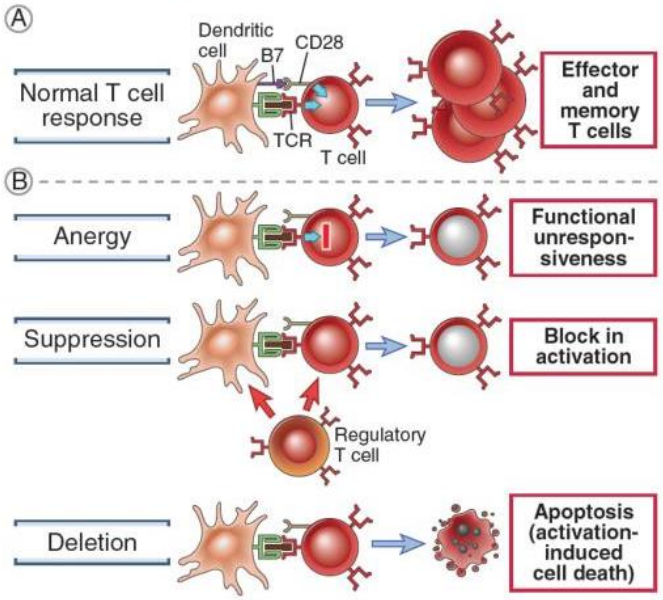

Figure 3. Mechanisms of peripheral tolerance. Three different mechanisms have been established to eliminate autoreactive T cells: anergy, inducing a hyporesponsive state, suppression, due to the action of regulatory T cells; and deletion, inducing apoptosis after a long-term exposition to the Ag. Image taken from Abbas, 2007 [26].

The key question to understand the etiology of ADs is to know how and what breaks the tolerance in certain subjects. It has been postulated that in certain cases the similarity between pathogenic Ags and self-Ags (known as Ag mimicry) can trigger the autoimmune process [27]. However, another layer of complexity resides in the fact that autoreactive T cells are found also in the healthy population that have encountered with same pathogenic Ags than AD patients, without manifesting the symptoms of the disease [28]. This reveals the complexity of the problematic of AD.

1.2. Requirements of new treatments for autoimmune diseases

Currently there is not a treatment able to restore the immune tolerance lost in AD patients. Therefore, ADs are not curable and only palliative therapies to treat the symptoms are available. Current treatments are classified into two categories: 1) symptomatic or replacement therapy and 2) immunosuppressive or immune-modulation therapy.

In many ADs the pathogenic symptoms are manifested when the target organ or target cells are mostly destroyed, and the remaining cells cannot support anymore the organ functions. For example, in T1DM patients the symptoms of the diabetes are manifested when the target cells (the pancreatic β cells, responsible for insulin secretion) are destroyed by around 90% [29]. These patients will need insulin supplementation during their whole life to restore the normoglycemia. This palliative approach does not solve the problem since it does not stop the immune attack over the β cells, thus preventing their recovery.

In other cases, when the replacement therapy is not possible such as in multiple sclerosis, the right approach is to induce immunosuppression, aiming at avoiding the activation of immune cells and thus reducing the inflammation [30]. This therapeutic approach involves the suppression of the entire immune system by the use of compounds such as cyclophosphamide [31,32], which is cytotoxic to the lymphoid population [33], or glucocorticoids [34], which can alter gene expression in the host cells by inhibiting the production of proinflammatory cytokines and inducing apoptosis in a variety of immune cells, systemically decreasing the inflammation [35,36]. A current view to treat ADs pretends to reduce the side effects of systemic immunosuppressors by targeting exclusively the pro-inflammatory cytokines and the T cells. One strategy that has been considered in this sense is the use of monoclonal antibodies [37–40]. For example, the antibody anti-CD3, is able to block the activation of T cells through binding to their CD3 receptor [41] or anti-TNFα which is able to block the soluble pro-inflammatory cytokine tumor necrosis factor α (TNFα). This cytokine has been found elevated in many ADs [42]. Initially, 60–70% of autoimmune patients respond to this therapy, but only a small percentage of the patients present a long-lasting period of remission.

The down side of using immunosuppressive drugs is that they increase the risk of infection by opportunistic organisms such as herpes or hepatitis, tumor development

due to the inability to stablish an immune response against malignant cells, cardiovascular diseases, attributed to the chronic inflammatory state as well as the hyperglycemic and hyperlipidaemic adverse effects [43–45]. Some novel small molecules that target particular proteins are being developed with the aim to increase the specificity of the anti-inflammatory therapy. This seems to be a good approach as it pretends to avoid general immunosuppression, but unexpectedly current molecules still do not increase significantly the remission rate despite the significant advances in last few decades. Hence, the ideal intervention for the treatment of ADs should be able to specifically remove the autoreactive T cell clones, without altering/damaging the rest of the immune cells thus avoiding the adverse secondary effects.

2. Type 1 diabetes mellitus as an autoimmune disease

2.1. Diabetes mellitus and glucose homeostasis

Diabetes mellitus (DM) is defined as a group of metabolic disorders characterized by the inability of the organism to maintain the homeostasis of blood glucose leading to hyperglycemia. The control of the blood glucose levels (glycaemia) relies in the pancreas.

This organ can be functionally divided into the exocrine pancreas, involved in digestive functions, and the endocrine pancreas, known as the islets of Langerhans or pancreatic islets, which regulates glucose homeostasis (Figure 4). The exocrine pancreas represents around 96% of the total pancreatic mass, while the endocrine pancreas constitutes the remaining 4% approximately [46]. Pancreatic islets are spherical clusters of cells scattered throughout the exocrine pancreas and in close proximity to fenestrated capillaries for the release of hormones into the bloodstream [47,48]. The most abundant cell types of the pancreatic islets are the insulin-producing β cells and the glucagon-producing α cells, however their proportion and distribution within the islets vary among species. In the case of human islets, β cells account for approximately 60% of the islet cells and appear to be randomly distributed throughout the islet and α cells represent approximately the 30%. The other cell types are: somatostatin-producing δ cells (~8%), ghrelin-producing ε cells (~1%) and pancreatic-polypeptide producing PP cells (0,3%) [49,50]. In the case of murine islets, the β cells represent approximately 80% of the islet cells and are located in the central core of the islet, surrounded by the α cells that account for 15-20% of islet cells. The other islet cell types include δ cells (less than 10%) and PP cells (less than 1%). ε cells are only present during embryonic development [51].

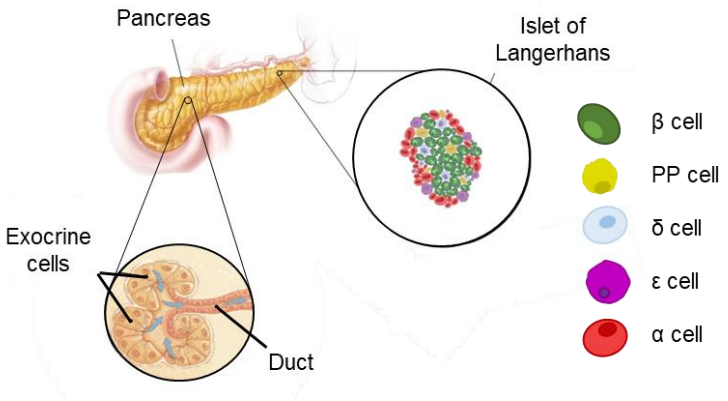

Figure 4. Endocrine and exocrine compartments of the pancreas. Almost all of the pancreas (~96%) consists of exocrine tissue that produces pancreatic enzymes for digestion. The endocrine pancreas or islets of Langerhans are clusters of 5 different types of cells (β, α, δ, PP and ε cells) that form spherical structures embedded in the exocrine pancreas.

Insulin and glucagon are the two main hormones involved in the glucose homeostasis (Figure 5). Upon increased blood glucose, β cells are stimulated and secrete insulin, which is the hormone that enhances the uptake of glucose by insulin target tissues, (liver, skeletal muscle and adipose tissue) decreasing the levels of glucose in the blood. In addition, insulin also suppresses the production of glucose by the liver [52]. As a counterpart, during a hypoglycemic status, α cells are stimulated and secrete glucagon that increases glucose release by the liver restoring the normal blood glucose levels [53–55].

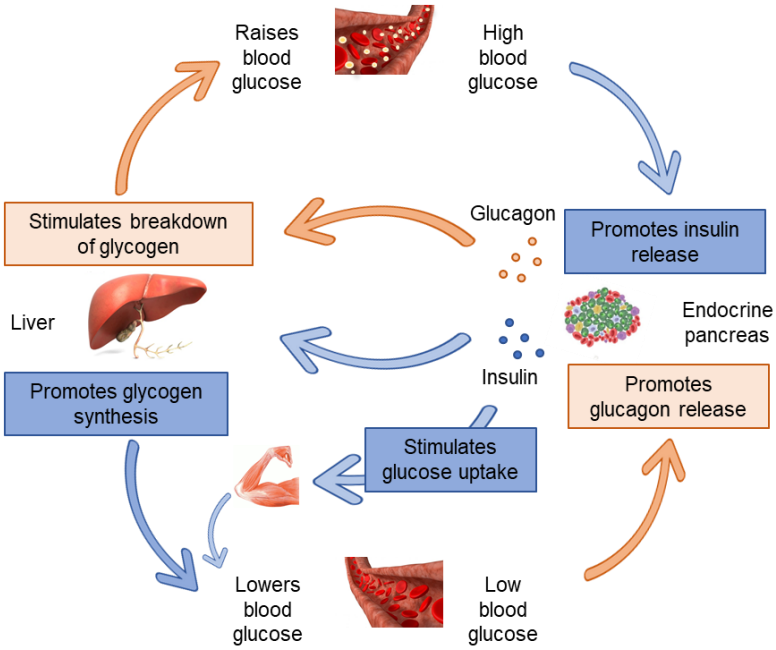

Figure 5. Regulation of glucose homeostasis by the endocrine pancreas. Based on blood glucose levels, 2 different scenarios are observed: high levels (blue arrows) and low levels (orange arrows). These conditions stimulate different pancreatic cell types to produce: insulin during hyperglycemic conditions or glucagon in hypoglycemic states. These hormones are secreted to the blood stream and act on their target tissues to regulate the glucose levels.

The insulin gene encodes a 110 aminoacid precursor, known as preproinsulin, which has a signal peptide (24 amino acids), a B-chain (30 residues), a connecting peptide (C-peptide) (35 residues), and an A-chain (21 aminoacids) (Figure 6). The signal peptide, which directs the protein to the lumen of the endoplasmic reticulum (ER), is cleaved to form the proinsulin that is then translocated to the Golgi [56,57]. The proinsulin is processed by the β cells specific enzymes convertases PC1 and PC2 and carboxypeptidase, specifically expressed in the β cells, producing the active two-chain mature insulin and free C-peptide (Figure 5). This proteolysis is initiated within the *cis*-Golgi and completed in the secretory vesicles, which derive from the *trans*-Golgi [58–65]. Insulin, together with the C-peptide, is stored in the secretory granules awaiting release on demand. Insulin is secreted primarily in response to glucose, which enters into β cells through the glucose transporter 2 (GLUT2). Within the β cell, glucose is metabolized increasing the levels of adenosine triphosphate (ATP), a signalling molecule needed for insulin release, and to close the ATP-sensitive K^+ channels. The closure of these channels causes the membrane depolarization, which allows the Ca^{2+} influx and thus the elevation of cytosolic Ca^{2+} concentration. This elevation rapidly increases the rate of insulin exocytosis by the fusion of the secretory granules with the plasma membrane [66,67].

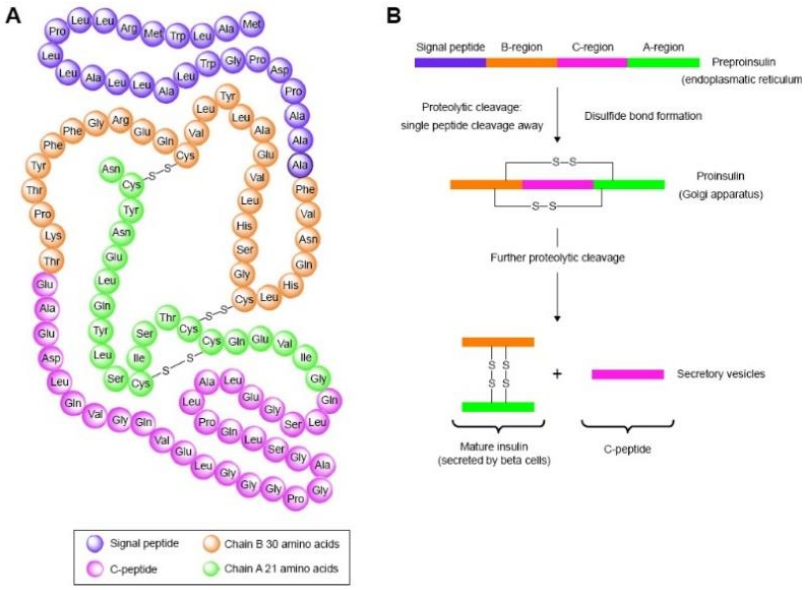

Figure 6. Schematic representation of human preproinsulin structure and its processing until biologically active insulin. Preproinsulin (A) contains four regions: signal peptide (blue), B chain (orange), C-peptide (magenta) and A chain (green). This protein is processed in ER and Golgi, producing the mature insulin (B). Image obtained from Matteucci 2015 [68].

Alterations in insulin production and/or secretion lead to the dysregulation of glucose homeostasis with the subsequent development of hyperglycaemia and DM. Blood glucose dysregulation suffered by diabetic patients is associated with long-term damage, dysfunction and failure of different organs, especially eyes, kidneys, nerves, heart, and blood vessels [2]. According to the last edition of the International Diabetes Federation Atlas published in 2017, 425 million people suffer diabetes and it is expected to rise to 592 million by 2035, reaching worldwide pandemic magnitudes[69]. Thus, DM is defined as a group of metabolic disorders, characterized by hyperglycemia due to defects in insulin secretion, insulin action or both [2]. Depending on its etiology, DM has been classified into 3 main forms: T1DM, due to autoimmune destruction of the insulin producing β cells; type 2 diabetes mellitus (T2DM), caused by insulin resistance in its target tissues and a progressive loss of insulin secretion due to the exhaustion of the β cells; and gestational diabetes mellitus (GDM) diagnosed during pregnancy [2]. There are other types of diabetes, much less common, which include those caused by genetic defects of β cell function.

Depending on the etiology of the diabetes different therapeutic approaches are used. In the case of T2DM, normally associated with obesity, the first approach includes changes in life style, such as healthy diet and physical activity, and oral medications such as metformin, which reduces the hepatic glucose production[70] not requiring, initially, treatment with exogenous insulin. However, insulin administration can be necessary for these patients at long-term due to the progression of the disease. On the other hand, T1DM patients need the administration of recombinant insulin from the moment of the diagnosis of the disease.

2.2. Type 1 diabetes mellitus

T1DM, also known as juvenile diabetes or insulin-dependent diabetes, is an AD predominantly diagnosed in children and young adults. T1DM is one of the most prevalent chronic illnesses diagnosed in these ages. In 2017, more than one million children and adolescents suffer this disease and each year around 130.000 new cases are diagnosed[69]. The increasing incidence is even higher than expected in children from 0 to 14 years. This is an alarming scenario, even more when the reason of the increase is unknown[71]. As previously indicated, T1DM is a chronic disease characterized by insulin deficiency and hyperglycemia due to the autoimmune destruction of the insulin producing β cells[2]. This T cell-mediated AD progresses

sequentially and begins prior to the symptomatic phase, when the patients lack β cells (around 90% loss), becoming insulin dependent [29].

The causing factor of T1DM is still unknown, but both genetic and environmental factors interact to trigger for the disease. The risk for T1DM in siblings of patients is 15-fold higher than in the general population, suggesting that genetic factors play an important role in disease susceptibility[72]. Linkage and association studies have identified several loci as T1DM susceptibility regions. One of them is the human leukocyte antigen (HLA) class II region (about 50% of genetic T1DM risk[73]) localized at chromosome 6p21, which contains genes involved in innate and adaptive immune functions [74,75]. The remaining loci correspond to several (close to 50) non-HLA loci. However, the fact that the monozygotic concordance rate is 50% demonstrates that environmental factors must play an important role [72]. Some proposed initiation factors are viral infections particularly the enteroviruses (for example, coxsackievirus), which can produce some islet injury and activate the diabetogenic response[76–78].

As explained above, in many ADs the symptoms appear only when the affected organs are severely damaged, as this is the case of T1DM. In the onset of T1DM the damage to the β cells is too high and thus exogenous insulin administration is absolutely required to maintain the normoglycaemia. Therefore, the identification of markers that would allow the diagnosis of the disease at an asymptomatic stage becomes a priority. Also this will be of help to increase our understanding of the disease progression in order to prevent complications such as diabetic ketoacidosis (DKA) at the diagnosis, and even to prevent the development of diabetes. In T1DM patients it has been identified antibodies that react against self-proteins such as antibodies against insulin, the major T1DM autoantigen[79–82], the glutamate decarboxylase (GAD65)[83,84], IA-2A (ICA512)[85], and ZnT8[86]. These antibodies can be easily measured and quantified from serum samples. They appear before the onset of diabetes and therefore could be used as T1DM markers. It has been proposed that the risk of developing hyperglycemia could be quantified through the determination of these biomarkers [87,88]. Also, their detection in relatives of T1DM patients together with the HLA genotype could predict the risk for developing the disease [89], increasing the possibilities proportionally with the number of different autoantibodies[90,91].

Based on the presence of autoantibodies, the progression of T1DM has been divided in three stages. Stages 1 and 2, during the presymptomatic phase of the disease, are defined by the presence of 2 or more islet autoantibodies (stage 1) and development of

glucose intolerance (stage 2). The 3rd stage, during the symptomatic phase, occurs with the development of hyperglycemia due to the β cell mass reduction. This last stage is accompanied by polyphagia, polyuria, polydipsia, weight lost and DKA (Figure 7) [2,92,93].

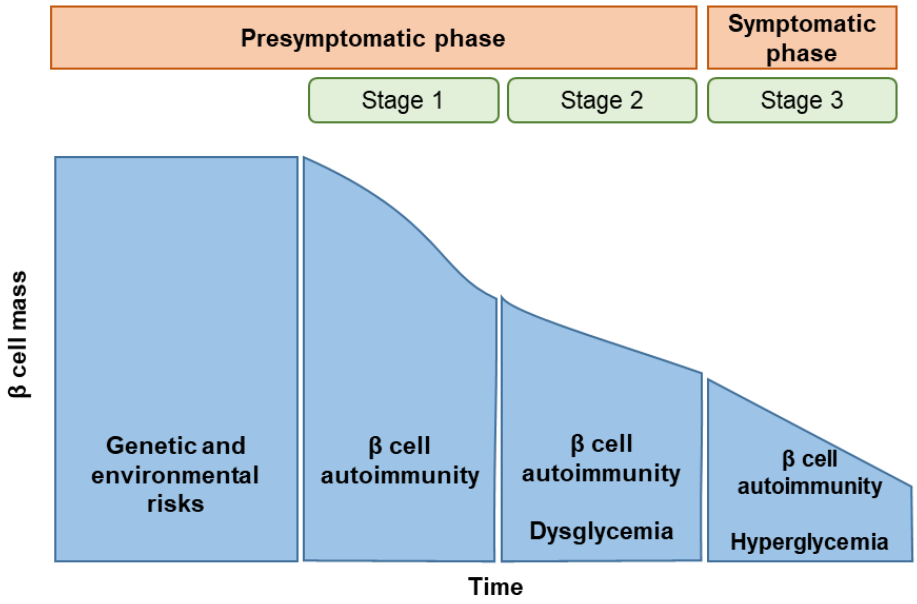

Figure 7. Different stages of T1DM progression. According to the development of symptoms, T1DM can be divided in presymptomatic and symptomatic phases. But also, and considering the markers, 3 main stages can be stablished. Those individuals with genetic and environmental risk can be initiated with the appearance of islet autoantibodies (during the presymptomatic phase) (stage 1). If the disease progresses, glucose intolerance appears (stage 2) reaching the symptomatic phase with the appearance of hyperglycemia when the β cell mass decreases until around 10% (stage 3).

2.3. Mechanism of immune attack in type 1 diabetes mellitus

The implication of the immune system in the development of T1DM was demonstrated by the use of cyclosporine (an immunosuppressor that decreases the production of pro-inflammatory cytokines by the T cells) that improves β cell survival[94]. However, as with basically all ADs, the etiology of the T1DM remains unknown.

T1DM begins with the loss of the self-tolerance to the β cell self-Ags that usually occurs early in life and can be triggered by a viral infection in the pancreas (Figure 8)[95]. These insults induce an initial damage of the β cells, which causes the release of self-Ags and pro-inflammatory cytokines. The APCs present within the pancreas uptake the released β cell Ags and migrate to the draining lymph nodes (LN), where they activate islet-specific $CD4^+$ and $CD8^+$ T cells[96]. Activated islet-specific T cells migrate to the pancreas thereby enhancing the inflammation and the insulitis[97]. The $CD4^+$ T cells

secrete pro-inflammatory cytokines, mainly interferon γ (IFNγ), and chemokines, resulting in the recruitment of more immune cells[98,99]. The released IFNγ increases the activation of APCs and enhances the production of pro-inflammatory cytokines, including interleukin-1β (IL-1β) and TNFα. Since β cells express high levels of IL-1 receptor this makes them more sensitive to the IL-1β released by APCs. IFNγ, IL-1β and TNFα also induce the expression of reactive oxygen species (ROS) including nitric oxide by β cells, which induces cell damage and apoptosis[95,100]. Apart from the APCs, $CD4^+$ T cells can also activate the $CD8^+$ T cells. Then, they migrate to the pancreas and the recognition of the Ag presented by the MHC class I from the β cells induces the secretion of granzymes and perforin with the subsequent cell death[101]. Interestingly, a reduced suppression capacity of Tregs can also participate in the activation of autoreactive T cells[102].

In a second stage, the immune attack against β cells is worsening. At the beginning, only few Ags are targeted by autoreactive T cells, however as a consequence of this initial β cell death together with the progression of insulitis, more and new Ags are released. This process is known as epitope spreading, that increasing the strength of the disease with the activation and recruitment of other autoreactive T cell clones specific to the new Ags[103,104]. Thus, the immune scenario is aggravated during the progression of T1DM.

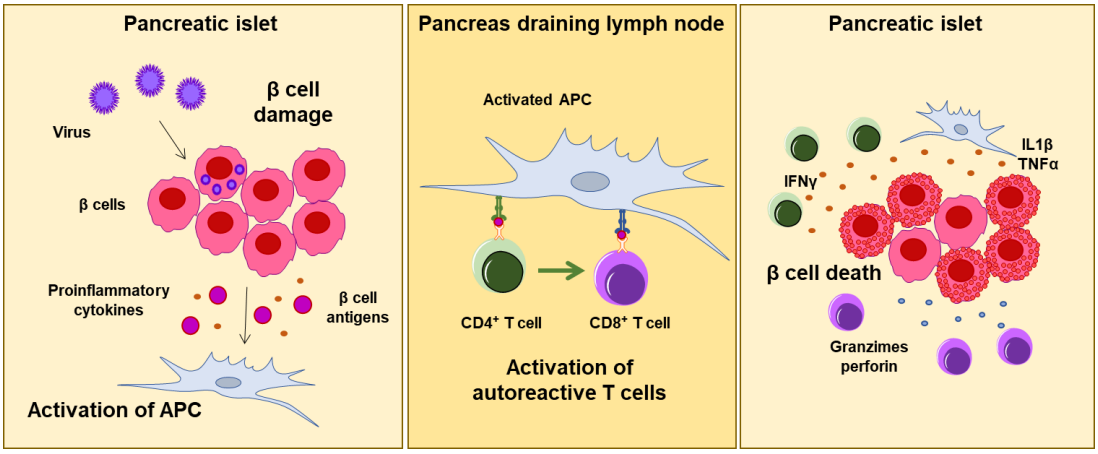

Figure 8. The immune components involved in the process that leads to β-cell destruction in T1DM. An initial β cell damage caused by pathogens such as virus can induce the secretion of proinflammatory cytokines as well as β cell Ags. This can trigger the maturation of the APCs that migrate to the pancreatic lymph nodes. There, these mature APCs activate the autoreactive $CD4^+$ and $CD8^+$ T cells which migrate to the pancreatic islets and promote the β cell death through the secretion of proinflammatory cytokines as well as granzimes and perforin.

2.4. Preclinical models to study type 1 diabetes mellitus in the laboratory

One approach to elucidate the etiology and the development of T1DM is the use of animal models. Mouse models are useful to study and understand the molecular bases of the β cell destruction, and to assay the effect of novel therapeutic agents. Here we briefly describe the most used mouse models to study T1DM:

- **Spontaneous model: non-obese diabetic (NOD) mice**

The NOD mouse is the most used model for T1DM studies since it shares several similarities with the human disease[105] such as structural analogies of class II MHC which can be responsible of the immune attack against β cells[106]. These varieties of class II MHC can cause T1DM susceptibility, producing a spontaneous infiltration that destroys the pancreatic β cells[107]. This infiltration is predominantly composed of $CD4^+$ and $CD8^+$ lymphocytes, macrophages, B cells and NK cells, mimicking the infiltration happening in human islets[108,109]. T1DM onset appears between 10 to 14 weeks of age, when approximately 90% of pancreatic β cells are destroyed. The incidence of hyperglycemia is higher in females with values that range between 60 to 80%, while in males is between 10 to 30%[110]. Despite that NOD mouse can be the T1DM model that better resembles the human disease, the onset of the disease cannot be accurately predicted, thus programming experiments becomes challenging. This is an important fact when it comes to test therapeutics agents.

- **Drug-inducible model: streptozotocin administration**

The administration of streptozotocin (STZ) specifically induces β cell death since this compound enters through the GLUT2 transporter[111].This model provides a simple, low-cost and easily controllable approach to induce hyperglycemia[112]. The protocol to induce T1DM employs multiple administrations of low-dose STZ which produce an initial β cell damage that triggers an inflammatory process causing the further loss of β cells that results in insulin deficiency and hyperglycaemia, mimicking human T1DM. Thus, low doses of STZ to mice for 5 consecutive days activate the immune attack against β cells. Two weeks after the first administration of STZ around a 40% of mice become hyperglycemic[113]. This model is appropriated for studying the pathological consequences of T1DM, but also to assess and evaluate experimental approaches for the treatment of this disease. The disadvantage of this model is that the use of these chemicals, despite their high specificity to β cells, can produce some toxicity in other

organs[113,114]. Hence, although the STZ administration to mice can be used as a T1DM model, it is not the best model to mimic this disease.

- **Pathogen-induced model: RIP-LCMV mice**

Some viruses, such as enteroviruses or rotaviruses, have been suggested to be associate with mediators of T1DM, based on the detection of enteroviral RNA in the sera of T1DM patients[115,116], and the increased levels of anti-rotaviruses antibodies that have been related to the appearance of β cell autoantibodies in children with T1DM risk[117–119]. Viral infections can induce direct β cell lysis, bystander activation of autoreactive T cells, loss of Tregs cells or molecular mimicry. Thus, several animal models use viruses to induce the β cell destruction[120]. An example is the RIP-LCMV mouse in which a defined viral Ag of the Lymphocytic Choriomeningitis Virus (LCMV) is expressed in β cells by the use of rat insulin promoter (RIP)[121]. This model is based on breaking the tolerance to a defined target autoantigen expressed by β cells through a viral infection. Thus, the infection of the mice with the LCMV activates the immune system against the viral Ags, which are also presented specifically in β cells. This induces the selective removal of β cells with an incidence of 90%[121]. However, because this model depends on the replication level of the virus and the timing of the infection the results obtained experience high variations.

- **DNA-inducible model: RIPB7.1 mouse model**

The RIPB7.1 is a transgenic model of experimental autoimmune diabetes (EAD) that presents a rapid development of diabetes after antigenic challenge with insulin. The RIPB7.1 mice express the T cell costimulatory molecule B7.1 (CD80) under the control of RIP, thus as before, only β cells express B7.1. Mice are immunized intramuscularly with a plasmid expressing the mouse preproinsulin II, that acts as the autoantigen that induces a selective T cell mediated immune response against the pancreatic β cells[122]. After the preproinsulin administration, 60 to 95% of these animals develop hyperglycemia within 3 to 5 weeks without gender deviation. The non-immunized animals do not develop EAD spontaneously. The presence of the costimulatory molecule B7.1 does not trigger the immune attack against β cells but it is necessary for the T cell effector function with the subsequent development of EAD[123].

This model presents some advantages. Due to the absence of gender deviation, entire litters can be used for experimentation, and, considering that only one allele of the B7.1 transgene is sufficient for the EAD development, the crossing with other transgenic

mice is possible without detrimental effects. The use of plasmid DNA to trigger the immune attack provides a better control of the induction of the disease obtaining more consistent results. This model shares some features with human T1DM: it is characterized by $CD4^+$ and $CD8^+$ insulitis that leads to β cell damage and insulin deficiency. The RIPB7.1 mice are suitable to evaluate therapeutic interventions based on the prevention of the autoimmune attack as well as to study mechanisms of insulin-specific T cell reactivity[122,123].

3. Treatments for type 1 diabetes mellitus

The actual treatment for T1DM patients is the administration of exogenous insulin or insulin analogues. Most patients are diagnosed when the lost of β cells is very significant and the pancreas cannot regulate the blood glucose levels. Patients must tightly and constantly control the glycaemia with glucometers and administer recombinant insulin their selves. A slight fail in glucose determination or insulin administration cause severe consequences due to the impossibility to simulate physiological glucose control and producing high variations of glycaemia. Thus, severe complications of diabetes cannot be avoided in most of cases. Insulin administration is a palliative treatment that does not avoid the development of life-threatening complications, and new therapies are needed.

3.1. Experimental therapies

3.1.1. Immunosuppression therapies

Immunosuppression consists in the removal or inactivation of self-reactive immune cells and the neutralization of the secreted pro-inflammatory cytokines. In T1DM patients, the use of monoclonal antibodies as anti-CD3 antibodies has been partially successful. The administration of anti-CD3 in T1DM patients showed a decreased demand for exogenous insulin together with an increased of endogenous production of insulin. However, the disease amelioration was short, even lower than two years[41,124–128]. Also, a reduction in the doses to decrease the undesired side-effects of immunosuppression, such as the reactivation of Epstein Barr virus, eliminated the benefits of this treatment[44]. Therefore, since T1DM is a T cell-mediated disease, other strategies such as the use of the antibodies Abatacept (anti-CTLA) or Alefacept (anti-CD2), have tried to target specifically the activation of these immune cells with only slight reduction on insulin requirements[129,130]. Other antibodies (Canakinumab – anti-IL-1β) or small molecules (Anakinra - IL-1β receptor antagonist) have been used to

neutralize the pro-inflammatory cytokines IL-1β. However, these treatments did not improve the levels of the C-peptide[131].

Therefore, the non-specific immunosuppression approaches can provide minimal benefits and a high risk of opportunistic infections.

3.1.2. Induction of tolerance by self-antigen presentation

As described above current treatments for AD are only palliative, since they do not target the root of the problem, and in addition entail risks associated to the treatment itself. Even without knowing what triggers T1DM, the activation of autoreactive T cells is the responsible of the tissue destruction. Therefore, the ideal therapeutic intervention to cure ADs will consist in the specific suppression of solely the autoreactive T cell clones that recognize the self-Ags. This process, known as antigen specific tolerance (AST) restoration, consists in the presentation of the implicated self-Ag to the autoreactive T cells in the absence of costimulatory molecules (CD40, CD80/86) and pro-inflammatory cytokines. In this scenario, instead of being activated, autoreactive T cells will become anergic or depleted, or they will become induced regulatory cells (iTregs) with suppressing capabilities. Therefore, the restoration of the AST will allow the specific blockage of the autoreactive T cells while maintaining the capacity of the immune system to clear non-self-Ags, protecting the organism from a pathogenic attack.

The restoration of AST has shown beneficial effects in animal models of ADs inducing a long-lasting tolerance without side effects[132–138], and in particular in T1DM[139,140]. In most of the cases, when the Ags are administered once the autoimmune attack has started, the AST has been only partially achieved. However, in other cases, mostly when the tolerogenic Ags were administered prophylactically (sometimes during the neonatal stage) the development of the inflammatory symptoms are avoided[141–143]. This is precisely one of the major limitations at the time of translating this therapeutic strategy into humans. Due to the absence of markers to detect the different stages of the AD, in most of the cases the intervention would be applied once that the autoimmune attack in ongoing, meaning that not only naïve but activated/memory T cells must be targeted, adding another layer of difficulty. Targeting activated/memory T cells increases the risk that the administration of the self-Ag involved in the AD can exacerbate the immune response instead of inducing tolerance. So far, the translation of AST into humans is challenging.

There are several factors such as the type of Ag and its nature, how is presented and the amount present that must be considered to achieve an efficient induction of AST. First, identify the major self-Ag. In the case of T1DM, insulin can be recognized as the major target of autoreactive T cells[79,80,144–146]. Also, the specific sequence of the Ag can also influence the outcome. Thus, different insulin isoforms have been tested to induce AST. For example, the administration of high doses of preproinsulin induced immunogenicity instead of tolerance[122,147,148]. However, the use of the non-secreted form, the proinsulin sequence, reduced the incidence of hyperglycemia[149]. Last, adequate levels of Ag expression in the proper cellular location are also important[150]. In the NOD mice, oral administration of 1 mg of insulin protected mice against the development of hyperglycemia while lower doses (0.01 to 0.1 mg) did not reach this protection[151].

AST has already been applied in clinical trials. For example, in T1DM patients vaccinated with a proinsulin encoding plasmid, C-peptide levels were preserved together with a reduction in proinsulin reactive $CD8^+$ population and islet autoantibodies. However, in these patients insulin requirements were maintained indicating this treatment needs further improvements[152,153].

Considering the current state of AST intervention, it is necessary to define more consistent protocols to efficiently restore the broken tolerance in patients and, in the case of T1DM patients, reestablish glucose homeostasis.

3.1.2.1. Organ targeting to induce antigen specific tolerance

The selection of the target organ for the delivery of the self-Ags influences the way that the tolerance is induced. Oral or mucosal administration delays T1DM onset in murine models[151,154,155]. However, the human translational trials have shown that nasal or oral insulin administration provided limited clinical benefits[156–161]. A possible explanation could rely on the Ag dose used. Based on the weight of mice the effective dose in mice was 100 fold higher than the dose administered in humans.

Alternatively, intramuscular administration allows an easy and efficient way to express the Ag in the host tissue. After intramuscular administration, the myocytes are the main target cells that express the Ag, although dendritic cells (an APCs) can also express it. In peripheral tissues such as the muscle, the DCs present a high phagocytic capacity and a low expression level of costimulatory molecules, rendering them poor initiators of

the immune response. Therefore, the uptake by DCs of apoptotic cells expressing the Ag in the absence of a danger signal induces cell tolerance[162,163]. Thus, the administration of an Ag by intramuscular vaccination avoids the activation of autoreactive T cells by deleting them via apoptosis, rendering them unresponsive (anergic) and/or inducing Tregs[164,165]. AST by intramuscular administration of the Ag has obtained encouraging results for the treatment of ADs such as multiple sclerosis or T1DM[153,166,167].

Liver has a great capacity to induce tolerance[168,169]. The liver is constantly exposed to external Ags that come from the food, inducing tolerance to these Ags. The liver presents a particular morphology with sinusoidal and fenestrated vessels that reduce the blood flux, allowing the blood to enter into the subendothelial space to facilitate the interaction of the carrying molecules from the intestine with the hepatocytes[170]. Thanks to the hepatic microcirculation and considering the number of times that the same T cell passes through the liver (around several hundred times per day), the interaction between T cells and liver cells is highly probable increasing the possibilities to encounter the Ag expressed by these liver cells. Once the T cell arrives, it is exposed to tolerogenic mediators such as interleukin-10 (IL-10) or TGFβ[171]. The interaction of T cells with liver cells (DC, Kupffer cells, sinusoidal endothelial cells and hepatocytes) in these non-inflammatory conditions induces the anergy state, their deletion or their differentiation to iTregs[172,173]. It has been shown that either by intravenous and intraportal administration of the Ag, AST is efficiently induced[174–176].

3.1.3. Gene therapy

Gene therapy is the use of a vector to deliver a gene to a target cell with a therapeutic aim. Due to the different nature of the vectors currently used in gene therapy, the expression of the Ag encoded into the delivered transgene can be modulated. Thus, the Ag expression can persist for a long period of time or can be for a short term, it can be expressed with low or high intensity and depending on the administration route and vector tropism, different cell types can be targeted. Therefore, gene therapy becomes a suitable way to induce AST.

3.1.3.1. Advantages and disadvantages of current vectors

The most used gene delivery vehicles for restoring tolerance approaches are based in naked DNA in the form of plasmids, or in viral vectors such as lentiviral vectors (LV) or adeno-associated viral vectors (AAV). A key question to choose a particular viral vector

to induce AST is the intrinsic immunogenicity of the vector particle. In the same way that adenoviral-based vectors are the vector particles of choice to induce immunogenicity[177,178], to induce AST, a vector that do not induce an immune response naturally, is needed, otherwise, even a small inflammation caused by the vector during the delivering of the self-Ags can exacerbate the immune response instead of inducing AST.

Plasmid DNA, if compared to viral vector particles, is easier and cheaper to manufacture under good manufacturing practice (GMP) conditions, is more stable and safer. As gene delivery vectors they are less efficient than viral vectors and repeated doses of plasmid are needed to obtain a comparable transduction efficacy. The use of naked DNA as vehicle to induce AST has been tested in the clinic for T1DM patients with relative success[152,153,179–181]. However, the current development of new polymers, which are being used to coat plasmid DNA molecules, can enhance their *in vivo* administration significantly.

On the other hand, viral vectors are more efficient than naked DNA to deliver genes to target cells, however, they are more expensive at manufacturing and more labile. Both LV and AAV transduce a wide variety of tissues. LV mediate a long-term expression *in vivo* because they are integrative, but precisely for this reason, *in vivo* administration of these vectors is not currently approved in humans. AAV-dervied vectors can also provide long term expression when the target cells have a very slow division rate, since they remain as episomes[182–186]. These vectors are able to induce AST in naïve hosts[137,187–190], however, they are not completely non-immunogenic and repetitive administrations can induce an immune response towards the vector particles, hence jeopardizing the AST. For example, around 60% of the population carries AAV antibodies as a consequence of previous infections with the native virus. This circumstance prevents the use of AAV-derived vectors [191].
Therefore, new viral vector systems that completely lack of immunogenicity are needed.

3.1.3.2. SV40 as a delivery system for gene therapy in T1DM

The use of replication deficient Simian Virus 40 particles (SV40) as a gene delivery vehicle can avoid many of the above mention problems without losing efficacy to induce AST. SV40 is a non-enveloped polyomavirus with an icosahedral capsid of 45 nm of diameter and with a double strand DNA molecule of 5.25 kb. The genome is

organized in a regulatory region that controls the expression of the early genes and the late genes. The early genes code for the non-structural proteins, the large T antigen (LTag) and small T antigen (STag) and the late genes code for the structural viral proteins (VP1, VP2, VP3)[192,193]. The viral particles replicate in macaques, the natural hosts, where it causes chronic asymptomatic infections[194]. As it enters cells through the caveola, viral particles avoid the proteasome degradation and evade the immune system[195,196].

The lack of immunogenicity of SV40 makes these vectors attractive for use in AST induction. Several reports have indicated that SV40 vector particles neither induce the generation of neutralizing antibodies nor a T cell reaction upon *in vivo* administration, considering then that humans are naïve for these viral particles[197–199]. Moreover, SV40 particles transduce efficiently a wide range of cells, increasing the possibilities of their application in the clinic[200–203].

These advantages of SV40 particles for their application in gene therapy have led to the generation of SV40 delivery vectors in which the removal of the early genes leaves 2.7 Kb of space to insert the transgene of interest. Nevertheless during the production process in the packaging cell line based in CV-1 cells (Cos1), wild type particles can emerge, making impossible to use these vector particles in the clinic[204]. The reason is a homology-dependent recombination process that happen between the chromosomally inserted SV40-specific DNA sequences and episomally replicating vector-specific DNA sequences. To solve this problem, at Amarna Therapeutics a new Vero-based packaging cell line has been developed by incorporating into the cells only the necessary DNA sequences to express exclusively the LTag preventing in this way the recombination process[205,206]. This SuperVero cell line produces comparable amounts of SV40 viral particles as the Cos1 cell line without contamination with replication-competent SV40 particles[206].

3.1.4. Drug therapy

3.1.4.1. BL001 as a novel T1DM therapy

Despite huge efforts to find an effective treatment for T1DM, there is still the necessity to identify compounds that can protect the β cells from the attack of the immune system, and at the same time can induce the proliferation of new β cells, to repopulate the lost tissue and the lost function. Some small drugs targeting proteins involved in

inflammation are in development phase, and although they are showing a good efficacy, comparable to glucocorticoids, they still cannot achieve long-term remission[207,208].

A potential target to treat T1DM is the liver receptor homolog 1 (LRH1). LRH1, also known as NR5A2, is a member of the NR5A family of nuclear receptors, which plays a pivotal role during early embryonic development. This gene is expressed in tissues derived from the endoderm including intestine, liver, immune cells and ovary as well as exocrine and endocrine pancreas[209–211]. In the exocrine pancreas, LRH1 regulates the expression of genes involved in digestive functions[212], while in the endocrine pancreas confers protection against stress-induced apoptosis and stimulates the production of enzymes involved in the glucocorticoids biosynthesis[213]. In the immune system, LRH1 is expressed in different cell types such as macrophages[210], primary and secondary lymphatic tissues, as well as in $CD4^+$ and $CD8^+$ T cells[211]. Several lines of evidence also support a role for LRH1 in the control of the inflammatory response by inducing an anti-inflammatory phenotype in macrophages[210], controlling the homeostasis of activated $CD4^+$ and $CD8^+$ T cells[211], regulating intestinal immunity in response to immunological stress[214] and modulating the hepatic acute-phase response[215]. Natural phospholipids have been described as activators of LRH1 and present therapeutic properties improving glucose homeostasis and decreasing hepatic steatosis[216–218]. According to that, an LRH1 agonist that potentiates the activity of this factor can be used as a therapeutic approach to treat T1DM. The initial β cell injury promotes the differentiation to proinflammatory macrophage phenotype (M1 subtype) which can induce β cell death through the secretion of the pro-inflammatory cytokines IL-1β and TNFα. These cells activate T lymphocytes which can produce other inflammatory cytokines such as IFNγ. As explained previously, the synergy of the action between these three cytokines can promote β cell death[219]. However, the presence of the anti-inflammatory macrophage subtype (M2 phenotype), promoted by a LRH1 agonist, can block the activation of M1 as well as the T lymphocytes, and the progress of the immune attack. This agonist would promote an islet anti-inflammatory environment that can prevent the progressive loss of pancreatic β cells, improving the glucose homeostasis. *In vitro* studies demonstrated the protective role of LRH1 against cytokines and streptozotocin-induced apoptosis by the overexpression of this transcription factor[213] as well as by BL001[220], a novel LRH1 agonist (patented by Dr. B. Gauthier WO 2011-144725-A2 and WO 2016-156531-A1). The experiments performed in our group have revealed that daily injections during 24 weeks did not produce macroscopic organ alterations in BL001-treated mice, with normal cholesterol and

triglycerides plasma levels up to 8 weeks of treatment. More importantly, our group showed that the treatment with BL001 significantly decreases the incidence of hyperglycemia. This action is mediated by the regeneration of β cells and, at the same time, the promotion of immune tolerance, skewing the pro-inflammatory M1 macrophages towards the M2 subtype, which is defined by the expression of distinct surface markers and the secretion of cytokines such as IL-10 or TGFβ (Figure 9). In agreement with this, isolated intraperitoneal macrophages treated *in vitro* with BL001 showed a dose-dependent increase in the transcription and secretion of IL-10 and TGFβ[220]. However, further experiments are needed to decipher whether these effects on macrophages of BL001 are mediated by LRH1. Similarly, the increase in the M2 population of macrophages has been associated with a protective effect in several preclinical models of autoimmune diseases[221,222]. Accordingly, a deficient function in M2 subpopulation from NOD mice, which present a reduced tolerogenic function, can play a role in the susceptibility of this model[221,223].

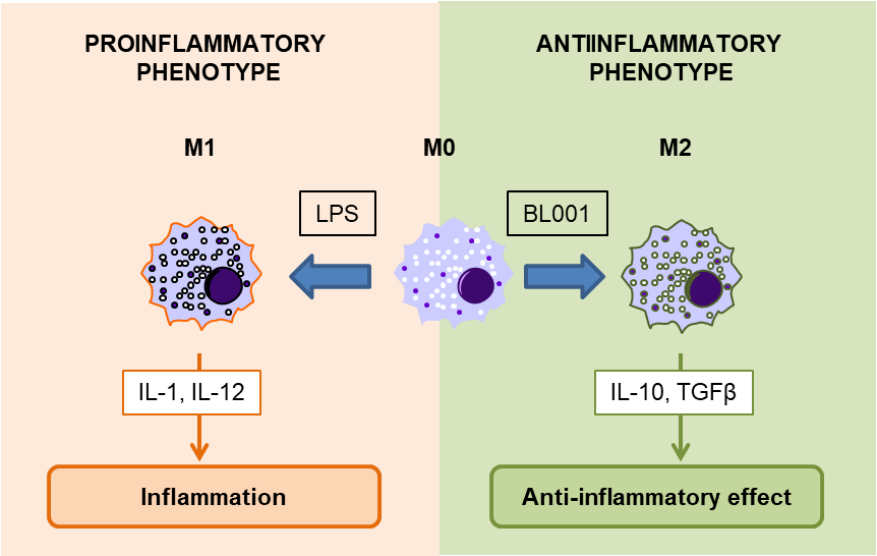

Figure 9. Macrophages differentiation to M1 or M2 profile after the treatment with LPS or BL001 respectively. Macrophages can differentiate into the proinflammatory phenotype (M1) or to the anti-inflammatory phenotype (M2). The production of cytokines by these subtypes can interact with other cells and modulate the immune response. Thus, based on our recent publication the presence of BL001 can induce the differentiation to the M2 phenotype meanwhile the treatment with lipopolysaccharide (LPS) prompts to the M1 subtype.

Despite the benefits of BL001, the translation into humans can present some limitations since BL001 cannot be administrated orally due to its lipophilicity. Hence, novel compounds that activate LRH1 and are well tolerated when they are administered orally should be developed.

II. HYPOTHESIS

The induction of a tolerogenic environment can preserve the survival and function of the pancreatic β cells, preventing the development of T1DM. For this end, two different approaches are investigated:

- The development of a vaccine based on SV40 viral vector that can restore the AST in T1DM by expressing the self- antigen insulin.

- An *in vitro* screening platform that can allow the identification of novel LRH1 agonists with an anti-inflammatory capacity.

III. OBJECTIVES

Objective 1. Induction of antigen specific tolerance in the transgenic RIPB7.1 mouse model by delivery of the insulin gene using SV40 vectors

1. **Determine the biosafety of the SV40-packaging cell line Super Vero**
2. **Validate the transduction efficiency of the SV40 vectors manufactured with SuperVero cells *in vivo***
3. **Setting up the conditions to induce antigen specific tolerance in the transgenic mice RIPB7.1**

Objective 2. LRH1 as a novel target to treat T1DM: Analysis of the effect of LRH1 agonist BL001 on the innate immune system and screening for a second generation of LRH1 agonists

1. **Effect of the LRH1 agonist BL001 in primary macrophages**
2. **Screening of novel LRH1 agonists**
   - **2.1. Development of a screening platform**
   - **2.2. Screening novel LRH1 agonists and their safety profile**
   - **2.3. Evaluation of the protective capacity of two novel LRH1 agonists *in vitro***

IV. MATERIALS AND METHODS

Mice

RIPB7.1 mice were housed in ventilated plastic cages under a 12 hours light/dark cycle, with food and water *ad libitum*. Mice experimentations were approved by the CABIMER Animal Committee, and performed in accordance with the Spanish law on animal use RD 53/2013. Mice genotyping was performed by PCR using a tail sample obtained at day 10 after birth. For genomic DNA extraction and transgene amplification from mouse tails the REDExtract-N-AmpTM Tissue PCR Kit (Sigma-Aldrich; #031M6112) was used. PCR was performed with the specific primers for the B7 transgene (Table I). Several in vivo parameters were assessed as described below:

a. Immunization.

For the EAD induction (immunization), 7 to 8 week-old RIP-B7.1 mice (males and females) were injected with 50µg of human ppINS expression pCI plasmid (Plasmid Factory GmbH, Germany) diluted in PBS into each of the two anterior tibialis muscles (50µl per injection). Glucose monitoring was performed weekly on blood samples from tail vein using on the Optium Xceed glucometer (Abbott Scientifica). Non-fasting blood glucose higher than 300mg/dl for two consecutive measurements was considered hyperglycemia. At the end of the experiments mice were euthanized and pancreases were extracted for immunohistochemistry assays.

b. DNA vaccination/ SV40 administration

DNA vaccines or SV40 vector particles were administrated diluted in PBS through different approaches: intramuscularly in the anterior tibialis muscles (in a final volume of 50µl), intravenously by the tail vein injection (in a final volume of 100µl) or hydrodynamically considering the 10% of weight (in grams) of the mice.

c. Alanine aminotransferase determination

Blood samples were collected from the facial vein of mice. Consecutively, serum alanine aminotransferase (ALT) was measured using the Reflotron GPT (ALT) Test (Roche, #10745138) according to the manufacturer's instructions.

d. *In vivo* imaging to assess biodistribution of viral/DNA particles harbouring luciferase

Pierce D-luciferin (Roche, #88293) was resuspended in PBS at a final concentration of 15mg/ml. Then, D-Luciferin was administrated into the tail vein, receiving each mouse 150 mg D-Luciferin/kg body weight. 5 minutes post-injection, luciferase signal was measured using the Xenogen *in vivo* imaging system (IVIS).

e. Measurement of anti-SV40 antibodies

Serum samples obtained from facial vein puncture from SV40 injected mice were heat-inactivated at 56°C for 30 min. As a control, serum of non-SV40 injected mice was included. All the samples were serially diluted with PBS (1/10, 1/50 to 1/100) and mixed with equal volumes of SVGFP viral particles. The mixtures (vector-serum dilutions) were incubated at 37°C for 30 min. One day after seeding of Cos1 cells in 96 multiwell plate, they were inoculated with 50 µl of vector–serum mixture and incubated for 4 hours at 37°C in a humidified 5% incubator. Fresh DMEM media was then added to each well and seven days after transduction, GFP was measured using Varioskan Flash spectrophotometer (Thermo Scientific). Every sample was tested in triplicate. A negative control without transduction was included in each experiment.

Islets isolation

Mice were sacrificed by cervical dislocation and pancreatic islets were isolated by collagenase perfusion (Sigma-Aldrich, #C9263) digestion, handpicked and maintained in RPMI 1640 at 11.1mM glucose (Sigma-Aldrich; #R0883) supplemented with 10% heat inactivated FBS (Sigma-Aldrich, #F7524), 2mM L-glutamine (Sigma-Aldrich, #G7513), 10mM HEPES (Gibco, #15630-056), 100 units/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich, #P4333), 1mM sodium pyruvate (Sigma-Aldrich, #S8636) and 50µM β-mercaptoethanol (Gibco, #31350-10).

Cytokines-induced apoptosis

Mouse islets were cultured in RPMI medium containing 10% charcoal-stripped FCS for 48 h. The latter is nearly free of endogenous steroids to avoid the deviation of the results. Islets were then cultured for 48 hours in the presence of different concentrations of the compounds (BL002 and BL003) at 0.05% of DMSO, alone or in

combination with a cocktail of cytokines (2 ng/ml IL-1β, 28 ng/ml TNFα and 833 ng/ml IFNγ) added 24 hours before ending the experiment.

Primary macrophages

a. Macrophage isolation

Intraperitoneal macrophages were isolated after CO_2 euthanasia to avoid excessive bleeding into the peritoneal cavity. Then, the skin was removed and 10ml of cold Dulbecco's phosphate-buffered saline F12/10 without calcium and magnesium (Sigma-Aldrich, #D8437) was injected into the intraperitoneal cavity. Subsequently, the peritoneum is gently messaged to remove any attached cells. The volume is harvested and collected in ice cold tubes which are centrifuged at 4°C for 10 minutes at 1500rpm. To eliminate the erythrocytes present in the sample, cell pellets are incubated with blood lysis buffer for 5 minutes at room temperature (Sigma-Aldrich, #R7757) and washed twice with supplemented media containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate and 50µM β-mercaptoethanol. Intraperitoneal macrophages were seeded in non-adherent plates for one week to obtain a purified macrophage population.

b. LRH1 silencing macrophages

Intraperitoneal macrophages were transfected with ON-TARGETplus SMARTpool siRNA targeted to mouse LRH1 (Dharmacon, #L003430) or a control luciferase siRNA (Sigma, #HA10574568) at 50nM using the transfection reagent Viromer Blue (Lipocalyx, #VB-01LB-00) according to the manufacturer´s instruction. 24 hours after transfection, primary cultures of intraperitoneal macrophages were treated with BL001 (10µM) or LPS (1µg/ml), which induces a pro-inflammatory profile in control macrophages. Then, 24 hours after treatment (48 hours after transfection), cells were collected for RNA extraction.

For immunocytochemistry analysis, macrophages were seeded in 12mm diameter glass coverslips (Menzel-Glaser, #J1800AMNZ) in 24 multiwell plates. After obtaining the pure macrophage culture, LRH1 was silenced as previously indicated and immunocytochemistry was performed 48 hours after silencing.

Cell culture

All cell lines were incubated in a humified (95%) atmosphere (5% CO_2) at 37°C in media supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS),

100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine. However, some exceptions have to be mentioned: SuperVero media was only supplemented by L-glutamine and HepG2 media contains 20% of FBS.

Monkey kidney fibroblast Vero and SuperVero cell lines (obtained from Amarna Therapeutics) were cultured in serum-free OptiPRO (Gibco, #12309019) medium. Cell monolayers at 90-95% confluency were digested by exposure to trypsin (Gibco, #15400054) to harvest the cells for sample preparation or culture passage. Passages were performed twice per week and media was changed every three days.

Murine macrophages Raw 264.7, monkey kidney fibroblast Cos1 and human embryonic kidney 293T cell lines were cultured in supplemented (as previously indicated) Dulbecco's modified eagle medium (DMEM) (25mM glucose). To harvest cells for sample preparation or culture passage, cell monolayers at 90-95% confluency were trypsinized. Media was changed every three days and passages were performed twices per week.

Human hepatoma HepG2 (kindly provided by Dr. Francisco Martin (GENYO-Granada)) and HeLa cell lines were cultured in supplemented Eagle's Minimum Essential Medium (EMEM) media. To harvest cells for sample preparation or culture passage, cell monolayers at 80-90% confluence were trypsinized. Passages were performed twice per week and media was changed every three days.

The rat insulinoma cell line Ins-1E (kindly provided by Claes Wollheim (University of Geneva, Switzerland)) was cultured in RPMI-1640 medium at 11.1mM glucose supplemented with 10mM HEPES, 1mM sodium pyruvate and 50µM β-mercaptoethanol. When confluence was approximately 80-90%, cells grown in TPP tissue culture flasks (TPP, #90076) were trypsinized. Passages were performed once weekly when cells reach 80-90% confluence and media was changed every three days.

Jurkat cell line was cultured in RPMI-1640 medium at 11.1mM glucose. A density of $1x10^5$ viable cells/mL was maintained during the culture. Every three days, density was corroborated to avoid exceed $3x10^6$ cells/ml and media was changed.

Primary macrophages were isolated and cultured in non-adherent plates (Greiner, #658170) in supplemented Dulbecco's phosphate-buffered saline F12/10 without

calcium and magnesium media supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin, 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate and 50µM β-mercaptoethanol. Media was replaced every three days.

Creation of the screening platform

For the creation of a stable cell line expressing GFP under the transcriptional control of the LRH-1 target SHP gene promoter, SHP-HepG2 (Figure 10), HepG2 cells were seeded in 24 multiwell plates and transduced with LV-SHP-GFP vectors with a multiplicity of infection (MOI) of 2. One month after transduction (10 passages post-transduction), GFP positive cells were analyzed by flow cytometry using FACSCalibur (BD Biosciences) showing a 50% of positive population. Then, LV-SHP-GFP vector copy number was determined by Q-PCR using the sequence Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) present in the vector, considering the DNA amount corresponding to 100.000 cells (600ng of genomic DNA from the cultured cells). The analysis showed that the culture has an average of 0.4 copies per cell, confirming the FACS data where, after the analysis of GFP population, around 50% of cells were positive for this fluorescence protein. GFP was measured every week during one month, keeping the percentage of GFP positive cells stable.

Then, these SHP-HepG2 cells were transduced with at MOI 1 using LV-EF1α-DsRed vectors. This promoter reporter construct was used as control non LRH-1 target. One week post-transduction, initial analysis using flow cytometry showed a 20% of GFP-DsRed-double positive cells. Two sorting processes using the FACSARIA I (BD) were necessaries to obtain a purity of 60% of GFP-DsRed-double positive cells. The EF1α-DsRed lentiviral copy number was determined by Q-PCR using specific primers for this transgene, showing 0.5 copies per cell. SHP/EF1α-HepG2 cells were expanded in culture medium, stored in liquid nitrogen and used for screening experiments. After 20 passages in culture, the expression of both transgenes remained constant revealing that these LV do not affect cell viability.

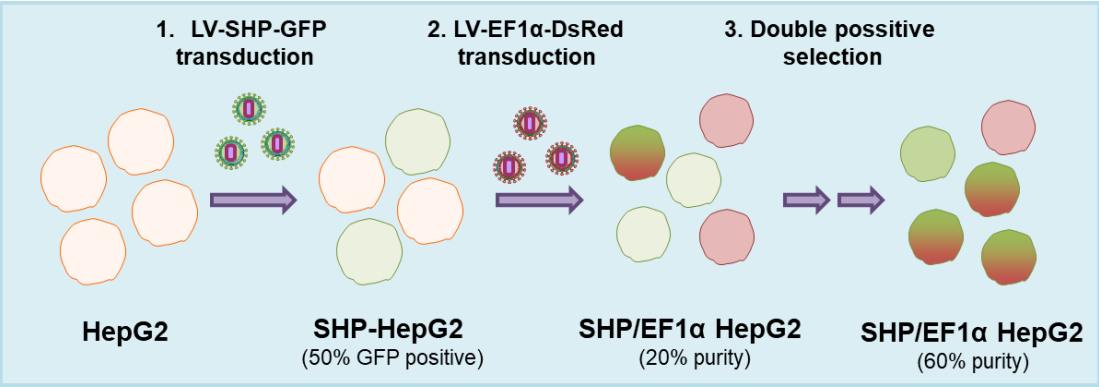

Figure 10. Production of the screening platform SHP/EFα HepG2 cell line. Schematic representation of the transduction steps using two different lentiviral vectors and subsequent selection of double positive cells by sorting. After transduction with LV-SHP-GFP, 50% of cells were GFP positive. Consecutively, the transduction with LV-EF1α-DsRed resulted in a 20% of double positive cells which were purified after two sorting processes to obtain a 60% of SHP/EF1α HepG2 cells.

DNA plasmid production

a. Cloning strategy

Human preproinsulin sequence from phppINS was used to create pSVPPI plasmid, which was cloned by Amarna Therapeutics BV.

In the case of proinsulin vectors, proinsulin was obtained from a purchased plasmid (GeneArt, ThermoFisher) containing proinsulin with the microRNA target sequence (miRT) 142·3p. The addition of this sequence prevents the expression of the transgene in hematopoietic cells such as APCs, which can improve the outcome of AST. First, proinsulin transgene together with the miRT-142·3p was cloned into pSV40 plasmid using ClaI (New England Biolabs, #R0197) and XbaI (New England Biolabs, #R0145), generating pSVProI-142·3p. Then, in order to produce the pSVGFP-142·3p or pSVLuc-142·3p plasmids, ProI transgene was removed using SpeI (New England Biolabs, #R0133) and XhoI (New England Biolabs, #R0146), being GFP or Luc inserted into these sites respectively. To delete the miRT sequence and obtain pSVProI, the enzyme AscI (New England Biolabs, #R0558) was used and generating this plasmid.
Afterwards, plasmids have been sequenced to confirm the correct insertion of the transgenes and the absence of mutations.

b. DNA plasmid amplification

Transformation of Stble3 bacteria

50ng of DNA was added to 50µl of Stble3 (Invitrogen, # C737303) competent bacteria for transformation. After incubation for 30 minutes on ice, a heat-shock at 42°C was performed for 45 seconds. Then, 450µl of Luria Broth (LB, Sigma-Aldrich; #L3022) medium was added and the suspension was incubated for 30 min at 37°C shaking. 100µl of bacterial suspension was plated on LB plates containing 1mg/ml ampicillin). Plates were incubated at 37 °C overnight.

Purification of DNA plasmids

For preparation of plasmid containing bacteria, single colonies were hand-picked and transferred to 5ml LB medium containing 1mg/ml ampicillin. Tubes were incubated shacking at 37°C overnight. Plasmid purification was performed according to the manufacturer´s instructions (Qiaprep Spin MiniPrep kit (Qiagen; #27106).

DNA plasmid amplification

Once the plasmids have been corroborated by restriction enzymes digestion, the positive clones were used to inoculated 500ml of LB medium containing 1mg/ml ampicillin and incubated at 37°C overnight shaking. Then, purification of plasmid DNA was performed according to the manufacturer´s instructions (Qiagen Plasmid Maxi Kit (Qiagen, #12963). DNA concentration was measured using NanoDrop (NanoDrop 2000c; Thermo Scientific).

Transfection of culture cells

To assess expression levels of pSVProI and pSVPPI, 293T were seeded in 12 multiwell plates and transfected using lipoD293 (Signagen, #SL100668) with these plasmids, including phppINS and pSVGFP according to the manufacturer´s instructions. Four hours post-transfection, media was replaced and cells incubated for 48 hours. Twelve hours prior to the end of the experiment, the proteasome inhibitor MG132 (Sigma-Aldrich, #M8699) was added to the cells at 10µM. Then, protein isolation was performed.

In the case of immunocytochemistry, 293T cells were seeded in 12mm diameter glass coverslips in 24 multiwell plates. Then, cells were transfected using lipoD293 with pSVPPI, pSVProI, phppINS and pSVLuc plasmids according to the manufacturer´s instructions. Four hours post-transfection, media was replaced and cells incubated for 48 hours for immunocytochemistry.

In order to evaluate whether the presence of miRT 142·3p can prevent the expression of the transgene in APCs and induce AST, the expression levels were assessed in different cell lines both hematopoietic and non-hematopoietic. HepG2, Raw 264.7 and Jurkat cells were seeded in 24 multiwell plate and 24 hours after, transfected with pSVGFP or pSVGFP-142·3p using Lipofectamine 2000 (Invitrogen, #11668-030) according to the manufacturer´s instructions. Intensity and percentage of GFP positive cells were measured through flow cytometry FACSCalibur (BD Biosciences) 48 hours post-transfection.

To validate if increasing amounts of LRH1 can enhance SHP promoter activity in the SHP/EFα HepG2 cell line, and, consequently increase the amount of GFP, they were seeded in a 24 multiwell plates and transfected with increasing amounts of pCMV-LRH1. Increasing amounts of plasmid was used to transfect these cells with Lipofectamine 2000 according to the manufacturer´s instructions. Four hours post-transfection, media was replaced. Intensity and percentage of GFP positive cells were measured through flow cytometry using FACSCalibur at 72 hours post-transfection.

Viral vector production

a. SV40 vector production

For the production of SV40 vectors, SuperVero cells were used. This procedure was carried out by Amarna Therapeutics B.V. In brief, SV40 plasmids were digested with NotI (New England, #R3189S) to remove the bacterial backbone. Then, these plasmids were isolated from agarose gels and re-circularized using T4 ligase (New England Biolabs, #M0202). SuperVero cells are transfected with these plasmids and viral vectors are harvested 72 hours post-transfection. This vector-containing media is used to subsequent transduction rounds. At least two cycles are performed using MOI 400. After harvest, vectors are cleared and concentrated by ultracentrifugation, and stored at 4°C until their use.

b. SV40 quantification

DNA was isolated from liver tissue using phenol:chlorophorm (Sigma-Aldrich, #77617). DNA concentration was measured using a spectrophotometer (NanoDrop1000; Thermo Scientific). 600ng corresponding to 10^5 cells was used to quantify the number of SV40 copies per cell. For the determination of number of vector particles, quantitative Taqman PCR was performed. The structural gene VP2 was used to determine the copy number (using the proof with the sequence:

5´- AATTGCTGCTATAGGCCTC-3´)). Samples were analyzed in duplicates, using Taqman mix (Applied Biosystem, #4304437).

c. Lentiviral vector production

The DNA sequence corresponding to the human SHP promoter was ordered and assembled from PCR products. The fragment was cloned into the pMA (ampR) plasmid (Life technologies) using KpnI (New England Biolabs, #R0142S) and SacI (New England Biolabs, #R0156S) cloning sites, obtaining the pMA-SHP plasmid.

To construct the vector LV-SHP-GFP, the plasmid pMA-SHP was digested using the restriction enzymes EcoRI (New England Biolabs, #R0101S) and BamHI (New England Biolabs, #R0136S). A band of an approximated size of 594 base pairs was recovered from the agarose gel and the DNA was isolated. The fragment was cloned into the lentiviral plasmid backbone CMV-GFP-WPRE that was previously digested with the restriction enzymes EcoRI and BamHI to release the CMV promoter. The result was the vector LV-SHP-GFP in the plasmid form. In the case of LV-EF1α-DsRed vector, plasmid construct was kindly provided by Dr. Francisco Martin (GENYO, Granada).

For the lentiviral production, these plasmids were cotransfected with the pCMVDR8.91 and pMDG. To do so, 293T cells were seeded in a 10cm Petri dish and transiently transfected 24 hours after using LipoD293 according to the manufacturer´s instructions. Plasmids used for transfection were: 1) 7.5µg of pSHP-GFP or pEF1α-DsRed, 2) 5µg of pCMVDR8.91 and 3) 2.5µg of pMDG to each plate. Four hours after transfection, media was replaced. Lentiviruses were harvested 48 and 72 hours post-transfection. Media was centrifuged for 5 minutes at 4°C at 1500rpm and filtered using 0.45 µm PVDF filters Millex- HV (Merk Millipore, #SLHV033RS) and concentrated by ultracentrifugation in an Optima L-100K ultracentrifuge at 22000rpm for 90 minutes at 4ºC in a swinging bucket rotor SW-28 (Beckman-Coulter). Pellets were resuspended in serum-free DMEM and viral particles were distributed in aliquots and stored at −80 °C.

d. Titration of lentiviral vectors

To determine the quantity of the lentiviral vector production, $5x10^4$ 293T cells were seeded in 24 multiwell plates. Increasing volume of the lentiviral suspension was added to the wells 24 hours post-seeding. Cells were harvested 72 hours post-

transfection to determine the percentage of GFP or DsRed positive cells by flow cytometry. To determine the titer, the number of positive cells was considered as well as the volume added to each well. Average of the different conditions was calculated.

Q-RT-PCR

a. RNA isolation

RNA from 293T cells was isolated using the RNeasy mini kit (Qiagen, #74106) according to the manufacturer's instructions. An intermediate treatment with Turbo DNAse (2U/µg RNA) (Ambion, #AM2238) for 30 minutes at 37ºC was included to remove traces of DNA contamination.

In the case of primary intraperitoneal macrophages, RNA was extracted using the commercial kit RNeasy Micro kit (Qiagen, #74004) according to the manufacturer´s instructions. RNA concentration was measured using a spectrophotometer (NanoDrop1000; Thermo Scientific). An Agilent 2100 Bioanalyser was used to determine the RNA integrity number (RIN). RNA samples with 260/280 ratios in the range of 2.0 to 2.2 and RIN 7-10 were selected for further analysis.

b. Complementary DNA synthesis:

To synthesize the cDNA, 1µg of total RNA was used in the case of the cell lines, and 600ng from the primary macrophages. These RNA samples were converted into cDNA using random hexamers (10x concentrated) (Roche, #11277081001) and Superscript II Reverse transcriptase with a concentration of 50U/µl (Invitrogen; #18064-014) following the manufacturer's instructions.

c. Q-RT-PCR

Quantitative PCR was performed using an ABI 7000 Sequence Detection System (Applera Europe) and using the FastStart Universal SYBR Green Master (Roche, #04913850001). The reactions were performed in duplicate for each sample, and normalized to the housekeeping gene, cyclophylin in the case of samples from human cell lines and β-actin for the murine primary cells. Primers are specified in table I.

	Forward	Reverse
B7	CAAACAACAGCCTTACCTTCGG	GCCTCCAAAACCTACACATCCT
hSHP1	ATCCTCTTCAACCCCGATGT	TCCAGGACTTCACACAGCAC
hLRH1	CCGACAAGTGGTACATGGAA	TCCGGCTTGTGATGCTATTA
hCYCLO	CCATTTGTGTTGGGTCCAGC	TACGGGTCCTGGCATCTTGT
hPROI	AGGCTTCTTCTACACACCCAAG	CACAATGCCACGCTTCTG
mβ-actin	GCTCACCCTTACCTGGAACA	GGACCAGATCCAAAAGGACA
SV40	ACTTGCATCTGTTGCTACTGTTGA	GCCCCAGATATCACAGCATAGG
mIL-10	CAGAGCCACATGCTCCTAGA	GGCAACCCAAGTAACCCTTA
mTGFB	GCCCTGGATACCAACTATTGC	AAGTTGGCATGGTAGCCCTT
mLRH1	ATGCCCTCTGACCTGACCATT	GGTTCAGAGGTAGGCCTTTGG
WPRE	CGCTGCTTTAATGCCTTTGT	GTTGCGTCAGCAAACACAGT
DsRed	CGACATCCCCCGACTACAAAGA	TTCACGCCGATGAACTTCAC

Table I. Primers used to perform the PCRs.

Protein isolation and Western Blot

For protein isolation, cells at 90% confluence were washed with PBS and frozen in liquid N_2. Then, cells were collected in RIPA buffer (Sigma-Aldrich, #R0278) containing 1X Protease Inhibitor Cocktail Tablets SigmaFast (Sigma-Aldrich, #S8820), incubated for 1 hour at 4°C with rotation and centrifuged at maximum velocity for 5 min at 4°C. Protein content of the supernatant was determined using the Quick Start Bradford 1X Protein Assay Kit (Bio-Rad, #500-0205) at 562 nm.

Then, 20µg of total protein was mixed with dithiothreitol (DTT) (Sigma-Aldrich, #43816) for a final concentration of 20mM and 1x loading buffer (250mM TrisHCl pH 6.8, 0.5% bromophenol blue/5% SDS/50% glycerol). Samples were heated at 95°C for 5 min and separated by 10% SDS-PAGE for 1 hour at 110mV. Afterwards, proteins were transferred by semi-dry transference to a PVDF membrane (Gibco, #RPN303F) using TransferBlot Turbo (BioRad). After that, the membrane was blocked with 5% Bovine serum albumin (BSA) (Sigma-Aldrich, #A3294) in TBST (NaCl 0.19 M/Tris-HCl pH 7.5 0.05M/0.1% Tween 20 (Sigma-Aldrich, #P2287)) for 1 hour at room temperature and subsequently incubated overnight at 4ºC with corresponding primary antibodies diluted in 3% BSA in TBST. Primary antibodies used are: 1:200 for rabbit polyclonal anti-insulin (Santa Cruz Biotechnology, #SC-9468), 1:1000 for goat polyclonal anti-GFP (Abcam, #ab6673), 1:5000 for mouse monoclonal tubulin (Sigma-Aldrich, #T9026), 1:200 for mouse anti-SV40 Tag (Abcam, #ab16879), 1:5000 for rabbit anti-VP1

(Abcam, #ab53977) and 1:5000 for mouse anti-GAPDH (Millipore, #ab2302). After primary antibody incubation, the membranes were washed with TBST and incubated with the corresponding HRP secondary antibody diluted in 1% BSA in TBST for 1 hour at room temperature. Secondary antibodies used are: anti-goat IgG peroxidase conjugated at 1:5000 (Sigma-Aldrich, #B8520), anti-rabbit IgG peroxidase conjugated at 1:5000 (Sigma-Aldrich, #A0545) and anti-mouse IgG peroxidase conjugated at 1:5000 (Sigma-Aldrich, #A9044). After washing, immunodetection was performed using the ECL Western Blotting SuperNova detection reagent (Cyanage, #XL53) by chemiluminescence (Chemidoc MP Imaging System).

Pancreas immunohistochemistry

Mice pancreas were fixed in 4% paraformaldehyde overnight at 4°C. After washing in PBS, the pancreases were dehydrated, embedded in paraffin and sectioned into 5µm slices at the Histology platform at CABIMER. Sections were mounted on SuperFrost Plus slides (Menzel-Glaser). Every 10 sections, one slide was stained with hematoxylin-eosin.

Sections heated at 60°C were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol, followed by several washes in water (Xylene 5min/2x; Ethanol 100% 1min/2x; Ethanol 90% 1 min; Ethanol 80% 1 min; Ethanol 70% 1 min; water). Then, sections were subjected to antigen retrieval using 10mM sodium citrate buffer (pH 6.0) in the autoclave (AES-8 Trade Raypa) and cold down for 20 minutes at room temperature. After washing twice with PBS and once with PBS/ 0.5% Triton X-100 (Sigma-Aldrich, #T8787), blocking was performed with PBS/0.1% Triton X-100 containing 3% BSA for 1 hour at room temperature. Primary antibodies were diluted in PBS/ 0.1% Triton X-100 containing 3% BSA and incubated for 1 hour at room temperature in a humid chamber. Concentrations of antibodies used were: 1:200 for rabbit polyclonal anti-insulin (Santa Cruz Biotechnology, #SC-9468) and 1:200 for mouse monoclonal anti-glucagon (Sigma-Aldrich, #G2654). After washing with PBS, sections were incubated with the corresponding secondary antibodies at 1:800 diluted in PBS/0.1% Triton-100 for 1 hour at room temperature in dark. Secondary antibodies used were: Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, #A11008) and Alexa Fluor 568 goat anti-mouse IgG (Invitrogen, #11004).Nuclear counterstaining was performed by DAPI at 1µg/ml (Sigma-Aldrich, #D9542) after 5 minutes of incubation, and sections were mounted using DAKO fluorescent mounting medium (Dako Diagnostics; #S3023).

Immunocytochemistry

Samples were fixed in 4% paraformaldehyde (Sigma-Aldrich, #P6148) for 10 min at room temperature. Then, permeabilization was performed using ice-cold methanol 100% for 2 minutes at -20ºC. After three washes with PBS/0.1% Tween 20, cells were exposed to rabbit polyclonal anti-insulin (Santa Cruz Biotechnology, #SC-9468) at 1:200 diluted, rabbit polyclonal anti-LRH1 (Abcam, #ab189876) or 1:200 for mouse anti-SV40 Tag (Abcam, #ab16879) in 3% BSA/0.1% Tween 20 in PBS for overnight at 4°C. Controls without primary antibodies were used for each experiment. Afterwards, to remove the excess of antibody, coverslips were washed three times in PBS/0.1% Tween 20 and incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, #A11008) or with Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, #A-11001) both at 1:500 in PBS/0.1% Tween 20 for 1 hour at room temperature in dark. Samples were then washed three times with PBS/0.1% Tween 20 for 5 min each. Nuclei staining was performed using 1µg/ml DAPI for 5 min at room temperature. Coverslips were mounted using DAKO fluorescent mounting medium and left to dry overnight at 4°C.Fluorescence microscopy images were acquired using a Leica DM6000B microscope (Leica Microsystem).

MTT assay

Ins-1E cells were plated in triplicates in 96 multiwell plates. On the next day, cells were cultured with fresh RPMI media containing various concentrations of compounds at 0.05% DMSO. Cellular viability were assessed by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay (Roche, #11465007001) following the manufacturer´s instructions after 24 or 48 hours post-treatment. The percentage of cell viability was calculated relative to untreated cells.

Proliferation assay

HepG2 cells were plated in 24 multiwell plates. One day after seeding, cells were treated at different concentrations with BL002 or BL003 at 0.05% of DMSO. Then, 48 hours after treatment, proliferation of cells was assessed using BrdU proliferation ELISA assay (Roche, #11647229001) according to the manufacturer´s instructions. The proliferation index was calculated relative to untreated cells.

Cell death assay

HepG2 cells were seeded in 24 multiwell plates. On the next day, cells were treated at different concentrations of BL002 or BL003 at 0.05% DMSO. Cell death ELISA (Roche, #11544675) assay was performed after 48 hours of treatment according to the

manufacturer´s instructions. The cell death index was calculated relative to untreated cells.

For the cytokines-induced apoptosis in primary mouse islets, after the treatment with BL002 or BL003 as well as the cytokines, cell death ELISA assay was performed according to the manufacturer´s instructions. The cell death index was calculated relative to non-cytokines-treated islets.

RTCA assay

HepG2 cells were plated in a 16 e-multiwell plate and incubated at 37°C using the X-cellygence (ACEA Bioscience). Twenty four hours after seeding, different range of concentrations of BL002 or BL003 were used, measuring the cell index constantly until the end of the experiment. Cell index relative to time point 0 hours, a quantitative measure of the status of the cells in an electrode-containing well, was calculated.

V. RESULTS

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VI. DISCUSSION

T1DM is an AD with an alarming increasing prevalence. During the development of this disease, the autoimmune destruction of the insulin producing β cells leads to a severe condition in which insulin deprivation causes a hyperglycemia with dangerous consequences. Currently the only therapy is based on the administration of insulin or insulin analogs upon a continuous surveillance of blood glucose levels. However, this is a palliative therapy that does neither cure the disease nor prevent secondary complications, leading to a severe health condition to the patients. Thus, the development of novel effective therapies is imperative. Because the intrinsic cause of T1DM as other ADs is the breakdown of the tolerance, the ideal therapeutic treatment must follow the restoration of the tolerogenic environment. In this thesis, two different approaches have been presented: a SV40-based gene therapy and a pharmacological therapy targeting LRH1.

1. Induction of antigen specific tolerance in the transgenic RIPB7.1 mouse model by delivery of the insulin gene using SV40-derived vectors

The delivery of self-Ags under non-inflammatory conditions is effective to restore the broken tolerance (if administered after the disease has started) or to prevent it (if administered before the disease has started) [152,153]. This is because this procedure resembles the clonal deletion process mediated by thymocytes in the "central tolerance" and the Ag presentation by APCs happening in the "peripheral tolerance" [237]. The key question is to find gene delivery vectors that do not induce an immune response by their selves, and therefore, do not induce an inflammatory process instead of a tolerogenic one. In humans, on the contrary as it can be done in preclinical models, AD can only be treated once they have started, because currently there are not available markers that, with 100% accuracy, can predict an imminent development of an AD in a person. This circumstance makes that auto-reactive T cell clones have been already activated. They are more sensitive to the presence of the Ag than naïve T cell clones, and therefore, easier to re-activate and more difficult to inactivate. Therefore, a completely inert or non-immunogenic vector is needed, to ensure the success of the AST induction in humans.

The lack of immunogenicity of SV40 shown by others and by us [238–240], pinpoints at this vector as the optimal candidate to be used to treat ADs by restoring the AST [241]. We have overcome the main problem that avoided the use of SV40-derived vector particles in the clinic[204,242–244], by the generation of SuperVero. SuperVero cells represent the only SV40 packaging cell line able to produce safe replication deficient SV40 vector particles, without the risk of contamination with recombination competent SV40 vectors [206]. SuperVero derives intentionally from Vero cells, which are permissive to SV40, and also they are accepted by the World Health Organization for human vaccines production [245]. The expression only of the LTag in SuperVero is sufficient for the replication and packaging of SV40 vector particles being enough to activate the transcription from the viral late promoter, resulting in the accumulation of capsid proteins as well as the SV40 vector production. The absence of STag improves the safety due to the reduction of SV40 viral genome necessary to produce the particles without interfering the high production of SV40 particles. The generation of SuperVero may represent a significant step forward in the treatment of ADs, as soon as the SV40 vector particles are proven to be effective for such end.

According to previous publications[197–199,225], our results also show that the *in vivo* administration of SV40 vectors does not interfere with the regular function of the liver, and more importantly, does no induce the activation of the immune system. The lack of immunogenicity resides in two main mechanisms. First, the pathway that SV40 particles follow during the entrance [196] that avoids the proteasome degradation and the MHC presentation, and second, the lack of the presence for long time the viral capsids in the target cells, as it has been described to happen for other vectors [246]. The absence of finding SV40 neutralizing antibodies in treated mice after the vector administration is in favor. We would agree that before to reach the clinical studies, more exhaustive toxicological/immunological experiments should be performed, but these are advantages that other gene therapy vectors such as AAV-based vectors do not display. Thus, the presence of AAV neutralizing antibodies in most of the half of the human population reduces the number of eligible candidates to be treated with AAV vectors[191]. Or as it has been done in some cases, a strong regime of immunosuppressors has been co-administered to the patients together with the vector particles, to prevent the immune response toward the vectors [247,248]. SV40 particles will avoid these problems, allowing also their re-administration, for those cases that it would be needed, adding another advantage over AAV.

The induction of AST does not need a continuous expression of the self-Ags [122,149,249,250]. Probably, the longer the Ag presentation the longer the risk, as an

independent inflammatory process happening at the same time could invert the beneficial effect of the self-Ag presentation from a tolerogenic restoration into an inflammatory relapsing stage. Thus, if the vector used is able to keep the expression of the Ag for a certain period of time would be suitable to efficiently induce AST. SV40 viral particles remain as episomes in infected cells[194,200,251] and the SV40-derived vector particles do the same. We show here that the duration of the expression of a gene (Luciferase) delivered by intravenous administration of SV40 particles is temporary. Others also have shown similar results with SV40, although the detection of the transgene was more prolonged than in our case, a circumstance that can be due to the dose or efficacy at the time of administration [225].

These results are interesting because the treatment of AD, compared to other diseases treated by gene therapy, includes a layer of complexity, which is based in what we can call: “opportunity”. The immune attack in ADs use to progress in “waves”, that is because usually the patients show relapsing-remitting stages. Therefore, the way, when and for how long the Ag is presented is crucial. Preclinical studies have shown that in some cases, a long-term expression of the Ag is necessary to induce AST[122,137,140,149,252] meanwhile in others, only a brief expression is enough[249,250]. This reveals the complexity of this therapy and the difficulty to find an effective protocol that can be translated into humans. Probably, the transient expression of Ags that could be provided by SV40 vectors may be enough to induce AST. To validate our hypotheses we expressed the selected self-Ag from the plasmid form to resemble what others did, but in the SV40 vector background.

As a model of T1DM we have used the RIPB7.1 mice, since this model provides more flexibility and consistency at the time of inducing the hyperglycemia[123,220,253,254]. Furthermore, what makes also this model very interesting is, as we have shown here, the use of human insulin to induce the development of the hyperglycemia. The degree of homology between the human and mouse preproinsulin II genes is very high, and then, this was reasonably expected. This circumstance allowed us to use the human version in our vectors, giving us the chance to validate our AST approach with the same construct that would be used in future clinic trials. However, these mice develop a strong immune attack that could cover the real effect of the AST therapy. For this reason, small decreases in the percentage of mice that develop hyperglycemia could mean a great therapeutic benefit.

Two different insulin variants have been considered for the AST: preproinsulin and proinsulin[147,152,255,256]. The difference between these variants is the presence of the signal peptide in the preproinsulin, which determines targeting the Ag to the ER, to be secreted. Probably, its secretion facilitates the presentation of insulin by the APCs

through the MHC II, previous its phagocytosis. Align with our hypotheses was the first positive outcome, to observe that insulin expressed in the SV40 background did not induced EAD in the RIPB7.1 mouse model. This was probably due to a lower expression levels of insulin, compared to the insulin-expressing plasmid used to induce EAD. This is consistent with a previous publication where a low Ag dose does not trigger immunogenicity but tolerance[257]. This may prelude that SV40 are safe, since a high Ag expression could trigger the activation of the immune system aggravating the situation of the patient. T1DM patients can present a susceptible state similar to the RIPB7.1, which only develop EAD after the administration of the high insulin expression plasmid and a ten times reduction of DNA avoids the development of EAD [122,258].

As it was shown by using other backbone plasmids[149], insulin expressed in the background of SV40 can also induce AST, when administered by the intramuscular route. In fact, we resembled others results also in the way that we decided to express insulin, as preproinsulin or proinsulin, corroborating that the expression of proinsulin was more effective than preproinsulin to induce AST, following this procedure. It was unfortunate that the results were not equally positive when both Ags were delivered to the liver, in a try to harness its great tolerogenic potential [172,173,259]. We could not analyze whether the Ags were being expressed properly *in vivo*, but a plausible reason about the fail to induce AST could be the capture of our insulin expressing plasmids by APCs and for some reason presented them under some pro-inflammatory environment [252,260], because when we avoided the expression of the Ags in the hematopoietic cells by adding the micro-RNA targeting sequence miRT-142·3p, our vectors efficiently induced AST, via liver administration. Our results are consistent with previous results that also showed the used of the miRT-142·3p to induce AST [261], and others that described that a low Ag expression in hepatocytes or liver endothelial cells can induce an anti-inflammatory environment promoting tolerance [262–264].

Here we show that genes carried by SV40 vectors can be efficiently expressed in liver, without inducing cytotoxicity, and more importantly, without inducing immunogenicity against the vector particles. Contrary to what happens with other viral vectors, these results mean that SV40 vector particles could be administered repeatedly, without detrimental circumstances neither for the patient or the therapeutic treatment. Equally relevant is that we show for the first time that self-Ags delivered and expressed in the background of SV40 are not able to initiate an autoimmune process, and that when the SV40 vector background is customized with sequences that target the expression to particular cells different from hematopoietic cells, AST is efficiently induced by upon liver administration. Now the challenge is to obtain proof of principle of SV40-

expressing insulin vectors as a treatment for EAD in the RIPB7.1 mouse model. These results, together with the availability of SuperVero packaging cells, that make possible the use of SV40 vectors in the clinic, we may predict that SV40 vector particles will make their niche in the gene therapy field.

2. LRH1 as a novel target to treat T1DM: Analysis of the effect of LRH1 agonist BL001 in the innate immune system and screening for a second generation of LRH1 agonists

For the development of a successful T1DM therapy, the balance between regulatory and self-reactive effector T cells should be reestablished as well as the β cell mass should be regenerated. Thus, identifying new targets that could combine both effects is necessary to design innovative drug-therapies. Here we propose LRH1 as a target for new T1DM pharmaceutical therapies. The overexpression of LRH1 protects islets from cytokines and streptozotocin-induced apoptosis, revealing the protective role of this factor[213]. This nuclear receptor can be activated by ligands, which allows a pharmacological treatment for its activation. Remarkably, endogenous and newly synthesized LRH1 agonists have been deciphered as anti-diabetic drugs [218,220,265]. Our previous data document that the administration of the LRH1 agonist BL001 reduces the incidence of hyperglycemia in different T1DM mouse models, preserving the β cell mass and inducing an anti-inflammatory environment [220]. Hence, this compound primes macrophages towards the immunosuppressive and tissue remodeling M2 phenotype resulting in an increased IL-10 expression and inducing the expansion of Tregs [220], which are essential in maintaining the self-immune tolerance [266]. Even in the presence of the strong pro-inflammatory LPS, BL001 is able to modulate the phenotype of these cells maintaining the expression of the anti-inflammatory cytokine IL-10, demonstrating its capacity to modulate the immune system [220]. Supporting the action of BL001 as an agonist of LRH1, BL001-treated LRH1-silenced primary macrophages cannot increase the secretion of this cytokine. Remarkably, the benefits of this compound are not only restricted to the immune system. BL001 can also increase the dual hormone glucagon/insulin cells in immunized mice by reducing the α cell markers, suggesting the transdifferentiation of α cells into β cells in an attempt to compensate the loss of β cell mass without a significant effect on cell proliferation [220]. Probably, Tregs and M2 macrophages contribute to this α-to-β cell trans-differentiation, considering these subsets of cells key players in tissue remodeling in muscle, bone and vasculature, promoting cell differentiation and expansion [267–270].

However, a drawback of BL001 for its use in the clinic is that BL001 is a lipophilic molecule that does not comply with the four rules of Lipinski limiting its human applicability. Therefore, novel compounds that can act as agonists of LRH1 similar to BL001 but with hydrophilic properties that fulfil the Lipinski rules need to be identified. For being able to analyze these compounds, we have generated a new and quick method to screen and test novel LRH1 agonists. This assay is based on HepG2 cell line stably transduced with two fluorescence reporter transgenes: one of them regulated by activated LRH1 (using SHP promoter) and the other one with constitutive expression (using EF1α promoter). The analysis of the fluorescent proteins is performed by flow cytometry that allows the combination of different fluorescence signals at the same time, being able to analyze simultaneously different promoters (SHP and EF1α). The selection of compounds was based on their capacity to activate the endogenous LRH1 expressed in HepG2 cells, which can mimic the *in vivo* situation, where no overexpression of LRH-1 can be induced. The use of this platform facilitates the screening of new LRH1 agonists, being the only limitation the time to detect the fluorescent proteins, which we have demonstrated is optimal at 72 hours post-treatment. Thus, considering the "unlimited" resource of a cell line, numerous compounds can be analyzed at the same time with a low cost and in a simple manner. Moreover, unlike previous reporter assay analysis, generally using transient transfections [233,271], the use of a stable cell line can produce more reproducible results, allowing the comparison between the different compounds tested. The validation of the SHP/EF1α HepG2 cells using BL001 determined the basal fluorescence level to select novel LRH1 agonists. Interestingly, overexpression of LRH1 itself do not reach the same fluorescence intensity than after BL001 treatment. A possible reason for this can be that the natural ligands of LRH1 present in the cells, the phospholipids, cannot increase the activity of this transcription factor as strong as BL001. Using this screening platform the novel LRH1 hydrophilic agonists have been compared to BL001, revealing two novel compounds with an improved activity: BL002 and BL003.

Besides the validation of their function, in our case as agonists of LRH1, for the development of new pharmaceutical compounds is important to study their potential toxicity, which is one of the most important causes of drug rejection in later stages of drug development. The toxicology studies that we have performed indicate that both, BL002 and BL003, are non-toxic at the doses required for the activation of LRH1, however BL002 at high doses, but not BL003, can impact negatively on cell growth. This difference between these two compounds could be due to the activation potency of BL002, which at 25μM not only increases the GFP fluorescence, but also increases the DsRed levels. These data suggest that high concentration of BL002 could activate

unspecific promoters or even induce toxicity, although further experiments are needed to analyze the specificity of this compound.

Previous reports have suggested a role of LRH1 in some tumours [272], however, the absence of increased proliferation after treatment with the BL compounds, together with our previous studies using BL001 both *in vitro* and *in vivo* [220] have questioned the tumorigenesis risk. However, further experiments are needed to corroborate the safety of the novel BL compounds. Based in our *in vitro* results with these compounds, we expect that the *in vivo* administration of BL002 and BL003 will act similarly to BL001, which after 6 months of diary administration does not induce tumour development [220]. Similar to the protection against cytokines and streptozotocin-induced apoptosis conferred by the overexpression of LRH1 [213] as well as by the activation of LRH1 by BL001 [220], the novel BL compounds protect mouse islets from cytokines-induced apoptosis. Remarkably, when compared to BL001, 100 times lower doses of BL002 and BL003 were enough to protect mouse islets from apoptosis, demonstrating their stronger effect. In the case of BL002, the lowest doses correlate the highest protection, being abrogated when the doses are increased, which are toxic for the cells. Probably, the reduction of cell death that we have observed at 25µM (but not at 12µM) is an artefact due to few cells surviving after the treatment. This is consistent with the BL002 basal cell death of islets observed at 12µM, which can even increase the cell death. Nevertheless this hypothesis needs to be corroborated. Interestingly, although in the screening platform we only detected the LRH1 activation at high concentrations (with apparently no effect with lower than 6 µM BL002), the highest protection against apoptosis was observed at lower values. This demonstrates the necessity of testing the compounds *in vivo,* since the concentration to identify the agonists in the stable cell line could be much higher than the concentration beneficial in a physiological scenario such as islets. Probably, it is not required a high activation of LRH1 *in vivo* to induce protection. However, further experiments are necessary to corroborate the *in vivo* validation and the mechanism of action. As a counterpart, BL003, which previously has been shown to be less toxic, protects mouse islets at all the concentrations tested and can even improve the survival of mouse islets reducing the basal cell death. Despite the requirement of further analysis *in vivo*, considering the encouraging results obtained with BL001, these novel compounds show an strong potential for their use in T1DM treatment. The absence of either toxicity or proliferation induction, as well as the wide range of concentrations able to protect pancreatic islet from apoptosis designate BL003 as a good candidate for a future T1DM therapy.

Here, a screening platform is presented with the objective of testing novel LRH1 agonists. As a result, an improved generation compounds of BL001 is presented emphasizing two of them, BL002 and BL003. These compounds can reduce the doses necessary for the protection of β cells by an enhanced binding affinity to LRH1 than BL001. Although *in vivo* experiments need to be performed, these results can augur their application as T1DM treatment.

VII. CONCLUSIONS

1. SV40 vectors as a tool to induce AST in a T1DM mouse model

1. The establishment of the packaging cell line SuperVero in combination with the absence of an immune response to vector particles will allow the safe use of SV40-derived vectors in the clinic
2. The use of human preproinsulin to induce EAD in the transgenic mouse model RIPB7.1 increases the translational value of this model
3. Intramuscular expression of self-antigens under the transcriptional control of SV40 early promoter induces immune tolerance to the self-antigen
4. By preventing expression of self-antigens by hematopoietic cells, upon intravenous administration of self-antigen-expressing SV40 vectors, the induction of inflammation is avoided and instead the induction of antigen specific tolerance is improved

2. LRH1 as a novel target to treat T1DM: Analysis of the effect of LRH1 agonist BL001 in the innate immune system and screening for a second generation

1. BL001 is capable of inducing IL10 production in peritoneal macrophages but not TGFβ
2. Silencing of LRH1, prevents induction of IL10 by BL001, validating the specificity of the BL001/LRH1 signaling pathway.
3. The generation of a cell-based screening platform to identify compounds that induces the SHP promoter is a valuable tool for the development drugs with potential anti-inflammatory effect
4. The preliminary *in vitro* profiles of the compounds BL002 and BL003 in terms of toxicology and islet protection, prelude their therapeutic benefit at the moment of being tested in the experimental autoimmune diabetes preclinical model

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