



available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim



REVIEW

Novel diagnostic and therapeutic approaches for autoimmune diabetes — A prime time to treat insulinitis as a disease



Juha Grönholm, Michael J. Lenardo*

Molecular Development of the Immune System Section, Laboratory of Immunology, NIAID Clinical Genomics Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Received 10 October 2014; accepted with revision 22 November 2014
Available online 5 December 2014

KEYWORDS

Autoantibody;
Insulinitis;
Type 1 diabetes;
Tolerance

Abstract Type 1 diabetes is a progressive autoimmune disease with no curative treatment, making prevention critical. At the time of diagnosis, a majority of the insulin secreting β -cells have already been destroyed. Insulinitis, lymphocytic infiltration to the pancreatic islets, is believed to begin months to years before the clinical symptoms of insulin deficiency appear. Insulinitis should be treated as its own disease, for it is a known precursor to autoimmune diabetes. Because it is difficult to detect insulinitic cellular infiltrates noninvasively, considerable interest has been focused on the levels of islet autoantibodies in blood as measurable diagnostic markers for islet autoimmunity. The traditional islet autoantibody detection assays have many limitations. New electrochemiluminescence-based autoantibody detection assays have the potential to overcome these challenges and they offer promising, cost-effective screening tools in identifying high-risk individuals for trials of preventive interventions. Here, we outline diagnostic and therapeutic strategies to overcome pancreatic β -cell destroying insulinitis.

© 2014 Elsevier Inc. All rights reserved.

Contents

1. Introduction	110
2. Autoantibodies in diagnosis of insulinitis	111
2.1. Autoantibody tests — conventional technologies	111
2.2. ECL assay as a next generation approach to measure islet specific autoantibodies	111

* Corresponding author at: Molecular Development of the Immune System Section, Laboratory of Immunology, NIAID Clinical Genomics Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 10, Room 11D14, 10 Center Drive, Bethesda, MD 20892-1892, USA.

E-mail addresses: juhapetrikristian.gronholm@nih.gov (J. Grönholm), lenardo@nih.gov (M.J. Lenardo).

3. Insulinitis treatment	112
3.1. Conventional immunosuppressants	112
3.2. New biological approaches	112
3.3. Antigen specific therapy	114
4. Concluding remarks and future perspectives	115
Conflict of interest statement	115
Acknowledgments	115
References	115

1. Introduction

Type 1 diabetes (T1D) is an autoimmune disease in which impaired tolerance to β -cell autoantigens leads to T cell-mediated destruction of insulin-secreting cells from the pancreatic islets of Langerhans. T1D usually manifests during childhood or adolescence. Diabetic individuals are dependent on lifelong exogenous insulin administration. The autoimmune β -cell destructive process usually begins years before hyperglycemic symptoms mark the onset of T1D. This destructive process, called insulinitis, is characterized by immune cell infiltrates into pancreatic islets, which include cells of both the innate and the adaptive immune system [1].

The immunopathology of insulinitis is challenging to study in humans because it requires pancreatic samples from prediabetic individuals. Only 100–200 cases of insulinitis have been described over the past century, most of them found from the post-mortem pancreatic samples from patients already diagnosed with the clinical T1D. A majority of those cases have not been studied with modern methods [2,3]. Immunohistochemistry of post-mortem pancreatic sections from newly T1D diagnosed individuals have revealed CD8⁺ cytotoxic lymphocytes (CTLs) to be the predominant leukocyte population in the islet infiltrates, regardless of the remaining number of insulin positive cells in the respective islets [4]. There is immunohistochemical evidence using in-situ tetramer stainings that these islet infiltrating CD8⁺ lymphocytes are islet-antigen reactive in some of the T1D patients [5]. Furthermore, CD8⁺ T cells are able to directly kill β -cells. HLA class I molecules are hyperexpressed on β -cells at the onset of T1D diagnosis, thus making the cells more susceptible to CTL-mediated cytotoxicity [6,7]. The second most abundant immune cell type within the insulinitis lesions is the macrophages, which are likely to produce inflammatory and chemotactic cytokines that promulgate insulinitis. CD4⁺ T lymphocytes are also found in islet infiltrates, but are less abundant when compared to CD8⁺ T cells. Their role in T1D pathogenesis is not well defined, but it is postulated that CD4⁺ T cells may induce and drive other effectors in the autoimmune process by cytokine secretion or directly mediate islet cell damage through toxic cytokine effects [1,4]. B cells are less prevalent, but the quantity of islet infiltrating B lymphocytes was found to increase when the level of insulin positive cells decreased [4]. In other words, B cells were more abundant in the late stage of insulinitis. Islet specific autoantibodies are characteristic of T1D, but they are not believed to play a major role in β -cell destruction [8]. Instead, B cells may contribute to insulinitis by secreting cytokines and by functioning as antigen presenting cells. Despite several differences in disease pathogenesis, non-obese diabetic (NOD) mice are

considered the best experimental animal model for T1D [9]. In the NOD mouse model, B cells take part in islet pathogenesis by presenting autoantigens to self-reactive CD8⁺ T cells [10]. Remarkably, only a very low number of Foxp3⁺ T regulatory cells and NK cells were found in insulinitis lesions [4].

The consensus criteria for the histopathological characteristics of insulinitis were defined in the meeting of JDRF Network for Pancreatic Organ Donors with Diabetes (nPOD) in 2013. One of the defining characteristics of patients with insulinitis is lymphocytic infiltration specifically targeting the islets of Langerhans. These infiltrating cells may be found in the islet periphery (peri-insulinitis) or may be disperse and present in the whole islet parenchyma (intra-insulinitis). Moreover, the infiltration mainly affects islets that contain insulin-positive cells and is always accompanied by the presence of atrophic islets that are devoid of β -cells. The lesion should be found from at least 3 islets and each insulinitic lesion should contain at least 15 CD45⁺ cells [2].

Insulinitis may not always be found in the pancreatic biopsies from recent onset T1D patients. Imagawa et al. [11] studied pancreatic biopsies from recent onset T1D patients (disease duration of about 3 months) collected by laparoscopic surgery and found that of the 29 patients tested, 17 had T cell predominant immune cell infiltration and increased MHC I expression in the islets of Langerhans either alone or in combination. Although these abnormalities were only observed in 59% of the cases, the true number is most likely higher since only a minute portion of the islets is represented in a single pancreatic biopsy [11]. The heterogeneity of remaining C-peptide levels in diabetic children can be explained by the preservation of insulinitis negative islets. In an additional study with cadaveric organ donors, Gianani et al. [12] found that 30% of T1D patients (with disease for 1–20 years) still had insulin positive β -cells in their islets. The islets that contained β -cells had one of two patterns: pattern A, lobular retention of areas containing abnormal β -cells that expressed the apoptosis inhibitor survivin and overexpressed HLA-Class I; or pattern B, normal appearance with significantly reduced number of β -cells [12]. In a meta-analysis of insulinitis by In't Veld [3], the general finding from all the published histopathological data was that insulinitis is a characteristic lesion in young patients (≤ 14 years) with short duration of T1D (≤ 1 month), whereas insulinitis is often absent after longer duration of the disease and in older patients with recent onset T1D [3]. These findings suggest that the pathogenesis of insulinitis and T1D varies between individuals.

Although the precise molecular process that initiates insulinitis is still unknown, it is apparent that both genetic and

environmental factors play an important role [13]. The genetic risk for T1D among white individuals is linked to HLA DR-DQ risk alleles *DRB1*04-DQA1*03:01-B1*03:02* (DR4-DQ8) and *DRB1*03:01-DQA1*05:01-B1*02:01* (DR3-DQ2), which either alone or in combination are present in nearly 90% of the T1D patients diagnosed before age 18 [14,15]. Protective HLA DR-DQ haplotypes such as *DRB1*15:01-DQA1*01:02-DQB1*06:02*, *DRB1*14:01-DQA1*01:01-DQB1*05:03* and *DRB1*07:01-DQA1*02:01-DQB1*03:03* have also been identified [16].

The incidence of T1D has been increasing in Western countries over the past few decades [17,18]. This must be attributable to environmental triggers such as viral infection, diet, or other influences since changes in allele frequency would not occur that rapidly [19]. It has been postulated that viral infection may trigger islet inflammation and β -cell destruction through pattern-recognition receptor activation. The inflammation is further amplified by cytokine and chemokine release from dying β -cells. Viruses may also initiate T1D through molecular mimicry by activating lymphocytes that recognize viral epitopes similar to β -cell specific autoantigens. After the viral infection has been eliminated, these activated lymphocytes can cause chronic autoimmune responses [20]. In a recent study in European populations, the coxsackievirus B1 (CVB1) subgroup was associated with T1D [21,22].

Development of new diagnostic tools that detect early onset of autoimmunity prior to insulin deficiency coupled with novel therapies that prevent the pathogenic autoimmune process in the pancreas is needed to overcome the lifelong burden for those who are susceptible to T1D.

2. Autoantibodies in diagnosis of insulinitis

One strategy to facilitate the prevention of diabetes is using a blood test that reliably diagnoses early stage autoimmunity. Typically, patients developing T1D have circulating islet autoantibodies in the preclinical phase of the disease. A correlation between the production and quantity of insulin autoantibodies and the onset and progression of insulinitis has been demonstrated in the NOD mouse model [23]. Autoantibodies detected in human include those specific for insulin (IAA), glutamic acid decarboxylase 65 (GADA), islet antigen-2 (IA-2A) and zinc transporter 8 (ZnT8A) [24,25]. Of these, the IAAs are usually the first islet specific antibodies to be generated and are present in almost all prediabetic individuals [26–28]. Because they provide the earliest signs of islet autoimmunity and can be measured in blood samples, autoantibodies have been extensively studied as biomarkers for prediabetic individuals in several prospective studies [29–32]. Individuals expressing two or more circulating islet autoantibodies combined with a genetic susceptibility allele or family history of the disease are at very high risk for developing T1D [25,32–36]. In addition, early seroconversion and rapid increases in autoantibody titers during childhood are strongly linked to T1D onset before puberty [30]. Insulinitis was found from two out of three non-diabetic adult organ donors (over 25 years of age) who were positive for three or four different autoantibodies and had susceptible HLA-DQ genotype [37]. The HLA-DQ genotype determines the type of correlation to different islet autoantibodies. For example, HLA-DQ8

correlates with T1D in IAA, GADA, and ZnT8A, whereas HLA-DQ2 is correlated with increasing risk of T1D with GADA, but a decreased risk of T1D with IA-2 autoantibodies [14]. Although most T1D patients are positive for IAA, not all IAA positive subjects develop T1D. This has been a challenge in using IAA as a required harbinger of autoimmune diabetes [38]. However, IAAs associated with T1D development recognize (pro)insulin epitopes with higher affinity than those found circulating in subjects who never progress to T1D [39]. Thus, a reliable blood test that can quantitatively measure antibody binding and specificity between different clinical centers could permit the diagnosis of insulinitis prior to diabetes onset.

2.1. Autoantibody tests — conventional technologies

Given the extensive testing of islet autoantibodies, it would appear to be a simple matter of serologically diagnosing insulinitis. However, several limitations have hindered the clinical implementation of this strategy to date. The most widely used traditional method to measure islet autoantibodies is the radioimmunoassay (RIA). The lengthy procedure calls for patient serum to be incubated with radiolabelled autoantigen (for example ^{125}I human insulin for IAA detection) for several days. After the incubation period, immune complexes are precipitated by adding sepharose gel to each sample and washing extensively. The radioactivity of the samples is measured by using a gamma counter. The results are then normalized to a standard curve that is constructed for every assay set using the same batch of radiolabelled autoantigen that was used with the clinical samples. Despite widespread utilization of this technique around the world, it has been hampered by several drawbacks. These include the undesirability of using radioactive materials, complex and time-consuming protocols, requirement of large serum volumes, significant variation in the results between laboratories, and relatively low specificity and sensitivity. A further problem is that these assays detect both disease relevant and non-relevant autoantibodies [40–42]. A micro-scale radio-binding assay was developed in the late 1990s for high throughput IAA screening and its modified versions are currently the most commonly used method for IAA testing [43]. This platform overcomes certain challenges, such as requirement for large serum volumes (the assay can be done from less than 50 μL of serum), but the assay still relies on radioactive labeling of the antigen. After the labeled insulin is bound to the autoantibody, an additional competition assay with unlabeled insulin is required to minimize the background noise. Reproducibility between different laboratories using this assay is poor [41,42]. There are dependable ELISA assays for GADA, IA-2A and ZnT8A, but a reliable ELISA assay for IAA has not been achieved [40,41,44,45].

2.2. ECL assay as a next generation approach to measure islet specific autoantibodies

Recently published novel electrochemiluminescence (ECL)-based assays for IAA and GADA provide a promising approach to overcome some of the limitations listed in Table 1 [23,46,47].

Table 1 Comparison of radioimmunoassay (RIA) and electrochemiluminescence (ECL)-based islet autoantibody assays.

	RIA	ECL
Antibody detection	Unable to distinguish disease-relevant and -irrelevant antibodies	Detects disease-relevant high-affinity autoantibodies, detects disease-relevant autoantibodies earlier in infancy ^a
Detection agent	Time-limited radiolabeled antigens	Non-radioactive sulfo-tagged antigens ^b
Reproducibility	Varies between laboratories	Needs to be studied in different laboratories
Standard curve	Requires a new standard curve for each assay	No need for a new standard curve for each assay

^a This has been shown with ECL assays for IAA and GADA published by Yu et al. [47] and Miao et al. [46], respectively.

^b ECL assays published by Yu et al. [47] and Miao et al. [46] utilizes sulfo-tagged antigen, while ECL-IA assay by Lo et al. [23] uses ruthenium-tagged anti-human IgG.

ECL-based assays have shown high sensitivity, excellent specificity, reproducibility, and flexibility for a wide range of analytes, leading to their usefulness in clinically deployed tests [48]. The ECL human insulin antibody assay published by Lo et al. [23], was found to be specific, reproducible, and highly correlated with RIA results in samples from diabetic individuals. The assay uses biotinylated insulin to pull down antibodies from the patient sera, which are then detected with ruthenium-labeled secondary antibody using an ECL detection instrument. Other recently published ECL assays for IAA and GADA use a different method [46,47] in which the IgG antibody in the serum bridges a biotinylated proinsulin or GAD65 molecule with a sulfo-TAG-labeled proinsulin or GAD65 molecule in fluid phase, respectively. The antigen–antibody complex is captured to a streptavidin-coated plate, followed by the ECL assay. The ECL-IAA assay was shown to be more sensitive and more specific for detecting diabetes-associated autoantibodies when compared to micro-RIA [27,47]. The sensitivity of the ECL-GADA assay was equivalent to that of the standard GADA-RIA, but the ECL assay was more specific [46]. The major advantage of these two assays compared to the traditional RIA is their ability to distinguish between disease-relevant high- and disease-irrelevant low-affinity antibodies. Furthermore, both of these assays were able to detect high-risk autoantibodies earlier in infancy, before the appearance of additional autoantibodies [27,46]. The assays can be performed in a 96-well plate of relatively small volume of patient serum (15 μ l in IAA-ECL assay). Taken together, these results provide a promising step forward towards an effective screening method for individuals at high risk of developing T1D.

3. Insulinitis treatment

These new diagnostics suggest that there may be a non-invasive way of detecting insulinitis and thus, early detection of T1D prior to extensive loss of insulin-secreting β -cells. If an immunosuppressive treatment were initiated early in the process of insulinitis, substantial insulin-producing cell capacity could be preserved. Therefore, treating insulinitis as a disease should serve as a novel prevention strategy for T1D. Next we will discuss some current and possible treatments aimed at preventing or ameliorating insulinitis.

3.1. Conventional immunosuppressants

As current therapies for T1D rely only on treating hyperglycemia, novel therapies targeting the underlying immune

response have now been under intensive investigation [14,49]. Since these therapies are commonly tested in children that already have diabetes, their ability to suppress the early stages of insulinitis that precede extensive β -cell destruction is untested. Furthermore, the extensive destruction of islets associated with clinically apparent diabetes cannot be reversed by simply suppressing the autoimmune process. Thus, it is not surprising that these have had limited impact on diabetes. Former and ongoing trials include several non-antigenic and antigen specific interventions.

Non-antigenic agents include conventional immunosuppressants, such as cyclosporine A, which was one of the first therapeutic agents tested to prevent β -cell destruction in the early 1980s. Cyclosporine A treatment was efficacious in preserving β -cell function in newly diagnosed T1D patients, but the side effects were severe [50,51]. A subsequent study using a lower dose of cyclosporine treatment (7.5 mg/kg/d for the first year, followed by a lower dosage) was tested in prediabetic children that had first-degree relatives with T1D. The metabolic and immunologic criteria used for categorizing prediabetes were positive islet cell antibodies (ICA) $\geq$ 20 Juvenile Diabetes Foundation (JDF) units, first phase insulin response below the 10th percentile, and impaired glucose tolerance. IAA levels were positive in half of the treated patients. After treatment, IAA levels decreased during the first follow-up year but were observed to vary over time. Cyclosporine treatment delayed, but did not prevent T1D onset in this study [52]. However, this study demonstrates how islet cell antibodies can be used to identify prediabetic children for prevention trials, suggesting that suppressing insulinitis may have a beneficial effect.

Another immunosuppressant, azathioprine, produced a significant, but an unfortunately short-lived improvement in fasting plasma C-peptide levels in newly diagnosed type 1 diabetics [53,54]. Both the histamine antagonist Ketotifen and nicotinamide monotherapy were tested as T1D therapeutics. Neither could preserve β -cell function or were not sufficient to prevent T1D [55–57]. Anti-thymocyte globulin (ATG) combined with prednisone was found to reduce insulin requirement of newly diagnosed T1D patients, but the treatment was found to cause thrombocytopenia, which ended the trial [58]. These studies suggest that different therapeutic timing or more effective immunosuppressants would be valuable to treat insulinitis.

3.2. New biological approaches

To date, trials with conventional immunosuppressants have failed to preserve long-term β -cell function. As our knowledge

of effective immune modulators has increased, a broader range of modern therapeutic interventions has been studied (Fig. 1). A promising result has been reported with a monoclonal anti-CD3 antibody engineered to reduce Fc receptor binding affinity [59]. Fc modifications reduce side effects caused by non-specific triggering of mitogenic signals and cytokine release through Fc receptors, but do not affect the immunosuppressant potency of the antibody [49]. Two humanized monoclonal anti-CD3 antibodies, teplizumab and otelixizumab, have been investigated in clinical studies with newly diagnosed T1D patients, therefore not targeting early autoimmune process.

In a phase II randomized placebo-controlled clinical trial, otelixizumab treatment was found to reduce exogenous insulin required for treating hyperglycemia in recent onset T1D patients over a four-year period. However, efficacy was dependent on age and β -cell mass at the time of treatment and therefore, was not beneficial to all patients [60]. Another humanized CD3 antibody, teplizumab, was found to slow the reduction of C-peptide levels for 2 years after T1D diagnosis in a subgroup of T1D patients with lower level HbA_{1c} and insulin

use at baseline [61,62]. Despite the ostensible improvement in these biochemical parameters, it is not clear whether a lasting impact on the natural history of disease was achieved. These findings emphasize the importance of identifying the correct responder group for immune modulatory treatment. Although both otelixizumab and teplizumab showed promising results in phase II studies, their effectiveness was not replicated in phase III trials [14,49]. Rituximab (anti-CD20 monoclonal antibody) monotherapy has been studied in a phase II clinical trial (TrialNet) in newly diagnosed T1D patients. Although T1D has primarily been considered to be a T cell-mediated disease, a four-dose course of rituximab treatment partially preserved islet β -cell function compared to placebo. Specifically, the mean area under the curve (AUC) for the C-peptide level after a mixed meal tolerance test was significantly higher in the rituximab group after one year [63]. Nevertheless, a recently published follow-up investigation found no difference in AUC, insulin dose, or HbA_{1c} after 30 months [63,64]. In addition, long term depletion in B cell counts by rituximab administration carries the risk of detrimental side effects, such as susceptibility to life-threatening infections. Thus, rituximab is

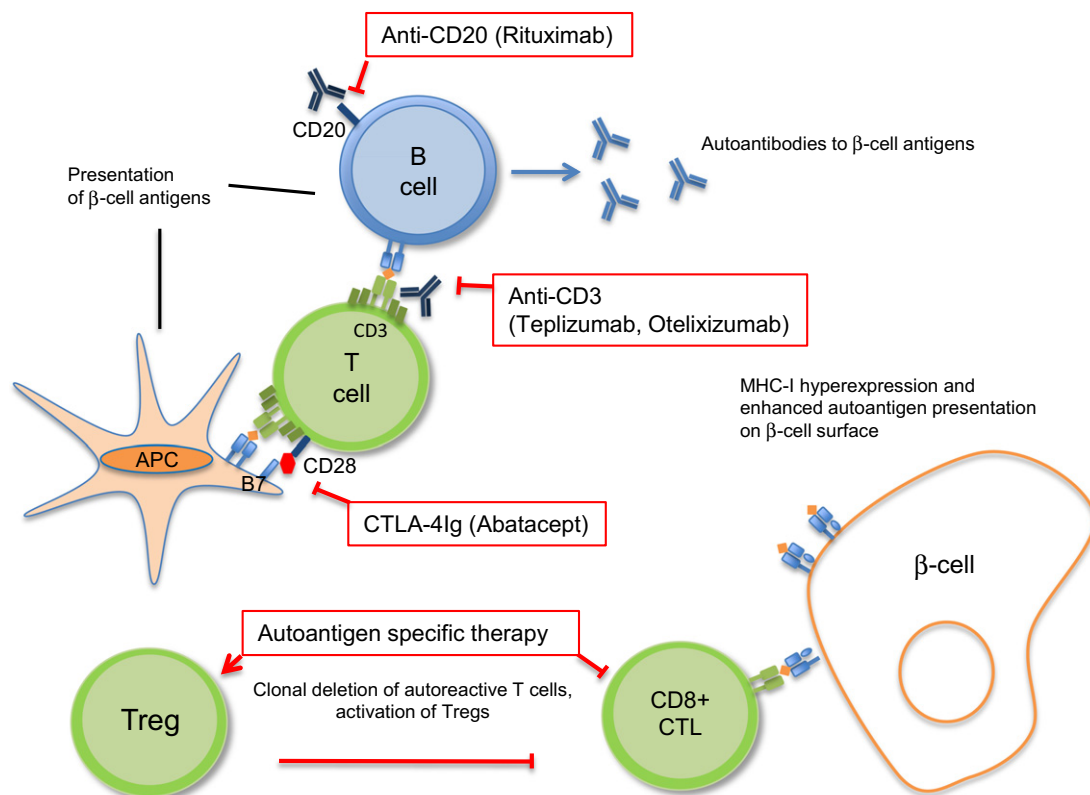


Figure 1 Pathogenesis and biological interventions in autoimmune diabetes. The class I MHC molecules are hyperexpressed on the β -cell surface in T1D patients making β -cells more susceptible to cytotoxic lymphocyte (CTL)-mediated destruction. B cells secrete autoantibodies against β -cell antigens (insulin, GAD65, ZnT8 and IA-2) and are also suggested to present autoantigens to diabetogenic T cells. Anti-CD20 (rituximab) induces B cell apoptosis and down regulates B cell receptor signaling. Humanized monoclonal anti-CD3 antibodies (teplizumab and otelixizumab) inhibit T cell activation by binding to CD3 in TCR complex. Cytotoxic T lymphocyte antigen 4 (CTLA-4) immunoglobulin fusion protein, abatacept, inhibits T cell activation by interfering the CD28–B7 interaction-mediated co-stimulatory signals. Antigen specific therapies can activate regulatory T cells (Treg), which inhibit the proliferation and activity of diabetogenic T cells. Antigenic interventions are also proposed to induce clonal deletion of specific diabetogenic T cell populations and thus ameliorate insulinitis.

Modified from Rydén et al. (2013) [49].

not an optimal therapeutic agent for a patient group that contains mainly children.

Other non-antigenic immune modulators studied in clinical trials include a cytotoxic T lymphocyte antigen-4 (CTLA-4) immunoglobulin fusion protein, abatacept, and interleukin-1 (IL-1) blocking drugs – anakinra and canakinumab. As a blocking agent for T cell activation responses (which does not eliminate T cells), abatacept is considered to be safer than other immunosuppressive agents. Modulation of T cell costimulation by antigen presenting cells with abatacept in a group of newly onset T1D patients was found to preserve C-peptide levels for only 9.6 months over a two year treatment span, indicating that there was still partial T cell activation at the time of T1D diagnosis [65]. Administration of abatacept in a differently structured time period may produce more effective results. Currently, abatacept is being investigated as a secondary means of prevention in prediabetic individuals [49]. Anakinra and canakinumab were studied in two randomized placebo controlled trials with recent onset T1D patients. Both treatments were found to be safe but unfortunately did not ameliorate diabetes [66].

3.3. Antigen specific therapy

A majority of the immunomodulatory drugs described above are tested in interventional trials to halt the disease. Extensive β -cell destruction during this stage causes an absolute insulin deficiency and the need for exogenous insulin. Hence, failure to achieve a major effect could result from the fact that there is nothing left to rescue or that the therapy has been misdirected at the wrong target of the pathogenetic process. Antigen-specific therapies provide a powerful approach to address the latter possibility.

Autoreactive T cells causing insulinitis have been shown to play a crucial role in T1D pathogenesis [1]. Low-dose tolerance induction or initiation of clonal deletion by repeated high-dose T cell receptor stimulation to suppress their function are both potential strategies for T1D prevention. However, such strategies rely on the identification of critical antigens that trigger insulinitis by stimulating autoreactive T cells. Pioneering studies strongly suggest that insulin is an early, if not the first, autoantigen that drives insulinitis [26,36,67]. Insulin autoreactivity appears to be an attractive target for antigen-specific treatment, as insulin autoantibodies are usually the first sign of insulinitis.

The antigen-specific approach has already been explored in experimental animal models of diabetes. For example, there are a myriad of ways to prevent T1D onset by using antigen preparations, including insulin by itself or strongly agonistic peptide mimotopes [68,69]. Insulin has been investigated in numerous secondary prevention studies with at-risk human populations, and has been administered by parenteral, oral, or intranasal routes [14]. Subcutaneous insulin was reported to delay the onset of T1D in a pilot study [70]. Later, it was found that some of the participants who showed delayed disease onset had protective HLA genotypes [70,71]. Parenteral insulin was investigated in a well-controlled Diabetes Prevention Trial (DPT-1), which studied a population of islet autoantibody positive, first- or second-degree relatives of

diabetes patients. The participants were estimated to have over 50% risk of developing diabetes within five years. The daily, low dose subcutaneous ultralente insulin treatment taken together with a four-day annual intravenous insulin administration did not significantly delay the T1D onset when compared to an observation group [72]. A significant problem with giving endocrinologically active insulin is that only minute amounts can be given to diabetic individuals because of hypoglycemia. If small amounts of insulin could effectively suppress the anti-islet immune response, then presumably diabetes would have been prevented early in the immune process. The insulin secreted during normal metabolism at the earliest insulinitis phase would have inhibited diabetes. On the contrary, most immunological tolerance is based on the fact that large doses of antigen are required to shut off the immune response.

Mouse studies have shown that both oral and nasal insulin administration can delay or prevent the onset of T1D [73,74]. These studies allowed the usage of higher amounts of insulin, since only a small portion of insulin reaches the blood stream from mucosal membranes. The findings prompted studies of mucosal membrane administration of insulin to prediabetic human cohorts. Oral insulin was studied in a randomized, double-blinded, placebo-controlled trial in a population of islet antibody positive, first- and second-degree relatives of T1D patients with a five-year risk of 26–50% to develop diabetes. Although there was no difference in T1D incidence between the placebo and insulin group, the disease onset was slightly delayed in the case of patients with high IAA titers that received treatment [75]. The level of persistence of orally administered protein (or its substituent peptide antigens) and its immunoreactivity was not documented.

Intranasal insulin has also been studied in human prevention trials. In the Intranasal Insulin Trial (INIT) study, Harrison et al. [76] showed that treatment with intranasal insulin provided some evidence of increased immune tolerance to insulin in T cell proliferation assays with no positive clinical effect [76]. Intranasal insulin has also been studied in Finland with the Diabetes Prediction and Prevention (DIPP) trial. Children with disease susceptibility HLA genotypes and were positive for islet cell and insulin antibodies were tested. Unfortunately, the treatment did not prevent or delay the onset of T1D [77]. Clinical effectiveness would depend on the establishment of tolerance to the appropriate islet antigens. Thus, the apparent failure of these approaches to halt insulinitis could be explained by the importance of antigens other than insulin, or by the fact that the quantity or route of administration was not sufficient to induce insulin-specific immunological tolerance.

It is worth mentioning that these clinical trials with insulin administration rely on low-dose tolerance induction that is speculated to attenuate autoimmune reaction by activating regulatory T cells or by driving autoreactive T cells into anergy. Since these trials have not met expectations so far, high-dose tolerance induction might be an effective alternative with carefully selected at-risk populations. Repeated stimulation of T cell receptor with high antigen doses leads to clonal deletion of antigen specific T lymphocytes through restimulation-induced cell death (RICD) [78,79]. By using repeated intravenous injections of the antigen, the power of RICD has been previously

demonstrated in the animal model of experimental autoimmune encephalomyelitis (EAE) with striking results [80]. In considering T1D, where insulin seems to be the earliest autoantigen, high dose tolerance induced elimination of insulin autoreactive T cells would hypothetically attenuate insulinitis before antigen spreading. To permit the administration of high doses of insulin antigen, endocrine activity would have to be inactivated, but the antigenic epitopes necessary for T cell receptor activation should be preserved. Hormonally dead insulin proteins have been previously studied in NOD mice with promising results. Repeated subcutaneous doses of the inactive insulin analog were shown to prevent T1D onset when compared to a vehicle treated group of NOD mice [69]. Although the study did not provide strong mechanistic data, it demonstrated the tolerogenic potential of the molecule. However, antigen administration could be a double-edged sword because it has the potential to provoke the immune response similar to a vaccine. Before high-dose tolerance induction in human trials, the safety of the dosage or other measures to limit T cell activation must be studied.

Of the other antigens, interesting results have been obtained with DiaPep277 (Andromeda Biotech Ltd), which is a peptide derived from heat-shock protein 60 (HSP60) with immunomodulatory potential. This chaperone protein was shown to be an important antigen for T1D induction in NOD mice [81]. Administration of HSP60 derived peptide (p277) protected NOD mice from diabetes [82]. DiaPep277 has been investigated in phase I through III clinical studies in humans. C-peptide levels of newly diagnosed T1D patients in this group were higher when compared to the placebo group. Further studies with DiaPep277 are ongoing [49,83].

GAD65 has been tested as a potential tolerogen in both murine models for diabetes and in human diabetes patients. In NOD mouse studies, both intraperitoneal and intranasal administration of GAD65 showed promising results in delaying or preventing the onset of diabetes [84,85]. The aluminum hydroxide adjuvant incorporated GAD65 (GAD-alum) has been studied in a phase II clinical study with late onset autoimmune diabetes (LADA) adult patients. The patients received two subcutaneous injections of GAD-alum. After a five-year follow up, fasting C-peptide levels were restored in groups who received subcutaneous injection of 4, 20, or 100 μ g of the drug when compared to placebo treated subjects [86]. In a subsequent trial on pediatric patients with recent onset diabetes treated with 20 μ g subcutaneous dose of GAD-alum or placebo, the patients receiving GAD-alum showed less reduction in C-peptide values when compared to the placebo group in a four year follow-up, but the difference was not statistically significant [87]. Thus, this pharmaceutical drug does not appear to have a significant disease-arresting activity. Currently, GAD-alum is under investigation as a secondary prevention study with pediatric patients who are positive to multiple autoantibodies [49]. The results from this study will be interesting, since the clinical studies with GAD as a tolerogen have not met the expectations from the mouse studies in delaying T1D disease progression. A significant problem with this medication is that tolerance typically results when there is lack of adjuvant or co-testamentary activity, so the rationale for adding alum is uncertain and may

actually be detrimental to the tolerogenic potential of the protein component.

4. Concluding remarks and future perspectives

The immunomodulatory drugs described above were mainly tested in interventional trials to halt immune process and presumably β -cell destruction in diabetics. Since this is at the stage when exogenous insulin is already needed, it is uncertain that islet metabolic function will be sufficiently revived even if complete suppression of the immune response is achieved. The Holy Grail for autoimmune T1D treatment would be a preventive therapeutic agent that could reverse the destructive autoimmune reaction and preserve the β -cell function prior to T1D onset. Recently described novel ECL-based islet antibody assays appear to be capable of identifying the disease-relevant autoantibodies with good reproducibility in the early stage of autoimmunity. In the mouse, this correlates with insulinitis [23]. However, these assays will need to be validated as a test for insulinitis in different laboratories before they can be reliably utilized in screening at-risk populations. Additionally, it would be beneficial to screen various autoantibodies simultaneously, since children with multiple autoantibodies have the highest risk of developing diabetes [25]. At the same time, new approaches to induce the tolerance for islet autoantigens may provide benefits not observed with conventional or biological methods. In particular, high-dose tolerance induction with endocrinologically inactive insulin or other islet cell antigens is a potential strategy to explore. The hope would be to eliminate insulinitis as early as possible and provide maximal islet β -cell protection, thereby preventing clinical diabetes.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

We thank Anthony Venida for the careful review of our manuscript as well as Prof. David Harlan and Dr. Kimberly Shafer-Weaver on their valuable comments on the manuscript. Dr. Samuli Rounioja is acknowledged for providing us the IAA-RIA protocol. This research was supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, Clinical Center, and National Human Genome Research Institute, National Institutes of Health. J.G. was supported by a fellowship grant from Sigrid Juselius Foundation and by Emil Aaltonen Foundation. Wellstat Diagnostics LLC and Novo Nordisk are acknowledged for their collaboration.

References

- [1] B.O. Roep, M. Peakman, Diabetogenic T lymphocytes in human type 1 diabetes, *Curr. Opin. Immunol.* 23 (2011) 746–753.
- [2] M.L. Campbell-Thompson, M.A. Atkinson, A.E. Butler, N.M. Chapman, G. Frisk, R. Gianani, B.N. Giepmans, M.G. von Herrath, H. Hyoty, T.W. Kay, O. Korsgren, N.G. Morgan, A.C. Powers, A. Pugliese, S.J. Richardson, P.A. Rowe, S. Tracy, P.A.

- In't Veld, The diagnosis of insulinitis in human type 1 diabetes, *Diabetologia* 56 (2013) 2541–2543.
- [3] P. In't Veld, Insulinitis in human type 1 diabetes: the quest for an elusive lesion, *Islets* 3 (2011) 131–138.
 - [4] A. Willcox, S. Richardson, A. Bone, A. Foulis, N. Morgan, Analysis of islet inflammation in human type 1 diabetes, *Clin. Exp. Immunol.* 155 (2009) 173–181.
 - [5] K.T. Coppieters, F. Dotta, N. Amiran, P.D. Campbell, T.W.H. Kay, M.A. Atkinson, B.O. Roep, M.G. von Herrath, Demonstration of islet-autoreactive CD8 T cells in insulinitic lesions from recent onset and long-term type 1 diabetes patients, *J. Exp. Med.* 209 (2012) 51–60.
 - [6] A. Skowera, R.J. Ellis, R. Varela-Calviño, S. Arif, G.C. Huang, C. Van-Krinks, A. Zaremba, C. Rackham, J.S. Allen, T.I. Tree, CTLs are targeted to kill β cells in patients with type 1 diabetes through recognition of a glucose-regulated preproinsulin epitope, *J. Clin. Invest.* 118 (2008) 3390.
 - [7] A. Foulis, M.A. Farguharson, A. Meager, Immunoreactive alpha-interferon in insulin-secreting beta cells in type 1 diabetes mellitus, *Lancet* 8573 (1987) 1423–1427.
 - [8] R.M. Hinman, M.J. Smith, J.C. Cambier, B cells and type 1 diabetes ... in mice and men, *Immunol. Lett.* 160 (2014) 128–132.
 - [9] J.P. Driver, D.V. Serreze, Y. Chen, Mouse models for the study of autoimmune type 1 diabetes: a NOD to similarities and differences to human disease, *Semin. Immunopathol.* 33 (2011) 67–87.
 - [10] E. Mariño, B. Tan, L. Binge, C.R. Mackay, S.T. Grey, B-cell cross-presentation of autologous antigen precipitates diabetes, *Diabetes* 61 (2012) 2893–2905.
 - [11] A. Imagawa, T. Hanafusa, S. Tamura, M. Moriwaki, N. Itoh, K. Yamamoto, H. Iwahashi, K. Yamagata, M. Waguri, T. Nanmo, S. Uno, H. Nakajima, M. Namba, S. Kawata, J.I. Miyagawa, Y. Matsuzawa, Pancreatic biopsy as a procedure for detecting in situ autoimmune phenomena in type 1 diabetes: close correlation between serological markers and histological evidence of cellular autoimmunity, *Diabetes* 50 (2001) 1269–1273.
 - [12] R. Gianani, M. Campbell-Thompson, S. Sarkar, C. Wasserfall, A. Pugliese, J. Solis, B. Hering, E. West, A. Steck, Dimorphic histopathology of long-standing childhood-onset diabetes, *Diabetologia* 53 (2010) 690–698.
 - [13] A. Lehuen, J. Diana, P. Zaccane, A. Cooke, Immune cell crosstalk in type 1 diabetes, *Nat. Rev. Immunol.* 10 (2010) 501–513.
 - [14] Å. Lernmark, H.E. Larsson, Immune therapy in type 1 diabetes mellitus, *Nat. Rev. Endocrinol.* 9 (2013) 92–103.
 - [15] P. Concannon, S.S. Rich, G.T. Nepom, Genetics of type 1A diabetes, *N. Engl. J. Med.* 360 (2009) 1646–1654.
 - [16] H. Erlich, A.M. Valdes, J. Noble, J.A. Carlson, M. Varney, P. Concannon, J.C. Mychaleckyj, J.A. Todd, P. Bonella, A.L. Fear, HLA DR-DQ haplotypes and genotypes and type 1 diabetes risk analysis of the type 1 diabetes genetics consortium families, *Diabetes* 57 (2008) 1084–1092.
 - [17] V. Harjutsalo, L. Sjöberg, J. Tuomilehto, Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study, *Lancet* 371 (2008) 1777–1782.
 - [18] A.T. Borchers, R. Uibo, M. Gershwin, The geoepidemiology of type 1 diabetes, *Autoimmun. Rev.* 9 (2010) A355–A365.
 - [19] H. Peng, W. Hagopian, Environmental factors in the development of type 1 diabetes, *Rev. Endocr. Metab. Disord.* 7 (2006) 149–162.
 - [20] D.L. Eizirik, M.L. Colli, F. Ortis, The role of inflammation in insulinitis and β -cell loss in type 1 diabetes, *Nat. Rev. Endocrinol.* 5 (2009) 219–226.
 - [21] O.H. Laitinen, H. Honkanen, O. Pakkanen, S. Oikarinen, M.M. Hankaniemi, H. Huhtala, T. Ruokoranta, V. Lecouturier, P. Andre, R. Harju, S.M. Virtanen, J. Lehtonen, J.W. Almond, T. Simell, O. Simell, J. Ilonen, R. Veijola, M. Knip, H. Hyoty, Coxsackievirus B1 is associated with induction of beta-cell autoimmunity that portends type 1 diabetes, *Diabetes* 63 (2014) 446–455.
 - [22] S. Oikarinen, S. Tauriainen, D. Hober, B. Lucas, A. Vazeou, A. Sioofy-Khojine, E. Bozas, P. Muir, H. Honkanen, J. Ilonen, M. Knip, P. Keskinen, M.T. Saha, H. Huhtala, G. Stanway, C. Bartsocas, J. Ludvigsson, K. Taylor, H. Hyoty, VirDiab Study Group, Virus antibody survey in different European populations indicates risk association between coxsackievirus b1 and type 1 diabetes, *Diabetes* 63 (2014) 655–662.
 - [23] B. Lo, A.D.E. Swafford, K.A. Shafer-Weaver, L.F. Jerome, L. Rakhlin, D.R. Mathern, C.A. Callahan, P. Jiang, L.J. Davison, H. E. Stevens, C.L. Lucas, J. White, R. von Borstel, J.A. Todd, M. J. Lenardo, Antibodies against insulin measured by electrochemiluminescence predicts insulinitis severity and disease onset in non-obese diabetic mice and can distinguish human type 1 diabetes status, *J. Transl. Med.* 9 (2011) 203.
 - [24] J.A. Todd, Etiology of type 1 diabetes, *Immunity* 32 (2010) 457–467.
 - [25] A. Ziegler, M. Rewers, O. Simell, T. Simell, J. Lempainen, A.K. Steck, G.S. Winkler, J. Ilonen, R. Veijola, M. Knip, E. Bonifacio, G.S. Eisenbarth, Seroconversion to multiple islet autoantibodies and risk of progression to diabetes in children, *JAMA* 309 (2013) 2473–2479.
 - [26] L. Yu, D.T. Robles, N. Abiru, P. Kaur, M. Rewers, K. Kelemen, G.S. Eisenbarth, Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes, *Proc. Natl. Acad. Sci. U. S. A.* 97 (2000) 1701–1706.
 - [27] L. Yu, F. Dong, D. Miao, A.R. Fouts, J.M. Wenzlau, A.K. Steck, Proinsulin/insulin autoantibodies measured with electrochemiluminescent assay are the earliest indicator of pre-diabetic islet autoimmunity, *Diabetes Care* 36 (2013) 2266–2270.
 - [28] H. Siljander, T. Härkönen, R. Hermann, S. Simell, A. Hekkala, R.T. Salonsaari, T. Simell, O. Simell, J. Ilonen, R. Veijola, M. Knip, Role of insulin autoantibody affinity as a predictive marker for type 1 diabetes in young children with HLA-conferred disease susceptibility, *Diabetes Metab. Res.* 25 (2009) 615–622.
 - [29] J.M. Barker, K.J. Barriga, L. Yu, D. Miao, H.A. Erlich, J.M. Norris, G.S. Eisenbarth, M. Rewers, Prediction of autoantibody positivity and progression to type 1 diabetes: diabetes autoimmunity study in the young (DAISY), *J. Clin. Endocrinol. Metab.* 89 (2004) 3896–3902.
 - [30] V. Parikka, K. Näntö-Salonen, M. Saarinen, T. Simell, J. Ilonen, H. Hyöty, R. Veijola, M. Knip, O. Simell, Early seroconversion and rapidly increasing autoantibody concentrations predict prepubertal manifestation of type 1 diabetes in children at genetic risk, *Diabetologia* 55 (2012) 1926–1936.
 - [31] K. Vehik, S.W. Fiske, C.A. Logan, D. Agardh, C.M. Cilio, W. Hagopian, O. Simell, M. Roivainen, J. She, T. Biese, Methods, quality control and specimen management in an international multi-center investigation of type 1 diabetes: TEDDY, *Diabetes Metab. Res.* 29 (2013) 2427.
 - [32] J.M. Sosenko, J.S. Skyler, J.P. Palmer, J.P. Krischer, L. Yu, J. Mahon, C.A. Beam, D.C. Boulware, L. Rafkin, D. Schatz, G. Eisenbarth, Type 1 Diabetes TrialNet Study Group, Diabetes Prevention Trial-Type 1 Study Group, The prediction of type 1 diabetes by multiple autoantibody levels and their incorporation into an autoantibody risk score in relatives of type 1 diabetic patients, *Diabetes Care* 36 (2013) 2615–2620.
 - [33] C.F. Verge, R. Gianani, E. Kawasaki, L. Yu, M. Pietropaolo, H. P. Chase, G.S. Eisenbarth, R.A. Jackson, Prediction of type 1 diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies, *Diabetes* 45 (1996) 926–933.

- [34] H. Siljander, R. Veijola, A. Reunanen, S. Virtanen, H. Åkerblom, M. Knip, Prediction of type 1 diabetes among siblings of affected children and in the general population, *Diabetologia* 50 (2007) 2272–2275.
- [35] S. Korhonen, M.M. Knip, P. Kulmala, K. Savola, H.K. Åkerblom, M. Knip, Autoantibodies to GAD, IA-2 and insulin in ICA-positive first-degree relatives of children with type 1 diabetes: a comparison between parents and siblings, *Diabetes Metab. Res.* 18 (2002) 43–48.
- [36] P. Achenbach, K. Warncke, J. Reiter, H.E. Naserke, A.J. Williams, P.J. Bingley, E. Bonifacio, A. Ziegler, Stratification of type 1 diabetes risk on the basis of islet autoantibody characteristics, *Diabetes* 53 (2004) 384–392.
- [37] P. In't Veld, D. Lievens, J. De Grijse, Z. Ling, B. Van der Auwera, M. Pipeleers-Marichal, F. Gorus, D. Pipeleers, Screening for insulinitis in adult autoantibody-positive organ donors, *Diabetes* 56 (2007) 2400–2404.
- [38] M. Schlosser, K. Koczwara, H. Kenk, M. Strebelow, I. Rjasanowski, R. Wassmuth, P. Achenbach, A. Ziegler, E. Bonifacio, In insulin-autoantibody-positive children from the general population, antibody affinity identifies those at high and low risk, *Diabetologia* 48 (2005) 1830–1832.
- [39] P. Achenbach, K. Koczwara, A. Knopff, H. Naserke, A.G. Ziegler, E. Bonifacio, Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes, *J. Clin. Invest.* 114 (2004) 589–597.
- [40] C. Törn, P. Mueller, M. Schlosser, E. Bonifacio, P. Bingley, Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2, *Diabetologia* 51 (2008) 846–852.
- [41] M. Schlosser, P. Mueller, C. Törn, E. Bonifacio, P. Bingley, Diabetes Antibody Standardization Program: evaluation of assays for insulin autoantibodies, *Diabetologia* 53 (2010) 2611–2620.
- [42] P. Bingley, A.J. Williams, Islet autoantibody testing: an end to the trials and tribulations? *Diabetes* 62 (2013) 4009–4011.
- [43] A.J. Williams, P.J. Bingley, E. Bonifacio, J.P. Palmer, E.A. Gale, A novel micro-assay for insulin autoantibodies, *J. Autoimmun.* 10 (1997) 473–478.
- [44] C.J. Greenbaum, J. Palmer, B. Kuglin, H. Kolb, Insulin autoantibodies measured by radioimmunoassay methodology are more related to insulin-dependent diabetes mellitus than those measured by enzyme-linked immunosorbent assay: results of the Fourth International Workshop on the Standardization of Insulin Autoantibody Measurement, *J. Clin. Endocrinol. Metab.* 74 (1992) 1040–1044.
- [45] A.J. Williams, R. Curnock, C.R. Reed, P. Easton, S. Rokni, P.J. Bingley, Anti-BSA antibodies are a major cause of non-specific binding in insulin autoantibody radiobinding assays, *J. Immunol. Methods* 362 (2010) 199–203.
- [46] D. Miao, K.M. Guyer, F. Dong, L. Jiang, A.K. Steck, M. Rewers, G.S. Eisenbarth, L. Yu, GAD65 autoantibodies detected by electrochemiluminescence assay identify high risk for type 1 diabetes, *Diabetes* 62 (2013) 4174–4178.
- [47] L. Yu, D. Miao, L. Scrimgeour, K. Johnson, M. Rewers, G.S. Eisenbarth, Distinguishing persistent insulin autoantibodies with differential risk nonradioactive bivalent proinsulin/insulin autoantibody assay, *Diabetes* 61 (2012) 179–186.
- [48] P.W. Rhyne, O.T. Wong, Y.J. Zhang, R.S. Weiner, Electrochemiluminescence in bioanalysis, *Bioanalysis* 1 (2009) 919–935.
- [49] A.K. Rydén, J.D. Wesley, K.T. Coppieters, M.G. Von Herrath, Non-antigenic and antigenic interventions in type 1 diabetes, *Hum. Vacc. Immunother.* 10 (2013) 838–846.
- [50] C. Stiller, J. Dupre, M. Gent, M. Jenner, P. Keown, A. Laupacis, R. Martell, N. Rodger, B. Von Graffenried, B. Wolfe, Effects of cyclosporine immunosuppression in insulin-dependent diabetes mellitus of recent onset, *Science* 223 (1984) 1362–1367.
- [51] H. Parving, L. Tarnow, F. Nielsen, P. Rossing, T. Mandrup-Poulsen, R. Osterby, J. Nerup, Cyclosporine nephrotoxicity in type 1 diabetic patients. A 7-year follow-up study, *Diabetes Care* 22 (1999) 478–483.
- [52] J. Carel, C. Boitard, G. Eisenbarth, J. Bach, P. Bougnères, Cyclosporine delays but does not prevent clinical onset in glucose intolerant pre-type 1 diabetic children, *J. Autoimmun.* 9 (1996) 739–745.
- [53] L.C. Harrison, P.G. Colman, B. Dean, R. Baxter, F. Martin, Increase in remission rate in newly diagnosed type I diabetic subjects treated with azathioprine, *Diabetes* 34 (1985) 1306–1308.
- [54] J.J. Cook, I. Hudson, L.C. Harrison, B. Dean, P.G. Colman, G. Werther, G.L. Warne, Double-blind controlled trial of azathioprine in children with newly diagnosed type I diabetes, *Diabetes* 38 (1989) 779–783.
- [55] K.P. Böhmer, H. Kolb, B. Kuglin, J. Zielasek, A. Hübinger, E.F. Lampeter, B. Weber, V. Kolb-Bachofen, H. Jastram, J. Bertrams, Linear loss of insulin secretory capacity during the last six months preceding IDDM: no effect of antiedematous therapy with ketotifen, *Diabetes Care* 17 (1994) 138–141.
- [56] E.A. Gale, European Nicotinamide Diabetes Intervention Trial (ENDIT): a randomised controlled trial of intervention before the onset of type 1 diabetes, *Lancet* 363 (2004) 925–931.
- [57] E.F. Lampeter, A. Klinghammer, W.A. Scherbaum, E. Heinze, B. Haastert, G. Giani, H. Kolb, The Deutsche Nicotinamide Intervention Study: an attempt to prevent type 1 diabetes. DENIS Group, *Diabetes* 47 (1998) 980–984.
- [58] G.S. Eisenbarth, S. Srikanta, R. Jackson, S. Rabinowe, R. Dolinar, T. Aoki, M.A. Morris, Anti-thymocyte globulin and prednisone immunotherapy of recent onset type 1 diabetes mellitus, *Diabetes Res.* 2 (1985) 271–276.
- [59] B. Keymeulen, E. Vandemeulebroucke, A.G. Ziegler, C. Mathieu, L. Kaufman, G. Hale, F. Gorus, M. Goldman, M. Walter, S. Candon, Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes, *N. Engl. J. Med.* 352 (2005) 2598–2608.
- [60] B. Keymeulen, M. Walter, C. Mathieu, L. Kaufman, F. Gorus, R. Hilbrands, E. Vandemeulebroucke, U. Van de Velde, L. Crenier, C. De Block, Four-year metabolic outcome of a randomised controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass, *Diabetologia* 53 (2010) 614–623.
- [61] K.C. Herold, S. Gitelman, C. Greenbaum, J. Puck, W. Hagopian, P. Gottlieb, P. Sayre, P. Bianchine, E. Wong, V. Seyfert-Margolis, Treatment of patients with new onset Type 1 diabetes with a single course of anti-CD3 mAb Teplizumab preserves insulin production for up to 5 years, *Clin. Immunol.* 132 (2009) 166–173.
- [62] K.C. Herold, S.E. Gitelman, M.R. Ehlers, P.A. Gottlieb, C.J. Greenbaum, W. Hagopian, K.D. Boyle, L. Keyes-Elstein, S. Aggarwal, D. Phippard, P.H. Sayre, J. McNamara, J.A. Bluestone, AbATE Study Team, Teplizumab (anti-CD3 mAb) treatment preserves C-peptide responses in patients with new-onset type 1 diabetes in a randomized controlled trial: metabolic and immunologic features at baseline identify a subgroup of responders, *Diabetes* 62 (2013) 3766–3774.
- [63] M.D. Pescovitz, C.J. Greenbaum, H. Krause-Steinrauf, D.J. Becker, S.E. Gitelman, R. Goland, P.A. Gottlieb, J.B. Marks, P. F. McGee, A.M. Moran, Rituximab, B-lymphocyte depletion, and preservation of beta-cell function, *N. Engl. J. Med.* 361 (2009) 2143–2152.
- [64] M.D. Pescovitz, C.J. Greenbaum, B. Bundy, D.J. Becker, S.E. Gitelman, R. Goland, P.A. Gottlieb, J.B. Marks, A. Moran, P. Raskin, H. Rodriguez, D.A. Schatz, D.K. Wherrett, D.M. Wilson, J.P. Krischer, J.S. Skyler, The Type 1 Diabetes TrialNet Anti-CD20 Study Group, B-lymphocyte depletion with rituximab and beta-cell function: two-year results, *Diabetes Care* 37 (2013) 453–459.

- [65] T. Orban, B. Bundy, D.J. Becker, L.A. DiMeglio, S.E. Gitelman, R. Goland, P.A. Gottlieb, C.J. Greenbaum, J.B. Marks, R. Monzavi, Co-stimulation modulation with abatacept in patients with recent-onset type 1 diabetes: a randomised, double-blind, placebo-controlled trial, *Lancet* 378 (2011) 412–419.
- [66] A. Moran, B. Bundy, D.J. Becker, L.A. DiMeglio, S.E. Gitelman, R. Goland, C.J. Greenbaum, K.C. Herold, J.B. Marks, P. Raskin, Interleukin-1 antagonism in type 1 diabetes of recent onset: two multicentre, randomised, double-blind, placebo-controlled trials, *Lancet* 381 (2013) 60023–60029.
- [67] S. Srikanta, A. Ricker, D. McCulloch, J. Soeldner, G. Eisenbarth, J. Palmer, Autoimmunity to insulin, beta cell dysfunction, and development of insulin-dependent diabetes mellitus, *Diabetes* 35 (1986) 139–142.
- [68] C. Daniel, B. Weigmann, R. Bronson, H. von Boehmer, Prevention of type 1 diabetes in mice by tolerogenic vaccination with a strong agonist insulin mimotope, *J. Exp. Med.* 208 (2011) 1501–1510.
- [69] D. Karounos, J. Bryson, D. Cohen, Metabolically inactive insulin analog prevents type I diabetes in prediabetic NOD mice, *J. Clin. Invest.* 100 (1997) 1344–1348.
- [70] R. Keller, R. Jackson, G. Eisenbarth, G. Eisenbarth, Insulin prophylaxis in individuals at high risk of type 1 diabetes, *Lancet* 341 (1993) 927–928.
- [71] A. Pugliese, R. Gianani, R. Moromisato, Z.L. Awdeh, C.A. Alper, H.A. Erlich, R.A. Jackson, G.S. Eisenbarth, HLA-DQB1* 0602 is associated with dominant protection from diabetes even among islet cell antibody-positive first-degree relatives of patients with IDDM, *Diabetes* 44 (1995) 608–613.
- [72] Diabetes Prevention Trial-Type 1 Diabetes Study Group, Effects of insulin in relatives of patients with type 1 diabetes mellitus, *N. Engl. J. Med.* 346 (2002) 1685–1691.
- [73] Z.J. Zhang, L. Davidson, G. Eisenbarth, H.L. Weiner, Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin, *Proc. Natl. Acad. Sci.* 88 (1991) 10252–10256.
- [74] L.C. Harrison, M. Dempsey-Collier, D.R. Kramer, K. Takahashi, Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes, *J. Exp. Med.* 184 (1996) 2167–2174.
- [75] J. Skyler, J. Krischer, J. Wolfsdorf, C. Cowie, J. Palmer, C. Greenbaum, D. Cuthbertson, L. Rafkin-Mervis, H. Chase, E. Leschek, Effects of oral insulin in relatives of patients with type 1 diabetes: The Diabetes Prevention Trial-Type 1, *Diabetes Care* 28 (2005) 1068–1076.
- [76] L.C. Harrison, M.C. Honeyman, C.E. Steele, N.L. Stone, E. Saruger, E. Bonifacio, J.J. Couper, P.G. Colman, Pancreatic β -cell function and immune responses to insulin after administration of intranasal insulin to humans at risk for type 1 diabetes, *Diabetes Care* 27 (2004) 2348–2355.
- [77] K. Nääntö-Salonen, A. Kupila, S. Simell, H. Siljander, T. Salonsaari, A. Hekkala, S. Korhonen, R. Erkkola, J.I. Sipilä, L. Haavisto, Nasal insulin to prevent type 1 diabetes in children with HLA genotypes and autoantibodies conferring increased risk of disease: a double-blind, randomised controlled trial, *Lancet* 372 (2008) 1746–1755.
- [78] J.M. Critchfield, M.J. Lenardo, Antigen-induced programmed T cell death as a new approach to immune therapy, *Clin. Immunol. Immunopathol.* 75 (1995) 13–19.
- [79] A.L. Snow, P. Pandiyan, L. Zheng, S.M. Krummey, M.J. Lenardo, The power and the promise of restimulation-induced cell death in human immune diseases, *Immunol. Rev.* 236 (2010) 68–82.
- [80] J.M. Critchfield, M.K. Racke, J.C. Zuniga-Pflucker, B. Cannella, C.S. Raine, J. Goverman, M.J. Lenardo, T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis, *Science* 263 (1994) 1139–1143.
- [81] D. Elias, D. Markovits, T. Reshef, R. van der Zee, I.R. Cohen, Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa heat shock protein, *Proc. Natl. Acad. Sci. U. S. A.* 87 (1990) 1576–1580.
- [82] D. Elias, T. Reshef, O.S. Birk, R. van der Zee, M.D. Walker, I.R. Cohen, Vaccination against autoimmune mouse diabetes with a T-cell epitope of the human 65-kDa heat shock protein, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 3088–3091.
- [83] B. Fischer, D. Elias, R.G. Bretzel, T. Linn, Immunomodulation with heat shock protein DiaPep277 to preserve beta cell function in type 1 diabetes—an update, *Expert. Opin. Biol. Ther.* 10 (2010) 265–272.
- [84] J.S. Petersen, A.E. Karlsen, H. Markholst, A. Worsaae, T. Dyrberg, B. Michelsen, Neonatal tolerization with glutamic acid decarboxylase but not with bovine serum albumin delays the onset of diabetes in NOD mice, *Diabetes* 43 (1994) 1478–1484.
- [85] J. Tian, M.A. Atkinson, M. Clare-Salzler, A. Herschenfeld, T. Forsthuber, P.V. Lehmann, D.L. Kaufman, Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes, *J. Exp. Med.* 183 (1996) 1561–1567.
- [86] C. Agardh, K. Lynch, M. Palmer, K. Link, Å. Lernmark, GAD65 vaccination: 5 years of follow-up in a randomised dose-escalating study in adult-onset autoimmune diabetes, *Diabetologia* 52 (2009) 1363–1368.
- [87] J. Ludvigsson, M. Hjorth, M. Chéramy, S. Axelsson, M. Pihl, G. Forsander, N. Nilsson, B. Samuelsson, T. Wood, J. Åman, Extended evaluation of the safety and efficacy of GAD treatment of children and adolescents with recent-onset type 1 diabetes: a randomised controlled trial, *Diabetologia* 54 (2011) 634–640.