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Antigen-based vs. systemic immunomodulation in type 1 diabetes

The pros and cons

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Abbreviations: Ab, antibody; Ag, antigen; GAD65, glutamic acid decarboxylase 65; HSP60, heat shock protein 60; L. lactis, Lactococcus lactis; NOD, non-obese diabetic; T1D, type 1 diabetes; Treg, regulatory T-cell; LADA, latent autoimmune diabetes in adults

In type 1 diabetic patients, insulin-producing pancreatic β-cells are destroyed by an orchestrated immune process involving self-reactive auto-antigen-specific $CD4^+$ and $CD8^+$ T cells. Efforts to reverse or prevent this destructive immunological cascade have led to promising results in animal models; however the transition to the clinic has yet been unsuccessful. In addition, current clinical studies lack reliable biomarkers to circumscribe end-point parameters and define therapeutic success. Here, we give a current overview of both antigen-specific and non-specific systemic immunomodulatory approaches with a focus on the therapies verified or under evaluation in a clinical setting. While both approaches have their advantages and disadvantages, rationally designed combination therapies may yield the highest therapeutic efficacy. In order for future strategies to be effective, new well-defined biomarkers need to be developed and the extrapolation process of dose, timing, and frequency from in vivo models to patients needs to be carefully reconsidered.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease with an alarming increase in incidence rate, especially in children under the age of 5 y.[1] This disease is caused by the specific destruction of the pancreatic insulin-producing β-cells by auto-reactive $CD4^+$ and $CD8^+$ T-cells. This autoimmune process is supported by auto-antigen presentation by antigen-presenting cells. The detection of auto-antibodies (auto-Abs) produced by B cells allows diagnosis during the symptom-free early stages of the disease. Once this destructive process is initiated, the β-cell itself does not stay neutral; due to chemokine signaling for example, it further attracts pathogenic mononuclear cells and initiates signaling cascades leading to apoptotic cell death.[2]

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To control the elevated blood glucose levels associated with the disease, patients mainly rely on insulin replacement therapy. However, the latter does not fully prevent the life-threatening complications including retinopathy, neuropathy, nephropathy and cardiovascular disease, occurring at later stages in the disease. Current approaches aimed at arresting disease progression focus on intervening in T-cell responses, such as T-cell specific monoclonal Abs (mAbs) like anti-CD3. Furthermore, β-cell replacement strategies are being implemented by transplanting patients with donor islets. However, such approaches do not address the ongoing autoimmune attack as the underlying cause of β-cell destruction. Therefore, strategies that specifically restore the tolerance against β-cell antigens (Ags) are being investigated to provide long-term disease protection.

Here, we aim to give an overview of strategies implementing systemic immunomodulators as well as current Ag-based approaches highlighting their advantages and disadvantages. Furthermore, we put forward a new concept of using combinational therapies in the treatment of T1D.

Systemic Immunomodulation

Up-to-date, systemic immunosuppression has given the most promising results in human trials. This approach aims to suppress or regulate the systemic immune responses in order to counteract the β-cell destructive autoimmune response. However this approach contains drawbacks due to its associated side effects. This class of drugs interacts usually with common signaling pathways used by different cell types from the immune system. When the body's immune system is being suppressed, reactivity against pathogenic Ags is being reduced which renders the body highly susceptible for infections and malignancies. This systemic approach contains two forms; either a general approach where the entire immune system is downregulated, like for cyclosporine A, or a more targeted one where a specific subset of immunological cells, like T-cells in the case of anti-CD3 mAbs, are being suppressed (**Table 1**).

Table 1. Overview of systemic immunomodulations as an intervention or prevention therapy for T1D in humans

Systemic approach	Study name	Treatment	Subjects	Clinical or immunological outcome	Reference
Cyclosporine A	The Canadian-European randomized control trial group	10 mg/kg/d po	• 9–35 y • < 6 w diagnosed	↑β-cell function ↑remission rate for 1 y	3
Cyclosporine A	French Multicenter Cyclo-sporine study	7.5 mg/kg/d po	• 15–40 y	↑remission rate	4
Cyclosporine A	French Pediatric Cyclo-sporine study	7.5 mg/kg/d po	• 7–15 y	↑remission rate	5
Anti-CD3	Phase II trial of Teplizumab for treatment of patients with recent onset T1D	14 d (34 mg/70kg) 12 d (41 mg/70kg) iv Teplizumab	• 9–30 y • < 4 mo diagnosed • stimulated C-peptide > 0.2 nM	better C-peptide response ↓HbA1c and insulin requirement	6
Anti-CD3	Phase II therapeutic trial with Otelixi-zumab in recently diagnosed T1D patients	6d (48 or 64 mg) iv Otelixizumab	• 12–39 y • < 4 w diagnosed	better C-peptide response ↓insulin requirement	7
Anti-CD3	The Protégé study	17 mg/70 kg (14 d) 5.6 mg/70 kg (14 d) 4.6 mg/70 kg (6 d) at entry and 26 w iv Teplizumab	• 8–35 y • < 12 w diagnosed	delayed ↓ C-peptide ↑glycaemic control	8
Anti-CD3	DEFEND-1	3.1 mg (8 d) iv Otelixizumab	• 12–45 y • < 90 d diagnosed • ICA+ • stimulated C-peptide >0.2 nM	No change in C-peptide	Tolerx and GSK
Anakinra	AIDA	100 mg daily Sc	• 18–35 y • GADA+ • < 12 w diagnosed	Still ongoing	9
Abatacept	Intravenous CTLA4-Ig treatment in recent onset T1D	D 1, d 14, every 28 d Iv 10 mg/kg	• 6–45 y • < 100 d diagnosed • ≥1 ICA+	Delayed β-cell decline	10
Rituximab	Effects of Rituximab on the progression of T1D in new onset subjects	D 1, d 8, d 15 and d 22 Iv 375 mg/m^2	• 8–45 y • ≥1 ICA+ • > 3 w and <3 mo diagnosed	↑ C-peptide ↓ Insulin need ↓ hbA1c	11
Vitamin D	Immuno-intervention with Calcitriol in new-onset T1D	0.25 μg 1,25(OH)$_2$D$_3$/d for 9 mo	• 19–39 y • ICA+ • < 2 min insulin treatment	No effect	12
Vitamin D	IMDIAB XI	0.25 μg 1,25(OH)$_2$D$_3$ every 2 d	• < 4 w diagnosed • ICA+	No effect	13
Vitamin D	LADA vitamin D study	0.5 μg/d for 1 y 1-α(OH)D3 + insulin	• LADA patients • < 5 y diagnosed • GADA+ • Fasting C-peptide > 200 pM	↑ β-cell function	14

For more information on advantages and disadvantages of a systemic immunomodulatory approach, see **Table 3**. d, day; mo, month; LADA, latent autoimmune diabetes in adults; Tregs, regulatory T cells; y, years; ICA, islet cell antibody; GADA, GAD-associated antibodies; po, per os; MMTT, mixed meal tolerance test; AHST, autologous nonmyeloablative hematopoietic stem cell transplantation; w, weeks.

Table 1. Overview of systemic immunomodulations as an intervention or prevention therapy for T1D in humans (continued)

Systemic approach	Study name	Treatment	Subjects	Clinical or immunological outcome	Reference
Vitamin D	Vitamin D in healthy individuals (MMTT and Tregs)	140 000 IU/m for 3 mo	Healthy non-diabetic	↑ Tregs	15
AHST	Safety and efficacy study of AHST for early onset T1D	cyclophosphamide (200 mg/kg) and ATG (4.5 mg/kg) + AHST	• 14–31 y • < 6 w diagnosed • GADA+ • No ketoacidosis	Different periods of insulin independence	65,66

For more information on advantages and disadvantages of a systemic immunomodulatory approach, see **Table 3**. d, day; mo, month; LADA, latent autoimmune diabetes in adults; Tregs, regulatory T cells; y, years; ICA, islet cell antibody; GADA, GAD-associated antibodies; po, per os; MMTT, mixed meal tolerance test; AHST, autologous nonmyeloablative hematopoietic stem cell transplantation; w, weeks.

Cyclosporine A. Cyclosporine A is one of the first immunosuppressive agents applied in humans to enhance renal transplantations and forestall rejections in the late 1970s.[16] It is a lipophilic polypeptide of 11 amino acids that inhibits calcineurin and in this way inhibits IL-2 production needed for T-cell activation, growth and proliferation.[16] The use of this immunosuppressive drug could prevent diabetes in the biobreeding (BB) rat and a pilot study in 41 newly-diagnosed T1D patients showed its therapeutic potential in humans.[17,18] Furthermore, a randomized double-blind placebo-controlled trial (the Canadian-European randomized control trial group) and a multicenter trial conducted by Feutren et al. could establish a higher remission rate coupled to a better preserved β-cell function in cyclosporine-treated patients.[3,4] However, these beneficial effects were only stable for 1 y. Similar results have been observed in children and in the Canadian open study, both of which could not show long-term efficacy.[5,19-21] Improved results were obtained in subgroups with faster diagnosis and subsequent faster treatment initiation.

Although these results were promising, concerns were raised by results from a 7-y follow-up study of the Canadian-European trial showing induction of nephrotoxicity in cyclosporine-treated patients.[22] Although the short-term renal side effects of cyclosporine A are completely reversible, long-term cyclosporine A treatment may induce irreversible structural renal damage. In contrast, another follow-up study did not see any long-term renal dysfunction not even after 8 to 13 y.[23]

Monoclonal anti-CD3 antibodies. The mAb anti-CD3 has been extensively studied in the context of T1D. As a specific T-cell-directed drug OKT3 (a pioneer of the anti-CD3 Abs) was first used in the context of transplantation where it could suppress acute graft rejection.[24] However the use of OKT3 in humans evoked a hazardous cytokine storm. This effect could be explained by the non-specific Fc receptor (FcR) binding by monocytes and the subsequent cross-linking of T-cell receptors (TCRs) followed by T-cell activation and cytokine release.[25-27] To avoid these toxic OKT3 effects two humanized non-mitogenic anti-CD3 mAb variants were produced. hOKT3γ1(Ala-Ala) (Teplizumab) preserves the OKT3 binding but has mutations in the Fc region, amino acids 234 and 235 are both changed to alanines, and as such FcR binding is decreased.[28] While with the aglycosylated ChAglyCD3 (Otelixizumab) amino acid 297 is replaced with an alanine resulting in the elimination of a glycosylation site necessary for FcR binding.[29]

In recent-onset NOD (non-obese diabetic) mice the administration of a low dose regimen anti-CD3 hamster mAb 145–2C11 (5 μg/d for 5 d), which is directed against the ε chain of the murine CD3/TCR complex, induced 80% diabetes remission.[30,31] This reversal is mediated in 2 phases. First a transient depletion of most probably recently activated T-cells occurs.[32,33] This depletion is accompanied by antigenic modulation (shedding and internalization of the TCR/anti-CD3 complex), which renders the T-cell blind for Ags.[34] In a second phase, anti-CD3 administration induces and stabilizes a Foxp3$^+$ Treg (regulatory T-cell) population.[32,33,35,36] This increase in the percentage of Tregs upon anti-CD3 administration could be attributed to the depletion of conventional T-cells while the absolute numbers of Foxp3$^+$ Tregs remain unchanged.[32] By rearranging the TCR-specific Treg ratio in the peripheral niche, where Ag-specific Tregs are normally selected and maintained at low frequencies, anti-CD3 therapy induces a complete reorganization of the Treg repertoires.[35] As such anti-CD3 therapy permits Tregs to reorganize and break through their normal peripheral constraints.[35] This Treg rearrangement seems to be independent of transforming growth factor β (TGF-β) since anti-TGF-β antibodies did not eliminate this effect.[35] Other studies however show that TGF-β is essential since anti-TGF-β antibodies could abolish anti-CD3 mediated diabetes remission.[36,37]

A recent study addressed the immunological discrepancies between mice and men by working with a humanized mouse model. Teplizumab-treatment of these animals induced a CD4$^+$CD25$^+$CCR6$^+$Foxp3$^+$ Treg population secreting IL-10 while migrating to the small intestine.[38] Although this specific Treg population later appears in the peripheral circulation, its intestinal migration seemed to be necessary for the Teplizumab-induced immunomodulatory effects. Another group created a transgenic mouse model expressing the human ε chain of the CD3 complex.[39] With these approaches the inconsistency between mice and men will be further narrowed down, and a more accurate understanding of the anti-CD3 mode of action in men should be obtained.

Herold and colleagues performed a first clinical trial using a 12 or 14-d course of Teplizumab administered to recent-onset T1D patients.[40] A single course of Teplizumab stabilized C-peptide

responses and improved insulin requirements for the first year of follow-up.[6,40] In the second year the C-peptide responses from the treated group gradually declined, although they remained significantly better than the control group.[6] Clinical responsiveness was associated with an increase in $CD8^+/CD4^+$ T-cell ratio at 30 and 90 d after treatment initiation.[6] A following trial, implementing a 40% higher dose, obtained similar therapeutic efficacy but individual subjects portrayed aggravated therapy-related side effects.[41] In this trial even after 5 y of follow-up slightly improved β-cell preservation was observed.[41] Another phase II study will examine whether Teplizumab can prevent or delay T1D in relatives at high risk of developing this disease (http://www.clinicaltrials.gov; NCT01030861).

Keymeulen et al. obtained a similar delay of β-cell destruction after a 6-d course of Otelixizumab in new-onset T1D patients.[7] At 48 mo the insulin requirements were still better than the placebo group.[42] In addition they stated that a younger age and higher basal β-cell function significantly correlated with improved clinical outcome.[42]

However, in the majority of patients receiving Otelixizumab a short-term transient reactivation of the Epstein-Barr virus (EBV) was observed.[7,42,43] This self-limited immune response was probably mediated through an activation of EBV-specific T-cells in combination with a short-term general immunosuppression. In the Teplizumab trial no clinical presentation of EBV reactivation was reported. This could be explained by the younger population leading to less healthy EBV carriers or by the lower cumulative dose used. Notably, EBV reactivation was completely self-limited and transient so it should not be considered as a limitation for further clinical applications. Adding to its safety profile is the absence of any chronic immunosuppression, since cellular immune responses against bacterial and viral Ags and tumors were preserved after Otelixizumab treatment.[44]

Due to these successful phase II trials several phase III studies with Otelixizumab and Teplizumab were launched. The DEFEND-1 (Durable Response Therapy Evaluation For Early or New-Onset Type 1 Diabetes) study used Otelixizumab in newly diagnosed T1D patients but did not meet its primary endpoint, being a beneficial change in C-peptide after 12 mo of treatment initiation (gsk website, press releases: http://www.gsk.com). Of note this study used a dose 16 times lower (3.1 mg) than the dose in the successful phase II study by Keymeulen et al. (48 mg). Further analyses of these data are underway. In addition, the phase III study using Teplizumab (the Protégé study) also missed its primary endpoint, being a significant change in clinical outcome at 1 y measured by insulin intake (< 0.5 U/kg) and HbA1c levels (< 6.5%).[8] Post-hoc analyses in patients treated with the 14-d high dose (2 × 17 mg/70 kg) revealed a preservation of C-peptide secretion: an effect which was enlarged in several subgroups being residents of the U.S., children between 8 and 11 y and patients less than 6 weeks diagnosed. With this treatment also improved glycaemic control (HbA1c level) was observed with using lower doses of exogenous insulin.

The failure of these phase III studies to deliver their primary endpoints highlights the importance of choosing the right validation parameters to evaluate therapeutic efficacy.

IL-1 blockade. Interleukin-1 (IL-1) is a pro-inflammatory cytokine that is produced especially by monocytes, macrophages and dendritic cells in the presence of inflammatory stimuli. IL-1β induces β-cell dysfunction in vitro and may contribute to diabetes pathogenesis.[9,45,46] In T2D a human recombinant IL-1 receptor antagonist (Anakinra) was able to improve glycaemia up to 39 weeks after treatment.[47] Also in the context of rheumatoid arthritis patients benefitted from daily Anakinra administrations.[48] In addition these studies have demonstrated the safety and well-tolerable profile of this immunotherapeutic drug.

Recently, a trial has been launched to evaluate the effects of Anakinra on the β-cell function of newly-diagnosed T1D patients.[9] Patients were allocated to subcutaneous daily doses of 100 mg Anakinra or placebo. A difference in stimulated C-peptide response was considered as the primary endpoint (NCT00711503).

Other approaches focus on neutralizing Abs, like Canakinumab, a human monoclonal Ab targeting IL-1β. This compound is currently evaluated in T2D patients for its effects on HbA1c levels and stimulated C-peptide responses (NCT00605475). Also with this agent a trial in new onset T1D patients has been conducted (NCT00947427).

Both the Anakinra and the Canakinumab trial were recently terminated and although a good safety profile was reported they both show the inability of IL-1β - blockade to alter the course of T1D.[49]

CTLA4-immunoglobulin fusion protein (Abatacept). For the complete activation of auto-Ag-specific effector T-cells, the main mediators in T1D, and T-cells in general two essential activation signals are needed. The first one is the interaction of the TCR with processed Ag presented by MHC-molecules on Ag-presenting cells (APC). The second trigger is provided by co-stimulatory signals, of which the most prominent one is the interaction of CD28 on T-cells with CD80 and CD86 on APCs. CD80 and CD86 are also natural ligands of cytotoxic T lymphocyte-associated antigen-4 (CTLA4) which is expressed by activated T-cells and serves as an inhibitory signal.[50] CTLA4 blocks the interaction with CD28 and subsequently suppresses early T-cell activation and proliferation.

This suppressive signaling interaction has already been studied in the context of autoimmunity while making use of a CTLA4-immunoglobulin fusion protein, Abatacept. This soluble molecule consists of the extracellular binding domain of CTLA4 fused to an immunoglobulin Fc domain.[51] In patients with stable psoriasis or rheumatoid arthritis Abatacept could significantly improve clinical outcome.[52,53] In NOD mice this antagonist of CD28-mediated costimulation could alter the onset of T1D if administered before overt disease but after insulitis onset.[54] A recent study administering Abatacept to newly-diagnosed patients monthly for a duration of 2 y could improve β-cell function and delay β-cell destruction.[10] Although β-cell decline was delayed, the decline rates for both placebo and Abatacept-treated groups remained parallel.

Recently, adaptations have been made to ameliorate the biologic avidity of Abatacept. A novel variant Belatacept contains

two amino acid substitutions, which improved the association with CD80 and CD86.[55]

Rituximab. Since T1D is characterized by a β-cell destruction mediated by auto-reactive $CD4^+$ and $CD8^+$ effector T-cells therapeutic approaches typically focus on this T-cell compartment. Recently another immune mediator, the B-lymphocyte, was reintroduced as a potential candidate for targeted immune intervention. Although B-lymphocytes provide the diagnostic auto-Ab profile and are partly responsible for T-cell activation following processing and presenting of Ags, they do not seem to be essential for the onset of T1D. One case report describes the development of T1D despite severe hereditary B-lymphocyte deficiency.[56] However, these APCs can exacerbate disease by presenting unexposed cryptic epitopes through altered Ag processing in the presence of necessary costimulatory molecules.

The transmembrane receptor CD20 is typically expressed on the surface of pre- and immature B-lymphocytes originating from the bone marrow and is lost upon plasma cell differentiation. In transgenic mouse models expressing human CD20 on B-lymphocytes T1D could be prevented and even delayed with anti-CD20 Abs (Rituximab) depleting the B-cell compartment.[57] A placebo-controlled phase II study was conducted in which recently-diagnosed T1D patients were administered a four-dose course of Rituximab.[11] After 1 y of follow-up significantly increased C-peptide levels were measured in addition to diminished insulin requirements and better glycaemic control. After 2 y, a parallel decline in C-peptide was noted however the difference between Rituximab and placebo groups was still significantly different.

Vitamin D. Vitamin D plays an essential role in the Ca^{2+} metabolism and subsequent in bone mineralization and mineral homeostasis. It can be synthesized in an endogenous way under the influence of UV radiation in the skin.[58] In addition, around 10% is coming from nutritive sources like fatty fish and eggs. It is converted to its active form, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), by two hydroxylation steps, first in the liver and successively in the kidney, after which it interacts with the vitamin D receptor located in a wide variety of cells. Besides its classic actions active vitamin D also demonstrated immunomodulatory effects. It generates a tolerogenic environment by altering the activation and maturation of APCs and B- and T-lymphocytes. Multiple studies already demonstrated the potential of active vitamin D, or its structural analogs, to delay the onset of T1D in the NOD mouse model.[59-61]

Birth-cohort studies were set up to address whether vitamin D supplementation could prevent or delay T1D in humans as well. Vitamin D supplementation (50 μg daily or 2000 IU daily) was associated with a diminished risk of developing T1D.[62] In parallel the EURODIAB substudy obtained a 33% reduction of T1D when children were supplemented with vitamin D in early life.[63]

Whether regular vitamin D can demonstrate efficacy in reversing established disease remains controversial. Some studies failed to observe an improved insulin requirement in newly diagnosed T1D patients treated with active vitamin D.[12,13] Whereas a small study showed improved preservation of β-cell function in LADA (latent autoimmune diabetes in adults) patients receiving $1\alpha(OH)D_3$ (0.5 μg/d for 1 y) in addition to subcutaneous insulin.[14] Another study focused on healthy individuals receiving a high dose of active vitamin D (140,000 IU) monthly for 3 mo. Here an improvement in β-cell function could not be confirmed but subjects featured an increase in Treg frequency in the peripheral blood.[15] In view of the clinical application of vitamin D, a balance between an optimal dose and calcaemic side effects needs to be investigated. Current research is focusing on vitamin D analogs that dissociate between an optimal immunomodulatory dose range and toxic calcaemic effects.

Autologous nonmyeloablative hematopoietic stem cell transplantation (AHST). In NOD mice bone marrow transplantation prevents T1D, although it cannot reverse established disease.[64] In humans preliminary successful interventional results were obtained. In a first prospective trial 15 recent-onset T1D patients were enrolled for an AHST preceded by a high dose immunosuppression consisting of cyclophosphamide and antithymocyte globulin (ATG).[65] In these patients an improved β-cell function translated into elevated C-peptide and lower HbA1c levels. Furthermore, 93% of the patients enjoyed a variable period of insulin-independency. Failure of a clinical response in the other 7% could be attributed to a very low β-cell mass at therapy start, as shown by prior ketoacidosis. In an enlarged follow-up study, with a mean of 29.8 mo, 20 out of 23 patients had a continuously (n = 12) or transiently (n = 8) insulin-independent period accompanied with a significant C-peptide increase.[66] The mechanisms underlying these beneficial effects remain unknown. However, a resetting of the immune system in support of a tolerant state is favored over the idea of β-cell regeneration since both animal and human studies have shown the inability of hematopoietic stem cells to regenerate into pancreatic β-cells.[64] Notwithstanding these positive results, there are still issues with this type of intervention considering the mayor risks attached when applying cell transplantations, in addition to the risk of undergoing immunosuppressive therapy.

Two Ag-Based Immunotherapy

What Ag to focus on? When using an Ag-specific approach the first question will automatically be what Ag to focus on? Several auto-Ags have been identified based on auto-Ab positivity in T1D patients or T1D-prone individuals. These include (pro)-insulin, glutamic acid decarboxylase 65 (GAD65), tyrosine phosphatase-like protein ICA512 (IA-2), islet cell auto-antigen 69 kDa (ICA69) and also the recently identified zinc transporter (ZnT8).[67-69] The serological presence of these auto-Abs in T1D relatives is being used as a diagnostic tool whereby the presence of multiple auto-Abs confers higher risk of progression to T1D.[68,70-72] The same auto-Ags are involved in the activation of self-reactive T-cells. Grouping of the already identified T1D-related T-cell epitopes revealed that GAD65, IA-2 and (pro)-insulin account for almost 75% of the already identified $CD4^+$ and $CD8^+$ T-cell epitopes in humans.[73] Islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), although less known, is also a T-cell auto-Ag involved in the further β-cell destruction, as are

pancreatic duodenal homeobox-1 protein (Pdx1) and the recently identified secretory granule proteins chromogranin A (ChgA) and islet amyloid polypeptide (IAPP).[74-77]

Interestingly, some studies proposed a model whereby one primary auto-Ag acts as an obligatory trigger for the activation and expansion of self-reactive T-cells, followed by β-cell destruction and more islet-specific Ag release with subsequent further activation of other self-reactive T-cells.[78] In support of this concept, a knockdown of insulin 1, which is only expressed in β-cells, almost completely prevented T1D in the NOD mouse model, although normal auto-Ab titers were maintained.[79] Such a proposed model, with insulin as a key auto-Ag in the development of T1D, could also apply for humans when the similarities in the immunological cascade underlying T1D in NOD mice and men are taken into account. More than 90% of the insulin-specific $CD4^+$ T-cells in NOD mice reacts with amino acids 9–23 of the insulin B-chain.[80] Alleva and colleagues demonstrated that B:9–23 is also an immunodominant epitope in human T1D.[81] Furthermore, insulin auto-Abs are among the first to appear in children developing T1D.[82]

On the other hand, a second model implies the simultaneous action of self-reactive T-cells reacting against various Ags. The necessity of GAD65 or IA-2 in NOD mice remains controversial since several studies state they are dispensable for the onset and development of T1D[83-85] whereas others claim they are essential.[86-88] In humans, however, the presence of multiple auto-Abs with different specificities confers a higher risk for developing T1D, a concept which favors the second model.[89,90] In addition, the susceptible human leukocyte Ag (HLA) DR4-DQ8 haplotype strongly correlates with insulin auto-Abs whereas the HLA DR3-DQ2 haplotype correlates with GAD auto-Abs.[91]

Whether the onset of T1D is mediated by one initial or various triggers, the autoimmune reaction will eventually be distributed over different Ags due to epitope spreading. The β-cell targeted autoimmune response quickly spreads to other epitopes from the same Ag (intramolecular spreading) and/or to epitopes from other Ags (intermolecular spreading) situated in the close proximity.[92,93] This process of epitope spreading contains a lot of similarities with the spreading of protection when bystander suppression is induced. In several autoimmune disease models, like experimental autoimmune encephalomyelitis (EAE) and T1D, administration of an auto-Ag through a tolerogenic route drives the downregulation of the immune response to that specific auto-Ag, mostly through the induction of a Treg compartment.[94,95] The extension of this suppression to immune responses against other disease-related auto-Ags is a phenomenon referred to as bystander suppression.[93,94,96] This is made possible partly by the suppressive cytokines secreted by Ag-non-specific Tregs. This phenomenon holds great promise for the future since any auto-Ag, capable of inducing a Treg compartment, could inhibit the destructive immunologic actions characteristic for an autoimmune disease when administered in a tolerogenic way. Various possible tolerogenic routes of administration (intravenous, subcutaneous, intranasal and oral) for several auto-Ags like pro-insulin, GAD65 and heat shock protein 60 (HSP60) for T1D have been suggested so far (**Table 2**).

Insulin. As mentioned above, insulin is being regarded as possibly the major auto-Ag in T1D. In the NOD mouse model numerous studies could prevent or delay the onset and development of T1D by administering insulin or an insulin peptide, like B:9–23, orally,[110,111] subcutaneously,[80] intravenously[112] or intranasally.[80,113,114] Nakayama and colleagues made a double insulin knockout in combination with an altered insulin B:9–23 transgene to preserve the insulin metabolic activity while abrogating the NOD T-cell responsiveness. In this model, T1D was prevented and insulin auto-Abs were almost completely absent.[115,116] Further studies are underway to elucidate whether B:9–23 is truly an essential auto-Ag in the NOD mouse model.

Based on these promising in vivo results an extensive Diabetes Prevention Trial–T1D (DPT-1) was conducted. This study contained two different arms either focusing on parenteral or oral insulin administration.[97,99] For the parenteral treatment both first- and second degree relatives of T1D patients were screened and enrolled based on age, ICA presence and their 5-y risk of developing T1D.[97] They received daily ultralente insulin by two subcutaneous injections (0.25 U/kg/d in total) in combination with an annual intravenous infusion, but in spite of this intensive regime and a median follow-up of 3.7 y, no prevention or delay of T1D was registered. Similar trials, such as EPPSCIT using 0.2 U/kg/d and a Belgian study using 0.1 U/kg/d, obtained comparable results.[98,100]

The oral arm of the DPT-1 trial assigned a similar population but with lower risk to oral insulin (7.5 mg/d) or placebo. This oral insulin treatment had no beneficial effect in the total cohort however in a subgroup of individuals with baseline insulin auto-Abs (IAA) ≥ 80 nU/ml a delay in diabetes onset was seen.[99] A follow-up study showed a prolongation of this effect which stopped as soon as therapy was discontinued.[117] However the data were suggestive enough to have led to a subsequent study in high Ab titer subjects which is still ongoing (http://www.clinicaltrials.gov; NCT00419562).

Two other trials focused on the preservation of C-peptide after diagnosis using a daily oral insulin dosage of 2.5 and 7.5 mg (ORALE) or 5 mg (IMDIAB VII) for 1 y. This preservation approach, rather than prevention, did not achieve any beneficial results.[101,102] Another oral insulin trial obtained a slower loss of C-peptide response in a subgroup of newly diagnosed diabetic patients older than 20 y receiving 1 or 10 mg oral insulin daily.[103] However this effect was not translated into a clinical improvement. Besides the diverse dosages a different study population can be a possible explanation for these discrepant outcomes since the former studies had in general a smaller group with a younger age distribution. Ergun-Longmire and colleagues further highlighted the link between better β-cell preservation and older age, C-peptide levels and number of auto-Abs at the start of therapy.[103]

An intranasal approach in HLA-conferred persons at risk, the T1D Prediction and Prevention study (DIPP), did not delay or prevent T1D, although this dose (1 U/kg/d) did induce immunological changes in the insulin Ab profile.[104,118] A similar approach in recent-onset diabetic patients also did not have a beneficial clinical outcome but diminished the level of insulin-specific Abs and IFN-γ secretion by T-cells in response to subcutaneous

Table 2. Overview of Ag-based approaches as an intervention or prevention therapy for T1D in humans

Ag	Study name	Treatment	Subjects	Clinical or immunological outcome	Reference
insulin	DPT-1 parenteral arm	0.25 U/kg/d sc + 4 d continuous infusion/y	• T1D relatives • ICA+ • 5-y risk > 50%	No effect	97
Insulin	Diabetes subcutaneous prevention trial in high risk relatives	2 × 0.05 U/kg/d Sc	• 5–39 y • IA-2 Ab+ • No protective haplotype	No effect	98
Insulin	DPT-1 oral arm	7.5 mg po	• T1D relatives • ICA+ • 5-y risk 26–50%	IAA ≥ 80 nU/ml delay onset	99
Insulin	EPPSCIT (European prediabetes prevention-sc insulin trial)	0.2 U/d sc	• T1D relatives • ICA+	No effect	100
Insulin	ORALE	2.5 or 7.5 mg/d po	• 7–40 y • auto-Ab+ • < 2 w diagnosed	No effect	101
Insulin	IMDIAB VII	5 mg/d po	< 4 w diagnosed	No effect	102
Insulin	Oral insulin	1 or 10 mg/d po	• ICA+ • < 4 w diagnosed	>20 y (+ normal baseline C-peptide) slower C-peptide loss no clinical benefit	103
Insulin	DIPP (T1D prediction and prevention study)	1 U/kg/d intranasal	≥ 2 auto-Abs	No effect	104
insulin	INIT (intranasal insulin trial)	40 U/d first 10 d, then 80 U/w 12 mo intranasal	• < 12 mo diagnosed • 30–75 y • GADA+ • fasting C-peptide ≥ 0.2 nM	Tolerance induction but no clinical effect	105
GAD-Alum	Subcutaneous GAD65	4, 20, 100 or 500 μg w 1 and w 4 sc	• LADA patients, • 30–70 y	20 μg; ↑ fasted and stimulated C-response	106
GAD-Alum	Swedish Diamyd	20 μg d 1 and d 30 sc	• 10–18 y • < 18 mo diagnosed • GADA+ • fasting C-peptide >0.1 nM	< 6 min diagnosed: ↑ fasted and stimulated C-response (30 min)	107
GAD-Alum	TrialNet GAD study	2 or 3 × 20 μg at d 1, 4 w and 12 w sc	• 3–45 y • < 100 d diagnosed • GADA+ • stimulated C-peptide > 0.2 nM	No effect	108
GAD-Alum	EU Diamyd	2 or 4 × 20 μg at d 0, d 30, d 90 and d 270 sc	• 10–20 y • < 3 min diagnosed • GADA+ • fasting C-peptide >0.1 nM	No effect	109

For more information on the advantages and disadvantages of an Ag-based approach, see **Table 4**. GAD65, glutamic acid decarboxylase 65; HSP60, heat shock protein 60; sc, subcutaneous; y, years; ICA, islet cell antibody; mo, months; d, day; GADA, GAD-associated antibodies; LADA, latent autoimmune diabetes in adults; w, weeks; po, per os.

Table 2. Overview of Ag-based approaches as an intervention or prevention therapy for T1D in humans (continued)

Ag	Study name	Treatment	Subjects	Clinical or immunological outcome	Reference
DiaPep277 (HSP60)	Trial 420	1 mg at entry, 1, 6 and 12 m sc	• Men • 16–58 y • < 6 mo diagnosed • basal C-peptide > 0.1 nM	Stable C-peptide response	136

For more information on the advantages and disadvantages of an Ag-based approach, see **Table 4**. GAD65, glutamic acid decarboxylase 65; HSP60, heat shock protein 60; sc, subcutaneous; y, years; ICA, islet cell antibody; mo, months; d, day; GADA, GAD-associated antibodies; LADA, latent autoimmune diabetes in adults; w, weeks; po, per os.

insulin or pro-insulin respectively.[105] So it seemed as if nasal insulin pushed the immune system toward a more tolerant state. The most recent effort administered a single intramuscular injection of human insulin B chain emulsified in incomplete Freund's adjuvant to recently-diagnosed T1D patients.[119] First results show a safe profile and an induction of Ag-specific Tregs, so further studies to elucidate the therapeutic efficacy are underway.

GAD65. The enzyme GAD produces the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) by decarboxylation of glutamic acid or glutamate. Besides its expression in the central and peripheral nervous system, GAD is also found in the pancreatic islets, the thymus, testes, ovaries and the stomach.[120] When β-cells get activated due to an increased glucose concentration, they secrete GAD in parallel with insulin.[121] Although the pancreatic function of GAD is not yet elucidated several suggestions have been made. These include a role for GAD as a possible regulator of pancreatic hormone release, a paracrine signal molecule between endocrine cells in the pancreatic islets or a negative regulator of first-phase insulin secretion.[122] To make it even more complex, this enzyme consists of two isoforms, GAD65 and GAD67 (65 kDa and 67 kDa respectively).[123]

Interestingly, several studies already demonstrated a therapeutic capacity of GAD65 in experimental T1D9 models.[124,125] For example, the nasal administration of GAD65 peptides into young NOD animals delayed the development of T1D, by steering T-cell reactivity toward a Th2 response.[126] Moreover, intravenous immunization with full GAD65 protein in insulitis-positive 12-week-old NOD mice was successful in preventing the progression of T1D and accompanied by a Treg induction.[127]

For human application a recombinant human GAD65 was formulated together with aluminum hydroxide (GAD-alum; Diamyd Therapeutics). This conventional adjuvant was chosen because it stimulates a humoral Th2 (T-helper 2) response rather than a cellular pathogenic immune response.[122] A phase two dose-titration study (Subcutaneous GAD65) was designed to assess the general safety profile of GAD-Alum and its possible toxic effect on β-cells.[106] GAD-alum was subcutaneously administered to GAD65 auto-Abs (GADA) LADA patients at week 1 and week 4 (4, 20, 100, or 500 μg). Even after 5 y of follow-up, β-cell function was not compromised, and there were no serious adverse events (SAE), only minor non-treatment related adverse events (AE).[128] As a suggestion for further studies, the 20 μg dose was selected since an increase in fasting C-peptide levels was observed compared with placebo.

The 20 μg dose of GAD-Alum was used in the Swedish Diamyd trial where 70 recently-diagnosed T1D children and adolescents (10–18 y) were assigned to 20 μg of GAD-Alum or placebo at day 1 and day 30.[107] Again no treatment-related SAE or AE could be determined, not even after 4 y of follow up.[129] However the primary endpoint, a change in fasting C-peptide after 15 mo in comparison to placebo, was not achieved. Nevertheless, at 30 mo post-trial initiation, this parameter was stable and significantly increased for a period up to 4 y.[129] Also the stimulated C-peptide secretion was significantly higher than the placebo group at both 15 and 30 mo. However, these differences were only seen in a subpopulation of patients treated within 6 mo of diagnosis.[107] This observation supports the hypothesis that early intervention at a time where sufficient initial β-cell mass is present, increases treatment efficacy. Although the clinical outcome of these patients, as measured by HbA1c levels and required daily insulin dosage, did not change, these study results were promising in that they showed a first proof of concept for GAD-Alum in T1D patients.

Another phase two study (http://www.clinicaltrials.gov; NCT00529399) injected 2 or 3 doses of 20 μg GAD-Alum subcutaneously at treatment start and after 1 and/or 3 mo in T1D patients aged 3–45 y.[108] However, this study failed to demonstrate efficacy as measured by stimulated C-peptide secretion.[108] Even in the subgroup aged 10–18 y no effect was achieved, in contrast to the results obtained by Ludvigsson et al. Also the EU Diamyd phase III study using four doses of 20 μg GAD-Alum did not achieve its endpoints.[109]

Heat shock protein 60 (HSP60). Heat shock proteins (HSP) function mainly as chaperone molecules in protein metabolism. However HSP60 also has modulatory effects on the immune system both in a pro- and anti-inflammatory way, mediated through toll like receptor (TLR) 4 on macrophages and TLR2 on T-cells respectively.[130] In this regard peptide 277, an immunomodulatory peptide derived from positions 437–460 of HSP60, only signals through TLR2 which preferentially drives anti-inflammatory pathways.[130] Furthermore, a single intraperitoneal injection with peptide 277 in 4-week old NOD mice could attenuate diabetes incidence, an effect possibly mediated by a transient Th2 burst.[131-133] These positive results in NOD mice and the HSP60 T-cell reactivity in recent-onset diabetic patients have highlighted peptide 277 as a possible candidate for Ag-specific clinical studies.[134] In order to stabilize peptide 277 chemically without interfering with its immunomodulatory

properties, 2 amino acid substitutions were made before formulating the final product in mannitol and vegetable oil, a combination which is referred to as Diapep277 (Peptor). Implementing this formulation, a phase II study recruited 35 recent-onset T1D patients and assigned them either subcutaneous injections of 1 mg DiaPep277 at entry and after 1 and 6 mo or three times placebo.[135] DiaPep277 treatment conserved β-cell function better than placebo as shown by stable vs. decreased stimulated C-peptide secretion and significantly diminished the exogenous insulin requirements. Raz and colleagues suggested a Th1 to Th2 shift as the underlying mechanism for protective efficacy.[135,136] An extension study was conducted in which a fourth additional dose at 12 mo was included. After a follow-up of 18 mo, the DiaPep277-treated subjects were reassigned to either placebo or an additional four dosages (months 0, 3, 6, and 9; second stage).[136] The stable stimulated C-peptide response could be maintained for the first follow-up period, however for the second stage improved clinical outcome could only be established for the subjects who continued Diapep277 treatment. Prolongation of the study pointed out that DiaPep277 treatment should be continuously administered at a certain interval to prohibit a setback. Several phase III trials to assess the long-term effect of DiaPep277 are now underway and will hopefully determine the value of this intervention.

Why do current approaches not meet their expectations in a clinical setting? Although both preventive and interventional Ag-specific settings seem to work consistently in mouse models, the translation to the clinic remains in general disappointing. There are many parameters that could explain the discrepancy between the promising results obtained in in vivo models and the discouraging results obtained in clinical trials. Although there are many similarities in the onset, development and pathogenesis of T1D between humans and the most studied in vivo models, the NOD mouse and the BB rat, there are as much significant differences.[137] In contrast to the human diversity, the NOD mice are all genetically identical, and besides their diabetic predisposition they are also very sensitive to inflammation and other autoimmune disorders. Another discrepancy is the difference in diabetic incidence between male and female NOD mice. In conclusion, it is a harsh challenge to extrapolate promising regimes from these models to patients. For example, in the parenteral arm of the DPT-1 study a dose of 0.125 U/kg was given twice daily whereas in NOD mice this dose was more than 200-fold higher.[138] However the extrapolation to a similar dose for humans would lead us far beyond the maximal tolerable dose. The anticipated pre-POINT trial (Primary Oral/Intranasal Insulin Trial) tries to address some of these questions by searching for a safe optimal dose and route of administration that could induce a beneficial immune response in genetically at risk children.[139] If an optimal dose would be obtained, the development of an adequate carrier would be inevitable to deliver the auto-Ag in the right concentration at its target organ. Especially the oral administration encounters problems herein, since gastric juices and bile acids degrade the auto-Ag before it can even reach its place of action, namely the mucosa of the gastrointestinal tract for the induction of oral tolerance.

Also the timing of treatment initiation and the frequency of administration are crucial parameters that define the therapeutic outcome. Several studies have already pinpointed that the higher initial β-cell mass, the higher the therapeutic efficacy.[107,136] Thus, the development of clinical biomarkers to diagnose T1D onset in the early stage is vital for a successful clinical approach.

The choice of adjuvant can also define a different outcome. In the GAD65-specific trials, Alum was used for its anti-inflammatory properties. Although Alum as adjuvant has been used before in conventional vaccines for children, it has strong immunomodulatory capacities that need to be considered.[140]

Combination Strategies

In order to restore tolerance, an Ag-specific therapy is by far the most preferred approach due to its oriented specific mode of action and consequently the absence of systemic side effects. To date the focus has been on single Ag vaccines; however, the discussion has started whether the simultaneous administration of multiple Ags would enhance efficacy. In addition, the combination with systemic immunomodulators would hold great potential since several pathways would be addressed simultaneously, increasing the chance of efficacy. In parallel, the systemic dosages linked to a toxic profile could be reduced to non-harmful levels. Different animal studies have already confirmed the great potential of this approach. Synergy was obtained when a low-dose of anti-CD3 was combined with GAD65-expressing DNA vaccine in the RIP-LCMV-GP mouse model. This viral-induced diabetes mouse model expresses the lymphocytic choriomeningitis virus glycoprotein (LCMV-GP) under the control of the rat insulin promoter (RIP).[141] Bresson and colleagues further demonstrated a higher diabetes remission when combining intranasal human pro-insulin II B24–C36 peptide with low doses of anti-CD3 both in the NOD and the RIP-LCMV mouse model.[142] Moreover both the GAD65 and pro-insulin strategy induced the expansion of GAD65 or pro-insulin-specific Tregs respectively.

Another approach demonstrated the potential of combining anti-CD3, cyclosporine A and a bioactive vitamin D_3 analog, TX527, in preventing diabetes remission using transplanted diabetic NOD mice as a recurrence model.[143] Combining these three agents at sub-therapeutic doses yielded a much longer diabetes-free period in comparison to either mono-therapies or dual therapies. Mechanistically they linked the beneficial outcome to reduced pro-inflammatory cytokine production and increased Treg frequencies.

It was postulated that IL-1β blocking could further enhance the therapeutic efficacy of anti-CD3, since IL-1β has a toxic effect on the β-cell and in addition alters T-cell activation and differentiation.[144] The simultaneous administration of anti-CD3 and IL-1β receptor antagonists significantly enhanced diabetes remission in hyperglycaemic NOD mice.[144] Other immune suppressors like Sirolimus (also referred to as Rapamycin), known to inhibit IL-2 mediated T-cell proliferation but not IL-2 mediated T-cell apoptosis, synergistically prevented T1D when combined with IL-2 in female NOD mice.[145] Due to this success a pilot

Table 3. Advantages and disadvantages of a systemic immunomodulatory approach

Advantages
• Due to general approach, efficacy increases
Disadvantages
• General immune suppression
• Systemic side effects
• Extrapolation dose, timing and frequency treatment administration

Table 4. Advantages and disadvantages of an Ag-based approach

Advantages
• Epitope spreading
• High specificity without systemic side effects
Disadvantages
• Time window for success
• Extrapolation dose, timing and frequency treatment administration
• Choice of adjuvants
• Tolerogenic route of administration requires adequate delivery system
• Can boost auto-reactivity

study with recombinant IL-2 (Proleukin) and Sirolimus was conducted in T1D patients diagnosed within the first 3–48 mo.[146] Although this combination therapy increased Treg numbers in the first month, it also transiently impaired β-cell function.

In view of β-cell regeneration exendin-4, an agonist of the glucagon-like peptide-1 (GLP-1) receptor, was also studied in a combinatory setting with anti-CD3 whereby the combination of the two enhanced diabetes remission in NOD mice, whereas exendin-4 alone did not.[147] However, in order for such efforts to bear fruit combination therapies should be rationally designed whereby previous obtained results in mono-therapies and safety profile play determinant roles.[148]

Whether the combination therapy contains auto-Ags, anti-inflammatory molecules or systemic immunomodulators, an optimal delivery of the substance needs to be guaranteed. Recently *Lactococcus lactis* bacteria (*L. lactis*) have been put forward as a new protein carrier to induce oral tolerance by delivering auto-Ags to the mucosa of the gastrointestinal tract. A recent study from our laboratory demonstrated that orally administered *L. lactis* genetically-modified to secrete human pro-insulin and the immunomodulatory cytokine IL-10 can induce long lasting diabetes remission in 59% of recent-onset diabetic NOD mice when combined with a subtherapeutic dose of anti-CD3.[149] This combination therapy induced a functional $CD4^+CD25^+Foxp3^+$ Treg population, located locally in the pancreas and pancreatic-draining lymph nodes. These Tregs were able to suppress both in vitro and in vivo in a diabetes adoptive transfer model. Moreover they could suppress in a pro-insulin-specific way while producing IL-10. Further studies, focusing on *L. lactis* secreting GAD65 or IA-2 peptides, are underway to address whether these Tregs provoke tolerance by further inducing bystander suppression. In addition this combination therapy did not induce a generalized immune suppression since T-cell responses against diabetes-unrelated Ags could be preserved. The use of live *L. lactis* as a carrier approach has been successfully studied before in other disease models and is currently under evaluation in a clinical setting in patients with mucositis. The combination of this save Ag-based strategy with the low-dose of anti-CD3 makes this therapy highly applicable for human studies.

Conclusion and Perspectives

Although several strategies obtained promising results in in vivo models, their clinical translation has been quite disappointing, the latter which at least in part could be attributed to the dissimilarities in T1D immunopathogenesis between in vivo models and patients, as well as the choice of Ag dose, timing, frequency and adjuvant formulation.

Additional questions that need to be addressed: (1) When to consider a study successful or failed? (2) Are the right clinical as well as immunological end-point parameters implemented? These are all parameters that need to be reconsidered for designing the future generation clinical studies.

New therapeutic strategies focusing on both the Ag-specific nature of T1D and the dissociation of systemic immunomodulation and its generalized side effects are very promising. The most appealing approach involves a rationally designed combination strategy using Ag-specific methods combined with immunosuppressive or anti-inflammatory drugs. Although rationale for combining these approaches is there, major hurdles for clinical translation exist, as regulatory bodies demand testing safety and even efficacy of individual therapies before allowing testing or claims on combination approaches. In view of combination strategies the safety profile and therapeutic efficacy of each individual compound should be fully documented and non-overlapping in order to obtain the best result and forestall undesired interactions.

Currently clinical studies lack reliable biomarkers to define therapeutic success. For example, current studies rely on the presence of auto-Abs, proliferative responses to the administered peptides or proteins or induced cell populations, while none of these parameters can be directly correlated with therapeutic success. Several studies focus on physiologic parameters, like C-peptide or blood glucose levels, to measure their induced efficacy. The setting of accurate clinical endpoints and the discovery of new biomarkers capable of identifying different stages of the disease are absolutely essential for optimizing future trial set-ups. Biomarkers will also aid in distinguishing responders from non-responders by setting clearer end-point parameters and can help distinguish a non-successful therapy from a non-successful therapy with immunological effects but no clinical impact. In conclusion, reliable biomarkers are absolutely crucial and currently hot topics in T1D research.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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