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Antigen-Specific Therapeutic Approaches in Type 1 Diabetes

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Development of strategies capable of specifically curbing pathogenic autoimmune responses in a disease- and organ-specific manner without impairing foreign or tumor antigen-specific immune responses represents a long sought-after goal in autoimmune disease research. Unfortunately, our current understanding of the intricate details of the different autoimmune diseases that affect mankind, including type 1 diabetes, is rudimentary. As a result, progress in the development of the so-called “antigen-specific” therapies for autoimmunity has been slow and fraught with limitations that interfere with bench-to-bedside translation. Absent or incomplete understanding of mechanisms of action and lack of adequate immunological biomarkers, for example, preclude the rational design of effective drug development programs. Here, we provide an overview of antigen-specific approaches that have been tested in preclinical models of T1D and, in some cases, human subjects. The evidence suggests that effective translation of these approaches through clinical trials and into patients will continue to meet with failure unless detailed mechanisms of action at the level of the organism are defined.

Current approaches toward a potential cure for Type 1 diabetes (T1D) have focused on three main targets: (1) ablation of the β -cell-specific autoimmune response; (2) β -cell replacement therapy using islet transplantation; and (3) potentiation of β -cell mass and function using pharmacologic agents capable of promoting β -cell proliferation, regeneration and/or repair.

Pancreas and islet-transplantation in the context of systemic immunosuppression are the only approaches that have afforded patients complete independence from exogenous insulin. However,

they have also highlighted the fact that, in the absence of immunosuppression, transplantation invariably meets with failure. For example, although 70% of islet-grafted patients remain insulin independent 1 year after transplantation, a significant fraction of them revert to insulin-dependency within 5 years, albeit with significantly lower insulin needs (Shapiro et al. 2000, 2006; Merani and Shapiro 2006; Robertson 2010). Because disease recurrence is typically associated with an anamnestic autoimmune response against the grafted tissue (Laughlin et al.

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2008; Monti et al. 2008; Velthuis et al. 2009), all approaches that aim at improving β -cell mass and function must be accompanied by some sort of immunosuppression.

This article provides an overview of antigen-specific strategies for the prevention and/or treatment of T1D, from their conception and testing in preclinical models of T1D (Table 1) to their translation into clinical trials (Table 2). We will discuss these approaches in order of increasing complexity, from peptide-based therapies, with or without adjuvants, through more exotic approaches such as administration of dendritic cell (DC)-targeted compounds, or DNA and peptide-major histocompatibility complex (pMHC)-based vaccines, to more elaborate antigen-specific cell transfer strategies (Fig. 1).

PROTEIN/PEPTIDE-BASED IMMUNOTHERAPIES

GAD

Glutamic acid decarboxylase (GAD) is the rate-limiting enzyme that converts glutamic acid into γ aminobutyric acid (GABA). Human and/or mouse pancreatic β cells express up to two isoforms of GAD (Christgau et al. 1991; Erlander et al. 1991; Bu et al. 1992); whereas human β cells only express the 64 kDa isoform (GAD65) (Karlsen et al. 1992), their murine counterparts predominantly express the 67 kDa isoform (GAD67) (Faulkner-Jones et al. 1993).

Early studies suggested that GAD might be an important autoantigen in T1D. Anti-GAD antibodies (Baekkeskov et al. 1982, 1987; Christie et al. 1992; De Aizpurua et al. 1992; Thivolet et al. 1992; Atkinson et al. 1993; Seissler et al. 1993; Tuomilehto et al. 1994; Verge et al. 1996) as well as GAD-reactive T cells (Honeyman et al. 1993; Panina-Bordignon et al. 1995; Rudy et al. 1995; Endl et al. 1997) were found in patients with preclinical or recent-onset T1D. In NOD mice, GAD autoreactivity was found at as early as 4 weeks of age (Kaufman et al. 1993; Tisch et al. 1993; Elliott et al. 1994). Notwithstanding the overwhelming evidence supporting a role for GAD autoreactivity in T1D, the role of GAD in diabetogenesis remains unclear. For example, whereas some

GAD-reactive CD4⁺ T-cell clones are diabetogenic, others afford T1D protection (Zekzer et al. 1998; You et al. 2004). Likewise, although suppression of β -cell-specific GAD65/67 expression via a GAD antisense RNA approach prevented T1D in NOD mice (Yoon et al. 1999), induction of GAD-specific tolerance by transgenic overexpression of GAD did not (Bridgett et al. 1998; Geng et al. 1998; Jaecel et al. 2003; Tian et al. 2009).

The identification of GAD as a prevalent autoantigen in T1D prompted several groups to attempt to inhibit the progression of disease by tolerizing GAD-reactive T cells. Young NOD mice treated intravenously with recombinant GAD65 (Kaufman et al. 1993; Ramiya et al. 1997; Tisch et al. 1999) or GAD67 (Elliott et al. 1994) were significantly protected from T1D, an effect that was ascribed to induction of GAD-specific T-cell tolerance. Administration of GAD through other routes, including intrathymic (Tisch et al. 1993; Cetkovic-Cvrlje et al. 1997), intraperitoneal (Pleau et al. 1995), intranasal (Tian et al. 1996), or oral routes (in the form of GAD67- [Ma et al. 1997] or GAD65/IL-4- transgenic plant tissues [Ma et al. 2004]) also had antidiabetogenic effects. Rather than inducing classical forms of T-cell tolerance, some of these approaches induced GAD-specific Th2 responses, elevating serum IgG1 levels while decreasing GAD-specific IFN γ production (Tian et al. 1996; Ma et al. 1997; Carter et al. 2006). Whether these antigen-specific Th2 responses were directly responsible for disease protection, however, was not formally established.

Other groups attempted to induce GAD-specific T-cell tolerance using GAD-derived peptides. Intraperitoneal injection of GAD65_{217–236}, GAD65_{247–265}, GAD65_{290–309}, and GAD65_{524–543} peptides emulsified in incomplete Freund's adjuvant (IFA) afforded disease protection when given at the onset of insulinitis, but were largely ineffective when given to prediabetic mice (Tisch et al. 1999). Intraperitoneal delivery of GAD_{206–220} and GAD_{221–235} in IFA elicited peptide-specific IFN γ /IL-10-secreting Tr1-like T cells capable of inhibiting disease transfer by NOD splenic T cells into NOD.scid hosts (Chen et al. 2003). Nasal or oral delivery of



Table 1. Summary of preclinical studies addressing the remission of hyperglycemic state

Immunotherapy	Antigen	Criteria for treatment	Protocol	Route ¹	Results	Reference	Clinical trial?
Peptide	hsp60 _{437–460} (p277)	15- to 17-wk-old female NOD (50%–80% diabetic mice >11.1 mM)	50 µg	s.c.	~100% survival at 40 wk. Mild hyperglycemia very few reverted to euglycemia	Elias et al. 1994	YES
Ig-peptide fusion	GAD65 _{206–220}	NOD females with BG between 8.9 mM and 13.9 mM for 2 wk were treated. Age of onset 14–30 wk	300 µg/d for 5 d, then 1 × 300 µg/wk until 52–56 wk	i.p.	Complete reversal of hyperglycemia with continuous weekly boosting. 60% remain diabetes free at 24 wk after treatment after withdrawal of boosting	Jain et al. 2008	NO
DNA vaccination	GAD65 and BAX	8-wk-old or older NOD females with fasting blood glucose >7.8 mM	50 µg/wk for 8 wk	s.c.	Mice considered cured if free of glycosuria and FBG <16.7 mM. 48% diabetes-free at 40 wk of age; 8% diabetes-free in control group. Treatment -induced GAD-specific CD4 ⁺ Tregs	Li et al. 2006	NO
Peptide/IFA	InsB _{9–23}	>10-wk-old NOD females with blood glucose 10–13.9 mM	100 µg 1 × at diagnosis	s.c.	52% diabetes-free at 35 wk. Induction of Tregs (not Ag-specific) that can transfer protection. Neutralization of either IL-10 or IFNγ abrogated protection	Fousteri et al. 2010	NO
Peptide/IFA	InsB _{9–23}	>10-wk-old NOD females with blood glucose 13.9–19.4 mM	100 µg 1 × at diagnosis	s.c.	No remission	Fousteri et al. 2010	NO

Continued

Antigen-Specific Therapies in T1D

Table 1. *Continued*

Immunotherapy	Antigen	Criteria for treatment	Protocol	Route ¹	Results	Reference	Clinical trial?
DNA vaccination	PPIs or PIns	NOD females with first BG 10.5–13.9 mM, and second BG of 9.4–16.7 mM	50 µg/wk or biweekly or monthly	i.m.	Weekly injection of PIns II-encoding high expression plasmid resulted in reversal of hyperglycemia in 70% of animals, while the original PIns II plasmid afforded 40% hyperglycemia reversal. PPIs I plasmids injection afforded marginal protection	Solvason et al. 2008	Phase I/II in progress
Peptide: MHC dimer	HA _{110–120}	RIP-HA/TCR-HA mice >11 mM	2 µg every 5 d up to age of 5 mo	i.v.	50%–60% reversal of hyperglycemia. IL-10-mediated suppression	Casares et al. 2002	NO
Peptide: MHC dimer	GAD65 _{217–230}	NOD females >13.9–19.4 mM	1 × or 4 × weekly 5 µg	i.v.	20% and 80% reversal in 1 × and 4 × groups, respectively	Lin et al. 2010	NO
Peptide: MHC dimer	BDC2.5 mimotope	NOD females >13.9 mM	10 µg	Retro-orbital	No protection	Masteller et al. 2003	NO
Peptide: MHC coated nanoparticles	NRP-V7, MimA2, IGRP _{265–273} , InsB _{10–18}	NOD or HLA-A2 NOD females (11–18 mM)	7.5 µg of Fe	i.v.	75% reversal of hyperglycemia and clearance of insulinitis, through IFNγ- and IDO-dependent mechanism and down-regulation/killing of APC	Tsai et al. 2010	NO
Ex vivo expanded Foxp3 ⁺ Tregs	BDC2.5	NOD females >16.6 mM	10 ⁷ Tregs	i.v.	60% reversal of hyperglycemia	Tang et al. 2004	NO
ECDI-fixed splenocytes	Insulin, GAD65 _{206–220} , GAD _{217–236} , GAD _{524–536} , NRP-V7	NOD females >13.9 mM	50 × 10 ⁶ splenocytes with 0.5 mg/mL antigen	i.v.	50% reversal of hyperglycemia in insulin ECDI-treated mice, but not other antigens	Fife et al. 2006	Planned

¹Abbreviations: s.c. subcutaneous; i.p. intraperitoneal; i.m. intramuscular; i.v. intravenous.



Table 2. Summary of clinical studies

Immunotherapy	Antigen	Criteria for treatment	Protocol	Route	Results	Reference	Tested in hyperglycemic NOD mice?
Whole Ag	Insulin	Recent onset diabetic patients <4 mo after diagnosis	5 mg/d for 12 mo	Oral	No effect in preventing c-peptide loss. In patients <15 yr c-peptide levels were lower in the treatment group at 9 and 12 mo. No differences in insulin requirement	Pozzilli et al. 2000	NO
Whole Ag	Insulin	Recent onset diabetic patients <2 wk after diagnosis	2.5 mg/d or 7.5 mg/d for 12 mo	Oral	No effect in preventing c-peptide loss. No differences in insulin requirement between treated and placebo	Chaillous et al. 2000	NO
Ag/IFA	Insulin B chain	Recent onset diabetic patients <3 mo after diagnosis	2 mg single injection	i.m.	No difference in stimulated c-peptide over 2 yr	Orban et al. 2010	NO
Ag/alum	GAD65	Recent onset diabetic patients <18 mo after diagnosis	20 µg on d1 and 30	s.c.	No effect in patients treated >6 mo after diagnosis. Decline in fasting and stimulated c-peptide is significantly slower in GAD-alum-treated group at 30 mo follow-up	Ludvigsson et al. 2008	NO
Ag/alum	GAD65	Recent onset diabetic patients <3 mo after diagnosis	20 µg on d1 and 30, or 20 µg on d1, 30, 90, and 270	s.c.	No effect in preserving stimulated c-peptide at 15 mo follow-up	Dyamid Medical, Press Release, June 1, 2011	NO
Peptide	hsp60 ₄₃₇₋₄₆₀ (p277)	Recent onset diabetic patients <6 mo after diagnosis	1 mg at 0, 1, and 6 mo; extension to 12 mo	s.c.	Maintains c-peptide production. Lower insulin requirements. At 18 mo c-peptide is maintained but same requirements of insulin as controls	Raz et al. 2001, 2007	YES

Continued

Antigen-Specific Therapies in T1D



Table 2. *Continued*

Immunotherapy	Antigen	Criteria for treatment	Protocol	Route	Results	Reference	Tested in hyperglycemic NOD mice?
Peptide	hsp60 _{437–460} (p277)	Diabetic patients. <42 mo after diagnosis	0.2, 1, 2.5 mg at 0, 1, 6, and 12 mo	s.c.	Maintains c-peptide in first 12 mo at 2.5 mg. No changes in insulin requirements	Huurman et al. 2007, 2008	YES
Peptide	hsp60 _{437–460} (p277)	Adult diabetic patients per group. <3 mo after diagnosis	0.2, 1, 2.5 mg at 0, 1, 6, and 12 mo	s.c.	At 18 mo, 0.1 and 1 mg groups had stable c-peptide. Stable insulin requirements	Schloot et al. 2007	YES
Peptide	hsp60 _{437–460} (p277)	Pediatric diabetic patients. <3 mo after diagnosis	0.2, 1, and 2.5 mg at 0, 1, 6, and 12 mo	s.c.	At 7 and 18 mo decrease in c-peptide and increase in insulin requirement	Schloot et al. 2007	YES
Peptide	hsp60 _{437–460} (p277)	Pediatric diabetic patients. <6 mo after diagnosis	1 mg at 0, 1, 6, and 12 mo	s.c.	At 6 mo and onward decrease in c-peptide and increase in insulin requirement	Lazar et al. 2007	YES
APL	InsB _{9–23}	Teen and 16 adults diabetic patients. <6 mo after diagnosis	0.1, 1, or 5 mg at 0, 2, 4, 6, and 8 wk	s.c.	During 25- wk follow-up, reduction of IFN γ responses against the APL or the natural peptide in the 5 mg group. Sporadic increase of IL-5 in the other doses	Alleva et al. 2006	NO
APL	InsB _{9–23}	Adolescent and adult diabetic patients. <6 mo after diagnosis	0.1, 0.5, and 1 mg at 0, 2, 4, and 23 more monthly injections	s.c.	After 24 months of follow-up, no differences in c-peptide, HbA _{1c} , or insulin requirements could be identified between treated groups and placebo	Walter et al. 2009	NO
DNA vaccine	PIns	Adult patients. <5 yr of diagnosis	1 mg weekly for 12 injections	i.m.	Preliminary at 6 mo of follow-up: prevention of the decline of C-peptide. Reduction of IAA and GAD autoantibodies	Gottlieb et al. 2008	YES

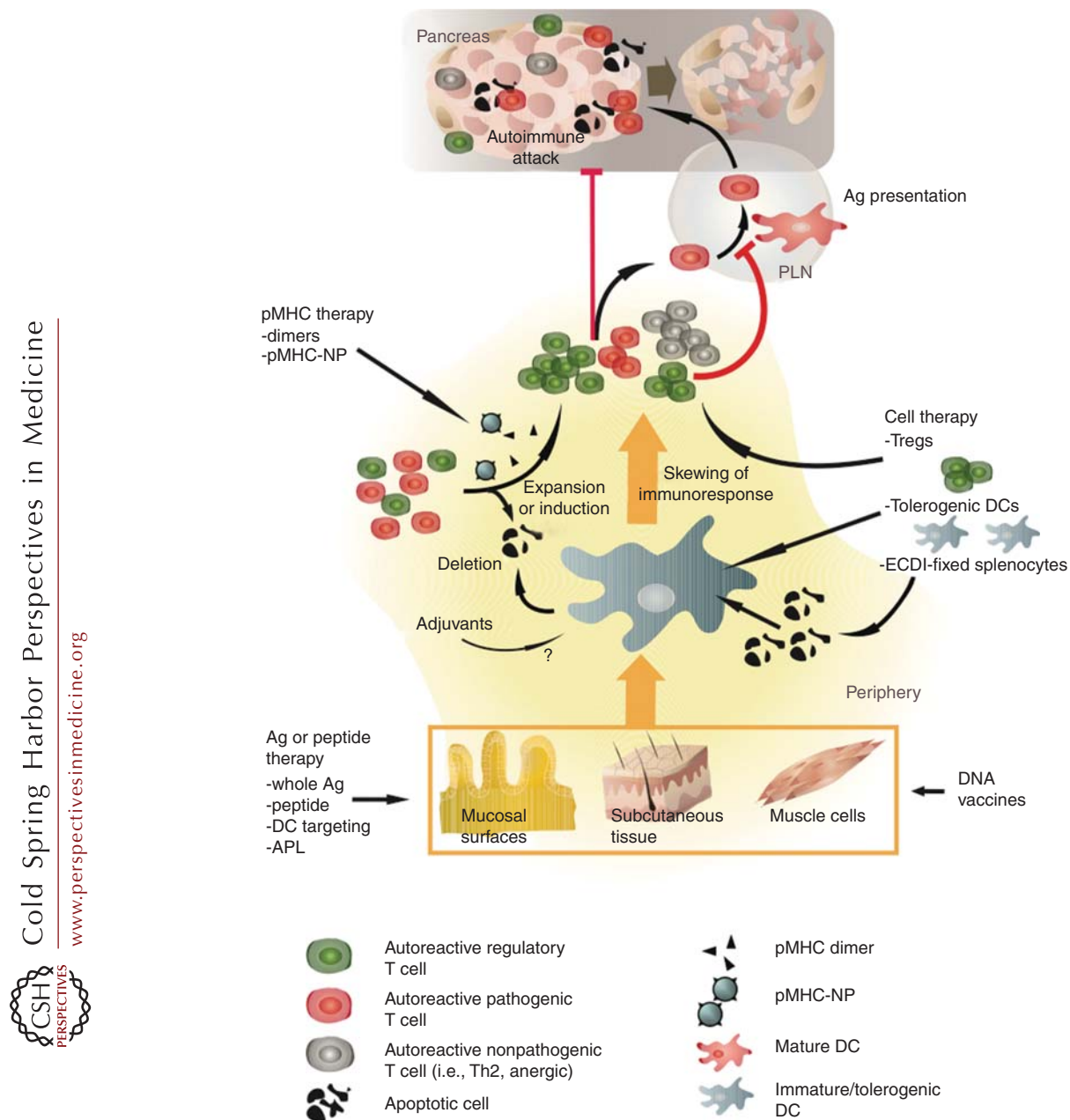


Figure 1. Putative mechanisms of action of antigen-specific therapies. Diverse mechanisms have been proposed to underlie the suppression of autoimmunity by antigen-specific approaches. This cartoon provides an overview of these mechanisms with the caveat that only a few of them have been documented formally. Capture of exogenous-derived autoantigens (i.e., naturally occurring peptides, APLs, proteins, ECDI-fixed splenocytes, or antigens encoded in DNA vaccines) in peripheral compartments by immature DCs can lead to the deletion of pathogenic effectors and/or induction of nonpathogenic/regulatory T cells. This tolerogenic effect can be enhanced with the use of apoptotic signals (ECDI) or adjuvants (IFA, alum). Strategies based on pMHC administration appear to function by activating autoreactive Treg subsets, which then go on to suppress the activation of pathogenic effectors through a number of mechanisms.

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GAD_{524–543} followed by injection of complete Freund's adjuvant (CFA) in the footpad resulted in the generation of Th2-like T cells that secreted IL-4, IL-10, and TGF β and could suppress diabetes transfer by NOD splenic T cells (Maron et al. 1999). Likewise, intranasal administration of GAD_{247–266}, GAD_{509–527}, and GAD_{524–543} delayed islet graft rejection in a syngeneic model of islet transplantation in NOD mice, in association with enhanced IL-10 and decreased IFN γ production in GAD antigen-induced recall responses (Ravanan et al. 2007).

Alum-formulated GAD has been tested in human clinical trials for safety and efficacy as an anti-T1D therapy. Preclinical studies and a phase I clinical trial sponsored by Diamyd Therapeutics found that administration of recombinant human GAD with or without adjuvants did not induce adverse side effects or exacerbate T1D in man and mice (Plesner et al. 1998; Uibo and Lernmark 2008). A subsequent phase II trial in LADA (Latent Autoimmune Diabetes in Adults) patients receiving placebo or two doses of alum-formulated GAD (Diamyd) injected subcutaneously at weeks 1 and 4, confirmed the safety of this approach. The 20 μ g dose was found to preserve insulin secretion/C-peptide production over the 24 week study period, and patients receiving Diamyd at this dose had increased CD4⁺ CD25⁺/CD4⁺ CD25[–] ratios in peripheral blood (Agardh et al. 2005). A subsequent phase IIb study tested the therapeutic effects of two doses of 20 μ g of Diamyd given 4 weeks apart to 10–18 year-old new onset diabetic patients (Ludvigsson et al. 2008). At 30 (but not 15) weeks after the initiation of treatment, fasting and stimulated C-peptide levels in the Diamyd-treated group were significantly higher than in the placebo-treated cohort. GAD treatment increased serum GAD autoantibody levels, Foxp3 and TGF β gene transcription, as well as IFN γ , IL-5, IL-10, IL-13, IL-17, and TNF α expression in PBMCs (Ludvigsson et al. 2008). Further analysis of the collected samples revealed an early (one month after treatment) and sustained (9 months after treatment) increase in GAD-specific IL-5 and IL-13 responses (Axelsson et al. 2010). GAD-alum treatment also led to increases in IgG3/IgG4 and decreases in

IgG1 (Cheramy et al. 2010), as well as increases in GAD65-specific CD4⁺ CD25^{hi} Foxp3⁺ T cells (Hjorth et al. 2011). Patients who had been treated with GAD-alum within 6 months of diagnosis had better preservation of fasting C-peptide levels than those that were treated later (Ludvigsson et al. 2011), although these beneficial effects did not translate into reduced insulin requirement.

Casting a shadow on these apparently encouraging results are two recent Phase II/III studies (performed independently by Diamyd and TrialNet), which failed to meet their primary efficacy endpoint in preserving insulin production (Wherrett et al. 2011). To the best of our knowledge, however, alum-formulated GAD has not been reported to reverse hyperglycaemia in NOD mice (Table 1). Thus, in the absence of adequate preclinical work in diabetic mice, it is not possible to rationalize whether lack of efficacy in the human trials was related to the choice of route of administration, antigen formulation, dosing or other factors.

Insulin

Insulin was the first β -cell autoantigen described in T1D patients (Palmer et al. 1983). The biologically active form of insulin is processed from its precursor, preproinsulin (PPIs), by sequential enzymatic cleavages that release the leader peptide (to make proinsulin; PIns) and the C-peptide. The role of insulin as a T1D autoantigen in both humans and NOD mice has been extensively reviewed elsewhere (Zhang et al. 2008). In humans, autoantibodies specific for both insulin and PIns are present in recent-onset diabetic patients (Kuglin et al. 1988; Yu et al. 2000), and T1D-associated T cells target multiple epitopes within insulin and its precursors (Lieberman and DiLorenzo 2003). In NOD mice, presence of insulin autoantibodies at 8 weeks of age is associated with high risk of early T1D onset (Yu et al. 2000), and prevalent intra-islet insulin-specific CD4⁺ T-cell responses have been documented in prediabetic NOD mice (Wegmann et al. 1994a,b). Importantly, there is substantial evidence pointing to insulin as an initiating autoantigen in T1D (Nakayama et al. 2005).

Administration of insulin through the oral (Zhang et al. 1991; Sai et al. 1996), parenteral (Atkinson et al. 1990), nasal (Harrison et al. 1996; Aspod and Thivolet 2002) and intravenous (Hutchings and Cooke 1995) routes has shown efficacy in preventing T1D in NOD mice. In some instances, conjugation of insulin to an adjuvant such as cholera toxin enhanced the therapeutic efficacy of insulin administration (Harrison et al. 1996; Bergerot et al. 1997; Aspod and Thivolet 2002). Subcutaneous immunization with insulin B chain in IFA at 4, 12, 16, and 22 weeks of age also afforded significant protection, although immunization with insulin A chain did not (Muir et al. 1995; Coon et al. 1999).

The efficacy of insulin-based vaccination using peptides, as opposed to full-length PPIs, PIs or insulin, has proven to be epitope-dependent and highly sensitive to the timing and route of administration. Certain combinations of routes and epitopes favor tolerance whereas others accelerate disease (Hutchings and Cooke 1998; Chen et al. 2001). For example, whereas intraperitoneal vaccination with PIs B₂₄-C₃₃ at 18 days of age reduced the incidence and delayed the onset of T1D, subcutaneous immunization with B₂₄-C₃₃ promoted disease acceleration (Chen et al. 2001). Intranasal delivery of insulin and PIs peptides has largely had antidiabetogenic effects, as shown by studies using B₂₄-C₃₃, B₂₄-C₃₂ and B₉-₂₃ (Martinez et al. 2003; Daniel and Wegmann 1996a,b). Furthermore, intranasal delivery of PIs B₂₄-C₃₃ (CTL-epitope mutated at C34) synergized with anti-CD3 mAb therapy to expand antigen-specific IL-10- and TGFβ-producing CD4⁺ Foxp3⁺ Tregs and reverse hyperglycemia in recent-onset diabetic animals (Bresson et al. 2006). Subcutaneous delivery of insulin peptides has yielded mixed results. For example, whereas immunization with the insulin-1 B₉-₂₃ peptide starting at 4 weeks of age accelerated T1D, immunization with insulin-2 B₉-₂₃ peptide afforded T1D protection (Devendra et al. 2004). In one study, subcutaneous injection of insulin B₉-₂₃ or B₁₃-₂₃ generated high titres of autoantibodies and caused fatal anaphylaxis in NOD mice after 6 weeks of treatment (Liu et al. 2002). Immunization with

insulin B₉-₂₃ emulsified in IFA delayed the onset and lowered the incidence of T1D in preinsulinitic or prediabetic NOD females (Muir et al. 1995; Daniel and Wegmann 1996a; Hutchings and Cooke 1998; Foustieri et al. 2010) but failed to reverse hyperglycaemia in recent onset diabetic mice (Foustieri et al. 2010). This treatment induced CD4⁺ CD25⁺ Tregs and relied on IL-10 and IFNγ for its protective function (Foustieri et al. 2010).

The human clinical trials conducted to date using insulin as a therapeutic or prophylactic immunotherapy have yielded disappointing results. In a safety-assessment trial, individuals at risk of developing T1D were treated with 1.6 mg of aerosolized insulin daily for 10 consecutive days, followed by 2 days/week of 1.6 mg for 6 months. Treatment did not generate adverse side effects or accelerate β-cell loss, but decreased insulin-specific T-cell responses (Harrison et al. 2004). A subsequent study in recent-onset diabetic individuals showed that intranasal insulin decreased serum insulin autoantibodies (IAAs) and, in a subset of patients, reduced insulin-specific T-cell (IFNγ) responses. The treatment, however, did not delay β-cell destruction (Fourlanos et al. 2011). In the Finnish T1D Prediction and Prevention Study (DIPP), intranasal insulin (1 unit/kg daily) did not delay or prevent T1D when given to infants carrying high risk HLA haplotypes or to siblings positive for two or more T1D-associated autoantibodies (Nanto-Salonen et al. 2008). As part of the T1D Prevention Trial (DPT-1), a study was conducted to test the efficacy of ultralente insulin (0.25 unit/Kg/d subcutaneously and one annual 4 day continuous intravenous infusion) as a prophylactic vaccine in at risk individuals presenting with a 5-year projected risk of >50%. The results showed that this protocol did not prevent or delay T1D development over a median 3.7 year follow-up period (Diabetes Prevention Trial—Type 1 Diabetes Study Group 2002). In another DPT-1 trial, first- or second-degree relatives with a 5-yr projected risk of 26%–50% (determined by metabolic, immunological, and genetic staging) received oral insulin (7.5 mg/d) or placebo. The median follow-up was 4.3 years. Overall, the treatment did not prevent or delay

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T1D (Skyler et al. 2005). However, subgroup analyses revealed that oral insulin had beneficial effects in patients with high IAA levels. Based on these results, TrialNet is conducting an Oral Insulin Prevention Trial enrolling subjects with similar characteristics to the subgroup mentioned above—relatives with normal glucose tolerance carrying at least two autoantibody specificities in serum, one of which must be anti-insulin, and presenting with 35% risk of T1D within 5 years (Skyler et al. 2008). In the immunotherapy T1D (IMDIAB) trial, oral insulin (5 mg/d) was given in conjunction with intensive subcutaneous insulin therapy to recent-onset diabetic patients within 4 weeks of diagnosis for 12 months. The results suggested that oral insulin failed to preserve C-peptide levels and reduce insulin requirement at 12 months of follow-up (Pozzilli et al. 2000). Furthermore, there was evidence of accelerated β -cell loss in patients younger than 15 years. In the T1D Insuline Orale study, daily oral insulin administration (2.5 mg or 7.5 mg/d) also failed to blunt established T1D in recent-onset diabetic patients (Chaillous et al. 2000). Lastly, a recent phase I clinical trial with 12 recent-onset diabetic subjects (within 3 months of diagnosis) undergoing treatment with a single intramuscular injection of insulin B chain/IFA showed that this approach elicits robust antibody and T-cell responses against insulin without causing adverse events (Orban et al. 2010). However, no significant differences in C-peptide level between the two groups could be detected. Clearly, unless the protocols that are tested in humans are previously tested in rodents at similar stages of disease progression, and the mechanisms of action explored fully, testing of similar immunotherapeutic approaches in clinical trials will be less likely to be successful.

Hsp60

In 1990, Cohen's group identified antibody and T-cell reactivity against the micobacterial heat shock protein 6 (hsp60) as well as its human and murine homologs during the prediabetic stage (Elias et al. 1990, 1991; Birk et al. 1996). Hsp60-reactive T-cell clones were found to

have diabetogenic properties (Elias et al. 1990, 1995). In humans, 87% of T1D patients showed a strong but transient T-cell reactivity toward multiple epitopes of hsp60 (Abulafia-Lapid et al. 1999, 2003).

Administration of hsp60 or an immunodominant hsp60 peptide spanning residues 437–460 (p277) in IFA or mineral oil prevented (Elias et al. 1991, 1997) and delayed (but did not reverse) the progression of hyperglycemia in NOD mice (Elias and Cohen 1994). This was associated with the induction of an antigen-specific shift from a Th1 (IFN γ) to Th2 (IL-5 and IL-10) response (Elias et al. 1991; Ablamunits et al. 1998) and a form of cell-mediated, transferable tolerance (Elias and Cohen 1995). Because hsp60 and p277 also bind to TLR2, promoting a series of anti-inflammatory events, such as up-regulation of SOCS3 leading to a reduction of T-cell chemotaxis and enhancement of Treg function (Zanin-Zhorov et al. 2005, 2006; Nussbaum et al. 2006), the mechanisms by which hsp60 might afford protection remain unclear.

Peptor and DeveloGen, currently known as Andromeda Biotech, performed several clinical trials to determine the tolerability and therapeutic efficacy of a variant of p277 in which the two cysteines (6 and 11) were mutated to valines to increase stability (referred to as Dia-pep277). These trials have been extensively reviewed elsewhere and are not discussed here in detail (Fischer et al. 2010). In initial phase II clinical trials, C-peptide production was maintained after several months of follow-up (Raz et al. 2001; Huurman et al. 2007, 2008), but the therapeutic effects were marginal overall. Another trial involving adult patients also showed a marginal trend toward improved maintenance of β -cell function for some of the doses that were tested (Schloot et al. 2007). None of the two trials involving pediatric patients was able to identify differences in C-peptide loss between groups (Lazar et al. 2007; Schloot et al. 2007). Patients with p277-specific responses prior to treatment (baseline responders) changed their cytokine responses toward IL-10 or showed a reduction in p277-specific responses. Both effects correlated with better preservation of β -cell function. Two additional clinical trials were subsequently

performed in LADA patients with inconclusive results (Fischer et al. 2010). The first phase III trial started in 2005, and the follow-up was for 24 months with an extended 21-month study (Fischer et al. 2010).

DC-targeted Peptide Therapy

In the steady state (i.e., absence of danger signals) DCs display an immature phenotype characterized by low expression of MHC and costimulatory molecules. These so-called immature DCs present antigens but do not generate an immunogenic response (Steinman et al. 2003). Rather, engagement of cognate pMHC by T-cells on immature DCs results in tolerogenic responses, including the deletion of cognate T-cells (Bonifaz et al. 2002) and/or the induction/expansion of regulatory T cells (Mahnke et al. 2003).

These observations predicted that targeting antigens to immature DCs should promote tolerance induction. One of the most widely used approaches to target antigens to DCs is to conjugate the antigen (a protein or peptide) to an antibody against DEC-205 (CD205), a C-type lectin that functions as an endocytosis receptor (Jiang et al. 1995). Antigen-coupled anti-DEC-205 antibodies are efficiently captured and processed by immature DCs. In 2005, Bruder et al. tested the ability of this approach to blunt diabetogenesis in a transgenic mouse model of T1D expressing the influenza A/PR/8/34 virus hemagglutinin (HA) under the control of the rat insulin promoter on β cells and the HA_{110–120}-specific 14.3.d TCR (RIP-HA/TCR-HA) on T cells (Bruder et al. 2005). Treatment of newborn mice with HA linked to anti-DEC-205 prevented the development of T1D, and this was associated with increased recruitment of T cells expressing Foxp3, CTLA-4, and IL-10. Subsequent application of this approach in a polyclonal system led to mixed results, with treatment outcome being a function of the autoantigen used (Petzold et al. 2011). For example, in mice treated with anti-DEC-205 mAb conjugated with a mimotope of the β -cell autoreactive BDC2.5 CD4⁺ T-cell clone, there was an increase in the number of cognate CD4⁺ T-

cells expressing Foxp3 but this had no effect on disease progression. In contrast, treatment with PIns-conjugated anti-DEC-205 mAb afforded significant protection from T1D, presumably because this antigen plays a dominant role in disease initiation. In another study, anti-DEC-205 mAb conjugated with a mimotope of the T1D autoantigen dystrophin myotonia kinase (MimA2) induced the deletion and anergy of cognate TCR-transgenic CD8⁺ T-cells (Mukhopadhyaya et al. 2008). Whether this approach can blunt diabetogenesis in nontransgenic NOD mice has not been determined.

A similar approach involves incorporating autoantigenic peptide sequences within the CDR3 region of an immunoglobulin molecule (Gregg et al. 2004; Jain et al. 2008; Tartar et al. 2010). In this case, binding of the autoantigen-encoding Ig molecule to Fc γ on APCs enables the uptake, processing and presentation of the autoantigen by immature DCs (Brumeanu et al. 1993; Zaghoulani et al. 1993). Intraperitoneal injections of aggregated but not soluble Ig-GAD65_{524–543} to preinsulinitic NOD mice expanded cognate Foxp3⁺ Tregs and afforded T1D protection. However, treatment was ineffective when initiated at the prediabetic or diabetic stages (Gregg et al. 2004). In another study, intraperitoneal injections of soluble Ig-GAD65_{206–220} failed to prevent T1D in 4–6 week-old NOD mice, but blunted disease progression in prediabetic and new-onset diabetic animals, in an IFN γ -dependent manner (Jain et al. 2008). These results suggest that the therapeutic efficacy of this approach is both epitope- and disease stage-dependent.

Altered Peptide Ligands (APLs)

APLs (Evavold and Allen 1991; Evavold et al. 1993; Sloan-Lancaster and Allen 1996) can be classified according to the type of T-cell response that they induce. Antagonists block T-cell activation by cognate agonists; partial agonists induce an incomplete or alternative activation state in responder T cells; and superagonists trigger stronger activation responses than the natural agonists. The potential ability of APLs to compete with agonist peptides for MHC

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binding, to trigger immune deviation, or to induce anergy or deletion of cognate T cells suggested that APLs might be useful tools as autoimmune disease therapeutic agents. Induction of disease exacerbation in MS clinical trials (Bielekova et al. 2000; Kappos et al. 2000), however, exposed the potential dangers of this approach. The clonal complexity (and TCR diversity) of the individual antigenic T-cell specificities targeted by specific APLs cannot guarantee a tolerogenic outcome; some clones may see the APL as a superagonist (Anderton et al. 1998).

In one of the first reports using T1D-relevant APLs, APLs of Imogen 38 were shown to block antigen-induced activation of a cognate human T-cell clone, albeit through unclear mechanisms (Geluk et al. 1998). The APL experience in the NOD model of T1D has been largely disappointing. In 2000, we reported that treatment of prediabetic NOD mice with agonistic mimotopes of IGRP_{206–214}-specific CD8⁺ T cells (NRP and NRP-A7) could effectively inhibit disease progression by blunting the avidity maturation of this T-cell subset (Amrani et al. 2000). We subsequently discovered that these mimotopes did not afford disease protection by selectively deleting high-avidity clonotypes, as we had proposed, but rather indirectly, by fostering the recruitment of their low-avidity counterparts (Han et al. 2005). We have recently shown that these low avidity clonotypes are a source of a negative feedback regulatory loop (memory-like autoregulatory CD8⁺ T cells) that aims to counter disease progression effected by high avidity clonotypes (Tsai et al. 2010). In fact, complete deletion of the IGRP_{206–214}-specific CD8⁺ T-cell pool (including low-avidity clonotypes) by high doses of a superagonistic mimotope (NRP-V7) failed to blunt disease progression in prediabetic NOD mice. Furthermore, nondeletion doses of partial (NRP-I4) or full agonists (NRP-A7) could also afford disease protection, but this effect was only seen over very narrow, APL-specific dose ranges in which treatment fostered the recruitment of low-avidity clonotypes. Thus, near complete deletion of a single antigenic-specificity in a complex autoimmune disease is unlikely to have therapeutic value, un-

less such antigenic specificity(ies) play(s) a critical role in disease initiation and treatment is initiated before secondary specificities become involved.

Neurocrine Biosciences, Inc. developed an APL of the dominant epitope of insulin B chain (residues 9–23) referred to as NBI-6024 (16,19 → A) (Alleva et al. 2002). In mice, this APL induced APL-specific Th2 cells that did not cross-react with insulin B_{9–23}. NBI-6024 delayed T1D onset in preinsulinitic mice and, when combined with IFA, in prediabetic mice (Alleva et al. 2002). When given to recently diagnosed T1D patients, NBI-6024 had no therapeutic effects (Walter et al. 2009).

DNA “VACCINES”

Plasmids encoding autoantigens have also been used as a mean to induce tolerance against specific autoantigens in autoimmune diseases. After cellular uptake (by immature DCs), the plasmids are maintained as episomes (and hence do not integrate into the host genome), although their expression efficiency is generally low. Protocols have been established to enhance gene transfer, including local electroporation (Prud’homme et al. 2007; Frelin et al. 2010), pretreatment with bupivacaine, cardiotoxin or other myotoxic drugs (Wolff et al. 1990; Danko et al. 1994), or delivery via a gene gun (Chen et al. 2002). Plasmids encoding several different β -cell antigens, including insulin and its precursors, GAD65, Hsp60, and the 2.5mi mimotope have been tested in the context of T1D through various routes, including intradermal, intramuscular, oral, and intranasal.

Insulin DNA Vaccination

Vaccination with PPIs, PIns or insulin-encoding plasmids produced mixed results depending on the type of antigen used, the route of administration, the expression efficiency, and the dosing regimen. Intramuscular vaccination with Insulin B chain- or Insulin B_{9–23}-encoding DNA afforded T1D protection in NOD and LCMV-NP models (Coon et al. 1999; Bot et al. 2001; Urbanek-Ruiz et al. 2001), whereas injections



of plasmids encoding Insulin A_{7–21} had no effects (Urbanek-Ruiz et al. 2001).

In one study, intramuscular delivery of PPIs II-encoding DNA was found to promote, rather than suppress, T1D development when given to prediabetic NOD mice (Karges et al. 2002). More recently, Solvason et al. tested the ability of PPIs I/II-, or PIs I/II-encoding plasmids to prevent disease in prediabetic mice or to restore normoglycemia in newly diagnosed diabetic animals (Solvason et al. 2008). Vaccination of hyperglycemic mice with a high-expression version of a PIs II-encoding plasmid blunted disease progression in a significant fraction of mice (Solvason et al. 2008). Although long-term PIs II vaccination induced IL-10- and IFN γ -producing T cells, the mechanism of action of this approach remains unresolved.

A Phase I/II study by Bayhill Therapeutics assessed the safety of 12 weekly doses of a human PIs-encoding plasmid (BHT-3201) in recent-onset diabetic patients (Gottlieb et al. 2008). Preliminary data from the vaccinated patients showed that BHT-3201 is well tolerated and may be associated with preservation of pancreatic β -cell function.

GAD65 DNA Vaccination

Intramuscular vaccination with DNA encoding GAD also yielded mixed results across different laboratories. Vaccination with rat GAD65 and GAD67 encoded in a plasmid under the control of the human cytomegalovirus promoter did not affect T1D incidence in NOD mice (Wiest-Ladenburger et al. 1998). Likewise, intramuscular vaccination with constructs encoding the full-length intracellular or truncated soluble forms of human GAD65 (Lieberman and DiLorenzo 2003), or a GAD-IgG Fc fusion protein (Tisch et al. 2001) enhanced Th1 responses and did not afford protection. In another study, a GAD-encoding DNA vaccine induced Th2 responses and promoted T1D (Gauvrit et al. 2004).

Administration of GAD-encoding DNA through the oral or intradermal routes had incremental protective effects (Lieberman and Di-

Lorenzo 2003). In some cases, injection of a control plasmid encoding an irrelevant protein also resulted in protection (Filippova et al. 2001). More promising results were obtained in a recent study in which a gene gun biolistic approach was used to deliver a GAD-IgG Fc-encoding plasmid through the skin. Gene gun delivery of this construct, as opposed to intramuscular delivery, induced IL-4-producing CD4⁺ T cells and delayed T1D onset in NOD mice (Goudy et al. 2008).

DNA Vaccination with Other Autoantigens

DNA vaccination of prediabetic NOD mice with a hsp60-encoding plasmid down-regulated hsp60-specific T-cell responses, up-regulated hsp60-specific autoantibodies of the IgG2b subclass, and inhibited diabetogenesis (Quintana et al. 2000). In another study, vaccination with a plasmid encoding a lysosome-targeted 2.5mi mimotope afforded T1D protection in a BDC2.5 TCR-transgenic CD4⁺ T-cell transfer model. Targeting the 2.5mi mimotope to the APC lysosomal compartment in which MHC class II loading takes place presumably enhances the efficiency of antigen presentation, hence the induction of tolerance (Rivas et al. 2011).

Codelivery of Antigen-Encoding DNA with Other Immunomodulatory Genes or Molecules

An advantage of DNA vaccination as an immunotherapeutic approach is the ease with which additional immunomodulatory genes and sequences can be codelivered with the target autoantigen. Plasmids encoding IL-4, IL-10, or a CTLA-4-binding ligand have been codelivered with insulin- or GAD-encoding plasmids to obtain synergistic effects (Tisch et al. 2001; Prud'homme et al. 2002; Chang et al. 2005; Glinka et al. 2006; Pop et al. 2007).

DNA vaccination has also been tested in combination with immunomodulatory monoclonal antibodies. Anti-CD3 mAb treatment enhanced the protective effect of vaccination with GAD-encoding DNA in the RIP-LCMV-

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GP (C57BL/6) model of T1D. However, this effect could not be reproduced in NOD mice, suggesting that the genetic background conditions the therapeutic efficacy of this approach (Bresson et al. 2010). Intranasal delivery of a PIns II-encoding plasmid in mice cotreated with a blocking anti-CD40L mAb elicited anti-diabetogenic CD4⁺ Tregs. In the absence of anti-CD40L blocking Abs, however, the treatment likely also primes pathogenic T cells, therefore rendering it ineffective in preventing T1D (Ev-ery et al. 2006).

PEPTIDE–MHC-BASED THERAPIES

Administration of autoantigenic pMHC class I or II complexes has also been explored as an approach to induce tolerance in autoimmune diseases. Different outcomes and mechanisms of action have been reported, depending on the structure that was used for therapy (monomers versus dimers or multimers coupled to nanoparticles) (reviewed in Clemente-Casares et al. 2011). Whereas pMHC class I monomers cannot activate T cells efficiently, higher-order structures can do so in a manner that is proportional to the valency, hence avidity, of the pMHC–TCR interaction (Herrmann and Mescher 1986; McCluskey et al. 1989; Abastado et al. 1995). Here, we briefly summarize the outcome of studies using dimers and nanoparticle-conjugated multimeric structures.

Dimers

Administration of dimeric pMHC have proved effective in the prevention of diabetes in spontaneous and transfer models (Masteller et al. 2003; Li et al. 2009; Lin et al. 2010) and reversal of hyperglycemia (Casares et al. 2002; Lin et al. 2010). These outcomes were attributed to the generation of anergic IL-10-producing CD4⁺ T cells. Although alternative mechanisms were not formally excluded, this interpretation of the data is consistent with the observation that in vitro stimulation of PBMCs from T1D patients and healthy controls with GAD65_{271–285} or PIns_{73–90}/DR4-IgG1 dimers elicits IL-10-producing T cells (Preda et al. 2005).

pMHC-Coated Nanoparticles

We have recently shown that iron oxide nanoparticles (NPs) coated with mono-specific, T1D-relevant pMHC class I monomers afford disease protection to prediabetic NOD mice and effectively restore normoglycemia to newly diagnosed diabetic animals (Tsai et al. 2010). We have shown that this approach functions by boosting a naturally occurring negative feedback regulatory loop that aims to blunt the progression of diabetogenic autoimmunity. This negative feedback loop consists of memory-like autoregulatory CD8⁺ T cells that arise (during disease progression, prior to pMHC-NP therapy) from low-avidity autoreactive T-cell clones in response to chronic autoantigenic stimulation. We have proposed that autoreactive T-cell memory in diabetogenesis primarily (albeit not exclusively) arises from low-avidity clones and thus is regulatory in nature. Because memory T cells are costimulation-independent, cross-linking of cognate TCRs on these memory-like autoregulatory T cells by the pMHC complexes coated onto the NPs induces autoregulatory T-cell expansion. In turn, these pMHC-NP-expanded mono-specific autoregulatory T cells blunt disease progression by suppressing the presentation of both cognate and noncognate autoantigens by autoantigen-loaded professional APCs to all other naïve autoreactive T cells partaking in the disease process, through a number of mechanisms that include APC-killing. Nonautoantigen-loaded APCs are spared. A more in-depth discussion of the significance of this new immunological paradigm and therapeutic approach can be found in the work of Clemente-Casares et al. (2011).

ANTIGEN-SPECIFIC CELL THERAPIES

DC-Based Therapies

Studies in the early nineties showed that transfer of DCs isolated from the pancreatic lymph nodes of NOD mice (autoantigen-loaded) could prevent the onset of T1D by inducing regulatory T cells (Clare-Salzler et al. 1992). Similar results were obtained with immature bone marrow-derived DCs (BMDCs) generated in the presence

of GM-CSF and IL-4 (Feili-Hariri et al. 1999; Morel et al. 1999), which presumably act by skewing the autoreactive T-cell response toward Th2 on autoantigen capture in vivo (Feili-Hariri et al. 2002). Another study reported that adoptive transfer of BMDCs generated in the presence of GM-CSF and IL-10 could prevent T1D almost completely (Tai et al. 2011). DCs generated in the presence of IL-10 express much lower levels of costimulatory molecules, produce high amounts of IL-10, and foster the expansion of memory and Foxp3⁺ CD4⁺ T cells. In humans, DCs generated from monocytes in the presence of GM-CSF, IL-4, IL10, and TGFβ were found to tolerize insulin-reactive CD4⁺ T cells in an antigen-specific manner (Torres-Aguilar et al. 2010). The tolerized cells produced low levels of IFNγ and IL-2 and significantly higher levels of IL-10 than CD4⁺ T cells challenged with control DCs. DCs genetically modified to express IL-4 (Feili-Hariri et al. 2003; Creusot et al. 2008; Ruffner and Robbins 2010), galactin-1 (Perone et al. 2006), PD-L1 (He et al. 2008) or treated with antisense oligonucleotides against costimulatory molecules (Machen et al. 2004) or NF-κB (Ma et al. 2003) have also been shown to have antidiabetic properties.

ECDI-Fixed Splenocytes

Another version of APC-based therapy is the administration of antigen-pulsed, ethylene carbodiimide-fixed splenocytes (ECDI-SP). It has been suggested that capture and processing of apoptotic ECDI-fixed splenocytes by endogenous DCs (Turley and Miller 2007) renders them tolerogenic (Xia et al. 2007; Marin-Gallen et al. 2010), in part by inducing the up-regulation of suppressive molecules such as PD-L1 (Fife et al. 2006, 2009; Turley and Miller 2007). Insulin- but not GAD or IGRP epitope-coupled ECDI-fixed splenocytes could reverse hyperglycemia in a fraction of newly diagnosed diabetic NOD mice (Fife et al. 2006). ECDI-fixed splenocytes coupled with CD8⁺ T-cell-associated epitopes of insulin and IGRP inhibited T1D development in a humanized NOD model (Niens et al. 2011). Curiously,

when the splenocytes were pulsed with one or more epitopes from a single autoantigen, only those coupled with IGRP, but not insulin epitopes prevented T1D, presumably by tolerizing cognate autoreactive T cells (Niens et al. 2011).

CONCLUDING REMARKS

Insulin independence is the ultimate goal of any therapeutic approach in T1D. However, from a pragmatic standpoint, the goal of treating T1D is to prevent the development of acute metabolic instability such as diabetic ketoacidosis and severe hypoglycemia, so as to enable normal growth and development in children, and to prevent the development of long-term complications without significantly impairing normal activity of living or quality of life. In this regard, insulin independence is not an absolute prerequisite for success. This concept is in part exemplified by the long-term outcome of islet transplantation. Transplanted patients with detectable endogenous insulin production maintained significantly lower plasma HbA1c levels than those who experienced graft failure (Shapiro et al. 2000, 2006; Merani and Shapiro 2006). In addition, patients who received islet transplants had a significant reduction in the incidence of hypoglycemic coma and reported an improvement in their quality of life (Merani and Shapiro 2006; Speight et al. 2010). Because a reduction in HbA1c levels is highly predictive of a reduction in T1D-related complication rates (The Diabetes Control and Complications Trial Research Group [1993]), any strategy that results in a simplified insulin regimen and a lower HbA1c may have clinical benefits. Indeed, restoration of β-cell mass to “normal” levels may not be necessary, as maintenance of modest β-cell activity has been shown to improve clinical outcome (Steffes et al. 2003). More importantly, the body appears to have “metabolic memory.” Results from the T1D Control and Complications Trial (DCCT) clearly showed that during a mean of 4–17 years of follow-up, patients who were originally assigned to the intense treatment group (who also had lower HbA1c levels) had a significant reduction in the progression



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of retinopathy and nephropathy (The Diabetes Control and Complications Trial Research Group [2000]), in the risk of cardiovascular disease, including the risk of death (Nathan et al. 2005), and in the progression of intimal-thickness (Nathan et al. 2003). These results suggest that improvement of metabolic control for only a few years (i.e., 6.5 years during the DCCT) is sufficient to provide long-term protection to the patients, despite worsening hyperglycemia later on.

The limited success of the clinical trials reviewed in this article shall not discourage us but rather heighten the hope that, with proper optimization of current and new therapies, and the development of well-designed clinical trials, clear beneficial effects in the diabetic population can be achieved. The aforementioned results of the DCCT study suggest that permanent insulin independence, although obviously the ideal outcome, should not be the only benchmark for deciding on the clinical utility of an intervention. Any intervention that can result in lower HbA1c levels, even if only for a short term, can potentially have a positive impact on the disease course and quality of life of the patients. These approaches, however, should be carefully developed and judiciously offered to our patients, to ensure that any associated side effects do not out-weigh the benefits derived from improved glycemic control and better quality of life.

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