Antigen-Specific Immunotherapy for Type 1 Diabetes: Maximizing the Potential

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The identification and study of autoimmune diseases has taught us that recognition of self-antigens can have devastating consequences. Yet there is a paradox to autoreactivity: when correctly balanced, it is at the heart of robust self-tolerance. This concept gives rise to several questions. Can this balance be manipulated? And if so, by what means and through which mechanisms? What are the rules that govern this opportunity to restore homeostasis?

Studies in a variety of animal models, which act as replicas of the major chronic inflammatory diseases that affect humans, have offered many answers to these questions. One of the clearer outcomes is that delivery of autoantigens, administered at different disease stages via a variety of routes, can provide robust, sustained health and protection from inflammatory autoimmune disease. The most appealing element to this approach, termed antigen-specific immunotherapy (ASI), has been that it not only provides an effective means of controlling the autoimmune response via induction or restoration of β-cell–specific tolerance, but that it may achieve these goals without major concerns over safety and certainly without the specter of immune suppression. Yet significant questions remain. Are we doing enough to realize the potential of this sacred cow? How do we move from concept to reality?

In this article, we provide an update on the mechanisms through which ASI is currently thought to operate. We discuss why, despite this body of knowledge, alternative, non-ASI approaches have emerged as the current vogue. We argue that more should be done to counter this trend and realize the potential of ASI, including strategies that combine its strengths with those of other complementary ways forward.

Mechanisms of therapeutic effect including evidence from human trials. Predicting the outcome of immunization with islet antigens is complex. The resulting immune response depends not only on the dose, frequency, and route of administration but also on the precise context, in which the use of suitable adjuvants and inflammation can profoundly influence the resulting immune response or lack thereof. In addition, one should expect interindividual variations in the autoreactive repertoire of T-cells: some islet-reactive T-cells might already be activated at the time of immunization and their avidities can be expected to vary depending on central (thymic) and peripheral tuning events, which in turn will influence the character (magnitude and cytokine production) of the resulting antigen-specific response. The underlying mechanisms are more apparent in murine studies, but evidence is also beginning to emerge from human studies in vivo, both in autoimmunity and comparable inflammatory settings. The two main outcomes involve the induction or augmentation of beneficial (regulatory) immune responses (1–3) and the elimination (deletion) of deleterious islet–specific effector responses (4–6), both of which are context-dependent and will be discussed below (Table 1). It is self-evident that both outcomes could be beneficial in type 1 diabetes (Fig. 1) where there may be both a regulatory T-cell (Treg) defect and effector cells that are relatively resistant to regulation (7).

Studies in mice have clearly documented the enhancement of Tregs and the skewing of cytokine responses (immune deviation) after mucosal (oral, nasal) or peripheral (peptide with and without adjuvant, DNA vaccination) insulin, proinsulin, or other autoantigenic peptide administration (8,9). In these experiments, repeated administration of β-cell autoantigens, most notably insulin or its peptides, led to increased interleukin (IL)-4, IL-10, and transforming growth factor (TGF)-β production by insulin-specific polyclonal T-cell populations isolated from the spleen, pancreatic lymph nodes, and, in some cases, the islets. Unfortunately the numbers of autoreactive T-cells in the blood are low and, therefore, little information is available about their frequency and function in peripheral blood, which would be very helpful to guide human biomarker efforts. The antigen-induced or enhanced cell populations can actively suppress effector immune responses as evidenced by adoptive transfer studies. Unless they are enabled by certain chemokines or chemokine receptors or integrins to home to solid organs, the antigen-induced modulating cells are usually found in lymph nodes and spleens following transfer into pre-diabetic recipient mice. Thus, antigenic immunization can endow islet-reactive T-cells with the ability to regulate and suppress deleterious effector responses, which entitles them to be called adaptive regulatory T-cells (aTregs). In some cases, the transcription factor forkhead box P3 (FoxP3), a good marker for naturally occurring Tregs (nTregs), shows increased expression. FoxP3 is of value as a bona fide Treg marker in the mouse, but its utility in humans is much more limited because it is also expressed on recently activated effector T-cells. Thus, successful ASIs that prevent type 1 diabetes in animal models are associated with the induction of cytokines, which can be considered as

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TABLE 1
Mechanisms of action of antigen-specific immunotherapy and predicted nature of response

Mechanism	Predicted outcomes	Tolerance induction	Durability
Immune regulation induced against β-cell antigen (typically associated with aTregs, IL-10, TGF-β induction)	Responses should be detectable (e.g., by cytokine production or functional read-out) Should offer benefit of linked suppression of response to other β-cell antigens	Operational tolerance should be achieved	Responses durable in the range of months up to 1 year
Immune deviation associated with change of dominant cellular phenotype (e.g., from T_H1 to T_H2)	Responses should be detectable (e.g., by cytokine production or functional read-out) May offer benefit of linked suppression	Operational tolerance should be achieved	Responses durable in the range of months
Immune deletion of β-cell antigen-specific T-cells	Difficult to detect deletion No benefit of linked suppression	Operational tolerance not guaranteed	Transient (weeks/months)

protective from type 1 diabetes (immune deviation) and are produced by CD4+ T-cells that can function as aTregs. In this context, the difference between immune deviation and Treg induction is mainly a semantic argument. The control of effector responses of various specificities (by-stander suppression) likely occurs through modulation of antigen-presenting cells (APCs) resulting in a lack of anti-islet effector T-cell expansion, but not their deletion. New alternative models have also been described recently in which APCs are perhaps incapacitated from their role of antigen presentation to autoreactive effector T-cells through a direct APC-lytic process mediated by Tregs both in vitro in man and in vivo in murine studies (10,11).

Similarly, immune deviation indicative of Treg generation has been documented in humans (for example, the induction of a proinsulin peptide–specific IL-10 response after low dose intradermal administration of the peptide in type 1 diabetic patients) (12), reminiscent of observations

FIG. 1. Beneficial effects of antigen-specific immunotherapy (ASI) on the pathological immune responses that result in type 1 diabetes. β-cell damage is a result of the combined actions of proinflammatory helper T cells (T_H1) and cytotoxic T lymphocytes (CTL), which are primed against islet autoantigens such as GAD65 (GAD) and preproinsulin (INS) by inflammatory dendritic cells (DCs). ASI has two predominant beneficial effects, namely deletion of T-cells and induction of regulation, either via priming of regulatory T-cells (Tregs) or immune deviation. The major benefit of Treg induction is linked suppression, the process by which Tregs induced to regulate in response to one autoantigen (e.g., GAD) can also regulate responses to other autoantigens (e.g., INS) presented by the same DC.

in studies of peptide immunotherapy in clinical allergy (13,14). Other examples are the significant increases in GAD-specific γ-interferon, IL-5,-13,-10,-17,-6, tumor necrosis factor-α, FoxP3, and TGF-β mRNA responses as well as autoantibody induction (15) following subcutaneous administration of 20 μg of recombinant human GAD65 adsorbed onto alum (in a classic prime-boost regimen, $n = 35$/group). In other clinical trials where the limited effects of ASI have been documented, for example after daily dosing of oral insulin (16) or after a proinsulin-expressing DNA vaccine (17), no such clear effects as of yet have been documented and will have to await future biomarker studies.

The strong clinical advantage of modulating the β-cell–specific immune response and redirecting its aggressive nature to a more regulatory function is that the resulting aTregs can suppress heterologous islet–specific effector responses, and they can do so in a site-specific manner because they are predicted to become active only at sites where islet antigens are being presented. Thus, in a sense, ASI offers a site-specific immune modulatory drug. The clinical disadvantage is that antigen-specific therapies may have less potency than directly immunosuppressive strategies, as evidenced by the fact that they tend only to work prior to onset of diabetes in preclinical models. Translation to man may therefore require deployment at the early stages of pre-diabetes or enhancement with other complementary strategies (see below). Thus, optimization of dosing and delivery regimens for ASI in parallel with the development of suitable adjunct therapies needs to be considered as a major priority area (see the detailed discussion below).

The current clinical trial landscape in type 1 diabetes: a question of balance. Given the many potential advantages of ASI over non-ASI discussed heretofore (safety, prospect of tolerance, site-specific regulation), it might be expected that clinical strategies based around antigens would be pursued vigorously and in many quarters. Paradoxically, however, this is far from being the case (Fig. 2). A snapshot view of major studies (as opposed to small-scale pilots) that have been completed and published, are currently in progress, or are at an advanced stage of planning indicates that at the stages of disease that are often referred to as primary and secondary prevention, ASI is indeed the dominant modality under investigation. However, there is much more clinical trial
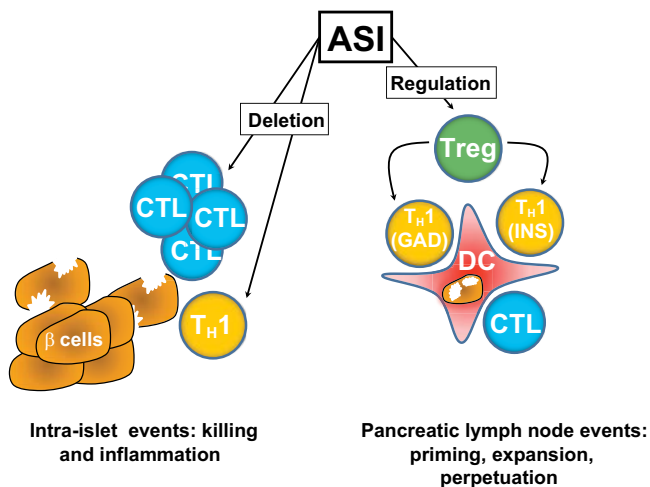
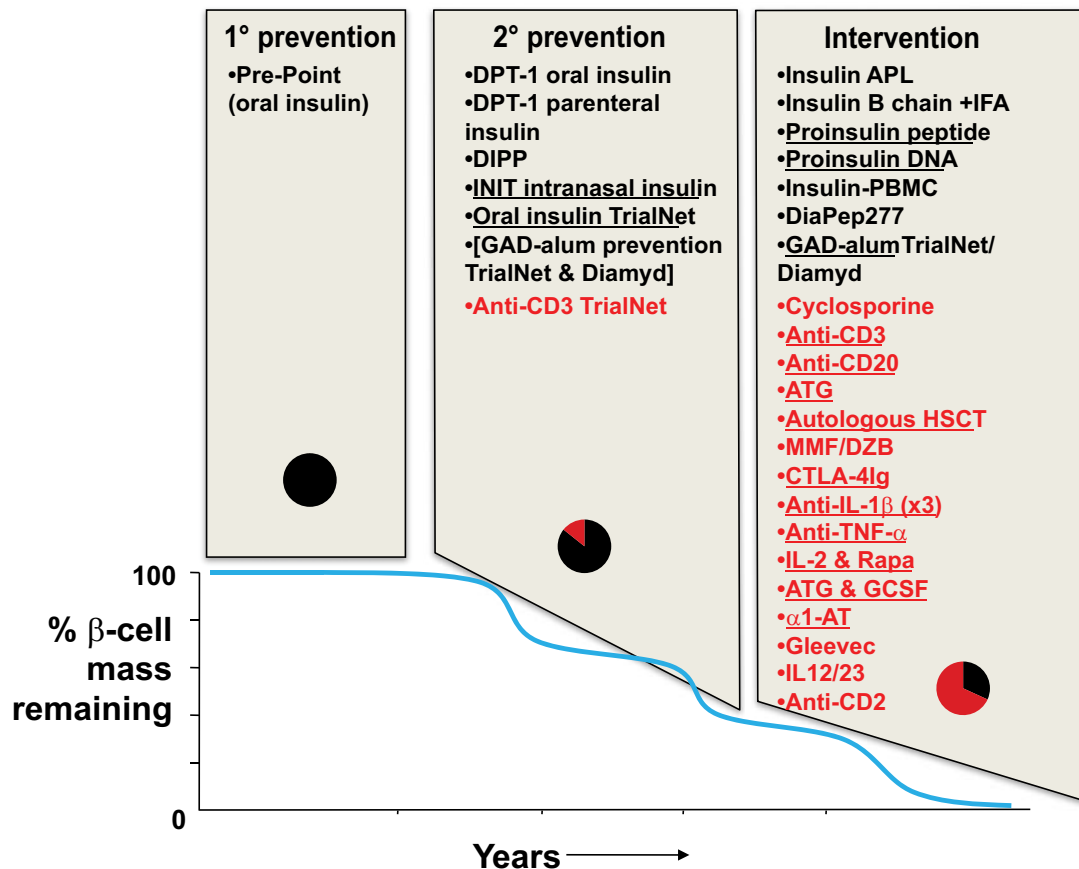| 1° prevention	2° prevention	Intervention
•Pre-Point (oral insulin)	•DPT-1 oral insulin •DPT-1 parenteral insulin •DIPP •<u>INIT intranasal insulin</u> •<u>Oral insulin TrialNet</u> •[GAD-alum prevention TrialNet & Diamyd] •Anti-CD3 TrialNet	•Insulin APL •Insulin B chain +IFA •<u>Proinsulin peptide</u> •<u>Proinsulin DNA</u> •Insulin-PBMC •DiaPep277 •<u>GAD-alum</u>TrialNet/ Diamyd •Cyclosporine •<u>Anti-CD3</u> •<u>Anti-CD20</u> •<u>ATG</u> •<u>Autologous HSCT</u> •MMF/DZB •<u>CTLA-4Ig</u> •<u>Anti-IL-1β (x3)</u> •<u>Anti-TNF-α</u> •<u>IL-2 & Rapa</u> •<u>ATG & GCSF</u> •<u>α1-AT</u> •Gleevec •IL12/23 •Anti-CD2

FIG. 2. Schematic representation of the balance of clinical trial activity in type 1 diabetes. Data are modeled onto a graphical representation of diabetes progression (adapted from reference [38]; reprinted with permission from Atkinson). Data are separated in two dimensions. First, according to stage of disease (primary prevention in the genetically at-risk before autoimmunity is apparent; secondary prevention when autoimmunity is present but no disease; and tertiary prevention or intervention when diabetes has been diagnosed but there is the opportunity to preserve C-peptide secretion); second, according to whether the therapy is antigen-specific (in black) or nonantigen-specific (in red). Underlined therapies are currently actively recruiting. The pie charts indicate the relative proportions of antigen-specific (black) and nonantigen-specific immunotherapy (red) in use at the different disease stages. DIPP, Diabetes Prediction and Prevention (39); APL, altered peptide ligand (40); ATG, anti-thymocyte globulin; CTLA-4Ig, cytotoxic T lymphocyte antigen-4 immunoglobulin; GCSF, granulocyte colony stimulating factor; HSCT, hematopoietic stem cell transplant; IFA, incomplete Freund's adjuvant; IL-1β, interleukin-1β; MMF/DZB, mycophenolate mofetil and daclizumab (41); α1-AT, α-1 antitrypsin; PBMC, peripheral blood mononuclear cell; TNF-α, tumor necrosis factor-α.

activity in the intervention arena (i.e., tertiary prevention, very close to diagnosis), and many more of the agents under evaluation are non-ASIs, especially when one considers the trials that are currently active. In general the ASI studies are low-risk and dominated by a single antigen, insulin. There is a sense that the desire to conduct studies in the prevention arena has led to trials of the very safest of drugs (e.g., injectable insulin), but these trials have not necessarily been fully optimized for efficacy.

There are numerous explanations for this evident bias toward evaluation of novel non-ASI reagents at the intervention stage (Table 2).

Perhaps most worrying are *1*) the limited involvement of the biotechnology and pharmaceutical industries in developing ASI and *2*) the fact that assessing ASI at the intervention stage and expecting favorable metabolic outcomes in order that a full program of development can be progressed is a very hostile environment for these "weaker" therapies. The upshot is that this important treatment modality is not being evaluated in sufficient depth at the safest stage of disease (because the patients already have diabetes) when the acquisition of subjects is the least expensive (because screening is not required). Given that the collective ability to conduct rationally designed biomarker analyses has improved markedly in

the last 10 years or so (18), it would make sense for ASI to be evaluated in the intervention setting more on the basis of its effect on biomarkers than on metabolic outcomes.

Risk analysis for antigen-specific immunotherapy. There are three major areas of concern for the use of ASI in type 1 diabetes: acceleration of disease, leading to more rapid β-cell loss; induction of life-threatening hypersensitivity; and induction of "off-target" autoimmunity. The first two of these will need to be discussed with prospective subjects being enrolled into any prevention or intervention study that uses an antigen-based approach, whereas the third area of concern is antigen-dependent and will therefore depend upon the nature of the trial.

The picture in relation to ASI and disease acceleration in nonclinical studies is generally reassuring. For example, an extensive analysis of the literature in relation to the nonobese diabetic (NOD) model of spontaneous autoimmune diabetes (in which, for example, approximately 100 published studies since 1996 have involved injection or ingestion of whole or peptide autoantigens, either as simple solutions or in conjunction with powerful adjuvants) has revealed that it is extremely unusual to accelerate disease; in most cases the maneuvers are protective or have no effect. In one reported study, disease was accelerated. In that case, two peptides of the β-cell au-

TABLE 2
Explanations for the paucity of antigen-specific immunotherapy studies in the intervention setting

	"Biologics" and other nonantigen-specific approaches	Antigen-specific immunotherapy
Biomarkers	Facile (e.g., reduction in B-cells during anti-CD20 therapy)	Emerging but remain typically site and study-specific; lack of consensus
Dosing and route of administration	Clear treatment pathways from Phase I studies and/or other diseases	Often difficult and complex in ASI; issues over use of adjuvants unresolved; optimal routes remain to be determined
Preclinical models	Generally robust and informative	Translation not always straightforward (e.g., is the intranasal route appropriate in humans; antigen or peptide choice; timing of therapy in relation to natural history)
Success in other autoimmune diseases	Yes	Not yet (but whole allergen and allergen peptide immunotherapy are effective)
Target population	All patients with type 1 diabetes	Inclusion criteria may require staging to presence of selected autoantibodies and their titre, and to HLA type for peptides
Efficacy	Often effective as interventions	Intervention is a tough arena for trials with metabolic outcomes (i.e., C-peptide preservation)
Safety	Variable but generally predictable	Good
Biotechnology/Pharmaceutical involvement	High	Variable; e.g., there is still no Good Manufacturing Practice (GMP) grade proinsulin; new Intellectual Property (IP) relies upon novel modes of delivery

toantigen GAD65 were administered intrathymically and caused a mild acceleration of diabetes onset in NOD mice (19). The authors pointed out that immunization with whole GAD65 does not induce CD4 T-cells reactive with either peptide used, implying that these are cryptic epitopes not naturally processed and presented by NOD mouse APCs. The route of administration may also be important in this outcome—our own studies injecting a single dose of one of these cryptic GAD65 epitopes intraperitoneally gave a strong protective effect (20). The use of naturally processed and presented epitopes or whole antigens may be an important safeguard against the danger of priming additional T-cells with cryptic peptides. Other examples of disease exacerbation by autoantigen administration in models of autoimmune demyelination (21) and autoimmune diabetes (22) have also been reported, but these appear to be rare occurrences. In clinical studies, the experience is obviously much less extensive. It is notable that in the study of oral insulin administration to first-degree relatives with insulin autoantibodies, the subgroup analysis of those who did not have confirmed insulin autoantibodies $\geq$80 nU/ml suggested a trend toward a detrimental effect of the treatment (16), reminding us that we may need to develop carefully argued selection algorithms for trial entry. The examples of disease exacerbation in multiple sclerosis (MS) using altered peptide ligands of the autoantigen myelin basic protein is yet another strong argument for using native peptide sequences or whole antigens (23,24). Apart from these examples, there has been no evidence of disease exacerbation from ASI trials in rheumatoid arthritis (25,26) or more recent studies of MS patients using native sequence peptides (27).

A second safety consideration raised by published nonclinical studies is the risk that antigen injection can result in hypersensitivity, systemic allergy, and anaphylaxis. The most relevant report here was of fatal anaphylaxis in NOD mice after repeated injections of an immunodominant peptide of insulin (residues B9–23). However, relatively large quantities (total >1 mg) and repeated (seven times)

dosing of peptide were used in these studies (28). These responses may have been idiosyncratic to this strain and were alleviated by the alteration of the peptide's isoelectric point (29). Such responses have not been seen to date in the setting of autoimmune disease in man (apart from the altered peptide ligand studies in MS discussed above, in which hypersensitivity responses were also observed). It may be that the induction of T helper 2 (T_H2)-like responses requires the achievement of a fine balance between those that may be beneficial (see the comments on immune deviation above) and those that are dangerous.

The final safety issue relates to the induction de novo of an autoimmune process, for example, when an ASI study uses a β-cell autoantigen that is expressed in other tissues as in the case of GAD65, which is expressed in the peripheral and central nervous systems. To date, the nonclinical and clinical experiences suggest that such a complication remains only a theoretical risk; notably there was no induction of neurological disease despite administration of GAD65 with adjuvant in a regime that boosted GAD-specific autoantibody titers to levels more typically seen in patients with stiff-man syndrome (15).

Synthesizing these comments, there are theoretical risks of ASI and for some of these risks, there are nonclinical studies to indicate that theory can become reality; but this is the exception rather than the rule, and clinical studies have proved extremely safe.

Optimizing antigen-specific immunotherapy for the clinic. Based on preclinical models, several factors are emerging as critical in determining the outcome of antigen-specific immunizations, most notably dose, route, adjuvant, and frequency of administration. Studies have shown that too frequent antigen administrations, as well as very high dosages, do not result in optimal induction of immune regulation and tolerance. In addition, there is no evidence that there is such a thing as "regulatory memory," and in most prevention studies so far, repeated administration of the antigen has been required. Exceptions arise, for example when certain adjuvants such as alum or incomplete Freund's adjuvant are used. Here, at least in
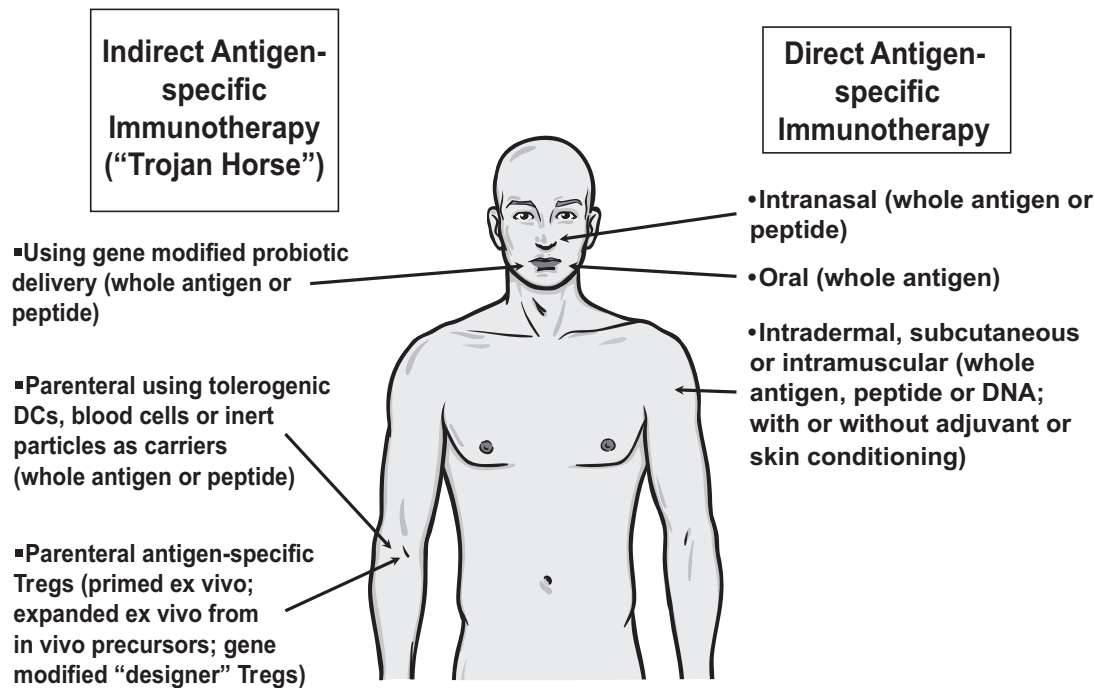
Indirect Antigen-specific Immunotherapy ("Trojan Horse")

▪Using gene modified probiotic delivery (whole antigen or peptide)

▪Parenteral using tolerogenic DCs, blood cells or inert particles as carriers (whole antigen or peptide)

▪Parenteral antigen-specific Tregs (primed ex vivo; expanded ex vivo from in vivo precursors; gene modified "designer" Tregs)

Direct Antigen-specific Immunotherapy

•Intranasal (whole antigen or peptide)

•Oral (whole antigen)

•Intradermal, subcutaneous or intramuscular (whole antigen, peptide or DNA; with or without adjuvant or skin conditioning)

FIG. 3. Approaches currently under evaluation for delivery of antigen-specific immunotherapy. DCs, dendritic cells.

animal models, a one-time administration of antigen with adjuvant is sufficient to prevent diabetes. In these cases, rather than augmentation of nTregs or de novo induction of iTregs or aTregs, an immune deviation to T_H2 and induction of T_H2 memory cells that produce IL-5, -4, and -13 might have occurred. This in itself might be very beneficial and the desired outcome of antigen-specific immunization in type 1 diabetes. We might not need to induce bona fide Tregs after all (i.e., the FoxP3+ CD127^{low}CD25^{high} type), but a mere T_H2 deviation could be sufficient. IL-4 is exquisitely protective in various animal models for type 1 diabetes and has a large therapeutic range, especially when delivered locally to the pancreatic islets or lymph nodes via β-cell antigen–specific Tregs (30). Thus, choosing the correct adjuvant (i.e., a T_H2-deviating compound) might be the key to antigenic immunization and long-term tolerance in type 1 diabetes (15). Tolerance-inducing adjuvants are not an area of large-scale research and are probably not in the development pipelines of many pharmaceutical companies.

In addition, several other factors need to be resolved. The most pressing issue is the precise dosing regimen. From the studies in various animal models, it is known that too high dosages might not be effective by leading to the deletion of Tregs rather than their augmentation. In murine models, for example, only oral insulin dosages between 0.2 and 2 mg are effective when given twice per week by oral gavage (31). Higher and lower dosages have no strong effect on preventing diabetes, therefore there is a strong need to translate dose regimens used in these animal models to humans as accurately as possible. This has not been achieved to date, at least in part because there is no fully validated formula. However, the currently utilized 7.5-mg dose in the oral insulin study of Diabetes TrialNet, which mirrors that used in the Diabetes Prevention Trial (DPT)-1 (16), is most likely too low comparatively and, based on animal models, less frequent dosing with a higher dose should greatly increase efficacy. This

factor could also explain the lack of efficacy in the Finnish nasal insulin diabetes prevention trial (39).

New strategies and creative approaches will be required to more rationally and rapidly translate from mouse studies to human trials, as we will discuss in the next section. **Emerging strategies for antigen-specific immunotherapy: implementation and bold steps.** The future is not all bleak: several new strategies are emerging that may well achieve success in enhancing the delivery and potency of ASI and when assembled together, the ASI portfolio offers a number of appealing options (Fig. 3). These options include strategies for the delivery of multiple epitopes from multiple antigens to mirror the approach that is proving successful in clinical allergy; the use of steroid hormone adjuvants (glucocorticoids and vitamin D) to modulate APCs presenting autoantigens both in vitro for adoptive transfer (32,33) and in vivo to enhance tolerance induction in the skin; new methods for the delivery of antigens to the gut using *Lactococcus lactis* gene modified to deliver islet autoantigens and cytokines; using soluble T-cell receptors specific for islet peptides (for details, see http://naimit.eu/); and antigens coupled to inert cells. As discussed above, these approaches tend to center on novel modes of antigen delivery, perhaps partially as a means to generate funding interest or intellectual property and thus to sustain the effort. Additional strategies will also be useful if conducted in parallel; notable among these strategies is the emerging interest in analyzing immune responses, tolerance, and ASI through investigation of disease models generated in silico (34).

Notwithstanding these fertile new areas, there is a sense that the progress made in getting antigens into the clinic has stalled and requires renewed invigoration. There is nothing intrinsically flawed about oral or nasal antigen administration or antigen injection. They have simply not been fully evaluated in a staged developmental program. As discussed above, much remains to be understood about dose, regimen, and route. We would advocate a return to

these questions, addressed in the context of small clinical studies with the emphasis on mechanistic outcomes. A second line approach will be the development of suitable combinations of antigens with immune modulators that have been specifically selected to foster Treg function and expansion while reducing the effector cell load. Such an initiative could be very beneficial in overcoming some of these issues and, most importantly, enabling antigen-specific Treg induction to be effective later during the disease process, for example in individuals at high risk of developing type 1 diabetes or in recently diagnosed patients (35). Animal studies using a combination of anti-CD3 and nasal proinsulin peptide strongly support this concept (36).

As a final comment, one advance that could facilitate rapid advances in these key areas relates to trial design. Perhaps, for example, the emphasis in relation to outcomes should shift away from metabolic recovery toward an intense focus on immunological biomarkers. This could be done in the context of small optimization trials. These trials, in turn, would be considerably better informed if another knowledge gap—namely the natural history of such immunological biomarkers in the 1- to 2-year period after diagnosis—were adequately mapped through longitudinal studies. Going forward, these efforts should enable the type 1 diabetes community to make informed decisions about the merits of ASI and hopefully realize its potential for "negotiating" with the immune system (37).

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