

## TYPE 1 DIABETES

# Humanizing Animal Models: A Key to Autoimmune Diabetes Treatment

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**Preclinical evaluation of antibody-based immunotherapies for the treatment of type 1 diabetes (T1D) in animal models is often hampered by the fact that the human antibody drug does not cross-react with its mouse counterpart. In this issue of *Science Translational Medicine*, researchers describe a new mouse model that expresses the human isoform of a molecule targeted by T1D antibody therapies that are currently being tested in clinical trials—the human epsilon chain of the CD3 complex expressed on T cells. Anti-CD3 is capable of reducing insulin needs in individuals with recently diagnosed T1D; however, the precise underlying mechanisms of action and the minimal effective dose have been difficult to define. The new humanized mouse model will be instrumental in optimizing anti-CD3-based therapies and accelerating their clinical realization.**

Type 1 (juvenile) diabetes (T1D) is one of the most widespread autoimmune disorders in the world. Nearly 20 million people suffer from T1D worldwide, and the incidence has been increasing by 2 to 5% in the past few years (1). The etiology of T1D is multifaceted and involves a complex interplay of genetic and environmental factors resulting in the destruction of insulin-producing  $\beta$ -cells in the islets of Langerhans of the pancreas. This wreckage causes high blood-sugar concentrations (hyperglycemia), the need for daily insulin injections, and, in the long term if not managed properly, severe vascular side effects.

The gradual loss of pancreatic  $\beta$ -cells over years is the result of an autoimmune response that likely involves a series of dysfunctions in patients' immune systems that unleash pathogenic autoreactive immune effector T cells ( $T_{\text{effs}}$ ) specific for  $\beta$ -cell antigens (2). In healthy individuals,  $T_{\text{effs}}$  are normally kept in check by various mechanisms. Regulatory T cells ( $T_{\text{regs}}$ ) play a key role in this process, but in patients who are in the process of developing T1D, their ability to suppress  $T_{\text{effs}}$  is inefficient, and this aberration facilitates the destruction of  $\beta$ -cells (3). Antibodies to CD3—a protein complex that is associated with the T cell receptor (TCR) and participates in T-cell activation by antigen—can restore normality to some of this immune dysregulation, because anti-CD3 antibodies (anti-CD3s) both reduce the number of  $T_{\text{effs}}$  and foster the development of  $T_{\text{regs}}$  (4, 5). However, the precise molecu-

lar mechanisms that underlie the effects of anti-CD3s on  $T_{\text{eff}}$  and  $T_{\text{reg}}$  functions are not fully understood, and unfortunately, not all anti-CD3s exhibit equal efficacy against T1D or uniformly favorable risk-benefit ratios in patients. These observations imply the need for animal models that can permit the evaluation and predict the behavior of humanized therapeutic anti-CD3s that are currently being tested in the clinic. In this issue of *Science Translational Medicine*, Kuhn *et al.* describe such a model (6).

The non-obese diabetic (NOD) mouse model for T1D recapitulates many of the immune imbalances as well as genetic and environmental influences present in T1D patients (7). As a consequence, the NOD mouse has been extensively used for preclinical testing of more than 100 candidate therapeutics for T1D (8). However, very few agents demonstrate a capacity to curb the autoimmune response after clinical onset of T1D. Antibodies that specifically target the human epsilon chain of the CD3 complex (huCD3 $\epsilon$ ) on T cells have rapidly emerged as potent immune regulators that reduce  $T_{\text{effs}}$  and augment  $T_{\text{regs}}$ ; these functions result in long-term tolerance—a physiological state in which T cells do not respond to a particular antigen—with respect to pancreatic  $\beta$ -cell proteins (9).

On the basis of these promising preclinical data, two clinical trials were launched using two different humanized monoclonal antibodies (mAbs) specific for huCD3 $\epsilon$  (teplizumab and otelixizumab). In both of these investigations, preservation of C-peptide (formed when proinsulin is cleaved to produce insulin) was achieved for more

than 3 years in patients with recent-onset T1D (10–12); however, cytokine release-related side effects occurred in many patients when the drug was administered and, in the European trial, all Epstein-Barr virus (EBV)-infected patients showed transient reactivation of the virus, which was rapidly controlled by an anti-EBV T cell response (13). Overall, the risk-benefit ratio was acceptable, but there was certainly room for improvement, especially if one considers that anti-CD3 might have to be administered to patients more than once. Other anti-CD3s, among them one called visilizumab (4, 14), exhibited less favorable risk-benefit ratios, and the clinical trials were discontinued.

In order to pinpoint, earlier in the translation process, which new anti-CD3 therapeutics are likely to display unwanted side effects in patients and to optimize dosing regimens for more promising candidates, researchers require robust preclinical testing systems that allow them to anticipate potential problems in the clinics. Although both mice and humans express the CD3 $\epsilon$  protein, their amino acid sequences differ, and the anti-huCD3 $\epsilon$  antibodies developed for clinical use do not bind to the mouse CD3 $\epsilon$  molecule (mCD3 $\epsilon$ ), making direct preclinical testing impractical. Now, Kuhn *et al.* (6) have developed a NOD mouse colony that expresses the huCD3 $\epsilon$  molecule on the surface of mouse T cells (NOD-huCD3 $\epsilon$ ). This study represents a crucial step toward (i) gaining a better understanding of the mechanisms by which humanized anti-huCD3 $\epsilon$  antibodies restore tolerance in vivo and (ii) having the ability to evaluate the dose-efficacy responses for safer and more effective clinical use of these biologics.

## IMPROVING THE RISK-BENEFIT RATIO

In the mid-1980s, a hamster anti-mCD3 $\epsilon$  mAb (clone 145-2C11) was derived by immunizing Armenian hamsters with a murine cytolytic T-cell clone (15). Then in 1994, Chatenoud and colleagues first demonstrated that this antibody could cure overt autoimmune diabetes in NOD mice (16). A 5-day treatment with 145-2C11 (5  $\mu$ g) just after disease onset was sufficient to cure T1D in 64 to 80% of NOD mice. However, the efficacy of intact 145-2C11 was associated with potent mitogenic activity that results from the ability of the fragment crystallized (Fc) domain of the mAb to interact with Fc receptors (FcR) on monocytes/macrophages, thus inducing a massive systemic cytokine release (17). This activity, shared by all intact anti-

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CD3 $\epsilon$ -specific mAbs, promotes extensive T cell proliferation and cytokine production, making nonmodified anti-CD3s unsuitable as interventions in T1D.

Indeed, the risk:benefit ratio for the development of any potential therapeutic drug for T1D has to be carefully weighed, because T1D frequently affects children and young adults, and life-long insulin therapy can afford a reasonable life expectancy for most patients. However, insulin cannot prevent all life-threatening long-term complications, and overall life expectancy can be reduced by 10 to 15 years as a result of such complications, which include retinopathy, nephropathy, cardiovascular diseases, and neuropathy. Thus, to move CD3-targeted therapies from bench to bedside, it was crucial to develop CD3 $\epsilon$ -specific mAbs that do not bind to FcRs. Preclinical studies demonstrated that the FcR-nonbinding F(ab')<sub>2</sub> fragment of 145-2C11 possesses anti-diabetic properties in NOD mice that are identical to the full-length parent mAb but without the dangerous mitogenic activity (18).

These data paved the way for the development, for clinical applications, of non-mitogenic anti-huCD3 $\epsilon$ -specific mAbs that resemble true human antibodies. The first attempt at synthesizing such a mAb occurred in 1979 (19). This mAb, called OKT3, was generated in mice, and although it was directed against huCD3 $\epsilon$ , it retained the ability to bind FcRs and thus displayed mitogenic properties in vitro (in human cells) and in vivo (inducing cytokine release in patients) (17). Therefore, researchers embarked on the following two-step process to improve the mAb. OKT3 was "humanized" by genetically engineering the mAb's constant and variable regions to transform the mouse antibody into a human one, so as to avoid a human anti-mouse antibody (HAMA) response in patients. Then, in order to eradicate the mitogenic properties of OKT3, punctual mutations of the Fc domain were made such that it no longer bound to FcRs.

This process yielded the huCD3 $\epsilon$ -directed mAb hOKT3 $\gamma$ 1 Ala-Ala, also known as teplizumab (20). In 2002, a U.S.-based phase I/II clinical trial was conducted with teplizumab in newly diabetic patients (within the first 6 weeks of diagnosis). A 14-day course of treatment with teplizumab halted progression of the disease in most patients, as shown by the stabilization of serum C-peptide concentrations and the amelioration of the defect in

glucose control reflected by lower amounts of glycosylated hemoglobin and exogenous insulin requirements in anti-CD3-treated patients relative to placebo controls (21). Because patients must receive daily insulin injections as a palliative therapy for T1D, insulin itself is not a reliable read out for  $\beta$ -cell function; however, C-peptide concentrations can reveal whether insulin is produced by  $\beta$ -cells endogenously, which is why this measure is used as an endpoint in clinical trials. In 4 of the drug-treated subjects, insulin production was preserved for up to 5 years after the 14-day course of treatment (11), and as intended, this antibody was associated with only mild and transient adverse effects.

A larger, concurrent phase II clinical trial based in Europe was conducted using a different drug, called ChAglyCD3 or oteelixumab, which is an aglycosylated non-mitogenic humanized IgG1 antibody directed against huCD3 $\epsilon$  and derived from the parental YTH12.5 mAb (22). A short-term therapy (6 consecutive days) with oteelixumab preserved residual  $\beta$ -cell function in patients with new-onset T1D (23). Importantly, the efficacy was more pronounced in younger individuals and correlated with pancreatic  $\beta$ -cell mass at trial entry. Seventy-five percent of the subset of patients with the highest  $\beta$ -cell mass (C-peptide concentrations in the >50th percentile) drastically reduced their insulin needs for up to 4 years after treatment (10). The main side effect observed in patients in the oteelixumab-treated group was a transient but generalized EBV reactivation (13), which could possibly be attributed to the higher dose of CD3 $\epsilon$ -specific mAb in the European-based trial relative to the U.S.-based trial (~685  $\mu$ g/kg versus ~500  $\mu$ g/kg, respectively).

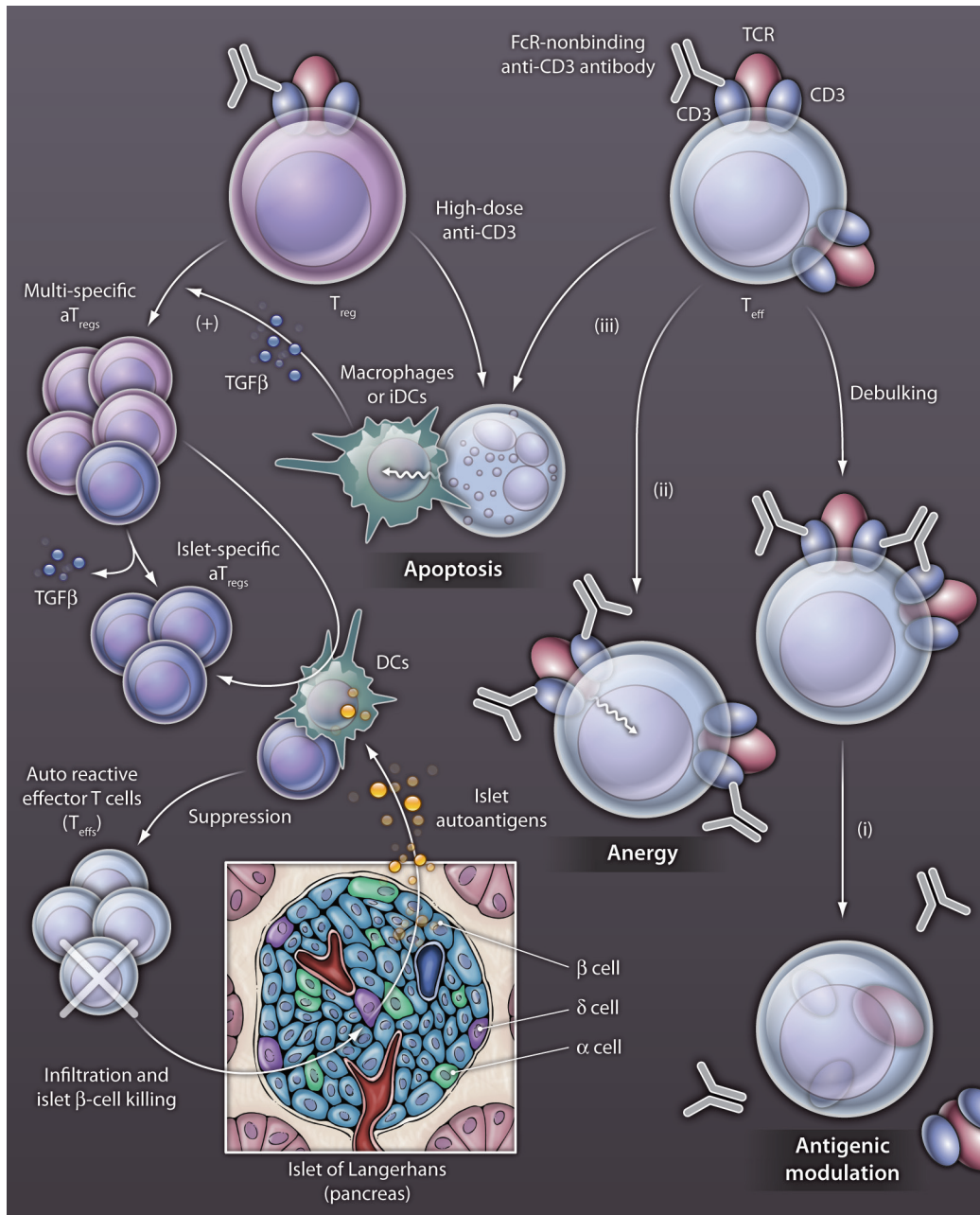
Despite these encouraging early successes with drugs that target the huCD3 $\epsilon$  molecule on T cells, there is a general consensus that the risk-benefit ratio could be improved further. This improvement is an important goal, because repeated antibody administration, combination with additional immune modulators, or ideally, antigen-specific tolerance (24) has to be contemplated for the future if we are to devise ways to further reduce the risk of long-term side effects associated with any immunosuppressive therapy (25). The new humanized mouse model might be useful for developing dosing regimens that permit a head-to-head comparison of different anti-CD3s. This point is especially timely,

because it was reported recently that the U.S.-based phase III trial with teplizumab did not meet its desired endpoint to improve glycemic control and reduce insulin needs (26).

## MODEL MECHANISMS OF TOLERANCE MEDIATION

There is no doubt that a precise delineation of the mechanisms by which the various anti-CD3s mediate their antidiabetic activity will be useful in the quest to improve drug tolerability and clinical outcomes. To this end, Kuhn *et al.* (6) engineered their NOD-huCD3 $\epsilon$  mouse model to co-express both the mCD3 $\epsilon$  and huCD3 $\epsilon$  chains at a 1:1 ratio selectively on the surface of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. These NOD-huCD3 $\epsilon$  mice developed spontaneous T1D with similar kinetics as the parental NOD mice. Using this model, the authors compared the efficacy and mechanisms of action of (i) mitogenic anti-mCD3 $\epsilon$  145-2C11 with anti-huCD3 $\epsilon$  YTH12.5 mAbs and (ii) non-mitogenic anti-mCD3 $\epsilon$  145-2C11 F(ab')<sub>2</sub> with anti-huCD3 $\epsilon$  oteelixumab. In vitro (using NOD-huCD3 $\epsilon$  T cells in culture) and in vivo (using NOD-huCD3 $\epsilon$  mice), these mAbs all displayed various similar activities that modulate T cell function and mediate their tolerogenic activity. Similar to the murine 145-2C11 F(ab')<sub>2</sub> (18), a 5-day treatment with oteelixumab cured newly diabetic NOD-huCD3 $\epsilon$  mice by lowering their blood glucose levels to normoglycemia and provided long-term tolerance toward pancreatic  $\beta$ -cell antigens. Both mAbs shared the ability to increase systemically the proportion of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>regs</sub> (Fig. 1). Oteelixumab-induced T<sub>regs</sub> actively suppressed  $\beta$ -cell autoimmunity in the NOD-huCD3 $\epsilon$  mice in a TGF $\beta$ - but not an IL-10-dependent manner, as previously observed with the nonmitogenic 145-2C11 F(ab')<sub>2</sub> in NOD mice (27).

However, when comparing the activity of oteelixumab used in the clinic to the other mAbs, some subtle but noteworthy differences were observed. Although it was known that anti-CD3 mAbs can modulate the TCR-CD3 complex (by internalization or shedding of the complex), rendering cells 'blind' to antigen (a process also referred to as antigenic modulation) (9), the oteelixumab-treated NOD-huCD3 $\epsilon$  mice showed very specific kinetics for this TCR modulation. In contrast to other anti-CD3 $\epsilon$  mAbs that exhibited peak antigenic modulation by 2 to 4 hours after treatment, the effect of oteelixumab was



**Fig. 1. mAb mechanisms.** Illustrated are the various mechanisms of FcR-nonbinding anti-CD3-specific mAbs in the restoration of immune tolerance in T1D. Efficacy of these anti-CD3-specific mAbs in reversing new-onset T1D relies on several nonmutually exclusive mechanisms that lead to  $T_{reg}$  expansion and immune tolerance induction. Two phases are commonly described. The first phase results directly from the presence of the anti-CD3-specific mAbs in the circulation and provides a short-term blockade of the autoreactive  $T_{effs}$ , called debulking (5, 9). A second phase begins when the mAbs have been cleared from the circulation, with a series of mechanisms involved in active immune tolerance that maintains the long-term efficacy of anti-CD3-specific mAbs. By binding to the TCR-CD3 complex on  $T_{eff}$  anti-CD3 mAbs induce a series of events that prevent  $T_{effs}$  from attacking pancreatic  $\beta$ -cells in the islets of Langerhans: (i) antigenic modulation of the CD3-TCR complex after its shedding or internalization, (ii) anergy of  $T_{effs}$  and (iii) apoptosis of  $T_{effs}$  and perhaps some  $T_{regs}$  with high-dose anti-CD3, anti-CD3 mAbs delivering a suboptimal activation signal, so that the cells die rather than expand. The apoptotic T cells can be endocytosed and degraded by macrophages or immature dendritic cells (iDCs), which in turn secrete large amounts of TGF $\beta$ . In the presence of TGF $\beta$ , stimulation with low amounts of anti-CD3 mAbs that remain in the circulation is sufficient to expand multi-specific  $aT_{regs}$ . If this expansion occurs in close proximity to mature DCs that present islet-specific autoantigens, the pool of  $aT_{regs}$  specific for the islet autoantigens increases, and these  $aT_{regs}$  suppress  $\beta$ -cell-specific autoreactive  $T_{effs}$ .

more prolonged and strongest 24 hours after administration. Interestingly, this differential antigenic modulation could not be observed in NOD-huCD3 $\epsilon$  mouse-derived T cells cultured in vitro, a finding that emphasizes the necessity of using humanized animal models to more accurately predict the potential human response to drugs. In addition, oteixizumab treatment induced less overall T cell activation (based on CD69 staining) than did the other mAbs. It is conceivable that any variation in the signal strength through the TCR-CD3 complex could lead to significant differences in T cell behavior and function in vivo. Indeed, one example described in the manuscript by Kuhn *et al.* (6) is the distinct cytokine expression profile found in the serum of oteixizumab-treated NOD-huCD3 $\epsilon$  mice, which was characterized by lower IL-6 but sustained IFN $\gamma$  and TNF concentrations. Because IL-6 is known to have deleterious effects in T1D, these findings suggest that the risk:benefit ratio of oteixizumab might be more favorable than those of the other mAbs. Thus, future efforts should focus on identifying the key molecular and cellular mechanisms specific to individual anti-CD3 $\epsilon$  mAbs as well as those shared by multiple therapeutic antibodies.

**A CURE ON THE HORIZON?**

Several mechanistic aspects should be comparatively addressed using the novel humanized CD3 mouse model. Such studies could allow for rational prioritization of individual anti-CD3s and aid in the development of new biomarkers that could monitor the intermediate success (or lack thereof) of the intervention.

For example, the U.S.-based intervention trial using teplizumab revealed the induction of Foxp3-, CD25-, and CTLA4-positive suppressor CD8+ T cells (28). Such suppressor popula-

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tions were never described in NOD mice treated with the 145-2C11 mAb. Therefore, it remains unclear whether induction of CD8<sup>+</sup> suppressor T cells is a common feature of all humanized anti-huCD3 $\epsilon$  mAbs in humans or whether this population is induced only by a specific signal delivered by teplizumab through the TCR-CD3 com-

plex. The use of NOD-huCD3 $\epsilon$  mice will be essential to address this issue by direct comparison of teplizumab and oteplizumab activities in vivo.

One can assume that slight changes in epitope recognition between the various anti-huCD3 $\epsilon$  mAbs can result in drastic modifications in the signal they deliver to T cells as is frequently observed with naturally occurring autoantibodies (29).

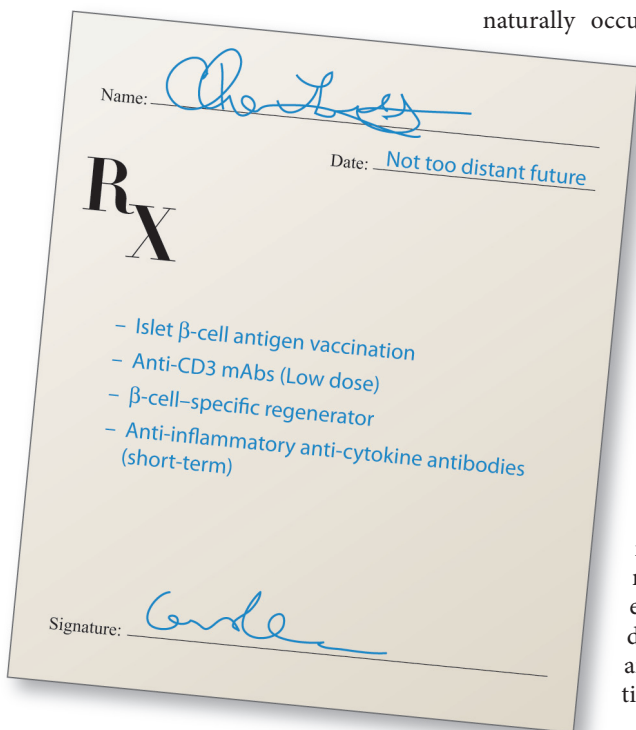
The crystal structure of huCD3 $\epsilon\gamma$  in complex with the antigen binding fragment (Fab) of OKT3 has been solved (30). The structure shows that OKT3 (teplizumab) interacts exclusively with a conformational epitope on the huCD3 $\epsilon$  subunit and has a low affinity for its antigen. Identification of the epitope recognized by oteplizumab is warranted to perform a side-by-side comparison with teplizumab in NOD-huCD3 $\epsilon$  mice and could reveal the relation between the selected epitopes recognized by different anti-huCD3 $\epsilon$  mAbs and their distinct modulation of T cell functions.

Treatment with FcR-non-binding humanized anti-CD3 $\epsilon$

mAbs can promote the generation of anti-idiotypic antibodies that bind to the variable regions of anti-CD3 $\epsilon$  mAbs, mimicking antigen binding and, therefore, blocking the activity of anti-CD3 mAbs (21, 23, 31). These antibodies that can appear a few weeks after mAb treatment and represent a potential therapeutic drawback if repeated treatments are needed to establish permanent tolerance. To overcome this problem, it is instrumental to develop a series of humanized FcR-nonbinding CD3 $\epsilon$ -specific mAbs with diverse variable heavy chain-variable light chain (VH-VL) pairings and sequences. One attractive approach is the use of random phage display antibody libraries to select the VH-VL pairings that show strong affinity for the antigen of interest by biopanning—successive rounds of selection and amplification—on huCD3 $\epsilon$  (32, 33). These antibody fragments could then be cloned, sequenced, and expressed to obtain a panel of humanized anti-CD3 $\epsilon$  mAbs. The NOD-huCD3 $\epsilon$  mice provide an excellent model to evaluate

such a panel of anti-CD3 $\epsilon$  mAbs with respect to their tolerogenic properties and their tendencies to diminish anti-idiotypic activity when injected sequentially.

Last, there is now a growing consensus that combination therapies will be needed to achieve both immune tolerance and  $\beta$ -cell regeneration in patients suffering from T1D. Because of their tolerability, therapeutic efficacy, and ability to induce or expand adaptive T<sub>reg</sub> (aT<sub>reg</sub>) functions, anti-CD3 $\epsilon$  mAbs are well poised for being a crucial component of combination therapies for T1D. The current vision (34) is that an ideal combination therapy prescription targeted to recent-onset T1D patients or prediabetic individuals at high-risk (for example, those patients who express multiple islet autoantibodies) will consist of an islet  $\beta$ -cell-derived antigen vaccine to induce and expand autoreactive aT<sub>regs</sub> (35, 36); a suitable immune modulator, such as anti-CD3s, that reduces T<sub>eff</sub> function immediately while fostering expansion of aT<sub>regs</sub> (24, 37); an anti-inflammatory drug (such as antibodies that neutralize IL-1, IL-6, or IL-12); and a compound that protects  $\beta$ -cells or ideally, supports their regeneration (Fig. 2). The novel humanized mouse model is an ideal system in which to rapidly and rationally optimize such combination therapies.



**Fig. 2. Prescription for combinatorial curing of T1D.** Several combination therapies have been proposed to induce, simultaneously or sequentially, immune tolerance and  $\beta$ -cell regeneration in patients suffering from T1D. Efficient combination therapies might consist of (i) an islet  $\beta$ -cell antigen vaccination to induce and expand autoreactive aT<sub>regs</sub> by using either direct immunization with islet autoantigen or tolerogenic dendritic cells loaded ex vivo with islet autoantigen peptide(s) or protein(s); (ii) a suitable immune modulator that rapidly reduces T<sub>eff</sub> function while fostering expansion of aT<sub>regs</sub>. Because of their acceptable tolerability, therapeutic efficacy, and ability to induce or expand aT<sub>reg</sub> functions, anti-CD3 $\epsilon$  mAbs are well poised for being this crucial immune modulator; (iii) a compound that protects pancreatic  $\beta$ -cells and ideally supports their regeneration, such as glucagon-like peptide-1/gastrin, exenatide, or alpha-1-antitrypsin; and (iv) an anti-inflammatory modulator (for example, antibodies to IL-1, IL-6, IL-12, or TNF) to reduce general inflammation. Quelling of inflammation might be required to enhance the expansion of aT<sub>regs</sub> and to restore  $\beta$ -cell secretory function.

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