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REVIEW

Reestablishing T Cell Tolerance by Antibody-Based Therapy in Type 1 Diabetes

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Abstract Type 1 diabetes (T1D) is an autoimmune disease in which the insulin-producing β cells are selectively destroyed. β cell-specific T cells are considered to be the major mediators of pathology. Accordingly, most immunotherapies tested in the clinic to date have focused on reestablishing self-tolerance within the T cell compartment. Monoclonal antibodies (Ab) targeting a variety of lymphocyte surface proteins have demonstrated benefits in preclinical and clinical settings. Indeed, the use of Ab to target T cells directly or indirectly has proven to be an effective strategy to rapidly suppress β cell autoimmunity and establish tissue-specific, long-term tolerance in rodent T1D models. In this review, we describe a number of these Ab-based immunotherapies, discuss associated immune regulatory mechanisms, and highlight results obtained in T1D clinical trials.

Keywords Autoimmunity · Immunotherapy · Immunoregulation · T cells

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Introduction

Type 1 diabetes (T1D) is characterized by the autoimmune destruction of the insulin-secreting β cells, which reside in the pancreatic islets of Langerhans. β cell autoimmunity is viewed as a chronic inflammatory response involving immune effector cell infiltration (i.e., insulitis) of the islets. Once initiated, the diabetogenic response may progress for a number of years until the majority of β cell mass is destroyed or rendered nonfunctional, at which time T1D is clinically diagnosed. Various types of immune effectors such as T and B cells, and innate cells such as NK cells, macrophages, and dendritic cells (DC) contribute to β cell autoimmunity. However, the general consensus is that both $CD4^+$ and $CD8^+$ T cells targeting multiple autoantigens are the critical drivers of β cell autoimmunity. In rodent models of T1D, such as the non-obese diabetic (NOD) mouse and biobreeding rat, $CD4^+$ and $CD8^+$ T cells are essential for mediating efficient β cell destruction and overt diabetes (Anderson and Bluestone 2005; Bach 1994; McDevitt and Unanue 2008). In T1D subjects, a strong genetic association with specific HLA class I and II alleles (Jahromi and Eisenbarth 2006), and an increased frequency of circulating β cell-specific T cells provide indirect evidence that T cells drive human T1D (Arif et al. 2004; Kronenberg et al. 2012). Indeed, islets of cadaveric pancreases from T1D patients typically contain T cells, consisting mostly of $CD8^+$ T cells (Coppieters et al. 2012; Mallone et al. 2007; Martinuzzi et al. 2008). Diabetic pancreases have also been observed without detectable T cell infiltration suggesting heterogeneity in the pathogenesis of human T1D (Arif et al. 2014; In't Veld 2014; Richardson et al. 2011; Rodriguez-Calvo et al. 2014).

The breakdown of β cell-specific tolerance in the T cell compartment is complex, influenced by environmental,

genetic, and age-dependent factors (Anderson and Bluestone 2005; Bach 1994; He et al. 2013; Todd 2010). Dysregulation of peripheral tolerance mechanisms is thought to favor the differentiation and expansion of pathogenic effector T cells (Teff) versus immunoregulatory T cells (Treg) (Tisch and Wang 2008). In NOD mice the pathogenicity of type 1 $CD4^+$ and $CD8^+$ Teff infiltrating the islets is initially suppressed by Foxp3-expressing $CD4^+$Treg ($Foxp3^+$Treg). However, due to insufficient local levels of interleukin (IL)-2, islet resident $Foxp3^+$Treg survival is impaired resulting in expansion of pathogenic Teff and efficient β cell destruction (Goudy et al. 2011; Tang et al. 2008). Notably, $Foxp3^+$Treg from T1D patients exhibit defects in expansion and suppressor function that are attributed to impaired IL-2 receptor (IL-2R) signaling (Garg et al. 2012; Long et al. 2010). These defects coupled with Teff that exhibit reduced sensitivity to Treg-mediated suppression in vitro also suggest aberrant peripheral tolerance in T1D subjects (Schneider et al. 2008).

Many immunotherapies in T1D clinical trials have focused on reestablishing the functional balance between Treg and Teff (Luo et al. 2010). Ideally, an immunotherapy would selectively tolerize islet infiltrating Teff, promote expansion and/or differentiation of β cell-specific Treg to maintain islet tolerance long-term, and would leave microbial and tumor immunity intact. In the clinic, immunotherapies can be used to prevent the onset of overt diabetes in at-risk individuals, as well as rescue β cell mass and ideally restore insulin independence in new-onset T1D subjects. The latter was formally demonstrated in early clinical studies assessing the efficacy of the immunosuppressive drug cyclosporine A (CsA) in recent-onset T1D patients (Assan et al. 1985; Bougneres et al. 1990; Canadian-European Randomized Control Trial Group 1988; Stiller et al. 1984). Depending on the time of intervention, dose and duration of CsA treatment, diabetes reversal was reported in 20–65 % of patients. However, the severe side effects of CsA precluded extended treatment, and recurrent diabetes was observed once drug administration was stopped. While diabetes reversal may be unachievable for patients who have managed T1D for a number of years, protection of residual β cell mass can still have a marked therapeutic benefit. For instance glucose control can be enhanced with reduced insulin use, thereby minimizing or delaying associated T1D complications.

There continues to be a need for immunotherapies that selectively suppress β cell-specific T cell reactivity long-term in at-risk or new-onset T1D subjects. Immunotherapies employing antibodies (Ab) have shown efficacy in the treatment of T1D. Ab-based immunotherapies can directly inhibit immunopathogenic Teff as well as modulate the expansion and function of Treg. Furthermore, once established, self-tolerance may persist long-term without subsequent treatments, which is not seen with the application of immunosuppressive drugs (e.g., CsA). For the purpose of this review we will discuss the application of different Ab-based immunotherapies, including the use of immunoglobulin (Ig) fusion proteins, to manipulate β cell-specific T cell reactivity with an emphasis on strategies tested in the clinic. We will focus on Ab approaches that directly and indirectly impact autoreactive Teff and Treg.

Ab-Based Therapies Directly Targeting T Cells

Direct targeting of T cells in preclinical and clinical T1D studies have employed Ab specific for various molecules including: (1) the T cell receptor (TCR) complex (e.g., CD3, TCR α and β chains), (2) co-stimulatory (e.g., CD2) and co-receptor (e.g., CD4, CD8) molecules, and (3) cytokine receptors [e.g., IL-2R (CD25), IL-7Rα (CD127)] (Table 1). In the limited number of clinical studies assessing T cell-specific Ab in T1D, outcome is dependent on the nature of the targeted molecule(s) and the subsequent effect(s) on T cells (Table 1). Two general approaches have been employed: therapies that broadly target T cells and most recently, strategies that target specific T cell subsets. The former embodies the "shotgun" approach, which enhances the likelihood that disease-relevant pathogenic (and regulatory) T cell subsets are modulated, but also increases the potential of unwanted effects on general immune function and homeostasis. The latter approach is expected to minimize deleterious effects on general immunity, but efficacy is dependent on whether targeting specific T cell subsets leads to sufficiently robust tolerance.

Suppressing β Cell Autoimmunity by Broadly Targeting T Cells

Anti-Thymocyte Globulin Therapy

Anti-thymocyte globulin (ATG) therapy has been used to deplete T cells in the transplantation arena. A polyclonal IgG cocktail, ATG exhibits reactivity to multiple antigens expressed by T cells as well as B cells, DC and other immune effectors (Mohty 2007). In NOD mice, treatment with ATG at a preclinical T1D stage prevents diabetes onset (Simon et al. 2008). However, only a modest effect is seen in newly diabetic NOD mice; ~30 % of animals receiving ATG undergo remission (Parker et al. 2009; Vargova et al. 2011). Interestingly, the effects of ATG are dependent on the activation status and subset of Ab-bound T cells. Naïve $CD4^+$ and $CD8^+$ T cells are preferentially depleted whereas memory T cells and $Foxp3^+$Treg are

Table 1 Ab-based therapies in T1D

Target	Clone	Recipient	Mechanism of action	Effect on type 1 diabetes	References
T cells					
ATG	Polyclonal	Human	T cell depletion	Ineffective	Gitelman et al. (2013)
ATG	Polyclonal	Mouse/NOD	T cell depletion/Treg mechanisms	Prevention	Simon et al. (2008)
CD3	145-2C11	Mouse/NOD	T cell depletion	Remission	Chatenoud et al. (1992, 1994)
CD3	Non-FcB 2C11	Mouse/NOD	T cell depletion	Remission	Belghith et al. (2003), Chatenoud et al. (1997), Penaranda et al. (2011)
CD3	OKT3	Human	T cell depletion/Treg mechanisms	Delay	Chatenoud et al. (1989)
CD3	ChAglyCD3	Human	T cell depletion	Delay	Friend et al. (1999), Keymeulen et al. (2005)
CD3	hOKT3γ1(Ala–Ala)	Human	T cell depletion/Treg mechanisms	Delay	Bisikirska et al. (2005), Herold et al. (2002, 2003)
TCR β chain	H57-597	Mouse/NOD	T cell depletion	Prevention/remission	Deng et al. (2014), Sempe et al. (1991)
α/β TCR	TOL101	Human	TCR signal inhibition	Unknown	Flechner et al. (2014)
T cell co-receptor					
CD4	GK1.5	Mouse/NOD	CD4 T cell depletion	Prevention/remission	Koike et al. (1987)
CD4/CD8	YTS177/YTS105	Mouse/NOD	Altered trafficking/Treg mechanisms	Prevention/remission	Hutchings et al. (1992), Yi et al. (2012)
T cell co-stimulation					
CD2	Polyclonal	Rat/BB	T cell depletion	Prevention	Barlow and Like (1992)
CD2	Alefacept	Human	T effector depletion	Delay	Cooper et al. (2003), Rigby et al. (2013)
CD28:CD80/86	CTLA4-Ig fusion/GL1	Mouse/NOD	Inhibition of T effector activation	Prevention	Lenschow et al. (1995)
CD28:CD80/86	Abatacept	Human	Inhibition of T effector activation	Delay	Orban et al. (2011)
CD127	28G9-mIgG2a/A7R34	Mouse/NOD	T cell inhibition/Treg mechanisms	Prevention/remission	Lee et al. (2012), Penaranda et al. (2012)
CD25	Mycophenolate Mofetil/Daclizumab	Human	Inhibition of T effector proliferation	Ineffective	Gottlieb et al. (2010)
B cells					
CD20	2H7	Mouse/NOD	B cell depletion	Prevention/remission	Hu et al. (2007)
CD20	MB20	Mouse/NOD	B cell depletion	Prevention	Xiu et al. (2008)
CD20	Rituximab	Human	B cell depletion	Delay	Pescovitz et al. (2009)

Nature of the targeted molecules and the effects on T cells

relatively resistant to the effects of ATG (Xia et al. 2012). An increase in Foxp3$^+$Treg coupled with the capacity of ATG to skew antigen-stimulated T cells towards protective IL-4- and IL-10-secreting Th2 and Tr1 cells, respectively, are thought to reestablish peripheral immunoregulation and suppress β cell autoimmunity (Xia et al. 2012). The effects of ATG binding to B cells and DC are also expected to indirectly modulate T cell reactivity (Monti et al. 2003; Zand et al. 2005).

Recently, a 12-month phase II trial was completed assessing a short course of ATG in new-onset T1D subjects (Gitelman et al. 2013). Subjects exhibited a number of adverse events associated with acute T cell depletion, as well as cytokine release syndrome (CRS) due in part to activation of ATG-bound T cells and other immune effectors. ATG failed to rescue residual β cell mass; levels of insulin C-peptide (which reflect endogenous insulin synthesis) were not preserved. In addition, no decrease was seen in glycated hemoglobin (HbA1c) levels or insulin use, which are additional metabolic indicators of improved β cell function. The frequency of circulating Foxp3$^+$Treg was increased but not maintained over time. The failure of ATG therapy to mediate a protective effect may be due to at least two key reasons. First, elevated levels of proinflammatory cytokines induced by ATG are expected to impair β cell survival and/or function; β cells are sensitive to the cytotoxic effects of interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IL-1β (Eizirik et al. 2009; Thomas and Kay 2000). In addition, high levels of proinflammatory cytokines may reduce Foxp3$^+$Treg survival and suppressor activity. Second, effector memory T cells are not depleted by ATG, which permits the re-activation of the diabetogenic T cell response. These findings suggest that generalized T cell depletion alone is insufficient to block ongoing β cell autoimmunity, particularly in the context of high levels of systemic inflammation.

Anti-CD3 Ab Therapy

To date, anti-CD3 Ab therapy in the clinic has proven to be the most effective at altering the diabetogenic response, and the most thoroughly studied Ab-based approach for the treatment of T1D (Chatenoud 2010; Chatenoud et al. 2012). Preclinical studies provided strong rationale for testing anti-CD3 Ab therapy in the clinic (Chatenoud et al. 1994, 1997). A short course of anti-CD3 Ab reverses diabetes long-term in ~60 % of newly diabetic NOD mice (Belghith et al. 2003). Mechanistic studies in mice indicate that disease reversal is associated with two key events. The first event entails inactivation and/or removal of pathogenic Teff (Penaranda et al. 2011). Upon anti-CD3 Ab binding, pancreatic Teff are rapidly depleted via induction of apoptosis; a significant frequency (e.g., 30–50 %) of peripheral T cells is also deleted albeit transiently, in a dose-dependent manner (Chatenoud et al. 1994, 1997). Anti-CD3 Ab can also induce long-term anergy in CD4$^+$ and CD8$^+$ T cells that is maintained via PD1–PDL1 interactions (Fife et al. 2006). The second event involves differentiation of β cell-specific adaptive Foxp3$^+$Treg in the periphery (Belghith et al. 2003). This process is supported by transforming growth factor (TGF)-β1, which is secreted by antigen-presenting cells (APC) in response to anti-CD3 Ab-induced apoptotic Teff (Perruche et al. 2008; You et al. 2007). In addition, natural Foxp3$^+$Treg are comparatively resistant to the depleting and inactivating effects of anti-CD3 Ab (Penaranda et al. 2011). The overall result is re-establishment of the functional balance between pathogenic Teff and protective Foxp3$^+$Treg, and suppression of β cell autoimmunity.

Testing the clinical efficacy of anti-CD3 Ab therapy for T1D has primarily focused on two humanized anti-CD3 Ab: hOKT3γ1(Ala–Ala) and ChAglyCD3, also known as teplizumab and otelixizumab, respectively (Bisikirska et al. 2005; Herold et al. 2002, 2005; Keymeulen et al. 2005, 2010b). Importantly the Fc regions of the respective human IgG1 molecules were mutated to limit Fc receptor binding to APC and NK cells. Earlier preclinical and clinical studies showed that native anti-CD3 Ab induced CRS, owing to robust activation of T cells, and Fc receptor-expressing cells following anti-CD3 Ab-mediated crosslinking (Abramowicz et al. 1989). Fc engineering of the anti-CD3 Ab significantly reduces these effects, although some degree of CRS is observed, especially following the first course. Regardless, a therapeutic benefit was demonstrated in phase II clinical trials assessing a short course of teplizumab or otelixizumab in recently diagnosed T1D subjects. Although reversal of diabetes was not achieved, some T1D subjects treated with teplizumab or otelixizumab showed improved C-peptide production, and reduced insulin use relative to control groups (Bisikirska et al. 2005; Herold et al. 2002, 2005; Keymeulen et al. 2005, 2010b). Nevertheless, protection was transient, lasting 2 to 4 years for teplizumab and otelixizumab, respectively (Herold et al. 2005; Keymeulen et al. 2010b). Furthermore, both Ab induced transient T cell depletion systemically, which in the case of otelixizumab was associated with recurrent Epstein-Barr virus infection in some patients (Keymeulen et al. 2010a).

The mechanism(s) involved in teplizumab- and otelixizumab-induced protection is ill-defined. The depletion of circulating T cells suggests a role for purging of islet Teff (Herold et al. 2013b). Observations also suggest that Treg contribute to protection. A unique subset of Foxp3$^+$CD8$^+$ T cells are elevated in peripheral blood of teplizumab-treated individuals (Bisikirska et al. 2005), as are CD4$^+$CD25^{hi}Foxp3$^+$ T cells expressing IL-10 and

CCR6 (Waldron-Lynch et al. 2012). The latter is particularly interesting in view of results showing that in humanized mice treated with teplizumab, human $CD4^+$ T cells expressing CCR6 traffic to the small intestine where IL-10 expression and a Treg-like phenotype are induced (Waldron-Lynch et al. 2012). Notably, teplizumab or otelixizumab treatment is most effective in T1D subjects exhibiting relatively elevated functional β cell mass at the time of treatment (Herold et al. 2013a; Keymeulen et al. 2010b). This suggests that likely responders to anti-CD3 Ab therapy are those that have "less aggressive" β cell autoimmunity and/or are treated at a relatively early stage of clinical T1D.

Despite initial promising results, recent phase III trials for teplizumab and otelixizumab in newly diagnosed T1D subjects have been underwhelming (Daifotis et al. 2013; Hagopian et al. 2013; Sherry et al. 2011). For both anti-CD3 Ab, primary endpoints were not achieved. However, key caveats need to be considered when interpreting these findings. The phase III study of teplizumab used a composite primary endpoint based on insulin requirements and HbA1c levels, which were arbitrarily selected and not validated by earlier studies. Indeed, post hoc analyses using proven endpoints showed efficacy for teplizumab consistent with earlier studies (Hagopian et al. 2013; Sherry et al. 2011). In an attempt to minimize adverse events, the dose of otelixizumab was reduced 15-fold relative to earlier phase II studies (Daifotis et al. 2013), which may have limited efficacy of the treatment in the phase III trial.

In view of results achieved with anti-CD3 Ab therapy, targeting other chains of the TCR may prove to be as (or more) effective in modulating β cell autoimmunity while exhibiting improved safety (Table 1). In NOD mice, treatment with Ab specific for the TCR β chain (clone H57-597) prevents diabetes onset and restores glycemic control if administered within one week of onset (Sempe et al. 1991). Furthermore, single dose administration of anti-TCRβ Ab protects islet allograft models through mechanisms of selective Teff depletion and expansion of alloantigen-specific $Foxp3^+$Treg (Deng et al. 2014; Miyahara et al. 2012). Notably, anti-TCRβ Ab induces considerably less cytokine release by T cells compared to anti-CD3 Ab. In addition, since anti-TCRβ (and/or α) Ab target only T cells that recognize peptide in the context of classical HLA molecules, the possibility of general T cell-induced immunosuppression is reduced compared to administration of anti-CD3 Ab, which also targets γδ T cells and NKT cells. Depending on results of a phase III trial assessing an anti-TCRαβ Ab (TOL101) in renal transplantation (Flechner et al. 2014), this approach may be attractive for trials aimed at T1D and other T cell-mediated autoimmune diseases.

Targeting the CD4 and CD8 Co-Receptors: A future Approach to Reestablishing β Cell-Specific Tolerance in the Clinic?

Recent work has shown that administration of non-depleting Ab specific for the CD4 and CD8 co-receptors is an effective strategy to selectively suppress β cell autoimmunity long-term in NOD mice. Treatment with a short course of non-depleting anti-CD4 (YTS177) and anti-CD8 (YTS105) Ab rapidly induces remission (e.g., as soon as 72 h post-treatment) in the majority (>80 %) of newly diabetic NOD mice that persists indefinitely (Yi et al. 2012). Numbers and the activation status of systemic T cells are unaffected by co-receptor therapy. Not surprisingly both anti-CD4 and -CD8 Ab are required to induce efficient diabetes reversal. Purging of $CD4^+$ and $CD8^+$ T cells residing in the islets is a key step in the functional recovery of β cells and the rapid induction of remission. T cell purging is independent of apoptosis and due instead to T cell egress from the pancreas. Such trafficking is likely attributed to a change in the islet microenvironment and/or the response of T cells to retention and/or egress cues. Strikingly, T cell purging is tissue specific; in addition to the islets, T cells are reduced in the draining pancreatic lymph nodes (PLN) but not in the spleen of anti-CD4/CD8 Ab-treated animals. Here it is believed that crosslinking of CD4 and CD8 has distinct effects on T cells in the context of ongoing inflammation versus homeostasis, thereby establishing the tissue specificity of co-receptor therapy. Long-term maintenance of remission on the other hand is attributed to increased β cell-specific $Foxp3^+$Treg that selectively "reseed" the PLN and exhibit enhanced suppressor function. This pool of $Foxp3^+$Treg is expected to suppress activation and differentiation of pathogenic Teff, reflected by the lack of insulitis in remission NOD mice (Yi et al. 2012). Consistent with the tissue-specific effects of co-receptor therapy, immunity to foreign antigens is unperturbed in remission NOD mice (Yi et al. 2012). Relative to anti-CD3 and other Ab-based therapies, the use of non-depleting co-receptor-specific Ab has important advantages including: (1) accelerated kinetics of remission induction, likely reflecting the distinct mechanisms in T cell tolerance and purging, and (2) the lack of systemic T cell activation and depletion, thereby minimizing deleterious effects on normal immune function and homeostasis.

Clinical studies have been limited to testing non-depleting Ab specific for human CD4. Therapeutic benefit was reported in patients with psoriasis (Philipp et al. 2006), and an ongoing phase II trial (NCT0148-1493) is testing a non-depleting anti-CD4 Ab in rheumatoid arthritis patients. Currently, there is no bona fide non-depleting Ab specific for human CD8. The robust tissue-specific effects seen in

preclinical work, however, provide rationale for further development and testing of non-depleting anti-CD4 and -CD8 Ab for the treatment of T1D in the clinic.

Suppressing β Cell Autoimmunity by Targeting Specific T Cell Subsets

Using Ab to selectively target T cells driving and/or regulating autoimmunity would be the ideal approach to treat disease. A conundrum, however, is identifying the appropriate molecules for the relevant T cell subsets. To validate this approach, basic investigations were carried out in which Teff were selectively targeted using a panel of Ab specific for CD44 (Weiss et al. 2000), a molecule that interacts with extracellular matrix proteins (specifically hyaluronan) and is preferentially expressed by activated T cells (Baaten et al. 2010; Huet et al. 1989). Anti-CD44 Ab administration significantly reduces insulitis and T1D development in a T cell transfer model without altering other T cell responses (Weiss et al. 2000). With this in mind, CD2 has garnered recent interest for the treatment of T1D. CD2 functions as a co-stimulatory and adhesion molecule expressed by T and NK cells. CD2 binds LFA-3 (CD58) expressed by APC, and engagement or blocking of this interaction by Ab influences T cell activation, proliferation, anergy, or apoptosis. Importantly, CD2 is upregulated on activated and memory T cells (Bockenstedt et al. 1988; Green et al. 2000). The latter has been exploited to treat psoriasis in the clinic by applying a humanized fusion protein consisting of the extracellular CD2-binding domain of LFA-3 linked to the Fc region of human IgG1 (LFA3-Ig; Alefacept) (Ellis et al. 2001). Efficacy of Alefacept in psoriasis patients correlates with selective depletion of circulating effector memory $CD4^+$ and $CD8^+$ T cells while the naïve T cell pool remains largely intact (Cooper et al. 2003; Ellis et al. 2001).

A 12-month phase II trial was recently completed in which new-onset T1D subjects received two 12-week courses of alefacept (Rigby et al. 2013). The primary endpoint, preservation of C-peptide relative to placebo controls, was not achieved. However, a number of secondary endpoints were met in the alefacept-treated subjects suggesting a modicum of therapeutic benefit. Insulin requirements and the frequency of hypoglycemic events were reduced, and the drug was well tolerated. Analogous to earlier clinical findings, effector memory T cells were reduced in the alefacept-treated subjects. In addition $Foxp3^+$Treg were unaffected suggesting a shift in the balance between pathogenic versus immunoregulatory T cells. These findings provide support for further work testing alefacept, and developing Ab and/or other drugs to target CD2 for the treatment of T1D.

Manipulating Diabetogenic T Cells Indirectly by Ab-Based Therapy

Ab-based strategies have been employed in preclinical and clinical studies to indirectly block pathogenic T cells and suppress β cell autoimmunity. These approaches have focused on professional APC, including B cells, macrophages, and DC. APC deliver critical signals needed for T cell activation, expansion, and effector cell differentiation by: (1) presenting peptide–MHC complexes (signal 1), (2) expressing co-stimulatory molecules (signal 2), and (3) secreting cytokines (signal 3). A recent study demonstrated that blocking APC-mediated "signal 1" via Ab specific for an insulin B9–23 peptide–IA^{g7} complex alters β cell autoimmunity in NOD mice (Zhang et al. 2014). Insulin-specific $CD4^+$ (and $CD8^+$) T cells play a key role in the diabetogenic response of NOD mice. Ab blocking of the insulin peptide–MHC class II complex in NOD mice is most effective at preventing the onset of diabetes when administered at early versus late preclinical T1D. Multiple β cell autoantigens and epitopes are recognized as the diabetogenic response progresses, so selectively blocking insulin-specific $CD4^+$ T cell priming would be expected to have only a limited effect at later T1D stages. To date, two approaches targeting APC have been tested in the clinic to treat T1D with some degree of success; namely co-stimulatory molecule blockade and B cell depletion.

Co-stimulatory Molecule Blockade

T cells generally become fully activated and proliferate upon TCR binding of peptide–MHC complexes (signal 1), coupled with signals transduced upon engagement of co-stimulatory molecules expressed by APC (signal 2). Depending on context, T cells receiving only signal 1 become anergic, undergo apoptosis, or differentiate into a regulatory subset (Chen and Flies 2013). Binding of CD28 expressed by naïve T cells to CD80 or CD86 on the surface of APC has provided the paradigm for the two signal model, which initially has been exploited in the clinic for inducing transplantation tolerance (Ford et al. 2014; Suntharalingam et al. 2006).

Preclinical studies in NOD mice showed that blockade of co-stimulatory molecules expressed by APC alters β cell-specific T cell reactivity (Herold et al. 1997; Lenschow et al. 1995). However, efficacy is dependent on a number of parameters including the identity of the co-stimulatory molecule that is targeted, subsequent effects on particular T cell subsets, and the stage of β cell autoimmunity at which therapy is initiated. For instance, treatment of NOD mice with a CTLA4-Ig fusion protein that binds to and blocks CD80 and CD86, or Ab specific for CD86 prevents the

onset of diabetes albeit with no marked effect on the frequency of insulitis. In contrast, anti-CD80 Ab therapy exacerbates β cell autoimmunity in NOD mice. Furthermore, the tolerogenic effect is only induced when CTLA4-Ig and anti-CD86 Ab are applied at early but not late preclinical T1D. This temporal effect may be due to inefficient priming of select β cell-specific clonotypes of naïve $CD4^+$ and $CD8^+$ T cells needed to efficiently drive later stages of the diabetogenic response. At late preclinical T1D, however, the impact of CD80/CD86 blockade may be reduced since the pancreatic infiltrate consists mostly of established Teff, which have only limited dependence on CD28 signaling (Tang et al. 2003). Notably, $Foxp3^+$Treg are also decreased by CD80/CD86 blockade due to the lack of CD28 signaling required for survival and effector function. $Foxp3^+$Treg are expected to play a more prominent role in achieving and maintaining tolerance under the stringent conditions encountered at later stages of β cell autoimmunity.

Treatment of recent-onset T1D subjects for two years with a humanized CTLA4-Ig chimeric protein (abatacept) in a phase II trial results in transient efficacy with minimal adverse effects (Orban et al. 2011). C-peptide is increased throughout the 2-year period in abatacept-treated subjects, with a marked delay in C-peptide reduction seen for the initial 10 months of therapy. After this time, however, the rate of loss of β cell function parallels that of the placebo group. Insulin use is also decreased but only for the first 12 months of abatacept therapy. Preservation of β cell function following abatacept therapy correlates with increased circulating naïve $CD4^+$ T cells, and a concomitant reduction in central memory $CD4^+$ T cells and $FOXP3^{+-}$ Treg relative to the placebo group (Orban et al. 2014). A stable naïve $CD4^+$ T cell pool, which serves as a source of diabetogenic Teff, is consistent with blockade of T cell activation. Interestingly, abatacept inhibits transmigration of central memory $CD4^+$ T cells across CD86-expressing microvascular endothelial cells in vitro (Lozanoska-Ochser et al. 2008). Based on this observation it was suggested that the decrease in circulating central memory $CD4^+$ T cells in the abatacept group is due to altered trafficking properties, possibly reflecting retention of these T cells in the lymph nodes (Lozanoska-Ochser et al. 2008). Notably, CD28 signals have recently been shown to regulate trafficking of murine autoreactive T cells into target tissues (Jain et al. 2013). These findings suggest that abatacept-induced efficacy is in part achieved by delaying expansion of the pool of diabetogenic Teff. The decrease in $Foxp3^+$Treg induced by abatacept further reflects the role for CD28 signaling in $Foxp3^+$Treg survival and maintenance. Importantly, failure to establish an expanded or enhanced $Foxp3^+$Treg pool likely hinders the duration and potency of the tolerogenic effect mediated by abatacept. Furthermore, targeting co-stimulatory molecules that regulate Teff and/or memory T cells (e.g., CD40L, 41BBL, OX40L, CD30L, CD70) (Chen and Flies 2013) may prove to be more effective at blocking β cell autoimmunity at late preclinical or clinical stages of T1D. Although insulin independence is an unlikely outcome of this approach, disease progression may be halted or slowed by targeting accessory signaling molecules.

B Cell Depletion

Evidence that B cells play a critical role in T1D comes from studies in which β cell autoimmunity and overt diabetes are prevented in genetically manipulated NOD mice deficient in B cells (Serreze et al. 1996). Although a strong predictive marker for the development of overt diabetes in both mice and at-risk individuals, β cell- and islet-specific autoantibodies are thought to have only limited pathogenicity (Holz et al. 2000; Martin et al. 2001). The consensus is that B cells serve primarily as APC directing autoantigen presentation via β cell-specific B cell receptors (Serreze et al. 1998; Silveira et al. 2004; Tian et al. 2006). Immunotherapies targeting B cells have proven to be effective at suppressing β cell autoimmunity in NOD mice (Hu et al. 2007; Xiu et al. 2008). Transient depletion of B cells via anti-CD20 Ab therapy at preclinical stages of T1D prevents the onset of overt diabetes in NOD mice (Xiu et al. 2008). Protection correlates with decreased T cell infiltration of the islets, believed in part due to reduced $CD4^+$ and $CD8^+$ T cell activation in the PLN. In a second study, anti-CD20 Ab-mediated B cell depletion was reported to reverse diabetes in a small percentage ($\sim$30 %) of new-onset NOD mice expressing a human CD20 transgene (Hu et al. 2007). A role for $Foxp3^+$Treg is suggested by a $\sim$ twofold increase in remission NOD mice. Here, restricting the pool of APC to DC and macrophages may favor the expansion and/or induction of $Foxp3^+$Treg and/or adaptive Treg. Interestingly, a subset of B cells with suppressor activity is detected after B cell reconstitution (Xiang et al. 2012), which may also contribute to β cell tolerance induced by anti-CD20 Ab therapy.

A phase II trial was carried out testing an anti-CD20 Ab (rituximab) in new-onset T1D patients. Subjects received four weekly injections of rituximab and were then monitored for 12 months (Pescovitz et al. 2009, 2014). Adverse events of limited severity are mostly seen after the first infusion of Ab, and no increase in infections is detected in rituximab-treated subjects likely reflecting preservation of memory B cells. Circulating $CD20^+$ B cells are rapidly depleted via cell- and complement-mediated cell lysis, and by 12 months levels return to $\sim$70 % of baseline values. Efficacy of rituximab, albeit transient, is indicated by a significant delay in C-peptide loss, and reduced HbA1c levels and insulin usage. Surprisingly, responders to

rituximab exhibit an increased frequency of $CD4^+$ and $CD8^+$ T cells specific for a panel of β cell autoantigens (Herold et al. 2011). The elevated β cell-specific reactivity is selective, since no difference is seen in T cell responses to control antigens. The phenotype of these β cell-specific T cells is undefined but may represent an expanded pool of adaptive Treg. Indeed, an increase in $Foxp3^+$Treg numbers is also detected within the first 10 weeks of therapy (Herold et al. 2011). An additional study shows that rituximab-induced B cell depletion has a marked effect on follicular helper T cells (Tfh) in T1D subjects (Xu et al. 2013). Tfh are potent regulators of B cell expansion and differentiation in lymphoid germinal centers mediated in part via IL-21 secretion (Nurieva et al. 2008; Vogelzang et al. 2008). The increased frequency of Tfh and elevated levels of serum IL-21 seen in T1D subjects are reduced following rituximab treatment (Xu et al. 2013). Together these clinical findings support the notion that Ab-based targeting of B cells can impact autoreactive T cell reactivity and T1D progression. The overall approach may be improved by employing various B cell-depleting agents, such as Ab or Ig recombinants specific for different B cell growth factors (e.g., BAFF, APRIL) (Marino et al. 2014; Zekavat et al. 2008) to target distinct B cell subsets and/or promote immunoregulatory B cells.

Combinatorial-Based Immunotherapies: The Next Step?

The lack of robust and durable β cell-specific tolerance and protection induced in the clinic by Ab-based or other "mono"-immunotherapies, has led to the idea that efficacy can be enhanced by combining different strategies. The aim is to develop combinatorial therapies that are synergistic and drive robust, long-term tissue-specific tolerance. An important benefit expected from such synergy is that dose and treatment intervals for respective therapeutics will be reduced thereby improving safety.

A study by the von Herrath group provided proof of principle that a combination of approaches can synergize to effectively suppress β cell autoimmunity (Bresson et al. 2006). Recent-onset diabetic NOD mice were given sub-optimal doses of anti-CD3 $F(ab')_2$ coupled with intranasal administration of proinsulin. Diabetes reversal and the frequency of proinsulin-specific $Foxp3^+$Treg and adaptive Treg are significantly increased by combining the two therapies versus either alone. Anti-CD3 $F(ab')_2$ purging of pathogenic Teff and subsequent quenching of ongoing inflammation is thought to establish a milieu favorable for the induction and expansion of proinsulin-specific Treg. This increased Treg pool then maintains β cell-specific tolerance under relatively less stringent conditions (i.e., reduced Teff numbers). Synergy has also been achieved by combining anti-CD3 Ab with cell-based therapy (Baas et al. 2014). The frequency and duration of islet allograft survival are increased in mice treated with anti-CD3 $F(ab')_2$ plus tolerogenic DC. Islet allograft tolerance is mediated by expanded alloantigen-specific $Foxp3^+$Treg. A recent study demonstrated that co-treatment with anti-CD20 Ab and orally administered anti-CD3 Ab increases diabetes prevention and remission in NOD mice expressing human CD20 (Hu et al. 2013). Protection correlates with increased $Foxp3^+$Treg exhibiting enhanced suppressor activity, and IL-10-secreting adaptive Treg. Coupling anti-CD3 Ab therapy with IL-1β blockade is another example of synergy being achieved to effectively suppress β cell autoimmunity (Ablamunits et al. 2012). As noted earlier, β cells are sensitive to the cytotoxic effects of IL-1β secreted by islet innate effectors. The combination of anti-IL-1β Ab and anti-CD3 $F(ab')_2$ enhances diabetes reversal in NOD mice by an increase in both Treg and anti-inflammatory APC. It is noteworthy that a recent phase II trial showed that treatment with either anti-IL-1β Ab (canakinumab) or an IL-1 receptor antagonist (anakinra) fails to preserve β cell function in recent-onset T1D subjects (Moran et al. 2013).

A combinatorial approach may also complement deficiencies associated with a given immunotherapy. Low-dose IL-2 therapy has been reported to selectively increase $Foxp3^+$Treg and suppress graft versus host disease in the clinic (Koreth et al. 2011). Accordingly, low-dose IL-2 may protect $Foxp3^+$Treg from the negative effects of co-stimulatory molecule blockade and, therefore, prolong protection in recent-onset T1D subjects treated with abatacept, for example. Low-dose IL-2 or IL-2–Ab complexes may serve as "adjuvants" to increase $Foxp3^+$Treg numbers, survival and/or function and in turn enhance the efficacy of various Ab-based strategies (e.g., anti-CD3 Ab, anti-CD20, alefacept).

Improved preservation of C-peptide production may be accomplished by not only targeting T cells and other immune effectors, but also by directly modulating β cell survival, function and/or replication. For instance, efficacy of anti-CD3 Ab therapy to reverse diabetes in new-onset NOD mice is improved by glucagon-like peptide 1 co-administration, which increases recovery of β cell function and insulin secretion (Sherry et al. 2007). Interestingly, remission induced in NOD mice treated with non-depleting anti-CD4 and -CD8 Ab is partly attributed to islet APC production of TGF-β1, which is thought to directly enhance β cell replication and insulin secretion (Yi et al. 2012).

Concluding Remarks

Clinical findings support the notion that progression of T1D can be altered via Ab-based immunotherapies that either directly or indirectly target T cells. However, the efficacy seen in the clinic has only been transient, indicating that more robust strategies are needed. An ever-growing list of targets has been explored in the various rodent models of T1D, including TCR and associated signaling molecules, co-stimulatory molecules, adhesion molecules, and cytokine and chemokine receptors. Specific Teff subsets have been targeted using both lytic and non-depleting approaches. Most of these Ab are effective in preventing disease progression while only a few reliably reverse hyperglycemia. Despite this extensive knowledge base, clinical efforts often do not reflect the level of scientific understanding in the arenas of rodent diabetes or human immunology. Trials have featured Ab that target T cells with the lowest level of precision (e.g., ATG), and the greatest risk of activation-associated adverse effects (e.g., anti-CD3 Ab). Because safer and more effective candidates than anti-CD3 Ab and ATG have been identified in animal studies, anti-T cell Ab remain a strategy deserving of clinical research efforts. Such efforts could be confounded by the fact that the diabetogenic response in humans is heterogeneous, possibly reflecting distinct "subsets" of T1D. It is, therefore, likely that combinatorial immunotherapies, targeting multiple effector cells and disease pathways will be required to treat T1D effectively. The challenge, however, is to identify the appropriate combination of monotherapies that promote potent synergy and long-term suppression of β cell autoimmunity. Advancement of T1D biomarker knowledge is paramount, with the understanding that biomarker patterns may differ from case to case and that different Ab will have distinct effects. As a side point, immunotherapies that generally block the production of proinflammatory cytokines (IFN-γ, TNF-α, IL-1β), starting from the first dose onward, while promoting anti-inflammatory/tissue repair cues hold particular promise. The key to reversing islet autoimmunity likely involves rapid elimination of the inflammatory mediators associated with autoimmunity and in response to broadly lytic Ab, as such molecules have toxic effects on the pancreas. Cessation of autoimmunity while promoting islet repair remains the main strategic goal. Results from clinical studies of Ab-based therapies targeting T cells provide for the first time, a foundation that can be exploited to improve T1D immunotherapy.

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