Chapter 23

Antibody-Based and and Cellular Therapies of Type 1 Diabetes

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1. Introduction

Type 1 diabetes (T1D) is an insulin-dependent diabetes because of insufficient insulin production by the pancreatic islet β cells. Although the pathogenic mechanism of T1D is not yet completely clear, the current view of T1D pathogenesis is that under certain genetic background, exogenous and/or endogenous factors trigger autoimmunity against islet β cells in the pancreas causing β cell damage and subsequent insufficiency of insulin production [1, 2]. About two decades ago, it was first demonstrated that T cells specific to β cell antigens were activated and participated in the pathogenesis of T1D [3, 4]. A great deal of work following these reports in both animal models and humans has provided convincing data further supporting T1D is a T cell-mediated autoimmune disease. On the other hand, the evidence showing that majority of T1D patients have high titers of autoantibodies against islet β cells [5, 6] suggests that self-reactive B cells must also be involved in the autoimmune process. The role of B cells in the pathogenesis of T1D was further supported by the recent research and clinical data demonstrating B cell depletion by anti-CD20 antibodies delayed the disease process.

The clinical presentation of T1D is preceded by a period of time of active autoimmune response occurring in the pancreatic islets. When overt diabetes occurs, approximately 95% of islets are destroyed. Therefore, tremendous efforts have been pulled in halting or slowing down autoimmune process for the purpose of preventing T1D. Several clinical trials in T1D prevention have been put forward. However, there is, thus far, no effective approach to the prevention of human T1D despite that many have shown promising results in T1D animal models. Further effort is needed to discover new ways to prevent T1D. Importantly, from a practical point of view, reversing overt diabetes is much needed. In this chapter, we will fo-

cus on recent research in immune intervention of disease process in T1D including modulation of T cells, B cells by antibodies as well as cellular therapies such as autologous hematopoietic stem cell transplantation (ASCT), treatment with mesenchymal stem cells (MSC) and cord blood transplantation.

2. Antibody-based therapies of type 1 diabetes

In this section, we will discuss antibody-based therapy in T1D including therapies using anti-CD20 and anti-CD3 antibodies as well as anti-thymocyte globulin.

2.1. Anti-CD20 antibody therapy in type 1 diabetes

B cells are immune cells that produce antibodies when stimulated by exogenous or endogenous antigens. Several autoimmune diseases are associated with self-reactive B cells such as systemic lupus erythematosus and idiopathic thrombocytopenia. Reports have shown that depletion of B cells ameliorates such autoimmune conditions [7-9]. CD20 is highly expressed on the surface of mature B cells and is the B cell-specific marker. Thus, anti-CD20 antibody has been employed for the *in vivo* depletion of B cells. The FDA-approved drug, anti-human CD20, Rituximab has been used clinically for CD20+ B cell lymphoma for over a decade and largely improved the survival of patients with B cell lymphoma. Recently, anti-CD20 therapy was tested in several clinical trials for the treatment of B cell-mediated autoimmune diseases and demonstrated promising efficacy including a clinical study for the new onset T1D patients [10]. We will get into details on how anti-CD20 therapy works, its efficacy in treating T1D, the potential adverse effects as well as the issues to be solved in the future.

2.1.1. The mechanisms of action of anti-CD20 therapy

It is generally believed that the effect of anti-CD20 therapy results from the depletion of B cells by the anti-CD20 antibodies *in vivo*. However, the mechanism of action of anti-CD20 therapy is largely beyond B cell depletion. Several mechanisms have been proposed concerning the action of anti-CD20 therapy.

2.1.1.1. Complement activation in anti-CD20 therapy

Complement activation by binding the Fc fragment of the antibody leads to cell lysis, or named complement-dependent cytoxicity (CDC). Complement-dependent cell lysis is controlled by the degree of complement activation and regulated by a series of complement inhibitory proteins, such as CD35, the complement receptor type 1; CD46, the membrane cofactor protein; CD55, the decay accelerating factor; and CD59, the membrane inhibitor of reactive lysis. It appears that the ability of CD20 to move into lipid rafts is needed for CDC to occur [11]. Some protein kinase pathways are also involved in regulating CDC activity, e.g. the activation of PKC, PKA and MEK is associated with B cell resistance to CDC [12]. Furthermore, complement activation has other effects besides cell lysis, such as depositing C3, C3b and additional CD3b breakdown products on the cell surface [13].

2.1.1.2. Antibody-dependent cellular cytoxicity (ADCC)

ADCC effect in anti-CD20 therapy represents killing of target cells (B cells) by the effector cells that are activated by binding the Fc fragment of anti-CD20 antibody bound on B cells. Members of the Fcγ receptor family are expressed on monocytes, macrophages and granulocytes, and include the activating high-affinity FcγRI (CD64) and low-affinity FcγRIIIA (CD16), as well as the inhibitory low affinity FcγRIIB (CD32). FcγRIIB is believed to be a key regulator on B lymphocytes [13-15]. ADCC was recently demonstrated as an important *in vivo* mechanism of anti-CD20 action [13].

2.1.1.3. CD20 binding induces B cell apoptosis

Although the major role of anti-CD20 therapy is cell and complement-mediated cell lysis, there is evidence that anti-CD20 antibody mediates B cell death through inducing B cells to undergo apoptosis [16]. Anti-CD20 antibody, such as Rituximab induces B cell apoptosis through activation of caspase-3 [17], whereas the FAS ligand/FAS death pathway does not seem necessary. Therefore, the mitochondrial-dependent pathway is likely the death pathway induced by anti-CD20. The role of bcl-2-dependent pathways remains unclear.

Most research on the mechanisms of action of anti-CD20 therapy was conducted in B cell lymphoma to study how anti-CD20 therapy kills lymphoma tumor cells but not normal B cells. Anti-CD20 antibody therapy may work differently when used to modulate normal mature B cells. Although the existing evidence shows that anti-CD20 therapy induces regulatory T cells or regulatory B cells in autoimmune settings [18-20], the mechanisms of action of anti-CD20 in modulating normal mature B cells are not fully understood and need to be further addressed. The insight into the mechanisms of action of anti-CD20 therapy in autoimmune settings is of great importance in guiding anti-CD20 therapy in autoimmune diseases.

2.1.2. Animal studies on anti-CD20 therapy in T1D

NOD (nonobese diabetes) mouse is an animal model of human T1D. In this strain of mice, diabetes starts to occur usually around 10 weeks of age. Tremendous measures have been tested for T1D prevention in NOD mice including anti-CD20 therapy. Hu reported for the first time that anti-CD20 therapy not only prevented but also reversed T1D in humanized NOD mice (Hu-NOD, CD20 transgenic mice). Furthermore, anti-CD20 therapy-modulated B cells can transfer diabetes-protective effect when co-transferred with diabetogenic spleen cells in NOD-scid mice, suggesting post anti-CD20 depletion the reconstituted B cells might acquire tolerogenic, or regulatory capacity. Additionally, the authors discovered that anti-CD20 therapy significantly induces CD4+CD25+Foxp3+ regulatory T cells [21]. It has been shown that NOD mice deficient for B cells from the birth fail to develop autoimmune diabetes [22]. Xiu, et al [23] directly tested how depletion of B cells influenced T1D pathogenesis in wild-type NOD mice with an intact immune system. NOD female mice at early and late pre-clinical stages of disease were treated with mouse anti-mouse CD20 mAbs. Short-term anti-CD20 mAb treatment in 5-week old NOD female mice reduced B cell numbers by 95%, decreased subsequent insulitis, and prevented diabetes in >60% of littermates. The treatment

in 15-week old NOD mice was unable to prevent T1D but significantly delayed the process. In contrast to the study described previously, this study failed to show any changes in T cells and regulatory T cells [22]. The results of anti-CD20 therapy in the above T1D animal models are encouraging. Further clinical studies are needed to determine whether anti-CD20 therapy has the same efficacy in human T1D.

2.1.3. Clinical studies on anti-CD20 therapy in T1D

A randomized, double blind clinical study testing the effect of Rituximab on new-onset T1D was conducted by the T1D TrialNet Anti-CD20 study group. The results were published in New England Journal of Medicine 2009 [10]. In this well-designed clinical study, 87 patients aged 8 to 40 years who had newly diagnosed T1D were assigned to either receive infusions of 375 mg/m^2 Rituximab (57 cases) or placebo (30 cases) on days 1, 8, 15, 22. The primary outcome assessed 1 year after the first infusion, was geometric mean area under the curve (AUC) for the serum C-peptide level during the first 2 hours of a mixed-meal tolerance test. The results showed that at 1 year post treatment, the mean AUC for the level of C peptide was significantly higher in the Rituximab group than in the placebo group. The levels of glycated hemoglobin and requirement of insulin were significantly reduced in Rituximab group as compared to placebo group. Peripheral blood B lymphocytes were quickly depleted in the Rituximab group, and slowly recovered with time. By the end of 1 year observation, the levels of B lymphocytes increased to 69% of baseline. It is noted that the rate of C-peptide loss did not accelerate with recovery of B cells between 6 months and 1 year. Patients in Rituximab group had more incidences of adverse events, displaying mostly grade 1 or grade 2 after the first infusion. The reactions appeared to be minimal with subsequent infusions. No increased frequency of infection or neutropenia with Rituximab was reported. In this study, the authors observed significant reduction of serum IgM but not IgG suggesting Rituximab may selectively deplete sub-populations of B cells. Based on the results of one-year follow-up, anti-CD20 therapy is a promising approach for T1D treatment. Whether repeating the course of anti-CD20 therapy is needed and whether this treatment leads to long-term protection need to be further investigated.

A follow-up study on the patients enrolled in the clinical study described above attempted to address how Rituximab infusion influences the levels of autoantibodies against islet antigens. Autoantibodies to insulin (IAAs), GAD65 (GADAs), insulinoma-associated protein 2 (IA2As), and ZnT8 (ZnT8As) were measured with radioimmunoassays. The results showed that Rituximab markedly suppressed IAAs compared with the placebo injection but had a much smaller effect on GADAs, IA2As and ZnT8As. A total of 40% (19 of 48) of Rituximab-treated patients who were IAA positive became IAA negative versus 0 patient out of 29 placebo-treated patients. In the subgroup (n=6) treated within 50 days of diabetes, IAAs were markedly suppressed by Rituximab treatment in all patients for 1 year and for four patients as long as 3 years despite of continuing insulin therapy. Independent of Rituximab treatment, the mean level of IAAs at study entry was markedly lower for patients who maintained C-peptide levels during the first year of follow up in both Rituximab-treated and placebo groups [24]. The results described above suggest that anti-CD20 therapy differen-

tially suppresses anti-islet autoantibodies. Further studies are needed to investigate whether autoreactive B cell clones against islet antigens are differentially depleted.

As mentioned earlier, mouse studies have demonstrated that anti-CD20 therapy alters T cells. It is of interest to know how Rituximab infusion affects T cell responses in human T1D. This was explored in a follow-up study on patients in the above-described clinical trial Surprisingly, it is noted that Rituximab treatment leads to enhanced proliferative responses of T cells to diabetes-associated islet-specific autoantigens, which are positively correlated with C-peptide levels [25]. It is still unclear why B cell depletion enhances T cell proliferation. It is likely to be related to the refill of immune system post B cell depletion by T cells through homeostatic proliferation. Further studies are needed to characterize those T cells phenotypically and functionally to determine whether those autoreactive T cells are beneficial or harmful for controlling autoimmunity.

Drug resistance is the major reason of failure in the treatment of B cell lymphoma by Rituximab [26, 27]. It had been unknown whether anti-CD20 therapy in autoimmune diseases, such as T1D can also lead to drug resistance until a recent report indicating a potential mechanism for the ineffectiveness of anti-CD20 therapy in T1D [28]. This animal study showed that anti-CD20 efficiently depleted follicular but not marginal zone B cells. Interestingly, the islet infiltrated B cells lost their CD20 expression, which might explain the ineffectiveness of anti-CD20 therapy in late stage of T1D in NOD mice. Gradual recovery of the antibodies against islet antigens further suggests that autoimmune B cells are unable to be completely wiped out by anti-CD20 therapy. New drugs targeting the islet-infiltrated $CD20^{neg}$ B cells, such as anti-CD19 antibodies may be needed to further improve the efficacy of immunotherapy targeting B cells.

One of the most concerned issues regarding anti-CD20 therapy is the potential infection arisen by B cell depletion [29]. Anti-CD20 therapy indeed leads to hypoimmunoglobulinemia [30-32]. However, there is no significant increase in the incidence of infection during anti-CD20 therapy, which is consistent with a recent report demonstrating that anti-CD20 therapy does not deplete memory B cells specific to the antigens previously encountered [33, 34]. Another biggest concern of anti-CD20 therapy is anaphylaxis to anti-CD20 antibodies [35] because the current clinically employed anti-CD20 antibodies like Rituximab are made from animals. Humanized anti-CD20 antibodies are being developed, and are expected to overcome this severe adverse effect.

2.2. Anti-CD3 antibody therapy in type 1 diabetes

Given that T1D is a T cell-mediated autoimmune disease, T cell depletion therapy is expected to be a promising approach in T1D therapy. Much attention to anti-CD3 therapy has been drawn to the researchers and clinicians in the field of T1D. Over the years of basic and clinical studies, enormous progress has been made in terms of mechanism of action and the optimization of anti-CD3 therapy. Several clinical trials in new onset T1D are under way. In the following section, we will discuss T1D anti-CD3 therapy including mechanism of action, animal studies, clinical studies, adverse effects as well as issues to be resolved, etc.

2.2.1. Mechanism of action of anti-CD3 therapy

As what has been described in anti-CD20 therapy, anti-CD3 therapy works eventually through non-selective depletion of T cells including CD4+ and CD8+ T cells. Currently, it is believed that the mechanism of action of anti-CD3 therapy is largely beyond T cell depletion. In this section, we will discuss how anti-CD3 therapy works to preserve islet β cells.

2.2.1.1. Activation-induced cell death in anti-CD3 therapy

It is known that in the initial stage of anti-CD3 therapy, all T cells including CD4+ and CD8+ T cells are activated as evidenced by the expression of CD69. Activation-induced cell death (AICD) is a major mechanism regulating central tolerance during T cell development in thymus. Yu et al reported that anti-CD3 triggered AICD in activated T cells *in vitro* [36], which might explain anti-CD3 therapy-induced immune tolerance. However, the *in vivo* data are controversial. While accumulating data showed that anti-CD3 therapy is able to induce T cell apoptosis *in vivo* as it behaves *in vitro*, but others provided evidence showing that anti-CD3 therapy does not induce T cell death but induce unresponsiveness to stimuli of mitogens [37]. Therefore, anti-CD3 therapy is not just T cell depletion, but likely other tolerogenic mechanisms participate in this process.

2.2.1.2. Anti-CD3 therapy promotes regulatory T cells

In 2003, an elegant study by Belghith, et al [38] reported that anti-CD3 treatment induced TGF-β-producing T cells which was indispensable for the anti-CD3-induced immune tolerance. A later study further confirmed this finding and demonstrated that anti-CD3 therapy induced regulatory T cells through TGF-β released from phagocytes phagocytosing apoptotic T cells induced by anti-CD3 therapy *in vivo* [39]. Neutralizing TGF-β or blocking phagocytosis abrogated the induction of regulatory T cells [39]. Additionally, anti-CD3 therapy might differentially deplete distinct subsets of T cells and preferentially preserve regulatory T cells. Consistent with this concept, a recent report demonstrated anti-CD3 therapy in NOD mice selectively depleted autoantigen-specific effector T cells but preserved regulatory T cells [40]. The increase of regulatory T cells was not due to the conversion of regulatory T cells from conventional T cells because all preserved regulatory T cells expressed helios which is a natural regulatory T cell marker [40]. The resistance of natural regulatory T cells to anti-CD3 depletion is not clear but may be associated with compromised activation of apoptotic pathways in regulatory T cells in response to anti-CD3 therapy. Recently, Bisikirska et al [41] reported that anti-CD3 treatment in human T1D patients expanded CD8+ T cells and induced Foxp3+ CD8+ regulatory T cells, suggesting that this type of regulatory T cells might contribute to immune tolerance induced by anti-CD3 therapy.

2.2.1.3. Anti-CD3 therapy induces T cell anergy

Another form of immune tolerance is T cell anergy. To dissect the mechanism of action of anti-CD3 therapy, it is interesting to know whether anti-CD3 therapy induces T cell anergy i*n vivo*. There are two forms of anti-CD3 antibodies based on their working principles, i.e.

mitogenic and nonmitogenic antibodies. Nonmitogenic anti-CD3 antibody is thought to induce T cell tolerance mainly through the induction of T cell anergy. Smith reported that nonmitogenic anti-CD3 antibody treatment only delivers partial T cell activation signals thereby inducing T cell unresponsiveness (anergy) [42]. This effect occurs not only in CD4+ T cells but also in CD8+ T cells. Research data from Bluestone's group show that *in vivo* treatment of anti-CD3 antibodies induces long-term CD8+ T cell anergy [43-45]. The mechanism underlying anti-CD3 therapy induced T cell anergy is not known until a recent report from Bluestone's group showing that PD1-PDL1 interaction is required for maintaining long-term T cell anergy and T1D protection [45]. Blocking PD1-PDL1 interaction quickly reverses T cell anergy and the anergic T cells become pathogenic effector T cells. Under blockade of PD1-PDL1 interaction, the protected NOD mice by anti-CD3 treatment quickly develop diabetes [45]. The above data suggest that maintenance of anergic state of autoimmune T cells is essential, and PD1-PDL1 may play a pivotal role in this process.

2.2.2. Anti-CD3 therapy in T1D animal models

There are currently two T1D animal models that spontaneously develop diabetes under genetic susceptibility, NOD mice and diabetes-prone biobreeding (BB) rat. Although these two T1D animal models have some similarities to human T1D, there are many differences between animal and human in terms of disease progression. Nevertheless, studies on animal models will definitely provide very useful information for the immunopathogenesis, prevention and treatment of T1D. Several clinical trials are based on promising results in animal models including anti-CD3 therapy in new onset diabetes.

Anti-CD3 therapy for T1D was first tested in NOD mice. Chatenoud, et al [46] reported that anti-CD3 treatment of adult NOD mice significantly inhibits the autoimmune process. Short-term low-dose anti-CD3 treatment (5 μg/day i.v. for 5 consecutive days) prevented the occurrence of an accelerated form of the disease induced by cyclophosphamide. When this regimen was administered in adult NOD females with newly diagnosed diabetes, 64-80% of treated mice obtained a complete remission of overt diabetes showing permanent normoglycemia. It was noted that this remission was durable (>4 months) and was not associated the disappearance of insulitis. Anti-CD3 treated mice failed to reject syngeneic islet graft but maintained normal response to allogeneic skin grafts, whereas control untreated diabetic NOD females rejected both, suggesting that anti-CD3 therapy reverses diabetes through inducing islet antigen-specific immune tolerance. This study also suggests that diabetes-reversing effect can be obtained by transient targeting of the CD3/T-cell receptor without massive T-cell debulking. As described earlier, this effect may be associated with diabetes-protecting regulatory T cells induced by anti-CD3 therapy.

To improve the effectiveness of anti-CD3 therapy, a strategy of combination with islet antigens has been proposed to more effectively restore self-tolerance to islet antigens. Bresson, et al [47] reported that anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes in comparison to monotherapy with anti-CD3 or antigen alone. Further studies demonstrated the expansion of CD25+Foxp3+ and insulin antigen-specific regulatory T cells producing IL-10, TGF-β and IL-4. When adoptively trans-

ferred, these cells could transfer immune tolerance to immunocompetent recent-onset diabetic recipients and suppressed autoaggressive CD8+ responsive T cells. This strategy would act more site-specifically thereby reducing the risk for systemic side effects. The same group employed a mathematical disease model, and revealed that preexisting autoantibodies predict efficacy of oral insulin in combination with anti-CD3 antibodies to cure autoimmune diabetes [48]. This study shows that NOD mice with higher pretreatment levels of serum insulin-associated antigens (IAAs) responded with a much higher likelihood to combination therapy but not anti-CD3 monotherapy, indicating that IAAs may be a good biomarker to predict a better capability of the mice in inducing insulin-specific regulatory T cells after oral insulin immunization. Ablamunits, et al [49] reported recently that co-administration of anti-CD3 and IL-1 receptor antagonist had synergistic effect on T1D reversal, which showed that the combinatorial therapy led to persistent remission from islet inflammation. Whether the resolution of islet inflammation leads to regeneration of islet β cells was not addressed in this report.

The outcomes from animal studies have provided very useful information for developing anti-CD3-based therapeutic strategies for human T1D. Although the results of anti-CD3 therapy in human T1D are mixed, the preservation of β cell function in the current clinical trial suggests that anti-CD3 therapy is a viable regimen for human T1D.

2.2.3. Anti-CD3 therapy in T1D clinical studies

The results of the first clinical trial using anti-CD3 therapy for human T1D were reported in New England Journal of Medicine in 2002. Herold, et al [50] studied the effects of a nonactivating humanized mnonclonal antibody against CD3 (hOKT3gamma1 (Ala-Ala) on the loss of insulin production in patients with recently diagnosed T1D. Within 6 weeks after diagnosis, 24 patients were randomly assigned to receive either a single 14-day course of anti-CD3 treatment, or no antibody and, were followed for one year. The results showed that anti-CD3 treatment maintained or improved insulin production after one year in 9 of the 12 patients in the treatment group whereas only 2 of the 12 controls had a sustained response. The treatment effect on insulin response lasted for at least 12 months after diagnosis. Glycated hemoglobin level and insulin dose requiement were reduced in the anti-CD3 treatment group. No severe adverse effect was observed, and the most common side effects were fever, rash, and anemia. Clinical responses were associated with a change of CD4+ T cells to CD8+ T cells ratios at 30 and 90 days after treatment with the responders showing reduced CD4/CD8 ratios. This study provides initial encouraging results. Longer period of follow-up would be needed to establish the long-term effectiveness of this therapy.

A clinical study was conducted using the similar protocol in the above-mentioned study but with different doses at different injection times during the treatment course. The results demonstrated significant improvement in C-peptide response to a mixed meal. The improved C-peptide responses were accompanied by reduced HbA1c and insulin requirements. These results indicate that treatment with anti-CD3 antibody, hOKT3γ1 (Ala-Ala), Teplizumab results in improved C-peptide responses and clinical parameters in T1D for at least 2 years in the absence of continued immunosuppressive medications. In this study, be-

cause of severe adverse effect of the increased dose of Teplizumab, the patient enrollment was stopped after 10 patients enrolled [51]. Among these patients, four drug-treated patients were followed up for 5 years. Results showed that C-peptide responses were maintained. During this study, it was found that increased dose of anti-CD3 antibodies caused severe adverse effects without gaining improved therapeutic effect [52]. Thus, the dosing may need to be further modified to gain the best benefit for the patients.

Recently, the results of a randomized and double blind clinical trial (clinicaltrial.gov, number NCT00385697) conducted by multi-centers from different countries on anti-CD3 therapy in treating new onset T1D was reported in Lancet journal [53]. In this 2-year trial, patients aged 8-35 years who had been diagnosed with T1D for 12 weeks or fewer were enrolled and treated at 83 clinical centers in North America, Europe, Israel and India. Participants received one of the three regimens of teplizumab infusions (14-day full dose, 14-day low dose, or 6-day full dose, or placebo). Patients and study staff remain masked through to study closure. 763 patients were screened, of whom 516 were randomized to receive 14-day full-dose teplizumab (n=209), 14-day low dose teplizumab (n=102), 6-day full-dose teplizumab (n=106), or placebo (n-99). Two patients in the 14-day full-dose group and one patient in the placebo group did not start treatment, so 513 patients were eligible for efficacy analysis. The primary outcome did not differ between groups at 1 year. Nonetheless, 5% (19/415) of patients in the teplizumab groups were not taking insulin at 1 year, compared with no patients in the placebo group at 1 year (p=0.03). All groups had similar incidences of adverse effects. The most common clinical adverse event in the teplizumab groups was rash (220/417 [53%] versus 20/99 [20%] in the placebo group). This study suggests that future studies of immunotherapeutic intervention with Teplizumab might have increased success in prevention of a decline in β-cell function and provision of glycemic control at reduced doses of insulin if they target patients early after diagnosis of diabetes and children.

From the results of the above clinical trials, anti-CD3 therapy is a promising regimen for human new onset T1D. However, its efficacy needs to be further improved. For this purpose, combinatorial therapy is a rational approach [54, 55]. As described above, recent animal studies demonstrated that anti-CD3 therapy combined with islet β cell antigens induced islet antigen-specific immune tolerance and significantly improved the effectiveness of anti-CD3 therapy in NOD mice. Anti-CD3 antibody combined with IL-1 receptor antagonist was tested in NOD mice and showed significant improvement of therapeutic efficacy. International multi-center clinical trials have tested the agents, anti-CD3, GAD, diapep227, insulin immunization and IL-1 receptor antagonist, anakinra, separately. There is, thus far, no clinical trial testing the efficacy of combinatorial therapy of the above-mentioned agents in treating T1D. Therefore, a phase 1 clinical trial may be needed in this respect.

2.3. Anti-thymoglobulin (ATG) therapy in type 1 diabetes

Simson, et al reported that ATG treatment (500 μg/mouse) at day 1 and day 3 attenuated T1D development. It was noted that this T1D protection only presented when NOD mice were at disease onset or in the late pre-diabetic phase (12 weeks of age). It was demonstrated that when provided at 12 weeks of age, ATG reversed pancreatic insulitis, improved met-

abolic responses to glucose challenge, and rapidly increased frequency of antigen-presenting cells in spleen and pancreatic lymph nodes. It was also found that ATG therapy dramatically increased the frequency and functional activity of CD4+CD25+ regulatory T cells. Adoptive transfer/cotransfer studies of T1D support that ATG therapy induces a stable and transferable immunomodulatory repertoire *in vivo*. This study indicates that an induction of immunoregulation, rather than simple lymphocyte depletion, contributes to the therapeutic efficacy of ATG therapy [56]. The same group reported that ATG therapy combined with granulocyte-colony-stimulating factor (G-CSF) was remarkably effective at reversing newly diagnosed diabetes in NOD mice and more efficacious than either agent alone. This combination also afforded durable reversal from disease (>180 days post-onset) in animals having pronounced hyperglycemia (i.e., up to 500 mg/dL). Mechanistically, this combination therapy resulted in both immunological and physiological benefits, showing increased CD4/CD8 ratios and splenic regulatory T cells, as well as increased pancreatic β cell area and attenuated pancreatic inflammation [57].

Our unpublished data show that ATG therapy preferentially depletes naïve T cells, and memory T cells are relatively preserved. In addition, ATG therapy largely spares CD4+CD25+ regulatory T cells. Of interest, ATG therapy does not deplete antigen-specific T cells but alters T cell responses to the previously experienced antigens, showing increased levels of Th2 and IL-10-producing Tr1 cells, which might contribute to ATG therapy-induced T1D protection. In addition, post-ATG therapy CD4+CD25+ regulatory T cells display memory-like T cells phenotypically, suggesting that those regulatory T cells might play an important role in ATG therapy-induced long-lasting T1D protective effect.

Based on the animal studies described above, a couple of clinical trials using ATG, or ATG combined with G-CSF in human T1D are ongoing (www.clinicaltrials.gov/NCT0116157, www.clinicaltrials.gov/NCT00515099). The assessment of the effectiveness of ATG therapy in human T1D await the outcomes from these clinical trials.

3. Cellular therapy of type 1 diabetes

T1D is characterized by the autoimmune destruction of insulin-producing β cells with loss of insulin secretion. Patients with T1D have absolute requirement of insulin for survival. While insulin is effective in lowering blood glucose, hypoglycemia, even life-threatening hypoglycemia, is almost unavoidable with insulin treatment, as exogenous insulin cannot exactly mimic the profile of physiological insulin secretion. Other limitations of insulin therapy include inconvenience of daily life, physical pain and high economic costs caused by recurrent insulin injections.

Therefore, other strategies have been explored to preserve or restore β cell function in the hope that endogenous insulin secretion will achieve better glycaemic control while reducing episodes of severe hypoglycemia. As discussed above, immunotherapy, in particular the use of immunomodulatory drugs has pulled much efforts. Both experimental and clinical data demonstrate that some agents like anti-CD20 and anti-CD3 antibodies are effective in delay-

ing the process of β cell autoimmune destruction. However, no drugs have demonstrated to prevent or reverse human T1D successfully in long-term.

More recently, many efforts have been focused on the use of stem cells as a potential therapeutic strategy for T1D. So far, accumulating data from both experimental and clinical trials have suggested that stem cell-based cellular therapy could be a promising approach for T1D treatment.

3.1. Hematopoietic Stem Cell Transplantation(HST) for T1D

The use of bone marrow transplantation (BMT) as a potential treatment for T1D was first proposed in animal study in 1985 [58], showing that allogenic bone marrow transplantation could prevent insulitis and overt diabetes in NOD mice. This concept was further substantiated by later animal study [59].

The first clinical trial to use hematopoietic stem cell transplantation in T1D patients was reported in 2003 [60]. The objective of the study was to stop autoimmune destruction of β cells with immunosuppressive drugs and to "re-set"the impaired immunologic system with a reconstituted one using autologous HSCs in the expectations of preserving residual β cell mass and facilitating endogenous mechanisms of β cell regeneration. With the above considerations, 15 newly diagnosed T1D patients were enrolled. All received high-dose immunosuppression followed by autologous hematopoietic stem cell transplantation (AHST) within 6 weeks of diagnosis. During a 7- to 36-month follow-up (mean 18.8 months), 14 patients became insulin-free. β cell function was improved as evidenced by the increase in C-peptide levels. No significant adverse effects were observed. The mechanism concerning the beneficial effects of HST is proposed to be associated with the generation of a more tolerant immune system, which blocks the autoimmune destruction of residual β cells. This hypothesis appears to be consistent with a recent clinical observation showing that intra-pancreatic autologous bone marrow infusion has no beneficial effects on long-standing T1D patients with absence of β cell function [61].

To date, it is unclear whether the beneficial effects of HST can be sustained because of the lack of long-term follow-up study. Second, it is not known whether the beneficial effects of HST are due to immune reconstitution *via* stem cell differentiation or modulating the function of existing immune cells. Therefore, randomized controlled trials with prolonged follow-up are needed to confirm the results of current studies and to evaluate the full potential of this regimen as a therapeutic option for T1D.

3.2. Umbilical cord blood (UCB) cell therapy for TID

Bone marrow is a rich source of stem cells, but its application is hampered by the limited availability of bone marrow donors and the invasive procedure for cell collection. Human umbilical cord blood (HUCB) is another source of stem cells. Compared to bone marrow, HUCB has some major advantages such as easy availability, absence of risk to the donor, low risk of graft-vs-host disease and tumorigenicity, high capacity for expansion [62]. UCB has been used successfully in transplantation for diseases like acute anemia, and sickle cell anemia [63]. There

have been both animal and clinical studies evaluating the use of UCB cells as a potential therapy for T1D. The rationale is based on experimental studies. *In vitro* cultures of HUCB can yield islet-like structures capable of insulin and C-peptide production [64]. *In vivo*, human cord blood-derived cells is also shown to be able to differentiate into islet cells when transfused into 2 day old NOD-scid mice [65]. A recent report demonstrated that cord blood-derived multipotent stem cells reversed T1D through islet β cell regeneration following immune modulation [66]. Second, UCB contains a population of immature unprimed functional regulatory T cells. Theoretically, these cells could limit inflammatory reaction and anergize effector T cells, which are believed to mediate cellular autoimmune processes. In addition, UCB stem cells may act as nurse cells to stimulate the proliferation of new islets from the remaining viable tissue [67]. Ende et al. [68, 69] reported in two separate studies that infusions of HUCB improved hyperglycemia and diabetic nephropathy in obesity-induced diabetic mice. In addition, nonobese diabetic (NOD) mice can be protected from developing insulitis and diabetes by HUCB dose-dependently. However, the results of available clinical study is disappointing. In a recently completed phase I clinical study [70], 24 children aged 3.4-6.9 years, with new onset T1D received a single autologous UBC infusion within 6 months of diagnosis. After 2 years of follow-up, there was no evidence of reservation of β cell function, as evaluated by the area under the curve C-peptide that was 2% of baseline 2 years after UBC infusion, despite that the numbers of regulatory T cells (Tregs) and naïve Tregs were increased 6 and 9 months after. In that study, there are several possibilities as to why UCB infusion may fail to preserve β cell function. First, the stem cell number is insufficient. Second, there exist memory T cells refractory to regulation by Tregs. Finally, it cannot be excluded that the UCB cells from the T1D patients may have intrinsic defects with compromised biological function. In future, autologous or allogeneic transplantation with expanded UBC Tregs either alone or in combination of immunomodulatory drugs may be worth trying. Importantly, randomized controlled studies are needed before definitive conclusions can be finally reached.

3.3. Mesenchymal stem cell therapy for TID

Mesenchymal stem cells (MSCs) were originally identified by Friedenstein et al. in 1976 [71] in the bone marrow as a fibroblast-like cell population capable of generating osteogenic precursors. MSCs from the bone marrow (BM) are a heterogeneous, stromal population of multipotent non-hematopoietic progenitor cells capable of differentiating into multiple mesenchymal lineages including bone, fat and cartilage. In addition to bone marrow, MSCs have been found to be present in other tissues such as adipose tissue, umbilical cord blood, synovial membrane, skeletal muscle, dermis, deciduous teeth, pericytes, trabecular bone, articular cartilage, umbilical cord, placenta, liver and spleen. It is now known that MSCs are able to differentiate into mesodermal and non-mesodermal cell lineages, including osteocytes, adipocytes, chondrocytes, myocytes, cardiomyocytes, fibroblasts, myofibroblasts, epithelial cells, and neurons [72].

In addition to their pluripotency to differentiate, MSCs have high immunomodulatory capacity. The immunomodulatory property of MSCs are associated with their inhibitory effects on the proliferation and differentiation of both T cells and B cells, as well as dendritic

cell (DC) [73]. Moreover, MSCs can modulate immune response through stimulating the production of $CD8^+$ Treg (regulatory T cells) [74]. MSCs are known to secrete a variety of trophic mediators such as growth factors and cytokines (M-CSF, IL-6, IL-11, IL-15, SCF, VEGF) that are involved in the regulation of immune response and hematopoieses. This could be a major mechanism underlying the immunomodulatory action of MSCs. Recently, MSCs have been used in clinical trials for the treatment of acute graft-versus-host disease (GVHD) following allogeneic HSC transplantation [75,76], and for autoimmune diseases such as multiple sclerosis and Crohn disease [77,78]. Another striking characteristic of MSCs is the ability to differentiate into insulin-producing cells (IPCs). *In vitro*, MSCs can be differentiated into IPCs when cultured under proper conditions. The types of MSCs that have been successfully induced to generate IPCs includes BM-MSCs, umbilical cord blood MSCs (UCB-hMSCs), pancreatic MSCs and adipose-derived MSCs,etc. [79].

By now, the use of MSCs for treatment of diabetes have been explored in two animal studies. In a model of murine STZ-induced diabetes, co-administration of BM cells with syngeneic or semi-allogeneic MSCs normalized blood glucose and serum insulin levels. The beneficial effect of this treatment does not seem due to the reconstitution of the damaged islet cells from the transplant since no donor-derived β cells were found in the recovered animals. Instead, the benefits may be due to the immunosuppressive effect of MSCs on the β cell-specific T cell response since MSCs injection caused the disappearance of beta-cell-specific T lymphocytes from diabetic pancreas, which may allow the regeneration of recipient-derived pancreatic insulin-secreting cells [80]. In another study [81], the mechanism underlying the beneficial effects of MSCs on blood glucose was investigated in a diabetic rat model induced by high-fat diet/streptozotocin (STZ) administration. Autologous MSCs were administered either 1 or 3 weeks after STZ injection. Infusion of MSCs during the early phase not only promoted β cell function but also ameliorated insulin resistance, whereas infusion in the late phase merely ameliorated insulin resistance. The improved insulin sensitivity induced by MSCs infusion is associated with an increase of GLUT4 expression and an elevation of phosphorylated insulin receptor substrate 1 (IRS-1) and Akt (protein kinase B) in insulin target tissues.

Taken together, these *in vitro* and *in vivo* experiments suggest that multiple mechanisms may be involved for the beneficial effect of MSCs on blood glucose control in T1D. Thus far, the use of MSCs to treat T1D is limited to animal studies. The efficacy of MSCs to treat patients with T1D needs to be further evaluated in well-designed clinical trials.

In conclusion, both anti-lymphocyte antibody-based and cellular therapies are promising in stopping ongoing autoimmunity against islet antigens and likely leading to a hopeful restoration of self-tolerance. The regimens combining anti-lymphocyte antibodies, islet antigens and cellular therapies could maximize the preventive and/or therapeutic efficacy for T1D.

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