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REVIEW

Immunotherapy in Autoimmune Type 1 Diabetes

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■ Abstract

Type 1 diabetes (T1D) is a chronic autoimmune disease affecting millions of people worldwide. The disease is characterized by the loss of self-tolerance to the insulin-producing β-cells in the pancreas, the destruction of β-cells, and finally the development of chronic hyperglycemia at diagnosis of T1D. Its incidence and prevalence are rising dramatically, highlighting the need for immunotherapeutic strategies able to prevent or treat the disease in a safe and specific manner. Immunotherapeutic strategies are being developed, and aim to restore immunological self-tolerance, thereby limiting unwanted immunity and β-cell destruction. Foxp3$^+$ regulatory T (Treg) cells exert essential functions to maintain and restore immunological self-tolerance. The identification of the transcription factor Foxp3 as the specification factor for the Treg cell lineage facilitated our understanding in the biology of Treg generation and function. This review highlights the current understanding of immunotherapeutic approaches as preventative and curative measures for autoimmune T1D. It includes an overview on early immunointervention studies, which made use of general immunosuppressive agents such as cyclosporin A, followed by a discussion on newly emerging clinical trials. Besides non-antigen-specific therapies, particular attention is given to antigen-specific generation of Foxp3$^+$ Treg cells and their potential use to limit autoimmunity such as T1D.

Keywords: type 1 diabetes · immunotherapy · autoimmunity · regulatory T cell · Foxp3 · mimetope · antigen · tolerance

Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disorder characterized by specific immune destruction of the insulin-producing pancreatic β-cells [1]. The inaccessibility of the human pancreas has led to relatively limited studies of its role in the biological mechanisms of T1D. Despite this limitation, several key studies have provided evidence for the infiltration of immune cells in the pancreatic islets (insulitis) before and at diagnosis of the disease [1]. Autoreactive T cells and other mononuclear cells infiltrate the islets initiating and maintaining insulitis, which ultimately leads to β-cell death, diabetes, and a lifelong requirement for insulin therapy [1]. However, detailed understanding of the precise mechanisms dictating the immunological events that control autoimmune destruction is still missing and needs to be elucidated.

The incidence of T1D is rapidly rising in children, with a predicted 70% increase in incidence over the next 15 years in Europe. Furthermore, the age of onset is also decreasing, with a predicted doubling of cases in children under the age of 5 years during the same period, which highlights the dramatic impact of T1D on future public health.

Despite refined treatment with insulin therapy, long-term complications, including nephropathy, retinopathy, neuropathy, and cardiovascular disease, can appear [2, 3]. It is believed that T1D de-

velops as a result of genetic predisposition and unknown environmental factors. Genetic susceptibility to T1D is largely conferred by the inheritance of the human leukocyte antigen (HLA) class II haplotypes of HLA-DR and HLA-DQ located within the major histocompatibility complex (MHC) on chromosome 6p21 [4]. Genome-wide association studies (GWAS) have revealed that 25 non-HLA-associated loci contribute additional risk to autoimmune T1D. The vast majority of these genes encode proteins that are involved in immune function and regulation [5, 6].

Recent longitudinal studies of a large cohort of monozygotic twins, initially discordant for T1D, demonstrated that 65% of twins ultimately develop T1D over a follow-up period of 43 years. These data support the suggestion that all identical twins will eventually become concordant providing they live sufficiently long [7]. The clinical definition of diabetes is determined by the appearance of glucose levels at which end-organ damage is known to occur. It does not account for the fact that the emergence of autoimmunity predates the diagnosis by several years [8].

Abbreviations:
AIDA - anti-interleukin-1 in diabetes action
CsA - cyclosporin A
CTLA4 - cytotoxic T lymphocyte antigen 4
DPT - Diabetes Prevention Trial
FoxP3 - forkhead box P3 protein (also known as scurfin)
GAD65 - glutamic acid decarboxylase 65
GWAS - genome-wide association studies
HLA - human leukocyte antigen
IAA - insulin autoantibody
IA-2 - insulinoma antigen
Ig - immunoglobulin
IL-1β - interleukin-1β
IMPDH - inosine monophosphate dehydrogenase
IPEX - immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
MHC - major histocompatibility complex
MMF - mycophenolate mofetil
MPA - mycophenolic acid
mTOR - mammalian target of rapamycin
NOD - non-obese diabetic
OKT3 - orthoclone muromonab CD3
PI3K - phosphatidylinositide 3-kinase
Rag - recombination activating gene
SCID - severe combined immunodeficiency (mouse model without functional T and B cells)
T1D - type 1 diabetes
TCR - T cell receptor
TGFβ - transforming growth factor beta
TNF - tumor necrosis factor
Treg - regulatory T (cell)
VNTR - variable number tandem repeat
ZnT8 - zinc transporter 8

The large number of identified genes supports the view that several signaling pathways are involved in the development of autoimmunity, finally resulting in the loss of tolerance to pancreatic islets in humans [9]. These findings have important implications for the development of immunomodulatory strategies for T1D. Since the disease is dependent on the individual, not all approaches are equally effective in all subjects. The chronic autoimmune process that usually precedes the development of clinical T1D can be monitored by autoantibody responses. Autoantibodies per se are not directly pathogenic, but they represent biomarkers for the development and status of the autoimmune disease [10]. It has been observed that individuals expressing specific autoantibodies reacting with 2 of 4 well-defined autoantigens (insulin, glutamic acid decarboxylase (GAD65), insulinoma antigen (IA-2), and islet zinc transporter (ZnT8)) eventually progress to the development of T1D [6].

Insulin-specific antibodies may be detected in some children as young as 6-12 months of age and may predate the development of T1D by up to 10 years [11]. The age of development of autoimmunity in T1D is related to the burden of susceptibility genes, with individuals at high genetic risk developing autoimmunity before the age of 5 years [12]. In line with this view, it became clear that young children can progress rapidly, within months after the appearance of autoantibodies, to overt diabetes. In contrast, some children take more than a decade to develop the disease, with evidence of chronic beta-cell destruction preceding diabetes over years, as discussed above [6]. Several studies have indicated that the appearance of autoantibodies is acute, and during the prediabetic period different autoantibodies rise and fall, but usually more than two are expressed, sometimes for decades. Similarly, in the setting of the non-obese diabetic (NOD) mouse model, the development of insulin autoantibodies occurs at an early age of around 15 days after birth. However, the characterization of other autoantibodies has met with challenges when measured with highly specific assays [1]. Alternatively, the characterization of T-cell-mediated autoimmunity to multiple autoantigens is more advanced in the NOD mouse model than in human T1D.

After the critical contribution of MHC in defining the risk of developing T1D, the second-most important locus determining T1D is the insulin gene. It has become evident that a polymorphism of a variable number tandem repeat (VNTR) 50 of the insulin gene, and not a variation in the insulin

gene sequence itself, contributes to the risk of developing T1D. The long variant of the VNTR is associated with both greater insulin message intrathymically and protection from the development of T1D [13, 14]. Insulin autoantibodies are usually, but not always, the first autoantibodies to appear in young children developing T1D [15]. Insulin autoantibody levels were found to inversely correlate with the age at which diabetes develops [6]. When detected at first, insulin autoantibodies are of high affinity and highly predictive. Insulin works as a critical target of both B cells and T cells in human T1D and in NOD mice [16-18]. In line with this observation, mice which express a mutant insulin gene product not recognized by T cells do not develop the disease [19]. In the context of human T1D, the extent of intrathymic insulin expression has been linked to the incidence of diabetes [13]. In mice and humans, the T cell response to insulin is highly focused on a segment of the B-chain encompassing residues 9-23 [16-18], and the human epitope is identical to that of the murine insulin.

It has been shown that the removal of insulin 1, which is mainly expressed in the islets, is associated with a blockade of disease progression [20]. Conversely, removal of the insulin 2 gene greatly promotes disease progression, most likely because of a deficiency in thymic insulin expression and subsequent loss of central tolerance [20-22]. Finally, retroviral introduction of T cell receptor (TCR) genes from insulin-reactive $CD4^+$ T cells into SCID NOD mice is sufficient to support the development of insulin autoimmunity in a significant proportion of mice [23].

Based on considerable evidence for the importance of insulin as an autoantigen in NOD and human T1D, and combined with the fact that in humans T1D incidence is rising dramatically, especially in young children, intensive efforts have been made to develop self-antigen-specific (e.g. insulin-specific) immunotherapies, with the ultimate goal of achieving a safe and specific prevention of T1D. Indeed, some advances have been made in the development of self-antigen-specific immunotherapy. However, translating these strategies from bench to bedside has met with challenges. Also, the success of clinical trials using natural self-antigens for induction of self-tolerance, e.g. natural insulin in T1D, has been limited. Thus far, three trials of secondary immunoprevention in T1D using whole insulin have been completed:

1. DPT-1 (oral [24] and parental [25])
2. Intranasal Insulin Trial I [26]
3. Intranasal Insulin for Prevention of T1D trial [27]

These studies using oral or intranasal insulin therapy in humans have had either no or limited clinical benefit in human T1D [28, 29]. Subsequent subgroup analysis of data from the DPT-1 trial supported the hypothesis that patients with high insulin-specific antibody titres who received oral insulin had a delay in progression to T1D [24]. The beneficial side-effect profile of oral and inhaled insulin in secondary immuneprevention trials has led to the establishment of a Phase I trial (named Pre-POINT) to assess the effects of mucosal insulin therapy for primary immunoprevention [30].

However, thus far the applied immunomodulatory approaches did not address the question of whether the choice of antigen, the time point and route of administration were suitable to induce tolerance in the respective population at risk of developing T1D. Furthermore, no specific protocols for the conversion of naïve T cells into Foxp3-expressing regulatory T (Treg) cells have been explored. Therefore, it has been proposed that disease state, antigen dose, route of administration, study cohort, and the choice of antigen, e.g. insulin vs. insulin B chain peptides, work as critical parameters in inducing tolerance [28, 29]. Moreover, an improved understanding of the requisites for an efficient induction of human self-antigen-specific $Foxp3^+$ Treg cells in an autoimmune setting such as T1D is needed.

When developing a novel T1D immunotherapeutic approach, it must build on known approaches for manipulating autoimmune mechanisms to devise novel therapeutic strategies that address these unmet medical requirements, as outlined above. In the context of the young patient population increasingly affected by T1D, the challenge is to achieve clinical efficacy in the absence of chronic immunosuppression to avoid compromising the host's defense against infections and tumors. Ultimately, the scientific efforts should result in a range of immunotherapeutic options that combine short-term β-cell preservation with long-term modulation of autoimmunity by the restoration of immunological self-tolerance. As in other immune-mediated diseases, it is unlikely that a single treatment will be effective in all patients. Biomarkers indicating clinical and immunologic efficacy will be required, along with the identification of combinatorial approaches that can guide immunotherapy for individual patients. In the

present review, we discuss the requisites and opportunities for immunotherapeutic strategies to interfere with autoimmunity such as T1D, with particular emphasis on the generation of self-antigen specific Foxp3$^+$ Treg cells.

Non-antigen-specific immunomodulative approaches

Based on the autoimmune etiology of T1D, early intervention strategies employed the use of immunosuppressive agents to interfere with the development of an autoreactive immune response. Some of these approaches were successful in inducing and prolonging clinical remission. However, protective effects were lost upon drug removal since their effect relied on general immunosuppression and was not based on long-term induction of tolerance. Moreover, the application of these drugs was frequently accompanied by deleterious side effects, limiting their broad application for prolonged time periods.

Cyclosporin A

One of the earliest compounds applied as an immunosuppressing agent in patients with T1D was cyclosporin A (CsA). CsA is an 11-amino acid cyclic peptide of fungal origin with a strong immunosuppressive capacity. It is a calcineurin inhibitor that interferes with TCR-mediated signal transduction, thereby inhibiting T cell activation, and the production of interleukin 2 (IL-2) by T cells, which is then able to limit the amplification of immune responses [31]. In T1D, CsA provided the first proof of concept that T-cell-directed immunosuppression was capable of preserving β-cell function and insulin production. Based on results from pilot studies [32, 33], two randomized phase II placebo-controlled studies were conducted [34, 35]. Based on these studies, it was concluded that treatment with CsA had no long-lasting effect on the course of T1D, persisting upon removal of compound administration [34, 36]. The need for chronic drug administration, the potential renal and pancreatic β-cell toxicity, and the cost for the drug led to the consensus that the risks outweighed the benefits, and the approach was dropped.

Mycophenolate mofetil

Mycophenolate mofetil (MMF) is an immunosuppressive drug which has been applied in the context of organ allograft rejection. It became clear that MMF possesses significant cytostatic effects on lymphocytes [37]. MMF functions as a pro-drug of mycophenolic acid (MPA), which is an inhibitor of inosine monophosphate dehydrogenase (IMPDH). IMPDH is the rate-limiting enzyme in the *de novo* synthesis of guanosine nucleotides, thereby playing a critical role in controlling the proliferation of T and B cells [38]. However, despite its efficacy in organ transplantation, a combination of MMF and a monoclonal antibody (daclizumab) targeting CD25, the alpha chain of the IL-2 receptor, which is widely used in transplantation [39], did not preserve β-cell function in newly diagnosed patients with T1D [40]. Also, there was no decrease in insulin requirement or improvement in metabolic control. One-third of patients treated with the combination of daclizumab and MMF suffered from serious adverse events. Although negative, these data are relevant as they highlight that it is not just any immunosuppressive regimen that can effectively treat T1D. The failure may be explained by the consideration that, by targeting CD4$^+$CD25$^+$ regulatory T cells (Tregs), daclizumab removes a cell subset from the immune system that plays an essential role in the maintenance of self-tolerance in T1D [41, 42].

Anti-CD20: rituximab

Rituximab is a chimeric antibody that targets the CD20 transmembrane receptor, which is expressed on all immature and mature B cells. Initially, rituximab was used for the treatment of non-Hodgkin's B cell lymphoma [43]. With respect to autoimmunity, rituximab has been combined with anti-proliferative agents to treat systemic lupus erythematosis and rheumatoid arthritis. However, these studies showed that both diseases present with relapses upon withdrawal of drug application. This result supports the concept that the effects are immunosuppressive but do not induce long-term tolerance [44, 45].

In a phase II clinical trial, rituximab was tested in patients with recent onset T1D. It became clear from these experiments that the treatment effect of rituximab was most prevalent within the first 3 months of application. Over this time period, the treatment was able to reduce the loss of C-peptide and insulin requirements. Later on, analyses revealed that the effects on C-peptide responses did not prevail. There was no statistically significant difference between patients that had received rituximab and the placebo-treated groups [46].

Cytotoxic T-lymphocyte-associated protein 4 immunoglobulin (CTLA4-Ig): abatacept and belatacept

CTLA4-Ig is a fusion protein consisting of the extracellular domain of CTLA4 and the Fc domain of an IgG1 antibody [47, 48]. It is well established that CTLA4 is expressed by activated $CD8^+$ T cells. However, CTLA4 exerts its main function as a negative costimulatory molecule leading to the inhibition of helper T cell activity and enhancement of Treg immunosuppression. Its ligands comprise CD80 and CD86, which bind to CD28, thereby delivering the costimulatory signal needed for T cell activation. Moreover, CTLA4 is a target gene of Foxp3. It was shown that a Treg-cell-specific CTLA4 knockout or blockade is able to inhibit the ability of Tregs to control autoimmune reactions and anti-tumor immunity.

In the NOD mouse model of T1D, the application of CTLA-Ig showed conflicting results with respect to the progression of diabetes, while in some settings the administration of murine CTLA4-Ig worsened the development of diabetes [49-51]. In humans, CTLA4-Ig (abatacept) has been successfully used to treat psoriasis and rheumatoid arthritis [51]. In rheumatoid arthritis, a combined application of abatacept and methotrexate was appropriate to treat patients who did not respond to anti-tumor necrosis factor (TNF) agents. Abatacept has no tolerogenic characteristics and monthly infusions are sufficient to maintain immunosuppressive properties [53-56]. Application of abatacept was tested in a multicenter, double-blind, randomized controlled trial with recent onset diabetes patients. The drug was applied at doses of 10 mg/kg on days 1, 14, and 28, followed by monthly injections for a total of two years [57]. Abatacept treatment resulted in an estimated delay in C-peptide reduction of about 10 months. A longer follow-up is necessary to determine whether there is a persisting treatment effect maintained after cessation of application.

Anti-TNF therapy

Anti-TNF therapy has been established for the treatment of chronic pro-inflammatory autoimmune diseases such as rheumatoid arthritis and Crohn's disease. In NOD mice, the effect of TNF blockade varies depending on the age at which treatment is applied. The development of autoreactive T cells was demonstrated to be modulated by TNF treatment, suggesting an effect on the development of the intrathymic autoimmune repertoire [58-60]. However, the critical factors, which delineated the timing of the TNF effects, are far from being understood in detail. Therefore, translation of these findings to the clinic remains a challenge.

Treatment with a soluble recombinant TNF receptor fusion protein that binds TNF (etanercept) did not prevent T1D in humans [61, 62]. In a double-blind, placebo-controlled pilot study, 18 children with new-onset T1D were included and patients received placebo or etanercept twice a week [63]. After 24 weeks, a clear reduction in the required insulin dose could be observed in the etanercept group. No serious adverse effects were seen in this group. Further studies in a larger set are required to confirm this promising result.

Anti-interleukin-1 (IL-1) therapy: anakinra

Anti-IL-1 therapy has primarily been used for the treatment of rheumatoid arthritis [64]. Results from studies using models of T1D support the concept that anakinra exerted direct protective effects on β-cells, rather than on insulin resistance, which led to the observed improvement in metabolic control [65]. The improvement in C-peptide responses persisted for up to 39 weeks after cessation of treatment [66]. It is hypothesized that the positive effects from anakinra result from the blockade of pro-inflammatory signals from immune cells and islet cells, thereby limiting the IL-1β-mediated induction of β-cell death.

Preliminary studies in NOD mice suggest that anti-IL-1 application is able to lower the incidence of T1D. Results from IL-1 receptor (IL-1R) deficient NOD mice showed a delay, but not protection, from T1D development. This observation supports the view that anakinra will probably not be sufficient as a single agent to achieve full treatment success [67]. The anti-interleukin-1 in diabetes action (AIDA) study is about to test feasibility, safety, and efficacy of anti-IL-1 therapy in maintaining and/or enhancing β-cell function in people with new-onset T1D [68].

Canakinumab (anti-interleukin-1β)

Canakinumab is a fully human anti-interleukin-1β (anti-IL-1β) monoclonal antibody (IgG-1 class). Canakinumab is designed to bind to human IL-1β resulting in the functional neutralization of this proinflammatory cytokine. In a recent trial, repeated injections of canakinumab were assessed for their ability to preserve β-cell function in patients with recent onset T1D.

Anti-CD3 therapy: teplizumab and otelizumab

Studies in mouse models of T1D were supportive of the concept that the application of CD3-specific mAbs is able to promote tolerance induction to β-cell antigens in the pancreas [69]. OKT3, also called muromonab-CD3, was the first generation of antibodies developed for the prevention of solid organ transplant rejection [70, 71]. However, its application was associated with a severe cytokine release syndrome, which resulted from crosslinking and activation of T cell receptors by the binding of the murine antibody Fc portion to Fc receptors on human cells [72]. To limit the side effects caused by the mitogenic potential of mAbs, humanized CD3-specific Fc mutated mAbs were developed. Teplizumab, also called hOKT3γl, a FcR-non-binding, and otelixizumab (chAglyCD3), an aglycosylated FcR non-binding CD3-specific Ab, were tested in clinical trials on patients with recent-onset T1D. They demonstrated significantly reduced side-effects [73, 74]. Based on preclinical studies in the NOD mouse setting, two phase II trials—one of which was placebo controlled—were performed and showed a clear effect, with best success seen in patients with higher functional β-cell mass before the start of otelixizumab treatment [75-77]. Following up on these findings, phase III clinical trials (otelixumab, tolerx, GlaxoSmithKline trial, and teplizumab, MacroGenics/Eli Lilly trial) were performed. However, the design of these studies differed significantly from those of the completed phase II clinical trials. Both studies failed to meet their primary end point at 1 year. Importantly, when a post-hoc analysis of the data of the teplizumab study was performed, using the conventional end points by previous studies (C-peptide production and insulin needs), a clear treatment effect became apparent [78].

Self-antigen-specific immunomodulative strategies

Foxp3$^+$ regulatory T (Treg) cells

Proper functioning of the immune system implies a tightly balanced regulation of effector mechanisms to avoid immune pathologies and autoimmunity. A variety of control mechanisms have been identified, including negative feedback circuits, which impact on activation, survival, or functioning of effector cells [79-82]. Treg cells, which can suppress self-reactive responses, are a vital and essential component in the maintenance of immunological self-tolerance [83, 84]. Early studies identified Treg cells as CD4$^+$CD25$^+$ T cells [85]. The identification of the X-chromosome-encoded transcription factor Foxp3 permitted a clearer understanding of Treg cell biology [86-88]. The pivotal impact of Foxp3$^+$ Treg cells in the maintenance of immunological self-tolerance was further highlighted by the fatal autoimmune syndrome found in patients with immunodysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, which develops in humans harboring mutations of the Foxp3 gene [89]. Likewise, scurfy mice, which lack a functional Foxp3$^+$ protein because of a natural mutation, suffer from various spontaneous and early-onset organ-specific autoimmune diseases due to hyperactivation of CD4$^+$ T cells [90]. It became clear from Treg adoptive transfer experiments in scurfy mice that Treg cells are essential in the avoidance of autoimmunity in mice. Moreover, enforced continuous expression of Foxp3 in conventional CD4$^+$ T cells was able to confer a phenotype and function resembling Treg cells. In turn, conditional deletion of the Foxp3 gene reprograms Treg cells into pathogenic T cells [91, 92]. These results supported the view that Foxp3 functions as a specific marker of Treg cells, and acts as their lineage specification factor or master regulator.

Extrathymic differentiation of Foxp3 expressing Treg cells

Recognition of agonist TCR ligand works as a key element for the initiation of Treg cell generation, a developmental program which can run intra- [93, 94] or extrathymically [95-100]. Studies on extrathymic Treg cell generation in the peripheral adult immune system can have an important therapeutic potential. Our group has carefully established protocols for antigen-specific Treg conversion. It became clear that extrathymic conversion of naïve CD4$^+$ T cells into Foxp3$^+$-expressing Treg cells can be achieved *in vivo* by the delivery of strong-agonist TCR ligands under subimmunogenic conditions [95-100] (Figure 1). The concept of *de novo* induction of Foxp3$^+$ Treg cells, rather than the expansion of already committed Treg cells, was further supported by the fact that Foxp3$^+$ Tregs were absent in Rag$^{-/-}$ TCR-transgenic animals expressing only one particular TCR in the absence of a co-expressed TCR-agonist ligand. Cellular proliferation was found to function as a limiting factor for Treg conversion since the best conversion is seen in T cells that undergo only limited proliferation. Whereas, higher doses of TCR agonists induce robust proliferation and diminished conver-

sion into Treg cells [99]. Recent data support the concept that such high doses of TCR ligands result in an activation of the PI3K/Akt/mTOR pathway [101], which could interfere with extrathymic Foxp3 induction [102]. Early blockade of PI3K signaling through the use of PI3K-mTOR inhibitors was found to promote Treg cell induction *in vitro* [98, 103] and *in vivo* [98]. In accordance with this concept, sustained Akt activation inhibited the stable induction of Foxp3 in peripheral Foxp3$^-$CD4$^+$ T cells [104].

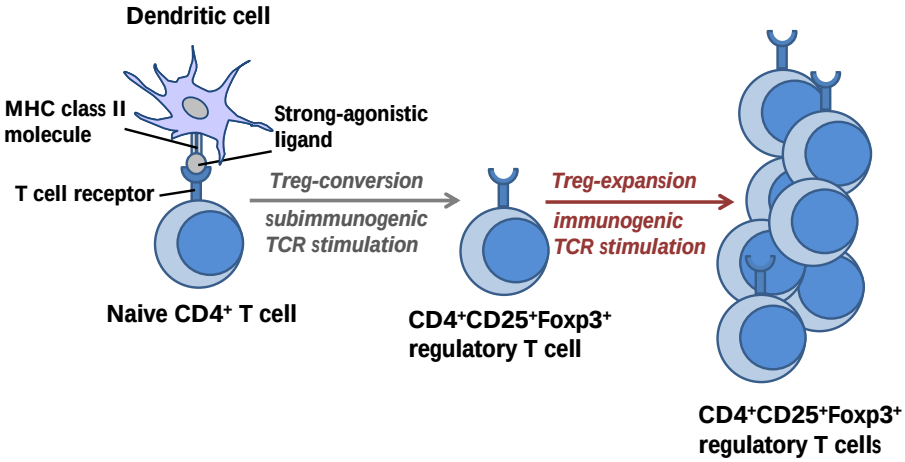

Figure 1. Generation of extrathymic regulatory T (Treg) cells. Efficient *in vivo* generation of Foxp3$^+$ Treg cells in the mouse system requires delivery of strong-agonistic T cell receptor (TCR)-ligands under subimmunogenic conditions and avoidance of activation of antigen-presenting and T cells. Immunogenic antigen stimulation is able to expand Treg cells once converted.

The induction of Foxp3 upon subimmunogenic antigen exposure *in vivo* requires TGFβ receptor signaling and is inversely correlated with cellular proliferation, as mentioned above [99]. Extrathymically induced Foxp3$^+$ Treg cells can prospectively be generated for suppression of unwanted immune responses since it has become clear that they are stable and independent of further antigen supply used to generate these cells [105, 106]. Recent studies support the concept that the extrathymically induced Foxp3$^+$ Treg cells are stable upon subimmunogenic antigen delivery [106], and maintain their function even in an immunogenic context [107]. Re-encounter of antigen under immunogenic conditions does not cause loss of Treg cells or loss of their activity [105], but permits the expansion of Treg cells [99].

Extrathymic induction of Treg cells in autoimmune T1D

In models unrelated to autoimmune disease, it was demonstrated that naïve CD4$^+$ T cells can be extrathymically converted into Foxp3$^+$ Treg cells *in vivo* if they are exposed to strong-agonistic antigens under subimmunogenic conditions [94-99]. Such extrathymic Treg cell induction strategies were used to prospectively generate transplantation tolerance in female mice to various male tissues [100]. However, initial studies investigating the feasibility of prospective extrathymic Foxp3 Treg generation in the prevention of the development of T1D by subimmunogenic delivery of natural insulin B chain epitopes in the NOD mouse showed only a short delay of disease progression.

As a possible scenario, it is believed that T1D and other autoimmune diseases develop when T cells with specificity for weakly binding TCR agonists, which may include self-antigens, escape thymic negative selection and evade into the periphery where they cause an autoreactive process [96, 108-112]. Specific modes of presentation and recognition have been proposed for certain self-antigens in the course of autoimmunity [108-116]. Also, higher epitope abundance in the respective organ (e.g. pancreatic islets) and local differences in peptide processing/truncating and/or presentation could possibly facilitate the activation of these self-reactive T cells in the periphery [114, 118].

It became clear from various studies that in autoimmune T1D insulin has a critical role as a self-antigen [19, 119]. As discussed above, in mice and humans, the T cell response to insulin is focused on the B-chain residues 9-23. In NOD mice, characterizing the relevant epitope(s) for the T cells within the insulin B:9-23 peptide has been a challenge. This difficulty underlines the significance of the concept that the insulin B-chain peptide may bind to the murine MHC class II mole-

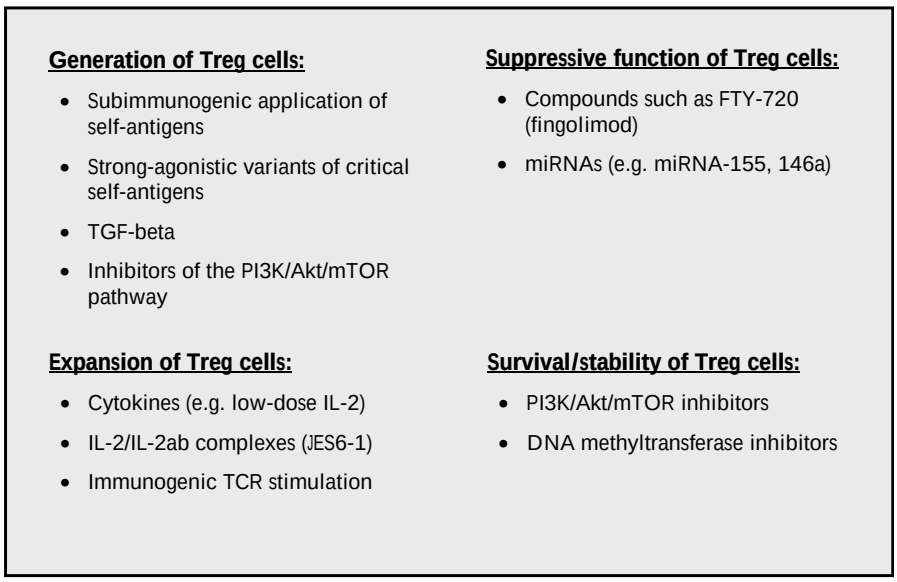

Figure 2. Approaches for the targeting of regulatory Foxp3$^+$ CD4$^+$ (Treg) cells. Studies in various model systems have helped to develop strategies suitable for the efficient manipulation of Foxp3$^+$ Treg cells. To achieve efficient Foxp3$^+$ Treg cell generation subimmunogenic application of antigens, e.g. strong-agonistic variants of self-antigens, is required. Enhancement of Treg induction can be achieved using transforming growth factor beta (TGF-beta), inhibitors of the phosphoinositide 3-kinase (PI3K)-Akt-mTOR pathways [98], or microRNAs [127]. Low doses of interleukin 2 (IL-2) [128, 129], specific IL-2/IL-2 antibody complexes, and immunogenic stimulation with antigen were found to help the expansion of Treg cells [98, 130]. Strategies for an increase of Treg cell suppressive function might be supported by the use of compounds such as fingolimod (FTY720) [131-134]. Additionally, an improvement in Treg cell survival and stability can be supported by PI3K-Akt/mTOR-inhibitors or by the use of DNA methyltransferase inhibitors [98].

cule I-A^{g7}, and may be recognized by T cells in several overlapping binding registers [17, 113, 114, 120-122].

In a model proposed by John Kappler, the insulin B chain peptide was presented by I-A^{g7} molecules in an unfavored low-affinity binding register, which resulted in weak agonistic activity of the peptide-MHC complex. The poor binding to I-A^{g7} molecules was the result of an incompatibility between the p9 amino acid of the insulin epitope (arginine = R) and the particular I-A^{g7} p9 pocket polymorphisms, which are highly linked with susceptibility to T1D [113, 114]. In this unfavored binding register, an arginine residue (=R) of the insulin epitope met another arginine (=R) in the positively charged p9 pocket of I-A^{g7}, thereby generating a highly unfavorable match [113, 114, 123, 124]. While these data represent a compelling model, it is challenged by other investigators who propose that binding of the insulin epitope to I-A^{g7} largely takes place in other low-affinity registers [122].

In human T1D, the susceptibility MHC class II allele, HLA-DQ8, shares very similar binding pockets for peptide presentation with the homologous molecule in NOD mice, I-A^{g7} [125]. The unique feature, which confers disease susceptibility in NOD mice, was equally identified in the peptide binding groove of human HLA-DQ8. It became clear that the polymorphism, which affects the presentation of insulin in NOD mice, i.e. the lack of one specific amino acid in the B chain of the MHC molecule, was identical in human HLA-DQ8 and I-A^{g7} of NOD mice [123, 125]. Therefore, both may exhibit similar peptide binding interactions and similar modes of antigen presentation.

We showed that upon subimmunogenic application of a strong-agonistic insulin B-chain variant to young NOD mice, efficient insulin-specific conversion of naïve T cells into Foxp3$^+$ Treg cells could be achieved. This approach has the ability to prevent the development of T1D [97]. In contrast to the natural insulin epitope, subimmunogenic Treg conversion with the strong-agonist mimetope resulted in high numbers of stable Foxp3$^+$ Treg cells. These findings are supportive of the concept that low doses of strong-agonistic antigens are able to induce stable Foxp3$^+$ Treg cells with high efficacy. Whereas, even high doses of poorly agonistic ligands fail to generate stable Foxp3$^+$ Treg cells. These results indicate that ligand density cannot compensate for diminished agonistic activity in determining the efficacy and stability of induced Foxp3$^+$ Treg cells [97, 126].

We studied the development of diabetes in NOD mice as a function of insulin autoantibody (IAA) indices at a young age before Treg-induction. We observed that autoantibody indices present in mice

at 4 weeks of age impact the development of T1D in these mice. A correlation became apparent in that mice with higher IAA indices developed T1D earlier. We showed that the natural insulin B chain epitope conferred only limited protection from T1D development irrespective of the applied dose. In contrast, application of subimmunogenic doses of the strong-agonistic insulin variant in NOD mice with moderate levels of IAA prevented T1D. In NOD mice with high IAA indices at a young age of 4 weeks, Treg generation using the insulin variant was not able to achieve complete prevention of disease development. These data support the view that NOD mice at an age of 4 weeks and with very high indices of IAA show insulin-specific T cell activation, which consequently impairs efficient Treg conversion [97].

Concluding remarks

In autoimmunity such as T1D, it became evident that efficient induction of insulin-specific Foxp3$^+$ Treg cells can be accomplished when strong-agonistic variants of insulin B chain epitopes are used under subimmunogenic conditions. The development of humanized mice models for the careful study of immunotherapeutic approaches *in vivo* will facilitate our understanding of these approaches in the presence of a human immune system. Based on the mechanistic insight that high doses of antigens promote cell proliferation and activation, it appears reasonable to develop approaches which are able to inhibit T cell activation. These approaches can also be combined with antigen-specific interventions for long-term tolerance induction. Such efforts could result in combinatorial strategy able to limit factors that interfere with the efficient generation of Foxp3$^+$ Treg cells, while maintaining antigen-specificity.

An overview on general approaches for the envisioned targeting of Foxp3$^+$ Treg cells is provided in Figure 2. Future studies will continue to improve our understanding of the involved molecular mechanisms of tolerance induction, with the goal to increase specificity and efficacy of therapies. This will be necessary to meet the complexity of the human immune system.

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