The Review of DIABETIC STUDIES

REVIEW

Chapter III.1

Type 1 Diabetes Therapy Beyond T Cell Targeting: Monocytes, B Cells, and Innate Lymphocytes

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■ Abstract

Recent clinical trials, investigating type 1 diabetes (T1D), have focused mainly on newly diagnosed individuals who have developed diabetes. We need to continue our efforts to understand disease processes and to rationally design interventions that will be safe and specific for disease, but at the same time not induce undesirable immunosuppression. T cells are clearly involved in the pathogenesis of T1D, and have been a major focus for both antigen-specific and non-antigen-specific therapy, but thus far no single strategy has emerged as superior. As T1D is a multifactorial disease, in which multiple cell types are involved, some of these pathogenic and regulatory cell pathways may be important to consider. In this review, we examine evidence for whether monocytes, B cells, and innate lymphocytes, including natural killer cells, may be suitable targets for intervention.

Keywords: type 1 diabetes · B cell · NOD · Treg cell · dendritic cell · ATG · CD3 · monoclonal antibody

1. Introduction

At the time of diagnosis of type 1 diabetes (T1D), many insulin-producing β-cells have been damaged in a process that has taken weeks, months, or even years to develop. There are multiple genetic loci predisposing individuals to disease [1], interacting with unknown environmental factors, in a slow process. It is well known that in prediabetes, the presence of one or two autoantibodies does not inevitably lead to diabetes, but when there are multiple autoantibodies, progression to diabetes is very likely [2]. Studies in animal models of diabetes have pointed to diverse cellular pathways that may be involved, and some of these have shown distinct parallels with the human disease. The potentially long course of time over which damage and destruction of pancreatic islet β-cells occurs suggests that many approaches might be successful if intervention could take place at an early time point. However, by the time T1D manifests, therapy is likely to be much more challenging since the immune response has diversified, with many cell types and autoantigens recognized by memory T cells, even if there is substantial β-cell function remaining.

After a long time of research into T1D pathogenesis, which will help to focus on rational interventions, we should acknowledge treatments that have already made the long journey to the clinical trial. Of the various antigen-specific interventions that have been aimed at the tolerization of T cells, targeting proinsulin by the use of insulin peptide [3], or insulin B chain in tolerogenic adjuvant [4], have been tested in early-phase trials. GAD-alum had reached the phase III clinical trial, but unfortunately clinical efficacy could not be demonstrated [5-7]. A phase III trial of HSP60 in the form of DiaPep277 was reported, at the ADA 72nd Scientific Sessions 2012, to show a 2 year reduc-

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tion in the loss of C-peptide on glucagon stimulation [8]. Another study using this approach is underway. Of the non-antigen-specific therapy, anti-CD3 mAb to target T cells (teplizumab, otelixizumab) was promising in the early trial stages [9-11], but stage III trials were terminated as the primary end-point was not met [12]. The reasons for this are discussed elsewhere in this special review series of *The Review of Diabetic Studies* [13, 14]. Anti-CTLA4 treatment (abatacept) showed initial delay in the loss of C-peptide over the first 6 months, but thereafter the decline in β-cell function was parallel to that seen in patients treated with placebo [15]. There are currently ongoing trials of anti-thymocyte globulin (ATG) and anti-CD2 which both target T cells [16].

How are we to decide what strategies are useful? For many years, animal models, both the non-obese diabetic (NOD) mouse and the BioBreeding (BB) rat have been used to test therapies. Some of these have moved into the clinical field and allow a semblance of rationality in the choice of therapy. It is clear that strategies for therapy that are successful in early stage disease, will not necessarily be translatable at this time, as most current clinical focus is on much later points in disease pathogenesis. We should hold this consideration in mind, should preventive measures for diabetes become more of a reality. However, therapies tested in disease models, which show promise at later phases in the disease models, could be useful. We need to build on these studies, with the investigation of the human immunology, and with the development and validation of biomarkers for efficacy. Clearly, having an outcome that will ultimately prevent T cells from causing damage to islet β-cells is the major goal. However, this does not mean that T cells are the only possible targets, as manipulations of other components of the immune response may aid in this process.

2. Monocytes, macrophages, and dendritic cells

Monocytes derive from hematopoietic stem cells in the bone marrow. They are precursors to both macrophages and myeloid dendritic cells in tissues where they are important in inflammation and defense against pathogens (reviewed by Auffray and colleagues [17]). There is considerable heterogeneity in the monocyte/macrophage subset of cells, with many surface markers defining the different types [18]. Dendritic cells (DCs) are key innate immune cells that direct the fate of T cells.

Abbreviations:
Ab – antibody
ADA – American Diabetes Association
APC – antigen-presenting cell
ATG – anti-thymocyte globulin
BAFF – B cell activating factor
BB – BioBreeding
BCMA – B cell maturation antigen
BCR – B cell receptor
BLyS – B lymphocyte stimulator
BMDC – bone marrow-derived dendritic cell
CCR5 – C-C chemokine receptor type 5 (CD195)
CD11b – complement receptor 3
CTLA-4 – cytotoxic T lymphocyte antigen 4
DC – dendritic cell
ER – endoplasmic reticulum
FO – follicular
Foxp3 – forkhead box P3
GAD – glutamic acid decarboxylase
GM-CSF – granulocyte-macrophage colony-stimulating factor
HLA – human leukocyte antigen
HSP60 – heat shock protein 60
IAA – insulin auto-antibody
IDIN – IRF7-driven inflammatory gene network
IFNγ – interferon gamma
Ig – immunoglobulin
IL – interleukin
iNKT – invariant natural killer T
iNOS - inducible nitric oxide synthase
i.p. – intraperitoneal
IRF7 – interferon regulatory factor 7
KIR – killer cell immunoglobulin-like receptor
lip-Cl_2MDP – liposome-encapsulated dichloromethylene diphosphonate
mAb – monoclonal antibody
MAP – mitogen-activated protein
MHC – major histocompatibility complex
MLR – mixed leukocyte reaction
MZ – marginal zone
MZB - marginal zone B (cell)
NCR – natural cytotoxicity receptor
NF-κB – nuclear factor kappa B
NK – natual killer
NKT – natural killer T
NO – nitric oxide
NOD – non-obese diabetic
NOR – non-obese resistant
PAMPS – pathogen-associated molecular patterns
PBMC – peripheral blood mononuclear cell
PLN – pancreatic lymph node
RNA – ribonucleic acid
siRNA - small interfering RNA
SLC11A1 – human solute carrier family 11 member A1
T1D – type 1 diabetes
T2MZB – type 2 marginal zone B (cell)
TCR – T cell receptor
TGF-β – transforming growth factor beta
TLR – toll-like receptor
TNF-α – tumor necrosis factor alpha
Treg – T regulatory
ZnT8 – zinc transporter 8

The many subsets of DCs all have an input into activating immune responses [19]. As antigen-presenting cells (APCs), DCs have a central role in early innate immune responses and directing the adaptive immune response. Thus, they may have a number of roles in disease processes, and are potentially important targets for therapy.

2.1 Genetics

Interestingly, there is a genetic association in the diabetes susceptibility locus Idd5.2 in the NOD mouse, which encodes *Slc11a1* (formerly called Nramp1). This gene codes for a lysosomal membrane protein that is involved in acidification within lysosomes, and therefore it is important in antigen presentation by APCs such as DCs. Silencing of the gene using lentivirus, encoding siRNA for the *Slc11a1* gene, reduced diabetes incidence in an NOD mouse cohort, and recapitulated the effect of a natural mutation of Idd5.2 [20]. In humans, the orthologous region *SLC11A1* encodes an evolutionary highly conserved protein. Although associations with a variety of immune-mediated diseases have been reported, a recent study did not observe changes in *SLC11A1* expression at the RNA level in whole blood samples from patients with T1D. However, the study did not exclude the possibility that genetic effects of polymorphisms in the gene may be seen in purified monocyte or macrophage populations [21]. In rats, Heinig and colleagues have identified an interferon regulatory factor 7 (IRF7)-driven inflammatory gene network (IDIN) [22]. In humans, there is a conserved equivalent of the rat IDIN genes expressed in monocytes [22]. IRF7 regulates the type 1 interferon response that has been linked to T1D. The genes encode proteins that are highly expressed in cells of the immune system, and the investigators suggested that these genes may regulate the innate immune response in macrophages, contributing to the risk of T1D.

2.2 Studies in animal models

In animal models of autoimmune diabetes, macrophages are amongst the earliest cells that infiltrate islets in the BB rat [23-25]. Similarly, in the NOD mouse, characteristic patterns of dendritic-like cells and macrophages infiltrate into islets before lymphocytes [26]. The macrophages in NOD mice are reported to have a defect in phagocytosis of apoptotic cells [27, 28]. Moreover, they have an abnormal inflammatory response, producing increased amounts of inflammatory cytokines that include IL-1β and TNF-α when encountering apoptotic cells, compared with non-obese resistant (NOR) or C57BL/6 mice [29]. Targeting macrophages was one of the earliest therapeutic strategies shown to inhibit diabetes in the NOD mouse. Antibody against the complement receptor 3 (CD11b) expressed on macrophages prevented diabetes development in an adoptive transfer system in sublethally irradiated NOD mice [30]. Studies using silica, or liposome-encapsulated dichloromethylene diphosphonate (lip-Cl_2MDP) to deplete macrophages also protected NOD mice from developing spontaneous diabetes [31]. The treatment reduced IL-12, IL-1β, and TNF-α in splenic macrophages, and there was a shift from a Th1 cytokine profile to Th2 in splenocytes and a reduction in the development of islet-reactive cytotoxic T cells [31].

Recent attempts at *in situ* macrophage-targeting using siRNA against Alox-15 have shown that this therapy is useful when given at an early phase in disease, similar to previous studies, but not effective when applied after 9 weeks of age [32].

2.3 Studies in humans

Macrophages are seen in human islet infiltrates in post-mortem sections of pancreas obtained from patients with diabetes, both at time of onset of disease [33] and later [34]. Furthermore, there have been many studies that focused on monocytes and their phenotypic changes in T1D. Differences in the maturation and function of monocyte-derived APCs have been suggested to be a reason for defective activation of regulatory cells in patients with diabetes [35]. In comparison with healthy first-degree relatives of T1D patients, there are raised serum cytokines from monocytes prior to the onset of diabetes [36]. Furthermore, monocytes from patients with T1D have an inflammatory phenotype, with increased IL-6 and IL-1β production. These cytokines can then stimulate the production of inflammatory IL-17-producing T cells [37]. The conversion to IL-17 cells in humans and mice appears to be different, with differentiation induced by IL-1β and IL-6, but not IL-12 or TGF-β, whereas TGF-β is critical for driving naïve CD4 T cells towards the Th17 lineage in mice [38].

Toll-like receptors (TLRs) are innate immune receptors that are expressed on both immune, especially APCs, and a variety of other cells in the body. They detect pathogen-associated molecular patterns (PAMPS). Interest has been generated in these receptors in connection with the activation of

immune cells in diabetes. Altered surface expression of TLR2 (recognizes components of gram-positive bacteria) and TLR4 (recognizes lipopolysaccharide) has been reported in monocytes from patients with T1D [39]. The monocytes have been studied in conditions of raised blood glucose. Increased secretion of the chemokine IP-10 was found, which is important in the homing of T cells, and it is associated with diabetes [40]. It has also been suggested that signaling via the TLR pathways is aberrant in patients with T1D [41], with increased production of IL-1β from monocytes and decreased IL-6 from myeloid DCs, following stimulation of monocytes through TLR4. Similar alterations have been observed in autoantibody-positive individuals at risk of developing T1D compared with those who are autoantibody negative [42].

These phenotypic studies have become more complex and wide-ranging with our increased abilities to investigate a very large range of cellular constituents in terms of gene expression profiles. Using a novel assay to examine gene expression signatures, Wang and colleagues took sera from newly diagnosed patients with diabetes, and tested this by incubating it with unrelated peripheral blood mononuclear cells (PBMC) to look for a gene expression signature induced in the PBMC by serum from the new-onset T1D patients [43]. Comparison was made with serum taken from healthy control subjects and patients with long-standing diabetes. They found that a unique expression signature was induced by the serum of new-onset patients that was also found in a small number of autoantibody-positive siblings, and that it was no longer present in long-standing patients [43]. Altered genes included IL-1 cytokine family members and chemokines amongst other molecules [43]. This has been further elaborated to indicate that the patterns are distinct from other inflammatory conditions [44]. The investigators suggested that detection of these changes have the potential to be used as unique disease identifiers that could improve disease prediction [44].

Using purified $CD14^+$ monocytes, Irvine and colleagues showed differences in monocyte gene expression patterns in children with newly-diagnosed diabetes compared with healthy control subjects, in addition to the finding of a reduction in $CD14^{hi}CD16^+$ monocytes and an increase in $CD14^{lo}/CD16^-$ monocytes in these patients [45]. The differences of gene expression include upregulation of endoplasmic reticulum (ER)-nuclear signaling pathways, negative regulation of caspase activity, together with cell adhesion genes and downregulation of negative regulators of NF-κB, again pointing to a distinct molecular signature [45].

Thus, currently, there are a variety of observations that suggest that the monocyte/macrophage/dendritic cell pathways have altered activity in T1D. Some of these appear to be intrinsic, i.e. potentially genetically determined, while others may be a response to inflammatory and metabolic changes. However, there is clearly involvement of these cells in the pathogenic process, and this suggests that there may be merit in considering them as potential targets for therapy.

2.4 Potential targets

Cytokines produced by macrophages. How might these important accessory cells be targeted? Macrophages are major producers of IL-1β that triggers the NF-κB and MAP kinase signaling pathways in pancreatic islets. IL-1β is toxic to β-cells *in vitro* as shown by a number of studies, particularly in combination with IFN-γ and TNF-α. However, this cytokine cocktail had different effects on human islets compared to rodent islets [46, 47]. Furthermore, IL-1β plays a synergistic role with other molecules, inducing nitric oxide (NO) that also damages islets [48, 49]. IL-1β clearly has a role in a number of inflammatory diseases, including arthritis and autoinflammatory syndromes. Various means of antagonizing the effects of IL-1 have been developed, of which IL-1 receptor antagonist (anakinra) and IL-1 trap (rilonacept), a long acting IL-1 blocking agent, have been approved for human use. Some of the data from animal models have suggested that antagonizing IL-1 may be beneficial, and that this could be done with either soluble IL-1 receptor or IL1 receptor antagonist. However, it was not very effective in the NOD mouse, but improved efficacy was seen when it was used with low dose anti-CD3 treatment [50]. It has also been shown that knocking out IL-1 in NOD mice had little effect on diabetes development [51, 52]. However, there has been success in the use of IL-1 antagonism in a variety of clinical conditions. It has been shown to be safe [53]. Two clinical studies were recently completed that targeted IL-1, one using IL-1 receptor antagonist (anakinra) and the other using an antibody to IL-1 (canakinumab), and neither had shown any efficacy when used at the time of diabetes onset (reported at ADA 72nd Scientific Sessions 2012 and Immunology of Diabetes Society meeting 2012). It is not known whether there may have been a different outcome if these agents could have been used at an earlier stage of disease. This sub-

ject will be addressed in more detail in another review by Thomas Mandrup-Poulsen in this series [54].

In vivo treatment to generate tolerogenic antigen-presenting cells. If the cytokine products of macrophages cannot be effectively neutralized, another option would be to alter the phenotype of the monocytes and macrophages by mutually targeting the cells. The aim of this approach would be to generate APCs that are programmed to tolerize T cells and stimulate the production of regulatory cells. Given the importance of these APCs in immune responses, this goal of inducing immune tolerance to unwanted effects, while at the same time maintaining effectiveness in dealing with infections, is a considerable challenge.

Vitamin D has an impact on many cells as the vitamin D receptor is expressed in nucleated cells. The best known effects are on bone and calcium metabolism. However, there are also effects on immune cells, including DCs and macrophages, and these may in turn alter the activation of T cells [55]. Treatment of prediabetic NOD mice from an early age, with vitamin D and an analogue of Vitamin D that did not induce hypercalcemia, considerably reduced the incidence of autoimmune diabetes. Cells from treated mice could also suppress diabetes development induced in an immunosuppressed host by adoptive co-transfer [56, 57]. There has been considerable interest in the role of vitamin D and its effects in the generation of tolerogenic DCs. Mouse DCs, generated in the presence of 1,25 dihydroxy vitamin D3, the active form, expressed lower levels of MHC class II and costimulatory molecules, together with increased chemokine receptor CCR5 and antigen-uptake receptor DEC205, compared with those DCs that are matured in the absence of 1,25 dihydroxy vitamin D3 [58]. Vitamin D can also alter the macrophage phenotype as treatment of murine peritoneal macrophages with 1,25 dihydroxy vitamin D3 reduces proinflammatory cytokines and other mediators that include IL-12p40, inducible nitric oxide synthase (iNOS), and TNF-α upon antigen stimulation [59]. These macrophages have a reduced capacity to activate antigen-specific BDC2.5 T cells. This was suggested to be partly dependent on IL-10 [59].

The immunomodulatory effects of 1,25 dihydroxy vitamin D3 in humans have been known for many years. When monocytes are cultured in the presence of 1,25 dihydroxy vitamin D3, CD14 remained high but CD1a, CD83, and HLA-DR were expressed at a low level, with concomitant changes in costimulatory molecules. The phenotype of the treated monocytes appeared to resemble unstimulated monocytes, providing a population of relatively immature DCs, which were less able to stimulate T cell proliferation [60].

Wide-ranging effects of vitamin D include effects on pancreatic β-cells where a decrease in expression of chemokines and cytokines was seen when NOD mice were treated with 1,25 dihydroxy vitamin D3 [61]. Moreover, vitamin D3 protects pancreatic β-cells from apoptotic death through modulating inflammatory cytokines on the cells, induction of A20 which is an apoptotic protein, and the reduction of Fas on human islets [62, 63].

Given the potential for immunomodulation of both DCs and macrophages derived from monocytes by vitamin D metabolites, could these be targeted by vitamin D or an analogue? These DCs and macrophages may then be able to stimulate regulatory T cells. The studies using vitamin D in humans have been relatively small scale (reviewed in [64]). There have been some effects on reducing the risk of T1D when vitamin D supplements are given in the first year of life. In recently diagnosed T1D patients, however, there was no improvement or delay of the decline in C peptide. Given these small effects, it is likely that, if vitamin D is effective, this would be at the earlier stage in prevention. Only a large scale trial would be able to prove this, and it remains to be seen whether this will be a viable option.

Tolerogenic dendritic cells. Could the beneficial effects of tolerogenic monocyte-derived macrophages/DCs be harnessed as a cellular therapy? DCs targeted in NOD mice have also been shown to be of critical importance. The activation state of the DCs is of major importance in determining whether T cells become activated or tolerized [65]. Immature DCs with the ability to tolerize T cells have been used to stimulate and maintain regulatory T cells. This role has major importance in the consideration of the use of tolerogenic APCs in immunotherapy. A variety of DC-based therapies have been reported in NOD mice that have had varying degrees of success in preventing diabetes. The best success has been achieved when transferred early in the disease process. We have previously shown that IL-10 conditioning induces the differentiation of tolerogenic bone marrow-derived dendritic cells (BMDCs) that inhibit spontaneous diabetes, and that can protect against diabetes in the NOD mouse when administered very early in disease, before 7 weeks of age [66]. The IL-10-conditioned BMDCs reduced antigen-specific responses *in vitro* and *in vivo*. They stimulated the accumulation of $B220^+$ plasmacytoid DCs in the is-

Table 1. Summary of non-antigen specific B cell depletion studies in NOD mice

Reagent used	Treatment age	Target cells	Treatment protocol	B cells after repopulation	Other cellular effects	Islet Abs	Effect on diabetes	Reference
Mouse anti-human CD20 (2H7, IgG2b)	4–5 wk	Immature and mature CD20+ B cells	4×250 µg i.v. at 3d interval)	T2 subset	Foxp3+ and CTLA4+ Treg. Increase in Gr1+ monocytes	Anti-insulin Ab	Delay and reduce	69, 77
	9–10 wk						Reduce	
	Diabetic mice						Euglycemia restored in 36%	
Hamster anti-mouse BLyS (10F4, hamster IgG)	4 wk	FO, MZ B cells	2×100 µg i.p., 5d interval), then 15µg biweekly	Transitional B cells	Foxp3+ Treg	Anti-insulin ab	Delay and reduce	72
	6–8 wk						Delay and reduce	
Mouse anti-mouse CD20 (MB20-11, IgG2c)	5 wk	CD20+ B cells (immature and mature B cells)	3×250 µg (iv, 2-wk interval)		No effect	Not done	Delay and reduce	70
	15 wk						Delay and reduce	
Calicheamicin-conjugated mouse anti-mouse CD22 (Cy34.1, IgG1)	10 wk	CD22+ B cells	2×160 µg/kg i.p. (5d interval),	Anergic B cells (T3 cells)	Foxp3+ Treg	Not done	Reduce	71
	Diabetic mice						100% reversal	
BCMA-huFc fusion protein	9-15 wk	FO and MZ B cells	12×150 µg i.p., twice/wk)	T2MZB cells and T1 cells in PLN	Foxp3+ Treg	Not done	100% protection	73, 133

Legend: Ab – antibody, Foxp3 – forkhead box P3, CTLA-4 – cytotoxic T lymphocyte antigen 4, Treg – T regulatory, BCMA – B cell maturation antigen, BLyS – B lymphocyte stimulator, Ig – immunoglobulin, MZ – marginal zone, FO – follicular, T2MZB - type 2 marginal zone B (cell), i.p. – intraperitoneal, PLN – pancreatic lymph node.

lets, and reduced insulitis [66]. Others have also shown that the transfer of DCs stimulated with GM-CSF/IL4 was able to prevent diabetes, but again this required treatment to be given when the mice were 5 weeks old [67]. If the DCs were first transduced with IL-4, delivered in adenovirus vectors, treatment was more effective up to 10 weeks of age [67]. The IL-4-transduced DCs altered the intra-pancreatic cytokines.

A phase 1 study has been carried out where "immunosuppressive" DCs were generated using autologous monocytes from elutriation cultured in the presence of IL-4 and GM-CSF together with phosphorothioate-modified antisense oligonucleotides targeting CD40, CD80, and CD86, and then administered 4 times over 2 months intradermally. There were no significant adverse effects. Although this was primarily a safety study, it was noted that serum IL-4 and IL-10 were increased together with $B220^+$ cells, involving a population that suppressed cytokine responses in mixed leukocyte reactions (MLRs). There was no generalized immunosuppression, as evidenced by the maintenance of responses in MLRs *in vitro*. PBMCs from the DC-treated patients responded in a similar manner to those seen at baseline, and there was

no alteration of responses to viral peptide antigens [68]. This may be a promising treatment although it is still in an early phase, and its efficacy is not currently known. The treatment would require specialist preparation, and is much more costly. These cellular interventions, requiring the infusion of pre-prepared tolerogenic cells, could certainly hold promise for some patients, but compared with other strategies which exert their effects *in vivo*, they are likely to benefit a smaller number of patients overall.

Thus, at present, there are some potentially interesting options when considering monocyte, macrophage, and dendritic cell targets for therapy. These could include targeting innate immune receptors to reduce inflammatory responses, and means of skewing dendritic cells and macrophages towards a more "tolerogenic" phenotype. Currently, treatment of this nature may be very effective *in vitro*, but would require a more basic understanding of the effects *in vivo* before they are likely to be translatable to human therapy. Preclinical studies directed at monocyte, macrophage, and dendritic cell targets appear to be most effective early in the pathogenesis of disease, and it is possible that a therapy targeting these cells would be more appropriately used in prevention regimens.

3. B cells

3.1 Studies in animal models

The B cell is a target of therapy in T1D. Anti-B cell therapy has both delayed and prevented diabetes in NOD mice when given at early stages. It restored normoglycemia in a proportion of mice after the onset of diabetes. The strategies have included the use of anti-CD20 [69, 70], calicheamicin toxin-conjugated anti-CD22 [71], anti-BAFF [72], and BCMA-Fc [73], as summarized in **Table 1**.

3.2 Studies in humans

Depletion of B cells using anti-CD20 (rituximab) has already had partial success in early phase clinical trials [74]. It is now believed that many individuals presenting with diabetes may have a significant number of remaining β-cells. Whilst not producing sufficient insulin, these may be preserved at the time of onset of diabetes, and although not functioning normally, they are not destroyed. Within the first 3 months after rituximab treatment, there was a greater preservation of C-peptide responses in the Rituximab group, whereas the rate of decline after this time was parallel to control subjects [74]. Since the original studies, further investigation of the effects following treatment, as well as refinements and new treatments, have continued to maintain interest in the targeting of B cells as a potential therapy for T1D. However, there is need for caution.

What happens after B cell depletion using anti-CD20, during reconstitution, and when B cells are finally restored? In the phase II rituximab study in T1D [74], 87 patients with new onset T1D were treated with rituximab weekly over 4 weeks. There was a slower decline in mixed meal tolerance-stimulated C-peptide levels of the rituximab treated group compared with the placebo group. The B lymphocytes took many months to reconstitute. At 12 months after the treatment period, naïve B cells, defined as $CD24^+$, IgD^+ $CD19^+$, $CD38^-$, and $CD10^-$, had returned to >80% of the baseline level. The switched memory B cells, defined as $CD19^+$, $CD27^+$, $CD1c^{+/-}$, $IgM^{+/-}$, and IgD^-, remained reduced at an average of 40% of the initial baseline [75]. This has raised concerns about the level of immunosuppression using this treatment. In a follow-up study to assess the effects on antibody responses, patients from both active treatment and placebo arms of the study were immunized with diphtheria/tetanus and hepatitis A vaccines 12 months after the initial rituximab treatment, and pre-existing antibody responses to measles, mumps, and rubella were assessed [75]. In addition, bacteriophage phiX174 was given during the depletion phase at 2 weeks, after the end of the rituximab infusion period, and again at 6 weeks after this, to examine responses to a T lymphocyte-dependent antigen. Plasma cells do not express CD20, and as expected, there was no effect on the circulating antibody responses that had been induced prior to rituximab treatment. Responses to immunization with the diphtheria/tetanus and hepatitis A vaccines were seen in the rituximab-treated group, and were considered to give adequate protection, although they were lower than those achieved in the control group [75]. There was no response to phiX174 at the early time point after rituximab treatment, and a reduced response when the treatment was given after one year. However, the responses to immunization returned to normal upon B cell recovery [75]. These results certainly have important implications regarding concerns about the length of time that it takes to repopulate B cells. The resulting immunosuppression may preclude general introduction of this type of treatment to deplete B cells. Interestingly, rituximab treatment suppressed anti-insulin auto-

antibodies (IAAs) more strongly than anti-GAD or anti-ZnT8 antibodies. This suppression continued for at least a year [76]. It was also observed that, independent of the treatment the subjects received, the level of IAAs was lower in those that maintained better C-peptide levels [76].

The effects on B cells immediately following depletion in the rituximab study have been well documented. Given the considerable interactions with T cells and other cells of the innate immune response, it is interesting to note other aspects of B cell treatment and effects after reconstitution. In mouse studies, following B cell depletion, when the B cells were restored, there was an increase in T regulatory cells [69, 71-73], a transitional population of B cells [69], and a regulatory population of monocyte-derived cells [77] (**Table 1**). In the rituximab trial, in those individuals who responded to the rituximab treatment, the number of $CD4^+$ T cells had increased. Moreover, the number of regulatory T cells, defined as $CD4^+CD25^+CD62L^+$ cells, was also greater 12 weeks after treatment, although this was not maintained over the first year. Interestingly, there was also a greater T cell proliferative response to 9 out of 12 antigens tested, which included neuronal, islet, and milk peptides in these individuals [78]. It is not clear in the human studies, what the underlying reason for this is. In the NOD mouse, when B cells were depleted in the TCR transgenic BDC2.5 mice (where the T cells recognize a peptide of chromogranin A), surprisingly, the BDC2.5 $CD4^+$ T cells were more aggressive during the period when the B cells were depleted [79]. This may indicate that, in addition to the depletion of pathogenic B cells, B cells that have a regulatory function are also depleted. During this phase, there may be some expansion of autoreactive T cells, while B cells are depleted, and this may account for the fact that the improvement in C-peptide was not sustained in the human study.

3.3 Other strategies for targeting B cells

B cell signaling pathway. Following on from these B cell depletion experiments, there have also been some newer preclinical studies, with strategies that may hold some promise for therapeutic targeting of B cells. The spleen tyrosine kinase (Syk), which is important for B cell signaling and $Fc\gamma R$-mediated responses, can be targeted using a selective inhibitor R788 (fostamatinib). This is an orally administered small molecule that is converted to R406, which has been used in phase II clinical trials in rheumatoid arthritis [80] and immune thrombocytopenic purpura [81]. To test whether this agent may show promise in diabetes, in a study in NOD mice, R788 reduced B cells to nearly half the number one to three months after treatment, with reduced follicular B cells and correspondingly increased marginal zone B cells, while activated B cells and plasma cells were unchanged [82]. IL-10-producing B cells, which have been shown to have regulatory properties, were increased in spleen and peritoneal cavities, although the absolute numbers did not change. Coincident with these changes, DC numbers were decreased in the spleen and pancreatic lymph nodes, and in parallel, regulatory T cells appeared to be decreased. The treatment with R788 at 6 weeks of age in NOD mice delayed and prevented diabetes in a dose-dependent manner [82]. When initiated at a later stage, after the onset of glucose intolerance detected by intra-peritoneal glucose tolerance test, progression of diabetes was also delayed. However, once diabetes was established, the drug did not restore glucose tolerance [82]. Therefore, this treatment would hold more promise for an early stage therapeutic.

Antigen-specific B cell therapy. Development of antigen-specific T cell therapy is still a subject for investigation, as its specificity is attractive. Could the same apply for B cells and might antigen-specific B cell-targeted therapy be a viable option? Antigen-specific B cells, like antigen-specific T cells, are present at a low frequency, and B cells as a total population only make up 5-10% of peripheral blood mononuclear cells in humans. In NOD mice, Henry and colleagues showed that when antigen-specific B cells recognizing insulin were targeted with a specific monoclonal antibody, commencing treatment at 3 weeks of age, then diabetes incidence was considerably reduced [83]. It was not effective when the frequency of insulin-specific B cells was greater than the low frequency found in wild type NOD mice. This was illustrated by the use of insulin antibody depletion in mice transgenic for the heavy chain of the 125 insulin-specific B cell, or 125 transgenic mice expressing both heavy and light chains of the 125 B cell receptor. In the transgenic mice, the anti-insulin antibody treatment did not have any effect on diabetes, and was not sufficient to fully deplete the insulin-reactive B cells. It is not known whether this type of antigen-specific anti-B cell therapy would be efficient if commenced at a later time point [83].

Tolerogenic B cells. A B cell gene therapy approach has been used to induce antigen-specific tolerance, where B cells are transduced with a retroviral vec-

tor encoding a target peptide antigen fused with an IgG heavy chain carrier. These B cells have been shown to be tolerogenic in a variety of preclinical models of human autoimmune diseases, including autoimmune diabetes in NOD mice [84]. Treatment with transduced activated B cells reduced the incidence of diabetes when treatment was commenced at 7 and 10 weeks, with constructs expressing either GAD or insulin B chain amino acids 9-23 peptide [84]. However, if treatment was commenced later at 14 weeks, there was no protection from diabetes. Further experiments have shown that transduction of antigen-specific B cells does not have any effect on ameliorating the disease. The results indicate that this means of generating tolerogenic B cells does not overcome the pathogenicity of B cells which have pre-existing antigen specificity [85].

Are the results seen thus far with anti-B cell therapy sufficient to continue to explore this therapeutic avenue, or will the limited time period over which the treatment is effective and the safety issues preclude anti-B cell therapy? This depends on the individual anti-B cell therapy used, and the repopulation characteristics of the treatment. Moreover, it will be particularly important to continue to explore therapies against antigen-specific B cells. This would complement means of targeting pathogenic B cells, while preserving or boosting any regulatory activity that may be present, rather than total depletion of all B cells.

4. Gamma delta T cells

Gamma delta (γδ) T cells are a small subset of T cells that express a distinct T cell receptor (TCR) compared with the majority of T cells that express α and β TCR chains, hence, αβ T cells [86, 87]. Unlike αβ T cells, the antigen recognition of γδ T cells is mostly not restricted to antigen processing and presentation by classical MHC molecules on APCs [87]. It has been hypothesized that γδ T cells recognize antigen patterns, similar to innate immune cells [88]. γδ T cells are abundant in tissues at the interface with the external environment including skin, respiratory tract, and intestine [89-94], and they could be considered to be innate T cells. Studies have shown that γδ T cells contribute to the immunopathogenesis of autoimmune diseases including T1D in both mouse models and in patients [95-97].

It is interesting that insulin B9-23-reactive γδ T cell clones were isolated from spleen and pancreatic draining lymph node (PLN) of NOD mice [98]. Other investigations have shown elevated levels of γδ T cells in both NOD mice and patients with T1D [95-97], although it is not clear whether the γδ T cells, in particular insulin-reactive γδ T cells, are pathogenic or regulatory. However, since γδ T cells are abundant in mucosal tissue, Harrison and colleagues showed that delivery of proinsulin by the respiratory or digestive tracts could induce potent regulatory γδ T cells that prevented diabetes development in NOD mice [99-101]. Moreover, these regulatory γδ T cells could also prevent diabetes development induced by diabetogenic cells through adoptive transfer [99-101]. The regulatory mechanism of these γδ T cells is likely to be mediated by IL-10 as these cells strongly resemble the induced regulatory Tr1 αβ T cells [99]. It is conceivable that the regulatory γδ T cells provide a new therapeutic approach for prevention and treatment of T1D. However, it is clear that more studies are needed, particularly to define their biology, in order to discover whether they may be important in humans and if so, how to target such cells to increase their number.

5. Natural killer cells

Natural killer (NK) cells have been investigated at both cellular and molecular levels, and the number of studies in recent years has significantly increased. The discovery of activating receptors, natural cytotoxicity receptors (NCRs), and the inhibitory killer cell immunoglobulin-like receptors (KIRs), on NK cells has further promoted the research in NK cell biology [102-109]. NK cells are innate immune cells that do not express gene-rearranged receptors such as TCR and B cell receptors (BCR) for specific antigen recognition. The central function of the adaptive immune response mediated by T and B cells is to react against "non-self" recognized via TCR or BCR. However, the expression of NCRs and especially KIRs on NK cells enables them to react against "altered self" including virally-infected cells in the host [110]. It is also possible that pancreatic β-cells could express "altered self" caused by viral infection or environmental stress. Therefore, it is possible that NK cells play a critical role in immune tolerance to autoimmunity.

The role of NK cells in the immunopathogenesis of T1D has been studied in both mouse models and patients with T1D [111-114]. Most of these studies showed that NK cells indeed play an important role in immmunopathogenesis of T1D, although some studies suggested that NK cells were not required for the development of disease [115]. It is interesting that Mandelboim and colleagues

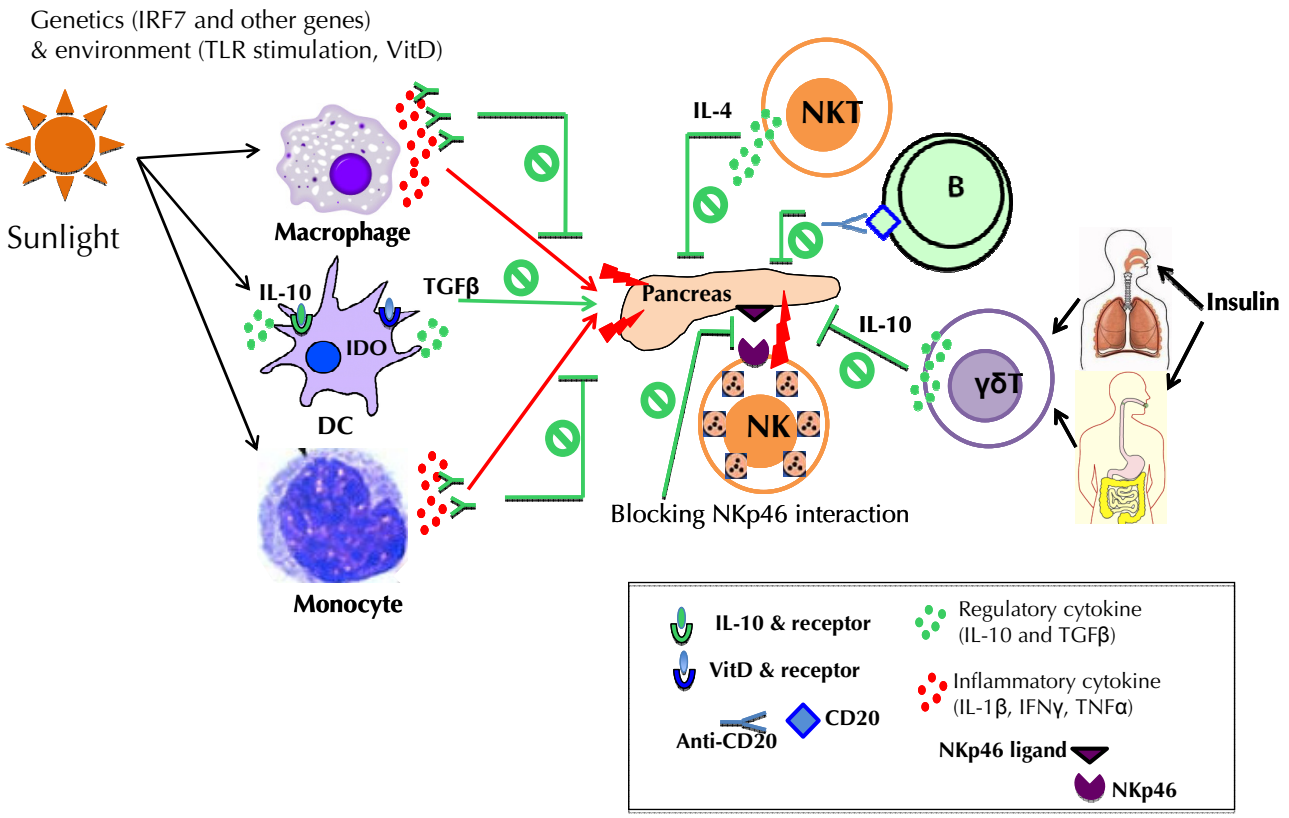

Figure 1. Summary of potential immune targets for immunotherapy in type 1 diabetes. The figure summarizes the immune targets highlighted in the text that could be manipulated for immunotherapeutic purposes. These targets include antigen-presenting cells (monocytes), macrophages, and dendritic cells (DCs), as well as B cells, innate lymphocytes (γδT cells), natural killer (NK) cells, and NKT cells. *Abbreviations*: IDO – indolamin-2,3-dioxygenase, IFNγ – interferon gamma, IL – interleukin, IRF7 – interferon regulatory factor 7, NKp46 – natural killer cell p46-related protein, TGFβ – transforming growth factor beta, TLR – toll-like receptor, VitD – vitamin D.

recently demonstrated that both mouse and human islet β-cells express an, as yet unidentified, ligand for the NKp46 activating receptor on NK cells [116]. As blocking the binding of NKp46 to β-cells prevented diabetes development in NOD mice the investigators suggested that binding the ligand on β-cells would lead to NK cell activation and β-cell destruction [116, 117]. Their study suggested that targeting NK cells might be a new and an additional therapeutic approach for prevention and treatment of T1D development.

Human studies have revealed that patients with T1D feature a reduction in NK cell numbers and impaired NK cell functions [113, 114]. The cause of the altered phenotype of NK cells in T1D is unclear. In a longitudinal study, Gillespie and colleagues demonstrated that an increased frequency of human KIR and HLA-C group 1 was significantly correlated with early onset of T1D and a sharp rise in the incidence over the past half century [118].

An interesting study using a T cell transgenic mouse model of T1D revealed that regulatory T cells exert tight control of the expansion and function of diabetogenic NK cells. This was illustrated by removing $Foxp3^+$ regulatory T cells which led to rapid diabetes development and considerable NK cell infiltration in pancreatic islets [119]. Regulatory T cell therapy is currently in clinical trial for treating patients with new-onset T1D [16]. Enhancing regulatory T cell number and function would also tame the diabetogenic NK cells. It will be interesting to observe whether NK cells are altered as a result of this treatment.

6. Natural killer T cells

There are a number of cells that fall into this category of innate lymphocytes. CD1d restricted natural killer T (NKT) cells have functions different from cytotoxic NK cells. Type 1 or invariant NKT cells (iNKT) express a TCR using an invariant alpha chain (Vα24-Jα18 in humans and Vα14-Jα18 in mice) together with a restricted set of β chains, and they produce a large amount of IL-4 and other cytokines upon activation [120]. They recognize glycolipid antigens presented by CD1d. Type 2 NKT cells are also CD1d-restricted but have more diverse T cell receptors [121], and they target sulphatide [122] amongst other lipid antigens.

A number of studies have shown that NKT cells in NOD mice are impaired in both number and function (reviewed in [123, 124]). Improving the function of this subset protects against diabetes development in NOD mice [125-129]. Targeting invariant NKT cells is potentially an attractive approach for an alternative immunotherapy. Invariant NKT cells are stimulated by the glycolipid α-GalactosylCeramide (α-GalCer), and initially this was proposed to be a possible therapeutic agent to boost these cells [130]. Very recently, sulphatide that stimulates type II NKT cells has also been proposed as a possible therapy [131].

Whilst the promotion of cytokine production, particularly IL-4, may be attractive to counteract the Th1 dominance in T1D, inducing such a change in cytokine balance also raises the risk of enhancing Th2-mediated immunopathology including allergy. Possible applications of therapy that stimulates NKT cells in T1D and other autoimmune diseases, and potential problems of this immunotherapy, have been very recently reviewed by Simoni and colleagues [132]. Whilst preclinical studies suggest that therapy directed at NKT cells is of potential clinical interest, there are considerations of safety and efficacy which need to be further explored if it were to be used for human immunotherapy in T1D.

7. Concluding remarks

It is clear that there are several potential immune therapies beyond T cell therapy (see **Figure 1**). However, more basic and applied research is required to understand the biological processes and to test for the safety and efficacy of new therapies. Many treatments highlighted in preclinical studies are only effective in early stages of the disease process; they are likely to be more effective in the prevention of human T1D. There are still insufficient biomarkers, other than multiple autoantibodies, to predict the future development of disease. Clearly, research should continue to be focused on this area. The smaller number of therapies that are effective at later stages in the preclinical models will require rigorous testing and understanding of how their effects may differ in humans compared with rodents. The results of the recent clinical trials provide us with some important messages. Firstly, a combination of different interventions may be more effective as there is unlikely to be a "magic bullet" (i.e., a monotherapy) for a multifactoral disease such as T1D. Secondly, the approaches that target immune cells other than T cells currently are likely to be more effective in prevention of T1D. We will need to continue the search for safe and effective agents that can be used in both early and later phases of disease.

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