Type 1 diabetes: Translating mechanistic observations into effective clinical outcomes

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The authors would like to dedicate this article to the remarkable contributions and memory of George Eisenbarth, who inspired all of us.

A model of the pathogenesis of type 1 diabetes (T1D) was originally proposed by George Eisenbarth in a landmark paper in 1986 (REF. 1). It followed earlier observations of a long pre-diabetic period identified by the presence of islet cell autoantibodies in diabetics with polyendocrine deficiencies and described a chronic autoimmune process, initiated by unknown factors, that proceeded over many years and involved the killing of insulin-producing β-cells by autoreactive lymphocytes. The bases for this highly original concept were observations from clinical studies. When patients with T1D received a pancreatic isograft from an identical twin, T cell infiltration was found in the isograft at the time of declining graft function. In addition, data from several intervention studies suggested that immunosuppressive therapies, such as anti-thymocyte globulin and cyclosporin A, could have a positive impact on T1D disease progression. Since then, extensive human and animal studies have strengthened the concept that this progressive disease is accompanied by β-cell destruction and also by β-cell dysfunction. At the time of onset, most clinical studies suggest that as much as 30% of β-cell mass is present, and in many cases residual insulin production can increase soon after disease diagnosis as the dysfunction improves with metabolic control (BOX 1). This level of residual function is by no means insignificant and warrants preservation. More than 90% of patients with new-onset disease, including children, have a level of stimulated C peptide that is at least 0.2 nmol L⁻¹; this level is found to be associated with improved glucose control and with reduced risk of severe hypoglycaemia and secondary end organ complications (for example, retinopathy and renal disease). However, there is a linear decline in functional β-cell mass. Thus, 5 years after the initial T1D diagnosis the proportion of subjects who maintain this level is small.

Studies in preclinical models have added to our understanding of the antigens, cells and mechanisms that are involved in T1D development and progression. Furthermore, recent clinical investigations have refined, and in some cases changed, these concepts. In this Review, we discuss the concepts that led to our current concepts of the disease mechanisms involved in T1D and the clinical studies promoted by these studies. The findings from preclinical and clinical studies support the original proposed model for how T1D develops but have also suggested that this disease is more complex than was originally thought and will require broader treatment approaches.
Type 1 diabetes (T1D) is one of the most common chronic diseases of childhood. The prevalence of T1D ranges from <5 in every 100,000 individuals in eastern countries to as many as 39.9 in every 100,000 individuals in European and other western countries. A substantial proportion (estimated to be approximately 10%) of adults who present with diabetes has T1D rather than the more common type 2 diabetes, which is not autoimmune in nature. There are strong genetic determinants of the disease (Box 3), but >90% of individuals who present with new-onset diabetes do not have a relative with T1D. More than 90% of individuals with T1D test positive for at least one autoantibody, and the presence of autoantibodies identifies relatives of patients who are at a high risk for the disease (typical targets of these autoantibodies include glutamic acid decarboxylase (GAD65), IA2 (also known as ICAS12 and PTTPRN), insulin, IGRP (also known as G6PC2), zinc transporter 8 (ZNT8) and islet cell autoantigen (ICA);47,109,119). The peak incidence of disease onset is between 6 and 15 years of age, and a second peak occurs later in adolescence. At the time of presentation, most patients have signs and symptoms of hyperglycaemia and insulin deficiency (such as polyuria, polydipsia, visual change, weight loss and elevated glycosylated haemoglobin A1c levels) or even more severe metabolic decompensation with ketoacidosis. However, some patients are identified on routine urine or blood tests, before β-cell destruction and insulin deficiency have resulted in symptoms. After presentation and metabolic stabilization, many patients enter a clinical ‘honeymoon’, in which time insulin secretion improves, and some patients can even discontinue the use of exogenous insulin. This period is invariably followed by a loss of insulin production and an increasing dependence on exogenous insulin therapy. Metabolic control is monitored by measurement of glycosylated haemoglobin A1c levels, and renal disease, as well as the acute complication of insulin-induced hypoglycaemia, remain common features.

Environmental factors leading to T1D

In many countries, the incidence of T1D has been increasing in younger children at a rate that is faster than can be accounted for by genetic change alone, highlighting a role for environmental factors. There is evidence of temporal changes over the past 20 or so years in those developing T1D, with increased incidences in the under-5 age group, as well as in individuals with lower risk HLA haplotypes, such as HLA-DQB1*0602. These disturbing findings have revived searches for environmental factors that may be responsible for triggering T1D, such as changes in exposure to infectious, environmental or nutritional agents.

Infectious agents and commensal organisms

Infectious agents, including viruses, bacteria and fungi, can have pathogenic or protective roles in T1D. Pathogenesis could be elicited through direct infection of β-cells, through a more generalized release of pro-inflammatory or cytotoxic cytokines in response to infection — especially at the pancreatic tissue site — or through antigen mimicry. Alternatively, it has been proposed that some infectious organisms, such as helminths, can shape the immune system in a manner that is of mutual benefit to the parasite and the host. These interactions might have historically prevented the onset of T1D, and the removal of these infections through increased public health measures might be responsible for the increased incidence of T1D that we see today (this idea is known as the hygiene hypothesis). It has been proposed for decades that enteroviruses are linked to T1D. The report of a 10-year-old patient who died with fulminant T1D and who showed acute and convalescent antibody titres against a coxsackie B4 virus — which was isolated and shown to cause β-cell destruction — supports this notion. A second case study also supports this: a sudden onset of T1D in an adult patient was found to be associated with coxsackie B4 virus infection and natural killer (NK)-cell-mediated insulin. Recent studies have shown that in response to viral infection, human islets secrete pro-inflammatory cytokines, such as interleukin-6 (IL-6), IL-8 and tumour necrosis factor (TNF), and chemokines, such as CXC chemokine 10 (CXCL10; also known as IP10), CC chemokine 3 (CCL3; also known as MIP1α) and CCL4 (also known as MIP1β). Moreover, phagocytosis of enterovirus-infected β-cells triggers innate immune responses in human dendritic cells (DCs). There is increasing evidence that commensal organisms have a role in moulding the host immune system and that alterations of the gut microbiome can have immunological, metabolic and pathological consequences (Figure 1). The polysaccharide component of the outer membrane vesicles of Bacteroides fragilis, which is a normal component of the human gut microbiota, has been shown to interact with host DCs, inducing anti-inflammatory cytokine production and generation of forkhead box P3 (Foxp3)+ regulatory T cells (Treg cells) that are capable of inhibiting inflammatory bowel disease. An increase in Bacteroides ovatus and the firmicute strain CO19 was found in the microbiota of patients with T1D in a case–control study, although it is not clear whether an associated polysaccharide component in these strains influences disease progression. Moreover, a relative decrease in B. fragilis was seen in patients compared with controls over time. Other commensal organisms have been shown to influence invaraint natural killer T cell (iNKT cell) activity. As Treg cells and iNKT cells have been shown to influence diabetes onset in model systems, the ability of exogenous infectious agents, as well as commensal organisms, to influence these regulatory cell types provides mechanisms by which environmental agents might influence the host immune response. However, the pathogenic effects of some commensal organisms may be disease-specific. For example, there is evidence that some species, such as segmented filamentous bacteria, can accelerate the onset of arthritis or experimental allergic encephalomyelitis by inducing helper T cells (T helper cells) in mice, but can inhibit the onset of autoimmune diabetes in non-obese diabetic (NOD) mice.

Studies of myeloid differentiation primary response 8 (MyD88)−/− mice underscored the importance of the gut microbiota and its interactions with the host innate immune system in modulating diabetes onset. This signalling pathway is required for autoimmune diabetes development in NOD mice under specific-pathogen-free conditions (SPF conditions). The way in which MyD88 signalling affects disease pathogenesis is through modulation of the gut microbiota, because...
Insulitis
Inflammation of the islets of Langerhans in the pancreas that comprises a complex cellular infiltrate that invades and destroys the islets of Langerhans. The cellular composition includes CD4+ T cells, CD8+ T cells, regulatory T cells, B cells, dendritic cells, natural killer cells and macrophages.

Gut microbiome
This is the collective community of bacteria in the small and large intestines.

Forkhead box P3 (FOXP3). A forkhead/winged helix family transcription factor that is a crucial master regulator of regulatory T cell development and function.

Figure 1 | Revision of the Eisenbarth model of type 1 diabetes. Our earlier concepts of the pathogenesis of type 1 diabetes (T1D) have been modified owing to the emergence of new information. During childhood, there is an increase in β-cell function (dotted line); as a result of this, patients who present with T1D at an older age have higher C-peptide responses compared with younger patients30,31,39. The immune process that leads to diabetes begins years before the clinical onset. Rather than a single event, antigenic epitopes are unmasked during the progression of disease, and this is associated with intra- and intermolecular epitope spreading. The decline in β-cell function may not be constant over time. A greater change may be more closely associated with the diagnosis than has previously been appreciated17. Nonetheless, the impairment seen at the time of diagnosis may stabilize (solid line) or even be partially reversed with immune therapy (dashed line), but then the decline in β-cell function continues with time.

MYD88−/− NOD germ-free mice develop rampant disease, but disease protection can be transferred with faeces from MYD88−/− NOD mice raised under SPF conditions. However, the precise role of the gut microbiota in this setting has not been clarified.

At least three other observations concerning the microbiota are relevant to this discussion. First, the microbiome of healthy children is more diverse and less stable than are the microbiomes from patients with autoimmune diseases25. Second, germ-free mice have incomplete immune systems, and colonization with microflora is needed for the development of Tc17 cell responses and Treg cell responses in the gut36. Third, the gut microbiota regulates intestinal permeability, and this may have a role in the initiation of insulitis by altering the transport of antigens and subsequent autoimmune-triggering antigens to the pancreas41.

Clinical testing targeting environmental factors. There are at least three challenges to identifying organisms that are causative of T1D development in humans. First, infection may not coincide with presentation with hyperglycaemia: these events may be separated by years. Second, most (>90%) patients who develop T1D do not have an affected relative with the disease. Therefore, identification of at-risk individuals requires a broad population-based search. Third, it is possible that the pathological event involves the absence of an immune response to an organism rather than the presence of a protective response and therefore cannot be identified. For instance, instead of looking for a virus or another environmental antigen that induces disease, the relative risk may increase as a result of failure to develop protective immunity, thereby making the causal linkage more difficult to identify. Studies such as The Environmental Determinants of Diabetes in the Young (TEDDY) and Diabetes Autoimmunity Study in the Young (DAISY) are screening high-risk individuals to identify viral and other pathogenic infections that may be associated with T1D.

Clinical data indirectly support the notion that early dietary manipulation may affect disease development. Epidemiological studies have shown that T1D incidence is lower in breast-fed versus bottle-fed offspring of parents with T1D, and the timing of exposure to cereal was also linked to T1D development42–44. The Trial to Reduce IDDM in the Genetically at Risk (TRIGR) study is testing whether exposure to cows’ milk is associated with the development of autoantibody responses that may trigger diabetes45,46.

Breaking tolerance to autoantigens
Creating the autoimmune repertoire: autoantigens, T cells and B cells. In the original model, the basis for the breakdown in tolerance to self proteins remained unclear, and this uncertainty persists today (BOX 2). The unresolved issues include the identity of the crucial self antigen (or antigens) that drives initiation and perpetuation of disease, the nature of the tolerance defect and the individual roles of central versus peripheral compartments in propagating diabetes. The discovery of autoantibodies in the serum of patients was direct evidence to suggest that T1D was an autoimmune disease1. Although autoantibodies identify the ongoing autoimmune response, the main way in which B cells contribute to T1D pathology appears to be through their antigen presentation functions37. Antibody-mediated depletion of B cells, even at the time of onset of hyperglycaemia, can prevent or reverse disease in NOD mice38,39.

Over the course of several years, the targets of these autoantibodies have been identified and include insulin, proinsulin, glutamate decarboxylase (GAD65; also known as GAD2), IGRP (also known as G6PC2), IA2 (also known as ICA512 and PTPRN) and, most recently, zinc transporter 8 (ZNT8), which is specifically expressed by β-cells40. A combination of human genome studies (BOX 3) and functional studies in animal models of disease has implicated insulin (or proinsulin)41,42 as the primary autoantigen for disease initiation, whereas other islet-specific molecules, such as IGRP and chromogranin A have been suggested to promote disease progression. Preclinical studies have highlighted the progression of disease through intramolecular and intermolecular spreading43,44. Although many autoantigens have been implicated as targets and drivers of T1D, there is limited direct evidence for the involvement of T1D.
**Box 2 | Mechanisms of tolerance relevant to type 1 diabetes**

Tolerance to self proteins is controlled by numerous checkpoints during the development of T and B cells — a process referred to as central tolerance — as well as by numerous checkpoints in the peripheral tissues to ensure that potentially autoreactive cells do not respond to the tissues, which is termed peripheral tolerance. Central tolerance purges the mature repertoire of T and B cells expressing autoreactive receptors in the thymus and bone marrow, respectively. This can occur by inducing apoptosis of T or B cells or, in the case of B cells, by altering the specificity of the B cell receptor (BCR) in a process termed receptor editing. These mechanisms are restricted to antigens that are presented or expressed in those compartments, and as a result potentially autoreactive T and B cells may escape into the periphery. Both cell-intrinsic and cell-extrinsic mechanisms are involved in controlling the activation of those cells. Cell-intrinsic mechanisms include modulators of BCR and T cell receptor (TCR) signalling, such as induction of inhibitory receptors (for example, cytotoxic T lymphocyte protein 4 (CTLA4), programmed cell death 1 (PD1) and lymphocyte activation gene 3 protein (LAG3)) or ubiquitin ligases (for example, CBL). Extrinsic factors involve restriction of the required co-stimulatory ligands on antigen-presenting cells (for example, the B7 molecules CD80 and CD86, which are required for CD28-mediated co-stimulation of T cells), limiting the availability of survival factors (such as BAFF (also known as TNFSF13B) and interleukin-17 (IL-17)), or exposure to inhibitory cytokines (such as IL-10) or regulatory populations (for example, regulatory T (T(reg) cells). T(reg) cells may develop in the thymus or in the periphery. T(reg) cells are characterized by the expression of the X-linked forkhead transcription factor FOXP3, which is a master regulator required for their maximal development and function. A genetic mutation of FOXP3 abolishes T(reg) cell function, leading in humans to the immunodeficiency, polyendocrinopathy enteropathy X-linked (IPEX) syndrome, which is a severe multi-organ autoimmune syndrome caused by uncontrolled immune activation.

Regulatory T cells (T(reg) cells). A rare subpopulation of CD4+ T cells that are endowed with potent suppressive capacity. They typically express the transcription factor FOXP3+. Both naturally occurring T(reg) cells (which develop in the thymus) and adaptive T(reg) cells (which acquire their regulatory activity in the periphery) have been described.

Invariant natural killer T cells (iNKT cells). Cells that share properties of T cells and natural killer (NK) cells and recognize the non-polymorphic CD1d molecule, which is an antigen-presenting molecule that binds self lipids and foreign lipids and glycolipids. They recognize α-galactosylceramide presented on CD1d molecules and have restricted T cell receptor (TCR) usage.

any single autoantigen in the development of the disease. Elimination of proinsulin or insulin completely prevents insulitis and diabetes in NOD mice, but the removal of IGRP — another self antigen that is targeted by T cells — did not show this protective effect. In humans, the primacy of insulin as the major autoantigen for diabetes initiation has not been proven, although in young children with diabetes, autoantibodies against insulin tend to appear before autoantibodies with other specificities. Furthermore, cytotoxic T cells isolated from a patient with TID were shown to kill β-cells through recognition of a glucose-related preproinsulin epitope.

Several studies continue to point to the potential importance of central tolerance mechanisms in preventing immune reactivity to autoantigens, and the failure of these mechanisms promotes the progression of autoimmune diabetes in mice and potentially in humans. A significant percentage of patients with autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) develops autoimmune diabetes. APECED is caused by mutations in the transcription factor autoimmune regulator (AIRE), which is expressed by thymic medullary epithelial cells and promotes their expression of tissue-specific antigens. This process facilitates the development of central tolerance to peripheral proteins, such as insulin, that are expressed only at restricted tissue sites. In mice, the insulin 1 (Ins1) gene is predominantly expressed by β-cells in the pancreatic islets, but the Ins2 gene is expressed both in the thymus and by islet β-cells. Although Ins1−/− mice exhibited reduced autoimmune diabetes, Ins2−/− mice showed markedly accelerated disease development, presumably owing to a defect in central tolerance. In an analogous manner in humans, the susceptibility alleles of the insulin-dependent diabetes mellitus 2 (IDDM2) gene locus, a polymorphism of the IRS promoter, cause lower thymic IRS expression compared with the alleles associated with diabetes resistance (Box 3).

Certain novel epitopes of disease-associated antigens may not be presented in the thymus, resulting in the escape of autoreactive T cells into the periphery. For instance, an insulin peptide (B12–20), which is distinct from the B13–21 peptide that is presented in the thymus, is presented by antigen-presenting cells in the islets to type B T cells. The antigen-presenting cells in the islets accomplish this without interaction with the chaperone molecule H2-DM, and the responding T cells, which can cause T1D, do not recognize peptides derived from the intracellular processing of the native protein. Moreover, Kappler and colleagues have shown that insulin peptides can bind to MHC molecules, such as I-Ak, in distinct registers owing to flexibility in the binding groove of the MHC class II molecule. The so-called ‘register 3’ binding is of low affinity and leads to the escape of the peptide-specific CD4+ T cells from the thymus. In the periphery, the unique tri-molecular complex of the T cell receptor (TCR), the MHC and a peptide can activate autoreactive CD4+ T cells. The absence of presentation and expression of these tissue-specific antigens in the thymus provides a potential mechanism that might be perturbed in autoimmune diseases, such as TID, although this has yet to be established.

Defects in both central and peripheral B cell tolerance mechanisms have also been identified in patients with TID. The frequency of polyclonal and HEP-specific autoantibodies is increased in these patients, suggesting failures of central and peripheral tolerance checkpoints, respectively. A similar increase in these types of autoantibodies is seen in patients with rheumatoid arthritis or in otherwise healthy subjects with the R620W variant of the protein tyrosine phosphatase, non-receptor type 22 (PTPN22; also known as LYP) allele, which is found on a gene locus that is associated with TID (namely, IDDM3). PTPN22 also acts in a complex with carboxy-terminal SRC kinase (CSK) to negatively regulate signalling from the TCR, and the R620W variant of PTPN22 has been shown to affect negative selection in the thymus by altering TCR signalling. Thus, these defects in central and peripheral T and B cell tolerance can establish an autoreactive lymphocyte repertoire that may drive TID development.

Mechanisms of β-cell destruction. In preclinical models, such as the NOD mouse and BB/W rat, β-cell destruction is caused by T cells. Two ‘checkpoints’ during the pathogenesis of the disease have been described. The first checkpoint is the recognition of islet antigens, and this has been associated with β-cell death, which can be developmentally programmed or can occur following cellular damage. The second checkpoint is the conversion from a non-destructive to a destructive insulitis, a process that is also enhanced by cellular damage. The second checkpoint may involve acquisition of
T helper 1 T cells

A subset of CD4+ T cells that is characterized by its expression of the transcription factors ROTy, ROYα and signal transducer and activator of transcription 3 (STAT3). They are involved in inflammatory responses and normally have an important protective role at epithelial and mucosal surfaces. Their development involves a combination of TCRβ, interleukin 21 (IL-21), IL-23 and IL-1β, and they secrete IL-17, IL-22, IL-23 and in some circumstances granulocyte macrophage colony-stimulating factor (GM-CSF) and/or interferon-γ (IFNγ).

Specific pathogen-free conditions

Mice raised under SPF conditions are guaranteed to be free of a defined list of mouse pathogens.

Germ-free mice

Also known as gnotobiotic mice, these are mice that do not harbour any bacteria, viruses or parasites.

Type B T cells

A term that has been used to describe CD4+ T cells that recognize and respond to unstable peptide–MHC complexes, which arise when peptides are loaded onto MHC class II molecules in the absence of H2-DM-mediated editing.

HEp-2

A human epithelial cell line that is commonly used as a target for immunofluorescent detection of a wide range of nuclear and cytoplasmic staining antibodies. Distinct staining patterns are associated with particular antibody specificities. For example, a homogeneous nuclear staining pattern is indicative of antibodies that react with small nuclear ribonucleoproteins.

Clinical testing of agents that target autoreactive lymphocyte responses

‘Natural history’ studies have shown that there is a strong association between the risk of developing T1D disease and the presence of autoantibodies to known islet antigens. Interestingly, the number of different antigens that were targeted by autoantibodies (including GAD65, IA2, IGRP, insulin and ZNT8) and islet cell autoantibodies (ICAs) rather than the overall titre of autoantibodies was the most important determinant of risk. Indeed, among unaffected first-degree relatives of T1D patients with positive islet cell antibody and ‘dysglycaemia’ or four autoantibodies, over 75% will develop T1D over the following 5–6 years with a median time to onset in those with dysglycaemia of 2.81 and 4.24 years for 8–17 and 18–45 years old, respectively.

Several studies have attempted to induce tolerance to specific antigens by parenteral or intranasal vaccination, or by oral administration (Fig. 2). The rationale for these approaches was to induce tolerance by affecting specific lymphocyte populations or to promote bystander suppression or even incessant tolerance. However, the results from trials of antigen-specific therapies have been disappointing. Trials to induce tolerance to insulin have largely been unsuccessful in altering disease progression after autoimmune destruction had ensued.

When high-risk relatives of patients with diabetes were treated with insulin parenterally (in the Diabetes Prevention Trial of Type 1 (DPT-1)) or even intranasally, they showed no evidence for modification of disease progression. Although a prevention trial to induce oral tolerance to insulin failed to show any significant delay in the onset of diabetes, the treatment did induce a significant delay in disease onset in individuals with the highest titres of anti-insulin antibodies. Another pilot study suggested a delay in the decline of C peptide levels when patients with T1D were administered GAD65 in alum adjuvant to generate autoantigen-specific regulatory T cells. The rationale for this study was that by modifying the immune response to this antigen through vaccination, the disease progression could be curtailed. However, two subsequent trials failed to corroborate
these findings, even though the immunization increased the titres of GAD65 autoantibodies and the frequency of T cells that produce inhibitory cytokines. These results have been disappointing but suggest that the selection of the antigen and patient may be paramount to the success of this strategy.

Some surprising results based on prior experiences with anti-thymocyte globulin and cyclosporin A were that neither co-treatment with the immunosuppressant mycophenolate mofetil and daclizumab (a monoclonal antibody that is specific for the α-subunit of the IL-2 receptor) nor treatment with anti-thymocyte globulin was able to delay the decline in C peptide levels seen in T1D patients with new-onset disease (S. E. Gitelman, L. Fisher, P. Gottlieb, M. Gottschalk, W. Moore, A. Moran, M. Rigby, S. Willi, L. Keyses-Elstein, L. Ding and M. Ehrler, unpublished observations). By contrast, eliminating B cells with a 4-week course of rituximab delayed the decline of C peptide levels at 1 year in patients with new-onset T1D. Insulin-specific and GAD65-specific autoantibodies, but not PTPRN-specific antibodies, were reduced by rituximab treatment, suggesting a kinetic hierarchy of antigens, but interestingly the T cell proliferative responses to islet antigens were not.

Figure 2 | Results of immunotherapy trials in type 1 diabetes. The figure illustrates the aspects of the immune response that have been targeted in type 1 diabetes (T1D; yellow text boxes). To date, therapies have been designed to target innate and adaptive immune responses that are thought to contribute to T1D pathology. Successful clinical trials have used: rituximab, which depletes B cells; cytotoxic T lymphocyte antigen–immunoglobulin (CTLA4–Ig) fusion protein, which blocks the delivery of CD80 and CD86 co-stimulatory signals to T cells; or CD3-specific monoclonal antibodies, which deplete T cells. Anti-thymocyte globulin (ATG) with glucocorticoids or autologous bone marrow transplant has also been successful. However, therapies that have involved administration of autoantigens (for example, glutamate decarboxylase 2 (GAD2)), targeting of T cells with immunosuppressive agents (for example, mycophenolate mofetil (MMF), daclizumab (DZB) or blocking pro-inflammatory cytokines (for example, anti-interleukin-1 (IL-1) reagents) have not had beneficial effects. A trial of rapamycin and IL-2 had a negative effect on patients who showed a transient decline in C peptide responses. A trial using treatment with heat-shock protein 60 (HSP60), which is thought to enhance regulatory T (TReg) cell functions by signalling via Toll-like receptor 2 (TLR2), has shown a beneficial effect on C peptide responses. A pilot trial of a soluble tumour necrosis factor (TNF) receptor (etanercept) resulted in lower blood glucose levels and increased insulin production in patients. In a trial of avoidance of cows’ milk, a reduced rate of new autoantibody formation was seen, but the mechanisms of this effect are not clear.

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Role of innate immune cells

Numerous studies have implicated cells of the innate immune system in both the initiation and the development of diabetes. Analysis of NOD mice and human diabetic islets has revealed the infiltration of macrophages, DCs and NK cells along with cells of the adaptive immune response. In addition to MHC class I-restricted killing of β-cells by cytolytic T cells and CD95–CD95L (also known as FAS–FASL) interactions, pathways mediated by innate immune cells have been implicated in the selective death of β-cells. These include interaction of NK-cell-expressed NKG2D or NKP46 (also known as NCR1) with β-cell-expressed RAE1 or NKP46 ligands, respectively, and cytokine-mediated effects, including reactive oxygen species (ROS) induction. Human and mouse β-cells express NKP46 ligands as well as ligands for NKG2D, which, together with the presence of CD107γ, NK cells in the diabetic mouse pancreas, has led to the suggestion that NK cells may play a part in β-cell death. However, recent depletion studies of NK1.1+ cells have questioned the importance of NK or NKT cells in NOD mice, as NKG2D is also expressed by activated T cells, and NKP46 is expressed by γδ-T cells and some αβ-T cells, as well as by innate lymphoid cells.

Blockade of macrophage entry into the pancreas or inhibition of macrophage function in mice prevents diabetes onset, suggesting a key role for this population in β-cell demise. Cytokines produced by cells of the innate immune system, including macrophages and DCs, have been implicated in β-cell dysfunction in the diabetic pancreas in mice and humans. IL-1β can inhibit insulin production and IL-1β, TNF and IFNγ may directly contribute to β-cell death. Treatment with IL-1-specific antibody or genetic deficiency in IL-1 receptor expression delays, but does not prevent, T1D in NOD mice.

Pro-inflammatory cytokines upregulate expression of MHC class I molecules on islets in vitro. Interestingly, in humans with T1D, islet cells show high levels of MHC class I molecule expression in both the presence and the absence of cellular infiltrates. This raises the possibility that a sustained inflammatory response within islets may be due to viral or other environmental insults, abnormalities in MHC class I peptide processing or the effects of systemic cytokines on the β-cell environment. The collective consequences of these immunological insults may make β-cells more susceptible to CD8+ T-cell-mediated killing. In addition to classical DCs, which have a key role in the initiation of diabetes through activation of autoreactive T cells, plasmacytoid DCs (pDCs) have also been implicated in diabetes development. These cells make large amounts of type 1 IFNs, as well as IL-12 and other pro-inflammatory cytokines, and there is evidence from several models that type 1 IFNs can enhance diabetes onset. Finally, nonspecific inhibition of inflammation with α1-antitrypsin (AAT), which inhibits enzymes that are released by innate immune cells such as neutrophils, was shown to reverse new onset diabetes in NOD mice. As a result of treatment with AAT, increased β-cell proliferation and improved insulin sensitivity were also seen in NOD mice.

Clinical testing based on innate immune cell targets

Preclinical studies had suggested that high doses of nicotinamide affected ADP ribosylation and other reactions in β-cells as well as in immune cells and the endothelium. These reactions are thought to have a role in signalling through certain TLRs and other innate inflammatory responses. Cell death pathways and gene expression patterns were modified, leading to improved β-cell survival and an altered immunoregulatory balance. This mechanism also prevented the depletion of NAD in β-cells. On the basis of these preclinical studies and of the notion that islet damage was an important driver of the disease, a trial of nicotinamide in autoantibody-positive relatives with dysglycaemia of patients with T1D was carried out. This study failed, however, to show any reduction in the high rate of progression among the active drug-treated participants compared with the placebo-treated participants.

A clinical trial of the IL-1 receptor antagonist (IL-1RA) anakinra in patients with type 2 diabetes showed an improvement in metabolic parameters, and these effects were largely attributed to a direct effect of anakinra on β-cells. A pilot trial of anakinra in patients with T1D showed biological efficacy. However, in two recent trials in patients with new-onset T1D, canakinumab (which is an IL-1β-specific antibody) and anakinra failed to affect the decline in C-peptide responses within the first year of the disease. These studies...
demonstrate the many variables that affect the translation of results in animal studies into effective therapies in humans. Such variables include differences in the drug itself, the timing and dosing of the interventions, and the lack of uniformity (that is, the possibility of disease subtypes) of the human disease compared with disease in animal models (Box 4).

Immunization with a peptide of heat-shock protein 60 (HSP60), DiaPeP277, has been postulated to enhance the function of CD4+CD25+ Treg cells (see below) by signalling through Toll-like receptor 2 (TLR2)107. DiaPeP277 was shown to reduce the decline in C peptide responses in patients with new-onset T1D, most recently in a Phase III trial108 (I. Raz, A. G. Ziegler, T. Linn, F. Bonnici, G. Schernthaner, L. A. Distiller, C. Giordano, F. Giorgino, L. de Vries, D. Mauricio, V. Procházká, J. Wainstein, D. Elias, A. Avron, M. Tamir, R. Eren, S. Dagan, I. R. Cohen and P. Pozzilli, unpublished observations). The mechanistic basis may involve the triggering of TLR2 in Treg cells, leading to activation of protein kinase C (PKC), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and p38 signalling pathways, and the secretion of transforming growth factor-β (TGFβ) and IL-10 (REF 105). Finally, studies are ongoing to test the effects of AAT in patients with new-onset T1D.

**Defects in control of tolerance by immune cells**

It has become increasingly evident over the past 10–15 years that the immune system is under tight control mediated by specialized cell subsets that suppress immune reactivity. The most prominent of the suppressive cell subsets is Treg cells. This rare T cell population, which is generally identified as CD4+CD25+CD127low CD25+ cells, is crucial for the maintenance of peripheral tolerance in many autoimmune diseases, including autoimmune diabetes.109-110 The maintenance of peripheral tolerance is dependent on Treg cells, and defects in Treg cell populations may contribute to disease pathogenesis in type 1 diabetes (Box 2).

Treg cells develop in the thymus with a unique TCR repertoire that is skewed towards the recognition of self antigens111. FOXP3 expression remains crucial throughout life to maintain the Treg cell populations and to prevent autoimmunity. In addition to its expression by Treg cells that develop in the thymus, FOXP3 can also be turned on during activation of conventional human T cells in the periphery. Under the right inflammatory conditions and cytokine milieu, the expression of FOXP3 is stabilized by demethylation of the conserved non-coding sequence 2 (CNS2), which is found within the FOXP3 promoter112. This results in the development of a peripheral Treg cell compartment

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**Box 4 | Pitfalls in translation**

There have been notable failures of effective therapies in preclinical models, most often in non-obese diabetic (NOD) mice, to achieve the same success in human type 1 diabetes (T1D) trials. Included among these are successful therapies of oral insulin, glutamate decarboxylase (GAD65) immunization and interleukin-1 (IL-1) blockade113. Effects on C peptide and insulin use were seen in clinical trials of CD3-specific monoclonal antibodies, but the permanent reversal of disease that was so striking in diabetic NOD mice was not achieved. Moreover, at least one therapy that was unsuccessful in NOD mice (namely, cytotoxic T lymphocyte protein 4–immunoglobulin fusion protein (CTLA4–Ig)) did improve C peptide responses in patients with new-onset disease114. These experiences have led some to question the value of preclinical studies in models and their necessity for the design of clinical studies.

However, a careful analysis of the preclinical studies suggests that their ability to predict outcomes is strong, but details concerning a broad number of variables, including dosage of agents (for example, in trials of oral insulin) and timing (for example, in trials of anti-IL-1 reagents), may not have been fully considered in the clinical trial design115. Nonetheless, there clearly are important differences between murine models and human autoimmune diabetes that complicate the translation. In fact, there may be differences among subsets of humans, even though T1D is lumped together as a single disease. It has been suggested that the ability of murine β-cells to regenerate may be more robust than human β-cells, but more studies are needed to confirm this.116,117 The kinetics of disease in NOD mice appears to be more abbreviated on the basis of the timing of metabolic decompensation. There may be differences between the development of T1D in younger versus older humans. When patients present with new-onset T1D, most retain a stimulated C peptide level of at least 0.2 nmol L⁻¹ and lose this clinically significant level only over a period of years after onset118. By contrast, β-cell function and mass in NOD mice are rapidly lost after the first appearance of hyperglycaemia; these are attributes that may be more similar in very young humans than in young adolescents and adults, in whom the therapies are often tested first. There are clearly differences in the innate and adaptive immune response pathways in mice and humans that are targeted by therapeutics, and therefore it is to be expected that there may be differences in their responses to therapies119. Finally, NOD mice are inbred and live in a protected pathogen-free environment. Patients with T1D live in the real world, but even this can vary enormously in different geographical locations and economic states. Thus the primary weakness may not be in the NOD mouse model per se but rather in our interpretation and use of the data derived from its use.

Where does this leave us? Clinical experience would suggest that therapies that have dramatic effects on diabetes in NOD mice may not achieve the same degree of therapeutic benefit in humans because of patient heterogeneity, differences in kinetics of disease, the responses of β-cells to stress and injury, and even subtle differences in immune responses. However, the animal models have been very effective in elucidating mechanisms of action that are relevant to human disease. The challenge is how to apply the data from preclinical models to human patients. End points of clinical trials should be carefully chosen to identify biological proof of efficacy and mechanism of action, giving outcomes that are important for the design of a combinatorial approach that will successfully achieve clinical end points. Considering the heterogeneity of human subjects, efforts to identify individuals that are most likely to respond to a particular intervention on the basis of clinical parameters or immunologic markers may be very valuable.
that has a repertoire overlapping that of conventional T cells\textsuperscript{111}. In NOD mice, depletion of CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{reg} cells greatly accelerates the development of diabetes\textsuperscript{113,114}. Similarly, removing crucial co-stimulatory or proliferative signals that are necessary for T\textsubscript{reg} cell development or survival, such as IL-2 or CD28, exacerbates diabetes in NOD mice\textsuperscript{114}. Thus, it is clear that T\textsubscript{reg} cells function as the major peripheral cells that control tolerance and immune homeostasis. However, it should be noted that there are multiple types of suppressor cell that have been identified and that may contribute to modulating autoimmune diabetes onset and/or progression. Examples include IL-10-producing regulatory B cells, suppressor macrophages, tolerogenic DCs and additional FOXP3\textsuperscript{+} TGF\textbeta\textsuperscript{+}dependent T\textsubscript{\textgamma}3 cells, IL-10-dependent T regulatory 1 (Tr1) cells and CD8\textsuperscript{+} regulatory cells\textsuperscript{115-120}. T\textsubscript{reg} cells can be subdivided into multiple subsets and tissue-specific subpopulations\textsuperscript{119}. In addition, it has been postulated that the T\textsubscript{reg} cell transcriptional programmes, and conceivably their suppression mechanisms, can be tailored to the nature of the effector response that they regulate\textsuperscript{121}. Thus, the collective magnitude of suppressive activities may reflect the functions of individual T\textsubscript{reg} cell subsets in different tissues with distinct dynamics and unique immunological effects.

The basis of T\textsubscript{reg} cell functional suppression is quite complex and includes several cell surface and soluble factors that directly control immune activation through bystander suppression. Some of their most prominent activities include production of IL-10, TGF\beta and IL-35, which are cytokines that shut down antigen-presenting DCs and activated T cells. Cell-surface molecules, including CTLA4, programmed cell death 1 (PD1) and lymphocyte activation gene 3 protein (LAG3) are also important for T\textsubscript{reg} cell-mediated suppression\textsuperscript{116,122-125}. For instance, CTLA4 can function by competing with CD28 for binding to CD80 and CD86, and in some studies CTLA4 was found to strip the molecules off the cell surface of the antigen-presenting cells (APCs) or to deliver a negative signal to APCs through those ligands. In addition, factors that are produced directly and indirectly as a result of T\textsubscript{reg} cell function (such as IL-10, IL-35, indoleamine 2,3-dioxygenase 1 (IDO) and TGF\beta) can promote the development of other regulatory cells in their vicinity, leading to so-called infected tolerance and a robust local regulation\textsuperscript{117,124,128,129}.

Several genetic loci that are important for T\textsubscript{reg} cell biology have been linked to increased susceptibility to T1D, including: CD25 and IL-2, which are the crucial growth factor receptor and ligand, respectively, for T\textsubscript{reg} cell growth and survival; CTLA4, which is a major functional receptor on T\textsubscript{reg} cells; and the HLA locus, which can alter T\textsubscript{reg} cell repertoires\textsuperscript{99,128,129} (BOX 2). In addition, PTPN22 alters TCR signalling, leading to less IL-2 production by effector T cells. This suboptimal IL-2 production by effector T cells in the islets could locally compromise T\textsubscript{reg} cell homeostasis. Furthermore, phosphorylation of the crucial IL-2-induced intracellular signalling molecule signal transducer and activator of transcription 5 (STAT5) is reduced in patients with T1D, which may be the reason for reduced T\textsubscript{reg} cell numbers in some patients with the disease\textsuperscript{130}. Reduced IL-2 receptor signalling has been linked to the IL-2RA susceptibility allele (rs12722495)\textsuperscript{130}. In fact, treatment of diabetic mice with IL-2 reverses diabetes\textsuperscript{131}.

T\textsubscript{reg} cells have been shown to be unstable in various autoimmune settings, including in mouse and human studies of T1D. In NOD mice, the lack of IL-2 expression in islets can lead to a loss of CD25 expression, reduction of FOXP3 expression and increased numbers of ‘exFOXP3\textsuperscript{+}’ cells; these exFOXP3\textsuperscript{+} T cells are potentially pathogenic as they can recognize islet antigens in a specific manner and can produce IFN\gamma (FIG. 5). Adoptive transfer of autoreactive exFOXP3\textsuperscript{+} T cells led to the rapid onset of diabetes\textsuperscript{111}. In patients, the frequency of T\textsubscript{reg} cells producing pro-inflammatory cytokines, such as IFN\gamma or IL-17, is elevated in T1D patients\textsuperscript{132}. A final point to consider is that, rather than being a primary defect that exists in T\textsubscript{reg} cell functions, it has been suggested that conventional effector T cells in patients with T1D are resistant to regulation mediated by T\textsubscript{reg} cells\textsuperscript{133}.

**Clinical testing based on targeting T\textsubscript{reg} cells.** Studies in NOD mice have shown that immunotherapies including CD3-specific, anti-thymocyte globulin and rapamycin may stabilize and expand T\textsubscript{reg} cell populations\textsuperscript{133-135}. More recent studies in conventional and humanized mouse identified a mechanism in which CD3-specific monoclonal antibody induces migration of T cells to the gut, where they acquire a regulatory phenotype and produce TGF\beta (in conventional mice) or IL-10 (in conventional and humanized mice)\textsuperscript{136,137}. Thus, several recent therapeutic opportunities have focused on altering the effector T cell and regulatory T cell balance in patients with T1D (FIG. 2).

Initial trials of two Fc receptor (FcR) non-binding CD3-specific monoclonal antibodies (namely, teplizumab and otelixizumab) showed that the decline in C peptide was reduced for up to 3 years after a single course of drug treatment in new-onset patients. Furthermore, these antibody therapies also showed efficacy in subgroups of patients with a longer-duration disease, importantly, without the need for continuous immunosuppression\textsuperscript{138-141}. Samples from the drug-treated subjects suggested that treatment with the CD3-specific antibodies induced CD8\textsuperscript{+} T cells with regulatory function\textsuperscript{140,141}. However, two Phase III studies with these drugs failed to meet their primary end points\textsuperscript{142}. In the study with otelixizumab, the drug administered was approximately one-tenth of the dose used in the previously successful Phase II trial. In the other trial with teplizumab, the primary end point (which was to achieve the number of subjects with haemoglobin A1c <6.5% using <0.5 U kg\textsuperscript{-1} d\textsuperscript{-1} of insulin) was not met, but an effect on preservation of C peptide secretion was still seen. Moreover, two recent trials have shown that teplizumab treatment can preserve C peptide levels in new onset patients and even in younger patients with a longer duration of disease\textsuperscript{145}.
Healthy individuals

Individuals with T1D

**Figure 3** | Regulatory T cells that have lost FOXP3 expression may contribute to autoimmune disease. In healthy individuals, developing thymocytes that do not express highly self-reactive T cell receptors (TCRs) mature and leave the thymus (left-hand panel). The forkhead box P3 (FOXP3) gene is methylated in these cells. By contrast, highly autoreactive T cells are deleted during development as a part of negative selection. Regulatory T (T_{reg}) cells also develop in the thymus and, compared with conventional mature T cells, express TCRs that show increased affinity for self antigens. Owing to their demethylation of the FOXP3 locus and expression of FOXP3 protein, T_{reg} cells have anti-inflammatory functions. However, in patients with type 1 diabetes (T1D) and other autoimmune diseases, T_{reg} cells may not maintain complete demethylation of FOXP3 owing to defects in interleukin-2 signalling or other mechanisms (right-hand panel). These ‘ex-T_{reg}’ cells remain autoreactive and, in the absence of FOXP3 expression, can produce potentially pathogenic pro-inflammatory cytokines. It has been suggested that such cells will participate in pathological immune responses to self antigens. In addition, failure to eliminate highly autoreactive T cells during thymic development may lead to the escape of potentially pathologic T cells into the periphery.


A pilot trial of IL-2 with rapamycin was carried out after preclinical studies and suggested that this combination therapy would increase the number of regulatory T cells and lead to disease reversal\(^{135,146}\). However, the treatment transiently worsened C peptide responses. This outcome has been attributed to the potentiating effects of IL-2 on pathogenic cells, such as NK cells. Further studies with lower doses of IL-2 without rapamycin are in progress.

**How can we improve treatments for T1D?**

T1D is a complex disease that is influenced by genetic and environmental factors and that involves innate and adaptive arms of the immune system. It is therefore likely that a multifaceted solution will be required for prevention, treatment and a durable cure. A key component of this process is to continue to develop a complete understanding of the disease process as well as to develop better biomarkers to identify as early as possible patients who will develop T1D in order to maximize the success of intervention (BOX 5). This will require better animal disease models as well as direct human experimentation.

There are some essential components that will need to be included in any therapy. First, it is likely that treatment will be given for short periods of time, or at best intermittently, to avoid long-term off-target effects on fundamental protective immune functions, which are likely to be seen with all except the safest therapeutics. Second, the primary rationale for modality selection should be approaches that are distinct but complementary and use data that support efficacy. Third, this should include therapies that engage or enhance regulatory mechanisms without the need for chronic immune suppression, which is often associated with long-term risks of infection and tumours. These tolerogenic therapies will be needed to reinstate robust central and peripheral tolerance. Examples might include targeting antigen-specific or other regulatory cells either through drugs or cell-based therapeutics. These efforts could build on current approaches, such as treatment with CD3-specific monoclonal antibodies, cytokines such as IL-2, drugs such as rapamycin and/or administration of ex vivo expanded T_{reg} cell populations. Future targeted therapies informed by the genetic pathways associated with T1D (such as the polymorphisms seen in IL-2 receptor-α (IL-2RA), which affect STAT5 signalling) or in phosphatases (such as PTPN22 and PTPN2) may prove to be effective for inducing immune tolerance.
In many cases, these pro-tolerogenic therapies may need to enhance regulatory mechanisms rather than simply to induce unresponsiveness in, or to delete, pathogenic T cells. Optimal combination therapies are those that can control multiple cells through mechanisms such as infectious tolerance and ensure the durability and stability of these regulatory populations. In addition, short-term treatment with drugs that silence pro-inflammatory responses and drugs that eliminate the effector and memory T cells, which are resistant to standard regulatory processes, could be used to stop the aggressive ongoing destruction and rapid deterioration of glucose tolerance that occurs in the first several months of the disease.

It is important to recognize that with all of these therapies, not all patients will respond, and therefore numerous strategies are needed. Data from clinical studies suggest that there are ‘responders’ and ‘non-responders’99. Therefore, in addition to biomarkers that can identify the biological efficacy of molecules in the short term, identification of the genetic, metabolic and immunological features that differentiate responders and non-responders may help to select therapies for subjects in order to improve efficacy and safety and to guide how combinations might be constructed.

The role of the β-cell in the progression of the disease is also an area that is not well understood. In response to immunological stress, β-cells secrete several pro-inflammatory factors, including cytokines such as IL-1β and chemokines such as IP10, which attracts pathological cells149,150. Dysfunction of β-cells probably has a role in the acute decline of insulin secretion seen at onset and the rapid recovery after metabolic stabilization151,152. Reversal of the recovery of metabolic stabilization most probably explains the ‘honeymoon’ that is seen soon after T1D diagnosis and metabolic stabilization. The rate of β-cell death is greater in patients with a new-onset disease, but analysis of immune therapies

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might consider the impact on cell death as well as function to identify those therapies that are most likely to have a lasting impact on the disease\(^\text{19}\). Moreover, β-cell dedifferentiation has been proposed as a mechanism that may explain β-cell function under conditions of metabolic or other stress\(^\text{14}\).

Finally, it is important to note that for hundreds of thousands of patients without any insulin-producing β-cells, it is essential that any immune-based therapy will also incorporate a β-cell replacement strategy. This may even be an issue in new-onset disease as there is increasing evidence that the ongoing assault of the islets by the immune response may initiate necrotic and apoptotic death pathways that may be irreversible following expression of pro-apoptotic genes\(^\text{15}\). Thus, efforts in the embryonic stem cell and induced pluripotent stem cell fields will be essential and complementary to ensure that after the immune problem has been ‘solved’, there will be an effective and ample supply of β-cells to replace the damaged tissue\(^\text{16}\).
Register shifting of an insulin peptide–MHC complex to T cell receptor transgenic mice for the transfer of diabetes in nonobese diabetic mice. \( R620W \) variant interferes with the removal of nonobese diabetic (NOD) mouse. to escape negative selection in the thymus but not in the periphery. 103–108 (2003).


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Competing interests statement

The authors declare competing financial interests: see Web version for details.