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Type 1 diabetes: translating mechanistic observations into effective clinical outcomes

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Abstract | Type 1 diabetes (T1D) remains an important health problem, particularly in western countries, where the incidence has been increasing in younger children. In 1986, Eisenbarth described T1D as a chronic autoimmune disease. Work over the past three-and-a-half decades has identified many of the genetic, immunological and environmental factors that are involved in the disease and have led to hypotheses concerning its pathogenesis. Clinical trials have been conducted to test these hypotheses but have had mixed results. Here, we discuss the findings that have 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.

Anti-thymocyte globulin
Polyclonal antibodies against human T cells that are produced by immunizing rabbits or horses.

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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[2] 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[3]. 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[4,5]. 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[6] (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)[7,8]. 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 clinical studies in patients with T1D and the results of testing those hypotheses in clinical trials. Similarly to the clinical observations that led to the original hypothesis that T1D is an autoimmune disease, translational research efforts in humans and animal models continue to be a source of new discoveries that shape the T1D field.

Box 1 | **Clinical aspects of type 1 diabetes**

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[157]. 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 disease do not have a relative with T1D[59]. 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 ICA512 and PTPRN), insulin, IGRP (also known as G6PC2), zinc transporter 8 (ZNT8) and islet cell autoantigen (ICA))[77,158,159]. 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[7,160]. Owing to the absolute deficiency in insulin production, replacement with exogenous insulin and dietary regulation are the mainstays of treatment. Retention of some endogenous insulin production, which is reflected by the level of C peptide (the by-product of processing of proinsulin that is synthesized by β-cells) of at least 0.2 nmol L^{-1}, has been associated with improved metabolic control and reduced risk of long-term complications, such as eye and renal disease, as well as the acute complication of insulin-induced hypoglycaemia[7,8]. Metabolic control is monitored by measurement of glycosylated haemoglobin A1c levels, which indicate blood glucose levels and reflect the glucose control over the previous 2–3 months.

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[9,10]. 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 parasites, viruses and bacteria, could 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[11]. 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)[12–15].

It has been proposed for decades that enteroviruses are linked to T1D[11,16]. 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[17]. 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 insulitis[18]. More 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)[19,20]. Moreover, phagocytosis of enterovirus-infected β-cells triggers innate immune responses in human dendritic cells (DCs)[21].

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[22,23] (FIG. 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 (T_{Reg} 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[24,25]. Other commensal organisms have been shown to influence invariant natural killer T cell (iNKT cell) activity[26]. As T_{Reg} 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 T helper 17 cells (T_H17 cells) but can inhibit the onset of autoimmune diabetes in non-obese diabetic (NOD) mice[27,28].

Studies of myeloid differentiation primary response 88 (*MYD88*)$^{-/-}$ NOD mice underscored the importance of the gut microbiota and its interactions with the host innate immune system in modulating diabetes onset[29]. 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

Cyclosporin A
An immunosuppressive drug that inhibits calcineurin, a Ca^{2+}-dependent serine/threonine phosphatase that is necessary for the nuclear translocation of the transcription factor nuclear factor of activated T cells (NFAT).

C peptide
The connecting peptide that joins the A chain and B chain of insulin in the proinsulin molecule.

Hygiene hypothesis
The theory that the lack of early childhood exposure to infectious agents, symbiotic microorganisms (for example, changes in gut microflora) and parasites increases susceptibility to allergic and autoimmune diseases by modulating immune system development.

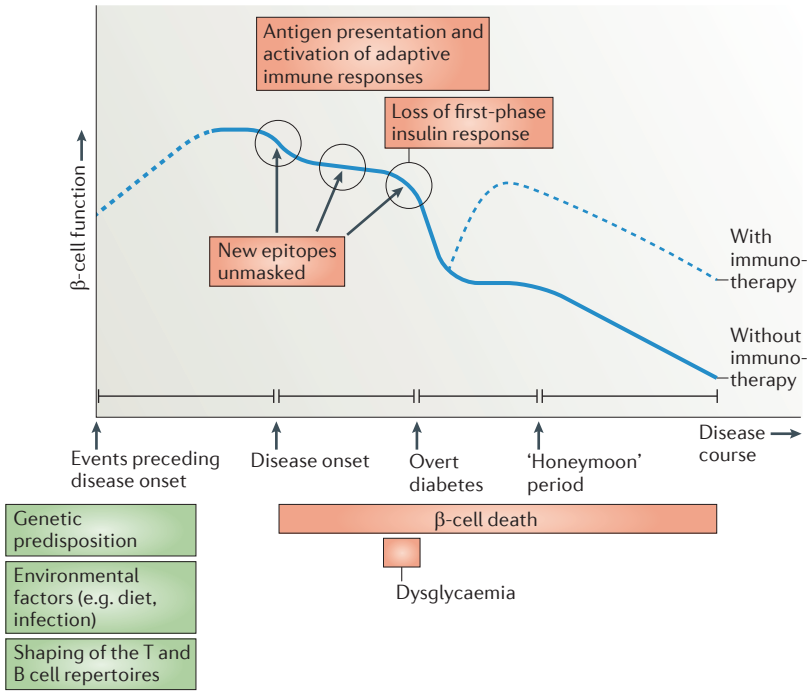

Figure 1 | **Revision of the Eisenbarth model of type 1 diabetes.** Our earlier concepts of the pathogenesis of type 1 diabetes (T1D) have been modified owing to the emergence of new information. During childhood, there is an increase in β-cell function (dotted line); as a result of this, patients who present with T1D at an older age have higher C peptide responses compared with younger patients[160,176]. 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 appreciated[177]. 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.

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.

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 diseases[25]. Second, germ-free mice have incomplete immune systems, and colonization with microflora is needed for the development of T_H17 cell responses and T_{Reg} cell responses in the gut[30]. 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 pancreas[31].

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 development[32–34]. 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 diabetes[35,36].

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 disease[2]. 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 functions[37]. Antibody-mediated depletion of B cells, even at the time of onset of hyperglycaemia, can prevent or reverse disease in NOD mice[38,39].

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-cells[40]. 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 spreading[43,44]. Although many autoantigens have been implicated as targets and drivers of T1D, there is limited direct evidence for the involvement of

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[161]. 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[162,163]. 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-7)), 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[164]. 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 immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome, which is a severe multi-organ autoimmune syndrome caused by uncontrolled immune activation.

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[41,42]. 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[45]. Furthermore, cytotoxic T cells isolated from a patient with T1D were shown to kill β-cells through recognition of a glucose-related preproinsulin epitope[46].

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[42,47,48]. A significant percentage of patients with autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) develops autoimmune diabetes[49,50]. 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[48,51,52]. In an analogous manner in humans, the susceptibility alleles of the insulin-dependent diabetes mellitus 2 (*IDDM2*) gene locus, a polymorphism of the *INS* promoter, cause lower thymic *INS* 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[53–55]. Moreover, Kappler and colleagues[55–57] have shown that insulin peptides can bind to MHC molecules, such as I-A^{g7}, 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[55–57]. In the periphery, the unique trimolecular 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 T1D, although this has yet to be established[41–45].

Defects in both central and peripheral B cell tolerance mechanisms have also been identified in patients with T1D[58]. The frequency of polyclonal and HEp-2-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 T1D (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[59]. Thus, these defects in central and peripheral T and B cell tolerance can establish an autoreactive lymphocyte repertoire that may drive T1D 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[60,61]. Two 'checkpoints' during the pathogenesis of the disease have been described[62]. 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

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.

Box 3 | **Genetics of type 1 diabetes**

There is a strong genetic basis for type 1 diabetes (T1D). Overall, the risk of disease for siblings of patients with T1D is ~6%, which is 15-fold higher than in the general population[165,166]. The risk for identical twins has been reported to be as low as 30%, but more recent data have suggested that with a longer observation period (to age 60), 65% are concordant[167]. The most important susceptibility alleles are within the MHC: the link to the MHC had originally suggested the autoimmune basis for T1D. The MHC has an odds ratio for disease of approximately 6.8 (REFS 59,168). The *HLA-DRB1*04–HLA-DQA1*0301–HLA-DQB1*0302* and *HLA-DRB1*03–HLA-DQA1*0501–HLA-DQB1*0201* haplotypes are the strongest T1D risk factors in European populations: heterozygosity for both risk haplotypes confers the greatest known genetic risk. Other alleles have been associated with T1D in non-European populations (for example, *HLA-DRB1*0405–HLA-DQB1*0401* and *HLA-DRB1*0901–HLA-DQB1*0303* in Japanese and Korean populations). In addition, strongly protective alleles (such as *HLA-DQB1*0602*) have a dominant effect[169]. Recently, the application of genome-wide single-nucleotide polymorphism (SNP) typing technology to large sample sets and comparisons with results from other immune-mediated diseases have provided convincing support for 19 non-MHC T1D loci, all with an allelic odds ratios of less than 1.3 (REF. 165). These include interleukin-2RA (*IL-2RA*), which has an odds ratio of ~1.6, protein tyrosine phosphatase, non-receptor type 22 (*PTPN22*), which has an odds ratio of 2.0, and cytotoxic T lymphocyte protein 4 (*CTLA4*), which has an odds ratio of ~1.25. Some of the immune-response-related loci are shared with other autoimmune diseases, whereas other susceptibility alleles appear to be disease-specific. For example, insulin-dependent diabetes mellitus 2 (*IDDM2*), which has an odds ratio of 2.1, is in the insulin promoter and thus may affect insulin expression in the thymus and negative selection.

T helper 17 cells
(T_H17 cells). A subset of $CD4^+$ T cells that is characterized by its expression of the transcription factors RORγ, RORα 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 TGFβ, interleukin 21 (IL-21), IL-23 and IL-1β, and they secrete IL-17, IL-22, IL-22 and in some circumstances granulocyte-macrophage colony-stimulating factor (GM-CSF) and/or interferon-γ (IFNγ).

Specific-pathogen-free conditions
(SPF 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 double-stranded DNA or chromatin, whereas a speckled nuclear-staining pattern is indicative of antibodies that react with small nuclear ribonucleoproteins.

new effector functions by T cells, a lack of negative signalling, the enhanced production of pro-inflammatory mediators, the exposure of previously inaccessible β-cell antigens that fuel the disease process, or abrogation of regulatory control caused by a defect in the number or function of T_{Reg} cells (see below for a more detailed discussion of this).

Both $CD4^+$ and $CD8^+$ T cell clones that are specific for islet antigens can transfer disease to naive recipients, and their destructive mechanisms involve the generation of cytokines, such as TNF and interferon-γ (IFNγ), in addition to their direct cytotoxic effects[63,64]. Although some have suggested that the $CD4^+$ T cells that infiltrate the islets of NOD mice possess a heterogeneous phenotype, in terms of TCR usage and antigen specificity[65,66], sequencing studies of early infiltrates suggested that TCR usage by islet-infiltrating T cells is fairly limited[67]. In humans, restricted and preferential TCR usage has been observed in intra-islet T cells from patients with T1D[68]. Some of this restricted usage may be due to the recognition of insulin peptides by germline-encoded TCR elements[69]. A crucial future goal will be to understand better the TCR usage and antigen specificity of the islet-infiltrating T cell repertoire. T cell retrogenic technology is a platform through which this can be accomplished in a reasonable time frame[70–73]. Studies using this technology have shown that islet antigen expression is a key factor in determining the ability of a given T cell population to accumulate in the islets. Cell-extrinsic mechanisms do not result in the accumulation of bystander cells, indicating that islet entry and accumulation is a cell-autonomous event[65]. The Network for Pancreatic Organ Donors with Diabetes (nPOD) and similar resources will probably prove to be invaluable for this work[74]. Such studies have shown multifocal infiltration in addition to widespread expression of MHC class I molecules on pancreatic islets. Using tetramer technology, it was furthermore possible to identify the islet antigen specificity of autoreactive $CD8^+$ T cells in insulitic lesions from recent-onset and long-term T1D patients[75].

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[76–78]. Interestingly, the number of different antigens that were targeted by autoantibodies (including GAD65, IA2, IGRP, insulin and ZNT8) and islet cell autoantibodes (ICAs) rather than the overall titre of autoantibodies was the most important determinant of risk[77]. 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 infectious tolerance[79]. 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[80,81]. 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[82]. 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[83]. 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[84]. 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

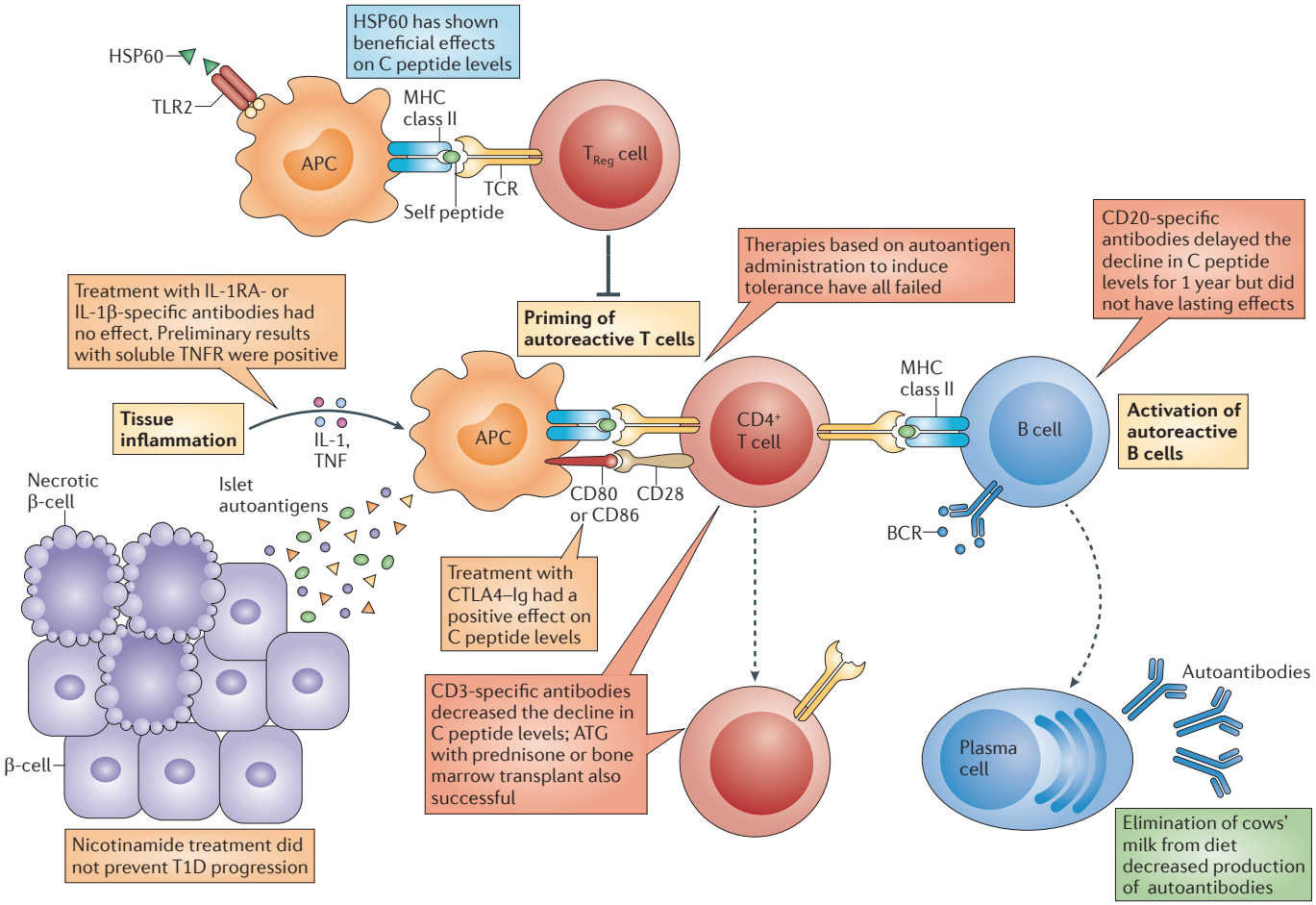

Figure 2 | **Results of immunotherapy trials in type 1 diabetes.** The figure illustrates the aspects of the immune response that have been targeted in type 1 diabetes (T1D; yellow text boxes). To date, therapies have been designed to target innate and adaptive immune responses that are thought to contribute 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[88,93,138–141]. Anti-thymocyte globulin (ATG) with glucocorticoids or autologous bone marrow transplant has also been successful[4,178,179]. However, therapies that have involved administration of autoantigens (for example, glutamate decarboxylase 2 (GAD2)), targeting of T cells with immunosuppresive 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[87,88,93,138,140–143]. A trial of rapamycin and IL-2 had a negative effect on patients who showed a transient decline in C peptide responses[146]. A trial using treatment with heat-shock protein 60 (HSP60), which is thought to enhance regulatory T (T_{Reg}) cell functions by signalling via Toll-like receptor 2 (TLR2), has shown a beneficial effect on C peptide responses[107]. A pilot trial of a soluble tumour necrosis factor (TNF) receptor (etanercept) resulted in lower blood glucose levels and increased insulin production in patients[180]. 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.

these findings, even though the immunization increased the titres of GAD65 autoantibodies and the frequency of T cells that produce inhibitory cytokines[84–86]. 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[87] (S. E. Gitelman, L. Fisher, P. Gottlieb, M. Gottschalk, W. Moore, A. Moran, M. Rigby, S. Willi, L. Keyes-Elstein, L. Ding and M. Ehlers, 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[88]. 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

reduced[89,90]. Despite its efficacy at 1 year, the beneficial effects of rituximab treatment were no longer detectable at year 2. Collectively, these studies suggest that induction of immune regulation, rather than cell depletion, may be a more effective strategy for inhibiting T1D disease in the long term (see below).

In addition to modulating specific autoreactive T cells directly, blocking their activation has been more successful. In mice, blockade of CD28–B7 co-stimulation signals with human cytotoxic T lymphocyte protein 4–immunoglobulin (CTLA4–Ig) fusion protein (also known as abtacept) at 2–4 weeks, but not later, prevented diabetes primarily owing to preventing CD86 (also known as B7.2)–CD28 signalling[91]. However, CTLA4–Ig transgenic NOD mice, or NOD mice treated with CD80 (also known as B7.1)-specific blocking antibody showed exacerbated disease[92]. In humans, treatment with CTLA4–Ig delayed disease progression (by 9.6 months) in a randomized placebo-controlled study in subjects with new-onset T1D who received 27 infusions of the drug over a 2-year period[93]. This finding suggests either that the timing of priming of diabetogenic cells is later than predicted in the NOD model or that CD80 and/or CD86-dependent mechanisms may be involved in the function of pathogenic T cells very late in the disease course. It should also be noted that in spite of continued treatment with CTLA4–Ig, the C peptide levels in the test group declined in parallel with the placebo-treated group after the first 6 months, possibly reflecting the action of costimulation-independent cells in this later stage or β-cell loss that is independent of CD80 and/or CD86-dependent immune mechanisms (FIG. 2).

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[94]. 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 $CD107^+$ NK cells in the diabetic mouse pancreas, has led to the suggestion that NK cells may play a part in β-cell death[95]. 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[96].

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[97,98]. Treatment with IL-1-specific antibody or genetic deficiency in IL-1 receptor expression delays, but does not prevent, T1D in NOD mice[99,100].

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[75,101]. 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[102]. 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[103]. 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[104]. 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[105]. A pilot trial of anakinra in patients with T1D showed biological efficacy[106]. 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

CD28–B7 co-stimulation
A crucial receptor–ligand interaction that is required for maximal T cell activation and survival.

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 $CD4^+CD25^+$ T_{Reg} 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 trial[108] (I. Raz, A. G. Ziegler, T. Linn, F. Bonnici, G. Schernthaner, L. A. Distiller, C. Giordano, F. Giorgino, L. de Vries, D. Mauricio, V. Procházka, 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 T_{Reg} 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 T_{Reg} cells. This rare T cell population, which is generally identified as $CD4^+CD25^+CD127^{lo}$ T 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 T_{Reg} cells, and defects in T_{Reg} cell populations may contribute to disease pathogenesis in type 1 diabetes (BOX 2).

T_{Reg} cells develop in the thymus with a unique TCR repertoire that is skewed towards the recognition of self antigens[111]. FOXP3 expression remains crucial throughout life to maintain the T_{Reg} cell populations and to prevent autoimmunity. In addition to its expression by T_{Reg} 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 promoter[112]. This results in the development of a peripheral T_{Reg} cell compartment

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) blockade[83,99,100,170,171,172]. 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–immunoglobin fusion protein (CTLA4–Ig)) did improve C peptide responses in patients with new-onset disease[91]. 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 design[173]. 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[174]. The kinetics of diabetes 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 onset[7]. 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 therapies[175]. 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[111]. In NOD mice, depletion of $CD4^+CD25^+$ T_{Reg} cells greatly accelerates the development of diabetes[113,114]. Similarly, removing crucial co-stimulatory or proliferative signals that are necessary for T_{Reg} cell development or survival, such as IL-2 or CD28, exacerbates diabetes in NOD mice[114]. Thus, it is clear that T_{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 $FOXP3^-$ TGFβ-dependent T_H3 cells, IL-10-dependent T regulatory 1 (Tr1) cells and $CD8^+$ regulatory cells[115–120]. T_{Reg} cells can be subdivided into multiple subsets and tissue-specific subpopulations[110]. In addition, it has been postulated that the T_{Reg} cell transcriptional programmes, and conceivably their suppression mechanisms, can be tailored to the nature of the effector response that they regulate[121]. Thus, the collective magnitude of suppressive activities may reflect the functions of individual T_{Reg} cell subsets in different tissues with distinct dynamics and unique immunological effects.

The basis of T_{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, TGFβ 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_{Reg}-cell-mediated suppression[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_{Reg} cell function (such as IL-10, IL-35, indoleamine 2,3-dioxygenase 1 (IDO) and TGFβ) can promote the development of other regulatory cells in their vicinity, leading to so-called infectious tolerance and a robust local regulation[117,124,126,127].

Several genetic loci that are important for T_{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_{Reg} cell growth and survival; CTLA4, which is a major functional receptor on T_{Reg} cells; and the HLA locus, which can alter T_{Reg} cell repertoires[59,128,129] (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_{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_{Reg} cell numbers in some patients with the disease[130]. Reduced IL-2 receptor signalling has been linked to the IL-2RA susceptibility allele (rs12722495)[128]. In fact, treatment of diabetic mice with IL-2 reverses diabetes[131].

T_{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 '$exFOXP3^+$' cells; these $exFOXP3^+$ T cells are potentially pathogenic as they can recognize islet antigens in a specific manner and can produce IFNγ (FIG. 3). Adoptive transfer of autoreactive $exFOXP3^+$ T cells led to the rapid onset of diabetes[111]. In patients, the frequency of T_{Reg} cells producing pro-inflammatory cytokines, such as IFNγ or IL-17, is elevated in T1D patients[132]. A final point to consider is that, rather than being a primary defect that exists in T_{Reg} cell functions, it has been suggested that conventional effector T cells in patients with T1D are resistant to regulation mediated by T_{Reg} cell[130].

Clinical testing based on targeting T_{Reg} cells. Studies in NOD mice have shown that immunotherapies including CD3-specific, anti-thymocyte globulin and rapamycin may stabilize and expand T_{Reg} cell populations[133–135]. More recent studies in conventional and humanized mice 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 TGFβ (in conventional mice) or IL-10 (in conventional and humanized mice)[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 immune suppression[138–141]. Samples from the drug-treated subjects suggested that treatment with the CD3-specific antibodies induced $CD8^+$ T cells with regulatory function[142,143]. However, two Phase III studies with these drugs failed to meet their primary end points[144]. 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 $<0.5\ U\ kg^{-1}\ d^{-1}$ 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[145]

Rapamycin
An immunosuppressive drug that, in contrast to calcineurin inhibitors (such as cyclosporin A and FK506), does not prevent T cell activation but blocks interleukin-2-mediated clonal expansion by blocking mammalian target of rapamycin (mTOR). It does not interfere with the function and expansion of naturally occurring regulatory T cells.

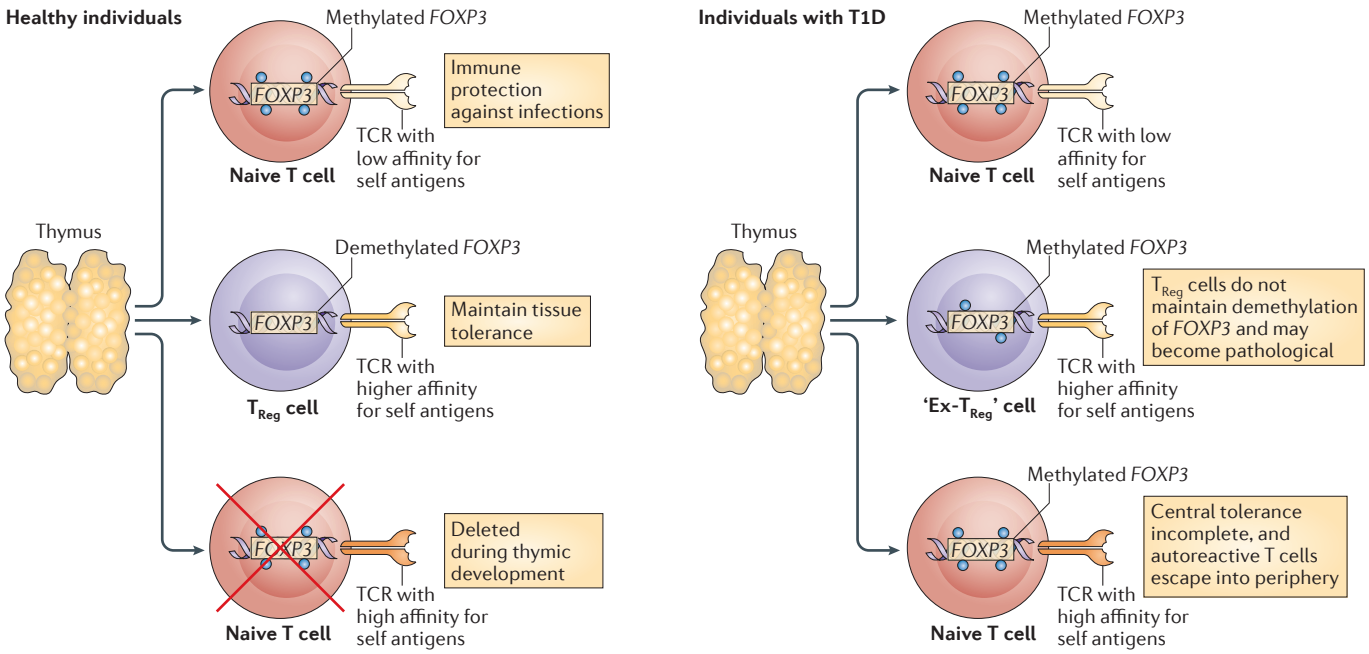

Figure 3 | **Regulatory T cells that have lost FOXP3 expression may contribute to autoimmune disease.** In healthy individuals, developing thymocytes that do not express highly self-reactive T cell receptors (TCRs) mature and leave the thymus (left-hand panel). The forkhead box P3 (*FOXP3*) gene is methylated 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.

(K.C.H., S. E. Gitelman, M. R. Ehlers, P. A. Gottlieb, C. J. Greenbaum, W. Hagopian, K. D. Boyle, L. Keyes-Elstein, S. Aggarwal, D. Phippard, P. H. Sayre, J. McNamara and J.A.B., unpublished observations)

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[133,146]. 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.

Box 5 | **Unresolved areas of translational investigation**

Although there has been much learned about the pathogenesis of type 1 diabetes (T1D) as a result of preclinical and clinical studies, several key questions have arisen and remain unanswered. Some of these are addressed here.

What are the initiating factors?

- Are viruses involved? Are these unique or common?
- Are any of these factors intrinsic to β-cells in T1D patients?
- Which antigens are presented, and does this change over time or in different patients?
- How does the microbiome affect the induction or progression of autoimmunity?
- How are innate responses involved?
- What is the role of epigenetic changes in the penetrance of disease?

How does the immune repertoire differ in patients who will develop T1D?

- What is the antigen specificity of pathogenic T cells, and how can these cells be identified?
- How much of the disease heterogeneity stems from stochastic variation in immune development versus exposure to natural pathogens versus normal responses to one's environment?
- Why does it take so long to destroy all of the β-cells?
- Are there unusual features of autoreactive T cell development pathways?
- How do immune response and other genes affect disease in general or the diabetogenic potential of T cells specifically?
- What is the role of cell-intrinsic regulatory mechanisms?
- What are the roles of thymus-derived and peripherally induced regulatory T cells?

What are the mechanisms of β-cell death?

- Which cells are involved?
- Do human β-cells regenerate, differentiate or divide, and does this differ in very young children versus adults?
- Can β-cell regeneration, transdifferentiation or division be induced?
- Why is β-cell death segmental and in a lobular distribution?

How can treatment be improved?

- What are the appropriate and realistic parameters for determining success in a clinical trial with a given modality (for example, should restoration of β-cell function be an expected outcome with a therapy that targets the autoimmune response if β-cell regeneration, transdifferentiation or division does not occur)?
- What are the mechanisms of long-term failure?
- Does the long-term failure of therapies reflect recurrence of the autoimmune response or failure of β-cells independently of immune attack?
- How does metabolic control affect responses to immune therapies?
- How can responders and non-responders be identified?
- When should interventions be initiated?
- Can any of the interventions prevent T1D?
- What combinations are optimal, and how can the regulatory path for the development of these combinations be optimized?

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[147] 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[147,148].

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'[89]. 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 cells[149,150]. Dysfunction of β-cells probably has a role in the acute decline of insulin secretion seen at onset and the rapid recovery after metabolic stabilization[151,152]. 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

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[153]. Moreover, β-cell dedifferentiation has been proposed as a mechanism that may explain loss of β-cell function under conditions of metabolic or other stress[154].

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[155]. 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[156].

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

FURTHER INFORMATION
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