Type 1 Diabetes: Etiology, Immunology, and Therapeutic Strategies

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Van Belle TL, Coppieters KT, von Herrath MG. Type 1 Diabetes: Etiology, Immunology, and Therapeutic Strategies. *Physiol Rev* 91: 79–118, 2011 doi:10.1152/physrev.00003.2010.—Type 1 diabetes (T1D) is a chronic autoimmune disease in which destruction or damaging of the beta-cells in the islets of Langerhans results in insulin deficiency and hyperglycemia. We only know for sure that autoimmunity is the predominant effector mechanism of T1D, but may not be its primary cause. T1D precipitates in genetically susceptible individuals, very likely as a result of an environmental trigger. Current genetic data point towards the following genes as susceptibility genes: HLA,

insulin, PTPN22, IL2Ra, and CTLA4. Epidemiological and other studies suggest a triggering role for enteroviruses, while other microorganisms might provide protection. Efficacious prevention of T1D will require detection of the earliest events in the process. So far, autoantibodies are most widely used as serum biomarker, but T-cell readouts and metabolome studies might strengthen and bring forward diagnosis. Current preventive clinical trials mostly focus on environmental triggers. Therapeutic trials test the efficacy of antigen-specific and antigen-nonspecific immune interventions, but also include restoration of the affected beta-cell mass by islet transplantation, neogenesis and regeneration, and combinations thereof. In this comprehensive review, we explain the genetic, environmental, and immunological data underlying the prevention and intervention strategies to constrain T1D.

I. INTRODUCTION

Type 1 and type 2 diabetes mellitus (T1D, T2D) have in common high blood glucose levels (hyperglycemia) that can cause serious health complications including ketoacidosis, kidney failure, heart disease, stroke, and blindness. Patients are often diagnosed with diabetes when they see a physician for clinical signs such as excessive thirst, urination, and hunger. These symptoms result from the underlying hyperglycemia that is in turn caused by insufficient insulin functionality. In T2D, which is usually associated with obesity or older age, this is mostly the result of insulin resistance: the muscle or adipose cells do not respond adequately to normal levels of insulin produced by intact beta-cells. T1D on the other hand usually starts in people younger than 30 and is therefore also termed juvenile-onset diabetes, even though it can occur at any age. T1D is a chronic autoimmune disorder that precipitates in genetically susceptible individuals by environmental factors (24). The body's own immune system attacks the beta-cells in the islets of Langerhans of the pancreas, destroying or damaging them sufficiently to reduce and eventually eliminate insulin production. On rare but increasing occasions, both T1D and T2D are diagnosed in patients.

According to the American Center for Disease Control, 23.6 million people, 7.8% of the population, have T1D or T2D, and 1.6 million new cases of diabetes were diagnosed in people aged 20 years or older in 2007. The prevalence of T1D for residents of the United States aged 0–19 years is 1.7/1,000. T1D incidence has been globally rising during the past decades by as much as a 5.3% annually in the United States. If present trends continue, doubling of new cases of T1D in European children younger than 5 years is predicted between 2005 and 2020, and prevalence of cases in individuals younger than 15 years will rise by 70% (328), characteristic of a left shift towards an earlier age (123). This suggests that whatever event triggers the onset is increasingly affecting susceptible individuals (115, 165). The search for such triggering factors has been ongoing for many years and has so far only yielded indirect evidence, predominantly implicating certain viral infections. It is now well established that a specific genetic constitution is required for such an event to cause diabetes. However, concordance rates between monozygotic twins amount to only 50%, whereas between dizygotic twins only ~10% (236). With longer follow-up, the majority of discordant identical twins of patients with T1D eventually express anti-islet autoantibodies and progress to diabetes, but anti-islet autoantibodies in the second twin may appear only 30 years after the first twin develops diabetes (354, 355). Thus it seems that genetic susceptibility persists for life, and progression to diabetes is usually preceded by a long prodrome of anti-islet autoantibody expression measured in years. Nevertheless, although the concordance rate for monozygotic twins is higher than previously thought, it is below unity, and there are strong divergences in terms of the time it takes to develop T1D. This implies a strong environmental component to contribute to the development of T1D.

Since the early 1920s, diabetes has been treated by insulin replacement, which, in the ideal case, will only shorten life expectancy by ~10 years. This sets a high safety bar for any immune-based intervention. Even more so, recent technology (continuous blood glucose monitors, slow release insulin, etc.) can reduce the chance for life-threatening hypoglycemic episodes from insulin overdoses. Therefore, immune-based interventions should ideally be effective, long-lasting, and have minimal side effects to replace substitutive insulin treatment with a cure. Today, despite the many remaining challenges in the field of T1D immunotherapy, good progress has been made.

With this review, we provide a comprehensive overview of the etiology and immunology of T1D and will discuss preventive or therapeutic biological strategies that have been tried or are currently undertaken.

II. THE GENETICS OF TYPE 1 DIABETES

A comprehensive overview of genetic data in mouse and human is beyond the scope of this article. Instead, we will focus on how the various susceptibility genes and environmental triggers can fit in a mechanistic model for T1D etiology.

A. Rare Monogenic Forms

Autoimmune diabetes is only rarely caused by mutational defects in a single gene. These monogenic forms are typically accompanied by multiple other autoimmune conditions due to the disruption of common regulatory

pathways. One such example is found in the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), in which mutations in the Foxp3 transcription factor lead to the dysfunction of regulatory T cells (Tregs) and wasting multiorgan autoimmunity (26, 77, 474). Approximately 80% of affected children develop autoimmune diabetes and generally succumb early due to overwhelming autoimmunity. Another example is autoimmune polyendocrine syndrome type 1 (APS-1, or APECED for autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy). Mutations in the transcription factor AIRE (autoimmune regulator) lead to severe autoimmune conditions, and ~20% of the cases develop T1D (455). Deficiencies in AIRE inhibit the expression of peripheral molecules, for example, insulin, in the thymus. This reduced expression allows autoreactive T cells to escape into the periphery, because it interferes with thymic deletion (16, 246). These rare monogenic forms represent a small minority of T1D cases, but highlight two main features related to its etiology. First, the observation that several well-characterized autoimmune conditions develop in parallel highlights the common tolerance mechanisms that prevent these diseases in healthy individuals. Second, although genetics and environment likely interact in a continuous spectrum in most autoimmune disease patients, the monogenic cause of IPEX and APS-1 illustrates that genetic constitution can dominate in certain extreme cases.

B. HLA Genes

Early studies indicated that the HLA region on chromosome 6p21 (commonly termed IDDM1, for insulin-dependent diabetes mellitus locus) is a critical susceptibility locus for many human autoimmune diseases, including T1D (305, 399). These initial findings revolutionized our understanding of T1D etiology in two ways, as stated by Nerup et al. (305) in conclusion of their 1974 report: *1*) T1D is a distinct disease entity, corroborating histopathological evidence; and *2*) an aberrant cellular immune response, potentially triggered by viral infection, instigates onset. Numerous new susceptibility loci have emerged since, but none of them matches the strong association found with the HLA region. It is unlikely that new loci will ever be discovered that confer such a dramatic risk to T1D development (96). In genetic studies, the odds ratio is the statistic used to calculate whether a single nucleotide polymorphism (SNP) given is associated with the disease. An odds ratio of one implies that the event is equally likely in both patient and control groups. Odds ratios of alleles predisposing to complex disorders are typically modest, often in the range of 1.2–1.3, and even the HLA region has a predicted value of only 6.8. This suggests that if genetic predisposition is indeed a

dominant factor in T1D development, a vast amount of common SNPs are still waiting to be discovered (96, 159). After several decades of continuous progress since the discovery of HLA association (for historical perspective, see Ref. 285), the class II genes remain the strongest genetic contributor (138, 323, 429, 433, 439). Several HLA class II genes are pivotal as their alleles were found to determine a susceptibility hierarchy ranging from protection to strongly at-risk (15, 73, 105, 134, 135, 237, 309, 393). The DRB1*1501-DQA1*0102-DQB1*0602 haplotype, found in ~20% of the population but only 1% of patients, confers dominant protection against T1D (134). At the susceptible end of this spectrum are individuals with the DR3/4-DQ8 heterozygous haplotype (DR3 is DRB1*03-DQB1*0201, DR4 is DRB1*04-DQB1*0302, DQ8 is DQA1*0301, DQB1*0302). It is important to note that only 30–50% of patients with T1D have the DR3/4-DQ2/8 genotype. A study in the Denver, Colorado area (15) identified this high-risk haplotype in 2.4% of newborns and more than 20% of the children affected by T1D, and its presence marks a 55% risk of developing overt diabetes by age 12. DR3/4-DQ2/8 siblings who are HLA identical to a diabetic proband have a risk as high as 80% for persistent anti-islet autoantibodies and 60% for progression to diabetes by age 15 (15).

An equally consistent, albeit substantially less prominent, association has been found for class I alleles (140, 192, 245, 302, 303, 308, 445, 446). A recent study by Nejentsev et al. (303) demonstrates that, after taking into account the dominating influence of class II genes, most of the residual association in the HLA region can be attributed to HLA-B and HLA-A genes (303). Most notably the presence of the HLA-B*39 allele was found to be a significant risk factor and is associated with a lower age at diagnosis of T1D. Additionally, HLA-A*02 increases the risk in individuals possessing the high-risk class II DR3/4-DQ8 haplotype (140, 360). HLA-A*0201 is one of the most prevalent class I alleles, with a frequency of >60% in T1D patients. There is accumulating evidence for the presence and functionality of HLA-A*02-restricted CD8 T cells reacting against beta-cell antigens such as insulin, glutamate decarboxylase (GAD), and IAPP in T1D patients and islet transplant recipients (325, 326, 337). Transgenic NOD mice have been generated expressing human HLA-A*02 molecules (271), and their accelerated diabetes onset provides functional evidence for the involvement of this particular class I allele.

C. The Insulin Gene

A lesser genetic predisposition to T1D is conferred by the IDDM2 locus on chromosome 11 containing the insulin gene region. A polymorphic region located 5′ of the insulin gene was first reported in 1984 to be associated with T1D in caucasoids (39). Now established as a pri-

mary autoantigen in T1D, it is not at all surprising that mutations in the insulin region could contribute to disease susceptibility. Detailed mapping showed that susceptibility resides in a variable number of tandem repeat (VNTR) polymorphisms in the promoter region of the insulin gene (42, 214, 225, 253). The magnitude of risk correlates with the number of these tandem repeats. VNTR type I (with shorter repeats) homozygous individuals in the highest risk category and VNTR type III (longer repeats) protects carriers against T1D. The predominant hypothesis is that these VNTR regulate the insulin expression levels in the thymus by affecting AIRE binding to its promoter region (16, 342, 442). The importance of insulin expression by Aire-expressing medullary thymic epithelial cells (mTECs) was recently underscored by the observation that mTEC-specific insulin deletion leads to diabetes in animals (137). As a consequence, VNTR type I will induce lower transcription of insulin and its precursors in the thymus, leading to reduced tolerance and T1D development. Conversely, insulin-reactive T cells are more efficiently eliminated by negative selection in the thymus from individuals with the protective VNTR type III allelic variant.

D. PTPN22

A relatively new member of the T1D susceptibility gene set is PTPN22, which encodes the lymphoid protein tyrosine phosphatase (LYP) (57, 402). The same allelic variant mediates risk in several other autoimmune diseases, suggesting the involvement of a crucial signaling axis (58). Indeed, the LYP protein is an important negative regulator of T-cell receptor signaling by way of dephosphorylation of Src family kinases Lck and Fyn, ITAMs of the TCRζ/CD3 complex, as well as ZAP-70, Vav, valosin-containing protein, and other key signaling molecules (476). Explanations for the mechanism are contradicting. A loss-of-function mutation can cause a lower threshold for autoreactive T-cell activation in the periphery. In contrast, a gain-of-function mutation that suppresses TCR signaling during thymic development can allow autoreactive T cells to escape negative selection (451).

E. IL2RA

Allelic variation in the interleukin (IL)-2 receptor-α gene (IL2RA) region accounts for another genetic risk factor implicated in T1D (252, 345, 453). The alpha chain of the IL-2 receptor complex (IL2Rα, CD25) is an essential molecule expressed on T cells upon activation and on natural Tregs at baseline. Tregs depend on IL-2 for their growth and survival. The presence of the IL2Rα subunit greatly enhances the affinity of the IL-2 receptor (266). In multiple sclerosis (MS) (263) and other autoimmune conditions (5, 157, 166), increased levels of soluble IL2Rα

(sIL2Rα) are found in circulation. Given the indispensible role of IL-2 in Treg function and the potential for sIL2Rα to neutralize IL-2, one could argue that IL2RA allelic risk variants impair Treg functionality by upregulation of sIL2Rα. However, it was recently found that IL2RA susceptibility genotypes in T1D are associated with lower levels of sIL2Rα (252, 262). Furthermore, in vitro stimulated peripheral blood mononuclear cells (PBMCs) from individuals with T1D make less sIL2Rα than those from control individuals (158). This could indicate a defect in the cellular subset that is the source of cleaved IL2Rα. An alternative explanation may be that, even in the presence of normal Treg frequencies in T1D (70), IL2RA polymorphisms account for functional defects in the Treg compartment (72, 346). In conclusion, it seems that although genetic variability in the IL2RA gene is associated with several autoimmune diseases including T1D, the mechanisms and extent to which sIL2Rα levels mediate these conditions differs significantly.

F. CTLA-4

Another confirmed T1D risk allele lies in the gene encoding cytotoxic T lymphocyte-associated protein 4 (CTLA-4) in the IDDM12 region (307, 437). CTLA-4 is by all accounts a vital molecule for proper negative regulation of immune responses, as evidenced by the severe lymphoproliferative disorders seen in knock-out mice (468). As with other regions, the risk association of allelic variants in the CTLA-4 region is again not exclusively confined to T1D, but was replicated in several other prevalent autoimmune disorders, including MS (283), systemic lupus erythematosus (SLE) (35), and rheumatoid arthritis (RA) (443). SNPs have been described in the human CTLA-4 promoter region and in exon 1. The A49G polymorphism is the only polymorphism that changes the primary amino acid sequence of CTLA-4. In vitro studies of A49G CTLA-4 have shown that this mutant form of CTLA-4 is aberrantly processed in the endoplasmic reticulum, leading to reduced surface expression (17). Exactly how these polymorphisms affect CTLA-4 function is still unclear. In addition to effects on processing and intracellular trafficking, they may affect oligomerization and surface retention (426). The predominant hypothesis in humans, however, is that the allelic variant lowers the mRNA levels of the soluble CTLA-4 splice variant (437).

G. Genome-Wide Association Studies and Rare Polymorphisms in the IFIH1 Gene

The advent of genome-wide association (GWA) studies has enabled high-throughput analysis of SNPs across the entire human genome at a resolution previ-

ously unattainable, in thousands of unrelated individuals, in a non-hypothesis-driven manner (333). Published results from multiple GWA studies and meta-analyses performed upon them have confirmed the association of the genes discussed above and identified a considerable number of new risk loci (75, 97, 164, 173, 174, 404, 434). The most recent GWA study focusing on T1D found that over 40 loci affect risk of T1D, including newly identified coding regions for immunoregulatory molecules such as IL-10 (36). It can be concluded from such comprehensive studies that autoimmune diseases indeed share many of the genetic risk factors, suggesting common underlying pathways. Further adding to that argument was the very recent discovery of predisposing SNP in the functional candidate gene TYK2, implicated in interferon (IFN)-α, IL-6, IL-10, and IL-12 signaling. This association had been previously demonstrated in MS, ankylosing spondylitis, SLE, and autoimmune thyroid disease (465). We will refrain from summarizing all these putative risk genes and speculating on their etiological implications for T1D. Instead, we highlight T1D-associated polymorphisms in the IFIH1 region (403), because they link genetic constitution and putative environmental factors.

Interferon-induced helicase 1 (IFIH1) codes for an IFN-induced helicase that contributes to recognition of dsRNA from picorna viruses. As such, IFIH1 serves as a cytoplasmic sensor for viral infection (221, 291). As we discuss below, one of the most prominent T1D-linked viruses is coxsackievirus B (CVB), an enterovirus belonging to the picornavirus family. Mouse studies confirm that IFIH1 and its adaptor molecule MAVS are critical for type I interferon responses to CVB (467), particularly during the late phase (495). Thus a genetic defect in IFIH1 could potentially interfere with proper detection and clearance of viral infections and lead to an abnormal, diabetogenic immune response. An independent study confirmed the presence of T1D-associated polymorphisms in the IFIH1 gene and showed that gene expression levels in PBMC are higher in individuals with the susceptible genotypes (248). A plausible hypothesis is that in these individuals with higher IFIH1 levels, viral infections may be primarily recognized by the IFIH1 pathway, leading to exacerbated antiviral immunity and production of type I interferons. The identification of rare protective IFIH1 variants in T1D is in line with this hypothesis (304). One of the protective variants is a nonsense mutation leading to a truncated protein, while two other variants probably disrupt normal splicing of the IFIH1 transcript. The initial prediction that these variants reduce the function of the IFIH1 protein, and thereby decrease the risk of T1D, has since been experimentally confirmed (395).

H. Parallels Between Human Genetics and Genetics of the NOD Mouse

The majority of mechanistic data on T1D pathogenesis and potential interventions have been derived from mouse studies. It is therefore important to understand the genetic predisposition of the most widely used mouse model, the NOD mouse. Similar to the human IDDM loci terminology, genetic regions that control progression to T1D in the NOD are designated as insulin-dependent diabetes (Idd) loci. One approach for detailed functional analysis of the risk loci is by creating congenic mice, in which specific disease susceptibility loci are replaced with protective genes derived from strains that are not diabetes-prone. Such studies confirm that, as in humans, major histocompatibility complex (MHC) class II (Idd1) genes in particular are the dominant genetic contributors to disease predisposition in NOD mice. In addition, over 20 non-MHC Idd regions have been found to mediate disease risk (264). We will focus on some of the susceptibility loci that are shared between human and mouse.

The first risk locus that shows correspondence between human and mouse is Ctla4 (Idd5.1) (472). Humans express two major splice variants coding for membrane-bound and soluble forms of CTLA-4. Mice express an additional variant lacking the B7 binding domain, termed ligand-independent CTLA-4 (liCTLA-4) (437). Genetic protection in the Idd5.1 congenic mouse strain was found to be mediated by higher expression levels of the liCTLA-4 isoform, which negatively modulates T-cell responses. This confirms the concept that polymorphisms affecting the levels of alternatively spliced CTLA-4 isoforms contribute to T1D susceptibility (454).

Genetic susceptibility in humans and mice also share variations in the IL-2 signaling pathway. But, whereas susceptibility in humans relates to the IL2RA gene region, variants in the NOD are in the IL-2 gene (in Idd3) (259, 339). Consistent with an indispensible role for proper IL-2 signaling to prevent T1D, several reports have documented the correlation of Idd3 with decreased IL-2 levels leading to impaired tolerance induction and Treg functionality (160, 269, 272, 332, 390). Taken together, this degree of similarity between two evolutionary distinct species emphasizes the critical role of the IL-2 pathway in maintaining self-tolerance. Although disruption occurs at different parts of the signaling cascade, the outcome could similarly be a breakdown of T-cell homeostasis and the expansion of a diabetogenic T-cell subset.

Some observations suggest that the mouse ortholog of *PTPN22*, *Ptpn8*, influences disease in the NOD, but this association has yet to be confirmed (473). As of today, no Idd locus has been revealed that regulates thymic insulin expression to the extent as demonstrated in humans. However, the concept of thymic insulin levels as a critical regulatory threshold during negative selection has been

confirmed by studying NOD mice with different degrees of insulin gene-dosage deficiency (92). Apart from the inherent evolutionary divergence of both species, some other limitations arise with regard to translation of genetic data from the NOD model to humans. For instance, NOD mice exhibit much stronger insulitis than that found in human islets (see also Fig. 3). Moreover, translation of immunomodulatory interventions from the NOD model to T1D patients is not straightforward (see below).

III. PRECIPITATING EVENTS

It is now established that clinical manifestation of T1D reflects the consequence of an underlying, sustained autoimmune process. For instance, autoantibodies against islet antigens are detected before the clinical onset of T1D. This suggests that a sequence of inciting events precedes the hyperglycemia for at least months, but most likely several years. This wide gap between initiation and detection of ongoing diabetogenic events poses a cardinal problem in the search for causative environmental triggers. Furthermore, the possibility exists that inciting agents may be of a "hit-and-run" type, leaving no detectable molecular trace. Alternatively, it may take multiple environmental insults to unleash autoimmunity, and different patients may incur them in divergent combinations. Having discussed the major genetic contributions to T1D susceptibility, we next discuss convincing epidemiological data indicating that the rising incidence in T1D has to be attributed to environmental changes (51, 156).

A. Viral Infections

A search on PubMed using the keywords "virus" and "type 1 diabetes" produced 1,355 titles at the time of writing, mirroring the extensive efforts to elucidate the role of viral infections in T1D. Publications dating back as far as 1926 document the seasonal variation of diabetes onset and make some link with viral infections (6). Despite all these efforts, there is still no direct evidence for a particular viral strain being causative.

1. Enteroviruses

Extensive circumstantial data designate enteroviruses, and more specifically coxsackieviruses, as prime viral candidates that can cause precipitation of T1D (142). The first papers suggesting a link between coxsackievirus infections and T1D were based on the finding of higher neutralizing antibody titers in serum from recent-onset patients versus healthy controls (152). These data were later confirmed using PCR technology (95). Some studies also tested for antibodies against other viruses in parallel, but coxsackievirus was usually found to be most preva-

lent (32). The possibility of a causal relationship has since been evaluated with varying success in both human studies (149, 436) and animal models (483). The question was approached from an interesting angle in a 1971 study that followed the diabetes incidence after an epidemic of Coxsackievirus B4 (CVB4) infection on the isolated Pribilof Islands. Five years after the epidemic, the incidence of diabetes in the CVB4-infected versus noninfected individuals was found to be similar, suggesting no link between CVB4 infection and T1D onset (117). Given our current knowledge on genetic contribution, this is a textbook case arguing that viral infection alone does not cause T1D in any given genetic background. Yoon et al. (484) later provided more direct support for CVB4 involvement by demonstrating that the virus could infect beta-cells and cause insulitis and diabetes in susceptible mouse strains (484). Furthermore, the same group isolated a CVB4 strain from a child with recent-onset T1D (482). Functional data show enhanced T-cell responses to CVB4 proteins in children with T1D after the onset of the disease (213). Among these CVB4-reactive cells, the effector/memory phenotype predominates around the time of diagnosis (452). Last, sampling within the Finnish population revealed associations between enterovirus infection and diabetes development in prospective studies (250, 251; recently reviewed in Ref. 425). Although enterovirus infection is unlikely to represent the exclusive cause for the extremely high and rising incidence of T1D in Finland, it may well be a significant contributing factor.

In their 1987 landmark paper, Foulis et al. (145) reported on the presence of abundant levels of HLA class I and IFN-α in the islets of recent-onset diabetic children, further fueling interest into the role of viruses in T1D (145). It is conceivable that beta-cell-tropic infection upregulates both HLA class I and IFN-α and leaves a molecular "viral signature." This also could explain why the immune response is directed specifically against the islets. A follow-up study probing for viral proteins in beta-cells disappointingly failed to detect any viral components (144), but the same group recently revised their conclusions after reanalysis of the same cohort using optimized methodology (356). Evidence of enterovirus presence was found in islets from 44 of 72 recent-onset patients versus 3 of 50 controls, offering the closest indications to a causal relationship to date. However, islets of 10 of 25 type 2 diabetics also showed traces of enterovirus presence, and concerns were raised regarding the specificity of the reagents that were used (363). Nevertheless, Dotta et al. (119) recently replicated the immunohistochemical detection of enterovirus protein and confirmed the results by sequencing. Unique pancreas samples such as those obtained by Foulis and co-workers (99) now rarely become available, because of the greatly improved clinical management of T1D. Therefore, replication of the results depends on initiatives such as the Juvenile Diabe-

tes Research Fund-funded Network for Pancreatic Organ Donors (nPOD), aimed at nationwide procurement of tissue relevant to T1D research (481).

Whereas the presence of enterovirus particles in pancreatic islets suggests that T1D is a consequence of selective viral infection of beta-cells, data favoring alternative mechanisms have been reported. The striking sequence similarities between the 2C protein from coxsackievirus and GAD, a major autoantigen in T1D, lead to the postulation of viral mimicry in the etiology of T1D (223). Subsequent results argued both in favor (22, 430) and against (195, 357, 382) such a mechanism, and some suggested an important contribution of HLA-DR3 in susceptibility to viral mimicry (464). Despite the sequence similarity between GAD and hsp60 (212), the association between autoimmunity to hsp60 and T1D turned out to be irreproducible (25, 357).

The timing of enterovirus infection in relation to T1D onset is a controversial issue. In addition to demonstrable traces of infection in recent-onset individuals, enterovirus infections have also been demonstrated before onset in prediabetic, autoantibody-positive children (374). Enterovirus infections during pregnancy were also identified as a risk factor for T1D (107, 126, 202). Together, these data suggest that viral infection instigates the autoimmune response. However, data from NOD mice show that pre-existing insulitis is required for coxsackievirus to induce diabetes (121, 196, 387). Translating this to the human situation, susceptible individuals may have ongoing subclinical insulitis for years until viral challenge triggers an acceleration of beta-cell destruction and hyperglycemia. Another interesting observation in this regard is that enteroviruses were recently found in intestinal biopsy samples in 75% of T1D cases versus 10% of control patients. This might reflect a persistent enterovirus infection of gut mucosa (315) that serves as a previously unidentified viral reservoir that may spill into pancreatic territory at later time points and cause insulitis.

CVB-induced upregulation of the chemokine CXCL10 on pancreatic beta-cells was recently exposed as one of the earliest consequences of infection (43). Hyperexpression and viral presence coincided in "fulminant" T1D, an extremely aggressive T1D variant found in the Japanese population (419). Animal studies have delineated a crucial role for CXCL10 in the islets during the recruitment of CXCR3-expressing autoreactive T cells following viral infection (93).

Cumulatively, the available data suggest the involvement of CVB in at least a subset of T1D cases and would warrant prevention of infection in susceptible individuals. Making matters complex, however, is the finding that under certain experimental conditions CVB can actually protect from disease (435), supporting the "hygiene hypothesis" (27). Our group has shown that this protection is mechanistically governed by two distinct pathways, including functional Treg enhancement and upregulation

of the coinhibitory receptor PD-L1 on lymphoid cells (141). These data shed light on the ambiguous role of viral infections in the context of autoimmunity.

2. Other viruses

Other viral infections have been associated with T1D, but a causal relation has not been proven.

The potential association of T1D and rotaviruses, the most common cause of childhood gastroenteritis, is based on possible molecular mimicry. Similarities were initially found between T-cell epitopes in GAD and IA-2 and viral protein (193). A subsequent Australian study found an association between rotavirus infection and islet autoantibody positivity in at-risk children (191), but a Finnish group failed to confirm this relationship (48). The same Finnish group later unsuccessfully sought an association between rotavirus-specific T-cell responses and the presence of T1D-associated autoantibodies (265). Thus the present status of rotavirus infection in the etiology of T1D remains unconfirmed. Likewise, many initial reports on the potential role of other viruses in T1D, such as cytomegalovirus (324), parvovirus (169, 220), and encephalomyocarditis virus (103), were challenged or remain to be confirmed in large patient populations.

A conceptually interesting case was made for the convincing relationship between congenital rubella infection and diabetes onset after birth, a topic recently reviewed by Gale (150). Congenital rubella syndrome consists of a wide range of both physical and behavioral disorders and hence is characterized as a multisystem disease. Interestingly, progression to diabetes was associated with a higher frequency of the HLA-A1-B8-(DR3-DQ2) T1D susceptibility haplotype (289), but direct evidence for islet-specific autoimmunity is extremely scarce (329). An alternative mechanism that the virus interferes with beta-cell mass development is now favored. As a consequence of these atypical features, some clinical consensus guidelines list congenital rubella diabetes in a distinct category of "other specific types" of diabetes (74). But the most important argument that rubella infection is not responsible in the rising global T1D incidence is that the virus has been largely eliminated in wealthier countries since the introduction of an efficient vaccine in 1969. A similar argument excludes mumps infection despite a recent report documenting a case of "fulminant" T1D (161, 190).

Because vaccinations did not reduce T1D incidence, it was questioned whether widespread vaccination programs could actually account for the observed rise. Indeed, introduction of general childhood immunizations and the growing prevalence of T1D in developed countries seemed to happen concurrently. However, multiple large studies found no support for any causal relation between childhood vaccination and T1D (47, 112, 136,

201), so the risk-to-benefit ratio remains strongly in favor of continued protection efforts against infectious diseases by means of immunization.

B. Bacteria

The bacterial composition of the intestine has long been acknowledged as an important variable affecting T1D development. Direct evidence exists in rodents, as diabetes is aggravated under specific pathogen-free conditions or upon administration of antibiotics (27). In other studies, however, administration of antibiotics prevents diabetes (68, 384). Perhaps autoimmunity ensues whenever the intricate microbial balance in the intestine is disturbed. Additionally, the intestinal wall does not seem to have the same capacity to form a coherent barrier separating luminal bacteria and the immune system in T1D models and patients versus controls. This so-called "leaky gut" phenotype is thought to enhance the exposure of bacterial antigens to the immune system (441). In the intestine of T1D patients, subclinical immune activation (380, 471) and evidence for an impaired Treg subset (431) were found. We have already mentioned the presence of enteroviruses in intestinal specimens, which may be one of the triggers of this inflammatory gut phenotype (315). Conversely, administration of specific probiotics may prevent islet autoimmunity under certain conditions (80), and clinical trials are ongoing to validate this hypothesis (249). Collectively, it appears that antibiotics and probiotics may influence T1D development by altering the balance of gut microbiota toward either a tolerogenic or nontolerogenic state, depending on constitution of the intestinal microflora at the time of administration (441).

A recent study by Wen et al. (469) sheds light on some of the mechanisms governing this delicate balancing act at the intestinal level. NOD mice lacking the essential TLR signaling component MyD88 were protected from diabetes. Furthermore, transferred $CD4^+$ T cells expressing the highly diabetogenic TCR receptor BDC2.5 failed to expand in the pancreatic lymph node (LN) of $MyD88^{-/-}$ NOD mice. It was postulated that abnormal recognition of certain gut bacteria may be indispensable for diabetes development in the regular NOD model, a pathway that is disrupted in the MyD88 deletion variant. Administration of broad-spectrum antibiotics to $MyD88^{-/-}$ NOD mice reintroduced the potential to develop disease, and germ-free $MyD88^{-/-}$ NOD mice showed an increased risk to develop T1D compared with $MyD88^{-/-}$ NOD mice housed under SPF environment. The latter findings indicate that at least some members of the microbial community in the intestine may protect from diabetes independently of MyD88. Although the mechanistic picture is far from complete, these results provide proof-of-principle in favor of a crucial role for intestinal immune homeostasis in T1D prevention.

Another recently discovered bacterial risk factor may be *Mycobacterium avium* subspecies paratuberculosis (MAP), which is the cause of paratuberculosis in ruminants. Of note, this bacterium is shed in the milk of infected cows and survives pasteurization. Clinically significant humoral responses to MAP antigens and whole cell lysates were detected in T1D patients (385). Moreover, the presence of MAP was confirmed in T1D patients by culture and was isolated from blood (367). It was subsequently reported that a polymorphism within the SLC11A1 gene was associated with the presence of MAP DNA in T1D patients. Since MAP persists within macrophages and is processed by dendritic cells, it was concluded that mutant forms of SLC11A1 may alter the processing or presentation of MAP antigens leading to diabetogenic responses (109). We place the footnote that all the studies referenced on this topic are from the same group documenting a relationship within the isolated Sardinian population. It remains to be seen whether this finding will be confirmed by others in different patient cohorts.

In conclusion, just as their viral counterparts, there is sufficient indirect evidence warranting a focus on bacterial agents as potential triggers in T1D.

C. Other Environmental Triggers

We have pointed out the apparent vulnerability of the intestinal immune homeostasis in T1D, implying a potential role for the bacterial flora. There are obviously many other substances that may disturb physiological responses at the site of the mucosal immune system, some of which have been under scrutiny as causal factors in T1D.

1. Cow's milk

Cow's milk, and in particular its albumin component, has been proposed to promote islet autoimmunity, because cross-reactivity was found between serum antibodies to albumin and ICA-1 (p69), a beta-cell surface protein (218). Some studies suggested early introduction of cow's milk as a predisposing factor as opposed to prolonged duration of breastfeeding during infancy (108, 233, 284). The emerging idea of cow's milk ingestion as a contributing parameter in T1D was soon challenged by reports that were unable to demonstrate any causal relevance (23, 52, 311). This topic has since been subject to controversy, as even in NOD mice and the BB rat strain contradicting outcomes were reported (110, 217, 267, 330). Support for a causal relationship in patients has predominantly come from the Finnish population. The TRIGR (Trial to Reduce IDDM in the Genetically at Risk) trial is particularly noteworthy in this respect. TRIGR will test whether hydrolyzed infant formula compared with cow's milk-based

formula decreases risk of developing T1D in children with increased genetic susceptibility (1). Early data suggest that hydrolyzed formula confers some protection and that maternal antibodies may offer breast-fed babies protection against enteroviruses (10, 373). Another recent Finnish study suggests that the PTPN22 polymorphism affects islet autoimmunity only if children are exposed to cow's milk during early infancy, providing a possible explanation for the many contradictory findings (244). As it stands today, convincing arguments in favor of a pathogenic role for cow's milk proteins in T1D are scarce. An interesting viewpoint put forward by Harrison and Honeyman (180) is that increased immunity to cow's milk proteins, rather than being a unique risk factor, may reflect a general impairment in mucosal immunity.

2. Wheat proteins

Albeit to a lesser extent than cow's milk, wheat proteins and more specifically gluten have received some attention (85, 260, 457). A milk-free, wheat-based diet produced higher diabetes frequency in BB rats and NOD mice (38), and in the latter this diet induced a Th1-type cytokine bias in the gut (143). In T1D patients, increased peripheral blood T-cell reactivity to wheat gluten was more frequently found than in healthy controls (232). It was further reported that timing of initial exposure to cereals in infancy may influence onset of islet autoimmunity in susceptible children (310, 497). This pattern is reminiscent of celiac disease (CD), an autoimmune disorder triggered by the ingestion of gluten in susceptible individuals. Overlap between both diseases has long been acknowledged, and the prevalence of (undiagnosed) CD within T1D and their relatives is higher than expected (199, 313, 414). Unlike in CD patients, however, there is no robust evidence to assume that gluten actually drives the initiation of autoimmunity in T1D (198). Analogous to cow's milk proteins, immune reactivity against wheat proteins in T1D could be the general consequence of an aberrant mucosal response rather than the specific driver of islet autoimmunity.

3. Vitamin D

Some dietary components might increase T1D development, but a substantial body of knowledge points towards the protective properties of vitamin D in T1D. Vitamin D is not only taken up through nutrition, but also synthesized in the skin upon exposure to sunlight (reviewed in Ref. 276). T1D has a clustered seasonal onset, and the monthly hours of sunshine and T1D incidence are inversely correlated (293). Vitamin D effectively inhibits dendritic cell differentiation and immune activation. Vitamin D metabolite levels were lower in plasma from T1D patients around onset (247), and increased vitamin D intake reduces incidence in mice (278) and humans (276).

The search for genetic polymorphisms in the vitamin D receptor (VDR) has yielded conflicting results on the correlation with T1D in the majority of studies (276), and a recent meta-analysis concluded that there was no such evidence (170). Recent studies have confirmed the absence of a relationship between VDR polymorphisms and beta-cell autoimmunity (297), but did identify T1D-associated polymorphisms in genes encoding enzymes involved in vitamin D metabolism (29). Interestingly, interaction was found between VDR and HLA alleles and is mediated by a vitamin D responsive element present in the promoter region of the HLA-DRB1*0301 allele. It can be reasonably envisioned that the absence of vitamin D in early childhood may contribute to T1D development due to poor expression of DRB1*0301 in the thymus (205). In sum, vitamin D can be considered an important environmental factor that is required for proper maintenance of self-tolerance and protection against autoimmunity. Of note, therapeutic use of vitamin D metabolites in humans is hampered by its profound effects on calcium and bone metabolism. Therefore, structural analogs have been designed that predominantly exert the immunomodulatory effects. Active treatment of T1D with vitamin D analogs remains a promising future avenue, and at-risk individuals should probably avoid vitamin D deficiency (277).

A wide array of other dietary compounds and environmental triggers have been shown to affect diabetes development in animal models, and for some of these such as omega-3 fatty acids (312), there is limited proof in human patients.

IV. TIMELINES FOR THE PATHOGENESIS OF TYPE 1 DIABETES

Several timeline models have been put forward to depict the outcome of the interplay between the genetic and environmental factors. Figure 1 provides a visual overview of some prominent hypotheses that have been proposed over the years. The linear beta-cell decline hypothesis postulated by Eisenbarth in 1986 remains the most widely referenced benchmark model for T1D (124). According to this model (Fig. 1A), genetically susceptible individuals at some point encounter certain environmental agents that initiate islet autoimmunity leading to a linear decay in beta-cell mass, development of autoantibodies, hyperglycemia, and eventually complete loss of C-peptide. While this view provides a unifying explanation for the sequence of events observed during the course of T1D, it does not integrate factors contributing to the variability along the time axis during the prediabetic phase. Some authors argue that disease progression in T1D is not a linear process, but rather proceeds at variable pace in individual patients (89). In the previous sections, we have discussed the impact of specific genetic
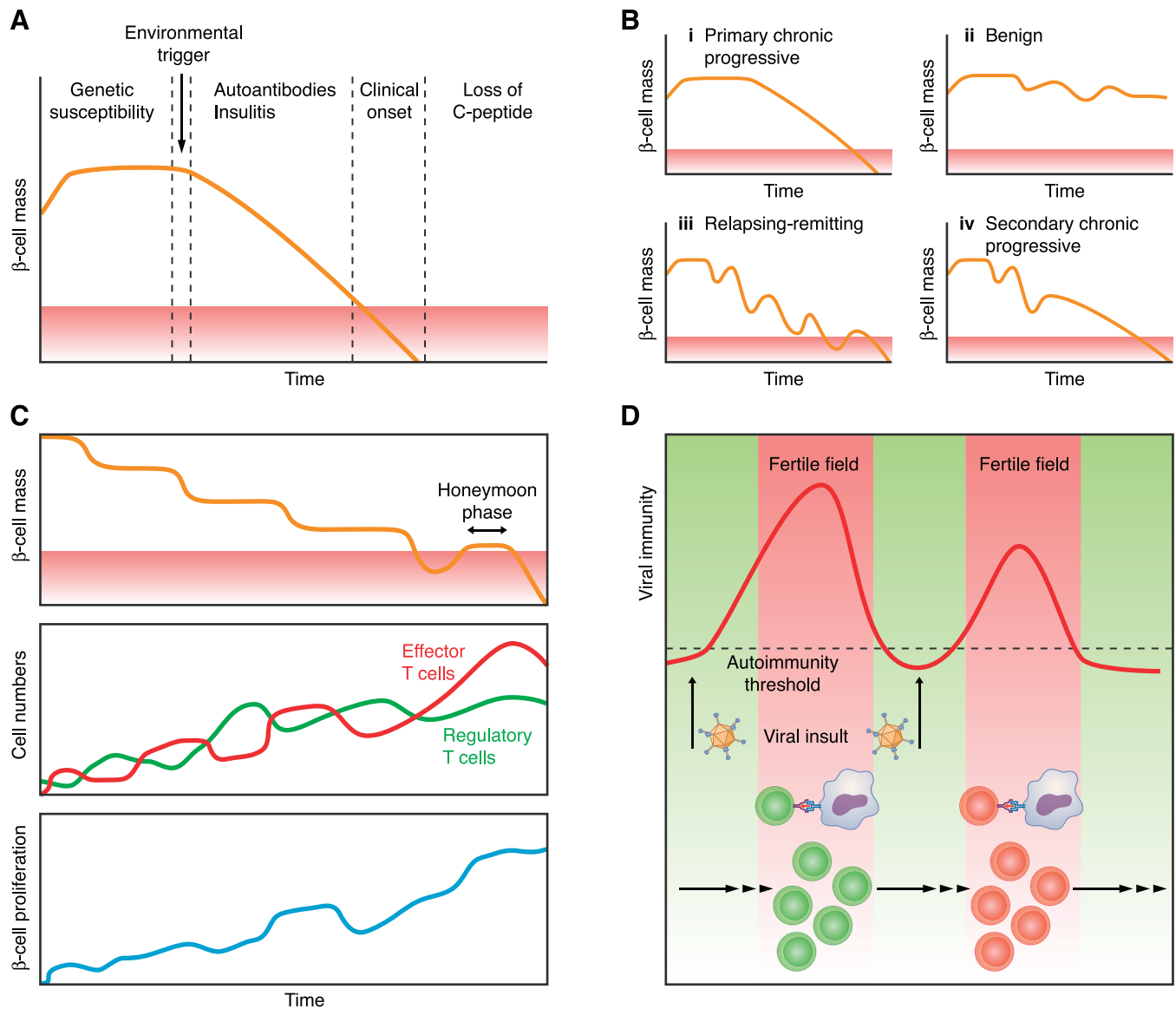
FIG. 1. Timelines for type 1 diabetes. *A*: model for linear beta-cell mass decay, as originally proposed by Eisenbarth (124). In the context of genetic predisposition, an environmental trigger induces islet autoimmunity and beta-cell death leading to a sequence of prediabetic stages and eventually clinical onset. *B*: it is widely acknowledged that the time window between initiation of autoimmunity and clinical onset is highly variable. Chatenoud and Bluestone (89) proposed some scenarios leading to the observed variability. Versatile interaction between genetic factors and environmental challenges such as viral infections likely contribute to the fluctuations in beta-cell mass before onset. *C*: we have introduced the concept of T1D as a relapsing-remitting disease, dependent on cyclical disruption and restoration of the balance between effector and Tregs and potentially counteracted by beta-cell proliferation (462). This model also provides a mechanistic rationale for its variable course. *D*: the fertile field hypothesis postulates the existence of a time window following viral infection during which at-risk individuals may develop autoimmunity (463). Infection with a certain virus would temporarily create a fertile field. Whereas initial exposure to virus (e.g., via APC presentation; purple cells) will generate a normal antiviral response (green T cells), subsequent generation of autoreactive cells (red T cells) may occur via cross-reactivity with viral antigens (molecular mimicry) or direct recognition of autoantigens (bystander activation). Bystander activation is thought to be mediated by APC that process and present self-antigens, with the potential to raise autoreactive T cells only in the presence of viral "danger" signals.

polymorphisms on disease susceptibility. It seems acceptable that on the extreme end of the genetic risk spectrum (see, e.g., IPEX and APS-1, but perhaps also HLA haplotype), the requirement for one or more environmental triggers is low, and patients will lose beta-cell mass linearly regardless of such encounters (Fig. 1*Bi*). On the other end, subtle predisposing mutations may by them-

selves never lead to T1D (Fig. 1*Bii*), or require some degree of environmental insult (e.g., IFIH1 and viral infection?) to culminate in hyperglycemia (Fig. 1*B*, *iii* and *iv*). Our group, in agreement with similar conclusions by Bonifacio et al. (55), proposed a more detailed version of the nonlinear model depicting T1D as a "relapsing-remitting" disease (Fig. 1*C*) (462). Specifically, we suggest that

disequilibrium between autoreactive effector T cells and Tregs could develop over time and eventually lead to a decline in beta-cell mass. Whereas the net balance shifts to islet autoimmunity, this effect is temporarily counteracted by the beta-cells' proliferative response, perhaps translating in a late transient phase of reduced insulin requirement called "honeymoon phase" (also see below, Fig. 2). In an attempt to fit the role of infectious agents into this temporal T1D model, we introduced the "fertile field" hypothesis (463). The fertile field (Fig. 1D) is described as a time window that follows virus infection, and which can vary depending on the type, anatomical loca-
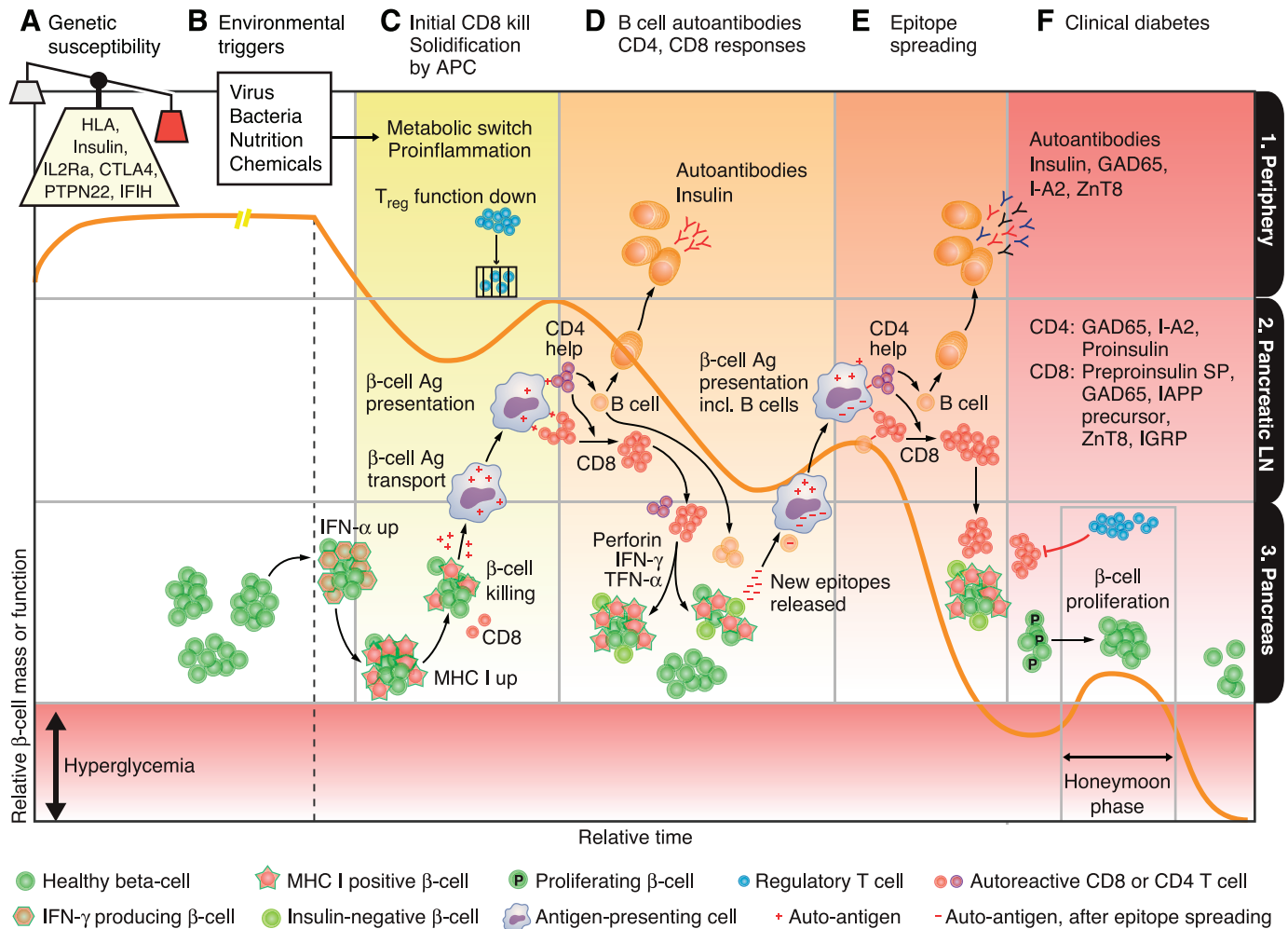

FIG. 2. How T1D might arise. This figure represents the beta-cell mass or function (represented by the orange line) as well as the different immunological phases (columns with alphabetized tabs on top) that occur in the relevant anatomical sites (rows with numerical tabs on the right). Specific events will be referred to via alphanumerical coordinates in the following explanation. Once the orange line of beta-cell function falls into the red zone, the individual is clinically diagnosed with type 1 diabetes. A complicated series of events precedes this and remains largely unnoticed. Initially, an unfortunate concurrence of genetic susceptibility (*a1*) and an environmental trigger (*a2*) sets an individual up for developing diabetes by causing two events. In the pancreas, beta-cells upregulate interferon (IFN)-α (*b3*) and subsequently MHC class I (*c3*). This exposes the beta-cells to attack by autoreactive CD8 T cells with specificity for antigens in the pancreas (*c3*). Consequently, the released beta-cell antigens are picked up by resident antigen-presenting cells (APC) (*c3*) and transferred to the pancreas-draining lymph node (LN) (*c2*). Meanwhile in the periphery (*c1*), the environmental trigger has caused a metabolomic switch creating a proinflammatory environment that favors effector T-cell responses over Treg function. Beta-cell antigens presented in this proinflammatory context and with CD4 help (*c2*) initiate conversion of B cells into plasma cells (*d2*) and the appearance of insulin autoantibodies (seroconversion) (*d1*). Also, autoreactive CD8 T cells are stimulated to proliferate (*d2*) and migrate into the pancreas (*d3*). The stress induced by this second wave of beta-cell killing (*d3*), which involves perforin, IFN-γ, and tumor necrosis factor (TNF)-α, causes some beta-cells to halt insulin production (pseudoatrophy). The killing also causes the release of new beta-cell antigens that are picked up by APCs, including migrated B cells (*d3*), and get shuttled to the pancreatic LN (*d3-d2*). This engages new specificities of CD4 and CD8 T cells (*e2*) and B cells (*e1*) in a process called epitope spreading. A subsequent wave of beta-cell killing is therefore more severe and usually results in severe depletion of beta-cell function and mass (*e3*). Surprisingly, the autoimmune inflammation can also stimulate some beta-cell proliferation (*f3*), so that the beta-cell mass temporarily resurrects. Also, Tregs can sometimes overpower and dampen the effector response (*f3*). The fluctuation between destructive autoreactive responses and the alleviation by immune regulation and beta-cell proliferation possibly creates a nonstop relapse-remitting profile of beta-cell mass (orange line). Eventually, the autoreactive response wins though, and T1D is diagnosed when only 10–30% of functional beta-cells remain. The remission after clinically diagnosed diabetes is termed the honeymoon phase (*f3*), a temporary state of relative self-sufficient insulin production.

tion, and duration of the virus-induced inflammatory response. This fertile field would allow autoreactive T cells to expand by mechanisms such as molecular mimicry or bystander activation and may lead to full-blown autoimmunity and T1D.

V. IMMUNE EVENTS IN TYPE 1 DIABETES

Several silent immune events occur before the clinical symptoms of type 1 diabetes become apparent. Most importantly, autoantibodies are produced and self-reactive lymphocytes become activated and infiltrate the pancreas to destroy the insulin-producing beta-cells in the islets of Langerhans (56). This persistent, targeted destruction may go undetected for many years, and the first clinical symptoms only become apparent after a majority of the beta-cells have been destroyed or rendered dysfunctional, making the individual dependent on insulin for survival (Fig. 2). Therefore, high priority is given to the search for "biomarkers" as whistleblowers of an ongoing autoimmune response. We will highlight some important immunological events here. Additional information on immune cell cross-talk in T1D can be found elsewhere (243).

A. Metabolic Changes: Linking Cause to Immune Events?

So far, seroconversion to autoantibody positivity is the first detectable sign of an ongoing autoimmune response. But Oresic et al. (320) recently suggested that metabolic dysregulation precedes overt autoimmunity in T1D. Elevated serum concentrations of lysophosphatidylcholine (lysoPC) precede the appearance of each islet autoantibody. In samples from the Finnish DIPP study cohort (235), characteristic changes in serum metabolites were found only in the children who later developed T1D. These changes included reduced serum succinate, PC, phospholipids, and ketoleucine, as well as elevated glutamic acid. These reactive lipid by-products are capable of activating proinflammatory molecules (286) that function as a natural adjuvant for the immune system (215). It remains unresolved whether these metabolic events trigger the initiation of the autoimmune period, or are just easier to detect. Nevertheless, these findings create an opportunity for earlier diagnosis.

B. B Cells Produce Diabetes-Associated Anti-islet Autoantibodies

The main autoantibodies in T1D are reactive to four islet autoantigens (islet cell autoantibodies or ICA): insulinoma-associated antigen-2 (I-A2, ICA512), insulin (micro IAA or mIAA), glutamic acid decarboxylase 65 (GAD65),

and zinc transporter 8 (ZnT8) (470). The early presence of autoantibodies implicates a role for antibody-producing plasma B cells in the initial immunological events. Indeed, B cells clearly contribute to the pathogenesis of human T1D (334). In the NOD model, B cells infiltrate the pancreas during the early stages of insulitis, and genetic or antibody-mediated ablation of B cells in NOD mice is protective (197, 386). How, when, and where B cells contribute to diabetes onset is still debated and discussed in further detail elsewhere (270, 397). In short, while B cell-derived autoantibodies might reflect a prelude to autoimmunity, B cells are likely active participants in the immune response because of their capacity to present antigen to diabetogenic CD4 and CD8 T cells.

C. Islet-Specific T Cells in the Periphery

A textbook definition of T1D could be "an autoimmune disease in which CD4$^+$ and CD8$^+$ T cells infiltrate the islets of Langerhans, resulting in β-cell destruction" (361). Indeed, T cells are considered to be the final executors of beta-cell destruction. This is evidenced by the precipitation or prevention of diabetes by transfer or elimination of CD4 or CD8 T cells, respectively. CD8 T cell-mediated beta-cell killing is likely a major mechanism of beta-cell destruction. CD8 T cells, found in insulitic lesions in NOD mice and in human (Fig. 3), can destroy beta cells upon activation via MHC class I expressed on beta cells. Indeed, deficiency in MHC class I due to lack of beta-2 microglobulin, or beta cell-restricted MHC class I deficiency is sufficient to arrest diabetes development and prevent beta-cell destruction in NOD (176). Mechanistically, beta-cell destruction can involve the release by CD8 T cells of cytolytic granules containing perforin and granzyme, or through Fas and Fas ligand-dependent interactions. CD4 T cells mostly provide help to both B cells and CD8 T cells by providing cytokines, such as IL-21, and a positive-feedback loop via CD40L-CD40 interactions to antigen-presenting cells, culminating in a proficient autoreactive CD8 T-cell response. Their presence in insulitic lesions suggests a beacon role and direct inflammatory properties.

The quest for autoreactive T-cell epitopes has been centralized around the four proteins that are also the major autoantibody targets: proinsulin (PI), GAD65, I-A2, and ZnT8 (114). So far, practical issues have made it difficult to determine systematically when autoantibodies and autoreactive T cells arise in the periphery relative to each other. An excellent source on this topic is the review by Di Lorenzo et al. (114) that takes stock of the large portfolio of epitopes identified to date. What is important to know is that, in general, the main known CD4$^+$ T-cell epitopes are derived from GAD65, IA-2, and PI in both human and mice. Additionally, smaller contributions from heat shock protein (HSP)-60 and islet-specific glucose-6-
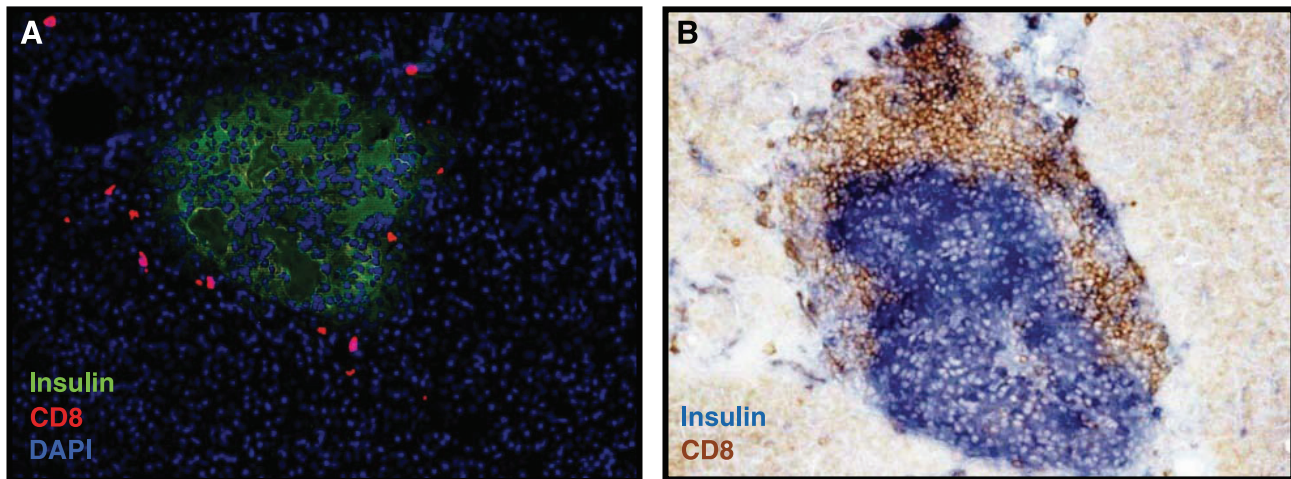
FIG. 3. The degree of pancreatic infiltration in T1D patients is limited compared with the insulitis in NOD mice around diabetes onset. Infiltration in or around the islets of Langerhans in the pancreas comprises CD8 T cells, CD4 T cells, but also B cells, macrophages and very few dendritic cells. In this respect, i.e., the type of cell infiltrating the islets, pancreatic section from human and NOD mice correspond. But the degree to which the pancreas and islets are inflamed, i.e., the number of infiltrating cells that can be found, is much more limited in humans than in NOD mice. *A*: typical degree of infiltration in recent-onset type 1 diabetic pancreas in humans. Staining of human pancreas sections for insulin (in green) and CD8 T cells (in red) indicates that only low levels of CD8 T cells can be seen in the proximity of the islets. A similar low degree of infiltration is observed for CD4 T cells and B cells in pancreatic sections of T1D patients (not shown). More information can be found in Reference 99. *B*: typical level of inflammation around diabetes onset in female NOD mice. Staining for insulin (in blue) and CD8 (in brown-red) shows a severe degree of infiltration by CD8 T cells in the islets. The type and degree of infiltration of CD4 T cells at this time point are usually similar (not shown).

phosphatase catalytic subunit-related protein (IGRP), as well as HSP70 in humans, complete the known range of CD4 epitopes. On the other hand, autoreactive CD8 epitopes in humans come mainly from preproinsulin signal peptide (400), and to a lesser extent IA2, human islet amyloid polypeptide (IAPP) precursor protein (325), IGRP, cation efflux transporter ZnT8 (Slc30A8) (470), and GAD65 (326). In mice, the CD8 epitopes are mainly derived from IGRP and GAD65/67, and ~30% from PI and 10% from dystrophia myotonica kinase (DMK). Interestingly, CD8 peptide epitopes were identified from the human preproinsulin signal peptide by elution from HLA-A2 molecules (400). This has led to the concept that beta-cells unwittingly contribute to their own demise, because they are targeted even more when stimulated to produce more insulin. Together, these data have provided strong evidence that CD8 T-cell autoreactivity is associated with beta-cell destruction in T1D in humans (337, 400).

D. Immunological Events in the Human Pancreas

We have recently reviewed the current status of our knowledge on T1D histopathology (99). We concluded that our fundamental understanding of what happens in the pancreas is mostly based on old observations, dating back as far as the 1960s.

In 1965, Willy Gepts first identified lymphocytic infiltrations in pancreatic islets. These have since then become a hallmark feature of T1D termed "insulitis" (155). In their 1985 case report, Bottazzo et al. (56) showed

dramatic upregulation of MHC class I molecules and pinpointed $CD8^+$ T cells as the predominant subset around the islets. These observations were confirmed in a large collection of samples by Foulis et al. (146). Several cornerstone insights were derived from subsequent analyses of these specimens. One such landmark paper documented that MHC class I upregulation is a common property of diabetic islets around time of onset (145). Of note, significant levels of IFN-α were found exclusively in beta-cells. This fueled interest in a possible viral etiology, because IFN-α is typically induced as a cellular response to viral infection (see above). Collectively, these findings cemented the idea of T1D being a multistep autoimmune disease (Fig. 2).

It has been widely acknowledged that the autoimmune process is quite variable, both between patients and within any given patient's pancreas over time. Foulis estimated the average number of lymphocytes associated with an inflamed islet to be ~85. About 23% of insulin-positive islets were affected versus only 1% of the insulin-deficient islets (147). Japanese studies, using a biopsy approach, found no (177) or very limited (2–62 mononuclear cells in 3–33% of islets) (206) signs of insulitis. These Japanese studies suffer from a lack of accurate sampling, as only a very limited pancreatic region was assayed.

Willcox et al. (475) recently reexamined many of the older findings using modern reagents on the samples collected by Foulis. $CD8^+$ T cells were confirmed to be a cellular component of the insulitic lesions. Also, the numbers of CD8s peak according to the degree of beta-cell

decay and disappear from pseudoatrophic (i.e., insulin-deficient) islets. B cells were found to follow the same pattern. Macrophages, on the other hand, are present only in moderate numbers during the entire process. Interestingly, Tregs were detected in the islets of only one patient. This suggests that the default territory of Tregs is confined to the pancreatic lymph nodes or spleen. Alternatively, the local absence of Tregs is the reason why insulitis could take place in the first place.

A logical consequence of beta-cell-directed autoimmunity is the induction of apoptosis. However, cross-sectional histology studies showed only very limited beta-cell apoptosis. The estimates ranged from none (296), 0.2 beta-cells per islet (78), to 6% of all beta-cells (287). The overall low rate of beta-cell apoptosis at any given time likely reflects a principal reason for the slow course of T1D. A potential role for survivin, a molecule involved in protection against apoptosis, was recently discovered in animal models (211) and T1D patients (358).

Beta-cells have a robust capacity to regenerate by proliferation, likely in response to immune inflammation (11), at least in the NOD mouse. Similar evidence in humans, using the Ki-67 proliferation marker, indicates no (79, 287), limited (204), or extensive levels (288) of beta-cell proliferation at various stages of disease. One of the possible explanations of this discrepancy between species may be the relative lack of inflammation in humans. Characterization of T1D in the pancreas by immunohistochemistry has traditionally been performed on sections from recent-onset individuals. It would obviously be very valuable to have data on the early disease stages in prediabetic patients. Because the presence of insulitis and autoantibody status around onset closely correlate (203), serum screening of healthy individuals might identify early immunologic abnormalities around the islets in autoantibody-positive, prediabetic patients. Only one study has systematically examined healthy autoantibody positive donors. This study revealed a remarkably low incidence of insulitis in only 2 of 62 cases, in <10% of the islets (204). This outcome may reflect the very subtle nature of the diabetogenic process in most human cases, possibly reflecting its relapsing-remitting course (462). Alternatively, the data may illustrate the pronounced lobular progression, making it easy to miss out on the affected regions.

E. The Honeymoon Phase: Do Beta-Cells Transiently Revive?

It is clear that patients are at the end stage of the disease course at the time of clinical presentation. Somewhere between 60 and 90% of the beta-cells are destroyed or dysfunctional. Exactly how many beta-cells remain around onset is still unknown because of the lack of accurate noninvasive imaging methods to quantify functional beta-cell mass in humans (98). In fact, assessment of beta-cell mass via detection of insulin by immunohistochemistry may considerably underestimate the amount of beta-cells left. Studies in the NOD mouse have found a substantial pool of nonfunctional, insulin-depleted beta-cells at the onset of hyperglycemia (394). Knowing how big the pool of compromised beta-cells is is important because it may be more achievable to revive than to regenerate beta-cells. An interesting window of opportunity in this respect is the honeymoon phase, a transient remission phase that occurs in up to 60% of T1D patients after initiation of insulin treatment (30) (Fig. 2). The honeymoon phase appears to occur more frequently with increasing age at onset (2, 298) and can often last 3–6 mo, but may continue for 2 years. In this period, insulin doses can be greatly reduced or even withdrawn completely (9, 257). The mechanisms governing improved beta-cell function are poorly understood, but it is thought that the constant hyperglycemic stimulus exhausts the beta-cells. Initiation of insulin treatment relieves this stress factor and temporarily allows dysfunctional beta-cells to replenish their insulin content. Our group has studied the remission phase from an immunological perspective. In a small cohort of patients, we focused on antigen-specific cytokine responses from T cells in the peripheral blood (377). Surprisingly, we found lower FoxP3 levels in $CD4^+CD25^+$ Tregs and lower numbers of IL-10 producing cells in remission patients versus new-onset cases. In a limited cross-sectional prospective study, we found that higher FoxP3 expression at diagnosis predicted worse glycemic control, but higher mean numbers of IL-10 cells were associated with better future glucose control. Additionally, others reported lower IFN-γ levels in remitters (12). Together, these data suggest that there may be an immunological component underlying the honeymoon phase, arguing for antigen-specific immunomodulation to curb autoimmunity soon after diagnosis. Finally, it remains to be seen whether these findings in the peripheral blood correlate with local events as they occur in the pancreas. This question is particularly difficult to address given the inaccessibility of patient samples.

F. The NOD Mouse: an Entirely Distinct Picture

The honeymoon phase does not occur in NOD mice. This already indicates the far more acute course of disease in the NOD mouse. As a matter of fact, comparing the typical histopathology from T1D patients and recent-onset NOD mice is like looking at two different diseases (Fig. 3).

Diabetes progression in the female NOD is characterized by nondestructive peri-insulitis, initially consisting of dendritic cells and macrophages, then followed by T

and B cells (209). This phase subsequently transgresses to a complete T-cell-mediated destruction of the beta-cell mass by 4–6 mo of age. The amount of inflammation eventually becomes so extensive that infiltrates develop into local tertiary lymphoid structures (254). These features are strikingly more aggressive than the subtle, chronic immune process in humans. Other distinctive parameters of the NOD include the potent ability of beta-cells to proliferate in the presence of inflammation and the considerable mass of nonfunctional beta-cells at onset. It is not known whether this also occurs in human T1D (394). Together, these differences may explain why the many preventive and therapeutic successes in the NOD model translate so inefficiently to the clinic.

VI. IDENTIFICATION OF PREDIABETIC INDIVIDUALS

Clinically, pre-T1D describes the period of ongoing islet beta-cell destruction in which sufficient beta-cell mass and functionality remains to preserve glucose homeostasis. Clinicians and researchers agree that silencing the diabetogenic attack at very early stages of beta-cell destruction is the most desired time of treatment of T1D. This is because it might be possible to keep treatment doses low and therefore cause less side effects. Diagnostically, determining "ongoing beta-cell destruction" is difficult, but individuals at high risk can nevertheless be identified by a combination of tests (Table 1).

A. Genetic Screening

We have discussed above the genetic component of T1D. The genetic susceptibility to T1D is determined by genes related to immune function with the potential exception of the insulin gene (434). The genetic susceptibility component of T1D allows some targeting of primary preventive care to family members of diagnosed T1D patients, but there is no complete inheritance of the disease. Nevertheless, the risk for developing T1D compared with people with no family history is ~10–15 times greater. Although ~70% of individuals with T1D carry defined risk-associated genotypes at the HLA locus, only 3–7% of the carriers of such genetic risk markers develop diabetes (3).

Therefore, the focus is on relatives of diabetic individuals, especially twins, and also on the genotype associated with the highest risk for T1D, namely, the DR3/4-DQ2/8 heterozygous genotype (15).

B. Diabetes-Associated Anti-islet Autoantibodies

The number of autoantibodies, rather than the specificity of the autoantibody, is thought to be most predictive of progression to overt diabetes. In the BABYDIAB study, almost no children expressing only one autoantibody progress to diabetes (3, 383). On the other hand, almost all individuals expressing multiple diabetes-associated autoantibodies progress to diabetes with long-term follow up (45, 353). Other cohort studies confirm that expression of two or more autoantibodies is associated with very high risk for type 1 diabetes and is rarely transient (33, 208). However, autoantibodies can fluctuate or even completely disappear. An example can be found in the American Diabetes Autoimmunity Study in the Young (DAISY), which is biased toward young children because of following children from birth. About 95% of

TABLE 1. *Available and future screening methods for T1D diagnosis*

Screening	Criterium	Concerns
Genetic	Relationship to diabetic individual (usually a first-degree relative) or identified to have high-risk HLA genotypes, like DR3/4-DQ2/8 (258)	Only 30-50% of T1D patients have the DR3/4-DQ2/8 genotype Only 50-80% of monozygous twins develop diabetes
Serologic	Serum autoantibodies associated with islet beta-cells (ICA): I-A2, IAA or mIAA, GAD65, ZnT8 (219, 470)	Presence of autoantibodies can fluctuate
Metabolic	First phase insulin production (by C-peptide) is low enough to give >50% risk for diabetes within the next 5 years and/or impaired fasting glucose or impaired glucose tolerance (226, 405)	Defective glucose control probably too late for most effective preventive treatment
T cell*	Cellular immunoblot, Elispot and tetramers	Reproducibility, standardization over multicenter is not yet complete enough
Metabolome*	Elevated serum concentrations of lysoPCs	Validation of specificity, access to mass spectrometry
β-Cell mass*	PET using radiolabeled IC2 Ab	Validation of specificity

Based on results from the Diabetes Prevention Trial-1 (DPT-1) trial, it was determined that the combination of *1*) the presence of two or more autoantibodies, with *2*) evidence of a defective first phase insulin response in *3*) individuals that are first-degree relatives to a type 1 diabetes (T1D) patient, increased the risk of developing diabetes to over 75% within 5 yr (319). In younger individuals, the risk approaches 90% within 7 yr (319). However, other and earlier detection methods could increase detection and detect disease prodromes earlier. *Not in clinical use yet. ICA, islet cell autoantibodies; I-A2, insulinoma-associated antigen-2, ICA512; IAA, insulin autoantibodies; GAD65, glutamic acid decarboxylase 65; ZnT8, zinc transporter 8.

prediabetic children express anti-insulin autoantibodies, but at onset only 50% continue to express insulin autoantibodies. Similarly in the NOD mouse, insulin autoantibodies can be transient during progression to diabetes (488). So, multiple specificities of autoantibodies should be checked. Screening for autoantibodies against ICA512, insulin, and GAD65 is accessible to primary care physicians, but ZnT8 is so far research or clinical trial use only. Further information can be found elsewhere (292).

C. Islet-Specific T-Cell Assays

Despite the strong contribution of T cells to disease pathogenesis, the presence of autoreactive T cells is not routinely assessed. An explanation for this is the poor knowledge of epitopes and the lack of robust assays to detect the low-affinity and/or low-frequency T cells (114). It is also uncertain that the peripheral blood provides access to the "right" T-cell pool. For instance, autoreactive T cells might selectively sequester to the islets or pancreatic lymph nodes, which makes consistent detection in peripheral blood difficult. Moreover, the microenvironment of the affected pancreas might alter the T-cell response. Recent data do indicate that cellular assays performed on peripheral blood have a high degree of accuracy (331, 389). T-cell assays can distinguish responses from T1D patients and healthy control subjects, but reproducibility appears to be limited, especially for CD4 responses (183). In combination with autoantibody assays however, the measurements reach a sensitivity of 75% with 100% specificity in distinguishing diabetic patients from nondiabetic controls (389). A possible explanation for this might be that T-cell responses to individual epitopes fluctuate over time, while autoreactivity as a whole persists in diabetic patients.

T-cell assays will become even more sensitive in the future, and systematic longitudinal data on epitope-specific T-cell responses will become available. This will facilitate efforts to determine the numbers and/or function of autoreactive T cells and other immune markers in the following settings.

1. Preventive screening

There is a vital need for biomarkers associated with T1D disease initiation and progression. Screening the T-cell self-specificities reveals the numbers, functional features, and specificities of the autoimmune reactivity and can expose disease activity. 1) "Numbers" show whether and how extensively autoreactive T cells are already involved. 2) "Functional features" are parameters such as proinflammatory versus immunoregulatory cytokines. For instance, HLA-DR4-matched subjects that contain IL-10-producing islet-reactive $CD4^+$ T cells develop disease 7 years later than those individuals not containing such

cells (19). 3) "Specificities" indicate whether and how many islet autoantigens for both CD4 and CD8 T cells have become targeted, as well as whether secondary epitopes (after epitope spreading) have emerged. The outcome of such T-cell profiling could allow tailoring of strategies aimed at redirecting immune responses. The optimal antigen for induction of Tregs can be selected, or more systemic approaches can be added. In this respect, some treatments prevent diabetes when given very early in the autoimmune process, but can aggravate the autoimmune response when administered at the time of demonstrable autoreactive CD8 T cells in the periphery (396, 448).

2. Transplantation

Islet transplantation and beta-cell replacement therapies provide unique opportunities to monitor recurrent autoimmune-mediated islet destruction within a relatively brief time window. Tracking T-cell responses before and after transplantation has shown that immune responses to islet allografts are associated with loss of beta-cell function (362). Such T-cell tracking can also identify factors involved in the attack (450) and distinguish the efficacy of different immune suppression protocols (361). Indeed, it is necessary to monitor closely the T-cell response in islet transplantation trials. For example, autoreactive T cells can homeostatically expand after some immunosuppression regimens used in islet transplantation (294), which explains why insulin independence is usually not sustainable under the Edmonton protocol (392).

3. Primary and follow-up markers for trial outcome

We propose that the immunological efficacy and safety of immune interventions is monitored by studying changes in T-cell autoreactivity. Assays are becoming available that allow sensitive, specific, and reproducible measurement of the disappearance or functional silencing of islet-autoreactive T cells, or the appearance of regulatory populations. T-cell ELISPOT analysis (ISL8Spot) showed that shifts, both in frequency and in immunodominance of $CD8^+$ T-cell responses, occur more rapidly than the changes in autoantibody titers in human T1D (273). Recently, HLA-A2 tetramer technology was used to show an increase in GAD65- and InsB-peptide reactive $CD8^+$ T cells upon anti-CD3 treatment of T1D patients (86). These tools might also help identify patients that will experience a honeymoon phase (377).

In conclusion, combining autoantibody and T-cell autoreactivity readouts with a panel of biomarkers provides a more complete picture of disease activity. A better evaluation of the patient's response should benefit treatment outcome and safety.

D. Metabolomic Screening

Efficacious prevention of T1D will require detection of the earliest events in the process. Autoimmunity is likely the predominant effector mechanism in T1D, but it is possibly not its primary cause. A recent interesting report by Oresic et al. (320) (see sect. VA) showed that elevated serum concentrations of lysophosphatidylcholine precede the appearance of each islet autoantibody, and thus overt autoimmunity, in T1D. If these results are validated in other well-characterized cohorts, like the German BABYDIAB (4), the United States-based DAISY (37) and PANDA (84) studies, and the multinational TEDDY study (172), metabolome screening could be added to the screening panel to effectively identify prediabetic individuals for preventive treatments.

E. Assessing β-Cell Mass

The number of islet beta-cells present at birth is mostly created by differentiation and proliferation of pancreatic progenitor cells, in a process called neogenesis (61). After birth, only a small fraction of cycling beta-cells remain capable of expanding. This might be sufficient to compensate for increased insulin demands, but probably not for regeneration after extensive tissue injury. Data from pathology samples indicate that only 10–30% of the beta-cell mass is left in long-term T1D patients. It is unclear what amount of beta-cell mass remains at diabetes onset. The deficit in beta-cells necessitates measurement of beta-cell mass in vivo, but tools are still lacking to do this accurately and sensitively in a non- or mildly invasive way.

Noninvasive beta-cell imaging using modern diagnostic equipment could provide very high sensitivity in humans and mice, but has some problems. First, single-cell resolution cannot yet be achieved to enable differentiation between scattered islets, single beta-cells, or surrounding tissue. Second, these imaging techniques rely on a specific molecular marker. Currently, the monoclonal IgM antibody IC2, which specifically binds to the surface of beta-cells, might be the only reliable marker for noninvasive imaging and quantification of native beta-cells (295, 378). Other candidates, like antibodies to ZnT-8 or the ligand to the vesicular monoamine transporter type 2 (VMAT-2), called dihydrotetrabenazine (DTBZ) and used in clinical trial (NCT00771576), are not as specific (375).

Knowledge of the amount of beta-cell mass can help with decisions on the type of therapy and in treatment follow-up. For instance, drugs that stimulate beta-cell proliferation could be chosen when sufficient β-cell mass remains, whereas very low remaining beta-cell mass would settle on islet transplantation, *trans*-differentiating drugs or stem cells. Such technology might also help answering a basic question: What is the minimal fraction of the initial beta-cell mass required to preserve glucose homeostasis? Lastly, early detection of beta-cell loss following islet transplantation might help to adjust immune modulation therapies in a timely and more targeted fashion.

VII. PREVENTIVE TRIALS

Successful prevention depends on 1) a good prediction/identification of at-risk individuals and 2) a very safe intervention that causes no harm in those individuals who would have never developed T1D. Knowledge of the primary cause of T1D might not be crucial, even at the preventive stage. This statement is based on the fact that immune modulation appears to work in a variety of T1D models and at different stages of the disease. However, many preventive trials are based on data from the NOD mouse model which has improved our understanding of disease pathophysiology. A comprehensive analysis by Shoda et al. (396) concluded that "some popular tenets regarding NOD interventions were not confirmed: all treatments do not prevent disease, treatment dose and timing strongly influence efficacy, and several therapies have successfully treated overtly diabetic mice." So, the good news is that some preventive strategies appear to have a good chance to cure the disease, even during an advanced status of beta-cell destruction. Examples of succesful treatments in NOD mice are ATG, anti-CD3, hsp, and proinsulin DNA vaccine (91, 129, 398, 440). Ideally, the balance between therapeutic efficacy and disease stage should be known prior to human trials.

A major problem with preventive trials is that it takes many years before conclusions can be drawn. As can be seen in Table 2, preventive trials divide in two main classes. The first category mainly contains non-antigen-specific nutritional supplements: vitamin D_3 (NCT00141986), hydrolyzed cow's milk (TRIGR; NCT00179777), and docosahexaenoic acid (DHA; NCT00333554). Other non-antigen-specific preventive approaches had no or limited effect using ketotifen (53), cyclosporine (83), nicotinamide (131, 151, 238), or combinations thereof (340). For instance, cyclosporine could delay onset, but not prevent T1D. The second category of preventive trials aims to induce antigen-specific oral tolerance. During the disease process in animal models and human T1D, T-cell autoimmunity progressively spreads intra- and intermolecularly among β-cell autoantigens such as insulin, GAD65, HSP, and IGRP (114, 322). Nevertheless, antigen-specific approaches use only one antigen (Table 2) to tolerize against multiple autoreactivities. The idea with this approach is to induce "bystander suppression" by Tregs as follows. Tregs induced against one autoantigen proliferate upon encountering their cognate autoantigen in the pancreatic lymph node and maybe also in the islets. These Tregs

Agent	Target/Mechanism	Development Phase, Clinical ID, Organizer
Vitamin D$_3$	Treg induction	Pilot, NCT00141986*, CDA
Omega-3 fatty acids	Anti-inflammatory	Phase II, NCT00333554*, NIDDK
Hydrolyzed cow's milk	Abnormal handling of intact foreign protein	TRIGR, Phase II, NCT00179777*, CHEO
Oral insulin	Ag-specific tolerance (oral)	Phase II, NCT00419562*, NIDDK
Nasal insulin	Ag-specific tolerance (mucosal)	Phase III, NCT00223613*, University of Turku
		Phase II, NCT00850161*, CPEX Pharma
		Phase II, NCT00336674*, Melbourne Health

* More information is available at http://www.clinicaltrials.gov/. CDA, Canadian Diabetes Association; CHEO, Children's Hospital of Eastern Ontario; NIDDK, National Institute of Diabetes and Digestive and Kidney Diseases.

also secrete cytokines that can dampen immune function or modulate APCs (421). The result is a localized antigen-specific immunosuppression and resolution of the autoimmune attack. However, translation to human T1D has been difficult, because there are no functional biomarkers that would indicate that the correct amount, route, or antigen has been used to achieve bystander suppression. For example, while the Diabetes Prevention Trial-1 (DPT-1) failed to demonstrate a benefit of oral (34, 401) or subcutaneous (120) insulin therapy in preventing T1D, post hoc subgroup analysis indicated a potential delay in T1D subjects with high insulin autoantibody titers who received oral insulin therapy (34, 319, 401, 406). A new clinical trial on the use of oral insulin in relatives at risk is underway (NCT00419562). Another example is the T1D Prediction and Prevention Study. This study could not demonstrate a beneficial effect of daily intranasal insulin treatment in preventing or delaying diabetes, even when treatment began soon after seroconversion (301). In contrast, the Intranasal Insulin Trial did show that intranasal insulin administration to individuals at high risk for developing T1D increased antibodies and decreased T-cell responses to insulin, and more clinical trials are initiated.

VIII. NEW-ONSET TYPE 1 DIABETES TRIALS

Intervention trials are more affordable than prevention trials, because potential subjects are readily identified and efficacy can be evaluated within a much shorter time frame. Current post-onset treatments are either substitutive (e.g., the many formulations of insulin), palliative (long-term use of anti-inflammatory and/or immune suppressive agents), antigen specific (islet antigen-induced Tregs), or combinations thereof.

A. Antigen-Specific Intervention Trials in T1D

In general, the idea behind antigen-based therapies is to induce Treg responses (active tolerance) or anergizing/deleting pathogenic T cells (passive tolerance) without having the side effects of long-term immune suppression.

Tolerizing against insulin or GAD65 has been rather effective in experimental settings (128, 222, 432). This approach has also led to success in T1D patients (87, 120, 133)(Table 3). Future trials should also target other peptides (114), because some investigations in diabetes-prone mice suggest that ignored determinants of beta-cell antigens are a more optimal choice to inhibit late-stage autoimmune disease (317).

Several clinical intervention trials target insulin, because it is the initiating antigen in the NOD model and it is also a major autoantigen in human T1D. A phase I trial has confirmed the data in animal models that incomplete Freund's adjuvant (IFA)-enhanced human insulin B-chain vaccination is safe and can induce insulin-specific Tregs for up to 2 years after vaccination (299). A follow-up trial will determine the effects on glycemic control (318). Another approach uses a CpG-free proinsulin-based DNA plasmid vaccine BHT-3021 (Bayhill Therapeutics). This vaccine is designed to tolerize the immune system to proinsulin by combining DNA codons for an immunodominant peptide of insulin (440) and immunomodulatory CpG oligonucleotides (189). Striking data in recent-onset diabetic NOD mice suggested that BHT-3021 induces proinsulin-specific Tregs (440) that can act as bystander suppressors. In recent-onset patients, the vaccine can preserve C-peptide and reduce HbA1c (162). This demonstrates the increased efficacy of the BayHill plasmid compared with the first generation of insulin B-expressing pCMV plasmids (94).

Another target for antigen-specific therapy is GAD65. GAD-Alum is an aluminum hydroxide (Alum) formulation of full-length recombinant human GAD65 (Diamyd Therapeutics). It was shown to be safe and to preserve residual insulin secretion in subjects with late-onset autoimmune diabetes of adulthood (LADA) (7). A subsequent phase II trial in recent-onset T1D showed significant preservation of residual insulin secretion and a GAD-specific immune response, both humoral and cell mediated (8). Currently, phase III studies are ongoing in Europe and the United States. The patients in all these trials were selected on the basis of elevated GAD65 autoantibodies. The formulation is crucial to Dyamid's GAD drug because

TABLE 3. *Intervention trials and Ag-specific monotherapy*

Agent	Target or Mechanism	Phase, ID, Organizer	Details	Reference Nos.
Diap277	Induction of Treg via TLRs	*Phase III (adults)*	Phase I: preserved C-peptide out to 18 mo	351
		NCT00615264	Phase II: no effect in T1D adults or children	240
		NCT00644501 Andromeda Biotech	Phase III: recruiting	381
Ins B in IFA	Tolerance vaccination to insulin B chain	*Phase I/II* NCT00057499 ITN	Ongoing	111, 299
NBI-6024	Tolerance vaccination to insulin	*Phase I /II*	Phase I: shift Th1 to protective Th2	14
(APL of insulin)		NCT00873561 Neurocrine	Phase II: no effect on residual β-cell mass and insulin needs	466
BHT-3021	Tolerance vaccination to insulin	*Phase I/II*	Reduced insulin autoAb titers, preserved C-peptide and reduced HbA1c	162
(Proinsulin vaccine)		NCT00453375 BayHill Therapeutics	No adverse effects	
GAD65-Alum	Tolerance to GAD65, skewing Th1 to Th2	*Phase II (LADA)*	Preservation of residual insulin secretion, GAD-specific humoral and	7
		NCT00456027	cellular immune response, but no protective effect of treatment ≥6 mo after	8
		Diamyd	diagnosis, no change in fasting C-peptide after 15 mo	256
		Phase II (T1D) NCT00435981	Ongoing	
		Phase III NCT00723411	Ongoing, Europe	
		Phase III NCT00751842	Ongoing, USA (DIAPREVENT)	
Islet autoAg-derived peptides eluted from human HLA class II	Regulated immune response	*Phase I* DVDC	N/A	400

APL, altered peptide ligand; IFA, incomplete Freund's adjuvant; ITN, immune tolerance network, DVDC, Diabetes Vaccine Development Center; PI, proinsulin.

1) adjuvant reduces the required quantity of antigen and *2)* aluminum salts preferentially induce a humoral rather than cellular immune response (255). Immune readouts show an increase in FoxP3 and transforming growth factor-β in cells from GAD-Alum-treated patients compared with placebo after 15 mo (255).

Despite these promising studies, it is too early to judge whether GAD65 and/or insulin are optimal target antigens to induce Tregs that can modulate the course of human T1D. Combination therapies with a short-term course of a suitable immune modulator are considered to enhance efficacy in recent-onset patients.

B. Non-antigen-Specific Intervention Trials in T1D

Since autoimmunity is the main effector mechanism in T1D, many intervention trials have used drug regimens to silence and/or modulate the immune response, preferably without negative effects on Tregs (Table 4). These immune-suppressive regimens will also prove valuable, if not critical, for the success of islet transplants and/or β-cell regenerative therapy.

The first immunosuppressive agent used in a placebo-controlled, double-blind clinical trial for T1D was cyclosporine A. Cyclosporine A inhibits calcineurin, which is responsible for the activation of IL-2 transcription. The lack of IL-2 and other cytokines reduces the function of effector T cells, but unfortunately also of Tregs. Cyclosporine treatment induced remission of T1D, but its chronic use was suspended because of unacceptable side effects (21, 59, 210). However, this limited success indicated that immunosuppression can reduce the autoimmune inflammation in T1D.

DiaPep277 was originally used in an antigen-specific therapy. The idea was that this peptide from HSP60 becomes an autoantigen in T1D because of cross-reactivity (127, 129). More recent insights indicate that Diap277 is a systemic modulator: HSP60 induces Treg via the Toll-like receptor (TLR)-2 (417, 490). A phase II trial showed that

DiaPep277 preserved C-peptide up to 18 mo in adult new-onset T1D patients (350). Beta-cell preservation was associated with IL-10 production before treatment and a decrease in autoantigen-specific T-cell proliferation after treatment (200). But so far, no DiaPep277-specific Tregs have been characterized in mice or humans. However, while a phase III study is ongoing in adults, no treatment effect was observed in children with T1D (240).

TABLE 4. *Intervention trials and Ag-nonspecific monotherapy*

Agent	Target/Mechanism	Phase, ID, Organizer	Details	Reference Nos.
Calmette-Guerin (BCG)	Hygiene hypothesis	*Phase I* NCT00607230 MGH	N/A new trial is recruiting Trial 10 yr ago: no effect	13
Diazoxide	Counteract chronic overstimulation of β-cells	*Phase IV* NCT00131755 V. Grill, MD	No effect on β-cell function Side effects (eliminated by lowering dose)	46, 168, 321
Ingested IFN-α	T1D is a type 1 IFN immunodeficiency syndrome	*Phase II* NCT00005665 NCCR	Safe Lower dose is more efficacious in preserving C-peptide Need better dosing	66, 369
Cyclosporin A	Immune suppression	*Completed*	Remission successful during treatment, but severe side effects	21, 59
Anti-CD3 (hOKT3) g1(Ala-Ala)	T-cell immunomodulation and Treg generation by FcR-nonbinding anti-CD3 mAb	*Phase I/II*	Remission out to 24 mo	182, 184-186
MGA031		Completed	36 mo positive effects on C-peptide levels	
Teplizumab (US based)		ITN (Herold) *Phase I/II*	Test: single 14-day course of Ab administered to recent-onset patients 4-12 mo postdiagnosis	
		NCT00378508 JDRF/NIDDK		
		Phase II AbATE	Multidose: 2 courses of Ab administered 1 yr apart	
		NCT00129259 ITN (Herold)	Read-out: C-peptide levels	
		Phase II/III Protégé NCT00385697 Macrogenics	Read out: insulin requirement, HbA1c	
Anti-CD3 mAb (ChAglyCD3)	T-cell immunomodulation and Treg generation by FcR-nonbinding anti-CD3 mAb	*Phase II*	6-day Tx: better maintenance of C-peptide levels, reduced insulin requirement out to 18 mo	91, 228
TRX4		Completed 2005	During Tx: headaches, nausea, body aches, and other flu-like symptoms; 6wk postTx: flare-up of EBV, sore throat, and swollen glands (temporarily and mild)	
Otelixizumab (Europe based)		*Phase II TTEDD* NCT00451321 JDRF/TolerRx	Test: multidose regimen	
		Phase III DEFEND-1 NCT00678886 JDRF/TolerRx	Test: 8-day treatment Recruiting	
		Phase I NCT00946257 GSK	Test: subcutaneous administration	
ATG Thymoglobulin/Atgam	T-cell depletion, generate Treg population	*Phase II START* NCT00515099 NIAID/ITN	Could cause cytokine release syndrome	398
Anti-CD20 mAb	B-cell depletion	*Phase II/III*	C-peptide AUC in MMTT better ≤12 mo	197, 334
Rituximab		NCT00279305 TrialNet	From 3-6 mo onwards: C-peptide AUC rate of decline similar to placebo	
CTLA4-Ig Abatacept, Belatacept	Costimulation blockade	*Phase II* NCT00505375 NIDDK	N/A, recruiting	50, 239

TABLE 4.—*Continued*

Agent	Target/Mechanism	Phase, ID, Organizer	Details	Reference Nos.
IL-1 antagonists *Anakinra*	Anti-inflammatory and improve β-cell survival	*Phase II/III (AIDA)*	N/A recruiting	116, 335
		NCT00711503 STENO/JDRF	http://www.aidastudy.org	
		Phase I/II NCT00645840 UTSMC	N/A, although completed	
Canakinumab		*Phase II* NCT00947427 NIDDK	N/A, pending	
Xoma 052		*Phase I/II* NCT00998699 Zurich University	N/A, pending	
Rilonacept Arcalyst	IL-1 beta trap	*Phase II* NCT00962026 UTSMC	N/A, pending	427
TNF-α blockade etanercept	Anti-inflammatory	*Phase I/II*	Lower HbA1C and insulin need, increased C-peptide AUC	274
		NCT00730392	No adverse effects, but TNF inhibitors have been associated with an increased risk of reactivation of latent tuberculosis	31, 94, 480
		Amgen		
GCSF (granulocyte colony stimulating factor) *Neulasta*	Enhance T regulatory cell numbers	*Phase I/II* NCT00662519 JDRF/NIH	N/A, recruiting	
Byetta, exendin-4, or exenatide	GLP-1 analog, stimulates insulin secretion	*Phase IV* NCT00456300	N/A, ongoing. In T2D: short half-life, GI side effects, development of antibodies	171, 370, 478, 479
		Baylor College		
Liraglutide	GLP-1 analog, stimulates insulin secretion	*Phase II/III* NCT00993720	N/A, ongoing In T2D: HbA1c lower, fewer side effects than exenatide	76, 104, 290, 341
		Hvidovre U. Hospital		
Sitagliptin	Inhibitor of DPP-4, GLP-1-degrading enzyme	*Phase I* NCT00813228 NIDDK	N/A, recruiting (immune function)	230, 231
		Phase IV NCT00978796 BDC	N/A, recruiting (glucose effects)	
Epidermal growth factor (EGF)	Islet neogenesis, β-cell proliferation	*Phase II (E1-INT)*	Daytime insulin reduced 35-75% in 3 of 4 patients. Reductions of daytime insulin usage evident after 28-day treatment and peak 1-2 mo posttreatment (stable BG control)	*Company website*
		Transition Therapeutics		
Pioglitazone	PPAR-γ stimulation	*Phase I*	N/A, recruiting (testing course of T1D)	44, 224, 491
		NCT00545857 Stony Brook Univ		
INGAP peptides	Islet cell regeneration	*Phase II*	Increase in C-peptide secretion in T1DM patients	122, 338, 366
		NCT00995540 Exsulin Corporation	HbA1c decreased (−0.4%)	

ATG, anti-thymocyte globulin; AUC, area under curve (for C-peptide levels in glucose tolerance test); BDC, Barbara Davis Center; DCCT, Diabetes Control and Complications Trial; DENIS, Deutsche Nicotinamide Intervention Study; EBV, Epstein-Barr virus, a herpesvirus that causes mononucleosis; ENDIT, European Nicotinamide Diabetes Intervention Trial; GSK, GlaxoSmithKline; ITN, Immune Tolerance Network; mAb, monoclonal antibody; MGH, Massachusetts General Hospital; MMF, Mycophenolate mofetil; NCCR, National Center for Research Resources; NIAID, National Institute of Allergy and Infectious Diseases; UTSMC, University of Texas Southwestern Medical Center.

An important class of biological immune modulators comprises antibodies that target receptors on T cells. For example, FcR-non-binding anti-CD3 monoclonal antibodies (mAbs) have shown the most promising results so far in T1D therapy. Anti-CD3 mAb acts at various levels. It causes a short-term internalization of the TCR-CD3 complex that makes the cell "blind" to antigen (88). Also, it alters TCR-mediated signal transduction so that anergy or apoptosis is induced preferentially in activated Th1 cells (91). The apoptosis is partially mediated by CD95-CD95L interactions with neighboring T cells. This might explain why effector T-cell death is most dramatic where the T-cell density is highest, i.e., at the site of inflammation. Moreover, anti-CD3 treatment also results in Treg development (486). It is thought that the Tregs may protect against damage by effector T cells long after the drug has been eliminated from the body. To obtain all these effects, optimal dosage is crucial. Too little mAb causes insufficient modulation and Treg generation, whereas too much mAb could lead to stimulation of the effector T cells and cytokine release. Multiple clinical trials have been initiated and were based on two different antibodies, both fully humanized IgG1, nonmitogenic and specific to human CD3 (Table 4): Teplizumab (United States trials) and TRX4/Otelixizumab (European trials) (54, 477). Teplizumab halted progression of recent-onset T1D for more than 1 year in most patients (phase II). Three years after treatment, the patients continued to have better preservation of C-peptide levels and a lower use of insulin compared with control groups (185, 186). TRX4/Otelixizumab also preserved beta-cell function very efficiently and decreased the insulin requirements drastically, even 18 mo after single course treatment (228). However, none of these treatments achieved euglycemia. The European study also revealed two side effects. First, anti-idiotypic antibodies were detected 2–3 wk after injection of the drug. This should only become a problem when repeated treatment is needed. Second, there was reactivation of Epstein-Barr virus, but this was transient, self-limiting, and isolated (227).

Other approaches using polyclonal anti-T cell antibody (ALS) (314) and anti-thymocyte globulin (ATG) might be able to temporarily eliminate a larger proportion of T cells from the bloodstream. In NOD mice, murine ATG can prevent diabetes in a late stage and can induce Tregs (398). It is unclear whether ATG will be as efficient as anti-CD3 (327). Treatment of T1D patients with rATG (ATG-Fresenius) prolonged the honeymoon period and improved the stimulated C-peptide levels up to 12 mo into the study (379). Consequently, ATG monotherapy is now tested in a phase II trial, the Study of Thymoglobulin to Arrest T1D (START). But ATG treatment also carries risks. ATG can cause cytokine release syndrome and maybe even a lymphopenia-induced outgrowth of autoreactive T cells, as was shown for other depletion-based immunosuppression (294, 392). Combination treatment with equine ATG and prednisone, a steroid that can counteract the cytokine release syndrome, led to a prolonged honeymoon phase in new-onset T1D (125). But most promising in the ATG trials was that some subjects went into complete remission and were insulin independent for at least 1 mo.

An immunomodulatory drug adopted from the transplantation arena is the CD20-specific mAb rituximab (Rituxan; Genentech and Biogen Idec). Rituximab aims to deplete a potentially potent antigen-presenting population of B cells without affecting long-lived antibody-producing plasma cells (197). Data from NOD mice clearly show that B cells are required for T1D (388). A phase II clinical trial showed some preservation of C-peptide levels for 3–6 mo (334), showing that B cells also contribute to pathogenesis in human T1D. However, the efficacy is small compared with anti-CD3 treatments (186, 228). Perhaps Ocrelizumab, a humanized anti-CD20 antibody successfully used in RA (154), might increase efficacy of B-cell depletion in T1D.

Anti-CD25 mAbs such as basiliximab (chimeric mouse-human monoclonal antibody) and daclizumab (humanized IgG1 mAb) do not cause a cytokine release syndrome. Therefore, they are increasingly being used in place of ATG as an induction therapy. However, in T1D, anti-CD25 mAbs are only used in combination therapies (see below).

Another class of targets consists of costimulatory molecules. CTLA-4-Ig (Abatacept), CTLA-4 fused to an immunoglobulin chain, interferes with costimulation of T cells. Classically, CD28/B7 interactions mediate costimulation and significantly enhance peripheral T-cell responses. In contrast, CTLA-4, interacting with the same B7 molecules, dampens T-cell activity. So, CTLA-4-Ig likely mediates its profound effects by preventing positive costimulation of CD28 by B7 during activation. This results in limited clonal expansion, induction of passive cell death, and IDO production in APCs (reviewed in Refs. 50, 376). The safety profile of CTLA-4-Ig treatment might be better than other immunosuppressive agents, because CTLA-4-Ig does not deplete T cells. However, because of the role of CD28 in Treg development and survival (376), CTLA-4-Ig may negatively affect Tregs. That said, CTLA-4-Ig therapy did not affect Tregs in renal transplantation (140). CTLA-4-Ig monotherapy is currently in a phase II clinical trial (data not published). Moreover, a high-affinity variant of CTLA4-Ig (LEA29Y, belatacept) (239) is being tested in islet transplantation in a phase I/II trial (NCT00501709). And the LEA29Y Emory Edmonton Protocol (LEEP, NCT00468403) phase II clinical trial combines CTLA-4-Ig with daclizumab or basiliximab (against acute transplant rejection) and mycophenolate mofetil (maintenance immunosuppressive therapy).

Manipulation of cytokines has seen a revival recently. Both the cytokines that affect T-cell responses (e.g., IL-2, IL-15) and the cytokines that play a role in inflammation and beta-cell death are considered as targets. It is known that IL-1 is selectively cytotoxic to rodent and human beta-cells in vitro. Anti-IL-1 therapies can reduce diabetes incidence in animal prevention models (41, 268, 279). As in RA, multiple clinical trials assess whether anti-IL1 therapy may be useful in the treatment of T1D. Results from a completed phase I/II trial using IL-1RA (anakinra, Kineret by Amgen) (116) in newly diagnosed T1D have not been released yet (335). In an ongoing phase II/III, patients will inject themselves with Anakinra once a day for two years, which requires a big commitment on their part. Also, three phase II trials are pending. One will test Canakinumab, a fully human anti-IL-1β monoclonal antibody, in new-onset T1D patients. Another one will test the anti-IL-1 mAb Xoma52 in established (>2 year), well-controlled T1D. The RID-1 study will test Rilonacept, a dimeric fusion protein that acts as cytokine trap for IL-1β, after satisfactory safety data in gouty arthritis (427).

The cytokine tumor necrosis factor (TNF)-α is a "master regulator" of the inflammatory response in many organ systems (139). TNF-α antagonists, such as etanercept (a soluble TNF-receptor) and infliximab (an antibody), are already used with success in RA. In T1D, phase II trial data showed that treatment with etanercept lowered HbA1C and increased endogenous insulin production, indicative of the preservation of beta-cell function (274). However, the story is not that simple. TNF-α might play a dual role in T1D. For instance, TNF and a TNFR2 agonist can selectively kill human autoreactive CD8 T cells (31). In animal models, TNF-α propels or lessens the diabetogenic response early or late in the T1D process, respectively (94, 207, 241, 480). Adding to the confusion are some clinical case reports documenting the development of T1D in arthritis (JIA or RA) patients following etanercept treatment (49, 60, 418), but also the resolution of T1D in patients requiring anti-TNF-α therapy for RA (18). These opposing outcomes need to be clarified soon. Of note, Bacille Calmette-Guerin (BCG) vaccination raises the systemic levels of TNF-α and was used in an unsuccessful interventional trial. A follow up study will determine better timing and dosage (13, 130).

Others have hypothesized that autoimmunity is due to lack of type I IFN. Type I IFNs can counteract type II IFN, which is likely a central factor in autoimmune inflammation (65). Clinical trials have so far shown that low-dose ingested rhIFN-α is safe and more efficacious at preserving C-peptide levels compared with high doses (66, 369). Mechanistically, ingested rhIFN-α reduced TNF-α levels in MS patients, indicating a link with TNF-α blockade therapy (67). However, this whole hypothesis is controversial. Others suggest that activation of TLRs by double-stranded RNA or poly I:C (viral mimic) through

induction of IFN-α may activate or accelerate immune-mediated beta-cell destruction (113).

Granulocyte colony stimulating factor (GCSF), a neutrophil mobilizing agent, prevents diabetes in NOD mice by induction of both tolerogenic dendritic cells and Tregs (216, 371). Safety and C-peptide preservation upon GCSF therapy (Neulasta) are currently being tested in a phase I/II clinical trial. A combination trial with ATG is also underway (see below).

Another angle of research aims to delay the demise of beta-cells by reducing the amount of insulin they secrete. This is anticipated to reduce beta-cell stress associated with the diabetic state and might also reduce the presented autoantigens, such as (pro)insulin. Diazoxide, an ATP-sensitive potassium channel opener, showed some preservation of residual insulin production in recent-onset T1D patients, but also substantial side effects (321). A recent phase IV trial indicated that doses that do not cause side effects are also inefficacious at preserving beta-cell function (168).

C. Cell-Based Tolerogenic Therapy

Propelled by evidence from animal models, cell-based tolerogenic immunotherapy has gained momentum (Table 5). The idea is to compensate a presumed deficiency by transferring cell types with immunomodulatory capacity.

Cellular immunotherapy with autologous Treg represents an attractive and feasible approach for curing T1D (69, 71). This was first indicated by the reestablished immune tolerance after adoptive transfer of autoantigen-specific Treg or Tr1 into NOD mice (422, 424, 485). Planned clinical trials aim to treat T1D by isolating the patient's Tregs for expansion outside the body and reinfusion of larger numbers (167). Experts in the field acknowledge the numerous technical problems that are likely to be encountered: a bona fide set of markers for "pure" human Tregs [currently set at CD4$^+$CD127$^{low/minus}$CD25$^+$ (343, 420)], a low frequency in the circulation ($\sim$5–7% of CD4$^+$ T cells)(28), the number of cells to be transferred, the frequency of transfers, in vitro expansion methods, the survival of these cells in vivo, correct homing to the target tissue, the inability to eliminate the transferred cells, and instability of the regulatory function (300, 496). Therefore, the field is divided into believers and nonbelievers. Some see cell-based tolerogenic therapy as a viable, routine clinical approach. Others prefer to target beta-cell antigens in conjunction with small molecules or mAb to augment islet-specific immunoregulatory cells directly in vivo.

Immunoregulatory dendritic cells (iDC) can also prevent diabetes in NOD mice (179, 261). In current clinical trials in Pittsburgh, autologous monocyte-derived DCs are treated ex vivo with antisense phosphorothioate-modified

TABLE 5. *Intervention trials and cell-based monotherapy*

Agent	Target/Mechanism	Phase, ID, Organizer	Details	Reference Nos.
T regulatory cell therapy	Augment Treg numbers	*Phase I (pending)*	Risk of conversion Treg to Teff (496)	343, 344, 422
Adult human mesenchymal stem cells (Prochymal)	Reduce inflammation and assist in tissue repair	*Phase II* NCT00690066 Osiris	Ongoing	
Autologous umbilical cord blood infusion	Enhance T regulatory cell numbers	*Phase I/II* Florida Univ, JDRF NCT00305344	No effect on C-peptide preservation No infusion-related adverse events	
Hematopoietic stem cells	Immune resetting effect	*Phase I/II*	Some insulin independence, C-peptide AUC increased, HbA1C lower	100, 101, 458
		NCT00315133	Bilateral nosocomial pneumonia, late endocrine dysfunction, oligospermia, but no mortality	
		Sao Paulo Univ		
Autologous dendritic cell therapy	Tolerogenic vaccine	*Phase I* NCT00445913 Pittsburgh Univ	Ongoing	179, 261

oligonucleotides that target the primary transcripts of the CD40, CD80, and CD86 costimulatory molecules. One concern is that instability of the antisense knockdown might allow reexpression of the targeted molecules. Also, the therapy rationale promotes the production of IL-7 by iDC as survival factor for Treg. But IL-7 is also important for naive and memory T cells (118, 179, 415), so presumably also for autoreactive T cells. Of note, B cells with a regulatory phenotype were augmented in some of the patients who received the immunomodulatory DCs (Massimo Trucco, personal communication).

D. Replacing Beta-Cell Shortage

The autoimmune attack in T1D reduces beta-cell mass and/or function to a critical point at which clinical diabetes becomes apparent (Figs. 1, *A* and *B*, and 2). Some treatments aim to directly compensate for this loss in beta-cells. In its simplest and most commonly used form, this is done by insulin injections. Other treatments increase beta-cell mass or function by exploiting the regenerative capacity that beta-cells display in response to the autoimmune attack (394) or nonautoimmune stimuli (11, 306). Five angles are considered to replace beta-cell shortage (Table 4, *bottom*): *1*) stimulation of insulin secretion, *2*) islet neogenesis from progenitor cells, *3*) islet regeneration from existing beta-cell mass, *4*) islet transplantation, and *5*) transplantation of stem cells. So far, all methods of beta-cell mass restoration encounter immunological problems. This is because the newly generated or transplanted beta-cells continue to provide antigens that are equally susceptible to autoimmune attacks (462). Islet transplantation and allogeneic stem cell approaches have the additional problem of an alloimmune response. This is why most islet grafts are lost within the first 4–5 years, despite the use of immunosuppressive cocktails (178, 372). These approaches will benefit from im-

proved strategies to control autoimmunity long-term, likely via combination therapies.

1. Stimulation of insulin secretion

Adapted from T2D treatment, analogs of the incretin hormone glucagon-like peptide-1 (GLP-1) (341) with sufficient half-life stimulate insulin secretion in the remaining beta-cells. Examples are Exenatide (Byetta by Amylin Pharmaceuticals and Eli Lilly), a synthetic version of the exendin-4 hormone found in the saliva of the Gila monster, and Liraglutide (Victoza by NovoNordisk), a GLP-1 analog that binds to albumin for slow release (76, 104). GLP-1 receptor activation modestly delayed diabetes onset in NOD mice (171). Mechanistically, exenatide not only stimulates insulin secretion, but might also enhance beta-cell replication and neogenesis in rats (478), protect against IFN-γ-mediated beta-cell death (102), and increase Treg frequency in NOD mice (479). However, the effects on beta-cell mass and the immune system are controversial. Exenatide monotreatment is already in phase IV trial, but a combination trial of exenatide with daclizumab yielded disappointing results (see below and Ref. 370). Liraglutide on the other hand supports the engraftment and function of syngeneic islet transplants in NOD mice (290), which has led a phase II/III trial testing Liraglutide monotherapy.

Another approach to increase insulin secretion is to slow down the physiological degradation of GLP-1. Sitagliptin inhibits the enzyme dipeptidyl peptidase-4 (DPP-4) that is responsible for the destruction of GLP-1. Sitagliptin can prolong islet graft survival (230, 231) and can partially reverse diabetes in NOD mice (408). Clinical trials will examine sitagliptin, either as stand-alone treatment or in combination with islet transplantation or antigen-specific therapy (see below). A phase I trial was also initiated recently to study the impact of DPP-4 inhibitors on the immune system (280).

2. Beta-cell neogenesis

In animal models, gastrin stimulates beta-cell neogenesis without increasing proliferation and hypertrophy, and without reducing beta-cell death (364, 365). Also, the beta-cell mitogenic properties of epidermal growth factor (EGF) can aid in restoring beta-cell mass. In a phase II trial, the EGF analog E1-INT (Transition Therapeutics) reduced daytime insulin usage by 35–75% and helped maintain stable blood glucose control as measured by HbA1c in some of the T1D patients. According to the company, the results were similar in NOD mice. However, others have shown that gastrin and EGF need to be combined to increase beta-cell mass and reverse hyperglycemia in recent-onset NOD mice (352, 412, 413). A phase I study (E1 + G1 INT, NCT00853151) is ongoing.

3. Islet cell regeneration

Islet neogenesis associated protein (INGAP) peptide therapy induces islet cell regeneration from progenitor cells residing in the pancreas in a manner that recapitulates islet development during normal embryogenesis (348). INGAP peptide can increase beta-cell mass and reverse hyperglycemia in animal models (338, 366). In phase I and II trials, INGAP peptide injections are safe and can increase C-peptide secretion, but hardly decrease HbA1c levels (122). A dose optimizing trial is ongoing.

4. Islet transplantation

In most developed countries, pancreas transplantation is the only accepted procedure to achieve normoglycemia (178). The Edmonton case series demonstrated that approximately two-thirds of the recipients enjoy insulin independence for 1 year after receiving their final islet infusion (391). The long-term results are unfortunately less encouraging. Islet function decreases over time so that by 5 years post-transplant <10% of the recipients remain insulin independent (178, 372). Why is this? In short, allosensitization against transplants from multiple donors can be controlled using immune suppression, but the current regimens might inadvertently propel autoreactivity in the long term and even negatively affect beta-cell functionality. In the original Edmonton protocol, patients infused with pancreatic islets from multiple cadaveric donors simultaneously received immune suppression in the form of a humanized anti-CD25 mAb (daclizumab) and continuous administration of low-dose rapamycin (sirolimus), which inhibits the response to IL-2, and FK-506 (tacrolimus), a calcineurin inhibitor blocking IL-2 production. However, this regimen was shown to cause lymphopenia and an elevation of the levels of homeostatic cytokines that drive the expansion of autoreactive CD8 T cells (294, 447). Consequently, the Edmonton protocol has been modified in several ways. For example, ATG plus et-anercept (TNF-α blockade) and maintenance immunosuppression using cyclosporine and everolimus (a derivative of sirolimus) rendered five of six recipients insulin independent at 1 yr, and four of six for an additional 3 yr (40, 391). A recent phase I/II trial will assess whether treatment with anti-CD3 mAb, sirolimus, and low-dose tacrolimus can prevent islet transplant rejection. However, animal studies have already shown that anti-CD3 no longer induces tolerance when tacrolimus was coadministered, even though it continues to immune suppress (90). Another phase II trial will test efficacy of a steroid-free, calcineurin inhibitor-free immunosuppression protocol for islet transplantation (NCT00315627), based on sirolimus, MMF, and Campath-1. Assessment of certain (auto)immune parameters before transplantation might also increase the success rate of islet transplantation. Indeed, T1D patients receiving intraportal islet cells under ATG-tacrolimus-MMF therapy have lower graft function if autoreactive T cells were detected before transplantation (187).

Current immune suppressive drugs can also interfere with beta-cell function (306, 359). More specifically, rapamycin (sirolimus) impairs engraftment (492), interferes with angiogenesis (81), induces insulin resistance (148), and inhibits β-cell replication (489). Rapamycin also, like corticosteroids, tacrolimus (306), and MMF, decreases insulin transcription (reviewed in Ref. 359). Finally, a recent study suggests that MMF also inhibits beta-cell neogenesis (153).

Human islet isolation techniques are still unsatisfactory (336, 407) to yield the ~12,000 islet equivalents per kilogram body weight required to restore insulin-independent normoglycemia in recipients (391). Only ~2,000 subjects in the United States can benefit from an islet transplant each year, because only half of the isolation efforts yield islets suitable for transplantation and recipients usually require islets from multiple donors (392). The use of xenogeneic islets, mostly from pigs or transgenic pigs (82, 181, 449), can fill the gap between supply and demand in islet transplantation. Porcine islets are recognized as the most physiologically compatible xenogeneic insulin-producing cells. Their xenogeneic nature likely requires immunoprotection in capsules that allow the inward passage of nutrients and glucose and the outward passage of insulin (see below).

5. Stem cells

Generation of beta-cells provides an exciting approach towards curing T1D (Table 5). This can be done by differentiation of embryonic stem (ES) cells (234) or pluripotent stem (IPS) cells (487), or the "reprogramming" of cells from their initial phenotype into beta-like cells (494). Stem cells can regenerate the beta-cell mass in vivo, as shown for bone marrow-derived stem cell transfers in immunodeficient mice with chemically induced pancreatic damage (498). But in a phase I/II trial, stem cells from umbilical cord blood assisted

the preservation of C-peptide levels poorly (175). Autologous nonmyeloablative hematopoietic stem cell transplantation (HSCT) yielded better results in newly diagnosed T1D (100, 459). Insulin independence was achieved, but was lost after 4–5 yr in most recipients, and side effects discourage the use of this approach for universal therapy (100).

Stem cells can potentially also be differentiated to beta-cells in vitro. The use of autologous stem cells avoids alloimmune, but not autoimmune, responses to the transplanted beta-like cells. A lot of effort is invested in encapsulation techniques to protect the transplanted cells or islets after implantation. For instance, Viacyte differentiates hESCs into pancreatic endoderm and has plans to subsequently encapsulate these cells in a protective permeable device for transplantation (Encaptra, see website) (106, 234). The idea is that isletlike structures form and become functionally responsive to glucose in vivo. A comparable encapsulation approach had been shown to protect human fibroblast allografts from rejection in rhesus monkeys (423). Also, implantation of primary murine beta-cells encapsulated in a similar device could ameliorate diabetes in NOD mice (242). Most importantly, Viacyte, formerly NovoCell, published that their differentiated hESCs generate glucose-responsive insulin-producing cells that can protect against streptozotocin-induced diabetes in mice (234). However, recent data examining this approach in athymic nude rats could not completely confirm this. Islet-like structures did develop from hESC differentiated to pancreatic endoderm, but the extent of endocrine cell formation and secretory function was considered insufficient to be clinically relevant (282). An alternative, the alginate microcapsule containing porcine islets from DiabeCell, allows some insulin production up to 9.5 years postimplant (132) and is currently being tested in phase I/II (NCT00940173, Living Cell Technologies). Likewise, the Cell Pouch is a device subcutaneously implanted prior to delivery of transplanted cells to allow tissue and blood vessel formation (Sernova). Last, the Sertolin technology codelivers Sertoli cells to provide an immune-protected environment for (islet) cell transplants (Sernova). A small-scale study in humans showed long-term transplant survival and positive effects on metabolic control (444), but extensive clinical trials have not yet begun. Although promising, two problems could arise: *1*) clogging can limit influx of nutrients and glucose and efflux of insulin, and *2*) soluble mediators that elicit beta-cell death can still reach the transplant.

E. Combination Intervention Trials in T1D

Like cancer treatment, T1D therapy might benefit from combining approaches that synergize to reverse autoimmunity, establish tolerance, and limit side effects. We repeat here our previous proposal that combination therapies should achieve three major goals (64): *1*) the "freezing" of the active immune response and dampening of any autoreactive response without strong side effects, *2*) generation of Tregs that can maintain long-term tolerance, and *3*) the regeneration of a critical beta-cell mass to maintain euglycemia without repetitive insulin injections.

We are convinced that combination treatments will become integral to T1D therapy and should therefore transcend licensing issues between the manufacturers of the individual compounds. Because of issues with regulatory affairs, initial steps will most logically use a combination of treatments with either a documented effect as monotherapy in T1D, or a combination treatment that has proven efficacy in another (auto)immune disease (Table 6).

To date, only two clinical trials of a combination therapy have released results, and they are disappointing. The recent phase III trial testing anti-CD25 mAb (daclizumab) in combination with MMF (mofetil, CellCept by Roche) reported no preservation of beta-cell function (163). Anti-CD25 mAbs block the IL-2 signaling pathway in activated T cells, but do not interfere with Tregs (456). MMF is an adjuvant drug that selectively inhibits T- and B-lymphocyte proliferation by suppressing the de novo purine synthesis. In BB rats, MMF and anti-CD25 mAb alone or in combination were shown to delay and prevent diabetes (438). Both systemic immunosuppressants have also proven efficacy in other autoimmune diseases and, in combination, in preventing acute graft rejection (275). Another unsuccessful trial, testing exenatide combined with daclizumab, showed no improved function of the remaining beta-cells in patients with long-standing T1D (21.3 ± 10.7 yr)(370).

A new Proleukin and Rapamune phase I trial will test the combination of IL-2 and rapamycin (which inhibits the response to IL-2). The rationale is that IL-2 promotes the expansion of Treg in favor of effector T cells if the expansion of effectors is simultaneously blocked by rapamycin (347). As a result, deletion of autoreactive Th1 cells causes a shift from Th1- to Th2- and Th3-type cytokine-producing cells. This approach is supported by promising preclinical results showing the prevention of spontaneous T1D onset in NOD (347).

A privately funded clinical trial (Helmsley Trust) will assess whether a combination of ATG and GCSF reverses new-onset diabetes in humans, based on data from NOD mice (327). The rationale is as follows: ATG temporarily reduces T cells in the bloodstream, while GCSF mobilizes granulocytes and hematopoietic stem cells from the bone marrow (428) and induces tolerogenic dendritic cells (216, 371). Additionally, both ATG and GCSF induce a Treg population to ensure long-term protection (216, 398).

The Diamyd-Sitagliptin-Lansoprazole phase II clinical trial is recruiting patients to test the combination of antigen-specific tolerance induction and beta-cell regeneration. GAD-Alum can prevent immune destruction and delay or prevent diabetes onset in NOD mice (see above) (7, 8, 256, 349). Sitagliptin indirectly stimulates insulin secretion (see

TABLE 6. *Intervention trials and combination therapy*

Agent	Target/Mechanism	Phase, ID, Organizer	Details	Reference Nos.
Exenatide + daclizumab	Stimulation of insulin secretion and blockade of IL-2 signaling pathway	*Phase II* NCT00064714 NIDDK	Combination of intensified insulin therapy, exenatide, and daclizumab did not induce improved function of remaining beta-cells	370
IL-2 + rapamycin *Proleukin and Rapamune*	Downregulate T effector while sparing T regulatory function Promote Treg	*Phase I* NCT00525889 NIAID, ITN	N/A (recruiting)	347, 420
MMF + anti-CD25 *Zenapax, Daclizumab*	Selectively inhibits T- and B-cell proliferation/blockade of IL-2 signaling pathway	*Phase III* NCT00100178 NIDDK/TrialNet	No preservation of beta-cell function	163
Thymoglobulin + *Neulasta*	Induce Treg and tolerogenic DCs, recruit granulocytes/HSCs	*Phase II* Helmsley Trust		
EGF and gastrin *(E1 +G1 INT)*	Beta-cell regeneration and islet neogenesis	*Phase I* NCT00239148 Transition Therapeutics	Completed, results not released	409, 412, 413
GAD alum (*Diamyd*), Lansoprazole, sitagliptin	GAD-specific immunomodulation, proton pump inhibitor DPP-4 inhibitor	*Phase II* NCT00837759 NIDDK	N/A (recruiting)	8, 230, 231, 256

ATG, anti-thymocyte globulin; AUC, area under curve (for C-peptide levels in glucose tolerance test); BDC, Barbara Davis Center; DCCT, Diabetes Control and Complications Trial; DENIS, Deutsche Nicotinamide Intervention Study; EBV, Epstein-Barr virus, a herpesvirus that causes mononucleosis; ENDIT, European Nicotinamide Diabetes Intervention Trial; GSK, GlaxoSmithKline; ITN, Immune Tolerance Network.

above). Lanzoprazole is a proton-pump inhibitor that provides partial reversal of diabetes in NOD mice, but strong reversal when combined with a DDP-4 inhibitor (408).

IX. PROMISING BENCH-SIDE THERAPEUTICS

Insights from preclinical research are crucial to therapy development, but translation is often intricate. A vast number of interventions have been tested preclinically, many with beneficial outcomes (396), but few have started clinical trials.

A. Insulin Substitution

The many formulations of insulin on the market have dramatically increased the T1D patient's quality of life. Insulin is not a cure, but it will nevertheless remain the major treatment in the short term and probably will be used as supplementation to other therapies in the long term. Continuous blood glucose monitors or closed-loop insulin-delivery systems, like the artificial pancreas, make diabetes management easier, but some problematic issues remain (188). A noteworthy approach with favorable preclinical data is SmartInsulin that consists of a layered, biocompatible, and biodegradable polymer-therapeutic that is bound to an engineered glucose-binding molecule. Insulin is released only when it is unbound by the presence of a specific glucose concentration.

B. Combination Therapies With Immune Modulators and Islet Antigenic Vaccines

We have shown that suboptimal doses of the FcR-nonbinding anti-CD3 F(ab')2 in conjunction with intranasal administration of proinsulin peptide can reverse diabetes in two mouse models of diabetes (63). Insulin-specific Tregs are induced, secrete immunomodulatory cytokines like TGF-β and IL-10, and confer dominant tolerance upon transfer to recent-onset diabetic recipient mice. Follow-up experiments showed the broader applicability of this approach: anti-CD3 in conjunction with a GAD65 plasmid vaccination could synergize strongly in a RIP-LMCV model of T1D (62). However, success depended on the genetic background, possibly due to how antigen is presented to Tregs (62).

C. Combination Therapies Using Immune Modulators and Compounds Enhancing Beta-Cell Mass or Function

Anti-CD3 in conjunction with exenatide combines induction of immune tolerance by FcR-non-binding anti-CD3 mAb with stimulation of insulin secretion of the remaining beta-cells. Given that this approach addresses two parts of the T1D problem, it is anticipated that this approach has a higher chance of success than the beta-cell regenerative agents gastrin and exenatide trials, which lack the immunomodulatory arm required to stop autoimmune attack of the pancreatic beta-cells. That said, favorable results in diabetic

NOD mice have led to a phase II trial of gastrin + exenatide (410, 411), while an anti-CD3 + exenatide trial is anticipated.

D. Cytokine-Based Therapeutics

Interference with cytokines in immune intervention is a complex matter, as underscored by a broad range of efficacy and dangerous side effects. The majority of cytokine manipulations also revealed a dual effect depending on the timing, dose, and route of administration. For instance, both IL-18 and TNF-α accelerate or inhibit T1D in NOD when administrated early or late, respectively (94, 316, 368). This level of complexity obstructs the clinical translation of cytokine targeting strategies. One strategy considered for clinical trial is the combined administration of rapamycin with agonistic IL-2-Fc and antagonistic IL-15-Fc fusion proteins, which has been shown to provide long-term engraftment/tolerance in allotransplant models (493). The idea is to limit effector T-cell activation and expansion (by blocking IL-15 signals) while promoting Tregs (IL-2 and rapamycin). Noteworthy is the essential role of the IL21/IL21R axis to autoimmune diabetes in NOD (416) and its part in genetic susceptibility to T1D (20).

X. OUR CONCLUSIONS

The incidence of T1D increases rapidly, especially in the developed world, and the time of onset shifts towards a younger age. T1D most likely results from an unfortunate combination of genetic susceptibility and exposure to an environmental trigger. The main effector mechanism is clearly an autoimmune reaction, which is also evident at time of clinical diagnosis. This implies two concepts for clinical treatment: *1*) knowledge of the cause is more critical for prevention than for at-onset therapy, and *2*) at-onset or near-onset therapy requires an immune silencing or modulating component. Our current opinion is that we should conduct trials with treatments like anti-CD3 that are effective at onset (alone or in combination), but advance their initiation based on early detection instead of waiting until overt hyperglycemia. Early detection is also required to maximally preserve the remaining beta-cell mass, because the ability to secrete even small amounts of insulin can make disease control easier and help minimize the complications of chronic inadequate glycemic control (194, 229). Screening efforts would ideally unify results from genetic (HLD-DR3/4-DQ2/8, family), metabolomic (lysophosphatidylcholine), and C-peptide release tests with autoantibody titers (insulin, GAD65, ICA512, ZnT08) and autoreactive T-cell assays (Proinsulin, GAD65, I-A2, and ZnT8).

Much of our current understanding of T1D comes from the NOD mouse model. For a more translational focus, it is necessary to look beyond the NOD mouse to take full advantage of the additional models available (461). And even

then, the majority of T1D treatment discovered in mouse models have not yet translated to viable treatments in humans. The landscape of possible treatment has been changed by the prospect that T1D progression may be blocked by the active stimulation of tolerance induced by (auto)antigen-specific immunization to generate Treg. Success will depend on correctly hitting the following four factors on target: *1*) the choice of protein or peptide that is delivered, *2*) the dosage, *3*) the disease stage, and *4*) the route of administration. A combination of computer-driven biosimulation (in silico) and "wet lab" experiments could increase the chance and reduce the time to reveal the sweet spot(s) of immune therapy.

The ultimate goal of autoimmune therapy is to silence the immune attack against self without sacrificing the patient's protective immune response to infections. This is most likely achieved by a therapy that combines a nonspecific immune suppressant (e.g., anti-CD3), antigen-specific induction of Tregs (e.g., Proinsulin, GAD65), and a suitable compound that increases beta-cell mass or function. For patients with C-peptide levels >0.5 (nM), a two-compound therapy might suffice, because preclinical research suggests that "natural" beta-cell regeneration still occurs (228). Patients with C-peptide levels in the 0.2–0.5 range might require additional beta-cell regenerative compounds, like gastrin and exenatide. C-peptide levels <0.2 indicate the need for pancreatic islet transplantation.

The unfortunate reality is that combination therapies using one or more nonapproved drugs are difficult to license (460). The current viewpoint is that each compound of a combination treatment needs to be efficacious and licensed on its own first (281). This should make place for safety trials of individual compounds and efficacy trials for the combination therapy. Also, competing interests obstruct the combination of drugs from different companies. So, major efforts on several fronts are still required to fully realize the benefits of the technological and scientific advances in autoimmune diabetes research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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