Chapter 24
Immunology of ß-Cell Destruction

Daria La Torre and Åke Lernmark

Abstract The pancreatic islet ß-cells are the target for an autoimmune process that eventually results in an inability to control blood glucose due to the lack of insulin. The different steps that eventually lead to the complete loss of the ß-cells are reviewed to include the very first step of a triggering event that initiates the development of ß-cell autoimmunity to the last step of appearance of islet-cell autoantibodies, which may mark that insulitis is about to form. The observations that the initial ß-cell destruction by virus or other environmental factors triggers islet autoimmunity not in the islets but in the draining pancreatic lymph nodes are reviewed along with possible basic mechanisms of loss of tolerance to islet autoantigens. Once islet autoimmunity is established the question is how ß-cells are progressively killed by autoreactive lymphocytes which eventually results in chronic insulitis. Many of these series of events have been dissected in spontaneously diabetic mice or rats, but controlled clinical trials have shown that rodent observations are not always translated into mechanisms in humans. Attempts are therefore needed to clarify the step 1 triggering mechanisms and the step to chronic autoimmune insulitis to develop evidence-based treatment approaches to prevent type 1 diabetes.

Keywords Islet autoimmunity · Autoantigens · Prediction · Prevention · Insulitis · Islet autoantibodies · CD4+ T cells · CD8+ T cells · T regulatory cells · Antigen-presenting cells · Dendritic cells

Abbreviations

APC Antigen-presenting cells
BB Bio breeding
BCR B-cell receptor

D. La Torre (B)
Lund University, CRC, Department of Clinical Sciences, University Hospital MAS, SE-205 02, MALMÖ, Sweden
e-mail: daria.la_torre@med.lu.se

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CTL Cytotoxic T lymphocytes
CTLA-4 Cytolytic T lymphocyte-associated antigen
cTreg Conventional regulatory T
DC Dendritic cells
Fas-L Fas-Ligand
FoxP3 Forkhead–winged helix
GABA Gamma-amino-butyric acid
GAD Glutamic acid decarboxylase
HLA Histocompatibility antigens
HSP Heat-shock protein
IA-2 Insulinoma-associated antigen-2
IAA Insulin autoantibodies
ICAM Intercellular adhesion molecule
IDO Indoleamine 2,3-dioxygenase
IFN Interferon
ICA Islet cell antibodies
ICSA Islet cell surface antibodies
IL Interleukin
iVEC Islet vascular endothelial cells
LFA-1 Leukocyte function-associated antigen-1
NF Nuclear factor
NK Natural killer lymphocyte
NKT Natural killer T
NO Nitric oxide
NOD Non obese diabetic
nTreg Natural regulatory T
PBMC Peripheral blood mononuclear cells
PD-1 Programmed death-1
pDC Plasmacytoid dendritic cell
pLN Pancreatic lymph node
pMHC Peptide-MHC
PRR Pattern recognition receptors
TCR T-cell receptor
TEDDY study The environmental determinants of diabetes in the young
TF Transcription factor
TGF Transforming growth factor
TLR Toll-like receptor
TNF Tumor necrosis factor
Treg Regulatory T cell
TSA Tissue-specific antigen
VNTR Variable nucleotide tandem repeat
ZnT8t Zinc transporter isoform-8

24.1 Background and Historical Perspectives

Immune-mediated selective destruction of the pancreatic islet ß-cells is the hallmark of type 1 diabetes mellitus (T1D), formerly known as insulin-dependent diabetes mellitus [1–4]. The immunogenetic feature of the disease is a polygenic inheritance of susceptibility, which is re ected in a highly polyclonal autoimmune response targeting several ß-cell antigens. The autoimmune response is associated with progressive ß-cell destruction that eventually leads to overt clinical disease. As attested by prospective studies of children at genetic risk for T1D (DIPP, DAISY, and BabyDIAB), the appearance of specific islet autoantibodies marks the initiation of islet autoimmunity and may be detectable for months to years [5, 6] during which time ß-cell dysfunction proceeds asymptomatically. T1D may therefore be viewed as a two-step disease. The first step is the initiation of islet autoimmunity, the second step is precipitation of diabetes when islet autoimmunity has caused a major ß-cell loss (>80%) [6], and insulin deficiency becomes clinically manifest.

At diagnosis, the typical histological finding of affected islets, first described in short-duration diabetes patients at the beginning of last century [7], and termed ‘insulitis’ [8, 9], consists of an infiltrate of in ammatory cells associated with a loss of the ß-cell endocrine subpopulation. The infiltrate consists of mononuclear cells [10, 11] and T and B lymphocytes [12]. Little is known about insulitis during the first step of the disease when subjects have preclinical islet autoimmunity. Recent studies suggest that the mere presence of an islet autoantibody does not predict insulitis [13].

The understanding of T1D etiology and pathogenesis is complicated by the lack of epidemiological data on the first step of the disease. In contrast, the epidemiology of T1D is developing rapidly through registers in many different countries. The incidence is different among age groups, highest among children [14–16], but the disease may occur at any age [17].

Annual incidence shows geographical variation among different countries and ethnic groups, from 0.1 per 100,000 children in parts of Asia and South America to the highest rate in Finland (64.2 per 100,000) [16, 18]. The mode of inheritance is complex as 80–85% of T1D is occurring sporadically [19] and the risk of becoming diabetic is approximately 7% for a sibling and 6% for the children of T1D parents [20].

An autoimmune etiology for T1D was suspected approximately 40 years ago from the association between diabetes and other autoimmune diseases [21–23]. The first attempt to identify an autoimmune reaction toward the endocrine pancreas dates back to 1973, when testing for leukocyte migration inhibition to islet antigens suggested that T1D patients might be sensitized to pancreatic antigens [24]. Nearly concomitantly, T1D was reported to be correlated to histocompatibility antigens (HLA) [25] that govern antigen recognition by immune effectors. Association studies have proved that the greatest contribution to genetic susceptibility to T1D is exerted by *HLA* class II alleles on chromosome 6, the HLA-DQ haplotypes DQ2 and DQ8, and DQ6.2 conferring the highest risk or protection [26–28]. The detailed

mechanisms by which different HLA molecules provide either risk or resistance to T1D is not fully understood [29]. It is possible that different conformations of the MHC molecules pocket yield different peptide-binding properties and in uence antigen presentation by antigen-presenting cells (APC) to effector T cells [30–32].

If genetic background appears to be a prerequisite for the development of ß-cell autoimmune destruction, a major role in the penetrance of a susceptible genotype is played by environmental factors. Virus infections have figured prominently in T1D epidemiological investigations [33–35]. The possible contribution of a virus infection to trigger islet autoimmunity (step 1 of the disease) or to the progression to clinical onset in islet autoantibody-positive individuals needs to be sorted out. The contribution of dietary factors is equally controversial [36–40]. Maternal factors [41], vaccinations [36, 42, 43], or toxins have also been considered [44]. Environmental factors likely account for the low concordance rate for T1D among monozygotic twins (30%) [45–47]. Similarly, the geographic distribution underscores the importance of the environment [3, 48, 49]. The multifactorial etiopathogenesis is also evident in the spontaneous diabetes in the nonobese diabetic (NOD) mouse and the bio breeding (BB) rat employed over the past three decades. These animals have given insights in the immunogenetics of T1D [50, 51], though the utility of these animals in preclinical trials to guide human research has been limited [52].

Although the event that initiates the autoimmune process (step 1) is not yet understood, the fact that it specifically targets the ß-cells promoted the attempt to find which ß-cell-specific antigens could give rise to the abnormal immunological recognition. The interest was initially focused on autoantibodies as useful tools in attempts to identify autoantigenic molecules (Table 24.1) and clarify the pathological immune response. The first description of pancreatic islet autoantibodies was in 1974, when indirect immuno uorescence on frozen human pancreas sections revealed circulating islet cell antibodies (ICA) in the serum of T1D patients with polyendocrine disease [53]. A few years later islet cell surface antibodies (ICSA) were demonstrated in newly diagnosed T1D patients using dispersed cell preparations of rodent pancreatic islets [54]. The molecular characteristics of islet autoantigens remained unknown until the demonstration in 1982 that sera from new-onset T1D patients had autoantibodies immunoprecipitating a 64 kDa protein in isolated human islets [55]. The 64 kDa immunoprecipitate proved in 1990

Table 24.1

to have gamma-amino-butyric acid (GABA)-synthesizing enzymatic activity [56]. Molecular cloning of human islet glutamic acid decarboxylase (GAD) showed that the ß-cells expressed the unique human isoenzyme GAD65 [57]. GAD65 is expressed in several cell types but, apart from some brain neurons, it is mainly localized to synaptic-like microvesicles in the ß-cells. GAD65 is in part responsible for the ß-cell-specific pattern of ICA [58]. Antigenic properties of insulin A and B chains and of the precursor proinsulin were postulated, as this autoantigen would explain ß-cell specificity and have possible physiopathological involvement. In 1983 autoantibodies reacting with insulin (insulin autoantibodies IAA) were demonstrated in T1D patients, uncorrelated to insulin administration [59]. In 1994 trypsin digestion of the 64 kDa immunoprecipitate revealed a 37/40 kDa autoantigen pair, recognized by sera of T1D patients [60]. This observation eventually led to the identification of two members of the tyrosine phosphatase family, sharing 74% of intracellular domain, insulinoma-associated antigen-2 (IA-2) and IA-2 beta (or phogrin) [61], which is probably less involved in T1D autoimmunity [62]. IA-2 is a transmembrane molecule of islet secretory granules and may be physiologically implicated in insulin secretion [63]. More recently, in 2007, autoantibodies to the zinc transporter isoform-8 (ZnT8t) were reported [64]. The ZnT8 protein mediates Zn^{2+} cation transport into the insulin granules, facilitating the formation of insulin crystals [64]. ZnT8 polymorphic variants [65] represent not only targets of islet autoimmunity but also a genetic marker for type 2 diabetes [65].

Continued study of serum samples from T1D patients identified additional candidate targets of the humoral immune response. Autoantigens reported so far have different tissue expression patterns and subcellular localizations, as DNA topoisomerase II [66], heat-shock protein 60 (HSP60) [67], HSP-70 [68], HSP-90 [69], vesicle-associated membrane protein-2 (VAMP2) and inhibitory neuropeptide Y (NPY) [70], carboxypeptidase H (CPH) [71], and others [72–80]. Further definition of this wide array of islet antigens is needed to define autoantigens with a pathogenetic role in islet destruction (step 1) from those that become secondarily available to immune system due to ongoing tissue damage. Thus, their relevance for the prediction of T1D is unclear. Many assays have been used to detect islet autoantibodies, but it was only from the mid-1990s, when recombinant human GAD65 cDNA became available, that simple and reproducible immunoprecipitation assays were developed with in vitro transcribed, translated, radiolabeled antigen [57, 81]. The novel in vitro labeling made large screenings and standardization workshops applicable [82–84]. Their high sensitivity and specificity and early appearance during the autoimmune process made autoantibodies useful clinical markers not only for diagnosis [85] but also for disease prediction [2, 86, 87]. Despite the attested association with T1D, there is no evidence that islet autoantibodies directly contribute to ß-cell damage, though the B lymphocytes producing islet autoantibodies may contribute as APC [88].

Adoptive transfer studies on the NOD mouse suggest that islet damage in T1D is mainly mediated by T-cell effectors [89]. The pivotal role in these mice of cytotoxic CD8+ T cells in the initiation and progression of destructive insulitis [51] and of CD4+ T cells that act as ‘helper’ cells [90] is well recognized. Studies of the

cellular arm in human T1D have detected CD4+ and CD8+ T cells that recognize the same autoantigens as targeted by the humoral arm [91, 92], sometimes with epitope overlapping [68, 93]. As expected, T-cell reactivity to minor autoantigens has also been reported [68, 94, 95]. However, cellular immunoreactivity to islet autoantigens is less easily assessed than the autoantibody response and is not yet applicable in the clinic. Most of the studies performed in the last decade to identify self-reactive T cells in the peripheral blood of T1D patients are based on the indirect detection of T-cell presence, through antigen-induced proliferation assays [96] or cytokine release (ELISPOT) analysis [97]. The latter analysis has limitations, as it does not allow precise enumeration of the cytokine-producing cells and may yield false-negative results if T cells are producing other cytokines than detected in the actual assay. In the last 5 years, the MHC tetramer technique has provided a novel insight into specific T cells and their precursor frequencies. The tetramer resembles the physiological MHC peptide/TCR interaction and offers phenotyping and selective isolation of antigen-specific T cells, upon a stimulation with the tetramer itself [98]. The technique is highly specific for the HLA type and the peptide lodged in the MHC peptide-binding groove. Attempts have been made in addition to further study the structural requirements in transgenic mice expressing the T1D-associated HLA-DR4 and DQ8 to identify peptides recognized by autoantigen-specific T cells [99]. As pointed out in a recent T-cell workshop, traditional in vitro proliferation assays suffer from methodological limitations [100] related to peculiarities of autoreactive T cells as low peripheral frequency [101], rapid number reduction [102, 103], ongoing modification of immunodominant specificity [103], and low TCR avidity [101]. As will be discussed later, these studies have produced inconsistent results [100] and have globally failed to detect marked differences on T cells from T1D patients and controls. The presence of autoreactive T cells in healthy subjects suggests that central tolerance is physiologically incomplete and that T-cell peripheral regulatory phenomena may be strongly involved in the development of the autoimmune process. From the early 1990s, immunoregulation of autoreactive T cells was viewed within the oversimplified model of Th1 and Th2 phenotypes [3]. The simplified notion was that progression of tissue-specific autoimmunity results from a functional imbalance between pathogenic Th1 cells and immunoregulatory Th2 cells. During the past several years it has become clear that a larger number of subsets of immunomodulating regulatory T cells (Treg) exists and contributes to the maintenance of peripheral tolerance [104, 105]. Recent advances point out at a crucial immunomodulating role of a subset of APC called dendritic cells (DC) [106]. It has been hypothesized that DC may be involved in the early breakdown of tolerance, as well as in the maintenance of ß-cell destruction [107].

The understanding of T1D has improved over the years since the rediscovery of insulitis in 1965 [108]. The recognition that T1D is a two-step disease characterized by a long prodrome of islet autoimmunity prior to clinical onset has allowed new hypotheses to be developed, as to the initiation of the ß-cell destructive process. The transition from islet autoimmunity to clinical T1D will also require a redefinition of the role of environmental factors triggering the disease. In this chapter we will review possible mechanisms of induction of ß-cell autoimmunity and the role of

environmental factors in this process. The reader is referred to other recent reviews on this or similar topics [2, 109–111]

24.2 Autoimmune ß-Cell Destruction

24.2.1 Genetic Etiology

The major genetic factor for T1D is HLA-DQ on chromosome 6 [112–114]. Both sib-pair analyses and association studies have indicated in Caucasians that the HLA-DQ A1-B1 haplotypes A1*0301-B1*0302 (DQ8) and A1*0501-B1*0201 (DQ2), alone or in combination (DQ2/8), confer the highest risk for T1D. Nearly 90% of newly diagnosed children carry DQ2/8 (about 30%), DQ8, or DQ2 in combination with other haplotypes [115]. Among the many haplotypes there are combinations, in particular with the DQA1*0201-B1*0602 (DQ6.2) haplotype, which is negatively associated (protective) with T1D. However, the effect is attenuated with increasing age [116]. The rising incidence of T1D is, however, puzzling as it is associated with a reduced overall contribution of high-risk HLA types in parallel with an increase in DQ8 and DQ2 combinations which did not confer risk 20 years ago [117–119]. The mechanisms by which DQ8, DQ2, or both increases the risk for T1D are not fully clarified. The function of the DQ heterodimers to present antigenic peptides to the immune system is well understood. It remains to be determined why the DQ2/8 heterozygocity is associated with a young age at onset [120]. It has been speculated that the DQ2 and DQ8 molecules are important to maintain central or peripheral tolerance to the ß-cell autoantigens GAD65, IA-2, insulin, or ZnT8. This possibility needs further exploration as it cannot be excluded that the primary association between T1D and HLA is the ‘step 1’ part of the disease rather than the progression to clinical onset. This hypothesis is supported by the observation that the presence in healthy subjects of GAD65 autoantibodies is associated with DQ2 and IA-2 autoantibodies with DQ8 [86, 121].

Several investigations suggest that HLA contributes about 60% to the genetic risk of T1D [122]. Major efforts have therefore been made to identify non-HLA genetic risk factors for type 1 diabetes [112]. These studies have been highly rewarding as more than 40 genetic factors (see examples in Table 24.2) have been found to contribute [112]. Interestingly enough, many of the genetic factors are important to the function of the immune system. *PTPN22* is a regulator of T-cell function and a genetic polymorphism results in a phosphatase variant that is increasing the risk not only for T1D but also for rheumatoid arthritis, juvenile rheumatoid arthritis, systemic lupus erythematosus, Graves’ disease, generalized vitiligo, and other human autoimmune diseases [123]. The *PTPN22* polymorphism seems in particular to affect progression from pre-diabetes to clinical disease [124] also in individuals with lower risk HLA genotypes [125]. The variable nucleotide tandem repeat in the promoter region of the insulin gene *INS VNTR* seems to contribute to T1D by the mechanisms of central tolerance [126]. In newly diagnosed T1D patients the presence of insulin autoantibodies is associated with the *INS VNTR* polymorphism [120]

Table 24.2

The many other genetic factors listed in Table 24.2 are all shown to be significantly associated with T1D [112]. The function of these genes is understood individually; however, it is not clear how these factors may interact to increase the risk for the development of islet autoimmunity (step 1), T1D (step two), or both. The majority of the genetic factors seem to be associated with the immune system (Table 24.2). It is therefore attractive to speculate that their contribution is related to the ability of the immune system to mount an autoimmune reaction specifically directed toward the islet ß-cells.

24.2.2 Immune Cells in Tolerance

Epitope presentation to T and B cells is the key step in the generation of tolerance, in its early failure, and during the maintenance of autoimmunity. The capacity to distinguish between self and nonself, which is the hallmark of a functional immune system, is lost when central and peripheral tolerization fail, leading to the development and expansion of autoreactive pathogenic effector cells. Central tolerance is induced at the site of lymphocyte development (the thymus and bone marrow, respectively, for T and B cells), while peripheral tolerance occurs at sites of antigen recognition, namely in lymphoid and non-lymphoid tissues. Central to the function of tolerance are APC.

24.2.2.1 APC

The recognition by T and B lymphocytes of antigens presented in the context of MHC surface of an APC is the first step of the adaptive immune response. Macrophages and particularly DC are the most efficient APC, as they show constitutive expression of MHC class II molecules, cytokine secretion, and migrating capacity [127]. APC have a dual role: uptake, processing, and presentation of antigens to T cells and regulating T-cell-driven responses through cytokine release. APC are involved in T-cell tolerance mechanisms at both central (clonal deletion) and peripheral level (clonal anergy). Negative selection of autoreactive clonotypes derived by random T-cell receptor (TCR) rearrangement is guided by T-cell affinity for self-peptide–MHC (pMHC) complexes presented in the thymus [128, 129]. An inadequate binding affinity spares self-reactive T cells from apoptosis [130]. The thymic expression of tissue-specific antigens (TSA) is regulated by the autoimmune regulatory (AIRE) transcription factor [131]. Insufficient level of expression and presentation of TSA-derived peptides is observed in subjects with a mutated *AIRE* gene. In mice, ß-cell-derived proteins have been found to be expressed on the surface of thymic epithelial (TEC) and medullary (mTEC) cells and DC [132, 128] . It is possible that some ß-cell autoantigens are not present in the thymus at sufficient concentrations to induce negative selection. This mechanism may explain the correlation of T1D protection with the ‘long form’ of *INS VNTR* [133, 134]. The number of ‘tandem repeats’ modulates thymic expression of this autoantigen and the ‘long variant’ results in increased insulin mRNA within the thymus

[133, 134]. This higher thymic insulin expression is thought to enhance the deletion of insulin-specific thymocytes and may account for the protective phenotype [135, 136].

Similarly, GAD65 expression in thymus may physiologically contribute to specific central tolerance, but its presence first suggested by immunochemical studies [137] has not been further confirmed [138].

Transcriptional modifications due to alternative splicing have been proposed to explain IA-2 immunogenicity, as IA-2 is not expressed full length in thymus, but in an alternatively spliced transcript derived from the deletion of exon 13 [139]. This may account for the escape of a subset of IA-2-reactive T cells. Interestingly, several B- and T-cell epitopes map to IA-2 exon 13 [140]. So far, it is not clear to what extent central tolerance and thymic expression are important to antigen presentation of the ZnT8 transporter protein. The efficiency of thymic negative selection can also be reduced in case of elevated threshold for clonal deletion, as may result from the single-nucleotide polymorphisms in the *PTPN22* gene associated with T1D [23, 133]. The mutation of this gene, that encodes for a negative regulator of TCR signaling [141], may increase the activation threshold needed for deletion of CD4+ and CD8+ T cells [133, 134, 141].

APC-T-cell interaction in the peripheral lymphoid organs is the key in peripheral tolerization. APC provide costimulatory molecules, such as CD40 [142], and adhesion molecules as leukocyte function antigen 1 (LFA-1) and intercellular adhesion molecule 1 (ICAM-1) that are necessary to activate naïve T cells [111] and molecules of the B7 family [143]. Without coactivation of B7-CD28 on the T-cell surface, the MHC-TCR signaling induces apoptosis of naïve T cells, mediated by upregulation of ‘Activation-Induced Cell Death’ [144]. Surviving T cells become anergic, i.e., unresponsive to subsequent antigen stimulation, through an active process involving a number of anergy factors [145]. T-cell reactivity is also controlled by negative regulatory receptors as cytolytic T lymphocyte-associated antigen (CTLA)-4 which binds to CD86 on the APC [146] and attenuates T-cell activation by competing for B7-CD28 ligation [147], and programmed death-1 (PD-1) [148]. The susceptibility to T1D associated to some splice variants of the human *CTLA-4* gene may be due to reduced expression of CTLA-4 and insufficient costimulatory molecule blockade [149] .

Among APC, DC are peculiar, highly specialized effectors with ontogenic, morphologic, and functional heterogeneity and can be mainly divided into conventional or myeloid DC (mDC) and plasmacytoid DC (pDC), upon superficial clusters of differentiation and secretive function [150]. pDC are potent productors of IFN-a and are connected to the innate immune system through the expression of toll-like receptors (TLR) specific for the detection of viral infections [150, 151]. Emerging evidence suggests a close relationship between pDC and autoimmune conditions [109]. In healthy subjects, autoantigen-bearing DC are physiologically found in blood, peripheral lymphoid organs, and thymus, where they are important source of TSA [137] . DC are also reported to display proinsulin epitopes through direct transcriptional events in a capture-independent way [152]. DC are thought to be the only APC effective in ‘cross-presentation’ [153], which is an

unconventional mechanism for processing and presenting exogenous antigens in the context of MHC I molecules directly to cytotoxic T lymphocytes (CTL) [153, 154]. Cross-presentation of parenchymal antigens is thought to be involved mainly in the detection of viral infections [154] but has also been proposed to contribute to peripheral tolerance [155]. The different outcome of the interaction between T cells and DC is ruled by their maturative status. Under homeostatic conditions, DC are in an immature status, which is predominantly tolerogenic and characterized by low expression of costimulatory factors as CD40, CD80, and CD86 [107, 156]. This homeostatic status may be maintained through the downregulation of nuclear factor- B(NF- B), which is a critical transcription factor for many genes involved in APC activation in mice [157]. After the activation by an antigen, DC undergo maturation, express pMHC complexes, and promote antigen-specific T-cell clonal expansion. At this mature stage, DC are generally immunogenic and produce costimulatory molecules and several cytokines. Among them, interleukin (IL) 12p70, which promotes differentiation of CD4+ T helper cells (Th0) and CD8+ effectors, IL-1-ß, tumor necrosis factor (TNF) alfa, and interferon (IFN)- [107]. Under specific conditions, such as transforming growth factor (TGF) beta and IL-10-enriched environment, mature DC can develop tolerogenic properties [158], secrete cytokines as IL-10 [159], which inhibits the activation of other APC, and promote antigen-specific expansion of Treg subsets [158–160]. Tolerogenic DC may also inhibit T-cell proliferation through the enzyme indoleamine 2,3-dioxygenase (IDO) [161] or directly induce T cells apoptosis through PD ligand 1 (PDL-1) [162]. In the NOD mouse, the binding of PDL-1 to the T-cell PD-1 receptor downregulates the priming of diabetogenic T cells in early stages of diabetes and inhibits islet destruction at a later phase [163].

The physiology of B cells as APC indicates that these cells are able to take up antigen at very low concentrations through their antigen-specific membrane-bound immunoglobulin [164] and to present it to T cells. The antigen presentation is enhanced in the presence of specific autoantibodies [165]. In the NOD mouse, antigen presentation by B cells may be important for the initiation of the autoimmune attack [166] and for the spreading of T- and B-cell determinants during the progression of the disease [167, 168]. A recent study on human B cells in T1D showed that B cells may regulate the autoimmune T-cell repertoire by enhancing the presentation of determinants located outside the B-cell immunodominant area [169]. Moreover, the minute amounts of antigen presented by B cells may be important for the maintenance of autoimmune reactivity in the later phase, once most of the target tissue has been destroyed [169]. Of interest, HLA-restricted B- and T-cell epitopes are in close proximity within the GAD65 molecule [170], and recently an overlapping within T and B IA-2 epitopes has been described [171]. These observations suggest that antigen–antibody complexes may in uence antigen presentation by APC and thereby T-cell reactivity [170]. The T- and B-cell synapse has been discussed in a recent review [111]. However, there are major gaps in our understanding of the possible importance of the T–B-cell synapse within the human islets of Langerhans. B cells are also the most frequent APC expressing CD1d, the restriction molecule responsible for antigen presentation to natural killer T (NKT) cells, a T-cell subset

linking the innate and adaptive immune system with a still controversial role in ß-cell destruction [172].

There is wide evidence from studies on T1D pancreas with insulitis that MHC class II expression is increased on islet vascular endothelial cells (iVEC) [11, 12, 173], as a result of de novo expression induced by the in ammatory cytokine IFN- [174]. The possibility that human VEC can act as APC and present exogenous antigens on HLA class II molecules to CD4+ T cells was reported previously [175]. More recent data on iVEC suggest that these cells are capable to internalize, process, and present disease-relevant epitopes from GAD65 [174] and insulin [176]. The in vivo acquisition of these autoantigens by iVEC is not clearly established. Since iVEC are physiologically exposed to very high insulin concentration, it is likely that these cells take up insulin and process it into peptides through endosomal degradation, rather than acquire peptides or pMHC complexes produced by ß-cells [176]. The mechanism is even more unclear for non-secreted antigens. Although it is uncertain whether islet vascular endothelium has any prominent role in the priming of autoreactive T cells, given the recognized importance of professional APC, it has been suggested that iVEC may be important for the trafficking of activated T cells providing antigen-driven homing specificity [176].

24.2.2.2 T Cells

Recent progress in studying peripheral tolerance has highlighted the importance of immunoregulation by Treg, co-expressing CD4 and the alfa-chain of the IL-2 receptor complex (CD 25) [177]. Treg are potent suppressors of organ-specific autoimmunity [105]. Natural Treg (nTreg) originate from intrathymic recognition of self-pMHC complexes [177, 178] and are functionally marked by the constitutive expression of forkhead–winged helix transcription factor (FoxP3) [179], while conventional Treg (cTreg) differentiate from naïve CD4+ T cells in the periphery [104]. Although FoxP3 plays a major role in Treg development and activity, as mutations in *FOXP3* gene in humans determine severe multi-organ autoimmunity (IPEX syndrome) [180], Treg function is complex (181) and involves other transcriptional signaling as TGF-ß, IL-2, and possibly others [182]. The possible dysregulation of IL-2 signaling in Treg suppressor activity is supported by the association of T1D and polymorphisms within the IL-2 receptor alfa gene region in humans [134, 183]. Immunoregulation by Treg affects T cells, B cells, and APC antigen-specific cellular responses in different manners, including production of anti-in ammatory cytokines (TGF-ß, IL-10, and IL-35) and contact-dependent mechanisms [184], possibly involving CTLA-4 and direct cytolysis [178]. The primary site at which nTreg control ß-cell autoimmunity is within the islet infiltrate on CTL and in ammatory cells [185]. In the secondary lymphoid tissue, nTreg regulate DC activation inducing DC secretion of IDO [161], by binding CD80/CD86 via CTLA-4, and prevent the priming of naïve autoreactive CD4+ and CD8+ T cells [186]. cTreg are distinguished based on their cytokine secretion pattern [104, 187] and can produce IL-4 (‘Th2-like’ cells), TGF-ß (‘Th3-like’ cells), and IL-10 [187, 188]. IL-10 is a potent systemic immune suppressor that regulates activation, proliferation, and

IFN- release by effector T cells [189] and indirectly controls DC activity [190]. IL-10 producing cTreg have also been reported to mediate a direct suppression by cell–cell contact, independently of IL-10 secretion [191].

24.2.2.3 B Cells

Little is known about self-tolerance mechanisms for B cells [192]. Immature B cells in the bone marrow are expressing a potentially polyreactive B-cell receptor (BCR), which results from stochastic gene recombination. B peripheral maturation process is thought to involve three checkpoints [193]. Twenty to fifty percent of autoreactive immature B cells undergo rearrangement of immunoglobulin light-chain genes and replacement of self-reactive BCR through a process called ‘receptor editing’; the remaining self-reactive B cells undergo peripheral deletion or peripheral anergy [193]. The extent to which deletion and anergy contribute to B-cell tolerance has not yet been determined. Although evidences of aberrant receptor editing have been associated with autoimmunity in mouse and human diseases [194], to what extent these defects participate in the establishment of autoimmunity is still unclear.

24.2.3 What Happens in the Islet?

It is presently unclear whether in humans the initiation of autoimmune ß-cell destruction requires autoreactive T cells simultaneously recognizing multiple ß-cell antigens, or if T cells primarily target a single antigen. In the mouse, the chronology of appearance of islet T-cell reactivity suggests GAD65 as a triggering antigen [195]. Knock-out mice indicate a key role for insulin or proinsulin [196]. At the time of diagnosis in human T1D, patients exhibit autoimmune responses to a number of islet-cell antigens [17]. These responses representing ‘antigen spreading’ may be explained with the release of previously sequestered immunogenic proteins during the ongoing ß-cell damage, as the clinical onset is manifest when more than 80% of the islets have been destroyed. The variability in reactivity to individual antigens may in turn be due to ‘epitope spreading,’ which consists of intramolecular shifting of the recognized epitopes with the progression of the autoimmune attack, and subsequent activation of new T-cell clonotypes. These events may provide an explanation for the widely diversified anti-islet immune response in T1D.

Priming of naïve CD4+ T cells by islets antigens presenting APC would be the first event in initiating islet autoimmunity (step 1) and diabetogenesis (step two) (Fig. 24.1). This event most likely takes place in the pancreatic lymph nodes (pLN). Islet antigen presentation in pLN has been demonstrated in the mouse [197, 198], but the detailed mechanisms in humans are unclear. What promotes the earliest event, namely uptake of antigen by APC in the islets, is still a matter of debate. Initial, still not fully characterized, insults (virus infection or other external damage, for example, environmental toxins) may elicit an innate immune response through the generation of exogenous or endogenous ligands for the pattern recognition receptors (PRR) on the ß-cell surface. The activation of these receptor triggers

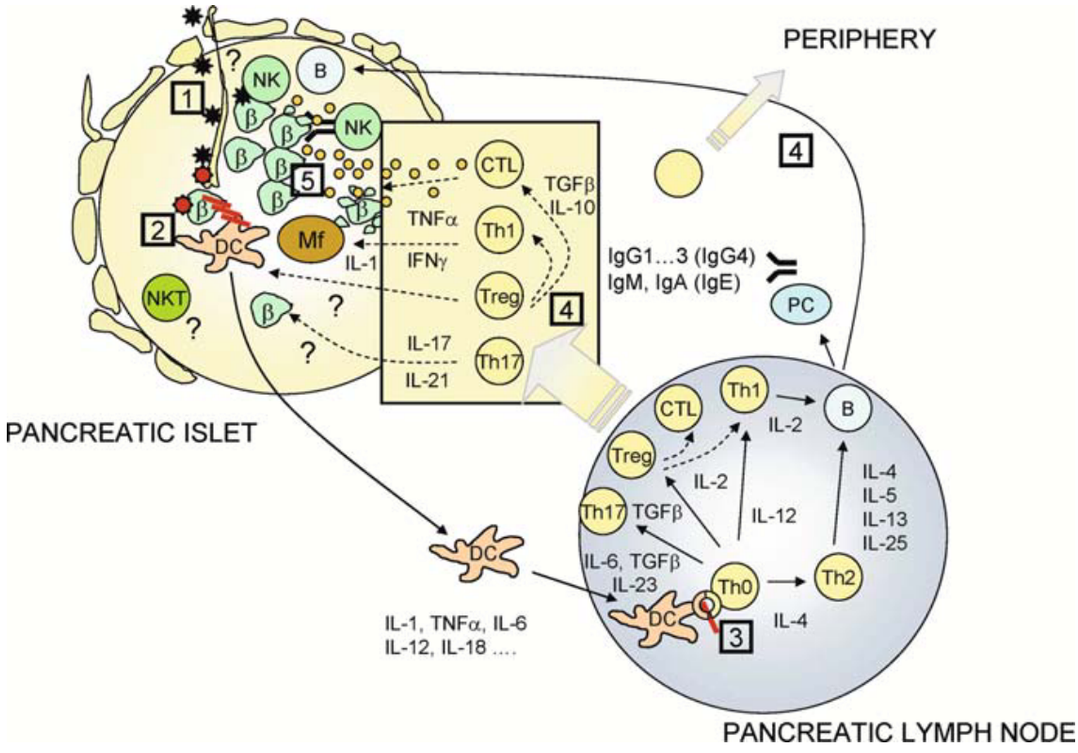

Fig. 24.1 Schematic view on possible immunopathogenesis of ß-cell destruction. **Steps of events:** (**1**) Environmental factors are conditioning the relevant milieu by activation of dendritic cells (DC), macrophages (Mf), natural killer (NK) cells, and natural killer T cells (NKT); (**2**) Intake of antigens or cross-reactive peptides by dendritic cells (DC); (**3**) Presentation of peptides to naive T helper (Th0) cells and subsequent activation and proliferation of type 1 (Th1) and type 2 (Th2) helper cells, IL-17-producing helper cells (Th17),regulatory T(Treg) cells, cytotoxic Tcells (CTL), B and plasma cells (PC), and activation of different cell subsets by cytokines; (**4**) Migration of activated cells from pancreatic lymph node to the islets, cross talk with periphery; (**5**) ß-cells destruction by cytokine- and perforin/granzyme-mediated mechanisms. – environmental factor (virus etc.); – islet antigens; – islet antigens or cross-reactive peptides; – islet-specific T cell; Ab – autoantibodies

intracellular responses including cytokine production, endoplasmic reticulum stress, and accumulation of misfolded proteins, which result in ß-cell apoptosis and promote local in ammation [110]. Notably, mouse experiments suggest that ß-cell apoptosis is a required step for T-cell activation [199]. Dying ß-cells may release immunostimulatory ‘danger’ signals, physiologically aimed at eliminating the initial harmful factor. This requires a transfer to adaptive immune response mediated by the enrollment of APC and the establishment of a pro-in ammatory local environment (IFN, IL-1-ß, and chemokines) to attract other immune cells. A defective resolution of the early in ammation results in a chronic destructive autoimmune reaction and may be dependent on the individual genetic background. For example, the DR3-DQ2 haplotype seems to be permissive of organ-specific autoimmunity [200, 201].

It is evident in the NOD mouse that DC are the first cells to infiltrate the islets [201]. Similar observations have been made in the BB rat [202] as well as in human

T1D [203]. The scavenging function of DC has been proven also in other mouse strains, in which physiological islet-cell death in pancreas remodeling [204, 205] is followed by DC in situ activation and migration to pLN [204]. Moreover, DC may cross-present peptides derived from apoptotic cells directly onto MHC class I molecules, without processing in the cytosol [206]. Taken together, it is likely that antigens derived from ß-cells dying upon external damage may be taken up by APC in the pancreatic islets and transported to pLN (Fig. 24.1). In humans, this has not been possible to fully demonstrate, though expression of the ß-cell autoantigens proinsulin, GAD65, and IA-2 has been detected in human peripheral DC [137]. Currently, our understanding on possible mechanisms of the very early events in islet autoimmunity relies on studies in animals.

24.2.3.1 Virus-Induced ß-Cell Killing

It has been amply demonstrated that CTL specific to viral antigens are able to kill ß-cells. Transgenic mice have been generated to express viral antigens on the ß-cell surface using the insulin promoter to achieve cell specificity [207, 208]. When the mice are infected with virus, the generated CTL effectively kill the ß-cells. These experiments are proof of principle of CTL-mediated ß-cell killing to generate useful mice for diabetes studies. However, these studies do not provide answers as to what possible pathways a virus may use to enter ß-cells, replicate, and express viral antigens on MHC class I molecules, thereby making the ß-cell a target for virus-specific CTL. Regardless of the numerous reports of TD1 onset following viral diseases [209, 210], no conclusive pathogenic connection has been found between viral infection and human islet autoimmunity [33, 209, 211]. Virus diabetogenicity has been studied in rodents [209] and may be sustained by an aberrant immune response toward the ß-cells. Interestingly, human pancreatic islets increase the expression of innate PRR when infected by virus or exposed to virus-related cytokines as IFN and IL-1-beta [212]. To be recognized by CTL virus antigen peptides need to be presented on MHC class I on the ß-cell surface [213]. The critical question is to what extent a virus-infected ß-cell is copresenting viral and ß-cell antigens on MHC class I molecules . Some viral antigen sequences are similar to self-peptides and may mislead T-cell responses. This phenomenon of ‘molecular mimicry’ has been proposed between PC-2 antigen from Coxsackie B and GAD65 [96], between Rotavirus and IA-2 [214], for rubella [215], and cytomegalovirus [33]. However, it is possible that these events are more relevant to the amplification of the autoimmune process [216] and its maintenance after the resolution of the viral infection, than to the initial triggering of autoimmunity. As previously described, virus may activate ß-cell intracellular signaling that induces altered expression of self-antigens on the ß-cell surface (‘neoantigens’ or ‘cryptic antigens’) and participates to the cascade leading to ß-cell apoptosis and insulitis [110]. Moreover, virus replication in the ß-cell may result in its necrosis and in release of previously sequestered cellular constituents (‘hidden antigens’), lacking induced thymic tolerance [217]. The uptake and presentation

of these self-antigens by APC to CD4+ T cells may eventually lead to the formation of specific autoantibodies. Coxsackie B4 has been isolated from the ß-cells of T1D new-onset patients [218]. Evidence for intracellular replication and induction of altered GAD65 islet expression [219] with production of specific autoantibodies has been reported in Coxsackie-infected mice [220]. In summary, there is wide evidence that virus infections may accelerate islet autoimmunity (step two) leading to a precipitation of clinical onset of T1D. The mechanism may be an increase in insulin resistance or a boost in ß-cell killing induced by the virus infection. The major question to be answered is whether a virus infecting and replicating in human ß-cells induces islet autoimmunity. The ongoing TEDDY (The Environmental Determinants of Diabetes in the Young) study may be able to answer this question [221, 222].

24.2.3.2 Cytotoxin-Induced ß-Cell Killing

Alloxan [223], streptozotocin [223, 224], and the rodenticide Vacor [225] are ß-cell cytotoxic agents. It is important to note that both alloxan and streptozotocin are more toxic to rodent than human ß-cells. Other chemicals that may be potential human beta-cytotoxins are nitrosamine derivatives as well as dietary microbial toxins [226]. Epidemiological data suggest that an increase in nitrate-treated food items enhances the risk for children to develop T1D [227]. Experiments in both rats and mice have given insights into possible mechanisms by which beta-cytotoxic agents may induce islet autoimmunity apart from inducing diabetes by direct ß-cell killing. First, low-dose streptozotocin treatment is inducing insulitis in a MHC and T-cell-dependent fashion [224, 228]. The use of streptozotocin in mice therefore offers a way to kill ß-cells with a toxin that initiate ß-cell autoimmunity. In rats treated with streptozotocin to induce ß-cell destruction [229] it has been possible to detect circulating immunoreactive GAD65 following the ß-cell killing, prior to hyperglycemia [230]. In previous rat studies with streptozotocin it was shown by electron microscopy that ß-cell remnants including insulin granules could be detected in islet macrophages, possibly representing islet APC [13, 231]. Other compounds structurally related to streptozotocin or alloxan have been implicated as possible environmental agents, contributing to human T1D. Most prominently these compounds include the rodenticide pyriminil (Vacor) [225] that induces islet-cell surface antibodies and confirms that ß-cell destruction in humans may cause islet autoimmunity [229].

In summary, several virus and chemical agents directly affecting islet cells may be causative in the initiation of an autoimmune ß-cell destructive process. Alternatively these factors may potentiate a process initiated by other environmental factors, which are currently under scrutiny in the TEDDY study [221, 222]. In individuals prone to develop TD1, environmental chemicals may play a detrimental role by repeat injuries to the pancreatic ß-cells over several years of life and in combination with a poor regenerative capacity of the ß-cell and islet autoimmunity eventually induce diabetes.

24.2.4 Antigen Presentation in Pancreatic Lymph Nodes

Although specific mechanisms in humans remain unclear, APC loaded with ß-cell antigens migrate from the islets to the pLN, where the processed antigens are presented to naïve CD4+ T cells (Th0) (Fig. 24.1). A recent study on human DC showed that antigen-specific DC/CD4+ T cells interaction allows DC migration through the dissolution of podosomes [232]. This led the authors to speculate that the same event may involve neighboring immature DC and induce their recruitment to the site of antigenic stimulation [232]. In the pLN, primed CD4+ T cells proliferate and differentiate into several subsets, as type 1 CD4+ T cells (Th1), IL-17-producing CD4+ T cells (Th17), and Treg cells, and activate naïve CD8+ T and B cells into CTL and plasma cells, respectively. The expansion of CD4+ T cells toward lineages of pro-in ammatory subtype (Th1 and Th17) is mainly promoted by the cytokine milieu, through IL-6, IL-12, and IL-23, whereas a balance toward IL-4 [233], IL-5, IL-13, and IL-25 would decrease the in ammation [234]. Th1 cells release IFN- , which activates macrophages, TNF-a, IL-12, and IL-18 [235]. The recent discovery of Th17 cells that are potent inducers of tissue in ammation and autoimmunity [236] is of interest, as they may have a role in islet destruction, as is reported for the NOD mouse [237]. Activation and differentiation of naïve CD8+ T cells to antigen-specific CTL is dependent on ‘cross-priming,’ namely the cognate recognition of the same antigen by the CD8+ and the CD4+ T cells on the same APC [238]. The interaction between CD40 on APC and CD154 on CD4+ T cells induces upregulation of costimulatory molecules for the activation of the CD8+ T cells [142] and increases the local production of pro-in ammatory cytokines such as TNF-a and IL-12 [239]. Alternatively, IFN- produced by CD8+ T cells could enhance CD4+ T-cell action [3]. The relative contribution of CD4+ and CD8+ T cells on diabetogenesis has been addressed by transfer experiments in mice [240, 241]. When cognate interaction occurs between B cells and activated CD4 + T cells, the B cells differentiate into plasma cells and start to secrete immunoglobulins with the same specificity of the previous membrane-bound immunoglobulin [242], upon stimulation of T-cell-released ‘Th2’ cytokines IL-4 and IL-5.

24.2.5 Homing of T Cells to Islets

Primed ß-cell-specific effector T cells gain access to peripheral non-lymphoid tissues, migrate to the pancreas, and reach the ß-cells [243] (Fig. 24.1). The molecular basis for this directed migration (homing) of autoreactive T cells to the islets and for endothelial transmigration is not completely clarified. The antigen specificity of infiltrating T cells has been amply demonstrated in mice [244, 245] and is reasonably postulated in humans, but the processes guiding islet autoantigen-specific T cells into islets are not known. In pancreas transplantations between monozygotic twins without immunosuppression, islets in the donor pancreas were infiltrated by CD8+ T cells in association with the loss of ß-cell function [246]. These experiments

demonstrate the immunologic memory of the recipient, as well as ß-cell killing by CTL, indicating that autoreactive CTL are reactivated. The mechanism of reactivation is unclear. It has been proposed that T cells can be programmed to a specific tissue tropism through a distinct ‘homing receptor pattern’ acquired at the site of priming [247]. In contrast to naïve cells, primed or memory T cells are significantly less dependent on a costimulatory signal and can proliferate with TCR engagement alone [248]. Upon second contact with cognate antigen in the islet, CTL are retained inside the islet tissue and initiate insulitis [197, 249] (Fig. 24.1). In the islets, ß-cell-specific CTL may recognize antigens expressed on ß-cells in association with MHC class I molecules. MHC class I overexpression on islet cells, previously described in pancreas with insulitis [12, 250, 251], is not likely to be involved in these early phases. It is noted in mouse studies that abrogation of MHC class I on ß-cell does not blunt T-cell activation in pLN [249], but it may contribute to the local retention of self-reactive clonotypes. In the NOD mouse, the early infiltrate consists of activated macrophages and CTL that lead the initial accumulation at the vascular entrance (peri-insulitis), probably under the effect of chemotactic mediators as IL-1 [252] and chemokines as CXCl10 and CCl2 [253] that direct leukocyte migration and activation during the transition to adaptive immunity [110]. Of notice increased islet levels of CXCl10, CCl2, CCl20, and IL-15 are detectable in the NOD mice during the pre-diabetic stage [254, 255]. Although the routine investigation of the early phase (step 1) is not feasible in humans, immunocytochemistry on pancreas biopsy specimens from new-onset T1D patients in Japan indicates the presence of CD8+ T cells and activated macrophages secreting in ammatory cytokines [256]. The ongoing in ammatory islet milieu expands the recruitment of autoreactive CTL through the expression of chemokines and homing ligands from the ß-cells. These may in turn secrete the chemokines as CXCL10, which specifically attracts autoreactive CD8+ T cells. Mouse ß-cells may also express the CXC chemokine receptor 3 [257] to further promote the recruitment of T cells and macrophages to the islets [258]. A study on the homing of human diabetogenic T cells reported that IFN- is crucial for diapedesis and penetration into islets [176]. As physiological response to the in ammation, islet endothelium upregulates the expression of surface adhesion molecules that increase vascular permeability and facilitate the recruitment of effector cells. Adhesion and diapedesis of T cells are feasible through the interactions of T-cell surface molecules (integrins) such as leukocyte function-associated antigen-1 (LFA-1) and very late activation antigen-4 (VLA-4) with their counter ligands on VEC, such as intercellular adhesion molecules (ICAM) and junctional adhesion molecules (JAM-1) [259] that play a major role in the homing of diabetogenic T cells to the pancreas in the NOD mouse [260, 261]. This hyperexpression of adhesion molecules is documented in new-onset diabetes pancreas [11, 262] but may not fully account for the observed enrichment of infiltrating autoantigen-specific T cells. It is now proposed that after migration from pLN, activated T cells require an additional upregulation of LFA-1 functional activity for the successful adhesion to VEC [263]. Data support that the triggering of TCR, achieved through peptide antigen presentation by iVEC, is an important component of integrin functional activation [264] and may provide an additional grade of antigen specificity in T-cell recruitment. The

hypothesis that iVEC may participate in T-cell selective recruitment and adhesion in an antigen-specific fashion is intriguing. A recent study reported that GAD65 presentation by iVEC markedly promotes the in vitro transmigration of GAD65-autoreactive T cells across iVEC monolayers in an LFA-1-dependent fashion [174]. In this process, CD4+ T cells may also intervene by secreting various lymphokines that attract and activate other cell types such as monocytes, eosinophils, and natural killer lymphocytes (NK) [173]. Whether islet-specific autoantibodies secreted by plasma cells take part in the islets destruction, or are merely recruited upon the ongoing discharge of autoantigens, it is still a matter of debate, since a defined pathogenetic effect has not been proven. A pathogenetic involvement of the autoantibodies may be suggested from NOD mouse experiments in which B-cell-deficient animals are protected from diabetes [265], but clinical evidences in humans do not support this hypothesis [266]. Nonetheless, the observation of a cytotoxic effect of autoantibodies on human ß-cell in vitro [267] may indicate possible harmful effects. Autoantibodies might exert either complement-mediated or antibody-dependent cellular cytotoxicity, but there is no clear evidence of these effects in vivo. Moreover, immunoglobulin deposits may [12] or may not be found in islets [3]. From previous experimental observations in mouse, the presence of islet autoantibodies does not seem to be sufficient [3] nor necessary [253] to ß-cell destruction, and do not clearly correlate with the T-cell responses [268]. However, recent insights acquired from NOD mice [88] suggested that B cells may be more important players than formerly considered, and their relevance in physiopathology of human ß-cell destruction is currently under investigation [269]. It is still matter of debate whether antibodies reacting to antigen-binding areas of autoantibodies (anti-idiotype) may be of relevance within the autoimmune process [270], through the blockade of circulating self-autoantibodies.

24.2.6 Insulitis and ß-Cell Destruction

The progression from the initiating phase to an adaptive immune response is thought to take place very early during the insulitis and determine the final outcome toward the generation of a prolonged devastating autoimmune reaction, or the resolution of in ammation and preservation of islet integrity. Target-cell death further activates PRR that in turn promote the progression of insulitis [271] through IFN-a-mediated upregulation of MHC class I molecules on pancreatic islet cells [110, 272]. Among TLR ligands, HSPs are reported to promote antigen presentation [273, 274] and shift DC toward immunogenic phenotype in vivo [273]. IFN and other macrophage-derived cytokines prompt NK activation. These cells exert nonantigen-specific cytotoxicity through the release of perforin, after the activation of surface receptors, as NKG2D that recognizes viral products and other specific ligands [275]. NKT cells on the other hand may be considered as innate-like lymphocytes, as they may co-express NK cell surface markers including NK1.1 (human CD161) and TCR [276]. Most NKT cells recognize glycolipid antigens presented on the MHC class

I-like molecule CD1d [276]. The majority of human NKT cells display an invariant TCR-a chain (Va24-Ja18) and limited number of beta chains [277], and it is often referred to as invariant NKT cells (iNKT) or ‘classical’ or ‘type 1’ NKT [276]. iNKT cells are potent producers of Th2-like reactivity in vivo [277] and are involved in autoimmune diseases in humans and mice [278]. The possible role of NK in ß-cell damage has not yet been clarified, since neither a protective nor a detrimental effect of these cells has been consistently reported in humans or in mice [279–282]. Similarly, an unequivocal role in islet autoimmunity for iNKT has not been established, though a predominant immunomodulatory function has been proposed in the NOD mouse [283]. The effect may be exerted by inducing DC tolerogenic differentiation [284, 285] and conditioning the cytokine environment of pLN or islets [286]. Conversely, the exacerbation of insulitis for an iNKT-mediated enhance of IFN- -producing CTL has also been reported [287].

As the islet invasion progresses, chemokines-attracted macrophages contribute to the recruitment of other immune cells that also release multiple chemokines and pro-in ammatory cytokines. These in ammatory signals create an overall immunoactivatory environment that modifies DC phenotype [107], shifts CD4+ T cells toward ‘Th1-like’ responses which promote the expansion of CTL, and shelters them from peripheral tolerance [154, 288]. If this vicious circle is not interrupted, the maintenance and amplification of insulitis evolve in accumulation of immune cells and their cytotoxic mediators that may act synergistically to destroy the islets [289] (Fig. 24.1). In the later stages, the destructive process may be worsened in the course of ß-cell failure as hyperglycemic environment may locally enhance insulin epitope presentation [290].

Apoptosis is probably the main form of ß-cell death in T1D and is regulated in parallel to the in ammation, through the activation of similar intracellular signaling pathways [110]. ß-cell injury in the course of insulitis is caused by both exposure to soluble mediators as cytokines and reactive oxygen species, secreted by infiltrating cells, and direct cell–cell contact with activated macrophages and CTL [252]. The role of cytokines in diabetes development was confirmed by the demonstration that suppression of cytokine signaling within ß-cells completely prevents mice from diabetes, despite the presence of insulitis [291]. Cytotoxic in ammatory cytokines IL-1-ß,TNF-a, and IFN- released by CTL and macrophages affect ß-cell gene regulatory networks, in uencing primarily transcription factors NFk-B, STAT-1, and AP-1, and activate apoptosis [292]. IL-1 exerts in vitro cytotoxic effects on islets [293] that express specific surface receptors [294]. Cytokine-induced ß-cell death seems to be preceded by a functional impairment, as IL-1 [295, 296] and TNF-a [297] inhibit insulin secretion from isolated cells. This effect may be mediated by the action of nitric oxide (NO) [297, 298]. TNF-a, IL-1, and IFN- activate ß-cell inducible NO synthase activity and enhance the production of endogenous NO [299]. For the lack of radical scavenging activity, ß-cells are highly susceptible to reactive oxygen species [300] that directly participate to cell death through DNA injury, activation of the DNA repair enzyme poly-ADP-ribose polymerase, and depletion of nicotinamide adenosine dinucleotide (NAD) (87). A central role in cytokine-mediated ß-cell death is ascribed to IFN- as observed in mouse

studies on anti-IFN- antibodies and IFN- transgenic expression [252]. In the islets INF- enhances T-cell cytotoxicity and participate to direct ß-cell damage, probably through the upregulation of receptor Fas on ß-cells [291]. Fas is a 45-kDa surface receptor which directly transduces the signal for apoptosis through translocation of phosphatidylserine, upon binding of its specific ligand (Fas-L) [301]. Although the precise mechanism is not defined in vivo, the Fas/Fas-L complex may act through caspase, which is thought to be a major effector enzyme in the apoptotic pathway [199, 204]. Effector T cells can trigger ß-cell death through direct contact between their surface Fas-L and membrane-bound TNF-a and apoptosis-inducing receptors on ß-cells or through the secretion of perforin [252]. Perforin acts facilitating the passage of protease (granzymes) and may be involved in more advanced stages of the destruction [87].

Further studies of early insulitis in humans will be needed to fully appreciate the initiating mechanisms of infiltration of immune cells. Effective prevention of T1D may require a better understanding of the early events of building chronic insulitis. We speculate that a chronic insulitis which includes APC presenting islet autoantigens within the islets as opposed to the pLN represents a refractory state to immunosuppression. This may explain why immunosuppression at the time of clinical diagnosis of T1D is ineffective. It cannot be excluded that immunosuppression therapy may be efficacious, provided that the treatment is used prior to chronic insulitis.

24.2.7 Is ß-Cell Destruction Re ected in the Blood?

Assaying the cells involved in ß-cell damage may give insights about the induction and maintenance of islet autoimmune destruction. Several possible immunological alterations have been searched in the peripheral blood of T1D patients and at-risk subjects to differentiate them from healthy subjects.

24.2.7.1 APC

Most alterations described in mice are consistent with the hypothesis of an increased DC capacity to activate CD4+ and CD8+ T cells [302], such as upregulation of costimulatory molecules, enhanced secretion of cytokines IL-12p70 and TNF-a [303], and downregulation of IDO [304]. An abnormal cytokine response by DC from T1D patients upon antigenic [305] or nonantigenic stimulation was proposed [306] but has not been confirmed by other studies [307]. More robustly, phenotypic characterization suggests that DC from recent-onset T1D patients exhibit an immature phenotype and may have a decreased T-cell stimulatory capacity, compared to controls [308]. DC may therefore indirectly participate to T1D autoimmunity through a reduced efficacy in stimulating Treg, as is also reported in mice [309] and BB rats [310]. This immature phenotype of T1D human DC may result from abnormal activation of the NF-kB pathway [311], consistently with the strong involvement of this transcription factor in the induction of self-tolerance in mice [157, 303].

Studies investigating the peripheral DC count reported a reduction in absolute number of blood DC in T1D children [307] and, more recently, a modest but significant increase in the relative frequency of pDC subset, strictly time-related with disease onset [312]. Individual, genetically determined antigen processing (internalization and proteasomic cleavage) has been demonstrated in different APC [313] and may account for disease-relevant epitope presentation in genetic susceptible individuals [314]. However, the present observations about DC in human diabetes rely upon studies on in vitro monocyte-generated DC that may not re ect the true in vivo situation (315).

24.2.7.2 T Cells

Plenty of studies on peripheral blood mononuclear cells (PBMC) of T1D patients aimed at detecting the presence of islet-specific CD4+ and CD8+ T cells, upon stimulation with synthetic peptides from islet antigens. The immunogenic epitopes are selected among putative immunodominant regions within the multiple diabetes-related islet autoantigens. Many of these studies report a higher frequency of islet-specific self-reactive T cells in T1D patients than in control subjects, when T cells are detected by either functional tests of antigen-induced proliferative [96] and cytokine-secretive response [91, 102, 316, 317] or tetramer staining [101, 318, 319].

CD8+ and CD4+ T cells from T1D patients target a wide array of epitopes within GAD65 molecule [92, 316, 318–322], insulin and proinsulin [91, 323–325], IA-2 [316, 317, 326], IGRP [323, 327, 328], I-A2b [327, 328], islet amyloid polypeptide (IAPP) [327–329], and glial fibrillary acidic protein (GFAP) [328] as comprehensively summarized in a review updated to the end of 2006 [330]. More recently, a few more epitopes have been described, as GAD536-545 [316], other IGRP fragments, among which IGRP 211–219 and 222–230 [331], and several novel insulin- and proinsulin-derived peptides targeted by CD8+ [323, 332, 333] and CD4+ T cells [334]. In conclusion, these investigations, mostly oriented toward epitope identification, provide evidence of multiple immunodominant ß-cell regions targeted by CTL in human T1D, but do not fully clarify the development of the T-cell-specific responses during the progression of the disease. In fact, no single epitope has proven to be highly discriminatory, though a hierarchy of T-cell responsiveness has been proposed among proinsulin peptides [102]. In some ways, the choice of the epitope may also be misleading. Candidate sequences are usually selected on the basis of predicted TCR–pMHC-binding motifs [335] or affinity algorithms [336], whereas the strength of the TCR–pMHC complex interaction may inversely correlate with immunogenicity [327, 337], in accordance with an insufficient negative thymic selection; this bias can be avoided through the analysis of multiepitope, multiantigen panels [317, 337]. Moreover, epitopes that have been proved of relevance in mice may guide the searching efforts in humans, as is recently happening with IGRP peptides [331]. As already mentioned, a precise, reproducible, and standardized method for detection and identification of ß-cell-specific autoreactive T cells to reliably identify the pathologic response of T1D patients is not available. However,

some authors reported that the use of multiple epitopes achieved more diagnostic sensitivity and discriminates T1D from controls [102, 324]. It is therefore still unclear to what extent all the data provided can be translated into evaluation of diabetes risk or disease condition. Moreover, autoreactive T-cells-specific responses for T1D self-antigens have been widely described in healthy individuals in stimulation assays with peptides from GAD65 [338, 339] and insulin [100, 102, 327]. Several differences have been proposed between self-reactive T CD4+ T cells from T1D and controls. Only GAD65-reactive T cells from T1D subjects seem to be fully autoantigen-experienced in vivo and express the memory T-cell marker CD45RA [338, 339] and are capable of activation in the absence of CD28/B7 costimulatory signals [248]. It was also recently proposed that CD4+ T cells from T1D subjects may have a lower threshold of activation, as compared to healthy controls [340].

Interestingly GAD65-specific T-cell TCR repertoire does not differ between T1D and controls [338], implying that central tolerance to GAD65 is the same among healthy and T1D subjects. Probably, in healthy individuals, self-reactive T cells are present but quiescent for the immunosuppressive action of Treg, as confirmed by the experimental observation that Treg in vitro depletion is followed by amplification of autoreactive T cells only in samples from healthy individuals [338]. Treg pool in human T1D has also been extensively investigated, and a deficiency in Treg peripheral frequency has been reported in patients compared to controls [341], but subsequent investigations have failed to uniformly replicate these findings [342] and have suggested that T1D nTreg may rather display an impaired immune suppressor function [342, 343]. Globally, it seems that a simple deficiency in the peripheral Treg repertoire is not confirmed [341], but a local impairment of Treg activity at the site of in ammation cannot be excluded. Notably, most defects in number and function of Treg observed in NOD mice [186, 344, 345] may be ascribable to the ogistic environment [346] that may actively inhibit Treg suppressive function through both the reduction in IL-2 [347, 348], mTGF-ß [349], and TNF-a [350] and the increase in IL-21 [351, 352]. Interestingly, the peripheral blood from T1D patients may be evidence for a misbalance toward in ammation. Autoantigen-driven cytokine secretion by CD4+ T cells from T1D patients may be polarized toward INF- , while HLA-matched healthy controls display IL-10+ cTreg-like responses [102]. This ‘regulatory phenotype’ skewed toward IL-10 has also been reported in association with T1D later onset [102] and better glycemic control [353]. Increased levels of ‘Th1 cell’-derived chemokines CCl3, CCl4, and CXCl10 [354, 355] and of adhesion molecules ICAM and L-selectin (CD62L) [356] have been found in serum of T1D patients. Several reports have addressed NK population in the peripheral blood of T1D patients [357] and have described a decrease in the peripheral frequency, in most cases temporally related to disease onset [4, 281], or a functional deficit [358], but these findings have not been universally replicated [357]. More recently, a larger study confirmed a functional impairment of NK cells in T1D patients, i.e., reduced surface expression of activating receptors and low levels of IFN- and perforin, and suggested that these alterations may be a consequence of T1D, since they are evident exclusively in long-standing disease [359]. It has also been reported that activated NK cells in T1D patients display a reduced expression

of NKG2D receptor [357]. It is possible that a downregulation of NKG2D receptor mediates the increased risk for T1D associated with polymorphisms of MHC class I chain-related (MIC) proteins [360] that are NKG2D natural ligands. Studies addressing a correlation between NKT cell levels in peripheral blood and T1D in humans have yielded variable results, since the reports of altered frequency [361] or cytokine secretion [224, 362, 363] have not been confirmed [364].

It must be noticed that the reported assays on cell repertoire and ogistic mediators in humans have been performed on peripheral blood samples. More disease-relevant alterations may be detected assessing the islets or the pLN, which would give a more realistic picture of locally generated signals. Some recent reports have tried to overwhelm this limit. Two studies in the mouse have suggested that, regardless the provenience of the T cells (periphery, islets, or lymph nodes), ß-cell antigen-specific CD8+ T-cell pool shares TCR chain usage [365] and show conserved patterns of epitope immunodominance [366]. Another study has performed micro array analysis of the cytokine pattern of PBMC from healthy subjects after the exposure to sera from new-onset T1D patients and has reported an enhanced secretion of pro-in ammatory factors as IL-1, CCl2, and CCl7 [367].

24.2.7.3 B Cells and Autoantibodies

The assessment of eventual disorders of humoral immunity in T1D relies on the monitoring of circulating islet-reactive autoantibodies (Table 24.2). Autoantibodies against at least one of islet-cell antigens GAD65, IA-2, insulin, and ZnT8t are present in more than 95% of T1D patients [34] and in only 1–2% of general population [368]. Radio-binding assay of these autoantibodies has replaced the ICA assay. GAD65 antibodies are found in 70–75% of T1D patients [369] and show a diagnostic sensitivity of 70–80% and a diagnostic specificity of 98–99% [40]. Of interest, the titer of GAD is usually low at time of diagnosis. The major antigenic epitopes of GAD65 are the middle- [370] and C-terminal region [371, 372] and are in close proximity to T-cell disease-relevant determinants [170]. Differential epitope specificities, as identified by monoclonal antibodies to GAD65 epitopes within the C-terminal region, align with distinct autoimmune disease phenotypes [170, 373], and the binding of N-terminal epitope is associated with slowly progressive ß-cell failure [374]. Finally, it was recently suggested that the presence of GAD65 antibodies in T1D patients may be the result of an ‘unmasking’ due to the lack of anti-GAD65-anti-idiotypic antibodies [375]. These anti-idiotypic antibodies are reported to highly discriminate T1D from healthy subjects and may be of some relevance in the pathogenesis of islet autoimmunity. IAA are found in approximately 50–70% of T1D patients [40, 140] and are first islet autoantibody to appear [376], suggesting an involvement of insulin as primary autoimmune triggering antigen, also in humans [377]. Epitopes targeted by IAA are placed within A and B chains and are shared between insulin and proinsulin [378]. IA-2 antibodies are detected in 60–70% of patients with new-onset T1D and tend to appear closer to the clinical onset [2, 40]. Epitopes for IA-2 antibodies are found exclusively within the cytoplasmic region of the molecule and predominantly within the

tyrosine phosphatase-like domain [379, 380]. Antibodies to ZnT8t are detected in 60–80% of newly diagnosed T1D [2, 65]. Finally, antibodies anti-alfa-2-amylase have been recently described in a subgroup of patients affected by autoimmune pancreatitis presenting with fulminant diabetes [381], a form that is commonly considered ‘non-autoimmune.’ In these patients, the lymphocyte infiltrate affecting the exocrine component in autoimmune pancreatitis is extended to the islets [381] and may reveal shared immune-mediated mechanisms, as seem to be suggested in the NOD mouse [382].

24.3 Prediction of ß-Cell Destruction

Standardized methods [83] have made islet autoantibodies the most useful marker for T1D prediction [2, 40] and for enrollment of subjects into clinical prevention trails. The most accurate single predictor is GAD autoantibodies [383] with a positive predictive value for T1D of about 60% [40, 384], followed by IAA (30%) that is a better predictor among children [59, 385]. To enhance the predictive power, more markers in combination are actually used [386, 387], and the prediction power for T1D reaches 100% in case of multiple positivity (Fig. 24.2). Similarly, in case of single autoantibody, the correlation between islet autoimmunity and histological evidence of insulitis is weak [251, 388]. Longitudinal studies investigating DC and T cells in at-risk subjects are lacking. Some reports have found poor in vitro maturation and pro-in ammatory cytokine response in DC from children at genetic risk for TID [389, 390] and a number of attested T-cell responses from PBMC of at-risk subjects toward islet-specific autoantigens GAD65 [318, 319] and insulin/proinsulin [318, 328]. Finally, increased chemokines, such as CXCl10 [354] and adhesion molecules [356], have been detected in the plasma of at-risk individuals; more

recently, in three at-risk subjects followed until diagnosis these alterations were present years before the clinical onset [367]. However, this data cannot to date be translated into risk stratification.

24.4 Concluding Remarks

In conclusion, the ß-cell in T1D is the major target for an autoimmune process that takes place in two steps. The first step is the development of an autoimmune reaction directed toward specific ß-cell antigens. This step is re ected by circulating autoantibodies to ß-cell autoantigens including GAD65, IA-2, ZnT8, and insulin. The number of autoantibodies predicts T1D risk. The second step is progression from islet autoimmunity to the clinical onset of T1D, which in humans is associated with a major loss of ß-cells and insulitis. Insulitis appears late in the autoimmune process and can be recapitulated in pancreas and islets transplantation. The immunological memory of ß-cell autoantigen is chronic. Efforts are needed both to detect intra-islet events that precede the development of autoantibodies and to disclose when islet autoantibody positivity is marking that the ß-cell destructive process of insulitis is about to be established. A better understanding of step 1 and two events will be necessary for the ultimate prevention of ß-cell destruction and of T1D.

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Table 24.1. Beta-cell autoantigens

Antigen	Mol weight Da	Autoantibody Abbreviation	Ref
Glutamic Acid Decarboxylase	65000	GAD65Ab	83
Insulin	5800	IAA	387
IA-2	4000	IA-2Ab	61
IA-2beta (Phogrin)	3700	IA-2βAb	62
Zinc Transporter ZnT8 R/W/Q variants	4100	ZnT8Ab	64

Table 24.2 Non-HLA genetic factors in type 1 diabetes

Gene (Syno.)	Name	Chromosome	Function	Association with other autoimmune diseases
PTPN22 (PEP, Lyp1, Lyp2, LYP, PTPN8)	Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)	1p13	Encodes tyrosine phosphatase May be involved in regulating CBL function in the T-cell receptor-signaling pathway.	T1D & 22 other diseases
CTLA-4 (DDM12, CELIAC3)	Cytotoxic T-lymphocyte-associated protein 4	2q33	Possible involvement in regulating T-cell activation.	T1DM & 99 other diseases
IFH1 (MDA5)	Interferon induced with helicase C domain 1	Chr.2q24	Proposed involvement in innate immune defense against viruses through interferon response.	T1DM association
IL2 (lymphokine, TCGF)	Interleukin 2	Chr.4q27	Encodes a cytokine important for T & B cells proliferation. Stimulate B-cells, Monocytes, killer & NK cells.	T1DM & 39 other diseases
ITPR3 (IP3R3)	Inositol 1,4,5-triphosphate receptor3	Chr.6p21.3	A second messenger that mediates the release of intracellular calcium	Strong T1DM association
BACH2 (BTB & CNC homology 1)	Basic leucine zipper transcription factor 2	Chr.6q15	Important roles in coordinating transcription activation and repression by MAFK (By similarity)	T1DM association
IL2RA (IDDM10, CD25)	Interleukin 2 receptor, alpha (chain)	Chr.10p15	Receptor for interleukin-2	Strong association with T1DM
INS-VNTR (proinsulin, ILPR,MODY)	Insulin II; insulin 2; insulin	Chr.11p15	Regulating glucose metabolism through adjusting central tolerance to insulin.	T1DM & 38 other diseases
TH (TYH, The)	Tyrosine hydroxylase	Chr.11p15	Encodes a protein that converts tyrosine to dopamine. Plays a key role in adrenergic neurons physiology.	T1DM & 35 other diseases
ERBB3 (c-erbB3, HER3, LCCS2)	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3	Chr.12p13	Encodes a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. Binds and is activated by neuregulins and NTAK.	T1DM & Multiple Sclerosis
C12orf30 (C12orf51, KIAA0614)	Similar to KIAA0614 protein	Chr.12q24	Not yet determined	T1DM association
CLEC16A/ KIAA0350 (Gop-1)	C-type lectin domain family 16, member A	Chr.16p13	Unknown. Proposed to be related to immune modulation mechanisms	Strong association with T1DM
PTPN2	Protein tyrosine phosphatase, non-receptor type 2	Chr.18p11	Encode a PTP family protein & may be related to growth factor mediated cell signaling.	T1DM association
BASH3A (TULA, CLIP4)	Ubiquitin-Associated and SH3 domain-containing protein A	Chr.21q22	Promotes accumulation of activated target receptors, such as T-cell receptors, EGFR and PDGFRB	T1DM association

FIGURE 24.1 Schematic view on possible immunopathogenesis of beta-cell destruction

FIGURE 24.2 Diagramatic presentation of the effect of multiple islet autoantibodies on the risk of developing T1DM in the Diabetes Prevention Trial-Type1 (DTP-1)
(*Courtesy of Jay Skyler*)