CHAPTER 16

IMMUNOBIOLOGY OF β-CELL DESTRUCTION

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Abstract: Type 1 diabetes is a chronic disease characterized by severe insulin deficiency and hyperglycemia, due to autoimmune destruction of pancreatic islets of Langerhans. A susceptible genetic background is necessary, but not sufficient, for the development of the disease. Epidemiological and clinical observations underscore the importance of environmental factors as triggers of type 1 diabetes, currently under investigation. Islet-specific autoantibodies precede clinical onset by months to years and are established tools for risk prediction, yet minor players in the pathogenesis of the disease. Many efforts have been made to elucidate disease-relevant defects in the key immune effectors of islet destruction, from the early failure of specific tolerance to the vicious circle of destructive insulitis. However, the events triggering islet autoimmunity as well as the transition to overt diabetes are still largely unknown, making prevention and treatment strategies still a challenge.

INTRODUCTION

The hallmark of Type 1 diabetes mellitus (T1DM) is chronic hyperglycemia resulting from severe insulin deficiency.[1] In this chapter, the term T1DM refers to subtype "1A" diabetes, characterized by autoimmune destruction of insulin-producing β cells in pancreatic islet of Langerhans.[2] The clinical onset occurs when extensive β-cell loss significantly impairs insulin secretion, after a months-to-years prodrome of asymptomatic islet autoimmunity that is marked by the appearance of specific islet autoantibodies.[3]

In the last two decades, epidemiology registries have attested a worldwide increase in T1DM incidence especially among younger children.[4] The predicted trend is equally troublesome, as the prevalence of new diagnoses before 15 years of age is expected to rise by 70% in the next ten years.[5]

Diabetes: An Old Disease, a New Insight, edited by Shamim I. Ahmad.

T1DM has an established genetic etiology that consists in inheritance of susceptibility to β-cell autoimmunity. However, 80-85% of T1DM cases are sporadic and concordance rate between monozygotic twins is as low as 30%.[6] The strongest genetic association is with human leukocyte antigens (HLA) DR and DQ within the class II major histocompatibility complex (MHCII) on 6p21. Several genes mapping outside MHC region have been linked to T1DM risk, as insulin gene (*ins*), protein tyrosine phosphatase, nonreceptor type 22 *(ptpn22)*, cytolytic T-lymphocyte-associated antigen (*ctla-4)* and several additional factors listed in Table 1 (recently reviewed in ref. 7). Additional susceptibility loci for T1DM are continuously discovered by genomewide association (GWA) studies performed with high-density single nucleotide polymorphism (SNP) genotyping platforms. More than 40 loci have been identified so far, some of which have immunoregulatory functions, as listed in Table 1.[8]

T1DM shows wide geographic variation with a ten-fold gradient within Europe.[14] Annual incidence ranges from 0.1 per 100,000 children in Asia and South America to 64.2 per 100,000 in Finland,[15] a six-fold higher rate than bordering Russian Karelia despite identical risk genes frequencies.[16] The largest contribution to rising incidence comes from traditionally low incidence regions.[17] Interestingly, T1DM risk among children born to immigrants to high risk regions increases, though not associated with the same HLA types of receiving land.[18]

These epidemiological observations support a crucial contribution from one or more environmental determinants (reviewed in ref. 19), which might account for the observed incidence raise, too steep for a genetic modification. Environmental factors possibly influencing T1DM risk have been widely investigated, as virus infections and vaccinations,[20] dietary products and toxins,[21] but their role is still controversial and currently under scrutiny.[22]

At clinical onset, T1DM patients exhibit an extensive autoimmune response towards the β-cell and islet histopathology shows inflammatory features of an "insulitis", with

Table 1. Candidate minor genetic factors with T1DM-relevant function

Gene	Name	Position	Function	Original Reference
CTLA4	Cytolytic T lymphocyte-associated antigen 4	*2q33*	Negative regulation of T-cell reactivity	Marron, Raffel et al 1997[8]
IL2	Interleukin 2	*4q27*	Lymphocytes proliferation and activation of B lymphocytes, monocytes and natural killers	Zhernakova, Alizadeh et al 2007[9]
IL2RA	Interleukin 2 receptor α (chain)	*10p15*	Receptor for interleukin-2	Lowe, Cooper et al 2007[10]
IFH1	Interferon-induced with helicase C domain 1	*2q24*	Modulation of IFN response for innate immunity to virus infection	Liu, Wang et al 2009[11]
PTPN2	Protein tyrosine phosphatase nonreceptor Type 2	*18p11*	Modulation of IFN γ-induced β-cell apoptosis	Moore et al 2009[12]

infiltrated mononuclear cells, T and B-lymphocytes.[23] Little is known about islet histology during preclinical autoimmunity. Nevertheless, signs of insulitis are not found in pancreas specimens from antibody-positive healthy donors except in case of multiple antibody positivity.[24] Because of the high sensitivity/specificity and early appearance during the autoimmune process, islet autoantibodies are established markers for T1DM diagnosis and prediction. However, since the events triggering autoimmunity and transition to clinical T1DM are still largely undefined, the precise disease risk estimation remains a challenge, even in genetically susceptible individuals.

In the last four decades, possible immunological defects have been widely investigated which might give insights into mechanisms of β-cell destruction. More recently, studies have focused on subsets of ambivalent immune effectors, as regulatory T (Treg) and natural killers T (NKT) lymphocytes, which have enriched the classic scenario of Th1/Th2 paradigm for autoimmune diseases.[25] Similarly, aberrant innate immune responses, first-line non-antigen specific defense to external threats, are figuring prominently as possible triggers of islet autoimmunity and T1DM.[26] The enrollment of adaptive immune system, that involves specialized antigen-driven responses, ultimately affects β-cells outcome, inducing either resolution of inflammation or prolonged detrimental attack and irreversible β-cell damage. In the context of a genetically determined autoimmune-prone environment, initial β-cell damage by environmental factors (e.g., virus) might trigger islet autoimmunity and progressive destruction by autoreactive T-lymphocytes.

In this chapter, the steps leading to immune-mediated β-cell death are reviewed together with possible early mechanisms of loss of tolerance to islet autoantigens and potential environmental triggers. The readers are referred to other recent reviews.[27,28]

IMMUNOGENETICS OF T1DM

HLA Genes

The main genetic factor for T1DM risk is HLA class II DQ, which contributes about 50% to overall risk.[29] HLA-DQ A1-B1 haplotypes A1*0301-B1*0302 (DQ8) and A1*0501-B1*0201 (DQ2), alone or in combination, confer the highest risk for T1DM in Caucasians[30] and are present in nearly 90% of newly diagnosed children.[31] Children carrying DQ2/8 heterozygocity show earlier disease onset.[32] Nonetheless, the global contribution of these high-risk haplotypes is decreasing in parallel with the rising in disease incidence.[33] Combinations of DQA1*0201-B1*0602 (DQ6.2) haplotype are reported negatively associated with T1DM, though this "protective" effect weakens with increasing age of patients.[34]

Although the role of DQ heterodimers in antigen recognition is well known, the mechanisms beneath T1DM risk or protection are not fully clarified. Possibly, distinct molecular conformations of peptide-binding pockets encoded by different haplotypes affect autoantigen presentation to immune effectors.[35] HLA genes might also be involved in the response to infections, since T1DM-related DR haplotypes correspond to more robust humoral reactivity to viral antigens.[36]

A minor part of T1DM association to MHC region reflects the contribution of class I (MHCI) alleles HLA-A and HLA-B regions.[37] An additional determinants of genetic susceptibility map to the MHC class I Chain-Related A (*mic-A*), probably involved in the regulation of innate immune responses.[38]

Non-HLA Genes

Outside MHC region, the strongest genetic T1DM associations map to *ins* on 11p15 and *ptpn22* on 1p13.[39]

Polymorphisms within the variable nucleotide tandem repeat of the promoter region in the insulin gene (*ins vntr*) have been reported in newly diagnosed T1DM in association with insulin autoantibodies.[32] *Ins vntr* is thought to have a direct role in the modulation of tolerance to insulin. Probably, the gene "long variant", that is negatively associated to T1DM,[34] yields the protective effect through the potentiation of central tolerance to insulin peptide.[40]

Polymorphisms of *ptpn22* increase T1DM risk irrespectively of HLA type.[41] *Ptpn22* gene may be a direct T-cell regulator and functional mutations of the encoded protein lymphoid tyrosine phosphatase (LYP) play a role in early islet autoimmunity and in the latest progression to clinical diabetes.[42] Missense mutation due to C1858T SNP (Arg620Trp) might result in a protein variant unable to bind the cellular signaling molecule necessary for negative regulation of TCR.[42]

Several other nonHLA genes associated to T1DM have been discovered, with known disease-relevant immunoregulatory functions (Table 1). Novel genetic factors have been proposed based on GWA studies, as BASH3A and BACH2,[43] ERBB3,[44] IKZF4[45] and CLEC16A,[46] yet of uncertain relevance to the induction and/or maintenance of islet cell autoimmune destruction.

ISLET CELL AUTOIMMUNITY

At diagnosis, T1DM patients show a broad, highly polyclonal immune response targeting several β-cell peptides. However, it is still not clear whether T-cell clonotypes initiating insulitis target one or multiple islet antigens. The extensive reactivity may be a late consequence of sequestered peptides leaking from damaged β cells ("antigen spreading"). Intramolecular shifting of recognized sequences ("epitope spreading") adds variability within the individual responses.[47] It is also possible that a genetically determined aptitude in antigen processing and presentation by APC accounts for the diversified disease-relevant epitope recognition.[48]

The first identification of T1DM-associated autoimmunity dates back to 1973 through tests for leukocyte migration inhibition to pancreatic antigens.[49] Since then, the detection of specific islet autoantibodies not only has guided the identification of main autoantigens, but also provided reliable tools for disease diagnosis and risk estimation.[50] Specific anti-β-cell cellular responses have also been investigated in T1DM and at-risk subjects, to gain insights into the pathogenetic mechanisms of the immune destruction, not easily dissectible in humans. Mostly, T-lymphocytes recognize the same antigenic peptides as autoantibodies, with possible epitope overlapping,[51] suggesting an interplay between antigen-antibody complexes and T-cell responses (human B and T epitopes are listed in Table 2). The spatial relationship between common B and T determinants has proven relevant for β-cell specific immunological events,[62,63] though its role in the destiny of human islets in T1DM in unknown. Possibly, B-lymphocytes modulate autoreactive T-cell repertoire through the presentation of nonimmunodominant β-cell determinants and participate in epitope spreading during the progression of the disease.[64]

Table 2. Confirmed minor β-cell autoantigens for B and T lymphocytes in humans

Antigen	Short Name	Recognizing Immune Cell	Original Reference
Amylase α-2A		B	Endo et al 2009[52]
ADP-ribosyl cyclase	CD38	B	Pupilli et al 1999[53]
Heat-shock protein 60	HSP60	B and T	Ozawa et al 1996[54]
Heat-shock protein 70	HSP70	B and T	Abulafia-Lapid et al 2003[55]
Heat-shock protein 90	HSP-90	B	Qin et al 2003[56]
Islet cell antigen 69	ICA69	B and T	Martin et al 1995[57]
Islet-specific glucose 6 phosphatase catalytic subunit related protein	IGRP	T	Jarchum et al 2008[58]
Islet cell antigen 12	ICA12/SOX13	B	Kasimiotis et al 2001[59]
Phogrin	IA-2β	B	Hawkes et al 1996[60]
Vesicle-associated membrane protein-2; Inhibitory neuropeptide Y	VAMP2 NPY	B	Hirai et al 2008[61]

Islet Autoantibodies

The appearance of autoantibodies marks the development of islet autoimmunity[65] and the risk for clinic diabetes.[66]

The first description of islet autoantibodies was in 1974 with the detection of islet cell antibodies (ICA) in the serum of T1DM patients by indirect immunofluorescence on frozen human pancreas.[67] In the last two decades, radio-binding assays with immunoprecipitation of in vitro transcribed-translated radiolabeled antigens provided a reliable and easily reproducible method, which allowed standardization programs.[68]

Antibodies Anti Glutamic Acid Decarboxylase

Glutamic acid decarboxylase isoenzyme 65 (GAD65) has been the first demonstrated islet autoantigen, as a 64 kDa immunoprecipitate in the serum of T1DM patients.[69] GAD65 is mainly located in the synaptic-like microvescicles of the β cells and synthesizes γ-amino butyric acid.

Antibodies anti GAD65 (GADA) show a diagnostic sensitivity of 70-80% and a specificity of 98-99%.[70] GADA are associated with a slower disease progression[71] and are the most frequent autoantibodies at diagnosis among older patients.[70] The major antigenic sites of GAD65 molecule are located in the middle and C-terminal regions[72] and N-terminal-binding autoantibodies imply slower progression of β-cell failure.[73] Interestingly, GADA have been reported to enhance GAD65 specific cellular responses.[74]

Finally, in one study the presence of antibodies against GADA (GADA anti-idiotypic antibodies, anti-ID) has successfully discriminated healthy from T1DM subjects.[75] The authors suggest that high GADA titers detected in T1DM samples rather result from an "unmasking" due to deficient anti-ID network.[75] Consistently, in a more recent study, GADA anti-ID titers are inversely correlated to the development of islet autoimmunity and β-cell loss.[76]

Insulin Autoantibodies

Insulin autoantibodies (IAA) was first demonstrated in T1DM patients in 1983.[77] Insulin is the only islet-specific antigen in T1DM and is related to the clinical manifestation of the disease. Hence, crucial involvement of IAA has been suggested in humans, as proved in animal models.[78] IAA are found in approximately 50-70% of patients,[70] and antibody titers are inversely related to age at diagnosis.[77] IAA are often the first autoantibody to appear in both T1DM and at-risk subjects.[65,79] Epitopes targeted by IAA are shared between insulin and proinsulin and are placed within A and B chains.[80]

Antibodies Anti Insulinoma-Associated Antigen-2A

A member of the transmembrane tyrosine phosphatase family, insulinoma-associated antigen-2 (IA-2), was identified in the serum of T1DM patients in 1996 from digestion of the 64 kDa antigen.[81] A secondary fragment named IA-2β is probably less involved in islet autoimmunity and is considered a minor autoantigen. IA-2 is a transmembrane molecule of islet secretory granules and may be physiologically implicated in insulin secretion.[82]

IA-2 autoantibodies (IA-2A) are detected in 60-70% of newly diagnosed patients.[70] Targeted epitopes are exclusively located within the C-terminal region and predominantly within the tyrosine phosphatase-like domain.[83]

Antibodies Anti-Zinc Transporter Isoform 8

The zinc transporter isoform-8 (ZnT8) transports zinc into the insulin granules for insulin crystallization. ZnT8 autoantibodies (ZnT8A) were firstly identified in 2007.[84] ZnT8A are detected in 60-80% of newly diagnosed T1DM,[50] especially among the youngest,[85] and show rapid decrease after clinical onset.[85]

Islet autoantibodies usually appear in sequence[86] and do not follow a precise pattern, though IAA appear earlier during the progression to islet cell autoimmunity and IA-2A closer to disease onset.[79] IAA and IA-2A are associated with DR4 and DQ8 genotypes whereas GADA are associated with DR3 DQ2 genotype.[87]

Globally, more than 95% of T1DM patients are positive for at least one among GADA, IAA, IA-2A and ZnT8A, with respect to only 1-2% of general population.[66] The most accurate single predictor of T1DM is GADA, with a 60% positive predictive value (PPV), followed by IAA (PPV 30%),[70] which are a better predictor among children.[88] PPV for T1DM increases with the combination of autoantibodies and approaches 100% in case of multiple positivity.[89] As already mentioned and consistent with these observational data, the correlation between single-islet autoantibody positivity and insulitis is weak.[24]

T-Lymphocytes and Islet Autoantigens

T-cell autoreactivity is associated with β-cell destruction in human T1DM.[90]

In recent years, several studies have addressed cellular immunoreactivity to islet autoantigens. These studies have used peripheral blood mononuclear cells (PBMC) from T1DM and at-risk subjects to uncover the presence and immune features of islet-specific $CD4^+$ helper (Th) and $CD8^+$ (effector) T-lymphocytes. Traditional antigen-induced proliferation assays have globally failed to identify significant abnormalities in T-cellular compartment,[91] probably because autoreactive T-lymphocytes show very low peripheral frequency and display

low peptide avidity and variation of immunodominant specificities.[92] Functional analyses of antigen-induced cytokine secretion, as the enzyme-linked immunosorbent spot assay (ELISPOT), have also been developed and achieved better discrimination between patients and controls.[93] Antigen-specific phenotyping and selective identification of T-lymphocytes is enhanced through the MHC tetramer technique, which resembles physiological interactions between the peptide-MHC complex (pMHC) and the T-cell receptor (TCR).[94] The candidate epitopes for the analysis are selected among immunodominant regions of relevant islet antigens, based on naturally proteasome generated sequences and pMHC-TCR binding predictions. The current approach is the application of multiantigen, multiepitope panels.[95]

Several studies report that T1DM patients show higher frequency of islet-specific $CD4^+$ and $CD8^+$ T-lymphocytes, targeting a wide array of epitopes within major islet autoantigens GAD65,[96] insulin,[97] IA-2[98] and proinsulin.[95] For a comprehensive review updated to 2007 see reference 99.

In conclusion, though easily reproducible and standardized methods for detection of β-cell-specific autoreactive T-lymphocytes are not yet available, the new assays have partly overwhelmed initial limitations. The last T-cell workshop has testified improved sensitivity and specificity in the discrimination between T1DM and healthy subjects.[100]

Although proof of principle for an extensive anti-islet cellular reactivity in human T1DM, these investigations do not clarify the development of T-cell specific responses during the progression of the disease, as no single epitope has proven to be discriminant.

A few studies have also attested T-cell responses from PBMC of at-risk subjects toward GAD65 and proinsulin.[101,102] However, it is still unclear to what extent these data can be used for T1DM risk stratification, since islet-specific autoreactive T-lymphocytes targeting GAD65 and insulin can be detected in healthy individuals.[103,106] Qualitative differences in T-lymphocytes have been described between T1DM and controls that may rather account for disease-relevant mechanisms (Table 3). Globally, evidences for altered T-cell function in T1DM patients are not robust and the assessment of T1DM-related cellular disorders are still not applicable for clinical purpose.

Loss of Tolerance to β-Cell Antigens

The immune system is programmed for the specific identification and elimination of potential threats to preserve integrity of the "self". Antigen recognition by T and B-lymphocytes on the surface of a professional antigen presenting cells (APC) is a key to every adaptive response, as well as to the induction of self-tolerance. The failure of tolerization produces survival and expansion of self-reactive effectors. Possible events leading to breakdown of tolerance in T1DM are reviewed in reference 119.

T and B-lymphocytes gain specific tolerance to self peptides through central and peripheral mechanisms. Central tolerization occurs at the sites of lymphocyte maturation, i.e., thymus and bone marrow respectively. Peripheral tolerance takes place in peripheral organs, as the result of a complex interplay between lymphocytes and other immune cells, under the essential contribution of local tissue microenvironment.

Central Tolerance

Immature T and B-lymphocytes express polyreactive and potentially autoreactive surface cell receptors (TCR and BCR), formed through the random gene recombination that physiologically guarantees a widespread peptide recognition.

In the thymus, thymocytes showing high affinity for self peptides presented on the surface of APC as medullary epithelial cells, dendritic cells (DC) and macrophages are eliminated ("clonal deletion").[120] This is consistent with the observed inverse correlation between the strength of pMHC-TCR complex and peptide immunogenicity in T-cell assays on T1DM.[95]

An adequate intrathymic expression of peripheral tissue specific antigens is prerequisite to central negative selection and is regulated by multiple genetic factors as the transcriptional autoimmune regulator AIRE, which is involved in severe human autoimmune disorders.[121]

Although not directly verified in humans, insufficient thymic expression of islet peptides might spare potentially diabetogenic T-lymphocytes. As mentioned, in the protective "long variant" of *ins vntr* gene,[34] multiple tandem repeats enhance thymic insulin expression and improve negative selection of insulin-reacting T-lymphocytes.[40] IA-2 peptide is expressed in the thymus after posttrascriptional deletion of amino acidic sequence encoded by exon 13. Thymic survival of IA-2-reactive clonotypes might result from an alternative transcript splicing,[122] as also supported by the observation that T epitopes map the region encoded by exon 13.[123] GAD65 thymic expression has been postulated[124] but not reliably reported. In fact, one study found the same GAD65-specific TCR repertoire in T1DM and controls,[106] suggesting that central tolerance to GAD65 does not differ between the two groups.

Thymic negative selection can also fail in case of thymocytes resistance to apoptosis. For example, inactivating mutations of *ptpn22* gene, that encodes for a negative regulator of TCR signaling, may result in increased threshold for deletion.[39]

Tolerization mechanisms of B-lymphocytes in humans are much less clarified (reviewed in ref. 125). In the bone marrow, about one-half of autoreactive B clonotypes rearrange the light-chains of membrane immunoglobulin receptors ('receptor editing'). It has been proposed that further gene rearrangements might contribute to human autoimmunity, but their role in T1DM is still to be determined.

Peripheral Tolerance

Central tolerance might be physiologically imperfect in humans, as proved in mice. Indeed, the mere presence of T-lymphocytes targeting islet antigens does not imply T1DM development.[103,106] The induction of peripheral tolerance is crucial in hampering organ specific autimmunity. T-lymphocytes peripheral tolerization occurs in the lymphoid and nonlymphoid tissues upon mutual interactions with professional APC as DC and immune regulators as regulatory T cells (Tregs).

Priming of naïve T-lymphocytes in the lymphoid tissue requires that a positive costimulation be provided by an APC concurrently with antigen presentation within the so-called "immunological synapse".[126] In the absence of appropriate cognate binding of T-cell surface receptor CD28 by APC-derived factor B7, TCR stimulation induces either T-cell apoptosis or unresponsiveness ("clonal anergy"). The best characterized factors for peripheral tolerance are B7,[127] the programmed death (PD-1)[128] and Ctla-4. Ctla-4 is a negative regulatory receptor that antagonizes B7-CD28 stimulatory binding.[129] T1DM-associated splice variants of *ctla-4* gene may result in impaired protein expression and inadequate neutralization of costimulatory signals.[130] For a complete review on T-cell tolerization see reference 131. Possibly, autoreactive B-lymphocytes undergo similar peripheral anergy mechanisms.

Table 3. Possible immunological defects reported in the serum of T1DM and at-risk subjects

Immune Cell/ Molecules	Defect	T1DM-Relevant Implication	Study Population	Ref
$CD4^+$ T cells	INF-γ secretory polarization	Establishment of autoimmune-prone milieu	ND T1DM subjects	Arif, Tree et al 2004[103]
Insulin-reactive $CD4^+$ T cells	Lower threshold for activation	Activation of islet specific responses	T1DM subjects	Yang, Danke et al 2008[104]
GAD65/ Insulin-reactive $CD8^+$T cells	Activation to CTL independent on CD28/B7	Lower need for activation to CTL	T1DM subjects	Viglietta, Kent et al 2002[105]
GAD65/ Insulin-reactive $CD8^+$T cells	Expression of CD45RO memory marker	Lower need for activation to CTL	T1DM subjects	Danke, Yang et al 2005;[106] Monti, Scirpoli et al 2007[107]
$CD4^+CD25^+$ T cells (Treg)	Weak Suppressive function in vitro and altered secretory phenotype	Inadequate suppression of tissue specific autoimmunity	ND T1DM subjects	Lindley, Dayan et al 2005[108]
DC	Secretive defect of antiviral cytokine IFN-α	Incomplete clearance of viral determinants	T1DM subjects	Summers, Marleau et al 2006[109]
DC	Absolute number reduction	Impaired immunoregulation	ND and LT T1DM subjects	Vuckovic, Withers et al 2007[110]
DC	Immature phenotype (abnormal NF-κB activation)	Inadequate Treg stimulation	ND T1DM subjects	Mollah, Pai et al 2008[111]
DC	Increased pDC-derived IFN-α; decreased mDC-derived IL-6	Altered innate response	ND T1DM	Meyers, Shah et al 2010[112]
DC	Poor in vitro maturation and pro-inflammatory cytokine response	Establishment of autoimmune-prone milieu	At-risk subjects	Skarsvik, Tiittanen et al 2004[113]
pDC	Increased frequency, enhanced presentation islet peptide to Th	Activation of islet specific responses	T1DM subjects	Allen, Pang et al 2009[114]
IFN-γ inducible cytokines (CXCL-10)	Increased serum level	Establishment of phlogistic milieu	T1DM and at-risk subjects	Nicoletti, Conget et al 2002[115]

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Table 3. Continued

Immune Cell/ Molecules	Defect	T1DM-Relevant Implication	Study Population	Ref
ICAM-1 and L-selectin	Increased serum level	Targeted migration and infiltration of flogistic mediators	T1DM and at-risk subjects	Lampeter, Kishimoto et al 1992[116]
NK	Reduced expression of activating receptors and reduced production of IFN-γ and perforin	Impaired cytotoxicity and possible reduced clearance of infected cells	ND and LT T1DM subjects	Rodacki, Svoren et al 2007[117]
iNK	Reduced frequency and Th-1 shifted cytokine secretive pattern	Establishment of proinflammatory milieu	ND and LT T1DM subjects	Kis, Engelmann et al 2007[118]

INF, interferon; GAD65, Glutamic acid decarboxylase isoenzyme 65; CTL, cytotoxic T-lymphocytes; ND, newly diagnosed; DC, dendritic cells; LT, long term; pDC, plasmacytoid dendritic cells; Th, helper T cells; ICAM, Intercellular adhesion molecule; NK, natural killers; iNK, invariant natural killers.

APC expressing GAD65, proinsulin and IA-2 have been described in human lymphoid organs, suggesting peripheral mechanisms of tolerance to islet antigens.[124]

Dendritic Cells

Dendritic cells (DC) are a subset of highly specialized APC displaying both tolerogenic and immunogenic properties, with potential effects in early failure of tolerance as well as in the persistence of autoimmune β-cell damage.[132]

DC are generally distinguished into conventional or myeloid (mDC) and plasmacytoid (pDC).[133] The surface expression of pattern recognition receptors (PRR) as toll-like receptors (TLR) and potent production of the anti-viral cytokine interferon (IFN)-α, pDC are effectors of the innate immune system.[133] As adaptive immune effectors, DC are able of "cross presentation" of exogenous peptides within MHCI complexes to effector T-lymphocytes,[134] a possible relevant mechanism in the very early stages of insulitis. As to islet cell antigens, one study reported that DC can present proinsulin epitopes through direct transcriptional events.[135]

Under homeostatic conditions, DC display low expression of costimulatory factors for naïve T-lymphocytes, as CD40.[133] Upon antigen uptake and processing, DC undergo maturation, acquire an overall immunogenic phenotype and promote antigen-specific T-cell clonal expansion via secretion of costimulatory molecules and cytokines. Among them, pro-inflammatory interleukins (IL) as IL12p70, that induces $CD4^+$ T and $CD8^+$ T-cell differentiation, IL-1β, IFN-γ and tumor necrosis factor (TNF)-α.[132] However, the ultimate DC mature phenotype as well as the outcome of DC-T-cell interaction largely depends on local cytokine milieu. In an anti-inflammatory environment, enriched with transforming growth factor (TGF)-β and IL-10, DC achieve tolerogenic properties[136] and dampen immunoreactivity via secretion of anti-inflammatory cytokines[137] and antigen-specific

expansion of Treg subsets.[138] DC may also directly induce T-cell apoptosis through the secretion of indoleamine 2,3-dioxygenase (IDO)[139] and PD-1.[128]

DC subsets from T1DM patients might be affected by either numeric or secretive defects that have been variously and not often consistently reported (summarized in Table 3). As to functional alterations, increased islet autoantigen presentation to T-lymphocytes has been proposed, though possible suboptimal T-cell activation capacity might equally ease autoimmunity hampering the expansion of Treg (Table 3). However, all these studies rely upon in vitro PMBC-derived DC that may not reflect the in vivo situation at the real sites of immunological events.

Regulatory T-Lymphocytes

Treg are a recently identified subpopulation of T-lymphocytes with immunoregulatory effects and play a major role in the maintenance of T-cell peripheral tolerance.[119] In vitro studies of Treg depletion suggest that in healthy individuals self-reactive T cells might be quiescent for peripheral immunosuppression by Treg.[106] Treg co-express CD4 and CD25, the α chain of the IL-2 receptor (ILR2A), which plays a role in maintaining islet immune tolerance is confirmed by the presence of T1DM-associated ILR2A polymorphisms[10] (Table 1).

Among Treg, the subset of "natural" Treg (nTreg) derives from intrathymic tolerization and is characterized by constitutive expression of the forkhead winged helix transcription factor (FoxP3).[140] The role of FoxP3 in the suppression of organ-specific autoimmunity is well attested by the severe systemic autoimmune disease resulting from *foxP3* mutations (IPEX). However, Treg function is very complex and possibly involving many other factors.[140] nTreg intervene at the site of lymphocytes priming,[141] where they participate to anti-inflammatory environment both directly through cytokine secretion and indirectly through the induction of DC tolerogenicity.[139] Although not proved in humans, nTreg may also act within the islet infiltrate directly on activated cytotoxic T-lymphocytes (CTL) and inflammatory cells, as observed in the mouse models.

Conventional Treg (cTreg) differentiate from naïve $CD4^+$ T cells in the periphery and consist of a heterogeneous population of lymphocytes producing anti-inflammatory cytokines,[142] among which IL-10 (Type 1 Treg).[143] IL-10 is a potent systemic immune suppressor that regulates effector T-cell activation, proliferation and IFN-γ secretion[143] and possibly inhibits further enrollment of APC to the site of inflammation.[144]

A simple deficiency in Treg peripheral frequency has not been constantly reported,[145,146] though several possible functional defects have been described in Treg repertoire of T1DM patients (Table 3). ELISPOT analysis has demonstrated that in healthy subjects autoantigen stimulation elicits IL-10^+ cTreg-like responses, whereas T1DM patients show a proinflammatory cytokine secretion.[103] Among T1DM patients, an IL-10-skewed secretive phenotype is described in association with later onset[103] and better glycemic control.[147]

Interestingly enough, Treg from children born to T1DM mothers exhibit an evident memory phenotype suggesting that fetal immunological activation might promote the expansion of regulatory T cells.[148]

Natural Killers T-Lymphocytes

Natural killers T-lymphocytes (NKT) are innate-like lymphocytes coexpressing NK cell surface markers and a TCR. Most NKT display an invariant α chain (Vα24-Jα18) (invariant NK or iNKT), are potent Th-2 mediators and are involved in several human autoimmune diseases.[149] NKT recognize glycolipid antigens in the context of the monomorphic restriction molecule CD1d, commonly expressed by B-lymphocytes.[150] Differently, NK are activated by IFN and related cytokines and exert nonantigen specific perforin-induced cytotoxicity, upon stimulation of surface receptor NKG2D.[151] The contribution of NK and NKT in β-cell autoimmune destruction is not known, though a prevalent immunomodulatory function can be hypothesized based on mouse studies.

Investigations of possible defects in iNKT from T1DM patients have not gained insights into possible pathogenetic roles, as the published studies variously support and disprove the hypothesis of altered frequency or functional impairment.[152-154] The evaluation of NK subsets in the serum of T1DM patients are equally mostly inconclusive (reviewed in ref. 155). Nevertheless, it is possible to postulate that reduced expression of NKG2D receptor mediates the increased risk for T1DM associated with mutations of *mic-A* gene, which encodes for NKG2D natural ligands.[38]

IMMUNOBIOLOGY OF β-CELL DESTRUCTION

The earliest event in islet autoimmunity is the priming of naïve Th by APC presenting islets antigens and likely takes place in the pancreatic lymph nodes (PLN) also in humans, as in mice (Fig. 1). The identification of the primary triggers of antigen uptake by APC in the islets has not been feasible in humans. However, it can be postulated that antigens discharged upon β-cell damage are captured and transported to PLN (Fig. 1).

In the islets, an initial external harmful event may primitively activate the innate immune system of the β cell and induce surface ligands for the PRR. The activation of innate receptors elicits intracellular "stress" responses as endoplasmic reticulum stress and accumulation of misfolded proteins, promoting β-cell apoptosis and the release of immunostimulatory signals for APC, first-line mediators of the adaptive response. Interestingly, human DC are able to crosspresent antigens derived from apoptotic cells within pMHC class I complexes[156] and might be the first to infiltrate the islets.[157]

In this condition, the local environment is enriched with inflammatory cytokines (IFN and IL-1β) and chemokines, aimed at recruitment and retention of immune effectors to eradicate the initial threat. Recent consistent evidence has been found for aberrant TLR-induced response in T1DM islets, with increased IFN-α and IL-1β responses.[112] IFN activates macrophage, polarizes T-cell responses and upregulates antigen expression on the dying cell. IL-1β is key innate immune effector and stimulates activated lymphocytes, inducing direct and TFN-α-mediated cytolisis.[158]

Physiologically, these events would create a self-limiting proinflammatory environment and allow the recovery of tissue homeostasis. Individual genetic background may hinder the effective resolution and promote a sustained, destructive immune reaction. The role of innate and adaptive responses in the induction and amplification respectively of β-cell loss is addressed in a recent review.[159]

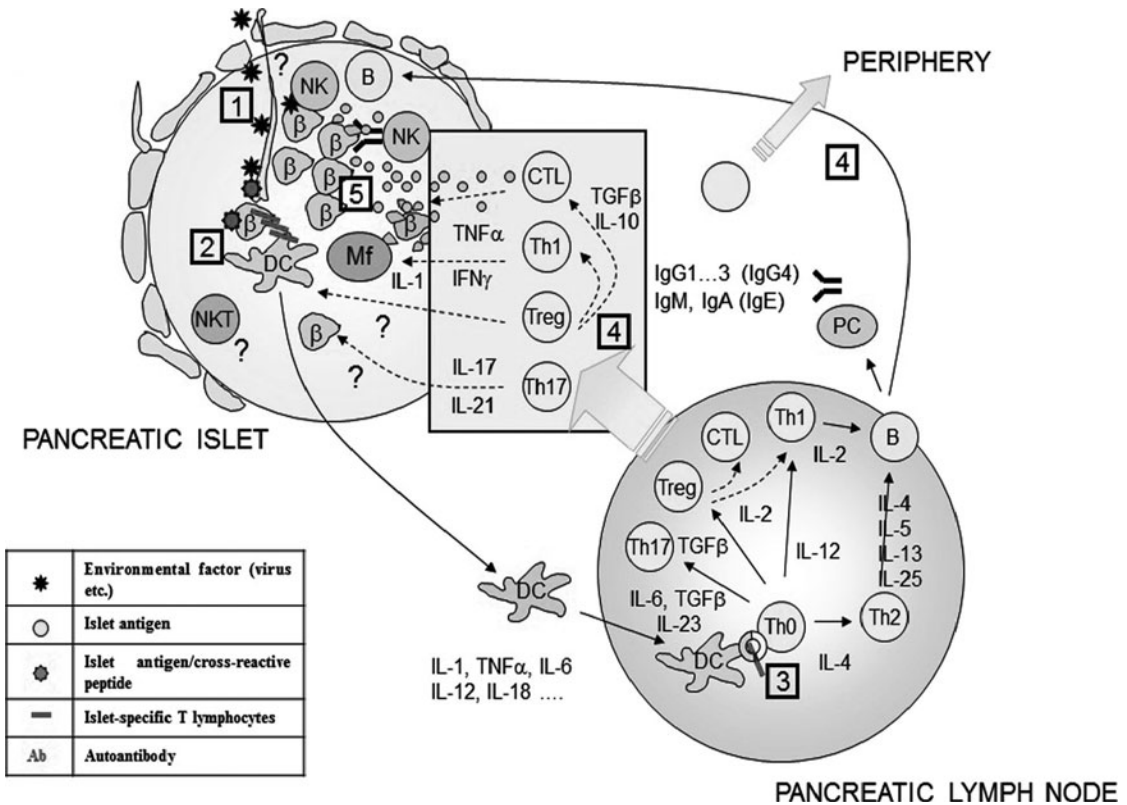

Figure 1. Pathogenesis of immune-mediated β-cell destruction 1. Triggering environmental factors initiate β-cell damage; 2. Dendritic cells take up cell antigens or cross-reacting peptides; 3. Dendritic cells, migrated to pancreatic lymph nodes, present peptides to naïve T helper (Th) cells and induce expansion of activated T-cell subsets and plasmacells through differentiated cytokine milieu; 4. Activated immune cells migrate to the islets, crosstalk with periphery; 5. β cells are destroyed by cytokine and cell-cell mediated mechanisms. β, β cell; B, B lymphocytes; CTL, cytotoxic T-lymphocytes; DC, dendritic cells; Mf, macrophages; NK, natural killer cells; NKT, natural killer T cells; PC, plasma cells; Th, T helper lymphocytes; Treg, regulatory T-lymphocytes. Reproduced from: La Torre D, Lenmark Å. Immunology of β-cell destruction. In: Islam, Md. Shahidul, ed. The Islets of Langerhans. New York: Springer Science+Business Media, 2010:Figure 24.1, pg. 551; ©2010 with kind permission of Springer Science+Business Media B.V.

Environmental Triggers

Environmental agents likely account for T1DM low concordance between monozygotic twins, geographical gradient and overall incidence increase.[14]

Viruses

Epidemiology observations demonstrate that TD1 onset may follow viral diseases,[160,161] as mostly suggested by studies on congenital rubella.[162] On the other hand, successful vaccination programs, decreasing the incidence of common infections among children in the last decades, have been paralleled by a raise in T1DM. Nevertheless, a potential causative role for vaccinations has been ruled out.[163] To complicate the picture, there is also evidence for an inverse correlation between infancy infections and T1DM development,[20] possibly explained by a protective effect that microbial antigens might exert by activating Treg and "diverting" immune system from autoantigens.[164] According to this view, the "hygiene hypothesis" proposes that the virtually sterilized environment resulting from widespread use of antibiotics and vaccinations may abolish the beneficial effect of infections and account for higher T1DM frequency in recent past, as well as in developed countries.[164]

Several viruses as mumps, rubella, enterovirus (EV), rotavirus, cytomegalovirus, Epstein-Barr virus and encephalomyocarditis virus have been linked to T1DM,[165,166] mostly through the detection of viral RNA in the blood of new onset T1DM patients.[167] More recently, immunostaining of EV capsid protein VP1 was detected in pancreas specimens from recent-onset T1DM and not in healthy controls.[168] However, this finding was common to Type 1 and Type 2, non autoimmune, diabetes samples, supporting that viruses may rather participate through a non immune-mediated β-cell damage to clinical hyperglycemia. A recent study reported the intriguing detection of EV in small intestinal biopsy specimens of T1DM patients through hybridization and immunohistochemistry.[169] The importance of intestine as an immunological site allows speculating that the virus might not only be retained and subsequently directly infect the islets, but also locally stimulate lymphocytes that initiate an aberrant immune response.[169] Agents of common gastroenteritis in children as EV and rotaviruses figure predominantly as candidate triggers of islet autoimmunity.[170] For a review on the presence of β-cell viral footprints in T1DM see reference 171.

Despite this evidence, no conclusive pathogenic connection has been found between virus infection and autoimmune β-cell destruction.[165,166,172] It is proved that human β cells increase PRR expression when infected by a virus or exposed to virus-related cytokines as IFN and IL-1β,[173] confirming the early local activation of specific innate responses. The hypersecretion of a wide array of pro inflammatory cytokines as IL-1 and IL-6 by human β cells under the effect of mums infection has been also demonstrated.[174] Mice studies clearly indicates that CTL are able to kill β cells expressing viral antigens,[175] but experimental evidences are lacking in humans to prove that upon a viral infection β cells express specific pMHCI complexes and become target for virus-specific CTL.

Viruses may participate in β-cell autoimmune destruction through different mechanisms at different levels, from the early induction of islet autoimmunity to the ultimate precipitation to clinical T1DM.

Viruses may activate β-cell intracellular signaling and induce altered expression of surface self-antigens ('neoantigens' or 'cryptic antigens'), which initiate β-cell

autoimmune attack.[165] Moreover, virus replication in the β cell may result in necrosis and release of previously sequestered immunogenic cellular constituents ('hidden antigens'), lacking thymic tolerance. Cross reactivity between viral and islet antigens due to similar amino acidic sequences ("molecular mimicry"), potentially misleading the immune response, has been proposed for Coxackie B/GAD65,[176] rotavirus/IA-2,[177] rubella[178] and cytomegalovirus.[166] Nevertheless, this event is probably more relevant to the amplification and persistence of the autoimmune process after the resolution of the viral infection, than to the initial trigger of autoimmunity.[179]

Viruses might also induce "bystander activation" of autoimmunity via local enrichment of proinflammatory mediators, that spare autoreactive T-lymphocytes from tolerization.[166]

Viral diabetogenicity might also be related to the precipitation of β-cell loss through direct lysis of the infected β cell or the aspecific impairment of local homeostasis due to insulin resistance.[166]

Genetic determined abnormal antiviral responses may provide explanation for T1DM associated variants of *ifih-1* gene, which might result in inappropriate detection and clearance of viral products and in exacerbated antiviral immunity[11] (Table 1).

Other Environmental Factors

As proved by experimental data on rodent β-cell toxin streptozotocin, toxin-induced islet destruction may develop through pMHC cell dependent insulitis. Similarly, the rodenticide Vacor induces islet cell surface antibodies in the rats confirming that β-cell toxic destruction may cause specific islet autoimmunity.

Several chemicals are potential β cytotoxins for humans, as dietary microbial toxins[180] and food nitrosamine derivatives.[181] It has been suggested that the consumption of food treated with additive compounds as nitrosamine,[181] proteins and carbohydrates[182] significantly enhances T1DM risk among children. European registries report that the timing of introduction of usual dietary factors in early infancy may also be crucial to later development of T1DM.[17] Long exclusive breastfeeding is reported inversely associated to both β-cell autoimmunity and T1DM,[183,184] whereas early exposure to cow milk proteins may increase T1DM risk,[185] possibly via molecular mimicry by bovine serum albumin.[186]

Apart from breastfeeding, the relevance of perinatal factors is largely unknown. Studies investigating maternal infections as a T1DM risk factor for the newborn have yielded contrasting conclusions.[187,188] The detection of islet autoantibodies in the cord blood is similarly controversial, as it does not seem to correlate with later development of T1DM,[189] but might be inversely correlated to the number of antibodies at disease onset.[190]

Antigen Presentation in Pancreatic Lymph Nodes

In the PLN, islet antigens are presented to naïve Th, $CD8^+$ T and B-lymphocytes (Fig. 1). Primed Th proliferate and differentiate into several subsets, in dependence on the cytokine milieu.[191] IL-6, IL-12 and IL-23 direct T-cell expansion towards Type 1 $CD4^+$ T cells (Th1) and IL-17 producing $CD4^+$ T cells (Th17), which in turn secrete a wide array of pro-inflammatory products as IL-17,IL-17F, IL-21, IL-22 and IL-26.[192] Th17 subset has been recently characterized as potent innate inducer of tissue inflammation and autoimmunity and expresses surface receptors for mDC-derived cytokines IL-23 and IL-1.[192]

Naïve $CD8^+$ T-lymphocytes differentiate into antigen-specific CTL upon coupled antigen recognition by a Th1 on the same APC ("cross-priming").[193] The interaction of CD40 on APC and CD154 on the Th1 yields the costimulation required for $CD8^+$ T-cell activation via local production of proinflammatory cytokines as IL-12.[194] IFN-γ produced by $CD8^+$ T cells, in turns, enhances Th1 activity.[25]

Upon stimulation by "Th2-like" cytokines IL-4 and IL-5, primed Th proliferate and differentiate to Th2 and in turn activate naïve B-lymphocytes presenting specific surface pMHC into plasma cells producing antibodies with identical peptide specificity. B cell activation also requires primitive contact signals as CD40 for the responsiveness to T-derived lymphokines, growth and differentiation factors.[195]

Homing of T-Lymphocytes and Insulitis

Partial pancreas transplant between monozygotic twins has attested that in T1DM β cells are damaged by CTL endowed with immunological memory of specific autoimmunity, rapidly infiltrating and destroying donor islets.[196] Immunocytochemistry on pancreas biopsies from new-onset T1DM patients has confirmed the presence of $CD8^+$ T cells and activated macrophages,[197] though the demonstration of antigen specificity has not been feasible in humans. Recent studies recording the evolution of human T1DM suggest a defined sequence of immune cell recruitment, where CTL and macrophages may contribute to β-cell death during early insulitis.[198]

In the islets, primed T-lymphocytes recognizing targeted β-cell antigens are directly activated to CTL without the need for costimulation, are retained and initiate insulitis (Fig. 1). Interestingly, the hyperexpression of adhesion molecules, reported not only in the blood[116] but also in pancreas specimens from new onset T1DM,[199] does not fully account for the massive recruitment of autoantigen-specific T cells to the islets. The mechanisms guiding the targeted migration ("homing") of primed T-lymphocytes to the islets are still not clear.[200] It is hypothesized that T-lymphocytes gain tissue tropism at the site of priming, through a specific "homing receptor pattern".[201]

In the inflammatory environment of insulitis, islet vascular endothelial cells (iVEC) might contribute to antigen-driven trafficking of autoreactive T-lymphocytes. This hypothesis is robustly supported by the evidence that T1DM iVEC show increased MHCII surface expression[202] and are able to promote in vitro transmigration of GAD65-autoreactive cells across endothelium monolayers via presentation of GAD65 epitopes.[203] Concurrently, iVEC upregulate surface adhesion molecules, ICAM (intercellular adhesion molecules) and JAM-1 (junctional adhesion molecules), which facilitate the penetration of effectors via interaction with T-cell surface integrins as LFA-1 (leukocyte function-associated antigen-1).[204] Antigen-driven lymphocyte activation by iVEC might also provide LFA-1 functional activation required for successful T-cell adhesion.[205] The ongoing inflammatory islet milieu in turn expands the trafficking of autoreactive CTL through the expression of chemokines and homing ligands from the β cells and promotes further enrollment of T-lymphocytes and APC. Among chemokines, CXCL-10 is a crucial mediator of inflammation in the distressed islet and has been recently described in T1DM islets along with infiltration by lymphocytes expressing the specific receptor CXCR3.[147]

At this stage, Th participate by secreting various lymphokines that attract and activate other cell effectors as monocytes, eosinophils and NKT.[202] Upon chemokines attraction, neighboring immature DC are also induced to migration toward the site of

antigenic stimulation, through the dissolution of podosomes.[206] The phlogistic environment hammers DC immunogenicity[132] and shifts Th towards "Th1-like" responses in which new T clonotypes develop, unreceptive to peripheral tolerization.[207]

Immunoglobulin deposits in T1DM human islet specimens are not constantly reported.[23,25] The pathogenetic involvement of islet-specific autoantibodies in β-cell destruction is still matter of debate, though autoantibody-mediated cytotoxicity on human β cells has been observed in vitro.[208] Clinical observations are also controversial, as severe deficiency of humoral response do not protect from T1DM,[209] nevertheless pharmacological B-lymphocyte depletion might preserve β-cell function.[210] Moreover, B-lymphocytes can uptake small amounts of antigens to present to T-lymphocytes, participating in the maintenance of autoimmune reaction after most tissue has been destroyed.[211] Recent advances seem to suggest that B-lymphocytes might play a greater role than traditionally assumed (reviewed in ref. 212).

β-Cell Destruction

Progressive islet invasion further contributes to the recruitment of chemokines-attracted immune effectors that release multiple proinflammatory mediators and synergistically maintain and amplificate insulitis. The intensification of these processes lead to progressive accumulation of immune cells, cytotoxic soluble mediators and reactive oxygen species secreted by infiltrating cells (Fig. 1). Apoptosis is probably the main form of β-cell death in T1DM[213] and in turn sustains insulitis through the secondary activation of innate responses inducing expression of surface pMHCI complexes and activation of PRR.[159] Cytotoxic inflammatory cytokines IL-1, TNF-α and IFN-γ produced by CTL and macrophages affect β-cell gene regulatory networks, primarily through transcription factors NFκB, STAT-1 and AP-1 and activate programmed cell death.[214] IL-1 exerts β-cell cytotoxicity in vitro[215] and inhibits insulin secretion by human islets,[216] possibly through the induction of nitric oxide release from the β cells.[217] Reactive oxygen species act through direct DNA damage and may be detrimental to β cells, which lack free radical scavenger activity.

Beta-cell damage also results from direct cell to cell contact with activated macrophages, lymphocytes and NK.[218] CTL trigger β-cell death directly through secretion of perforin that induces intracellular access of proteases (granzymes) and indirectly through specific ligands for apoptosis-inducing receptors on β cells.[213] Essential role is played by Fas ligand-Fas complex, that directly transduces the signal for apoptosis activation also in human T1DM.[219] Upon stimulation by IFN and other macrophage-derived cytokines, NK exert non-antigen specific cytotoxicity through the release of perforin, after the activation of surface receptors NKG2D.[218] At late stages, β-cell functional impairment may worsen the picture, as insulin epitope presentation seems to be enhanced in hyperglycemic local environment.[220]

As a final point, it must be noticed that most investigations on T-cell repertoire and phlogistic mediators in humans have been performed on peripheral blood samples (Table 3). Disease-relevant alterations might rather display at the sites of locally generated imbalances, islets and PLN. As recently addressed by a comprehensive review, data on gene expression profiling in T1DM partly overwhelming these limitations have documented an overexpression of inflammatory and innate immune response genes in target organs.[221]

CONCLUSION

The islet of Langherhans is the major target in the autoimmune attack in T1DM. Autoimmune β-cell destruction develops through a subclinical long prodrome, reflected by the presence of autoantibodies to specific β-cell antigens. The progression from islet autoimmunity to overt T1DM results from the establishment of chronic insulitis and extensive β-cell loss. Several environmental factors are proposed to have permissive role in the penetrance of susceptible genotypes. The understanding of T1DM etiology and pathogenesis is limited by the lack of data on events initiating islet autoimmunity as well as factors precipitating destructive insulitis. Searching efforts are needed to clarify early pathogenetic mechanisms to develop evidence-based approaches for the prevention and the treatment of T1DM.

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