DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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AUTOIMMUNE DIABETES:
an immunological study of type 1 diabetes in humans and in a model of experimental diabetes (in RIP-B7.1 mice)

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ABBREVATIONS

AAb	autoantibody
APC	antigen presenting cell
DMK	dystrophia myotonica kinase
EAD	experimental autoimmune diabetes
GAD	glutamate decarboxylase
GADA	glutamate decarboxylase autoantibodies
HA	haemagglutinin
HLA	human leukocyte antigen
IAA	insulin autoantibodies
IA-2A	tyrosine phosphatase autoantibodies
ICA	islet cell antibodies
IFN-α	interferon-alpha
IFN-γ	interferon-gamma
IFNAR	interferon type 1 receptor
IGRP	islet-specific glucose-6-phosphatase catalytic subunit–related protein
IL-10	interleukin-10
INS	insulin gene
KO	knockout
LADA	latent autoimmune diabetes of adults
LCMV	lymphocytic choriomeningitis virus
LCMV-GP	LCMV glycoprotein
LCMV-NP	LCMV nucleoprotein
mAb	monoclonal antibodies
MHC	major histocompatibility complex
NOD mice	nonobese diabetic mice
PBMC	peripheral blood mononuclear cells
pins	proinsulin
ppins	preproinsulin
RIP	rat insulin promoter
SCID	severe combined immunodeficiency
T1D	type 1 diabetes
TLR	toll-like receptor
TNF-α	tumour necrosis factor-alpha
VNTR	variable number of tandem repeats
wt	wild-type

1. INTRODUCTION

Type 1 diabetes (T1D) develops in genetically predisposed individuals as a consequence of autoimmune destruction of pancreatic β-cells. Direct evidence of the autoimmune nature of T1D dates back to more than 30 years ago when Bottazzo et al. demonstrated the presence of circulating islet cell antibodies (ICA) in the sera of patients with diabetes mellitus (Bottazzo et al., 1974). Since then studies in affected humans and in animal models of T1D have concentrated on elucidation of the genetic determinants and environmental triggers of the disease, and on characterisation of pathogenic immune responses and auto-antigenic targets in its pathogenesis (Gianani and Eisenbarth, 2005).

The aetiology of T1D is multifactorial and involves both environmental and genetic factors (Devendra et al., 2004a). The main environmental agents proposed to contribute to development of T1D include viral infections and dietary components but their role in the pathogenesis of the disease has remained poorly understood (Knip et al., 2005). Genetic studies of families with two or more affected individuals have described more than 20 gene loci associated with T1D (Kelly et al., 2003). Several determinants of genetic susceptibility have been identified; the best studied among them being the major histocompatiblity complex (MHC) genes encoding human leukocyte antigens (HLA) and the insulin gene region (INS), designated as IDDM1 and IDDM2, respectively (Gianani and Eisenbarth, 2005).

Following the initial description of ICA, multiple diabetes-associated autoantibody (AAb) specificities have been identified. Most frequently, at disease onset AAb against glutamic acid decarboxylase (GADA), tyrosine phosphatase-like protein IA–2 (IA–2A) and insulin (IAA) – all widely expressed in β-cells – are found (Pihoker et al., 2005). Data from a few populations indicate that the AAb profile at disease onset is influenced by particular susceptibility genotypes at IDDM1 and IDDM2, probably reflecting the modifying effect of genes on development of immune responses against β-cell autoantigens in the natural course of T1D (Pihoker et al., 2005).

In T1D, final β-cell destruction is believed to be mediated by diabetogenic autoreactive T cells (Roep, 2003). Pancreatic islets are not easily accessible in humans, and, therefore, rodent models of T1D have been an indispensable experimental tool to get a more profound insight into the pathogenesis of the disease (Leiter and von Herrath, 2004). Mice develop diabetes either spontaneously in the nonobese diabetic mouse (NOD) model (Anderson and Bluestone, 2005), or in response to transgene-encoded "neo-self" antigens selectively expressed in pancreatic beta cells under rat insulin promoter (RIP) control (Oldstone, 2005). Examples of the latter approach include induction of diabetes by LCMV infection or immunisation against LCMV proteins in mice expressing LCMV antigens under RIP control (Oldstone, 2005). These models reflect the diabetogenicity of high avidity anti-viral T cells rather than the

diabetogenicity of low avidity autoreactive T cells specific for natural beta cell antigens.

In RIP-B7.1 mice, spontaneous development of diabetes is rarely observed but following immunisation with β-cell autoantigens a high proportion of animals are affected by the disease (Karges et al., 2002; Pechhold et al., 2003; Devendra et al., 2004b). In RIP-B7.1 (H-2^b) mice diabetes develops after immunisation with preproinsulin (ppins) but not glutamic acid decarboxylase (GAD) expressing plasmid DNA. It is plausible that β-cell damage is mediated by (prepro)insulin-specific autoreactive T cells in this experimental autoimmune diabetes (EAD) model, however, the characterisation of these cells at the subpopulation level and their fine autoantigenic specificity have not been addressed so far.

In the current study, different immunological aspects of T1D were addressed with a focus on the role of insulin and glutamic acid decarboxylase as the target autoantigens. In the clinical part of the study, the prevalence of AAb and the frequency of major HLA class II alleles and INS polymorphisms in patients with newly diagnosed T1D in Estonia were determined, and the associations between genetic factors, particularly INS polymorphisms and appearance of AAb, were studied. In the experimental part of the study, the RIP-B7.1 mouse model (Karges et al., 2002) was used to investigate the role of different T cell subpopulations in development of T1D, to determine the epitope specificity of the major diabetogenic T cell subset and to elucidate the molecular mechanisms of β-cell destruction.

2. REVIEW OF THE LITERATURE

2.1. Human type 1 diabetes

T1D is a multifactorial autoimmune disease resulting from interactions between genetic and environmental determinants (Devendra et al., 2004a). The incidence rate of T1D ascertained in the age group of 0–14 year-old children varies widely ranging from 1.7/100,000 per year in Japan to more than 40/100,000 in Finland (Onkamo et al., 1999; Podar et al., 2001). Importantly, analysis of the incidence trends of T1D shows a worldwide incidence increase of 3–4% annually (Onkamo et al., 1999; EURODIAB ACE Study Group, 2000), and the largest rate of increase is observed in children under 5 years of age (EURODIAB ACE Study Group, 2000). In Estonia, the incidence of childhood-onset T1D has risen from 12.3 per 100,000 a year in 1991–1993 to 14.9 in 1999–2003 (Podar et al., 2001; Tillmann et al., 2004). The data of the incidence of T1D in older age groups are available from a few populations. In general, they suggest that development of T1D in adults is as common as in children (Molbak et al., 1994; Vandewalle et al., 1997).

Studies of monozygotic twins have found the concordance rate for T1D being about 30–40% which is the best evidence of involvement of both genetic and non-genetic factors in disease development (Redondo et al., 2001). Importantly, studies of monozygotic twins have also demonstrated age-dependence of the effect of genetic determinants on disease penetration. If diabetes occurs before the age 5 in the first monozygotic twin, the diabetes risk for the second twin is 50%, whereas diabetes development after the age 25 in the first twin confers the risk of only 5% for the second twin (Redondo et al., 2001).

2.1.1. Environmental agents

Mainly, viral infections and dietary components have been suggested as possible environmental factors driving autoimmunity against β-cells (Gianani and Eisenbarth, 2005). The role of viral infections in emerging autoimmunity is supported by the observation that both presentation with T1D (Mooney et al., 2004) and the appearance of AAb in prediabetic individuals (Kimpimaki et al., 2001) show seasonal variation being the most common in the cold season. In the Finnish Diabetes Prediction and Prevention (DIPP) study the peak of laboratory-confirmed enteroviral infections was shown to precede the seasonal peak of AAb occurrence (Knip et al., 2005). Paradoxically, at the same time, the frequency of enteroviral infections has decreased over the last decades in the background population in Finland. This apparent paradox can be explained by the decreased maternal transfer of protective antibodies, which results in the vulnerability of young children to the diabetogenic effect of enteroviruses (Knip et al., 2005). Indeed, when the prevalence of maternal enterovirus antibodies in

populations with different T1D incidence was studied, antibody frequency was significantly higher in countries with low and intermediate incidence of T1D compared with high-incidence countries (Viskari et al., 2005). The viruses are thought to trigger autoimmunity either by T-cell activation through local or systemic infection or by molecular mimicry between viral and β-cell antigenic sequences (Yang and Santamaria, 2003). Some viruses can infect and destroy human β-cells, as demonstrated by studying pancreata from children with fatal viral infections (Jenson et al., 1980). Furthermore, the viruses have been shown to induce the damage of human β-cells *in vitro* (Roivainen et al., 2000), and to initiate islet inflammation and hyperglycemia in mouse models of diabetes through local (Horwitz et al., 1998) or systemic effects (Lang et al., 2005). Another explanation of how the viruses can trigger autoimmunity is molecular mimicry between the viral sequences and the β-cell autoantigens, e.g. between the P2-C protein of the Coxsackie B4 virus and glutamate decarboxylase (Kaufman et al., 1992). However, it has been suggested that the viruses and other environmental encounters may serve as modifiers rather than triggers of the disease in that they either promote or attenuate diabetes development depending on timing and quantity of exposures (Atkinson and Eisenbarth, 2001).

Dietary antigens are another good candidate for modifying factors in the pathogenesis of T1D due to an early and abundant exposure of children to various dietary components (Knip et al., 2005). The Childhood Diabetes in Finland Study Group has reported that early infant exposure to cow's milk is associated with seroconversion to positivity for β-cell AAb and with progression to the clinical disease (Virtanen et al., 1998). However, other studies (Norris et al., 1996; Hummel et al., 2000) have failed to demonstrate the relationship between early cow's milk consumption and T1D. Ongoing large clinical studies, i.e. the trial to reduce IDDM in the genetically at risk (TRIGR) (Knip et al., 2005) and the environmental determinants of diabetes in the young (TEDDY) (Hagopian et al., 2006) have been introduced to identify major environmental determinants of T1D.

2.1.2. Genetic susceptibility

Two major determinants of genetic susceptibility to T1D are the HLA genes, located within the MHC complex on chromosome 6p21, and the INS on chromosome 11p15, designated as IDDM1 and IDDM2, respectively (Davies et al., 1994; Kelly et al., 2003). The genome screen has demonstrated that the IDDM1 locus accounts for approximately 40% and the IDDM2 locus contributes a further 10% of familial inheritance of T1D (Kelly et al., 2003). Other identified diabetes-susceptibility genes include the cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Ueda et al., 2003), the lymphoid protein tyrosine phosphate (LYP encoded in PTPN22) (Bottini et al., 2004), the small ubiquitin-like modifier 4 (SUMO4) (Guo et al., 2004) and the interleukin-2 receptor

(IL2RA/CD25) (Vella et al., 2005) genes. Genome-wide association studies represent a powerful approach to the identification of the genes involved in common human diseases including type 1 diabetes (The Wellcome Trust Case Control Consortium, 2007).

2.1.2.1. Major histocompatibility complex

The genes encoded in MHC are arranged in three subregions: class I, class II, and class III. Class I genes encode the α-chain of HLA class I molecules, and class II genes encode the loci of both α- and β-chains of heterodimeric HLA class II molecules (HLA-DQ, -DR and -DP). Therefore, the MHC class I and class II subregions encode the key molecules of the adaptive immune system involved in the presentation of antigenic sequences to the $CD8^+$ and $CD4^+$ T cells, respectively. Class III genes encode a variety of molecules including the components of complement, and tumour necrosis factor-alpha (TNF-α) (Kelly et al., 2003). Identification of the primary disease susceptibility determinants within the MHC region has been hampered by the strong linkage disequilibrium of the genes. However, fine mapping studies have confined major T1D predisposition to the HLA class II genes, DRB1 and DQB1 (Herr et al., 2000). In Caucasians, the two main HLA-DQ heterodimers encoded by the DQA1*0301, DQB1*0302 (DQ8) and DQA1*0501, DQB1*0201 (DQ2) alleles have the strongest association with T1D. These haplotypes are in linkage disequilibrium with the HLA-DR4 and -DR3 alleles, respectively (Gianani and Eisenbarth, 2005). More than 90% of patients with T1D have either DR3-DQ2 or DR4-DQ8, while less than 40% of normal controls have these haplotypes (Devendra et al., 2004a). The heterozygous combination DR3-DQ2/DR4-DQ8 confers the highest risk for T1D. In the United States, approximately 50% of the children below age 5 years and 20–30% of adults presenting with T1D have this genotype compared with a population prevalence of 2.4% (Devendra et al., 2004a). Studying French patients with the childhood-onset (<15 years of age) T1D of Caucasian origin, Caillat-Zucman et al. found that 74.6% and 59.2% of the patients compared to 34.4% and 11.6% of healthy controls carried HLA-DQB1*0201 and DQB1*0302, respectively. The relative risk (RR) for development of T1D was 5.6 for HLA-DQB1*0201 and 11.5 for DQB1*0302. The HLA-DQB1*0302/0201 heterozygosity was observed in 33.6% of the patients and 2.3% of the controls (Caillat-Zucman et al., 1992). In Estonian patients with childhood-onset T1D, the most frequent HLA-DQB1-allele was *0302 (in 66% of the patients vs. 17.8% of the controls) followed by *0201 (in 54.6% of the patients vs. 31.1% of the controls) (Adojaan and Podar, 1998).

At the subtype level the overall disease risk is determined by both the HLA-DQ and -DR, and some DR-alleles (*0403, *0406) have been found to confer protection from the disease (Kelly et al., 2003). Moreover, the protective effect

of DRB1*0403 can prevail over the disease susceptibility conferred by the highest risk DR3-DQ2/DR4-DQ8 genotype (Roep et al., 1999).

The most common protective HLA molecule is the DQB1*0602. In the study of Caillat-Zucman et al., 35% of the controls carried the protective DQB1*0602 or *0603 alleles compared to 2.8% of the patients with T1D (Caillat-Zucman et al., 1992). In Estonia, these two alleles were present in 44.2% of the controls versus 2.1% of the patients with childhood-onset T1D (Adojaan and Podar, 1998). Weaker protection is associated with HLA-DQB1*0301 found in 34.4% of the controls and 11.3% of the patients in the study of Caillat-Zucman et al. (Caillat-Zucman et al., 1992). Similar results were obtained in Estonian study in which 33.1% of the controls and 10.3% of the patients carried HLA-DQB1*0301 (Adojaan and Podar, 1998).

More detailed genetic studies have indicated that genes in other MHC subregions including the MHC I can modify the risk for T1D (Kelly et al., 2003). In a study of Nejentsev et al., the HLA-B39 allele was found significantly more often in the patients with the high-risk DRB1*0404-DQB1*0302 haplotype than in the controls with the same haplotype (Nejentsev et al., 1997). Moreover, Tait et al. demonstrated in their study with 452 Australian families affected by T1D that while the appearance of the AAb in the preclinical phase of T1D is determined by the HLA II risk alleles, progression from preclinical AAb positivity to the overt disease is associated with particular HLA class I alleles (Tait et al., 2003).

2.1.2.2. Insulin gene

The primary association between T1D and polymorphisms within the INS is thought to be determined by a variable number of tandem repeats (VNTR) region about 0.5 kb upstream of the insulin gene (Bennett et al., 1995). Three classes of VNTR alleles have been identified segregated according to the number of the repeats of a 14–15 bp consensus sequence: class I alleles consist of 20–63 repeats, class II, of 64–139 repeats, and class III, of 140–210 repeats (Kelly et al., 2003). Homozygosity for class I alleles confers a 2–5-fold increase in T1D risk, while class III alleles are dominantly protective (Bennett et al., 1996). Class II alleles are very rare in the Caucasoid population (Kim and Polychronakos, 2005). Marginally lower insulin mRNA level in the human pancreas in association with class III alleles suggested that the VNTR have transcriptional effects on insulin expression (Bennett et al., 1995). Subsequently, studying insulin mRNA expression in the human thymus, 2–3 times higher levels were found in the presence of the protective class III VNTR compared to the predisposing class I alleles (Pugliese et al., 1997; Vafiadis et al., 1997). This finding suggested that the higher level of insulin in the thymus of individuals with class III VNTR may facilitate tolerance induction to insulin – one of the major autoantigens in T1D (Pugliese et al., 1997; Vafiadis

et al., 1997; Park, 2007). Thymic expression of autoantigens may alter the autoreactive T cell repertoire by a negative selection of potentially pathogenic insulin-specific T cells (Kelly et al., 2003), or by a positive selection of T cells with a regulatory phenotype (Durinovic-Bello et al., 2005).

2.1.3. Autoantibodies

In most cases of T1D, the disease-associated AAb as a hallmark of β-cell autoimmunity can be found at the clinical onset of the disease. They were first described in 1974 in the sera of patients with polyendocrine autoimmunity as cytoplasmic islet cell autoantibodies (ICA) reacting with frozen sections of the human pancreas (Bottazzo et al., 1974). The presence of IAA in the sera from patients treated with animal insulin was known since the 1950s but in 1983 Palmer et al. discovered that IAA are present in the sera from patients with new-onset T1D prior to initiation of treatment (Palmer et al., 1983). Subsequently, several studies have demonstrated the appearance of IAA in the preclinical phase of T1D (Verge et al., 1996; Kulmala et al., 1998; Knip, 2002). Additionally, in 1982 Baekkeskov et al. reported that a high number of sera from patients with newly diagnosed T1D recognise the 64 kD protein (Baekkeskov et al., 1982). This protein was identified as glutamic acid decarboxylase (GAD), the biosynthesizing enzyme of inhibitory neurotransmitter gamma-aminobutyric acid (Baekkeskov et al., 1990). In the early 1990s Christie et al. characterised another autoantigen which co-precipitated with GAD in 64 kD-positive sera and was shown by them to be related to the tyrosine phosphatase-like protein IA-2 (ICA512) (Christie et al., 1993; Payton et al., 1995).

In the following years assays for these four AAb (ICA, GADA, IA-2A and IAA) have been in the process of standardisation at international workshops (Pihoker et al., 2005).

The T1D is considered primarily a T cell mediated disease (Devendra et al., 2004a) although it has been hypothesized that the AAb may play a role in the disease pathogenesis by mediating the capture of autoantigens by antigen presenting cells (APC) and thereby facilitating the pathogenic T cell responses (Pihoker et al., 2005). However, β-cell AAb are useful markers for diagnostic purposes and for prediction of the disease both in first-degree relatives of the patients and in general population (Atkinson and Eisenbarth, 2001).

The diagnostic sensitivity of AAb varies with age at diagnosis of the disease. The diagnostic sensitivity of ICA between 80–90% has been reported in children and adolescents with newly diagnosed T1D (Strebelow et al., 1999; Sabbah et al., 2000) while ICA prevalence tends to decline with older age at diagnosis, being between 60–70% in the age group of 20–30 years (Sabbah et al., 2000; Graham et al., 2002) and less than 50% thereafter (Sabbah et al., 2000). The GADA are found in 70–80% of the patients of Caucasian origin at diagnosis of T1D and the

frequency of GADA is not affected by age (Pihoker et al., 2005). Vandewalle et al. have reported that the prevalence of GADA at diagnosis of T1D remained at 65–85% in adults between 20 and 40 years of age (Vandewalle et al., 1995). For IA-2A, the diagnostic sensitivity of 68–80% has been reported in patients with disease onset below age of 15 years (Strebelow et al., 1999; Sabbah et al., 2000; Graham et al., 2002) but the prevalence of this AAb decreases to 30–48% if the disease is diagnosed in adulthood (Sabbah et al., 2000; Graham et al., 2002). The diagnostic sensitivity of IAA varies most with age being about 70% in the very young (below age of 7 years) and decreasing already in the age groups of 7–13 and 14–20 years to 41% and 24%, respectively (Graham et al., 2002).

Efficient prediction of T1D is an important prerequisite for development of preventive treatment strategies of the disease. Several studies have followed the appearance of AAb in first-degree relatives, particularly in siblings, of patients with T1D. These studies have suggested that there is no single sensitive disease marker but rather the combined testing of AAb helps to predict diabetes development (Pihoker et al., 2005). In a study with 882 first-degree relatives of patients, Verge et al. showed that among individuals developing diabetes, the increase of the risk for the disease was associated with the number of biochemically measured AAb (IAA, GADA, or IA-2A) present (Verge et al., 1996). In a similar study Kulmala et al. reported that siblings of T1D patients with multiple (two or more of four) AAb had a risk of 55% for progression to T1D within 7.7 years compared with a risk of only 0.8% in those with one or no AAb (Kulmala et al., 1998). These results indicate that single AAb positivity may represent harmless nonprogressive β-cell autoimmunity, whereas two or more AAb reflect progressive β-cell destruction (Kukko et al., 2005). A few studies carried out in general population have demonstrated the feasibility of prediction of T1D by determination of AAb in subjects with no family history of T1D (Kimpimaki et al., 2002; Kukko et al., 2005).

2.1.4. Heterogeneity of T1D

The T1D can manifest at any age. A peak of incidence is found around puberty but about 50% of cases are diagnosed in adulthood (Molbak et al., 1994; Vandewalle et al., 1997). At diagnosis, adult-onset T1D is characterized by a longer symptomatic period, milder signs of metabolic decompensation, and a better preservation of residual β-cell function compared to T1D that begins in childhood (Karjalainen et al., 1989; Sabbah et al., 2000). The prevalence of various AAb depends on age at onset of T1D as discussed above, and this may reflect varieties in the pathogenesis of the disease (Pihoker et al., 2005). For instance, high proportion of very young children has IAA at diagnosis, and it has been hypothesized that IAA are a marker for a rapid β-cell loss (Falorni and Brozzetti, 2005). Moreover, several studies have reported an age-dependent

genetic heterogeneity of T1D, mainly a significant decrease of HLA-DR3/4 and DQB1*0302/02 heterozygosity and an increase of non-DR3/non-DR4 and DQB1*non-0302/non-02 genotypes in adult-onset compared to the childhood-onset disease (Karjalainen et al., 1989; Caillat-Zucman et al., 1992; Sabbah et al., 2000; Cerna et al., 2003; Jahromi and Eisenbarth, 2007).

In order to further dissect the heterogeneity of T1D, several studies have estimated the effect of genetic factors on humoral β-cell autoimmunity in different age groups. The HLA-DQA1*0501, DQB1*0201 (DQ2) haplotype was shown to be associated with appearance of GADA (Sanjeevi et al., 1996; Graham et al., 2002), and this effect was stronger in younger than in older patients (Graham et al., 2002). The IAA, IA-2A and ICA were observed significantly more frequently in patients carrying the HLA-DQA1*0301, DQB1*0302 (DQ8) haplotype (Graham et al., 2002; Vandewalle et al., 1993). Furthermore, the association between the INS VNTR I/I risk genotype and presence of IAA in patients with newly diagnosed T1D (Graham et al., 2002) and in children at risk for development of T1D (Hermann et al., 2005) suggests that allelic variations at the INS gene locus may modify insulin-specific autoimmunity. Altogether, these observations imply heterogeneity of T1D and indicate that the genes modify selection of autoantigenic targets in T1D.

Another group of patients with autoimmune diabetes is identified by presence of AAb, particularly GADA, in adult patients initially diagnosed with T2D. Clinical characteristics of these patients include lower body mass index (BMI), lower C-peptide level, and early need for insulin treatment compared to AAb-negative patients with T2D (Turner et al., 1997; Stenstrom et al., 2005). In the literature this subtype of diabetes is referred to as latent autoimmune diabetes in adults (LADA) (Falorni and Brozzetti, 2005; Stenstrom et al., 2005). The GADA have been found as being the most prevalent AAb in patients with LADA. A majority of studies have reported the prevalence of GADA between 9–12% in patients with the initial diagnosis of T2D (Turner et al., 1997; Tuomi et al., 1999; Pietropaolo et al., 2000). A few genetic studies have demonstrated a similar prevalence of high-risk HLA-DQB1 alleles in patients with LADA and classical adult-onset T1D (Hosszufalusi et al., 2003), and a low frequency of protective HLA-DQB1 genotypes in LADA patients (5%) compared to AAb-negative patients with T2D (42%) (Stenstrom et al., 2002). One study, however, has shown that LADA deviates from the classical T1D in terms of risk and protective HLA-DQB1 genotypes (Tuomi et al., 1999). Moreover, in the above study, in patients with LADA the frequency of the INS VNTR I/I genotype was similar to that of healthy controls but not to that of patients with T1D (Tuomi et al., 1999). Possible diversity of underlying pathogenetic mechanisms between LADA and T1D remains elusive (Leslie and Delli Castelli, 2004).

In Estonia, the frequency of HLA class II risk alleles and the prevalence of AAb in patients with newly diagnosed T1D were studied a decade ago in the age group of children younger than 15 years (Adojaan and Podar, 1998) and recently, the prevalence of the HLA and other T1D-associated genes has been

investigated in patients with the long-lasting disease (Haller et al., 2007). There are neither studies of genetic and autoimmunity markers in Estonian adult patients with newly diagnosed T1D, nor have age-dependent differences in the prevalence of the determinants of genetic susceptibility at the IDDM1 and IDDM2 loci and in the presence of AAb been investigated. The knowledge of the diagnostic sensitivity of AAb at the diagnosis of T1D at various ages is important for diagnostic purposes in clinical practice; also it helps plan future screening programme for individuals at increased risk for T1D as well as for general population. Studies on associations between the determinants of genetic susceptibility and AAb may reveal important data about the influences of genetic factors on selection of autoantigenic targets in the pathogenesis of T1D.

2.1.5. Cellular immunity in human T1D

Beta-cell destruction in T1D is believed to be mediated by pathogenic autoreactive T cells (Devendra et al., 2004a). Circulating peripheral blood mononuclear cells (PBMC) are the only relatively unlimited resource of disease-associated autoreactive T cells in humans. However, the low frequency of these cells in PBMC is a major obstacle to development of reliable T cell assays for studying human autoimmune diseases (Roep, 2002). In recent years, novel approaches, using flow cytometry (Brusko et al., 2005; Lindley et al., 2005; Endl et al., 2006), enzyme-linked immunosorbent spot (ELISPOT) assay (Arif et al., 2004; Toma et al., 2005) and MHC-tetramers (Reijonen et al., 2002; Pinkse et al., 2005), have been applied for characterization of autoreactive T cell and other immune cell subsets in T1D. "Humanised" transgenic mice carrying human high-risk HLA-DR*0401 or -DQ8 alleles have served as a useful tool to identify naturally processed $CD4^+$ T cell epitopes from relevant autoantigens including GAD65 and insulin (Patel et al., 1997; Congia et al., 1998; Herman et al., 1999). Subsequently, presence of T cells recognising these determinants has been demonstrated in human PBMC (Durinovic-Bello et al., 2002; Reijonen et al., 2002), indicating that mice transgenic for human genes are a suitable tool to study human autoreactive T cells. Studies in humans have shown that autoreactive T cells recognising β-cell autoantigens can be detected in patients with T1D as well as in HLA-matched healthy individuals but T cells from patients and healthy subjects differ in their phenotypic characteristics. For example, using the ELISPOT assay, Arif et al. demonstrated that T cell response to proinsulin and IA-2 exhibited a proinflammatory phenotype in patients as shown by high interferon-gamma (IFN-γ) production, whereas regulatory interleukin-10 (IL-10) response predominated in healthy individuals (Arif et al., 2004). In a study of Reijonen et al., $CD4^+$ T cells, expanded *in vitro* with an autoantigenic peptide from GAD65 and stained with MHC II tetramers, showed an activated phenotype in the patients but not in the controls (Reijonen et al., 2002).

Similarly, in a study of Endl et al. T cell activation markers ($CD25^+$, $CD134^+$) allowed discrimination between the proinsulin and GAD65 specific $CD4^+$ T cells from the patients and those from the healthy subjects (Endl et al., 2006). In more recent studies the $CD8^+$ T cells specific for β-cell autoantigens, including ppins, GAD and the islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP), have been detected in patients with new-onset T1D (Ouyang et al., 2006; Unger et al., 2007; Blancou et al., 2007; Mallone et al., 2007).

2.2. Animal models of type 1 diabetes

Human studies in individuals affected by T1D and in those at an increased risk for the disease have provided vast amounts of data on genetic and immune elements associated with the disease. However, the primary sites of immunopathologic events in T1D – the pancreatic islets and the regional pancreatic lymph nodes – are normally not accessible in humans. Therefore, investigation on animal, particularly rodent, models of T1D has been of utmost importance in understanding the immunopathogenesis of T1D. The most extensively studied spontaneous models of diabetes include the NOD mouse and the BioBreeding Diabetes-Prone (BB-DP) rat (Leiter and von Herrath, 2004). Additionally, a number of transgenic models have been developed. Particularly, the expression of transgenes under the control of the RIP has been employed to allow tissue (β-cell) specific expression of a transgene of interest. Neoantigens under the control of the RIP, such as proteins from the LCMV, can serve as targets of T cell attack triggered upon the viral infection (Oldstone et al., 1991), while RIP-driven expression of stimulatory molecules from the immune system, such as B7.1 (CD80) or TNF-α, increases the susceptibility of β-cells to autoimmune destruction (Guerder et al., 1994; Harlan et al., 1994; von Herrath et al., 1995).

2.2.1. NOD mice

The NOD mouse represents one of the best studied animal models of T1D (Gianani and Eisenbarth, 2005). Insulitis is found as early as around 5–7 weeks of age in NOD mice and overt diabetes develops in about 70% of female and 40% of male animals by 30 weeks of age (Wicker et al., 1987).

2.2.1.1. Genetic predisposition

The genetic predisposition of NOD mice to T1D has been mainly attributed to the unique $H2^{g7}$ MHC haplotype (A^{g7}, E^{null}, K^d, D^b) (DiLorenzo and Serreze, 2005). Studies in NOD mice, transgenic for MHC alleles derived from

haplotypes other than $H2^{g7}$, have demonstrated that the allelic combination of NOD mice is particularly permissive for diabetes development. T1D occurrence in NOD mice requires homozygous expression of H2-A^{g7} and transgenes encoding H2-A variants other than H2-A^{g7} or those encoding H2-E inhibit T1D development (DiLorenzo and Serreze, 2005). Structurally, H2-A^{g7} is unusual in that unlike most other allelic variants, it lacks aspartic acid in position 57 (Acha-Orbea and McDevitt, 1987). It is noteworthy that similar distinctiveness characterises also the human HLA-DQB1*0302 allele (Nepom and Kwok, 1998). The mechanisms by which non-Asp-57 alleles contribute to diabetogenesis are not entirely clear but may include impaired tolerance induction to the self-antigens in the thymus (Yang and Santamaria, 2003). In contrast, the MHC class I alleles of the NOD mouse – K^d and D^b – are quite usual and present also in diabetes-resistant mouse strains. However, in diabetes development in NOD mice these alleles seem to be as crucial as H2-A^{g7} because transgenic replacement of them by other MHC class I molecules prevents T1D (DiLorenzo and Serreze, 2005). Thus, most probably, in NOD mice MHC class I and class II alleles act in concert to generate the diabetogenic $CD8^+$ and $CD4^+$ T cell repertoire. Indeed, it has been shown that the diabetes resistance conferred by the heterozygous expression of the MHC II genes other than H-$2A^{g7}$ involves the anergy of autoreactive $CD8^+$ T cells (Serreze et al., 2004). The implication of both HLA class II and I alleles in T1D susceptibility in humans suggests that like in NOD mice, not a single susceptibility-allele but rather an unfavourable combination of genetic determinants may establish the pathogenic T cell repertoire leading to T1D.

2.2.1.2. Cellular requirements in the pathogenesis of T1D

During the early stages of insulitis macrophages and dendritic cells are the major immune cell populations infiltrating the islets in NOD mice followed by invasion of T-lymphocytes, NK cells and B-lymphocytes (Jun et al., 1999). Insulitis in NOD mice is preceded by a phase of β-cell remodelling and increased apoptosis which has been shown to represent a physiological process in the β-cells of neonatal rodents (Trudeau et al., 2000). Studies in NOD mice have indicated that clearance of apoptotic β-cells by macrophages (O'Brien et al., 2002) and presentation of self-antigens by dendritic cells (Peng et al., 2003) might be abnormal in this mouse strain and lead to priming and activation of autoreactive T cells in the pancreatic lymph nodes (DiLorenzo and Serreze, 2005). Activated T cells are believed to be the final effector cells mediating β-cell destruction upon recognition of their cognate self-antigens expressed on β-cells. Indeed, diabetes can be transmitted by adoptive transfer of purified T cells from diabetic NOD donors into neonatal or irradiated recipients (Bendelac et al., 1987; Miller et al., 1988a). Strikingly, diabetes transfer has been reported in

man after bone marrow transplantation from a HLA-identical diabetic sibling (Lampeter et al., 1993; Vialettes and Maraninchi, 1993).

For successful transfer of T1D from diabetic NOD donors to healthy neonates (Bendelac et al., 1987) or irradiated young adults (Miller et al., 1988a), both $CD4^+$ and $CD8^+$ T cells are necessary. However, to NOD mice with severe combined immunodeficiency (SCID), diabetes can be transferred with isolated $CD4^+$ or $CD8^+$ T cell clones specific for a β-cell autoantigen (Peterson and Haskins, 1996; Wong et al., 1996). Thus, both $CD4^+$ and $CD8^+$ T cells can function as final effectors in NOD mice causing β-cell damage that ultimately leads to diabetes (Yang and Santamaria, 2003).

2.2.1.3. $CD4^+$ T cells and their target autoantigens

Strong genetic association between H2-A^{g7} molecules and the disease has led to the investigation of $CD4^+$ T cells as the major pathogenic T cell population in T1D in NOD mice (Lieberman and DiLorenzo, 2003). Autoreactive $CD4^+$ T cells have been shown to recognise a variety of islet autoantigens, including insulin, glutamic acid decarboxylase (GAD) 65 and GAD67, tyrosine phosphatase-like IA-2, phogrin, ICA69, and heat shock protein 60 (Yang and Santamaria, 2003). Early studies have suggested GAD65 as a major autoantigen in the pathogenesis of T1D. Kaufman et al. demonstrated in NOD mice that at the onset of insulitis there develop pathogenic T cell responses to a confined region of GAD65. At the later stages there follow both intramolecular spreading of the immune response to additional GAD determinants and intermolecular spreading to other autoantigens like heat-shock protein 65 and insulin (Kaufman et al., 1993). The findings arguing the role of GAD65 as a key autoantigen in T1D have been opposed in later studies in genetically manipulated NOD mice. Kash et al. demonstrated that diabetes developed normally in GAD65 knockout NOD mice (Kash et al., 1999), and Jaeckel et al. showed that NOD mice tolerant to GAD65 show a normal incidence of diabetes (Jaeckel et al., 2003).

Another autoantigen proposed to be a trigger of pathogenic T cell responses in NOD mice is insulin. Mice have two preproinsulin (ppins) genes – ppins I (chromosome 19) and ppins II (chromosome 7) – both providing a metabolically active gene product.

The IAA represent a marker of autoimmunity in NOD mice (Bonifacio et al., 2002), and early studies on islet-infiltrating T cells showed that the majority of T cell clones derived from islet-infiltrating $CD4^+$ T cells of 4–12 wk old NOD mice recognise the insulin B-chain peptide 9–23 (Wegmann et al., 1994). Further evidence for the role of ppins as a key autoantigen in T1D comes from studies with ppins-I and ppins-II knockout NOD mice. It has been demonstrated that the disruption of either ppins-I or ppins-II has opposite effects on diabetes penetrance. Ppins-I knockout NOD mice are protected from the disease (Moriyama et al., 2003), whereas lack of ppins-II accelerates diabetes

development (Thebault-Baumont et al., 2003). The striking difference in diabetes outcome between these two knockouts has been ascribed to differences in the expression pattern of ppins-I and ppins-II in the thymus and in the pancreatic β-cells – the sites of tolerance induction and autoimmune attack, respectively. In the pancreatic islets both ppins-I and ppins-II are expressed (Deltour et al., 1993), while in the thymus preferential expression of ppins-II has been found (Heath et al., 1998). Accordingly, it has been hypothesised that in NOD mice, ppins-II may play a role in induction of central tolerance, while in β-cells, insulin-specific T cells preferentially target ppins-I (Gianani and Eisenbarth, 2005). Strikingly, when the native ppins genes of the NOD mice were replaced by a proinsulin transgene with a single amino acid substitution at position B_{16}, the mice were completely protected from diabetes indicating that the insulin B-chain$_{9–23}$ might be the main target of diabetogenic T cells in this mouse model (Nakayama et al., 2005).

2.2.1.4. $CD8^+$ T cells and their target autoantigens

The $CD8^+$ T cells are also absolutely necessary for development of diabetes in NOD mice as evidenced by the finding that β_2-microglobulin deficient and hence MHC class I- and $CD8^+$ T cell-deficient NOD mice are diabetes and insulitis resistant (Serreze et al., 1994). Unlike the $CD4^+$ T cells that depend on autoantigen presentation by antigen presenting cells (APC), the $CD8^+$ T cells can directly recognise target autoantigens presented by MHC class I molecules on pancreatic β-cells (DiLorenzo and Serreze, 2005), and it has been proposed that initial β-cell insult in T1D might be mediated by $CD8^+$ T cells (Yang and Santamaria, 2003). Early studies on diabetogenic $CD8^+$ T cells were based on the analysis of T-cell receptor (TCR) gene usage of $CD8^+$ T cell clones propagated from the pancreatic islets of 5–7 wk old NOD mice (Santamaria et al., 1995; Wong et al., 1996; DiLorenzo et al., 1998). During the last decade precise autoantigenic targets of pathogenic $CD8^+$ T cells have been identified. By screening of an organ-specific cDNA library Wong et al. identified the target epitope of $CD8^+$ T cell clone G9C8 as insulin B-chain residues 15–23 restricted by H2-K^d (Wong et al., 1999). Upon adoptive transfer into irradiated NOD or NOD-SCID recipients the G9C8 clone was highly diabetogenic eliciting diabetes in a complete absence of $CD4^+$ T cells (Wong et al., 1996). Interestingly, the $CD8^+$ T cell epitope of the G9C8 clone overlaps completely with the above described insulin B-chain$_{9–23}$ recognised by diabetogenic $CD4^+$ T cells. Two other antigenic specificities have been identified as islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP) residues 206–214 (Lieberman et al., 2003), and dystrophia myotonica kinase (DMK) residues 138–146 (Lieberman et al., 2004), the former being an islet-specific and the latter a ubiquitously expressed protein. The IGRP$_{206–214}$ exhibits good

binding to H2-K^d and the $IGRP_{206–214}$ tetramers can be used to track this diabetogenic T cell clone in NOD islets and peripheral blood (Trudeau et al., 2003; Lieberman et al., 2004). Importantly, it has been demonstrated that modulation of anti-$IGRP_{206–214}$ $CD8^+$ T cell response by treatment with altered peptide ligands of $IGRP_{206–214}$ prevents diabetes in NOD mice (Han et al., 2005). As demonstrated in tetramer studies, insulin B-$chain_{15–23}$, $IGRP_{206–214}$ and $DMK_{138–146}$ specific $CD8^+$ T cell clones can account for a remarkable proportion (up to 60%) of all islet-infiltrating $CD8^+$ T cells. However, despite being in the same stage of insulitis, individual mice show their unique pattern of reactivities to these three determinants (Lieberman et al., 2004).

In summary, recent extensive research on diabetogenic $CD8^+$ T cells in NOD mice has demonstrated that this cell subset is clearly involved in β-cell loss in T1D and may represent an attractive target for immunotherapy of T1D (DiLorenzo and Serreze, 2005). The observation that in humans with recent-onset T1D, β-cell islets are predominantly infiltrated by $CD8^+$ T cells (Bottazzo et al., 1985; Itoh et al., 1993), supports the idea that the cytotoxic $CD8^+$ T cells may be responsible for destruction of a majority of β-cells.

2.2.2. RIP-LCMV and RIP-B7.1 mouse models of T1D

In parallel with understanding the complexity of interactions between the genetic determinants defined by MHC and ppins genes and autoreactive T cells, leading to T1D in NOD mice, another mouse model of T1D – RIP-LCMV mice – provided a similar concept of immunopathogenesis of T1D in which the thymic expression of self-antigens controls the fate of self-reactive and potentially diabetogenic T cells. In RIP-LCMV mice, the transgenic expression of viral proteins from the LCMV virus as neo-autoantigens in β-cells is not pathogenic per se but after a challenge with the LCMV virus most animals develop autoimmune diabetes characterised by the infiltration of the islets by mononuclear cells, mainly $CD8^+$ T cells (Oldstone et al., 1991). Thus, the LCMV-specific T cells escape thymic deletion in RIP-LCMV mice and are activated in the immune periphery upon a neo-autoantigenic challenge. Detailed studies in different RIP-LCMV mouse lines documented that both the MHC genotype of the mice and the nature of the antigen determine the disease susceptibility of the mice (von Herrath et al., 1994). In RIP-LCMV (H-2^b) mice expressing the LCMV glycoprotein (LCMV-GP) under the control of the RIP no self-antigen expression was found in the thymus, the LCMV-GP specific $CD8^+$ T cell repertoire was equal with that of their wild-type littermates, and after infection with LCMV the mice rapidly (within 10–14 days) developed CD4+ T cell-independent T1D. In contrast, if the LCMV nucleoprotein (LCMV-NP) was expressed under the control of the RIP, the product of the transgene was found both in the thymus and in the β-cells, which yielded a low-

affinity and low-avidity anti-LCMV-NP $CD8^+$ T cell population that induced a slow-onset $CD4^+$ T cell-dependent T1D after a viral challenge (von Herrath et al., 1994). Moreover, diabetes susceptibility varied in different mouse strains. In H-2^d (BALB/c) mice with the LCMV-NP transgene diabetes developed within 1–2 months, while in H-2^b (C57BL/6) mice 3–5 months were required. After LCMV inoculation, $CD8^+$ T cells were more readily detected in H-2^d than in H-2^b mice indicating that in H-2^d mice a higher number of anti-LCMV-NP T cells escaped thymic deletion (von Herrath et al., 1994). The differences observed in diabetes susceptibility and in the diabetogenic T cell repertoire in different subtypes of RIP-LCMV mice is a further evidence that the quality of interaction between autoreactive T cells and their cognate self-antigens presented by MHC in the thymus may be a central determinant that establishes the potentially diabetogenic T cell repertoire (von Herrath et al., 1994).

One of the key findings of studies on the RIP-LCMV mouse model is that autoreactive T cells peacefully coexist with β-cells expressing their cognate self-antigen, while autoimmunity is avoided by peripheral tolerance (von Herrath et al., 1995). A recent study demonstrated that even higher numbers of LCMV-GP specific $CD8^+$ T cells are relatively insufficient to induce diabetes in absence of viral components from LCMV (Lang et al., 2005). Initiation of diabetogenesis by a viral trigger was shown to use toll-like receptor (TLR) signalling, inducing systemic interferon-alpha (IFN-α) and an upregulation of MHC class I on the β-cells, resulting in a rapid development of hyperglycemia (Lang et al., 2005). It was concluded that engagement of innate immune system by unspecific viral components may be crucial in converting autoreactivity into overt autoimmune disease (Lang et al., 2005). These observations provide explanation of how viruses as environmental triggers can be involved in the pathogenesis of T1D.

Treatment with natural β-cell autoantigens is not pathogenic in non-diabetes-prone (H-2^b or H-2^d) mice (Karges et al., 2002; Devendra et al., 2004b). However, the diabetes-susceptibility of these mouse strains is greatly enhanced if β-cells express stimulatory elements of the immune system such as the B7.1 (CD80), normally expressed on professional APCs, or proinflammatory cytokine TNF-α (Guerder et al., 1994; von Herrath et al., 1995). In RIP-LCMV mice, the coexpression of B7.1 along with LCMV-GP in pancreatic β-cells breaks peripheral tolerance to GP and leads to spontaneous diabetes development (von Herrath et al., 1995). In single RIP-B7.1 transgenic mice (H-2^b or H-2^d), spontaneous disease appearance is rarely observed but diabetes can be induced upon immunisation with natural β-cell autoantigens in an immune stimulating formula, e.g. insulin B-chain$_{9–23}$ with poly(I:C) in RIP-B7.1 (H-2^d) (Devendra et al., 2004b), or insulin as a DNA vaccine in RIP-B7.1 (H-2^b) mice (Karges et al., 2002; Pechhold et al., 2003).

The mechanisms by which the RIP-B7.1 transgene contributes to diabetes development after an autoantigenic challenge are not entirely understood but may involve amplification of ongoing T cell response if β-cells present self-antigens in

presence of appropriate costimulation (von Herrath et al., 1995). The cytokines secreted by islet-infiltrating T cells may play a role because in the RIP-LCMV model, T cells recovered from the pancreas of RIP-LCMV/B7.1 double transgenic mice showed a pro-inflammatory cytokine profile towards Th1-type cytokines (interferon-gamma, interleukin-2) as opposed to a more pronounced anti-inflammatory Th2-type (interleukin-4- and interleukin-10-secreting) immune response in single transgenic RIP-LCMV mice (von Herrath et al., 1995).

Immunisation with ppins-II DNA has been shown to induce $CD4^+$ and $CD8^+$ T cell insulitis and diabetes in most RIP-B7.1 ($H\text{-}2^b$) mice (Karges et al., 2002) indicating that thymic expression of ppins-II in mice (Moriyama et al., 2003) does not lead to the negative selection of ppins-II specific T cells in the thymus in $H\text{-}2^b$ mice. It is not known whether ppins-I has a similar effect in EAD in RIP-B7.1 ($H\text{-}2^b$) mice. Immunisations with plasmid DNA encoding exogenous antigens from pathogens have been demonstrated to generate efficient protective antibody and T cell responses against these pathogens, particularly $CD8^+$ cytotoxic T cells producing proinflammatory Th1 cytokines, such as IFN-γ (Gurunathan et al., 2000; Schirmbeck and Reimann, 2001). The effect of DNA vaccines can be based, at least in part, on unmethylated cytosine-phosphate-guanosine (CpG) motifs in plasmid backbone known to stimulate directly numerous types of immune cells including macrophages, dendritic cells, B cells and T cells (Gurunathan et al., 2000). Diabetes development observed in RIP-B7.1 ($H\text{-}2^b$) mice after immunisation with ppins-II DNA is consistent with the proinflammatory character of cellular immune responses elicited by DNA vaccines (Karges et al., 2002). Moreover, exacerbation of diabetes upon delivery of DNA vaccines encoding ppins-II was demonstrated in female NOD mice and in partly diabetes-resistant male NOD mice (Karges et al., 2002). In contrast, the GAD65 DNA vaccine did not induce diabetes in RIP-B7.1 mice and conferred partial protection against the disease in NOD mice (Karges et al., 2002). The reverse outcome in the case of immunising mice with ppins-II or GAD65 may reflect a higher diabetogenic potential of ppins-II specific T cells.

The above presented data from the experiments with NOD mice that are genetically manipulated at the ppins genes (Nakayama et al., 2005) support the notion that ppins may be a central target of pathogenic T cell responses in the natural course of T1D. Moreover, IDDM2, which confers 10% of the genetic risk in human T1D, is most probably implicated in the pathogenesis of the disease due to its effect on induction of insulin tolerance (Pugliese et al., 1997; Vafiadis et al., 1997). Insulin is associated with humoral and cellular immune responses both in mouse and human diabetes. In NOD mice, IAA can distinguish diabetes-prone mice from non-diabetes prone mice (Yu et al., 2003), and in humans, IAA are usually the first AAb appearing in very young children who develop T1D before 5 years of age (Ziegler et al., 1999). The T cells recognising the ppins epitopes have been identified both in NOD mice (Wegmann et al., 1994; Wong et al., 1999) and in humans (Arif et al., 2004; Toma et al., 2005; Pinkse et al., 2005; Mallone et al., 2007).

The EAD in RIP-B7.1 mice is a defined model system which enables to identify the factors leading to activation of autoantigen-specific T cells and to develop the preventive therapeutic strategies interfering with diabetogenic T cell responses. The possibility to investigate the interplay between insulin – a central autoantigen in the pathogenesis of T1D – and insulin-specific auto-reactive T cells is of particular importance. The prerequisite for further studies on this EAD mouse model is the identification of the major diabetogenic T cell populations and their exact autoantigenic targets.

2.2.3. Mechanisms of β-cell damage in T1D

In different mouse models of type 1 diabetes, FasL (Itoh et al., 1997), perforin (Kagi et al., 1996; Kagi et al., 1997), TNF-α (Pakala et al., 1999), and IFN-γ (von Herrath and Oldstone, 1997; Seewaldt et al., 2000) have been considered as effector molecules mediating the apoptosis of a majority of pancreatic β-cells. However, the exact contribution of different effector mechanisms to β-cell death has remained elusive (Santamaria, 2003). Early studies in Fas-deficient NOD-*lpr/lpr* mice demonstrated that these mice are diabetes-resistant and suggested that Fas/FasL interaction is required for development of diabetes (Itoh et al., 1997). These findings were opposed by studies showing the lack of protection in NOD-*lpr/lpr* pancreata grafted into diabetic NOD mice (Kim et al., 1999). Furthermore, perforin-deficient NOD mice have a reduced incidence and delayed onset of diabetes indicating that perforin-dependent mechanisms play a role in β-cell death (Kagi et al., 1997). The data obtained from the study of RIP-LCMV mice suggest that IFN-γ is able to induce injury of β-cells. The RIP-LCMV mice deficient for IFN-γ are diabetes-resistant (von Herrath and Oldstone, 1997), which is probably due to the interaction of IFN-γ with its receptor on β-cells as RIP-LCMV mice with a mutated IFN-γ-receptor are also protected from the disease (Seewaldt et al., 2000). Additionally, various other inflammatory mediators such as TNF-α, interleukin-1β, interleukin-6, interleukin-18 and certain chemokines may be involved in the pathogenesis of T1D through local or systemic effects (Mandrup-Poulsen, 2003; Kristiansen and Mandrup-Poulsen, 2005). In the RIP-LCMV mouse model, IFN-α has been shown to enhance the susceptibility of β-cells to immune attack by autoreactive T cells (Lang et al., 2005).

Conclusively, it can be hypothesized that several effector pathways are acting in concert in β-cell death in T1D, and a number of factors, including the nature of the target autoantigens, the type of effector cells, and changes in immune surveillance in the pancreatic islets during disease progression critically influence the activity of a given pathway (Santamaria, 2003). In EAD in RIP-B7.1 (H-2^b) mice, the contribution of various effector mechanisms to β-cell damage has not been investigated so far.

3. AIMS OF THE STUDY

1. To estimate the prevalence of the main HLA-DQB1 alleles and INS polymorphisms in children and adults with newly diagnosed T1D in Estonia.
2. To analyse in patients associations between age at diagnosis of T1D and presence of AAb, between age at diagnosis and genetic markers as well as the influence of genetic factors on presence of β-cell AAb.
3. To establish an adoptive diabetes transfer system in the EAD (RIP-B7.1) mouse model in order to confirm the central role of cellular immunity in disease pathogenesis.
4. To analyse the contribution of the main T cell subpopulations ($CD4^+$ and $CD8^+$ T cell subsets) to diabetes development in EAD in RIP-B7.1 ($H\text{-}2^b$) mice.
5. To determine the immunogenic region(s) of ppins in RIP-B7.1 ($H\text{-}2^b$) mice and to identify autoantigenic determinant(s) for ppins-specific diabetogenic T cells.
6. To investigate the role of type 1 IFN-mediated innate immunity response in the pathogenesis of T1D in EAD in RIP-B7.1 ($H\text{-}2^b$) mice as well as the mechanisms of β-cell killing in this model.

4. MATERIALS AND METHODS

4.1. Human type 1 diabetes

4.1.1. Study subjects

The study group consisted of 92 patients (median age 20 years, range 2–62 years, 49 females) with newly diagnosed T1D. The patients were enrolled in the study between 2001 and 2003 from the two main children's hospitals and from the two main adult inpatient endocrinology and diabetes units in Estonia. The diagnosis of T1D was based on clinical characteristics including rapid onset of symptoms, weight loss, polydipsia, polyuria, ketosis and necessity for insulin therapy. Particular attention was paid to adults to exclude patients with type 2 diabetes and with diseases of the exocrine pancreas. Blood samples from all patients were collected within one week of diagnosis.

For subgroup analysis the patients were divided into three groups. There were 31 children <15 years of age (mean age ± SD, 8.3 ± 3.3 years, 15 female), 33 adolescents and young adults 15–30 years of age (21.2 ± 4.8 years, 20 female), and 28 adults >30 years of age (38.6 ± 8.5 years, 14 female).

The control group comprised 251 individuals and was used for the risk evaluation of the HLA-DQB1 and INS VNTR alleles. One hundred and sixty of them were healthy blood donors and the remaining 91 subjects were the patients hospitalised for various reasons, who did not have diabetes as an accompanying illness (median age 45 years, range 13–85 years, 151 female).

The study was approved by the Ethics Committee of the University of Tartu, and informed consent was obtained from the adult participants and from the parents of the children involved.

4.1.2. Autoantibody assays

4.1.2.1. ICA

The ICA were detected by a standard indirect immunofluorescence assay on cryosections of the human pancreas from a donor of blood type 0 (Bottazzo et al., 1974). The end-point titres of ICA were converted to Juvenile Diabetes Foundation Units (JDFU). The titres equal or larger than 8 JDFU were considered positive (Greenbaum et al., 1992).

4.1.2.2. GADA and IA-2A

The GADA and IA-2A were measured by the fluid-phase ^{125}I-antigen binding assay (Strebelow et al., 1999) at the Institute of Pathophysiology, University of Greifswald, Germany. The levels of GADA and IA-2A were expressed as arbitrary Karlsburg units (KU/l) derived from an in-house standard serum pool. The cut-off limit for antibody positivity was defined as the 98th percentile of the laboratory's control group, being 2.14 KU/l for GADA and 0.53 KU/l for IA-2A. In the 4th Diabetes Antibody Standardization Program (DASP) in 2005, the assay for GADA reached a sensitivity of 82% and a specificity of 96% and the assay for IA-2A reached a sensitivity of 66% and a specificity of 100%.

4.1.2.3. IAA

The IAA were also determined at the University of Greifswald, Germany, using the competitive fluid-phase antigen binding assay with A14 mono-^{125}I-insulin (Aventis Pharma Deutschland GmbH, Frankfurt am Main, Germany) with and without the addition of unlabelled insulin (Schlosser et al., 2002). The IAA level of 55.37 μU/l corresponding to the 98th percentile of the laboratory's control group was chosen as the cut-off limit for IAA positivity. In the 4th DASP in 2005, the assay reached a sensitivity and a specificity of 58% and 97%, respectively.

4.1.3. Genotyping

4.1.3.1. HLA-DQB1 alleles

HLA-DQB1 typing was performed using the hybridisation of lanthanide-labelled allele-specific oligonucleotide probes with a PCR amplified gene product from blood spots (DELFIA®, Wallac, PerkinElmer Life Sciences, Boston, MA). Five HLA-DQB1-alleles associated with susceptibility to (HLA-DQB1*0302 and *02) or protection from T1D *(*DQB1*0301, *0602 and *0603) were tested.

4.1.3.2. INS polymorphisms

For genotyping INS polymorphism, the genomic DNA was purified by the salt extraction method (Miller et al., 1988b). The INS VNTR was identified by its surrogate marker HphI A/T single nucleotide polymorphism at the locus –23 (rs689) (Lucassen et al., 1993). Class I and III alleles of INS VNTR were determined by –23 HphI A and T alleles, respectively. The HphI –23 A/T was genotyped by restriction fragment length polymorphism analysis (Bennett et al., 1995).

4.1.4. Statistics

The R 2.3.1 A Language and Environment (Free Software Foundation, Boston, MA) was used for linear and logistic regression analyses. The regression coefficients and the odds ratios (OR) were calculated. A p value < 0.05 was considered statistically significant.

4.2. Experimental autoimmune diabetes

4.2.1. General outline of the experiments

Task	Approach
Characterisation of the immunogenic forms of ppins-II.	Transient transfection of HEK293 cells with ppins-II tagged with the haemagglutinin determinant (pCI/ppins-HA) and immunoprecipitation of cells with HA-specific mAb.
Comparison of diabetogenicity of ppins-I and ppins-II.	Immunisation of RIP-B7.1 (H-2^b) mice with ppins-I and ppins-II plasmid DNA.
Confirmation of the central role of cellular immunity.	Adoptive transfer of diabetes with spleen cells from diabetic RIP-B7.1 mice.
Evaluation of the role of different T cell subpopulations in diabetogenesis.	Adoptive diabetes transfer with sorted T cell subpopulations. *In vivo* depletion of $CD8^+$ and $CD4^+$ T cells. Histological examination of islets from prediabetic and diabetic animals.
Mapping and identifying the $CD8^+$ T cell epitope(s) of ppins-II.	Immunisation studies with the deletion-mutant vectors containing the domains of ppins-II. Restimulation of the spleen cells from diabetic RIP-B7.1 mice with overlapping insulin A-chain peptides *in vitro*.
Investigation of the role of effector molecules in EAD.	Immunisation studies in RIP-B7.1 mice lacking the expression of IFN-γ, perforin or type I IFN receptor.

4.2.2. Mice

RIP-B7.1 mice were backcrossed to the C57BL/6 (H-2^b) background as described (Harlan et al., 1994). Heterozygous RIP-B7.1 animals were used for the experiments. Double transgenic mice that express RIP-B7.1 and are IFN-γ- or perforin- or IFN type I receptor (IFNAR)-deficient were generated by crossing homozygous RIP-B7.1 mice with respective knockout (KO) mice and

then intercrossing F1 animals. Animal studies were conducted in Ulm University with institutional board approval in accordance with the German Federal Animal Protection Law.

4.2.3. Genotyping

Genotyping of the mice was performed by PCR of the DNA extracted from tail specimens. The primer pair used for RIP-B7.1 was 5' CAAACAACAGCCTTACCTTCGG and 5' GCCTCCAAAACCTACACATCCT and PCR-amplification resulted in a product of 642 bp separated on a 1.5% agarose gel (Fig. 1A, B). Wild-type and mutated IFN-γ (Fig. 1A) and perforin (Fig. 1B) and IFNAR (data not shown) were determined following the genotyping protocols of The Jackson Laboratory (Bar Harbor, Maine, USA).

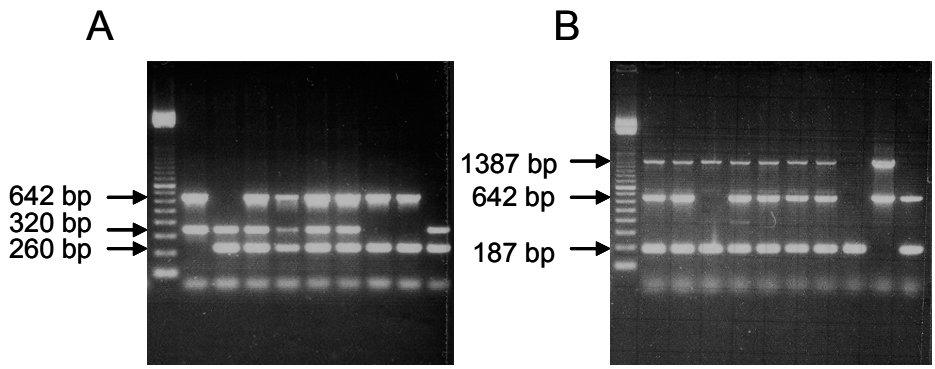

Figure 1. Genotyping of the RIP-B7.1 mice deficient for IFN-γ (A) or perforin (B). The positions of PCR products for RIP-B7.1 (642 bp), IFN-γ wt (260 bp), IFN-γ KO (320 bp), perforin wt (187 bp) and perforin KO (1387 bp) are indicated. Each line represents one individual mouse.

4.2.4. Immunisation and diabetes screening

For immunisation studies, 6–12 week-old mice were injected intramuscularly 50 µg of plasmid DNA (in 50 µl PBS) into each tibialis anterior muscle. In some experiments, the mice were boosted with the same amount of plasmid DNA two weeks later. Diabetes development was monitored for a 1–2-week interval by blood glucose measurements (Glucometer Freestyle, TheraSense, Alameda, CA) and diagnosed if two consecutive readings exceeded 13.8 mmol/l (250 mg/dl).

4.2.5. Construction of DNA expression vectors

Mouse ppins-I (Genbank X04725) and ppins-II (Genbank X04724) cDNA were generated from murine islet cell RNA and subcloned into the EcoRI and XbaI sites of pCI (cat. no. E1731, Promega, Mannheim, Germany) generating pCI/ppins-I and pCI/ppins-II vectors, respectively. Mouse pCI/pins-II was generated from the pCI/ppins-II DNA by PCR using site-specific primers. Mouse pCI/BA-II (linear insulin II vector) was constructed by fusion PCR of insulin B- and A-chain PCR products generated from the pCI/ppins-II DNA template using site-specific primers. A synthetic DNA construct encoding the haemagglutinin-tagged ppins-II (ppins-HA) was synthesised by GeneArt, Regensburg, Germany. The ppins-HA sequence was cloned into the XhoI/NotI sites of pCI generating the pCI/ppins-HA vector. The deletion mutants encoding domains of ppins-II were generated from the pCI/ppins-II DNA by PCR using site-specific primers. The construct pCI/sp encodes the ppins 1–36 sequence (i.e. the entire 24-residue signal peptide (sp) and 12 aa of the B-chain); pCI/B encodes the ppins 15–66 sequence (i.e. 10 aa of sp, the B-chain and 10 aa of the C-peptide); pCI/C encodes the ppins 47–97 sequence (i.e. 8 aa of the B-chain, the C-peptide and 8 aa of the A-chain); and the pCI/A encodes the ppins 80–110 sequence (i.e. 8 aa of the C-peptide and the A-chain) (Fig. 2). Plasmids were transformed into XL-1 blue MRF' E. coli (Stratagene) and isolated with the Plasmid Mega Prep Kit (Qiagen).

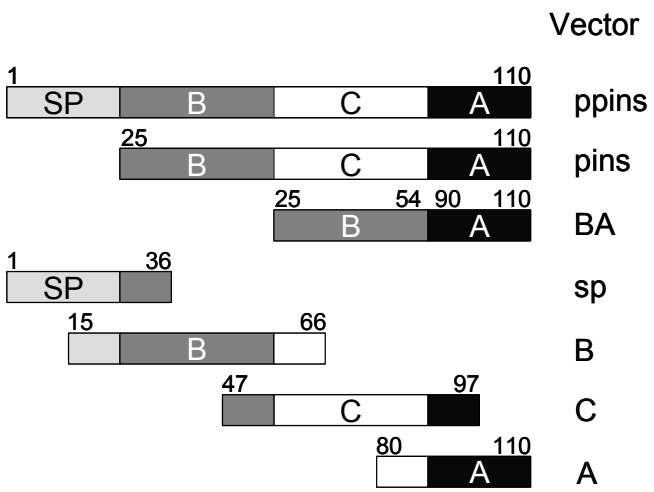

Figure 2. Vectors used in immunisation studies. Sequences of preproinsulin-II (ppins), proinsulin (pins), linear insulin (BA), and domains of ppins-II with extension(s) to neighbouring sequences such as the signal peptide (sp), B-chain (B), C-peptide (C) and A-chain (A) were subcloned into pCI vector and used in *in vivo* studies in the RIP-B7.1 mice. The included amino acid positions are shown above sequences.

4.2.6. Expression of ppins-II in non-pancreatic cells

Human embryonal kidney cells (HEK293) were transiently transfected with pCI/ppins-HA DNA (encoding the ppins-II fused C-terminally with the HA sequence YPYDVPDYA), labelled with ^{35}S-methionine/cysteine and analysed by HA-specific immunoprecipitation.

4.2.7. Adoptive transfer of splenocytes or isolated CD8$^+$ or CD4$^+$ T cells

Following immunisation or diabetes induction with the pCI/ppins-II vector, splenocytes were isolated from the diabetic RIP-B7.1 mice, pooled (2 spleens per group), and 0.2, 0.5, 1 or 2x10^7 cells in 300 µl of RPMI 1640 medium were injected intravenously into sublethally irradiated (650 rad) RIP-B7.1 or wt recipients. In selected transfer experiments, CD8$^+$ or CD4$^+$ T cell subsets were isolated by negative selection using the CD8a$^+$ or CD4$^+$ T cell isolation kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instruction. Briefly, spleen cells from three diabetic RIP-B7.1 donors were pooled and 10^8 cells were incubated with an appropriate amount of the non-CD8a$^+$ or non-CD4$^+$ biotin-conjugated antibody cocktail followed by incubation with anti-biotin MicroBeads. The cells were passed through the LS column in the magnetic field of a MACS Separator (both from Miltenyi Biotec). The viability of the cells, as determined by trypan blue exclusion, was >95%, and the purity of the isolated cell populations reached at least 94%, as assessed by flow cytometry. 0.8x10^6 CD8$^+$, 1.4x10^6 CD4$^+$ T cells, or, in the control group, 1x10^7 whole spleen cells, were injected into the tail vein of the irradiated B7.1 recipients.

4.2.8. In vivo T cell subset depletion

In vivo CD4$^+$ or CD8$^+$ T cell subset depletion was performed with monoclonal antibodies (mAb) YTS 191.1.2 or YTS 169.4.2.1 (both IgG2b,(Cobbold et al., 1984)), respectively. The mice were injected intraperitoneally 300 µg of antibody in 300 µl phosphate buffered saline (PBS) 2 days and 1 day before and 1 day after (a total of 900 µg mAb per mouse) intramuscular injection of 100 µg of ppins-II in pCI vector. The efficacy of depletion, as determined by the flow cytometry of PBMC from the peripheral blood of the mice five days after the last injection of antibodies, was nearby 100%.

4.2.9. Histology

The pancreata were snap-frozen in liquid nitrogen. For obtaining cryosections, the pancreata were embedded in an OCT compound (Leica, Nussloch, Germany) and cut into 5 µm serial sections. The sections were fixed in aceton and processed for hematoxylin-eosin staining, or incubated with fluorescent antibodies. The primary antibodies were FITC-conjugated rat anti-mouse CD4 (RM4–5), Alexa Fluor 647-conjugated rat anti-mouse CD8a (53–6.7), hamster anti-mouse CD3E (500A2), all purchased from BD Biosciences Pharmingen, and guinea-pig anti-insulin obtained from Sigma-Aldrich. As the secondary antibodies, rhodamine-conjugated rabbit anti-hamster IgG (Abcam) and TRITC-conjugated rabbit anti-guinea-pig IgG (Sigma-Aldrich) antibodies were used.

4.2.10. T cell assays

4.2.10.1. Peptides

A library of 9-meric peptides overlapping by 8 aa from the insulin A-chain (13 peptides) was applied in T cell assays. Between residues 11 and 21 of the insulin A-chain, additionally 7-mers LYQLENY, YQLENYC, QLENYCN; 8-mers CSLYQLEN, SLYQLENY, LYQLENYC, YQLENYCN, 10-mer SLYQLENYCN and 11-mer CSLYQLENYCN were used.

The synthetic peptides were obtained from Thermo Electron GmbH (Ulm, Germany) or JPT Peptide Technologies GmbH (Berlin, Germany). The peptides were dissolved in DMSO at a concentration of 1–20 mg/ml and stored at –20°C for further use.

4.2.10.2. Flow cytometry

After lysing the red cells, the splenocytes from the diabetic RIP-B7.1 mice were washed and resuspended in the RPMI 1640 medium with GlutaMAX™ (Invitrogen) supplemented with 10% FCS (PAA Laboratories GmbH, Linz, Austria) and 1% Penicillin-Streptomycin (Invitrogen). The 1.5 x 10^6 splenocytes were plated per well and peptides were added at 25 µg/ml in 96-well round-bottom plate. The plates were incubated at 37°C, 6% CO_2 overnight in the presence of brefeldin A at 7.5 µg/ml. Before staining for flow cytometry, the unspecific binding of monoclonal antibodies was blocked with anti-mouse CD16/CD32 mAbs from the 2.4G2 cell line from the American Type Culture Collection (Manassas, VA, USA). Surface-staining was performed with PE-conjugated anti-mouse CD3ε and PerCP-conjugated anti-mouse CD8a. For

intracellular staining, the cells were fixed with 2% paraformaldehyde in PBS, permeabilised in the buffer containing 0.5% saponin, 0.5% bovine serum albumine (BSA) and 0.05% sodium azide, and stained with APC-conjugated anti-mouse IFN-γ. All antibodies were purchased from BD Biosciences Pharmingen. For flow cytometry, FACS Calibur (Becton Dickinson) was used and the data were analysed with the WinMDI 2.8 software.

4.2.10.3. H-2^b stabilisation assay

The 5 x 10^5 transporter associated with antigen processing (TAP)-deficient RMA-S cells (American Type Culture Collection) were pulsed with peptides at 40 µg/ml and incubated at 37°C for 4 h. H-$2K^b$ was stained with PE-conjugated mAb CTKb (Caltag Laboratories) and H-$2D^b$ was detected with biotinylated mAb 28–14–8 followed by streptavidin-FITC (both from BD Biosciences Pharmingen). The analysis was performed on the FACS Calibur.

5. RESULTS

5.1. Human type 1 diabetes (Paper 1)

The prevalence of the HLA-DQB1*0302, *02, *0301, *0602 and *0603 alleles and of the INS VNTR genotypes in the patients with T1D and in the healthy controls is presented in Table 1. The HLA-DQB1*0302 and *02 alleles conferred a strong risk for T1D with OR of 5.51 ($p < 0.001$) and 2.10 ($p = 0.002$), respectively; while the HLA-DQB1*0301 and *0602–03 alleles were associated with protection from the disease with OR of 0.48 ($p = 0.016$) and 0.12 ($p < 0.001$), respectively (Table 1).

Table 1. The prevalence (%) and odds ratios (OR) of HLA-DQB1 alleles and INS VNTR genotypes in patients with type 1 diabetes and healthy controls

	Patients (%) N = 92	**Controls (%) N = 251**	**OR (95% CI)**
HLA-DQB1			
***0302**	51.1	15.9	5.51 (3.24–9.36)*
***02**	52.2	33.9	2.10 (1.31–3.46)#
***0301**	17.4	30.7	0.48 (0.26–0.87)#
***0602–03**	8.7	44.2	0.12 (0.06–0.26)*
INS VNTR			
I/I	69.2	45.8	2.66 (1.60–4.44)*

* $p < 0.001$; # $p < 0.05$

The data were analysed using logistic regression analysis. For HLA-DQB1 alleles the lack of the particular allele was considered the reference genotype and for INS VNTR the VNTR I/III and III/III genotypes were considered the reference genotype. Eight percent of the controls but none of the patients had the INS VNTR III/III genotype.

The INS VNTR I/I genotype was present in 69.2% of the patients and 45.8% of the controls (OR = 2.66, $p < 0.001$) (Table 1). The association between INS VNTR I/I and the disease remained highly significant regardless of adjusting for the HLA-DQB1*02/0302 genotype (adjusted OR = 2.72, $p < 0.001$), or for the HLA-DQB1*0302 (adjusted OR = 2.65, $p < 0.001$) or *02 alleles (adjusted OR = 2.74, $p < 0.001$).

In logistic regression analysis, the prevalence of the HLA-DQB1*02/0302 genotype, the HLA-DQB1*0302 allele, the DQB1*02 allele or the INS VNTR I/I genotype was not associated with age at diagnosis of T1D in our study group (Table 2).

Table 2. The association between genetic susceptibility markers and autoantibodies and the age at onset of T1D

	Median age (range)	OR (95% CI)
HLA-DQB1		
***02/0302**	20 (1–42)	0.98 (0.95–1.02)
***0302**	17 (1–58)	0.99 (0.96–1.02)
***02**	23 (1–62)	1.01 (0.98–1.04)
INS VNTR		
I/I	24 (1–62)	1.03 (0.99–1.06)
Autoantibodies		
IAA	11 (1–41)	0.92 (0.88–0.96)*
IA-2A	16 (1–58)	0.95 (0.92–0.99)#
ICA	17 (2–53)	0.97 (0.94–1.00)#
GADA	17 (1–58)	0.97 (0.94–1.00)

* $p < 0.001$; # $p < 0.05$

The data were analysed using logistic regression analysis. The patient group missing the genotype, allele or autoantibody under study was considered the reference group.

The IAA, IA-2A and ICA were negatively associated with age at diagnosis of T1D while no significant association was revealed between GADA and age at diagnosis of T1D (Table 2). Linear regression analysis showed a highly significant negative association between number of AAb and age at diagnosis of T1D (regression coefficient –4.66, $p < 0.001$).

In age subgroup analysis, the children <15 years of age were more often positive for 3–4 AAb (71%) than the adolescents and the young adults 15–30 years of age (45.5%) and the adults >30 years of age (28.6%) (Table 3). One child and 3 individuals in the age group of 15–30 years were negative for all four AAb, whereas in the group of adults >30 years of age 7 individuals (25%) had none of the AAb studied. Combined testing of AAb increased the diagnostic sensitivity of AAb for T1D. The combination of GADA and IA-2A yielded the highest diagnostic sensitivity in all three age groups. Positivity for GADA and/or IA-2A was found in 96.8, 87.9 and 67.9% of the patients in the age groups of <15, 15–30 and >30 years, respectively (Table 3). In children, the only patient without GADA or IA-2A, had none of the studied AAb. In the age group of 15–30 years, one patient had IAA only, and in the age group of >30 years, one patient had IAA only and one had ICA only.

Table 3. Prevalence (%) of β-cell autoantibodies in three age groups with newly diagnosed type 1 diabetes.

	Age groups		
	<15 years N = 31	**15–30 years N = 33**	**>30 years N = 28**
Autoantibodies			
GADA	83.9	69.7	60.7
IA-2A	80.6	63.6	50.0
GADA and/or IA-2A	96.8	87.9	67.9
ICA	67.7	54.5	46.4
IAA	64.5	27.3	14.3
Number of AAb			
3–4	71.0	45.5	28.6
1–2	25.8	45.5	46.4
0	3.2	9.0	25.0

Next, association between the T1D susceptibility genes and AAb was sought using logistic regression analysis. The GADA, IA-2A and ICA were not associated with the HLA-DQB1*02/0302 genotype, or the DQB1*0302 or *02 alleles in our study population. However, the presence of IAA showed a tendency to be associated with the high risk HLA-DQB1*02/0302 genotype (OR = 2.45; $p = 0.08$), or the DQB1*0302 allele (OR = 2.22; $p = 0.07$).

Logistic regression analysis of the association between AAb and the INS VNTR genotype revealed positive association between GADA and the protective INS VNTR I/III genotype (crude OR = 4.79; $p = 0.018$). This association remained significant after adjustments for the (1) HLA-DQB1*02/0302 genotype and age at diagnosis of T1D, (2) HLA-DQB1*0302 allele and age at diagnosis of T1D, and (3) HLA-DQB1*02 allele and age at diagnosis of T1D (Table 4).

We failed to reveal association between IAA and the INS VNTR I/I genotype in our study population regardless of the fact of whether the whole study group (OR = 1.21; $p = 0.69$) or only the children <15 years of age (OR = 1.81; $p = 0.45$) were included. There was no association between IA-2A or ICA and the INS VNTR I/I genotype (data not shown).

Table 4. Frequency of GADA in patients with INS VNTR I/III and INS VNTR I/I according to age or HLA-DQB1 genotype

	INS VNTR I/III	INS VNTR I/I
Age		
< 20	16 (100.0)	18 (66.7)
≥ 20	9 (75.0)	22 (61.1)
All	25 (89.3)	40 (63.5)
HLA-DQB1		
***02/0302**	5 (100.0)	10 (62.5)
***0302**	6 (100.0)	10 (83.3)
***02**	8 (88.9)	9 (69.2)

The data are *n* (%). In the logistic regression analysis the positive association between INS VNTR I/III and GADA remained significant after adjustments for the (1) HLA-DQB1*02/0302 genotype and age at diagnosis of T1D (adjusted OR = 4.39; 95% CI 1.17–16.42), (2) the HLA-DQB1*0302 allele and age at diagnosis of T1D (adjusted OR = 4.75; 95% CI 1.26–17.99), and (3) the HLA-DQB1*02 allele and age at diagnosis of T1D (adjusted OR = 4.40; 95% CI 1.18–16.40). The patient group missing the genotype or allele under study was considered the reference group.

5.2. Experimental autoimmune diabetes (Papers 2 and 3)

5.2.1. Characterisation of ppins-II expression in non-pancreatic cells

Intramuscular injection of murine ppins-II encoding DNA (pCI/ppins-II) induced hyperglycemia in RIP-B7.1 mice, but the immunogenic form(s) of insulin-protein(s) are undefined. In intramuscular DNA vaccination, ppins is expressed by non-pancreatic APC. We tested if expression of ppins-II in non-pancreatic cells produces proinsulin and/or insulin. We tagged ppins to facilitate detection of its expression patterns by C-terminally fusing an antibody-defined haemagglutinin determinant (HA-tag) in frame to the insulin A-chain (Fig. 3). This modification did not alter the antigenicity of ppins (data not shown). The HA-tagged intermediates that could be expressed from the pCI/ppins-HA construct are: 14.5 kD ppins-HA, 11.5 kD pins-HA and 3.5 kD A-chain-HA. The HEK293 cells were transiently transfected with pCI/ppins-HA and immunoprecipitated with HA-specific mAb (Schirmbeck et al., 2006). SDS-PAGE analyses revealed only the 11.5 kD pins-HA band (Fig. 3). In non-pancreatic cells, ppins was hence expressed and efficiently processed into pins

in the ER (by removing the signal peptide) but further downstream processing of pins into bioactive insulin was not detected. This result is consistent with the observation that immunisation with ppins DNA has no metabolic effect in RIP-B7.1 mice.

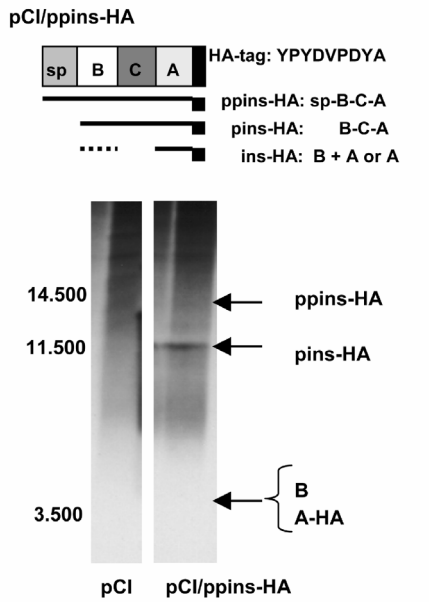

Figure 3. Expression of ppins-II in non-pancreatic cells. Human embryonal kidney cells (HEK293) were transiently transfected with pCI/ppins-HA DNA, labelled with ^{35}S-methionine/cysteine and analysed by HA-specific immunoprecipitation. The position of pins-HA and the expected positions of ppins-HA and A-HA (comigrating with the B chain) are indicated.

5.2.2. Murine ppins-I and ppins-II are equally diabetogenic in EAD

To compare the diabetogenic potential of murine ppins-I and ppins-II in RIP-B7.1 mice a single immunisation with 50 µg of ppins-I (n = 11) and ppins-II (n = 13) plasmid DNA into each tibialis anterior muscle of the mice was performed. In both groups diabetes developed rapidly (median of onset, 3 weeks) and with an incidence of 100% (data not shown). Similar diabetes development with both murine ppins isoforms suggests shared epitope(s) for diabetogenic T cells.

5.2.3. Diabetes can be adoptively transferred with splenocytes from diabetic RIP-B7.1 mice into irradiated syngeneic recipients

After the adoptive transfer of $2x10^7$ and $1x10^7$ splenocytes from diabetic RIP-B7.1 mice, all recipient animals developed hyperglycemia with onset of the disease at 8±0.8 and 10±2.8 weeks, respectively. However, injecting $0.5x10^7$ and $0.2x10^7$ spleen cells from diabetic donors resulted in a significant delay of disease development in recipient mice (15±7.3 and 30±7.5 weeks, respectively) (Fig. 4). These results demonstrate that EAD can be readily transferred into untreated conditioned recipients in a dose-dependent fashion.

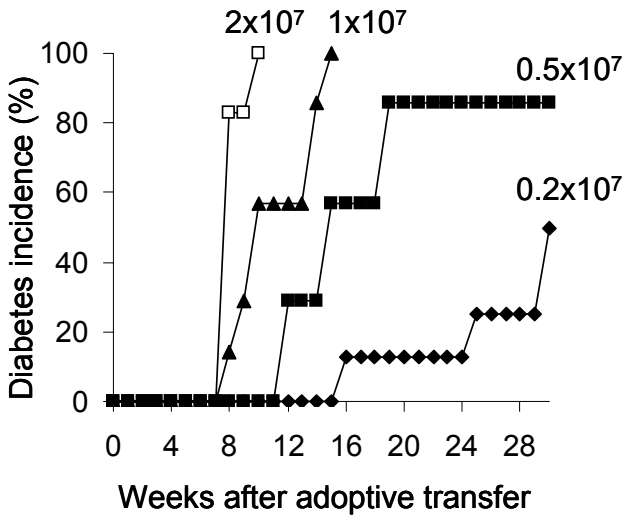

Figure 4. Adoptive diabetes transfer in EAD. Irradiated RIP-B7.1 recipients received intravenous injection of $2x10^7$ (n = 6) $1x10^7$ (n = 7), $0.5x10^7$ (n = 7), or $0.2x10^7$ (n = 8) syngeneic splenocytes from diabetic donors.

5.2.4. Diabetogenesis in EAD depends on $CD8^+$ T cells

To define the exact cellular requirements necessary for diabetognesis in EAD, diabetogenic potential of bulk splenocytes and isolated $CD8^+$ and $CD4^+$ T cell subsets was compared transferring them from hyperglycemic RIP-B7.1 mice into irradiated RIP-B7.1 recipients. Diabetes occurred with similar kinetics and high incidence in recipients grafted with bulk splenocytes and isolated $CD8^+$ T cells, whereas $CD4^+$ T cell transfer resulted in hyperglycemia in only 2 out of 7 mice during the extended follow-up of 18 weeks (Fig. 5).

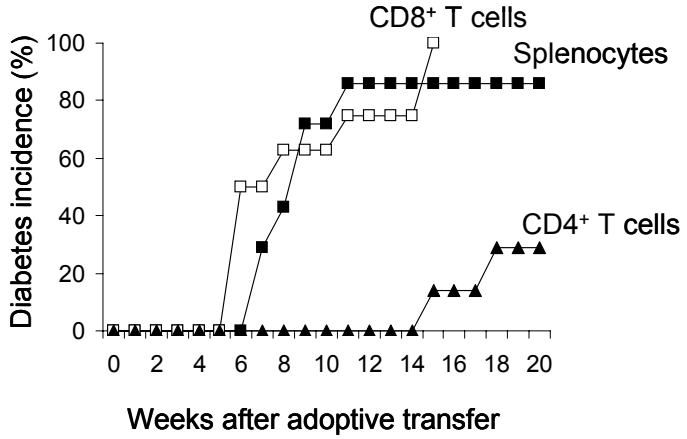

Figure 5. Adoptive diabetes transfer with sorted T cell subsets. Irradiated RIP-B7.1 recipients received $1x10^7$ bulk splenocytes (n = 7), or 1.4 x 10^6 $CD4^+$ (n = 7), or 0.8 x 10^6 $CD8^+$ T cells (n = 8) from diabetic RIP-B7.1 donors.

The necessity of $CD8^+$ T cells for diabetogenesis was confirmed when immunisation with ppins-II was performed in mice undergoing *in vivo* depletion of $CD8^+$ or $CD4^+$ T cells. Within 3 weeks of immunisation all 3 mice lacking $CD4^+$ T cells and 2 out of the 3 control mice (not treated with depleting antibodies) but none of the 3 mice lacking $CD8^+$ T cells had developed diabetes (data not shown).

5.2.5. The islets of prediabetic and diabetic RIP-B7.1 mice are predominantly infiltrated by $CD8^+$ T cells

Histological examination of the islets of ppins-II immunised RIP-B7.1 mice was carried out 2 weeks after immunisation and at diabetes onset (Fig. 6). Two weeks after immunisation in most animals (5 out of 7) the majority of islets had been attacked by $CD8^+$ and to lesser extent by $CD4^+$ T cells. Consistent with normoglycemia in these prediabetic mice, in many islets insulin staining was still intense. Freshly diabetic mice showed progressive insulitis with $CD8^+$ and $CD4^+$ T cell infiltration and nearly absent insulin staining. In the islets of the unimmunised control mice no T cell infiltration was observed.

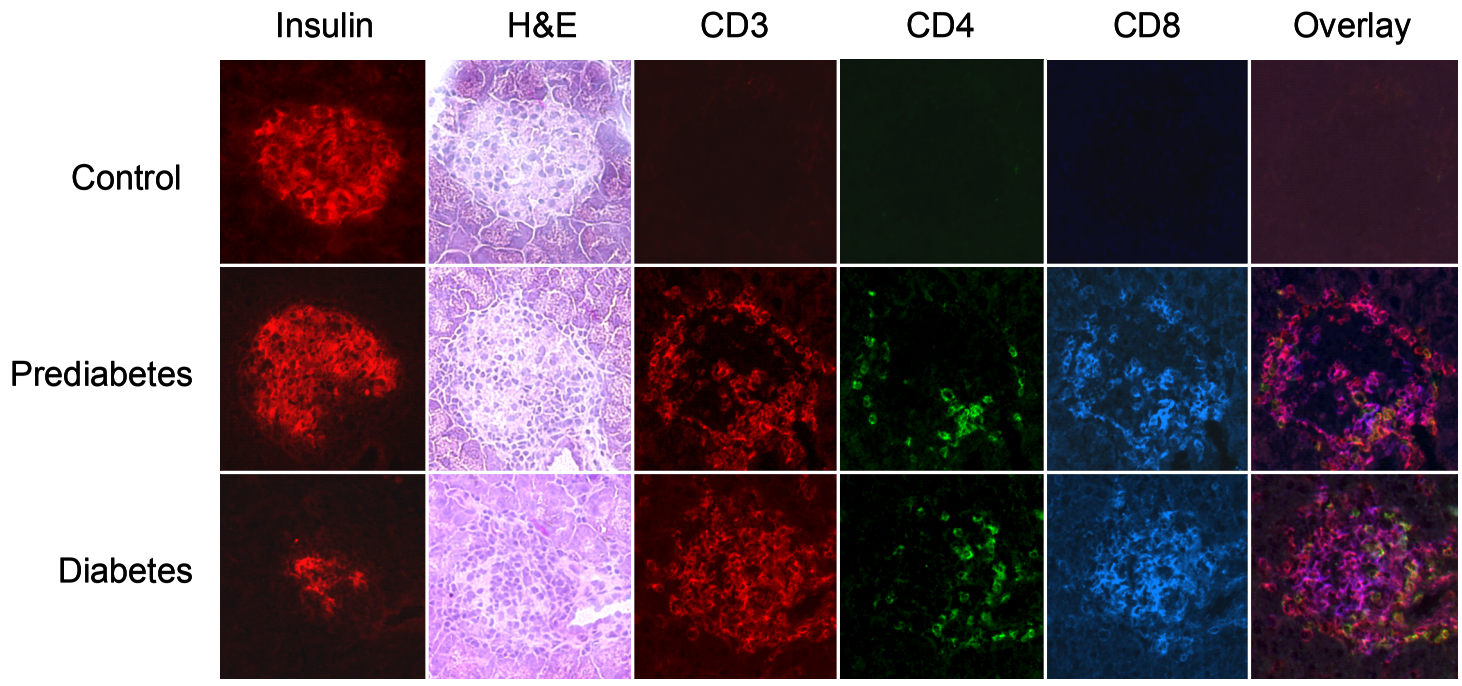

Figure 6. Insulitis in EAD with a predominance of $CD8^+$ T cells. The histology was performed in non-immunised (control) and pCI/ppins-II immunised prediabetic and diabetic mice. Serial pancreatic sections were stained for insulin, with hematoxylin-eosin (H&E), for $CD3^+$, for $CD4^+$ and for $CD8^+$ T cells. The last column represents an overlay of staining for $CD3^+$, $CD4^+$ and $CD8^+$ T cells.

5.2.6. Diabetogenic CD8$^+$ T cells are specific for a C-terminal epitope of the insulin A-chain

5.2.6.1. Candidate epitope(s) for pathogenic T cell recognition map to the insulin A-chain

To narrow the relevant immunogenic region(s) of ppins-II, immunisation studies with deletion-mutant vectors of ppins-II were performed. The DNA vectors lacking the signal sequence (pins II) or both the signal sequence and C-peptide ("linear" insulin II) caused diabetes with 100% incidence but with a slightly delayed kinetics (Fig. 7). Next, a set of DNA vectors encoding the functional domains of ppins-II with an extension of 8–12 amino acids to the neighbouring domain(s) was generated (pCI/sp, pCI/B, pCI/C and pCI/A). Strikingly, only the insulin A-chain vector (pCI/A) induced diabetes development in RIP-B7.1 mice (Fig. 7). The overlap of 18 amino acids with the neighbouring extended C-peptide vector (pCI/C) that was not diabetogenic, indicated that the CD8$^+$ T cell determinant(s) map to the insulin A-chain (ppins-II 90–110, 21 amino acids).

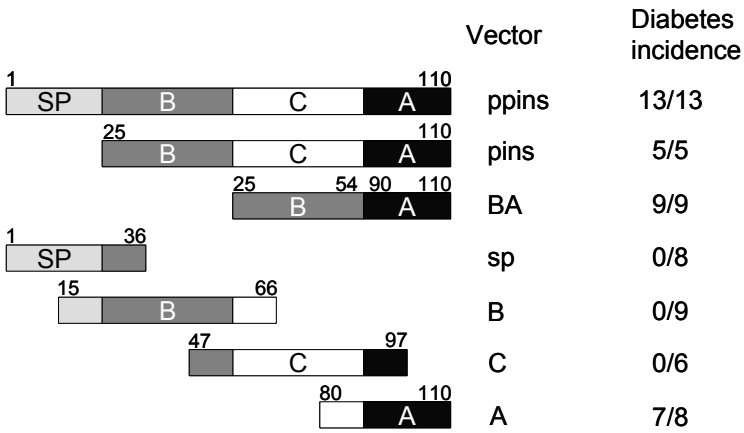

Figure 7. Diabetes incidence in RIP-B7.1 mice immunised with ppins-II or its domains. The mice were injected intramuscularly 100 μg of plasmid DNA and diabetes development was monitored for a 1–2-week interval.

5.2.6.2. $CD8^+$ T cells from diabetic RIP-B7.1 mice recognise the insulin A-$chain_{12–21}$

In order to identify dominant $CD8^+$ T cells epitope(s) in the insulin A-chain, spleen cells from the diabetic RIP-B7.1 mice were restimulated with a panel of overlapping peptides spanning the entire insulin A-chain and the responses were detected by flow cytometry. The IFN-γ^+ $CD8^+$ T cells were detected in diabetic but not in healthy RIP-B7.1 mice, after *in vitro* recall with the ppins peptide $A_{12–21}$ (Fig. 8). No additional IFN-γ $CD8^+$ T cell responses or $CD4^+$ T cell responses were detectable.

The immunogenicity of the K^b-restricted epitope insulin A-$chain_{12–21}$ in C57BL/6 (H-2^b) mice has been reported earlier (Ma et al., 2000). Like these authors, we observed that this epitope barely stabilised K^b molecules on the surface of TAP-deficient RMA-S cells (data not shown). This may indicate low avidity binding of the antigenic, insulin-derived peptide to K^b and inefficient stimulation of $CD8^+$ T cells allowing their escape from thymic negative selection.

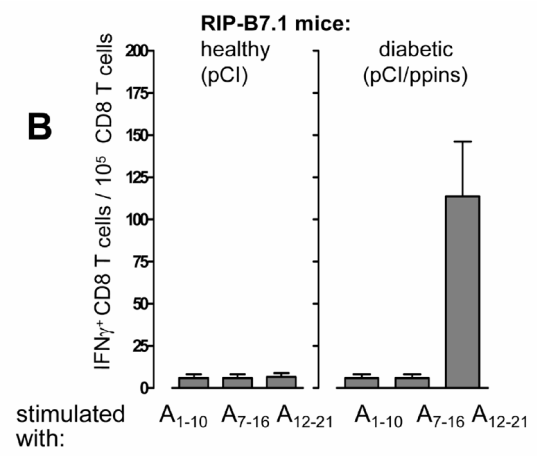

Figure 8. *In vitro* $CD8^+$ T cell reactivities. Spleen cells obtained from diabetic or healthy RIP-B7.1 mice were restimulated *ex vivo* with the $A_{1–10}$, $A_{7–16}$ or $A_{12–21}$ peptides. Specific IFN-γ^+ CD8 T cells were determined by flow cytometry. The mean numbers of IFN-γ^+ $CD8^+$ T cells /10^5 splenic CD8 T cells $\pm$ SD of four mice per group are shown.

5.2.7. Diabetogenesis depends on IFN-γ but not on perforin or type 1 IFN in EAD

In order to investigate the role of the two main cytotoxic molecules – perforin and IFN-γ – in β-cell destruction in EAD, the RIP-B7.1 mice lacking either perforin or IFN-γ expression were generated. Both mouse lines remained otherwise healthy and did not develop diabetes spontaneously. In the perforin-deficient RIP-B7.1 mice, immunisation with ppins-II plasmid DNA induced diabetes with similar kinetics in comparison to heterozygous and wt littermates indicating that in EAD, perforin-mediated apoptosis is not the main factor for β-cell destruction (Fig. 9A). In contrast, although most of the RIP-B7.1 IFN-γ wt and heterozygous mice developed diabetes within 8 weeks after single immunisation with ppins-II DNA, none of the RIP-B7.1 IFN-$\gamma^{-/-}$ mice had developed diabetes by week 12, and even after the prolonged follow-up of 20 weeks all mice retained normoglycemia (Fig. 9B).

The RIP-LCMV/gp33 mice lacking the type I IFN receptor (IFNAR$^{-/-}$) do not develop diabetes after LCMV infection (Lang et al., 2005). In this model diabetes induction is facilitated by co-stimulating toll-like receptor (TLR)-induced interferon-α (IFN-α) production that upregulates the MHC class I molecules on the pancreatic β cells thereby facilitating susceptibility of the target cells to CD8 T cells. We crossed RIP-B7.1 mice with IFNAR$^{-/-}$ mice. The IFNAR$^{-/-}$ RIP-B7.1 mice as well as the IFNAR$^{+/-}$ RIP-B7.1 littermates efficiently developed EAD after immunisation with pCI/ppins-II (data not shown). Development of EAD in the RIP-B7.1 mice upon an autoantigenic challenge is thus not dependent on type I IFN.

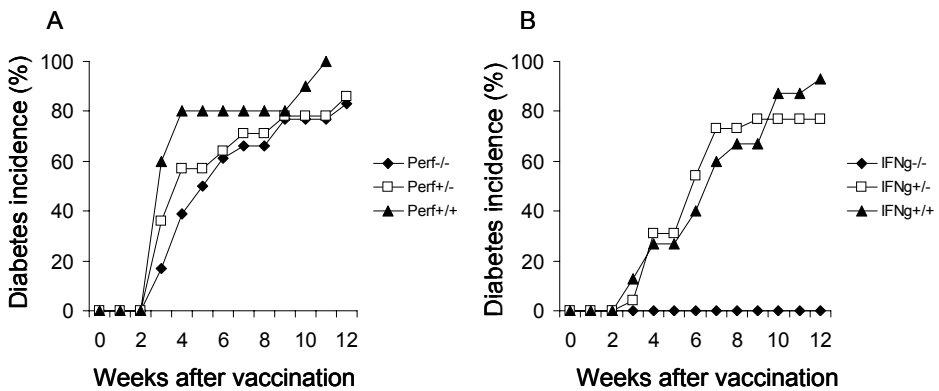

Figure 9. Diabetogenesis depends on IFN-γ but not on perforin in EAD. A. Diabetes development in perforin$^{-/-}$ (n = 18), in perforin$^{+/-}$ (n = 14) and in perforin$^{+/+}$ (n = 10) RIP-B7.1 mice. **B.** Diabetes development in IFN-$\gamma^{-/-}$ (n = 22), in IFN-$\gamma^{+/-}$ (n = 26) and in IFN-$\gamma^{+/+}$ (n = 15) RIP-B7.1 mice.

6. DISCUSSION

6.1. Age at diagnosis and genetic factors are associated with autoimmunity markers in human T1D

In the current study, the prevalence of the major markers of genetic susceptibility to T1D at the IDDM1 and IDDM2 loci and of β-cell AAb in Estonian patients with newly diagnosed T1D was analysed. Patients with a broad range of age at diagnosis were included in the study. The phenotypic characteristics of the patients were carefully evaluated at enrolment to exclude individuals with diabetes other than that of autoimmune origin. At least one β-cell AAb was found in 96.8% of the patients less than 15 years of age and in 91% of the patients between 15 and 30 years of age at diagnosis. Among the patients over 30 years of age, 25% were negative for all four commonly measured AAb. These results are similar with data for newly diagnosed T1D in different age groups reported by other authors (Sabbah et al., 2000; Graham et al., 2002). The number of AAb in Estonian patients was consistent with a typical age-dependent profile. The prevalence of IAA, IA-2A and ICA decreased with increasing age at diagnosis, while the prevalence of GADA was not significantly influenced by age (Vandewalle et al., 1995; Sabbah et al., 2000; Graham et al., 2002).

The GADA were the most frequent AAb in all three age groups and the diagnostic sensitivity of AAb testing for T1D could be further increased by a combined testing of GADA and IA-2A. Among the children under 15 years of age all but one patient (96.8%) were positive for GADA and/or IA-2A. Similar high diagnostic sensitivity of a combined testing of GADA and IA-2A (95.5%) has been reported in a German study in children between 6–17 years of age with new-onset T1D (Strebelow et al., 1999). In older patients, the diagnostic sensitivity of the combination of GADA and IA-2A was also higher than that of GADA alone and only in a very few cases the patients had IAA or ICA but no GADA or IA-2A. These results favour the combined testing of GADA and IA-2A to confirm the autoimmune origin of diabetes in clinical and research settings. Indirectly, our data also indicate that measurements of GADA and IA-2A could be used in future screening programme for estimation the risk for T1D in relatives of patients with T1D and in general population. The existing high throughput assays for GADA and IA-2A (Verge et al., 1996; Kulmala et al., 1998) significantly simplify population screening for T1D.

The IAA, suggested to be a marker for rapid β-cell loss characteristic of childhood-onset T1D (Pihoker et al., 2005), were present in 10 out of the 11 children under 7 years of age (90.9%) and in 50% of the children between 7 and 14 years of age. Therefore, the prevalence of IAA in the studied group was even higher than that reported earlier elsewhere (Sabbah et al., 2000; Graham et al.,

2002). Since the appearance of IAA may precede that of GADA and IA-2A in children at risk for development of T1D (Kimpimaki et al., 2002; Wasserfall and Atkinson, 2006), this result favours additional determination of IAA in screening programme involving children. High prevalence of IAA in children at onset of T1D supports the concept of primary role of insulin as an autoantigen in the pathogenesis of the disease (Gianani and Eisenbarth, 2005).

In the healthy controls, the prevalence of the HLA-DQB1*0302, *02, *0301, *0602 and *0603 alleles in the present study was similar to the prevalence of these alleles found in previous studies of nondiabetic population, conducted in Estonia (Nejentsev et al., 1998) and elsewhere (Caillat-Zucman et al., 1992; Hosszufalusi et al., 2003). Similarly, the distribution of the INS VNTR genotypes in healthy subjects corresponded to the expected frequencies in Caucasoid individuals (Bennett et al., 1996; Graham et al., 2002). Logistic regression analysis of the data on allele and genotype frequencies in the patients and healthy controls confirmed that the HLA-DQB1*02/0302 genotype, the HLA-DQB1*0302 and *02 alleles, and the INS VNTR I/I genotype were all independent risk factors of T1D. Several studies have shown that the high-risk HLA-DQB1*02/0302 genotype is associated with early onset of the disease (Karjalainen et al., 1989; Caillat-Zucman et al., 1992; Sabbah et al., 2000). Although the prevalence of the DQB1*02/0302 genotype decreased in the older age groups also in our study population, in logistic regression analysis this association did not reach statistical significance, which is probably due to the insufficient statistical power of our study (< 20%).

Several previous studies have demonstrated the effect of HLA class II alleles on humoral β-cell autoimmunity in patients with T1D. These observations include the associations between the HLA-DQA1*0501, DQB1*0201 (DQ2) haplotype and appearance of GADA in newly diagnosed T1D (Sanjeevi et al., 1996; Graham et al., 2002), and between the HLA-DQA1*0301, DQB1*0302 (DQ8) haplotype and IAA (Vandewalle et al., 1993; Graham et al., 2002). Furthermore, there is mounting evidence that insulin-specific autoimmunity is influenced by allelic variations at the INS locus. Graham et al reported that the INS VNTR I/I risk genotype contributed to the appearance of IAA in patients with T1D (Graham et al., 2002) and Hermann et al demonstrated an association between INS VNTR I/I and IAA in children with an increased risk for T1D (Hermann et al., 2005). These results are in line with experimental data showing that the INS VNTR class I alleles may confer disease susceptibility by lower insulin expression in the thymus and decreased induction of central tolerance to this β-cell autoantigen (Vafiadis et al., 1997).

We failed to show significant relationship between INS VNTR I/I and IAA in our patients. Instead, we found that the INS VNTR I/III genotype was associated with the presence of GADA. Indeed, only 10.7% of the patients with the INS VNTR I/III genotype were negative for GADA, whereas 36.5% of those having the INS VNTR I/I genotype lacked these AAb. It is important to emphasise (on the basis of logistic regression analysis) that the observed

phenomenon was not influenced by possible confounding factors such as the presence of HLA-DQB1*02/0302 heterozygosity, or presence of HLA-DQB1*0302 or *02 alleles. To our knowledge, this is the first study demonstrating association between the INS VNTR I/III genotype and GADA in patients with newly diagnosed T1D. An observation similar to our finding was made by Walter et al in children with an increased risk for T1D. These authors reported that the offspring of parents with T1D, developing AAb to a number of antigens, had an increased prevalence of high risk genotypes at both the IDDM1 and IDDM2 loci, while the offspring with GADA only displayed increased frequencies of high risk IDDM1 and protective IDDM2 genotypes (Walter et al., 2003).

The importance of our data regarding development of the disease cannot be easily explained. However, we suggest that in absence of the INS VNTR I/I genotype the effect of other susceptibility factors prevails in T1D, and this may promote the targeting of autoantigens other than insulin. Unlike IAA and IA-2A, GADA is a common AAb in adult-onset T1D (Pihoker et al., 2005), and it is a hallmark of autoimmune diabetes with slowly progressive β-cell failure, designated as latent autoimmune diabetes of adults (LADA) (Stenstrom et al., 2005). The data reported by Tuomi et al (Tuomi et al., 1999) and those obtained by our own group (Haller et al., 2007) suggest that in patients with LADA, the frequency of the protective INS VNTR I/III genotype is similar to that in general population. Altogether, this supports the idea that in presence of the INS VNTR I/III genotype glutamic acid decarboxylase might be the preferential target of autoimmunity against β-cells. However, it is important to note that considering the relatively small number of patients in our study, confirming studies of the association between INS VNTR I/III and GADA in larger patient groups are necessary.

In summary, the current work was the first study on HLA-DQB1 and INS genes and on β-cell AAb in Estonian patients with newly diagnosed T1D, which involved the patients within a broad range of age (2−62 years) at diagnosis. The AAb profile in different age groups was similar to that demonstrated in other populations (Vandewalle et al., 1995; Sabbah et al., 2000; Graham et al., 2002; Pihoker et al., 2005), and a high diagnostic sensitivity of the combined testing of GADA and IA-2A was revealed, particularly in the patients aged 2−30 years.

The novel finding about the association between the INS VNTR I/III genotype and presence of GADA is of potential interest. Together with earlier observations of the associations between the genetic and β-cell autoimmunity markers, it reflects the heterogeneity of T1D and underlines the significance of disease-associated genes possibly modifying the hierarchy of autoantigenic targets in the pathogenesis of T1D.

6.2. EAD in RIP-B7.1 mice is caused by $CD8^+$ T cell immunity against an insulin A-chain epitope

Rodent models of autoimmune diabetes with spontaneous or inducible disease development are of great value to elucidate the pathogenesis of T1D and to investigate the perspectives of immune intervention treatments (Leiter and von Herrath, 2004).

Previous research of experimental autoimmune diabetes (EAD) in RIP-B7.1 mice has shown that EAD shares several features of T1D in NOD mice and humans, including infiltration of the pancreatic islets by $CD4^+$ and $CD8^+$ T cells, progressive β-cell damage and insulin deficiency (Karges et al., 2002). Diabetes occurred specifically after immunisation with ppins-II and not with GAD65 plasmid DNA in EAD (Karges et al., 2002), supporting the idea that ppins can be a central target of pathogenic T cell responses in T1D.

The current study demonstrated that the two ppins isoforms – ppins-I and ppins-II – were equally diabetogenic in EAD indicating shared autoantigenic determinant(s) of diabetogenic T cell reactivities. To address the contribution of different T cell subpopulations in EAD, an adoptive transfer system was established. After transplantation of syngeneic spleen cells from diabetic donors into irradiated RIP-B7.1 recipients, diabetes developed readily in a dose-dependent fashion in recipient animals. The experiments with sorted T cell subsets demonstrated that isolated $CD8^+$ T cells are sufficient for adoptive diabetes transfer, while isolated $CD4^+$ T cells do not induce disease development in recipient mice. These results were confirmed by *in vivo* T cell depletion experiments using anti-CD4 and anti-CD8 antibodies. Altogether, these findings demonstrated that in RIP-B7.1 ($H\text{-}2^b$) mice, the ppins-specific $CD8^+$ T cells are the major diabetogenic T cell subpopulation and that this EAD model is a suitable experimental tool to study $CD8^+$ T cell mediated diabetogenesis.

In order to narrow down the immunogenic region(s) of ppins-II containing the epitope(s) for diabetogenic $CD8^+$ T cells, an *in vivo* experimental system was used where RIP-B7.1 mice were immunised with deletion-mutant vectors of ppins-II. In these experiments, the putative autoantigenic determinant(s) for diabetogenic $CD8^+$ T cells were confined to the insulin A-chain. In following T cell assays, testing the reactivities of spleen cells from diabetic RIP-B7.1 mice against a library of insulin A-chain peptides by flow cytometry, the C-terminus of the insulin A-chain ($A_{12–21}$) was identified as the major autoantigenic determinant recognised by diabetogenic $CD8^+$ T cells. The amino acid sequence of the insulin A-$chain_{12–21}$ is identical in mouse ppins-I and ppins-II. This explains why the DNA vaccines containing ppins-I or ppins-II have comparable immunogenicity in EAD.

In NOD mice, insulin has been suggested as a central autoantigenic target in the pathogenesis of T1D. Overexpression of insulin in the thymus but not that of glutamate decarboxylase leads to a remarkable reduction in diabetes

occurrence in NOD mice (Jaeckel et al., 2003; Jaeckel et al., 2004). Deficiency of either ppins-I or ppins-II in NOD mice has opposite effects on diabetes penetrance in these mice. Ppins-II knockout NOD mice develop diabetes in an accelerated manner (Thebault-Baumont et al., 2003) while ppins-I knockout mice are protected from the disease (Moriyama et al., 2003). It suggests that ppins-II may play a role in induction of central tolerance in the thymus while in β-cells ppins-I is preferentially targeted. Replacement of natural ppins isoforms by a proinsulin transgene in which tyrosine was replaced with alanine at position 16 of the insulin B-chain resulted in a complete diabetes-resistance of female NOD mice (Nakayama et al., 2005). These results suggested that the insulin B-chain$_{9–23}$, containing the epitopes for both CD4+ and CD8+ T cells, might be an essential target of immune destruction in NOD mice and, if identical $B_{9–23}$ is expressed both in the thymus and β-cells, tolerance to insulin is induced.

Although it can be assumed in EAD in RIP-B7.1 (H-2^b) mice that the insulin A-chain$_{12–21}$ is expressed both in the thymus and in the β-cells, $A_{12–21}$-specific CD8$^+$ T cells nevertheless escape negative selection in the thymus and become diabetic upon autoantigenic challenge. Our preliminary data confirmed the observations of Ma et al (Ma et al., 2000; Ma and Kapp, 2001) that the insulin $A_{12–21}$-specific CD8$^+$ T cells were inducible also in wild-type C57BL/6 mice. Moreover, these cells are capable of homing to the pancreatic islets, however, islet invasion and β-cell destruction do not occur in non-transgenic mice (unpublished data). Thus, C57BL/6 (H-2^b) mice harbour the insulin-specific autoreactive CD8$^+$ T cells which are activated upon immunisation with plasmid DNA. Yet progression to clinical autoimmune disease in EAD depends on local susceptibility within the target organ, as afforded by co-stimulatory B7.1 under the control of RIP.

The immunogenicity of the insulin A-chain$_{12–21}$ in C57BL/6 mice has been reported earlier (Ma et al., 2000). Blocking reactivities with anti-H-2K^b or anti-H-2D^b antibodies, H-2K^b was proposed as a restriction element of $A_{12–21}$ by those authors. In our hands, insulin $A_{12–21}$ barely stabilised H-2K^b expression on TAP-deficient RMA-S cells, suggesting a weak binding affinity to H-2K^b. It is plausible that the weak interaction between the autoantigen and MHC may facilitate positive selection of autoreactive T cells in the thymus. Data from the RIP-LCMV mouse model suggest that the affinity of “neo”-self-peptide to the MHC may influence the quality of the self-reactive CD8$^+$ T cells escaping negative selection in the thymus. Upon restimulation, the quality of these different CD8$^+$ T cell clones determines the kinetics of diabetes development (von Herrath et al., 1994).

In NOD mice, three epitopes for diabetogenic CD8$^+$ T cells have been characterised so far (Wong et al., 1999; Lieberman et al., 2003; Lieberman et al., 2004). Among them the H-2K^d-restricted insulin B-chain 15–23 (LYLVCGERG) has an expected dominant anchor residue for H-2K^d-binding at

position 2 but not at position 9 (Falk et al., 1991) and, as a consequence, it demonstrates poor binding to H-2K^d (Wong, et al., 2002). Nevertheless, the insulin $B_{15–23}$-specific CD8$^+$ T cells are believed to play an important role in spontaneous diabetes development in NOD mice. They can be readily found in islet infiltrates of diabetic NOD mice (Wong et al., 1999; Lieberman et al., 2004). Also the G9C8 CD8$^+$ T cell clone recognising the insulin $B_{15–23}$ is highly diabetogenic upon adoptive transfer to irradiated NOD mice (Wong et al., 1996). Thus, the epitopes of autoreactive CD8$^+$ T cells may deviate from typical motifs for the given MHC class I molecules, and this may facilitate positive selection of these potentially pathogenic T cells in the thymus (Wong, et al., 2002). In contrast, $IGRP_{206–214}$ (VYLKTNVFL) – the cognate epitope of another highly diabetogenic CD8$^+$ T cell clone in NOD mice – harbours dominant anchor residues for binding H-2K^d both at positions 2 and 9 and a good binding affinity to H-2K^d can be observed (Lieberman et al., 2003). Therefore, in diabetes-prone NOD mice poor MHC class I binding is not required for the self-peptides recognised by autoreactive CD8$^+$ T cells (Lieberman et al., 2003). In RIP-B7.1 (H-2^b) mice, however, the low affinity interaction between the self-peptides and MHC I may play an important role in natural diabetes-resistance.

In different mouse models of type 1 diabetes, FasL (Itoh et al., 1997), perforin (Kagi et al., 1996; Kagi et al., 1997), TNF-α (Pakala et al., 1999) and IFN-γ (von Herrath and Holz, 1997; Seewaldt et al., 2000) have been claimed to mediate the apoptosis of the majority of pancreatic β-cells. Based on this, it has been hypothesised that there is no single exclusive β-cell apoptosis pathway in T1D (Santamaria, 2003). Therefore, in order to fully understand the pathogenesis of T1D and to be able to develop therapeutic approaches interfering with β-cell death in end-stage T1D, further investigation in different experimental models of T1D is worthwhile. In RIP-LCMV transgenic mice, diabetes development critically depends on IFN-γ (von Herrath and Oldstone, 1997) but the participation of different T cell effector mechanisms in β-cell damage in RIP-B7.1 mice had not been investigated so far. Therefore, we generated the RIP-B7.1 mice lacking expression of perforin or IFN-γ. Immunisation experiments with these mice convincingly demonstrated that in EAD, diabetogenesis depends on IFN-γ and not on perforin. Whether the effects of IFN-γ are mediated by up-regulation of the MHC class I on beta-cells or by IFN-γ-dependent β-cell cytotoxicity, as has been suggested for the LCMV model (Seewaldt et al., 2000), is currently unknown.

A recent study demonstrated that diabetes development in RIP-LCMV mice requires not only generation of autoreactive T cells but also toll-like-receptor (TLR)-mediated inflammation that enhances target organ susceptibility to immune attack (Lang et al., 2005). Coupling of the innate immune system for breaking peripheral tolerance was shown to use signalling through TLR3 and TLR7, which induces systemic IFN-α response and thereby promotes upregulation of MHC class I molecules on pancreatic β-cells. In order to

investigate the contribution of systemic type 1 IFN upregulation to diabetes development in our EAD model, the RIP-B7.1 mice, deficient in type I IFN receptor, were generated. These mice efficiently developed EAD after immunisation with ppins plasmid DNA, indicating no role for type 1 IFN mediated innate immunity. Whether the alternative pathways of innate immune system, e.g. signalling through TLR9 recognising unmethylated cytosine-phosphate-guanosine (CpG) motifs in plasmid backbone (Donnelly et al., 2005), are involved in the diabetogenicity of DNA vaccines in EAD, is currently unknown.

The $CD8^+$ T cells exert cytotoxic damage in adaptive immune responses recognising the relevant antigenic peptides presented by MHC class I molecules. As the latter are expressed on all nucleated cells, $CD8^+$ T cells have a great potential to induce tissue damage in autoimmune diseases (Liblau et al., 2002). Recent experimental data suggest that the $CD8^+$ T cells, recognising the autoantigenic peptides expressed in their target tissues, contribute substantially to tissue damage in autoimmune diseases including T1D, multiple sclerosis and others (Huseby et al., 2001; Liblau et al., 2002). In NOD mice, three major diabetogenic $CD8^+$ T cell clones can account for a considerable proportion (up to 60%) of the $CD8^+$ T cells infiltrating islets in NOD mice at various stages of disease development (Lieberman et al., 2004). These results suggest that in spontaneous autoimmune diabetes, a restricted number of relevant $CD8^+$ T cell clones are involved in disease development. In RIP-LCMV mice, $CD8^+$ T cells, specific for a single immunodominant epitope of LCMV-GP, induce the autoimmune diabetes (Ludewig et al., 1998; Lang et al., 2005).

There is accumulating evidence that $CD8^+$ T cells contribute significantly to development of T1D in man. In humans with recent-onset T1D, β-cell islets are predominantly infiltrated by $CD8^+$ T cells (Bottazzo et al., 1985; Itoh et al., 1993). Furthermore, in recent studies, $CD8^+$ T cell epitopes have been identified in various β-cell autoantigens, including ppins, GAD and IGRP (Ouyang et al., 2006; Blancou et al., 2007; Mallone et al., 2007; Unger et al., 2007). Studying $CD8^+$ T cell reactivities against these epitopes allowed discrimination between patients with new onset T1D and healthy controls with high accuracy (Mallone et al., 2007).

Mounting evidence from humans with T1D and from experimental models of the disease suggests that insulin as an autoantigen may be a trigger in the pathogenesis of T1D (Kent et al., 2005; Nakayama et al., 2005; von Herrath, 2005). Therefore, attempts to modify insulin-specific immune response in experimental models and in humans with increased risk for the disease may ultimately lead to prevention of T1D. In RIP-B7.1 mice, we transferred the elements of the classic LCMV system to insulin as a natural islet autoantigen. Upon autoantigenic challenge, $CD8^+$ T cells recognising the insulin A-$chain_{12–21}$ were identified as the major diabetogenic T cell population. Considering the key elements of diabetogenesis in RIP-B7.1 ($H\text{-}2^b$) mice, this model provides an attractive experimental approach allowing to investigate the mechanisms of

$CD8^+$ T cell mediated β-cell damage as well as to develop therapeutic interventions interfering with diabetogenic, particularly insulin-specific $CD8^+$ T cell responses.

In ongoing clinical studies the effectiveness of anti-CD3 mAb (Keymeulen et al., 2005) and alum-formulated GAD65 (Lernmark and Agardh, 2005) in immunomodulation in patients with T1D is being evaluated. Study of the complexity of interactions between the genes and both humoral and cellular immunity in humans with T1D and of the immunopathogenesis of autoimmune diabetes in rodent models will open up new avenues for further development of the immunotherapy of the disease.

7. CONCLUSIONS

1. In human type 1 diabetes, the HLA-DQB1*02/0302 genotype, the HLA-DQB1*0302 and *02 alleles, and the INS VNTR I/I genotype were all risk factors of T1D in our study population. In logistic regression analysis, the association between INS VNTR I/I and the disease remained highly significant regardless of adjusting for the HLA-DQB1*02/0302 genotype, or for the HLA-DQB1*0302 or *02 alleles, confirming that INS VNTR I/I was an independent risk factor of T1D.
2. The prevalence of the HLA-DQB1*02/0302 genotype, the HLA-DQB1*0302 allele, the DQB1*02 allele or the INS VNTR I/I genotype was not associated with age at diagnosis of T1D in our study group. The IAA, IA-2A and ICA but not GADA were negatively associated with age at diagnosis of T1D. Linear regression analysis showed a highly significant negative association between number of AAb and age at diagnosis of T1D. Analysis of the associations between the genetic markers and AAb revealed, as a novel finding, the association between the INS VNTR I/III genotype and presence of GADA. There was also a tendency of IAA to be associated with the HLA-DQB1*02/0302 genotype or the HLA-DQB1*0302 allele.
3. In EAD in RIP-B7.1 (H-2^b) mice, the transfer of diabetes with spleen cells from the hyperglycaemic mice in a dose-dependent manner into the irradiated RIP-B7.1 recipients confirmed the central role of cellular immunity in the pathogenesis of diabetes in this mouse model.
4. Diabetes transfer experiments with sorted T cell subpopulations and *in vivo* depletion experiments demonstrated convincingly that $CD8^+$ T cells mediate diabetogenesis in this model. Further evidence was provided by histological studies where predominant infiltration of β-cell islets by $CD8^+$ T cells in prediabetic and diabetic mice was found.
5. Using the deletion-mutant vectors of ppins-II in immunisation studies, the immunogenic $CD8^+$ T cell determinant(s) mapped to the insulin A-chain. The *in vitro* experiments demonstrated that the insulin A-$chain_{12–21}$ was the immunodominant epitope for diabetogenic $CD8^+$ T cell reactivities in EAD.
6. The EAD in RIP-B7.1 mice was independent of systemic type 1 IFN response. Beta-cell killing in this model required the expression of IFN-γ but not of perforin.

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SUMMARY IN ESTONIAN

Autoimmuunne diabeet: immunoloogiline uuring 1. tüüpi diabeediga haigetel ja eksperimentaalse diabeedi mudelis (RIP-B7.1 hiirtel)

Esimest tüüpi diabeet on autoimmuunhaigus, mida iseloomustab rakuliste immuunmehhanismide poolt vahendatud kõhunäärme β-rakkude hävimine. Haiguse etioloogia on mitmeteguriline, hõlmates ühelt poolt geneetilist eelsoodumust ja teiselt poolt haigust vallandavaid keskkonnamõjureid. Viimaste kohta on suhteliselt vähe teada, kuid eelkõige seostatakse 1. tüüpi diabeedi teket viirusinfektsioonide ja toitumise iseärasustega.

Esimest tüüpi diabeedi geneetiline eelsoodumus on määratud rohkem kui 20 erineva geenilookusega. Neist kõige olulisemad ja paremini iseloomustatud on suures koesobivuskompleksis (MHC) kodeeritud HLA II klassi geenid (6p21) ja insuliini geeni regioon (11p15). HLA II klassi geenialleelide hulgas kannab kõige suuremat diabeediriski heterosügootne kombinatsioon HLA-DQB1*0302/0201, samuti HLA-DQB1*0302 või *0201 olemasolu homosügootsena või kombinatsioonis mõnede teiste alleelidega. Kõige sagedamini esinevad kaitsvad alleelid on HLA-DQB1*0602 ja *0301. Insuliini geeni alleelivariantide seost 1. tüüpi diabeediga seostatakse kordusjärjestuste arvuga (VNTR) insuliini geeni 5' otsas. I klassi alleelid koosnevad 20–63 kordusjärjestusest ja III klassi alleelid 140–210 kordusjärjestusest. II klassi alleele esineb europiidse rassi esindajatel harva.

Esimest tüüpi diabeedile on iseloomulik spetsiifiliste autoantikehade olemasolu, seda juba enne haiguse kliinilist avaldumist. Kõige sagedamini leitakse saarekeste rakkude vastaseid autoantikehi (ICA) ning autoantikehi glutamaadi dekarboksülaasi (GADA), türosiini fosfataasi (IA-2A) ja insuliini (IAA) vastu.

Esimest tüüpi diabeet võib manifesteeruda igas vanuses, kusjuures HLA-DQB1 alleelide ja autoantikehade levimus võib olla vanusest sõltuv. Eestis on 1. tüüpi diabeedi geneetilisi ja immuunmarkereid uuritud alla 15-a. esmastel haigetel ning on leitud, et põhiliste HLA II klassi alleelide levimus Eestis vastab teiste populatsioonide näitajatele. Eesti täiskasvanud esmastel 1. tüüpi diabeediga patsientidel pole HLA II klassi alleelide ega haigusspetsiifiliste autoantikehade levimust uuritud. Lisaks sellele puuduvad andmed insuliini geeni polümorfismide levimuse kohta esmastel 1. tüüpi diabeediga haigetel. Eesti patsientidel pole analüüsitud ka geneetiliste tegurite ja autoantikehade omavahelisi seoseid.

Kõhunäärme β-rakkude kahjustus 1. tüüpi diabeedi korral on vahendatud põhiliselt T-lümfotsüütidega ja 1. tüüpi diabeedi keerulisse immunopatogeneesi on haaratud kogu immuunsüsteem. Seetõttu on 1. tüüpi diabeedi loommudelid asendamatuks uurimismaterjaliks haiguse patogeneesi väljaselgitamisel. Katseloomadest kasutatakse põhiliselt hiiri, kusjuures diabeet tekib nendes mudelites

kas spontaanselt nagu NOD (*nonobese diabetes mouse*) hiirtel või immuniseerimise tulemusel. Viimane eeldab β-rakkude geneetilisi manipulatsioone nagu näiteks LCMV (*lymphocytic choriomeningitis virus*) valkude transgeeni RIP-i (*rat insulin promoter*) kontrolli all. Beeta-rakkude tundlikkust autoimmuunse kahjustuse tekke suhtes suurendab ka RIP-B7.1 (RIP-CD80) transgeen. Preproinsuliini plasmiidse DNA lihasesisene süstimine vallandab RIP-B7.1 (H-2^b) transgeensetel hiirtel diabeedi, mis oma kulult sarnaneb 1. tüüpi diabeediga inimestel. Praeguseks ajaks on nii eksperimentaalsetest kui ka kliinilistest uuringutest kogunenud rohkelt andmeid selle kohta, et (prepro)insuliinil on β-rakkude spetsiifilise autoantigeenina keskne koht 1. tüüpi diabeedi patogeneesis. Eksperimentaalne autoimmuunne diabeet (EAD) RIP-B7.1 (H-2^b) transgeensetel hiirtel võimaldab uurida preproinsuliini vastaste immuunreaktsioonide rolli 1. tüüpi diabeedi patogeneesis ja hinnata 1. tüüpi diabeedi preventiivse ravi võimalusi. Nende uurimissuundade edendamise eelduseks on diabeeti põhjustavate T-lümfotsüütide ja nende autoantigeensete epitoopide identifitseerimine selles eksperimentaalse autoimmuunse diabeedi mudelis.

Töö eesmärgid

1. Hinnata põhiliste HLA-DQB1 alleelide ja insuliini geeni polümorfismide levimust Eesti esmastel lapse- ja täiskasvanueas haigestunud 1. tüüpi diabeediga patsientidel.
2. Analüüsida geneetiliste tegurite ning autoantikehade seost haigestumise eaga Eesti 1. tüüpi diabeediga patsientidel, samuti geneetiliste tegurite mõju autoantikehade esinemisele.
3. Töötada välja diabeedi adoptiivse ülekande süsteem EAD (RIP-B7.1) hiiremudelis, et saada kinnitust rakulise immuunsuse kesksele rollile haiguse patogeneesis selles mudelis.
4. Hinnata põhiliste T-lümfotsüütide alarühmade ($CD8^+$ ja $CD4^+$ alarühmade) rolli diabeedi arengus EAD mudelis RIP-B7.1 (H-2^b) hiirtel.
5. Määratleda preproinsuliini molekuli immunogeensed regioonid RIP-B7.1 (H-2^b) hiirel ja teha kindlaks preproinsuliini spetsiifiliste T-lümfotsüütide autoantigeensed epitoobid.
6. Hinnata 1. tüüpi interferoonide poolt vahendatud loomuliku immuunsuse rolli diabeedi patogeneesis RIP-B7.1 (H-2^b) hiirel, samuti β-rakkude hävimise mehhanisme selles mudelis.

Materjal ja meetodid

Kliiniline uuring

Uuringugrupp koosnes 92 patsiendist esmase 1. tüüpi diabeediga (vanus 2–62 a., vanuse mediaan 20 a., 49 naissoost isikut), kelle haigus diagnoositi aastatel 2001–2003. Earühmade analüüsiks jaotati patsiendid 3 rühma: alla 15-a. lapsed (31 patsienti, keskmine vanus ± SD, 8,3 ± 3,3 a., 15 naissoost), noorukid ja noored täiskasvanud vanuses 15–30 a. (33 patsienti, 21,2 ± 4,8 a., 20 naissoost) ja üle 30-a. täiskasvanud (28 patsienti, 38,6 ± 8,5 a., 14 naissoost). Kontrollgruppi kasutati geneetiliste markerite levimuse võrdlemiseks patsientide ja üldrahvastiku vahel. Grupp koosnes 160 tervest veredoonorist ja 91 erinevatel põhjustel hospitaliseeritud patsiendist, kellel ei esinenud kaasuva haigusena diabeeti (vanus 13–85 a., vanuse mediaan 45 a., 151 naissoost).

Saarekeste vastased antikehad (ICA) määrati kaudsel immunofluorestsentsmeetodil, kasutades antigeensubstraadina 0 veregrupiga inimese pankreasekude. GADA, IA-2A ja IAA määrati radioimmunopretsipitatsiooni meetodil Saksamaa Greifswaldi Ülikooli patofüsioloogia instituudis.

HLA alleelid DQB1 lookuses määrati hübridisatsioonireaktsiooniga, kasutades lantaniididega märgistatud oligonukleotiidseid sonde ja fluoromeetrilist detekteerimist. Testimine hõlmas HLA-DQB1 alleele *02, *0302, *0301, *0602 ja *0603. Insuliini geeni kordusjärjestuste arvu (*VNTR*) polümorfismid määrati surrogaatmarkeri *HphI* A/T üksiku nukleotiidi polümorfismi (*SNP*) genotüpeerimise alusel.

Statistiline analüüs teostati lineaarse ja logistilise regressiooni analüüsi meetodil ja selleks kasutati vabavara *The R 2.3.1 A Language and Environment*.

Eksperimentaalne uuring

RIP-B7.1 hiired olid C57BL/6 (H-2^b) geneetilise taustaga. IFN-γ-, perforiin- või I tüüpi IFN retseptori defitsiitsed RIP-B7.1 hiired saadi homosügootsete RIP-B7.1 hiirte ja IFN-γ-, perforiin- või I tüüpi IFN retseptori defitsiitsete (H-2^b) hiirte ristamise ja F1 põlvkonna omavahelise ristamise teel.

Immuniseerimiseks süstiti 6–12 nädala vanustele hiirtele mõlemasse *m. tibialis anterior*'i 50 μg plasmiidset DNA-d. Diabeedi teket jälgiti veresuhkru mõõtmise abil ja seda diagnoositi siis, kui veresuhkur oli kahel järjestikusel korral üle 250 mg/dl (13,8 mmol/l).

Kõik plasmiidse DNA-ga immuniseerimiseks vajalikud DNA lõigud klooniti pCI vektorisse. DNA lõigud kodeerisid järgmisi polüpeptiide: hiire preproinsuliin I ja II (ppins-I ja -II), spetsiifilise hemaglutiniini antikeha epitoobiga märgistatud hiire preproinsuliin II (ppins-HA), hiire preproinsuliin II signaalpeptiid koos 12 aminohappega B-ahelast (pCI/sp), insuliini B-ahel koos 10 aminohappega signaalpeptiidist ja 10 aminohappega C-peptiidiist (pCI/B), C-

peptiid koos 10 aminohappega B-ahelast ja 8 aminohappega A-ahelast (pCI/C), insuliini A-ahel koos 8 aminohappega C-peptiidist (pCI/A).

Preproinsuliini ekspressiooni uurimiseks mittepankreaatilistes rakkudes transfitseeriti inimese embrüonaalsed neerurakud (HEK293) ppins-HA DNA-ga, märgistati ^{35}S-metioniini/tsüsteiiniga ja analüüsiti HA-spetsiifilise immunopretsipitatsiooni teel.

Põrnarakkude siirdamiseks eraldati põrnarakud diabeediga RIP-B7.1 hiirte põrnast ning 0,2; 0,5; 1 või 2x10^7 rakku 300 µl RPMI-söötmes süstiti kiiritatud (650 rad) RIP-B7.1 hiirtele sabaveeni. $CD8^+$ või $CD4^+$ T-lümfotsüütide siirdamiseks eraldati põrnarakud diabeediga RIP-B7.1 hiirte põrnast, isoleeriti vastavad $CD8a^+$ või $CD4^+$ rakupopulatsioonid, misjärel süstiti kiiritatud (650 rad) RIP-B7.1 hiirte sabaveeni 0,8x10^6 $CD8^+$ või 1,4x10^6 $CD4^+$ T-lümfotsüüti või kontrollgrupis 10^7 põrnarakku. $CD4^+$ või $CD8^+$ T-lümfotsüütide rakurühma eemaldamiseks *in vivo* kasutati vastavaid monoklonaalseid antikehi YTS 191.1.2 või YTS 169.4.2.1.

Histoloogiliseks uuringuks kasutati 5 µm koelõike, mis värviti kas hematoksüliini-eosiiniga või fluorestseerivate märgistega tähistatud CD4, CD8a, CD3E ja insuliini vastaste antikehadega.

T-lümfotsüütide stimuleerimiseks *in vitro* kasutati insuliini A-ahela osaliselt kattuvaid peptiide (n = 22). Peale 16-tunnist inkubeerimist värviti rakud fluorestseerivate märgistega tähistatud CD8a, CD3ε ja IFN-γ vastaste antikehadega. H-2^b stabiliseerimise uuringus kasutati TAP- (*transporter associated with processing*) defitsiitset RMA-S rakuliini, insuliini A-ahela peptiide ja fluorestseerivate märgistega tähistatud H-$2K^b$ ja H-$2D^b$ vastaseid antikehi. Rakke uuriti voolutsütomeetrilisel meetodil ja tulemuste analüüsimiseks kasutati vabavara WinMDI 2.8.

Tulemused ja arutelu

Kliiniline uuring

HLA-DQB1*0302 ja *02 alleelide olemasolu tõstab Eesti populatsioonis 1. tüüpi diabeeti haigestumise riski (šansside suhe vastavalt 5,51 ja 2,1) ning HLA-DQB1*0602–03 ja *0301 kaitsevad 1. tüüpi diabeeti haigestumise eest (šansside suhe vastavalt 0,016 ja 0,48). Kõik need seosed olid statistiliselt olulised. INS VNTR I/I genotüüp tõstab samuti haigestumise riski (šansside suhe 2,66; $p < 0,001$). Logistilise regressiooni analüüsis jäi INS VNTR I/I ja haiguse vaheline seos väga oluliseks vaatamata kohandamisele HLA-DQB1*02/0302 genotüübile või HLA-DQB1*0302 või *02 alleelile.

HLA-DQB1*0302/02 genotüübi, HLA-DQB1*0302 ja *02 alleelide ning INS VNTR I/I genotüübi levimus ei olnud seotud patsientide eaga diabeeti haigestumisel.

Logistilise regressiooni analüüs tõi ilmsiks negatiivse seose IAA, IA-2A ja ICA levimuse ja haigestumise ea vahel, kuid GADA levimuse ja haigestumise ea vahel ei olnud olulist seost. Lineaarse regressiooni analüüs näitas väga olulist negatiivset seost autoantikehade arvu ja haigestumise ea vahel.

Autoantikehade levimuse analüüs earühmade kaupa näitas GADA ja IA-2A kombineeritud testimise kõrget diagnostilist tundlikkust kõigis kolmes rühmas. GADA ja/või IA-2A testid olid positiivsed 96,8%-l alla 15-a. lastel, 87,9%-l 15–30-a. patsientidel ja 67,9%-l üle 30-a. täiskasvanutel. GADA, IA-2A ja ICA levimus ei olnud meie patsientidel seotud HLA-DQB1*0302/02 genotüübi ega HLA-DQB1*0302 või *02 alleeliga, kuigi täheldati tendentsi IAA levimuse ja HLA-DQB1*0302/02 genotüübi või HLA-DQB1*0302 alleeli esinemise vahel.

Logistilise regressiooni analüüsil ilmnes seos GADA ja kaitsva INS VNTR I/III genotüübi vahel (šansside suhe 4,79; p=0,018). See seos jäi oluliseks, kui logistilise regressiooni analüüsis arvestati muid mõjufaktoreid nagu HLA-DQB1*0302/02 genotüüpi ja vanust haigestumisel, HLA-DQB1*0302 alleeli ja vanust haigestumisel või HLA-DQB1*02 alleeli ja vanust haigestumisel. Tegemist on uudse tulemusega, mis võib viidata asjaolule, et INS VNTR I/I riskigenotüübi puudumisel on autoimmuunreaktsioonide teke β-rakkude vastu määratud teiste geneetiliste tegurite poolt ja sihitud eelistatult glutamaadi dekarboksülaasi vastu.

Eksperimentaalne uuring

Varasematest uuringutest RIP-B7.1 (C57BL/6) hiirtel oli teada, et hiirte immuniseerimine ppins-II plasmiidse DNA-ga vallandab enamusel hiirtel inimese 1. tüüpi diabeedile sarnase haiguse.

Selleks et näidata ppins-II plasmiidse DNA ekspressiooni mittepankreaatilistes rakkudes ja teha kindlaks ekspresseeritav antigeen, viidi läbi HEK293 rakkude transfektsioon ppins-HA konstruktiga, millele järgnes immunopretsipitatsioon HA-spetsiifilise monoklonaalse antikehaga. SDS-PAGE analüüsil leiti ainult 11,5 kD produkt, mis vastab proinsuliin-HA-le. Puudusid 14,5 kD ja 3,5 kD produktid, mis oleks vastanud preproinsuliin-HA-le ja A-ahel-HA-le. Seega on mittepankreaatilistes rakkudes ppins DNA-vektori põhiline produkt proinsuliin.

Kui RIP-B7.1 hiiri immuniseeriti ppins-I (n = 11) või ppins-II (n = 13) plasmiidse DNA-ga, siis arenes kõigil katseloomadel kiiresti diabeet (mediaaniga 3 nädalat). Tulemus viitab sellele, et tõenäoliselt jagavad ppins-I ja ppins-II ühist T-lümfotsüütide epitoopi.

$2x10^7$ ja $1x10^7$ põrnaraku siirdamisel diabeediga hiirtelt kiiritatud (650 rad) RIP-B7.1 hiirtele arenes enamusel retsipientidel 8–10 nädala jooksul diabeet. $0,5x10^7$ ja $0,2x10^7$ põrnaraku siirdamisel arenes retsipientidel haigus oluliselt aeglasemalt. Seega on RIP-B7.1 mudelis võimalik üle kanda diabeeti põrnarakkude siirdamisega, mis viitab eelkõige haiguse rakulisele geneesile.

Kui võrreldi diabeedi ülekandmise efektiivsust põrnarakkude ja isoleeritud $CD8^+$ ning $CD4^+$ rakupopulatsioonidega, osutusid $CD8^+$ T-lümfotsüüdid haiguse vallandamisel sama efektiivseks kui sortimata põrnarakud. Selle tulemuse alusel võib järeldada, et $CD8^+$ T-lümfotsüüdid on EAD mudelis põhiline diabetogeenne rakupopulatsioon. Tulemus leidis kinnitust ka eksperimendis, mille käigus eemaldati *in vivo* monoklonaalsete antikehadega kas $CD8^+$ või $CD4^+$ T-lümfotsüüdide rakurühm. $CD8^+$ T-lümfotsüütide eemaldamisel hiirtel diabeeti ei tekkinud, $CD4^+$ T-lümfotsüütide eemaldamisel arenes haigus aga normaalse kiirusega.

Pankrease saarekeste histoloogilisel uuringul oli katseloomadel 2 nädalat peale immuniseerimist ppins-II DNA-ga enamus saarekesi infiltreeritud $CD8^+$ ja väiksemal määral $CD4^+$ T-lümfotsüütide poolt. Selles staadiumis oli β-rakkudes näha veel küllaldaselt insuliini ja hiirtel esines normoglükeemia. Diabeedi staadiumis oli histoloogiliselt näha väljendunud insuliit $CD8^+$ ja $CD4^+$ T-lümfotsüütide infiltratsiooniga ja insuliin β-rakkudes praktiliselt puudus.

Kui hiirte immuniseerimiseks kasutati preproinsuliini domeene sisaldavaid vektoreid, siis arenes diabeet ainult pCI/A konstruktiga (insuliini A-ahel koos 8 aminohappega C-peptiidist). Kuna pCI/C konstrukt (C-peptiid koos 10 aminohappega B-ahelast ja 8 aminohappega A-ahelast) diabeeti ei vallandanud, siis võis järeldada, et $CD8^+$ T-lümfotsüütide epitoop või epitoobid paiknevad täielikult insuliini A-ahelas.

Stimuleerides diabeediga RIP-B7.1 hiirte põrnarakke insuliini A-ahela osaliselt kattuvate peptiididega ja uurides neid voolutsütomeetriliselt selgus, et ainus peptiid, mis stimuleerib $CD8^+$ T-lümfotsüütide IFN-γ vastust, on insuliini A-ahela C-terminaalne peptiid $A_{12–21}$. Tegemist on identse peptiidiga hiire ppins I-l ja ppins II-l, mis seletab ppins I ja ppins II võrdset immunogeensust RIP-B7.1 hiirel. $A_{12–21}$ immunogeensust C57BL/6 ($H\text{-}2^b$) hiirtel on varem kirjeldatud ja on näidatud, et peptiidi esitab $H\text{-}2K^b$ molekul. $A_{12–21}$ praktiliselt ei stabiliseerinud K^b ekspressiooni TAP-defitsiitsetel RMA-S rakkudel, mis viitab peptiidi nõrgale afiinsusele K^b suhtes. Nõrk afiinsus peptiidi ja seda esitava MHC I molekuli vahel võib põhjendada $A_{12–21}$ vastaste $CD8^+$ T-lümfotsüütide positiivset selektsiooni tüümuses.

Perforiini defitsiitsed RIP-B7.1 hiired haigestusid diabeeti peale ppins-II DNA-ga immuniseerimist tavalise sagedusega. Seevastu IFN-γ-defitsiitsed RIP-B7.1 hiired olid diabeedi suhtes resistentsed, mis viitab sellele, et β-rakkude apoptoos on selles EAD mudelis vahendatud peamiselt IFN-γ poolt. I tüüpi IFN retseptori defitsiitsetel RIP-B7.1 hiirtel arenes diabeet immuniseerimise järgselt tavalise sagedusega. Seega ei osale 1. tüüpi IFN poolt vahendatud loomuliku immuunsuse mehhanismid diabeedi tekkes RIP-B7.1 hiirtel.

Peamise diabetogeense rakupopulatsiooni ja selle sihtmärkepitoobi, samuti β-rakkude apoptoosi mehhanismide tundmine EAD loommudelis on oluliseks eelduseks diabeedi patogeneesi ja preventiivse ravi võimalusi käsitlevate edasiste uuringute läbiviimisel.

Järeldused

1. 1. tüüpi diabeedi riskiteguriteks osutusid Eesti populatsioonis HLA-DQB1*02/0302 genotüüp, HLA-DQB1*0302 ja *02 alleelid ning INS VNTR I/I genotüüp, mis kinnitab varasemaid andmeid. Logistilise regressiooni analüüsis jäi INS VNTR I/I ja haiguse vaheline seos väga oluliseks vaatamata kohandamisele HLA-DQB1*02/0302 genotüübile või HLA-DQB1*0302 või *02 alleelile, mis näitab, et INS VNTR I/I on 1. tüüpi diabeedi sõltumatu riskitegur.
2. HLA-DQB1*02/0302 genotüüp, HLA-DQB1*0302 alleel, HLA-DQB1*02 alleel ega INS VNTR I/I genotüüp polnud meie patsientidel seotud haigestumise vanusega. IAA, IA-2A ja ICA levimuse ning haigestumise vanuse vahel leiti negatiivne seos, kuid GADA polnud seotud vanusega T1D haigestumisel. Lineaarse regressiooni analüüs näitas väga olulist negatiivset seost autoantikehade arvu ja haigestumise vanuse vahel. Genotüüpide ja alleelide ning autoantikehade seoste analüüsis ilmnes uudse tulemusena seos INS VNTR I/III genotüübi ja GADA esinemise vahel. Lisaks sellele esines seose tendents IAA ning HLA-DQB1*02/0302 genotüübi ja IAA ning HLA-DQB1*0302 alleeli vahel.
3. EAD loommudelis RIP-B7.1 (H-2^b) hiirtel näitasid põrnarakkude siirdamise eksperimendid, et diabeeti on võimalik üle kanda hüperglükeemilistelt hiirtelt kiiritatud RIP-B7.1 hiirtele, mis kinnitab, et selles loommudelis on diabeedi patogeneesis keskne roll rakulisel immuunsusel.
4. Eksperimendid põrnarakkude alarühmade siirdamise ja alarühmade *in vivo* eemaldamisega demonstreerisid veenvalt, et diabeedi teke RIP-B7.1 hiirtel on vahendatud $CD8^+$ T-lümfotüütide poolt. Seda kinnitasid ka histoloogilised uuringud, mis näitasid eelistatult $CD8^+$ T-lümfotsüütide infiltratsiooni prediabeedi ja diabeedi staadiumis.
5. Preproinsuliini domeene sisaldavate vektorite kasutamisel immuniseerimise eksperimentides ilmnes, et $CD8^+$ T-lümfotsüütide epitoop või epitoobid asuvad insuliini A-ahelas. *In vitro* katsed näitasid, et diabeeti tekitavate $CD8^+$ T-lümfotsüütide immunodominantne epitoop on insuliini A-ahela C-terminaalne peptiid $A_{12–21}$.
6. EAD RIP-B7.1 hiirtel ei sõltu süsteemsest 1. tüüpi IFN vastusest. Beetarakkude apoptoos toimub selles loommudelis IFN-γ ja mitte perforiiniga seotud mehhanismide kaudu.

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PUBLICATIONS

I

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Insulin VNTR I/III genotype is associated with autoantibodies
against glutamic acid decarboxylase in newly diagnosed type 1 diabetes.
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INSULIN VNTR I/III GENOTYPE IS ASSOCIATED WITH AUTOANTIBODIES AGAINST GLUTAMIC ACID DECARBOXYLASE IN NEWLY DIAGNOSED TYPE 1 DIABETES

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ABSTRACT

BACKGROUND: In type 1 diabetes (T1D), the influence of age at diagnosis and of the IDDM1 and IDDM2 genetic susceptibility loci on the profile of β-cell autoantibodies has been demonstrated. We studied these associations in a group of 92 patients (children, adolescents and adults, age range 2–62 years) with newly diagnosed T1D. METHODS: The prevalence of the *HLA-DQB1**02 and *0302 alleles and of the classes of variable number of tandem repeats (VNTR) of the insulin gene (*INS*), and of β-cell autoantibodies (GADA, IA-2A, ICA and IAA) was determined. Statistical analysis was performed using linear and logistic regression models. RESULTS: The presence of IAA, IA-2A and ICA, but not of GADA was negatively associated with age at diagnosis. Younger patients were more likely to have multiple autoantibodies. There was a tendency of a higher prevalence of IAA in patients with the *HLA-DQB1**02/0302 genotype or with the *DQB1**0302 allele compared to patients lacking these markers. As a novel observation, the *INS* VNTR I/III genotype was significantly associated with the presence of GADA (OR = 4.79; p = 0.018). CONCLUSION: The association between the *INS* VNTR I/III genotype and GADA may suggest that in patients with T1D lacking the *INS* VNTR I/I genotype, the effect of other susceptibility factors prevails, which promotes the development of autoimmunity to β-cell antigens other than insulin.

Key words
Type 1 diabetes, *HLA-DQB1*, insulin gene VNTR, β-cell autoantibodies, GADA, IAA

INTRODUCTION

Type 1 diabetes (T1D) develops in genetically predisposed individuals as a consequence of autoimmune destruction of pancreatic β-cells. The hallmarks of autoimmunity in individuals at increased risk of T1D and in patients with the overt disease are antibodies against β-cell autoantigens, particularly islet cell antibodies (ICA), and autoantibodies (AAb) against glutamic acid decarboxylase (GADA), tyrosine phosphatase-like protein IA-2 (IA-2A) and insulin (IAA) [1]. The major determinants of genetic susceptibility to T1D include the MHC complex and the insulin gene region, designated as IDDM1 and IDDM2, respectively [2]. At the IDDM1 locus, susceptibility to the disease is confined to *HLA-DRB1* and *HLA-DQB1*, which are in tight linkage disequilibrium with each other [2]. The IDDM2 susceptibility locus has been identified as allelic variation of variable number of tandem repeats (VNTR) 5' of the insulin gene (*INS*) [3]. Homozygosity for class I VNTR is associated with a 2–5 fold higher risk for T1D, while class III VNTR alleles are dominantly protective [3,4].

Association studies in patients with T1D and in subjects at increased risk for the disease have indicated that the profile of AAb is influenced by particular susceptibility alleles and genotypes at IDDM1 and IDDM2, probably reflecting the modifying effect of genes on the development of immune responses against β-cell autoantigens in the natural course of T1D [1]. For example, in newly diagnosed T1D, an association between the *HLA-DQA1**0501, *DQB1**0201 (DQ2) haplotype and appearance of GADA has been observed [5,6], and in patients with the *HLA-DQA1**0301, *DQB1**0302 (DQ8) haplotype, the IAA has been found significantly more often than in patients without this haplotype [6,7]. At IDDM2, an association between the *INS* VNTR I/I genotype and higher prevalence of IAA in patients with newly diagnosed T1D [6] and in subjects at increased risk for development of T1D [8] has been demonstrated.

We are reporting a novel observation made by studying the associations between the main genotypes and alleles at IDDM1 and IDDM2 and diabetes-related AAb in Estonian paediatric and adult patients with newly diagnosed T1D. The patients carrying the protective *INS* VNTR I/III genotype were positive for GADA significantly more often. Logistic regression analysis confirmed that this association was independent of the *HLA-DQB1* alleles and age at diagnosis of T1D. Our observation supports the notion that the genes modify the β-cell specific autoimmunity in T1D.

MATERIALS AND METHODS

Subjects

The study group consisted of 92 patients (median age 20 years, range 2–62 years, 49 females) with newly diagnosed T1D. The patients were enrolled in the study between 2001 and 2003 from the two main children's hospitals and from the two main adult inpatient endocrinology and diabetes units in Estonia. The diagnosis of T1D was based on clinical characteristics including rapid onset of symptoms, weight loss, polydipsia, polyuria, ketosis and necessity for insulin therapy. Particular attention was paid to adults to exclude patients with type 2 diabetes and with diseases of the exocrine pancreas. Blood samples from all patients were collected within one week of diagnosis.

The control group comprised 251 individuals and was used for the risk evaluation of the *HLA-DQB1* and *INS* VNTR alleles. One hundred and sixty of them were healthy blood donors and 91 subjects were patients hospitalised for various reasons who did not have diabetes as an accompanying illness (median age 45 years, range 13–85 years, 151 females).

The study was approved by the Ethics Committee of the University of Tartu, and informed consent was obtained from the adult participants and from the parents of children involved.

Genotyping

HLA-DQB1 typing was performed using the hybridisation of lanthanide-labelled allele-specific oligonucleotide probes with a PCR amplified gene product from blood spots (DELFIA®, Wallac, PerkinElmer Life Sciences, Boston, MA). Five *HLA-DQB1* alleles associated with susceptibility to (*HLA-DQB1**0302 and *02) or protection from T1D *(DQB1**0301, *0602 and *0603) were tested.

For genotyping *INS* polymorphism, the genomic DNA was purified by the salt extraction method [9]. The *INS* VNTR was identified by its surrogate marker *Hph*I A/T single nucleotide polymorphism at the locus -23 (rs689) [10]. Class I and III alleles of *INS* VNTR were determined by -23 *Hph*I A and T alleles, respectively. The *Hph*I -23 A/T was genotyped by restriction fragment length polymorphism analysis [3].

Autoantibody measurements

The ICA were detected by a standard indirect immunofluorescence assay on cryosections of the human pancreas from a donor of blood type 0 [11]. The end-point titres of ICA were converted to Juvenile Diabetes Foundation Units (JDFU). The titres equal to or larger than 8 JDFU were considered positive [12].

The GADA and IA-2A were measured by the fluid-phase ^{125}I-antigen binding assay [13] at the University of Greifswald, Germany. The levels of GADA and IA-2A were expressed as arbitrary Karlsburg units (KU/l) derived

from an in-house standard serum pool. The cut-off limit for antibody positivity was defined as the 98th percentile of the laboratory's control group, being 2.14 KU/l for GADA and 0.53 KU/l for IA-2A. In the 4th Diabetes Antibody Standardization Program (DASP) in 2005, the assay for GADA reached a sensitivity of 82% and a specificity of 96% and the assay for IA-2A reached a sensitivity of 66% and a specificity of 100%.

The IAA were also determined at the University of Greifswald, Germany, using the competitive fluid-phase antigen binding assay with A14 mono-^{125}I-insulin (Aventis Pharma Deutschland GmbH, Frankfurt am Main, Germany) with and without the addition of unlabelled insulin [13]. The IAA level of 55.37 μU/l corresponding to the 98th percentile of the laboratory's control group was chosen as the cut-off limit for IAA positivity. In the 4th DASP in 2005, the assay reached a sensitivity and a specificity of 58% and 97%, respectively.

Statistics

The R 2.3.1 A Language and Environment (Free Software Foundation, Boston, MA) was used for linear and logistic regression analyses. The regression coefficients and the odds ratios (OR) are provided. A p value < 0.05 was considered statistically significant.

RESULTS

Profile and age-dependency of genetic markers and autoantibodies

The prevalence of the *HLA-DQB1**0302, *02, *0301, *0602 and *0603 alleles and of the *INS* VNTR genotypes in the patients with T1D and in the healthy controls is presented in Table 1. The prevalence of the *INS* VNTR genotypes both in the patients and in the controls conformed to the Hardy-Weinberg equilibrium. Eight percent of the controls but none of the patients had the *INS* VNTR III/III genotype.

The risk for T1D determined by the *HLA-DQB1* alleles and by the *INS* VNTR I/I genotype was estimated using logistic regression analysis and the respective OR values are shown in Table 1. The association between *INS* VNTR I/I and the disease remained highly significant regardless of adjusting for the *HLA-DQB1**02/0302 genotype, or for the *HLA-DQB1**0302 or *02 alleles (data not shown).

To analyse the associations between genetic susceptibility markers and age at diagnosis of T1D, and between AAb and age at diagnosis of T1D, logistic regression analysis was used. The prevalence of the *HLA-DQB1**02/0302 genotype, the *HLA-DQB1**0302 allele, the *DQB1**02 allele or the *INS* VNTR I/I genotype was not associated with age at diagnosis of T1D in our study group (Table 2). The prevalence of all four studied AAb decreased with age at diagnosis of T1D. Logistic regression analysis confirmed that IAA, IA-2A and

ICA were negatively associated with age at diagnosis of T1D while no significant association was revealed between GADA and age at diagnosis of T1D (Table 2). Linear regression analysis showed a highly significant negative association between the number of AAb and age at diagnosis of T1D (regression coefficient -4.66, $p < 0.001$).

Associations between genetic markers and presence of autoantibodies

We next sought an association between the T1D susceptibility genes and AAb using logistic regression analysis. The GADA, IA-2A and ICA were not associated with the *HLA-DQB1**02/0302 genotype, the *DQB1**0302 or *02 alleles in our study population. However, the presence of IAA showed a tendency to be associated with the high risk *HLA-DQB1**02/0302 genotype (OR = 2.45; $p = 0.08$), or the *DQB1**0302 allele (OR = 2.22; $p = 0.07$).

Logistic regression analysis of association between AAb and the *INS* VNTR genotype revealed a positive association between GADA and the protective *INS* VNTR I/III genotype (crude OR = 4.79; $p = 0.018$). This association remained significant after adjustments for the (1) *HLA-DQB1**02/0302 genotype and age at diagnosis of T1D (adjusted OR = 4.39; $p = 0.028$), (2) *HLA-DQB1**0302 allele and age at diagnosis of T1D (adjusted OR = 4.75; $p = 0.022$), and (3) *HLA-DQB1**02 allele and age at diagnosis of T1D (adjusted OR = 4.40; $p = 0.027$) (Table 3). We were not able to reveal an association between IAA and the *INS* VNTR I/I genotype in our study population regardless of the fact of whether the whole study group (OR = 1.21; $p = 0.69$) or only the children <15 years of age (OR = 1.81; $p = 0.45$) were included. There was no association between IA-2A or ICA and the *INS* VNTR I/I genotype (data not shown).

DISCUSSION

In this study we analysed the prevalence of the major markers of genetic susceptibility to the T1D at IDDM1 and IDDM2 loci and of β-cell AAb in a patient group with newly diagnosed T1D with a broad age range at diagnosis. In addition, the impact of the susceptibility alleles and genotypes at IDDM1 and IDDM2 on formation of AAb was determined. The number of AAb in our patients was consistent with a typical age-dependent profile. The prevalence of IAA, IA-2A and ICA decreased with increasing age at diagnosis [6,14,15], while the prevalence of GADA was not significantly influenced by age [1]. In adults, 21% of the patients were negative for all four commonly measured AAb. This is in agreement with data for newly diagnosed T1D in adults reported by other authors [6,14].

In the healthy controls, the prevalence of the *HLA-DQB1**0302, *02, *0301, *0602 and *0603 alleles was similar to the prevalence found in previous studies of the nondiabetic population conducted in Estonia [16] and elsewhere

[13,17,18]. Similarly, the distribution of *INS* VNTR genotypes in healthy subjects corresponded to the expected frequencies in Caucasoid individuals [4,6]. Logistic regression analysis confirmed that the *HLA-DQB1**02/0302 genotype, the *HLA-DQB1**0302 and *02 alleles, and the *INS* VNTR I/I genotype were all independent risk factors of T1D. Several studies have shown that the high-risk *HLA-DQB1**02/0302 genotype is associated with early onset of the disease [14,17,19]. We failed to confirm this association, which is probably due to the insufficient statistical power of our study (< 20%).

Several previous studies have demonstrated the effect of HLA class II alleles on humoral β-cell autoimmunity in patients with T1D including the associations between the *HLA-DQA1**0501, *DQB1**0201 (DQ2) haplotype and appearance of GADA in newly diagnosed T1D [5,6], and between the *HLA-DQA1**0301, *DQB1**0302 (DQ8) haplotype and IAA [6,7]. Furthermore, there is mounting evidence that insulin-specific autoimmunity is influenced by allelic variations at the *INS* locus. Graham et al reported that the *INS* VNTR I/I risk genotype contributed to the appearance of IAA in patients with T1D [6] and Hermann et al demonstrated an association between *INS* VNTR I/I and IAA in children with an increased risk for T1D [8]. These results are in line with experimental data showing that *INS* VNTR class I alleles may confer disease susceptibility by lower insulin expression in the thymus and decreased induction of central tolerance to this β-cell autoantigen [20].

We failed to show a significant relationship between *INS* VNTR I/I and IAA in our patients. Instead, we found that the *INS* VNTR I/III genotype was associated with the presence of GADA. Indeed, only 10.7% of the patients with the *INS* VNTR I/III genotype were negative for GADA, whereas 36.5% of those having the *INS* VNTR I/I genotype lacked these AAb. It is important to emphasise (on the basis of logistic regression analysis) that the observed phenomenon was not influenced by possible confounding factors such as the presence of *HLA-DQB1**02/0302 heterozygosity, or presence of *HLA-DQB1**0302 or *02 alleles. To our knowledge, this is the first study demonstrating the association between the *INS* VNTR I/III genotype and GADA in patients with newly diagnosed T1D. An observation similar to our finding was made by Walter et al in children with an increased risk for T1D. These authors reported that the offspring of parents with T1D, developing AAb to multiple antigens, had an increased prevalence of high risk genotypes at both the IDDM1 and IDDM2 loci, while the offspring with GADA only displayed increased frequencies of high risk IDDM1 and protective IDDM2 genotypes [21]. The importance of our data regarding the development of the disease cannot be easily explained. However, we suggest that in absence of the *INS* VNTR I/I genotype the effect of other susceptibility factors prevails in T1D, and this may promote the targeting of autoantigens other than insulin. Unlike IAA and IA-2A, GADA is a common AAb in adult-onset T1D [1], and it is a hallmark of autoimmune diabetes with slowly progressive β-cell failure, designated as latent

autoimmune diabetes of adults (LADA) [22]. The data reported by Tuomi et al [23] and those of our own [24] suggest that in patients with LADA, the frequency of the protective *INS* VNTR I/III genotype is similar to that in the general population. Altogether, this supports the idea that in the presence of the *INS* VNTR I/III genotype glutamic acid decarboxylase might be the preferential target of autoimmunity against β-cells. However, it is important to note that considering the relatively small patient numbers in our study, confirmatory studies on the association between *INS* VNTR I/III and GADA in larger patient groups are necessary.

In summary, we have reported a novel association between the *INS* VNTR I/III genotype and GADA in patients with newly diagnosed T1D irrespective of age at diagnosis of the disease. This finding underlines the significance of disease-associated genes possibly modifying the hierarchy of autoantigenic targets in the pathogenesis of T1D.

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Table 1. The prevalence (%) and odds ratios (OR) of *HLA-DQB1* alleles and *INS* VNTR genotypes in patients with type 1 diabetes and healthy controls

	Patients (%) N = 92	Controls (%) N = 251	OR (95% CI)
HLA-DQB1			
***0302**	51.1	15.9	5.51 (3.24–9.36)*
***02**	52.2	33.9	2.10 (1.31–3.46)#
***0301**	17.4	30.7	0.48 (0.26–0.87)#
***0602–03**	8.7	44.2	0.12 (0.06–0.26)*
***INS* VNTR**			
I/I	69.2	45.8	2.66 (1.60–4.44)*

* $p < 0.001$; # $p < 0.05$

The data were analysed using logistic regression analysis. For *HLA-DQB1* alleles the lack of the particular allele was considered the reference genotype and for *INS* VNTR the VNTR I/III and III/III genotypes were considered the reference genotype.

Table 2. The association between genetic susceptibility markers and autoantibodies and the age at onset of T1D

	Median age (range)	OR (95% CI)
HLA-DQB1		
***02/0302**	20 (1–42)	0.98 (0.95–1.02)
***0302**	17 (1–58)	0.99 (0.96–1.02)
***02**	23 (1–62)	1.01 (0.98–1.04)
***INS* VNTR**		
I/I	24 (1–62)	1.03 (0.99–1.06)
Autoantibodies		
IAA	11 (1–41)	0.92 (0.88–0.96)*
IA-2A	16 (1–58)	0.95 (0.92–0.99)#
ICA	17 (2–53)	0.97 (0.94–1.00)#
GADA	17 (1–58)	0.97 (0.94–1.00)

* $p < 0.001$; # $p < 0.05$

The data were analysed using logistic regression analysis. The patient group missing the genotype, allele or autoantibody under study was considered the reference group.

Table 3. Frequency of GADA in patients with *INS* VNTR I/III and *INS* VNTR I/I according to age or *HLA-DQB1* genotype

	***INS* VNTR I/III**	***INS* VNTR I/I**
Age		
< 20	16 (100.0)	18 (66.7)
≥ 20	9 (75.0)	22 (61.1)
All	25 (89.3)	40 (63.5)
HLA-DQB1		
*** 02/0302**	5 (100.0)	10 (62.5)
*** 0302**	6 (100.0)	10 (83.3)
*** 02**	8 (88.9)	9 (69.2)

The data are *n* (%). In the logistic regression analysis the positive association between *INS* VNTR I/III and GADA remained significant after adjustments for the (1) *HLA-DQB1**02/0302 genotype and age at diagnosis of T1D (adjusted OR = 4.39; 95% CI 1.17–16.42), (2) the *HLA-DQB1**0302 allele and age at diagnosis of T1D (adjusted OR = 4.75; 95% CI 1.26–17.99), and (3) the *HLA-DQB1**02 allele and age at diagnosis of T1D (adjusted OR = 4.40; 95% CI 1.18–16.40). The patient group missing the genotype or allele under study was considered the reference group.

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