NON-MHC genes in type 1 diabetes

Family-based association studies and functional studies of disease-associated polymorphisms

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INTRODUCTION

Type 1 diabetes mellitus (T1DM), formerly called insulin-dependent diabetes mellitus (IDDM), is a serious chronic disorder characterised by absolute insulin deficiency caused by an immune-mediated selective destruction of the insulin-producing β-cells in the islets of Langerhans. The immune-mediated β-cell destruction is thought to be initiated by interaction between yet unknown environmental factors and T1DM susceptibility gene variants (Atkinson et al 2001).

The *incidence of T1DM* varies in ethnic groups and countries, and the incidence rates increase in particular in very young children (Onkamo et al 1999, Karvonen et al 2000, Green 2001, Svensson et al 2002). In Denmark, the incidence rate for the 0-14 years age group is 19.5/100,000 person-years and annually ~200 children under the age of 15 years develop T1DM (Svensson et al 2002). The prevalence of T1DM is reported to be 0.4% in Denmark based on paediatric materials (Pociot 1996), likely to be an underestimate as 44% of all T1DM cases have onset of T1DM above the age of 30 years, and the lifetime risk of T1DM may be as high as 1.5% in the Danish population (Mølbak et al 1994).

Insulin replacement therapy dramatically increases life expectancy, but T1DM is still associated with increased relative mortality and morbidity due to micro- and macro-vascular complications including kidney failure, blindness and cardiovascular diseases (Borch-Johnsen 1989) – complications with severe socio-economic consequences (Ng et al 2001). Stringent control of blood glucose levels reduces the risk of developing complications (The Diabetes Control and Complications Trial Research Group 1993). Currently, the only causal treatment of the disease is replacement of insulin producing β-cells by pancreas or islet transplantation. These treatment modalities are limited by the availability of donor organs and high risk of morbidity related to the use of potent immunosuppressants (Robertson et al 2000, Atkinson et al 2001, Robertson et al 2003).

Identification of ubiquitous *environmental factors* that trigger the initial process leading to β-cell destruction and subsequent T1DM has been unsuccessful, and causality between identified environmental factors or modifiers and T1DM remains to be demonstrated (Akerblom et al 2002).

T1DM has a *genetic basis* and more than 30 different chromosomal loci have been linked to T1DM susceptibility. The major genetic contributor(s) of susceptibility to T1DM map to the major histocompatibility complex (MHC) on chromosome 6p21.3 which has been denoted *IDDM1* (ECIGS 2001, Cox et al 2001a, Field 2002, Pociot et al 2002). *IDDM1* harbours several HLA and non-HLA genes that may confer susceptibility to T1DM, and accounts for ≥ 40% of the familial clustering of the disease (Risch 1987, Noble et al 1996). Genes outside the MHC locus also confer susceptibility to T1DM, but apart from the preproinsulin gene (*INS*) at *IDDM2* and the CTLA-4 gene (*CTLA4*) at *IDDM12*, these remain to be identified and characterised (Pociot et al 2002, Ueda et al 2003).

Immune-mediated mechanisms are involved in the *pathogenesis of T1DM*. Both cell-mediated (Roep 2003) and humoral autoimmunity (LaGasse et al 2002) have been detected in T1DM patients, although humoral autoimmunity is not a prerequisite for development of immune-mediated T1DM development in man (Martin et al 2001a) and islet cell autoantibodies (ICAs) do not appear to be the immune effector molecules (Mandrup-Poulsen 1996). The histopathological lesion in recent onset T1DM patients resembles a delayed type 4 hypersensitivity reaction with mononuclear cell infiltration in the islets and selective β-cell destruction (Gepts 1965, Foulis et al 1991, Itoh et al 1993). Moreover, T-cell immuno-suppression preserves β-cell function compared to placebo (Feutren et al 1986, The Canadian-European Randomized Control Trial Group 1988). These observations underscore the important role of cell-mediated autoimmunity.

It is possible to predict risk of T1DM development in first degree relatives (Bingley et al 1994, Verge et al 1996) and in the general population (LaGasse et al 2002) by measurement of ICAs. Unfortunately, no preventive treatments are known in man and as two recent prevention studies failed to demonstrate effects on T1DM development (Diabetes Prevention Trial – Type 1 Diabetes Study Group 2002, ENDIT Group 2004), the prospects of one or combined prevention modalities seem distant. One lesson learned from these studies is that the current knowledge of the pathogenic mechanisms leading to β-cell destruction in man is still incomplete. Thus, before launching new prevention studies more insight in the pathogenic mechanisms – including the genetic factors – causing β-cell destruction is in demand (Schatz et al 2003).

The purpose of this thesis is to review studies of candidate genes selected through a pathogenetic model presented in the following chapter, with the perspective of using a genetic approach to elucidating pathogenetic mechanisms and thereby identifying putative therapeutic targets.

2. AN INFLAMMATORY MODEL FOR THE PATHOGENESIS OF T1DM

Immune mediated mechanisms play a major role in the pathogenesis of T1DM in man (Atkinson et al 2001). However, detailed knowledge of both the relative and specific roles of different immunological cell subsets, of the HLA class I and II molecules, of islet autoantibodies, β-cells antigen specificities, cytokines, adhesion molecules, co-stimulatory molecules, of T-cell receptors (TCR) and other T-cell surface receptors, of apoptosis in immune competent cells and β-cells, of protective or deleterious mechanisms specific for the β-cell and of transcription factors in the initiating and perpetuating processes leading to β-cell destruction and subsequent T1DM in humans is scarce, and most of our current insight has been obtained from animal models and *in vitro* experiments.

The investigation of T1DM pathogenesis in man is hampered by; 1) the technical and *ethical* inaccessibility of the target β-cell for studies in time and space during disease development, and 2) the fact, that the patient phenotypically presents at a very late stage of the pathogenetic process (Atkinson et al 2001). Thus, observation of the initiating and the perpetuating processes in man is difficult as it has generally only been possible to study the end-stage islet lesion. For these reasons, most hypotheses on T1DM pathogenesis in man are based on: 1) the observation of β-cell destruction and insulitis in autopsies and biopsies from patients with recent onset T1DM (Gepts 1965, Foulis et al 1991, Itoh et al 1993), and evidence of

improved β-cell function in recent onset T1DM patients treated with T-cell immuno-suppression (Feutren et al 1986, The Canadian-European Randomized Control Trial Group 1988) and 2) experiments in a) spontaneous animal models of T1DM – particularly the Non-Obese Diabetic (NOD) mouse model (Leiter et al 1987) and the Diabetes-Prone Bio-Breeding Worchester (DP-BB) rat (Mordes et al 1996), b) in animal models of induced diabetes, c) in islet transplantation models, d) in transgenic and knockout animal models and e) *in vitro* experiments on cultured islets, β-cells, T-cells and macrophages. These studies will not be reviewed here as they have been reviewed extensively elsewhere (Helqvist 1994, Mandrup-Poulsen 1996, Pociot 1996, Christensen 1996, Bach 1996, Reimers 1998, Andersen 1999, Eizirik et al 2001a, Rabinovitch et al 2003, Sparre 2004).

In 1987, Nerup and co-workers suggested a hypothesis for the pathogenetic (i.e. the earliest events and mechanisms affecting the target organ) events leading to T1DM (Nerup et al 1987). It has been revised (Nerup et al 1994) based on accumulating scientific evidence, and is shown in a contemporary form (Bergholdt et al 2003) in **Figure 1**.

In the pathogenetic T1DM model it is hypothesised that the efficacy of antigen processing and presentation, the efficacy of intercellular co-stimulation, the regulation of the T cell response, the level of cytokine production and the balance between them as well as the β-cell response to cytokine action – regulated at the molecular level – are all determinants of the magnitude of β-cell destruction. It also hypothesises that genetic susceptibility to T1DM at the individual level is not conferred by one single gene variant, but rather by the unfortunate combination of a number of *frequently* occurring normal gene variants with quantitative functional impacts that in concert increase the magnitude of β-cell destruction. A specific combination of genetic susceptibility genes is unlikely to be shared by all individuals, and differences in the genetic T1DM susceptibility profiles are therefore likely to exist within and between populations.

This model has served as the pathophysiological framework within which the candidate genes presented in the following chapters have been selected.

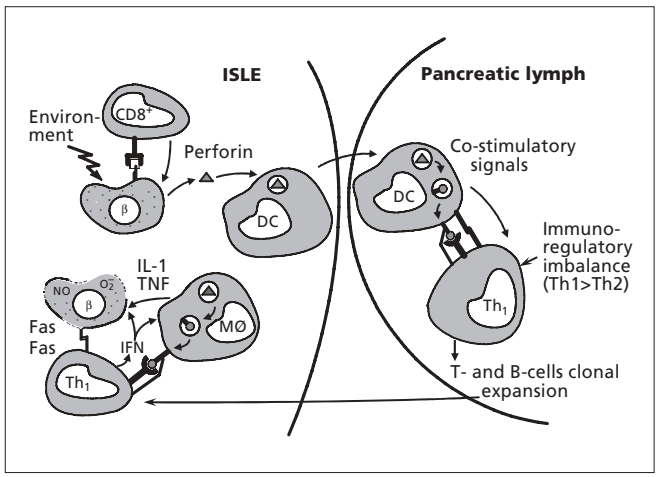

Figure 1. An inflammatory model for the pathogenesis of T1DM. The model suggests that environmental factors, most likely in the form of common viruses, induce a MHC Class I restricted presentation of β-cell antigen. This antigen is recognized by $CD8^+$ T-cells that cause a limited MHC Class I restricted β-cell damage, either via cytotoxic cytokines as secreted IFNγ and/or secreted TNFα/membrane-bound TNFα or the perforin/granzyme system. Liberated β-cell components, such as insulin or GAD possibly in glycosylated immature forms not previously "seen" by the immune system are taken up by dendritic cells in the islets and transported to regional pancreatic lymph nodes, where the antigens are processed and presented to autoreactive $CD4^+$ T-cells that have escaped negative selection and normal immunoregulation. After clonal expansion the $CD4^+$ T-cells will home to the islets, tracing antigen gradients, chemokines and endothelial adhesion molecules induced by the early $CD8^+$ T-cell mediated inflammatory response. The activated $CD4^+$ T-cells will recruit and activate specific as well as non-specific inflammatory cells that then build up the inflammatory insulitis infiltrate. The effector phase of the β-cell destruction is mediated by cytokines via induction of proapoptotic signaling selectively in β-cells and/or by inducing β-cell expression of Fas, marking the β-cells for MHC Class II non-restricted $CD4^+$ T-cell mediated killing via interaction between the Fas ligand on $CD4^+$ T-cells and Fas on the β-cells.

3. RATIONALE, HYPOTHESIS AND AIMS

The genetics of T1DM cannot exclusively be explained by the genetic susceptibility conferred by the genes harboured in the MHC (*IDDM1*) locus. This was evident prior to the initiation of the experimental work underlying this thesis and based on evidence 1) from the comparison of crude probandwise concordance rates of T1DM in monozygotic twins of 23-54% (Barnett et al 1981, Japan Diabetes Soc 1988, Kaprio et al 1992, Kumar et al 1993, Kyvik et al 1995) with the concordance rates in HLA-identical siblings of ~15% (Thomson et al 1988), 2) from estimates of the role HLA in T1DM implying that the HLA contribution to the total familial clustering were 44% (Risch 1987) and 3) from the two initial genome-wide T1DM scans (Hashimoto et al 1994, Davies et al 1994). Hence, we decided to undertake investigation of selected candidate genes outside the MHC locus using the robust Transmission Disequilibrium Test (TDT) (Spielman et al 1993) in order to identify contributing genetic variants or evidence for such variants in linkage disequilibrium (LD) with the investigated polymorphisms.

The *rationale* for identifying the genes conferring susceptibility to T1DM is that through an understanding of the genetic variance underlying T1DM, insight in the genetically regulated mechanisms leading to β-cell destruction may facilitate strategies for prevention of the disease.

The *hypothesis* of the experimental work behind this thesis was that by selecting candidate genes outside *IDDM1* and genetic variants in these genes and investigate for linkage and association between these candidate gene variants and T1DM by use of TDT analysis in a population based, ethnically homogeneous cohort of Danish T1DM families, it would be possible to identify gene variations conferring risk to T1DM. Subsequent functional studies of associated variants were designed to enable assessment of the putative impact of the genetic variant in the T1DM pathogenesis.

Thus, the aims of the experimental work behind this thesis have been:

1. To investigate for linkage in the presence of linkage disequilibrium and/or association between T1DM and genetic polymorphisms in genes encoding proteins with putative impact on the process leading to T1DM and identified as possible candidate genes for T1DM development based on the above model, Figure 1.
2. To investigate for functional significance of genetic variants displaying linkage and association to T1DM.

4. CANDIDATE GENE SELECTION AND DELIMITATIONS IN THIS THESIS

Since the first observations of association between HLA and (Type 1) diabetes mellitus (Singal et al 1973, Nerup et al 1974), the main focus within T1DM genetics has been on the genes encompassing the MHC locus, and in particular HLA gene variants. This thesis deals with genetic variants in T1DM outside the MHC locus. In consequence, the role of MHC genes in T1DM will only be briefly reviewed in section 7.4.

The criteria for the selection of *candidate genes* were primarily that the gene encoded protein was potentially involved in the processes leading to β-cell destruction either by playing a role in the immune system or in the β-cell destruction/defence, and that altered function, expression and/or production of the gene product would theoretically either promote or inhibit the outcome of the process(es) outlined in the pathophysiological model, Figure 1.

Genes previously demonstrating association to T1DM or other autoimmune diseases and positional candidate genes, i.e. genes

mapping to loci that have shown some linkage (MLS ≥1.2) to T1DM in man, in the rodent T1DM disease models, or in both, were considered candidate genes of particular interest. Candidate genes mapping outside identified T1DM loci were also considered, as the initial genome-wide T1DM scans were under-powered to identify minor T1DM loci (Lander et al 1995). The individual genes focused on in the experimental work underlying this thesis are outlined in **Table 1**.

In addition to the genes detailed in Table 1, we further evaluated genetic markers in three previously identified T1DM loci for linkage and association to T1DM in Danes (Larsen et al 1999, Kristiansen et al 2000a).

A review of T1DM loci identified through whole and partial genome scans in man is detailed in section 7.3, whereas a general review of identified T1DM loci in the T1DM animal models was outside the scope of this thesis since animal models were not studied to any significant degree.

The criteria used for *genetic variant* selection in chosen candidate genes were *generally* as detailed in **Table 2**.

By nature, mutation screening cannot predict the frequency, mapping and putative functional implication of an identified genetic variant, and such variants may not fulfil any of the criteria in Table 2. However, using these criteria, the experimental work behind this thesis has investigated genetic variants fulfilling the criteria for selection of candidate gene variants proposed for association studies in complex diseases (Risch 2000, Tabor et al 2002).

Selection of variants with common alleles was based on the assumption that the genetic variants conferring risk to T1DM are common in the population as suggested in the disease model (Figure 1). "The common allele – common complex disease theory" is controversial (Lander 1996, Terwilliger et al 1998, Weiss et al 2000, Risch 2000, Wright et al 2001, Lohmueller et al 2003). Recent progress within the genetics of complex diseases has shown that minor susceptibility alleles may be both rare (Hugot et al 2001, Ogura et al 2001) and common (Bennett et al 1995, Horikawa et al 2000, Lohmueller et al 2003, Ueda et al 2003). Hence, selection of common genetic variants exclusively for evaluation of disease association carries a risk of missing detection of rare variants conferring risk or protection to the investigated disease. The importance of power to detect distorted transmission in the Danish cohort was another reason for selecting common genetic variants (Risch et al 1996).

The intention of the experimental work was not to identify genetic variants for prediction of T1DM risk and genetic counselling, but rather to identify genetic polymorphisms involved in the pathogenesis of T1DM in order to contribute to an understanding of the process leading to β-cell destruction.

It has not been the aim of the experimental work to demonstrate a direct causality between identified T1DM linked/associated gene variants and the pathogenetic events leading to β-cell destruction in man. Rather, the assumption was that linked and associated genetic variants demonstrating a functional impact putatively explaining or pointing to mechanisms promoting to β-cell destruction in the above model (Figure 1) were likely genetic T1DM culprit variants. Genetic variations not demonstrating linkage/association were not tested further for functional impact. It should be clearly stated that in case of lack of association between a gene variant and T1DM, the gene encoded product may well play a role in T1DM pathogenesis, and furthermore other non-investigated (or non-identified) gene variants in that gene may indeed confer susceptibility or protection to T1DM development.

5. METHODOLOGICAL CONSIDERATIONS

The technology and methodology of the experimental work are not described here as the principles of these are well-established and detailed in the papers underlying this thesis.

Table 1. Selected candidate genes.

Gene	Association to autoimmunity or T1DM	Positional candidate	Putative functional role
GALNT3[1]	No	Yes	O-glycosylation of β-cell antigens, changed antigenicity
IL1B[2]	Yes	No	β-cell destruction and T-cell stimulation
IL1RN[2]	Yes	No	β-cell protection. Antagonises IL-1β action on β- and T-cells
IL1RT1[2]	Yes	Yes	β-cell destruction. Receptor for IL-1β
CD4[3,4]	Yes	Yes	T-cell regulation. Enhance T-cell response
ICAM1[5]	Yes	Yes	T-cell regulation and adhesion, β-cell destruction
CTLA4[6]	Yes	Yes	T-cell regulation, peripheral and central tolerance
FAS[7]	Yes	No	T-cell regulation, peripheral tolerance. T-cell and β-cell apoptosis
FasL[8]	Yes	No	T-cell regulation, peripheral tolerance. T-cell and β-cell apoptosis
IL6[9]	Yes	No	T-cell regulation and/or β-cell destruction/defence
NQO1[10]	No	Yes	β-cell defence. Protects against redox cycling and oxidative stress

1) Kristiansen et al 2000a; 2) Kristiansen et al 2000b; 3) Kristiansen et al 1998a; 4) Kristiansen et al 2004; 5) Kristiansen et al 2000c; 6) Larsen et al 1999; 7) Nolsøe et al 2000, 8) Nolsøe et al 2002, 9) Kristiansen et al 2003, 10) Kristiansen et al 1999.

Table 2. Criteria used for variant selection in chosen candidate genes.

Gene variant	Investigated gene variants fulfilling criteria
Suggested to have "functional" implication	*IL1B+3953C>T, IL1RN (VNTR), IL6-174G>C, NQO1* (P187S)
Maps to transcription site	*FAS-670G>A*
Causes amino acid substitution	*ICAM1* (K469E), *CTLA4+49A>G* (*Thr→Ala*), *NQO1* (P187S)
Genetic association with T1DM in other cohorts	*IL1B+3953C>T, CD4-1188 (TTTTC)$_{5-14}$, ICAM1* (K469E), *IL6-174G>C, NQO1* (P187S)
Genetic association with other autoimmune disease	*IL1B+3953C>T, IL1RN (VNTR), ICAM1* (K469E), *CTLA4+49A>G, IL6-174G>C*
Maps to promoter (5'UTR)	*IL1B-511C>T, IL1RI (Pst I), CD4-1188 (TTTTC)$_{5-14}$, CD4-1050T>C, CD4-521C>G, CD4-181C>G, CTLA4+49A>G, FAS-690T>C, FAS-670G>A, FASL-843C>T, IL6-174G>C*
Maps to exon	*IL1B+3953C>T, ICAM1* (K469E), *CTLA4+49A>G, FAS+154C>T, NQO1* (P187S)
Maps to splice site	–
Maps to 3'UTR	*GALNT3+2183T>A*
Allele not rare (>5% in the population)	All
High heterozygousity (preferably >40%)	*GALNT3+2183T>A, IL1B-511C>T, IL1B+3953C>T, IL1RN (VNTR), CD4-1188 (TTTTC)$_{5-14}$, CD4-1050T>C, CD4-181C>G, ICAM1* (K469E), *CTLA4+49A>G, FAS-670G>A, FASL-843C>T, IL6-174G>C*

5.1. MUTATION SCREENING

For mutation screening the *single strand conformational polymorphism* (SSCP) method was used. Albeit sensitive, the SSCP method may not have identified all existing sequence variations in the sequences investigated (Nolsøe et al 2000, Nolsøe et al 2002, Kristiansen et al 2004). In-house validation of the SSCP-heteroduplex analysis demonstrated a sensitivity of ~90-95% by evaluation of its ability to detect known SNPs (Nolsøe et al 2000, Johannesen et al 2001). Furthermore, the screening panel comprised only subjects of Danish ancestry and the number of screened individuals ranged from 30-40 of which 20 had T1DM (Nolsøe et al 2000, Nolsøe et al 2002, Kristiansen et al 2004). Using a screening cohort of ≥ 30 individuals will allow the detection of variants with alleles as rare as 5% with a probability of more than 95% when using direct sequencing for mutation screening (Kruglyak et al 2001).

5.2. MATERIAL

The linkage and association analyses were performed by means of TDT-based analyses as described in section 6.3 in a Danish T1DM family cohort identified through two population-based epidemiological studies (Pociot et al 1993, Lorenzen et al 1998).

The sample sizes of the materials genotyped in the individual studies show minor differences between studies (**Table 3**) explained by 1) availability of individual gDNA at the time of genotyping 2) ongoing collection of families and individuals, and 3) later exclusion of four families diagnosed with Type 3 maturity onset of diabetes in the young (MODY-3) (Møller et al 1998).

Replication of positive findings of linkage/association in the Danish cohort was not pursued in other T1DM cohorts for reasons detailed in the relevant sections.

5.3. REPORTER ASSAY

The reporter assay approach for evaluation of functional implications of T1DM linked and associated variants in cultured cell lines (Kristiansen et al 2003, Kristiansen et al 2004) was chosen as this approach allows evaluation of the effect of the individual promoter gene variant(s) on expression. The use of reporter assay in cell lines compared to the use of mRNA and protein expression *in vitro* of cells harvested from peripheral blood in genotyped individuals limits putative confounders such as 1) the putative presence and functional impact of other genetic variants not identified in and around the promoter variant in question, 2) influence of inter-individual variation in other genes, 3) differences in the ratio between *in vivo* stimulated and unstimulated cells harvested from different individuals and 4) day-to-day intra-individual heterogeneity (e.g. undetected sub-clinical viral infections) in harvested cell populations. The cell line approach, however, has its own limitations, three of the most important being 1) genetic regulatory mechanisms may differ from that of naïve cells, 2) the selected cell line is not likely to fully resemble the relevant naïve cell type and 3) the regulation of the promoter activity in question may vary in different relevant naïve cells types in which the gene encoded protein is expressed and of functional importance.

Systematic confounding factors in reporter assay systems mainly include differences in inter- and intra-assay transfection efficiency, inter-assay differences in reporter signal, impurities in the plasmid DNA (pDNA) preparations and pDNA-strand breaks affecting the reporter signal of the individual variant, and finally inadequate pDNA quantitation (Protocols and Applications Guide 1996, Kingston 1997, Manufacturer's Instruction 1999a, Manufacturer's Instruction 1999b). These putative caveats were controlled to the extent possible (Kristiansen et al 2003, Kristiansen et al 2004).

Table 3. Material in individual studies.

Gene(s) investigated	Sibpair families (N)	Trio families (N)	Total T1DM and non-T1DM offspring (N)
GALNT3[1]	138	103	392/233
IL1B, IL1RN, IL1RT1[2]	140	105	398/233
CD4[3]	115	105	343/203
CD4[4]	150	103	416/250
ICAM1[5]	150	103	414/250
CTLA4[6]	151	103	413/244
FAS[7]	138	103	392/233
FasL[8]	154	103	420/252
IL6[9]	150	103	416/250
NQO1[10]	144	103	405/249

1) Kristiansen et al 2000a; 2) Kristiansen et al 2000b; 3) Kristiansen et al 1998a; 4) Kristiansen et al 2004; 5) Kristiansen et al 2000c; 6) Larsen et al 1999; 7) Nolsøe et al 2000; 8) Nolsøe et al 2002; 9) Kristiansen et al 2003; 10) Kristiansen et al 1999.

6. METHODS USED TO IDENTIFY THE GENES PREDISPOSING TO T1DM

This chapter briefly outlines the methods that have been used to dissect the genetics of T1DM, and describes the methods used in the scientific work behind this thesis. The methods anticipated to be used in the future dissection of the genetic susceptibility underlying T1DM are discussed in section 16.3.

6.1. METHODS USED FOR DETECTION OF T1DM GENES

The dissection of complex genetic traits such as T1DM in which several genetic variants modify risk of disease has been considered a genetically "Gordian knot" (Ott 1990). Knowledge of penetrance, disease allele frequencies, number of disease modifying gene variants and their interaction, within and between population genetic heterogeneity, etiological heterogeneity, gene-environment interaction(s), disease phenocopies and mode of inheritance is lacking in complex diseases (Terwilliger et al 2000).

The genetic approaches used to dissect the genetics underlying T1DM fall into four major categories:

1. Candidate gene based association studies using:
   a. cohorts of matched cases and controls
   b. family-based association studies of genes and loci
2. Random marker genome-wide and partial genome scans using ASP/allele sharing analyses
3. Classical linkage studies in extended family pedigrees
4. Genetic analysis of experimental crosses in T1DM rodent models.

The theoretical backgrounds for these methods have been described in detail elsewhere (Spielman et al 1993, Spielman et al 1996, Strachan and Read 1999, Terwilliger et al 2000) and will not be further discussed here. However, the TDT is discussed in section 6.3. Topics such as power, limitations and putative caveats of the individual methods are discussed where relevant in the sections and chapters to follow.

6.2. METHODOLOGY USED IN THE INVESTIGATIONS UNDERLYING THIS THESIS

The experimental work of this thesis used a model-based candidate gene approach. Initially to investigate for linkage/association by use of TDT methods between T1DM and selected known and identified genetic variants in non-MHC candidate genes chosen on the basis of a proposed model of the pathogenesis leading to T1DM (Figure 1) in a Danish cohort of T1DM families. Genetic variants demonstrating evidence for linkage/association to T1DM were subsequently investigated in reporter assays studies.

The obvious disadvantage of applying a model-based candidate gene approach is that in lack of complete and solid evidence for the disease pathogenesis the selected candidate gene may be irrelevant, and hence likely to fail in demonstrating linkage/association to the disease. Selecting positional candidate genes and/or genes associated with the disease in question in other populations or other related

disease entities may increase the likelihood of disease association. Replication of genetic disease association (and linkage) in other populations is increasingly requested (Editorial 1999, Hirschhorn et al 2002, Lohmueller et al 2003) and is important to establish additional evidence of the correctness of the initial finding(s) (Hirschhorn et al 2002, Lohmueller et al 2003). Particularly, family-based association analyses are in demand to validate or reject observations in case-control studies which may be flawed by population admixture, selection biases or "spurious" association due to other reasons (Ewens et al 1995, Altshuler et al 1998, Editorial 1999, Cardon et al 2001, Hirschhorn et al 2002, Lohmueller et al 2003).

Using this approach, observation of no association indicates that the genetic variant and gene variants in strong LD with the investigated variant are unlikely to have an impact on disease susceptibility, or that the risk conferred is too small for detection in the investigated population. However, it does not exclude the gene encoded product from being involved in the disease pathogenesis, and neither does it exclude other genetic variants in the same or nearby mapping genes, not in LD with the investigated gene variant, from playing a role in the disease pathogenesis.

The Danish family cohort has been used in other candidate gene investigations in addition to the investigations behind this thesis, and these studies will be mentioned in subsequent sections where relevant.

6.3. THE TRANSMISSION DISEQUILIBRIUM TEST

The genetic analyses (Spielman et al 1993, Sham et al 1995, Martin et al 1997a, Spielman et al 1998) used in the experimental work behind this thesis are all based on the principles of the initially suggested Transmission Disequilibrium Test, TDT (Spielman et al 1993). These analyses test for linkage in the presence of association in family cohorts with multiple affected offspring and test for association in families with only one affected offspring included (Spielman et al 1996, Spielman et al 1998). One of the TDT analyses used, the T_{sp} variant (Martin et al 1997a), utilises data from multiple affected members in each family when testing for association.

The TDT tests are intra-familial tests evaluating transmission from heterozygous parents, and the untransmitted alleles from these heterozygous parents are used as control alleles (Spielman et al 1993). The strength of the TDT tests compared to the classical case-control studies is that "spurious" association due to selection bias or population admixture is avoided (Ewens et al 1995). Statistical modelling demonstrates family-based association studies to possess the power needed for detection of minor genes with genetic sibling risk ratios (λ_s) as low as 1.05 in complex diseases (Risch et al 1996, Risch 2000, Wang et al 2003). However, the assumptions used for these calculations have been questioned (Terwilliger et al 1998, Weiss et al 2000, Pritchard et al 2001).

Before searching for the genetic variants underlying a disease it should be established that genetic factors indeed do play a role for the unfolding of the disease – the topic of the following chapter.

7. T1DM – A POLYGENETIC COMPLEX DISEASE

The aim of this chapter is to review the epidemiological, twin study based and genetic evidence for genetic factors, in particular non-*IDDM1* genetic factors, in the aetiology of T1DM in man – not to establish the mode of inheritance or a genetic model for T1DM. *IDDM1* and *IDDM2* are established T1DM loci harbouring genes affecting risk of T1DM. Hence, the genetic and functional implications for T1DM susceptibility of the genes mapping to these two loci are briefly reviewed in sections 7.4. and 7.5., respectively.

7.1. EPIDEMIOLOGY OF FAMILIAL T1DM RISK

Epidemiological studies of recurrence risk in families may identify patterns indicating genetic susceptibility of a disease. Two main well-established epidemiological observations indicate genetic susceptibility in T1DM.

First, *offspring* of T1DM parents has higher risk of T1DM than the general population, 1.3-10.5% vs. ~0.4% (Wagener et al 1982, Chern et al 1982, Warram et al 1984, Bleich et al 1993, Lorenzen et al 1994, Tuomilehto et al 1995, el Hashimy et al 1995, Lorenzen et al 1998). Remarkably, higher recurrence risk in offspring of T1DM fathers than T1DM mothers is consistently observed, **Table 4**.

Cross-sectional population-based studies in Europe and Denmark support this pattern since T1DM offspring has increased prevalence of T1DM fathers compared to mothers; 3.4-6.1% vs. 1.8-2.1%, respectively (Pociot et al 1993, Green et al 1998).

The background of this difference between mothers and fathers is currently unknown. The proposed but yet undocumented explanation of this phenomenon is miscarriage of T1DM-prone fetuses in T1DM mothers, maternally induced protection against T1DM in T1DM-prone fetuses of T1DM mothers or genetic imprinting (Warram et al 1984, Lorenzen et al 1998, Gale et al 2001a).

Second, *siblings* of T1DM probands have higher risk (~6% at age 30) of T1DM than the general population (Spielman et al 1980, Wagener et al 1982, Thomson et al 1988, Spielman et al 1989, Pociot et al 1993, Lorenzen et al 1994, Gillespie et al 2002). Estimates vary between studies due to the design and time of observation. Due to uncertainties with regard to the study designs some early investigations (for review see Wagener et al 1982) are not cited here.

The age at onset in the T1DM proband sibling affects risk in siblings; younger age at onset increases risk in siblings (Chern et al 1982, Allen et al 1991, Gillespie et al 2002), although some controversy exists, properly due to differences in study designs (Wagener et al 1982, Lorenzen et al 1994, Green et al 2000). The increased sibling recurrence risk is – at least in part – conferred by increased prevalence of T1DM high risk HLA haplotypes (*DRB1*03-DQA1*0501-DQB1*02* and *DRB1*04-DQA1*0301-DQB1*0302*) in families with early onset T1DM siblings (Gillespie et al 2002).

T1DM recurrence risk in *HLA identical siblings* is 13-19% and 19% for DR3/DR4 siblings (Thomson et al 1988). Compared to the overall recurrence risk of ~6% in siblings, it is clear that variants in the HLA class II are important for T1DM susceptibility, but cannot explain the entire genetic risk load (Spielman et al 1980, Risch 1987). Importantly, approximately 7% of all T1DM cases are unable to encode a DQαβ susceptibility heterodimer, and 1-6% of non-T1DM control subjects are DR3/DR4 heterozygous (reviewed in Pociot 1996).

Recurrence risks in *monozygotic, first-, secondary- and tertiary-degree relatives* show that a single locus model of inheritance fails to account for the non-linear nature of familial risk of T1DM, which is also indicative of additional non-*IDDM1* gene susceptibility in T1DM (Rich 1990).

The facts that ~85% of newly diagnosed T1DM patients have no first-degree relatives with T1DM and less than 25% of all offspring in families with a T1DM sibling develop T1DM even in studies with long time of observation speak against simple Mendelian inheritance (Dahlquist et al 1989, Tuomilehto et al 1992, Pociot et al 1993, Lorenzen et al 1994).

To summarise, genetic susceptibility facilitates T1DM development and HLA genes play a major role, but notably not all T1DM patients carry a HLA risk haplotype. Hence, HLA risk is not sufficient, nor is it necessary for developing T1DM. The remaining component of susceptibility is conferred by other genetic and en-

Table 4. Recurrence risk (%) in offspring of T1DM parents.

Father T1DM	Mother T1DM	Risk up to age (y)	References
6.1	1.3	20	Warram et al 1984
8.9	3.4	20	Bleich et al 1993
5.1	2.1	20	el Hashimy et al 1995
7.6	3.5	20	Tuomilehto et al 1995
5.7	2.3	30	Lorenzen et al 1998

vironmental factors. Since the vast majority of T1DM patients are "sporadic" and T1DM is a common disease, the genetic variants conferring susceptibility are likely to be frequent in the population. The mode of inheritance and the degree of penetrance in T1DM susceptible individuals remain to be elucidated.

7.2. T1DM IN TWINS

A classical way of evaluating disease heritability is by studying concordance in monozygotic (MZ) and dizygotic (DZ) twins (Smith 1974, Martin et al 1997b).

Crude probandwise cross-sectional T1DM concordance rates are 23-54% in MZ and 0-14% in DZ twins and the differences in concordance rates between MZ and DZ twins have been confirmed by actuarial analyses, **Table 5**.

The concordance rate in MZ twins exceeds that of DZ twins in all studies investigating both MZ and DZ twins (Table 5). Moreover, heritability estimates, i.e. the proportion of overall, phenotypic variance attributable to genetic factors, of 0.72-0.88 for T1DM were found in one Danish and two Finnish studies, underscoring a considerable impact of genetic factors in the risk of T1DM development (Kaprio et al 1992, Kyvik et al 1995, Hyttinen et al 2003). Studies in discordant siblings, in MZ and in DZ twins also suggest that genetic factors are important for β-cell autoimmunity (Redondo et al 1999, Gale et al 2001b).

The concordance rate of ~50-58% in DR3/DR4 and DQ8/DQ2 heterozygous MZ twins is higher than the concordance rate in MZ twins with lower risk HLA haplotypes, again emphasising the genetic impact of the HLA class II gene variants (Johnston et al 1983, Redondo et al 1999, Redondo et al 2001).

Comparison of the lifetime cumulative T1DM risk of ~50% in MZ twins (Table 5) and ~15% concordance in HLA-identical siblings (Thomson et al 1988) strongly suggests the presence of non-*IDDM1* genetic susceptibility in T1DM. Although differences in both the nature and the timing (prenatal and postnatal) of environmental exposure between HLA-identical siblings and MZ twins may exist – these differences are unlikely to explain the entire difference in concordance rates.

The importance of non-genetic factors is underscored by the lack of 100% concordance in MZ twins. Incomplete penetrance, the extent of which is yet uncovered in T1DM, may also contribute to the lack of complete genotype-phenotype correlation in MZ twins. It has been argued that difference in the degree of shared environmental factors is the cause of differences in concordance rates of T1DM in DZ twins and non-twin sibling (Kyvik et al 1995). This is challenged by two observations; DZ twins and non-twin siblings of T1DM probands do not significantly differ in 1) progression to diabetes, although argued differently by the authors of the Danish twin study* (Lorenzen et al 1994, Kyvik et al 1995*, Redondo et al 1999), and 2) expression of islet autoantibodies (Redondo et al 1999).

In summary, twin studies indicate a major genetic basis of T1DM and that genetic factors in addition to HLA variance exist. The T1DM phenotype is not generally a simple genetic disease with 100% penetrance. Susceptibility to T1DM and development of autoimmunity towards islet antigens are mainly genetically determined, but environmental factors modify the risk of developing T1DM. Twin studies have not identified genetic variants or loci conferring risk to T1DM. The search for T1DM susceptibility loci by genome-wide and partial scans in T1DM families is detailed below.

7.3. GENOME-WIDE AND PARTIAL SCANS IN T1DM

A large number of T1DM genome-wide and partial genome scans utilising ASP families has been performed. Six genome-wide scans have been performed in Caucasian T1DM families from France (Hashimoto et al 1994), UK (Warren Repository see Bain et al 1990), the US (HBDI families see Lernmark et al 1990) and Scandinavia* (Hashimoto et al 1994, Davies et al 1994, Mein et al 1998, Concannon et al 1998, ECIGS 2001*, Cox et al 2001a) and two studies have undertaken genome-wide linkage analysis in large pedigrees with high prevalence of presumed monogenic or oligogenic T1DM (Verge et al 1998, Vaessen et al 2002).

Table 5. T1DM concordance rates in twins.

Population	MZ twins	DZ twins	References
Crude probandwise cross-sectional concordance rates (%)			
UK[a]	54	NA	Barnett et al 1981
J[a]	45	0	Japan Diabetes Soc 1988
FIN[b,c]	23	13	Kaprio et al 1992
FIN[b,c]	43	7	Hyttinen et al 2003
US[a]	29	14	Kumar et al 1993
DK[b]	53	11	Kyvik et al 1995
Estimated cumulative probandwise risk (%)			
US[a,d]	32[e], 44[f]	16[e], 19[f]	Kumar et al 1993
UK/US[a,d]	50[g]	NA	Redondo et al 2001
DK[b]	70[h]	11[h]	Kyvik et al 1995

NA: not available. a: Not population based; b: population based; c: Finnish cohort without overlap; d: US MZ cohorts without overlap; e: life-time risk and proband age at onset 0-30 years; f: life-time risk and proband age at onset 0-14 years; g: risk from birth to 40 years after onset in proband; h: risk from birth to age 35 years and proband age at onset 0-40 years. Thus, part of the differences observed between the studies above is likely caused by differences in ascertainment, sampling, follow-up and observation time.

The *IDDM1* and *IDDM2* loci were originally identified through candidate gene case-control approaches (Singal et al 1973, Nerup et al 1974, Bell et al 1984) and are dealt with separately in this chapter. Partial genome scans of chromosomal loci syntenic to chromosomal regions conferring risk to diabetes and/or to insulitis in rodent T1DM disease models have led to identification of *IDDM12* (*CTLA4*), *IDDM13* and *IDDM18* (Morahan et al 1996, Nistico et al 1996, Morahan et al 2001).

Loci identified with nominal evidence for linkage to T1DM (MLS >1.2, equalling $P_{nominal} = 0.01$, Lander et al 1995) are listed in **Table 6A** and **Table 6B**. The MLS threshold of >1.2 was chosen in order 1) to exclude a large number of loci presumably identified by chance and 2) to allow inclusion of all currently denoted *IDDM* loci. Related references regarding the individual loci are included in the table for completeness. Please note that not all related references support the observation of linkage.

A large number of loci (n = 34, excluding loci only found in pedigrees) demonstrates nominal evidence for linkage (MLS ≥ 1.2) to T1DM in one or more scans. However, independent replication of linkage of loci outside the *IDDM1* region has proven very difficult (ECIGS 2001, Cox et al 2001a), even for loci demonstrating signifi-

Table 6A. *IDDM* denoted T1DM loci with evidence for linkage at MLS ≥ 1.2 (P ≤ 0.01).

Locus	Genome wide scans Chromosome	Maximal with MLS ≥ 1.2	MLS reported*	References‡
IDDM1	6p21.3	1, 2, 3, 4, 5, 6†	65.8	See section 7.4.
IDDM2	11p15.5	2, 4, 5, 6†	4.3	See section 7.5
IDDM3	15q26		2.6	7†, 8-10
IDDM4	11q13	1, 2	3.9 (5.0)	7, 9, 10†, 11-14
IDDM5	6q25	2, 3, 6	4.5 (4.6)	9, 10†, 15-17
IDDM6	18q21	4	3.7 (1.6)	9, 18†, 19-21
IDDM7	2q31	6†	2.6	9, 10, 22-26
IDDM8	6q27	2, 3, 4, 6	3.6 (5.0)	9, 10†, 15-17, 27
IDDM9	3q21-q25	4	1.2	28, 29†
IDDM10	10p13-q11	1, 2, 4† ,6	4.7	30-32
IDDM11	14q24-q31	–	4.0	33†, 34, 35
IDDM12	2q33	6	3.4	25, 36†, 37-45
IDDM13	2q34	6	3.3	25, 38, 46†, 47
IDDM15	6q21	3, 5†, 6	9.4 (23.3)	16†, 17
IDDM16	14q32.3		NA	48†, 49
IDDM17	10q25	6	5.0	50†, 51
IDDM18	5q33-q34		1.8	52†, 53-58

See Table 6B for references.

Table 6B. Unnamed T1DM loci with evidence for linkage at MLS ≥1.2 (P ≤0.01).

Marker(s)	Chromosome	Genome wide scans with MLS ≥1.2	Maximal MLS reported*	References‡
D1S1617	1q42	3,6	2.4	59†
D2S113	2q11	5†	2.1	
D4S2366-D4S418	4p14-p16.1	4†,5	1.3	
D5S405-D5S432	5p15	4†	1.2	
D5S407	5p11-q13	5†	1.9	
GCK	7p13		NA	60†
D8S522-D8S261	8p24-p21	1,4†	1.3	29
D8S198/D8S556	8q23-q22	2†,	2.6	29
D8S1128	8q24		2.4	61, 62†
D12S99	12p13-pter	4,5†	1.7	63, 64
D14S70-D14S276	14q12-q21	4†	2.0	
D16S420-D16S261	16p11-q12#	4†,6	1.4	65, 66
D162405-D16S207	16p#	5†,6	2.4	65, 66
D16S515-D16S520	16q22-q24	4,6†	3.9	13
D17S2059	17q24		NA	61†
NA	17q25	6†	1.8	
D19S247-D19S226	19p13	4†	1.7	67-70
D19S225	19q11-q13	4,6†	1.8	
DXS991	Xp13p11	2†	1.4	71

NA: not available. *: In unconditioned analyses. ‡: References (in addition to refs. 1-6) investigating linkage of markers or gene variants in the region. Note that not all references support the observation of linkage. †: Reporting the highest MLS (number in brackets = pooled data). #: Some overlap between these regions. GCK: glucokinase gene. 1) Hashimoto et al 1994; 2) Davies et al 1994; 3) Concannon et al 1998; 4) Mein et al 1998; 5) ECIGS 2001; 6) Cox et al 2001a; 7) Field et al 1994; 8) Zamani et al 1996; 9) Luo et al 1995a; 10) Luo et al 1996; 11) Nakagawa et al 1998; 12) Eckenrode et al 2000; 13) Cordell et al 1995; 14) Twells et al 2003; 15) Davies et al 1996; 16) Delepine et al 1997; 17) De Nanclares et al 2001; 18) Merriman et al 1997; 19) Merriman et al 1998; 20) Merriman et al 2001; 21) Hall et al 2003; 22) Copeman et al 1995; 23) Owerbach et al 1995; 24) Luo et al 1995b; 25) Esposito et al 1998; 26) Kristiansen et al 2000a; 27) Owerbach 2000; 28) Paterson et al 1999; 29) Cucca et al 1998a; 30) Wapelhorst et al 1995; 31) Reed et al 1997; 32) Johnson et al 2002; 33) Field et al 1996; 34) Corder et al 2001; 35) Pociot et al 2001; 36) Nistico et al 1996; 37) Marron et al 1997; 38) Larsen et al 1999; 39) Marron et al 2000; 40) Chistiakov et al 2001; 41) McCormack et al 2001; 42) Guja et al 2002; 43) Turpeinen et al 2003; 44) Ueda et al 2003; 45) Case-control studies, for details see chapter 12. Studies published before 2000 are reviewed in Kristiansen et al 2000d; 46) Morahan et al 1996; 47) Fu et al 1998; 48) Field et al 2002; 49) Veijola et al 1996; 50) Verge et al 1998; 51) Babu et al 2003; 52) Morahan et al 2001; 53) Johansson et al 2001; 54) Dahlman et al 2002; 55) Nistico et al 2002; 56) Davoodi-Semiromi et al 2002; 57) Bergholdt et al 2004; 58) Holm et al 2003; 59) Ewens et al 2002; 60) Rowe et al 1995; 61) Vaessen et al 2002; 62) Sale et al 2002; 63) Kristiansen et al 1998a; 64) Kristiansen et al 2004; 65) Bugawan et al 2003; 66) Maier et al 2003; 67) Guja et al 1999; 68) Nejentsev et al 2000a; 69) Kristiansen et al 2000c; 70) Nejentsev et al 2003; 71) Cucca et al 1998b.

cant linkage in one cohort according to the guideline criteria for claim of linkage of MLS ≥3.6 ($P \leq 2 \times 10^{-5}$) in genome-wide scans (Lander et al 1995). The genome-wide T1DM scans have been successful in identifying minor T1DM loci and have confirmed the major genetic susceptibility gene(s) to map to the MHC locus (Hashimoto et al 1994, ECIGS 2001, Cox et al 2001a) – but unsuccessful in confirming loci found in other cohorts.

The reasons for these discrepancies may be several: 1) low of power of the allele sharing method(s) (Lander et al 1995, Risch et al 1996), 2) low genetic risk contribution (λ_s) to T1DM susceptibility by the minor genes/loci – λ_s for the individual minor T1DM loci has been estimated to be less than 1.6 (ECIGS 2001, Cox et al 2001a) and the two non-HLA genes so far implicated in T1DM disease have λ_s values less than 1.1 (Lucassen et al 1993, Ueda et al 2003), 3) inadequate sample size (Lander et al 1995, Altmuller et al 2001, Cox et al 2001a, Wang et al 2003), 4) heterogeneity within and between populations – e.g. the genetic non-MHC basis of T1DM may differ between and within populations (Altmuller et al 2001), 4) loci are likely to have been identified by chance (Lander et al 1995), 5) the phenotype "immune-mediated T1DM" may arise through several different genetically influenced mechanisms leading to immune-mediated β-cell destruction and 6) differences in gene-gene and environment-gene interactions between populations (Altshuler et al 1998). Recent studies suggest that the lack of consistency between genome scans mainly arises from inadequate sample sizes and use of "mixed" populations (Altmuller et al 2001, Cox et al 2001a).

The genome scans have identified loci putatively harbouring T1DM susceptibility genes, but they have not identified the *in sensu strictu* etiological T1DM variants in these loci. Some loci are likely to have been linked by random chance as a consequence of use of insufficient (MLS <3.6) statistical significance levels for claiming linkage (Lander et al 1995, ECIGS 2001, Cox et al 2001a). On the other hand, regions harbouring etiological gene variants may not have been identified as a consequence of sample heterogeneity, cohort size and limited genetic contribution of the etiological variant in the investigated population.

The genome scans, however, have provided evidence that genetic variants outside the MHC region contribute to the genetic basis of T1DM. Identification of these genes and gene variants and clarification of their role disease pathogenesis are the future challenges.

The *IDDM1* and *IDDM2* loci were not investigated in the experimental work behind this thesis. However, these loci are briefly described in the following sections as *IDDM1* has a major impact on risk of T1DM and *IDDM2* was the first established non-MHC locus.

7.4. *IDDM1* AND THE HLA GENES IN T1DM

The aim of this section is to briefly summarise the current knowledge on *IDDM1* genes and in particular the HLA class II genes in T1DM in man. It is beyond the scope of this thesis to review the entire literature on MHC genes in T1DM, the detailed genetic structure of the human HLA locus on chromosome 6p21.3 and the function of the HLA genes as these genes were not investigated in the experimental work underlying this thesis.

The HLA locus contains >200 genes, of which >40 encode leukocyte antigens involved in antigen presentation (Klein et al 2000). The locus is characterised by a strong degree of LD between the neighbouring genes in the HLA complex (Carrington 1999, Herr et al 2000, Walsh et al 2003). Despite the large genetic impact of *IDDM1* on T1DM susceptibility, strong LD has contributed to tremendous difficulties in the identification of the etiological T1DM variant(s) in the locus (Undlien et al 2001).

HLA Class I variants were associated with T1DM approximately 30 years ago (Singal et al 1973, Nerup et al 1974). Today, it is well-established that *IDDM1* (HLA) confers up to 50% (λ_{HLA} ~3.4) of the genetic clustering of T1DM (Risch1987) and that *IDDM1* is the major T1DM locus (ECIGS 2001, Cox et al 2001a).

There is convincing evidence for the involvement of particular alleles and combined haplotypes of the Class II *HLA-DQA1* and *-DQB1* and *-DRB1* genes in the susceptibility to and protection from T1DM, **Table 7** (reviewed in Undlien et al 2001 and Pociot et al 2002). Evaluation of the individual impact of the *HLA-DQ* and *-DR* genes has proven extremely difficult because of the strong LD between the two loci (Undlien et al 2001).

Combination of the susceptibility genes into genotypes affects the degree of T1DM risk, the highest risk is found in *DQ2(DQA1*05-DQB1*02)/DQ8(DQA1*03-DQB1*0302)* heterozygous individuals (Undlien et al 1997).

Interestingly, some haplotypes – in particular *DQA1*0102-DQB1*0602-DRB1*1501(DQ6)* – strongly protect against T1DM development in a dominant fashion, i.e. even in combination with a susceptibility haplotype (Baisch et al 1990, Nepom 1990). Moreover, the *HLA-DQB*0602* allele seems to protect against T1DM even in the presence of islet autoantibodies (Pugliese et al 1995).

The *HLA-DRB1*4* alleles found in combination with the DQ8 haplotype confer variable risk to T1DM (**Table 8**, Undlien et al 1997, Pociot et al 2002).

These differences in T1DM risk are considered to be determined

Table 7. T1DM associated HLA class II alleles, haplotypes and genotypes.

HLA-DQ alleles	HLA-DR alleles	RR
Alleles and haplotypes		
A1*0301-B1*0302	DRB1*04	2.5-9.5
A1*0501-B1*0201	DRB1*301	2.5-5.0
A1*0301-B1*0201	DRB1*701	8.0-13.0
A1*0301-B1*0201	DRB1*901	5.5
A1*0301-B1*0401	DRB1*04	3.5-4.5
A1*0301-B1*0303	DRB1*901	2.0-4.5
A1*0102-B1*0602	DRB1*1501	0.03-0.2
A1*0103-B1*0603	DRB1*1301	0.05-0.25
A1*0301-B1*0301	DRB1*04	0.2-0.5
A1*0501-B1*0301	DRB1*1101	0.05-0.5
Genotype		**RR**
Genotypes		
A1*0501-B1*0302-DRB1*301/DRB1*04		12.0-32.0
A1*0301-B1*0201-DRB1*301/DRB1*04		
A1*0301-B1*0402-DRB1*04/DRB1*801		4.0-15.0

Modified from Pociot and McDermott 2002. The relative risk is for the combined DQ-DR haplo- and genotypes in high-risk populations. There is significant variation between different ethnic populations.

Table 8. Impact of HLA-DRB1*04 β-chain variant residues on risk of T1DM.

	Polymorphic residues							
DRβ1*	37	57	67	70	71	74	86	T1DM risk
0401	Tyr	Asp	Leu	Gln	Lys	Ala	Gly	↑
0402	Tyr	Asp	Ile	Asp	Glu	Ala	Val	→
0403	Tyr	Asp	Leu	Gln	Arg	Glu	Val	↓
0404	Tyr	Asp	Leu	Gln	Arg	Ala	Val	→
0405	Tyr	Ser	Leu	Gln	Arg	Ala	Gly	↑
0406	Ser	Asp	Leu	Gln	Arg	Glu	Val	↓
0407	Tyr	Asp	Leu	Gln	Arg	Glu	Gly	↓

Modified from Undlien et al. 1997 and Pociot and McDermott 2002.
↑ high, → neutral and ↓ low T1DM susceptibility, respectively.

by the structure and the action of the peptide binding "pockets", P4 and P8, of the DRB1 molecule (Cucca et al 2001a). It is widely accepted that the susceptibility to and protection against T1DM is caused by particular amino acid changes at sites critical for the peptide-binding function of the HLA-DQB1 and -DRB1 molecules (Table 8, Chao et al 1999, Latek et al 2000, Stratmann et al 2000, Lee et al 2001, Cucca et al 2001a). Amino acid substitution at residue 57 in the "pocket" P9 of DQB1 molecule has attracted particular attention since the protective alleles encode aspartic acid, whereas the susceptibility alleles encode alanine, serine and valine (Todd et al 1987). Residue 57 amino acid substitutions have profound effects on the peptide binding function of the class II molecules (Kwok et al 1996, Corper et al 2000), but structural and genetic data indicate that other residues in the P9 pocket – in addition to the amino acid changes at residue 57 – contribute to susceptibility to T1DM (Awata et al 1990, Yamagata et al 1991, Lee et al 2001, Cucca et al 2001a).

A large number of studies have undertaken the investigation of other MHC Class II, Class I and Class IV genes, but the specific genetic and functional roles of these genes in T1DM are not yet clear. However, there is evidence for at least two HLA complex T1DM susceptibility genes apart from the *HLA-DQA1, -DQB1* and *DRB1* genes (Johansson et al 2003). The Class II *HLA-DPB1* gene may play a role although it is debated (Erlich et al 1996, Lie et al 1997, Noble et al 2000, Valdes et al 2001, Cucca et al 2001b) and one or more genes in the Class I and Class IV regions may be involved (Moghaddam et al 1997, Nejentsev et al 1997, Lie et al 1999, Nejentsev et al 2000b, Zavattari et al 2001, Cordell et al 2002, Johansson et al 2003).

In summary, gene variants in *HLA-DQA1, -DQB1* and *-DRB1* with implication for peptide binding in the peptide binding groove of these Class II molecules affect risk of T1DM. The specific role of these variants remains to be fully characterised and other genes in the HLA complex affecting susceptibility to T1DM still need identification and functional characterisation. Since *IDDM1* accounts for less than 50% of the inherited risk of T1DM, identification of genes outside this locus is also important for the understanding of the genetic background of T1DM.

7.5. *IDDM2* – THE PREPROINSULIN GENE (INS) AND T1DM

The *IDDM2* locus mapping to chromosome 11p15.5 was identified through a candidate gene approach since INS was considered a candidate gene for T1DM. A *VNTR* polymorphism mapping 5' to *INS* was found to be associated with T1DM in Caucasians, but not in Blacks (Bell et al 1984). The T1DM association with the *INS VNTR* in Caucasians, but not in Black and Orientals, was reproduced in other studies (reviewed in Undlien 1997). Additional studies mapped (Julier et al 1991, Owerbach et al 1993, Lucassen et al 1993, Julier et al 1994, Undlien et al 1995) and identified (Bennett et al 1995) the *INS VNTR* as the *IDDM2* etiological polymorphism.

The *INS VNTR* maps to the *INS* 5' regulatory region and arises from a tandem repetition of a 14-15 oligonucleotide sequence. The *VNTR* is highly polymorphic and the allelic variants are divided into 3 classes based on the number of repeats; class I (26-63 repeats), class II (mean of 80 repeats and virtually absent in Caucasians) and class III (141-209 repeats) (Bell et al 1982, Rotwein et al 1986, Bennett et al 1995). The class I and III frequencies are approximately 0.71 and 0.29, respectively, in Caucasian populations (Owerbach et al 1982, Julier et al 1994, Bennett et al 1996a).

Class I/I homozygosity is associated with two-to-five fold increased risk of T1DM development (Owerbach et al 1982, Julier et al 1991, Owerbach et al 1993, Lucassen et al 1993, Julier et al 1994, Bennett et al 1995, Undlien et al 1995, Bennett et al 1996a, Bennett et al 1997). Variable degrees of susceptibility are associated with each of the *INS VNTR* alleles (Bennett et al 1996b). Adding further to the complexity, the T1DM risk conferred by transmission of the 814 (42 repeat) *INS VNTR* class I allele is dependent on the parent-of-origin and type of non-transmitted allele in fathers (Bennett et al 1997). The genetic mechanisms underlying the latter observations – imprinting and/or an epigenetic phenomenon – are yet unresolved. The class III *VNTR* alleles are dominantly protective and are associated with a 60-70% reduction in risk of T1DM (Bennett et al 1995, Bennett et al 1996a). Despite these impressive figures of risk and protection, it has been estimated that *INS VNTR* contribution to familial clustering in T1DM has a marginal λ_{INS} of ~1.1 (Cox et al 2001a). It is unlikely that the *IDDM2* locus would have been found in a random linkage genome-wide screen using cohorts of the current size (Cox et al 2001a, Field 2002).

Several lines of evidence support a functional role of the *INS VNTR* in preproinsulin synthesis: 1) the *VNTR* class I and II alleles affect the *in vivo* fasting and stimulated C-peptide levels in a gene-dose manner (class I high and class III low) and class I and III homozygous individuals have significant differences (class I/I higher than class III/III) in glucose stimulated insulin levels (Cocozza et al 1988), 2) the *VNTR* alleles differently affect the function and physical state of this region through variable formation of inter- and intra-molecular G-quartet formations due to differences in number tandem repeats between the *VNTR* alleles (Hammond-Kosack et al 1992, Hammond-Kosack et al 1993, Lew et al 2000), 3) the *INS VNTR* affects *in vivo INS* mRNA expression in both the fetal and adult pancreas with high expression of class I compared to class III alleles (Bennett et al 1995, Vafiadis et al 1996, Bennett et al 1996a) and these data are supported by studies using transient transfection in rodent β-cells although data are inconsistent (Lucassen et al 1995, Kennedy et al 1995, Owerbach et al 1996) and 4) finally and most intriguingly, *in vivo* fetal thymic mRNA expression is affected by the class I and III *VNTR* variants with high mRNA expression of the *INS* class III variants (Pugliese et al 1997, Vafiadis et al 1997, Vafiadis et al 2001).

The latter has led to the leading hypothesis regarding the mechanism of the *INS VNTR* in T1DM development; that dominant protection of the *INS VNTR* class III alleles is caused by high expression of (pro)insulin in cells in the thymic medulla leading to negative selection of T-cells reactive against (pro)insulin. Conversely, *INS VNTR* class I/I homozygosity related low thymic (pro)insulin expression results in lack of negative selection of T-cells specific for anti-insulin and thereby increased anti-β-cell reactivity (Werdelin et al 1998, Hanahan 1998).

Thus, it is generally accepted that the *INS VNTR* is the etiologic variant at *IDDM2* and that differences in the class I and III allele encoded (pro)insulin levels in medullary thymic cells and the resulting "lack of" or "normal" negative selection, respectively, of (pro)insulin autoreactive T-cells are the likely genetically encoded mechanism conferring different degrees of risk of T1DM.

7.6. CONCLUSION

The twin studies, the epidemiological studies and the genome scans individually and in concert have provided substantial evidence that T1DM genetic loci outside the HLA complex exist. The "established" T1DM loci cannot explain the entire genetic susceptibility behind T1DM. The genome scans have provided limited and conflicting suggestions of where to look for these additional T1DM risk modifying genes, and are very likely to have missed regions harbouring T1DM susceptibility genes. Moreover, some of the loci reported to be T1DM loci are likely not to be "real" T1DM loci as the criteria for significant/suggestive linkage were not reached. Studies of the candidate genes and T1DM loci investigated in the experimental work behind this thesis are discussed in the following chapters.

8. THE *IDDM7* LOCUS AND *GALNT3* ON CHROMOSOME 2q

One of the regions outside *IDDM1* having attracted most attention in T1DM genetics has been chromosome 2q and in particular the 2q31-q35 and the 2q12-q21 regions. This is based on three observations. First, the early demonstration of β-cell damage by interleukin-1 (Mandrup-Poulsen et al 1986, Bendtzen et al 1986, Mandrup-Poulsen 1988) and mapping of the human "interleukin-1 gene cluster" to 2q12-21 (Webb et al 1986, Lafage et al 1989, Copeland et al 1991, Lennard et al 1992, Steinkasserer et al 1992). Second, the NOD mouse susceptibility locus *Idd5* on the murine chromosome 1 (Cornall et al 1991, Garchon et al 1991, Ghosh et al 1993) harbours genes syntenic to human genes on 2q, including the IL-1 receptor genes (Schurr et al 1990, Copeland et al 1991). Third, the demonstrations of "marginal" linkage to the 2q31 region in an early T1DM genome-wide scan (Davies et al 1994). Six of the candidate genes evaluated in the work behind this thesis map to chromosome 2q12-33. This chapter deals with investigations of the *IDDM7* locus at 2q32 and the *GALNT3* candidate gene. Investigations of the "interleukin-1 gene cluster genes" in T1DM are discussed in chapter 9 and studies of *CTLA4 (IDDM12)* and the *IDDM13* locus are detailed in chapter 12.

8.1. *IDDM7* – A T1DM SUSCEPTIBILITY LOCUS ON 2q31-32?

The first T1DM genome scans in UK patients found "borderline" (MLS = 1.2) evidence for linkage to a locus on 2q31 (*D2S326*) in ASPs not sharing HLA (Davies et al 1994) and much attention was directed to this particular locus due to the reasons detailed above. The *ASP analyses* of this locus in T1DM are listed in **Table 9**.

The study most convincingly supporting a T1DM locus at 2q31-32 found linkage to T1DM in a ~21 cM large region from 2q24 to 2q32 by unconditional ASP analysis (Owerbach et al 1995). This region harbours *D2S152* (the marker assigned for *IDDM7*). Strengthened linkage was found by conditioning for HLA and *INS VNTR* risk, suggesting interaction between the three loci (Owerbach et al 1995). This latter observation was substantiated in an enlarged but overlapping cohort (Buhler et al 1997). The Scandinavian genome-wide scan was unable to exclude minor ($\lambda_s \leq 2$) susceptibility genes at the *IDDM7* and *IDDM12* loci (ECIGS 2001). The two studies demonstrating suggestive evidence for linkage in unconditioned ASP analyses are overlapping in cohorts (Owerbach et al 1995, Cox et al 2001a). Thus, linkage by means of ASP analysis has not been formally established.

As linkage disequilibrium mapping, i.e. TDT analysis, is more powerful than the allele-sharing methods, several studies have investigated the *IDDM7* marker *D2S152* by TDT analyses in T1DM cohorts (Copeman et al 1995, Luo et al 1995b, Luo et al 1996, Esposito et al 1998, Kristiansen et al 2000a). The marker *D2S152* is highly polymorphic. Thus, the authors of the first investigation using the TDT approach chose to assess the frequency of transmission of the three most frequent alleles and found increased transmission of the 228 mu (migrating unit) allele in some but not all populations (**Table 10**, Copeman et al 1995). The transmission of the 228 mu allele in all reported materials is detailed in Table 10.

Thus, the most frequent and *initially* T1DM associated 228 mu allele demonstrates a marginal evidence for association with an overall 52% transmission in T1DM siblings. However, before rejecting *IDDM7* as a T1DM locus, the approach applied should be criticised for analysing transmission of the most frequent alleles exclusively and combining the data from several populations of different ethnic backgrounds.

Was it appropriate to evaluate transmission distortion of only *one* allele in several populations of different *ethnic* backgrounds? The ETDT analysis demonstrated significant linkage and association of *D2S152* to T1DM in the Danish T1DM families (Kristiansen et al 2000a) – but the 228 mu allele of *D2S152* was not, despite overall linkage of *D2S152*, preferentially transmitted in Danish T1DM siblings (Table 10). In contrast, the 275 bp allele (~232 mu allele) transmission rate was 62% in T1DM offspring, suggesting that this allele is in LD with the *IDDM7* gene variant in Danes. However, this interpretation should be taken with a note of caution due to the limited number (N = 101) of transmissions and the number of alleles evaluated (Kristiansen et al 2000a). Although speculative and an "experiment of thoughts", if the *D2S152* data of the Danish (Kristiansen et al 2000a) and the initial UK cohort (Copeman et al 1995)

Table 9. Linkage between T1DM and the *IDDM7* locus by ASP analysis.

Population(s)	MLS	Marker(s)	Distance from *D2S152* (cM)	Locus	References
UK/US/DK/SD*	1.5	*D2S152*	0	2q31-33	Copeman et al 1995
UK/US	2.6	*HOXD8*	5	2q31	Owerbach and Gabbey 1995
US/I	0.2	*D2S152*	0	2q32	Luo et al 1996
UK	1.1	*D2S326-D2S152*	0-10	2q31-32	Esposito et al 1998
DK/S/N	0.8	*HOXD8-CTLA4*	NA	2q31-33	ECIGS 2001
UK/US	2.6	*D2S1391*	~2	2q31	Cox et al 2001

Only the two most recent genome wide T1DM scans are included. There is a major overlap in materials between most studies as parts of the HBDI and the Warren Repository cohorts is included in the US and UK cohorts investigated, respectively. Only data obtained in unconditioned analyses are included. Conventional national codes used. *: Sardinia.

Table 10. Transmission of the *D2S152* 228 mu allele in 8 populations.

Data sets	Families (n)	T1DM offspring T	NT	%T	N P≤0.05	non-T1DM offspring T	NT	%T
SD	180	82	55	60	0.01	54	58	48
US	294	268	207	56	0.005	24	31	44
I	162	70	53	57		42	58	42
UK	390	278	288	49		NA	NA	NA
DK*	251	134	146	48		77	81	49
FIN	215	92	97	49		54	51	51
F	82	64	51	56		50	39	56
RO	204	65	70	48		43	47	48
Total	1748	1055	967	52	0.05	344	365	49

Modified from Esposito et al 1998 with the inclusion of data from *: Kristiansen et al 1999. NT: not transmitted; T: transmitted; %T: transmission rate. NA: not available. Apart from the Italian cohort (P = 0.03) no significant differences in transmission patterns in T1DM and non-T1DM offspring were observed. Conventional national codes used. SD: Sardinia.

had been combined first, a significant 55% transmission rate of the 230 mu had emerged as the only significant transmission distortion among the three most prevalent alleles. This would have turned the focus to the 230 allele. But the 230 mu allele transmission rate is 43% in the T1DM offspring of the combined US and Sardinian cohorts (Copeman et al 1995). These data indicate that simply selecting the three most informative alleles, i.e. in terms of number of observable transmissions, is an insufficient approach for TDT analysis in several populations – important information is lost. On the other hand, ETDT analysis in the combined materials would probably have been negative as the population specific transmission distortions would be "neutralised". Conversely based on the available data (Copeman et al 1995, Esposito et al 1998, Kristiansen et al 2000a), it is likely that ETDT analysis in the individual populations had demonstrated linkage and association in several populations. These observations are suggestive of a T1DM susceptibility gene at *IDDM7* but the causal variant is in LD with different *D2S152* alleles in populations of different ethnicity. Support for this hypothesis was obtained by comparison of the transmission rates of 228 mu alleles in the populations of Mediterranean and Non-Mediterranean ancestry, respectively, and omitting the US population due to admixture of Northern-European and Mediterranean ethnicity, **Figure 2**.

If the pattern of "population specific" heterogeneity in transmission distortion of particular *D2S152* alleles can be confirmed, this may facilitate the identification of the *IDDM7* culprit variant.

Future studies aiming at the identification of the *IDDM7* variant should ideally involve complete mutation scanning of this region, establishment of haplotype blocks (if possible) and LD patterns, and a comprehensive linkage disequilibrium mapping of all SNPs and selected markers within few cM of *D2S152* in large cohorts of different ethnicity, initially analysed individually. A re-analysis of the *D2S152* data using an ETDT-like analysis in individual populations is strongly recommended.

Recent studies in congenic mouse strains have reduced the 34 cM large *Idd5* locus (Cornall et al 1991, Garchon et al 1991, Ghosh et al 1993) to a 9.4 cM large region with two susceptibility genes *Idd5.1* and *Idd5.2* (Hill et al 2000, Lamhamedi-Cherradi et al 2001), and have excluded *IDDM7* as the syntenic region to both *Idd5.1* and *Idd5.2,* as the two homologous human regions map to 2q33 and 2q34-q37, respectively.

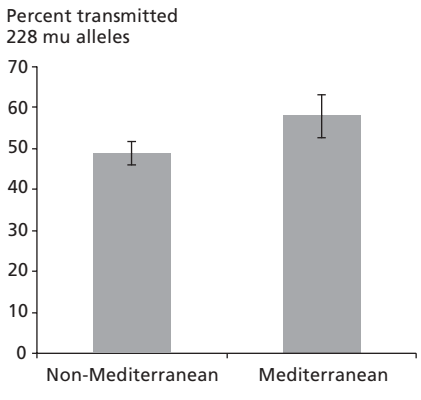

Figure 2. Transmission rates of the *D2S152* 228 mu allele in T1DM offspring of Mediterranean and non-Mediterranean ancestry. Comparison of transmission patterns: P = 0.003.

Despite this lack of supportive evidence from the NOD mouse and the lack of consistent and replicated strong evidence for linkage and association to T1DM in man, the *IDDM7* locus should be considered a candidate locus in T1DM worthwhile pursuing in further investigations.

In addition to our evaluation of *IDDM7 (D2S152)* in Danish T1DM patients, another aim was to evaluate a novel candidate gene, *GALNT3,* mapping to the *HOXD8-IDDM7* locus, in relation T1DM as described in the following section.

8.2. *GALNT3* A CANDIDATE GENE FOR T1DM?

In the T1DM pathogenetic model, Figure 1, one mechanism for perpetuation of β-cell destruction is the liberation β-cell antigens, possibly in glycosylated or in post-translationally modified forms not previously "seen" by the immune system.

Post-translational modifications include various forms of glycosylation, which have important biological implications (Varki 1993). The addition of the N-acetylgalactosamine (GalNAc) to serine and threonine amino acid residues, the so-called mucin-type O-glycosylation, is one of the most abundant forms of O-glycosylation. Mucin-type O-glycosylation is initiated and controlled by a family of UDP-GalNAc: polypeptide N-acetyl-galactosaminyltransferases (GalNAc-transferases) (Clausen et al 1996, Ten Hagen et al 2003). The gene family is highly evolutionarily conserved and it is estimated that there are more than 20 isoforms in the human genome (Ten Hagen et al 2003). Mucin-type O-glycosylation serves important biological functions such as shielding of the protein core against proteases and thermal changes (Sauer et al 2000, Garner et al 2001), protein folding (Jentoft 1990, Hilkens et al 1992), intracellular protein transport and sorting (Spodsberg et al 2001, Breuza et al 2002) and leukocyte homing and trafficking (McEver 2002). Glycopeptides associated with MHC class I molecules can use GalNAc to anchor the peptide in the antigen presenting groove and enable high-affinity binding (Apostolopoulos et al 2003), and glycopeptides are retained, processed and presented on MHC class II molecules by dendritic cells (Vlad et al 2002). Thus, glycopeptides are believed to play critical roles in antigen presentation and recognition. GalNAc-transferase type 3, GalNAc-T3, is differentially expressed in different organs and predominantly expressed in pancreas and testis (Bennett et al 1996c), and the GalNAc-T3 gene (*GALNT3*) is expressed in isolated human islet cells (Bennett et al 1998).

The *GALNT3* microsatellite marker *D2S2363* maps to chromosome 2q24-q31 between the markers *D2S124* and *D2S138* (Bennett et al 1998), and the two latter markers map within the linked *HOXD8/IDDM7* region (Owerbach et al 1995).

Thus, we hypothesised that variants in regulatory or coding regions of *GALNT3* may result in quantitatively or qualitatively changes in GalNAc-T3 activity, respectively, leading to changes in antigenicity of "self"-molecules and promotion of β-cell destruction in T1DM (Kristiansen et al 2000a). Evidence for such a mechanism in human T1DM is lacking. But an example hereof has recently been demonstrated in patients with rheumatoid arthritis, in which the T-cell reactivity against type II collagen is affected by the degree of glycosylation of an important type II collagen epitope (Backlund et al 2002).

Therefore, we evaluated the marker *D2S2363* and a novel 3'UTR SNP identified through mutation screening of the *GALNT3* gene for linkage and association to T1DM as outlined in the next section.

8.3. *GALNT3* VARIANTS IN T1DM

Mutation scanning identified only one SNP in *GALNT3*, a novel *GALNT3+2183T>A* SNP (denoted T284A in Kristiansen et al 2000a) mapping 281 bp downstream of exon 10 in the 3'UTR region. LD between the *D2S2363* microsatellite and *GALNT3+2183T>A* was strong.

The allele-wise ETDT evaluation of the two genetic variants in the Danish cohort did not support a role of *GALNT3* in susceptibility T1DM, neither in all T1DM offspring nor after conditioning for HLA risk.

Interestingly, a nominally significantly increased (59%) transmission of the *D2S2363* 193-bp allele was observed in the non-HLA-DR3/DR4 subset of T1DM siblings, although this was not significant after conservative Bonferroni correction. In addition, borderline ("uncorrected") differences in the transmission patterns of the 193-bp allele and the *GALNT3+2183T>A* alleles to T1DM offspring were observed between the two HLA conditioned subset.

Thus, data supporting the identified *GALNT3+2183T>A* SNP as the etiological *HOXD8/IDDM7* gene variant was not provided. However, the combination of the HLA conditioned data and the observation of an effect of HLA conditioning on linkage between this locus and T1DM in an independent cohort (Owerbach et al 1995) implies that the *GALNT3+2183T>A* SNP or another genetic variant in or close to *GALNT3* may confer risk of T1DM in HLA low-risk patients. The possibility of other genetic variants in *GALNT3* cannot be excluded as only a small number of individuals were mutation-scanned and thus rare alleles may not have been identified (Kristiansen et al 2000a).

The number of HLA "low-risk" trios needed to demonstrate association at $P < 0.05$ with a power of 80% given the observed transmission pattern of 55% is approximately 1,600. Thus, currently the material to demonstrate such a putative association does not exist. So far association studies of this variant in other T1DM cohorts have not been reported and investigation of this gene was not pursued further in the Danish cohort.

8.4. OTHER CANDIDATE GENE STUDIES AT THE *HOXD8/IDDM7* LOCUS

Two other genes mapping to 2q31-32 have been evaluated in relation to T1DM. A polymorphism in *HOXD8* was not associated with T1DM in Caucasians (Owerbach et al 1997). Genetic variants in *BETA2*, primarily a non-synonymous Ala45Thr (*+182G>A*) SNP, have been investigated, **Table 11**. *BETA2/NEUROD1* encodes a member of the basic helix-loop-helix family of transcription factors of importance for insulin-gene transcription playing a pivotal role in pancreatic islet development and differentiation, and apoptosis of entero-endocrine cells (Madsen et al 1997, Naya et al 1997, Mutoh et al 1998).

The data in Table 11 indicate that the *BETA2* 45Thr allele is a susceptibility allele for T1DM in the Japanese population, whereas none of the *BETA+182G>A* variants confer susceptibility to T1DM in Caucasians. A non-autoimmune, rapid onset, insulinopenic form of diabetes has been described in Japanese diabetes patients (Imagawa et al 2000). Whether the *BETA2* 45Thr variant is involved in the pathogenesis of this type of diabetes through its impact on β-cell development and/or apoptosis is unknown.

Table 11. Association studies of the *BETA2* Ala45Thr polymorphism.

	Genotypes			
Population/Group	**Ala/Ala**	**Ala/Thr**	**Thr/Thr**	**P<0.05***
Case-control association studies				
Japanese[1-4]/T1DM	356	112	11	<0.0001
Japanese[1-4]/Control	678	117	5	
Caucasian[5,6]/T1DM	132	125	32	NS
Caucasian[5,6]/Control	93	128	32	

	A45T allele transmission		
Population/Group	**Ala**	**Thr**	**P_{tdt} < 0.05**
TDT association studies			
US[7]	114	121	NS
DK[8]	115	81	0.02
US[6]	88	85	NS
European/US[9,†]	1494	1510	NS
Studies[6,8,9]	1697	1678	NS

1) Iwata et al 1999; 2) Awata et al 2000; 3) Yamada et al 2001; 4) Mochizuki et al 2002; 5) Dupont et al 1999; 6) Malecki et al 2003; 7) Owerbach et al 1997; 8) Hansen et al 2000; 9) Vella et al 2004. *: Comparison between genotypes of T1DM and controls was performed by 2 × 3 contingency-tables. †: European families were from UK, Northern Ireland, Norway, Romania and Finland. The Japanese studies (1-4) and the Caucasian (French/US, 4 and 5) studies were combined, respectively. Significant differences between the genotype and the allele distributions in Japanese and Caucasian T1DM cohorts were observed. The Thr-allele was strongly associated with T1DM in the combined Japanese studies. Data from Owerbach et al 1997 were omitted from the combined TDT analysis due to putative overlap with US data from Vella et al 2004.

8.5. CONCLUSION

The *IDDM7* marker *D2S152* demonstrated linkage and association to T1DM in the Danish cohort, implying the presence of a T1DM gene at chromosome 2q31. Re-analysis of reported *D2S152* data implied that *IDDM7* is likely to be a T1DM locus. However, the *IDDM7* etiological T1DM gene seems to be in LD with particular *D2S152* alleles in different populations. Further and more comprehensive evaluations of the *IDDM7* locus in enlarged family cohorts and trans-racial studies are required to identify the *IDDM7* culprit gene. Evaluation of two common genetic variants in *GALNT3* did not provided evidence for *GALNT3* as a T1DM gene.

9. THE ROLE OF "INTERLEUKIN-1 GENE CLUSTER" VARIANTS IN T1DM SUSCEPTIBILITY

The genes encoding IL-1α (*IL1A*), IL-1β (*IL1B*), the IL-1 receptor antagonist (*ILRN*) and the IL-1 type II (*IL1RT2*) and type I (*IL1RT1*) receptors map to chromosome 2q12-21 (Webb et al 1986, Lafage et al 1989, Copeland et al 1991, Lennard et al 1992, Steinkasserer et al 1992, Patterson et al 1993, Nothwang et al 1997, Dale et al 1999, Nicklin et al 2002). This region was named "the interleukin-1 gene cluster". IL-1α and IL-1β are prominent pro-inflammatory cytokines, and in particular IL-1β action and/or lack of antagonism of IL-1 action on the insulin-producing β-cell have been proposed to play a key role in the pathogenesis of T1DM. It is beyond the scope of this thesis to review the literature on the IL-1 system and the role of the IL-1 related polypeptides/receptors in T1DM pathogenesis and in particular β-cell destruction in detail as it has been reviewed extensively elsewhere (Mandrup-Poulsen 1988, Mølvig 1992, Helqvist 1994, Mandrup-Poulsen 1996, Bach 1996, Reimers 1998, Dinarello 1998, Andersen 1999, Eizirik et al 2001a, Donath et al 2003, Rabinovitch et al 2003).

9.1. THE IL-1 SYSTEM

The purpose of this section is to briefly illustrate the relative roles of

the two agonists (IL-1α and IL-1β), the IL-1 receptor antagonist (IL-1Ra), the two membrane bound (type I: IL-1RI and type 2: IL-1RII) and soluble (IL-1sRI and IL-1sRII) forms of IL-1 receptors, and the IL-1 receptor accessory protein (IL-1R AcP) in IL-1 signalling (**Figure 3**). For more detailed features of the genetic regulation of production and secretion, and the biological properties of the various members of the IL-1 system in health and disease, see Dinarello 1998.

The IL-1 agonists, IL-1α and in particular IL-1β, are pro-inflammatory cytokines affecting almost every cell type by signalling via the IL-1RI/IL-1/IL1R AcP complex. The major sources of IL-1α and IL-1β are monocytes, macrophages, dendritic cells, B-lymphocytes and fibroblasts (Dinarello 1998), but other cell types including the β-cells produce IL-1β *in vivo* and *in vitro* upon various stimuli (Heitmeier et al 2001, Maedler et al 2002). IL-1 production and secretion in immune related cells are induced by microbial and viral products, cytokines and other stress factors (Dinarello 1996).

IL-1 released from cells of the monocytic lineage provides an important co-stimulatory activating signal to proximal T-cells, macrophages and dendritic cells leading to increased antibody production, increased lymphokine synthesis, development of human Th_2 T-cell clones and inhibition of tolerance to protein antigen. IL-1 is also comitogenic for T- and B-cells (Dinarello 1996). The effects of *in vitro* exposure of IL-1 to non-immune cells are highly dependent on the species, cell type, presence of other inflammatory substances and the metabolic state of the cell (Dinarello 1996). The IL-1 system has been suggested to play a key role in the destruction of β-cells leading to T1DM – the topic of the following section.

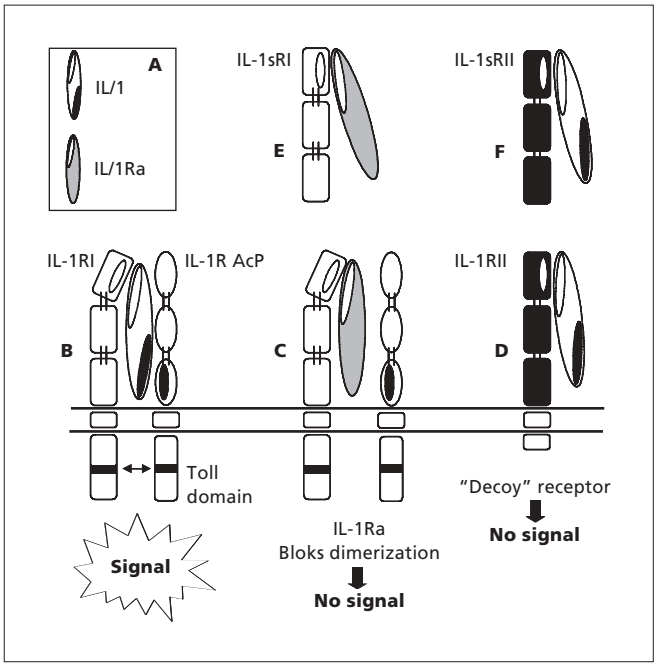

Figure 3. Interactions and regulation of signalling in the IL-1 system. Modified from Dinarello 1998 and Dinarello 2003. **A:** IL-1α and IL-1β are represented by the same symbol. IL-1α is generally expressed as a surface molecule on monocytes (and to a lesser degree by other cells) and is not secreted as mature IL-1α. IL-1α is released as proIL-1α from dying cells and is cleaved by extracellular proteases. In contrast, IL-1β is mainly secreted by monocytes (or other cells) as the mature IL-1β after the cleavage of proIL-1β by the IL-1 converting enzyme (ICE ~caspase-1). IL-1Ra, the IL-1 receptor antagonist, is secreted from all cells upon IL-1 action on these cells, but mainly from monocytes. **B:** The IL-1RI is the signal transducing receptor. IL-1 and IL-1Ra compete for binding to the IL-RI. After low-affinity binding of IL-1 to IL-1RI, the IL-1R Accessory Protein (AcP) forms a high-affinity complex with IL-1/IL-1RI leading to signal transduction. **C:** In contrast, binding of IL-1Ra to IL-1RI inhibits high-affinity complex formation with the IL-1R AcP and consequently no signal is induced. Thus, the IL-1Ra has an anti-inflammatory action. **D:** The membrane bound IL-1RII lacks a signal transducing cytosolic domain and has high affinity for IL-1β and low affinity for IL-1Ra. Thus, this receptor acts as a "decoy" receptor dampening the pro-inflammatory action of IL-1β. **E:** The soluble IL-1sRI receptor acts as a pro-inflammatory molecule due to preferential binding of IL-1Ra compared to the IL-1 agonists. **F:** The IL-1sRII has higher affinity for IL-1β and IL-1α than for IL-1Ra and is considered having anti-inflammatory function.

9.2. THE ROLE OF IL-1 IN β-CELL DESTRUCTION

It is well-established that *in vitro* exposure of physiological concentrations IL-1β by itself is cytotoxic to rat β-cells and that IL-1β in combination with IFNγ and/or TNFα/β is cytotoxic to human and murine β-cells *in vitro*. Moreover, IL-1Ra antagonises IL-1β induced reduction in DNA content in rat β-cells *in vitro* (Eizirik et al 1992). IL-1Ra protects human β-cells against IL-1β-induced NO formation and reduced viability (Heitmeier et al 2001) and protects against NO induced Fas-triggered β-cell apoptosis (Giannoukakis et al 1999) *in vitro*. Finally, both human and rat islet cells express IL-1RI (Nielsen et al 1994, Scarim et al 1997) and the rat insulinoma cell line Rinm5F expresses both types of IL-1 receptors (Bristulf et al 1994). Thus, *in vitro* studies point to a *putative* β-cell deleterious effect of IL-1β and a protective role of IL-1Ra in T1DM in man.

The remainder of this section will briefly discuss the indications for a *putative* role of the IL-1 system in T1DM in man arising from *in vivo* studies in the *spontaneous* rodent T1DM models and from human studies.

A prerequisite for the validity of the hypothesis of IL-1 as an effector molecule in T1DM is the demonstration of IL-1 in the insulitis infiltrate. IL-1 mRNA and protein is highly expressed in the insulitis infiltrate of DP-BB rats and NOD mice (reviewed in Mandrup-Poulsen 1996), but *IL1A* and *IL1B* mRNAs were not detected in the islets of pancreatic biopsies from two cases of longstanding (10 years) and recent onset T1DM, respectively (Somoza et al 1994). Whether this is due to the late stage of β-cell destruction is yet unknown as these are the only cases investigated for IL-1 expression.

Systemic IL-1 treatment in NOD mice and DP-BB rats has lead to controversial outcomes on diabetes incidence and onset. In the NOD mouse diabetes onset is delayed and incidence is unchanged (Satoh et al 1989, Jacob et al 1990) or decreased (Formby et al 1992). In the DP-BB rat, high dose IL-1 administration accelerates onset (Wilson et al 1990, Vertrees et al 1991), but the effect of IL-1 on incidence was unchanged (Wilson et al 1990, Reimers et al 1994) and IL-1 low-dose administration reduced incidence (Wilson et al 1990). Thus, high systemic IL-1 levels do not consistently increase T1DM in DP-BB rats and NOD mice.

IL-1Ra treatment delays diabetes onset in DP-BB rats (Dayer-Metroz et al 1992) but reports on the effect of IL-1Ra treatment in the NOD mouse have not been published. Both sIL-1R (Nicoletti et al 1994) and IL-1 antibody (Cailleau et al 1997) treatments reduce diabetes incidence in cyclophosphamide treated NOD mice in a dose dependent fashion, but sIL-1R treatment does not affect the degree of insulitis or T-cell reactivity (Nicoletti et al 1994), suggesting protection at the β-cell level.

NOD mice deficient in the signal-transducing IL-1RI have a reduced (~75%) incidence of diabetes at 350 days and the onset of disease is delayed compared to wild-type littermates (Thomas et al 2004). Combined IL-1, TNF and IFNγ NOD knock-outs have not been investigated. Caspase-1 (i.e. ICE) deficient NOD mice develop diabetes at the same rate and with the same incidence as wild-type NOD-mice (Schott et al 2004), properly due to redundant effects of IL-1α which does not require ICE for processing (Dinarello 1998).

Attempts to demonstrate that IL-1α, IL-1β, IL1Ra and sIL-1RTI levels in serum and IL-1 stimulatory capacity of mononuclear cells were associated with T1DM predisposition and/or T1DM development have emerged (Luger et al 1988, Mølvig et al 1990, Sahdev et al 1992, Ohno et al 1993, Ciampolillo et al 1993, Espersen et al 1993, Mandrup-Poulsen et al 1994, Hussain et al 1996, Netea et al 1997, Hussain et al 1998, Bergholdt et al 2000), but the data are conflicting. Thus, a consistent association between any of these parameters and T1DM has not been demonstrated.

In summary, the evidence for a key *in vivo* role of the IL-1 system in human T1DM pathogenesis is scarce – if not absent. However, *in vitro* studies show that human β-cells can be destroyed by mechanisms involving IL-1β and are protected by IL-1Ra. Inhibition of IL-1 action *in vivo* in the spontaneous disease models indicates that IL-1 action facilitates but is not an essential prerequisite for T1DM development. These observations qualify the genes encoding the IL-1 related molecules detailed in Figure 3 as T1DM candidate genes. Further, early case-control studies suggested association between SNPs in these genes and particular T1DM subsets in some studies. Thus, these and novel variants were investigated by intra-familial analysis as detailed in the next section.

9.3. "THE IL-1 GENE CLUSTER" VARIANTS MAY AFFECT RISK AND TIME OF ONSET OF T1DM

Several variants in *IL1A, IL1B, IL1RN* and *IL1RT1* have been evaluated for association to T1DM in various populations (**Figure 4**, **Table 12** and **Table 13**). A total of seven SNPs in *IL1B* and *IL1RT1* (Figure 4) and the *IL1RN* intron 2 86-bp repeat (Tarlow et al 1993) have been evaluated for association in the Danish T1DM cohort (Kristiansen et al 1998b, Bergholdt et al 2000, Kristiansen et al 2000b). The gene encoding IL-1R AcP maps to chromosome 3q28 and has not been evaluated in relation to T1DM.

Evaluation of *multi-allelic polymorphic markers* for *IL1A, IL1B, IL1RN* and *IL1RT1* has not consistently supported presence of etiological T1DM variants in any of these genes (Copeman et al 1995, Esposito et al 1998, Ogunkolade et al 2000), but the presence of genetic variants with $\lambda_s < 1.7$ could not be excluded (Esposito et al 1998).

The investigated intra-genic *IL1A* polymorphisms and markers in LD with this gene are not linked or associated with T1DM (Table 12, Copeman et al 1995, Esposito et al 1998, Ogunkolade et al 2000). Thus, there is no evidence for genetic contribution to T1DM risk from an *IL1A* variant.

9.3.1. IL1B

We undertook the investigation of the *IL1B-511C>T* and *IL1B+3953C>T* SNPs (denoted *IL1B AvaI* and *IL1B TaqI*, respectively, in Kristiansen et al. 2000b) in the Danish T1DM family cohort because of the controversial observations of association of the *IL1B+3953C>T* SNP in subsets of Danish T1DM patients in case-control studies and the lack of intra-familial T1DM association between the *IL1B-511C>T* and *IL1B+3953 C>T* SNPs in 92 UK T1DM families (Table 12). We found no overall T1DM linkage or association of the SNPs individually or of *IL1B-511C>T – IL1B+3953 C>T* haplotypes (Kristiansen et al 2000b). We were unable to show an impact of HLA-conditioning as suggested in a previous Danish case-control study (Pociot et al 1992). Moreover, an enlarged study supported the negative overall T1DM association in UK patients (Esposito et al 1998). Recently, association between the *IL1B+3953* T allele (*IL1B*1*) carrier status and T1DM was reported in Hungarian patients (Krikovszky et al 2002), but a disproportionately high number of *IL1B+3953C>T* heterozygous individuals was found among T1DM cases and the Hardy-Weinberg criteria were not fulfilled in the T1DM cohort, suggesting that genotyping errors or poor DNA quality in this cohort may have contributed to the positive association. However, this may also be attributed to strong T1DM association.

The *IL1B+3953C>T* SNP maps to exon 5 in *IL1B* and causes a silent mutation (Pociot et al 1994). Nonetheless, the *IL1B+3953C>T* alleles are associated with an allele dose-dependent impact on LPS stimulated IL-1β secretion from monocytes *in vitro* (Pociot et al 1992). Transfection experiments with chloramphenicol acetyltransferase (*CAT*) gene/*IL1B* full-length and truncated 3'UTR constructs in THP-1 cells have demonstrated a repressive effect of the full-length *IL1B* 3'UTR on CAT activity in resting cells and a LPS responsive and mRNA stabilising element in the terminal 177 bp of the 3'UTR (Kern et al 1997). Thus, we hypothesised that a genetic variant in the 3'UTR in LD with the *IL1B+3953C>T* might explain the observed effect of the latter SNP on LPS stimulated IL-1β secretion in monocytes. We SSCP mutation screened the full-length IL1B 3'UTR and identified an *IL1B+6912G>C* SNP as the only variant in the *IL1B* 3'UTR (Kristiansen et al 1998b). Interestingly, this SNP maps within the terminal 177 bp of the *IL1B* 3'UTR and is in com-

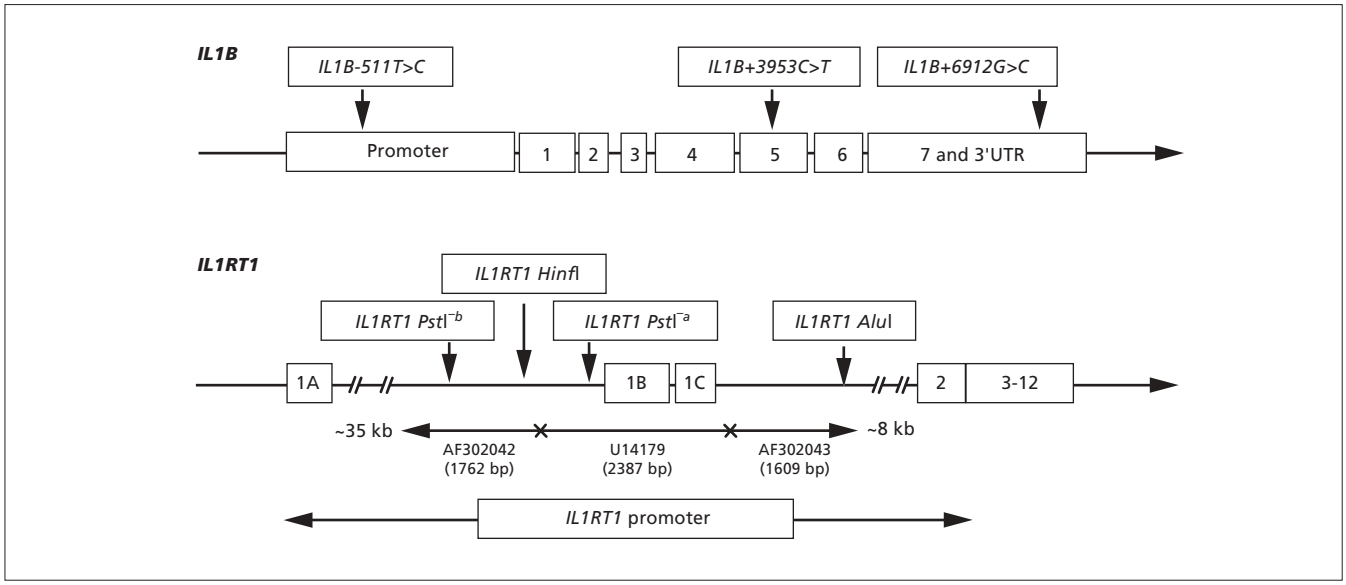

Figure 4. *IL1B* and *IL1RT1SNPs* evaluated for intra-familial association to T1DM. The SNPs in *IL1B* were previously denoted *IL1B Ava*I (IL1B-511T>C), *IL1B Taq*I (*IL1B+3953C>T*) and *IL1B* 3'UTR SNP (*IL1B+6912G>C*) in Kristiansen et al 1998b and Kristiansen et al 2000b. The latter SNP maps 97 bp upstream the start of the poly-A-signal site. The *IL1RT1PstI*$^{-a}$ (C>T) SNP was investigated in Kristiansen et al 2000b. The other SNPs detailed in IL1RT1 were identified and evaluated in Bergholdt et al 2000. Numbers in boxes refer to exon numbers. The three promoters in *IL1RT1* direct the transcription of three different 5'UTRs encoded by exons 1A, 1B and 1C respectively. The GenBank accession numbers AF302042, U14179 and AF302043 completes a sequence of 5,758 bp. In this combined sequence the position of the SNPs and exons are: *IL1RT1 PstI*$^{-b}$ (G>A) 709, *IL1RT1 Hinf*I (G>A) 1,622, *IL1RT1 PstI*$^{-a}$ (C>T) 2,732, exon 1B 2,847 – 3,320, exon 1C 3,647 – 3,885 and *IL1RT1 Alu*I (T>C) 5,647. Additional SNPs have been observed in the 5,758 bp sequence; C>T at 2,758 and T>C at 2,896 (Bergholdt et al 2000), A>G at 3,372 and T>A at 3,786 (Sitara et al 1999), C>A at 3,698 (Sitara et al 2000b), G>A at 3,746 (Sitara et al 2000a) and T>C at 5,125 (Bergholdt et al 2000). These SNPs and the very large number of common (40 common SNPs identified in European Americans) SNPs recently reported (Carlson et al 2004) have not been evaluated in Danish T1DM families.

Table 12. T1DM association studies of *IL1A, IL1B* and *IL1RN* variants.

Polymorphism	Population	Study-design C-C[1] or TDT	Associated allele	Comment	References
IL1A-889C>T	UK	TDT	–		Copeman et al 1995
IL1A+5540 (AC)$_n$	DK/UK/US	TDT	–	48 DK families included	Copeman et al 1995
	India	TDT	–		Ogunkolade et al 2000
IL1B-511C>T	UK	TDT	–		Copeman et al 1995
	DK	TDT	–		Kristiansen et al 2000b
IL1+3953C>T	DK	C-C	IL1B*1 (T)	T1DM HLA ÷ DR3/ ÷ DR4 patients only	Pociot et al 1992
	DK	C-C	IL1B*1 (T)	IL1B1*1 carrier status in familial T1DM	Pociot et al 1994
	UK	TDT	–		Esposito et al 1998[2]
	DK	TDT	–		Kristiansen et al 2000b
	H	C-C	IL1B*1 (T)	T1DMs not in H-W[3]	Krikovszky et al 2002
IL1+6912G>C	DK	TDT	G	T1DM patients with onset >10 years	Kristiansen et al 1998b
IL1RN (86bp)$_{2-6}$	DK	C-C	A1 (86bp)$_4$	A1/A1 genotype in familial T1DM	Pociot et al 1994
	DK	TDT	–		Kristiansen et al 2000b

1) C-C: case-control studies. 2) Part of the UK cohort was also investigated in Copeman et al. 1995. 3) Hardy-Weinberg equilibrium. The large number of recently identified common SNPs found by complete mutation scanning of *IL1B* and *IL1RN* (Carlson et al 2004) have not been evaluated in relation T1DM.

Table 13. T1DM association studies of *IL1RT1* variants.

Polymorphism	Population	Study-design C-C[1] or TDT	Associated allele	Comment	References
*IL1RT1 Pst*I^{-a}	DK	C-C	–	Low heterozygosity in T1DM patients	Pociot et al 1994
	DK	C-C	–	Low heterozygosity in T1DM patients	Bergholdt et al 1995
	UK	C-C	T		Metcalfe et al 1996
	FIN	C-C	T	T1DM HLA ÷ DR3/ ÷ DR4 patients only	–
	India	C-C	–		–
	E	C-C	–		Mato et al 1997
	DK	TDT	–		Kristiansen et al 2000b
	India	TDT	–		Ogunkolade et al 2000
*IL1RT1 Pst*I^{-b}	DK	TDT	–		Bergholdt et al 2000
*IL1RT1 Hinf*I	DK	TDT	G		Bergholdt et al 2000
*IL1RT1 Alu*I	DK	TDT	–		Bergholdt et al 2000

1) C-C: case-control.

plete LD with the *IL1B+3953C>T* SNP in our families – suggesting that the *IL1B+6912G>C* SNP rather than the *IL1B+3953C>T* SNP may be the cause of the allele dose-dependent effect on LPS stimulated IL-1β production in monocytes (Pociot et al 1992), admitting that we have not formally demonstrated this.

Not surprisingly – in the light of the LD between the two SNPs – the *IL1B+6912G>C* SNP did not demonstrate overall linkage and association to T1DM (Kristiansen et al 1998b). However, a *post-hoc* analysis of the allelic transmission patterns in groups stratified for mean age at onset of T1DM in the cohort (10 years) revealed two interesting features (**Table 14**). First, there was significant association with the LPS low-responder *IL1B+6912* G-allele in offspring with "later" onset T1DM and second, significant differences in transmission patterns between the two groups were observed.

As this is a *post-hoc* analysis, interpretation of these data in terms of functional importance for T1DM development should be done with caution. It is, however, tempting to speculate that in genetically predisposed individuals the high-responder C-allele may accelerate β-cell destruction whereas the low-responder G-allele increases the time needed before a sufficient number of β-cells are destroyed for T1DM to develop.

In summary, the combined data of the genetic variants in *IL1B* (Table 12) do not indicate an overall effect of any of these variants on risk of T1DM development. However, our *post-hoc* analyses (Kristiansen et al 1998b) should instigate evaluation of the impact of these variants on the age at onset of T1DM. Finally, it cannot be excluded that this variant may play a role in some subsets of T1DM patients, e.g. patients without T1DM HLA risk alleles.

Table 14. Transmission of the *IL1B+6912G>C* alleles in T1DM – effect on age at onset?

Age at onset	G (%T)	C (%T)	$\chi^{2,\ TDT}$	PTDT
<10 years	63 (44)	79 (56)	1.80	NS
≥10 years	66 (62)	40 (38)	6.38	0.01

Comparison of transmission pattern in the two groups: $\chi^2 = 7.09$ (P = 0.008).

9.3.2. IL1RN

The *IL1RN* 2nd intron 86-bp repeat A1/A1 genotype was initially shown to associate with "familial" T1DM in a case-control study (Pociot et al 1994). By intra-familial association analysis we did not find significantly increased transmission of the A1 allele to T1DM offspring in the Danish T1DM families (Kristiansen et al 2000b). This polymorphism has not been evaluated in other T1DM populations – and since the initial finding (Pociot et al 1994) caries a substantial risk of being "spurious" due to the limited number of cases and controls evaluated there is no evidence supporting that this variant or variants in LD with this polymorphism confer risk to T1DM.

9.3.3. IL1RT1

The case-control studies of the *IL1RT1 Pst*I^{-a} SNP (Figure 4) – one of the two first identified SNPs in the *IL1RT1* promoter (Bergholdt et al 1995) – demonstrated significant allelic association in the UK population and in Finnish non-DR3/non-DR4 patients (Table 13, Metcalfe et al 1996). The associations in the "all" UK T1DM and "non-DR3/non-DR4" Finnish T1DM were highly significant with estimates of the relative risks conferred by the *IL1RT1 Pst*I^{-a} C allele of 2.51 and 2.62, respectively (Metcalfe et al 1996). Conversely, allelic association was not found in the remaining case-control studies (Table 13). The highly positive association found in one UK population was contradicted by the lack of association with *D2S1473* – a marker mapping close to *IL1RT1* – in 352 UK families (Esposito et al 1998). Moreover, two additional family-based studies including one in Danes found no T1DM association with the *IL1RT1 Pst*I^{-a} SNP (Ogunkolade et al 2000, Kristiansen et al 2000b).

In a subsequent study, we obtained new sequence up- and down-

stream of the known sequence of the exon 1B and 1C (Figure 4: AF302042 and AF302043, respectively) and identified four novel SNPs (Bergholdt et al 2000). Three of these were evaluated in the Danish T1DM families (Figure 4 and Table 13). The fourth SNP (T>C) mapping 522 bp upstream *IL1RT1 Alu*I was not tested as it is in complete LD with the *IL1RT1 Alu*I SNP.

The *IL1RT1 Hinf*I was significantly linked and associated with T1DM. The transmission rate of the frequent *IL1RT1 Hinf*I G allele was 57% in T1DM offspring. Interestingly, the *IL1RT1 Hinf*I was in LD with the novel *IL1RT1 Pst*I^{-b} SNP, but not with the known *IL1RT1 Pst*I^{-a} and the two SNPs downstream exon 1C (Figure 4, Bergholdt et al 2000). This provides the explanation of 1) the association of the *IL1RT1 Hinf*I and 2) the lack of association of the *IL1RT1 Pst*I^{-a} SNP in the same T1DM families of two SNPs mapping merely 1,100 bp apart.

An *in vivo* impact of this variant was suggested since serum levels of sIL-1RTI were related to the *IL1RT1 Hinf*I genotype in a allele dose-dependent fashion in cross-sectionally collected plasma samples from healthy individuals and patients with longstanding T1DM (Bergholdt et al 2000). The highest sIL-1RTI levels were found in *IL1RT1 Hinf*I G/G homozygous individuals and the the lowest levels in A/A individuals. The level of sIL-1RTI was slightly (but insignificantly) higher in T1DM patients compared to control subjects and was explained by increased numbers of T1DM patients with the GG genotype. An increased level of sIL-1RTI leads to increased pro-inflammatory activity as the sIL-1RTI has a higher affinity for IL-1Ra than for IL-1β (Figure 3, Dinarello 1998). These findings suggest that the *IL1RT1 Hinf*I or a gene in LD with this SNP confers susceptibility to T1DM in man and further provides the *plausible* functional effect encoded by the allelic variants of this SNP.

9.4. CONCLUSION

The genetic studies evaluating variants in *IL1A, IL1B* and *IL1RN* have not convincingly demonstrated linkage or association to T1DM. A *post-hoc analysis* implies that the putatively functional *IL1B+6912G>C* SNP may have an impact on the age at onset of T1DM. Of the four SNPs mapping to the *IL1RT1* promoter, the *IL1RT1 Hinf*I SNP is a likely risk T1DM variant and the alleles of this SNP are associated with the *in vivo* levels of the sIL-1RTI. The case-control observations on the highly investigated *IL1RT1 Pst*I^{-a} SNP are conflicting and not supported by family-based studies. It is therefore unlikely that this variant is a general T1DM susceptibility variant. The findings for *IL1B+6912G>C* and *IL1RT1 Hinf*I should be substantiated in enlarged family cohorts and functional studies evaluating the impact of the *IL1B+6912G>C* SNP on IL-1β production and *IL1B* mRNA stability are in demand. Recently, most – if not all – SNPs in *IL1RT1* and *IL1B* were identified and this facilitates the future genetic evaluation of these genes in relation to T1DM and other diseases.

10. ARE *CD4* PROMOTER POLYMORPHISMS RISK VARIANTS FOR DEVELOPING T1DM?

The chapter briefly summarises the evidence for a role of CD4$^+$ T-cells in T1DM in man and discusses the key importance of CD4 receptor expression for T-cell functions, the genetic studies on *CD4* promoter variants in T1DM and the functional investigations of CD4 promoter studies. Finally, it is discussed how these findings relate to the proposed pathogenic model in Figure 1.

10.1. A ROLE OF CD4$^+$ T-CELLS IN T1DM?

It is beyond the scope of this review to detail the entire literature on the role of T-cells in T1DM. This has recently been extensively reviewed elsewhere (Roep 2003). As outlined in **Table 15** there is an accumulating body of evidence in support of a role of T-cells and in particular CD4$^+$ T-cells in T1DM in man.

Studies of the NOD mouse also indicate a major role of CD4$^+$ T-cells in NOD diabetes as NOD CD4^{null} mice are completely diabetes resistant (Wong et al 1998, Graser et al 2000) and NOD mice treated with depleting anti-CD4 antibodies do not develop diabetes (Koike et al 1987, Shizuru et al 1988).

Table 15. Support for a role of T-cells in the pathogenesis of T1DM.

– Presence in the insulitis infiltrate
– Delay of progress in disease with immunosuppressive drugs
– Preservation of β-cells at clinical onset of T1DM after anti-CD3 monoclonal antibody therapy
– Recurrent selective β-cell destruction in pancreas graft transplanted to diabetic MZ twin
– "Adoptive transfer" of T1DM with bone marrow (not depleted for T-cells) from T1DM donor to non-diabetic recipient
– Circulating autoreactive T-cells in T1DM patients
– Concordance between islet graft failure and increased in T-cell autoreactivity
– Lack of benefit from plasmapheresis and intravenous immunoglobulin therapy
– Development of *autoimmune* T1DM in B-cell and antibody deficient patient with intact T-cell immunity

Modified from Roep 2003.

Thus, there is considerable supportive evidence that T-cells and in particular CD4$^+$ T-cells are involved in the immuno-pathogenesis of T1DM in man. These observations do not necessarily qualify the gene encoding CD4 as a candidate gene in T1DM. However, as discussed below the CD4 molecule has several important implications for CD4$^+$ T-cell functions.

10.2. THE IMPACT OF CD4 SURFACE EXPRESSION ON CD4$^+$ T-CELL ACTIVATION AND REGULATION

The CD4 gene encodes a molecule of vital importance for several immuno regulatory mechanisms such as the thymic CD4$^+$ T-cell development and thymic selection, signal transduction in CD4$^+$ T-cells, peripheral T-cell homeostasis and the survival of naïve T-cells in the absence of antigenic stimulation.

The best characterised role of CD4 is its function as a co-receptor during TCR:antigen:MHC class II interaction (reviewed in Konig et al 2004). Initially, it was thought that CD4 was mainly an adhesion molecule leading to stabilisation of the contact between the T-cell and the APCs, but recent studies have shown that CD4 does not interact directly with the TCR (Wang et al 2001a), and affinity to the MHC class II binding site is very weak (Xiong et al 2001, Davis et al 2003). The events upon antigen stimulation are believed to be as follows: 1) the TCR-antigen/MHC class II interaction initiates the formation of a specialised junction between the T-cell and the APC, the *immunological synapse,* the TCR/CD3ζ complexes cluster in the cSMAC (*c*entral zone of the *s*upramolecular *a*ctivation *c*luster) and adhesion molecules including LFA-1 and ICAM-1 (see chapter 11) form a ring – the peripheral SMAC (pSMAC) – surrounding the central area (Monks et al 1998), 2) CD4 is rapidly recruited to the CD3ζ complex (Zal et al 2002) docking the p56^{lck}, a member of the Src family of tyrosine kinases which is important for TCR signalling, into the cSMAC (Holdorf et al 2002), 3) a few minutes after docking to the TCR/CD3ζ complex, CD4 translocates to the periphery, while the TCR/CD3ζ complex stabilises within the cSMAC (Krummel et al 2000). Although only present in the cSMAC for a few minutes and in spite of the weak affinity for the MHC class II molecule, the interaction of CD4 with the CD3ζ and the MHC class II molecule strongly reduces the threshold for T-cell activation (Irvine et al 2002). In the presence of CD4, a single MHC class II molecule presenting antigen suffices to induce Ca^{2+} flux, ten MHC class II molecule/antigen complexes cause the formation of an immunological synapse and finally blockade of CD4 reduces sensitivity to antigen stimulation more than three-fold (Irvine et al 2002). Moreover, expression of CD4 lowers the activation threshold allowing detection of low-affinity TCR reactivity (Feito et al 1997), and the level of CD4 affects the T-cell response to endogenous ligands (Vidal et al 1996). The combined initial signalling through the TCR and CD4 pro-

motes a long-lasting elevation in intracellular Ca^{2+} (Zhou et al 2003) which is required for T-cell activation (Wulfing et al 1997).

Inhibition of CD4 and MHC class II interaction by administration of non-depleting anti-CD4 monoclonal antibodies re-establishes tolerance to β-cells in NOD mice *in vivo* (Hutchings et al 1992) and leads to *in vivo* induced antigen specific tolerance (Laub et al 2002). Anti-CD4 antibody treatment of T-cells recognising a wild-type peptide-MHC class II ligand leads to disproportionate inhibition of interleukin-2 relative to interleukin-3 (Madrenas et al 1997), the same pattern seen using TCR partial agonist/antagonists, suggesting that decreased CD4-MHC class II interaction may result in tolerance induction.

Lack of CD4 and MHC class II interaction in the absence of antigen leads to apoptotic cell death of resting naïve $CD4^+$ T-cells in mice expressing a mutant MHC class II receptor inhibiting CD4-MHC interaction (Maroto et al 1999, Shen et al 2001) and in CD4 deficient mice (Strong et al 2001).

Interruption of the CD4-MHC class II interaction by MHC mutagenesis does not inhibit maturation of $CD4^+$ T-cells to the single-positive state, but the frequency of $CD4^+$ T-cells is reduced threefold compared to wild-type transgenic mice (Gilfillan et al 1998).

The genetic regulation of CD4 expression during thymocyte maturation in man and mice is complex and involves several enhancers and silencers of *CD4* expression. These are of key importance for normal development of $CD4^+$ T-cells (reviewed in Siu 2002 and Kioussis et al 2002). Moreover, transcription factor binding sites in the CD4 promoter are important for CD4 mRNA expression during thymocyte development (Siu et al 1992, Nakayama et al 1993, Duncan et al 1995, Adlam et al 1997), and the *CD4* promoter seems to be a key regulatory region of *CD4* expression in mature T-cells (Siu et al 1992, Hanna et al 1994, Adlam et al 1997, Uematsu et al 1997, Rushton et al 1997).

In summary, the expression CD4 on $CD4^+$ T-cells is of major importance for the functions, development and homeostasis of this T-cell subset. Thus, *CD4* is an obvious T1DM candidate gene and as outlined below in the next section *CD4* is a positional candidate gene as well.

10.3. GENETIC STUDIES OF *CD4* VARIANTS IN RELATION TO T1DM

The human and mouse CD4 genes map to chromosomes 12p13 (Isobe et al 1986, Ansari-Lari et al 1996) and 6 (Seldin et al 1988), respectively. The human *CD4* spans a region of 31 kb comprising the promoter, 10 exons and the 3'UTR (Ansari-Lari et al 1996). Three polymorphisms in the human *CD4* gene were described prior to our investigations: 1) a penta-nuclotide repeat mapping at position -1188 upstream the transcription start site, here denoted *CD4-1188(TTTTC)$_{5\text{-}14}$* (Edwards et al 1991), 2) a 256 bp deletion polymorphism of a 285 bp *Alu* element mapping 9.8 kb upstream the *CD4-1188(TTTTC)$_{5\text{-}14}$* repeat in the first intron of *CD4* (Edwards et al 1992). There is a high degree of LD between *CD4-1188(TTTTC)$_{5\text{-}14}$* alleles and the *Alu* deletion alleles (Alu^+ and Alu^-). Hence, the Alu^- is only found with the *A7 (TTTTC)$_6$* allele, whereas all other repeat alleles, including the frequent *A4 ((TTTTC)$_3$(CTTTC)$_1$(TTTTC)$_6$)* and *A8 (TTTTC)$_5$* alleles associates with the Alu^+ in European populations (Edwards et al 1991, Tishkoff et al 1996), and 3) a C→T SNP that leads to an $Arginine^{240}$ to $Tryptophan^{240}$ substitution in the CD4 molecule. This variant is extremely rare in Europeans, but more frequent in African Americans and Japanese (Lederman et al 1991, Hodge et al 1991).

10.3.1. The CD4-1188(TTTTC)$_{5\text{-}14}$ A4 allele in T1DM

The 12p12-pter region was not linked to T1DM in the first T1DM genome scans (Hashimoto et al 1994, Davies et al 1994), but in two subsequent studies the marker *D12S99* mapping ~4 cM pter of *CD4* was linked to T1DM (Mein et al 1998, ECIGS 2001). In support of theses findings, a very recent re-analysis of the Scandinavian T1DM genome scan data (ECIGS 2001) using data mining and neural network analysis suggested a ~14 cM large (*D12S99-D12S358*) region harbouring *CD4* to be involved in T1DM susceptibility (Pociot et al 2004). Moreover, a case-control study in Belgian T1DM patients indicated association of the A4/A4 genotype with T1DM (Ghabanbasani et al 1994). Investigations in NOD and NON (non-obese non-diabetic) mice also indicate 1) that the NOD *Idd6* susceptibility locus maps close (~11cM) to *Cd4* (http://www.informatics.jax.org/, Ghosh et al 1993), and 2) that a 42 cM large region of the NON mouse chromosome 6 comprises a diabetes susceptibility gene with maximal linkage at the marker *D6Mit52* mapping 61.4 cM from the telomere – with *Cd4* mapping at 60.2 cM (http://www.informatics.jax.org/, McAleer et al 1995). Thus, in addition to our studies detailed below there is substantial suggestive evidence for a T1DM susceptibility gene harboured at the telomere part of the human chromosome 12p.

Our first investigation (Kristiansen et al 1998a) aimed at substantiating the observations in the Belgian population (Ghabanbasani et al 1994) of association of the *CD4-1188(TTTTC)$_{5\text{-}14}$* A4 allele to T1DM. Indeed, both linkage and association of the A4 allele were demonstrated by TDT analysis in the Danish cohort – with an A4 allele transmission rate of 58% in T1DM offspring (Kristiansen et al 1998a). The allele-wise ETDT analysis also indicated linkage between the *CD4-1188(TTTTC)$_{5\text{-}14}$* polymorphism (P = 0.04, data not shown in the original paper), whereas the less powerful extended sib pair analysis (ESPA) was unable to demonstrate linkage when testing exclusively the sib pair families. The A4 allele was randomly transmitted to unaffected siblings. A number of sub-stratifications based on HLA-risk, gender, age at onset, paternal/maternal transmission and type of families (ASP families and parent-offspring families) did not demonstrate differences in the transmission patterns between relevant subsets (Kristiansen et al 1998a). Thus, our data indicated that a general T1DM susceptibility gene variant – either the A4 allele or a genetic variant in LD with this allele – conferred susceptibility to T1DM (Kristiansen et al 1998a).

10.3.2. CD4 promoter haplotypes in T1DM

As this *CD4-1188(TTTTC)$_{5\text{-}14}$* variant is situated in the *CD4* promoter and as genome scans suggested a T1DM susceptibility gene at chromosome 12p12-pter, we decided to follow-up on our initial findings by 1) mutation screening of the *CD4* promoter, 2) evaluation of identified variants and haplotypes for linkage and association to T1DM and 3) investigations of the impact of various variants on the *CD4* promoter activity in reporter-studies (Kristiansen et al 2004), the latter issue will be detailed in the section 10.5.

We identified three common SNPs in the *CD4* promoter; *CD4-1050T>C, CD4-521C>G* and *CD4-181C>G* (Kristiansen et al 2004). None of the SNPs mapped within the minimal tissue specific promoter or in reported transcription factor binding sites (Salmon et al 1993, Zhao-Emonet et al 1998).

Linkage disequilibrium and allelic association between the SNP alleles and the *CD4-1188(TTTTC)$_{5\text{-}14}$* alleles were profound. Of the nine full-length *CD4* promoter haplotypes observed, four were frequent in our cohort; *A4$_{-1188}$T$_{-1050}$G$_{-521}$C$_{-181}$, A7TGC, A8CGG* and *A8CCG.*

The transmission rate of the *A4TGC* haplotype was 58% in T1DM siblings, but as a consequence of almost complete allelic association with the SNP variants we were unable to determine whether association was conferred by the haplotype or by the A4 allele in itself (Kristiansen et al 2004). None of the SNPs or the two and three SNP haplotypes displayed a transmission rate in T1DM offspring more skewed than the *A4TGC* haplotype (Kristiansen et al 2004). However, the *G$_{-521}$C$_{-181}$* haplotype and the *CD4-181 C* allele transmission rates were comparable with that of the *A4TGC* haplotype.

These observations indicated that the *A4TGC* haplotype, the A4 allele or a gene variant in LD with the A4 allele confers risk of T1DM. As described below, our reporter assay data of the *CD4* pro-

moter haplotypes and gene variants implied that the investigated variants may have functional impact on CD4 expression and thus strengthen the candidature of these variants as causal T1DM gene variants. However, other genes may harbour the culprit variant (Kristiansen et al 2004) and as detailed in **Figure 5** several genes map close to *CD4* – some of which may be likely candidates.

The TPI promoter variants and an intron 5 G/A SNP are in *linkage equilibrium* with the *CD4-1181 VNTR* alleles (Humphries et al 1999) implying that if the *A4TGC* haplotype is not the causal T1DM variant, the culprit T1DM variation most likely locates upstream of *TPI*. Two putative T1DM candidate genes map upstream *CD4* (Figure 5): *LAG3* encodes LAG-3, a MHC class II ligand evolutionarily related to CD4, expressed exclusively in activated T-cells and natural killer lymphocytes and involved in regulating the evolving immune response (Bruniquel et al 1997, Bruniquel et al 1998), and *TNFRSF1A* encoding the tumor necrosis factor receptor 1. A functional promoter SNP allele, *TNFRSF1A-383C* which encodes increased *TNFRSF1A* mRNA compared to the A allele *in vitro*, was recently associated with T1DM in Japanese (Nishimura et al 2003).

In summary, the *CD4-1188(TTTTC)*$_{5\text{-}14}$ A4 allele is associated with T1DM in both Belgian and Danish patients and this allele is part of a frequent *CD4* promoter haplotype *A4TGC*. The functional implications of the *CD4* variants on *CD4* promoter activity have not been investigated previously. Thus, we undertook an evaluation of the functional impact of the identified frequently occurring haplotypes on *CD4* promoter by reporter assays as described below.

10.4. FUNCTIONAL IMPLICATIONS OF THE *CD4* PROMOTER VARIANTS

In the light of the importance of CD4 surface expression detailed above there is a striking lack of studies investigating the correlation between *CD4* promoter activity ~*CD4* transcriptional activity (= *CD4* mRNA levels) and the degree of CD4 surface expression on the individual T-cell. In developing thymocytes, CD4 surface expression is transcriptionally regulated (Takahama et al 1992, Siu et al 1994). Antigen stimulation of TCR transgenic mature CD4$^+$ T-cell clones leads to down-regulation of TCR, CD3 and CD4 surface expression (Viola et al 1997). Non-antigen specific stimulation of human T-cells with soluble anti-CD3 + PMA, PMA, plate-bound anti-CD3 or anti-TCR in leads to 1) initial CD4, CD3 and TCR surface down-regulation and a subsequent up-regulation of these molecules after 20-72 hours dependent on the stimulation modality (Acres et al 1986, Wiegers et al 2000) and 2) an initial decrease in the TCR, CD3 and CD4 mRNAs caused by concomitant inhibition of transcription and destabilisation of the mRNAs followed by an up-regulation of the mRNAs through recovery of transcription and increased mRNA stability (Paillard et al 1988, Paillard et al 1990). The increase in protein tyrosine phosphorylation observed after TCR antigen stimulation can be mimicked by treatment of Jurkat cells with PMA (Fernandez et al 1999), and the *CD4* promoter is known to be active in CD4$^+$ Jurkat cells (Zhao-Emonet et al 1998). These studies suggested evidence for a transcriptional regulation of CD4 surface expression upon stimulation and *CD4* promoter activity in CD4$^+$ Jurkat cells. Thus, the constitutive and the stimulated activities of full-length and truncated *CD4* promoter constructs were evaluated by reporter assays in CD4$^+$ Jurkat cells in order to characterise the impact of the individual *CD4* promoter variants on *CD4* promoter activity (Kristiansen et al 2004).

The stimulation modalities detailed in **Figure 6** were initially investigated for dose-dependent effects on *CD4* promoter activity, and stimulation doses providing the half-maximal stimulation index of the individual modality were included in the time-response investigations in Figure 6 (data not presented in Kristiansen et al 2004).

There was no significant effect of the time of incubation between related data sets. Since PMA/Phytohemagglutinin (PHA) stimulation was superior in providing a higher stimulation index, this stimulus and 24 hours of incubation were chosen for the final studies.

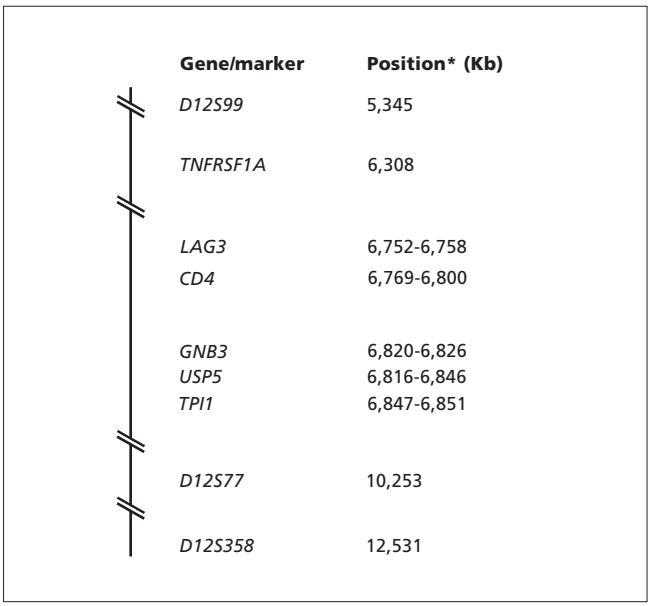

Figure 5. Markers and genes at the *CD4* locus. The map constructed from http//www,ncbi.nlm.nih.gov/entrez/uniSTS and Ansari-Lari et al 1996. *) GenBank accession # NC_000012, the complete human chromosome 12 sequence. Genetic distances from 12pter (Marshfield Center for Medical Genetics: http//www.research.marshfieldclinic.org/genetics/): *D12S99* (12.6 cM) – *CD4* (16.4 cM) – *D12S77* (20.3 cM) – *D12S358* (26.3 cM). *TNFRSF1A:* the tumor necrosis factor receptor 1 gene (OMIM # 191190), *LAG3:* lymphocyte activation gene 3 (OMIM # 153337), *GNB3:* guanine nucleotide-binding protein BETA-3 gene (OMIM # 139130), *USP5:* ubiquitin-specific protease 5 gene (OMIM # 601447) and *TPI1:* triosephosphate isomerase 1 gene (OMIM # 190450).

Our study – the first to evaluate the functional impact of *CD4* promoter variants on constitutive and non-antigen specific stimulated *CD4* promoter activity – revealed a number of intriguing observations.

First, a high *constitutive* activity of the T1DM associated *CD4* promoter haplotype *A4TGC* was observed. The activity of the *A4TGC* promoter haplotype exceeded that of the other three frequently occurring *CD4* promoter haplotypes *A7TGC, A8CCG* and *A8CGG* with 13%, 16% and 48%, respectively – implying that the *A4TGC* promoter *may* lead to higher levels of cell membrane bound CD4 in the non-stimulated state.

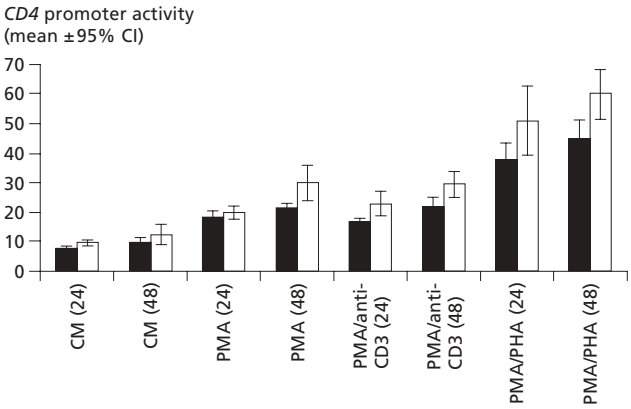

Figure 6. Effects of time of incubation and stimulation modalities on *CD4* promoter activity. Columns: 1320*CD4* (black) and 1141*CD4* (white) constructs. In each of the three experiment one preparation of each of the four different 1320*CD4* and the three different 1141*CD4* constructs were included in triplicates and a mean activity for the 1320*CD4* and the 1141*CD4* constructs variants were calculated for each experiment. *CD4* promoter activity = mean *CD4*$_{\text{normalised for Renilla activity}}$ Firefly-activity/Basic Vector$_{\text{normalised for Renilla activity}}$ Firefly constitutive-activity. Numbers in brackets indicate hours of incubation.

Second, a generalised functional impact of the *CD4-181C>G* SNP on stimulated activity of the *CD4* promoter. The stimulated activities of *CD4-181G* promoter constructs were higher than those of the *CD4-181C* constructs, irrespective of the length of the inserted *CD4* promoter. The ratio between the *stimulated* activity of the *CD4-181G* and the *CD4-181C* construct variants consistently demonstrated ~10-35% higher activity in the *CD4-181G* constructs – 10% in full-length constructs.

Third, in addition to the impact of the *CD4-181C>G* SNP, functional effects of the *CD4-521C>G* and the *CD4-1050T>C* SNPs, but not of the *CD4-1188(TTTTC)$_{5-14}$* variant on stimulated *CD4* promoter activity were suggested as illustrated in **Figure 7**.

Fourth, there were no significant differences in the *absolute* stimulated activities between the frequently occurring full length *CD4* promoter haplotypes, which can be explained by the combination of the various alleles in the haplotypes and the regulatory effects of these alleles on stimulated *CD4* promoter activity, Figure 7. This suggests that the *stimulated* activities of the four frequent haplotypes observed in Danish Caucasians are identical, and this may reflect the importance of tight control of *stimulated CD4* transcription and CD4 expression. We found no differences in the absolute stimulated capacity of the four frequent *CD4* promoter haplotypes (Kristiansen et al 2004).

Although these studies were performed in the Jurkat cell line and with antigen non-specific stimulation, the results are suggestive of functional impacts of the four variants on *CD4* promoter activity and putatively on CD4 surface expression on T-cells. However, in order to fully appreciate the pathophysiological impact of these variants, investigations of more physiologically relevant stimuli of the *CD4* promoter activity such as antigen stimulation (Viola et al 1997) and interaction with MHC class II molecules in the absence of antigen (Maroto et al 1999, Konig et al 2002) should be undertaken – both in well-characterised CD4$^+$ T-cell subsets and in thymocytes. Moreover, evaluation of the correlations between the identified *CD4* promoter variants and the *in vivo* CD4 receptor expression and the *CD4* mRNA expression is warranted.

10.5. HOW DOES THE CONTRIBUTION OF THE *CD4* VARIANTS "FIT" INTO THE PATHOGENETIC MODEL OF T1DM?

In spite of the limitations of our functional studies detailed above, these investigations may, however, clarify how the *CD4* promoter variants protect and predispose to T1DM.

The high constitutive activity of the *CD4 A4TGC* promoter may render previously non-stimulated autoreactive CD4$^+$ T-cells more sensitive to stimulation by a low number of *"self"*-antigens (Irvine et al 2002), and thus elicit an autoimmune process. In contrast, such cells constitutively expressing low levels of CD4 may upon discrete antigenic stimulation turn into an anergic state leading to tolerance induction (Madrenas et al 1997, Laub et al 2002). Moreover, high CD4 surface expression on naïve, putatively autoreactive T-cells may render these less susceptible to undergo apoptosis due to lack of CD4-MHC class II interaction in the absence of antigenic stimulation (Maroto et al 1999, Wang et al 2001b, Konig et al 2002).

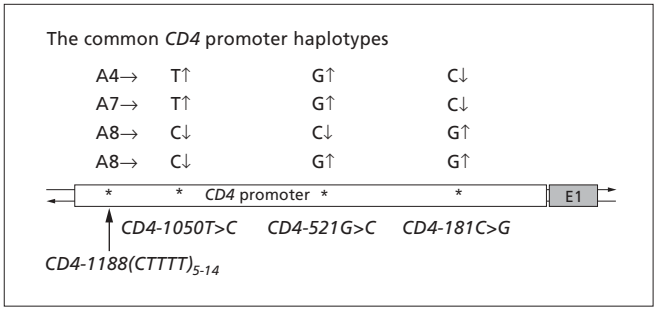

Figure 7. Impact of the *CD4* promoter variants on stimulated promoter activity. PMA/PHA stimulation in CD4+ Jurkat cells: ↑enhanced and ↓decreased *CD4* promoter activity, respectively.

Since human CD4$^+$CD25^{high} T$_r$ cells only maintain suppressor function for ~15 h upon strong TCR stimulation, whereas weaker TCR stimuli induce suppressive effects until 60 h after activation *in vitro* (Baecher-Allan et al 2002), it may be that increased CD4 expression in these cells reduces their suppressive potential, whereas low CD4 expression may allow optimal suppression even at higher levels of antigenic TCR stimulation. How these *CD4* variants affect the maturation and survival of developing thymocytes is unknown, but differences in CD4 expression may affect the process of negative selection.

10.6. CONCLUSION

There are several lines of evidence implying that a causal T1DM gene is harboured on chromosome 12p13. The *CD4-1188 (CTTTT)$_{5-14}$* A4 allele is linked and associated with T1DM in two independent populations. We found that the A4 allele is part of the *CD4 A4TGC* promoter haplotype which has a high constitutive activity in reporter assay studies in CD4$^+$ Jurkat cells. Our data imply that either the *CD4 A4TGC* promoter haplotype or a gene variant in LD with this haplotype is the culprit T1DM variant on chromosome 12p13. It is hypothesised that the *CD4 A4TGC* promoter is the causal variant. However, confirmation of the genetic data in large independent cohorts of T1DM families is in demand. Moreover, the implications of the identified *CD4* promoter haplotypes need further functional characterisation in various T-cell subset and thymocytes – both *ex vivo* and *in vitro*.

11. *ICAM1* – AN ETIOLOGICAL T1DM SUSCEPTIBILITY GENE?

Several studies have indicated that the intercellular adhesion molecule 1 (ICAM-1) could play a pivotal role in autoimmune diseases, including T1DM, via its roles in the immune response, leukocyte migration and expression on cytokine exposed target cells. Studies published after our investigation of ICAM-1 have provided further evidence for its importance in T1DM. This chapter briefly outlines the function of ICAM-1 in the immune system, discusses the evidence for a role of ICAM-1 in T1DM, and finally deals with the genetic studies of *ICAM1* variants in human T1DM and the putative functional impact of associated variants.

11.1. FUNCTIONS OF ICAM-1 IN THE IMMUNE RESPONSE

ICAM-1 was originally identified as the ligand for the lymphocyte function-associated antigen-1 (LFA-1) on T-cells (Rothlein et al 1986) and further shown to provide an important co-stimulatory signal for TCR-mediated activation of resting T-cells (Van-Seventer et al 1990). A large number of studies has revealed the importance of ICAM-1 expression in the regulation of the immune response, in leukocyte trafficking, and putatively as a signal transducing molecule in ICAM-1 expressing cells, including target cells in immune-mediated diseases (McMurray 1996, Hubbard et al 2000, Anderson et al 2003) as briefly outlined below. The action of ICAM-1 in β-cell destruction may be at the level of the target β-cell or through its role in leukocyte trafficking and/or as a co-stimulatory molecule for T-cells.

ICAM-1 is a member of the immunoglobulin supergene family (Springer 1990) of receptors/ligands, and its natural ligands/receptors are the αβ-integrin members LFA-1 expressed on leukocytes and Mac-1 (macrophage differentiation-antigen-1) expressed on monocytes and neutrophils (McMurray 1996). ICAM-1 is a transmembrane glycoprotein with five Ig-like domains. The first and the third domains are responsible for binding to LFA-1 and Mac-1, respectively (Hubbard et al 2000, Anderson et al 2003). The intracellular domain comprises 29 amino acid residues (Simmons et al 1988, Staunton et al 1988).

In contrast to LFA-1 and Mac-1, ICAM-1 is constitutively ex-

pressed only at low levels on vascular endothelial cells and on lymphocyte and monocyte subsets (Rothlein et al 1986, Hubbard et al 2000), but can be expressed on multiple cells types, including β-cells, by exposure to inflammatory agents such as cytokines and reactive oxygen species (ROS) (Roebuck et al 1999, Hubbard et al 2000). ICAM-1 expression is regulated at the transcriptional level and induced by a large number of stimuli (Roebuck et al 1999), **Table 16**.

The ICAM-1/LFA-1 interaction has major implications for the TCR response upon antigen presentation (Anderson et al 2003). LFA-1 on the T-cell and ICAM-1 on the APC are of importance for both the formation and stabilisation of the immunological synapse (Grakoui et al 1999). Further, LFA-1 provides important co-stimulatory signals to the T-cell (Anderson et al 2003), including signals inducing IL-2 and inhibiting IL-10 production, thus favouring Th_1 rather than Th_2 cell differentiation (Labuda et al 1998). Moreover, optimal T-cell responses require both ICAM-1 and B7-1 co-stimulation (Dubey et al 1995, Camacho et al 2001). Inhibition of the LFA-1/ICAM-1 interaction upon antigen stimulation of $CD4^+$ T-cells results in T-cell anergy, leading to tolerance (reviewed in Anderson et al 2003). Thus, ICAM-1 is of major importance, not only for the amplitude of the T-cell response but it also directs the response towards a Th_1 profile, whereas inhibition of LFA-1/ICAM-1 binding induces tolerance.

Expression of ICAM-1 on the endothelial cell is a vital mechanism in leukocyte trafficking and homing to inflammatory sites (Springer 1990, McMurray 1996).

Accumulating evidence indicates that ICAM-1 acts as a signal transducing molecule. Although the signalling mechanisms are not fully elucidated, a number of interesting observations on cytokine production, cell membrane protein expression and production of ROS induced by ICAM-1 signalling have been reported (reviewed in Hubbard et al 2000). ICAM-1 cross linking leads to increased *IL1B* mRNA expression and IL-1β protein production in rheumatoid synovial cells and increased MHC class II expression in mouse B-cells. Moreover, cross-linking of ICAM-1 on human monocytes leads to substantial ROS production (Rothlein et al 1994) and on mouse splenic macrophages to expression of M150, a macrophage specific molecule, mainly responsible for activation of Th_1 cells (Suvas et al 2003). Thus, signalling through ICAM-1 is likely to induce production of both ROS and cytokines which may further promote an inflammatory response.

A soluble form of ICAM-1 (sICAM-1) has been detected in human sera (Rothlein et al 1991). The pro- or anti-inflammatory significance of sICAM-1 remains to be fully elucidated (Gearing et al 1993), but data from NOD mice and human T1DM indicate that sICAM-1 may have anti-inflammatory properties (Roep et al 1994, Martin et al 1998).

11.2. ICAM-1 IN T1DM – A ROLE IN THE β-CELL, IN THE ENDOTHELIAL WALL, AS AN IMPORTANT CO-STIMULATOR OF THE TCR RESPONSE – OR A COMBINATION?

Studies directly demonstrating an *in vivo* role of ICAM-1 in human T1DM are lacking. However, circumstantial evidence arising from *in vitro* and *in vivo* studies in rodent T1DM models and from human studies is accumulating.

ICAM1 mRNA has been detected in infiltrated islets from one recent onset T1DM patient dying in ketoacidosis (Somoza et al 1994) and in two of nine pancreatic biopsy specimens from recent onset T1DM patients (Itoh et al 1993). ICAM-1 expression has consistently been demonstrated in spontaneous NOD mice infiltrated islets, but predominantly on infiltrating and endothelial cells (Prieto et al 1992, Lo et al 1993, Picarella et al 1993, Linn et al 1994, Faveeuw et al 1994, Hasegawa et al 1994, Yagi et al 1995, Martin et al 1996, Hunger et al 1997, Papaccio et al 2001). Most of these investigators were unable to detect ICAM-1 expression on β-cells, whereas some found ICAM-1 positive β-cells in the infiltrate (Yagi et al 1995, Papaccio et al 2001). However, β-cells can express ICAM-1 upon various stimuli *in vitro* and *in vivo* (Table 16). ICAM-1 is not expressed in non-inflamed human islets (Vives et al 1991) and NOD islets do not express ICAM-1 or LFA-1 prior to infiltration with mononuclear cells, implying that epithelial expression of these molecules does not initiate the mononuclear homing in T1DM, or initiates the β-cell destructive process (Martin et al 1996). Collectively, these *in vivo* studies indicate that ICAM-1 may be in involved in the process leading to T1DM and that ICAM-1 is likely to be important for leukocyte homing after the β-cell destructive process has been initiated.

Table 16. Cell type-specific induction of ICAM-1 expression.

Cell type	Stimuli
Endothelial cell	TNF-α, IL-1, IL-6, IFNγ, estradiol, oxidised LDL, H_2O_2
Epithelial cell	TNF-α, IL-1, LPS, some viruses, histamine
Fibroblast	TNF-α, IL-1, IL-4, IFNγ, PGE_2
Hepatocyte	TNF-α, IL-1, IL-6, IFNγ
Leukocyte	TNF-α, IL-1, IFNγ, IL-3, GM-CSF
Smooth muscle cell	TNF-α, PDGF
Beta-cell	IL-1*, IFNγ, TNF-α, IL1** + INFγ, IL-6** + INFγ

Modified from Roebuck et al 1999 with inclusion data on β-cell studies of ICAM-1 expression: Campbell et al 1989a, Vives et al 1991, Prieto et al 1992, Cardozo et al 2001, Todaro et al 2003, Wachlin et al 2003. LDL: low-density lipoprotein, GM-CSF: granulocyte-monocyte colony-stimulating factor, PDGF: platelet-derived growth factor.
*: Not alone in human β-cells. **: In combination in human β-cells.

Inhibition of LFA-1/ICAM-1 binding inhibits or reduces the incidence of spontaneous diabetes in the NOD mouse. Anti-ICAM-1 antibody treatment alone or in combination with anti-LFA-1 antibodies prevents/strongly reduces the incidence of T1DM and the grade of insulitis when administrated 1) from 5-12(30) weeks of age (Hasegawa et al 1994), 2) for 6 consecutive days at 2 weeks of age (Moriyama et al 1996, Chowdhury et al 2002) or 3) from 12-30 weeks of age (Yagi et al 1995 – note overlap in authors of the latter four references) – and early treatment induces tolerance to β-cell specific antigens (Moriyama et al 1996). *In vitro* priming of TCR transgenic $CD4^+$ T-cells with $B7\text{-}1^+/ICAM\text{-}1^-$ APC and subsequent adoptive transfer to the RIP-mOVA (transgenic recipient mouse expressing membrane ovalbumin under the control of the rat insulin promoter) diabetes model leads to generation of T effector cells migrating to the islets, but not to diabetes. In contrast, priming with $B7\text{-}1^+/ICAM\text{-}1^+$ APC leads to extensive β-cell destruction and rapid onset diabetes, and the pathogenicity was associated with T-cell production of the macrophage attracting chemokines CCL3 and CCL4 (Camacho et al 2001). Treatment of pre-diabetic 35 days old NOD mice with sICAM-1 or dimeric recombinant murine ICAM-1-Ig reduces the incidence of diabetes and the degree of insulitis (Martin et al 1998). Moreover, transient expression of the dimeric recombinant murine ICAM-1-Ig leads to stable diabetes remission in 50% of recent onset diabetes NOD mice without signs of immuno-suppression, indicating actively induced tolerance (Bertry-Coussot et al 2002). Finally, *Icam1*-deficient NOD mice are dominantly resistant to diabetes development (Balasa et al 2000, Martin et al 2001b), have only mild peri-islet infiltration (Martin et al 2001b), and are resistant to islet intra-islet infiltration and diabetes upon adaptive transfer of *in vitro* primed TCR β-cell specific $CD4^+$ BDC2.5 NOD splenocytes (Balasa et al 2000) – a compelling indication of involvement of ICAM-1 in T1DM. The above studies demonstrate that ICAM-1 expression is essential for the pathogenesis of diabetes in the NOD mouse.

Recent onset T1DM patients and high risk T1DM individuals have elevated serum levels of sICAM (Lampeter et al 1992, Mysliwiec et al 1999, Toivonen et al 2001). This likely reflects an ongoing chronic inflammation leading to shedding of ICAM-1 and sICAM-1 is not thought to be involved in β-cell destruction (Martin 1997).

On the contrary, sICAM-1 has been demonstrated to inhibit antigen specific proliferation of human insulin-secretory granule specific T-cells *in vitro* (Roep et al 1994), suggesting – as in the NOD mouse – that the function of sICAM-1 is to dampen ongoing inflammation.

In summary, ICAM-1/LFA-1 binding is of paramount importance in T1DM pathogenesis – at least in mice – and a major impact of ICAM-1 is as a co-stimulatory molecule for T-cell activation. Moreover, ICAM-1 may act as a signal transducing molecule inducing membrane proteins and cytokines that promote inflammation, stimulate T-cells and facilitate leukocyte homing to the islet. It is unresolved whether β-cells express ICAM-1 *in vivo* and if so, what the functional implications are. The high levels of shed sICAM-1 in patients at risk of T1DM and in recent onset T1DM patients are most likely a reflection of an ongoing chronic inflammation and sICAM-1 does not promote β-cell destruction. Based on these studies, it is obvious that *ICAM1* is a candidate gene in T1DM. Several studies evaluating *ICAM1* in relation to human T1DM have been undertaken as detailed in the following section.

11.3. GENETIC STUDIES OF *ICAM1* IN T1DM

The human *ICAM1* (OMIM # 147840) maps to chromosome 19p13.3-13.2 (Trask et al 1993). ICAM1 spans ~17kb with a total of 7 exons (Simmons et al 1988, Staunton et al 1988, Carlson et al 2004) and maps within a region with some (MLS ~1.7) evidence for linkage to T1DM in the UK/US T1DM cohorts (Mein et al 1998, Cox et al 2001a). *Icam1* maps to the murine chromosome 9 (Ballantyne et al 1991) close to the NOD susceptibility locus *Idd2* (McAleer et al 1995). Thus *ICAM1* is also a positional candidate gene.

Prior to our investigation (Kristiansen et al 2000c), two SNPs in the coding regions of *ICAM1* had been reported in Caucasians (Vora et al 1994): a rare G→A SNP in exon 4 (G241R) encoding a glycine to arginine change in the third Ig domain of ICAM-1, reported to have minor allele (A ~241R) frequency of ~0.03-0.1, and a frequent A→G SNP in exon 6 (K469E) encoding a lysine to glutamine change in the fifth Ig domain. An additional non-conservative A→T SNP in exon 2 (K29M) causing a lysine to methionine change was known (Fernandez-Reyes et al 1997). This SNP is extremely rare in Caucasians of Northern European ancestry (Zimmerman et al 1997, Fernandez-Reyes et al 1997). Recently, 61 *ICAM1* SNPs were reported, 39 SNPs – of which 19 are common – were identified in European Americans (Carlson et al 2004).

We (Kristiansen et al 2000c) and others (Guja et al 1999, Nishimura et al 2000, Nejentsev et al 2000a, Nejentsev et al 2003) have evaluated the K469E variant in T1DM, and one group further evaluated the G241R in two independent cohorts (Nejentsev et al 2003).

In the Danish cohort, we (Kristiansen et al 2000c) found no overall linkage or association to T1DM of the 469K variant (49% transmission) in contrast to a preliminary report (Guja et al 1999) showing association of this allele with T1DM in a Romanian family cohort with a 63.5% 469E transmission rate. A Finnish investigation was also unable to associate the 469E variant with T1DM by both intra-familial and case-control approaches (Nejentsev et al 2000a) and moreover, a Japanese case-control study failed to associate this variant to T1DM overall whereas association of the 469E variant was detected in T1DM patients with late (25-61 years of age) onset (Nishimura et al 2000). We were unable to confirm the latter observation in Danish "late-onset" T1DM patients (Kristiansen et al 2000c).

We further evaluated the combined transmission data from the Danish, Finnish and Romanian studies (N = 728 T1DM offspring) and found overall association with a 55% transmission rate of the 469K variant, but the increased transmission was carried exclusively by the transmission distortion in the Romanian material. Further, we observed heterogeneity in transmission patterns between the Nordic (Finnish and Danish) and Romanian cohorts. We concluded that association was found – but only due to the transmission distortion in the Romanian material (Kristiansen et al 2000c). A very recent investigation of the K469E variant in several T1DM populations (Nejentsev et al 2003; 3695 families and 4603 T1DM offspring) including the Finnish and the Romanian families described above found that this variant alone was not associated with T1DM (49.6% transmission of the 469K variant). Thus, there is no evidence that *ICAM1* exon 6 A→G SNP leading to the ICAM-1 K469E protein polymorphism in itself confers risk of T1DM, and this observation is in full agreement with the data obtained in the Danish family material (Kristiansen et al 2000c).

The study by Nejentsev et al (2003) further evaluated the "rare" G241R variant for association to T1DM in their cohort of families from Finland, Great Britain, US, Norway, Romania and Northern Ireland. Interestingly, significantly decreased transmission of 47.5% of the rare 241R variant was observed with a resulting relative risk of 0.91 (0.83-0.99). The 241R allele was transmitted randomly (49%) in unaffected offspring. Moreover, this under-transmission was validated in an independent UK T1DM family cohort of 446 families with a 241R transmission rate of 37.5% in T1DM offspring. Interestingly and in support of our observation of heterogeneity at the *ICAM1* locus between populations of Northern European ancestry and the Romanian population, heterogeneity in transmission patterns between populations was observed, and a trend for *increased* transmission of the 241R variant to Romanian T1DM offspring was observed (Nejentsev et al 2003). This study strongly indicates that the A allele of *ICAM1* exon 4 G→A SNP encoding the 241R ICAM-1 variant confers protection to T1DM.

Interestingly, a recent study evaluating both *ICAM1* SNPs in multiple sclerosis found that the 241R/469K haplotype was *not* transmitted (zero of seven possible transmissions) to affected offspring (Cournu-Rebeix et al 2003), suggesting that this is a rare protective haplotype. It is tempting to speculate that this is the explanation for the observed under-transmission of the 241R allele to T1DM patients (Nejentsev et al 2003). With the same "expected" frequency of this haplotype in the parents of the T1DM patient cohort as in the French parents to the multiple sclerosis patients (Cournu-Rebeix et al 2003) this would account for approximately 70 non-241R transmissions.

It was decided not to genotype for the *ICAM1* exon 4 SNP in the Danish families because the minor allele was reported to be very rare (Vora et al 1994) and lack of power was inferred. Studies of other loci (Copeman et al 1995, Esposito et al 1998, Larsen et al 1999, Kristiansen et al 2000a, Kristiansen et al 2000b, Ueda et al 2003) in the Danish and UK families have demonstrated very similar transmission patterns, but even with the transmission distortion of the 241R variant found in the combined UK material of 57% (53-61%) and an estimated 90 transmissions, the power to confirm this association in the Danish families would only be ~3-20%.

It cannot be excluded that the observed negative association is due to LD with one or more variants in *ICAM1* (Carlson et al 2004) or variants in neighbouring genes, and as suggested in the above, the A allele of the ICAM1 exon 4 G→A SNP may be one of several variants in a protective haplotype.

If the A allele of the *ICAM1* exon 4 G→A SNP is a culprit protective T1DM variant how does the 241R protect against T1DM or autoimmunity in general? The 241G variant is present in the analogous position in several species (Vora et al 1994), implying functional importance of glycine at this site. Amino acid residue 241 is located in the third Ig domain involved in binding to Mac-1 (Hubbard et al 2000) and not to LFA-1. However, domains 3, 4, and 5 are also important for the accessibility of the LFA-1 binding site (Staunton et al 1990), and the 241R variant may affect LFA-1 binding. Another putative mechanism is sterical hindrance of the important ICAM-1 dimerisation by the 241R variant.

11.4. CONCLUSION

The *ICAM1* exon 6 A→G SNP encoding the ICAM-1 K469E variant is not in itself associated with T1DM. In contrast, the A allele of the

ICAM1 exon 4 G→A SNP encoding the ICAM-1 241R variant is associated with protection against T1DM. The mechanism by which this protection is conferred remains to be demonstrated experimentally.

12. *CTLA4 (IDDM12)* AND *IDDM13* – A GENE AND A LOCUS CONFERRING RISK TO T1DM

As described in the introduction to chapter 8, much focus in T1DM genetics has been directed to chromosome 2q12-35 due to the homology of this region with the *Idd5* susceptibility locus in the NOD mouse. This instigated two partial genome linkage scans which identified two T1DM loci, *IDDM12* at 2q33 with the CTLA-4 gene as the prime T1DM candidate gene and *IDDM13* at 2q34-35. This chapter discusses the role of CTLA-4 in T1DM, the genetic studies of the CTLA-4 gene in T1DM, the consequences of genetic studies of the CTLA-4 gene for future genetic studies of complex diseases, and finally the studies evaluating the *IDDM13* locus and the T1DM candidate genes investigated at this locus.

12.1. CTLA-4 – A NEGATIVE REGULATOR OF T-CELL ACTIVITY

Autoimmune diseases arises from the failure of maintaining tolerance to "self"-proteins via deletion or neutralization of autoreactive T-cells (Kamradt et al 2001). In addition to negative thymic selection, peripheral tolerance and active regulation are of importance in autoimmunity (Kamradt et al 2001, Walker et al 2002, Bluestone et al 2003).

The cytotoxic T lymphocyte antigen-4 (CTLA-4) is a member of the B7-CD28 superfamily of co-stimulatory molecules of major importance in T-cell regulation (Sharpe et al 2002). The CTLA-4 molecule is a key negative regulator of T-cell activity and proliferation, and of major importance in the maintenance of peripheral tolerance (Walunas et al 1994, Tivol et al 1995, Waterhouse et al 1995, Lee et al 1998, Takahashi et al 2000, Salomon et al 2001, Manzotti et al 2002, Eagar et al 2002, Chikuma et al 2003). CTLA-4 is expressed on $CD4^+$ T-cells upon CD28/B7 engagement (Alegre et al 1996, Walunas et al 1996) and is constitutively expressed on $CD4^+CD25^+$ T_r cells (Takahashi et al 2000). Signalling via CTLA-4 augments the suppressive functions of the T_r cells (Read et al 2000, Takahashi et al 2000, Manzotti et al 2002), and this suppressive function is critical for the prevention of autoimmune diseases (Sakaguchi et al 1995, Asano et al 1996, Chatenoud et al 2001, Bluestone et al 2003, Alyanakian et al 2003). A soluble CTLA-4 splice variant lacking the exon-3 encoded transmembrane region is produced in man (Magistrelli et al 1999, Oaks et al 2000a, Oaks et al 2000b). CTLA-4 auto-antibodies have been demonstrated in several human autoimmune conditions (Matsui et al 1999, Matsui et al 2001) and are believed to antagonise CTLA-4/B7 interaction.

12.2. A ROLE OF CTLA-4 IN T1DM?

CTLA-4 has been suggested to be involved in diabetes development in the NOD mouse. Early (<12 days of age) anti-CTLA-4 mAb treatment of BDC2.5 transgenic NOD mice, that carry the rearranged TCR genes from a $CD4^+$, β-cell specific, diabetogenic T-cell clone isolated from a NOD mouse, precipitates diabetes rapidly, and the T-cells in the islet infiltrate have a more aggressive phenotype. Late treatment (>17 days of age) does not affect incidence or onset (Luhder et al 1998). The time of critical action of CTLA-4 in dampening the autoimmune reaction seems to be when activated $CD4^+$ T-cells migrate to the target tissue and re-encounter antigen (Luhder et al 2000). Several studies have manipulated the CTLA-4/CD28:B7 co-stimulatory system in the NOD mouse and these studies point to an important role of this system in the pathogenesis of diabetes in the NOD mouse (reviewed in Salomon et al 2001).

The general functions of CTLA-4 and its putative involvement in β-cell destruction qualify *CTLA4* as candidate gene in relation to the pathogenetic model in Figure 1. Moreover, *CTLA4* is a positional candidate gene. *Ctla4* encoding the murine CTLA-4 maps to the *Idd5.1* locus (Hill et al 2000, Lamhamedi-Cherradi et al 2001) – a locus also linked to a NOD mouse apoptosis resistant lymphocyte phenotype, which resembles the T-cell apoptosis resistance observed in CTLA-4 knockout mice (Colucci et al 1997, Bergman et al 2001). *CTLA4* maps to *IDDM12* (Nistico et al 1996) and *CTLA4* variants are associated with several autoimmune diseases (Kristiansen et al 2000d). Thus, we and others have investigated variants in *CTLA4* in relation to T1DM as described in the next section.

12.3. STUDIES OF THE *IDDM12/CTLA4* LOCUS IN T1DM

After the initial observation of linkage of the *D2S72-CTLA4-D2S116* region in Italian T1DM families (Nistico et al 1996), numerous investigations of the relation between T1DM and variants in and close to *CTLA4* were undertaken (**Table 17** and **Table 18**). These studies focused on a *CTLA4+49A>G* SNP, encoding a threonine to alanine substitution at codon 17 in the CTLA-4 leader peptide and a *CTLA4+5,378(AT)$_n$* in the 3'UTR. As the two polymorphisms are in strong LD (Marron et al 1997, Esposito et al 1998, Larsen et al 1999, Ueda et al 2003), only the family-based TDT data and the case-con-

Table 17. TDT studies of the *CTLA4+49A>G* SNP in T1DM.

Population	Families (N)	Transmitted (T) allele *+49A*	Transmitted (T) allele *+49G*	*+49G* (%T)	P<0.05	References
Chinese	31	10	8	44		Marron et al 1997
Danish	254	145	166	53		Larsen et al 1999
French	44	27	40	60		Marron et al 1997
French	70	27	43	61		Fajardy et al 2002
German	109	39	71	65	0.002	Donner et al 1998
Italian	187	75	114	60	0.004	Nistico et al 1996
Korean	41	3	13	77	0.03	Marron et al 1997
MA*	99	26	42	62		Marron et al 2000
N. Ireland	297	133	173	57	0.02	McCormack et al 2001
Romanian	204	84	102	55		Guja et al 2002
Russian	56	11	39	78	<0.0001	Christiakov et al 2001
Sardinian	123	46	45	49		Nistico et al 1996
Spanish	44	18	40	69	0.004	Nistico et al 1996
Spanish	39	26	17	40		Larsen et al 1999
UK	284	253	264	51		Nistico et al 1996
US	301	265	304	53		Marron et al 1997
Combined	2183	1188	1481	55	2.1×10^{-7}	All studies

All available TDT data reported excluding Ueda et al 2003. *: Mexican-American. Due to overlap between some study populations, the studies with the largest number of investigated families are included. Thus, some studies reporting TDT data on this SNP have been omitted. Other *CTLA4* variants investigated in T1DM prior to 2000 are reviewed in Kristiansen et al 2000d.

Table 18. Case-control studies of the *CTLA4+49A>G* SNP in T1DM.

	Genotypes				
Population/Group	**GG**	**GA**	**AA**	**P<0.05**	**References**
Belgian/T1DM	75	269	181	<0.0013	Nistico et al 1996 and Van der Auwera et al 1997
Belgian/Control	51	242	236		
Chinese/T1DM	74	166	110	<0.0001	Osei-Hyiaman et al 2001
Chinese/Control	42	177	201		
Chinese/T1DM	81	82	17	–	Marron et al 1997
Chinese/Control	196	148	35		
Chinese/T1DM	150	85	18	<0.01	Lee et al 2000
Chinese/Control	37	45	9		
Chinese/T1DM	15	11	5	<0.01	Ma et al 2002
Chinese/Control	8	9	19		
Czech/T1DM	57	125	123	–	Cinek et al 2002
Czech/Control	50	133	106		
Filipinos/T1DM	37	35	18	<0.02	Klitz et al 2002
Filipinos/Control	21	43	30		
French/T1DM	34	41	37	<0.03	Djilali-Saiah et al 1998
French/Control	16	37	47		
French/T1DM	17	76	41	–	Fajardy et al 2002
French/Control	31	146	96		
French/T1DM	49	10	3	<0.0001	Ongagna et al 2002
French/Control	14	27	43		
German/T1DM*	55	147	91	<0.03	Donner et al 1997
German/Control*	41	149	135		
Japanese/T1DM	72	80	21	–	Awata et al 1998
Japanese/Control	170	197	58		
Japanese/T1DM	54	42	21	–	Hayashi et al 1999
Japanese/Control	72	47	21		
Japanese/T1DM	33	25	16	–	Takara et al 2000
Japanese/Control	30	43	34		
Japanese/T1DM	57	62	6	0.005	Kikuoka et al 2001
Japanese/Control	78	88	34		
Japanese/T1DM	44	36	17	–	Mochizuki et al 2003
Japanese/Control	21	27	12		
Korean/T1DM	57	38	2	–	Marron et al 1997
Korean/Control	56	44	12		
Moroccan/T1DM	7	52	59	–	Bouqbis et al 2003
Moroccan/Control	8	47	59		
Polish/T1DM	60	95	37	<0.003	Krokowski et al 1998
Polish/Control	21	76	39		
Spanish/T1DM	6	37	46	–	Marron et al 1997
Spanish/Control	5	29	23		
Tunisians/T1DM	32	38	4	<0.01	Kamoun Abid et al 2001
Tunisians/Control	11	28	10		
W. African/T1DM	9	67	106	–	Osei-Hyiaman et al 2001
W. African/Control	11	61	129		
All – T1DM	1075	1619	979	1×10^{-11}	All studies above
All – Control	990	1843	1388		

Studies including patients from family-based studies were excluded and only studies reporting the full genotype frequencies were included. Heterogeneity between genotypes in control and T1DM cohorts evaluated by 2 × 3 contingency-tables. *: The T1DM and the control cohorts comprised approximately $^2/_3$ Germans and $^1/_3$ Canadians. The final two rows represent the "crude" sum of the above studies. As "significant" association-studies have better chances of publication these numbers may be marginally biased. All other *CTLA4* variants investigated in T1DM prior to 2000 were reviewed in Kristiansen et al 2000d.

trol data of the *CTLA4+49A>G* SNP are detailed in Table 17 and Table 18, respectively.

The T1DM intra-familial association of the *+49G* allele to T1DM is evident from Table 17, although significant intra-familial association was found in less than 40% of the cohorts. All cohorts, including the Danish (Larsen et al 1999), evaluating in excess of 100 *CTLA4+49A>G* transmissions have *nominally* increased *+49G* transmission. A similar (52.1%) increased transmission pattern of *+49G* was found in 3,671 Caucasian T1DM families with some overlap (~1,050 families) between the cohorts in Table 17 (Ueda et al 2003). The *CTLA4+5,378(AT)$_n$* alleles in LD with the *+49G* variant also associate to T1DM in intra-familial studies (Nistico et al 1996, Esposito et al 1998, Donner et al 1998, Larsen et al 1999, Marron et al 2000, Turpeinen et al 2003).

The "crude" combined association analysis in Table 18 strongly indicates association between the *+49G* and T1DM. T1DM relative risks of 1.15 (1.11-1.19) and 1.18 (1.11-1.25) were estimated for the *+49G* allele and *+49G* carrier-status, respectively (Table 18). These relative risk estimates are comparable to the T1DM relative risk of 1.09 conferred by *+49G* in another cohort Caucasians (Ueda et al 2003).

We observed *+49G* transmission rates of 53% and 47% in T1DM offspring and unaffected siblings, respectively, similar to findings in other high-to-medium T1DM risk European populations (Nistico

et al 1996, Marron et al 1997, McCormack et al 2001, Ueda et al 2003), but we were unable to show linkage and association of this variant to T1DM independently in Danes (Larsen et al 1999). Our *CTLA4+5,378(AT)$_n$* data were also comparable with those found in other populations (Nistico et al 1996, Marron et al 1997, Esposito et al 1998, Larsen et al 1999, Marron et al 2000). The *(AT)$_{16}$* and *(AT)$_{17}$* alleles were in LD with the *+49G* allele and both alleles displayed a nominally increased transmission – the *(AT)$_{17}$* allele significantly – but with a limited number of transmissions. Thus, we were unable to demonstrate independent linkage and association of the two *CTLA4* variants in Danes (Larsen et al 1999). Nevertheless, our findings support a role of genetic variants in or close to *CTLA4* in T1DM Caucasian Danes as our observations are equivalent to those of ethnically comparable populations (Table 17, Ueda et al 2003). The most likely explanation of why we did not demonstrate linkage and association with the variants is lack of power as a consequence of the limited number of families. Moreover, our data contribute to the "overall" evidence for a real *IDDM12* T1DM risk gene variant in or close to *CTLA4.*

The results of the studies in Tables 17 and 18 are indicative of a etiological T1DM gene variant close to or in *CTLA4* at *IDDM12* locus on 2q33, but only one study (Marron et al 2000) aimed at identification of the *IDDM12* etiological variant or a more narrow delimitation of *IDDM12.* Hence, the *IDDM12* T1DM risk variant could map to any part of this locus, including any of the three candidate genes – *CD28* (the gene encoding CD28, an important T-cell co-stimulatory receptor and receptor for the B7 (CD80 and CD86) ligands/receptors on APCs, Salomon et al 2001), *CTLA4* and *ICOS* (the gene encoding the *inducible co-stimulator,* Coyle et al 2000, Tafuri et al 2001) – all mapping to *IDDM12* at 2q33 (Ling et al 2001). Thus, in a comprehensive effort to identify the general etiological autoimmune risk variant (Kristiansen et al 2000d) at the *IDDM12* locus and functional characterisation of the variant, 108 SNPs were identified in 330 kb region including *CTLA4, CD28* and *ICOS* (Ueda et al 2003). The SNPs in *CD28* and *ICOS* were excluded as T1DM and autoimmunity risk variants (Ueda et al 2003) and the exclusion of *CD28* was supported (Marron et al 2000). Eight SNPs mapping to two Graves' disease association-peaks in and around a *CTLA4,* the *CTLA4+49A/G* mapping between the peaks and two markers 3' of *ICOS* were tested in 3,671 T1DM Caucasian families. Independent association was found with four SNPs in or close to *CTLA4,* but not with the *ICOS* markers. Association was strongest with the markers *rs1863800* and *MH30* from the first peak mapping 20-53 kb 5' of *CTLA4* and the haplotypes comprising the *CT60* G allele in the second peak mapping 0.2-6.3 kb 3' of the end of the *CTLA4* transcript. The markers from the 5'-peak could not be excluded as causal markers by regression analysis, but the functional studies and the Graves' disease association data suggested the common etiological autoimmune (and T1DM) variant to be situated in the 6.1 kb region 3' of *CTLA4,* with the *CT60* G allele being the best marker for the risk haplotype (Ueda et al 2003). The *+49A>G* SNP was excluded as the culprit variant, but was in LD with the *CT60 (CTLA4+6230G>A)* mapping 279 bp distal to the polyadenylation (polyA) site of the predominant 2 kb transcript of *CTLA4.* The effect of the *CT60* variants on T1DM risk was limited with a relative risk of 1.14 (1.07-1.21) conferred by the G-allele. Importantly and in line with the observations of the *+49G/A* variant in the Danish T1DM families (Larsen et al 1999), transmissions rates of the *CT60* G risk allele to T1DM offspring and unaffected siblings were 53% and 47%, respectively (Ueda et al 2003). This strengthens the association data and rules out preferential segregation distortion.

An explanation of the discrete effect of the *CTLA4* 3' 6.1 kb region variants on T1DM risk may reside in the populations investigated (Ueda et al 2003) as UK, Finish, US, Romanian or Norwegian T1DM cohorts have not individually demonstrated association with the *+49A/G* SNP (Table 17, Turpeinen et al 2003). The transmission distortion of the *+49G* allele is more profound in populations of Mediterranean-European ancestry, in Mexican-Americans and in some African and Asian populations compared to Northern European and US families (Tables 17 and 18, Nistico et al 1996, Marron et al 1997, Marron et al 2000). Therefore, investigations of the *CTLA4* 3' 6.1 kb region variants in these populations are warranted.

Functionally, the *CT60* G allele associates with decreased steady-state levels of mRNA encoding the soluble CTLA-4 molecule (*sCTLA4*) in CD4$^+$ T-cells. In *in vivo* unstimulated CD4$^+$ T-cells from "disease-susceptible" *CT60* G/G individuals the ratio of *sCTLA4* to *flCTLA4* (encoding the full length membrane bound CTLA-4 molecule) mRNA isoforms was 50% lower compared to that in A/A "protected" individuals, and an allele-dose effect of the *CT60* SNP variants on this ratio was demonstrated (Ueda et al 2003).

Thus, the common autoimmune susceptibility variant(s) at 2q33 in man maps to a non-coding 6.1 kb 3' region of *CTLA4* and the marker for disease susceptibility, i.e. the *CT60* G allele, associates with lower mRNA levels of the soluble alternative splice-form of CTLA-4 (Ueda et al 2003).

These findings naturally raise some important questions. If the *sCTLA4* mRNA levels are reflected in a corresponding 25-50% reduction in soluble CTLA-4 produced and secreted by T-cells *in vivo* in individual hetero- and homozygous for the *CT60* G allele, respectively – which is yet to be demonstrated – then how does it relate to the effect of *CTLA4* variants in T-cells, and by which mechanisms do decreased levels of soluble CTLA-4 affect T-cell regulation, autoimmunity and T1DM development?

First, *sCTLA4* mRNA is translated, secreted and circulating in human serum (Magistrelli et al 1999, Oaks et al 2000a, Oaks et al 2000b) and recombinant soluble CTLA-4 inhibits the mixed leukocyte response in a dose-dependent manner (Oaks et al 2000a), but the *in vivo* functional impact of the soluble CTLA-4 molecule on T-cell regulation and autoimmunity remains to be elucidated. In contrast to what might be anticipated from the findings by Ueda et al, soluble CTLA-4 is found at higher concentrations in individuals with autoimmune thyroid disease (Oaks et al 2000b), but is also found in healthy individuals (Magistrelli et al 1999).

Second, functionality of *CTLA4* variants has been reported. The +49G and the *(AT)$_{16\text{-}17}$* 3'UTR alleles, all in strong LD with the *CT60* G allele, associate with increased T-cell activation and proliferation *in vitro* (Kouki et al 2000, Huang et al 2000, Maurer et al 2002, Takara et al 2003), decreased number of peripheral blood mononuclear cells (PBMC) expressing surface CTLA-4 (~decreased number of CD4$^+$CD25$^+$ T$_r$ cells), decreased unstimulated cell surface CTLA-4 expression in CD4$^+$ T-cells, decreased *CTLA4* mRNA expression in PBMC (Ligers et al 2001), decreased cell surface/total CTLA-4 ratio and incomplete glycosylation of CTLA-4 in *CTLA4* transiently transfected COS-1 cells (Anjos et al 2002).

Third and more hypothetically, reduction in soluble CTLA-4 levels may lead to reduced blockade of the B7 (CD80/CD86) receptors, causing increased co-activation through CD28 in T-cells and/or less stimulation of the B7 receptors on immature DC, leading to decreased tryptophan catabolism in these cells resulting in reduced inhibition of proliferation and decreased apoptosis of proximal T-cells (Finger et al 2002). Accordingly, CTLA4-Ig induces indoleamine 2,3 dioxygenase (IDO) expression in DC via B7 receptor interaction leading to increased catabolism of tryptophan *in vivo* (Grohmann et al 2002, Mellor et al 2003).

The subset(s) of T-cells expressing *sCTLA4* mRNA and secreting soluble CTLA-4 in the unstimulated state (Ueda et al 2003) has yet to be defined. The CD4$^+$CD25$^+$ T$_r$ cells constitutively expressing membrane CTLA-4 (Takahashi et al 2000) are a likely source. Moreover, CD4$^+$CD25$^+$ T$_r$ cells initiate tryptophan catabolism in DC through a membrane-bound CTLA-4 dependent mechanism *in vitro* (Fallarino et al 2003).

Interestingly, a homologous variant to the *CT60* SNP is not present in the NOD mouse; Rather a translationally-silent change in exon 2 resulting in a *Ctla4* mRNA splice form encoding a trans-

membrane isoform of CTLA-4 without the CD86/CD80 binding domain was found in a diabetes-resistant strain and not in NOD mice. The ligand-independent isoform is expected to protect against autoimmunity by raising the threshold for activation of T-cells (Ueda et al 2003).

12.4. CONSEQUENCES OF THE GENETIC STUDIES OF *CTLA4* IN T1DM FOR FUTURE GENETIC STUDIES IN COMPLEX DISEASES

The study by Ueda and al is a major break-through for positional cloning in common diseases in man, although several functional and genetic details remain to be elucidated. In addition to the actual findings, this study has implications for the planning of future study of the genetics of T1DM and other complex diseases. The *IDDM12* locus was not identified by the genome-wide scans (ECIGS 2001, Cox et al 2001a) using the requested criteria for genome-wide "suggestive" linkage of MLS ≥ 2.2 (Lander et al 1995). Thus, the T1DM susceptibility variant in *CTLA4* may have been missed if a candidate gene approach had not been applied in a population with strong disease association (Nistico et al 1996). This underscores the fact that other loci – not identified in the genome scans – may harbour minor T1DM genes and that even discrete association or transmission distortion may be of importance. The latter has been substantiated for genes investigated in other complex diseases (Lohmueller et al 2003).

Of even more concern is the number of families needed to demonstrate significant association with a minor – but well-qualified – T1DM gene variant. Even in this large material, it was impossible to determine the culprit variant by positional cloning. This underscores the need for establishing large scale internationally consortia-based T1DM study groups, which should preferably include animal facilities, access to patient materials and facilities for functional studies.

The location of the culprit variants in both mouse and man indicates that no SNP should initially be short of suspicion and points to splice-variants as "suspects" in common diseases (Cartegni et al 2002). The importance of screening the regulatory regions in search for candidate gene variants for complex traits has been documented. The *IDDM12* and *Idd5.1* variants both affect *CTLA4* splicing, but in different ways. Still, both lead to increased risk of β-cell destruction. Thus, caution must be observed before drawing parallels from causal disease variants in rodents to disease variants in humans.

The *CT60* SNP has two frequent alleles providing additional support of the "common allele – common disease" hypothesis (Lander 1996, Lohmueller et al 2003), keeping in mind that rare alleles or haplotypes may also contribute to common diseases.

12.5. THE *IDDM13* LOCUS ON 2q34 – A T1DM LOCUS?

This locus was identified as a positional candidate locus due to its homology to the *Idd5* locus, now the *Idd5.2* homologue (Hill et al 2000).

Linkage to the region *D2S137-D2S301-D2S164* was initially found in 98 ASP (Morahan et al 1996). Increased sharing was mainly found in ASP not sharing HLA and in families with excess affected females. Genome-wide (ECIGS 2001, Cox et al 2001a) and partial scans (Copeman et al 1995, Esposito et al 1998) were unable to confirm linkage by ASP analysis, but some markers at this locus displayed distorted transmission in UK/US T1DM offspring (Copeman et al 1995, Esposito et al 1998), and association between T1DM and the frequent *D2S137* 137 mu allele was observed in Japanese patients (Fu et al 1998). We evaluated five previously T1DM associated markers at the locus and found association between T1DM and frequent alleles of the three markers mapping at the central part of the region; *D2S137, D2S164* and *D2S1471* (Larsen et al 1999). The central marker *D2S164* was evaluated in a Spanish T1DM family material and distorted transmission of the two most frequent alleles was found (Larsen et al 1999). Although transmission distortion of the two alleles was oppositely directed in the two family materials, these observations provided supportive evidence for *IDDM13* as a T1DM locus. We and others have not been able to confirm the initially observed effect of HLA and gender (Esposito et al 1998, Larsen et al 1999), whereas the strongest association in Japanese patients was observed in the HLA low(er) risk groups (Fu et al 1998). Our study suggests that the causal T1DM gene maps to the ~3.5 cM between *D2S137* and *D2S1471* (Larsen et al 1999).

Association studies of candidate genes and microsatellite markers for candidate genes mapping to *IDDM13* have been performed (**Table 19**), but so far no T1DM significant gene association has been reported. However, the *NRAMP1* gene, encoding a protein with pleitropic effects on macrophage function (Blackwell 1996), is still considered a candidate gene as two investigations demonstrated trends for association to T1DM (Esposito et al 1998, Bassuny et al 2002).

12.6. CONCLUSION

The *IDDM12* is a confirmed T1DM locus and in addition a common autoimmunity locus. The genetic culprit variant maps to a 6.1 kb region 3' of *CTLA4* and the common allelic variant affects *CTLA4* mRNA splicing and results in decreased levels of *sCTLA4* mRNA. This is thought to cause a reduction in peripheral T-cells tolerance leading to autoimmunity. Studies clarifying several of the functional issues are still in demand. Although association has not formally been demonstrated, this variant is thought to confer risk of T1DM in Danes. The main lessons learned from the genetic studies evaluating *CTLA4* in T1DM are that very large cohorts of families and functional studies are in needed for the future dissection of the genetics of complex diseases.

Accumulating evidence is in favour of *IDDM13* on chromosome 2q34 as an etiological T1DM locus. The search for candidate genes has been inconclusive, and an approach as the one used for *IDDM12* is required. A genetic variant in or close to *NRAMP1* affecting some aspect of NRAMP1 action or expression is considered to be the prime "suspect".

13. THE ROLES OF THE HUMAN FAS AND FAS LIGAND GENES IN T1DM

We have identified and evaluated genetic variants in the human Fas (*FAS,* also known as *TNFRSF6, APT1, APO1, CD95,* OMIM # 134637) and Fas ligand (*FASL,* also known as *TNFSF6, APT1LG1, CD95L, CD178,* OMIM # 134638) genes as candidate genes in T1DM. This chapter therefore discusses the role of apoptosis in T1DM, the role of Fas-Fas ligand (FasL) interaction in apoptosis, the "pro's" and "con's" for involvement of the Fas-FasL interaction in the pathogenesis of T1DM and finally the results of our genetic studies.

13.1. APOPTOSIS – THE FATE OF MOST β-CELLS IN T1DM, AND OF T-CELLS DURING THYMIC SELECTION AND UPON ACTIVATION-INDUCED CELL DEATH

Eukaryotic cells may die in two conceptually different ways: one is necrosis induced by thermal, physical, ischemic or chemical cell injury and the other is apoptosis (Kerr et al 1972, Wyllie 1997).

The term apoptosis, or programmed cell death, is used to describe the regulated energy requiring disposal of a cell (Kerr et al 1972, Wyllie 1997). All mammalian cells have the machinery required for

Table 19. Candidate genes investigated at the *IDDM13* locus.

Candidate gene	OMIM #	Polymorphism	Reference
PTPRN/IA2	601773	*D2S1753E* (intron 2)	Nishino et al 2001
PTPRN/IA2		*D2S1753E* and *D2S31F23*	Esposito et al 1998
IGFBP5	146734	$(CA)_n$-repeat (intron)	Owerbach et al 1997
NRAMP1	600266	*Bsr* I RFLP (intron 13)	Copeman et al 1995
NRAMP1		$(GT)_n$-repeat (promoter)	Bassuny et al 2002
NRAMP1		$(GT)_n$-repeat (promoter)	Esposito et al 1998

apoptosis, and apoptosis is required for the normal maintenance of development and homeostasis in a cell-system, an organ or an individual as a whole (Jacobson et al 1997, Vaux et al 1999, Meier et al 2000).

During the apoptotic cell death, as opposed to necrotic cell death, the cell does not swell and burst, but rather "wraps up" all the cellular components into so-called apoptotic bodies that are phagocytised before the intracellular content leaks, thereby strongly reducing or preventing the risk of evoking an autoimmune response (Wyllie 1997). The latter, however, may not always be the case (Casciola-Rosen et al 1994, Utz et al 1997, Kurts et al 1998) and therefore apoptosis may lead to autoimmunity. Apoptosis may be induced by signals delivered by cell surface ligands, viral infections, cytokines, absence of supporting growth factors and other stimuli (Nagata 1997, Wyllie 1997, Hengartner 2000).

When thymocytes are deleted by negative selection in the thymus they die by apoptosis (Surh et al 1994, Savill et al 2000) and apoptosis – mainly regulated by Fas-FasL interaction – has a pivotal role in peripheral lymphocyte homeostasis, both in activation-induced cell death in the down-regulation of an immune response and in peripheral clonal deletion of autoreactive T-cells (Krammer 2000, Green et al 2003).

Beta-cell apoptosis is a physiological feature during normal development in both mouse (Trudeau et al 2000), rats (Scaglia et al 1997, Petrik et al 1998, Trudeau et al 2000) and humans (Kassem et al 2000). Human, rat and mouse neonates have a period of physiologically increased β-cell apoptosis (Scaglia et al 1997, Petrik et al 1998, Trudeau et al 2000, Kassem et al 2000). This period of increased β-cell apoptosis has been hypothesised to contribute to the autoimmune reaction against the β-cells in genetically susceptible individuals (Trudeau et al 2000, Mathis et al 2001).

Apoptosis is the mode of β-cell death responsible for the development of spontaneous diabetes in the NOD mouse (O'Brien et al 1997, Augstein et al 1998) and in the DP-BB rat (Lally et al 2001). Apoptosis of β-cells in pancreatic biopsies from recent onset T1DM patients has not been demonstrated (Moriwaki et al 1999), but apoptosis was demonstrated in Fas^+ β-cells adjacent to $FasL^+CD3^+$ cells in pancreatic autopsies from two recent onset T1DM patients dying in ketoacidosis (Stassi et al 1997). A more formal proof of apoptosis as the mode of β-cell death in man will be difficult to establish considering the rapid clearance (a few minutes) of apoptotic β-cells (Kurrer et al 1997, O'Brien et al 1997) and the time span of months to years of β-cell destruction.

The cellular pathways leading to apoptosis in cells in general and in β-cells in particular have been reviewed by others (Hengartner 2000, Mandrup-Poulsen 2001, Eizirik et al 2001a, Eizirik et al 2001b, Gaur et al 2003) and will not be detailed further in this review.

13.2. THE ROLES OF FAS AND THE FASL IN THE INDUCTION OF APOPTOSIS

Fas and FasL are two key surface molecules involved in the induction of apoptosis and in this section their functions are briefly sketched.

Interaction between membrane bound Fas and the surface bound FasL induces pro-apoptotic signals in the Fas bearing cell, **Figure 8**.

Although Fas mediated signals are important for apoptosis induction, it is by no means the only surface molecule able to induce pro-apoptotic signalling, and many pathways and molecules with anti-apoptotic properties exist. Hence, the cell fate is determined by a balance between pro- and anti-apoptotic components (Hengartner 2000, Gaur et al 2003).

13.3. FAS-FASL INDUCED APOPTOSIS IN T1DM – IMPACT ON IMMUNE REGULATION, IMPACT ON THE TARGET β-CELL OR BOTH?

The role of the Fas/FasL system in T1DM will be reviewed briefly here based on studies in humans, in spontaneous T1DM models and models with "*controlled*" genetic modifications, **Table 20**.

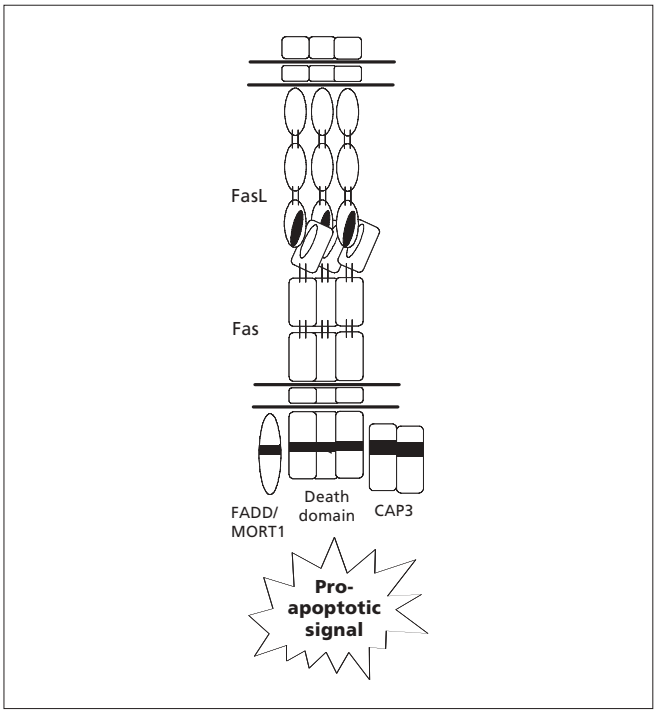

Figure 8. Fas and FasL interaction induces pro-apoptotic cellular signalling in the Fas expressing cell. The human Fas molecule is a widely expressed type I transmembrane cell surface receptor molecule. The cytoplasmic tail of Fas – the death domain is essential for transduction of the apoptotic signal. Fas expression is boosted by cytokines, viral infection of cells and upon activation of lymphocytes (Leithauser et al 1993, Krammer 2000). The FasL, the natural ligand for Fas, is a type II membrane protein belonging to the tumor necrosis factor (TNF) family of molecules. FasL is predominantly expressed in activated in activated T-lymphocytes, in NK cells and in immune privileged sites such as the testes and the eye (Suda et al 1993, Nagata 1997), but can be expressed in other cells, including human β-cells (Loweth et al 1998, Maedler et al 2001). Trimerisation of FasL and Fas, respectively, is required for signal transduction. Upon FasL-Fas ligation a complex of proteins associates with the activated Fas trimere within seconds. This complex – designated the death-inducing signalling complex, induces a pro-apoptotic signal in the Fas-bearing cell (Nagata 1999, Hengartner 2000, Krammer 2000). For more detailed information on the downstream pro- and anti-apoptotic cellular pathways see the above references.

The studies in Table 20 strongly imply that the Fas-FasL system plays a role in T1DM, even in the light of a number of controversial observations. However, 1) the exact mechanism(s) by which it contributes to β-cell destruction still remains to be clarified, 2) it seems well-established that expression of Fas on β-cells is not necessary for, but it facilitates β-cell destruction; 3) it is still unclear whether β-cells can commit suicide or fratricide via Fas-FasL interaction and 4) the impact of the Fas-FasL system on the developing and acting immune system in T1DM is not fully characterised.

In spite of these limitations, the Fas and FasL genes are strong candidate genes in T1DM and we have screened *FAS* and *FASL* for variations and evaluated identified common variants in relation to T1DM as detailed in the following section.

13.4. COMMON *FAS* AND *FASL* VARIANTS IN T1DM SUSCEPTIBILITY

The human Fas and FasL genes map to chromosomes 10q24.1 (Inazawa et al 1992) and 1q23 (Takahashi et al 1994), respectively. The T1DM genome-scans published prior to (Hashimoto et al 1994, Davies et al 1994, Mein et al 1998, Concannon et al 1998) and after (ECIGS 2001, Cox et al 2001a) our investigations of *FAS* (Nolsøe et al 2000) and *FASL* (Nolsøe et al 2002) did not qualify the two genes as positional candidate genes. However, the recent neural network and data mining evaluation of the Scandinavian genome-scan data (Pociot et al 2004) identified a susceptibility locus on chromosome 10q24 mapping 115 cM (*D10S583*) from the 10ptel with the nearest

Table 20. Pro's and Con's for an impact of the Fas/FasL system in T1DM pathogenesis.

Pro	β/T*	Con	β/T*
Fas expression is induced on mouse (1, 2), rat (3) and human (4, 5) β-cell upon exposure to cytokines	β	Fas is not expressed on β-cells isolated from NOD mice spontaneously developing diabetes evaluated by flow cytometry, but on β-cells in accelerated NOD mouse models (2, 6)	β
Fas is expressed on human β-cells in inflamed islets from newly diagnosed patients and infiltrating T-cells express FasL (7, 8). β-cell apoptosis in Fas^+ β-cells close to $FasL^+$ T-cells is found by some (8) but not others (7).	β	Fas is not expressed on β-cells *in situ*, but on infiltrating cells during spontaneous diabetes development in NOD mice (9, 10)	β
Fas is expressed a higher levels in islets of 15 weeks old female NOD mice compared to males (11). Fas is progressively expressed on NOD β-cells from week 3 through week 12 (12)	β		
Soluble FasL induces apoptosis in mouse β-cells expressing cytokine induced Fas *in vitro* (2)	β		
$CD4^+$ 4.1 β-cell specific TCR^+ T-cells kill Fas^+ but not Fas- β-cells *in vitro* (13)	β		
FasL is expressed on human β-cells *in situ* (14, 15)	β	FasL is not expressed on human β-cells in infiltrated islets from newly diagnosed patients, but on infiltrating cells (7, 8)	β
FasL is expressed constitutively on NOD β-cells *in situ* (9)	β	FasL not expressed on NOD β-cells from "old" mice evaluated by cytometry (2) or in infiltrated islet of NOD mice (10, 13), but on flow glucagon-producing α-cells and not on infiltrating cells (10)	β
RIP-FasL (16-19) and HIP-FasL (20) transgenic NOD mice displays increased diabetes incidence and rapid onset, in two cases with (17, 18) and two cases without (19, 20) neutrophilic infiltration	β		
RIP-FasL (19) and HIP-FasL (20) NOD *Scid* transgenic, who have normal neutrophil counts but no lymphocytes, do not develop diabetes	T		
Viral infection can induce expression of both Fas and FasL in β-cells with a resulting Fas/FasL-mediated β-cell fratricide and diabetes (21)	β		
β-cell specific expression of a defective Fas receptor inhibiting Fas trimerisation reduces diabetes incidence in NOD transgenic mice in a dose dependent fashion (19)	β	β-cell specific Fas death-domain deficient TCR-HA Ins-HA mice develop autoimmune diabetes (22)	β
NOD mice display a deficit in central tolerance induction due to diminished deletion of semi-mature thymocytes *in vivo* and *in vitro*, and the defect applies to both Fas-independent and Fas-dependent pathways of apoptosis (23)	T		
NOD mice T lymphocytes are more resistant to activation induced cell death than T-cell from NOR and C57BL/6T mice and express lower levels of *Fas* and *Fasl* mRNA (24)	T		
NOD mice carry a low activity FasL molecule (25)	T	NOD mice carry a low activity FasL molecule (25)	β
Reduced T-cell FasL expression prevents NOD diabetes (26)	T		
Human $CD4^+$ and $CD8^+$ T-cells from T1DM patients have defective (low) expression of Fas *in vivo* and upon anti-CD3 stimulation *in vitro* (27)	T		
Anti-FasL treatment from 2-4 weeks of age and from 5-15 weeks of age prevents and delays onset of diabetes, respectively (28). Due to deletion of $CD4^+CD45RB^{low}$ memory cells (29) or action at the target organ? Remains to be elucidated	T/β		

Studies utilising a) transplantation of islets, b) adoptive transfer of primed and un-primed immune cells, c) chemically accelerated or induced diabetes, and d) the $NOD^{-lpr/-lpr}$ mouse (a transgenic NOD mouse with a defective Fas gene (Chervonsky et al 1997, Itoh et al 1997) are not included as these studies are potentially flawed: a-c) do not necessarily reflect the mechanisms underlying the spontaneous development of diabetes in rodents, and 4) the pleiotropic effect the *lpr* mutation on the immune system and the β-cells render these studies inconclusive at to whether the effect is exerted at the target organ or in the immune system (Kim et al 2000). *: Site of action. β: β-cells and T: T-cells. RIP: rat insulin promoter, HIP: human insulin promoter, HA: (influenza) hemagglutinin, NOR: non-obese diabetes resistant mouse. 1) Yamada et al 1996; 2) Thomas et al 1999; 3) Kuttler et al 2003; 4) Stassi et al 1995; 5) Loweth et al 2000; 6) Darwiche et al 2003; 7) Moriwaki et al 1999; 8) Stassi et al 1997; 9) Reddy et al 2003; 10) Signore et al 1997; 11) In gelsson et al 1998; 12) Walter et al 2000; 13) Amrani et al 2000; 14) Maedler et al 2001; 15) Loweth et al 1998; 16) Chervonsky et al 1997; 17) Kang et al 1997; 18) Allison et al 1997; 19) Savinov et al 2003; 20) Silva et al 2003; 21) Christen et al 2004; 22) Apostolou et al 2003; 23) Kishimoto et al 2001; 24) Decallonne et al 2003; 25) Kayagaki et al 1997; 26) Su et al 2000; 27) Giordano et al 1995; 28) Nakayama et al 2002; 29) Kim et al 2000.

markers mapping 13 (*D10S201*) and nine (*D10S192*) cM up- and down-stream, respectively. *FAS* maps 109-113 cM (*D10S541-D10S564*) from 10ptel. Moreover, unique familiar *FAS* variants which encode defective Fas protein variants were found in rare autoimmune/lymphoproliferative diseases (Rieux-Laucat et al 1995, Cheng et al 1995, Drappa et al 1996, Pensati et al 1997, Jackson et al 1999). Variation in *FASL* was also found in a patient with SLE and lymphoproliferative disease (Wu et al 1996) and finally the NOD mouse carries a *Fasl* variant encoding a low activity FasL molecule (Kayagaki et al 1997). Thus, functional variants in the two genes are clearly capable of conferring risk of autoimmunity.

13.4.1. Genetic variants in FAS – identification and relation to T1DM

The human *FAS* has nine exons spanning 26 kb (Behrmann et al 1994, Cheng et al 1995). The complete genomic sequence was not known at the time of our SSCP mutation scan (Cheng et al 1995), and therefore we scanned the promoter, all exons including intron-exon boundaries and the known 5'UTR (Nolsøe et al 2000). Although our SSCP method is sensitive (90-95%), we may have failed in identifying variants in the scanned regions, and furthermore, the intronic sequences are likely to harbour genetic variants.

We identified 15 variants (Nolsøe et al 2000), of which four (*-1377 G>A, -670A>G, g250A>G* (codon 58 in Fas) and *g154C>T* (codon 198)) were previously reported (Fiucci et al 1994, Huang et al 1997). Allele frequencies were similar to those previously reported for these variants. A total of eight promoter variants were found; two very rare variants, a *-1194A>T* SNP and a *-295Ains* altering transcription factor binding consensus sites for c-Myb, SP-1 and nuclear factor-κB (NF-κB), respectively (Nolsøe et al 2000).

The common *-690T>C, -670A>G* and *g154C>T* SNPs as well as the haplotypes arising from these SNPs were evaluated for linkage and association to T1DM in the Danish family cohort (Nolsøe et al 2000). None of the SNPs demonstrated linkage or association to T1DM. The two-SNP promoter haplotypes and the three-SNP haplotypes were also randomly transmitted to T1DM offspring. Thus, we found no suggestive evidence for a role of these variants in T1DM susceptibility. Due to limited power of our study to detect marginal transmission distortions, it cannot be excluded that these variants may confer risk to T1DM, although the likelihood of this is limited. Neither can it be excluded that the more rare variants identified or the *-1377G>A* SNP may confer susceptibility to T1DM as these variants were not evaluated (Nolsøe et al 2000).

We also genotyped a "*FAS* microsatellite marker" reported to map within 48.5 kb of *FAS* (Sangthongpitag et al 1998). Subsequent sequencing, however, has disclosed that this marker maps some 86 million bp upstream of the *FAS* promoter. The data on this marker are therefore not discussed further.

In summary, there is yet no evidence indicating that genetic variants in *FAS* confer susceptibility to T1DM, but enlarged cohorts are needed to confirm this observation. Whether the less frequent variants are associated with T1DM was not investigated.

13.4.2. FASL variants are not linked to T1DM in Danes

The human *FASL* has four exons spanning 9 kb (Takahashi et al 1994). The promoter, the four exons, intron-exon boundaries and the 5'UTR were mutation scanned by SSCP (Nolsøe et al 2002). We identified two novel SNPs in the *FASL* promoter, a *-843C>T* and a *-475A>T* SNP. These SNPs are located in a negative regulatory region of the *FASL* promoter (Holtz-Heppelmann et al 1998), but do not alter any transcription factor binding sites. Genetic variants in exons or in the 3'UTR were not observed despite an estimated power of approximately 80 and 97% for the detection of SNPs with allele frequencies of 1% and 5%, respectively (Kruglyak et al 2001).

The *FASL-843C>T* SNP and a *FASL* GT-repeat microsatellite mapping within 600 bp of exon 4 in the 3'UTR (Sunden et al 1996) and the haplotypes rising from these loci were evaluated in relation to T1DM in Danish families by (E)TDT analysis. The transmission rates of the common *-843C* allele were 54% (48-60%), 53% (46-60%) and 54% (50-58%) in T1DM offspring, unaffected offspring and all offspring, respectively. TDT analysis did not demonstrate linkage or association to T1DM. Rather, these data suggested a putative, although not significant, preferential meiotic segregation of the *-843C* allele in our cohort (Nolsøe et al 2002) similar to that observed for the *INS* VNTR alleles (Eaves et al 1999). A similar pattern of segregation distortion was observed for the *FASL* microsatellite, which can be considered as a di-allelic variant as the $(GT)_{16}$ and $(GT)_{17}$ alleles accounts for ~80 and 18% of the alleles in the parents (Nolsøe et al 2002). The allele-wise ETDT indicated linkage and association to T1DM due to an increased transmission of the $(GT)_{16}$ allele; 58% (51-65%) of the informative $(GT)_{16}$ alleles were transmitted to the T1DM offspring. Although random transmission by TDT analysis was observed in non-T1DM siblings, transmission of the $(GT)_{16}$ allele (54%) exceeded that of the $(GT)_{17}$ allele, and furthermore, the overall transmission of the $(GT)_{16}$ allele to all siblings was 57% (52-62%) which implied preferential meiotic segregation. It should, however, be noted that the major contributors to this distorted transmission were the affected offspring (Nolsøe et al 2002). Evaluation of the haplotypes arising from the two loci gave no indication of linkage or association to T1DM by ETDT. Rather, a striking pattern emerged with regard to the preferential transmission of the *-843C* and $(GT)_{16}$ alleles (Nolsøe et al 2002). These alleles were preferentially transmitted to T1DM (and unaffected) offspring exclusively when in combination, whereas nominally decreased or neutral transmission of these alleles were found when in combination with the $(GT)_{17}$ and the *-843T* alleles, respectively. Moreover, transmission distortion of the *-843C*/$(GT)_{16}$ haplotype was only borderline ($\chi^2 = 3.5$ (1 df.); $P = 0.06$) when evaluated in all offspring, with the major contribution from the T1DM offspring. Therefore, it cannot be excluded that this haplotype contributes to T1DM susceptibility, although we were unable to establish formal evidence for this in the Danish cohort. Our study also illustrates the pivotal importance of genotyping unaffected offspring in order to exclude preferential transmission distortion as the causal explanation of an identified linkage and association to disease when TDT analysis is employed.

13.5. CONCLUSION

Our studies of *FAS* and *FASL* led to the identification of 13 novel variants in the two genes. These studies were unable to establish evidence for linkage or association of common variants in *FAS* and *FASL* to T1DM. However, our observations suggest that variants at the *FASL* locus may be linked to T1DM, or alternatively preferential meiotic segregation of these variants is present at the *FASL* locus. This warrants further investigation of these polymorphisms in T1DM and in the population in general in enlarged cohorts.

14. THE *IL6-174G>C* PROMOTER SNP – A T1DM CULPRIT VARIANT IN FEMALES?

This chapter discusses in brief the functions of IL-6, the evidence for a pathogenetic role of IL-6 in T1DM, genetic studies of the *IL6-174G>C* SNP in T1DM, and finally the functional implication of this SNP on IL-6 production and *IL6* promoter activity with focus on the implications of the presence of 17β-estradiol.

14.1. IL-6 – A PLEIOTROPIC CYTOKINE

A complete review of the pleiotropic biological effects of IL-6 on different cells, tissues and organs is beyond the scope of this thesis and has been one of the topics of a recent review (Kamimura et al 2003).

IL-6 can induce signalling in all cells expressing the ubiquitous gp130 receptor (also denoted the IL-6 signal transducer, IL-6Rβ, or CD130), as the other IL-6 receptor subunit, the IL-6Rα, acts as an agonist in both its soluble and membrane bound forms (Kamimura et al 2003, Heinrich et al 2003). Upon formation of the IL-6/IL-6Rα/gp130 hexameric signalling complex two distinct signalling pathways are activated (Kamimura et al 2003, Heinrich et al 2003): the Janus Kinase (JAK)/Signal Transducers and Activator of Transcription (STAT) and the Src homology 2-containing tyrosine phosphatase (SHP-2)/Extracellular-signal Regulated Kinase (ERK)/Mitogen Activated Protein Kinase (MAPK) pathways. The cellular response to IL-6 signalling depends on which of the signalling pathways that prevails in the individual cell type. The balance between the two pathways of signalling may further depend on the metabolic state of the cell and on the combination of other external stimuli. Thus, the biological outcome resulting from IL-6 exposure is complex and may result in a variety of physiological events such as cell proliferation, differentiation, survival and apoptosis (Kamimura et al 2003).

Although IL-6 acts on most tissues, only the major effects of IL-6 on the immune system are detailed here and the effects of IL-6 on the β-cell are detailed in section 14.2.

IL-6 is involved in both the amplification of and protection against the inflammation in response to infection and tissue injury (Baumann et al 1994, Kamimura et al 2003). The IL-6 system aggravates local inflammation by amplification of leukocyte recruitment (Romano et al 1997) and in chronic inflammation IL-6 production contributes to polyclonal B-cell activation and antibody/autoantibody production (Muraguchi et al 1988, Ishihara et al 2002), but also stimulates the expression and delays the degradation of anti-apoptotic factors (Kovalovich et al 2001). IL-6 produced by DC acts on responder T-cells and makes them refractory to T_r cell mediated suppression (Pasare et al 2003). IL-6 prevents anti-CD3 activation-induced apoptosis and it affects the Th_1/Th_2 balance of cytokines towards a Th_2 pattern although data are conflicting (reviewed in Kamimura et al 2003). Finally, IL-6 switches differentiation of monocytes from DC to macrophages (Kamimura et al 2003).

IL-6 induces expression of hepatic acute phase reactants (Baumann et al 1994), directly stimulates the adrenal gland cells to steroid secretion *in vitro* (Path et al 1996), stimulates the hypothalamic-pituitary-adrenal axis (Wilder 1995), is an essential, corticotropin-releasing hormone-independent stimulator of the adrenal axis during immune system activation (Bethin et al 2000), and induces synthesis of the tissue inhibitor of metalloproteinases-1 (Lotz et al 1991), the circulating interleukin-1 receptor antagonist and the soluble tumor necrosis factor p55 (Tilg et al 1994). These actions are thought to mediate mainly anti-inflammatory effects.

Several cell types, including T-cells, cells of the monocytic lineage, fibroblasts, endothelial cells and pancreatic β-cells synthesise IL-6 (Campbell et al 1989b, Akira et al 1993). Hence, inappropriate regulation of IL-6 may play a role in immune-mediated diseases.

14.2. IL-6 – A ROLE T1DM PATHOGENESIS?

The role of IL-6 in human and rodent T1DM is debated and evidence arising from human studies is lacking. The action of IL-6 in T1DM pathogenesis may be exerted at the level of the target β-cell (Campbell et al 1989b, DiCosmo et al 1994, Campbell et al 1994), immune regulation (Ishihara et al 2002) or the hypothalamic-pituitary-adrenal axis (Wilder 1995, Bethin et al 2000).

An independent cytotoxic effect of IL-6 on (rat) pancreatic β-cells *in vitro* has only been reported once (Buschard et al 1990) and other studies have been unable to show such an effect, but IL-6 potentiates β-cell toxicity of pro-inflammatory cytokines *in vitro* (reviewed in Rabinovitch et al 2003). IL-6 can be produced by β-cells upon various stimuli *in vivo* and *in vitro* (Campbell et al 1989b, Pilstrom et al 1995, Cardozo et al 2001), but the implications of this in β-cell destruction remains to be elucidated.

Data on *IL6* mRNA expression in human infiltrated islets of newly onset T1DM patients are conflicting (Somoza et al 1994, Huang et al 1995, Yamagata et al 1996), but collectively they seem to suggest a *low IL6* mRNA expression compared to controls. IL-6 is expressed in islets both prior to and during immune infiltration in NOD mice (Anderson et al 1993, Pilstrom et al 1995, Faulkner-Jones et al 1996, Teros et al 2000) and interestingly islet IL-6 expression is higher in NOD females than in males (Anderson et al 1993). Moreover, IL-6 is expressed in infiltrated islets of DP-BB rats (Jiang et al 1991). Thus, *in vivo* data on spontaneous autoimmune diabetes indicate that IL-6 may play a role at the level of the target β-cell.

β-cell specific over-expression of *Il6* promotes islet inflammation in both the NOD mouse (DiCosmo et al 1994) and in non-diabetes prone mouse strains (Campbell et al 1994), but surprisingly delays overt diabetes development (DiCosmo et al 1994). Development of autoimmune diabetes or disturbed glucose-homeostasis did not result from universal over-expression of the *Il6* gene in *Il6*-transgenic mice (Suematsu et al 1992, Woodroofe et al 1992, Fattori et al 1994, Katsume et al 1997). Studies investigating *Il6* knock-out NOD mice have not been reported. Anti-IL-6 administration in cyclophosphamide treated NOD mice reduces incidence of T1DM (Campbell et al 1991). Hence, local over-expression of IL-6 promotes islet inflammation, but apparently additional factors are needed for diabetes development. Furthermore, excessive local IL-6 production retards onset in NOD mice which indicates a protective effect of high local levels of IL-6. Moreover, universally increased IL-6 does not induce autoimmune diabetes in mice.

Studies comparing serum levels of IL-6 in T1DM patients and controls are conflicting (Cavallo et al 1991, Ng et al 1995, Geerlings et al 2000, Mohamed-Ali et al 2001, Erbagci et al 2001). *In vitro* LPS-stimulated monocytes from T1DM patients secrete lower levels of IL-6 than control subjects (Ohno et al 1993, Geerlings et al 2000).

These studies provide some evidence for a role of IL-6 in human T1DM, although the pathogenetic implications – protective or deleterious – and the site of action of IL-6 in T1DM pathogenesis are unclear.

A case-control study suggested association between a functional (Fishman et al 1998) *IL6* promoter SNP and T1DM (Jahromi et al 2000), and as *IL6* is a positional T1DM candidate gene based on studies in the NOD mouse (McAleer et al 1995), we and others evaluated the *IL6-174G>C* SNP in T1DM as described following section.

14.3. GENETIC STUDIES OF THE *IL6-174G>C* PROMOTER SNP IN T1DM

The human *IL6* (OMIM#147620) maps to chromosome 7p21 (Bowcock et al 1988, Ferguson-Smith et al 1988), has five exons and covers a region of 5.7 kb (Yasukawa et al 1987). *IL6* has a high degree of sequence homology with the murine *Il6,* in particular in regulatory sequences – including the proximal promoter (Tanabe et al 1988). The murine *Il6* maps to chromosome 5 (Mock et al 1989) close to the NOD susceptibility locus *Idd15* (McAleer et al 1995). The *IL6* locus was not linked to T1DM in the human genome-wide scans (Hashimoto et al 1994, Davies et al 1994, Mein et al 1998, Concannon et al 1998, ECIGS 2001, Cox et al 2001a). Gender stratified analysis (Paterson et al 1999) of 356 T1DM UK sib-pairs (Mein et al 1998) identified differences in LOD scores between the markers on chromosome 7, but was not significant for the marker *D7S629* mapping ~47 kb downstream of *IL6.* Thus, the human genome scans did not qualify *IL6* as a positional candidate gene.

There are several polymorphisms in and close to *IL6* (Bidwell et al 1999, Bidwell et al 2001, Carlson et al 2004). The LD between the *IL6* variants is strong in populations of European Caucasoid ancestry (Terry et al 2000, Jordanides et al 2000, Tiret et al 2002, Carlson et al 2004). The number of *IL6* polymorphisms is higher in African Americans (Carlson et al 2004) and there are considerable differences in allelic frequencies of the individual *IL6* polymorphisms between populations (Bowcock et al 1988, Schotte et al 2001, Zhai et al 2001, Cox et al 2001b, Lim et al 2002, Carlson et al 2004). The *IL6-174G>C* promoter *SNP* (Olomolaiye et al 1998) which has been suggested to functionally affect *IL6* promoter activity (Fishman et al 1998) – an issue discussed further in section 14.4 – and which has been associated with several autoimmune diseases (Haukim et al 2002) including T1DM (Jahromi et al 2000) is an excellent haplotype marker for the common *IL6* promoter polymorphisms. These include a frequent *-597A/G* SNP, a rare *-572G/C* SNP and a *-373*A_n T_m polymorphism (Terry et al 2000). In Scottish (Jordanides et al 2000) and UK (Terry et al 2000) population the *IL6-174C* allele is a marker for the promoter haplotype *597A-572G-373*A_8/T_{12}*-174C* in 91 of 94 informative chromosomes, whereas the *IL6-174G* allele combines with the *-597G* and *-572G* alleles in ~92% of the informative chromosomes. This haplotype does not harbour the -373A_8/T_{12} variant but other -373A_nT_m variants (Terry et al 2000). Hence, *IL6-174G>C* SNP genotyping provides considerable genetic information on the promoter haplotype and putatively for all common genetic variants in the regulatory and coding regions of *IL6* (Tiret et al 2002).

We (Kristiansen et al 2003) and others (Jahromi et al 2000, Siekiera et al 2002, Eerligh et al 2004) have evaluated the *IL6-174G>C SNP* in relation to T1DM with widely differing results, **Table 21**.

The Polish study is not discussed further due to the low number of cases (N = 36) and controls (N = 36) (Siekiera et al 2002). We estimated the transmission of the *IL6-174G* allele from heterozygous parents to the UK T1DM patients to be within the range of 79-89% (Kristiansen et al 2003) based on the UK case-control data (Jahromi et al 2000), this is high transmission rate for a minor gene variant (Bennett et al 1996a, Ueda et al 2003). Thus, the power of family-based TDT analyses in the UK (Jahromi et al 2000), the Danish (Kristiansen et al 2003) and in the Dutch (Eerligh et al 2004) cohorts to confirm this finding at the 5% significance level can be estimated to be 80-99%, >99% and >99%, respectively. Since all studies failed in confirming this association, it is likely that the observation in the UK case-control material is "spurious". This is substantiated by the observation that the genotype distribution of the *IL6-174G>C* SNP in UK T1DM patients from the Oxford area (Gillespie et al 2005) is significantly different from that of the T1DM patients ($P < 10^{-5}$) and similar to that of the control subjects (P = 0.2) in the study by Jahromi et al. The power of the Dutch study to confirm the observation of the *IL6-174C* allele transmission rate of ~56% in Danish patients (Kristiansen et al 2003) is ~35%. Hence, the two studies may be in agreement, but transmission data on the *IL6-174G>C* SNP was not reported in the Dutch study (Eerligh et al 2004). Based on the power of the three relevant studies and their design, the observation in the Danish T1DM families (Kristiansen et al 2003) is favoured as the most plausible, and this is substantiated by our findings in the gender stratified analyses discussed below.

Highly significant linkage in the presence of association and association alone were found in females exclusively, with *IL6-174C* transmission rates of 63 and 66%, respectively (Kristiansen et al 2003). In contrast, random transmission was observed in males and moreover, the transmission patterns in T1DM females and males were significantly different. Preferential meiotic segregation in females as the cause of association was ruled out, and furthermore heterogeneity in the transmission patterns between affected and unaffected female offspring was demonstrated. Thus, our study strongly indicated that linkage and association between the *IL6-174G>C* SNP was a phenomenon in females exclusively (Kristiansen et al 2003).

To further explore the impact of the two *IL6-174G>C* alleles in conferring susceptibility to T1DM in females, families with female offspring in which one parent was homozygous (CC or GG) and the other parent was heterozygous (GC) were identified. This approach allowed evaluation of both the parental transmission and the putative effect of the non-informative (homozygous) parent's genotype. The transmission patterns in these families are shown in **Figure 9**.

These analyses demonstrated an effect of both the genotype of the homozygous parent (*IL6-174G* protects) and a striking effect of the transmitted allele from the heterozygous parent – *IL6-174C* and *IL6-174G* conferred risk and protection, respectively, if the other parent was CC homozygous.

We further evaluated the impact of the *IL6-174G>C* SNP on the ages at onset in males and females (Kristiansen et al 2003) as this SNP affects ages at onset in other diseases (Papassotiropoulos et al 1999, Pascual et al 2000). We demonstrated that the mean age at onset in CC homozygous females was lower than in CC males: 8.8 and 14.0 years, respectively. We have subsequently confirmed this observation in a large population-based UK T1DM material (Gillespie et al 2005). Moreover, our data suggested that the *IL6-174C* allele in a recessive fashion affected the age at onset differently in males and females.

Table 21. Studies of the *IL6-174G>C* SNP in T1DM.

Population	Study design	Associated allele	References
UK	C-C	*IL6-174G*	Jahromi et al 2000
UK	TDT	–	Jahromi et al 2000
PL	C-C	–	Siekiera et al 2002
DK	TDT	*IL6-174C*	Kristiansen et al 2003
NL	TDT	–	Eerligh et al 2004

C-C: case-control study. TDT: family-based TDT design.

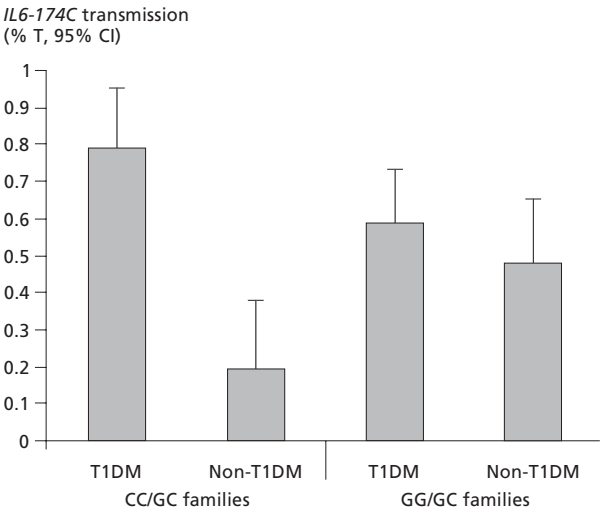

Figure 9. Transmission of the *IL6-174C* allele in females from families with homozygous/heterozygous parent combinations.

To summarise, our genetic investigation indicated that the *IL6-174C* allele is highly associated with T1DM in females, that transmission of one *IL6-174G* allele provides a considerable degree of protection in females, whereas the transmission of two *IL-174C* alleles confers significantly increased risk of T1DM in females. Moreover, we showed that the *IL6-174G>C* SNP affects the age at onset of T1DM in females and this has been confirmed in an independent cohort. However, independent confirmation of our TDT data is strongly in demand and ~1500 T1DM trio families with a T1DM female offspring are required to confirm the data (power 90%, statistical significance level of 5% and an anticipated transmission of 56%, which is the lower end of the confidence interval of transmission in females). Further, it cannot be excluded that the association is due to LD with another genetic variant in or close to *IL6*, although our functional studies discussed below suggest that the *IL6-174G>C* SNP is a plausible etiological T1DM variant. Our observations also underscore the necessity of evaluating genetic linkage and association data in T1DM not only in analyses grouped by HLA class II genotype or HLA class II sharing, but also stratified by gender and age at onset in order to identify genes that confer T1DM risk, in particular when analysing genes and gene variations or markers close to genes that are likely to be affected by gender or age. Hopefully, novel analytical tools for the investigation of complex traits like the recently reported neural network/data mining methodology (Pociot et al 2004) will be able to include parameters such as gender and age at onset in the analyses.

14.4. THE *IL6-174G* AND *IL6-174C* PROMOTER ACTIVITIES ARE DIFFERENTLY AFFECTED BY 17β-ESTRADIOL

The *IL6-174G>C* SNP has been suggested to be of importance for *IL6* promoter activity based on *in vivo* serum IL-6 levels (Fishman et al 1998, Burzotta et al 2001, Hulkkonen et al 2001) and reporter assay studies (Fishman et al 1998). These studies implied higher *IL6* promoter activity and higher serum levels of IL-6 encoded by the *IL6-174G* allele. These findings, however, have been challenged (Terry et al 2000, Kilpinen et al 2001, Bonafe et al 2001, Nauck et al 2002, Unfried et al 2003). Moreover, 17β-estradiol (E_2) has been claimed to suppress stimulated activity of the *IL6* promoter in human estrogen-receptor positive (hER^+) cells (Pottratz et al 1994,

Galien et al 1996, Ray et al 1997). None of these studies, however, investigated the effect of E_2 on different *IL6* promoter variants.

In light of our genetic observations (Kristiansen et al 2003) and the above studies, it was decided to investigate the hypothesis that the activities of the *IL6-174G* and *IL6-174C* promoters were differently affected by E_2. For this purpose the *IL6-174G* and *IL6-174C* promoter variants were cloned into reporter constructs and transfected into the hER^+ human Ishikawa endometrial adenocarcinoma cells. Promoter activity was evaluated with and without PMA treatment in the presence and absence of E_2. The *constitutive* activities of the two promoters were similar in activity and unaffected by the presence or absence of E_2. Under E_2-free conditions PMA treatment was unable to stimulate the *IL6-174G* promoter activity, and the stimulated activity of the *IL6-174C* promoter exceeded that of the *IL6-174G* promoter by ~70%. The inability of PMA to stimulate the *IL6-174G* promoter and the stimulated capacity of this promoter variant was reverted by E_2 preincubation, whereas the PMA stimulated activity *IL6-174C* promoter variant was unaffected by E_2. This suggests that in "E_2-free" settings (prior to puberty) the *IL6-174C* promoter activity exceeds that of the *IL6-174G* promoter, whereas after the menarche the stimulated activities of two promoter variants are identical in females. In contrast to a previous study (Ray et al 1997), we did not find decreased *IL6* promoter activity upon E_2 treatment of Ishikawa cells, which may be due to hER down regulation (Ray et al 1997), or differences in assay conditions. Most notably, pre-incubation not co-incubation with E_2 was used in our study (Kristiansen et al 2003). The SNP maps to a negative regulatory domain in the *IL6* promoter (Stein et al 1995). This site, however, has not been directly implicated in the E_2 regulation of *IL6* promoter activity which is most likely mediated through a direct binding of NF-IL6 and NF-κB to the hER, thereby preventing these transcription factors from binding to the more downstream *IL6* promoter (Ray et al 1997).

Interestingly, *in vitro* exposure of lymphocytes and monocytes to PMA stimulates and inhibits *IL6* expression, respectively (Costanzo et al 1999). Hence, *in vivo* regulation of *IL6* promoter activity in various immune-competent cells is likely to be cell-type dependent. To fully appreciate the functional impact of E_2 on the activity of the two *IL6* promoter variants, experiments investigating the impact of E_2 and more physiological stimuli should be pursued in specific cell types such as DC, macrophages, T-cell (subsets), endothelial cells and β-cells.

14.5. CONCLUSION

Our investigation of the *IL6-174G>C* promoter SNP demonstrated a strong linkage and association to T1DM in young females, but not in males, and showed that the *IL6-174C* and *IL6-174G* alleles confer susceptibility and protection, respectively, in females. Moreover, the *IL6-174C* allele affects the age at onset in females in a recessive way with low age at onset in *CC* homozygous females. The functional studies suggested evidence for a negative impact of the absence of E_2 on the stimulated activity of the *IL6-174G* variant which was reverted by E_2. In contrast, the stimulated activity of the *IL6-174C* variant is E_2 insensitive and higher than the stimulated activity of the *IL6-174G* variant without the presence of E_2. Although, our study does not provide a direct mechanistical explanation for the role of the *IL6-174G>C* SNP in T1DM development, it suggests that higher *IL6* promoter activity may confer risk to T1DM in very young females. This excess risk is negated with increasing age, possibly by the increasing E_2 levels in puberty. Confirmation of our genetic data is strongly warranted as some conflicting data on this SNP in T1DM exist. A very large family cohort is needed for this purpose (~1500 trios with T1DM female offspring). The impact of the SNP on the age at onset of T1DM has been confirmed in an independent population-based cohort. Additional investigations of the functional impact of E_2 on the promoter activity of two *IL6* promoter variants in other cell types including human immune cells should be undertaken with other relevant stimuli.

15. GENETIC STUDIES OF THE FUNCTIONAL NQO1 P187S VARIANT IN T1DM

A cornerstone in the pathogenetic model (Figure 1) is the hypothesis that the target β-cell is not a passive bystander in the process leading to β-cell destruction – rather the β-cell dies because it is a β-cell. As implied in the lower left corner of Figure 1 the β-cell is thought to generate inappropriately high levels of reactive oxidative species (ROS) upon exposure of cytokines during the immune attack and hereby the β-cell itself contributes to its own demise.

It is beyond the scope of this thesis to review the extensive literature on *in vitro* studies of β-cells and ROS, which have been parts of the topics of two earlier Danish theses (Helqvist 1994, Andersen 1999).Therefore, this chapter will briefly discuss the *in vivo* evidence for a role of reactive oxidative species (ROS) in the pathogenesis of T1DM arising from studies in the rodent T1DM models, the function of the NAD(P)H:quinone oxidoreductase 1 (NQO1) as an important molecule in protection against oxidative stress and its putative role in β-cell protection, and genetic studies of the functional NQO1 P187S polymorphism in T1DM.

15.1. THE ROLE OF LIPID PEROXIDATION IN β-CELL DESTRUCTION

There is currently no *in vivo* evidence for lipid peroxidation arising from the chemical interaction between lipids and free oxygen radicals or nitric oxide (NO) in human β-cell destruction. Studies in rodents, however, have strongly implied that lipid peroxidation is likely to contribute to β-cell destruction (Rabinovitch et al 1996, Suarez-Pinzon et al 1997), and the β-cell itself is capable of producing significant levels of ROS (Suarez-Pinzon et al 1997, Andersen 1999). The first indications came from studies demonstrating that both Alloxan, a generator of free oxygen radicals, and Streptozotocin, a generator of NO, induce or accelerate β-cell destruction in rodents (Like et al 1976, Matsushita et al 1989). Expression of the inducible nitric oxide synthase (iNOS) in macrophages and β-cells is associated with spontaneous β-cell destruction in NOD mice (Rabinovitch et al 1996) and further, development of spontaneous autoimmune diabetes in NOD mice is associated with formation in β-cells of peroxinitrite ($ONOO^-$) – a highly reactive oxidative agent produced by the reaction of superoxide O_2^- with NO (Suarez-Pinzon et al 1997). *In vivo* local cytokine challenge of pancreatic islets also involves lipoperoxidation as a mechanism of β-cell destruction (Tabatabaie et al 2003, Todaro et al 2003). Moreover, *in vivo* treatment with antioxidants such as vitamin-E (Hayward et al 1992) and guanidinoethyldisulphide, a selective iNOS inhibitor and a scavenger of peroxinitrite (Suarez-Pinzon et al 2001), decreases or prevents spontaneous autoimmune diabetes in NOD mice, respectively. The *iNOS* deficient NOD mouse develops diabetes (Mathis et al 2001) which argues against an essential impact of lipid peroxidation induced by iNOS up-regulation, but it does not argue against lipid peroxidation in β-cell destruction as NO production catalysed by the constitutive nitric oxide synthase is unaffected or even compensatorily up-regulated by iNOS deficiency.

The expression of antioxidant enzymes is lower in murine pancreatic islets compared to other murine tissues suggesting that the islet β-cells are particularly sensitive to oxidative stress (Lenzen et al 1996). It is, however, important to note that differences in antioxidative capacity exist between murine, rat and human islets, since human islets express higher levels of some antioxidant enzymes (Welsh et al 1995) implying that human islet cells are less vulnerable to oxidative stress.

15.2. FUNCTION OF NQO1 AND ITS PUTATIVE ROLE IN T1DM PATHOGENESIS

One-electron reduction of quinones and their derivatives by enzymes such as cytochrome P450 reductase, ubiquinone oxidoreductase, xanthine oxidoreductase, and cytochrome b_5 reductase generates unstable semi-quinones which undergo redox cycling in

the presence of molecular oxygen. This leads to the formation of ROS which in turn results in oxidative cellular stress, DNA damage and lipid peroxidation (O'Brien 1991, Monks et al 1992, Joseph et al 2000).

The NQO1 enzyme (also known as DT-diaphorase) is a homodimeric flavoprotein that acts as an obligatory two-electron reductase of quinones catalying reductive detoxification of these, thereby protecting cells against redox cycling and oxidative stress (O'Brien 1991, Riley et al 1992, Ross et al 2000, Joseph et al 2000). Among the endogenous quinones, NQO1 has been demonstrated to reduce both ubiquinone to ubiquinol and vitamin-E quinone to vitamin-E hydroquinone, respectively, and these reduced quinones inhibit lipid peroxidation (Bindoli et al 1985, Beyer et al 1996, Siegel et al 1997, Landi et al 1997, Ross et al 2000). As vitamin E also is able to decrease the incidence of diabetes in NOD mice (Hayward et al 1992), and since quinones and quinone isomers are present in β-cells and affect insulin secretion *in vitro* (MacDonald 1991, McInerney et al 1996), the *NQO1* was considered a candidate gene in T1DM. However, *Nqo1* deficient mice do not develop diabetes, indicating that lack of NQO1 activity by itself is not sufficient of inducing β-cell destruction (Radjendirane et al 1998). As detailed in the following section, not only is a functionally important SNP present in the NQO1 gene (*NQO1*) and a preliminary report had suggested an impact of this SNP on T1DM in Japanese patients, but *NQO1* was also a positional candidate gene.

15.3. THE NQO1 P187S POLYMORPHISM – NO ASSOCIATION TO T1DM

NQO1 maps to chromosome 16q22.1, spans approximately 17 kb and has 6 exons (Jaiswal et al 1988, Chen et al 1991, Jaiswal et al 1999, Nebert et al 2002). NQO1 maps approximately 87 cM from chromosome 16ptel between the markers *D16S3031* and *D16S2139*), and is thus located approximately 20 cM from the *D16S3098* (at 108 cM) marker displaying the largest MLS at the chromosome 16q22-24 (*D16515-D16S520*) T1DM locus identified in UK T1DM families (Mein et al 1998) and subsequently consolidated in an enlarged cohort (Cox et al 2001a). This locus was not linked to T1DM in the Scandinavian T1DM families (ECIGS 2001).

Only two SNPs leading to amino acid substitutions are known; a frequent and functional *NQO1 c609C>T* SNP in exon 5 leading to a proline to serine substitution at codon 187 (P187S) of the NQO1 molecule (Ross et al 1996, Traver et al 1997) and a rare *NQO1 c465C>T* SNP in exon 4 leading to an arginine to tryptophan substitution at codon 139 (Pan et al 1995). The NQO1 139P variant is rare with an allele frequency of 4% in Caucasians and is always found in combination with the NQO1 P187 polymorphism (Gaedigk et al 1998). Several rare and mainly intronic SNPs have recently been identified in *NQO1* (Nebert et al 2002).

The NQO1 P187 variant has full NQO1 activity, whereas the 187S variation results in loss of NQO1 activity (Ross et al 1996, Misra et al 1998, Ross et al 2000) due to rapid degradation of the NQO1 187S protein by the ubiquitin proteasomal pathway (Siegel et al 2001) as a consequence of decreased binding to heat shock protein 70 (Anwar et al 2002). The NQO1 187S variant has been associated with benzene induced bone marrow toxicity, some types of cancers (Moran et al 1999, Nebert et al 2002) and a protective effect against ozone induced asthma (David et al 2003).

The genotype distribution of the NQO1 P187S variant demonstrated differences between T1DM cases and controls in a preliminary Japanese study (Hirai et al 1998). Decreased P187 homozygosity and increased 187S variant frequency were found in T1DM patients.

We investigated the NQO1 P187S polymorphism and found no indication of linkage or association to T1DM, as only 51% of the informative alleles transmitted to the T1DM offspring were NQO1 187S alleles (Kristiansen et al 1999). Random transmission was also observed to unaffected offspring. Conditioning for HLA-risk did not disclose any evidence for disease association in these T1DM subsets. Thus, our observations did not suggest an impact of the NQO1 187S variant on T1DM susceptibility in Danes. The *a priori* assumption of 40% NQO1 P187S heterozygous parents was not fulfilled as we only had 28% such parents in our cohort (Kristiansen et al 1999). A *post hoc* estimate suggested that our study only had a power of ~60% to find an association similar to that observed in the Japanese cohort (Hirai et al 1998, Kristiansen et al 1999). Thus, it cannot be excluded that the negative finding is due to limited power of our study, but since transmission distortion was completely absent in the Danish T1DM offspring in Danish patients, it is unlikely that the NQO1 P187S has a major impact on the genetic risk of T1DM in Danes (Kristiansen et al 1999). "Spurious" association may explain the finding in the Japanese case-control study (Hirai et al 1998), but genetic heterogeneity in the predisposition to T1DM in the two investigated populations cannot be excluded. Although the *NQO1 c609C>T* SNP had not previously been investigated in Danes, a lower frequency of the 187S encoding allele and therefore a lower heterozygosity frequency in the Danish population compared to Japanese might have been expected based on the available genetic epidemiological data (Kelsey et al 1997, Gaedigk et al 1998).

15.4. CONCLUSION

Genetic studies of the functional NQO1 P187S polymorphism in relation to T1DM susceptibility have provided conflicting results: association of the NQO1 187S variant in Japanese patients, but no linkage or association in Danish T1DM patients. Given the design of the studies, the NQO1 P187S should not be considered a causal T1DM variant until large studies prove otherwise.

16. CONCLUSIONS AND PERSPECTIVES

This chapter summarises the lessons learned from studies undertaking evaluation of T1DM genetics in general, the lessons learned from the studies behind this thesis and finally the perspectives of future genetic studies in complex human diseases and T1DM are discussed.

16.1. GENERAL CONCLUSIONS

Today, some 30 years after the initial observations of association between genetic variants at the HLA locus and T1DM (Singal et al 1973, Nerup et al 1974) this finding still stands (ECIGS 2001, Cox et al 2001a). However, the T1DM susceptibility variants at the MHC locus are not sufficient or necessary for developing T1DM. Moreover, they only account for ~40-50% of the genetic susceptibility to T1DM.

Thus, etiological genetic variants mapping outside MHC contribute to T1DM susceptibility, but factors such as the number of contributing genes, the frequency of culprit variants (rare or common), the balance between protective and risk alleles, the extent of gene-gene interaction, the degree of environmental-gene interaction, penetrance, impact of gender, impact of within and between population genetic heterogeneity and the impact of imprinting still need to be analysed in detail.

Genome-wide and partial genome scans have suggested more than 30 minor T1DM loci demonstrating nominal (MLS>1.2) to significant (MLS >3.6) linkage to T1DM. Independent confirmation of the minor loci by means allele-sharing analyses has rarely been possible (ECIGS 2001, Cox et al 2001a). The reasons for the latter are several. Some loci reported to be linked with T1DM using insufficient criteria for claiming linkage may have arisen by random chance (Lander et al 1995, Altmuller et al 2001). The impact of the individual susceptibility gene is very low (Lander et al 1995, Bennett et al 1995, Ueda et al 2003), the power of the allele-sharing methods is low (Lander et al 1995), the number of families investigated is inadequate and use of "mixed" population decreases power (Altmuller et al 2001, Cox et al 2001a). Additional *putative* reasons are mentioned in the above. An "established" T1DM etiological gene variant identified through an initial genome-wide scan and sub-

sequent classical positional cloning is yet to be reported. Moreover, it is highly conceivable that several T1DM loci have not been identified by the genome-wide scans.

The candidate gene approach has been more successful in identifying T1DM susceptibility gene variants. Both *IDDM1* and the two "established" minor T1DM susceptibility genes, *INS* (Bell et al 1984, Bennett et al 1995) and *CTLA4* (Nistico et al 1996, Ueda et al 2003), were found this way.

The important lessons learned from the genetic studies of *INS* and *CTLA4* in T1DM including the "final" identifications of the etiological variants in theses genes (Bennett et al 1995, Ueda et al 2003), and from the studies of *ICAM1* in T1DM (Kristiansen et al 2000c, Nejentsev et al 2003) are: 1) the impact of the individual minor T1DM variants on risk is likely to be very small, 2) very large family cohorts are in demand for the detection of minor T1DM gene variants both in genome-wide, family-based and case-control studies, 3) rare as well as common alleles may affect T1DM susceptibility, 4) complex patterns of dominant protective alleles and susceptibility alleles are also found in minor T1DM genes, 5) complete mutation screening of candidate genes and neighbouring genes and sequences and genotyping of most – if not all, genetic variants, is essential for a) the identification of the causal candidate gene variant, b) the exclusion of association due to LD with other variants and c) concluding that a candidate gene is in fact *not* a candidate gene and 6) even with very large cohorts it may not be possible by means of LD mapping to identify the culprit variant, pointing to the requirement of functional characterisation of candidate gene variants for the final identification of the etiological variant.

Studies in the NOD mouse (Todd et al 2001, Cordell et al 2001) and in humans (Cordell et al 1995, Bennett et al 1996a) point to the need of analytical tools enabling detection of gene-gene interaction and identification of protective gene variants in T1DM. Methods using data mining and neural networks (Pociot et al 2004) may prove able to disclose T1DM loci with such characteristics.

16.2. LESSONS LEARNED FROM THE STUDIES BEHIND THIS THESIS AND RELATED STUDIES

The experimental work underlying this thesis has undertaken a model-based candidate gene approach: initially to investigate – in a Danish cohort of T1DM families – for linkage/association by use of TDT methods between T1DM and selected known and identified genetic variants in non-MHC candidate genes chosen on the basis of a proposed model of the pathogenesis of T1DM. Gene variants demonstrating evidence for linkage/association to T1DM were investigated in reporter assay studies.

The pathogenetic T1DM model has proven to be an excellent framework for the selection of qualified T1DM candidate *genes.* Genetic variants in *IL1RT1, CD4, ICAM1, CTLA4* and *IL6,* five of eleven candidate genes evaluated, have been associated to T1DM in family-based studies (Kristiansen et al 1998a, Bergholdt et al 2000, Kristiansen et al 2003, Ueda et al 2003, Nejentsev et al 2003). However, independent confirmations of association of the genetic variants in *IL1RT1* and *IL6* to T1DM are warranted.

In contrast, the criteria used for selection of *gene variants* for evaluation in chosen candidate genes have proven insufficient. Selected gene variants in *ICAM1* and *IL1RT1* showed no association (Kristiansen et al 2000b, Kristiansen et al 2000c), but subsequent studies found significant association of other variants in these genes (Bergholdt et al 2000, Nejentsev et al 2003). As detailed in the above section – more comprehensive approaches are required for evaluation of candidate genes.

Studies using the Danish T1DM families have demonstrated linkage in the presence of association of variants in several genes and loci: *IL6* (Kristiansen et al 2003), *CD4* (Kristiansen et al 1998a, Kristiansen et al 2004), *IDDM7* (Kristiansen et al 2000a) and *IDDM13* (Larsen et al 1999) in the studies behind this thesis and *IL1RT1* (Bergholdt et al 2000), *NOS2* (Johannesen et al 2001), and *IDDM16* (Field et al 2002) in other studies. However, we now know that transmission distortion of minor T1DM gene variants is marginal. Thus, TDT studies using materials of sizes similar to the Danish T1DM family cohort are prone to type 2 errors. Therefore, more families are in demand for future genetic studies of T1DM.

The use of reporter assays for functional characterisation of promoter variants using one cell line and non-specific stimulation is a much too "reductionistic" approach (Kristiansen et al 2003, Kristiansen et al 2004). To fully appreciate the functional implication of genetic promoter variants a battery of *in vitro* and *in vivo* studies employing both cell lines and various naïve cell types are needed.

The studies behind this thesis demonstrated linkage and association in two of the investigated candidate genes. The *IL6-174G>C* promoter SNP was linked to and associated with T1DM exclusively in young females (Kristiansen et al 2003). Reporter assay studies showed differences in stimulated activities of the T1DM "protective" *IL6-174G* and the T1DM "risk" *IL6-174C* promoters dependent on the presence or absence of 17β-estradiol (Kristiansen et al 2003). How this SNP confers susceptibility to T1DM in young females remains to be elucidated, but this study underscores the importance evaluating linkage and association in gender conditioned analyses. Linkage and association of a *CD4* promoter haplotype with T1DM was demonstrated (Kristiansen et al 1998a, Kristiansen et al 2004) and functional studies suggested high promoter activity of the T1DM associated haplotype putatively leading to increased CD4 surface expression, which is important for the T-cell antigen response.

The genes investigated in the studies behind this thesis demonstrating association in either the Danish cohort, *CD4* (Kristiansen et al 1998a, Kristiansen et al 2004), *IL6* (Kristiansen et al 2003) and *IL1RT1* (Bergholdt et al 2000), or in other cohorts, *CTLA4* (Ueda et al 2003) and *ICAM1* (Nejentsev et al 2003), are involved in the co-stimulation of the T-cell response to antigen or make responder T-cells refractory to T_r suppression. Although several details of the functional implication of these variants remain to be elucidated, it is tempting to hypothesise that these human T1DM gene variants encode quantitative and qualitative changes, which in combination promote a more aggressive response of potentially autoreactive T-cells, rendering these refractory to T_r suppression and leading to β-cell destruction and development of T1DM. If *in vivo* and *in vitro* investigations substantiate this hypothesis, preventive strategies aiming at the induction of specific tolerance to β-cell antigens by inhibition of co-stimulation in patients with ongoing β-cell destruction may be an avenue to prevent T1DM.

16.3. FUTURE PERSPECTIVES FOR THE UNRAVELLING OF THE GENETICS BEHIND T1DM

The future strategies in the dissection of T1DM genetics in humans narrow down to three main approaches when excluding the classical linkage studies in extended T1DM pedigrees, studies in population isolates and studies in animal models (Risch 2000, Terwilliger et al 2000, Tabor et al 2002, Botstein et al 2003): 1) additional genome-wide and partial ASP analyses studies with subsequent positional cloning, 2) family-based or case-control based genome-wide and partial association studies and 3) case-control or family-based candidate gene association studies, all approaches to be performed in very large cohorts.

Linkage studies, using allele sharing analysis in randomly selected ASPs, are unable to detect all minor loci with the current number of available T1DM ASPs. Approximately 4,300 ASP families are required to achieve 80% power to detect significant (MLS>3.6) linkage with levels of genetic sibling risk ratios (λ_s) ranging from 1.05-1.30 (Lander et al 1995). Thus, collection of large ethnically homogenous and well-ascertained cohorts are in demand (Terwilliger et al 2000) – and ongoing (www.t1dgc.org).

Family and case-control based genome-wide and partial association studies in large cohorts performed by genotyping thousands

of SNPs in each individual is believed to be the methods for the future dissection of the genetics of complex diseases (Botstein et al 2003). Statistical modelling indicates that association studies have the power to detect of minor susceptibility genes with λ_s as low as 1.05 in complex diseases (Risch et al 1996, Risch 2000, Wang et al 2003), but the assumptions used for these calculations are controversial (Terwilliger et al 1998, Weiss et al 2000, Pritchard 2001). Moreover, a series of issues remains to be clarified prior to performing such studies using SNPs in genome-wide scans (Botstein et al 2003); 1) which and how many SNPs are needed for such studies? 2) should all SNPs in the investigated sequence be known prior to investigation? 3) is mutation re-scanning of the gene harbouring regions of the genome in an enlarged and more ethnically diverse cohort of individuals necessary for identification of all SNPs? 4) informations on the extent LD in all regions of the genome must be established, 5) can haplotype mapping reduce the number SNPs needed to be genotyped? 6) should a map- or a sequence-based approach be undertaken? Studies evaluating these topics are under way and starting to emerge in the public domain.

Case-control studies have often reported positive association between gene variants and T1DM, but have rarely been confirmed. This situation is not unique for T1DM but an overwhelming problem for common diseases with complex genetic inheritance (Hirschhorn et al 2002). In order to avoid reports on "spurious" association and inconsistent replication of "real" positive association in case-control studies there is an urgent requirement of larger cohorts, improved genetic and epidemiological matching of control cohorts and more stringent criteria for claiming association (Cardon et al 2001, Hirschhorn et al 2002, Lohmueller et al 2003, Cardon et al 2003).

Model-based candidate gene association studies are also believed to be methods capable of "cracking" some of the codes underlying complex diseases (Risch 2000, Tabor et al 2002, Botstein et al 2003). These should, however, as detailed above, undertake a very comprehensive approach to be conclusive. The very recent report of considerable intra-genic common haplotype diversity between populations and significantly higher numbers of intra-genic SNPs compared to previously reported (Carlson et al 2004, Crawford et al 2004) has considerable implications for future candidate-genes studies: 1) the candidate gene must be completely mutation screened, 2) a significantly higher number of SNPs needs to be genotyped to enable evaluation of all common haplotypes and SNPs and 3) importantly, collection of patients from several populations may facilitate the identification of the causal gene variant.

Novel analytical tools enabling incorporation of non-genetic parameters such as environmental factors, gender and other factors, with the ability not only to detect risk alleles but also to identify protective alleles, and to investigate for gene-gene and gene-environment interactions, are in demand.

The studies performed so far have strongly shown that future studies of the genetics of T1DM and complex diseases in general require the establishment of large scale internationally consortia based T1DM study groups with access to well-characterised and very large cohorts of T1DM families , case-control materials, animal facilities and facilities for functional studies.

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