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Immunoregulatory differences between adult onset type 1 diabetes and latent autoimmune diabetes in adults (LADA)

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Abstract

Autoimmune diabetes is characterized by cell mediated autoimmunity against insulin producing beta cells in pancreatic islets. Type 1 diabetes is the major cause of diabetes in children and adolescents and is believed to be mediated by a Th1 driven destruction of beta cells. The much more prevalent type 2 diabetes is not considered as an autoimmune disease but diabetes specific autoantibodies (ICA or GADA) are found in 5-15 % of the cases. These subjects are referred to as latent autoimmune diabetes in adults (LADA). It has been questioned if LADA is a unique entity or should be considered as type 1 diabetes. Subjects with LADA have a better preserved insulin secretion and a slower progression to beta cell failure at clinical onset compared to subjects with type 1 diabetes. Furthermore, there are adult onset type 1 diabetic patients that share the rapid progression to beta cell failure with juvenile onset diabetes. The hypothesis was therefore that type 1 diabetes and LADA have different autoimmune activity. The aim was to identify some of the immunoregulatory events that could explain differences in disease progression between the groups. Heterozygosity of the diabetes associated high risk HLA haplotypes DR3/DQ2-DR4/DQ8 was considered as a risk factor for both adult onset type 1 diabetes and LADA. Also homozygosity for the microsatellite marker TNFa2 conferred an increased risk for both type 1 diabetes and LADA even though this was probably due to linkage disequilibrium with the HLA haplotypes. However, presence of either one of HLA DR3/DQ2 or DR4/DQ8 conferred an increased risk of type 1 diabetes but not LADA. Autoantibodies directed against glutamic acid decarboxylase 65 (GADA) were predominantly of the IgG1 subclass in both adult onset type 1 diabetes and LADA. Moreover, the IgG4 subclass was the second most common subclass in LADA but below detection limit in type 1 diabetes at clinical onset. This suggests a larger involvement of Th2 cells in LADA compared to in type 1 diabetes. The levels of all the GADA IgG subclasses decreased in type 1 diabetes three years after clinical onset while LADA remained their subclass profiles indicating a sustained difference in autoimmune response against the beta cell antigen GAD65 between the groups. The slower progression of beta cell failure in LADA might be due to a more balanced T cell response that reduces the cytotoxic Th1 cell mediated response. Type 1 diabetes and LADA could thus be considered as different variations of autoimmunity.

Key words: Autoimmune diabetes, type 1 diabetes, autoantibodies, LADA, isotype class switch

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To my wife Susanne and my daughters Michelle, Rebecka and Emelie for their patience and gift of time

To scientists of different areas for inspiration

A great discovery is a fact whose appearance in science gives rise to shining ideas, whose light dispels many obscurities and shows us new paths

Claude Bernard (1813-1878)
An Introduction to the Study of Experimental Medicine, pt. I, ch. 2, sect. ii (translated by H. C. Greene)

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LIST OF PAPERS

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I. Törn C, **Hillman M**, Sanjeevi C.B and Landin-Olsson M. *Polymorphisms of TNF microsatellite marker a and HLA-DR-DQ in diabetes mellitus-a study in 609 Swedish subjects*. Hum Immunol, 2006, 67(7); p 527-534 .

II. **Hillman M**, Törn C, Thorgeirsson H and Landin-Olsson M. *IgG(4)-subclass of glutamic acid decarboxylase antibody is more frequent in latent autoimmune diabetes in adults than in type 1 diabetes*. Diabetologia, 2004, 47; p 1984-1989.

III. **Hillman M**, Törn C and Landin-Olsson M. *Determination of GADA IgG subclasses – A comparison of three immunoprecipitation assays (IPAs).* Submitted manuscript

IV. **Hillman M**, Törn C and Landin-Olsson M. *The GAD_{65}Ab IgG subclass profile differs between T1DM and LADA up to three years after clinical onset*.

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LIST OF ABBREVIATIONS

APCs	Antigen Presenting Cells
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CPM	Counts Per Minute
DSM	Disease Susceptibility Motif
ELISA	Enzyme Linked Immunosorbent Assay
GAD65	Glutamic Acid Decarboxylase 65
GADA	GAD65 autoantibodies
HLA	Human Leukocyte Antigen (the human MHC)
IA-2A	Insulinoma Associated protein- 2 Autoantibodies
IA	Insulin Antibodies
IAA	Insulin Autoantibodies
ICA	Islet Cell Autoantibodies
ICA_{512}	*see* IA-2A
IL	Interleukin
IFN	Interferon
IPA	Immunoprecipitation Assay
LADA	Latent Autoimmune Diabetes in Adults
LPS	Lipopolysaccharide
LTA	Gene encoding for TNFβ (lymphotoxin α)
LTB	Gene encoding for lymphotoxin β
MHC	Major Histocompatibility Complex
MS	Multiple sclerosis
NHS	N-hydroxysuccinimide
NOD	Non-Obese Diabetic (mice)
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PRR	Pattern Recognition Receptor
RIA	Radio Immuno Assay
SNP	Single Nucleotide Polymorphism
TLR	Toll-like Receptor
TNFA	Gene encoding for TNFα
TNFa	Microsatellite marker (a) of the TNFA gene
TNFα	Tumor Necrosis Factor alpha

BACKGROUND

History of diabetes

Characteristic symptoms for diabetes mellitus were already documented for more that 3,000 years ago and it was in particular the description of abnormal urine excretion (polyuria) in the *Ebers Papyrus* that led to the interpretation that it was diabetes. The treatment of recommendation was a four day diet on decoction of lead, grain, gravel and bone [1]. During the 2nd century, the first clinical description of diabetes was written by the Turkish physician *Aretaeus* – a disciple of *Hippocrates* - as a state of immense thirst, massive urine excretion and weight loss. He even introduced the term "*diabetes*" from the Greek *dia* (through) and *bainein* (go to) for siphon [2, 3]. Another follower of Hippocrates, the roman physician *Galen* described the condition as rare, as he had only seen two cases ever [4].

Diabetes before the discovery of insulin

Far later than Aretaeus and Galen, in 1674, the English, physician, *Thomas Willis*, studied the composition of urine in diabetic subjects and found out it had a sweet taste [5] even though the ancient *Hindus* used the term "honey urine" a thousand years earlier to describe the ailment [4], maybe due to the observation that the urine from diabetic subjects attracted ants and flies. Almost a century after Willis, the Scottish clinician, consultants and educator *William Cullen* suggested the additional name *mellitus* to diabetes[6], which is the Latin word for honey. This was after consultation with *Matthew Dobson* who confirmed that the sweet taste was the result of excess sugar in urine [7] and moreover showed an excess of sugar in blood of diabetic patients. Dobson also made the observation that in some cases the diabetes was fatal within a few weeks, while it in other cases was more of a chronic ailment (diabetes, type 1 and type 2)[8]. The distinction between obese and non-obese diabetes was also commented in 1866 by *George Harley* [9] and some later also by the French clinicians *Bouchardat* [10] and *Lancrereaux* [11].

Some years after Dobson's report, alterations in pancreas of a recently deceased 34-year old man was observed during an autopsy by *Thomas Cawley* [12]. Since Dr. Cawley like many others at that time believed that diabetes was a disease of the kidney, he did not realize that the observation was related to the real cause of type 1 diabetes – destruction of pancreatic

islets. In fact, the islet cells were not seen until 1869, when the young student *Paul Langerhans* made the first detailed description of nine different cell types in the structure of pancreas with a primitive light microscope [13]. Just like Cawley, Langerhans did not realize the importance of his finding and five years after his death, the French histologist *Edouard Languesse* named the pancreatic cell clusters to "*islots de Langerhans*" [14, 15].

A major milestone in diabetes began with *Oskar Minkowski's* and *Joseph von Mering's* demonstration that fatal diabetes was developed in dogs when pancreas was removed [16]. This is considered as the first experimental proof of the relationship between diabetes and pancreas [17]. About ten years later, *Eugine Opie*, a recent graduate pathologist at John Hopkins Medical School, reported that Diabetes mellitus was caused by the destruction of the islet of Langerhans [18, 19], based on the presence of lesions in islets of subjects with clinical diabetes. This was basically the same observation that Thomas Cawley noticed 112 years earlier. However, without Minkowski's and Mering's evidence that diabetes was a disease of pancreas it was not reasonable to make the same conclusion as Opie did.

The new data placed pancreas in focus of diabetes research and gave birth to new ideas. The German, *George Ludwig Zuelzer*, reported in 1908 a study in which he injected a pancreatic extract into five patients with clinical diabetes. Blood glucose levels were reduced but the experiment had to be abandoned due to severe cramps [20], that was probably caused by hypoglycaemia [21]. Nevertheless, at that time, the side effects of the pancreatic extract were interpreted as toxic reactions and Dr Zuelzers funding was withdrawn. Similar studies were performed in United States [22-24] and in France [25] but it was finally the work of the Canadians *Frederick Banting* and *Charles Best* that led to the discovery of insulin [26].

The discovery of insulin

Frederick Banting who was a surgeon took a part-time job as educator in physiology at the local University, although he was hardly qualified for the position [17]. During a preparation for a lecture in pancreas physiology, Banting studied an article by *Moses Barron*, describing the relation between diabetes and the islet of Langerhans [27]. Barron reported a remarkable case where the main pancreatic duct was obstructed by stone formation and the similarity to when the ducts were blocked experimentally by ligation [28]. The paper gave Banting an idea and a week later, Banting visited the professor of physiology, *John James Richard Macleod*,

and discussed it. Macleod was not enchanted by the idea at first but finally supplied Banting with both material and laboratory. Furthermore, he sent a couple of assistants, one of them, *Charles H Best* who was a senior in physiology and biochemistry had some knowledge in experimental diabetes and blood glucose analyses [2, 29, 30]. During the following months, Banting and Best worked hard and made some fast progress.

After a while, Macleod expanded the team with the biochemist *James B Collip* to work with the task of purification. Collip already had some experience of making extracts of glandular secretions [31]. Different proteins are soluble at different concentrations of alcohol and acidity. Banting and Best knew that their pancreatic substance was soluble at 50%. Collip discovered a limit slightly above 90% in where the active substance precipitated. So he purified it by adding alcohol, close to 90% were most of the contaminants had precipitated. He moved the supernatant and increased the alcohol to just above 90% to precipitate the active substance [17]. After evaporation he had a white powder of the extract that was purified enough to test on humans. One week before Collip's purification, a young physician at the Toronto General Hospital injected an extract made by Banting and Best into a human patient. The extract was a light brown liquid containing a considerable amount of sediment. The patient was the 14-year old boy *Leonard Thompson*. A 25% fall in blood sugar level was observed but no clinical benefit was evidenced. Twelve days later, daily injections of Collip's purified extract were made and this time immediate improvements were observed [26]. They called the new substance for *insulin* and the finding granted Banting and Macleod the Nobel Prize in physiology and medicine in 1923.

Diabetes after the discovery of insulin

Before 1923, 86% of children under 16 years of age died in ketoacidosis [32] while older and more obese patients survived for years. This was probably the basis for the early classification that was mentioned previously [7, 9, 11]. In the 1930s *Dr. Harold Himsworth* found that a major difference between these two groups of patients was their sensitivity for insulin. The short title for the paper, published in Lancet, 1936 even included "*Two types of diabetes mellitus"* [33]. However, the concepts of type 1 and type 2 diabetes as we know them today were not introduced until 1951 by *John Lister* [34] and by one of his collaborators again in 1976 [35].

In 1901 *Paul Ehrlich* postulated the *horror autotoxicus* and that *"organisms possess certain contrivances by means of which the immune reaction is prevented from acting against its own elements."* However, the idea of autoimmunity was not established until the late 1950s as it became clear that animals could produce autoantibodies against their own thyroid tissue when thyroglobulin antigen was incorporated into an adjuvant and reinjected into the animals [36, 37]. Some groups started to confirm that autoantibodies were found in Hashimoto's disease [38, 39] as well as in Addison's disease [40].

Possible autoimmunity in several diseases was now investigated, among them diabetes [41-43] but it was not until around 1970 that a possible relation between type 1 diabetes and auto-immunity was reported [44, 45]. The problem at this time was to demonstrate the presence of diabetes specific autoantibodies. This was later solved independently by *Dr. Gian Franco Bottazzo* in and *A.C MacCuish* in 1974 [46, 47] who discovered the islet cell autoantibodies (ICAs). ICA served as a valuable tool in diagnosis of type 1 diabetes for many years later. During the same year, *Dr. Jørn Nerup* showed that cell mediated immunity was involved in the destruction of pancreatic islets [48] as well as an association between human major histocompatibility complex (MHC) haplotypes and autoimmune diabetes [49]. Further progress made that some subsets of islet cell cytoplasmic antigens were identified as 65 kD glutamic acid decarboxylase GAD_{65} [50-53] and tyrosine phosphatase IA-2 [54-57]. Detection of autoantibodies directed against GAD_{65} (GADA) and IA-2 (IA-2A) have been used as a tool to distinguish between type 1 and type 2 diabetes ever since.

In 1977 it was reported that about 11% of patients with type 2 diabetes were ICA positive and these patients required insulin more rapidly than ICA negative subjects [58]. This particular appearance of diabetes was suggested the term *latent autoimmune diabetes in adults* (LADA) [59, 60] more than a decade later. The term LADA was quickly introduced to the world health organization (WHO) proposal for the criteria for diabetes as a group separated from type 1 and type 2 [61]. However, it has not yet been included as a separate type and there is still some disagreement concerning LADA as a unique disease entity [62].

Epidemiology of diabetes mellitus

As previously mentioned, the ancient Galus estimated diabetes as a rare disease, based on the experience of only two cases ever. Today, diabetes is currently affecting over 170 million people globally [63] and is hardly regarded as rare. Type 1 diabetes approximately counts for 10-20 million cases [64] which represents between 5-10% of the total diabetes prevalence. Although a well-known correlation between the susceptibility for type 1 diabetes and certain genes exists, genetics is not sufficient to cause the disease by itself. The incidence rate of type 1 diabetes is extremely variable between different geographical regions and Sweden has one of the highest incident rates in the world together with the other Nordic countries [65]. About 45,000 cases of type 1 diabetes are known in Sweden [66] and a seasonal variation in incidence among children was noticed during the mid 1900s [67]. One peak of increased incidence was observed during the winter and another peak during late summer indicating the importance of environmental factors. Certain virus infections have been suggested to trigger the disease either by inducing autoimmunity or by cytolytic destruction of beta cells [68]. In addition, dietary proteins have been proposed as a possible contributing factor for the development in genetic predisposing children [69-71] as well as nitrate [72] and certain toxins [73]. However, in adult onset type 1 diabetes, possible environmental factors are not that established [74].

Type 2 diabetes is a multifactorial disease affecting roughly 150 million people [75]. There is no genetic correlation with the HLA-system but the disease shows an apparent familiar aggregation. Type 2 diabetes seems to be the result of several combined gene defects and does not segregate in classical Mendelian fashion [76]. The majority of cases are associated with sedentary life style and obesity and is predominantly an adult onset disease. Nevertheless, increasing incidents are also seen in younger subjects [77]. In contrast to type 1 diabetes that has the highest incidence rates in the industrialized countries, there is a powerful increase of type 2 diabetes in developing countries [75]. The number of diagnosed cases of type 2 diabetes in Sweden is ~250,000. Besides, approximately 200,000 more cases are believed to have undiagnosed type 2 diabetes [66]. The estimated prevalence of type 2 diabetic subjects that presents with diabetes specific autoantibodies is 10%. That would indicate a group that is at least as large as type 1 diabetes. The importance of understanding the pathophysiology in this group should therefore not be underestimated.

Definition and classification of diabetes mellitus

Diabetes mellitus is defined as a group of metabolic disorders with the appearance of chronic hyperglycemia resulting from disturbances in insulin action [78]. The hyperglycemia often causes the symptoms mentioned earlier and recognized by Aretaeus; immense thirst (*polydipsia*), massive urine excretion (*polyuria*) and weight loss. It is also associated with long-term damage to the microvascular system in several organs. Such as the eyes (*retinopathy*), the kidneys (*nephropathy*), and peripheral nerves (*neuropathy*) but also larger blood vessels (*angiopathy*) that might cause for example heart failure [79]. Several different types of diabetes mellitus exist in addition to type 1 and type 2. Some of them are related to genetic defects, others can be induced by chemicals or drugs [80, 81]. With the exception of LADA and gestational diabetes the contribution from the other types is considered as small (<5%) and will not be further discussed here.

Type 1 diabetes

Type 1 diabetes is an organ specific, T cell mediated autoimmune disease. The occurrence of beta cell associated autoantibodies indicates the presence of autoimmunity in these subjects. In children and young adults, type 1 diabetes is related with rapid progression to absolute insulin deficiency (*insulinopenia*) and severe ketoacidosis [82, 83]. Younger age at diagnosis is associated with a more rapid progression to disease and children below seven years of age have often lost approximately 80% of their islets at clinical onset [84]. Also adult subjects in practically any age can be affected by type 1 diabetes. Since the majority of beta cells are destroyed, patients with type 1 diabetes always require immediate insulin treatment. Thus, type 1 diabetes previously went by the acronym IDDM for *insulin dependent diabetes mellitus* [85]. However, the perplexity with this term was that classification of diabetes was based on treatment rather than etiology and is no longer recommended [78].

It is worth mentioning, that at least one form of type 1 diabetes exists with unknown etiology called *idiopathic type 1 diabetes*, *non-autoimmune type 1 diabetes* or *diabetes type 1B*. Some of these patients are prone to ketoacidosis and have permanent insulinopenia. They are not normally of Caucasian origin and have no genetic association with the HLA-region [79].

Type 2 diabetes

Type 2 diabetes is not considered to be an autoimmune disease. The development of the ailment is associated with insulin resistance, that is, diminished effectiveness of insulin in lowering plasma glucose levels [86]. The appearance of insulin resistance is also due to other factors like age, low physical activity, smoking etc [87, 88] but could be temporary in like for example pregnancy (gestational diabetes) or during inflammatory responses [88, 89]. Individuals with insulin resistance need higher concentrations of insulin to maintain glucose homeostasis. When the beta cells no longer have the capacity to provide the cells with the increased requirement of insulin, type 2 diabetes arise [90-92]. The long-term damage to the vascular system is often worse than in type 1 diabetes [92]. The majority of the affected patients are above 40 years of age but the disease is becoming more prevalent also among younger ages. There is even a increase in the prevalence of childhood type 2 diabetes [77, 93].

The hereditariness plays an important part for the development of type 2 diabetes. So far, only a few genes have been identified to correlate with the disease [80, 94] but the risk to develop the disease could be as high as 40% in children or siblings to individuals with type 2 diabetes [90]. The treatment of type 2 diabetes often benefits of weight reduction to increase the sensitivity for insulin if overweight or obesity is present. Oral agents, like insulin sensitizers [95] or insulin secretagogues [96] could also be useful to increase the effect of the self produced insulin. Nevertheless, the need for insulin is in many patients inevitable and the term non insulin dependent diabetes mellitus (NIDDM) is no longer used to avoid confusion with the classification.

Latent autoimmune diabetes in adults (LADA)

The appearance of islet cell autoantibodies in patients that was clinically classified with type 2 diabetes raised the idea of a new subgroup of diabetes with unique features distinguishing it from type 1 and type 2 diabetes [97]. This subgroup was named latent autoimmune diabetes in adults (LADA) [59, 60] and was restricted to patients above 35 years of age [98] and without the need of insulin therapy for at least six months after clinical onset [99, 100]. However, as the prevalence of type 2 diabetes in children and adolescents increase, cases similar to LADA are also discovered in this group [101, 102] and LADA should thus not be restricted only to patients after a certain age. Besides, the criteria for insulin independency during the first half year maybe should be reconsidered. Several things affects the period prior

to insulin independency, the subjective opinion of the treating physician, the progression of the disease and the time of diagnosis in relation to disease duration [103]. GADA seems to be the most prevalent autoantibody since IA-2A and autoantibodies to insulin (IAA) have been found to be less frequent in LADA [104, 105]. Nevertheless, one study reported that most of the ICA signal could be blocked by the addition of GAD and IA-2 to sera from subjects with type 1 diabetes but not from LADA [106] indicating the presence of an immune response to other antigens than GAD and IA-2. One argument to include LADA as part of the spectrum of type 1 diabetes is the higher frequency of the susceptibility haplotypes HLA-DR3/DQ2 and DR4/DQ8 in LADA compared to in healthy non-diabetic subjects [60].

Immunity and autoimmunity

The contrivance that Paul Ehrlich mentioned in his postulation in 1901 is what we today identify as tolerance. The immune system has developed complex ways to efficiently combat infections and still maintain self-tolerance and prevent damage to the own tissue. In some individuals these ways are inadequate and the immune system fails to discriminate between self and non-self molecules. T lymphocytes recognize antigens only when they are associated with MHC molecules on a cellular surface [107].

The major histocompatibility complex (MHC)

In humans the MHC molecules are known as human leukocyte antigens (HLA) and are located on chromosome 6p21. This is the most polymorph region in the human genome and represents the major locus for autoimmune diabetes [108]. The polymorphisms are fundamental to diversify the structure of MHC molecules and consequently allow interaction with different processed antigens [109]. MHC class I molecules, consist of an α-chain and an additional subunit (β2-microglobulin) and are expressed on all nucleated cells. MHC class I present intra cellular peptides to $CD8^+$ T cells. MHC class II molecules, consist of two chains (α and β) and are mainly expressed by antigen presenting cells (APCs). Peptides on MHC class II originates from inter cellular surroundings and are presented to $CD4^+$ T cells [110]. The majority of alleles predisposing for diabetes in Caucasians are the MHC class II alleles; HLA-DR and DQ. The DR3 and DR4 are in linkage disequilibrium with the DQ-alleles, DQ2 and DQ8 [111], yielding haplotypes DR3/DQ2 and DR4/DQ8 (Table 1). Heterozygosity for the DR3 and DR4 alleles confers the highest risk followed by homozygosity for DR4 and DR3 respectively [112-114]. Nevertheless, high risk haplotypes are inversely related to age at onset, which means that even though the majority of children diagnosed with type 1 diabetes

have high risk HLA haplotypes, these are not as frequent in adulthood [115-119]. However, some studies have shown significantly increased frequencies of high risk haplotypes in adult onset diabetes as well [104, 120-123]. Other alleles have been found to be protective against autoimmune diabetes, for example DR2 [124], DR6 and DR11 [125, 126], some of which have been suggested to increase with age at diagnosis [127]. HLA alleles that seem protecting against diabetes might predispose for other disorders, for example DR2 have an increased susceptibility for multiple sclerosis (MS) [128]. Different theories of the mechanism behind the increased susceptibility for autoimmunity among certain HLA haplotypes have been suggested [128-130]. The molecular structure of the peptide binding pocket of MHC molecules varies and creates different avidity/affinity for self-peptides. This leads to the theory of *disease susceptibility motifs* (DSM) [131]. Which means that the MHC expressed either generates a certain resistance or an increased susceptibility depending on

Table 1. HLA haplotypes, associated with autoimmune diabetes. Susceptibility in the DR genotypes is associated with the β-chain while the susceptibility in the DQ genotypes is associated with both chains of the MHC class II molecule. Haplotypes DR3/DQ2 and DR4/DQ8 is associated with increased susceptibility for autoimmune diabetes. Interesting is however, that some DR4/DQ8 seems to be protective against diabetes depending on the DR β-chain. Another remarkable thing is that high risk DR3 and low risk DR7 shares the same DQ β-chain (0201). However, they are associated with different DQ α-chains.

HLA-DR	*DR β-chain*	**HLA-DQ**	*DQ α-chain*	*DQ β-chain*	Risk
DR2	*DRB1*1501*	**DQ6.2**	*DQA1*0102*	*DQB1*0602*	—
DR3	*DRB1*0301*	**DQ2**	*DQA1*0501*	*DQB1*0201*	**
DR4	*DRB1*0401*	**DQ8**	*DQA1*0301*	*DQB1*0302*	**
DR4	*DRB1*0402*	**DQ8**	*DQA1*0301*	*DQB1*0302*	*
DR4	*DRB1*0403*	**DQ8**	*DQA1*0301*	*DQB1*0302*	—
DR4	*DRB1*0404*	**DQ8**	*DQA1*0301*	*DQB1*0302*	*
DR4	*DRB1*0405*	**DQ8**	*DQA1*0301*	*DQB1*0302*	**
DR6	*DRB1*0701*		*DQA1*0201*	*DQB1*0303*	—
DR7	*DRB1*0701*		*DQA1*0201*	*DQB1*0201*	—

— Protecting haplotypes
* Predisposing haplotype
** High risk haplotype

their capacity to bind self-peptides with high affinity in the thymus. MHC molecules in individuals with HLA-DR3/DQ2 and DR4/DQ8 would in this theory be less competent to present diabetes specific antigens during central tolerance and thus fail the negative selection. HLA-DR2 in contrast would be excellent presenting diabetes specific antigens but less competent presenting antigens involved in MS. An example of particular interest has been shown to the DQ β-chain, that sometimes lacks a aspartic acid at position 57 (Asp57) in diabetes predisposing HLA haplotypes, suggesting that this amino acid is critical in peptide binding and presentation to autoreactive T cells [132]. A motif on the DQ α-chain containing an arginine at position 52 (Arg52) has also been correlated to diabetes susceptibility [133].

TNFA gene polymorphisms

In addition to the MHC molecules, other genes are also located in the HLA region [134]. Tumor necrosis factor (TNF) gene cluster (Fig. 1) was found in the MHC class III region during the mid 1980s [135, 136] and polymorphisms in this cluster have been noticed in extended HLA haplotypes [137].

Five different microsatellite polymorphisms in the TNF locus have been described in European populations, TNF(a),(b),(c),(d) and (e) [138] and a sixth in Japanese population TNF(f) [139]. Microsatellite marker *TNFa* has at least 13 different alleles based on differences in GT-repetitions and associations to diabetes have previously been suggested [140, 141]. Also one single nucleotide polymorphism (SNP) in the promoter region (-308) of TNFA, known as *TNF2* has been associated with higher TNFα production in some studies [142, 143] although not consistently [144]. One study indicated a lower frequency of TNF2 in patients with LADA compared to in patients with type 1 diabetes and in healthy controls [121].

It is however difficult to interpret the contribution from single polymorphisms in areas with such strong linkage disequilibrium as the HLA-region. Certain polymorphisms of the TNF(a) microsatellite have shown associations with altered TNFα production, TNFa2/c2 [145], TNFa2/b3 [146] and TNFa2 and a9 [141]. These microsatellites, either in combinations or separately have been established in extended haplotypes with HLA class I and class II genes as well as TNF2 SNPs [146, 147] (Table 2).

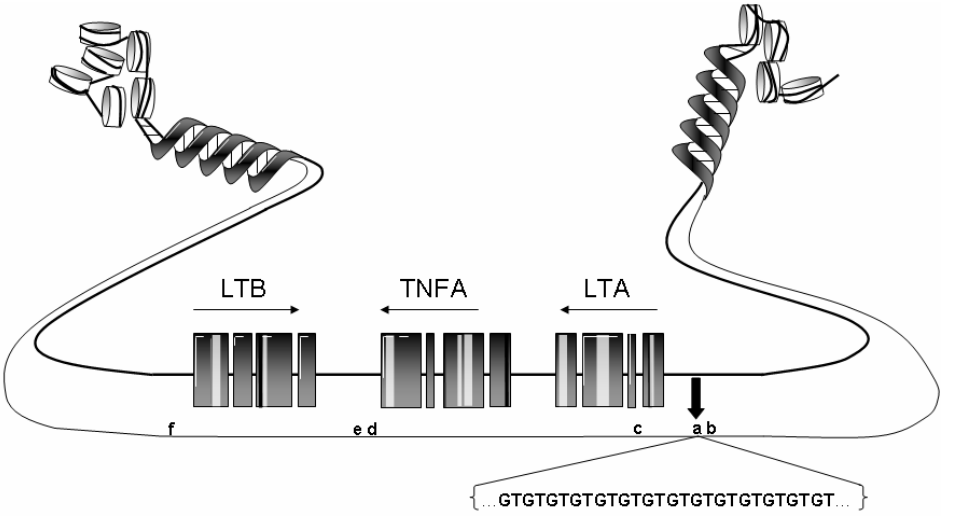

Figure 1. A schematic depiction of the TNF gene cluster on chromosome 6. Each gene has 4 introns (boxes) and 3 exons (space between boxes). The TNFβ gene (LTA) is located close to the telomeric end of the MHC class III region and followed by TNFα (TNFA) and lymphotoxin β (LTB) genes. Microsatellite marker polymorphisms have been found within the TNF locus. TNF(**a)** (bold arrow) and (**b)** are located 3.5 kb upstream of the LTA gene and contains at least 13 $(GT)_n$ and 7 $(G/A)_n$ alleles respectively. TNF(**c)** is located within the first exon of LTA and contains 2 $(GA)_n$ alleles. TNF(**d)** and (**e)** are located between the LTB and TNFA gene and contains 7 $(GA)_n$ and 3 $(GA)_n$ alleles respectively. TNF(f) has been described in Japanese population and 10 $(CA)_n$ alleles have been found so far.

Table 2. Some studies have reported a linkage between TNF locus (MHC class III) and MHC class II. TNF2 is a single nucleotide mutation (G→A) in the TNFA promoter (-308). Microsatellite markers in TNFA locus have also been associated with diabetes but it is difficult to interpret the contribution of each gene since they are inherited in complex extended haplotypes. Mitchell et al reported 2001 that the 8.1 ancestral haplotype was associated with increased TNFα production.

MHC II haplotype	*DRB1*	TNF2	TNF microsatellite marker alleles				
			(a)	(b)	(c)	(d)	(e)
DR3/DQ2			1	5	2	3	4
DR3/DQ2†	*0301*	A	2	3	1	1	3
DR4/DQ8	*0404*		11	4	1	3	3
	0401		6	3	1	3	3
	0401	G	2	1	2	4	1

† 8.1 Ancestral haplotype (HLA-A1, B8, TNF2, TNFa2b3, DR3/DQ2)

Source Ref [138, 146-147]

Diabetes associated autoantibodies/autoantigens

The identification of ICA in the 1970s enabled the recognition of subjects with ongoing autoimmune process against the islet cells of Langerhans [46, 47]. At that time, the pathophysiological role of the antibodies was unknown but would eventually be considered as secondary markers of a cell mediated disease [148] and diabetes can develop even in absence of B-lymphocytes [149]. Several autoantigens have been suggested to be of importance in autoimmune diabetes, most of them part of ICA[148, 150] but insulin, GAD_{65} and IA-2 are considered as the major autoantigens [151].

Insulin

Insulin could seem like the obvious autoantigen in diabetes. Insulin autoantibodies (IAA) are detected in 50-70% of children with type 1 diabetes [152, 153] but are not as frequent in adult onset patients [151]. Levels of IAA seems to correlate with HLA-DR4/DQ8 [154] and an immunodominant peptide of the insulin B-chain has been demonstrated in complex with the HLA-DQ molecule [155] suggesting a disease susceptibility motif for insulin on the DQ8. It is important to discriminate between IAA and insulin antibodies (IA) that appears a few weeks after the start of insulin therapy and is related to immunity rather than autoimmunity.

Glutamic acid decarboxylase (GAD)

The GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) is considered to be one of the strongest candidate antigens in autoimmune diabetes. Two isotypes exists (GAD_{65} and GAD_{67}) and are located on different chromosomes [156]. Both isotypes are synthesized as cytoplasmic soluble proteins but GAD_{65} is anchored to the membrane after posttranslational modification [157] The GAD_{65} isotype is the most frequently occurring in diabetes and is not influenced by age [158]. Hence, it is a common feature in both adult onset type 1 diabetes and LADA. GAD_{65} is expressed in almost all human islet cells [150], central nervous system, testis and ovary. GAD_{65} restricted T cells have been identified in human diabetes [159, 160] but why only beta cells are destroyed is unknown. Autoantibodies directed against GAD_{65} (GADA) are found in about 70% of patients with autoimmune diabetes and in 1-2% in healthy individuals using radioimmunoassays (RIA)[161-165] or enzyme linked immunosorbent assays (ELISA) [166]. The prevalence of GADA in the general background population thus exceeds the frequency of the disease which of course reduces the predictive value [148].

However, the GADA titers in background population are normally not as high as for the diseased subjects, and presence of GADA in addition to other autoantibodies have a high predictive value [167, 168].

Insulinoma associated protein 2 (IA-2)

The transmembrane protein tyrosine phosphatase IA-2 is also a major autoantigen in type 1 diabetes [169]. It is sometimes referred to as islet cell antigen 512 (ICA_{512}) and is enzymatically inactive due to amino acid substitution at conserved sites, critical for tyrosine phosphatase activity [170]. IA-2 is widely expressed in dense core secretory vesicles throughout many neuroendocrine cells. It has been suggested to influence on insulin secretion by increasing the number of insulin-containing dense core vesicles in pancreatic beta cells [171]. Autoantibodies to the tyrosine phosphatase (IA-2A) are present in 75-80% of newly diagnosed children with type 1 diabetes and are less common in adults [172]. There also seems to be a correlation between IA-2A and the HLA-DR4 genotype [173].

Central T cell tolerance

The learning of immune cells begins with *central tolerance* in primary lymphoid organs. Type 1 diabetes is considered to be a T cell mediated disease and thymus is the organ for development of T cells. Both classes of MHC molecules are expressed by cortical thymus epithelial cells (cTECs). T cells bearing the T cell receptor (TCR) with appropriate affinity for the peptide-MHC complexes are positively selected (Fig 2, a). This certifies that the T cells selected have at least some affinity for MHC molecules and is known as *MHC restriction*. However, if the TCR fails to interact with the MHC complex, death receptor CD95 (FAS) expression is not interrupted and the cell dies (Fig 2, b).

The MHC restricted T cells migrates towards the medulla and interacts with medullary thymic epithelial cells (mTECs). These cells have the ability to present antigens that are normally expressed outside the thymus, for example insulin and GAD [109]. T cells that interacts with "high affinity" for self antigens are not accepted, even though interaction in thymus is much weaker than the interaction required to induce mature T cell activation in the periphery [174, 175]. High affinity interacting cells are killed in the "negative selection" which basically is a clearance of auto reactive T cells [176] (Fig 2, c).

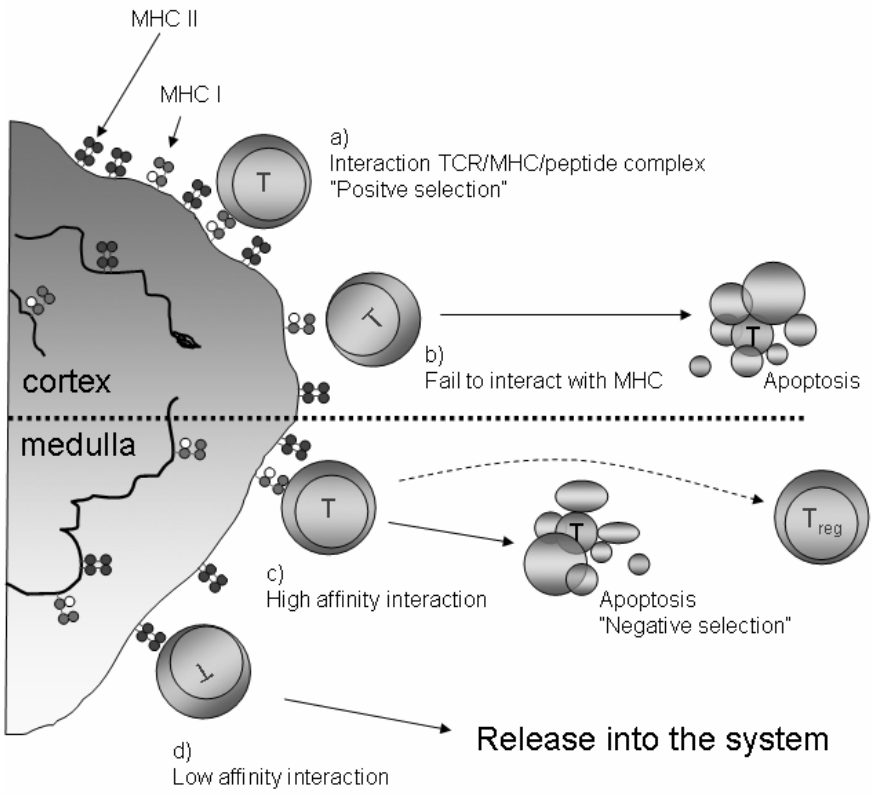

Figure 2. The principle of central tolerance. Most of the human self antigens are expressed in the thymus. Interaction between the T cell receptor and the MHC/self peptide complex in thymus cortex leads to positive selection (a). Malfunction of the T cell receptor in cortex will lead to apoptosis (b). Functional thymocytes migrate to thymus medulla where high affinity interaction with self peptides on MHC molecules is considered hazardous. Those cells are killed /negatively selected (c). However, some of the high affinity interacting cells might differentiate into a subgroup of T cells that is specialized in reducing immune system activity – regulatory T cells (T_{reg}). Low affinity interacting thymocytes leaves thymus as naive T cells (d).

Nevertheless, it is worth mentioning that not all of the high affinity interacting cells are killed Some of them appears to differentiate into another subgroup of T cells with regulatory functions [177, 178]. The low affinity interaction of the MHC restricted T cells in thymic medulla is not considered sufficient to cause autoreactivity and they are released into the system to fight infections (Fig 2, d).

Peripheral T cell tolerance

Even though the central tolerance is strictly regulated, autoreactive T cells will escape into the peripheral tissues. It has been shown that even in non-diabetic healthy subjects, GAD reactive T cell lines can be raised from normal peripheral blood lymphocytes [179]. Thus, a peripheral tolerance mechanism operating outside thymus is required [180]. In contrast to the central tolerance this tolerance occurs on already mature T cells. Since naive T cells cannot enter peripheral tissue other than lymphoid organs they will not induce autoimmunity during normal conditions [181]. Nevertheless, peripheral self-antigens are captured by APCs and presented to T cells (*cross-presentation*) in secondary lymphoid organs [182]. Interaction of TCR and MHC/peptide complex will bring the cell from G_0-phase and prepare the cell for proliferation and differentiation [183] but is by it self insufficient to activate the immune response. Further stimulation is required from membrane bound as well as soluble mediators.

One of the first costimulatory mediators demonstrated was CD28, an integral membrane protein homodimer that interacts with B7.1/B7.2 (CD80/86) expressed on APCs [184]. CD28 signaling seems to increase the expression of IL-2 and the IL-2 receptor α-subunit (CD25) to enhance TCR induced proliferation but also IL-4 and IFNγ [185] which might promote differentiation to certain T helper cell subsets [186]. In addition, CD28 has been shown to stimulate antiapoptotic actions in T cells [187, 188]. As a consequence, engagement of the TCR without receiving a costimulatory signal from CD28 results in a transient activation with extremely low IL-2 production followed by a sharp decline in activation [189] Instead of proliferation or differentiation the event will lead to cell death or anergy [190, 191]. This is also the basic principle of peripheral tolerance. Without proinflammatory stimulation of antigen presenting cells, no B7.1 and only very low concentrations of B7.2 are expressed on the surface[183] and the APCs are considered as immature [192].

Presentation of self antigens to auto reactive T cells by immature APCs will not give the appropriate stimulation to the T cell and tolerance is induced. Another costimulatory molecule that was identified during a screening for genes involved in T cell mediated cytolysis [193], cytotoxic T lymphocyte antigen 4 (CTLA-4) also binds to B7.1/B7.2 molecules. CTLA-4 is antagonistic to CD28 and works as an inhibitor of T cell proliferation. Blockade of CTLA-4 has been shown to accelerate autoimmune diabetes in non-obese diabetic (NOD) mice [194]. In addition, several other costimulatory signaling molecules as CD40 [195-198] and OX40L

[199] are necessary as well as homing receptors to stabilize the interaction between the APC and the T cell during antigen presentation [200].

Breaking the tolerance

One major potential mechanism in the failure of tolerance against beta cell autoantigens is probably the thymic deletion of beta cell specific T cell clones [201], and the extent of the deletion could vary among individuals [202]. Genetic variation has been suggested to influence the intrathymic expression of for example insulin [203]. Furthermore, the level of cross-presentation could influence on the peripheral tolerance [204, 205]. Low levels of self-antigen would mean minimal cross-presentation and autoreactive (although ignorant) T cells could remain in the repertoire. High levels of self-antigens would indeed increase the cross-presentation but the fate of the T cell in this event is coupled to the maturation of the APC. It has been reported that maturation of APCs is associated with antigen release by necrosis but not by apoptosis [206, 207].

If the release of self-antigen occurs in absence of proinflammatory cytokines inducing the expression of costimulatory molecules, then tolerance would probably occur. However, if it takes place in the presence of proinflammatory signals the tolerance could be broken and autoimmunity arise (bystander activation). Other factors that might influence on the maturation of APCs are the cells ability to resist immunosuppression by IL-10 [208], and produce IL-12 [209, 210] but also chemokine expression [211, 212] and recognition of pathogen associated molecular patterns (PAMPs) [213]. Quite recently, a certain type of receptors called pattern recognition receptors (PRRs) were found to recognize PAMPs and mediate signals to leukocytes, connecting innate and adaptive immunity. Toll-like receptors (TLRs) are among the best characterized PRRs. They interacts with different pathogen derived components as for example double stranded RNA (TLR-3), LPS (TLR-4), flagelline (TLR-5) and CpG DNA (TLR-9) [214, 215]. TLR-4 and TLR-9 have been showed to induce maturation of APCs [216] and at least LPS has been shown to interfere with peripheral tolerance [217, 218].

The contribution of TLRs in autoimmune diabetes has not yet been established but might explain some of the links between virus infections and diabetes in a near future.

Other mechanisms have been proposed such as *molecular mimicry* in which some viral structures with striking resemblance to self-antigen structures causes' autoimmunity by activating autoreactive cells by "mistake" [219]. For example, Coxsackie B virus infections have been reported in epidemiological studies to be a common event in patients who ultimately suffer from type 1 diabetes [220-222]. Coxsackie protein P2-C and human GAD_{65} have a common linear epitope [223] and antibodies to P2-C have been reported to cross react with GAD_{65} [224]. Cytomegalovirus (CMV) has also been suggested to induce type 1 diabetes [225] and GAD_{65} contains an amino acid sequence similar to a protein in CMV [226]. However, it seems like cross reactive immune responses between viruses and host are relatively common [227, 228] even in cases were autoimmunity is not induced. The relevance of molecular mimicry in diabetes remains to be further investigated but have so far not been evident.

T cell subsets and IgG subclasses

Cytokine expression by different T cell subsets was first described in mice ([229, 230] and later in humans [231]. The majority of T helper cells (CD4+) belong to either T_h1 or T_h2 subsets [232] with different functions and cytokine patterns (Figure 3). T_h1 cells are known to induce cytotoxic and inflammatory functions [186] as well as delayed type hypersensitivity [233, 234] with a cytokine pattern including IFNγ, TNFα, TNFβ and IL-12. T_h2 cell cytokine pattern of IL-4, IL-5 and IL-13 is associated with antibody production and histamine release and dominates in for example allergic reactions [186, 235, 236].

T_h1 cells also have the capacity of stimulating antibody production of the IgG_1 subclass even though this B-lymphocyte help might be suppressed at very high numbers of T_h1 cells [231, 237]. Type 1 diabetes was early considered as a T_h1 mediated disease due to the presence of IL-1 [238] and IFNγ [239-241] observed in pancreatic islets while IL-4 was reported as decreased in patients with type 1 diabetes [242]. T_h2 related cytokines were also reported to reverse the diabetes onset in NOD mice [243, 244]. Furthermore, the immune response to GAD_{65} was reported to be IgG_1 dominated [245-247] while IgG_4 autoantibodies against IA-2A were associated with protection from type 1 diabetes [248], suggesting a $T_h1$mediated response. Other studies suggest a T_h2 involvement in the pathogenesis of type 1 diabetes [249].

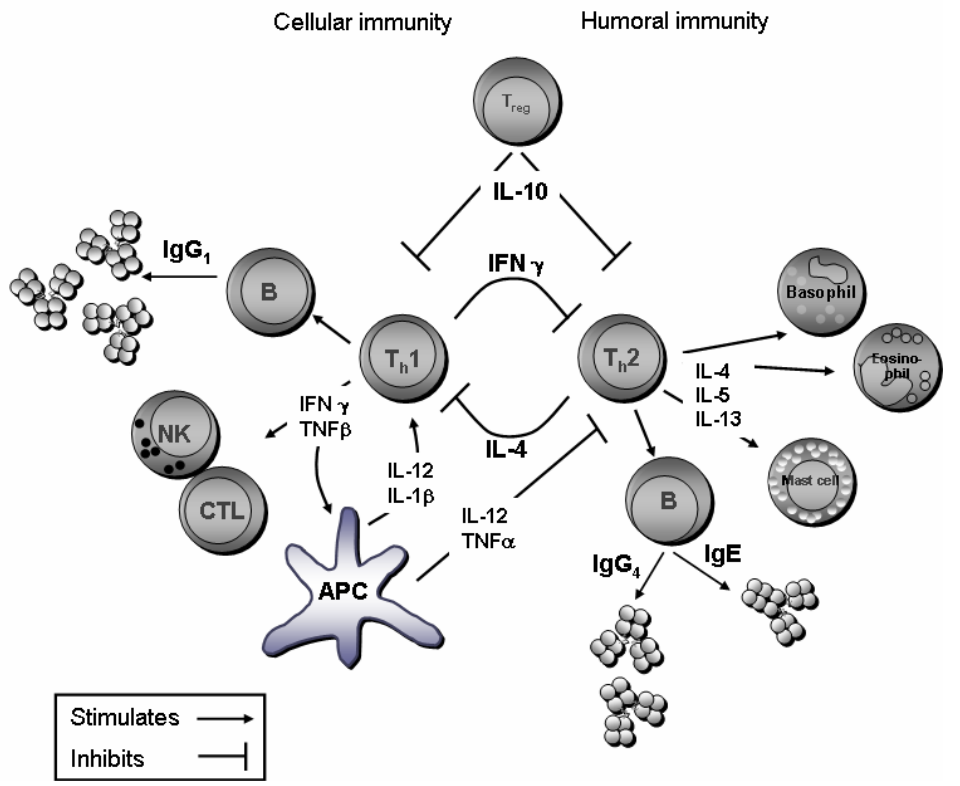

Figure 3. The basic principle of the interplay between different cytokines belonging to T_h1 or T_h2 lymphocytes in humans. Cellular immunity is mediated through T_h1 related cytokines, i.e. IFNγ, TNFα, TNFβ, IL12 and IL-1. Humoral immunity is mediated through T_h2 related cytokines, i.e. IL-4, IL-5 and IL-13. T_h1 specific cytokines suppress proliferation of T_h2 cells and vice versa. Regulatory T-cells further controls regulation by suppressing both T_h1 and T_h2 cells with IL-10. Cellular immunity involves antibody class-switch to IgG1, while humoral immunity mediates class-switch to IgG_4 and IgE. One way to study the response of a T cell subset in a tissue would be to investigate the antibody subclasses directed against antigens from that certain tissue.
APC=antigen presenting cell, B =B-lymphocyte, CTL = cytotoxic T lymphocyte, NK = Natural Killer Cell

The T_h2 involvement was based on the fact that T_h2 cytokines in NOD mice did not inhibit the progression of type 1 diabetes [250, 251]. But also due to the presence of T_h2 cytokines in pancreatic tissue [252, 253] or pancreatic homing of T_h2 cells [253, 254]. Some studies even reported that T_h2 related cytokines accelerated the destruction of insulin producing cells in NOD mice [255, 256]. All studies suggesting T_h2 mediated destruction of beta cells were performed in NOD mice and conclusions were based on the presence of IL-10 rather than

IL-4. IL-10 is a T_h2 related cytokine in mice together with IL-4 [232] but in humans IL-10 is primarily released by regulatory T cells [257]. However, since none of the observations included IL-4 as well it is not obvious whether this effect was unique to IL-10 or a comprehensive feature of T_h2 related cytokines [258].

It is reasonable to assume that both subsets of T cells mediate pancreatic lesions in more or less balance with each other. T_h1 mediated destruction has been reported to yield a more confined insulitis that consists of mostly cytotoxic T lymphocytes. The beta cell death was also performed by apoptosis [259]. In contrast, the insulitis observed in T_h2 mediated destruction has been reported to be more dispersed. The immune cells consisted primarily of eosinophils, macrophages and fibroblasts. The lesions were also caused by necrosis [260] which might be the result of nitric oxide (NO) and reactive oxygen species (ROS) release by macrophages.

There are no reported observations of T cell subset involvement or characterization of the immune response in adult onset type 1 diabetes or in subjects with much slower progression of beta cell failure (LADA).

AIMS

The aim of the study was to define possible immunoregulatory differences between adult onset type 1 diabetes and LADA.
Studies were performed to:

- Examine the importance of TNFa microsatellite polymorphisms and compare these findings with HLA DR3/DQ2 and DR4/DQ8 between adult onset type 1 diabetes and LADA.

- Compare the frequency of GADA IgG subclasses between patients with adult onset type 1 diabetes and LADA at clinical onset.

- Acquire a high-quality assay for GADA IgG subclass determination by comparing the more established use of streptavidin/biotin conjugated system with an N-hydroxysuccinimide (NHS) binding assay.

- Compare the GADA IgM and IgG subclass levels during three years after clinical onset in adult onset patients with type 1 diabetes and LADA

MATERIAL AND METHODS

Subjects

All patients included in the studies were diagnosed with diabetes according to the WHO criteria [261] and all papers focused on adult onset diabetes. Blood samples were collected at local hospitals and sent to us by mail for autoantibody- and C-peptide analyses. Information about each patient such as civic number, date of diagnosis, blood glucose (fasting or non-fasting), body weight, height and clinical classification accompanied the sample, written on a standardized form. The samples were collected as close to onset as possible and therefore both fasting and non-fasting samples were included. Patients that were not clinically classified as type 1 diabetes but positive for at least one of ICA or GADA were considered as LADA in **paper I** and **II**. In **paper IV**, patients with LADA were further restricted to the recommended minimal age of 30 years at clinical onset [103] and without insulin requirement for at least six months.

Patients in **paper I** ,**II**, **III** and **IV**, were identified from a study in a defined area in southern Sweden (former Malmöhus Läns Landsting) where autoantibodies were determined in 1557 newly diagnosed diabetic patients [262]. In **paper IV**, additional patients were included from the Diabetes Incidence Study in Sweden (DISS) since the number of subjects that donated yearly samples was insufficient in the Malmöhus Läns Landsting study. DISS included subjects 15-34 years of age and consequently lowered the median age among LADA in **paper IV** (Table 3).

The subjects included in **paper III** were all LADA patients pre-screened for GADA IgG subclasses. All subjects were positive for GADA IgG_1 plus at least one of IgG_2, IgG_3 or IgG_4.

Non-diabetic controls consisted of healthy blood donors that donated an extra vial for diabetes research. These samples were used to determine the cut-off level for positivity for IgG subclasses and IgM and were also genetically typed for HLA DR and DQ as well as the microsatellite TNFa.

All studies were approved by the Ethical committee at Lund University.

Table 3. The number of subjects in **paper I** – **paper IV** and their median age at onset.

	Paper I Number Age(min-max)	**Paper II Number Age(min-max)**	**Paper III Number Age(min-max)**	**Paper IV Number Age(min-max)**
Autoimmune Type 1 diabetes	n=63 30 (9-67)	n=45 31 (16-67)	None	n=40 28 (18-65)
Latent autoimmune diabetes in adults	n=54 49 (21-79)	n=60 49 (23-80)	n=25 54 (28-79)	n=43 36 (30-79)
Non-autoimmune Type 1 diabetes	n=35 50 (17-89)	None	None	None
Type 2 diabetes	n=340 64 (50-89)	None	None	None
Non-diabetic Control subjects	n=117 35 (19-65)	n=119 35 (19-65)	n=25 43 (25-65)	n=119 35 (19-65)

C-peptide analysis

C-peptide was analysed with a commercial kit (MD315, Euro-Diagnostica, Malmö, Sweden) at the Department of Clinical Chemistry, Lund University Hospital. The intra assay variation in the measurement interval 0.5-3.5 nM was 5% and the sum of the intra- and inter assay variation was 7% (total variation) in the same measure interval (**paper III** and **IV**).

Preparation of DNA genotyping

DNA extraction of human blood samples

After the removal of EDTA plasma, blood cells were transferred to sterile tubes and lysis of erythrocytes was performed in 4x volumes of RBC buffer (155 mM NH_4Cl, 5 mM EDTA and 50 mM TRIS, pH 8.0) for 30 min on ice. The supernatant was removed after centrifugation of the samples (10 min, +4˚C, 1000 x g), followed by a second incubation of 3x volumes of RBC lysis buffer for 15 min on ice. After the second centrifugation (10 min, +4˚C, 1000 x g), 2 ml of SET buffer (150 mM NaCl, 5 mM EDTA, 50 mM TRIS, pH 8.0), 125 µl 10% SDS and 25µl Proteinase K (67031-100, Merck, Germany) were added to solve the pellet. The samples were incubated over night at 37˚C. Next morning, 1 ml of 6 M NaCl was added followed by vigorous shaking for 10 s and centrifugation (15 min, +4˚C, 2000 x g). The supernatants were transferred to sterile 15 ml tubes followed by the addition of 7 ml ethanol. DNA precipitated as the tube was carefully turned upside down a few times. The DNA was washed in ice cold

70% ethanol and air dried under a 60 W bulb. The DNA was solved in sterile nuclease free water (608-274-4330, Promega, Madison WI) and the preparations were quantified with spectrophotometer at 260 nm and purity was estimated at 260/280 nm (**paper I**).

Amplification of TNFa microsatellite markers

In **paper I** the TNF gene was amplified with polymerase chain reaction (PCR) with the reverse primer labeled with a fluorescent 6-HEX or 6-FAM (Amersham-Pharmacia Biotech, Uppsala, Sweden). The reaction was performed in 25 µl tubes containing 250 ng extracted DNA, 2.5 U Taq-polymerase (N808-0244, Amplitaq®Gold, Roche, New Jersey, NY), 2.5 µl PCR buffer (15 mM $MgCl_2$) and a mix of deoxyribonucleotides (dNTP)(Promega, Madison, WI). The first denaturation was performed in 96 C in 10 min followed by 34 cycles of denaturation at 95 C for 40 s, annealing at 60 C for 40 s and strand synthesis at 72 C for 40 s. The PCR ended with the final extension of the PCR product at 72 C for 10 min and identification by electrophoresis at 85V for 40 min in 2% agarose gel with 0.02% ethidium bromide. The number of basepairs (Table 3) was determined by a DNA sequencer (ABI-Prism 373, Perkin-Elmer, Norwalk, CT), measuring the FAM and HEX content at Cybergene Novum Geneotyping Laboratory in Huddinge, Sweden (Fig 4).

Table 2. TNFa microsatellite markers in the HLA region (MHC class III) are constructed of different repetitive sequences $(GT)_n$ corresponding to different alleles. At least 13 different alleles are known in European Caucasian populations (TNFa1 - TNFa13).

Allele	Number of basepairs
TNFa1	98
TNFa2	100
TNFa3	102
TNFa4	104
TNFa5	106
TNFa6	108
TNFa7	110
TNFa8	112
TNFa9	114
TNFa10	116
TNFa11	118
TNFa12	120
TNFa13	122

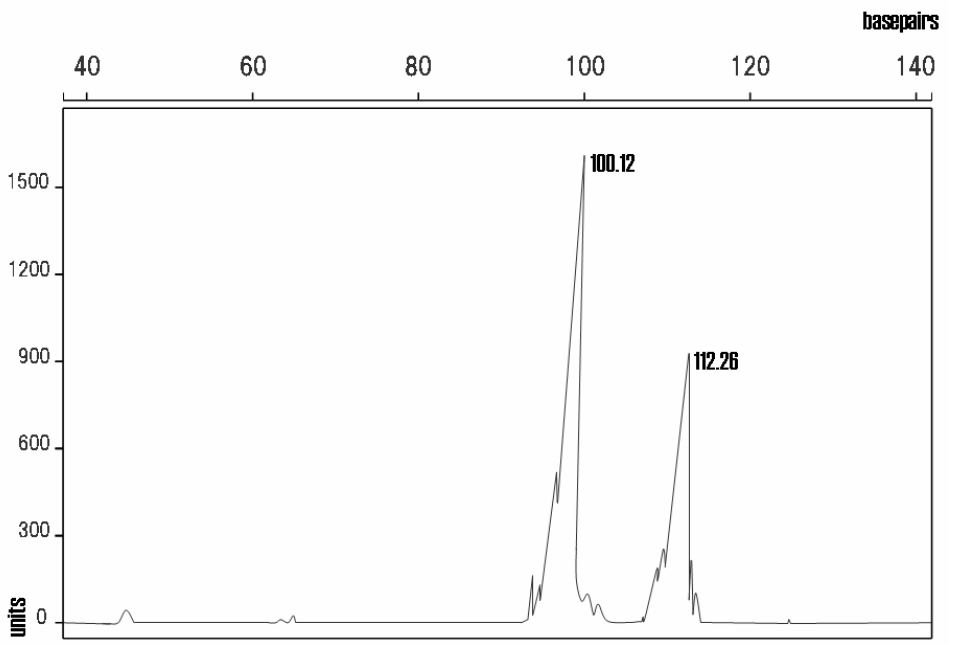

Figure 4. Results of the TNFa genotyping was obtained as peaks from where the number of basepairs (bp) could be identified. The subject in this case had oneTNFa2 allele (100 bp) and one TNFa8 allele (112 bp).

HLA-DR and DQ typing

Amplification of the polymorphic exons of the DQ A- and B-chains as well as the DR B-chain was performed using PCR with specific primers for each region. The amount of 250 ng of extracted DNA was used for the each separate amplification reaction. Activation of Taq-polymerase was performed in 96˚C for 10 min followed by 30 cycles of denaturation at 94˚C for 60 s followed by annealing at 62˚C for the DQA1 primer and 55˚C for the DQB1 primer, both at 60 s. Strand synthesis was performed at 72˚C for 60 s and final extension at 72˚C followed by identification by electrophoresis as described for the TNFa microsatellite amplification. The amplified DNA was dotted onto nylon membranes and hybridization was done with sequence specific oligonucleotides (SSO-probes) 3´end-labelled with ^{32}P-deoxyCTP and washed under specific stringency conditions [263] before exposure to x-ray film. Hybridisation temperature for both probes was 62˚C. Results were manually read from the autoradiograph. The SSO probes used for hybridization of DQB1-genes were done as described by Rønningen et al [111] (**paper I**).

Radioimmunoprecipitation assays of GADA IgG, IgM and IgG subclasses.

The antibodies and subclasses directed against GAD_{65} were measured with ^{35}S-labeled immunoprecipition assays (IPAs). The determination of total GADA was done as previously described by Grubin and Falorni [161, 162]. Briefly, ^{35}S-labeled in-vitro translated recombinant GAD_{65} was prepared using human GAD_{65} DNA from plasmid pGAD65cDNAII [264] (provided by Dr Å Lernmark as a glycerol stock) and TNT® Sp6 Coupled Reticulocyte System Kit (L4600, Promega, USA) according to manufacturers´ instructions. Additional RNasin®, 2500 µl (N211A, Promega, USA) and ^{35}S-methionine (20-7149-50, Amersham Biosciences, Germany) were also used in the preparation (**paper II, III** and **IV**).

Determination of GAD_{65} antibodies (GADA)

Human EDTA plasma (5 µl) incubated with ^{35}S -labeled GAD_{65} (10.000 cpm/well) overnight at +4˚C on a plate shaker. Duplicates of the plasma-^{35}S-GAD_{65} (50 µl/well) then incubated with 50% protein A Sepharose (50 µl) on precoated (1% BSA) microfilter plates (MADVN 6550, Molsheim, France) for 50 minutes at +4˚C. Since protein A interacts with the Fc region of IgG_1, IgG_2 and IgG_4, antibodies of these subclasses precipitated in the Sepharose. The activity was determined in a beta-counter (Packard Tri-carb 2100 TR, Meriden, CT) after dissolving the precipitates in scintillation liquid (**paper II** and **IV**). The lower level for postitivity was an index of 0.08 (97.5 percentile) and defined using 833 healthy controls. The sensitivity and specificity was 81% and 95% respectively [168].

Determination of GADA IgG subclasses and IgM

Most of the studies on GADA IgG subclasses so far have been performed with immuno-precipitation assays based on the same principles as for the total GADA assay described by Grubin and Falorni [161, 162]. Even though assay procedures differs between different reports the precipitation of antibody/antigen complex has been performed by immobilizing biotin conjugated antibodies directed against specific human IgG subclasses on avidin or streptavidin Sepharose [245, 247, 265-268]. Beside this type of assay with solid phase binding principles one report used a liquid phase binding assay [269]. In **paper III** comparisons between the solid phase binding assay and the liquid phase binding assay (Fig 5) were performed to see if the stability and accuracy differed. We also tried another kind of solid phase binding assay (NHSBA) with purified antibodies and N-hydroxy succinimide (Fig 6)

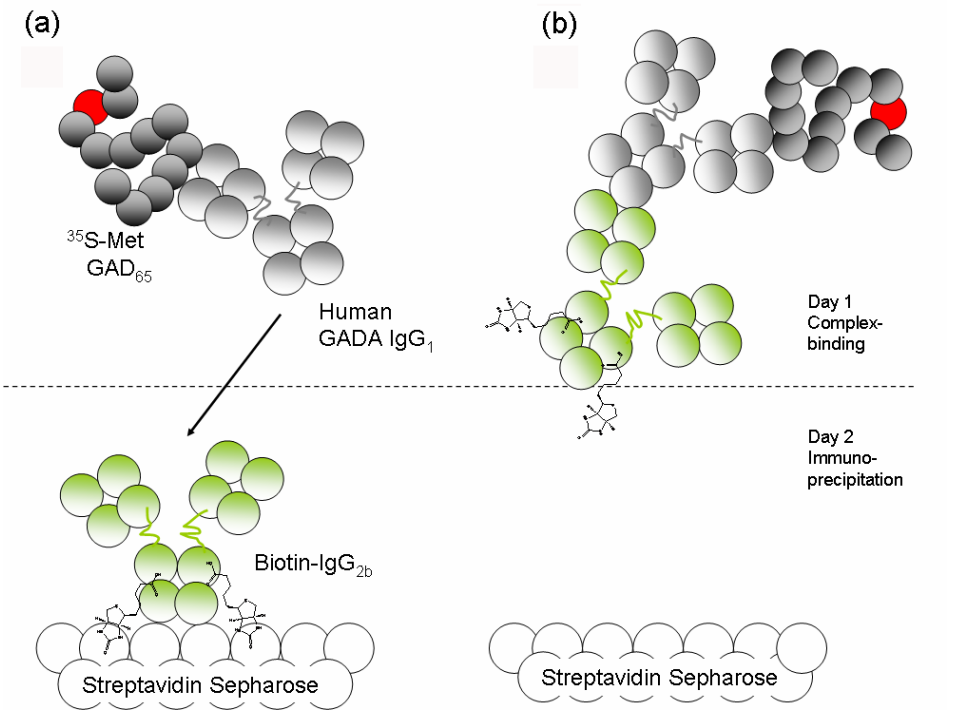

Figure 5. The basic principles of the solid phase binding assay (SPBA) and the liquid phase binding assay (LPBA)**.** Panel A shows the SPBA in which biotin conjugated mouse-antihuman antibodies (green) directed against human IgG_1 (light grey) are immobilised on streptavidin Sepharose before precipitation of the immunocomplex with ^{35}S-labeled recombinant GAD_{65} (dark grey and red). Panel B shows the LPBA in which the interaction between the mouse antibody and the human immunocomplex occurs before precipitation in streptavidin Sepharose.

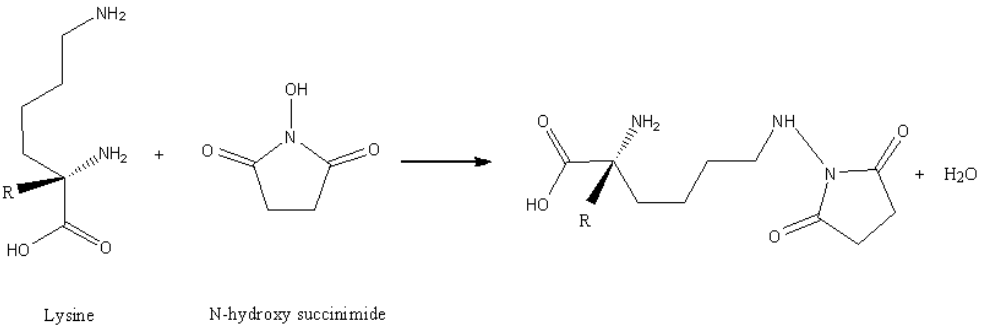

Figure 6 . Lysine residues on mouse antihuman-antibodies directed against specific human IgG subclasses were bound to activated N-hydroxy succinimide sepharose 4 fast flow (Amersham biosciences). Immunocomplex of the subclass and recombinant GAD_{65} was then captured followed by determination of activity in beta counter.

Statistical methods

All statistical calculations were performed with MedCalc version 7.4 for Windows and the Statistical Package for Social Sciences (SPSS) version 12 for Windows. P values <0.05 were considered as significant.

Test for Normal distribution

Kolmogorov-Smiornov test was used to test for normal distribution in all papers and is a well suited test for small sample size as well as large sample size [270]. Normality was rejected when p<0.05 and non-parametric tests were used further on.

Difference plot

The *Bland-Altman plot* was used to assess the agreement between different IgG subclass assays. The differences between the methods are plotted against the means. If the two assays agree, then the mean difference should be close to zero [271]. Also, a regression line inside the Bland-Altman plot should have a slope close to zero or would otherwise be considered as a proportional bias [272]. Nevertheless, the bias itself does not provide enough information about the agreement between the methods. In addition, the "95% limits of agreement" should be included and is calculated as follows;

$$\bar{d} \pm 1.96\sigma$$

Where $\bar{d}$ is the mean difference, and σ is is the standard deviation of the differences. The value 1.96 is the standardized normal deviate corresponding to two-sided α-value of 5% [271]. The distance between the limits of agreement should be as narrow as possible to the mean difference in assays with high extent of agreement (**paper III**)

Comparisons between groups

Frequencies of antibodies or subclasses between groups were compared using the χ^2*-test* or *Fishers exact test* were appropriate (**paper I**, **II** and **III**). Differences in antibody levels between groups were estimated using *Mann-Whitney U-test* (**paper II**, **III** and **IV)**. *Friedman's analysis* was used to test for changes in IgM and IgG subclass levels during the three years follow up (**paper IV**).

Odds ratio (OR) was used for testing genetic risk of TNFa microsatellites and HLA-types. If the lower limit of the 95% confidence interval (CI_{95}) of the OR was above 1, an increased risk

was considered and if the upper limit for the CI_{95} was below 1, a decreased risk was considered (**paper I**).

Correlations

The *Spearman rank correlation test* (r_s) was used to study possible correlations between the levels of total GADA and GADA IgM or IgG subclasses (**paper II** and **IV**).

Regression analysis

Logistic regression analysis is a technique for problem analysis when one or several independent variables determine the result of a dependent variable with only two possible outcomes (dichotomous variable) in this case $^{0)}$*not-diabetic* or $^{1)}$*diabetic*. (**paper I**). Results were expressed as OR (**paper I**).

Deming regression analysis was used to study bias between different methods (**paper III**). In contrast to ordinary least square (OLS) regression, Deming regression assume a random error in both dependent and independent variables [273]. Since it is a method of linear regression;

$$y = kx + m$$

two exactly related methods would generate k=1 and m=0. The CI_{95} of the slope (k) and intercept (m), can be used to test this hypothesis. The hypothesis is accepted if the CI_{95} for the slope ($CI_{95,k}$) contains the value 1 and if the CI_{95} of the intercept ($CI_{95,m}$) contains the value 0. If the $CI_{95,k} \neq 1$, a proportional bias is assumed and if the $CI_{95,m} \neq 0$, a fixed bias is assumed.

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RESULTS AND DISCUSSION OF PAPERS I – IV

Paper I

Since the TNFa2 allele was present in most of the subjects with the high risk or high susceptibility HLA haplotypes, it is reasonable to believe that TNFa2 is in linkage disequilibrium with certain HLA-types. Homozygosity for TNFa2 was present in half of the subjects with the high risk haplotype DR3/DQ2-DR4/DQ8 while total absence of TNFa2 was only found in 3% of the subjects with this haplotype (Fig 7, panel a). Furthermore, TNFa2 was found in 87% of subjects with DR3/DQ2 and just above 50% in subjects with DR4/DQ8 (Fig 7, panel b and c) suggesting a particular linkage with DR3/DQ2 which have also been reported in other studies [146, 147] (Table 2). Nevertheless, more than one third of the subjects without the high susceptibility haplotypes had at least one TNFa2 allele indicating its ordinariness in the background population (Fig 8).

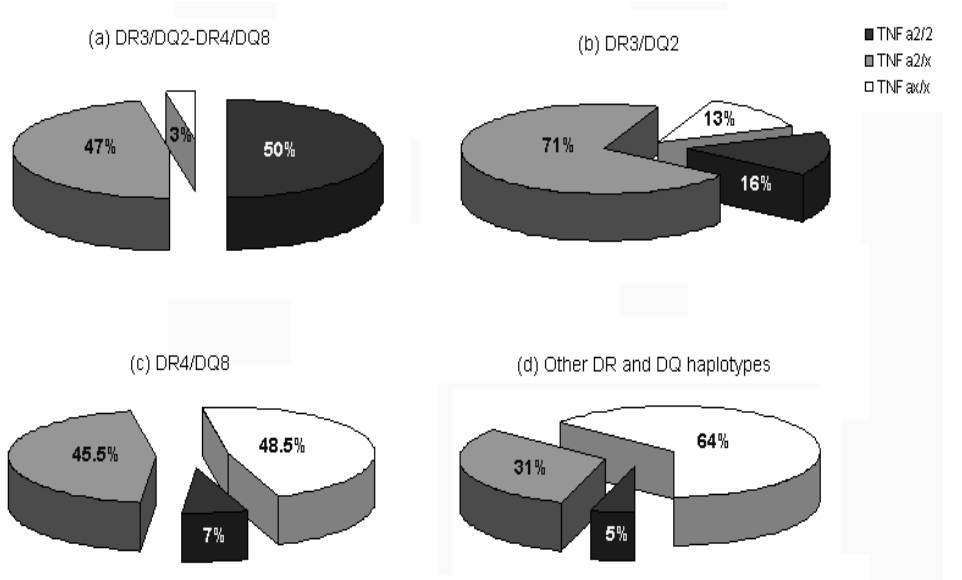

Figure 7. Frequencies of TNFa2 in all subjects (n=609) with different HLA-types. The TNFa2 was the most frequently occurring allele in patients as well as in controls. Homozygosity for TNFa2 is illustrated in black, heterozygosity for TNFa2 is illustrated in grey while absence of TNFa2 is illustrated in white. Panel (a) shows the TNFa2 frequency in the heterozygotic high risk haplotype DR3/DQ2-DR4/DQ8 (n=31). Panel (b) and (c) shows the TNFa2 frequency in high susceptibility haplotypes DR3/DQ2 (n=128) and DR4/DQ8 (n=150) respectively while the frequency in other DR and DQ haplotypes (n=299) are shown in panel (d).

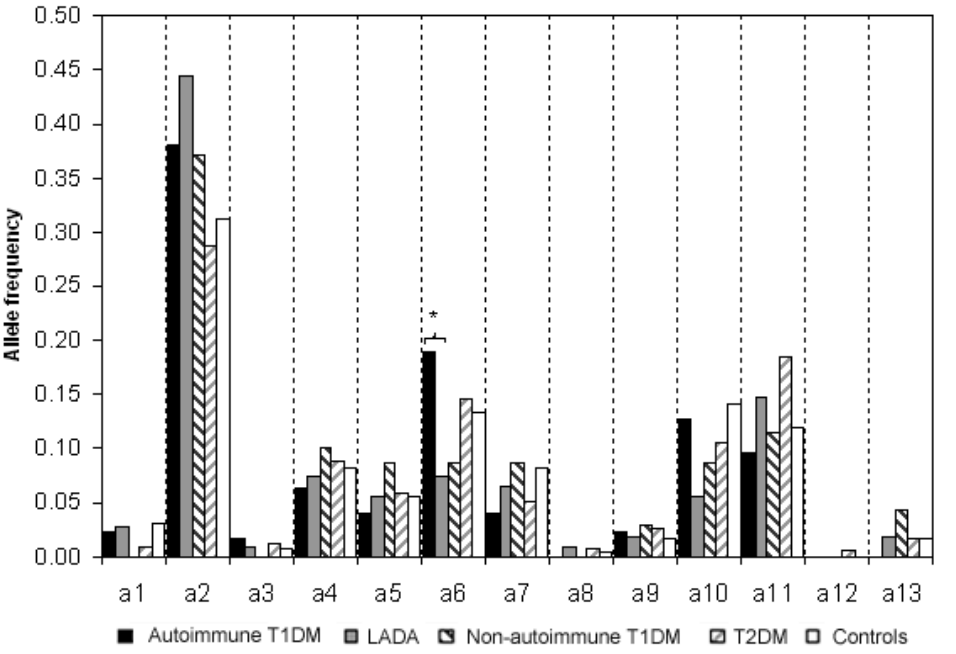

Figure 8. TNFa allele frequencies in 609 Swedish subjects. TNFa2 was the most common allele in all groups. TNFa6 was significantly more frequent in autoimmune type 1 diabetes (19%) compared to in LADA (7%). TNFa10 also appeared to be more frequent in type 1 diabetes (13%) than in LADA (6%) but was not statistically significant.
T1DM = type 1 diabetes, T2DM = type 2 diabetes and LADA = latent autoimmune diabetes in adults. * = $p<0.05$

The TNFa6 allele was more frequent in patients with autoimmune type 1 diabetes compared to in LADA ($p<0.05$) but the rest of the individual alleles were fairly similar between all groups (Fig 8).

Combined genotypes and univariate risk estimation

Homozygosity for TNFa2 allele (Fig 9) appeared to be more common in type 1 diabetes and LADA compared to in type 2 diabetes and controls. In the univariate risk analysis homozygosity for the TNFa2 allele (TNFa2/2) fell out as a significant risk factor in autoimmune type 1 diabetes (OR=5.82, CI_{95}=1.97-17.2) and LADA (OR=3.90, CI_{95}=1.21-12.5) but also in non-autoimmune type 1 diabetes (OR=4.63, CI_{95}=1.32-16.2). This was quite interesting because

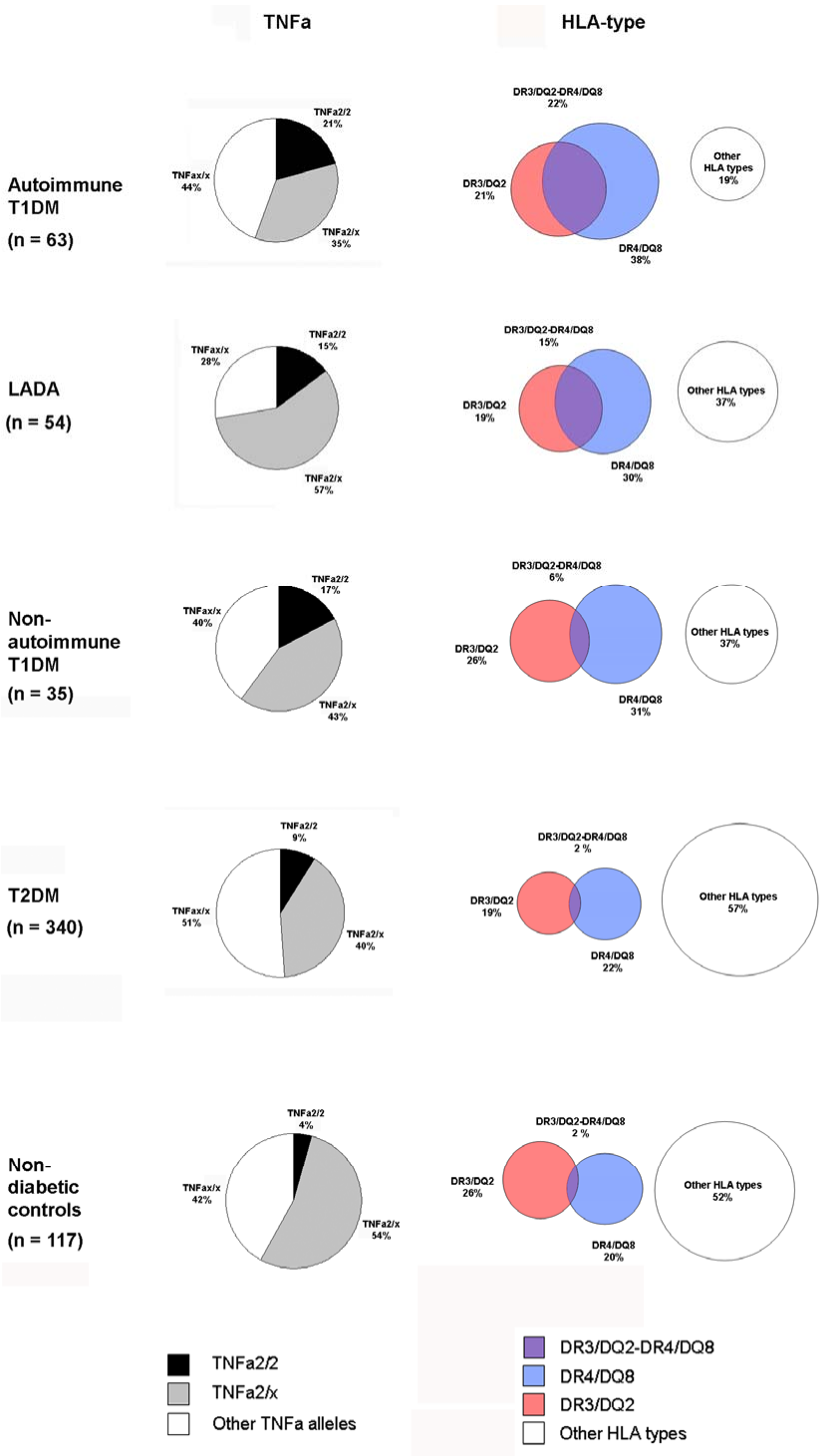
TNFa
HLA-type
Autoimmune T1DM (n = 63)
TNFa2/2 21%
TNFax/x 44%
TNFa2/x 35%
DR3/DQ2-DR4/DQ8 22%
DR3/DQ2 21%
DR4/DQ8 38%
Other HLA types 19%
LADA (n = 54)
TNFa2/2 15%
TNFax/x 28%
TNFa2/x 57%
DR3/DQ2-DR4/DQ8 15%
DR3/DQ2 19%
DR4/DQ8 30%
Other HLA types 37%
Non-autoimmune T1DM (n = 35)
TNFa2/2 17%
TNFax/x 40%
TNFa2/x 43%
DR3/DQ2-DR4/DQ8 6%
DR3/DQ2 26%
DR4/DQ8 31%
Other HLA types 37%
T2DM (n = 340)
TNFa2/2 9%
TNFax/x 51%
TNFa2/x 40%
DR3/DQ2-DR4/DQ8 2 %
DR3/DQ2 19%
DR4/DQ8 22%
Other HLA types 57%
Non-diabetic controls (n = 117)
TNFa2/2 4%
TNFax/x 42%
TNFa2/x 54%
DR3/DQ2-DR4/DQ8 2 %
DR3/DQ2 26%
DR4/DQ8 20%
Other HLA types 52%
TNFa2/2
TNFa2/x
Other TNFa alleles
DR3/DQ2-DR4/DQ8
DR4/DQ8
DR3/DQ2
Other HLA types

Figure 9. (Left) Frequencies of combined genotypes of TNFa2 microsatellite allele (pie chart) and diabetes susceptibility HLA haplotypes (intersection bubble plots). Homozygosity for the TNFa2 allele was generally more common in type 1 diabetes and LADA compared to in type 2 diabetes and in healthy controls. Heterozygosity for DR3/DQ2-DR4/DQ8 was more associated with autoimmune diabetes as expected. Non-autoimmune type 1 diabetes had rather high frequency of the TNFa2 homozygosity even though this was not associated with increase in HLA DR3/DQ2-DR4/DQ8. However it is not excluded that this could be affected by the rather low number of subjects.
T1DM = type 1 diabetes, T2DM = type 2 diabetes, LADA = latent autoimmune diabetes in adults.

there was no significant risk associated with certain HLA-types in this group, suggesting the possibility that the contribution from TNFa alleles could influence separately from the DR/DQ haplotypes. Nevertheless, the number of subjects in this group was rather low which will broaden the confidence interval and overestimate the risk. The well known association of heterozygosity for HLA DR3/DQ2-DR4/DQ8 as the most significant risk factor for autoimmune type 1 diabetes (OR=16.4, CI_{95}=3.6075.0) was confirmed but was also the most significant risk factor in LADA (OR=10.0, CI_{95}=2.05-48.9). The frequency of heterozygosity of DR3/DQ2-DR4/DQ8 in autoimmune type 1 diabetes (22%) was similar to other reports in Caucasian populations [119, 122]. In our study HLA DR4/DQ8 was considered as risk factor for autoimmune type 1 diabetes (OR=2.52, CI_{95}=1.27-4.98) also in the absence of DR3/DQ2 but not in LADA. We could not find any associations with TNFa12 and LADA and neither could we find any protective effect of TNFa13 which both have been reported in a Japanese population [141, 274]. Both TNFa12 and a13 were rather infrequent in our study although heterozygosity for TNFa2 (TNFa2/x) seemed to be protective in non-autoimmune diabetes (OR=0.571, CI_{95}=0.374-0.873).

Genetic risk determined by multivariate risk estimation

When studying the risk factors in a multiple logistic regression, no significance of TNFa2 was demonstrated in any of the groups. Heterozygosity for DR3/DQ2-DR4/DQ8 remained significant in both autoimmune type 1 diabetes (OR=48.2, CI_{95}=8.04-289) and LADA (OR=7.59, CI_{95}=1.38-41.8). However, only autoimmune type 1 diabetes showed individual contribution to risk by DR3/DQ2 (OR=3.36, CI_{95}=1.18-9.62) and DR4/DQ8 (OR=6.79, CI_{95}=2.75-16.8). The contribution of the HLA DR/DQ region is thus stronger than the possible contribution from the TNFa2 allele.

Paper II

GADA IgG subclasses were analyzed with biotin/streptavidin solid phase binding assay (discussed in detail in **paper III**). Cut-off level for positivity was determined by the average subclass level plus three standard deviations in 119 healthy control subjects. Results were expressed as indexes and were calculated as average counts per minute (cpm) for the samples and duplicates of negative and positive standard in the following way:

$$\frac{Sample - NegStd}{PosStd - NegStd} = INDEX$$

In accordance with other reports of GADA IgG subclasses, IgG_1 was the most frequently occurring subclass [245-247, 266] in both type 1 diabetes (96%) and LADA (87%) at clinical onset. However, GADA IgG_4 was the second most frequent subclass in LADA while it was

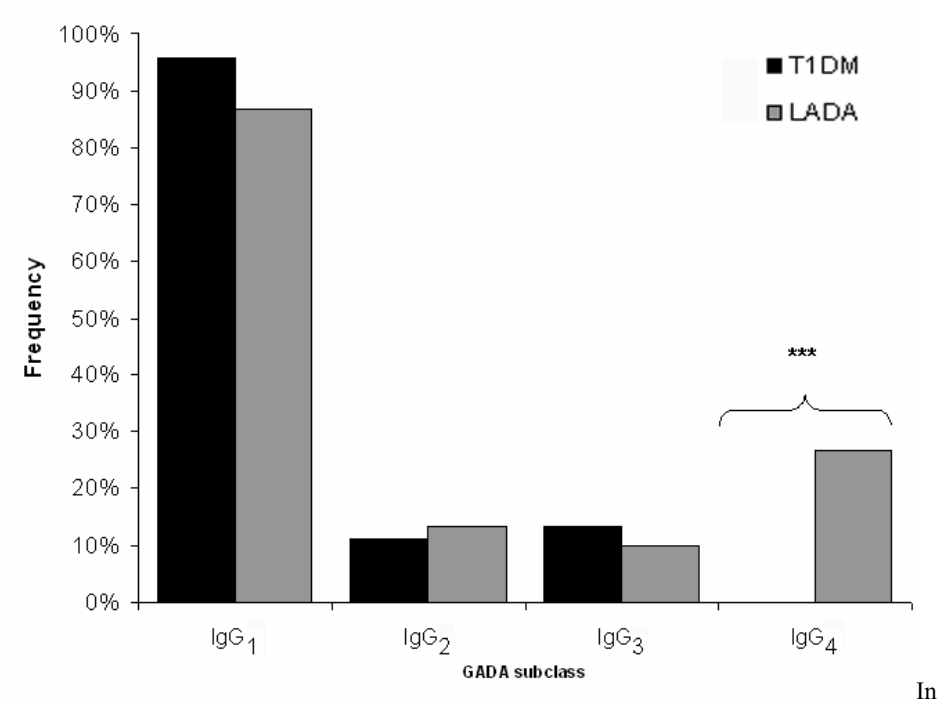

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Figure 10. GADA subclass distribution in subjects with adult onset type 1 diabetes (T1DM) and LADA. IgG_1 was the most frequent IgG subclass in both groups while IgG_4 was only present in LADA ($p<0.001$).

totally absent in type 1 diabetes (Fig 10). Levels of GADA subclasses IgG_2 and IgG_3 were similar between groups. Moreover, IgG_2 and IgG_3 subclasses were rather high in cut-off levels and were hence harder to separate between antibody positive and negative subjects (table 4, SPBA). It has been suggested that presence of subclasses besides IgG_1 is titer dependent rather than disease dependent [275]. If the frequency of subclasses was titer dependent, then subjects with LADA should have higher levels of total GADA compared to in type 1 diabetes. In our study there was no significant difference of total GADA titers between the groups (Fig 11, panel e). Spearman rank correlation of total GADA against each subclass showed a positive correlation with IgG_1 (Fig 11, panel a), r_s=0.82, p<0.001), basically reflecting that this is the major GADA subclass. No significant correlation was found between total GADA and the other subclasses (Fig 11, panel b-d) even though IgG_2 (p=0.12) and IgG_4 (p=0.06) indicated a tendency. The same pattern was observed by Bonifacio et al in subjects with juvenile diabetes [245]. It is reasonable to assume that some patients with high levels of GADA present with high titers due to a polyclonal activation of proliferating B lymphocytes. This could be associated with higher involvement of T_h2 cells since very high numbers of T_h1 cells appeared to inhibit antibody production [231]. Thus a correlation between levels of total GADA and IgG subclasses would not exclude that high GADA IgG subclasses are related to the disease. Couper and colleagues also found that the GADA IgG_4 subclass was associated with slower progression to beta cell failure in prediabetic subjects [246]. In the same study, patients expressing only GADA IgG_1 were younger than patients expressing other subclasses. Subjects with IgG_4 were generally older than subjects without IgG_4. Whether the non-progressors developed clinical diabetes or not is unknown but it is likely that the GADA IgG_4 positive subjects would be classified as LADA in case of developing diabetes considering the older age at clinical onset. One possible explanation to the increased frequency of IgG_4 in LADA would address the question whether this subclass is more frequent as a consequence of the old age rather than differences in immune reaction. One study suggested that increase of total serum immunoglobulins was associated with aging [276] while another study instead reported a decrease of IgG_2 in elderly men and all the IgG subclasses in elderly women [277]. In both cases the results referred to the amount of total immunoglobulins and not to antibodies directed against specific antigens. Furthermore, Petersen and colleagues found no associations between the total amount of immunoglobulin isotypes and GADA IgG subclasses [247]. It is more likely that the higher presence of GADA IgG_4 in LADA reflects a more balanced immune response (T_h1 and T_h2 cells) against pancreatic islet beta cells while the response in type 1 diabetes is restricted to T_h1 cells.

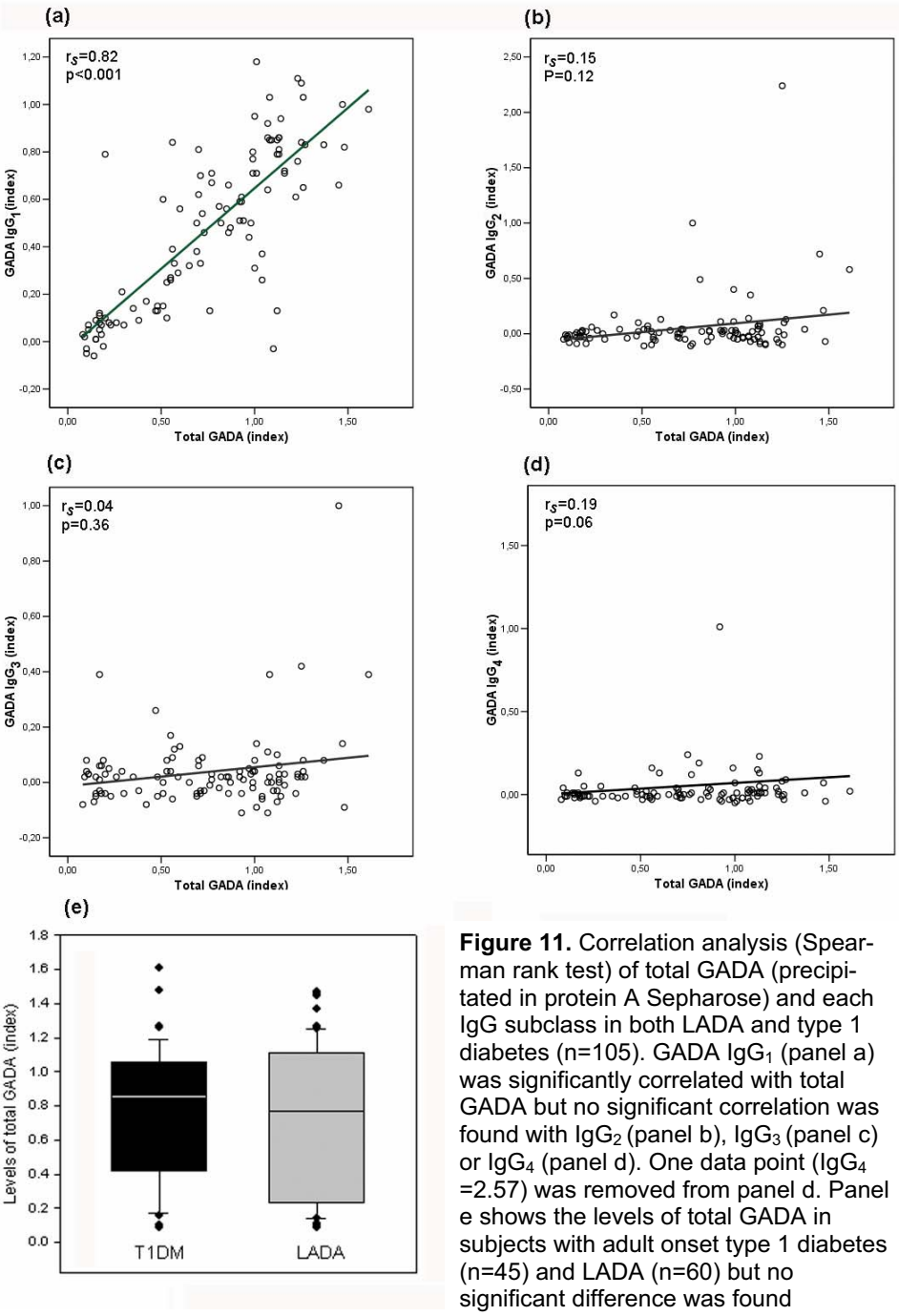

Figure 11. Correlation analysis (Spearman rank test) of total GADA (precipitated in protein A Sepharose) and each IgG subclass in both LADA and type 1 diabetes (n=105). GADA IgG_1 (panel a) was significantly correlated with total GADA but no significant correlation was found with IgG_2 (panel b), IgG_3 (panel c) or IgG_4 (panel d). One data point (IgG_4 =2.57) was removed from panel d. Panel e shows the levels of total GADA in subjects with adult onset type 1 diabetes (n=45) and LADA (n=60) but no significant difference was found

Paper III

Solid phase binding assay (SPBA)

The solid phase binding assay is the most frequently used assay in published reports of GADA IgG subclasses so far. The cut-off levels for positivity of IgG_2 and IgG_3 were relatively high (Table 4) making it difficult to separate subclass positives from subclass negatives in some cases. Especially since even control subjects showed rather high levels. That degree of unspecific binding was not observed with subclasses IgG_1 and IgG_4. The SPBA recognized the IgG_1 subclass in all cases and other subclasses in 16-24% of the patients (Table 5). The coefficient of variation reached between 14-26% for this assay (Table 6). The required antibody concentrations for optimal binding capacity were 15 µg/ml (IgG_1), 30 µg/ml (IgG_2), 22 µg/ml (IgG_3) and 20 µg/ml (IgG_4).

Liquid phase binding assay (LPBA)

The liquid phase binding assay had similar cut-off levels between the subclasses (Table 4). GADA IgG_1 was detected in all of the patients while the other subclasses were found in 24-84% of diabetic cases (Table 5). The coefficient of variation attained between 11-17% (Table 6). Antibody concentrations for optimal binding capacity were 15 µg/ml (IgG_1), 25 µg/ml (IgG_2), 10 µg/ml (IgG_3) and 25 µg/ml (IgG_4).

Table 4. The cut-off levels (indexes) as determined by mean+3SD of GADA IgG-subclass levels in GADA negative control subjects (n=25**)**. The LPBA obtained the lowest cut-off for all GADA IgG-subclasses but in particular for IgG_2 and IgG_3.

	SPBA	**LPBA**	**NHSBA**
IgG_1	0.05	0.04	0.10
IgG_2	0.14	0.05	0.13
IgG_3	0.11	0.03	0.15
IgG_4	0.04	0.03	*

* Unable to detect the subclass

Table 5. The frequency of GADA IgG-subclasses found by the assays of GADA positive diabetic subjects (n=25). Not all subjects were positive for all four subclasses . The LPBA detected higher frequency of IgG_2 and IgG_3 ($p<0.001$) compared to the other assays. This was related to significantly lower cut-off levels and unspecific binding with this assay.

	SPBA	LPBA	NHSBA	p (χ^2-test)
IgG_1	25 (100%)	25 (100%)	25 (100%)	NS
IgG_2	4 (16%)	17 (68%)	5 (20%)	< 0.001
IgG_3	6 (24%)	21 (84%)	5 (20%)	< 0.001
IgG_4	5 (20%)	6 (24%)	*	NS

*Unable to detect the subclass

N-hydroxysuccinimide binding assay (NHSBA)

The NHSBA was unable to detect any IgG_4 at all but the cut-off levels for the other subclasses were rather similar (Table 4). The NHSBA detected IgG_1 in all of the diabetic subjects and IgG_2 and IgG_3 in 20% (Table 5). The coefficient of variation was between 43-49% with NHSBA which is far from acceptable. The required antibody concentration for optimal binding capacity was 20 µg/ml (IgG_1), 40 µg/ml (IgG_2) and 25 µ/ml (IgG_3). Attempts to bind IgG_4 were made with antibody concentrations up to 100 µg/ml without any indication on increased binding activity whatsoever (data not shown). Alternation with acidic and alkaline buffers before blocking with TRIS did not improve the binding capacity at all.

Comparison between the assays

All three assays handled the detection of the IgG_1 subclass well. The LPBA had the lowest unspecific binding compared to SPBA and NHSBA and consequently detected significantly higher frequency of IgG_2 and IgG_3 in diabetic subjects. Furthermore, LPBA had the lowest coefficient of variation and was hence consistently the most stable assay for the determination of GADA IgG subclasses. SPBA and LPBA handled the IgG_4 subclass equally (Fig 12, panel A and B). The agreement between SPBA and LPBA was decent for IgG_1 and IgG_4 but the NHSBA totally disagreed with the two biotin/streptavidin interacting assays.

Table 6. The coefficients of variation (CV) were calculated for each subclass and assay based on repeated measurements (n=18) of the same subclass positive sample. The LPBA had continously least variation while the largest variation was found in the NHSBA. The IgG_4 subclass could not be determined for the NHSBA since the binding to this matrix repeatadly failed for IgG_4.

	SPBA	**LPBA**	**NHSBA**
IgG_1	0.14	0.12	0.45
IgG_2	0.26	0.17	0.43
IgG_3	0.25	0.17	0.49
IgG_4	0.16	0.11	*

* Unable to detect the subclass

Some of the IgG_4 positive samples were notably higher in the LPBA compared to in the SPBA (Figure 12, panel A and B, indicated by arrows). The difference plot (Bland-Altman) on IgG_4 was therefore not reliable because the differences were not normally distributed when these samples were included. The Deming regression was used to study bias between SPBA and LPBA (Table 7). A proportional bias between the SPBA and LPBA was indicated for IgG_1 since the upper limit for CI_{95} was below 1. That means that the agreement is better at low antibody concentrations but the divergence will increase at higher concentrations. For IgG_2, IgG_3 and IgG_4, the CI_{95} was too extensive for bias estimation. This was due to that only a few samples had really high levels for these subclasses and a lot of data was clustered at low concentrations – affecting the linear regression. But it was also due to low sample size (n=25) that will broaden the confidence interval. The CI_{95} for the intercept indicated a systematic or fixed bias for the IgG_2 assay which was expected since LPBA was superior analyzing this subclass, as shown by the lower CV and background. This was also expected for the IgG_3 subclass although surprisingly no fixed bias was detected by the Deming regression. Published results on GADA IgG subclasses agree that IgG_1 dominates the subclass profile directed against GAD_{65} but the contribution from the other subclasses is conflicting in some cases. This could partly be explained by the use of different assays and in particular by the extended use of the solid phase binding principle which was poor for IgG_2 and IgG_3. Our recommendation would be to use the liquid phase binding principle for GADA IgG subclasses.

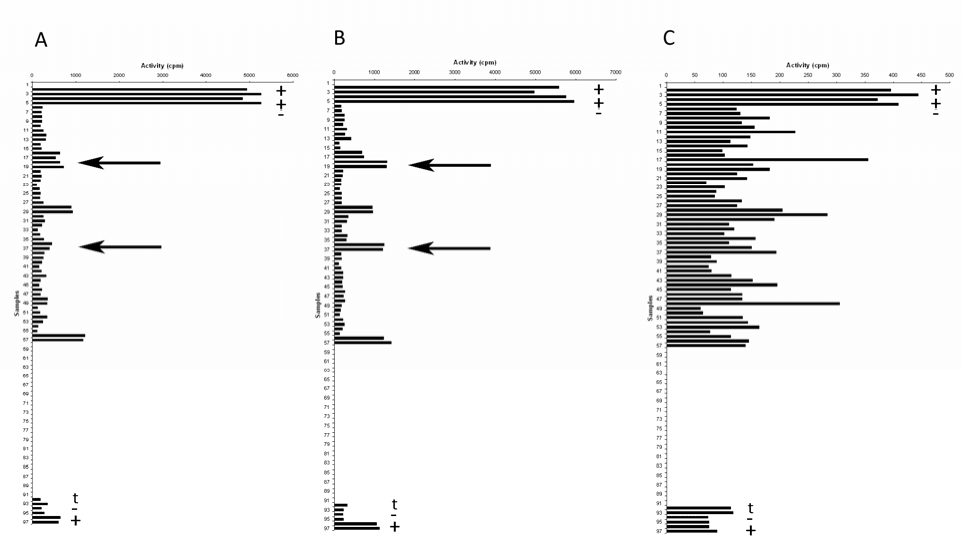

Figure 12. GADA IgG_4 subclass screening in 25 GADA positive diabetic subjects with SPBA (panel A), LPBA (panel B) and NHSBA (panel C). Each sample was analysed in duplicates. NHSBA showed instability with high background and irregular duplicates. SPBA and LPBA performed equally but two of the IgG_4 positive samples (indicated by arrows) were considerably higher in the LPBA even after repeating the analyses. The four lines on the top indicated by plus signs are the duplicates of the two positive standards and the two lines at the bottom indicates the positive control. Negative standards are depicted by minus signs and the tracer is depicted by a **t** at the lower section of the plot.

Table 7. Deming regression analysis of LPBA (y) and SPBA (x) for GADA IgG subclasses. The linear equation is given by y = kx + m, followed by the 95% confidence intervals for the slope (k) and the intercept (m). Proportional bias was assumed if CI_{95},k ≠ 1‡ and fixed bias was assumed if CI_{95},m ≠ 0 ‡‡

	$y = kx + m$	CI_{95}, k	CI_{95}, m
IgG_1	0.82x + 0.04	0.73 to 0.90 ‡	-0.03 to 0.11
IgG_2	0.82x + 0.09	0.26 to 1.38	0.05 to 0.12 ‡‡
IgG_3	0.82x – 0.01	0.34 to 1.30	-0.05 to 0.03
IgG_4	1.38x + 0.01	0.44 to 2.31	0.00 to 0.02

Paper IV

The slower progression to beta cell failure in LADA is often associated with a better preserved beta cell function at clinical onset and consequently higher C-peptide levels compared to in classical type 1 diabetes. Eventually, LADA will come to a period (often within three years) where the remaining beta cells cannot provide sufficient insulin production to maintain glucose homeostasis regardless of oral agents [278]. This would be characterized by a decrease in C-peptide level (Fig 13). In **paper II** we showed that the IgG_4 subclass of GADA was more prevalent in LADA, perhaps reflecting a greater involvement of T_h2 cells. Thus, we wanted to see if the GADA subclasses were more similar to type 1 diabetes three years after clinical onset and maybe show a higher degree of T_h1 restriction.

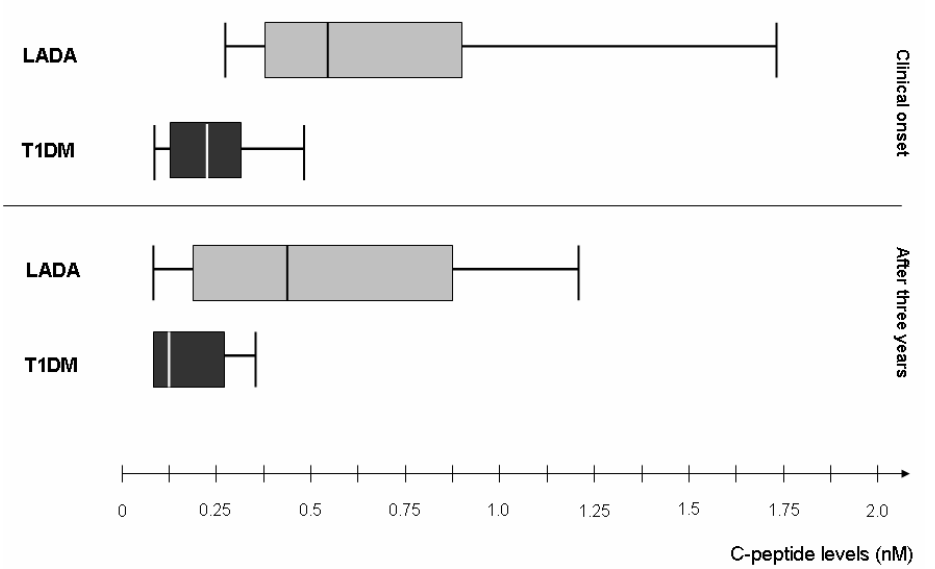

Figure 13. C-peptide levels were significantly lower in patients with type 1 diabetes compared to in LADA both at clinical onset (above)($p<0.001$) and three years after clinical onset(below) ($p<0.001$) as indicated by the Mann-Whitney U-test. However, levels of C-peptide decreased significantly in patients with LADA ($p<0.05$) three years after clinical onset as indicated by the Wilcoxon signed rank test.

In this study we used the LPBA instead of the SPBA used in **paper II**. Levels of GADA IgG_4 were significantly higher in LADA at clinical onset ($p<0.05$) confirming the results of **paper II**. This time we also found significantly higher levels of GADA IgG_3 in subjects with LADA ($p<0.01$) compared to in type 1 diabetes at clinical onset which might be due to the use of LPBA. Total GADA levels correlated significantly with the levels of the IgG_1 subclass (r_s= 0.63, $p<0.001$) and IgM (r_s= 0.24, $p<0.05$). No significant correlation was found between total GADA and the other subclasses this time either (data not shown).

Friedman analysis showed that levels of GADA IgG_1, IgG_2 and IgG_3 decreased significantly ($p<0.001$) in the group with type 1 diabetes three years after clinical onset. An interesting observation was that even though levels of IgG_4 in most cases of type 1 diabetes were below the cut-off limit for positivity at clinical onset, levels still decreased significantly three years after clinical onset ($p<0.05$). In contrast to type 1 diabetes no decrease in IgG subclass levels was observed in the group with LADA three years after clinical onset. Instead, there was a slight increase in GADA IgG_2 levels ($p<0.05$). Furthermore, no significant decrease in levels of total GADA was observed in any of the groups.

Some of the patients had fluctuations in their GADA IgM levels during the three years after clinical onset (data not shown) suggesting that the immune response against GAD_{65} is not a single event leading to disease. It is more likely that the immune system carry on with frequent reactivation against new epitopes during the entire disease process. This was evident in patients with both type 1 diabetes and LADA. However, the levels of GADA IgM decreased significantly in both type 1 diabetes ($p<0.001$) and in LADA ($p=0.001$) three years after clinical onset.

As a consequence of decreasing subclass levels in type 1 diabetes while LADA sustained their profile, a significant difference in levels of IgG_2, IgG_3 and IgG_4 between the groups became apparent already one year after diagnosis. However, even though the same pattern was manifested for IgG_1, the levels of this subclass were never significantly different between type 1 diabetes and LADA.

CONCLUSIONS

- Heterozygosity for DR3/DQ2-DR4/DQ8 is a risk factor for adult onset autoimmune type 1 diabetes as well as in LADA. Homozygosity for the TNFa2 allele is also associated with an increased risk for type 1 diabetes and LADA but the biological significance is still uncertain. The risk is probably related to linkage disequilibrium with the HLA DR3/DQ2-DR4/DQ8.

- DR3/DQ2 and DR4/DQ8 contribute individually as risk factors in adult onset type 1 diabetes but not in LADA.

- The antibody response to GAD_{65} is dominated by the T_h1 cell associated IgG_1 subclass in both adult onset type 1 diabetes and LADA. The T_h2 associated IgG_4 subclass is the second most frequent subclass in LADA but not detected in type 1 diabetes at clinical onset suggesting a more balanced T cell response in LADA.

- The liquid phase binding assay (LPBA) is recommended for GADA IgG subclass analysis due to lower background and higher precision compared to solid phase binding assay (SPBA) and NHS binding assay (NHSBA).

- The decline in GADA IgG subclass levels is significant in type 1 diabetes up to three years after clinical onset. In contrast, the subclass profile in LADA is sustained. The immune response seems to be more balanced in LADA also three years after clinical onset.

POPULÄRVETENSKAPLIG SAMMANFATTNING

VAD ÄR DIABETES?

Diabetes mellitus – en av vår tids stora folksjukdomar, är egentligen inte per definition en sjukdom utan snarare en grupp metabola störningar som leder till kroniskt förhöjd blodsockernivå (*hyperglykemi*). Historiskt sett är diabetes en sedan länge känd åkomma som beskrevs redan för tusentals år sedan. Man visste tidigt att det fanns två typer av diabetes, en som drabbade barn och förknippades med en näst intill hundraprocentig dödlighet och en som drabbade äldre vilka kunde leva flera år med sjukdomen. Diabetes betraktades som ovanligt delvis för att överlevnaden bland drabbade barn var obefintlig och delvis eftersom kosten skiljde sig avsevärt ifrån den kost som genererar vår tids fetma epidemier. Idag vet vi att det finns flera typer av diabetes och sjukdomen betraktas knappast som ovanlig. Orsaken till diabetes är att hormonet *insulin* som tillverkas i bukspottskörteln och reglerar blodsockernivån antingen inte frisätts i tillräcklig mängd eller har en försämrad funktion. Blodsockernivån kommer då att stiga och kroppen kompenserar genom att utsöndra socker i urinen. Detta leder till ökade urinmängder med ökad törst som följd.

Till de vanligaste typerna av diabetes räknas **typ 1 diabetes** som är en autoimmun sjukdom det vill säga, det egna immunförsvaret angriper av ”misstag” insulinproducerande celler i den egna vävnaden. Denna form drabbar ofta barn och ungdomar men förekommer i alla åldrar. Ganska vanligt är att typ 1 diabetes uppträder i samband med vissa infektioner. Nästan alla barn som drabbas av typ 1 diabetes har en speciell uppsättning *gener* (arvsmassa). Dessa gener kallas HLA och bestämmer till viss del vilka proteiner som immunförsvaret ska omge sig med. Det är sannolikt att dessa proteiner bidrar till ”misstaget” som leder till att de insulinproducerande cellerna dör. Hos patienter med typ 1 diabetes kan man hitta antikroppar mot proteiner från insulinproducerande celler som påvisar att det är immunförsvaret som angripit de egna cellerna (*autoantikroppar*). I princip alla med typ 1 diabetes måste behandlas med injicerat insulin. I Sverige har cirka 45 000 personer typ 1 diabetes och är ett av de länder i världen som har den största förekomsten och ökningen av sjukdomen.

En ännu vanligare form är **typ 2 diabetes** som företrädesvis drabbar äldre, inaktiva och överviktiga personer. Denna typ av diabetes orsakas av att kroppen inte känner av det insulin som tillverkas. Detta är en långsammare process som kan pågå under en lång tid innan

diagnosen ställs. Ärftligheten för typ 2 diabetes är ännu starkare än vid typ 1 diabetes men exakt vilka gener som bidrar till sjukomen är inte helt klarlagda. Fler än 250 000 personer är diagnostiserade med typ 2 diabetes i Sverige och nästan lika många till beräknas ha typ 2 diabetes utan att ha fått sjukdomen diagnostiserad. Till skillnad från typ 1 diabetes är denna typ inte förknippad med autoimmunitet. Trots detta hittar man diabetes relaterade autoantikroppar i 5-10% av patienterna med typ 2 diabetes vilket tyder på att immunförsvaret gjort ett ”misstag” här i alla fall. Denna grupp av patienter har kommit att kallas *latent autoimmun diabetes hos vuxna* eller **LADA**.

Det har tvistats om LADA är en separat typ av diabetes eller om det tillhör typ 1 diabetes. LADA skiljer sig från typ 1 diabetes genom att patienternas insulin produktion vid diagnos är betydligt bättre vilket gör att de åtminstone till en början inte nödvändigtvis måste behandlas med insulin. Det kan tyckas oviktigt att tvista om i fall LADA är typ 1 diabetes eller inte men det finns goda kliniska skäl för detta. Det händer att vuxna patienter insjuknar i vad som utan tvivel är den typ 1 diabetes som drabbar barn med ett snabbt insjuknande och inkluderar alla kliniska drag tillhörande denna typ. Dessa patienter förväxlas aldrig med LADA. Förväxlingen sker i stället med de patienter som är diagnostiserade med typ 2 diabetes. Detta indikerar på ett immunsvar mot insulinproducerande celler som helt skiljer sig mellan typ 1 och LADA. **I denna avhandling görs ett försök att förstå skillnaden i immunsvar mellan typ 1 diabetes som uppträder i vuxen ålder och LADA**.

IMMUNSVAR

Till skillnad från andra vävnader i kroppen där celler sitter samman och bildar organ cirkulerar *immunceller* (vita blodkroppar) i blodet och letar efter främmande liv som bakterier, virus, svamp och parasiter. Flera olika typer av immunceller existerar och en del är specialiserade på att fånga upp proteiner i vävnaderna och föra dessa till lymfnoder där de visas upp för andra immunceller med olika specialområden. En typ av celler är specialiserade på att angripa t.ex. virusinfekterade celler eller parasiter. En annan typ producerar antikroppar. Antikroppar är lösliga proteiner som själva kan känna av främmande strukturer i kroppen. De klistrar sig fast och underlättar för immunceller att städa undan främlingen. Dessa antikroppar finns i flera olika typer och klasser (t.ex. IgG_1, IgG_2, IgG_3, IgG_4, IgM och IgE) med något skilda funktioner. Till skillnad från antikroppar klistrar sig autoantikroppar till egen vävnad i tron att denna är främmande. Vid diabetes är detta till strukturer i insulinproducerande celler kallade GAD, IA-2 men också mot insulin.

T-hjälpar lymfocyter är en grupp av vita blodkroppar som har till uppgift att styra kommunikationen mellan de övriga immuncellerna och bestämmer till viss del vilken typ eller klass av antikropp som ska frisättas. Två grupper dominerar, T_h1 stimulerar ett immunsvar och leder bland annat till produktion av IgG_1 medan T_h2 stimulerar till produktion av IgE och IgG_4. Genom att studera typen av autoantikroppar vid typ 1 diabetes och LADA kan man se om dessa stimuleras av olika immunsvar eller om de är resultatet från samma typ av angrepp.

RESULTAT

I den första studien visades att HLA generna är viktiga riskfaktorer för både typ 1 diabetes och LADA men att risken för vissa individuella HLA-typer är mer uttalade vid typ 1 diabetes.

Den andra studien visade att immunsvaret mellan typ 1 diabetes och LADA skiljer sig något åt vid diagnos. Vid både typ 1 diabetes och LADA var den dominerande GAD antikroppen av IgG_1 typ vilket är en indikation på att T_h1 celler har en viktig styrande roll vid båda typerna. Vid LADA var även GAD antikroppar av IgG_4 typ relativt vanlig medan nivåerna av denna antikropp inte kunde detekteras vid typ 1 diabetes. Detta skulle peka på att immunsvaret vid LADA är mer balanserat av T_h2 celler än vid typ 1 diabetes.

Den tredje studien var ett arbete för att förbättra metoden att påvisa vilken klass av antikroppar som involveras i immunsvaret mot GAD. Tre olika försök gjordes och det som visade sig bäst var att binda upp antikropparna i en löslig fas. I studie två hade detta gjorts i en fast fas vilket eventuellt kunde försämra metodens upplösning något.

I den fjärde studien jämfördes därför antikroppklasserna igen med den nya metoden och denna gång var syftet även att följa utvecklingen upp till tre år efter diagnos. Liksom vid första studien var nivåer av IgG_4 högre i patienter med LADA jämfört med typ 1 diabetes. Nivåerna av samtliga antikroppklasser IgG_1, IgG_2, IgG_3 och IgG_4 sjönk vid typ 1 diabetes men höll sig stabila över tre år i patienter med LADA.

Resultaten pekar på skillnader i immunsvar mellan typ 1 diabetes och LADA och dessa har sannolikt olika sjukdomsförlopp. Det är således rimligt att räkna LADA till en variation av autoimmunitet som skiljer sig från klassisk typ 1 diabetes.

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