Association between immune reactivity and disease status of type 1, type 2 diabetes and latent autoimmune diabetes in adults

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Düsseldorf, den 23.02.2012

Thi Minh Nguyet Pham

“Learn from yesterday, live for today, hope for tomorrow.

The important thing is not to stop questioning”.

Albert Einstein

to my parents,

Tien Thanh, Ngoc Tran,

Duy Vu, Chieu Anh,

Phuoc Tuy, Ngoc Dung, Dominik An, Andre Thien,

Huu Minh, Thanh Dung, Maria Huong,

Firdevs, Fariba and Abdelouahid for their love, faith and support

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Chapter 1

Introduction

Diabetes mellitus

Diabetes mellitus is a chronic metabolic disease characterized by hyperglycemia resulting from the defects of insulin secretion, insulin action, or both.[1]

According to recently published data from the International Diabetes Federation's (IDF) 5th edition of the Diabetes Atlas, 366 million persons worldwide have diabetes mellitus in 2011.[2] Given the population growth, aging, obesity, physical inactivity, altered eating behaviour and environmental changes, the prevalence of diabetes mellitus is continuously increasing (Table 1). Currently, the highest prevalence of diabetes mellitus cases is found in Middle East, the United States of America (USA) and India.[3] The IDF expects that the current prevalence with 366 million will rise to 552 million by 2030.[2]

In 2004, the World Health Organization (WHO) estimated 3.4 million cases of death associated with diabetes mellitus.[4] The major complications of diabetes mellitus are cardiovascular disease (CVD), diabetic nephropathy, neuropathy and retinopathy as shown in Table 1.[2-4]

Diabetes mellitus is generally classified in two major types with different underlying pathophysiologies, type 1 and type 2 diabetes, and other forms of secondary diabetes mellitus.[1,2] Numerous recent studies have described a subgroup of diabetes mellitus called latent autoimmune diabetes in adults (LADA).[5-8]

TABLE 1: Risk factors, warning signs, complications and management in diabetes mellitus

Risk factors	Warning signs	Complications	Management
Obesity	Frequent urination	Cardiovascular disease	Increase of physical activity
Diet	Excessive thirst	Diabetic neuropathy	
Physical inactivity	Increased hunger	Diabetic nephropathy	
Increasing age	Weight loss	Diabetic retinopathy	Reduction of body weight
Insulin resistance	Tiredness		
Family history of diabetes	Vomiting		Healthy eating
	Stomach pain		Avoid tobacco
Ethnicity	Blurred vision		Monitoring complications
	Frequent infections		
	Slow-healing wounds		

Source: Adpated from IDF's 5th edition of the Diabetes Atlas

Type 1 diabetes

Type 1 diabetes, formerly named insulin dependent diabetes mellitus (IDDM) or juvenile onset diabetes, is a chronic immune-mediated disease resulting in the destruction of autologous pancreatic β-cells and absolute insulin deficiency.[9-11]

Epidemiology

An estimated proportion of 5-10% of all diabetes cases account for type 1 diabetes.[1] In the past decades a worldwide increase of type 1 diabetes incidence was observed in young children.[12-18] This incidence is variable among different ethnic population: 0.1/100,000 per year in China, 36.8/100,000 in Sardinia and 36.5/100,000 per year in Finland.[19,20] The observation of high incidence in northern countries and lower incidence in southern countries points to a north-south gradient of the disease.

The aetiology of type 1 diabetes appears to be multifactorial including a variety of environmental factors, which are still not completely known (Table 2).

TABLE 2: Potential risk factors for type 1 diabetes

Environment	Diet components	Genes	Antigens
Bacterial infections	Cow's milk	HLA DR/DQ	ICA
Enterovirus	Gluten	Insulin VNTR	IA2
Congenital rubella	Vitamin D	PTPN22	IAA
Rotavirus	Protein	CTLA4	GAD
Cytomegalie virus			ZnT8
Mumps			

Clinical manifestation

Symptoms for type 1 diabetes are hyperglycaemia, polyuria, polydipsia, weight loss and ketoacidosis.[1,2] Chronic complications of type 1 diabetes are predominantly seen in patients with insufficient control of blood glucose concentrations resulting in hyperglycaemia.[21-23] Optimal insulin treatment should therefore aim on near-normo-glycaemic blood glucose.[21-23]

In the first year after the initiation of insulin therapy around 30-60% patients with type 1 diabetes develop a transient and remission phase known as the "honeymoon phase" (Figure 1).[24] During the remission phase patients with type 1 diabetes require no or low amounts of exogenous insulin to maintain normoglycaemia.[25] This period generally extends over a few weeks to a year and has been observed to be more common in adult and adolescent patients

compared to young children with type 1 diabetes, who display a more aggressive disease progression.[26,27]

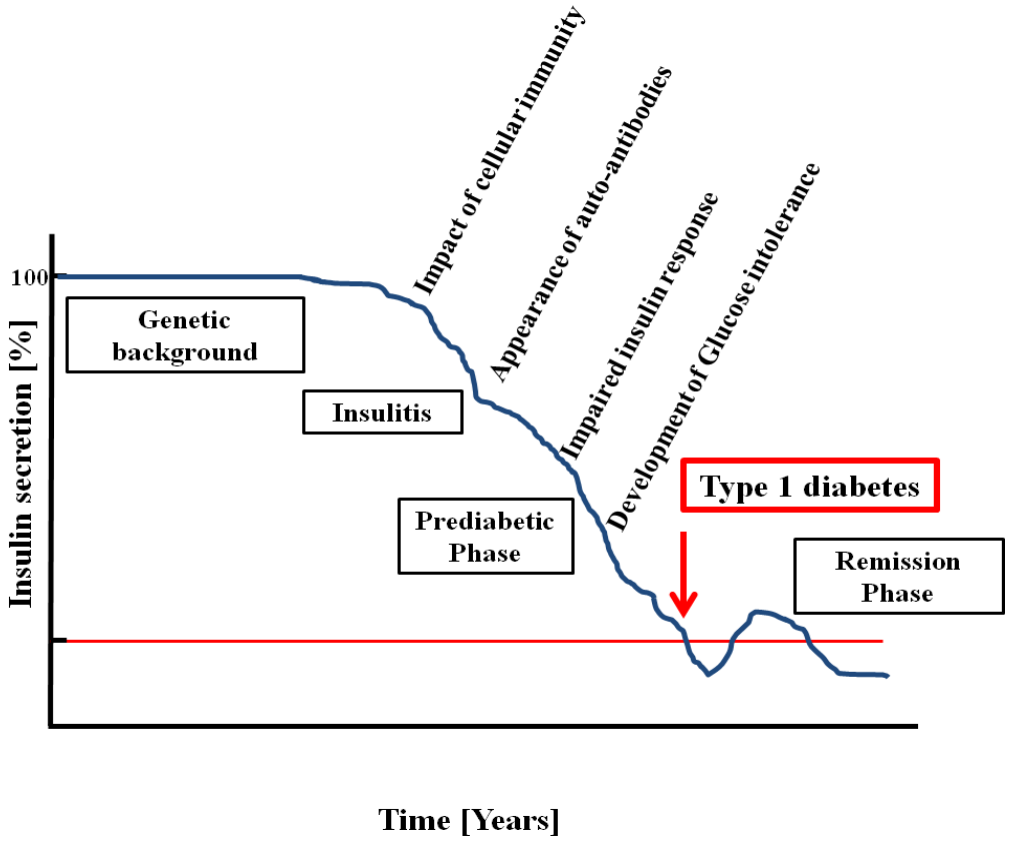

FIGURE 1: Progression of type 1 diabetes
Source: Adapted from Eisenbarth GS. N Engl J Med 1986

Genetic risk factors of type 1 diabetes

Susceptibility to type 1 diabetes is largely determined by genetic factors.[28-30] Siblings of patients have a 3-7% higher risk for the development of type 1 diabetes (Table 3).[31] Offspring of diabetic fathers had a higher risk (~7%) than offspring of diabetic mothers (~3%) (Table 3).[32] Studies in monozygotic twins showed a 30-50% concordance of developing type 1 diabetes whereas dizygotic twins revealed a 20-30% concordance as shown in Table 3.[33-41]

Genome wide association studies (GWAS) have demonstrated that human leukocyte antigen (HLA) genes are associated with the development of type 1 diabetes.[42-49] The class II loci HLA-DR and –DQB show the highest association with susceptibility to type 1 diabetes.[50,51]

High risk of DR/DQ haplotypes for the development of type 1 diabetes are HLA-DR3 (DRB1*03) and HLA-DR4 (DRB1*04) which are detected in ~80% in patients with type 1 diabetes whereas ~40% of healthy subjects carry these risk genes (Table 4).[52-57]

TABLE 3: Likelihood for developing type 1 diabetes

Family history with type 1 diabetes	Relative risk [%]
No family history with type 1 diabetes	0.4
Siblings	3-7
Mother	1-3
Father	3-7
Both parents	20-40
Dizygotic twins	20-30
Monozygotic twins	30-50

Source: Adapted from American Diabetes Association 2011

The DR3/DR4 heterozygous genotype shows the highest risk for type 1 diabetes, followed by DR4 and DR3 homozygosity. Siblings with the HLA-DR3/DR4 genotype have a risk of ~20% for the development of type 1 diabetes. The HLA-DR2 (DRB1*1501) genotype show a protective effect against type 1 diabetes.[52,55] Table 4 shows an overview of high-risk and protective haplotypes.

Meanwhile, outside the HLA-region more than 40 genes have been identified which modulate the risk for type 1 diabetes.[43,58-60] Figure 2 shows some of these genes and their odds ratios for the development of type 1 diabetes. Specific alleles of non-HLA genes with higher risk for the onset of type 1 diabetes are described for variable number of tandem of repeats (VNTR) of the insulin gene, protein tyrosine phosphatase non-receptor type 22 (PTPN22) and polymorphic cytotoxic T-lymphocyte-associated antigen-4 (CTLA4)[61-65].

TABLE 4: HLA types and type 1 diabetes susceptibility risk

HLA types	Risk
DR3	High
DR4	High
DR3/DR4	Very high
DR2	Low→ protective
DR6	Low→ protective

Source: Adapted from Pugliese A, Eisenbarth GS. Chapter 7. 2011. Barbara Davis Center

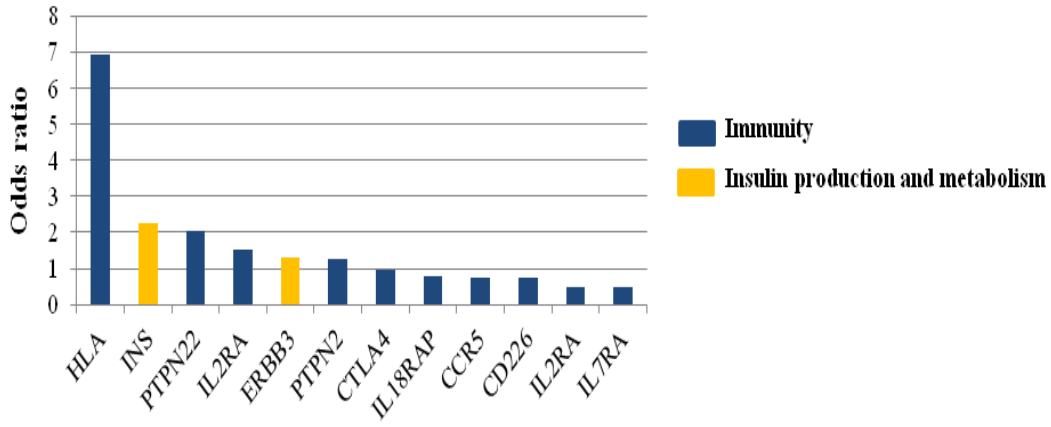

FIGURE 2: Odds ratio for identified HLA and non-HLA susceptibility genes from GWA studies

Source: modified from Concannon P, Rich SS, Nepom GT. N Engl J Med 2009

Environmental factors in type 1 diabetes

Currently, environmental factors are discussed as trigger for the progression of immune reactivity toward overt type 1 diabetes in genetically predisposed individuals (Table 2). Detailed knowledge about the impact of environmental factor on the progression of type 1 diabetes can contribute to develop strategies for the prevention of the disease.

TABLE 5: Exposures related to infant nutrition implicated as factors modulating β-cell autoimmunity or type 1 diabetes

Exposure	Outcome	Effect
Short breast feeding (<3 months)	Type 1 diabetes	Predisposing
Early introduction of cow's milk	Type 1 diabetes	Predisposing
Longer duration of breastfeeding (>4 months)	β-cell autoimmunity	Protective
Early introduction of cow milk	β-cell autoimmunity	Predisposing
Weaning to a highly hydrolyzed formula	β-cell autoimmunity	Protective
Early introduction of cereals (<12 months of age)	β-cell autoimmunity	Predisposing
Early introduction of fruit and berries (<4 months of age)	β-cell autoimmunity	Predisposing
Use of cod liver oil during the first year of life	Type 1 diabetes	Protective
Supplementation with vitamin D in infancy	Type 1 diabetes	Protective

Source: Adapted Knip M, Virtanen SM, Åkerblom HK. Am J Clin Nutr 2010

Impact of diet in the pathogenesis of type 1 diabetes

An increasing number of studies implicate that dietary factors are likely to influence risk of both the emergence of the β-cell autoimmunity and the development of type 1 diabetes as summarized in Table 5 and 6.[66-73] Prospective studies on the diet role in type 1 diabetes have been carried out in the last decades. The outcomes of these studies are inconsistent. Short breastfeeding, early cow's milk intake or early introduction of cereals increased the risk of type 1 diabetes and the presence of auto-antibodies to the dominant antigens.[74] Other studies including Diabetes Autoimmunity Study in the Young (DAISY) and BABYDIAB did not find an association of breastfeeding or early intake of cow's milk with the development of islet auto-antibodies.[75,76]

Recent data from the Trial to Reduce Insulin-dependent diabetes mellitus in the Genetically at Risk (TRIGR) showed that among children with an HLA genotype conferring increased risk of type 1 diabetes, weaning to a highly hydrolysed formula was associated with fewer signs of β-cell directed autoimmunity up to 10 years of age compared to children with intake of cow's milk.[77] Although the mechanism by which hydrolysed formula decreases the risk of diabetes-predictive auto-antibodies remains to be elucidated. These data clearly demonstrated the impact of dietary factors on the development of type 1 diabetes.

TABLE 6: Proposed mechanisms for nutrition-related exposures during infancy modulating the risk of β-cell autoimmunity and type 1 diabetes

Factor	Proposed mechanisms
Breastfeeding	• decreased intestinal permeability
Early introduction of cow's milk proteins	• inflammation in intestinal mucosa • dysregulated immune response to cow milk proteins • increased intestinal permeability
Early introduction of cereal	• inflammation in intestinal mucosa
Weaning to a highly hydrolysed formula	• decreased intestinal permeability? • induction of regulatory T cells in gut-associated lymphoid tissue? • increased diversity of gut microflora?
Vitamin D deficiency	• decreased suppression of pathologic Th1 immune responses

Source: Adapted from Knip M, Virtanen SM, Åkerblom HK. Am J Clin Nutr 2010

Sunlight, vitamin D and type 1 diabetes

As vitamin D levels are dependent on the exposure to sunlight, it has been suggested that the observed north-south gradient of the prevalence of type 1 diabetes may in part be caused by different levels of vitamin D. Increased levels of vitamin D have been proposed to protect from the development of autoimmunity and type 1 diabetes via reduction of the production of lipopolysaccharide-induced interleukin (IL-) 12 and interferon-gamma (IFN-γ) and arrest Th1 cell infiltration and disease progression (Table 6).[78,79] However, further studies are required to characterize the role of vitamin D.

Viral role in the aetiology of type 1 diabetes

Several studies immunohistochemical have identified human viruses in the pancreatic islets of children with newly diagnosed type 1 diabetes.[80-82] Therefore, it has been suggested that infection with viruses such as Coxsackie B virus, mumps virus, cytomegalovirus (CMV), Epstein-Barr virus (EBV), rotavirus and parvovirus are associated with the development of type 1 diabetes and could trigger islet autoimmunity (Table 2).[83-93] Molecular mimicry and T-cell cross-reactivity to beta-cell auto-antigens and environmental agents with sequence similarities have been a proposed mechanism underlying the pathogenesis of type 1 diabetes.[86-88] Further investigations are needed to clarify the role of viruses in the aetiopathogenesis of type 1 diabetes.

The hygiene hypothesis

The hygiene hypothesis explains why type 1 diabetes incidence is paradoxically higher in industrialized countries despite the high sanitary standards and broad availability of antibiotics.[94] Kondrashova et al. supported the hygiene hypothesis and demonstrated that the neighbouring populations of Finland and Russian Karelia, that have different standard of hygiene, display a significant difference in the incidence of type 1 diabetes, even though their genetic profiles are similar.[95]

According to the hygiene hypothesis, viral infections during childhood would protect individuals from the risk of developing type 1 diabetes or would delay disease onset.[94,96] This finding implicates that under conditions favouring enterovirus infections young children rise strong immune responses.[97] Recently, Wen et al. provided further support for the hygiene hypothesis by their observation that mice which grew up in a germ-free environment showed higher risk for the onset of insulin-deficient diabetes.[98]

Immunopathogenesis of type 1 diabetes

Diabetes-associated auto-antibodies

Auto-antibodies against various autologous antigens were found to be associated with type 1 diabetes. The most dominant of those described are islet cell auto-antibodies (ICA), insulin auto-antibodies (IAA), antibodies directed against 65 kDa isoform of the glutamic acid decarboxylase protein (GADA), antibodies against tyrosine-phosphatase-related IA-2 molecule (IA-2A) and antibodies against the Zinc transporter molecule 8 (ZnT8).[99] A list of the major auto-antibodies in type 1 diabetes is given in Table 7.

TABLE 7: Major auto-antibodies in type 1 diabetes

Auto-antibodies	Abbreviation	Implication in diagnosis
Islet cell antibodies	ICA	• In 80-90% of patients with newly diagnosed type 1 diabetes • Titre reduction is associated with increasing age
Glutamic acid decarboxylase antibodies	GAD	• In 70-80% of patients with newly diagnosed type 1 diabetes • Detectable even in patients with longer diabetes duration
Anti-bodies against Tyrosine-phosphatase-related IA-2 molecule	IA-2	• In 60-70% of patients with newly diagnosed type 1 diabetes • 2-5% in 1st degree relatives
Zinc transporter 8 antibodies	ZnT8	• In 60-80% of patients with newly diagnosed type 1 diabetes • In 30% of patients with other autoimmune disease associated with type 1 diabetes

Source: Adapted from Simon MC, Pham MN, Schloot NC. Der Diabetologe 2011

Auto-antibodies as markers for the prediction of type 1 diabetes

Auto-antibodies are used as markers for the prediction of the onset of type 1 diabetes and reflect the progression and severity of the autoimmune process.[99,100] The appearance of type 1 diabetes-associated auto-antibodies and their predictive value in the development of type 1 diabetes has been extensively studied in prospective follow-up cohorts such as the German BABYDIAB study, the Finnish Diabetes Prediction and Prevention (DIPP) study and in the

American Diabetes Autoimmunity Study in the Young (DAISY).[99-102] Relatives of patients with type 1 diabetes presenting have a risk of 68% to manifest the disease within 5-years.[103-106] Individuals with three auto-antibodies have an almost 100% risk for disease manifestation within 5-years.[103-106]. The findings suggest that the velocity of diabetes progression is determined by the number of auto-antibodies. The Karlsburg Type 1 Diabetes Risk Study detected 821 (6.9%) from a total number of 11,840 schoolchildren with positivity for a single auto-antibody whereas 83 (0.7%) children had multiple auto-antibodies.[107]

Auto-antibodies as diagnostic markers

If diagnosis of recent onset of type 1 diabetes by clinical parameters does not allow a decision or patients are adults and obese without ketoacidosis, testing auto-antibodies is meaningful in order to distinguish these patients from patients with type 2 diabetes, Maturity onset diabetes of the young (MODY) or other secondary forms of diabetes. It has been proposed to determine multiple auto-antibodies in order to improve diagnosis by sensitivity and specificity.[107] Several studies have further shown that the presence of auto-antibodies depends on the age at diabetes onset.[108-110]

Immune cells in the pathogenesis of type 1 diabetes

T cells subsets in type 1 diabetes

During the process of islet inflammation ("insulitis") the first cells infiltrating the islets have been described to be macrophages, dendritic cells and natural killer (NK) cells.[111,112] This process is followed by the infiltration of T cells that are thought to be key players in β-cell destruction (Table 8, Figure 3).[111,112]

T lymphocytes can be divided into two major phenotypes depending on their cytokine secretion profile: Th1 and Th2 cells (Table 10). The Th1 phenotype produces IL-2, IFN-γ and TNF-α/β and was found be able to transfer insulin-deficient diabetes in animal model.[113-115]

T lymphocytes of the Th2 phenotype secrete IL-4, IL-5, IL-10 and IL-13 and are rather non-destructive, acting anti-inflammatory and suppressing autoimmune reactivity.[116-123] The development of naïve T cell to a Th1 or Th2 phenotype is cytokine driven and therefore potentially modifiable.[124] IL-12 is the key factor for the differentiation into a Th1 phenotype under the control of the transcription factor signal transducer and activator of transcription-4 (STAT4) and the transcription factor T-box expressed in T cells (T-bet).[125]

Subtypes of T cells as defined by their functional activities are T helper cells (T_H cells) known as cluster of differentiation 4+ (CD4+) Tcells, cytotoxic T cells known as CD8+ T cells, memory T cells and natural killer T cells (NKT).[113] Previous studies in the non-obese diabetic (NOD) mouse revealed that both, autoreactive CD4+ and CD8+ T cells, respond to pancreatic β-cell auto-antigens and that both cell types can destroy pancreatic β-cells; in addition, both subsets are necessary for the effective transfer of diabetes into NOD mouse model (Table 8).[126-139] Autoreactive T cells also play a central role in the pathogenesis of human type 1 diabetes (Table 8).[140,141] Furthermore, it has been shown that treatment with a monoclonal antibody directed against CD3, a general marker of T cells , delays the decline in β-cell function in recently diagnosed patients.[142,143]

TABLE 8: Role of T cells in the pathogenesis of type 1 diabetes/ insulin-deficient diabetes

Evidence
• Presence in inflammatory lesion (insulitis)
• Delay of progress in disease with immunosuppressive drugs
• Preservation of β-cells at clinical onset of disease after anti-CD3 monoclonal antibody therapy
• Adoptive transfer of diabetes with bone marrow from diabetic donor to non-diabetic recipient
• Circulating autoreactive T cells in type 1 diabetes patients
• Development of autoimmune type 1 diabetes in B cell and antibody-deficient patient with intact T cell immunity

Source: Adapted from Roep BO. Semin Immunopathol 2002

Recently, a new subgroup of T cells, the regulatory T cells (Treg) which are defined by the co-expression of CD4+, CD25+, CTLA-4 and forkhead box P3 (Foxp3) have been demonstrated to control the balance between tolerance and immunity and suppress auto-reactive CD4+ and CD8+ T cells responses (Figure 3)[144-153.]. The deficiency of Treg cells in peripheral immune systems has been suggested to accelerate tumor-directed immunity, allograft rejection and autoimmune diseases such as type 1 diabetes.[144-153] In the NOD mouse model CD4+CD25+ Tregs control development of insulin-deficient diabetes. Mice without CD80/CD86-CD28 costimulation are devoid of CD4+CD25+ Tregs and develop insulin deficiency much faster.[154] The disease phenotype of these mice is rescued by adoptive transfer of CD4+CD25+ Tregs.[154-156]. Some groups describe decreased numbers and functions of CD4+CD25+ Tregs in NOD mice, particularly in their pancreatic lymph nodes, and within the islets while others found no differences or even expansion of these cells in pancreatic lymph nodes or islets.[154-162]. Studies on Treg number and function in patients with type 1 diabetes are difficult to interpret and have recently led to contradictory results.[154] One study found a reduced number of CD4+CD25+ Treg cells in new onset and long standing type 1 diabetes whereas three other studies could not confirm the findings.[163-166]

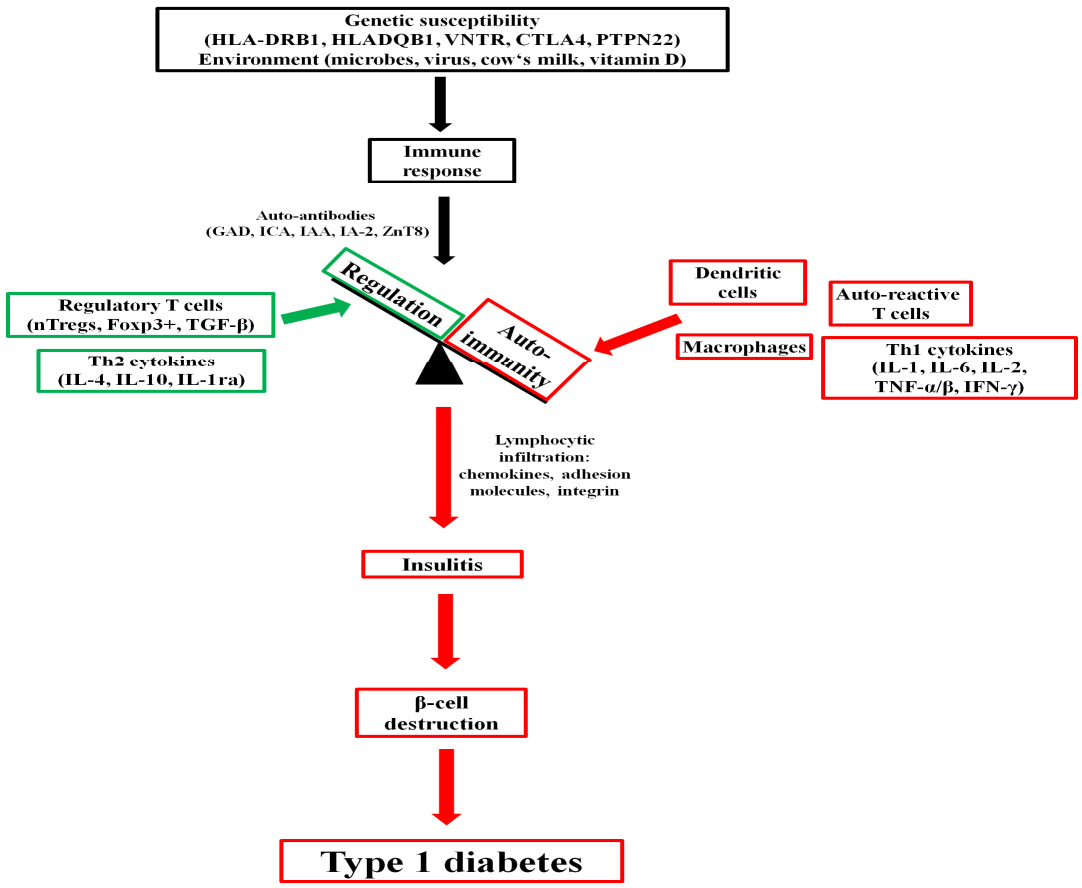

FIGURE 3: Breakdown of the immune regulation in type 1 diabetes

Animal models and human studies of T cell mediated type 1 diabetes

Our current impediment in type 1 diabetes research is the limited availability of essential human samples such as routinely biopsies of pancreas, the target organ in this disease. However, much of our current understanding about the disease progression is driven from animal models.[167] We also know that no single animal model perfectly mimics humans and that not all results obtained in animals relate humans.

Rodent models

The main rodent models of spontaneous type 1 diabetes are the NOD mouse and the diabetes-prone BioBreeding (BB) rat.[169-173] It has been demonstrated that development of insulin deficiency in rodent models is primarily dependent on CD4+ and CD8+ autoreactive T cells.[173-177] These findings show the ability to transfer disease with purified CD4+ and CD8+ T

cells from NOD donors.[173,178,179] Some studies showed that after T cell modulating therapies for example injection of antibodies against CD4+ T cells delays the onset of type 1 diabetes in rodent model.[180-184] Diabetes can also be transferred from affected animals by passive transfer of splenocytes.[173] Furthermore, it has been reported that adoptive transfer of CD4+CD25+ Treg cells could prevent disease in 80% of BB rats.[185] To date, many studies have shown several prevention strategies in both NOD mouse and BB rat model.[185-191]

Human studies

The evidence of a putative role of CD4+ and CD8+ T cells in the development of type 1 diabetes has also been indicated in humans with type 1 diabetes.[192-194] With the aid of histological examinations we have identified that T cells infiltrate the islets of patients who have recently developed type 1 diabetes.[194] The major role of CD4+ T cells in patients with type 1 diabetes is emphasized by investigations of HLA alleles that are associated with the risk of type 1 diabetes.[35,45,49,50]

At the moment several research groups are aiming to measure islet antigen-specific T cell response from a sample of human blood.[192] Current assays for measuring islet antigen-specific T cell responses measure cytokine production (*enzyme-linked immune spot technique (ELISPOT), CD4/CD8+ T cell ELISPOT, CSA (analysis of antigen-specific T cells by cytokine secretion assay*), T cell proliferation (*cellular immunoblot, 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-proliferation assay*) or the frequency of epitope-specific T cells using HLA-peptide multimers, with or without *in vitro* expansion (*analysis of antigen-specific CD8+ T cells using class I pentamers, analysis of antigen-specific CD4+ T cells*).[194-199] However, peripheral blood is the only practical source of initial material for use in islet-specific T cell assays. In peripheral blood, the frequency of islet-specific T cells is very low and newly diagnosed patients with type 1 diabetes are often lymphopenic.[201-203] The next obstacle in this strategy is the technical difficulty of isolating and expanding auto-antigenic T cells.[204] Despite the technical obstacles recent investigations have demonstrated that patients with type 1 diabetes showed positivity to the T cell reactivity of β-cell associated antigens.[193-195,198]

However, the results of islet antigen-specific T cell responses were not always consistent between studies. As yet there have been no standardized T cell assays. Consequently, the T-Cell Workshop Committee of the Immunology of Diabetes Society (IDS) is focusing on identification of suitable antigens and the development of standardized assays that will be described in **Chapter 6** and **7**.

Glucagon test and mixed meal tolerance test for measurement of β-cell function in type 1 diabetes

Type 1 diabetes is an immune mediated disease resulting in selective destruction of insulin producing β-cells. Glucagon test and mixed meal tolerance test (MMTT) are commonly used for measurement of β-cell function in patients with type 1 diabetes.[205] In both methods C-peptide levels represent as a valuable marker for the residual β-cell function of patients.[205] Figure 4 illustrates the performance, advantages and disadvantages of both methods.

Glucagon test

For the glucagon test, adult patients get an intravenous (i.v.) bolus injection of 1mg glucagon, a hormone secreted by pancreatic β-cell, aiming to raise the blood glucose levels. The level of application of glucagon to children is dependent on their weight (0.03 mg/kg, maximum 1mg). Immediately before and 6 min after glucagon injection blood samples should be drawn for measurement of baseline and stimulated C-peptide levels. Normal C-peptide values are between 0.88 and 2.7 ng/ml. C-peptide values below 0.76 ng/ml (baseline) or 1.82 mg/ml (postglucagon) are seen as potential signs of insulin dependence.

The advantages using the glucagon test are the possibility of the stimulation of the pancreatic β-cell function, the reproducibility, the sensitivity of the test, and its short duration. The disadvantage is the low tolerance rate for the glucagon test (Figure 4).

Mixed meal tolerance test (MMTT)

For the MMTT, patients will be given 6 ml/kg of a standardized liquid meal in the form of a high boost protein drink up to maximum of 360 ml, which will be ingested within 5 minutes. Before and during the 2 hours after the intake of MMTT, blood samples will be taken to measure fasting and stimulated C-peptide levels (Figure 4).[205]

The advantages of using MMTT are the possibility of the stimulation of pancreatic β-cell function, the reproducibility, the sensitivity of the test, and the good tolerance. The disadvantage is the long duration of the test.

Recently, Greenbaum et al. compared the MMTT and glucagon tests in two different study groups Type 1 Diabetes TrialNet Research Group (Tria lNet) and European C-peptide Trial (ECPT).[205] In both study groups patients completed the MMTT and glucagon tests on separate days in randomized sequences.

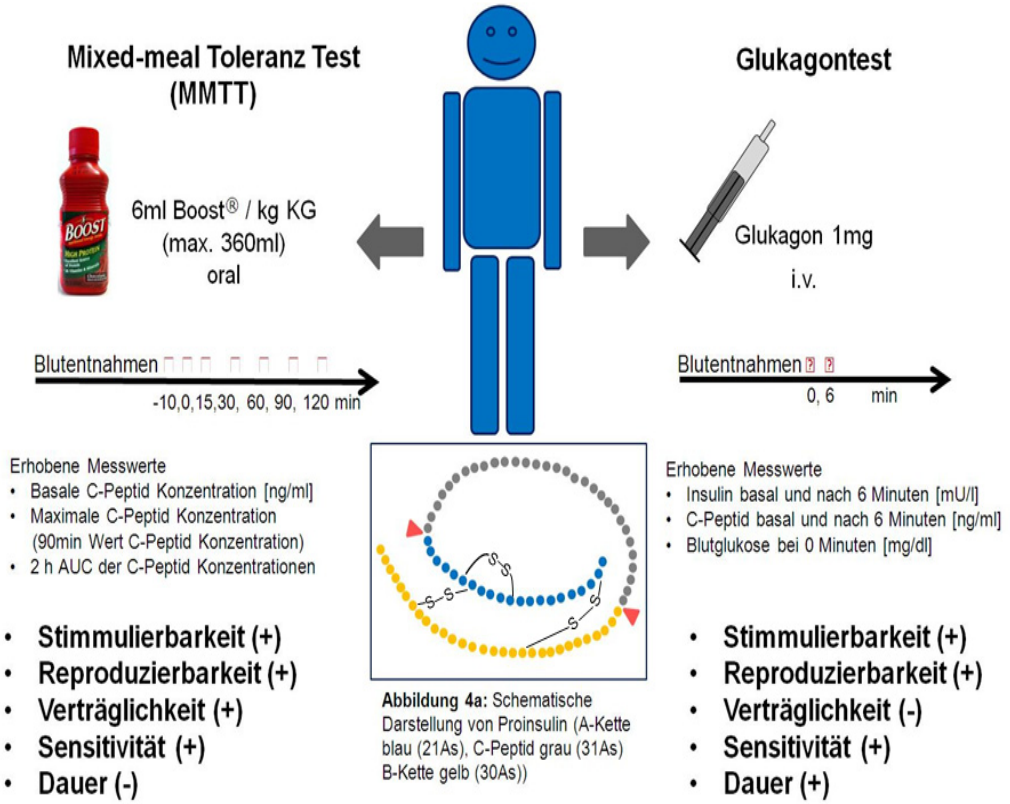

FIGURE 4: Mixed meal tolerance test vs glucagon test

Source: Adapted from Simon MC, Pham MN, Schloot NC. Der Diabetologe 2011

Interestingly, both studies provided clear, concordant results and showed that both tests were highly reproducible for measuring stimulated C-peptide responses.[205] Furthermore, both studies showed that MMTT is a more sensitive test of residual β-cell function, with the peak C-peptide response being significantly greater than in the glucagon test.[205] In the MMTT, the peak response occurred at about 90 minutes compared with 6 minutes for the glucagon test.[205] In comparison to glucagon test, the MMTT is more reproducible and better tolerated and thus is the preferred method to measure residual β-cell function. Greenbaum et al. suggested that if clinicians interpret results from clinical trials to arrest the type 1 diabetes disease process, they should be aware that these two commonly used outcome measures are not directly comparable.[205]

Type 2 diabetes

This form of diabetes, formerly known as non-insulin dependent diabetes mellitus (NIDDM), is an interaction between several genetic and environmental factors resulting in insulin resistance and β-cell dysfunction.[1-4,206] The major contributors to the development of insulin resistance and impaired glucose tolerance (IGT) are overweight and obesity.[207,208] The manifestation of type 2 diabetes is indicated due IGT in which the pancreas cannot secrete sufficient insulin to overcome insulin resistance.[207-209]

Epidemiology

Type 2 diabetes is the most common form and accounts around 90% of those with diabetes.[1] The worldwide prevalence of diabetes mellitus among adults was 285 million in 2010.[2,210] It is expected that this number will increase to 552 million by the year 2030.[2,210] In developing countries the prevalence of type 2 diabetes is growing, linked to changes towards a western lifestyle (high-energy diets with reduced physical activity) and the rise in the prevalence of overweight and obesity.[211-213] These facts indicate that type 2 diabetes is becoming a pandemic disease.[214,215] To date, it is the fifth leading cause of death worldwide resulting in the increased expenditure on health care.[214,215]

Diagnosis

According to recommendation of the WHO and the American Diabetes Association (ADA) a glycated haemoglobin A_{1c} (HbA_{1c}) level of 6.5% or higher can be used to diagnose diabetes.[1,216]

The symptoms for type 2 diabetes are polydipsia, polyuria, weight loss, sight disorder, fatigue and glucosuria.[1,2] The major chronic complications of type 2 diabetes are mostly macrovascular (e.g. myocardial infarction, atherosclerosis or stroke) and microvascular (e.g. retinopathy, neuropathy, nephropathy) diseases[206,217-219].

Genetic factors

The contribution of genetic features in the pathogenesis of type 2 diabetes has been demonstrated in several genetic studies.[220-229]

Individuals with a family history of diabetes have 2.4 fold higher risks for type 2 diabetes.[206] The maternal and paternal conferred risk of type 2 diabetes to their offspring is similar.[220] If

both parents are type 2 diabetes patients, their offspring have around a 60% risk of developing type 2 diabetes by the age 60 years.[221] The first degree relatives of type 2 diabetes patients have 15-25% risk of developing impaired glucose tolerance and type 2 diabetes.[222] In monozygotic twin studies type 2 diabetes appears 50-70% more than in dizygotic twins, who display an incidence of 20-30%.[223,224]

Moreover, results from GWA studies have demonstrated that several genetic risk loci are associated with the onset of type 2 diabetes.[230-234] These loci influence the pancreatic β-cell function (*KCNJ11, TCF7L2, HNF1B, SLC30A8, CDKAL1, IGF2BP2, CDKN2A, CDKN2B, NOTCH2, MTNR1B, GIPR*), insulin sensitivity (*PPARG, IRS1, FTO*), obesity (*FTO*), incretin secretion and sensitivity (*KCNQ1, WFS1, TCF7L2, GIPR*).[235-242]

Environmental factors

The adoption of westernised lifestyles, including a high calorie, high fat diet, increased consumption of sugar and increased physical inactivity, is associated with the increased prevalence of type 2 diabetes.[243-248] This evidence is observed in developing countries such as China and India.[249] The outcomes of the adoption of westernised lifestyles are accompanied with increased risk for chronic complications.[1,2] Wang et al. demonstrated the outcomes of the adoption of westernised lifestyles in their meta-analysis of the Chinese population and reported that from 1992 to 2002 the obesity rate increased from 20% to 29.9% and obesity-and diet-related chronic diseases such as hypertension from 14.4% to 18.8%, cardiovascular disease from 31.4% to 50% and type 2 diabetes from 1.9% to 5.6%.[250]

Several studies have found that the imbalance of micronutrient intake including deficiency in vitamin D, vitamin B12 and increased body iron is related to the development of type 2 diabetes.[251-253]

Currently, the involvement of the gut microbiota in the onset of type 2 diabetes is being discussed. The changes in the gut microbiota result from changes in infant feeding, increased use of antibiotics and westernised food intake.[254] Some studies demonstrated that this alteration of the gut microbiota is associated with higher risk of obesity, insulin resistance and type 2 diabetes.[255-257]

β-cell dysfunction

Pancreatic β-cell dysfunction is present in patients with type 2 diabetes. This process is thought to be associated with hyperglycemia.[206] The hyperglycemia is a consequence of an

interplay between insulin sensitivity and secretion resulting in failure of pancreatic β-cells, which cannot compensate sufficiently for the increased insulin requirement.[258] The outcome of hyperglycemia is insulin resistance.[259]

Furthermore, several studies have observed an association of development of β-cell dysfunction with long-term high-fat diet and higher levels of endogenous free fatty acid (FFA), particularly saturated FFA which affect lipotoxic to pancreatic β-cells resulting in β-cell death (apoptosis).[260] Many studies revealed a positive association between increased consumption of saturated FFA and higher risk for type 2 diabetes.[261,262]

Insulin resistance

Insulin resistance involves liver, muscle and adipose tissue and predicts the onset of glucose intolerance and overt type 2 diabetes.[263-270] Individuals with NGT (non-glucose tolerance) first-degree relatives of patients with type 2 diabetes and individuals with IGT are also shown insulin resistance[269-272]. Interestingly, data from the Whitehall study demonstrated the effect of insulin secretion and insulin sensitivity several years before diagnosis and reported that the diabetic group showed a decrease of HOMA (homeostasis model assessment) insulin sensitivity up to 86.7% during the 5 years before diagnosis, a linear increase in fasting and 2-h postload glucose 3 years before diagnosis, an increase of HOMA β-cell function 3-4 years before diagnosis and then a decrease up to 62.4% until diagnosis.[273]

The development of insulin resistance involves also genetic factors, pregnancy, lifestyle, high-fat diet (FFA) and various medications (e.g. steroids, estrogens, nicotinic acid) are related to the progression of insulin resistance.[274-278]

Adipose tissue and inflammation

Adipose tissue has fat storing capacity and contains two types: white adipose tissue (WAT) and brown adipose tissue (BAT).[279-281] Currently, adipose tissue has been recognized as major endocrine organ which secretes hormones and adipokines such as adiponectin, leptin, cytokines, chemokines and reactive oxygen (ROS).[279-281]

In type 2 diabetes or metabolic syndrome, WAT is abnormal in multiple ways: reduction of adiponectin expression and secretion, higher expression and production of inflammatory cytokines for example TNF-α, IL-1β, monocyte chemoattractant protein (MCP) -1, increased tissue inflammation (e.g. macrophages infiltrates) and diminished adipocyte differentiation.[211,282,283]

Investigations with obese animals and humans showed bone-marrow-derived macrophages are recruited to the fat pad under the influence of proteins secreted by adipocytes including MCP-1.[279,284,285] Ablation of either MCP-1 or its receptor diminishes macrophage infiltration of fat depots and improves insulin sensitivity.[279,286] Moreover, investigations with New Zeeland obese (NZO) mouse model have demonstrated that heat shock protein 60 (Hsp60) is able to induce the pro-inflammatory mediators (IL-6, MCP-1 and IL-8) of adipocytes via toll-like lipopolysaccharide (LPS) receptor TLR4, which has been discussed as being related to insulin resistance.[287,288]

Several cross-sectional and prospective studies have demonstrated elevated systemic levels of acute phase protein (e.g. C-reactive protein (CRP), haptoglobin, fibrinogen, plasminogen activator inhibitor (PAI), serum amyloid A), cytokines and chemokines in patients with type 2 diabetes.[289,290] Increased systemic concentrations of IL-1β, IL-6 and CRP could act as prediction marker for the subclinical inflammation in type 2 diabetes.[289-293] Patients with a higher risk of obesity and prediabetes show higher circulating concentration of interleukin-1 receptor antagonist (IL-1ra).[292-294]. The elevation of IL-1ra levels has been observed 6 years before diagnosis of type 2 diabetes.[294]

Latent autoimmune diabetes in adults

Definition

The term latent autoimmune diabetes in adults (LADA) describes adult patients with diabetes, who present with type 2 diabetes symptoms but have GAD auto-antibodies associated type 1 diabetes.[5-8] The Expert Committee of the WHO and ADA describes LADA as a slowly progressive form of type 1 diabetes.[1,4] Some authors suggest clinical steps for the characterization of patients with LADA as shown in Figure 5.[6,8] However, patients with LADA are different from those with type 1 diabetes as they do not require insulin in the first months or years (Table 8).[295,296] In spite of the classification of LADA into the subgroup of type 1 diabetes it is still unknown why patients with LADA lose their β-cell mass and function more slowly than patients with type 1 diabetes and more quickly than patients with type 2 diabetes.[297] The committee of the IDS recommends three criteria for discriminating patients with LADA from type 1 and type 2 diabetes: 1) adult age at onset (>30 years), 2) presence of at least one of the circulating auto-antibodies (GADA, ICA, IAA, IA-2), and 3) initial insulin independence for 6 months.[298]

Prevalence

The prevalence of LADA based on auto-antibodies amongst adults with diabetes is approximately 10-30% in Caucasian and Asian.[5,8,296,299-305] These patients commonly have a personal or family history of other autoimmune disease such as Grave's disease, Hashimoto's thyroiditis, celiac disease and others.[306-310]

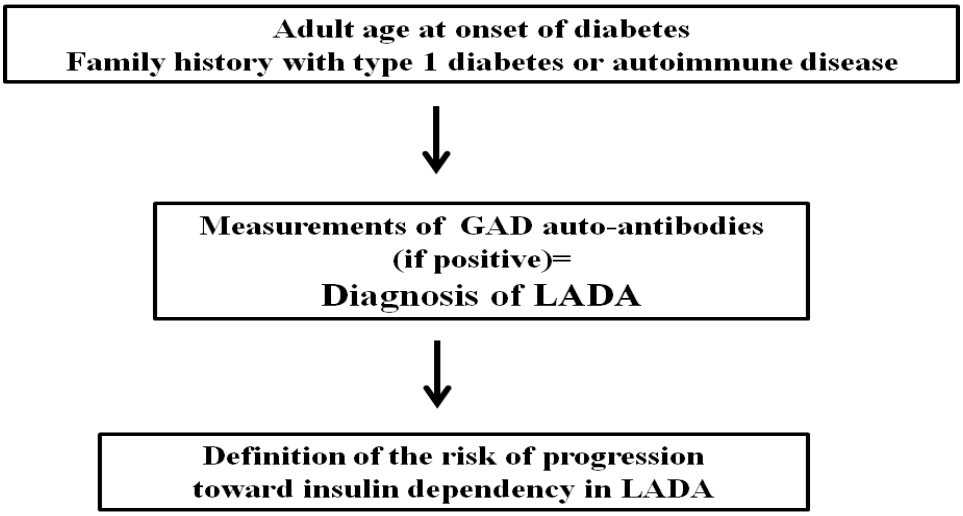

FIGURE 5: Suggested steps for characterization of LADA

Source: Pozzilli P, Di Mario U. Diabetes Care 2001

Therapy

Diet and oral anti-diabetic therapy are efficacious in patients with LADA.[1,3,8] However, these treatments cannot halt the β-cell destruction.[295] As a result, more than 80% of patients with LADA will become insulin dependent within 5 years after diagnosis (Table 8).[1,6,295,311-314]

Genetic susceptibilities

There are currently few genetic epidemiological studies for LADA. However, some studies have reported that patients with LADA share genetic features with both type 1 and type 2 diabetes.[315-320]

In patients with LADA the risk of the onset of diabetes is positively associated with HLA class II alleles DR3, DR4 and their associated DQB1*0302 and DQB1*0201.[315,321-326]. These genes have also been implicated in the susceptibility to type 1 diabetes.[52-57] The HLA alleles DQB1*0602 and DR2 show a strong protective role against the susceptibility to type 1

diabetes and are rarely seen in type 1 diabetes.[329,340] Interestingly, these alleles are relatively common in patients with LADA.[316,327]

In type 1 diabetes the short-chained VNTR could be associated with disease susceptibility and the long-chained VNTR seem to be protective against disease development.[62,65,328,329] Likewise, the existence of short-chained VNTR is more frequent in patients with LADA than in control subjects.[330,331] Furthermore, the CTLA-4 gene, which could be one of the genetic risk factors in patients with type 1 diabetes and involved in the repression of T cell activation, has been found in patients with LADA.[326,332-334] Some investigations have also found the PTPN22 in patients with LADA.[335-337] In patients with type 2 diabetes transcription factor 7-lie 2 (TCF7L2) variants are common and have been shown to be associated with the pathogenesis.[239] Some studies have also found this gene in patients with LADA.[338-341]

TABLE 8: Comparisons between type 1 diabetes, LADA and type 2 diabetes

Characteristic	Type 1 diabetes	LADA	Type 2 diabetes
Age at onset	children, all ages	adults	adults mostly
Onset	rapid (days to weeks)	slow	slow, without symptoms
Body habitus	lean/fit	normal/overweight	overweight/obese
Disposition for ketoacidosis	often	low	missing/low
Insulin secretion	diminished/deficient	defective/low	defective
Insulin resistance	no	present	present
C-peptide level	low	low/normal	normal/high
Metabolic syndrome	no	sometimes	yes
Islet antigens	90-95% at diagnosis (GAD, IA-2, IAA)	present at diagnosis (GAD, IA-2, IAA, ZnT8)	no
Biomarkers	blood glucose, HbA1c, auto-antibodies, cytokines, C-peptide, ketone bodies	blood glucose, HbA1c, auto-antibodies, cytokines, C-peptide	blood glucose, HbA1c, adipokine, cytokines
Insulin therapy	essential	in >80% of patients with LADA need insulin injection within 5 years after diagnosis	when oral medications is insufficient, insulin injections is needed

Source: Modified from Simon MC, Pham MN, Schloot NC. Der Diabetologe 2011

Immunology

Auto-antibodies are also appear in patients with LADA.[3,5,6,8,295] Previous studies have frequently found single positivity for GAD auto-antibody in patients with LADA (Table 8).[305,342-347] The circulating auto-antibodies IAA and IA-2 are also detectable in patients with LADA (Table 8).[344,346] Auto-antibodies to ZnT8 are described as an additional marker of type 1 diabetes.[348-350] Recent investigations were also able to detect ZnT8 in patients with LADA (Table 8).[351-353]

Some studies have revealed that the clinical characteristics of patients with LADA are associated with the titre and number of diabetes-associated auto-antibodies.[301,354-359] Interestingly, the presence of multiple auto-antibodies and higher titre of GAD in patients with LADA could be related to an early age at onset, low fasting C-peptide values and low prevalence of markers of the metabolic syndrome.[321,355] Moreover, patients with LADA show also positivity for other non-diabetes-specific auto-antibodies including thyroid peroxidase (TPO) and antibodies against gliadin.[307,321,360,361]

Similarly to patients with type 1 diabetes islet reactive T cells in LADA patients with and without auto-antibodies can respond to multiple islet proteins.[363-369]. The recognition of islet proteins by T cells is different between patients with LADA and those with type 1 diabetes.[334,362,369] These differences have been identified using multiple islet proteins.[362] Furthermore, one study has demonstrated that patients with LADA, who show T cell reactivity, had a significantly lower glucagon stimulated C-peptide compared to those patients with no T cell reactivity.[370] This finding suggests T cell reactivity in patients with LADA correlated with β-cell failure.[370]

In type 1 diabetes regulatory T cells characterized by the expression of CD4+ and CD25+ (CD4+CD25+Foxp3+Treg) are thought to be contributors to the development of autoimmune disease.[144-153] Yang et al. detected a reduction of CD4+ regulatory T cells and decreased expression of Foxp3 mRNA in CD4+ T cell in patients with LADA.[372]

Metabolic features and chronic complications of patients with LADA

Previous studies reported that higher BMI, obesity and hypertension are frequently in patients with LADA and that those patients are phenotypically indistinguishable from those with type 2 diabetes.[3,5,8,372-375] Patients with LADA, who did not need insulin injection, revealed similar age, C-peptide and glucose levels and metabolic syndrome as those with type 2 diabetes.[373,374]

Furthermore, it has been observed insulin resistance was more prevalent in patients with type 2 diabetes and LADA compared to healthy control subjects.[375] One observation demonstrated a similar risk of chronic complications and death among patients with LADA and type 2 diabetes.[376] However, these findings are not consistent between all investigations.

Other studies have also reported a lower BMI, waist/hip ratio, lower high blood pressure, lower total triglyceride levels and higher HDL in patients with LADA compared to patients with type 2 diabetes.[323,377-380] Hawa et al. revealed similar prevalence for metabolic syndrome among patients with type 1 diabetes and LADA, which was lower than type 2 diabetes.[377] The authors suggested that metabolic syndrome is not characteristic for autoimmune disease.[377] Patients with LADA showed a lower clinical examination score and cardiorespiratory reflex index and fewer features of diabetic neuropathy than patients with type 2 diabetes.[381]

Gottsäter et al. have demonstrated that the level of insulin secretion in LADA was intermediate between type 1 and type 2 diabetes.[382] Its decline of C-peptide levels was faster than in type 2 diabetes but slower than type 1 diabetes.[382]

Several studies found similarities between patients with type 1 diabetes and LADA regarding their clinical features and chronic complications. Two studies detected a similar degree of insulin resistance among patients with type 1 diabetes and LADA.[301,383] Furthermore, the prevalence of retinopathy, nephropathy and neuropathy has been described as similar among patients with LADA and type 1 diabetes.[384]

Soluble cytokines, chemokines, adhesion molecules and adipokines

Immunologic alterations are involved in the pathogenesis of type 1 and type 2 diabetes.[111,112,385] Immune mediators including cytokines, chemokines, adhesion molecules or adipokines has been described to act as important factors in accelerating or preventing β-cell destruction in type 1 diabetes as well as inflammation and insulin resistance in type 2 diabetes.[291,294,386-392] A better understanding of the role of these immune mediators might be helpful for future prevention strategies and therapies.

Cytokines

Cytokines are small secreted molecules by numerous of cells of the immune system and are associated with cell-mediated immunity responses.[113,393] They are classified into subfamilies as shown in Table 9. The classification of cytokines families is based on binding to receptor family.[113] Cytokines can act autocrine, paracrine or endocrine and are pleiotropic.[113] Interleukins interact directly with surrounding cells whereas chemokines as regulatory molecules affect on migration, adhesion and activation of leukocytes into damaged tissue.[113,394] Furthermore, cytokines have an important role in cellular and metabolic processes with linking to innate and adaptive immune system.[395-398]

Currently it is possible, based on cellular immunoassays including enzyme-linked immunosorbent assay (ELISA) and multiplex bead based technology, to measure circulating concentrations of soluble cytokines in patients with diabetes mellitus.[387-392] The possibility has been discussed of using cytokines as biomarkers in diabetes mellitus to determine the immune and inflammation status of patients.[399-401]

TABLE 9: Cytokine families

Family	Cytokine
Haematopoietic (type I cytokines)	IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-11, IL-15, IL-21
Interferon (type II cytokines)	IL-10, IL-20, IL-22, IL-24, IL-26, IL-28, IL-29, IFN
TNF	TNF-α, TNF-β, RANKL
IL1/ Toll superfamily	IL-1, IL-18, IL-33
Chemokines	C, CC, CXC, CX3C
Tyrosine kinase	RAS/Raf-pathway, Jak/STAT-pathway
Cysteine	TGF-beta, IL-17, IL-12, IL-23, IL-25

Source: Adapted from Taniguchi T, Science 1995

Several studies with type 1 diabetes patients have shown that cytokines such as IL-2, TNF-α or IFNγ initiate a cascade of immune-inflammatory processes in the pancreatic islet resulting in production of pro-inflammatory cytokines e.g. IL-1β and IL-6, with a cytotoxic effect via increased nitric oxide (NO) production (Table 9 and 10).[400,402-409] Furthermore, it has been considered that IL-1β and TNF-α act as key cytokines for the β-cell destruction, whereas IL-1ra has a protective effect by blocking the receptor of IL-1.[402,403,407-409]

Type 2 diabetes is associated with higher obesity and low-grade chronic inflammation resulting in the activation of the innate immune system.[280,281,289-294] These two factors accelerate disease progression and the release of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, that inhibit the insulin signaling and its homeostasis and action leading to higher insulin resistance.[385,407,408,410-413] Elevated levels of IL-6, IL-8, TNF-α and IL-1β have been found in patients with type 2 diabetes.[414-418]

TABLE 10: Th1/Th2 cytokines and their effects

Th1 cytokines	Th2 cytokines
• IL-2, IFN-γ/α, TNF-β/α, IL-12, IL-1 • pro-inflammatory effect • induction of chronic low-grade inflammation • cytotoxic effect on pancreatic β-cells • activation of macrophages and auto-reactive T cells • induction of insulitis • inhibition of insulin synthesis and secretion • impairment of insulin action • acceleration of insulin resistance • negative association with β-cell function in type 1 diabetes	• IL-4, IL-5, IL-10, IL-13, IL-1ra • anti-inflammatory effect • regulatory/ protective cytokines • inhibitory effect on activated macrophages • down-regulation of Th1 cytokines • correlation with benign of insulitis • stimulation of IgM, IgG and IgE synthesis by B cells • association between low concentrations of Th2 cytokines and higher risk for insulin resistance • positive association with β-cell function in type 1 diabetes

Chemokines

Chemokines as part of the cytokine family are proteins with chemottractant activity to induce migration and recruiting of potent leukocytes into site of inflammation (Table 11).[113] Several studies have shown that some chemokines are inflammatory and their expression is activated by pro-inflammatory cytokines such as IL-1.[419,420] Some studies with humans have shown the important role of chemokines in the regulation of inflammatory processes during the insulitis, and have detected higher systemic concentrations of chemokines and their receptors in new onset of type 1 diabetes.[388,421] In type 2 diabetes higher concentrations of chemokines are

positively associated with the incidence of type 2 diabetes, insulin resistance, and chronic inflammation grade.[392,422] Chemokines also act also as a key factor in β-cell failure and dysfunction and the development of micro- and macro-vascular complications.[423]

TABLE 11: Chemokines families and their effects

Chemokines families	Effects of chemokines
CXC chemokines CXCL8 (IL-8), CXCL9 (Mig), CXCL10 (IP-10) **C chemokines** Lymphotactin α/β **CX3C chemokines** CX3CL1 (Fractalkine) **CC chemokines** CCL2 (MCP-1) CCL3 (MIP-1α) CCL4 (MIP-1β) CCL5 (Rantes) CCL17 (Tarc)	• chemotactic activity • regulation of immune system • recruitment of effector lymphocytes into inflammation • recruitment of cytotoxic T cells to β-cell • inflammatory/homeostatic function • induction of T cells proliferation • increasing production IFN-γ secretion • correlation between high levels of IFN-γ levels in the islets of Langerhans and the expression of chemokines • up-regulation of Th1 cytokines • down-regulation of Th2 cytokines • angiogenesis • involvement in the pathogenesis of virus-induced immune diabetes • accelerating chronic inflammation in type 2 diabetes • vital roles in the development of atherosclerosis, CVI and endothelial dysfunction

Adhesion molecules

Adhesion molecules including ICAM-1, VCAM-1, E-/L-/-P-Selectin are proteins and may serve as important biomarkers for inflammatory processes on platelets and endothelium and for the development of cardiovascular disease, atherosclerosis and diabetes mellitus (Table 12).[113,424,425] These molecules are involved in the regulation of the immune system and the migration of leukocytes into target organs and pancreatic islets resulting in β-cell destruction.[426] Adhesion molecules are also positively correlated with insulin resistance and adiposity in patients with type 2 diabetes.[427]

TABLE 12: Adhesion molecules and their effects

Adhesion molecules	
Subgroups	**Effects**
ICAM-1 VAM-1 E-/L-/P-Selectin	• regulation of the immune system • activating migration of leukocytes and auto-reactive T cells into inflammatory loci and pancreatic islets • up-regulation of inflammation • acceleration of β-cell destruction and dysfunction • elevated serum concentrations of adhesion molecules in type 1, type 2 and obese patients with micro- and macro-vascular diseases • positive relation to insulin resistance, metabolic abnormalities and endothelial dysfunction • hyperglycemia increases circulating concentrations of adhesion molecules • negative association with β-cell function

Adipokines

Adipokines are proteins secreted by adipose tissue which regulate energy homeostasis, immunity and inflammation (Table 13).[113,280,282,293,387] Adipocytes and adipose tissue-infiltrated macrophages induce secretion of adipokines including chemerin, IL-6, plasminogen activator inhibitor-1 (PAI-1), retinol binding protein 4 (RBP4), TNF-α, visfatin, leptin, adiponectin and apelin leading to chronic subinflammatory.[113,280] The releases of adipokines are associated with the development of insulin resistance, type 2 diabetes and increased risk for cardiovascular disease associated with obesity.[275,280,293,390] Previous studies have also shown that adipokines are involved in the immunopathogenesis and β-cell destruction of type 1 diabetes and partake in cytokine-mediated up-regulation of the β-cell toxic pro-inflammatory cytokines IL-1β, TNF-α and IL-6.[428]

TABLE 13: Adipokines and their effects

Adipokines	
Subgroups	**Effects**
Adiponectin Leptin Resistin Visfatin Vaspin IL-6 TNF-α RBP4 PAI-1 Chemerin	• regulation of lipid metabolism, energy balance, inflammation and angiogenesis • involvement in the insulin synthesis, signaling and secretion • influence on the development of insulin resistance • association with β-cell function in patients with type 1 diabetes • effect on cytokine secretion

Aims of the thesis

Type 1 diabetes is an immune mediated disease in which T cells and cytokines play a major role in the pathogenesis. LADA (latent autoimmune diabetes in adults), that is characterized by clinical type 2 diabetes with positivity for type 1 diabetes related auto-antibodies, is similar but not identical to type 1 diabetes, as β-cell destruction is less progressive in LADA. So far, it is not well understood why type 1 diabetes and LADA differ and whether cellular immune responses or systemic immune status is related to diabetes forms and β-cell function.

The aims of this thesis were:

- to compare systemic concentrations of cytokines, chemokines, adhesion molecules and T cell responses between patients with type 1, type 2 diabetes and LADA (**Chapter 2-4**)

- to investigate the association of systemic concentrations of adipokines with β-cell function and acute food intake in patients with type 1 diabetes (**Chapter 5**)

- to validate of T cell assays for measurement of autoreactivity in patients with type 1 diabetes (**Chapter 6**)

- to compare of cryopreservation methods on T cell responses to islet and control antigens from type 1 diabetic patients and controls (**Chapter 7**)

Chapter 2

Pro- and anti-inflammatory cytokines in latent autoimmune diabetes in adults, type 1 and type 2 diabetes

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ARTICLE

Pro- and anti-inflammatory cytokines in latent autoimmune diabetes in adults, type 1 and type 2 diabetes patients: Action LADA 4

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Abstract
Aims/hypothesis Systemic pro- and anti-inflammatory cytokines are associated with both type 1 and type 2 diabetes, while their role in latent autoimmune diabetes in adults (LADA) is unclear. Therefore, we compared cytokine concentrations in patients with LADA, type 1 or type 2 diabetes and healthy individuals to test the hypothesis that differences of cytokine concentrations between all groups are attributable to diabetes type and BMI.

Methods The pro-inflammatory cytokines IL-6 and TNF-α, and the anti-inflammatory cytokines IL-1 receptor antagonist (IL-1RA) and IL-10 were measured in 90 participants with type 1 diabetes, 61 with LADA, 465 with type 2 diabetes and 41 control participants using multiple regression models adjusted for BMI, sex, age, blood pressure and diabetes duration.
Results Patients with type 2 diabetes had higher concentrations of systemic IL-1RA, IL-6 and TNF-α cytokines

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than patients with either LADA or type 1 diabetes ($p<0.0001$ for all differences). Cytokine concentrations in controls were lower than those in all diabetes types ($p<0.04$). Increased BMI was positively associated with higher systemic cytokine concentrations in all diabetes types ($p<0.0001$). Despite the association of cytokines with anthropometric data, differences between diabetes forms persisted also after adjusting analysis for the confounders BMI, age, sex, disease duration and blood pressure ($p<0.04$).

Conclusions/interpretation Although body mass associates positively with pro- and anti-inflammatory cytokine levels, patients with type 2 diabetes have higher cytokine levels independent of the prevailing BMI. LADA and type 1 diabetes could not be distinguished by systemic cytokines.

Keywords Body mass index · Interleukin-6 · Interleukin-10 · Interleukin-1 receptor antagonist · Latent autoimmune diabetes in adults · Tumour-necrosis factor alpha · Type 1 diabetes · Type 2 diabetes

Abbreviations

GADA	Glutamic acid decarboxylase antibody
IL-1RA	IL-1 receptor antagonist
LADA	Latent autoimmune diabetes in adults

Introduction

Type 2 diabetes is characterised by impaired beta cell function and insulin sensitivity and is often accompanied by other metabolic abnormalities [1, 2]. These features are accompanied by alterations of the immune system [3–6]. Previous studies found an elevation of systemic pro- and anti-inflammatory cytokine concentrations in patients at risk for diabetes and with overt type 2 diabetes [7, 8]. Obese patients with or without type 2 diabetes also have increased levels of systemic cytokines [9, 10]. In particular, IL-6 and TNF-α are produced in abundance in adipose tissue and are thought to contribute to both development of type 2 diabetes and insulin resistance [11–14]. Serum concentrations of IL-10 and IL-1 receptor antagonist (IL-1RA), anti-inflammatory cytokines, are also secreted in adipose tissue and are associated with obesity and disease progression of type 2 diabetes [15, 16]. Treatment with IL-1RA in long-term type 2 diabetes patients has shown to improve HbA_{1c} and to increase endogenous insulin secretion [17].

Type 1 diabetes results from immune-mediated beta cell destruction and is accompanied by islet-directed antibodies, while T cell-mediated and cytokine-mediated cytotoxicity is thought to lead to beta cell destruction [18, 19]. It has been shown that during insulitis of type 1 diabetes invading immune cells produce cytokines such as IL-1β, TNF-α and IFN-γ, which are known to be cytotoxic to beta cells [20–26]. After manifestation of type 1 diabetes, T cell reactivity and systemic cytokines such as IL-1RA are associated with endogenous insulin secretion and have been shown to relate to disease progression [27–29].

Latent autoimmune diabetes in adults (LADA) has some clinical features of type 2 diabetes but shows immunological abnormalities similar to those in type 1 diabetes, such as glutamic acid decarboxylase antibody (GADA) [30, 31]. So far it is not understood why disease progression in LADA is slower than in type 1 diabetes despite the immunological similarities. Insulin secretion was reported to be intermediate in LADA compared with type 1 and type 2 diabetes, whereas metabolic syndrome was similar in type 1 diabetes and LADA [32, 33]. The role of pro- and anti-inflammatory cytokines in LADA has not yet been investigated.

We tested and compared circulating concentrations of pro-inflammatory cytokines IL-6 and TNF-α, and anti-inflammatory cytokines IL-10 and IL-1RA in patients with LADA, type 1 or type 2 diabetes and healthy participants and analysed their associations with body mass, age and sex.

Methods

The study population consisted of 657 individuals aged from 30 to 70 years, 616 of whom had been diagnosed with diabetes within 5 years before entering this cross-sectional study from the Action LADA cohort. The Action LADA multicentre study was performed to identify immune and clinical risk factors for adult-onset autoimmune diabetes, including its epidemiology, genetic susceptibility, metabolic characteristics and clinical progression [33]. Serum samples were selected on basis of availability and age range. We included 61 individuals with LADA, 90 with type 1 diabetes, 465 with type 2 diabetes and 41 healthy individuals.

Patients with type 1 diabetes were GADA-positive and received insulin treatment after diabetes diagnosis. GADA-positive patients aged from 30 to 70 years who did not use insulin treatment for at least 6 months after diagnosis were termed LADA. GADA-negative patients who did not use insulin at least for the first year after diagnosis were defined as having type 2 diabetes. Blood withdrawal from all participants was carried out in the fasting state. The local ethics committee of each study centre approved the study protocol, in accordance with the Declaration of Helsinki. All patients gave written informed consent for the study.

Serum cytokine measurements Serum was collected and stored at −80°C and thawed only once for cytokine analysis. Circulating cytokine concentrations of IL-1RA, IL-6, TNF-α and IL-10 were measured by multiplex-bead

technology using commercially available kits (Fluorokine MAP; R&D Systems, Wiesbaden, Germany). The detection limits of the assays were 9.56 pg/ml for IL-1RA, 0.1 pg/ml for IL-6, 0.08 pg/ml for TNF-α and 0.25 pg/ml for IL-10. For cytokine concentrations lower than the detection limit a value half of the detection limit was assigned (IL-6, n=46; IL-1RA, n=0; TNF-α, n=0). Concentration of cytokine IL-10 was only detectable in 44% of the samples. Immunoassays showed inter-assay variations <20% and intra-assay variations <10%.

Statistical methods Analyses were performed using SAS Enterprise Guide version 4.2 (SAS Institute, Cary, NC, USA) and GraphPad Prism version 4 for Windows (GraphPad Software, La Jolla, California, USA). Continuous variables are presented as medians and interquartile ranges (Q1, 25th percentile; Q3, 75th percentile). First, Gaussian distribution of data was assessed using the Kolmogorov–Smirnov test. The Kruskal–Wallis and Mann–Whitney U tests were used to compare continuous variables. Fisher's exact test or the χ^2 test was performed to evaluate the differences in categorical data with two or more classes. Circulating concentrations of IL-10 were estimated as categorical variables (detectable or not detectable). Data on these analyses were not corrected for multiple comparisons and are therefore descriptive. Univariate correlations between circulating concentrations of cytokines of all groups and BMI, age, sex, blood pressure and diabetes duration were described by Spearman correlation coefficients (r). Associations between tested circulating concentrations of cytokines were carried out with regression analysis adjusted for BMI, age and sex. In addition, we used multivariate regression models to investigate differences of log-transformed cytokine concentrations (dependent variables) in different participant groups adjusted for BMI, age, sex, systolic and diastolic blood pressure and duration of diabetes (independent variables). We tested five models, which adjusted for an increasing number of variables: model 1, unadjusted; model 2, sex and age; model 3, age, sex and BMI; model 4, age, sex, BMI and blood pressure; and model 5, age, sex, BMI, blood pressure and diabetes duration. For the analysis of IL-10 logistic regression was performed using the same independent variables as in multiple linear regression models. BMI was calculated as body weight in kilograms divided by the square of the height in meters (kg/m^2). For all these descriptive statistical analyses, p<0.05 was considered to indicate a statistically significant difference.

Results

The median age of all patients with diabetes was 54.3 years (Q1: 44.3, Q3: 60.9). Groups differed regarding the median age (Table 1). Patients with type 1 diabetes were younger than those with type 2 diabetes and LADA (p<0.0001). There were no differences in the median ages of the type 1 diabetes and healthy groups or the LADA and type 2 diabetes groups. As expected, the median age at onset in patient groups was different (p<0.0001): type 1 diabetes patients were younger than type 2 diabetes and LADA patients (p=0.002, Table 1). The median duration of diabetes was similar between individuals with type 2, type 1

Table 1 Clinical characteristics of participants with type 1 diabetes, LADA and type 2 diabetes and control individuals

Characteristic	Type 1 diabetes	LADA	Type 2 diabetes	Control	p value
n (women/men)	90 (28/62)	61 (35/26)	465 (202/263)	41 (25/16)	**
Age (years)	43.2 (35.5–53.3)	49.1 (39.0–58.5)	56.3 (48.1–62.1)	47.7 (39.6–53.1)	***
Duration of diabetes (years)	0.1 (0.02–1.6)	0.1 (0.04–2.3)	0.1 (0.03–1.6)	–	NS
Age at onset (years)	44.3 (36.9–52.0)	49.0 (41.50–58.0)	54.10 (46.9–59.0)	–	***
BMI (kg/m^2)	26.1 (22.8–29.2)	25.5 (23.0–28.8)	30.3 (27.0–34.3)	23.5 (21.0–27.3)	***
Systolic blood pressure (mmHg)	120 (110–139)	130 (119–140)	133 (117–145)	112 (105–131)	***
Diastolic blood pressure (mmHg)	80 (68–85)	80 (71–85)	82 (72–89)	80.0 (67–81)	NS
IL-1RA (pg/ml)	927.3 (616.1–1757.0)	942.7 (603.4–1261.3)	1167.3 (728.6–1972.0)	855.5 (570.3–1016.0)	***
IL-6 (pg/ml)	0.6 (0.3–1.4)	0.6 (0.3–1.4)	1.1 (0.5–2.3)	0.3 (0.03–0.5)	***
TNF-α (pg/ml)	2.4 (1.4–3.4)	2.2 (1.4–3.4)	2.9 (1.8–4.2)	1.8 (1.1–2.5)	***
IL-10 (pg/ml)	0.01–17.4	0.01–1.9	0.01–49.9	0.1–2.3	NS

Data are presented as medians and interquartile range (Q1–Q3) if not otherwise indicated

As 56% of IL-10 serum concentrations were below the detection limit, range (minimum to maximum) is shown

Individual cytokine data are depicted in Fig. 1

p values are derived from comparison of all four groups

**p<0.01; ***p<0.001

diabetes and LADA. The median BMI was higher in type 2 diabetes patients than in those with type 1 diabetes or LADA and healthy individuals (all $p<0.0001$), while the type 1 diabetes and LADA groups were similar in their median BMI ($p=0.72$). The healthy individuals had a normal median BMI.

Comparison of circulating cytokine concentrations in different diabetes types The median circulating concentrations of IL-1RA, IL-6 and TNF-α were different between all four groups (all $p<0.0001$), though we observed an extensive overlap between groups (Fig. 1, Table 1). Type 2 diabetes patients had increased median levels of the anti-inflammatory cytokine IL-1RA, and the pro-inflammatory mediators IL-6 and TNF-α compared with type 1 diabetes patients, those with LADA and healthy participants (all $p<0.03$, Table 2). Compared with healthy participants, type 1 diabetes and LADA patients showed higher median concentrations of IL-1RA, IL-6 and TNF-α (all $p<0.04$, Table 2). Interestingly, group-by-group comparisons revealed no differences in median cytokine concentrations of IL-1RA, IL-6 and TNF-α between LADA and type 1 diabetes patients (Fig. 1, Table 2). Systemic concentrations of IL-10 showed statistically significant differences between all groups ($p=0.049$, Table 1). In healthy individuals, IL-10 concentrations showed a trend to be lower than in type 1 diabetes ($p=0.06$, Table 2) and were lower than in patients with LADA ($p=0.003$, Table 2) and type 2 diabetes ($p=0.007$, Table 2). There were, however, no differences between the diabetes groups, though only 44% of sera samples had measurable IL-10 concentrations.

Associations of circulating cytokine concentrations with potential confounders Systemic concentrations of IL-1RA, IL-6 and TNF-α in all groups correlated positively with BMI (IL-1RA, $r=0.35$; IL-6, $r=0.30$; TNF-α, $r=0.20$; all $p<0.0001$; Table 3). Circulating concentration of TNF-α in healthy participants did not correlate with BMI after classification of all individuals in groups.

In addition, waist circumference data, as a more representative measure of abdominal obesity, were available for a subgroup ($n=495$, 70% of total cohort) of patients. In this subgroup, a positive correlation between BMI and waist circumference was seen ($r=0.85$, $p<0.0001$). Waist circumference also showed a positive correlation with IL-1RA, IL-6 and TNF-α (IL-1RA: $r=0.36$, IL-6: $r=0.33$, TNF-α: $r=0.23$,

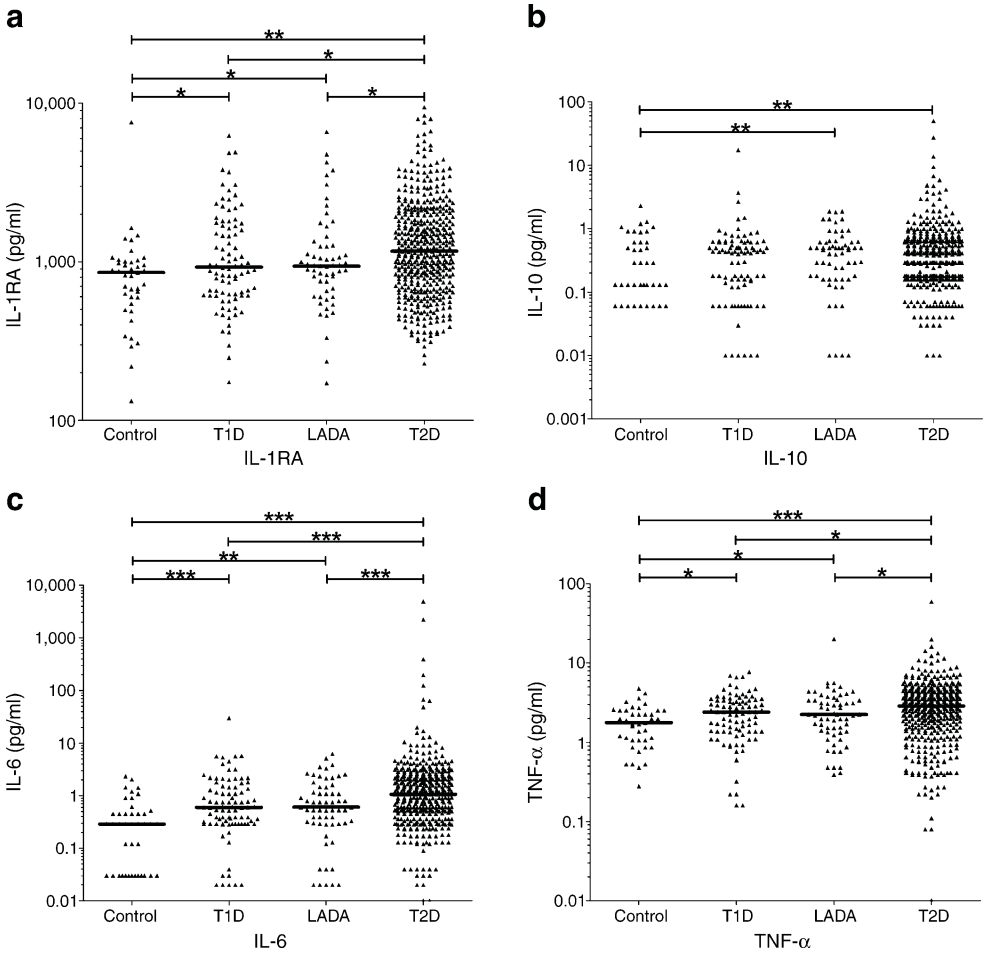

Fig. 1 Circulating concentrations of cytokines in control, type 1 diabetes, LADA and type 2 diabetes participants: (**a**) IL-1RA; (**b**) IL-10; (**c**) IL-6; and (**d**) TNF-α. Each point represents the measured cytokine concentrations of an individual. Horizontal lines depict medians. *p* values of IL-1RA, IL-6 and TNF-α were calculated with multiple linear regression models (model 1). *p* values of IL-10 were estimated with logistic regression. *$p<0.05$; **$p<0.01$; ***$p<0.001$. T1D, type 1 diabetes; T2D, type 2 diabetes

Table 2 Adjusted comparisons between LADA, type 1, type 2 diabetes and control groups

Cytokine/ model	LADA vs type 1 diabetes (β)	LADA vs type 2 diabetes (β)	Type 1 diabetes vs type 2 diabetes (β)	Control vs LADA (β)	Control vs type 1 diabetes (β)	Control vs type 2 diabetes (β)
IL-1RA						
1	0.06	0.17*	0.04*	0.02*	0.05*	0.14**
2	0.06	0.17*	0.03*	0.01*	0.05*	0.16**
3	0.05	0.18*	0.04*	0.01*	0.02*	0.12*
4	0.06	0.33**	0.01*	0.01*	0.01*	0.21***
5	0.07	0.33**	0.01*	–	–	–
IL-6						
1	0.05	0.31***	0.26***	0.13**	0.45***	0.36***
2	0.06	0.33***	0.21*	0.13*	0.46***	0.35***
3	0.04	0.23*	0.13*	0.09*	0.38**	0.27***
4	0.01	0.32*	0.31*	0.12*	0.41**	0.35***
5	0.02	0.31*	0.33**	–	–	–
TNF-α						
1	0.01	0.08*	0.09*	0.04*	0.11*	0.19***
2	0.01	0.06*	0.05*	0.04*	0.11*	0.09*
3	0.01	0.04*	0.04*	0.02	0.10	0.07*
4	0.03	0.08*	0.08*	0.01	0.01	0.06*
5	0.04	0.08*	0.08*	–	–	–
IL-10						
1	0.04	0.05	0.03	0.08**	0.16	0.09**
2	0.03	0.04	0.05	0.08**	0.17	0.09**
3	0.04	0.04	0.06	0.08**	0.16	0.09**
4	0.03	0.12	0.04	0.08**	0.16	0.07
5	0.03	0.12	0.04	–	–	–

Multiple linear regression models were performed for IL-1RA, IL-6 and TNF-α

Cytokines were entered into the models as log-transformed variables; logistic regression analyses were applied for IL-10

Model 1: unadjusted; model 2: age and sex; model 3: age, sex and BMI; model 4: age, sex, BMI and blood pressure (diastolic, systolic); model 5: age, sex, BMI, blood pressure and duration of diabetes

*$p<0.05$; **$p<0.01$; ***$p<0.001$

all $p<0.0001$), similar to the association reported for these cytokines with BMI.

To visualise this effect in all individuals with diabetes we categorised BMI according to the clinical classification of normal weight (18–24.9 kg/m^2), overweight (25–29.9 kg/m^2), and obesity (≥30 kg/m^2) in accordance with WHO definitions. As the number of healthy individuals investigated was low, we display only the results for type 2 diabetes and

Table 3 Correlation between circulating cytokine concentrations and metabolic variables for all groups combined

Variable	IL-1RA (*r*)	IL-10 (*r*)	TNF-α (*r*)	IL-6 (*r*)
Age	−0.003	−0.03	0.18***	0.16***
Sex	0.16***	0.07	−0.03	0.02
Diabetes duration	−0.04	0.04	0.08	0.004
Systolic blood pressure	0.25***	−0.04	0.12*	0.25***
Diastolic blood pressure	0.17***	0.05	−0.01	0.08
BMI	0.35***	0.09	0.20***	0.30***
Type 1 diabetes	0.39***	0.07	0.19*	0.31**
LADA	0.38**	0.19	0.17*	0.23**
Type 2 diabetes	0.31***	0.01	0.19***	0.29***
Control	0.20*	0.26	0.18	0.45**

Correlation analyses between anthropometric variables and IL-1RA, IL-10, TNF-α and IL-6 were performed using Spearman's test

*$p<0.05$; **$p<0.01$; ***$p<0.001$

the merged patient group type 1 diabetes and LADA, named 'autoimmune diabetes', as individuals with type 1 diabetes and LADA were similar in their cytokine level. Participants with type 2 diabetes, as well as those with autoimmune diabetes, showed increased median cytokine concentrations associated with BMI in the case of TNF-α, IL-1RA and IL-6 but not for IL-10 (Fig. 2). In both groups, the highest median systemic cytokine concentrations of IL-1RA, IL-6 and TNF-α were in individuals with obesity, followed by overweight patients and with the lowest values in patients with normal weight. However, comparison of median IL-6 concentrations between normal weight and overweight individuals in the autoimmune diabetes/type 2 diabetes group did not reveal significant differences (Fig. 2).

In addition, we found higher median concentrations of IL-1RA in women and a positive correlation of serum concentrations of IL-1RA with systolic and diastolic blood pressures (Table 3). Pro-inflammatory cytokines IL-6 and TNF-α were also positively correlated with age and systolic blood pressure. Circulating concentrations of IL-10 did not reveal any correlation with potential confounders (Table 3). Likewise we did not detect an association of waist circumference with IL-10 when analysing the subgroup of n=495 (r=0.04, p=0.28).

Associations of circulating concentrations of cytokines Table 4 shows the results of association analysis between cytokines adjusted for BMI, age and sex. All statistically significant associations were positive in all groups with diabetes but not in healthy participants. The strongest associations were between the anti-inflammatory cytokine IL-1RA and pro-inflammatory cytokines IL-6 and TNF-α (Table 4). Serum concentrations of IL-10 in healthy individuals were positively associated with TNF-α (β=0.7, p=0.03, Table 4), but only 44% of sera had detectable IL-10.

Increased concentrations of cytokines in type 2 diabetes after adjustment for confounders As circulating concentrations of cytokines were significantly associated with anthropometric variables we investigated the influence of potential confounding factors on differences in levels of cytokines between the groups. We employed multiple

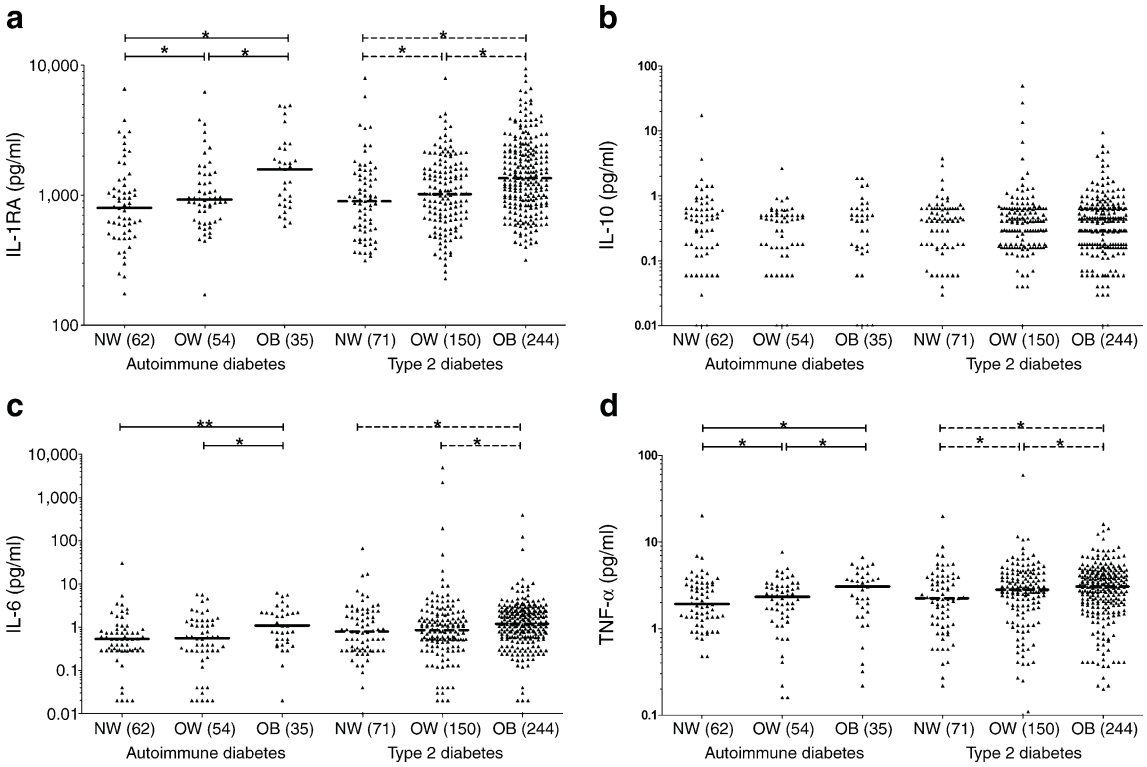

Fig. 2 Circulating cytokine concentrations by BMI of all patients with type 2 diabetes and autoimmune diabetes: (**a**) IL-1RA; (**b**) IL-10; (**c**) IL-6; and (**d**) TNF-α. We pooled type 1 diabetes and LADA participants in one group named 'autoimmune diabetes'. Each point represents the measured cytokine concentrations of an individual. Horizontal lines represent medians. For IL-10 no medians were calculated because too many values were below the detection limit. Normal weight (NW) 18–24.9 kg/m^2; overweight (OW) 25–29.9 kg/m^2; obesity (OB) ≥30 kg/m^2. Number in brackets depicts group size. Continuous lines exhibit comparisons of circulating cytokine concentrations between each subgroup with autoimmune diabetes. Broken lines show comparisons of circulating cytokine concentrations between each subgroup with type 2 diabetes. Kruskal–Wallis test resulted in p<0.0005 for IL-1RA, IL-6 and TNF-α. Fisher's test for IL-10 gave p=0.69. p values for IL-1RA, IL-6 and TNF-α were obtained by Mann–Whitney U test: *p<0.05; **p<0.01. Additional comparisons between groups revealed further significant p values that are not reported for reasons of clarity

Table 4 Associations between pro- and anti-inflammatory cytokines adjusted for BMI, age and sex

Types/cytokine	IL-1RA (β)	IL-6 (β)	TNF-α (β)
LADA			
IL-1RA	–		
IL-6	0.51**	–	–
TNF-α	0.39**	0.54*	−0.20
IL-10	−0.04	−0.06	
Type 1 diabetes			
IL-1RA	–		
IL-6	0.35*	–	–
TNF-α	0.35*	0.15**	−0.08
IL-10	0.06	−0.03	
Type 2 diabetes			
IL-1RA	–		
IL-6	0.53***	–	–
TNF-α	0.28***	0.86***	−0.72
IL-10	0.03	−0.001	
Control			
IL-1RA	–		
IL-6	0.12	–	–
TNF-α	0.35	0.06	0.7*
IL-10	−0.11	−0.12	

Adjusted association analyses between IL-1RA, IL-6, IL-10 and TNF-α were performed with multiple regression analyses

*$p<0.05$; **$p<0.01$; ***$p<0.001$

regression models to estimate whether differences in IL-1RA, IL-6, TNF-α and IL-10 concentrations between groups, detected in the univariate analysis, persisted after stepwise adjustment for BMI, age, sex, blood pressure and duration of diabetes (Table 2). The similarity of participants with LADA and those with type 1 diabetes and their differences compared with type 2 diabetes patients were maintained after stepwise adjustments. Only the differences for systemic concentrations of TNF-α between healthy control participants vs LADA/type 1 diabetes disappeared after additional adjustments for confounders.

Discussion

Increased concentrations of systemic cytokines were observed in patients with type 2 diabetes, while levels in patients with LADA and type 1 diabetes were similar. Higher BMI was positively associated with higher systemic cytokine concentrations in all groups. Furthermore, patients with type 2 diabetes exhibited a BMI-independent elevation of systemic cytokines which was not explained by sex, age or blood pressure. We therefore confirmed the hypothesis that increased BMI affects systemic cytokine concentrations in patients with type 2 diabetes and extended these observations to patients with type 1 diabetes and LADA. Similar findings were obtained in healthy control individuals; however, as this group was small and the focus or our investigation was on comparison of type 1, type 2 diabetes and LADA, further studies are warranted to confirm this finding. Several studies have shown an association between overproduction of pro- and anti-inflammatory cytokines and weight, obesity, adipose tissue and metabolic syndrome [16, 34, 35]. In the present study BMI was used to study obesity, as this marker correlates tightly with waist circumference and both variables are robust risk factors associated with diabetes [36, 37]. In our study, all individuals with higher BMI simultaneously had higher circulating pro- as well as anti-inflammatory cytokine concentrations, regardless of diabetes type. These data point to a positive influence of obesity on the secretion of systemic cytokines independent of diabetes group and it is an additional risk factor for impairment of disease progression in autoimmune diabetes as well as type 2 diabetes.

Our study is the first investigation comparing systemic cytokine concentrations in LADA with type 1 and type 2 diabetes. It has some limitations that should be mentioned. The number of LADA patients compared with type 2 diabetes is relatively low and our definition of LADA as clinical ‘type 2 diabetes patients positive for GADA’ (and not for other type 1 diabetes related autoantibodies) may be oversimplistic, though similar to that used in previous studies [31–33]. The study design was cross-sectional. A longitudinal follow-up study would be more appropriate and enable the investigation of different stages in progression from healthy control to prediabetic states and overt diabetes. Finally, possible associations between glucose and lipid toxicity, beta cell function and circulating cytokine concentrations in different diabetes groups could not be investigated because suitable data were not collected. As adjustment for the confounding variables age, sex and BMI did not change the associations, it would be of interest to implement measures of glycaemia (fasting glucose, HbA_{1c}), lipids, insulin secretory capacity and insulin resistance in such patients in the future.

However, in the present study we showed that circulating pro- and anti-inflammatory cytokine concentrations in LADA and type 1 diabetes were similar, but lower compared with type 2 diabetes. This finding is consistent with the proposal that type 1 diabetes and LADA are immunologically similar, occupying different ends of an immunophenotypic spectrum [38]. The present data are in line with our previous observation that the prevalence of metabolic syndrome in type 2 diabetes patients was higher than in patients with LADA or type 1 diabetes [33].

Our current results show that systemic cytokines are positively associated with BMI in addition to diabetes type.

Previous studies have reported that the elevation of pro- and anti-inflammatory cytokine levels is linked to higher risk of diabetes development [39–41]. We extend these observations as we describe that in addition of BMI, diabetes type is associated with systemic cytokine concentrations. Contrary to our expectation, differences in cytokine concentrations in type 2 diabetes compared with type 1 diabetes and LADA persisted after adjustment for BMI, age, sex, diabetes duration and blood pressure. Overall, these results illustrate that increased circulating concentrations of pro- and anti-inflammatory cytokines are generally affected by BMI and other potential confounders. This observation did not hold true for IL-10, which was detected in 44% of individuals only, similar to the findings in previous studies [42–45]. Differences in cytokine concentrations between those with type 2 diabetes and those with both autoimmune diabetes types are not solely explained and influenced by these potential confounders, suggesting a diabetes-type associated immune dysregulation.

Unfortunately, additional variables of the metabolic syndrome according to the IDF definition such as waist-to-hip ratio, triacylglycerols, HDL-cholesterol and fasting plasma glucose were not available for this study and we cannot conclude that differences of cytokines between types of diabetes relate, in fact, to disease pathogenesis. Measurement of systemic cytokine concentrations is unlikely to reflect the local inflammatory milieu around disease-related tissue.

Of note, IL-1RA, TNF-α and IL-6 were positively associated in all patients groups, even after adjusting for BMI, age and sex. We speculate that this positive correlation between pro- and anti-inflammatory cytokines may reflect a counter-regulatory attempt to ameliorate inflammation in patients with diabetes, associated with a general upregulation of immune responses. Interestingly, the Whitehall II study on IL-1RA also reported an upregulation of both pro- and anti-inflammatory mediators 6 years before the diagnosis of type 2 diabetes [7, 8].

In conclusion, we confirmed that greater systemic cytokine concentrations associate with body mass in type 2 diabetes. Further, systemic cytokines were similarly lower in both type 1 diabetes and LADA, but higher than in control participants. Thus, factors other than cytokines are responsible for the difference of the clinical phenotype between type 1 diabetes and LADA.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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Chapter 3

Increased serum concentrations of adhesion molecules but not of chemokines in patients with type 2 diabetes patients compared to patients with type 1 diabetes and latent autoimmune diabetes in adults

Increased serum concentrations of adhesion molecules but not of chemokines in patients with type 2 diabetes compared to patients with type 1 diabetes and latent autoimmune diabetes in adult age: Action LADA 5

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Abstract

Aims Systemic concentrations of adhesion molecules and chemokines are associated with increased risk of cardiovascular complications. We compared these factors between patients with type 2 diabetes versus type 1 diabetes or latent autoimmune diabetes in adults (LADA).

Methods Serum concentrations of adhesion molecules sE-selectin, sICAM-1 and sVCAM-1, and chemokines CCL2, CCL3 and CCL4 were measured in 61 patients with LADA, 90 with type 1, 465 with type 2 diabetes and in 41 control subjects, using multiple regression models to adjust for possible confounders.

Results Type 2 diabetes patients exhibited greater concentrations of adhesion molecules (p<0.02) than type 1 diabetes, LADA and control subjects. These differences persisted upon adjustments for age, sex, body mass index (BMI), blood pressure and diabetes duration (p<0.04). Higher BMI positively correlated with concentrations of adhesion molecules in all subjects (p<0.0001). Concentrations of sE-selectin positively related to diastolic (β=0.31) and systolic (β=0.28) blood pressure in the adjusted model (p<0.04). Concentrations of the chemokines, CCL2 and CCL4, did not differ between groups, while CCL3 was higher in LADA and type 1 diabetes patients than in type 2 diabetes and control subjects (p<0.05).

Conclusions Systemic concentrations of adhesion molecules, but not chemokines, relate to cardiovascular risk factors, but remain higher after adjustments in type 2 diabetes suggesting a diabetes-type specific effect without difference between LADA and type 1 diabetes, despite their dissimilar phenotype.

Introduction

Patients with diabetes mellitus have an increased risk of cardiovascular disease (CVD) compared to nondiabetic persons [1]. Previous studies reported that CVD is characterised by fatty and fibre-like deposits in endothelial vessel accompanied by endothelial dysfunction [2]. This pathogenic process is associated with the recruitment and infiltration of circulating leukocytes into endothelial lesions [3]. Adhesion molecules and chemokines are involved in this infiltration process and serve as biomarkers of endothelial dysfunction and CVD [4,5]. Several studies have shown increased concentrations of cellular adhesion molecules (such as sE-Selectin, sVCAM, sICAM) and chemokines (CCL2, CCL3, CCL4, CXCL8) in patients with higher risk for CVD, obesity, hypertension and diabetes mellitus, particularly type 1 and type 2 diabetes [6-9]. Elevated body mass index (BMI), longer diabetes duration, higher

blood pressure, age and sex are attributable risk factors for the development of CVD in patients with diabetes mellitus [10].

Adhesion molecules and chemokines have also been shown to be associated with disease progression [11-13]. For type 1 diabetes an association of adhesion molecule sICAM-1 and chemokine CCL3 (MIP-1α) with diabetes progression and β-cell function has been reported [11,14]. Chemokine CCL2 (MCP-1) is predictive for later development of type 2 diabetes [12].

Latent autoimmune diabetes in adults (LADA) is etiologically assigned to type 1 diabetes [15]. However, patients with LADA are often diagnosed clinically as type 2 diabetes despite positive type 1 diabetes associated auto-antibodies [16]. Some studies showed that the prevalence of parameters of the metabolic syndrome such as waist circumference, blood pressure, triglycerides and HDL-cholesterol, chronic complications, coronary heart disease (CHD) and cardiovascular mortality are similar between LADA and type 2 diabetes, others have shown that waist circumference, blood pressure and cholesterol concentrations as features of the metabolic syndrome in LADA is more similar to type 1 diabetes compared to type 2 diabetes [16-20]. Due to their immunological similarities, LADA and type 1 diabetes are often classified as autoimmune diabetes.

Our aim for this study was to investigate whether type 2 diabetes, LADA and type 1 diabetes have similar concentrations of adhesion molecules and chemokines. Therefore, we measured systemic chemokines CCL2, CCL3 and CCL4 (MIP-1β), and adhesion molecules sICAM-1, sVCAM-1 and sE-Selectin in patients with LADA, type 1 and type 2 diabetes. In addition, we determined the influence of confounders BMI, systolic and diastolic blood pressure, sex, age and diabetes duration on these systemic immune mediators.

Patients and methods

Subjects

Our cross-sectional study included 41 healthy control subjects, 90 type 1 diabetes, 61 LADA and 465 type 2 diabetes patients from the Action LADA cohort. The study population has been described elsewhere [21]. Patients aged 30-70 years, positive for GADA (glutamatic acid decarboxylase antibody) who did not inject insulin within the first 6 months after diagnosis were defined as LADA. Adult patients with type 1 diabetes were GADA-positive and were treated with insulin after diabetes diagnosis. For some analyses LADA and type 1 diabetes patients were investigated as a combined group named “autoimmune diabetes”. Type 2 diabetes patients were GADA-negative and without insulin treatment for at least one

year after diagnosis. Serum samples of patients with duration of diabetes less than 5 years after diagnosis were included in this study.

Type 1, type 2 diabetes and LADA patients had similar median duration of diabetes with median diabetes duration of 1 year (Table 1). Patients with type 2 diabetes were older than type 1 diabetes and LADA (Table 1). Type 1 diabetes and LADA patients were similar in their median age and BMI. Subjects with type 2 diabetes showed higher median BMI than all other groups. Median systolic blood pressure of patients with type 2 diabetes and LADA was similar and higher compared to type 1 diabetes patients and healthy subjects. Median diastolic blood pressure was similar in all groups (Table 1).

Measurement of adhesion molecules and chemokines

Circulating concentrations for sICAM-1, sVCAM-1, sE-Selectin, CCL2, CCL3 and CCL4 were determined with commercially available multiplex-bead technology kits (Fluorokine MAP; R&D Systems, Wiesbaden, Germany). Intra- and interassay coefficients of variations were <5% and <11%, respectively. The detection limits of the assays were 13.8 ng/ml for sICAM-1, 69.0 ng/ml for sVCAM-1, 17.1 ng/ml for sE-Selectin, 40.3 pg/ml for CCL2, 2.4 pg/ml for CCL3 and 0.4 pg/ml for CCL4. At least 95% of serum concentrations were above the detection limit for all markers except for CCL3 levels, which were detectable in 72% of all samples. Determinations of immune mediators concentrations lower than the detection limit were assigned a value half of the detection limit as described [21,22]. Serum samples were retrieved from freshly drawn blood samples from fasting subjects in the morning hours, and were stored at -80°C until the time of the assay.

Statistical analyses

Data are expressed as median with interquartile range (Q1: 25^{th} percentile; Q3: 75^{th} percentile). Normal distribution of each variable was carried out using Kolmogorov-Smirnov test. Comparisons of continuous data within all subjects groups were performed using non-parametric Kruskal-Wallis test followed by Mann-Whitney test in case of significance to investigate differences between each groups. Categorical variables with ≥ 2 classes were compared using Fisher's exact test and χ^2-test. These tests were not adjusted for multiple comparisons and are therefore descriptive. Comparisons of circulating concentrations of chemokines and adhesion molecules between all groups adjusted for sex, age, BMI, blood pressure (systolic, diastolic) and diabetes duration were assessed using multivariate regression model. We used five models with increasing number of variables adjusting for model 1 - unadjusted; model 2 - sex and age; model 3 - age, sex and BMI; model 4 - age,

sex, BMI and blood pressure (diastolic, systolic); and model 5 - age, sex, BMI, blood pressure and duration of diabetes. BMI was calculated as body weight in kilograms divided by the square of the height in meters (kg/m^2).

All statistical analyses described above were conducted using SAS Enterprise Guide version 4.2 (SAS Institute, Cary, NC, USA) and GraphPad PRISM version 4 for Windows (GraphPad Software, La Jolla, California, USA). $P<0.05$ was considered to indicate statistically significant differences.

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of the Medical Faculty at the Heinrich-Heine University Duesseldorf, Germany. Written informed consent was obtained.

Results

Serum adhesion molecules and chemokines

Firstly, comparisons of serum concentrations between all groups showed that circulating concentrations of adhesion molecules (all $p<0.0001$) and chemokine CCL3 ($p<0.04$) were significantly different (Table 1) whereas serum concentrations of chemokines CCL2 and CCL4 were similar in four groups (Table 1). We detected higher median levels of sVCAM-1 and sE-Selectin in patients with type 2 diabetes compared to type 1 diabetes, LADA and healthy subjects (all $p<0.04$) (Figure 1). Similarly, median circulating concentrations of sICAM-1 were slightly increased in patients with type 2 diabetes compared to LADA and healthy subjects (all $p<0.02$). All adhesion molecules tested had similar median levels in patients with LADA and type 1 diabetes.

The comparison of chemokines between diabetes types showed that patients with type 1 diabetes and LADA had higher median circulating concentration of CCL3 compared to healthy subjects and also compared to patients with type 2 diabetes (all $p<0.03$). As for adhesion molecules, median circulating concentrations of CCL3 in patients with LADA did not differ from type 1 diabetes. CCL2 and CCL4 were not different between the groups tested (Figure 1, Table 3). These findings were persistent when we compared age, BMI and sex matched pairs (n= 16, type 2 diabetes versus LADA; n= 19 type 1 versus type 2 diabetes, n=4 type 1 versus LADA, data not shown).

Associations between immune mediators and anthropometric parameters

Figure 2 shows the influence of BMI on circulating concentrations of adhesion molecules and chemokines for patients with diabetes for each individual. We classified patients in groups of normal weight (18-24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obesity (≥ 30 kg/m^2) in accordance with the WHO definition. Patients with type 1 diabetes and LADA were pooled and named "autoimmune diabetes" as both groups were similar in their immune mediators levels. We observed a positive association of BMI with circulating concentrations of adhesion molecules but not for chemokines in this classified analysis (Figure 2).

These observations are in line with results from regression analysis showing a positive relation of concentrations of sICAM-1, sVCAM-1 and sE-Selectin with BMI in patients with autoimmune diabetes, type 2 diabetes and control subjects (sICAM-1: β = 0.34, sVCAM-1: β=0.15, sE-Selectin: β=0.38, all p<0.0008) (Table 2) while chemokines did not show such association. Serum concentrations of adhesion molecules were strongly associated with BMI even after adjusting for potential confounders age and sex in patients with diabetes and healthy subjects (Table 2). In contrast, CCL3 was negatively associated with BMI (p=0.03) (Table 2) in a combined group with all subjects.

In addition, waist circumference data as a more representative measure of abdominal obesity, were available for a subgroup of n = 496 (70%) of the total cohort. In this subgroup, a positive correlation between BMI and waist circumferences (r=0.85, p<0.0001) was observed. In line with the observed association between BMI and serum concentrations of adhesion molecules, we detected positive associations between circulating concentrations of adhesion molecules and waist circumferences (sICAM-1: β=0.29, sVCAM-1: β=0.17, sE-Selectin: β=0.43, all p<0.0001). For chemokines we did not find significant association with waist circumference.

Systolic and diastolic blood pressure was positively associated with sE-Selectin for all groups without adjustment (all p<0.03) (Table 2). After adjustment for age, sex and BMI these positive associations were observed for patients with type 1, type 2 diabetes and LADA (all p<0.05).

Interestingly, serum levels of chemokines CCL3 and CCL4 in patients with type 2 diabetes showed a negative association with diastolic blood pressure after adjustment (p<0.03) (Table 2). The chemokine CCL4 and the adhesion molecule sVCAM-1 were positively associated with age (p<0.02). sVCAM-1 was negatively associated with duration of diabetes (p=0.0003).

Soluble adhesion molecules and chemokines according to diabetes type

Above, we determined whether differences of adhesion molecule and chemokine levels between control, LADA, type 1 and type 2 diabetes subjects occur in univariate analysis (Figure 1). As circulating concentrations of adhesion molecules and chemokines were associated with anthropometric parameters, we also carried out multiple regression models to compare type 1, type 2 diabetes and LADA with each other. The differences observed in univariate comparison persisted after stepwise adjustment for age, sex, BMI, blood pressure (systolic, diastolic) and diabetes duration (Table 3). Interestingly, adjustment for these confounders did not influence the findings made in univariate analysis, e.g. the differences between groups for sICAM-1, sVCAM-1, and sE-Selectin and CCL3 were maintained.

Discussion

In the present study type 2 diabetes patients had higher median serum levels of adhesion molecules compared to autoimmune diabetes e.g. type 1 diabetes and LADA patients and control subjects. Circulating levels of adhesion molecules and chemokines were similar in type 1 diabetes and LADA patients and were not different compared to control subjects. To our knowledge this is the first study, directly comparing circulating concentrations of adhesion molecules and chemokines in type 2 diabetes, LADA and type 1 diabetes.

Based on previous observations that adhesion molecules serve as biomarkers of CVD and endothelial dysfunction and that type 2 diabetes patients have higher risk for CVD than other groups we showed in our study increased systemic concentrations of adhesion molecules in patients with type 2 diabetes compared to control, type 1 diabetes and LADA subjects [4,5]. In contrast to previous studies, patients with autoimmune diabetes in our cohort showed systemic levels of adhesion molecules similar to control subjects and were overall lower compared to patients with type 2 diabetes [23]. This fits well with the observation that type 2 diabetes patients are clinically known to be more susceptible to CVD and vascular complications because of their higher BMI and higher blood pressure compared to type 1 diabetes and healthy subjects [24,25]. However, longitudinal clinical studies and mechanistic studies are required, to establish a causal relationship between adhesion molecules and increased CVD in type 2 diabetes.

As expected, we found positive association of adhesion molecule concentrations with age, BMI and blood pressure, similar to previous studies in type 2 diabetes [26]. Even after adjustment of these confounders the significant differences of type 2 diabetes compared to

autoimmune diabetes persisted suggesting that factors not identified in our study contribute to increased concentrations of adhesion molecules in type 2 diabetes. Since adjustment for BMI in our cohort did not alter the significant difference between LADA and type 2 diabetes in Action LADA, obesity related insulin resistance is unlikely to be cause for the differences observed.

Four points need to be taken into account for the interpretation of our data 1) although median concentrations differed statistically, levels of adhesion molecules and chemokines did considerably overlap between groups and therefore cannot be used as individual surrogate markers, 2) other CVD confounding parameters that were not measured in this cohort (cholesterol, triglycerides, HbA1c, insulin resistance, smoking history, past hypertension, stroke, hyperlipidemia) may have been non-analysed confounders, and 3) diabetes duration may have been underestimated in patients with type 2 diabetes, 4) measures of endothelial function was not available that should be used as clinical surrogate for increased risk for CVD. These confounding and surrogate factors would need to be evaluated in future studies on this topic.

Studies about chronic complications in LADA have shown that the prevalence of chronic (cardio) vascular complications between LADA and type 2 diabetes was similar [17,18]. However, the confirmed CVD risk marker hs-CRP in Chinese LADA was not significant different compared to type 2 diabetes, interestingly hs-CRP was higher in LADA compared to type 1 diabetes [27]. Our results showed in contrast, that LADA patients had similar concentrations of adhesion molecules compared to type 1 diabetes and lower concentrations compared to type 2 diabetes. The different results regarding chronic complications, hs-CRP concentrations and our findings on adhesion molecules are likely to result from the analysis of different disease markers on one hand and on the difference of ethnicity, age and diabetes duration (<1 year in the Chinese study, 4 years in the Australian Freemantle study and 13 years in the Finnish Botnia Study) on the other hand [17, 18,27]. The similarity of adhesion molecule concentrations between LADA and type 1 diabetes did not change after adjustment for confounders including BMI. Actually, the LADA patients in our Action LADA cohort are more similar regarding metabolic syndrome to type 1 diabetes than to type 2 diabetes despite the clinical appearance with regard to hyperglycemic treatment options resembling more to type 2 diabetes [19]. Interestingly, previous studies have shown that increased sICAM-1 concentrations are associated with the development of autoimmune diabetes compared to control subjects [11,20,28,29]. We could not confirm this observation as serum concentrations of sICAM-1 between type 1 diabetes, LADA and healthy subjects were similar. This discrepancy likely relates to different disease duration and age groups in the different studies.

In conclusion, we demonstrated similar serum concentrations of adhesion molecules in autoimmune diabetes and control subjects, whereas type 2 diabetes patients had higher median concentrations of adhesion molecules compared to healthy subjects. This finding is in line with previous observations that patients with type 2 diabetes due to their higher prevalence for metabolic features are especially prone to develop coronary chronic complications; in addition however, the systemic immune status may be differentially regulated in the different diabetes groups.

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Competing interests

Nothing to declare.

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Figure legends

FIGURE 1 Circulating concentrations of adhesion molecules and chemokines in control subjects, type 1 diabetes, LADA and type 2 diabetes patients.

Each point represents the measured concentration of a subject. Horizontal lines depict medians. *P*-values were obtained from Mann-Whitney test. *P*-values for Kruskal-Wallis test and interquartile range of immune mediators are given in Table 1. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

FIGURE 2 Classification of circulating concentrations of adhesion molecules and chemokines by BMI of all patients with type 2 diabetes and autoimmune diabetes.

We pooled type 1 diabetes and LADA subjects in one group named autoimmune diabetes. Each point represents the measured concentrations of a subject. Horizontal lines represent medians. Normal weight (NW) = 18-24.9 kg/m^2; Overweight (OW) = 25-29.9 kg/m^2; Obesity (OB) ≥ 30 kg/m^2. Kruskal-Wallis test resulted $P \leq 0.0001$ for sICAM-1, sVCAM-1 and sE-Selectin and $P = 0.04$ for CCL3. Continuous lines exhibit comparisons of circulating cytokine concentrations between each subgroup with autoimmune diabetes. Broken lines show comparisons of circulating cytokine concentrations between each subgroup with type 2 diabetes. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

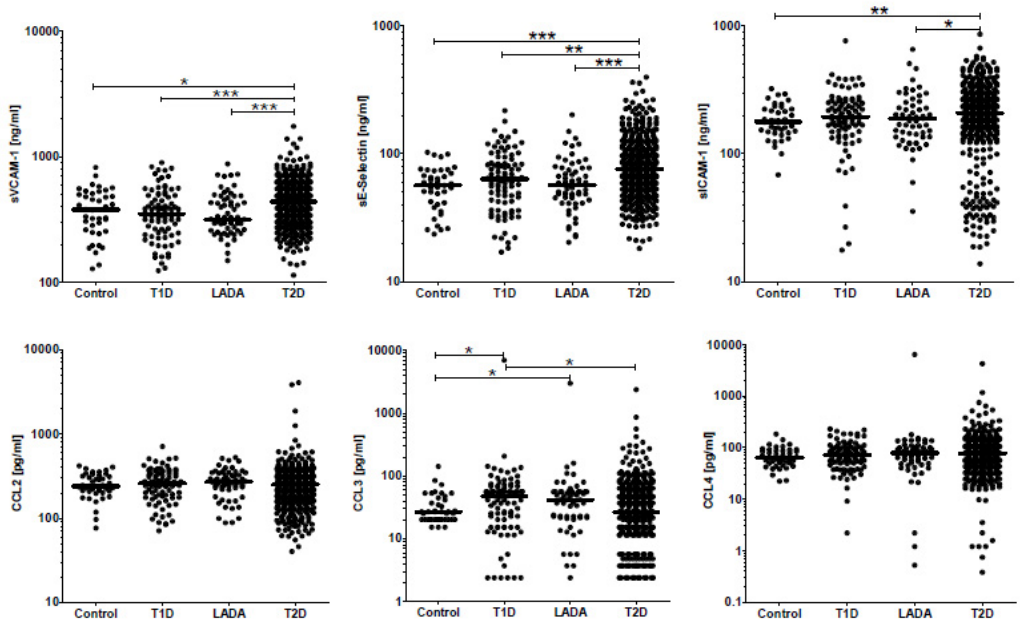

FIGURE 1

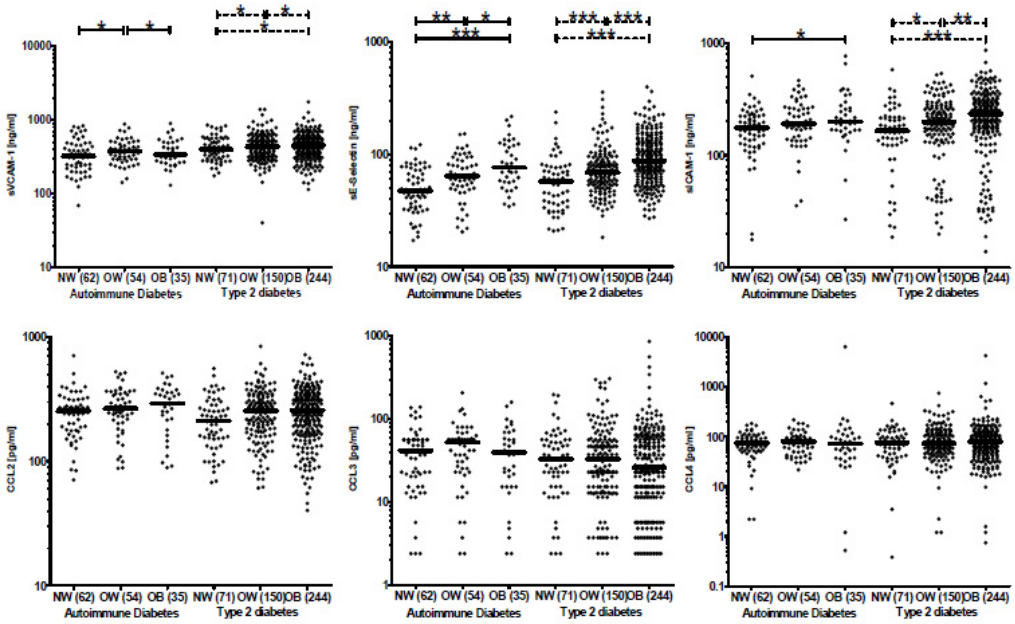

FIGURE 2

TABLE 1 Clinical data of control, type 1 diabetes, LADA and type 2 diabetes subjects.

	Control	Type 1 diabetes	LADA	Type 2 diabetes	*P*-value
N (female/male)	41 (25/16)	90 (28/62)	61 (35/26)	465 (202/263)	**
Age (years)	47.7 (39.6-53.1)	43.2 (35.5-53.3)	49.1 (39.0-58.5)	56.3 (48.1-62.1)	***
Duration of diabetes (years)	-	1.0 (0.2-1.6)	1.0 (0.5-2.3)	1.0 (0.3-1.6)	ns
BMI (kg/m^2)	23.5 (21.0-27.3)	26.1 (22.8-29.2)	25.5 (23.0-28.8)	30.3 (27.0-34.3)	***
Systolic blood pressure (mmHg)	112 (105-131)	120 (110-139)	130 (119-140)	133 (117-145)	***
Diastolic blood pressure (mmHg)	80 (67-81)	80 (68-85)	80 (71-85)	82(72-89)	ns
CCL2 [pg/ml]	234.2 (196.9-315.3)	259.1 (181.9-338.8)	268.4 (228.9-342.2)	250.3 (168.1-323.7)	ns
CCL3 [pg/ml]	25.4 (20.3-34.8)	44.5 (15.3-64.2)	41.8 (21.1-56.0)	26.9 (13.0-56.6)	*
CCL4 [pg/ml]	62.7 (49.7-87.4)	71.6 (48.6-96.5)	79.3 (50.4-99.4)	76.3 (47.0-113.6)	ns
sVCAM-1 [ng/ml]	379.8 (262.1-500.3)	349.2 (247.1-454.1)	318.6 (263.2-454.4)	436.7 (321.3-556.8)	***
sE-Selectin [ng/ml]	56.7 (41.5-70.3)	63.8 (41.6-85.0)	54.2 (43.7-74.8)	75.8 (53.8-105.1)	***
sICAM-1 [ng/ml]	165.2 (144.7-223.2)	190.1 (140.0-256.9)	185.0 (135.6-227.5)	207.5 (154.9-275.2)	*

Data are shown as medians and interquartile range. *P* –values indicate the differences between four groups. *P* -values were calculated using Kruskal-Wallis analysis (continuous variables) and χ^2-test (categorical variables). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = not significant; See individual immune mediators data in Figure 1.

TABLE 2 Association between circulating concentrations of immune mediators and confounders for all groups

Parameter	CCL2	CCL3	CCL4	sVCAM-1	sE-Selectin	sICAM-1
Sex	ns	ns	ns	ns	ns	ns
Age	0.05 (0.18)	0.06 (0.17)	**0.12 (0.02)**	**0.28 (<0.0001)**	0.004 (0.93)	0.05 (0.20)
Diabetes duration	0.05 (0.27)	0.03 (0.53)	-0.03 (0.41)	**-0.15 (0.0003)**	-0.01 (0.88)	-0.04 (0.31)
BMI	0.10 (0.45)	**-0.21 (0.03)**	-0.21 (0.73)	**0.15 (0.0004)**	**0.38 (<0.0001)**	**0.34 (<0.001)**
- Type 1 Diabetes	0.10 (0.33)	-0.05 (0.63)	-0.10 (0.31)	**0.11 (0.001)**	**0.32 (0.002)**	**0.29 (0.0002)**
- LADA	0.12 (0.38)	-0.07 (0.38)	-0.09 (0.46)	**0.11 (<0.0001)**	**0.31 (0.001)**	**0.30 (<0.0001)**
- Type 2 Diabetes	0.07 (0.11)	-0.15 (0.09)	-0.03(0.59)	**0.18 (0.0001)**	**0.36 (<0.0001)**	**0.25 (<0.0001)**
- Control	0.10 (0.39)	-0.03 (0.34)	0.05 (0.30)	**0.12(<0.0001)**	**0.21 (<0.0001)**	**0.25 (<0.0001)**
BMI with adjustment for age and sex						
-Type 1 diabetes	0.16 (0.49)	-0.08 (0.61)	-0.15 (0.53)	**0.13 (0.03)**	**0.30 (<0.0001)**	**0.39 (0.02)**
- LADA	0.21 (0.27)	-0.12 (0.14)	-0.07 (0.83)	**0.11 (0.001)**	**0.25 (0.01)**	**0.39 (0.0004)**
- Type 2 diabetes	0.29 (0.26)	-0.08 (0.71)	-0.16 (0.61)	**0.17 (<0.0001)**	**0.34 (<0.0001)**	**0.79 (<0.0001)**
- Control	0.16 (0.34)	0.09 (0.69)	-0.07 (0.81)	**0.10 (0.01)**	**0.23 (0.0003)**	**0.32 (0.01)**
Diastolic blood pressure	0.12 (0.35)	0.12 (0.14)	0.12 (0.26)	0.08 (0.67)	**0.27 (0.01)**	0.10 (0.23)
- Type 1 Diabetes	0.12 (0.39)	0.01 (0.89)	0.20 (0.15)	0.06 (0.69)	**0.21 (0.03)**	0.006 (0.96)
- LADA	0.07 (0.70)	0.02 (0.93)	0.08 (0.63)	0.14 (0.42)	**0.21 (0.02)**	0.02 (0.89)
- Type 2 Diabetes	0.09 (0.24)	0.09 (0.20)	0.08 (0.26)	0.02 (0.81)	**0.17 (0.03)**	0.08 (0.28)
- Control	0.13 (0.43)	0.10 (0.43)	0.15(0.38)	0.07 (0.59)	0.12 (0.18)	0.09 (0.45)
Diastolic blood pressure with adjustment for age, sex and BMI						
- Type 1 diabetes	0.19 (0.44)	-0.15 (0.48)	0.17 (0.37)	0.15 (0.42)	**0.31 (0.03)**	0.17 (0.38)
- LADA	0.19 (0.35)	0.09 (0.89)	0.08 (0.92)	0.12 (0.86)	**0.35 (0.03)**	0.15 (0.41)
- Type 2 diabetes	0.21 (0.23)	**-0.29 (0.03)**	**-0.36 (0.01)**	0.08 (0.85)	**0.35 (0.01)**	0.15 (0.39)
- Control	0.12 (0.51)	0.12 (0.51)	0.11 (0.53)	0.05 (0.85)	0.09 (0.12)	0.09 (0.51)
Systolic blood pressure	0.12 (0.39)	0.20 (0.21)	0.30 (0.25)	**0.13 (0.03)**	**0.47 (0.01)**	0.04 (0.86)
- Type 1 Diabetes	0.12 (0.38)	0.21 (0.27)	0.26 (0.06)	-0.002 (0.99)	**0.47 (0.01)**	0.04 (0.78)
- LADA	0.002 (0.99)	0.19 (0.26)	0.05 (0.76)	0.03 (0.86)	**0.20 (0.02)**	0.09 (0.56)
- Type 2 Diabetes	0.08 (0.31)	0.08 (0.33)	0.005 (0.95)	0.09 (0.28)	**0.13 (0.03)**	0.08 (0.34)
- Control	0.09 (0.34)	0.19 (0.20)	0.10 (0.87)	0.07 (0.28)	**0.11 (0.02)**	0.09 (0.42)
Systolic blood pressure with adjustment for age, sex and BMI						
- Type 1 diabetes	0.10 (0.62)	0.12 (0.69)	0.20 (0.19)	0.17 (0.34)	**0.28 (0.04)**	0.11 (0.79)
- LADA	0.16 (0.36)	0.12 (0.79)	0.07 (0.83)	0.21 (0.41)	**0.33 (0.04)**	0.20 (0.54)
- Type 2 diabetes	0.09 (0.80)	0.21 (0.35)	0.09 (0.89)	0.21 (0.23)	**0.29 (0.04)**	0.16 (0.34)
- Control	0.10 (0.34)	0.18 (0.20)	0.10 (0.85)	0.15 (0.32)	0.12 (0.07)	0.13 (0.35)

Association analyses were performed for adhesion molecules and chemokines with multivariate regression analysis. This table shows the β- value (regression coefficients) and *P*-value which is in bracket. Significant *P* -values and corresponding β-values are indicated in bold. ns=not significant (no correlation).

TABLE 3 Adjusted comparisons of circulating adhesion molecules and chemokines concentrations between all groups

	Model	LADA vs Type 2 diabetes		Type 1 diabetes vs Type 2 diabetes		Type 1 diabetes vs LADA		Control vs Type 2 diabetes		Control vs Type 1 diabetes		Control vs LADA	
		β	*P*	β	*P*	β	*P*	β	P	β	*P*	β	P
sVCAM-1	**1**	**0.41**	**0.001**	**0.49**	**0.001**	0.05	0.87	**0.28**	**0.04**	0.09	0.64	0.08	0.73
	2	**0.41**	**0.01**	**0.43**	**0.002**	0.05	0.93	**0.28**	**0.03**	0.09	0.58	0.08	0.77
	3	**0.31**	**0.01**	**0.43**	**0.002**	0.05	0.86	**0.21**	**0.03**	0.10	0.47	0.08	0.77
	4	**0.31**	**0.01**	**0.33**	**0.03**	0.13	0.27	**0.28**	**0.02**	**0.35**	**0.0002**	0.12	0.09
	5	**0.31**	**0.02**	**0.33**	**0.03**	0.13	0.27	/	/	/	/	/	/
sE-Selectin	**1**	**0.53**	**<0.0001**	**0.35**	**0.002**	0.11	0.13	**0.47**	**0.0003**	0.23	0.06	0.10	0.59
	2	**0.53**	**<0.0001**	**0.35**	**0.002**	0.11	0.17	**0.47**	**0.0001**	0.17	0.12	0.10	0.61
	3	**0.49**	**0.001**	**0.21**	**0.01**	0.06	0.33	**0.43**	**0.0004**	0.08	0.70	0.08	0.75
	4	**0.49**	**0.0003**	**0.33**	**0.01**	0.06	0.39	**0.43**	**0.001**	0.08	0.82	0.11	0.48
	5	**0.49**	**0.0003**	**0.33**	**0.01**	0.06	0.43	/	/		/		/
sICAM-1	**1**	**0.35**	**0.02**	0.15	0.24	0.09	0.89	**0.39**	**0.01**	0.18	0.16	0.15	0.21
	2	**0.35**	**0.02**	0.05	0.82	0.09	0.81	**0.30**	**0.04**	0.18	0.20	0.15	0.21
	3	**0.32**	**0.04**	0.09	0.68	0.09	0.96	**0.30**	**0.04**	0.08	0.49	0.15	0.23
	4	**0.32**	**0.05**	0.11	0.36	0.11	0.23	**0.29**	**0.04**	0.12	0.32	0.08	0.57
	5	**0.32**	**0.05**	0.11	0.37	0.11	0.21	/	/		/		/
CCL2	**1**	0.05	0.96	0.08	0.72	0.10	0.63	0.12	0.46	0.12	0.29	0.15	0.13
	2	0.12	0.59	0.18	0.34	0.10	0.60	0.14	0.25	0.12	0.34	0.15	0.12
	3	0.12	0.51	0.18	0.29	0.12	0.49	0.14	0.18	0.11	0.43	0.15	0.16
	4	0.16	0.26	0.19	0.17	0.10	0.64	0.14	0.08	0.13	0.07	0.14	0.26
	5	0.16	0.26	0.19	0.18	0.10	0.67	/	/	/	/	/	/
CCL3	**1**	0.07	0.95	**-0.29**	**0.03**	0.08	0.74	0.11	0.65	**0.24**	**0.02**	**0.20**	**0.03**
	2	0.07	0.99	**-0.29**	**0.03**	0.13	0.43	0.11	0.58	**0.24**	**0.02**	**0.20**	**0.03**
	3	0.07	0.99	**-0.28**	**0.04**	0.13	0.51	0.11	0.53	**0.24**	**0.03**	**0.20**	**0.04**
	4	0.13	0.19	**-0.28**	**0.04**	0.11	0.62	0.15	0.13	**0.24**	**0.03**	**0.20**	**0.03**
	5	0.13	0.19	**-0.28**	**0.05**	0.11	0.60	/	/		/	/	/
CCL4	**1**	0.15	0.45	0.17	0.39	0.14	0.22	0.09	0.50	0.09	0.31	0.12	0.38
	2	0.15	0.38	0.13	0.53	0.10	0.41	0.09	0.51	0.11	0.25	0.12	0.32
	3	0.15	0.35	0.17	0.49	0.14	0.29	0.09	0.45	0.09	0.32	0.10	0.44
	4	0.17	0.11	0.17	0.34	0.14	0.38	0.10	0.38	0.10	0.26	0.10	0.42
	5	0.17	0.11	0.17	0.32	0.13	0.36	/	/	/	/	/	/

Multiple regression models were performed for sICAM-1, sVCAM-1, sE-Selectin, CCL2 and CCL4. Logistic regression analysis was done for CCL3. Immune mediators entered the models as log-transformed variables. Significant *P* -values and β- values (regression coefficients) are indicated in bold. Model 1: unadjusted; Model 2: age and sex; Model 3: sex, age and BMI; Model 4: sex, age, BMI and blood pressure (diastolic, systolic); Model 5: sex, age, BMI, blood pressure and diabetes duration.

Chapter 4

Cellular interferon-γ and interleukin-13 immune reactivity in type 1, type 2 and latent autoimmune diabetes

Cellular interferon-γ and interleukin-13 immune reactivity in type 1, type 2 and latent autoimmune diabetes: Action LADA 6

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Abstract

Objective Type 1 diabetes and latent autoimmune diabetes in adults (LADA) are thought to result from immune-mediated ß-cell destruction. It remains unclear why LADA is clinically less severe compared to type 1 diabetes. This study aimed to compare the pro-inflammatory (interferon-γ, INF-γ) and anti-inflammatory (interleukin-13, IL-13) T-cell responses in humans with LADA and type 1 diabetes.

Research design and methods INF-γ and IL-13 T-cell responses to a panel of 16 (auto)-antigens were tested using an enzyme linked immune-spot technique and peripheral T-cells from 35 patients with type 1 diabetes, 59 patients with type 2 diabetes, 23 LADA patients, and 42 control subjects.

Results LADA and type 1 diabetes patients did not display any statistically significant differences in the frequency of INF-γ or IL-13 responses to auto-antigenic stimuli or positive control. IL-13 responses but not INF-γ responses to recall antigen tetanus toxoid were higher in healthy control subjects compared to patients with type 1 or type 2 diabetes or LADA ($P < 0.05$). Diabetes, independent of type, was associated with weaker response to recall antigen tetanus toxoid.

Conclusions LADA patients are indistinguishable from type 1 diabetes patients for cellular INF-γ and IL-13 responses upon islet and recall antigen stimulation. These results extend previous findings showing that systemic cytokine/chemokine and humoral responses in type 1 diabetes and LADA are similar.

Keywords

Diabetes, LADA, T-cell response, IL-13, INF-γ, ELISPOT

Introduction

The term latent autoimmune diabetes of adults (LADA) was introduced in 1995 by P.Z. Zimmet to define a subgroup of adult diabetes patients who were classified clinically as type 2 diabetes subjects but tested positive for GAD auto-antibody [1]. Five years after diagnosis, 80% of LADA patients progress to insulin dependence [2, 3]. When LADA patients progress to insulin treatment their phenotype is similar to type 1 diabetes. LADA also resembles type 1 diabetes immunogenetically. LADA patients can share genetic similarities with type 1 diabetes and type 2 diabetes patients, but the predominant genetic association is with type 1 diabetes [4]. On the other hand, LADA patients have increased frequency of HLA-DQB1 and PTPN22 risk genotypes and alleles which distinguish them from patients with type 1 diabetes diagnosed after 35 years of age [5]. Immunologically type 1 diabetes and LADA patients have similar characteristics of auto-antibodies [6-9] and systemic cytokines such as IL-1RA, IL-6, and TNF-α [10].

Only few data are available comparing T-cell reactivity from patients with LADA, type 1 and type 2 diabetes. In a small Chinese study T-cell reactivity to GAD65 was compared between LADA and type 2 diabetes. There was low level reactivity for both IFN-γ and IL-4, and the number of IFN-γ producing T-cells was higher in patiens with LADA [11]. A study by Brooks-Worrell *et al.* reported that unfractionated mononuclear blood cells from LADA and type 1 diabetes patients responded more strongly with proliferation to islet proteins blotted onto nitrocellulose than cells from patients with type 2 diabetes [12]. The quality of the immune response in terms of cytokines produced was not determined.

In the present study we aimed to evaluate autoimmune T cell responses in LADA and type 1 diabetes patients in comparison to patients with type 2 diabetes. We recruited patients from the Action LADA trial that have been diagnosed with diabetes within 5 years [13]. In addition we evaluated healthy unrelated control subjects performing enzyme linked immunosorbent-spot assays (ELISPOT).

Material and methods

Subjects

The study population consisted of 159 German individuals from the Action LADA cohort [10] and included 35 patients with type 1 diabetes, 59 with type 2 diabetes, 23 with LADA, and 42 healthy control subjects. Diabetes patients had been diagnosed with diabetes for a maximum of 5 years. Design and characteristics of the Action LADA study have been described in detail before [10, 13]. LADA was defined as diabetes occurring at the age of 30-70, not requiring insulin for the first 6 months after diagnosis and having autoantibodies to GAD65. The study protocol was approved by the local ethics committee in accordance with the Declaration of Helsinki. All patients gave written informed consent for the study.

Enzyme-linked immunosorbent spot assay

ELISPOT was performed as described [14, 15] using the U-CyTech Assays (Utrecht, The Netherlands) for INF-γ and IL-13. Briefly, venous blood was drawn into K^+-EDTA tubes, shipped to the institute and stored over night at RT. Antigens were pipetted into the appropriate wells of 48-well plates. To each antigen-containing well 3.5×10^6 PBMC's were added and the plate was incubated at 37 °C for 18 h. Next, 0.5 ml of supplemented RPMI 1640 containing 10% human AB serum were added to each well and the plates incubated for additional 22 h.

Following the stimulation the non-adherent PBMC's were collected, washed, suspended in 300 µl medium and transferred as 100 µl aliquots in triplicate wells into 96-well Nunc Maxisorp plates (Merk, UK). U-CyTech INF-γ and IL-13 ELISPOT assays were used to determine the number of INF-γ and IL-13 producing cells. Detected spots were counted using the automated reader system Bioreader 3000LC (BioSys, Germany).

Stimuli

We used 16 different stimuli including mitogens, recall- and auto-antigens, islet hormones, and peptides. Medium alone was used as negative control. PI, a mixture of phorbol-myristate-acetate (PMA) [10 ng/ml] and ionomycin [1 µM] (Sigma, Germany), was used as positive control, while tetanus toxoid (TT) [1.5 Lf/ml] (SVM, The Netherlands) was included as a recall antigen. The other stimuli were human GAD65 [1 µg/ml] (Diamyd,

Sweden), pro-insulin [10 µg/ml] (Eli Lilly and Company, USA), insulin [10 µg/ml] (Novo-Nordisk, Denmark), ghrelin [5 µg/ml] (American Peptide, USA), hsp60 [0.5 µg/ml] (gift from Peptor, Israel), DiaPep277 [10 µg/ml] (gift from Peptor, Israel); GAD and IA-2 peptides according to Arif *et al.* [16] - GAD_3.1 (aa 335-352) [5 µg/ml], GAD_4.11 (aa 554-575) [5 µg/ml], GAD_4.7 (aa 270-292) [5 µg/ml], human pIA-2 (p25, p26) [10 µg/ml], pIA-2_R2 (aa 853-872) [5 µg/ml], pIA-2_R3 (aa 752-775) [5 µg/ml], pIA-2_R5 (aa 709-736) [5 µg/ml], and Insulin peptide ins B11-23 (aa 11-23) [5 µg/ml] (all peptides were synthesized at the Leiden University Medical Center, The Netherlands).

Statistics

ELISPOT results are reported as stimulation index (SI), dividing mean spots upon stimulation by mean background [BG] spots as described [16,17]. Descriptive and inferential statistics were applied where appropriate applying SAS Enterprise Guide v4.2 (SAS Institute, USA) and GraphPad Prism v.4 (GraphPad Software, USA). Continuous variables are displayed as medians and interquartile ranges (IQR = Q3[75%] - Q1[25%]). To determine differences within all groups we used Kruskal-Wallis test and Mann-Whitney *U* test to compare single groups. For the evaluation of categorical data with two or more classes Fisher's exact test was applied. Results were considered significant when the *P* value was < 0.05.

Results

The baseline characteristics for sex, age, BMI, diabetes duration and blood glucose of the groups are presented in Table 1. All groups differed in sex, BMI, and blood glucose (for all $P < 0.0001$). In addition, diabetes groups had different diabetes duration ($P < 0.05$).

ELISPOT response to 16 stimuli was evaluated using INF-γ and IL-13 ELISPOT assays. Immune responses in BG samples were low (median spot numbers [IQR] for INF-γ = 1 [1.7] and for IL-13 = 0.7 [1.2]), T-lymphocyte responses to mitogen PI were high (Figure 1A). No differences between the groups in the INF-γ and IL-13 response to the PI mitogen (Figure 1A) or background responses were present.

Statistically significant differences between the groups were observed in the anti-inflammatory (IL-13) immune response to the recall-antigen TT (Figure 1B) and to the peptide IA-2_R3 (Table 2). TT responses were lower in the diabetes groups than in the control group ($P < 0.05$, Kruskal-Wallis test). Type 1, type 2 diabetes and LADA patients had similar responses to TT (Figure 1B). Upon further analysis applying linear regression we

found that INF-γ and IL-13 responses to tetanus toxoid were not influenced by diabetes duration (r = -0.00277; P = 0.9766), confirming our previous findings [15]. Furthermore, tetanus response did not significantly relate to age (r = -0.1207; P = 0.1949) or BMI (r = -0.0247; P = 0.7924).

For IA-2_R3 the immune response (IL-13) was slightly stronger in the type 2 diabetes group compared to the other groups ($P < 0.05$, Kruskal-Wallis test). Again, T1D and LADA were indistinguishable. For the majority of antigenic stimuli responses were low and mostly did not exceed background responses in line with previous studies [14, 15]. We performed descriptive comparison of groups for all stimuli using the Mann-Whitney U test (Table 2). IL-13 showed higher responses upon hsp60 stimulation in the control group compared to LADA and a higher response for IA-2_R2 in type 2 diabetes patients compared to LADA ($P < 0.05$). IFN-γ, showed higher responses to GAD65 and TT in the control group compared to type 1 diabetes, and higher response for GAD65, GAD peptide 4.7 and insulin in type 2 diabetes patients compared to type 1 diabetes patients (all $P < 0.05$).

Discussion

In the present study we used ELISPOT to detect differences towards auto-antigenic and antigenic stimuli in LADA, type 1 and type 2 diabetes. Despite strongly increased responses to mitogen and recall antigen over background responses, the number of spots in our assay upon antigen stimulation was generally low and auto-antigenic responses did not differ between diabetes patients groups and control subjects. Reasons for not seeing differences could be dependent on the different nature of the assay, the antigenic stimuli or the ethnic background of patients [11,12].

The low responses obtained in our assays are in line with data from the recent T-cell workshop applying the same type of ELISPOT assay [17] and are likely to result from immune regulation occurring *in vitro* in the first phase of incubation with antigen and peripheral blood mononuclear cells including macrophages, T-cells and T_{reg} cells.

When we investigated immune-reactivity towards the recall antigen tetanus toxoid, responses in patients and controls were considerably higher for IL-13 and IFN-γ compared to auto-antigenic responses. Interestingly, IL-13 responses to tetanus toxoid were significantly lower in patients with type 1, type 2 diabetes or LADA compared to healthy subjects consistent with a metabolic effect on these responses independent of diabetes type. Our data extend previous observations by Casey *et al.* [18] who were using proliferation assays demonstrating lower responses to tetanus toxoid in type 2 diabetes patients compared to

control subjects. Interestingly, several studies have demonstrated that type 1 diabetes patients have a less sufficient immune response compared to healthy subjects upon vaccination [19-24] which would be in line with a compromised immunological function.

Conclusion

These findings suggest that there is no difference in IL-13 and IFN-γ response between type 1 diabetes and LADA patients. Our cellular immunology data are in line with previous findings that islet auto-antibodies and systemic cytokines/chemokines concentrations are similar in LADA and adult type 1 diabetes patients. We also provide the first evidence that LADA patients could have impaired immune response similar to type 1 diabetes patients.

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N. Schloot is currently on leave of absence from the German Diabetes Center.

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Figure Legends

Figure 1. Cellular IL-13 and INF-γ responses to PI (**A**) and TT (**B**). Shown are individual Stimulation indices (SI). The horizontal lines depict the medians. (SI – stimulation index; n.s. – not significant)

Figure 1

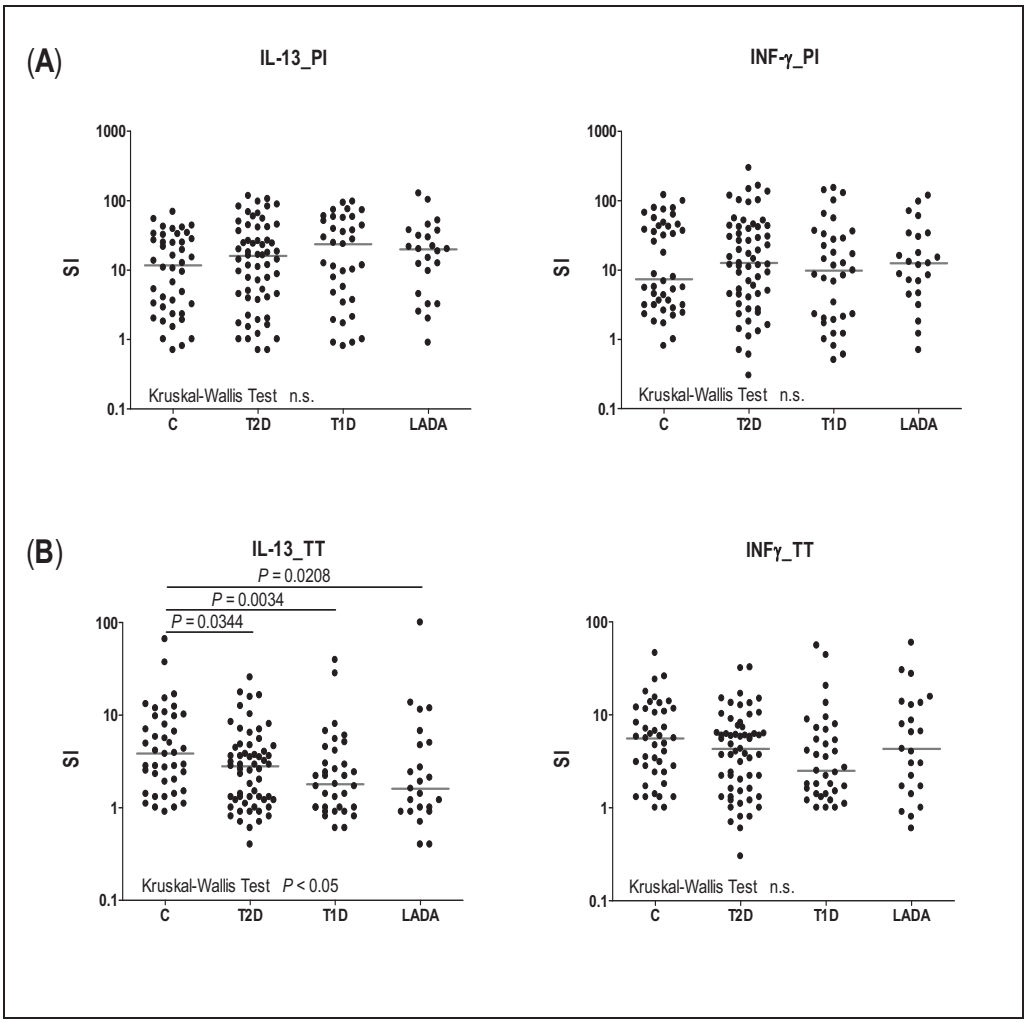

Table 1 Baseline characteristics of the study participants

Characteristic	Control	T2D	T1D	LADA	*P* value
Subjects, n	42	59	35	23	
Men, n (%)	17 (40.5)	43 (72.9)	23 (65.7)	6 (26.1)	< 0.0001
Median age (IQR), years	48.2 (24.6)	49.9 (19.2)	44.3 (13.8)	49.1 (17.2)	n.s.
Median BMI (kg/m^2) (IQR)	23.12 (6.94)	30.3 (7.1)	25.1 (6.3)	26.6 (7.5)	< 0.0001
Median glucose [mg/dL] (IQR)	83.0 (14.5)	122.0 (54.0)	126.5 (59.0)	123.0 (52.0)	< 0.0001
Median diabetes duration (IQR), months	-	12.5 (34.5)	4.0 (24.0)	27.0 (32.0)	< 0.05

Data are presented as number (n) and percentage or as medians and interquartile range (IQR). *P*-values are corresponding to the comparison of all groups using Kruskal-Wallis test or Fisher's exact test.

Table 2 Descriptive Mean Stimulation index for T1D, T2D, LADA and Control subjects

Stimuli	T1D	T2D	LADA	Control
INFγ				
TT	**6.72 (±11.59)**	6.03 (±6.41)	9.86 (±13.52)	**7.96 (±8.56)[1]**
GAD65	**0.95 (±0.35)**	**1.27 (±0.65)[1]**	1.14 (±0.57)	**1.24 (±0.60)[1]**
GAD_4.7	**1.17 (±1.16)**	**1.33 (±0.76)[1]**	1.16 (±0.76)	1.18 (±0.55)
IL-13				
Hsp60	1.14 (±0.43)	1.25 (±0.58)	**1.00 (±0.46)**	**1.23 (±0.48)[3]**
pIA-2_R2	1.25 (±0.63)	**1.40 (±0.68)**	**1.06 (±0.48)[2]**	1.27 (±0.68)
pIA-2_R3*	1.10 (±0.48)	**1.39 (±0.78)**	**1.03 (±0.56)[2]**	**1.02 (±0.53)[2]**

The stimulation index data are expressed as mean SI (±SD). Data are depicted in bold in case of significant difference between groups. *For all comparisons but pIA-2R3 ($p < 0.05$), Kruskal-Wallis test was not significant. Data comparison by unpaired testing was significant for [1] – Control subjects or T2D compared with T1D ($p < 0.05$); [2] – LADA and control subjects compared with T2D ($p < 0.04$); [3] – Control subjects compared with LADA ($p < 0.04$).

Chapter 5

Leptin and resistin positively associate with β-cell function in type 1 diabetes, in contrast to adiponectin

Leptin and resistin positively associate with beta-cell function in type 1 diabetes, in contrast to adiponectin

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Abstract

We aimed to investigate a possible association of adipokines adiponectin, leptin and resistin with ß-cell function in type 1 diabetes patients.

118 type 1 diabetes patients underwent mixed meal tolerance test (MMTT) over 2h. Baseline and stimulated C-peptide, blood glucose and A1c were tested in addition to circulating concentration of adiponectin, leptin and resistin at 0-120 min. Differences of C-peptide levels between patient groups were carried out using multivariate regression test adjusted for sex, age, diabetes duration, BMI, A1c and blood glucose.

Serum concentrations of the three adipokines varied little over the 120 min of the MMTT. Patients were divided by their adipokine levels in subgroups above or below the median level ("higher versus lower"). Higher adiponectin levels (>10.6µg/ml) were associated with lower fasting and stimulated C-peptide concentrations than seen in the lower adiponectin <10.6µg/ml) subgroup ($p<0.03$). Conversely, higher leptin or resistin levels associated positively with fasting and stimulated C-peptide concentrations ($p<0.04$ for all comparisons). All differences remained significant after adjustment for baseline metabolic parameters, including baseline C-peptide when analyzing stimulated C-peptide (all $p<0.05$). In conclusion, serum adiponectin levels negatively associate with ß-cell function in patients with type 1 diabetes. Contrary to what is known for type 2 diabetes, adiponectin does not seem to exert a protective effect on disease development in type 1 diabetes. Surprisingly, both leptin and resistin levels correlated with better preservation of ß-cell function in early type 1 diabetes which may be mediated by modulation of innate immunity and regulatory T-cell functions by the two adipokines.

Introduction

Adipokines are peptides secreted by adipocytes, correlate with white adipose tissue mass and play a role in insulin resistance [1-3]. Data from animal models and human studies suggest that increased fat mass and associated adipokines not only play a role in chronic inflammation in type 2 diabetes but are thought to be associated with disease pathogenesis and ß-cell destruction of type 1 diabetes [4-9]. It is thought that adipokines leptin and adiponectin are involved in cytokine upregulation of ß-cell toxic IL-1ß, TNF-α and IL-6 and thereby indirectly associate with disease activity [9-11]. Interestingly, patients with type 2 diabetes have decreased concentrations of adiponectin that is associated positively with insulin sensitivity [12]. Previous studies in human type 1 diabetes have shown that higher systemic adiponectin concentrations are associated with diminished ß-cell function and that higher adiponectin concentrations are found in type 1 diabetes and LADA patients compared to type 2 diabetes patients [7,8,13].

Whereas to our knowledge no data on leptin, resistin and type 1 diabetes in human subjects are available*, in vivo* administration of leptin improves inflammatory and metabolic parameters in animal models suggesting a protective role of leptin in animal models for type 1 diabetes [14]. Indeed, leptin receptors are expressed by pancreatic ß-cells from mice and resistin receptors have been detected on mouse as well as on human pancreatic islet as well as on macrophages [15,16]. It has been shown that pretreatment of ß-cells with pathological concentration of resistin for 24 hours significantly reduced insulin receptor expression [17].

Food, especially high-fat meal as well as high-carbohydrate consumption has shown to associate with increased postprandial circulating concentrations of cytokines (IL-18, IL-8, IL-6, CRP) in type 2 diabetes patients and showed decreased postprandial concentrations of adiponectin in healthy subjects [18-20]. Therefore, food intake may also play a role in influencing adipokines such as leptin and resistin that may directly or indirectly be involved in disease pathogenesis or progression of type 1 diabetes.

In the present study we aimed to determine the effect of standardized liquid mixed meal tolerance test (MMTT) on ß-cell function and circulating concentrations of adipokines in type 1 diabetes. Furthermore, we tested the hypothesis that systemic concentrations of adipokines are associated with ß-cell function in patients with type 1 diabetes. We investigated the serum concentrations of the adiponectin, leptin and resistin as these immune mediators have shown associations with immunpathogenesis of type 1 diabetes, regulation of food intake, obesity and insulin resistance [1-3,7-9,14,19,21-23].

Research Design and Methods

Study population

In our cross-sectional study we included 118 patients with type 1 diabetes from the European C-peptide Trial (ECPT) with diabetes duration less than 5 years that received a standardized liquid mixed meal tolerance test (MMTT). The study protocol and study population have been described elsewhere [24]. The MMTT is a boost high protein drink (containing 33 g carbohydrate, 15 g protein, 6 g fat, 10 mg cholesterol, 27g sugars, 170 mg sodium, 240 kcal; Mead Johnson) applying 6 ml/kg body weight (maximum of 360 ml) that was performed at 10 am according a standardized procedure [30]. Blood samples from overnight fasting subjects were drawn before and after the intake of MMTT over 120 minutes for analyzing serum adipokines and metabolic parameters. The ethics committee approved the study protocol which is in accordance with the Declaration of Helsinki. All patients gave written informed consent for the study.

Serum adipokine measurements

For our study we investigated serum samples from MMTTs at -5, 0, 30, 90 and 120 minutes after ingestion of the mixed meal [24]. The blood samples were stored for coagulation at room temperature for 1 hour and centrifuged for 10 minutes at 3000 rpm. Serum samples were stored at -20°C and thawed only once for adipokine measurements. We accounted for our measurements of systemic adipokine concentrations of adiponectin, leptin and resistin by multiplex-bead technology using commercially available kits (Fluorokine MAP; R&D Systems, Wiesbaden, Germany) the serum samples of time points -5, 0, 30, 90 and 120 minutes [25,26]. The detection limits of the assays were 83.15 pg/ml for adiponectin, 418.08 pg/ml for leptin and 2864.48 pg/ml for resistin. The assays of adipokines showed an inter-assay variation of 8% and intra-assay of 11% variation.

Metabolic parameters

The central laboratory at Steno Diabetes Center, Copenhagen measured blood glucose, C-peptide and A1c as described elsewhere [24]. C-peptide represents ß-cell function in type 1 diabetes and was analyzed by fluoroimmunometric assay (AutoDELFIA; Perkin Elmer

Wallac). The inter-assay coefficient of variation was <6%. The range of C-peptide kit was calculated 0.01-6 pmol/l [24].

Statistical methods

For our analyses we used data of time points -5, 0, 30, 90 and 120 minutes. The mean was calculated for time points -5 and 0 minutes and referred to as baseline.

Clinical data and systemic concentrations of adipokines of patients are expressed as medians and interquartile ranges (Q1: 25 th percentile, Q3: 75th percentile).

Firstly, data were tested for normal distribution by Kolmogorov-Smirnov test.

Furthermore, we divided the patients in subgroups below or above the adipokine median levels in order to investigate the negative or positive association of adipokine levels with C-peptide or glucose concentrations. For this step we used the multivariate regression analysis adjusted for sex, age, diabetes duration, BMI, A1c and blood glucose.

With Spearman correlation test we analyzed associations between circulating concentrations of adipokines and metabolic parameters as well as the associations between investigated immune mediators.

Comparisons of circulating adipokine concentrations between time points after MMTT were carried out by Friedman-test followed by Wilcoxon paired test.

Our analyses were descriptive and were not corrected for multiple comparisons. We defined $p<0.05$ as statistically significant.

All data were processed using SAS Enterprise Guide version 4.2 (SAS Institute, Cary, NC, USA) and GraphPad Prism version 4 for Windows (GraphPad Software, La Jolla, California, USA).

Results

Clinical characteristics of patients

48 women and 70 men with type 1 diabetes were included in our study (Table 1). Their median age was 19.40 years and median diabetes duration 2.32 years. We calculated a median A1c of 7.40% and median BMI of 21.30 kg/m^2 (Table 1). Median fasting blood glucose concentration was 8.70 mmol/l and C-peptide 77.00 pmol/l.

Association of adipokine concentrations with C-peptide levels

We investigated how ß-cell function relates to adipokine concentrations during the MMTT if these subjects have high or low concentrations of systemic adipokines. Two subgroups above and below the median adipokine baseline level were assigned and then analyzed for differences in C-peptide (Figure 1).

Patients with lower level of adiponectin had significantly higher concentrations of fasting C-peptide compared to patients with higher adiponectin level (p=0.03) (Figure 1). This trend remained significant after the intake of MMTT (all p<0.05) (data not shown). Conversely, leptin and resistin associated positively with fasting C-peptide concentrations (leptin p=0.02, resistin p=0.042) (Figure 1). Patients with higher concentrations of leptin and resistin showed also increased level of stimulated C-peptide (all p<0.03) (data not shown). Interestingly, all differences persisted significantly after adjustment for sex, age, BMI, diabetes duration, A1c and baseline glucose when analyzing fasting and stimulated C-peptide (Figure 1).

Associations between circulating adipokine concentrations and metabolic parameters

Table 2 shows the correlation analysis between adipokines and metabolic parameters performed by Spearman correlation test.

We detected negative correlations between fasting and stimulated C-peptide levels and systemic concentrations of adiponectin (p<0.028) (Table 2) whereas circulating concentrations of leptin and resistin revealed positive correlations with fasting and stimulated C-peptide concentrations (p<0.037) (Table 2). These findings confirmed the previous described results presented in Figure 1.

Systemic concentrations of adiponectin correlated positively with stimulated blood glucose (p<0.0001). Leptin and resistin did not exhibit correlations with fasting and stimulated blood glucose (Table 2).

Adipokines are fat tissue derived peptides with regulatory functions on metabolism [1,4,8,9,28]. We observed that systemic concentrations of adiponectin correlated negatively with BMI (p<0.0001) (Table 2). Circulating concentrations of leptin revealed positive correlation with BMI as expected (p<0.0001) (Table 2). For A1c we encountered positive correlations with adiponectin, leptin and resistin (all p<0.011) (Table 2).

We noted that female subjects showed significantly higher systemic concentrations of adipokine than male (all p<0.03) (data not shown). Systemic concentrations of adiponectin

correlated negatively with age (p<0.0001, r=-0.463) (data not shown) and diabetes duration (p<0.031, r=-0.102) (data not shown) whereas leptin and resistin did not show these correlations.

In addition, we carried out a Spearman correlation test to examine whether the investigated immune mediators show associations with each other. Resistin exhibited positive correlations with leptin (p=0.004, r=0.27) (data not shown). Adiponectin presented no correlations with leptin and resistin (data not shown).

Circulating concentrations of adipokines over 120 minutes

After the intake of MMTT we revealed a significant decrease of serum concentrations of leptin and resistin over 120 minutes of MMTT (all p<0.0005) (Figure 2). Circulating concentrations of adiponectin remained stable over 120 minutes.

Discussion

In the present study in type 1 diabetes we detected a positive correlation of leptin and resistin with fasting and stimulated C-peptide that is contrasted by the negative association of systemic adiponectin with ß- cell function [7,8]. These associations were still maintained after adjustment for anthropometric and metabolic parameters thereby suggesting an effect at least partly independent of sex, age, diabetes duration, BMI, A1c and blood glucose.

The role of leptin in type 1 diabetes has so far been investigated predominantly in animal models. Matarese et al. found in non-obese diabetic mice (NOD) increased serum leptin concentrations before the onset of hyperglycemia [23]. When they injected leptin, autoimmune destruction of insulin-producing ß-cells was accelerated leading to increase IFN-γ production in peripheral T-cells. This is suggestive of an accelerating effect for diabetes progression or an unsuccessful counter regulatory attempt [23]. In mice that became diabetic upon high dose streptozotocin, administration of leptin led to improve glucose homeostasis and insulin sensitivity suggestive of a positive metabolic leptin effect [14,27,28]. Earlier studies demonstrated that leptin with its insulin-sensitizing effect acts directly on murine ß-cells because it induces the hyperpolarization of cell membranes by opening K_{ATP} channels leading to release of insulin vesicles out of the ß-cell [29]. The latter data are in line with our observations suggestive of a protective role of leptin in type 1 diabetes as it was positively associated with endogenous c-peptide. Immunological investigations in other models such as rheumatoid arthritis or multiple sclerosis reported that

leptin have been suggestive of disease promoting role – in contrast to our findings [30,31]. Whether leptin receptors known to be located on monocytes, lymphocytes, leukocytes and macrophages play a role in promoting or ameliorating islet directed immune activation still needs to be determined.

Resistin is known to impair insulin secretion of islets by inducing SOCS3 expression and inhibiting Akt phosphorylation [32]. Furthermore it modulates cell viability in rodent pancreatic ß-cells and is known to associate with insulin resistance in type 2 diabetes while its role in the immunopathogenesis of type 1 diabetes needs to be clarified [33]. Data from our study show that resistin is positively related to C-peptide and could thereby be protective. Celi et al. observed higher resistin concentration in recently diagnosed type 1 diabetes children compared to non-diabetic control subjects, however no C-peptide data were available for this study [34]. Fehlmann et al. found similar systemic resistin concentrations in patients with type 1, type 2 diabetes and healthy control subjects again without detailed metabolic data [35]. Several human studies regarding on other autoimmune disease showed that levels of resistin have been associated with disease progression [36]. However, it has been suggested that further studies are needed to investigate and clarify the role of resisitn in the pathogenesis of autoimmune diseases.

Adiponectin has been investigated more thoroughly; systemic concentrations have been shown to be associated negatively with ß-cell function, confirmed by our data [7,8]. Previous studies have shown that adiponectin has receptors on ß-cells and influences insulin gene expression and secretion by ERK and Akt activation [37]. It has been discussed that adiponectin may be protective for ß-cells as the insulin deficiency in patients with type 1 diabetes might induce adiponectin secretion that itself then leads to increased insulin secretion [11,38]. Similarly, Forsblom et al. reported on increased concentration of adiponectin in patients with long standing type 1 diabetes and increased urinary albumin excretion (AER) [39]. It has been pointed out that cardiovascular mortality in patients with type 1 diabetes is positively associated with adiponectin concentration [39].

Nutrients are known to have an effect on metabolism, risk for development of diabetes and secretion of immune mediators [18-20]. In the present study we investigated how ingestion of a standardized mixed meal influences blood glucose and C-peptide in patients with type 1 diabetes and whether this is associated with systemic adipokine concentrations.

Our data show overall rather stable adipokine concentrations for resistin, adiponectin and leptin. Although we observed a statistically significant decrease for leptin and resistin 30 minutes after mixed meal ingestion, the absolute differences were minor and the overall range was extensive so that we hesitate to assign a biological effect of this finding. Previous studies showed significantly increased adiponectin concentrations upon meal intake in patients with type 2 diabetes, although these results seem discordant with our data at first

sight, it needs to be kept in mind, that we used the mixed meal test with different and lower fat content than the other studies [18-20], which might to be the cause for the lack of postprandial increase of adipokines. To our knowledge, resisitin and leptin have not been investigated upon food ingestions in patients with type 1 diabetes. Studies with C57BL/6J mice found that plasma leptin increased after consumption of high-fat diet [40]. One study investigated the effect of low-energy diet on serum adiponectin, leptin and resistin levels in patients with type 2 diabetes and nephropathy. It has been reported that fat mass decreased followed by lower level of resistin whereas adiponectin and leptin correlated positively with fat mass [41]. Further studies with intake of high fiber content revealed an increase of adiponectin concentrations in patients with type 2 diabetes [42,43]. Our data for resistin showed highly significant decreased upon MMTT upon paired analysis. However, median data showed a decrease of less than 12% and only mechanistic studies could reveal whether this is a biological meaningful change.

As it is known from patients with type 2 diabetes and obesity, resistin and leptin are associated with anthropometric (sex, age, diabetes duration) and metabolic (BMI, A1c, C-peptide and glucose) parameters [2,3,10,22]. When we analyzed our data from type 1 diabetes patients we also detected that leptin and resistin are correlated with A1c and C-peptide [22,44,45]. Furthermore, we confirmed the negative correlation between BMI and adiponectin [7,8]. Consistent with other studies with type 1 diabetes, we encountered that adiponectin is not only negatively associated with fasting and stimulated C-peptide levels but also positively associated with HbA1c [7,8,34].

To our knowledge, this is the first study investigating the association between adipokines and fasting and stimulated ß-cell function in type 1 diabetes patients. When interpreting the results we need to take the cross-sectional study design into account. As it is likely that the intra-individual development of diabetes progresses differently, a longitudinal study would give more information on disease progression. Although our study investigates the influence of meal intake on systemic cytokines in type 1 diabetes, the comparison to studies obtained in type 2 diabetes or healthy control subjects seems difficult, as composition of the meals differed in that usually high fat or high carbohydrate meals were tested and not directly compared with type 1 diabetes patients [20-22,46,47].

In conclusion, we confirmed our hypothesis that systemic concentrations of adipokines leptin and resistin are positively associated with ß-cell function whereas serum concentrations of adiponectin were negatively associated with ß-cell function in patients with type 1 diabetes as expected. Leptin and resistin showed positive association with better preservation of ß-cell function in early type 1 diabetes. Our findings point out that adipokines especially adiponectin do not qualify as individual markers for the ß-cell function in patient with type 1

diabetes but may play a role in diabetes progression and islet function. Therefore, further studies are required to investigate the role of adipokines in the ß-cell destruction.

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Table 1: Clinical data of study population.

Parameter	Absolute
n (female/male)	118 (48/70)
Age (years)	19.40 (13.79-25.78)
Diabetes duration (years)	2.32 (1.35-3.30)
A1c (%)	7.40 (6.50-8.20)
BMI (kg/m^2)	21.30 (19.46-23.30)
Glucose baseline [mmol/l]	8.70 (6.95-12.80)
C-Peptide baseline [pmol/l]	77.00 (22.00-157.00)

Data are presented as medians with interquartile range (Q1-Q3).

Table 2: Associations between circulating adipokine concentrations and metabolic parameters.

Parameter	fasting C-peptide	stimulated C-peptide	fasting glucose	stimulated glucose	BMI	A1c
Adiponectin	***-0.103*** ***(0.028)***	***-0.146*** ***(0.002)***	0.129 (0.230)	***0.219*** ***(<0.0001)***	***-0.437*** ***(<0.0001)***	***0.121*** ***(0.011)***
Leptin	***0.110*** ***(0.018)***	***0.119*** ***(0.025)***	0.118 (0.213)	0.026 (0.583)	***0.255*** ***(<0.0001)***	0.064 (0.176)
Resistin	***0.141*** ***(0.025)***	***0.096*** ***(0.037)***	-0.026 (0.789)	0.012 (0.797)	0.040 (0.399)	***0.257*** ***(<0.0001)***

Correlation analyses were carried out by Spearman test. This table shows the Spearman correlation coefficients (r) and *p*-values that are in bracket. Significant *p*-values and corresponding r-values are indicated in bold and italic.

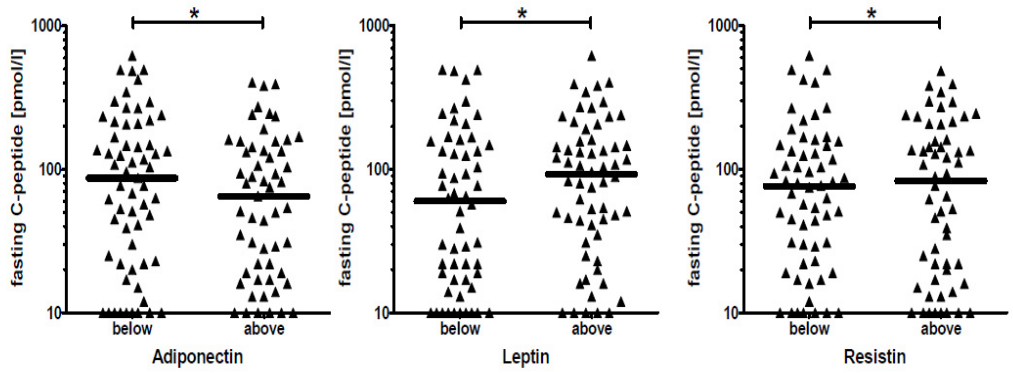

Figure 1: Comparisons of circulating adipokine concentrations of patients with low and high concentrations of adipokines.
Patients were divided in subgroups below or above the adipokine median levels in order to investigate the association of adipokine levels with fasting C-peptide concentrations. Similar results were obtained for stimulated C-peptide (data not shown). *P*-values were assessed by multivariate regression analyses and exhibit differences between time points adjusted for sex, age, diabetes duration, BMI, A1c and baseline glucose level. Bars represent the medians. *p<0.05.

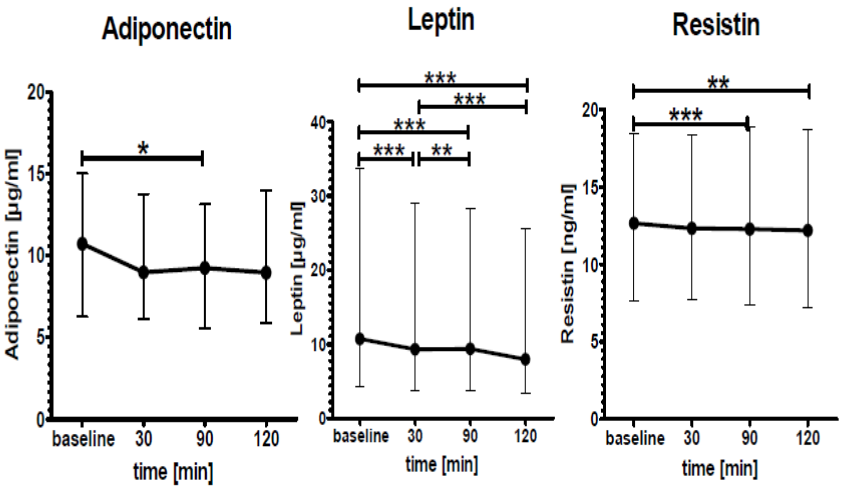

Figure 2: Circulating adipokine concentrations of patients with type 1 diabetes at baseline and 30, 90 and 120 minutes after intake of MMTT.
Median and interquartile range (Q1-Q3) are shown. P-values were assessed by Wilcoxon paired test and exhibit differences between time points. Friedman-test resulted in p<0.005 for leptin and resistin. *p<0.05. **p<0.01. ***p<0.001.

Chapter 6

Immunology of Diabetes Society T-Cell Workshop: HLA Class II Tetramer-Directed Epitope Validation Initiative

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RESEARCH ARTICLE

Immunology of Diabetes Society T-Cell Workshop: HLA class II tetramer-directed epitope validation initiative

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Abstract

Background Islet-antigen-specific CD4+ T cells are known to promote auto-immune destruction in T1D. Measuring T-cell number and function provides an important biomarker. In response to this need, we evaluated responses to proinsulin and GAD epitopes in a multicentre study.

Methods A tetramer-based assay was used in five participating centres to measure T-cell reactivities to DR0401-restricted epitopes. Three participating centres concurrently performed ELISPOT or immunoblot assays. Each centre used blind-coded, centrally distributed peptide and tetramer reagents.

Results All participating centres detected responses to auto-antigens and the positive control antigen, and in some cases cloned the corresponding T cells. However, response rates varied among centres. In total, 74% of patients were positive for at least one islet epitope. The most commonly recognized epitope was $GAD_{270-285}$. Only a minority of the patients tested by tetramer and ELISPOT were concordant for both assays.

Conclusions This study successfully detected GAD and proinsulin responses using centrally distributed blind-coded reagents. Centres with little previous experience using class II tetramer reagents implemented the assay. The variability in response rates observed for different centres suggests technical difficulties and/or heterogeneity within the local patient populations tested. Dual analysis by tetramer and ELISPOT or immunoblot assays was frequently discordant, suggesting that these assays detect distinct cell populations. Future efforts should investigate shared blood samples to evaluate assay reproducibility and longitudinal samples to identify changes in T-cell phenotype that correlate with changes in disease course. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords T cells; antigens/peptides/epitopes; validation; type 1 diabetes; GAD65; proinsulin

Abbreviations: AB – autoantibodies; AIM-V – serum free cell expansion medium; BDC – Barbara Davis Center; BRI – Benaroya Research Institute; DR – HLA-DR locus; EC – effective concentration; ELISPOT – enzyme-linked immunospot; FACS – fluorescence activated cell sorting; GAD – glutamic acid decarboxylase; GDC – German Diabetes Center; HLA – human leucocyte antigen; HSV – herpes simplex virus; IA-2 – tyrosine phosphatase-2; IC50 – inhibitory concentration: concentration of a peptide that elicits 50% of a maximal functional response; IGRP – islet-specific glucose-6-phosphatase catalytic subunit-related protein; PBMC – peripheral blood mononuclear cell; PBS – phosphate buffered saline; PPI – preproinsulin; proINS – proinsulin; SDS-Page – sodium dodecyl sulfate polyacrylamide gel electrophoresis; T1D – type 1 diabetes; TDEVI – tetramer directed epitope validation initiative

Introduction

There is a clear need for accurate measurements of auto-reactive T-cell activity in T1D. Established genetic markers (including HLA), islet-specific auto-antibodies, and glucose tolerance tests provide standardized criteria for estimating disease risk and defining disease onset [1]. However, none of these markers are well suited for monitoring the auto-immune destruction which precedes diagnosis or the changes caused by therapies intended to prevent or cure the disease. It is well established that CD4+ T cells which recognize islet-antigen epitopes promote auto-immune destruction of pancreatic β cells. In general, such auto-reactive T cells are part of a natural T-cell repertoire, even in healthy individuals who possess one or more of the subsets of HLA class II alleles that are strongly associated with T1D susceptibility [2]. However, islet-specific T cells in patients with T1D have been shown to preferentially carry phenotypic markers of T helper 1, memory, chronic activation, and high avidity [3–7]. Especially in the light of these characteristics, it is considered likely that these auto-reactive T cells mediate islet β-cell damage [8]. Indeed, the contribution of CD4+ T cells to β-cell damage has been clearly demonstrated in the non-obese diabetic mouse and implicated in diabetes pathogenesis in humans [9]. Therefore, assays which measure islet-antigen-specific T-cell number and function in the periphery have the potential to provide a good correlate for pancreatic β-cell destruction.

The strong association of a small number of HLA class II alleles with T1D susceptibility has led to focused investigation of T-cell epitopes by a wide variety of techniques and approaches, utilizing blood samples from human subjects or cells obtained from HLA-transgenic mice, and either whole auto-antigens [10,11] or peptides [12–14]. This has generated a growing array of CD4+ T-cell epitopes from the major islet auto-antigens, which has been summarized in recent publications [8,15,16]. Despite this considerable knowledge, there is currently no standardized assay available to assess T-cell activity against pancreatic antigens. Previous studies have utilized diverse methodologies to measure islet-specific T-cell responses, with some success [17,18]. However, efforts to improve and standardize T-cell assays have in some cases proven more difficult than expected [19,20]. These difficulties prompted one recent report to conclude that the limited reproducibility of current assays and difficulty in distinguishing patients with T1D and healthy controls may simply reflect the low overall frequency of self-reactive T cells and variability in their appearance in peripheral blood [21]. Cumulatively, these findings underscore both the importance and promise of CD4+ T-cell monitoring and the legitimate technical challenges that must be surmounted.

This current report describes a collaborative strategy for epitope validation and assessment of T-cell responses. Its approach is based on independent, blinded tetramer testing combined with local assays performed in multiple laboratories on local blood samples. This study analysed responses to four DRB1*04:01-restricted epitopes from two major auto-antigens in T1D, PPI and GAD, which were chosen using a combination of algorithm prediction, literature survey, and confirmatory HLA-binding assays. In particular, the goal was not to distinguish patients from controls (as was the case in previous studies conducted by the Immune Tolerance Network and TrialNet), but rather to standardize the detection of epitope-specific responses in individual patients.

Methods

Research design

Five participating centres performed HLA class II tetramer analysis and optional local assays (ELISPOT or cellular immunoblot) to measure T-cell reactivity to selected HLA-DRB1*04:01-restricted peptide epitopes. Assays were performed using freshly drawn samples from individual subjects. Peptides and tetramer reagents were distributed by the BRI tetramer core facility in a blind-coded fashion. This code was broken after local testing of each blood samples from patients with T1D and data analysis had been performed.

Human subjects

Adult patients diagnosed with T1D according to the criteria of the American Diabetes Association were recruited with informed consent as part of Institutional Review Board approved studies at each participating centre. Subjects were less than 40 years of age at diagnosis, not obese (body mass index $< 30\ kg/m^2$) and recruited within 5 years of diagnosis. Each subject was confirmed by high-resolution HLA typing to have a DRB1*04:01 haplotype.

Peptide selection and synthesis

Epitope selection was performed by the participating centres based primarily on epitope prediction algorithms and published literature (Table 1). This panel of epitopes was also reviewed and approved by A. Sette and colleagues of the La Jolla Institute of Allergy and Immunology, acting as external independent experts. Approved epitopes included influenza $HA_{306-318}$ (positive control), $HSV_{465-484}$ (negative control), $GAD_{270-285}$, $GAD_{555-567}$, PPI_{90-104}, PPI_{76-90}, and $PPI_{76-90,88S}$. The latter peptide carries an amino acid substitution at position 88 which has been shown to increase its binding affinity for DRB1*04:01, thereby improving the stimulation and detection of low-avidity T cells [7,22]. These peptides were synthesized (Genscript) for evaluation by in vitro HLA-binding affinity measurement.

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Table 1. Selection of DRB1*0401-restricted GAD65 and proinsulin epitopes

Position in protein[a]	Sequence	IEDB score[b] Score[2]	SYFPEITHI score[c] Score[3]	PROPRED score[d] Score[4]	Measured IC_{50} IC_{50} (μM)
$GAD_{555-567}$	VNFFRMVISNPAAT	0.15	28	4.3	0.03
$GAD_{270-285}$	LPRLIAFTSEHSHFSLKK	1.1	26	−0.3	0.11
$PPI_{76-90(C22-A1)}$	SLQPLALEGSLQKRG	3.0	12	1.1	9.1
$PPI_{76-90,\ 88S}$	SLQPLALEGSLQSRG	4.9	12	3.9	0.19
$HA_{306-318}$	PKYVKQNTLKLAT	0.81	14	4.5	0.16

[a]Position of first amino acid of peptide from insulin precursor protein.
[b]IEDB consensus percentile rank of the corresponding 15mer sequence; low rank represents strongly predicted binding.
[c]SYFPEITHI binding score, scores above 21 represent strongly predicted HLA-binding peptides.
[d]PROPRED binding score; highest score achievable by any peptide 8.6.

Class II tetramer reagents

HLA-DRB1*04:01 tetramers were produced by the BRI Tetramer Core Facility as previously described [23]. Briefly, HLA-DRA1/DRB1*04:01 protein was expressed and purified from insect cell culture supernatants. Following *in vitro* biotinylation, class II monomers were loaded with either peptide pools or individual peptides by incubating for 48 h at 37 °C with 25-fold molar excess peptides (total) in phosphate buffer, pH 6.0 in the presence of 0.2% *n*-octyl-D-β-glucopyranoside. Tetramers were formed by incubating class II molecules with phycoerythrin-labelled streptavidin for 6–18 h at room temperature at a molar ratio of 8:1. Tetramer quality was confirmed by staining PPI, GAD65, and haemagglutinin epitope-specific T-cell lines and clones.

HLA class II peptide-binding competition assay

Various concentrations of each peptide were incubated in competition with 0.01 μM biotinylated $HA_{306-318}$ peptide in wells coated with HLA-DRB1*04:01 protein as previously described [24]. After washing, the biotin-haemagglutinin peptide was labelled using europium-conjugated streptavidin (Perkin Elmer) and quantified using a Victor2 D time-resolved fluorometer (Perkin Elmer). Peptide-binding curves were simulated by nonlinear regression with Prism software (Version 4.03, GraphPad Software Inc.) using a sigmoidal dose–response curve. IC_{50} binding values were calculated from the resulting curves as the peptide concentration needed for 50% inhibition of reference peptide binding.

CD4+ T-cell stimulation and tetramer staining

PBMCs were isolated from heparinized fresh blood by Ficoll underlay. CD4+ T cells were isolated from PBMCs using a 'no touch' CD4+ T-cell isolation kit (Miltenyi Biotec), cultured, and analysed by tetramer staining as previously described [25]. CD4+ T cells were seeded in 48-well plates at 2.0×10^6 cells/well in T-cell medium (RPMI-1640 with 10% pooled human serum, L-glutamine, and penicillin/streptomycin) and stimulated with autologous antigen-presenting cells (i.e. the CD4-negative fraction obtained after magnetic sorting) pulsed with 10 μg/mL peptide. After 1 week, a dose (50 μL/well) of T-cell growth factor (Hemagen) was added to each well. Cells were split and fed with fresh T-cell medium and interleukin-2 as needed. On day 12–15, tetramer staining was performed on at least 50 000 cells by incubating 50 μL cell suspension with 1 μL of tetramer-phycoerythrin (10 μg/mL final) for 2 h at 37 °C. After incubation, cells were stained using anti-CD4 and antibodies for other surface markers (such as anti-CD3, CD14, CD19, CD25) for gating and analysis, washed twice with FACS buffer (PBS 1 × +1% foetal bovine serum + 0.1% sodium azide) and analysed by flow cytometry. A positive tetramer response was defined as ≥1% (i.e. twice the staining level of the negative control tetramer).

CD4+ T-cell cloning

Tetramer-positive cells were single-cell sorted in 96-well round bottom plates, each well containing 10^5 non-DR-matched irradiated PBMCs in AIM-V medium (Invitrogen). The following day, T-cell growth factor (10 U/mL) and phyto-haemagglutinin-P (5 μg/mL) were added. After 10 days, a second stimulation cycle was performed by adding 10^5 DR*0401+ irradiated PBMCs pulsed with 10 μg/mL peptide to each well. T-cell growth factor was added again after 24 h and cells re-fed and/or expanded as needed before tetramer staining at day 20.

ELISPOT assays

ELISPOT assays were performed as previously described [26–28]. Briefly, 2×10^6 PBMCs per well were stimulated with 10–20 μmol/L peptide, dimethyl sulfoxide (assay negative control), or 0.16 units of tetanus toxoid (assay positive control) in 48-well plates in 0.5 mL media (RPMI-1640 medium supplemented with antibiotics and 10% human AB serum) and incubated at 37 °C, 5% CO_2 with plates inclined at 5°, fed with 0.5 mL pre-warmed medium with 10% AB serum after 24 h. ELISPOT plates

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were pre-coated with anti-interferon-γ capture antibody (U-Cytech) and blocked with 1% bovine serum albumin in PBS. After 2 days of culture, non-adherent cells were resuspended at a concentration of 10^6 cells/300 μL in pre-warmed medium with 2% AB serum, washed, and plated in triplicate into wells of 96-well Nunc Maxisorp plates at 100 μL/well. Plates were incubated at 37 °C, 5% CO_2 for 18 h, manually washed three times in PBS followed by eight times in PBS/Tween-20 using an automated plate washer, and spots developed according to the manufacturer's instructions (U-Cytech). Developed plates were dried and spots of 80–120 μm diameter counted using a BioReader 4000 (BioSys, Karben, Germany). Response indexes were calculated as the number of spots of experimental wells/number of spots in background wells (media alone).

Cellular immunoblotting

Cellular immunoblotting was performed as previously described. Briefly, normal human islet cell preparations (from the National Institute of Health Islet Cell Resource Centers) were subjected to preparative one-dimensional 10% SDS-PAGE and electroblotted onto nitrocellulose. The nitrocellulose was cut into molecular weight regions (blot sections) and then solubilized to form nitrocellulose particles. The nitrocellulose particles containing islet proteins were used to stimulate PBMC, at a concentration of 3.5×10^5 cells/well. To control for inter-assay variation of the islet antigen preparations, the quantity and quality of islets were held constant among preparations and new antigen preparations were compared with and run alongside older preparations. Results were reported as the number of blots with positive responses determined to be T-cell proliferative responses to >4 blot sections [27,29].

Results

Confirmation of peptide and tetramer reagents

On the basis of multiple epitope prediction methods and a review of the pertinent literature, a panel of candidate epitopes derived from PPI and GAD was selected (Table 1). The selected peptides were synthesized and tested for their ability to bind to DRB1*0401 in an *in vitro* binding assay. As summarized in Figure 1A, while the PPI_{90-104} peptide did not bind to DRB1*0401 with appreciable affinity, the remaining peptides bound with measurable affinities. The observed IC_{50} values varied, but all were within the appropriate range for producing stable HLA class II tetramers. These peptides were used to generate HLA class II tetramer reagents. The quality and specificity of these tetramer reagents were confirmed by staining PPI and GAD epitope-specific T-cell lines and clones (Figure 1B). These peptides and tetramers were divided into coded aliquots, distributed to each participating centre and utilized in the tetramer assay and laboratory-specific 'local' T-cell assays (summarized in Table 2) to assess islet-specific T-cell responses using local blood samples from patients with T1D. Further documenting staining specificity, tetramer-positive cells detected after peptide-specific stimulation were in some cases sorted and cloned at the INSERM U986 in Paris, leading to CD4+ T cells recognizing the original stimulating peptide (Figure 1C).

HLA class II tetramer assays

Among the participating centres, samples from a total of 38 patients with T1D were analysed using tetramers. For this analysis, a positive tetramer response was defined as $\geq$1% (i.e. greater than or equal to twofold the staining level of the negative control tetramer). Figure 2A summarizes the individual responses for each subject. The most commonly recognized epitope was $GAD_{270-285}$, with 45% (17/38) of the patients reacting, followed by $GAD_{555-567}$, recognized by 37% (14/38), and PPI_{76-90} recognized by 34% (13/38). As shown in Figure 2B, 29% of the patients (11/38) reacted only to GAD epitopes, 16% of the patients (6/38) reacted only to PPI epitopes, and 29% of the patients (11/38) reacted to the epitopes of both auto-antigens. Taking all epitopes together, 74% (28/38) patients responded to at least one islet epitope. Surprisingly, the positive control $HA_{306-318}$ antigen was recognized by only 58% (22/38) of the patients. However, this was not a result of an overall failure of the tetramer assay because 11 of the 16 patients who failed to respond to $HA_{306-318}$ did respond to at least one self-epitope. As shown in Figure 3, some notable differences in response rate were observed between the participating centres. $GAD_{270-285}$ had the most variation in response rate for individual centres, ranging from 0% (University of Washington) to 100% (BDC). Similarly, responses to the positive control $HA_{306-318}$ antigen ranged from 20% (BDC) to 90% (BRI).

ELISPOT assays

In two of the participating centres, samples from a total of ten patients with T1D were tested by interferon-γ ELISPOT in conjunction with class II tetramer staining. A summary of these ELISPOT results is shown in Table 3. A positive ELISPOT response was defined as greater than or equal to twofold the background response similarly to the cutoff used for tetramer assays. In agreement with the tetramer assay data, the most commonly recognized epitope in the ELISPOT was $GAD_{270-285}$, recognized by 30% (3/10) of the patients. Responses to the remaining epitopes were less frequent. $GAD_{555-567}$ was recognized by 10% (1/10) of the patients. While the $PPI_{76-90,88S}$ agonist peptide was recognized by 20% (2/10) of the patients, the wild-type PPI_{76-90} was not recognized by any. In close agreement with the tetramer results, the

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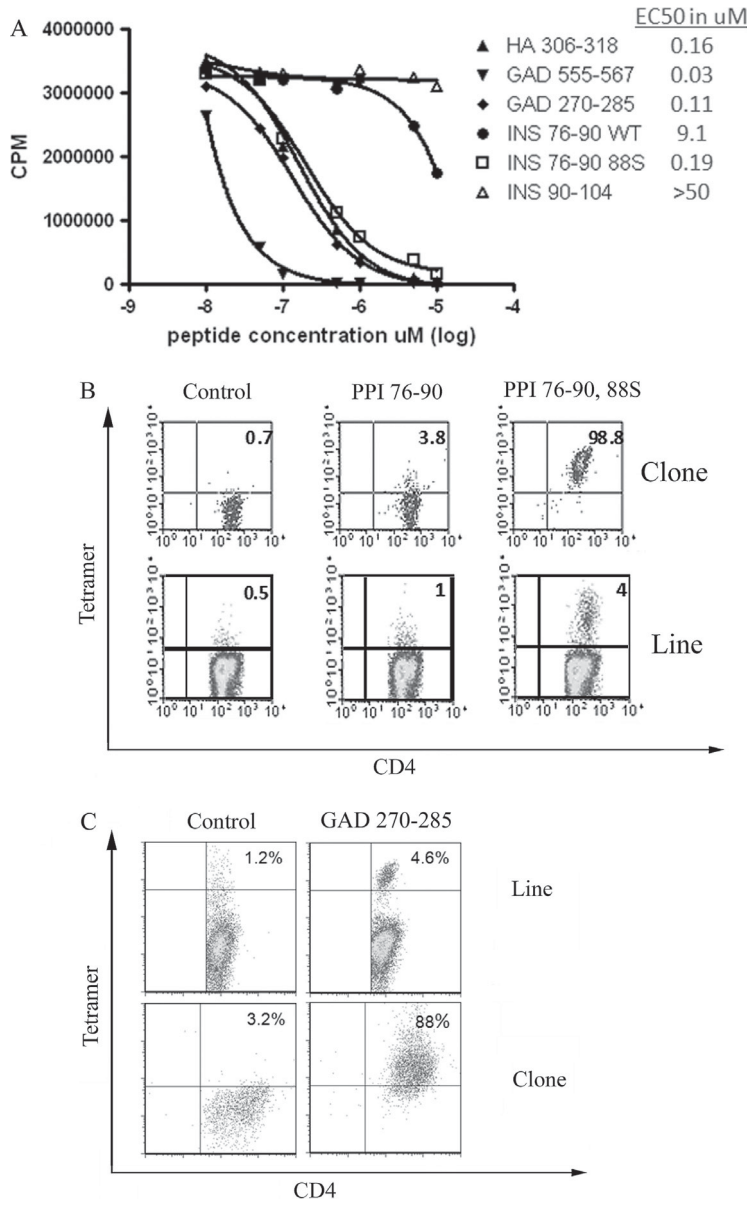

Figure 1. Peptide binding and tetramer quality. (A) The binding of each peptide to DRB1*04:01 was measured in an *in vitro* competition assay. Increasing concentrations of each peptide displaced the labelled reference peptide, allowing calculation of EC_{50} values for each peptide (with the exception of PPI_{90-104}, which failed to bind). (B) Quality of the tetramer reagents was verified by staining T-cell clones and lines. In this example, a PPI_{76-90}-specific clone (upper panels) and line (lower panels) were stained using streptavidin-phycoerythrin-labelled tetramers loaded with wild-type or 88S substituted peptide. (C) $GAD_{270-285}$ and control tetramer staining of a T-cell line after $GAD_{270-285}$-specific stimulation (first row) and a T-cell clone following sorting and expansion of tetramer-positive cells (second row)

positive control $HA_{306-318}$ antigen was recognized by 50% (5/10) of the patients.

Cellular immunoblot assays

One of the participating centres performed cellular immunoblot assays on samples from five out of eight patients in conjunction with their tetramer analysis (for the remaining samples there were insufficient cells for both assays). Although the immunoblot assay does not measure a precise, known specificity, this assay provides a sensitive and accurate assessment of T-cell auto-reactivity [17]. Of the five subjects tested, four had positive immunoblot scores.

Concordance of class II tetramer assays and local assays

A total of 15 subjects were concurrently tested in class II tetramer assays and a local T-cell assay. As shown in the upper portion of Table 4, ten subjects were tested by ELISPOT and five subjects were tested by immunoblot. For the control haemagglutinin epitope, five subjects had positive ELISPOT results, while three had a positive

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Table 2. Centres participating in TDEVI class II study

Centre	Code	Subjects	Local assay
University of Washington, DVA Puget Sound Health Care System, Seattle, WA B. Brooks-Worrell	UW	8	Cellular immunoblot
Benaroya Research Institute, Seattle, WA I. Durinovic-Belló, E. James	BRI	10	Not done
Barbara Davis Center, Denver, CO P. Gottlieb	BDC	5	ELISPOT
INSERMU986, DeAR Lab *Avenir*, St Vincent de Paul Hospital, Paris, France R. Mallone	INSERM	10	Not done
German Diabetes Center, Heinrich-Heine University, Düsseldorf, Germany N. Schloot	GDC	5	ELISPOT

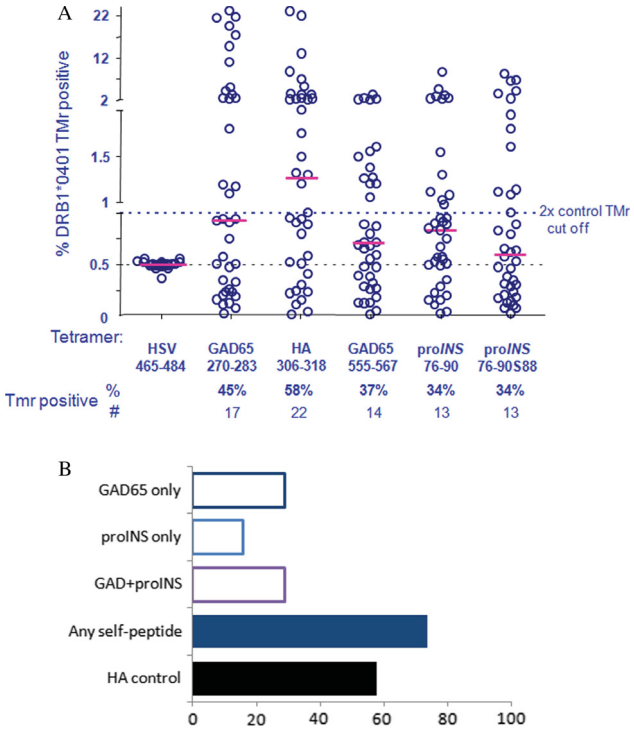

Figure 2. Tetramer-positive responses to islet epitopes (A). Individual responses for 38 patients with T1D analysed by DRB1*04:01 tetramer staining after *in vitro* stimulation with GAD65 and PPI epitopes, along with a positive control ($HA_{306-318}$) epitope. Irrelevant tetramer staining (indicated by the dashed line) was adjusted to 0.5% for each sample. Responses more than twofold the negative control (≥1%) were considered positive (indicated by the dotted line). For each column, the horizontal line represents the median staining for all samples. The percentage and number of tetramer-positive responses were as indicated. (B) Response rates to GAD65 and proINS epitopes for all patients with T1D. Open bars indicate the percentage of the patients who responded to GAD65 epitopes only (dark blue), proINS epitopes only (light blue), or to both GAD65 and proINS epitopes (purple). Solid bars indicate the percentage of the patients that responded to any self-peptide (dark blue) or the haemagglutinin control (black)

tetramer result. Surprisingly, only one sample was jointly $HA_{306-318}$ positive by tetramer and ELISPOT. Although the ELISPOT had a higher detection rate for the control antigen, tetramers had a higher detection rate for β-cell epitopes. For β-cell epitopes, all the ten subjects had at least one positive tetramer result, while three out of ten tested by ELISPOT had a positive result. Looking at individual β-cell epitope responses, 25 out of 40 pairs of assay measurements were jointly positive or negative, a total concordance rate of 62.5%. However, considering only positive results, only 6 out of 30 assay measurements were jointly positive, indicating a positive concordance rate of only 20%. Among discordant measurements, 13 were tetramer positive and ELISPOT negative while 2 were ELISPOT positive and tetramer negative. As shown in the lower portion of Table 4, four of the five subjects tested by immunoblot had positive responses. Among these, one had a positive tetramer result for a β-cell epitope. However, four of the five subjects responded to the control haemagglutinin epitope, suggesting that the negative responses were not a result of failure of the tetramer assay.

Discussion

This study utilized independent, blinded tetramer testing in combination with local assays performed in multiple laboratories to assess T-cell responses in subjects with T1D. In contrast to other recent efforts, our objective was to standardize the detection of epitope-specific responses in individual patients, rather than distinguishing patients from controls. For both logistical and technical reasons, assays were performed using fresh, local blood samples. This study design had inherent limitations, most notably difficulties in evaluating assay reproducibility and estimating the rate of false-positive and false-negative results. In spite of these limitations, this study provided an opportunity to advance the field by assessing the feasibility of conducting a standardized assay across multinational centres. The most prominent success of this study was the successful distribution of blinded, centralized reagents to facilitate testing in local centres. The implementation of a complex, multistep T-cell assay in multiple laboratories with little or no previous experience was ambitious and to some degree fell short of our best hopes for the project. Because the study design did not utilize shared samples, the current results leave room for doubt about whether the tetramer assay was performed with equal success in each laboratory. However, each of the participating centres was able to detect responses both to auto-antigens

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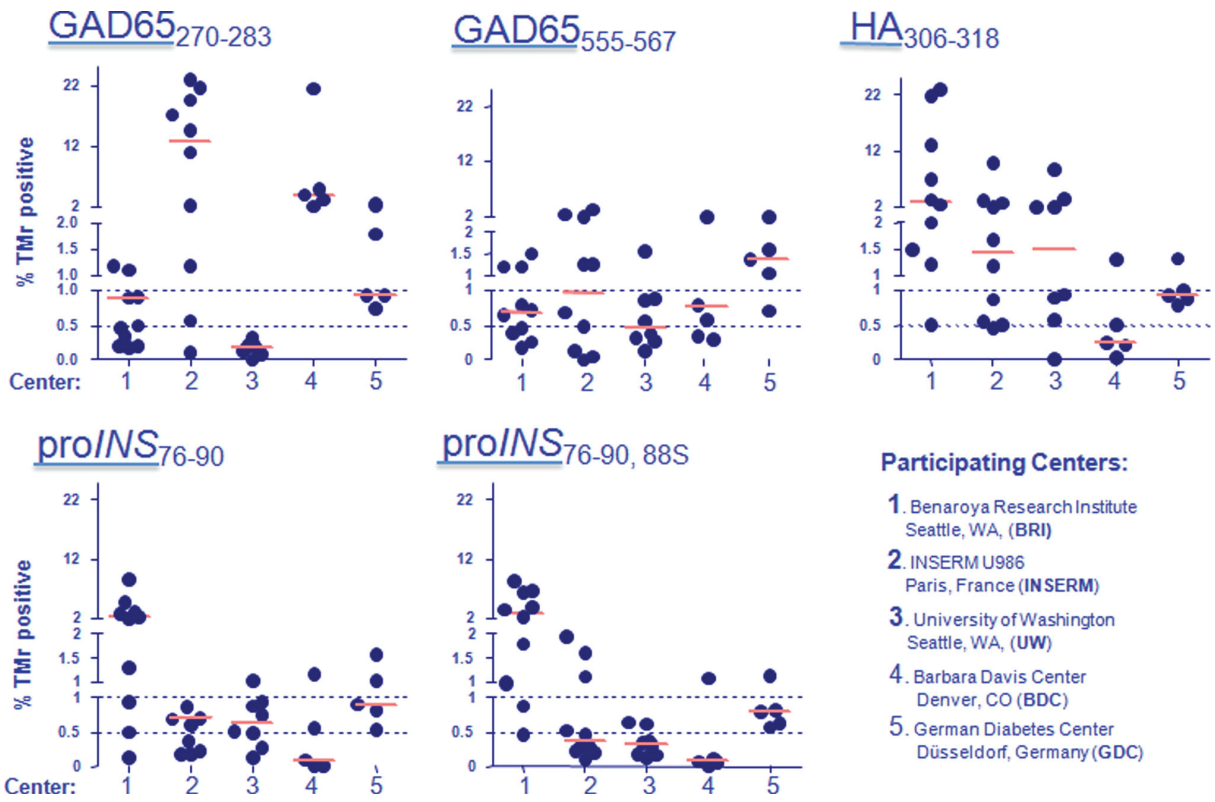

Figure 3. TDEVI class II tetramer results by each participating Centre. T-cell responses analysed by DRB1*04:01 tetramer staining by each participating centre (numbered as indicated in the inset). For each subject, irrelevant (HSV control) tetramer staining (indicated by the dashed line) was adjusted to 0.5% for each sample. Responses more than twofold the negative control (≥1%) were considered positive (indicated by the dotted line). For each column, the horizontal line represents the median staining for all samples

Table 3. ELISPOT assay results

	$HA_{306-318}$	$GAD_{270-285}$	$GAD_{555-567}$	PPI_{76-90}	$PPI_{76-90,88S}$
Positive	5	3	1	0	2
Negative	5	7	9	10	8
Response rate (%)	50	30	10	0	20

and the positive control antigen using tetramers. The majority of these subjects (74% of patients) responded to at least one β-cell epitope. Responses to the most prevalent epitope ($GAD_{270-285}$) were seen in 45% of the subjects tested. These results are similar to a recent single-centre study, in which 61% (11/18) of patients with T1D were positive for one of three GAD65 or PPI-containing tetramers [30]. Surprisingly, 40% of the subjects failed to respond to the positive control haemagglutinin epitope in spite of the fact that this is reported to be an immunodominant epitope. These results could indicate that some assay results were false negatives. Alternatively, some subjects could lack T-cell responses to this epitope because of the lack of recent influenza immunization or exposure to one of the H3N2 strains that correspond to this epitope. In any case, the inclusion of additional epitopes (such as conserved epitopes from the structural proteins of influenza) will likely be necessary to achieve complete coverage for all subjects with DRB1*04:01 haplotypes.

While each centre successfully detected auto-reactive T cells using tetramers, response rates for each epitope varied significantly among centres. Because assays were performed using fresh, local blood samples, it was not possible to conclude whether these response rates result from lack of assay reproducibility, sampling variation because of the low overall frequency of self-reactive T cells, variability in their appearance in peripheral blood, and/or real differences in the patient populations tested by centres in different geographic locations. These are important questions that should be specifically addressed in follow-up studies. Clearly, these future studies should utilize shared, distributed PBMC samples in order to effectively resolve these questions.

Among the subset of patients tested by ELISPOT, 56% (10/18) of the subjects tested had at least one positive result for a β-cell epitope and 94% (17/18) had at least one positive tetramer result. At the level of single epitopes, the total concordance of individual assay measurements (results that were jointly positive or negative) was 62.5%

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Table 4. Concordance of tetramer and local assay results[a]

	$HA_{306-318}$		$GAD_{270-285}$		$GAD_{555-567}$		PPI_{76-90}		$PPI_{76-90,88S}$		
Subject	Tmr	ELISPOT	Tmr	ELISPOT	Tmr	ELISPOT	Tmr	ELISPOTq	Tmr	ELISPOT	Immunoblot
GDC-1	−	+	−	−	−	−	+	−	−	−	Not done
GDC-2	+	−	+	−	+	−	−	−	−	−	Not done
GDC-3	−	+	−	−	+	−	−	−	−	−	Not done
GDC-4	+	−	+	−	+	−	+	−	+	−	Not done
GDC-5	−	−	−	−	+	−	−	−	−	−	Not done
BDC-1	−	−	+	−	−	−	−	−	−	−	Not done
BDC-2	+	+	+	+	+	−	+	−	+	+	Not done
BDC-3	−	+	+	+	−	−	−	−	−	−	Not done
BDC-4	−	−	+	−	−	−	−	−	−	−	Not done
BDC-5	−	+	+	+	−	+	−	−	−	+	Not done
University of Washington −1	−	Not done	−	Not done	−	Not done	−	Not done	−	Not done	+
University of Washington −2	+	Not done	−	Not done	−	Not done	+	Not done	−	Not done	+
University of Washington −3	+	Not done	−	Not done	−	Not done	−	Not done	−	Not done	−
University of Washington −4	+	Not done	−	Not done	−	Not done	−	Not done	−	Not done	+
University of Washington −5	+	Not done	−	Not done	−	Not done	−	Not done	−	Not done	+

[a]+ indicates a positive response, − indicates a negative response, discordant results shown in boldface.

(25/40). However, accounting for positive results only the concordance was only 20% (6/30). This rate of agreement for positive results is quite low and raises questions about the combined false-positive and false-negative rate of the two assays. However, some of the observed discordance may have a biological basis. Among discordant measurements, 13 were tetramer positive and ELISPOT negative. Notably, the tetramer assay includes a 14-day amplification culture, while the ELISPOT assays utilize a shorter 2-day culture to activate the cells without expanding them. Given this technical difference, it is not unexpected that the tetramer assay would be able to uncover responses that could not be detected by ELISPOT assays. Independent of this, at least some samples that were tetramer positive and ELISPOT negative may contain high avidity islet-reactive cells that do not produce interferon-γ. Samples such as the two that were ELISPOT positive and tetramer negative may contain low avidity epitope-reactive T cells or epitope-reactive T cells that are restricted by an HLA other than DRB1*04:01.

Although relatively few subjects were tested, the significant discordance between tetramer and immunoblot assay results was surprising. The specificity of cellular immunoblotting for the detection of T-cell responses to islet proteins from diabetic patients has been previously validated in two separate workshops conducted by the Immune Tolerance Network and TrialNet [17,21]. One possibility is that the epitopes responsible for the positive immunoblot seen in these subjects were disparate from those tested using tetramers. This explanation is plausible as the immunoblot assesses responses to all possible islet proteins, whereas the tetramer assay measures only the responses to specific epitopes within proinsulin and GAD65. Alternatively, it is possible that some of these tetramer results may have been false negatives. However, it should be noted that several other subjects tested by that centre did have positive tetramer responses.

In spite of its limitations and differences, our study demonstrated the feasibility of distributing centrally blinded reagents to interrogate T-cell responses in patients with T1D. The discordant results observed for the various assays employed indicate that assay reproducibility and biological variation because of the low frequency of self-reactive T cells are important issues that must be addressed through studies that utilize shared, distributed blood samples. In addition, it is likely that multiple epitopes will be needed for each class II allele to approach complete patient coverage. This should be feasible through the inclusion of additional epitopes from other known islet antigens such as IA-2 and IGRP. Such a panel used in the correct assay or combination of assays would be extremely useful for immune monitoring studies examining the frequency and phenotype of islet-specific T cells in subjects with T1D and auto-antibody positive individuals over time or in response to therapy. Even taking into account the likelihood of false-positive and false-negative results for the various assay methods used, our results suggest

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that each assay probably detects a different subset of auto-reactive T cells; therefore, it may be important to utilize multiple assays to obtain a robust understanding of auto-reactive T-cell behaviour. Naturally, efforts moving forward should include optimization of existing assays and the development of novel assays with improved sensitivity and specificity. Given the low overall frequency of self-reactive T cells and possible variation in their appearance in peripheral blood, repeated sampling may also be important. It would be of particular interest to investigate assays in a longitudinal study to identify clear shifts in the phenotype of auto-reactive T cells that precede disease onset. These could include imbalances in particular T-cell subsets, variations in the magnitude or character of cytokine responses, or other changes related to T-cell activation or homing. Such studies are likely to provide important insights into disease mechanisms and opportunities for monitoring disease progression and therapeutic intervention by identifying changes in T-cell phenotype that correlate with changes in disease course.

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Conflict of interest

None declared.

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Chapter 7

Comparison of Cryopreservation Methods on T cell Responses to Islet and Control Antigens from Type 1 Diabetes Patients and Controls

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RESEARCH ARTICLE

Comparison of cryopreservation methods on T-cell responses to islet and control antigens from type 1 diabetic patients and controls

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Abstract

Background Type 1 diabetes (T1D) is a cell-mediated autoimmune disease characterized by destruction of the pancreatic islet cells. The use of cryopreserved cells is preferable to the use of freshly isolated cells to monitor clinical trials to decrease assay and laboratory variability.

Methods The T-Cell Workshop Committee of the Immunology of Diabetes Society compared two widely accepted T-cell freezing protocols (warm and cold) to freshly isolated peripheral blood mononuclear cells from patients with T1D and controls in terms of recovery, viability, cell subset composition, and performance in functional assays currently in use in T1D-related research. Nine laboratories participated in the study with four different functional assays included.

Results The cold freezing method yielded higher recovery and viability compared with the warm freezing method. Irrespective of freezing protocol, B cells and CD8+ T cells were enriched, monocyte fraction decreased, and islet antigen-reactive responses were lower in frozen *versus* fresh cells. However, these results need to take in to account that the overall response to islet autoantigens was low in some assays.

Conclusions In the current study, none of the tested T-cell functional assays performed well using frozen samples. More research is required to identify a freezing method and a T-cell functional assay that will produce responses in patients with T1D comparable to responses using fresh peripheral blood mononuclear cells. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords T cells; proliferation; ELISPOT; tetramer; cryopreservation; CFSE; islet antigens; type 1 diabetes

Introduction

The pathogenesis of type 1 diabetes (T1D) is believed to be T-cell-mediated, as T cells, but not autoantibodies, are necessary to transfer disease in animal models and human T1D [1–3]. Therefore, having T-cell assays to complement studies into the pathogenesis of T1D and clinical trials investigating potential therapies for T1D would be desirable. However, to date, the only T-cell assays validated in blinded multi-centre workshops for use in distinguishing T-cell responses from patients with T1D compared with controls [4,5] have involved the use of freshly isolated peripheral blood mononuclear cells (PBMCs) from patients with T1D. Using freshly isolated PBMCs in clinical trials is logistically difficult and potentially introduces inter-laboratory and inter-assay variability.

Cryopreservation of cells from patients in clinical trials would ensure that longitudinal patient samples could be run in the same

assay in the same laboratory, thus decreasing introduction of laboratory and assay variability. Unfortunately, throughout the literature, significant differences between fresh and frozen PBMCs have been documented although the extent of the changes is highly dependent on the antigenic stimulus, cytokine response tested, and the patient population [6]. In T1D studies, the effects of freezing using a multiplex enzyme-linked immunoassay (ELISA) have demonstrated that frozen PBMCs spontaneously secrete higher levels of interleukin-6, interferon (IFN)-γ, interleukin-10, interleukin-12, interleukin-13, and monocyte chemoattractant protein-1 compared with fresh cells from the same patients [7]. Using an IFN-γ CD8+ T-cell enzyme-linked immunospot (ELISPOT), Mallone *et al*. [8] demonstrated that basal IFN-γ secretion was much higher in cryopreserved PBMCs *versus* fresh PBMCs; however, the net antigen-specific CD8+ T-cell responses remained comparable between fresh and frozen cells.

In 1999, the Cryopreservation Working Group of the Pediatric Human Immunodeficiency Virus Clinical Trials Group was formed to optimize methods of cryopreserving PBMCs, adapting immunologic assays to frozen cells, and establishing quality control parameters for immunologic assays with cryopreserved PBMCs [9]. Although this group was formed primarily to optimize processing methods of cryopreserved cells for use with human immunodeficiency virus-infected patients, this group observed that PBMCs have stable viability and function over prolonged periods of time when adequately stored. However, they also observed differences in function and distribution of T-cell subpopulations between fresh and frozen PBMCs [9].

In 2010, the Immunology of Diabetes Society T-Cell Workshop Committee undertook a similar approach to optimize methods for cryopreserving PBMCs for use in T1D-related T-cell studies and trials. Our approach was to compare fresh and frozen PBMCs from patients with T1D and normal controls using various assays currently utilized in T1D-related research [10]. PBMCs were isolated from patients with T1D and cryopreserved using both a 'cold' or a 'warm' method for comparison. After a month, the frozen cells were thawed and the responses of the frozen cells using the two freezing methods were compared with fresh PBMCs from the same subjects. Comparisons were made using carboxyfluorescein succinimydyl ester (CFSE) proliferation assay, cellular immunoblot, ELISPOT, and a human leucocyte antigen class II tetramer (TMr) assay [10]. PBMC recovery, viability, and cell subset distribution were also compared.

Methods

Subjects

Each participating laboratory obtained blood from up to six patients with T1D diagnosed within 3 years and six healthy age- and sex-matched controls. Samples were obtained by each individual laboratory through informed consent obtained under the approval of local Ethics committees.

Isolation of PBMCs

PBMCs were isolated from whole blood using density gradient centrifugation [6] and divided into three aliquots. One aliquot was immediately subjected to a functional T-cell assay, while the other two aliquots were frozen using either the 'cold' or 'warm' protocol. Fresh and thawed specimens were counted and viability assessed by Trypan blue dye exclusion. PBMC separation and freeze–thaw procedures were performed identically in all participating laboratories, following a centrally distributed standard operating procedure.

Freeze–thaw protocols

The two freezing protocols studied differed by the temperature of the freezing medium used (ice-cold *versus* room temperature; hereby referred to as 'cold' and 'warm'). Freezing medium was human AB serum and dimethyl sulfoxide (DMSO) at a final 10% concentration and was centrally distributed. Cryovials yielding cell recoveries <50% at thawing were excluded from analysis along with the corresponding fresh samples.

Cold freeze–thaw

All steps of the 'cold' protocol were performed on ice and all cryovials were cooled prior to placing cells into them. Cells were resuspended to 20×10^6 cells/mL in cold freezing medium A (100% human pooled AB+ sera; PAA Laboratories, Yoevil, UK). The cells were mixed gently by tapping the tube to ensure single cell suspension. Drop by drop an equal volume of freezing medium B (80% human pooled AB+ sera/20% Hybri-Max® DMSO; Sigma-Aldrich, Dorset, UK) was added to a final cell concentration of 10×10^6 cells/mL. The PBMCs were then aliquoted into cryovials on ice. The cryovials were placed into a Nalgene Cryofreezing Container with isopropanol. The container was placed into a $-80\,^{\circ}$C freezer for a minimum of 12–18 h before cryovials were transferred into liquid nitrogen. Samples remained in liquid nitrogen for at least 1 month after which the cryotubes were transferred to a $37\,^{\circ}$C water bath and gently agitated until the contents are almost thawed. One millilitre (mL) of complete medium + 10% human AB serum was added dropwise to the vial. The cells were transferred to a 15-mL sterile tube and complete medium + 10% human AB serum was added dropwise. Cells were centrifuged, washed with medium + 10% serum, and resuspended at 4×10^6 cells/mL. Cells were allowed to rest for 1 h prior to fluorescence activated cell sorting (FACS) analysis or placing into functional assays. One participating laboratory further tested a cold freeze–thaw protocol using serum-free AIM-V medium

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(Medium, Invitrogen, Life Technologies, Carlsbad CA) with 10% DMSO for freezing and AIM-V alone for washing steps at thawing [11].

Warm freeze–thaw

The warm freeze method was the same as stated above for the cold freezing method with the exception that the freezing medium A was brought to room temperature prior to use. This method was adapted from the protocol published on the Immune Tolerance Network website (www. immunetolerance.org/sites/files/ITN_protocol_PBMC-CPT). Also, for the warm freeze method, after the cryotubes were removed from liquid nitrogen, they were placed in a 37 °C water bath. When the tubes started to thaw, their contents were placed into a tube containing four volumes of complete medium with 10% human AB serum at room temperature. The AB serum used was from a single batch and centrally distributed. The cells were centrifuged, washed, and allowed to rest for 1 h prior to FACS analysis or placing into functional assays.

PBMC phenotyping

A small aliquot of the isolated PBMCs (fresh or thawed) was stained using antibodies specific to CD4, CD3, CD8, CD19, CD14, and CD56 and subjected to FACS acquisition. Staining, acquisition, and analyses were performed according to centrally distributed standard operating procedures.

Antigens

Antigens were blinded in a central laboratory and distributed back to the laboratories prior to testing. Antigens used for the ELISPOT and CFSE assays were Pediacel (Sanofi Pasteur Ltd, Toronto, Canada), a pentavaccine consisting of purified diphtheria toxoid, tetanus toxoid, acellular pertussis, inactivated poliovirus, and *Haemophilus influenza* type b polysaccharide at 0.2 and 2.0 μg/mL, as well as peptides or whole proteins derived from glutamic acid decarboxylase autoantibody (GAD)65 (Diamyd Medical AB, Stockholm, Sweden), proinsulin, insulin, insulinoma antigen 2 autoantibody (IA2), and heat-shock protein-60 (HSP-60). Antigenic peptides used in the HLA-DR0401 TMr assays were preproinsulin (pro*INS*PPI) 76–90, PPI pro*INS* 76-90S88 (agonist) 78(88S), GAD65 270–283, GAD65 555–567, and the recall antigen influenza A haemagglutinin 306–318. Laboratories used their own antigens in concentrations that had been applied for previous studies [10].

Blinding of antigens

Test antigens have been blinded centrally. Blinding was achieved using randomly generated codes. Along with the blinded antigens a central negative control phosphate buffered saline (PBS) was blinded in the same fashion and provided to each lab.

TMr class II assay

The TMr class II assay was performed as previously described [6,10,12–15]. In brief, CD4+ T cells were isolated from PBMC using Miltenyi-negative selection microbeads followed by 14-day culture with autologous antigen-presenting cells (the CD4-negative fraction after magnetic sorting) pulsed with peptides and expansion of the cultures with addition of interleukin-2 (Hemagen) as described [12,14]. TMr staining was performed on day 14. Expansion of CD4+ cells for class II TMr detection has been shown to be necessary because of their low frequency in peripheral circulation [15]. Results are reported as: percent $CD4^+$ T cells specific for TMr. TMr+ responses were defined as two times the response of negative TMr (negative quadrants adjusted to set TMr− to 0.5%).

Cellular immunoblot assay

In brief, cellular immunoblot was performed as described [6,10,16,17]. Normal human islet cell preparations were subjected to preparative one-dimensional 10% SDS-polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose, the nitrocellulose cut into molecular weight regions (blot sections), and then solubilized to form nitrocellulose particles. The nitrocellulose particles containing islet proteins were used to stimulate PBMCs, at a concentration of 3.5×10^5 PBMCs per well. Human pancreatic islets were obtained from the National Institutes of Health supported Islet Cell Resource Centers. The specificity of the T-cell responses to islet proteins from diabetic patients has been demonstrated previously [16,18–20]. Cellular immunoblot has been previously validated in two separate workshops conducted by the Immune Tolerance Network [18] and TrialNet [19]. These two workshops documented a sensitivity for separating patients with T1D from controls of 94 and 74% and a specificity of 83 and 88%, respectively. Results are reported as number of blots recognized by T cells, and positive responses defined as proliferation to $\geq$4 blot sections [17].

CFSE proliferation assay

CFSE assay was performed as previously described [6,10]. In brief, PBMCs were diluted to 10×10^6/mL and an aliquot set apart for use in establishing optimal flow cytometry settings. The remaining cells were placed into a 50-mL conical bottom tube, CFSE added to a final concentration of 0.1 μM. The cells were incubated for 5 min at 37 °C, after which 5 mL of serum-containing culture media was added. The cells were then resuspended to 2.0×10^6 PBMC/mL in culture

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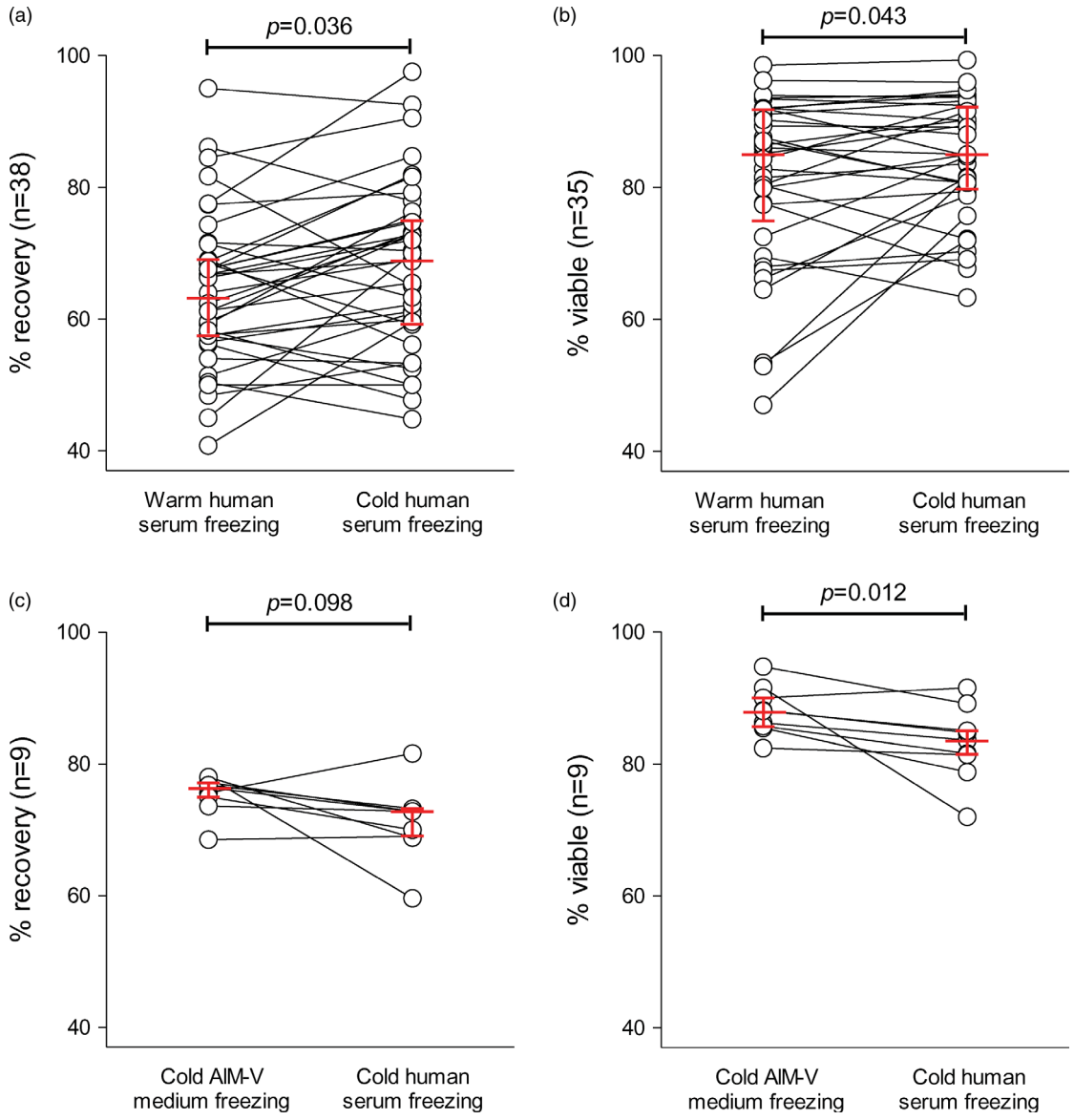

Figure 1. Percent recovery (A) and viability (B) of frozen peripheral blood mononuclear cells (PBMCs) for warm and cold freeze–thawing protocols compared with freshly isolated PBMCs. Percent recovery (C) and viability (D) of frozen PBMCs for cold AIM-V and cold human serum freeze–thawing protocols compared with freshly isolated PBMCs. Pooled data from patients with type 1 diabetes and healthy controls are presented, as no difference was observed between the two groups. Red bars show the median and inter-quartile range for each distribution. *P* values comparing different protocols are given

medium and 1.5–4.5 mL of cell suspension was added to tubes for antigen stimulation and incubated for 7 days at 37 °C 5% CO_2. After 7 days, the supernatant was discarded, fluorochrome-labelled monoclonal antibody against human CD4 added, and the cells incubated on ice for 15–25 min. The cells were then washed once in 1 mL 0.1% bovine serum albumin/PBS, resuspended in 400 µL 0.1% bovine serum albumin/PBS, 1 µL of propidium iodide (0.1 mg/mL in PBS) added and the samples analysed. The magnitude of the response can be expressed as the number of cells that have proliferated (diluted their CFSE) for each treatment, or expressed as the ratio of the number of cells that have proliferated in response to antigen: the number of cells that have proliferated in the absence of antigen for the same number of $CFSE^{bright}$ $CD4^+$ T cells. Results are reported as cell division index, i.e. the ratio of $CFSE^{dim}$ cells with and without antigen.

CD4+ T-cell ELISPOT

ELISPOT was performed as previously reported [6,10,21–24] for IFN-γ and interleukin-13. In brief, PBMCs are cultured in 48-well plates at a density of 2×10^6 in 0.5-mL RPMI-1640 medium (Gibco, Life Technologies, Carlsbad CA) with antibiotics (Antibiotic-Antimycotic, Gibco) and 10% human AB serum (Harlan Sera-Lab, Leicestershire, UK) together with 10–20 µmol/L peptide and incubated at 37 °C,

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5% CO_2 with the culture plates tilted at 5 °C. Control wells contain culture medium together with an equivalent amount of peptide diluent (DMSO) as a negative assay control, or 0.16 IU of tetanus toxoid (Diftavax, Aventis Pasteur MSD, UK), as a positive assay control. The next day (day 1), 0.5 mL of pre-warmed medium with 10% AB serum was added. After another 24 h, non-adherent cells were resuspended at 10^6/300 µL and plated in triplicate into wells of 96-well ELISA plates (100 µL/well; Nunc Maxisorp; Merck, Poole, UK) pre-blocked with 1% bovine serum albumin in PBS and coated with anti-IFN-γ or anti-interleukin-13 capture antibody (U-Cytech, Utrecht, The Netherlands). After 18-h incubation at 37 °C, 5% CO_2, the ELISA plates were washed, spots developed, and counted. The plates are dried and spots of 80–120 µm diameter are counted in a BioReader 4000 (BioSys, Karben, Germany). Responses are shown as stimulation index (number of spots of experimental wells/number of spots in background wells). Polyclonal stimulation was evaluated using PMA and PHA, control recall antigens were Measles vaccine and tetanus toxoid. Test antigens used in this assay were GAD 4.11, IA2-R2, DiaPep277, proinsulin (C19-A3), IA2, GAD65, IA-2 (709–736), IA-2 (752–775), and HSP-60. Antigens used for the ELISPOT were prepared in Dusseldorf and distributed centrally (Pedicel 0.2, Pedicel 2.0, PMA/ionomycin, PBS). Other antigens were provided by Peptor Ltd (HSP-60, DiaPep277) or were prepared in Leiden (GAD 4.11, IA2R2 according to M. Peakman). Background was calculated using wells containing cells alone with media.

Statistics

Results were sent to a central laboratory with data locked prior to revealing the identity of antigens used. Paired Student's *t*-test and Wilcoxon matched pairs test were used to determine significant differences between groups, according to sample size.

Results

Cell recovery and viability

Results from samples of T1D and healthy subjects did not show significant differences and are therefore presented as pooled data. The cold freeze–thawing yielded significantly higher PBMC recovery (median 68.9%, inter-quartile range 59.3–74.9 *versus* 63.2%, 57.5–69.0%; $p = 0.036$) and viability (85.0, 79.9–92.4 *versus* 85.1%, 75.0–91.9%; $p = 0.043$) compared with the warm method (Figure 1A). The cold serum-free AIM-V freeze–thawing (Figure 1B) resulted in a significantly increased viability (88.0, 85.7–90.0 *versus* 83.6, 81.4–85.0; $p = 0.012$) compared with the cold serum freeze–thaw protocol.

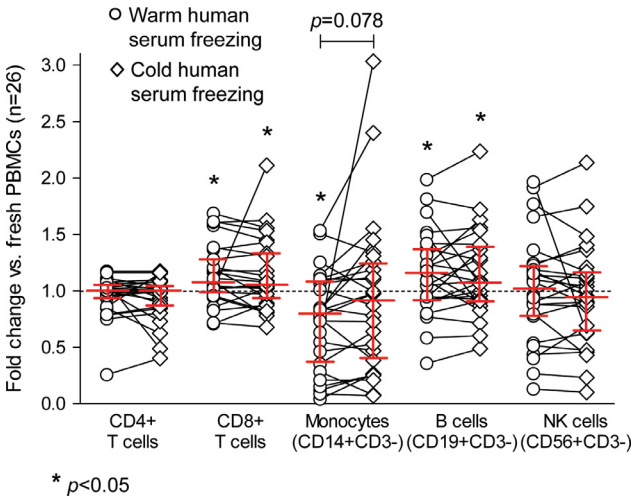

Figure 2. Fold changes in different peripheral blood mononuclear cell subsets between frozen–thawed and fresh peripheral blood mononuclear cells. Pooled data from patients with type 1 diabetes and healthy controls are presented, as no difference was observed between the two groups. Asterisks indicate significant ($p < 0.05$) differences compared with freshly isolated peripheral blood mononuclear cells. $P = 0.078$ for comparison of monocyte fractions obtained after cold and warm human serum freeze–thawing

Cell populations

The composition of recovered cell populations using both freezing methods compared with fresh cells is shown in Figure 2. Recovered PMBC cell populations were divided into CD4+, CD8+, monocytes, B cells, and natural killer cells. No significant changes were observed using either freezing protocol compared with fresh cells for recovered CD4+ cells and natural killer cells. However, both cold and warm freeze–thaw protocols resulted in an increased B-cell recovery (median fold change *versus* fresh PBMCs 1.08, range 0.91–1.39; $p = 0.047$; and 1.16, 0.92–1.37; $p = 0.046$, respectively) and increased CD8+ T cells (1.06, 0.94–1.33; $p = 0.03$; and 1.08, 0.99–1.28; $p = 0.016$). Warm freeze–thawing also led to a significant decrease in the monocyte subset (0.80, 0.38–1.08; $p = 0.005$).

Functional assays

CD4+ ELISPOT

Comparison of ELISpot analysis in fresh and frozen PBMC samples is shown in Figure 3. Responses are shown as stimulation index of IFN-γ ELISPOT responses of patients with T1D in response to control (a, f, and k) and islet antigens (b–e, g–j, and l–o). Results from panels a–e, f–j, and k–o were generated in three separate laboratories. The responses to islet antigens also varied, with no changes observed stimulating with some antigens (GAD4.11, IA2-R2, p277), whereas frozen cells gave sometimes lower but sometime also higher

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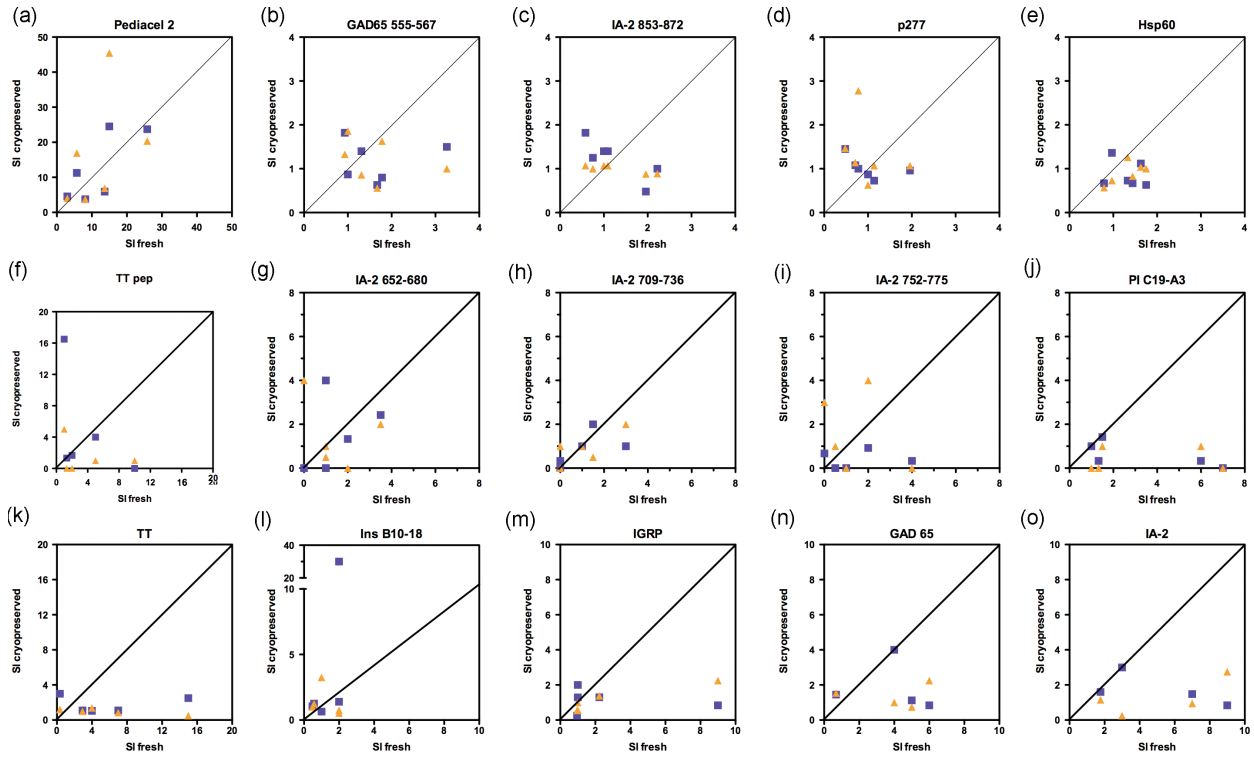

Figure 3. Comparison of enzyme-linked immunospot analysis in fresh and frozen peripheral blood mononuclear cell samples. Stimulation index of interferon-γ enzyme-linked immunospot responses of patients with type 1 diabetes in response to control (a, f, and k) and islet antigens (b–e, g–j, and l–o). Results from panels a–e, f–j, and k–o were generated in three separate laboratories. In all cases responses in peripheral blood mononuclear cell frozen by cold method are shown in blue squares and responses in peripheral blood mononuclear cell frozen by warm method are shown in orange triangles. Diagonal line represents the line of identity

response than fresh PBMCs with other antigens [HSP-60, IA-2 (709–736), IA2 (752–775), proinsulin (C19-A3), GAD65] compared with fresh PBMCs. Although freezing overall did affect cellular responses measured by ELISPOT, there was no clear trend regarding cells derived from patients or control subjects and type of assay (IFN-γ or interleukin-13) on whether stimulation with frozen or fresh cells gave higher or lower responses. However, the warm and the cold freezing protocol almost always affected results in the same direction.

CFSE proliferation

Figure 4 illustrates the comparisons of cell division index of patients with T1D in response to control (a–b) and islet antigens (c–d) measured by CFSE dye dilution assay in fresh and frozen PBMC samples. Patients with T1D in CFSE responses to insulin A peptide and tetanus demonstrated a good correlation between both freezing protocols and fresh cells.

Cellular immunoblotting

Positive T-cell responses to islet proteins were observed for the patients with T1D using fresh cells. However, irrespective of the freezing protocol used, the T-cell responses to islet proteins became negative when frozen cells were used (data not shown).

Class II tetramer

TMr responses to the control antigen Flu haemagglutinin 306–318 demonstrated reproducible responses when comparing fresh and frozen samples (Table 1). However, frozen samples responded similarly irrespective of freezing protocol and gave generally lower responses compared with fresh cells to islet antigens (preproinsulin and GAD).

Discussion

Cryopreservation of PBMCs has helped to lessen the variability associated with measuring human lymphocyte responses allowing for longitudinal studies to be performed in many infectious diseases [9]. However, differences have been reported in the freezing of PBMCs with respect to viability and cell populations by many researchers [23,25,26]. On the basis of this literature, the Immunology of Diabetes Society T-Cell Workshop Committee decided to identify an optimal freezing protocol that would demonstrate minimal changes from responses to fresh cells but also a protocol that would allow for the highest and most reproducible responses to be obtained in assays currently being utilized in T1D research studies [6,10,27].

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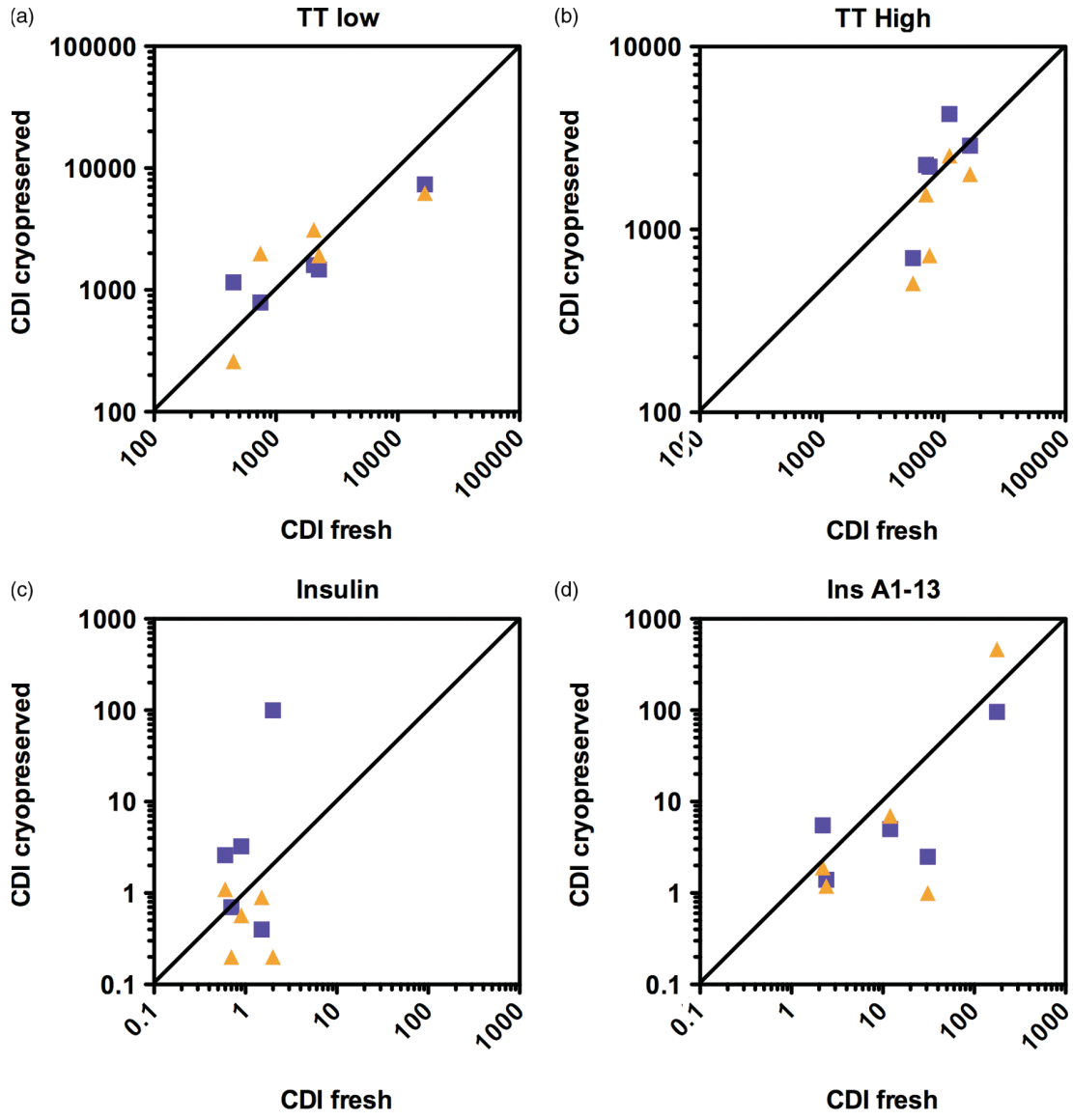

Figure 4. Comparison of proliferative responses measured by carboxyfluorescein succinimydyl ester dye dilution assay in fresh and frozen peripheral blood mononuclear cell samples. Cell division index of patients with type 1 diabetes in response to control (a–b) and islet antigens (c–d). In all cases responses in peripheral blood mononuclear cell frozen by cold method are shown in blue squares and responses in peripheral blood mononuclear cell frozen by warm method are shown in orange triangles. Diagonal line represents the line of identity

Table 1. Tetramer staining of fresh CD4+ T cells compared with frozen cells using two different freezing protocols

	Haemagglutinin 306–318		Pro*INS* *76-90*	Pro*INS* *76-90S88*	GAD65 270–283	GAD65 555–567	Any self-antigen	
	N positive (total *N* = 4)	Response recovery (%)	*N* positive (total *N* = 4)				*N* positive (total *N* = 16)	Response recovery (%)
Fresh	3	–	3	2	3	2	10	–
Cold[a]	3	100	2	0	1	1	4	40
Warm[a]	3	100	1	1	1	1	4	40

Positive response has been defined as two times the response of negative tetramer.
[a]Freezing protocol tested.

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Comparing PBMCs frozen with the warm freezing protocol and the cold freezing protocol to freshly isolated cells, the cold freeze–thaw method produced slightly higher cell recoverability and viability than the warm freeze–thaw method. The serum-free AIM-V medium resulted in a significantly higher viability compared with the cold freeze–thaw method. However, compared with fresh cells, recovery ranged from <40 to >90% demonstrating a large variation among laboratories and patient samples. Irrespective of freezing protocol, the CD8+ T cells and B cells appeared to be slightly enriched while cell losses were observed for monocytes. Lower responses were also observed in the T-cell functional assays with the frozen cells for both control and islet antigens irrespective of the assay tested.

The results of this study demonstrate that the cold protocol may be slightly better for use with T1D patient responses to islet autoantigens and the AIM-V medium method may prove to be a better method for use with cells from patients with T1D though more testing is needed to confirm these results. In this study, neither the cold nor the warm freezing protocol appeared to be adequate for maintaining responses in established and validated assays utilized currently used in T1D-related research. Therefore, a search for a different freezing protocol is a necessity if cryopreserved cells are going to be incorporated into longitudinal T1D trials. An alternative solution would be to develop new T-cell assays with responses not affected by cryopreservation. There are a number of new T-cell functional assays under development that may be of use for future studies.

On the basis of these results, the Immunology of Diabetes Society T-Cell Workshop Committee recommendation is that either fresh or cryopreserved cells should be used consistently throughout a study to allow for a sound comparison of results among different subjects and/or time points. Identification and optimization of a freezing protocol applicable to PBMCs from patients with autoimmune disease remain a very important component missing from current research.

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Conflict of interest

The authors declare no conflicts of interest.

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Chapter 8

Summary, discussion and conclusion

Similar systemic concentrations of cytokines, chemokines, adhesion molecules and T cell responses between patients with type 1 diabetes and LADA

Summary

In **Chapter 2-4** we observed that patients with type 1 diabetes and LADA did not differ in their circulating concentrations of immune mediators including cytokines, chemokines and adhesion molecules and their pro-inflammatory IFN-γ and anti-inflammatory IL-13 T cell responses upon islet and recall antigen stimulation. Increased concentrations of systemic immune mediators were observed in patients with type 2 diabetes when compared to healthy control subjects and patients with type 1 diabetes and LADA.

As expected systemic concentrations of cytokines, chemokines and adhesion molecules correlated significantly with anthropometric parameters including sex, age, blood pressure and diabetes duration. Higher BMI was positively associated with higher systemic concentrations of cytokines, chemokines and adhesion molecules in all diabetic groups.

Interestingly, even after adjustment for sex, age, BMI, blood pressure and diabetes duration patients with type 2 diabetes maintained significantly higher circulating concentration of immune mediators compared to control subjects and both autoimmune diabetes groups.

Discussion

We have shown that systemic concentrations of cytokines, chemokines and adhesion molecules and T cell responses were similar between patients with type 1 diabetes and LADA, which were lower than in patients with type 2 diabetes. Our data are consistent with the proposal that patients with type 1 diabetes and LADA are immunologically alike in spite their different clinical characteristics and the slower process of C-peptide loss in LADA compared to type 1 diabetes.[295,429] In the cohort studied the prevalence of metabolic syndrome in patients with type 1 diabetes and LADA was similar but lower than patients with type 2 diabetes.[377]

Previous studies have shown an association between overproduction of immune mediators and weight, obesity, adipose tissue and metabolic syndrome.[430,431] In our study BMI was used to study obesity, as this maker correlates tightly with waist circumference and both variables are robust risk factors associated with diabetes.[432,433] As expected patients with higher BMI simultaneously had higher circulating concentrations of cytokines, chemokines and adhesion molecules. Our data indicate the positive influence of obesity on the expression of circulating

concentrations of cytokines, chemokines and adhesion molecules, interestingly also anti-inflammatory cytokines such as IL1ra and IL-10 were positively associated with BMI suggesting a general upregulation of systemic immune mediators potentially resulting from a counterbalancing reaction. Our data suggest that obesity would be an additional risk factor for impairment of disease progression not only in patients with type 2 diabetes but also in patients with type 1 and LADA.

The elevation of cytokines, chemokines and adhesion molecules has been thought to be associated with higher risk of diabetes onset.[290-294,401,422,434] We extended these observations as we described that in addition to BMI, diabetes type is associated with systemic cytokine concentrations. Interestingly, differences in cytokine concentrations in patients with type 2 diabetes compared to patients with type 1 diabetes and LADA persisted after adjustment for BMI, age, sex, diabetes duration and blood pressure. Our data demonstrate that higher systemic concentrations of immune mediators are influenced by BMI and other potential confounders including sex, age, blood pressure and diabetes duration. However, our data indicate also that differences in systemic concentrations of immune mediators between those with type 2 diabetes and those with LADA and type 1 diabetes are not only explained and influence by these potential confounders and are therefore suggestive of a diabetes-type associated immune dysregulation.

Conclusions

Our findings indicate that patients with LADA and type 1 diabetes cannot be distinguished by systemic concentrations of immune mediators and T cell responses tested in our setting. Thus, factors other than these immune mediators and T cell are responsible for the difference of the clinical phenotype between LADA and type 1 diabetes. Indeed, a recent study of LADA patients in Chinese showed differences of high sensitive (hs)-CRP and Lipocalin 2 (LCN-2).[435]

The increased systemic cytokines, chemokines and adhesion molecules in patients with type 2 diabetes are reflective of subclinical inflammation that contributes to micro- and macrovascular complications in type 2 diabetes. As the differences of immune mediators between diabetes types were maintained also after adjustment for confounding parameters such as BMI, age, blood pressure, diabetes duration and sex we conclude that additional factors not investigated in our study contribute to subclinical inflammation.

Systemic concentrations of adipokines, β-cell function and acute food intake in patients with type 1 diabetes

Summary

In **Chapter 5** we reported on systemic immune status in patients with type 1 diabetes and detected positive associations between circulating concentrations of adipokines leptin and resistin and fasting as well as stimulated C-peptide, that is used as gold standard for measuring β-cell function. In contrast to leptin and resistin, systemic concentrations of adiponectin associated negatively with fasting and stimulated β-cell function in line with previous reports.[387,389] The associations of adipokines with C-peptide persisted after adjustment for the anthropometric and metabolic parameters sex, age, diabetes duration, BMI, A1c and blood glucose.

In addition, we found that acute meal intake in form of a high boost protein drink was associated with slightly but significantly altered systemic concentrations of adipokines including leptin and resistin in patients with type 1 diabetes.

Discussion

In type 1 diabetes the role of leptin has so far been investigated predominantly in animal models.[436-439] Several studies showed in mice, which became diabetic upon high dose streptozotocin, that exogenous application of leptin improved glucose homeostasis and insulin sensitivity.[436,438,439] The authors suggested that leptin may have a positive effect on metabolism.[436,438,439] One earlier study reported an insulin-sensitizing effect of leptin, which affect directly on murine β-cells.[440] In our study circulating concentrations of leptin in patients with type 1 diabetes are positively associated with β-cell function well in line with the observations in mice. We suggest that leptin could have a protective role in patients with type 1 diabetes. However, further studies still need to investigate the role of leptin in the pathogenesis of type 1 diabetes.

Systemic concentrations of resistin are positively associated with insulin resistance and obesity in patients with type 2 diabetes.[441,442] The impact of resistin in the pathogenesis of type 1 diabetes is still unknown and needs to be clarified. Contrary to our expectation, we found in our study positive association between systemic concentrations of resistin and β-cell function in patients with type 1 diabetes suggesting a protective role against disease progression.

The receptors of adiponectin have been found on β-cells, which induce insulin gene expression.[443] In patients with type 2 diabetes higher circulating concentrations of adiponectin correlated positively with lower insulin resistance and improved insulin sensitivity suggesting an anti-inflammatory and protective effect on the pathogenesis.[444] In contrast, one study with long-standing type 1 diabetes patients showed that increased systemic concentration of adiponectin associated positively with higher urinary albumin excretion and cardiovascular mortality.[445] Likewise, our finding in the present study revealed negative association with higher systemic concentrations of adiponectin and lower β-cell function in patients with type 1 diabetes. Our present data are in line with our previous observations, which showed an elevation of adiponectin in patients with poor metabolic control after 6 and 12 months after diagnosis.[387,389]

Consumption of food has an effect on metabolism and differently composed nutrition is likely to affect the risk for development of diabetes and secretion of immune mediators.[446-448] Studies with type 2 diabetes and healthy control subjects showed an increase of postprandial circulating concentrations of cytokines (IL-18, IL-8, IL-6, CRP) and adhesion molecules (ICAM-1, VCAM-1) and a decrease of adiponectin from baseline concentrations after consumption of high-fat meals as well as high-carbohydrate consumption.[446-448] Currently, investigations about the effect of adiponectin, resistin and leptin on metabolism upon food ingestions in patients with type 1 diabetes is scarce. Observations with C57BL/6J mice reported that plasma concentrations of leptin increased after intake of high-fat diet.[449] In our study we found contrary to the other studies with type 2 diabetes and healthy control subjects that systemic concentrations of adiponectin remained overall stable after mixed meal ingestion. Although our finding appears discordant with other studies, it needs to be kept in mind, that we used a standardised mixed meal test with lower fat content, which likely causes the lack of postprandial changes of adiponectin. After 30 minutes of food intake we observed a statistically significant decrease for leptin and resistin in patients with type 1 diabetes, however the absolute decreases of resistin and leptin after food consumption were minor. Our data indicate that the acute food intake could affect on the metabolism of patients with type 1 diabetes. However, only mechanistic studies may reveal whether this is a biological meaningful change.

Conclusions

Circulating concentrations of adiponectin were negatively associated with pancreatic β-cell function in patients with type 1 diabetes. Contrary to what is known for type 2 diabetes, adiponectin does not seem to act a protective effect on the pathogenesis of patients with type 1 diabetes.

Systemic concentrations of leptin and resistin correlated with better preservation of β-cell function which may be mediated by modulation of innate immunity and regulatory T cell functions by the two adipokines.

Acute food intake can modulate the secretion of adipokines and metabolism in patients with type 1 diabetes.

We suggest that not only cytokines and chemokines but also adpokines such as adiponectin, leptin and resistin could be players in the development of type 1 diabetes.

Validation of T cell assays for measurement of autoreactivity in patients with type 1 diabetes

Summary

In **Chapter 6** we applied a tetramer-based assay in an international multicenter approach to measure T-cell reactivities to DR0401-restricted epitopes. Three participating centres concurrently performed ELISPOT or immunoblot assays. All participating centres detected T cell responses to auto-antigens and the positive control antigen. The most commonly recognized epitope was $GAD_{270-285}$, followed by $GAD_{555-567}$ and PPI_{76-90}. 74% of all patients responded at least one islet epitope. Responses rates using HLA class II tetramer assay varied among centres. Only a minority of the patients tested by HLA class II tetramer and ELISPOT was concordant for both assays.

Discussion

This study is one of several performed by the T cell workshop comittee of the Immunology of Diabetes Society. The goal was standardise the detection of epitope-specific responses in patients with type 1 diabetes rather than distinguishing patients from controls.

Although each participating centre could successfully measure auto-reactive T cells using tetramer assay, the response rates were different among centres. As a study design was chosen using fresh local blood samples and no sharing samples, we cannot conclude whether the variability of response rates result from lack of assay reproducibility and sampling variation. Current findings leave room for doubt about whether the tetramer assay was performed with equal success in all laboratories. Future investigations should consider this question and use shared, distributed PBMC samples aiming to resolve this problem.

In the present study only a minority of the patients tested showed the same response for both tetramer and ELISPOT assays. This observation was not unexpected because technical operating is different between both assays suggesting tetramer assays could achieve different responses than ELISPOT assays.[192]

In our study all participating centres could detect T cell responses to both auto-antigens and the positive control antigen using tetramer assay. Study by Oling et al. could determine 61% positivity rate for one of the three GAD65 or PPI.[450] Similar to the latter study, we observed that 45% of all tested subjects responded mostly to the prevalent epitope $GAD_{270-285}$. Contrary to our expectation, 40% of tested subjects did not response to control haemagglutinin epitope. We suppose that some assays were interpreted as false negative or some patients did not show T cell responses to this epitope because of the lack of recent influenza immunization. The inclusion of additional epitopes will be helpful to achieve complete coverage for all subjects with DRB1*0401 haplotypes.

Conclusions

Despite the variability of results and difficulties of validation of T cell assays and the unexpected incongruence of recognition of specific epitopes we encourage to optimize existing assays and to develop novel assays with improved sensitivity and specificity, as it is considered crucial to monitor disease activity in type 1 diabetes during naturally occurring pathogenesis or accompanying immune intervention trials.

Investigating assays in a longitudinal study would be of interest to identify clear shifts in the phenotype of auto-reactive T cells that precede disease onset. These could include imbalances in T cell subsets, variations in the magnitude or character of cytokine responses or other changes related T cell activation or homing.

Future efforts should investigate shared blood samples to evaluate assays reproducibility and longitudinal samples to identify changes in T cell phenotype that correlate with changes in disease course.

Comparison of cryopreservation methods on T cell responses to islet and control antigens from type 1 diabetic patients and controls

Summary

In **Chapter 7** we report on results obtained by the T-Cell Workshop Committee of the Immunology of Diabetes Society (IDS), that compared two widely accepted T-cell freezing protocols (warm and cold) to freshly isolated peripheral blood mononuclear cells from patients with T1D and controls in terms of recovery, viability, cell subset composition, and performance in functional assays currently in use in type 1 diabetes related research. Nine laboratories including ours participated in the study with four different functional assays included. The cold freezing method yielded higher recovery and viability compared with the warm freezing method. Irrespective of freezing protocol, B lymphocytes and CD8+ T cells were enriched, the monocyte fraction decreased, and islet antigen-reactive responses were overall lower in frozen versus fresh cells. However, overall response to islet auto-antigens was low in most assays.

Discussion

The findings of the present study show that the cold-freeze thaw protocol could be better slightly for use with type 1 diabetes patient responses to islet autoantigens compared to the warm protocol. However, more testing is needed to testing these results.

In this study, neither the cold nor the warm protocol seemed to be adequate for maintaining responses in established and validated assays utilized currently in type 1 diabetes related research. Thus, we suggest searching for adequate freezing protocol as cryopreserved cells are going to be incorporated much easier into longitudinal studies than working with freshly isolated cells. It would be of interest to investigate new T cell assays with responses not affected by cryopreservation. Future studies should also consider new T cell functional assays under development.

Conclusions

None of the T cell functional assays performed well using frozen samples, T cell responses measured by different assays often yielded different results. Therefore, more investigations should be carried out to obtain a freezing method and a T cell functional assay that are reproducible, sensitive and reliable enough to study autoreactive T cell responses in patients with type 1 diabetes.

Appendices

Summary

Type 1 diabetes is an immune mediated disease resulting from selective destruction of pancreatic β-cells. The involvement of T lymphocytes and immune mediators including cytokines, chemokines and adhesion molecules in the pathogenesis and β-cell destruction of type 1 diabetes has been described in previous studies. Latent autoimmune diabetes in adults (LADA) has some clinical features of type 2 diabetes but shows similar immunological abnormalities as type 1 diabetes such as positivity for glutamic acid decarboxylase antibodies (GADA). So far, it is not well understood why disease progression in patients with LADA is slower than in patients with type 1 diabetes despite immunological similarities and whether cellular immune response or systemic immune status is related to diabetes forms and β-cell function.

The aims of this thesis were 1) to compare systemic concentrations of cytokines, chemokines, adhesion molecules and T cell responses between patients with type 1, type 2 diabetes and LADA, 2) to investigate the association of systemic concentrations of adipokines with pancreatic β-cell function and acute food consumption in patients with type 1 diabetes, 3) to standardize T cell assays for measurement of autoreactivity in patients with type 1 diabetes and 4) to compare of cryopreservation methods on T cell responses to islet and control antigens from type 1 diabetes patients and controls.

In the studies presented in this thesis we found that patients with type 2 diabetes showed increased systemic concentrations of cytokines, chemokines and adhesion molecules compared to patients with LADA and type 1 diabetes and healthy control subjects. Patients with LADA and type 1 showed similar concentrations of systemic concentrations of immune mediators and pro-inflammatory IFN-γ and anti-inflammatory IL-13 T cell reactivity upon islet and recall antigen stimulation. Circulating concentrations of immune mediators in control subjects were lower than in patients. Higher systemic concentrations in all patients with diabetes were positively correlated with increased body mass index (BMI). Despite associations of immune mediators with anthropometric parameters, differences between all groups persisted after adjustments for BMI, age, sex, diabetes duration and blood pressure suggesting a diabetes-type associated immune dysregulation

Furthermore, we detected positive associations between systemic concentrations of leptin and resistin and fasting and stimulated C-peptide levels, that serve as measure for β-cell function. In contrast, adiponectin showed negative association with β-cell function in patients with type 1 diabetes. These associations maintained also after adjustment for sex, age, BMI, A1c, blood glucose and diabetes duration. We observed that the acute ingestion of a high boost protein

drink decreased slightly the circulating concentrations of leptin and resistin and increased blood glucose and C-peptide in patients with type 1 diabetes.

In collaboration with the T-Cell Workshop Committee of the Immunology of Diabetes Society (IDS) we used a tetramer-based assay to measure T cell reactivity. All participating centres could detect T cell responses to auto-antigens and positive control antigen. Dual analysis by tetramer and ELISPOT assays was frequently discordant suggesting that these assays detect different cell populations.

Moreover, we could show that PBMCs frozen with the freezing protocol using cold medium had higher recovery and viability than with freezing method using room temperature medium and that B-cells and CD8+ T cells were enriched and monocyte fraction reduced. We found that the islet antigen-reactive responses were lower in frozen than fresh cells. However, these results need to take into account that the overall response to islet antigens was low in some assays.

In conclusion, results of this thesis indicate that patients with LADA and type 1 diabetes from the cohort investigated are immunologically similar, despite their different rate of islet destruction and disease progression towards insulin deficiency. Type 1 diabetes and LADA patients could not be distinguished by systemic concentrations of immune mediators and T cell responses measured in our study. As expected, patients with type 2 diabetes had increased systemic concentrations of cytokines, adhesion molecules and chemokines.

Furthermore, our results suggest that not only cytokines, chemokines or adhesion molecules but also adipokines might have a putative role in type 1 diabetes and β-cell function. In spite of variation of results and difficulties of validation of T cell assays and development of appropriate freezing method of PBMCs we encourage to continue to search for validated T cell assays and continue developing reliable freezing protocols as standardized assay as both are considered necessary to monitor disease activity during natural disease progression and immune intervention trials aiming to halt islet destruction.

Zusammenfassung

Der Typ 1 Diabetes ist eine immun-mediierte Erkrankung, der eine selektive Zerstörung der pankreatischen β-Zellen zugrunde liegt. Frühere Studien beschrieben die Beteiligung von T-Lymphozyten und Immunmediatoren wie Zytokinen, Chemokinen und Adhäsionsmolekülen in der β-Zell-Zerstörung und der Pathogenese des Typ 1 Diabetes. Der latente auto-immune Diabetes im Erwachsenenalter (LADA) hat einige klinische Merkmale des Typ 2 Diabetes, zeigt aber ähnliche immunologische Abnormitäten wie der Typ 1 Diabetes wie z.B. die Positivität für Antikörper gegen das Enzym Glutaminsäure-Decarboxylase (GADA). Bisher ist es ungeklärt, warum die Krankheitsprogression bei Patienten mit LADA langsamer als bei Patienten mit Typ 1 Diabetes verläuft trotz der immunologischen Ähnlichkeiten der beiden Krankheitsformen und ob zelluläre Immunantworten und/oder der systemischen Immunstatus mit den Diabetesformen und β-Zellfunktion assoziiert sind.

Ziele dieser Dissertationsarbeit sind 1) die Aufklärung der Assoziationen von systemischen Konzentrationen von Zytokinen, Chemokinen, Adhäsionsmolekülen und T-Zellantworten mit Typ 1, Typ 2 Diabetes und LADA, 2) die Aufklärung der Assoziation von systemischen Konzentrationen von Adipokinen mit der Funktion der pankreatischen β-Zellen und der akuten Nahrungsaufnahme in Patienten mit Typ 1 Diabetes, 3) die Standardisierung der Methode zur Messung der Autoreaktivität von T-Zellen bei Patienten mit Typ 1 Diabetes und 4) den Einfluss des Kryokonservierungsverfahren auf T-Zellantworten zu Insel- und Kontroll-Antigene von Patienten mit Typ 1 Diabetes und gesunden Kontrollpersonen zu vergleichen.

In der vorliegenden Arbeit dargestellten Studien zeigten Patienten mit Typ 2 Diabetes erhöhte systemische Konzentrationen von Zytokinen, Chemokinen und Adhäsionsmolekülen im Vergleich zu Patienten mit LADA und Typ 1 Diabetes und gesunden Kontrollpersonen. Patienten mit LADA und Typ 1 Diabetes zeigten vergleichbare Konzentrationen von systemischen Immunmediatoren und ähnliche pro-inflammatorische IFN-γ und anti-inflammatorische IL-13 T-Zellreaktivität. Die Konzentrationen zirkulierender Immunmediatoren bei Kontrollpersonen waren im Vergleich niedriger als bei allen Patienten. Bei allen Patienten mit Diabetes waren die Konzentrationen systemischer Immunmediatoren dem Körpermasseindex (BMI) assoziiert. Trotz der Assoziationen der Immunmediatoren mit anthropometrischen Parametern blieben die Unterschiede zwischen allen Gruppen nach der Adjustierung für BMI, Alter, Geschlecht, Diabetesdauer und Blutdruck bestehen, was vermuten lässt, dass die Immundysregulation mit der Diabetesform assoziiert ist.

In der vorliegenden Arbeit wurden positive Assoziationen von systemischen Konzentrationen von Leptin und Resistin mit nüchtern und stimulierten C-Peptid Spiegeln, welche als Maß für die β-Zellfunktion dienen. Adiponectin wies dagegen eine negative Assoziation mit der β-

Zellfunktion bei Patienten mit Typ 1 Diabetes auf. Diese Assoziationen blieben auch nach Adjustierung für Geschlecht, Alter, BMI, HbA1c, Blutglucose und Diabetesdauer bestehen. Die einmalige Einnahme eines Protein-reichen Getränk erniedrigte die zirkulierenden Konzentrationen von Leptin und Resistin und erhöhte, wie erwartet die Blutglucose und C-Peptid Spiegel bei Patienten mit Typ 1 Diabetes.

Im Rahme einer Kooperation mit dem T-Zell Workshop Komitee der „Immunology of Diabetes Society (IDS)“ wurde eine Tetramer-basierte Methode für die Messung von T-Zell Reaktivität erprobt. Dabei wurden die T-Zellantworten zu Auto-Antigenen und Kontroll-Antigenen bestimmt. Die vergleichende Analyse mit Tetramer-basierte und ELISPOT Methode zeigten eine Diskordant, was darauf hinweist, dass diese beiden Methoden unterschiedliche Zell-Populationen detektieren.

Darüber hinaus konnte gezeigt werden, dass die eingefrorenen PBMC mit dem Einfrierungsprotokoll unter Verwendung von kaltem Medium höhere Ausbeute und Viabilität im Vergleich zu dem Einfrierungsprotokoll mit Raumtemperaturen-warmen Medium und dass die B-Zellen und CD8+ T Zellen angereichert und die Monozyten-Fraktion reduziert waren. Zudem wurde festgestellt, dass die Antworten auf Insel-Antigenen bei eingefrorenen Zellen niedriger waren als bei frisch-isolierten Zellen. Bei der Interpretation dieser Ergebnisse sollte allerdings beobachtet werden, dass die allgemeine Antwort zu Insel-Antigenen bei einigen Ansätzen sehr niedrig war.

In der Schlussfolgerung, deuten die Ergebnisse der vorliegenden Arbeit darauf hin, dass die Patienten mit LADA und Typ 1 Diabetes in der untersuchten Kohorte immunologisch ähnlich sind trotz ihrer unterschiedlichen Raten der Insel-Zerstörung und Krankheitsprogression in Richtung einer Insulindefizienz. Patienten mit Typ 1 Diabetes und LADA konnten durch die gemessenen systemischen Konzentrationen der Immunmediatoren und der T-Zellantworten nicht unterschieden werden. Wie erwartet, hatten Patienten mit Typ 2 Diabetes erhöhte systemische Konzentrationen von Zytokinen, Ahäsionsmolekülen und Chemokinen. Ferner weisen die Ergebnisse darauf hin, dass nicht nur Zytokine, Chemokine oder Adhäsionsmoleküle sondern auch Adipokine eine Rolle im Typ 1 Diabetes und in der β-Zellfunktion haben. Trotz der Variation der Ergebnisse und der Problematik der Validierung von T-Zell gestützten Verfahren sowie der Entwicklung einer geeigneten Einfriermethode von PBMC ist es empfehlenswert weiterhin T-Zell gestützte Verfahren zu validieren und zuverlässige Einfriermethoden zu erstellen. Beide Methoden bilden eine wichtige Grundlage für die Entwicklung von Standardverfahren zur Bestimmung der Krankheitsaktivität während des Krankheitsverlaufs und während einer Immunintervention, die auf den Erhalt der β-Zellfunktion abzielt.

List of Abbreviations

BB rat	BioBreeding rat
ADA	American Diabetes Association
BAT	Brown adipose tissue
BMI	Body mass index
CD	Cluster of differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CDKN2B	Cyclin-dependent kinase inhibitor 2B
CFSE	5,6-carboxyfluorescein diacetate succinimidyl ester
CMV	Cytomegalie virus
CRP	C-reactive protein
CTLA4	cytotoxic T-lymphocyte-associated antigen 4
CVD	Cardiovascular disease
DAISY	Diabetes Autoimmunity Study in the Young
DIPP	Finnish Diabetes Prediction and Prevention
EBV	Epstein-Barr virus
ECPT	European C-peptide Trial
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immune spot technique
FFA	Free fatty acid
Foxp3	Forkhead box P3
FTO	Fat mass and obesity-associated protein
GAD	Glutamic acid decarboxylase
GIPR	Gastric inhibitory polypeptide receptor
GWAS	Genome wide association studies
HbA_{1c}	Glycated haemoglobin A_{1c}
HLA	Human leukocyte antigen
HNF1B	Hepatocyte nuclear factor 1 homeobox B
HOMA	Homeostasis model assessment
Hsp60	Heat shock protein 60
i.v.	Intravenous
IA2	Insulinoma-associated-2 antigen
IAA	Insulin auto-antibodiess
ICA	Islet cell antigen
IDDM	Insulin dependent diabetes mellitus
IDF	International Diabetes Federation
IDS	Immunology of Diabetes Society
IFN-gamma	Interferon gamma
IGF2BP2	Insulin-like growth factor 2 mRNA-binding protein 2
IGT	Impaired glucose tolerance
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IRS1	Insulin receptor substrate 1

LADA	Latent autoimmune diabetes in adults
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
MODY	Maturity onset diabetes of the young
MTNR1B	Melatonin receptor 1B
NGT	Non-glucose tolerance
NIDDM	Non-insulin dependent diabetes mellitus
NK-cell	Natural killer cell
NO	Nitric oxide
NOD mouse	Non-obese diabetic mouse
NOTCH2	Neurogenic locus notch homolog protein 2
NZO	New Zeeland obese
PAI	Plasminogen activator inhibitor
PPARG	Peroxisome proliferator-activated receptor gamma
PTPN22	Protein tyrosine phosphatase non-receptor type 22
ROS	Reactive oxygen
SLC30A8	Solute carrier family 30 (zinc transporter) member 8
STAT4	Signal transducer and activator of transcription-4
T-bet	T-box expressed in T-cells
TCF7L2	Transcription factor 7-like 2
T_H cell	T helper cell
TLR4	Toll-like receptor 4
TPO	Thyroid peroxidase
Treg	Regulatory T cell
TrialNet	Type 1 Diabetes TrialNet Research Group
TRIGR	Trial to Reduce Insulin-dependent diabetes mellitus in the Genetically at Risk
USA	United States of America
VNTR	Variable number of tandem of repeats
WAT	White adipose tissue
WFS1	Wolframin protein
WHO	World Health Organization
ZnT8	Zinc transporter 8

Curriculum Vitae

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1 **Pham MN**, Hawa MI, Pfleger C, Roden M, Schernthaner G, Pozzilli P, Buzzetti R, Scherbaum W, Seissler J, Kolb H, Hunter S, Leslie RD, Schloot NC; Action LADA Study Group. Pro- and anti-inflammatory cytokines in latent autoimmune diabetes in adults, type 1 and type 2 diabetes patients: Action LADA 4. *Diabetologia* 2011;54:1630-1638; IP 6.973

2 **Pham MN**, Hawa MI, Roden M, Schernthaner G, Pozzilli P, Buzzetti R, Scherbaum W, Seissler J, Kolb H, Hunter S, Leslie RD, Schloot NC; Action LADA Study Group. Increased serum concentrations of adhesion molecules but not of chemokines in patients with type 2 diabetes compared to patients with type 1 diabetes and latent autoimmune diabetes in adult age: Action LADA 5. *Diabetic Med* 2011 [accepted, in press]; IP 3.026

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7 Simon MC, **Pham MN**, Schloot NC. Biomarker und Typ 1 Diabetes. *Der Diabetologe* 2011 Review [in press]; IP 0.237

Presentations

- 4th Congress of the Central European Diabetes Association, Salzburg, Austria (2009)
- 46th European Association for the Study of Diabetes (EASD) Annual Meeting, Stockholm, Sweden (2010)
- 11th International Congress of Immunology of Diabetes Society (IDS), Incheon, South-Korea (2010)
- 46th German Diabetes Foundation (DDG) Annual Meeting, Leipzig, Germany (2011)
- 71st Scientific American Diabetes Association Sessions (ADA), San Diego, USA (2011)
- 47th EASD (European Association for the Study of Diabetes) Annual Meeting, Lisbon, Portugal (2011)

Awards and Honors

- Travel award for 4th Congress of the Central European Diabetes Association, Salzburg, Austria
- Travel award for 46thEASD Annual Meeting, Stockholm, Sweden
- Travel award for 11th International Congress of IDS, Incheon, South-Korea
- Travel award for 46th DDG Annual Meeting, Leipzig, Germany
- Travel award for 47th EASD Annual Meeting, Lisbon, Portugal
- "Meine Heine Frau 2010" (Honour Award for outstanding women students) Heinrich-Heine University, Düsseldorf, Germany

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- German Diabetes Foundation "Das zuckerkranke Kind": *Systemic concentrations of regulatory cytokines transforming growth factor (TGF-) beta and anti-inflammatory interleukin-1 receptor antagonist (IL1RA) in patients with type 1 diabetes, latent autoimmune diabetes in adults and type 2 diabetes* (10,000€, 2010)
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Contributions to Chapter 2-7

Besides my own contribution, the published data presented in this thesis involved the contributions from colleagues of the laboratory of Priv.- Doz. Dr. med. Nanette C. Schloot and external collaborators. In the following paragraphs, my specific contribution to each publication is outlined.

Chapter 2

- Measured circulating concentrations of pro- and anti-inflammatory cytokines using multiplex beads based technology
- Analysed data using SAS 9.2 program
- Created the statistical models for the analysis
- Wrote manuscript
- Presented data as poster at Congress of the Central European Diabetes Association (2009) in Austria, the 46th European Association for the Study of Diabetes (EASD) Annual Meeting in Sweden (2010) and the 11th International Congress of Immunology of Diabetes Society (IDS) in South-Korea (2010)

Chapter 3

- Measured circulating concentrations of adhesion molecules and chemokines cytokines using multiplex beads based technology
- Analysed data using SAS 9.2 program
- Created the statistical models for the analysis
- Wrote manuscript
- Presented data as poster at the 11th International Congress of Immunology of Diabetes Society (IDS) in South-Korea (2010), the 46th German Diabetes Foundation (DDG) Annual Meeting in Germany (2011) and the 71st Scientific American Diabetes Association Sessions (ADA) in USA (2011)

Chapter 4

- Contributed to the statistical analysis of data using SAS 9.2 program
- Reviewed the manuscript
- Edited the manuscript

Chapter 5

- Measured circulating concentrations of adipokines using multiplex beads based technology
- Analysed data using SAS 9.2 program
- Created the statistical models for the analysis
- Wrote manuscript
- Presented data as poster at the 47th EASD (European Association for the Study of Diabetes) Annual Meeting in Portugal (2011)

Chapter 6

- Learned the technique of tetramer analysis during a 4-weeks stay in Seattle at Benaroya Research Institute (BRI) and implemented the technique at the Institute for Clinical Diabetology at the German Diabetes Center
- Recruited probands for the T-Cell Workshop Committee of the Immunology of Diabetes Society (IDS) in Germany
- Responsible for PBMC separation and implementation of tetramer-based and ELISPOT assays
- Measured T cell responses with tetramer-based assay using fluorescence activated cell sorting (FACS)
- Measured T cell responses by ELISPOT
- Analysed German data
- Reviewed manuscript

Chapter 7

- Learned the technique of tetramer analysis during a 4-weeks stay in Seattle at Benaroya Research Institute (BRI) and implemented the technique at the Institute for Clinical Diabetology at the German Diabetes Center
- Recruited probands for the T-Cell Workshop Committee of the Immunology of Diabetes Society (IDS) in Germany
- Responsible for PBMC separation and implementation of tetramer-based and ELISPOT assays
- Measured T cell responses with tetramer-based assay using fluorescence activated cell sorting (FACS)
- Analysed German data
- Reviewed manuscript