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Cellular Immunological Mechanisms Contributing to Diabetes Pathogenesis

MILJANA V. RADENKOVIC

DEPARTMENT OF CLINICAL SCIENCES, MALMÖ | LUND UNIVERSITY 2016



Cellular Immunological Mechanisms Contributing to Diabetes Pathogenesis

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Miljana V. Radenkovic



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DOCTORAL DISSERTATION

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Cellular Immunological Mechanisms Contributing to Diabetes Pathogenesis		
<p>Abstract</p> <p>Dysregulated activation of immune cells is linked to the development of Type 1 diabetes (T1D) and Latent autoimmune diabetes of the adult (LADA). Although type 2 diabetes mellitus (T2D) is mostly associated with metabolic dysregulation, islet reactive T cells and islet-related autoantibodies have been recently identified in T2D patients. The work in this thesis aimed to characterize the major lymphocyte and T cell subsets that have been associated with the cellular immunological pathogenesis of diabetes. Firstly, we showed that the phenotype and frequency of circulating peripheral CD4⁺CD25⁺ T cells was different in LADA patients compared to healthy individuals. A lower frequency and lower number of CD4⁺ T cells expressing intermediate levels of CD25 (CD4⁺CD25^{int}) were demonstrated in LADA patients compared to controls. However, no changes were found in the frequency and total numbers of CD4⁺ T cells expressing CD25 (CD4⁺CD25^{hi}) in LADA patients. We further characterized the lymphocyte populations in isolated islets from non-diabetic organ donors. We found that CD8⁺ T cells were the most abundant cell type in human pancreatic islets followed by TCD4⁺ cells. Most CD4⁺ and CD8⁺ T cells displayed a central memory (T_{cm}) and effector memory (T_{em}) phenotype. Finally, we investigated the distribution and phenotypes of CD4⁺ and CD8⁺ T cells in pancreatic islets of type 2 diabetes donors. T cells were the most dominant lymphocyte population within T2D as described for non-diabetic islets. Likewise, higher percentage of CD8⁺T cells than CD4⁺T cells was demonstrated in T2D. CD8⁺T cells were mostly of central and effector memory phenotype. In conclusion, these studies illustrate the complexity of the immune network (both in peripheral blood and the pancreas) and demonstrate possible mechanisms contributing to the pathogenesis of diabetes. Investigations of these mechanisms in the pancreas, can deepen our understanding of the initial triggering events of diabetes and hopefully impact the design novel immunotherapy.</p>		
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Cellular Immunological Mechanisms Contributing to Diabetes Pathogenesis

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To my parents Vukomir and Stojanka

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Populärvetenskaplig sammanfattning

Diabetes är en endokrin sjukdom som orsakar högt blodsocker. I bukspottskörteln finns de Langerhanska öarna (uppkallade efter den tyske patologen Paul Langerhans som tyckte att dessa cellanhopningar såg ut som öar) som består av bland annat alfa- och betaceller. Betacellerna producerar hormonet insulin medans alfacellerna producerar hormonet glukagon. Insulin kontrollerar hur mycket glukos levern ska producera och hjälper också cellernas upptag av glukos från blodet och reglerar på så vis blodsockernivåerna. Glukagon stimulerar produktionen av glukos i levern vilket leder till att blodsockernivån ökar. Vid obalans mellan dessa två hormoner uppstår en ökad nivå av blodsocker, ett första tecken på diabetes. Diabetes är en heterogen och mångfacetterad sjukdom, de vanligaste är typ 1 diabetes och typ 2 diabetes.

Typ 1 diabetes, vilket oftast diagnosticeras hos barn och ungdomar, karaktäriseras som autoimmun då det egna immunförsvaret förstör de insulin producerande cellerna vilket leder till brist på insulin i kroppen. När immunförsvaret förstör de kroppsegna cellerna, som vid t ex typ 1 diabetes, kallas det för en autoimmun sjukdom. Detta beror på en obalans i immunsystemet. Orsaken till att immunförsvaret attackerar kroppens egna insulinproducerande celler är inte känd, kanske finns det en defekt i mekanismer som skall balansera immunförsvaret som ger upphov till sjukdomen.

Typ 2 diabetes utvecklas vanligtvis hos vuxna vars insulinproduktion är reducerad eller vars celler är insulinresistenta. De mekanismer som ligger bakom typ 2 diabetes är inte fullt kända men kan kopplas till livsstil, övervikt och arv.

Det finns ytterligare en form av diabetes som benämns LADA (Latent Autoimmune Diabetes in Adult) vilken orsakas av en autoimmun process men yttrar sig mer som en typ 2 diabetes.

Vi har studerat olika cellmekanismer som skulle kunna orsaka diabetes.

I vår första studie undersökte vi förekomst och egenskaper av de cirkulerande regulatoriska T-celler i blodprover från nyligen diagnosticerade LADA-patienter. För att undersöka dessa celler använde vi oss av flödescytometri som baseras på detektion av specifika cellmarkörer med hjälp av fluorescerande antikroppar. Regulatoriska T-celler (Tregs-celler) är kända för att tillhöra den beskyddande delen av kroppens immunförsvaret vilket innebär att de kontrollerar immunförsvaret genom att åstadkomma tolerans. Ett förfaringssätt som de har är att kväsa de celler i immunförsvaret som är involverade i förstörelsen av de kroppsegna insulinproducerande betacellerna. Vanligtvis karaktäriseras de regulatoriska T-cellerna som T-celler som uttrycker proteinerna CD4 och CD25 på cellens yta samt att de har transkriptionsfaktorn FoxP3 inuti cellen. Vid typ 1 diabetes har man kunna

relatera sjukdomen till defekta Tregs-celler men i LADA-patienter är de regulatoriska T-cellernas funktion dåligt undersökta. I vår studie fann vi dock en minskad förekomst av Tregs-celler med cellytemarkörerna CD4 och CD25 hos LADA-patienter jämfört med friska individer. Likväl visade det sig att den för Tregs-celler specifika transkriptionsfaktorn FoxP3 fanns i liknande nivåer både hos LADA-patienter och friska individer i T-celler med höga nivåer av CD4 och CD25. Men intressant nog visar vår studie att de regulatoriska T-celler som har mellanliggande nivåer av CD4 och CD25 förekom i mindre antal hos LADA-patienter vilket kan medverka till försämrad kontroll av immunförsvarets reaktion, och därmed bidra till autoimmunitet hos LADA-patienter.

Nästa studie inriktar sig på att undersöka vad som kännetecknar de immunförsvarsceller som man kan finna i bukspottskörteln, och då främst i de Langerhanska öarna där de insulinproducerande betacellerna befinner sig, hos icke-diabetiska organdonatorer. Då bukspottskörteln har en relativt undangömd plats i kroppen är möjligheterna att studera detta organ vid sjukdom, t ex diabetes, mycket begränsade. Tidigare studier har använt blodprover för att undersöka oegentligheter i immunförsvaret vid diabetes men dessa prover återspeglar dåligt det egentliga sjukdomsförloppet eftersom det är i bukspottskörteln Langerhanska öar som sjukdomen visar sig. Med hjälp av flödescytometri och isolerade Langerhanska öar från organdonatorer utan diabetes, analyserade vi de immunologiska celltyperna: T-, B- och NK-celler samt de insulin producerande betacellerna och de glukagon producerande alfacellerna. En undergrupp av T-celler, CD8 uttryckande T-celler, som kallas cytotoxiska celler anses vara delaktiga vid immunförsvarets nedbrytning av betacellerna vid typ 1 diabetes. Vi fann att dessa cytotoxiska T-celler dominerade i antal i de undersökta Langerhanska öarna. Andra immunologiska celler förekom i lågt antal. Vidare utforskade vi T-cellernas egenskaper utifrån cellytemarkörer som indikerar deras status och funktion. Naiva T-celler är en typ av T-celler som ännu inte stött på några sjukdomsalstrande celler eller mikroorganismer, patogener. När en naiv T-cell träffar på ett patogen så utvecklas de till att bli mer specialiserade (effektor-T-celler) för att kunna aktivt ingripa och avlägsna det som är sjukdomsalstrande men dessa effektor-T-celler kan också, när de är felaktigt reglerade, reagera och agera mot kroppsegna vävnader och ger då upphov till autoimmunitet. De flesta effektor-T-celler dör när de gjort sitt jobb, dock överlever en mycket liten del av dessa celler och kallas då för minnes-T-celler vilka är redo att snabbt och effektivt reagera nästa gång de stöter på samma patogen. I denna studie fann vi att de T-celler som befann sig i de Langerhanska öarna var av typen minnes-T-celler. Intressant nog visade det sig att det i den friska bukspottskörteln finns T-celler som är hemmahörande där och att de skiljer sig från den profil cirkulerande T-celler har vilket tydliggör vikten av att studera förloppet i bukspottskörteln för att kunna förstå skeendet vid utvecklandet av diabetes.

I den tredje studien, som bygger på föregående, undersökte vi om det är någon skillnad i förekomsten av de immunologiska cellerna T- B- och NK-celler i de Langerhanska öarna hos organdonatorer med typ 2 diabetes. Typ 2 diabetes har man trots utvecklades på grund av brister i ämnesomsättningen (metabolismen) men nyligen har man funnit att även immunologiska celler är involverade i uppkomsten av typ 2 diabetes. Genom att tillämpa samma analysmetod som i föregående studie, flödescytometri, fann vi att T-celler var mest förekommande och att dessa T-celler var av typen cytotoxiska T-celler, CD8 uttryckande T-celler, och att de flesta av dessa T-celler hade tillståndet minnes-T-celler. Vidare fann vi att dessa T-celler hade egenskap av att vara hemmahörande i vävnaden, i detta fall bukspottskörteln och dess Langerhanska öar och därmed inte återfinns ute i cirkulationen. Vi utvärderade dessutom förekomsten av alfa- och betaceller och upptäckte att hos organdonatorer med typ 2 diabetes fanns det mer glukagon producerande alfaceller än insulin producerande betaceller. Hos organdonatorer som inte hade typ 2 diabetes var förhållandet det motsatta dvs mer betaceller än alfaceller. Genom att studera det immunologiska nätverket som finns i bukspottskörteln hos diabetiker och icke-diabetiker kan vi få en bättre förståelse för de processer som gör att man utvecklar diabetes och därmed också i förlängningen kanske kunna förhindra och råda bot på diabetes.

List of original publication

- I. **Altered regulatory T cell phenotype in latent autoimmune diabetes of the adults (LADA).**, Radenkovic M, Silver C, Arvastsson J, Lynch K, Lernmark Å, Harris RA, Agardh CD, Cilio CM., Clinical and Experimental Immunology. 2016 June 30. doi: 10.1111/cei 12834
- II. **Characterization of resident lymphocytes in human pancreatic islets.**, Radenkovic M, Uvebrant K, Skog O, Sarmiento L, Arvastsson J, Storm P, Vikman P, Bertilsson P-A, Fex M, Korsgren O, Cilio CM., Experimental Immunology. (accepted for publication)
- III. **Characterization of pancreatic islets associated lymphocytes in type 2 diabetes.**, Radenkovic M, Uvebrant K, Skog O, Sarmiento L, Arvastsson J, Storm P, Bertlsson P-A, Korsgren O, Cilio CM. (submitted for publication)

Abbreviations

T1D	Type 1 diabetes
T2D	Type 2 diabetes
LADA	Latent Autoimmune Diabetes
Tregs	Regulatory T cells
nTregs	Natural T regs
iTregs	Inducible Tregs
TGF- β	Transforming growth factor- β
ZnT8	Zinc transporter 8
APC	Antigen presenting cell
CTL	Cytotoxic T lymphocyte
FoxP3	Forkhead box p3
HLA	Human leucocyte antigen
IA-2A	Insulinoma-associated protein 2
INF- γ	Interferon γ
IL-1 β	Interleukin-1 β
GAD65	Glutamic carboxylase acid
DC	Dendritic cells
GLUT	Glucose transporter
TCA	Tricarboxylic cycle
CTLA-4	Cytotoxic T lymphocyte associated-4
PPI	Preproinsulin
PLNs	Peripheral lymph nodes
IL-17	Interleukin 17
TCF7L2	Transcription factor 7 like 2
GSIS	Glucose stimulated insulin secretion
TLR4	Toll like receptor 4
ICA	Islet cell autoantibodies
NRAID	Insulin Requiring Autoimmune Diabetes
BMI	Body mass index

The history of diabetes

Diabetes mellitus is a group of heterogeneous diseases all sharing an increased blood glucose concentration due to insufficient insulin secretion, insulin resistance or both. Historically, this complex disease was generally classified as type 1 diabetes (T1D; insulin-dependent diabetes mellitus) and type 2 diabetes (T2D; non-insulin dependent diabetes mellitus) [1]. T1D is considered a disease most developing at a young age and with an autoimmune origin, which implicates a major role of the immune system in the pathogenesis. In contrast T2D, which generally develop at an older age and is believed to be caused primarily by metabolic perturbations and insulin resistance [1].

The first cases believed to be T1D, were described for the first time in an Egyptian manuscript from around 1500 BC, explaining that these patients suffered from polyuria. The word diabetes derived from the Greek word *diabainein*- “to pass through” (dia-trough, betes-to go) and as a disease, diabetes was described around 250 BC by the Greek physician Arateus as “meltdown of flesh and limbs into urine”[2]. In 1674, Dr Thomas Willis at Oxford University discovered that urine from T1D patients was sweet. Subsequently, the disease was termed diabetes mellitus (Latin word mellitus means sweet as honey). Long after, glucose in urine was used as a diagnostic marker for T1D [2].

The islets of Langerhans, were first described in 1869 by the German medical student Paul Langerhans in his theses entitled “*Beitrag zur mikroskopischen Anatomie der Bauchspeicheldrüse*” (Contribution to the microscopic anatomy of the pancreas). Although he described the islands of cells clusters throughout the pancreas, he did not explain their function [3]. In 1889, a German physician Josef von Mering and Professor Oscar Minkowski discovered that all symptoms and signs of T1D were developed after the pancreas was removed from dog [4]. In 1893, a French pathologist Gustave-Edouard named the tiny clusters of cells in the pancreas, as *les îlots de Langerhans* (Islets of Langerhans) and postulated that these cells produce secretions that are responsible for blood glucose control [3].

In 1921, a Canadian medical scientist and physician Dr. Frederic Banting and his assistant Dr. Charles Best isolated functional insulin for the first time and treated pancreatectomized dogs with bovine pancreatic islets extracts, which resulted in diabetes remission and a prolonged life span for the dogs [5]. The effect of purified pancreatic extract preparation was also tested on a fourteen years old boy, Leonard

Thompson, in 1922 at University General Hospital of Toronto in collaboration with Professor James Macleod and a chemist named James Collip. Thompson died at the age of 27, not from diabetes related complications, but from pneumonia [6]. In first human clinical trial seven patients with diabetes have been involved who received purified and concentrated pancreatic extract, what resulted with in disappearing subjective symptoms of the disease [5]. They together with pharmaceutical company Eli Lilly and Company made improved commercially available insulin in 1923 in United States and Canada. The Nobel Prize in Physiology or Medicine in 1923 was given to Banting and Best, which they shared with Best and Collip [2].

The first recombinant insulin was produced in 1982 by Genentech and Eli Lilly and since then all recombinant forms of insulin are based on recombinant technology in yeast or bacteria [2]. In 1922 Dr August Krogh from Denmark and his wife Marie paid a visit to the United States, where they learned about the discovery of insulin. Since Marie Krogh was diagnosed with diabetes, they visited Professor John MacLeod in Toronto and soon after returning to Denmark, Krogh and H.C. Hagedorn founded an insulin producing company, Nordisk Insulin Company together with Novo Company [6], today called Novo Nordisk.

T1D and type 2 diabetes (T2D) were first termed as different disorders by the Indian physicians Sushruta and Charaka in 400-500 CE [6], but a clear distinction between T1D and T2D was later described (in 1936) by Harold Percival [7].

Classification of diabetes

The general belief is that T1D arises as a consequence of an autoimmune destruction of insulin producing beta cells in the pancreas [8]. It is characterized by genetic, immunological and metabolic features, among which are the genes within the major histocompatibility complex (HLA), circulating autoantibodies against autoantigens and severe loss of insulin secretion, ketoacidosis and death if life-long treatment with insulin is not administered. T1D is mainly diagnosed in children but adults can also develop the disease [8]. It requires lifelong administration of exogenous insulin.

T2D is more common among adults and associated with ageing, family history, obesity and physical inactivity. T2D results from a combination of insulin resistance and beta cell dysfunction. This results in hyperglycemia [9]. Treatment of T2D involves diet, exercise life style changes and antidiabetic drugs, such as metformin, sulfonylureas [10].

According to the World Health Organization around 90% of all patients diagnosed with diabetes suffer from T2D and 5 to 10% from T1D [11]. Since there are cases with a T2D phenotype, which require insulin treatment and have autoantibodies, the percentage of T1D can be underestimated. Beside autoantibodies and HLA genes linked to the development of T1D, the disease can occur in adults and this form is named as Latent Autoimmune Diabetes of the Adults (LADA) [12, 13]. LADA is diagnosed by the presence of autoantibodies against beta cell antigens, in particular, glutamic acid decarboxylase autoantibodies (GADA), striking biomarkers of autoimmunity, which are reported in 5-15% of T2D patients. Interestingly, a higher prevalence of LADA patients has been documented compared to classical childhood onset of T1D in the European Study Action LADA 7 [14].

Beside these complex and multifactorial types of diabetes, a number of rare monogenic forms of this disease have been described. The most common type of monogenic inherited diabetes is named Maturity Onset Diabetes of the Young (MODY) and results in primary defects in insulin secretion with variable clinical presentations depending on the gene involved [15]. At least 13 genes have been found to be responsible for MODY phenotypes but are only 6 genes that account for the majority of cases. The most common forms of MODY are due to a mutation of the hepatocyte nuclear factor 4 α (HNF4A; MODY1), glucokinase (GCK; MODY2), HNF1A (MODY3), pancreatic and duodenal homeobox 1 (PDX1; MODY4), transcription factor 2 (TCF2) or HNF1B (MODY5), neurogenic

differentiation 1 (NEUROD1; MODY6), Kruppel-like factor 11 (KLF11; MODY 7), carboxyl ester lipase (CEL; MODY8), paired-box-containing gene 4 (PAX4; MODY9), insulin (INS; MODY10), B-lymphocyte kinase (BLK; MODY11), adenosine triphosphate (ATP)-binding cassette, sub-family C (CFTR/MRP), member 8 (ABCC8; MODY12), and potassium channel, inwardly rectifying subfamily J, member 11 (KCNJ 11; MODY13) [16]. However, in about 20% of patients with phenotypes suggestive of MODY, no mutations in these 6 genes are evidenced.

The pancreas- Islets of Langerhans Architecture

Embryologically the pancreas originates from the endodermal layer. During development the ventral pancreas together with the common bile duct rotate toward dorsal pancreas and at some point both components fuse both form the pancreas. This position ensures pancreatic and biliary secretions entering the duodenum through the ventral pancreatic duct [17]. The body and tail develop from dorsal pancreas while the head of the pancreas originates from both ventral and dorsal pancreas. The pancreas is a gland with an endocrine and exocrine part. The endocrine part is composed from small cellular clusters termed islets of Langerhans. Meanwhile, the exocrine part of pancreas secretes digestive enzymes into the duodenum involved in nutrient uptake and digestion. The islets of Langerhans contain different cell types; the insulin-producing beta cells, glucagon-producing alpha cells, somatostatin-producing delta cells, pancreatic polypeptide-producing cells and ghrelin producing epsilon cells. Human and rodent islets are different with regards to the number and distribution of insulin and glucagon producing cells. In humans, they are scattered throughout the islets along small blood vessels, while in mouse alpha cells are situated around islets and beta cells in the core. Under normal physiological conditions insulin and glucagon are the main product of endocrine part of pancreas. Both hormones play a critical role in maintaining systemic glucose level within narrow range [18].

Beta cells function and insulin secretion

The most abundant cell types in the islets of Langerhans are beta cells. They are surrounded by a dense capillary network, which is involved in dynamic interactions with beta cells pivotal for secretion of insulin into circulation. Treatment approaches directed towards beta cells are common, especially in T2D to correct inadequate function. They constitute about 60% of the endocrine pancreatic mass and they secrete insulin which is secreted in response to an increased glucose level in the circulation [18]. Insulin secretion occurs on biphasic pulsatile manner and insulin has anabolic effect in muscle, liver and adipose tissue [19]. After glucose absorption

from the food through intestine, glucose enters the beta cells by a glucose transporter (GLUTs), initiating insulin secretion process [20]. Further, glucose is metabolized to pyruvate via glycolysis reaction which enters the mitochondria and is metabolized into the tricarboxylic cycle (TCA) and mitochondrial oxidative metabolism is activated [21]. This leads to an increased ADP/ATP ratio in the cytoplasm of beta cells, what closes K^{+} channels and depolarizes the membrane, followed with opening voltage dependent calcium channels (VDCC) as consequence. As a result, influx of Ca^{2+} from extracellular into intracellular is raising, triggering exocytosis in insulin granules and releasing insulin into circulation. During this first phase of insulin secretion, insulin comes from the ready releasable pool (RRP), which is replenished from the resting pool (RP). A sustained second phase of insulin secretion is maintained through amplifying the response to glucose and transferring remaining insulin granules into RRP mediated by ATP dependent reactions [22, 23].

Alpha cells function and glucagon secretion

The glucagon-secreting alpha cells account for about 30% of the endocrine mass of the pancreas [18]. Hypoglycemia (decreased level of glucose in the blood) is the main stimulus of alpha cells to secrete glucagon. Glucagon acts on the liver to stimulate glucose release from stored glycogen through actions on gluconeogenesis and glycogenolysis. Glucagon secretion can also be stimulated by amino acids such L-arginine, gastric inhibitory polypeptide and activation of the autonomous nervous system. Beside beta cell dysfunction and deficiency of insulin, alpha cell dysfunction was also observed as a component of pathogenic mechanisms of diabetes, but this is still poorly understood [24].

Type 1 diabetes

T1D results from T-cell mediated autoimmune destruction of the insulin-producing beta cells in the pancreatic islets. The etiology of the disease remains largely unknown [8, 25]. The disease is more common in childhood but can also develop in adults [26]. The classic symptoms of T1D are polydipsia, polyphagia and polyuria [8]. Although treatable with injected insulin, glycemic control is imperfect and many patients ultimately will develop macrovascular and microvascular complications such as stroke, angina, myocardial infarction, retinopathy, nephropathy and neuropathy [8]. The incidence of the disease is increasing at a rate of 2-3% per year in the developed world with the highest incidence in Finland, Sweden, Norway, United Kingdom and Sardinia (more than 20/100000 patients per year). United States has an incidence rate of 17,8/100000 per year while Cuba has less than 3/100000 per year. China and South America has the lowest incidence (1/100000) [27-31].

Environmental factors and T1D

The autoimmune destruction of islets beta cells that leads to T1D results from a combination of genetic and environmental factors [8]. Disease-associated genes are clearly important, but numerous studies, especially those on monozygotic twins, show that heritable factors alone account for only 30-50% of disease susceptibility. In addition, the constant and rapid increase of T1D incidence rates worldwide cannot be explained by changes in the genetic background of any particular populations. Interest has therefore focused on environmental factors that might trigger and/or accelerate disease. These environmental factors include: nutritional factors (cow's milk consumption, cereals), geographic latitude, sun exposure, mean temperature breast feeding, prenatal and perinatal risk factors, national prosperity (infant mortality rate, life expectancy at birth, and national human development index) [32-34].

The environmental factors that have received most attention are viruses, some of which have a proven role in some forms of diabetes (eg, diabetes associated with congenital rubella). Other viruses, particularly the Human enteroviruses (HEVs) are

considered by many investigators to be associated with T1D, but the evidence for this remain controversial. A variety of associations with other viruses have been reported for human T1D, including mumps, rotavirus and cytomegalovirus infections [35-38].

Genetics of T1D

Human Histocompatibility Complex (HLA) locus

The first gene described to impact T1D development in the highest extent is the Human Leukocyte Antigen complex (HLA). HLA is located on chromosome 6p21. To date, are described HLA class I and class II genes where class II is associated with the pathogenesis of T1D [39]. HLA class I, is a protein expressed as a single chain and presents intracellular antigens to CD8⁺ T cells, while HLA class II molecule is expressed as heterodimer on thymic epithelial cells and antigen presenting cells that presents extracellular antigens to TCR receptor on CD4⁺ T cells. HLA molecules are important for T cell education during their development in thymus to distinguish self from non-self, ensuring peripheral T cell repertoire mediate immune response against potentially harmful foreign antigens [40].

Most of HLA genes are highly polymorphic, and in different combinations of alleles, haplotypes and genotypes HLA II are linked to the risk of T1D development. Around 30% of T1D patients have both HLA-DR3 and HLA-DR4 class II antigens with the highest risk for T1D [39, 41]. Also homozygosity for DR3 or DR4 contributes to the risk for development of T1D but in less degree compare to heterozygous. Moreover, the DQA1*0102-DQB1*0602 is linked with the protection from development of T1D [39].

Non-HLA genes

Other non-HLA loci clearly contribute to various extent to the susceptibility of T1D [41]. The major non-HLA risk gene for T1D is Interleukin 2 receptor alpha (IL2RA) located on chromosome 10p15 [42]. It encodes the alpha chain of the IL-2 receptor (CD25) and mutations as variations in expression of the IL2 receptor (IL2RA or CD25), are associated with development of autoimmune process in T1D [41]. CD25 is expressed on regulatory T (Tregs) cells and binding IL2 is responsible for proliferation of Tregs. IL2/IL2RA signaling pathway is crucial for regulation of immune response and CD25 regulates proliferation of effector T cells (Teff) in response to antigenic stimuli through CD25 signaling pathway inside Tregs [42, 43].

Another non HLA gene, IDDM2 locus with a variable number of tandem repeats (INS VNTR) is located upstream of the insulin gene found on chromosome 11p15.5 [44]. While INS VNTR class III accounts for protective effect, INS VNTR class I was associated with increased risk for T1D [45]. Moreover, the mechanism, which connect VNTR and the risk of T1D, is not clearly understood. It has been reported that VNTR class I is associated with increased expression of insulin mRNA in pancreas and thymus, while the opposite was observed for VNTR class III [45]. Next non-HLA gene was discovered 15 years ago, cytotoxic T lymphocyte associated-4 (CTLA-4) as the negative regulator of T cell mediated response [46]. CTLA-4 is located on chromosome 2q33 and a number of studies confirmed its association with T1D [47]. It shares two ligands, CD80 and CD86 and blocks their interaction with CD28, a homologous receptor [48]. CTLA-4 is expressed on both CD4+ and CD8+ T cells but on activated cells in mice and humans [49, 50]. Contrary to conventional T cells, CTLA-4 is constitutively expressed in Tregs with continuous upregulation after antigen stimulation and activation [51, 52]. Different studies have provided evidence that CTLA-4 play a role in Tregs suppressive function as well as its importance in regulating the autoimmune islets destruction in an in vivo adoptive transfer model of diabetes [53, 54]. PTPN22, located on chromosome 1p13, encodes the protein tyrosine phosphatase LYP (PEP in mice). It is expressed on T cells and negatively regulates TCR proximal signaling. Mutations of this, have been associated with susceptibility to T1D and other autoimmune diseases [55]. It has been shown that PTPN22 gene mutation in T1D patients play a role in the destruction of beta cells, by modulating cytokine secretion balance and by modulating intracellular signaling, while in Crohn's disease patients, it modulates the innate immune responses [56]. Its allelic variant C1858T is considered as the strongest non-HLA genetic risk factor for development of T1D [57].

Autoantibodies in T1D

One of major hallmarks of T1D is the reactivity to molecularly defined islet autoantigens: insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA), insulinoma-associated-antigen-2 autoantibodies (IA-2) and Zinc transporter 8 (ZnT8). Autoantibodies to other antigens have been reported, but either occur infrequently or have been inadequately validated [58-62]. These autoantibodies have been demonstrated to be markers of the islet autoimmunity that precede clinical onset of T1D [63, 64].

Insulin is a critical autoantigen specifically expressed on the beta islet cells, which is perceived as the target antigen to cause autoimmune diabetes for a long time [59]. GAD exists in two isoforms, GAD-65 and GAD-67. However, only GAD65 is expressed in the beta cells of human islets, the autoantibody response is primarily

to this isoform [65]. IA-2, is a transmembrane protein-tyrosine phosphatase-like protein belonging to an evolutionarily conserved family [66]. ZnT8 is an islet beta cell secretory granule membrane protein recently identified as an autoantibody antigen in T1D [67].

IAs are the first to appear, GADs and IAs are the most frequent islet autoantibodies in childhood, GADA is the hallmark of adult-onset type 1 diabetes, and IA-2 antigens are very specific for the development of diabetes. However, IAs and GADs are heterogeneous. Unlike GAD and IA2, ZnT8 is highly beta cell specific, and thus, ZnT8 antibodies measurements may be useful in monitoring islet destruction after onset [68-70].

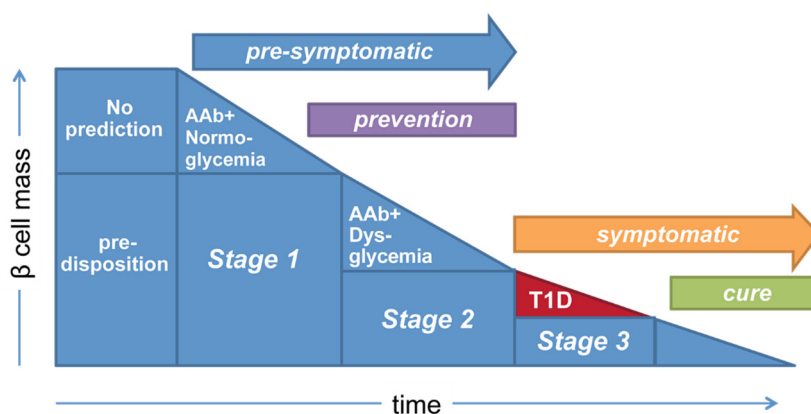


Figure 1. New T1D staging according to JDRF, the Endocrine Society and the American Diabetes Association. Adapted from Foustier *et al.*, 2016

Autoreactive T cells in T1D

Major efforts in T1D research are focused on the detection of T cell response against islet specific autoantigens in order to track ongoing autoimmune process in individuals at high risk to develop T1D and to follow disease progression as well as a biomarker to monitor the immunological effect of immunotherapy.

The main triggering event in the destruction of beta cells is still unclear. However, it has been suggested that APCs, as dendritic cells, macrophages and B cells, present autoantigens to naïve T cells through T1D susceptible HLA molecules complex, leading to priming and expansion of diabetogenic T cells and generating of autoreactive CD4+ T cells in pancreatic draining lymph nodes. Further, CD4+T cells contribute to the activation of autoreactive CD8+ T cells, which after recruitment to the islets participate in the destruction of beta cells [71]. Autoreactive

CD4⁺ and CD8⁺ T cells are present in very low frequency both in peripheral blood as well as in peri-pancreatic lymph nodes and, are capable of rapid clonal expansion upon autoantigens stimulation. These cells are part of the total T cell repertoire and they have been detected in T1D patients but also in healthy individuals [72-74]. Several differences have been described between autoreactive T cells in healthy subjects and T1D patients. The main difference is that the pool of autoreactive T cells in healthy individuals tend to have a more naïve phenotype while in T1D is more shifted towards memory phenotype [75], which, together with telomere shortening, terminal differentiation and the expression of oligoclonal T cell receptor (TCR) indicate an antigen driven expansion of autoreactive T cells [73, 76]. In line with the hypothesis that autoreactive T cells in T1D are functionally different when compared to the one detected in healthy subjects, Arif S. et al. showed that stimulation of autoantigen-specific CD4⁺ T cells isolated from blood of recent-onset T1D patients lead to a prominent pro-inflammatory response characterized with the production of INF- γ , while in healthy subjects autoreactive CD4⁺ T cells show a predominant production of IL-10 and therefore a possible immune-regulatory response [77].

To date, a variety of different assays have been developed to detect and measure the number and frequency of autoreactive T cells in patients with T1D and to evaluate their function such as cytokine production, T cell proliferation or the frequency of epitope-specific T cells using HLA-peptide tetramers and multimers. However, the standardization of such assays has been intensively debated. The difficulties to obtain pancreatic tissue and draining lymph nodes from T1D patients and high-risk subjects, the very low frequency of these cells in peripheral blood, the complexity of the assays, the inability to get enough amount of blood from pediatric samples to perform these assays as well as the low avidity of autoreactive cells are all limiting factors that challenge a comprehensive detection and evaluation of autoreactive T cells in T1D [78, 79].

More attention has been given to CD8⁺ autoreactive T cells as the main actor in the pathogenesis of T1D [80-82]. HLA class I tetramers have been used as the most valuable tools to deeper knowledge about autoreactive CD8⁺ T cells [83, 84]. Circulating CD8⁺ T cells, which recognized human preproinsulin signal peptide sequence have been isolated from recent-onset T1D patients [85]. It has been suggested that CD8⁺ T cells are implicated in beta cell damage through the secretion of perforin and granzyme B from cytotoxic granules [86]. CD8⁺ T cell reactivity against glutamic acid decarboxylase 65 (GAD65) in HLA-A*0201 subjects has been also reported [87].

Recently, simultaneous detection CD8⁺ T cells reactive against multiple HLA-A2 restricted beta cell epitopes using a combinatorial quantum dot major histocompatibility complex multimer technique (qDots), demonstrated that insulin

B (10-18), IA-2 (797-805), IGRP (265-273) were predominant epitopes recognized with islet autoreactive CD8⁺ T cells while PPI epitope (15-24) was the most sensitive [88]. Beside detection of autoreactive CD8⁺ T cells specific for different beta cell epitopes associated to T1D, a recent study performed a deeper phenotypic characterization of beta cell specific CD8⁺ T cells using tetramers and multicolor flow cytometry and demonstrated that the frequency of CD8⁺ T cells for five different HLA-A*0201 restricted specificities tested, was not different between T1D patients and healthy non-diabetic control [76]. On the other hand, deeper investigations of phenotypic characteristics of CD8⁺ T cells in T1D, revealed antigen driven expansion of beta cell specific CD8⁺ T cell compartment [76]. In addition, examining of TCR repertoire of beta cell specific CD8⁺ T cell population specific for pre-proinsulin epitope PPI₁₅₋₂₄, showed skewed oligoclonal beta cell specific CD8⁺ T cell receptor repertoire [76].

Evidences for autoreactive CD8⁺ T cell responses have been demonstrated also against other T1D-associated autoantigens like PPI, GAD65ZnT8 and IGRP [89].

A number of studies using MHC class II tetramers have shown the presence of low frequency of autoreactive CD4⁺ T cells specific for proinsulin, GAD65 and IA2 in peripheral blood of T1D patients and healthy subjects [90]. In contrast, a recent study by Yang J et al, demonstrated that HLA-DQ8 restricted CD4⁺ T cells responses against a crucial insulin epitope B:9-23 were detected only in T1D patients [91]. This finding was confirmed in another recent study by Pathiraja V et al, implicating DQ8-restricted CD4⁺ T cells in pathogenesis of T1D and indicating the importance of specific epitopes in the expansion and activation of autoreactive CD4⁺ T cells [92]. Another T1D associated autoantigen found to elicit a proliferative response of CD4⁺ T cells is ZnT8 [93]. T cells that specifically respond to ZnT8 showed a dominant Th1 cytokine profile in T1D patients while in healthy controls a more pronounced Th2 response was observed [94].

Different markers have been used to study the memory differentiation profile of T cells (i.e. CD62L, CD45RA, CD45RO, CD27 and CCR7). The T cell memory repertoire is made of central memory (T_{cm}), effector memory (T_{em}) and terminal effector subset of T cells (T_{te}) [95]. T_{cm} retains the expression of homing receptors CCR7 and CD62L which promote migration of these cells to secondary lymph nodes, while T_{em} lose expression of these markers and they are able to migrate to inflamed tissue. Autoreactive T cells with memory phenotype have been detected as a key contributor promoting recurrent autoimmunity in pancreas transplantation [96]. Moreover, the discovery of resident T cells in diverse non-lymphoid tissue showing a distinct memory phenotype from circulating T cells pool [97, 98].

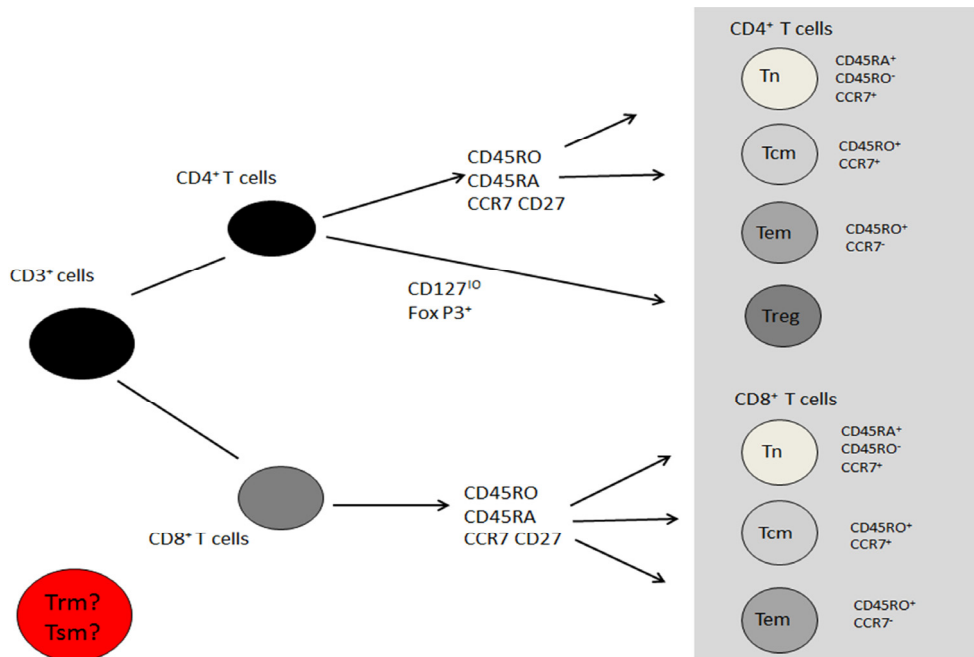


Figure 2.
Illustration of the proposed classification of memory T cells

Regulatory T cells

Regulatory T cells (Tregs) account for 5-10% of peripheral CD4⁺ T cells and are phenotypically recognized by the presence of multiple markers between which CD4, CD25 (IL-2Ra) [99], expression of the transcription factor forkhead box protein 3 (Foxp3) and constitutive expression of the activation marker CTLA-4 (CD4⁺CD25^{hi}CTLA-4⁺Foxp3⁺) are the most widely used. Tregs represent a special arm of the cellular immune response, which contribute to maintenance of immune homeostasis and immunological self-tolerance [100, 101]. In human it has been shown that most of the Tregs detected in peripheral blood express high levels of CD25 [102], however, Foxp3 has been depicted as one of the most specific markers for Tregs. Foxp3 is a crucial transcription factor important for the development and the functional capacity of Tregs [101] and was discovered in humans as the gene on the X chromosome responsible for the IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) and the phenotype of scurfy mice. Both patients and mice develop a multi-organ autoimmune disease as consequence of absence or lack of functional Foxp3 and a lack of a normal Tregs repertoire [103]. The observation that beside CD25, also

Foxp3 can be expressed on recently activated T effector cells (Teff) cells [104], rendering the identification of canonical Tregs in peripheral blood more challenging, has led to the identification of additional markers to be employed for the characterization and isolation of Tregs like CD127 [105], CD62L [106], CD45RA [107], CD27 [108], CTLA-4 [53]. In addition, the discovery that, the Foxp3 gene is heavily demethylated on Treg (Tregs- specific demethylated region, TSDR), but methylated on Teff cells, has enabled a better characterization of these cells at the molecular level [109].

The regulatory functions through which Tregs suppress the activation and proliferation of autoreactive Teff cells appear to occur via different direct and indirect mechanisms [110]. Beside the suppressive effect on autoreactive cells, they inhibit or regulate immune response via other mechanisms like competing for growth factors, cell-cell contact, secreting of suppressive cytokines like TGF- β , IL-10 and IL-35 [111, 112], killing or modification of professional antigen presenting cells [113] and converting Teff into inducible Tregs after stimulation with immunomodulatory molecules like TGF β [114]. Most of the Tregs are naturally occurring and differentiate in the thymus are called natural Tregs (nTregs) to differentiate them from a subset of Tregs which is generated in the periphery (pTregs) or induced *in vitro* from CD4+CD25- T cells stimulated with tolerogenic cytokines such as TGF β (iTregs) [115].

Beside phenotypic characterization, assessment of Tregs function is generally evaluated with different assays for measuring the capacity to inhibit proliferation or secretion of pro-inflammatory cytokines of Teff cells *in vitro* [116]. Disruption of balance between immune regulation and immune response due to impaired function and number of Tregs leads to development of autoimmune diseases [100, 117].

Regulatory T cells in T1D

To date the role and involvement of Tregs in T1D pathogenesis is not clearly elucidated and there are controversial data published regarding their number and function during the natural history of the disease. For example, low number of resting CD4+CD25+ T cells with immunoregulatory properties in PBMC of T1D patients has been reported in one study [118], while another was not able to demonstrate any difference in the frequency of these cells in T1D as in control healthy subjects. However, the suppressive capacity of those cells to inhibit T cell proliferation *in vitro*, was diminished [119]. In contrast to both previous studies, Putnam Al et al. did not find significant differences in the number and function of CD4+CD25^{high} population between T1D patients and healthy individuals [120]. In line with the latter study, no altered frequency of CD4+CD25+Foxp3+ T cells was observed between T1D and first-degree relatives [121]. On the hand, transient defect

in suppressive capacity of CD4+CD25+Foxp3+ T cells was observed 9-12 months after diagnosis of T1D, while elevated proliferation of these cells correlated with increased IL-17 and TNF- α but not INF- γ , suggesting two independent dysfunction within this population of cells in T1D patients[122].

Recently many efforts have been made to characterize human tissue-associated Tregs and Teff cells in pancreatic islets and lymph nodes of T1D patients to better understand the role of Tregs in the disease process. A recent study performed a phenotypical and functional characterization of Th17 cells and Tregs residing in lymph nodes (PLNs) of T1D patients and healthy control subjects [123]. The results of this important investigation, revealed a functional defect of CD4+CD25hi Tregs and an expansion of pro-inflammatory Th17 cells of T1D subjects with long disease duration, which was not observed in peripheral blood, suggesting that while Th17 T cell responses predominates at a later stage of the disease Tregs function decrease with time [123].

Cumulatively, these findings indicate that the results obtained in peripheral blood do not necessarily mirror the phenotype and function of Tregs and Teff locally in the target tissue and draining lymph nodes and that this should be taken into consideration when evaluating these T cell phenotypes as biomarkers of disease progression.

Another very intriguing the hypothesis that in autoimmune diabetes an increased sensitivity to apoptosis could contribute to Tregs deficiency, has been challenged by Jilwala et al., who reported an higher induction of apoptosis *in vitro* of peripheral blood Tregs of T1D patients [124]. These data suggest that in T1D Tregs might be intrinsically more susceptible to apoptosis-induced signals and resulting in a progressive impairment of immune-regulation during chronic to self-antigen exposure. The finding is also strengthened by similar experiments performed on intra-islets Tregs of NOD mice, demonstrating that pancreatic Tregs were more sensitive to apoptosis, resulting in diminished control of autoimmune responses [125].

Recently using a pool of six genes, inclusive Foxp3, a genetic signature of Tregs has been proposed to be associated with recent-onset T1D [126] representing an important progress in the field that may lead to the possibility of better monitor Tregs in T1D.

Tregs therapy in T1D

IL-2 is a fundamental cytokine for the survival, expansion and function of Tregs, which is a very important cytokine that promote Tregs expansion and function and it has been shown to be decreased in peripheral blood of T1D patients [127]. This

abnormality was ascribed to a defect in signaling via IL-2R [127]. Based on this discovery, several investigators have proposed to test the administration of low doses of IL-2 in clinical trials with the aim to stimulate and selectively expand of Tregs *in vivo*. In a first pilot study, administration of low doses of IL2 to adult patients with T1D induced a dose dependent increase of CD4+ and CD8+T cells and Foxp3+ Tregs with increased CD25, CTL4, GTLR and STAT5 markers, therefore obtaining a proof of concept of the safety and efficacy of these treatment in T1D [128].

A non-cell-based approach to increase Tregs function *in vivo* could be represented by the administration of Rapamycin (Sacrolimus), which has been shown to increase the suppressive activity of these cells [129], however this treatment has also been implicated in the development of relevant clinical adverse events and its use in T1D pediatric populations has been criticized.

Cell-based therapy is a more promising approach in the treatment of autoimmune disease. Infusion of autologous Tregs expanded *ex vivo* in T1D patients was recently tested showing a transient increase of long-lived Tregs in the recipient patients [130].

Insulinitis in T1D

Although insulinitis (the presence leucocytes infiltrate in and surrounding the pancreatic islets) has been classically defined as hallmark of autoimmune diabetes [131], in humans this pathological finding is still debated [132]. One of the main reason for the lack of systematic analysis of the histological features of T1D arise from the peculiar anatomical localization of the pancreas in the retroperitoneal cavity, which has hindered the use of longitudinal biopsies during the natural history of the disease. Most of the *in situ* studies have been performed using retrospective samples post mortem or biopsies during surgery of T1D patients, therefore the information about the initial pathological cellular process and the type of inflammatory components in the pancreas of T1D patients is very limited.

Insulinitis was described for the first time in 1965 by a study of Willy Gepts on autoptotic material from patients under 30 years of age newly diagnosed with insulin-deficient diabetes [133]. In this study, he reported a predominant lymphocytes infiltration within the islets of 68% of the cases. A reduced number of B cells was observed in the islets of Langerhans which were often large size, while B cells were almost in all cases absent in patients with long duration of diabetes and the islets of Langerhans were smaller with atrophic cells [133]. Similar findings about insulitis were later reported by Foulis et al. also using autoptotic specimens from recently diagnosed T1D patients [134]. He observed that insulitis was present in 23% of islets containing insulin, but only in 1% of islets which were insulin deficient, suggesting

that insulinitis is a sign of immune mediated destruction of beta cells [134]. Bottazzo et al. reported that lymphocytes with predominant CD8+ T cells were found in the insulinitic lesions of a 12-year-old girl died within 24 hours of diagnosis with heterogeneous insulinitis distribution through the whole pancreas, and in line with previous studies mostly affecting insulin containing islets [135].

Applying modern immunohistology technique in order to gain deeper understanding of a quantitative and phenotypic cellular composition of insulinitis and its relation with beta cell destruction, Willcox et al. reanalyzed pancreatic specimens investigated in a previous cohort [136]. In line previous studies, he reported CD8+ T cells as predominant cells in insulinitic lesions, showing positive correlation with beta cell decay (measured based on the percentage of insulin positivity in islets) and their absence after destruction of all functional beta cells. Interestingly, B cells were at low numbers in early insulinitis, but increased through all stages of beta cell decline [136]. Macrophages CD68+ were described in large number but constantly, as CD4+ T cells although at lower number. Foxp3+ Tregs cells were detected only in islets of one single donor [136]. Since immune cell infiltration in the pancreas can be caused after prolonged life support of patients in intensive care units without having diabetes [137], one important issue has been to define insulinitis according to the number of immune cells present in the lesions in order to distinguish inflamed from non-inflamed pancreatic tissue. Recently a new consensus paper defined insulinitis as three or more islets containing 15 or more lymphocytes often focally aggregated (peri-islets infiltration) or/and within the islets (intra-islets infiltration) [138]. The DiViD study (Diabetes Virus Detection) used another approach to investigate the pathology of the pancreas by using pancreatic biopsies from living patients at the onset of the T1D [139, 140], demonstrating that all six patients included in the study had insulinitis according to the new definition. Another study from Imagawa et al, confirmed this finding but in larger cohort in which, 17 of 29 new onset T1D patients included, had a predominant T cell infiltration of the islets [141]. Although these findings are in line with previous reports on autopsy specimens, one limitation of this approach could be relate to the limited pancreatic region analyzed. One old study from Wagner et al, examined pancreata from non-diabetic patients positive for GADA and ICA autoantibodies without finding evidence of leucocytes infiltration in the islets [142]. Recently, a Japanese study using biopsies of living recent new-onset T1D patients, showed a positive correlation between insulinitis and the presence of a single autoantibody, GADA or IA2 and demonstrated that CD8+ T cells and macrophages were the predominant cell inside the insulinitic lesions, with a low number CD4+ and CD20+ B cells [141].

Although, most of the previous studies have observed a predominance of CD8+ T cells in islet infiltrates, their role in destruction of beta cells remain rather unclear. A recent study on pancreatic tissue from a US-based large bio-bank of pancreas and diabetes related tissues from organ donors with T1D patients and autoantibody-

positive subjects (The Network for Pancreatic Organ donors with Diabetes, nPOD) demonstrated the presence in the islets of T1D patients of beta cell-associated autoantigens-specific CD8+ T cells *in situ* using MHC class I tetramer staining, supporting the notion of CD8+ T cell-mediated destruction of beta cells [143]. In another study, Pathiraja et al, using isolated CD4+ T cell clones from pancreatic islets of an organ donor with T1D, demonstrated their autoreactive nature against HLA class II-restricted proinsulin epitopes [92]. Although, less attention has been given to B cells and their role in diabetes pathogenesis, a recent study on autptic samples from recently diagnosed T1D patients, while confirming the enrichment of CD8+ T cells within the isulitic lesions also highlighted two different type of insulitis based on the number of B cells [144]. Donors with high numbers of islets-associated B cells (CD20Hi) correlated with a more aggressive autoimmune pathology ad early age of diabetes onset in contrast to donors with low numbers (CD20low) who were diagnosed at later age and showed a less aggressive disease phenotype (aged 13 years and older) [144]. It is well known that individuals with multiple autoantibodies have a higher risk to develop T1D compare to one with single autoantibody [145]. Since autoantibody-positive donors are rare in the general population, previous studies were conducted on small sample size giving scarce information about relation of autoantibody response and pancreas pathology. In order to gain deeper understanding how autoantibodies are related to the presence or absence of insulitis, the nPOD consortia reported that from recovered pancreata of 18 collected autoantibodies positive donors, 13 donors a single positive (GADA) did not have insulitis and high risk HLA genes. In other 5 donors with multiple autoantibodies, the presence of insulitis and high risk HLA genes for development T1D were recorded [146].

A large retrospective study screened 1507 islet donors for the presence of circulating diabetes-related autoantibodies ICA, GADA, IA2A and IAA [147]. The investigators were able to identify 62 autoantibodies positive donors and showed that insulitis was present only in 2 donors positive for ICA, GADA and IA2A and carrying the HLA-DQ high risk genotype [147]. In contrast, a recent Scandinavian study reported that out 32 autoantbody-positive donors among which 9 were multiple autoantibodies positive, none of them showed sign of insulitis [148].

Another important confirmatory finding generated through the nPOD consortia is the presence of insulitis mostly in insulin-positive islets (33%) while rarely in insulin-negative (2%) [146]. Interestingly, both the nPOD and DiViD consortia confirmed that in young children with recent onset of T1D 40-60% of pancreatic islets were found to contain insulin positive cells, while in adult between 18%-66% were stained positive for insulin. This data clearly challenge the common dogma that 90% of the beta cell mass is lost at the time of diabetes onset [146].

Recently, Rodriguez-Calvo et al. for the first time reported the presence of inflammatory infiltration also in the exocrine tissue of T1D patients, T2D and autoantibody positive subjects [149]. CD8+ T, CD4+ T and CD11c+ dendritic cells were present in high number in exocrine tissue but without visible sign of insulitis in T1D and autoantibodies positive individuals. Interestingly, CD8+ T cells were the dominant cell population in all groups except in autoantibodies positive individuals and T1D with a short duration of disease where a dominant proportion of CD11c+ cells and CD4+ T cells [149]. This is in line with results published by Wilberg et al. and pinpoint a possible role of the exocrine pancreas in the early stage of diabetes pathogenesis [148].

Type 2 diabetes

T2D, is the most common form of diabetes and the disease is a worldwide health problem. According to the latest estimate by The International Diabetes Federation (IDF) (2013), 382 million people had diabetes and by the year 2035 this number will rise to 592 million [150]. T2D is a complex disease influenced by the interaction of predisposing genes and an unhealthy lifestyle associated with obesity [151].

T2D is considered a metabolic disease characterized by hyperglycemia, insulin resistance of peripheral glucose-sensitive tissues and at later stages even defective pancreatic insulin secretion and altered suppression of glucagon production contribute to disease [11, 152-154]. Since hyperglycemia develops gradually, T2D is often diagnosed long after the first symptoms appear clinically evident. The risk to develop T2D increases with aging, in obese and physically inactive people [155, 156]. In patients affected by insulin resistance, islet beta cells compensate the metabolic demand by expanding their mass to be able to secrete more insulin and overcome hyperglycemia [157, 158]. As a result blood glucose is more or less maintained until beta cells failure will develop [157, 159]. However, beta cells may still be present in the islets of Langerhans, and it has been hard to prove that the pancreas of T2D patients have decrease beta cell mass although beta cell function can be impaired. Consequently, a decreased number or non- functional beta cells are not able to compensate for insulin resistance, thus hyperglycemia evolves [156, 160] and T2D progresses. Insulin resistance may be improved by weight reduction, exercise and/or pharmacological treatment of hyperglycemia [161-163]. Beta cell dysfunction has been largely investigated in T2D, the role of glucagone-secreting alpha cells is less clear. Already in 1975 Unger and Orci observed that altered glucose homeostasis in T2D may also involve alpha cell dysfunction [24]. Usually in T2D insulin deficiency is accompanied by increased glucagon levels in the circulation, thereby possibly participating in the development of glucotoxicity (toxic effects of hyperglycaemia) [164, 165]. So far, mechanisms involved in defective glucagon secretion are not clearly understood. It has been suggested that increased glucose concentrations elicit an increased insulin secretion from beta cells, which in turn cause suppression of increased glucagon secretion after meal. Other stimuli have been described to elicit elevated production of glucagon such as protein-rich food and arginine [166, 167]. The etiology of T2D is still not completely understood and autoimmune destruction of beta cells as a component of the disease process has

not been shown yet, but increasing evidences points to a local inflammation as a key component in the pathogenesis of T2D [168].

Genetics of T2D

T2D is a polygenic disease. The results of large genome wide association studies (GWAS) identified more than 40 genes associated with increased risk to develop T2D and highlighted the complexity of the genetic inheritance and of the possible gene-environment interaction involved in the disease process [151]. Depending on the genetic predisposition, around 20% of obese insulin resistant individuals develop T2D [156]. A strong association to a specific susceptibility genetic locus predisposing to T2D, the transcription factor 7-like 2 (TCF7L2) gene was first identified in 2006 [169]. TCF7L2 regulates proglucagon gene expression in entero-endocrine cells via the Wnt signaling pathway [169]. This discovery is confirmed by other studies in Europe, Asia and Afrika [170, 171]. Although this gene does not seems to be associated with childhood T1D [172], it has been suggested that TCF7L2 gene product influence beta cell survival, proliferation and insulin secretory capacity [173, 174].

Several other studies have discovered other genes, which are associated with T2D risk. For example, a polymorphic allelic variant of the MTNR1B gene (rs10830963), which encodes a melatonin receptor expressed in the brain but also in pancreatic islets, has been shown to predict the development T2DM [175]. Another important gene polymorphism involved in T2D risk is located within the GCK gene (rs1799884), which encodes for a glucokinase. GCK gene variants was also shown to associate with impaired glucose regulation [176, 177]. Furthermore, the G-allele polymorphism of the GCKR gene (rs780094) encoding for a regulatory protein of GCK have been also associated with T2D risk [178]. Some of the identified genes are important for beta cell mass and other for beta cell function, but underlying molecular mechanisms how they predispose T2D are still not entirely clear [179].

Islet inflammation in T2D

Several different mechanisms including glucotoxicity, lipotoxicity, oxidative stress and endoplasmic reticulum stress underlies impaired insulin secretion and beta cell dysfunction [180]. All of these processes are also connected to inflammation [181]. Recent studies have brought attention to islet inflammation as a component of T2D

pathology in rodents and humans [182, 183]. Histological studies in human pancreatic islets has demonstrated inflammatory changes, like amyloid deposition [184], immune cell infiltration [182], cell death and fibrosis [185] associated with T2D development. A better understanding of the local pancreatic islets immunity in T2D subjects and their association with beta cell function was previously limited by difficulties to access pancreatic tissue of T2D for research. While the concept of insulinitis was well established long time ago in T1D, recently there are increasing evidence pointing to insulinitis in T2D patients [186]. One of the original first publication which suggested that islet inflammation contribute to T2D pathogenesis, described non-autoimmune mechanisms, where high glucose concentration *in vitro* induced secretion of IL-1 β from isolated human islets. In fact, increased IL-1 β protein expression was observed in islets from pancreatic sections of T2D human subjects and from the hyperglycaemic T2D model, *Psammomys obesus* gerbil [187]. An up-regulation of pro-inflammatory cytokine IL-12 as well as chemokines CCL2 and CCL13 has been described in islets and peripheral blood of T2D patients [187, 188], supporting the hypothesis about connection inflammation to the pathogenesis of T2D.

The pathogenic role of the immune system in the development of T2D is supported by published clinical studies where beta cell function in T2D patients was improved by IL-1 inhibition [168] and later supported by another study that found that interleukin-1 receptor antagonist (IL1Ra) administration improves beta cell function without affecting insulin resistance [189, 190].

The presence of macrophages within islets of human and mice was reported under normal physiological condition [191] and suggested that they might contribute to proper expansion of beta cells mass during embryonic development and after birth [192, 193]. Moreover, two recent studies revealed the presence of macrophage infiltration in T2D patients compared to non-diabetic controls and similar findings was observed in glucose intolerant mice (a high-fat diet C57BL/6) and in other rodent models [182, 194]. Interestingly, it has been demonstrated in the Cochen rats, an animal model for T2D that decreased glucose stimulated insulin secretion (GSIS) in isolated perfused pancreas is a consequence of lipid accumulation and macrophage infiltration in the exocrine pancreas [195]. Macrophage and granulocyte infiltration in and around islets followed by islets fibrosis was also detected in the Goto-Kakizaki rat (GK), a spontaneous model of T2D [196]. All together, these suggest that an inflammatory islets milieu may promote macrophage accumulation for instance via toll-like receptor 4 (TLR4) signaling and secretion of chemokines [197]. A more detailed characterization of immune cell types in the pancreas of T2D donors, revealed an increased number CD45+ cells with predominant CD20+ B lymphocytes [198]. Individuals with T2D have an increased absolute number of CD11b+ myeloid cells as CD3+T cells [198]. In another study,

T2D and control healthy subjects did not differ regarding CD68⁺ macrophage numbers, but differences may be due to sample size [199].

Circulating T helper 17 (Th17) and Th1 cells are increased in T2D patients, but Tregs cells are decreased which indicate that pro-inflammatory cytokines participate in development of inflammation in T2D [200]. Recent discoveries of the islets-specific T cells as autoimmune components in T2D patients indicate the need for stratification of diabetes based on autoimmune status [201]. The presence of circulating autoantibodies, what was considered as a hallmark of autoimmunity has been reported in T2D patients [12]. Moreover, recently the presence of islet reactive T cells [201, 202] in T2D patients have been associated with more severe beta cell dysfunction, compared to beta cell dysfunction found in islet-autoantibody positive T2D subjects without reactive T cells detected [203]. This suggests that T2D could be considered an auto-inflammatory disease [180]. Subset T2D negative for autoantibodies but positive for islet reactive T cells have been selected [204]. To date, it is not known how islet autoimmunity associates with T2D pathogenesis, which subset of islet reactive T cells are involved in destruction of T2D islets as their memory status and relation to beta cell function.

Latent Autoimmune Diabetes of the Adult

Latent autoimmune diabetes of the adult (LADA) describe a form of diabetes observed in patients with T2D, which are positive for islet-associated autoantibodies do not require insulin treatment at diagnosis but progress towards insulin deficiency within few years [205, 206]. Indeed, at the time of diagnosis LADA patients are almost clinically identical to T2D patients, although they are generally younger than T2D patients. Beside lower residual beta cell function in LADA patients compared to T2D patients, it has been also observed that the patients that achieve good metabolic control, do not exhibit declined beta cell function reflecting the strong metabolic component of the disease [207]. Moreover, LADA patients exhibit lower BMI, blood pressure and triglyceride levels as well as less insulin resistance compared to T2D [208-210]. To date, a specific therapeutic strategy for LADA patients does not exist and so far is based on clinical opinion. However, several international efforts are ongoing to establish better guidelines for LADA treatment that take into consideration not only insulin resistance but also insulin deficiency in order to preserve beta cell function, treat hyperglycemia and decrease the development of severe complications in LADA patients.

As described in more details below, LADA is associated with the same HLA genes as T1D reinforcing the immunological basis of the disease [211, 212]. The frequency of LADA among adult-onset diabetes in Caucasian populations have been calculated to be around 4 to 12% [14, 213, 214], while childhood and adult onset T1D have similar incidence in European countries with high incidence of T1D [215]. It is very interesting that in China, where is the incidence of T1D low, LADA is the most common entity of diabetes [216]. The frequency of LADA is higher in northern Europe [206, 217] than in southern Europe, Asia and North America [218, 219].

Probably the first description of this form of diabetes was reported in 1977 by Irvin et al., in a group of patients with diabetes treated with oral hypoglycemic agents that resulted positive for the presence of islet cell autoantibodies (ICA) other organ specific autoantibodies that required insulin treatment [220]. A slow autoimmune mediated form of diabetes of the adults, which initially does not require insulin treatment was thereafter been confirmed by Groop et al. [221]. However, it was in

1994 that Zimmet et al. and Toumi et al. coined the term Latent Autoimmune Diabetes (LADA) for this group of diabetic patients, which shows clinical features of both T1D and T2D [205, 206]. From this point, many scientists have proposed diagnostic criteria for LADA [222, 223].and different terms have been used to describe this type of diabetes like 1,5 diabetes, double diabetes, slow progressive insulin dependent diabetes and adult-onset autoimmune diabetes. Today LADA is diagnosed based on three major criteria: 1) the presence of circulating islets autoantibodies, to separate LADA from T2D 2) age at onset of diabetes above 30 years; 3) insulin independence for at least 6 months after diagnosis, to distinguish LADA from typical T1D [211, 224]. However, the timing of initiation of insulin treatment as criteria in order to separate LADA from T1D is still debated since the decision of the treating doctors to initiate insulin therapy depend more on clinical opinion not always reflecting the pathophysiology of the disease [225].

Although, there is no clearly defined consensus to the definition of LADA, the disease, beside the presence of autoantibodies, a period without insulin treatment (at least six months) and age at onset less than 30 years of age seem to be the strongest features for the diagnosis of the disease [226].

Genetics of LADA

Beside similarities regarding autoantibody status between T1D and LADA, genetic are shared with T1D but also with T2D in LADA patients. This again rise the questions if the pathogenic mechanisms of LADA are unique or a mixture of T1D and T2D forms. The transcription factor 7-like 2 (TCF7L2) the strongest T2D association, and was also observed as a genetic risk factor for development of LADA. Bakhtadze et al., showed that common variants of this gene was useful to separate autoimmune from non-autoimmune diabetes in the age range of 15-34 years old patients age but not in groups of patients aged 40-59 [227]. On the other hand, LADA shared genetic features with T1D where HLA, PTPN22, STAT4, CTL4, IL2RA and INS were detected during genotyping of LADA subjects [228, 229].

With the aim to identify a more sensitive marker for prediction of early insulin treatment, Maioli et al., tested LADA subjects for the presence of GAD65 and other organ specific autoantibodies including HLA classification. The data from this study suggest that autoantibodies in association with a high-risk HLA genotype that predicts development of insulin dependence. This is in contrast to the low risk HLA genotype in LADA [219]. The major determinant as high risk genes for T1D, HLA class II HLA-DRB1*03 and HLA-DRB1*04 are described in most LADA cases [230]. Another study revealed that LADA had an increased frequency of HLA-

DQB1 and PTPN22 risk genotypes and alleles compared with T2D subjects, but the frequency was significantly lower compared with T1D (>35y) patients [231].

The risk genotype in TCF7L2 correlates with low GAD65 titers, suggesting the possibility to use GAD65 titer to classify LADA in two subgroups with high/low titer of GAD65 with specific autoimmune and genetic signatures for each group [232]. Moreover, IA-2 was identified as a sensitive marker for detection of LADA subjects and was positively correlated with susceptible HLA haplotypes and a higher frequency of autoimmunity [229]. Non-Insulin Requiring Autoimmune Diabetes (NRAID) Study 6 reported a higher frequency of organ-specific antibodies in LADA with high titer GAD65 autoantibodies [233]. Zhou et al., reported in a large multi-centric study of LADA subjects that patients with low GAD65 titers were similar to T2D regarding sex, age, HbA1c, beta cell function and other metabolic components at diagnosis. They differ from LADA patients with high GAD65 titer that had lower beta cell function and BMI, were younger and associated with T1D risk HLA genetic features. This study brings focus to the need to define new criteria to distinguish LADA from other forms of diabetes, since single antibody at low titer can be caused by false positivity and insufficient sign of beta cell autoimmunity.

Immunology of LADA

A critical immunological parameter that defines LADA patients is the presence of autoantibodies. ICA (Islet Cell Autoantibodies) was the first type of autoantibodies discovered in the serum of LADA patients and it has been shown to poor clinical application since the assay standardization for the measurement of this antibody is very difficult. In general, autoantibody criteria alone are not specific to identify LADA patients within the large T2D population since they are also strongly linked to T1D. Today the major autoantibodies used for separation of LADA from other form of diabetes together with other clinical and metabolic criteria are GADA and IA-2A and in a lesser extent ZnT8A [234-236]. Overall however, GADA65 is the most common and persistent autoantibodies compared to other autoantibodies, which tend to disappear with time (IA-2A and ZnT8), and it can be detected up to 12 years after diagnosis of LADA [237, 238]. Recently, it has been described that LADA patients with high titers and affinity of GADA develop a more rapid decline of beta cell function compared to patients with low GADA affinity, and it has been proposed that early insulin treatment might be beneficial to protect beta cell loss in LADA patients with high GADA titer [239].

Cellular immunology has not been widely investigated in LADA. An interesting study described a significant decrease of the expression of FoxP3 mRNA in CD4+

T cells in LADA proposing a similar possible defective function of Tregs as proposed in T1D [240]. Beside defective expression of FoxP3 at RNA level, epigenetic mechanisms can also contribute to the defective expression of this important transcription in Tregs of LADA patients. To support this hypothesis Li et al. demonstrated a hyper-methylation the FoxP3 promoter region in CD4⁺ T cells of LADA patients [241].

The European action LADA cohort study (Action LADA 6) points to that LADA patients are indistinguishable from T1D regarding cellular INF- γ and IL-13 response upon mitogen and recall stimulation of T cells [242]. This is in line with previous findings showing no difference in systemic cytokine like IL-1RA, IL-6, TNF- α [243] and chemokine concentration between T1D and LADA [244]. Another study compared T cell reactivity to GAD65 and reported higher levels of INF- γ producing cells against GAD65 in LADA patients in comparison to T2D [245]. The importance of detecting T cell reactivity in LADA subjects was emphasized with recent discoveries where autoantibody negative T2D patients had a significantly increased T cell reactivity to islets antigens [204]. But so far, there is no evidence of a pathophysiological significance of T cells. Recently, using pancreatic scintigraphy in order to detect the existence of insulinitis, it was demonstrated that pancreatic uptake of radiolabeled IL-2 with (99m)Tc ((99m)Tc-IL-2) was higher in LADA patients than in T2D. This was similar to what was observed in T1D subjects at diagnosis [246]. The role of T cell in LADA has been further substantiated by the study of the pancreas of a 65-years old woman diagnosed with T2D and presenting high titers of GAD65 autoantibody and a significant remaining beta cell mass [247]. T cell infiltration were clearly observed in the pancreas of this patient with, CD4⁺ T cells been more frequent than CD8⁺ T cells, a low numbers of NK cells and macrophages and no detectable B cells, again pointing towards a cell-mediated pathogenesis of LADA [247]. Finally, our group revealed a decrease in NK cell frequency in peripheral blood of LADA patients and an aberrant expression of the activation receptor NKG2D of the inhibitory receptor KR3DL1 suggesting that defective frequency and function of NK cells might contribute to the cellular immunological mechanisms underlying the pathogenesis of LADA [248].

Aims of the thesis

Specific aims:

- To investigate the phenotype and frequencies of peripheral circulating regulatory T cells in LADA patients prior to insulin deficiency.
- To characterize the phenotype and frequency of islets-associated lymphoid cells in human pancreatic islets of non-diabetic organ donors.
- To characterize the memory differentiation profile of islets-associated CD8+ and CD4+ T cells in human pancreatic islets of non-diabetic organ donors.
- To investigate the phenotype and frequencies of islets-associated lymphoid cells in T2D organ donors.

Material and methods

Human pancreatic islets

Significant progress has been made in understanding of pathogenesis of diabetes by using new methods for isolation and characterization of islets from non-diabetic and diabetic organ donors. In our studies, pancreatic islets from brain dead organ donors, were obtained from the Nordic Center for Clinical Islets Transplantation in Uppsala, Sweden through Human Tissue Laboratory at Lund Diabetes Center, Malmö, Sweden. The islets were isolated at Uppsala University by using a digestion-filtration method described previously [249] and approved by the Local Ethics Committee. All subjects included in this study had given consent to donate organs for medical research. Islets were cultured prior RNA and single cell suspension preparation. Human islets were dissociated to single cell suspension and used for flow cytometry analysis to characterize phenotype of immune cells residing islets.

Flow Cytometry

Flow cytometry is an advanced laser-based technology that is used to phenotype and characterized different immune cells based on their expression of cellular markers. Flow cytometer is defined as an instrument that can measure physical and multi-color fluorescence properties of the single cells from suspension flowing in the stream. The major component of flow cytometry is a fluidic system, which is responsible for transportation of the sample through the instrument by hydrodynamic focusing where the single cells are aligned in the core stream surrounded by sheath fluid stream while traveling. When the focused laser beam hit the single cells within the sample stream, light and fluorescence is generated. Afterwards, this signal is converted by the electronic system, into electronic signals.

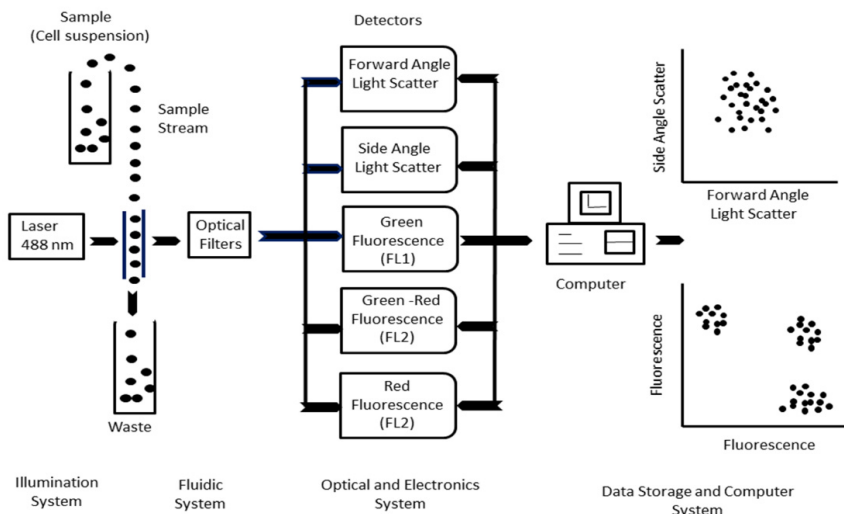


Figure 3.
A schematic interpretation of flow cytometry components.

Generated light after the laser beam strikes a single cell, can be scattered by the cell in all directions or absorbed by surface molecules on the cells or inside the cells. Reflected light is detected in two different directions: forward direction of the light beam (FSC, forward scatter) providing information about cell size and 90° from the axis of light beam (SSC, side scatter) gives information about granularity and complexity of the cells. If the cell is labeled with fluorochrome, after the laser beam hits the cell, the fluorochrome can absorb the light energy and emit it in the form of light but with a higher wavelength. A series of mirrors and filters are employed to optically separate different fluorescent emissions and fluorescence is detected simultaneously as FSC and SSC. The filter collects light within the range of wavelengths specific for each fluorescent channel and after direct to PMT for conversion into electronic signal, which is processed and analyzed digitally by computer software. The ability to detect cell surface and intracellular components using fluorochrome-conjugated fluorescent reagents, allow us to analyze a specific population of cells inside heterogeneous populations. Moreover, because of spectral overlap during multicolor analyses, spillover in a non-specific channel is often an issue, which can be solved by using compensation. Compensation is the process that electronically eliminates spectral overlap between different channel, using mathematical algorithms. After compensation, heterogeneous populations of cells can be visualized in a single dot plot and dissected morphologically and immunologically based on fluorescently labeled surface/intracellular markers [250, 251].

Immunohistochemistry

In paper I we used immunohistochemistry to determine the presence and distribution of CD8+ T cells in human pancreatic islets. Immunohistochemistry is a method used to detect proteins or antigens in different tissues. The principle of the method (indirect immunofluorescence) is to add a primary antibody binding specifically to a protein, followed by addition of secondary antibody conjugated to a fluorescent molecule with specificity for the species in which the primary antibody was produced. From non-diabetic donors, pancreatic biopsies were embedded in paraffin before being sectioned into thin slices. The sections were stained for CD8+ as described in Paper I. Fluorescence images presented in Paper I was captured using fluorescence microscopy.

RNA sequencing

RNA-sequencing (RNAseq) refers to the use of the new well established deep sequencing technologies. In general, RNA molecules are converted to a library of cDNA fragments with adaptors anchored to one or both ends. Each molecule is then sequenced to obtain short fragments from one end (single-end sequencing) or both ends (pair-end sequencing). Following sequencing, the resulting reads (typically 30-400 bp) are either aligned to a reference genome or reference transcripts, or assembled *de novo* without the genomic sequence to produce a genome scale transcription map that consists of both the transcriptional structure and/or level of expression for each gene. In paper II, we measured RNA expression for insulin and glucagon genes from non-dissociated islets by RNA seq. In these studies, total RNA extracted from human islet donors was converted to a library of cDNA fragments. RNAseq library was sequenced on an Illumina HiSeq 2000 to a specific depth (an average depth of 32m read pairs/sample). These reads were aligned to the reference transcriptome and gene expression was estimated as Fragments per Kilobase of transcripts per Million mapped reads (FPKM) by the Rsem software.

Glucose-stimulated insulin secretion assay in human pancreatic islets

In paper II and paper III, beta-cell function of human pancreatic islets was assessed by determining of insulin secretion *in vitro*. Isolated islets were handpicked under a stereo-microscope and stabilized for 1h at 37 °C, and then incubated under the same

conditions with either 1mM to assess basal insulin secretion or 16.7 mM glucose to determine stimulated insulin secretion. The islets then were incubated additional 1 hour at 37 °C and immediately after incubation insulin release was measured by colorimetric ELISA (Mercodia, Uppsala, Sweden) using two different antibodies against insulin, one plate-bound and one labeled with peroxidase. Glucose stimulated insulin secretion was expressed as ng/islet/hour.

Statistical analyses

Statistical analysis was performed using Prism 6 (Graphpad software, San Diego, CA, USA) and SPSS 20 for Windows (SPSS, Chicago, IL, USA). In paper II and III, linear regression analysis was used to analyze an impact of purity as covariate on p-values. Correlations were analyzed using parametric Pearson correlation test as well non-parametric Spearman correlation test. In paper I, Mann-Whitney two-tailed U test was used for unpaired observation. For comparing several groups, Bonferoni post hoc test was used. A p-value of less than 0.05 was considered significant. All the data are presented as mean \pm SD.

Results and discussion

Altered regulatory T-cell phenotype in latent autoimmune diabetes of the adults (LADA) (Paper I)

While in T1D Tregs have been described as an important player in the immune dysregulation underlying T1D [120] these cells have been poorly investigated in LADA patients and most importantly in the early stages of the disease, before full blown insulin deficiency will develop.

In this study, we investigated the frequency and phenotype of circulating peripheral Tregs in blood samples of LADA patients by flow cytometry and compared the data to healthy controls. All LADA individuals were non-insulin dependent and without anti-inflammatory treatment.

While we could not detect any differences in the percentage and total number of CD4+ and CD8+ T cells a significant lower total CD4+CD25+ T cells was observed in LADA patients. Since in human peripheral blood canonical nTregs with the highest suppressive function has been shown to a high level of CD25 expression [120], we decided to stratify the CD4+CD25+ T cell population into CD4+CD25neg (negative) CD4+CD25int (intermediate) and CD4+CD25hi (high) T cell populations. The results surprisingly revealed that the significant decrease of total CD4+CD25+ T cells was attributable to the relative decrease of the CD4+CD25int T cell population. Tregs with low expression of CD25 has been described to possess a detectable suppressive function [99, 252], suggesting that CD4+CD25int likely could represent an early differentiation stage of the Tregs population. Thus, we believe that a decrease of these cells can cause a disruption of immune regulation and maintain peripheral tolerance. This could contribute to the underlying pathology in LADA patients.

The transcription factor FOXP3 has been described as a canonical marker of Tregs cells [101]. Therefore, since we were interested to study Tregs cell population more in-deep, we analyzed the expression of this marker in CD4+CD25+ T cell subpopulation and divided it according to the CD25 level of expression. We show that there were no differences between LADA subjects and healthy controls regarding the percentage of FOXP3 inside CD4+CD25hi population of cells. In contrast, the percentage of CD4+CD25- and CD4+CD25int expressing Foxp3 are increased in LADA subjects compare to healthy controls. Our result fits well with

previous finding of transient Foxp3 expression on non-suppressive CD4+CD25- T cells after activation [252]. On the same note, a low expression of Foxp3 has been described within CD4+CD25^{int} as subpopulation of Th17 specific T effector cells in T1D [253]. Additionally, it has also been reported that CD4+CD25- and CD4+CD25^{int} T cell populations that transiently express Foxp3, can function as suppressor cells [104]. However, another report observed a decrease in the fraction of the CD4+CD25⁺ cells in LADA patients [240]. A controversial observation has been published regarding the expression of Foxp3, where decreased mRNA of Foxp3 was described in CD4+ T cells in LADA patients [241]. These conflicting data could nevertheless be explained by the differences in diabetes duration involving LADA patients in different studies. Our data were further strengthened by the analysis of surface markers CD69, CCR4, CD45RO and CTL-4 alone or in combination with Foxp3 in order to determine memory and differentiation status of the CD4+ subpopulation. Based on the expression CD69, early activation markers alone or co-expressed with Foxp3, we show an increased activation status on all CD4+ T cell subpopulations indicating a general T cell activation. We show the same observation for CTLA-4 co-expressed with Foxp3, which is proposed to be involved in down regulation of abnormal T cell activation thus suppressing autoimmune destruction of pancreatic islets in animal models [53]. We were also able to detect that the chemokine receptor CCR4 and memory marker CD45RO have similar patterns of expression and interestingly no significant difference has been observed in CD4+CD25^{hi} population of cells, while CD4+CD25^{int} positive for CCR4 and CD45RO was decreased in LADA patients compare to healthy controls. The percentage of cells analyzed following both markers but co-expressed with FOXP3, was increased in CD4+CD25- not only in CD4+CD25^{int} as was the case using both markers alone.

In conclusion, our data suggest that the frequency and number of CD4+CD25^{int}, which are decreased in LADA, could ultimately contribute to disruption of peripheral immune regulation and progression of diabetes to insulin dependency.

Characterization of resident lymphocytes in human pancreatic islets (Paper II)

T1D is a chronic inflammatory disease, caused by the autoimmune mediated destruction of beta cells of the pancreatic islets leading to insulin deficiency [8]. Previous studies have shown a higher frequency of CD8+T cells in inflammatory lesions and infiltration of the pancreatic islets with autoreactive CD8+ T cells in T1D patients [136, 146, 254]. Moreover, by investigating pancreatic islets of autoantibodies positive donors compared to non-diabetic islet donors, no differences

have been reported regarding T cells, which were found in both groups of subjects [142, 147, 148, 255]. The characterization of immune cells infiltrating pancreatic islets have been performed using immunohistology and recently published study used flow cytometry as a new approach to characterize immune cells present in pancreatic islets of T2D donors [198]. In this project we wanted to further characterize the frequency and phenotype of pancreatic islets associated immune cells in non-diabetic autoantibody negative organ donors using flow cytometry. We performed immunophenotyping of T, B and NK cells. In line with published data on non-diabetic and T2D islet donors [198], we found that T cells were the dominant population of cells. Furthermore, we dissected T cell compartment and demonstrated CD8⁺ T cells present at a higher percentage compared to lower frequency CD4⁺ T cells. These data were further strengthened using immunohistology. We found CD8⁺ T cells localized within the islets but also scattered around the islets. In order to further elucidate memory differentiation status of detected CD8⁺ and CD4⁺ T cells, we performed immunophenotyping applying CD27 and CD45RO markers. Interestingly, this experiment revealed that the majority of CD8⁺ as CD4⁺ T cell belong to central memory (CD45RO⁺CD27⁺) as opposed to effector memory (CD45RO⁺CD27⁻) phenotype. We speculate that memory CD8⁺ T cells residing in pancreatic tissue are potentially involved in maintenance of immune tolerance, but further studies need to be performed to confirm this. CD8⁺ T cells with regulatory properties have been demonstrated in the lung [256]. In line with recent published observations where analysis of different tissues demonstrates the existence a permanent resident non-circulating T cells [257-259], we were able to detect higher percentage of CD8⁺T cells positive for both markers characteristic for tissue residence (CD69 and CD103). Next we were able to show a significant linear correlation between the percentage of insulin and glucagon positive cells measured by flow cytometry with RNA expression from non-dissociated islets by RNA sequencing, indicating that our flow cytometry approach is a suitable method for detection of alpha and beta cells. In conclusion, we show the presence of CD8⁺ T cells with a dominant memory and resident phenotype in pancreatic islets of non-diabetic organ donors. Our finding suggests that underlying mechanism driving CD4⁺ and CD8⁺ T cells into a memory phenotype is not related to autoimmunity. Unfortunately, we did not perform functional studies, which would have revealed their role in immune homeostasis in pancreas. In the future, we will continue to characterize the TCR receptor and determine antigen specificity of these resident memory cells.

Characterization of pancreatic islets associated lymphocytes in type 2 diabetes (Paper III)

An islet reactive T cell and associated autoantibodies as markers of autoimmunity, have been recently been observed in T2D patients [201, 202]. In the present study we investigate the phenotype of infiltrating lymphocytes with the focus on T cells residing in the pancreas and a possible association of their memory differentiation status with beta cell dysfunction in isolated islets from T2D organ donors. Our results revealed no differences in the percentage of residing lymphocytes T, B and NK cells in T2D compared to non-diabetic islet donors. A higher number of CD45+ T cells with elevated proportion of B cells and resident T cells analyzed in T2D donors using a flow cytometry, has been previously reported [198]. We performed a deeper phenotyping of T cells using our modified flow cytometry approach. We were able to detect a higher frequency of CD8+ T cells present in islets from T2D donors as compared to non-diabetic islet donors. The low frequency of CD4+ T cells as other lymphocytes characterized in this study were very low with no differences compared to non-diabetic controls. We have recently revealed central memory T cells as the most dominant cell population in non-diabetic donors (data not published). Additionally, we investigated the memory compartment using CD45RO and CD27 as markers to dissect a deeper phenotype of residing T cells in pancreatic islets from T2D organ donors. We show no differences regarding the percentages of each subpopulation inside the memory compartment compared to controls with the dominance of T cells with a central memory phenotype. An existence of non-circulating resident immune cells in different tissue have been discovered [258]. In line with this, we observe as we described in our previous study, a higher frequency of resident CD8+ T cells being positive for both CD103 and CD69 markers. We further, investigated the percentage of alpha and beta cells using flow cytometry. Interestingly, although no differences could be observed in the percentages of beta cells, we show significantly higher percentage of glucagon secreting alpha cells in T2D and no differences in insulin secreting beta cells. Further we investigated functional capacity of beta cells and for that we used insulin secretion assay. We show as previously published observation that stimulated insulin secretion was decreased in T2D donors in comparison with non-diabetic as controls while at base level we did not observe any differences [260].

In conclusion, our data suggests that the presence and similar distribution of memory CD4+ and CD8+ T cells in pancreatic islets of T2D donors as in non-diabetic donors indicate that might altered TCR receptor and dysfunctional CD8+ T cells contribute to the pathogenesis of T2D.

Summary, conclusions and future perspectives

The pathophysiology of diabetes is varied and complex. The majority of investigations conducted over the years have highlighted the role of peripheral immune dysregulation in the pathogenesis of Diabetes. However, the mechanisms involved in the cellular immunological pathogenesis of diabetes are not fully understood.

In study I, we investigated the phenotype and frequency of circulating peripheral CD4⁺CD25⁺ T cells in LADA patients compared to healthy individuals. No changes were observed in the total T cells and CD4⁺ T cells expressing CD25 in LADA patients compared to healthy controls. However, the frequency and total numbers of CD4⁺ T cells expressing an intermediate level of CD25 were decreased in LADA patients. Additionally, the expression of the activation/memory markers CD69, CTLA-4, CCR4 and CD45RO, were increased in CD4⁺ CD25⁺ T cells of the LADA patients. The specific signature of peripheral T cells and circulating potential Tregs in LADA patients could contribute to the slow process leading to insulin-dependent diabetes in these patients. Further studies are warranted to evaluate whether defective Tregs frequency, phenotype and function contribute to LADA pathogenesis

In study II, we applied a flow cytometry-based approach to characterize the lymphocyte populations in isolated islets from non-diabetic organ donors. This study demonstrated a relative preponderance of central and effector CD8 memory T cells with a “resident memory T cell” phenotype (CD69⁺/CD103⁺) in human pancreatic islets. CD8⁺ T cells were present to the greatest extent in human pancreatic islets followed by TCD4⁺ cells. CD8⁺ T cells were scattered in both pancreatic tissue surrounding the islets and within the islets. Further analysis using markers CD45RO and CD27 showed that majority of the CD4⁺ and CD8⁺ displayed a central memory (T_{cm}) and effector memory (T_{em}) phenotype. Importantly, CD4⁺ T_{te} showed negative correlation with beta cells and CD8⁺ T_{cm} correlated negatively with alpha cells, whereas T_{em} correlated positively with both alpha and beta cells. Further functional characterization of these cells will be required to unravel the role of immune cells associated with islets

In study III, we focused on the distribution and phenotypes of CD4⁺ and CD8⁺ T cells within T cell compartment in non diabetic and T2D human pancreatic islets by using the same flow cytometry approach. We detected no differences in the frequency and distribution of investigated immune cells in human islets from organ donors with or without T2D. T cells were the most dominant lymphocyte population within both T2D and non- diabetic islets leukocytes. NK and B cells were present to a lesser extent in the two cohorts. A deeper characterization of T cells showed a significantly higher percentage of CD8⁺T cells than CD4⁺T cells in both T2D and non diabetic donors. The majority of CD8⁺ T cells in T2D donors displayed a Tcm phenotype. The detection of resident lymphocytes in non-diabetic and type 2 diabetic human islets may provide insight into the physiologic role of immune cells associated with islets

Collectively, in this theses I have undertaken the characterization and differentiation of the of major lymphocyte and T cell subsets that have been associated with the cellular immunological pathogenesis of diabetes. These findings may prove important in the nascent understanding about the identity of immune cells residing in the human pancreas and would help us to understand the already complicated pathogenesis of Diabetes. Further studies are needed, particularly to clarify the tissue resident T cell function. This knowledge will ultimately pave the way for more effective immunotherapy for the treatment of diabetes in the future.

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I am a medical doctor (M.D) graduated from University of Kragujevac, Kragujevac, Serbia, in July 2005. All my life, my intention has been to make a difference in the field of healthcare. By finishing this PhD I believe that I will be a step closer to achieve this goal.

