ISLET ANTIGEN-SPECIFIC T CELLS AND REGULATORY T CELLS IN TYPE 1 DIABETES

by

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To my loved ones

ABSTRACT

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T cells are the key players in the development of type 1 diabetes (T1D), mediating autoimmune reactions leading to the destruction of insulin producing β cells in the islets. We aimed to analyze the role of different T-cell subtypes in the autoimmunity and pathogenesis of T1D. The frequency of islet antigen-specific (GAD65-, proinsulin-, and insulin-specific) CD4$^+$ T cells was investigated *in vitro* in T1D patients, at-risk individuals (diabetes-associated autoantibody positive), and in controls, using MHC class II tetramers. An overall higher frequency of CD4$^+$ T-cells recognizing the GAD65 555−567 peptide was detected in at-risk individuals. In addition, increased CD4$^+$ T-cell responses to the same GAD65 epitope displaying a memory phenotype were observed in at-risk and diabetic children, which demonstrate a previous encounter with the antigen *in vivo*. Avidity and phenotypic differences were also observed among CD4$^+$ T-cell clones induced by distinct doses of GAD65 autoantigen. T-cell clones generated at the lowest peptide dose displayed the highest avidity and expressed more frequently the TCR Vβ5.1 chain than low-avidity T cells. These findings raise attention to the antigen dose when investigating the diversity of antigen-specific T cells. Furthermore, an increased regulatory response during the preclinical phase of T1D was also found in genetically at-risk children. Higher frequencies of regulatory T (Treg) cells (CD4$^+$CD25^{high}, HLA-DR$^-$/CD69$^-$) and natural killer T (NKT) cells (CD161$^+$Vβ11$^+$) were observed in children with multiple autoantibodies compared to autoantibody-negative controls. Taken together, these data showed increased frequency of islet-specific CD4$^+$ T-cells, especially to the GAD65 555-567 epitope, and Treg and NKT cell upregulation in children at-risk for T1D, suggesting their importance in T1D pathogenesis.

Keywords: autoimmunity, autoreactive T cells, GAD65, insulin, natural killer T cells, proinsulin, regulatory T cells, type 1 diabetes

TIIVISTELMÄ

Viveka Öling

Saarekesoluspesifiset T-solut ja säätelijä-T-solut tyypin 1 diabeteksessa

T-soluilla on keskeinen merkitys tyypin 1 diabeteksen (T1D) kehittymiseen johtavassa autoimmuunireaktiossa, mikä myös johtaa insuliinia tuottavien saarekesolujen (β-solujen) tuhoutumiseen. Tutkimuksen tavoitteena oli selvittää erilaisten T-solutyyppien merkitys diabetekseen liittyvässä autoimmuniteetissa ja patogeneesissä. Saarekesoluspesifisten (GAD65-, proinsuliini- ja insuliini-spesifisten) CD4$^+$ T-solujen määrää verrattiin äskettäin diabetekseen sairastuneiden lasten, riskissä olevien lasten (positiivisia diabetekseen liittyviä autovasta-aineita) ja terveiden kontrollilasten välillä käyttäen MHC luokka II tetrameerejä. Voimakkaampi *in vitro* CD4$^+$ T-soluvaste GAD65 555-567 peptidille oli havaittavissa riskilapsilla verrattuna kontrolleihin. Voimakkaampi CD4$^+$ T-soluvaste samalle GAD65-epitoopille havaittiin myös muistisolujen fenotyypin omaavilla soluilla sekä riskilapsilla että T1D potilailla, mikä viittaa antigeenien aiempaan *in vivo* kohtaamiseen. Erilaisia GAD65-peptidikonsentraatioita käyttäen valmistettiin myös spesifisiä CD4$^+$ T-soluklooneja, joilla oli eroja aviditeetissa ja fenotyypissä. Alhaisilla peptidipitoisuuksilla aikaansaadut ja ylläpidetyt T-soluklooniilinjat omasivat korkeimman aviditeetin ja ilmensivät useammin T-solureseptorin Vβ5.1 ketjua verrattuina korkeissa peptidipitoisuuksissa viljeltyihin T-soluihin. Lisääntyneitä säätelyvasteita oli myös todettavissa lapsilla, joilla oli korkea geneettinen riski sairastua diabetekseen. Korkeampi määrä säätelijä-T-soluja (Treg) (CD4$^+$CD25^{high}, HLA-DR$^-$ tai CD69$^-$) ja luonnollisia tappaja-T-soluja (NKT) (CD161$^+$Vβ11$^+$) havaittiin lapsilla, joilla oli useita autovasta-aineita verrattuna autovasta-ainenegatiivisiin lapsiin. Saadut tulokset lisääntyneestä saarekesoluspesifisten, erityisesti GAD65 555-567 epitoopille reagoivien CD4$^+$ T-solujen, sekä Treg ja NKT solujen määrästä T1D riskilapsilla viittaavat näiden T-solujen merkitykseen T1D:n patogeneesissä.

Avainsanat: autoimmuniteetti, autoreaktiiviset T-solut, GAD65, insuliini, luonnolliset tappaja-T-solut, proinsuliini, säätelijä-T-solut, tyypin 1 diabetes

ABSTRAKT

Viveka Öling

Öcells-antigen-specifika T-celler och reglerande-T-celler i typ 1-diabetes

T-celler har en nyckelroll i utvecklingen av typ 1-diabetes (T1D), genom att de förmedlar autoimmuna reaktioner som leder till att de insulinproducerande öcellerna (β-cellerna) förstörs. Vårt mål var att undersöka betydelsen av olika T-celler vid autoimmuniteten och patogenesen av T1D. Förekomsten av öcell-antigen-specifika (GAD65-, proinsulin- och insulinspecifika) $CD4^+$ T-celler undersöktes i blodprov från T1D patienter, från barn med ökad risk att insjukna i T1D (positiva för diabetesassocierade autoantikroppar) och från kontroller, genom användning av MHC klass II tetramerer. En allmänt högre nivå av $CD4^+$ T-celler som uppvisade respons för GAD65 555-567 peptiden detekterades hos barn med ökad risk för T1D. En förhöjd $CD4^+$ minnes-T-cellrespons för samma GAD65-epitop observerades också hos barn med ökad risk för T1D och hos barn som insjuknat i T1D, vilket påvisar en tidigare kontakt med antigenet *in vivo*. Skillnader i aviditeten och fenotypen hos $CD4^+$ T-cellskloner inducerade med olika doser av GAD65-autoantigenen observerades likaså. T-cellskloner etablerade med den lägsta peptiddosen visade den högsta aviditeten och uttryckte oftare TCR Vβ5.1 kedjan än T-celler med låg aviditet. Dessa resultat fäster uppmärksamheten på antigendosen vid undersökning av antigen-specifika T-cellers aviditet. Dessutom observerades även en ökad reglerande immunologisk respons under den prekliniska fasen av T1D hos barn med genetisk risk att insjukna i T1D. Högre nivåer av reglerande-T-celler (Treg) ($CD4^+CD25^{high}$, HLA-$DR^-/CD69^-$) och naturliga mördar-T-celler (NKT) ($CD161^+Vβ11^+$) detekterades hos barn med fler autoantikroppar jämfört med autoantikroppsnegativa barn. Sammanfattningsvis kan konstateras att dessa resultat påvisade en högre nivå av öcell-specifika $CD4^+$ T-celler, speciellt mot GAD65 555-567 epitopen, och en uppreglering av Treg och NKT celler hos barn med ökad risk för T1D, vilket förespråkar deras betydelse i patogenesen av T1D.

Nyckelord: autoimmunitet, autoreaktiva-T-celler, GAD65, insulin, naturliga mördar-T-celler, proinsulin, reglerande-T-celler, typ 1-diabetes

CONTENTS

ABBREVIATIONS

AAb+	Autoantibody positive
AICD	Activation-induced cell death
AIRE	Autoimmune regulator
APC	Antigen presenting cell
Apc	Allophycocyanin
aTreg	Adapted regulatory T cell
BB rats	BioBreeding diabetes-prone rats
CBV4	Coxsackie B virus 4
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen-4 gene
CMV	Cytomegalovirus
cpm	Counts per minute
DASP	Diabetes Autoantibody Standardization Program
DIPP	Type 1 Diabetes Prediction and Prevention study
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead/winged-helix transcription factor box protein 3
GABA	γ-aminobutyric acid
GADA	Glutamic acid decarboxylase antibodies
GAD65	65 kDa isoform of glutamic acid decarboxylase
GATA-3	GATA binding protein 3 (AGATAG binding site)
GITR	Glucocorticoid-induced tumor necrosis factor receptor
HLA	Human leukocyte antigen
HSV	Herpes simplex virus
IA-2A	Antibodies to the protein tyrosine phosphatase-related IA-2 protein
IAA	Insulin autoantibodies
ICA	Islet cell antibodies
ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IL	Interleukin
iNKT	Invariant Natural Killer T cell
INS	Insulin gene
JDFU	Juvenile Diabetes Foundation units
kDa	Kilodalton
LADA	Latent autoimmune diabetes in adult

LAG-3	Lymphocyte activation gene-3
LFA-1	Lymphocyte functional antigen-1
LYP	Lymphoid tyrosine phosphatase
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorter
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
mTEC	Medullary thymic epithelial cell
NK	Natural killer cell
NKT	Natural killer T cell
NOD	Non-obese diabetic
nTreg	Natural regulatory T cell
NSI	Non-structural protein of influenza virus
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phychoerythrin
PerCP	Peridinin chlorophyll protein
PHA	Phytohemagglutinin-P
PRR	Pattern-recognition receptor
PTPN22	Protein tyrosine phosphatase N22 gene
RNA	Ribonucleic acid
RU	Relative units
SNP	Single nucleotide polymorphism
TCR	T-cell receptor
TGF-β	Transforming growth factor-β
Th	T helper cell
TLR	Toll-like receptor
Treg	Regulatory T cell
T1D	Type 1 diabetes
VNTR	Variable number of tandem repeats
VP	Virus protein
ZnT8	Zinc transporter T8
ZnT8A	Zinc transporter T8 antibodies

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by corresponding Roman numerals I-IV.

I Öling V, Marttila J, Ilonen J, Kwok WW, Nepom G, Knip M, Simell O, Reijonen H. GAD65- and Proinsulin-Specific CD4+ T Cells Detected by MHC Class II Tetramers in Peripheral Blood of Type 1 Diabetes Patients and At-Risk Subjects. *J. Autoimmun.* 25:235-243, 2005.

II Öling V, Reijonen H, Simell O, Knip M, Ilonen J. GAD65- and (Pro)insulin-Specific CD4+ T Cells Detected by MHC Class II Tetramers in Diabetes-Associated Autoimmunity. *Submitted*

III Öling V, Geubtner K, Ilonen J, Reijonen H. A Low Antigen Dose Selectively Promotes Expansion of High-Avidity Autoreactive T cells with Distinct Phenotypic Characteristics: A Study of Human Autoreactive CD4+ T Cells Specific for GAD65. *Autoimmunity.* Early Online 1-10, 2010.

IV Öling V, Marttila J, Knip M, Simell O, Ilonen J. Circulating CD4+CD25high Regulatory T Cells and Natural Killer T Cells in Children With Newly Diagnosed Type 1 Diabetes or With Diabetes-Associated Autoantibodies. *Ann. N.Y. Acad. Sci.* 1107:363-372, 2007.

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1. INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of the insulin producing β cells in the pancreas. It is a chronic disease, with life-long insulin treatment as an aftermath. The incidence of type 1 diabetes has been steadily increasing throughout the world and Finland has the highest incidence.

The pathogenic mechanisms of the disease have so far not been unraveled, but genetic susceptibility exists and susceptible genes in combination with environmental factors are considered the most likely cause. Several viral infections and nutritional factors have been suggested to play a role in triggering the autoimmune destruction of the β-cells. T cells have a pivotal role in mediating the autoimmune processes and both CD4+ T helper cells and CD8+ cytotoxic T cells are involved. Additionally, immunosuppressive T cells represent a subset regulating harmful immune responses.

The aim of this study was to investigate the role of immunosuppressive T-cell subsets and islet-specific autoreactive CD4+ T cells during the preclinical phase of T1D, and at the time of onset. The aim was also to evaluate the use of the MHC class II tetramer technique in the prediction of T1D. More knowledge about the role of these T cells in T1D could help us better understand how the autoimmune reactions occur and could open new possibilities for the development of therapeutic approaches, which can be used when the first signs of β-cell destruction appear.

2.   REVIEW OF THE LITERATURE

2.1   Type 1 diabetes

2.1.1 Clinical manifestation

The classical symptoms of diabetes are polyuria, polydipsia, loss of weight and fatigue (Levy-Marchal *et al.*, 2001). Patients who have autoimmune T1D are typically diagnosed at a young age ($\leq$ 15 years) with acute onset and with risk for ketoacidosis at diagnosis. They usually have several circulating autoantibodies to β-cell antigens and their C-peptide levels are low, referring to very low insulin secretion. No evidence of insulin resistance can be found and these patients are rarely obese. All subjects who develop T1D need lifelong insulin treatment with parenteral insulin. In the long-term, T1D can cause micro- and macrovascular complications. The most common diabetes-related complications are nephropathy, neuropathy, retinopathy and higher risk for cardiovascular diseases. Also other autoimmune conditions, such as Hashimoto's thyroiditis, Graves' disease, Addison's disease, and Celiac disease are more common in T1D patients (Daneman, 2006).

2.1.2 Pathogenesis

T1D is the final result of the destruction of insulin producing β cells in the pancreatic islets of Langerhans. The abnormal T-cell-mediated immune reactions in susceptible individuals lead to an inflammatory response in the islets (insulitis) and humoral (B-cell) responses with the production of autoantibodies to β-cell antigens. A continuation of the β-cell destruction leads to a progressive loss of the insulin-secretory reserve, followed by a clinical onset of diabetes when insulin secretion falls down to a critical point. At this time only 10-20% of the β cells remains (Fig. 1). A short honeymoon or remission phase takes place soon after clinical diagnosis and the introduction of insulin therapy, when some insulin secretion is restored in the remaining β cells. Ultimately a state of absolute insulin deficiency emerges in the majority of cases (Eisenbarth, 1986).

The knowledge of the disease progression and the pancreatic infiltration is largely based on animal models of T1D, non-obese diabetic (NOD) mice and BioBreeding (BB) diabetes-prone rats (Yang & Santamaria, 2006), and only limited data are available in human beings. A recent study (Willcox *et al.*, 2009) with a large collection of post-mortem pancreatic organs from T1D patients confirm old findings (Bottazzo *et al.*, 1985; Hänninen *et al.*, 1992; Somoza *et al.*, 1994) that the infiltrating cells consist of CD8^{+} cytotoxic T cells, CD4^{+} T helper cells, macrophages, B cells, and in some cases, natural killer (NK) cells. In the large set of post-mortem pancreas samples CD8^{+} cytotoxic T cells were the most abundant cell population during insulitis, and increased in number as insulin-positive β cells decreased, while macrophages were present at lower and more

constant numbers. CD20^{+} B cells were frequently detected in all patients, while CD4^{+} T helper cells were less abundant than CD8^{+} cytotoxic T cells and NK cells were rare (Willcox *et al.*, 2009).

The β-cell proteins, such as (pro)insulin, glutamic acid decarboxylase 65 (GAD65), and the protein tyrosine phosphatase-related IA-2, are likely to become exposed to the immune system due to viral infection in the islets or local stress. Further, antigen-presenting cells [macrophages, dendritic cells (DC), B cells] internalize and process the autoantigens and transport them to pancreatic draining lymph nodes, where they can interact with and stimulate autoreactive T cells (Roep, 2003). CD8^{+} cytotoxic T cells are mainly thought to mediate destruction of the β cells (DiLorenzo & Serreze, 2005), although some investigators emphasize the role of macrophages (Yoon *et al.*, 1998; Horwitz *et al.*, 2004). The activated antigen-presenting cells (APC) and lymphocytes secrete cytokines that regulate the immune response into a T helper 1 or T helper 2 direction. The β cells are destroyed by apoptosis through the Fas ligand-Fas receptor pathway, or by granzymes and perforin released from CD8^{+} cytotoxic T cells, or by secretion of pro-inflammatory cytokines [interleukin (IL)-1β, tumor necrosis factor α, interferon (IFN)-γ] by cells infiltrating the islet. Overall, the fate of the β cells is dependent on the balance between autoreactive and regulatory responses (Yoon & Jun, 2001; Pirot *et al.*, 2008).
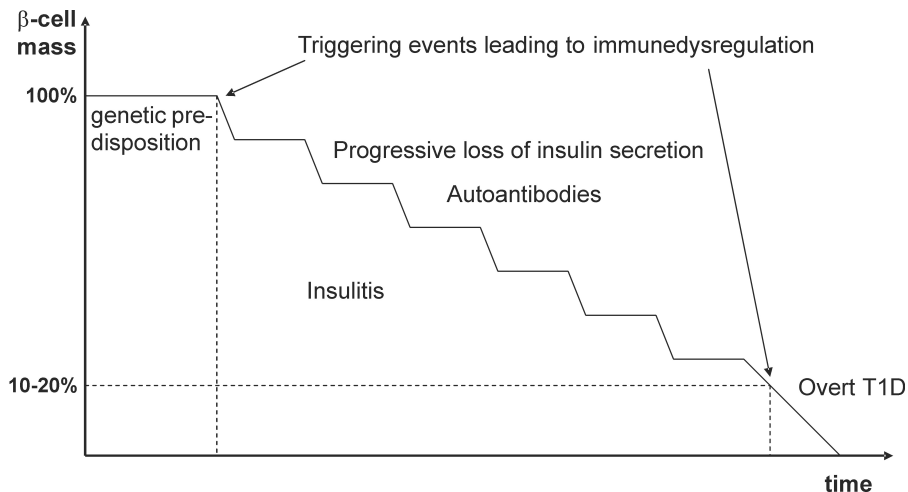
Figure 1. Progression of type 1 diabetes (T1D). Modified from Eisenbarth 1986 (Eisenbarth, 1986).

2.1.3 Autoantibodies and prediction

Islet autoantibodies are present in most T1D patients and are widely used for the prediction of the disease. The most common autoantibodies associated with T1D are islet cell antibodies (ICA), glutamic acid decarboxylase antibodies (GADA), tyrosine phosphatase-related protein antibodies (IA-2A), insulin antibodies (IAA), and zinc transporter 8 antibodies (ZnT8A) (Lieberman & DiLorenzo, 2003; Wenzlau *et al.*, 2007).

Autoantibodies to islet cells were the first antibodies described in diabetes patients (Bottazzo *et al.*, 1974). ICA in sera can be detected with indirect immunofluorescence using pancreatic sections as antigen. ICA include autoantibodies to GAD65, IA-2, and additional other unidentified antigens (Atkinson *et al.*, 1993; Bonifacio *et al.*, 1995b; Myers *et al.*, 1995; Månsson *et al.*, 2001). ICA are reported to be present in approximately 90% of T1D patients at the onset of the disease and up to 3% in the general population (Bonifacio *et al.*, 1995a; Bingley *et al.*, 1997; Kulmala *et al.*, 2001; Schlosser *et al.*, 2002). Glutamic acid decarboxylase (GAD) is expressed in pancreatic islets, but also in the central and peripheral nervous system. GAD is an important enzyme in the translation of glutamic acid to γ-aminobutyric acid (GABA), which is a neurotransmitter. The function of GAD in the pancreatic islets is however not known (Karlsen *et al.*, 1991). The 65-kilodalton (kDa) isoform of GAD is found in 70% of newly diagnosed T1D patients and in only 0.5-4% in controls (Hagopian *et al.*, 1993; Bingley *et al.*, 1997; Kulmala *et al.*, 2001; Schlosser *et al.*, 2002). IA-2 (also known as ICA512), is a member of the protein tyrosine phosphatase family, and is enriched in the membrane of insulin secretory granules of the β cells, and like GAD65, IA-2 is found in the central nervous system (Payton *et al.*, 1995). Autoantibodies to IA-2 have been found in 66-75% of T1D patients (Lan *et al.*, 1996; Bingley *et al.*, 1997), and in 2% or less in the general population (Bingley *et al.*, 1997; Kulmala *et al.*, 2001; Schlosser *et al.*, 2002). Autoantibodies to insulin, which is produced by the β cells, are detected in a lower number of recently diagnosed T1D patients (18-69%) (Palmer *et al.*, 1983; Bingley *et al.*, 1997; Yu *et al.*, 2000), and about 1% in non-affected schoolchildren (Kulmala *et al.*, 2001; Schlosser *et al.*, 2002). A recently discovered autoantibody to ZnT8, which is a zinc transporter expressed in pancreatic β cells, has been a promising new diabetes-associated autoantibody. Autoantibodies to ZnT8 were found in 60-80% of new-onset T1D patients, and less than 2% in controls (Wenzlau *et al.*, 2007).

Up to 90% of recently diagnosed T1D patients express autoantibodies to one or more of the common diabetes-associated antigens (Notkins & Lernmark, 2001; Pihoker *et al.*, 2005). GADA, IA-2A, IAA together with newly discovered autoantibodies to ZnT8 increases the autoimmunity detection rate to 98% (Wenzlau *et al.*, 2007). Combined analysis of autoantibodies clearly improves the prediction of T1D (Bingley *et al.*, 1994; Verge *et al.*, 1994; Kulmala *et al.*, 1998; Hummel *et al.*, 2004; Wenzlau *et al.*, 2007).

The first signs of β-cell autoimmunity are usually detected months or years prior to clinical onset. Children developing T1D under the age of 10 years have been shown to express circulating autoantibodies very early in life. In the BABYDIAB study, around 4% of the offspring of parents with T1D, and around 6% of genetically at-risk infants recruited from the general population in the Finnish DIPP study, have developed autoantibodies by the age of 2 years (Kimpimäki *et al.*, 2002; Hummel *et al.*, 2004). IAA is usually the first autoantibody to appear in young children, whereas both ICA and GADA usually appear before IA-2A (Kimpimäki *et al.*, 2001b). Children carrying high-risk genotypes for T1D have also been reported to develop autoantibodies earlier and more frequently than children with moderate-risk genotypes (Schenker *et al.*, 1999; Kimpimäki *et al.*,

2001b; Kimpimäki *et al.*, 2002; Siljander *et al.*, 2009). Among first-degree relatives, the risk of developing T1D within 5 years are 3-15% if one autoantibody is present and 40-100% if positive for multiple autoantibodies (Verge *et al.*, 1996; Hummel *et al.*, 2004).

2.1.4 Epidemiology

The incidence of T1D in children younger than 15 years varies largely throughout the world. The lowest incidence is found in Venezuela (0.1/100 000 per year), low to intermediate in many African populations (1/100 000 to 9/100 000 per year) and high to very high in North America and in several European countries (10/100 000 to over 20/100 000 per year) (DIAMOND, 2006). The incidence of T1D has steadily been increasing during the last decades. Finland has the highest incidence rate in the world, followed by Sardinia, Sweden, the United Kingdom, Norway and Denmark (Patterson et al. 2009). In Finland, the incidence was 18/100 000 children per year in 1965, gradually increasing to 36/100 000 children per year in the late 1980's. The incidence increase became even steeper during the 1990's (45/100 000 per year in 1996) and reached 64/100 000 children per year in 2005 (Tuomilehto *et al.*, 1991; Tuomilehto *et al.*, 1999; Harjutsalo *et al.*, 2008). The annual increase of the disease is estimated to be 2.7% in Finland and 3.9% overall in European countries. Fast rates of increase have been reported in low-incidence countries, especially in Eastern Europe, in the Czech Republic, Poland, Romania and Slovakia. The increase of incidence has also been the most significant in very young children. In European countries the incidence increase is 5.4% for children between 0-4-years-old, 4.3% for children at 5-9-years-old and 2.9% in children with the age 10-14-years (Patterson *et al.*, 2009).

2.1.5 Genetic susceptibility

It is well known that T1D is a genetic disease. This is seen in familial clustering and in identical (monozygotic) twins. The incidence of T1D in monozygotic twins of an affected individual is reported to be 27-53% (Barnett *et al.*, 1981; Kyvik *et al.*, 1995; Redondo *et al.*, 2001; Hyttinen *et al.*, 2003). In non-identical (dizygotic) twins the risk is similar to the risk observed in siblings (5-10%) which, in turn, is approximately 10-fold higher than in the general population (0.2-0.4%) (Risch, 1987; Harjutsalo *et al.*, 2005; Kim & Polychronakos, 2005; Hemminki *et al.*, 2009). This demonstrates the role of genetic factors in the etiology of T1D.

2.1.5.1 Human leukocyte antigen (HLA) gene region

The most important genes predisposing to T1D are located in the human leukocyte antigen (HLA) region on chromosome 6p21.3 (Risch, 1987). HLA is the human major histocompatibility complex (MHC) and the HLA region encodes for the class I (HLA-A, -B, -C), and class II (HLA-DP, -DQ, -DR) molecules (Klein & Sato, 2000b). The HLA class II region has been found to strongly associate with T1D and especially the DQA1, DQB1 and DRB1 genes. In Caucasians the most common T1D-predisposing haplotypes

are DRB1*0301-DQA1*0501-DQB1*0201 (DR3, DQ2), and DRB1*04- DQA1*0301-
DQB1*0302 (DR4, DQ8). Heterozygosity for DR3-DQ2/DR4-DQ8 predisposes even a
higher risk for T1D [Odds ratio (OR) 37.85; 95% confidence interval (CI) 16.59−86.37
in Finnish children] than homozygosity for either haplotypes (DR3-DQ2 OR 4.08;
95% CI 1.50−11.13 and DR4-DQ8 OR 11.93; 95% CI 4.21-33.78 in Finnish children)
(She, 1996; Cucca *et al.*, 2001; Ilonen, 2004; Lambert *et al.*, 2004). Only certain DR4-
associated DRB1 alleles are prevalent in T1D patients. DRB1*0401, *0402, *0404, and
*0405 are positively associated with T1D, while DRB1*0403 is negatively associated
even in combination with DQB1*0302 (Undlien *et al.*, 1997; Cucca *et al.*, 2001). In the
Finnish population, the DRB1*0401-DQB1*0302 haplotype is the most common one
in T1D patients (OR 5.64; 95% CI 4.37−7.29), followed by the DQA1*05-DQB1*02
(OR 3.40; 95% CI 2.65−4.36) and DRB1*0404-DQB1*0302 haplotypes (OR 3.07;
95% CI 2.14−4.41), while the DRB1*0402 and *0405 haplotypes are rare in the general
population (Hermann *et al.*, 2003). Protection against T1D is associated with the
haplotype DRB1*1501-DQA1*0102-DQB1*0602 in all populations (OR 0.07; 95% CI
0.04−0.12 in Finnish children). Also several other DQA1, DQB1 and DR alleles modify
the risk and protection of T1D (She, 1996; Cucca *et al.*, 2001; Hermann *et al.*, 2003;
Lambert *et al.*, 2004).

The HLA class I gene region has also been found to contribute to T1D susceptibility and
multiple predisposing and protective alleles have been localized mainly to the HLA-A,
and -B gene regions. HLA-A*24 (OR 1.66; 95% CI 1.22−2.24 [#] = conditioned on HLA-
DQB1 and -DRB1) and HLA-B*39 (OR 2.53; 95% CI 1.57−4.07 [#]) alleles predispose
for T1D and both have been associated with a lower age-at-onset of T1D (Reijonen *et
al.*, 1997; Nejentsev *et al.*, 2000; Noble *et al.*, 2002; Valdes *et al.*, 2005; Nejentsev *et
al.*, 2007). HLA-A*24 has also been associated with a higher rate of β-cell destruction.
Protection from T1D has instead been found to associate with HLA-A*01 (OR 0.67;
95% CI 0.53−0.84 [#]) and -A*11 (OR 0.44; 95% CI 0.29−0.66 [#]), as well as with the
HLA-B*27 allele (OR 0.55; 95% CI 0.36−0.82 [#]) (Nejentsev *et al.*, 2007).

2.1.5.2 Non-HLA genes

The strongest non-HLA loci with increased susceptibility to T1D are the insulin gene
(*INS*) on chromosome 11p15.5, the cytotoxic T-lymphocyte antigen-4 gene *(CTLA-4)*
on chromosome 2q33, and the lymphoid protein tyrosine phosphatase nonreceptor type
22 gene (*PTPN22*) on chromosome 1p13 (Kim & Polychronakos, 2005). The insulin
gene consists of a variable number of tandem repeats (VNTR) polymorphism in the
promoter region of the insulin gene (Bennett *et al.*, 1995). *INS*-VNTR polymorphism
is associated with lower expression of insulin in the thymus, which could influence
the negative selection of T cells, resulting in a less efficient deletion of insulin-specific
autoreactive T cells (Pugliese *et al.*, 1997; Vafiadis *et al.*, 1997). *CTLA-4* gene encodes
for the cell surface co-receptor on T cells that negatively regulates T-cell activation
(Scalapino & Daikh, 2008). Several polymorphisms in the *CTLA-4* region have been
associated with T1D, such as the single nucleotide polymorphism (SNP) CT60. CD4^{+}

T cells from subjects carrying the CT60 G/G disease-susceptibility haplotype were shown to produce a lower amount of CTLA-4 mRNA compared to individuals with protective CT60 A/A genotype (Ueda *et al.*, 2003). CT60 A/A genotype has also been associated with the elevated frequency of CD4$^+$CD25$^+$ regulatory T cells in peripheral blood (Atabani *et al.*, 2005). The *PTPN22* gene displaying T1D susceptibility, encodes LYP, an intracellular lymphoid-specific phosphatase that inhibits T-cell receptor (TCR) signaling (Bottini *et al.*, 2004). A SNP in the PTPN22 gene (C1858T), resulting in an arginine to tryptophan substitution at position 620, is associated with increased risk for T1D and other autoimmune diseases. This PTPN22 620Trp disease variant has been demonstrated to reduce IL-2 production and calcium mobilization in activated CD4$^+$ T cells (Vang *et al.*, 2005; Rieck *et al.*, 2007; Aarnisalo *et al.*, 2008a). In addition, several other minor T1D-associated genes have been described in other chromosome regions, such as region 10p15 that contains the gene for IL-2 receptor α chain, the gene region on 12p13.31 that encodes for the T-cell activation marker CD69, and the gene region 1q32.1 encoding for the immunoregulatory cytokines IL-10, IL-19, and IL-20 (Todd *et al.*, 2007; Barrett *et al.*, 2009).

2.1.6 Environmental risk factors

The rapid increase in the incidence of T1D indicates the significance of environmental and lifestyle changes (Knip *et al.*, 2005; Åkerblom *et al.*, 2005). Migration studies also emphasize the impact of environmental factors, demonstrating an increase of T1D incidence in individuals moving from a low-incidence to a high-incidence region (Siemiatycki *et al.*, 1988; Bruno *et al.*, 2000; Zung *et al.*, 2004; Delli *et al.*, 2010). Another evidence of environmental impact on T1D is the seasonal patterns observed in the onset of T1D, with peaks during the autumn and winter months and decreases during the summer months. These seasonal variations of T1D have been mostly associated with viral infections (Padaiga *et al.*, 1999; Moltchanova *et al.*, 2009).

2.1.6.1 Viral infections

The role of viral infections triggering the development of autoimmunity and T1D can be broadly divided into three mechanisms: 1) some viruses can directly infect pancreatic tissues and induce cytolysis and β-cell death (Roivainen *et al.*, 2002; Ylipaasto *et al.*, 2004) ; 2), inflammation caused by the virus could enhance bystander activation of autoreactive T cells (Horwitz *et al.*, 1998; Horwitz *et al.*, 2004) ; 3), and viral proteins with a close similarity to host tissue, could induce cross-reactivity responses against the self-antigen, the "molecular mimicry hypothesis" (Hiemstra *et al.*, 2001; Härkönen *et al.*, 2002). Infections are not necessary only contributing to the development of T1D, but could possibly also prevent autoimmune diseases. Parasites and bacteria, particularly those that chronically infect the host, have been shown to have a protective effect against T1D in animal models (Cooke, 2009). For example, helminth infections with *Schistosoma mansoni* or its antigens have been reported to effectively reduce T1D incidence in NOD mice, probably due to a shift more toward Th2-mediated responses (Cooke *et al.*, 1999;

Zaccone *et al.*, 2003). These findings emphasize the "hygiene hypothesis". This hypothesis proposes that lesser exposure to infections early in life, due to improved living standards, may lead to a less experienced immune system causing dysfunctional immune responses to environmental triggers, and resulting in autoimmunity or allergies (Bach, 2001).

Human enteroviruses have been implicated as triggers of T1D, but the results have been controversial (Tracy *et al.*, 2010). The first connection with enteroviruses and T1D were done in the late 1960's, when T1D patients were found to have higher antibody titers to the Coxsackie B4 virus (CBV4) than controls (Gamble *et al.*, 1969). Since then, many studies have supported this finding (Banatvala *et al.*, 1985; Frisk *et al.*, 1992; Helfand *et al.*, 1995). CBV4 has also been associated with T-cell cross-reactivity with islet autoantigen GAD65. A sequence similarity between the Coxsackie B4 virus P2-C protein (PEVKEK) and GAD65 was identified in the beginning of the 1990's (Kaufman *et al.*, 1992; Atkinson *et al.*, 1994), and peptides containing the PEVKEK sequence were shown to induce T-cell proliferation, supporting the notion of PEVKEK sequence as an immunogenic epitope (Schloot *et al.*, 1997; Marttila *et al.*, 2001). Moreover, some enteroviruses were shown to infect the pancreatic islets and Coxsackie B viruses have been isolated from the pancreas of children at the onset of T1D (Yoon *et al.*, 1979; Champsaur *et al.*, 1980). Enterovirus RNA and enterovirus protein have also been detected in pancreatic islets of T1D patients or autoantibody-positive individuals (Ylipaasto *et al.*, 2004; Dotta *et al.*, 2007). Furthermore, enterovirus infection preceding the appearance of T1D-associated autoantibodies has been observed in several Finnish prospect studies (Lönnrot *et al.*, 2000a; Lönnrot *et al.*, 2000b; Sadeharju *et al.*, 2001; Salminen *et al.*, 2003). Maternal enterovirus infections during pregnancy have also been associated with increased risk of T1D development in the offspring (Dahlquist *et al.*, 1995; Hyöty *et al.*, 1995). However, contradictory studies not observing any relationship between enteroviruses and T1D have also been reported (Palmer *et al.*, 1982; Mertens *et al.*, 1983). In more recent German and American prospect studies, no associations between enterovirus infection and β-cell autoimmunity were detected (Füchtenbusch *et al.*, 2001; Graves *et al.*, 2003), and in a Finnish study, maternal enteroviral infections during the first trimester were not associated with higher T1D-risk in the offspring (Viskari *et al.*, 2002).

Rotavirus infections, a common cause of diarrhea in children, have been shown to correlate with the appearance of GAD and IA-2 autoantibodies in children at risk of developing T1D (Honeyman *et al.*, 2000). However, this was not observed in the Finnish DIPP study with young children genetically at risk for T1D (Blomqvist *et al.*, 2002). Subsequently, enteral virus infections, including rotavirus, were linked to increased immunization to insulin in the DIPP cohort (Mäkelä *et al.*, 2006). Cross-reactivity between the rotavirus VP7 protein and pancreatic islet autoantigens has also been suggested to trigger autoimmunity. The rotavirus VP7 protein is highly similar to a sequence in IA-2 (805-817), and one in GAD65 (115-128) (Honeyman *et al.*, 1998). Recent findings support the hypothesis of molecular mimicry by demonstrating that human T cells expanded in the presence of the IA-2 805-817 peptide express a similar response to both cognate and rotavirus VP7 (40-52) peptides (Honeyman *et al.*, 2010).

Cytomegalovirus (CMV) has been shown to infect β cells, neuronal tissue and peripheral blood mononuclear cells in humans (Jenson *et al.*, 1980; Gilbert *et al.*, 1989; Poland *et al.*, 1994). Usually the infection is asymptomatic, but in some cases CMV can cause severe complications during the first trimester of pregnancy (Malm & Engman, 2007). CMV has been suggested to be involved in the pathogenesis of T1D, and this has been supported by the correlation observed between the presence of the CMV genome and ICA in newly diagnosed T1D patients (Pak *et al.*, 1988), and high titers of CMV IgG-antibodies were associated with ICA appearance in healthy siblings of T1D patients (Nicoletti *et al.*, 1990). Also, CMV has been associated with T-cell cross-reactivity with GAD65. Similarities between a peptide of human cytomegalovirus major DNA-binding protein (674-687) and GAD65 (339-352) have been identified, and the CMV peptide was described to stimulate GAD65-specific T-cell clones (Hiemstra *et al.*, 2001; Roep *et al.*, 2002). Persistent CMV infections have been linked to the initiation of β-cells destruction in a pancreas-transplanted patient (Zanone *et al.*, 2009), and in an adult patient with the rapid-onset of T1D (Osame *et al.*, 2007). On the other hand, evidence of no association between primary CMV infection and T1D has also been reported in several studies (Ivarsson *et al.*, 1993; Hiltunen *et al.*, 1995; Aarnisalo *et al.*, 2008b).

Congenital rubella and mumps can both infect human β cells and have been suggested to induce islet-cell antibodies and eventually diabetes (Ginsberg-Fellner *et al.*, 1984; Hyöty *et al.*, 1988), but successful vaccination programs against these viruses make them an unlikely factor contributing to the increasing frequency of T1D in the developed countries (Hyöty *et al.*, 1993).

2.1.6.2 Dietary factors

Various nutritional factors have been implicated with the increased risk of T1D, such as early exposure to cereals, cow's milk and the reduced intake of vitamin D. Lower vitamin D serum levels have been detected in newly diagnosed T1D patients (Baumgartl *et al.*, 1991; Littorin *et al.*, 2006; Greer *et al.*, 2007; Borkar *et al.*, 2009). In a meta-analysis of five studies, oral vitamin D supplementation in infancy was demonstrated to reduce the risk of T1D later in life (Zipitis & Akobeng, 2008). However, there is a generally implemented intake of vitamin D drops during infancy in Northern European countries. Low T1D incidence in some areas of Northern Europe (e.g. Russian Karelia), with strong seasonal variation in sunshine hours and a partly similar HLA background to the Finnish population, argues against the role of vitamin D deficiency in T1D β-cell autoimmunity (Knip *et al.*, 2005).

Exposure to cereals in infancy has been shown to increase the risk for developing diabetes-associated autoantibodies. An American study (Norris *et al.*, 2003) and a German study (Ziegler *et al.*, 2003) on the early (< 4 months of age) exposure to cereals and the American study on the late exposure (> 7 months or later) suggested an association with increased risk of β-cell autoimmunity. In another Finnish study increased T-cell reactivity to gluten was observed in T1D patients at diagnosis (Klemetti *et al.*, 1998). In addition, gliadin

has been associated to trigger intestinal inflammation in T1D patients, suggesting that intestinal immune responses may enhance the autoimmune reaction against the β-cells (Auricchio *et al.*, 2004).

Cow's milk has been proposed to be a possible trigger of the autoimmune process leading to T1D. The early introduction of supplementary milk feeding in infancy and short breast-feeding duration have been associated with an increased risk for T1D (Mayer *et al.*, 1988; Virtanen *et al.*, 1993; Kimpimäki *et al.*, 2001a). Savilahti *et al.* were the first to observe increased levels of antibodies to cow's milk proteins in recently diagnosed T1D children (Savilahti *et al.*, 1988). A reduced risk for T1D in children that had been breast-fed longer was also reported around the same time (Borch-Johnsen *et al.*, 1984; Mayer *et al.*, 1988). Both findings have been confirmed in later studies (Virtanen *et al.*, 1991; Dahlquist *et al.*, 1992; Virtanen *et al.*, 1993; Verge *et al.*, 1994; Vaarala *et al.*, 1999). In the Finnish DIPP study, a short-term breast-feeding of less than two months, were reported to increase the risk of diabetes-associated autoantibody appearance in genetically susceptible children, as compared to HLA-matched controls. This suggest that long-term breastfeeding is needed to protect children who are genetically at risk from progressing to β-cell autoimmunity during the first years of life (Kimpimäki *et al.*, 2001a). However, no association between short-term breastfeeding or early exposure to cow's milk formula and an increased risk of T1D and diabetes-associated autoimmunity has also been reported (Hummel *et al.*, 2000; Ziegler *et al.*, 2003; Virtanen *et al.*, 2006).

The protein composition between human breast milk and cow's milk differ, with a higher casein content in cow's milk than in breast milk, and β-lactoglobulin is not present in breast milk (Darke, 1976). Bovine insulin has only a three amino acid difference from the human insulin (aa 8 and 10 in the A chain and aa 30 in the B chain), and has been shown to be immunogenic (Knip *et al.*, 2005), inducing insulin-binding antibodies in humans when treated with bovine insulin for T1D (Kurtz *et al.*, 1980). Furthermore, oral exposure to cow's milk formulas before three months of age have been reported to induce bovine insulin-binding antibodies that cross-react with human insulin (Vaarala *et al.*, 1998; Vaarala *et al.*, 1999). Higher T-cell reactivity to bovine insulin and human insulin in infants exposed to cow's milk formula before three months of age compared to fully breast-fed infants has also been reported (Paronen *et al.*, 2000). In the pilot study of the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) in which infants are exposed to highly hydrolyzed or conventional cow's milk-based formula during the first 6-8 months of life, a lower incidence of diabetes-associated autoantibodies was detected in the highly hydrolyzed formula group, suggesting the possibility to manipulate β-cell autoimmunity responses through early dietary intervention (Åkerblom *et al.*, 2005).

2.1.7 Prevention

The slow progression of the β-cell destruction in some individuals and the possibility for prediction by combined screening of several T1D-associated autoantibodies in genetically at-risk individuals, offers possibilities for intervention with immunotherapy

(Goudy & Tisch, 2005). The aim of intervention in autoimmune diabetes before the onset of the disease is to delay or prevent the clinical disease. At the time of diagnosis, about 80-90% of the β-cells are destroyed and intervention at the disease onset is aiming to arrest the destruction of the remaining β-cell mass. This approach has the potential to reduce the severity of the disease progression (Goudy & Tisch, 2005; Skyler, 2007). Immunomodulation of T1D can broadly be divided into antigen-specific (Tab. 1), or non-antigen-specific immunotherapy (Tab. 2). A common approach of antigen-specific therapy is to target the autoreactive T cells and to tolerize them with soluble and formulated islet-cell antigens. Antigens that have attracted the most attention for this purpose are insulin, GAD65, and heat shock protein (Hsp) 60 (Sia, 2004; Cernea & Herold, 2006).

In the Diabetes Prevention Trial-Type 1 (DPT-1), the therapeutic potential of subcutaneous and oral insulin effects were investigated in first- and second-degree relatives of T1D patients (DPT-1, 2002; Skyler *et al.*, 2005), and in the Finnish DIPP trial, nasal insulin was administrated to children with an increased risk for T1D (Näntö-Salonen *et al.*, 2008). However, neither trial was able to delay or prevent T1D development. In another antigen-specific trial, alum-formulated recombinant human GAD65 was shown to slow down the loss of fasting C-peptide levels in newly diagnosed T1D patients. However, the treatment had no effect on the insulin requirement (Ludvigsson *et al.*, 2008). Similar results have been obtained with the GAD-alum vaccination in latent autoimmune diabetes in adult (LADA) patients (diagnosed within 5 years). After a 24 week study-period and a 5 year follow-up, LADA patients receiving 20 μg GAD-alum had increased fasting C-peptide levels compared to the placebo group (Agardh *et al.*, 2005; Agardh *et al.*, 2009). Antibodies and T-cell responses to Hsp60 have been shown to precede the onset of T1D (Ozawa *et al.*, 1996; Horvath *et al.*, 2002). Hsp60 is not a β-cell-specific autoantigen, but is expressed on the membranes of β-cell granules (Brudzynski *et al.*, 1992). DiaPep277 is a peptide derived from the Hsp60, and treatment with DiaPep277 in new-onset T1D patients showed improved C-peptide levels and lower insulin requirement (Raz *et al.*, 2001; Elias *et al.*, 2006). However, the effect was not seen in younger patients (Lazar *et al.*, 2007; Schloot *et al.*, 2007).

Monoclonal antibodies to CD3 are an example of a non-antigen-specific therapy used in T1D prevention trials. Anti-CD3 antibodies are a potent inducer of cytokine release and T-cell depletion, but also enhance immune regulation (Kaufman & Herold, 2009). Anti-CD3 antibody treatment in new-onset T1D patients showed maintained or improved C-peptide responses. Hemoglobin A1c levels and insulin requirement were also reduced in the drug-treated subjects, but the adverse effects are common and are an obstacle that need to be overcome (Herold *et al.*, 2002; Herold *et al.*, 2005; Keymeulen *et al.*, 2005; Herold *et al.*, 2009b; Keymeulen *et al.*, 2010). Another ongoing non-antigen-specific intervention is the Trial to Reduce IDDM in Genetically At-Risk (TRIGR), aiming to evaluate the role of early exposure to cow's milk protein as a risk factor for T1D (Åkerblom *et al.*, 2005; TRIGR, 2007).

Table 1. T1D prevention trials using antigen-specific approaches.

Agent	Route	Results	References
Insulin	S.c., oral	No effect	DPT-1, 2002; Skyler *et al.*, 2005
Insulin	Intranasal	No effect	Näntö-Salonen *et al.*, 2008
GAD65	S.c.	Improved C-peptide levels in new-onset T1D patients and in LADA patients	Ludvigsson *et al.*, 2008; Agardh *et al.*, 2005; Agardh *et al.*, 2009
DiaPep277 (Hsp60 peptide)	S.c.	Improved C-peptide levels and lower insulin requirements in new-onset T1D patients	Ras *et al.*, 2001; Elias *et al.*, 2006; Lazar *et al.*, 2007; Schloot *et al.*, 2007

S.c., subcutaneous; LADA, latent autoimmune diabetes in adult.

Table 2. T1D prevention trials using non-antigen-specific approaches.

Agent	Route	Results	References
Anti-CD3	I.v.	Improved C-peptide levels and reduced insulin requirements in new-onset T1D	Herold *et al.*, 2002; Keymeulen *et al.*, 2005; Herold *et al.*, 2005; Herold *et al.*, 2009b; Keymeulen *et al.*, 2010
Hydrolysed cow's milk	Oral	Ongoing	Åkerblom *et al.*, 2005; TRIGR, 2007

I.v., intravenous.

2.2 T cells and autoimmunity

2.2.1 Introduction of the immune responses

The immune system protects the body against harmful pathogens (e.g. virus, bacteria, parasites), but at the same time it must recognize its own and other beneficial microbes in order to maintain normal tissue and organ function. Immunological responses can be divided into innate and adaptive immunity. Innate immune responses are the first and rapid line of defense against pathogens, composed of external barriers (e.g. skin, mucous membranes), phagocytosis, NK cells, the complement system and cytokines. Innate immunity recognizes the presence of pathogens with a number of germline-encoded receptors called pattern-recognition receptors (PRR) that bind, for example, lipopolysaccharides, proteins, and nucleic acids expressed by pathogens. Recognition of these pathogen patterns by PRR leads to the activation of antigen-presenting cells and adaptive immunity. Adaptive immunity is more sophisticated, consisting of B and T cells (lymphocytes), and allows specific recognition of self and non-self structures via antigen presentation. T cells are the effectors of cellular immune responses, while the antibody-producing plasma cells derived from B cells mediate adaptive humoral immunity after receiving signals from T cells and other cells. Adaptive immunity is also capable of generating immunological memory to antigens, leading to a stronger and more efficacious immune response when encountering the same antigen. The innate immune system is, on the other hand, not able to remember earlier antigenic encounters (Delves

& Roitt, 2000; Kim & Lee, 2009; Chaplin, 2010). Innate immunity has a key role in the initiation phase of the autoimmune process of T1D and one of the best characterized PRR, the Toll-like receptors (TLR) have been implicated with altered innate immune functions in T1D (Kim & Lee, 2009). The activation of self-reactive T cells is believed to result from malfunctions in the adaptive immune response (Chaplin, 2010), and in the following chapters the focus will be placed on T cells in adaptive immune responses.

2.2.2 T-cell development

T cells are derived from bone marrow stem cells and their differentiation process occurs in the primary (thymus) and secondary lymphoid organs (lymph nodes, spleen) (Zlotoff *et al.*, 2008). Thymic development consists of several processes, during which double-negative CD4$^-$CD8$^-$ precursor T cells become double-positive CD4$^+$CD8$^+$ T cells in the cortex, and differentiate further into single-positive CD4$^+$ or CD8$^+$ T cells in the medulla, and finally mature T cells are exported to the periphery (Starr *et al.*, 2003). During the developmental process the T cell receptor (TCR) is assembled, consisting of αβ or γδ heterodimers. Each α, β, γ, and δ chains contains a constant domain and a variable domain. The variable domain is generated by the recombination of the V (variable), D (diversity), and J (joining) gene segments, giving the TCR its great diversity in recognizing different antigens (Delves & Roitt, 2000). V and J segments are present in all variable domains of TCR, whereas the D segment is only present in the β and δ chains. The TCRαβ receptor is found on 95% of T cells in the peripheral blood and only a small fraction express the TCRγδ receptor. T cells expressing TCRγδ are more commonly found in the gastrointestinal epithelium (Bonilla & Oettgen, 2010). In order to become activated and to gain their full function, circulating naïve T cells need to encounter their specific antigen peptide bound to the MHC molecule on the surface of an APC. The T-cell activation occurs through the TCR binding to the peptide-MHC complex. The α and β chains of the TCR associate with invariant accessory chains of the CD3 complex (consisting of transmembrane CD3γ, CD3δ, CD3ε chains and a intracytoplasmic homodimer of two CD3ζ chains) that transduce the signal when the TCR binds to a peptide-MHC complex (Chaplin, 2010).

2.2.3 T-cell activation and antigen recognition

The initiation of adaptive immune responses requires the activation of T cells. The CD4 or CD8 co-receptors on T cells form together with CD3 an essential part of the T-cell receptor complex. CD4$^+$ T cells recognize antigen peptides presented on a cell expressing the MHC class II molecule and CD8$^+$ T cells bind to antigen peptides presented by cells expressing the MHC class I molecule (Delves & Roitt, 2000; Klein & Sato, 2000b). The T-cell activation is performed according to a two-step model (Bretscher & Cohn, 1970), and the first step is the interaction between TCR and its specific peptide-MHC complex (signal 1). Essential additional signals come from co-stimulatory molecules, the second step in T-cell activation (signal 2). In the absence of co-stimulatory signals, T cell receptor recognition leads to anergy. The most potent co-stimulatory molecules are the B7-1 (CD80) and B7-2 (CD86) expressed on APC. Their ligands are CD28 and

CTLA-4 (CD152). CD28/B7 interaction is needed when naïve T cells become activated, whereas the CTLA-4/B7 interaction is needed for inactivation of T cells. CD28 also promotes interleukin-2 (IL-2) production and T-cell survival (Lenschow *et al.*, 1996; Sharpe & Freeman, 2002). Another co-stimulatory molecule involved is the intercellular adhesion molecule ICAM-1 and its ligand lymphocyte functional antigen-1 (LFA-1), contributing to the formation of a stable contact between the T cell and the corresponding APC, known also as an immunological synapse (Fig. 2) (Lebedeva *et al.*, 2005). A third cytokine-dependent signal is also important for a strong T-cell activation and clonal expansion. IL-12 and IFN-α/β have been reported to function as a third signal for CD8$^+$ T cells, while it is less clear for CD4$^+$ T cells. Recent findings suggest that IL-1 could act as a third signal for CD4$^+$ T cells (Curtsinger & Mescher, 2010).

A strong TCR stimulation is important in the development of long-lived memory T cells, especially for CD4$^+$ memory T cells. Memory T cells, arisen from activated naïve T cells, are more readily triggered and produce a more rapid response upon stimulation with the specific antigen compared to a naïve T cell. Signaling through both the IL-7 and IL-15 receptors, but not MHC is required for memory T-cell survival and proliferation. Memory T cells can be distinguished from naïve T cells by their expression of different variants of the CD45 molecule. CD45RA is expressed on naïve cells and CD45RO is expressed on memory cells (Tanel *et al.*, 2009; van Leeuwen *et al.*, 2009).

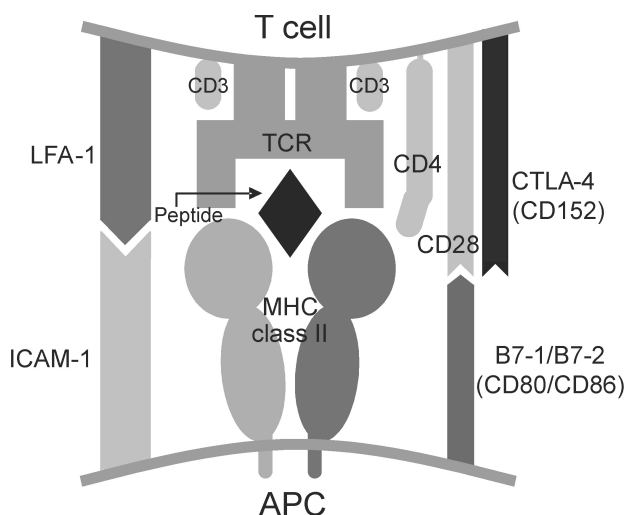**Figure 2.** Schematic picture of the interaction between a CD4$^+$ T cell and an antigen-presenting cell (APC).

2.2.4 MHC, antigen processing and presentation

MHC class I molecules are widely expressed on all nucleated cells, but class II molecules are only expressed on B cells, activated T cells, macrophages, DC, and thymic epithelial cells. The MHC class I molecule consists of a polymorphic α-chain with two peptide-

binding domains, one immunoglobulin-like domain, transmembrane region, cytoplasmic tail, and a non-polymorphic β2-microglobulin encoded outside the MHC. The MHC class II α- and β-chains consists of a peptide-binding domain, an immunoglobulin-like domain, the transmembrane region, and the cytoplasmic tail (Klein & Sato, 2000a). MHC class I molecules present short peptides of 9-11 amino acids, while the MHC class II molecule, which has a open-ended peptide-binding groove, can present peptides of 13-24 amino acids (Bonilla & Oettgen, 2010).

The processing and presentation of the peptides to the cell surface can take different routes, depending on the MHC class I- or II-type of cell. MHC class I cells present antigens synthesized within the cell, whereas MHC class II cells present extracellular antigens. In the MHC class I pathway, worn-out or defective proteins in the cytosol are digested to peptides by proteasomes. The peptides are then transported to the endoplasmic reticulum (ER), where they are loaded onto the MHC class I molecule. The peptide-MHC class I complex is then transported by the Golgi apparatus to the cell membrane where it can be presented to CD8^{+} T cells. For the MHC class II pathway self or foreign proteins are picked up by endocytosis or phagocytosis into an endosome and degraded by lysosomal enzymes. In the ER, newly synthesized MHC class II molecule associate with the invariant chain, which protects the peptide-binding groove while the MHC class II molecule is transported in a membranous vesicle to the endosome containing the extracellular peptides. The transporting vesicle and the endosome fuse, the invariant chain is degraded, and the peptide-MHC class II complex is formed (with the help of the HLA-DM molecule). Thereafter, the complex is ready to be transported to the surface of the cell for recognition by CD4^{+} T cells. In some cases, exogenous antigens that have been internalized by endocytosis can also be presented in HLA class I molecules. This is known as "cross-presentation" (Klein & Sato, 2000a; Chaplin, 2010).

2.2.5 T-cell effector subsets

During the maturation process in the thymus and secondary lymphoid organs, αβ T cells differentiate into subpopulations with defined repertoires and effector functions, as well as with memory functions. The two major subsets are, as mentions earlier, CD4^{+} and CD8^{+} T cells. CD4^{+} T cells are designated helper cells (Th cells) that stimulate both humoral and cellular immune responses. CD8^{+} T cells express cytotoxic activity on cells infected with intracellular microbes and on tumor cells, but they also show suppressive activity. They are referred to as CD8^{+} cytotoxic T cells (CTL) and CD8^{+} suppressive T cells (Bonilla & Oettgen, 2010).

CD4^{+} T helper cells can be divided into several different subsets depending on which cytokines they produce (Fig. 3). The two main subsets are the Th1 and Th2 cells (Mosmann *et al.*, 1986; Mosmann & Sad, 1996). Th1 and Th2 cells develop from naïve Th0 precursor cells that express cytokines of both patterns. Th1 cells secrete IL-2 and IFN-γ and their development from Th0 cells is stimulated by IL-12 and IFN-γ. Th1 cells produce pro-inflammatory cytokines and activate mononuclear phagocytes, NK cells,

and CTL for the killing of intracellular infected target cells (Mosmann & Sad, 1996; Wan & Flavell, 2009; Bonilla & Oettgen, 2010). On the other hand, Th1 cells can also cause tissue damage and promote unwanted autoimmune diseases. Th1 cells and IFN-γ have been associated with inflammatory bowel disease (Davidson *et al.*, 1996; Parronchi *et al.*, 1997), rheumatoid arthritis (Leung *et al.*, 2000) and T1D (Wang *et al.*, 1997; Pakala *et al.*, 1999). Th2 cells produce IL-4, IL-5, IL-10, and IL-13 and are shown to differentiate from Th0 precursors under the influence of IL-4 and transcription factor GATA-3. Th2 cytokines promote antibody-production, allergic reactions (atopy, asthma), and parasite-induced immune responses (Wan & Flavell, 2009; Bonilla & Oettgen, 2010).

Th17 is a newly identified subset of effector T cells that produce mainly IL-17A, IL-17F, IL-21, and IL-22. The cytokines, IL-1β, IL-6, IL-21, and IL-23, are essential for the development of human IL-17 producing cells (Crome *et al.*, 2009; Torchinsky & Blander, 2010). Some early findings suggested that the transforming growth factor-β (TGF-β) was not needed for human Th17 cell development (Acosta-Rodriguez *et al.*, 2007), but more recent reports have shown that TGF-β is important for their differentiation (Manel *et al.*, 2008; Volpe *et al.*, 2008; Yang *et al.*, 2008b). IL-17A and IL-17F are potent pro-inflammatory cytokines, and Th17 cells are believed to be important in the host's defense against infections, as well as in autoinflammatory disorders (Crome *et al.*, 2009; Torchinsky & Blander, 2010), including inflammatory bowel disease (Fujino *et al.*, 2003), rheumatoid arthritis (Chabaud *et al.*, 1999) and multiple sclerosis (Hofstetter *et al.*, 2009).

CD4$^+$ T cells with suppressive functions, the regulatory T (Treg) cells, are another important subset controlling the immune responses. This T-cell subset consists of naturally occurring and inducible Treg cells. Naturally occurring CD4$^+$ T cells coexpress high levels of CD25 (IL-2 receptor α chain), and arise from the thymus. Inducible CD4$^+$ Treg cells develop in the periphery during activation, such as type 1 regulatory T (Tr1) cells and T helper 3 (Th3) cells (Liu & Leung, 2006). CD4$^+$CD25$^+$ Treg cells are also characterized by their expression of forkhead/winged-helix transcription factor box protein 3 (FoxP3), and they produce the immunomodulatory cytokines TGF-β and IL-10. Their development is stimulated by IL-2, TGF-β and by transcription factor FoxP3. Tr1 and Th3 cells do not express FoxP3 (Corthay, 2009). Tr1 cells are characterized by their high production of IL-10, while Th3 cells preferentially produce high levels of TGF-β (Liu & Leung, 2006). The best-characterized subset of CD4$^+$ Treg cells, the CD4$^+$CD25$^+$ Treg cells, will be more extensively discussed in the next chapter, as well as their role and function in T1D.

Natural Killer T (NKT) cells are an unusual subset of TCRαβ expressing T cells that simultaneously express NK cell markers. In humans, a large fraction of the NKT cells is characterized by their expression of a unique TCR combination of Vα24-Jα18 together with the Vβ11 chain (Bonilla & Oettgen, 2010). NKT cells links the innate and adaptive immune systems and they are suggested to have a key regulatory role in T1D (Novak *et al.*, 2007), which will be further discussed in chapter 2.2.7.
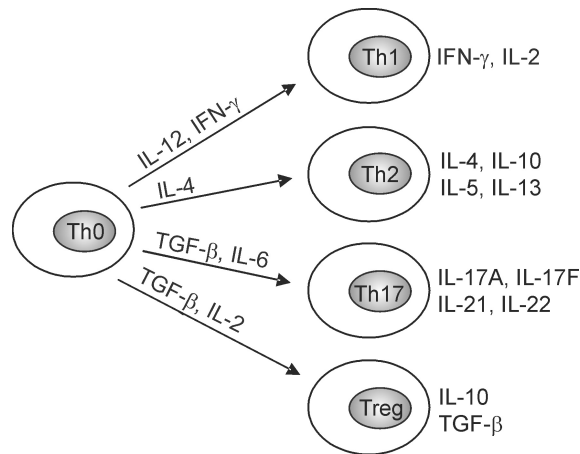
Figure 3. CD4^{+} T-cell subsets. Depending on the APC and the cytokine stimulation at the site of the antigen encounter, the Th0 cell can develop into several different subsets expressing specific cytokine profiles and functions.

2.2.6 Regulatory T cells

Since the mid-1990's, when Sakaguchi *et al.* described a small population of the CD4^{+} T cells expressing the α chain of the IL-2 receptor (CD25) with an immune suppressive function (Sakaguchi *et al.*, 1995), a large amount of interest has been focused on these Treg cells. Recent evidence suggests that the Treg cells can be divided into two main groups. The first group, known as natural Treg (nTreg) cells, develop in the thymus and are mainly characterized by their expression of high levels of CD25, the transcription factor FoxP3, CTLA-4, glucocorticoid-induced tumor necrosis factor receptor (GITR), lymphocyte activation gene-3 (LAG-3), and low levels of the IL-7 receptor α chain (CD127) (Liu & Leung, 2006; Sakaguchi *et al.*, 2006; Corthay, 2009). Their regulatory activity is cell-contact dependent and can be mediated through several suppressive mechanisms, such as B7/CTLA-4 interaction, cytolysis, and the secretion of immunosuppressive cytokines (e.g. IL-10, TGF-β) (Nakamura *et al.*, 2001; von Boehmer, 2005; Wan & Flavell, 2009). Natural (CD4^{+}CD25^{high}) Treg cells comprise 6-7% of peripheral CD4^{+} T cells in humans (Torgerson, 2006). The second group of Treg cells differentiates in the periphery from naïve CD4^{+} T cells, following TCR activation by specific antigen. These are named adaptive Treg (aTreg) cells. Their expression of FoxP3 can be variable and they produce large amounts of TGF-β and IL-10 (Stassen *et al.*, 2004; Curotto de Lafaille & Lafaille, 2009).

The primary function of Treg cells is to maintain self-tolerance and to prevent autoimmune diseases. Their role in the immune system is complex and the findings are somewhat controversial, mainly because no single specific marker exclusively expressed on Treg cells is known (Corthay, 2009). CD25, FoxP3, CTLA-4, GITR and LAG-3 are all up-regulated on T cells following activation (Triebel *et al.*, 1990; Allan *et al.*, 2007). CD127 has been used to discriminate between CD127^{dim} Treg cells and CD127^{high} cells in humans (Liu *et al.*, 2006; Seddiki *et al.*, 2006). More recently, it has been shown that most CD4^{+}

T cells downregulate CD127 upon activation (Allan *et al.*, 2007; Aerts *et al.*, 2008). However, the transcription factor FoxP3 is clearly important for Treg development, in the absence of FoxP3 CD4$^+$CD25$^+$ Treg cells do not develop. FoxP3 is considered to be the most reliable marker for Treg cells, although it is not restricted to them (Fontenot *et al.*, 2003; Fontenot *et al.*, 2005).

Treg cells appear to have a critical role in autoimmunity both in animal models and in human diseases, including inflammatory bowel disease, rheumatoid arthritis, and T1D (Boden & Snapper, 2008; Walker, 2008; Boissier *et al.*, 2009). A clear indication that Treg are important for the regulation of pancreas autoimmunity in humans is the early onset of T1D in patients with immune dysregulation, polyendocrinopahty, enteropathy, X-linked (IPEX) syndrome, in whom mutations of FoxP3 leads to a severely dysregulated immune system (Torgerson, 2006). Early work suggested a decrease in CD4$^+$CD25$^+$ Treg cells in T1D (Kukreja *et al.*, 2002), but subsequent studies have not observed any differences in the numbers of Treg cells between T1D patients and healthy controls (Brusko *et al.*, 2005; Lindley *et al.*, 2005; Putnam *et al.*, 2005; Liu *et al.*, 2006; Brusko *et al.*, 2007). Analysis of Treg function in T1D has generated conflicting evidence of defective Treg function (Brusko *et al.*, 2005; Lindley *et al.*, 2005; Putnam *et al.*, 2005; Putnam *et al.*, 2009). Several groups have found an impaired suppression function of Treg cells isolated from T1D patients, the CD4$^+$CD25$^+$ Treg cells showed impaired suppression of the autologous CD4$^+$ responder T cells (Brusko *et al.*, 2005; Lindley *et al.*, 2005), but others have contested these results (Putnam *et al.*, 2005; Putnam *et al.*, 2009). Further studies have suggested an increased resistance of T-effector cells for Treg-mediated suppression as a mechanism for the defective regulation of autoimmunity in T1D patients (Lawson *et al.*, 2008; Schneider *et al.*, 2008). Recently, islet-specific CD25$^+$FoxP3$^+$ adaptive Treg cells have been generated from human peripheral CD4$^+$CD25$^-$ T cells from both T1D patients and controls, showing that functional adaptive Treg response to islet-antigens is possible. However, no difference in the suppressive function of these antigen-specific adoptive Treg cells in patients and non-affected individuals was observed (Long *et al.*, 2009). Further, new approaches and more specific Treg markers should illuminate the Treg role in autoimmune diabetes.

2.2.7 Natural Killer T cells

Natural killer T cells express both T cell and NK cell characteristics. Their immunomodulatory effect is mediated by changing the cytokine milieu at the inflammatory site, contributing to both innate and adaptive immunity. A major group of NKT cells, known as invariant NKT (iNKT) cells, express a TCR formed of Vα24-Jα18 and Vβ11 chains in humans (Vα14-Jα18 and Vβ8.2/Vβ7/Vβ2 in mice), and can be segregated into three subsets depending on their CD4$^+$, CD8$^+$ or CD4$^-$CD8$^-$ expression (Novak *et al.*, 2007; Wu & Van Kaer, 2009). They also express CD161 and CD56 NK specific markers in their late stages of differentiation (Exley *et al.*, 1998). iNKT cells recognize glycolipid antigens presented by the MHC class I-like protein CD1d (Brigl *et al.*, 2003), and they are present in very low numbers in humans, 0.01-1% in peripheral blood (Montoya *et al.*, 2007). Another subset consists of CD1d-restricted NKT cells with a more diverse

TCR, named non-invariant or type II NKT cells, and are not as well defined as the iNKT (Novak *et al.*, 2007; Wu & Van Kaer, 2009).

Activated iNKT cells respond with a rapid production of cytokines, including IFN-γ, tumor necrosis factor-α, IL-2, IL-4, and IL-13. Human CD4$^+$ iNKT subsets produce both Th1 and Th2 cytokines, while human CD4$^-$CD8$^-$ iNKT subsets have a more Th1 cytokine profile (van der Vliet *et al.*, 2004). iNKT have been associated with a variety of regulatory responses, such as tumor rejection, infectious diseases, as well as involvement in allergic responses and autoimmune diseases (van der Vliet *et al.*, 2004). Evidence of iNKT cells immune regulation have been seen in non-obese diabetic (NOD) mice protected from T1D by increasing the iNKT cell number followed by an α-galactosylceramide (synthetic glycolipid ligand) treatment, transgenic induction of Vα14-Jα28 expression or transgenic expression of CD1d in NOD islets (Lehuen *et al.*, 1998; Sharif *et al.*, 2001; Falcone *et al.*, 2004). In human T1D, the frequency of iNKT cells has been reported to be decreased (Wilson *et al.*, 1998; Kukreja *et al.*, 2002; Kis *et al.*, 2007), increased (Oikawa *et al.*, 2002) or unaltered (Lee *et al.*, 2002; Tsutsumi *et al.*, 2006) in the peripheral blood of T1D patients compared to non-affected individuals. Interestingly, a higher expansion of CD4$^-$CD8$^-$ iNKT cells associated with a pro-inflammatory profile has been described in individuals at risk for T1D compared to controls (Montoya *et al.*, 2007). In two other studies, higher production of Th1 cytokines in iNKT cells were observed in T1D patients (Wilson *et al.*, 1998; Kis *et al.*, 2007), while others found a general suppression of IFN-γ and IL-4 in iNKT cells in T1D patients (Kukreja *et al.*, 2002). Also no impaired iNKT cell production of IFN-γ and IL-4 has been observed in diabetes patients and at-risk subjects (Lee *et al.*, 2002). In these studies, different detection methods were used for identification of iNKT, and may be the source of the variable results or they are describing different iNKT subsets mediating either susceptibility or resistance to T1D.

2.2.8 Self-tolerance

The ability of the immune system to avoid attacking its own tissues is referred to as self-tolerance. Thus, it is induced when immature lymphocytes encounter self antigens in the primary lymphoid organs (central tolerance), and later on when mature lymphocytes come across self antigens in peripheral or secondary lymphoid organs (peripheral tolerance) (Starr *et al.*, 2003).

The mechanism for central T-cell tolerance is complex and involves different checkpoints in the thymus. Positive selection is a crucial step allowing only those T cells showing TCR recognition and binding specificity to encountered peptide-MHC complexes to differentiate further. T cells not showing any affinity for the expressed peptide-MHC complexes are directed to apoptosis. The selected T cells then migrate from the cortex to the medulla, where they interact with DC and medullary thymic epithelial cells (mTEC). At this checkpoint, T cells showing high affinity for self-peptides are eliminated, known as negative selection (Starr *et al.*, 2003; Romagnani, 2006). Dendritic cells and especially mTEC, have a critical role in negative selection, as mTEC can express a large scale of

tissue-restricted self-peptides on their surface for T-cell recognition. This is possible due to promiscuous gene expression during mTEC development, which is regulated by the transcription factor called autoimmune regulator (AIRE) (Kyewski & Klein, 2006). Eventually, only T cells equipped with TCR showing low affinity for self-peptides survive and migrate from the thymus to secondary lymphoid organs (Starr *et al.*, 2003; Romagnani, 2006).

Peripheral tolerance completes the central tolerance in regulating the expansion of low-affinity autoreactive T cells, or T cells showing high-affinity for other tissue-restricted self-peptides not expressed in the mTEC, that escape negative selection. The three main mechanisms of peripheral T-cell tolerance are anergy, peripheral deletion and immune suppression. Anergy follows if co-stimulatory signaling is missing or if CTLA-4 instead of CD28 interacts with CD80/CD86 co-stimulatory molecules. Peripheral deletion is based on activation-induced cell death (AICD) and is mediated through the Fas/Fas-Ligand signaling. The immune suppression of effector T cells is carried out by different types of immunoregulatory T cells, such as the Treg and NKT cells discussed earlier (Romagnani, 2006; Mueller, 2010).

Failure of self-tolerance is the underlying cause of autoimmune diseases, misdirecting immune responses that result in tissue damage. T cells with low-affinity for tissue-restricted self-peptides, which escape the negative selection process, may later develop into aggressive autoreactive T cells which have an important role in autoimmune development (Fig. 4) (Romagnani, 2006; Zehn & Bevan, 2006).
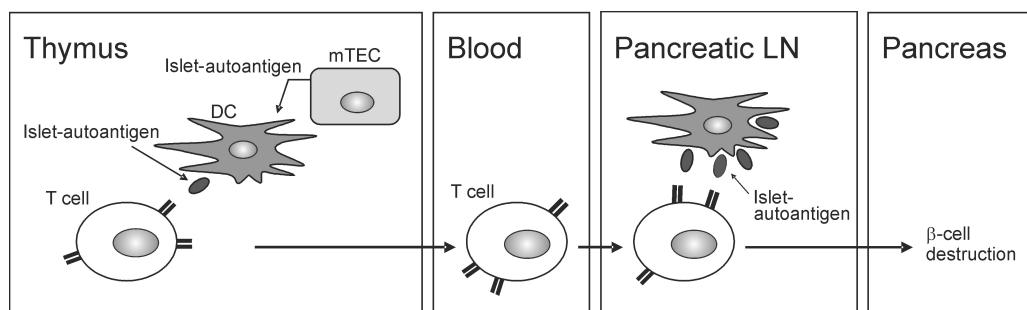
Figure 4. Low-affinity epitopes fail to negatively select T cells. For example, islet-cell specific proteins are expressed by the medullary thymic epithelial cells (mTEC) and islet-autoantigens with a low expression are inefficiently presented to thymic T cells, resulting in the partial failure of the negative selection of self-reactive T cells. In the pancreatic lymph node (LN), islet-autoantigens are expressed at a higher level in pancreatic β-cells, and can activate the specific self-reactive T cells that escaped negative selection in the thymus. T-cell activation can lead to β-cell destruction and type 1 diabetes. Modified from (von Boehmer & Melchers, 2010).

2.2.9 Autoreactive CD4$^+$ T cells in T1D

Independent of the individual triggers of T1D, or the underlining pathogenic mechanisms, destruction of pancreatic β cells is the final coordinated effort of lymphocytes in the

immune system. Self-reactive CD4^{+} and CD8^{+} T cells have both a key role in the disease development. MHC class II-restricted CD4^{+} T cells have been shown to be involved in the development of diabetes in NOD mice (Kanagawa *et al.*, 1998; Wen *et al.*, 2000), and in studies showing strong association between T1D and expression of MHC class II molecules, the human leukocyte antigen (HLA)-DR3 and -DR4 and associated HLA-DQ2 and -DQ8 molecules (She, 1996; Cucca *et al.*, 2001; Lambert *et al.*, 2004) emphasize the importance of CD4^{+} T cells in T1D development.

2.2.9.1 Glutamic acid decarboxylase 65-specific T cells

The islet-cell protein, 65 kDa isoform of GAD, has been extensively studied as a potential autoantigen for T cells. The recognized human GAD65-specific T cell epitopes are many and map primarily to the middle and C-terminal domain of the protein. T1D-associated GAD65-epitopes have been reviewed recently (Panagiotopoulos *et al.*, 2004; Di Lorenzo *et al.*, 2007).

The GAD65 247–266 and 260–279 epitopes described by Atkinson *et al.* approximately 15 years ago, are recognized by diabetes-associated HLA-DR4 and -DQ8 restricted CD4^{+} T cells. This region has a sequence similarity with the P2-C protein of Coxsackie B viruses and may result in molecular mimicry responses. T-cell responses to these two peptides in at-risk individuals and in newly diagnosed T1D patients have been described (Atkinson *et al.*, 1994). T cells recognizing another epitope of close proximity, GAD65 270–283, have been derived from T1D patients with a high-risk gene combination (Endl *et al.*, 1997). Other GAD65 epitopes 115–127, 274–286, and 554–566 have also been shown to be processed and presented by HLA-DRB1*0401 in transgenic mice (Wicker *et al.*, 1996). In another study, three very similar peptides (GAD65 116–130, 271–285, and 551–565) were also described to be immunodominant in HLA-DR4 transgenic mice (Patel *et al.*, 1997). GAD65 555–567, has been shown to be naturally processed by the extraction of HLA-DR4 molecules on human APCs (Nepom *et al.*, 2001). The presence of GAD65 555–567 CD4^{+} T cells was further demonstrated in peripheral blood from HLA-DRB1*0401 or DRB1*0404 positive T1D patients, and from at-risk individuals, using the MHC class II tetramer technique (Reijonen *et al.*, 2002; Reijonen *et al.*, 2004). In a recent study, human TCR derived from GAD65 555–567 specific T cells injected into HLA-DR4 transgenic mice were shown to enhance early autoimmune responses in pancreatic islets (Gebe *et al.*, 2008). These observations strongly support the role of GAD65-specific T-cell responses in the pathogenesis of human T1D.

2.2.9.2 Proinsulin- and insulin-specific T cells

Proinsulin and insulin are β-cell specific autoantigens associated with T1D. Mature insulin consists of an A and B chain and is processed from its precursor molecule preproinsulin first to proinsulin, and then to insulin once the C-peptide region has been cleaved off (Steiner *et al.*, 2009). Increasing evidence suggests that proinsulin and insulin may be an early autoantigen in the pathogenesis of T1D, and overviews of T-cell specific

proinsulin and insulin epitopes have been presented recently (Panagiotopoulos *et al.*, 2004; Di Lorenzo *et al.*, 2007).

CD4^{+} T-cell reactivity to insulin was first reported in pre-diabetic NOD mice, in which CD4^{+} T-cell clones specific to the insulin B9–23 epitope were isolated from infiltrated islets (Daniel *et al.*, 1995). The insulin B9–23 region is identical both in mice and humans, and was subsequently described to induce HLA-DR4 and -DQ8 restricted T-cell responses in new-onset T1D patients and in at-risk subjects (Alleva *et al.*, 2001). A larger peptide from the same region, insulin B11-27, was also reported to stimulate T-cell responses in recently diagnosed T1D patients (Schloot *et al.*, 1998). Other T1D-associated insulin epitopes have been reported from the insulin A chain. Enhanced T-cell responses to insulin A1–12 have been described in HLA-DR3/DR4-heterozygous T1D patients and in at-risk children (Marttila *et al.*, 2008), and in other studies insulin A1–13 specific CD4^{+} T-cell clones have been isolated from autoimmune diabetes patients (Mannering *et al.*, 2005; Mannering *et al.*, 2009). Insulin A1–15 specific T cells expanded from pancreatic lymph nodes of deceased T1D patients presented additional evidence for the possible role of insulin-specific CD4^{+} T cells in pancreatic autoimmunity (Kent *et al.*, 2005). The overlapping insulin A6–21 peptide has been associated as well with increased T-cell responses in T1D patients and in individuals with T1D-associated autoantibodies compared to controls (Durinovic-Bello *et al.*, 2002).

Regions spanning the B–C junction seem to be the most frequently recognized in proinsulin and insulin. The proinsulin B24–36 (B24–C4) peptide has been reported to stimulate an increased T-cell response in individuals at-risk for T1D compared to healthy controls (Rudy *et al.*, 1995). This region has similarities to the GAD65 peptide 506–518 (Rudy *et al.*, 1995). T-cell reactivity in the spleen and pancreatic lymph nodes of NOD mice to a similar epitope (proinsulin B24–33) was described by Chen *et al*. (Chen *et al.*, 2001). Intranasal administration of proinsulin B24–36 has been shown to induce regulatory T cells in NOD mice, but it was not able to prevent or delay the onset of the disease. However, treatment with proinsulin B24–32 or B24–33 reduced the incidence of diabetes in NOD mice (Martinez *et al.*, 2003). Another immunogenic region is spanning from C-chain to the A-chain, including preproinsulin 73–90 (proinsulin C17–A1), preproinsulin 74–90 (C18–A1), preproinsulin 69–88 (C13–C32), preproinsulin 75–92 (C19–A3), and preproinsulin 77–94 (C21–A5) (Congia *et al.*, 1998; Durinovic-Bello *et al.*, 2002; Arif *et al.*, 2004; Durinovic-Bello *et al.*, 2004; Durinovic-Bello *et al.*, 2006). Many of these peptides contain the preproinsulin 76–90 (proinsulin C20–A1) region, which has been shown to contain a HLA-DR*0401 binding motif, and to be a naturally processed epitope (Marshall *et al.*, 1995; Arif *et al.*, 2004). Recently reports have described an increased response to the preproinsulin 75-92 (C19-A3) (Thrower *et al.*, 2009), and preproinsulin 76-90 (C20-A1) peptides in T1D patients (Durinovic-Bello *et al.*, 2010), and a lower threshold of activation in T1D patients to the same peptide compared to healthy individuals (Yang *et al.*, 2008a).

3. AIMS OF THE STUDY

The main aim of this study was to investigate islet-specific T-cell autoimmunity mediated and regulated by distinct T-cell lineages in T1D, to obtain more knowledge of their role in T1D pathogenesis.

The specific aims of this study were:

- To analyze the prevalence of GAD65-, proinsulin- and insulin-specific CD4$^+$ T cells in T1D and during the pre-clinical phase of the disease, and to evaluate the use of MHC class II tetramers in the prediction of T1D.

- To compare memory and naïve CD4$^+$ T-cell responses to GAD65, proinsulin and insulin in children with T1D, in children with diabetes-associated autoantibodies, and in control individuals. To distinguish if T1D patients more often express autoantigen-specific T cells of memory phenotype compared to controls.

- To determine how antigen dose influences the diversity of autoreactive GAD65-specific CD4$^+$ T cells. To test the hypothesis if low doses of antigens can contribute to autoreactive CD4$^+$ T cells with high avidity.

- To study the role of circulating CD4$^+$CD25^{high} regulatory T cells and natural killer T cells in T1D. To determine if any differences in the frequency of regulatory T-cell subsets can be observed when the first signs of autoimmunity are present or at clinical onset.

4.   SUBJECTS, MATERIALS AND METHODS

4.1   Subjects

Subjects in reports I, II and IV were participants in the Type 1 Diabetes Prediction and Prevention (DIPP) study that is ongoing at three university hospitals in Finland (Turku, Tampere and Oulu University hospitals). DIPP is a population-based birth cohort trial and all newborns at these three university hospitals are invited to participate in genetic screening for susceptibility to T1D. Children at risk are further invited to take part in the follow-up study. In Turku, the children are followed-up in 3-month intervals until 2 years of age and then every 6 months. In Tampere and Oulu, the follow-up visits are at 3, 6, 12, 18 and 24 months of age, and then annually. During every visit, blood samples are drawn to measure β-cell autoimmunity and information about health, vaccinations, breastfeeding and cow's milk formula usage are obtained (Kupila *et al.*, 2001). Additionally, a subcohort of the multiple autoantibody-positive subjects included in reports I, II and IV were participants in a placebo-controlled, double-blind intervention trial, aimed at assessing whether it is possible to decrease the progression rate to clinical disease by daily nasal administration of human short-acting insulin, described in detail (Näntö-Salonen *et al.*, 2008). The local university and hospital ethical committees approved the DIPP study protocol and informed consents were obtained from the guardians of the participants. The demographics of the subjects in report I, II and IV are presented in table 3.

A volunteer subject positive for HLA-DQB1*0302/-DR*0404 (negative for T1D associated autoantibodies) with a known response to both wild type GAD65 555-567 and GAD65 555-567 (557I) peptide contributed to report III.

Table 3. Demographics of the subjects included in the study. Report IIa = subjects in PBMC stimulations, Report IIb = subjects in the study with T cells of memory and naïve origin.

	Report I	Report IIa	Report IIb	Report IV
T1D patients (n=)	18	26	15	25
Median age, years	11.0	7.5	8.5	8.0
Range, years	3.0−16.0	1.5−15.0	2.0−15.0	3.0−13.0
Multiple AAb+ (n=)	20	48	20	21
Median age, years	6.0	9.0	10.0	6.0
Range, years	3.0−13.0	3.0−17.0	4.0−15.0	2.0−11.5
3-4 AAb+ (n=)				14
Median age, years				5.0
Range, years				2.0−9.0
2 AAb+ (n=)				7
Median age, years				8.0
Range, years				5.0−11.5
Controls (n=)	21	70	31	39
Median age, years	5.5	7.5	10.0	6.5
Range, years	3.0−12.0	2.0−16.5	1.0−14.0	2.0−13.0
Age at diagnosis of T1D	4.5 years	≤ 7 days	≤ 7 days	≤ 7 days
Participants in the insulin trial (n=)	16	34	12	16
AAb+, later developed T1D (n=)	2	11	3	3

4.2   Methods

4.2.1 Genetic screening

Children were eligible for participating in the DIPP study based on the presence of HLA-DQA1*05-DQB1*02 and/or DQB1*0302 alleles/haplotypes, and lack of protective DQB1 alleles. HLA-DR4 subtyping (DRB1*0401, *0402, *0403/6, *0404, *0405, *0407, *0408) was also determined in all subjects in reports I-III. Genetic screening of HLA-DR-DQ haplotypes was carried out using a PCR-based lanthanide labeled oligonucleotide hybridization and the time-resolved fluorometry method as described (Sjöroos *et al.*, 1995; Nejentsev *et al.*, 1999; Laaksonen *et al.*, 2002; Hermann *et al.*, 2003).

4.2.2 Autoantibody assays

Autoantibody detection of ICA was done with the immunofluorescence method, and detection of GADA and IA-2A with radiobinding assays previously described in detail (Savola *et al.*, 1998a; Savola *et al.*, 1998b). IAA detection was done with a modified version of microassay described by Williams *et al.* (Williams *et al.*, 1997). The detection limit in the ICA assay was 2.5 Juvenile Diabetes Foundation units (JDFU), and sensitivity of the test was 100%, and specificity was 96%. For the GADA, IA-2A, and IAA assays, the cut-off limit for positivity was set at the 99th percentile in 371 or more non-diabetic Finnish children. Subjects were considered to be positive when the specific binding was equal or higher than 5.35 RU (relative units) for GADA, 0.429 RU for IA-2A, and 1.55 RU for IAA. The disease sensitivity and specificity for GADA was 76% and 96%, for IA-2A 58% and 100%, for IAA 30% and 100% in the 2001 Diabetes Autoantibody Standardization Program (DASP) workshop (Kimpimäki *et al.*, 2002).

4.2.3 Isolation of cells from peripheral blood

Peripheral blood mononuclear cells (PBMC) were isolated from 5−30 ml (80 ml in report III) of heparinized blood by Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden; GE healthcare, Piscataway, NJ, USA) gradient centrifugation according to the manufacturer's instructions. The cells were suspended in RPMI-1640 (Invitrogen Corporation, Paisley, UK) and supplemented with 10% human AB serum (SPR, Helsinki, Finland; Sigma-Aldrich Cheme GmbH, Steinheim, Germany). Except for the cells in report IV, which were suspended in 1 x PBS. In report II, CD4$^+$CD45RO$^+$ enriched T cells and CD45RA$^+$ naïve cells were isolated from 4-40 x 10^6 PBMC with the MACS (magnetic activated cell sorter) Memory CD4$^+$ T cell isolation kit (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions with the VarioMACS separator (Miltenyi Biotec). Only CD4$^+$CD45RO$^+$ T-cell fractions exceeding 90% purity and CD4$^+$CD45RA$^+$ T-cell fractions exceeding 80% purity were used in subsequent analysis. In report III, CD4$^+$ cells (including both naïve and memory cells) were isolated from 50 x 10^6 PBMC with the MACS CD4$^+$ T-cell isolation kit II (Miltenyi Biotec, Auburn, CA, USA). The

separation was done according to the manufacturer's instructions with the autoMACS (Miltenyi Biotec).

4.2.4 Stimulation of T cells

Stimulation of antigen-specific T cells was done in flat bottom 24/48-well plates. 5×10^6 cells were cultured in the presence of GAD65 274-286, GAD65 555-567 557I, proinsulin B24-36, insulin A1-15, and insulin A6-21 peptides (10 μg/ml) shown in table 4. In report I, only one peptide per well was added, while in report II, two to three peptides per well were added, due to limited cell amounts. (GAD65 peptides were added to the same well and insulin peptides to a separate well, and if the subject was DR3/DR4-heterozygous the proinsulin peptide was added together with the GAD65 peptides). Cells were then stimulated primarily for 10-11 days, followed by a secondary stimulation on plates coated with 10 μg/ml HLA-DR*0401, DR*0404 or DR*0301 monomers containing the same peptide used in the primary stimulation. Secondary stimulation continued for 3 days with 1 μg/ml of the anti-CD28 antibody (BD Pharmingen, San Jose, CA, USA).

In report II, 0.5×10^6 CD4$^+$CD45RO$^+$ enriched T cells were stimulated with 10 μg/ml GAD65, proinsulin and insulin peptides (Tab. 4), and with irradiated 2.5×10^6 CD45RA$^+$ (non-adherent CD45RA$^+$, CD4$^+$, CD8$^+$, γδ T cells, B cells, NK cells, DC, monocytes). CD45RA$^+$ cells (2×10^6/well), from the same sample, were also separately stimulated with the same peptides used for CD4$^+$CD45RO$^+$ enriched T cells. The cells were cultured for 13-14 days, with the addition of IL-2 on day 7 (5 U/ml) and 10 (10 U/ml).

4.2.5 Preparation of HLA-DR*0401, -DR*0404 and -DR*0301 monomers and tetramers

The construction of the expression vectors for the generation of the soluble DRB1*0401, DRB1*0404 and DRB1*0301 molecules was prepared at Benaroya Research Institute at Virginia Mason (Seattle, WA, USA), as described in detail (Novak *et al.*, 1999). A general description is provided here. Soluble class II molecules with leucine zipper regions and flexible linkers were produced in the *Drosophila* system. For the production of DR molecules, cDNA encoding the extracellular domains of DRα and DRβ chains were respectively attached to the acidic and basic portion of the leucine zipper cDNA. (The leucine zippers stabilize the pairing of the soluble DRα and DRβ chains.) To the 3′ end of the DRβ leucine zipper cassette, a site-specific biotinylation sequence was added and the chimeric cDNA were subcloned into a Cu-inducible Drosophila expression vector pRMHa3. DRα and DRβ expression vectors were cotransfected into Schneider S-2 cells. Clones that expressed large amounts of class II molecules were further expanded for class II purification. Soluble DR molecules were purified by affinity chromatography with L243 antibody and biotinulation of the class II molecules were preformed with the Bir A enzyme. The empty class II molecules were loaded with the specific peptides of interest for 48-72 h at 37°C, in the presence of *n*-octyl-β-D-glucopyranoside, and a high molar ratio of peptide to MHC molecule were used to improve the efficiency of

the peptide loading process. Tetramers were assembled by the incubation of class II molecules with PE-labeled streptavidin, which has four biotin binding sites and serves as the cross-linking agent for the class II molecules, and provides a fluorochrome for flow cytometry (Fig. 5) (Novak *et al.*, 1999).
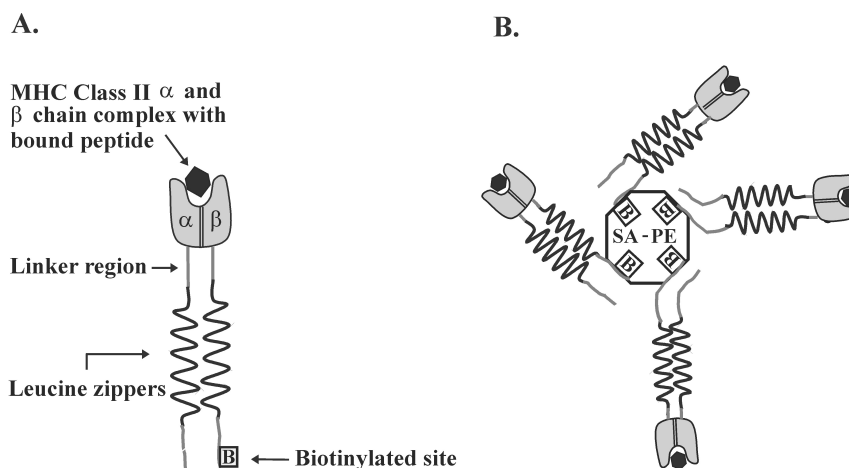

A.

MHC Class II α and
β chain complex with
bound peptide

α β

Linker region →

Leucine zippers

B ← Biotinylated site

B.

SA - PE

Figure 5. MHC class II tetramers. Soluble recombinant MHC class II α and β chains assembled as biotinylated dimers using leucine zipper and linker sequences. Schematic picture of the monomer structure (A) and its tetramerized form (B) via assembly with streptavidine (SA) and fluorochrome phycoerythrin (PE)-label. Modified from (Nepom *et al.*, 2002).

4.2.6 Tetramer binding technique

MHC class II tetramers allow for the direct detection of antigen-specific CD4$^+$ T cells by flow cytometry. This method is based on the specific interaction between the peptide-MHC complex and the corresponding T-cell receptor. Cross-linking four peptide-MHC complexes with streptavidin increases the avidity of the interaction (Nepom *et al.*, 2002). Because of the relatively low frequency of autoreative CD4$^+$ T cells, an *in vitro* amplification is usually needed for detection with tetramers. Previously, results have showed that tetramer positive cells are usually CD25$^+$ and often CD4^{high}. A positive tetramer staining result is compared to the staining of the same cells with a control tetramer, since the level of background staining can vary.

Following peptide stimulation in reports I-III, one half of the cells were stained with 10 µg/ml (15 µg/ml in report I) PE-labeled HLA-DR*0401 or *0404 GAD65 274–286, DR*0401 or *0404 GAD65 555–567 557I, DR*0301 proinsulin B24–36, DR*0401 or *0404 insulin A1−15 or DR*0401 insulin A6−21 tetramers, and the other half of the cells were stained with 10 µg/ml (15 µg/ml in report I) PE-labeled control tetramers HLA-DR*0401 or *0404 herpes simplex virus (HSV) 2 p61 VP16 465−484 or DR*0301 non-structural protein of influenza virus (NSI) p32−45 (Tab. 4). Tetramer staining was performed for 2.5 h at 37ºC, 5% CO$_2$.

Table 4. Peptides used for antigen-specific T-cell stimulation and monomer and tetramer production.

Peptides	Amino acid sequence
GAD65 274−286	IAFTSEHSHFSLK
GAD65 555−567 557I	NFIRMVISNPAAT
Proinsulin B24−36 (B24−C4)	FFYTPKTRREAED
Insulin A1−15	GIVEQCCTSICSLYQ
Insulin A6−21	CCTSICSLYQLENYCN
Control peptides	**Amino acid sequence**
HSV-2 p61 VP16 465−484	YGALDVDDFEFEQMFTDAMG
NSI p32−45	FLDRLRRDQRSLRG

4.2.7 Flow cytometric analysis

In this study two-, three- and four-color stainings were done with FITC-, PE-, PerCP- and Apc-labeled monoclonal antibodies (mAb). The cells were stained for 20 minutes on ice (30 minutes at room temperature in report IV), with mAb and washed twice with PBS containing 2% FCS and 0.1% NaN_3; in report IV cells were also fixed with PBS containing 1% formaldehyde solution (Merck, Darmstadt, Germany).

Following tetramer staining, the cells were stained with mAb for CD4, CD14 and CD25 in report I, and in report II anti-CD8 was also added. In report II a small aliquot of CD45RO^{+} and CD45RA^{+} cells were also stained with anti-CD4, anti-CD45RO and anti-CD45RA after separation with the MACS Memory CD4^{+} T-cell isolation kit (Miltenyi Biotec).

T-cell receptor expression was analyzed in GAD65-specific T-cell clones in report III with mAb against TCRαβ, TCR Vβ-1, -2, -3, -5.1, -5.2, -7, -8, -11, -12, -13.1, -13.6, -14, -16, -17, -20, -22 (Serotec, Oxford, UK) and against CD4 (BD). If the clone did not stain with any of these TCR Vβ-specific monoclonal antibodies, additional monoclonal antibodies against TCR Vβ-4, -5.3, -9, -13.2 and Vβ-21.3 were used (Immunotech, Beckman Coulter, Fullerton, CA, USA). A total of 1.5 x 10⁵ cells were stained with each TCR antibody. Additional cell-surface markers to tetramer staining in report III were anti-CD3 and anti-CD4. For Treg detection in report IV a three-color combination was used with CD4, CD25, HLA-DR or CD62L or CD69 mAb. As NKT cell markers, anti-CD161, anti-Vβ11 and anti-Vβ24 were used. A total of 1 x 10⁵ cells/tube were used in the Treg and NKT analysis.

The flow cytometric analyses were done on a FACSCalibur flow cytometer (BD) in reports I-III, and on a FACScan flow cytometer (BD, Mountainview, CA, USA) in report IV. FACS data were analyzed with the CellQuest (BD), and WinMDI (Stanford University) software programs in reports I and IV, and with the FlowJo (Tree Star, Ashland, OR, USA) software program in reports II and III. Live lymphocyte cell population was gated on their forward- and side-scatter properties, followed by specific gating, described in detail in the respective reports (I-IV). The positive tetramer staining value in report I was set to $\geq 1.03\%$ (based on the 90th percentile for the tetramer staining in the control group). In report II and III, a response was considered positive if the tetramer binding was twice of higher than the background staining with a control tetramer.

4.2.8 Single-cell sorting and expansion of T-cell clones

In report III, on day 14 the GAD65 555−567 (557I) stimulated cells (10, 1 and 0.1 μg/ml of peptide) were single-cell sorted into round bottom 96-well plates (containing RPMI-1640 media complemented with 30% pooled human AB serum), using a FACSVantage cell sorter (BD). Irradiated HLA-unmatched PBMCs were added (1 x 10^5/well) to the wells. The next day, 5 μg/ml phytohemagglutinin (PHA-P) (Sigma-Aldrich, St. Louis, MO, USA) and 10 U/ml of IL-2 (Endogen, Woburn, MA, USA) were added, and the cells were cultured further for 9 days at 37°C, 5% CO_2. On day 10, the cells were stimulated with irradiated HLA-DR*0404 matched PBMCs pulsed for 2−3 h at 37°C, 5% CO_2 with 10, 1 or 0.1 μg/ml GAD65 555−567 (557I) peptide. The following day, IL-2 was added at 10 U/ml. After 10−12 days, the clones were selected on the basis of their growth for further unspecific expansion with PHA/IL-2 as described above.

4.2.9 Proliferation assay

Resting GAD65-specific T-cell clones (5 x 10^4 T cells/well) were tested first for specificity by stimulation with irradiated HLA-DR*0404-matched PBMCs (1.5 x 10^5/well) pulsed with GAD65 555−567 (557I) peptide. PBMCs without the antigen were used as a negative control. Functional avidity of the clones was tested at decreasing concentrations of the GAD65 555−567 (557I) peptide (10, 1, 0.1, 0.01, 0.001 μg/ml) in triplicate wells. Stimulation with the lowest peptide dose (0.001 μg/ml) was not performed for all clones due to the limited number of cells. The cells were incubated for 72 hrs at 37°C, 5% CO_2 and labeled with [^{3}H] tritiated thymidine (2 μCi/ml, Amersham, Buckinghamshire, UK) for the last 18 h. The cells were harvested on a glass fiber filter (Wallac, Turku, Finland) with Tomtec 93 Mach Manual Harvester (Tomtec, Orange, CT, USA) and thymidine incorporation was measured with a Micro Beta scintillation counter (Wallac, Turku, Finland). The mean of the counts per minute (cpm) of tritiated thymidine incorporation in triplicate wells was calculated.

4.2.10 Cytokine secretion assay

Secretion of IFN-γ, IL-4, -5, -10, -13 and -17 was measured with Luminex 100IS xMAP technology, using Milliplex human cytokine immunoassay (Lot# 1546198) (Millipore Corporation, Billerica, MA, USA). Supernatants of 25 μl from proliferation assays at three concentrations of GAD65 peptide (10, 1, 0.1 μg/ml) were used for the measurements on a 96-well filter plate according to the manufacturer's instructions.

4.2.11 Statistical analysis

Statistical analysis was carried out with StatView and PASW statistics software. The Mann-Whitney U-test was used for the comparison between the two groups (reports I–IV). Distributions of positive and negative values were analyzed with the Chi-square test (reports I, II, IV) and Fisher's exact test was used when the expected value was less than five (report I). The Spearman rank test was used for correlation analysis (report II). *P*-values lower than 0.05 were considered statistically significant.

5. RESULTS

5.1 Autoreactive CD4$^+$ T cells in T1D patients, autoantibody-positive subjects and in controls

In report I, blood samples from 18 T1D patients, 20 multiple autoantibody-positive subjects and 21 HLA-matched controls were collected. The isolated PBMC were stimulated with GAD65 274−286, GAD65 555−567 (557I) and proinsulin B24−36 peptides for 10−11 days. Secondary stimulation for an additional three days was performed with plate-bound monomers loaded with the same peptides used in the primary stimulation. The frequency of autoreactive CD4$^+$ T cells against the peptides were detected with specific HLA-DR*0401/0404 or DR*0301 MHC class II tetramers. In this study, 61.0% of the T1D patients, 35.0% of the autoantibody-positive subjects, and only 9.5% of the controls, had positive levels of tetramer binding cells to at least one of the investigated peptides. The frequency of tetramer positive subjects was significantly higher among T1D patients and at-risk subjects than among control subjects ($P < 0.001$ and $P = 0.049$, respectively) (Report I, Tab. 2 corrigendum). The number of GAD65 or proinsulin activated CD4^{high}CD25$^+$ T cells was also increased in T1D patients and autoantibody-positive subjects compared to controls. The difference between T1D patients and control subjects was statistically significant ($P = 0.02$) (Report I, Fig. 2).

A larger cohort of subjects was analyzed in report II. PBMC's were stimulated with GAD65 274−286, GAD65 555−567 (557I), proinsulin B24−36, insulin A1−15 and insulin A6−21 peptides from a total of 26 children with recently diagnosed T1D, 48 multiple autoantibody-positive children, and 70 HLA- and age-matched control children. The same detection methods for antigen-specific CD4$^+$ T cells were used as in report I, except that in report II, two or three peptides were pooled together in the primary stimulation. Thirteen of the 26 T1D patients (50.0%), and 30 out of the 48 (62.5%) autoantibody-positive subjects, responded positively to at least one of the five investigated peptides. Similarly, more than half of the controls (54.3%) had a positive response towards at least one of the tested peptides (Report II, Tab. 1). Autoantibody-positive subjects recognized more frequently the GAD65 555−567 peptide, when the epitopes were separately analyzed. Twenty-two autoantibody-positive subjects out of the 42 analyzed (52.4%) had a positive response to the GAD65 555−567 peptide, compared to 30.5% of the controls ($P = 0.027$). In the T1D patient group, four of the 18 (22.2%) analyzed had a positive response to GAD65 555−567. T1D patients were also observed to have a more frequent positive response to insulin A1−15 (35.7%) than autoantibody-positive (15.4%) and control subjects (18.8%) (not statistically significant). T1D patients (40.0%) and autoantibody-positive subjects (42.9%) showed a tendency to more often respond to the insulin A6−21 peptide than the control individuals (18.2%), although these differences did not reach statistical significance (Report II, Tab. 1).

5.2 Fluctuations in the frequency of tetramer binding CD4^{+} T cells in autoantibody-positive and -negative subjects

Three autoantibody-positive subjects in report I, and 34 autoantibody-positive and 13 controls in report II, have been analyzed with GAD65-, proinsulin- and insulin-specific tetramers on two or more occasions. In report I, one subject displayed similar levels of tetramer binding (17.9 and 18.1%) in both samplings (time duration 10 months). For the second subject, a decrease of the GAD65 555−567 (557I) specific T cells was detected nine months later (9.0% and 4.2%). The third subject displayed a very high GAD65 555−567 (557I) tetramer binding result in the first sampling (53.6%), but three months later no tetramer binding cells were detected.

In report II, follow-up time between samples varied between 3 to 54 months. A majority (65%) of the samples were collected between 3 to 12 months, and more sporadically after 15 months. Only one follow-up sample was collected from the control subjects, usually after 6 months from the first sampling. Results from eleven subjects in report I were included in the follow-up studies in report II, to obtain a longer time of follow-up. From four autoantibody-positive subjects, at least one sample was obtained prior to the diagnosis of T1D, and an additional sample was collected within one week after the T1D diagnosis. Three out of four subjects had tetramer positive T cells, at least at one point, before the diagnosis (Report II, Tab. 2). However, differences in the level of tetramer binding was observed in each individual during the follow-up (Report II, Fig. 1), suggesting fluctuation in the number of tetramer binding cells in circulation between the given time points. Therefore, no statistically significant correlations were observed between the longitudinally collected samples, neither when the samples were organized according to time (3 month interval), or according to collecting order without time adjustment. No significant correlations were observed between autoantibody levels and the tetramer staining.

5.3 Increased GAD65 555−567 specific T-cell response of memory origin in T1D patients and autoantibody-positive subjects

Autoreactive T-cell responses of naïve or memory origin against the same GAD65, proinsulin and insulin peptides were further evaluated in a smaller group of children in report II. Directly after lymphocyte isolation from peripheral blood, CD45RA^{+} naïve cells and CD4^{+}CD45RO^{+} enriched T cells from the same sample were separated and stimulated with the five peptides. The frequency of autoreactive T cells was analyzed with specific and control tetramers after 14 days of stimulation.

T1D patients and autoantibody-positive subjects displayed more frequently T-cell responses of memory origin against the GAD65 555−567 peptide, which was not seen in any of the 27 control subjects ($P = 0.029$ and $P = 0.028$, respectively). Additionally, GAD65 274−286 and insulin A6−21 peptides induced stronger T-cell responses of memory phenotype in T1D patients (33.3% and 25.0%, respectively) than in the

autoantibody-positive (17.7% and 20.0%, respectively), and in control children (16.0% and 6.3%), but the differences were not statistically significant (Report II, Tab. 3).

Furthermore, in T-cell stimulations of naïve origin, CD4$^+$ T-cell responses to GAD65 555–567 were more often seen in T1D patients (30.0%) than in control subjects (4.6%) ($P = 0.044$). A strong response to GAD65 274–286 was also observed in T1D patients (5 of 10 subjects, 50.0%), in contrast to 19–22% in autoantibody-positive and control subjects ($P = 0.076$). Only one of the ten children with T1D had a positive T-cell response to insulin A1–15, and one out of six T1D patients responded to insulin A6–21. Not one of the autoantibody-positive subjects showed a T-cell response of naïve origin against the insulin A1–15 and A6–21 peptides. Two of the 15 control children (13.3%) responded to the insulin A1–15, while no T-cell responses to insulin A6–21 was detected in the twelve analyzed control children (not statistically significant). Altogether, in T-cell stimulations of naïve origin the total CD4$^+$ T-cell responses to the investigated peptides were strongest in the T1D patients, since 75% of the subjects were positive for at least one of the GAD65, proinsulin or insulin peptides (not statistically significant) (Report II, Tab. 3).

5.4   Inverse correlation between the antigen dose and avidity of GAD65-specific T cells

In report III, the aim was to investigate how the antigen dose can influence the avidity of GAD65-specific T cells. Enriched CD4$^+$ T cells (including both naïve and memory T cells) from a healthy HLA-DR*0404 positive individual were stimulated with GAD65 555–567 (557I) peptide at 10, 1 and 0.1 µg/ml for 14 days, and thereafter stained with a specific and irrelevant tetramer. The frequency of GAD65 555–567 specific CD4$^+$ T cells decreased with a lower peptide dose used (10 µg/ml: 1.82%, 1 µg/ml: 0.74%, 0.1 µg/ml: 0.43%), whereas the geometric mean fluorescence intensity (MFI) of the tetramer binding tended to be higher for the cells stimulated with the lowest peptide dose (10 µg/ml: 369, 1 µg/ml: 331, 0.1 µg/ml: 664). In cultures with the low concentration of GAD65 peptide, a preferential proliferation of high-avidity T cells were observed (Report III, Fig. 1 A and B).

To further investigate the avidity differences at a single-cell level, tetramer positive cells were isolated by single-cell sorting. Five CD4$^+$ T-cell clones were derived from cultures with 0.1 µg/ml of peptide, nine clones from the 1 µg/ml cultures, and eight from the 10 µg/ml cultures. Tetramer staining and proliferation with the specific peptide were used for determining the specificity of these clones. The tetramer staining results and functional avidity data of the investigated clones were in concordance. T-cell clones that were derived from the low antigen cultures expressed the strongest tetramer-binding affinity in comparison to the T-cell clones derived from high antigen cultures ($P = 0.026$) (Report III, Tab. I). Functional avidity was determined by half maximal proliferation at decreasing concentrations of the GAD65 555–567 (557I) peptide (10–0.001 µg/ml).

T-cells clones isolated from the cultures stimulated with a low antigen dose (0.1 and 1 µg/ml) displayed a greater half maximal proliferation response in comparison to the highest antigen dose (10 µg/ml) -induced clones ($P = 0.003$ and $P = 0.006$, respectively). The clones established at the lowest peptide concentration also displayed a greater proliferation response than T-cell clones established with the intermediate peptide dose ($P = 0.003$) (Report III, Tab. I).

Furthermore, variations in the TCR-Vβ usage were detected between the T-cell clones stimulated with high-, medium- or low-peptide dose of GAD65 555−567 epitope, displaying distinct avidities. Three of five (60.0%) high-avidity T-cell clones from the low antigen dose (0.1 µg/ml) stimulations, and four of nine (44.0%) T-cell clones derived from the intermediate antigen dose (1 µg/ml) stimulations, expressed the Vβ5.1 chain. In contrast, only one out of eight (12.5%) low-avidity T-cell clones from the cultures with a high antigen dose (10 µg/ml) was Vβ5.1 positive (Report III, Tab. I). Even though some of the T-cell clones did not stain with any of the commercially available TCR-Vβ specific antibodies, they all stained with TCRαβ antibody. The lack of staining observed could therefore not be due to TCR down-regulation.

Cytokine (IFN-γ, IL-4, IL-5, IL-13 or IL-17) secretion was measured in the supernatants from the proliferation assays performed at three different concentrations (10, 1, 0.1 µg/ml) of GAD65 555−567 (557I) peptide. All the clones expressed either a Th0 or Th2 cytokine profile determined by the ratio of Th1 (IFN-γ) and Th2 (IL-4, IL-5, IL-10 and IL-13) cytokines. (A clone was considered to be Th2 if its IL-4 production was at least two times higher, ratio ≥2, than its IFN-γ production). Secretion of IFN-γ, IL-4, IL-5, IL-13 or IL-17 was not different between the three groups of clones (Report IV, Fig. 3). IL-13 was secreted in high amounts in all clones, which may suggest an anti-inflammatory profile of GAD65-specific T cells in this healthy individual (Report III, Fig. 3E). The intermediate avidity T-cell clones also expressed a significantly higher level of IL-10 in comparison to the low-avidity T-cell clones ($P = 0.021$). The IL-10 level remained reduced by the low-avidity T-cell clones when compared to the intermediate- and high-avidity T-cell clones grouped together ($P = 0.032$) (Report III, Fig. 3D).

5.5   Circulating regulatory T cells and natural killer T cells during the prediabetic phase of type 1 diabetes

In a similar set of individuals as in report I and II, the frequency of $CD4^+CD25^{high}$ regulatory T cells and natural killer T cells were investigated in peripheral blood. A total of 25 children recently diagnosed with T1D, 21 multiple autoantibody-positive children and 39 HLA- and age-matched controls were analyzed.

Since the $CD4^+CD25^{high}$ T-cell population is a heterogeneous group of cells, including activated T cells as well as Treg, additional activation markers (HLA-DR, CD69 and CD62L) were added to the analysis. HLA-DR is described as a constant marker of activation (Ko *et al.*, 1979), while CD69 is expressed very early in the activation process

(Sancho *et al.*, 2005). CD62L, also known as L-selectin, is downregulated upon activation (Jung *et al.*, 1988). When considering only the CD4$^+$CD25^{high} T cells, no differences were observed between the three study groups. With the additional activation markers, a higher level of CD4$^+$CD25^{high}HLA-DR$^-$ T cells was detected in multiple autoantibody-positive children compared to controls ($P = 0.021$). The frequency of CD4$^+$CD25^{high}CD69$^-$ and CD4$^+$CD25^{high}CD62L$^+$ cells did not differ between the three study groups. Furthermore, the multiple autoantibody-positive children were divided into two subgroups, depending on their expression of either ICA and one additional autoantibody (GADA, IAA or IA-2A), or ICA and two or three autoantibodies. Interestingly, children expressing three to four autoantibodies had a significantly higher proportion of CD4$^+$CD25^{high}HLA-DR$^-$ and CD4$^+$CD25^{high}CD69$^-$ T cells than the controls ($P = 0.004$ and $P = 0.048$, respectively) (Report IV, Fig. 2A and B). Three of the children expressing three to four autoantibodies developed diabetes after this study was completed, and all of them had increased levels of CD4$^+$CD25^{high}HLA-DR$^-$ T cells compared to controls ($P = 0.016$). The frequency of CD161$^+$Vβ11$^+$ and Vα24$^+$Vβ11$^+$ natural killer T cells was investigated in these subjects. Subdivision of the multiple autoantibody-positive subjects implied a higher level of CD161$^+$Vβ11$^+$ NKT cells in children positive for only two autoantibodies in comparison to control children ($P = 0.002$) (Report IV, Fig. 2D), whereas, Vα24$^+$Vβ11$^+$ NKT cells proportions were similar in all three study-populations.

6.   DISCUSSION

6.1   GAD65- and (pro)insulin-specific CD4^{+} T-cell responses in autoimmune diabetes

Identification of the antigenic targets of β-cell-reactive T cells in T1D has still only been partially unraveled. The development of tetramer-based techniques allows more precise identification of the peptide-MHC specificity and characterization of the functional properties of T-cells involved in autoimmunity. By using the MHC class II tetramers, we investigated self-reactive CD4^{+} T-cell responses *in vitro* in patients with T1D, and in multiple autoantibody-positive children. In report I, we found an increased frequency of GAD65 274−286, GAD65 555−567 and proinsulin B24−36 specific T cells in cultures of peripheral lymphocytes from T1D patients and autoantibody-positive children compared to controls. Furthermore, using the same GAD65, proinsulin peptides and additionally two insulin peptides (insulin A1−15, insulin A6−21) in an enlarged cohort, we detected a high frequency of positive responses (report II). The autoantibody-positive children showed an overall stronger CD4^{+} T-cell response to the GAD65 555−567 peptide compared to the controls. However, we could not detect any significant differences in the number of tetramer positive cell recognizing any other of the investigated epitopes in the enlarged cohort. In a subcohort of autoantibody-positive subjects, we showed further that T cells with GAD65 555−567 specificity displayed memory phenotype. However, in T1D patients T-cell responses of both memory and naïve origin were detected, implying increased autoreactivity to GAD65 555−567 in both subsets in newly diagnosed T1D patients. It is also possible that the T-cell cultures of naïve origin contained some GAD65-specific memory T cells in the samples from T1D patients, since the purity of some CD45RA separations was ~80% allowing some residual carry-over of CD45RO^{+} (memory) T cell. Even so, in cases with lower purity of the CD45RA separation, no tendency of higher tetramer binding was observed, suggesting no carry-over. Overall, these findings are in agreement with earlier published results. In a study by Danke *et al.,* GAD65 555−567 reactive T cells were generally detected in the naïve T-cell pool in control individuals, and both memory and naïve T cells were observed in T1D individuals (Danke *et al.*, 2005). In another study, similar results in new-onset T1D patients were reported with whole GAD65 and insulin (Monti *et al.*, 2007).

A tendency of higher recognition of the insulin A1−15 epitope in newly diagnosed T1D patients and of the insulin A6−21 epitope in both T1D patients and autoantibody-positive subjects was observed in comparison to controls. This is concurrent with earlier published findings, showing a higher T-cell response to a similar insulin peptide (insulin A1−12) in at-risk and autoimmune diabetic children expressing HLA-DR3/4 (Marttila *et al.*, 2008). However, it has to be noted that insulin treatment in the T1D patients could influence the observed increased responses. T-cell responses to insulin have also been shown to correlate with residual β-cell function and lower insulin requirements at the

onset of the disease (Mayer *et al.*, 1999). It would be interesting to evaluate the insulin requirements and C-peptide levels in the subjects with T-cell response to the insulin peptides in report II, to see wheather a similar trend could be observed.

In report I, we observed that only a low number of control subjects (2 out of 21, 9.5%) displayed T-cell responses to islet-autoantigen peptides. In contrast, a more frequent recognition of GAD65, proinsulin and insulin peptides were observed in control children in a larger cohort in report II. The appearance of self-reactive T cells in non-affected individuals is well known (Lohmann *et al.*, 1996; Lohse *et al.*, 1996; Semana *et al.*, 1999), and our findings are consistent with earlier published results with whole GAD65 or with the same GAD65 555–567 epitope (Viglietta *et al.*, 2002; Danke *et al.*, 2004). This indicates that the normal T-cell repertoire does include islet-autoantigen-specific CD4^{+} T cells potentially capable of mediating autoaggressive responses. Most of the control individuals showed a low T-cell response of both memory and naïve origin to the investigated peptides, except for the proinsulin B24–36 epitope. Proinsulin B24–36 stimulated strong T-cell responses of both memory and naïve origin, as well as a strong total CD4^{+} T-cell response. The narrow selection of collected individuals expressing high-risk genes associated with T1D may be one explanation for the increased T-cell recognition of the investigated peptides in control subjects. (The HLA-criteria was HLA-DQB1*0302 and/or DQA1*05-DQB1*02 alleles/haplotypes and lack of the protective alleles HLA-DQB1*0603 or -DQB1*0604 in report II). It could suggest a possible higher reactivity to these epitopes in genetically high-risk individuals, which became more visible in the larger control group in report II. Another possible explanation could be the pooled peptide stimulations used in the second report (due to limited amounts of cells and increased numbers of peptides), compared to only single peptide cultures in the first report, even though the following monomer stimulations in the PBMC cultures were always done separately. In report II, GAD65 peptides were added to the same well and insulin peptides to a separate well, and if the subject was DR3/DR4-heterozygous the proinsulin peptide was added together with the GAD65 peptides. The proinsulin peptides pooled together with GAD65 peptides could eventually trigger a higher background in DR3/DR4-heterozygous subjects, and also in control subjects, although no statistically significant differences were observed in proinsulin-specific T-cell responses between DR3- and DR3/DR4-subjects. Furthermore, an up-regulation of adaptive Treg cells could also possibly explain the frequent response seen in controls, since the expression of Treg markers of these GAD65-, proinsulin-, and insulin-specific T cells have not been addressed in these specific cultures.

6.2　Longitudinal analysis of autoreactive T-cell responses in autoantibody-positive and -negative subjects

We found substantial variation in the levels of tetramer binding GAD65-, proinsulin-, and insulin-specific T cells in the peripheral blood during a longitudinal follow-up of autoantibody-positive and control children (report II). The same phenomenon was also

seen in children who progressed to T1D during the follow-up. Comparable fluctuations in the levels of tetramer binding cells have been reported, both in NOD mice (Trudeau *et al.*, 2003) and in humans (Herold *et al.*, 2009a). Tetramer binding diabetogenic CD8^{+} T cells were shown to appear in cycles in peripheral blood before the onset of hypoglycemia in NOD mice, and not all mice that developed diabetes expressed detectable levels of autoreactive T cells (Trudeau *et al.*, 2003). A recent multicenter TrialNet study showed variable detection levels of islet-autoantigen-specific T cells in PBMC from T1D patients tested on two occasions of a maximum of 28 days apart by using MHC class II tetramers (Herold *et al.*, 2009a).

Our findings, together with earlier published results, suggest that the observed variations reflect the number of these autoreactive T cells in the circulation at the time of blood draw. No obvious differences in the handling of the blood samples or quality of research reagents have been identified in our study. However, timing from the blood draw to lymphocyte isolation varied up to 24 hours, which may possibly have an effect on the cells with a very low precursor frequency. The interval between the sequential samples, which was at least three months in our study, could also contribute to the high degree of fluctuations observed. It has previously been shown that there is heterogeneity on the detection of peripheral T cells (proliferation, immunoblot, ELISPOT) between repeated samples, which increases with time (Herold *et al.*, 2009a). This longitudinal follow-up could not explain if the observed variations of GAD65-, proinsulin-, and insulin-specific T cells in the peripheral blood reflect disease progression and target organ homing during the inflammatory process prior to development of autoimmune diabetes. Nevertheless, islet-cell specific CD4^{+} T cells were detected in three out of four autoantibody-positive subjects who progressed to T1D at least at one point prior to diagnosis.

6.3 Antigen dose-dependent avidity in GAD65-specific CD4^{+} T cells

Phenotypic characteristics of the human T-cell repertoire are influenced by several different factors including the antigen dose, which is important in shaping the avidity of antigen-specific T cells. Self-reactive T cells are believed to undergo avidity maturation over time, triggered by repeated exposure to their specific autoantigen (Rees *et al.*, 1999; Amrani *et al.*, 2000), or by progressive antigen waning (Speiser *et al.*, 1992; Alexander-Miller *et al.*, 1996a). Limited amounts of peptide on the surface of APC available to specific T cells may also induce T cells with higher avidity as a result of the competition between T cells with similar recognition patterns (Alexander-Miller *et al.*, 1996b). In report III, we investigated how the antigen dose influences the diversity of GAD65 555−567 specific CD4^{+} T cells with the same MHC class II restriction in a healthy individual. Enriched CD4^{+} T cells were stimulated with three different concentrations of the GAD65-peptide and T-cell clones were established.

Our results showed that T-cell clones established with the lowest antigen dose, displayed a higher affinity for the specific tetramer and higher proliferative response upon *in vitro* stimulation at decreasing concentrations of the peptide. It is well known that the tetramer-binding efficiency of an individual T cell correlates with the TCR affinity (Casanova *et al.*, 1991; Kedl *et al.*, 2000). The number of tetramer positive cells reflects the strength of tetramer binding to TCR, which further correlates with MFI. Our findings in report III, suggested a direct link between the MFI of tetramer binding and the functional avidity of the derived T-cell population. For some T-cell clones, no tetramer binding was detected although they proliferated upon peptide stimulation. Most likely, these T cells displayed an extremely low affinity TCR to the peptide-MHC class II ligand, as the functional data also showed in report III. As other groups have similarly reported, functionally competent T cells can express TCR with affinity to the specific ligand that is lower than the threshold required for successful tetramer binding (Rubio-Godoy *et al.*, 2001; Laugel *et al.*, 2007). It has been previously shown that the lack of tetramer binding is not due to TCR down-regulation in similar GAD65-specific T-cell clones (Reijonen *et al.*, 2004). This was confirmed by positive TCRαβ-antibody staining in this report.

In addition, we found a prevalent expression of the Vβ5.1 chain in higher avidity T-cell clones established with the lower doses of GAD65 peptide. Similar findings have been previously obtained, describing a preferential expression of the Vβ5.1 chain in GAD65 555-567 specific T cells from HLA-DR4 positive T1D and control individuals (Reijonen *et al.*, 2004; Danke *et al.*, 2005). These findings suggest a selection for higher affinity receptors against the investigated epitope, which might be more limited in their use of TCR variable chain region repertoire. However, a more detailed sequencing of the clone's TCR expression is required for a stronger confirmation of this finding.

The equally high IL-13 production by all T-cell clones is interesting and implies an anti-inflammatory profile in this individual who showed no signs of autoimmunity. It has been shown that IA-2 and proinsulin-specific CD4^{+} T-cells from T1D patients display a pro-inflammatory cytokine profile (IFN-γ), in contrast to an anti-inflammatory profile (IL-4) in control individuals (Arif *et al.*, 2004). The more predominant IL-10 secretion of higher avidity T-cell clones could simply be a result of an activated adaptive Treg cell. Furthermore, it is well known that a low dose of antigens promote Th2 response, while a high dose promotes Th1 responses (Adorini *et al.*, 1996; Constant & Bottomly, 1997). It would be interesting to confirm these cytokine findings in a cohort of both autoimmune and non-affected individuals.

These stimulations with enriched CD4^{+} T cells included both naïve and memory T cells, which may have contributed to a more complex result. However, these findings bring the attention to the antigen dose in the evaluation of the T-cell repertoire, and how well the experimental data reflects the overall T-cell diversity if only one antigen dose is used in these settings. The antigen dose should be carefully considered when investigating the diversity of antigen-specific T-cells.

6.4   Treg and NKT cells in children with T1D and in at-risk children

Regulatory T cells and natural killer T cells have a key role in suppressing autoimmune pathogenic responses, but their importance and functions in the onset of diseases like T1D is not clear (Torgerson, 2006; Novak *et al.*, 2007; Corthay, 2009; Wu & Van Kaer, 2009). The available evidence suggest that there is no numerical deficit in Treg and NKT cells in T1D patients (Lee *et al.*, 2002; Brusko *et al.*, 2005; Lindley *et al.*, 2005; Tsutsumi *et al.*, 2006), and that altered Treg and NKT function (Wilson *et al.*, 1998; Brusko *et al.*, 2005; Lindley *et al.*, 2005; Kis *et al.*, 2007) might be present in some patient groups, or only during a certain phase of the development of the disease.

We investigated the frequency of CD4$^+$CD25^{high} Treg and NKT cells in the peripheral blood from autoantibody-positive at-risk children and recently diagnosed T1D patients. In agreement with the majority of published results (Lee *et al.*, 2002; Brusko *et al.*, 2005; Lindley *et al.*, 2005; Tsutsumi *et al.*, 2006), we found no difference in the levels of CD4$^+$CD25^{high} Treg and NKT cells between T1D patients and the control children. Interestingly, we detected an increased frequency of CD4$^+$CD25^{high} Treg cells negative for activation markers HLA-DR and CD69 in children expressing three to four diabetes-associated autoantibodies compared to the control children. We also found an increased frequency of Vβ11$^+$CD161$^+$ NKT cells in at-risk children expressing two diabetes-associated autoantibodies than in autoantibody-negative controls, suggesting an up-regulation of Treg and NKT cells in at-risk children. Recently, an increase of CD4$^+$FoxP3$^+$ Treg cells in peripheral lymph nodes of NOD mice at the time of disease onset has been reported, and that disease progression was associated with the imbalance of Treg:T-effector cells (Tang *et al.*, 2008). Furthermore, Montoya and colleagues observed an increased expansion of CD4$^-$CD8$^-$ iNKT cells in the peripheral blood of high-risk individuals compared to controls, using a novel monoclonal antibody for the Vα24Jα18 TCR α-chain (Montoya *et al.*, 2007).

The increased frequency of Treg and NKT cells in at-risk children seen here in report IV, might as well reflect an ongoing "battle" between diabetogenic T-effector cells and Treg cells, with a possible defeat of the Treg cells and diabetes development. However, the lack of more specific markers for Treg and NKT cells, and the absence of functional studies of these T-cell populations to confirm their suppressive capacity, are two potential limitations to these findings. In order to investigate Treg cell's suppressive function and cytokine release and the resistance of T-effector cells to Treg suppression, it would be important to use the recent and more specific markers, FoxP3 (Fontenot *et al.*, 2005) and CD127 (Seddiki *et al.*, 2006) in combination with CD25, for the detection of Treg cells. This would give a better overview of an eventual attempt to regulate ongoing autoimmune responses in at-risk children. Investigating the expression of CD4$^+$, CD8$^+$ or CD4$^-$CD8$^-$ phenotype of the NKT cells (Montoya *et al.*, 2007) would also be informative, and could explain if the difference seen in the NKT cell population in at-risk subjects is characterized by a CD4$^+$ NKT cell subset associated with a immune-regulatory phenotype or a CD4$^-$CD8$^-$ NKT cell subset with a more pro-inflammatory phenotype (Wilson & Delovitch, 2003).

6.5 Tetramer methodology and future aspects of islet-specific autoreactive T-cell assays

MHC class II tetramers allow for the detection of antigen specific CD4^{+} T cells by flow cytometry. This method relies on the high specific interaction between the peptide-MHC complex and the corresponding TCR. Cross-linking peptide-MHC complexes with streptavidin increase the avidity of the interaction compared to only a single peptide-MHC molecule (Kwok *et al.*, 2002; Reijonen & Kwok, 2003). T-cell expansion prior to flow cytometry analysis is usually needed, because of the relatively low frequency of autoreactive CD4^{+} T cells in peripheral blood. The MHC class II tetramer based islet antigen-specific T-cell assay used in our study were not completely successful in distinguishing the at-risk subjects from the non-affected controls. On the other hand, the MHC class II islet antigen-specific T-cell assay has been shown to display high specificity also in comparison to other T-cell assays, including proliferation and immunospot assays based on a wider array of antigens (Herold *et al.*, 2009a). Therefore, MHC class II tetramers are a useful tool in the detection of very specific subpopulations of CD4^{+} T cells and monitoring antigen-specific cells in the circulation, which also were shown in our study (reports I-III).

Overall, the development of T-cell assays for early diagnosis and therapeutic follow-up of T1D are still very challenging, but new techniques and novel modifications of the current methodologies are in progress. Findings reported by Chen and colleagues, emphasize the importance of molecular modeling-based analysis in evaluating function and prediction of potential T-cell epitopes. Structural variations can lead to different MHC-peptide conformation, which may activate different T-cell functions and be the trigger for protective or diabetogenic activations (Chen *et al.*, 2009). Other promising new approaches are the new fluorescent nanoparticles quantum dots (Pinaud *et al.*, 2010) and measurements of islet antigen-specific CD8^{+} T-cell responses with MHC class I tetramers and with ELISPOT-based assays (Martinuzzi *et al.*, 2008). However, MHC class II-restricted CD4^{+} T-cells are important antigen-specific players in T1D development, and the further development and improvement of the MHC class II tetramer T-cell assay is ongoing. The focus is set on increasing the specificity in control individuals, by distinguishing phenotypic differences between progressors and non-progressors. New MHC class II epitopes are also explored and reagents affecting tetramer binding stability and specificity are evaluated. New approaches, a combination of relevant epitopes, and further advanced methodologies, will hopefully shed more light on the ongoing autoimmune processes prior to T1D onset.

7. CONCLUSIONS

I-II. In the first report, islet-specific CD4$^+$ T cells were detected in peripheral blood from T1D patients, multiple autoantibody-positive children and controls by MHC class II tetramers. The results suggested an increased level of GAD65- and proinsulin-specific CD4$^+$ T cells in T1D patients and autoantibody-positive children, using *in vitro* PBMC peptide stimulations. In the second report, GAD65-, proinsulin- and insulin-specific CD4$^+$ T cells were further analyzed using PBMC and T cells of memory and naïve origin in a larger cohort of recently diagnosed T1D patients, children expressing multiple autoantibodies, and in autoantibody-negative control children. A significantly increased level of GAD65 555−567 specific T cells was observed in autoantibody-positive children in comparison to control children, while no significant differences in the T-cell responses for the other MHC class II epitopes were observed. Autoantibody-positive children also displayed positive T-cell responses to GAD65 555−567 of memory phenotype, while none of the control children showed a response of memory origin to this peptide. T1D patients expressed T-cell responses to the GAD65 555−567 epitope of both memory and naïve origin, suggesting enhanced autoreactivity to GAD65 555−567 in recently diagnosed T1D patients. Additionally, levels of GAD65- and (pro)insulin-specific T cells were followed longitudinally in individuals with or without autoantibodies. Tetramer positive cells were detected in three out of four subjects who eventually progressed to T1D at least at one point prior to diagnosis. The results showed substantial fluctuation in the frequency of the autoreactive T cells in the peripheral blood, and most likely reflect biological variations in the circulation at given time points. Although, this tetramer based islet-specific T-cell assay was not completely successful in distinguishing the at-risk subjects from the controls, the MHC class II tetramers are a useful tool in the detection of specific subpopulations of CD4$^+$ T cells and in the monitoring of antigen-specific cells. In the future, it is important to improve the tetramer technique further and increase the specificity of the assay. The observation of increased GAD65 555-567 T-cell responses of memory phenotype in new-onset T1D patients and autoantibody-positive subjects could potentially be used in the monitoring of at-risk subjects progressing to diabetes and in evaluating the effect of immunomodulatory antigen-specific intervention trials.

III. We tested the hypothesis that antigen dose influences the diversity of the T-cell repertoire, by investigating circulating GAD65-specific CD4$^+$ T cells from a healthy individual. GAD65-specific CD4$^+$ T-cell clones expanded by *in vitro* stimulation with variable antigen doses displayed functional and phenotypic differences. The lowest peptide dose generated high-avidity T cells that showed higher proliferation responses upon stimulation with a decreasing dose of the peptide, supporting the hypothesis of this study. The high-avidity T-cell clones also frequently expressed TCR Vβ5.1 chain, which has previously been reported to be preferentially used by GAD65-specific T cells identified in T1D patients. Inversely, T-cell clones established with intermediate and high peptide doses displayed a lower tetramer staining and proliferation. Lower doses of

peptide stimulated also a higher IL-10 production in the high- and intermediate-avidity T-cell clones. These results emphasize the importance of the peptide dose when designing autoantigen-specific assays for the evaluation of autoreactive CD4$^+$ T cells in functional studies, as well as in clinical trials.

IV. No difference was detected in the frequency of regulatory T cells and NKT cells between new-onset T1D patients and controls, which was in concordance with other reported findings. Interestingly CD4$^+$CD25^{high}HLA-DR$^-$ regulatory T cells were detected in a higher frequency in children expressing multiple-autoantibodies, in particular in children with three to four autoantibodies, in comparison to autoantibody-negative control children. This difference was also observed with the CD4$^+$CD25^{high}CD69$^-$ marker combination. The frequency of NKT (CD161$^+$Vβ11$^+$) cells was also increased in children expressing two autoantibodies in comparison to the control children. These results suggest an increased immunoregulatory response during the preclinical phase of T1D in genetically at-risk children. This could possibly reflect an ongoing struggle between diabetogenic T-effector cells and Treg cells that eventually leads to the onset of diabetes. Further investigations in at-risk individuals with more specific markers, especially for regulatory T cells, and complemented with functional analysis would elucidate the dynamics and interaction of these cell populations in the pathogenesis of T1D.

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