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VIRUS INFECTIONS IN EARLY CHILDHOOD, COW'S MILK FORMULA EXPOSURE AND GENETIC PREDISPOSITION IN THE DEVELOPMENT OF DIABETES-ASSOCIATED AUTOIMMUNITY

by

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ABSTRACT

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Virus Infections in Early Childhood, Cow's Milk Formula Exposure and Genetic Predisposition in the Development of Diabetes-Associated Autoimmunity

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Type 1 diabetes (T1D) is an autoimmune disease caused by the destruction of insulin-producing pancreatic β cells. Although both genetic and environmental factors affect the disease susceptibility, the exact disease pathogenesis is not known. We aimed to analyse the effect of various environmental factors during early childhood on the appearance of autoimmunity associated with T1D as well as clinical diabetes, with special emphasis on the interplay between different environmental factors and gene-environment interactions.

Cytomegalovirus and enterovirus infections were not found to predispose to β-cell autoimmunity, but early-acquired rotavirus infection was observed to enhance the appearance of β-cell-specific autoantibodies in a cohort of subjects with HLA-conferred T1D risk. Interestingly, among subjects exposed to cow's milk (CM) –based formula nutrition in early infancy, early-acquired enterovirus infection enhanced the appearance of T1D-associated autoantibodies, suggesting an interaction between these two environmental factors in T1D autoimmunity.

PTPN22 C1858T gene polymorphism was found to be associated with altered $CD4^+$ T-cell activation and proliferation response, indicating an altered T-cell signalling among subjects with 1858T allele associated with T1D risk. Moreover, the presence of the T allele was associated with the development of humoral signs of β-cell autoimmunity and overt T1D. Interestingly, this phenomenon was restricted to subjects exposed to CM-based formula before six months of age, suggesting a gene-environment interaction.

These data suggest that the effect of various environmental triggers on the induction of T1D-associated autoimmunity is altered by other environmental factors and genetic predisposition.

Keywords: Autoimmunity, autoantibody, cow's milk-based formula, cytomegalovirus, enterovirus, insulin*, PTPN22,* rotavirus, type 1 diabetes

LYHENNELMÄ

Johanna Lempainen

Varhaislapsuuden virusinfektioiden, lehmänmaitopohjaisen äidinmaitovastikeen ja geneettisen alttiuden merkitys diabetekseen liittyvän autoimmuniteetin kehittymisessä

Virusoppi ja Immunogenetiikan laboratorio, Turun yliopisto, ja Turun biolääketieteellinen tutkijakoulu, Turku
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Tyypin 1 diabetes on autoimmuunisairaus, joka syntyy haiman insuliinia tuottavien β-solujen tuhouduttua elimistön oman immuunipuolustusjärjestelmän hyökkäyksen seurauksena. Sekä perimän että ympäristötekijöiden arvellaan vaikuttavan tautiprosessiin, mutta taudin tarkkaa syntymekanismia ei tunneta. Tutkimuksen tarkoituksena oli selvittää varhaislapsuuden ympäristötekijöiden vaikutusta β-soluautoimmuniteetin syntyyn, erityispaino tutkimuksessa oli ympäristötekijöiden yhteisvaikutuksessa sekä geneettisten riskitekijöiden ja ympäristötekijöiden vuorovaikutuksessa.

Varhaislapsuudessa sairastettu sytomegalovirus- tai enterovirusinfektio ei lisännyt β-soluautoimmuniteetin riskiä lapsilla, joilla on geneettisesti kohonnut riski sairastua tyypin 1 diabetekseen. Ennen puolen vuoden ikää sairastettu rotavirusinfektio lisäsi hieman tyypin 1 diabetekseen liittyvän autoimmuniteetin riskiä. Tarkemmassa analyysissa varhaislapsuuden enterovirusinfektio osoittautui kuitenkin autovasta-aineiden muodostumisen riskitekijäksi niiden lasten joukossa, jotka olivat saaneet lehmänmaitopohjaista äidinmaidon vastiketta ensimmäisten elinkuukausien aikana. Tämä löydös viittaa enterovirusinfektion ja lehmänmaitopohjaisen vastikkeen yhteisvaikutukseen tyypin 1 diabetekseen liittyvän autoimmuniteetin synnyssä.

Löydösten mukaan *PTPN22* geenin C1858T polymorfismi vaikuttaa $CD4^+$ T solujen aktivaatioon ja proliferaatiovasteeseen, 1858T alleeliin liittyy alentunut T-solureseptorivälitteinen aktivaatio. 1858T alleelin kantajuuteen liittyy lisäksi lisääntynyt autovasta-aineiden ja kliinisen diabeteksen ilmaantuvuus. Tämä yhteys rajoittui yksilöihin, jotka olivat altistuneet lehmänmaitopohjaiselle vastikkeelle ennen kuuden kuukauden ikää.

Tulosten mukaan sekä ympäristötekijöiden väliset yhteisvaikutukset että perimä vaikuttavat yksittäisen ympäristötekijän merkitykseen tyypin 1 diabetekseen liittyvän autoimmuniteetin synnyssä. Nämä yhteisvaikutukset ympäristötekijöiden kesken ja perimän ja ympäristötekijöiden välillä selittävät aiemmin julkaistujen tulosten ristiriittaisuutta tutkimuksissa, joissa on analysoitu vain yhden ympäristötekijän vaikutusta diabeteksen ilmaantuvuuteen.

Avainsanat: Autoimmuniteetti, autovasta-aine, enterovirus, insuliini, lehmänmaitovastike, *PTPN22*, rotavirus, sytomegalovirus, tyypin 1 diabetes

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ABBREVIATIONS

ANOVA	Analysis of variance
ATCC	American Type Culture Collection
APC	Antigen presenting cell
BB	BioBreeding
BBDP	Diabetes-prone BioBreeding
BI	Bovine insulin
BSA	Bovine serum albumin
CBV4	Coxsackievirus B4
CD	Cluster of differentiation
CF	Complement fixation
CFSE	Carboxyfluorescein succinimidyl ester
CI	Confidence interval
CM	Cow's milk
CMV	Cytomegalovirus
cpm	Counts per minute
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DIPP	Type 1 Diabetes Prediction and Prevention study
DPT-1	Diabetes Prevention Trial – Type 1
DNA	Deoxyribonucleoside triphosphate
EIA	Enzyme immunoassay
EM	Electron microscopy
ENDIT	European Nicotinamide Diabetes Intervention Trial
FoxP3	Forkhead/winged helix transcription factor
GABA	γ-aminobutyric acid
GAD65	65 kD isoform of glutamic acid decarboxylase
GADA	Glutamic acid decarboxylase antibodies
HLA	Human leukocyte antigen
HR	Hazard ratio
HSP	Heat shock protein
HSV	Herpes Simplex virus
IA-2A	IA-2 molecule antibodies

IAA	Insulin autoantibodies
ICA	Islet cell antibodies
ICAM-1	Intracellular adhesion molecule 1
IFNγ	Interferon-γ
IL	Interleukin
INS	Insulin gene
JDFU	Juvenile Diabetes Foundation units
kDa	Kilodalton
LYP	Lymphoid tyrosine phosphatase
MWU-test	Mann-Whitney *U* -test
NOD	Non-obese diabetic
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEP	PEST domain-enriched tyrosine phosphatase
PHA	Phytohemagglutinin-P
PTPN22	Protein tyrosine phosphatase 22 gene
RBA	Radio-binding assay
RNA	Ribonucleic acid
RSV	Respiratory syncytial virus
RT	Room temperature
SI	Stimulation index
STRIP	Special Turku Coronary Risk Factor Intervention Project
T1D	Type 1 diabetes
TCR	T-cell receptor
Th	T helper cell
TNFα	Tumor necrosis factor-α
TRIGR	Trial to Reduce IDDM in the Genetically at Risk
VNTR	Variable number of tandem repeats
VP	Virus protein
VZV	Varicella-Zoster virus

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, referred to in the text by the Roman numerals I to V.

I. Aarnisalo J, Ilonen J, Vainionpää R, Volanen I, Kaitosaari T and Simell O: Development of antibodies against Cytomegalovirus, Varicella-zoster Virus and Herpes Simplex Virus in Finland during the first eight years of life: a prospective study. *Scand J Infect Dis* 35: 750-753, 2003.

II. Aarnisalo J, Veijola R, Vainionpää R, Simell O, Knip M and Ilonen J: Cytomegalovirus infection in early infancy: risk of induction and progression of autoimmunity associated with type 1 diabetes. *Diabetologia* 51: 769-772, 2008.

III. Aarnisalo J*, Treszl A*, Svec P, Marttila J, Öling V, Simell O, Knip M, Körner A, Madacsy L, Vasarhelyi B, Ilonen J and Hermann R: Reduced CD4+ T cell activation in children with type 1 diabetes carrying the *PTPN22*/Lyp 620Trp variant. *J Autoimmun* 31:13-21, 2008.

IV. Lempainen J **, Vaarala O, Mäkelä M, Veijola R, Simell O, Knip M, Hermann R and Ilonen J: Interplay between *PTPN22* C1858T polymorphism and cow´s milk formula exposure in type 1 diabetes. *Journal of Autoimmunity*, in press

V. Lempainen J**, Tauriainen S, Vaarala O, Mäkelä M, Honkanen H, Marttila J, Veijola R, Simell O, Hyöty H, Knip M and Ilonen J: Interaction of enteral virus infection and cow´s milk-based formula nutrition in type 1 diabetes-asssociated autoimmunity. Submitted

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** Née Aarnisalo

1. INTRODUCTION

Type 1 diabetes (T1D) is a chronic disease resulting from the destruction of insulin-producing pancreatic β cells through an autoimmune mechanism. After the loss of insulin production life-long insulin treatment is required. The prevalence of T1D is increasing world wide, with the highest frequency of autoimmune diabetes being observed in Finland.

There is a strong genetic predisposition associated with the disease. However, only a part of subjects with inherited T1D risk develop the disease, indicating the importance of environmental factors in the pathogenesis of T1D. Although several virus infections including enteroviruses, cytomegalovirus and rotavirus have been suggested to trigger β-cell autoimmunity, the results have remained controversial. In addition, also the effect of nutritional factors, mainly cow's milk-based formula exposure and/or short breastfeeding have been proposed to have a role in the autoimmunity.

The aim of this study was to explore the effect of various viral infections and nutritional patterns in early infancy on the development of T1D. In addition, the aim was to analyse the effect of the *PTPN22* 1858T variant, associated with increased type 1 diabetes risk, on cellular functions crucial for the disease process, and to determine the combined effect of this genetic variant and cow's milk exposure on the emergence of T1D-associated autoimmunity.

2. REVIEW OF THE LITERATURE

2.1. Characterisation of type 1 diabetes

2.1.1. Clinical manifestation of type 1 diabetes

Insulin and its counter-actor glucagon are the main effectors in the glucose metabolism. Insulin is produced by β cells in the pancreatic islets. The function of insulin is to decrease blood glucose by transporting glucose molecules into cells. Type 1 diabetes is caused by the selective destruction of the insulin-producing β cells leading to loss of insulin secretion, and thus to an elevated blood glucose level. The high blood glucose level induces the escape of glucose molecules into urine and results in osmotic polyuria. This cascade eventually results in polydipsia, ketotic metabolism, loss of weight, and finally ketoacidosis. At the time when symptoms appear the production of insulin in pancreatic β cells is 10-20% of the normal level, and during the final disease process the insulin formation ends completely. Thus, a subject with T1D requires life-long insulin replacement.

2.1.2. Epidemiology

T1D is one of the most common chronic diseases appearing in childhood. The overall incidence of T1D varies markedly among different populations, being highest in Finland and in Sardinia, and lowest in China and Venezuela, presenting an over 400-fold difference in disease frequency (1). However, the incidence of T1D is increasing worldwide. In Finland, an incidence of 18 / 100 000 children under the age of 15 years per year was observed in 1965, while in 2005, the corresponding number was 64 / 100 000 (2; 3). The rise in the disease appearance is most rapid among children under the age of 5 years (2). During the years 1989-1994 in Europe, an incidence increase of 6.3% was observed among 0-4-year-old children, whereas a 3.1% and 2.4% increase was observed among 5-9- and 10-14-year-old children, respectively (4).

A ten-fold difference in the disease appearance has been observed among different European populations (1). The high incidence countries (>20 per 100 000 children per year) are Finland, Sardinia, Sweden, Norway, Portugal and the United Kingdom (5). In contrast, in Eastern European countries, the disease emergence is generally low (5.7 in Latvia, 5.9 in Romania, and 6.0/100 000 children per year in Poland) (5). However, in Europe, the highest proportional increase in the disease appearance is currently taking place in countries of low T1D incidence (1).

2.1.3. Pathogenesis

T1D is an autoimmune disease characterised by lymphocyte infiltration in the pancreatic islets and T-cell-mediated destruction of insulin-secreting β cells. T1D is associated with the appearance of several islet-specific autoantibodies, and the disease onset can be prevented or delayed by immunosuppressive or immunomodulative treatment (6). The current understanding of the mechanism of the autoimmune destruction of β cells is largely based on animal models of human T1D, non-obese diabetic (NOD) mice and BioBreeding (BB) rats. The role of T cells as mediators of T1D autoimmunity has been shown in the NOD mouse model where T1D can be transferred from a diabetic to an unaffected mouse with lymphocytes (7). In addition, autoimmune diabetes does not occur in genetically athymic or T lymphopenic NOD mice or in mice thymectomised at birth (8). T-cell reactivity against several β-cell autoantigens has been shown to precede the onset of diabetes in the NOD mouse model (9; 10). Moreover, in NOD mice, T-cell reactivity against GAD was shown to appear first, followed by the appearance of responsiveness against other β-cell autoantigens such as insulin and heat shock protein 65. In addition to autoantigen specificity, the quality of the immune response towards autoantigens is critical for the consequence of the immune autoreactivity. In an animal model, insulitis characterised by Th1-type lymphocytes with high IFNγ secretion was shown to lead to autoimmune diabetes, whereas islets infiltrated by Th2-type lymphocytes with strong IL-4 response and low IFNγ secretion were not destroyed (11). The balance between Th1 and Th2-type immune responses is thought to be crucial in the formation of tolerance or autoimmunity. Accordingly, when autoantigens have been used for tolerance induction a switch from Th1 to Th2 type response has been observed (12).

The activation of autoreactive T cells is dependent on the presentation of autoantigens by MHC II molecules. In islet autoimmunity, β-cell-specific autoreactive T cells can be activated via β-cell autoantigens by antigen-presenting cells (APC) in pancreatic lymph nodes. These activated autoreactive T cells may then invade the islets where they become re-activated by β-cell autoantigens and initiate insulitis. The increased expression of intracellular adhesion molecule 1 (ICAM-1) has been observed in vascular endothelium of islets, supporting the increased infiltration of mononuclear cells from the circulation to pancreatic islets (13). The insulitis is characterised by the infiltration of mononuclear cells, mainly $CD8^+$ T cells, accompanied by macrophages, $CD4^+$ T cells and B cells (14).

The dominant role of β-cell-specific autoreactive T cells in T1D suggests that individuals with this disease possess a defect in the induction or maintenance of T-cell tolerance towards β-cell autoantigens. An antigen-independent deficit in the induction of central tolerance including incomplete deletion of autoreactive T cells differentiating in the

thymus has been suggested in NOD mice (15; 16). Moreover, an antigen-dependent defect has been observed in the thymus. The formation of tolerance towards insulin among subjects with insulin gene polymorphism associated with decreased thymic insulin expression resulted in enhanced insulin autoimmunity (17; 18). In addition, evidence of ineffective induction and/or maintenance of peripheral tolerance in NOD mice has been reported (19-22). This defect in the formation of peripheral tolerance may reflect aberrances in numbers and/or activity of regulatory T-cell populations (23-26).

β-cell autoimmunity is known to emerge at any age, although the majority of the process starts during early childhood. The appearance of humoral signs of β-cell autoimmunity precedes the clinical onset of T1D. This autoimmunity process is asymptomatic and the duration of this period varies widely (27). The schematic figure shows the pathogenetic model of T1D including the genetic susceptibility, emergence of autoimmunity and finally the β-cell destruction leading to the lack of insulin (Fig 1). However, the initiation of the β-cell autoimmunity does not lead to clinical T1D in all individuals, but the mechanism regulating the progression of autoimmunity is not well defined. The hypothesis of the essentiality of environmental triggers in addition to the genetic susceptibility is widely accepted. Supporting this hypothesis, about 20% of the Caucasian population carry the HLA-genotype associated with T1D risk, but the cumulative incidence of T1D among this population remains under 1%, indicating that only approximately 5% of the genetically at-risk subjects develop clinical T1D. In addition, the importance of environmental factors is indicated by the rising incidence of T1D worldwide, suggesting a rapid change in the prevalence of factors triggering autoimmunity. Moreover, the frequency of T1D-associated autoantibodies except IA-2A was reported to be equal among children in Russian Karelia and Finland, whereas a four-fold emergence of IA-2A and a six times higher progression rate to clinical T1D was observed among Finnish children (28), suggesting a difference in an exogenous factor fortifying the autoimmunity process or lack of protective environmental factors. Similar results were observed when analysing the prevalence of GAD and IA-2 autoantibodies among clinically healthy school children in England and Lithuania; the frequency of autoantibody positivity did not differ significantly between the two populations with twofold to threefold difference in the disease appearance (29). Thus, these findings suggest that the early autoimmunity process is equally common among populations with both high and low diabetes incidence, but that the autoimmunity process progresses more frequently to clinical disease in counties with high disease incidence.

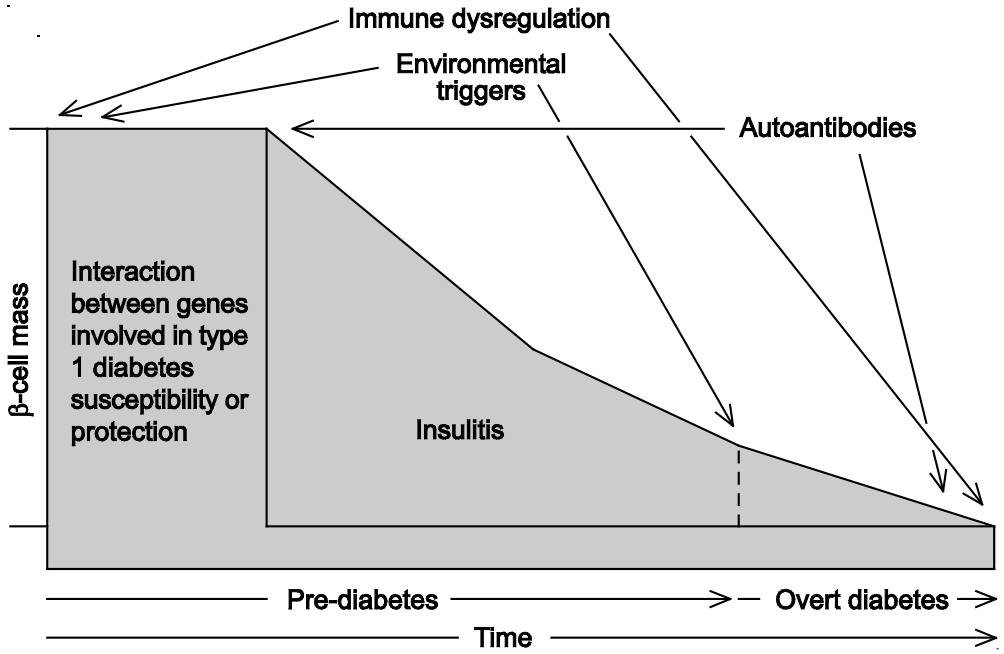

Figure 1. The different pathogenetic factors affecting the loss of β-cell mass during the development of T1D. Modified from Atkinson and Eisenbarth (30).

Molecular mimicry between environmental triggers and β-cell autoantigens has been implicated as a mechanism leading to the initiation of autoimmunity. Here, a T cell specific for an external antigen, e.g. virus or nutritional antigen, is thought to fail to differentiate between the original and the self-antigen and to react with an autoantigen sharing similar stretches of amino acids with the external protein (31). The cross-reacting determinants need not be fully identical, but a sufficient amount of conformational similarity must be present to provide a similar antigenic surface (32-35). Molecular mimicry between enteroviruses, especially coxsackie B4 virus (CBV4) and GAD65, and between cytomegalovirus (CMV) and GAD65 has been described (9; 36).

Alternatively, a bystander activation of pre-existing autoreactive T cells after virus infection has been suggested. Virus infections activate strong immune responses, especially CBV4 infection in pancreatic islets is reported to induce strong inflammation within the islets (37). The β-cell cytolysis promoted by the infection is suggested to lead to increased expression of MHC I molecules by β cells, to tissue damage and release of sequestered islet antigens, enhanced secretion of cytokines, and ultimately to enhanced autoantigen presentation by APCs. This autoantigen presentation in the context of inflammation would lead to the activation of the pre-existing β-cell-specific autoreactive T cells (38). Indeed, CBV4 infection has been shown to enhance the development of T1D in the mouse model carrying a diabetogenic T-cell receptor specific for an islet granule molecule other than GAD65 (38), and CBV4-infection induced T1D has been reported to be associated with the phagocytosis of virus-infected β cells by macrophages,

leading to enhanced presentation of β-cell autoantigens by macrophages promoting the development of T1D (39). Similarly to the CBV4 infection, β-cell injury induced by streptozotocin has been shown to promote the development of T1D in BDC2.5 mice by the release of β-cell autoantigens and induction of autoantigen presentation by APCs (40).

2.1.4. Autoantibodies

Several types of autoantibodies associated with T1D have been described. The most important of those described are islet cell antibodies (ICA), insulin autoantibodies (IAA), antibodies specific for 65 kDa isoform of GAD protein (GADA), antibodies against tyrosine-phosphatase-related IA-2 molecule (IA-2A), and antibodies against a Zinc transporter molecule ZnT8.

The cytoplasmic islet cell autoantibodies were discovered in 1974 (41). ICAs are directed against islet cell cytoplasmic antigens and they can be detected from serum samples by indirect immunofluorescence using pancreatic tissue sections and fluorochrome labelled by anti-human immunoglobulins. ICAs have later been shown to include autoantibodies against GAD65, IA-2 and insulin. However, ICA reactivity does not always correlate with reactivity towards these autoantigens, suggesting that additional autoantigens exist (42). Insulin and its precursor, proinsulin, are β-cell-specific autoantigens. IAAs were first described in 1983 among newly-diagnosed T1D patients before treatment with exogenous insulin (43). Glutamic acid decarboxylase (GAD65 and GAD67) is an enzyme crucial for the synthesis of γ-aminobutyric acid (GABA) within the pancreatic islets and the central nervous system. In the islets, GAD65 is expressed not only in β cells, but also in α and δ cells. GAD-specific autoantibodies have been demonstrated in patients with stiff-man syndrome and later in patients with new-onset T1D (44-46). Autoantibodies against the protein thyrosine phosphatase-related IA-2 molecule were described in 1995 (47). Compared to other autoantibodies described so far, antibodies against IA-2 (IA-2A) present with the best predictive value for the development of T1D (48-50). Recently, autoantibodies against Zinc transporter specific for β cells, ZnT8, have been described (51). ZnT8 is localised on the membrane of insulin secretory granules, but its precise role in autoimmunity is still unclear.

The appearance of T1D-associated autoantibodies and their predictive value in the development of clinical T1D has been clarified in prospective follow-up cohorts such as the German BabyDIAB study, the Finnish Diabetes Prediction and Prevention (DIPP) study and in the Diabetes Autoimmunity Study in the Young (DAISY) in Colorado, USA (52-54). The T1D-associated autoantibodies have been shown to appear typically in clusters within some months after the emergence of the first autoantibody (52). In the emergence of humoral autoimmunity against β cells, IAA most commonly appears as the first autoantibody and is followed by GADA, ICA, and subsequently IA-2A (52).

However, any of these autoantibodies can occasionally appear as the first autoantibody. The appearance of diabetes-associated autoantibodies increases the risk for the development of clinical T1D, and the risk increases with the number of autoantibodies emerging (52; 55-60). In family studies, among first-degree relatives, positivity for two to four autoantibodies provided an estimated 60-100% 5-year risk for clinical T1D (61). Recently, ZnT8-specific autoantibodies were found in 60-80% of newly-diagnosed T1D patients, less than 2% of control subjects, and 30% of patients with other autoimmune disease associated with T1D (51).

In addition to the number of autoantibodies, the antibody titers and subclasses are associated with the predictive value. Among young children, high ICA titers increase the predictive value of the autoantibody positivity, and ICA titers above 28 JDFU were reported only among subjects with multiple autoantibodies (52). Moreover, ICA-positive subjects subsequently turning ICA-negative during follow-up were single autoantibody-positive and presented with low ICA titers, while ICA-positive (52). In addition, the especially high insulin IAA levels are almost exclusively observed in T1D patients diagnosed by five years of age whereas over half of the patients diagnosed after the age of five years have insulin autoantibody levels similar to healthy controls (62).

The isotype-specific humoral response towards antigens may reflect the Th1/Th2 balance of the immune response towards a specific antigen (63; 64). In humans, Th1-dominated immunity is defined by interferon-γ (IFNγ), tumor necrosis factor –α (TNFα) and IL-2 and synthesis of IgG1 subclass antibodies, whereas Th-2 dominated immune response is characterized by generation IL-4 and IL-10, and IgG4 and IgE subclass antibodies (64). The ICA response has been shown to appear dominantly in the IgG1 subclass (64). When analysing the isotypes of insulin autoantibodies among prospectively followed subjects with HLA-conferred T1D susceptibility, strong IgG1 and IgG3 subclass antibody responses to insulin were associated with progression to clinical T1D, whereas an absent IgG3 subclass response was protective against the development of T1D (64). Similarly, among GAD autoantibodies, IgG2 and IgG4 subclasses predominate in GADA-positive subjects not progressing to clinical T1D (65), and IgE class antibodies against IA-2 have been shown to provide protection from or delay progression to T1D (66).

Although providing predictive value when assessing the individual risk for T1D development, the presence of T1D-associated humoral autoimmunity is not critical for the autoimmunity process leading to the destruction of the β cells. Martin et al described the development of T1D in a patient with agammaglobulinemia, indicating that neither autoantibodies nor B-cell function is critically involved in the pathogenesis of T1D (67). In accordance with this finding, islet-reactive $CD4^+$, Haskins's BDC2.5 and $CD8^+$ T-cell clones can induce autoimmune diabetes in immunocompromised NOD.SCID mice

without any help from B cells, indicating that β-cell autoimmunity in NOD mouse is T-cell, but not B-cell dependent (68-71).

2.1.5. Intestinal immunity and T1D

The gastrointestinal mucosa constitutes the largest surface area of the body, and the intestinal tract is constantly exposed to microbes and food antigens. The microbiota of the intestine is known to contain over 400 species of microbes (72). As it is suggested that the development of T1D autoimmunity is triggered by environmental factors, and the intestinal mucosa supply the largest area for the interaction between environmental factors and the immune system, it can be hypothesised that changes in the intestinal microflora may alter the predisposition to the development of autoimmunity. Interestingly, in the development of allergy, changes in the gut microbiota have been observed to precede the appearance of clinical symptoms (73). Moreover, the effect of probiotics as an immunomodulator preventing the development of allergies has been shown (74; 75).

Increased permeability of the gut mucosa has been suggested in T1D patients, a phenomenon that would allow a greater exposure of the intestinal immune system to foreign antigens. This hypothesis has been supported by findings on diabetes-prone BB (BBDP) rats and also in human studies. Increased gastric and intestinal permeability has been shown to appear in BBDP rats (76). Interestingly, no differences in the permeability between BBDP and BB rats was observed at the time of weaning (21-28 days of age) but later, on day 50, a marked increase in the intestinal permeability was apparent in BBDP animals. This difference was observed before the appearance of insulitis or overt diabetes. Recently, low levels of claudin, a major intracellular tight junction protein, and a highly permeable intestine have been observed in BBDP rats before the onset of T1D (77). In human studies, increased permeability has been reported among subjects at-risk for the development of T1D or with clinical T1D (78-80), and changes in the intraepithelial junctions in electron microscopy have been observed among T1D patients (80). Moreover, a high level of the protein increasing the permeability of the intestine, zonulin, was reported in BBDP rats (81) and, similarly, high serum levels of zonulin correlating with increased gut sugar permeability results were observed in T1D patients (79).

Enhanced immunological activation in the intestine has been implicated in the aetiology of T1D. Increased presence of MHC II positive cells, enhanced expression of ICAM-1 on the epithelium and increased expression of α4β7-integrin positive cells in the lamina propria have been reported among subjects with T1D, with no signs of celiac disease (82; 83). In addition, activation of cytokine signalling was also reported in lamina propria, where especially increased numbers of cells positive for IL-4 and IL-1α, and cells expressing IFNγ mRNA were observed. Interestingly, low densities of Foxp3-positive T

cells and low activation of Foxp3 transcripts in small intestinal biopsies in subjects with T1D have recently been reported (84). These results suggest a decreased activation of regulatory T cells in the intestine, offering a possible explanation for the altered tolerance to dietary antigens among subjects developing T1D-associated autoimmunity.

2.1.6. Induction of tolerance

Maintenance of tolerance towards self-antigens is central in evasion of autoimmunity. Tolerance is defined as a state in which the immune system does not react destructively against self-molecules, cells or tissues. The checkpoints in the induction of tolerance aim to maintain the balance between prevention of autoimmunity and harmful impairment of immunity to foreign pathogens.

The lack or loss of tolerance towards self-antigens results in autoimmune responses leading to cellular destruction and eventually to clinical autoimmune disease. Tolerance towards self-antigens is induced in the thymus (central tolerance) (85). The positive selection of T cells requires the recognition of self-peptide:self-MHC complexes by the developing T cells to proceed to $CD4^+$ or $CD8^+$ cells. Thereafter, the cells undergo negative selection, in which T cells recognising self-peptide:self MHC complexes with high affinity in the thymus undergo apoptosis and thus the potentially self-reactive T cells are eliminated. The negative selection of T cells recognising tissue-specific autoreactive T cells, like insulin-specific T cells, is based on the thymic expression of proteins with otherwise tissue-restricted expression.

The thymic selection has been considered as an effective tolerogenic mechanism for self-molecules widely expressed in the thymus. However, tolerance towards proteins not available for presentation in the thymus is achieved through mechanisms in the periphery (85). The principal mechanisms of peripheral tolerance are anergy (functional unresponsiveness of T cells recognising self-antigen:MHC complex on APC not expressing co-stimulatory molecules), deletion (apoptotic cell death) and suppression by regulatory T cells.

Induction of tolerance towards orally administered antigens requires immunoregulatory and suppressive immunological events, and failure in the regulatory cascade may lead to the development of allergic or autoimmune disease. The balance between formation of tolerance (suppression) and sensitisation depends on several factors including genetic background, nature and dose of antigen, frequency of administration, age at first exposure, immunological status of the individual, and antigen transmission via breast milk (86).

$CD4^+CD25^+FoxP3^+$ regulatory T (Treg) cells are considered to be major regulators of the immune system (87; 88). Tregs arise in the thymus during early development.

Tregs express high levels of IL-2 receptor, CD25. In addition, the forkhead/winged helix transcription factor gene, *FoxP3*, is strongly linked to the regulatory function of these cells. Elimination of Tregs within the first few days after birth leads to a systemic autoimmune response characterised by inflammatory infiltration into tissues, destruction of the tissues by autoreactive T cells and, thus, to loss of immune homeostasis. Treg function depends on the presence of IL-10 and TGFβ, and Treg-mediated suppression can be abrogated by antibodies against IL-10 and/or TGFβ.

2.2. Genetic susceptibility

The inherited genetic risk for T1D is well known. Studies on monozygotic twins have shown the 35% to 50% appearance of T1D in the sibling of an affected subject (89). Moreover, in dizygotic twins, the disease frequency and the frequency of β-cell autoimmunity is similar to the frequency observed among siblings, suggesting that genetic factors play an important part in the determination of islet cell autoimmunity (90).

The human leukocyte antigen (HLA) region was the first locus found to be strongly associated with T1D (91; 92). Later, several other loci have been associated with increased emergence of T1D. Among these, the most widely described are insulin gene (*INS*), cytotoxic T-lymphocyte antigen -4 gene (*CTLA-4*) and protein tyrosine phosphatase 22 gene (*PTPN22*). Recently, SNPs in the interleukin-2 (IL-2) receptor (IL2RA) region encoding CD25 molecule (93), in immune response gene *CD226* and in interferon-induced helicase (*IFIH1*) have been described (94; 95).

2.2.1. HLA

HLA (in humans used as a synonym for the major histocompatibility complex, MHC) molecules are a cluster of homologous cell-surface proteins divided into class I (A, B, C) and class II (DP, DQ, DR). Class I HLA molecules are ubiquitiously expressed and present an intracellular antigen to $CD8^+$ T cells. In contrast, HLA class II molecules are localised on the cell membrane of antigen-presenting cells (macrophages, dendritic cells and B cells) and present endocytosed peptides in their peptide-binding cleft. These peptide-HLA class II complexes are subsequently recognized by the T cell receptor (TCR) of $CD4^+$ T cells (Fig 2). The HLA proteins are more polymorphic that any other protein in the human genome, having the ability to bind a wide range of peptides. Different HLA molecules have slightly different peptide-binding clefts and different HLA molecule thus bind to and present different peptides to T cells. Animal studies have suggested that certain MHC alleles present self-peptides less effectively to the maturing T cells in the thymus, leading to failure in negative selection (96; 97).

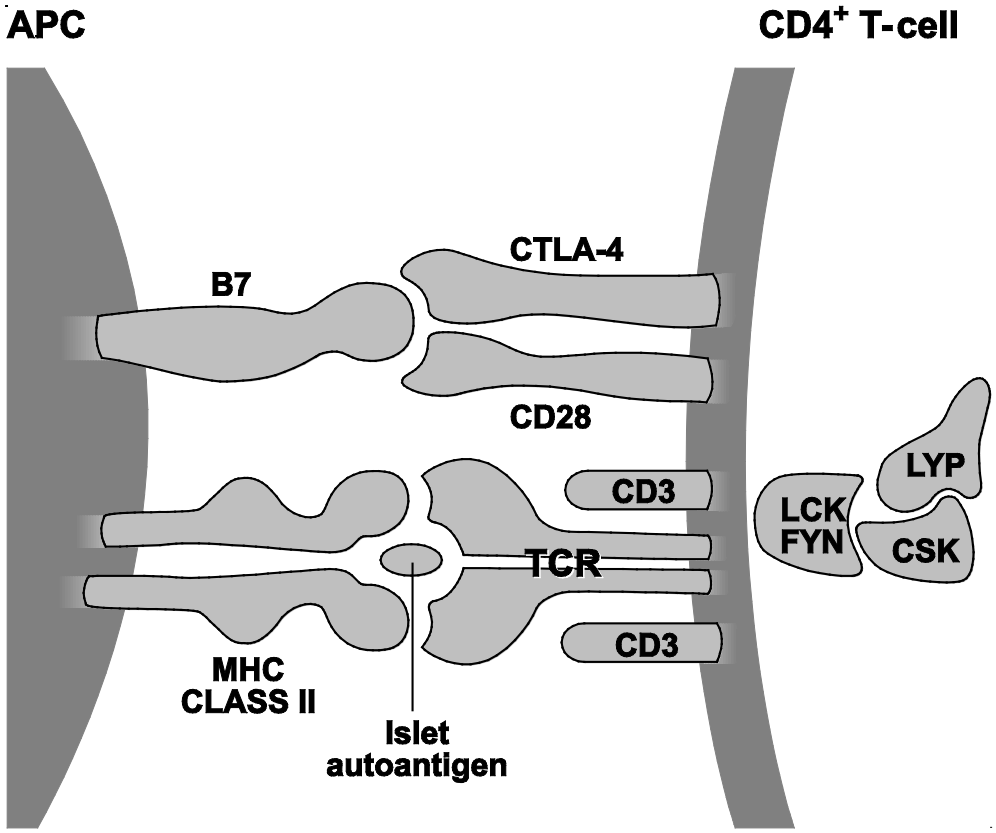

Figure 2. Schematic picture of the interaction between antigen-presenting cell (APC) and $CD4^+$ T cell.

The first report on an HLA-associated disease was published in 1967 describing the association between the HLA antigen 4C and Hodgkin's disease (98). Some years later the HLA association of T1D was published for the first time (99). Later, associations between HLA and several other autoimmune diseases, like Celiac disease, Grave's disease, Myasthenia gravis, Addison's disease and Rheumatoid arthritis have been described (98).

The HLA class II region on the short arm in chromosome 6p21 comprises the most important genes affecting T1D susceptibility, and provides approximately 40-50% of the inheritable T1D risk (100). The dominant role of the HLA region in the pathogenesis of T1D is also demonstrated by the fact that the concordance rate for HLA identical siblings is approximately 15% to 25% and only 1% for siblings differing at both HLA haplotypes (98), and that the concordance among monozygotic twins is influenced by the HLA genotype; the concordance increases with high HLA risk and decreases with low HLA risk (101). The genes in the HLA II region provide susceptibility or protection, whereas some appear neutral in relation to the disease risk. The susceptibility and protection are mainly determined by DR and DQ molecules (102-106). The strongest susceptibility among Caucasian population is found in subjects heterozygous for DR3,DQ2/DR4,DQ8 (HLA

*DRB1*0301* with *DQB1*02,DQA1*0501/ DRB1*04* with *DQB1*0302,DQA1*0301*) genotype, but the risk also remains increased for subjects carrying either of these haplotypes (107). In Finland, among T1D patients diagnosed during childhood approximately 24% carry the DR3,DQ2/DR4,DQ8 haplotypes (108), while the frequency of this combination in the background population is approximately 2%. Protection against the development of T1D is associated with *DQB1*0602,DQA1*0102, DQB1*0503,DQA1*0104* or *DQB1*0303,DQA1*0201* or the DR4 subtype *DRB1*0403/6* and the protective effect is often dominant. Thus, people carrying any of these alleles seldomly develop diabetes even if they carry the DR3 or DR4 risk genotype (109).

2.2.2. Insulin gene polymorphism

The human insulin gene (*INS*) is located on chromosome 11p15. Besides the HLA gene region, the insulin gene region has been associated with T1D predisposition, the variable number of tandem repeats (VNTR) and the single nucleatide polymorphisms (SNPs) described in strong linkage disequilibrium with the VNTRs have been shown to be a susceptibility locus for T1D (110-112). VNTR in the *INS* gene consist of repeat units of 14-15 base pairs (112; 113). Class I VNTR alleles have been shown to contain 28 to 44 repeats and class III alleles, 138 to 159 repeats. The intermediate class II alleles are rare in non-African populations (112; 114). Class I alleles are associated with T1D, while class III alleles are shown to have a dominant protective effect on the development of T1D but both classes have subclasses with a converse or neutral effect on T1D (115; 116). Of the SNPs described, the -23HphI A allele is mostly transmitted linked to class I VNTR, whereas the T allele is linked to class III VNTR (114).

The thymus has been found to express a multitude of genes encoding self-molecules with tissue-restricted expressions (117) and, in addition to pancreatic β cells, *INS* is the actively transcribed in the thymus. It has been suggested that the thymic expression of self-antigens is crucial for the development of tolerance during the maturation of the immune system. The negative selection of autoreactive T cells in the thymus is known to be dose-dependent. In line with this, class I VNTR alleles predisposing to T1D are transcribed at lower levels in the thymus compared to the class III VNTR alleles transcribed at higher levels (117; 118; 119). Thus, the increased transcription levels detected in the thymus seem to provide the protective effect associated with class III VNTR alleles since the higher insulin levels in the thymus may improve the negative selection of the insulin-specific autoreactive T cells. The homozygosity for class I VNTR alleles and low levels of insulin expression in the thymus may, on the contrary, lead to non-effective deletion of insulin-specific T cells. This hypothesis has been supported by studies in a mouse model. Unlike humans, mice have two insulin genes, Ins1 and Ins2 (120). Of these, Ins2 is almost exclusively expressed in the thymus. In a transgenic mouse model, the low thymic insulin expression levels have been shown to present detectable peripheral

reactivity to insulin, whereas mice with normal thymic insulin expression levels showed no significant response (17).

2.2.3. CTLA-4 gene polymorphism

The cytotoxic T-lymphocyte antigen-4 (*CTLA-4*) gene encodes a receptor expressed on T cells (121), and the expression of *CTLA-4* on $CD4^+$ T cells is up-regulated during TCR activation. *CTLA-4* ligates the members of the B7 family (CD86 and CD80) on APCs, and *CTLA-4* competes with CD28, a positive costimulatory molecule expressed on T cells and activated by B7 in the presence of antigen-MHC II signalling though the TCR (Fig 2). In T-cell activation, *CTLA-4* limits the T-cell proliferation response. The function of *CTLA-4* is critical for the regulation of peripheral self-tolerance and for prevention of autoimmunity, and has thus been considered as a candidate gene for autoimmunity (122). It has been suggested that *CTLA-4* may downregulate the T-cell proliferation responses by inhibiting production of IL-2, expression of IL-2 receptor and progression of the cell cycle (123; 124). Loss of *CTLA-4* function has been shown to lead to excessive lymphoproliferation (125).

The human *CTLA-4* gene (2q33) contains an A/G polymorphism at position +49 in exon 1 leading to amino acid exchange (alanine to threonine) in the encoded protein (126). The disease-associated G allele is shown to reduce the *CTLA-4*-driven negative regulation of T-cell activation (126; 127). In addition, another polymorphism described in the *CTLA-4* and associated with autoimmunity, CT60, has been shown to alter the splicing of the *CTLA-4* gene leading to increased production of the soluble form of *CTLA-4* (128).

The association between the *CTLA-4* gene region and T1D was first reported by Nistico et al (129). Later, the *CTLA-4* +49 A/G polymorphism has been associated with increased appearance of T1D (130). Marron et al showed an association between the polymorphism and clinical T1D in several ethnic groups, especially of South European origin, but failed to detect an association between the SNP and the disease among populations especially of British origin (131). An extensive meta-analysis has confirmed the association between the polymorphism and T1D (132). Moreover, the meta-analysis reported a significant ethnic variation in the SNP prevalence. In addition to T1D, the *CTLA-4* +49 A/G polymorphism has been associated with Grave's disease, autoimmune thyroid disease, SLE, autoimmune Addison's disease, MS, rheumatoid arthritis, celiac disease and vitiligo (133).

2.2.4. PTPN22 gene polymorphism

The protein tyrosine phosphatase 22 gene, *PTPN22* is located on chromosome 1p13 and it encodes a lymphoid tyrosine phosphatase (LYP, a human homologue to murine protein tyrosine phosphatase PEP) that is a non-receptor protein tyrosine phosphatase expressed

in cells of hematopoietic origin, notably in T cells (134-137). The main function of LYP is to decrease T-cell receptor-mediated signalling. The target of LYP is Lck, a protein tyrosine kinase activating the TCR signal transduction pathway but also other targets of LYP have been reported (135; 136; 138). LYP forms a complex with the C-terminal Src kinase (CSK), which regulates the activation state of Lck (138). The significance of the negative regulatory function has been shown in the PEP-/- knock-out mouse model on a non-autoimmune background (139). The lack of PEP led to T-cell hyperresponsiveness in this mouse model with a non-autoimmune background.

A single nucleotide polymorphism in the *PTPN22* gene (C1858T) causes arginine to tryptophan substitution in codon 620 in the phosphatase. This polymorphism was first shown to be associated with increased appearance of T1D by Bottini et al (140). The association between the polymorphism and T1D has been confirmed in several populations (141-147). In addition, the polymorphism has since then been associated with several other autoimmune diseases like rheumatoid arthritis, juvenile rheumatoid arthritis, SLE, Grave's disease, and recently, with celiac disease (148; 149).

There is a wide variation in the *PTPN22* gene 1858T allele distribution among different populations (150). In European countries, there is a south to north gradient in the allele frequencies, varying between a 1858T allele frequency of 2-3% in Italian and Sardinian populations, 7-8% in Western populations, over 10% among Scandinavians and 15% in the Finns. In the European-American population in the US there are differences in the allele frequency reflecting the geographic origin of the original emigrants from different locations in Europe. Among African and Asian populations, the *PTPN22* 1858T disease allele seems to be absent.

The *PTPN22* 620Trp disease variant has been shown to lead to decreased calcium mobilisation and IL-2 production of $CD4^+$ T cells upon TCR activation (151). Moreover, a reduced responsiveness of $CD4^+$ memory T cells, indicated by decreased calcium mobilisation, expression of CD25 and IL-10 production has been described (152). In addition, the disease variant was also associated with a decreased number of memory B cells and diminished B-cell responsiveness upon B-cell receptor activation. However, the disease variant was shown to be associated with an increased number of circulating memory T cells.

An increased risk for invasive gram-positive bacterial diseases, especially invasive pneumococcal diseases among carriers of the *PTPN22* 1858T allele has been reported (153), but the disease variant has also been shown to protect against tuberculosis (154). At the time of T1D diagnosis and during the first year after the diagnosis the disease allele is associated with lower residual β-cell function and poorer metabolic control (155).

2.3. Environmental triggers

Although there is a clear genetic predisposition, several of the observed phenomena support the importance of environmental factors in the development of T1D. A dramatic increase in the prevalence of T1D during recent decades particularly in Europe indicates the pivotal role of environmental factors. Such an increase cannot be a consequence of changes in the genetic disease susceptibility, but is most likely related to changes in the environment and probably also life-style. In addition, data from a migrant study emphasized the influence of changing environment (156). Moreover, a seasonal variation in the appearance of autoantibodies associated with T1D supports the role of environmental factors (157).

As environmental triggers of β-cell autoimmunity, viral infections and dietary factors are commonly mentioned. The viral infections proposed include enterovirus, rotavirus, cytomegalovirus, rubella virus and mumps infections, while cow's milk (CM) –based formula, short breastfeeding, dietary glutein and vitamin D deficiency are among the possible nutritional triggers most often suggested.

Rubella virus infection *in utero* has been reported to increase the risk for T1D; almost 20% of the infected subjects developed T1D (158-163). Interestingly, 50-80% of the patients with congenital rubella virus infection were also positive for ICA and/or IAA (164) and an increased frequency of HLA-DR3 allele predisposing to T1D was observed among these patients, whereas the frequency of the protective HLA-DR2 allele was reduced (162). However, a recent study failed to show an association between rubella virus infection *in utero* and the development of diabetes-associated autoantibodies (165), suggesting a non-autoimmune background of congenital rubella virus-induced diabetes. Although a persistent rubella virus infection has been detected in the pancreas of a part of the subjects with congenital infection (166; 167), no signs of direct destruction of β cells have been detected after rubella infection of human islets (168). An alternative mechanism suggested is molecular mimicry; T cells of patients with T1D after congenital rubella infection were reported to cross-react with rubella virus peptides and GAD antigen (169).

Mumps virus infection has been suggested to induce β-cell autoimmunity; mumps infection has been associated with increased appearance of islet cell autoantibodies and clinical T1D (170). Moreover, an increase in T1D incidence two to four years after a mumps outbreak has been described (171). Mumps has been shown to infect human β-cells, but without β-cell lysis during the infection (172; 173). Induction of IL-1 and IL-6 release in a human insulinoma cell line and increased expression of HLA class I and II antigens has been detected during mumps infection, suggesting an alteration in β-cell tolerance (173). However, due to extensive vaccination programmes against rubella virus

and mumps in developed countries the effect of these infections on the development of T1D apparently remains marginal; it does not explain the increasing incidence of T1D.

Early exposure to cereals has been reported to enhance the appearance of humoral signs of β-cell autoimmunity. Both early (before four months of age) and late (after the age of seven months) exposure to cereals, both gluten-containing and non-glutein-containing, were associated with increased risk of β-cell autoimmunity in an American report (174), whereas a German study reported an increased risk for the appearance of autoantibodies if exposed to cereals before three months of age (175). Interestingly, dietary gliadin is suggested to trigger intestinal inflammation in subjects with T1D (176). This phenomenon proposes a gliadin-induced activation of the gut immune system that may enhance the autoimmune attack against pancreatic β cells.

Lack of vitamin D in infancy has been shown to increase the risk of T1D in some studies (177-179). However, in Northern Europe, with strong seasonal variation in the amount of daylight, there is a widely implemented recommendation for oral vitamin D substitution in the form of daily drops in infancy. Morever, there are low T1D incidence areas in Northern Europe; in the Baltic countries and in Russian Karelia with a HLA background partly similar to that of the Finnish population.

2.3.1. Enteroviruses

Human enteroviruses are members of the *Picornaviridae* family. They are non-enveloped viruses with single-stranded RNA genome. The virus consists of four structural proteins, VP1-3 are on the virion surface and VP4 is internal. In addition, seven non-structural proteins, 2A-C and 3A-D are responsible for virus replication (180). Enteroviruses are divided into four genetic clusters according to the similarities in the capsid-encoding region. Cluster A contains 11 coxsackievirus A (CAV) serotypes and enterovirus 71, cluster B contains coxsackievirus B (CBV) serotypes, CAV9 and echoviruses, cluster C contains polioviruses and 11 CAV serotypes, and cluster D contains enteroviruses 68 and 70 (181-183). Enteroviruses are mainly transmitted by faecal-oral or respiratory routes and the primary replication occurs mostly in the epithelium of the small intestine. The clinical outcome of an enterovirus infection ranges from sub-clinical infection to mild respiratory illness (common cold), hand, foot and mouth disease, acute hemorrhagic conjunctivitis, gastroenteritis, aseptic meningitis, myocarditis, severe neonatal sepsis-like disease, and acute flaccid paralysis. The epidemiology of enteroviruses is affected by the variation in the serotype and geographic location. In Finland, the peak incidence in the enterovirus infections usually occurs in late summer and early autumn. Laboratory diagnosis of enteroviruses can be preformed by PCR detection, serology, and virus isolation. Humoral immunity towards enteroviruses includes the appearance of IgM, IgG and IgA class antibodies, the first of which normally disappears within the first six

months, whereas IgG and IgA class antibodies persist for longer, IgG class antibodies for years. Serological cross-reactivity exists between different enterovirus serotypes.

Enterovirus infections, especially coxsackie B4 (CBV4) virus infections, have been suggested as triggers of T1D-associated autoimmunity and clinical T1D. The association was first proposed when higher CBV4-specific antibody titres were observed among subjects with T1D compared to healthy controls (184). This finding has since then been supported by several other reports (185-188), but also contradictory results have been published (189). Moreover, enterovirus RNA has been detected in peripheral blood or stool samples from T1D patients more ofter than in controls (190-192). In addition, enterovirus infection, detected by serological means or the PCR method, has been shown to precede the appearance of T1D-associated autoantibodies in several prospective follow-up studies among at-risk subjects in Finland (193-200), but no temporal association could be detected in other studies (201; 202).

The HLA-genotype is shown to affect the strength of the humoral immune response towards CBV4, being stronger among subjects with HLA-genotypes associated with T1D risk (DR3 and/or DR4) and weaker among subjects with protective alleles (DR2) (203), and differences in the cellular responsiveness to CBV4 have been reported between subjects carrying the predisposing alleles DR3 or DR4; here the DR3 allele was associated with decreased CBV4-specific responsiveness compared to subjects carrying the DR4 allele (204; 205), indicating the importance of the case-control –matching of the study subjects. Moreover, an Autralian group reported an association between enterovirus RNA in blood and/or stool samples at the time of T1D diagnosis among subjects with low-risk HLA genotype, but not among subjects carrying the HLA-genotype associated with elevated T1D risk (192).

Enterovirus infection during pregnancy has also been implicated as a possible trigger of β-cell autoimmunity. Higher levels of enterovirus antibodies at delivery have been reported among pregnant mothers giving birth to a child subsequently developing T1D (194; 206; 207), but no association with enterovirus infection during pregnancy was observed in other studies (201; 208).

Contradictory results on the enterovirus-specific T-cell responsiveness in patients with T1D have been reported. Stronger PBMC proliferation responses to CBV4 lysate antigen were observed among clinically healthy subjects with T1D-associated autoantibodies compared to healthy controls in a Finnish study on subjects with an unselected HLA-background (209). In contrast, in a cohort with subjects carrying HLA-confirmed T1D risk, the CBV4-induced PBMC proliferation responses were higher in subjects with T1D analysed months after the T1D diagnosis compared to newly-diagnosed subjects or healthy controls, whereas CBV4-specific PBMC responses in newly-diagnosed subjects did not differ from controls (210). When measuring several activation markers but not

proliferation, Skarsvik et al reported a decreased type 1 immune response upon CBV4 stimulation among subjects with T1D months after diagnosis compared to healthy subjects with or without HLA risk genotype (211), suggesting an impaired response to enterovirus infection that might lead to delayed clearance of the virus. Interestingly, a persistent enterovirus infection has been observed in the intestine of diabetic patients with normal mucosal morphology (212).

Although CBV4 is most commonly associated with the pathogenesis of T1D, also other enterovirus infections including other CBV serotypes have been associated with β-cell autoimmunity in serological studies (187; 213). Moreover, several enterovirus serotypes including CBV3, 4 and 5 and coxsackievirus A9 have been shown to infect human β-cells *in vitro* (214). Coxsackie B viruses typically cause a lytic infection, but only a part of the β cells are immediately killed (214; 215). However, enterovirus infections in islets caused e.g. by coxsackievirus A9 may also proceed without cell lysis *in vitro*, suggesting the establishment of persistent infection (216; 217). Currently, there is no exact evidence on which type of the β-cell infection in human pancreas predisposes to the development of β-cell autoimmunity. Both lytic and non-lytic infections are suggested to promote β-cell destruction by stimulating expression of cytotoxic proinflammatory cytokines, and thus facilitating immune-mediated β-cell damage (218; 219).

A six-amino-acid sequence (PEVKEK) is shared by the islet cell autoantigen GAD65 and the CBV4 protein P2C (9; 36), and peptides containing PEVKEK have been shown to stimulate T cells (220). Interestingly, PBMCs from individuals at risk of T1D responding to adjacent GAD65 peptides also responded to coxsackie viral peptide sharing the same amino acid sequence, suggesting a molecular mimicry in this response (221). In addition, antibody cross-reactivity between GAD65 and 2C proteins has been shown, although no cross-reactivity could be observed after natural infection (222-224).

Besides molecular mimicry, bystander activation during CBV4 infection has been implicated in the β-cell destruction. A local CBV4 infection in the pancreas has been shown to induce the development of T1D in a mouse model carrying diabetogenic T-cell receptor specific to β-cell autoantigen other than GAD65 (38). In accordance with this finding, injury of β cells by streptozotocin was found to equally induce T1D in this mouse model, suggesting the non-specific nature of the phenomenon and the importance of the release of β-cell autoantigens (40). Moreover, the induction of bystander activation by CBV4 infection has been shown to depend on the presence of a β-cell-specific autoreactive T-cell population implicating the importance of the timing of the CBV4 infection for the β-cell destruction (225).

2.3.2. Rotavirus

Rotaviruses are members of the *Reoviridae* family of viruses (226). The genome of the rotavirus consists of 11 segments of double-stranded RNA and is surrounded by three protein layers (capsids). Viral protein (VP) 2 forms the core, VP6 constitutes the middle capsid, and VP7 and VP4 form the outer capsid. VP6 determines the serogroup antigen specificity (A-G), and it is the most immunogenic protein of rotavirus. Serogroups A, B and C have been identified in humans; of these groups, A is the most common cause of a rotavirus infection (227; 228). Group A can be differentiated by serotype, determination of which depends on the antigens expressed on the outer viral capsid, VP7 or VP4. The segmented genome enables the gene re-assortment during infection of a cell by two different rotavirus strains, and thus creates serotype diversity.

In children, the clinical picture of rotavirus gastroenteritis includes febrile illness, vomiting and watery diarrhea lasting for four to seven days (226). In contrast, the infection in adults is mostly mild or sub-clinical. Rotavirus is a common cause of diarrheal illness worldwide, being responsible for about 5% to 10% of all diarrheal episodes in infants and children under five years of age, but for 30% to 50% of severe diarrheal episodes in the same age group. In the US over 90% of children are shown to have antibodies against the virus at the age of four years. Rotavirus is mostly transmitted through the faecal/oral route, but data indicating transmission in respiratory droplets have also been reported (229). In the gastrointestinal tract, the virus infects enterocytes in the small intestine. Virus particles replicate in the cell cytoplasm and are then shed, damaging the cell. The damage to the enterocytes prevents the effective uptake of fluids and nutrients. In response to the cell damage, secretory crypt cells proliferate and enhance the secretion of fluid and electrolyte in the gut lumen. In addition, damage to the infected cells prevents the production and secretion of digestive enzymes (230). Thus, the pathophysiology of rotavirus diarrhea is a combination of osmotic and secretory mechanisms (231).

The laboratory diagnosis of an acute rotavirus infection is based on VP6 antigen detection in faeces. In addition to antigen detection, electron microscopy (EM) can be employed to detect rotavirus from stool samples. Also rotavirus-specific IgG- and IgA-class antibodies can be found in serum after the infection. These serological tests are known to detect more cases of rotavirus infection than antigen detection (232). However, due to the slow development of antibody positivity, serology is not commonly applied in clinical settings. Besides antigen detection, EM and serology, RT-PCR can also be employed for detection of the virus from faeces.

During an acute rotavirus infection, oral rehydration therapy is applied to treat the dehydration caused by the gastroenteritis; in severe cases the rehydration can be performed intravenously. A vaccination against rotavirus for infants is available. This vaccination will be included in the Finnish national vaccination programme during 2009.

Rotavirus infection during childhood has been associated with the appearance of GAD and IA-2 specific autoantibodies in an Australian birth-cohort of children genetically at risk for T1D (233). Moreover, a temporary association between rotavirus infection and the first appearance of IAA, GADA and IA-2A in children with a first-dergee relative with T1D was reported. In addition, ICA levels were reported to increase during repeated rotavirus infections. In contrast, no association between rotavirus infection and the appearance of humoral signs of β-cell autoimmunity was observed in a Finnish prospective study from the DIPP cohort (234). However, Mäkelä et al have later shown an association between enteral virus infections, including rotavirus infection, in infancy and elevated bovine insulin-binding antibody levels in early childhood, indicating an association between these virus infection and enhanced formation of immunity towards insulin (235).

Interestingly, a sequence homology between the VP7 protein of rotavirus and the tyrosine phosphatase IA-2 has been observed; the VP7 and the IA-2 peptides bind to HLA-DR4 (*DRB1*0401*) and are presented identically to the TCR (236). In addition, the amino-terminal region of VP7 shows a high-degree similarity with a sequence of GAD65 (236). Both the VP7 regions similar to GAD and IA-2 act as immunogenic epitopes and are capable of binding to HLA class II molecules and induce rotavirus-specific immune responses, indicating possible significance for molecular mimicry between the virus and these two T1D-associated autoantigens, GAD and IA-2.

Alterations in the gut permeability during rotavirus infection have been reported (237-239). IFNγ and TNFα produced during rotavirus infection alter the function of tight junctions in the gut epithelium. In addition, a subunit of rotavirus VP5 protein, VP8, has been shown to modulate the gate and fence function of tight junctions in gut epithelial cells (238). Orally given VP8 was also shown to allow the enteral administration of insulin to diabetic rats in the absence of rotavirus-induced symptoms like diarrhea or fever, indicating its capacity to facilitate the passage of molecules through gut epithelia. Thus, epithelial permeability of the gut is increased during rotavirus infection, a phenomenon which may enable the transit of nutritional components through gut mucosa, thereby increasing their immunogenicity.

2.3.3. The Herpesviridae family and cytomegalovirus

Human herpes group viruses, a part of the *herpesviridae* family, have a genome consisting of double-stranded DNA, which is enveloped in a proteinaceous matrix (tegument), surrounded by a lipid bilayer containing viral glycoproteins (envelope) (240). Herpes group viruses in humans include Herpes Simplex virus (HSV-1 and HSV-2), Cytomegalovirus (CMV), Varicella Zoster virus (VZV), Epstein-Barr virus (EBV) and Human herpes viruses 6, 7 and 8 (HHV-6, HHV-7 and HHV-8). Characteristic for the herpes group viruses is the ability to remain latent in their natural host after primary

infection. In cells harbouring the latent virus the viral genome is in the form of closed circular molecules and only a small subset of the viral genome is expressed. The latent genome retains the capacity to replicate and cause disease on reactivation.

Human cytomegalovirus (CMV, also called Human herpes virus 5, HHV5) is the largest known human herpes virus, with a genome of about 230 kb (241). CMV can be transmitted via genital secretions during delivery, saliva, breastfeeding, placental transfer, sexual contact, blood transfusion, solid-organ transplantation or haematopoietic stem-cell transplantation (242). CMV is commonly shed into breast milk during lactation in CMV seropositive women; amounts of CMV secretion vary widely between different reports (from 27% to 96%) (243-246). CMV infects epithelial cells and lymphocytes. After primary infection, CMV remains latent within T cells, endothelial cells and macrophages. Overt reactivation of the virus with clinical symptoms rarely occurs unless the immune system is suppressed. CMV-induced immune response is considered to favour Th1 type response with increased production of IL-2, TNFα and IFNγ by T cells and small amounts of IL-4 (247).

A primary infection of a seronegative woman during pregnancy causes a 40% risk of congenital CMV infection of the fetus (248). Congenital CMV infection is a common congential infection affecting 1% of newborns. After birth, the clinical feature of an intrauterine CMV infection ranges from non-symptomatic to the development of hearing defects, symptoms of central nervous system involvement, intrauterine growth retardation, hepatosplenomegaly, thrombocytopenia, petechiae and hepatitis (249). Severe complications are caused by infection during the first trimester. In contrast, perinatally acquired CMV infection is mostly asymptomatic, but in up to 30% of perinatal infections the infected infants display short-term, self-limiting symptoms of hepatosplenomegaly, lymphadenopathy, hepatitis or pneumonia (249). Later in life, primary CMV infection is mostly sub-clinical but in some cases it can cause symptoms similar to EBV infection, including mononucleosis/glandular fever-like syndrome with prolonged fever and mild hepatitis but, compared to EBV infection, there are rarely signs of tonsillopharyngitis or great splenomegaly (249).

Transmission of CMV depends on direct contact with infected body secretions, so hygiene remains an important determinant of virus transmission patterns (242). Developing countries typically exhibit widespread transmission early in life, whereas developed areas show a broader range of patterns. In general, the prevalence of CMV infection increases with age. In Western countries, the most rapid rise in CMV-specific antibodies in childhood is seen before two years of age, while the second period for the rapid appearance of CMV infection is seen at teen age (250-254). Among adults, a range of 40% to 90% seroprevalence has been reported among US citizens.

Laboratory diagnosis of CMV infection can be performed by detection of CMV-specific IgM and IgG class antibodies from serum samples (242). In addition to serological means, virus isolation can be performed, but the technique is time-consuming, taking over two weeks to detect the cytopathic effect specific for CMV. In contrast, rapid CMV isolation and detection of immediate early antigens by monoclonal antibodies allows the detection of the virus within 24 to 48 hours. Currently, real-time PCR is widely performed to detect CMV infection, the benefit of the method is the time consumption similar to serology, providing the potential to obtain the result within one day.

Cytomegalovirus infection has been implicated in the development of T1D. Pak et al reported an increased prevalence of CMV genome in lymphocytes of T1D patients positive for β-cell autoantibodies compared to healthy control individuals (255). A correlation between high CMV-specific IgM and IgG antibodies and ICA among first-degree relatives of T1D patients has been reported, suggesting that chronic CMV infection may be associated with the development of β-cell autoimmunity (256). However, no temporal association between primary CMV infection and the appearance of β-cell-specific autoantibodies or clinical T1D could be observed in a prospectively followed-up cohort (257). Nor did the authors observe any difference in the seroprevalence of CMV IgG and IgM antibodies in pregnant mothers of children subsequently developing T1D-autoimmunity compared to control subjects, and nor did they observe differences in the levels of β-cell-specific autoantibodies and CMV titers among newly-diagnosed T1D patients. Similarly, no effect of congenital CMV infection on the T1D incidence was observed in a Swedish cohort (258), and the CMV genome could not be detected in pancreatic tissue in patients who died at the onset of T1D (259).

Asymptomatic CMV infection has been reported to be associated with increased risk of T1D among adults after renal transplantation (260). Moreover, Osame et al published a case report of an adult patient with rapid-onset T1D and appearance of GAD autoantibodies after CMV infection (261), suggesting a role of CMV infection in the emergence of β-cell autoimmunity in a subset of cases. Interestingly, T-cell cross-reactivity between CMV and GAD65 has been reported (262). In addition, the mimicking epitope in the major DNA-binding protein of CMV has been shown to be naturally processed by dendritic cells and recognized by GAD65-reactive T cells restricted by HLA-DR3 molecule (263).

2.3.4. Cow's milk formula and breastfeeding

In the 1980s, many studies on the effect of cow's milk (CM) on the development of β-cell autoimmunity were performed in BB rats and the NOD mouse model. Elliot et al reported an effect of the manipulation of nutritional proteins on the emergence of diabetes in BB rats (264). They showed that the replacement of proteins by semi-synthetic amino acids in the diet decreased the incidence of diabetes from 52% to 15%.

This prevention of diabetes was supported by the report on NOD mice with decreased diabetes incidence following a purified casein hydrolysate diet (265; 266). Moreover, a narrow time-window for the effect of CM proteins on the diabetes induction was reported (267). Finally, the predisposing effect of short breastfeeding on the emergence of T1D in humans was reported (268). Since these findings, a series of reports on the role of CM-based formula nutrition and the length of breastfeeding on the appearance of T1D-associated autoimmunity has been reported.

2.3.4.1. The exposure to cow's milk-based formula nutrition in infancy and the length of breastfeeding

Both early exposure to CM-based formula nutrition in infancy and short exclusive breastfeeding have been suggested to take part in the induction of the T1D-associated autoimmunity, but the results have remained controversial. A vast number of studies on the role of either of these factors on the emergence of T1D or β-cell autoimmunity has been conducted, many of them supporting the role of early CM exposure or short breastfeeding in the initiation of T1D autoimmunity (268-284), but several studies have also failed to find association between these suggested triggers and T1D (285-297). However, these studies have been performed retrospectively, possibly biasing the result and many of the studies also lack appropriate controls matched for the HLA genotype conferring the T1D risk.

In a Finnish prospectively followed-up cohort of children with HLA-conferred T1D risk, the short exclusive breastfeeding and early introduction of CM-based infant formula was shown to predispose to the appearance of humoral signs of β-cell autoimmunity (298). Moreover, a prospective population-based follow-up study in Sweden reported an association between short exclusive breastfeeding or early introduction of CM-based formula and the appearance of T1D-associated autoantibodies (299). In contrast, no association between the length of exclusive breastfeeding or early CM-formula exposure and the appearance of T1D-associated autoimmunity or clinical T1D was detected in an extensive Finnish prospective cohort of subjects with HLA genotypes conferring T1D susceptibility (300), and no association could be observed in a German prospective follow-up cohort of offspring of a parent with T1D (301), or in an Australian cohort of children with a first-degree relative with T1D (302).

Interestingly, the pilot study of the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) among infants with high HLA risk and having a first degree relative with T1D reported a decreased appearance of T1D-associated autoantibodies and a decreased cellular and humoral response towards bovine insulin among subjects receiving hydrolysed casein formula during the first months of life compared to subjects receiving regular cow's milk-based formula nutrition (303). A study on a larger cohort of the TRIGR study with the aim of confirming the results from the pilot study is underway.

2.3.4.2. Immune response to insulin

There are differences in the protein composition between human breast milk and cow's milk, the protein concentration in CM being higher due to the larger casein content. In CM, the main whey protein is β-lactoglobulin, which is not found in human milk. In addition, the primary serum albumin amino acid sequence differs between CM and human milk in small area. Importantly, the structure of the bovine insulin molecule differs from human insulin only by three amino acids, the substitutions of threonine by alanine at A8, isoleucine by valine at A10, and threonine by alanine at B30 (Fig 3.). Cow's milk has been shown to contain small amounts of bovine insulin (303).

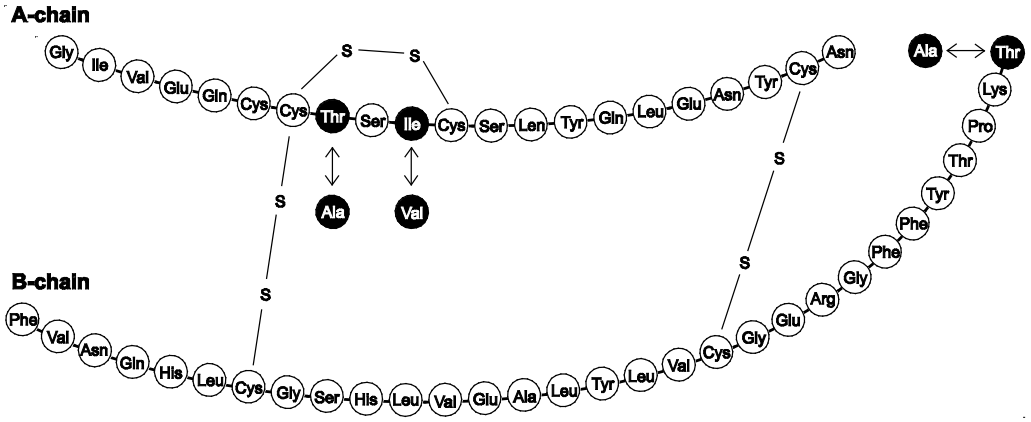

Figure 3. Schematic picture of the structure of human insulin. Bovine insulin differs from human insulin by three amino acids: the substitutions of threonine by alanine at A8, isoleucine by valine at A10, and threonine by alanine at B30.

Insulin is the only β-cell-specific autoantigen known so far. Insulin is first synthesised as a large precursor molecule, pre-proinsulin, which is then processed to proinsulin and finally to insulin once the C-peptide has been cleaved. Insulin is stored in the secretory granules of the pancreatic β cells and it is secreted in response to increasing blood glucose levels.

Insulin-binding antibodies (IAA) detected by liquid-phase radio-binding assay (RBA) are strongly associated with the development of T1D. IAA is often the first autoantibody to appear during the pre-diabetic phase (157; 305; 306), and it is commonly detectable at the time of diabetes diagnosis in young patients (157; 305; 307-309). In addition, IAA levels at the time of T1D diagnosis correlate inversely with age, the highest IAA levels being observed in children presenting with overt diabetes before the age of five years (308). The predictive value of IAA together with other autoantibodies for the development of T1D is described in section 2.1.4.

Dietary bovine insulin has been shown to induce insulin-specific immunity in infants. Bovine insulin-binding antibodies detected by enzyme immunoassay (EIA) are not

specific for T1D autoimmunity, but they indicate sensitisation to insulin and are found in healthy individuals without increased risk for T1D (310-313). The insulin antibody response declines with age, indicating the formation of immune tolerance towards insulin (314). IgG-class antibodies against bovine insulin and human insulin are strongly cross-reactive. Interestingly, increased levels of bovine insulin-binding antibodies are observed among infants exposed to CM before three months of age compared to infants exclusively breastfed until that age (310-313), indicating the stronger immunogenicity of bovine insulin in cow's milk compared to insulin in breast milk. Moreover, maternal ingestion of bovine-insulin-containing food during lactation has been shown to enhance the formation of tolerance towards insulin in her offspring (315). Finally, increased levels of bovine-insulin-binding antibodies have been reported among subjects with HLA-conferred T1D risk and subsequently presenting with β-cell autoantibodies, suggesting a failure in the formation of tolerance towards dietary insulin among the progressors (312).

Although T1D is considered a T-cell mediated disease, currently the only methods to reliably detect β-cell autoimmunity are based on autoantibodies. The difficulties in the development of specific T-cell assays for the detection of responsiveness against autoantigens are probably related to the use of peripheral blood mononuclear cells (PBMCs) of affected individuals, while no access to the lymphocytes in the pancreatic lymphnodes is available. However, Marttila et al recently reported an enhanced responsiveness of PBMCs to bovine insulin pepetide (A1-12) and human insulin peptide (A1-12) among newly-diagnosed T1D patients and multiple autoantibody positive subjects with HLA-conferred disease risk compared to clinically healthy, autoantibody negative subjects with the same HLA risk, indicating detectable insulin autoreactivity associated with the β-cell destruction (316). Moreover, Kent et al have shown that insulin-specific T cells exist in lymphoid organs near the pancreas in long-standing T1D patients, but not in healthy controls nor in patients with type 2 diabetes (317), and they were unable to isolate insulin specific T cells from the spleen of the T1D patients, suggesting the pancreas-specific localisation of the insulin-autoreactive cells.

2.4. Prevention

Prevention of T1D requires understanding of the autoimmune disease process in pancreatic β cells. Since the autoimmune attack often starts during the first years of life, the prevention strategy requires intervention at early stages in infancy or childhood. The prevention can include intervention prior to autoimmunity appearance, at the appearance of the T1D-associated autoimmunity before clinical disease, or after the diagnosis of the clinical disease. Intervention before clinical disease requires the identification of the population at risk for diabetes. Currently, this includes screening for the genetic risk markers, mainly the HLA class II genotypes associated with T1D risk. The benefit of the

intervention after the appearance of humoral autoimmunity targeted against pancreatic β cells is the remarkably decreased number of subjects requiring prevention. However, once the autoimmune response has started to evolve it may be difficult to stop or slow down.

To prevent the formation of β-cell autoimmunity in the first place, an intervention on the environmental factors is the main target. Currently, the TRIGR study aims to clarify the role of cow's milk formula in the pathogenesis of T1D (see section 2.3.4.1). In addition to intervention in diet, the prevention of autoimmunity-triggering virus infection with vaccination would be of interest. Several attempts have been made to stop the progression of β-cell autoimmunity after the autoantibodies have appeared. The Diabetes Prevention Trial-type1 (DPT-1) tested the efficacy of subcutaneus and oral insulin (318; 319) and the DIPP study in Finland determined the efficacy of nasal insulin (320) for the prevention of or delay in the development of T1D but both studies failed to slow down the autoimmunity process. The European Nicotinamide Diabetes Intervention Trial (ENDIT) assessed the effect of nicotinamide on the progression to T1D, but no difference in the T1D frequency was detected between the nicotinamide and placebo groups (321).

At the moment of T1D diagnosis approximately 80% to 90% of the β-cell mass has been lost. The remaining islets fail to secrete sufficient amounts of insulin, leading to the need for insulin treatment. However, although exogenous insulin therapy is needed, preserving the residual β-cell function has been shown to lower the GHbA1C levels, to reduce the incidence of hypoglycemia, and to delay the development of complications (322). Thus, interventions aiming at the prolongation of β-cell function have been undertaken. Monoclonal antibodies against CD3 receptor are thought to mediate the β-cell protection by inducing clonal deletion or anergy in pathogenic β-cell-specific T cells, which may aid the differentiation and expansion of regulatory T cells (323). The treatment in newly-diagnosed T1D patients has been shown to decrease the decline of stimulated C-peptide, to lower HbA1C levels and lower the insulin requirement of the patients (324-326). However, the adverse effects of this treatment are challenging its clinical use. Moreover, an immunogenic peptide 277 (p277) from the heat shock protein (HSP) 60 has been shown to slow down the decline of C-peptide production and presented with no adverse effects (327-329), but this beneficial effect could not be observed among young patients (330). Finally, a trial to assess the potential of alum-formulated human recombinant GAD65 showed a decelerated decline in C-peptide after GAD65 treatment in pediatric T1D patients with no severe adverse effects, but the treatment did not change the insulin requirement (331).

3. AIMS OF THE STUDY

The purpose of the study was to obtain more information on viral factors possibly participating in the type 1 diabetes-associated autoimmune process and to search for their interactions with genetic and nutritional factors.

The specific aims were:

1. To determine the prevalence of Herpes group virus infections in childhood in Finland, and to analyse the possible association between CMV infection early in life and the appearance of T1D-associated autoimmunity.

2. To characterise the effect of the *PTPN22* C1858T polymorphism on cellular responsiveness among patients with T1D and clinically healthy subjects.

3. To analyse the combined effect of *PTPN22* C1858T polymorphism and exposure to cow's milk-based formula nutrition on the formation of β-cell autoimmunity.

4. To explore the effect of enteral virus infections during the first year of life on the pathogenesis of T1D with respect to nutritional patterns in early infancy.

4. SUBJECTS AND METHODS

4.1. Subjects

4.1.1. Study I

The subjects were participants in the Special Turku Coronary Risk Factor Intervention Project (STRIP) study, which is a prospective follow-up study of children born in Turku from 1989 to 1992. The aim of the project is to explore the cardiovascular disease risk factors during childhood and adolescence. In total, 1062 children were recruited to the study and divided into an intervention group receiving regular counselling by a physician, nutritionist and nurse regarding lifestyle and nutritional advice, and a control group. Both groups were followed-up at 1- to 12-month intervals according to the study protocol (332). The local ethical committee approved the study.

In the study I, the cohort comprised 199 participants from the STRIP study. In the study, subjects were analysed for the frequency of CMV, HSV and VZV IgG class antibodies at the age of seven months, 13 months, two years and yearly thereafter until the age of six years, and at the age of eight years (misprinted in report I).

4.1.2. Studies II-V

The subjects in the studies II, III, IV and V were participants in the Diabetes Prediction and Prevention Study (DIPP) which is a prospective population-based follow-up study in Turku, Oulu and Tampere University Hospitals in Finland (333). According to the study protocol, the new-born infants are invited to take part in a screening for the genetic risk factors of T1D. Subjects carrying the T1D-associated HLA risk genotypes, i.e. carriers of HLA-*DQB1* genotypes **02/*0302, *0302/x* (x≠02, *0301 or *0602) and boys born in Turku with genotype *DQB1 *02/y-DQA1*05/z* (y≠*0301, *0302, *0602, *0603; z≠*0201) are then recruited for the study. In Turku, the participants were followed-up at three months intervals until the age of two years and at six months intervals after that. In Oulu and Tampere, the visits were scheduled at the age of 3, 6, 12, 18 and 24 months, and yearly thereafter. Information on breastfeeding and cow's milk formula nutrition was obtained at all visits during the first year of life. In addition, information on participants' health and vaccinations were obtained and blood samples drawn during the visits. The local ethical committees have approved the study and informed consent was obtained from the guardians of the participant.

To detect signs of humoral immunity towards β cells the presence of T1D-associated autoantibodies was analysed from serum samples obtained during regular visits. Until the end of year 2002, the serum samples were analysed for the presence of ICA and if

positive, for IAA, GADA and IA-2A. From the beginning of 2003, all follow-up samples were analysed for the presence of the four autoantibodies. The development of clinical T1D among the study participants was diagnosed according to the WHO criteria.

All subjects developing positivity for at least two of the T1D-associated autoantibodies were invited to take part in a double-blinded placebo-controlled intervention trial on the efficacy of nasal insulin in the prevention of the T1D (320). No effect of the administration of nasal insulin on the progression to clinical diabetes could be observed in the study.

In addition to the participants in the DIPP project, study III included 15 T1D patients diagnosed under the age of 15 years, recruited from the pediatric diabetes clinic in Budapest, Hungary. All these patients were diagnosed according to the WHO criteria.

Study II aimed to analyse the association between cytomegalovirus infection in infancy with the development of T1D-associated humoral immunity and clinical T1D. The study cohort consisted of 169 case subjects (67 girls, age 0.5 – 2.0 years, median 1.3 years) who developed the first autoantibody by the age of two years and who subsequently developed positivity for at least two of the T1D-associated autoantibodies during the follow-up. In addition, 791 control subjects (326 girls, age 0.5-2.0 years, median 1.3 years, 3-5 controls per case, median 5 controls) matched for the HLA-*DQB1* genotype, gender, birth date and place of birth (Turku or Oulu) were analysed. During further follow-up (2.7-12.3 years, median 7.5 years) 90 of the case subjects developed clinical T1D. CMV IgG antibodies were analysed from serum samples at the time of the appearance of the first autoantibody or three to six months after it.

Study III aimed to analyse the effect of the *PTPN22* C1858T polymorphism on the lymphocyte responsiveness. For this purpose, the $CD4^+$ T cell proliferation, IL-2 production and intracellular calcium mobilisation were analysed in 11 subjects with established T1D. The frequency of various *PTPN22* genotypes in the T1D patients was 1858TT in two cases, 1858CT in four cases and 1858CC in five cases (age 5.2-9.8 years, median 8.6 years). The $CD4^+$ T cell proliferation and IL-2 production analysis were similarly performed in 18 autoantibody negative healthy control subjects from the DIPP study group (age 1.0-6.1 years, median 3.3 years, 7 subjects with 1858 CT genotype and 11 subjects with 1858 CC genotype). For these analyses in the healthy control group we used PBMC samples that were collected earlier during DIPP follow-up and stored frozen until the analyses.

In addition, peripheral blood mononuclear cell (PBMC) proliferation tests and cytokine secretion analysis were performed in clinically healthy subjects positive for at least two T1D-associated autoantibodies and in autoantibody negative healthy controls from the DIPP cohort and in newly-diagnosed T1D patients. The multiple autoantibody positive study cohort comprised 68 subjects. In this group, four subjects were *PTPN22* 1858TT

homozygous (age 3.2-8.5 years, median 5.5 years), 21 subjects were *PTPN22* 1858CT heterozygous (age 2.5-15.7 years, median 6.1 years), and 43 carried the wild type 1858CC genotype (age 2.1-15.7 years, median 6.4 years). Cytokine secretion analysis was performed in a subgroup of 38 multiple autoantibody positive subjects. Among the autoantibody negative healthy controls (n=95) 17 subjects carried the 1858CT genotype (age 1.2-15.2 years, median 5.6 years) and 78 subjects carried the 1858CC genotype (age 1.4-15.8 years, median 6.4 years), cytokine secretion was analysed in a subgroup of 62 autoantibody negative subjects. The *PTPN22* C1858T genotype distribution among the 35 newly-diagnosed T1D patients collected from the Turku area, Finland, was 13 1858CT genotypes (age 1.2-15.4, median 6.1 years, 5 girls) and 22 1858CC genotypes (age 1.7-14.3 years, median 11.4 years, 7 girls). Cytokine secretion was analysed in a subgroup of 15 subjects with newly-diagnosed T1D.

In study IV our objective was to explore the effect of the genetic polymorphisms, associated with enhanced appearance of T1D, on the development of T1D-associated autoimmunity, bovine-insulin-specific humoral immunity and progression to clinical T1D, with special emphasis on the interaction with the effect of early cow's milk-based formula nutrition. The study population comprised 719 subjects (320 girls) born between December 1994 and June 2002 and taking part in the DIPP study in two cities in Finland, Turku and Oulu. The study population comprised 156 subjects who developed positivity for at least two of the T1D-associated autoantibodies. Eighty-three of these subjects developed clinical T1D during the follow-up. In addition, 563 autoantibody-negative subjects with a similar distribution of HLA-*DQB1* genotypes, gender, date and place of birth were analysed. The median follow-up time for the autoantibody appearance was 9.0 years (range 0.5-14.0 years), and for the development of clinical T1D, 10.4 years (range 6.2-13.8 years). One-hundred of the 156 children who developed multiple autoantibodies took part in the placebo-controlled intervention trial on the efficacy of nasal insulin in the prevention of T1D. Fifty-one of these subjects received intranasal insulin. The distribution of the studied gene polymorphisms was 15 subjects with *PTPN22* 1858TT genotype, 175 subjects heterozygous for 1858CT genotype, and 529 subjects homozygous for the *PTPN22* 1858CC wild-type genotype. Four hundred and forty-four children were homozygous for the *INS* -23HphI AA genotype, 240 were AT heterozygous, and 31 subjects carried the TT genotype. In the study cohort, the distribution of the *CTLA4* $^{+}$49 A/G polymorphism was as follows; 189 subjects were homozygous for GG, 396 heterozygous for GA and 133 homozygous for AA. Four subjects could not be successfully genotyped for the -23HphI polymorphism and one participant for the *CTLA4* +49 polymorphism. Data on the exposure to CM formula in early infancy were available in 586 children. Altogether four hundred and forty-five of these subjects were exposed to CM before the age of six months, and 141 were exposed to CM at the age of six months or later.

The cohort in study V comprised 99 subjects showing positivity for at least two of the T1D-associated autoantibodies and 477 autoantibody-negative controls (3-5 controls per case, median 5 controls) matched for HLA-*DQB1* genotype, gender and birth date and place of birth (Turku or Oulu) and who were not included in the pilot study group published earlier (235). During the later follow-up (4.5-12.0 years), 23 of the controls developed positivity for single autoantibody and eight controls appeared with multiple autoantibodies. Controls developing single autoantibody positivity were excluded from further analysis, and controls emerging with multiple autoantibodies were analysed with the case group. Fourty-seven of the case subjects developed clinical T1D during the follow-up. Information on the exposure to cow's milk-based formula feeding was available in 472 study subjects: 251 subjects were exposed to cow's milk-based formula feeding before three months of age and 221 subject at the age of three months or later. In this study the aim was to analyse the effect of enteral virus infections in infancy on the development of humoral immunity against bovine insulin and the appearance of T1D-associated humoral autoimmunity and clinical T1D. Moreover, the combined effect of early introduction of cow's-milk based formula feeding and early enteral virus infection on the development of autoimmunity was explored. Respiratory syncytial virus (RSV) infection during infancy was analysed as a control infection.

4.2. Methods

4.2.1. Genetic analysis

Blood spots dried on filter paper and stored at room temperature (RT) were used as starting material for all genotypings. For HLA analyses a 3-mm disk was punched directly into 96 well PCR plates where the amplification mix was added. Polymorphic parts of second exon of HLA-*DQB1* and –*DQA1* genes were amplified using primer pairs with biotinylated 3´primer. The biotinylated PCR products were transferred to streptavidin-coated microtitration plates, denatured and hybridized with sequence-specific probes labelled with lanthanide chelates of europium (Eu), terbium (Tb) or samarium (Sm). After incubation, enhancement and washing steps, three-colour time-resolved fluorescence was measured to detect specific hybridization to bound PCR products. Details of the procedure including used probes and primers are described in earlier publications (334; 335).

For SNP assays, DNA was extracted by sodium hydroxide from disks punched from blood spots. Similarly to HLA assays, the principle of microtitration-plate-bound biotinylated amplification products and lanthanide labelled probes was used in SNP assays for *INS* -23A/T (rs689) and *CTLA-4* +49A/G (rs231775) polymorphisms (336; 337). For *PTPN22* +1858C/T (rs2476601) assay the one-step assay based on asymmetric amplification and subsequent time-resolved fluorescence measurement was used. Upon hybridizing to the

PCR-product the probes dehybridize from their complementary quenchers and become capable of emitting fluorescence (143; 338).

4.2.2. Antigens

4.2.2.1. CMV, HSV, VZV and adenovirus antigens

Cytomegalovirus, herpes simplex virus and varicella-zoster virus lysate antigens were prepared as described earlier (339). The adenovirus hexon protein antigen was prepared as previously described (340).

4.2.2.2. Enterovirus, rotavirus and RSV antigens

Coxsackie B4 (CBV4) [J.V.B, American Type Culture Collection (ATCC)] was grown in ML-2 LLC cells (ATCC). When cells detached freely from the flask surface the infected cells and supernatant were frozen and thawed three times. The purified antigen was obtained from the supernatant with sucrose gradient centrifugation (341). The antigen was inactivated by β-propiolactone.

The Nebraska calf diarrhea virus (NCDV) that is serologically largely cross-reactive with human rotavirus has been conventionally used as antigen to detect rotavirus-specific antibodies in human sera. For this purpose, NCDV (G serotype, P serotype 6) was grown in rhesus monkey kidney epithelial cells (LLC-MK2, ATCC) with trypsin (342). After the cytopathogenic effect was complete the virus was released by repeated freezing and thawing cycles, scraped into the supernatant and pelletted by centrifugation. The protein concentration was measured with Pierce BCA protein assay reagent (Pierce, Rockford, IL, USA).

Respiratory syncytial virus (RSV) A (Randall strain, isolated by Beem et al) was grown in Vero cells (343). After the cytopathogenic effect was complete the virus was released by freezing and quickly thawing repeatedly and then scraped into the supernatant and pelletted by centrifugation. The suspension was homogenized. The homogenate was centrifuged, the supernatant was collected and further centrifuged. The pellet was resuspended in PBS, sonicated, and the protein concentration was measured.

4.2.2.3. Other antigens

Phytohemagglutinin-P (PHA) (Becton Dickinson, Sparks, MD, USA) was used at a concentration of 25 μg/ml. For antigen-specific T-cell stimulation, purified protein derivative of tuberculin (2.5 μg/ml; Statens Serum Institut, Copenhagen, Denmark) and tetanus toxoid (1 μg/ml; National Public Health Institute, Helsinki, Finland) were used.

4.2.3. Antibody assays

4.2.3.1. The analysis of CMV, VZV, HSV, rotavirus and RSV IgG antibodies

The CMV, VZV, HSV, rotavirus and RSV-specific IgG antibodies were analysed by EIA. The virus antigens were prepared as described above. Microstrip 96-well plates (Immunoplate, Nunc, Roskilde, Denmark) were coated with the antigen diluted in PBS and incubated overnight at RT. Plates were washed with wash buffer (1% Tween in PBS) and serum samples diluted in 1:1000 (for detection of CMV, VZV and HSV-specific antibodies), 1:200 (for detection of rotavirus-specific IgG antibodies) and 1:100 (for detection of RSV-specific antibodies) assay buffer (5% pork serum, 0.5% Tween in PBS) were added to the plate and incubated at +37°C for 2h. Each sample was tested in duplicate and serial samples from one subject were tested on the same plate. After washes, peroxidase conjugated rabbit anti-human IgG antibody (Dako, Copenhagen, Denmark) diluted in PBS was added and the plates were incubated at +37°C for 1 h. O-phenylene-diamine tablets (Kem-En-Tec Diagnostics, Copenhagen, Denmark) in a buffer [7.3% $C_6H_8O_7xH_2O$, 11.9% $Na_2HPO_4xH_2O$ (pH 5.0)] were used as substrate. The reaction was stopped by 1 M HCl and the absorbance at 490 nm (A_{490}) was measured by spectrophotometer. A positive result was defined as an absorbance ≥ 3 times higher than the negative control exceeding the cut-off level (0.015 optical density units), and for serial samples, a ≥ 2-fold increase in the absorbance between two consecutive samples.

4.2.3.2. The analysis of rotavirus IgA antibodies

Microstrip 96-well plates were coated with rotavirus lysate antigen diluted in 100 µl of PBS and incubated overnight at RT. Plates were washed with wash buffer (0.1% Tween in PBS) and residual-coated with 1% bovine serum albumin (BSA) in PBS. After washes, serum samples diluted 1:10 in assay buffer (0.2% BSA and 0.05% Tween in PBS) were added to the plate and incubated at RT for 2 h. Each sample was tested in duplicate. After washes, biotinylated goat anti-human IgA (Vector Laboratories, Burlingame, CA, USA) in 1:100 dilution in assay buffer was added to the plates. After incubation of 1.5 h at RT, plates were washed and AP-streptavidin (Zymed, San Francisco, CA, USA) diluted 1:1000 in assay buffer was added to the plate and incubated at RT for 1 h. After washes, 4-nitrophenyl phosphatase substrate (Sigma, St. Louis, MO, USA) diluted in carbonate buffer [0.05 M $NaHCO_3$, 0.05 M Na_2CO_3, 0.02% $MgCl_2$, 0.02% NaN_3 (pH9.8)] 2 tabl/10 ml was added to the plate. The reaction was developed at RT and stopped with 100 µl 5 N NaOH. The absorbance at 405 nm (A_{405}) was measured by spectrophotometer. A positive result was defined as a ≥ 2–fold increase in the absorbance between consecutive samples, or a ≥ 3-fold absorbance compared to a negative control specimen, exceeding the cut-off level (0.015 optical density units).

4.2.3.3. The analysis of enterovirus and adenovirus IgG and IgA antibodies

IgG and IgA antibodies against purified coxsackievirus B4 antigen and adenovirus hexon protein were measured by EIA as described earlier (195; 235). The positive result was defined as a ≥2-fold increase in absorbance between consecutive samples, exceeding the cut-off level (0.200 optical density units).

4.2.3.4. The analysis of bovine insulin-binding IgG antibodies

Bovine insulin-binding IgG antibodies were detected by EIA (310). Briefly, microtitre plates (Combiplate Enhanced Binding, Labsystems, Helsinki, Finland) were coated with bovine insulin (Sigma, St. Louis, MO, USA) (1 µg/well in PBS) and incubated at +4°C overnight. The plates were washed with buffer containing 0.05% Tween 20 in PBS and residual-coated with 1% human serum albumin. Samples were diluted 1:10 in PBS containing 0.2% human serum albumin and 0.05% Tween 20 and incubated at RT for 2h. After washes, alkaline phosphatase-conjugated rabbit anti-human IgG antibody (Vector Laboratories, Burlingame, CA, USA) was added in a 1:100 dilution and the plates were incubated at RT for 90 min. P-nitrophenyl phosphatase buffer (Sigma) was used as substrate and the absorbance was read with a spectrophotometer.

4.2.3.5. Detection of autoantibodies

The antibody assays have been described in detail earlier (344). The detection limit for ICA was 2.5 Juvenile Diabetes Foundation Units (JDFU; sensitivity 100%, specificity 98%). The cut-off limits for IAA, GADA and IA-2A positivity were 1.56 RU, 5.36 RU and 0.43 RU, respectively, representing the 99th percentiles in a series comprising more than 370 non-diabetic Finnish children and adolescents.

4.2.4. Cell culture assays

4.2.4.1. Isolation of PBMCs and PBMC proliferation test

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation. The PBMCs were washed and resuspended in RPMI 1640 medium supplemented with 7.5% human AB serum (Human Sera Type AB, Lot Number 01104581, Cambrex Bio Science, Rockland, ME, USA), glutamine, HEPES and gentamycin 10 µg/ml. PBMCs (50,000/well) were incubated in quadruplicate with antigens in a final volume of 200 µl in 96-well round-bottomed microtitre plates for 6 days. Tritiated thymidine (2 µCi/ml, GE Healthcare, Chalfont St.Giles, Bucks, UK) was added 18 hours before harvesting. The cultures were harvested on glass fibre filters using a Tomtec 93 Mach III Manual Harvester (Tomtec, Orange, CT, USA) and incorporated radioactivity was measured with a Micro-Beta scintillation counter (Wallac, Turku,

Finland). Stimulation indices (SI) were calculated by dividing the median cpm value of antigen-stimulated quadruplicate wells by the median cpm of quadruplicate control wells. Cell culture medium served as baseline spontaneuos proliferation and negative control.

4.2.4.2. Cell cultures for cytokine secretion analysis

For cytokine secretion analyss, 100,000 PBMCs in a final volume of 200 µl were incubated at +37 ºC in 5% CO_2 for 72h in medium alone and with antigens in duplicate in 96-well round-bottomed microtitre plates (Corning Incorporated, Corning, NY, USA). After stimulation the supernatants of the PBMC cultures were collected and stored at –70°C for cytokine analysis.

4.2.4.3. PBMC cytokine secretion measurements

For the analysis of cytokine secretion the measurement of IL-2, IL-5, IL-10, IL-17, IFNγ and TNFα were done by Luminex 100IS xMAP technology, Luminex100 IS software 2.3, using the human cytokine LINCOplex kit (LINCO Research Inc., St. Charles, MO, USA) 96-well plate assay according to the manufacturer's protocol. 6.25 µl of bead mixture, detection antibody cocktail and streptavidin-phycoerythrin diluted in 18.75 µl of assay buffer were used in each well. LINCOplex human cyto/chemo standard cocktail (Lot #800-17K, LINCO Research Inc.) was used to establish standard curves for the assays.

4.2.4.4. T-cell proliferation analysis with flow cytometry

For the CFSE staining, PBMCs were labelled with CFSE (Molecular Probes) and stimulated with anti-CD3/anti-CD28 beads (Dynal Inc., Lake Success, NY, USA) with 1.0 bead/cell ratio for 48 h in 96-well round-bottomed microtitre plates. At the end of the culture period, cells were stained with APC-labelled anti-CD4 (BD Pharmingen, Erembodegem, Belgium) for 30 min in the dark and then run immediately on the flow cytometer. The proportion of cells divided at least once was evaluated. Gates were set to the deflection point between two histogram peaks.

4.2.4.5. Intracellular cytokine staining

PBMCs were plated at 2 x 10^5 cells/well into 96-well round-bottom plates (Sarstedt, Nürnbrecht, Germany) supplemented with RPMI-1640 culture medium and stimulated for 24 h with anti-CD3/anti-CD28 coated beads (Dynal) at 0, 0.1, 0.3, and 1 bead/cell ratios. Finally, the cells were stained with APC-labelled CD4 antibodies. After washes, cells were permeabilised and incubated with monoclonal antibodies (FITC anti-IL-2 and anti-IFNγ, and PE anti-IL-4; PharMingen, San Diego, CA, USA). The BD FACSAria flow cytometer was used for analysis; 10 000 cells per test were analysed. The data were processed using the BD FACSDiva software (BD Biosciences, San Jose, CA, USA), and

mean fluorescence values of cytokines in the total $CD4^+$ subpopulation were analysed. Mean fluorescence values were normalised to the unstimulated (control) values, and a three-parameter logistic model was applied after Box-Cox normalisation. Data were analysed as described (345; 346).

4.2.4.6. Intracellular calcium-flux measurements

Fluo-3-AM (Cat. No. F-1242) and Fura Red-AM (Cat. No. F-3021) were obtained from Molecular Probes (Eugene, OR, USA). PBMCs were resuspended in HBSS at 10^6-10^7 cells/ml and loaded with dyes (4 µg/ml Fluo-3 AM and 10 µg/ml Fura Red AM supplemented with Pluronic-F127) for 30 min at +30°C. Cells were washed once, and stained with APC anti-CD4. After washing, cells were kept at RT in the dark. A 500 µl aliquot was warmed to +37°C prior to fluxing. First, a baseline (30 s) level was recorded. Then, the tube was removed, 25 µg/ml PHA added and the tube replaced. Recording was commenced as soon as cells traversed the laser line and continued for up to 10 min (600 s). Data were saved as FCS 3.0 files, and analysed with the Bioconductor rflowcyt package (347). The baseline-normalised ratio of mean fluorescence intensity of Fluo-3 to Fura Red was plotted against time, and median fluorescence values per second were extracted and put into a matrix according to the patient's *PTPN22* genotype (CC, CT, and TT). From each matrix median values per time units were created and plotted against time, with a lowest smoothing of f=0.2.

4.2.4.7. Intracellular FoxP3 measurement

For the analysis of Foxp3 expression at the single-cell level, PBMCs were first stained for the expression of CD4 and CD25 surface molecules with anti-CD4 APC and anti-CD25 FITC, respectively. Cells were fixed and stained with PE-labelled FoxP3 antibody according to the manufacturer's recommendations (eBioscience, San Diego, CA, USA). A PE-labelled isotype control was used to evaluate the ratio of anti-FoxP3 positive T cells according to the manufacturer's recommendations.

4.2.5. Statistical analysis

Statistical analysis for studies II and III were performed using StatView 5.0.1 (SAS Institute Inc., Cary, NC, USA), and for studies IV and V with SPSS 15.0 (SPSS Inc., Chicago, Illinois). Additional analyses on specific measurements in study III were carried out as mentioned in the corresponding sections. *P* values lower than 0.05 were considered statistically significant. Statistical significance of differences between the groups was tested using non-parametric tests: Mann-Whitney *U* -test (MWU-test) for comparisons between two groups and Kruskal-Wallis rank sum test for comparison among three groups. ANOVA for repeated measurements was

used to compare the antibody levels between the groups over time. The Log Rank –test (Mantel-Cox) was used in the Kaplan-Meier survival analysis to compare the appearance of autoantibodies or T1D between the groups. Cox regression analysis was employed to analyse the combined effect of the analysed factors on the appearance of autoimmunity.

5. RESULTS AND DISCUSSION

5.1. The prevalence of CMV, VZV and HSV infections in childhood

We aimed to analyse the prevalence of CMV, HSV and VZV infections during childhood in Finland. For that purpose, the CMV, HSV and VZV specific IgG class antibodies were analysed in a prospectively followed-up cohort of 199 children in the Turku area, Finland, from the age of seven months until the age of eight years. In this cohort, perinatal CMV infection and CMV infection during infancy were common, at the age of seven months 27% of the subjects had CMV-specific IgG antibodies (Study I, Table I and Fig I). The seroprevalence of CMV antibodies increased slowly during childhood, reaching 41% by five years of age, suggesting infections acquired through vertical transmission, e.g. in day-care centres. During the last three years of the follow-up, the seroprevalence increased only marginally. Interestingly, 75% of the CMV infection acquired during childhood occurred during the first year of life. This transmission pattern reflects the importance of CMV shedding into genital secretions during delivery and into breast milk as the main transmission route during the first years of life.

Compared to results reported earlier, the incidence of CMV infection seems to have remained constant or slightly increased in Finland. In the 1970s, a CMV prevalence of 20% to 34% before one year of age was reported when analysing the CMV-specific antibodies with the complement fixation (CF) technique, and the prevalence of antibodies then decreased slowly reflecting the rarity of newly-acquired infections (253). In another Finnish study, the prevalence of CMV at two years of age varied between 10% and 35% during the follow-up period (254). In a Swedish study, a 40% to 46% prevalence of CMV antibodies has been reported between one and nine years of age (348).

VZV infection is one of the most contagious diseases, the infection being transmitted from 80% to 90% of uninfected subjects within a household (349). There is wide variation in the VZV prevalence worldwide (350). In the UK, a prevalence of 23% has been reported at 0.5 to 4.0 years of age, and the prevalence increased to 52% by the age of 4.1-12.0 years. In Hong Kong, the prevalence was higher compared to the UK; 32% at 0.5 to 4.0 years of age and 83% at the age of 4.1-12.0 years. According to our results, VZV infection in infancy is a rare event in Finland. By seven months of age, 2.8% of subjects had VZV-specific IgG antibodies, and the seroprevalence at 13 months of age was 4.5%. However, the prevalence increased rapidly during childhood, reaching 80% at the age of six years. In accordance with our findings, Koskiniemi et al recently reported a high seroprevalence of VZV at the end of the first decade of life in Finland; over 90% of subjects at ten years of age were VZV-seropositive (351). These results suggest that

the VZV incidence during childhood is particularly high in Finland compared to other Western populations, possibly due to the widely used day-care system.

HSV antibodies were less frequently observed in the follow-up series analysed. At seven months of age, 1.8% of the subjects had HSV-specific IgG class antibodies. During the follow-up the prevalence increased slowly, reaching 11% by three years and 17% by eight years of age. The prevalence of HSV antibodies was particularly low compared to other Western populations (352). In the UK, at the age of four years, over 50% of children have HSV-specific antibodies, and the prevalence further increases with age (353). Moreover, HSV seroprevalence in Finland has been reported to vary between 22% and 25% at four to six years of age (254), indicating a decreasing incidence of primary HSV infection during childhood. Thus, the proportion of uninfected individuals is increasing.

5.2. The association between early-acquired CMV infection and development of T1D-associated autoantibodies and clinical T1D

Cytomegalovirus infection in childhood has been suggested to play a role in the initiation of β-cell autoimmunity, but the results have remained contradictory (255-256). We aimed to analyse the effect of CMV infection acquired during the perinatal period and infancy on the appearance of T1D-associated autoantibodies and clinical T1D during subsequent follow-up. The CMV IgG antibodies were analysed at the time of autoantibody seroconversion or within the following six months in 169 subjects presenting with the first β-cell autoantibody by two years of age and turning positive for at least two of the autoantibodies during later follow-up. In addition, 791 autoantibody negative, clinically healthy control subjects matched for gender, age and HLA-*DQB1* genotype were analysed. At the time of the first autoantibody appearance, 22.5% of the case subjects and 26.0% of the control subjects were positive for CMV-specific autoantibodies (p=0.38 χ^2 test with continuity correction) (Study II, Table 1). In addition, no effect of the early-acquired CMV infection on the emergence of any of the autoantibodies analysed (ICA, IAA, GADA or IA-2A) could be observed (data not shown). Moreover, no difference in the prevalence of CMV antibodies could be observed among subjects with HLA DR4-DQ8 haplotype, subjects with DR3-DQ2 haplotype, and subjects heterozygous for both haplotypes (p=0.61).

In addition, the effect of CMV infection during infancy on the progression to clinical T1D was analysed. During the follow-up, the emergence of clinical T1D was enhanced among subjects without CMV antibodies, 16 (6.6%) of the 244 CMV-positive subjects developed clinical T1D compared to 74 (10.3%) of the 716 subjects without CMV-specific antibodies, although the difference remained non-significant (p=0.098, χ^2 test with continuity correction). Similarly, in the Kaplan-Meier survival analysis, the progression rate to clinical T1D did not significantly differ between the CMV-

seropositive and CMV-seronegative groups (p=0.078, Mantel Cox) (Study II, Fig 1a). Finally, the progression to clinical T1D was then analysed among the subjects positive for multiple autoantibodies. Here, 16 (42.1%) of the CMV-seropositive subjects and 74 (56.5%) of the CMV-seronegative subjects progressed to overt disease (p=0.17, χ^2 test with continuity correction) (Study II Fig 1b).

Contradictory findings on the role of CMV infection in the development of T1D have been observed (255-259). Molecular mimicry between CMV and an autoantigen significant in the autoimmune process leading to the development of T1D, GAD65, has been suggested. Jones et al have described a molecular mimicry between GAD65 and sequences of the US22 gene family encoding proteins HHLF5 and HWLF in CMV (354). These homologies were defined on the basis of linear similarities in peptide sequences, and it has not been confirmed that these sequences actually act as immunologic epitopes presented by the MHC II molecule. However, a T-cell cross-reactivity between GAD65 and a peptide from the major DNA-binding protein has been described (262). Supporting the immunologic relevance of this cross-reaction, this peptide could also be processed by the dendritic cells.

The most common transmission route for CMV infection in early childhood is thought to be breast milk. Thus, the length of breastfeeding, a suggested trigger of the development of T1D, may confound the effect of early acquired CMV infection on the T1D-associated autoimmunity. Therefore, we analysed the effect of breastfeeding on the appearance of CMV-specific IgG antibodies and autoantibodies in a subgroup of children included in the original analysis. Here, no effect of the length of breastfeeding on the autoantibody appearance was observed (data not shown). Although no significant difference in the CMV seroconversion was observed when children were categorised according to the feeding patterns, CMV infection was markedly rare in the group with a very short breastfeeding period (0-4 weeks); in this group, only one of the 11 subjects was CMV-seropositive.

According to these results, early acquired CMV infection is not associated with early humoral signs of β-cell autoimmunity. In contrast, in a group of children carrying the T1D-risk genotype and showing early positivity for T1D-associated autoantibodies, CMV infection was less prevalent among subjects progressing to overt diabetes compared to children remaining clinically healthy. These results suggest that CMV infection in infancy may in fact be protective against the development of clinical T1D among children carrying the HLA-conferred T1D risk. This finding might be related to the hygiene hypothesis. Early microbial contacts are important for the maturation of the immune system and reduced diversity of microbial contacts and lack of early immune stimulation might be related to the increased incidence of allergy and autoimmune diseases. Moreover, there is epidemiologic data showing a north-south gradient in the

T1D frequency, indicating an inverse correlation between 'hygiene' and the incidence of autoimmune diseases (and allergy) (1-5). Interestingly, an interaction of CMV and EBV infection in childhood in the formation of allergic response has been suggested (355). CMV infection during childhood was reported to enhance IgE antibody response to airborne and food allergens. However, an antagonism of EBV infection towards this phenomenon during the first years of life was observed, suggesting an interplay between these virus infections in the maturation of the immune system. CMV and EBV both cause extensive systemic immune stimulation, of which the type induced by CMV infection is mainly of Th1 (247), whereas the EBV-induced immune response seems to favour the Th2-type response including induction of IL-10 production, B-cell proliferation and polyclonal antibody production (356). According to these results, analysis of several immunologic stimuli such as co-infection with two viruses might be of interest when determining the risk factors of T1D autoimmunity.

In conclusion, our results do not suggest a role for CMV infection acquired during the perinatal period and infancy in the development of T1D-associated autoimmunity or clinical T1D among subjects with the HLA-genotype associated with increased T1D risk.

5.3. The effect of *PTPN22* C1858T polymorphism on T-cell activation

The *PTPN22* gene encodes LYP that is an inhibitor of TCR signal transduction. LYP is known to dephosphorylate the autophosphorylation sites on the protein tyrosine kinases Lck, Fyn and Zap70 (357). Interaction with CSK is shown to enhance the inhibitory function of LYP on TCR signalling (138). The *PTPN22* C1858T polymorphism changes arginine to tryptophan in codon 620 and is associated with an increased risk for the development of T1D (140-147). Importantly, this amino acid substitution occurs in the region crucial for the LYP-CSK interaction. Thus, variation in the amino acid sequence at this position may be expected to alter the inhibitory effect of LYP on TCR signalling.

In study III, the effect of the *PTPN22* C1858T polymorphism on the lymphocyte activation and responsiveness was analysed in groups of subjects with established T1D and clinically healthy controls. In addition, the effect of the 1858T disease variant on the cellular responsiveness to microbial recall antigens was analysed in a larger cohort of clinically healthy subjects positive for multiple autoantibodies associated with T1D and healthy autoantibody-negative subjects from the DIPP cohort, and in subjects with new-onset T1D.

CFSE staining was employed to evaluate the effect of the LYP 620Trp variant on the $CD4^+$ T-cell responsiveness upon anti-CD3/anti-CD28 stimulus. Interestingly, the presence of the 1858T disease allele was associated with reduced proliferation response

of $CD4^+$ cells among diabetic patients, although the difference remained non-significant (p=0.08) (Study III, Table 1, Fig 1). Similarly, among healthy controls, the $CD4^+$ T cell-responsiveness upon anti-CD3/anti-CD28 stimulation was also found to be lower among subjects carrying the T variant compared to subjects homozygous for the C variant (p=0.0006) (Study III, Table 1). Moreover, when analysing the effect of the polymorphism on the $CD4^+$ T-cell in IL-2 production after stimulation with anti-CD3/anti-CD28 beads, a decreased IL-2 production was observed among diabetic patients (trend p=0.02) (Study III, Fig 2), although no significant difference was observed when the observation points were analysed separately (mean fold change in fluorescence intensity, bead/cell ratio 0.1: 1.30 in subjects with T allele compared to 1.27 in subjects with CC genotype bead/cell ratio 0.3: 1.48 vs. 1.87 and bead/cell ratio 1.0: 1.63 vs. 2.15, respectively). However, among healthy controls, no significant difference in the IL-2 production of the $CD4^+$ cells could be observed among subjects with the 1858CT/TT or CC genotypes (trend p=0.22).

The effect of the LYP 620Trp variant on the calcium mobilisation of $CD4^+$ T cells was then analysed among subjects with established T1D. The calcium-flux of the PHA-activated $CD4^+$ cells differed significantly among subjects with the 1858TT, CT or CC genotypes (Study III, Fig 3). Here, the fold changes for the peak median values (25%, 75% quartiles) were 1.233 (1.223-1.243) in subjects with the 1858TT genotype, 1.427 (1.417-1.445) in subjects with the CT, and 1.571 (1.438-1.604) in subjects with the CC genotype. Moreover, subjects with the 1858CT or TT genotype had significantly lower peak calcium-flux values (p=0.004, comparison of fold-change), suggesting a delayed or entirely reduced TCR-mediated response of the $CD4^+$ T cells upon PHA stimulation.

The effect of the 1858T allele on the PBMC responsiveness upon stimulation with microbial recall antigens was analysed in a cohort of clinically healthy subjects with multiple T1D-associated autoantibodies, healthy autoantibody-negative controls with HLA-conferred T1D risk, and newly-diagnosed T1D patients. No effect of the 1858T allele on the spontaneous proliferation of PBMCs could be observed. Similarly, no differences in the tuberculin or tetanus-toxoid-induced PBMC proliferation were detected between the group carrying the T allele and subjects with the CC genotype (data not shown). Accordingly, the tuberculin or tetanus-toxoid-induced PBMC secretion of IL-2, IL-5, IL-10, IL-17, IFNγ and TNFα did not differ among the three groups. The effect of the presence of the 1858T allele on the PBMC activation was not affected by the HLA-*DQB1* genotype.

Finally, the effect of the 1858T disease allele on the proportion of $FoxP3^+$ $CD4^+CD25^+$ T cells among the PBMCs was analysed in subjects with established T1D. No significant difference was observed among subjects with the 1858TT, CT or CC genotype [median 5.85 (range 5.01-9.05), 6.12 (4.02-7.28) and 8.03 (6.21-9.85), respectively].

Recently, Vang et al have reported an increase in the enzymatic activity of LYP among subjects with the 620Trp substitution associated with T1D risk (151). They reported decreased calcium-flux and reduced IL-2 secretion upon anti-CD3 stimulus in human T cells carrying the disease allele. In accordance with these findings, Rieck et al have shown that the 620Trp disease variant is associated with diminished calcium mobilisation of $CD4^+$ T cells upon anti-CD3 stimulation in T1D patients and healthy controls. Interestingly, when they stimulated the $CD4^+$ T cells with ionomycin, no difference in calcium mobilisation was observed among subjects with TT or CC genotypes, indicating that the defect in the regulation of the TCR signal transduction among subjects with the 620Trp variant is in the proximal TCR signalling pathway. Moreover, they reported a decrease in the percentage of $CD4^+$ T cells expressing CD25 among subjects with the disease variant. However, the variant was not associated with alterations in proliferation responses among the groups. The IL-10 secretion of $CD4^+$ T cells upon anti-CD3/anti-CD28 stimulation was reduced among subjects with the 620Trp variant, but there was no significant effect of the variant on the secretion of IL-2, IL-4, IL-5, INFγ or TNFα. Finally, they also described altered $CD4^+$ T cell and B lymphocyte profiles in subjects with the 1858T allele: the disease allele was associated with an increased proportion of $CD4^+$ memory T cells and a decreased proportion of memory B cells. The decreased calcium mobilisation associated with the 1858T allele was also observed in the B-cell compartment, indicating the importance of the polymorphism in the lymphoid cells other than $CD4^+$ T cells.

Our findings on the role of the *PTPN22* 1858T allele on the T-cell activation and responsiveness are consistent with the results reported on the two above-mentioned studies. The disease allele seems to alter the TCR-dependent T-cell activation and to some extent also B-cell activation. However, exactly how the 620Trp disease variant predisposes to autoimmunity is currently not known. The association of the variant with several autoimmune diseases suggests that the function altered by it affects the mechanism shared among these diseases. According to the results reported by Vang et al, the variant decreases the IL-2 production of $CD4^+$ T cells (151). Similarly, we observed a diminished IL-2 production of $CD4^+$ T cell after stimulus with antiCD3/antiCD28 beads among diabetic patients, but the result in healthy controls remained non-significant. However, in contrast to the samples obtained from the diabetic patients, the samples collected from healthy controls were stored frozen before the analysis, procedure which was observed to decrease the cellular responsiveness to some extent, and may thus hamper the possibility to detect differences between the groups with different genotypes. IL-2 is shown to be crucial for the survival of regulatory T cells (358). In this respect, the effect of the variant may be related to the development or maturation of regulatory T cells, and thus affect the formation of peripheral tolerance. It is of interest to note that also another regulator of T-cell activation, *CTLA-4*, presenting with polymorphisms associated with

T1D and several other autoimmune diseases, is crucial for the normal function of the regulatory T cells (359). Thus, the gene polymorphisms in factors altering T-cell activation and responsiveness associated with a multitude of autoimmune disorders may play a role in the maturation of regulatory T cells and formation of peripheral tolerance without specificity for any single autoimmune process. In our study, we did not observe a difference in the proportion of regulatory T cells between the diabetic patients carrying different *PTPN22* C1858T genotypes. However, the number of samples studied was low and larger case-control panels are needed to explore the hypothesis.

In contrast to other findings, the proliferation response and cytokine secretion of PBMCs in response to stimulus with naturally processed antigen is not affected by the 620Trp variant. This finding may be related to the increased memory T-cell population among the variant carriers reported earlier (152). The possibly decreased antigen-specific T-cell proliferation in the PBMC culture may be compensated for by the increased proliferating memory T-cell compartment.

To summarise these results, we showed that the LYP 620Trp variant enhancing the appearance of autoantibodies and progression to clinical T1D is associated with reduced T-cell responsiveness reflected by decreased calcium mobilisation, IL-2 production, and proliferation response of the $CD4^+$ T cells. This is likely to contribute to the development of an autoimmune response leading to β-cell destruction and overt T1D.

5.4. Interplay between *PTPN22* 1858T allele and cow's milk-based formula exposure on the development of β-cell autoantibodies

Insulin is a β-cell-specific autoantigen and IAA most commonly appears as the first autoantibody in young children. Immunisation to dietary bovine insulin in CM-based formulas is suggested to be essential in the early steps of the development of insulin autoimmunity (310; 312; 360). IAA has been shown to bind to non-human insulin and a portion of IAA is of the IgA subclass, suggesting its mucosal origin (361).

Thus, the induction of insulin immunity in infancy precedes the formation of insulin autoimmunity. The *PTPN22* polymorphism has been shown to play a role especially in the formation of insulin autoimmunity (143). Moreover, the *PTPN22* 620Trp variant is shown to alter T-cell function and production of IL-2 crucial for the development of regulatory T cells. Therefore, it may alter the function of regulatory T cells and thus affect the maintenance of peripheral tolerance of β-cell autoantigens required for islet survival. In addition, the *INS* gene HphI -23 A/T polymorphism is associated with decreased transcription and expression of insulin in the thymus, a phenomenon that has been suggested to favour the escape of insulin-autoreactive T cells from the thymus (17; 110; 116; 118; 119; 362-364) suggesting that the effect of exposure to CM in infancy

may be altered by the *INS* variant. Finally, *CTLA-4* regulates the TCR-dependent T-cell activation by competing with the stimulator of TCR signal transduction, the CD28 molecule, for the signal transmitted by B7 (CD80 and CD86) in the ACP (Fig 2) (122). Thus, when activated, *CTLA-4* sends a negative signal inhibiting T-cell activation. *CTLA-4* plays a role especially in the maturation of regulatory T cells crucial for the development of tolerance. In Study IV, we aimed to analyse the effect of the *PTPN22* C1858T, *INS* gene -23 A/T and *CTLA-4* +49 A/G polymorphisms on the appearance of signs of T1D autoimmunity in regard to different exposure patterns of CM-based formula feeding.

In our prospectively followed-up cohort of children with HLA-conferred T1D risk, the *INS* -23AA genotype and *PTPN22* 1858CT and TT genotypes were associated with the appearance of autoantibodies and clinical T1D, whereas no association between the *CTLA-4* +49 GG genotype and T1D autoimmunity could be observed (Study IV, Table 1). Interestingly, the association between the *PTPN22* and *INS* gene polymorphisms and signs of autoimmunity were restricted to subjects exposed to CM-based formula nutrition before six months of age (Study IV, Figs 1 and 2). In the Kaplan-Meier survival analysis subjects carrying the 1858T allele had enhanced emergence of ICA together with any of the biochemically defined autoantibodies, for IAA, GADA, IA-2A and overt T1D if exposed to CM before the age of six months ($p<0.001$, <0.001, 0.001, <0.001, <0.001, respectively, Log Rank test), but not among the group of subjects exposed to CM later in infancy (p=0.73, 0.85, 0.84, 0.26 and 0.33, respectively). Moreover, Cox-regression analysis revealed an interaction between the *PTPN22* 1858T allele and early CM exposure on the appearance of ICA, IAA or IA-2A (for the interaction term p=0.03, 0.04 and 0.02, respectively) (Study IV, Table 2). When analysing the effect of *INS* gene polymorphism on the appearance of ICA, IAA, GADA, IA-2A and clinical T1D, the effect of this polymorphism on the emergence of autoimmunity differed similarly to that of *PTPN22* C1858T polymorphism according to the CM exposure patterns in infancy. In the survival analysis, the autoantibodies and clinical disease appeared more frequently among the carriers of the *INS* -23 AA genotype in the early CM exposure group (p=0.02, 0.02, 0.01, 0.04 and 0.006, respectively), whereas no significant difference was observed in the group exposed to CM later in infancy (p=0.38, 0.32, 0.91, 0.83, 0.22, respectively). However, in Cox regression analysis, there was no significant interaction between the AA genotype and CM exposure. *CTLA-4* +49 A/G polymorphism did not show an association with T1D autoimmunity or overt T1D in any of the nutritional groups. In accordance with this finding, the effect of this polymorphism on the T1D risk is weak in populations of Northern European origin although it seems to provide a clear contribution in other populations (365).

The effect of the CM exposure on the appearance of humoral signs of T1D and overt diabetes was then analysed separately among subjects carrying the 1858TT and CT

genotypes, and subjects with the 1858CC genotype. Interestingly, the early exposure to CM-based formula seemed to be a risk factor for enhanced appearance of ICA with any of the biochemically defined autoantibodies, for IAA or IA-2A, among subjects with the T allele (p=0.03, 0.03, 0.02, respectively, Log Rank test) (Study IV, Fig 3). In contrast, no effect of early CM exposure on the emergence of signs of β-cell autoimmunity was observed among subjects homozygous for the 1858CC genotype.

Bovine insulin-binding IgG class antibodies were analysed in this cohort at the age of 3, 6, 12, 18 and 24 months. CM exposure before three months of age was shown to enhance the insulin-specific antibody response at three months of age ($p<0.001$, MWU-test), and the insulin antibody levels were higher over the total observation period (p=0.007, ANOVA for repeated measurements). Bovine insulin-binding antibody levels were higher among subjects turning multiple autoantibody-positive during the subsequent follow-up compared to subjects remaining autoantibody-negative ($p<0.001$, ANOVA for repeated measurements).

Finally, we analysed the effect of the three genetic polymorphisms on the bovine insulin-binding antibody levels during the first two years of life. The bovine insulin antibody levels did not differ between subjects carrying the *INS* -23 HphI AA genotype compared to subjects with the AT or TT genotype (p=0.49, ANOVA for repeated measurements), and the result remained non-significant when the two dietary groups were analysed separately (p=0.29 and 0.11 for CM exposure before six months of age and CM exposure later in infancy, respectively). Similarly, the *CTLA-4* +49 A/G polymorhism did not affect the bovine insulin-binding antibody levels during the period analysed (data not shown). In contrast, the *PTPN22* 1858T allele was associated with elevated bovine insulin antibody levels over time compared to subjects with the 1858CC genotype (p=0.02, ANOVA for repeated measurements) (Study IV, Fig 4a). Moreover, this finding was restricted to subjects exposed to CM before six months of age (p=0.001) (Study IV Fig 4b), whereas no effect of the gene polymorphism on the antibody levels could be observed among subjects exposed to CM later in infancy (p=0.77) (Study IV, Fig 4c).

The effect of the *PTPN22* C1858T polymorphism on the enhanced emergence of T1D has been shown in several populations (140-143; 145-147). Moreover, *PTPN22* has been shown to be involved primarily in the regulation of insulin autoimmunity (143). Dietary insulin has been shown to induce insulin-specific tolerance; this process is affected by the form of dietary insulin, and the tolerance induction fails in some cases, leading to excess immunoresponse towards dietary bovine insulin (310; 312; 313). The failure in the formation of tolerance towards dietary insulin is thought to be a risk for the development of immune responsiveness towards autologous insulin. According to our results, the *PTPN22* 1858T allele is a risk factor for the development of β-cell autoimmunity only among subjects exposed to CM formula before the age

of six months. Early CM exposure was also found to be associated with enhanced humoral β-cell autoimmunity among subjects carrying the 1858T allele. Finally, the disease allele was associated with enhanced antibody response towards bovine insulin; this finding was also restricted to subjects exposed to CM formula in early infancy. These findings support the role of the *PTPN22* disease allele in the formation of insulin autoimmunity. Moreover, they suggest that the development of tolerance in the maturating gut is affected by the functional changes caused by the disease variant. As shown earlier, cow's insulin in CM-based formula triggers immunity towards insulin in a large majority of cases, finally inducing tolerance. Our results suggest that among subjects with predisposing factors, e.g. the *PTPN22* disease allele affecting the cellular immune responses, the exposure to oral insulin may fail to induce the tolerance towards nutritional insulin but may lead to deranged immunity characterised by insulin autoantibodies, autoantibodies to multiple islet antigens by antigenic spreading and immune attack on pancreatic ß-cells.

The *INS* gene polymorphism has been shown to be associated with decreased insulin expression in the thymus (17; 110; 116; 118; 119; 362-364). In the thymus, negative selection deletes autoreactive T cells and it has been suggested that the *INS* gene HphI -23 AA genotype leading to low thymic expression of insulin would lead to the escape of insulin-autoreactive T cells (119). Alternatively, the reduced insulin expression may also affect the development of regulatory T cells in the thymus. Interestingly, in this cohort the enhancing effect of the *INS* polymorphism on the appearance of T1D autoimmunity was observed only among subjects exposed to CM before six months of age. This finding may be related to the *INS*-related increased presence of insulin-specific autoreactive T cells in the periphery, the effect of which may be more critical in early infancy, but the finding requires further investigation.

These results suggest an interplay between genetic and nutritional factors in the initiation of β-cell-specific autoimmunity leading to overt T1D. Moreover, the findings indicate that the effect of environmental factors on the disease process is dependent on the genetic background of the individual. The results suggest that not all environmental risk factors may be involved in the disease process in all cases, as is also supported by the individual variety of β-cell-specific autoantibodies, their combinations and order of appearance. Finally, the results provide an explanation for the contradictory findings on the role of CM-based formula exposure and the length of breastfeeding in the emergence of T1D autoimmunity. Early CM exposure seems to act as a risk factor for β-cell autoimmunity among subjects carrying the *PTPN22* 1858T allele and probably to some extent also among subjects with the *INS* gene -23 AA genotype. Interestingly, the frequency of both the *PTPN22* and *INS* gene risk alleles is particularly high in Finland compared to other European populations (143; 336).

5.5. Interaction between cow's milk-based formula nutrition in early infancy and enteral virus infections during the first year of life and their effect on the development of T1D-associated autoimmunity

In addition to genetic risk factors and CM exposure in infancy, viral infections have also been suggested to be involved in the initiation of T1D autoimmunity. An association between early enteral virus infection and elevated bovine insulin-binding antibody levels has recently been reported by our group (235). This effect of enteral virus infection on the formation of bovine insulin-specific immunity was more common among subjects exposed to CM-based formula during early infancy suggesting that the effect of these triggering factors may depend on each other in the induction of islet cell autoimmunity. Thus, in Study V, our purpose was to confirm this finding in a larger cohort and to explore the combined effect of early acquired enteral virus infection and CM formula exposure on the development of T1D-associated autoimmunity.

We analysed the effect of enterovirus, rotavirus, adenovirus and RSV infection by six or 12 months of age on the bovine insulin-binding IgG class antibodies at six, 12, 18 and 24 months. In the study group, 15 subjects with enterovirus, 28 subjects with rotavirus, 13 subjects with adenovirus and 42 subjects with RSV infection were detected by six months of age. By the age of 12 months 83, 97, 68 and 152 subejcts had signs of these infections, respectively. Among subjects with enterovirus, rotavirus or adenovirus infection by six months of age the bovine insulin-binding IgG antibodies were higher over the observation period ($p=0.01$, ANOVA for repeated measurements) and at the age of six months when each time point was analysed separately ($p<0.001$, MWU-test) (Study V, Fig 1a). The occurrence of any of these enteral virus infections by 12 months of age did not alter the bovine insulin antibody levels at subsequent follow-up. When the virus infections were analysed separately, a tendency for elevated bovine insulin antibody levels was observed throughout the subsequent follow-up period after rotavirus infection by six months of age ($p=0.03$) and at the age of six, 12 and 18 months when the time points were analysed separately ($p=0.02$, 0.09 and 0.05, respectively) (Study V, Fig 1b). Rotavirus infection acquired by 12 months of age was also associated with a tendency for elevated antibody levels during the rest of the follow-up ($p=0.05$), and also at 12 and 18 months when observation points were analysed separately ($p=0.09$ and 0.03, respectively). In addition, adenovirus infection before six months of age was associated with a trend for higher antibody titers over time ($p=0.06$) and at six and 12 months of age when analysed separately ($p=0.003$ and 0.06, respectively) (Study V, Fig 1c) but no effect of adenovirus infection before 12 months of age on the antibody levels was detected. When the effect of enterovirus or RSV infection by six or 12 months of age was analysed no effect on the bovine insulin-binding antibody levels was observed (Study V, Fig 1 d and e).

The effect of these viral infections on the appearance of autoantibodies associated with T1D and progression to clinical T1D was analysed in the cohort. We observed no effect of enterovirus, adenovirus or RSV infection by six or 12 months of age on the appearance on humoral signs of β-cell autoimmunity or on T1D (Study V, Table 1). Moreover, we observed no effect of CM-based formula exposure before three months of age on the appearance of T1D-associated autoimmunity or clinical disease (data not shown). On the contrary, our results revealed a trend for an enhanced appearance of ICA with any of the biochemically defined autoantibodies, for GADA and clinical T1D among subjects with rotavirus infection by six months of age (p=0.05, 0.04 and 0.09, Log Rank test, Kaplan-Meier survival analysis) (Study V, Table 1). Rotavirus infection during later infancy did not alter the emergence of signs of autoimmunity.

We proceeded to define the effect of the various viral infections combined with the nutritional patterns in infancy. The enhancing effect of early-acquired rotavirus infection on the appearance of T1D autoimmunity was restricted to subjects exposed to CM formula before three months of age (Study V, Table 2). However, Cox regression analysis did not show significant interaction between early rotavirus infection and CM formula exposure before three months of age. In addition, none of the other virus infections acquired by six months of age showed an interaction with CM exposure. Interestingly, when making this comparison at the age of 12 months, we observed an enhancing effect of enterovirus infection on the appearance of T1D-associated autoantibodies in subjects exposed to CM formula nutrition before three months of age (p=0.001, <0.001, <0.001 and 0.001 for the appearance of ICA with any of the biochemically defined autoantibodies, for IAA, GADA and IA-2A respectively, Log Rank test) (Study V, Table 3). On the other hand, no significant association between these environmental factors was observed in the group of subjects exposed to CM later in infancy (p=0.08, 0.08, 0.10, 0.42, respectively). Moreover, the predisposing effect of enterovirus infection was strongest among subjects who experienced the infection at six to 12 months of age compared to subjects with the infection before six months of age and subjects without signs of enterovirus infection during the first year of life (p<0.001, 0.001, <0.001, <0.001 and 0.09 for the appearance of for the appearance of ICA with any of the biochemically defined autoantibodies, for IAA, GADA, IA-2A and clinical T1D, respectively, Log Rank test). The CM-based formula exposure did not clearly alter the effect of adenovirus, RSV or rotavirus infection before 12 months of age on the appearance of signs of autoimmunity (Study V, Table 3).

In accordance with these findings, Cox Regression analysis for the combined effect of early exposure to CM-based formula and enterovirus infection during the first year of life revealed a significant interaction between these two factors on the formation of signs of β-cell autoimmunity [for ICA with any of the biochemically defined autoantibodies p=0.001, hazard ratio (HR) for interaction term 7.4, 95% CI 2.2-25.4,

for IAA p=0.002, HR 9.5, 95% CI 2.3-38.4, for GADA p=0.001, HR 9.7, 95% CI 2.5-38.2, for IA-2A p=0.013, HR 5.2, 95% CI 1.4-18.7]. However, the effect on progression to clinical T1D remained non-significant (p=0.15, HR 3.8, 95% CI 0.60-24.1).

Results on the role of enterovirus infection in the early stages of β-cell autoimmunity have been contradictory. Enterovirus infections have been suggested to be involved in the pathogenesis of T1D autoimmunity in several studies both at the time when T1D-associated autoantibodies appear for the first time and at the onset of clinical disease (185-187; 196; 199; 200), but several studies have also failed to find an association between these infections and T1D autoimmunity (189; 201; 202; 233). Interestingly, a persistent or recurrent enterovirus infection in gut mucosa has been described recently among subjects with clinical T1D, suggesting a reduced clearance of enterovirus among diabetic patients (212). Rotavirus infection has also been temporally associated with increases in β-cell autoantibodies in an Australian study (233), but no association between rotavirus infection and development of T1D-autoimmunity was observed in a Finnish prospective cohort (234). However, enteral virus infections, especially rotavirus infections enhance the humoral immune response towards bovine insulin, suggesting a role for the infection in the induction of insulin immunity (235). In addition, the timing of rotavirus infection is shown to be essential for the effect on the β-cell autoimmunity (366; 367).

The role of early exposure to CM-based formula in infancy and the effect of extended breastfeeding have been frequently debated, but the findings in studies in various populations have been contradictory, so the role of CM remains unclear. Association between CM exposure or short exclusive breastfeeding and emergence of T1D-autoimmunity have been observed in several studies (268; 274; 277; 298; 303), but numerous other studies have concurrently reported absence of the relation between these triggering factors and T1D (175; 292; 301; 302; 368). However, a prospective intervention study among newborns in families with a member with T1D and carrying the HLA-conferred T1D risk revealed a protective effect of hydrolysed casein formula on the appearance of T1D-associated autoantibodies compared to conventional CM-based formula nutrition (303).

Our results suggest an interplay between CM formula nutrition and enteral virus infections, enterovirus and rotavirus in early life, as revealed by specific antibody data. Rotavirus infection during the first six months of life was associated with enhanced emergence of T1D autoimmunity among subjects with early introduction of CM-based formula nutrition. This finding is likely to be explained by the increase in the permeability of the gut mucosa, leading to increased absorption of and enhanced immunity to food proteins (369), a phenomenon which may facilitate the generation of autoimmunity towards autologous insulin. This mechanism in the autoimmunity

process seems to differ from the role of enterovirus infection in the pathogenesis of T1D since enterovirus infection is not known to affect the gut permeability and, in our results, no effect of the enterovirus infection on bovine insulin-specific humoral immunity was observed. However, humoral response towards bovine insulin has been shown to be enhanced by enterovirus-specific cellular responses (311).

According to these results, enterovirus infection during the first year of life does enhance the appearance of T1D-associated autoimmunity among subjects carrying the HLA-*DQB1* genotype associated with increased T1D risk, but only if another triggering factor, exposure to CM formula in early infancy, is present. This result suggests a more complex role for enterovirus infection in the pathogenesis of autoimmunity in affected subjects.

A bystander effect of coxsackievirus infection in the development of autoimmune diabetes has been described (38). According to the results obtained by a mouse model carrying a diabetogenic T-cell receptor specific for islet granule other than GAD65, autoimmune diabetes induced by CBV4 infection is a result of local infection in pancreatic islets. The authors suggested that the resting autoreactive T cells are activated by islet antigens released due to tissue damage during the infection, eventually leading to ultimate destruction of β cells. Similar induction of β-cell destruction was observed in this mouse model when the islet damage was induced by streptozotocin (40), indicating the necessity and non-specificity of the lytic process in islets during the enterovirus infection triggering β-cell autoimmunity. Moreover, CBV4 infection is shown to produce this bystander effect only if a threshold level of autoreactive T cells has accumulated (225). This finding suggests that the timing of the infection is critical for the role of the infection in the pathogenesis of T1D. Interestingly, the results indicated that if the infection occurred before the critical autoreactivity has developed, the CBV4 infection may in fact block the formation of autoimmunity through a non-specific immunostimulatory mechanism. Moreover, the role of macrophages in the pathogenesis of T1D has been shown (39). After CBV4 infection, APCs harbour β-cell specific antigens and APCs isolated from an infected mouse are shown to transfer the destruction of β cells and the development of autoimmune diabetes into a non-infected mouse with the diabetogenic T-cell receptor.

We observed an enhancing effect of enterovirus infection during the first year of life, and to some extent, of rotavirus infection before six months of age among subjects carrying HLA-conferred T1D risk. These findings were restricted to subjects exposed to CM-based formula in early infancy. This observation suggests a more complex pathway of the environmental factors in the initiation of T1D-associated autoimmunity. In the case of enteroviruses, the infection of pancreatic islets may trigger the risk for β-cell autoimmunity in susceptible individuals if the necessary predisposition to a primary antigen and the formation of autoreactive T cells exists.

It has to be noted that we did not observe a significant effect of the two risk factors in the formation of clinical T1D. However, the trend observed between the two triggering factors and progression to clinical disease was similar to the appearance of autoantibodies, so the finding is likely to be explained by the lower number of cases, and the longer follow-up time needed to detect the significant effect on the progression to clinical disease.

5.6. General discussion

The development of T1D is a complex process affected by several different factors, genetic and environmental, during different stages of the disease pathogenesis. The genetic factors known so far are mainly those affecting the immunological responses induced by foreign antigens and self-antigens and, particularly in the case of HLA genes, the peptide patterns presented by APCs. Genetic factors are also shown to alter the formation of immune responses. In addition, insulin gene polymorphism associated with enhanced appearance of T1D autoimmunity is associated with altered expression of insulin in the thymus, leading to impaired formation of central tolerance towards insulin.

A rapid increase in the disease frequency has been observed during recent decades, but still only a small proportion of subjects at genetic risk of T1D present with clinical disease. Thus, the role of environmental factors in the disease pathogenesis is crucial. However, the findings on the effect of environmental factors on disease appearance between population and also within populations have been contradictory. Enterovirus infection and early cow's milk formula exposure or short breastfeeding have been repeatedly observed to enhance the development of T1D-associated autoimmunity or overt disease, but also negative findings have been reported. This phenomenon suggests variability in the effect of specific triggering factors on populations with different genetic backgrounds. Moreover, the effect of various environmental risk factors may be modified by other factors in the environment varying between populations and within populations.

Studies on monozygotic twins have proposed a differing significance of genetic susceptibility on the disease emergence depending of the age at which the individual presents with the disease (370). If the first subject develops T1D by five years of age the risk for T1D in the second twin is 50%. In contrast, if T1D emerges after the age of 25 in the first subject, the subsequent T1D risk for the second twin is 5%. Thus, the significance of the genetic susceptibility seems to be higher among subjects developing the disease during the early years, whereas the genetic predisposition remains less prominent when the disease develops later in life. In addition, the differences in the allele frequencies of

predisposing genetic polymorphisms between different populations indicate variability in the predisposing factors between populations.

Our findings suggest an interplay between genetic and environmental factors and also between different environmental factors in the development of β-cell autoimmunity and clinical T1D among subjects emerging with autoimmunity during childhood (Fig 4). The results indicate that exposure to cow's milk formula modifies the T1D risk associated with the *PTPN22* 1858T allele and that exposure to cow's milk formula in infancy may be a risk factor for the development of β-cell autoantibodies. However, this finding was observed in a specific subgroup of subjects with HLA-conferred T1D risk and carrying *PTPN22* T allele predisposing to T1D, whereas CM exposure may lack the predisposing effect in the absence of this genetic susceptibility. How the 1858T allele increases T1D risk seems to be related to the TCR-dependent activation of T cells. *PTPN22* encodes LYP, an important regulator of T-cell activation and the *PTPN2*2 C1858T polymorphism leads to a substitution of arginine by tryphophan in codon 620 at the active site of the phosphatase. The effect of this substitution was shown to lead to impaired activation, proliferation and IL-2 production of $CD4^+$ T cells. Moreover, the finding on the interaction between CM exposure and *PTPN22* C1858T polymorphism is of particular interest since the polymorphism has been previously shown to contribute most strongly to the development of insulin autoimmunity (143).

An interaction between two environmental factors, early CM exposure and enterovirus infection during early life was observed among subjects with HLA-confirmed T1D risk. This finding proposes a more complex interplay in the role of environmental factors for the development of autoimmunity, and indicates the need for combined analysis of multiple factors in the development of T1D. The results suggest that exposure to CM serves as a primary trigger for β-cell autoimmunity but the ultimate β-cell destruction occurs if another predisposing factor, enterovirus infection, occurs during early life. Importantly, the increased intestinal permeability observed in a mouse model and among patients with T1D (76-81) suggests an altered exposure of intestinal immune cells to dietary insulin that may lead to impaired induction of tolerance towards dietary insulin and, ultimately, to a breakdown in tolerance to autologous insulin. In addition, a persistent enterovirus infection in pancreatic islets is suggested to be a relatively common phenomenon, but the infection seems to fail to induce β-cell destruction in the majority of subjects (371). According to our findings, not only the presence or absence of the enterovirus infection is essential for the disease pathogenesis, but also the timing of the infection related to other triggering factors seems to be crucial for the outcome. A similar time-dependent variability in the effect of enterovirus infection on the development of diabetes has been reported earlier in a mouse model (225). Our findings suggest that at least on early age, enterovirus infection enhances the existing β-cell specific autoimmunity induced by early exposure to CM.

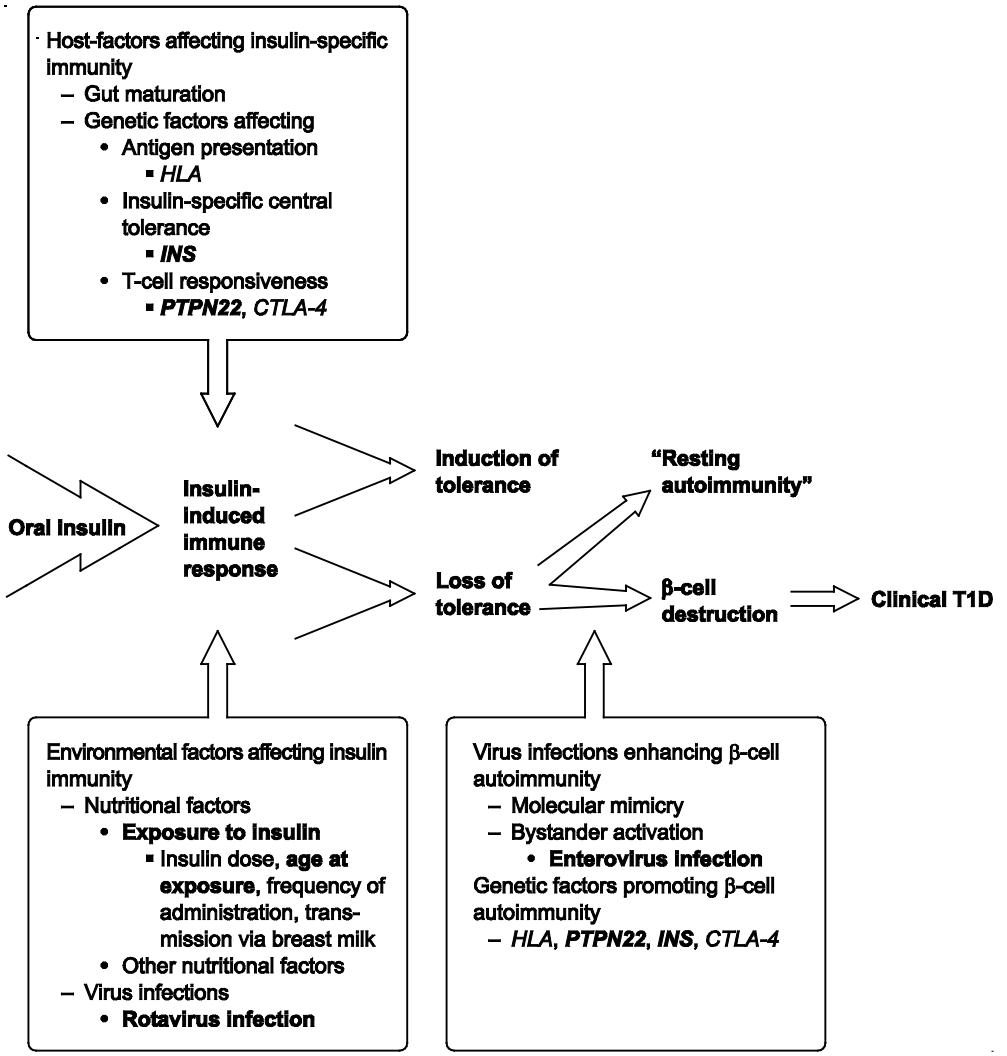

Figure 4. Schematic figure of the role of genetic and environmental factors in the pathogenesis of T1D.

We failed to detect an association between CMV infection during infancy and early appearance of β-cell autoimmunity or subsequent development of T1D among subjects with HLA-conferred T1D risk. However, an association between CMV infection and emergence of autoimmune diabetes in adult patients has recently been reported (260; 261). Interestingly, the effect of the HLA risk defined by traditional means on the development of T1D decreases with age, and, thus the risk factors at an older age seem to differ from those observed at a young age. Moreover, GAD-specific autoantibodies are the most common autoantibodies in newly-diagnosed T1D patients progressing to clinical disease at teen age or later in life, suggesting the increasing importance of GAD autoimmunity in these subjects (372). Thus, it can be hypothesised that although no effect of early acquired CMV infection was observed in our material, the role of GAD-

specific autoimmunity and the role of CMV infection as a triggering factor may be more relevant for autoimmunity appearing at an older age.

Our results indicate the diversity of the genetic and environmental factors in the development of T1D-associated autoimmunity. Both gene-environment interaction and the interplay between different environmental factors were observed in the studies performed. The results suggest that not only the presence or absence of a potential trigger of autoimmunity is crucial, but the consequence of the trigger is affected by the timing of the exposure and by the genetic background of the individual. Thus, to obtain more definitive results, a large-scale cross-analysis of the various environmental triggers and genetic factors in a long-term follow-up cohort is required.

6. CONCLUSIONS

I. In early childhood, the Herpes group virus infections, especially CMV and VZV infections, are relatively common. The prevalence of CMV infection has remained constant or even increased in Finland during the last few decades. During the early years of life, the infection is most commonly acquired in infancy indicating the essential role of breastfeeding in the virus transmission. In our material, the frequency of CMV infection during the perinatal period and early infancy was 27%. The frequency of VZV infections in childhood is particularly high in Finland compared to other Western populations, the vast majority of children being affected by school age. In contrast, the occurrence of HSV infection has decreased among children, suggesting a delayed time-point of primary infections.

II. CMV infection acquired during the perinatal period and infancy is not a risk factor for the development of T1D-associated autoantibodies or clinical disease in Finland among subjects with HLA-*DQB1*-genotypes conferring the T1D risk.

III. The *PTPN22* 1858T allele encoding an Arg to Trp substitution on LYP, an important regulator of T-cell activation, is associated with decreased calcium mobilisation, IL-2 production and proliferation response of $CD4^+$ T cells, suggesting an altered immune response upon T-cell activation in affected individuals.

IV. The *PTPN22* 1858T allele is associated with the development of T1D-associated autoantibodies and clinical T1D only in subjects exposed to cow's milk based-formula nutrition before six months of age suggesting an interplay between these factors in the initiation of T1D autoimmunity. Thus, cow's milk formula nutrition may be a risk factor for T1D-associated autoimmunity among carriers of the 1858T allele. Moreover, the results indicate that the effect of various environmental factors on the pathogenesis of T1D may be altered by the genetic background of the individual.

V. Enterovirus infection and probably also rotavirus infection during infancy are a risk factors for T1D-associated autoimmunity in subjects exposed to cow's milk-based formula nutrition in early infancy. These results suggest an interaction of two environmental factors in the development of T1D. Furthermore, the results provide an explanation for the contradictory findings on the role of one of these factors in the initiation of autoimmunity.

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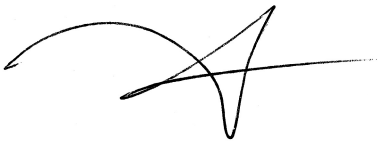

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