NATIONAL INSTITUTE
FOR HEALTH AND WELFARE

RESEARCH

Kristiina Luopajärvi

The Development of Immune Responses and Gut Microbiota in Children at Genetic Risk of Type 1 Diabetes

RESEARCH 90

Kristiina Luopajärvi

The development of immune responses and gut microbiota in children at genetic risk of type 1 diabetes

ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Medicine, University of Helsinki, in the Niilo Hallman Auditorium of the Children's Hospital, Stenbäckinkatu 11,
on November 2nd, 2012, at 12 noon

Immune Response Unit, Department of Vaccination and Immune Protection,
National Institute for Health and Welfare, Helsinki, Finland
and
Children's Hospital, University of Helsinki, Finland

Helsinki 2012

NATIONAL INSTITUTE
FOR HEALTH AND WELFARE

ISBN 978-952-245-740-0 (printed)
ISSN 1798-0054 (printed)
ISBN 978-952-245-741-7 (pdf)
ISSN 1798-0062 (pdf)
http://urn.fi/URN:ISBN 978-952-245-741-7

Juvenes Print – Finlands University Print
Tampere, Finland 2012

Supervisors
Professor Outi Vaarala, MD, PhD
Immune Response Unit
Department of Vaccination and Immune Protection
National Institute for Health and Welfare
Helsinki, Finland

Emeritus Professor Hans K. Åkerblom, MD, PhD
Children's Hospital
University of Helsinki
Helsinki, Finland

Reviewers
Docent Jussi Kantele, MD, PhD
Department of Medical Microbiology and Immunology
University of Turku
Turku, Finland

Docent Aaro Miettinen, MD, PhD
Department of Bacteriology and Immunology
University of Helsinki
Helsinki, Finland

Opponent
Docent Arno Hänninen, MD, PhD
Department of Medical Microbiology and Immunology
University of Turku
Turku, Finland

To my family

ABSTRACT

Kristiina Luopajärvi. The development of immune responses and gut microbiota in children at genetic risk of type 1 diabetes. National Institute for Health and Welfare (THL). Research 90. 148 pages. Helsinki, Finland 2012.
ISBN 978-952-245-740-0 (print); ISBN 978-952-245-741-7 (pdf)

Type 1 diabetes (T1D) is an autoimmune disease that results from the destruction of insulin-producing pancreatic β-cells caused by attack from the body's own immune system. The exact disease pathogenesis is unknown, but genetic and environmental factors have a significant effect on the development of the disease. The aim of this study was to determine how the immune system develops in children with a T1D-associated genetic risk.

We compared the differentiation of cord blood T cells cultured in type 1 and type 2 cytokine environments between infants with a diabetes-associated HLA risk genotype and infants without a risk genotype. In infants with a diabetes-associated HLA risk genotype, the expression of transcription factor GATA-3 and chemokine receptor CCR4 was reduced in T cells differentiated in a type 2 cytokine environment. Thus, infants with a T1D-associated risk genotype may develop an aberrant immune response to environmental factors. This may indicate susceptibility to developing a more prounounced cytotoxic immune response locally in tissues such as the pancreatic islets.

We examined the early development of the antibody response to cow's milk proteins in children with a diabetes-associated HLA risk genotype. IgG-class antibodies to beta-lactoglobulin at 6 months of age and IgA antibodies to cow's milk-based infant formula at 9 months of age were enhanced in those children who later progressed to T1D as compared with children who remained healthy. The results indicate that children who progress to diabetes have a stronger antibody response to cow's milk proteins during the first year of life than children without signs of beta-cell autoimmunity, whereas no differences were observed in the responses to tetanus toxoid. An increased immune response to antigens encountered in the intestinal immune system may already reflect T1D-associated alterations in the gut immune system during early life. The results may suggest an early dysregulation of oral tolerance in children who later progress to T1D.

Previous epidemiological studies have demonstrated a reduced risk of T1D in the offspring of mothers with T1D when compared with children of affected fathers. We examined whether exposure of offspring to maternal insulin therapy *in utero* induces insulin-specific regulatory mechanisms. In cord blood the expression of transcription factor FOXP3 in regulatory T cells was higher in the offspring of mothers with T1D than in infants of unaffected mothers. After *in vitro* insulin stimulation, the expression of FOXP3 in $CD4^{+}CD25^{+}$ regulatory T cells, and up-regulation of immunological response related genes (FOXP3, IL-10, TGF-β, NFATc2 and STIM1) was only

increased in the offspring of mothers with T1D. The results suggest that maternal insulin therapy specifically increases regulatory T cell activation and the development of tolerance to insulin in the fetus. This may explain the lower risk of diabetes in children with maternal vs. paternal diabetes.

We investigated the composition of the intestinal microbiota in fecal samples from children with T1D-related autoantibodies and from autoantibody-negative children. An increased abundance of the genus *Bacteroides* and low abundances of *Bifidobacteria* and butyrate-producing species were found in the children with T1D-related autoantibodies. In this study we also confirmed previous research indicating that breastfeeding and the age of the child additionally influenced the composition of the gut microbiota.

In this thesis study, we found several indications that abnormalities in the intestinal immune system, such as an enhanced immune response to dietary protein as well as imbalanced gut microbiota, are associated with the development of T1D. In addition, we found that maternal insulin treatment appears to modify the regulatory T cells and insulin tolerance in the fetus.

Keywords: antibodies to cow's milk, cord blood, insulin, microbiota, regulatory T cells, type 1 diabetes

TIIVISTELMÄ

Kristiina Luopajärvi. Immunivasteen ja suoliston mikrobiflooran kehittyminen lapsilla, joilla on perinnöllinen alttius sairastua tyypin 1 diabetekseen. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 90. 148 sivua. Helsinki, Finland 2012.
ISBN 978-952-245-740-0 (painettu); ISBN 978-952-245-741-7 (pdf)

Tyypin 1 diabetes on autoimmuunisairaus, joka syntyy haiman insuliinia tuottavien β-solujen tuhouduttua elimistön oman immuunipuolustusjärjestelmän hyökkäyksen seurauksena. Taudin tarkkaa syntymekanismia ei tiedetä, mutta perimällä ja ympäristötekijöillä on merkittävä vaikutus taudin kehittymiseen. Tutkimuksen tarkoituksena oli selvittää, miten immuunijärjestelmä kehittyy lapsilla, joilla on tyypin 1 diabetekseen liittyvä riskiperimä.

Tutkimuksessa selvitettiin, eroaako napaveren T-solujen erilaistuminen ns. tyypin 1 ja tyypin 2 sytokiiniympäristössä lapsilla, joilla on diabetekseen liittyvä HLA-riskiperimä verrattuna lapsiin, joilla ei ole tällaista riskiperimää. T-solujen erilaistuminen tyypin 2 sytokiiniympäristössä poikkesi ns. riskilapsilla siten, että GATA-3 transkriptiofaktorin ja CCR4 kemokiinireseptorin ilmentyminen jäi alhaiseksi. Tyypin 1 diabeteksen riskiperimän omaavan lapsen immuunivaste ympäristötekijöitä kohtaan voi olla näin ollen poikkeava. Tämä voi merkitä alttiutta kehittää voimakas sytotoksinen immunoaktivaatio paikallisesti kudoksessa kuten haiman saarekkeissa.

Tutkimuksessa selvitettiin varhaista vasta-aineiden kehittymistä lehmänmaidon proteiineja kohtaan lapsilla, joilla on diabeteksen riskiperimä. IgG-luokan vasta-aineet beta-laktoglobuliinia kohtaan 6kk iässä ja IgA-luokan vasta-aineet lehmänmaitopohjaisen äidinmaidonkorvikkeen proteiineja kohtaan 9kk iässä olivat koholla lapsilla, jotka sairastuivat myöhemmin diabetekseen verrattuna lapsiin, jotka pysyivät terveinä. Tulokset osoittavat, että lapsilla, jotka sairastuvat myöhemmin diabetekseen, on poikkeavan voimakas vasta-ainemuodostus ensimmäisen elinvuoden aikana lehmänmaidon proteiineja kohtaan, mutta ei rokotuksena annettavaa tetanus toksoidia kohtaan, verrattuna autovasta-aine negatiivisiin lapsiin. Lisääntynyt immuunivaste suoliston immuunijärjestelmän kautta tulevia antigeeneja kohtaan voi heijastaa varhaista merkkiä diabetekseen liittyvästä suolen puolustusjärjestelmän häiriöstä jo imeväisiässä. Tulokset viittaavat oraalisen toleranssin häiriöön diabetekseen sairastuvilla lapsilla ennen varsinaista taudin puhkeamista.

Aikaisempien epidemiologisten tutkimusten perusteella on havaittu, että lapsilla, joiden äidillä on diabetes, on alhaisempi riski sairastua diabetekseen kuin diabetesta sairastavien isien lapsilla. Tutkimuksessa selvitettiin, miten sikiöaikainen äidin insuliinihoito vaikuttaa lapsen insuliinispesifisen immuunivasteen säätelyyn. Napaveressä transkriptiotekijä FOXP3:n ilmentyminen regulatorisissa T-soluissa oli suurempi vastasyntyneillä lapsilla, joiden äidillä oli tyypin 1 diabetes verrattuna lapsiin, joiden äidillä ei ollut tyypin 1 diabetesta. Lisäksi insuliinistimulaation jälkeen havittiin diabeetikon lapsen lymfosyyteissä enemmän transkriptiotekijä

FOXP3:n ilmenemistä $CD4^+CD25^+$ soluissa ja immunivasteen aktivaatioon liittyvien geenien (FOXP3, IL-10, TGF-β, NFATc2 ja STIM1) esiintymistä. Tulokset viittaavat siihen, että äidin insuliinihoito lisää regulatoristen T-solujen aktivaatiota spesifisesti ja lapselle kehittyy toleranssi insuliinia kohtaan jo sikiökaudella. Tämä voi selittää sen, että jos äidillä on diabetes, lapsen riski sairastua diabetekseen on pienempi kuin jos esim. isällä on diabetes.

Olemme tutkineet suoliston mikrobiflooran koostumusta ulostenäytteistä lapsilla, joille on ilmaantunut tyypin 1 diabetekseen liittyviä autovasta-aineita sekä lapsilla, joilla ei ole autovasta-aineita. Tutkimuksen perusteella *Bacteroides*-lajia esiintyy enemmän autovasta-aine positiivisilla lapsilla, kun taas tiettyjen *Bifidobakteerien* sekä voihappoa tuottavien bakteerikantojen määrä on alhaisempi. Vahvistimme myös aikaisemmat tutkimukset, joiden mukaan rintaruokinta ja lapsen ikä vaikuttavat myös normaalin bakteeriflooran kehittymiseen.

Väitöskirjatyössä löysimme useita viitteitä siitä, että suoliston puolustusjärjestelmän poikkeavuudet, kuten voimakas varhainen immuunivaste ravinnon proteiineja kohtaan sekä suoliston mikrobiston epätasapaino, liittyvät tyypin 1 diabeteksen kehittymiseen. Lisäksi havaitsimme, että sikiöaikana äidin insuliinihoito näyttää muokkaavan regulatorisia T-soluja ja niiden toleranssia insuliinia kohtaan.

Avainsanat: lehmänmaitovasta-aine, napaveri, insuliini, mikrobifloora, regulatoriset T-solut, tyypin 1 diabetes

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

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

I Luopajärvi K, Skarsvik S, Ilonen J, Åkerblom HK, Vaarala O. Reduced CCR4, interleukin-13 and GATA-3 up-regulation in response to type 2 cytokines of cord blood T lymphocytes in infants at genetic risk of type 1 diabetes, *Immunology*, 121(2), 189-196, 2007.

II Luopajärvi K, Savilahti E, Virtanen SM, Ilonen J, Knip M, Åkerblom HK, Vaarala O. Enhanced levels of cow's milk antibodies in infancy in children who develop type 1 diabetes later in childhood, *Pediatr Diabetes*, 9(5), 434-441, 2008.

III Luopajärvi K, Nieminen JK, Ilonen J, Åkerblom HK, Knip M, Vaarala O. Expansion of CD4+CD25+FOXP3+ regulatory T cells in infants of mothers with type 1 diabetes, *Pediatr Diabetes*, 13(5), 400-407, 2012.

IV de Goffau MC*, Luopajärvi K*, Knip M, Ilonen J, Ruohtula T, Härkönen T, Orivuori L, Hakala S, Welling GW, Harmsen HJ, Vaarala O. Fecal microbiota composition differs between children with β-cell autoimmunity and those without (Submitted).

*Both authors equally contributed to this study.

Publication (I) was published earlier in Skarsvik S (2005): Aberrancies associated with dendritic cells and T lymphocytes in type 1 diabetes, Linköping: University Medical Dissertations No. 920.

ABBREVIATIONS

APC	Antigen-presenting cell
Apc	Allophycocyanin
BB	Biobreeding
BI	Bovine insulin
BLG	beta-lactoglobulin
CBMC	Cord blood mononuclear cells
CM	Cow's milk
ELISA	Enzyme-linked immunosorbent assay
FINDIA	Finnish Dietary Intervention Trial for Prevention of Type 1 Diabetes
FITC	Fluorescein isothiocyanate
FOXP3	Forkhead/winged-helix transcription factor box protein 3
GADA	Glutamate decarboxylase autoantibody
HI	Human insulin
HLA	Human leukocyte antigen
IA-2A	Antibodies to the protein tyrosinase phosphatase-related IA-2 protein
IAA	Insulin autoantibody
ICA	Islet cell antibody
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin

MHC	Major histocompatibility complex
NOD	Non-obese diabetic
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cell
PE	Phycoerythrin
PHA	Phytohemaglutinin
RT-PCR	Reverse transcriptase-polymerase chain reaction
TCR	T cell receptor
T1D	Type 1 diabetes
Th cell	T helper cell
Treg	Regulatory T cell
TRIGR	Trial to Reduce IDDM in the Genetically at Risk
TT	Tetanus toxoid

1 INTRODUCTION

Type 1 diabetes (T1D) is the second most common chronic disease of childhood in Finland after allergies. In T1D, pancreatic insulin-producing β-cells are selectively destroyed in individuals at genetic risk. T1D is thought to have an autoimmune etiology. It develops as a consequence of a combination of genetic predisposition, environmental factors and stochastic events. T1D may occur at any age, but over half of the patients are diagnosed under the age of 15. The highest incidence of T1D among children younger than 15 years exists in Finland (EURODIAB ACE Study Group 2000, Patterson *et al.* 2009). Approximately 600 new children in Finland are affected annually by T1D.

The incidence of T1D has increased considerably since the 1950s among children and adolescents, especially in children less than five years old. The reasons for this are not known. Genetic factors alone can hardly explain the rapid increase. There have been numerous alterations in the environment, such as changes in the pressure of infections, healthcare habits, and dietary factors. These factors could affect the development of the immune system and thus the risk of T1D. Their influence can already be seen *in utero*, during the first years of life and later in childhood.

The focus of this study was to assess the possible alterations in the early maturation of the immune system related to the risk of T1D. We studied the *in vitro* differentiation of cord blood Th1 and Th2 cells in children at genetic risk of T1D. We analyzed the regulatory T cells in infants with maternal T1D and examined whether exposure of the offspring to maternal insulin therapy *in utero* induces insulin-specific regulatory mechanisms. The factors reflecting oral tolerance were also evaluated, such as the development of antibodies to cow's milk proteins in infancy in children who later developed T1D, and the gut microbiota in relation to β-cell autoimmunity was studied. The ultimate goal was to better understand the risk factors and mechanisms for the development of T1D and autoimmune diseases in general.

2 REVIEW OF THE LITERATURE

2.1 Immune system: innate and adaptive immunity

The immune system is designed to defend the host against potentially pathogenic microorganisms (e.g. virus, bacteria, parasites) while maintaining immunological homeostasis. Another function of the immune system is to clear apoptotic cells. It should be able to discriminate between foreign antigens and antigens expressed in the tissues of the host. Thus, failure in the function of the immune system can result in auto-inflammation or the development of autoimmune disease. Autoimmune diseases result from failure in establishing immunological tolerance or the unusual presentation of self-antigens that allow the development of aberrant tissue-damaging immune responses. In other words, the immune system attacks its own cells and tissues. The immune system is divided into innate and adaptive immunity. Innate immunity is evolutionarily conserved and provides an immediate immune defense against antigens without previous contact with the antigen. The slower adaptive immune response follows the innate response, and one of its characteristics is the generation of immunological memory. Adaptive immunity is capable of responding to variable targets (Murphy *et al.* 2008). The two major subgroups of T cells in adaptive immunity with separate immune functions are $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells.

2.2 The innate immune system

The principal effector cells of innate immunity include natural killer (NK) cells, neutrophils, eosinophils, basophils, mast cells, macrophages, and professional antigen-presenting cells (APC). External barriers (e.g. skin, mucosal epithelium), the phagocyte system, the complement system, and cytokines are also involved in innate immunity. Innate immune recognition is mediated by a number of germline-encoded receptors called pattern recognition receptors (PRRs). PRRs bind structurally conserved molecules expressed by microbes known as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) of Gram-negative bacteria, or viral nucleic acids. The best-characterized molecules of PRRs are Toll-like receptors (TLR) expressed on various cell types including epithelial cells, dendritic cells and macrophages. Some TLR are located on the cell surface of DC, where they are able to detect extracellular pathogen molecules. TLR located intracellularly can recognize microbial components, such as DNA. The binding of microbial ligands to PRRs leads to the activation of APC and of adaptive immunity (Barton and Medzhitov 2002, Janeway and Medzhitov 2002, Medzhitov 2007, Chaplin 2010, Takeuchi and Akira 2010). The recognition of symbiotic microorganisms by the innate im-

mune system has an important role in maintaining intestinal homeostasis (Rakoff-Nahoum *et al.* 2004). The mechanisms that allow the innate immunity system to distinguish between pathogenic and non-pathogenic (symbiotic) microorganisms are not well understood.

Innate cells that activate naive T cells are known as antigen-presenting cells (APC), and dendritic cells (DC), macrophages, and B cells are the main cell types involved. DC are professional APC that serve as a major link between the innate and adaptive immune systems. DC are able to activate several types of immune effector cells, including B cells, T cells, and NK cells. DC are comprised of several subpopulations. Immature DC are located in peripheral tissues, where they encounter invading pathogens. Langerhans cells are immature DC that take up antigen in the skin in response to infection. DC can be divided into two different functional groups: myeloid and plasmacytoid DC (mDC and pDC, respectively). pDC are derived from lymphoid progenitor cells, whereas mDC originate from myeloid precursor cells. mDC migrate as precursor cells to the sites of potential entry of pathogens. They express TLR1-7 and are thus capable of recognizing several bacterial and viral components via these receptors. pDC express intracellular receptors TLR7 and selectively TLR9, which recognize viral RNA and DNA, respectively. pDC produce large amounts of IFN (interferon) α, especially in response to viral infections. The peripheral blood monocytes can differentiate into DC (Sallusto and Lanzavecchia 1994). In the intestine, several types of DC in Peyer's patches have been characterized based on their functionality and phenotype (Scott *et al.* 2011).

The antigen capture activity of DC is dependent on the expression of several surface receptors such as Fc receptors, receptors for heat-shock proteins and lectins. Activated DC begin to express high levels of major histocompatibility complex (MHC) class II molecules and co-stimulatory receptors such as CD80/86 that are critical for different T cell effector functions. In addition, the expression of chemokine receptors CCR2 and CCR7 is induced in DC after stimulation. Thus, stimulated DC are sensitive to signals from the chemokines CCL19 and CCL21, which direct them to the draining lymphoid tissues, and they produce cytokines including IL-12, IL-23, and IL-6. DC are able to migrate into the T cell zone in the defined draining lymph nodes, where they present antigens to T cells for the induction of immunity or tolerance (Banchereau and Steinman 1998). DC activation plays an important role in the differentiation of antigen-specific functional phenotypes of T helper cells. mDC secrete IL-12 and IL-27, which alters the Th-cell balance in the Th1 direction, while pDC, physiologically residing in primary and secondary lymphoid organs, secrete type I interferons (IFN) (de Jong *et al.* 2005).

2.3 $CD4^+$ T cells

$CD4^+$ cells are important regulators of adaptive immunity, and form the majority of T cells. They express the surface molecule CD4, which interacts with MHC class II molecules. During TCR activation, naive CD4 T cells can differentiate into distinct T helper cell lineages that secrete restricted sets of cytokines. However, plasticity of T helper cells may also take place, and the phenotype of T helper cells may change due to environmental stimuli (O'Shea and Paul 2010).

2.3.1 Th1 and Th2 cells

Historically, T helper cells were divided into T helper 1 (Th 1) and helper 2 (Th 2) cells. $CD4^+$ effectors were viewed in the context of Th1-Th2 polarization. It was primarily proposed by Tada that $CD4^+$ T cells are divided into at least two subtypes (Tada *et al.* 1978). Mosmann and Coffman discovered that naive mice $CD4^+$ T helper cells, after antigenic stimulus, differentiate into two very different subsets determined by their cytokine production and function (Coffman and Carty 1986, Mosmann *et al.* 1986). These subsets, Th1 and Th2 cells, are responsible for cell-mediated and humoral immune responses (Paul and Seder 1994, Rengarajan *et al.* 2000). Similar types of functional phenotypes were also described for human Th cells (Del Prete *et al.* 1991). IL-12 and IL-4 are the most important cytokines directing Th1/Th2 polarization, respectively (O'Garra 2000). Moreover, recent studies have demonstrated that epigenetic remodeling (altering the structure of chromatin and DNA methylation) of cytokine loci is central in establishing effector T cell lineage commitment (Ansel *et al.* 2003).

Th1 and Th2 cells have been associated with specific roles in immune responses. When innate immune cells recognize invasion by intracellular pathogens such as protozoa, intracellular bacteria or viruses, naive CD4 T cells differentiate into Th1 cells. Th1 cells are effective inducers of cell-mediated immunity against intracellular pathogens such as *Mycobacterium* tuberculosis and viral infections by activating macrophages, NK cells, and $CD8^+$ cytotoxic cells. On the other hand, when innate immune cells recognize extracellular bacteria or parasites such as helminths, the naive $CD4^+$ T cells differentiate into Th2 cells. They modulate B cells to induce immunoglobulin class switching and epithelial cells to enhance their mucus production. Th1 cells have been implicated in organ-specific autoimmune diseases, whereas the development of Th2 cells is an important mechanism in the development of allergy.

Th1/Th2 differentiation is regulated by the cytokine milieu, which is created under the influence of the particular pathogens as well as the dose of antigens and the genetic background of the host (Abbas *et al*. 1996). IL-12 and IFN-γ are two major cytokines in Th1 differentiation. IL-12 is a heterodimer composed of two subunits, p35 and p40, and is secreted by activated APC, including macrophages, monocytes, and dendritic cells. IL-12 signals through the IL-12 receptor

complex composed of the IL-12Rβ1 and IL-12Rβ2 chains. TCR activation is needed to induce both IL-12Rβ1 and IL-12Rβ2 chains and the formation of a functional IL-12R complex (Presky *et al.* 1996). IL-12Rβ2 expression is maintained on the differentiating Th1 cells. Naive CD4 T cells lacking IL-12R expression are unresponsive to IL-12 (Rogge *et al.* 1997). In humans, IL-12 binds to the IL-12 receptor on the cell surface, which induces the activation of signal transducer and activator of transcription 4 (STAT4). Activated STAT4 can directly induce the IFN-γ production and expression of IL-12Rβ2 and T-bet during Th1 differentiation (Szabo *et al.* 2003, Usui *et al.* 2003, Yang *et al.* 2007). In humans with mutations in components of the IL-12R signaling pathway, severely impaired immune responses to infectious agents are seen.

IFN-γ is secreted by DC, macrophages, Th1 cells and NK cells and binds to IFN-γ receptor. IFN-γ induces the expression of transcription factor T-bet through the activation of STAT1. T-bet is a T-box transcription factor that is regarded as the master regulator for Th1 cell differentiation. Once expressed, T-bet induces IL-12R expression, resulting in enhanced IL-12-STAT4 signaling (Szabo *et al.* 2000, Commins *et al.* 2010). Individuals with mutations in the IFN-γ receptor-signaling pathway are prone to infections caused by intracellular pathogens such as uncontrolled mycobacterial infections. T-bet inhibits the transcription of genes related to the induction and function of Th2 cells. In addition, cytokines IL-18 (Xu *et al.* 1998) and IL-27 induce Th1 development (Pflanz *et al.* 2002) (Figure 1).

Th2 cells are critical for the elimination of extracellular pathogens such as helminths and are effective in promoting antibody production by B cells such as IgE and IgG1 (Murphy *et al.* 2008). The differentiation of naive T cells into Th2 cells requires both TCR- and IL-4-mediated signals. IL-4R signals are transduced by the transcription factor STAT6 in naive $CD4^+$ T cells. In this signaling pathway, STAT6, together with NFAT (nuclear factor of activated T cells), AP-1, NF-κB, and other TCR-induced signals, activates the transcription of Th2-type genes. GATA-3 is master transcription factor responsible for Th2 differentiation (Takeda *et al.* 1996, Zhang *et al.* 1997, Zhu *et al.* 2001). Th2 cells secrete IL-4, IL-5, IL-10, IL-9, IL-13, and IL-25.

Th1 and Th2 cells cross-regulate each other through the action of the cytokines secreted. For example, IFN-γ and the IL-12 signaling pathway through T-bet activation have been implicated in the suppression of GATA-3 expression and inhibition of the production of Th2 cytokines (Szabo *et al.* 2000). IL-4-induced expression of GATA-3 has been shown to inhibit the production of IFN-γ (Ouyang *et al.* 1998). In addition, GATA-3 and T-bet are able to silence the opposing Th lineage by directly regulating each other's gene expression.

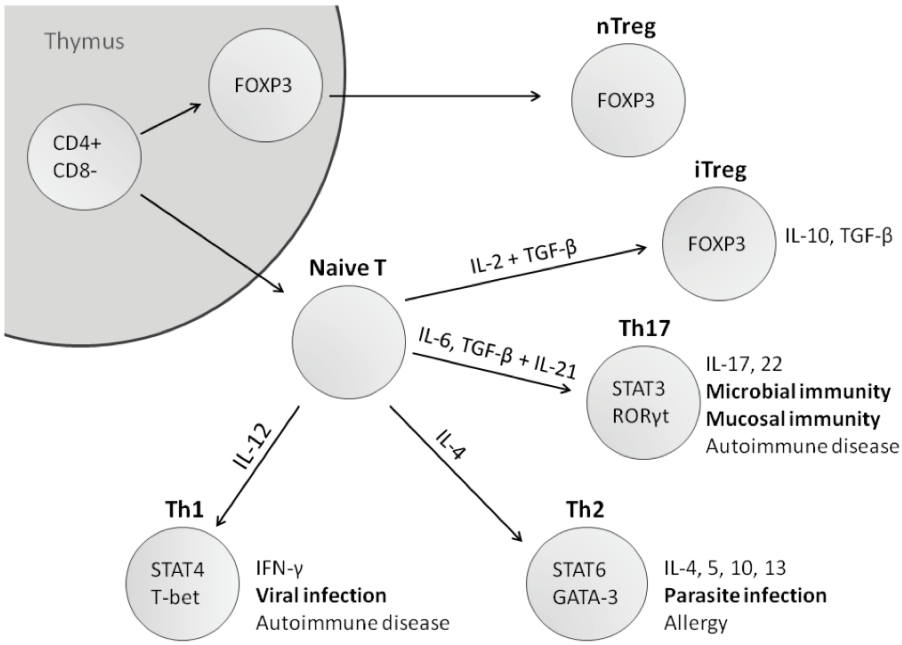

Figure 1. The network of transcription factors in $CD4^+$ T cells. Naive $CD4^+$ T cells can differentiate into various subsets of T helper (Th1, Th2 and Th17) cells. However, in the presence of TGF-β, naive T cells convert into FOXP3-expressing induced Treg (iTreg) cells. Transcription factors (T-bet, GATA-3 and RORγt) have been identified as master regulators. Differentiated T helper cells are characterized by a combination of specific effector cytokines that orchestrate functions of the adaptive immune system.

2.3.2 Th17 cells

Th17 immunity has an important role in host defense against specific extracellular bacteria and fungi such as *Candida albicans* and *Borrelia burgdorferi* (Korn *et al.* 2009). Th17 cells are characterized by the production of selected cytokines, including IL-17A, IL-17F, IL-21, IL-22, and IL-26 (humans). Th17 cells recruit neutrophils to the site of inflammation, but have also been described as uniquely pathogenic in multiple inflammatory diseases. Thus, Th17-mediated immune responses are very important in promoting chronic inflammation, and their importance has been suggested in several autoimmune diseases (Torchinsky *et al.* 2009) such as multiple sclerosis, inflammatory bowel disease, T1D, and rheumatoid arthritis. It has been shown that a combination of the immunoregulatory cytokine TGF-β and the proinflammatory cytokine IL-6 is required to induce the expression of IL-17 in $CD4^+$ T cells that are being activated through their TCR (Veldhoen *et al.* 2006, Bettelli *et al.* 2006). Transcription factor RORγt has been indicated as the master regulator for Th17 cells, and its

up-regulation is dependent on transcription factor STAT3 signaling (Ivanov *et al.* 2006). IL-23 has been shown to have a role in the maturation and maintenance of Th17 cells. IL-17 has been shown to protect mucosal barrier function due to stimulation of tight junction formation and mucin secretion (Kinugasa *et al.* 2000, Blaschitz and Raffatellu 2010).

In addition, Th17 cells produce IL-22, which acts co-operatively with IL-17, for instance to induce the expression of antimicrobial peptides such as β-defensin on mucosal surfaces. Studies have additionally shown that the induction of two intestinal antimicrobial peptides, RegIIIβ and RegIIIγ, is also dependent on IL-22 production. IL-22 expression is restricted to cells of lineages of the innate and adaptive immune responses, whereas its target cells expressing IL-22 receptors are widespread. The functional IL-22R seems to be restricted to nonhematopoietic cells of the skin, pancreas, intestine, liver, lungs and kidneys. Specific Th22 cells have also been identified as a new T helper cell population. Th22 cells express a special set of chemokine receptors (CCR4, CCR6, CCR10) and have a high expression of the aryl hydrocarbon receptor, but a low expression of RORγt and T-bet (Witte *et al.* 2010, Sonnenberg *et al.* 2011).

2.3.3 Th9 cells

It has been suggested there is a specialized subset of T cells dedicated to producing IL-9 (Th9 cells). IL-9 production was first associated with the Th2 phenotype. However, other Th subsets also appear to have a potential for IL-9 production. Human Th17 cells can secrete IL-9, and long-term Th17 cultures have the ability to coexpress IL-17A and IL-9. In contrast, IL-23, a cytokine required for maintenance of the Th17 phenotype, has inhibitory effects on IL-9 production. Naive $CD4^+$ T cells primed in the combination of TGF-β and IL-4 produce high levels of IL-9. The transcription factors that regulate Th9 development include TGF-β-induced Sfpi1 and IL-4-induced STAT6, which induces IRF4 as it represses FOXP3 and T-bet (Jabeen and Kaplan 2012).

2.3.4 Regulatory T cells

Regulatory T cells (Tregs) are a subset of $CD4^+$ T cells thought to actively suppress the immune system, maintaining immune system homeostasis and tolerance to self-antigens, and thereby preventing pathological self-reactive inflammation and autoimmunity. There are at least two major types of regulatory T cells: natural and induced Tregs. Naturally occurring $CD4^+CD25^+$ $FOXP3^+$ Tregs (nTregs) develop in the thymus after recognition of medium-affinity self-antigens. The nTregs express the high-affinity α chain of the IL-2 receptor, CD25, and represent 5–10% of the $CD4^+$ T lymphocytes in healthy adult mice and humans (Sakaguchi *et al.* 1995). Induced Tregs (iTregs) are produced in the secondary lymphoid tissues after antigen stimulation of naive $CD4^+$ cells in the

presence of TGF-β (Fontenot *et al.* 2003, Fontenot and Rudensky 2005, Sakaguchi 2005, Yagi *et al.* 2004).

Sakaguchi *et al.* discovered that depletion of $CD4^+CD25^+$ T cells from normal mice leads to the spontaneous development of various autoimmune diseases, such as autoimmune gastritis, thyroiditis, sialoadenitis, adrenalitis, glomerulonephritis and polyarthritis. When $CD4^+$ cell suspensions were depleted of $CD25^+$ cells prepared from BALB/c nu/+ mice lymph nodes and spleens and then inoculated into BALB/c athymic nude (nu/nu) mice, all recipients spontaneously developed autoimmune diseases. Co-transfer of a small number of $CD4^+CD25^+$ cells inhibited the development of autoimmunity (Sakaguchi *et al.* 1995, Sakaguchi 2005). Furthermore, $CD25^+CD4^+$ nTregs not only inhibit autoimmune responses, but also suppress a variety of physiological and pathological immune responses to non-self antigens (Shimizu *et al.* 2002).

No single characteristic surface marker to Tregs has been identified. nTregs express high levels of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and the glucocorticoid-induced tumor necrosis factor receptor (GITR) (Takahashi *et al*. 2000, Shimizu *et al.* 2002), and low levels of the IL-7 receptor, CD127 (Liu *et al.* 2006). The transcription factor Forkhead box 3 (FOXP3) is considered to be a master regulator of Treg development and function and is activated through STAT5 signaling. nTregs functions are dependent on IL-2 and TGF-β (Sakaguchi *et al.* 2009). Patients with mutations in the FOXP3 gene develop a fatal autoimmune disorder termed immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (Bennett *et al*. 2001), which is characterized by the manifestation of several immune-mediated diseases such as T1D, thyroiditis, recurrent infections, eczema and allergic inflammation. Naive T cells in the periphery can also acquire FOXP3 expression and Treg function when naive T cells *in vitro* are stimulated in the presence of TGF-β activation (iTregs) (Chen *et al.* 2003, Zheng 2008). IL-2 facilitates the differentiation of naive $CD4^+$ T cells into $FOXP3^+$ Tregs.

Both nTreg and iTreg cells have the ability to suppress the function of effector T cells (Shevach 2009). The regulatory activity of FOXP3-expressing Tregs is indicated to be cell-contact dependent. Tregs can kill T effector cells by the cytolytic or apoptotic pathway. Tregs have been shown to lyse target cells by granzyme B-dependent or perforin-dependent mechanisms (Cao *et al.* 2007). CTLA-4 expressed by Tregs cells can downmodulate CD80 and CD86 expression by DC and thereby inhibit the activation of T effector cells. In addition, Treg-mediated suppression involves the secretion of immunosuppressive cytokines such as IL-10 and TGF-β (von Boehmer 2005), and IL-35 (Collison *et al.* 2007). It has also been suggested that several members of the galectin family of the carbohydrate-binding proteins are involved in Treg functions. For example, galectin-9 preferentially induces apoptosis of activated CD4-positive T cells. It has recently been demonstrated that Gal-9 is a ligand of T cell immunoglobulin-

and mucin domain-containing molecule 3 (TIM-3), which was selectively expressed on terminally differentiated Th1 cells, and that Gal-9 induces apoptosis of TIM-3-expressing cells *in vitro* and *in vivo* (Zhu *et al.* 2005).

In humans, FOXP3 is upregulated in $CD4^+CD25^-$ cells upon TCR stimulation, and this does not always lead to the development of Tregs with suppressive activity. It has been shown that FOXP3 expression is strongly associated with the hyporesponsiveness of activated T cells. However, FOXP3 expression is transient in this nonsuppressive T cell population, while it is stably expressed in activated T cells with a suppressive function and in nTregs. It has been indicated that FOXP3 may be insufficient to induce Tregs activation or to identify them (Wang J. *et al.* 2007).

Besides $FOXP3^+$ Tregs, other types of Tregs cells can be induced from naive T cells, such as Tr1 (Groux *et al.* 1997) and Th3 cells (Weiner 2001). Tr1 cells are typically found in the intestinal mucosa and they express IL-10 and TGF-β. Tr1 cells are produced *in vitro* by the antigenic stimulation of naive T cells in the presence of IL-10 (Vieira *et al.* 2004). Th3 cells are primarily induced from naive $CD4^+$ T cells and have a regulatory function in oral tolerance. Th3 cells are located in the peripheral immune compartment and are triggered by TCR signaling in the gut by oral antigens. Following triggering in the gut, the Th3 cells secrete TGF-β. TGF-β maintains naturally occurring $CD4^+CD25^+FOXP3^+$ Tregs, suppresses Th1 and Th2 responses, and together with IL-6 may induce Th17 responses. TGF-β from Th3 cells also acts on $CD4^+FOXP3^-$ cells and converts them to iTregs. Activated Th3 cells are then able to suppress systemic autoimmune and inflammatory responses and are associated with the induction of oral tolerance. In the gut, exposure to lower doses of antigen favors the induction of Tregs such as Th3 cells that are able to inhibit inflammation by secreting high levels of TGF-β (Chen *et al.* 1994), whereas higher doses of antigen exposure favor anergy/deletion as a mechanism of tolerance induction (Chen *et al.* 1995).

2.3.5 Plasticity of the T cell lineage

Helper T cell subsets have been viewed as lineages, defined by the expression of selective signature cytokines and master regulator transcription factors. In recent years, the view of T cell differentiation has altered. There is increasing evidence for substantial phenotypic flexibility in helper T cells, and subsets also appear to be more plastic than originally recognized. $CD4^+$ T cells may not differentiate into rigidly defined Th1, Th2, Th17 and Treg cell lines, as was originally thought (Nakayamada *et al.* 2012). It is now clear that $CD4^+$ T cells can change their phenotype and profile of cytokine production, and there are circumstances in which the expression of master regulators is transient or T cells express more than one master regulator. For example, IL-10 is now recognized to be produced

by multiple cell subsets: Th1, Th2, Tregs, and Th 17 cells. It has been reported that IL-17-producing cells could be repolarized to either the Th1 or Th2 phenotype in the presence of IL-12 or IL-4. Moreover, it has been shown that Th17 cells can be changed to a population of T cells with IFN-γ production (O'Shea and Paul 2010). Indeed, studies now suggest that plasticity results from a broad range of epigenetic states in transcription factors, allowing re-activation in already differentiated $CD4^+$ cells (Wei *et al*. 2009).

2.4 $CD8^+$ cells

$CD8^+$ cytotoxic T cells are able to recognize and kill cells infected with viruses or other intracellular microbes. These cells are also important in the regulation of activation and differentiation of $CD4^+$ T cells. $CD8^+$ T cells recognize antigens presented in MHC class I molecules. Practically all nucleated cells express MHC class I molecules, and if they become infected they can therefore present antigens to $CD8^+$ cells. They may kill target cells by one of at least three distinct pathways. First, they can eliminate target cells using a perforin-dependent mechanism where perforin is inserted in the target-cell membrane and forms pores on the cell, and then granzyme enzymes A and B mediate apoptosis. Alternatively, by a FasL/Fas mechanism, $CD8^+$ cells are able to upregulate Fas ligand (CD95L) on T cells that bind to Fas molecules (CD95) on the target cell, leading to caspase-mediated cell death by apoptosis. In addition, $CD8^+$ cytotoxic T cells produce cytokines such as IFN-γ and TNF-α. TNF-α triggers the caspase cascade, leading to target-cell apoptosis. IFN-γ induces the upregulation of MHC class I and Fas expression on target cells, leading to the enhanced presentation of endogenous peptides by MHC class I molecules, and increases Fas-mediated target-cell lysis (Murphy *et al.* 2008, Andersen *et al.* 2006).

2.5 Immune tolerance

The immune system is able to discriminate between antigenic determinants expressed on foreign substances such as microbes, and antigenic determinants of the host. This ability of the immune system to avoid attacking its own tissues and the elimination of potentially self-reactive T and B cells is referred to as immunological tolerance (Starr *et al.* 2003). Tolerance to self-molecules is established and maintained through mechanisms taking place in both the thymus (central tolerance) and peripheral lymphoid organs (peripheral tolerance).

The mechanisms for central tolerance consist of multiple stages and checkpoints in the thymus. T cell precursors arise from hematopoietic stem cells and migrate to the thymus from bone marrow. Upon entry into the thymus, T cell precursors lack the expression of TCR chains, CD4 and CD8 molecules. These T cells are known as double-negative (DN). Thymocytes develop from these DN cells into cells that express both $CD4^+$ and $CD8^+$ coreceptors (double-positive

stage, i.e. DP cells). Once TCR chains are expressed, these DP cells undergo two important selection processes within the thymus, namely positive and negative selection. First, in positive selection, DP cells further differentiate into mature thymocytes that express CD4 or CD8 cells (single-positive stage) and high levels of the TCR-CD3-complex. Only a minority of T cells capable of weak affinity for self-peptides presented in the context of self-MHC molecules are selected (i.e. they undergo positive selection). This process involves the interaction of DP thymocytes with peptides bound to class I or II MHC molecules on accessory cells, where CD4 binds to class II and CD8 to class I receptors. This interaction determines the commitment of DP thymocytes to either $CD4^+$ or $CD8^+$ lineages (Takahama 2006).

In negative selection, the selection is determined by the affinity of TCR to the peptide−MHC complex. T cells migrate from the cortex to the thymic medulla, where they interact with DC and medullary thymic epithelial cells (mTEC). T cells with high affinity TCR for self-peptides receive signals from APC to undergo apoptosis. In addition, T cells with TCR that do not show any affinity for expressed peptide-MHC complexes are eliminated. However, those thymocytes expressing TCR with a low affinity for self-peptides survive and migrate from the thymus to secondary lymphoid organs.

Gene expression during mTEC development is regulated by the transcription factor known as autoimmune regulator (AIRE). The *AIRE* gene has the ability to induce the expression of an extensive selection of peripheral tissue antigens such as insulin (Anderson *et al.* 2005). Mutations in the *AIRE* gene cause autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, also known as autoimmune polyendocrine syndrome type 1). This is a severe autoimmune disease characterized by the loss of self-tolerance in multiple endocrine organs, and affects central tolerance in the thymus (Finnish-German APECED Consortium 1997).

It has been suggested that failure of self-tolerance leads autoimmune diseases. The role of central tolerance is crucial, but despite the function of central tolerance, T cells with a low affinity for self-peptides escape the negative selection process and may later develop into autoreactive T cells, leading to autoimmunity. Self-reactive B cells are usually deleted, but a subset of short-lived autoreactive B cells provides protection from infection, because the B cell receptor cross-reacts strongly with foreign antigen (von Boehmer and Melchers 2010).

Peripheral tolerance supplements the central tolerance in regulating the expansion of low-affinity autoreactive T cells or T cells escaping negative selection. The main mechanisms of peripheral tolerance are anergy, deletion and immune suppression. Anergy follows the unresponsiveness of T cells recognizing the self-antigen–MHC complex on APC in the absence of co-stimulatory molecules. Peripheral deletion is based on programmed apoptotic cell death. Peripheral tolerance is also maintained by distinct subsets of T cells with regula-

tory functions. $CD4^+CD25^+FOXP3^+$ regulatory T cells are considered to be major regulators of the immune system.

2.6 MHC, antigen processing and presentation

T cells play a key role in adaptive immunity. They derive from hematopoietic stem cells and undergo differentiation in the thymus (Hernandez *et al.* 2010). These cells can be further divided into two distinct classes based on the cell surface receptors they express: helper T cells expressing CD4 ($CD4^+$ T cells) and cytotoxic T cells expressing CD8 ($CD8^+$ T cells). The majority of T cells express antigen-binding receptors (TCR) consisting α and β chains that recognize short linear peptides in major histocompatibility complex (MHC) molecules on antigen-presenting cells (APC).

The major histocompatibility complex (MHC) is known in humans as the human leukocyte antigen (HLA) system. The HLA genes involved in immune recognition fall into two structurally and functionally different classes: class I (HLA-A, B, and C) and II (HLA-DP, DQ, and DR). The MHC class molecules present peptides to the TCR. Class I genes are expressed by most somatic cells, and class I molecules present peptides derived from intracellular proteins to $CD8^+$ cells. In contrast, class II genes are normally expressed by APC, i.e. DC, B lymphocytes, macrophages and thymic epithelial cells, and class II molecules present peptides derived from exogenous proteins to $CD4^+$ cells (Klein and Sato 2000, Delves and Roitt 2000).

The MHC class I molecule consist of a polymorphic α-chain with peptide-binding domains (α1 and α2), one immunoglobulin-like domain (α3), a transmembrane region, sytoplasmic tail, and polymorphic β2-microglobulin encoded outside the MHC. The MHC II α- and β-chains consist of a peptide binding domain (α1 or β1), an immunoglobulin-like domain (α2 or β2), a transmembrane region, and a cytoplasmic tail (Klein and Sato 2000). MHC class I molecules present shorter peptides (usually 9–11 amino acids) than MHC class II molecules (13–17 amino acids) (Bonilla and Oettagen 2010).

The pathways by which antigenic peptides are processed and presented differ between MHC class I and II molecules. MHC class I molecules present antigens synthesized within the cells, whereas class II molecules present extracellular antigens. In the MHC I class pathway, proteins in the cytosol are degraded into short peptides by proteosomes. The resulting peptides are transported by molecules known as the Transporter associated with Antigen Processing (TAP1 and 2) into the endoplasmic reticulum (ER). The peptides are then loaded onto the MHC class I molecules. Finally, peptide–MHC class I complexes are transported by the Golgi apparatus to the cell membrane for antigen presentation to $CD8^+$ cells (Murphy *et al.* 2008).

In the basic MHC class II antigen presentation pathway, self and foreign proteins are taken up by endocytosis or phagocytosis into an endosome and degraded by lysosomal enzymes. MHC class II α- and β-chains associate with the polypeptide called invariant chain (Ii) in the ER, which protects and blocks the peptide-binding groove of the class II molecule. The transporting vesicle (containing the Ii-MHC class II heterodimer) and the endosome (containing exogenous antigen) fuse. The invariant chain is degraded, and the peptide–MHC class II complex is formed with the help of the HLA-DM molecule, allowing peptides derived from exogenous proteins to bind. The complex is delivered to the surface of the cell for recognition by $CD4^+$ T cells (Neefjes *et al.* 2011).

The distinction between antigen presentation to $CD8^+$ and to $CD4^+$ T cells is not definitive. APC are able to process peptides that are derived from exogenous antigens onto MHC class I molecules. DC take up antigen by endocytosis and present it to $CD4^+$ T cells through MHC II molecules, and cross-present it to $CD8^+$ T cells through MHC I molecules. Activated $CD4^+$ T cells can stimulate $CD8^+$ cells by secreting IL-2 and stimulate DC for cross-priming through CD40L–CD40 interactions. DC up-regulate co-stimulatory molecules (CD80, CD86) and downregulate inhibitory molecules (programmed cell death ligand). TLR further activate DC and increase the cross-presentation activity. Thus, cross-presentation can lead to the induction of cross-priming and cross-tolerance (Kurts *et al.* 2010).

2.7 T cell activation

Activated APC have a role in activating T cells to become effector or memory cells. The maturation process of T cells occurs in the primary (thymus) and secondary lymphoid organs (lymph nodes, spleen). T cell receptors (TCRs) are composed of a αβ heterodimer, which is found on 95% of T cells, or a γδ heterodimer. All of the α, β, γ, and δ chains have an amino-terminal variable (V) region and carboxyl-terminal constant (C) region. The variable region is generated by the somatic recombination of variable (V), diversity (D), and joining (J) gene segments during the development of the T cell. Each T cell consists of a different combination of these genes, giving huge diversity in TCR structures and antigen recognition by TCR. A smaller fraction of T cells (5%) consists of γ and δ chains instead of α and β chains. T cells bearing γ and δ chains are a distinct lineage of T cells. γδT cells are commonly found in the gastrointestinal epithelium and have been suggested to have a role in innate immune responses (Murphy *et al.* 2008, Bonilla and Oettegen 2010).

The first step in activating a T cell is recognition of the appropriate peptide antigen bound to the groove of the HLA class I or class II molecule by the T cell receptor/CD3 complex in $CD8^+$ or $CD4^+$ cells. This antigen-specific signal is referred to as signal 1. Before the process of T cell activation can continue, the T

cell requires an activation signal from the APC. This signal is antigen independent and provided by co-stimulatory signals, which along with signal 1 induces T cell proliferation, differentiation, and development into memory cells. Without these signals the cell will either become anergic or die by programmed cell death. The main co-stimulatory molecules expressed on APC are CD80 (B7-1)/CD86 (B7-2) and CD40, which respectively bind CD28 and CD40 ligand (CD40L) on the T cell. CTLA-4 can competitively bind CD80 and CD86 and results in an inhibitory signal to the activated T cell. ICOS is also upregulated upon T cell activation. To further facilitate this interaction, adhesion structures on both the APC and the T cell interact. For example, intracellular adhesion molecule-1 (ICAM) on the APC interacts with leukocyte function-associated antigen-1 (LFA-1) on the T cell. A third signal comes from cytokines, which induce the development of distinct types of T effector cells (Figure 2).

The coreceptors CD4 or CD8 enhance TCR recognition by stabilizing the TCR-MHC complex. CD4 binds to MCH II. This brings the cytoplasmic tyrosinase kinase LCK into the signaling complex and activates it. LCK initiates the phosphorylation of all tyrosines in the cytoplasmic tails of the CD3 complex. This phosphorylation activates kinase ZAP-70, which activates signaling pathways that culminate in the activation of transcription factors in the nucleus. A rapid increase in intracellular calcium levels is induced in ER. In addition, activation of the T cell receptor results in the release of Ca^{2+} ions from endoplasmic reticulum (ER) Ca^{2+} stores. This calcium flux activates a calcium release-activated calcium (CRAC) channel. Calcium entering the cytosol from the ER or extracellular space binds to the regulatory protein calmodulin, which in turn activates the enzyme calcineurin. Dephosphorylation of NFAT by calcineurin allows NFAT to enter the nucleus, which results in differentiation, proliferation and the effector function of T cells (Hogan *et al.* 2003, Murphy *et al.* 2008, Smith-Garvin *et al.* 2009) (Figure 2).

When naive T cells encounter APC bearing a peptide-MHC complex for which its TCR has high affinity, most of these cells rapidly die, but a small fraction of effector cells develop into long-lived memory cells. These cells have the ability to quickly react to previously encountered specific antigens. This differentiation is influenced by factors such as T cell antigen receptor (TCR) signal strength, IL-7 and IL-15. Memory cells express CD45RO and naive cells express CD45RA (Jameson and Masopust 2009, Sprent and Surh 2011).

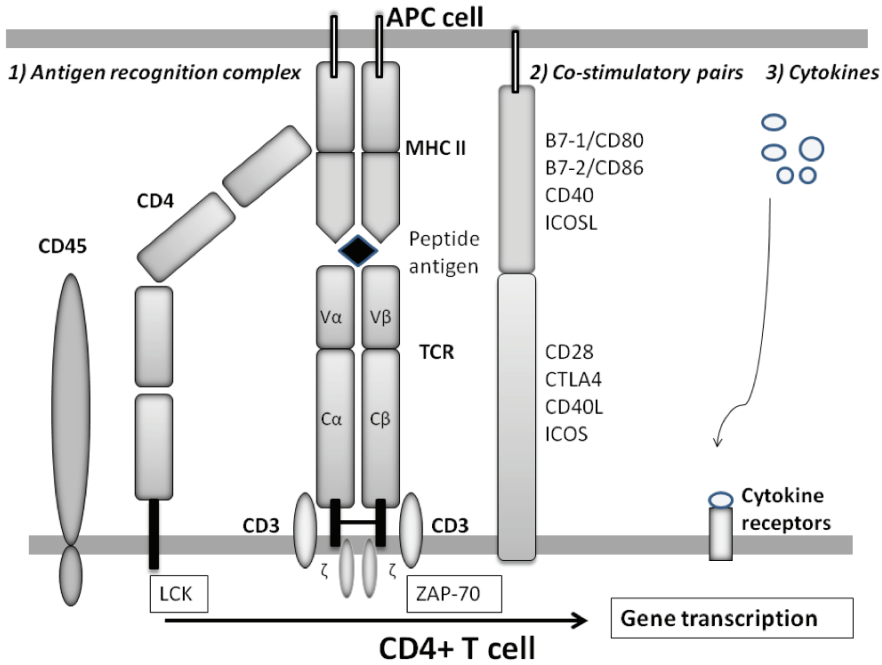

Figure 2. Interaction between antigen-presenting cells (APC) and $CD4^+$ T cell leading to the development of activated $CD4^+$ cells (modified from Murphy *et al.* 2008). Signal 1: The TCR α and β chain recognize peptide/MHC complexes on APC, the interaction is stabilized by binding CD4 to MHC II. Signaling is initiated by CD3 chains through the cytoplasmic tail, which are phosphorylated by kinases such as LCK, leading to the recruitment of signaling molecules, including ZAP-70. The tyrosine phosphatase CD45 activates LCK. Signal 2: The second signal is delivered through co-receptors. The main co-stimulatory molecules expressed on APC are CD80 (B7-1)/CD86 (B7-2) and CD40, which respectively bind CD28 and CD40 ligand (CD40L) on the T cell. CTLA-4 can competitively bind CD80 and CD86, which results in an inhibitory signal to the activated T cell. ICOS is also upregulated upon T cell activation. Signal 3: The third signal comes from cytokines secreted by APCs.

2.8 Chemokines and chemokine receptors

Chemokines are a group of small (8-14kD) chemotactic cytokines that regulate the migration of leukocytes from the blood between various tissues. To date, over 50 different chemokines and over 20 chemokine receptors have been identified. Chemokines are classified according to the position of two cystein (C) residues that lie close to the N-terminus region of the protein. The four chemokine subgroups are CXC, CC, XC, and CX3 (Onuffer and Horuk 2002). CXC chemokines attract neutrophils, whereas CC chemokines are less selective and attract lymphocytes, monocytes, basophils, and eosinophils. Chemokines are generally classified as either inducible (inflammatory), expressed under inflammatory stimuli, or constitutive (homeostatic), such as controlling cell trafficking

and homing. Chemokines have a crucial role in guiding the migration of various cell types. Together, both physiological and pathological properties have suggested potential chemokine-based therapeutic possibilities (Luster 1998, Rossi and Zlotnik 2000).

Chemokines have a central role in inflammatory responses. Chemokines are locally retained on cell-surface heparan sulfate proteoglycans, establishing chemokine concentration gradients surrounding the inflammatory stimulus, as well as on the surface of the overlying endothelium. Leukocytes rolling on the endothelium in a selectin-mediated process are brought into contact with chemokines. Chemokine signaling activates leukocyte integrins, leading to firm adherence and extravasation. The recruited leukocytes are activated by local proinflammatory cytokines and may become desensitized to further chemokine signaling because of high local concentrations of chemokines. Chemokines are removed from the circulation, which helps in the maintenance of a tissue–bloodstream chemokine gradient.

Th1 cells more frequently express CXCR3, CXCR6, and CCR5 chemokine receptors than Th2 cells. CXCR3 expression is dependent on T-bet expression, and has mostly been associated with Th1 immune responses and Th1-associated diseases. In contrast, Th2 cells are associated with the increased expression of CCR3, CCR4, and CCR8 (Bonecchi *et al.* 1998). Many chemokine receptors have been identified on $CD4^+$ regulatory T cells, including CCR4, CCR5, and CCR8 (Sallusto *et al.* 2000). All the Th17 cells express CCR6, which is associated with mucosal homing (Singh *et al.* 2008).

2.9 Gut immune system

The gut-associated lymphoid tissue (GALT) is the largest immune system compartment in the body. The physiological role of the GALT is the ingestion of nutritionally important molecules and protection of the host from ingested pathogens. The microbiota in the intestine is an additional major source of natural antigenic stimulation. The sites important for the development of immune responses in the gut are Peyer's patches, which are organized lymphoid tissues in the submucosa, and mesenteric lymph nodes. In addition, lymphocytes exist throughout the epithelium and lamina propria of the mucosa. A single layer of epithelial cells separates the gut microflora from the gut immune system. Antigens must cross the layer of mucus and then the intestinal epithelial cell barrier to induce a mucosal immune response. Antigens are taken up through a variety of mechanisms, including specialized epithelial cells called M cells associated with Peyer's patches, and columnar epithelial cells. In addition, DC sample the luminal content by extending their processes through the epithelium (Mowat 2003). It is now well established that oral (mucosal) antigen administration induces Tregs such as $CD4^+CD25^+$ FOXP3 expressing iTregs, $CD25^+Foxp3^+$ natu-

ral Tregs, Tr1 cells, Th3 TGF-β dependent Tregs, and $CD8^+$ Tregs, which suppress immune responses to mucosal antigens.

Humans live in a symbiotic relationship with a number of microorganisms, such as the mucosal microbiota. The majority of these organisms are bacteria, and it is estimated that the average human microbiome contains 10^{14} bacteria. The epithelial surfaces (skin, airways) and especially the intestine are colonized by the largest number of bacteria. The human intestinal microbiota is mainly composed of the Gram-positive *Firmicutes* and *Actinobacteria*, and the Gram-negative *Bacteroidetes* and *Proteobacteria.* The *Firmicutes* is the largest bacterial phylum, comprising over 200 genera, including *Lactobacillus* and *Clostridium* species. *Firmicutes* and *Bacteroides* are the two most prominent phyla and represent 90% of the total gut microbiota. However, differences in the proportions of these bacterial phyla exist between individuals, as well as over time in an individual (Rajilic-Stojanovic *et al*. 2007). The functions of the microbiota include metabolic functions such as the fermentation of non-digestible dietary substances and vitamin synthesis, the barrier effect, which protects against pathogens, and the control of the immune homeostasis of the gut.

2.10 Maturation of the immune system

The immune system undergoes a huge transition at birth, when adapting from the sheltered intra-uterine entity into a new environment followed by age-dependent maturation. The fetal and neonatal immune system is under physiological demands such as protection against infection, including viral and bacterial pathogens at the maternal-fetal interface (McDonagh *et al.* 2004), avoidance of potentially harmful pro-inflammatory/Th1-cell-polarising responses that could induce alloimmune reactions between the mother and fetus (Halonen *et al*. 2009), and the transition of infant from the normally sterile intrauterine environment to the foreign antigen-rich environment of the outside world, including colonization of the skin and intestinal tract by microorganisms (Karlsson *et al*. 2002).

2.10.1 Prenatal maturation

A successful pregnancy requires that the maternal immune system does not reject a genetically different fetus. It is not fully understood how immunological unresponsiveness is achieved during pregnancy. Normal fetal development occurs in a Th2-biased environment at the maternal–fetal interface. Excessive production of IFN-γ at the feto–maternal interface is associated with fetal loss. Immunosuppression is mediated by the activation of Th2 cytokine production and the suppression of Th1 cytokine production for the maintenance of successful pregnancy (Lin *et al*. 1993, Wegmann *et al*. 1993).

It is well established that maternal IgG molecules are actively transferred to the fetus. Maternal immunoglobulin G (IgG) concentrations in fetal blood increase during the pregnancy, most antibodies being acquired during the third trimester. It is also known that IgG1 is the most efficiently transported subclass and IgG2 the least (Simister 2003). The mechanisms of IgG transport are not fully understood (Simister and Story 1997). The transport pathway across the syncytiotrophoblasts of the chorionic villi (first cellular layer) is dependent on neonatal Fc receptors. Immune complexes are absorbed in the stroma of the villi. T cells in humans can be detected as early as from the gestational week 10 in the primary lymphoid organs, thymus and bone marrow. T cells mature in the thymus and B cells mainly in the liver and bone marrow. Mature lymphocytes migrate to secondary lymphoid tissues (spleen, lymph nodes, tonsils, Peyer's patches and lamina propria), where they respond to antigens. In contrast, in mice the adaptive immune system only starts to develop around birth (Haynes *et al.* 1988, Holt and Jones 2000).

It has been considered for a long time that a neonate is immunologically naive and the development of antigen-specific immune responses is restricted to the period after birth. However, *in utero* exposure to environmental antigens has been documented in both cord blood and amniotic fluid. A number of studies have reported that newborn infants can already perform antigen-specific T cell reactivity to exogenous antigens such as dietary and inhalant allergens and microbial antigens at birth (Szépfalusi *et al*. 2000, Holloway *et al.* 2000, Warner and Warner 2000, Legg *et al.* 2002). It has been demonstrated that placental transport of ovalbumin and β-lactoglobulin takes place (Edelbauer *et al.* 2004). Lymphocyte stimulation studies have shown that cord blood mononuclear cells are able to produce cytokines in response to specific allergens (Prescott *et al.* 1998). These immune responses indicate intrauterine sensitization and priming of the fetal immune system. In addition, antigen priming has been implicated to occur in the fetal gut (Jones *et al*. 2001).

Regulatory T cells are now considered as key mediators of immunological tolerance in the fetus. Mold *et al.* showed that human T cells arise from different hematopoietic stem and progenitor cell populations during different stages of development and that fetal $CD4^+$ T cells are biased towards immune tolerance (Mold *et al.* 2010). In the fetus, $CD4^+CD25^+$ thymocytes already have the potential to suppress the proliferation of $CD25^-$ cells. After leaving the thymus, $FOXP3^+CD4^+CD25^+$ Tregs enter the fetal lymph nodes and spleen, where they acquire a primed/memory phenotype and play an immunoregulatory role in intrauterine life (Takahata *et al*. 2004, Cupedo *et al.* 2005, Michaëlsson *et al.* 2006).

2.10.2 Postnatal maturation

During early life, the immune responses are characterized by immature cellular and humoral functions, and neonates are particularly susceptible to infections that only seldom affect adults. This is related to deficient activation of the adaptive immune system, including the function of effector and memory cells (Bach 2002, Adkins *et al.* 2004). A preponderance of Th2 responses in neonates is perhaps one of the best-characterized immunological differences when compared to adults. After birth, APC are critical for balancing the postnatal Th2 response and Th1 immune responses. The co-stimulatory signals for the induction of Th1 cells are defective. APCs secrete little IL-12 and inefficiently drive the immune response towards Th1 type cells. It has been indicated that selective IFN-γ gene expression in T cells in early postnatal life is due to hypermethylation of CpG sites in the proximal promoter, which results in a reduced capacity to transcribe IFN-γ-specific mRNA. In contrast, hypermethylation of the IFN-γ promoter region in adult cells has not been detected (White *et al.* 2002).

Tregs are suggested to be less mature in early life than in adulthood. Cord blood $CD25^+$ T cells differ from adult $CD25^+$ T cells in their expression markers of naive T cells (90%), including $CD45RA^+$ and CD62L (Wing *et al.* 2002). Freshly isolated cord blood $CD25^+$ T cells are able to suppress T cell proliferation (Takahata *et al.* 2004). It has been demonstrated that cord blood-derived Tregs are able to become highly suppressive upon antigen exposure (Mayer *et al*. 2012). In cord blood, FOXP3 demethylation is very low in $CD4^+CD25^-$ T cells and high in $CD4^+CD25^{hi}$ T cells. Thus, from early life, FOXP3 methylation seems to be a stable parameter for Treg assessment (Liu *et al.* 2010). In addition, Th17 cells have been shown to have regulatory properties early in life (Schaub *et al.* 2008).

Early in the maturation of immune system, innate responses are important because of the natural unresponsiveness of the adaptive response. Neonates have a lower percentage of DC and specific $CD14^+$ monocyte subsets with a lower number of co-stimulatory signals (Encabo *et al*. 2007). In addition, the phenotype of fetal and infant DC is skewed towards immaturity when compared with adult DC (Holloway et al. 2009). Furthermore, it has been found that CB DC secrete less TNF-α and IFN-α after stimulation than PB DC. Plasmacytoid and mDC responses to TLR4 agonists (bacterial lipopolysaccharide) and TLR9 agonists (CpG oligonucleotides) are lower in the neonate and infant when compared to adult responses and increase during the first year of life (Nguyen *et al.* 2010). Other studies, however, have suggested that CB DC have better antigen-presenting capabilities than peripheral blood DC. The mature Th1/Th2 balance is acquired by the naive immune system through exposure to microbial antigens during infancy.

2.10.3 Postnatal development of the gut immune system

Both endogenous and exogenous factors drive the development and maturation of the intestinal immune system. The formation of secondary lymphoid structures, such as Peyer's patches and mesenteric lymph nodes, occurs before birth. Later development depends on the postnatal microbial colonization of the gut. The gut harbors multiple subsets of lymphocytes, including Tregs. Therefore, different bacterial strains may differently stimulate lymphocytes that exist in the gut lymphoid tissues (Ivanov *et al.* 2008, Bilate and Lafaille 2012). Oral and nasal administration of antigens has been shown to favor the induction of tolerance to specific antigens.

The bacterial colonization of the infant gut occurs in the first year of life. It is influenced by several factors such as the route of delivery. Vaginal delivery exposes the neonate to the mother's vaginal and intestinal flora. In contrast, the intestinal microbiota of infants born by caesarean section is characterized by skin flora, and shows a lower abundance of *Bifidobacterium* and *Bacteroides* species and more frequent colonization by *Clostridium difficile* (Bennet and Nord 1987, Grönlund *et al.* 1999). Other factors that can have an impact on the composition of the intestinal microbiota in infants are prematurity, the general level of hygiene, antibiotics, and the infant diet. *Bifidobacteria* almost always dominate the microbiota of breast-fed infants. Exclusively formula-fed infants were more often colonized with *E. coli*, *C. difficile*, *Bacteroides*, and *lactobacilli*, as compared with breast-fed infants (Penders *et al.* 2006).

2.11 Type 1 diabetes

2.11.1 Historical aspects

Diabetes mellitus has been known for thousands of years to be a lethal disease in which the patient's flesh and limbs melt into urine that has a sweet taste. The first reference to diabetes, the disease associated with polyuria, goes back to 1552 BC to ancient Egyptian physicians (the Ebers Papyrus). The name diabetes was first used around 250 BC and is a Greek word meaning "to siphon". In 1675, the term Diabetes Mellitus was first used by Thomas Willis in England after finding the sweet taste in the urine of patients with the disease. Mellitus means "honey" in Latin and refers to the glucose in the urine. In 1889, the German physicians von Merring and Minkowski demonstrated that removal of the pancreas from a dog results in diabetes. The surgeon Frederick Banting, with help from the medical student Charles Best in the laboratory of Professor John Macloed in Canada, succeeded in isolating pancreatic extracts from dogs. The life expectancy of diabetic patients was less than a year from diagnosis until insulin was identified. In 1921, insulin was isolated from a dog's pancreas and one year later the first diabetic teenage patient successfully received insulin

treatment in Toronto. In 1923, Banting and Macleod received the Nobel Prize for the discovery for insulin (Banting *et al.* 1922, Bliss 2007). During the next decades, the heterogenic nature of diabetes was discovered. The immunological response that generates an inflammation (insulitis), followed by the death of insulin-secreting β-cells in the pancreas, was eventually proposed by Gepts in 1965 (Gepts 1965). By 1974, T1D as an autoimmune disease was confirmed by the presence of islet cell-specific autoantibodies (Bottazzo *et al.* 1974, MacCuish *et al.* 1974) and the genetic association of T1D with the human leukocyte antigen (HLA) in T1D patients and in individuals susceptible to the disease (Nerup *et al.* 1974).

2.11.2 Classification of diabetes in children

According to the World Health Organization (WHO), diabetes mellitus is defined as "a group of metabolic diseases characterised by chronic hyperglycemia with disturbance of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both." According to current classifications recommended by the WHO Expert Committee and the American Diabetes Association, diabetes mellitus can be divided into four clinical categories: type 1 diabetes, type 2 diabetes, gestational diabetes mellitus and other specific types such as genetic defects of beta-cell function, diseases of the exocrine pancreas, and diabetes induced by drugs, chemicals, or infections (American Diabetes Association 2010, Craig *et al.* 2009).

T1D can be classified into two subclasses: type 1A diabetes and rare type 1B diabetes. Type 1A diabetes results from the autoimmune destruction of β-cells in the pancreas. Polyuria, polydipsia, fatigue, and weight loss are the classic symptoms of diabetes. The function of insulin is to transport glucose into the target cells. The symptoms appear when the insulin-secretion capacity is 20% or less of normal level. If symptoms are present, elevated plasma glucose can be measured randomly, and a concentration of ≥11.1 mmol/l is considered diabetic. A fasting glucose concentration of ≥7.0 mmol/l in a plasma sample also indicates diabetes. In addition, the diagnosis of diabetes can be based on the oral glucose tolerance test (OGTT), in which the 2-h plasma glucose level is ≥11.1 mmol/l following the oral ingestion of 75 g of glucose. For an asymptomatic patient, the hyperglycemia should be measured on at least two separate occasions. Finally, the individuals have an absolute deficiency of insulin secretion, and life-long insulin replacement is required (American Diabetes Association 2010, Daneman 2006). Type 1B diabetes is referred to as idiopathic diabetes because the etiology of the β-cell destruction is unknown. It has been reported in individuals from Africa and Asia. These patients lack immunological evidence of β-cell autoimmunity, and no HLA associations exist, but they have insulin deficiency and are prone to ketoacidosis.

In addition to T1D, there are several other rare forms of diabetes diagnosed during the first decades of life. Maturity-onset diabetes of the young (MODY) is characterized by impaired insulin secretion, an autosomal dominant mode of inheritance and onset before the age of 25 years (Fajans *et al.* 2001). Another monogenic defect in the β-cell function is mitochondrial diabetes. It is characterized by point mutations in mitochondrial DNA. It is maternally inherited and associated with deafness (Reardon *et al.* 1992, van den Ouweland *et al.* 1992). Transient or persistent neonatal diabetes (TNDM and PNDM, respectively) is insulin-requiring diabetes that is usually diagnosed in infants before the age of six months (Aguilar-Bryan and Bryan 2008).

2.11.3 Epidemiology of type 1 diabetes

The highest incidence of T1D among children younger than15 years in the world is found in Finland (Onkamo *et al.* 1999, EURODIAB ACE Study Group 2000, Karvonen *et al.* 2000, Patterson *et al*. 2009). In Finland, the first nationwide study in 1953 showed an annual rate of 12/100 000 (Somersalo 1954). Since then, the annual incidence has gradually increased, from 31 per 100 000 per year in 1980 to 64 per 100 000 per year in 2005 (Harjutsalo *et al.* 2008). Approximately 600 new children in Finland are affected annually by T1D.

The global variation in incidence of T1D in children under the age of 15 years is significant. According to the WHO DIAMOND report covering the time period 1990–1999, over 400-fold differences have been reported from different populations: the lowest incidence from China and Venezuela (0.1/100 000 per year) and highest incidence from Sardinia (37.8/100 000 per year) and Finland (40.9/100 000 per year) (DIAMOND Project Group 2006). In Europe, the incidence of T1D differs between countries by more than ten-fold (Soltesz *et al.* 2007). The EURODIAB registry data during the years 1989–1994 demonstrated a high incidence (>20 per 100 000 children per year) in Finland, Sardinia, Sweden, Norway, and the United Kingdom. In contrast, in Eastern Europe the incidence is less than in other parts of Europe (3.2 in Macedonia, 6.7 in Poland and 6.6/ 100 000 children per year in Latvia) (EURODIAB ACE Study Group 2000, Patterson *et al.* 2009). The incidence varies significantly from one country to another in the same geographical region. In Estonia, for example, the incidence is 2–4 times less than in the Nordic countries (Podar *et al.* 2001). A study in the Karelian Republic of Russia indicated a six-fold higher incidence of T1D in Finland than in Russian Karelia (Kondrashova *et al.* 2005).

Altogether, the incidence of T1D has risen significantly over the last decades and the average increase per year has been 3.0% worldwide (DIAMOND Project Group 2006, Onkamo *et al.* 1999). Previously the peaks of onset occurred in two age groups: at 5 to 7 years and at the time of puberty (12–13 years of age). Strikingly, the most rapid increase in incidence rate was seen in the youngest age

group: an incidence increase of 5.4% was observed among 0- to 4-year-old children, whereas 4.3% and 2.1% increases were observed among 5- to 9- and 10- to 14-year-old children, respectively. However, this rapid rise of T1D among the youngest age group was particularly marked for regions in Central and Eastern Europe (Patterson *et al.* 2009). In Finland (during the period 1980–2005), the age-specific rates per 100 000 per year were 31.0, 50.5, and 50.6 at ages of 0–4 years, 5–9 years, and 10–14 years, respectively (Harjutsalo *et al.* 2008). In addition, in young adults (15- to 39-year-old population), an increasing trend of 3.9% per year in the incidence of T1D has been reported in Finland (Lammi *et al.* 2007, Lammi *et al*. 2008). Thus, the risk of T1D in the Finnish population extends into adulthood. In high-incidence countries, as in Finland, T1D also occurs more frequently in boys than in girls. The male/female ratio is reported to be 1.1 (1.7 at the age of 13 years) (DIAMOND Project Group 2006, Harjutsalo *et al.* 2008). Seasonal variation in the incidence of T1D also exists, with a peak in the winter months, whereas lower incidence rates are more typical during the warm summer months, both in the northern and southern hemisphere (Levy-Marchal *et al.* 1995, Moltchanova *et al.* 2009).

2.11.4 Autoantibodies in T1D

Already before the onset of clinical T1D, autoantibodies against β-cell antigens can be detected. The most common β-cell autoantibodies associated with T1D are islet cell antibodies (ICA), insulin autoantibodies (IAA), antibodies specific to the 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 first beta-cell autoantibody detected was ICA in 1974 (Bottazzo *et al.* 1974, MacCuish *et al.* 1974) using frozen, unfixed human pancreas sections and indirect immunofluorescence. IAA were first found in 1983 among patients with newly diagnosed T1D before treatment with exogenous insulin (Palmer *et al.* 1983). Glutamic acid decarboxylase (GADA65) is an enzyme that catalyzes the synthesis of γ-aminobutyric acid (GABA). There are two distinct isoforms, GADA65 and GADA67. The enzyme is expressed in the pancreatic islets and the central nervous system (Baekkeskov *et al.* 1990). GADA are found in patients with newly diagnosed T1D and also in patients with stiff-man syndrome. GADA are more prevalent in diabetic children with the HLA DR3 genotype, T1D patients diagnosed in adolescence and in patients with LADA (Rowley *et al.* 1992). Autoantibodies against tyrosine phosphatase-related protein (IA-2A) were reported in 1995 (Payton *et al.* 1995). The majority of newly diagnosed T1D patients have IA-2A, and since these autoantibodies usually appear near the diagnosis, IA-2A can be used to predict the onset of the disease. Autoantibodies specific against zinc transporter 8 are the latest discov-

ery, and their role in the prediction of diabetes is not yet understood (Chimienti *et al.* 2004).

The number of detectable autoantibodies is related to the risk of progression to T1D both in family studies and also in general population cohorts. In family studies, among first-degree relatives, positivity for two to four autoantibodies is associated with a risk of developing clinical T1D in the range of 60–80% over the next 5 years (Verge *et al.* 1996). Several studies have shown that β-cell autoimmunity may be induced early in life. Data from the Finnish Diabetes Prediction and Prevention (DIPP) Study have shown that the first autoantibodies may already appear before the age of 3 months, although this is not common. The autoantibodies are shown to appear in clusters, and IAA most commonly emerges as the first autoantibody, usually followed in order by GADA, ICA, and IA-2A (Kupila *et al.* 2002). In the general population, in children with a HLA-associated genetic T1D risk, the combination of persistent ICA and IAA positivity resulted in the highest positive predictive value (91.7%) and 100% specificity. A young age at seroconversion, high ICA level, multipositivity, and persistent positivity for IAA are indicated as significant risk markers for T1D (Siljander *et al.* 2009).

During pregnancy, T1D-related IgG antibodies are actively transported through the placenta. Accordingly, newborn infants of mothers with T1D have been shown to have insulin antibodies (IA) and other disease-associated antibodies in their circulation because of transplacental transfer from the maternal circulation (Tingle *et al.* 1979, Ziegler *et al.* 1993). Most of the transplacentally transferred antibodies disappear from the infant's circulation during the first months of life, so that only 20–30% of the antibodies are still detectable at the age of 3 months and very low levels at 9 months, but antibodies can occasionally be seen up to the age of 12 months. Also, the higher the initial antibody level, the longer is the elimination time of maternal autoantibodies in infants (Martikainen *et al.* 1996, Hämäläinen *et al.* 2000).

2.11.5 Immune response to insulin

Insulin is a protein hormone with a molecular weight of 5.8 kD. It is composed of two separate chains, the A chain with 21 amino acids and the B chain comprised of 30 amino acids, which are joined by two disulfide bridges. The initial mRNA transcript for insulin is for preproinsulin. After the signal sequence is cleaved, proinsulin is packaged into secretory granules for secretion from β-cells. Insulin is processed from proinsulin, consisting of A- and B-chains and a C-peptide. Inside secretory granules, insulin is formed when C-peptide is cleaved from the proinsulin molecule. Insulin is stored in the secretory granules of β-cells in the pancreas, and its secretion is stimulated by increased glucose levels in the blood and the parasympathetic nervous system. Insulin is the only

known β-cell-specific autoantigen (Jasinski and Eisenbarth 2005). In addition, it is expressed in lymphoid tissues, including the thymus, where insulin is expressed as a self-antigen without hormonal importance.

Several facts support the view that insulin may be an important and even crucial antigen in the initiation of β-cell autoimmunity. Firstly, insulin autoantibodies (IAA) have been observed to often appear as the first autoantibody in the preclinical period of T1D (Kimpimäki *et al.* 2001). Secondly, the age at onset of T1D has been demonstrated to correlate inversely with IAA. IAA often appear in children who develop diabetes prior to the age of five (Vardi *et al.* 1988). Thirdly, insulin is the only β-cell-specific autoantigen identified so far. T cell responses to human insulin and proinsulin have been reported in prediabetic individuals and in patients with recently diagnosed T1D (Gottlieb and Eisenbarth 2002).

Both cellular and humoral autoimmune responses to insulin have been described in NOD mice. IAA are detected at an early age and has been associated with the early development of spontaneous autoimmune diabetes in a NOD mouse model (Yu *et al.* 2000). Insulin-specific T cells from infiltrated islets can transfer the disease to the recipient mice (Daniel *et al.* 1995). There is also strong evidence that insulin is targeted by CD4 and CD8 cells in the early stages of NOD diabetes, and T cells reactive against proinsulin and insulin epitopes have been described. Various experiments have identified the following CD4 epitopes in mice: B-chain peptide including amino acids 2–16 (B:2–16) (Halbout *et al.* 2002), A:7–21 (Daniel and Wegmann 1996), A:1–15 (Halbout *et al.* 2002), and B:9–23 (Abiru *et al.* 2000). Many putative epitopes of proinsulin/insulin have recently also been identified in humans (Di Lorenzo *et al.* 2007, Zhang *et al.* 2008) (Figure 3). $CD8^+$ T cells as well as $CD4^+$ T cells are required for the development of T1D, and CD8 T cells may be particularly important for the initiation of the disease. CD8 T cell clones that bind B:15–23 can transfer disease in mouse models (Wong *et al*. 1999).

Interestingly, the administration of insulin or insulin peptides via a nasal (Harrison *et al.* 1996), subcutaneous (Daniel and Wegmann 1996), or oral route (Zhang *et al.* 1991) has been reported to prevent or reduce the incidence of autoimmune diabetes in young NOD mice. On the other hand, acceleration of diabetes has also been observed when insulin is given via mucosal surfaces. In animal models, oral insulin has been studied as an inducer of oral tolerance in the strategy to prevent T1D. Mucosal autoantigen administration is a double-edged sword and can lead not only to regulatory and protective immunity but also to pathogenic, tissue-destructive immunity and exacerbation of autoimmune disease (Blanas *et al.* 1996, Hänninen *et al*. 2001, Hänninen and Harrision 2004).

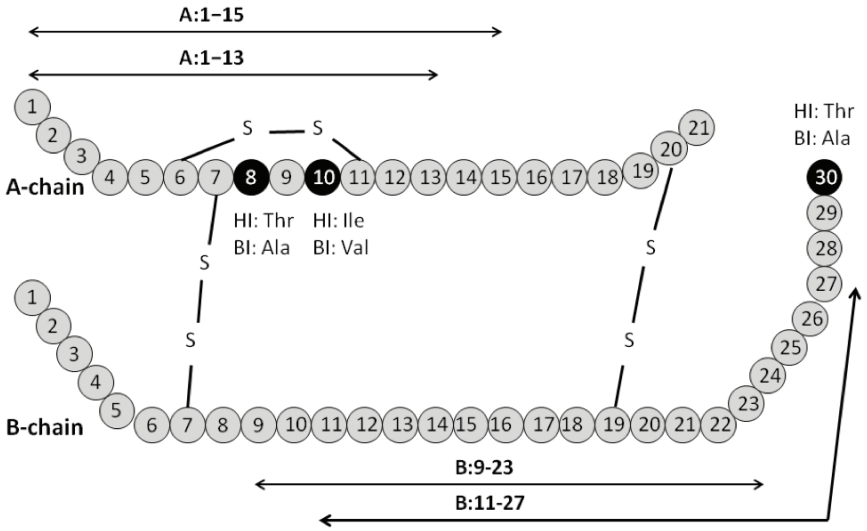

Figure 3. Schematic diagram of the structure of human insulin. Bovine insulin differs from human insulin by three amino acids: human insulin (HI) has threonine and bovine insulin (BI) alanine in position 8 of the A-chain, the substitutions of isoleucine (HI) by valine (BI) in position 10 of the A-chain, and theorine (HI) by alanine (BI) in position 30 of the B-chain. The CD4 T cell epitopes for human insulin are shown.

In humans, it has been difficult to develop T cell assays for the detection of auto-antigen-specific responses. Disease specificity of peripheral blood T cell responses to insulin has not been demonstrated. However, T cells from pancreatic lymph nodes have been reported to consist of insulin-specific T cells in T1D patients but not in controls (Kent *et al.* 2005).

Dietary bovine insulin has been shown to induce insulin-specific immunity in infants. Bovine insulin is immunogenic in humans when used in the treatment of patients with T1D. Bovine insulin differs from human insulin by three amino acids (A8, A10, and B30) (Figure 3). It has been reported that exposure to bovine insulin in CM induced insulin-binding antibodies and T cell responses to insulin in formula-fed infants when compared with exclusively breast-fed infants. The immune responses declined with age, indicating the development of tolerance towards insulin. Also, the antibodies binding to bovine insulin cross-reacted with human insulin. In children carrying a HLA risk genotype, who later developed β-cell autoantibodies, bovine insulin-binding antibodies increased steadily towards the age of 18 months, and there was no indication of the devel-

opment of tolerance. This suggested that the early insulin-specific immune response is not normally regulated in those children who develop islet cell-related autoimmunity (Vaarala *et al*. 1999, Paronen *et al*. 2000).

2.11.6 Genetic risk factors of T1D

It has been known for a long time that the genetic background of an individual is essential in the development of T1D. Disease concordance is relatively high among identical twins, and the risk of developing T1D is higher in the first-degree relatives of affected individuals than in the general population. In the monozygotic twins of T1D patients the concordance rate for T1D has been around 40−65% depending on the follow-up time (Kyvik *et al.* 1995, Redondo *et al.* 2008) and the genetic risk of T1D (Johnston *et al.* 1983, Metcalfe *et al.* 2001). It has been shown that monozygotic twins with the HLA DR3/DR4 genotype have a higher risk of progression to diabetes and also of developing islet cell autoantibodies (Johnston *et al.* 1983, Redondo *et al.* 1999). An early onset of diabetes increased the risk in co-twins (Kumar *et al.* 1993, Hyttinen *et al.* 2003). In contrast, in dizygotic twins the average risk has been estimated to be 6−10%. This is similar to the risk observed among siblings of an affected child (Hyttinen *et al.* 2003, Harjutsalo *et al.* 2005), which is more than 10 times higher than the lifetime risk (0.4−1.0%) for T1D among the general public (Risch 1987).

TID is clustered in families. The risk of developing T1D in the offspring of affected fathers is greater than the risk in offspring of mothers with T1D. It has been reported that the offspring of affected mothers have a risk of about 2−3%, whereas the risk is 5−6% in the offspring of affected fathers (Warram *et al.* 1984, El-Hashimy *et al.* 1995, Lorenzen *et al.* 1998, Harjutsalo *et al.* 2006, Harjutsalo *et al.* 2010). However, only a minority of children diagnosed with T1D (about 10%) have an affected family member at the time of diagnosis of T1D.

2.11.6.1 Human leukocyte antigen (HLA) gene region

A recent genome-wide association study combined with meta-analysis of previous studies has shown that there are more than 40 genetic polymorphisms conferring susceptibility to T1D. The most important part of genetic susceptibility to T1D is associated with the HLA class II region on chromosome 6p21, and these genes are responsible for approximately 40–50% of the genetic T1D risk (Barrett *et al.* 2009). The HLA region is one of the most polymorphic regions of the human genome. Certain HLA class II haplotypes are strongly associated with genetic susceptibility to T1D. Some haplotypes confer weak disease predisposition and some are protective, whereas some are considered as neutral in relation to the disease risk. The complex influence of HLA class II gene products on the risk of T1D is not exactly known, but it is probably related to their peptide-binding characteristics in antigen presentation and the activation of T

cells. The strongest susceptibility has been linked to HLA-DQB1 and HLA-DQA1 genes. In addition, the risk is modified by HLA-DR alleles that are in linkage disequilibrium with HLA-DQ alleles. The ultimate risk of T1D is determined by the HLA genotype defined by the combination of susceptible and protective alleles inherited from both parents (Ilonen *et al.* 2002).

The highest risk genotype of T1D among the Caucasian population is heterozygous for DR3,DQ2/DR4,DQ8 (HLA-DRB1*0301 with DQB1*02,DQA1 *0501/HLA-DRB1*04 with DQB1*0302,DQA1*0301). The risk is increased for subjects carrying either of the haplotypes, DR4,DQ8 showing a higher risk than DR3,DQ2 (Sheehy *et al.* 1989). Disease protection is associated with the DQB1*0301, DQB1*0602, and *0603 alleles. Strong genetic disease susceptibility is associated with an earlier appearance of autoimmunity to islet cell antigens and earlier progression of T1D. It has been reported that the proportion of patients carrying protective haplotypes (including DQB1*0602 and *0603) is significantly higher among patients diagnosed during recent years than among those diagnosed 50 years ago (Hermann *et al*. 2003). This may reflect an increasing environmental pressure allowing the presentation of clinical disease with a weaker genetic predisposition than previously.

2.11.6.2 Insulin gene polymorphism

The human insulin gene (*INS*) is located on chromosome 11p15, and its polymorphism has been linked with susceptibility to T1D (Bell *et al.* 1984). The susceptibility gene locus has been localized to a region comprised of variable nucleotide tandem repeats (VNTR) in the promoter of the INS gene, which is important for regulating how much INS is produced. Alleles in this region are divided into three classes distinguished by the number of DNA base pair repeats. There is a higher frequency of class I alleles in patients with T1D, whereas protection from T1D is associated with class III alleles. The intermediate class II alleles are rare in non-African populations (Bennett *et al*. 1995). Of the single nucleotide polymorphisms (SNPs) described, the -23HphI A allele is linked to class I VNTR, whereas the T allele is linked to class III VNTR. The relative risk ratio of the INS VNTR I/I genotype vs. the heterozygous INS VNTR I/III or protective III/III has been found to be moderate (in the range 3–5), and it accounts for about 10% of the familial clustering of T1D.

In studies on INS gene expression, it has been shown that the class I allele is associated with higher INS expression in the pancreas when compared with class III alleles. In contrast, in the thymus, the INS VNTR III genotype is associated with a high expression of proinsulin in thymic medullary epithelial cells, whereas class I alleles are expressed at lower levels. This is likely to alter the selection of T cells in the thymus. Self-antigens are expressed during negative selection of the lymphocytes in the thymus, under the control of the AIRE gene (Villasenor *et al.* 2005). Reduced insulin expression by thymic medullary

epithelial cells has been suggested to have a role in T1D through the impaired deletion of autoreactive T cells and decreased induction of insulin-specific nTregs and insulin tolerance (Vafiadis *et al*. 1997, Pugliese *et al.* 1997, Pugliese *et al*. 2001). It has been reported that the number of detectable proinsulin tetramer positive T cells was increased (79% vs. 29%, respectively) in peripheral lymphocytes of subjects carrying INS VNTR I/I when compared to subjects with INS VNTR III/III (Durinovic-Bello *et al*. 2010).

2.11.6.3 PTPN22 gene polymorphism

The protein tyrosine phosphatase 22 gene, PTPN22, is located on chromosome 1p13 and encodes a lymphoid protein tyrosine phosphatase (LYP). It was found to be associated with susceptibility to T1D, as well as with other autoimmune diseases (Cohen *et al*. 1999). The main function of LYP is to reduce TCR-mediated signaling. It is accomplished by direct dephosphorylation of the Src family kinases Lck and other signaling molecules, such as Fyn, ITAMs of the TCRζ/CD3 complex, as well as ZAP-70 (Bottini *et al.* 2006). The autoimmunity-predisposing allele of PTPN22 is a missense C–T mutation at position 1858, which changes amino acid residue 620 from arginine to tryptophan in the encoded LYP protein (Bottini *et al.* 2004). This T1D-associated allele is relatively common in the general population. Approximately 10% of healthy subjects in Northern European populations carry a polymorphism of the PTPN22 gene (1858C/T), whereas it is present in 17% of T1D patients. The PTPN22 620Trp disease variant has been demonstrated to reduce IL-2 production and calcium mobilization in activated $CD4^+$ cells (Vang *et al*. 2005, Rieck *et al*. 2007, Aarnisalo *et al*. 2008). It has been reported to be associated with an increased risk of Gram-positive bacterial diseases, especially pneumococcal diseases, in subjects with the PTPN22 1858T allele (Chapman *et al.* 2006). On the other hand, a reduced risk of tuberculosis has been suggested to be linked with the T1D-associated variant of the PTPN gene (Gomez *et al*. 2005).

2.11.7 T1D and autoimmunity

T1D is caused by autoimmune destruction of the insulin-producing β-cells in the pancreas. Insulitis, i.e. infiltration of the pancreatic islets by cells of the immune system, was first described by Gepts in 1965 in the pancreatic islets of young patients with T1D (Gepts 1965). The pathogenesis of T1D is characterized by infiltration of mononuclear cells such as $CD8^+$ cytotoxic T cells, $CD4^+$ helper cells, macrophages, monocytes, natural killer cells, and B cells into the islets, of which the $CD8^+$ cells seem to predominate in the infiltrations (Bottazzo *et al* 1974, Itoh *et al*. 1993). In the 1970s, the autoimmune nature of T1D was confirmed when both autoantibodies and autoreactive T cells against cells were demonstrated in the circulation of patients with T1D (Bottazzo *et al.* 1974, MacCuish *et al.* 1974). The aberrant hyperexpression of MHC Class I and II

molecules on cells of insulin-positive islets has also been shown, suggesting the immunological mechanisms in the pathogenesis of T1D (Bottazzo *et al*. 1985, Foulis *et al*. 1987). The β-cells are considered to be destroyed by autoreactive cytotoxic effector T cells using granzyme- and perforin-dependent mechanisms of apoptosis or the secretion of pro-inflammatory cytokines (IL-1β, TNF-α, IFN-γ) by cells infiltrating the islets (Moriwaki *et al.* 1999).

T1D is considered to be a T cell-mediated autoimmune disease. Animal models strongly support the T cell-mediated initiation of β-cell destruction shown in non-obese diabetic (NOD) mice and BioBreeding (BB) diabetes-prone rat models, in which the spontaneous development of autoimmune diabetes is seen. In studies on the pathogenetic mechanisms of autoimmunity, it was first demonstrated that the transfer of splenocytes from diabetic animals could induce diabetes in healthy NOD mice (Wicker *et al*. 1986). Later, it was shown that in NOD mice T1D can be transferred from a diabetic to an unaffected mouse with T-lymphocytes (Bendelac *et al*. 1987). Autoimmune diabetes does not develop in NOD mice that are athymic or lymphopenic, or are thymectomized at birth (Adorini *et al.* 2002). In an animal model, insulitis was characterized by the infiltration of Th1-type lymphocytes with high IFN-γ secretion. In contrast, islets infiltrated by Th2-type lymphocytes with a strong IL-4 response and low IFN-γ secretion resulted in non-destructive peri-insulitis with no β-cell death (Healey *et al*. 1995). In addition, diabetes was prevented by administering antibodies targeting cytokines mainly responsible for modulating T cell responses, particularly Th1-polarizing cytokines such as IFN-γ and IL-12, thus supporting the role of a T cell-mediated autoimmune process (Rabinovitch 1994).

In humans, T1D has been reported to be transferred from a diabetic individual to an unaffected individual by autoreactive cells in bone marrow transplantation (Lampeter *et al*. 1993). T cells infiltrating the pancreatic islets are activated, as shown by increased expression of HLA class II molecules and interleukin IL-2 receptors on their surface. The role of T cells in the pathogenesis of T1D is supported by the observation that in the treatment of T1D, monoclonal antibodies against CD3 induce tolerance and prolong insulin production after diagnosis (Herold *et al.* 2002). However, the role of $CD4^+$ and $CD8^+$ cells during the pathogenesis of T1D is not fully understood. It has been thought that $CD4^+$ Th cells are responsible for the initiation of the pathogenic process in the islets, but cytotoxic $CD8^+$ lymphocytes are responsible for the destruction of β-cells.

Recently, Th17 cells have been demonstrated to be associated with the development of autoimmune diabetes. The exact effect of IL-17 on T1D is still unclear. In NOD mice it has been shown that IL-17 promotes pancreatic inflammation and is up-regulated (Martin-Orozco *et al.* 2009). Moreover, treatment with IL-25, which inhibits Th17 cells, and with neutralizing IL-17 antibodies have been shown to prevent the development of diabetes when given from 10 weeks of age to NOD mice (Emamaullee *et al*. 2009). In humans, upregulation

of Th17 immunity has been demonstrated in peripheral blood T cells from children with T1D. This was characterized by increased IL-17 secretion and expression of IL-17, IL-22, and retinoic acid-related orphan receptor C isoform 2, but also FOXP3 transcripts, upon T cell activation *in vitro*, suggesting the role of IL-17 immunity in the pathogenesis of human T1D (Honkanen *et al*. 2010, Marwaha *et al*. 2010).

2.11.8 Environmental risk factors

The major environmental contributors to the risk of T1D are associated with intestinal immune activation due to dietary or microbial factors and viral infection. The use of a cow's milk-based formula, a short period of breastfeeding and dietary gluten are among the possible nutritional triggers most often suggested as environmental triggers of β-cell autoimmunity.

2.11.8.1 Breastfeeding and cow's milk formula

In the 1980s, the association of cow's milk proteins and T1D was studied in BB rats and the NOD mouse model. Elliot and Martin reported an effect of the manipulation of nutritional proteins on the emergence of diabetes in BB rats (Elliott and Martin 1984). The study showed that the replacement of proteins by semi-synthetic amino acids in the diet reduced the incidence of diabetes from 52% to 15% in contrast to BB rats on a diet supplemented with cow's milk (CM). The exposure to intact CM protein as a risk factor for autoimmune diabetes is further supported by studies on NOD mice. The diabetes incidence was lowered following a hydrolyzed CM protein-based diet (Elliott *et al.* 1988, Coleman *et al.* 1990). Moreover, a relatively narrow time window (weaning period) has been reported for the effect of CM proteins on diabetes induction (Daneman *et al.* 1987). In both animal models, contradictory findings also exist, showing no effect from CM proteins on the incidence of diabetes (Malkani *et al*. 1997, Paxson *et al.* 1997).

The mechanisms by which hydrolyzed formula reduces and foreign proteins (like cow's milk proteins) increase the risk of diabetes-predictive autoantibodies are not known. A recent study showed that casein hydrolyzed formula was associated with a decrease in autoimmune diabetes in the disease-prone BB rat, in association with improved integrity of the intestinal barrier and higher production of regulatory cytokine IL-10 in the ileum, as well as beneficial changes in the gut microflora (increased *Lactobacilli* and reduced *Bacteroides* levels). This suggested that the presence of specific peptides has a beneficial effect in the prevention of autoimmune diabetes (Visser *et al.* 2012).

Several reports have attempted to establish a link between breastfeeding and protection against T1D, but the results have been controversial. There have been studies showing a protective effect, but also reports of no or a positive association (Fort *et al.* 1986, Kyvik *et al*. 1992). In addition, it has been indicated that a

short breastfeeding period (<3 mo) in infancy may be associated with the appearance of β-cell autoimmunity (Gerstein 1994, Holmberg *et al.* 2007). There may be reasons for the contradictory findings with regard to the effect of breastfeeding on the development of β-cell autoimmunity and T1D. Most studies have been performed retrospectively and looked at overall breastfeeding without any differentiation between exclusive and total breastfeeding. In addition, there are differences in breastfeeding practices between various countries, and studies have also lacked appropriate controls matched for the HLA genotype.

The early introduction of CM proteins has also been studied in relation to the risk of β-cell autoimmunity and T1D. In the nationwide "Childhood Diabetes in Finland" (DiMe) study, the age at introduction of supplementary formula feeding was related to the risk of diabetes independently of duration of breastfeeding in the case-control study (Virtanen *et al*. 1993). However, some studies have demonstrated no association between the early introduction of CM and the risk of β-cell autoimmunity (Wadsworth *et al*. 1997, Couper *et al.* 1999). There may be several explanations for the discrepant results. Although CM-based formula is the first complementary food an infant is exposed to, there is variation between countries in the types of complementary foods, e.g. CM formula or cereals that infants are first exposed to.

Increased circulating antibody levels to CM proteins were reported in children with T1D for the first time in 1988, when Savilahti observed that diabetic children had significantly higher levels of serum IgA antibodies to CM and beta-lactoglobulin and of IgG antibodies to beta-lactoglobulin than age-matched control subjects (Savilahti *et al*. 1988). The immunological reactivity to CM proteins may be enhanced in children who progress to T1D, or their gastrointestinal permeability to CM proteins might be increased.

Interestingly, a pilot intervention study, the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) among infants with at least one affected family member and HLA-conferred susceptibility to T1D, reported a decreased cumulative incidence of diabetes-associated autoantibodies in children receiving hydrolyzed casein formula during the first months of life when compared to subjects receiving a CM-based formula (Åkerblom *et al.* 2005, Knip *et al.* 2010). Based on the pilot results, a larger randomized double-blinded trial of the TRIGR is underway.

2.11.8.2 Wheat proteins

Gluten or other cereal-derived proteins have been implicated as potential driving antigens in T1D. An association between T1D and celiac disease has been reported (Mäki *et al.* 1984). Pilot intervention studies have been performed to assess whether gluten elimination modifies the natural course of β-cell autoimmunity. Accordingly, this trial indicated that a gluten-free diet has no effect on the signs of β-cell autoimmunity in first-degree relatives of affected patients

(Hummel *et al.* 2002). However, other studies have shown that the risk of development of islet autoimmunity is increased in children who were exposed to cereal proteins (Norris *et al*. 2003, Ziegler *et al*. 2003) during the first 3 mo of life compared with children who received gluten between 3 and 6 mo of age. Furthermore, the removal of gluten in children positive for diabetes-associated autoantibody did not prevent progression to T1D (Hummel *et al*. 2002).

2.11.8.3 Viral infections

Viral infections have for a long time been implicated in the etiology of T1D. There is some epidemiological evidence that seasonal variation exists in the emergence of β-cell autoimmunity, which could reflect the seasonality of viral infections (Moltchanova *et al.* 2009). Enterovirus infections appear to be the most probable trigger of β-cell autoimmunity. In 1979, a study reported a 10-year-old previously healthy boy presenting with diabetic ketoacoidosis within 3 days of onset of flu-like symptoms. He died seven days later, and Coxsackie B4 virus was isolated in the islets of Langerhans and necrosis of β-cells was demonstrated in the pancreas in the post-mortem examination. Inoculation of the virus into mice produced hyperglycemia, infiltration of inflammatory cells in the islets and β-cell necrosis (Yoon *et al.* 1979). Furthermore, enterovirus protein was detected in histological sections of islets of a beta-cell autoantibody-positive patient (Oikarinen *et al.* 2008). The virus appeared to be located in the islets, while the exocrine pancreas was mostly uninfected.

Several case–control studies have reported increased levels of enterovirus antibodies and enterovirus RNA in the peripheral circulation in patients with T1D (reviewed by Tauriainen *et al.* 2011). Two prospective studies (BABYDIAB in Germany and the Diabetes Autoimmunity Study in the Young [DAISY] in Denver) have failed to demonstrate any association between enterovirus infections and β-cell autoimmunity (Fuchtenbusch *et al.* 2001, Graves *et al.* 2003). Thus, further studies are required to establish a causal association between recent or ongoing enterovirus infection and T1D.

2.11.9 Prevention of T1D

Primary prevention aims to minimize the effect of the factors that initiate the pathogenic autoimmunity towards β-cells in individuals with or without genetic disease susceptibility. The TRIGR study is an example of a primary prevention study. It is a randomized, double-blind intervention trial designed to answer the question of whether weaning to an extensively hydrolyzed formula over the first 6–8 months of life will reduce the risk of T1D later in childhood. The second TRIGR pilot study performed among Finnish infants showed that weaning to a highly hydrolyzed formula reduced by approx. 50% the cumulative incidence of one or more diabetes-associated autoantibodies. This finding was confirmed in a recent follow-up analysis to 10 years of age. The international TRIGR study was

initially started in 2002 to clarify the role of cow's milk formula in the pathogenesis of T1D.

Another primary prevention trial was the Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes (FINDIA) pilot study, which was a randomized, double-blind pilot trial designed to answer the question of whether weaning to a bovine insulin-free CM formula reduces T1D-associated autoantibodies in children at genetic risk. It was performed in Finnish children and in comparison with ordinary CM formula, weaning to an insulin-free CM formula reduced the cumulative incidence of autoantibodies.

Secondary prevention aims to stop or delay progressive β-cell destruction before the manifestation of clinical disease. Several trials have been initiated in high-risk individuals with β-cell autoantibodies to stop the progression of β-cell autoimmunity. The Diabetes Prevention Trial–Type1 (DPT-1) tested the effect of subcutaneous and oral insulin administration (Skyler *et al.* 2005), and the DIPP study in Finland determined the effect of nasal insulin in preventing or delaying the progression of T1D (Näntö-Salonen *et al.* 2008), but these studies could not be shown to prevent or delay T1D. The European Nicotinamide Diabetes Intervention Trial (ENDIT) included ICA-positive first-degree relatives up to the age of 40 years, who received daily either a high dose of oral nicotinamide or placebo to test whether the treatment is able to reduce the incidence of T1D. No difference was recorded between the two groups in the progression rate to clinical T1D (Gale *et al.* 2004).

After the clinical diagnosis, tertiary prevention aims to preserve the residual β-cell function and/or to prevent the clinical complications of the disease. Monoclonal antibodies against CD3 receptors have been studied in tertiary prevention trials. Anti-CD3 antibodies mediate β-cell protection by the induction of antigen-nonspecific regulators such as $CD25^{high}$ cells, as well as anergy or deletion of activated aggressive lymphocytes. In the GAD-Alum Prevention Study, a primary objective was to determine whether GAD-Alum vaccine will prevent or delay the development of clinical T1D in nondiabetic relatives of patients with T1D, age 3–45, who are positive for GAD autoantibodies but not for IAA. The treatment did not change the insulin requirement (Skyler 2008). The majority of the intervention studies have been organized by the Trial Network.

It has been reported in animal models that a combination treatment with anti-CD3-specific antibody and intranasal proinsulin peptide can reverse recent-onset diabetes with much greater efficacy than monotherapy (with anti-CD3 or antigen) alone. The expansion of $CD25^{+}FOXP3^{+}$ and insulin-specific Tregs producing IL-10, TGF-β, and IL-4 was enhanced. Thus, the combination of a systemic immune modulator and antigen-specific Treg induction may be more efficacious in reverting diabetes (Bresson *et al.* 2006).

3 AIMS OF THE STUDY

The Table 1 presents the specific hypotheses and objectives of the present thesis.

Table 1. The specific hypotheses and objectives of the present thesis

Hypothesis-based research question	Objectives
Do T1D-associated HLA risk genotypes modulate the development of T cell phenotypes and thus the development of autoimmunity?	To study the possible differences in T cell polarization of cord blood T cells *in vitro* in relation to T1D-associated HLA risk genotypes.
Is aberrant early immunoreactivity to cow's milk proteins, as a marker of tolerance development, seen in children who develop T1D?	To compare the levels of antibodies to cow's milk proteins in children who later developed T1D and in healthy children.
Does exposure of offspring to maternal diabetes and insulin therapy result in tolerization to insulin *in utero* and reduce the risk of T1D by this mechanism?	To compare the number of Treg cells and *in vitro* insulin-induced $CD4^+CD25^+FOXP3^+$ cells in neonates born to mothers with T1D with neonates born to mothers without T1D.
Are there signs of dysbiosis of intestinal microbiota in children with β-cell autoimmunity?	To compare whether the composition of the gut microbiota differs between children with β-cell autoantibodies and autoantibody-negative children.

4 SUBJECTS AND METHODS

4.1 Subjects

4.1.1 Study I

Cord blood samples were received from newborn infants participating in the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) study having at least one first-degree relative (mother, father, or full-sibling) with T1D (n = 27), or in the Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes (FINDIA) pilot study with no affected family member (n = 40). The families were recruited at the Department of Obstetrics, University of Helsinki, the Helsinki City Maternity Hospital, and Jorvi Hospital in the TRIGR study, and at Jyväskylä Central Hospital and Kuopio University Hospital in the FINDIA study. The gestational age of all participants was ≥35 weeks. In the cord blood the T1D-associated HLA risk alleles were detected and infants carrying the T1D-associated HLA-DQ risk genotype [HLA-DQB1*0302/*02, or *0302/x (x≠*0301, or *0602), HLA DQA1*05-DQB1*02/y or HLA DQA1*03-DQB1 *02/y (y≠ DQA1*0201-DQB1*02, DQB1*0301, DQB1*0302, DQB1*0602, or DQB1*0603) were recruited for the follow-up study. In study I, the cohort consisted infants carrying the DR4-DQ8 (DQB1*0302) haplotype (n = 18), and infants with the DR3-DQ2 (HLA DQA1*0501-DQB1*02) haplotype (n = 12). The number of newborn infants carrying neither the DR4-DQ8 nor the DR3-DQ2 haplotype was 37. The Ethics Committee for Pediatrics, Adolescent Medicine and Psychiatry (Helsinki, Finland), the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (Helsinki, Finland), and the Ethics Committee of Kuopio University Hospital (Kuopio, Finland) approved the study protocol. The families gave their written informed consent.

4.1.2 Study II

The subjects were participants in the TRIGR pilot study. Newborn infants with at least one first-degree relative with T1D (mother, father, or sibling) were recruited to the study between 1995 and 1997, but only individuals having an increased genetic risk (HLA-DQB1*02/*0302, *0302/x, or *02/y, where x≠*02, *0301,*0602, or *0603, and y≠*0301,*0302, *0602, or *0603) were included in the intervention study. The TRIGR pilot study aimed to evaluate the effect of supplementing breast milk with highly hydrolyzed milk formula on the appearance of diabetes-associated autoantibodies by the age of 10 years. Infants were randomized into two groups: after exclusive breast-feeding, whenever breast

milk was not available, the groups were to receive either a regular CM protein control formula (Enfamil®, Mead Johnson, Evansville, IL, USA) or an extensively hydrolyzed casein-based test formula (Nutramigen®, Mead Johnson) until the age of 6–8 months, depending on when the formula was started. The control formula included 20% casein hydrolysate formula to eliminate the flavor and smell difference between the two formulas. According to the study protocol, the minimum exposure time to the study formula was 2 months. Exclusive breastfeeding was encouraged. During the intervention period, the children avoided all infant food products containing bovine protein, but the diet of the lactating mothers was unmodified.

Study II included those children who were randomized to be weaned to the CM-based infant formula (n = 118). A total of 14 subjects were excluded from the analyses because of incomplete feeding data, and 10 subjects were excluded because of no exposure to the study formula at all. The cohort consisted of 94 children. In this series, eight subjects progressed to T1D by the age of 7 years, and the median age at diagnosis of T1D was 57 months (range 20–88 months). Fifteen subjects developed positivity for at least one T1D-associated autoantibody without progression to T1D, and 71 subjects were autoantibody-negative during the observation period. Serum samples for screening of ICA, IAA, GADA and IA-2A were obtained from the subjects at the age of 3, 6, 9, 12, 18, and 24 months, and subsequently at the age of 3, 5, and 7 years. Serum samples at the age of 3, 6, 9, 12, 18, 24, and 36 months were analyzed for IgG and IgA class antibodies to CM proteins, IgG antibodies to bovine insulin, and tetanus toxoid. Among the 15 autoantibody-positive children, the first autoantibody appeared at median age of 36 months (range 9–84 months). Written informed consent was obtained from the mother before enrolment. The study was approved by the Joint Ethics Committees of the participating hospitals. The characteristics of the participants are presented in Table 2.

Table 2. Characteristics of subjects in study II.

Study II	T1D (n = 8)	AAB+ (n = 15)	Controls (n = 71)
Female/male	4/4	7/8	32/39
T1D in family f/m/s/fs/ms/mf	5/-/1/1/1/-	5/6/4/-/-/-	29/29/12/-/-/1
HLA-DQB1			
*02/*0302	3 (37.5%)	3 (20%)	17 (24%)
*0302/x	4 (50%)	5 (33%)	27 (38%)
*02/y	1(12.5%)	7 (47%)	27 (38%)

First-degree family member with T1D: f, father; m, mother; s, full-sibling;
x ≠ DQB1*02, *0301, *0602, *0603; y ≠ DQB1*0301, *0302, *0602, *0603

4.1.3 Study III

CBMC and plasma samples were received from newborn infants taking part in the TRIGR study who had a first-degree relative (full-sibling, mother, or father) with T1D, or from infants born in the Department of Obstetrics, Helsinki University Central Hospital. We studied CB samples from 20 infants (eight females) with maternal T1D and 20 infants (eight females) of unaffected mothers. The inclusion criterion was a gestational age of ≥35 weeks. The infants with signs of infection and infants of mothers with gestational diabetes were excluded from the study. There was no difference in the maternal age between mothers with T1D (28.9 years; range 23.8–44.7 years) and unaffected mothers (30.4 years; 24.6–42.1 years). The duration of diabetes in mothers with T1D ranged from 2 to 30 years, the median being 18 years.

There were differences in gestation age at delivery, in birth weight and in birth order between mothers with T1D compared to mothers without diabetes. The gestation age at the delivery was longer in mothers without diabetes than in mothers with T1D [$p < 0.001$; median 39.6 weeks (range 37.1–42.3) vs. median 37.4 weeks (range 35.4–39.9), respectively]. The birth weight was greater in infants with maternal diabetes than in infants with no maternal diabetes [$p < 0.05$; median 3870 g (range 2550–5335 g) vs. median 3528 g (range 2710–4270 g), respectively]. Caesarean sections were more common in mothers with T1D ($n = 15$) when compared to mothers without diabetes ($n = 8$) ($p < 0.05$).

The median daily insulin dose was 0.88 IU/kg (range 0.58–1.48 IU/kg) at the end of pregnancy in the T1D mothers. The mothers were treated during pregnancy with NPH insulin or long-acting analogue insulin glargine together with rapid-acting human insulin analogs (lispro or aspart). The Ethics Committee for Pediatrics, Adolescent Medicine and Psychiatry and the Coordinating Ethics Committee of the Hospital District of Helsinki and Uusimaa (Helsinki, Finland) approved the study protocol, which was in accordance with the Declaration of Helsinki. Written parental consent was obtained from the parents of all children. Characteristics of the infants are presented in Table 3.

Table 3. Genetic characteristics of offspring of mothers with and without type 1 diabetes (T1D). The data are the number of subjects (n). HLA genotype indicates the presence of HLA-DQ haplotypes associated with the T1D risk. DR3-DQ2 = DQA1*05-DQB1*02, DR4-DQ8 = DQB1*0302, x = non-DR3-DQ2, y = non-DR4-DQ8, z = Neither risk haplotype.

Study III, GENETICS	Maternal T1D (n = 20)	No maternal T1D (n = 20)
HLA genotype		
DR3-DQ2/DR4-DQ8	2	2
DR4-DQ8/x	8	7
DR3-DQ2/y	4	6
z/z	6	5
INS -23 HphI A/T		
AA	13	16
AT/TT	7	4
PTPN22 1858C/T		
CT	7	6
CC	13	14

4.1.4 Study IV

The study included 18 children with an HLA-associated genetic risk of T1D who had developed signs of progressive β-cell autoimmunity, i.e. tested positive for at least two T1D-associated autoantibodies (cases). The control children were matched for age, gender, and HLA-DQB1 genotype, as well as for the time of exposure to and the type of infant formula. The study subjects were derived from two intervention studies performed in Finland: the TRIGR (n = 20) or the FINDIA pilot study (n = 16) (Knip *et al*. 2010, Vaarala *et al.* 2012). In the TRIGR pilot study, subjects were followed until the age of 10 years, and in the on-going FINDIA pilot study the follow-up time was from 4 to 7 years. The control children remained negative during the follow-up for all four autoantibodies analyzed.

In the TRIGR pilot study, infants with a first-degree relative having T1D were randomized to receive either a regular CM formula (Enfamil®, Mead Johnson, Evansville, IN, USA) or an extensively hydrolyzed casein-based test formula (Nutramigen®, Mead Johnson) until the age of 6–8 months. In the FINDIA study infants, were randomized to receive a standard CM-based formula (Tutteli®, Valio), a whey-based hydrolyzed formula (Peptidi-Tutteli®), or a whey-based FINDIA formula from which bovine insulin had been removed. In both studies, exclusive breast-feeding was encouraged. No difference was observed in the duration of exclusive breast-feeding between case and control groups [N.S., median 2.9 months (range 0−5.5 months) vs. median 4.0 months

(range 0.1−6.0 months), respectively], but the total duration of breast-feeding was longer in controls when compared with cases [$p < 0.05$; median 10.5 months (range 5.1−19.3) and median 8.1 months (range 1.6−16.5), respectively]. In this study, the number of cesarean-delivered children was seven (four in cases and three in controls). The study was approved by the Ethics Committees of the participating hospitals, and the families gave their written informed consent. The characteristics of the subjects in study IV are presented in Table 4.

Table 4. Characteristics of the study subjects. Cases are children positive for at least two diabetes-associated autoantibodies and control subjects are negative for β-cell autoantibodies. Data are n or medians (with range). x ≠ *0301, or *0602, y ≠ *0301, *0602*, *0603.

Study IV	Cases (n = 18)	Controls (n = 18)
Female/Male	7/11	7/11
T1D in first degree relatives	10	10
Age (years)		
TRIGR	13.3 (11.7−14.2)	12.8 (11.9−13.6)
FINDIA	5.1 (4.9−6.0)	5.0 (3.9–7.0)
HLA-DQB1 genotype		
*02/0302	7	7
*0302/x	8	8
*02(DQA1*05)/y	2	2
*02(DQA1*0201)	1	1
Study formula		
Cow's milk formula	10	10
Hydrolyzed casein based formula	4	4
Hydrolyzed whey based formula	3	3
Insulin-free whey-based formula	1	1

4.2 Methods

Table 5. Laboratory methods used in this thesis

Cell preparations	**Method**	**Publication**
CBMC isolation from cord blood	Ficoll gradient centrifugation	I, III
Monocyte depletion	Plastic adherence	I
Cell stimulation		
Cytokine stimulation of PHA activated CB T cells	Cell culturing	I
Insulin stimulation of CBMC	Cell culturing	III
Flow cytometry analysis		
CD4, CD8, IL-12Rβ2, IL-18R, CXCR4, CXCR3	Extracellular staining	I
CXCR6, CCR2, CCR3, CCR4, CCR5, CCR7	Extracellular staining	I
CD4, CD8, CD25	Extracellular staining	III
FOXP3	Intracellular staining	III
Detection of secreted cytokines		
IFN-γ, IL-13	ELISA	I
IL-6, IL-4, IL-2, IL-10, IFN-γ, TNF-α	CBA	III
Quantitative measurements of mRNA		
CCR4, T-bet, GATA-3	RT-PCR, TaqMan	I
FOXP3, NFATc2, STIM-1, TGF-β, IL-10	RT-PCR, TaqMan	III
Detection of antibodies		
Detection of autoantibodies (IAA, GADA, IA-2A)	Radioimmunoassay	I-IV
Detection of ICA	Immunofluorescence	I-IV
Insulin antibodies	ELISA	III
Antibodies to cow's milk proteins	ELISA	III
Antibodies to tetanus toxoid	ELISA	III
Fecal sample analyses		
IgA, Calprotectin	ELISA	IV
DNA extraction	Repeated Bead Beating method	IV
Pyrosequencing	Roche FLX Genome Sequencer	IV

4.2.1 Cell preparations (I, III)

In studies **I** and **III**, cord blood mononuclear cells (CBMC) were isolated from EDTA cord blood samples by Ficoll gradient centrifugation (Amersham Biosciences, Uppsala, Sweden). After centrifuge isolation, the cells were washed three times with PBS and suspended in RPMI 1640 (Gibco/Life Technologies, Paisley, U.K.) containing 5% inactivated human AB serum

(obtained in study I from the Finnish Red Cross Blood Transfusion Service, Helsinki, Finland, and in study III from Sigma, St. Louis, MO, USA), L-glutamine (2 mmol/l; Gibco) and gentamicin (25 µg/ml; Sigma).

4.2.2 *In vitro* stimulation of CBMC (I, III)

Stimulation of T cells in type 1 or type 2 cytokine environments (**I**). In study I, plastic adherence was used to deplete monocytes from the CBMC fraction (CBMC at the concentration of 1 x 10^6 in a 50 ml plastic cell culture bottle for 1 h at 37 °C in a CO_2 incubator). The non-adherent lymphocytes were stimulated (2 x 10^6 cells/well) with PHA and type 1 or type 2 cytokines in RPMI 1640 culture medium (Gibco) containing 5% inactivated human AB serum (the Finnish Red Cross Blood Transfusion Service) and gentamicin (25 µg/ml; Sigma). For type 1 stimulations, 200 pg/ml IL-12 (BD PharMingen, San Diego, CA) and 50 ng/ml anti-IL-4 (BD PharMingen) were used, and for type 2 stimulations, 400 pg/ml IL-4 (Sigma) and 1 µg/ml anti-IL-12 (BD PharMingen) were used. On day 2, the T-lymphocyte lines were expanded by the addition of IL-2 (32 pg/ml; BD PharMingen). After culturing the cells for 4 days, supernatants were collected and stored at −70 ºC for further analysis of the cytokine profile. Furthermore, half of the medium was replaced and cytokines (IL-12 or IL-4), antibodies (anti-IL-4 or anti-IL-12), and IL-2 were added again. After six days of cultures the cells were collected and the expression of chemokine and cytokine receptors was analyzed by flow cytometry. In addition, lymphocytes were collected and stored at −70 ºC in lysis buffer (GenElute Mammalian Total RNA Miniprep kit; Sigma) until RT-qPCR analysis.

Insulin stimulation of CBMC (**III**). In study III, CBMC were cultured in U-bottomed 24-well cell culture plates at 2 x 10^6 cells (2 ml) per well for flow cytometry analysis and in U-bottomed 96-well culture plates, 2 x 10^5 cells per well in quadruplicates, for RT-qPCR analysis with and without antigen. Human insulin 300 µg/ml (sterile yeast recombinant protein from Boehringer Mannheim, Mannheim, Germany) was used as antigen. After culturing the cells for 72 h, the supernatants were collected and stored at −70 ºC for the detection of secreted cytokines, and cells were also collected and lysed with the lysis buffer of a total RNA purification kit (Sigma) for mRNA analysis. Lysed cells were stored at −70 ºC until use.

4.2.3 Flow cytometry analyses (I, III)

In study **I**, flow cytometry was used for analyses of cell surface molecules CD4 and CD8, as well as chemokine and cytokine receptors. CB T cells stimulated in the type 1 or type 2 cytokine environment for six days were collected and resuspended in PBS with 0.5% bovine serum albumin (BSA). Approximately 300000 cells were stained with FITC-anti-CXCR3, FITC-anti-CXCR4, FITC-anti-

CCR3, FITC-anti-CCR7, PE-anti-IL-18R, PE-anti-CCR2, and PE-anti-CXCR6 (R&D Systems, Minneapolis, MN), as well as FITC-anti-CCR5, PE-anti-IL-12Rb2, PE-anti-CCR4, PerCP-anti-CD8, and APC-anti-CD4 (BD PharMingen). Appropriate isotype controls were also used. After staining for 30 min, cells were incubated with 2 ml of fluorescence-activated cell sorter (FACS®) Lysing Solution (Becton Dickinson, SanJose, CA) for 10 min at room temperature to lyse erythrocytes and were then washed with PBS with 0.5% BSA. The labeled cells were analyzed with four-color flow cytometry using FACSCalibur and CellQuest software (BD).

In study **III**, flow cytometry was used for the analysis of cell surface molecules CD4, CD8 and CD25 and intracellular molecule FOXP3. The fresh CBMC and 72 h cultured cells were first stained for surface antigens CD4, CD25, and CD8 with anti-CD4 (PerCP), anti-25 PE, and anti-CD8 APC, respectively. Cells were fixed and stained with Alexa488-labeled FOXP3 antibody using the FOXP3 staining kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. BD FACSCalibur flow cytometry was used for analysis; at least 1 x 10^6 cells were tested from each sample. The data were analyzed using FACSDiva (BD) software.

4.2.4 Antibody assays

4.2.4.1 Disease-associated autoantibodies (I–IV)

The antibody assays have been described in detail earlier (Kimpimäki *et al.* 2002, Kukko *et al*. 2005). The concentrations of autoantibodies to GAD65 (GADA), the IA-2 molecule (IA-2A), and insulin (IAA) were measured by specific radiobinding assays, and islet cell antibodies (ICA) by a standard immunofluorescence method. The detection limit for ICA was 2.5 Juvenile Diabetes Foundation Units (JDFU). The cut-off limits for IAA, GADA, and IA-2A positivity were 1.56 RU, 5.36 RU, and 0.43 RU, respectively. According to the 2005 Diabetes Antibody Standardization Program (DASP) workshop, the disease sensitivity and specificity of the assay were, respectively, 58% and 98% for IAA, 82% and 96% for GADA, 100% and 98% for ICA, and 72% and 100% for IA-2A (Schlosser *et al*. 2010, Torn *et al.* 2008). The autoantibodies were analyzed in the laboratory of Professor Mikael Knip.

4.2.4.2 IgG- and IgA-class antibodies to CM proteins (II)

In study **II**, IgG and IgA class antibodies to CM formula, beta-lactoglobulin (BLG), bovine serum albumin (BSA), and alpha-casein (CAS) were analyzed in the Research Laboratory of the Hospital for Children and Adolescents, University of Helsinki, by ELISA as previously described (Savilahti *et al.* 1993, Saukkonen *et al*. 1994, Vaarala *et al*. 1995). The levels of antibodies were com-

pared with a standard serum sample with very high titers of CM and BLG antibodies, and the levels of antibodies were expressed as arbitrary units (AU).

4.2.4.3 IgG-class antibodies to bovine insulin (II)

In study **II**, bovine insulin-binding IgG antibodies were measured with a in-house ELISA method (Paronen *et al.* 2000). Briefly, polystyrene plates were coated with bovine insulin (Sigma, St Louis, MO, USA) (1 μg/well in PBS) and the plates were incubated at 4 ºC overnight. The plates were washed with buffer containing 0.05% Tween 20 in PBS and blocked for unspecific binding with 1% human serum albumin. Samples were diluted 1:20 in PBS containing 0.2% human serum albumin and 0.05% Tween and incubated at room temperature for 2 h. After washes, alkaline phosphatase-conjugated rabbit anti-human IgG antibodies (Jackson Immunoresearch, West Grove, PA, USA) were used as the secondary antibody and P-nitrophenyl phosphatase (Sigma) as a substrate, and absorbance was read on a spectrophotometer. Results were expressed as optical density (OD) units.

4.2.4.4 TT antibody assay (II)

Antibodies to TT were measured by ELISA in study **II**. Maxisorb® plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with TT (1 μg/ml). The plates were washed with buffer containing 0.05% Tween 20 in PBS and blocked with 1% human serum albumin. Plasma samples were diluted 1:800 in 0.2% human serum albumin and 0.05% Tween-PBS and incubated for 2 h at room temperature. Alkaline phosphatase-conjugated rabbit anti-human IgG antibodies (Jackson Immunoresearch) were used as the secondary antibody and diluted 1:3000 in 0.2% human serum albumin and 0.05% Tween-PBS. P-nitrophenyl phosphate (Sigma) was used as a substrate. Results were expressed as optical density (OD) units.

4.2.4.5 Calprotectin and IgA detections in feces (IV)

A beaker in the bottom cap of the extraction device was filled with a thawed and homogenized stool sample, avoiding seeds and grains. The beaker contained approximately 100 mg of sample. The extraction tube was filled with 4.9 mL extraction buffer (volume ratio 1:50) and vortexed for 30 s. Mixing was continued in a shaker at 1000 rpm for 3 min or until only solid particles remained. The particles were allowed to settle before a 10-min centrifugation at 10000 g at room temperature. Supernatant was collected and stored at -20 °C.

IgA values were measured with the modified ELISA method from Lehtonen *et al.* (1984). The polystyrene plates (Nunc MaxiSorp®, Thermo Fisher Scientific, Roskilde, Denmark) were incubated at +4 °C overnight. The antibody used for the primary coating was rabbit anti-human IgA (DakoCytomation, Glostrup, Denmark) 1:1000 in carbonate buffer, and 0.5% Tween 20 in PBS was used for

washing the plates. The plates were blocked with 1% BSA in PBS, 1h at 37 °C. Peroxidase-conjugated rabbit anti-human IgA (DakoCytomation) 1:5000 in PBS was used as a conjugate, and tetramethylbenzidine as a substrate. The reaction was stopped with 1M sulfuric acid and optical density was read at 450 nm. The immunoglobulin concentrations were calculated from a human IgA control curve (InvivoGen; San Diego, CA, USA).

Calprotectin levels in stool samples were determined using a Calpro Extraction Device and Calprolab calprotectin ELISA tests (Lot. CALP-Pilot3, Calpro AS, Oslo, Norway). Altogether, 20 µL of extract was mixed with 980 µL of sample dilution buffer. Standard and positive controls were performed in duplicate. The optical density was read at 405 nm. using an ELISA reader.

4.2.5 Cytokine analyses

4.2.5.1 Cytokine levels in *in vitro* studies (I, III)

In study **I**, ELISA was used to analyze IFN-γ concentrations in the culture supernatants. In brief, Maxisorb® plates (Nunc) were coated with monoclonal anti-human IFN-γ (M-700 A; Endogen, Woburn, MA, USA) (2 µg/ml, 50 µl/well). The plates were incubated overnight at +4 °C, washed with PBS-Tween, and then blocked with 1% BSA in PBS for 30 min. A standard curve was prepared by using recombinant human IFN-γ (Pharmingen, San Diego, CA). Supernatant samples (100 µl/well) and standards were incubated for 2 h at room temperature. After washing with PBS-Tween, biotinylated monoclonal anti-human IFN-γ (Endogen) was added (0.5 µg/ml, 50 µl/well), and the plates were incubated for 90 min. Streptavidin–alkaline phosphatase complex (Zymed, San Francisco, CA) was added. For the color development, P-nitrophenyl phosphate (Medix, Kauniainen, Finland) was added and the plates were analyzed using a plate reader. The detection level of the assay was 50 pg/ml. IL-13 concentrations were determined with a human IL-13 ELISA Kit (Pelikine Compact, Amsterdam, Netherlands) with a detection limit of 0.5 pg/ml.

In study **III**, the Cytometric Bead Array (CBA Kit, BD) was used to analyze the IL-2, IL-4, IL-6, IL-10 TNFα, and IFNγ concentrations of the culture supernatants after 72 h stimulation with or without insulin.

4.2.5.2 Cytokine levels in plasma (III)

In study **III**, for the analysis of cytokines in cord blood plasma samples, the measurements of IL-2, IL-4, IL-6, IL-10 TNFα, and IFNγ were performed by Cytometric Bead Array according to the manufacturer's instructions (CBA Kit, BD).

4.2.6 Quantitative real-time PCR (I, III)

In study **I**, the mRNA level of GATA-3, T-bet and chemokine receptor CCR4 was detected by quantitative real-time RT-PCR from CB T cells stimulated in the type 1 or type 2 cytokine environment for six days. Total RNA was extracted from frozen (−70 °C), lysed cells using a Total GenElute Mammalian RNA Miniprep kit (Sigma) and a reverse transcription reaction was performed. Quantitative RT-PCR was carried out with commercial primers and probes, using an ABI Prism 7700 Sequence Detection System (Applied Biosystems Sequence Detector, Foster City, CA) in triplicate wells. Ribosomal RNA 18S was applied as an endogenous control.

In study **III**, the mRNA level of FOXP3, NFATc2, STIM-1, IL-10, and TGF-β was analyzed by quantitative real-time RT-PCR from CBMCs stimulated for 72 h with insulin or medium alone. The total RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantitative real-time (RT) PCR was performed as previously described in study **I**.

PHA-stimulated peripheral blood mononuclear cells for 48 h from healthy subjects were used as a calibrator sample that was included in every plate. The comparative threshold cycle (C_T) method was used to quantitate the gene transcription in CB T cells. ΔC_T stands for the difference between C_T of the target gene and C_T of the 18S gene, whereas $\Delta\Delta C_T$ is the difference between ΔC_T of the analyzed sample and ΔC_T of the calibrator. The results are expressed as relative units, based on calculations $2^{-\Delta\Delta CT}$, and then give the relative amount of the analyzed sample compared to the calibrator, both normalized to an endogenous control (18S).

4.2.7 DNA extraction and pyrosequencing (IV)

The fecal samples from children were collected using stool collection vials and immediately stored in home freezers (-20 °C). Families delivered the frozen samples to the study center and the samples were stored at −80 °C until processing.

Total DNA was extracted from 0.25 g fecal sample using the Repeated Bead Beating method described in detail by Wu *et al.* (2010) with a number of modifications. In brief, four 3-mm glass beads were added during the homogenization step. Bead beating was performed using a Precellys 24 tissue homogenizer/grinder (Bertin Technologies, Montigny le Bretonneux, France). Protein precipitation with 260 μL ammonium acetate was performed twice. Elution of DNA from the purification columns was performed twice. Columns from the QiaAmp Stool kit were replaced with those from the QIAamp DNA Mini Kit (Qiagen).

From each sample, the 16S rRNA genes were amplified using a primer set corresponding to primers 27F-degS (van den Bogert *et al.* 2011) and 534-R (Wu

et al. 2010). These PCR primers target the V1, V2, and V3 hypervariable regions of the 16 rRNA. Pyrosequencing was carried out using a Roche FLX Genome Sequencer at DNAvision (Liège, Belgium) according to their standard protocol (De Filippo *et al.* 2010).

Pyrosequencing produced a total of 461 874 reads of 16S rDNA. Sequences were assigned to samples according to sample-specific barcodes. Sequencing Quality Control was carried out by using the Galaxy Tools website (Goecks *et al.* 2010). SFF files from the 454 Genome Sequencer FLX were converted into FASTA files and FASTA quality files. FASTA - formatted files contained an average (± SD) of 12 830 ± 4888 reads per sample. The RDP pyrosequencing pipeline (Cole *et al.* 2009) (RDP 10 database, Update 17) was subsequently used to check the FASTA sequence files for the same criteria as described by De Filippo *et al.* (2010), and that the average experimental quality score was at least 20. After this quality check, the FASTA files contained an average (± SD) of 8024 ± 3136 high quality reads.

Taxonomy (phylum, family and genus level) was assigned using RDP classifier v.2.01 (Wang Q. *et al*. 2007). Richness and diversity analyses were performed as described by De Filippo *et al.* (2010). Identification down to the species level was carried out using ARB software (Ludwig *et al.* 2004). For this, an SSU reference database (SSURef_106_ SILVA_19_03_11) was downloaded from the SILVA website (Pruesse *et al.* 2007). From this database, only sequences of cultured and identified isolates were used. From these sequences, a "PT server" database was built, which was subsequently used to find the closest match for each of our high-quality sequences imported from the FASTA files. For this, the "search next relatives of listed species in PT server" function was applied with the following settings: Oligo length: 12; mismatches: 0; match score: relative; Min score: 10. The average (± SD) match score was 75.2 ± 18.5. Sequences that were identified as being from different strains but belonging to the same species were grouped together. Species that represented more than 0.005% of all sequences were included in statistical analysis, together representing 99.2% ± 0.35 of all high quality FASTA reads per sample.

4.2.8 Genetic analyses (I–IV)

HLA genotyping was performed according to the screening protocol in the TRIGR study. The initial HLA-DQB1 typing for risk-associated (DQB1*02, DQB1*0302) and protective (DQB1*0301, DQB1*0602, DQB1*0603) alleles was complemented with DQA1 typing for DQA1*0201 and DQA1*05 alleles in those with DQB1*02 without protective alleles or the major risk allele DQB1*0302. This two-step screening technique is based on the hybridization of PCR products with lanthanide-labeled probes detected by time-resolved fluoro-

metry as described in earlier publications (Sjöroos *et al.* 1995, Laaksonen *et al.* 2002).

Similarly to the HLA assays, the principle of microtitration-plate-bound biotinylated amplification products and lanthanide-labeled probes was applied in the single nucleotide polymorphism (SNP) assays for the INS -23A/T (rs689) polymorphism (Laine *et al.* 2007). For PTPN22 1858C/T (rs2476601) analysis, a 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 (Kiviniemi *et al.* 2003, Hermann *et al.* 2006).

4.2.9 Statistical analysis

Statistical analysis for studies **I–III** was performed using SPSS 10.0–15.0 for Windows (SPSS Inc, Chicago, IL, USA). P-values below 0.05 were considered significant. The data were not normally distributed and non-parametric statistical methods were therefore used. The statistical significance of differences between the groups was tested using non-parametric tests: the Mann-Whitney U-test for comparisons between two unrelated groups and Kruskal-Wallis test for comparisons among three unrelated group. The Wilcoxon Signed-Ranks Test was used for the comparison of two related parameters, and Spearman's rank correlation test for correlation analysis of variables. In study **II**, the chi-squared test was used to compare different groups with regard to gender, HLA genotypes, and diabetes in the family. In study **II**, the area-under-the-curve (AUC) method was used to compare the CM protein antibody levels between the groups (Matthews *et al*. 1990).

In study **IV**, initial analysis of the samples showed that bacterial populations were most often not normally distributed. Mann-Whitney U and Spearman's rho tests were therefore used. P values of less than 0.05 were considered significant. In addition, principal component analysis (PCA) was performed to find clusters of similar groups of samples or species. PCA is an ordination method based on multivariate statistical analysis that maps the samples in different dimensions. All tests were performed with PASW Statistics 18.

5 RESULTS AND DISCUSSION

5.1 Association between decreased expression of the chemokine receptor CCR4 on CD4 cells and the genetic risk genotype of type 1 diabetes (I)

T cells play a role in the pathogenesis of T1D, and proper polarization of T cells is important in the regulation of immune responses. Chemokines and chemokine receptors are important in T cell migration and directing T cells to inflammatory sites. Different T cell subsets are associated with the expression of certain chemokine receptors. The HLA class II haplotypes are associated with several autoimmune diseases, in which the target autoantigens are different. The HLA DR3-DQ2 haplotype is associated with autoimmune diseases such as T1D, celiac disease, Addison's disease, and SLE, and HLA-DR4 haplotypes are associated with both T1D and rheumatoid arthritis. The aim of this study was to characterize the *in vitro* regulation of T cell polarization in the newborn infants at genetic risk of T1D. The expression of cytokine and chemokine receptors on T cells cultured with PHA in a type 1 or 2 cytokine environment was investigated in cord blood. Differentiation of Th2 cells depends on IL-4 signaling and STAT6 activation, which induces the expression of the transcription factor GATA-3, and this in turn mainly binds to promoters of IL-5 and IL-13. It has also been reported that GATA-3 may be the main factor determining the expression level of IL-13 (Zhou *et al.* 2003).

Relative mRNA expression levels of transcription factors that direct Th1 and Th2 differentiation indicated successful polarization in the cell cultures. In the type 1 culturing conditions, Th1-specific transcription factor T-bet was expressed at higher levels when compared to Th2 type cultures ($p < 0.001$, Wilcoxon Signed Rank Test). In type 2 cell culture conditions, GATA-3 was expressed at higher levels when compared to type 1 cell culture conditions ($p < 0.001$, Wilcoxon Signed Rank Test). In addition, the expression of IL-12Rβ_2-chain ($p < 0.001$) and the secretion of IFN-γ ($p < 0.001$) were higher, and the secretion of IL-13 was lower ($p < 0.001$) when CB lymphocytes were cultured in a type 1 cytokine environment when compared to a type 2 cytokine environment. IL-13 was used as marker for Th2 cytokines because IL-4 was present in cultures for the induction of deviation.

In infants carrying the DR3-DQ2 or DR4-DQ8 haplotype, when compared to infants with no genetic risk of T1D, we observed a decreased percentage of CD4 T cells expressing the chemokine receptor CCR4 ($p < 0.01$) (original publication I, Figure 3b). In our study we also found that infants carrying the DR4-DQ8

haplotype expressed lower levels of CCR4mRNA and lower mRNA levels of the Th2-associated transcription factor GATA-3 after culture of cord blood lymphocytes in a type 2 cytokine environment than infants with no genetic risk of T1D (Figure 4).

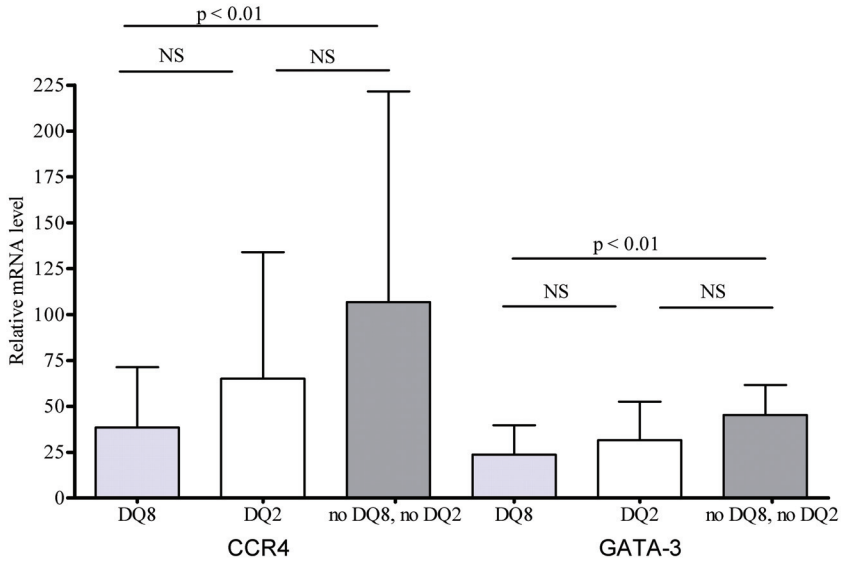

Figure 4. Relative levels of CCR4 and GATA-3 mRNA. CCR4 and GATA-3 mRNA level in CB lymphocytes from infants carrying DR4-DQ8, DR3-DQ2, or no DR4-DQ8 and DR3-DQ2 haplotypes after culture in a type 2 cytokine environment. The expression of CCR4 and GATA-3 differed between three groups (p = 0.008 and p= 0.004 Kruskal-Wallis test). p-values from the Mann–Whitney U-test are shown in the figure. The median and interquartile range are shown. NS, not significant.

CB lymphocytes cultured in a type 2 cytokine environment from infants carrying the DR4-DQ8 haplotype produced reduced levels of IL-13 when compared to infants without a genetic risk ($p < 0.05$) (original publication I, Figure 5). There was no difference in the expression of IFN-γ between the groups.

Our results indicate that the *in vitro* T cell response to type 2 cytokine stimulation is less pronounced at birth in infants carrying the DR4-DQ8 haplotype. This was demonstrated as a poor induction of GATA-3, IL-13 and CCR4 by the type 2 cytokine environment. In newborn infants the immune system is normally deviated towards Th2 (Bryson *et al.* 1980), but our results suggest that this deviation is less pronounced in infants at genetic risk of T1D. Th2 cells are needed in the suppression of Th1 immune responses, and impairments in Th2 cell differentiation may contribute to an imbalance of the immune response, which favors the development of Th1 responses or may lead to difficulties in the regulation of autoimmune responses.

Similar results have reported in patients with T1D. The expression of CCR4 on freshly isolated T cells was reduced in patients with newly diagnosed and longstanding T1D, and this correlated with a low PHA-induced production of IL-4 (Lohmann *et al.* 2002). Furthermore, in patients with T1D, reduced mRNA levels of IL-4 in unstimulated peripheral blood mononuclear cells (Halminen *et al*. 2001) have been reported, giving further support for a defect in Th2 cell differentiation in patients with T1D. In subjects at high risk of T1D, the secretion of IL-13 in whole blood cultures stimulated with PHA was lower in first-degree relatives of T1D patients with an increased genetic risk of T1D, and auto-antibody positive when compared to controls (with a low genetic risk of T1D and no auto-antibodies) (Kretowski *et al*. 2000).

Impairments in T cell responses to the type 2 cytokine environment associated with certain HLA risk haplotypes for T1D may not imply a specific risk of β-cell autoimmunity, but rather be a marker of more general immune aberrancy, which contributes to the development of immune-mediated diseases. The HLA-DR4 allele is associated with an increased risk of developing T1D, but also with an increased risk of rheumatoid arthritis. The development of celiac disease is also associated with partly the same HLA risk alleles as for T1D. It is possible that HLA-associated aberrancies in T cell development underlie immune-mediated diseases by allowing autoimmunity to develop, but other disease-specific factors are involved in triggering the disease.

After culture of CB lymphocytes in type 1 and type 2 cytokine environments, infants carrying DR4-DQ8 or DR3-DQ2 haplotype had increased numbers of CD4 T cells expressing CXCR4 as compared to infants with no DR4-DQ8 or DR3-DQ2 haplotype. CXCR4 is a G-protein-coupled chemokine receptor with a major role in lymphocyte homing. Its ligand, CXCL12, is a highly efficient chemotactic factor for T cells, monocytes, pre-B cells, dendritic cells and myeloid bone marrow-derived cells. CXCR4 has been reported to be expressed on naive T cells (Sallusto *et al.* 1998), and our results may reflect general impairments in the differentiation and maturation of T cells in infants with the DR4-DQ8 or DR3-DQ2 haplotype.

In conclusion, individuals with the HLA DR4-DQ8 haplotype appear already at birth to have poor *in vitro* maturation of T cells in response to type 2 cytokines. This may, at least partly, explain why these infants are prone to developing pronounced Th1 responses and autoimmunity later in their life when they encounter the disease-specific triggers that drive autoimmunity, for example against β-cells.

5.2 The development of the humoral immune response to dietary CM proteins and bovine insulin in early childhood and its relation with developing T1D (II)

The role of the gut immune system in the pathogenesis of T1D has been indicated in several studies showing that diet modulates the development of autoimmunity in animal models, and that lymphocytes activated in the gastrointestinal tract home to the islets. It has been demonstrated that oral antigens are capable of activating antigen-specific T cells in pancreatic lymph nodes (Turley *et al.* 2005), and that the interaction between the endothelium and T cells is controlled by shared homing receptors in inflamed islets and the gut (Hänninen *et al.* 1996, Yang *et al.* 1997, Hänninen *et al.* 2007). T1D is associated with increased permeability of the small intestine, which in turn may alter antigen responses and cause intestinal inflammation, leading to autoimmunity.

Previously, children with newly diagnosed T1D have been reported to have significantly higher concentrations of serum IgA antibodies to cow's milk (CM) protein and to beta-lactoglobulin (BLG), and of IgG antibodies to BLG (Savilahti *et al.* 1988, Savilahti *et al.* 1993, Dahlquist *et al.* 1992). The differences in CM protein antibodies as well as BLG antibodies are more pronounced among children with an early onset of T1D (Savilahti *et al.* 1993, Dahlquist *et al*. 1992). However, levels of antibodies to ovalbumin did not differ between diabetic and control children (Saukkonen *et al.* 1994), although infants are exposed to eggs much later than CM. We aimed to examine the association between the early development of humoral immune responses to dietary CM proteins and later progression to T1D in children who took part in the TRIGR pilot study.

Our study revealed that those children who later progressed to T1D had elevated concentrations of IgG antibodies to BLG from 3 to 18 months of age, and IgA antibody titers to cow milk formula at the age of 9 months in infancy. The IgG antibody levels to BLG differed between groups at the age of 6 months ($p < 0.05$). The IgG antibody levels to BLG were higher in children diagnosed with T1D than in controls (median 218.3 vs 32; $p < 0.05$), but not at other time points. In addition, based on AUC analysis, the IgG antibody levels to BLG were increased during the time period from 3 to 18 months of age among the children who later progressed to T1D compared to the controls ($p < 0.05$) (original publication II, Figure 2). The IgA antibody levels to CM formula differed between the three groups at the age of 9 months ($p < 0.05$). The IgA antibody levels to CM formula were increased in children who later developed T1D as compared to control children (median 3.14 vs 0.97; $p < 0.05$) (original publication II, Figure 1). This finding indicates that either the immune response to CM proteins is dysregulated or the intestinal permeability is increased in infancy among those children who present with clinical T1D later in childhood.

Factors such as dietary exposure, breastfeeding, gut permeability, and genetics are considered to affect the mucosal immune responses. The nature of the dietary antigens and the timing of exposure to these antigens may modulate the oral immune response. CM is often the first food introduced, and the introduction of dietary CM proteins in infancy is a strong immunogenic stimulus. In our study, despite continuous oral antigenic stimulus, cellular responses declined from the time of the introduction of the CM formula up to the age of 12 months, and IgG levels to BLG decreased by the age of 2 years in all children who received the CM based formula before the age of 6–8 months. This is in agreement with earlier reports showing that feeding with CM formula leads in healthy children to stimulation of the humoral immune response to CM proteins and the development of oral tolerance (Tainio *et al.* 1988, Vaarala *et al.* 1995).

Increased early antibody responses to CM proteins in infants who later progressed to T1D could be a marker of an intestinal maturation defect or increased permeability. However, antibody data are only an indirect indicator of gut permeability. Increased intestinal permeability has been shown with the lactulose-mannitol test in patients with T1D (Mooradian *et al*. 1986, Carratu *et al*. 1999) and in prediabetes (Bosi *et al.* 2006). In a biobreeding-rat model, weaning to a hydrolyzed casein diet instead of a whole protein diet reduced the development of autoimmune diabetes by 50%. Moreover, rats with a low prediabetic intestinal permeability developed autoimmune diabetes later or were protected against the disease (Visser *et al*. 2010).

In our study, decreased levels of antibodies to tetanus toxoid were seen at the age of 12 months in subjects who were autoantibody positive when compared with those later diagnosed with T1D or controls ($p < 0.05$). The significance of this finding remains open. In contrast to TT antibodies, antibodies to different dietary antigens were systemically increased in children at risk of T1D, which strengthens the observation of aberrant oral immune responses in such children.

It has earlier been reported that dietary bovine insulin (BI) induces antibodies in infants, and the levels of antibodies binding to BI detected by ELISA were increased during the first 2 years of life in children who developed at least two diabetes-associated autoantibodies. In contrast, the levels tended to decrease during the follow-up in autoantibody-negative children (Vaarala *et al.* 1999). In our cohort, CM exposure before three months of age was shown to enhance the insulin-specific antibody response at 18 months of age in children who developed at least one disease-associated autoantibody compared with autoantibody-negative subjects (median 0.58 vs. 0.34, $p < 0.05$) (original publication II, Figure 4). The results of the TRIGR pilot study in which hydrolyzed formula was used during the first 6–8 months of life showed that elimination of CM protein during infancy reduced the development of β-cell autoimmunity (Knip *et al*. 2010). It is possible that CM contains a diabetogenic antigen, for example BI. Immunisation

to dietary bovine insulin in CM-based formulas is suggested to be essential in the early steps of development of insulin autoimmunity.

5.3 Exposure of offspring to maternal insulin therapy affects $CD4^+CD25^+$ T cells *in utero* (III)

A number of studies have reported a reduced T1D risk in the offspring of affected mothers when compared to the offspring of fathers with T1D (Harjutsalo *et al*. 2006, Warram *et al*. 1991). The exposure to maternal diabetes *in utero* has been implicated to be important in modifying the risk of development of T1D and to represent a protective factor in the offspring. It has been thought that this may be due to the induction of immune tolerance to β-cell antigens presented *in utero* by exposure to maternal diabetes. We hypothesized that exposure to insulin due to maternal insulin treatment could induce regulatory T cells and explain the reduced risk.

We showed that the percentage of FOXP3-positive cells among $CD4^+CD25^{high}$ T cells was increased in freshly isolated CBMC from the offspring of mothers with T1D as compared to infants of unaffected mothers ($p < 0.05$) (original publication III, Figure 1). Interestingly, the percentages of FOXP3-positive $CD4^+CD25^{high}$ T cells tended to be higher in those infants of diabetic mothers who carried the INS 23HphI AT genotype of the insulin gene when compared to the infants who carried the T1D-associated AA genotype variant ($p = 0.07$, NS). None of the children had the TT genotype. The presence of the T allele has been reported to associate with higher expression levels of insulin in the thymus, and is known to be protective for T1D (Pugliese *et al.* 1997). The INS gene polymorphism has been shown to be associated with decreased insulin expression in the thymus. In the thymus, negative selection deletes autoreactive T cells, and it has been suggested that the INS 23HphI AA genotype, leading to low thymic expression of insulin, would lead to the escape of insulin autoreactive T cells (Vafiadis *et al.* 1997). Moreover, the reduced expression level of insulin in the thymus may affect the induction of natural insulin-specific Tregs. Our results lend support to this view, because the highest numbers of Tregs were associated with the AT genotype in infants with maternal diabetes, but the finding requires further investigation.

It has been implicated that insulin antibodies (IA) observed in neonates appear to have been transferred transplacentally from the maternal circulation during pregnancy in mothers treated with exogenous insulin. IA in insulin-treated patients are of IgG class and are actively transported through the placenta (Potter and Wilkin 2000). It is possible that either transplacentally transferred insulin bound to IA or IA as such are responsible for the induction of insulin-specific tolerance in the infants of diabetic mothers (Menon *et al*. 1990). The importance of insulin tolerization is supported by the observation that the risk for T1D in

infants born before the maternal onset of diabetes was higher than in those born in mothers diagnosed with T1D earlier, and had consequently been exposed to maternal diabetes and insulin therapy *in utero* (Warram *et al*. 1991). The present results of enhanced regulatory activity in the infants of diabetic mothers are also in agreement with previous report of decreased insulin-specific T cell proliferation responses at the age of 9 months in the offspring of diabetic mothers (Paronen *et al*. 2000). Accordingly, early exposure to human insulin could induce FOXP3-expressing Tregs with the potential to downregulate β-cell autoimmunity later in life.

In our study we found increased expression of FOXP3 in $CD4^{+}CD25^{+}$ cells after stimulation with insulin in infants with maternal T1D. The increase in the percentage of $FOXP3^{+}$ cells in $CD4^{+}CD25^{high}$ cells among insulin-stimulated CBMC was higher than in unstimulated CBMC cultured *in vitro* for 72 h ($p < 0.001$; Wilcoxon test). In infants of non-diabetic mothers there was no difference between FOXP3 expression in $CD4^{+}CD25^{high}$ T cells after stimulation with insulin compared with unstimulated CBMC (NS, Wilcoxon test).

We found up-regulation of FOXP3, NFATc2, STIM1, IL-10, and TGF-β transcripts in CBMC in infants with maternal diabetes. FOXP3, NFATc2, STIM1, IL-10, and TGF-β specific mRNA increased significantly in CBMC in response to insulin (Wilcoxon test $p < 0.001$, $p < 0.01$, $p < 0.01$, $p < 0.01$, and $p < 0.05$, respectively) in infants with maternal diabetes (original publication III, Figure 3A), whereas no such increase was seen in the infants of non-diabetic mothers (original publication III, Figure 3B). This suggests that *in vitro* stimulation with insulin resulted in further expansion of insulin-specific Tregs induced *in utero*.

In the infants with maternal diabetes and the disease-associated T allele at the PTPN22 1858 position, the up-regulation of STIM1 and NFATc2 specific mRNA in CB cells upon insulin stimulation was reduced compared to infants carrying the CC genotype ($p < 0.01$ and $p < 0.05$) (Figure 5). No such associations with the PTPN22 genotypes were seen in the infants of non-diabetic mothers.

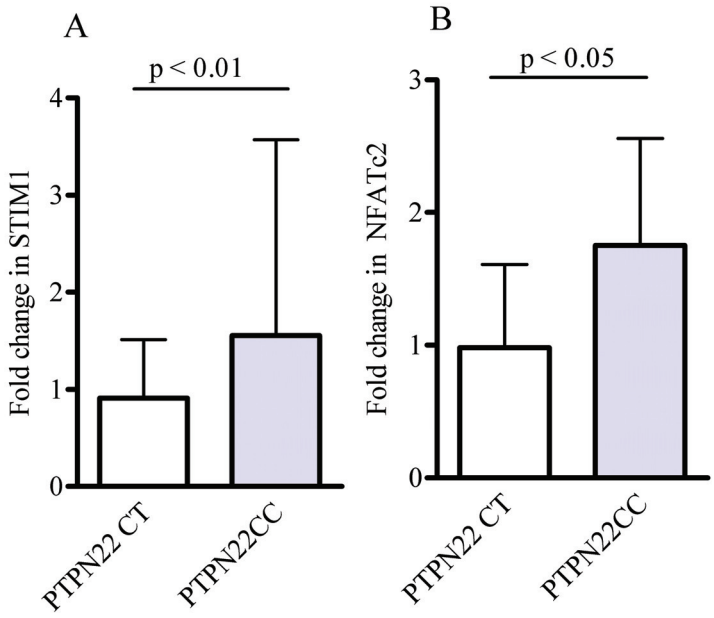

Figure 5. Up-regulation of STIM1 and NFATc2 specific mRNA expressed as the fold change in relation to the PTPN22 genotype in infants of mothers with type 1 diabetes. The fold change in STIM1 (A) and NFATc2 (B) was calculated by dividing the relative mRNA level of insulin-stimulated (72 h) CBMC by the relative mRNA level of non-stimulated CBMC. The median and range are shown. p values comparing the groups (Mann-Whitney U-test) are presented in the figure.

The impaired insulin-induced upregulation of STIM1 and NFATc2 was associated with the predisposing PTPN22 polymorphism in the infants with maternal T1D. The 620Trp variant of the LYP protein, encoded by the lymphoid tyrosine phosphatase 22 gene (PTPN22), is associated with autoimmunity. The PTPN22-coded protein is a downstream signaling molecule in TCR-mediated T cell activation (Vang *et al.* 2005), and impaired T cell activation has been associated with the T1D-predisposing PTPN22 polymorphism (Aarnisalo *et al.* 2008). The PTPN22 gene encodes LYP, which is an inhibitor of TCR signal transduction. LYP is known to dephosphorylate the protein tyrosinase kinases Lck, Fyn, and Zap70. The PTPN22 C1858T polymorphism causes arginine to tryptophan substitution in codon 620 and is associated with an increased risk of developing of T1D (Douroudis *et al.* 2008, Hermann *et al.* 2006, Zheng 2005). STIM1 is a sensor of endoplasmic reticulum calcium in T cell activation and tolerance (Oh-Hora *et al.* 2008). Interestingly, the strength of TCR signaling altered thymic T cell selection, regulatory T cell function and the autoimmune phenotype in experimental studies (Tanaka *et al.* 2010). On the basis of these

findings, weak TCR signaling could lead to decreased regulatory T cell induction in the thymus and/or defects in Treg function (Stanford *et al*. 2010). We suggest that the PTPN22 risk variant of T1D could be associated with the impaired activation of insulin-specific regulatory T cells because of impaired TCR signaling, and thus contribute to T1D.

To conclude, infants of diabetic mothers showed expansion of Tregs occurring *in utero* that is associated with the insulin genotype conferring protection against T1D. This suggests tolerization to insulin in those exposed to maternal diabetes/insulin therapy *in utero*. These results may explain the epidemiological data of a lower risk of T1D in the offspring of diabetic mothers than of diabetic fathers. Accordingly, early induction of autoantigen-specific immunological tolerance might protect against the induction of autoimmunity and progression to diabetes later in life. Our results are encouraging in terms of attempts to develop insulin-specific immune interventions for the prevention of T1D.

5.4 Differences in the intestinal microbiota between children with at least two type 1 diabetes-associated autoantibodies and autoantibody-negative children (IV)

Previous studies have indicated that factors affecting the gut, such as alterations in the intestinal microbiota, are capable of modulating the development of autoimmune diabetes. Several animal studies have provided information regarding the influence of the intestinal microbiota on the development of autoimmune diabetes. The gut microbiota differs between biobreeding diabetes-prone (BB-DP) and diabetes resistant (DR) rats. Stool samples from BB-DR rats studied before the onset of diabetes contained higher populations of *Lactobacillus* and *Bifidobacterium*, whereas BB-DP rats had higher number of *Bacteroides*, *Eubacterium*, and *Ruminococcus* (Roesch *et al.* 2009). Antibiotics, which modulate gut microbiota, can also prevent autoimmune diabetes in BB-DP rats. In a non-obese diabetic mouse model (NOD), the pathogen-free mice lacking MyD88, a TLR signaling molecule, did not develop diabetes, which emphasizes the role of the intestinal microbiota as a regulator of autoimmune diabetes (Wen *et al.* 2008). The aim of this study was to compare the composition of the gut microbiota between children with β-cell autoimmunity and autoantibody-negative children matched for age, gender, HLA risk genotype, and early feeding history.

In our study, the analysis of all samples at the phylum level showed that *Firmicutes* (58.1%), *Actinobacteria* (36.2%), and *Bacteroidete*s (3.4%) were the dominant phyla. The most common families were the *Bifidobacteriaceae* (32.8%) (*Actinobacteria*), *Lachnospiraceae* (18.4%) (*Firmicutes*), and *Ruminococcaceae* (17.1%) (*Firmicutes*). *Bifidobacterium* was the most frequent genus (34.2%). There were differences in the composition of intestinal microbiota between the autoantibody-positive and autoantibody-negative children. In

autoantibody-positive children, the phylum *Bacteroidetes*, family *Bacteroidaceae* (2.5%) and the genus *Bacteroides* (3.1%) were more common than in autoantibody-negative children ($p < 0.05$, $p < 0.05$, and $p < 0.05$, respectively). There have only been a few previous studies on the intestinal microbiota in relation to T1D in humans. However, in accordance with our results, an earlier follow-up study including four case-control pairs suggested that the ratio of *Bacteroidetes* to *Firmicutes* increased over time in those children who eventually progressed to clinical T1D, while it decreased in children who remained non-diabetic (Giongo *et al.* 2011). A role of the genus *Bacteroides* in the development of autoimmune diabetes has been implicated in both animal models and humans (Brown *et al.* 2011).

Principal component analysis (PCA) on the species level showed various correlations with β-cell autoimmunity. The first principal component (PC) correlated positively with *Bifidobacterium adolescentis* (11%), *Faecalibacterium prausnitzii* (5.6%), *Clostridium clostridioforme* (2.3%), and *Roseburia faecis* (0.94%), which are considered as short-chain fatty acid-producing species. The abundance of lactate and butyrate-producing bacteria was also inversely related to the number of β-cell autoantibodies in children; the lowest levels were observed in children positive for three or four autoantibodies (original publication IV, Figure 1).

The second and the third PC were strongly related to the abundance of both *B. adolescentis* and *Bifidobacterium pseudocatenulatum* (original publication IV, Figure 2*). B. adolescentis* was the most common species (15.8%) amongst the children in the TRIGR pilot study (older group), whereas *B. pseudocatenulatum* (younger group) was most frequent (15.8%) in the children from the FINDIA study. Samples from children with autoantibody-positivity from both age groups were overrepresented, in which the combined abundance of *B. adolescentis* and *B. pseudocatenulatum* was below 12% (see Figure 2 in original publication IV).

At level of single bacterial species, *Roseburia faecis* (0.94%) was present in greater proportions in autoantibody-negative than -positive children, whereas the butyrate-producing bacterium *Eubacterium hallii* (6.0%) inversely correlated with the total number of autoantibodies. *Bifidobacterium animalis* (0.18%), *Lactobacillus acidophilus* (0.03%), and *Clostridium perfringens* (0.03%) were more abundant in children with β-cell autoimmunity than in those without.

Butyrate is thought to be beneficial, as it is the main energy source for colonic epithelial cells (Hague *et al*. 1996). Butyrate-producing bacteria represent a functional group within the microbial community of the human large intestine. Despite their heterogeneity, butyrate plays a key role in maintaining human gut health, as the major source of energy to the colonic mucosa, and as an important regulator of gene expression, inflammation, differentiation, and apoptosis in host cells (Louis and Flint 2009). Furthermore, butyrate has been shown to regulate

the assembly of tight junctions and gut permeability (Peng *et al*. 2009). In our study, *Roseburia faecis*, which produces butyrate using acetate (Duncan *et al.* 2002), showed the most apparent inverse relation with autoantibody positivity. In addition, *E. hallii* (6.0%), which produces butyrate from lactate and acetate, was inversely correlated with the numbers of autoantibodies. A recent study including four pairs of cases who developed T1D and autoantibody-negative controls suggested that higher proportions of butyrate-producing and mucin-degrading bacteria were observed in autoantibody-negative subjects than in T1D cases (Brown *et al*. 2011).

The administration of butyrate has been tested in the prevention of autoimmune diabetes in a BB rat model. Although the enteral administration of sodium butyrate shortly after birth during the weaning period in BBDP rats did not reduce the subsequent development of autoimmune diabetes, it modulated the gut inflammatory response (Li *et al*. 2010).

In this cohort, the total duration of breastfeeding, but not the duration of exclusive breastfeeding, showed an inverse association with autoantibody positivity. The duration of breastfeeding correlated positively with the abundance of *B. adolescentis* and *Blautia hansenii*. *Coprococcus comes* were more common in children who received CM formula before the age of 6 months than in exclusively breast-fed children. The abundance of the genus *Bifidobacterium* correlated with the total duration of breastfeeding although the children in the TRIGR pilot study were studied several years later. This is in accordance with previous reports that indicated the long-lasting effects of breastfeeding on the intestinal microbiota in children (Harmsen *et al*. 2000).

Interestingly, *B. animalis* and *L. acidophilus* were found to be extremely abundant in autoantibody-positive children who had received a hydrolyzed whey-based infant formula, whereas their corresponding controls, who had received the same infant formula, did not have a high abundance of either.

The diversity of the intestinal microbiota was significantly higher in the children in the TRIGR pilot cohort (older age group) than in the children in the FINDIA pilot cohort (younger age group). Furthermore, there was a trend towards a higher diversity per sample in the autoantibody-negative than the autoantibody-positive children, especially in the children from the TRIGR pilot study. This is in agreement with an earlier study showing that microbial diversity decreased with increasing age in the four children who developed T1D and was lower in autoantibody-positive than in autoantibody-negative children (Giongo *et al.* 2011).

It is known that the 16S method in studies provides information on the number of known species in the microbiome, but no information is provided on the function of these bacteria. Moreover, the methods whereby intestinal bacterial samples are collected have an affect on the results and must be standardized. Thus, larger cohort studies are needed to further define alterations in the compo-

sition of the intestinal microbiome in humans and the underlying mechanisms that lead to autoimmunity and diabetes development.

To conclude, our study indicated the importance of bifidobacteria and lactate and/or butyrate-producing species in relation to the development of β-cell autoimmunity. Bifidobacteria not only supply butyrate-producing species with lactate and acetate, but they also enhance the intestinal epithelial barrier function by modulating the gut mucosa. In children with β-cell autoimmunity, the higher proportion of bacteria of the phylum *Bacteroides* was confirmed. The findings demonstrate specific changes in the composition of intestinal bacteria in children with β-cell autoimmunity, and suggest that dysbiosis is seen at the prediabetic stage of the disease.

6 CONCLUSIONS

I. In healthy infants with a T1D-associated HLA-mediated risk, altered T cell differentiation was observed in cord blood-derived cells. The expression of transcription factor GATA-3 and chemokine receptor CCR4 was reduced in the Th2 environment in children at risk. This suggests that infants with a T1D-associated risk genotype may develop altered immune responses to environmental factors, and this could be associated with the risk of T1D.

II. Enchanced levels of antibodies to CM proteins were observed in infancy in those children who later progressed to T1D when compared with children who remained healthy. The results indicate that children who progress to autoimmune diabetes have increased antibody responses to the cow's milk proteins during the first year of life. This may reflect a dysfunctional gut immune system in early infancy. The results suggest that dysregulation of oral tolerance is present years before the actual onset of the disease in children who later progress to T1D.

III. We demonstrated that the expression of transcription factor FOXP3 in cord blood regulatory T cells was higher in the offspring of mothers with T1D when compared with the infants of unaffected mothers. After *in vitro* insulin stimulation, the expression of FOXP3 in $CD4^{+}CD25^{+}$ regulatory cells, and up-regulation of transcription factors (FOXP3, IL-10, TGF-beta, NFATc2, and STIM1) were only increased in the offspring of mothers with T1D. The results suggest that maternal insulin therapy specifically increases regulatory T-cell activation and triggers the development of tolerance to insulin in the fetus. This may explain the lower risk of diabetes in children with maternal vs. paternal diabetes.

IV. Principal component analysis indicated that a low abundance of lactate- and butyrate-producing species was associated with β-cell autoimmunity. Furthermore, a low abundance of two *Bifidobacterium* species, *B. adolescentis* and *B. pseudocatenulatum*, but an increased abundance of the *Bacteroides* genus was observed in the children with β-cell autoimmunity. The low abundance of bifidobacteria and butyrate-producing species could adversely affect the intestinal epithelial barrier function and inflammation, whereas the apparent importance of the genus *Bacteroides* in T1D, possibly as an immunomodulator, is insufficiently understood.

7 ACKNOWLEDGEMENTS

The present work was carried out over the years 2003–2012 at the Immune Response Unit, the National Institute for Health and Welfare, and Children's Hospital, University of Helsinki.

I am deeply grateful to all people who have contributed to this work. In particular, I warmly thank:

Professor Pekka Puska, the Director of the National Institute for Health and Welfare, and Terhi Kilpi, the Head of the Department of Vaccines and Immune Protection, the National Institute of Health and Welfare, for providing excellent research facilities.

Docent Jari Petäjä (Director of the Department of Gynecology and Pediatrics, Helsinki University Central Hospital), Professor Mikael Knip (Chair of the Children's Hospital, University of Helsinki), and Docent Eero Jokinen (Head of the Department of Pediatrics, Helsinki University Central Hospital) for providing excellent facilities to carry out this study.

Professor Markku Heikinheimo, Head of the Institute of Clinical Medicine and former Head of the National Graduate School of Clinical Investigation, and former Head of the Pediatric Graduate School at University of Helsinki, and Docent Jussi Merenmies, Head of the Pediatric Graduate School, for their valuable work in bringing together and supporting young investigators.

All the children and their families, for participating in these long-lasting studies.

Professor Outi Vaarala, my supervisor, for teaching me the principles of the scientific research, sharing her knowledge with me in the field of diabetes immunology, friendship, and support at various stages of this work.

Emeritus Professor Hans Åkerblom, the first Principal Investigator of the Trial to Reduce IDDM in Genetically at Risk, my supervisor, for the opportunity to participate in the diabetes intervention study and continuous encouragement. His lifework dedication to diabetes research has made my work possible.

Docent Aaro Miettinen and Docent Jussi Kantele, for their thorough review of the thesis and for providing constructive comments.

Emeritus Professor Erkki Savilahti and Docent Jukka Rajantie, for their valuable advice and sincere support as members of the follow-up group in the Pediatric Graduate School.

All the co-authors, Professor Mikael Knip for his encouraging attitude towards scientific work and never-ending enthusiasm, Professor Jorma Ilonen for providing excpertise in genetics during these years, Professor Suvi Virtanen, Professor Gjalt Welling, Professor Hermie Harmsen, Taina Härkönen, Susanne

Skarsvik, Marcus de Coffau, Terhi Ruohtula, Janne Nieminen, Laura Orivuori, and Saara Hakala, for valuable contribution to this study.

Anneli Suomela, Harry Lybeck, Maria Kiikeri, Sinikka Tsupari, and Pirjo Mäki, for their skilful technical assistance, and Tarja Alander, for her administrative assistance in the Immune Response Unit.

All my former and present colleagues in our lab, Jarno Honkanen, for inspiring discussions and support during these years, Terhi Ruohtula, for great times shared at work and on several conference trips, Minna Tiittanen, Paula Klemetti, Johanna Paronen, Heli Siljander, Riikka Nissinen, Harri Salo, Emma Marschan, Emma Savilahti, Saara Aittomäki, Janne Nieminen, Veera Hölttä, Linnea Hartwall, Laura Orivuori, and Monika Paasela, for their support and companionship in and out of lab.

Marja Salonen, Hilkka Puttonen, Tarja Tenkula, Kristiina Merentie, Heli Suomalainen, Katriina Koski, Matti Koski, Mila Hyytinen, Eeva Pajakkala and Päivi Kleemola, for their excellent collaboration in the TRIGR Study. To work and travel with you over the years has been a great pleasure.

Sirpa Nolvi and Anne Björk, for their excellent collaboration.

All the doctors, nurses and other personnels of the TRIGR study in Finland, for pleasant collaboration, and the personnel of the Research Laboratory of Children's Hospital, for skilfull assistance.

My friends outside research, especially Marjaana and her family, for their friendship.

My dear parents, Marja and Aarne, and godmothers, Anja and Eine, for their endless support throughout my life, and my brother Matti, and his family, for their friendship and encouragement. I wish my mother could have been here today to see me reach this goal.

Finally, with all my heart, I want to thank my children Anni and Olli, for their love, and inspiration, and Juha, without your love, unfailing support and patience through the years, this work would never have been completed.

This study was financially supported by the National Graduate School of Clinical Investigation, the Päivikki and Sakari Sohlberg Foundation, the Finnish Medical Society Duodecim, the Finnish Diabetes Foundation and Finska Läkaresällskapet.

Helsinki, October 2012

Kristiina Luopajärvi

8 References

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(1), 13-21, 2008.

Abbas, A.K., Murphy, K.M. and Sher, A., Functional diversity of helper T lymphocytes, *Nature*, 383(6603), 787-793, 1996.

Abiru, N., Wegmann, D., Kawasaki, E., Gottlieb, P., Simone, E. and Eisenbarth, G.S., Dual overlapping peptides recognized by insulin peptide B:9-23 T cell receptor AV13S3 T cell clones of the NOD mouse, *J Autoimmun*, 14(3), 231-237, 2000.

Adkins, B., Leclerc, C. and Marshall-Clarke, S., Neonatal adaptive immunity comes of age, *Nat Rev Immunol*, 4(7), 553-564, 2004.

Adorini, L., Gregori, S. and Harrison, L.C., Understanding autoimmune diabetes: insights from mouse models, *Trends Mol Med*, 8(1), 31-38, 2002.

Aguilar-Bryan, L. and Bryan, J., Neonatal diabetes mellitus, *Endoc Rev*, 29(3), 265-291, 2008.

American Diabetes Association, Diagnosis and classification of diabetes mellitus, *Diabetes Care*, 33(Suppl 1), S62-9, 2010.

Andersen, M.H., Schrama, D., Thor Straten, P. and Becker, J.C., Cytotoxic T cells, *J Invest Dermatol*, 126(1), 32-41, 2006.

Anderson, M.S., Venanzi, E.S., Chen, Z., Berzins, S.P., Benoist, C. and Mathis, D., The cellular mechanism of Aire control of T cell tolerance, *Immunity*, 23(2), 227-239, 2005.

Ansel, K.M., Lee, D.U. and Rao, A., An epigenetic view of helper T cell differentiation, *Nat Immunol*, 4(7), 616-623, 2003.

Bach, J., The effect of infections on susceptibility to autoimmune and allergic diseases, *N Engl J Med*, 347(12), 911-920, 2002.

Baekkeskov, S., Aanstoot, H.J., Christgau, S., Reetz, A., Solimena, M., Cascalho, M., Folli, F., Richter-Olesen, H. and De Camilli, P., Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase, *Nature*, 347(6289), 151-156, 1990.

Banchereau, J. and Steinman, R.M., Dendritic cells and the control of immunity, *Nature*, 392(6673), 245-252, 1998.

Banting, F.G., Best, C.H., Collip, J.B., Campbell, W.R. and Fletcher, A.A., Pancreatic extracts in the treatment of Diabetes Mellitus, *CMAJ*, 12(3), 141-146, 1922.

Barrett, J.C., Clayton, D.G., Concannon, P., Akolkar, B., Cooper, J.D., Erlich, H.A., Julier, C., Morahan, G., Nerup, J., Nierras, C., Plagnol, V., Pociot, F., Schuilenburg, H., Smyth, D.J., Stevens, H., Todd, J.A., Walker, N.M., Rich, S.S. and The Type 1 Diabetes Genetics Consortium, Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes, *Nat Genet*, 41(6), 703-707, 2009.

Barton, G.M. and Medzhitov, R., Control of adaptive immune responses by Toll-like receptors, *Curr Opin Immunol*, 14 (3), 380-383, 2002.

Bell, G.I., Horita, S. and Karam, J.H., A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus, *Diabetes*, 33(2), 176-183, 1984.

Bendelac, A., Carnaud, C., Boitard, C. and Bach, J.F., Syngeneic transfer of autoimmune diabetes from diabetic NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells, *J Exp Med*, 166(4), 823-832, 1987.

Bennet, R. and Nord, C.E., Development of the faecal anaerobic microflora after caesarean section and treatment with antibiotics in newborn infants, *Infection*, 15(5), 332-336, 1987.

Bennett, C.L., Christie, J., Ramsdell, F., Brunkow, M.E., Ferguson, P.J., Whitesell, L., Kelly, T.E., Saulsbury, F.T., Chance, P.F. and Ochs, H.D., The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3, *Nat Genet*, 27(1), 20-21, 2001.

Bennett, S.T., Lucassen, A.M., Gough, S.C., Powell, E.E., Undlien, D.E., Pritchard, L.E., Merriman, M.E., Kawaguchi, Y., Dronsfield, M.J. and Pociot, F., Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus, *Nat Genet*, 9(3), 284-292, 1995.

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L. and Kuchroo, V.K., Reciprocal developmental pathways for the generation of pathogenic effector Th17 and regulatory T cells, *Nature*, 441(7090), 235-238, 2006.

Bilate, A.M. and Lafaille, J.J., Induced CD4+Foxp3+ regulatory T cells in immune tolerance, *Annu Rev Immunol*, 30(1), 733-758, 2012.

Blanas, E., Carbone, F.R., Allison, J., Miller, J.F. and Heath, W.R., Induction of autoimmune diabetes by oral administration of autoantigen, *Science*, 274(5293), 1707-1709, 1996.

Blaschitz, C. and Raffatellu, M., Th17 cytokines and the gut mucosal barrier, *J Clin Immunol*, 30(2), 196-203, 2010.

Bliss, M., *The discovery of insulin*, 25th Anniversary Edition, the University of Chicago Press, US, 2007.

Bonecchi, R., Bianchi, G., Bordignon, P.P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P.A., Mantovani, A. and Sinigaglia, F., Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s, *J Exp Med*, 187(1), 129-134, 1998.

Bonilla, F.A. and Oettgen, H.C., Adaptive immunity, *J Allergy Clin Immunol*, 125(2), S33-S40, 2010.

Bosi, E., Molteni, L., Radaelli, M.G., Folini, L., Fermo, I., Bazzigaluppi, E., Piemonti, L., Pastore, M.R. and Paroni, R., Increased intestinal permeability precedes clinical onset of type 1 diabetes, *Diabetologia*, 49(12), 2824-2827, 2006.

Bottazzo, G.F., Florin-Christensen, A. and Doniach, D., Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies, *Lancet*, 2(7892), 1279-1283, 1974.

Bottazzo, G.F., Dean, B.M., McNally, J.M., MacKay, E.H., Swift, P.G. and Gamble, D.R., In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis, *N Engl J Med*, 313(6), 353-360, 1985.

Bottini, N., Musumeci, L., Alonso, A., Rahmouni, S., Nika, K., Rostamkhani, M., MacMurray, J., Meloni, G.F., Lucarelli, P., Pellecchia, M., Eisenbarth, G.S., Comings, D. and Mustelin, T., A functional variant of lymphoid tyrosine phosphatase is associated with type 1 diabetes, *Nat Genet*, 36(4), 337-338, 2004.

Bottini, N., Vang, T., Cucca, F. and Mustelin, T., Role of PTPN22 in type 1 diabetes and other autoimmune diseases, *Semin Immunol*, 18(4), 207-213, 2006.

Bresson, D., Togher, L., Rodrigo, E., Chen, Y., Bluestone, J.A., Herold, K.C. and von Herrath, M., Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs, *J Clin Invest*, 116(5), 1371-1381, 2006.

Brown, C.T., Davis-Richardson, A.G., Giongo, A., Gano, K.A., Crabb, D.B., Mukherjee, N., Casella, G., Drew, J.C., Ilonen, J., Knip, M., Hyoty, H., Veijola, R., Simell, T., Simell, O., Neu, J., Wasserfall, C.H., Schatz, D., Atkinson, M.A. and Triplett, E.W., Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for

for the development of autoimmunity for type 1 diabetes, *PloS one*, 6(10), e25792, 2011.

Bryson, Y.J., Winter, H.S., Gard, S.E., Fischer, T.J. and Stiehm, E.R., Deficiency of immune interferon production by leukocytes of normal newborns, *Cell Immunol*, 55(1), 191-200, 1980.

Cao, X., Cai, S.F., Fehniger, T.A., Song, J., Collins, L.I., Piwnica-Worms, D.R. and Ley, T.J., Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance, *Immunity*, 27(4), 635-646, 2007.

Carratu, R., Secondulfo, M., de Magistris, L., Iafusco, D., Urio, A., Carbone, M.G., Pontoni, G., Carteni, M. and Prisco, F., Altered intestinal permeability to mannitol in diabetes mellitus type I, *J Pediatr Gastroenterol Nutr*, 28(3), 264-269, 1999.

Chaplin, D.D., Overview of the immune response, *J Allergy Clin Immunol*, 125(2), S3-23, 2010.

Chapman, S.J., Khor, C.C., Vannberg, F.O., Maskell, N.A., Davies, C.W., Hedley, E.L., Segal, S., Moore, C.E., Knox, K., Day, N.P., Gillespie, S.H., Crook, D.W., Davies, R.J. and Hill, A.V., PTPN22 and invasive bacterial disease, *Nat Genet*, 38(5), 499-500, 2006.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G. and Wahl, S.M., Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor Foxp3, *J Exp Med*, 198(12), 1875-1886, 2003.

Chen, Y., Kuchroo, V.K., Inobe, J., Hafler, D.A. and Weiner, H.L., Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis, *Science*, 265(5176), 1237-1240, 1994.

Chen, Y., Inobe, J., Marks, R., Gonnella, P., Kuchroo, V.K. and Weiner, H.L., Peripheral deletion of antigen-reactive T cells in oral tolerance, *Nature*, 376(6536), 177-180, 1995.

Chimienti, F., Devergnas, S., Favier, A. and Seve, M., Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules, *Diabetes*, 53(9), 2330-2337, 2004.

Coffman, R.L. and Carty, J., A T cell activity that enhances polyclonal IgE production and its inhibition by interferon-gamma, *J Immunol*, 136(3), 949-954, 1986.

Cohen, S., Dadi, H., Shaoul, E., Sharfe, N. and Roifman, C.M., Cloning and characterization of a lymphoid-specific, inducible human protein tyrosine phosphatase, Lyp, *Blood*, 93(6), 2013-2024, 1999.

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A.S., McGarrell, D.M., Marsh, T., Garrity, G.M. and Tiedje, J.M., The Ribosomal Database Project: improved alignments and new tools for rRNA analysis, *Nucleic Acids Res*, 37, D141-145, 2009.

Coleman, D.L., Kuzava, J.E. and Leiter, E.H., Effect of diet on incidence of diabetes in non-obese diabetic mice, *Diabetes*, 39(4), 432-436, 1990.

Collison, L.W., Workman, C.J., Kuo, T.T., Boyd, K., Wang, Y., Vignali, K.M., Cross, R., Sehy, D., Blumberg, R.S. and Vignali, D.A.A., The inhibitory cytokine IL-35 contributes to regulatory T-cell function, *Nature*, 450(7169), 566-569, 2007.

Commins, S.P., Borish, L. and Steinke, J.W., Immunologic messenger molecules: Cytokines, interferons, and chemokines, *J Allergy Clin Immunol*, 125(2), S53-S72, 2010.

Couper, J.J., Steele, C., Beresford, S., Powell, T., McCaul, K., Pollard, A., Gellert, S., Tait, B., Harrison, L.C. and Colman, P.G., Lack of association between duration of breast-feeding or introduction of cow's milk and development of islet autoimmunity, *Diabetes*, 48(11), 2145-2149, 1999.

Craig, M.E., Hattersley, A. and Donaghue, K.C., Definition, epidemiology and classification of diabetes in children and adolescents, *Pediatr Diabetes*, 10(Suppl 12), 3-12, 2009.

Cupedo, T., Nagasawa, M., Weijer, K., Blom, B. and Spits, H., Development and activation of regulatory T-cells in the human fetus, *Eur J Immunol*, 35(2), 383-390, 2005.

Dahlquist, G., Savilahti, E. and Landin-Olsson, M., An increased level of antibodies to beta-lactoglobulin is a risk determinant for early-onset type 1 (insulin-dependent) diabetes mellitus independent of islet cell antibodies and early introduction of cow's milk, *Diabetologia*, 35(10), 980-984, 1992.

Daneman, D., Fishman, L., Clarson, C. and Martin, J.M., Dietary triggers of insulin-dependent diabetes in the BB rat, *Diabetes Res*, 5(2), 93-97, 1987.

Daneman, D., Type 1 diabetes, *Lancet*, 367(9513), 847-858, 2006.

Daniel, D., Gill, R.G., Schloot, N. and Wegmann, D., Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice, *Eur J Immunol,* 25(4), 1056-1062, 1995.

Daniel, D. and Wegmann, D.R., Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23), *Proc Natl Acad Sci USA*, 93(2), 956-960, 1996.

De Filippo, C., Cavalieri, D., Di Paola, M., Ramazzotti, M., Poullet, J.B., Massart, S., Collini, S., Pieraccini, G. and Lionetti, P., Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa, *Proc Natl Acad Sci USA*, 107(33), 14691-14696, 2010.

de Jong, E.C., Smits, H.H. and Kapsenberg, M.L., Dendritic cell-mediated T cell polarization, *Springer Semin Immun*, 26(3), 289-307, 2005.

Del Prete, G.F., De Carli, M., Mastromauro, C., Biagiotti, R., Macchia, D., Falagiani, P., Ricci, M. and Romagnani, S., Purified protein derivative of Mycobacterium tuberculosis and excretory-secretory antigen(s) of Toxocara canis expand in vitro human T cells with stable and opposite (type 1 T helper or type 2 T helper) profile of cytokine production, *J Clin Invest*, 88(1), 346-350, 1991.

Delves, P.J. and Roitt, I.M., The immune system, *N Engl J Med*, 343(1), 37-49, 2000.

DIAMOND Project Group, Incidence and trends of childhood type 1 diabetes worldwide 1990-1999, *Diabet Med*, 23(8), 857-866, 2006.

Di Lorenzo, T.P., Peakman, M. and Roep, B.O., Translational mini-review series on type 1 diabetes: systematic analysis of T cell epitopes in autoimmune diabetes, *Clin Exp Immunol*, 148(1), 1-16, 2007.

Douroudis, K., Prans, E., Haller, K., Nemvalts, V., Rajasalu, T., Tillmann, V., Kisand, K. and Uibo, R., Protein tyrosine phosphatase non-receptor type 22 gene variants at position 1858 are associated with type 1 and type 2 diabetes in Estonian population, *Tissue Antigens*, 72(5), 425-430, 2008.

Duncan, S.H., Barcenilla, A., Stewart, C.S., Pryde, S.E. and Flint, H.J., Acetate utilization and butyryl coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large intestine, *Appl Environ Microbiol*, 68(10), 5186-5190, 2002.

Durinovic-Bello, I., Wu, R.P., Gersuk, V.H., Sanda, S., Shilling, H.G. and Nepom, G.T., Insulin gene VNTR genotype associates with frequency and phenotype of the autoimmune response to proinsulin, *Genes Immun*, 11(2), 188-193, 2010.

Edelbauer, M., Loibichler, C., Nentwich, I., Gerstmayr, M., Urbanek, R. and Szépfalusi, Z., Maternally delivered nutritive allergens in cord blood and in placental tissue of term and preterm neonates, *Clin Exp Allergy*, 34(2), 189-193, 2004.

El-Hashimy, M., Angelico, M.C., Martin, B.C., Krolewski, A.S. and Warram, J.H., Factors modifying the risk of IDDM in offspring of an IDDM parent, *Diabetes*, 44(3), 295-299, 1995.

Elliott, R.B. and Martin, J.M., Dietary protein: a trigger of insulin-dependent diabetes in the BB rat? *Diabetologia*, 26(4), 297-299, 1984.

Elliott, R.B., Reddy, S.N., Bibby, N.J. and Kida, K., Dietary prevention of diabetes in the non-obese

diabetic mouse, *Diabetologia*, 31(1), 62-64, 1988.

Emamaullee, J.A., Davis, J., Merani, S., Toso, C., Elliott, J.F., Thiesen, A. and Shapiro, A.M., Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice, *Diabetes*, 58(6), 1302-1311, 2009.

Encabo, A., Solves, P., Carbonell-Uberos, F. and Miñana, M.D., The functional immaturity of dendritic cells can be relevant to increased tolerance associated with cord blood transplantation, *Transfusion*, 47(2), 272-279, 2007.

EURODIAB ACE Study Group, variation and trends in incidence of childhood diabetes in Europe, *Lancet*, 355(9207), 873-876, 2000.

Fajans, S.S., Bell, G.I. and Polonsky, K.S., Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young, *N Engl J Med*, 345(13), 971-980, 2001.

Finnish-German APECED Consortium, An autoimmune disease, APECED, caused by mutations in a novel gene featuring two PHD-type zinc-finger domains, *Nat Genet*, 17(4), 399-403, 1997.

Fontenot, J.D., Gavin, M.A. and Rudensky, A.Y., Foxp3 programs the development and function of CD4+CD25+ regulatory T cells, *Nat Immunol*, 4(4), 330-336, 2003.

Fontenot, J.D. and Rudensky, A.Y., A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3, *Nat Immunol*, 6(4), 331-337, 2005.

Fort, P., Lanes, R., Dahlem, S., Recker, B., Weyman-Daum, M., Pugliese, M. and Lifshitz, F., Breast feeding and insulin-dependent diabetes mellitus in children, *J Am Coll Nutr,* 5(5), 439-441, 1986.

Foulis, A.K., Farquharson, M.A. and Hardman, R., Aberrant expression of class II major histocompatibility complex molecules by B cells and hyperexpression of class I major histocompatibility complex molecules by insulin containing islets in type 1 (insulin-dependent) diabetes mellitus, *Diabetologia*, 30(5), 333-343, 1987.

Fuchtenbusch, M., Irnstetter, A., Jager, G. and Ziegler, A.G., No evidence for an association of coxsackie virus infections during pregnancy and early childhood with development of islet autoantibodies in offspring of mothers or fathers with type 1 diabetes, *J Autoimmun*, 17(4), 333-340, 2001.

Gale, E.A., Bingley, P.J., Emmett, C.L., Collier, T. and European Nicotinamide Diabetes Intervention Trial (ENDIT) Group, European Nicotinamide Diabetes Intervention Trial (ENDIT): a randomised controlled trial of intervention before the onset of type 1 diabetes, *Lancet*, 363(9413), 925-931, 2004.

Gepts, W., Pathologic anatomy of the pancreas in juvenile diabetes mellitus, *Diabetes*, 14(10), 619-633, 1965.

Gerstein, H.C., Cow's milk exposure and type 1 diabetes mellitus. A critical overview of the clinical literature, *Diabetes Care*, 17(1), 13-19, 1994.

Giongo, A., Gano, K.A., Crabb, D.B., Mukherjee, N., Novelo, L.L., Casella, G., Drew, J.C., Ilonen, J., Knip, M., Hyöty, H., Veijola, R., Simell, T., Simell, O., Neu, J., Wasserfall, C.H., Schatz, D., Atkinson, M.A. and Triplett, E.W., Toward defining the autoimmune microbiome for type 1 diabetes, *ISME J*, 5(1), 82-91, 2011.

Goecks, J., Nekrutenko, A., Taylor, J. and Galaxy Team, Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences, *Genome Biol*, 11(8), R86, 2010.

Gomez, L.M., Anaya, J.M. and Martin, J., Genetic influence of PTPN22 R620W polymorphism in tuberculosis, *Hum Immunol*, 66(12), 1242-1247, 2005.

Gottlieb, P.A. and Eisenbarth, G.S., Insulin-specific tolerance in diabetes, *Clin Immunol*, 102(1), 2-11, 2002.

Graves, P.M., Rotbart, H.A., Nix, W.A., Pallansch, M.A., Erlich, H.A., Norris, J.M., Hoffman, M., Eisenbarth, G.S. and Rewers, M., Prospective

study of enteroviral infections and development of beta-cell autoimmunity. Diabetes autoimmunity study in the young (DAISY), *Diabetes Res Clinical Pract*, 59(1), 51-61, 2003.

Groux, H., O'Garra, A., Bigler, M., Rouleau, M., Antonenko, S., de Vries, J.E. and Roncarolo, M.G., A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis, *Nature*, 389(6652), 737-742, 1997.

Grönlund, M.M., Lehtonen, O.P., Eerola, E. and Kero, P., Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery, *J Pediatr Gastroenterol Nutr*, 28(1), 19-25, 1999.

Hague, A., Butt, A.J. and Paraskeva, C., The role of butyrate in human colonic epithelial cells: an energy source or inducer of differentiation and apoptosis? *Proc Nutr Soc*, 55(3), 937-943, 1996.

Halbout, P., Briand, J.P., Becourt, C., Muller, S. and Boitard, C., T cell response to preproinsulin I and II in the nonobese diabetic mouse, *J Immunol*, 169(5), 2436-2443, 2002.

Halminen, M., Simell, O., Knip, M. and Ilonen, J., Cytokine expression in unstimulated PBMC of children with type 1 diabetes and subjects positive for diabetes-associated autoantibodies, *Scand J Immunol*, 53(5), 510-513, 2001.

Halonen, M., Lohman, I.C., Stern, D.A., Spangenberg, A., Anderson, D., Mobley, S., Ciano, K., Peck, M. and Wright, A.L., Th1/Th2 patterns and balance in cytokine production in the parents and infants of a large birth cohort, *J Immunol*, 182(5), 3285-3293, 2009

Harjutsalo, V., Podar, T. and Tuomilehto, J., Cumulative incidence of type 1 diabetes in 10,168 siblings of Finnish young-onset type 1 diabetic patients, *Diabetes*, 54(2), 563-569, 2005.

Harjutsalo, V., Reunanen, A. and Tuomilehto, J., Differential transmission of type 1 diabetes from diabetic fathers and mothers to their offspring, *Diabetes*, 55(5), 1517-1524, 2006.

Harjutsalo, V., Sjöberg, L. and Tuomilehto, J., Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study, *Lancet*, 371(9626), 1777-1782, 2008.

Harjutsalo, V., Lammi, N., Karvonen, .M. and Groop, P.H., Age at onset of type 1 diabetes in parents and recurrence risk in offspring, *Diabetes*, 59(1), 210-214, 2010.

Harmsen, H.J., Wildeboer-Veloo, A.C., Raangs, G.C., Wagendorp, A.A., Klijn, N., Bindels, J.G. and Welling, G.W., Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods, *J Pediatr Gastroenterol Nutr*, 30(1), 61-67, 2000.

Harrison, L.C., Dempsey-Collier, M., Kramer, D.R. and Takahashi, K. Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes, *J Exp Med*, 184(6), 2167-2174, 1996.

Haynes, B.F., Martin, M.E., Kay, H.H. and Kurtzberg, J., Early events in human T cell ontogeny. Phenotypic characterization and immunohistologic localization of T cell precursors in early human fetal tissues, *J Exp Med*, 168(3), 1061-1080, 1988.

Healey, D., Ozegbe, P., Arden, S., Chandler, P., Hutton, J. and Cooke, A., In vivo activity and in vitro specificity of CD4+ Th1 and Th2 cells derived from the spleens of diabetic NOD mice, *J Clin Invest*, 95(6), 2979-2985, 1995.

Hermann, R., Knip, M., Veijola, R., Simell, O., Laine, A.P., Åkerblom, H.K., Groop, P.H., Forsblom, C., Pettersson-Fernholm, K., Ilonen, J. and FinnDiane Study Group, Temporal changes in the frequencies of HLA genotypes in patients with type 1 diabetes-indication of an increased environmental pressure? *Diabetologia*, 46(3), 420-425, 2003.

Hermann, R., Lipponen, K., Kiviniemi, M., Kakko, T., Veijola, R., Simell, O., Knip, M. and Ilonen, J., Lymphoid tyrosine phosphatase (LYP/PTPN22) Arg620Trp variant regulates insulin autoimmunity and progression to type 1 diabetes, *Diabetologia*, 49(6), 1198-1208, 2006.

Hernandez, J.B., Newton, R.H. and Walsh, C.M., Life and death in the thymus − cell death signaling during T cell development, *Curr Opin Cell Biol*, 22(6), 865-871, 2010.

Herold, K.C., Hagopian, W., Auger, J.A., Poumian-Ruiz, E., Taylor, L., Donaldson, D., Gitelman, S.E., Harlan, D.M., Xu, D., Zivin, R.A. and Bluestone, J.A., Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus, *N Engl J Med*, 346(22), 1692-1698, 2002.

Hogan, P.G., Chen, L., Nardone, J. and Rao, A., Transcriptional regulation by calcium, calcineurin, and NFAT, *Genes Dev*, 17(18), 2205-2232, 2003.

Holloway, J.A., Warner, J.O., Vance, G.H., Diaper, N.D., Warner, J.A. and Jones, C.A., Detection of house-dust-mite allergen in amniotic fluid and umbilical-cord blood, *Lancet*, 356(9245), 1900-1902, 2000.

Holloway, J.A., Thornton, C.A., Diaper, N.D., Howe, D.T. and Warner, J.O., Phenotypic analysis of circulating dendritic cells during the second half of human gestation, *Pediatr Allergy Immunol*, 20(2), 119-125, 2009.

Holmberg, H., Wahlberg, J., Vaarala, O., Ludvigsson, J. and ABIS Study Group, Short duration of breast-feeding as a risk-factor for beta-cell autoantibodies in 5-year-old children from the general population, *Br J Nutr*, 97(1), 111-116, 2007.

Holt, P.G. and Jones, C.A., The development of the immune system during pregnancy and early life, *Allergy*, 55(8), 688-697, 2000.

Honkanen, J., Nieminen, J.K., Gao, R., Luopajärvi, K., Salo, H.M., Ilonen, J., Knip, M., Otonkoski, T. and Vaarala, O., IL-17 Immunity in human type 1 diabetes, *J Immunol*, 185(3), 1959-1967, 2010.

Hummel, M., Bonifacio, E., Naserke, H.E. and Ziegler, A.G. Elimination of dietary gluten does not reduce titers of type 1 diabetes-associated autoantibodies in high-risk subjects, *Diabetes Care*, 25(7), 1111-1116, 2002.

Hyttinen, V., Kaprio, J., Kinnunen, L., Koskenvuo, M. and Tuomilehto, J., Genetic liability of type 1 diabetes and the onset age among 22,650 young Finnish twin pairs: a nationwide follow-up study, *Diabetes*, 52(4), 1052-1055, 2003.

Hämäläinen, A.M., Ronkainen, M.S., Åkerblom, H.K. and Knip, M., Postnatal elimination of transplacentally acquired disease-associated antibodies in infants born to families with type 1 diabetes. The Finnish TRIGR Study Group. Trial to Reduce IDDM in the Genetically at Risk, *J Clin Endocrinol Metab*, 85(11), 4249-4253, 2000.

Hänninen, A., Salmi, M., Simell, O. and Jalkanen, S., Mucosa-associated (beta 7-integrinhigh) lymphocytes accumulate early in the pancreas of NOD mice and show aberrant recirculation behavior, *Diabetes*, 45(9), 1173-1180, 1996.

Hänninen, A., Braakhuis, A., Heath, W.R. and Harrison, L.C., Mucosal antigen primes diabetogenic cytotoxic T-lymphocytes regardless of dose or delivery route, *Diabetes*, 50(4), 771-775, 2001.

Hänninen, A. and Harrison, L.C., Mucosal tolerance to prevent type 1 diabetes: can the outcome be improved in humans? *Rev Diabet Stud*, 1(3), 113-121, 2004.

Hänninen, A., Nurmela, R., Maksimow, M., Heino, J., Jalkanen, S. and Kurts, C., Islet beta-cell-specific T cells can use different homing mechanisms to infiltrate and destroy pancreatic islets, *Am J Pathol*, 170(1), 240-250, 2007.

Ilonen, J., Sjöroos, M., Knip, M., Veijola, R., Simell, O., Åkerblom, H.K., Paschou, P., Bozas, E., Havarani, B., Malamitsi-Puchner, A., Thymelli, J., Vazeou, A. and Bartsocas, C.S., Estimation of genetic risk for type 1 diabetes, *Am J Med Genet*, 115(1), 30-36, 2002.

Itoh, N., Hanafusa, T., Miyazaki, A., Miyagawa, J., Yamagata, K., Yamamoto, K., Waguri, M., Imagawa, A., Tamura, S. and Inada, M., Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex an-

tigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients, *J Clin Invest*, 92(5), 2313-2322, 1993.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J. and Littman, D.R., The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper Cells, *Cell*, 126(6), 1121-1133, 2006.

Ivanov, I.I., de Frutos Lanos R., Manel, N., Yoshinaga, K., Rifkin, D.B., Sartor, R.B., Finlay, B.B. and Littman, D.R., Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine, *Cell Host Microbe*, 4(4), 337-349, 2008.

Jabeen, R. and Kaplan, M.H., The symphony of the ninth: the development and function of Th9 cells, *Curr Opin Immunol*, 24(3), 303-307, 2012.

Jameson, S.C. and Masopust, D., Diversity in T cell memory: an embarrassment of riches, *Immunity*, 31(6), 859-871, 2009.

Janeway, C.A., Jr and Medzhitov, R., Innate immune recognition, *Annu Rev Immunol*, 20, 197-216, 2002.

Jasinski, J.M. and Eisenbarth, G.S., Insulin as a primary autoantigen for type 1A diabetes, *Clin Dev Immunol*, 12(3), 181-186, 2005.

Johnston, C., Pyke, D.A., Cudworth, A.G. and Wolf, E., HLA-DR typing in identical twins with insulin-dependent diabetes: difference between concordant and discordant pairs, *BMJ*, 286(6361), 253-255, 1983.

Jones, C.A., Vance, G.H., Power, L.L., Pender, S.L., MacDonald, T.T. and Warner, J.O., Costimulatory molecules in the developing human gastrointestinal tract: a pathway for fetal allergen priming, *J Allergy Clin Immunol*, 108(2), 235-241, 2001.

Karlsson, H., Hessle, C. and Rudin, A., Innate immune responses of human neonatal cells to bacteria from the normal gastrointestinal flora, *Infect Immun*, 70(12), 6688-6696, 2002.

Karvonen, M., Viik-Kajander, M., Moltchanova, E., Libman, I., LaPorte, R. and Tuomilehto, J., Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group, *Diabetes Care*, 23(10), 1516-1526, 2000.

Kent, S.C., Chen, Y., Bregoli, L., Clemmings, S.M., Kenyon, N.S., Ricordi, C., Hering, B.J. and Hafler, D.A., Expanded T cells from pancreatic lymph nodes of type 1 diabetic subjects recognize an insulin epitope, *Nature*, 435(7039), 224-228, 2005.

Kimpimäki, T., Kupila, A., Hämäläinen, A.M., Kukko, M., Kulmala, P., Savola, K., Simell, T., Keskinen, P., Ilonen, J., Simell, O. and Knip, M., The first signs of beta-cell autoimmunity appear in infancy in genetically susceptible children from the general population: the Finnish Type 1 Diabetes Prediction and Prevention Study, *J Clin Endocrinol Metab*, 86(10), 4782-4788, 2001.

Kimpimäki, T., Kulmala, P., Savola, K., Kupila, A., Korhonen, S., Simell, T., Ilonen, J., Simell, O. and Knip, M., Natural history of beta-cell autoimmunity in young children with increased genetic susceptibility to type 1 diabetes recruited from the general population, *J Clin Endocrinol Metab*, 87(10), 4572-4579, 2002.

Kinugasa, T., Sakaguchi, T., Gu, X. and Reinecker, H.C., Claudins regulate the intestinal barrier in response to immune mediators, *Gastroenterology*, 118(6), 1001-1011, 2000.

Kiviniemi, M., Nurmi, J., Turpeinen, H., Lovgren, T. and Ilonen, J., A homogeneous high-throughput genotyping method based on competitive hybridization, *Clin Biochem*, 36(8), 633-640, 2003.

Klein, J. and Sato, A., The HLA system, *N Engl J Med*, 343(10), 702-709, 2000.

Knip, M., Virtanen, S.M., Seppä, K., Ilonen, J., Savilahti, E., Vaarala, O., Reunanen, A., Teramo, K., Hämäläinen, A.M., Paronen, J., Dosch, H.M., Hakulinen, T., Åkerblom, H.K. and Finnish TRIGR Study Group, Dietary intervention in infancy and later signs of beta-cell

autoimmunity, *N Engl J Med*, 363(20), 1900-1908, 2010.

Kondrashova, A., Reunanen, A., Romanov, A., Karvonen, A., Viskari, H., Vesikari, T., Ilonen, J., Knip, M. and Hyöty, H., A six-fold gradient in the incidence of type 1 diabetes at the eastern border of Finland, *Ann Med*, 37(1), 67-72, 2005.

Korn, T., Bettelli, E., Oukka, M. and Kuchroo, V.K., IL-17 and Th17 Cells, *Annu Rev Immunol*, 27, 485-517, 2009.

Kretowski, A., Mysliwiec, J. and Kinalska, I., In vitro interleukin-13 production by peripheral blood in patients with newly diagnosed insulin-dependent diabetes mellitus and their first degree relatives, *Scandinavian J Immunol*, 51(3), 321-325, 2000.

Kukko, M., Kimpimäki, T., Korhonen, S., Kupila, A., Simell, S., Veijola, R., Simell, T., Ilonen, J., Simell, O. and Knip, M., Dynamics of diabetes-associated autoantibodies in young children with human leukocyte antigen-conferred risk of type 1 diabetes recruited from the general population, *J Clin Endocrinol Metab*, 90(5), 2712-2717, 2005.

Kumar, D., Gemayel, N.S., Deapen, D., Kapadia, D., Yamashita, P.H., Lee, M., Dwyer, J.H., Roy-Burman, P., Bray, G.A. and Mack, T.M., North-American twins with IDDM. Genetic, etiological, and clinical significance of disease concordance according to age, zygosity, and the interval after diagnosis in first twin, *Diabetes*, 42(9), 1351-1363, 1993.

Kupila, A., Keskinen, P., Simell, T., Erkkilä, S., Arvilommi, P., Korhonen, S., Kimpimäki, T., Sjöroos, M., Ronkainen, M., Ilonen, J., Knip, M. and Simell, O., Genetic risk determines the emergence of diabetes-associated auto-antibodies in young children, *Diabetes*, 51(3), 646-651, 2002.

Kurts, C., Robinson, B.W. and Knolle, P.A., Cross-priming in health and disease, *Nat Rev Immunol*, 10(6), 403-414, 2010.

Kyvik, K.O., Green, A., Svendsen, A. and Mortensen, K., Breast feeding and the development of type 1 diabetes mellitus, *Diabet Med*, 9(3), 233-235, 1992.

Kyvik, K.O., Green, A. and Beck-Nielsen, H., Concordance rates of insulin dependent diabetes mellitus: a population based study of young Danish twins, *BMJ*, 311(7010), 913-917, 1995.

Laaksonen, M., Pastinen, T., Sjöroos, M., Kuokkanen, S., Ruutiainen, J., Sumelahti, M.L., Reijonen, H., Salonen, R., Wikstrom, J., Panelius, M., Partanen, J., Tienari, P.J. and Ilonen, J., HLA class II associated risk and protection against multiple sclerosis-a Finnish family study, *J Neuroimmunol*, 122(1-2), 140-145, 2002.

Laine, A.P., Holmberg, H., Nilsson, A., Ortqvist, E., Kiviniemi, M., Vaarala, O., Åkerblom, H.K., Simell, O., Knip, M., Ludvigsson, J., Ivarsson, S.A., Larsson, K., Lernmark, A., Ilonen, J. and Finnish Paediatric Diabetes Registry, Two insulin gene single nucleotide polymorphisms associated with type 1 diabetes risk in the Finnish and Swedish populations, *Dis Markers*, 23(3), 139-145, 2007.

Lammi, N., Taskinen, O., Moltchanova, E., Notkola, I.L., Eriksson, J.G., Tuomilehto, J. and Karvonen, M., A high incidence of type 1 diabetes and an alarming increase in the incidence of type 2 diabetes among young adults in Finland between 1992 and 1996, *Diabetologia*, 50(7), 1393-1400, 2007.

Lammi, N., Blomstedt, P.A., Moltchanova, E., Eriksson, J.G., Tuomilehto, J. and Karvonen, M., Marked temporal increase in the incidence of type 1 and type 2 diabetes among young adults in Finland, *Diabetologia*, 51(5), 897-899, 2008.

Lampeter, E.F., Homberg, M., Quabeck, K., Schaefer, U.W., Wernet, P., Bertrams, J., Grosse-Wilde, H., Gries, F.A. and Kolb, H., Transfer of insulin-dependent diabetes between HLA-identical siblings by bone marrow transplantation, *Lancet*, 341(8855), 1243-1244, 1993.

Legg, J.P., Jones, C.A., Warner, J.A., Johnston, S.L. and Warner, J.O., A hypothesis: antenatal sensitisation to respiratory syncytial virus in viral

bronchiolitis, *Arch Dis Child*, 86(6), 431-433, 2002.

Lehtonen, O.P., Grahn, E.M., Ståhlberg, T.H. and Laitinen, L.A., Amount and avidity of salivary and serum antibodies against Streptococcus mutans in two groups of human subjects with different dental caries susceptibility, *Infect Immun*, 43(1), 308-313, 1984.

Levy-Marchal, C., Patterson, C. and Green, A., Variation by age group and seasonality at diagnosis of childhood IDDM in Europe. The EURODIAB ACE Study Group, *Diabetologia*, 38(7), 823-830, 1995.

Li, N., Hatch, M., Wasserfall, C.H., Douglas-Escobar, M., Atkinson, M.A., Schatz, D.A. and Neu, J., Butyrate and type 1 diabetes mellitus: can we fix the intestinal leak? *J Pediatr Gastroenterol Nutr*, 51(4), 414-417, 2010.

Lin, H., Mosmann, T.R., Guilbert, L., Tuntipopipat, S. and Wegmann, T.G., Synthesis of T helper 2-type cytokines at the maternal-fetal interface, *J Immunol*, 151(9), 4562-4573, 1993.

Liu, J., Lluis, A., Illi, S., Layland, L., Olek, S., von Mutius, E. and Schaub, B., T regulatory cells in cord blood-FOXP3 demethylation as reliable quantitative marker, *PLoS ONE*, 5(10), e13267, 2010.

Liu, W., Putnam, A.L., Xu-Yu, Z., Szot, G.L., Lee, M.R., Zhu, S., Gottlieb, P.A., Kapranov, P., Gingeras, T.R., Fazekas de St Groth, B., Clayberger, C., Soper, D.M., Ziegler, S.F. and Bluestone, J.A., CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells, *J Exp Med*, 203(7), 1701-1711, 2006.

Lohmann, T., Laue, S., Nietzschmann, U., Kapellen, T.M., Lehmann, I., Schroeder, S., Paschke, R. and Kiess, W., Reduced expression of Th1-associated chemokine receptors on peripheral blood lymphocytes at diagnosis of type 1 diabetes, *Diabetes*, 51(8), 2474-2480, 2002.

Lorenzen, T., Pociot, F., Stilgren, L., Kristiansen, O.P., Johannesen, J., Olsen, P.B., Walmar, A., Larsen, A., Albrechtsen, N.C., Eskildsen, P.C., Andersen, O.O. and Nerup, J., Predictors of IDDM recurrence risk in offspring of Danish IDDM patients. Danish IDDM Epidemiology and Genetics Group, *Diabetologia*, 41(6), 666-673, 1998.

Louis, P. and Flint, H.J., Diversity, metabolism and microbial ecology of butyrate-producing bacteria from the human large intestine, *FEMS Microbiol Lett*, 294(1), 1-8, 2009.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A. and Schleifer, K.H., ARB: a software environment for sequence data, *Nucleic Acids Res*, 32(4), 1363-1371, 2004.

Luster, A.D., Chemokines-chemotactic cytokines that mediate inflammation, *N Engl J Med*, 338(7), 436-445, 1998.

MacCuish, W.J. Irvine W.J., Barnes E.W. and Duncan L.J., Antibodies to pancreatic islet cells in insulin-dependent diabetics with coexistent autoimmune disease, *Lancet*, 2(7896), 1529-1531, 1974.

Malkani, S., Nompleggi, D., Hansen, J.W., Greiner, D.L., Mordes, J.P. and Rossini, A.A., Dietary cow's milk protein does not alter the frequency of diabetes in the BB rat, *Diabetes*, 46(7), 1133-1140, 1997.

Martikainen, A., Saukkonen, T., Kulmala, P.K., Reijonen, H., Ilonen, J., Teramo, K., Koskela, P., Knip, M. and Åkerblom, H.K., Disease-associated antibodies in offspring of mothers with IDDM, *Diabetes*, 45(12), 1706-1710, 1996.

Martin-Orozco, N., Chung, Y., Chang, S.H., Wang, Y. and Dong, C., Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells, *Eur J Immunol*, 39(1), 216-224, 2009.

Marwaha, A.K., Crome, S.Q., Panagiotopoulos, C., Berg, K.B., Qin, H., Ouyang, Q., Xu, L., Priatel, J.J., Levings, M.K. and Tan, R., Cutting edge: Increased IL-17-secreting T cells in children with new-onset type 1 diabetes, *J Immunol*, 185(7), 3814-3818, 2010.

Matthews, J.N., Altman, D.G., Campbell, M.J. and Royston, P., Analysis of serial measurements in medical research, *BMJ*, 300(6719), 230-235, 1990.

Mayer, E., Bannert, C., Gruber, S., Klunker, S., Spittler, A., Akdis, C.A., Szepfalusi, Z. and Eiwegger, T., Cord blood derived CD4+ $CD25^{high}$ T cells become functional regulatory T cells upon antigen encounter, *PloS One*, 7(1), e29355, 2012.

McDonagh, S., Maidji, E., Ma, W., Chang, H., Fisher, S. and Pereira, L., Viral and bacterial pathogens at the maternal-fetal interface, *J Infect Dis*, 190(4), 826-834, 2004.

Medzhitov, R., Recognition of microorganisms and activation of the immune response, *Nature*, 449(7164), 819-826, 2007.

Menon, R.K., Cohen, R.M., Sperling, M.A., Cutfield, W.S., Mimouni, F. and Khoury, J.C., Transplacental passage of insulin in pregnant women with insulin-dependent diabetes mellitus. Its role in fetal macrosomia, *N Engl J Med*, 323(5), 309-315, 1990.

Metcalfe, K.A., Hitman, G.A., Rowe, R.E., Hawa, M., Huang, X., Stewart, T. and Leslie, R.D., Concordance for type 1 diabetes in identical twins is affected by insulin genotype, *Diabetes Care*, 24(5), 838-842, 2001.

Michaëlsson, J., Mold, J.E., McCune, J.M. and Nixon, D.F., Regulation of T cell responses in the developing human fetus *J Immunol*, 176(10), 5741-5748, 2006.

Mold, J.E., Venkatasubrahmanyam, S., Burt, T.D., Michaëlsson, J., Rivera, J.M., Galkina, S.A., Weinberg, K., Stoddart, C.A. and McCune, J.M., Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans, *Science*, 330(6011), 1695-1699, 2010.

Moltchanova, E.V., Schreier, N., Lammi, N. and Karvonen, M., Seasonal variation of diagnosis of type 1 diabetes mellitus in children worldwide, *Diabet Med*, 26(7), 673-678, 2009.

Mooradian, A.D., Morley, J.E., Levine, A.S., Prigge, W.F. and Gebhard, R.L., Abnormal intestinal permeability to sugars in diabetes mellitus, *Diabetologia*, 29(4), 221-224, 1986.

Moriwaki, M., Itoh, N., Miyagawa, J., Yamamoto, K., Imagawa, A., Yamagata, K., Iwahashi, H., Nakajima, H., Namba, M., Nagata, S., Hanafusa, T. and Matsuzawa, Y., Fas and Fas ligand expression in inflamed islets in pancreas sections of patients with recent-onset type 1 diabetes mellitus, *Diabetologia*, 42(11), 1332-1340, 1999.

Mosmann, T.R., Cherwinski, H., Bond, M.W., Giedlin, M.A. and Coffman, R.L., Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins, *J Immunol*, 136(7), 2348-2357, 1986.

Mowat, A.M., Anatomical basis of tolerance and immunity to intestinal antigens, *Nat Rev Immunol*, 3(4), 331-341, 2003.

Murphy, K, Travers, P. and Walport, M., *Janaway's Immunobiology*, 7th edn, Garland Science, Taylor and Francis Group, New York, 2008.

Mäki, M., Hällstrom, O., Huupponen, T., Vesikari, T. and Visakorpi, J.K., Increased prevalence of coeliac disease in diabetes, *Arch Dis Child*, 59(8), 1984.

Nakayamada, S., Takahashi, H., Kanno, Y. and O'Shea, J.J., Helper T cell diversity and plasticity, *Curr Opin Immunol*, 24(3), 297-302, 2012.

Neefjes, J., Jongsma, M.L.M., Paul, P. and Bakke, O., Towards a systems understanding of MHC class I and MHC class II antigen presentation, *Nat Rev Immunol*, 11(12), 823-836, 2011.

Nerup, J., Platz, P., Andersen, O.O., Christy, M., Lyngsoe, J., Poulsen, J.E., Ryder, L.P., Nielsen, L.S., Thomsen, M. and Svejgaard, A., HL-A antigens and diabetes mellitus, *Lancet*, 2(7885), 864-866, 1974.

Nguyen, M., Leuridan, E., Zhang, T., De Wit, D., Willems, F., Van Damme, P., Goldman, M. and Goriely, S., Acquisition of adult-like TLR4 and TLR9 responses during the first year of life, *PLoS One*, 5(4), e10407, 2010.

Norris, J.M., Barriga, K., Klingensmith, G., Hoffman, M., Eisenbarth, G.S., Erlich, H.A. and Rewers, M., Timing of initial cereal exposure in infancy and risk of islet autoimmunity, *JAMA*, 290(13), 1713-1720, 2003.

Nänto-Salonen, K., Kupila, A., Simell, S., Siljander, H., Salonsaari, T., Hekkala, A., Korhonen, S., Erkkola, R., Sipilä, J.I., Haavisto, L., Siltala, M., Tuominen, J., Hakalax, J., Hyöty, H., Ilonen, J., Veijola, R., Simell, T., Knip, M. and Simell, O., Nasal insulin to prevent type 1 diabetes in children with HLA genotypes and autoantibodies conferring increased risk of disease: a double-blind, randomised controlled trial, *Lancet*, 372(9651), 1746-1755, 2008.

O'Garra, A, Commit ye helpers, *Nature*, 404(6779), 719-720, 2000.

Oh-Hora, M., Yamashita, M., Hogan, P.G., Sharma, S., Lamperti, E., Chung, W., Prakriya, M., Feske, S. and Rao, A., Dual functions for the endoplasmic reticulum calcium sensors STIM1 and STIM2 in T cell activation and tolerance, *Nat Immunol*, 9(4), 432-443, 2008.

Oikarinen, M., Tauriainen, S., Honkanen, T., Vuori, K., Karhunen, P., Vasama-Nolvi, C., Oikarinen, S., Verbeke, C., Blair, G.E., Rantala, I., Ilonen, J., Simell, O., Knip, M. and Hyöty, H., Analysis of pancreas tissue in a child positive for islet cell antibodies, *Diabetologia*, 51(10), 1796-1802, 2008.

Onkamo, P., Väänänen, S., Karvonen, M. and Tuomilehto, J., Worldwide increase in incidence of Type 1 diabetes-the analysis of the data on published incidence trends, *Diabetologia*, 42(12), 1395-1403, 1999.

Onuffer, J.J. and Horuk, R., Chemokines, chemokine receptors and small-molecule antagonists: recent developments. *Trends Pharmacol Sci*, 23(10), 459-467, 2002.

O'Shea, J.J. and Paul, W.E., Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells, *Science*, 327(5969), 1098-1102, 2010.

Ouyang, W., Ranganath, S.H., Weindel, K., Bhattacharya, D., Murphy, T.L., Sha, W.C. and Murphy, K.M., Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism, *Immunity*, 9(5), 745-755, 1998.

Palmer, J.P., Asplin, C.M., Clemons, P., Lyen, K., Tatpati, O., Raghu, P.K. and Paquette, T.L., Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science*, 222(4630), 1337-1339, 1983.

Paronen, J., Knip, M., Savilahti, E., Virtanen, S.M., Ilonen, J., Åkerblom, H.K. and Vaarala, O., Effect of cow's milk exposure and maternal type 1 diabetes on cellular and humoral immunization to dietary insulin in infants at genetic risk for type 1 diabetes. Finnish Trial to Reduce IDDM in the Genetically at Risk Study Group. *Diabetes*, 49(10), 1657-1665, 2000.

Patterson, C.C., Dahlquist, G.G., Gyürüs, E., Green, A., Soltesz, G. and the EURODIAB Study Group, Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study, *Lancet*, 373(9680), 2027-2033, 2009.

Paul, W.E. and Seder, R.A., Lymphocyte responses and cytokines, *Cell*, 76(2), 241-251, 1994.

Paxson, J.A., Weber, J.G. and Kulczycki, A.,Jr, Cow's milk-free diet does not prevent diabetes in NOD mice, *Diabetes*, 46(11), 1711-1717, 1997.

Payton, M.A., Hawkes, C.J. and Christie, M.R., Relationship of the 37,000- and 40,000-M(r) tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512), *J Clin Invest*, 96(3), 1506-1511, 1995.

Penders, J., Thijs, C., Vink, C., Stelma, F.F., Snijders, B., Kummeling, I., van der Brandt, P.A. and Stobberingh, E.E., Factors influencing the composition of the intestinal microbiota in early infancy, *Pediatrics*, 118(2), 511-521, 2006

Peng, L., Li, Z.R., Green, R.S., Holzman, I.R. and Lin, J., Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers, *J Nutr*, 139(9), 1619-1625, 2009.

Pflanz, S., Timans, J.C., Cheung, J., Rosales, R., Kanzler, H., Gilbert, J., Hibbert, L., Churakova, T., Travis, M., Vaisberg, E., Blumenschein, W.M., Mattson, J.D., Wagner, J.L., To, W., Zurawski, S., McClanahan, T.K., Gorman, D.M., Bazan, J.F., de Waal Malefyt, R., Rennick, D. and Kastelein, R.A., IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4+ T cells, *Immunity*, 16(6), 779-790, 2002.

Podar, T., Solntsev, A., Karvonen, M., Padaiga, Z., Brigis, G., Urbonaite, B., Viik-Kajander, M., Reunanen, A. and Tuomilehto, J., Increasing incidence of childhood-onset type 1 diabetes in 3 Baltic countries and Finland 1983-1998, *Diabetologia*, 44(Suppl 3), B17-20, 2001.

Potter, K.N. and Wilkin, T.J., The molecular specificity of insulin autoantibodies, *Diabetes Metab Res Rev*, 16(5), 338-353, 2000.

Prescott, S.L., Macaubas, C., Holt, B.J., Smallacombe, T.B., Loh, R., Sly, P.D. and Holt, P.G., Transplacental priming of the human immune system to environmental allergens: universal skewing of initial T cell responses toward the Th2 cytokine profile, *J Immunol*, 160(10), 4730-4737, 1998.

Presky, D.H., Yang, H., Minetti, L.J., Chua, A.O., Nabavi, N., Wu, C.Y., Gately, M.K. and Gubler, U., A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits, *Proc Natl Acad Sci USA*, 93(24), 14002-14007, 1996.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J. and Glockner, F.O., SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB, *Nucleic Acids Res*, 35(21), 7188-7196, 2007.

Pugliese, A., Zeller, M., Fernandez, A.,Jr, Zalcberg, L.J., Bartlett, R.J., Ricordi, C., Pietropaolo, M., Eisenbarth, G.S., Bennett, S.T. and Patel, D.D., The insulin gene is transcribed in the human thymus and transcription levels correlated with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes, *Nat Genet*, 15(3), 293-297, 1997.

Pugliese, A., Brown, D., Garza, D., Murchison, D., Zeller, M., Redondo, M.J., Diez, J., Eisenbarth, G.S., Patel, D.D. and Ricordi, C., Self-antigen-presenting cells expressing diabetes-associated autoantigens exist in both thymus and peripheral lymphoid organs, *J Clin Invest*, 107(5), 555-564, 2001.

Rabinovitch, A., Immunoregulatory and cytokine imbalances in the pathogenesis of IDDM. Therapeutic intervention by immune-stimulation? *Diabetes*, 43(5), 613-621, 1994.

Rajilic-Stojanovic, M., Smidt, H. and de Vos, W.M., Diversity of the human gastrointestinal tract microbiota revisited, *Environ Microbiol*, 9(9), 2125-2136, 2007.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S. and Medzhitov, R., Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis, *Cell*, 118(2), 229-241, 2004.

Reardon, W., Ross, R.J., Sweeney, M.G., Luxon, L.M., Pembrey, M.E., Harding, A.E. and Trembath, R.C., Diabetes mellitus associated with a pathogenic point mutation in mitochondrial DNA, *Lancet*, 340(8832), 1376-1379, 1992.

Redondo, M.J., Rewers, M., Yu, L., Garg, S., Pilcher, C.C., Elliott, R.B. and Eisenbarth, G.S., Genetic determination of islet cell autoimmunity in monozygotic twin, dizygotic twin, and non-

twin siblings of patients with type 1 diabetes: prospective twin study, *BMJ*, 318(7185), 698-702, 1999.

Redondo, M.J., Jeffrey, J., Fain, P.R., Eisenbarth, G.S. and Orban, T, Concordance for islet autoimmunity among monozygotic twins, *N Engl J Med*, 359(26), 2849-2850, 2008.

Rengarajan, J., Szabo, S.J. and Glimcher, L.H., Transcriptional regulation of Th1/Th2 polarization, *Immunol Today*, 21(10), 479-483, 2000.

Rieck, M., Arechiga, A., Onengut-Gumuscu, S., Greenbaum, C., Concannon, P. and Buckner, J.H., Genetic variation in PTPN22 corresponds to altered function of T and B lymphocytes, *J Immunol*, 179(7), 4704-4710, 2007.

Risch, N., Assessing the role of HLA-linked and unlinked determinants of disease, *Am J Human Genet*, 40(1), 1-14, 1987.

Roesch, L.F., Lorca, G.L., Casella, G., Giongo, A., Naranjo, A., Pionzio, A.M., Li, N., Mai, V., Wasserfall, C.H., Schatz, D., Atkinson, M.A., Neu, J. and Triplett, E.W., Culture-independent identification of gut bacteria correlated with the onset of diabetes in a rat model, *ISME J*, 3(5), 536-548, 2009.

Rogge, L., Barberi-Maino, L., Biffi, M., Passini, N., Presky, D., H., Gubler, U. and Sinigaglia, F., Selective expression of an interleukin-12 receptor component by human T helper 1 cells, *J Exp Med*, 185(5), 825-831, 1997.

Rossi, D. and Zlotnik, A., The Biology of chemokines and their receptors, *Annu Rev Immunol*, 18(1), 217-242, 2000.

Rowley, M.J., Mackay, I.R., Chen, Q.Y., Knowles, W.J. and Zimmet, P.Z., Antibodies to glutamic acid decarboxylase discriminate major types of diabetes mellitus, *Diabetes*, 41(4), 548-551, 1992.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M., Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases, *J Immunol*, 155(3), 1151-1164, 1995.

Sakaguchi, S., Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self, *Nat Immunol*, 6(4), 345-352, 2005.

Sakaguchi, S., Wing, K., Onishi, Y., Prieto-Martin, P. and Yamaguchi, T., Regulatory T cells: how do they suppress immune responses? *Int Immunol*, 21(10), 1105-1111, 2009.

Sallusto, F. and Lanzavecchia, A., Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med*, 179(4), 1109-1118, 1994.

Sallusto, F., Lenig, D., Mackay, C.R. and Lanzavecchia, A., Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes, *J Exp Med*, 187(6), 875-883, 1998.

Sallusto, F., Mackay, C.R. and Lanzavecchia, A., The role of chemokine receptors in primary, effector, and memory immune responses, *Annu Rev Immunol*, 18(1), 593-620, 2000.

Saukkonen, T., Savilahti, E., Vaarala, O., Virtala, E.T., Tuomilehto, J. and Åkerblom, H.K., Children with newly diagnosed IDDM have increased levels of antibodies to bovine serum albumin but not to ovalbumin. Childhood Diabetes in Finland Study Group. *Diabetes Care*, 17(9), 970-976, 1994.

Savilahti, E., Åkerblom, H.K., Tainio, V.M. and Koskimies, S., Children with newly diagnosed insulin dependent diabetes mellitus have increased levels of cow's milk antibodies, *Diabetes Res*, 7(3), 137-14, 1988.

Savilahti, E., Saukkonen, T.T., Virtala, E.T., Tuomilehto, J. and Åkerblom, H.K., Increased levels of cow's milk and beta-lactoglobulin antibodies in young children with newly diagnosed IDDM. The Childhood Diabetes in Finland

Study Group, *Diabetes Care*, 16(7), 984-989, 1993.

Schaub, B., Liu, J., Schleich, I., Höppler, S., Sattler, C. and von Mutius, E., Impairment of T helper and T regulatory cell responses at birth, *Allergy*, 63(11), 1438-1447, 2008.

Schlosser, M., Mueller, P.W., Torn, C., Bonifacio, E., Bingley, P.J. and Participating Laboratories, Diabetes Antibody Standardization Program: evaluation of assays for insulin autoantibodies, *Diabetologia*, 53(12), 2611-2620, 2010.

Scott C.L., Aumeunier A.M. and Mowat A.M., Intestinal CD103+ dendritic cells: master regulators of tolerance? *Trends Immunol*, 32(9), 2011.

Sheehy, M.J., Scharf, S.J., Rowe, J.R., Neme de Gimenez, M.H., Meske, L.M., Erlich, H.A. and Nepom, B.S., A diabetes-susceptible HLA haplotype is best defined by a combination of HLA-DR and -DQ alleles, *J Clin Invest*, 83(3), 830-835, 1989.

Shevach, E.M., Mechanisms of Foxp3+ T regulatory cell-mediated suppression, *Immunity*, 30(5), 636-645, 2009.

Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y. and Sakaguchi, S., Stimulation of CD25+CD4+ regulatory T cells through GITR breaks immunological self-tolerance, *Nat Immunol*, 3(2), 135-142, 2002.

Siljander, H.T., Simell, S., Hekkala, A., Lähde, J., Simell, T., Vähäsalo, P., Veijola, R., Ilonen, J., Simell, O. and Knip, M., Predictive characteristics of diabetes-associated autoantibodies among children with HLA-conferred disease susceptibility in the general population, *Diabetes*, 58(12), 2835-2842, 2009.

Simister, N.E. and Story, C.M, Human placental Fc receptors and the transmission of antibodies from mother to fetus, *J Reprod Immunol*, 37(1), 1-23, 1997.

Simister, N.E., Placental transport of immunoglobulin G, *Vaccine*, 21(24), 3365-3369, 2003.

Singh, S.P., Zhang, H.H., Foley, J.F., Hedrick, M.N. and Farber, J.M., Human T cells that are able to produce IL-17 express the chemokine receptor CCR6, *J Immunol*, 180(1), 214-221, 2008.

Sjöroos, M., Iitiä, A., Ilonen, J., Reijonen, H. and Lövgren, T., Triple-label hybridization assay for type-1 diabetes-related HLA alleles, *BioTechniques*, 18(5), 870-877, 1995.

Skyler, J.S., Krischer, J.P., Wolfsdorf, J., Cowie, C., Palmer, J.P., Greenbaum, C., Cuthbertson, D., Rafkin-Mervis, L.E., Chase, H.P. and Leschek, E., Effects of oral insulin in relatives of patients with type 1 diabetes: The Diabetes Prevention Trial, *Diabetes Care*, 28(5), 1068-1076, 2005.

Skyler, J.S. and Type 1 Diabetes TrialNet Study Group, Update on worldwide efforts to prevent type 1 diabetes, *Ann N Y Acad Sci*, 1150, 190-196, 2008.

Smith-Garvin, J.E., Koretzky, G.A. and Jordan, M.S., T cell activation, *Annu Rev Immunol*, 27, 591-619, 2009.

Soltesz, G., Patterson, C.C., Dahlquist, G. and EURODIAB Study Group, Worldwide childhood type 1 diabetes incidence-what can we learn from epidemiology? *Pediatr Diabetes*, 8(Suppl 6), 6-14, 2007.

Somersalo, O., Studies of chilhood diabetes I. Incidence in Finland, *Acta Paediatr Fenn*, 1, 239-249, 1954.

Sonnenberg, G.F., Monticelli, L.A., Elloso, M.M., Fouser, L.A. and Artis, D., CD4+ lymphoid tissue-inducer cells promote innate immunity in the gut, *Immunity*, 34(1), 122-134, 2011.

Sprent, J. and Surh, C.D., Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells, *Nat Immunol*, 12(6), 478-484, 2011.

Stanford, S.M., Mustelin, T.M. and Bottini, N., Lymphoid tyrosine phosphatase and autoimmunity: human genetics rediscovers tyrosine phosphatases, *Semin Immunopathol*, 32(2), 127-136. 2010.

Starr, T.K., Jameson, S.C. and Hogquist, K.A., Positive and negative selection of T cells, *Annu Rev Immunol,* 21(1), 139-176, 2003.

Szabo, S.J., Kim, S.T., Costa, G.L., Zhang, X., Fathman, C.G. and Glimcher, L.H., A novel transcription factor, T-bet, directs Th1 lineage commitment, *Cell*, 100(6), 655-669, 2000.

Szabo, S.J., Sullivan, B.M., Peng, S.L. and Glimcher, L.H., Molecular mechanisms regulating Th1 immune responses, *Annu Rev Immunol*, 21, 713-758, 2003.

Szépfalusi, Z., Pichler, J., Elsässera, S., van Duren, K., Ebner, C., Bernaschek, G. and Urbanek, R., Transplacental priming of the human immune system with environmental allergens can occur early in gestation, *J Allergy Clin Immunol*, 106(3), 530-536, 2000.

Tada, T., Takemori, T., Okumura, K., Nonaka, M. and Tokuhisa, T., Two distinct types of helper T cells involved in the secondary antibody response: independent and synergistic effects of Ia- and Ia+ helper T cells, *J Exp Med*, 147(2), 446-458, 1978.

Tainio, V.M., Savilahti, E., Arjomaa, P., Salmenperä, L., Perheentupa, J. and Siimes, M.A., Plasma antibodies to cow's milk are increased by early weaning and consumption of unmodified milk, but production of plasma IgA and IgM cow's milk antibodies is stimulated even during exclusive breast-feeding, *Acta Paediatr Scand*, 77(6), 807-811, 1988.

Takahama, Y., Journey through the thymus: stromal guides for T-cell development and selection, *Nat Rev Immunol*, 6(2), 127-135, 2006.

Takahashi, T., Tagami, T., Yamazaki, S., Uede, T., Shimizu, J., Sakaguchi, N., Mak, T.W. and Sakaguchi, S., Immunologic self-tolerance maintained by CD25+CD4+ regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4, *J Exp Med*, 192(2), 303-310, 2000.

Takahata, Y., Nomura, A., Takada, H., Ohga, S., Furuno, K., Hikino, S., Nakayama, H., Sakaguchi, S. and Hara, T., CD25+CD4+ T cells in human cord blood: an immunoregulatory subset with naive phenotype and specific expression of forkhead box p3 (Foxp3) gene, *Exp Hematol*, 32(7), 622-629, 2004.

Takeda, K., Tanaka, T., Shi, W., Matsumoto, M., Minami, M., Kashiwamura, S., Nakanishi, K., Yoshida, N., Kishimoto, T. and Akira, S., Essential role of Stat6 in IL-4 signalling, *Nature*, 380(6575), 627-630, 1996.

Takeuchi, O. and Akira, S., Pattern recognition receptors and inflammation, *Cell*, 140(6), 805-820, 2010.

Tanaka, S., Maeda, S., Hashimoto, M., Fujimori, C., Ito, Y., Teradaira, S., Hirota, K., Yoshitomi, H., Katakai, T., Shimizu, A., Nomura, T., Sakaguchi, N. and Sakaguchi, S., Graded attenuation of TCR signaling elicits distinct autoimmune diseases by altering thymic T cell selection and regulatory T cell function, *J Immunol*, 185(4), 2295-2305, 2010.

Tauriainen, S., Oikarinen, S., Oikarinen, M. and Hyöty, H., Enteroviruses in the pathogenesis of type 1 diabetes, *Sem Immunopathol*, 33(1), 45-55, 2011.

Tingle, A.J., Lim, G., Wright, V.J., Dimmick, J.E. and Hunt, J.A., Transplacental passage of islet cell antibody in infants of diabetic mothers, *Pediatr Res*, 13(12), 1323-1325, 1979.

Torchinsky, M.B., Garaude, J., Martin, A.P. and Blander, J.M., Innate immune recognition of infected apoptotic cells directs Th17 cell differentiation, *Nature*, 458(7234), 78-82, 2009.

Torn, C., Mueller, P.W., Schlosser, M., Bonifacio, E., Bingley, P.J. and Participating Laboratories, Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2, *Diabetologia*, 51(5), 846-852, 2008.

Turley, S.J., Lee, J.W., Dutton-Swain, N., Mathis, D. and Benoist, C. Endocrine self and gut non-self intersect in the pancreatic lymph nodes, *Proc Natl Acad Sci USA*, 102(49), 17729-17733, 2005.

Usui, T., Nishikomori, R., Kitani, A. and Strober, W., GATA-3 suppresses Th1 development by

downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet, *Immunity*, 18(3), 415-428, 2003.

Vaarala, O., Saukkonen, T., Savilahti, E., Klemola, T. and Åkerblom, H.K., Development of immune response to cow's milk proteins in infants receiving cow's milk or hydrolyzed formula, *J Allergy Clin Immunol*, 96(6), 917-923, 1995.

Vaarala, O., Knip, M., Paronen, J., Hämäläinen, A.M., Muona, P., Väätäinen, M., Ilonen, J., Simell, O. and Åkerblom, H.K., Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes, *Diabetes*, 48(7), 1389-1394, 1999.

Vaarala, O., Ilonen, J., Ruohtula, T., Pesola, J., Virtanen, S.M., Härkönen, T., Koski, M., Kallioinen, H., Tossavainen, O., Poussa, T., Jarvenpää, A.L., Komulainen, J., Lounamaa, R., Åkerblom, H.K. and Knip, M., Removal of bovine insulin from cow's milk formula and early initiation of beta-cell autoimmunity in the FINDIA Pilot Study, *Arch Pediatr Adolesc Med*, 166(7), 608-614, 2012.

Vafiadis, P., Bennett, S.T., Todd, J.A., Nadeau, J., Grabs, R., Goodyer, C.G., Wickramasinghe, S., Colle, E. and Polychronakos, C., Insulin expression in human thymus is modulated by INS VNTR alleles at the IDDM2 locus, *Nat Genet*, 15(3), 289-292, 1997.

van den Bogert, B., de Vos, W.M., Zoetendal, E.G. and Kleerebezem, M., Microarray analysis and barcoded pyrosequencing provide consistent microbial profiles depending on the source of human intestinal samples, *Appl Environ Microbiol*, 77(6), 2071-2080, 2011.

van den Ouweland, J.M., Lemkes, H.H., Ruitenbeek, W., Sandkuijl, L.A., de Vijlder, M.F., Struyvenberg, P.A., van de Kamp, J.J. and Maassen, J.A., Mutation in mitochondrial $tRNA^{(Leu)(UUR)}$ gene in a large pedigree with maternally transmitted type II diabetes mellitus and deafness, *Nat Genet*, 1(5), 368-371, 1992.

Vang, T., Congia, M., Macis, M.D., Musumeci, L., Orru, V., Zavattari, P., Nika, K., Tautz, L., Tasken, K., Cucca, F., Mustelin, T. and Bottini, N., Autoimmune-associated lymphoid tyrosine phosphatase is a gain-of-function variant, *Nat Genet*, 37(12), 1317-1319, 2005.

Vardi, P., Ziegler, A.G., Mathews, J.H., Dib, S., Keller, R.J., Ricker, A.T., Wolfsdorf, J.I., Herskowitz, R.D., Rabizadeh, A. and Eisenbarth, G.S., Concentration of insulin autoantibodies at onset of type 1 diabetes. Inverse log-linear correlation with age, *Diabetes Care*, 11(9), 736-739, 1988.

Veldhoen, M., Hocking, R.J., Atkins, C.J., Locksley, R.M. and Stockinger, B., TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells, *Immunity*, 24(2), 179-189, 2006.

Verge, C.F., Gianani, R., Kawasaki, E., Yu, L., Pietropaolo, M., Jackson, R.A., Chase, H.P. and Eisenbarth, G.S., Prediction of type 1 diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies, *Diabetes*, 45(7), 926-933, 1996.

Vieira, P.L., Christensen, J.R., Minaee, S., O'Neill, E.J., Barrat, F.J., Boonstra, A., Barthlott, T., Stockinger, B., Wraith, D.C. and O'Garra, A., IL-10-secreting regulatory T cells do not express Foxp3 but have comparable regulatory function to naturally occurring CD4+CD25+ regulatory T cells, *J Immunol*, 172(10), 5986-5993, 2004.

Villasenor, J., Benoist, C. and Mathis, D., AIRE and APECED: molecular insights into an autoimmune disease, *Immunol Rev*, 204, 156-164, 2005.

Virtanen, S.M., Räsänen, L., Ylönen, K., Aro, A., Clayton, D., Langholz, B., Pitkäniemi, J., Savilahti, E., Lounamaa, R. and Tuomilehto, J., Early introduction of dairy products associated with increased risk of IDDM in Finnish children. The Childhood in Diabetes in Finland Study Group, *Diabetes*, 42(12), 1786-1790, 1993.

Visser, J.T., Lammers, K., Hoogendijk, A., Boer, M.W., Brugman, S., Beijer-Liefers, S., Zandvoort, A., Harmsen, H., Welling, G., Stellaard, F., Bos, N.A., Fasano, A. and Rozing, J., Restoration of impaired intestinal barrier function by

the hydrolysed casein diet contributes to the prevention of type 1 diabetes in the diabetes-prone BioBreeding rat, *Diabetologia*, 53(12), 2621-2628, 2010.

Visser, J.T., Bos, N.A., Harthoorn, L.F., Stellaard, F., Beijer-Liefers, S., Rozing, J. and van Tol, E.A., Potential mechanisms explaining why hydrolysed casein based diets outclass single amino acid based diets in the prevention of Autoimmune Diabetes in Diabetes Prone BB rats, *Diabetes Metab Res Rev*, 28(6), 505-513, 2012.

von Boehmer, H., Mechanisms of suppression by suppressor T cells, *Nat Immunol*, 6(4), 338-344, 2005.

von Boehmer, H. and Melchers, F., Checkpoints in lymphocyte development and autoimmune disease, *Nat Immunol*, 11(1), 14-20, 2010.

Wadsworth, E.J.K., Shield, J.P.H., Hunt, L.P. and Baum, J.D., A Case-control study of environmental factors associated with diabetes in the under 5s, *Diabet Med*, 14(5), 390-396, 1997.

Wang, J., Ioan-Facsinay, A., van der Voort, E.I., Huizinga, T.W. and Toes, R.E., Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells, *Eur J Immunol*, 37(1), 129-138, 2007.

Wang, Q., Garrity, G.M., Tiedje, J.M. and Cole, J.R., Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy, *Appl Environ Microbiol*, 73(16), 5261-5267, 2007.

Warner, J.A. and Warner, J.O., Early life events in allergic sensitisation, *Br Med Bull*, 56(4), 883-893, 2000.

Warram, J.H., Krolewski, A.S., Gottlieb, M.S. and Kahn, C.R., Differences in risk of insulin-dependent diabetes in offspring of diabetic mothers and diabetic fathers, *N Engl J Med*, 311(3), 149-152, 1984.

Warram, J.H., Martin, B.C. and Krolewski, A.S., Risk of IDDM in children of diabetic mothers decreases with increasing maternal age at pregnancy, *Diabetes*, 40(12), 1679-1684, 1991.

Wegmann, T.G., Lin, H., Guilbert, L. and Mosmann, T.R., Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today*, 14(7), 353-356, 1993.

Wei, G., Wei, L., Zhu, J., Zang, C., Hu-Li, J., Yao, Z., Cui, K., Kanno, Y., Roh, T., Watford, W.T., Schones, D.E., Peng, W., Sun, H., Paul, W.E., O'Shea, J.J. and Zhao, K., Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells, *Immunity*, 30(1), 155-167, 2009.

Weiner, H.L., Induction and mechanism of action of transforming growth factor-β-secreting Th3 regulatory cells, *Immunol Rev*, 182(1), 207-214, 2001.

Wen, L., Ley, R.E., Volchkov, P.Y., Stranges, P.B., Avanesyan, L., Stonebraker, A.C., Hu, C., Wong, F.S., Szot, G.L., Bluestone, J.A., Gordon, J.I. and Chervonsky, A.V., Innate immunity and intestinal microbiota in the development of type 1 diabetes, *Nature*, 455(7216), 1109-1113, 2008.

White, G.P., Watt, P.M., Holt, B.J. and Holt, P.G., Differential patterns of methylation of the IFN-γ Promoter at CpG and non-CpG sites underlie differences in IFN-γ gene expression between human neonatal and adult CD45RO- T cells, *J Immunol*, 168(6), 2820-2827, 2002.

Wicker, L.S., Miller, B.J. and Mullen, Y., Transfer of autoimmune diabetes mellitus with splenocytes from nonobese diabetic (NOD) mice, *Diabetes*, 35(8), 855-860, 1986.

Wing, K., Ekmark, A., Karlsson, H., Rudin, A. and Suri-Payer, E., Characterization of human CD25+ CD4+ T cells in thymus, cord and adult blood, *Immunology*, 106(2), 190-199, 2002.

Witte, E., Witte, K., Warszawska, K., Sabat, R. and Wolk, K., Interleukin-22: A cytokine produced by T, NK and NKT cell subsets, with importance in the innate immune defense and tissue protection, *Cytokine Growth Factor Rev*, 21(5), 365-379, 2010.

Wong, F.S., Karttunen, J., Dumont, C., Wen, L., Visintin, I., Pilip, I.M., Shastri, N., Pamer, E.G. and Janeway, C.A., Jr, Identification of an MHC class I-restricted autoantigen in type 1 diabetes by screening an organ-specific cDNA library, *Nat Med*, 5(9), 1026-1031, 1999.

Wu, G.D., Lewis, J.D., Hoffmann, C., Chen, Y.Y., Knight, R., Bittinger, K., Hwang, J., Chen, J., Berkowsky, R., Nessel, L., Li, H. and Bushman, F.D., Sampling and pyrosequencing methods for characterizing bacterial communities in the human gut using 16S sequence tags, *BMC Microbiol*, 10(206), 1-14, 2010.

Xu, D., Chan, W.L., Leung, B.P., Hunter, D., Schulz, K., Carter, R.W., McInnes, I.B., Robinson, J.H. and Liew, F.Y., Selective expression and functions of interleukin 18 receptor on T helper (Th) type 1 but not Th2 cells, *J Exp Med*, 188(8), 1485-1492, 1998.

Yagi, H., Nomura, T., Nakamura, K., Yamazaki, S., Kitawaki, T., Hori, S., Maeda, M., Onodera, M., Uchiyama, T., Fujii, S. and Sakaguchi, S., Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells, *Int Immunol*, 16(11), 1643-1656, 2004.

Yang, X.D., Sytwu, H.K., McDevitt, H.O. and Michie, S.A., Involvement of beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in the development of diabetes in obese diabetic mice, *Diabetes*, 46(10), 1542-1547, 1997.

Yang, Y., Ochando, J.C., Bromberg, J.S. and Ding, Y., Identification of a distant T-bet enhancer responsive to IL-12/Stat4 and IFNγ/Stat1 signals, *Blood,* 110(7), 2494-2500. 2007.

Yoon, J.W., Austin, M., Onodera, T. and Notkins, A.L., Isolation of a virus from the pancreas of a child with diabetic ketoacidosis, *N Engl J Med*, 300(21), 1173-1179, 1979.

Yu, L., Robles, D.T., Abiru, N., Kaur, P., Rewers, M., Kelemen, K. and Eisenbarth, G.S., Early expression of antiinsulin autoantibodies of humans and the NOD mouse: Evidence for early determination of subsequent diabetes, *Proc Natl Acad Sci USA*, 97(4), 1701-1706, 2000.

Zhang, D., Cohn, L., Ray, P., Bottomly, K. and Ray, A., Transcription factor GATA-3 is differentially expressed in murine Th1 and Th2 cells and controls Th2-specific expression of the interleukin-5 gene, *J Biol Chem*, 272,(34), 21597-21603, 1997.

Zhang, L., Nakayama, M. and Eisenbarth, G.S., Insulin as an autoantigen in NOD/human diabetes, *Curr Opin Immunol*, 20(1), 111-118, 2008.

Zhang, Z.J., Davidson, L., Eisenbarth, G. and Weiner, H.L., Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin, *Proc Natl Acad Sci USA*, 88(22), 10252-10256, 1991.

Zheng, S.G, The Critical Role of TGF-β1 in the development of induced Foxp3+ regulatory T cells, *Int J Clin Exp Med*, 1(3), 192-202, 2008.

Zheng, W. and She, J.X. Genetic association between a lymphoid tyrosine phosphatase (PTPN22) and type 1 diabetes, *Diabetes*, 54(3), 906-908, 2005.

Zhou, M. and Ouyang, W., The function role of GATA-3 in Th1 and Th2 differentiation, *Immunol Res*, 28(1), 25-37, 2003.

Zhu, C., Anderson, A.C., Schubart, A., Xiong, H., Imitola, J., Khoury, S.J., Zheng, X.X., Strom, T.B. and Kuchroo, V.K., The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity, *Nat Immunol*, 6(12), 1245-1252, 2005.

Zhu, J., Guo, L., Watson, C.J., Hu-Li, J. and Paul, W.E., Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion, *J Immunol*, 166(12), 7276-7281, 2001.

Ziegler, A.G., Hillebrand, B., Rabl, W., Mayrhofer, M., Hummel, M., Mollenhauer, U., Vordemann, J., Lenz, A. and Standl, E., On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth, *Diabetologia*, 36(5), 402-408, 1993.

Ziegler, A., Schmid, S., Huber, D., Hummel, M. and Bonifacio, E., Early infant feeding and risk of

developing type 1 diabetes–associated autoantibodies, *JAMA*, 290(13), 1721-1728, 2003.

Åkerblom, H. K., Virtanen S. M., Ilonen J., Savilahti E., Vaarala O., Reunanen A., Teramo K., Hämäläinen A.-M., Paronen J. and National TRIGR Study Groups, Dietary manipulation of beta cell autoimmunity in infants at increased risk of type 1 diabetes: a pilot study, *Diabetologia*, 48(5), 428-437, 2005.