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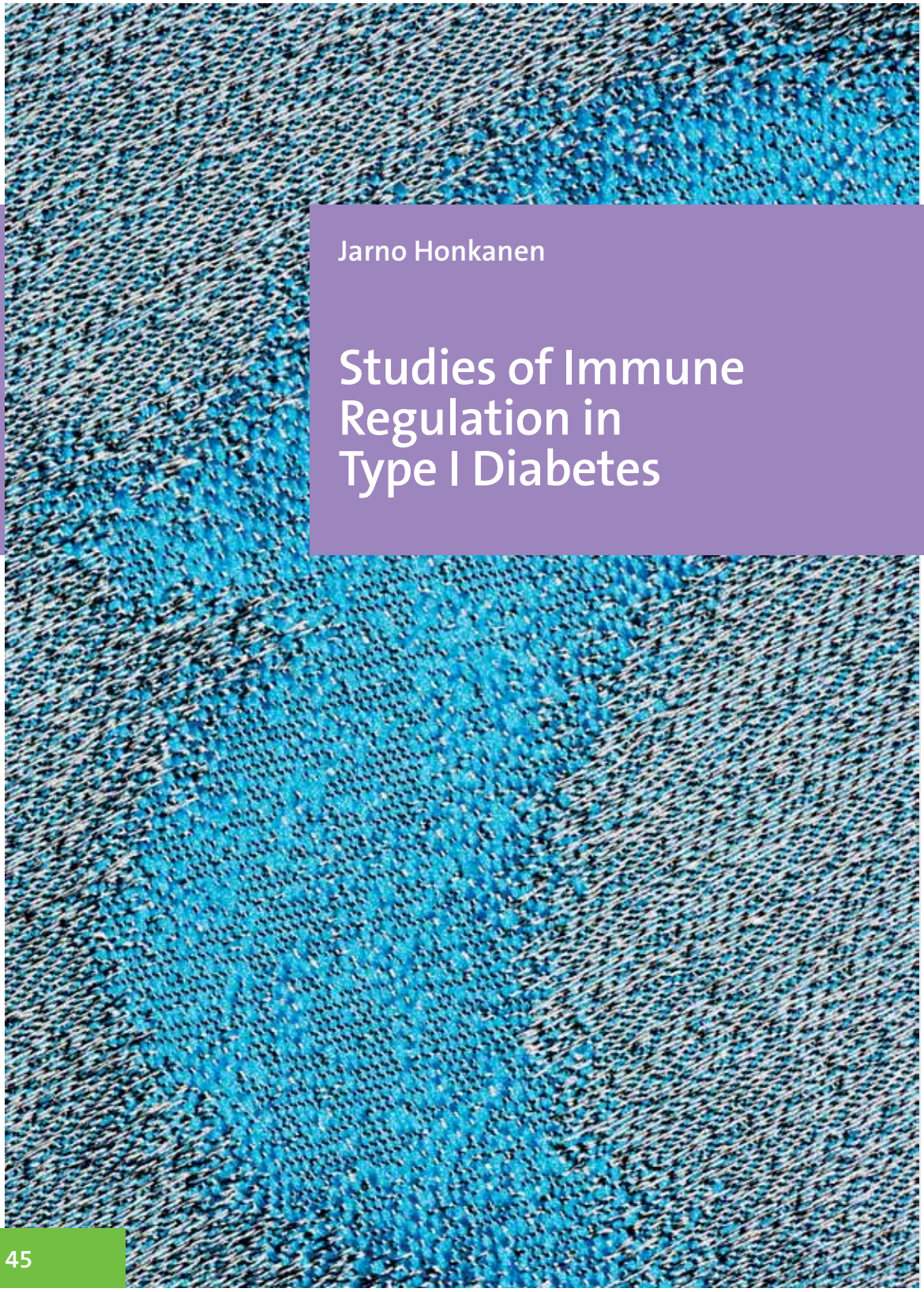

RESEARCH

Jarno Honkanen

Studies of Immune Regulation in Type I Diabetes

45

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Studies of Immune Regulation in Type 1 Diabetes

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Medicine of the University of Helsinki, for public examination in Niilo Hallman auditorium, Hospital for Children and Adolescents
on 3 December 2010, at 12 noon.

Immune Response Unit, Department of Vaccines and
Immune Protection,
National Institute for Health and Welfare,
Helsinki, Finland
and
Hospital for Children and Adolescents, University of Helsinki

RESEARCH 45/2010
Helsinki 2010

NATIONAL INSTITUTE
FOR HEALTH AND WELFARE

Layout: Raili Silius

ISBN 978-952-245-371-6 (printed)
ISSN 1798-0054 (printed)
ISBN 978-952-245-372-3 (pdf)
ISSN 1798-0062 (pdf)

Helsinki University Print
Helsinki, Finland 2010

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Abstract

Jarno Honkanen. Studies of Immune Regulation in Type 1 Diabetes. National Institute for Health and Welfare. Research 45/2010. 159 pages. Helsinki, Finland 2010. ISBN 978–952–245–371–6 (print); ISBN 978–952–245–372–3 (pdf)

Type 1 diabetes (T1D) is considered to be an autoimmune disease. In T1D insulin producing pancreatic β cells are destroyed. The disease process begins years before the clinical diagnosis of T1D. During the pathogenesis of T1D, pancreatic islets are infiltrated by cells of the immune system and T-lymphocytes are considered to be the main mediators of the β-cell destruction. In children with an active β-cell destruction process, antibodies against β-cell antigens appear in the blood. Individuals at increased risk of developing T1D can often be identified by detecting serum autoantibodies against β-cell antigens.

Immunological aberrancies associated with T1D are related to defects in the polarization of T cells and in the function of regulatory mechanisms. T1D has been considered as an organ-specific autoimmune disease mediated by uncontrolled Th1 – responses. In human T1D, the evidence for the role of overexpression of cytokines promoting cytotoxicity is controversial. For the past 15 years, regulatory T cells (Tregs) have been recognized as having a key role in the initiation and maintenance of tolerance, limiting harmful autoantigen-specific inflammation processes. It is possible that, if regulatory mechanisms fail to be initiated, the subtle inflammation targeting β cells lead to insulitis and eventually to overt T1D in some individuals.

In the present thesis, we studied the induction of Tregs during the generation of T-cell responses in T1D. Impaired up-regulation of Treg-associated genes was observed in activated T-cells cultured in type 1 cytokine environment. We found no defect in the induction of genes directing polarization of Th1 or Th2 cells. Our data suggest, that in T1D the induction of regulatory mechanisms is aberrant in cytotoxic cytokine environment, which may contribute the pathogenesis of T1D.

T1D has a strong heritable component. However, genetic risk explains only a fraction of the overall risk, and environmental factors must therefore contribute to the risk of developing T1D. One of the most studied risk factors is enterovirus infections. Interestingly, we found children with T1D to have lowered type 1 response against coxsackie virus B4 (CVB4). Children with T1D may be more susceptible to subtle and/or prolonged CVB4 infections, and they may thus have an increased frequency and proportion of CVB-infected β cells. This may cause priming of autoreactive T cells and β-cell autoimmunity in the pancreatic lymph nodes via antigen presentation by the antigen presenting cells (APCs).

Recently, a novel T helper cell subset called Th17 has been discovered. Animal models have associated Th17 cells and especially co-producers of IL-17 and IFN-γ with the pathogenesis of T1D. We aimed to characterize the role of Th17 immunity in human T1D. We found increased secretion of IL-17 and increased mRNA expression of IL-17, retinoic acid-related orphan receptor C isoform 2, (RORC2) and IL-22 in stimulated peripheral blood mononuclear cells (PBMCs) of T1D patients. In addition, we observed increased expression of transcription factor Forkhead box 3 (FOXP3) in stimulated PBMCs of T1D patients. This suggests simultaneous up-regulation of regulatory mechanisms and the IL-17 pathway in T1D. We observed similar expression levels of interferon-γ (IFN-γ) and T-box expressed in T cells (T-bet) in stimulated PBMCs of T1D patients and healthy children. However, IFN-γ expression correlated with IL-17 secretion and co-producers of IL-17 and IFN-γ were found in children with T1D. In another set of T1D blood samples similar up-regulation of Th17-associated genes and FOXP3 were seen in peripheral $CD4^+$ memory T cells. Moreover, IL-17 alone or in synergy with interleukin-1β (IL-1β) and IFN- γ was shown to be detrimental for human pancreatic islets *in vitro*. Our results suggest that IL-17 immunity may be involved in the pathogenesis of T1D. Th17 activation may increase cytokine mediated apoptosis of β cells, and thus, the release of β-cell derived auto-antigens, which would accelerate the development of β-cell specific autoimmunity.

As a conclusion, the findings of the present thesis provide evidence for the enhanced activation of IL-17/IL-22 pathways in association with aberrancies in the FOXP-related regulatory pathways in children with T1D. These findings may have therapeutic implications for the treatment and prevention of T1D.

Keywords: type 1 diabetes, immune regulation, T helper cells, regulatory T cells, interleukin-17

Tiivistelmä

Jarno Honkanen. Studies of Immune Regulation in Type 1 Diabetes [Immuunivasteen säätely tyypin 1 diabeteksessa]. Terveyden ja hyvinvoinnin laitos. Tutkimus 45/2010. 159 sivua. Helsinki, Finland 2010.
ISBN 978–952–245–371–6 (painettu); ISBN 978–952–245–372–3 (pdf)

Tyypin 1 diabetesta (T1D) pidetään autoimmuunisairautena. T1D:ssa elimistön immuunijärjestelmä tuhoaa haiman insuliinia tuottavat β-solut. Tautiprosessi alkaa vuosia ennen taudin puhkeamista. T1D:n patogeneesin aikana, haiman saarekkeisiin tunkeutuu immuunjärjestelmän soluja, ja β-solukuoleman aiheuttajana ovat todennäköisesti kehon omia rakenteita tunnistavat T-lymfosyytit. Lapset joilla on suurentunut riski sairastua T1D:een voidaan usein tunnistaa havaitsemalla seerumista β-solujen rakenteita kohtaan tuotettuja autovasta-aineita jo vuosia ennen kliinistä diagnoosia.

T1D:een liitetyt immunologiset häiriöt ovat T-solujen vinoutunut erilaistuminen ja poikkeava T-soluvasteiden säätelykyky. T1D:een on liitetty liiallinen Th1 -solujen toiminta. Näyttö Th1 -solujen osallisuudesta ihmisen tautipatogeneesiin on kuitenkin ristiriitainen. Viimeisen 15 vuoden aikana säätelevien valkosolujen toiminta on tunnistettu keskeiseksi mekanismiksi (auto)immuunivasteiden säätelyssä. Jos säätelevien valkosolujen aktivaatio ja toiminta on vajavaista, on mahdollista että immuunijärjestelmä ei kykene taltuttamaan β-solujen rakenteita vastaan kohdistuvia immuunivasteita. Etenevä β-solutuho johtaa lopulta T1D:n puhkeamiseen joillekin lapsille.

Tässä väitöskirjassa tutkittiin T-soluvasteiden käynnistymistä, kehittymistä ja säätelevien valkosolujen aktivaatiota immuunivasteen käynnistymisen aikana. Havaitsimme että Th1-tyyppinen sytokiiniympäristö estää säätelevien valkosolujen aktivaatiossa keskeisen transkriptiotekijä FOXP3:n (Forkhead box 3) ilmentymisen tyypin 1 diabeetikoilla. Th1- ja Th2-tyyppisten effektorisolujen erilaistumista ohjaavien transkriptiotekijöiden, T-bet ja GATA-3, aktivaatio oli normaali tyypin 1 diabeetikoilla verrattuna terveisiin lapsiin.

Alttius sairastua tyypin T1D:een on voimakkaasti perinnöllisten tekijöiden määräämä. Perinnöllinen alttius selittää kuitenkin vain osan sairastumisen kokonaisriskistä, ja siten ympäristötekijöillä on merkitystä T1D:n synnyssä. Eräs eniten tutkituista ympäristötekijöistä ovat enterovirusinfektiot. Havaitsimme että tyypin 1 diabeetikoilla on heikentynyt kyky coxsackievirus B4:n (CVB4) vastustuskyky. Tyypin 1 diabetespotilaiden virushäätö näyttäisi olevan heikentynyt, jolloin toistuvat ja pitkittyneet β-solujen virusinfektiot voivat johtaa lisääntyneeseen

β-solukuolemaan. β-solurakenteiden vapautuminen kuolevista soluista voi puolestaan johtaa kiihtyneeseen antigeeniesittelyyn haiman imusolmukkeissa, ja aikaansaada β-soluja tunnistavien T–solujen syntymisen.

Hiljattain on löydetty uusi T-auttajasolujen alaluokka, Th17-solut. Erityisesti interlukiini-17:a ja interferoni-γ:aa tuottavat Th17-solut ovat osoittautuneet patogeenisiksi T1D:n eläinmalleissa. Tavoitteemme oli kuvata Th17-solujen osuutta ihmisen T1D:ssä. Havaitsimme että tyypin 1 diabeetikoiden stimuloidut valkosolut ilmensivät runsaammin IL-17 sytokiiniä, sekä lähetti-RNA– että proteiinitasolla. Lisäksi havaitsimme Th17-solujen toimintaan liittetyn interleukiini-22 ja transkriptiotekijöiden ROR-C2 (retinoic acid-related orphan receptor C isoform 2) ja FOXP3 voimakkaamman lähetti-RNA ilmentymisen tyypin 1 diabeetikoiden stimuloiduissa valkosoluissa. Samanaikainen voimakkaampi FOXP3 ilmentyminen stimuloiduissa valkosoluissa Th17-solujen merkkiaineiden kanssa on yllättävä havainto, mikä saattaa viitata häiriintyneeseen Th17-soluvasteiden säätelyyn tyypin 1 diabeetikoilla. Th1-tyyppisten solujen kehittymistä ohjaavan T-bet:in ja liukoisen merkkiaineen IFN-γ ilmentyminen oli samalla tasolla sekä terveillä että tyypin 1 diabetekseen sairastuneilla lapsilla. IFN-γ tasot kuitenkin korreloivat IL-17 erityksen kanssa diabeteskseen sairastuneilla lapsilla. Lisäksi havaitsimme joidenkin T1D:een sairastuneiden lasten stimuloitujen valkosolujen ilmentävän samassa solussa IL-17 ja IFN-γ merkkiaineita. Tutkimme Th17 immuniteettiin liitettyjen geenien ilmentymistä myös perifeerisen veren muistivalkosoluissa. Havaitsimme että IL-17, IL-22 ja FOXP3 lähetti-RNA ilmentyminen oli koholla jo kiertävissä perifeerisen veren T –auttajamuistisoluissa T1D:ssa. Osoitimme lisäksi että IL-17 yksin tai yhdessä muiden tulehdusreaktiota voimistavien merkkiaineiden, kuten IL-1β ja IFN-γ, kanssa lisää saarekesolujen kuolemaa *in vitro*. Th17 aktivaatio voisi siten kiihdyttää β-solukuolemaa, ja edesauttaa β-soluihin kohdistuvan autoimmuniteetin syntymistä.

Johtopäätöksenä tämän väitöskirjan tuloksista on että voimistunut IL-17/IL-22 välitteinen immuniteetti ja häiriintynyt FOXP3 välitteisten säätelymekanismien aktivaatio saattaa olla osallisena T1D:een johtavassa patogeneesissä. Löydöksillä saattaa olla hoidollista merkitystä T1D:ssa.

Avainsanat: tyypin 1 diabetes, immuunivasteen säätely, T-auttajasolut, regulatoriset T-solut, interleukiini-17

Contents

List of original publications

This thesis is based on the following publications, which have been reprinted with permission of the copyright holders:

(I) Honkanen J., Skarsvik S., Knip M., Vaarala O., Poor *in vitro* induction of FOXP3 and ICOS in type 1 cytokine environment activated T cells from children with type 1 diabetes. Diabetes Metab Res Rev. 2008 Nov-Dec;24(8):635-41.
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(II) Skarsvik S., Puranen J., Honkanen J., Roivainen M., Ilonen J., Holmberg H., Ludvigsson J., Vaarala O., Decreased *in vitro* type 1 immune response against coxsackie virus B4 in children with type 1 diabetes. Diabetes. 2006 Apr;55(4):996-1003.
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(III) Honkanen J., Nieminen J. K., Gao R., Luopajarvi K., Salo M., Ilonen J., Knip M., Otonkoski T., Vaarala O., IL-17 immunity in human type 1 diabetes. J Immunol. 2010 Aug 1;185(3):1959-67.
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(IV) Honkanen J., Sirkiä M., Korpela M., Vaarala O., Lahdenperä., J Screening method of modulators of FOXP3 gene expression in human regulatory T-cells. (Submitted)

Abbreviations

Ag	antigen
Ahr	aryl hydrocarbon receptor
Aire	autoimmune regulator
APC	antigen-presenting cell
B2M	beta-2-microglobulin
BSA	bovine serum albumin
CBA	cytometric bead array
CCR	chemokine receptor,
COX-2	cyclo-oxygenase 2
CTL	cytotoxic lymphocyte
CTLA-4	cytotoxic T -lymphocyte antigen 4
CVB4	coxsackie virus B4
CXCR	chemokine, cxc motif, receptor
DTH	delayed type hypersensitivity
EAE	experimental encephalomyelitis
ELISA	enzyme linked immunosorbent assay
HLA	human leukocyte antigen
FACS	fluorescence activated cell sorter
FICZ	6-formylindolo[3,2-b]carbazole
FOXP3	forkhead box 3
GADA	antibodies to the glutamic acid decarboxylase
GAPDH	glyceraldehyde-3-phosphate
GITR	glucocorticoid-induced TNF-receptor
IA-2	islet antigen 2 autoantibodies
IAA	insulin autoantibodies
ICA	islet cell autoantibodies
ICAM-1	intracellular adhesion molecule-1
ICOS	inducible co-stimulator
IFN	interferon
IFN-γR	interferon gamma receptor
IL-2R	interleukin-2 receptor
IL-4R	interleukin-4 receptor
IL-6R	interleukin-6 receptor
IL-12Rβ2	interleukin-12 receptor β subunit
IL-1β	interleukin-1β
IL-2	interleukin-2
IL-4	interleukin-4
IL-10	interleukin-10
IL-12	interleukin-12

IL-18	interlukin-18
IL-21	interleukin-21
IL-22	interleukin-22
IL-23	interleukin-23
IL-27	interleukin-27
IL-35	interleukin-35
IPEX	immune dysregulation polyendocrinopathy enteropathy X-linked
iTreg	inducible regulatory T-cell
MDA5	melanoma differentiation-associated gene 5
MHC I, MHC II	major histocomatibility complex
MIN-6	mouse insulinoma cell line 6
MODY	maturity-onset diabetes of the young
NFATc2	nuclear factor of activated T cells, calcineurin-dependent
NK	natural killer cell
NOD	non-obese diabetic
NOS2A	nitric oxide synthase 2A
nTreg	natural regulatory T -cell
OGTT	oral glucose tolerance test
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PFA	paraformaldehyde
PHA	phytohemagglutinin
RNA	ribonucleic aid
RORC2	retinoic acid-related orphan receptor C isoform 2
RT-qPCR	reverse transcription quantitative polymerase chain reaction
STAT	signal transducer and activator of transcription
T1D	type 1 diabetes
T2D	type 2 diabetes
T-bet	T-box expressed in T cells
TCR	T-cell receptor
TGF-β	transforming growth factor β
Th1	T helper 1
Th17	T helper 17
Th2	T helper 2
Th3	T helper 3
Thp	T helper precursor
TLR-4	toll-like receptor 4
TNF-α	tumor necrosis factor alpha
Tr1	regulatory T-cell, Tr1 type
Treg	regulatory T-cell
VNTR	variable nucleotide tandem repeat
ZnT8	zinc transporter 8

1 Introduction

Type 1 diabetes (T1D) is a life-long chronic disease. In type 1 diabetes insulin-producing β cells in the islets of Langerhans are destroyed, resulting in total lack or insufficient production of insulin. This leads to hyperglycemia, glycosuria and ketonuria and eventually, if not treated with exogenous insulin, to death.

Symptoms resembling those of T1D were characterized for the first time in 1552 BC in Egyptian writings. However, it was not until 1674 that T. Willis discovered sugar in the urine of patients with diabetes mellitus. Over a hundred years later, in 1776, M. Dobson also found sugar in the blood of diabetic patients.

In 1889, Minkowski and von Mering discovered that dogs subjected to the surgical removal of the pancreas developed diabetes. Pancreas extract was used as a treatment for diabetes in the year 1908, and the treatment reduced the blood glucose level of diabetes patients. Insulin was isolated for the first time from a dog's pancreas in 1921, and a year later insulin was successfully used to treat a diabetic patient for the first time.

T1D is perceived as an autoimmune disease caused by immune cell mediated destruction of pancreatic β cells. In 1965, Willy Gepts published an article showing the infiltration of immune cells in the pancreatic tissue [1]. The autoimmune nature of T1D was ultimately confirmed when the genetic association of T1D with the HLA gene region [2] and the presence of islet cell-specific autoantibodies [3] was demonstrated in patients with T1D. T cells of the adaptive immune system have been considered to be the main mediators of the β-cell destruction [4], and defects in T-cell polarization and immune regulation have been associated with T1D. The pathogenesis of T1D has been considered to result from a breakdown of immunologic tolerance towards β-cell antigens. Immunologic tolerance is maintained by two major phenomenon; central and peripheral tolerance. Peripheral tolerance is dictated by the fine balance between effector T cells and so called regulatory T cells (Treg). Recent advances in T cell research have provided new insights into the dynamics and regulation of T cell responses. Not only the balance between fully committed effector T cells and regulatory T cells, but also the plasticity between these phenotypes seems to play a role in autoimmunity and tolerance [5-7]. Novel T-cell subsets, like Th17 cells, have been characterized, and the function of Tregs has been demonstrated to be more dynamic and plastic than previously thought. Better understanding of the mechanisms generating and regulating T-cell responses is required for the development of effective therapies for T1D. The work presented in this thesis aimed at assessing the alterations related in generation and regulation of T-cell responses in T1D.

2 Review of the literature

2.1 Classification of diabetes in children

Diabetes mellitus is a heterogeneous group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The current classification of diabetes mellitus includes four main categories; type 1 diabetes, type 2 diabetes, diabetes with known monogenic defect and gestational diabetes. Type 1 diabetes can be further divided into two subclasses; type 1A, and a rare type 1B diabetes [8]. T1D is caused by a deficiency in insulin secretion due to the loss of pancreatic β cells, and the disease requires life-long treatment with exogenous insulin. Without the body's own insulin production the body loses its ability to utilize carbohydrates as an energy source.

T1DA diabetes is considered as an autoimmune disease, which is developed due to the T-cell-mediated destruction of β cells in the islets of Langerhans of the pancreas. In children with an active β-cell destruction process, autoantibodies against β-cell structures appear in the circulation. In T1DB the cause for β-cell destruction is not known. This type of diabetes is a very rare condition in Caucasian populations. However, in Japan it comprises of 10% of all T1D cases. T1DB differs from the T1DA in that it has no HLA –association and the mechanisms of β-cell destruction do not include β-cell autoimmunity.

Type 1A diabetes (T1D hereafter) is the most common form of diabetes in children worldwide, and approximately 80% of the children with diabetes has T1D [9]. In addition, there are several rare forms of diabetes diagnosed in the early childhood or in late youth/early adulthood. These types of diabetes are typically monogenic, and caused by an inherited mutation in certain genes involved in the insulin secretion of β cells (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003; American Diabetes Association 2006). These forms of diabetes are characterized by the onset of hyperglycaemia during childhood or early adulthood (<25 years of age), and are referred to as maturity-onset diabetes of the young (MODY). MODY is characterized by impaired insulin secretion with minimal or no defects in insulin action [10-12]. Patients with MODY are divided into different categories according to the specific mutations in the genes regulating the functions of β cells [13]. In another form of diabetes caused by point mutations in the mitochondrial DNA, deafness has been associated with diabetes mellitus [14, 15]. An A to G transversion mutation in the tRNALeu(UUR) gene is found in about 1.5% of the diabetic patients worldwide [16]. In some cases, diabetes is caused by genetic defects in insulin action. Functional insulin is cleaved from the precursor molecule proinsulin. Some types of diabetes are caused by mutations interfering the enzymatic cleavage of proinsulin molecules into functional insulin molecules. Such diabetes types are inherited in an autosomal dominant pattern

[17, 18]. In the much more prevalent type 2 diabetes (T2D), the disease is caused by a combination of tissue resistance to insulin and deficiency in insulin secretion from the pancreas. Individuals with T2D have relative rather than absolute insulin deficiency [19, 20]. Such individuals with this type of diabetes may survive, at least initially, without insulin replacement therapy (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003; American Diabetes Association 2006). The etiology of T2D is largely unknown, but obesity and reduced physical activity have been associated with the appearance of T2D. In the past, T2D was considered a disease of adults and older individuals. Recently, however, the incidence of T2D has been increasing even among children and adolescents [19, 20].

2.2 Epidemiology of T1D

Finland has the highest incidence of T1D in the world [21, 22], and the prevalence is continuously rising [23]. The overall incidence of T1D in Finnish children doubled between 1980 and 2005, and the increase was particularly steep in children aged 0-4 years. The incidence of T1D was 64.2 per 100 000 children under the age of 15 in the year 2005 [24]. The incidence rate of T1D was predicted to reach 50 per 100 000 children in 2010, if the increase had remained linear from 1950s [25].

The incidence of T1D is highest in Western societies, and there are clear geographical and ethnic differences. Particularly high incidences (>20 per 100 000 per year in 1994) have been reported in Finland, Sweden, Norway, Portugal, Canada and New Zealand [26]. Moderately high incidences are found in the United States and in European countries. For example, the incidence rate in France was 9.3 per 100 000 in 1995, and in the United States 14.8 per 100 000 in 1994. Lower incidences have been recorded in Eastern Europe countries than in other parts of Europe. For example, the incidence is 3.5 times higher in Nordic countries than in Estonia [26, 27]. Even more striking difference can be seen between Finland and Russian Karelia; the incidence of clinical type 1 diabetes is six times higher in Finland [28]. The lowest incidence globally (<1/100,000 per year) has been detected in populations from China and South America [26]. A low incidence (1-10 per 100 000) has been found in some parts of Asia, such as in Japan, where it is about 1.5 per 100 000 [29], as well as in South America [30] and in Africa [31, 32].

2.3 Clinical features of autoimmune T1D

2.3.1 Diagnostic criteria

The clinical signs of diabetes are associated with hyperglycaemia and osmotic diuresis. Untreated insulin deficiency leads to the use of fats as an energy source and to elevated levels of ketoacids in the body. This results in nausea and hyperventilation as clinical symptoms. Unexplained weight loss also often accompanies the above-mentioned symptoms. If blood glucose level exceeds 11.1mM or the fasting glucose concentration is above 7.0 mM, diabetes is clinically diagnosed. Diabetes may also be diagnosed by performing an oral glucose tolerance test (OGTT), in which the plasma glucose level is measured 2 hours after orally ingesting 75 g of glucose. If the plasma glucose level exceeds 11.1 mM the subject is considered diabetic. (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003; American Diabetes Association 2006). T1D and T2D can be distinguished from each other by detecting autoantibodies against the islet cell structures, typically found in 90% of patients with T1D at the time of diagnosis. About 50% of patients with T1D are diagnosed under the age of 14 years. Most patients with T1D have no detectable fasting C -peptide secretion remaining. The diagnosis of other specific diabetes syndromes than T1D requires the gathering of more detailed clinical information such as the ethnic background of the patient, age of onset, personal history of organ-specific autoimmunity, obesity and acute versus subtle onset of the disease.

2.3.2 Autoimmunity in T1D

The first evidence for the immune mediated pathogenesis of T1D was presented in the mid-1960s when Willy Gepts published a study characterizing the infiltration of immune cells in the inflamed islets of dead patients with acute onset diabetes [1]. The pathogenesis of T1D is characterized by the infiltration of mononuclear cells such as $CD4^+$ helper cells and cytotoxic $CD8^+$ cells, macrophages, natural killer cells and B cells into the pancreatic islets [33, 34]. The autoimmune nature of the pathogenesis of T1D became more evident when the association of T1D with other autoimmune diseases such as autoimmune thyroiditis and autoimmune adrenalitis was recognized [35-37]. The autoimmune nature of T1D was ultimately confirmed when the genetic association of T1D with HLA gene region [2] and the presence of islet cell-specific autoantibodies was demonstrated in patients with T1D [3, 38]. The aberrant hyperexpression of both MHC I and MHC II molecules in the pancreas further suggested the participation of immunological mechanisms in the pathogenesis of T1D [34, 39, 40]. Increased expression of MHC Class I molecules A, B and C has been observed in the pancreatic islets cells of acute onset immune-

mediated diabetes [39, 40]. Infiltrating immune cells are activated and express increased levels of MHC Class II and interleukin (IL)-2 receptor molecules on their surface. Endocrine cells in the normal pancreas do not express Class II MHC molecules [41-43]. Animal models of T1D strongly suggest that T lymphocytes are the main mediator of β-cell destruction [4]. It seems most likely that in T1D, tolerance to β-cell antigens is broken and insulin producing β cells in the pancreas are destroyed by the host's own immune system.

In children with an active β-cell destruction process, antibodies against β-cell structures appear in the peripheral blood. Furthermore, individuals at increased risk of developing T1D can often be identified by detecting antibodies against β-cell structures in the peripheral blood. Autoantibodies reflecting the β-cell destruction include islet cell antibodies (ICAs), insulin autoantibodies (IAA), glutamic acid decarboxylase (GAD65) autoantibodies, and autoantibodies to the tyrosine phosphatases IA-2 and IA-2beta [44-52]. Recently, antibodies to the cation efflux transporter ZnT8 have been detected in 60-80% of new onset T1D patients [53]. Of the T1D related autoantibodies, insulin is the only protein with limited expression in β-cells and thymic medullar epithelial cells. Several clinical studies have shown the appearance of autoantibodies in the course of T1D pathogenesis. However, there is no evidence that autoantibodies related to T1D play a direct role in the pathogenesis of the disease.

Animal models also support the T-cell rather than B-cell-mediated initiation of β-cell destruction. Two animal models, the NOD mouse and BB rat, have been especially valuable in studying the pathogenetic mechanisms of autoimmune diabetes. The NOD mouse is an inbred mouse strain that spontaneously develops autoimmune mediated diabetes [54]. Wicker et al. showed in the 1986 that transferring splenocytes from diabetic NOD mice to healthy irradiated mice >6wks of age initiates the diabetes in the healthy mice [55]. Later, it was shown that the transfer of T cells from diabetic NOD mice to healthy NOD mice transfers the disease, without autoantibodies being present [4, 56]. Autoimmune-mediated diabetes can be prevented in NOD mice by the administration of antibodies against T cells, supporting the role of T cells in the pathogenesis of T1D [57]. Prediabetic NOD mice were also protected from overt diabetes when antibodies targeted the molecules involved in the antigen recognition [58, 59], cellular activation [60] or pancreas homing receptors [61] were administered to the mice. Rabinovitch et al. succeeded in preventing disease in NOD mice by administering antibodies targeting cytokines mainly responsible for orchestrating T -cell responses, particularly Th1-directing cytokines such as IFN-γ, IL-12 and TNF-α [62]. In BB rats neonatal thymectomy reduced the frequency of spontaneous diabetes mellitus from 27% to 3% emphasizing the role of T-cell-mediated autoimmune processes in the pathogenesis of autoimmune diabetes [63].

The role of T cells in the pathogenesis of T1D is also supported by the observation that drugs down regulating T-cell activity prolong the survival of islet

transplants in patients with T1D. Immune suppressors such as cyclosporine A and rapamycin have been used in animal models and in clinical trials for immune suppression to protect transplanted islets. However, in humans, the side effects of these drugs have been severe, and these treatments are consequently not in clinical use. In the treatment of T1D, modified antibody targeting the CD3 (hOKT3γ1) molecule on the surface of T lymphocytes is already undergoing clinical trials. The anti-CD3 infusion induces tolerance, and prolongs insulin production after diagnosis [64].

In humans, allogeneic bone marrow transplantation transferred T1D from affected sibling to the non-diabetic patient [65]. T-cell involvement in the course of pathogenesis of T1D is evident. However, the detailed roles of $CD4^+$ and $CD8^+$ cells during the pathogenesis of T1D remain unresolved. Some studies have demonstrated that $CD4^+$ T helper cells are responsible for the initiation of the pathogenic process in pancreatic tissue, but the ultimate destruction of β cells is carried out by cytotoxic $CD8^+$ lymphocytes (CTLs) [66, 67]. Animal models have shown that the transfer of $CD4^+$ T helper cells initiates autoimmune diabetes, but the transfer of $CD8^+$ T cells alone does not. It has also been demonstrated in NOD mice that the parallel transfer of both $CD4^+$ and $CD8^+$ T -cells greatly increases the rate and the proportion of healthy recipient mice developing autoimmune diabetes [68]. During insulitis, T cells are the predominant cell type infiltrating the islets of Langerhans. IFN-γ-secreting Th1 cells prepared from diabetic NOD mice have been shown to initiate pathogenic events in the pancreatic islets, whereas the transfer of Th2 type cells secreting mainly IL-4 resulted in non-destructive peri-insulitis with no β-cell death [69]. The infiltration of type 1 cytokines secreting lymphocytes is associated with the destruction of β cells in the inflamed islets of NOD mice. In diabetes-prone NOD mice, elevated levels of IL-12, IL-18, and IFN-γ were observed, supporting the role of Th1-type cells in the pathogenesis of diabetes [70]. In the same study it was recognized that diabetes-resistant NOD mice did not show up-regulation of Th1-related cytokines, suggesting the Th1-cytokine milieu to be involved in the pathogenetic processes of autoimmune diabetes. Moreover, sustained IL-4 expression in the pancreas of diabetes-resistant NOD mice suggested that the local cytokine milieu plays a critical role in the regulation of local immune responses.

According to Foulis et al. [71], over 40% of the mononuclear cells infiltrated in the pancreatic islets of patients with T1D expressed IFN-γ, a hallmark cytokine for Th1-type cells. Increased expression of IFN-α mRNA was observed from the pancreas biopsy of a newly diagnosed T1D patient, supporting the idea that cytotoxic T-cell responses participate in the pathogenesis of T1D [72, 73]. Higher IFN-γ secretion of phytohemagglutinin (PHA)-stimulated PBMCs of T1D patients has also been observed when compared to healthy children [73]. However, there are studies suggesting impaired Th1 responsiveness in PBMCs from children with T1D, and the results from human studies are thus contradictory.

2.4 Immune system

2.4.1 Immunological tolerance

Immunological self-tolerance is critical for the prevention of autoimmunity and maintenance of immunological homeostasis. Immunological homeostasis is mainly maintained by two mechanisms; central and peripheral tolerance. Central tolerance refers to the deletion of T cells with an excessively high T-cell receptor (TCR) affinity towards the body's own peptides presented in MHC class I and class II molecules in the thymus [74-76]. It has been suggested that central tolerance is aberrant in patients suffering from autoimmune disease and that autoreactive T cells escape from the thymus and prime the immunological systems towards autoimmunity. However, autoreactive T cells are also released from the thymus in healthy people, but in healthy people these potentially pathogenic autoreactive T cells are controlled in the periphery. This phenomenon is known as peripheral tolerance.

T cells arise from the hematopoietic precursor cells that are born or generated in the bone marrow. These precursor cells then migrate to the thymus and begin the maturation process before they can enter into the peripheral blood stream. In order to produce T cells that have appropriate responses to discriminate between self and non-self structures in the periphery, the development of T cells takes place in a specialized organ, the thymus [77]. In the thymus T cells recognize their specific antigens bound to self-MHC molecules via their TCR. The early maturation process of T cells consists of several sequential checkpoint events [78]. In the later stages of development, thymocytes are double positive regarding $CD4^+$ and $CD8^+$ surface antigens. These developing $CD4^+CD8^+$ thymocytes are the subject of positive and negative selection. During positive selection the affinity of TCR is tested on the Ag-MHC complex, and only those thymocytes that possess a high enough affinity are allowed to live. Thymocytes with too low an affinity are directed to the apoptotic pathway, and thymocytes that pass the positive selection do not to have an excessively high affinity towards the body's own peptides. At this stage, developing thymocytes are differentiated to express only either $CD4^+$ or $CD8^+$ antigens on their surface. After the selection, fully matured, naïve T cells migrate to secondary lymphoid organs where they meet their specific antigen and are activated.

For the past 15 years, thymus derived natural regulatory T cells (nTregs), expressing the signature transcription factor Forkhead box 3 (FOXP3), have been recognized as key moderators in maintaining immunological homeostasis, extinguishing inflammation processes initiated by the autoreactive T cells. The role of Tregs in shutting off unwanted, harmful immune reactions has been described in many diseases [reviewed in [79-83]]. By far the most significant evidence for the role of Tregs in immunological homeostasis is the fact that both humans and mice deficient in Tregs suffer from widespread autoimmune diseases [84]. Tregs are known to develop from thymocytes with a moderately high TCR affinity to the

body's own peptides. It is not known why some high affinity thymocytes escape and differentiate into Treg cells. Neither is the antigen specificity of the cells entering the Treg development pathway known [85]. It is assumed that Treg cells are polyclonal populations and that they are able to recognize a wide repertoire of both self-derived and exogenous antigens [85]. The autoimmune regulator gene (Aire) promotes the expression of tissue-specific antigens in the medullary epithelial cells in the thymus [86]. The deletion of self-reactive thymocytes is critically regulated by the expression of the Aire gene [74, 87].

2.4.2 T lymphocytes

The human adaptive immune system consists of T and B lymphocytes. T cells can be divided into two main categories, $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. The cells of the acquired immune system have the capability to form a memory of a pathogen that they have encountered. Thus, the immune system can dramatically improve its potential to resist pathogens. T lymphocytes recognize their specific target antigen bound to HLA molecules. Cytotoxic $CD8^+$ T cells recognize peptides bound to HLA I class molecules, while $CD4^+$ T helper cells recognize peptides bound to HLA II molecules. MHC I molecules are expressed in all cells of the body, whereas MHC II molecules are only expressed by professional antigen-presenting cells such as dendritic cells (DCs), monocytes, macrophages and B-cells.

$CD4^+$ T helper cells provide help for other cell types by secreting soluble cytokines. These cytokines direct the responses of the recipient cells, either stimulating or inhibiting them.

Cytotoxic $CD8^+$ T cells kill the body's own cells infected with intracellular pathogens such as viruses. $CD8^+$ cells recognize their specific antigenic peptides presented on HLA class I molecules. When the engagement of the TCR-Ag-MHC I complex occurs, $CD8^+$ cells initiate their death-mediating processes. $CD8^+$ cytotoxic T cells kill their target cells by perforin and/or granzyme killing. They can also induce apoptosis in their target cells by FAS-FASL interaction or by secreting soluble cytokines such as TNF-α and IFN-γ.

2.4.3 T helper cells

The first observation that $CD4^+$ T cells are divided into at least two distinct subtypes originally came from mouse studies [88]. Soon after, the functional dichotomy of T helper cells was confirmed to also be present in humans [89]. $CD4^+$ T cells were originally divided in two functionally different categories, Th1 and Th2 cells, both of which have a different potential to augment B-cell responses *in vitro* [90]. Nowadays, further $CD4^+$ T-cell subsets have been defined. In addition to Th1 and

Th2, novel effector $CD4^+$ T cell subsets called Th17 cells and a $CD4^+$ T-cell class mediating immune regulation functions, known as Tregs, have been characterized. T helper cell precursors originate from the bone marrow and mature in the thymus. From the thymus, mature T cells migrate into the peripheral blood circulation. Activation of Th cells takes place in the lymph nodes after the interaction of their antigen-specific T-cell receptor with the antigen-MHC class II complex presented by the APCs. Figure 1 illustrates the differentiation of $CD4^+$ T-cell subsets, and factors contributing to the activation and differentiation process of these subsets in humans. The differentiation of Th cells into either Th1, Th2 or Th17 effector or the Treg phenotype is mainly controlled by the cytokine stimuli of their environment and the strength of the MHC II-Ag-TCR interaction. APCs are the most important regulators of the cytokine environment in the tissue. Several factors influence the differentiation process, including the type of APCs, the strength and quality of antigen exposure, ligation of co-stimulatory molecules and the local cytokine environment. Dendritic cells (DC) have been suggested to be the primary, if not exclusive, type of APCs presenting peptides to naïve $CD4^+$ T cells in an MHC II restricted fashion [91].

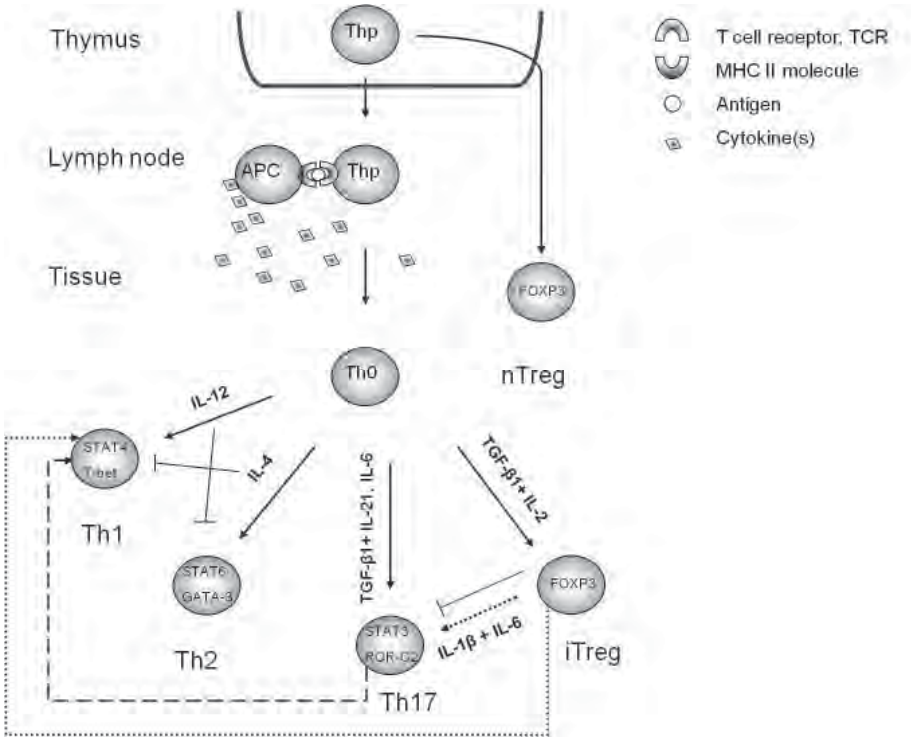

Figure 1. Differentiation of human CD4⁺ T helper cell subsets. T helper cell precursors (Thp) are generated in the bone marrow. From there they migrate to to the thymus and undergo through the positive and negative selection. Naïve T cells then migrate to the peripheral blood circulation. Th cells are activated in the lymph nodes when APCs present peptides bound onto their HLA class II molecules. After the activation of Th cells, cytokines secreted mainly by the APCs direct the differentiation process either towards effector Th1, Th2, or Th17 phenotypes or towards the iTreg phenotype. nTregs are already committed to the regulatory phenotype in the thymus. Tregs have been characterized to show significant plasticity under certain experimental conditions, and are able to convert into either Th1 or Th17 phenotypes [7, 92] (dotted lines in the figure). Th17 cells may also begin to express IFN-γ, a hallmark cytokine of Th1 cells (dashed line in the figure). Th17 cells expressing IFN-γ are potentially highly pathogenic, and in islet cell antigen-specific T cell transfer experiments, these cells have been shown to initiate β-cell autoimmunity in naïve mice recipients [5].

The activation and differentiation of CD4⁺ T helper cells requires at least two signals. The first is delivered through the engagement of TCR, and the second signal is delivered through the co-stimulatory molecules such as CD28 and ICOS (Figure 2).

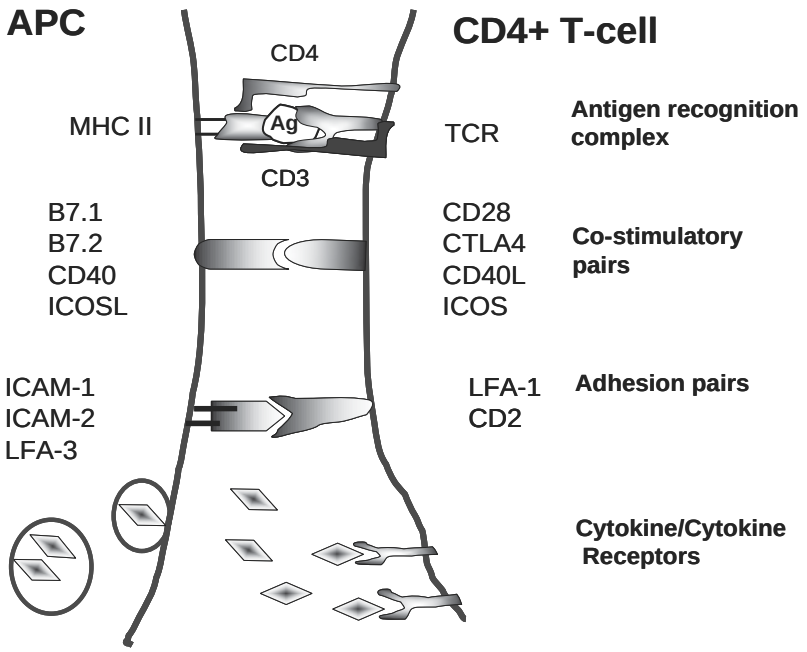

Figure 2. Activation scheme of CD4⁺ T cells. TCR engagement is the first requirement for the activation of CD4⁺ T-cells. APCs present peptides bound onto MHC II class molecules. For full activation, the second signal needed, which is delivered through the interaction of co-stimulatory molecules on the surface of APCs and their ligands on the surface of CD4⁺ T cells. The binding of adhesion molecules such as ICAM-1 provides stability for the cell-cell interaction and enables the endothelial transmigration of activated CD4⁺ T cells to the site of inflammation. Cytokines secreted by the APCs direct the development of activated CD4⁺ T cells to fully functional effector T cells or Tregs.

2.4.4 Th1 and Th2 cells

The most critical cytokines regulating the Th1/Th2 polarization of the Th cells are IL-12 and IL-4, directing Th1 and Th2 polarization, respectively. Uncontrolled or unbalanced Th helper cell responses have been associated with allergy and autoimmune disease pathogenesis. Th1 cells have been linked to many chronic autoimmune diseases whereas Th2 –cells are often linked to atopic and allergic conditions [93].

Th1 cells predominantly release IFN-gamma and IL-2, TNF-α, and lymphotoxin, and direct the immune response towards cell-mediated immunity, delayed-type hypersensitivity (DTH) and macrophage activation. Thus, Th1 cells promote the clearance of intracellular pathogens, e.g. *Leishmania major* and *Listeria monocytogenes*. Th1 responses have been associated with many autoimmune diseases. Th1 cells characteristically express the IFN-γ receptor-β chain, IL-12 receptor β

chain [94], IL-18 receptor [95], CXCR3 and CCR5 chemokine receptors [96] on their surface, whereas Th2 cells preferentially express CCR3 and CCR4 cell surface receptors [97, 98]. The STAT4 cascade is activated when IL-12 binds to the IL-12 receptor on the cell surface. An activated STAT4 homodimer molecule is actively transported to the nucleus, where it recognizes specific DNA sequences and activates transcriptional events of its target genes, IFN-γ, IL-12Rβ2 and T-box expressed in T cells (T-bet). IFN-γ binding to its receptor leads to activation of the STAT1 signaling cascade. The STAT1 homodimer eventually binds to the promoter region of T-bet and induces the transcriptional activity of T-bet, thus enhancing the Th1 differentiation events in the cell [99, 100]. Increased susceptibility to microbial pathogens and viruses has been observed in mice deficient in IFN-γ, IFN-γR1, IFN-γR2 or the signaling molecule STAT1 [101-106]. Humans with mutations in IFN-γ signaling pathway components have been characterized to have profound immunodeficiencies, resulting in severe intracellular bacterial infections [107-110]. T-bet is a master regulator of the induction and maintenance of the Th1 phenotype of $CD4^+$ T cells [111]. T-bet belongs to a conserved family of transcription factors known as the T-box family. This family of transcription factors is conserved throughout diverse species, and they play a key role in directing early developmental processes. TCR engagement has a central role in the control of T-bet expression. After TCR-Ag-MHC II interaction, the IFN-γR/STAT1 signaling cascade is recruited and the expression of T-bet is rapidly up-regulated [112, 113]. IFN-γ secretion by NK cells, macrophages and DCs induce the expression of T-bet, which in turn causes chromatin remodeling of the IFN-γ locus [114]. One of the key mechanisms by which T-bet induces and drives Th1 differentiation is up-regulation of the IL-12Rβ2 subunit of the IL-12 receptor. Up-regulation of the IL-12 receptor leads to increased STAT4 signaling and thus optimizes the IFN-γ production, further strengthening the development of the Th1 phenotype [112, 114]. In activated and differentiating T cells, T-bet efficiently inhibits the transcription of genes related to the induction and functions of Th2 cells (Figure 3).

Th2 cells typically secrete cytokines activating B cells, such as IL-4, IL-5, and IL-13, to produce antibodies providing protection against extracellular pathogens [115, 116] including helminths and nematodes. IL-4 is the cytokine responsible for directing the maturation of the Thp cells towards the Th2 direction. The development of the Th2 phenotype is controlled by the activation of the STAT6 signaling cascade. Binding of IL-4 to its receptor recruits STAT6 molecules to bind to the cytoplasmic tail of the IL-4 receptor. STAT6 molecules are then phosphorylated by Jak kinases and are homodimerized and translocated to the nucleus [117]. In the STAT6 signaling pathway, the transcription factor mainly responsible for the induction of Th2-type genes is GATA-3, and the recognition sequence G/A/T/A can be found at least in the promoter region of IL-5, IL-13 and IL-4R genes (Figure 4). GATA-3 also has a role in chromatin remodeling of the Th2 loci in chromosome five. Th1 and Th2 cell development rapidly diverges after the antigenic recognition.

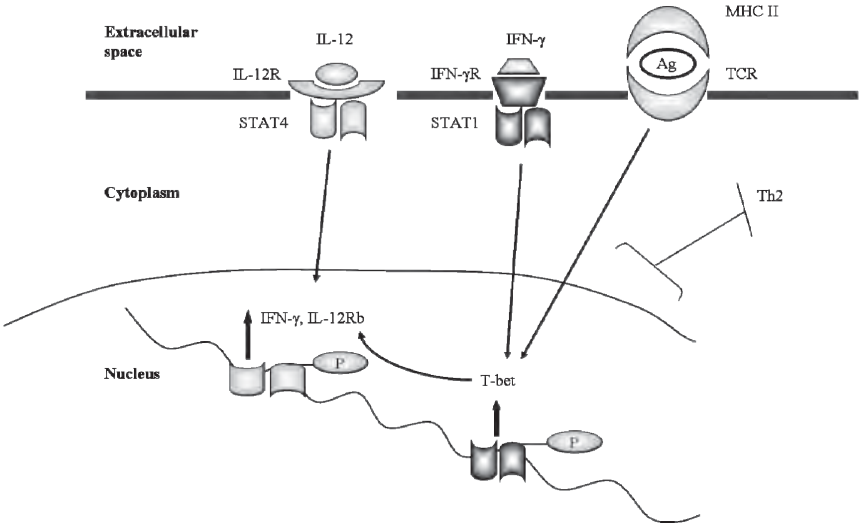

Figure 3. Signaling directing the differentiation of activated naïve T helper cells towards the Th1 phenotype. After TCR-Ag-MHC II interaction, the IFN-γR/STAT1 signaling cascade is recruited and induces the expression of transcription factor T-bet. IL-12 ligation leads to STAT4 dimerization and phosphorylation and induces the expression of the IL-12Rβ2 subunit and IFN-γ. IFN-γ strengthens its own expression through a positive feedback loop, which leads to rapid Th1 phenotype stabilization. Th1 type signaling efficiently inhibits the induction of the Th2 phenotype.

The signaling pathways directing the differentiation process of distinct subsets are robust and counter-regulatory. Th1-type cytokines have counter-regulatory effects on Th2 cells, and vice versa [115, 118].

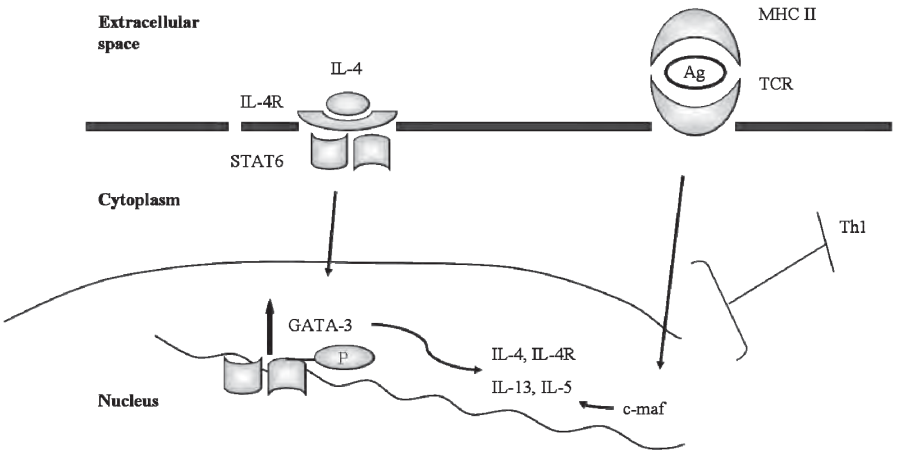

Figure 4. IL-4 is the most critical cytokine directing the differentiation of activated naïve T cells towards the Th2 phenotype. IL-4 ligation induces STAT6 signaling and GATA-3 transcription. Transcription factor GATA-3 promotes the transcription of Th2 type cytokines and their receptor counterparts. TCR signaling gives rise to the expression of c-maf, promoting the expression of Th2 type genes. GATA-3 is an efficient inhibitor of Th1 phenotype development in the activated naïve T cells.

2.4.5 nTreg, Tr1, and Th3 cells

The importance of $CD4^+CD25^+FOXP3^+$ Tregs has been recognized since 1995. During that year, Sakaguchi et al. demonstrated that a small proportion of activated $CD4^+CD25^+$ cells contributed to the maintenance of self-tolerance by down-regulating the immune response to self and non-self antigens in an Ag-nonspecific manner. Tregs were able to suppress the proliferation of $CD8^+$ T cells and established Th1 and Th2 cells by secreting soluble cytokines but also by direct cell-cell contact. Inoculation of thymus-and spleen-derived $CD25^+$ depleted $CD4^+$ cells from Balb/nu/+ mice into BALB/c athymic nude (nu/nu) mice caused a variety of autoimmune diseases such as thyroiditis, gastritis, insulitis, sialoadenitis, adrenalitis, oophoritis, glomerulonephritis and polyarthritis in all recipients [80]. Relatively soon after the rediscovery of 'suppressor' T cells by Sakaguchi et al., it was demonstrated that $CD4^+$ cells expressing high levels of $CD25^+$ on their surface had the potential to inhibit the proliferation of stimulated $CD4^+CD25^-$ cells *in vitro* in mice [119].

The best available marker for Tregs is the expression of transcription factor FOXP3, which was first characterized in 'scurfy' mice carrying the X chromosomal mutation leading to severe autoimmunity. These mice suffered from a wide spectrum of autoimmune diseases. Mutation in the FOXP3 gene coding region leads to premature termination of the transcription process, resulting a truncated and

biologically inactive protein lacking an NH2 terminus [120]. The same mutation was earlier described in humans in a *FOXP3*-like coding sequence called *JM2* [12]. Patients with mutations in that particular genomic region develop a severe, fatal systemic autoimmune disorder called immune dysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome. In these patients, IPEX causes the enlargement of secondary lymphoid organs, lymphocyte infiltration into several organs and the manifestation of several immune-mediated diseases, such as autoimmune-mediated diabetes, eczema and food allergies.

Tregs are divided into naturally occurring $CD4^+CD25^+FOXP3^+$ Tregs (nTreg), that develop in the thymus after recognition of high-affinity self antigen, and inducible Tregs (iTreg), which are induced from naïve $CD4^+$ T cells following antigenic stimulation in the periphery [121-125] (Figure 5). nTregs comprise ~5-10% of $CD4^+$ T cells in the peripheral blood [126]. nTregs characteristically express a high affinity IL-2 receptor α subunit (CD25) on their surface [127]. Unlike effector T cells that upregulate CD25 upon activation through TCR, nTregs constitutively express CD25 at very high levels. The thymus-induced nTregs characteristically express cytotoxic lymphocyte activation antigen (CTLA-4), inducible co-stimulator (ICOS), and the glucocorticoid-induced TNF receptor family-related protein TNFRSF18 (GITR). The observation that forced FOXP3 expression in T cells, leads to acquisition of the regulatory potential of these cells underlines the importance of FOXP3 for the Treg functioning [121, 128]. nTregs are anergic and they do not secrete IL-2 or proliferate when activated *in vitro*. In $CD4^+CD25^+$ T cells the minimal 300-bp proximal promoter does not undergo DNA demethylation of CpG or deacetylation of core histones, thus preventing transcription of the IL-2 gene [129]. However, nTreg functions are dependent on IL-2 and TGF-β. FOXP3 is essential for the differentiation and maintenance of Tregs in the periphery, and TGF-β together with IL-2 is required for FOXP3 induction through SMAD and STAT5 signaling (Figure 5). It should be noted, however, that FOXP3 is also upregulated in human $CD4^+CD25^+FOXP3^-$ T cells upon TCR stimulation and thus FOXP3 cannot be considered alone as a marker of Tregs [130].

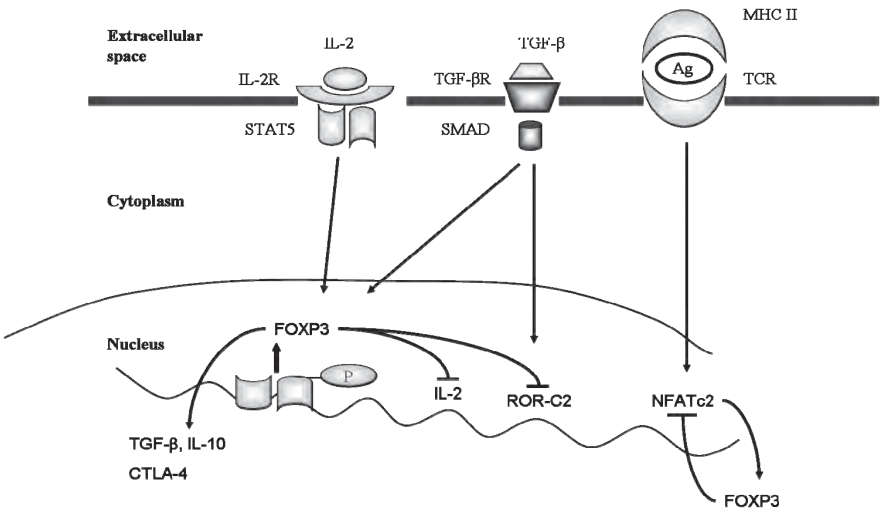

Figure 5. FOXP3 is the master regulator of Tregs. TGF-β and IL-2 ligation to their receptors leads to the activation of FOXP3 through SMAD3 and STAT5 dependent mechanisms. TCR-Ag-MHC II interaction leads to the activation of NFATc2, a transcription factor promoting FOXP3 activation. High concentrations of FOXP3 inhibit NFATc2 expression, thus providing negative feedback. FOXP3 represses the production of IL-2. FOXP3 activates the expression of suppressive cytokines TGF-β and IL-10 and co-stimulatory molecule CTLA-4, mediating the inhibitory signal to the activated T cells.

The basic mechanisms of action of Tregs include suppression by inhibitory cytokines, suppression by causing target cell cytolysis or apoptosis, suppression by metabolic disruption and suppression by modulation of APC functions. Treg functioning is considered to mainly be mediated through the secretion of inhibitory cytokines TGF-β, IL-10, and recently discovered IL-27 and IL-35 [131]. The role of IL-27 and IL-35 is not, however, firmly established in humans.

TGF-β has a critical role in maintaining immunological homeostasis. Its role in immune regulation *in vivo* is evident, despite the controversial results from *in vitro* studies [83, 119, 132, 133]. TGF-β down-modulates T-cell responses directly and indirectly. It is produced by several immune and non-immune cell types, and it acts in both an autocrine and paracrine manner [134, 135]. It can exert its antiproliferative effects by inhibiting the production of IL-2 and up-regulating cell-cycle inhibitors [136]. TGF-β also down-regulates the expression of T-bet and GATA-3, thus inhibiting the development of Th1 and Th2 subsets of $CD4^+$ T cells [137, 138]. Moreover, TGF-β inhibits macrophage activation and their production of pro-inflammatory cytokines. In addition, TGF-β down-regulates the expression of MHC II class molecules and prevents the maturation of dendritic cells [136].

IL-10 is an essential anti-inflammatory cytokine produced by Tregs, and it has anti-inflammatory and suppressive effects on most hematopoietic cells. In addition, it limits the antigen-presenting capacity of the APCs thus down-modulating

the adaptive response generated by $CD4^+$ T cells [139-141]. IL-10 down-regulates the expression of co-stimulatory molecules and the expression of pro-inflammatory cytokines produced by the APCs and $CD4^+$ T cells. IL-2 and TNF-alpha production by $CD4^+$ T cells is directly inhibited by IL-10 [142]. IL-10 suppresses both naïve and memory T cells [143-145].

Tregs can also direct their target effector T cells to the cytolytic or apoptotic pathway by direct cell-cell contact. Cytotoxic activity has been considered to be a feature of NK cells and cytotoxic $CD8^+$ T cells, but $CD4^+$ T cells may also exhibit cytotoxic activity. Tregs have shown to be able to kill their target cells by granzyme B and perforin killing [146]. The activity of T cells is also modulated by co-stimulatory pathways. CD28 ligation with its ligands CD80 and CD86 co-operates with signals mediated by TCR engagement to favor the activation of T cells instead of anergy and apoptosis [147]. Two structurally related cell surface molecules are selectively expressed by T cells, namely CTLA-4 and ICOS. The expression of these molecules is dependent on the activation status of the T cells [148]. CTLA-4 binds to the same ligands as CD28, but with a higher affinity. CTLA-4 ligation with its ligands mediates negative signals to the activated T cells, thus switching them off. ICOS ligates members of a different B7 family compared to CD28 or CTLA-4, i.e. B7h and B7H. ICOS regulates cytokine production in recently activated T cells, and has only a weak effect on naïve T cells [149-152]. In humans, it has been shown that ICOS favors the differentiation of effector T cells when operating together with an appropriate stimulus, such as anti-CD3 and anti-CD28, whereas it predominantly supports the differentiation of Tregs when co-stimulatory signals are suboptimal [153].

Several distinct subpopulations of Tregs have been characterized so far in humans. Several antigen-specific Treg cells have been characterized that are induced after exposure to specific exogenous antigens [154]. Stock et al. (2004) suggested that there could be a spectrum of iTregs with slightly different characteristics, and they reported a Th1-cell-like Treg population that expressed cytokines IFN-γ, IL-10, and transcription factors T-bet and Foxp3 in mice [155]. At that time, Th2-like Tregs had already been reported by Akbari et al. in 2002 [154].

In addition to $CD4^+CD25^+FOXP3^+$ Tregs, two other subsets of Tregs have been characterized so far, namely Th3 and Tr1 cells. TGF-β is the cytokine inducing and maintaining the regulatory functions of Th3 cells [156], while IL-10 is able to induce the development and maintain the functions of Tr1 cells [145, 157]. Th3 cells mediate their suppression by secreting TGF-β, and Tr1 cells by secreting IL-10, respectively. These different subsets of Tregs do share some features, such as the expression of cell surface molecules CD25, CTLA-4, GITR, CD62L and $CD45RBl^o$ [158], and the potential to inhibit the proliferation and cytokine production of effector T cells. Classical nTregs express CD25 at high levels, but Th3 and Tr1 cells at intermediate to low levels.

Th3 cells express the transcription factor FOXP3 upon stimulation, whereas Tr1 cells do not, suggesting that Tr1 cells are distinct in origin from Th3, and nTregs in particular [157]. Both Th3 and Tr1 cells have been shown to be able to prevent the development of autoimmune disease [159-161]. The function of Th3 cells has been associated with the induction of oral tolerance, which has presumably evolved to prevent hypersensitivity reactions to food proteins and bacterial antigens present in the normal mucosal flora [156]. Continuous exposure to low dose antigens eventually leads to the induction of regulatory mechanisms such as the emergence of Th3 cells capable of inhibiting inflammation by secreting high levels of TGF-β [162], whereas high dose exposure induces anergy and deletion [163, 164]. Th3 cells are particularly important in a variety of disease settings and in maintaining tolerance to antigens present in the intestinal tissue [165]. Th3 cell induction is suggested to involve interaction with APCs, DCs in particular [166, 167]. Intestinal $CD103^+$ cells play a key role in the induction of Foxp3 expressing Tregs in oral tolerance induction [168].

Tr1 cells can be induced from naïve $CD4^+$ T cells in the periphery by chronic exposure to antigens and in the presence of IL-10 secreted by immature dendritic cells (iDCs), tolerogenic myeloid DCs and plasmacytoid DCs (pDCs). In mice and humans, it has been shown that the induction of Tr1 cells is associated with the development of tolerance in transplant settings, and in response to allergens, pathogens and tumor antigens [169, 170]. The main mechanism for Tr1 cells to mediate suppression is the secretion of inhibitory cytokine IL-10. The importance of IL-10 in immune regulation was realized when IL-10 deficient mice were shown to develop ulcerative colitis [171]. Since the first observation, several studies have associated IL-10 aberrancies in autoimmune and allergic diseases. Mucosal administration of antigens induced tolerance and prevented autoimmune disease in experimental autoimmune uveitis [172], experimental autoimmune myasthenia gravis [173], arthritis in rats [174-176]. Intranasal administration of insulin peptide B: 9-23 induced IL-10 secretion has been shown to protect NOD mice from diabetes [177].

2.4.6 Th17 cells

Th17 cells provide protection against bacterial and fungal infections by recruiting neutrophils and activating their migration to the site of infection. Th17 cells secrete pre-dominantly IL-17A and IL-17F. These cytokines induce multiple pro-inflammatory mediators including chemokines, cytokines and metalloproteinases, from epithelial and fibroblast cells. Th17 cells also secrete IL-22, which induces epithelial cells to produce anti-microbial peptides RegIIIβ and RegIIIγ [178]. IL-17 producing activated T cells were originally discovered in the mid-1990s. The key observation was that IL-17 introduced to fibroblasts induced IL-6 and IL-8 production and upregulated cell surface expression of the intracellular adhesion mol-

ecule 1 (ICAM-1). IL-17 transcripts were only detected in stimulated T cells, and not in other cell types [179, 180]. In 2005 it was recognized that IL-17 producing T helper cells were an independent and separate lineage from Th1, Th2 or Treg cells. Interestingly, IFN-γ, IL-12 and IL-4 inhibited the induction and development of IL-17-producing cells in mice. Accordingly, inhibition of IFN-γ enhanced the development of pathogenic IL-17-producing cells. Thus, Harrington et al. considered that Th17 cells have developed via a lineage distinct from the T helper type 1 and type 2 lineages [181]. Autoantigen-specific and dysregulated Th1 responses have been associated with organ-specific autoimmunity. The concept of an organ-specific autoimmune response being caused by excessive Th1 responses was, however, challenged by the observation that IL-12p40 knockout mice showed clinical signs of EAE. IL-23 is comprised of the p40 subunit of IL-12 but a different p19 subunit, and the absence of IL-23 made symptoms of EAE milder in IL-12p19-deficient mice [182]. When elevated levels of IL-17 were found from the synovial fluid of patients with rheumatoid arthritis, it was evident that IL-17 may play a role in human autoimmunity [183].

Antibodies against IL-17 inhibited chemokine expression in EAE, whereas overexpression of IL-17 in the lung epithelium resulted in up-regulation of chemokines and lymphocyte infiltration. Thus, it was reasoned that IL-17 is a critical regulator of tissue inflammation [184].

Th17 cells have been intensively studied during recent years. However, the molecular mechanisms directing the induction and development of Th17 are still poorly understood. *In vivo* studies have revealed that the induction and maintenance of Th17 cells requires TCR engagement and cytokines TGF-β and IL-6 in mice [185, 186]. Originally, IL-23 was thought to play a central role in the induction of Th17 cells, but more recently the role of IL-23 has been shown to be the expansion and stabilization of activated and Th17-primed T cells, since naïve T cells do not have a receptor for the IL-23 cytokine [185]. In the absence of IL-6, Th17 cells can still develop, and IL-21 has been demonstrated to be essential for the induction of IL-23R and RORC2, and IL-17 [187]. In synergy with TGF-β, IL-21 has been demonstrated to inhibit TGF-β-mediated FOXP3 up-regulation, and the subsequent Treg development of activated naïve T helper cells [188]. The observation that TGF-β is a critical cytokine for Th17 cell development was controversial, since TGF-β has been considered as an anti-inflammatory cytokine through its inhibitory effect on effector T-cell induction. TGF-β induces distinct subpopulations of IL-17- and FOXP3-expressing T cells. Thus, TGF-β has a dualistic role on activated naïve T cells [189].

The induction and development of Th17 cells is independent of the transcription factors or other signaling molecules that are required for the induction and maintenance of Th1, Th2, and Treg cells. RORC2 is specifically expressed by human Th17 cells [190]. In humans TGF-β together with IL-21 and/or IL-6 induces the expression of RORC2 in naïve T cells. (Figure 6) [191]. Up-regulation of

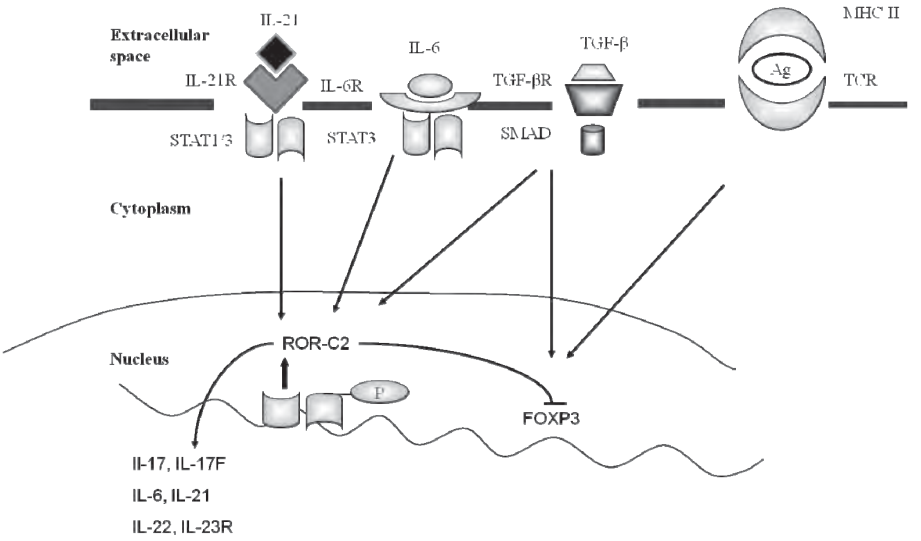

Figure 6. During the initial activation of CD4+ lymphocytes, continued TGF-β and IL-21 and IL-6 signaling activates the expression of RORC2 through STAT1/3- and SMAD-dependent mechanisms. A high level of IL-6 silences FOXP3 and skews development towards the Th17 phenotype. RORC2 expression promotes the expression of IL-23R, which is necessary for the expansion and stabilization of the Th17 cell population.

RORC2 is dependent on the STAT3 signaling [192]. RORC2 expression leads to FOXP3 pathway down-regulation, and Treg development is thus inhibited in activated naïve T cells. In humans, IL-1β together with IL-6 induce IL-17 response in memory T cells [193].

Transcription factor FOXP3 antagonizes the function of Th17-specific RORC2 by binding physically the RORC2 via the motif encoded by exon 2 of FOXP3. Simultaneous expression of FOXP3 and RORC2 has been related to the counter-acting role of IL-6 in FOXP3-mediated inhibition of the IL-17 response [185, 194, 195]. The dynamic balance of Treg cells and Th17 cells seems to be delicately controlled by several means. In addition to the reciprocal functions of FOXP3 and RORC2, the transcription factor RUNX1 is also required for the development of both Tregs and Th17 cells [196, 197]. RORC2 can bind directly to RUNX1, and together they can bind to the promoter region of IL-17A, thus enhancing mRNA transcription of IL-17A. Moreover, FOXP3, together with RUNX1, can bind RORC2 and inhibit IL-17A transcription [196]. RUNX1 mediates the interaction between FOXP3 and RORC2 in a cytokine-dependent manner, and mediates the balance between activating or repressing activities of the activated CD4+ T cells. Another factor in the regulation of the Treg/Th17 balance is the Aryl hydrocarbon receptor (Ahr), and Th17 cells and iTregs express Ahr at high levels. Ahr is a ligand-dependent transcription factor and mediates the effects of environmental toxins such as dioxins or FICZ (a UV photoproduct of tryptophan) [198]. The Ahr

functions in a ligand-specific fashion, and it has been reported to support the development of Th17 when FICZ is present, and Treg development when dioxin is present [199]. However, the results from different studies have been contradictory, since another study reported the opposite results for the role of Ahr ligands in the induction and development of Treg/Th17 cells. Experiments with Ahr-deficient animal models have suggested Ahr to be essential for IL-22 expression [199, 200].

The Th17 subset of $CD4^+$ T cells provides help for extracellular microbial eradication and fungus eradication. Th17 cells promote the secretion of a variety of cytokines, metalloproteinases and other inflammation mediators. The recruitment of granulocytes is an important function of Th17 cells [201, 202]. IL-17Ra-deficient mice are more prone to bacterial and fungal infections than wild type littermates. Also, the blocking of IL-17 signaling by antibodies directed against the IL-17 receptors leads to reduced resistance of these animals to bacterial and fungal infections [203]. In humans, the role of Th17 cells in the pathogenesis of infectious and autoimmune diseases has just begun to be elucidated.

IL-17-secreting T cells may convert into Th1-like cells when TGF-β1 is not present [204]. Recent reports from animal models indicate that beside nTregs, natural Th17 (nTh17) cells also exist, and they develop in the thymus. nTh17 cells share developmental relationship with $FOXP3^+$ nTregs, showing self-reactivity and mucosal homing properties [205]. These natural Th17 cells also expressed α4β1 integrins and the chemokine receptor CCR6, and were recruited to the lung, gut and liver. Thus, self-ag-specific Th17 cells occur, providing novel insights into the immunological mechanisms in organ-specific autoimmune diseases such as T1D.

2.5 Risk factors for T1D

2.5.1 Genetic risk

T1D is aggregated in families, and the risk of developing T1D, being inheritable, is well known. The genetic component of T1D is polygenic, and several loci have so far been recognized to contribute to the overall risk of T1D.

2.5.1.1 HLA polymorphism

Approximately 50% of the total risk is associated within the genomic region containing genes for HLA molecules (Human Leucocyte Antigen, human analog for MHC). HLA molecules are expressed on the cell surface. The function of HLA molecules is to present peptides bound into their peptide binding cleft and to display them on the cell surface for recognition by the appropriate T cells. HLA molecules are divided in two classes that differ from each other by the structure and

the expression signature. HLA class I molecules are ubiquitously expressed. They present peptides for the cytotoxic $CD8^+$ T cells. In contrast, the expression of HLA class II molecules is restricted to the APCs.

Major determinants of HLA-related T1D risk are HLA class II alleles. HLA-DQ is a heterodimeric molecule encoded by DQA1 and DQB1 genes. HLA-DQB1 and HL-DQA1 are highly polymorphic genes, and the variation especially affects the conformation of the peptide-binding groove of these molecules. This reflects in the antigen presentation process, affecting the binding affinity of peptides to HLA-DQA1 and HLA-DQB1 molecules. The variation in the antigen presentation properties of different HLA molecules is the explanation for the fact that some forms of the molecules are protective against T1D, while others are risk factors for T1D [206]. HLA-DQB genotyping determines a large proportion of the T1D risk, since HLA-DQA1 is in linkage disequilibrium with the HLA-DQB1 locus. The actual risk is, however, more complicated to determine, since other loci, such as like HLA-DR, modify the overall risk of T1D. The highest risk genotypes for T1D are DQA1*0301-DQB1*0302 and DQA1*0501-DQB1*0201 [207]. Some alleles within the HLA-DQ region have a protective effect on the HLA-related T1D risk. DQB1*0602 has the strongest protective effect, whereas DQB1*0301 and DQB1*0603 have a moderate protective effect.

DQB1*0302 and DQB1*0201 are common in the Finnish general population and approximately 40% of Finns have either one of the high risk alleles, while approximately half of all individuals with one high risk allele have no protective alleles. The importance of HLA-DQB1 molecules is underlined by the fact that 90-95% of the diabetic children carry either the DQB1*0302 allele or the DQB1*0201 allele, or both.

2.5.1.2 Insulin gene polymorphism

The insulin gene promoter is the second gene region known to contribute the genetic risk of T1D [208]. The promoter of the insulin gene contains variable nucleotide tandem repeat polymorphimsms. Polymorphisms within this region have been divided into three categories according to the number of 14-15 bp oligonucleotide repeats (VNTR I, II and III) [209]. The VNTR I allele has been associated with lower insulin expression in the thymus [210-212]. Self-antigens are expressed during the negative selection of the lymphocytes in the thymus, under the control of the Aire gene [213]. Reduced insulin expression by the thymic medullary epithelial cells may play a role in T1D through the impaired deletion of autoreactive T cells [211, 212], and decreased induction of insulin-specific nTregs.

2.5.1.3 CTLA-4 gene polymorphism

Polymorphism in the cytotoxic T –lymphocyte antigen-4 gene region in chromosome 2 (CTLA-4) was the third gene region found to be associated with the T1D risk (IDDM3). CTLA-4 is expressed on the surface of both $CD4^+$ and $CD8^+$ T cells after the TCR-mediated stimulation [214, 215]. In T-cell activation, CTLA-4 interacts with B7 molecules expressed on the surface of APCs. In resting T cells, the second signal needed for activation is delivered through the surface molecule CD28. The function of CTLA-4 is to down-regulate T-cell responses, and it has been suggested to play a role in the induction and maintenance of peripheral tolerance. Loss of function models have shown CTLA-4 to be essential for down-regulation of the proliferation of activated T cells [216]. An amino acid-changing nucleotide polymorphism has been characterized at position +49 in exon 1 of the CTLA-4 gene [217]. Since the first observation of the association of the CTLA-4 gene region and T1D by Nistico et al. [218], the +49 A–> G polymorphism has been associated with T1D in several studies [219, 220]. However, considerable ethnic variation exists in the association of the CTLA-4 polymorphism and T1D [221]. The contribution of CTLA-4 to the genetic risk of T1D has been reported in a study in which non-coding polymorphism of the CTLA-4 promoter was significantly associated with T1D [222].

2.5.1.4 PTPN22 gene polymorphism

PTPN22 gene product, LYP, is a lymphoid-specific protein phosphatase, expressed mainly in cells of lymphoidal origin. PTPN22 is notably expressed on $CD4^+$ T cells, and it's function is to negatively regulate transcriptional activity of T cells induced by the engagement of T-cell receptor and co-stimulatory molecule CD28 [223]. Animal models have associated the dysfunction of PEP, that is homologous to human LYP, to increased antigen independent proliferation and cytokine secretion [224].

The PTPN22 gene has several polymorphic sites [225]. Polymorphisms in the PTPN22 gene region have been associated with several autoimmune diseases, including T1D. In 2004 Bottini et al. [226] reported the association of an amino acid-changing nucleotide transition (C to T) at position 1858 with T1D. Since then, several reports have confirmed this association with T1D [227-232], and other autoimmune diseases as well. A recent report demonstrated 23 single nucleotide polymorphisms and 1 deletion insertion polymorphism. In the same study, polymorphic site 1858T was confirmed to be primarily associated with T1D [225].

The prevalence of the predisposing allele variant 1858T of the PTPN22 gene varies greatly: 15% of the Finnish general population carries the 1858T allele, compared to over 10% of the other Scandinavian populations, 7-8% of the Western populations and 2-3% in Italian and Sardinian populations.

PTPN22 1858T polymorphism is a gain of function mutation. The 1858T allele has been shown to have a higher catalytic activity, and this has been associated with reduced IL-2 secretion and intracellular calcium release [233]. In addition, lowered activation of $CD4^+$ memory T cells, decreased expression of CD25 and IL-10 has been associated with PTPN22 1858T polymorphism [234].

2.5.1.5 IFIH1 gene polymorphism

IFIH (interferon induced with helicase C domain 1, also known as MDA5) is an intracellular protein recognizing virus RNA and mediating the innate immune response. Genome wide association studies have associated gene region containing IFIH1, FAP, GCA and KCNh7 genes in chromosome 2 with T1D [235]. Further genotyping revealed several variants across the gene region in high linkage disequilibrium. However, functional properties of IFIH1 gene product suggested IFIH1 to have possible role in T1D. Recently four low frequency variants were shown to be protective for T1D [236]. In allele specific expression analysis, protective haplotype correlated with reduced IFIH1 transcription in interferon-β stimulated PBMCs [237].

2.5.2 Environmental risk factors

2.5.2.1 Virus infections

T1D is a complex disease with a largely unknown aetiology. The disease has a strong genetic component. However, despite the fact that risk alleles for T1D are common in the Finnish general population, the risk for newborn baby to develop T1D is less than 1% [238]. Furthermore, the concordance rate between monozygotic twins for developing T1D is 50%. Thus, environmental factors must be involved in triggering the disease in genetically susceptible individuals.

Acute virus infections may induce autoimmunity, and the role of viruses has been suspected in chronic autoimmune diseases. Virus infections are widely studied, and yet their role in the pathogenesis process of T1D is still poorly understood. Certain viruses have long been associated with T1D. Mumps viruses and congenital rubella viruses are known to be able to induce diabetes in some infected individuals. However, these viruses have been eradicated from western societies due to intensive vaccination programs, and these viruses cannot therefore play a role in T1D pathogenesis in developed countries. Studies have occasionally associated cytomegalovirus, Epstein-Barr virus and retroviruses with T1D, but solid evidence for their contribution is still lacking [239].

2.5.2.2 Enteroviruses

Enteroviruses were associated with T1D for the first time in 1969 when a patient with recent onset T1D was shown to have higher antibody titers against the coxsackievirus B4 strain than unaffected control subjects [240]. Coxsackieviruses isolated from the pancreas of a child who died in ketoacidosis induced diabetes when inoculated in susceptible mice, causing hyperglycemia, infiltration of immune cells into the pancreatic tissue and β-cell necrosis [241]. The presence of enteroviruses has been associated with the appearance of autoantibodies, suggesting that enteroviruses have a possible role in the initiation of autoimmunity. Antibodies against enteroviruses were more often detected from the serum of subjects who were positive regarding one or more T1D related autoantibodies, indicating that enteroviral infections may have a causal role in the pathogenesis of the disease [242, 243]. Several studies have shown children with T1D and prediabetic children to have more often coxsackievirus RNA or antibodies against coxsackieviruses in their blood circulation [239, 240, 242, 244-248]. Thus, children with T1D or at genetic risk for T1D may be more prone to coxsackievirus infections. The role of coxsackieviruses in the course of T1D is further supported by the reports that show CVB4 to be able to infect human β cells both *in vitro* [249] and *in vivo* [250], which may in turn induce β-cell autoimmunity or support it. Recent studies have supported the role of enteroviruses in the pathogenesis of T1D. The presence of CVB capsid protein vp1 has been demonstrated in the pancreatic tissue of T1D patients [251]. Moreover, enteroviruses have been found in the intestine of T1D patients, whereas the enteroviruses were not detected in healthy children [252]. So far, several epidemiological studies have repeatedly shown an association between enteroviruses and T1D. However, the causal relationship of enterovirus infections and T1D is still unclear.

2.5.2.3 Nutritional factors

The early signs of the initiation of β-cell autoimmunity emerge during the first years of life. Despite the strong genetic predisposition to T1D, there is accumulating evidence supporting the idea that exogenous factors contribute to the initiation of β-cell autoimmunity, gradually leading to overt diabetes. Nutrition is an essential part of the exogenous antigen exposure load, but so far, no specific factor has been identified as unequivocally related to the risk of initiation of autoimmunity.

2.5.2.4 The length of breastfeeding and early exposure to cow's milk proteins

The effect of the length of breast feeding has been studied, but the results have been controversial. Some studies have shown that a short breastfeeding period in infan-

cy may be associated with the appearance of β-cell autoimmunity [253, 254]. However, other studies have indicated that the length of breastfeeding does not contribute to the individual's risk of developing β-cell autoimmunity [255, 256] and T1D.

Early exposure to cow's milk proteins has been considered as one of the possible factors priming β-cell autoimmunity in genetically predisposed individuals. Early exposure to cow's milk-derived protein has been associated with an elevated risk of the initiation of β-cell autoimmunity [253, 254] and T1D [257, 258], whereas some studies have found no such associations [259, 260]. The role of early exposure to cow's milk-derived proteins in the priming of β-cell autoimmunity is supported by the observation that when hydrolyzed formula instead one containing cow's milk protein was given to children during the first months of their life, hydrolyzed formula significantly reduced the appearance of T1D-associated autoantibodies in those individuals genetically at risk of developing T1D [261]. Interestingly, exposure to bovine insulin in cow's milk formula induces an immune response to bovine insulin, which crossreacts with human insulin, and this primary immunization to insulin, when it takes place during the early months of life, could explain the link between cow's milk and T1D. Early exposure to bovine insulin has been proposed to be associated with impaired insulin tolerance [262, 263].

2.5.2.5 Wheat proteins

Wheat gluten has been considered as one of the nutritional risk factors for T1D. Indeed, an association between T1D and celiac disease has been reported [264-266]. Gliadin may be the specific trigger for subclinical inflammation in T1D patients. When jejunal biopsies from T1D patients and those with celiac disease were stimulated with gliadin, up-regulation of T-cell activation markers CD25 and ICAM-1 was observed *in vitro*. This up-regulation was not associated with active celiac disease, but with T1D [267]. Animal models have shown that dietary wheat gluten modulates the intestinal immune system, and gluten may therefore also participate in the pathogenesis of T1D [268, 269].

2.5.3 Immunological aberrancies in T1D

In health, the immune system delicately balances between the two states: it efficiently recognizes non-self structures and prevents pathogen invasion, and at the same time tolerates self-structures and antigenic structures derived from food and commensal bacteria. In T1D, this balance is disturbed and insulin producing β-cells are progressively destroyed in the pancreas by the body's own immune system. Immune responses against foreign structures are generated by the function of the innate and adaptive immune systems. The innate immune system provides an immediate response against pathogens based on the recognition of foreign

structures by the germ-line-encoded specific receptors such as Toll-like receptors (TLRs). The pathogen structure recognition of the adaptive immune system, however, is based on usage of variable antigen-specific receptors produced by gene segment re-arrangements. The involvement of adaptive immune responses in the pathogenesis of T1D is firmly established, as discussed earlier. The development of T cells specific for β-cell structures leads to the generation of pathogenic autoimmunity targeting insulin-producing β cells. If regulatory mechanisms fail to be initiated, then subtle inflammation targeting β cells leads to insulitis and eventually to overt T1D in some individuals. For the past 15 years, Tregs have been recognized as having a key role in the initiation and maintenance of tolerance, limiting harmful autoantigen-specific inflammation processes. The differentiation of activated naïve $CD4^+$ and $CD8^+$, as well as the function of fully committed $CD4^+$ and $CD8^+$ is tightly controlled by the innate immune system through the cytokine network and cell-cell interaction of APCs and T lymphocytes. Thus, the possible flaws in the regulation of the adaptive immune system may also reflect aberrancies in the innate immune system.

IFN-γ-secreting Th1 cells prepared from diabetic NOD mice have shown to initiate pathogenic events in the pancreatic islets, whereas the transfer of Th2-type cells mainly secreting IL-4 resulted in non-destructive peri-insulitis with no β–cell death [69]. Infiltration of type 1 cytokine-secreting lymphocytes is associated with the destruction of β cells in the inflamed islets of NOD mice. In parallel, IL-4 secretion was reduced in insulitis-associated mononuclear cells from diabetic female NOD mice, suggesting that the β–cell destruction is mediated by cytokines promoting cytotoxicity [270]. In diabetes-prone NOD mice, elevated levels of IL-12, IL-18 and IFN-γ were observed, supporting the role of Th1-type cells in the pathogenesis of diabetes [70]. In the same study it was recognized that diabetes-resistant NOD mice did not show up-regulation of Th1-related cytokines, suggesting the Th1 cytokine milieu to be involved in the pathogenetic processes of autoimmune diabetes. Moreover, sustained IL-4 expression in the pancreas of diabetes-resistant NOD mice suggests that the local cytokine milieu plays a critical role in the regulation of local immune responses. According to Foulis et al. [71] over 40% of the mononuclear cells that infiltrated the pancreatic islets of patients with T1D expressed IFN-γ, a hallmark cytokine for Th1-type cells. Increased expression of IFN-α mRNA was observed from the pancreas biopsy of a newly diagnosed T1D patient, supporting the idea that cytotoxic T-cell responses participate in the pathogenesis of T1D [72]. When PBMCs from patients with T1D were stimulated, significantly increased levels of IFN-γ secretion were observed when compared to healthy control subjects or patients with Grave's disease [73]. In contrast, some studies have indicated a decreased type 1 response in patients with T1D. Reduced levels of intracellular IFN-γ in $CD4^+$ T cells were reported in the study by Kukreja et al., when PBMCs of both recent-onset and long-lasting T1D patients were stimulated with PMA and ionomycin [271]. Antigen-induced IFN-γ secretion has also

been observed to be lower in T1D patients than in healthy children when their PBMCs were stimulated either with GAD65 or IA-2 antigens [272].

Recently, IL-17-producing T cells have been associated with the pathogenesis of T1D. In NOD mouse elevated IL-17 transcript levels were found to correlate with the progression of insulitis to diabetes both in the pancreas and blood circulation [273]. In mouse insulinoma cells (MIN6), IL-17 enhances the generation of inducible nitric oxide synthase, and elevates the release of NO from the pancreatic β cells. IL-17-induced NO is toxic to β cells, thus driving the β cells to the apoptotic and necrotic pathway. The administration of IL-17-neutralizing antibody reduced the release of NO from the pancreatic cells, and diminished the damage of insulinoma cells and pancreatic islets induced by activated T cells [274]. More recently, IL-17 neutralization has been demonstrated to prevent T1D development in NOD mice. The treatment was, however, only effective if given after the initiation of the autoimmunity process [275]. IL-17 neutralization reduced the islet-specific inflammatory T-cell infiltration and increased the proportion of $Foxp3^+$ Tregs around the islets, suggesting that Th17 inhibition leads to the activation of Tregs [275]. The pathogenic nature of Th17 cells in T1D was further supported by an experiment in which Th17 cells transferred diabetes from mouse strain BDC2.5 with transgenic islet-cell specific TCR to healthy NOD/SCID recipients. In the recipients, the highly purified Th17 cells rapidly turned into Th1-like cells [5]. Moreover, these cells were able to express Th1-specific transcription factor T-bet and to secrete IFN-γ *in vitro* when stimulated with IL-12. These data indicate substantial plasticity of Th17 cells. Plasticity of fully committed memory T cells may contribute to the pathogenesis of T1D.

The role of Th17 cells in the pathogenesis of human T1D is unknown. Monocytes from patients with T1D have shown spontaneous activation of IL-6 and IL-1β, and the monocytes isolated from diabetic patients were able to induce IL-17 deviation in allogenic memory T cells *in vitro* [276]. This observation indirectly supports the idea that IL-17 mediated immunity may be involved in the pathogenesis of human T1D.

The aberrancies of Tregs have been associated with T1D in both humans and animal studies. In patients with newly-diagnosed T1D, impaired functioning of Tregs has been reported [277]. The defects were demonstrated as an inability of Tregs to inhibit T-cell proliferation, as well as an increased ratio of IFN-gamma/IL-10 in the co-cultures of Tregs and T effector cells [277]. In contrast, the number and function of Tregs was normal in T1D, according to the study by Putnam et al. [278]. Evidence from animal models indicates that dynamic changes in the functional capacity of Tregs emerge over time in NOD mice spontaneously developing autoimmune diabetes. Tregs are present in the inflamed islets in NOD mice, and TGF-beta signaling is critical for the control of β-cell destruction [279]. Tregs from young NOD mice inhibit autoimmune diabetes, and the effect is mediated by membrane-bound TGF-β [280]. However, Tregs become functionally defective,

i.e. a decline in membrane-bound TGF-β is seen, and they do not regulate β-cell autoimmunity when IAA seroconversion occurs in the mouse model. Moreover, Treg-associated IL-10 was shown to protect NOD mice from developing diabetes. The effect of the exogenous IL-10 was potentiated when administered together with the immunosuppressive drug rapamycin [281]. Tregs may also lose their ability to express FOXP3, and begin to secrete inflammatory cytokines IFN-γ or IL-17. If FOXP3 expression is lost, then the effector functions may be promoted in the so-called ex-regulatory T cells. These exFOXP3 cells have been reported to be able to transfer diabetes in an animal model [7].

3 Aims of the study

In T1D, immunological tolerance towards β-cell antigens is broken and the immune system specifically destroys insulin-producing β cells in the islets of Langerhans of the pancreas. The broken peripheral tolerance is probably a manifestation of flaws in immune regulation mechanisms. The aim of the studies presented in this thesis was to exmine the induction and differentiation of T helper cells in T1D, and to characterize possible flaws in the regulation of T-cell responses that may contribute to the pathogenesis of T1D. An additional aim was to develop a methodology that could be used for the screening of small molecule entities to enhance the function of regulatory T cells in order to identify potential therapeutic candidates for the treatment of autoimmune disorders.

I The aim of the first study was to characterize whether the impaired induction of regulatory mechanisms during the activation of T-cell responses is associated with T1D.

II The aim of the second study was determine whether the T-cell response to coxsackievirus, which has been associated with T1D, is aberrant in patients with T1D when compared to the healthy subjects with or without a genetic risk for T1D.

III In the third study the aim was to characterize whether Th17 immunity is associated with T1D.

IV In the fourth study we aimed to develop a methodology for the screening of small molecule entities modulating the dynamic balance between effector and regulative functions, as measured by the up-regulation of FOXP3.

4 Subjects and methods

4.1 Subjects

Publication I

For gene expression and cytokine secretion analyses of cultured PBMCs, blood samples were taken from 18 recently diagnosed diabetic patients (0-14 days from diagnosis; mean age 9.3 years), 11 children who had had T1D for 1 year (mean disease duration 384 days, SD 36.9, mean age 10.6 years, SD 5.3) and 14 non-diabetic children (mean age 8.1 years, SD 3.3) were used.

The proliferation inhibition assays were carried out by using blood samples from six healthy individuals. Flow cytometry analyses of FOXP3 protein expression in cultured Th1-type cells were performed using heparinized blood samples from three healthy individuals.

The study was approved by the local Ethics Committee. The parents and/or the study subjects gave their informed consent before the withdrawal of the blood sample.

Publication II

A venous blood sample was collected from 15 children with type 1 diabetes (mean age 11 years, range 6-17 years, disease duration 1-48 months), and from 13 healthy children with HLA risk genotype for T1D (mean age 11 years, range 10-18 years) and from 14 children without HLA risk genotype for T1D (mean age 12 years, range 10-16 years). The HLA risk genotype was defined as DQA1* / DQB1* (DR3-DQ2) and/or DQAB1 (DR4-DQ). All the children without HLA risk genotype were also negative for autoantibodies against insulin, GAD and IA-2A. One of the children with HLA risk genotype was positive regarding autoantibodies against the IA-2A, and another child in this group was positive for insulin autoantibodies. All the other children with HLA risk genotype were negative for autoantibodies.

The study was approved by the Regional Ethics Committee for Human Research at the Faculty of Sciences of Linköping University in Sweden.

Publication III

Peripheral blood samples were studied from 24 children with type 1 diabetes (mean age 8.7 years, SD 4.4, median disease duration of diabetes 12.5 days, range 0-415 days) and 20 healthy children (mean age 8.2 years, SD 4.4) to compare Th17 immunity in PBMCs stimulated *in vitro* and in memory T cells *ex vivo*. The IL-6 levels were also determined from plasma withdrawn by centrifugation from 15 T1D patients and 12 healthy children. Both T1D patients and healthy control children were genotyped for T1D risk alleles. Genotyping was not performed for three diabetic patients or for two healthy control children. Two diabetic children were neg-

ative for HLA risk alleles, whereas all the remaining diabetic patients carried the risk alleles DR3/DQ2 and/or DR4/DQ8. Seven healthy control children were negative for HLA risk alleles, while 11 were positive for either DR3/DQ2 or DR4/DQ8, or both.

For the determination of peripheral blood monocyte activation, frozen PBMCs from eight recently diagnosed patients with T1D and eight healthy siblings of diabetic children were studied.

The study was approved by the Ethics Committees of the participating hospitals. The parents gave their informed consent and children aged 10 years or older provided their informed assent before the venous blood samples were collected.

To study the effects of pro-inflammatory cytokines IL-1β, IFN-γ and IL-17 on human pancreatic islet cells, seven islet preparations from donated human pancreata were prepared at the Central Laboratory of the Nordic Network for Clinical Islet Transplantation in Uppsala. All procedures were approved by the institutional Ethics Committees in Sweden and Finland. We also examined the effects of pro-inflammatory cytokines on mouse insulinoma cell lines (MIN6) in three separate *in vitro* experiments.

Publication IV

For the development of the screening methodology we studied $CD4^+CD25^+$ cells isolated from the peripheral blood of adult (age between 18 and 65 years) volunteer blood donors. The volunteers were first tested regarding their blood hemoglobin levels. An acceptable hemoglobin level of the donor had to be 130g/l or above. After the informed consent was received, a venous blood sample up to 300ml was withdrawn. The study was approved by the local Ethics Committee.

4.2 Methods

Table 1. Laboratory methods used in this thesis

Cell preparations & Purification of Target Cells	**Method**	**Publication**
PBMC separation from peripheral blood	Ficoll gradient centrifugation	I, III, IV
PBMC separation from peripheral blood	Cell Preparation Tubes™	II
Monocyte depletion	Plastic adherence	I
Isolation of naive CD4 -cells	Magnetic bead technique	I
$CD4^+CD25^+$ regulatory T cell separation	Magnetic micro beads	IV
Isolation of monocytes from PBMC	Magnetic micro beads	IV
Human islet preparation	Ficoll gradient centrifugation	III
Cell Stimulation Tests		
Cytokine stimulation of PHA activated naive T cells	Cell culturing	I
Coxsackievirus B4 stimulation of PBMC for 7 days	Cell culturing	II
$\alpha CD3^+\alpha CD28$ stimulation of PBMC 40h	Cell culturing	III
Cytokine stimulation of MIN6 cells 72hαCD3	Cell culturing	III
Cytokine stimulation of human islets in vitro 72h	Cell culturing	III
Stimulation and chemical treatment of Treg cells	Cell culturing	IV
T -cell Suppression Test		
$CD4^+CD25^+$ cell co-culture	Thymidin incorporation	I
Flow Cytometry Analyses		
CD3, CD4, CD25, CD45RA, CD45RO	Extracellular staining	I
CD4, CD8, CXCR3, CXCR4, CXCR6, CCR2, CCR3, CCR4, CCR5, CCR7, IL-18R, IL-12Rβ2	Extracellular staining	II
CD3, CD4, CCR4, CCR6, TCRα/β, TCRγ/δ	Extracellular staining	III
CD3, CD4, CD25	Extracellular staining	IV
IFN-γ, IL-17	Intracellular staining	III
FOXP3	Intranuclear staining	I, IV
Cytokine Analyses		
Culture supernatant levels of IL-10	Cytometric Bead Array	I
Plasma levels of IL-6	Cytometric Bead Array	III
Culture supernatant levels of IFN-γ, IL-5, TGF-β1	ELISA	I
Culture supernatant levels of IFN-γ	ELISA	II
Culture supernatant levels of IL-17	ELISA	III
Other Protein Analyses		
Quantitative analysis of T-bet and GATA-3 protein	Western blot analysis	II
RNA Isolation		
Total RNA isolation from polarized T cells	TRIzol extraction method	I
Total RNA isolation from virus stimulated PBMCs	Filtration technique	II, III
mRNA purification from $CD4^+CD25^+$ cells	Affinity capture of mRNA	IV
Gene Expression Analyses		
T-bet, GATA-3, FOXP3, ICOS and NFATc2,18s	RT-PCR & TaqMan	I
T-bet, GATA-3, c-maf, IL-4R and IL-12R,18s	RT-PCR & TaqMan	II
IL-17, RORC2, IL-22, FOXP3, IFN-γ ,T-bet,BCL-2, COX2, NOS2A, SOD2,18s	RT-PCR & TaqMan	III
FOXP3,B2M	RT-PCR & TaqMan	IV
Detection of Antibodies		
Detection of GADA & IA-2A autoantibodies from plasma	Immunoprecipitation	II
Detection of IAA autoantibodies from plasma	Radioimmunoassay	II
Detection of CVB4 neutralizing antibodies from plasma	Microneutralization assay	II

4.2.1 Cell preparations

In studies **I, III** and **IV**, PBMCs, were isolated from heparinized blood samples by Ficoll gradient centrifugation. Human islets were purified from the pancreatic preparations by Ficoll gradient centrifugation. In study **II**, the PBMC fraction was isolated from heparinized blood samples with Cell Preparation TubesTM according to the manufacturer's instructions (Becton Dickinson). After centrifuge isolation (studies **I, III** and **IV**), the cells were washed three times with PBS (Lonza) and finally re-suspended in RPMI 1640 (Gibco/Life Technologies, Paisley, U.K.) supplemented with 5% inactivated human male AB serum (obtained in studies **I** and **II** from the Finnish Red Cross Blood Transfusion Service, Helsinki, Finland, and in study **III** from Innovative Research, USA), L-glutamine (2mmol/l; Invitrogen, Stockholm, Sweden) and gentamicin (25 µg/ml; Sigma-Aldrich, Stockholm, Sweden). In study **IV**, isolated PBMCs were re-suspended after the washing steps into the X-VIVO15 culture medium (Lonza, Verviers, Belgium). In studies **I** and **III**, isolated cells were re-suspended at a concentration of 1x106 cells/ml in RPMI-1640 culture medium supplemented with inactivated human AB serum (5%), L-glutamine (2mmol/l; Invitrogen, Stockholm, Sweden), and gentamicin (25 µg/ml; Sigma-Aldrich, Stockholm, Sweden). In study **IV** the isolated PBMC fraction was re-suspended in X-VIVO15 culture medium (Lonza) for the further processing.

4.2.2 Purification of target cells

In study **I**, plastic adherence was used to deplete monocytes from the PBMC fraction (PBMC at a concentration of $1x10^6$ in a 50 ml plastic cell culture bottle for 1h at 37°C in a CO_2 incubator). Naïve T cells were purified from the monocyte depleted PBMC preparations by depleting cells positive for CD45RO with a magnetic particle purification technique according to the manufacturer's instructions (Dynal Corp., Oslo, Norway). In study **III**, $CD4^+$ memory T cells and monocytes from PBMC of T1D patients and healthy children were purified for the gene expression analyses. Memory T cells were purified from the freshly isolated PBMC by a magnetic separation technique. Firstly, $CD4^+$ T cells were purified using a CD4 Multisort Kit (Miltenyi). Naïve T cells positive for the CD45RA isoform were then depleted using CD45RA microbeads according to the manufacturer's instructions (Miltenyi Corp., Bergisch Gladbach, Germany). $CD45RO^+$ memory T cells were lysed in Qiagen RLT Buffer for RT-qPCR analysis. The purity of the isolated memory T cells was assessed by flow cytometry. The memory T cell purity of the preparations was constantly greater than 90%. Monocytes from frozen and thawed PBMC were isolated with a magnetic purification technique (Miltenyi). Monocyte Isolation Kit II and LS columns were used according to the manufacturer's instructions

(Miltenyi). The purity of isolated $CD14^+$ monocytes was assessed by flow cytometry. After the isolation of $CD4^+$ memory T cells and monocytes, cells were washed with PBS and lysed with RLT buffer (Qiagen), supplemented with 1% 2-mercaptoethanol. The lysate was stored at -80°C prior to RNA extraction.

In study **IV**, $CD4^+CD25^+$ cells were purified from PBMC fractions by using the MACS (Miltenyi Corp., Bergisch Gladbach, Germany) magnetic separation technique. $CD4^+$ cells were negatively selected by incubating PBMCs with magnetic particles coated with antibodies targeting cells other than $CD4^+$ T helper cells and collecting the flowthrough fraction. Negatively-selected $CD4^+$ cells were washed with 0.5% BSA in PBS with EDTA. Cells were briefly spun down and incubated for 20 minutes at 4°C with magnetic microbeads coated with antibodies targeting the CD25 molecule. $CD4^+CD25^-$ cells were first washed out from the column attached to a magnetic stand. Then the column was removed from the stand and placed in a 15 ml Falcon tube and the magnetically labeled $CD4^+CD25^+$ cells were eluted from the column with the elution buffer according to the manufacturer's instructions. The purity of the eluted $CD4^+CD25^+$ cells was confirmed by flow cytometry.

4.2.3 Cell culture methods

*Stimulation of $CD45RA^+$ T cells in type 1 or type 2 cytokine conditions (**I**).* $CD45RA^+$ T cells were stimulated with PHA and type 1 or type 2 cytokines in RPMI-1640 culture medium (Invitrogen, Paisley, UK) supplemented with heat-inactivated human AB serum (5%), L-glutamine (2mmol/l; Invitrogen, Stockholm, Sweden), and gentamicin (25 μg/ml; Sigma-Aldrich, Stockholm, Sweden). For type 1 stimulations, 200 pg/ml IL-12 (Sigma) and 50 ng/ml anti-IL-4 (BD Pharmingen, San Diego, CA, USA) were used, and for type 2 stimulations, 400 pg/ml IL-4 (Sigma) and anti-IL-12 were used (1 μg/ml, Serotec, Oxford, UK). After culturing cells for 72h, cells were harvested and homogenized in the TRIzol reagent (Invitrogen). *Stimulation of PBMCs with coxsackievirus B4 strain (**II**).* PBMCs were stimulated by culturing $2x10^6$ cells per well in a 24 well cell culturing plate. Coxsackievirus B4 strain (1μg/ml; obtained from the American Type Culture Collection, Manassas, VA) was used for the stimulation of isolated PBMCs. Viruses were inactivated by incubating them at 56°C for 30 min. IL-2 (BD PharMingen, Stockholm, Sweden) was added to the cell cultures on the third day at the concetration of 32 pg/ml. On the seventh day of the cell culture, supernatants were collected and stored at -70°C for later cytokine analyses. For mRNA, Western blot and flow cytometry analyses cells were collected on day seven. For mRNA analysis, cells were collected and lysed with the lysis buffer of a total RNA purification kit (Sigma-Aldrich, St.Louis, MO, USA). Lysed cells were stored at -70°C until use. Cells for Western blot analyses were collected and stored in liquid nitrogen until use. *PBMC stimulation with anti-CD3 and anti-CD28 (**III**).* T-cell stimulations were performed by culturing 2×10^5 PBMCs in

a total volume of 200µl for 40 h as three replicates in 96-well round-bottom culturing plates (NUNC) pre-coated with anti-CD3 antibody (50 µl of anti-CD3 at 5µg/ml in PBS) (BD Pharmingen) and in the presence of soluble anti-CD28 antibody at the concentration of 0.5µg/ml (BD Pharmingen). After the incubation, cells were collected and lysed in Qiagen RLT lysis buffer for RT-qPCR analyses. Prior to collecting cells, supernatants were collected for cytokine detection. *Stimulation of islet cells (**III**).* Batches of islets were incubated in non-adherent 24-well plates for 24 h or 72 h in RPMI 1640 medium (Gibco) supplemented with antibiotics and 10% FCS (Lonza) and treated without (as negative control) or with one or more of following recombinant human cytokines: IL-17 (100 ng/ml, eBioscience), IL-1β (5 ng/ml, PeproTech) and IFN-γ (50 ng/ml, PeproTech). MIN6 cells (a mouse insulinoma cell line) were cultured in 12-well tissue culture plates with or without glass coverslips in D-MEM medium (4.5 g/l glucose, Lonza) supplemented with antibiotics, beta-mercaptoethanol (0.25 µmol, Gibco) and 15% FCS (Lonza). MIN6 cells were treated with cytokines similarly to human islets. *Stimulation of Tregs (**IV**).* The cell attachment test was performed by culturing 4000 $CD4^+CD25^+$ and $CD4^+CD25^-$ cells on plates with or without anti-CD3 coating (BD Pharmingen, San Diego, California, USA). U-bottomed 96-well culture plates (NUNC, Roskilde, Denmark, Cat. No. 163320) were incubated with anti-CD3 at the concentration of 5µg/ml in PBS (30 µl/well) for 4 hours at 37°C in the presence of 5% of CO_2. After the coating step, cell culture plates were washed three times with 100µl of PBS. The cells were then spread onto the plate and cultured for 16 h at 37°C and in 5% CO_2 in the following test conditions: cells in culture medium, cells incubated in wells with plate-bound anti-CD3; and cells incubated in wells with plate-bound anti-CD3 and soluble anti-CD28 (BD Pharmingen, San Diego, California, USA, Cat.No. 555725) at the concentration of 5µg/ml and TGF-β1 (R&D Systems, Minneapolis, USA, Cat. No. 240-B) at the concentration of 20 ng/ml in a total volume of 100µl of X-VIVO15 culture medium (Lonza, Verviers, Belgium). In the pilot chemical screening of study **IV**, $CD4^+CD25^+$ cells were prepared as previously described and incubated at + 37°C for 72 h with different members of the Spectrum Collection chemical library (MicroSource Inc., CT, USA) at the concentration of 1 µM. In the pilot screen, we performed the experiment in two 96-well chemical plates each containing 80 different members chosen randomly from the chemical library. As a positive control $CD4^+CD25^+$ cells were cultured with plate-bound anti-CD3 and soluble anti-CD28 at the concentration of 5µg/ml and TGF-β1 at the concentration of 20 ng/ml for 72 h. Cells were incubated for 72 h and mRNA samples were collected. The inter-individual variation in the response to the compound was analyzed by using $CD4^+CD25^+$ cells from six different donors. The dose-response curve was tested for anti-CD3-stimulated and phenylbutazone-treated $CD4^+CD25^+$ cells at concentrations of 0.1 µM, 1.0 µM and 10 µM by using $CD4^+CD25^+$ cells from two different donors. Throughout study **IV**, mRNA samples were collected similarly after the incubation. First, the culture supernatants were gently removed with a Biomek FX

(Beckman Coulter, CA, USA) at the speed of 5 µl/second after centrifugation of the culture plates for 5 minutes at 800*g. The cells were then lysed with 50 µl of mRNA Lysis/Binding buffer of the Bio-Nobile QuickPick™ mRNA kit (Bio-Nobile Ltd., Turku, Finland) and stored at -80°C prior to mRNA extraction.

4.2.4 T-cell suppression assay

In study **I**, the suppressive capacity of $CD4^+CD25^+$ cells generated upon stimulation of naïve T cells with the type 1 condition was tested with the thymidin incorporation assay. Briefly, $CD4^+CD25^+$ and $CD4^+CD25^-$ effector cells were separated from T cells stimulated with type 1 cytokine conditions for 72 h using the magnetic bead separation technique MACS (Miltenyi Corp., Bergisch Gladbach, Germany). Fifty thousand $CD4^+CD25^-$ cells were stimulated with soluble anti-CD3 (5 µg/ml) and anti-CD28 (5 µg/ml) in the presence or absence of purified $CD4^+CD25^+$ cells at a ratio of 1:1 for 48 h. Tritiated thymidin was then added into the culture wells and the plates were incubated for 18h and harvested on a glass fibre mat with a Tomtec 93 Mach III manual cell culture harvester (Tomtec, Orange, CT, USA). The incorporated radioactivity was measured with a Micro-Beta scintillation counter (Wallac, Turku, Finland).

4.2.5 Flow cytometry analyses

In study **I**, flow cytometry was used for the analysis of cell surface molecules CD3, CD4, CD25 and the intranuclear molecule FOXP3. T cells stimulated in a type 1 cytokine environment for 72 h were harvested and fixed with 1% paraformaldehyde and 0.05% Tween-20 overnight at 4oC, and then treated twice with DNAse I (Roche Diagnostics, Mannheim, Germany). The cells were then incubated with monoclonal mouse-anti-human FOXP3 (clone 150D/E4) (a gift from Alison Banham, University of Oxford, UK), and Alexa-Fluor 488® goat anti-mouse IgG (Invitrogen). Intracellular IFN-γ was stained using mAb PE mouse anti-human IFN-γ (BD Pharmingen). After the staining of intracellular and nuclear molecules, cell surface antigens were stained. The cells were then incubated with PerCP anti-human CD4 (BD Pharmingen) and APC anti-human CD25 (BD Pharmingen) for 20 minutes at room temperature and washed with 0,5% BSA in PBS. Binding of antibodies to their target molecules was analyzed with FACSCalibur™ instrumentation and CellQuestTM software (Becton Dickinson, San Jose, CA, USA) was used to analyze the stained cells. In study **II**, approximately 300,000 cells were stained with APC-anti-CD4, PCP-anti-CD8, PE anti-IL-12R-β2 chain, PE-anti-CCR4, FITC- anti-CCR5 (BD PharMingen, San Jose ,CA), FITC-anti-CXCR4, FITC-anti-CXCR3, PE-anti-IL-18R, FITC-anti-CCR7, PE-anti-CCR2, FITC-anti-CCR3, and PE-anti-

CXCR6 (R&D Systems, Minneapolis, MN). Appropriate isotype controls were used to determine the specific binding for each fluorescent channel. Cells were incubated with conjugated antibodies for 30 min and then washed with PBS (Medicago, Uppsala, Sweden) supplemented with 0.5% BSA (Difco Laboratories, Detroit, MI). After a short centrifugation the cells were resuspended in 160 µl PBS supplemented with 0.5% BSA. The labeled cells were analyzed with four-color flow cytometry using FACSCalibur and CellQuest software (Becton Dickinson, San Jose, CA).

In study **III**, intracellular cytokines IFN-γ and IL-17 were analyzed in addition to the cell surface markers CD3, CD4, CCR4, CCR6, TCRα/β, and TCRγ/δ. Prior to harvesting cells for staining, GolgiStop reagent (BD Biosciences) was added for at least the last 4 h of T-cell activation of PBMCs. Collected PBMCs were washed once with 0.5% BSA in PBS and briefly centrifuged. The PBMCs were stained for surface antigens using anti-CD4-FITC (Becton Dickinson), anti-CCR4-PE (BD Pharmingen), anti-CCR6-PE (BD Pharmingen), anti-TCRα/β-FITC (Becton Dickinson), and anti-TCRγ/δ-FITC (Becton Dickinson). The cells were then fixed and permeabilized using the Cytofix/Cytoperm Plus Fixation and Permeabilization Kit (BD Biosciences). Intracellular IFN-γ and IL-17 were stained with anti-IFN-γ-PE (Becton Dickinson) and anti-IL-17-Alexa 647nm (eBioscience). Appropriate isotype controls were used to determine specific binding for each fluorescent channel: IgG1-FITC (Becton Dickinson), IgG1-PE (Becton Dickinson) and IgG1-Alexa 647nm (eBioscience). In study **IV**, the purity of the isolated $CD4^+CD25^+$ cells was analyzed by flow cytometry. Extracellular markers CD3, CD4, and CD25 were stained and detected similarly to study **I**.

4.2.6 β-cell viability and apoptosis assay

In study **III**, the proportion of necrotic and apoptotic cells was determined by nuclear double staining with 5 µg/ml Hoechst 33342 (HO, Sigma) and 5 µg/ml propidium iodide (PI, Sigma). After the incubation, the cells were exposed to the dyes for 30 min at 37°C, and then washed with PBS. The human islet cells were further dissociated with 0.05% trypsin and 0.02% EDTA and centrifuged to the slides before fixation. However, MIN6 cells on cover slips were directly fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. A fluorescence microscope was used to observe the staining of the cells and photographs were taken for later counting.

4.2.7 Cytokine analyses

In studies **I** and **II**, an in-house ELISA was used to analyze IFN-γ concentrations in the culture supernatants. In brief, Maxisorb plates (Nunc, Roskilde, Denmark) were coated with monoclonal anti-human IFN-γ (M-700 A; Endogen, Woburn, MA) at the concentration of 2 μg/ml (50μl/well). Plates were incubated overnight at +4°C, and washed with PBS-Tween. Plates were blocked in PBS containing 1% BSA for 30 min. Recombinant human IFN-γ (Pharmingen, San Diego, CA) was used to prepare a standard curve. Next, 100μl of supernatant samples and standards were added onto plates and incubated for 2 h. The plates were then washed with PBS-Tween and biotinylated monoclonal anti-human IFN-γ (Endogen) was added at the concentration of 0.5 μg/ml (50 μl/well). After incubation for 90 minutes, a streptavidin-alkaline phosphatase complex was added. For the color development, p-nitrophenyl phosphate (Medix, Kauniainen, Finland) was added and the plates were analyzed using a plate reader. In study **I**, a commercially available ELISA kit was used to assess the culture supernatant levels of IL-5 and TGF-β1 in concordance with the manufacturer's instructions (R&D Systems). A commercially available CBA kit (Cytometric Bead Array) was used to analyze the IL-10 levels of the culture supernatants in study **I** and IL-6 levels of the plasma in the study **III**.

4.2.8 Western blot analyses for T-bet and GATA-3

In study **II**, protein levels of transcription factors T-bet and GATA-3 were analyzed using the Western blot technique. First, total protein was isolated from the CVB4-stimulated PBMCs and 5 μg of total protein was run on a 10% SDS-polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA). Then the gel was blotted onto a polyvinylidene difluoride nitrocellulose blotting membrane (Amersham Biosciences UK, Buckinghamshire, U.K.). The membrane was blocked by incubating it for 1 h in PBS-Tween (Medicago) supplemented with 5% nonfat dried milk (Bio-Rad Laboratories) and 0.2% sodium fluoride (Merck, Damstadt, Germany). Antibodies against T-bet and GATA-3 (Both antibodies were obtained from the Santa-Cruz Biotechnology, CA, USA) were then added onto the membrane as 1:1000 dilutions followed by washes. The membrane was subsequently incubated with a 1:15000 dilution of the rabbit anti-mouse IgG-conjugated horseradish peroxidase (Bio-Rad laboratories). The membrane was blocked again as previously described. GAPDH antibody (final dilution 1:1,000; Santa Cruz Biotechnology) was then incubated with the membrane. The membrane was washed a few times and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (final dilution 1:15,000; Bio-Rad Laboratories). The proteins were detected with an ECL Plus kit (Amersham Biosciences) and chemiluminescence films (Amersham Bio-

sciences). The immunoblots were quantitated by densiometric scanning (Gel-PRO analyzer; Media Cybernetics, Silver Spring, MD).

4.2.9 RNA isolation methods for RT-qPCR

In study **I**, the chloroform/phenol extraction method TRIzol (Invitrogen) was used according to the manufacturer's instructions for the isolation of the total RNA from the stimulated T-cell cultures. In studies **II** and **III**, a column filtration technique was used for the isolation of total RNA from stimulated cell cultures and from the isolated monocytes. In study **II**, total RNA was isolated from the samples by using a Genelute total RNA isolation kit (Sigma-Aldrich, St. Louis, MO). In study **III**, total RNA from the cultured human PBMCs and $CD4^+$ memory T cells and human β cells was isolated with a Qiagen RNeasy Mini kit with the on-column DNase I treatment option of the RNA isolation kit. From the purified human monocytes, total RNA was isolated using a Qiagen RNeasy Micro Kit with the on-column DNase I option. In study **IV**, mRNA was isolated from stimulated $CD4^+CD25^+$ cells with QuickPick™ mRNA chemistry in accordance with the manufacturer's instructions (Bio-Nobile Ltd, Turku, Finland). Binding of mRNA molecules to oligo-dT-coated magnetic particles took place during a 10-min incubation in a shaker at the revs of 500 rpm, which was followed by two washes with wash buffer A in a volume of 100 µl and one wash with the wash buffer B. Finally, particles were eluted in 100 µl of Elution Buffer provided with the kit. All the particle transfers were carried out with a Magro 8-M automated robotic workstation (Bio-Nobile Ltd, Turku, Finland).

4.2.10 Reverse transcription of the isolated RNA

In studies **I** and **II**, isolated total RNA was subjected to additional DNase I (Roche Diagnostics, Mannheim, Germany) treatment to eliminate genomic DNA contamination from the sample. In studies **I-III**, first strand cDNA synthesis was primed using the random hexamer option of the High Capacity cDNA Archive kit according to manufacturer's instructions (Applied Biosystems, California, USA). In study **IV**, we designed reverse transcription utilizing solid phase elution of the mRNA. mRNA synthesis was primed using the oligo-dT priming option of the Transcriptor kit (Roche Corporation, Mannheim, Germany, Cat. No. 4379012), following the guidelines given by the manufacturer of the kit, with the exception that the concentration of oligo-dT was two times higher in the final reaction mix. Supplemental oligo-dT(15) was obtained from the Roche Corporation. (Cat. No.814270). Magnetic particles with the bound mRNA were spun slightly down (100*g for 1 minute) and a Biomek FX robotic workstation was used to remove the excess elution buffer. Next, 5 µl of elution buffer suspension containing the mRNA-magnet-

ic particle complexes was applied to the reverse transcription reaction already pipetted into the 96-well PCR. The RT reaction was performed in an MJ-200 thermal PCR cycler using Thermo-Fast 96 Skirted PCR plates (Cat. No.AB-0800-L, Abgene, Epsom, UK). Oligo-dT-primed reactions were incubated at +55˚C for 55 min and at +85˚C for 5 min. The cDNA was stored at -20˚C until use.

4.2.11 Quantitative PCR

In studies **I-IV**, readily designed and optimized TaqMan Gene Expression assays spanning over the exon-exon boundaries were used for the real time detection of the target gene cDNA amplification (see Table 1. for a list of genes). In studies **I-III**, an assay specific for the ribosomal 18s subunit was used as an endogenous control for the normalisation of the amount of RNA in the real time PCR. In study **IV,** a VIC-labeled assay specific for beta-2-microglobulin was used as an endogenous control. Assay for the amplification of beta-2-microglobulin was designed to span over exon-exon boundary. In studies **I, II** and **IV**, real time PCR was carried out according to the manufacturer's protocols using a TaqMan Universal PCR master mix with AMPerase UNG (Applied Biosystems). In study **III,** a TaqMan Fast Master Mix with no AMPerase UNG was used for the sequence-specific amplification. In studies **I** and **II**, an ABI Prism 7700 sequence detector instrumentation (Applied Biosystems) was used for signal detection, whereas a StepOne Plus real time PCR instrument (Applied Biosystems) was used for the detection in study **III** and an ABI 7900 HT real time PCR instrument was used for the detection in study **IV**. For the estimation of the original copy number at the beginning of each reaction, a comparative threshold cycle method (ΔΔCt –method) was used in studies **I-IV**. The quantitative value obtained from the TaqMan run is a threshold cycle Ct, indicating the number of PCR cycles at which the amount of an amplified target molecule exceeds a predefined fluorescence threshold. The difference value (ΔCt) is the normalised quantitative value of the expression level of the target gene achieved by subtracting the Ct value of the housekeeping gene from the Ct value of the target gene. Control cDNA pool (calibrator) was considered as an inter-assay standard, to which the normalised samples were compared. In studies **I-III**, calculations for the relative gene expression are expressed as follows: ΔΔCt(sample 1) =ΔCt(sample 1) – ΔCt(calibrator). The difference in expression level between the sample and the calibrator is given by the formula: $2^{(-\Delta\Delta Ct)}$. For graphical reasons relative expression levels were multiplied by a constant factor. The calibrator was made by preparing cDNA from RNA derived from PHA-stimulated PBMCs of healthy donors. In study **IV**, the mean ΔCt value of the anti-CD3 stimulated $CD4^+CD25^+$ culture wells (n=4 per plate) was considered as a reference within each plate. Relative FOXP3 expression levels of the anti-CD3 stimulated and chemical treated $CD4^+CD25^+$ cells were calculated with the formula: relative expression of

sample 1 $=2^{-[\Delta Ct(sample1) - \Delta Ct (mean\ anti\text{-}CD3)]}$.

4.2.12 Detection of antibodies

In study **II**, autoantibody concentrations of GAD and IA-2A in plasma were analyzed by immunoprecipitation as previously described [282, 283]. Positivity for GAD and tyrosine phosphatase autoantibodies was determined as antibody levels above the 98th percentile. The Diabetes Autoantibody Standardization Program 2003 was used to set the specificity and sensitivity levels. For the GAD autoantibody assay, the specificity was 98% and the sensitivity was 78%, and for the tyrosine phosphatase autoantibody assay, the specificity was 100% and the sensitivity was 48%.

CVB4 antibodies were determined by a microneutralization assay in green monkey kidney cells. First, 75 µl of the virus, corresponding to 30–300 TCID_50 units was mixed with 75 µl fourfold dilution of plasma specimens on microtiter plates (96-well Nunclon Microtest plates). The plates were incubated for 1 h at 36°C and then left overnight at 4°C. The next day, 30,000 cells were added to each well, and the plates were incubated at 36°C for 6 days before staining with crystal violet. The highest dilution with complete inhibition of the virus-induced cytopathic effect was considered as the end point titer.

4.2.13 Statistical analyses

In studies **I** and **II**, groupwise comparisons were performed using the non-parametric Kruskal-Wallis test. In studies **I-III**, pairwise comparisons were carried out by using the non-parametric Mann-Whitney U-test. Variable correlations were analyzed in studies I-IV with the Spearman rank correlation test. In study **III**, expression levels of the target gene mRNA in memory $CD4^+$cells were compared between the two groups with the two-tailed Fisher's exact test. The changes between untreated and cytokine treated human islets or MIN6 cells were analyzed with a paired t-test. In study **IV**, the changes in the expression level of FOXP3 upon chemical treatment of stimulated $CD4^+CD25^+$ cells were analyzed with the Wilcoxon Signed Rank test. Linear regression was used to analyze the relationship between variables in study **IV**. Statistical analyses were performed using Graph Pad Prism 4 software (San Diego, CA, USA).

5 Results and discussion

5.1 No differences in factors directing T-cell polarization in T1D

Naïve T cells from recently diagnosed type 1 patients, patients who had had T1D for one year and healthy control children were stimulated with phytohemagglutin (PHA) and polarized towards either the type 1 or type 2 phenotype with IL-12 or IL-4 cytokines, and neutralizing antibodies against IL-4 or IL-12 respectively. Higher IFN-γ secretion was seen in cells stimulated in the type 1 environment than in those stimulated in the type 2 environment (median values 296 ng/ml versus 0.3ng/ml). Higher IL-5 secretion was seen in cells stimulated in the type 2 environment than in those cells stimulated in the type 1 environment (median values 330 pg/ml versus 32 pg/ml). IFN-γ, IL-5, IL-10, and TGF-β1 secretion did not differ between diabetics and healthy controls.

Relative mRNA expression levels of Th1 and Th2 differentiation directing transcription factors in respective cell cultures indicated successful polarization. In the type 1 culturing condition, Th1-specific transcription factor T-bet was expressed at higher levels when compared to type 2 cell cultures (Figure 7. panel A vs. B). No difference in the expression of T-bet was observed between patients and healthy control subjects in T cells cultured in type 1 or in type 2 environments (Figure 7, panels A and B). In type 2 cell culture conditions, GATA-3 was expressed at a higher level when compared to type 1 cell culture conditions, as shown in Figure 7 (Panel C vs. D). No difference was observed between patients and healthy control children in GATA-3 expression in T cells cultured in the type 2 conditions (Figure7, panel D).

Prior to study, we studied the kinetics of the transcription factors T-bet, GATA-3 and FOXP3 in cell cultures with type 1 and type 2 cytokine environment. We chose the incubation time of 72h due to the observation that all three transcription factors showed maximal mRNA expression by the third day of cytokine stimulation.

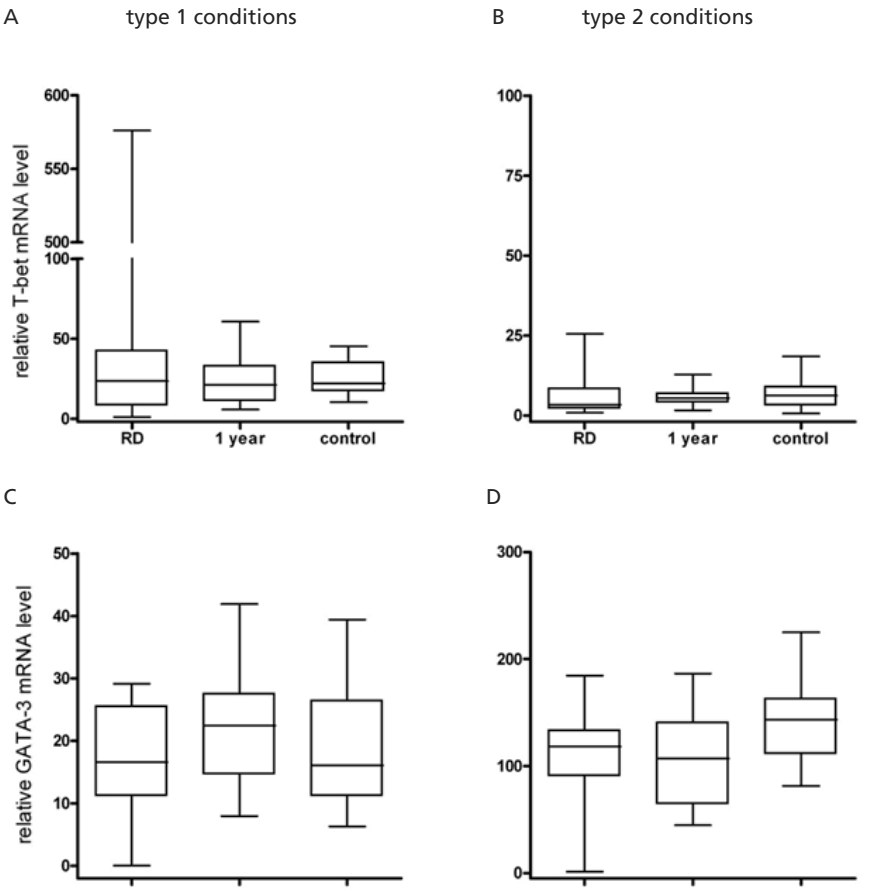

Figure 7. mRNA expression levels of T-bet and GATA-3 in T cells cultured in type 1 (panels A and C) or type 2 (panels B and D) environment from patients with recently diagnosed T1D (RD), patients with long-lasting T1D (1 year), and in healthy controls (control). No difference in factors directing Th1 and Th2 polarization was seen when T1D patients were compared with healthy children. Data are shown as box plots (median, 25% and 75% quartile with whiskers denoting maximal and minimal data points) and expressed as arbitrary relative units.

T1D is has been considered to be an organ-specific autoimmune disease mediated by uncontrolled Th1 responses. Animal models of T1D have shown increased IFN-γ levels to be associated with the inflammation process in the pancreas. However, we did not observe up-regulation of factors related to Th1-type responses in patients with T1D, when naïve T cells were activated with PHA and stimulated with IL-12 and anti-IL-4. Patients with T1D and healthy control children similarily up-regulated the Th1-related factors T-bet and IFN-γ upon stimulation, indicating normal T-cell activation in T1D patients. No difference in the expression of Th2-

related markers GATA-3 and IL-5 was observed in type 1 or type 2 cells generated *in vitro*, further supporting the normal T-cell activation in T1D patients.

5.2 Impaired up-regulation of Treg-associated genes in T cells of T1D patients stimulated in a type 1 cytokine environment

The T cells of patients with newly diagnosed T1D stimulated in a type 1 cytokine environment showed decreased expression of FOXP3 when compared to healthy controls, with median values of 22.2 versus 38.6 (Figure 8, panel B). A similar trend was seen when comparing patients with longer duration T1D and healthy control children (Figure 8, panel B). The differences in FOXP3 expression between the patients with newly diagnosed T1D, patients with longer-duration T1D, and healthy control children were already seen in naïve T cells, the median values being 3.8 versus 9.8 versus 8.0 respectively (Figure 8, panel A).

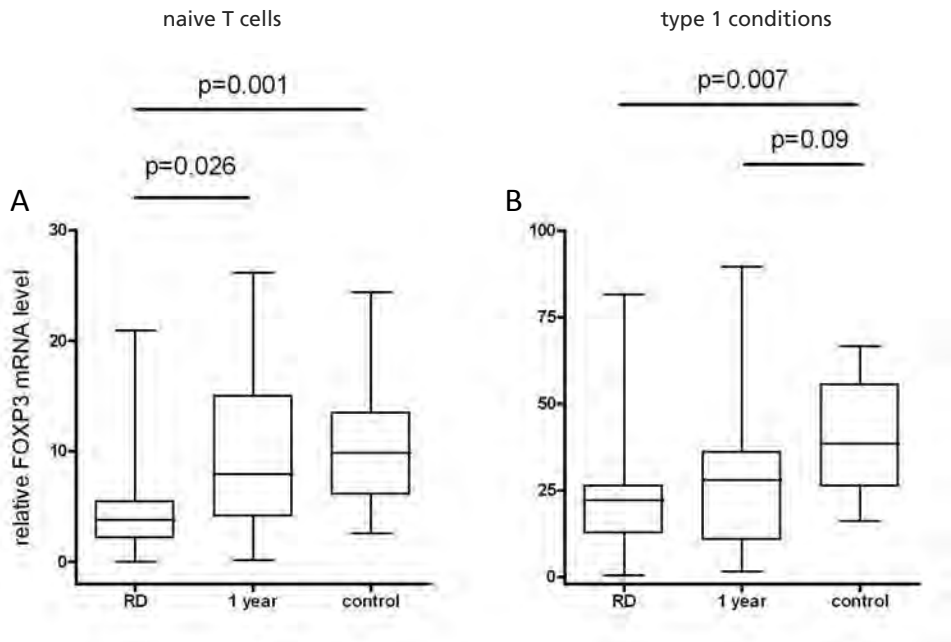

Figure 8. mRNA expression levels of FOXP3 (panels A and B) in naïve T cells (panel A), and in T cells cultured in a type 1 environment from patients with recently diagnosed T1D, patients with long-lasting T1D and in healthy controls. The expression of FOXP3 differed between the three groups in naïve T-cells and in T cells activated in a type 1 cytokine environment (p=0.003 and p=0.02 Kruskall-Wallis test). Significant p-values for comparisons between two groups are shown in the figure. Data are shown as box plots (median, 25% and 75% quartile with whiskers denoting maximal and minimal data points) and expressed as arbitrary relative units.

In T cells stimulated in a type 1 cytokine environment, inducible co-stimulator (ICOS) showed higher expression in patients with recent-onset T1D than in healthy children, the median values being 50.8 versus 91.6 (p=0.001). No difference in ICOS expression was observed between patients with longer-duration T1D and healthy children. Higher expression of NFATc2 was observed in type 1 cytokine stimulated T cells of healthy control children than in the T cells of patients with recent-onset T1D, with median values of 7.8 versus 3.8 (p=0.02). NFATc2 showed no difference in the expression in T cells stimulated by type 1 cytokines between healthy control children and patients with T1D of prolonged duration, with median values 7.8 versus 5.1. Between 3-8% of $CD4^+CD25^{high}$ gated cells showed FOXP3 protein expression in a subgroup of T cells stimulated in a type 1 cytokine environment (Figure 9).

The $CD4^+CD25^+$ cells isolated from the T cells stimulated in a type 1 cytokine environment showed inhibition of proliferation in one of six individuals studied, when co-cultures of $CD4^+CD25^+$ and $CD4^+CD25^-$ cells mixed at ratio 1:1 were stimulated with mitogen.

FOXP3 is a signature transcription factor for Tregs, which have a central role supporting peripheral tolerance towards the body's own structures. Our observation of decreased FOXP3 in diabetic patients in a type 1 cytokine milieu suggests that mechanisms mediating the induction and maintenance of peripheral toler-

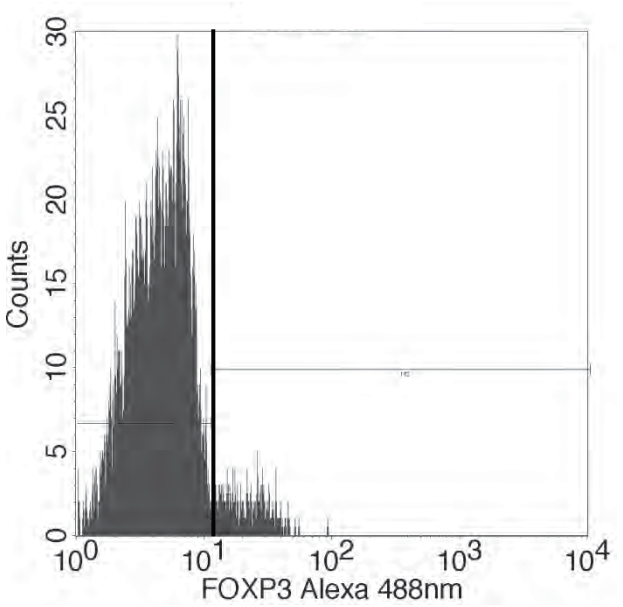

Figure 9. Flow cytometry analysis of FOXP3 in $CD4^+CD25^{hi}$ cells generated from naïve T cells in a type 1 cytokine stimulation for 72h.

ance may be disrupted by the cytotoxic response associated with tissue destruction in the pancreas. Tregs have been considered to mediate the anti-inflammatory response by inhibiting the proliferation of effector T cells by secreting inhibitory cytokines TGF-β1 and IL-10, but also by delivering an inhibitory signal via direct cell-cell contact. In direct cell-cell contact, cell surface molecules such as CTLA-4 and ICOS mediate the down-regulatory signal to the effector T cells.

Recent studies have suggested FOXP3 to down-regulate TCR-mediated T-cell activation leading to the hyporesponsiveness of activated T cells [284, 285]. This mechanism negatively down-regulates T-cell responses after Ag-specific activation, limiting collateral tissue damage. TCR engagement leads to up-regulation of the NFATc2 transcription factor [286]. Together with general transcription factors, NFATc2 binds to the FOXP3 promoter region and promotes FOXP3 transcription. An increase in the concentration of FOXP3-specific transcripts eventually leads to the activation of negative feedback loop, and thus to the down-regulation of NFATc2 and FOXP3 [287, 288]. In patients with T1D, the type 1 cytokine environment seems to inhibit the up-regulation of NFATc2. This provides a possible explanation for the decreased FOXP3 expression; NFATc2 levels in T1D patients do not support the transcription of FOXP3 at similar levels as in healthy children.We also found decreased induction of ICOS in T cells stimulated in a type 1 cytokine environment *in vitro*. Diabetic patients already showed decreased ICOS mRNA levels in the naïve T -cell population. According to a study by Mesturini et al., ICOS favors the induction and differentiation of Th effector cells when CD3 and CD28 or an IL-2 stimulus is present whereas a sub-optimal stimulus would support the differentiation of Treg cells [153]. We could not find a difference in TGF-β1 or IL-10 secretion between the study groups. This may reflect the relatively early time point of the T-cell culture originally designed for the detection of Th–subtype-specific transcription factors at the mRNA level. In mice, ICOS-dependent Treg functioning in the pancreas during insulitis has been described. In BDC2.5 TCR transgenic mice, the inhibition of ICOS accelerates the disease process and turns insulitis into overt diabetes [289]. During the insulitis, both effector T cells and Tregs are infiltrated in the inflamed tissue, and the dynamic balance of these two opposing Th subsets dictates the outcome of the pathogenesis process. This balance is disturbed if ICOS signaling is lost, and ICOS-mediated IL-10 production is decreased in the pancreatic lesions. ICOS-dependent IL-10 regulation has been shown in airway hypersensitivity model. Antigen-specific Treg induction is also dependent on the ICOS-ICOS-ligand interaction [154]. However, contrasting reports on the role of ICOS in the pathogenesis of T1D do exist. Hawiger et al. reported in 2008 that ICOS is the mediator of the β-cell destruction rather than the mediator of tolerance in pancreatic lesions [290]. In a study by Hawiger et al. heterozygous wild type mice developed β-cell autoimmunity more aggressively than ICOS deficient littermates. However, the design of this study does not only focus on the role of ICOS function in Tregs but on other cell types as well. A dynamic and precise bal-

ance between co-stimulatory pathways is crucial for the phenotype development of the activated T cells, and manipulations leading to a total lack of some factor of the scheme may cause an aberrant response. Thus, the role of ICOS in the induction and maintenance of regulatory mechanisms in human T1D needs to be further addressed.

The function of $FOXP3^+$ Tregs has been linked to inhibition of the proliferation of effector T cells. $CD4^+CD25^+$ cells purified from the type 1 cells generated *in vitro* only showed an inhibitory function in one of the six individuals studied. Isolated cell populations are inevitably heterogeneous, and the proportion of $FOXP3^+$ Treg cells in the $CD4^+CD25^+$ cell population may not be sufficient to produce efficient inhibition. Another possible explanation for the poor inhibition potential of the $CD4^+CD25^+$ T cells might be that FOXP3 expression upon stimulation is functionally related to down-modulation of TCR-mediated activation, instead of promoting an inhibition function in the cells expressing FOXP3. Thus, the expression of FOXP3 may be insufficient to induce 'classic' regulatory T cells or to identify them [284].

The evidence provided so far on the role of Tregs in T1D is controversial. The function of Tregs has been reported to be altered in human T1D [277, 291]. Lindley and associates have shown defects in patients with newly diagnosed T1D as incompetence in inhibiting the proliferation of effector T cells upon stimulation. IFN-γ secretion was also higher in co-cultures prepared using $CD4^+CD25^-$ and $CD4^+CD25^+$ cells from diabetic patients. In addition, IL-10 secretion was decreased in T1D patients in the co-cultures. However, Putnam et al. have reported the proportion and function of Tregs to be normal in T1D [278].

Our data show a decreased expression of Treg-related markers in the naïve T cell population. A circulating population of naïve Tregs (abbreviated as NnTreg by the authors) have been characterized [292]. These cells are nTreg precursors, cells that have met their specific antigen in the thymus. In contrast to nTregs, natural naive Tregs are able to proliferate in response to autologous APCs, indicating autoreactive T cells to be present in the population of NnTregs.

Thus, we conclude that poor induction of Treg-associated genes FOXP3, NFATc2 and ICOS upon TCR mediated activation of T cells in the type 1 cytokine environment may contribute to the pathogenesis of T1D.

5.3 Impaired Th1 response against coxsackievirus B4 in T1D

When PBMCs were stimulated with CVB4, children with T1D showed less IFN-γ secretion than healthy children with or without HLA risk genotype (Figure 10) , a hallmark cytokine for type 1 cells [293].

In our CVB4 stimulation of PBMCs children with T1D showed impaired up-regulation of T-bet measured at the mRNA level when compared to healthy children with (Figure 11) or without HLA risk genotype (Figure 11). However, when the T-bet protein was measured, children without HLA risk genotype showed higher T-bet levels (p=0.048) than healthy children, but only a slight tendency was observed between healthy children with HLA risk and diabetic children.

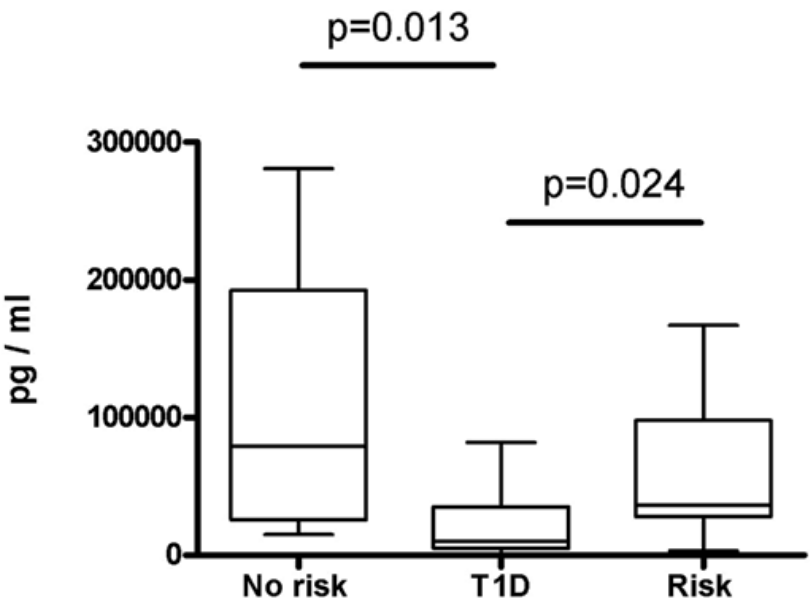

Figure 10. Secretion of IFN-γ from PBMCs stimulated with CVB4 for 7 days in healthy children with no HLA risk genotype, children with T1D, and healthy children with HLA risk genotype. P-value was calculated with Mann-Whitney U-test. Data are shown as box plots (median, 25% and 75% quartile with whiskers denoting maximal and minimal data points) and expressed as pg/ml.

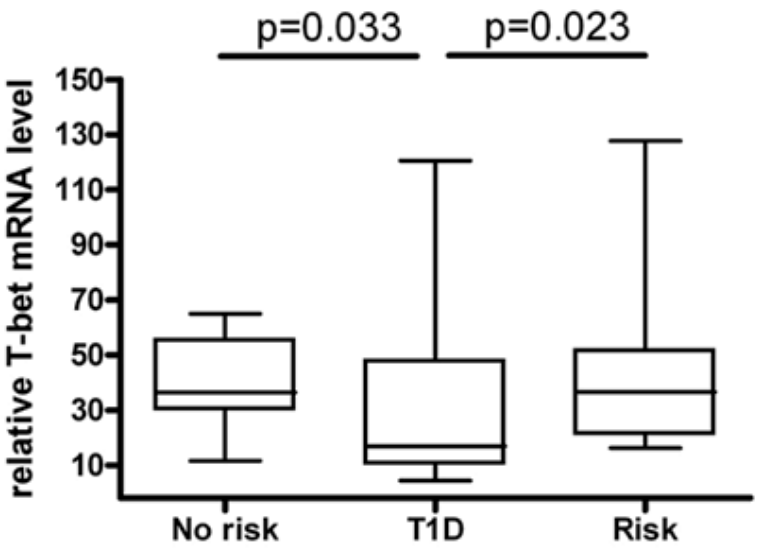

Figure 11. mRNA expression of T-bet in PBMCs stimulated with CVB4 for 7 days from healthy children without HLA risk genotype, children with T1D, and healthy children with HLA risk genotype. Data are shown as box plots (median, 25% and 75% quartile with whiskers denoting maximal and minimal data points) and expressed as arbitrary relative units.

CXCR6 showed lower expression in $CD4^+$ and $CD8^+$ T cells in children with T1D than in healthy children with (p=0.043 and p=0.024) or without HLA-risk genotype (p=0.022 and p=0.014). In addition, a lower proportion of $CD8^+$ T cells expressing IL-12R was observed in patients with T1D in comparison to healthy children without HLA risk (p=0.026).

Our data imply that patients with T1D have an impaired response against CVB4 *in vitro*. Diabetic children showed impaired up-regulation of factors closely related to the induction and maintenance of cytotoxic responses, when their PBMCs were cultured with CVB4. This may cause a slow or incomplete eradication of CVB4, and the virus may spread throughout the body. Thus, children with T1D may be more susceptible to CVB4 infections, and they may have coxsackieviruses infecting pancreatic β cells more often than healthy children. The type 1 response is elementary in the eradication of viruses from the human body. Moreover, efficient virus clearance from infected tissue demands interaction with chemokines and chemokine receptors of Th1 and cytotoxic type 1 $CD8^+$ cells (Tc1), allowing these cells to migrate to the site of inflammation. The induction of IFN-γ expression is regulated by the transcription factor T-bet. Up-regulation of IL-12Rβ2 by T-bet further strengthens the development of the type 1 phenotype of activated T

cells, rapidly leading to the establishment of fully committed type 1 T cells. IFN-γ production is synergistically induced by the engagement of IL-12 receptors. Low expression of T-bet may be responsible for the reduced percentage of T cells expressing the IL-12Rβ2 chain. Our finding of lowered T-bet expression in the PBMCs of T1D patients, but not in healthy control children, refers to impaired virus responses in T1D patients. CVB4 induces the expression of IFN-γ, IL-1β, TNF-α, IL-2, and IL-10 in healthy donor PBMCs [294]. Proper interferon signaling has been shown to be crucial for β-cell protection in CVB4 infection [295].

General impairment in the generation of the type 1 response generation *in vitro* has been observed in T1D in some studies. Reduced levels of intracellular IFN-γ in $CD4^+$ T cells was reported in a study by Kukreja et al., when PBMCs of both recent-onset and long-lasting T1D patients were stimulated with PMA and ionomycin [271]. In another study, decreased IFN-γ and IL-10 secretion of PHA-stimulated PBMCs was observed in children and young adults with T1D in comparison to healthy control subjects [296]. Fresh PBMCs of children with newly diagnosed T1D showed decreased secretion of IFN-γ and TNF-α in a study by Lohmann et al. [297]. In addition, anti-CD3 and antiCD28 stimulation of effector T cells resulted in a decreased production of IFN-γ in T1D patients when compared to healthy controls [291]. HLA loci determine approximately 50% of the genetic risk of T1D. HLA molecules play a central role in the presentation of antigens to $CD4^+$ and $CD8^+$ T cells. It has been shown that HLA molecules modify the outcome of T-cell responses against different pathogens and antigen structures. The HLA haplotype is known to modify immune responses against CVB4. Children positive for HLA-DR3 showed a decreased frequency of T lymphocytes responding to CVB4, whereas HLA-DR4-positive children showed an increased frequency of T cells responding to CVB4 [298]. Sadeharju et al. have shown that children with the T1D risk haplotype HLA-DR3 and/or HLA-DR4 have a higher antibody response towards CVB4 than healthy children with the protective HLA-DR2 haplotype [299]. This may mirror the decreased type 1 responses in T1D patients, and thus the increased activity of Th2 cells providing help for B cells. However, in our study, no differences were seen between healthy children with or without the HLA risk genotype regarding the expression levels of markers associated with type 1 responses. However, a difference was seen between HLA-DR4-DQ8 matched diabetic patients and healthy control children. In accordance with the results of our study, Juhela et al. have shown a decreased proliferation of T cells against purified CVB4 in children with T1D compared with healthy control children [300].

We conclude that children with T1D appear to have an impaired immune response against CVB4. A poor type-1 response seems not to be associated with HLA risk genotypes at least *in vitro*. Children with T1D may be more susceptible to subtle and prolonged CVB4 infections, and thus an increased frequency and proportion of β-cell death. This may cause the priming of autoreactive T cells, and thus β-cell autoimmunity in the pancreatic lymph nodes via antigen presentation by the

APCs. Whether the impaired type 1 responses are restricted to CVB4, or whether it is an impairment of antigen-specific activation in general needs to be further evaluated.

Enteroviruses, and particularly coxsackieviruses, are considered as one possible environmental trigger in the pathogenesis of T1D. Enteroviruses were associated with T1D for the first time in 1969, when patients with recent-onset T1D were shown to have higher antibody titers against the coxsackievirus B4 strain than unaffected control subjects [240]. Additionally, enterovirus infections have been associated with the appearance of β-cell autoimmunity [301, 302]. Several studies have shown children with T1D and prediabetic children to more often have coxsackievirus RNA or antibodies against coxsackieviruses in their blood circulation [239, 240, 242, 244-248]. Thus, children with T1D or at genetic risk of T1D may be more prone to coxsackievirus infections. The role of coxsackieviruses in the course of T1D is further supported by reports that show CVB4 being able to infect human β cells both *in vitro* [249] and *in vivo* [250], which may in turn induce the β-cell autoimmunity or support it.

5.4 Increased Th17 activation in human T1D

When PBMCs from T1D patients and healthy children were stimulated with anti-CD3 and anti-CD28, enhanced IL-17 secretion (Figure 12, panel A) in children with T1D was observed. IL-17 secretion correlated strongly with the measured mRNA levels of IL-17 in T1D patients.

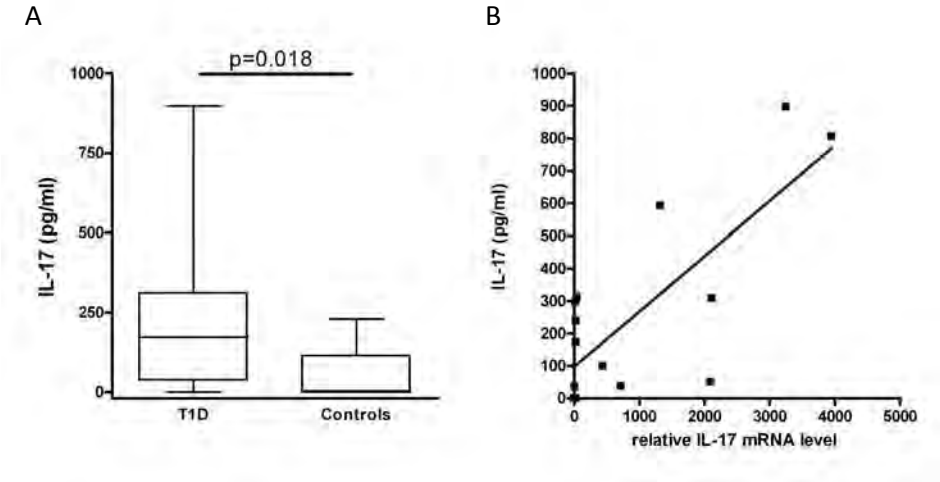

Figure 12. Overwhelming IL-17 secretion upon T-cell activation in human T1D. IL-17 levels in culture supernatant samples (panel A). IL-17 protein levels and IL-17 mRNA levels correlated strongly in stimulated PBMCs from T1D patients (panel B) (Spearman R=0.72, p=0.0026). For pairwise comparisons, p -values were calculated with the Mann-Whitney U-test. The correlation co-efficient of IL-17 protein levels and IL-17 mRNA levels was calculated using the Spearmank rank correlation test. On the panel A data are shown as box plots (median, 25% and 75% quartile with whiskers denoting maximal and minimal data points) and expressed as pg/ml.

Transcription factor RORC2 also showed higher mRNA expression in stimulated PBMCs of T1D patients than healthy control children (Figure 13, panel A). Similarly, Treg-associated transcription factor FOXP3 was expressed at higher levels in response to T-cell activation in children with T1D than in healthy children (Figure 13, panel B). The levels of RORC2 and IL-17 transcripts induced upon T-cell activation correlated with secreted IL-17 in children with T1D (rho=0.599, p=0.018 and rho=0.717, p=0.003). Th17-associated cytokine IL-22 was expressed at higher levels in stimulated PBMCs of T1D patients in comparison to healthy children. The induction of IL-22 has been reported to be Ahr-dependent. However, we did not find a difference between the study groups in the expression of Ahr in stimulated PBMCs (data not shown).

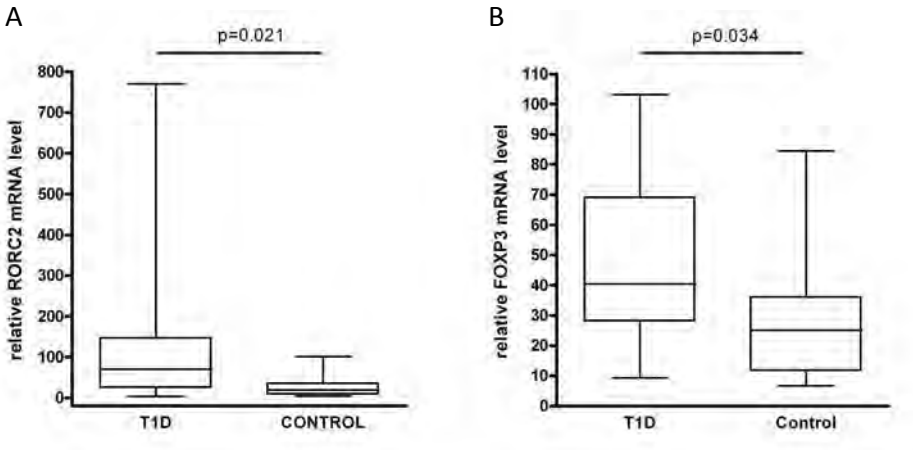

Figure 13. Upon stimulation, PBMCs of diabetic children showed increased up-regulation of Th17 signature transcription factor RORC2 (panel A) and Treg signature transcription factor FOXP3 (panel B). p -values were calculated with the Mann-Whitney U-test. Data are shown as box plots (median, 25% and 75% quartile with whiskers denoting maximal and minimal data points) and expressed as arbitrary relative units.

IFN-γ and T-bet mRNA up-regulation in diabetic and healthy children did not differ between the study groups. The existence of IL-17 and IFN-γ co-producing T cells has been reported in autoimmune diseases [303], and we thus examined the relation between the IL-17 and IFN-γ response in T1D. We found that in children with T1D, the IFN-γ response, both at the mRNA and protein level, correlated with IL-17A secretion of the anti-CD3 and anti-CD28 stimulated PBMCs (rho=0.652, p=0.006 and rho=0.735, p=0.002).

Anti-CD3 and anti-CD28 stimulation of T cells caused an overwhelming Th17 response in children with T1D, but not in healthy children. To determine whether increased Th17 activity could be seen *in vivo* in patients with T1D, we examined the mRNA levels of Th17-related genes from the circulating memory $CD4^+$ cells of nine children with T1D and eight healthy children. IL-17A and IL-22 mRNA were expressed at higher levels in the population of freshly purified peripheral blood $CD45RO^+CD4^+$ memory cells from children with T1D. Minimal or undetectable expression levels were seen in healthy children (6/8 vs 0/8, p=0.007 for IL-17 and 5/8 vs 0/7, p=0.026 for IL-22).

IFN-γ mRNA expression in $CD45RO^+CD4^+$ cells showed no difference between T1D patients and healthy children. Higher mRNA levels of FOXP3 were observed in the $CD45RO^+CD4^+$ cell compartment of T1D patients when compared to healthy children (5/8 vs. 0/8, p=0.026).

According to flow cytometry analysis, PBMC stimulation with anti-CD3 and anti-CD28 increased the percentage of $CD4^+$ T cells positive for IL-17, whereas $CD4^-$ T cells positive for IL-17 were rarely observed (Figure 14).

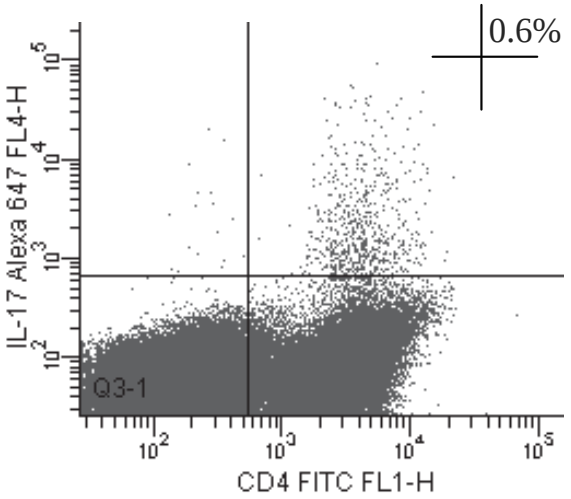

Figure 14. Phenotype characterization of Th17 cells generated upon T-cell stimulation. An increase in IL-17-positive cells was observed most often among $CD4^+$ cells, whereas $CD4^-$ T cells did not show IL-17 production.

Over 90% of the $CD4^+$ T cells expressing IL-17 upon stimulation were also expressing CCR6 (Figure 15, panel B, median 95.2%, range 81-99.4%: n=7 diabetic children). IL-17-positive $CD4^+$ T cells were $TCR\alpha\beta^+$ (Figure 15, panel D).

When anti-CD3 and anti-CD28 stimulated PBMCs of T1D patients were studied by flow cytometry regarding the expression of IL-17 and IFN-γ, double-positive cells were observed in 4 children out of 11 (Figure 16, panel B). We did not screen systematically healthy children for IL-17 and IFN-γ double-positive cells.

Th17 immunity has been associated with the development of autoimmune diabetes in animal models. In NOD mice elevated IL-17 transcript levels were found to correlate with the progression of insulitis to diabetes both in the pancreas and blood circulation [273]. It has also been shown that in NOD mice the inhibition of Th17 cells controls autoimmune diabetes, but the role of IL-17 immunity seems to be associated rather with the progression of the diabetic disease process during β-cell destruction than with the initiation of β-cell autoimmunity [275, 304]. Convincing evidence for the role of Th17 cells in the pathogenesis exists from animal models of T1D. However, direct evidence for the role of Th17 cells in human T1D is so far scarce. Anti-CD3 and anti-CD28 stimulation of PBMCs from T1D patients significantly up-regulated IL-17 at both the mRNA and protein level in association with Th17-specific transcription factor RORC2 and Th17-associated cytokine IL-22 when compared to healthy children. A similar difference was also seen at the mRNA level in $CD4^+CD45RO^+$ memory cells of T1D patients in comparison

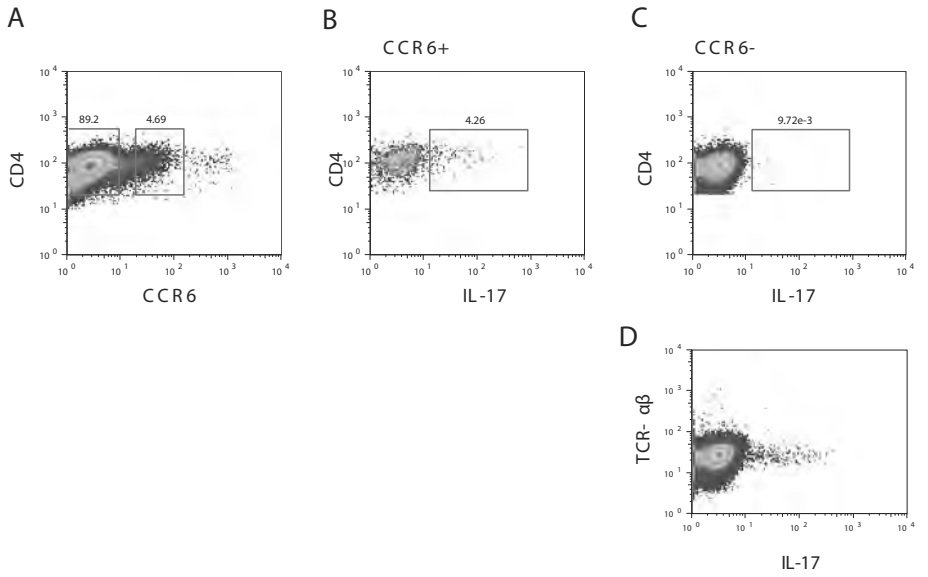

Figure 15. Phenotype analysis of IL-17-positive cells in T1D. Gating strategy for CCR6$^-$ and CCR6$^+$ CD4 cells (panel A). A representative dot plot of IL-17-expressing cells among CD4+ CCR6$^+$ cells (panel B) and CD4$^+$CCR6$^-$ cells (panel C) from anti-CD3 plus anti-CD28 stimulated peripheral blood mononuclear cells from a child with newly diagnosed T1D. (panel D) A representative dot plot of anti-CD3 plus anti-CD28 stimulated peripheral blood mononuclear cells from a child with newly diagnosed T1D showing that CD4$^+$ T cells expressing IL-17 are TCRαβ positive.

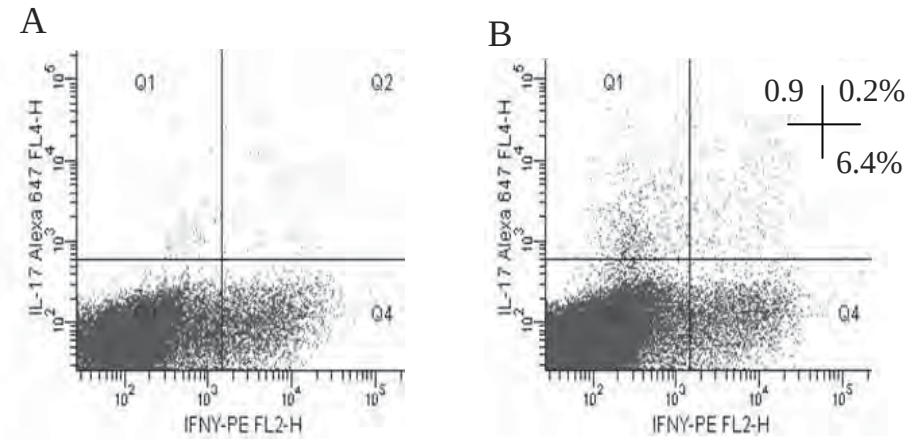

Figure 16. Phenotype analysis of IL-17-positive cells in T1D. IL-17 and IFN-γ-positive cells in CD4-gated lymphocytes in unstimulated (panel A) and anti-CD3 plus anti-CD28 stimulated (panel B) peripheral blood mono-nuclear cells from a representative child with T1D.

to those of healthy children, suggesting that the activation of IL-17/IL-22 pathways is a major alteration in T1D.

Th17 immunity is considered as an important function of the mucosal barrier, and the activation of Th17 cells provides protection against bacterial and fungal infections [204]. In this function, the IL-22 cytokine has a central role. IL-22 is preferentially expressed by the Th17 cells, both in the early stages of polarization of naïve $CD4^+$ T cells towards the Th17 phenotype, and when Th17 cells are restimulated. IL-22 acts directly on epithelial cells, and some fibroblast cells in peripheral tissue also respond to IL-22 and it induces an acute phase response both *in vitro* and *in vivo* [305-310]. Thus, IL-22 seems to coordinate innate immunity, but it also contributes to the modulation of tissue organization, and this may be an important function of IL-22 in respect to autoimmune diseases. IL-22 in conjunction with IL-17 synergistically induces the expression of antimicrobial peptides [311]. The IL-22 receptor is not expressed on immune cells, and thus the IL-22 cytokine is used for communication between immune cells and tissues. There is no direct evidence for the role of IL-22 in human T1D. However, a recent report has shown that IL-22R1 is expressed in pancreatic β cells. Thus, T-cell-mediated immunity may modulate the function of human pancreatic islets via IL-22 signaling [312]. We observed higher expression of IL-22 in stimulated PBMCs of T1D patients. In animal models, the induction of IL-22 has been reported to be Ahr-dependent [199, 200]. When we measured Ahr levels from the stimulated PBMCs, we could see no difference in the mRNA levels of Ahr between T1D patients and healthy children (Figure 14, panel B). Thus, the difference we observed in the IL-22 expression levels may not be explained by abnormalities in signaling pathways associated with the Ahr expression. However, functional abnormalities in transcriptional complex regulating IL-22 gene expression, which Ahr is a part of, may still be involved in the pathogenesis of T1D, and need to be evaluated in future studies.

Interestingly, in addition to higher expression of IL-17, RORC2, and IL-22 we also observed increased expression of FOXP3 transcripts in both activated T cells and memory $CD4^+$ cells, while IFN-γ and transcription factor T-bet showed similar expression both in stimulated PBMCs and memory T cell compartment levels when T1D patients were compared to healthy children. This suggests simultaneous up-regulation of regulatory mechanisms and the IL-17 pathway in T1D. Tregs may lose their ability to express FOXP3, and begin to secrete inflammatory cytokines IFN-γ or IL-17, blurring the picture of the Treg/Th17 scheme even more. FOXP3 is a key molecule in maintaining the regulatory functions of $CD4^+$ T cells. If FOXP3 expression is lost, effector functions may be promoted in the so-called ex-regulatory T cells. These exFOXP3 cells, which formed a significantly higher percentage in the autoimmune diabetes setting, were able to transfer diabetes [7]. Accumulating evidence shows that human Tregs may differentiate into IL-17-producing cells [313-315]. Some studies suggest that pathogenic T cells in autoimmune conditions are co-producers of IL-17 and IFN-γ, or that a shift from Th17 to Th1 cells makes

IL-17 cells pathogenic [6, 303]. Highly purified Th17 cells with specificity for an islet cell antigen were diabetogenic when converted into IFN-γ secreting Th1-like cells [5]. Because co-producers of IL-17 and IFN-γ have been observed in autoimmune diseases, we examined the relation between the IL-17 and IFN-γ response in T1D. When PBMCs from diabetic patients were stimulated, simultaneous protein expression of IFN-γ and IL-17 was seen in a small proportion of $CD4^+$ T cells. It seems that in T1D some $CD4^+IL\text{-}17^+$cells are also IFN-γ co-producers. The production of IL-17 and IFN-γ by the same T-cell population in children with T1D further suggests that at least some of the IL-17-secreting T-cells associated with T1D are not functionally committed T-cells.

TGF-β1 has been thought to be primarily an anti-inflammatory cytokine. TGF-β1 induces the expression of FOXP3 *in vitro*, and thus differentiates activated naïve T cells to Tregs. Recent progress in the field of T-cell biology has brought controversial knowledge on the role of TGF-β1. On the other hand, TGF-β1 has broad inhibitory effects on the immune system, but it is absolutely required for the induction of a pro-inflammatory response to eradicate certain bacteria and fungi. The reciprocal relationship between Tregs and Th17 cells was first demonstrated by Bettelli et al. [185], and further elucidated by Littman and colleagues [191]. Although TGF-β1 induces FOXP3 expression in naïve T cells, IL-21 inhibits this regulation and together with TGF-β1 drive the development of Th17 cells. In synergy with TGF-β1, IL-1β, and IL-6 drives the re-call of memory Th17 cells [193]. The role of Th17 immunity in human T1D was indirectly supported by the observation that monocytes from patients with T1D showed spontaneous activation of IL-6 and IL-1β, and the monocytes isolated from diabetic patients were able to induce IL-17 deviation in allogenic memory T-cells *in vitro* [276]. However, we could find no differences in IL-6 levels when the plasma of T1D patients and healthy children was analyzed. To further characterize the possible role of IL-6 and IL-1β in T1D, we analyzed the mRNA levels of IL-6 and IL-1β of the eight T1D patients and eight non-related auto-antibody negative siblings of T1D patients. We did not confirm the up-regulation of IL-6 and IL-1β in T1D patients. Contradictory results regarding the IL-6 levels may be explained by the age composition of the study groups, since our study only included children under the age of 15, whereas the study by Bradshaw et. al. also included adult subjects [276].

The IL-17 producing $CD4^+$ T cells in children with T1D expressed CCR6, an established chemokine receptor for Th17 cells [316, 317]. Peripheral blood and lymphoid tissue-derived CCR6-expressing Tregs have been shown to produce IL-17 upon activation [318]. CCR6 is typically expressed on T lymphocytes showing homing properties to mucosal surfaces [316, 317]. Th17 immunity is considered as an important function of the mucosal barrier, and the activation of Th17 cells provides protection against bacterial and fungal infections [204]. Overwhelming activation of Th17 immunity has been associated with autoimmune conditions and inflammatory bowel disease [319]. Both human and animal models of T1D

have shown that lymphocytes infiltrating into the pancreas express mucosal homing properties [320-322]. Aberrancies of the gut immune system, such as subclinical intestinal inflammation [267, 323] and increased gut permeability [324, 325], have also been associated with human T1D. At least TCRγ/δ-expressing T cells have been shown to be IL-17 producers in mice [326, 327]. However, in children with T1D, IL-17-expressing $CD4^+$ T cells were positive for TCRα/β. Nevertheless, aberrant mucosal immunity may be involved in the course of T1D. Factors contributing to the regulation of the mucosal barrier in the gut are probably the next interesting field in T1D research. For example, the effect of environmental toxins on the function of the mucosal immune system may play a part in T1D pathogenesis. It has already been shown that the Treg/Th17 balance can be modulated by dioxin via a mechanism mediated by Ahr [199, 200], providing a link between environmental pollutants and altered immune regulation [200].

Tregs expressing CCR6 can produce IL-17 upon activation and simultaneously express both FOXP3 and RORC2 [318]. Interestingly Tregs induced to express IL-17 are still potent inhibitors of the proliferation of $CD4^+$ effectors. It has been demonstrated that CCR6-negative FOXP3-expressing cells can differentiate into IL-17-producing cells when stimulated and treated with exogenous IL-2 and IL-15. The differentiation could be further enhanced if additional IL-1β, IL-23 or IL-21 were also present [315].

We observed overwhelming upregulation of Th17 immunity in T1D patients upon T cell stimulation. According to flow cytometry analysis, IL-17 was produced in $CD4^+$ T cells, and not by $CD8^+$ cells, or other cell types. Our data on isolated memory $CD4^+$ cells support the view that memory T cells, and not naive differentiating cells were the origin of upregulated Th17 immunity. We used 'classical' anti-$CD3^+$ antiCD28 stimulation for the activation of PBMC. The relatively short incubation time of 40h may not be long enough to result in differentiation of IL-17 secreting cells with $CCR6^+$ expression. Simultaneous up-regulation of FOXP3 and Th17-related genes suggests that dysregulation of the balance of Tregs and Th17 cells may play a role in T1D pathogenesis. Alternatively, FOXP3 upregulation may be feedback activation of regulatory mechanisms as response to overwhelming Th17 immunity in patients with T1D. In conclusion, our results indicate that dysregulated Th17 activation may be involved in T1D pathogenesis.

5.5 IL-17 effects on pancreatic islets

Up-regulation of IL-17 related genes upon T-cell stimulation in T1D patients raised the question of the effects of IL-17 on pancreatic β cells. The effects of IL-17 on human β cells *in vitro* were studied as such or in combination with other inflammatory mediators, namely IL-1β and IFN-γ. Both IL-17 receptor variant A and C mRNA transcripts were detected in human islet RNA preparations by RT-qPCR.

IL-17 treatment up-regulated the transcription of superoxide dismutase (SOD2), and down-regulated the transcription of anti-apoptotic gene BCL-2 (Figure 17, panel A) in human islets. In synergy with IL-1β and IFN- γ, IL-17 enhanced the transcription of the inducible isoform of nitric oxide synthase (iNOS), and cyclo-oxygenase (COX-2) in human islets (Figure 17, panel B).

We also analyzed the potential apoptotic effect of IL-17, and observed an enhanced apoptotic effect of IL-17 with IL-1β and IFN-γ on human islet cell prep-

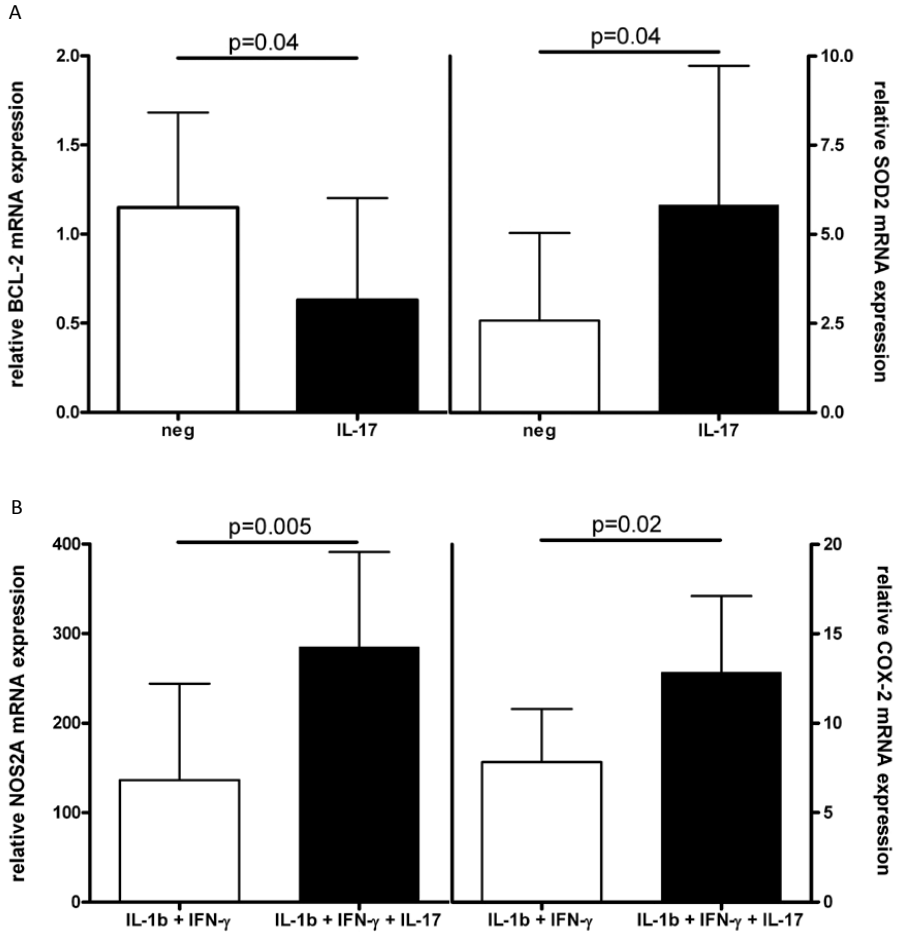

Figure 17. IL-17 contributes to the activation of the inflammatory and pro-apoptotic responses in human islets. IL-17 inhibits the expression of BCL-2 and up-regulates the expression of SOD2 in human islets treated with IL-17 (panel A, black bars) in comparison to negative treatment (panel A, white bars). In combination with IL-1β and IFN-γ (panel B, white bars), IL-17 enhances the expression of nitric oxide synthase (NOS2A) and cyclo-oxygenase (COX-2) (panel B, black bars). Bars represent mean, and whiskers standard deviation. Student's t-test for paired samples was used for calculation of p-values.

arations, and on the MIN6 cell line. Images were produced showing the necrotic plus apoptotic cells identified by the white nuclear staining and fragmented nuclei (arrows indicating positive cells) in a human islet cell preparation (Figure 18, left panel) and in MIN6 cells (Figure 18, right panel) when treated with IL-17 together with IL-1β and IFN-γ (Figure 18, lower images) and an untreated control (Figure 18, upper images). The p -values were calculated with the paired t-test.

IFN-γ secretion by Th17 cells may result in additive β-cell damage due to the synergistic effects mediated by IFN-γ and IL-17 on human islet cells as we demonstrated here. IL-17R is expressed on human islets and IL-17 potentiated the inflammatory and apoptotic response in human islet cells *in vitro*. The up-regulation of iNOS and SOD results in the increased production of free-radicals that interfere with β-cell functioning and cause β-cell damage. IL-17 triggers iNOS activation in endothelial cells, and endothelial cells participate in the regulation of the T-cell-dependent inflammatory response [328]. IL-17 has been demonstrated to augment iNOS expression and subsequent NO production by different combinations of IFN-γ, TNF-α, and IL-1β. In addition, a significant increase in blood IL-17 levels was observed in a multiple low-dose streptozotocin model of diabetes, sug-

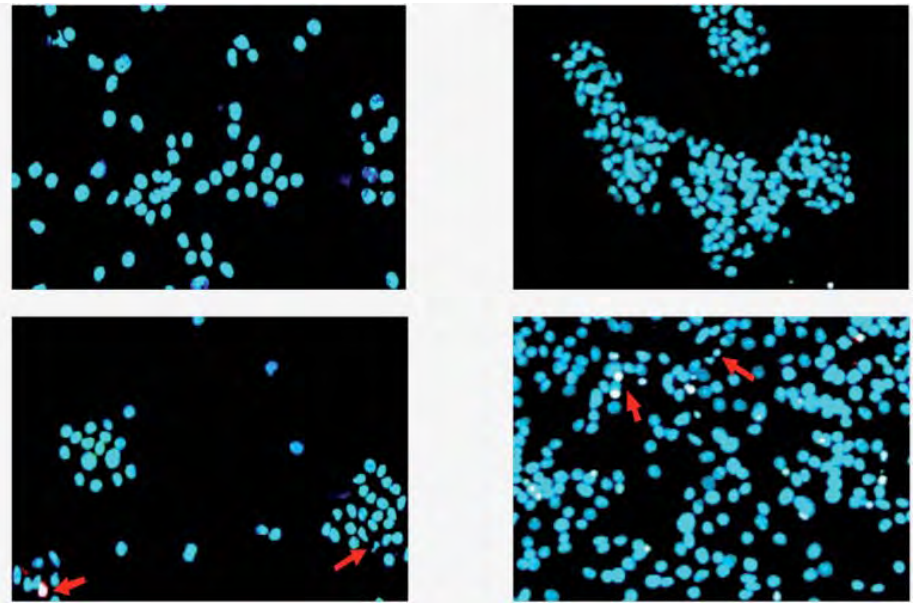

Figure 18. Examples of necrotic plus apoptotic cells identified by white nuclear staining and fragmented nuclei (arrows indicating positive cells) in human islet cell preparation (left panel) and in MIN6 cells (right panel) when treated with IL-17 together with IL-1β and IFN-γ (lower images) and untreated control (upper images). Pictures were originally taken at 20x magnification.

gesting that T-cell-derived IL-17 might be involved in the NO-dependent damage of β cells in T1D [274].

Down-regulation of the anti-apoptotic gene BCL-2 was seen, as well as increased apoptosis of islet cells when treated either with IL-17 alone or together with other inflammatory cytokines, such as IL-1β and IFN-γ[329]. Thus, the synergistic effect of IFN-γ and IL-17 in the induction of inflammation and apoptosis in the human islet cells indeed suggests that the co-producers of IL-17 and IFN-γ could be of clinical importance. Furthermore, the results could indicate that antigen-specific T-cell-mediated attack is not necessarily needed for β-cell destruction, which could be mediated by other factors such as cytokines including IL-17. The detrimental effect of IL-17 on human islet cells emphasizes the role of IL-17 in the pathogenesis of β-cell destruction without the prerequisite of an autoantigen-specific attack against insulin-producing β cells. However, autoantigen-specific T cells producing IL-17 may exist. Indeed, GAD specific T-cell clone has been reported to produce IL-17, thus providing link to the pathogenesis of T1D.

Our results here suggest that IL-17 is detrimental to human β cells. Th17 activation may increase apoptosis of β cells, and thus the release of β-cell-derived autoantigens, which would accelerate the development of β-cell specific autoimmunity.

5.6 Screening method for FOXP3 gene expression modulators in human Tregs

From the PBMC fraction, $CD4^+CD25^+$ target cells were purified with a MACS separation technique, and the purity of the $CD4^+CD25^+$ cell population was constantly greater than 90%. Magnetic particle technology was used for mRNA purification from the isolated target cells. Using purified mRNA as a template, cDNA was prepared and TaqMan technology was used for the analysis of beta-2-microglobulin (B2M) and FOXP3 expression levels. The effect of the anti-CD3 coating on B2M and FOXP3 expression levels was analyzed. Anti-CD3 coating of the cell culturing wells improved the signal levels and reduced the observed variation for both B2M and FOXP3. Mean Ct values for B2M were 25.2 (SD=0.37) and 25.9 (SD=1.1) for eight cell culturing wells with and without anti-CD3 coating, respectively. The mean Ct values for FOXP3 were 28.8 (SD=0.28) and 31.1 (SD=0.51) , respectively, for eight cell culturing wells with and without anti-CD3 coating. When different numbers of $CD4^+CD25^+$ cells were used for analysis, we observed that Ct values of B2M and FOXP3 were dependent on the cell numbers used for analysis when linear regression analyses were performed (R^2=0.77, $p<0.001$ for B2M and R^2=0.72, $p<0.001$ for FOXP3), whereas dCt values were independent of the cell numbers used for analysis (R^2=0.02, p=0.55) (Figure 19).

When relative FOXP3 expression analysis was performed from the untreated $CD4^+CD25^+$ cells, and from the $CD4^+CD25^+$ cells stimulated with either the anti-CD3 alone or in combination with anti-CD28 and cytokine TGF-β1, we observed the relative FOXP3 expression levels presented in Figure 20. Spearman's rank correlation coefficient was calculated for the dCt and Ct values for both the FOXP3 and reference gene B2M for different cell populations and treatments. Ct values for FOXP3 expression correlated with the dCt values ($r=0.94$, $p<0.0001$). No correlation ($r=-0.02$, $p=0.93$) was seen between Ct values for the reference gene B2M and dCt values.

Functional defects in Tregs have been associated with autoimmune diseases [330]. The proportion of Tregs in peripheral blood circulation is limited, and the detection method for gene expression should thus be sensitive and robust with a broad dynamic range. RT-qPCR matches these requirements [331, 332]. Quantitative reverse transcription PCR (RT-qPCR) in combination with a magnetic-particle-based mRNA capture technique was used for the quantitation of FOXP3 gene expression in $CD4^+CD25^+$ human Tregs. We detected FOXP3 expression even from ~1200 $CD4^+CD25^+$ cells, and changes in the Ct values of the reference gene B2M and FOXP3 were linear from ~1200 cells to 20 000 cells. However, the dCt

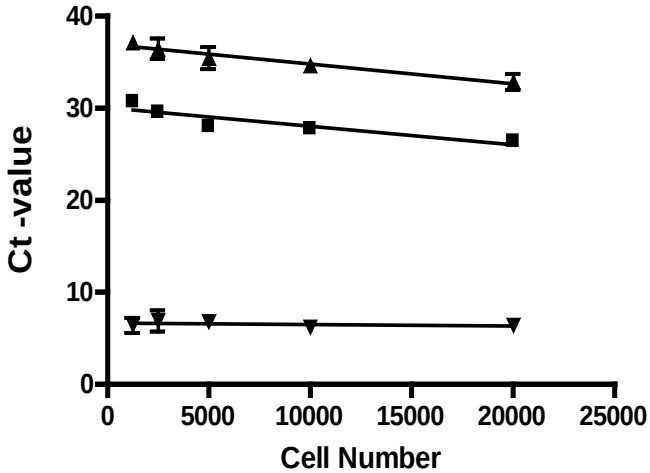

Figure 19. Linear regression of Ct values for B2M , FOXP3 and dCt determined from various quantities of unstimulated $CD4^+CD25^+$cells (quadruplicates). The dCt –value of the gene expression analysis is not dependent on the cell number used in the analysis in the range from 1250 to 20 000 cells. B2M, $R2=0.77$, $p<0.001$, FOXP3, $R2=0.72$, $p<0.001$, dCt, $R2=0.02$, $p=0.55$. Ct(B2M)=▲, Ct(FOXP3)=■, dCt=▼. Mean±SD is shown in the figure.

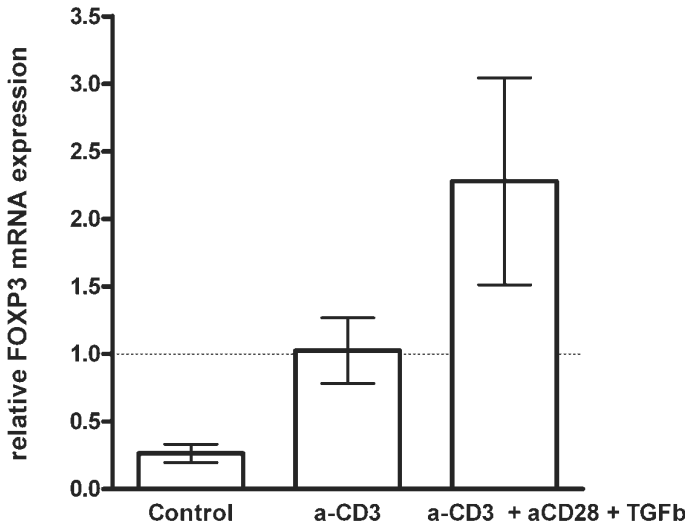

Figure 20. Comparison of relative expression levels of FOXP3 mRNA in $CD4^+CD25^+$ cells (control) and in $CD4^+CD25^+$ cells stimulated with anti-CD3 or with anti-CD3 and anti-CD28 and FOXP3 promoting cytokine TGF-β1. The horizontal line shows the average FOXP3 expression of the anti-CD3-stimulated $CD4^+CD25^+$ cells..

value remained constant across the range of cell quantities. Thus, we decided to use approximately 5000 $CD4^+CD25^+$ cells in the FOXP3 gene expression analyses in later studies. When $CD4^+CD25^-$ and $CD4^+CD25^+$ cells and different biological treatments given to these cells were analyzed, we observed that $CD4^+CD25^+$ cells almost exclusively showed spontaneous FOXP3 expression. This is in concordance with an earlier report (271), which also showed that $CD4^+CD25^-$ cells up-regulate FOXP3 upon stimulation to some extent. Spontaneous FOXP3 expression was, however, markedly higher in $CD4^+CD25^+$ cells, and FOXP3 expression above the cut-off criteria (mean+3*SD) can be considered to have originated from $CD4^+CD25^+$ cells. Our methodology successfully distinguished different biological treatments given to the $CD4^+CD25^+$ and $CD24^+CD25^-$ cells. When the relationship between the B2M and FOXP3 dCt values was examined in $CD4^+CD25^+$ and $CD4^+CD25^-$ cells with or without stimulation, a strong correlation was observed between the Ct values of FOXP3 and dCt values. Thus, we concluded that the normalized relative expression estimate of FOXP3 is dependent on the changes in the Ct values of FOXP3. This validates the use B2M for mRNA input normalization.

5.7 Results of the pilot screen

In the pilot screen for the FOXP3 up-regulation potential of 160 small molecules, the mean expression level plus 3*SD units of the anti-CD3 treated cells was considered as a cut-off for the up-regulation of FOXP3 expression. For the first screened plate (80 molecules), the mean + 3*SD FOXP3 expression for the anti-CD3-stimulated $CD4^+CD25^+$ cells was 2.3 relative units (mean = 1.05, SD = 0.41), and for the second plate (80 molecules) 1.5 (mean = 1.01, SD = 0.12). Using this cut-off criterion, from the 160 different chemicals screened, six fulfilled the criterion and were considered as potential FOXP3 up-regulators (See Figure 21, Panels A and B, for Plates 1 and 2 respectively).

Four chemicals with FOXP3 up-regulation potential were purchased and reanalyzed using $CD4^+CD25^+$ cells from six different donors. Table 2 shows the observed inter-individual variation in the FOXP3 up-regulation induced by the four different molecules.

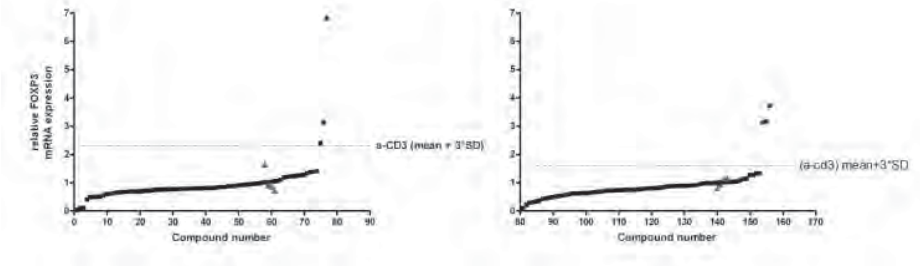

Figure 21. mRNA expression levels of the FOXP3 in $CD4^+CD25^+$ cells induced by 160 drugs or drug-like molecules included in the pilot screen. Observations within a plate are arranged in order according to the FOXP3 expression levels. Red triangles represent the FOXP3 expression levels of cell culturing wells with bare anti-CD3-stimulated $CD4^+CD25^+$ cells.

Table 2. Table 2. FOXP3 expression in chemically treated (1.0μM) $CD4^+CD25^+$ cells from six different donors. The expression of FOXP3 induced by chemical treatment is relative to the expression of FOXP3 in anti-CD3 stimulated $CD4^+CD25^+$ cells.

Tested individual	Inter-individual variation of FOXP3 expression in chemical stimulated Tregs			
	Phenylbutazone	Resveratrol	Fluphenazine hydrochloride	Oxyphencyclimine
A	1.34	0.65	0.94	0.65
B	0.67	0.53	1.33	0.91
C	1.61	0.62	0.46	0.56
D	1.62	2.61	1.58	1.54
E	1.75	1.21	1.36	0.31
F	1.38	1.03	1.10	0.99

Phenylbutazone (PB) showed a FOXP3 up-regulation potential in five out of six tested individuals. PB-induced up-regulation of FOXP3 was significant ($p = 0.03$, Wilcoxon Signed Rank Test). In addition, the dose response of FOXP3 up-regulation induced by PB was examined. The average FOXP3 expression for anti-CD3-stimulated and 0.1μM, 1.0μM, and 10μM phenylbutazone treated $CD4^+CD25^+$ cells was 1.3 RU (SD = 0.18), 1.6 (SD = 0.007) and 2.7 RU (SD = 0.29), respectively (Figure 22).

The biological relevance of the transcription factor FOXP3 is rather well documented. However, the pathways regulating the expression of FOXP3 are still unclear. The functioning of Tregs has been shown to mainly be initiated by FOXP3-dependent mechanisms [286], and FOXP3 dysfunction has been characterized in many autoimmune diseases [330]. It has been demonstrated that marginal FOXP3 up-regulation in purified $CD4^+CD25^+$ Tregs leads to a significant increase in the suppressive capacity of Tregs [286].

The chemical genomics strategy is a system biology research approach used successfully in industrial drug discovery. Chemical genomics applies a cell-based screening methodology, leaving a high degree of freedom for the model system to iterate a new balance in response to the applied chemical. Thus, we aimed to develop a method for the screening of small molecule entitites (small molecule < 1000 Daltons) up-regulating FOXP3, using primary human $CD4^+CD25^+$ cells as our target cell model. The screening strategy to characterize molecules with a FOXP3 up-regulating potential is presented in Figure 23.

After the automation and optimization of the FOXP3 quantification using limited primary cell resource of $CD4^+CD25^+$ cells, the methodology was applied on

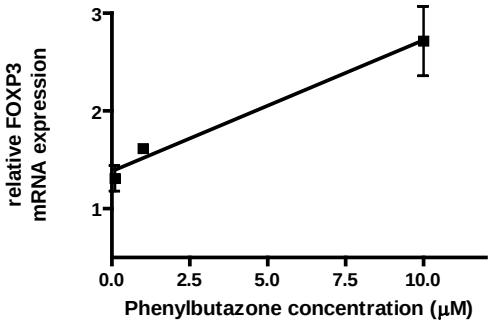

Figure 22. PB up-regulated FOXP3 in a concentration-dependent manner in anti-CD3-stimulated $CD4^+CD25^+$ cells. Three phenylbutazone concentrations (0.1μM, 1.0μM, and 10μM) were tested on $CD4^+CD25^+$ cells from two donors. $R2 = 0.87$, $p = 0.007$.

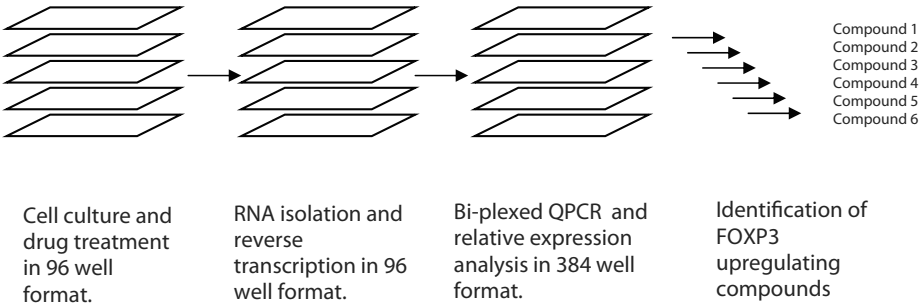

Figure 23. Strategy for the screening of small molecules up-regulating FOXP3 transcription in human $CD4^+CD25^+$ cells derived from peripheral blood.

a small-scale pilot screening of FOXP3 modulators. The method to monitor the up-regulation of FOXP3 in anti-CD3 stimulated and chemical-treated cells was proven to be feasible, and we found modulators of FOXP3 gene expression. The sensitivity of the screening method enables the use of small cell numbers in the screening, and the methodology is thus particularly suitable for screening approaches targeting human blood-derived primary cells. As anti-CD3 stimulation increased FOXP3 gene expression in $CD4^+CD25^+$ cells, the screening method could be used in the search for FOXP3 down-regulators.

In the pilot screen, PB showed a FOXP3 up-regulation potential in five out of the six individuals studied. In addition, PB up-regulated FOXP3 in a dose dependent manner with three tested concentrations and the dose dependency of FOXP3 up-regulation by PB was seen in $CD4^+CD25^+$ cells from two different donors. PB is a strong anti-inflammatory agent, that has been shown to inhibit pro-inflammatory cytokines IL-6 and GM-CSF of anti-CD3 and anti-CD28 stimulated PBMCs [333], and IL-6 production by adherent cells stimulated with lipopolysaccharide (LPS) [334]. The molecular mechanism through which PB mediates its anti-inflammatory action is not known. FOXP3 mediates the repression of cytokine expression, and PB may therefore inhibit cytokine expression by up-regulating FOXP3.

In conclusion we have developed a screening method that is feasible for the screening of modulators of FOXP3 gene expression in human Tregs. Our methodology can easily be applied to other similar approaches where the target cell population is limited. Such an approach would be the characterization of FOXP3 expression in recently discovered Th17 cells, which have been shown to be closely related to the human Tregs. The screening of drug molecules that modulate the dynamic balance of Tregs and Th17 cells could provide new insights for drug development to cure immune-mediated diseases.

6 Conclusions

Rapidly accumulating knowledge suggests that defective regulation of T-cell responses plays an essential role in the pathogenesis of autoimmune diseases such as T1D. The development of T1D is a long and complex process depending on a fine balance between pathogenic and tolerogenic immunological pathways. Recent advances in the field of T-cell biology have revealed new and more dynamic mechanisms in the control of immune reactions. Advances in Treg research, in particular, have revolutionized the concept of immune regulation. Tregs can lose their regulatory features and turn into self-antigen-recognizing effector T cells producing pro-inflammatory cytokines such as IFN-γ and IL-17. The work presented in this thesis aimed at assessing the alterations in the regulation of T-cell responses generated in T1D. The results suggest that the generation of regulatory mechanisms and effector mechanisms upon T-cell activation is aberrant in children with T1D. In our studies, an *in vitro* cytotoxic environment inhibited the induction of genes associated with regulatory functions, namely FOXP3, NFATc2, and ICOS, upon T-cell activation. We also found T1D patients to have an impaired cytotoxic response against coxsackievirus B4. Ineffective virus clearance may increase the apoptosis of β cells, and thus the risk of β-cell specific autoimmunity, due to the increased presentation of β-cell-derived peptides by APCs to T cells in pancreatic lymph nodes.

IL-17 immunity has been shown to contribute the pathogenesis of T1D in animal models. We demonstrated IL-17 activation to be a major alteration in T1D patients in comparison to healthy children. Moreover, alterations related to the FOXP3-mediated regulatory mechanisms and to the generation of a Th1 response was associated with the IL-17 up-regulation seen in T1D patients.

T1D is believed to result from an imbalance between auto-reactive effector T cells and Tregs. This concept is built on the assumption that T-cell subsets are static entities. In the light of current knowledge, this view seems oversimplified. The plasticity of activated T cells as well as Tregs seems to be a critical mechanism for the human body to 'edit' immunological functions over time in order to generate an adequate immune response against invading pathogens without causing harmful tissue destruction.

Responses that do not extinguish the infection are not desired, and shutting them off as well as initiating a modulated response is thus an important feature of T cells. In conclusion, we suggest that in T1D this 'editing function' of T cells is disrupted and the phenotype commitment of Tregs and effector T cells is disturbed. This, in turn may lead to the activation of self-antigen-specific effector T cells that were initially released from the thymus as natural Tregs. This hypothesis provides a topic for the future studies based on the results of this doctoral thesis as well as findings presented by others.

7 Acknowledgements

This study was initiated at the Department of Molecular Medicine of the National Public Health Institute in 2003, then carried on at the Department of Viral Diseases and Immunology, and finalized in 2010 at the Department of Vaccines and Immune Protetction of the National Institute for Health and Welfare. I wish to thank the former head of the National Public Health Institute Professor Jussi Huttunen and the former head of the National Public Health Institute and the present director of the National Institute for Health and Welfare Professor Pekka Puska. I also would like to thank the former heads of the departments, Leena Palotie, Tapani Hovi and Ilkka Julkunen and the present head of the Department of Vaccines and Immune Protection Terhi Kilpi for providing excellent facilities needed to complete this study.

I owe my deepest gratitude to the study subjects and their families for participating in these studies. Without their willingness this type of research would not be possible.

I am deeply grateful to my supervisor Professor Outi Vaarala whose guidance, never-ending optimism and patience have been invaluable. Whenever I encountered difficulties in my project(s), it was her positive attitude and encouragement that made me to try harder and finally to complete this thesis.

I want to thank all the co-authors for collaboration in the original papers. I owe a special thanks to Professor Mikael Knip for fruitful collaboration, and giving invaluable clinical aspects to this thesis. I also would like to owe a special thanks to Professor Jorma Ilonen for collaboration during these years. I also wish to thank Professor Johnny Ludvigsson for collaboration.

I acknowledge Professor Timo Otonkoski for collaboration with human islets experiments, and PhD Ru Gao for designing and performing cytokine stimulation experiments with human islet preparations.

I also thank PhD Susanne Skarsvik for collaboration. I am deeply grateful for her help with my first T cell experiments in Helsinki.

I would like to thank Docent Arno Hänninen and Professor Heikki Hyöty for reviewing my thesis and for giving precious comments and constructive criticism.

My warmest thanks go to all the former and present colleagues with whom I have had splendid time doing research in Outi's lab. I wish to thank Minna Tiittanen, Riikka Nissinen, Janne Nieminen, Terhi Ruohtula, Veera Hölttä, Linnea Reinert, Emma Savilahti, Laura Orivuori, Marko Sirkiä, Harri Salo, Tomi Pihlajavaara, Saara Aittomäki, Paula Klemetti and Guillermo Kociubinski for the past years and their direct and indirect contribution to this thesis. I especially want to thank Kristiina Luopajärvi for sharing the office with me several years and giving precious help and mental support. I also would like to express my deep gratitude to Harry Lybeck, Maria Kiikeri, Anneli Suomela, Sinikka Tsupari, Pirjo Mäki and

Tarja Alander, for their skilful technical and administrative assistance and practical help.

I also would like to thank all the people of the 'old ROIM'. I have felt most warmly welcome among them.

My work would have been impossible without good friends. When I was buried into the laboratory, for too long and too deep, someone has always contacted me and suggested some re-creational activity. That is the reason why I still might be sane. I want to express my warmest thanks to Lauri for being my friend from the first day of high-school. Jussi and Karoliina Kuusela are thanked for the warm friendship beginning from the days of '*G. salaris* diagnostics'. Special thanks need to be addressed to the members of 'Prostata –group', Jussi, Janne, Juhani, Jalo, Jouni and Juhana. I will never forget our robber-steak weekends, spear-fishing trips and pseudo-science meetings. I believe these happenings have helped me to understand 'structure of reality'. Also, all the people with whom I managed to avoid studying cryptogam taxonomy Toni, Jesse, Anab, Anu, Kimmo, Mike, Nearco, Iita, Emilia and Salla are thanked for great company.

The family Mäenpää is thanked for keeping my/our wheels rolling for three decades.

I also want to express my deep gratitude to Petri Grönfelt for being my friend.

My hearty thanks go to Riitta and Jyrki. They have made me feel like being at home whenever I have stayed in Siilinjärvi or Tuusniemi. Visits to the 'Savonmua' have re-charged my batteries and helped me to complete this thesis.

I am deeply grateful to my parents. They have always encouraged and supported me, whatever decisions I have made in life.

My most loving thanks go to Riikka, Lempi and Santra. After the day at work they gently remind me what is the most important thing in life: a little evening walk with them.

This study was financially supported by the Academy of Finland, the Sigrid Juselius Foundation, the Finnish Diabetes Research Foundation and the Finnish Funding Agency for Technology and Innovation.

November 2010

Jarno Honkanen

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