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MAIRE LINK

Transcription factors FoxP3 and AIRE: autoantibody associations

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Department of General and Molecular Pathology, University of Tartu, Estonia

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Supervisors: Raivo Uibo, MD, PhD, Professor of Immunology, University of Tartu, Estonia
Kai Kisand, MD, PhD Senior Research Fellow at the Department of General and Molecular Pathology, University of Tartu, Estonia

Reviewers: Aavo-Valdur Mikelsaar, MD, PhD, Professor of Human Biology and Genetics, University of Tartu, Estonia
Martti Laan, MD, PhD, Extraordinary Senior Researcher at the Department of General and Molecular Pathology, University of Tartu, Estonia

Opponent: Seppo Kalevi Meri, MD, PhD, Professor of Immunology, University of Helsinki and Helsinki Central Hospital, Finland

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I. Link M, Salur L, Kisand K, Rajasalu T, Tillmann V, Uibo R. Higher FoxP3 mRNA expression in peripheral blood mononuclear cells of GAD65 or IA-2 autoantibody positive persons compared with autoantibody-negative persons. APMIS 2008; 116:896–902

II. Reimand K, Perheentupa J, Link M, Krohn K, Peterson P, Uibo R. Testis-expressed protein TSGA10 – an auto-antigen in autoimmune polyendocrine syndrome type I. Int Immunol 2008; 20: 39–44

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Paper I: participation in study design, performing the experiments and statistical analysis, writing the paper

Paper II: participation in performing the experiments, statistical analysis, participating in writing the paper

Paper III: participation in performing the experiments and data analysis

ABBREVIATIONS

AIRE	autoimmune regulator
APECED	autoimmune polyendocrinopathy-candidosis-ectodermal dystrophy
CYP11A1	cytochrome p450 side chain cleavage enzyme
CYP17	cytochrome p450 17α-hydroxylase
FoxP3	forkhead box P3
GADA	65 kD glutamic acid decarboxylase autoantibodies
IAA	insulin autoantibodies
IA-2A	tyrosine phosphatase-like insulinoma antigen 2 autoantibodies
ICA	islet cell autoantibodies
IFN	interferon
IL	interleukin
IPEX	immunodysregulation, polyendocrinopathy and enteropathy, X-linked
ISG	interferon-stimulated gene
MHC	major histocompatibility complex
moDC	monocyte-derived dendritic cells
mTEC	medullary epithelial cell
PBMC	peripheral blood mononuclear cells
pDC	plasmocytoid dendritic cells
RIU	radioimmunoprecipitation unit
RT-PCR	reverse-transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TGFβ	transforming growth factor β
Treg	regulatory T cell
T1D	type 1 diabetes

1. INTRODUCTION

Since Paul Erlich first described autoimmunity, in his words "horror autotoxicus", numerous autoimmune diseases have been described. A lot of progress has been made in understanding the pathogenesis of autoimmunity. As a result, there are multiple treatment options available, immunosuppression in systemic autoimmune diseases, replacement therapy for different autoimmune diseases of endocrine organs etc. Still, up to date there is no curative treatment for any autoimmune diseases.

For development of such treatment in the future, we need to deepen our knowledge on the basic mechanisms underlying the development of autoimmune reactions and the connection between the trigger/cause of autoimmunity and the resulting phenotype of patient with autoimmune disease.

2. BACKGROUND OF THE STUDY

2.1. Monogenic autoimmune syndromes

To date there have been described several autoimmune syndromes, associated with a defect in a single gene. Great efforts are made to understand the basic mechanisms of autoimmunity, and these syndromes and genes involved are of great interest in this process. Most studied in this field are two transcirption factors, involved in the maintenance of self-tolereance: FoxP3 and Aire. These transcription factors are related to two different stages in avoiding the autoimmunity: Aire is necessary for central deletion of self-reactive T-cells [1, 2] and FoxP3 participates in maintaining peripheral tolerance via regulatory T cells (Tregs) [3]. Defect in either of these genes is associated with multiorgan autoimmunity: FoxP3 deficiency leads to the immunodysregulation, poly-endocrinopathy and enteropathy syndrome (IPEX; OMIM 304790) and homozygous Aire mutations cause the autoimmune polyendocrinopathy-candidosis-ectodermal dystrophy syndrome (APECED; OMIM 240300).

2.2. FoxP3

FoxP3 (forkhead box P3) belongs to the forkhead/winged-helix family of transcriptional regulators. It’s gene (GeneID: 50943) is located on chromosome X (Xp11.23). The protein contains DNA-binding winged-helix/forkhead domain, responsible for the transport of protein to nucleus; zinc finger domain; leucine zipper domain, required for homodimerisation and a domain in the N-terminal part of the protein, required for repression of the transcription [4].

FoxP3 acts as both transcriptional repressor and transcriptional activator. Genes regulated by FoxP3 include genes encoding proteins involved in T-cell receptor signalling cascade, cytokines, immunosupressive molecules, proteins downregulating T-cell activation, molecules defining Treg surface phenotype, and also non-coding miRNA. Significant part of FoxP3-regulated gene expression is likely to be regulated in indirect manner, via other transcription factors. [5, 6]

2.2.1. FoxP3 and regulatory T cells

FoxP3 is expressed in CD4+CD25+ regulatory T cell lineage, it is essencial for Treg development [7, 8] and their peripheral maintenance [9].

In addition to CD4+CD25+ Tregs, selected in thymus (so called natural Tregs), FoxP3 expression can be induced in CD4+CD25- cells by transforming growth factor (TGF) β both *in vivo* and *in vitro*, the cells gain suppressive

capacity [10–13]. Such induction is suppressed by interleukin (IL) 4, secreted by CD4+CD44high memory cells and enhanced by retinoic acid, which counteracts the suppression by IL4 [14, 15]. FoxP3 is also induced in effector T cells upon activation, which does not lead to suppressive phenotype, indicating that in humans FoxP3 alone is not sufficient for inducing regulatory function in T cells [16, 17].

There are two different isoforms of FoxP3 expressed in human regulatory T cells, full length isoform and Δ2 isoform, lacking the second exon. Both isoforms are functional repressors of CD4+ cell activation and are co-expressed in Tregs [18, 19]. It has been shown *in vitro*, that overexpression of either isoform can convert the CD4+CD25- cells into Tregs with regulatory capacities and T cells transduced with only full length or Δ2 isoform are similar both phenotypically and functionally [20]. In contrast to this, only full length isoform is capable of interacting with retinoic acid receptor-related orphan receptor (ROR) α and suppressing ROR-mediated transcriptional activation; Δ2FoxP3 lacks this ability, as the RORα-binding domain is located in the second exon [21]. However, the possible influence of this interaction on the function of Tregs remains unclear.

The function of Tregs is to suppress immune reaction: they control immune response in infectious diseases [22, 23], allergy [24], autoimmunity [3], allograft rejection/tolerance [25] and immune reactions against tumors [26]. The actual mechanism of suppression by Tregs is still somewhat unclear. Recent studies have confirmed, that TGFβ is needed for the suppressive function of Tregs [24, 27, 28], but the data about whether the effect of TGFβ is dependent on signalling via TGFβ receptor type II is controversial [24, 27]. TGFβ may act by cell-cell contact, mediated by membrane bound TGFβ [28, 29]. In addition to TGFβ, IL10 is shown be necessary for suppressive capacity of Tregs [24, 30], though there is some controversy [31]. This discrepancy may be explained by the presence of two Treg subsets, exploiting different cytokines for suppression [32]. For peripheral induction of Tregs upon activation, IL12 signalling through IL12Rβ2 is needed, lack of such signalling leads to reduced suppressive capacity of Tregs [33]. Cytotoxic T-lymphocyte associated antigen (CTLA) 4 expressed by Tregs is proposed to suppress the immune reaction by downregulating the expression of costimulatory molecules CD80/CD86 on antigen presenting cells and thus decreasing their capacity of eliciting immune response [34, 35].

Tregs are also involved in the regulation of antibody production. They induce the production of IgG4 through IL10 and cell-cell interaction via glucocorticoid-induced tumor necrosis factor receptor related protein (GITR) and it's ligand [36]. In addition to inducing IgG4 production, Tregs suppress the production of IgE, presumably by secreting IL10 [37]. Suppressive function of Tregs is not confined to adaptive immune system, they also inhibit mast cell degranulation via OX40-OX40L ligation [38].

2.2.2 FoxP3 and autoimmunity

2.2.2.1 IPEX

In 1982 Powell *et al* described for the first time a syndrome including diarrhea, eczematoid rashes, haemolytic anemia, exaggerated responses to viral infections and autoimmune destruction of endocrine glands [39].

IPEX (OMIM 304790) is a X-linked syndrome, which usually manifests in infancy with type 1 diabetes (T1D), followed by dermatitis, autoimmune enteropathy and chronic inflammation. The syndrome can include different autoimmune manifestations, for example thyroiditis, autoimmune haemolytic anaemia, autoimmune neutropenia, ulcerative colitis. Also, the presence of different autoantibodies is characteristic. Without treatment, the syndrome leads frequently to death within first two years of life, due to growth retardation or sepsis. [40, 41]

In 2001 mutations in FoxP3 were identified as the cause of the syndrome [42]. IPEX patients have been shown to have normal range of Tregs in their peripheral blood, but the function of Tregs is defective at varying degrees, depending on the mutation and the strength of TCR stimuli [43].

There is no good correlation between the particular FoxP3 mutations and clinical picture (symptoms and severity) of the IPEX, nor with the expression of FoxP3 in peripheral blood [41].

2.2.2.2. FoxP3 polymorphisms in autoimmunity

Several studies on the association of different FoxP3 polymorphisms and autoimmune diseases have been conducted. A Japanese study involving 199 diabetic patients and 289 controls evaluated microsatellite polymorphisms (GT)n in intron zero and (TC)n in intron five, and found significantly higher frequency of the $(GT)_{15}$ genotype in T1D patients [44]. This finding, however, was not confirmed in later studies, which were not able to show association between the (GT)n microsatellite polymorphism and T1D in Japanese [45] or Norwegian population [46]. Similarily, there was no association between the 12 studied FoxP3 polymorphisms and T1D in Sardinian cohort of patients [47]. In a recent study in Japanese population, the $(GT)_{16}/(GT)_{16}$ genotype in (GT)n microsatellite polymorphism was associated with adult onset T1D, especially in females and in female patients with slow progression of the diabetes; there was also association with low titers of 65 kD glutamic acid decarboxylase autoantibodies (GADA) [48].

The (GT)n microsatellite polymorphism has been studied in sarcoidosis patients, there was no association with the disease risk, but higher prevalence of $(GT)_{15}$ allele was found in patients without skin lesions [49].

The (TC)n microsatellite polymorphism has been studied in patients with thyroiditis. In autoimmune thyroiditis patients, there was significant association between the (TC)n polymorphism and disease risk in US Caucasian males, but not in Caucasian females or Japanese patients; the same study found also association with DXS573 polymorphism in Caucasian females [50]. The association with (TC)n, however, has not been supported by a study involving Caucasian patients (from northeastern England) with Graves' disease [51].

The A allele at intron one rs3761548 is associated with higher risk for psoriasis, and also with severer form of the disease and higher incidence of concomitant autoimmune diseases [52]. In this study, AA genotype proved to be associated with lower frequency of Tregs in T cells and lower FoxP3 expression in CD4+CD25+ cells, probably because of the impaired binding of transcription factors E47 and c-Myb to the rs3761548 site.

In primary biliary cirrhosis patients, the frequency of single nucleotide polymorphism IVS9+450 (T to C) is higher, compared with healthy controls [53].

So far, no association has been shown between FoxP3 polymorphisms and juvenile idiopathic arthritis [54], celiac disease [46, 55], autoimmune Addison's disease [51], systemic lupus erythematosus [55], rheumatoid arthritis [55], inflammatory bowel disease [55] or Crohn's disease [53, 55].

2.2.2.3. FoxP3 expression levels and autoimmunity

There are accumulating data about the FoxP3 expression levels in different autoimmune diseases. In most of the studies, FoxP3 levels have proven to be lower in patients with autoimmune diseases, compared with healthy subjects.

Both the numbers of CD4+CD25+FoxP3+ Tregs and the expression level of FoxP3 in CD4+CD25+ cells in peripheral blood was found to be lower in myasthenia gravis patients, the function of Tregs was found to be defective [56]. Also, in the thymuses of myasthenia gravis patients, CD4+CD25+ cells showed decreased FoxP3 expression and defective function, compared with normal thymuses; the number of CD4+CD25+ cells was normal in the patients' thymuses [57].

In a study by Frisullo *et al*, involving untreated patients suffering from relapsing-remitting multiple sclerosis, both the number of CD4+CD25+Foxp3+ cells in peripheral blood and the FoxP3 protein level in CD4+CD25+ cells were dependent on the phase of disease, being markedly lower at the time of relapse, compared with remission; this decrease was accompanied by impaired Treg suppression [58]. In the study by Frisullo *et al*, there was no difference in Treg numbers or FoxP3 expression in remitting patients, compared with healthy controls. In contrast to that, another study on patients with relapsing-remitting multiple sclerosis showed reduced numbers of Tregs and lower FoxP3 protein

level in Tregs at the time of remission, the FoxP3 expression level correlated with *in vitro* suppressive capacity of Tregs [59].

Lower FoxP3 expression levels in peripheral blood cells have been described in systemic lupus erythematosus [60], Graves disease [61], autoimmune hepatitis [62] and primary Sjögren's syndrome [63] patients.

In contrast to the studies mentioned above, Grant *et al* found higher expression of FoxP3 protein in the Tregs of patients with T1D [64].

Higher FoxP3 expression levels have also been detected in autoimmune diseases at the sites of inflammation, for example in portal tracts of patients with primary biliary cirrhosis [65]. Higher expression of FoxP3 on mRNA level has been detected in small bowel mucosa from patients with celiac disease and patients with coexisting celiac disease and T1D, compared to healthy controls; this difference could possibly be due to higher accumulation of Tregs in patients' mucosa [66].

2.3. AIRE

Gene encoding Aire (autoimmune regulator) protein is located on chromosome 21 (21q22.3). Aire protein includes several functional domains – a N-terminal homogeneously staining region (HSR)/CARD domain, a nuclear localisation signal (NLS), a SAND domain, two plant homodomains (PHD), separated by proline-rich region (PRR) and four LXXLL nuclear receptor motifs. HSR region is thought to be necessary for forming cytoplasmic filaments [67], formation of nuclear speckles [67, 68] and homomultimerisation [68, 69]. However, a recent study demonstrated CARD-domain in the region previously designated as HSR; CARD domain was shown to be involved in nuclear localisation and transactivation capacity of Aire [70]. NLS domain is associated with the formation of characteristic nuclear speckles [67]. SAND domain is involved in DNA binding [71], nuclear compartmentalisation [67] and homomultimerisation [68]. PHD domains are associated with formation of nuclear dots [67], PHD1 mediates polyubiquitylation by it's E3 ligase activity [72]. PHD2 domain and COOH-terminal region of the protein, containing LXXLL and PXXPXP motifs, have shown to possess transactivational properties [72, 73].

Both in human organism and mice, Aire is expressed mainly in thymus. Aire is present on protein level in rare (about 0.005% of whole thymus) population of epithelial cells in thymic medulla and cortico-medullary junction. These cells are charaterised by the high expression level of MHC II and several costimulatory molecules, like CD80/CD86, CD40, PD-L1 and PD-L2. [74, 75] Aire mRNA is present in thymic antigen presenting cells [75] and low levels of Aire mRNA have been detected in thymocytes [76].

Besides thymic medulla, some level of Aire expression has also been demonstrated in peripheral tissues. In humans, Aire expression has been seen in

lymph node medulla and paracortical region, spleen and foetal liver; however, there is no Aire expression in target organs for APECED-related autoimmune diseases (e.g. adrenal cortex, pancreas, thyroid glands) [74, 77]. In peripheral tissues, the expression of Aire on mRNA level has been shown in lymphocytes [76, 78], monocytes and monocyte-derived dendritic cells [79–81]. It has been suggested, that Aire is expressed in monocytes and monocyte-derived dendritic cells (moDC) in protein level [79], but the presence of Aire protein in peripheral tissues is still considered debatable [82].

In adult mice, in addition to the thymus, the expression of Aire protein and mRNA (though in lower level, compared with thymus) is shown in lymph node, spleen, kidney, lung, brain, ovary, [83, 84], bone marrow [84], gut [83] and testis [84], more specifically in developing spermatocytes [83, 85]. However, the presence of Aire in peripheral lymphoid tissues is disputable, as a recent study showed the absence of Aire protein both in spleen and lymph nodes and in dendritic cells isolated from these tissues [75].There is also some controversy in the data about the expression of Aire in adult murine liver [83, 84]. It has been claimed that though in mice Aire mRNA is present in many different tissues, at the protein level, Aire is present only in thymic medulla [86].

At the subcellular level, the localisation of Aire protein has two different forms, depending on the cell cycle: nuclear dots or homogeneous distribution in nucleoplasm [87]. The Aire protein can also be present in cytoplasm, colocalising with microtubular cytoskeleton [72, 74].

2.3.1. Negative selection in thymus

Studies have proven, that Aire is involved in the process of negative selection of autoreactive T cells in thymus. Aire regulates the thymic expression of several antigens, otherwise restricted to periphereal tissues.

In humans, Aire-dependent expression of several tissue restricted antigens (α-subunit of the nicotinic acetylcholine receptor, IA-2, retinal S-antigen and H+/K+ATPase) have been shown in mTECs. This expression has wide interindividual variability, possibly depending on Aire expression levels and genetic variations in tissue-specific antigens. [88]

In mice, Aire upregulates the expression of several autoantigens, known to be associated with APECED, for example insulin 1, insulin 2, cytochrome P450 1A2, tryptophan hydroxylase [89, 90]. In addition to APECED-related autoantigens, Aire regulates (either activates or represses) the expression of genes, involved in posttranslational modifications [89] or antigen presenting (major histocompability complex (MHC) class II,) [91, 92], also cytokines (IL9, IL1Ra) [91, 92], chemokines (CCL5, CCL9, CCL25) [91] and transcription factors [89].

Both *in vitro* experiments and mouse models have proven the association between Aire expression and apoptotic death of thymocytes in the process of

negative selection in thymus [1, 2]. In Aire-deficient thymus, thymocytes specific for tissue restricted antigens are not eliminated by apoptosis, allowing the release of high-affinity autoreactive cells to peripheral circulation [93, 94]. At the same time, negative selection of thymocytes specific for some autoantigens, known to have Aire-independent expression in thymus, is not influenced [94].

Although advances have been made in the understanding of negative selection and Aire's role in it, several questions remain. As Aire –/– mice develop autoimmunity against α-fodrin, an Aire-independent antigen [95], there has to be additional role for Aire in the process of negative selection, apart from inducing promiscuous gene expression in mTECs. This idea is also supported by findings by Anderson *et al*, in their transgenic mouse model autoimmune reactions appeared against a peptide under the control of Aire, despite the normal expression levels of the given antigen in thymus [94]. Moreover, not all tissue specific antigens are regulated by Aire: for example, GAD67 expression is not abolished in Aire –/– mice [90]. This suggests mechanisms of negative selection, that are independent of Aire.

In addition to the process of negative selection of autoreactive T cells, it is shown, that Aire-expressing mTECs are able to initiate the generation of Tregs in thymus [96]. The effect of the lack of Aire on peripheral pool of Tregs is not clear. APECED patients appear to have normal numbers of $CD25^{high}$ Tregs, but their FoxP3 expresssion is lower and function impaired to some extent [97]. Somewhat controversely, there appears to be no defect in the positive selection of Tregs in Aire –/– mice, both the numbers and function of Tregs are normal in Aire-knockout mice compared with wild type mice [93, 94].

Recent studies have shown that Aire is also involved in the development of normal thymic morphology. Being expressed in a subset of mTECs, Aire influences the differentiation program of these cells; Aire deficient mice have altered morphology and distribution of mTECs, also the number of terminally differentiated mTECs is reduced [98, 99]. It is hypothesised, that Aire influences the differentiation of mTECs, ensuring capability of terminally differentiated, nonproliferating mTECs to promiscuously express tissue-specific antigens [98, 100].

2.4. Aire and autoimmunity

2.4.1. APECED

APECED (OMIM 240300) is a rare monogenic disease with autosomal recessive inheritance pattern.

Characteristic for the syndrome is the presence of chronic mucocutaneous candidosis, hypoparathyreoidism and Addison's disease; for diagnosis, the presence of 2 out of 3 is required, though it has been suggested, that in siblings

of APECED patients, the precence of one component could be sufficient for the diagnosis [101]. It is also proposed, that APECED could be diagnosed in the presence of one typical disease component, if a mutation in Aire gene is detected [102]. In addition to chronic mucocutaneous candidosis and different endocrine manifestations (see Table 1), signs of ectodermal dystrophy (dental enamel and nail dysplasia, keratopathy) are part of the syndrome [101, 103, 104].

There are three populations with somewhat higher prevalence of the disease – Finnish (prevalence 1:25,000) [101], Sardinian (prevalence 1:14,400) [105], Iraninan Jews (prevalence 1:6500 to 1:9000) [104]. In Europe, smaller patient cohorts are described in Norway [103], Sweden [106, 107], United Kingdom [108], Slovenia [109], Poland [110], Slovakia [111], Ireland [112] and continental part of Italy [113].

In 1997, mutations in Aire gene were identified as the cause of APECED [114]. Up to date, there are at least 70 Aire mutations described according to the Human Gene Mutation Database® [115]. There are predominant mutations in all abovementioned three populations with higher prevalence of APECED, suggesting a common ancestor in each population. In Finland, the most prevalent mutation is R257X, which is present in 89% of patients and 72% of patients are homozygous for this mutation [116]. The Y85C mutation appears to be specific for Iranian Jews and predominant in this population [116]. In Sardinian cohort of patients, R139X is the prevalent mutation [105].

One mutation, inherited in dominant fashion (G228W) has also been described [117]. Functional analysis of the mutation revealed the dominant negative effect of the mutation: the mutant protein interacts with the normal Aire protein and completely blocks the formation of nuclear dots, normally seen in Aire-expressing cells and disrupts the transcriptional activation capacity of the wild-type Aire [117].

2.4.1.1. Manifestations of APECED

As mentioned above, the three most frequent disease components in APECED are chronic mucocutaneous candidosis (frequency in different populations 17–100%), hypoparathyroidism (85–96%) and Addison's disease (22–84%) [103, 104, 118].

The disease phenotype varies greatly. The number of disease components can vary from one (patient with known mutations in Aire gene) to ten [118, 119], median number of components is five [118]. Onset of disease (most often the first manifestation is candidosis [103, 118]) can be from the first year of life up to age over forty [119] and new manifestations can appear throughout the life [104, 118, 119]. Common manifestations of APECED are shown in Table 1. In addition to these disease components, several other disease components can be present, like pernicious anaemia, autoimmune hepatitis, chronic diarrhoea

and less common manifestations like asplenia, nephritis, exocrine pancreas insufficiency etc [104, 118].

There are implications, that there may be correlation between the disease phenotype and underlying Aire mutation. In the population of Iranian Jews, where most patients carry the Y85C mutation (in a sample of 13 patients, all were homozygous for the mutation) [116], the hypoparathyreoidism is present in almost all patients (96%) and the prevalence of mucocutaneous candidosis is very low (17%) [104]. Higher incidence of chronic mucocutaneous candidosis is associated with the mutation R257X (most prevalent mutation in Finnish patients), lower incidence of candidosis is shown in patients without nonsense mutation [120].

The association between HLA alleles and APECED phenotype has been studied as well. There appears to be higher risk to Addisons disease in carriers of the DRB1*03 allele, alopecia is associated with the DRB1*04 allele and DQB1*0602, known to be protective for T1D, is also protective in APECED patients. [120]

Table 1. Common manifestations of APECED. nr – not reported; * – among patients in reproductive age; # – percent of gonadal failure among patients of both genders

	Frequency (%)			
	Zlotogora 1992 [104]	Myhre 2001 [103]	Betterle 2002 [121]	Perheentupa 2006 [118]
Candidosis	17	85	79	100
Hypoparathyroidism	96	85	88	88
Addison's disease	22	80	100	84
Keratopathy	0	10	12	22
Alopecia	13	40	38	39
Vitiligo	nr	25	22	31
Ovarian failure*	38	31#	61#	69
Testicular failure*	33			28
Diabetes mellitus	4	0	6	33
Hypothyroidism	4	10	13	31

2.4.1.2. Autoantibodies and APECED

In APECED patients, the presence of several serum autoantibodies (both organ specific and non-organ specific) is characteristic [122, 123]. One patient can have up to eight different autoantibodies, most patients have 2 to 3 different autoantibodies [123], but the actual numbers of autoantibodies may be even higher, as recently novel autoantibodies with very high prevalence (NALP5 and type 1 interferon autoantibodies) have been discovered [107, 124]. Most common autoantibodies are against type 1 interferons (97–100%) [119, 124], cytochrome p450 side chain cleavage enzyme (CYP11A1; present in 52–63% patients), cytochrome p450 21-hydroxylase (63–66%), aromatic L-amino acid

decarboxylase (51–52%) and tryptophane hydroxylase (45–52%) [120, 123]. Autoantibodies can be present without clinical manifestation of related disease, for example islet cell autoantibodies (ICA), GADA and tyrosine phosphatase-like insulinoma antigen 2 autoantibodies (IA-2A) are not necessarily related to T1D [122, 123].

While most autoantibodies present in APECED patients are also common in isolated autoimmune diseases, there are autoantibodies, which are considered to be relatively specific for APECED. These antibodies include aromatic L-amino acid decarboxylase autoantibodies, associated with vitiligo in APECED patients [125, 126]. In addition to APECED, these autoantibodies are also present in a subset of patients with Addison's disease [127]. Autoantibodies against hepatic antigen cytochrome P450 1A2 [128], tyrosine hydroxylase [129], tryptophan hydroxylase [126, 130] and parathyroid antigen NALP5 [107] are present in APECED, but not in patients with isolated autoimmune diseases.

Recently, neutralising autoantibodies against type 1 interferons (IFNα, IFNω, to lesser extent also IFNβ) were shown to be present in APECED sera [124]. Virtually all APECED patients are positive for these autoantibodies, while patients with isolated autoimmune diseases and healthy controls are negative [124, 131, 132]. Besides APECED patients, these autoantibodies are also present in myasthenia gravis patients and in more than half of patients with thymoma, but in lower titers [124, 133, 134]. It is proposed that autoantibodies against type 1 IFN-s might be used in the diagnosis of APECED, as they are highly specific and sensitive for the syndrome [131, 132, 135].

2.4.2. Aire polymorphisms and autoimmunity

In patients of Caucasian origin, several associations between autoimmune diseases and Aire polymorphisms have been found. There appears to be higher risk for vitiligo [136] and alopecia areata [137] in individuals with T7215C allele. In vitiligo patients with other associated autoimmune diseases there is also association with T11787C allele [136]. In subgroup of alopecia areata patients (early onset alopecia universalis/ophialis) there is association with C4144G polymorphism [137].

In systemic sclerosis patients with associated autoimmune thyroiditis, there is significantly higher prevalence of G11107A polymorphism, compared with healthy subjects or patients with systemic sclerosis alone [138].

The lack of association between Aire polymorphisms and Addisson's disease, autoimmune polyendocrine syndrome II [139] or T1D [140] has been reported.

Heterozygousity for APECED-related mutations of Aire has been studied in different patient populations: patients with autoimmune hepatitis [141], sporadic idiopathic hypoparathyroidism [142], inflammatory bowel disease [143], T1D,

autoimmune thyroid diseases [144, 145] and Addisons' disease [144], but so far, no significant associations have been found.

2.4.3. Aire expression levels and autoimmunity

The expression levels of Aire in thymus have been studied in thymoma patients. Thymoma, epithelial cell tumor in thymus, is frequently associated with myasthenia gravis or other autoimmune diseases. There is evidence, that in thymoma patients, the expression of Aire in thymus is absent or lower than in healthy individuals, accompanied by smaller numbers of Tregs in thymus and lower FoxP3 expression in Tregs. The data about the correlation between the Aire expression level and coexistence of myasthenia gravis is still controversial, but there seems to be no association with the presence of autoantibodies against acetylcholine receptors. [146–148]

In thymomas, the loss of Aire expression is accompanied by the significantly reduced expression of some Aire-regulated genes (insulin, CYP1A2, IL4, some chemokines), in the same time the expression of other Aire-regulated genes is not influenced (CHRNA1) [133, 147]. Despite that, autoimmune diseases characteristic for APECED and autoantibodies related to these diseases are uncommon in thymoma patients, with the exception of IFNα and IFNω autoantibodies, which are present in more than half of thymoma patients [133].

3. AIMS OF THE STUDY

The aim of this study was to examine the influence of two genes, FoxP3 and Aire, known to be associated with the development of autoimmune diseases, on the phenotype of different autoimmune conditions. We aimed to evaluate the association of two FoxP3 isoforms, expressed in regulatory T cells, with newly diagnosed T1D as an example of an actively ongoing autoimmune process. Also, we wanted to assess the correlation between autoantibodies, possibly specific to Aire-deficiency and phenotype of APECED patients.

Accordingly, the specific aims were as follows:

1. To determine the frequency of FoxP3 positive regulatory T cells and the expression of FoxP3 in peripheral blood mononuclear cells in newly diagnosed type 1 diabetes patients, to evaluate the association between the regulatory T cell numbers and the FoxP3 expression and the levels of autoantibodies in patients' sera
2. To detect the presence and titers of TSGA10 autoantibodies in APECED patients, to evaluate the possible association with clinical parameters in APECED and to compare the frequency of these autoantibodies in APECED, in healthy persons and in a disease not related to Aire-deficiency (Addison's disease)
3. To assess the *in vivo* and *in vitro* influence of autoantibodies against type 1 interferons in APECED patients to the expression of different genes, regulated by type 1interferons.

4. MATERIAL AND METHODS

4.1. Patients and controls

T1D group. Peripheral blood mononuclear cells (PBMC) from 26 T1D patients (Table 2) were used in the study. All patients were newly diagnosed and the blood was drawn within the first week of the insulin treatment. In all patients, the presence of 4 types of autoantibodies (GADA, IA-2A, ICA and insulin autoantibodies (IAA)) was detected. The autoantibody assays were conducted in the Tartu University Clinics laboratory or Department of Internal Medicine I, University of Ulm (Germany).

The corresponding control group consisted of 17 healthy controls (Table 2)

Table 2. Characteristics of the T1D patients group and the corresponding control group.

	T1D patients	*Controls*
n	26 (14 M, 12 F)	17 (8 M, 9 F)
Mean age (min;max)	25.6 (1.75; 58)	26.9 (3; 60)
Autoantibody positivity:		
GADA	21/26	1/17
IA-2A	17/26	0/17
ICA	13/26	0/17
IAA	11/26	1/8

APECED group I. Sera of 66 APECED patients of Finnish origin were used, 40 males and 26 females (Table 3). Gonadal dysfunction (azoospermia or testicular atrophy/primary or secondary amenorhhoea) was diagnosed in 5 men and 12 women. The patients with gonadal dysfunction were tested for autoantibodies against steroidogenic enzymes, cytochrome p450 17α-hydroxylase (CYP17) and CYP11A1 using radioimmunoprecititation [149]. Either or both autoantibodies were found in 1 of 5 men with hypogonadism and in all 12 women.

Table 3. Characteristics of the APECED patients in the APECED group I. * among patients in reproductive age

	Males	*Females*
n	40	26
Gonadal insufficiency*	5/40	12/26
Autoantibody positivity		
CYP17	8/26	18/22
CYP11A1	13/26	12/22

Two control groups were used for the study: healthy controls and patients with isolated Addison's disease:

i. sera of 20 healthy men of Finnish origin and 96 blood donors – 50 males and 46 females
ii. sera of 32 patients, diagnosed with autoimmune Addison's disease – 13 males and 19 females. All patients were of Estonian origin.

APECED group II.
For gene array analysis, peripheral blood monocytes from an APECED patient of New-Zealand origin and two matching healthy controls were used.

For quantitative RT-PCR and cell stimulation experiments, sera and peripheral blood cells of 8 APECED patients of Norwegian and Slovenian origin (4 males and 4 females; Table 4) and 9 age-matched controls (2 males, 7 females) were used. None of the patients was taking immunosuppressive treatment at the time of the sampling. All patients were characterised for the presence of neutralising type I interferon autoantibodies (IFNα2, IFNα8, IFNω, IFNβ). All patients were positive for IFNω autoantibodies; one patient was negative for IFNα-autoantibodies, one had low titers and the rest had high titers of IFNα-autoantibodies. None of the controls had neutralising autoantibodies against any of the tested type 1 interferons.

For cytokine measurements, sera from Norwegian APECED and Addison disease collection, the Finnish APECED collection, a Sardinian APECED and unaffected heterozygous relative cohort and some sera from APECED patients of US origin and SLE sera from Tartu University Clinics serum bank were used.

Table 4. Characteristics of the patients in the APECED group II

		Males	*Females*
n		4	4
Age range (y)		17–58	20–56
Neutralising interferon-autoantibodies			
	interferon α	4/4	3/4
	interferon β	0/4	1/4
	interferon ω	4/4	4/4

All studies have obtained approval from the local ethics committees (including the committees in the patients' land of origin) and every patient has given informed consent to participate in the study.

4.2. Autoantibody detection

Radioimmunoassay. In vitro translated TSGA10 was purified through the Sephadex TM G-25 DNA Grade column (Amersham Biosciences, Piscataway, USA). Antigen (~40 000 cpm) was suspended in 50 µl RIP buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA, 0.05% Tween 20 and 1% gelatin hydrolysate). 50 µl of 1:10 diluted human serum was added and incubated for 15 min at room temperature. 50 µl of Protein G Sepharose TM (Amersham Biosciences) 15% (v/v) suspension in RIP buffer was added and incubated for 45 min at room temperature on a overhead shaker. The samples were washed six times with RIP buffer. 3 ml of liquid scintillation cocktail (Optiphase „HiSafe" 3, PerkinElmer TM life sciences) was added and signal was counted on Wallac Guardian 1414 liquid scintillation counter (Wallac OY, Turku, Finland). All tests were run in duplicates. As positive control, a serum of an APECED patient, known to have autoantibodies against TSGA10 was used. A TSGA10-autoantibody negative serum of an APECED patient was used as a negative control. Results were expressed as radioimmunoprecipitation index units $\{RIU=[(cpm_{sample} - cpm_{negative\ control})/(cpm_{positive\ control} - cpm_{negative\ control})]x100\}$. The cutoff level was set at 6 RIU.

*Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot.*Purified GST-fused TSGA10 was boiled for 5 minutes at 95º C in the sample buffer (0.0625 mM Tris-HCl, pH 6.8, 3% sodium dodecylsulphate, 10% glycerol, 0.02% bromophenol blue and 0.1 M DTT). The antigen samples were separated in 10% SDS-PAGE using the Mini-Protean II electrophoresis system (Bio-Rad, Hercules, USA) in the running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS; pH 8.3). Low range molecular weight markers (BioRad) were run on every gel.

The separated proteins were transferred to the nitrocellulose membrane (Hybond-ECL 0.45µm pore, Amersham Biosciences) using Mini Trans-Blot tank transfer system (Bio-Rad) in the transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol; pH 8.3) for 1 hour at 150 mA. The membrane was blocked in 4% skimmed milk (Põlva Piim, Põlva, Estonia) and 0.05% Tween20 in Tris-buffered saline (25 mM Tris, 150 mM NaCl; pH 7.5) for 1 hour at room temperature. In order to avoid bacterial growth, 0.02% sodium azide was added to the blocking solution. The membrane was stripped and the strips were incubated in human sera (diluted 1:100 in blocking solution) overnight at +4º C. The bound antibodies were detected using alkaline-phosphatase conjugated secondary rabbit antibodies against human IgG (Dako, Glostrup, Denmark) diluted 1:500 in 0.05% Tween20 in Tris-buffered saline. The reaction was visualised using 0.0025% 5-bromo-4-chloro-3-indolyl phosphate and 0.0025% nitro blue tetrazolium in substrate buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$; pH 9.5).

4.3. Isolation and differentiation of cells

Isolation of PBMC. Mononuclear cells were isolated from peripheral blood by Ficoll-Hypaque Plus (Amersham Biosciences) density gradient centrifugation. Peripheral blood was diluted in 2 volumes of phosphate buffered saline (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl; pH 7.4) and layered over Ficoll-Hypaque Plus and centrifuged 30 minutes at 20ºC at 400 g. The interface layer containing PBMC was collected and washed twice in phosphate buffered saline. The cells were suspended in RPMI 1640, 20% human serum, 10% dimethyl sulphoxide (Sigma, St Louis, USA) and stored in liquid nitrogen.

Isolation of monocytes and pDC. PBMC were isolated from peripheral blood by gradient centrifugation. Monocytes were isolated from PBMC, using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), using manufacturers protocol for positive sorting. The purity of isolated cells was evaluated by flow cytometry, using fluorochrome-conjugated antibodies against CD14 and CD3, the purity was greater than 99%.

Plasmocytoid dendritic cells (pDC) were isolated from PBMC by positive sorting using anti-BDCA-4-conjugated magnetic microbeads (Miltenyi Biotec). The purity of isolated cells, evaluated by flow cytometry (using BDCA-2 antibodies), was greater than 95%.

Generation of moDC. Isolated monocytes were cultured in 6-well tissue culture-plates at the density of 10^6 cells/ml in RPMI1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% foetal calf serum (PAA laboratories, Linz, Austria) in the presence of 50 ng/ml granulocyte-macrophage colony-stimulating factor and 25 ng/ml interleukin-4. The cells were cultured for 6 days, every other day the medium was changed and fresh cytokines were added. The phenotype of differentiated cells was evaluated by flow cytometry, using fluorochrome-conjugated antibodies against CD14 and DC-specific intercellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN).

Cell incubation with APECED sera. Freshly isolated monocytes from healthy donors were incubated for 18 hours in the presence of autologous plasma and 2% APECED sera either positive or negative for neutralising type 1 IFN autoantibodies or control sera. The cells were cultivated at the density of 10^6 cells/ml in RPMI1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (PAA laboratories).

4.4. Flow cytometry

Flow cytometry. Defrosted PBMC ($3x10^5$ per tube) were incubated with fluorochrome-conjugated antibodies against CD4, CD25, CD127 (BD Biosciences, Franklin Lakes, USA) for 20 minutes at room temperature. Intracellular staining of FoxP3 was undertaken with the monoclonal FoxP3 antibody (clone PCH 101; eBioscience, San Diego, USA) using manufacturer's buffers and protocol. Cells were permeabilized/fixed for 30 min at 4° C, non-specific binding was blocked with 2% normal rat serum for 15 min at 4° C, and cells were stained for 30 min at 4° C. Isotype control (rat IgG_{2a} κ) was included. Cells were studied on FACS Calibur flow cytometer (BD Biosciences) and results analysed with CellQuest software.

4.5. Gene expression analysis

RNA isolation. Total RNA was isolated from PBMC, monocytes, plasmocytoid dendritic cells (pDC) and moDC, using Trizol® Reagent (Invitrogen, Carlsbad, USA). The cells were lysed in 1 ml Trizol® Reagent and incubated 5 minutes at room temperature. 200 µl chloroform was added and incubated for additional 5 minutes at room temperature. The samples were centrifuged 15 minutes at 12000 g at +4ºC. The liquid phase was removed and 500 µl isopropanol added. After 10 minutes incubation at room temperature, the RNA was precipitated by centrifuging 10 minutes at 12000 g at +4° C. The precipitate was washed in ethanol and diluted in RNase free water.

Affimetrix GeneChips The RNA was labeled and Affymetrix GeneChip hybridisations were done by the Australian Genome Research Facility, using Megascript T7 kit (Ambion, Austin, USA), GeneChip IVT Labeling kit and the Human Genome U133 Plus 2.0 array slides. The slides were scanned, using GeneChip scanner 3000 (Affimetrix). For analysis, the intensities for each probe set were normalised and summarised, using the Robust Multi-array Analysis algorithm [150]. Further data analysis was performed using Microarray Data Analysis system, version 2.19 and Multi Experiment Viewer, version 4.0.

cDNA synthesis. The SuperScript III Reverse Transcriptase kit (Invitrogen) was used for cDNA synthesis. 1 µl dNTP mix (10mM) and 1µl oligo dT20 (50 µM) were added to the RNA (diluted in 11 µl RNase free water). The samples were incubated 5 minutes at +65° C, followed by incubation on ice for 1 to 5 minutes. 4 µl 5x RT buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$], 1 µl DTT, 1 µl RiboLock RNase inhibitor (40 U/µl, Fermentas, Burlington, Canada), 1 µl SuperScript™ reverse transcriptase (200 U/µl) were added to each reaction. The samples were incubated 60 minutes at 50º C, followed by 15 minutes at 65° C.

Table 5. Primers used in the quantitative RT-PCR analysis. F–forward; R– reverse.

Transcript		*Primer sequence*
β-actin	F	CTGGAACGGTGAAGGTGACA
	R	CGGCCACATTGTGAACTTTG
FoxP3	F	ATGCACCAGCTCTCAACGCT
	R	GTGTGAGGCTGATCATGGC
Δ2 FoxP3	F	TTCCAGGGCCGAGATCTTC
	R	TCCACCGTTGAGAGCTGCAG
CCL5	F	CTGCCTCCCCATATTCCTCG
	R	TAGAAATACTCCTTGATGTGGGCAC
CIITA	F	CCTTGGAAACCCTCAATCTG
	R	CAGTTATTGTACAAGCTTAGCC
CXCL10	F	TTCCTGCAAGCCAATTTTGT
	R	TTCTTGATGGCCTTCGATTC
CXCL9	F	GGGAGAAACAGGTCAGCCAA
	R	GACGAGAACGTTGAGATTTTCGAA
IFI44	F	TGTGGCATTTGTATTTGATGCC
	R	CCAGCGTTTACCAACTCCCTTC
IFI44L	F	GTGGCCTTGCTTACTAAAGTGGATG
	R	CCCGGCTTTGAGAAGTCATAGA
IFIT1	F	ATCCACAAGACAGAATAGCCAGATCT
	R	TGTACTCATGGTTGCTGTAAATTAGGC
IFIT3	F	ACACAGAGGGCAGTCATGAGTG
	R	TGAATAAGTTCCAGGTGAAATGGC
IFNAR1	F	CGCAAAGCTCAGATTGGTCCT
	R	CCATCCAAAGCCCACATAACACT
Mx1	F	AGGCTCGGTGGCTGAGAA
	R	ACCTAGAGCCCGCAGGGAG
OAS2	F	CACCAGCTCCAATCAGCGAG
	R	TCAGCCATTGCCAGCATATTTTATC
TNFSF10	F	GAAGCAACACATTGTCTTCTCCAA
	R	TTGCTCAGGAATGAATGCCC

Quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). The relative expressions of transcripts were determined using qPCR Core kit for Sybr® GreenI (Eurogentec, Seraing, Belgium). Samples were run in triplicate, all results were normalised with respect to β-actin. The relative expression ($2^{-\Delta\Delta Ct}$) was determined as the relative amount of the analysed sample compared to the calibrator. Used primers are given in the Table 5.

4.6. Cytokine measurements

IP-10 (CXCL10) Duo Elisa kit (R&D systems, Minneapolis, USA) was used according to the manufacturers instructions to detect the serum levels of CXCL10 in the patient and control sera.

4.7. STAT1 phosphorylation

Normal human PBMC or U937 monocytic cell line were used. PBMC were rested for two hours in RPMI1640 supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% foetal calf serum (PAA laboratories). The U937 cells were kept in serum-free medium for 12 hours. The cells were pelleted $2x10^5$ per tube and resuspended in 100 µl of medium with 1000 U/ml recombinant human IFNα (PBL Biomedical Laboratories, Piscataway, USA) and various concentrations (2%, 5% or 10%) of test sera. After 15 minutes at 37ºC and 5% CO_2, the cells were fixed with an equal volume of Cytofix buffer (BD Biosciences) and permeabilised in cold Perm Buffer III (BD Biosciences) for 30 min on ice. The cells were stained with 5 µl anti-phospho-STAT1 (Y701)-Alexa Fluor 488 at room temperature for 1 hour, washed and analysed using FACSCalibur (BD Biosciences). The mean Alexa Fluor 488-fluorescence intensity (MFI) of the cells was analysed using CellQuest software (BD Biosciences).

4.8.Statistics

The statistical analysis was conducted using R, a language and environment for statistical computing [151].

For normalisation of data, taking natural logarithms, common logarithms or square roots was used.

Differences between the groups were evaluated using χ^2 test, Student's t test or Wilcoxon test. Correlations between the variables were evaluated by Pearson's correlation coefficient and Kendall's rank correlation coefficient tau. Probability levels less than 0.05 were considered significant.

The statistical analysis of Affymetrix GeneChip data was performed by Ken Simpson using empirical Bayes moderated t-statistics from the LIMMA package [152].

R and GraphPad Prism® software were used for generating the figures.

5. RESULTS

5.1. FoxP3 positive regulatory T cells and FoxP3 expression in type 1 diabetes (Paper I)

5.1.1. The frequency of Foxp3 positive regulatory T cells

We analysed the frequency of different CD4 positive T cell subgroups in PBMC by flow cytometry. The results are shown in Table 6. We found a median of 4.60% (range 0.67–9.30%) CD4+FoxP3+CD127low cells in CD4+ cells in control group. The corresponding values for T1D patients were 2.8% (1.2–7.0%), the difference was not statistically significant. Similarily, when we defined the Tregs as CD4+CD25+Foxp3+ lymphocytes, there was no difference between the T1D patients and control group. The frequencies of CD4+CD25+Foxp3+ lymphocytes and CD4+FoxP3+CD127low lymphocytes were in good correlation ($r=0.86$, $p<0.001$).

We also analysed the association of presence of autoantibodies and their titers (GADA, IA-2A, IAA and ICA) and the number of Tregs in peripheral blood. We could not detect any statistically significant difference between the autoantibody positive *vs* negative study subjects. There was no significant correlation between the cell subtype frequencies and the titers of tested autoantibodies.

Table 6. The frequencies of different lymphocyte subpopulations in PBMC. The results are expressed as percentages of total lymphocytes. No statistically significant differences could be detected. T1D – type 1 diabetes patients; C – control group

	Mean %		*Median (Q1; Q3) %*	
	T1D	C	T1D	C
CD4+	39.2	39.0	40.3 (33.2; 45.7)	39.0 (34.6; 42.3)
CD4+CD25high	0.76	0.97	0.61 (0.42; 1.04)	0.96 (0.53; 1,25)
CD4+CD25highFoxP3+	0.38	0.45	0.31 (0.23; 0.42)	0.44 (0.18; 0.55)
CD4+FoxP3+	1.02	0.87	0.92 (0.72;1.16)	0.81 (0.69; 1.13)
CD4+CD127lowFoxP3+	0.57	0.71	0.59 (0.49; 0.67)	0.67 (0.53; 0.95)

5.1.2. The expression of FoxP3 in PBMC

The expression of two FoxP3 isoforms – full length FoxP3 and Δ2FoxP3 (lacking the second exon) was determined on mRNA level by quantitative RT-PCR. There was no statistically significant difference between the T1D patients and healthy persons in the expression levels of either FoxP3 isoform or their ratio (Figure 1).

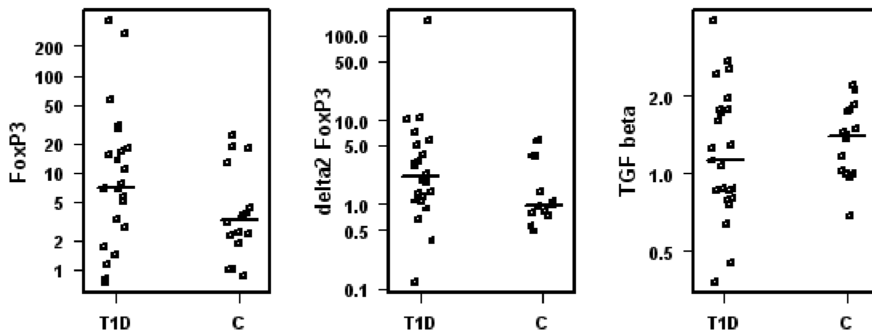

Figure 1. Relative expression of FoxP3 isoforms and TFGβ on mRNA level in T1D patients and controls. Median expression is indicated by lines.

When we compared the expression levels of FoxP3 between the autoantibody positive and negative study subjects, we found significant difference between the GADA or IA-2A positive and negative persons (Figure 2). The expression of both FoxP3 isoforms was higher in subjects positive for either of those autoantibodies, compared to autoantibody-negative study subjects (Table 7). We did not see similar association with either IAA or ICA positivity. Also, the ratio of two isoforms did not differ between the autoantibody positive or negative study subjects.

There were no significant correlations between the autoantibody titers and expression levels of the FoxP3 isoforms.

We also measured the expression of TGFβ by quantitative RT-PCR. There was no significant difference between the T1D patients and healthy controls (Figure 1), nor was there any difference in TGBβ expression between autoantibody positive or negative study subjects. The expression of TGFβ did not correlate with the expression of either full-length FoxP3 ($\tau = -0.08$, p=0.46) or Δ2 FoxP3 ($\tau = -0.15$, p=0.21).

Table 7. The relative expression of two FoxP3 isoforms (median (Q1;Q3)) on mRNA level in PBMC.

	GADA			*IA-2A*		
	Positive	Negative	*p*-value	Positive	Negative	*p*-value
FoxP3	9.3 (5.5;18.1)	2.4 (1.2; 4.1)	0.004	13.1 (5.9; 25.7)	3.4 (1.7; 6.9)	0.017
Δ2FoxP3	2.9 (1.3; 5.4)	1.0 (0.72; 1.8)	0.007	3.0 (1.4; 6.7)	1.1 (0.8; 3.0)	0.03
Ratio	3.9 (2.0; 7.8)	3.1 (2.0; 4.9)	0.8	4.7 (2.4; 7.9)	3.1 (2.0; 4.7)	0.71

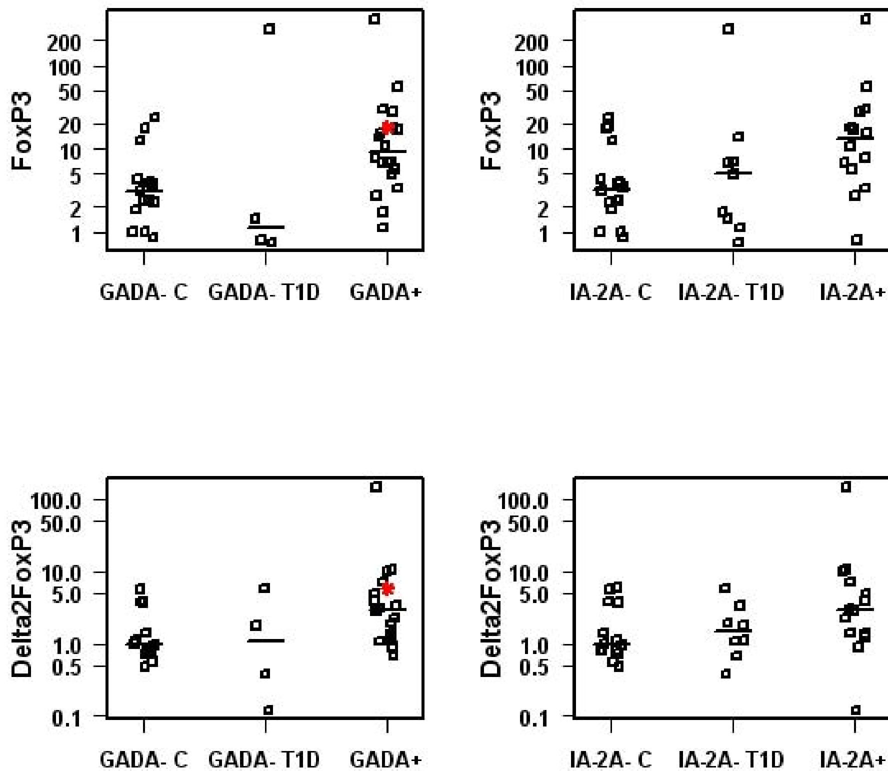

Figure 2. Relative expression of FoxP3 isoforms in study subjects by their autoantibody-positivity and negativity. Median expression is indicated by lines.

GADA – C: GADA negative control subjects; GADA – T1D: GADA negative T1D patients; GADA + : GADA positive study subjects. The GADA positive healthy control is indicated by red asterisk.

5.2. TSGA10 autoantibodies and APECED (Paper II)

5.2.1. The frequency of TSGA10 autoantibodies in studied groups

Using radioimmunoprecipitation, we found TSGA10 (GeneID 80705; testis specific, 10) autoantibodies in 5 APECED patients' sera (3 males, 2 females), Table 8. No autoantibodies were detected in the sera of healthy controls, the difference of the frequency of autoantibody positivity between two groups was statistically significant (p=0.005). Also, the difference between the RIU values was significantly different between APECED patients and healthy controls (p=0.039), Figure 3. No TSGA10 autoantibodies were found in Addisson disease patients.

Using SDS-PAGE and immunoblot analysis we could not detect TSGA10 autoantibodies in either APECED patients or in control group.

Table 8. The results of TSGA10 autoantibody analysis by radioimmunoprecipitation. AD – Addison's disease.

Group	*n*	*Presence of TSGA10 auto-antibodies (cut-off value 6 RIU)*	*RIU values: median (Q1;Q3)*
APECED (males)	40	3 (7.5%)	0.17 (0.07; 0.46)
APECED (females)	26	2 (7.7%)	0.39 (0.15; 0.64)
Healthy controls (Finnish males)	20	0	0.22 (0.10;0.33)
Healthy controls (Estonian)	96	0	0.16 (0.01; 0.38)
AD patients	32	0	0.47 (0.30; 0.86)

Figure 3. The results of TSGA10 autoantibody analysis by radioimmunoprecipitation. The results are shown in radioimmunoprecipitation units. The cut-off value (6 RIU) is indicated by line. C– control group; AD – Addison's disease group

5.2.2. The association of TSGA10 autoantibodies with clinical characteristics

The precence of TSGA10 autoantibodies was not associated with infertility. Among the APECED patients, five males had clinical signs of hypogonadism, but none of those had TSGA10 autoantibodies. None of the TSGA10 autoantibody-positive male APECED patients had clinical signs of testicular failure.

Several serum samples were available from three patients. One patient had four serum samples from 26-year follow-up period, they all tested positive. Another patient had three positive sera samples from 18-year period. One patient developed TSGA10 autoantibodies at the age of 33, last serum from the follow up period of 25 years tested positive. None of those patients developed signs of gonadal insufficiency during the follow-up period.

One of the two TSGA10 autoantibody-positive females had gonadal insufficiency and secondary amenorrhoea at the age of 18.5 years. None of the other 11 female APECED patients with gonadal insufficiency had TSGA10 autoantibodies.

The TSGA10 autoantibody RIU values did not differ between the APECED patients with or without gonadal insufficiency (p=0.28). Similarily, there was no difference in the RIU values between the CYP17 autoutoantibody positive *vs* negative APECED patients (p=0.36) or CYP11A1 positive *vs* negative patients (p=0.21).

9

5.3. Neutralising type 1 interferon autoantibodies and APECED (Paper III)

5.3.1. The expression of interferon-stimulated genes in APECED

We analysed gene array data (Affymetrix Human Genome U133 Plus 2.0 Array) on monocytes and moDC from one APECED patient, known to have high titer of type I interferon (IFN) autoantibodies and two healthy controls. Genes showing more than 1.5-fold difference between the APECED patient and healthy controls in both of two experiments, were considered to be differentially expressed.

We selected 285 genes, that are up regulated by IFNs (ISGs, interferon stimulated genes) according to the literature [153, 154]. In monocytes, 60 of these genes were downregulated in APECED patients compared with healthy controls (Figure 4). Among the rest of the approximately 47,000 transcripts included in gene chip (non-ISGs), 301 were downregulated. The difference between ISGs and non-ISGs is statistically significant (p=3.9x10^{-123}). When monocytes were differentiated into immature and mature moDC, the number of downregulated genes decreased (10 and 13 ISGs remained downregulated, respectively), but the difference between the ISGs and non-ISGs was still significant (p=5.7x10^{-5} for immature and p=1.9x10^{-5} for mature moDC).

To confirm these results, we chose 8 ISGs that had shown downregulation in gene array experiments (CXCL10, IFI44, IFI44L, IFIT1, IFIT3, Mx1, OAS2, TNSF10) and 4 non-ISGs (CIITA, CCL5, IFNAR1, CXCL9) and evaluated their expression on mRNA level by quantitative RT-PCR, using PBMC from 5 APECED patients and 5 controls. All ISGs showed significant downregulation in APECED patients with high titers of neutralising IFNα autoantibodies, compared with healthy controls, similar to results from array analysis. One patient, who had low titers of IFNα autoantibodies had the highest expression levels of 7 ISGs out of 8 tested among the APECED patients. In contrast to these results, the patient who had only INFω autoantibodies showed strong increase in tested ISGs (Figure 5). There was no significant difference between the APECED patients and healthy controls in the expression levels of non-ISGs.

Further we analysed the expression levels of 8 ISGs listed above in freshly isolated monocytes (7 patients and 7 controls), freshly isolated pDC (2 patients and 2 controls) and *in vitro* differentiated moDC (3 patients and 3 controls). Both patients' monocytes and pDC, but not moDC showed downregulation of ISGs, similar to PBMC (Figure 5).

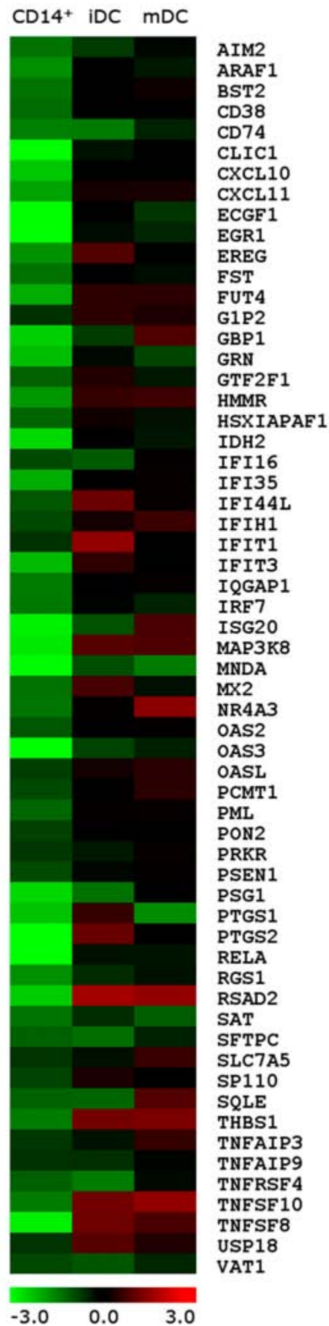

Figure 4. Microarray analysis of APECED and control monocyte-derived DC. The gene expression profiles of 61 ISGs differentially expressed in APECED patients and controls are shown. CD14+ monocytes; iDC immature moDC, mDC mature moDC

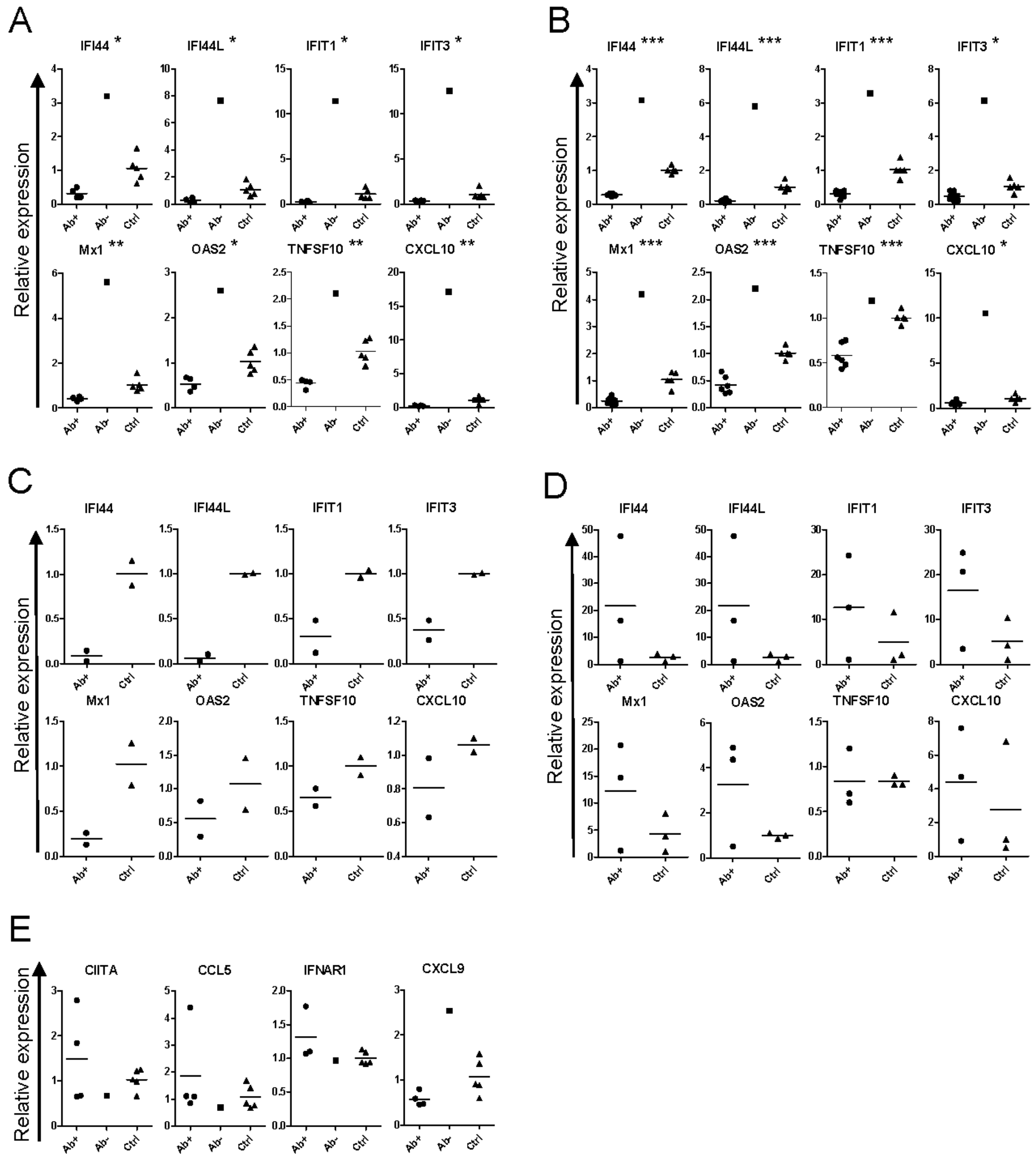

Figure 5. The relative expression of ISGs and non-ISGs in APECED patients and healthy controls. (A) and (E) freshly isolated PBMC; (B) freshly isolated monocytes (C) freshly isolated pDC; (D) monocyte-derived DC The expression levels in PBMC are evaluated by quantitative RT-PCR. Bars represent averages. Ab+ APECED patients positive for IFNα autoantibodies; Ab- APECED patients negative for IFNα autoantibodies; Ctrl control group. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, comparison between autoantibody-positive patients and healthy controls.

5.3.2. The influence of APECED patients' sera to the expression of interferon-stimulated genes

We tested *in vitro* the influence of the sera, containing the neutralising type I IFN autoantibodies, on monocytes from healthy donors. The monocytes were incubated for 18 hours in medium containing 2% sera from an APECED patient or healthy control and 20% autologous plasma. The expression of ISG-s was evaluated by quantitative RT-PCR.

All tested ISGs were significantly downregulated in the presence of APECED patients' sera containing IFNα autoantibodies. The serum containing only IFNω autoantibodies appeared not to have similar effect (Figure 6).

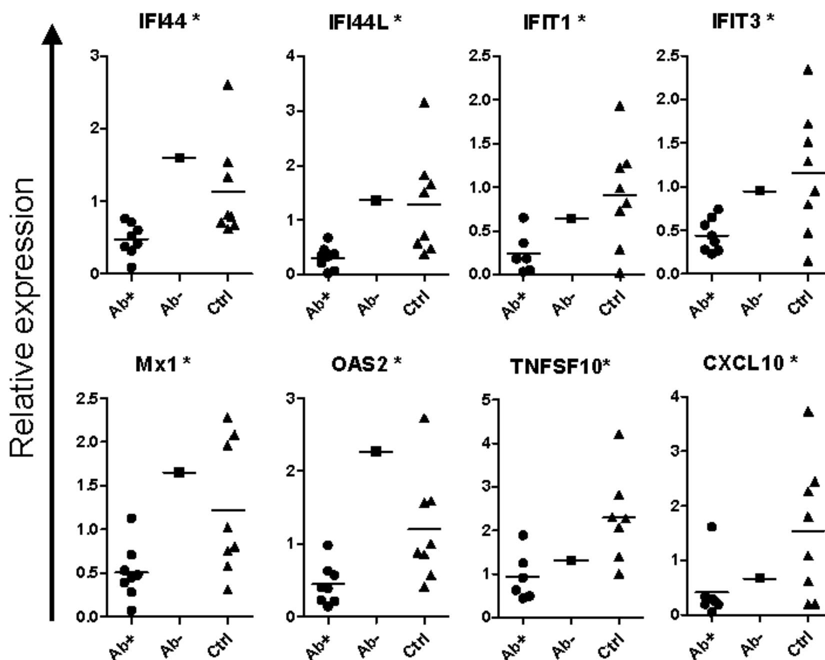

Figure 6. Relative expression of ISGs on mRNA level in monocytes after incubation in the presence of autologous plasma and APECED patients' sera or control sera. Ab+ sera from APECED patients positive for IFNα autoantibodies; Ab- sera from APECED patients negative for IFNα autoantibodies; Ctrl control sera. * $p<0.05$

When the autologous plasma was substituted with foetal calf serum, there was no downregulation of ISGs, suggesting that human plasma contains low levels of type I IFNs, which can be blocked by the patients' neutralising antibodies.

To test the influence of APECED patients' sera on IFN signalling, we assessed the phosphorylation of STAT1 protein, a crucial and early event in IFN

10

signalling. U937 cells, treated with IFNα showed significant up-regulation of STAT1 phosphorylation. This upregulation was inhibited to baseline level by APECED sera containing high titers of IFNα autoantibodies and not control sera, this effect was dose-dependent (Figure 7). One APECED serum containing low titers of IFNα autoantibodies had weak effect on the STAT1 phosphorylation. Interestingly, one serum containing only IFNω autoantibodies induced the phosphorylation.

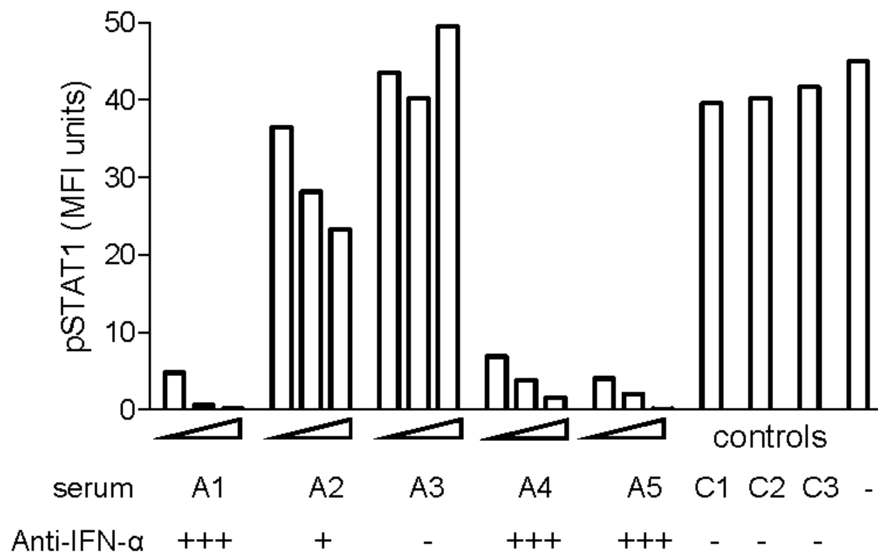

Figure 7. The effect of APECED patients and control sera on the STAT1 phosphorylation in PBMC form healthy controls. A1-A5 APECED patients. C1-C3 healthy controls. The presence of IFNα autoantibodies in the sera is shown below the graph. APECED sera were used in three different concentrations (2%, 5%, 10%).

5.3.3. The levels of CXCL10 in the APECED patients' sera

As our results show decreased expression of CXCL10 in PBMC, we decided to detect it's expression on protein level. We compared the levels of CXCL10 in the sera of APECED patients with healthy controls, heterozygous relatives and patients suffering of diseases not related to AIRE (autoimmune polyglandular syndrome 2 and Addison's disease).

Our results show, that despite the low mRNA level in blood cells, the APECED patients have increased levels of CXCL10 in their peripheral blood (Figure 8). The levels were significantly higher than in healthy controls ($p<0.01$), heterozygous relatives ($p<0.001$) or patients with diseases not related to Aire deficiency.

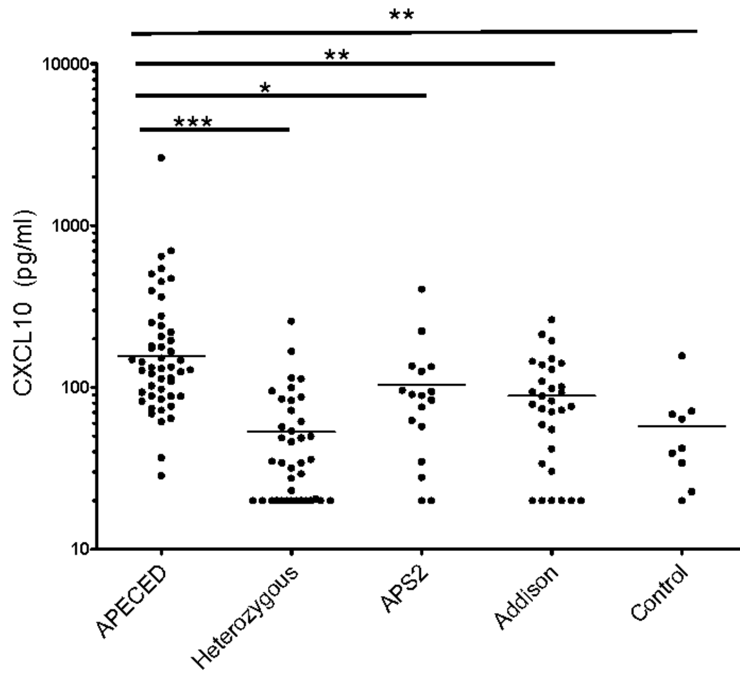

Figure 8. CXCL10 levels in APECED and control sera. Geometric means are indicated by lines. * $p<0.05$; ** $p<0.01$; *** $p<0.001$

6. DISCUSSION

6.1. Regulatory T cells and FoxP3 expression in type 1 diabetes

In our study we evaluated the frequency of Tregs in peripheral blood of newly diagnosed T1D patients (within the first week of insulin treatment), compared with healthy controls. We used two different ways of defining the Tregs: either CD4+CD25+FoxP3+ or CD4+FoxP3+CD127low. Independent of the definition of Tregs, we found similar frequency of Tregs in PBMC of T1D patients and healthy controls. This finding is in good correlation with some previous results. Though it has been suggested that T1D patients have lower frequency of CD4+CD25+ cells in their peripheral blood [155] or increased population of both CD4+CD25+FoxP3+ and CD4+FoxP3+CD127low cells in long-lasting diabetes, most of the studies have not been able to show any difference in peripheral blood Treg numbers between T1D patients and healthy controls, regardless of the duration of disease [156–161].

When we divided the study subjects according to the presence or absence of GADA, IA-2A, IAA or ICA in their sera, the Treg frequencies did not differ between the groups.

Interestingly, we found higher expression of both FoxP3 isoforms (full-length and Δ2) in PBMC of study subjects, positive for either GADA or IA-2A, compared with subjects negative for those autoantibodies. There was no similar difference, when we divided the study subjects by the presence of IAA or ICA or as T1D patients/healthy controls. As the frequency of CD4+FoxP3+ cells did not differ between the study subjects positive/negative for either GADA or IA-2A (data not shown), it is plausible that higher FoxP3 expression in those subjects derives from higher expression on single-cell level.

There is little evidence in literature on FoxP3 levels in diabetic patients. Grant *et al* found higher FoxP3 protein expression on single-cell level in patients' Tregs, compared with healthy controls in a study involving diabetic patients with well-established T1D and full metabolic control [64]. In contrast to that, a study on Swedish cohort of diabetic children showed no difference in the FoxP3 expression in PBMC, compared with healthy children [162]. The discrepancy between the study by Grant *et al* and our study may be due to difference in patients characteristics (patients with well established disease vs newly diagnosed patients), also there was no information about the patients' autoantibody positivity or negativity in the study by Grant *et al*.

In recent years, evidence has emerged, that the mechanism of action of FoxP3 is dose-dependent, rather than in on-and-off manner. Using targeted gene „knock-in model“ it has been shown, that decreased FoxP3 expression leads to abrogation of Treg suppressive activity, leading to aggressive autoimmune disease, phenotypically similar to FoxP3 deficient scurfy mice [163]. In a

mouse model of allograft transplantation (orthotopic corneal transplantation), the Treg cell numbers in draining lymph nodes were unchanged after the transplantation, but the FoxP3 levels in CD4+CD25+ Tregs isolated from lymph nodes were 2-fold higher in allograft acceptors, compared with rejectors; the Tregs from acceptors proved to be more efficient in terms of suppressive activity and cytokine production [25]. In another mouse model using FoxP3-transduced cells in the treatment of collagen-induced arthritis, the subset of cells with high FoxP3 expression had better suppressive potential both *in vitro* and *in vivo*, compared with FoxP3low subset [164]. Several studies have shown decreased FoxP3 expression accompanied by defective Treg function in patients with autoimmune diseases [56, 59].

In the light of these findings, it may be argued that Tregs in autoantibody-positive persons might have better regulatory potential, compared with Tregs in autoantibody-negative persons. This could reflect compensatory process, trying to downregulate the process of autoimmune destruction in tissues. This is in line with recent experiments on non-obese diabetic mice model [165], and data on diabetes patients [166] which suggest, that in development of diabetes, the autoagression to β-cells develops due to resistance of effector T cells to the suppression, rather than due to defective function of Tregs.

The question remains, whether the differential expression of FoxP3 in autoantibody positive and negative patients could influence the clinical course of the disease. So far, there is little evidence about the association of GADA or IA-2A with prognosis of T1D. It has been suggested, that positivity for IA-2A is associated with lower C-peptide levels and with the need of higher doses of insulin [167, 168], blood glucose levels have been shown to be higher in IA-2A positive patients [169]. On the other hand, in one study there was no association between the IA-2A and C-peptide [170]. There is also data about the association of lower C-peptide levels with GADA [167, 170], but other studies have not confirmed this finding [168, 169]. In newborns to diabetic mothers, the number of cord blood Tregs is positively correlated with the level of GADA in cord blood plasma [171] and the risk of developing diabetes is smaller in infants positive for either GADA or IA-2A at birth [172].

Analysing the study results, it has to be taken into consideration, that our results may at least partly be influenced by the treatment of patients. There is evidence, that insulin induces higher FoxP3 expression on mRNA level in PBMC of T1D patients, both after *in vitro* stimulation [162] and *in vivo*, after treating newly diagnosed patients with insulin for up to 21 days (median 8 days) [173]. Thus it can be argued, whether starting insulin treatment (the samples were collected within the first week of treatment) may have influenced the FoxP3 expression in our samples. It has to be underlined though, that difference seen in our study was associated with autoantibody positivity/negativity, not with the mere presence of diabetes (and thus insulin treatment).

In future perspective, functional capacity of Tregs in patients positive/ negative for GADA or IA-2A needs to be studied and possible clinical

associations between FoxP3 expression, Treg functionality and disease outcome evaluated.

6.2. TSGA10 autoantibodies

We have described a novel autoantigen in APECED patients, TSGA10 (see Paper II). The TSGA10 gene was identified in 2001, it was found to be expressed in testis and foetal tissues in humans [174, 175]. The precise localisation and function of the TSGA10 protein in testicular tissue is studied using mouse homologue (89% similarity with human gene, 94% identity on amino acid level). The gene was found to encode 65 kD protein, expressed in testis, in postmeiotic phase of spermatogenesis. Second isoform, likely result of posttranslational modification, is 27 kD protein containing N-terminal part of the full length TSGA10. The shorter isoform is predominant variant of the TSGA10 in mature spermatocytes. On subcellular level, the protein is located in the fibrous sheath in the principal piece (short isoform) and midpiece (full-length isoform) of sperm tail. It appears to be structural protein, forming cytoplasmic filaments. [175, 176].

We aimed to evaluate the frequency of TSGA10 autoantibodies in APECED patients and their possible association with the disease phenotype. Infertility is a frequent component of APECED syndrome. The most prevalent cause is hypogonadism, described in 31–61% patients [103, 121], but agglutinating sperm autoantibodies have also been described [177]. Given the expression pattern of TSGA10, we decided to analyse possible association with infertility and TSGA10 autoantibodies.

For detection of the TSGA10 autoantibodies, two different assays were used: radioimmunoprecipitation and Western blotting. The antibodies were detectable only by radioimmunoassay. Such discrepancy between two assays suggests conformational epitope, as in Western blotting the protein is in primary structure and conformational epitopes are not detectable. This explanation is likely, because most autoantibodies are thought to react with conformational epitopes [178].

Using radioimmunoprecipitation, we found TSGA10 autoantibodies in 7.6% of APECED patients, they are present both in men (7.5%) and women (7.7%). In other tested groups, healthy controls and patients with isolated Addison's disease, no TSGA10 autoantibodies were present.

In the group of APECED patients, we evaluated associations between TSGA10 autoantibodies and infertility. Out of 16 patients known to have gonadal insufficiency, only one female patient had TSGA10 autoantibodies. At the same time, from 5 patients positive for TSGA10, only one female had signs of gonadal insufficiency.

In APECED patients, autoantibodies against steroid cells and steroidogenic enzymes are associated with gonadal failure, sometimes preceding the clinical

manifestation for years [179, 180]. In the light of that, we analysed long-term data on our patients. It appeared that TSGA10 autoantibodies can be present for longer periods (up to 26 years in our cohort), without any clinical signs of gonadal failure.

The autoantigens among steroidogenic enzymes, found to be associated with gonadal failure in APECED patients are members of cytochrome P450 family, expressed in reproductive tissues – CYP11A1 and CYP17 [180–182]. In our patient group, autoantibodies against CYP11A1 and CYP17 were measured and potential association with TSGA10 autoantibodies evaluated. There was no statistically significant difference in the levels of TSGA10 autoantibodies between the patients positive or negative for CYP11A1/CYP17 autoantibodies.

As it is known, that TSGA10 is expressed in various malignant tissues in humans and TSGA10 autoantibodies are detected in cancer patients (in 3 patients out of 345) [183], we investigated the possible presence of malignancies in our patient group. No cases of malignant tumors occurred in the period of follow-up.

Noteworthily, antibodies against TSGA10, an antigen known to be expressed in adult humans only in males, were equally frequent in males and females. One possible explanation is, that TSGA10 may be expressed in other tissues besides testis and spermatocytes in adult humans. Supporting this, in adult mice different TSGA10 isoforms are expressed in several tissues, including brain, kidney, liver [176]. Other possibility is, that TSGA10 antibodies are not of autoimmune nature in women, but resemble antibodies directed against sperm antigens, seen in infertile women [184, 185].

We hypothesise, that TSGA10 antibodies may be specific for Aire-deficiency, similar to autoantibodies against type 1 interferons, NALP5, tyrosine hydroxylase, tryptophan hydroxylase and cytochrome P450 1A2 (see p. 19). To confirm this, additional studies are needed, involving patients with isolated autoimmune disorders and especially autoimmune infertility. Also, considering the rarity of TSGA10 autoantibodies, their possible associations with clinical manifestations of APECED syndrome need to be studied in larger patient cohorts.

6.3. IFN autoantibodies

The family of type 1 interferons (IFNα, IFNβ, IFNω and others) is produced mainly by plasmocytoid dendritic cells upon stimulation through toll-like receptors (TLR7, TLR9). Type 1 IFNs are designated to fight viral infections and intracellular parasites. Signalling through type 1 IFN receptor results in activation of the expression of hundreds of genes, leading to so called „antiviral state“. Antiviral effect is achieved by direct antiviral activity, enhancing antiviral T-cell reactivity and antibody production as well as sensitising infected cells to apoptosis. [186, 187]

Recent studies have shown the presence of neutralising autoantibodies against type 1 interferons in APECED patients' sera [124, 131, 132]. Our aim was to investigate the role of those autoantibodies *in vivo*.

Compared with healthy controls, we found in APECED patients significantly lower expression of interferon-stimulated genes in monocytes and freshly isolated plasmocytoid dendritic cells. In contrast to that, SLE patients, known to have higher levels of type 1 IFNs in their peripheral blood [188, 189], expressed the ISGs on significantly higher level, compared with APECED patients and healthy controls.

To further confirm the results from gene array analysis and quantitative RT-PCR, we evaluated the influence of APECED patients' sera, containing neutralising type 1 IFN antibodies to the expression of ISGs in monocytes, isolated from healthy donors. We found that patients' sera were able to inhibit the expression of ISGs *ex vivo*. Additionally, patients' sera were capable of inhibiting IFN signalling pathway.

After differentiating monocytes into moDC *in vitro* without presence of APECED sera containing IFN autoantibodies, the difference in gene expression decreases when evaluated by gene array and the expression of some transcripts was even higher in APECED patients when evaluated by quantitative RT PCR. This fact further confirms, that immediate presence of type 1 IFN auto-antibodies is necessary for downregulation of ISGs, seen in APECED patients monocytes and pDC.

Remarkably, the inhibitory potential seems to rely on neutralising antibodies against IFNα rather than other type 1 interferons. One patient without neutralising antibodies against IFNα had higher expression of all tested ISGs in PBMC, compared with patients positive for IFNα autoantibodies or healthy controls, and serum from this patient did not inhibit the expression of ISGs *ex vivo*.

In the light of the function of type 1 IFNs and our findings on down-regulation of ISGs in APECED patients, it would be expected that frequency of viral infections is increased in patients. However, this is not the case. Though a patient with recurrent herpes simplex virus has been reported [190], there is no general propensity to infections in APECED patients, apart from the chronic mucocutaneous candidosis [104, 118, 119, 121].

We next aimed to evaluate the association between downregulated expression of ISGs and corresponding protein levels in APECED patients. For this purpose, serum levels of CXCL10 in APECED patients were measured. CXCL10 is one of ISGs, downregulated in APECED patients, as shown by gene array analysis and confirmed by quantitative RT-PCR. CXCL10 is involved in antiviral response as a chemoattractant for antigen-specific T cells [191–193] and NK cells [193, 194], and plays a role in autoimmune responses [195] and anti-tumor immunity [196]. Surprisingly, significantly higher serum levels of CXCL10 were detected in APECED patients, compared with healthy controls and patients with autoimmune polyglandular syndrome type 2 or isolated

Addison's disease. There was approximately five fold difference in the average serum levels.

There is accumulating evidence about secretion of CXCL10 in target tissues of autoimmune destruction, induced mainly by IFNγ and partly also by type 1 IFNs [197–201]. Hence it is likely, that sites of autoimmune inflammation are the source of higher CXCL10 levels in APECED patients' sera and it is induced by IFNγ or IFNβ or other type 1 IFNs, which are recognised infrequently by autoantibodies [124]. Higher expression of ISGs in one patient without IFNα autoantibodies, compared to healthy controls, hints local overproduction of type 1 IFNs in APECED patients. As mainly IFNα antibodies appear to mediate the downregulation of ISGs, such local overproduction of other type 1 IFNs might contribute to the higher expression of CXCL10 protein in tissues.

There is another interesting aspect regarding the possible overproduction of type 1 IFNs in APECED patients. It has been shown, that exposure to high levels of IFNα during antiviral treatment leads frequently to thyroid autoimmunity [202, 203]. Noteworthily, one patient in our group, who did not have neutralising antibodies against IFNα, has thyroiditis, otherwise rare among Norwegian APECED patients [103, 119] and one patient with low titers of IFNα autoantibodies has thyroid peroxydase autoantibodies. Hence the production of type 1 IFNs in inflamed tissues in APECED patients, it's possible association with thyroid autoimmunity and protective effect mediated by neutralising IFNα autoantibodies are an appealing field for further studies.

We can conclude, that neutralising autoantibodies against type 1 IFNs, particularly against IFNα in APECED patients lead to downregulation of ISGs in several cell types in peripheral blood. This downregulation may though be compensated by the effect of INFγ and type 1 interferons other than IFNα in peripheral tissues, involved in autoimmune inflammation. The downregulation of ISGs is not associated with higher risk for viral infections. However, the type 1 IFN autoantibodies may contribute to the phenotype of APECED patients by mediating protective effect against thyroid autoimmunity.

7. CONCLUSIONS

1. The frequency of FoxP3 positive regulatory T cells in peripheral blood does not differ between newly diagnosed type 1 diabetes patients and healthy controls. The relative expression of FoxP3 isoforms (full length and Δ2 isoform lacking second exon) and their ratio in peripheral blood mononuclear cells is similar in diabetes patients and healthy controls. Positivity of either GAD65 or IA-2 autoantibodies is associated with higher expression of both FoxP3 isoforms in PBMC, but the ratio of the isoforms is not changed.
2. TSGA10 autoantibodies could be detected only in limited number (7.6%) of APECED patients and had no verifiable clinical associations.
3. Autoantibodies to IFNα, that are present in almost all of the APECED patients, cause the downregulation of interferon-stimulated genes in peripheral blood mononuclear cells, monocytes and plasmocytoid dendritic cells *in vivo*.
4. *In vitro* IFNα autoantibodies are able to inhibit phosporylation of STAT1, a crucial event in the intracellular interferon signalling pathway. IFNα autoantibodies, contained in APECED patients' sera, are able to block the interferons present in normal plasma and reduce the expression of interferon-stimulated genes in normal monocytes.

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SUMMARY IN ESTONIAN

Transkriptsioonifaktorid FoxP3 ja Aire: seos autoantikehadega

Autoimmuunsuse tekkemehhanismid ei ole praeguseks hetkeks veel lõplikult selged. Autoimmuunsuse patogeneesi uurimisel on väärtuslikuks materjaliks monogeensed autoimmuunhaigused. Enimuuritud on selles valdkonnas transkriptsioonifaktorid FoxP3 ja Aire ning nende defektist põhjustatud sündroomid IPEX (ingl k. *immunodysregylation, polyendocrinopathy and enteropathy, X-linked*) ja APECED (ingl k. *autoimmune polyendocrinopathy-candidosis-ectodermal dystrophy*).

FoxP3 on inimesel seotud regulatoorsete T rakkude tekke ja funktsiooniga. Selle defektist on tingitud IPEX, X-liiteline sündroom, mille puhul esinevad erinevate organite autoimmuunne kahjustus, enteropaatia, dermatiit ning sagedased nakkushaigused. FoxP3 erinevaid polümorfisme on seostatud mitmesuguste autoimmuunhaigustega. Uuritud on ka FoxP3 ekspressioonitaset ja FoxP3+ regulatoorsete rakkude hulga muutusi autoimmuunhaiguste korral, kuid ühest seost ei ole leitud.

Aire ekspresseerub inimesel eeskätt tüümuses, medullaarsetes epiteelirakkudes, ning on seotud perifeersetele kudedele spetsiifiliste antigeenide ekspressiooniga tüümuses. Aire defitsiidist on põhjustatud APECED, autosoomne retsessiivne sündroom, mille korral esineb erinevate endokriinorganite autoimmuunne kahjustus, ektodermaalset päritolu kudede düstroofia ning krooniline naha ja limaskestade kandidoos.

Uurimistöö eesmärgiks oli:

Määrata regulatoorsete T rakkude hulk ja FoxP3 ekspressioonitase 1. tüüpi suhkurtõve haigete perifeerses veres; hinnata seost FoxP3 ekspressioonitaseme, regulatoorsete T rakkude hulga ning autoantikehade taseme vahel patsiendide veres.

Määrata TSGA10 autoantikehade olemasolu ja tiiter APECED patsientide seerumis ning hinnata võimalikku seost APECED sündroomi kliinilise avaldumisega.

Hinnata APECED patsientidel esinevate neutraliseerivate 1. tüüpi interferoonide vastaste autoantikehade mõju geeniekspressioonile *in vivo* ja *in vitro*.

Materjal ja metoodika.

Uuringu esimeses osas kasutati seerumit ning perifeerse vere mononukleaarseid rakke, mis olid kogutud 1. tüüpi suhkurtõve haigetelt (n=26) vahetult pärast diagnoosimist (esimesel ravinädalal). Sama materjal koguti soo ja vanuse poolest sarnase koostisega kontrollgrupilt, kuhu kuulus 17 tervet isikut.

Seerumites määrati diabeedile iseloomulike autoantikehade esinemine. Perifeerse vere mononukleaarsetes rakkudes hinnati regulatoorsete T rakkude

(CD4+CD25+FoxP3+ T lümfotsüütide) esinemissagedust ning FoxP3 ekspressiooni mRNA tasemel.

Uuringu teises osas kasutati 66 APECED patsiendi seerumit, kontrollgruppi kuulusid 20 tervet meest, 96 tervet veredoonorit, 32 patsienti isoleeritud Addisoni tõvega.

Seerumites määrati TSGA10 vastaste autoantikehade esinemine, kasutades kahte erinevat meetodit (radioimmunopretsipitatsioon ja immunoblot).

Kolmandas uuringuetapis kasutati geenikiibi analüüsiks ühe APECED patsiendi ning kahe terve kontrollisiku monotsüüte ning *in vitro* monotsüütidest diferentseeritud dendriitrakke.

Edasisteks uuringuteks kasutati APECED patsientide seerumeid (n=8) ning perifeerse vere rakke. Kontrollgruppi kuulus 9 tervet isikut. Hinnati interferoonide poolt reguleeritavate geenide ekspressiooni monotsüütides, plasmotsütoidsetes dendriitrakkudes ning *in vitro* monotsüütidest diferentseeritud dendriitrakkudes.

APECED patsientide seerumites sisalduvate IFN autoantikehade mõju geeniekspressioonile hinnati *in vitro*, inkubeerides tervetelt kontrollisikutelt isoleeritud monotsüüte APECED patsientide või tervete kontrollisikute seerumite juuresolekul.

Et teha kindlaks IFN autoantikehade mõju interferoonide signaali ülekandele, inkubeeriti U937 monotsütaarse rakuliini või tervete isikute perifeerse vere rakke patsientide või tervete seerumitega ja mõõdeti rakusisest STAT1 fosforüleerimist.

CXCL10 taseme määramiseks kasutati APECED patsientide, nende Aire mutatsiooniga heterosügootsete tervete sugulaste, isoleeritud Addisoni tõvega patsientide, süsteemse luupus erütematoosusega patsientide ja tervete isikute seerumeid.

Tulemused ja arutelu.

CD4+CD25+FoxP3+ regulatoorsete T rakkude sagedus 1. tüüpi suhkurtõve haigete perifeersete lümfotsüütide hulgas ei erine oluliselt nende rakkude sagedusest tervetel (mediaan vastavalt 0,31% ja 0,44%). Samuti ei erine oluliselt FoxP3 ekspressioon perifeersetes mononukleaarsetes rakkudes patsientide veres ja tervetel, sarnaselt olid ekspresseeritud mõlemad inimese regulatoorsetes rakkudes esinevad FoxP3 isovormid.

Käesolevas uuringus ilmnes oluliselt kõrgem FoxP3 (nii täispika kui Δ2 isovormi) ekspressioon perifeerses veres neil uuritavatel, kellel esinesid kas GAD65 või IA-2A autoantikehad. Sarnast seost ei olnud insuliini autoantikehade ega Langerhansi saarekeste vastaste autoantikehadega (ICA).

Kuna viimastel aastatel on näidatud FoxP3 toime sõltuvust tema ekspressioonitasemest, võib oletada, et kõrgema FoxP3 ekspressioonitasemega uuritavate perifeerses veres kaasneb ka nende regulatoorsete T rakkude võime

tõhusamalt immunvastust pärssida. Praeguseks pole selge, milline võiks olla GAD65 või IA-2 autoantikehadega isikutel kõrgema FoxP3 ekspressiooni mõju suhkurtõve kliinilisele kulule. Senised andmed GAD65 ja IA-2A autoantikehade seosest diabeedi prognoosiga on olnud vastukäivad.

Kasutades APECED patsientide seerumeid, on identifitseeritud uus autoantigeen, TSGA10 (vt. artikkel II). Kahe erineva meetodi, radioimmunopretsipitatsiooni ning immonobloti abil määrati TSGA10 autoantikehade sagedus APECED patsientidel, isoleeritud Addisoni tõvega patsientidel ning tervetel. Antikehi leiti 7,6% APECED patsientidest, isoleeritud Addisoni tõve korral ega tervetel kontrollidel neid ei esinenud. Hinnati ka seost TSGA10 autoantikehade ning APECED sündroomi kliinilise pildi vahel. Arvestades TSGA10 ekspressiooni testises ning spermatotsüütides, oli peamiseks hüpoteesiks autoantikehade seos viljatusega. Antud uuritavate grupis ei õnnestunud näidata seost hüpogonadismi ja TSGA10 autoantikehade esinemise vahel.

TSGA10 autoantikehi esines uuritavate hulgas ainult APECED patsientidel, isoleeritud Addisoni tõvega patsientidel ega tervetel isikutel autoantikehi ei leitud. On võimalik, et tegu on Aire defitsiidile spetsiifiliste autoantikehadega, mida ei esine isoleeritud autoimmuunhaiguste korral. Sarnaselt on APECED sündroomile spetsiifilised näiteks 1. tüüpi interferoonide vastaste autoantikehad, tsütokroom p450 1A2, türosiini hüdroksülaasi, trüptofaani hüdroksülaasi ja kõrvalkilpnäärme antigeeni NALP5 vastased autoantikehad. See hüpotees vajab siiski kontrollimist suuremal patsientide grupil ja erinevate isoleeritud autoimmuunhaiguste korral.

Et hinnata 1. tüüpi interferoonide vastaste neutraliseerivate autoantikehade mõju geeniekspressioonile, kasutati geenikiibi analüüsi ja kvantitatiivset RT-PCR analüüsi. Võrreldes tervetega oli interferoonide poolt stimuleeritavate geenide (ISG) ekspressioon APECED patsientide monotsüütides ning plasmotsütoidsetes dendriitrakkudes oluliselt madalam. IFN autoantikehad patsientide seerumis olid võimelised *in vitro* inhibeerima ISG ekspressiooni monotsüütides ning samuti pärssima interferoonide kaudu toimuvat signaaliülekannet perifeerse vere mononukleaarsetes rakkudes.

1. tüüpi interferoonide ülesanne immuunsüsteemis on eeskätt viirustevastane kaitse. IFN toimel muutub sadade geenide ekspressioon, mille tulemuseks on nn „viirustevastane seisund“. Kuna APECED patsientidel on ISG ekspresseeritud madalamal tasemel, võiks oletada sagedasemat viirushaiguste esinemist nendel patsientidel. Senised kliinilised andmed seda siiski ei kinnita, kirjeldatud on vaid ühte patsienti korduvate herpesviirusnakkustega. Üldist viirusinfektsioonide sagenemist APECED sündroomi korral näidatud ei ole.

Käesolevas töös hinnati ka IFN autoantikehade mõju geeniekspressioonile valgu tasemel. Patsientide veres määrati CXCL10 tase. CXCL10 geen kuulub interferoonide poolt reguleeritavate geenide hulka ning selle ekspressioon on APECED patsientidel madalam kui tervetel.

On märkimisväärne, et CXCL10 valgu tase patsientide seerumites oli oluliselt kõrgem kui tervetel. Tõenäoline selgitus sellisele erinevusele geeniekspressiooni taseme ja valgu hulga vahel veres on valgu pärinemine perifeersetest kudedest. Autoimmuunse põletiku koldes on näidatud CXCL10 sekretsiooni, mida indutseerib IFNγ ning vähemal määral 1. tüüpi interferoonid. Seega on tõenäoline, et APECED patsientidel nähtud kõrge CXCL10 taseme allikaks on selle tootmine autoimmuunsest protsessist haaratud kudedes.

Järeldused:

1. FoxP3+ regulatoorsete rakkude hulk perifeerses veres ja FoxP3 ekspressioon perifeerse vere mononukleaarsetes rakkudes ei erine 1. tüüpi diabeediga patsientidel ja tervetel isikutel. GAD65 või IA-2A autoantikehade olemasolu organismis on seotud kõrgema FoxP3 ekspressioonitasemega perifeerse vere mononukleaarsetes rakkudes.
2. TSGA10 autoantikehi esineb piiratud hulgal APECED patsientidest (7,6%), nende seost sündroomi kliinilise avaldumise eripäradega ei õnnestunud näidata.
3. APECED patsientide seerumis leiduvad IFNα vastased autoantikehad pärsivad interferoonide poolt reguleeritavate geenide ekspressiooni perifeersete vere mononukleaarsetes rakkudes, monotsüütides ning plasmotsütoidsetes dendriitrakkudes *in vivo*.
4. IFNα autoantikehad on võimelised *in vitro* inhibeerima rakusisest interferoonide signalisatsioonirada ning pärssima interferoonide poolt reguleeritavate geenide ekspressiooni normaalsetes monotsüütides.

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CURRICULUM VITAE

MAIRE LINK

Born: February 13, 1979, Tartu, Estonia
Citizenship: Estonian
Address: University of Tartu, Department of General and Molecular Pathology, Ravila 19, Tartu, Estonia
Phone: +372 7 374 239
E-mail: maire.link@ut.ee

Education

1994–1997 Gustav Adolf Gymnasium, Tallinn, Estonia
1997–2004 University of Tartu, Faculty of Medicine, medicine
2004–2009 University of Tartu, Faculty of Medicine, PhD student in medical science

Scientific work

Research fields: type 1 diabetes, cellular immunity in autoimmune poly-endocrinopathy-candidosis-ectodermal dystrophy, the role of Langerhans' cells in the immunity against *Candida albicans*.
4 publications, 8 presentations at international congresses

Memberships

Estonian Society for Immunology and Allergology
Ethics Review Committee on Human Research

CURRICULUM VITAE

MAIRE LINK

Sündinud: Tartus, 13. veebruaril 1979
Kodakondsus: Eesti
Aadress: TÜ Üld- ja Molekulaarpatoloogia Instituut, Ravila 19, Tartu
Telefon: +372 7 374 239
E-mail: maire.link@ut.ee

Haridus

1994–1997 Gustav Adolfi Gümnaasium, Tallinn
1997–2004 Tartu Ülikool, Arstiteaduskond, arstiteaduse eriala
2004–2009 Tartu Ülikool, Arstiteaduskond, arstiteaduse eriala doktorantuur

Teadustegevus

Uurimisvaldkonnad: 1 tüüpi diabeet, rakulised immuunreaktsioonid autoimmuunse polüendokrinopaatia-kandidoosi-ektodermaalse düstroofia korral, Langerhansi rakkude roll *Candida albicansi* vastases immuunsuses
4 publikatsiooni, 8 ettekannet rahvusvahelistel teaduskonverentsidel

Kuuluvus erialaseltsidesse ja muudesse organisatsioonidesse

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Tartu Ülikooli inimuuringute eetika komitee

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