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JAANIKA KÄRNER

Cytokine-specific autoantibodies in AIRE deficiency

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Contribution of Jaanika Kärner to original publications:

Study I: Performed the experiments and the data analysis, prepared the figures and wrote the paper.

Study II: Performed the Luciferase Immunoprecipitation assays (LIPS) experiments and analyzed the data.

Study III: Performed the real-time PCR experiments and data analysis, participated in paper writing.

Study IV: Performed the experiments and data analysis, prepared the figures and wrote the paper.

Study V: Performed the LIPS experiments for determining type I IFN-specific autoantibodies and cell-based type I IFN neutralizing assays, participated in the data analysis and figure preparation.

ABBREVIATIONS

aa	amino acid
AADC	aromatic L-amino acid decarboxylase
AChR	acetylcholine receptor
AD	Addison's disease
AIH	autoimmune hepatitis,
AIRE/Aire	autoimmune regulator, gene designation in human/mouse
AIRE/Aire	autoimmune regulator, protein designation in human/mouse
AL	alopecia
AP	alkaline phosphatase
APECED	Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy
APRIL	A proliferation-inducing ligand
ATF7ip	activating transcription factor 7 interacting protein
BAFF	B cell activating factor
BPIFB1	bactericidal/permeability-increasing fold-containing B1
CARD	caspase recruitment domain
CaSR	calcium-sensing receptor
CBP	CREB-binding protein
CD	cluster of differentiation
CMC	chronic mucocuteneous candidiasis
CRMP	collapsing response mediator protein
CYP11A1	side chain cleavage enzyme
CYP17A1	steroid 17-α-hydroxylase
CYP1A2	cytochrome P450 1A2
CYP21	steroid 21-hydroxylase
CYP2A6	cytochrome P450 Family 2 Subfamily A Member 6
DC	dendritic cell
DEFA5	defensin, alpha 5
EBI-1	Epstein-Barr virus-induced G-protein coupled receptor 1, CCR7
EC	extracellular
ED	enamel dysplasia
EpCAM	epithelial cell adhesion molecule
Ets	E26 transformation-specific
GAD	glutamic acid decarboxylase
GID	gastro-intestinal dysfunction
Gluc	*Gaussia* luciferase
HDC	histidine decarboxylase
HLA	human leucocyte antigen
HP	hypothyroidism
HSR	homogeneously stained region
HT	hypothyroidism
IA-2	insulinoma-associated tyrosin phosphatase–like protein
IC	intracellular

IF	intrinsic factor
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
ILD	interstitial lung disease
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
ISG	interferon stimulated genes
ISRE	IFN-stimulated response element
JMJD6	Jumonji Domain Containing 6, arginine demethylase and lysine hydroxylase
KCNRG	potassium channel-regulating protein
KO	knock-out
KRT8	keratin 8
M	membrane
MAdCAM	mucosal addressin cell adhesion molecule-1
MAGEB2	melanoma antigen family B 2
MBD1	methyl-CpG-binding domain protein 1
MG	myasthenia gravis
MHC	major histocompatibility complex
MuSK	muscle-specific kinase
mTEC	medullary epithelial cell
NAA	nuclear autoantigens
NLS	nuclear localization signal
NMT-	neuromyotonia
NNA-1	neuronal nuclear antigens
NOD	non-obese diabetic
NU	neutralizing units
OF	ovarian failure
PA	pernicious anemia
PBMC	peripheral mononuclear cells
PDIA2	protein isomerase A2
PDILT	protein disulfide isomerase-like testis expressed
PHD	plant homeodomain
PRR	proline rich region
P-TEFb	positive transcription elongation factor
RANK	receptor activator of nuclear factor κ B
RANKL	receptor activator of nuclear factor kappa-B ligand
rh	recombinant human
RU	relative luciferase units
RyR	ryanodine receptor
SAND	Sp100, AIRE, NucP41/75, DEAF-1
SLE	systemic lupus erythematosus
SOX	SRY-Box

STAT	signal transducer and activator of transcription
T1D	type 1 diabetes
TCR	T cell receptor
TDRD6	tudor domain containing protein 6
TG	thyroglobulin
TGM4	transglutaminase 4
TH	tyrosine hydroxylase
TIN	tubulo-interstitial nephritis
TNF	tumor necrosis factor
TPH1	tryptophan hydroxylase 1
TPO	thyroid peroxidase
TRP-1	tyrosinase related protein-1
Treg	regulatory T cell
TSA	tissue restricted antigen
TSGA10	testis-specific gene 10 protein
TSHR	thyroid-stimulating hormone receptor
VGKC	voltage-gated potassium channel complex
VIT	vitiligo

1. INTRODUCTION

Many Greek physicians believed that the human soul is located in the thymus, the latter being the basis of one's existence. In the context of immune system, the thymus is indispensable, as defects in thymus lead to severe malfunctions of the whole body. One of the key factors needed in normal thymus function, and in the development of central tolerance, is the autoimmune regulator (AIRE), which is conserved from cartilaginous fish to all jawed vertebrates. The best known function of AIRE is the regulation of the tissue-specific autoantigen (TSA) expression in thymus. These TSAs are presented to T-cell progenitors and needed to negatively select the autoreactive ones.

Mutations in AIRE directly cause a severe autoimmune disease – autoimmune-polyendocrinopathy-candidiasis-ectodermal-dystrophy (APECED). Besides having severe dysfunctions of many endocrine organs, the patients show a unique pattern of autoantibodies. The ones specific to type I IFNs and Th17 related cytokines are shared with thymus tumor patients, who often also lack AIRE expression in their tumors. The mechanism leading to the development of these cytokine-specific autoantibodies has remained unexplained, as AIRE is not directly linked to type I interferons (IFNs) and Th17 related cytokine production.

In the current thesis, we focus on studying APECED and its mouse model's autoantibody profile, their dynamics, function/significance *in vivo*, and the characterization of the patients' specific immunogenic epitopes of the cytokines. Additionally, we describe thymoma patients with similar autoantibodies as APECED, and study the tissue-restricted antigen expression in their thymi as a potential model, in order to better understand the processes taking place in APECED thymus.

2. REVIEW OF THE LITERATURE

2.1. AIRE

The autoimmune regulator gene was cloned almost 20 years ago (Nagamine et al. 1997, Finnish-GermanAPECEDConsortium 1997), whereas the syndrome APECED (Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy) caused by mutations in the former, was characterized already in the 1980s (Ahonen 1985). *AIRE* gene is located in the long arm of the 21st chromosome (21q22.3). It is 13kb long, has 15 exons, and bears a 71% similarity with the respective mouse gene (Mittaz et al. 1999, Nagamine et al. 1997, Finnish-GermanAPECEDConsortium 1997). *AIRE* is mainly expressed in medullary thymic epithelial cells (mTECs), and localizes in nuclear dots or diffusely, although in some cases, cytoplasmic accumulation has also been noted (Björses et al. 1999).

2.1.1. AIRE protein

AIRE protein consists of four main domains and bears resemblance to transcriptional regulator (Figure 1). At N-terminus, the protein has CARD (caspase recruitment domain)/ HSR (homogeneously stained region), which is needed to oligomerize and interact with the DNA (Maslovskaja et al. 2015, Ferguson et al. 2008). AIRE protein oligomerization is important to increase the overall avidity towards histone 3 lysine 4 (H3K4) (Kumar et al. 2001, Koh et al. 2008). The CARD domain is followed by nuclear localization signal (NLS) region. The only active NLS in 113-114 amino acids that is needed for functional interaction with nuclear pore transport receptors and for effective translocation of AIRE (Saltis et al. 2008, Ilmarinen et al. 2006). Although the SAND (Sp100, AIRE, NucP41/75, DEAF-1) domain has lost the traditional DNA-binding motif, the β-pleated sheet enables protein-protein interactions with repressive complex of transcription (Gibson et al. 1998, Waterfield et al. 2014).

In the C-terminal part of the protein lie two plant homeodomain fingers (PHD), separated by a proline rich region (PRR) (Saltis et al. 2008). PHD1 domain is negatively charged and necessary for the interaction with the hypomethylated H3K4, and may recruit other protein complexes to chromatin structures (Org et al. 2008, Gaetani et al. 2012). In contrast, the PHD2 has a positive electrostatic surface that, in theory, could allow direct interactions with DNA. In both PHD domains, zinc ions are essential for the proper conformation, and are also indispensable for subcellular localization and AIRE transcriptional activity (Gaetani et al. 2012, Perniola and Musco 2014). In addition, AIRE comprises four LXXXLL motifs, which may influence transcription, as they interact with nuclear receptors (Savkur and Burris 2004).

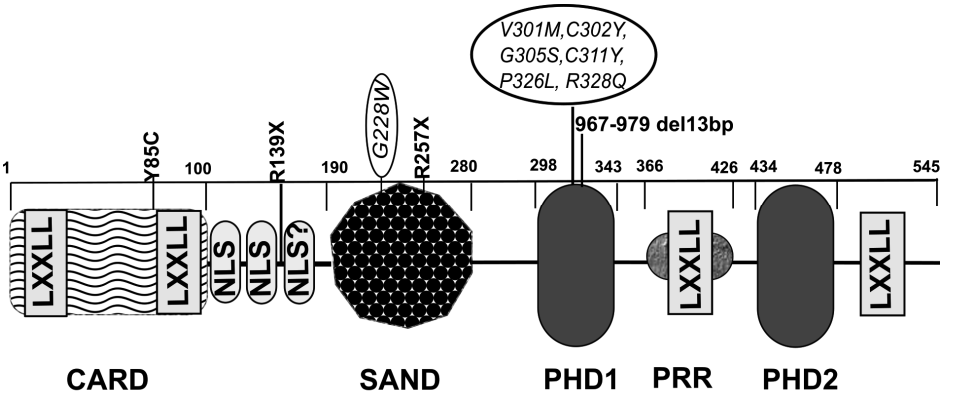

Figure 1. AIRE protein structural domains and the most common mutations. Mutations in circles are autosomal dominant. CARD (caspase-recruitment domain); NLS (nuclear localization signal), SAND (SP100, AIRE1, NucP41/P75, and DEAF1 domain); PHD (plant homeodomain), PRR-(proline rich region).

AIRE is indispensable for the regulation of the TSA expression, the medullary thymic epithelial cells (mTEC) maturation and differentiation (Nishikawa et al. 2014, Wang et al. 2012, Yano et al. 2008, Matsumoto 2011). AIRE expression is present in fetus before gastrulation (Nishikawa et al. 2010), and is needed for the maintenance of self-renewal and proliferation in embryonic stem cells (Gu et al. 2010). AIRE mRNA is found in lymph nodes, testes, ovary, keratinocytes, spleen and fetal liver (Anderson et al. 2002, Halonen et al. 2001, Schaller et al. 2008, Heino et al. 1999, Heino et al. 2000, Poliani et al. 2010, Hobbs et al. 2015).

Interestingly, a certain population of thymic B cells, as well as the peripheral lymph node dendritic cell-like population ($MHCII^{hi}$, $CD45^{lo}$, $EpCAM^{hi}$, $CD11c^{lo}$, $CD80^{lo}$, $CD86^{lo}$), can acquire *AIRE* expression. *AIRE* expression in these populations is several times lower than in the thymus, and the upregulated TSA repertoire is also different, but is necessary for tolerance induction in specific CD4+ T cells (Yamano et al. 2015a, Gardner et al. 2008, Gardner et al. 2013, Poliani et al. 2010, Yamano, Steinert and Klein 2015b).

For *AIRE* expression, its promoter has a functional TATA box, a GC box and inverted CCAAT, plus several transcription factor binding sites (e.g. Ets-family proteins, activator protein-1) (Murumägi, Vahamurto and Peterson 2003, Murumägi, Silvennoinen and Peterson 2006). The timing of *AIRE* expression is controlled through discrete time points and dependent on the differentiation of mTECs (Metzger et al. 2013). During mTEC differentiation, *AIRE* promoter region H3K4 is fully methylated, and the proximal CpG island is hypomethylated (Org et al. 2009, Kont et al. 2011, Murumägi et al. 2003, Murumägi et al. 2006). *AIRE* expression in the thymus and lymph nodes is also regulated by a conserved noncoding sequence that has two functional NF-κB-sites (LaFlam et al. 2015, Haljasorg et al. 2015). It has been shown that tumor necrosis factor

(TNF) superfamily members contribute to creating functional thymic environment, whereas the RANK-RANKL signaling directly mediates the upregulation of *AIRE* and its dependent genes (Khan et al. 2014, Bichele et al. 2016, Akiyama et al. 2005, Akiyama et al. 2008). *AIRE* mRNA splicing is important to obtain functional protein, which is controlled by dioxygenase JMJD6 (Yanagihara et al. 2015).

2.1.2. AIRE function in immune tolerance

AIRE is best characterized as being the key figure controlling the promiscuous tissue-specific antigens' expression in mTECs and presenting them to maturing thymocytes. A single mTEC is able to express ~4000 genes, which are presented directly or via dendritic cells (DC) to maturing T cells. This is necessary for the negative selection of autoreactive T cells (Anderson et al. 2002, Liston et al. 2003, Sansom et al. 2014, Derbinski et al. 2005). AIRE+ mTECs upregulate the different chemokines needed for thymocyte migration and for the accumulation of the thymic dendritic cells that are indispensable for regulatory T cells (Treg) induction (Laan et al. 2009, Lei et al. 2011). In addition, the absence of AIRE disturbs the correct differentiation of Tregs – leading to a situation where the conventional T cell pool includes T cell receptors (TCRs) that would be regulatory T cells (Malchow et al. 2016, Malchow et al. 2013, Pomié et al. 2011).

The exact mechanism whereby AIRE controls the TSA expression in mTECs is largely unknown, but can be deduced by studying the interacting partners (Abramson et al. 2010). Despite having some features of a transcription factor, activation through AIRE is non-traditional, meaning it does not bind to the specific DNA motif in promoter, intron or polyadenylation sequences (Maslovskaja et al. 2015, Mathis and Benoist 2009). AIRE seems to bind transcriptional start sites of many genes and to interact with stalled RNA polymerase II (Giraud et al. 2012). However, only transcriptionally inactive genes (carrying unmethylated H3K4) are able to stabilize AIRE on chromatin and recruit AIRE interacting partners, e.g. DNA topoisomerase 2-alpha, which leads to DNA double strand breaks and recruits DNA repair proteins (most importantly DNA-dependent protein kinase), and leads to chromatin opening and to transcription elongation with the help of bromodomain-containing protein 4, factor P-TEFb and coactivator CBP binding (Abramson et al. 2010, Liiv et al. 2008, Zumer et al. 2012, Abramson and Goldfarb 2016, Chuprin et al. 2015, Oven et al. 2007, Yoshida et al. 2015). Recently, deacetylase Sirtuin-1 was found to be an activator of AIRE transcriptional activity (Chuprin et al. 2015). In addition, AIRE binds to methylated CpG dinucleotides of inactive genes through interaction with repressive MBD1-ATF7ip complex, and leads to TSA expression (Waterfield et al. 2014). It should be noted that AIRE is probably not solely responsible for TSA expression in mTECs. This is supported by the newly described transcription factor Fez that regulates TSAs directly and independently from AIRE (Takaba et al. 2015).

AIRE has been shown to interact with the nuclear matrix and has been suggested to influence the chromatin looping, and to bring together transcriptional machinery and enhancer regions (Johnnidis et al. 2005, Razin, Iarovaia and Vassetzky 2014). Furthermore, AIRE associates with splicing factors and RNA helicases needed to process pre-mRNA (Abramson et al. 2010, Giraud et al. 2014). At the same time, cells expressing AIRE are more prone to apoptosis, which could be an additional mechanism to amplify TSAs presentation via DC. It is plausible that apoptosis may result from intensive TSA expression, but post-AIRE cells have also been described by many groups (Colome et al. 2010, Liiv et al. 2012, Matsumoto 2011, Wang et al. 2012, Metzger et al. 2013).

2.2. APECED

As *AIRE* gene is indispensable in controlling the induction of central tolerance, mutations affecting the function of AIRE protein cause rare, mostly recessive autoimmune disease APECED (Nagamine et al. 1997, Finnish-German-APECEDConsortium 1997). Chronic mucocutaneous candidiasis, hypoparathyroidism and adrenal failure are the three classic APECED components (Husebye et al. 2009). According to the Human Gene Mutation Database, over 100 different mutations have been found in AIRE gene with the prevalence of 1:100,000 [HGMD® Professional 2016.1;(Kahaly 2009)]. However, human populations that have undergone founder effects and/or geographic isolation, carry preferably the disease causing mutations [e.g. Finns 1:25,000 (R257X); Iranian Jews 1:9,000 (Y85C), Sardinian 1:14,400 (R139X)], although prevalence is also higher in Norway [1:80,000 (967–979del13bp)] and Slovenia [1:43,000 (R257X)] (Peterson and Peltonen 2005, Podkrajsek et al. 2005, Myhre et al. 2001, Zlotogora and Shapiro 1992, Rosatelli et al. 1998).

AIRE gene mutations are mostly recessive, emerge throughout the protein, although concentrating more in functional domains (Fig. 1) and result in the interference in correct nuclear localization and full transcriptional activity (Björses et al. 2000). Most common mutations are 13 base-pair deletion (967–979del13bp) in 8 exon and R257X in exon 6: responsible for truncated protein, and exhibiting correlation with chronic *Candida* infection (Wolff et al. 2007, Björses et al. 2000). The autosomal dominant mutations that have been found tend to locate in the SAND and PHD1 domains (Fig. 1), interact and disturb the function of AIRE (Oftedal et al. 2015, Su et al. 2008). Mutations outside the protein domains have been reported, including complete deletion, insertions, splicing sites (Podkrajsek et al. 2008, Cihakova et al. 2001, Mora et al. 2014, Bruserud et al. 2016b). Also, changes in *AIRE* gene regulatory regions could alter the *AIRE* transcription (Lovewell et al. 2015, LaFlam et al. 2015, Haljasorg et al. 2015).

The prevalence of dominant mutations seems to be higher than previously thought. These are present also in mixed populations and patients with these mutations demonstrate non-classical and milder APECED symptoms (Oftedal et

al. 2015, Bruserud et al. 2016b). The best known phenotype is glycine change to tryptophan in SAND domain (at the position of 228) with pronounced tendency to hypothyroidism (Cetani et al. 2001, Su et al. 2008, Oftedal et al. 2015). Interestingly, among recessive mutations, there is no good genotype-phenotype correlation aside of *Candida* infection, which is 100% present in patients with the mutations R257X and R139X, rare in patients with Y85C and 967–979del13bp (Kisand et al. 2010, Kisand et al. 2011, Björses et al. 2000). Instead, some haplotypes of human leucocyte antigen (HLA) show correlation with clinical manifestations [e.g. Addison's disease (HLA-DRB1*03); alopecia (HLA-DRB1*04- DQB1*0302)] (Halonen et al. 2002).

Interestingly, a study in Norwegian population showed that HLA class II haplotypes that are otherwise protective or rare in known diseases [Addison disease (AD), type 1 diabetes (T1D)] have no protective effect in the case of AIRE deficiency (Bruserud et al. 2016a).

2.2.1. Organ-specific autoantibodies and clinical manifestations

APECED clinical picture and disease progression is quite variable between patients, even with the same mutation and between close relatives. The emergence of first symptoms can take up to 18 years, although it mostly starts in the first months/years of life with the chronic mucocutaneous candidiasis that is followed by parathyroid and adrenocortical failures (Perheentupa 2006, Ahonen et al. 1990). APECED patients usually have three to five medical conditions over lifetime; however, diagnosis is made if at least two of the above-mentioned symptoms are present. In addition, type I IFN-specific autoantibodies and sequencing of AIRE gene also contribute to final diagnosis (Meloni et al. 2008, Perheentupa 2006, Husebye et al. 2009).

Only two thirds of patients develop the classic APECED triad; others may display for many years the more rare symptoms (e.g. autoimmune hepatitis, vitiligo, alopecia, rash, keratoconjunctivitis, periodical fevers, gastrointestinal dysfunction), which may hinder diagnosis (Perheentupa 2006, Kisand and Peterson 2015, Ahonen et al. 1990). This is especially seen in USA populations, who are genetically more diverse (e.g. compound heterozygosity) (Ferre et al. 2016). Developing autoimmune failures is sometimes correlated with high-titer organ-specific autoantibodies (Table 1).

Table 1. Organ-specific autoantigens and the associated autoimmune disease in APECED patients. Modified from (Kisand and Peterson 2015, Landegren et al. 2015, Landegren et al. 2016).

Autoantibody targets	**%**	**Associated with**	**Expressed in**
steroid 21-hydroxylase (CYP21A2)	55–69	AD	adrenal, IC
steroid 17-α-hydroxylase (CYP17A1)	24–58	AD, OF	adrenal, IC
side chain cleavage enzyme (CYP11A1)	38–68	OF	adrenal, ovary, testis, IC
NACHT leucine-rich-repeat protein 5 (NALP5)	32–49	HP	ovary, parathyroid, breast, testis, IC
calcium-sensing receptor (CaSR)	86	HP	parathyroid, pancreas, kidney, IC, M
thyroglobulin (TG)	15–21	HT	thyroid, IC, EC
thyroid peroxidase (TPO)	15–36	HT	thyroid, IC, EC
islet antigen-2 (IA-2)	7	T1D	pancreas, IC, M
glutamic acid decarboxylase (GAD65)	27–42	T1D	pancreas, brain, IC, M
testis-specific gene 10 protein (TSGA10)	8		testis, brain, IC
transglutaminase 4 (TGM4)	52–78	prostatitis	prostate, IC, EC
protein disulfide isomerase-like testis expressed (PDILT)	30	gonadal insufficiency?	gonadal germ cells, IC
melanoma antigen family B 2 (MAGEB2)	34	gonadal insufficiency?	gonadal germ cells, IC
tudor domain containing protein 6 (TDRD6)	49		testis, brain, IC, M
intrinsic factor (IF)	15–30	PA	stomach, IC, M, EC
aromatic L-amino acid decarboxylase (AADC)	39–68	AIH, VIT	kidney, intestine, brain, liver, pancreas, IC, EC
cytochrome P450 1A2 (CYP1A2)	6–8	AIH	liver, IC
Cytochrome P450 Family 2 Subfamily A Member 6 (CYP2A6)		AIH	liver, IC
tryptophan hydroxylase 1 (TPH1)	28–61	GID, AIH	multiple, IC

Autoantibody targets	%	Associated with	Expressed in
histidine decarboxylase (HDC)	37	GID	brain, stomach, lung, IC
tyrosine hydroxylase (TH)	44–50	AL, ED	brain, adrenal, IC, M
SOX9/SOX10	15–22	VIT	nervous system, breast, IC
potassium channel-regulating protein (KCNRG)	6	ILD	lung, cervix, IC
bactericidal/permeability-increasing fold-containing B1 (BPIFB1)	10	ILD	lung, stomach, esophagus, cervix, EC
Defensin, alpha 5 (DEFA5)	27	GID	Paneth cells, IC, EC

AD: Addison's disease, AIH: autoimmune hepatitis, AL: alopecia, CMC: chronic mucocutaneous candidiasis, EC: extracellular, ED: enamel dysplasia, GID: gastro-intestinal dysfunction, HP: hypoparathyroidism, HT: hypothyroidism, IC: intracellular, ILD: interstitial lung disease, M: plasma membrane, OF: ovarian failure, PA: pernicious anemia, T1D: type 1 diabetes, TIN: tubulo-interstitial nephritis, VIT: vitiligo

As AIRE is involved in the mechanisms of self-tolerance and APECED patients develop a wide number of autoimmune diseases, one would expect that APECED patients are prone to a large variety of autoimmune diseases. Interestingly, mainly endocrine organs are affected: e.g. parathyroid (77–96 %), adrenal cortex (63–92%), ovary (60%), pancreatic islets (up to 30%) (Perheentupa 2006, Wolff et al. 2007, Meloni et al. 2012, Kisand and Peterson 2015).

In addition, APECED patients share some organ-specific autoantibodies with other known autoimmune diseases: e.g. anti-CYP21A2 with AD and APS-2; anti-GAD65 with T1D. At the same time, they develop also quite unique pattern of organ-specific autoantibodies – many of those target hormone-/neurotransmitter- synthesizing enzymes, which are *in vivo* protected by their intracellular location (Table 1) (Peterson and Peltonen 2005, Kluger, Krohn and Ranki 2013, Kisand and Peterson 2015, Ahonen et al. 1990).

2.2.2. Cytokine-specific autoantibodies

One of the most peculiar features of APECED patients are high-titer cytokine-specific autoantibodies, as AIRE is not directly regulating cytokine gene expression in mTECs. Autoantibodies against type I IFN are used as diagnostic markers, because they are so prevalent and present before any clinical symptoms (Meloni et al. 2008, Meager et al. 2006, Toth et al. 2010). APECED patients' sera recognize and neutralize IFN-ω (100%) and IFN-α (95%), and IFN-β (22%) and IFN-λ (14%). Although the autoantibodies inhibit the biological function of interferons *in vitro* and downregulate IFN-dependent genes, the susceptibility towards viral infections is not increased (except for very rare

cases of viral encephalitis) and IFN-producing dendritic cell numbers are unaffected. This is probably because IFNs affect cells locally and other interferon types may compensate (Meager et al. 2006, Kisand et al. 2008, Kisand et al. 2011).

The other group of cytokines targeted in APECED is the one produced by Th17 cells – interleukin (IL)-22 (91%), IL-17F (75%) and IL-17A (41%) (Puel et al. 2010, Kisand et al. 2010). Interestingly, IL-22 and IL-17F antibodies correlate with *Candida* infection (Kisand et al. 2010). In addition, stimulated peripheral mononuclear cells (PBMCs), steady-state and skin memory CD4+ T cells of autoantibody positive APECED patients' show decreased IL-22 production (Laakso et al. 2014, Ahlgren et al. 2011, Kisand et al. 2010). While the amount of IL-17F and IL-22 producing PBMCs in APECED patients is reduced, the IL-17A expression can even be increased, indicating that the immune response is more towards IL-22 producing cells and pathways (Kisand et al. 2010, Ahlgren et al. 2011).

The mechanism by which cytokine-specific autoantibodies are provoked in AIRE deficiency is unknown. Both type I IFN and Th17 specific autoantibodies are seen in patients with thymic epithelial tumors that are known to have tertiary lymphoid organ-like formation in thymus, containing plasma cells producing autoantibodies against IFN-α and IL-12 (Shiono et al. 2003, Kisand et al. 2010). So it is plausible that, if AIRE is not expressed in mTECs, the microenvironments of thymus will change and possibly initiate active autoimmunization against these cytokines (Kisand et al. 2011, Meager, Peterson and Willcox 2008). As the type I IFN, IL-17A/F and IL-22 are expressed in the thymus by macrophages, γδT cells, natural killer cells and innate lymphoid cells (ILCs), the potential for autoimmunization there exists (Dudakov et al. 2012, Meager et al. 2006, Shibata et al. 2008, Cupedo et al. 2009, Wolk et al. 2010). In the periphery, locally produced IL-17, IL-22 and/or type I IFNs could constantly restimulate germinal center responses and autoantibody production (Meager et al. 2006, Mitsdoerffer et al. 2010).

The intrathymic autoimmunization hypothesis is also supported by the findings in young *myasthenia gravis* (MG) patients, who have autoantibodies specific to acetylcholine receptor (AChR) and ongoing anti-AChR antibody production by thymic plasma cells (Hill et al. 2008). In addition, their thymi are under chronic inflammation: they have dysfunctional regulatory T cells (able to produce inflammatory cytokines) and conventional T cells that are unresponsive to suppression by Tregs (Gradolatto et al. 2014).

2.3. Aire deficient mouse

AIRE/Aire proteins are 71% identical and contain same domains. Creating Aire deficient mouse strains was only possible after cloning and mapping mouse *Aire* gene, two years after human's. A possible breakthrough was hoped for explaining the mechanism of APECED (Mittaz et al. 1999, Saltis et al. 2008).

Indeed, the importance of Aire in thymic architecture, and its function as one of the drivers of negative selection, has been unveiled during the following years (Zuklys et al. 2000, Anderson et al. 2002, Kuroda et al. 2005, Matsumoto et al. 2013, Liston et al. 2003).

The four main mouse models that have been created replicate the most common APECED mutations, leading to: truncated protein or deletions in SAND, CARD, PRR, PHD1 and PHD2 domains (Jiang et al. 2005, Hubert et al. 2009). The original Aire deficient mouse is on C57BL/6 background, but as mouse strains carry different susceptibilities to autoimmune diseases, they have been crossed with mice on different backgrounds (NOD, BALB/c, SJL/J) (Hubert et al. 2009, Jiang et al. 2005, Anderson et al. 2002, Ramsey et al. 2002, Kuroda et al. 2005). Conspicuously, the Aire deficient mice on NOD or SJL background develop more frequently exocrine pancreatitis and thyroiditis, respectively; BALB/c mice are predisposed to gastritis (Jiang et al. 2005). The phenotype in C57BL/6 mice is rather mild; these mice have been known to suffer from keratoconjunctivitis and uveitis (Taniguchi et al. 2012, DeVoss et al. 2006, Yeh et al. 2009). However, despite infiltrations in the organs, they remain functional and their life expectancy is overall unaffected (Jiang et al. 2005, Hubert et al. 2009, Hässler et al. 2006). Interestingly, the NOD.Aire deficient mice stay normoglycemic. However, these mice show intensive autoreactivity, accompanied by autoantibodies and infiltrations in liver, stomach, reproductive organs, lungs and exocrine pancreas (Jiang et al. 2005). In addition, NOD.Aire-/- mice have high mortality (~79%), which is preceded by severe weight loss between 5–15 weeks of age. This correlates best with lung lesions and generalized pneumonitis (Jiang et al. 2005). Surprisingly, the autosomal dominant mutation, G228W, results in a more severe phenotype in mice than in humans, with multiple infiltrations in thyroid and retina, salivary and lacrimal glands. In addition, on NOD background, the mice develop progressive peripheral neuropathy (Su et al. 2008).

Overall, the generated mouse models display several times milder autoimmune manifestations and lack the classical APECED triad. Nevertheless, the T cell population show alterations in peripheral and thymic TCR repertoire; also, the size of activated and memory cell compartments is increased (Anderson et al. 2002, Hubert et al. 2009, Malchow et al. 2016, Ramsey et al. 2002). Infertility, organ infiltration and the destruction of ovaries, eyes, lungs, stomach, thyroid, liver, lacrimal and salivary glands are all common manifestations (Anderson et al. 2002, Ramsey et al. 2002, Hubert et al. 2009, Kuroda et al. 2005). Furthermore, in the ageing process, the changes in thymus morphology of Aire deficient mice become more pronounced (e.g. increase in B cells, contracted medullary compartment, atrophy) (Gillard et al. 2007, Ramsey et al. 2002, Milicevic et al. 2010, Hässler et al. 2006, Pöntynen et al. 2006).

The phenotypic differences between Aire deficient mice and APECED patients could emerge because of the differences in the development of the immune system in the two species. Newborn mice are lymphopenic and in Aire deficient mice, this is further amplified by the dysregulation of the chemokines

needed for thymocyte migration (Chang 2012, Guerau-de-Arellano et al. 2009, Kisand, Peterson and Laan 2014, Laan et al. 2009). Although this extended lymphopenic situation causes the proliferation of existing autoreactive T cells in the periphery, further failures in peripheral tolerance mechanisms are needed for it to lead to autoimmune manifestation (King et al. 2004, Kekäläinen et al. 2011, Kisand et al. 2014). Aire deficient mice have also decreased numbers of regulatory T cells; this possibly promotes peripheral autoreactivity (Malchow et al. 2013, Yang et al. 2015).

2.3.1. Autoantibodies

Aire deficient mouse sera have autoantibodies that correlate with organ infiltration. Also, the autoantibody patterns are strain-specific and suggest oligoclonal autoimmune responses (Jiang et al. 2005, Pöntynen et al. 2006). Interestingly, the autoantigens are mostly different from those of APECED patients, even the ones that are directly under Aire control (Pöntynen et al. 2006). This is in agreement with the lack of any endocrinopathies in *Aire* deficient mice, although mild infiltrations have been described (Pöntynen et al. 2006, Hubert et al. 2009).

Autoantigens [BPIFB1 and vomeromodulin (human orthologue LPLUNC1)] characteristic to rare interstitial lung disease, are shared by the mouse model and APECED patients (Shum et al. 2009, Shum et al. 2013). In addition, two prostate-specific autoantigens – seminal vesicle secretory protein 2, (human homolog of semenogelin) and TGM4 – are shared by the patients and Aire mouse model. These help to explain fertility problems and prostatitis seen in both (Hou et al. 2009, Landegren et al. 2015). The lack of Aire also causes the development of autoreactive T cells specific to melanocyte antigen – tyrosinase related protein-1 (TRP-1). Recognition of TRP-1 by T cells correlates with development of vitiligo (Zhu, Nagavalli and Su 2013).

Aire deficient mice exhibit destruction in pancreas and stomach, and have two specific autoantigen targets: mucin 6 expressed in stomach mucosa; and protein isomerase A2 (PDIA2) that is present both in stomach and pancreas (Gavanescu et al. 2007, Kurisaki et al. 2013). In addition, as previously mentioned, the autoimmune infiltrations in the eyes, lacrimal and salivary glands are prevalent manifestations in Aire deficient mice. Autoantibodies against the odorant binding protein 1a and α-fodrin are well correlated with lacrimal gland destruction leading to Sjögren's syndrome like condition (Kuroda et al. 2005, DeVoss et al. 2010, Ulbricht, Schmidt and Witte 2003). Those against α-fodrin correlate with several exocrine gland infiltrations (parotid, submandibular glands), due to its ubiquitous expression (Kuroda et al. 2005). Another autoantigen interphotoreceptor-retinoid-binding-protein has been described in spontaneous autoimmune uveitis (DeVoss et al. 2006).

Aire deficient mouse strains completely lack the type I IFN specific autoantibodies characteristic of APECED patients (Hubert et al. 2009). Regarding the

Th17 related cytokines, which are targeted in APECED and correlate with chronic mucocutaneous candidiasis, no spontaneous *Candida* infection nor IL-17A, IL-17F or IL-22 autoantibodies have not yet been described in the mouse model. Artificial *Candida* infection has given contradictory results, for instance Hubert *et al.* noticed no difference between Aire wild-type and knock-out, while Ahlgren *et al.* described increased vulnerability towards mucosal candidiasis and difficulty in the clearing of infection in knock-out (Ahlgren et al. 2011, Hubert et al. 2009). In the latter model, Aire knock-out (KO) mice had enhanced responses of Th1 and Th17 cells with high IFN-γ and IL-17A production, yet the immune memory development towards *Candida albicans* was defective (Ahlgren et al. 2011).

Surprisingly, Aire regulates neither type I IFNs nor α-fodrin and PDIA2 expression, meaning TSA expression and negative selection is not the only mechanism by which Aire induces tolerance (Kisand et al. 2011, DeVoss et al. 2010, Kurisaki et al. 2013).

2.4. Thymoma

2.4.1. Clinical features and diagnosis

Thymomas are rare (occurrence rate <1% of all tumors) slow-growing tumors of thymic epithelial cells that usually arise in the anterior mediastinum (Scorsetti et al. 2016, Weis et al. 2015). However, the capability of turning into a clinically aggressive tumor lies in all the subtypes of the disease (Moran et al. 2012). The risk of thymoma is low before age 20, increases in middle age, is the highest in the seventh decade of one's life, and does not differ in men and women (Engels 2010, Weis et al. 2015). However, onset takes place earlier if the patients have *myasthenia gravis* (Scorsetti et al. 2016, Lewis et al. 1987, Marx et al. 2010). In the absence of MG, thymoma may be discovered by chance (routine X-rays) or even go undetected, as 30–50% of the patients with thymomas are asymptomatic (Scorsetti et al. 2016, Riedel and Burfeind 2006). The "thoracic symptoms" noted by others, which include cough, dysphagia, chest pain, and/or superior vena cava syndrome, are associated with local invasion (Scorsetti et al. 2016, Lewis et al. 1987). Approximately 30% of all patients have systemic symptoms (e.g. fever, night sweats, fatigue, or weight loss), which might still be difficult to distinguish from those of lymphoma (Scorsetti et al. 2016, Thomas, Wright and Loehrer 1999, Lewis et al. 1987).

2.4.2. Histology and genetics

Thymomas are heterogeneous (Table 2); the World Health Organization (WHO) classifies them based on the epithelial cells' shape and the amount of thymocytes they generate. Types AB, B2 and B3 are soft, friable, and with numerous thymocytes. Other types are firm or hard, and there may be some calcification

in any type (Thomas et al. 1999). Type B is further divided into subclasses based on the proportion of increase in lymphocytes [B1 (predominantly cortical); B2 (cortical); B3 (well-differentiated thymic carcinoma)], and the presence of atypical epithelial cells (Table 2) (Inoue et al. 2003, Marx et al. 2015, Moran and Suster 2008). The corticosteroid treatment used for accompanying autoimmune diseases may deplete lymphocytes (Willcox et al. 1987), converting type AB towards type A or type B2 towards B3.

Table 2. The main WHO thymic tumor histotypes and their relative prevalence. Modified from (Scorsetti et al. 2016, Weis et al. 2015, Suzuki et al. 2008, Scarpino et al. 2007, Ströbel et al. 2007, Marx et al. 2015)

	Main component(s)	**Previous name/behavior**	**% of all thymomas**
Type A	Spindle/ oval shaped TEC; no nuclear atypia; almost no thymocytes. No Aire expression, MG (26%).	'spindle cell' 'medullary' thymoma, benign	Asia 6 Europe 15 USA 14
Type AB	Admixed areas of type A TEC with and without abundant thymocytes, segregated either sharply or indistinctly. Rare AIRE expression. MG (25%)	'mixed' thymoma, benign	Asia 27 Europe 23 USA 18
Type B1	Resemble normal thymus, most areas cortical, some small/ medullary; sparse TEC/ little atypia. ~50% of B1 thymomas express AIRE. MG (35%)	predominantly 'cortical' 'organoid' thymoma, local invasion uncommon	Asia 16 Europe 17 USA 20
Type B2	Plump TEC (vesicular nuclei, conspicuous nucleoli), either single or in small clusters, enmeshing abundant thymocytes. Rarely express AIRE. MG (49%)	'cortical' thymoma, often invade locally, sometimes via pleura, rarely via blood	Asia 20 Europe 31 USA 32
Type B3	Round or polygonal TEC, with mild atypia, and fewer thymocytes. Rarely express AIRE. MG (40%)	well-differentiated 'thymic carcinoma' 'epithelial' thymoma, invasion as for B2	Asia 32 Europe15 USA16

The neoplastic TEC rarely show the atypia seen in thymic carcinomas, and evidently retain the ability to select polyclonal thymocytes, and export their progeny to the periphery (Buckley et al. 2001b). Although all thymomas are potentially malignant (Moran et al. 2012, Hoffacker et al. 2000), types A and AB very rarely invade outside their capsule (Scorsetti et al. 2016, Weis et al. 2015). By contrast, types B2 and B3 often invade locally, and sometimes more distantly via the pleura, but rarely via the blood stream or lymphatics (Thomas et al. 1999). The frequency of invasion/stage seems to have gradual progression A~AB<B1<<B2≤B3 (Weis et al. 2015, Kondo et al. 2004).

The prevalent treatment strategy is thymectomy, followed by prophylactic radiotherapy for types B2 and B3, or preceded by radio- and/or chemotherapy if invasion is already evident before surgery (starting from stages II) (Ströbel et al. 2004). Many autoimmune symptoms accompanying thymomas tend to disappear after resection (Bernard et al. 2016). However, the MG typically deteriorates over the subsequent years, frequently requiring immunosuppressive therapy (e.g. corticosteroids, azathioprine). In severe cases of *myasthenia gravis,* plasmapheresis and immunoglobulin treatments are also applied (Bernard et al. 2016, Romi 2011). However, the corticosteroid usage may increase the overall susceptibility for infections that some thymoma patients contract (Holbro et al. 2012).

Genetic changes are not easy to study in the neoplastic TEC, because of the large numbers of 'contaminating' lymphocytes (Inoue et al. 2002). Nevertheless, some recurrent aberrations have been identified. These are histotype-dependent and are usually associated with the thymoma stage and survival rate. Malignant tumors tend to have more mutations, deletions, duplications, changes in tumor suppressor, growth hormone receptor, epigenetic regulator, cell-cell interaction and cell cycle genes (Scorsetti et al. 2016, Petrini et al. 2014, Inoue et al. 2003, Marx et al. 2015). Interestingly, all histotypes share a loss of heterozygosity in several regions on chromosome 6. One of these is situated in the major histocompatibility complex region (Inoue et al. 2002). That might help tumor cells to escape immune surveillance, and impair self-tolerance induction in developing thymocytes, or may promote concurrent autoimmune manifestations (Inoue et al. 2002).

2.4.3. Paraneoplastic autoimmune syndromes with organ-specific autoantibodies

Thymomas are associated with a great variety of autoimmune disorders, whether neuromuscular [MG (30–44%), neuromyotonia (NMT) (~3%), polymyositis (1–5%)], hematological [pure red cell aplasia (4%) or hypo-γ-globulinemia (5–20%)], cutaneous [pemphigus, alopecia areata or totalis, vitiligo (0.5–17%)] or systemic [systemic lupus erythematosus (SLE) (2%)], or other, more rare examples. The accompanying autoantibodies recognize a restricted subset of known targets (Table 3) (Klein et al. 2013, Marx et al. 2010).

Table 3. Most common autoantigens in thymoma patients modified from (Marx et al. 2010).

Autoantigen	**Accompanying disease**
Acetylcholine receptor (AChR)	*myasthenia gravis***, Rippling muscle disease, autonomic neuropathy, gastrointestinal dysmotility, NMT
Titin	myasthenia gravis
Ryanodine receptor (RyR)	*myasthenia gravis*, Rippling muscle disease*, polymyositis*, myocarditis*, NMT
Voltage-gated potassium channel complex (VGKC)	Morvan's syndrome, myocarditis, encephalitis (limbic and cortical), gastrointestinal dysmotility
Glutamate decarboxylase (GAD65)	encephalitis (limbic and cortical), cerebellar degeneration, T1D
Collapsing response mediator protein (CRMP-5)	autonomic neuropathy, cranial nerve impairment, sensory neuropathy
CRMP3,4,5	encephalitis (limbic and cortical)
Neuronal nuclear antigens (NNA-1)	sensory neuropathy, encephalitis (limbic and cortical), cerebellar degeneration
Thyroglobulin	Hashimoto's thyroiditis, Graves' disease
Thyroid peroxidase	Hashimoto's thyroiditis, Graves' disease
Thyroid-stimulating hormone receptor (TSHR)	Graves' disease
Insulin	Type 1 diabetes
Plakin family	paraneoplastic pemphigus
Desmoglein1 and 3	pemphigus vulgaris; foliaceus
Nuclear autoantigens (NAA)	minimal change >membranous >other glomerulonephritis, SLE
(ds)DNA	minimal change >membranous >other glomerulonephritis, SLE

NMT: neuromyotonia; SLE: systemic lupus erythematosus; **definitely, *debatably, or possibly pathogenic

These associations might result from: (a) failed induction of self-tolerance of the T cells developing in these disorganized microenvironments, where the neoplastic TEC show greatly reduced expression of both HLA-class II (Willcox et al. 1987, Marx et al. 2010) and AIRE (Scarpino et al. 2007, Ströbel et al. 2007), and/ or (b) a ‘dangerous climate’ where, because of these reductions,

local autoantigens are more likely to autoimmunize than to induce self-tolerance (Kisand et al. 2011, Liu et al. 2014, Okumura et al. 2008).

Patients with thymomas also show altered T cells in the periphery, altered ratios of CD4/CD8 cells; also increased numbers of recent thymic emigrants [especially from types AB and B2; (Buckley et al. 2001a)] and CD8+CD45RA+ T cells (Hoffacker et al. 2000), which disappear after thymectomy. In the case of thymoma, both a decrease in numbers of regulatory T cells and unaltered numbers have been reported (Scarpino et al. 2007, Ströbel et al. 2004).

The autoimmune disease most frequently associated with thymoma is MG, which is in most cases caused by autoantibodies against acetylcholine receptor. These autoantibodies impair neuromuscular transmission (Lindstrom et al. 1976, Vrolix et al. 2010). MG patients often have striational autoantibodies against several intracellular muscle antigens e.g. titin and/or the skeletal muscle calcium release channel [ryanodine receptor (RyR)] (Gautel et al. 1993, Mygland et al. 1994, Lindstrom et al. 1976, Vrolix et al. 2010, Williams and Lennon 1986, Marx et al. 1989). In one study, RyR autoantibodies were associated with more severe MG (Mygland et al. 2000). These antibody subclasses were IgG1 and IgG3, so they could activate complement and reduce both the complexity of the post-synaptic folds and AChR numbers, thus causing long-lasting muscle weakness (Lindstrom et al. 1976, Gomez et al. 2010, Marx et al. 2010).

In very rare cases of thymoma – such as the thymoma associated-multiorgan-autoimmunity generalized failure of negative selection leads to graft-versus-host-like disease, where autoreactive T cells attack multiple peripheral organs (Wadhera et al. 2007).

2.4.4. Autoantibodies against cytokines

In addition to various autoimmune manifestations, many thymoma patients have neutralizing autoantibodies to certain cytokines, especially type I IFNs and/or IL-12 (Meager et al. 1997, Burbelo et al. 2010, Meager et al. 2003). Those specific to type I IFNs are highly selective for IFN-αs and IFN-ω, as IFN-β neutralization is rare and IFN-γ recognition is completely absent (Meager et al. 2003). Moreover, they are not found in numerous other autoimmune, infectious or neoplastic diseases, the two exceptions being with late-onset MG patients without evident thymoma (Meager et al. 2003, Burbelo et al. 2010), and APECED patients (Meager et al. 2006). Interestingly, these autoantibodies are usually present at diagnosis, persist long after thymectomy, and are poorly controlled by corticosteroids. Their titers tend to rise, often strikingly, around the time when thymomas recur (Meager et al. 2003, Hapnes et al. 2012). Furthermore, thymoma tissue from seropositive patients has the capacity to produce these antibodies spontaneously *in vitro*; suggesting the presence of terminally differentiated plasma cells (Shiono et al. 2003).

The prevalent cytokine-specific autoantibody class is IgG (Meager et al. 2003). It has been shown *in vitro* that IL-12 autoantibodies inhibit Th1 polarization of naïve CD4+Tcells producing IFN-γ (Zhang et al, 2003); by interfering in the signal transducer and activator of transcription 4 (STAT)-dependent signaling pathway, they also halt the positive feedback loop of IL-12 production (Burbelo et al. 2010). However, their IL-12-neutralizing capacity is influenced by their titer and epitope-specificity (Burbelo et al. 2010). Similarly, the type I IFN autoantibodies affect the STAT1 pathway (Meager et al. 1997, Hapnes et al. 2012, Meager et al. 2003, Zhang et al. 2003, Burbelo et al. 2010). It has been proposed that such antibodies can suppress inflammatory immune responses (Montoya et al. 2002, Smyth, Taniguchi and Street 2000): their neutralization might thus favor tumor growth, possibly also by inhibiting the anti-angiogenetic effects of IL-12 (Strasly et al. 2001).

Occasional BAFF-, IL-1β-, TNF-α-, IL-6-, IFN-ε-, IL-18-, APRIL- and EBI1-specific binding, and in some cases, also neutralization, have been noted in thymoma patients, but without any clear clinical correlates (Burbelo et al. 2010, Meager et al. 2003).

The strong neutralizing capacity of the cytokine-specific autoantibodies might possibly predispose the patients to opportunistic infections, though they are infrequent. Susceptibility to several infections, e.g. *Cryptococcal*, is associated with IL-12 neutralization (Burbelo et al. 2010, Meager et al. 1997, Rowland, Griffiths and Kabat 1965). However, this is not seen with autoantibodies specific to type I IFN or IL-1β (Burbelo et al. 2010, Meager et al. 2003, Meager et al. 1997, Holbro et al. 2012).

The evidence is much stronger for the correlation between anti-IL-17/22 and mucocutaneous *Candida* infection (Kisand et al. 2010, Burbelo et al. 2010) (see Chapter 2.2.2). In most of these autoantibody positive patients, susceptibility to other infections is not increased, most likely because of differences in epitope, titer, in effects on epithelial barriers *in vivo,* and possibly, yet unknown factors that influence immune defenses (Burbelo et al. 2010).

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In recent years, AIRE has been studied thoroughly, and much has been discovered that has broadened researchers' views on its function and regulation. The understanding on how AIRE promotes self-tolerance in thymus is based on experiments using transgenic mouse models, and it is only assumed that similar events occur in humans. AIRE is still considered to be above all a transcriptional regulator of thymic TSAs, in spite of its additional functions as mTECs lifespan and differentiation/maturation regulator, and an indispensable shaper of thymic microenvironment. Moreover, the development of autoantibodies to cytokines in APECED patients is not in line with the defective negative selection theory in AIRE deficiency. The exact mechanisms by which AIRE functions remain yet to be discovered, since the development of APECED phenotype is still unexplained due to its surprising differences from the Aire deficient mouse model. Whether or not Aire deficient mice lacked also autoantibodies to Th17 cell related cytokines, was unknown before this study.

Type I IFN specific autoantibodies are useful for APECED diagnostics, but their effect on APECED manifestations has remained obscure. At the same time, the Th17 cell related antibodies correlate directly with CMC. Some additional mechanisms influencing the Th17/Th22 cell populations and leading to CMC need to be taken into account. As similar autoantibodies are found in thymomas, which themselves are often AIRE deficient, the involvement of thymus in the development of cytokine-specific autoantibodies seems plausible. These similarities should be studied further.

3. AIMS OF THE STUDY

The general purpose of this study was to characterize cytokine-specific autoantibodies in APECED, thymoma patients and in the Aire deficient mouse model.

More specifically, the aims were:

1. to characterize the isotypes of cytokine-specific autoantibodies in adult as well as very young APECED patients;
2. to determine whether APECED and thymoma patients have neutralizing autoantibodies against cytokines important for Th17 cell differentiation or maintenance;
3. to find the immunodominant epitopes of IFN-α2a and IL-22;
4. to test the Aire deficient mice for immunoreactivity towards autoantigenic cytokines;
5. to study the AIRE-dependency of the main organ-specific APECED autoantigens in thymoma tissue in comparison to thymic remnants and normal pediatric thymus;
6. to test the hypothesis that type I IFN neutralization capacity of APECED patient sera is inversely correlated with development of T1D.

4. MATERIAL AND METHODS

4.1. Patients and controls

All samples (Table 4) were taken with informed consent and an ethics committee approval in each referral center (in Bergen, Ljubljana, London, Oxford, and Tartu). All experiments were conducted in accordance with the Helsinki Declaration, with informed consent and local Ethics Committee approval. Blood sera were collected in parallel from patients and controls, and stored at –20°C until used.

The APECED diagnosis was confirmed by both: mutations analysis of *AIRE* gene and the presence of autoantibodies to IFN-ω and IFN-α2.

Thymomas were removed surgically or biopsied and examined by routine histopathology (hematoxylin-eosin, additional keratin and anti-CD3 or anti-CD1 staining) in nearly every case. In very rare exceptions, where surgery was unfavorable, tumors were noted on X-rays or scans. The MG was diagnosed based on the typical clinical features, electromyography and positivity for serum AChR autoantibodies in every case.

Table 4. Overview of the study groups

Study groups		
Study I: **Anti-cytokine antibodies in APECED and Aire deficient mice**	19 APECED patients 4 controls 40 thymoma patients 6 controls	IgG and IgA isolation from 9 APECED patients and 7 controls
	Aire deficient mice (BALB/c; B6) 69 KO + 43WT 6–8 weeks, 1.5–2 years old	
Study II: **Anti-cytokine antibodies in young APECED**	11 APECED patients 2 asymptomatic relatives	
Study III: **AIRE and tissue restricted antigen expression in thymoma patient**	26 thymoma patients (31 samples) 5 thymus samples from healthy children	
Study IV: **IL-6-specific autoantibodies among APECED and thymoma patients**	41 APECED patients 56 controls 104 thymoma patients	
Study V: **Type I IFN autoantibodies as possible protecting factor against type 1 diabetes in APECED patients**	81 APECED patients	21 APECED patients: 5 GAD65+T1D+ 3 GAD65-T1D+ 13 GAD65+T1D-

Study I
19 APECED patients of Finnish, Norwegian and Slovenian origin (age 29±16 years), among whom 15 had *Candida* infection. Patients were matched with healthy controls (N=6) for age and nationality. Immunoglobulins were isolated from 9 patient and 7 control sera. Sera from 40 UK thymoma patients (age 51±14) were studied.

Study II
For this study, we collected sera and clinical information from 11 very young APECED patients and 2 asymptomatic siblings from Finland, Hungary, Norway, Italy and USA (Table. 5).

Table 5. Characteristics of young APECED patients

Patient ID	CMC	Age at sampling and follow-ups	Sample origin
S1 (Fam1)	–	1.5m–48m	Hungary
S2 (Fam2)	15m	5m–30 m	Sardinia
P1 (Fam3)	54m	53m–43yr	Finland
P2 (Fam4)	9yr	62m–16yr	Finland
P3 (Fam5)	–	72m	USA
P4 (Fam6)	41m	36m–60m	Sardinia
P5 (Fam7)	34m	24–72m	USA
P6 (Fam8)	30m	40m–36yr	Finland
P7 (Fam9)	36m	36m–17yr	Norway
P8 (Fam9)	24m	6.5yr–20yr	Norway
P9 (Fam10)	18m	60m–84m	Hungary
P10 (Fam10)	42m	72m–120m	Hungary
P11 (Fam12)	–	60m–144m	USA

CMC: chronic mucocutaneous candidiasis; Fam: Family number; m: months, yr: years; P: patients, S: yet unaffected siblings

Study III
Thymoma tissue samples (N=31) were snap-frozen as blocks from 26 patients and stored at –80˚C until use. Nearly all thymomas were encapsulated and could be clearly separated from any adjacent thymic remnants (n=5), which were often minimal or absent in older or steroid pre-treated cases. Five pediatric thymi, removed during cardiac surgery were used as controls.

Study IV

We used sera from Finnish and Slovenian APECED patients (n=41) and healthy controls (n=56; 51±15.9 years old) (Table 6). 104 patients with thymomas were from UK, 99 of them with *myasthenia gravis* plus acetylcholine receptor antibodies. About 50% of the thymoma patients were first sampled pre-treatment, but most eventually needed corticosteroids (alternate-day dosing) and/or azathioprine for their MG, at varying times and doses.

Table 6. Characteristics of APECED and thymoma patients

	APECED patients (N=41)	**Thymoma patients (N=104)**
Mean age (min; max)	35 (4; 73)	52 (20;80)*
CMC	39	3
Autoantibody positivity:		
IL-17A	16	8
IL-17F	34	2
IL-22	41	14
IFN-α2	41	76
IL-12	0	91

*data on the age of only 79 thymoma patients were available; CMC: chronic mucocutaneous candidiasis

Study V

81 APECED patients from Finland, Italy, Norway, and Slovenia were assayed for type I IFNs and GAD65-reactivity, and then further compared with their T1D status. All GADA+T1D+ (N=5), GAD-T1D+ (N=3) and GAD+T1D- patients (N=13) were selected for further analysis. Eight patients with T1D (mean age: 48±11 years) were compared with an available cohort of thirteen patients without T1D but with strong reactivity to GAD65 (relative luciferase units >5) (mean age: 31±12 years).

4.2. Mice

Aire-mutant (967–979 del13) mice were used, originally on the C57BL/6 background [12]; one subline was backcrossed onto the BALB/c background for at least ten generations. Homozygous *Aire*-mutant (N=69) and wild-type (N=43) littermates were bred and maintained at the mouse facility of the Institute of Molecular and Cell Biology (Tartu University, Tartu, Estonia). Test sera were collected from mice aged either 6–8 weeks or 1.5–2 years, in accordance with the European Communities Directive 86/609/EEC.

4.3. Purification of immunoglobulins

Total IgG fractions were separated with fast protein liquid chromatography using Protein G Sepharose 4 Fast Flow (GE Healthcare), then concentrated and buffer-exchanged for PBS with iConTM Concentrator 7ml/20K tubes (Pierce Biotechnology). Total IgA was separated using agarose-bound Jacalin lectin (Vector Laboratories Inc), and dialyzed against 1xPBS using Spectra/Por Dialysis Membrane (MWCO 12 – 14 000) (Spectrum Laboratories). Protein concentrations were determined with the Bio-Rad Protein Assay, based on the Bradford method and using bovine gamma globulin as standard (Bio-Rad Laboratories Inc).

The purities of the isolated IgGs and IgAs were assessed by SDS-PAGE and Western blotting. The identity of the IgH bands was confirmed with specific antibodies (data not shown). The mean (±SD) protein concentration of IgG samples was 3.581 μg/ml ± 2.570 (range 514.5 – 10.158 μg/ml) and for IgA 533 μg/ml ± 278; (range 177.5–1.012 μg/ml). We detected ≤3% IgG contamination in isolated IgA preparations by immune-turbidimetry with Cobas Integra 400 Plus (Hoffmann-La Roche). Binding ELISA for detecting type I IFN-specific IgG in purified fractions (25μg/ml) was done as previously described (Meager et al. 2006).

4.4. Autoantibody detection

4.4.1. ELISA

Serum autoantibodies binding to mouse cytokines were assayed by ELISA. Microtiter wells were coated with carrier-free recombinant mouse IL-17A, IL-17F or IL-22 (Biolegend) or IFN-α4 (PBL InterferonSource) at 1-2 μg protein/ml (PBS, pH 7.0), overnight at 4°C. After blocking, mouse sera diluted 1:10 were incubated overnight at 4°C, before washing and development with either anti-mouse IgG [γ-chain-specific] -alkaline phosphatase (AP)-conjugate (Sigma-Aldrich Corporation) or anti-mouse IgG subclass-specific (IgG1, IgG2b, IgG2b, IgG3) biotinylated antibodies (Biolegend) followed by streptavidin-conjugated horseradish peroxidase and appropriate enzyme substrate and OD reading.

4.4.2. Constructs encoding luciferase (LUC) fused to cytokines

An overview of all full-length and truncated human IFN-α2a and IL-22 proteins and the primer sequences described in (Kärner et al. 2013). Human IFN-α2a and IL-22 coding sequences were amplified by PCR without the signal sequences. The PCR products were ligated into the BamHI/NotI site of pPK-CMV-F4 (PromoCell GmbH) mammalian expression vector using T4 ligase (Invitrogen).

IFN-α1, -α2, -α4, -α5, -α6, -α7, -α8, -α10, -α14, -α16, -α17, -α21, -ω, IL-6, IL-1β, IL-21, IL-23A (p19), IL-12A (p35) and IL-12B (p40), TGF-β3 or GAD 65 sequences (without signal peptide) were cloned into modified pPK-CMV-F4 fusion vector (PromoCell) downstream of naturally secreted *Gaussia* luciferase (Gluc) that was substituted in the plasmid for Firefly luciferase.

All plasmids containing correct inserts (as confirmed by DNA sequencing) were propagated in *E. coli* NOVA XG cells, amplified, extracted and purified using conventional methods. Finally, HEK 293 cells were transfected with the plasmids; after 48h the crude protein extracts were prepared using 1x passive lysis buffer (Promega). In the case of *Gaussia* luciferase, the fusion proteins were obtained by collecting the cell culture medium. All solutions were stored at –20°C.

4.4.3. Luciferase immunoprecipitation assay

Serum samples were incubated with fusion proteins solutions ($2x10^6$ luminescence units) overnight at +4°C. The next day, Protein G Agarose beads (25 µl of 4% suspension, Exalpha) were added and incubated at room temperature for 1 h in 96-well microfilter plates (Merck Millipore) to capture antibodies and immune complexes to the beads. After washing to remove unbound fusion proteins, luciferase substrate was added (coelenterazine GAR-2B, Targeting Systems), and luminescence intensity (LU) measured in VICTOR X Multilabel Plate Readers (PerkinElmer Life Sciences). The results were expressed as relative luciferase units

$$(\mathrm{RU}) = \frac{LUsample}{average\ LU\ of\ healthy\ control\ samples}$$

The positive/negative discrimination level was set to the mean plus 3 standard deviations of the healthy control samples.

Patients with the highest binding values were selected for IL-6 and IL-23 blocking experiments. Briefly, the serum samples were pre-incubated with 40µg/ml of recombinant human (rh) IL-6, (PeproTech EC Ltd) or IFN-γ (Miltenyi Biotec). To test for IL-23 blocking, the thymoma serum samples were pre-incubated with 40µg/ml of rhIL-23 (PeproTech EC Ltd), IL-12 (PeproTech EC Ltd) or rhIL-6 (PeproTech EC Ltd). The samples were rotated for two hours at room temperature and centrifuged for 15 minutes at 16,000g and supernatants were transferred into new Eppendorf tubes before performing LIPS assay as above.

To render the assay IgG subclass-specific, agarose beads coupled with streptavidin (25µl of 4% solution, Life Technologies) were coated with 10µl of biotin-conjugated human subclass-specific antibodies (1:100 dilution, anti-IgG1, anti-IgG2, anti-IgG4, from BD Pharmingen, anti-IgG3 from Life Technologies) for 1 hour in microfilter plates (Merck Millipore), to capture any subclass-specific immune complexes formed during the standard overnight pre-incubation, before washing and readout as above.

4.5. Autoantigen expression in thymus

4.5.1. RNA extraction from thymomas and real-time PCR

Snap-frozen tissue samples were homogenized in Trizol (Thermo Scientific) using AutoMACS with M-tubes (Miltenyi Biotech), followed by RNA extraction according to the manufacturer's protocol. RNA concentrations were measured with NanoDrop (Thermo Scientific); 5 mg of total RNA was reverse transcribed using Superscript III (Invitrogen), 10mM dNTP Mix, RiboLock RNase inhibitor and random hexamers (Thermo Scientific).

4.5.2. Real-time quantitative PCR

qPCR was performed using Applied Biosystems ViiA 7 Real-Time PCR System with 384-Well Block (Life Technologies) and Maxima SYBR Green /ROX qPCR Master Mix (Thermo Scientific). Every sample was run in three parallel reactions in two separate series of experiments; their results were broadly consistent and have been combined. We detected reliable signals for all transcripts tested, except NALP-5. Every transcript signal was expressed as $2^{-\Delta\Delta Ct}$ (where Ct represents the threshold cycle), and normalized relative to the value for β-actin in the same sample, and then to its (β-actin-normalized) KRT8 value to adjust for the highly variable TEC content. The resulting AIRE or TSA values were next expressed relative to that in one control infant thymus. Primers are listed in Wolff et al. 2014.

4.6. Western blot

IFN-ω and IL-6 luciferase fusion proteins or human IFN-α2 (PBL InterferonSource) were heated at 95°C for 4 min in reducing sample buffer [3% SDS, 10% glycerol, 0.1 M dithiothreitol, 0.02% bromophenol blue and 6.25 mM Tris–HCl, pH 6.8], run in 12% SDS-PAGE and blotted onto polyvinylidene difluoride filters. After blocking, strips of the filter were incubated with patient or control sera (1:100) or rabbit anti-luciferase antibody (1:1000 New England Biolabs) or mouse anti-IFN-α2b antibody (1:1000, Abcam) followed by secondary antibodies (anti-human HRP 1:10,000 or goat anti-rabbit-HRP 1:5000; anti-mouse-HRP 1:30,000, Jackson ImmunoResearch), and visualization by enhanced chemiluminescence using the manufacturer's protocol (GE Healthcare).

4.7. Cell-based cytokine neutralization assays

4.7.1. IL-17A, IL-17F and IL-22 neutralization assays

For IL-17A and IL-17F, NCTC 2544 keratinocytes (Interlab Cell Line Collection) preincubated with 0.1 ng/ml TNF (Biolegend) were seeded at 1 x 10^4 cells/well, in which IL-17A or IL-17F (2 ng/ml, R&D Systems) had been preincubated with serially diluted IgG or IgA for 2 h. After incubation at 37°C for 16–20h, supernatants were collected and assayed by ELISA (R&D Systems) for CXCL1. For human IL-22, we used the cell line Colo205. Cells were seeded at 3 x 10^4 cells per well in which IL-22 (2 ng/ml, R&D Systems) had been preincubated with serially diluted patient sera or IgGs or IgAs for 2h. After incubation at 37°C for 24–30h, supernatants were collected and analyzed for IL-10 by ELISA (R&D Systems). Results from all the cytokine neutralization assays were estimated from graphs of ELISA absorbances as the ED50s – the concentration of Ig needed to halve the cytokine activity of the test sample – and represented graphically as cytokine neutralization units (NU) per µg of protein.

4.7.2. Type I IFN neutralizing assay using reporter cell-line

Interferon neutralization capacity was tested using reporter cell line: HEK-Blue™ IFN-α/β cells (InvivoGen), which expresses alkaline phosphatase (AP) under the inducible ISG54 promoter after IFN-stimulated-gene-factor binding to the IFN-stimulated response elements (ISRE) in the promoter,aspreviously described (Breivik et al. 2014).

The cells were grown in DMEM (Naxo), heat inactivated 10% FBS and supplemented with 30g/ml blasticidin (InvivoGen) and 100g/ml Zeocin (InvivoGen). IFN-α2a was used at concentration of 12.5U/ml (Miltenyi Biotech). IFN-α4b and IFN-α5 (PBL assay science) were used at final concentrations of 37.5 U/ml. IFNA-α1, IFN-α6, IFN-α7, IFN-α8, IFN-α10, IFN-α14, IFN-α16, IFN-α17, IFN-α21, IFN-ω fusion proteins cloned for LIPS were also used for neutralization assays. Serial dilutions were made from the antigen preparations to determine the optimal dilution. The dilution that induced approximately the same AP concentration in the stimulated reporter cell supernatant as 12.5 U/ml recombinant IFN-α2a was selected for neutralizing assays.

50µl of patients' plasmas were three-fold serial diluted in 96 well flat bottomed cell culture plates (BD Biosciences) and coincubated with interferons for 2 hours at 37°C, 5% CO_2. Then 10^5 reporter cells were added and incubated 21 hours at 37°C, 5% CO_2. QUANTI-Blue™ (Invivogen) colorimetric enzyme assay was used to determine AP in the cell culture supernatants. 180µl of warm (37°C) substrate was pipetted into 96 well flat-bottomed microtiter plate and 20 µl of cell culture supernatant was added and incubated in 37°C for 30 minutes. OD was measured at 620nm with Multiscan MCC/340 (Labsystems) ELISA reader. IC50 was calculated from the dose-response curves.

4.8. Statistical analysis

Mann-Whitney or Kruskal-Wallis or One-Way ANOVA with Bonferroni's Multiple Comparison tests were used to assess the differences between studied groups using GraphPad Software. For TSA transcript values, the threshold for significance was set at p=0.01. Differences between thymoma and thymus remnant expression of AIRE were evaluated using paired t-tests. We also calculated z-scores to show the number of standard deviations by which each thymoma TSA signal differed from the corresponding mean of the five infant control thymi, either above the mean (positive z-scores) or below (with minus signs).

5. RESULTS

5.1. Anti-cytokine antibodies in APECED and Aire deficient mice (Study I, II)

5.1.1. IgG is responsible for binding and neutralizing type I IFNs and Th17 related cytokines in APECED patients

One of the aims of this study was to characterize the isotypes of cytokine autoantibodies in APECED patients. In order to do this, we looked to the main immunoglobulin class of autoantibodies. An autoantibody class gives suggestions as to the site and the type of immune cells involved in the autoantibody responses. For example, *Candida* infection is on the mucosa and a lot of cytokines (especially Th17 related) are produced to suppress the infection (Netea et al. 2015). Therefore, it is plausible that emerging cytokine-specific autoantibodies would belong to the IgA class, as a mechanism to alleviate the inflammation.

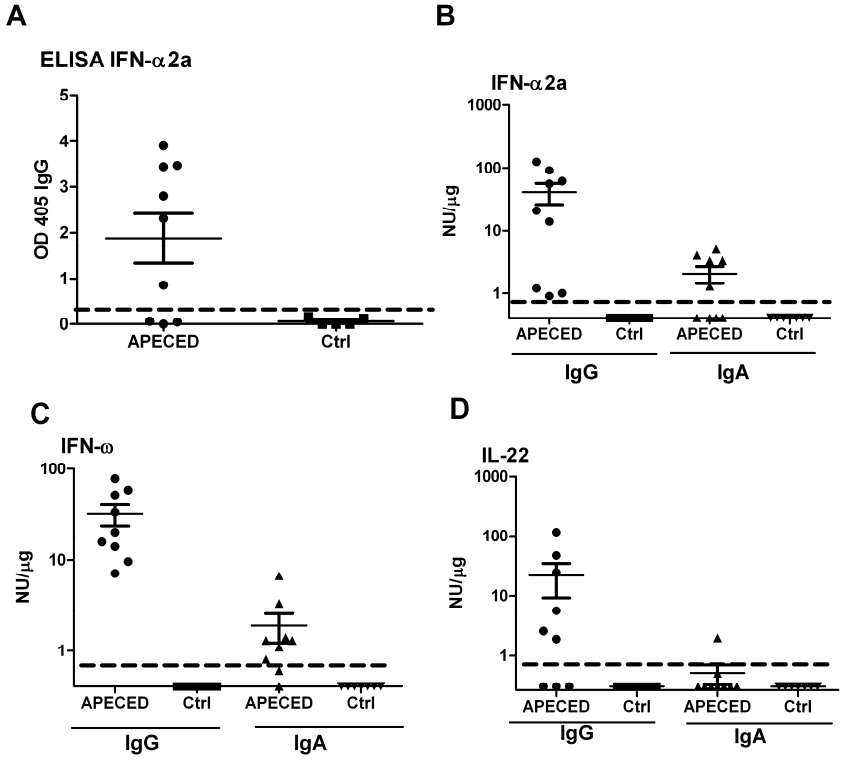


Figure 2. Binding and neutralizing activities of immunoglobulin (Ig) isolated from APECED and control sera. (a) Enzyme-linked immunosorbent assay (ELISA) was used to test for anti-interferon (IFN)-α2a autoantibodies in patient and control (ctrl) IgG fractions. The neutralizing activity of the isolated IgG and IgA fractions towards IFN-α2a, (b), IFN-ω (c) and interleukin (IL)-22 (d) in cell-based assays. Cytokine-neutralizing units (NU) per mg of protein are shown. Nine patient and seven control samples were tested.

In order to verify this, the IgG and IgA fractions from APECED patients' and healthy control sera were isolated. First of all, an ELISA test confirmed the presence of type I IFN specific binding only in APECED patients, and not in healthy control purified IgG isolates (Fig 2a). Thereafter, the cell-based neutralization tests with purified IgGs and IgAs proved that the IgG fraction carries the biological neutralization capacity of IFN-α2a, IFN-ω and Th17 cell-associated cytokines, as concentrations as low as 40 ng/ml were functionally active (Fig. 2, Table 7). On the other hand, some purified IgA samples from several APECED sera also showed weak neutralizing activity against IFN-α2a and IFN-ω (Fig. 2b,c). Per μg of protein, however, this was only ~3% (for IFN-α2a) and ~7% (for IFN-ω) of that measured in the respective IgGs. Although this reaction was positive, it is likely, that the IgA preparations were contaminated by a small amount of IgG. As for IL-22, the neutralization by the IgAs was even weaker, and was seen only in two out of six patient samples (Fig. 2d, Table 7). Although the IL-17A and IL-17F specific antibodies were rarer among APECED patients, the neutralization activity was mostly detectable in the IgG fraction (Table 7).

Table 7. The presence of neutralizing autoantibodies in 9 APECED sera, and IgGs and IgAs purified from them, against Th17 cytokines (P–positive, N–negative). ED50=the concentration of Ig needed to halve the cytokine activity of the test sample.

	Serum antibodies against			**IgG neutralizing activity ED_{50} (ng/ml) against**			**IgA neutralizing activity ED_{50} (ng/ml) against**		
Sample	**IL-22**	**IL-17A**	**IL-17F**	**IL-22**	**IL-17A**	**IL-17F**	**IL-22**	**IL-17A**	**IL-17F**
A1	P	N	P	43	>25000	5540	2500	>25000	>25000
A2	P	P	P	200	2200	11890	10000	>25000	>25000
A3	P	P	P	1900	1000	>25000	>16700	>25000	>25000
A4	P	N	N	105	>25000	>25000	>16700	>25000	>25000
A5	P	N	P	900	>25000	>25000	>16700	>25000	>25000
A6	P	N	N	2600	>25000	>25000	>16700	>25000	>25000
A7	N	N	N	>16670	>25000	>25000	>16700	>25000	>25000
A8	N	N	N	>16670	>25000	>25000	>16700	>25000	>25000
A9	N	N	N	>16670	>25000	>25000	>16700	>25000	>25000

5.1.2. IgG1 and IgG4 are the dominant subclasses against IFN-α2a and IL-22

For investigating the environment that leads to autoimmunization, we looked further into the IgG subclasses that hint at the cell types that orchestrate B cell class switch. The organ-specific immunoglobulins described above are mainly of IgG1 type (Brozzetti et al. 2010, Bøe et al. 2004). Interestingly, in APECED patients, the anti-cytokine antibodies showed high binding in the IgG4 fraction (Fig. 3a). The signals for IgG1 were overall higher than for IgG4, except for two APECED patients, who completely lacked IgG1 reactivity towards IFN-α2a and IL-22 (Fig. 3a,b) but had substantial neutralization capacity.

Both IL-17A/F and IL-22 antibodies have been described in rare thymoma patients (Meager et al. 1997, Kisand et al. 2008); we analyzed the IgG subclass distribution in these patients. The results corroborated that the IFN-α2a and IL-22 specificity lies in IgG1 and IgG4 fractions. Remarkably, the autoantibody positive sera showed even stronger IgG4 dominance, especially against IL-22 (Fig. 3b), where the binding was almost exclusively by IgG4 in 4 out of 15 sera. However, both subclasses were detected in anti-IFN-α2a fractions in every positive thymoma serum.

IgG4 is often associated with allergic diseases, as a sign of tolerance induction and decrease in IgE titer. Both patient groups presented rather low values of autoantibodies of IgE subtype: APECED patients exceeded the controls significantly only against IL-22 (Fig. 3a) and thymoma patients only against IFN-α2a (Fig. 3b). In addition, IgA-type autoantibody levels were low in both syndromes, confirming the previous results with isolated Ig isotypes. Collectively, the similar subclass/isotype distributions of their anti-cytokine autoantibodies suggest Th2 and/or regulatory T cell involvement in their induction in these two syndromes.

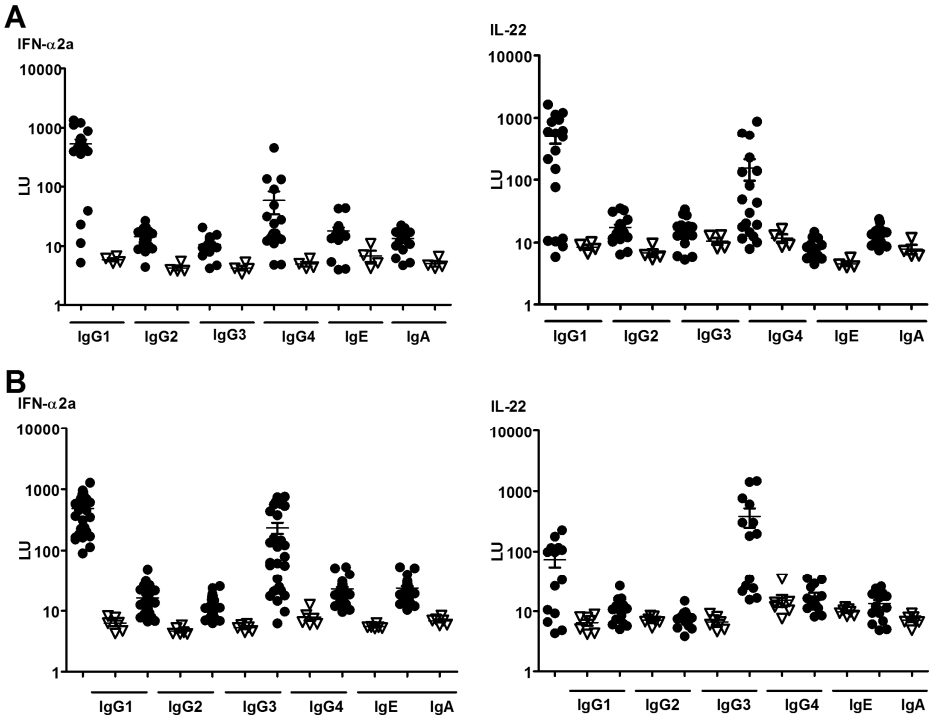


Figure 3. Immunoglobulin subclasses against IFN-α2a and IL-22 in APECED (a) and thymoma (b) patients. Antibody levels are shown in luminescence units (LU). Mean values with standard error of the mean are indicated. Thymoma sera were selected by prior testing for autoantibody positivity (n=30 for anti-IFN-α2a and n=15 for IL-22). All the sera were tested at least twice in three different experiments. Patient samples are indicated with filled circles, control samples with open triangles.

5.1.3. The preference towards IgG4 antibodies is present already before the onset of APECED (Study II)

We had the opportunity to study the cytokine-specific autoantibodies in some very young APECED patients and their asymptomatic siblings. We saw that, again, IgG1 was prevalent in all the autoantibodies detected in six of the studied subjects (Table 8). Surprisingly, against IL-22, there were already substantial proportions of IgG4 at 7 months in S1 (with traces of IgG2 and IgG3). What is more, in patient P7, the IgG4 already exceeded IgG1 as early as 42 months. Against IFN-α2a, IgG4 constituted, once again, a substantial proportion in S1 (at 30 months) and P4 (30 months), but not in the two other seropositive patients.

Table 8. IgG subclasses of autoantibodies against IL-22 and IFN-α2 in young APECED patients

IL-22	**age**	**IgG1**	**IgG2**	**IgG3**	**IgG4**	**Ratio IgG1 : IgG4**	
S1	7m	94208	6011	1439	**17607**	5.4	
S1	25m	48435	2113	1332	**83035**	0.6	**IgG4>IgG1**
S1	30m	4761	257	148	**7242**	0.7	**IgG4>IgG1**
S2	6m	551	235	128	707	0.8	**IgG4>IgG1**
P4	36m	7858	271	159	755	10.4	
P5	42m	2093	402	131	**10240**	0.2	**IgG4>IgG1**
P5	**6yr**	2236	360	144	**11518**	0.2	**IgG4>IgG1**
P9	48m	5784	195	186	282	20.5	
P9	60m	8041	204	173	392	20.5	
P11	**5yr**	137	319	122	154	0.9	**IgG4>IgG1**
IFN-α2	**age**	**IgG1**	**IgG2**	**IgG3**	**IgG4**	**Ratio IgG1 : IgG4**	
S1	30m	2249	178	123	**1471**	1.5	
S2	6m	1596	161	128	144	11.1	
P4	36m	2973	189	169	**1284**	2.3	
P5	42m	111	113	110	117	0.9	**IgG4>IgG1**
P5	**6yr**	189	135	111	125	1.5	
P9	48m	528	145	158	139	3.8	
P9	60m	2241	121	121	107	20.9	
P11	**5yr**	108	152	106	102	1.1	

High IgG4 values are marked in bold. *different substrate, m: months; yr:years

5.1.4. Immunodominant epitopes of IFN-α2a and IL-22 are conformational or C-terminal

Next, we attempted to identify the conformational requirements and immuno-dominant epitope(s) of IFN-α2a and IL-22. For that, we first cloned three shorter cDNA fragments from IFN-α2a (corresponding to amino acid sequences: 24–69aa; 67–124aa, 123–188aa) or IL-22 (34–76aa, 74–114aa, 113–179aa) into CMV vectors. After the LIPS analysis, we did not find autoantibody binding to IFN-α2a, nor to IL-22 fragments (data not shown), indicating that

any specific epitopes had apparently been lost from these constructs. As these fragments might have been too short to form conformational epitopes, we used the same primers to create longer fragments. APECED patients still failed to recognize N-terminal fragments of IFN-α2a (24–124aa) and IL-22 (34–114aa). However, the C-terminal fragments (67–188aa, 74–179aa) showed some binding, although the reactivity was much weaker than to full-length cytokines (Fig. 4a). These findings suggest that the major immunodominant epitopes are conformational. These results were corroborated further using recombinant human IFN-α2 that was denatured in reducing conditions before SDS-PAGE. We found that the APECED sera that bound exclusively to the full-length construct in LIPS assays (e.g. P3 in Fig. 4b) did not recognize denatured IFN-α2 in Western blots, unlike other sera which had additional binding to the C-terminal construct (P1 and P2 in Fig. 4b).

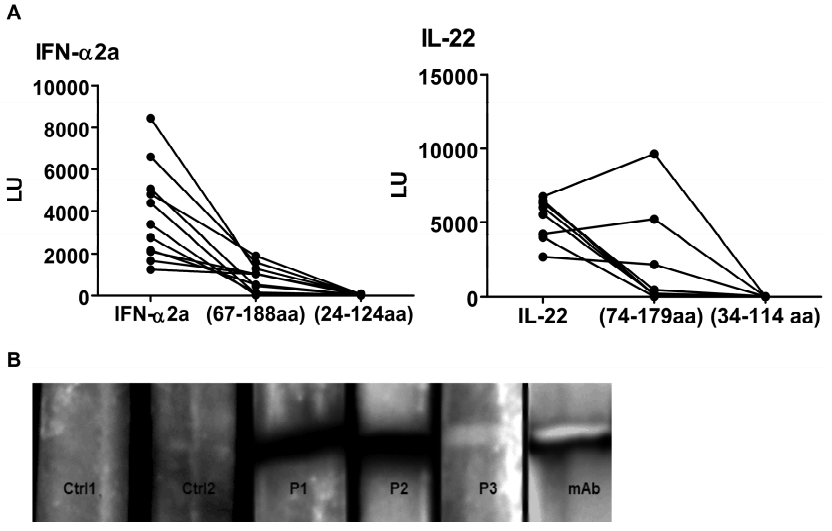


Figure 4. Epitope mapping of IFN-α2a and IL-22. (a) APECED sera were tested against full-length and N- and C-terminal fragments of IFN-α2a and IL-22 using luciferase-conjugated polypeptides as antigens. (b) Western blot against denatured human IFN-α2 revealed that APECED sera had exclusively conformation- (P3) or C-terminus-specific (P1 and P2) autoantibodies. Monoclonal antibody (mAb) to IFN-α2 was used as a positive control.

5.1.5. Cytokine-specific autoantibodies in Aire deficient mice

Although different Aire deficient mouse models have been created, only a few overlapping organ-specific autoantigen targets have been reported (Ali-mohammadi et al. 2008, Landegren et al. 2015, Pöntynen et al. 2006, Shum et al. 2009, Shum et al. 2013). Interestingly, Aire deficient mice, on both BALB/c

and C57BL/6 backgrounds, completely lack the type IFN autoantibodies, the major hallmark in APECED patients (Meloni et al. 2008, Hubert et al. 2009). We confirmed this by testing 1.5–2 year old BALB/c mice sera (Fig. 5).

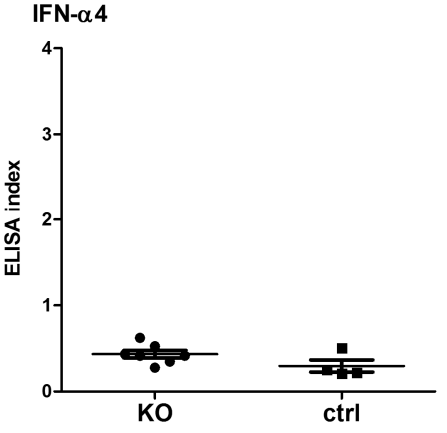


Figure 5. Aire deficient mice have no type I IFN specific antibodies

Aire deficient mouse sera had not been tested for autoreactivity towards IL-17A/F or IL-22. We chose mice from C57BL/6 and BALB/c backgrounds and two different age groups (6–8weeks, 1.5–2years old). Notably, sera from 22 of the 24 aged Aire deficient BALB/c mice (92%) bound IL-17A *versus* only three of the eight young mice (38%; all very weak, Fig. 6a). In sharp contrast, we found weak binding in only one of the 12 (8%) aged AIRE deficient C57BL/6 mice *versus* none of their young counterparts (Fig. 6a). Furthermore, 9 of the 13 available aged BALB/c sera neutralized IL-17A bioactivity (Fig. 6c) *versus* none of those from young BALB/c or old C57BL/6 mice (data not shown). Their neutralizing activity correlated broadly with the ELISA binding values (Fig. 6a,c). Finally, we tested the subclasses of the mouse autoantibodies to IL-17A. Interestingly, they were mainly IgG1 (Fig. 6b), implying a Th2 bias even more clearly than in the human syndromes.

We also found autoantibodies against IL-17F in 7 of the 19 old (37%) and 5 of the 10 young (50%) BALB/c mouse sera (Fig. 7a). Signals were mainly weaker than against IL-17A, and almost all sera from the C57BL/6 mice were negative (Fig. 7a). Very few BALB/c or C57BL/6 sera showed even moderate binding of IL-22; it was detected mainly in the mice positive against IL-17F, but age differences were again less obvious (Fig. 7a,b). In addition, their sera did not neutralize IL-22 detectably (data not shown).

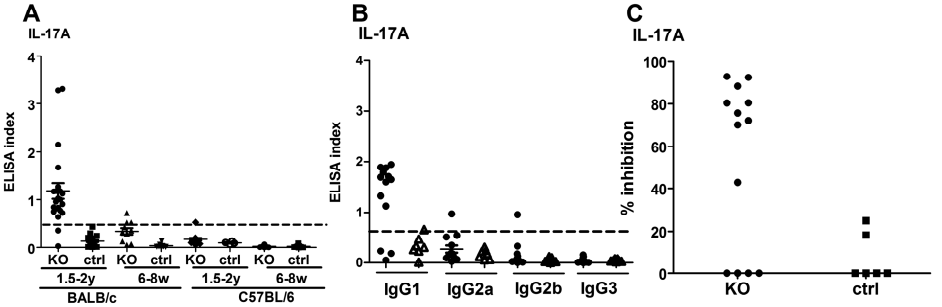


Figure 6. Aire deficient mice develop neutralizing antibodies against IL-17A. Autoantibody subclasses versus IL-17A are shown in (b).

KO: Aire deficient mice; ctrl: combined Aire wild-type and heterozygotes. Mean values with standard error of the mean are indicated. Horizontal lines indicate positive–negative discrimination levels drawn according to the old BALB/c group (mean + 2 standard deviations).

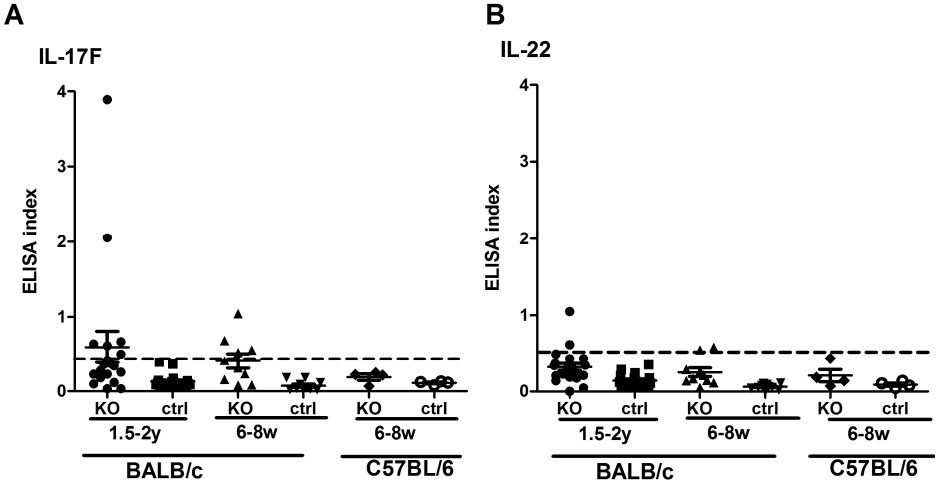


Figure 7. Murine IL-17F and IL-22 specific antibodies in Aire deficient mice

KO: Aire deficient mice; ctrl: combined Aire wild-type and heterozygotes. Mean values with standard error of the mean are indicated. Horizontal lines indicate positive–negative discrimination levels drawn according to the old BALB/c group (mean + 2 standard deviations).

Thus, in contrast with APECED patients, neutralizing and/or binding autoantibodies to Th17 cell-associated cytokines reached higher prevalences and titers in aged than in younger Aire deficient mice. Their higher prevalences on the more severely affected BALB/c background, and in older mice, possibly reflect the progression of the pathological changes.

5.2. AIRE and tissue restricted antigen expression in thymoma patients (Study III)

We wanted to test whether the autoimmune manifestations present in APECED and thymoma patients are caused by decreased expression of AIRE and TSA transcripts (Anderson et al. 2002), or alternatively, by some other mechanism, e.g. active autoimmunization against certain common targets present in aberrant thymic tissue (Kisand et al. 2011). We studied TSA expression in AIRE-negative thymoma tissue as a surrogate for APECED thymus.

We analyzed *AIRE* and 16 TSA transcripts in the available thymoma, remnant or control thymus blocks. As thymic epithelial cell content varies greatly between thymomas, we normalized qPCR results by using keratin-8 (KRT-8) signals, which correlate negatively with thymocyte content (Willcox et al. 1987).

Relative *AIRE* transcript levels were low in almost all thymoma subtypes, but there were large individual differences (Fig. 8a). Values were high in one of the two available type B1 thymomas, in line with previous reports (Scarpino et al. 2007, Ströbel et al. 2007). As expected, *AIRE* expression was much lower in the thymomas than in the adjacent autologous thymic remnants in four of the five available pairs (one was non-MG; Fig. 8b).

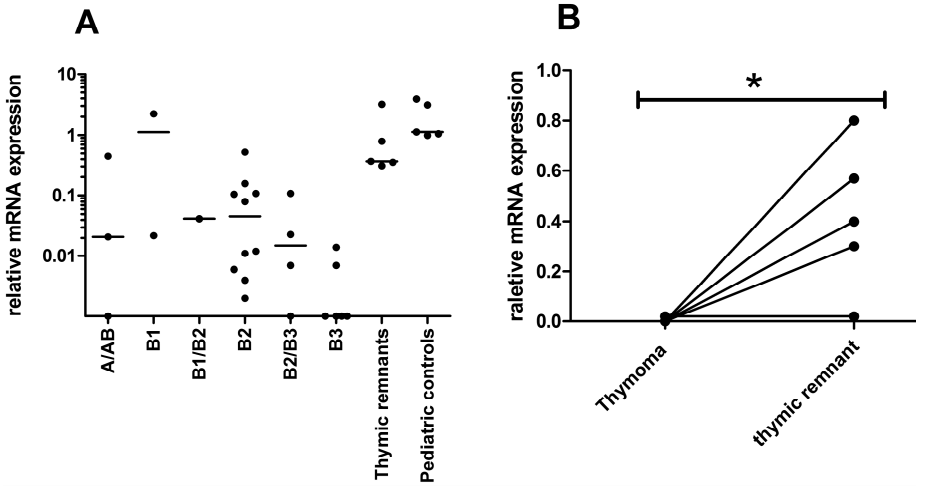


Figure 8. Relative *AIRE* expression in thymoma types and adjacent thymic remnants. (a) *AIRE* expression in blocks from different thymoma types using keratin-8 (KRT-8) to adjust for epithelial cell content, and a pediatric control sample as calibrator. (b) *AIRE* expression in paired thymomas or thymic remnants for five informative patients. Expression was calculated as for (A). *$p<0.05$.

Data from these paired thymomas or thymic remnants also illustrate the variability between different patients and different TSAs (Table 9). TDRD6 and

H+/K+ ATPase transcript values were lower in most of the thymomas than in the remnants, as we had expected. In contrast, the steroidogenic enzyme transcripts mostly showed similar or even higher values in the thymomas.

Table 9. Tissue-specific autoantigen transcript values in paired thymoma divided by autologous thymic remnant

Gene	Patient no.				
	P1 non-MG	**P2**	**P3**	**P4**	**P5**
AIRE	0.02	0.04	0.10	0.02	0.00
21OH	**4.87**	0.11	0.38	0.22	**13.90**
17OH	**7.87**	0.11	0.26	0.88	1.17
SCC	0.22	0.32	0.49	1.44	0.12
AADC	0.00	1.40		0.00	0.04
TPH-1	0.40	0.15	0.19	0.11	
HDC	0.02	0.01	0.04	0.37	0.65
TG	0.58	0.11	0.14	0.46	
TPO	0.01	0.32	0.20	1.55	0.03
GAD65	0.01	0.06	0.00	**38.30**	
INS	0.31	0.19	0.02	2.31	0.07
IA-2	0.16	0.10	1.54	2.17	2.63
TDRD6	0.07	0.06	0.05	0.17	0.37
H/K ATPase	0.01	0.05	0.01	0.04	
SOX9					1.51
AChR-α	0.00	0.35		3.49	

Each number is the TSA transcript value in the patient's thymoma block divided by the value in the autologous thymic remnant. Numbers in bold are 4-fold higher in thymomas than in thymic remnants, regular font indicates transcript values 4-fold lower, those underlined are intermediate transcript values (0.26–4.00).

The 26 thymomas (including two non-MGs) showed the most striking variability in TSA transcripts, even when AIRE expression was low (black circles in Fig. 9). Values for AADC, H+/K+ ATPase and AChR-α clearly followed the AIRE expression pattern: highest in control thymi, intermediate in remnants, and low in thymomas (Fig. 9). The expression levels of insulin, HDC, TDRD6 and GAD65 in thymoma tissue each showed significant differences from combined remnants and

control thymi ($p<0.01$; Mann–Whitney U test) (Fig. 9). For the above TSAs, values were all higher in non-neoplastic tissue than in thymoma tissue.

Surprisingly, even when AIRE values were very low, we found higher TSA transcript values per epithelial cell in many thymomas (number of thymoma samples, with z-scores >3 are shown in brackets) than any of the control thymi for: 21OH (10/26), 17OH (1/26), TG (3/26), TPO (5/26), TH (1/26), HDC (2/26), TDRD6 (2/26), IA-2 (1/26), SOX9 (11/26) and TPH-1 (3/26). These TSAs appear AIRE-independent, despite their frequent recognition by autoantibodies in thymoma and APS-I patients.

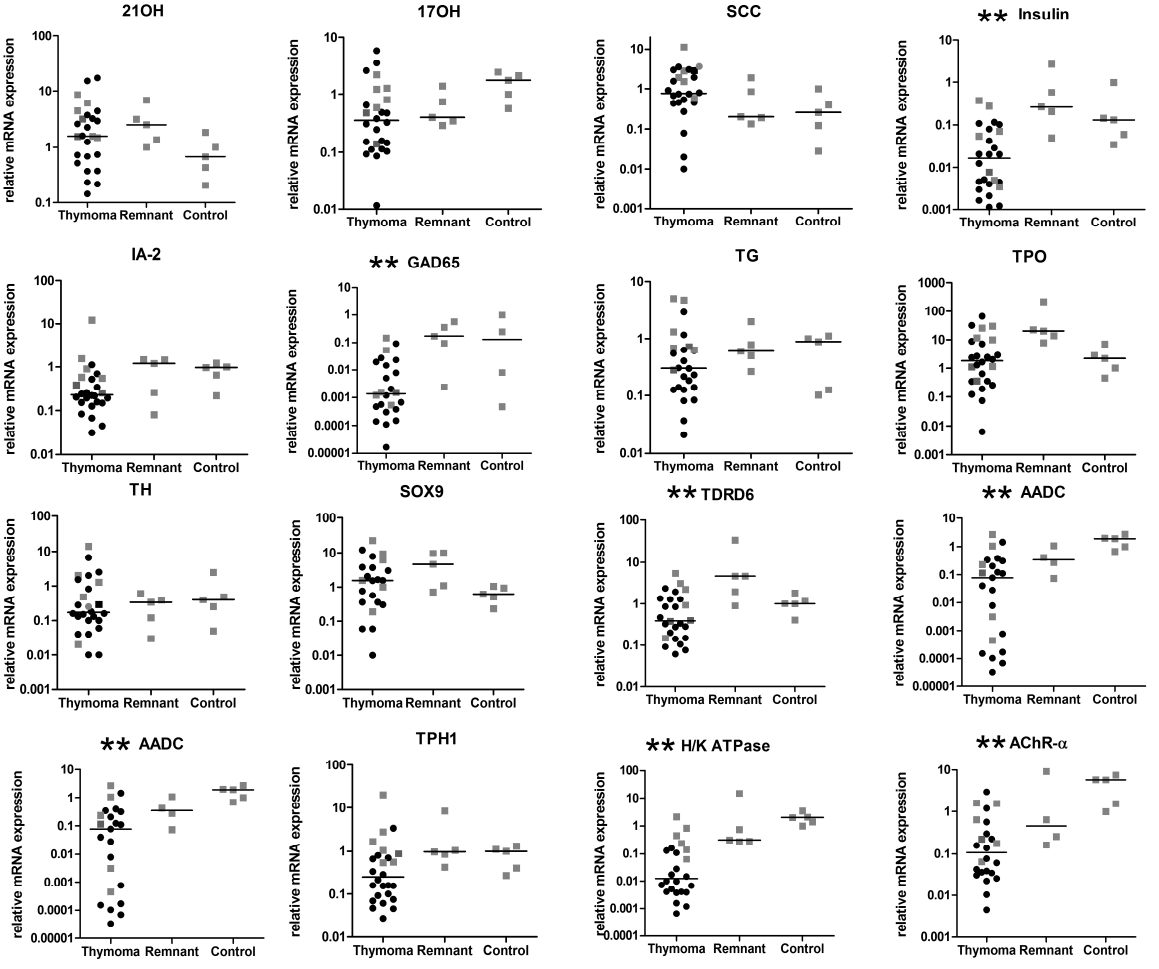


Figure 9. Relative transcript signals for APS-I target autoantigens (normalized to keratin-8) and shown as fold change compared with one pediatric control sample. The bars for each group represent the medians. The gray squares and black circles indicate samples with AIRE expression >0.1 or <0.1, respectively. Thymomas differed significantly from the combined remnants plus controls ** $p< 0.01$ (Mann–Whitney U tests).

5.3. IL-6-specific autoantibodies among APECED and thymoma patients (Study IV)

The effect of Th17 cytokine-neutralizing autoantibodies predisposing to *Candida* infection is not clear, as APECED and thymoma patients' PBMCs show also severely impaired IL-22 and IL-17F production (Laakso et al. 2014, Ng et al. 2010, Ahlgren et al. 2011, Kisand et al. 2010). Interestingly, IL-17A secretion by APECED T cells can range from almost absent to supra-normal in the patients (Kisand et al. 2010, Ng et al. 2010, Ahlgren et al. 2011). This led us to hypothesize that neutralizing autoantibodies to Th17-driving cytokines (IL-1β, IL-6, IL-21, IL-23 and/or TGF-β (Veldhoen et al. 2006, Stockinger and Veldhoen 2007, Deenick and Tangye 2007) might be involved in shaping or stunting Th17 responses.

5.3.1. APECED and thymoma patients share autoantibodies specific for IL-6 but anti-IL-23 is present only in thymoma patients

Sera from APECED patients, thymoma patients and healthy controls were tested for autoantibodies against IL-6, IL-1β, IL-21, IL-23 (p19 + p40) or TGF-β3 using LIPS assays that preserve the natural cytokine conformations. We could not detect any significant autoantibody binding to IL-1β, IL-21 and TGF-β3 except for single borderline reactivities to IL-1β and IL-21 (Fig. 10a-c). We found autoantibodies to IL-6 in 8 of the 41 APECED patients' sera (19.5%, Fig. 10d; Table 6); also in 13 (12.5%) of 104 thymoma patients, though mostly at moderate levels (Fig. 10d; Table 6). However, these antibodies did not correlate with any clinical symptoms (including CMC). The infections seen in thymoma patients were probably due to corticosteroid use for their MG. As the number of IL-6 positive patients was small, correlations with other clinical or demographic parameters, or tumor histology were not justified.

Notably, 2 out of 56 (3.6%) control sera showed moderate but consistent anti-IL-6 levels (Fig. 10d).

Autoantibodies in thymoma patients recognize IL-12 by its p40 subunit or the p35/p40 heterodimer [A. Meager, unpublished, (Meager et al. 1997, Meager et al. 2003)]. Therefore, it was not surprising to find autoantibodies also to IL-23, as it shares the p40 subunit with IL-12.

Notably, they were evident only in patients with thymoma but not with APECED. Indeed, p19 + p40 were bound by 28 of the 92 anti-IL-12 positive thymoma patient sera (Fig. 10e). However, none of them showed reactivity against the IL-23-specific chain p19 alone (Fig. 11). IL-23 autoantibodies did not correlate with CMC in thymoma patients.

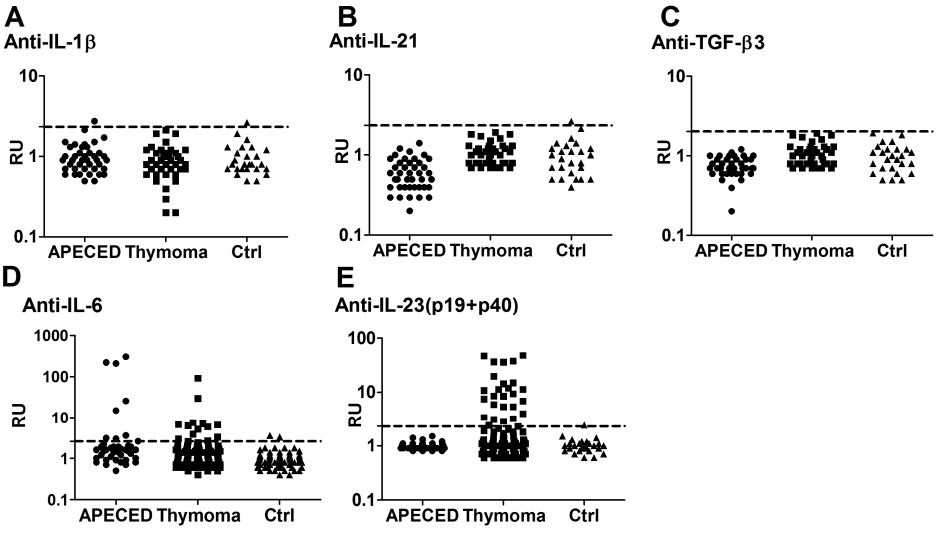


Figure 10. Cytokines related to Th17 maintenance and differentiation were tested with LIPS analysis for autoantibodies in APECED (41), thymoma (104) patients and 56 healthy controls. The dashed lines indicate the mean of controls + 3SD

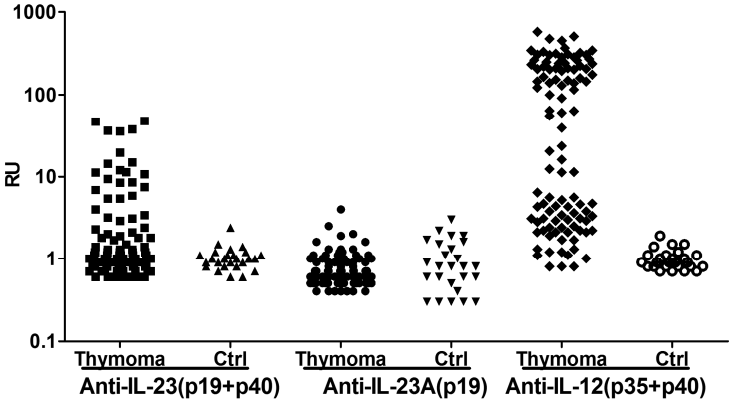
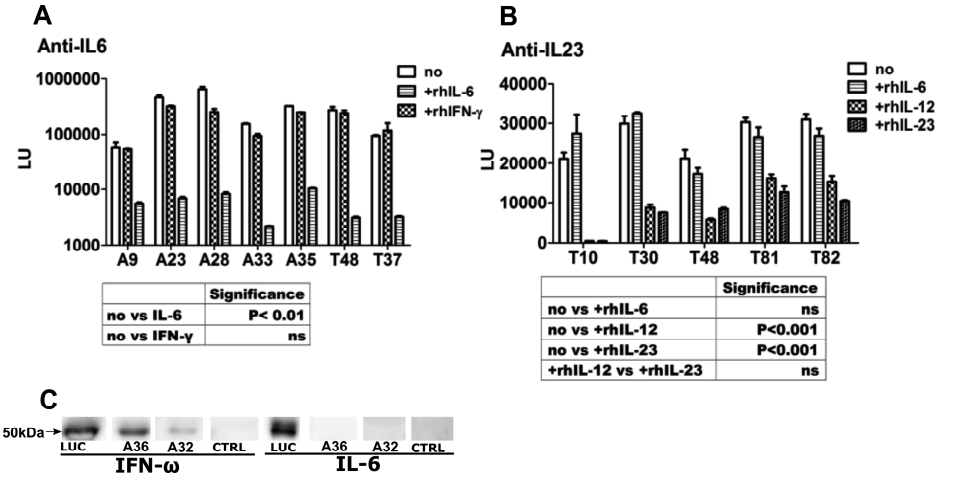


Figure 11. Binding of IL-23(p19+p40), IL-23A(p19) and IL-12(p35+40) by thymoma sera. RU-relative units.

To confirm the specificity of the detected autoantibodies, we pre-incubated positive APECED or thymoma patients' sera with recombinant human cytokines before assaying them by LIPS. Indeed, the binding of luciferase-tagged IL-6 to autoantibodies was completely blocked by rhIL-6 ($p<0.01$) but not by rhIL-IFN-γ ($p>0.05$), which was used as a control cytokine (Fig. 12a). Further supporting the p40-specific cross-reactivity between IL-12 and IL-23 noted

above, they both strongly blocked binding by the anti-IL-23 antibodies ($p < 0.0001$ for each), and with almost equal potency (Fig. 12b).

We also used Western blotting to test for recognition of conformation-independent epitopes by the strongest APECED sera. Binding was not detected to denatured IL-6: however, it was still strong against IFN-ω (Fig. 12c), which we used as a positive control, as autoantibodies are known to recognize linear epitopes in type I interferons too (Kärner et al. 2013).



	Significance
no vs IL-6	P< 0.01
no vs IFN-γ	ns

	Significance
no vs +rhIL-6	ns
no vs +rhIL-12	P<0.001
no vs +rhIL-23	P<0.001
+rhIL-12 vs +rhIL-23	ns

Figure 12. Binding specificities of IL-6 and anti-IL-23 antibodies in patients with APECED (A) and thymoma (T). (a), (b) Blocking of serum antibodies by pre-incubation with recombinant human (rh) IL-6, IFN-γ or/and rhIL-23 or IL-12, or buffer only (no); ns- not significant; (c) IFN-ω and IL-6 Gluc fusion proteins were denatured in SDS buffer, separated with PAGE and analyzed by Western blot using patient or control sera. A-APECED, T-Thymoma

5.3.2. IL-6 antibodies develop later in life and are mainly IgG1

In APECED, titers against both type I IFNs and Th17 cytokines are nearly maximal at diagnosis (Meager et al. 2006, Kisand et al. 2010, Wolff et al. 2013), and are found even before the onset of CMC in informative cases and even in infancy (Puel et al. 2010, Kisand et al. 2010). Moreover, they tend to decline subsequently (Fig. 13). Likewise, in most seropositive thymoma patients, IFN-α and/ or IL-12 antibodies are usually also found at diagnosis (Meager et al. 1997), though their titers usually rise sharply if the thymoma recurs (Buckley et al. 2001b), and may vary with changes in doses of immunosuppressive drugs (Meager et al. 2003).

In sharp contrast, the IL-6 autoantibodies – when present – were low initially but apparently increased over time in both patient groups, and were maximal in samples taken decades after disease onset (Fig. 13).

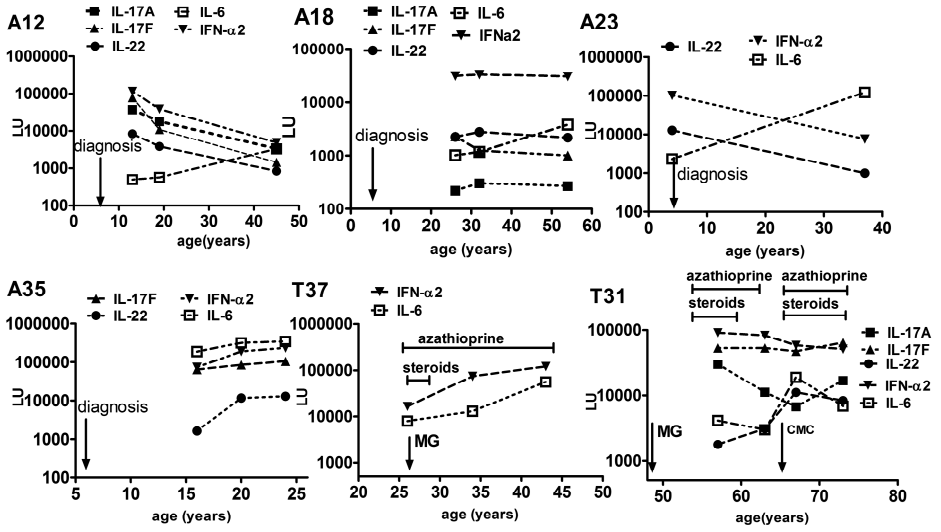


Figure 13. Changes in IL-6, IFN-α2, IL-17A, IL-17F and IL-22 specific autoantibody levels over time in APECED and thymoma patients. We detected no antibodies against Th17 related cytokines in patient T37. A: APECED, T: thymoma, LU: luminescence units

Next, we studied the IgG subclasses of the antibodies in the six strongest IL-6-binding sera (3 patients with APECED and 3 with thymoma). Reactivity was mainly in the IgG1 or in some IgG3, except T1, who showed reactivity in all four subclasses (Table 10).

Table 10. IL-6-specific IgG subclasses in APECED and thymoma patients

APECED	**IgG1**	**IgG2**	**IgG3**	**IgG4**
A23	**25**	1	**3**	1
A28	1	1	**29**	1
A35	**31**	1	1	1
Thymoma				
T1	**2**	**3**	**2**	**3**
T37	**6**	1	1	1
T48	**27**	1	1	1

The values are expressed as RU (fold changes over the average luminescence of healthy control samples). Positive values are marked in **bold**. A-APECED patients; T-Thymoma patients

5.3.3. Autoantibodies fail to neutralize IL-6, but may increase its stability

To help understand the biological significance of these autoantibodies against IL-6, we next tested their capacity to inhibit signaling from its receptor via phosphorylation of STAT3, which acts downstream in this pathway. FACS analysis clearly showed no inhibition by purified IgGs from six APECED patients; indeed, mean signals were even marginally higher than with the six control IgGs (3.927 vs 3.584, $p > 0.05$; Fig. 14a). This is well in line with the rarity of staphylococcal infections in APECED patients and argues strongly against our hypothesis that anti-IL-6 autoantibodies are involved in the pathogenesis of CMC in APECED patients.

Next, we studied the serum levels of IL-6 and of the Th17 cytokines IL-17A, IL-17F and IL-22 in the available unthawed APECED and healthy control serum aliquots. IL-17F and IL-22 levels were below the detection limit in most of these sera, but IL-6 and IL-17A levels were both higher in the two anti-IL-6 positive patients than in the nine negatives or the ten healthy controls ($p<0.001$, Fig. 14b and c). This suggests that IL-6-specific autoantibodies could prolong the half-life of IL-6 *in vivo*, thus indirectly enhancing IL-17A production by Th17 cells. So as to confirm this finding, the experiment should be repeated, as the number of anti-IL-6 positive patients' was too small.

In summary, we demonstrated novel IL-6 antibodies in APECED and thymoma patients. These antibodies develop later in life, are mainly IgG1, recognize conformational epitopes, and fail to neutralize the biological effect of IL-6, rather they seem to stabilize it *in vivo*.

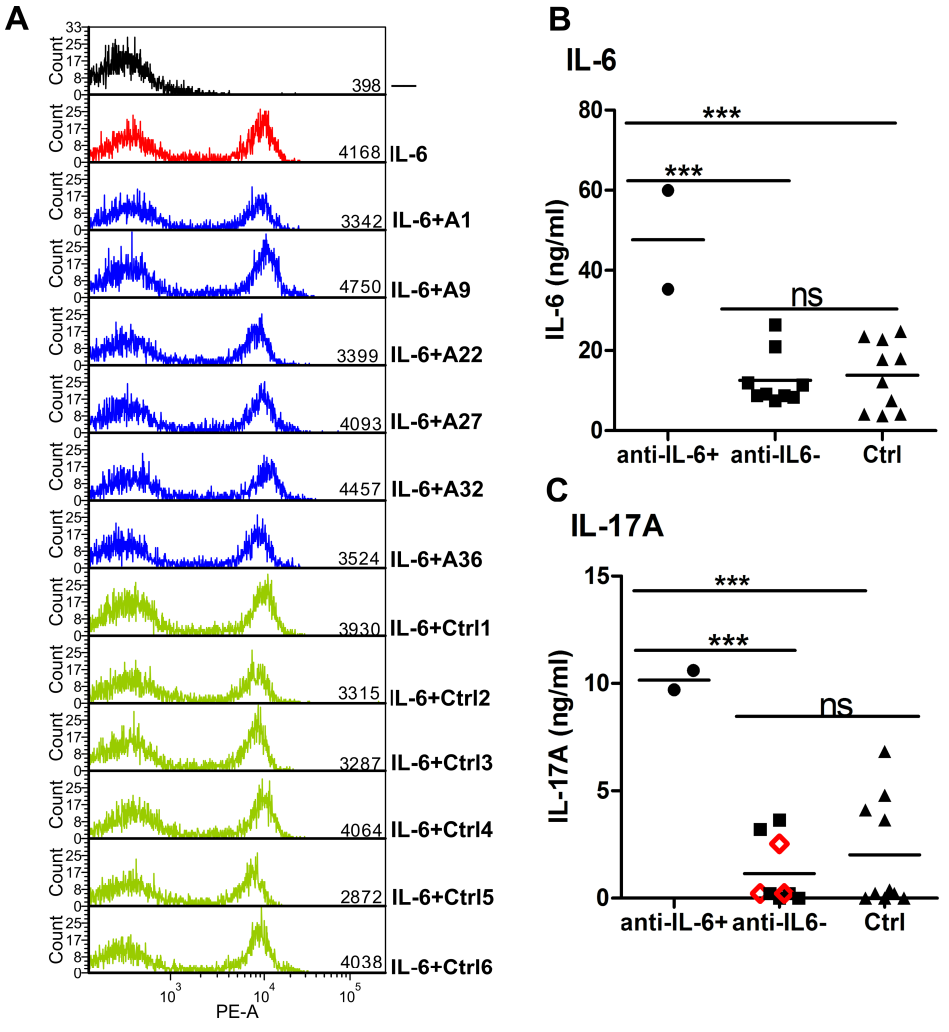


Figure 14. Testing for biological effects of IL-6 antibodies.

(a) Intracellular phospho-STAT3 staining in cells incubated with medium alone (black), or plus IL-6 (red) or plus IL-6 pre-incubated with either APECED patients' IgG (blue) or controls' IgG (green). Serum concentrations of IL-6 (b) and IL-17A (c) in APECED patients with or without IL-6-specific autoantibodies and in age-matched healthy controls. APECED patients positive for anti-IL-17A autoantibodies are indicated with red open diamonds.

5.4. Type I IFN autoantibodies as possible protecting factor against T1D in APECED patients (Study V)

The biological significance of neutralizing type I IFN specific autoantibodies in APECED patients have been puzzling for a long time. The studies in mouse models have suggested that type I IFNs contribute to the development of T1D (Carrero et al. 2013, Downes et al. 2010, Ferreira et al. 2014, Kallionpää et al. 2014, Foulis, Farquharson and Meager 1987). Although APECED patients by definition suffer from polyendocrinopathy, T1D affects only ~10–20% of patients and manifests primarily in adulthood (Husebye et al. 2009, Kisand and Peterson 2015). This is surprising, because radioimmunoassay analyses have shown that clinically-applied biomarker for the likely onset of T1D, GAD65 specific autoantibodies, are present in higher proportions of APECED patients' sera (Ziegler et al. 2013). Our LIPS analyses show that many patients carried GAD65 antibodies, but among them relatively few presented clinically with T1D (Fig. 15).

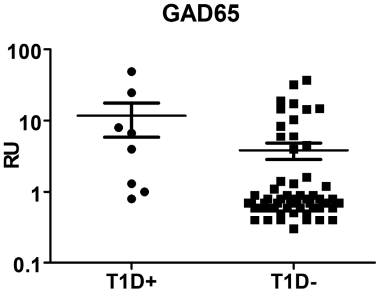


Figure 15. Autoantibodies against the T1D specific sensitive marker, GAD 65, in APECED patients.

Considering the possibility of the protecting role for type I IFN-autoantibodies, we analyzed the patients further for the ability to neutralize type I IFNs. Consistent with the fact that T1D onset in APECED patients is usually in adulthood, GAD65-reactivities almost invariably arose post-adolescence, so the patients comprised 20 adults and one 8-year-old girl. In common with almost all APS1/APECED patients, each of the 81 patients harbored autoantibodies to IFN-α and IFN-ω, as assayed by LIPS (Fig. 16).

However, when those antibodies were tested for their capacity to neutralize a series of IFN-α subtypes (using a HEK-Blue IFN-α/β/reporter cell line expressing an alkaline phosphatase reporter from an ISRE), they showed a striking, statistically significant segregation with the patients' clinical status (Fig. 17). In sum, the eight patients presenting with T1D showed negligible or very low capacity to neutralize any of the 12 IFN-α subtypes tested, whereas patients presenting without T1D collectively neutralized each IFN-α subtype.

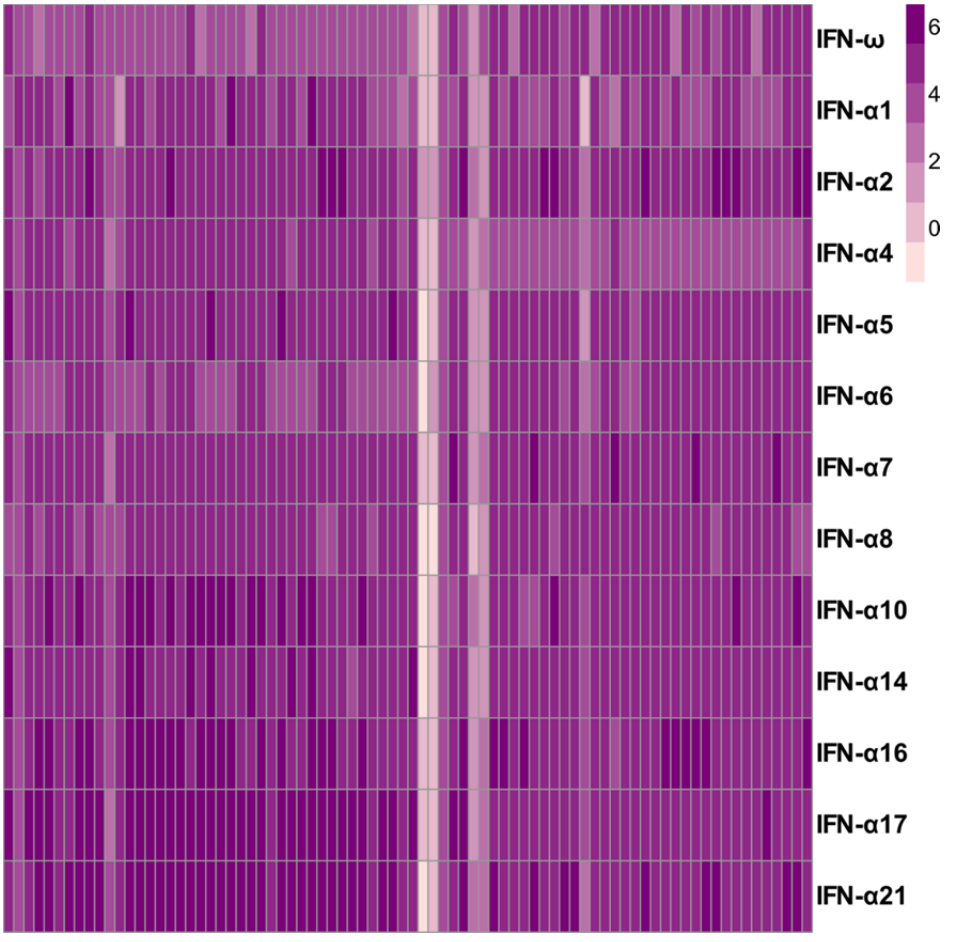


Figure 16. Heatmap for type I IFN binding by APECED patients' sera.

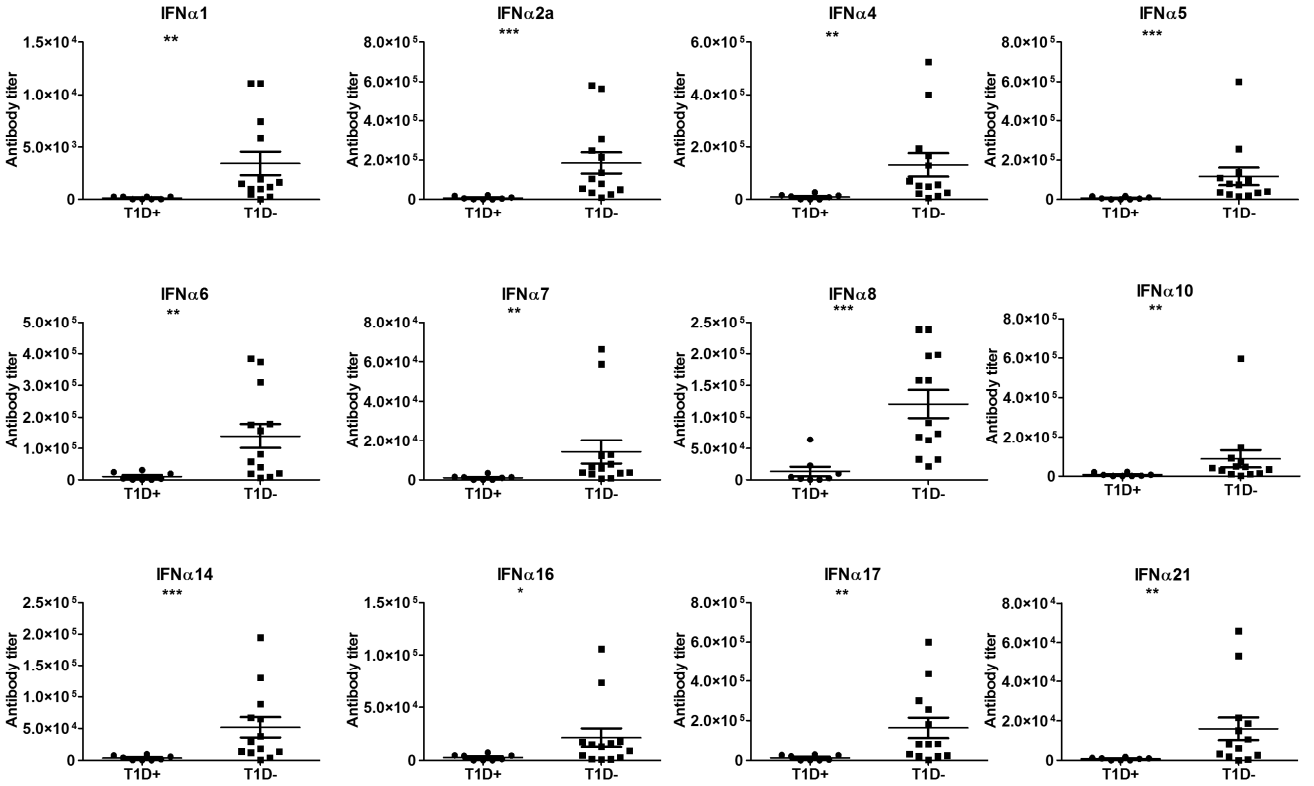


Figure 17. IFN-α neutralizing titers in APS1/APECED patients with T1D (n=8) and anti-GAD65 seropositive APS1/APECED patients without T1D (n=13). Y axis represents the neutralizing capacity in inhibitory concentration IC50 showing the serum dilution in which the activity of the respective IFN was reduced to its half. Exact significance values were calculated by Mann-Whitney test using GraphPad Prism v.6, with * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

6. DISCUSSION

APECED syndrome is the best known example of large-scale failure of central tolerance, caused by defective AIRE function, leading to aberrant negative selection of autoreactive T cells. Nevertheless, a large portion of the clinical and serological manifestations seen in APECED patients cannot only be explained by failures in negative selection: the preference for failures in endocrine organs, weak genotype-phenotype correlation, and high titer cytokine-specific autoantibodies. This thesis looked further into the peculiarities and varieties of autoantibodies seen in AIRE/Aire deficiency.

6.1. AIRE deficient mice differ from APECED patients

Aire deficient mice do not display the APECED specific parathyroid and adrenal failures or CMC, but preferentially have immune cell infiltration in thyroid, exocrine pancreas, stomach, eye and lacrimal/ salivary glands. Furthermore, all Aire knock-out models lack the IFN-specific binding that is distinctive to APECED patients (Hubert et al. 2009, Pöntynen et al. 2006).

For the first time, we describe Th17 related cytokine-specificity in Aire deficient mice. However, the generation of these autoantibodies in mice is different from APECED patients, as they recognize mostly IL-17A and their capacity to neutralize develops in older mice (~2years). As reported earlier, the background of the Aire deficient mouse influences significantly the development of autoantibodies. Aire deficient mice on BALB/c background have overall higher serum autoreactivity towards different organs (especially stomach) and develop more infiltrations than B6 mice (Jiang et al. 2005, Hubert et al. 2009). We also saw higher titers and stronger neutralization of IL-17A, IL-17F and IL-22 autoantibodies on BALB/c background.

We can speculate that variability in autoantibodies is the reason for different outcome of *Candida* susceptibility (Hubert et al. 2009, Ahlgren et al. 2011). In addition, the skin composition of mice and humans is different, as humans have more of interfollicular epidermis compared to hair follicle epithelium, and there are differences in the skin immune cell populations as well (Pasparakis, Haase and Nestle 2014). The important phenotypic differences of AIRE deficiency between the two species call into question the relevance of the mouse model in understanding the disease mechanisms in APECED patients. Therefore, it is important to study patients in every possible way. What complicates studying the disease pathology in humans, though, is that there is currently no APECED thymus available for research.

6.2. Thymoma as a surrogate for APECED thymus

One of the most peculiar phenomena in human AIRE deficiency are the cytokine-specific autoantibodies that develop early in life and are predictors of disease progression. The ability to bind and neutralize type I IFNs and Th17 related cytokines also emerges in the presence of thymus tumors (Kisand et al. 2010, Meager et al. 1997, Meager et al. 2006).

As thymi from APECED patients were unavailable, we studied the changes in the expression of TSAs in thymomas, which also present deranged thymic architecture and often lack *AIRE* expression, while the production of T cells continues, especially in type B (Scarpino et al. 2007, Ströbel et al. 2007, Marx et al. 2010). 40% of thymoma patients showed APECED typical antibodies [e.g. 21-hydroxylase, IFN-α, GAD65, side-chain cleavage enzyme; (Table II from Wolff et al. 2014). However, these antibodies did not always correlate with their corresponding diseases. The typical APECED clinical manifestations [e.g. autoimmune thyroiditis, T1D, alopecia, CMC, vitiligo; (Table 1, Wolff et al. 2014)] were present in only 8% of thymoma patients and often appeared long after thymoma.

Nevertheless, some TSAs were clearly underexpressed in thymomas, notably AChR-α, H+/K+ ATPase, AADC, insulin, GAD65, HDC, and TDRD6. This partially fits with previous reports of AIRE-dependent, but variable expression of insulin, AChR-α, IA-2, and H+/K+ ATPase (Ströbel et al. 2007, Kisand et al. 2011, Taubert, Schwendemann and Kyewski 2007, Giraud et al. 2007). Notably, AChR-α transcript levels were high in some thymoma patients, implying that underexpression is not the sole cause of their MG. This also seems unlikely because none of our APECED patients had MG or detectable autoantibodies against AChR or titin. Other AChR subunits can be important targets, too (Maclennan et al. 2008), and so can pre-existing peripheral tolerance to any of the subunits. In striking contrast, transcript values for 21OH, TPO, and SOX9 were higher (with positive z-scores) in 40–65% of our thymoma samples than in the control thymi – even in the tumors in which AIRE transcripts were almost undetectable.

We recognize that measuring only the expression of TSA transcripts is probably not sufficient to describe changes in their protein levels; however, in our current study we were unable to quantify TSA proteins, as their levels were low. In addition, several factors could have possibly affected AIRE and TSA expressions, for example changes in thymoma, blood supply and hormone levels that occur over time (Willcox et al. 1987). Also, the clonal nature of TECs (Inoue et al. 2003) suggests that expressions are different for each TEC (Derbinski and Kyewski 2010, Taubert et al. 2007), so they could also vary between and within individual tumors. However, we could not see striking variations with our duplicate samples (Fig. 8b). Finally, thymomas seem to have one common progenitor with maturation and differentiation defects, which differs between thymoma types (Willcox et al. 1987, Ströbel et al. 2014). We believe that the influence of some of these restrictions could be reduced if single TECs were to be analyzed.

Our data also suggest AIRE-independent expression of 17OH, SCC, TG, TH, and TPH-1. Likewise, AIRE-independent expression has been reported for TG, TPO, and GAD67 in control human TECs (Taubert et al. 2007, Li et al. 2009), and for 17OH and 21OH in AIRE-negative thymomas (Ströbel et al. 2007). What is more intriguing, type I IFNs, IL-17A, IL-17F, IL-22, thyroglobulin and steroidogenic enzymes are not expressed in mTECs (Kisand et al. 2010, Li et al. 2009, Pazirandeh et al. 1999), meaning that negative selections naturally continue in AIRE deficiency. However, as these autoantibodies and organ pathogeneses still occur in the latter, there seem to be other mechanisms that contribute.

Taking into account all the similarities that have hitherto been found between thymoma and APECED patients, and the emerging knowledge about the additional roles of AIRE in mTEC maturation and differentiation, tissue and blood samples from thymoma patients can be accepted as substitutes for those of APECED patients. However, as with the Aire deficient mouse model, several factors have to be considered when results from thymomas are interpreted.

6.3. Autoantibodies to IL-6 are not neutralizing the biological effect

Our study characterized autoantibodies specific to IL-6 in APECED and thymoma patients. Interestingly, autoantibodies to other cytokines needed for the maintenance and induction of Th17 were not present in APECED patients. Thymoma patients had autoantibodies that bound to IL-23, although it was due to cross-reaction with the shared IL-12p40 subunit (Fig. 10e), as there was no significant difference between binding efficiencies of the two (Fig. 11). Surprisingly, the IL-6 antibodies lacked neutralizing capacity (Fig. 14a), meaning that impaired IL-22 and IL-17F production, seen in both syndromes with CMC (Kisand et al. 2010, Laakso et al. 2014, Ng et al. 2010), have other causes than disturbed Th17 induction. Non-neutralizing autoantibodies are also in line with the absence of staphylococcal infections, otherwise characteristic of disturbance in IL-6 signaling pathway (Puel et al. 2008). Interestingly, studies on large numbers of healthy blood donors have revealed IL-6-binding reactivity in up to 9% of the cases, and also, in human IgG preparations, using either RIA or ELISA in earlier studies (Galle et al. 2004, Hansen et al. 1991). Only 0.1% of the sera showed high binding levels and were able to neutralize the biological function of IL-6 without any sign of disease in the respective donors (Galle et al. 2004).

The anti-IL-6 autoantibodies seem to produce the opposite effect in systemic sclerosis patients, as they are able to stabilize IL-6 *in vivo*, leading to higher IL-6 levels in autoantibody positive patients. These autoantibodies are believed to work as carriers, as IL-6 activity was preserved in these immune complexes (Suzuki et al. 1994). This kind of cytokine-stabilizing effect has been described for anti-IL-2 (Spangler et al. 2015). According to current opinions, antibodies may increase the persistence of the cytokine in the circulation through neonatal

Fc receptor (FcRn). FcRns are expressed on vascular endothelium and on myeloid cells that take up IgG, which is then pinocytozed with plasma and binds to FcRn in late endosomes. Finally, it is recycled back into the plasma still retaining its bound cytokine (Pyzik et al. 2015). Our data also indirectly suggest that the IL-6 autoantibodies can potentiate Th17 development, as the two informative anti-IL-6 positive patients had the highest IL-17A levels in their sera (Fig. 14b and c). Both patients' sera lacked reactivity towards IL-17A, as did six of the nine anti-IL-6-negative patients (Fig. 14c). Furthermore, we saw the highest percentage of IL-17A producing T cells (Fig.3c from Ref.Kisand et al. 2010) in one of the patients (A35) with IL-6 antibodies. However, as the sample size was too small, given that we had only two anti-IL-6 positive patients, further analysis on a larger scale is required to repeat and confirm this finding.

6.4. Autoantibodies bind cytokines by their conformational epitopes

In order to understand the neutralizing mechanisms of the anti-cytokine autoantibodies, we attempted to map their immunodominant epitopes in IFN-α2 and IL-22. In many cases, these proved to be conformational, as none of the shorter truncated polypeptides or denatured IFN-α2 was recognized by these patients' sera. Other sera were able to bind to their longer C- but not N-terminal polypeptides (encompassing approximately two-thirds of their lengths). These results were corroborated further using recombinant human IFN-α2 that was denatured in reducing conditions before SDS-PAGE. IL-22 and IL-6 seem to be even more conformation-sensitive, because adsorption to plastic for ELISAs reduces or even abolishes antibody binding [Fig. 12c; (Kisand et al. 2010)]. Autoantibodies to IL-12 and IL-23 seem to be less conformation-dependent: many can be detected using protein arrays (Rosenberg et al. 2006), ELISAs (Meager et al. 2003), or even immunoblotting (A. Meager, unpublished data), though LIPS must be more sensitive for detecting low autoantibody levels.

These C-terminal regions of IL-22 and IFN-α2 contain amino acids that are more prone to make β-turns, and are hydrophilic: two important qualities for evoking specific antibodies that recognize intact proteins (Grant 2002). As receptor-binding of both IL-22 and IFN-α2 is via sites closer to their C-termini (Quadt-Akabayov et al. 2006, Bleicher et al. 2008), any autoantibodies against these are likely to neutralize. The C-terminus of IL-6 has been described to form an α-helix, and to be indispensable for biological activity (Brakenhoff et al. 1990). However, as IL-6 signaling can be mediated both in *cis* (forming tetrameric complex: $IL\text{-}6_1$*$IL\text{-}6R_1$*$gp130_2$) and in *trans* (hexameric complex $IL\text{-}6_2$*$IL\text{-}6R_2$*$gp130_2$), blocking the binding of one of these does not abolish all signaling (Lacroix et al. 2015, Scheller and Rose-John 2006). It is plausible that IL-6 specific autoantibodies in APECED and thymoma patients allow one of the receptor signaling pathways to continue. Furthermore, as the serum levels of

IL-6 are higher in patients with antibodies, these autoantibodies could potentially mediate stabilization through FcRn. As conformation sensitivity seems to prevail among cytokine-specific autoantibodies, tests should be carried out in solution. There, the natural conformation and superior sensitivity is preserved (neutralization, LIPS, bead-based assays) (Kisand et al. 2010, Puel et al. 2010, Burbelo et al. 2010, Kisand and Peterson 2015).

6.5. Autoantibody isotypes may reveal the sites and environment of autoimmunization

Determining the cytokine-specific immunoglobulin classes and their subclasses helps to eliminate the possibility of other interfering serum components in neutralization assays, e.g. soluble receptors (Xu et al. 2001, Novick et al. 1995) and also hints at the possible cytokines, environment and cells involved in these antibody responses (van Zelm 2014, Jackson, Wang and Collins 2014).

The predominance of the IgG class in these cytokine autoantibodies eliminates the possibility of mucosal autoimmunization against them, as in this case, the potency should lie in IgA fraction. IgA autoantibodies would be quite an expected outcome, as they are produced at high concentrations in mucosal sites, for protection against invading pathogens. APECED patients suffering from chronic *Candida* infection could overstimulate the cytokine production and autoantibodies could emerge. In addition, some gastrointestinal autoimmune diseases are often characterized by IgA autoantibodies in the blood sera. For example, anti-tissue transglutaminase specific IgA is highly specific, used as a diagnostic marker of coeliac disease (Teesalu et al. 2009, Dieterich et al. 1997). Interestingly, in primary biliary cirrhosis, the major autoantigen, pyruvate dehydrogenase, is recognized and inhibited by both the IgG and IgA fractions (Kisand et al. 1998). However, as in the case of APECED and thymoma patients, the high IgG prevalence suggests a different place for the autoimmunization.

Subclass analysis of autoantibodies against type I IFN, Th17 related cytokines and IL-6 added another surprising finding – a high proportion of autoantibodies recognizing IFN-α and IL-22 belonged to IgG4 subclass, in addition to the expected IgG1 that is the dominant subclass of organ-specific autoantibodies (Brozzetti et al. 2010, Bøe et al. 2004). This prevalence of IgG4 was not seen in the less frequent IL-6 antibodies (Table 10). As IL-6 antibodies are mainly IgG1, this result correlates well with the idea of the IL-6 stabilizing effect by the FcRn, which preferentially binds IgG1 (Roopenian and Akilesh 2007). In addition, the anti-IL-17A subclass analysis of the Aire deficient mouse emphasizes even more the dominance of mIgG1, implying a Th2 bias even more clearly than in the human syndromes. However, one should keep in mind that the BALB/c strain, which had the highest reactivity in our experiments, is Th2 biased (Locksley et al. 1987).

The early emergence of type I IFNs and IL-22 autoantibodies seems to involve Th2 differentiation, in addition to Th1 cytokines. High IgG4 serum levels are usually associated with a decrease in allergic reactions and IgE antibodies (Aalberse et al. 2009, Tomicic et al. 2009). In our study, the levels of IgE were minimal (Fig. 3). Thus, it is plausible that IL-10 produced by Tregs, and possibly a specific subset of B cells, as well as chronic antigen exposure, are involved in generating high IgG4 levels (Mobs et al. 2008, van de Veen et al. 2013). The IgG4 is known to moderate IgE hypersensitivity by competing with both high and low affinity receptors and possibly engaging the inhibitory receptor FcγRIIb (Platts-Mills et al. 2004, James and Till 2016, Lambin et al. 1993). IgG4 is induced during hyposensitizing therapy by injecting intradermally small amounts of allergen (Aalberse 2011, Müller 2005). The IgG4 antibodies do not activate complement pathways, and therefore, may help to inhibit IgG1-antigen complex binding to the complement factors. This ability could be involved in regulation of immune inflammation (van der Zee, van Swieten and Aalberse 1986, Schroeder and Cavacini 2010). For example, the anti-inflammatory characteristics may lie in the potential of exchanging a heavy chain and attached light chain with another IgG4 molecule, resulting in bispecific antibodies (van der Neut Kolfschoten et al. 2007, Aalberse et al. 2009). Although we had two patients' sera that contained only IgG4 autoantibodies to IFN-α and IL-22, their neutralizing capacity was as high as in those with IgG1 autoantibodies.

Interestingly, IgG4 is prevalent in certain autoimmune diseases affecting the epidermis, e.g. pemphigus (Aoki, Sousa and Diaz 2011), or other epithelial tissues, e.g. IgG4-related sclerosing disease (Zen and Nakanuma 2011). In our view, these suggest autoimmunization in epithelial tissues, such as the skin, or the thymus, where AIRE-expressing medullary epithelial cells show maturation markers similar to those in the epidermis (Kisand et al. 2011, Wang et al. 2012). This is supported by a strikingly similar IgG4 autoantibody bias in patients with thymomas (Fig. 3b), wherein the key feature shared with APECED appears to be the generation of new T cells in the absence of AIRE (Kisand et al. 2011, Kisand et al. 2010). Also, one subset of *myasthenia gravis* patients have mainly IgG4 autoantibodies that target the muscle-specific kinase (MuSK) (Vincent, McConville and Newsom-Davis 2005). However, the thymus appears to be less involved in this subgroup (Leite et al. 2005). Although IgG4 antibodies do not activate complement, their pathogenicity is generally accepted in pemphigus (Sitaru and Zillikens 2005), and has now gained ground in MuSK-MG, where it might result from interference in MuSK dimerization or other interactions (Vincent et al. 2005). So it is plausible that the IgG4 bias in APECED and thymoma patients might reflect active regulatory mechanisms rather than inflammatory responses.

6.6. Dynamics of cytokine autoantibodies

The cytokine-specific autoantibodies have become the hallmark of APECED, because of the early onset and prolonged presence in the patients' blood sera. In APECED patients the autoantibodies against type I IFNs reach high levels, which remain stable for up to 30 years (Meager et al. 2006, Meloni et al. 2012, Toth et al. 2010). As to thymoma patients, their anti-IFN-α/IL-12 titers are persistent and a sudden increase in titer is a marker of tumor recurrence (Meager et al. 1997, Buckley et al. 2001b). The titers of Th17 specific autoantibodies also rise very early in life in APECED patients, but the titer seems rather to decrease over time (Kisand et al. 2010). In sharp contrast, the IL-6 autoantibodies – when present – were initially low, but apparently increased over time in both of our patient groups, and were maximal in samples taken decades after disease onset (Fig. 13).

During this study, we had the opportunity to study IgG subclasses of anti-IFN-α and IL-22 antibodies, starting from very young ages. In addition to finding substantially high autoantibody titers, we also saw high IgG4 levels, especially to IL-22, already at an early age (Table 8). This is rather surprising, as the constant region of IgG4 is known to be the minority subclass and the most distal of IgG subclasses, therefore rearrangements of Cγ4 would be more likely to take place later in life, as seen after vaccination (Hendrikx et al. 2011). On the other hand, the immune system of infants is considered to be tuned more towards Th2 responses, and our finding of high IgG4 titers conforms to this theory, as IgG4 antibodies, like IgE, need IL-13 and IL-4 (Punnonen et al. 1993).

The overall high autoantibody titers against cytokines in APECED children are rather peculiar, as vaccination of healthy infants (before 2 years of age) usually results in rather weak immune responses (Siegrist and Aspinall 2009, Truck and Pollard 2010). This may hint that the immune system of APECED patients goes through more rapid maturation, similarly seen in IPEX (immunodysregulation polyendocrinopathy enteropathy X-linked syndrome), where patients often suffer from type 1 diabetes already at birth (Xavier-da-Silva et al. 2015). Nevertheless, the mechanism of APECED seems to involve more immune cells and functions than IPEX, which is largely mediated by T cells.

6.7. Thymus as a possible site for autoimmunization towards cytokines

Although thymus is regarded to be a tolerogenic organ, tertiary lymphoid organ-like structures, and plasma cells specific to AChR, IL-12 and/or type I IFNs, are present in the thymus of thymoma patients with MG (Hill et al. 2008, Willcox et al. 2008, Shiono et al. 2003). Also, B cell numbers are increased in mouse thymus in the absence of AIRE (Hässler et al. 2006). Magri et al. 2014 have shown that RORγt+ ILCs can interact with spleen marginal zone B cells that are

T cell independent, and give necessary survival signals (CD40L, BAFF, Delta-like 1). In addition, they get survival signals (e.g. IL-7) from MAdCAM-1+ marginal reticular cells and influence them via tumor necrosis factor and lymphotoxin (Magri et al. 2014). It can be conceived that distorted micro-environments emerge in AIRE deficient thymi and that AIRE-independent autoantigens are available there for bias selection. This may create pro-inflammatory environments, where cytokine expressions are high, creating an altered cross-talk between ILCs and mTECs (Kisand et al. 2011). So, it is plausible that T and B cells could be primed and actively autoimmunized against cytokines and/or target organs, as seen in thymomas (Shiono et al. 2003, Kisand et al. 2011). Given that cytokine-specific autoantibodies persist for decades, the ILCs, that are present in peripheral lymphoid tissue, could produce cytokines and thereby boost the autoimmunization against cytokines (Kim et al. 2009, Meager et al. 2006, Kisand et al. 2010). Persistent secretion of type I IFNs in homeostatic conditions have been already reported in the normal thymic medulla and in germinal centers (Meager et al. 2006).

On the one hand, autoimmunization towards cytokines could be mediated by AIRE-expressing thymic B cells, on the other, it seems improbable, as these type I IFNs, Th17 cytokines, and additional self-protein specific autoantibodies are also found in thymomas, where B cells are rare, and also, AIRE sufficient (Yamano et al. 2015a, Kisand et al. 2011, Meager et al. 1997). This casts doubt on the participating role of extrathymic Aire-expressing cells, whose tolerance induction functions have only been shown in mice (Gardner et al. 2008, Poliani et al. 2010). Moreover, AIRE seems to be dispensable in tolerance induction in specific dendritic cell populations (Crossland et al. 2016). So it is possible that AIRE deficiency influences thymic B cell tolerance indirectly through functionally competent thymic γδ-cells or ILCs, dysregulated in thymomas and capable of creating pro-inflammatory priming environments (Ribot et al. 2009, Dudakov et al. 2012, Meager et al. 2006).

6.8. Autoantibodies can have protective roles

Although the autoantibodies specific to type I IFNs have been known for a long time, the direct effects had remained inexplicable. Susceptibility to viral or other infections is not increased in APECED patients (Kisand and Peterson 2011), in contrast with children that have genetically impaired type I IFN pathway (Ciancanelli et al. 2015). In our study, we analyzed APECED patients for GAD65 autoantibodies, which are considered to be a potential sign of β cell autoimmunity (Ziegler et al. 2013), and of the capacity of the patient serum to neutralize type I IFNs. Intriguingly, the patients whose sera lacked the ability to interfere with type I IFNs biological function, or only moderately inhibited it, had T1D. As type I IFNs have been known to be important in the development of T1D (Foulis et al. 1987, Downes et al. 2010), neutralizing them could potentially have a protective effect. Recently, the connection between autoantibodies

to IFN-γ and *Aspergillus spp.* protein was found. The cross-reaction was linked to the shared linear epitope (Lin et al. 2016). According to one hypothesis, T1D development is probably triggered by enterovirus infection (Hober and Sauter 2010, Filippi and von Herrath 2008). However, in our case, the epitope that APECED patients recognize is conformational, and thereby discovering similar molecular mimicry is almost impossible.

We here propose a new concept that in certain cases, the naturally-arising autoantibodies can be protective against some of the autoimmune diseases. The existence of protective autoantibodies is not widely accepted, although studies in humans and animals have shown that naturally occurring IgM autoantibodies that recognize senescent and pro-apoptotic cells help to clear cell debris, protecting thus against autoimmunity (Nagele et al. 2013, Binder 2010, Tuominen et al. 2006). Protective effect of IgG autoantibodies is not widely acknowledged, despite the widespread use of therapeutic monoclonal antibodies. Systemic sclerosis, Sjögren's syndrome, multiple sclerosis or SLE – pathologies known to develop through interplays of IL-17/Th17 and type I IFNs – are absent in APECED patients, making it a potentially elegant example of the beneficial effect of these cytokine-neutralizing autoantibodies (Ambrosi, Espinosa and Wahren-Herlenius 2012). Likewise, Th17-driven psoriasis was diagnosed in only two of our patients, each of whom lacked auto-antibodies to IL-17A, IL-17F and IL-22 (our unpublished data). Furthermore, atopy/allergy is seemingly rare among APECED patients, although whether anti-IL-5 antibodies are causative for this phenotype requires more study. For now, the data presented by this study strongly suggest that antibodies prevalent in APECED patients include several with profound therapeutic and diagnostic potential.

In conclusion, the studies on cytokine autoantibodies in APECED and thymoma have advanced the knowledge about central tolerance mechanisms in thymus. They support the view that AIRE should have more roles than solely being the regulator of transcription and thereby being the key factor of T cell negative selection (Anderson and Su 2011). As AIRE has other roles in terminal differentiation of mTEC (Matsumoto 2011, Matsumoto et al. 2013), in intrathymic thymocyte migration (Laan et al. 2009), in thymic architecture (Gillard et al. 2007) and in mTEC ultrastructure (Milicevic et al. 2010), it may have essential function in normal thymic homeostasis.

7. CONCLUSIONS

1. High proportions of type IgG4 autoantibodies that develop already at a very young age in APECED patients and the lack of IgA autoantibodies indicate non-mucosal sites and regulatory T cell involvement in the autoimmunization process against type I IFNs and IL-22.
2. In contrast, IL-6-specific autoantibodies develop late in APECED and thymoma patients' life, are mainly of IgG1 type and fail to neutralize the biological effect of IL-6. Instead, they might mediate the stabilization of IL-6 *in vivo*.
3. The autoantibody binding to type I IFNs, Th17 related cytokines and IL-6 is mostly conformation-sensitive.
4. Most of the major organ-specific autoantigens that are shared by APECED and thymoma patients appeared to be AIRE-independent, supporting the idea of multiple roles of AIRE in thymic tolerance induction.
5. Aire deficient mouse model is not suitable for studying the mechanisms of autoimmunization against cytokines.
6. Our data suggest that high titers of neutralizing autoantibodies to type I IFNs are protective against T1D in APECED patients.

8. SUMMARY IN ESTONIAN

Tsütokiinide spetsiifilised autoantikehad AIRE puudulikkuse korral

Autoimmuunne polüendokrinopaatia-kandidoos-ektodermaalne düstroofia (APECED) on haruldane autoimmuunhaigus, mis esineb ennekõike väikestes, isoleeritud populatsioonides (Iraani juudid, soomlased, sardiinlased). APECED on põhjustatud ainult ühe geeni – autoimmuunsuse regulaatori (*AIRE*) – mutatsioonidest. *AIRE* geen ekspresseerub eelkõige tüümuse säsirakkudes, kus selle üheks peamiseks teadaolevaks ülesandeks on reguleerida koespetsiifiliste antigeenide avaldumist. AIRE mutatsioonide tagajärjel häirub arenevate tümotsüütide negatiivne selektsioon, mille tagajärjel pääsevad potentsiaalselt autoreaktiivsed T rakud perifeeriasse, põhjustades autoimmuunkahjustusi.

APECED patsientide haiguspilt on väga kompleksne, kuid peamiselt saavad kahjustada endokriinorganid. Enamasti algab haigus lapseeas kroonilise naha ja limaskestade kandidoosiga (KMK), millele järgnevad neerupealiste ja kõrvalkilpnäärme puudulikkused. Hiljem võib lisanduda väga erinevaid autoimmuunseid organikahjustusi, näiteks pankrease β-rakkude hävitamise tagajärjel kujunev esimest tüüpi diabeet. APECED patsientidele on iseloomulik kõrges tiitris autoantikehade esinemine. Nende hulgas on kõige suurema esinemissagedusega tsütokiinide vastased autoantikehad.

I tüüpi interferoonide (IFNide) spetsiifilised autoantikehad on olemas kõigil patsientidel ning need on kujunenud usaldusväärseks diagnostiliseks markeriks. Kuigi antud antikehad on enamasti kõrges tiitris ja neutraliseerivad, ei ole täheldatud patsientide kõrgenenud vastuvõtlikkust viirushaigustele, mis olnuks ootuspärane, kuna I tüüp IFNid on olulised kiire viirusvastuse tekkeks. Lisaks esinevad 40–90% patsientidest interleukiin (IL-)17A, IL-17F ja IL-22 spetsiifilised autoantikehad, mis otseselt korreleeruvad kroonilise *Candida albicans'*i infektsiooniga. Nii I tüüpi IFNide kui IL-17A, IL-17F ja IL-22 vastaste antikehade tekkemehhanism ning -keskkond AIRE puudulikkuse korral on jäänud veel seletuseta, kuna antud tsütokiinide ekspressiooni ei reguleeri AIRE.

Aire uurimiseks on loodud ka erineva taustaga Aire-defitsiitseid hiireliine, kuid mudel-haiguse fenotüüp pole nii tõsine ning puuduvad ka enamik APECED patsientidele iseloomulikest autoimmuunkahjustustest ning autoantikehadest. Samas on leitud tüümuse kasvaja – tümoomiga – patsientidel nii I tüüpi IFNide kui IL-17A/F kui IL-22 spetsiifilisi antikehasid, millest viimasena mainitud on korrelatsioonis *C. albicans'* i esinemisega. Osadel neist patsientidest jätkub tüümuses T-rakkude küpsemine, kuid kuna normaalne homöostaas koos AIRE ekspressiooniga on häirunud, satub perifeeriasse ka autoreaktiivseid T-rakke, mis võivad põhjustada mitmeid autoimmuunilminguid. Lisaks on tümoomi patsientide tüümuses kirjeldatud idukeskuse sarnaseid struktuure, milles toimub autoantikehade tootmine.

Uurimistöö eesmärgid

Käesoleva töö põhiliseks eesmärgiks oli iseloomustada APECED patsientides, tümoomihaigetes ja Aire puudulikus hiiremudelis tsütokiinispetsiifilisi antikehasid:

- iseloomustada tsütokiinivastaseid antikehasid täiskasvanud ja väga noortel APECED patsientidel,
- uurida, kas APECED ja tümoomi patsientides on autoantikehasid Th17 toovate rakkude diferentseerumiseks ja säilitamiseks vajaminevate tsütokiinide vastu;
- leida IFN-α2a ja IL-22 immunodominantsed epitoobid;
- uurida Aire-puudulikes hiirtes tsütokiinivastaste autoantikehade esinemist;
- uurida, kas põhilised APECED patsientide organspetsiifilised autoantigeenid on tümoomi, tüümuse jäänukis ja normaalses tüümuses AIRE-sõltuvad;
- uurida, kas APECED patsientide vereseerumis esinevate I tüüpi interferoonide spetsiifiliste autoantikehade neutraliseerimisvõime võiks olla negatiivses korrelatsioonis 1. tüüpi diabeedi (T1D) esinemisega.

Materjal ja metoodika

Uurimistöös olid kasutusel viis erinevat patsientide gruppi: 1) 19 APECED ja 40 tümoomi patsienti; 2) 11 APECED patsienti ja 2 diagnoosita sugulast (vanuses 15 kuud – 9 aastat); 3) 26 tümoomi patsienti ja 5 südameoperatsiooni käigus eemaldatud lapse tüümust; 4) 41 APECED ja 104 tümoomi patsienti; 5) 81 APECED patsienti. APECED patsientide vereseerumid pärinesid Norrast, Sloveeniast, Soomest, USAst, Ungarist ja Eestist; tümoomi patsientide vereseerumid ja tüümuse koeproovid Suurbritanniast ning tervete laste tüümuse koeproovid Eestist. Kontrollgruppide vereseerumid pärinesid tervetelt perekonnaliikmetelt ja/või tervetelt veredoonoritelt. Kõik teostatud uuringud on kooskõlas Helsingi deklaratsiooniga, omavad kohalike eetikakomiteede luba ning uuritavad olid allkirjastanud informeeritud nõusoleku. Aire-puudulikud hiired olid 967–979 del13 mutatsiooniga ning C57BL/6 või BALB/c taustal, uuritav vereseerum eraldati neilt 6–8 nädala või 1,5–2 aasta vanuselt. Kõik hiirtega tehtud tööd olid kooskõlas Euroopa Liidu direktiiviga 86/609/EEC.

Vereseerumist isoleeriti immunoglobuliin (Ig) G ja IgA vedelikkromatograafia abil ning seejärel kontsentreeriti lahused ja hinnati nende puhtust SDS-PAGE'i ja Western blotiga. Autoantikehade seondamisvõimet hiirtel tuvastati ensüüm-immunosorptsioon-analüüsi (ELISA). Lutsiferaasil-põhinevat-immunosadestamis-analüüsi (LIPS) kasutati inimeste vereseerumi tsütokiinispetsiifilise seondamisvõime ja IgG alaklasside määramiseks. IFN-α2a ja IL-22 immunogeensete epitoopide hindamiseks jagati need fragmentideks, mis kloneeriti kokku lutsiferaasiga ning teostati seejärel LIPS analüüs. Samuti testiti Western blot analüüsiga patsientide vereseerumi seondamisvõimet pärast IFN-α2a, IFN-ω, IL-6 ja IL-22 denatureerimist SDS-geelil. Autoantigeenide ekspressiooniks eraldati külmutatud tümoomide ja kontrollisikute koetükkidest RNA, millest

seejärel tehti cDNA ning teostati reaalaja PCR. Seerumite/väljapuhastatud immunoglobuliinide neutraliseerimisvõime hindamiseks kasutati rakupõhiseid neutralisatsiooniteste.

Uurimistöö peamised tulemused ja järeldused:

1) immunoglobuliini G fraktsioon, mitte IgA, neutraliseerib APECED patsientide vereseerumis tsütokiine. See tähendab, et tsütokiinivastane autoimmuniseerimine ei toimu limaskestadel ja pole ilmselt otseselt *Candida* infektsiooni tagajärg. APECED ja tümoomi patsientidel on IgG1 ja IgG4 põhilised IFN-α ja IL-22 seondavad alaklassid. Kuna IgG4 tase vereseerumis on tavaliselt kõige madalam ning APECED patsientidel on seda tüüpi autoantikehade tase kõrge väga varajases eas, siis võib järeldada, et autoimmuniseerumises nende tsütokiinide vastu osalevad ka regulatoorsed T-rakud.
2) APECED ja tümoomi patsientidel on vereseerumis IL-6 spetsiifilised antikehad, mis tekivad hilisemas eas, on eelistatult IgG1 alaklassist ning ei suuda IL-6 bioloogilist funktsiooni neutraliseerida, vaid näivad selle taset vereseerumis stabiliseerivat.
3) autoantikehade seondumine I tüüp IFNide, Th17 tsütokiinide ja IL-6ga toimub peamiselt konformatsiooniliste epitoopide kaudu: tsütokiinide lineariseerimise või nende molekulaarse terviklikkuse häirumisel autoantikehade seondumine väheneb või kadus hoopis.
4) Aire-puuduliku hiire vereseerumis, sarnaselt APECED patsientidele, on olemas Th17 tsütokiinide vastased autoantikehad. Samas on nende dünaamika vastupidine APECED patsientidele: tiitrid tõusevad ja neutraliseerimisvõime tekib hiire vananedes ning need sõltuvad hiireliini taustast. Seega ei ole võimalik Aire-puudulikku hiiremudelit kasutada tsütokiinde vastase autoimmuniseerumise mehhanismide uurimiseks.
5) kuigi tümoomi koes on *AIRE* ekspressioon madalam kui normaalses tüümuses, ei järgi peamised APECEDi ja tümoomi patsientide poolt jagatud autoantigeenid sama mustrit. See tähendab, et paljude autoantigeenide avaldumine tüümuses ei ole AIRE-st sõltuv ning viitab sellele, et AIRE-l on tüümuses kanda mitmekesisem roll kui vaid koespetsiifiliste geenide transkriptsioonilise regulaatori oma.
6) esimest tüüpi diabeet kujuneb vaid väikesel osal APECED patsientidest, ning enamasti alles täiskasvanueas. Samas on GAD65 spetsiifilised autoantikehad, mis on isoleeritud T1D markeriks, määratavad ka neil APECED patsientidel, kellel T1D puudub, mis viitab pankreasesaarekeste vastasele autoimmuunsusele. Meie töö tulemused lubavad oletada, et APECED patsientide vereseerumite võime neutraliseerida I tüüpi IFNe kõrges tiitris kaitseb neid T1D avaldumise eest.

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Teadusorganisatisooniline ja -administratiivne tegevus

2010–… Eesti Immunoloogide ja Allergoloogide Selts

Publikatsioonid:

1. Kärner, J., A. Meager, M. Laan, J. Maslovskaja, M. Pihlap, A. Remm, E. Juronen, A. S. B. Wolff, E. S. Husebye, K. T. Podkrajšek, N. Bratanic, T. Battelino, N. Willcox, P. Peterson and K. Kisand. (2013), 'Anti-Cytokine Autoantibodies Suggest Pathogenetic Links with Autoimmune Regulator Deficiency in Humans and Mice', *Clinical & Experimental Immunology* Vol. 171, No. 3, pp. 263–272.
2. Wolff, A. S. B., A. K. Sarkadi, L. Marodi, J. Kärner, E. Orlova, B. E. V. Oftedal, K. Kisand, E. Olah, A. Meloni, A. G. Myhre, E. S. Husebye, R. Motaghedi, J. Perheentupa, P. Peterson, N. Willcox and A. Meager. (2013), 'Anti-Cytokine Autoantibodies Preceding Onset of Autoimmune Polyendocrine Syndrome Type I Features in Early Childhood', *Journal of Clinical Immunology* Vol. 33, No. 8, pp. 1341–1348.
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4. Rebane, A., T. Runnel, A. Aab, J. Maslovskaja, B. Rückert, M. Zimmermann, M. Plaas, J. Kärner, A. Treis, M. Pihlap, U. Haljasorg, H. Hermann, N. Nagy, L. Kemeny, T. Erm, K. Kingo, M. Li, M. P. Boldin and C. A. Akdis. (2014), 'Microrna-146a Alleviates Chronic Skin Inflammation

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