Immune regulatory mechanisms in endocrine autoimmune disorders

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Scientific environment

The work of this thesis was conducted between 2020 and 2023 at the Department of Clinical Science and KG Jebsen Center for Autoimmune Disorders, Faculty of Medicine, University of Bergen, and Department of Medicine, Haukeland University Hospital, Bergen, Norway. Main supervisor was Professor Anette Susanne Bøe Wolff and co-supervisor was researcher Bergithe Eikeland Oftedal. Financial support was provided by Western Norway Health Authorities (Helse Vest), the KG Jebsen Foundation and Gades Foundation. Travel grants were received from the Scandinavian Society of Immunology.

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cooked meals and you taking care of the laundry I always bring with me. Without your support, this work would not have been possible. To my best friend Anne: I truly appreciate our (almost) weekly FaceTime talks, “Feite Fredager” and our discussions about the Real Housewives and the Kardashians. Our trip to London was one of the best, especially the glass floor walk at Tower Bridge, and a much needed distraction from a busy PhD-life. Thank you for always listening to all my frustrations and for keeping me motivated. I could not have done this without you. To Ingeborg, I have really enjoyed our afternoon breaks that always lasts a tiny bit longer than planned.

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Thea

Abstract

The immune system is, through various regulatory mechanisms, taught to discriminate self from non-self, known as immunological tolerance. Sometimes breakdown of tolerance occur, leading to autoimmune disease in susceptible individuals. Of note, patients with immunodeficiencies also commonly present with autoimmune components, indicating that impaired regulatory mechanisms might be a shared risk factor. Cells with suppressive function, particularly regulatory T cells (Tregs), are crucial for regulation of tolerance and alterations in the Treg compartment can contribute to disease development. Another shared factor is circulating autoantibodies targeting cytokines, which have been described in both immunodeficiency and autoimmunity. However, details on how these mechanisms come together and regulate immune tolerance still remain elusive. In the present work, several approaches have been used to study immune regulatory mechanisms in human model disorders of autoimmunity.

In paper I, we aimed at investigating the proteome and transcriptome of *in vitro* expanded Tregs from patients with the monogenic autoimmune polyendocrine syndrome type 1 (APS-1). Single-cell and T cell receptor (TCR) sequencing was performed to examine differential gene expression and analyze the TCR repertoire. Overall, the expanded Treg transcriptome and TCR repertoire were found to be similar between patients and controls. Multipanel flow and mass cytometry revealed a significantly reduced CD4+CD25+FOXP3+CD127- population in patients compared to healthy donors. An optimized flow cytometry based Treg suppression assay revealed that APS-1 expanded Tregs could suppress responder cell proliferation to the same degree as expanded Tregs from healthy controls.

In paper II, we wanted to examine the frequency of Tregs in PBMC and explore *in vitro* expanded Treg suppressive function, transcriptome and mitochondrial function in autoimmune primary adrenal insufficiency (PAI), an endocrine autoimmune disorder with a polygenic inheritance pattern. No differences were observed in the frequency of Tregs in PBMC when comparing patients and healthy controls. The Treg

suppression assay showed that expanded PAI Tregs were able to suppress proliferation of responder cells similar to expanded healthy control Tregs. However, the RNA sequencing revealed an upregulation of mitochondrial genes in the patient group. To further examine mitochondrial function, a Seahorse mitochondria stress test assay was performed. Patient cells displayed a basal, maximal and non-mitochondrial respiration, in addition to proton leak and spare capacity, comparable to that of healthy controls.

In paper III, we wanted to examine whether screening of patients with known endocrine disorders for cytokine autoantibodies could identify individuals with a possible monogenic or oligogenic cause of disease. An enzyme-linked immunosorbent assay (ELISA) was optimized and performed to screen patients with autoimmune PAI for autoantibodies against interleukin (IL) 22, while an established radio-immuno assay (RIA) was used to screen patients with a variety of endocrine autoimmune disorders for autoantibodies against interferon (IFN)-ω. Positive patients were subsequently sequenced with an immune deficiency pipeline at Haukeland University Hospital. This strategy identified four new patients with APS-1 and two patients with disease-causing mutations in *NFKB2* and *CTLA4*, respectively. Further, rare variants were detected in the genes *IKZF2*, *LAT*, *LCK*, *STAT1*, *JAK3*, *RAG1*, *TERC*, *TNFAIP3* and one variant of *AIRE* associated with autoimmune PAI, all with potential roles as causative factors for the clinical manifestations seen in these patients.

Together, the findings from this thesis furthers our knowledge on immune regulatory mechanisms in autoimmune disease and highlights the use of autoantibody screenings as a part of the diagnostic toolbox. Our findings open for future studies involving further characterization of monogenic diseases with cytokine autoantibodies and of immune regulatory mechanisms.

Sammendrag

Ulike mekanismer eksisterer for at immunforsvaret skal lære å skille det som er eget fra det som er fremmed, også kjent som immunologisk toleranse. I noen tilfeller brytes denne toleransen, noe som kan føre til at enkelte utvikler autoimmune sykdommer. Det som er interessant er at pasienter med immunsvikt også ofte utvikler autoimmunitet. Dette kan tyde på at en feil i immunforsvarets regulerende mekanismer kan være en felles risikofaktor. Regulatoriske T celler (Tregs) har evnen til å dempe en immunrespons og er veldig viktige for å opprettholde toleranse. Et annet fellestrekk er sirkulerende autoantistoffer rettet mot cytokiner, som er funnet i både immunsvikt og autoimmunitet, men hvordan disse mekanismene virker sammen for å regulere toleranse er fortsatt uvisst. I denne avhandlingen har ulike metoder blitt brukt for å studere regulatoriske mekanismer i to modellsykdommer for autoimmunitet.

I artikkel I undersøkte vi transkriptomet og proteomet til *in vitro* ekspanderte Tregs fra pasienter med den monogene sykdommen autoimmunt polyendokrint syndrom type 1 (APS-1). Vi brukte enkelt-celle og T celle reseptor (TCR) sekvensering for å se på forskjell i genuttrykk mellom pasienter og kontroller. Samlet viste resultatene få forskjeller i transkriptomet til ekspanderte Tregs og TCR repertoar mellom pasienter og kontroller. Analyse med flow- og massecytometri viste at CD4+CD25+FOXP3+CD127- populasjonen var signifikant redusert hos APS-1 pasienter sammenlignet med friske kontroller. For å undersøke Tregs sin evne til å dempe proliferering av responder celler, satte vi opp et flowcytometri-basert suppresjonsassay, som viste at ekspanderte Tregs fra APS-1 pasienter kan hemme celledeling like bra som ekspanderte Tregs isolert fra friske kontroller.

I artikkel II ville vi undersøke frekvensen av Tregs i PBMC og den suppressive kapasiteten til ekspanderte Tregs i pasienter med den endokrine autoimmune sykdommen primær binyrebarksvikt, med polygent arvemønster. I tillegg har vi sett på forskjeller mellom Tregs fra disse pasientene og friske kontroller på transkripsjonsnivå og studert mitokondriell respirasjon. Ingen forskjeller ble funnet i frekvensen av Tregs i PBMC når pasienter og kontroller ble sammenlignet. Suppresjonsassayet viste at det

ikke er forskjeller mellom pasienter med primær binyrebarksvikt og friske kontroller når det kommer til å dempe proliferering av responder celler. Vi gikk videre med RNA-sekvensering for å se om vi kunne finne forskjeller på gennivå. Igjen viste resultatene få forskjeller mellom pasienter og kontroller, men flere gener uttrykt i mitokondriene var oppregulert i pasientgruppen. For å studere mitokondriell funksjon, satte vi opp et Seahorse metabolisme assay for å måle raten av oksygenforbruk. Assayet viste at Tregs fra pasienter hadde en basal-, maksimal-, og ikke-mitokondriell respirasjon, i tillegg til proton lekkasje og reservekapasitet på nivå med målinger for friske kontroller.

I artikkel III ønsket vi å finne ut om cytokin-autoantistoff-screening kan brukes til å identifisere pasienter med mono- eller oligogen årsak til sykdom. Et enzyme-linked immunosorbent assay (ELISA) ble satt opp for å måle autoantistoffer mot interleukin (IL) 22, mens et etablert radioaktivitetsassay ble brukt til å mål autoantistoffer mot interferon (IFN)-ω. Positive pasienter ble deretter sekvensert med et panel bestående av immunerelaterte gener etter standard protokoll fra Medisinsk avdeling, Haukeland Universitetssykehus. Med denne strategien identifiserte vi fire nye pasienter med APS-1 og to pasienter med sykdomsfremkallende mutasjoner i henholdsvis *NFKB2* og *CTLA4*. I tillegg fant vi sjeldne varianter i *IKZF2*, *LAT*, *LCK*, *STAT1*, *JAK3*, *RAG1*, *TERC*, *TNFAIP3* og en variant av *AIRE* som er assosiert med primær binyrebarksvikt, varianter som kan være forårsakende for de kliniske manifestasjonene hos pasientene.

Resultatene fra denne avhandlingen bidrar med økt kunnskap om regulatoriske mekanismer involvert i immunologisk toleranse og viser at screening for autoantistoffer er et verdifullt diagnostisk verktøy. Videre åpner funnene for studier som karakteriserer monogene sykdommer med autoantistoffer mot cytokiner og andre regulatoriske mekanismer.

Abbreviations

21OH	21-hydroxylase
AIRE	Autoimmune regulator
AITD	Autoimmune thyroid disease
AP-1	Activator protein 1
APC	Antigen presenting cell
APECED	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy
APS	Autoimmune polyendocrine syndrome
ATP	Adenosine triphosphate
BCR	B cell receptor
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CD	Cluster of differentiation
Cdk	Cyclin dependent kinase
CID	Combined immunodeficiency
CMC	Chronic mucocutaneous candidiasis
CpG	5'-Cytosine-phosphate-Guanine-3'
CTLA4	Cytotoxic T lymphocyte associated protein 4
CyTOF	Cytometry by time-of-flight
CVID	Common variable immunodeficiency
DC	Dendritic cell

DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
FOXP3	Forkhead box P3
GRB2	Growth factor receptor-bound protein 2
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
HP	Hypoparathyroidism
IL	Interleukin
IFN	Interferon
IPEX	Immunodysregulation polyendocrinopathy enteropathy X-linked
ITAMs	Immunoreceptor tyrosine-based activation motifs
iTreg	Inducible Treg
KIR	Killer-cell immunoglobulin-like receptor
KLRC	Killer-cell lectin-like receptor C
LAG	Lymphocyte activating gene
LAT	Linker for activation of T cells
LCK	Lymphocyte protein tyrosine kinase
MAIT	Mucosal-associated invariant T cell
MAPK	Mitogen-activated protein kinase

MHC	Major Histocompatibility Complex
mTEC	Medullary thymic epithelial cell
NALP5	NACHT leucine-rich repeat protein 5
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor kappa light-chain enhancer of activated B cells
NK cell	Natural killer cell
NOD	Non-obese diabetic mice
PAI	Primary adrenal insufficiency
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PHD	Plant homeo-domain
PLCγ1	Phospholipase C gamma 1
POI	Primary ovarian insufficiency
pTreg	Peripheral Treg
RAG	Recombination-activating gene
RIA	Radio-immuno assay
RNA	Ribonucleic acid
ROAS	Registry for Organ-Specific Autoimmune Diseases
SAND	*S*p100, *A*IRE-1, *N*ucP41/75 and *D*EAF-1
SCC	Side-chain cleavage enzyme

SCID	Severe combined immunodeficiency
SLE	Systemic lupus erythematosus
SLP-76	Scr-homology 2 domain containing 76-kDa leukocyte protein
STAT	Signal transducer and activator of transcription
TGF-β	Transforming growth factor beta
Th cell	T helper cell
Tr1	Type 1 regulatory T cell
Treg	Regulatory T cell
Tresp	Responder T cell
TSDR	Treg-specific demethylated region
tTreg	Thymic-derived Treg
ZAP-70	Zeta-chain associated protein kinase 70

List of publications

Paper I

Thea Sjøgren, Shahinul Islam, Igor Filippov, Adrianna Jebrzycka, André Sulen, Lars E. Breivik, Alexander Hellesen, Anders P. Jørgensen, Kari Lima, Liina Tserel, Kai Kisand, Pärt Peterson, Annamari Ranki, Eystein S. Husebye, Bergithe E. Oftedal and Anette S. B. Wolff (2023).

Single-cell transcriptomic and proteomic analyses reveal functional regulatory T cells in autoimmune polyendocrine syndrome type 1.

Submitted manuscript.

Paper II

Thea Sjøgren, Jan-Inge Bjune, Bergithe E. Oftedal, Eystein S. Husebye and Anette S. B. Wolff (2023).

Regulatory T cells in autoimmune primary adrenal insufficiency.

Manuscript.

Paper III

Thea Sjøgren, Eirik Bratland, Ellen C. Røyrvik, Marianne Aa. Grytaas, Andreas Benneche, Per M. Knappskog, Olle Kämpe, Bergithe E. Oftedal, Eystein S. Husebye and Anette S. B. Wolff (2022).

Screening patients with autoimmune endocrine disorders for cytokine autoantibodies reveals monogenic immune deficiencies.

Journal of Autoimmunity, Volume 133, December 2022, 10.1016/j.jaut.2022.102917.

Related publications

Amund Holte Berger, Eirik Bratland, **Thea Sjøgren**, Marthe Heimli, Torgeir Tyssedal, Øyvind Bruserud, Stefan Johansson, Eystein Sverre Husebye, Bergithe Eikeland Oftedal and Anette Susanne Bøe Wolff (2021).

Transcriptional changes in regulatory T Cells from patients with autoimmune polyendocrine syndrome type 1 suggest functional impairment of lipid metabolism and gut homing.

Frontiers in Immunology, Volume 12, August 2021, 10.3389/fimmu.2021.722860.

Anette S. B Wolff, Lena Hansen, Marianne Aa. Grytaas, Bergithe E. Oftedal, Lars Breivik, Fan Zhou, Karl Ove Hufthammer, **Thea Sjøgren**, Jan Stefan Olofsson, Mai Chi Trieu, Anthony Meager, Anders P. Jørgensen, Kari Lima, Kristin Greve-Isdahl Mohn, Nina Langeland, Rebecca Jane Cox and Eystein S. Husebye (2023).

Vaccination prevents severe COVID-19 outcome in patients with neutralizing type 1 interferon autoantibodies.

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Contents

1. Introduction

The immune system is a complex network of cells and molecules working together to protect the host from a variety of invasive, disease-causing agents (1). Any dysregulation of immune homeostasis can lead to autoimmunity on one side or cancer and immunodeficiency on the other side. Autoimmune disease affect around eight percent of the population in Europe and the United States (2), with a seemingly higher prevalence in women, and collectively cause significant morbidity and mortality (3, 4). Identifying and describing mechanisms that establish and maintain tolerance by examining conditions where tolerance is broken could pave the way for generating future therapy for immunological conditions.

While autoimmune conditions are characterized by an overactive immune system targeting self-components, an immunodeficiency is the opposite, where the immune system fails to respond adequately to infections. Intriguingly, patients with immunodeficiencies also acquire autoimmune components (5), pointing to improper regulatory mechanisms as a joint risk factor (Fig. 1). One important regulatory mechanism is regulatory T cells (Tregs), vital for the maintenance of tolerance (6). Another shared feature between autoimmunity and immunodeficiencies is autoantibodies, which are present in a variety of autoimmune disorders, and target molecules ranging from tissue-specific to more or less ubiquitously expressed proteins like deoxyribonucleic acid (DNA), ribonucleic acid (RNA) and cytokines (7). Cytokine autoantibodies are also described in a number of immunodeficiencies, such as in patients with *recombination-activating gene* (*RAG*) gene deficiencies (8) and in patients with immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (9). Even though most autoimmune disorders are mainly caused by autoreactive T cells, autoantibodies produced by B cells might contribute to establishment, exaggeration or maintenance of autoimmune disorders, and are nevertheless of high diagnostic value, as they are often present in sera and plasma even before disease onset (10).

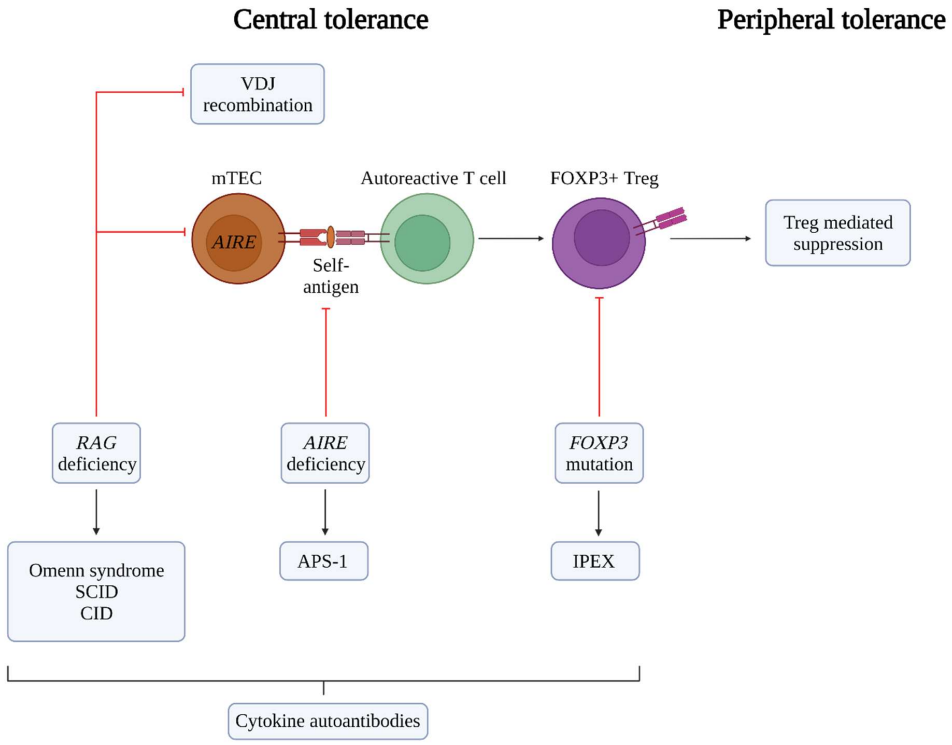

Figure 1. Deficiency of central and peripheral tolerance mechanisms can cause autoimmunity and immunodeficiency. *Impaired VDJ recombination during T cell development in the thymus, caused by mutations in RAG genes, leads to Omenn syndrome, severe combined immunodeficiency (SCID) and combined immunodeficiency (CID). A missing or non-functional autoimmune regulator (AIRE) gene interrupts thymic tolerance and clinically manifests as autoimmune polyendocrine syndrome type 1 (APS-1), while mutations in the Treg master regulator forkhead box P3 (FOXP3) causes failure of peripheral tolerance and immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome. Patients with IPEX do not have functional Treg cells. In all of these disorders patients have cytokine autoantibodies. Figure created with BioRender.com. Adapted from Schmidt et.al. (5).*

In the following, a brief introduction to the adaptive immune system, with focus on T cells, is provided. This is followed by a description of tolerance mechanisms, focusing on Tregs. Finally, autoimmune disease is covered, with emphasis on autoimmune primary adrenal insufficiency (PAI) and autoimmune polyendocrine syndrome (APS) type 1 (APS-1).

1.1 Adaptive immunity

An adaptive immune response is highly specific and will lead to generation of immunological memory (11, 12). Consequently, this type of immune response will improve upon reencountering the same pathogen. The adaptive response is mediated mainly by two different subsets of lymphocytes, B cells expressing B cell receptors (BCR) and T cells expressing T cell receptors (TCR). BCR and TCR rearrangement processes taking place in the bone marrow and thymus, respectively, will give each B and T cell a distinct receptor with a unique antigen specificity (13).

1.1.1 B cells and autoantibodies

B cells exert their effector functions by acting as antigen presenting cells (APCs) and by becoming plasmablasts and plasma cells with the ability to produce antibodies. Antibodies produced by B cells are highly specific and important for eliminating and neutralizing pathogenic substances, however, they can also cause tissue damage, rejection of organ transplants and autoimmune disease (14, 15). Several autoimmune conditions are hallmarked by the presence of autoantibodies targeting self-antigens (16). These autoantibodies can be useful markers of autoimmune conditions, as they are often present in serum long before a patient develops clinical symptoms of disease (10, 17). In autoimmune disease, autoantibodies can for instance be stimulating, such as autoantibodies activating the thyroid-stimulating hormone receptor in Graves' disease (18), inhibiting, such as antibodies against the acetylcholine receptor in myasthenia gravis (19), or neutralizing, as exemplified by type I interferon (IFN) and interleukin (IL) 22 autoantibodies in APS-1 (20, 21) (Section 1.5.2). However, B cells are not able to produce autoantibodies or become memory cells without help from T cells (22, 23).

1.1.2 T cells

T cells make up the cellular part of adaptive immunity and are able to directly kill invaders or secrete cytokines to help other immune cells to exert their defending potential. T cells can be divided into two major subsets based on the expression of co-receptors cluster of differentiation (CD) 8 and CD4, which interact with major histocompatibility complex (MHC) class I and II, respectively (24, 25). Almost all nucleated cells express MHC I, while MHC II is found on APCs, such as dendritic cells (DCs), macrophages and B cells. CD4 positive T cells are known as T helper (Th) cells and are producers of cytokines important for the differentiation and activation of other cell types (26). In addition, they are required for the initiation of a CD8 positive T cell mediated immune response, and these cells will become cytotoxic and can directly kill a pathogen upon activation (27).

1.1.3 T cell activation and downstream signalling

To eliminate and prevent a pathogen from causing disease, T cells need to be activated in a process consisting of three steps. The first step is the interaction between the TCR and MHC I/II-antigen complex on the APC. Secondly, the T cell requires a co-stimulatory signal through its CD28 molecule, interacting with CD80/86 on the APC (28, 29). Lack of co-stimulation through CD80/86 will leave cells in a state of unresponsiveness, also known as anergy (30). Further, production of cytokines have been shown to provide a third activation signal (31). In addition, T cells will start to produce IL2 upon activation, promoting T cell clonal expansion (32)

Post-activation, T cells upregulate cytotoxic T-lymphocyte-associated protein 4 (CTLA4), which acts as an inhibitor of T cell activation and downregulates the immune response by out-competing CD28 for its ligands (33-35). The importance of CTLA4 as an immune checkpoint is shown in mice with homozygous *Ctla4* mutations, who develop fatal lymphoproliferative disease (36, 37). The T cell activation process is

shown in Fig. 2. Most activated T cells will eventually die by apoptosis, but a small fraction will survive and become memory T cells, providing long lasting immunological memory (12).

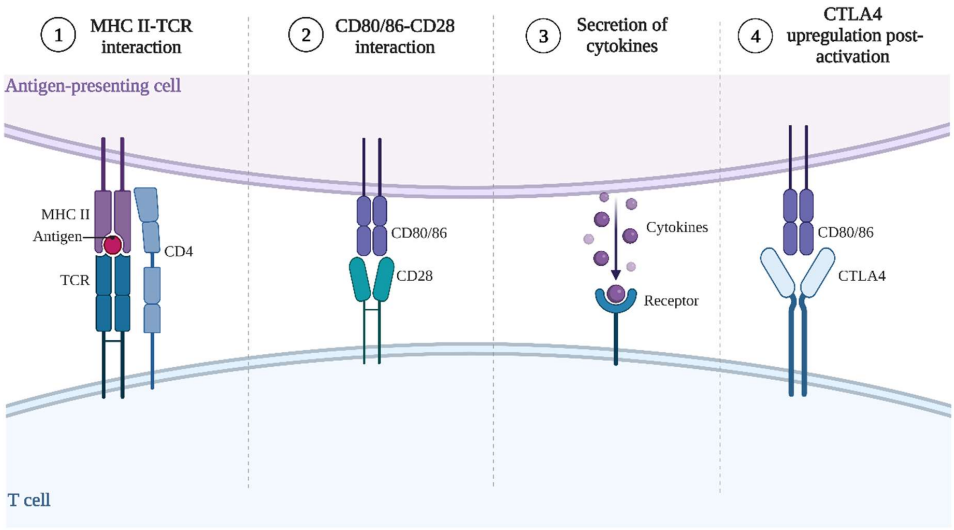

Figure 2. T cell activation. *Three signals are required for complete T cell activation. (1) MHC-antigen-TCR interaction for activation, (2) CD80/86-CD28 interaction for survival and (3) production of cytokines from the APC for T cell differentiation and clonal expansion. Stimulation of CD28 by CD80/86 induces (4) production and upregulation of CTLA4, which regulates T cell activation by attenuating TCR signalling through competitive inhibition with CD28 for their shared ligands. Adapted from "Three signals required for T cell activation" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.*

The initiation of a T cell mediated immune response requires antigen presentation through MHC I or II (38). A fragment of antigen is presented on the cell surface of an APC, and a T cell harbouring a specific TCR will recognize the antigen and initiate an immune response. A small fraction of T cells have a TCR consisting of γ/δ chains, but for the majority of T cells, it consists of an α- and a β-chain, forming a TCR-complex with CD3 and a ζ-chain, containing a cytoplasmic tail necessary for downstream signalling and activation (39-41) (Fig. 3).

Activation of the TCR after interaction with MHC/peptide complex initiates various downstream signalling pathways within the cell (Fig. 3). One of the earliest events occurring is the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs), located on the cytosolic part of the TCR/CD3 complex, by lymphocyte protein tyrosine kinase (LCK) (42). Further, zeta-chain associated protein kinase 70 (ZAP-70) is recruited to the complex and is activated by LCK-mediated phosphorylation (43). Activation of ZAP-70 leads to recruitment and activation of several signalling molecules, including linker for activation of T cells (LAT) and Src-homology 2 domain-containing 76-kDa leukocyte protein (SLP-76) (43).

Phosphorylated LAT will then recruit growth factor receptor-bound protein 2 (GRB2) and phospholipase C gamma 1 (PLCγ1), leading to the activation of RAS, mitogen-activated protein kinase (MAPK) and Ca^{2+} signalling pathways (44). These events will subsequently activate downstream transcription factors, including nuclear factor of activated T cells (NFAT), which forms a complex with activator protein 1 (AP-1) transcriptional factors JUN/FOS, and nuclear factor kappa light-chain enhancer of activated B cells (NF- κB) (44). Together, this will lead to transcription of specific genes necessary for T cell activation, survival, proliferation and differentiation into different effector cell subsets (44).

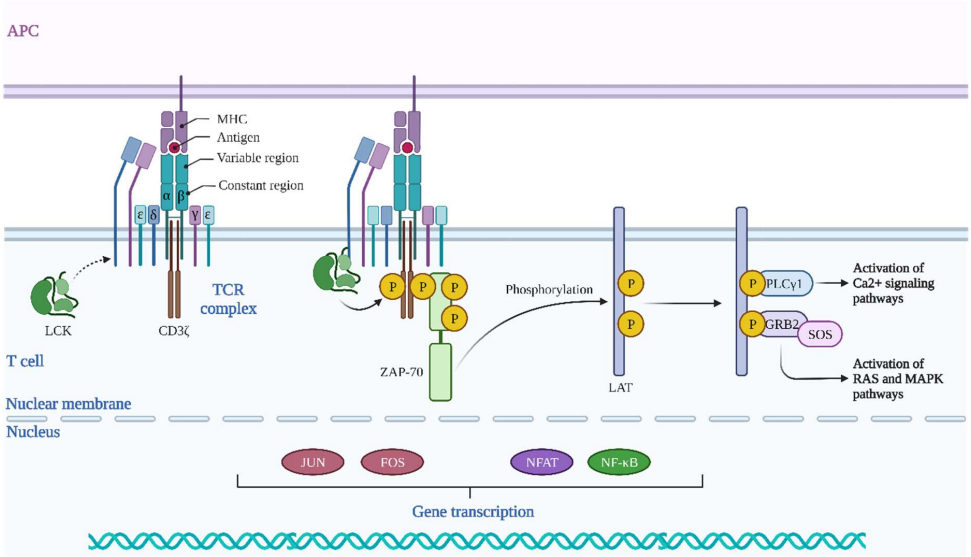

Figure 3. T cell receptor and downstream signalling. *The TCR consists of a constant and variable region, with a α/β chain expressed by most T cells. TCR activation by interaction with the MHC/antigen complex causes phosphorylation of ITAMs by LCK. This will in turn lead to recruitment of ZAP-70, which is activated by LCK-mediated phosphorylation. ZAP-70 recruits LAT to the complex. Phosphorylated LAT recruits PLCγ1 and GRB2. This will activate Ca^{2+}, RAS and MAPK pathways and in turn activate nuclear transcription factors, leading to transcription of genes necessary for T cell survival, proliferation and differentiation. Adapted from "TCR Downstream Signalling" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.*

1.1.4 T cell differentiation

CD4 T cells can differentiate into several different effector subsets, including Th1, Th2, Th17, T follicular helper cells or Tregs (26). The differentiation of these subsets is in part determined by different cytokines in the nearby environment (Fig. 4). For instance can Th17 cells be generated in a milieu of transforming growth factor beta (TGF-β), IL6 and IL21, while the conversion of naïve/effector T cells into Tregs depend on the presence of both IL10 and TGF-β (26). Further, generation of Th1 cells depend on IFN gamma (γ) and IL12, while Th2 cells depend on IL2 and IL4 (26) (Fig. 4).

T cell subsets generate different cytokines and thereby initiate a variety of effector mechanisms. For instance, Th17 cells produce IL17 and IL22, which are important in the defence against fungal infections, while Tregs produce the suppressive cytokines IL10 and TGF-β (45) (Fig. 4). The Th1 subset makes IFN-γ and tumor necrosis factor (TNF) alpha (α), and are important in the defence against intracellular pathogens and cancer, while the Th2 fraction produce IL4, IL5 and IL13, and are important in the fight against extracellular microorganisms and in allergy responses (45, 46). However, Th cells are not constrained to their differentiated state, as many of them have the ability to re-differentiate and start producing effector cytokines specific for another Th subset under appropriate conditions (26). Other Th subpopulations, such as Th9 and Th22, also have their characteristic effector cytokines, IL9 and IL22, respectively (45), but these will not be further described here.

Since T cells are highly effective once activated, it is important to keep their function under tight control in order to maintain immunological tolerance (47).

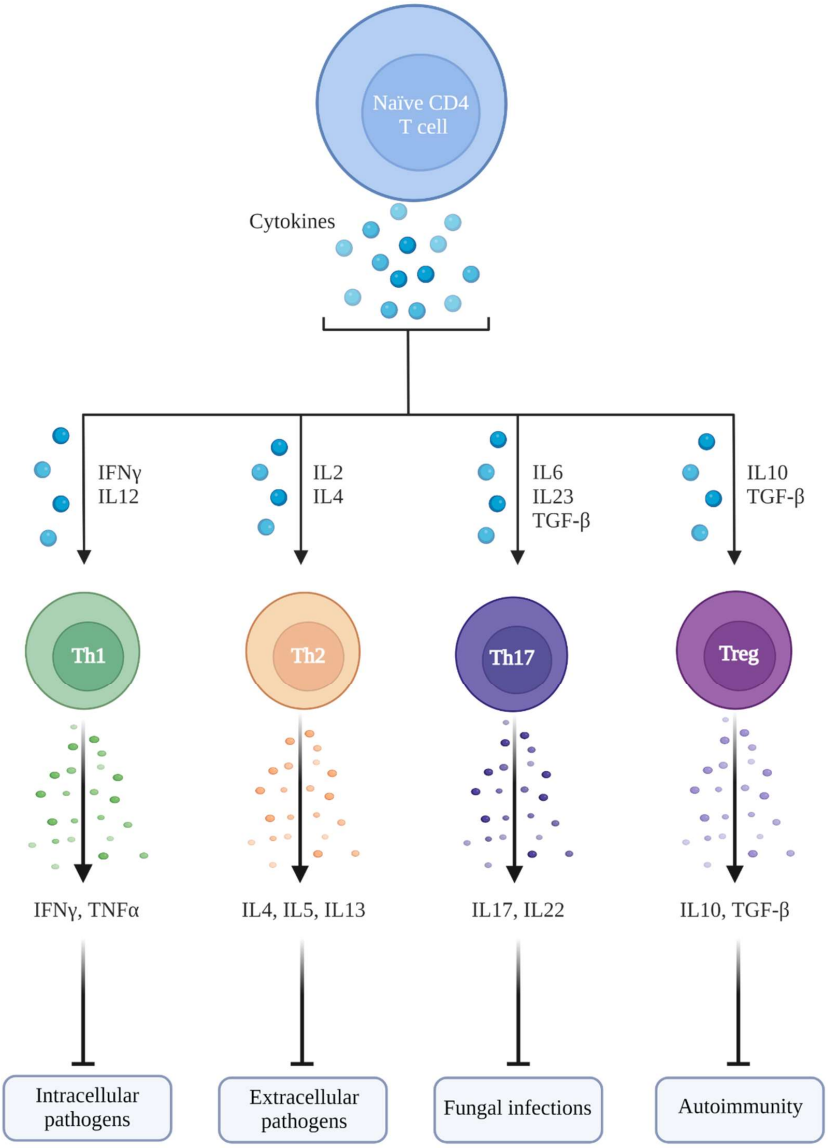

Figure 4. T cell differentiation. *A naïve CD4 T cell can differentiate into different T helper cell subsets depending on the surrounding cytokine environment. Each helper cell will further start to produce cytokines specific for the particular subgroup; IFN-γ and TNF-α for Th1 cells, IL4, IL5 and IL13 for Th2 cells, IL17 and IL22 for Th17 cells and IL10 and TGF-β for Tregs. Th1 and Th2 cells are important for the immune response targeting intra- and extracellular pathogens, respectively. Th17 cells respond to fungal infections, while Tregs can prevent an autoimmune response. Adapted from "T cell activation and differentiation" by Biorender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.*

1.2 Immunological tolerance

The initial immune repertoire is originally incredibly diverse, with abilities to respond to a vast array of antigens, including self-molecules. Being able to discriminate self from non-self is an essential property of a well-functioning immune system, termed immunological tolerance. Depending on the place of origin, it is divided into central (thymus or bone marrow) or peripheral tolerance (tissues, lymph nodes and blood).

1.2.1 Central tolerance

The term central tolerance refers to the mechanisms by which T and B cells learn to differentiate self from non-self during early developmental stages. B cells develop and learn this in the bone marrow, where B cells interacting with self-peptides at high affinity will be programmed to change their specificity, by BCR rearrangement, in a process known as receptor editing (48, 49). Self-reactive B cells that fail to rearrange their BCR will further be programmed to die by apoptosis (49).

The site of T cell development and maturation is the thymus, an organ located behind the chest bone, which consists of a capsule, a cortex and a medulla. In the cortex, random somatic recombination of variable (V), diversity (D) and joining (J) segments, under control of the *RAG* genes and RAG proteins, results in the generation of a highly diverse TCR (50, 51). Double positive thymocytes, expressing both CD4 and CD8 co-receptors, and a TCR capable of binding to a self-peptide/MHC-complex on cortical thymic epithelial cells with a suitable affinity, will receive a survival signal in a process known as positive selection (52). Cells that do not bind the self-peptide/MHC-complex will die by apoptosis (53). During positive selection, developing T cells interacting with MHC I will become CD8 positive cytotoxic cells, while those responding to MHC II will mature into CD4 positive Th cells (54).

Thymocytes that survive positive selection and differentiation into CD4 or CD8 single-positive T cells, will migrate to the thymic medulla. Here, cells will encounter self- and tissue-restricted antigens presented by MHCs on the surface of medullary thymic epithelial cells (mTECs) (52). The expression of tissue-restricted antigens, such as insulin, is mediated, at least in part, by the autoimmune regulator (AIRE) protein, which is expressed in mTECs and encoded by the *AIRE* gene (55, 56) (Fig. 5A). In a process known as negative selection (Fig. 5B), T cells that interact with the self-peptide/MHC-complex with a high affinity will receive a signal for apoptosis, while cells that interact with low affinity will exit the thymus and migrate to the periphery as mature naïve T cells (55, 57, 58). However, T cells harbouring a TCR that interacts with self-peptides presented by MHC molecules with intermediate affinity can differentiate into Tregs (59-62) (Fig. 5B), further described in section 1.3. Central tolerance is of major importance for a proper functioning immune system. If it fails, self-reactive cells might escape to the peripheral blood stream and initiate autoimmune destruction of tissues and organs (Fig. 5C), as seen in patients with *AIRE* mutations and autoimmune polyendocrine syndromes (63).

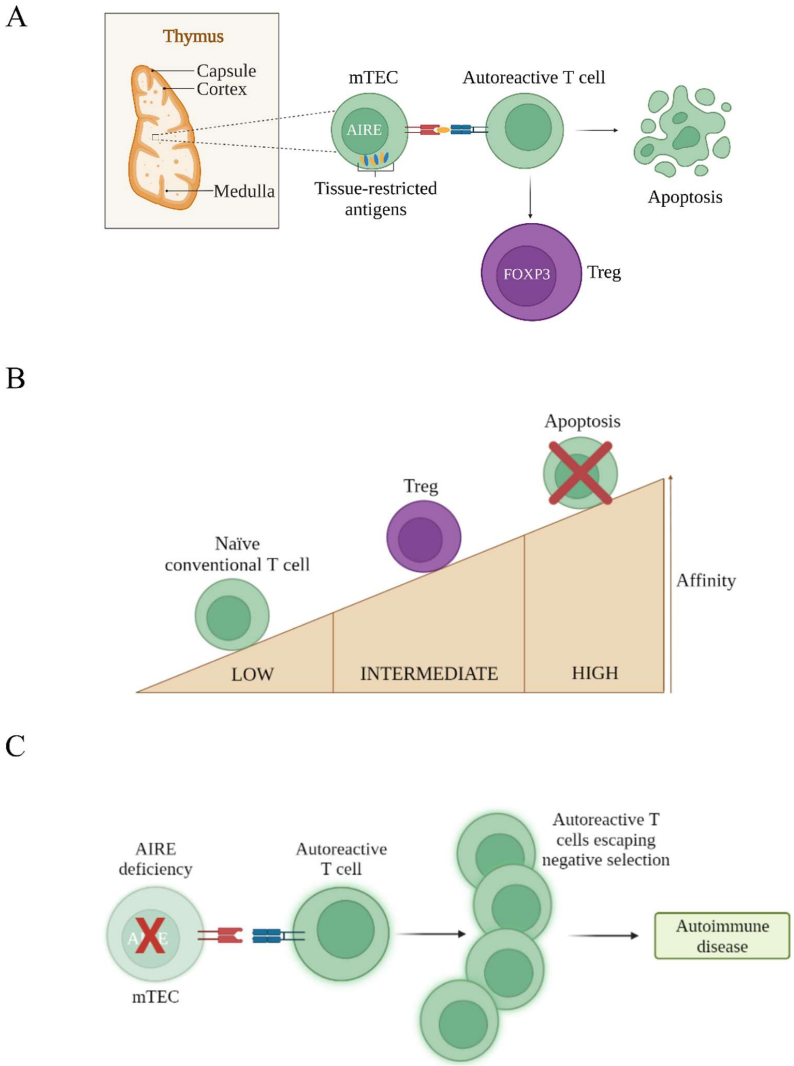

Figure 5. Central tolerance. *(A) AIRE mediates the expression of tissue-restricted antigens to developing T cells. Autoreactive cells displaying high to intermediate affinity for self-antigens presented on the MHC molecule either die by apoptosis or become Tregs expressing FOXP3. Adapted from Husebye et.al. (63). (B) Negative selection. T cells with a TCR that binds to the self-MHC complex with high affinity will receive a signal for apoptosis, while cells that respond to the same complex with low affinity will migrate to the circulation and secondary lymphoid organs as mature naïve T cells. Cells responding with intermediate affinity will be directed into the FOXP3+ Treg cell lineage. Adapted from Santamaria et.al. (64). (C) Failure of central tolerance. If AIRE is missing or non-functional, self-antigen presentation in the thymus is impaired and autoreactive cells can escape negative selection. Once present and circulating outside the thymus, these cells might initiate an attack directed at self-tissues and cause autoimmune disease. Adapted from Husebye et.al. (63). Figure created with BioRender.com.*

1.2.2 Peripheral tolerance

Peripheral tolerance comprises a set of mechanisms that work to eliminate self-reactive T and B cells that have escaped negative selection processes, with the aim of avoiding development of autoimmune disease (47). This type of tolerance will also prevent immune responses directed against harmless food antigens and allergens, and includes clonal deletion, induction of anergy, activation-induced cell death and Tregs (47). The latter will be the focus in the following section.

1.3 Regulatory T cells (Tregs)

The majority of human Tregs are generated in the thymus, are known as thymic-derived Tregs (tTregs) and play a crucial role in maintaining tolerance by dampening disadvantageous immune responses taking place outside the thymus (65, 66). Tregs are hallmarked by the expression of the transcription factor forkhead box P3 (FOXP3), which is of major importance for Treg development and function (65, 67, 68), in addition to constitutive expression of CD25 (high-affinity IL2 receptor alpha chain) and CTLA4 (6, 69). For a cell to commit to the Treg cell lineage, a series of independent events are necessary: appropriate TCR/self-MHC affinity during thymic negative selection, co-stimulatory signalling through CD28, and signalling via the IL2/CD25/Signal transducer and activator of transcription (STAT) 5 axis (61, 70-72). Together, this will induce expression of FOXP3 and generate a Treg-specific cytosine-phospho-guanine (CpG) hypomethylation pattern in a conserved region within intron 1 of the *FOXP3* gene, known as the Treg-specific demethylated region (TSDR), important for both lineage stability and suppressive function (73-75).

1.3.1 Treg functional and phenotypic diversity

In addition to tTregs, Tregs can also be induced from CD4+ naïve T cells outside the thymus, under appropriate cytokine conditions, these are known as peripheral Tregs

(pTregs) (76-78), and *in vitro* in the presence of TGF-β and IL2, which are known as inducible Tregs (iTregs) (79). At mucosal surfaces, exposed to commensal antigens, food and other environmental antigens, pTregs are induced as they interact with non-self antigens (77). The most frequent populations of pTregs are type 1 Tregs (Tr1) and Th3 cells (80). Tr1 cells are identified by the production of IL10, and through co-expression of CD49b and lymphocyte activating gene 3 (LAG3) in both mice and humans (81). However, a subpopulation of IL10-producing Tregs were found not to express Foxp3, although harbouring suppressive capacities similar to that of CD4+CD25+ Treg cells (82). Th3 cells develop from TGF-β stimulated CD4+ T cells and are involved in oral tolerance mechanisms (83). Both tTregs and pTregs are important for peripheral tolerance, as the interplay between Treg subsets have been shown important in preventing autoimmunity (84).

The expression of CD4+ T cell markers CD45RA and CD45RO have been used to define three phenotypically and functionally distinct populations of CD4+FoxP3+: resting/naïve Tregs, effector/memory Tregs and cytokine producing non-suppressive T cells (85). Multiple other molecules are involved in Treg function, including CD25, CTLA4, GITR, OX40 and MHC class II determinants (HLA-DR) (6, 69, 86-89). In addition, expression of Helios and neuropilin 1 have been suggested as markers to discriminate between tTregs and pTregs (90, 91), but the use of these markers remain debatable (92, 93). Interestingly, mutations in the *IKZF2* gene, encoding HELIOS, have been shown to not affect Treg suppressive abilities (94) or Treg numbers (95), but instead might have an effect on mucosal-associated invariant T (MAIT) cells (94) and T follicular helper cells (95).

Tregs are considered heterogeneous and can lose their FOXP3-expression in the presence of pro-inflammatory cytokines. Then they might acquire effector T cell properties, for instance by acquiring a Th1- or Th17-like phenotype, through the expression of transcription factors T-bet and ROR-γt, respectively (96, 97).

Furthermore, transient expression of FOXP3 can occur in naïve T cells after stimulation of the TCR in humans, but this did not induce regulatory function in these cells (98, 99). The expression of Th2 signature transcription factor GATA3, identified yet another subpopulation of Tregs, as GATA3+ Tregs were found at barrier surfaces in the gut and skin (100). Further, expression of GATA3 has been shown to be important for Treg function, as deletion of Gata3 in Tregs caused spontaneous inflammatory disease in mice (101).

1.3.2 Treg mediated suppression – mechanisms of action

There are several proposed mechanisms as to how Tregs mediate immune suppression, which includes cell-cell contact, cytokine secretion, production of granzymes and competition for growth factors (102) (Fig. 6). Despite no production of IL2 by Tregs (103-105), this cytokine is of major importance for Treg homeostasis and suppressive function (106-108). When effector T cells become fully activated outside the thymus, they upregulate CD25. This establishes a positive feedback loop of IL2-signalling, which creates a signal for the T cells to start proliferating (32). If Tregs are present in the cellular microenvironment, they are thought to compete with effector T cells for IL2 consumption (109, 110). For Tregs to deprive an effector T cell of IL2, they need to be within a few tens of micrometres from each other (110). In addition to compete for IL2, Tregs can mediate suppression of effector cell functions through the production of granzymes (111, 112) and the suppressive cytokines TGF-β, IL10 and IL35 (113, 114).

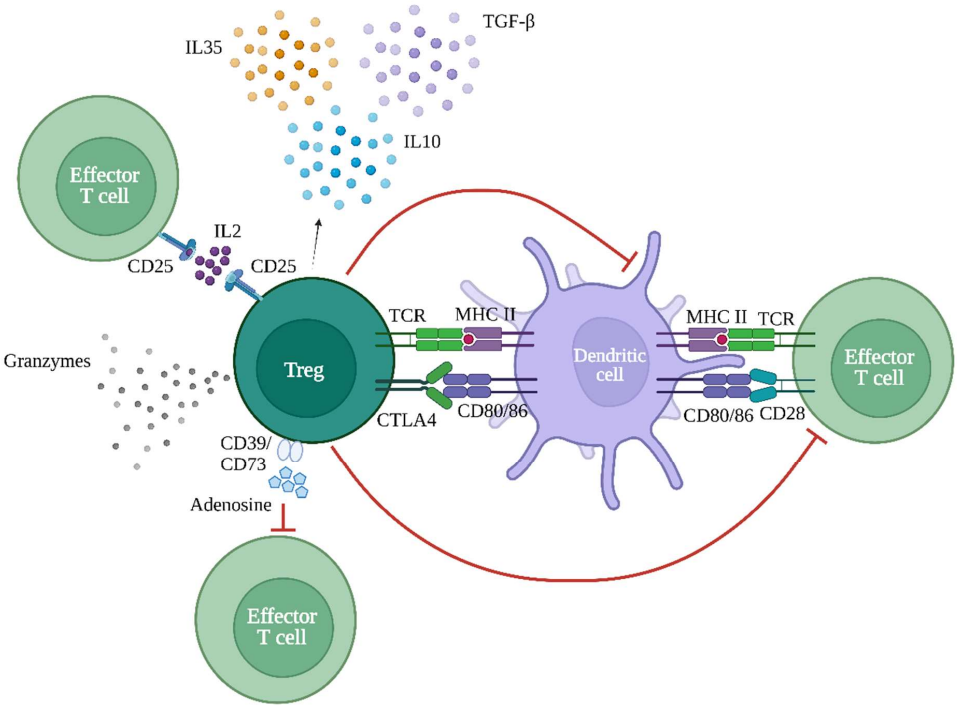

Figure 6. Treg mediated suppression. *Tregs can suppress an unwanted immune response through cell-cell contact dependent mechanisms, production of granzymes and inhibitory cytokines, and by depriving conventional T cells of IL2. This will lead to suppression of MHC and co-stimulatory molecules, antigen-presenting cell function, inflammatory cytokines and effector T cell proliferation. Adapted from "Treg suppress dendritic cells and effector T cells" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates.*

Cell-cell contact dependent suppression mechanisms are controlled by surface marker CTLA4, competing with the co-stimulatory receptor CD28 on binding CD80/86 on the APC, and will outcompete CD28 due to a higher affinity and avidity for the ligands (115). It has been suggested that CTLA4 works through a process known as trans-endocytosis, where it relocates ligands from the opposing cells into the cells expressing CTLA4, resulting in impaired co-stimulation through CD28 (116). CTLA4 can also act by attenuating the threshold necessary for signalling through the TCR (117) or by initiating indoleamin-2, 3-dioxygenase production in DCs, leading to degradation of

tryptophan (118). Without tryptophan, cell cycle progression is inhibited and the T cell response is consequently reduced (119). In addition to CTLA4, expression of LAG3 has also been suggested to contribute to Treg suppressor activity (120) and it has been shown that Tregs can inhibit dendritic cell maturation through the interaction of LAG3 with MHCII on DCs (121). Further, Tregs can also mediate conversion of adenosine 5'-triphosphate (ATP) into immunosuppressive adenosine, which causes changes in T cell metabolic environment, through the production of the ectoenzymes CD39 and CD73 (122).

1.3.3 Treg metabolism

Treg function is not only dependent on lineage marker expression, they also harbour a distinct metabolic profile, with an increased mitochondrial metabolism under steady-state conditions and following activation *in vitro*, in addition to a dependency on the mitochondrial respiratory chain (123-125). In addition, mitochondrial metabolism is important for both Treg suppressive abilities and their stability (126, 127). Previous studies have shown that *in vitro* generated Tregs are less dependent on glycolysis compared to other T cell subsets and use oxidative phosphorylation for production of energy (128, 129). On the other hand, glycolysis is of importance for natural occurring tTregs, which were shown to display glycolytic activity to the same extent as conventional T cells (130). Furthermore, oxidative phosphorylation is also critical for Treg generation of ATP, proliferation and suppressive function (131). It has also been shown that Foxp3 controls Treg metabolic adaptations through induction of oxidative phosphorylation by increasing the NAD:NADH ratio, and further by promoting Treg survival in environments with high levels of lactate and low levels of glucose (123). In mice with experimental autoimmune encephalomyelitis (EAE) and human subjects with systemic lupus erythematosus (SLE) and rheumatoid arthritis, altered Treg mitochondrial function was found, leading to increased production of mitochondrial reactive oxygen species, cell death and breakdown of self-tolerance (132).

1.4 When immunological tolerance fails

If breakdown of self-tolerance mechanisms occur, either central or peripheral, disease can arise as a consequence of imbalance between regulating and effector functions of the immune system. Monogenic and polygenic disorders leading to immune dysregulation and autoimmunity are important to study as it can uncover regulatory mechanisms and possibly new targets for immune therapy. This is exemplified by the discovery of *AIRE*, which has provided insights as to how developing T cells are taught to discriminate self from non-self (55, 56). Mutations in the *AIRE* gene, causing the monogenic APS-1, is an apt example of breakdown of central tolerance (133) (Section 1.5.2). Another example of monogenicity as a result of failure of central tolerance is Omenn syndrome, caused by mutations in the *RAG* genes (134). Over 400 different types of primary immunodeficiencies have been described and are thought to affect more than 1 in 10000 individuals around the world (135-137). Interestingly, patients suffering from immunodeficiencies often acquire autoimmune components and it is not always easy to separate the two, especially since many of the same signalling pathways and immune regulatory mechanisms are involved (5).

One important peripheral immune regulatory mechanism is Tregs. As these cells are needed to control autoreactive cells in the periphery, both functional and numerical abnormalities in the Treg population, in addition to effector T cell resistance to Treg mediated suppression, can contribute to disease development in susceptible individuals (138-140). In humans, the importance of Tregs in the prevention of autoimmune disease is best exemplified by patients with mutations in the *FOXP3* gene, who lack functional Treg cells and present with IPEX syndrome (66, 141, 142). Patients with IPEX develop multiple autoimmune manifestations, including early-onset enteropathy and type 1 diabetes (141). A similar syndrome is seen in scurfy mice, with non-functional Foxp3, who acquire a similar autoimmune profile (143). Notably, transfer of Tregs from healthy to Foxp3-deficient and athymic mice, respectively, have been shown to restore tolerance (6, 65). A monogenic disease resulting in a deficient Treg

population is sometimes referred to as Tregopathy (144), and immunodeficiency and autoimmunity have been described in patients with mutations in the Treg signature genes *BACH2* (145)*, CD25* (146-149) and *CTLA*4 (150, 151).

In more common polygenic autoimmune disorders, such as type 1 diabetes, multiple sclerosis and rheumatoid arthritis, both quantitative and functional changes in the Treg population have been observed (152-154). Defective Treg function has also been reported in APS-1 and APS-2, both characterized by several autoimmune features, but where APS-1 is caused by mutations in the *AIRE* gene, APS-2 is of polygenic aetiology (155-159). For Tregs to efficiently inhibit effector T cell activation, it also requires the latter cell type to be prone to Treg mediated suppression. In both rheumatoid arthritis and type 1 diabetes, effector T cell resistance to suppression by Tregs have been described, which might play a role in autoimmune disease pathogenesis (160, 161).

1.5 Autoimmune disease

Why some individuals develop autoimmune disease is largely unknown, but the aetiology is thought to be a combination of genetic predisposition and environmental factors. Several human leukocyte antigen (HLA) class II subtypes are associated with autoimmune disease development, such as HLA-DR3-DQ2 and HLA-DR4-DQ8 in type 1 diabetes and HLA-DQ2/DQ8 in celiac disease (162). Further, epigenetic changes, exemplified by DNA methylation, can alter gene expression profiles without changing the DNA sequence, and has been linked to the development of autoimmune conditions (163). Exposure to toxins, viruses and/or changes in the microbiome have been suggested as environmental factors that can contribute to autoimmune disease in genetically susceptible individuals (164). There are two main types of autoimmune conditions, depending on the type of response the disease leads to; systemic and organ-specific. In organ-specific autoimmunity, an immune attack targets self-antigens present in specific organ(s) or tissue(s). One example is type 1 diabetes, characterized

by an immune reaction against the self-antigens insulin, zinc transporter 8 and glutamate decarboxylase 65, leading to T cell mediated destruction of insulin producing beta cells in the pancreas (165). Another example is autoimmune primary adrenal insufficiency (PAI) (Section 1.5.1), where the enzyme 21-hydroxylase (21OH) is considered to be the main autoantigen (166, 167), while self-antigens found in several affected endocrine glands or organs are targeted in autoimmune polyendocrine syndromes (63, 168-170).

1.5.1 Autoimmune primary adrenal insufficiency (PAI)

The main cause of PAI in industrialized countries is an autoimmune attack on hormone-producing cells in the adrenal cortex. This is known as autoimmune PAI and clinically manifests as skin hyperpigmentation, salt cravings, fatigue, muscle weakness, weight loss, nausea and depression due to a deficiency of the life-essential hormones cortisol and aldosterone (171-173). Autoimmune PAI is rare, and the prevalence have been reported in Nordic countries to be approximately 15-22 individuals per 100000 (174-176), while it has been estimated to be around 10 individuals per 100000 in other European countries (177, 178).

Most patients with autoimmune PAI present with autoantibodies directed against 21OH, an enzyme important for the generation of glucocorticoids and mineralocorticoids (166, 179). These autoantibodies can precede diagnosis by decades and are still present in more than 90% of patients even after 30 years of disease (180). Antibody levels seem to decrease with disease duration, but does not reach negativity in most cases (180). As autoimmune PAI causes destruction of hormone-producing cells in the adrenals, patients are deficient in glucocorticoids. Despite this, it has recently been shown in a Norwegian cohort, that one-third of the patients still produce small amounts of cortisol decades after being diagnosed (181). Autoreactive T cells specific for 21OH have been found in patients with autoimmune PAI, indicating that

these cells might be mediating the autoimmune attack taking place in the adrenals (182-184).

The PAI aetiology is thought to be a combination of genetic predisposition and environmental factors, including viral infections and/or the use of checkpoint inhibitors (173, 185, 186). It is becoming clear that autoimmune PAI has a complex pattern of inheritance, dominated by HLA-DQB1*02:01 and HLA-DQB1*03:02 (serotypes DR3-DQ2 and DR4-DQ8) (179, 187-189). The strong heritability of autoimmune PAI have been confirmed in studies on monozygotic twins in the Swedish Twin Registry (190, 191). Several variants in immune cell related genes further contribute to the susceptibility of autoimmune PAI, many of which are shared with other autoimmune diseases (192). Autoimmune PAI is often seen concomitant with other autoimmune disorders, such as autoimmune thyroid disease (AITD) and/or autoimmune primary ovarian insufficiency (POI) (193, 194), as part of an autoimmune polyendocrine syndrome (63). Where APS-1 is caused by mutations in one single gene (*AIRE*) (133), isolated PAI and APS-2 have similar polygenic inheritance patterns.

1.5.2 Autoimmune polyendocrine syndrome type 1

APS-1, also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED; Online Mendelian Inheritance in Man [OMIM] number 240300), is a rare disease with an estimated prevalence of 1:90000 in Norway (195), but has a higher prevalence in the Finnish (1:25000) (169, 196) and Sardinian (1:14000) (197, 198) populations, as well as in Persian Jews living in Israel (1:9000) (199).

The AIRE gene and disease-causing mutations

The disease causing gene for APS-1, *AIRE*, is located on chromosome 21q22.3, consists of 14 exons, and encodes the 545 amino acid long AIRE protein, with a molecular weight of 57.5 kD (133, 200) (Fig. 7). In addition to mainly being expressed in the thymus (55, 56), AIRE has also been found in peripheral lymphoid tissue DCs in humans and in extrathymic cells in mice (201, 202). Clinically, mutations in *AIRE* manifests as APS-1, and as of today, more than 100 different mutations have so far been discovered (203). Pathogenic mutations are found in different sites of the gene, where the most common is the Finnish major mutation (p.R257X/c.769C>T) in the Sp100, AIRE-1, NucP41/75, DEAF-1 (SAND) domain (203). This mutation is found, not only in Finland, but also in Russia and Eastern Europe (204, 205). Another common APS-1 mutation is the 13-base pair deletion (c.967_979del13) found in the plant homeodomain (PHD) 1 region, with a high prevalence in Norway (170), North America (206) and Great Britain (207). While APS-1 most commonly follows a recessive pattern of inheritance, dominant mutations have also been described (208-211). In these cases, the mutated gene is thought to affect the function of wild type *AIRE* (dominant negative) due to changes in critical amino acid sequences (208). Compared to the recessive phenotype, patients with dominant mutations often present with a milder form of disease (208-211).

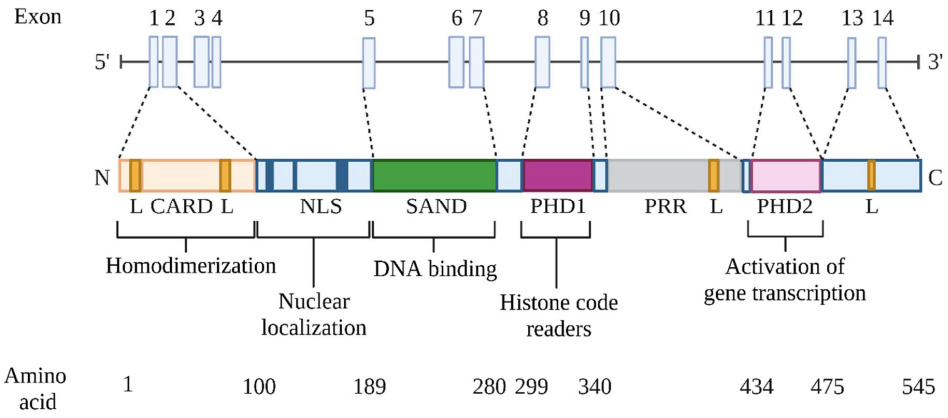

Figure 7. AIRE gene structure. *AIRE is a transcriptional regulator, encoding the AIRE protein. It is 545 amino acids long and consists of 14 exons. The gene has four main subdomains; CARD, SAND, PHD1 and PHD2. In addition, it contains four LXXLL domains capable of activating nuclear receptors. Corresponding exons and amino acids are provided in the figure. Figure created with BioRender.com. Adapted from Bruserud et.al. (203).*

Clinical features of APS-1

A clinical diagnosis of APS-1 requires the presence of two out of three main components: autoimmune PAI, hypoparathyroidism (HP) and/or chronic mucocutaneous candidiasis (CMC) (63). However, a great variety of other endocrine and ectodermal manifestations are also seen (196) (Fig. 8). In addition, the diagnosis can also be determined based on two pathogenic *AIRE* mutations (133, 200) or by the presence of one main component if a sibling also has the disease (212). APS-1 can manifest in susceptible individuals from early infancy to old age, as the number of acquired manifestations varies greatly between patients and new disease components may develop throughout life (63). The clinical variance makes it challenging to diagnose, and although the disease usually present during childhood, many patients get the diagnosis late in life and some patients never get diagnosed at all (195). Even siblings with the same *AIRE* mutations varies a lot in their clinical presentation of the syndrome (170).

Enamel dysplasia is one of the most common manifestations and is probably one of the first to appear, although it commonly goes unnoticed clinically (170). Out of the three main components, CMC usually manifest first, followed by HP, affecting 80% of the patients (212), and then autoimmune PAI, which is present in 60-80% of the patients (170, 196, 197, 213). Of the ectodermal components, alopecia is the most common, while some patients also present with vitiligo (212). Gastrointestinal symptoms, such as autoimmune gastritis leading to vitamin B12 deficiency, are also reported in patients with APS-1 (212). On the other hand, type 1 diabetes is not commonly seen (212). As a consequence of the different disease components, patients with APS-1 have a higher mortality rate compared to the general population, often caused by cancer of the oral cavity or oesophagus due to uncontrolled CMC, or adrenal crisis, which is a life-threatening complication of autoimmune PAI (63, 214, 215).

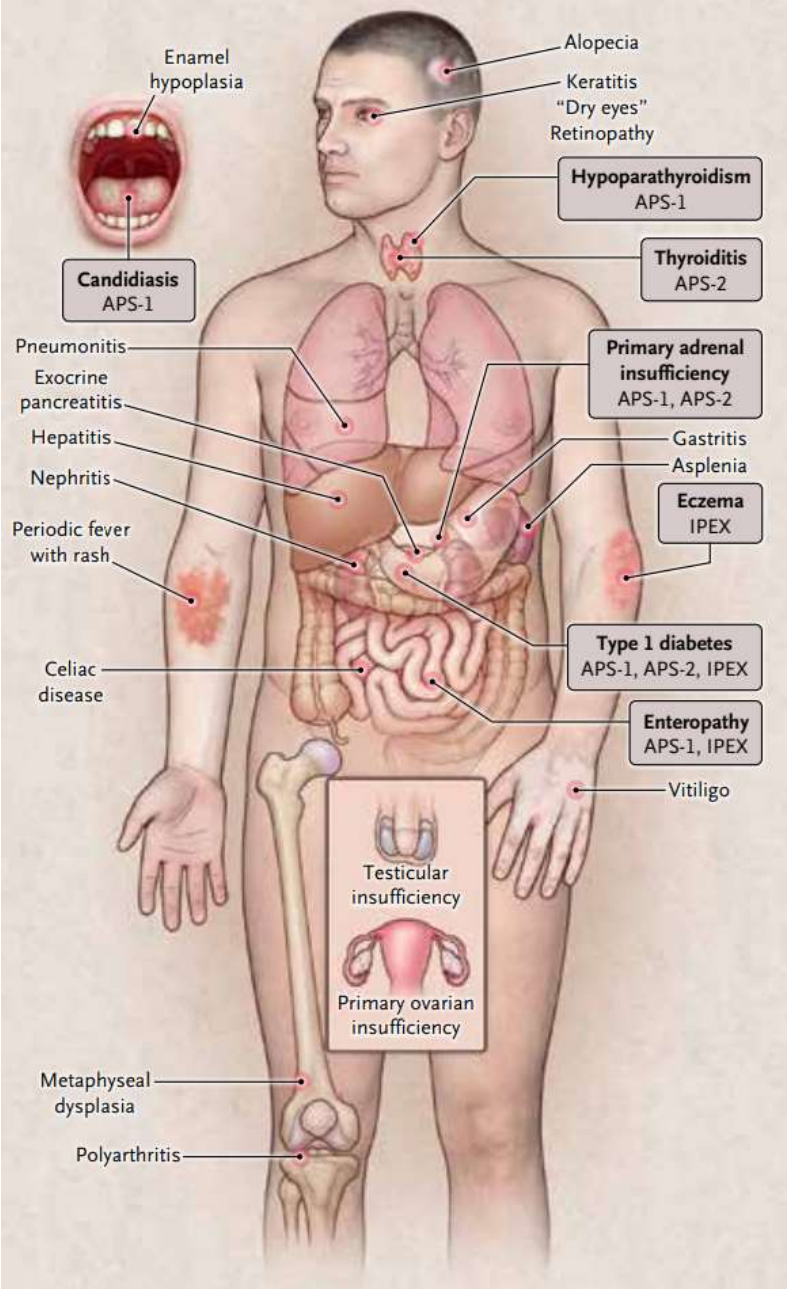

Figure 8. Autoimmune and ectodermal manifestations of APS-1. *The syndrome is characterized by presence of two out of three hallmark features; chronic mucocutaneous candidiasis (CMC), hypoparathyroidism (HP) and autoimmune primary adrenal insufficiency (PAI). However, multiple other manifestations are also seen. Reproduced with permission from Husebye et.al. (63). Copyright Massachusetts Medical Society.*

Autoantibodies in APS-1

Patients with APS-1 harbour autoantibodies directed at proteins in affected organs and these can be useful as diagnostic markers (168, 182). This includes autoantibodies directed at 21OH in the adrenal glands (167, 168), side-chain cleavage enzyme (SCC) in the adrenal glands and ovaries (167), and NACHT leucine-rich repeat protein 5 (NALP5) in the parathyroid gland (216). In addition, patients with APS-1 present with high titre neutralizing autoantibodies against type I IFNs ω and α, and ILs secreted by Th17-cells, including IL17A, IL17F and IL22 (20, 21, 217, 218). These autoantibodies are present in almost all patients with recessive *AIRE* mutations described and can be detected even before disease onset (218-220). However, they are not commonly seen in patients with dominant *AIRE* mutations (208, 211). Autoantibodies are useful diagnostic tools, as screening of the Swedish and Norwegian patient registries of autoimmune PAI have led to the discovery of previously undiagnosed APS-1 patients, presenting with disease-causing mutations in *AIRE* (221, 222). This is important, as diagnosis will initiate proper follow-up, treatment, early detection of possible new disease components and help to prevent complications.

1.5.3 Cytokine autoantibodies in other diseases

While autoantibodies against IFNs ω and α are considered hallmark features of pathogenic *AIRE* mutations, such autoantibodies have also been described in patients with mutations in the genes *FOXP3* (9), *RAG1* and *2* (8), *IKZF2* (94) and *NFKB2* (223, 224). In addition, the presence of these autoantibodies have been described in a fraction of the general population, where the prevalence seems to be increasing with age (225). Autoantibodies against type I IFNs have also been described in patients with thymoma and/or late-onset myasthenia gravis (226), in addition to low-titre IFN autoantibodies found in systemic autoimmunity, such as SLE (227-229). Even though initial studies claimed that pre-existing type I IFN autoantibodies were large risk determinants for severe COVID-19 disease, the role of these markers have later been diminished, and the biological relevance of type I IFN autoantibodies is still an enigma (230-233).

1.6 Treg-based therapy in autoimmune disease

The use of Tregs to treat human disease involves both Treg promoting and inhibiting strategies (138). In the case of cancer, depletion of Tregs in order to enhance the anti-tumour immune response would be favourable, while in an autoimmune setting it would be advantageous to generate, expand or enhance Treg suppressive function (138). There are several proposed ways to expand the Treg fraction in autoimmune disease, including *in vitro* expansion of natural Tregs, low-dose IL2 therapy, adoptive Treg transfer, and conversion of conventional T cells into Tregs (138). Using low-dose IL2 to specifically activate and expand Tregs have great potential, as Tregs constitutively express the high affinity IL2 receptor alpha (CD25) and respond to much lower levels of IL2 compared to other cell subsets (234). It is also possible to block CD28 co-stimulation by using CTLA4-Ig/abatacept, however, such use has shown a decrease in circulating Tregs in rheumatoid arthritis and type 1 diabetes (235-237). The negative effect on the Treg fraction was recently shown to be restored if CTLA4-Ig/abatacept treatment was combined with low-dose IL2, as this treatment regimen protected humanised mice from developing autoimmune diabetes (238).

The use of adoptive Treg therapy, where the patient's Tregs are expanded *in vitro* and transferred back to the patient, has been proven achievable and safe in both type 1 diabetes and graft-versus-host disease (239-241). The addition of low-dose IL2 to the adoptive Treg therapy regimen tested in type 1 diabetes led to an increased frequency of Tregs, but did also cause adverse expansion of cytotoxic T cells (242). Efforts have been put into identifying substances with the ability to selectively increase Tregs, and through a library screening, AS2863619, a dual Cdk8/19 inhibitor, was identified (243). This molecule had the ability to convert antigen-specific effector/memory T cells into Foxp3 expressing Tregs (243). Interestingly, when mice with EAE and non-obese diabetic (NOD) mice were treated with AS2863619 reduced incidence and severity of spontaneous diabetes in NOD mice and reduced severity of EAE was

observed (243). Manipulating Tregs to restore tolerance is a promising approach for the future treatment of autoimmune disease (244).

1.7 A role for Tregs in autoimmune PAI and APS-1?

Tregs are crucial mediators of peripheral tolerance and a lack of functional Tregs can cause fatal autoimmune disease, as seen in IPEX syndrome patients (66, 141). Although mice and humans are different in biology and physiology, work using animal models have provided insights as to how Aire affects Treg fate. Notably, mice double-deficient of Aire and Foxp3 succumb to fulminant autoimmunity early in life, being more affected by disease than Aire and Foxp3 single-knockout animals (245). Further, Aire knockout mice show a reduced Treg fraction and have even been found to lack a specific Treg subpopulation early in life (246-248). How AIRE affects Treg specificities in humans is still not known. However, the most frequent Treg specificities in mice are generated independent of Aire, while those that are Aire-dependent might contribute to the autoimmune phenotype seen in Aire depleted mice (248). In addition, Aire is also thought to be involved in Treg recirculation between the thymus and the blood stream (249).

Ectopic expression of CTLA4 was found in Aire-deficient mTECs, causing depletion of CD80/86 on thymic DCs, in turn affecting the ability of DCs to deliver a co-stimulatory signal and self-antigen transfer from mTECs to DCs, both of which are necessary for the development of Tregs (250). By removing CTLA4 from Aire-deficient mTECs, Treg production was restored and autoimmune disease rescued in affected animals (250). Further, since Aire is also believed to be involved in the generation of Tregs (248, 251, 252), AIRE deficiency can cause reduced numbers or dysfunctionality of these suppressive cells, as shown in several studies performed on human APS-1 patients, where both reduced Treg frequencies and impaired suppressive function have been reported (155-158). In addition, a dysregulated anticommensal

response in APS-1 was associated with a systemic Treg malfunction (253). Further, fewer clones of common TCR-β sequences were found in APS-1 Tregs, but were instead present in the regular T cell pool (254). Reduced expression of FOXP3 have also been found in APS-1 patients, in addition to an accelerated Treg turnover, causing a depleted pool of recent thymic emigrants (156). Also, findings point to a failure of Treg gut homing, caused by downregulation of *GPR15*, and increased Treg metabolic activity, due to upregulation of *FASN,* in APS-1 (255). However, whether this is a cause or consequence of APS-1 is still unknown.

Autoimmune PAI is one of the main components in both APS-1 and APS-2, and while APS-1 is known to be caused by a failure of central tolerance, the aetiology behind isolated PAI and APS-2 is currently unknown. A defective Treg suppressor function have been described in APS-2 (159) and mice depleted of CD4+CD25+ regulatory cells develop a resembling syndrome, where several endocrine glands are affected (6). A recent genome-wide association study (GWAS) in patients with autoimmune PAI showed that several of the major risk genes associated with higher risk for disease development are genes involved in Treg function, including *CTLA4*, which is constitutively expressed on Tregs and activated conventional T cells, and functions to downregulate an immune response, and *BACH2*, which is involved in regulating Treg responses (192). Interestingly, polymorphisms in the *CTLA4* gene have been associated with an increased risk of developing autoimmune disease, including autoimmune PAI (256). In addition, the mentioned GWAS also showed a strong association for autoimmune PAI development in polygenic settings with a coding variant (p. Arg471Cys) in the *AIRE* gene (192).

Despite increased knowledge on Treg function and their vital role in avoiding autoimmune disease, the extent to which Treg dysfunction contributes to the autoimmune phenotype seen in PAI and APS-1 remains poorly understood.

2. Aims

The principal aim of this thesis was to elucidate immune regulatory mechanisms in endocrine autoimmune disorders.

The detailed objectives were

I. To decipher the Treg immune (TCR) repertoire, transcriptome, proteome and suppressive properties on a single-cell level in patients with APS-1.
II. To elucidate the Treg transcriptome, suppressive capacity and metabolomic properties in patients with autoimmune PAI.
III. To determine whether cytokine autoantibody screenings can be used to identify patients with monogenic and/or oligogenic immune disorders.

3. Methodological considerations

In this section, key aspects of the main methods used in this thesis are covered, in addition to ethical aspects. An overview of the experimental pipeline is provided below (Fig. 9). Experimental details can be found in the individual papers.

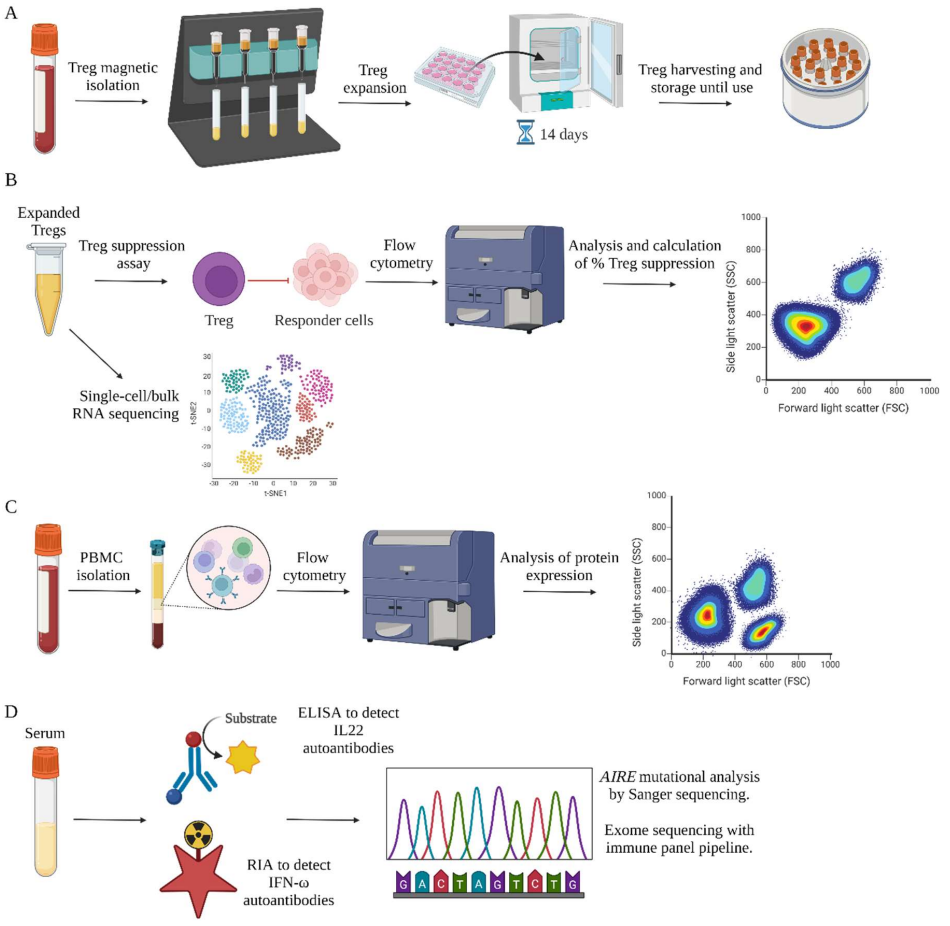

Figure 9. Overview of methods. *(A) Tregs were magnetically isolated from whole blood and expanded for 14 days, prior to harvesting and storage until use (paper I and II). (B) Expanded Tregs were single-cell (APS-1, paper I) or bulk (adrenal insufficiency, paper II) RNA sequenced and their suppressive properties were examined in a Treg suppression assay. (C) PBMCs were used to examine the expression of T cell and Treg markers (paper II). (D) An ELISA assay was used for measuring autoantibodies against IL22, while an established radio immune assay was used to detect autoantibodies against IFN-ω. Positive patients were AIRE and/or exome sequenced (paper III). Figure created with BioRender.com.*

3.1 Registry for Organ-Specific Autoimmune Diseases (ROAS)

The Norwegian National Registry for Organ-Specific Autoimmune Diseases (ROAS) was established in 1996 and is a part of the Section for Endocrinology, Department of Medicine, at Haukeland University Hospital in Bergen, Norway. ROAS is one of the world's largest registries of autoimmune PAI. The registry also includes small cohorts of patients with other endocrine diagnoses, including type 1 diabetes, autoimmune thyroid disease, hypoparathyroidism and adrenal insufficiency of unknown aetiology (Table 1) and the registry also has national coverage, meaning that patients from all over Norway are recruited and subsequently included. As of 3rd of May 2023, the registry included 2696 patients, whereof 962 patients have autoimmune PAI and 53 patients have APS-1 (Table 1). The registry also includes a biobank, containing serum samples, cells, DNA and blood from the included patients. New samples are obtained when patients see their endocrinologist for a yearly follow-up. All participants have signed an informed consent upon registry inclusion and have consented to be part of research connected to the registry and biobank. As a part of the routine, all new ROAS patients are screened for autoantibodies against 21OH and IFN-ω.

3.2 Patients and controls

Peripheral blood mononuclear cells (PBMC), serum and Tregs from patients with APS-1, autoimmune PAI and other autoimmune endocrine diseases were obtained from the ROAS registry. PBMC, serum and Tregs from healthy donors were obtained from Haukeland University Hospital Blood Bank, and all donors gave their informed consent for participating in research. Controls were considered matched with a particular patient if their sex was the same and their age within $\pm$ 10 years the age of the patient.

Table 1: Patient groups included in ROAS as of May 2023.

Disease	Number of patients*
Autoimmune PAI	962
Adrenal insufficiency of unknown aetiology	126
APS-1	53
APS-2	503
Hypothyroidism	568
Hyperthyroidism	673
Type 1 diabetes	242
Alopecia	87
Vitiligo	177
Celiac disease	110
Vitamin B12 deficiency	170
Hypoparathyroidism	415
Chronic mucocutaneous candidiasis	68

*Some patients are counted in more than one disease group.

3.3 Ethical aspects and considerations

This project was approved by the regional committee for medical and health research ethics (REK) west, with REK-numbers 2013/1504 (biobank), 2009/2555 and 2018/1417 and was conducted in accordance with the Declaration of Helsinki. Sensitive data containing patient information was stored on a secure Helse Vest server and human transcriptomic data was stored and handled on secure servers (SAFE, University of Bergen, Norway; TSD, University of Oslo, Norway).

Since the cytokine autoantibodies characterizing APS-1 can be detected even before clinical signs of disease (219, 257), these can be used as predictors for disease development. Hence, in populations predisposed to autoimmune disease, such as close family members, autoantibodies could be used as valuable screening tools. However, this raises major ethical concerns as screenings can only predict a possibility of disease development and autoantibody positive individuals may never acquire clinical signs of disease. Further, if otherwise healthy at-risk individuals are positive for autoantibodies, it might raise the question as to whether treatment should be started in these individuals. As there are currently no strategies to stop the progression of autoimmune disease, the results of autoantibody screenings, could have a great impact on quality of life for the affected individual. However, while diseases such as autoimmune PAI can be life-threatening, it can also easily be treated with hormone-replacement therapy, and early diagnosis and subsequent treatment could potentially save lives, in addition to reducing social and economic burden (258). All of the above is especially difficult if healthy controls are found positive for the autoantibodies in question, as they are completely anonymous and cannot be identified in any way.

When performing genetic studies, there is always a chance of genetic variants appearing without being connected to the phenotype in question. Whether to inform study participants about the findings is an ethical dilemma, as they might cause severe

distress. However, in the ROAS consent form, included patients have the opportunity to choose if they would like to be informed if a genetic variant giving high risk for inherited disease is discovered by chance. If needed, and wanted, patients and close relatives can be referred to genetic counselling. ROAS and the research group have a close collaboration with the Department of Medical Genetics, Haukeland University Hospital, if such cases are discovered.

3.4 User perspectives

Given the rarity of APS-1, the Norwegian cohort consists of a limited number of patients. This means that patients are frequently asked to participate and donate blood and/or urine to several new or ongoing studies. For some patients this can be a burden, especially since most of the current research projects will not benefit them, but rather future generations. The ROAS network and the research group is in constant contact with the patient organization and have meetings gathering ROAS physicians from all over Norway and patient representatives every year. In these meetings, the patient organization has the opportunity to impact the focus of research by presenting which areas the patients are interested in. Patient perspectives are important and always taken into consideration when new projects are being planned and applications are written.

3.5 Enzyme-linked immunosorbent assay (ELISA) (Paper I, II, and III)

In papers I and II, commercial ELISA kits were used to detect production of Treg-specific cytokines IL10 (RnD Systems), TGF-β (Abcam) and IL35 (Nordic Biosite) in Treg suppression assay supernatants. Routine radio-immuno assays (RIAs) are established in our lab for measuring autoantibodies against IFN-ω, IFN-α_2, IL17F and IL22 (259-261). However, these assays involve the use of ^{35}S radiolabelled methionine,

which represents a major disadvantage, and currently they are not verified for dealing with pooling of serum samples. In paper III, an in-house ELISA was used to screen patients' sera for autoantibodies against IL22. In this assay pooling of serum samples was introduced and optimized, in order to screen a large number of samples simultaneously. To verify the usefulness of the assay, pre-screened sera from patients with APS-1 (N=49) with known antibody-levels from a RIA were compared to sera from healthy controls (N=90). Samples were considered positive with a signal greater than the mean plus three times the standard deviation of the signals from 12 healthy controls included on each plate.

3.6 *In vitro* Treg expansion and Treg suppression assay (Paper I and II)

As Tregs constitute approximately five to ten percent of the entire CD4+ T cell pool (6, 262, 263), studies are hampered by their low frequency. However, by isolating and expanding Tregs *in vitro*, it is possible to obtain a high enough cell count for downstream analysis, which was the chosen approach for the experiments in this thesis (paper I and II). The downside of using expanded Tregs is that it might affect the phenotype and stability of these cells, as repetitive *in vitro* stimulation have been shown to cause loss of FOXP3 expression in Tregs (264). In addition, no perfect combination of cell surface and intracellular markers of Tregs exist, especially since FOXP3 and CD25 is also transiently expressed by activated conventional T cells (98, 99, 265, 266).

In paper I, Tregs were isolated from APS-1 patients (N=18) and matched healthy controls (N=18), while in paper II, Tregs were isolated from patients with adrenal insufficiency (N=24) and matched healthy controls (N=27). In all cases, Tregs were expanded for 14 days in the presence of IL2 and anti-CD3/28. FOXP3 expression was evaluated by flow cytometry.

The suppressive abilities of Tregs has traditionally most commonly been assessed by measuring their ability to inhibit responder (Tresp) cell proliferation, either by using incorporation of ^{3}H-thymidine or based on flow cytometry (267). In paper I and II, a flow cytometry based assay was established, making it possible to distinguish Tregs from responder cells by labelling Tresp cells with the proliferative dye CellTrace Violet (Invitrogen). In addition, Tresp cells were analysed by flow cytometry prior to the suppression assay in order to determine which peak represented the undivided cells. Thus, it was made sure that non-proliferating cells were excluded in the calculation of Treg suppression. Further, all cells were stained with conjugated mouse anti-human antibodies against CD3, CD4, CD8 and CD25. Treg suppression was assessed for four different Tresp-to-Treg ratios; 1:1, 2:1, 4:1 and 8:1. After five days of co-culture in the presence of IL2 and anti-CD3/CD28, %Treg suppression was calculated as (%Tresp alone-%Tresp treated with Tregs/%Tresp alone)*100%. In an attempt to increase the level of Treg suppression, AS2863619 (Cayman Chemicals) and CD152 [CTLA4]:Fc (Chimerigen) were added to the co-culture wells, using a pool of Tresp cells (Paper II). For all suppression assays, patients and their corresponding age- and sex-matched healthy controls were run simultaneously to reduce batch effects. Cells were fixed and permeabilized prior to analysis using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Data was analysed by BD LSR Fortessa or S6 Cell Analysers (BD) and the BD FACSDiva Software (BD).

3.7 Phenotypic characterization of immune cells (paper I and II)

In paper I, *in vitro* expanded Tregs from APS-1 patients (N=17) and the corresponding age- and sex-matched healthy controls were characterized by multi-parametric flow cytometry. Tregs were stained with a 13-colour antibody panel, based on and modified from previous work by Santegoets *et.al* (268), against different T cell markers, including the Treg lineage marker FOXP3. Tregs were gated as the CD4+CD25+FOXP3+ fraction. In paper II, flow cytometry was used to examine the

frequency of and expression of blood Treg functional markers in patients with PAI and polyendocrine syndromes (N=15). Cells were stained with a 10-colour antibody panel against different markers of T cells, including FOXP3, CTLA4 and CD25. In both papers, cells were fixed and permeabilized prior to intracellular staining using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience).

To complement the flow cytometric characterization (Paper I), polyclonal Tregs isolated from APS-1 patients (N=17) and healthy controls (age- and sex-matched) were prepared for time-of-flight mass cytometry (CyTOF) by an established in-house protocol. The panel used included 27 antibodies against human immune cell markers conjugated to metal isotopes. Seventeen of the applied markers were Treg specific, while 10 were markers of other immune cells (B cells, natural killer (NK) cells, monocytes, granulocytes and CD8 T cells). The use of CyTOF gave us the opportunity to simultaneously examine the expression of a higher number of markers, as compared to conventional flow cytometry. In addition, spill-over between channels is avoided due to low spectral overlap between the metal isotopes (269). All samples were frozen in CRYO#20 (Cytodelics) at -80℃ until acquisition on the CyTOF XT instrument (Fluidigm). After acquisition, samples were debarcoded using the Fluidigm Debarcoding Software (7.0.8493.0) with a 20-plex-debarcoding key (Fluidigm) and bulk normalized in the fluidic CyTOF Software v8.0 using EQ four-element calibration beads. Samples were run in four batches, where the same standard control was included in all four. To reduce batch effects, all samples were batch corrected using Cydar with ncdFlow v.2.36.0 (270) and flowCore v.2.2.0 (271) as dependency packages (272, 273) in RStudio 2022.12.0+353. FlowJo v10.2 and v10.8 CL (BD) was used to analyse flow and mass cytometric data.

3.8 Single-cell, bulk and TCR sequencing (Paper I and II)

With the use of single-cell sequencing techniques, it is possible to uncover cell-to-cell variations, identify subpopulations and detect new cell populations, as opposed to bulk sequencing which generates an average of cell expression profiles (274). Further, since each T cell expresses a single receptor sequence and have paired α and β chains, the use of single-cell techniques can resolve both the sequence and the pairing information for each cell (275). Therefore, in paper I, single-cell sequencing was used to explore the transcriptome (N=9) and TCR repertoire (N=8) of APS-1 *in vitro* expanded Tregs. In addition, naïve Tregs from Finnish APS-1 patients (N=4) were also single-cell sequenced. For the Norwegian APS-1 cohort, we chose to use a targeted Human Immunology Panel (10X Genomics) for the analysis, which include genes related to innate and adaptive immunity, inflammation and immune-oncogenics, in order to examine the expression of immune focused genes and to reduce background noise. To reduce the impact of batch effects, gene expression and TCR libraries from patients and their matching healthy controls were generated simultaneously. All libraries were sequenced on a NovaSeq SP flow cell using an Illumina NovaSeq 6000 instrument. The use of single-cell techniques are costly and only a limited number of cells are captured and sequenced: the input in paper I was 10000 cells per sample. Since the expanded Tregs sequenced in paper I appeared homogenous, we chose to bulk RNA-sequence *in vitro* expanded Tregs from patients with adrenal insufficiency (N=16) and healthy controls (N=16) in paper II. Sequencing was performed using an Illumina paired-end 150 bp sequencing on the NovaSeq 6000 system, with an average sequencing depth of 101 million reads per sample.

3.9 Seahorse mitochondria respiration assay (Paper II)

In paper II, a Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies) was used to assess mitochondrial respiration in polyclonal expanded Tregs from autoimmune PAI patients (N=11) and healthy controls (N=11). Cells were seeded at 250000 cells

per well in quadruplicates on a Seahorse XFe96/XF Pro PDL Cell Culture Microplate (Agilent Technologies) one day prior to the assay. Mitochondrial respiration was measured by the oxygen consumption rate using the Seahorse XF Cell Mito Stress Test Kit and the Seahorse XFe96 Analyzer (Agilent Technologies). Inhibitors of the mitochondrial respiration chain was added in the following order: 2 μM oligomycin (inhibits ATP synthase), 2 μM Carbonyl cyanide m-chlorophenylhydrazone (CCCP) (uncouples protein gradient), 2 μM rotenone (inhibits complex I) and 2 μM antimycin A (inhibits complex III). Three rounds of measurements were conducted between adding of the inhibitors. OCR measurements were normalized relative to gDNA content, extracted using 5% Chelex (BioRad), Proteinase K (Qiagen) and RNase A (Qiagen).

3.10 Statistical analyses (Paper I, II and III)

Differences in cytokine production, Treg marker expression, mitochondrial respiration and levels of Treg suppression between APS-1 patients, autoimmune PAI patients and healthy controls (age- and sex-matched) were assessed by an unpaired, parametric t-test (Paper I and II). A non-parametric t-test (Mann-Whitney test) was used to compare differences in the TCR repertoire diversity between APS-1 patients and healthy controls (Paper II). Correlations between RIA and ELISA (Paper III) were measured by the Pearson correlation coefficient and a 95% confidence interval. All statistical analyses were performed using GraphPad Prism v.9.1.0 (GraphPad Software). For the single-cell data (Paper I), all genes expressed with a $-0.15>\log2FC>0.15$ were retained. The adjusted p value was calculated based on Bonferroni correction using all genes in the dataset. Differences in VDJ gene usage were estimated with t-tests, an ANOVA test and post-hoc t-tests. For RNA sequencing data (Paper II), p values were adjusted using the Benjamini and Hochberg's approach for controlling false discovery rate. For all statistical tests, a p or adjusted p value<0.05 was considered significant.

4. Summary of results

4.1 Paper I: "Single-cell transcriptomic and proteomic analyses reveal functional regulatory T cells in autoimmune polyendocrine syndrome type 1"

The aim of this study was to explore the transcriptome and proteome of *in vitro* expanded Tregs, on the single cell level, to examine whether patients with APS-1 have impaired Treg function. We recruited in total 18 Norwegian APS-1 patients and 18 age- and sex-matched healthy controls for the expanded Treg protocol, and a cohort of four Finnish patients and four healthy controls, from whom naïve Tregs were freshly sorted and sequenced. Tregs from the Norwegian patients were isolated from whole blood and expanded for 14 days in the presence of anti-CD3/CD28 and IL2, before subjected to single-cell sequencing and characterization by flow cytometry and CyTOF. The ability of Tregs to suppress responder cell proliferation was assessed with a flow cytometry based Treg suppression assay.

We found an upregulation of several *HLA*-genes and the proliferation marker *MKI67* in APS-1 patients compared to healthy controls in polyclonal Tregs. However, the log2 fold change differences were small, indicating that the differences between the populations were very small. Further, sequencing experiments using freshly sorted Tregs from a small cohort of APS-1 patients (N=4) and healthy controls (N=4) did not reveal differences between patients and controls. TCR sequencing revealed an overall similar repertoire when comparing APS-1 patients and controls, although some patients harboured a more restricted TCR repertoire. Treg characterization by flow and mass cytometry revealed high expression of FOXP3 (> 90%) in both groups, but a lower frequency of CD4+CD25+FOXP3+ cells in the patient cohort ($p = 0.0009$). This finding was confirmed by the CyTOF experiments, where the CD4+CD25+CD127- fraction was significantly lower in patients compared to controls ($p = 0.0282$). A

rigorous Treg suppressive assay was established to examine the suppressive abilities of *in vitro* expanded Tregs from APS-1 patients. Overall, we found that Tregs from patients were able to suppress CD4+ responder cell proliferation to the same extent as Tregs from healthy controls. Taken together, our findings show that Treg suppressive abilities were similar between patients and controls, and no major differences in the expression of Treg functional markers were observed.

4.2 Paper II: "Regulatory T cells in autoimmune primary adrenal insufficiency"

For the second study, we aimed at exploring Treg suppressive function and possible transcriptional changes in patients with autoimmune PAI compared to healthy controls. From the ROAS registry, we recruited 23 patients with autoimmune PAI and one with secondary adrenal insufficiency. Forty one healthy controls were recruited from the Haukeland University Hospital Blood Bank. First, Treg frequency within the CD4+ population in cryopreserved PBMCs, in addition to Treg functional markers, were compared between patients and controls using flow cytometry. In addition, Tregs were isolated from whole blood and expanded for two weeks in the presence of anti-CD3/28 and IL2, with subsequent Treg suppression assay, bulk RNA sequencing and mitochondria respiration assay.

No differences were seen when the frequency of Tregs and the markers HLA-DR, Helios, CTLA4, CD45RA and CD31 within Tregs were compared in PBMC from patients (N=15) and healthy controls (N=15). When generating expanded Tregs, a significantly higher number of Tregs were made from patients versus healthy controls, which might indicate high proliferation capabilities and possibly faster Treg turnover. Treg suppressive capacity was further assessed in a flow cytometry based Treg suppression assay using expanded Tregs. Preliminary findings in five patients and five

controls indicated a significantly lower percentage of Treg suppression in patients compared to controls for ratios 1:1, 2:1, 4:1 and 8:1. Based on this, we included 15 additional patients and controls. Overall, there was no statistically significant difference in Treg ability to suppress responder T cell proliferation between patients and controls at Tresp-to-Treg ratios 1:1 ($p = 0.1870$) and 4:1 ($p = 0.0507$). We additionally used a pool of responder cells and Tregs from individual patients (N=5) and controls (N=5) to test whether responder cells were resistant to Treg mediated suppression. No differences were observed between patients and controls, indicating that patient responder cells are not Treg resistant. In an attempt to increase Treg suppression in patients and controls, we added AS2863619 (N=3) and CD152 [CTLA4]:Fc (N=2) to the co-culture assays, however, no differences in Treg suppression were observed.

Initial findings from the suppression assay made us suspect that there might be differences in the *in vitro* expanded Treg pool on a transcriptional level. Hence, Tregs from 16 patients and 16 controls were RNA sequenced. The results showed an upregulation of several mitochondrial and killer-cell lectin-like receptor C (*KLRC*) genes in the patient cohort. Further, 12444 genes were shared between the two groups, while 342 and 161 genes were found to be uniquely expressed in the patient and control group, respectively. Impaired mitochondrial function in autoimmune PAI expanded polyclonal Tregs was not found, as no significant differences in basal or maximal respiration, ATP production or spare capacity were observed when comparing expanded Tregs from patients and healthy controls.

Taken together, the results showed that Tregs from patients with autoimmune PAI had a high proliferative capability and were able to inhibit responder T cell proliferation to the same extent as cells from healthy controls. Sequencing revealed an upregulation of mitochondrial genes in the patient cohort, but the oxygen consumption rate measured

by a Seahorse XF Mito stress test was found comparable to healthy controls, suggesting that patients with autoimmune PAI harbour functional *in vitro* expanded Tregs.

4.3 Paper III: "Screening patients with autoimmune endocrine disorders for cytokine autoantibodies reveals monogenic immune deficiencies"

In this study, patients with endocrine autoimmune disorders in the ROAS registry were screened for cytokine autoantibodies, focusing on IFN-ω and IL22 autoantibodies, with the aim of identifying patients with undiagnosed APS-1 or other genetic causes of disease. A newly established ELISA, capable of dealing with pooling of serum samples, was used to screen patients with autoimmune PAI (N=675) for autoantibodies against IL22, while a routine RIA was used to detect autoantibodies against IFN-ω in patients with different endocrine disorders (N=1778). The threshold for positivity in both assays were based on indices from healthy controls. Positive subjects were subsequently sequenced to search for mutations in immune related genes.

All together, we were able to find 29 patients with autoantibodies against IFN-ω and/or IL22. Further, the study showed that autoantibodies against IFNs and ILs can be used to detect patients with *AIRE*-mutations, as four new patients with APS-1 were identified. Based on autoantibody screenings and subsequent exome sequencing, we found eight patients with rare mutations in genes involved in the T cell receptor signalling pathway. These genes were *CTLA4*, *NFKB2, LAT, LCK, JAK3, STAT1, RAG1, TNFAIP3*, *TERC* and *IKZF2*. The heterozygous mutations discovered in *CTLA4* and *NFKB2* were both confirmed as disease-causing, but the others need further confirmation. We also identified a patient with an *AIRE* variant known to predispose for development of autoimmune PAI. Based on the findings from this study, we conclude that it is possible to identify patients with disease of previously unknown

mono- or oligogenic cause based on cytokine autoantibody screenings. This is of importance for follow-up of both patients and their close relatives.

5. General discussion

In the present work, we have used several approaches to study human immune regulatory mechanisms with a focus on Tregs and cytokine autoantibodies. Our findings show that expanded Tregs in both APS-1 and autoimmune PAI can suppress responder cell proliferation to the same extent as Tregs expanded from healthy controls. Further, we show that it is possible, based on type I IFN and IL22 autoantibody screenings, to identify patients with a mono- or oligogenic cause of disease. In the next section follows a general discussion of the main findings, pointing to the importance of studying regulatory mechanisms and rare diseases.

5.1 The strenght of disease registries and biobanks

Elucidating biological underlying factors of diseases generally requires large cohorts to achieve statistical power and robustness. GWAS studies are examples of this: to identify risk genes with sufficient power, thousands of subjects are needed. This has pushed large international initiatives, e.g. the Psychiatric Genomic Consortium have managed to collect >400000 subjects from 36 countries and studies on type 1 diabetes have included >500000 subjects (276, 277). However, such large efforts are not possible for rarer disorders, but it may be even more crucial to gather rare patients in registries and biobanks and to collaborate across borders. Only then will enough information be collated to draw conclusions. This is the success factor for identification of causative genes (e.g. *AIRE* for APS-1), biomarkers and improved treatment for rare immune disorders. This is also the reason why the work conducted in this thesis was possible. Good registries (meaning that the information that has been captured from patients is accurate, informative data is collected and that longitudinal follow-up is possible) might facilitate further biological studies. This is exemplified by the identification of *AIRE* as a susceptibility factor for PAI in a polygenic setting, which would not have been possible without strict inclusion criteria to secure a homogenous cohort (only 21OH-positive patients), inclusion of patients for several decades,

dedicated clinicians and researchers, contact with patient organizations and close collaboration between Norway and Sweden (192).

The main limitation of studying rare diseases, such as APS-1 and autoimmune PAI, is the restricted access to an adequate volume of patients, which might compromise statistical power. Several of the undertaken experiments in this thesis were dependent on fresh blood samples, which requires willingness and collaboration from both patients and their consulting physician, and the work in this thesis would not have been possible without their cooperation. The ROAS registry is associated with one of the world's largest biobanks containing PBMC, blood and sera from patients with autoimmune PAI, APS-1 and other autoimmune endocrine disorders, and the registry has national coverage. Through collaborations within the ROAS network, we were able to overcome geographical challenges of getting fresh blood samples from APS-1 patients living in other parts of Norway, thereby increasing the statistical power of the study (paper I). When working with rare diseases, international connections are important, and in collaboration with Estonia and Finland, we were able to include results involving additional APS-1 patients (paper I). However, one must keep in mind that the use of different cohorts might be affected by different genetic background in the population(s) and healthy controls with the same genetic background should therefore be included.

5.2 Monogenic diseases; powerful experiments of nature

The aetiology of autoimmune disorders rely on complex contributions from both genetic and environmental factors (278). A few informative disorders exist where mutations in only one gene causes autoimmunity, such as APS-1 and IPEX (66, 133). These rare monogenic disorders are important to study for several reasons. The first is of direct benefit for the patient and close family: identification of causative mutations can predict clinical outcome, foresee new disease components and point to treatment

and follow-up regimes. A monogenic cause further enables genetic screening to be carried out in siblings and parents. Another justification to use large resources on monogenic immune disorders is that genetic faults can pinpoint immune pathways which may be targets for curative therapy. An apt example of this is the development of gene therapy for severe combined immunodeficiency (SCID) caused by adenosine deaminase-mutations (279). The largest impact of monogenic studies might be related to the fact that genetic variants causing strong autoimmune phenotypes most often encode important factors that regulate immune function or immune tolerance. Knowledge of such mechanisms is important as it might take us one step closer to elucidate the pathogenesis of more common and complex autoimmune conditions, such as autoimmune PAI, that in the future can aid in the development of immune therapy.

APS-1, as investigated in this thesis (paper I), is an excellent model disease for combined autoimmunity and immune deficiency, as the disease-causing gene, *AIRE*, is a major tutor of developing T cells to discriminate self from non-self (55, 63). In depth knowledge of thymic tolerance has been revealed from studies examining either mice or humans with *AIRE* mutations, and these studies have additionally improved diagnostic toolboxes (with autoantibodies and *AIRE* in genetic immune panels) for APS-1 and isolated entities of the APS-1 clinical spectra (without *AIRE* mutations, but with organ-specific autoantibodies).

The intriguing finding of cytokine autoantibodies in close to all APS-1 patients and in other monogenic syndromes is another step to understand the balance between autoimmunity and immune deficiency (8, 9, 218). Indeed, IFN-ω is used in Norway to screen for potential APS-1 cases, being incorporated in the clinic at Haukeland University Hospital, and we propose that cytokine autoantibody screenings combined with panels of next generation sequencing may help to identify other monogenic endocrine syndromes as well (paper III). Although anti-IL22 antibodies are not as prevalent in other diseases than APS-1 (to our knowledge), this marker might be a good

candidate to also be included in cytokine autoantibody screening panels, as it has been (and is) used by the Swedish Addison Registry to screen for APS-1 (221), and we also discovered patients with exclusively anti-IL22 serology in our effort to identify rare immune gene variants (paper III). Identification of novel gene variants responsible for autoimmunity and immune deficiency might pave the way for improved knowledge of the immune system. Of notice, the use of an adaptation of a well-defined genetic immune panel in paper III revealed several new patients with rare variations in immune regulating genes, which means that this specific pipeline and panel is useful for the purpose. *IKZF2* was one of the very few genes that were added to the otherwise diagnostic routine pipeline, and our finding of two patients with in total two possible disease-causing mutations in this gene indicate that *IKZF2* should be included in the panel on a regular basis.

5.3 Cytokine autoantibodies and their implications in immunological diseases

In addition to APS-1, cytokine autoantibodies are described in a number of immunodeficiencies, including patients with mutations in *RAG*, *IKZF2* and *NFKB2* genes, patients with a malignancy of the thymus and in myasthenia gravis (8, 94, 224, 280, 281) (paper III). Further, type I IFN autoantibodies are also described in patients with IPEX syndrome caused by *FOXP3* mutations (9). We here add new patients with both previously known and novel rare variants in *CTLA4, IKZF2, RAG, NFKB2, LAT, TNFAIP3, STAT1, TERC, JAK* and *LCK* to the list of patients where anti-type I IFN and/or anti-IL22 antibodies are serologically present (paper III). Hence, these autoantibodies are found in disease states caused by failure of both central (e.g. APS-1, *RAG* mutations) and peripheral tolerance (e.g. IPEX). Autoantibodies are not always seen in disease states, as type I IFN autoantibodies were also found in a fraction of healthy individuals, where the levels were highest in people over 70 years of age (225).

Even though it seems like the cytokine autoantibodies might be very good markers for a collection of monogenic immune deficiencies/autoimmune disorders, as shown in paper III, it is not clear what their functions are *in vivo*. Two studies have shown that the type I IFN profile in whole blood and isolated PBMCs, plasmacytoid dendritic cells, monocytes and monocyte-derived dendritic cells were affected in APS-1 patients with IFN-α autoantibodies, but not in the very few patients without these specific autoantibodies (282, 283). Further, a study by Meyer *et.al* recently suggested that presence of high levels of neutralizing anti-IFN-α antibodies could protect against type 1 diabetes in APS-1 (284).

During the COVID-19 pandemic, presence of type I IFN autoantibodies were shown to predispose for serious disease requiring admission to intensive care units (230, 231), making a new "renaissance" for how cytokine autoantibodies might interfere with immunity and viral immunity in specific. Patients with type I IFN autoantibodies were later found not to always become critically ill, which contradicts the first observations of the pandemic, and these patients also showed a normal vaccine response (232, 233). APS-1 patients are further more susceptible to severe herpesvirus infections (285), which may also be due to the high levels of neutralizing type I IFN autoantibodies. Looking at the presence of autoantibodies targeting the Th17 cell-specific cytokines IL17 and IL22 in APS-1 patients, this may indicate that CMC as a part of the syndrome is not a "classic" immune deficiency component, but rather a part of an ongoing response against the immune system itself (20, 217).

5.4 Immunological tolerance in AIRE deficiency

Achieving and maintaining immunological tolerance is important in order to avoid disease development. Mechanisms regulating the balance between health and disease consists of both central (i.e. bone marrow and thymus) and peripheral (i.e. blood and

tissues) branches. APS-1 is known to be caused by a breakdown of central tolerance and studies of Aire have taught us important lessons regarding negative selection and the induction of Tregs (55, 56, 59, 252). The finding of the p.R471C variant of *AIRE* as associated with PAI, and other autoimmune conditions (192, 277, 286), raises questions as to whether central tolerance mechanisms might be involved in the pathogenesis of these diseases as well, which could possibly also affect the Treg population. Hence, studying immune mechanisms in individuals with different anticipated AIRE dosage potentiate even more insight into autoimmunity and immune tolerance, in addition to insights into the aetiology of other disorders than APS-1. This thesis includes patients with different types of mutations in *AIRE* (recessive, nonsense, missense, splice, dominant), and in addition patients with PAI, in which common *AIRE* variants may impact on AIRE dosage/function. Although AIREs role in central tolerance is established (55, 56), the role in Treg generation and function, and thereby its role in peripheral tolerance, is still controversial. Breakdown of peripheral tolerance mechanisms is also known to cause autoimmune disease, as seen in patients with IPEX, who harbour cytokine autoantibodies and lack functional Tregs due to mutations in the *FOXP3* gene (9, 66, 142). As there are limited measures to repair the failure of central tolerance, we look to peripheral mechanisms in this thesis, with a focus on Tregs, to identify disease mechanisms and possible ways of restoring immunological tolerance.

5.5 Tregs and endocrine autoimmunity

Tregs are crucial mediators of peripheral tolerance and alterations in the Treg compartment with regards to frequency and function are seen in several autoimmune conditions. AITD, most commonly hypothyroidism, is often seen concomitant with autoimmune PAI and APS-1 (170, 194). In AITD, no difference was found by Glick *et.al* in the frequency of Tregs compared to healthy controls, but a reduced rate of suppression was reported (287). However, Tregs were only found partially dysfunctional, as the suppression was unchanged when using allo-PBMC as responder cells and reduced when auto-PBMC made up the responder cell fraction (287). Another

autoimmune component seen together with PAI and APS-1 is POI (63, 193). In POI, both a reduced frequency of Tregs and a lower expression of FOXP3 have been described (288, 289). Also in type 1 diabetes, several studies have found deficient Tregs (161, 290-294). However, a study by Brusko *et.al* suggested that the frequency of FOXP3+ Tregs was unchanged in subjects with type 1 diabetes compared to first-degree relatives and healthy controls (295), while Viisanen *et.al* reported increased Treg frequencies in paediatric patients with type 1 diabetes (154). In sum, there are numerous studies published with results both implying differences in Treg function/numbers and the opposite for this disorder. These observations probably rely on different patient cohorts with a variety of e.g. antibody profiles, clinical course and maybe even with different aetiology and on methodological issues. For type 1 diabetes, studies have also shown increased Treg apoptosis in recent-onset type 1 diabetes (290), as well as increased effector cell resistance to Treg-mediated suppression (161, 296), which may contribute to impaired peripheral tolerance and risk for autoimmunity.

Regarding Tregs in PAI and APS-1, there is a large agreement in the APS-1 research field of lower frequencies of these regulatory cells in blood compared to healthy controls (155-158), also supported by our findings (paper I). Further, defective suppressor function of Tregs have previously been described in both APS-1 and APS-2 by Kekäläinen *et.al* and Kriegel *et.al*, respectively (157, 159), which is opposed to our findings (paper I and II). The conclusion of paper II, showing functional Tregs in isolated PAI is the first study to our knowledge on Tregs in this disorder. While we could not detect any large disturbances in Tregs of neither PAI patients nor APS-1 patients (paper I and II), both studies revealed a higher proliferation of Tregs in patients *ex vivo*, which should be further studied.

A neglected important point is that thymic, blood and “other” tissue Tregs are probably not alike in number and function. Most studies have been conducted on circulating immune cells, as this thesis is also a proof of. The reason is the difficulty of obtaining

tissue biopsies from informative cases, as it requires an invasive procedure. It also raises major ethical concerns, as a surgical procedure is always associated with a risk of complications. Hence, our best option is to perform *in vitro* experiments using cells isolated from blood, which might not accurately represent the mechanisms taking place *in vivo*, as the majority of T cells are tissue-resident (297). Currently, no mouse models are available for studying autoimmune PAI and studies must be performed on humans with these obvious limitations. There are however several mouse models that correspond to the human APS-1 and Aire-knockout mice have provided valuable insights as to how Aire mediates and maintains immunological tolerance (55, 56, 298, 299). Mice deficient in Aire suffer from multi-organ autoimmune disease, however, the main components of APS-1 are not always seen and the autoantibody repertoire is also different (55, 298-301). A previous study by Yang *et.al* have shown that Aire knockout mice lack a specific subpopulation of Tregs (247) and by using single-cell sequencing (paper I) we were hypothesising to observe differences in the Treg compartment also in human subjects with APS-1. However, our APS-1 cohort included patients with a large age range and differences in clinical phenotype (paper I), and relied on Tregs from blood, while the previous study on the missing Treg subpopulation was found in neonatal mice (247), also highlighting the difference between mice and humans and the importance of having access to tissue and possibly study subjects of different ages.

5.6 Treatment possibilites and challenges in the immune modulative era

One of the thoughts behind the work in this thesis was to bring forward knowledge to aid future therapy, and the focus was on Tregs. Several approaches have been studied in other autoimmune conditions with regards to Tregs in both human subjects and mice, including co-stimulation blockade with abatacept/CTLA4-Ig, low-dose IL2 therapy, adoptive Treg transfer and inhibition of Cdk8/19 (236, 238, 239, 242, 243). Since the

level of Tregs in APS-1 is low, indicated by both lower FOXP3 expression and by the frequency of FOXP3+ cells within the CD4+ subset (155-158) (paper I), a possible strategy would be to expand the already existing Treg pool. This has been proven both achievable and safe in 1 diabetes, where Bluestone *et.al* and Marek-Trzonkowska *et.al* showed that Treg treatment did not cause toxicity or other adverse events in patients receiving Treg therapy (239, 241).

In autoimmune PAI/APS-2, we tried to increase the rate of Treg suppression in an *in vitro* cell culture system by using AS2863619, which have been shown to convert effector cells into regulatory cells in mice (243), and CD152[CTLA4]:Fc, analogous to CTLA4-Ig, which can maintain the Treg population and protect against autoimmune disease development in mice when combined with IL2 treatment (238). However, the addition of these small-molecules did not seem to affect the rate of Treg suppression in patients or healthy controls (paper II). Our study only included a limited number of patients and further studies are required to fully determine the effect of the two different components. The use of Tregs to treat autoimmune disease require cells to be functional, stable, being able to survive, and they should be specific in order to avoid undesired and non-specific immune suppression (302). Desired Treg specificity could be obtained by generating chimeric antigen receptor Tregs expressing a fragment of the antigen-binding region specific for a particular self-antigen (302). For PAI and APS-1; a possible candidate would be 21OH, but the required knowledge on epitopes on this protein for CD4+ T cells are not yet known. When manipulating the Treg fraction for therapy, one also has to take immune homeostasis into consideration. As autoimmune features, including adrenal insufficiency, are seen as a side-effect of cancer treatment with checkpoint inhibitors (303, 304), one could speculate that adverse events, such as cancer, might be a consequence of trying to cure autoimmunity.

While Tregs are potential therapeutic candidates, there is also a question as to whom might benefit from Treg therapy. This is especially relevant as autoimmune disease

have often progressed beyond tissue destruction when a patient is diagnosed. As of today, no measures exist that can prevent autoimmune disease development and at-risk individuals from progressing to clinical signs of disease. We therefore need tools to identify and monitor patients and relatives with an increased risk for autoimmunity. Recently, a polygenic risk score was developed to identify persons at risk for developing autoimmune PAI (305). This is the first step to find individuals at risk. Cytokine and 21OH autoantibodies, in addition to other autoimmune disease-specific autoantibodies, have been found present in relatives of patients with PAI (306), and these biomarkers can be detected several years before the first clinical signs (7, 258). Together, these approaches and established patient registries offer great opportunities for detecting individuals with a higher risk for developing disease. Despite the development of different screening tools and sequencing methods, further work is required with regards to the treatment/clinical part, to come up with strategies to re-establish tolerance before overt disease develops. If targeted treatment existed, one could argue for the inclusion of *AIRE* mutational analysis in the new-born screening program for early detection of APS-1. In Finland, new-borns are screened for increased genetic risk for type 1 diabetes, enabling longitudinal follow-up of at-risk individuals harbouring specific *HLA* genotypes (307, 308). Since *HLA* is a risk loci strongly associated with autoimmune PAI (192), a similar screening method could be used for early detection of individuals with a higher risk for developing the disease. However, this is hampered by ethical considerations, as the presence of a specific *HLA* type in a new-born does not necessarily imply that they will develop the disease, but it could either way cause distress and affect quality of life in close family members. Also, PAI is much rarer than type 1 diabetes, which will limit the number of identified “at risk PAI patients”, which again may challenge ethical discussions of burden versus benefit for patients and the society.

6. Final conclusions

In accordance with the specific aims of this thesis, the following conclusions can be drawn:

- Patients with both APS-1 and PAI had an *in vitro* increased proliferative capability of Treg generation which might point to faster Treg turnover.
- Polyclonal Tregs from APS-1 and autoimmune PAI patients are overall capable of suppressing responder T cell proliferation to the same extent as polyclonal Tregs from healthy controls.
- The *in vitro* expanded Treg repertoire was overall similar between patients with APS-1 and healthy controls, however, some patients did show a more restricted TCR repertoire.
- Flow and mass cytometric analyses revealed a reduced CD4+CD25+FOXP3+ population among *in vitro* expanded Tregs from patients with APS-1 compared to healthy controls.
- Several mitochondrial and *KLRC* genes were upregulated in expanded Tregs from patients with autoimmune PAI.
- Polyclonal Tregs in autoimmune PAI displayed functional mitochondrial basal respiration, non-mitochondrial respiration, maximal respiration, ATP-production and spare capacity similar to healthy controls.
- Cytokine autoantibody screenings identified four novel APS-1 cases with disease-causing mutations in the *AIRE* gene and mutations in several genes involved in the T cell signalling pathway were identified in eight patients with different endocrine disorders.
- Cytokine autoantibody screenings can be used to identify patients with immunological disease of previously unknown monogenic or oligogenic cause.

7. Future perspectives

Patients with APS-1 have autoantibodies targeting antigens in affected organs and tissues, and as already emphasised these are of great diagnostic value. Patients positive for APS-1-specific autoantibodies, but without pathogenic *AIRE* mutations, were here identified with other genetic variants in immune related genes. An obvious next step to continue the work from paper III would be to determine the disease-causing potential of these variants, assess disease penetrance and further perform functional cell-based assays. Since the patients, and some of their close relatives, are included in ROAS, it is possible to get access to sample material. From a few vials of blood PBMCs, DNA and RNA can be isolated. In addition, the isolation of Tregs gives the opportunity to examine how Treg function and numbers are affected by the various mutations, both in naïve and expanded cells. The already established suppression assay can then be used to assess Treg suppressive function.

Since the suppression assay from papers I and II only measures general suppression in response to anti-CD3/CD28 activation, future experiments should involve activation with disease-specific antigens, such as 21OH, to measure antigen-specific responses. Further, trying to block different Treg mechanisms of action, such as inhibiting cytokine production, could be performed in order to determine if specific mechanisms are impaired, although general suppression were comparable to that of healthy controls. It would also be interesting to follow activation pathways in non-expanded Tregs using for instance phosphor-flow. Further, it would be interesting to study tissue resident Tregs and especially try to identify adrenal-specific Tregs. However, obtaining tissue from patients is not feasible and the development of a mouse model for autoimmune PAI is necessary. Mitochondrial function in PAI, and APS-1, could be further elucidated by isolating mitochondria from Tregs and other cell types to further assess respiration and metabolic activity, including measuring production of mitochondrial reactive oxygen species. Subsequent isolation of mitochondrial DNA, followed by

sequencing, can then be used to search for disease-associated variants and lead to identification of possible therapeutical targets.

The ultimate goal in the study of autoimmune diseases is to understand underlying mechanisms and find new possible targets for therapy that could skew an intolerant immune reaction towards tolerance and reverse a starting autoimmune reaction before overt disease is evident. However, re-establishing already broken tolerance is difficult, especially since tissue-destruction often starts before patients progress to clinical signs of disease. Tregs show great potential in the treatment of autoimmune disease and the generation of chimeric antigen receptor Tregs opens for the opportunity to produce antigen-specific Tregs, possibly with 21OH as a target in PAI. Also, mapping which peptide sequence(s) of 21OH that are targeted by autoreactive CD4+ T cells would be of great interest. This might be used to identify 21OH-specific Tregs, which could possibly be used in a therapeutic setting.

Even though PAI and APS-1 are rare diseases, they still have great impact on patients' quality of life and is a burden to their close relatives. Early diagnosis, prevention of life-threatening complications and monitoring patients for development of additional disease components are of major importance. This can be achieved by autoantibody screenings and by keep searching for new therapy targets that can lead to improved follow-up and personalised treatment for patients and close relatives. Since it is believed that the pathogenesis of PAI has similarities to type 1 diabetes, our studies will also have implications for the understanding of other endocrine autoimmune disorders.

8. References

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9. Appendix

Paper II

Regulatory T cells in autoimmune primary adrenal insufficiency

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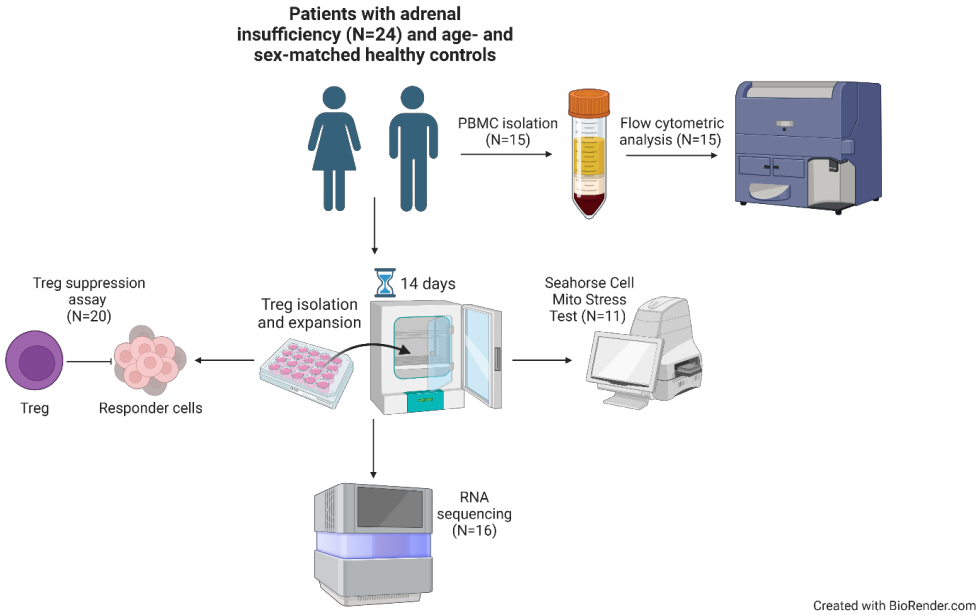
Graphical abstract



Abstract

Background: Primary adrenal insufficiency (PAI) is most often caused by an autoimmune destruction of the adrenal cortex resulting in failure to produce the life-essential hormones cortisol and aldosterone. The aetiology is thought to be a combination of genetic and environmental risk factors, leading to breakdown of immunological tolerance. Regulatory T cells (Tregs) are deficient in function or number in many autoimmune disorders, but it is not known whether these suppressive cells contribute to development or progression of PAI.

Objectives: We aimed to investigate the frequency and function of naïve and expanded Tregs from patients with PAI and polyendocrine syndromes compared to age- and gender-matched healthy controls.

Methods: Flow cytometry was used to assess the frequency and characterise functional markers of blood Tregs in PAI (N=15). Expanded Treg suppressive abilities were then assessed with a flow cytometry based suppression assay (N=20), while bulk RNA-sequencing was used to examine transcriptomic differences between patients' and controls' Tregs (N=16) and oxygen consumption rate was measured by Seahorse cell metabolic assay (N=11).

Results: Treg frequency within CD4+ cells was similar between patient and controls. Tregs from patients with PAI had a similar suppressive capacity to healthy controls, but an increased expression of killer-cell leptin-like receptors and mitochondrial genes. PAI patients' Tregs did not display signs of mitochondrial dysfunction, however, trends toward lower oxygen consumption rates in the patient group compared to healthy controls was observed.

Conclusion: Patients with autoimmune PAI, with our without the co-existence of other autoimmune conditions, harbour Tregs with suppressive capacities similar to that of healthy controls.

1. Introduction

The main cause of primary adrenal insufficiency (PAI) in industrialised countries is an autoimmune attack targeting hormone-producing cells in the adrenal cortex. This is known as autoimmune PAI, hereby referred to as PAI, and clinically manifests as fatigue, nausea, depression and muscle weakness, caused by deficiency in the life-essential hormones cortisol and aldosterone [1]. This disease can appear as an isolated entity in patients, but is most often seen in concert with other autoimmune components. When present together with type 1 diabetes and/or autoimmune thyroid disease it is defined as autoimmune polyendocrine syndrome (APS)-2, with a similar polygenic inheritance pattern as isolated PAI, while the additional components hypoparathyroidism and chronic mucocutaneous candidiasis define the monogenic APS-1, caused by mutations in the *AIRE*-gene [2]. Except for PAI in APS-1, the aetiology of the disorder is still an enigma, but is thought to include genetic factors in combination with unknown environmental triggers [3], which will lead to breakdown of immunological tolerance. At diagnosis, >90% of PAI patients have autoantibodies against the key enzyme for generation of corticoids and mineralocorticoids, namely 21-hydroxylase (21OH), which is used in the diagnostic toolbox for PAI [4, 5]. However, autoreactive T cells are thought to have a dominant role in the pathogenesis [6-8].

A key player in the maintenance of self-immune tolerance is T cells with suppressive capacity, i.e. regulatory T cells (Tregs), which have the ability to dampen possibly damaging immune responses in the blood stream and peripheral tissues [9-11]. Several autoimmune disorders have been found with altered levels or impaired function of Tregs [12-14]. In addition, failure of effector T cells to respond to Treg mediated suppression have also been described [15, 16]. Importantly, the stability and function of Tregs is dependent on mitochondrial metabolism [17, 18] and processes taking place in the mitochondria are also important for Treg survival in lactate-rich environments [19].

How Tregs appear in PAI and APS is not yet clear, but one study previously claimed that APS-2 patients have lower Treg suppressive capacity than healthy controls [20], while Treg number is clearly impaired in APS-1, although the functional properties of Tregs in this syndrome are controversial [21-23]. Recently, our genome-wide association study (GWAS) identified HLA-haplotypes and several Treg-related genes, including *CTLA4*, *BACH2* and *AIRE* to be associated with a higher risk of developing PAI [24], which might point to Treg disturbances in PAI.

Here, we hypothesized that Tregs may be involved in the pathogenesis of PAI. To this end, we examined Treg frequency in peripheral blood mononuclear cells and further studied the suppressive capacity, the transcriptional activity by RNA-sequencing and mitochondrial

function of expanded Tregs isolated from patients with adrenal insufficiency with or without comorbidities and compared them with Tregs from healthy controls. Our results can help to establish knowledge on how Tregs are affected in PAI and whether or not Tregs may be suitable future targets or vehicles for therapy for this disorder.

2. Methods

2.1 Patients, controls and ethical considerations

Six patients with isolated PAI, 16 patients with APS-2, one patient with PAI and primary ovarian insufficiency (POI) and one patient with secondary adrenal insufficiency due to adrenolectomy enrolled in the Norwegian National Registry for Organ Specific Autoimmune Diseases (ROAS) were included in the study (16 female, 8 male, mean age 51.6, range (19-78) years, Table 1). All patients have given their written informed consent for participating. Whole blood from 41 sex- and age-matched healthy donors (25 female, 16 male, mean age 49.1, range (22-74) years) were obtained from the Blood Bank at Haukeland University Hospital (Bergen, Norway) and donors gave their informed consent for research when donating blood. This study is approved by the Regional Committee for Medical and Health Research Ethics (REK), with REK-number 2018/1417, and was conducted in accordance with the Declaration of Helsinki. From 15 patients and matched controls, we had access to cryopreserved peripheral blood mononuclear cells (PBMC) and Treg frequencies and functional markers were assessed. Expanded Tregs were generated from all included patients (N=24) and most healthy controls (N=27). These expanded cells were then applied to suppression assays, RNA transcriptomics and metabolic assays (Table 1).

2.2 Peripheral blood mononuclear cell (PBMC) isolation

PBMCs were isolated using Ficoll density gradient centrifugation. Cells were frozen in human AB serum supplemented with 10% dimethyl sulfoxide (DMSO), stored at -80℃ for 2-3 days and further at -150℃ until use.

2.3 Flow cytometric analysis for PBMCs

PBMCs from 15 patients (mean age 48.5 (range 22-78) years, 53% females) and 15 healthy controls (mean age 46.8 (22-68) years, 53% females) were thawed, stained with Live/dead Fixable Yellow Dead Cell Stain Kit according to the manufacturer's instructions (Invitrogen,

cat. L34959) and Fc-blocked with BD Pharmingen Human BD Fc Block (BD, cat.564219). Subsequently, cells were stained with the following antibodies: V500 anti-human CD3, PerCP-Cy5.5 anti-human CD4, PE-Cy5 anti-human CD8, PE-Cy7 anti-human CD25, APC-H7 anti-human CD45RA, BV421 anti-human CTLA4, BV785 anti-human CD31, BV650 anti-human HLA-DR, biotin anti-human TCRγ/δ, biotin anti-human CD1c and biotin anti-human CD14. Cells were then stained with FITC Streptavidin (BioLegend, 1:500, cat. 405202) (Supplementary Table 1). Fixation and permeabilization was achieved using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, cat. 00-5523-00) according to instructions from the manufacturer. Cells were intracellularly stained with APC anti-human Helios and PE-CF594 anti-human FOXP3 (Supplementary Table 1). Cells were analysed using the BD LSRFortessa Cell Analyser and the BD FACSDiva Software. FlowJo v10.2 (BD) was used to analyse flow cytometric data.

2.4 Treg isolation and *in vitro* expansion

Tregs were isolated from whole blood using the MACSxpress Treg Isolation Kit Human according to instructions from the manufacturer (Miltenyi Biotec, cat. 130-109-557). Next, Tregs were expanded in TexMACS medium (Miltenyi Biotec, cat. 130-097-196) supplemented with 500 U/mL recombinant (r) IL2 (Miltenyi Biotec, cat. 130-097-744) and 5% human AB serum for 14 days at 37℃ and 5% CO_2, according to the Treg Expansion Kit (Miltenyi Biotec, cat. 130-095-345). Cell culture medium was exchanged every 2-3 days and cells were split when necessary. After expansion, cells were harvested and frozen in human AB serum supplemented with 10% DMSO. Cells were cryopreserved and kept at -150℃ until use.

2.5 Treg suppression assay of expanded Tregs

The PAN T Cell Isolation Kit Human (Miltenyi Biotec, cat. 130-096-535) was used to obtain the untouched T cell fraction (here referred to as responder cells, Tresp) from 20 patients (mean age 50.2 (range 22-78) years, 75% females) and 20 controls (mean age 49.3 (range 22-74) years, 75% females), according to instructions from the manufacturer (Miltenyi Biotec). Tresp cells ($1x10^6$ cells/mL) were rested overnight in TexMACS medium containing 5% human AB serum and 50 U/mL rIL2 (Miltenyi Biotec) at 37℃ and 5% CO_2. These cells were then stained with the CellTrace Violet Cell Proliferation Kit according to instructions from the manufacturer (Invitrogen, cat. C34557). Tresp and Tregs were dissolved to a concentration of $5x10^5$ cells/mL in TexMACS medium supplemented with 50 U/mL rIL2, 5% human AB serum and 1% penicillin-streptomycin (Treg suppression medium).

For the suppression assay, cells were activated with 3 μL/mL Immunocult Human CD3/CD28 T cell Activator (Stemcell Technologies, cat. 10971) and co-cultured in Tresp-to-Treg ratios 1:1, 2:1, 4:1 and 8:1 for five days at 37℃ and 5% CO_2.

For five patients and five healthy controls, the suppressive capacity of Tregs was additionally assessed using a pool of Tresp from five new healthy controls (3 female, 2 male, mean age 47.8, range (41-59) years) to evaluate whether there was inherent impairment of patients' Tresp. Furthermore, for three patients and three controls, 100 nM AS2863619 (Cayman Chemicals, cat. 30976) was added to the co-culture wells (concentration chosen based on work by Akamatsu et.al. [25]). For two patients and two controls, 20 g/mL CD152 [CTLA4] (human):Fc (human)(Chimerigen, cat. CHI-HF-220A4-C500) was added to the co-culture wells (concentration chosen based on titrations with concentrations ranging from 0.65 to 20 g/mL). Both molecules were added to wells with a 1:1 ratio of Tresp pool and Tregs. The culturing conditions were the same as for the regular suppression assay.

Cells were stained with the Live/Dead Fixable Yellow Dead Cell Stain Kit (Invitrogen, cat. L34959) according to the manufacturer's protocol, and directly conjugated mouse anti-human antibodies against V500 anti-CD3, PerCP-Cy5.5 anti-CD4, PE-Cy5 anti-CD8 or APC anti-CD8 and PE-Cy7 anti-CD25 were added (Supplementary Table 1). Fixation and permeabilization was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, cat. 00-5523-00) according to instructions from the manufacturer. Suppressive capacity was assessed using flow cytometry (BD LSRFortessa and S6 Cell Analysers and the BD FACSDiva Software). Percentage of Treg suppression was calculated as %Treg suppression=((Tresp alone-Tresp treated with Tregs)/Tresp alone)*100%. FlowJo v10.2 CL and v.10.8 CL (BD) were used to analyse flow cytometric data.

2.6 Enzyme-linked immunosorbent assay

Treg-specific cytokines were measured in supernatant from Treg suppression assay at end of culturing using Quantikine HS ELISA Human IL-10 (RnD Systems, cat. HS100C), Human TGF beta 1 ELISA Kit (Abcam, cat. AB100647) and Human Interleukin 35 (IL-35) ELISA Kit (Nordic BioSite, cat. EKX-6FHVKH-96) according to instructions from the manufacturers. Absorbance was read at A450 nm (IL-35 and TGF-β), and A490 nm (IL-10) using a SpectraMax plus 384 Microplate Spectrophotometer and the SoftMax Pro 7.1 software (Molecular Devices).

2.7 RNA sequencing and bioinformatic analysis

RNA was isolated from *in vitro* expanded Tregs 16 patients (mean age 52.1 (range 19-78) years, 69% females) and 16 healthy controls (mean age 51.4 (range 24-71) years, 62% females) using the RNeasy Mini Kit according to instructions from the manufacturer (Qiagen, cat. 74106), except for the final elution step, which was repeated twice. Further library preparation, sequencing and bioinformatic analysis was done by Novogene (Cambridge, UK). In short, sequencing was performed on poly-A enriched mRNA libraries using Illumina paired-end 150 bp sequencing by the NovaSeq 6000 system. Raw data was processed with a Novogene in-house perl script. Adaptors and low quality reads (Phred.score=10, meaning an error rate of 90%) were then removed. The clean sequencing reading depth per sample was mean 101 million reads (range 78-105 million reads). Paired-end high quality reads were mapped to the reference Homo sapiens GRCh38/hg38 using Hisat2 v.2.0.5. The program featureCounts v.1.5.0-p3 was then used to count the read numbers mapped to each gene. Differential expression analysis was subsequently performed using the DESeq2 R package (1.20.0) and p-values adjusted with Benjamini and Hochberg's FDR method. Genes with an adjusted p-value≤0.05 were considered differentially expressed. Differentially expressed genes were loaded onto String (https://string-db.org/cgi/network) to perform pathway analysis [26].

2.8 Seahorse cell metabolic assay

Cellular mitochondrial respiration was assessed in 11 patients (mean age 41.1 (range 19-24) years, 73% females) and 11 healthy controls (mean age 42.5 (range 22-64) years, 82% females) by oxygen consumption rate (OCR) using the Seahorse XF Cell Mito Stress Test kit (Agilent Technologies, cat. 103015-100) and the Seahorse XFe96 Analyzer (Agilent Technologies). On the day before the assay, 250,000 *in vitro* expanded Tregs were seeded per well in quadruplicates in a poly-D-Lysine coated Seahorse cell plate (Agilent Technologies, cat. 103799-100) before centrifugation at 300xg for 5 with zero braking. The cells were allowed to attach o/n at 37℃ in a 5% CO2 incubator. On the day of assay, the culture medium was carefully replaced with Seahorse XF RPMI assay medium (Agilent Technologies, cat. 103576-100) supplemented with 2 mM L-glutamine (Sigma, cat. G7513), 2 mM sodium pyruvate (Sigma, cat. P5280) and 25 mM glucose (Sigma, cat. G7021). The cells were incubated at 37℃ in an incubator without CO_2 infusion for 1 h before a second medium change followed by 30 min incubation before starting the assay. After measuring baseline OCR, 2 μM oligomycin, 2 μM CCCP and 2 μM rotenone/antimycin A were added in successive order block ATP production, uncouple oxygen consumption from ATP production and blocking the electron transport chain, respectively. Three measurement cycles were performed between additions

of each inhibitor. After the assay, gDNA was extracted by incubation with 5% Chelex (BioRad, cat. 1421253), 200 µg/mL Proteinase K and 50 µg/mL RNase A (both from Qiagen, cat. 19131 and 19101) at room temperature for 5 minutes, followed by 56℃ for 4h. OCR values were normalized to relative gDNA contents quantified by the QIAxpert spectrophotometer (Qiagen).

2.9 Statistical analysis and figures

An unpaired parametric t-test was used to examine differences between patients and healthy controls for the Treg suppression assay, ELISA and Seahorse experiments. For non-normal distributed data, with few samples per group, a Mann-Whitney test was used. In all cases, a p-value less than 0.05 was considered statistically significant. All statistical analyses and figures were made using the GraphPad Prism 9.1.0 software (GraphPad Software).

3. Results

3.1 No difference in Treg frequencies between patients and controls

PBMCs from 15 patients and 15 healthy controls were analysed by flow cytometry to search for differences in the frequency of Tregs from cryopreserved PBMCs. These cells were gated as CD25+FOXP3+ cells within the CD4+ compartment (Supplementary Fig. 1A). In addition, within the Treg subset, we examined the frequency of HLA-DR, Helios, CTLA4, CD45RA and CD31 (Supplementary Fig. 1B). We did not find differences in the frequency of Tregs, based on the expression of CD25 and FOXP3, nor in the expression of the other aforementioned markers when comparing patients and healthy controls (Fig. 1A-F).

3.2 Treg isolation and *in vitro* expansion

Tregs from 24 patients and 27 healthy controls were isolated and expanded *in vitro* for 14 days. While the average number of Tregs obtained from patients and controls at day 0 was highly similar (265 000 (range 150 000-400 000) for patients and 267 222 (range 115 000-500 000) for controls, p=0.9314, Supplementary Fig. 2A), the number of cells post-expansion was significantly higher for patients ($15x10^6$ (range $2.0x10^6$-$38.0x10^6$) compared to healthy controls ($9.8x10^6$ (range $1.7x10^6$-$19.7x10^6$), p=0.0081, Supplementary Fig. 2B).

3.3 Treg suppression in patients and controls

To assess whether a functional impairment could be observed in the patients' Tregs, we used a flow cytometry based Treg suppression assay to measure the ability of *in vitro* expanded Tregs to inhibit Tresp proliferation. Tregs were distinguished from the Tresp population based on CellTrace Violet and CD25 (Supplementary Fig. 3). Four different Tresp-to-Treg ratios were initially tested in five patients and matched controls (Table 1, patient 2, 3, 10, 13 and 15), and we observed a significantly lower level of Treg suppression for the patient group at all ratios (Supplementary Fig. 4).

Based on these initial findings, we included a total of 20 patients and 20 healthy controls, and chose to examine Treg suppression with only a 1:1 and 4:1 ratio of Tresp and Tregs. Overall, patients' Tregs showed trends toward lower ability to inhibit Tresp proliferation at both 1:1 (mean patients 63.4%, mean controls 71.0%, p=0.1870, Fig. 2A) and 4:1 (mean patients 31.2%, mean controls 44.0%, p=0.0507, Fig. 2B), but without reaching statistical significance. We did the same analysis looking at the CD8+ responder cell population and observed the same trend (Supplementary Fig. 5). Adjusting for APS-2 /isolated PAI, disease duration, HLA risk type for PAI, presence of the AIRE p.R471C risk allele (rs74203920) (present in three of the included patients), age and sex did not reveal differences when comparing the groups, although this may be related to the few subjects tested.

Further, we examined the production of the Treg-specific cytokines IL-10, IL-35 and TGF-β in suppression assay supernatant. While IL-35 production was detected in most samples, both IL-10 and TGF-β were undetectable in the majority of samples, indicating low or non-existing production of these cytokines. Taken together, we did not find differences in cytokine production in Treg suppression cultures between patients and controls (Supplementary Fig. 6). Further, we looked at proliferation and expansion indices for Tresp:Treg ratios 1:0 and 1:1 for all samples, without revealing any differences when comparing patients and controls (Supplementary Fig. 7).

Next, we wanted to examine whether the slightly lower Treg suppression observed in patients was caused by Tresp resistance to Treg mediated suppression and therefore examined Treg suppression by using a pool of responder cells. The responder cell pool was prepared by mixing responder cells from five different healthy controls, followed by co-culture with Tregs from five patients (Table 1, #5-8 and 14) and five controls at ratios 1:1 and 4:1. We found that Tregs from patients and healthy controls were equally efficient in suppressing the proliferative response generated by the responder cell pool (Supplementary Fig. 8A and B). In an attempt to increase Treg suppression, we added 100 nm of the dual Cdk8/19 inhibitor AS2863619 (three patients and three controls, Table 1 #5, 6, 14) and 20 g/mL CD152 [CTLA4]:Fc (two patients, two controls, Table 1 #5, 14) and assessed Treg inhibition of Tresp

pool proliferation. However, no differences were observed with or without these *in vitro* treatments (Supplementary Fig. 8C and D).

3.4 Differential gene expression analysis in patients versus healthy controls

As the suppression assay pointed at trends towards lower Treg suppressive abilities in patients compared to healthy controls, we performed RNA sequencing on expanded Tregs to search for differences on the transcriptomic level. The cohort included 11 patients with APS-2, one patient with secondary adrenal insufficiency and four patients with isolated PAI (Table 1). The two groups, patients and controls, co-expressed 12444 genes, while 342 genes were found uniquely expressed in the patient cohort, and 161 genes were selectively found in the control group (Fig. 3A). In total, 1541 and 648 genes were up- and downregulated, respectively ($p<0.05$,$|log2FC| > 0$, Fig. 3B). Principal component analysis was used to evaluate intergroup relationships and although we observed differences between individual samples, the sample distribution was overall similar at the group level (Supplementary Fig. 9).

In sum, RNA sequencing indicated very small differences between patients and healthy controls, as only 60 genes were found significantly differentially expressed (adjusted $p<0.05$ and $|log2FC| > 0.1$). Among these, only six genes were significantly downregulated in patients, while 54 were significantly upregulated (Supplementary Table 2). Among the upregulated genes were several killer cell lectin like receptor C (KLRC) family genes, including *KLRC4* (log2FC 3.314), *KLRC3* (log2FC 3.286) and *KLRC2* (log2FC 2.984) and multiple mitochondrial genes (Supplementary Table 2). The most downregulated gene in the patient group was the monoamine oxidase B (*MOAB*) gene (log2FC -3.305), encoding an enzyme belonging to the flavin monoamine oxidase family, which localizes to the mitochondrial outer membrane (Fig. 2C). The six downregulated genes were loaded onto String to search for any functional links between them, however, none were identified (Fig. 4A). The analysis was repeated for the 54 upregulated genes and, as expected, functional links were found between several of the mitochondrial and *KLRC* genes, respectively (Fig. 4B), pointing to mitochondrial pathways and cell killer responses to be differentially active in patients' versus controls' Tregs.

3.5 Expanded Treg metabolic respiration in PAI was similar to healthy controls

Because we found mitochondrial genes to be differentially expressed in expanded Treg cells between patients and controls, we sought to assess whether this would manifest as differences in mitochondrial function. To this end, we measured cellular mitochondrial

respiration in expanded Tregs using a Seahorse assay in 11 patients and 11 controls. We observed large variations in the oxygen consumption rates within both groups (Fig. 5A) and consequently there were no significant differences between the groups, except from non-mitochondrial respiration, which was lower in the patients (Fig. 5B). Overall, Tregs from patients with PAI did on average not display significant mitochondrial dysfunction compared to healthy controls, however, there was a trend towards lower oxygen consumption rate amongst patients (Fig. 5).

4. Discussion

Tregs are crucial in the maintenance of self-tolerance and decreased frequencies and/or dysfunctional Tregs can lead to development of autoimmune disease. In this study, we have investigated Treg frequency in PBMC, while transcriptional changes, metabolic function and suppressive capacity were investigated in *in vitro* expanded Tregs in patients with adrenal insufficiency.

The aetiology of PAI is still not completely understood. However, both genetic susceptibility and environmental factors are thought to be involved [3]. PAI is one of three features characterizing APS-1, where patients acquire several autoimmune and ectodermal manifestations due to mutations in the *AIRE* gene [27]. AIRE has a major tutor role in induction and maintenance of immunological central tolerance, in addition to a more uncharacterised function in Treg generation [28, 29]. As a coding variant of *AIRE* (p.R471C) was recently found to be associated with PAI [24], there is a possibility of central tolerance mechanisms being involved in the pathogenesis of PAI, possibly implicating a loss of peripheral suppression of autoreactive cells. Of note, the p.R471C variant of *AIRE* have also been associated with other autoimmune conditions, such as type 1 diabetes and pernicious anaemia [30, 31]. Patients with two recessive *AIRE* mutations (and hence with a full APS-1 diagnosis) have been shown to have defective and reduced numbers of Tregs [21-23, 32]. In APS-2, however, Tregs were not found to be changed in numbers, but they were defective in their suppressive function [20]. Here, we did not find alterations in the Treg suppression when comparing PAI or APS-2 patients and healthy controls, nor did we see any pattern related to presence of the AIRE p.R471C risk allele.

Increased Treg cell death due to increased mitochondrial oxidative damage have been found in both humans and mice in autoimmune settings [33], which might be the cause of the increased expression of a set of mitochondrial genes in PAI patients. However, Treg numbers obtained on the day of isolation were similar between patients and controls, arguing against increased cell death. In addition, the mitochondria stress test, even though some patients had

a low response, in sum revealed similar oxygen consumption rate related to basal and maximal respiration, in addition to a proton leak and spare capacity in patients comparable to healthy controls. We furthermore observed that patient Tregs displayed a significantly higher proliferative capacity from day 0 to day 14 of the expansion period compared to healthy controls, also pointing against reduced viability of these cells and rather towards increased proliferative capabilities. This may indeed be an interesting observation, which points to accelerated turnover of Tregs, as have also been suggested for Tregs in autoimmune polyendocrine syndromes in the past [32]. This should be investigated further in new experimental setups.

We also found an upregulation of several *KLRC* genes in the patient cohort, which are normally expressed by natural killer (NK) T cells. Interestingly, other NK cell receptor genes, such as *KIR*, were found expressed in CD8+ T cells with regulatory properties in blood and inflamed tissue in patients with autoimmune disease [34]. Whether these markers are really expressed on expanded Tregs under certain conditions, or if we possibly have some "carry over cell subtypes" in the *in vitro* culture system which pollutes the Tregs is not known. How an upregulation of *KLCR* genes affect Treg function in our patients also require further investigations. Tregs are known to be able to kill cells with the granzyme and perforin pathways [35, 36], but we have not found any published information about *KLRC* expression in Tregs.

Due to the limited amount of Tregs in blood, we were not able to control how the expansion period affected or altered the composition of the Treg population, which is a major limitation of the study. However, analysis of cryopreserved Tregs obtained directly from blood did not indicate Treg disturbances in PAI patients, supporting the results from expanded Tregs. Another limitation is the focus on Tregs from whole blood and not from the relevant tissues from PAI patients, e.g. the adrenals. This rely on the fact that sampling of adrenal tissue biopsies is unfeasible. Our study only includes 24 patients, and even though the prevalence of autoimmune PAI in Norway is rare, indicated by 144 per million (2009) [37], and we thereby have included a high relative number of patients, the low number of patients still leads to underpowered analyses.

We conclude that patients with PAI, with or without the co-existence of other autoimmune conditions (APS-2), harbour Tregs with suppressive and metabolic capacities similar to those of healthy controls. However, upregulation of mitochondrial genes and accelerated expansion rate of polyclonal Tregs could still indicate altered functional properties, which could possibly contribute to the autoimmune phenotype seen in these patients.

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Table 1: Patient characteristics

Patient	Sex[a]	Disease duration [years][b]	Manifestations[c]	21OH[d]	PAI HLA risk[e]	APS-2	Flow cytometry	Suppression assay	RNA-seq	Seahorse
1	F	12	PAI, HypoT	+	-	x		x	x	
2	F	24	PAI, HypoT	+	Low	x		x	x	
3	M	29	PAI, HypoT	+	Intermediate	x	x	x		
4	F	13	PAI, HyperT, T1D, vitiligo	+	Intermediate	x		x		
5	F	6	PAI	+	-			x		x
6	F	23	PAI	+	Low		x	x		x
7	M	30	PAI, HypoT	+	Intermediate	x	x	x		x
8	F	3	PAI	+	-		x	x		x
9	M	39	PAI, HypoT, T1D	+	Low	x	x	x		x
10	F	27	PAI, HypoT, T1D	+	Low	x	x	x	x	
11	F	30	PAI, POI	+	Intermediate			x	x	x
12	M	23	PAI, HypoT, T1D, vitiligo	+	High	x	x	x	x	x
13	F	39	PAI, HypoT, HyperT	+	Low	x		x		
14	F	11	PAI, HypoT, T1D	+	Intermediate	x	x	x	x	x
15	F	7	PAI, HypoT, vitiligo	+	Intermediate	x	x	x	x	x
16	F	9	PAI, T1D	+	Intermediate	x	x	x	x	
17	F	55	PAI, HypoT	+	Intermediate	x	x	x	x	
18	F	11	PAI, HypoT	+	-	x	x	x	x	x
19	F	2	PAI	+	-			x	x	x
20	M	14	PAI, HypoT	+	Low	x	x	x	x	
21	M	9	PAI	+	High		x		x	
22	M	37	PAI, HyperT, T1D	+	Low	x	x		x	
23	F	-	PAI	-	-				x	
24	M	14	Adrenolectomy	-	-				x	

[a] F, female; M, male.

[b] -; not known.

[c] HyperT, hyperthyroidism; HypoT, hypothyroidism; PAI, primary adrenal insufficiency; POI, primary ovarian insufficiency; T1D, type 1 diabetes.

[d] +; positive, -; negative.

[e] -; not known. Risk categories based on Wolff et.al., European Journal of Endocrinology, https://doi.org/10.1530/EJE-20-1268, 2021 [5].

Figure legends

Fig. 1 Flow cytometric analysis of PBMCs from patients with PAI (N=20) and healthy controls (N=20). (A) Treg frequencies in a representative patient and healthy control. Expression of (B) HLA-DR, (C) Helios, (D) CTLA4, (E) CD45RA and (D) CD31) within the CD4+CD25+FOXP3+ population. All flow figures are shown for a representative patient and healthy control. The p value was calculated using an unpaired, parametric t-test. ns; non-significant.

Fig. 2 Treg suppression assay with expanded Tregs for patients with PAI (N=20) and healthy controls (N=20). CellTrace Violet labelled responder cells were co-cultured with Tregs, in the presence of anti-CD3/28 and IL2 at ratios (A) 1:1 for 20 patients and 20 controls, (B) 4:1 for five patients and five controls. The five patients used for optimizing the assay are also included in the figure and statistical analysis. FlowJo figures are shown for a representative patient and control for each ratio. *p<0.05 and **p<0.01, calculated using an unpaired, parametric t-test. ns; not significant.

Fig. 3 Distribution of differentially expressed genes in expanded Tregs for PAI patients (N=16) and healthy controls (N=16). (A) Venn diagram showing the number of genes uniquely expressed within each group, with the overlapping region showing the number of genes that are co-expressed in the two groups; patients and controls. (B) Volcano plot showing the overall distribution of differentially expressed genes for patient vs. control. Red dots represent upregulated genes and green dots represent downregulated genes. The dotted line indicates the threshold for differential gene screening criteria (p<0.05,$|log2FC| > 0.1$). Figures A and B were created by Novogene. (C) Significantly differentially expressed genes between patients and healthy controls presented in a volcano plot. Genes were set as significantly differentially expressed if the adjusted p value<0.05 and $|log2FC| > 0.1$.

Fig. 4 String pathway analysis of differentially expressed genes in expanded Tregs from PAI patients (N=16) and healthy controls (N=16). (A) Functional relationships between significantly downregulated genes in the patient cohort compared to healthy controls. (B) Functional relationships between significantly upregulated genes in the patient cohort compared to healthy controls. Requirements for differential gene expression were adjusted p value<0.05 and $|log2FC| > 0.1$.

Fig. 5 Measurement of mitochondrial respiration in expanded Tregs from patients with PAI (N=11) and healthy controls (N=11). (A) Cellular oxygen consumption rate (OCR) measured by a Seahorse mitochondrial stress test. After determining basal respiration, inhibitors of mitochondrial respiration were added as followed: Oligo (oligomycin) to inhibit ATP synthase, CCCP (carbonyl cyanide m-chlorophenylhydrazone) to uncouple oxygen consumption from production of ATP, AA (antimycin A) to block mitochondrial complex III and rotenone to block mitochondrial complex I. (B) The addition and effect of inhibitors made it possible to calculate non-mitochondrial, basal and maximal respiration, ATP production, spare capacity and proton leak, as shown with individual values and mean±SEM. *$p<0.05$, calculated with an unpaired parametric t-test (maximal respiration, ATP production, spare capacity, non-mitochondrial respiration, proton leak) and a Mann-Whitney test for non-normal distributed data (basal respiration). ns; non-significant.

Fig. 1

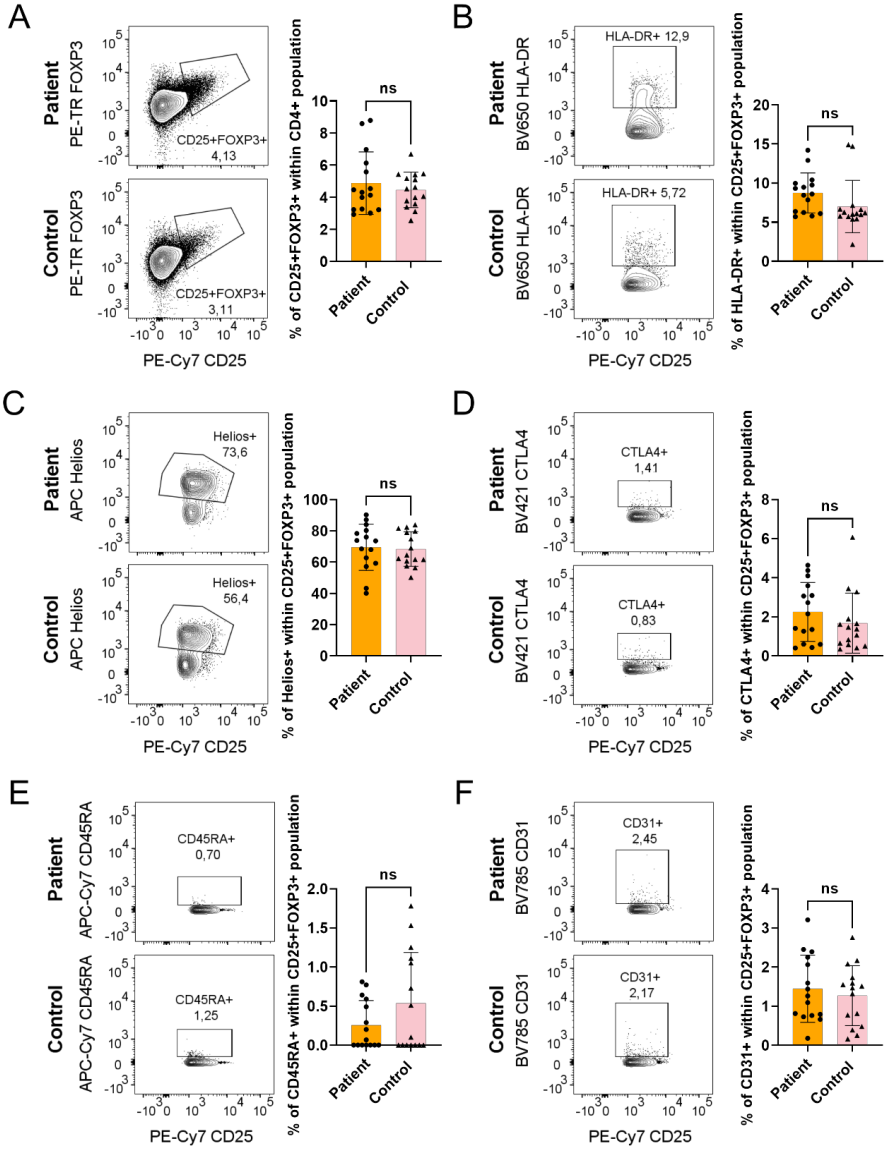

A
Patient
PE-TR FOXP3
CD25+FOXP3+
4,13
Control
PE-TR FOXP3
CD25+FOXP3+
3,11
PE-Cy7 CD25
% of CD25+FOXP3+ within CD4+ population
ns
Patient
Control
B
Patient
BV650 HLA-DR
HLA-DR+ 12,9
Control
BV650 HLA-DR
HLA-DR+ 5,72
PE-Cy7 CD25
% of HLA-DR+ within CD25+FOXP3+ population
ns
Patient
Control
C
Patient
APC Helios
Helios+
73,6
Control
APC Helios
Helios+
56,4
PE-Cy7 CD25
% of Helios+ within CD25+FOXP3+ population
ns
Patient
Control
D
Patient
BV421 CTLA4
CTLA4+
1,41
Control
BV421 CTLA4
CTLA4+
0,83
PE-Cy7 CD25
% of CTLA4+ within CD25+FOXP3+ population
ns
Patient
Control
E
Patient
APC-Cy7 CD45RA
CD45RA+
0,70
Control
APC-Cy7 CD45RA
CD45RA+
1,25
PE-Cy7 CD25
% of CD45RA+ within CD25+FOXP3+ population
ns
Patient
Control
F
Patient
BV785 CD31
CD31+
2,45
Control
BV785 CD31
CD31+
2,17
PE-Cy7 CD25
% of CD31+ within CD25+FOXP3+ population
ns
Patient
Control

Fig. 2

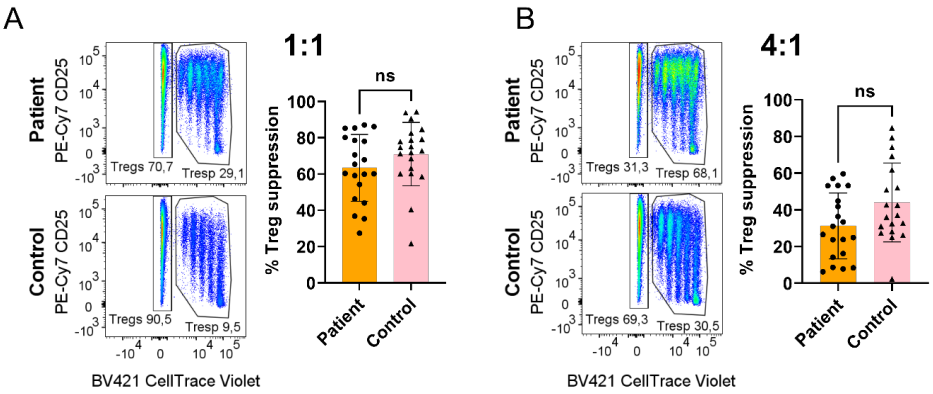

A
Patient
PE-Cy7 CD25
Tregs 70,7
Tresp 29,1
Control
PE-Cy7 CD25
Tregs 90,5
Tresp 9,5
BV421 CellTrace Violet
1:1
ns
% Treg suppression
Patient
Control
B
Patient
PE-Cy7 CD25
Tregs 31,3
Tresp 68,1
Control
PE-Cy7 CD25
Tregs 69,3
Tresp 30,5
BV421 CellTrace Violet
4:1
ns
% Treg suppression
Patient
Control

Fig. 3

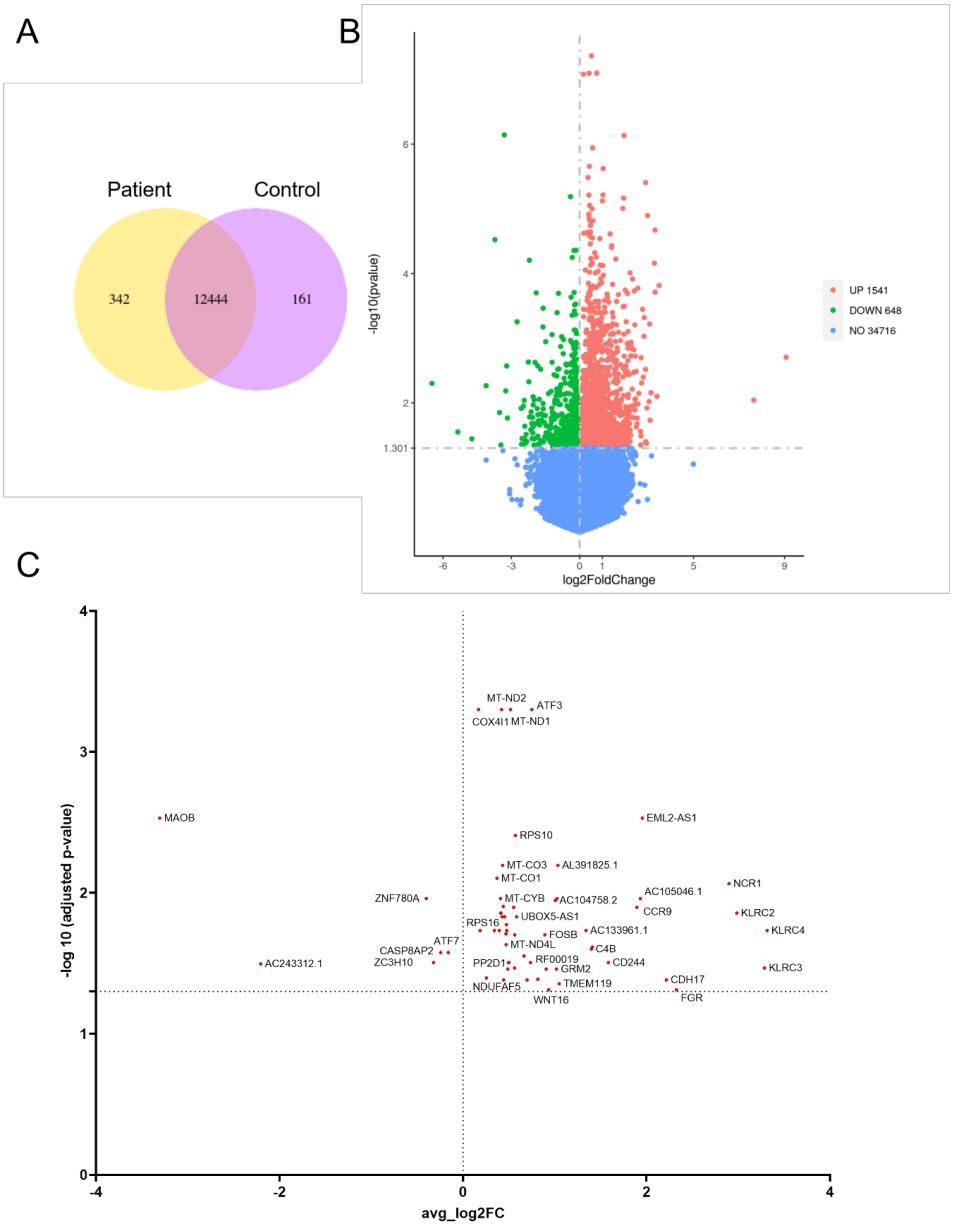

A
Patient
Control
342
12444
161
B
-log10(pvalue)
log2FoldChange
UP 1541
DOWN 648
NO 34716
C
-log 10 (adjusted p-value)
avg_log2FC
MT-ND2
ATF3
COX4I1
MT-ND1
MAOB
EML2-AS1
RPS10
MT-CO3
AL391825.1
MT-CO1
NCR1
AC105046.1
ZNF780A
MT-CYB
AC104758.2
CCR9
KLRC2
UBOX5-AS1
RPS16
AC133961.1
KLRC4
FOSB
ATF7
MT-ND4L
C4B
CASP8AP2
AC243312.1
ZC3H10
RF00019
CD244
KLRC3
PP2D1
GRM2
CDH17
NDUFAF5
TMEM119
FGR
WNT16

Fig. 4

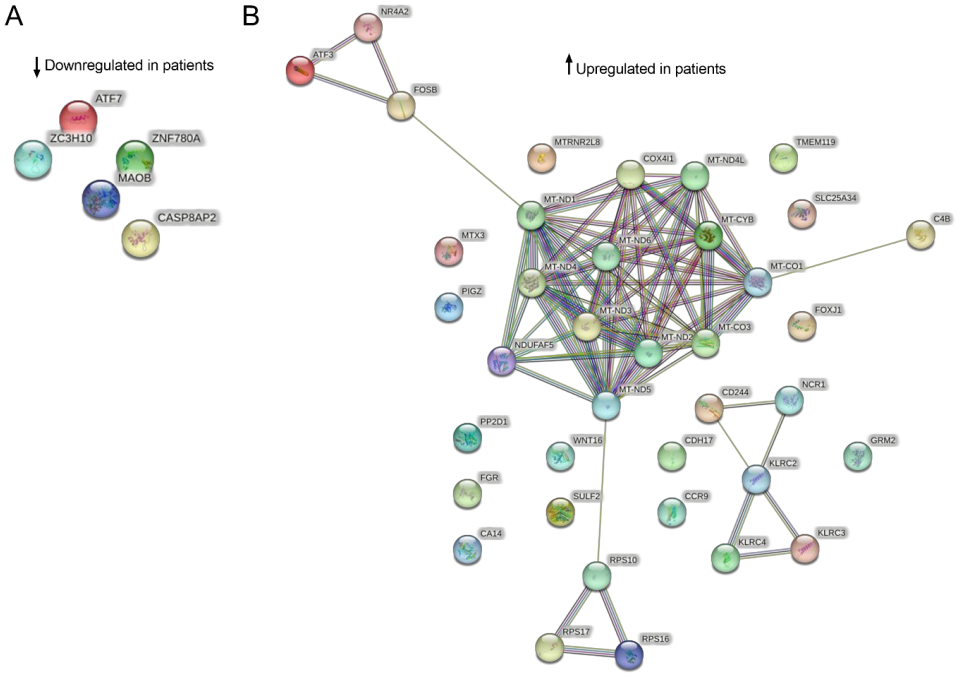
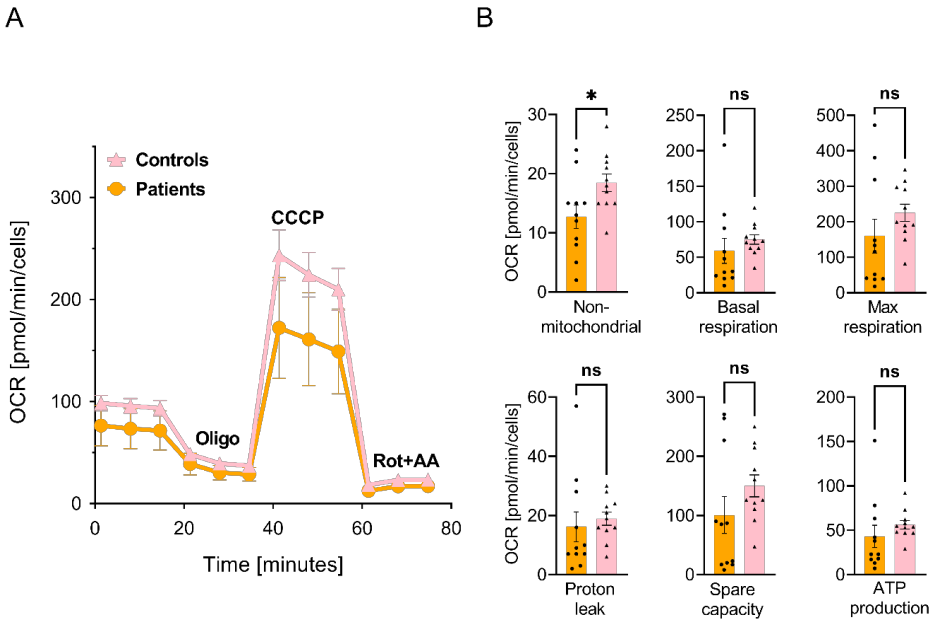

A
Downregulated in patients
ATF7
ZC3H10
ZNF780A
MAOB
CASP8AP2
B
Upregulated in patients
NR4A2
ATF3
FOSB
MTRNR2L8
COX4I1
MT-ND4L
TMEM119
MT-ND1
SLC25A34
MT-CYB
C4B
MTX3
MT-ND6
MT-ND4
MT-CO1
PIGZ
MT-ND3
FOXJ1
MT-CO3
MT-ND2
NDUFAF5
MT-ND5
CD244
NCR1
PP2D1
WNT16
CDH17
GRM2
KLRC2
FGR
SULF2
CCR9
CA14
KLRC4
KLRC3
RPS10
RPS17
RPS16

Fig. 5


A
B
Controls
Patients
CCCP
Oligo
Rot+AA
OCR [pmol/min/cells]
Time [minutes]
*
ns
Non-mitochondrial
Basal respiration
Max respiration
Proton leak
Spare capacity
ATP production

Regulatory T cells in autoimmune primary adrenal insufficiency

Supplementary material

SUPPLEMENTARY TABLES

Supplementary Table 1: Flow cytometry panel.

Marker	Fluorochrome	Clone	Cat. number	Dilution
CD4 (BioLegend)	PerCP-Cy5.5	RPA-T4	300530	1:100
CD25 (BD)	PE-Cy7	2A3	335824	1:40
CD8 (BD)	PE-Cy5	RPA-T8	555368	1:100
CD8a (BioLegend)	APC	HIT8a	300912	1:100
CellTrace Violet Cell Proliferation Kit (Invitrogen)	BV421	-	C34557	-
CD3 (BD)	V500	UCHT1	561416	1:20
CTLA4 (BioLegend)	BV421	BNI3	369606	1:20
CD45RA (BD)	APC-H7	HI100	560674	1:80
CD31 (BD)	BV785	L133.1	744757	1:160
HLA-DR (BD)	BV650	G46-6	564231	1:100
Streptavidin (BioLegend)	FITC	-	405202	1:500
Biotin TCR γ/δ (BioLegend)	-	B1	331206	1:500
Biotin CD1c (BioLegend)	-	L161	331504	1:500
Biotin CD14 (BioLegend)	-	63D3	367106	1:500
FOXP3 (BD)	PE-CF594	236A/E7	563955	1:10
HELIOS (BioLegend)	APC	22F6	137222	1:40
Live/dead Fixable Yellow Dead cell Stain kit (Invitrogen)	Q-dot585/BV570	-	L34959	1:1000

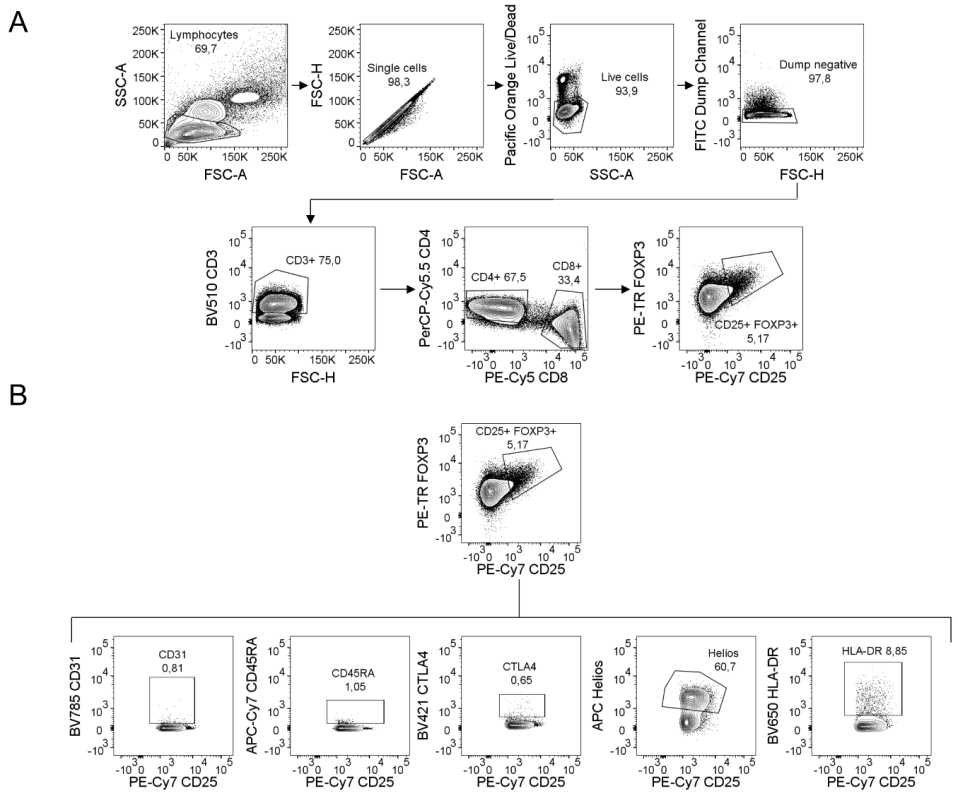
Supplementary Table 2: Expression of significantly up- or downregulated genes in PAI patient Tregs (N=16) compared to healthy control Tregs (N=16) (adjusted p<0.05, $|log2FC|$>0.1).

Gene name	ENSG	log2FoldChange	p-value	p-value adjusted
MAOB	ENSG00000069535	(-)3,305360	7,200055E-07	0,002948
AC243312.1	ENSG00000261866	(-)2,204717	6,241214E-05	0,031901
ZNF780A	ENSG00000197782	(-)0,401077	6,492466E-06	0,010985
ZC3H10	ENSG00000135482	(-)0,321421	5,649236E-05	0,031257
CASP8AP2	ENSG00000118412	(-)0,243528	4,417967E-05	0,026533
ATF7	ENSG00000170653	(-)0,160917	4,373101E-05	0,026533
KLRC4	ENSG00000183542	3,314105	2,126323E-05	0,018511
KLRC3	ENSG00000205810	3,285654	6,950647E-05	0,034077
KLRC2	ENSG00000205809	2,983797	1,274401E-05	0,013916
NCR1	ENSG00000189430	2,900063	3,939767E-06	0,008604
FGR	ENSG00000000938	2,326428	1,216572E-04	0,048710
CDH17	ENSG00000079112	2,216660	9,786782E-05	0,041392
EML2-AS1	ENSG00000267757	1,954111	7,363874E-07	0,002948
AC105046.1	ENSG00000261026	1,932893	6,858832E-06	0,010985
CCR9	ENSG00000173585	1,894791	9,881420E-06	0,012660
CD244	ENSG00000122223	1,584457	5,985205E-05	0,031257
FOXJ1	ENSG00000129654	1,410116	3,729747E-05	0,024216
C4B	ENSG00000224389	1,399587	3,964433E-05	0,025063
AC133961.1	ENSG00000251009	1,340851	2,442667E-05	0,018511
TMEM119	ENSG00000183160	1,048845	1,066236E-04	0,044162
AL391825.1	ENSG00000229808	1,034632	2,389183E-06	0,006377
NR4A2	ENSG00000153234	1,025526	6,104834E-06	0,010985
GRM2	ENSG00000164082	1,018331	7,525672E-05	0,034767
AC104758.2	ENSG00000259792	1,004122	7,536786E-06	0,011316
WNT16	ENSG00000002745	0,933428	1,203942E-04	0,048710
SNORD59A	ENSG00000207031	0,906866	7,412478E-05	0,034767
FOSB	ENSG00000125740	0,891473	2,892862E-05	0,019856
SULF2	ENSG00000196562	0,815460	9,211153E-05	0,040978
ATF3	ENSG00000162772	0,750061	8,000158E-08	0,000500
RF00019	ENSG00000252367	0,735540	5,590020E-05	0,031257
CA14	ENSG00000118298	0,697153	9,821298E-05	0,041392
MIRLET7BHG	ENSG00000197182	0,665729	4,781383E-05	0,028015
UBOX5-AS1	ENSG00000235958	0,585261	1,536314E-05	0,014763
RPS10	ENSG00000124614	0,571529	1,142849E-06	0,003922
AC093525.8	ENSG00000279520	0,564164	2,826691E-05	0,019856
MT-RNR2	ENSG00000210082	0,561659	6,903513E-05	0,034077
LINC00672	ENSG00000263874	0,552218	1,001315E-05	0,012660
MT-ND1	ENSG00000198888	0,517656	4,319262E-08	0,000500
AC092045.1	ENSG00000239557	0,499930	5,946322E-05	0,031257

PP2D1	ENSG00000183977	0,495777	5,842107E-05	0,031257
NDUFV2-AS1	ENSG00000266053	0,488278	7,415353E-05	0,034767
SLC25A34	ENSG00000162461	0,476666	2,268679E-05	0,018511
PIGZ	ENSG00000119227	0,473174	1,818663E-05	0,016804
MT-ND4L	ENSG00000212907	0,470581	3,492576E-05	0,023306
MT-ND3	ENSG00000198840	0,468169	2,686774E-05	0,019559
AP000254.1	ENSG00000273271	0,453353	1,519272E-05	0,014763
LINC00641	ENSG00000258441	0,442086	9,527355E-05	0,041392
MT-ND4	ENSG00000198886	0,438090	8,836679E-06	0,012487
MT-CO3	ENSG00000198938	0,431413	2,203612E-06	0,006377
MTCO1P12	ENSG00000237973	0,425719	1,435861E-05	0,014763
MT-ND2	ENSG00000198763	0,421032	8,043170E-08	0,000500
MT-ND6	ENSG00000198695	0,413419	1,204939E-05	0,013916
MT-ND5	ENSG00000198786	0,409363	1,243493E-05	0,013916
MT-CYB	ENSG00000198727	0,408158	6,134239E-06	0,010985
RPS17	ENSG00000182774	0,394488	2,465825E-05	0,018511
MT-CO1	ENSG00000198804	0,369892	3,280604E-06	0,007881
MTX3	ENSG00000177034	0,342266	2,319064E-05	0,018511
NDUFAF5	ENSG00000101247	0,254139	8,861146E-05	0,040164
RPS16	ENSG00000105193	0,186165	2,385673E-05	0,018511
COX4I1	ENSG00000131143	0,168047	8,323283E-08	0,000500

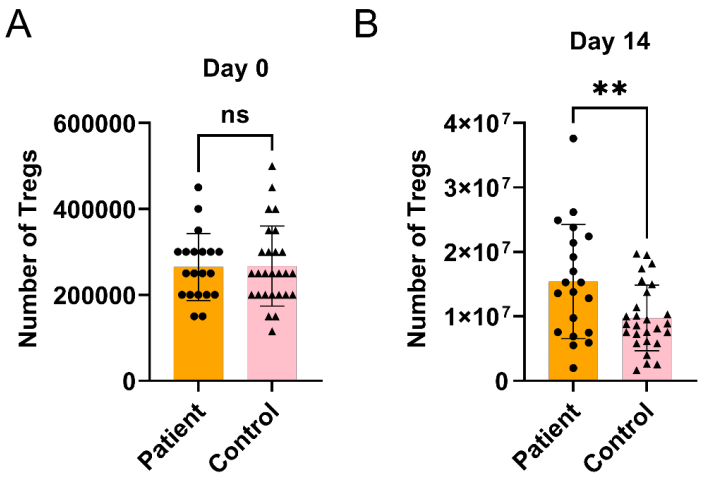
SUPPLEMENTARY FIGURES

Supplementary Figure 1. Gating strategy for PBMC flow cytometric analysis.



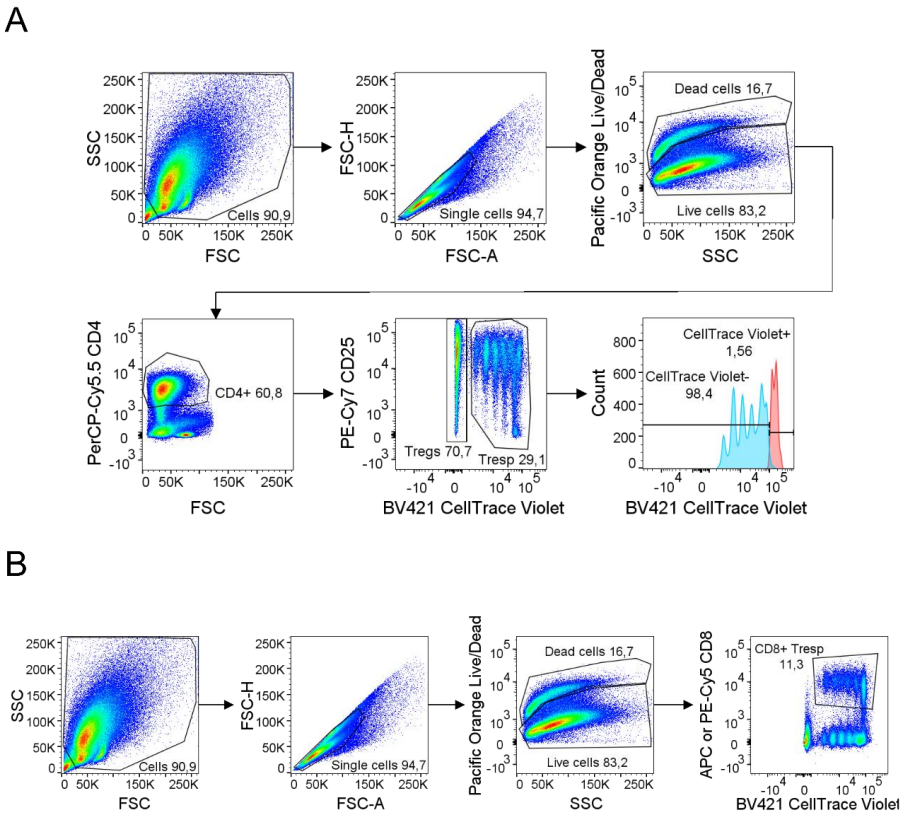
(A) General gating strategy for the analysis of PBMCs by flow cytometry. Tregs were gated as the CD4+CD25+FOXP3+ population. (B) Within the CD4+CD25+FOXP3+, we gated for markers CD31, CD45RA, CTLA4, Helios and HLA-DR:

Supplementary Figure 2. Treg numbers at the day of isolation (day 0) and after expansion (day 14) for PAI patients (N=24) and healthy controls (N=27).



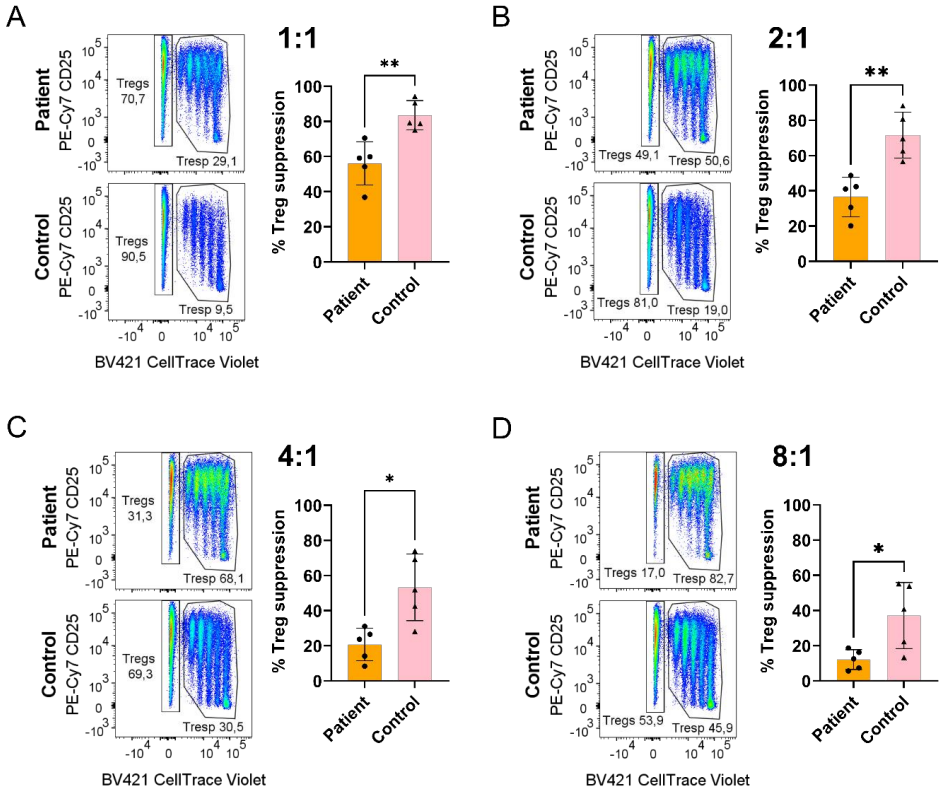
(A) Treg numbers obtained at the day of isolation (day 0) and used for in vitro Treg expansion for patients and controls. (B) Treg count after expansion for 14 days for patients and controls. All cells were isolated according to the same protocol, although on different days, but patients and healthy controls were in general isolated on the same day. **p<0.01, calculated using an unpaired, parametric t-test.

Supplementary Figure 3. Treg suppression assay gating strategy.



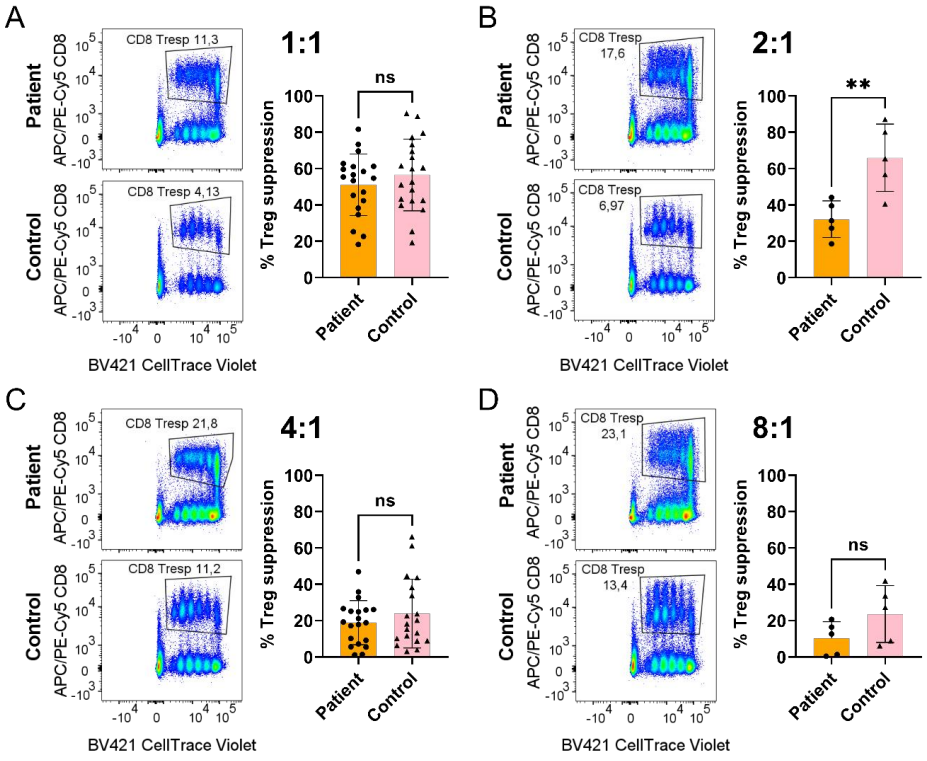
Gating strategy shown for a representative patient sample. (A) Samples were gated as single, live cells and further as CD4 positive cells. The expression of CD25 and CellTrace Violet were used to differentiate Tregs and Tresp cells, as only the Tresp fraction were stained with the proliferation dye. To ensure that the Tresp gate contained only dividing cells, the expression of CellTrace Violet versus count at day 0 were examined. (B) CD8 positive responder cells were gated from the live cell population, and was separated from the CD4 positive responder cells by using the CD8 vs. CellTrace Violet channels.

Supplementary Figure 4. Preliminary Treg suppression of CD4+ responder cell proliferation for PAI patients (N=5) and healthy controls (N=5) to optimize Tresp-to-Treg ratios.



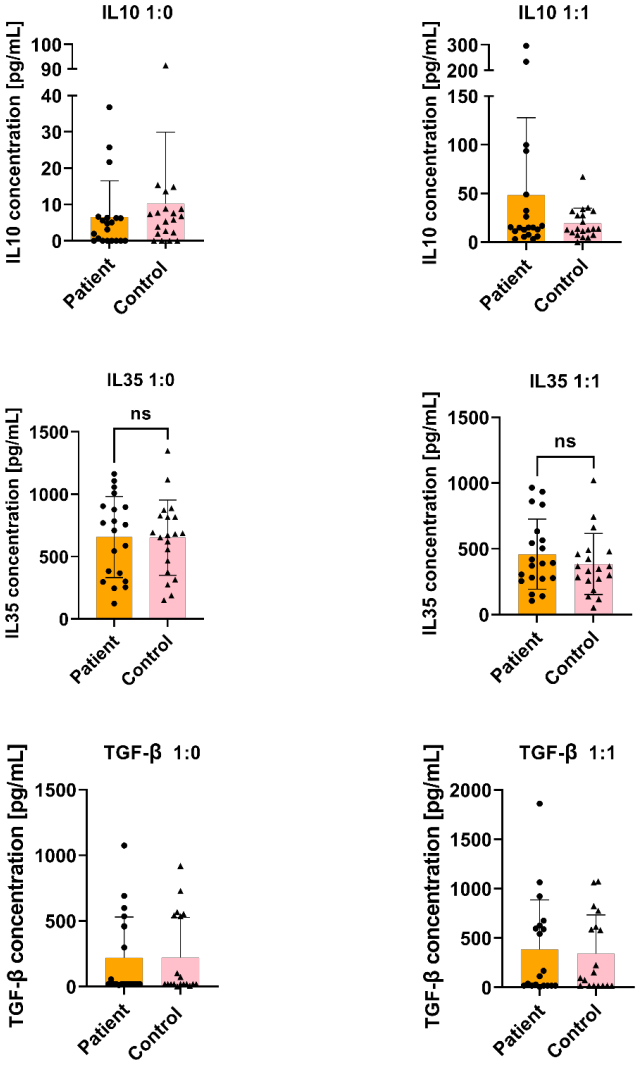
CellTrace Violet labelled responder cells were co-cultured with expanded Tregs, in the presence of anti-CD3/28 and IL2 at ratios (A) 1:1 for five patients and five controls, (B) 2:1 for five patients and five controls, (C) 4:1 for five patients and five controls and (D) 8:1 for five patients and five controls. FlowJo figures are shown for a representative patient and control for each ratio. *p<0.05 and **p<0.01, calculated using a Mann-Whitney test.

Supplementary Figure 5. Treg suppression of CD8+ responder cell proliferation for PAI patients (N=20) and healthy controls (N=20).



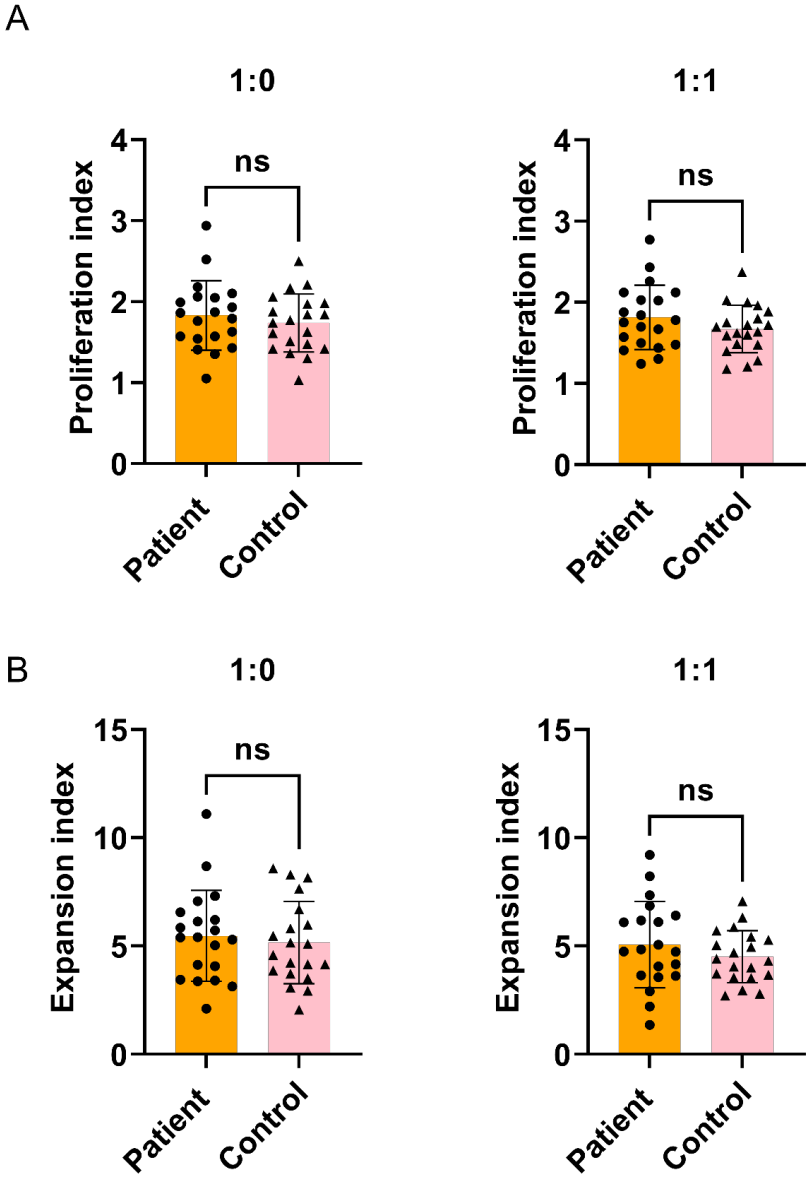
CellTrace Violet labelled CD8+ responder cells were co-cultured with expanded Tregs at different ratios for five days in the presence of anti-CD3/28 and IL2. (A) Tresp:Treg 1:1, (B) Tresp:Treg 2:1, (C) Tresp:Treg 4:1 and (D) Tresp:Treg 8:1. Flow figures are shown for a representative patient and control for each ratio. 1:1 and 4:1 includes 20 patients and controls, while 2:1 and 8:1 includes five patients and controls. BV421 CellTrace Violet vs. PE-Cy5 CD8 was used to separate CD8 from CD4 responder cells and Tregs. P-values were calculated with an unpaired, parametric t-test,(1:1 and 4:1) and a Mann-Whitney test (2:1 and 8:1) and $p<0.05$ was set as the threshold for significance. **p<0.01. ns; not significant.

Supplementary Figure 6. Production of Treg-specific cytokines in suppression assay supernatant for PAI patients (N=20) and healthy controls (N=20).



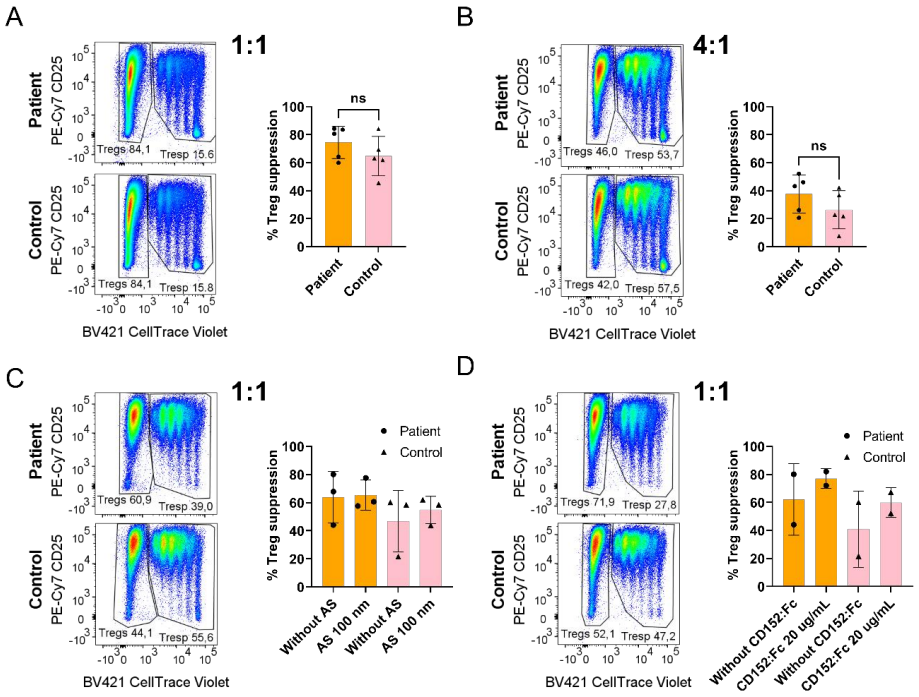
For IL-10, 7 patients and 4 controls had non-detectable concentrations for the Tresp:Treg 1:0 samples, while 1 control had non-detectable concentrations at 1:1. These samples were given the minimum detectable dose concentration of 0.03 pg/mL. For IL-35, all samples had detectable concentrations at 1:0, while 20 patients and 19 controls had detectable concentrations at 1:1. For TGF-β, 10 patients and 10 controls had non-detectable concentrations at 1:0, while 7 patients and 7 controls had non-detectable concentrations at 1:1. These samples were given the minimum detectable dose concentration of 18 pg/mL. No statistical analysis was performed on these samples. P-values were determined by an unpaired, parametric t-test. A p-value<0.05 was considered significant. ns; not significant.

Supplementary Figure 7. Suppression assay proliferation and expansion indices for PAI patients (N=20) and healthy controls (N=20).



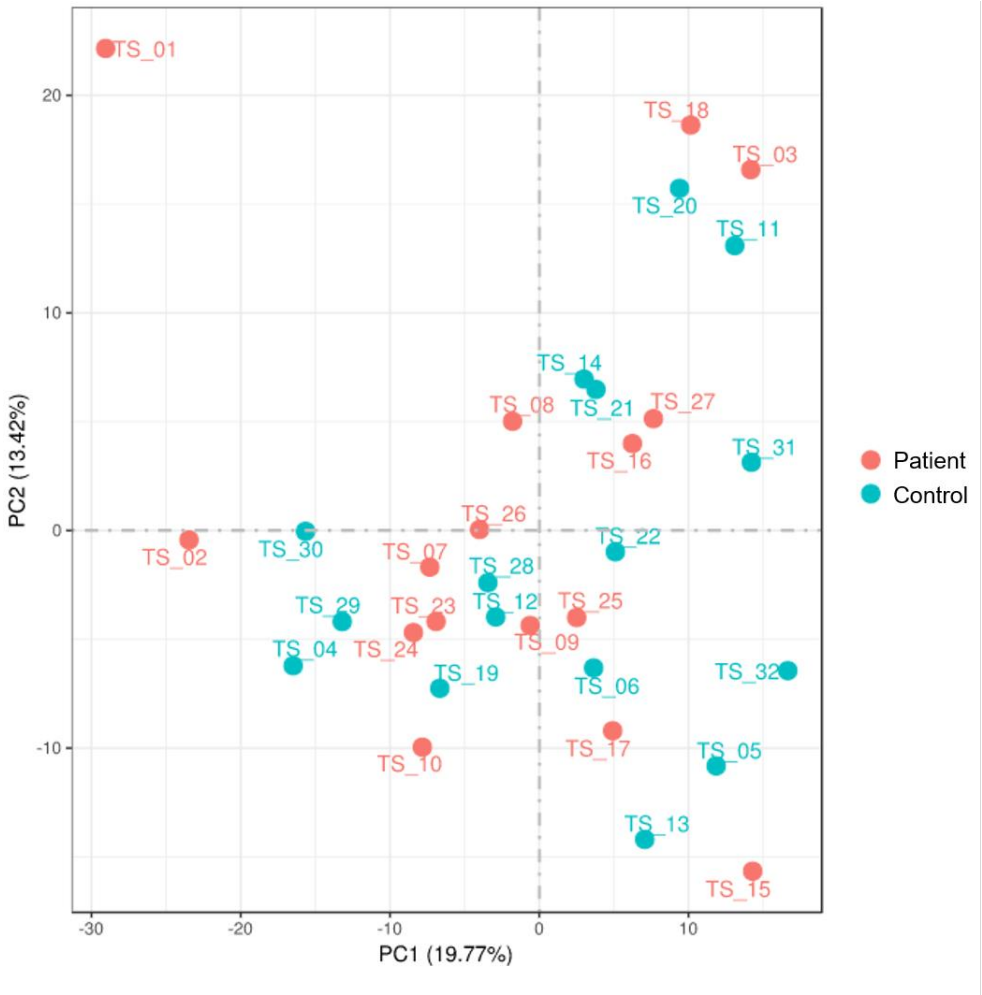
Analysis of proliferation and expansion indices for Tresp:Treg ratios 1:0 and 1:1 did not show any differences between patients and controls. P-values were calculated using an unpaired, parametric t-test, and $p<0.05$ was considered significant. ns; not significant.

Supplementary Figure 8. Treg suppression assay with pool of responder cells for patients (N=5) and healthy controls (N=5) and with addition of two drugs (AS2863619 and CD152 [CTLA4]:Fc).



Responder cells from a pool of PBMCs were co-cultured with expanded patient (N=5) and control (N=5) Tregs at ratios (A) 1:1 and (B) 2:1 in the presence of anti-CD3/28 and IL2. (C) 100 nm AS2863619 (D) 20 ug/mL CTLA4-Ig was added to the responder cell pool and Treg co-culture, with the same culturing conditions as for the regular suppression assay. No statistical analysis was performed for (C) and (D) due to low sample numbers. Flow figures are shown for a representative patient and control for each ratio. A p-value was calculated using a Mann-Whitney test. ns; not significant. AS; AS2863619.

Supplementary Figure 9. Principal component analysis for patients (N=16) and controls (N=16).



Principal component (PC) analysis was used to evaluate intergroup differences. Red circles show patient samples and green circles denote control samples. The figure was created by Novogene.

Paper III

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Screening patients with autoimmune endocrine disorders for cytokine autoantibodies reveals monogenic immune deficiencies

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ABSTRACT

Background: Autoantibodies against type I interferons (IFN) alpha (α) and omega (ω), and interleukins (IL) 17 and 22 are a hallmark of autoimmune polyendocrine syndrome type 1 (APS-1), caused by mutations in the autoimmune regulator (*AIRE*) gene. Such antibodies are also seen in a number of monogenic immunodeficiencies.
Objectives: To determine whether screening for cytokine autoantibodies (anti–IFN–ω and anti-IL22) can be used to identify patients with monogenic immune disorders.
Methods: A novel ELISA assay was employed to measure IL22 autoantibodies in 675 patients with autoimmune primary adrenal insufficiency (PAI) and a radio immune assay (RIA) was used to measure autoantibodies against IFN-ω in 1778 patients with a variety of endocrine diseases, mostly of autoimmune aetiology. Positive cases were sequenced for all coding exons of the *AIRE* gene. If no *AIRE* mutations were found, we applied next generation sequencing (NGS) to search for mutations in immune related genes.
Results: We identified 29 patients with autoantibodies against IFN-ω and/or IL22. Of these, four new APS-1 cases with disease-causing variants in *AIRE* were found. In addition, we identified two patients with pathogenic heterozygous variants in *CTLA4* and *NFKB2*, respectively. Nine rare variants in other immune genes were identified in six patients, although further studies are needed to determine their disease-causing potential.
Conclusion: Screening of cytokine autoantibodies can efficiently identify patients with previously unknown monogenic and possible oligogenic causes of autoimmune and immune deficiency diseases. This information is crucial for providing personalised treatment and follow-up of patients and their relatives.

1. Introduction

Organ-specific autoimmune disorders, such as type 1 diabetes, Hashimoto thyroiditis, Grave's disease and autoimmune primary adrenal and ovarian insufficiencies, collectively cause significant morbidity and mortality [1–3]. The aetiology is thought to be a combination of unfavourable genetic variants in immune genes, environmental triggers and stochastic events following T- and B-cell receptor rearrangement. *HLA*-genes are major contributors to the risk of endocrine autoimmune conditions, exemplified by the strong association with specific *HLA*-alleles in type 1 diabetes and autoimmune primary adrenal insufficiency (PAI) [4,5]. Of note, several joint modifier loci have also been discovered, including *CTLA4*, *PTPN22* and *BACH2* [4,6–8]. Although most autoimmune disorders are considered polygenic, monogenic forms exist, e.g. autoimmune polyendocrine syndrome type 1 (APS-1) caused by *AIRE* mutations [9–11] and immune dysregulation polyendocrinopathy,

Abbreviations: APS-I, Autoimmune polyendocrine syndrome type 1; IFN, interferon; AIRE, Autoimmune regulator.

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enteropathy, x-linked (IPEX) syndrome caused by *FOXP3* mutations [12]. Identifying patients with monogenic causes is important in order to provide targeted treatment and personalised follow-up aimed at preventing complications.

For more than a decade it has been known that patients with pathogenic *AIRE* mutations are hallmarked by neutralizing autoantibodies against type I interferons (IFN), in particular IFN-ω, and against cytokines of the Th17-effector pathway, including interleukin (IL) 22 [13–15]. Intriguingly, these are present in almost all patients reported [16–18], and can be detected years before disease onset [19,20]. Neutralizing autoantibodies against IFN-α/ω have also been described in patients with mutations in *FOXP3* [21], *RAG 1* and *2* [22], *NFKB2* [23,24] and *IKZF2* [25]. Low-titre autoantibodies directed against IFNs can also occur in patients with systemic autoimmunity, such as systemic lupus erythematosus (SLE) [26–28], and even in a fraction of the general population, with frequencies increasing with age [29]. Anti-IL22 is more restricted to APS-1, where their neutralizing property together with impaired IL22 and IL23 secretion upon *C.albicans* stimulation have been connected with chronic candidiasis [13–15,30]. IL22 has further been reported to be involved in the development and pathogenesis of several other autoimmune diseases, including rheumatoid arthritis, SLE and Sjøgren's syndrome [31].

Several of the aforementioned endocrine autoimmune conditions are commonly aggregated in the same individuals or in families, and patients with one condition have an increased risk of acquiring additional organ-specific autoimmune diseases. Some of the monogenic disorders in this category also have increased risk of infections and malignancies, which argues for close and personalised follow-up. However, since endocrine disorders collectively are common, it is time-consuming and costly to check all individuals by DNA sequencing. Since cytokine autoantibodies have been described in a number of immunodeficiencies [32,33], we hypothesised that screening a registry of autoimmune endocrine diseases could be an efficient way of identifying patients with monogenic endocrinopathies and immunodeficiencies. In addition to a routine assay for measuring autoantibodies against IFN-ω, we aimed at establishing a high throughput-screening tool for anti-IL22 autoantibodies, capable of dealing with pooling of serum samples. To further test our hypothesis, IFN-ω and/or IL22 autoantibody positive cases were sequenced for all coding exons of the *AIRE* gene to verify or exclude APS-1. If no *AIRE*-mutations were found, we performed next-generation sequencing (NGS) to search for mutations in immune genes that could explain the clinical and serological features. Certainly or possibly disease-causing variants were found in several such genes in patients with endocrinopathies.

2. Methods

2.1. Patients and controls

We have collected samples from patients with endocrine autoimmune disorders in the Norwegian Registry for Organ-Specific Autoimmune Disorders (ROAS) since 1996. The registry contains mostly samples from patients with autoimmune PAI and polyendocrine syndromes, but also small cohorts with other endocrine diagnoses [34], including non-autoimmune PAI, hypoparathyroidism, primary ovarian insufficiency (POI), autoimmune thyroid disease, type 1 diabetes, celiac disease and vitiligo (Fig. 1A). All patients with endocrine deficiencies included in ROAS have since 2016 been routinely screened for IFN-ω antibodies at inclusion. We have also screened the majority of patients included since 1996 retrospectively (N = 1778). Known APS-1 cases (with *AIRE* mutations) have also been screened, and all of the included APS-1 patients were positive for anti–IFN–ω antibodies. The APS-1 patients have been reported on previously [35]. In addition, 675 autoimmune PAI (419 female, 256 male, age range 12–109, mean age 60.4) patients and 49 APS-1 (23 female, 26 male, age range 5–77, mean age 49.5) patients consecutively enrolled in the ROAS registry were screened for autoantibodies against IL22. Patients found to have IFN-ω autoantibodies, who did not have APS-1, were included in the anti-IL22 screening. Fig. 1A shows a flow chart of the enrolled patients and Fig. 1B the screening pipeline.

2.2. Ethics

All subjects were included in the Norwegian registry and biobank for organ-specific autoimmune disorders (ROAS), and they all gave informed consent for participating (Biobank approval number from the Regional Committee for Medical and Health Research Ethics 2013/1504). Sera from healthy donors were obtained from the Blood Bank at Haukeland University Hospital (Bergen, Norway). This study is approved by the Regional Committee for Medical and Health Research Ethics (2009/2555 and 2018/1417), and was conducted in accordance with the Declaration of Helsinki.

2.3. Autoantibody screening using a radio immune assay (RIA)

Autoantibodies against IFN-ω and IFN-α_2 were assayed by routine radioimmunoassays (RIA), as previously described by Oftedal et al. [36] and Hapnes et al. [37], respectively. Autoantibodies against IL22, IL17F and the endocrine/APS-1 specific antigens 21-hydroxylase (21OH), side-chain cleavage enzyme (SSC), 17-hydroxylase (17OH), glutamic acid decarboxylase (GAD), tryptophan hydroxylase (TPH), tyrosine hydroxylase (TH), NACHT leucine-rich-repeat protein 5 (NALP5) and aromatic I-amino acid decarboxylase (AADC) were also measured in cytokine antibody-positive patients by routine radioimmunoassays, as previously described [36,38]. For all antigens, an index-value is generated from the raw data of each individual, which is based on the sample result compared to a standard negative and positive sample (*1000). The threshold for positivity was set as the mean of healthy controls plus three standard deviations (at least 100 healthy controls were used to calculate the threshold, e.g. N = 150 for IFN-ω).

2.4. Establishment of an anti-IL22 screening tool using an enzyme-linked immunosorbent assay (ELISA)

A 96-well flat-bottomed (F96 Maxisorp NUNC-immuno plate, Thermo Fisher Scientific, Waltham, MA) plate was coated with 2 μg/mL recombinant human IL22 protein (Bio-Techne, Minneapolis, MN) in phosphate buffered saline (PBS) and incubated at 4°*C* overnight. The wells were blocked with PBS, 3% BSA and 0.1% Tween-20 (block buffer), at room temperature, on a plate shaker, for 1 h. Subsequently, 3 washes with PBS-Tween-20 (0.1%, wash buffer) were performed. Next, patient serum (1:100 in block buffer), was added to the plate, followed by incubation for 1 h. Washing was repeated. The secondary antibody, alkaline phosphatase-conjugated anti-human IgG (1:2500 in block buffer) (Sigma-Aldrich, Saint Louis, MO), was then added, before a new incubation of 1 h.

Washing was repeated and SigmaFast p-Nitrophenyl phosphate (Sigma-Aldrich, Saint Louis, MO) was added according to manufacturer's instructions. Absorbance was read at A405 nm after 30 min using a SpectraMax plus 384 Microplate Spectrophotometer and the SoftMax Pro 7.1 software (Molecular Devices, San Jose, CA).

To evaluate the usefulness of the newly developed ELISA, sera from patients with APS-1 (N = 49) and known anti-IL22 indices from RIAs were first ran and compared to 90 healthy blood donors, to our knowledge, without endocrine disorders. To efficiently screen 675 PAI patients, two consecutive screenings of 360 and 315 patients in pools of five, were then performed. Serum from two APS-1 patients were used as positive controls and repeated on all plates. Samples were considered positive if the signal was greater than the mean plus three standard deviation signal generated by sera from 12 different healthy controls added on each plate. Hence, each plate has its own threshold for positivity. In all ELISA assays, two wells containing only PBS were included

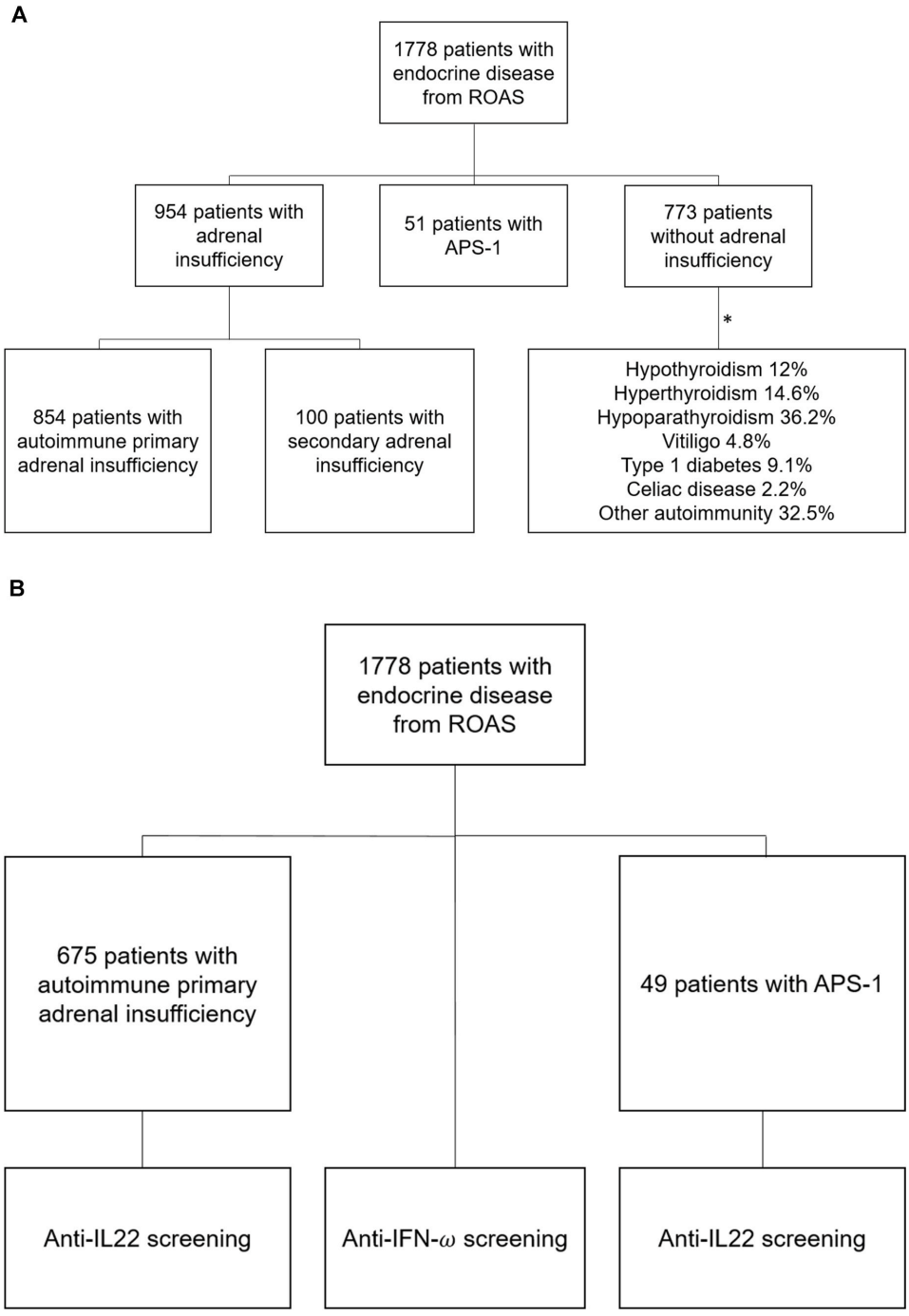


Fig. 1. Study design. (A) Flow chart describing the patients enrolled in the study. *Patients might be counted in more than one autoimmune disease subgroup. **(B)** Flow chart describing the screening pipeline.

to measure background absorbance. All individual samples from positive serum-pools were re-screened individually in duplicates to verify which sample(s) contained anti-IL22 antibodies.

2.5. NGS sequencing

DNA was purified from whole blood with EDTA additive using the MasterPure™ DNA purification kit version II B1 (Epicenter, Madison, WI) at the HUNT Laboratory (Levanger, Norway). We then used two different approaches and pipelines of NGS sequencing.

Libraries for whole exome sequencing (WES) were prepared using SEQCap EZ HyperCap (v1.2) or KAPA HyperCap (v3.0) library preparation kits, with SeqCap EZ MedExome- or KAPA HyperExome target enrichment kits, respectively (all kits and reagents are from Roche, Basel, CH). Libraries were sequenced on Illumina NextSeq500 or Illumina NovaSeq6000 instruments using paired-end 150 bp or 100 bp reads, respectively. Data processing, alignment to GRCh37 and variant-calling were performed essentially as previously described [39], except that Genome Analysis Toolkit (GATK) v.3.8.1 were used according to GATK's Best Practices guidelines [40,41]. Variant annotation and interpretation were performed using Alissa Interpret (Agilent Technologies, Santa Clara, CA) and Alamut Visual 2.15 (Sophia Genetics, Boston, MA). Gene panel based infiltration of variants were performed using the expert curated Primary Immunodeficiency (v2.12) panel from Genomics England PanelApp, supplemented with an in-house designed panel of additional candidate genes. Altogether, 311 genes were included in the panel-based analysis (see supplementary Table 1 for list of all gene IDs).

All variant calls with Call Quality (CQ) $\leq$ 200, Depth of Coverage (DP) $<$ 10 and Mapping quality (MQ) $\leq$ 20 were filtered out. Furthermore, variants with allele frequencies $\geq$ 0.1% (autosomal dominant model) or $\geq$ 2.0% (autosomal recessive model) in any ethnic subpopulation in the public database gnomAD (versions v.2.1.1 and v.3.1.2) were excluded. Only presumed loss-of-function (LoF) variants (indel-inframe, indel-frameshift, start-lost, nonsense, stop-lost, synonymous splice-site (within first or last two bases of the exon), and canonical splice-site (within first or last two bases of the intron)) and missense mutations predicted to be damaging by at least one *in silico* (see below) were retained for downstream genetic analyses. The potential damaging impact of missense variants was assessed using the following *in silico* algorithms: CADD (CADD - Combined Annotation Dependent Depletion (washington.edu)), using a score $\geq$ 20 as threshold for potentially damaging variants, as this corresponds to the top 1% most deleterious variants of the human genome, SIFT (SIFT - Predict effects of non-synonmous/missense variants (a-star.edu.sg)), PolyPhen2 HumVar (PolyPhen-2: prediction of functional effects of human nsSNPs (harvard.edu)) and MutationTaster (MutationTaster). Clinical classification of the identified variants was based on the American College of Medical Genetics and Genomics (ACMG) guidelines [42].

For 150 bp paired-end whole genome sequencing (WGS), performed at the National Genomics Infrastructure (NGI) at Science for Life Laboratory (Stockholm, Sweden), libraries were prepared with Illumina TruSeq PCR-free kits, with 350 bp inserts, then sequenced on a NovaSeq6000 platform. Data were handled using Sarek [43] pipeline for alignment, QC, processing and variant calling. The samples had a coverage between 27X and 36X, with 90% > Q30 (>Q30 = aggregated % of bases with quality score above 30). The same bioinformatic pipeline as above was followed to search for known or possible disease-causing variants.

2.6. AIRE, Helios (IKZF2), LAT and TNFAIP3 sequencing

DNA from anti-IL22 and anti–IFN–ω positive patients was sequenced for all 14 exons of the *AIRE* gene either using a diagnostic pipeline at the Department of Medical Genetics, Haukeland University Hospital (Bergen, Norway) or an in-house protocol. The in-house protocol uses the AmpliTaq Gold DNA Polymerase with Gold buffer & MgCl2 kit (Applied Biosystems, Waltham, MA) for PCR. A master mix consisting of 10X PCR gold buffer, 25 mM $MgCl_2$, 10 mM dNTP, betaine, ddH_2O, 10μM forward primer, 10μM reverse primer and DNA polymerase enzyme was prepared, and transferred to PCR tubes. One μl DNA template (20–100 ng) was added to each PCR tube. The samples were run on a Veriti 96 well thermal cycler (AppliedBiosystems, Waltham, MA) with the following settings: one step at 98°*C*for 10 min; 10 cycles at 98°*C* for 15 s, 69°*C* for 15 s and 72°*C* for 45 s; 30 cycles at 95°*C* for 15 s, 50°*C* for 15 s and 72°*C* for 45 s, and finally for 7 min. Purification of the PCR-products were carried out using the illustra ExoProStar 1-step enzymatic PCR and Sequence Reaction Clean-up Kit according to the manufacturer's protocol (GE Healthcare Life Sciences, Chicago, IL).

Sequencing was performed using the BigDye 3.1 v1.1 kit, with standard protocol. Samples were sequenced at the Department of Medical Genetics, Haukeland University Hospital (Bergen, Norway) and analysed using either Sequence Pilot Software (JSI Medical systems) or the CLC Main Workbench 8.0.1 software (Qiagen, Aarhus, DK). *AIRE* primer sequences can be found in supplementary Table 2. Two patients and the parents of one of them were sequenced for one exon of the *Helios* gene (*IKZF2*) according to the same protocol. DNA from one of these patients and his parents were also typed for c.1186 in *TNFAIP3* and c.167 in *LAT* by Sanger sequencing to verify findings from the exome sequencing. Primer sequences can be found in supplementary Table 3.

2.7. Statistics

The Pearson correlation coefficient was used to measure linear correlation between RIA and ELISA anti-IL22 data, using the GraphPad Prism 9.1.0 software (GraphPad Software, San Diego, CA). A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Anti–IFN–ω screening of patients with autoimmune endocrine diseases

As a part of our routine analysis, we screened 1778 patients with endocrine diseases included in ROAS since 1996 for autoantibodies directed against IFN-ω using RIA. By using this approach, we identified 63 (3.5%) positive patients. Of these, 12 (19%) were anti–IFN–ω positive without being diagnosed with APS-1 (Table 1). Fifty-one known APS-1 patients were all found positive for this marker.

3.2. Anti-IL22 screening of patients with autoimmune PAI and anti–IFN–ω positive non-APS-1 patients

In addition to our routine RIAs for measuring IFN-ω and IL22 autoantibodies [36], we established a fast and reliable ELISA screening tool for IL22 autoantibodies, capable of dealing with pooled serum samples. The specificity and usefulness of the more efficient ELISA assay for anti-IL22 was determined by comparing results with the well-established RIA for formerly screened ASP-1 sera and found satisfactory (Pearson correlation coefficient 0.8442 [0.7524, 0.9039], p $<$ 0.0001 (Suppl. Fig. 1)). With this ELISA, we screened 675 patients with autoimmune PAI and 90 healthy controls, in pools of five, for autoantibodies against IL22 in two major consecutive screenings. We found in total 13 (1.9%) autoimmune PAI patients (Table 1, patient 12–24) positive for anti-IL22 from 11 serum-pools, although the absorbance values were much lower than for positive APS-1 patients (Fig. 2). Further, we found 2 (2.2%) anti-IL22 positive healthy controls (Suppl. Fig. 1). In addition to patients with autoimmune PAI and APS-1, nine non-APS-1 patients previously found to have interferon-ω autoantibodies, but without *AIRE* mutations, were checked for anti-IL22 autoantibodies, using the same ELISA assay. All were negative, with signals clearly below the cut-off (results not shown). One patient (Table 1,

Table 1
Patient clinical, serological and genetic manifestations.

Patient	Sex[a]	Autoantibodies[b]						Manifestations[c]	Other autoantibodies[d]	Genetics[e]
		IFN-ω RIA	IFN-α_2 RIA	IL17F RIA	IL22 RIA	IL22 ELISA	21OH RIA			
1	M	Pos	Pos	NA	Neg	Neg	Pos	Autoimmune PAI	SSC	–
2	M	Pos	Pos	Neg	Neg	NA	Pos	Al, CMC, B12, MG	GAD, 17OH, TH	–
3	M	Pos	Pos	Neg	Neg	Neg	Neg	Secondary adrenal insufficiency due to pituitary gland failure, HGG	–	*NFKB2*: c.2557C > T, p. Arg853Ter
4	M	Pos	Pos	NA	Neg	Neg	Neg	Postoperative HP	–	*LCK*: 8G > c, p.Cys3Ser
5	M	Pos	Pos	NA	Pos	Neg	Pos	Al	–	*STAT1*: c.1727 + 15T > A
6	M	Pos	Neg	NA	Pos	Neg	Pos	Adrenolectomy	AADC, TPH, NALP5	*IKZF2*: c.982C > G, p. Leu328Val JAK3: c.2979-6C > T
7	M	Pos	Pos	Neg	Neg	Neg	Neg	T1D	GAD, TPH	*CTLA4*: c.82dup, p. Leu28ProfsTer32
8	F	Pos	Neg	NA	NA	Neg	Neg	HypoT, B12, V, POI, AAG	–	–
9	M	Pos	Pos	Neg	Neg	Neg	Pos	T1D, CD	–	–
10	F	Pos	Neg	Neg	Neg	Neg	Pos	Autoimmune PAI, HypoT	–	–
11	F	Pos	Neg	NA	Pos	NA	Pos	Autoimmune PAI, HyperT, CD	–	*TERC*: n.*545_*548del
12	F	Neg	Neg	Neg	Neg	Pos	Pos	Autoimmune PAI, T1D, HypoT	AADC	*RAG1*: c.3115G > A, p. Asp1039Asn
13	F	Neg	Pos	Neg	Neg	Pos	Pos	Autoimmune PAI, HypoT	SSC, TPH, NALP5	*AIRE*: c.1411C > T, p. Arg471Cys
14	M	Neg	Neg	Pos	Neg	Pos	Pos	Autoimmune PAI, T1D	–	*TNFAIP3*: c.1186 A > T, p. Met396Leu *LAT*: c.167C > T, p. Thr56Met *IKZF2*: c.104 A > G, p. Asn35Ser *IKZF2*: c.982C > G, p. Leu328Val
15	M	Neg	Neg	NA	NA	Pos	Pos	Autoimmune PAI	–	–
16	F	Neg	NA	NA	NA	Pos	Pos	Autoimmune PAI, V, T1D, B12	–	–
17	M	NA	NA	NA	NA	Pos	Pos	Autoimmune PAI, HypoT	–	–
18	M	Neg	NA	NA	NA	Pos	Pos	Autoimmune PAI, CD	–	–
19	M	Pos	NA	NA	NA	Pos	Pos	Autoimmune PAI, HypoT	–	–
20	F	Neg	NA	NA	NA	Pos	Neg	Autoimmune PAI	–	–
21	M	Neg	NA	NA	NA	Pos	Pos	Autoimmune PAI	–	–
22	M	Neg	NA	NA	NA	Pos	Neg	Autoimmune PAI, HyperT	–	–
23	F	Neg	NA	NA	NA	Pos	Pos	Autoimmune PAI, HypoT	–	–
24	M	Neg	NA	NA	NA	Pos	Neg	Autoimmune PAI	–	–
25	F	Neg	Pos	Neg	Pos	NA	Neg	HypoT, HP, POI	–	No variants to explain clinical phenotype
26	M	Pos	Pos	Neg	Pos	NA	Pos	Autoimmune PAI, CMC, V, B12, Al	–	*AIRE*: c.22C > T/ c.967_979del
27	F	Pos	Pos	NA	Neg	NA	Pos	Autoimmune PAI, HP	SSC, NALP5	*AIRE*:c.22C > T/ c.1163_1164insA
28	M	Pos	Neg	NA	Pos	NA	Pos	Autoimmune PAI	–	*AIRE*: c.347C > T/c.1411C > T
29	F	Pos	Pos	NA	NA	NA	NA	HypoT	–	*AIRE*: c.1336C > G/ c.967_979del

[a] F, female; M, male.
[b] 21-OH, 21-hyroxylase; ELISA, enzyme-linked immunosorbent assay; IFN-ω, interferon-omega; IFN-α_2, interferon-alpha 2; IL17F, interleukin 17F; IL22, interleukin 22; neg, negative; pos, positive; PAI, primary adrenal insufficiency; RIA, radio immune assay.
[c] AAG, autoimmune atrophic gastritis; Al, alopecia; B12, vitamin B12 deficiency; CD, celiac disease; CMC; chronic mucocutaneous candidiasis; HGG, hypogammaglobulinemia; HP, hypoparathyroidism; hyperT, hyperthyroidism; hypoT, hypothyroidism; MG, myasthenia gravis; NA, not analysed; POI, primary ovarian insufficiency; T1D, type 1 diabetes mellitus; V, vitiligo; -, not found.
[d] 17OH, 17-hydroxylase; AADC, aromatic I-amino acid decarboxylase; GAD, glutamic acid decarboxylase; NACHT leucine-rich-repeat protein 5; SSC, side-chain cleavage enzyme; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; -, not found.
[e] *AIRE* sequencing performed for all; NGS performed for all except for those marked with (−).

patient 25) was included in the study due to anti-IL22 positivity found in a RIA assay.

3.3. Single gene AIRE and NGS sequencing reveals monogenic immune deficiencies

All together, we identified 29 patients that were anti–IFN–ω and/or anti-IL22 positive (Table 1). To determine if autoantibody positive patients were genetically susceptible for APS-1, they were investigated for *AIRE* mutations by Sanger-sequencing (N = 10) or NGS (N = 19). Sequencing revealed four new APS-1 cases (Table 1, patients 26–29) with biallelic mutations in *AIRE*. In addition, we found a heterozygous c.1411C > T (p.Arg471Cys) variant in *AIRE* for patient 13, who was mildly positive for anti-IL22. This variant has previously been found to associate with increased risk of autoimmune PAI [4], pernicious anaemia [44], and type 1 diabetes [45]. This patient clinically presented with autoimmune PAI, hypothyroidism and autoantibodies directed against 21OH, IFN-α_2, SSC, TPH and NALP5.

For patients without confirmed deleterious *AIRE* mutations, we searched for other rare variants that could help explain the clinical phenotype seen in these patients by NGS and a diagnostic pipeline for immunodeficiencies. We sat the criteria for NGS-sequencing inclusion to 1) the patient had to be alive and 2) RIA index IFN-ω > 300 (threshold 200) and/or 3) ELISA IL22 endpoint absorbance >0.1 or positivity with

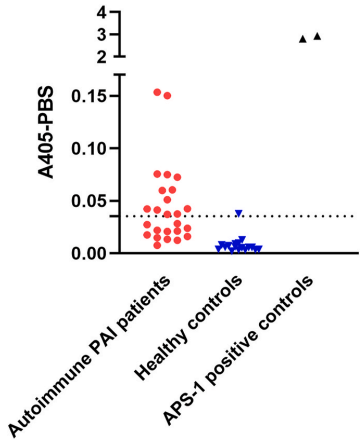


Fig. 2. Anti-IL22 screening using the newly established high-throughput assay. Screening of 675 patients with autoimmune PAI identified 13 (1.9%) patients with autoantibodies against IL22. The dotted line represents the threshold for positivity (mean healthy controls + 3SD).

the "over the mean healthy controls plus three standard deviations" criteria, in addition to having other APS-1 like organ-specific antibodies. Using this approach, we found rare gene variants in 8 out of 10 patients (Table 1). The previously reported c.2557C > T (p.Arg853Ter) nonsense mutation (MAF gnomAD genomes 0, CADD score 36) in the *NFKB2* gene was found in patient 3, providing a truncated protein. This patient presented with secondary adrenal insufficiency, due to pituitary gland failure, and hypogammaglobulinemia. Patient 7 was heterozygous for a frame-shift mutation, c.82dup (p.Leu28ProfsTer32), in *CTLA4*, also predicting a LoF deleterious mutation (MAF gnomAD genomes 0). This patient clinically presented with type 1 diabetes and autoantibodies directed against TPH and GAD.

By using the screening-criteria for identification of immune variants described in the method section, we further identified rare nucleotide shifts in *TNFAIP3* and *LAT* in patient 14, *IKZF2* in patients 6 and 14, *RAG1* in patient 12 and *LCK* in patient 4. The following three variants are in addition of potential interest based on their rareness and the biological function of the relevant genes: *STAT1* in patient 5, *JAK3* in patient 6 and *TERC* in patient 11 (see Tables 1 and 2, Fig. 3 for details).

Of interest, exome-sequencing revealed two mutations in *IKZF2*, encoding the translational activator and repressor Helios, in patient 14 (c.104 A > G, p.Asn35Ser, MAF gnomAD genomes 0.0022, CADD score 24.6, and c.982C > G, p.Leu328Val, MAF gnomAD genomes 0.000092, CADD score 23.6). This patient also had rare variants in *TNFAIP3* (c.1186 A > T, p.Met396Leu, MAF gnomAD genomes 0, CADD score 22.3) and *LAT* (c.167C > T, p.Thr56Met, MAF gnomAD genomes 0.000079, CADD score 12.1). In addition to early onset autoimmune PAI and type 1 diabetes, he presented with 21OH-, IL17F-, and IL22 autoantibodies. As we also had access to samples from his parents, we sequenced and confirmed the c.104 A > G (p.Asn35Ser) *IKZF2*-, *TNFAIP3*- and *LAT*-variants in his mother and the c.982C > G (p.Leu328Val) *IKZF2* variant in his father. The *IKZF2* variant c.983C > G was also found in patient 6, together with a rare *JAK3*-variant (c.2979-6C > T, MAF gnomAD genomes 0, CADD score 12.2). This patient also had high indices of several other autoantibodies, including AADC, NALP5 and TPH. For patient 25, no variants in immune related genes were found.

4. Discussion

Here, we have identified several patients with probable monogenic or oligogenic causes of organ-specific autoimmunity by screening a large endocrine patient biobank for anti-cytokine antibodies. We identified four new APS-1 patients with pathogenic *AIRE* mutations in both alleles and eight patients with certain and/or probable disease-causing monoallelic heterozygous rare variants. We used RIA for anti–IFN–ω measurements and a newly developed ELISA assay for anti-IL22 where we introduced pooling of samples to make it high-throughput. We confirmed the usefulness of the ELISA assay by comparing results with a previously validated RIA-assay for anti-IL22 antibodies, showing good correlation between assays. Some patients fall above the threshold for one, but not for the other assay. These borderline positive or borderline negative samples are in contrast to the high antibody levels we usually find in APS-1 patients. Nevertheless, this new assay may contribute to earlier diagnosis of patients with other monogenic autoimmune syndromes.

Even though the clinical criteria for some of the known monogenic autoimmune syndromes, like APS-1, are known, the phenotypic variation is vast, and the diagnosis is often delayed. Screening of anti-cytokine and organ-specific antibodies is easy to perform, and as these antibodies are reliable biological markers of the disease, they should be brought into clinical practice. Here, we identified four new APS-1 cases, with confirmed *AIRE* mutations, using anti–IFN–ω screening, although only two of them were confirmed to be anti-IL22 positive. They all showed a typical APS-1 autoantibody profile, presenting with autoantibodies directed against IFN-ω and IFN-α_2, as well as several of the other organ-specific autoantibodies typical of APS-1, e.g. NALP5, AADC and TPH (Table 1, patient 26–29). In most APS-1 patients, autoantibodies against type I IFNs and IL22 are neutralizing [13,14,46], but the functional impact remains obscure. Clinically, the patients did not fulfil the classical criteria of two of the main components, adrenal insufficiency, hypoparathyroidism and chronic mucocutaneous candidiasis. They are now followed up more closely since they have a significant risk of developing new and potentially life-threatening manifestations [9]. In a similar study in Sweden where only anti-IL22 RIA and 21OH-antibodies RIA were used for screening, four autoimmune PAI patients with undiagnosed APS-1 were identified [47]. Importantly, if we had omitted IFN-ω screening, we would have failed to detect one of the identified APS-1 patients, and several of the patients with other probable monogenic cause would remain unnoticed.

Eleven of our included patients, with different autoimmune and/or endocrine manifestations, were positive for IFN-ω autoantibodies, but without detectable *AIRE* mutations. In addition, 13 autoimmune PAI patients were positive in the anti-IL22 ELISA assay, in addition to one anti-IL22 positive patient from former RIA screenings. Sequencing with an immune panel pipeline identified rare variants in eight patients. Among these were pathogenic variants in *NFKB2* (p.Arg853Ter) and *CTLA4* (p.Leu28ProfsTer32), in two patients. None of these variants has been reported to public genomic databases, such as gnomAD, and both are predicted to cause dysfunctional gene products.

The *NFKB2* p.Arg853Ter variant will lead to the synthesis of a truncated protein lacking amino acids critical for nuclear translocation of the NFKB2 protein (CADD score 36). Other variants in the *NFKB2* gene have previously been associated with common variable immunodeficiency (CVID) [48] and pituitary insufficiency [49–51], as was seen in our patient (OMIM: CVID10, David syndrome). Terminating mutations in the C-terminal part of one of the alleles encoding the NFKB2-protein do not result in mRNA decay, but in a non-functional product interfering with the wild type NFKB2-protein, ultimately causing a dominant negative effect [52]. Defects in the function of natural killer cells have been revealed in individuals with *NFKB2*-mutations, in line with the immune deficient clinical manifestations [52]. Furthermore, our patient suffered from recurrent respiratory tract infections from early childhood until he received substitutions with

Table 2
Genetic variant predictions and frequencies.

Gene (Transcript)	Genomic DNA	cDNA	Protein	Reference SNP[a]	MAF gnomAD exomes[b]	MAF gnomAD genomes[c]	SIFT[d]	PolyPhen-2 HumVar[e]	MutationTaster[f]	CADD[g]
NFKB2 (NM_001288724.1)	Chr10 (GRCh37): g.104161895C > T	c.2557C > T	p. (Arg853Ter)	rs397514332	0	0	NA	NA	NA	36
STAT1 (NM_007315.4)	Chr2 (GRCh37): g.191844483 A > T	c.1727 + 15T > A	p.?	NA	0	0	NA	NA	NA	5.6
CTLA4 (NM_005214.4)	Chr2 (GRCh37): g.204732747dup	c.82dup	p. (Leu28Profs*32)	NA	0	0	NA	NA	NA	NA
TNFAIP3 (NM_001270508.2)	Chr6 (GRCh37): g.138199768 A > T	c.1186 A > T	p. (Met396Leu)	NA	0	0	Tolerated	Benign	Disease causing	22.3
LAT (NM_014387.3)	Chr16 (GRCh37): g.28997459C > T	c.167C > T	p. (Thr56Met)	NA	0.000091	0.000079	Deleterious	Benign	Benign	12.1
IKZF2 (NM_016260.2)	Chr2 (GRCh37): g.214012467T > C	c.104 A > G	p. (Asn35Ser)	NA	0.0023	0.0022	Deleterious	Probably damaging	Benign	24.6
IKZF2 (NM_016260.2)	Chr2 (GRCh37): g.213872683G > C	c.982C > G	p. (Leu328Val)	NA	0.00018	0.000092	Deleterious	Probably damaging	Benign	23.6
JAK3 (NM_000215.3)	Chr19 (GRCh37): g.17941435G > A	c.2979-6C > T	p.?	NA	0	0	NA	NA	NA	12.2
RAG1 (NM_000448.3)	Chr11 (GRCh37): g.36597969G > A	c.3115G > A	p. (Asp1039Asn)	NA	0.00016	0.000026	Deleterious	Benign	Benign	23.2
TERC (NR_001566.1)	Chr3 (GRCh37): g.169481850_169481853del	n.*545_*548del	NA	NA	0	0	NA	NA	NA	NA
LCK (NM_001042771.2)	Chr1 (GRCh37): g.32739938G > C	c.8G > C	p. (Cys3Ser)	NA	0	0.0000066	Deleterious	Probably damaging	Disease causing	28.1

[a] NA not found.
[b] MAF, minor allele frequency; gnomAD, The Genome Aggregation Database.
[c] MAF, minor allele frequency; gnomAD, The Genome Aggregation Database.
[d] NA, no missense variant found; SIFT, sorting intolerant from tolerant.
[e] HumVar, human genome variation; NA, no missense variant found; PolyPhen 2, polymorphism phenotyping v2.
[f] NA, no missense variant found.
[g] CADD, combined annotation dependent depletion; NA, not found.

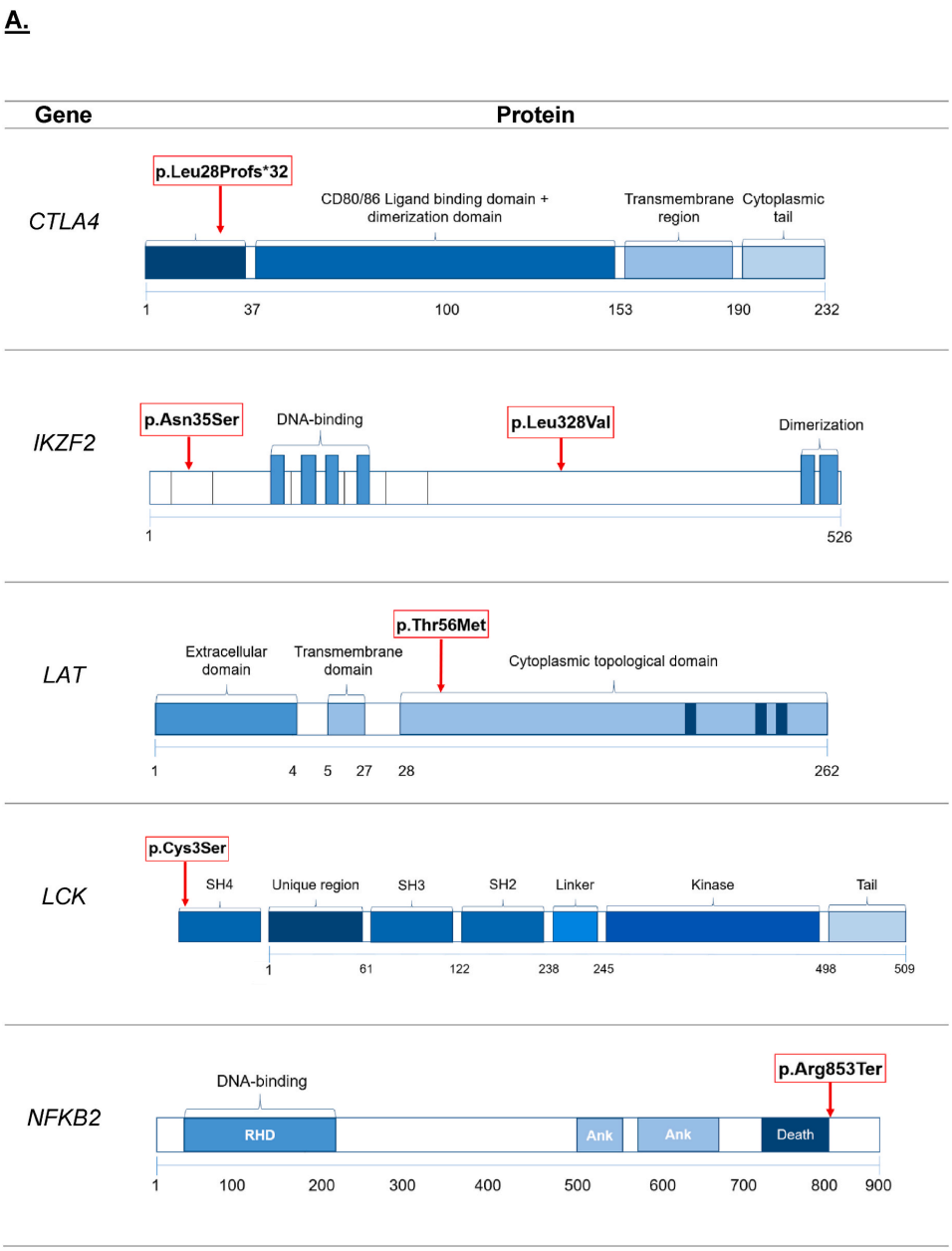
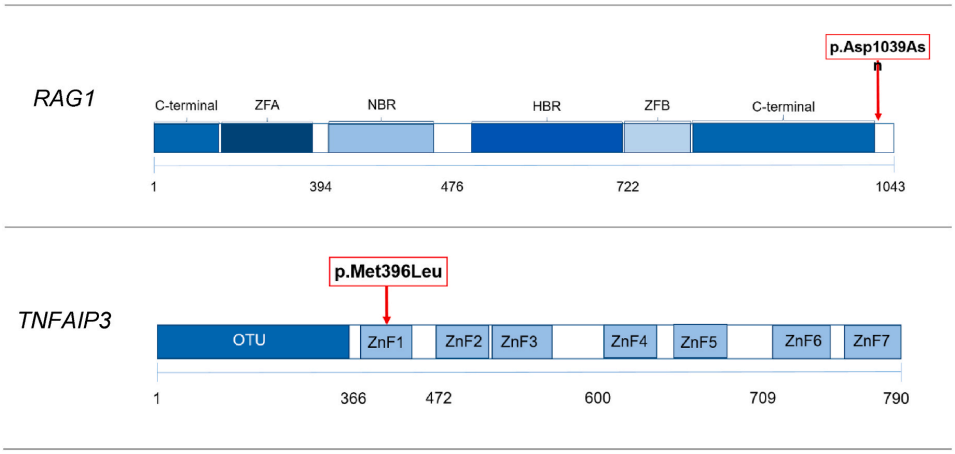


Fig. 3. Genetic variants and their corresponding protein locations. (A) The genes that were found to contain rare genetic variants in patients with various endocrinopathies are shown on the left side and the corresponding protein domain architectures and marked location of variants on the right side. **(B)** Rare variants of potential interest with uncertain functional impact. *TERC* is missing in the figure because it is a long non-coding RNA, which does not have amino acid domains. The blue boxes in the figures represent different important functional domains. The line under each figure represents the amino acid positions.

immunoglobulins. His brother died of pneumonia at 6 years, but we have not been able to clarify if he or other family members harbour the same *NFKB2* variant.

The *CTLA4* variant reported on here is predicted to cause a non-functional gene product (CTLA4 p.Leu28ProfsTer32 probably results in nonsense-mediated decay of the mRNA). Since CTLA4 usually functions as a dimer, this will imply haploinsufficiency; a dose effect on CTLA4 concentration that provides large risk for autoimmune disease.



B.

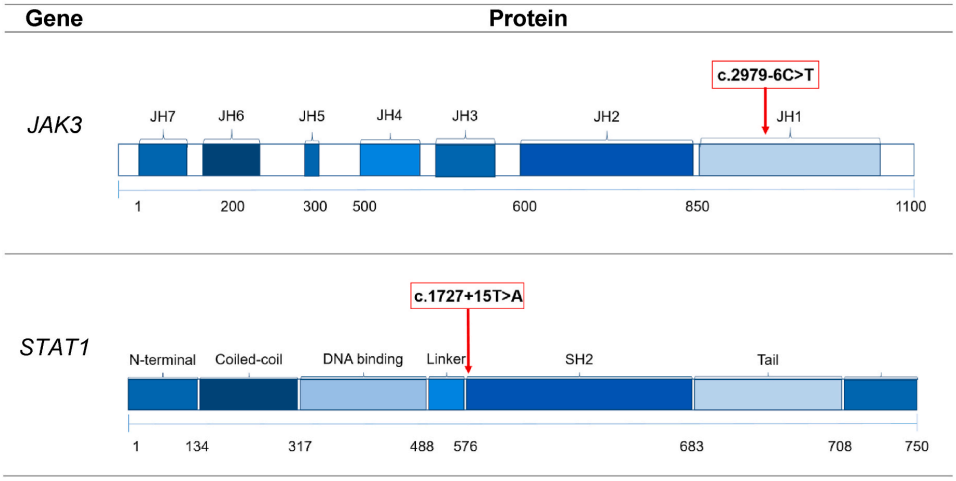


Fig. 3. (*continued*).

Further, CTLA4 is an important inhibitory immune checkpoint, upregulated on conventional T cells after activation, to suppress further cell proliferation and activation [53,54]. Interestingly, it is thought to be constitutively expressed by regulatory T cells (Tregs) and important for their suppressive function [55]. Immune dysregulation and lowered production of CTLA4 has been shown in patients with *CTLA4* mutations, leading to an IPEX-like syndrome [56,57]. In addition, neutralizing autoantibodies directed against type I interferons have been described in IPEX patients [21]. Our patient was also positive for autoantibodies against TPH and GAD. A similar *CTLA4* mutation was previously reported in a patient with CVID (c.75delG, p.L28Ffs*44), resulting in reduced levels of CTLA4 mRNA and protein, with deficiencies of T cells, particularly the Treg suppressive function and B cell activity [56]. Further functional studies are required to formally show that CTLA4 production and Treg function is directly impaired in our patient.

As we did not have access to informative first-degree relatives to these two cases, we were not able to check for disease penetrance. However, similarly to many other autosomal dominant immunodeficiencies, penetrance of disease has been reported to be variable for heterozygous mutations in *NFKB2* [52,58] and *CTLA4* [57]. Indeed, patients with mutations in these genes have previously been shown to harbour autoantibodies against type I interferons [23,24], but no functional role of the antibodies has been revealed.

Interestingly, one patient in our cohort, patient 14, had a rare variants in the *IKZF2* gene, where the c.982C > G, p.Leu328Val variant was inherited from the father and the c.104 A > G, p.Asn35Ser variant was

inherited from the mother. Thus, patient 14 was found combined heterozygous for this gene. In the same patient, we also found rare variants in genes *LAT* and *TNFAIP3*, inherited from the mother. Patient 14 was negative for type I interferon autoantibodies, but positive for anti-IL17F and –IL22 autoantibodies and diagnosed with autoimmune PAI and type 1 diabetes (Table 1). One of the *IKZF2* variants in patient 14 was also found in one of the other included patients (Table 1, patient 6). This patient had high titres of several autoantibodies, including IFN-ω. Of interest, neutralizing autoantibodies against type I interferons were described in a small cohort of Finnish patients with mutations in *IKZF2* [25] presenting with CVID-phenotypes, and mutations in this gene were also described by Shahin et al. [59]. Collectively, they showed that mutations in *IKZF2* could interfere with multimerization of the protein (depending on where the mutation is located), which again results in increased proinflammatory signalling and impairment of follicular T cells, natural killer cells and mucosal-associated invariant T (MAIT) cells [25,59]. The product of the *IKZF2* gene, Helios, is highly expressed in effector T cells [60], and is important for Treg functional stability, as it averts Il2 production through epigenetic silencing [61]. However, no evidence of large effects on Tregs have been found [25,59]. Whether the *IKZF2* mutations affect the function of Tregs and other T cells in our patients needs additional investigations.

The functional impact of the other nine genetic variants that we report here are more uncertain, but they are rare and could theoretically lead to altered protein function. However, variants in some of these genes have been associated with autoimmune conditions previously. *LAT* has been shown to be important in T cell development and TCR signalling, as *LAT* knockout mice lack peripheral T cells [62]. In addition, a severe combined immunodeficiency (SCID) phenotype was described in patients with biallelic *LAT* mutations [63]. Recently, *TNFAIP3* mutations have been shown to cause A20 haploinsufficiency with a lupus-like phenotype [64] and Behçet-like disease [65]. Autoantibodies against type I IFNs α and ω have been reported in Omenn syndrome patients presenting with mutations in genes *RAG1* and/or *RAG2* [22], characterized by SCID and autoimmunity due to impaired V(D)J recombination activity during lymphocyte development [66]. *STAT1* is involved in the IFN signalling pathway, and gain-of-function mutations in this gene is associated with development of chronic mucocutaneous candidiasis (CMC) [67,68]. Furthermore, the autosomal recessive inherited $T^{-}B^{+}$ SCID is associated with mutations in the *JAK3* gene [69,70]. The *TERC* gene is important for the generation of telomerase and mutations have been described in patients with dyskeratosis congenita and aplastic anaemia [71,72].

In conclusion, we show that screening for autoantibodies against cytokines can effectively identify previously unknown monogenic and possible oligogenic causes of autoimmunity and immunodeficiency, information crucial for the clinician to provide personalised follow-up of the patients and close relatives.

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Author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Screening patients with autoimmune endocrine disorders for cytokine autoantibodies reveals monogenic immune deficiencies

Supplementary material

SUPPLEMENTARY TABLES

Supplementary Table 1. Immune panel sequencing pipeline gene IDs.

Gene IDs						
ACD	CD3G	DNMT3B	IKZF4	MYD88	RAC2	TAP2
ACP5	CD40	DOCK2	IL10	MYO5B	RAG1	TAPBP
ADA	CD40LG	DOCK8	IL10RA	MYSM1	RAG2	TAZ
ADAM17	CD46	ELANE	IL10RB	NBN	RASGRP1	TBX1
ADAR	CD55	EPG5	IL12B	NCF1	RBCK1	TCF3
AICDA	CD59	ERCC6L2	IL12RB1	NCF2	RFX5	TCN2
AIRE	CD70	EXTL3	IL17F	NCF4	RFXANK	TERC
AK2	CD79A	F12	IL17RA	NCSTN	RFXAP	TERT
AP1S3	CD79B	FADD	IL17RC	NDNL2	RHOH	TICAM1
AP3B1	CD81	FAM105B	IL1RN	NFKB1	RIPK1	TINF2
ARPC1B	CD8A	FAS	IL21	NFKB2	RLTPR	TLR3
ATM	CDCA7	FASLG	IL21R	NFKBIA	RMRP	TMC6
ATP6AP1	CEBPE	FAT4	IL2RA	NHEJ1	RNASEH2A	TMC8
B2M	CECR1	FCGR3A	IL2RG	NHP2	RNASEH2B	TMEM173
BACH2	CFB	FERMT3	IL36RN	NLRC4	RNASEH2C	TNFAIP3
BCL10	CFH	FOXN1	IL7R	NLRP1	RNF168	TNFRSF11A
BLM	CFHR1	FOXP3	INO80	NLRP12	RORC	TNFRSF12A
BLNK	CFHR3	FPR1	IRAK4	NLRP3	RPSA	TNFRSF13B
BTK	CFHR4	G6PC3	IRF3	NOD2	RTEL1	TNFRSF13C
C1QA	CFHR5	G6PD	IRF7	NOP10	SAMD9	TNFRSF1A
C1QB	CFI	GATA1	IRF8	NRAS	SAMD9L	TNFRSF25
C1QC	CFP	GATA2	ISG15	ORAI1	SAMHD1	TPP2
C1R	CFTR	GATA3	ITCH	PARN	SBDS	TRAC
C1S	CHD7	GFI1	ITGB2	PEPD	SERPING1	TREX1
C2	CIITA	GINS1	ITK	PGM3	SGPL1	TRNT1
C3	CLPB	GUCY2C	JAGN1	PIK3CD	SH2D1A	TTC37
C4A	COPA	HAX1	JAK3	PIK3R1	SKIV2L	TTC7A
C4B	CORO1A	HELLS	KRAS	PLCG2	SLC29A3	TYK2
C5	CR2	HPS1	LAMTOR2	PMS2	SLC35C1	UNC13D
C6	CSF2RA	HPS4	LAT	PNP	SLC37A4	UNC93B1
C7	CSF2RB	HPS6	LCK	POLA1	SLC46A1	UNG
C8A	CSF3R	HTRA2	LIG4	POLE	SMARCAL1	USB1
C8B	CTC1	ICOS	LPIN2	POMP	SP110	USP18
C9	CTLA4	ICOSLG	LRBA	PRF1	SPINK5	VPS13B
CARD11	CTPS1	IFIH1	LYST	PRKCD	SPPL2A	VPS45
CARD14	CTSC	IFNGR1	MAGT1	PRKDC	STAT1	WAS
CARD9	CXCR4	IFNGR2	MALT1	PSENEN	STAT2	WDR1

CASP10	CYBA	IGHM	MAP3K14	PSMA3	STAT3	WIPF1
CASP8	CYBB	IGKC	MBL2	PSMB4	STAT5B	XIAP
CCBE1	DCLRE1B	IGLL1	MCM4	PSMB8	STIM1	ZAP70
CD19	DCLRE1C	IKBKB	MEFV	PSMB9	STING1	ZBTB24
CD247	DKC1	IKBKG	MOGS	PSTPIP1	STK4	
CD27	DNAJC21	IKZF1	MSN	PTEN	STX11	
CD3D	DNASE1L3	IKZF2	MTHFD1	PTPRC	STXBP2	
CD3E	DNASE2	IKZF3	MVK	RAB27A	TAP1	

Supplementary Table 2. *AIRE* primers used for PCR and Sanger sequencing.

Oligo name (Primers 14 exons)	Sequence (5'-3')
AIRE_Ex_1Fw	CAAGCGAGGGGCTGCCAGTG
AIRE_Ex_1Rv	GGATCTGGAGGGGCGGGGTC
AIRE_Ex_2Fw	ACCACCTGACTCCACCACAAGCC
AIRE_Ex_2Rv	TCAGGGTTTTCTCCAGGGGTAGGG
AIRE_Ex_3Fw	GTGATGTTCCAGGACCGTCTTG
AIRE_Ex_3Rv	AGACCCGCCCGCCTACTT
AIRE_Ex_4Fw	TGAAGTAGGCGGGCGGGTCTC
AIRE_Ex_4Rv	CAGGGGCGACTGGCAAGATCA
AIRE_Ex_5Fw	TTGGGTGCACACACGAACA
AIRE_Ex_5Rv	GGCAGAAACTCTGGCTACCTGA
AIRE_Ex_6Fw	CACCCTGGGGCCTACACGACT
AIRE_Ex_6Rv	GAAGAGGGGCGTCAGCAATGG
AIRE_Ex_7Fw	CCAGGAACAGCGTTGCCTC
AIRE_Ex_7Rv	CGGTGCTCATCCCTGAGTGCC
AIRE_Ex_8Fw	CAGGTGGTCAGGGCAGAATTTCA
AIRE_Ex_8Rv	AGGCTGGGCAGCAGGTGTG
AIRE_Ex_9Fw	ATCTCTCTGCTGTGCCTCGGTTC
AIRE_Ex_9Rv	TGGGCATGGGGGACATAGTG
AIRE_Ex_10Fw	TGCCACAGCCTTTCCCACTCAGT
AIRE_Ex_10Rv	TGCCACAGCCTTTCCCACTCAGT
AIRE_Ex_11Fw	GCCTGAGGGTGCTTGGGTCG
AIRE_Ex_11Rv	GGGGTGTGGTTGTGGGCTGTATG
AIRE_Ex_12Fw	CCCCCACTCACCACCCACG
AIRE_Ex_12Rv	GGGAGCCCTGGCAGGACTCTC

AIRE_Ex_13Fw	CCCCAGCCCCATCATGCC
AIRE_Ex_13Rv	TGGTGGGTGGAGCAGGGACAG
AIRE_Ex_14Fw	TGGATGGTGACTTCTTGTAACGA
AIRE_Ex_14Rv	ACCTCCCGAGTTCAAGTGATTC

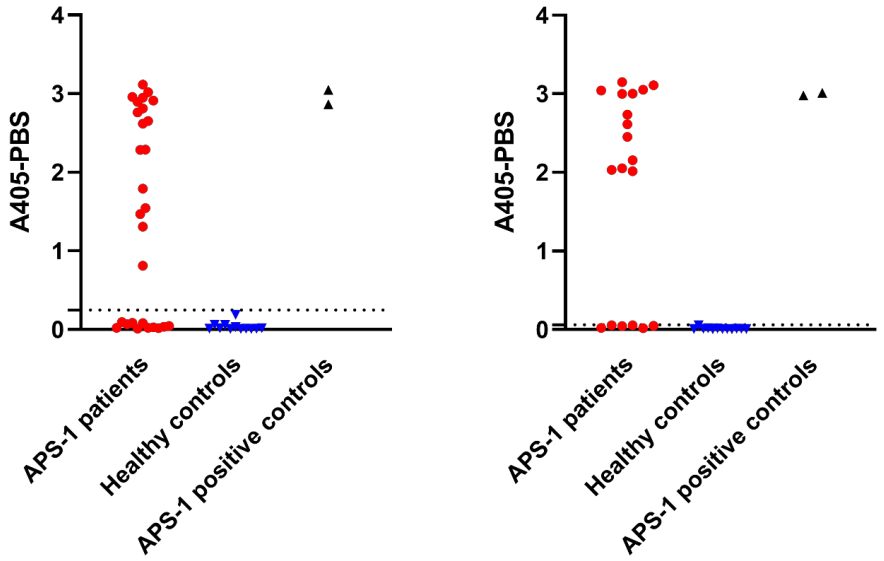
Supplementary Table 3. ***IKZF2 (*****Helios*****), LAT and TNFAIP3 primers used for PCR and Sanger sequencing.***

Nucleotide change	**Oligo name**	**Sequence (5'-3')**
IKZF2: c.104A>G	IKZF2-Helios_seq_primer_fw	TGCATGCCATTGCGTGTATAA
	IKZF2-Helios_seq_primer_rv	ACCACAATGTGAGAGGACCC
IKZF2: c.982C>G	IKZF2-Helios_seq_primer_fw	TCTTTCACCTCCCACTCCCTT
	IKZF2-Helios_seq_primer_rv	AGCTGAGCTTATAACTGGGGC
LAT: c.167C>T	LAT_seq_primer_fw	AGTGAGCGTGAACCTTCAGA
	LAT_seq_primer_rv	GAGCAGCTACTCACGGGAT
TNFAIP3: c.1186A>T	TNFAIP3_seq_primer_fw	AATGGCAGGAAAACAGCGAG
	TNFAIP3_seq_primer_rv	AAGGGCTCATAGGCTTCTCC

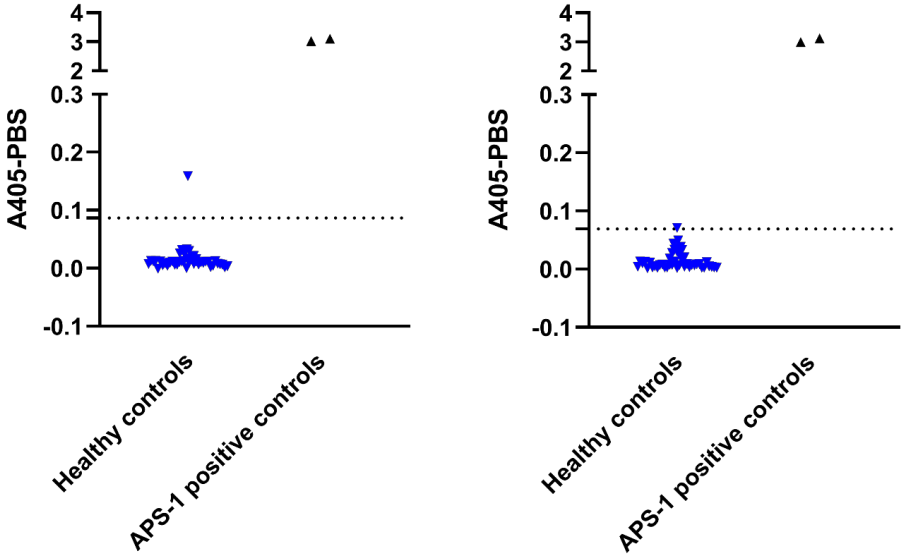
SUPPLEMENTARY FIGURES

Supplementary Figure 1. Anti-IL22 screening of patients with APS-1 and healthy controls to verify the newly established high-throughput anti-IL22 ELISA.

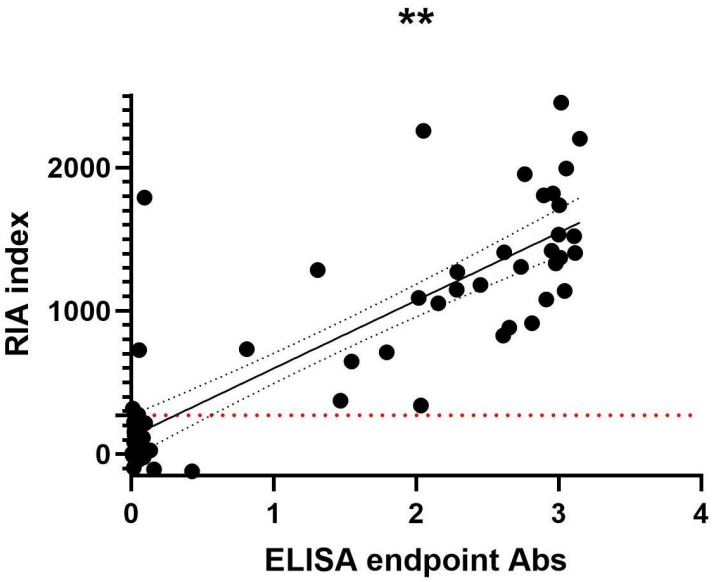
A.



B.



C.



(A) Thirty-two (65%) of the 49 analysed APS-1 patients had anti-IL22 indices over the threshold, compared to (B) two (2.2%) of the 90 included healthy controls. Importantly, the end-point absorbance level of the positive APS-1 patients were much higher than the few positive healthy controls. (C) Correlation analysis between RIA and ELISA assays for measurement of autoantibodies against IL22. ELISA endpoint absorption measurements are shown on the x-axis, while the RIA index is shown on the y-axis. The Pearson test was performed to assess correlation between ELISA and RIA for IL22 autoantibodies (Pearson correlation coefficient 0.8442 [0.7524, 0.9039], $p < 0.0001$). The dotted horizontal lines represent the threshold for anti-IL22 positivity for each graph (mean healthy controls + 3SD). The black dotted line represents the confidence interval. ** represents $p < 0.05$.

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