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Research Article

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Research Article

Regulatory T cells in autoimmune primary adrenal insufficiency

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Abstract

Primary adrenal insufficiency (PAI) is most often caused by an autoimmune destruction of the adrenal cortex resulting in failure to produce 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 many autoimmune disorders, but it is not known whether they contribute to development of PAI. We aimed to investigate the frequency and function of naive and expanded Tregs in patients with PAI and polyendocrine syndromes compared to age- and gender-matched healthy controls. Flow cytometry was used to assess the frequency and characterize functional markers of blood Tregs in PAI ($N = 15$). Expanded Treg suppressive abilities were assessed with a flow cytometry based suppression assay ($N = 20$), while bulk RNA-sequencing was used to examine transcriptomic differences ($N = 16$) and oxygen consumption rate was measured by a Seahorse cell metabolic assay ($N = 11$). Our results showed that Treg frequency and suppressive capacity were similar between patients and controls. An increased expression of killer-cell leptin-like receptors and mitochondrial genes was revealed in PAI patients, but their expanded Tregs did not display signs of mitochondrial dysfunction. Our findings do not support a clear role for Tregs in the contribution of PAI development.

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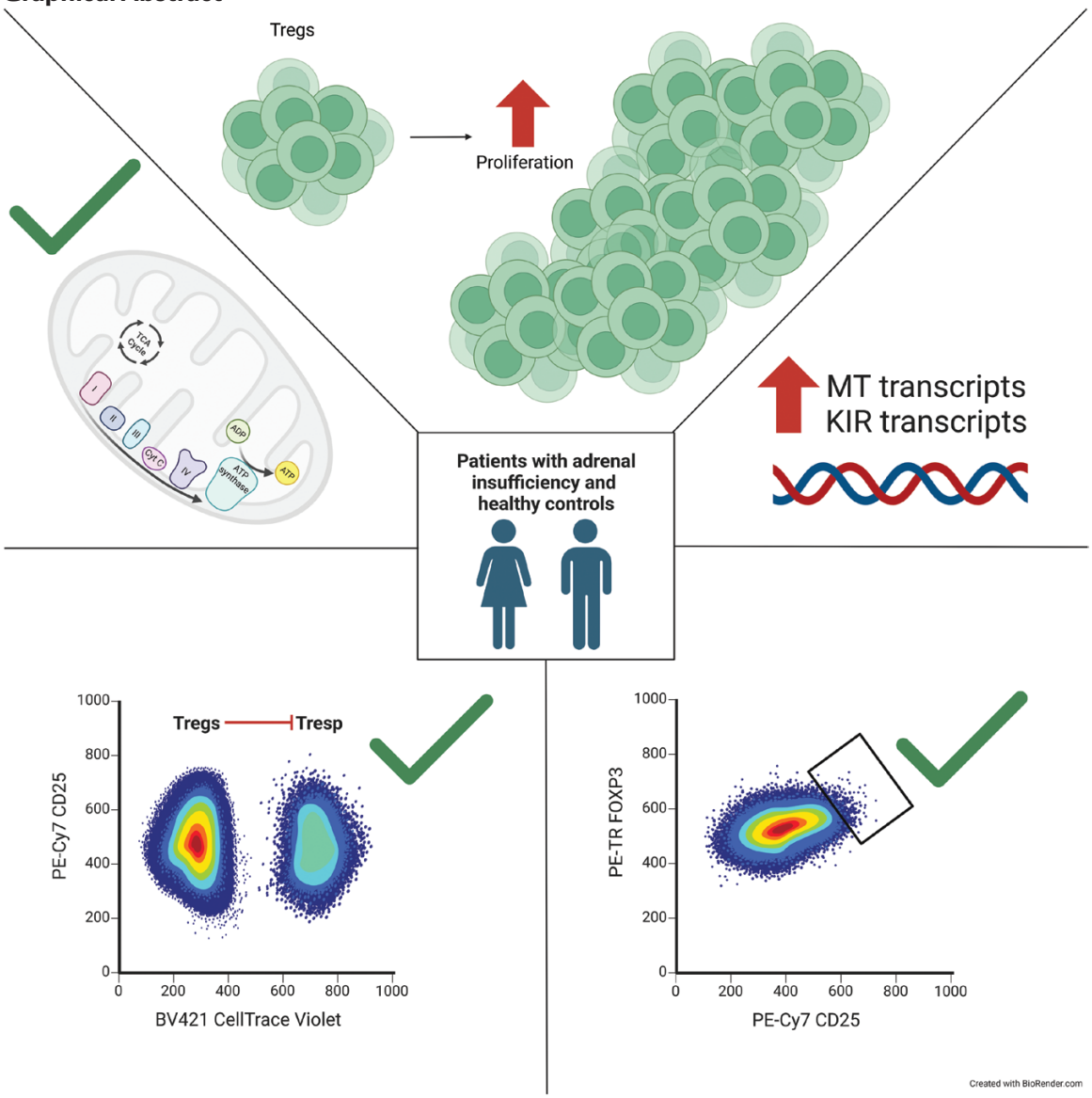
Graphical Abstract



Keywords: regulatory T cells (Tregs), autoimmune disease, adrenal insufficiency, RNA sequencing, Treg suppression assay

Abbreviations: AIRE: autoimmune regulator; APS: autoimmune polyendocrine syndrome; PAI: primary adrenal insufficiency; PBMC: peripheral blood mononuclear cells; Tregs: regulatory T cells.

Introduction

The main cause of primary adrenal insufficiency (PAI) in industrialized 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 of hypoparathyroidism and chronic mucocutaneous candidiasis define the monogenic APS-1, caused by mutations in the *autoimmune regulator* (*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 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 has also been described [15, 16]. Importantly, the

stability and function of Tregs are 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 (PBMC) 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.

Methods

Patients, controls, and ethical considerations

Six patients with isolated PAI, 16 patients with APS-2, 1 patient with PAI and primary ovarian insufficiency (POI), and 1 patient who had undergone an adrenolectomy, all enrolled in the Norwegian National Registry for Organ Specific Autoimmune Diseases (ROAS), were included in the study [16 females, 8 males, mean age 51.6, range (19–78) years, Table 1]. All patients have given their written informed consent for participating. The patients were treated with hormone substitution therapy based on the individuals' endocrine manifestations, i.e. patients with PAI are treated with hormone substitution using cortisone acetate or hydrocortisone in physiological doses, while patients with hypothyroidism are treated with levothyroxine. Whole blood from 41 sex- and age-matched healthy donors [25 females, 16 males, mean age 49.1, range (22–74) years] was obtained from the Blood Bank at Haukeland University Hospital

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].

(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 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).

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells were isolated using Ficoll density gradient centrifugation. Cells were frozen in human AB serum supplemented with 10% dimethyl sulfoxide (DMSO), stored at −80°C for 2–3 days, and further at −150°C until use.

Flow cytometric analysis for PBMCs

Peripheral blood mononuclear cells 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 TCR γ/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 were 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 Analyzer and the BD FACSDiva Software. FlowJo v10.2 (BD) was used to analyse flow cytometric data.

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), where non-CD4 and CD127 + cells are removed before the CD25 + population is enriched. The obtained cells are CD4 + CD25 + $CD127^{dim}$. 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°C 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°C until use.

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 (1×10^6 cells/ml) were rested overnight in TexMACS medium containing 5% human AB serum and 50 U/ml rIL2 (Miltenyi Biotec) at 37°C 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 5×10^5 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 of 1:1, 2:1, 4:1, and 8:1 for 5 days at 37°C 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 females, 2 males, 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 µg/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 µg/ml). Both molecules were added to wells in 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 were 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 Analyzers 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.

Enzyme-linked immunosorbent assay

Treg-specific cytokines were measured in supernatant from the suppression assay at the 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).

RNA sequencing and bioinformatic analysis

RNA was isolated from *in vitro* expanded Tregs from 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 were 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 were 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]. PANTHER GO analysis was applied on the upregulated genes to point at specific molecular pathways (https://pantherdb.org).

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 300×g for 5 min with zero braking. The cells were allowed to attach o/n at 37°C in a 5% CO_2 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°C 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 to block ATP production, uncouple oxygen consumption from ATP production, and block 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 min, followed by 56°C for 4 h. Oxygen consumption rate values were normalized to relative gDNA contents quantified by the QIAxpert spectrophotometer (Qiagen).

Statistical analysis and figures

An unpaired parametric *t*-test was used to examine differences between patients and healthy controls for the flow cytometry characterization, suppression assay and ELISA, while a Mann–Whitney test was used for Seahorse experiments with fewer samples. A *P* value less than 0.05 was considered statistically significant, except for flow cytometric and cell metabolic assays, where the significance threshold level was set to $P < 0.01$. All statistical analyses and figures were made using the GraphPad Prism 9.1.0 software (GraphPad Software).

Results

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).

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 [15×10^6 (range 2.0×10^6–38.0×10^6] compared to healthy controls [9.8×10^6 (range 1.7×10^6–19.7×10^6], $P = 0.0081$, Supplementary Fig. 2B). These observations do not support the initial hypothesis of fewer Tregs in PAI patients but could instead point to different regulation of their Tregs.

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 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, patients 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 towards lower ability

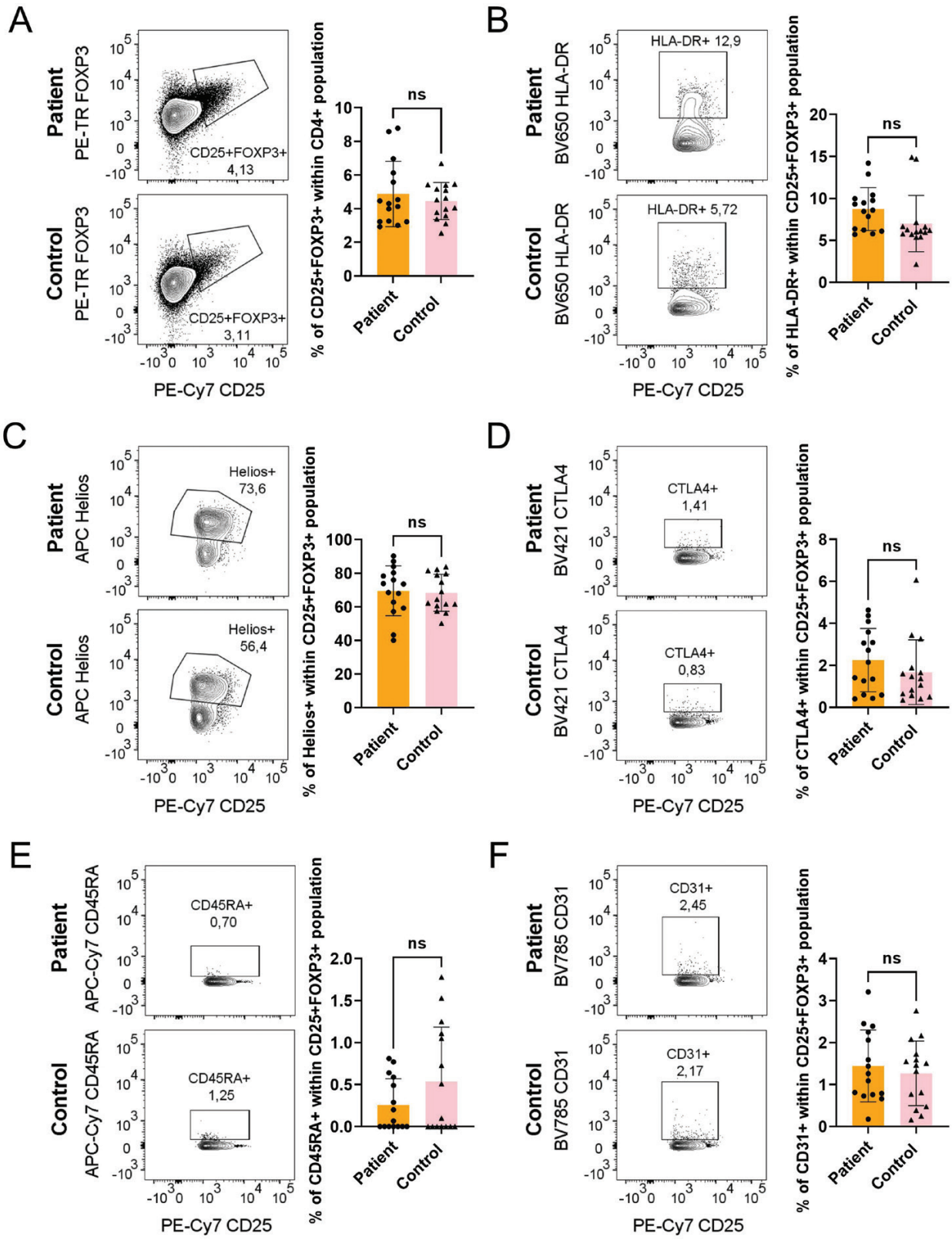


Figure 1. Flow cytometric analysis of PBMCs from patients with PAI ($N = 20$) and healthy controls ($N = 20$). (A) Treg frequencies in a representative patient and healthy control. Expression of (B) HLA-DR, (C) Helios, (D) CTLA4, (E) CD45RA, and (D) CD31 within the CD4 + CD25 + FOXP3 + population. All 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.

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 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 µg/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).

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, 1 patient who had undergone an adrenolectomy, and 4 patients with isolated PAI (Table 1). 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). Sixty genes were found significantly differentially expressed (adjusted $P < 0.05$ and $|\log2FC| > 0.1$). Among these, 54 were significantly upregulated, while only 6 genes were significantly downregulated in patients compared to controls (Fig. 3A; Supplementary Table 2). Panther GO ontology revealed strong enrichment for genes involved in mitochondrial electron transport and natural killer (NK) cell-mediated cytotoxicity among the upregulated genes (Supplementary Table 3). Genes in the latter category included several killer cell lectin-like receptor C (KLRC) family genes, including *KLRC4* (log2FC 3.314), *KLRC3* (log2FC 3.286), and *KLRC2* (log2FC 2.984). 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. The six downregulated genes were loaded onto String to search for any functional links between them; however, none were identified (Fig. 3B). 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. 3C), pointing to mitochondrial

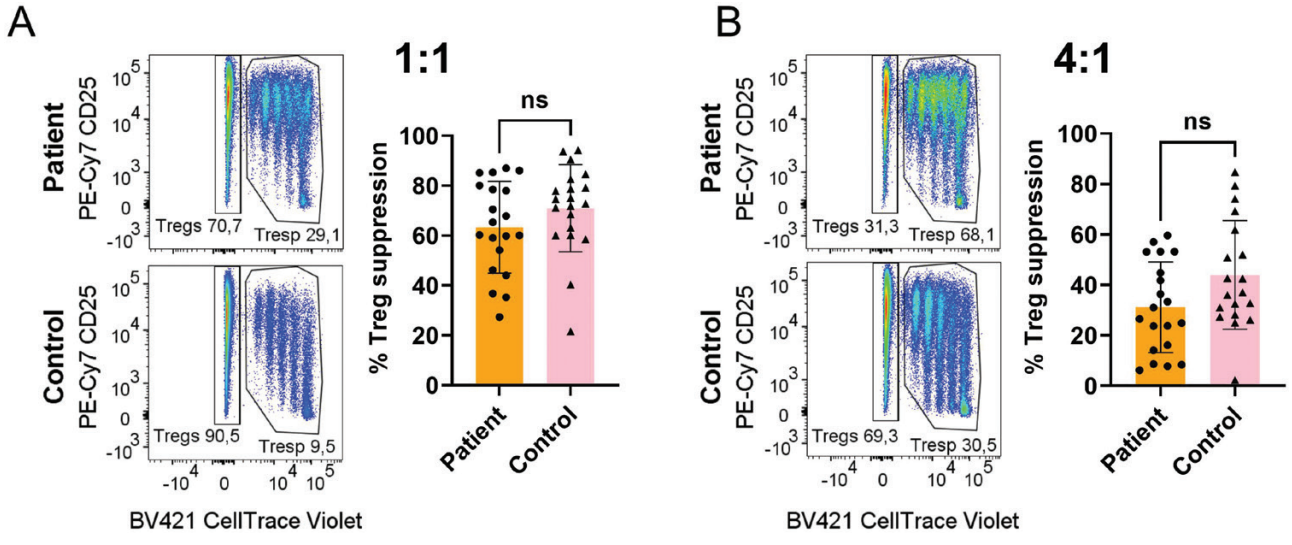


Figure 2. Treg suppression assay with expanded Tregs for patients with PAI ($N = 20$) and healthy controls ($N = 20$). CellTrace Violet labelled responder cells were co-cultured with Tregs, in the presence of anti-CD3/28 and IL2 at ratios (A) 1:1 for 20 patients and 20 controls, (B) 4:1 for 5 patients and 5 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.

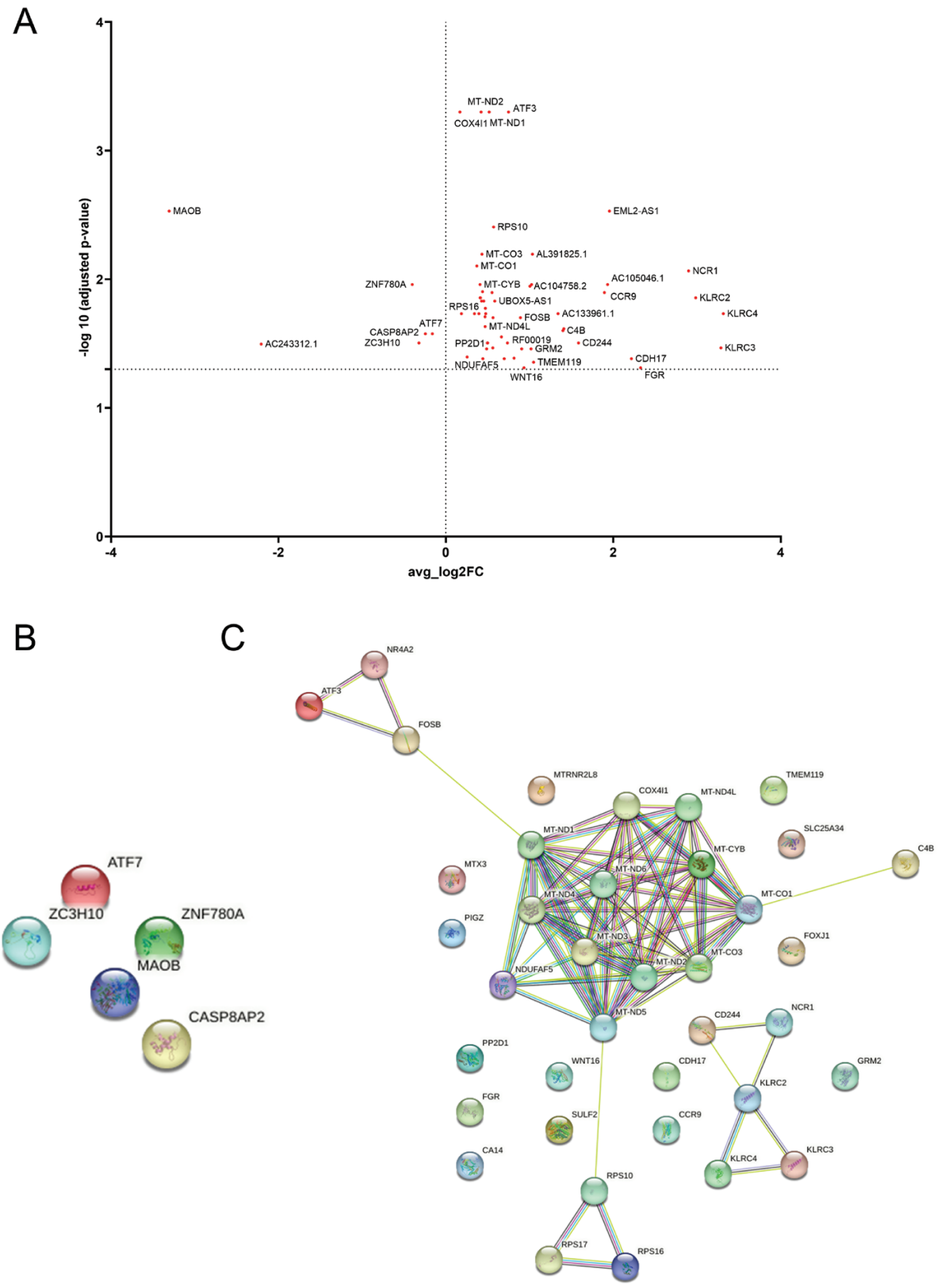


Figure 3. (A) Distribution of differentially expressed genes in expanded Tregs for PAI patients ($N = 16$) and healthy controls ($N = 16$). Significantly differentially expressed annotated genes between patients and healthy controls presented in a volcano plot. Genes were set as significantly differentially expressed if the adjusted P value < 0.05 and $|\text{log2FC}| > 0.1$. (B and C) String pathway analysis of differentially expressed genes (B) Functional relationships between significantly downregulated genes in the patient cohort compared to healthy controls. (C) 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 $|\text{log2FC}| > 0.1$.

pathways and cell killer responses to be differentially active in expanded Tregs from patients versus controls.

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 OCRs within both groups (Fig. 4A) and consequently there were no significant differences between the groups (Fig. 4B). 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 OCR amongst patients (Fig. 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 uncharacterized 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.

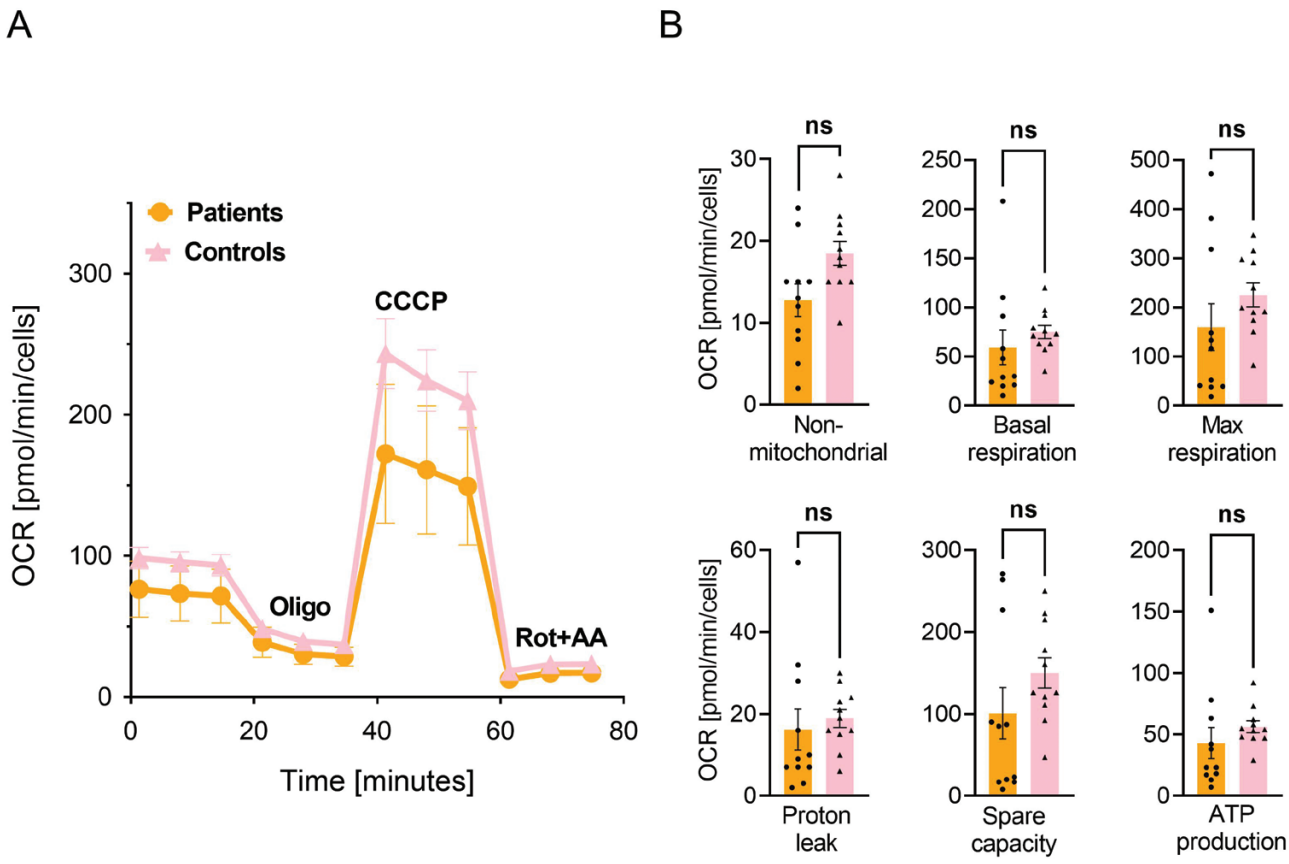


Figure 4. Measurement of mitochondrial respiration in expanded Tregs from patients with PAI ($N = 11$) and healthy controls ($N = 11$). (A) Cellular oxygen consumption rate measured by a Seahorse mitochondrial stress test. After determining basal respiration, inhibitors of mitochondrial respiration were 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. The *P* value was calculated with a nonparametric Mann–Whitney test. ns, non-significant.

Increased Treg cell death due to increased mitochondrial oxidative damage has 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 OCR 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 indicates 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 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 pollute the Tregs is not known. How an upregulation of *KLCR* genes affects Treg function in our patients also requires further investigation. 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 number, 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.

Supplementary data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Ethical approval

Donors gave their informed consent. 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.

Conflict of interests

The authors declare no conflicts of interest.

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Data availability

Data are available in the Supplementary Information (Tables 2 and 3) for differential expression in the RNA sequencing experiment. Additional data can be obtained by approaching the corresponding author Anette Wolff (anette.boe@uib.no).

Author contributions

T.S., B.E.O., and A.S.B.W. had the idea for the study; T.S. and E.S.H. collected the patient and control samples; T.S., J.I.B., and A.S.B.W. performed laboratory work and analysed data; T.S., J.I.B., E.S.H., B.E.O., and A.S.B.W. interpreted data into results and conclusions; T.S. and A.S.B.W. drafted the manuscript. All authors read the final version of the manuscript and approved submission.

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