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ORIGINAL ARTICLE

# Expanded CD4$^+$ Effector/Memory T Cell Subset in APECED Produces Predominantly Interferon Gamma

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**Abstract**
*Purpose* Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) is a rare human autoimmune disorder caused by mutations in the *AIRE* (autoimmune regulator) gene. Loss of AIRE disrupts thymic negative selection and gives rise to impaired cytotoxic and regulatory T cell populations. To date, CD4$^+$ T helper (Th) cells remain little studied. This study aims to elucidate their role in APECED pathogenesis.
*Methods* Th cells were explored in ten APECED patients and ten healthy controls using cell culture assays, multiparameter flow cytometry, and transcriptome analysis.
*Results* The proportions of effector/memory populations were increased while the fraction of naive cells was diminished. The naive population was abnormally activated, with an increased number of cells expressing characteristic Th1, Th2, and Th17 cytokines. No clear deviation to any Th subclass was observed, but transcriptome analysis suggested abnormalities in the Th1 cytokine interferon gamma (IFN-γ) pathway and flow cytometry showed that INF-γ had the highest expression. The augmented INF-γ signaling may promote the function of the putative pathogenic CD8$^+$ cytotoxic population in the patients. In addition, the frequency of CD4$^+$ recent thymic emigrants (RTEs) was decreased in the patients, and RTEs also contained cytokine-producing cells at an increased frequency.
*Conclusion* These data reveal abnormalities in the Th population and suggest that they may in part be traced to premature activation already in the thymus.

**Keywords** APECED · CD4$^+$ T cells · naive T cells · effector/memory T cells · Th subclasses · recent thymic emigrants

## Introduction

Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) is a rare, recessively inherited, monogenic human autoimmune syndrome caused by loss-of-function mutations in the autoimmune regulator (*AIRE*) gene (OMIM 240300). It is characterized by autoimmune destruction of multiple endocrine organs and chronic mucocutaneous candidiasis. Besides the most common endocrine components, hypoparathyroidism and Addison's disease, hypogonadism, diabetes, and gastrointestinal manifestations are also common [1]. The clinical presentation is highly variable, and disease manifestations differ even between siblings with a similar genetic background, but, interestingly, all patients have autoantibodies against type I interferons [2].

AIRE is a transcriptional regulator mainly expressed in medullary thymic epithelial cells (mTECs) and, to a lesser extent, in peripheral lymphoid organs. Murine experiments have linked AIRE to negative selection in the thymus by showing that it promotes ectopic transcription of tissue-restricted antigens in mTECs. The original model of APECED pathogenesis thus suggests that AIRE deficiency impairs the presentation of these antigens to developing thymocytes, allowing self-reactive T cells to escape deletion and migrate to the

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periphery [3, 4]. However, autoimmunity can also be directed against antigens not regulated by AIRE (e.g., α-fodrin, 21-hydroxylase, and SOX9), and conversely, only a fraction of AIRE-regulated tissue-specific antigens are targeted [5, 6]. Because these phenomena are difficult to explain by failed negative selection alone, alternative pathogenetic models including disrupted thymic microenvironment have been proposed [7, 8]. Nevertheless, the expression patterns of AIRE, as well as murine models, indicate that abnormal T cell responses are a key pathogenetic event in APECED.

Previous studies of APECED patients have identified a defect of regulatory T cells (Treg) manifested by decreased frequency of $CD25^+$ cells, decreased FoxP3 expression, and defective regulatory function [9–11]. $CD8^+$ T cells are also abnormal. The patients have an increased frequency of highly differentiated $CD45RA^+CCR7^-$ effector/memory-like cells, which express the cytotoxic effector molecule perforin. Interestingly, the patients seem to have an almost complete lack of a normal naive $CD8^+$ population since markers of aberrant activation are also found in $CD8^+$ cells expressing markers associated with recent emigration from the thymus [12]. This may indicate that the cells are already activated in the thymus, as has been previously suggested [7].

In contrast, very little is known of $CD4^+$ T helper (Th) cells in APECED. Most patients have neutralizing autoantibodies against the Th17 cytokines IL-17 and IL-22, which leads to a functional Th17/Th22 defect associated with the candidiasis, but whether Th17 cells themselves are affected remains unclear [13, 14]. IL-17A production after stimulation was reported either normal or increased, while IL-17F response was reduced [13, 15]. One study of five patients reported increased frequency of Th17 cells after stimulation with *Candida* [16], while another suggested a normal frequency of circulating cells co-expressing CXCR3 and CCR6, surrogate markers for IL-17A-producing cells [17]. Recently, we have studied Th17 responses in APECED patients in vivo and found a predominant defect in IL-22 production both in antigen-challenged skin and unexposed skin [18]. Similarly, the composition of the $CD4^+$ population has been reported to be either normal or skewed, and the full pattern of functional Th differentiation has not been studied [17, 19, 20]. Here, we report the characteristics of the circulating Th cell population in Finnish APECED patients.

## Methods

### Subjects

The study included ten adult APECED patients (mean age = 41.7 years, range = 23–65 years, 6/10 female); nine were homozygous for the Finnmajor mutation R257X and one patient had R257X mutation and 1085/1097 deletion. The most common disease components were chronic mucocutaneous candidiasis (10/10), hypoparathyroidism (8/10), Addison's disease (8/10), acute hepatitis, keratopathy, ovarian failure, diabetes, hypothyroidism, severe chronic constipation, alopecia, and vitiligo (ESM S1). At the time of sampling, the patients did not receive systemic immunosuppressive treatment, had no acute infections, and none was pregnant. The control group consisted of ten sex- and age-matched healthy individuals (mean = 40.5 years, range = 22–54 years, 6/10 female). The study was conducted according to The Declaration of Helsinki principles and was approved by the Ethics Committee of Helsinki University Hospital. Written informed consent was obtained from the subjects.

### Samples and Flow Cytometry

Blood samples were drawn into EDTA Vacutainer tubes (BD Biosciences), plasma was separated by centrifugation, and peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque (GE Life Sciences) gradient centrifugation. Cells were stained freshly or after overnight anti-CD3 stimulation. The monoclonal anti-CD3 antibody (clone MEM-57 from ImmunoTools) was bound on 24-well plates and cells were incubated in +37 °C in RPMI-based media containing 10 % human AB serum, 1 % HEPES, penicillin–streptomycin, L-glutamine, and 2-mercaptoethanol. Primary mouse and rat antibodies detecting extracellular markers were added in a single step. The direct fluorescent antibodies used were CD4-APC-Cy7, CCR7-PeCy7 (both from BD Biosciences) and CD45RA-FITC (ImmunoTools). Biotinylated CD31 (eBioscience) was added simultaneously with the direct fluorescent antibodies and coupled to streptavidin–PE-Texas Red (BD Biosciences) in the next step. Cells were permeabilized using a FoxP3 permeabilization kit (eBioscience) and stained with the following intracellular antibodies: IFN-γ-APC (eBioscience), IL-4-PE, and IL-17A-PerCP-Cy5.5 (both from BioLegend). In cell sorting, the following direct fluorescent antibodies were used: CD4-Pe (BD Biosciences), CD45RA-FITC, and CD31-APC (both from ImmunoTools). Cells were analyzed with Cyan ADP instrument (Beckman Coulter) and cell sorting was performed with FacsAria instrument (BD Biosciences). Data analysis was done with Summit software (Beckman Coulter).

### Real-Time PCR

Total DNA was extracted with DNeasy kit (Qiagen). The quantity of T cell receptor excision circles (TRECs) was determined with real-time polymerase chain reaction (PCR) using primers 5′-CACATCCCTTTCAACCATGCT-3′ and 5′-GCCAGCTGCAGGGTTTAGG-3′ and probe 5′-6FAM-GACACCTCTGGTTTTTGTAAAGGTGCCCACT-3′. The results were normalized against the signal from exon 2 of

the SOCS1 gene (TaqMan assay from Applied Biosystems), representing the genomic DNA.

### Transcriptome Analysis

The transcriptome of patient and control lymphocytes was determined adapting the previously described single-cell reverse transcription method for 5′-end RNAseq [20] and upscaling the reaction volumes for microtiter plates. Total bulk RNA was extracted from about $4 \times 10^5$ cells with RNeasy kit (Qiagen) and diluted to 4 ng/μL concentration. Two microliters of RNA lysis buffer was added to 1 μL of each RNA sample on a 96-well plate, incubated at 71 °C for 3 min, and then cooled to 4 °C for 10 min. A volume of 3.6 μL of the reverse transcription mix was added on the samples and incubated at 42 °C for 90 min and subsequently at 70 °C for 10 min. The acquired cDNA was amplified by PCR and quantified by Qubit Fluorometer (Life Technologies).

Six nanograms of cDNA was simultaneously fragmented and barcoded by Tn5 tagmentation, isolated with beads, and quantified by KAPA Library Quant (Kapa Biosystems). Sequencing was performed on an Illumina HiSeq 2000 instrument generating 50-bp reads, including a 6-bp unique molecular identifier along with 8-bp index reads corresponding to the cell-specific barcode. Components of the library reaction mixes have been described earlier [20, 21], and details of the modified protocol are available on request. Sequence reads were filtered and trimmed as described earlier [20, 21] and aligned to the human UCSC reference genome. PCR bias was eliminated by unique molecular identifiers and counting of unique molecules rather than reads.

### Statistics

Statistical analysis was performed with SPSS software (SPSS Inc.). The results are shown as the mean ± standard deviation (SD). Normality of the data was tested with Shapiro–Wilk test. Statistical significance was tested with Student's two-tailed *t* test for normally distributed datasets; otherwise, Mann–Whitney *U* test was used for non-dependent datasets and Wilcoxon signed-rank test for dependent datasets. The limit for statistical significance was $p < 0.05$.

The differential expression of RNA sequences was calculated in Chipster open-source data analysis platform (chipster.csc.fi) using edgeR with a *p* value cutoff of 0.05, Bonferroni–Hochberg multiple testing correction, tagwise dispersion method, and dispersion value at 0.16. Transcriptional networks were assessed with QIAGEN's Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City; www.qiagen.com/ingenuity). IPA recapitulates the effect of different genes affecting the same transcriptional pathway and represents it as a *z* score, which predicts the activation status of a transcriptional regulator in a functional pathway. The analysis counted molecular relationships that have been experimentally observed and published in peer-reviewed journals.

## Results

### Skewed Composition of the $CD4^+$ T Cell Population

Previous studies have shown that the composition of the $CD8^+$ T cell population in APECED patients is skewed, but data on $CD4^+$ T cells are conflicting [12, 19, 20]. In our cohort, the frequencies of $CD4^+$ and $CD8^+$ T cells and the CD4/CD8 ratio were similar in patients and controls (Table 1). However, the frequency of naive $CD4^+$ T cells, defined as $CD45RA^+CCR7^+$ cells, was significantly decreased in the patients (25.7 ± 14.7 vs. 41.5 ± 10.3 %, $p = 0.01$). Conversely, the frequency of the $CD45RA^-$ effector/memory population was higher in patients, but with considerable interindividual variation. When the $CD45RA^-CCR7^+$ central memory and $CD45RA^-CCR7^-$ effector memory cells were analyzed separately, the latter subset was significantly increased in the patients. These results indicate that also the $CD4^+$ T cell population in APECED patients is biased, with accumulation of effector-type cells. Notably, the terminally differentiated $CD45RA^+CCR7^-$ effector subset, also termed effector memory $RA^+$, highly enriched within the

**Table 1** Frequencies of $CD8^+$ and the main $CD4^+$ helper T cell populations

| Population | Definition | Frequency (%) | | *p* |
|---|---|---|---|---|
| | | Patient | Control | |
| $CD4^+$ | | 38.9 ± 13.7 | 41.3 ± 7.1 | NS |
| $CD8^+$ | | 28.3 ± 7.3 | 26.0 ± 4.6 | NS |
| CD4/CD8 ratio | | 1.4 | 1.6 | NS |
| Naive | $CD4^+CD45RA^+CCR7^+$ | 25.7 ± 14.7 | 41.5 ± 10.3 | 0.01 |
| Effector memory | $CD4^+CD45RA^-CCR7^-$ | 32.0 ± 14.2 | 19.4 ± 7.4 | 0.01 |
| Central memory | $CD4^+CD45RA^-CCR7^+$ | 24.8 ± 9.8 | 22.3 ± 6.7 | NS |
| Effector memory RA | $CD4^+CD45RA^+CCR7^-$ | 5.3 ± 4.7 | 5.1 ± 4.2 | NS |

*NS* non-significant

$CD8^+$ compartment, was not increased in $CD4^+$ T cells (Fig. 1).

### Naive-Like Cells Produce Elevated Amounts of Th Cytokines

An integral part of $CD4^+$ T cell function is their functional differentiation upon activation. To determine whether APECED patients have a skewed pattern of differentiation, we used flow cytometry to measure the frequency of cells producing the key Th cytokines IFN-γ, IL-4, and IL-17. Overall, the frequency of $CD4^+$ T cells producing any Th effector cytokine was higher in the patients, a phenomenon most notable in the naive population (3.4 ± 2.3 vs. 1.3 ± 0.9 %, $p = 0.04$). In the memory/effector subsets, the difference was not significant (Fig. 2a).

The cytokine with the highest expression in both patients and controls was IFN-γ, whereas cells producing IL-4 or IL-17 were less frequent. Individual variation, however, was high, especially in the patients. IFN-γ expression was higher in patients than in controls throughout the naive, effector, and memory cell populations, with the biggest difference found in the naive subset (3.0 ± 2.9 vs. 1.0 ± 0.8 %, $p = 0.05$). Similarly, IL-4 expression was elevated in several patient cell subsets, especially the naive population (Fig. 2b, c). T cells producing IL-17 were very rare in both controls and patients, with no significant difference between the groups (data not shown).

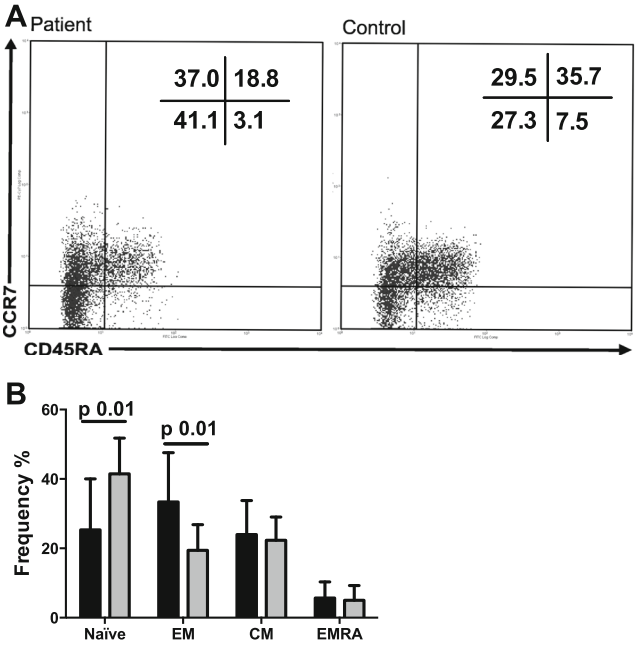


**Fig. 1** Naive cell frequency is diminished and effector/memory frequencies are augmented in APECED. The frequencies of naive and effector/memory populations are gated from $CD4^+$ lymphocytes. **a** Dot plots of the median patient and control. **b** Diagram of the mean population frequencies and standard deviations. *Black bars* represent patients; *gray bars* represent controls. Populations defined as naive $CD45RA^+CCR7^+$, effector memory (*EM*) $CD45RA^-CCR7^-$, central memory (*CM*) $CD45RA^-CCR7^+$, and effector memory RA (*EMRA*) $CD45RA^+CCR7^-$

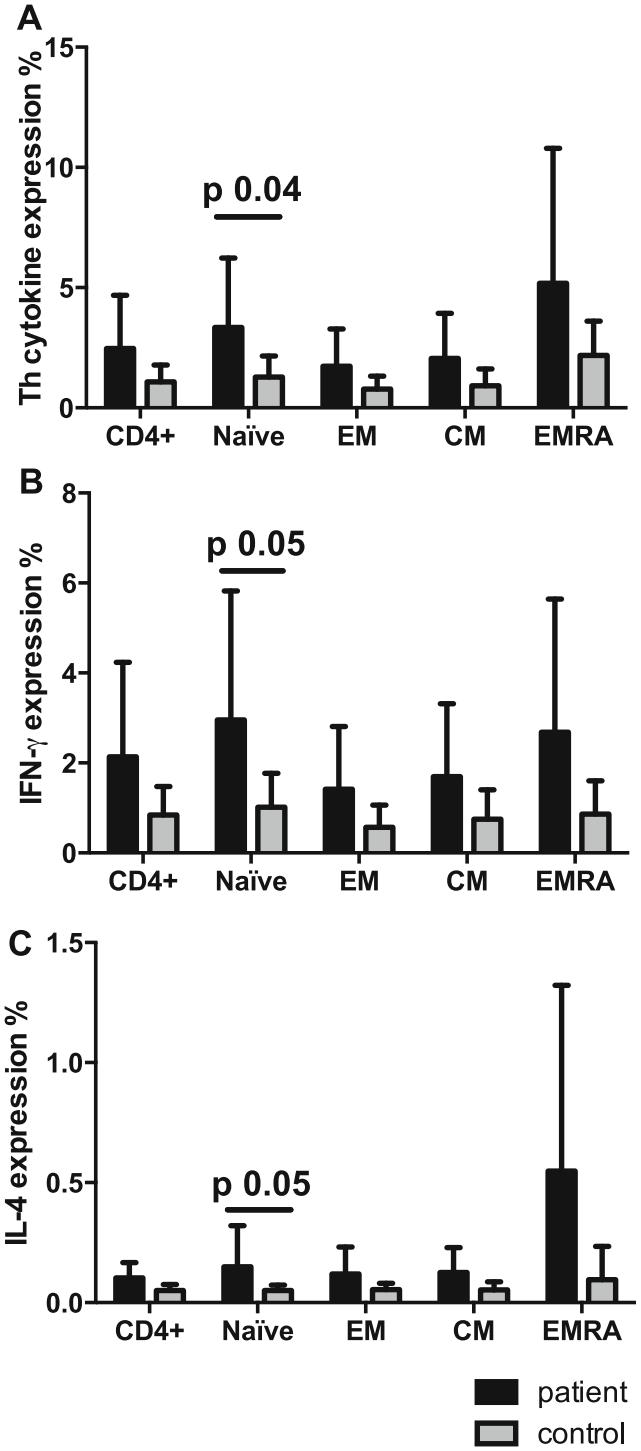


**Fig. 2** Th cytokine production is upregulated in APECED. The proportions of cells expressing any Th cytokine (**a**), INF-γ (**b**), and IL-4 (**c**) in all $CD4^+$ cells, naive, EM, CM, and EMRA populations are shown. *Black bars* represent patients; *gray bars* represent controls

To further characterize the functional pattern in T cells from APECED patients, we performed a transcriptome analysis of circulating T cells from three patients and five healthy controls. These data supported the abnormal activation status, with emphasis on the IFN-γ pathway. Network analysis of gene pathways in IPA suggested the IFN-γ pathway to be activated in patient cells with an activation $z$ score of 3.821 ($p = 2.47 \times 10^{-24}$). In a more detailed analysis, 218 of 393 differentially expressed genes in the IFN-γ pathway showed expression consistent with the activation of the pathway in the patients (Fig. 3 and ESM S2). There was no significant upregulation in the IL-4 or the IL-17 pathway in either the patients or controls.

## Patient Helper T Cells Show Increased Plasticity

A functionally committed T cell can gain the properties of another T cell lineage, losing or preserving the markers of its previous commitment. This phenomenon, called plasticity, is frequently associated with inflammatory conditions [21]. In our analysis, $CD4^+$ T cells expressing simultaneously two characteristic helper T cell cytokines were very rare, but nonetheless, they were more common in APECED patients than in healthy controls. In particular, a small subset of cells with overlapping Th1/Th17 characteristics was detected in the patients, mostly in the $CD45RA^+$ population (0.20 vs. 0.06 %, $p = 0.04$), again consistent with the increased activation of the naive population (Fig. 4).

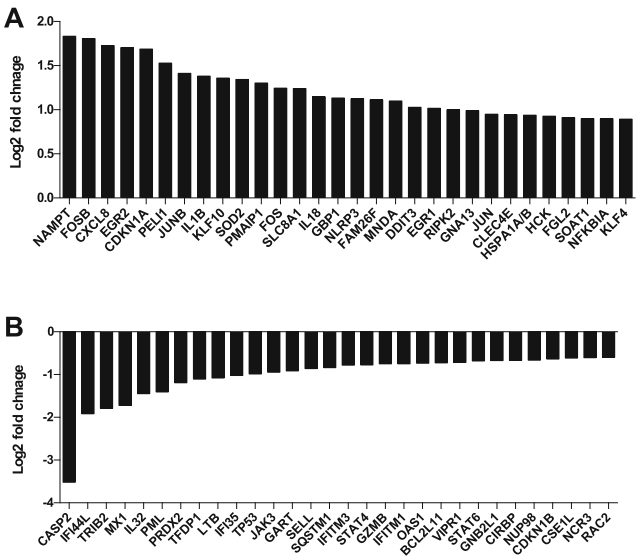


**Fig 3** IFN-γ pathway-associated genes are differentially expressed, suggesting IFN-γ upregulation in patients. Shown are the top 30 differentially expressed genes whose activation (**a**) or inhibition (**b**) is related to IFN-γ pathway upregulation in patients. Data are shown as log2 fold change. All 393 differentially expressed genes on the IFN-γ pathway in patients are listed in ESM S2

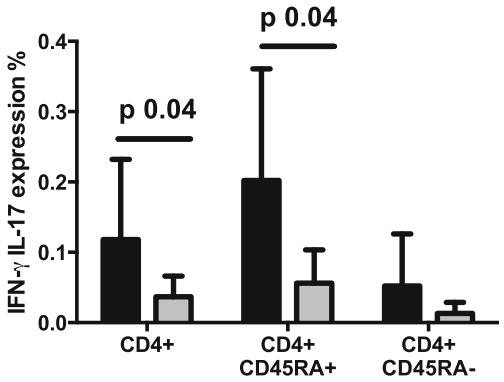


**Fig. 4** The $CD4^+CD45RA^+$ population contains cells with overlapping Th1/Th17 characteristics in APECED. Proportions of cells expressing simultaneously IFN-γ and IL-17 in $CD4^+$, $CD4^+CD45RA^+$, and $CD4^+CD45RA^-$ populations are shown. *Black bars* represent patients; *gray bars* represent controls

## In Vitro Stimulation of T Cells Attenuates the Differences in Cytokine Expression Between Patients and Controls

We then analyzed the polarization of cells cultured overnight with the mitogenic anti-CD3 antibody, which mostly stimulates memory T cells when used alone, without ligation of co-stimulatory molecules. As expected, in healthy controls, the stimulation resulted in the upregulation of IFN-γ, mainly in the effector memory and central memory T cell populations, while in naive $CD4^+$ T cells, there was little overall change (Fig. 5a). The expression of IL-4, in contrast, was increased in both memory and naive T cells (Fig. 5b). Despite the stimulation, IL-17 expression remained very low. Analysis of IL-4 and IL-17 showed no significant difference between patients and controls.

Surprisingly, in the patients, the stimulation through CD3 failed to produce a consistent IFN-γ response. This was particularly marked in the naive subset in which the stimulation resulted in a decreased frequency of IFN-γ-producing cells (Fig. 5a). These data were supported by transcriptome analysis. The effect of anti-CD3 stimulation on lymphocyte gene expression was calculated with edgeR. Following the stimulation, the expression of 206 genes was differentially expressed in patients, whereas in controls 437 genes were differentially expressed (ESM S3 and S4 for patients and controls, respectively). Only 93 of the differentially expressed genes were shared between patients and controls. In control samples, the anti-CD3 stimulation augmented or suppressed the expression of many genes linked to the IFN-γ pathway. In contrast, in patients, the overall effect of stimulation on the IFN-γ pathway was clearly suppressive, with an activation $z$ score of −4.718 ($p = 4.18 \times 10^{-22}$). Stimulation had no clear net effect on the expression of the two other helper cytokines in either patients or controls.

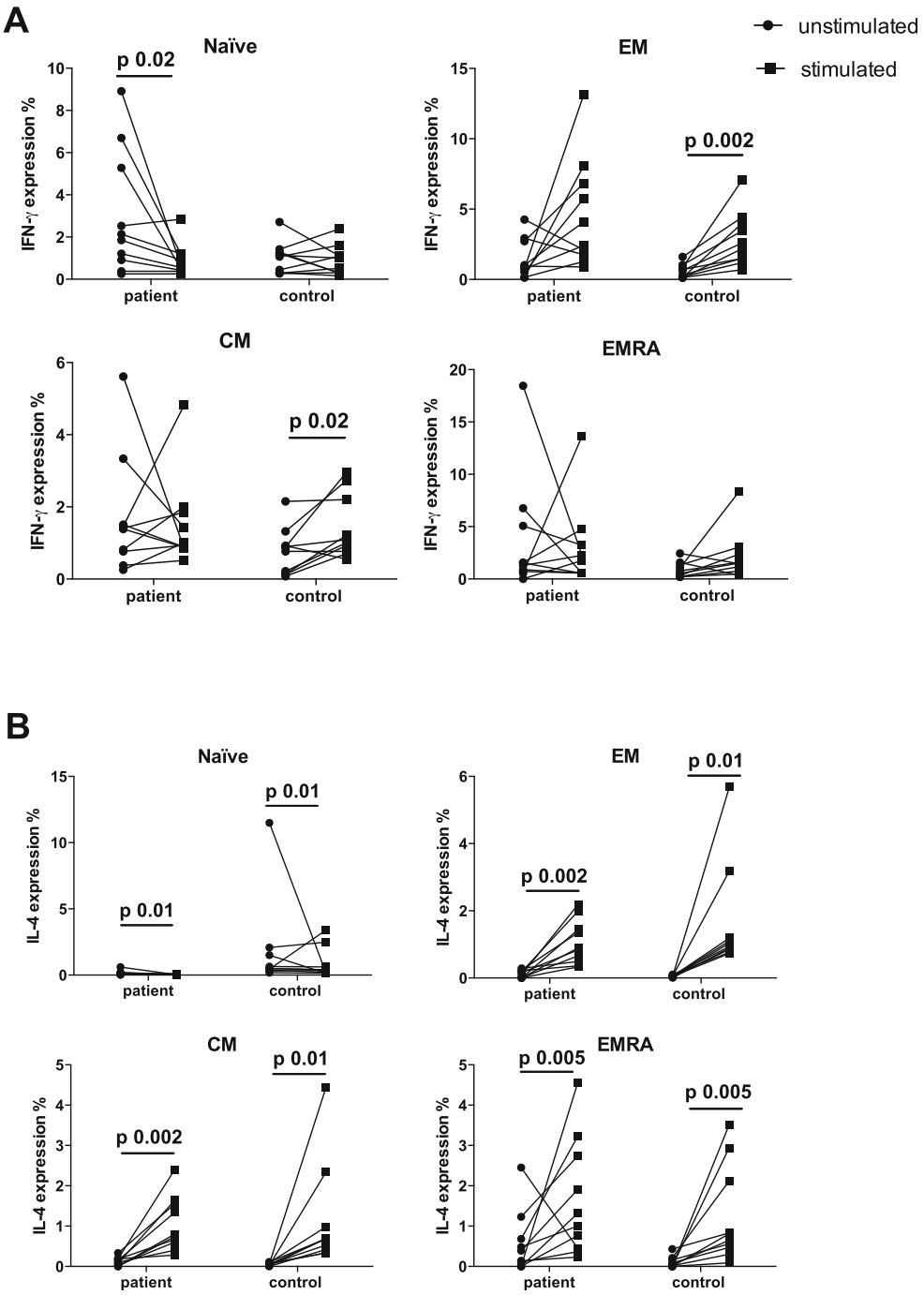


**Fig. 5** Effector memory and central memory populations in APECED patients fail to upregulate IFN-γ production following anti-CD3 stimulation. The effects of anti-CD3 stimulation on the proportions of IFN-γ- (**a**) and IL-4 (**b**)-producing cells in naive, EM, CM, and EMRA populations are shown

### Recent Thymic Emigrants Within the Helper T Cell Population Are Also Affected

The highest expression of AIRE is found in the thymus, suggesting that the T cell abnormalities may originate already in the thymus [4]. Since thymic tissue was not accessible in APECED patients, a surrogate had to be used. Several studies have shown that CD31 is expressed by recent thymic emigrants (RTEs), which still retain TRECs. As extrachromosomal DNA elements, these remnants of thymic TCR rearrangements are not replicated during mitosis and become gradually rarer [22]. To establish that CD31 is associated with RTEs also in APECED patients, we isolated $CD4^+CD45RA^+CD31^+$ and $CD4^+CD45RA^+CD31^-$ cells from four patients and five controls and used qPCR to measure their TREC content. No TRECs were detected in naive $CD31^-$ cells from either patients or controls. In contrast, all five $CD31^+$ samples from the controls were $TREC^+$, and in the patients, three in four of the $CD31^+$ samples contained TRECs. The relative TREC expression was slightly lower in the $CD31^+$ cells isolated from the

patients, with a difference of 1.7 in normalized mean delta cycle (Fig. 6). These data indicate that $CD31^+$ is an RTE marker also in the patients, but suggest abnormalities in the dynamics of the naive population. Indeed, the frequency of $CD45RA^+$ RTE cells was reduced in patients (17.1 ± 9.5 vs. 25.6 ± 8.6 %, $p = 0.01$; Fig. 7a, b). The frequency of $CD4^+$ RTE cells producing IFN-γ or IL-4 was also higher in the patients, although the variation was high and only IL-4 showed a statistically significant difference (Fig. 7c).

## Discussion

Our current results identify abnormalities in the $CD4^+$ helper T cell population in APECED patients, suggesting a role in the pathogenesis of the endocrine autoimmunity. In the healthy controls studied here, the frequencies of the main subsets of naive and effector/memory cells matched well those reported in a recent study on organ donor T cell subsets [23]. In contrast, in APECED patients, we found an expanded effector memory population and a diminished naive population. In addition, the patient $CD4^+$ population produced elevated amounts of Th cytokines, but no clear deviation to one of the helper classes was detected. Apart from the previously recognized defect of Treg and Th17 cells, little is known about other $CD4^+$ Th populations in APECED. Earlier reports on $CD4^+$ and $CD8^+$ proportions have been contradictory, but a flow cytometric study with 19 patients found $CD4^+$ and $CD8^+$ populations to be normal, a finding consistent with our results [17]. Previous data on the $CD4^+$ naive and effector/memory population frequencies are likewise unclear, as one study reported unaltered frequencies and a cohort of four patients even found a diminished effector memory subset [15].

The Th cytokine production and differentiation in APECED has been assessed only in a few studies. Although the autoimmune manifestations in $AIRE^{-/-}$ mice have been connected to Th1 responses, a human study found no bias in IFN-γ production [15]. Another human study even described reduced IFN-γ levels in stimulated lymphocyte culture supernatants in four female APECED patients, thus evoking an idea of Th2 dominance [19]. Our results show increased activation of both the Th1 and Th2 subsets, although the frequency of $IL\text{-}4^+$ cells was very low. T cells with simultaneous expression of IFN-γ and IL-17 were also found at a low yet increased frequency in the patients. These data are in contrast with earlier results reporting the same Th1/Th17 subset to be decreased in APECED, but these data were based on cell surface markers and the production of IFN-γ or IL-17 was not evaluated [17].

In relative terms, both IFN-γ and IL-4 were similarly increased in the patients, and no apparent Th bias could be detected. Even so, IFN-γ and the IFN-γ pathway in general were emphasized as the most abundant cytokine both in flow cytometric and transcriptome analyses. Transcriptome analysis was performed on unsorted PBMCs, so we cannot rule out sources other than Th cells for the increased expression. $CD8^+$ T cells, natural killer (NK) cells, and natural killer T (NKT) cells are all able to produce significant amounts of IFN-γ [24]. However, the FACS data indicate that the $CD4^+$ population is likely to be mainly responsible for the upregulation of the IFN-γ pathway in APECED as $CD8^+$ cells did not produce increased amounts of IFN-γ (data not shown). The NK and NKT cells only form a minor population in the peripheral blood and their frequencies are unaltered in APECED [17].

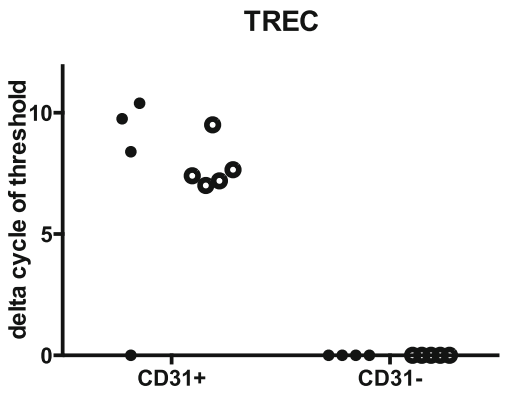


**Fig. 6** TREC is detected in $CD4^+CD45RA^+CD31^+$ cells in APECED. Data are shown as TREC-PCR delta cycle of threshold in $CD4^+CD45RA^+CD31^+$ and $CD4^+CD45RA^+CD31^-$ populations. *Full circles* represent patients; *open circles* represent controls

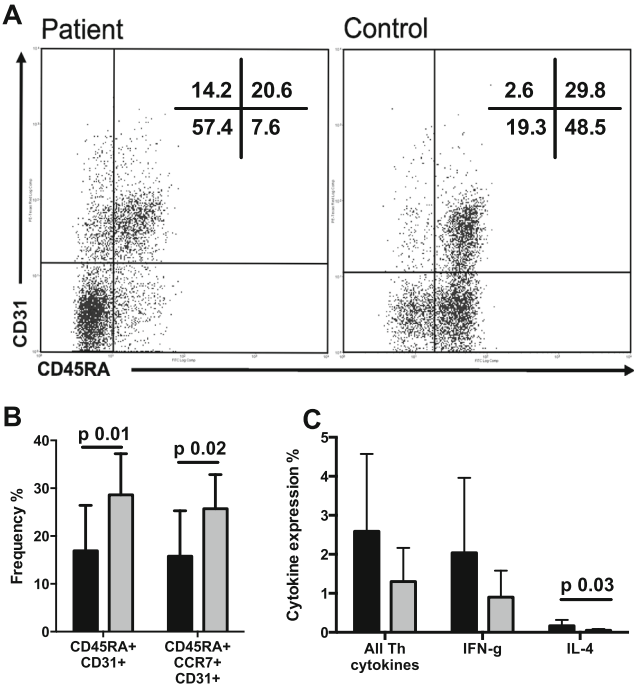


**Fig. 7** $CD4^+$ RTE ($CD45RA^+CD31^+$) population is diminished in patients and the production of Th cytokines is augmented among them. **a** RTE population gated from $CD4^+$ cells in median patient and median control. **b** Mean frequencies and standard deviations of RTE and naive-RTE populations. **c** Th cytokine production in $CD4^+$ RTE cells. *Black bars* represent patients; *gray bars* represent controls

Our data thus indicate the presence of an expanded $CD4^+$ effector memory subset with the characteristics of Th1 cells in APECED patients. Together with the presence of a highly expanded perforin-expressing subset of cytotoxic $CD45RA^+CD8^+$ effector/memory cells, this suggests a Th1-driven axis of cell-mediated autoimmunity as an important part of the pathogenesis of APECED. Although the cytotoxic lymphocytes may be responsible for the organ-specific tissue damage, the Th1 subset is likely to be important in providing activating signals and help for the cytotoxic $CD8^+$ population.

The transcriptome analysis also indicated a clear repression of the IFN-γ pathway upon in vitro stimulation. Flow cytometric analysis showed that $CD4^+$ T cells from the APECED patients were not able to increase IFN-γ production after stimulation, from the high levels observed already ex vivo. Since a substantial fraction of the T cells expressing IFN- γ already before stimulation is likely to be autoreactive, these data suggest a previously unidentified functional defect in normal, non-autoreactive Th1 responses. A further indication of possibly impaired cellular immune responses are the high levels of neutralizing antibodies against type I interferons, found virtually in every patient [2]. The significance of these abnormalities remains to be determined since no clear clinical immunodeficiency consistent with impaired cellular immunity has been reported.

The presence of an expanded Th1 subset expressing effector memory markers obviously cannot be separated from the chronic inflammatory conditions and prolonged autoimmune process, however important it may be for maintaining the disease process. Studying pediatric subjects with shorter disease duration might be useful in differentiating the effect of chronic inflammation from the disease itself. Indeed, many characteristic autoantibodies, e.g., against type 1 interferons, are present in patients already long before clinical autoimmune manifestations. It is thus possible that pathogenetic alterations in the Th population are also an early event and might be more easily distinguished in pediatric patients. However, results from adults and children are not directly comparable as the proportions of naive, effector, and memory populations and even the production of Th effector cytokines are age-dependent [25].

However, the increased cytokine production found in the naive and RTE compartments and the decreased frequency of $CD4^+$ RTE cells suggest that the abnormal activation may also be part of the original pathogenetic process in the thymus. Peterson and colleagues have suggested that AIRE deficiency in the thymus may lead to aberrant thymic microenvironment, with formation of tertiary lymphoid nodes, cytokine imbalance, and precocious activation of autoreactive T cells already in the thymus. Although our data are by necessity indirect, they are compatible with such a scenario. They thus add to the previously identified abnormalities in the RTE subset of both $CD8^+$ and Treg cells in APECED patients.

In conclusion, we report here a disruption of Th homeostasis in APECED, with decreased and abnormally activated naive-like population and expanded effector memory population, mostly with Th1 characteristics. These results identify an IFN-γ-associated pathway in maintaining the autoimmune process, but also suggest that the changes may partly be of thymic origin. The data thus provide further indirect support for defective thymic microenvironment as an important factor in APECED pathogenesis.

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**Authorship Contributions** N.H. and T.P.A. designed the study, interpreted the results, and wrote the article. N.H., S.M.L., H.M., and P.S. collected and analyzed the data. E.K., S.M.L., P.S., and H.J. critically revised the article.

**Compliance with Ethical Standards** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

**Conflicts of Interest** The authors declare no conflict of interest.

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