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Longitudinal Immune Profiling in Autoimmune Polyendocrine Syndrome Type 1

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ABSTRACT

Autoimmune polyendocrine syndrome Type-1 (APS-1) is a rare, but severe organ-specific autoimmune disease caused by mutations in the autoimmune regulator (AIRE) gene. Lack of AIRE causes autoreactive T cells to escape negative selection and alters the T regulatory cell subset. However, little is known about how the immune cell subsets vary across the lifespan in APS-1. Here we analysed the peripheral distribution of 13 immune cell subsets along the lifespan using epigenetic quantification. We found the largest discrepancy in immune cells to appear early in APS-1 patients' lives, coinciding with the time point they obtained most of their clinical symptoms. We further revealed longitudinal changes in cell compositions both within the adaptive and the innate arms of the immune system. We found that cell frequencies of B cells, T-cell subgroups, nonclassical monocytes, and Natural Killer cells to be reduced in young APS-1 patients. We also found B-cell frequencies to decrease with ageing in both patients and healthy controls. Our results suggest that Tregs, follicular helper T, and natural killer cells have opposing trends of cell frequencies during life, indicating the importance of considering the age profiles of cohorts which could otherwise lead to conflicting conclusions.

1 | Introduction

Autoimmune polyendocrine syndrome type-1 (APS-1) is a rare, monogenic disease caused by mutations in the *autoimmune regulator (AIRE)* gene [1–3]. The AIRE protein upregulates intrathymic expression of a wide range of tissue-specific self-antigens in medullary thymic epithelial cells and has a key function in the negative selection of T cells [4, 5]. Dysfunctional elimination of self-reactive immune cells in APS-1 patients causes autoimmune disease starting in the early stages of life, with gradually progressing clinical symptoms over time [1].

APS-1 is diagnosed either by detecting disease-causing mutations in *AIRE* or clinically by the presence of at least two components of the classical triad, which consists of primary adrenal insufficiency (AAD), chronic mucocutaneous candidiasis (CMC) and hypoparathyroidism (hypoPTH) [1, 6]. For individuals having a sibling previously diagnosed with APS-1, the presence of one classical triad component is enough for APS-1 diagnosis [6]. In addition to the classical triad, patients typically present with a wide range of other manifestations associated with autoimmunity throughout life, such as vitiligo, hypothyroidism, Type 1 diabetes, enamel hypoplasia and alopecia [1].

Abbreviations: AAD, autoimmune Addison's disease; APRIL, A proliferation-inducing ligand; APS-1, autoimmune polyendocrine syndrome Type-1; BAFF, B-cell activating factor; Bmem, B memory cell; ELISA, enzyme-linked immunosorbent assay; f-SMA, smoothed moving average of cell frequencies; HC, healthy Control; ncMonocyte, nonclassical monocyte; NK, natural killer cell; pDC, plasmacytoid dendritic cell; SMA, smoothed moving average; TFH, T follicular helper cell; Treg, T regulatory cell.

Several studies have looked at immune cell compositions within different cohorts of APS-1 patients, and most agree that both B cells and regulatory T cells are reduced in peripheral blood mononuclear cells (PBMCs) [7–14]. The autoimmune tissue destruction is thought to mainly be T cell-driven, while high levels of autoantibodies produced by B cells serve as excellent diagnostic markers [15–17]. However, immune cell composition throughout a lifetime has not been previously investigated.

Epigenetics explores inheritable changes in gene function or expression that occur without altering the DNA sequence itself, including mechanisms like DNA methylation [18]. Quantification of immune cell subsets can be done by targeting cell type-specific unmethylated DNA regions in whole blood by bisulfite conversion [19, 20], opening possibilities for using stored EDTA blood instead of cryopreserved PBMCs for immune phenotyping. We here ask the question of how the immune system in APS-1 patients compares to healthy controls in a life-time perspective, using EDTA blood samples from 27 Norwegian APS-1 patients collectively spanning from childhood to old age.

2 | Methods

2.1 | Experimental Design

Patient data and samples were obtained from the Norwegian registry for organ specific autoimmune diseases (ROAS) biobank. All patients have provided informed consent, and the study was approved by the Regional Committee for Medical and Health Research Ethics (REK no. 2009/255 and 2018/1417).

Twenty-seven patients (14 males and 13 females), all fulfilling the clinical diagnostic criteria for APS-1 with confirmed recessive mutations in the *AIRE* gene, were included in this study. Longitudinal blood samples (range: 3–7) were used from each patient, with 10 (±5) year intervals between each time point, in total 106 samples (55 males, 51 females) (Figure 1A, Table 1). 48 blood donors were recruited from the Haukeland Hospital blood bank and used as healthy controls (HC). From each HC, a blood sample was taken at one point. The patient and control cohorts were divided into six age groups: 0–19, 20–29, 30–39, 40–49, 50–59, and 60–69. Each age group consisted of four female HC and four male HC samples (Figure 1A, Table 1). 26 of 27 APS-1 patients had longitudinal serum samples in ROAS, stored simultaneously with whole blood. A total of 130 serum samples (84 APS-1 samples, 46 HC samples) were used for ELISA analysis of BAFF and APRIL (Table 1). Different HC samples were used as controls in the whole blood (epigenetic quantification) and serum (ELISA) analyses. Most of these patients have been previously reported by us [6, 21], and have also been previously included in studies of blood and PBMCs by flow cytometry and RNA sequencing, although only at a single time point [10, 14, 22, 23].

Venous peripheral whole blood samples were collected in K_2EDTA tubes and stored at-80°C until analysis. Patient and control samples were randomly grouped in batches for epigenetic analysis. Each batch included patient and control samples from both females and males. All longitudinal samples from the same patient were analysed in the same batch.

Epigenetic quantification was performed on all blood samples ($n = 154$), with a panel of 13 immune cell types, established from the available cell types (Epimune GmbH): $CD3^+$ T, $CD4^+$ T, $CD8^+$ T cells, regulatory T cells (Treg), T helper 17 (Th17), T follicular helper cells (TFH), B ($CD19^+$) and memory B cells (Bmem), monocytes ($CD14^+$) and nonclassical monocytes (ncMonocyte)($CD14^-CD16^+$), natural killer cells (NK)($CD16^+CD56^{dim}$), plasmacytoid dendritic cells (pDC) and neutrophils. Cell type-specific regions of the bisulfite-converted DNA (bisDNA) have been identified and quantified for the established panel (Table S1) [24].

2.2 | Epigenetic Quantification: Bisulfite Conversion and qPCR

Cell lysis, DNA extraction and bisulfite conversion by i.Mune Prep kit (Epimune GmbH) were performed using 40 μL of blood, according to the manufacturer's instructions. qPCR was then performed with bisDNA materials using a customised amplification assay kit from Epimune GmbH, including two positive and one negative control. Samples were run on the QuantStudio 5 Real-Time PCR System (Thermo Fisher), using instructions provided by Epimune GmbH (15 s at 95°C and 1 min at 61°C, 45 cycles in PCR stage). The collected data was processed by QuantStudio Design & Analysis Software (v1.5.3, Thermo Fisher), followed by analysis in Analysis Tool qPCR1&2 and QuantStudio File Converter (both Epimune GmbH). After initial analysis and quality control, data were further analysed using R (v4.3.0), RStudio (v2023.12.1 + 402), *zoo* package (v1.8–12) and GraphPad Prism (v9.5.1).

2.3 | Flow Cytometry

For an in-depth study of B-cell subsets, flow cytometry analysis on PBMC was performed with a B-cell panel. PBMC isolation was performed on heparin blood using Ficoll Paque Plus (Cytiva), and cells were then frozen in human AB-serum (Sigma-Aldrich, Darmstadt, Germany) with 10% DMSO (Sigma-Aldrich) at −150°C until use. Nine APS-1 patients (average age 44.7 years (±11.04 years), 22.2% females) and 9 age- and sex-matched controls were included. On the day of analysis, cells were thawed and transferred to 37°C Dulbecco's modified Eagle medium GlutaMAX (DMEM) (Gibco, Thermo Scientific), supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% Penicillin–Streptomycin (Sigma-Aldrich). Samples were centrifuged and subsequently resuspended in 4 mL PBS with 2% FBS and filtered using a MACS SmartStrainer 70 μm (Miltenyi Biotec).

Two microliters undiluted Fc-block (BD, Franklin, New Jersey, US) were added, and cells were incubated for 20 min before being stained with 1 μL live/dead marker (Zombie Violet BV421, BioLegend). After washing, samples were subsequently incubated in the dark at 4°C for 20 min with a panel of antibodies to identify B-cell subsets (Table S2). Acquisition was conducted on the LSR Fortessa Flow Cytometer (BD), and analysis was performed using FlowJo (v10.7.1, BD) by the gating strategy outlined in Figure S1, based on Clavarino et al. [25].

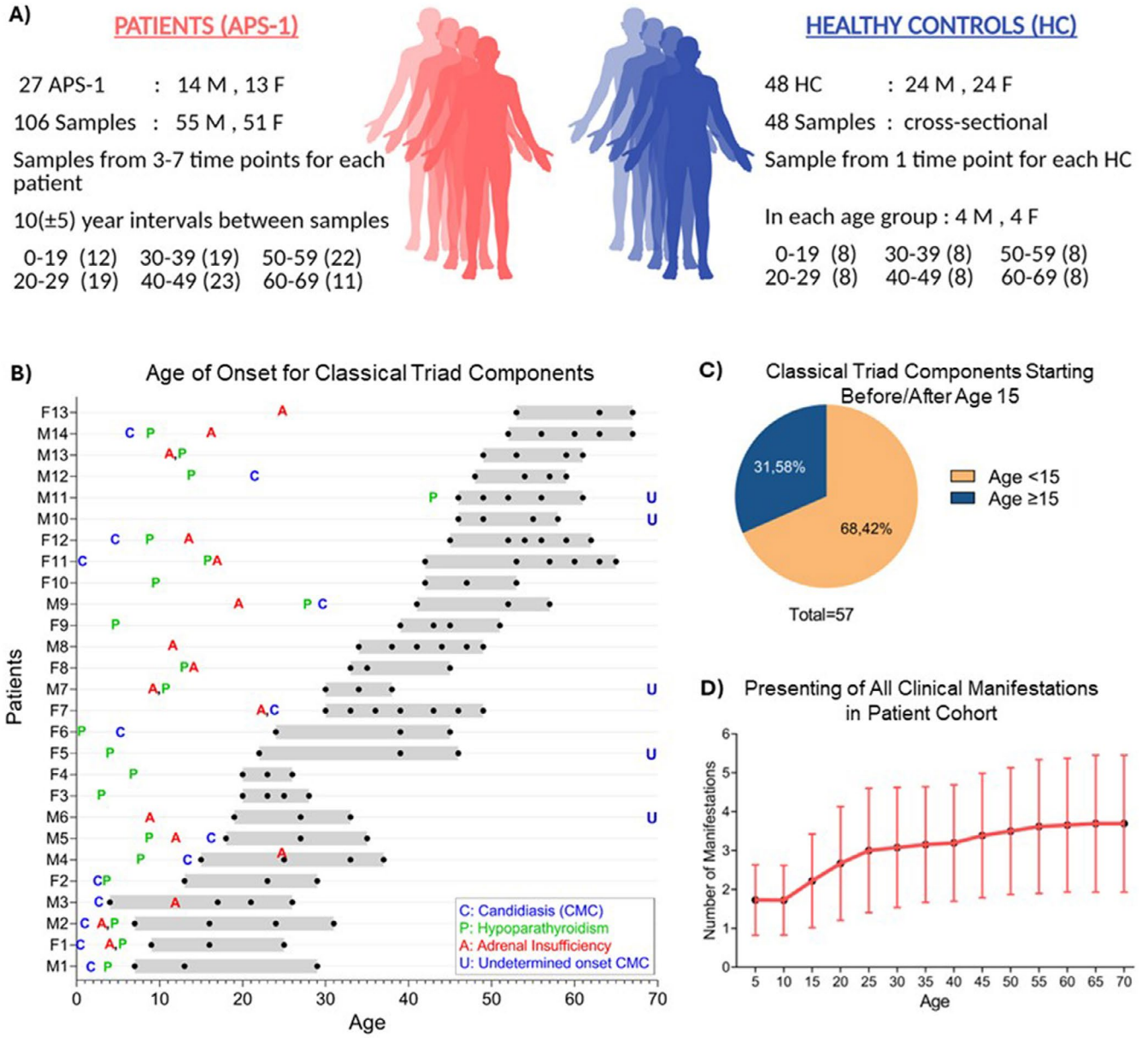

FIGURE 1 | Experiment design and characteristics of patient cohort. (A) Study cohort of APS-1 and healthy controls, age and sex profiles. (B) Sex and age distribution of APS-1 patient samples included in the study, shown together with onset age of classical triad components. Each row represents one APS-1 patient. Patient naming on *y*-axis includes sex (F: Female, M: Male). The black points on horizontal bars represent the age of patients when the sample was taken. Letters on the graph indicate the age of patients when each component of the clinical diagnosis criteria was first presented clinically (onset). A, primary adrenal insufficiency; C, chronic mucocutaneous candidiasis; P, hypoparathyroidism; U, candidiasis with undetermined age of onset. The components separated by comma indicate that age of onset is mutual. (C) Distribution of number of classical triad components in APS-1 cohort, starting before or after age 15. (D) Mean ± SD of number of all clinical manifestations in APS-1 cohort through ageing. *Y*-axis shows the total number of manifestations, including classical triad and other manifestations. Mean ± SD of number of manifestations were calculated with 5-year intervals. Figure A created in BioRender. Figures B–D created in GraphPad Prism.

2.4 | ELISA of BAFF and APRIL

Effects on B-cell activity and survival were measured by BAFF (B-cell activating factor) and APRIL (a proliferation-inducing ligand) concentrations in longitudinal serum samples using ELISA. BAFF and APRIL levels were analysed in 25 μL serum from 26 patients longitudinally (84 samples) and 46 single-point samples from healthy controls (Table 1) by the Human BAFF/Blys/TNFSF13B Quantikine ELISA kit (DBLYS0B) and Human APRIL/TNFSF13 DuoSet ELISA (DY884B), both from R&D systems (Abingdon, United Kingdom) in accordance with the manufacturer's protocol.

2.5 | Statistical Analysis

Analysis of epigenetic quantification and ELISA were performed using R (version 4.3.0), RStudio (version

TABLE 1 | Patient and control cohorts, including their distribution into sex and age groups.

			Number of samples							
			Age groups (Person's age at sampling)							
	Sex	Number of people	<20	20–29	30–39	40–49	50–59	60–69	Total	Total
Blood										
Patients ($n=27$)	Female	13	3	12	9	12	9	6	51	106
	Male	14	9	7	10	11	13	5	55	
Controls ($n=48$)	Female	24	4	4	4	4	4	4	24	48
	Male	24	4	4	4	4	4	4	24	
Serum										
Patients ($n=26$)	Female	13	4	6	7	11	8	6	42	84
	Male	13	5	6	8	8	10	5	42	
Controls ($n=46$)	Female	24	4	4	4	4	4	4	24	46
	Male	22	2	4	4	4	4	4	22	

2023.12.1 + 402 'Ocean Storm' Release for Windows), and GraphPad Prism (version 9.5.1 for Windows). The differences between APS-1 and HC within each age group were analysed by Mann–Whitney *U* test followed by Holm–Šídák method for multiple testing adjustments using GraphPad Prism. The threshold for *p* value comparisons was set to 0.05. Simple moving average (SMA) method was used for plotting the cell frequencies over a long-term timeline. The dot plots displaying the moving average of cell frequencies were created in R using the zoo package (version 1.8–12). For SMA analysis of epigenetic quantification and ELISA, the cell frequency and serum concentration data were sorted by the age of individuals at the time of sampling. For the SMA of cell frequencies (f-SMA), the window range of 15–19 data points were included in calculation, with the aim of using the smallest possible window size for each cell type that provides a smoothed transition in the plots. A window size of 19 data points was used for each cell type except TFH, consisting of a central value and 9 neighbouring data points before and after. A window size of 15 data points was used for TFH, with 7 neighbouring data points before and after a central point. For the SMA of serum BAFF and APRIL concentrations, a window size of 9 data points was used, consisting of a central value and 4 neighbouring data points before and after.

In flow cytometry experiments, differences between groups were analysed by the Student *t*-test in GraphPad prism (version 10 for Windows).

3 | Results

3.1 | Clinical Manifestations Throughout Life

Our patient cohort included 27 APS-1 patients of various ages, from which blood and serum samples were collected at multiple timepoints ranging between 5 and 67 years of age. We analysed a total of 154 EDTA blood samples for epigenetic quantification and 130 serum samples for ELISA. 11 patients presented with all three components of the classical triad, 11 patients had two of the three components, and five patients had one component together with a sibling previously diagnosed with APS-1(Figure 1B). Patients acquired most of the classical components before age 15 (Figure 1C), with the earliest age of debut being at newborn stage. Ageing was significantly associated with the total number of manifestations, causing a linear trend of increase ($p<0.0001$) where the total number of clinical manifestations showed a rapid increase until age 25 (Figure 1D). No changes were found when accounting for gender.

3.2 | Longitudinal Patterns of Immune Cells in APS-1

To identify changes in immune cell composition that might reflect the clinical picture in APS-1, a longitudinal study of the immune cell subsets was done. In CD3+ T, CD4+ T, and CD8+ T cell populations, no significant differences between patients and controls were found (Figure S2A–C). In CD4 + T cells, we observed that the f-SMA levels fluctuated between 14% and 17% in healthy controls throughout life, with values above 15% before age 40 and below 15% after age 40 (Table S3, Figure S2B). In patient samples, the fluctuations followed the opposite directions across the lifespan. In CD8+ T cells, f-SMA levels in the patient cohort decreased after age 40, whereas the decline in the control cohort was observed across the lifespan (Table S3, Figure S2C). In the 0–19 age group, patients had lower f-SMA levels compared to controls; however, the difference in CD8 + T cell frequencies between the groups was not significant at any age. The cell frequencies of memory B cells (Bmem), monocytes, plasmacytoid dendritic cells (pDC) and neutrophils did not show significant variation between patients and controls across any age group (Figure S1D–G). In the analysis of f-SMA values in APS-1 patients, we observed an increasing trend until age 40 years in Bmem and monocytes,

followed by a decline thereafter. In both patients and control cohorts, neutrophil f-SMA values were observed to rise until age 40, with a decline seen beyond this age (Figure S2F), whereas the f-SMA values of pDCs exhibited a decline across the lifespan (Figure S2G).

3.3 | B Cells in APS-1

We observed a significant difference in B cell frequencies of patients and controls throughout life. In the 0–19 age group, the mean frequency of HC (21.5 ± 9.84 SD) was more than double that of the mean frequency of APS-1 (7.78 ± 3.98 SD). Both in patients and controls, the percentage of B cells in blood decreased with ageing, and the gap between APS-1 and HC groups narrowed upon increasing age (Figure 2A,B; Table 2). In the 40–49 age group, APS-1 and HC cohorts had the most similar B-cell frequency profiles (APS-1: 3.62 ± 3.04 SD; HC: 3.81 ± 1.42 SD). Analysing B-cell subsets by flow cytometry confirmed a decrease, mainly within the transitional B cells in APS-1 patients, including an upregulation of the activation marker CD69, as shown in other APS-1 cohorts (Figure 2C–F). To further elucidate the discrepancies in B-cell frequencies, serum levels of the B cell activation factors BAFF and APRIL were analysed longitudinally, revealing a trend towards elevated levels in all age groups for the APS-1 patients (Figure 2G–J, Figure S3).

3.4 | T-Cell Subsets Are Aberrant Early in Life of APS-1 Patients

Looking into the different T cell subsets, Tregs in the 0–19 age group were three times higher in HC compared to APS-1 (mean ± SD, APS: 1% ± 0.53%; HC: 3.24% ± 1.08%, $p = 0.0004$), and the gap between HC and APS-1 Treg frequencies narrowed with age (Figure 3A,B; Table 2). In the f-SMA analysis, the healthy controls showed a decreasing trend until age 45, where they reached the f-SMA of 1% of the leukocytes and remained stable after this age. In patient samples, however, the f-SMA was below 1% until age 40, then surpassed 1% up to 1.25% until age 50, followed by a decrease back to 1% by age 60. The Th17 cell subset of APS-1 patients was furthermore significantly lower than controls in the 0–19 age group (APS-1: 0.82% ± 0.45%, HC: 2.07% ± 1.22%, $p = 0.005$) (Figure 3C,D). Similarly, in the 0–19 age group, the frequency of TFH cells was reduced in APS-1 patients compared to controls (APS-1: 2.01% ± 1.13%, HC: 8.4% ± 2.42%, $p = 0.000095$) (Figure 3E,F). Looking at the f-SMA values, both TFH and Th17 cell populations showed an upward trend until age 40 in the APS-1 patients followed by a decline thereafter, while the healthy controls had the highest f-SMA values early in life, with a continuous decline until age 40, where it levelled off subsequently.

3.5 | Innate Immune Cells Change in Early Stages of Life

The ncMonocytes also showed a similar trend in patients with the lowest frequency in the youngest age group (APS-1: 0.41% ± 0.2%, HC: 0.73% ± 0.11%, $p = 0.039$) increasing with age, to have a similar f-SMA profile as controls after age 40 (Figure 4A,B). NK cells presented with a larger gap between APS-1 and controls in early stages of life, with the mean frequency of controls being significantly higher than patients (0–19 age group, APS: 2.54% ± 1.62%, HC: 7.89% ± 4.2%, $p = 0.001$) (Figure 4C,D). Until 40–50 years of age, NK frequency in controls remained greater than in patients; however, with a diminishing difference. After the 40–49 age group, NK cell frequencies in the blood of APS-1 and HC remained at a similar level with a slight increase upon ageing (Table 2).

4 | Discussion

In this study, we examined the peripheral immune composition of APS-1 patients using samples collected over a 28-year period. We analysed the cell frequencies in whole blood samples collected at various time points throughout the study. This highlights the possible use of long-term stored EDTA blood samples for longitudinal immune cell composition studies, especially when the quantity or quality of blood or sampling and storage facilities are insufficient for flow cytometry analysis. With the aim of diagnosing APS-1 patients as early in life as possible and considering the need for the ability to work with limited volumes of blood obtained from newborns, epigenetic quantification has a crucial benefit of enabling work with small sample volumes with similar efficiency as flow cytometry. Our epigenetic quantification results both confirm previous findings and add to our knowledge about immune cell subsets and mechanisms by describing differences in the immune cell compartment in patients with APS-1.

The onset of disease and the appearance of the different components varied between the patients, with an overweight appearing before the age of 15 years. Interestingly, we also found most changes in the immune cell frequencies within the group below 20 years of age, and one could speculate if these changes in immune composition might hint towards pathological mechanisms and possible points of intervention. Looking at the trends during a lifetime, several studies have investigated immune cell composition in the general population by different methods like flow cytometry, RNA sequencing and single-cell sequencing [26, 27]. We have been able to replicate these studies using stored whole blood and observed similar trends, such as decreased B-cell activity with age [26, 28]. Several studies have looked at the gender impact of immune cell subsets during ageing in the normal population [26, 28], and at single-cell levels, it was found that NK cells and Tregs had sex-associated differences while B-cell populations were similar in males and females [28]. We show the main differences to be sourced from ageing, with males and females of the same age groups having similar trends in immune profiles [26, 27]. However, we have few samples included in the over 69-years cohort, and the majority of the age-related changes were most prominent above this age.

Looking at the differences between APS-1 patients and controls before the age of 40, the frequencies of TFH, Th17 and B-cell populations are lower in patients, most prominently under age 19 years. This trend reversed after 40 years of age, except for Tregs, which converged around 45 years of age, portraying

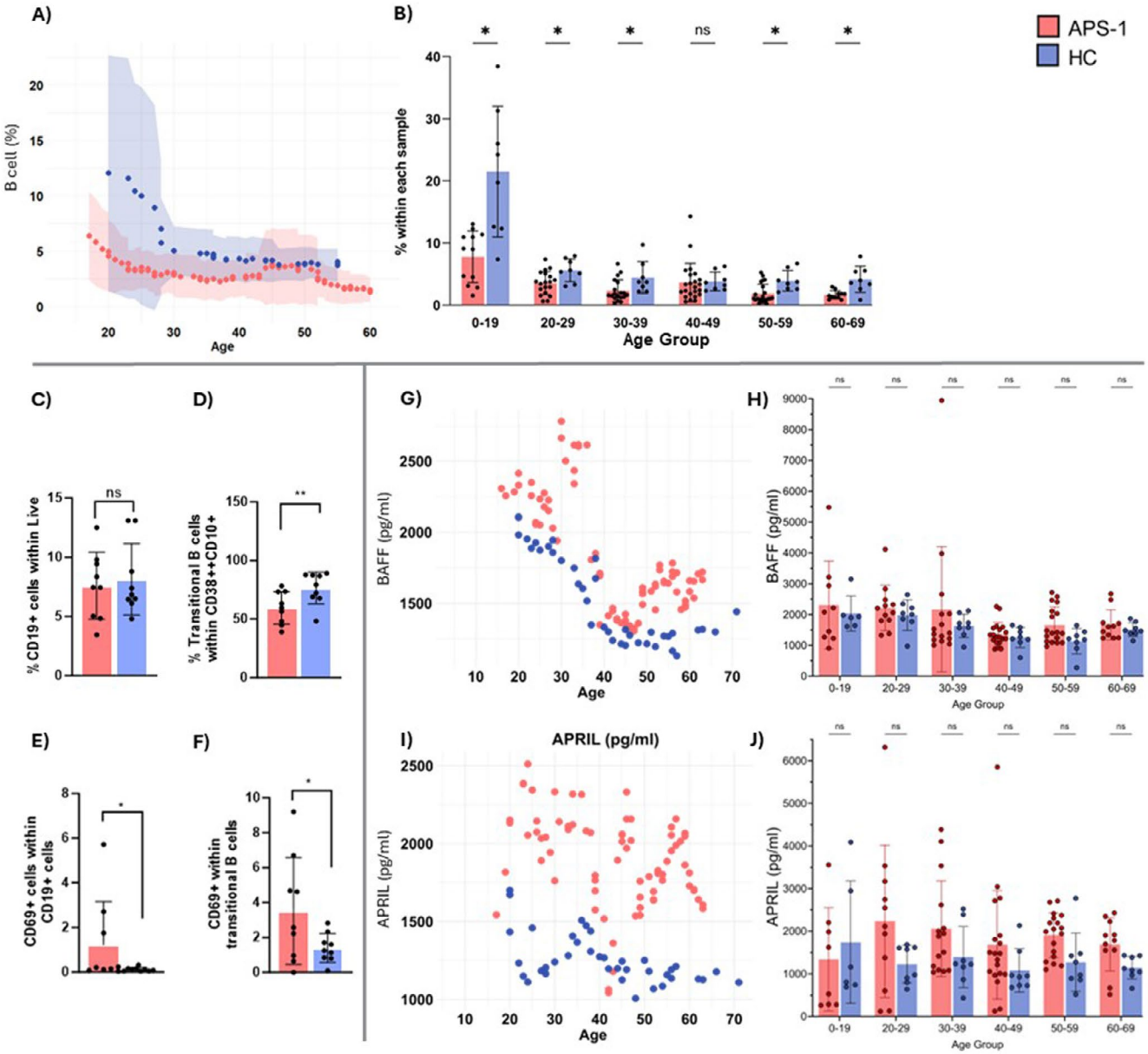

FIGURE 2 | B-cell characterisation in APS-1 versus HC: Cell frequencies, flow cytometry analysis and BAFF-APRIL levels. Epigenetic quantification (A,B): (A) Longitudinal simple moving average (sma) of B-cell frequencies (rolling mean ± SD) for APS-1 patients (red) versus controls (blue) and the frequencies within the different age groups (B). (C) Frequency of CD19+ cells within the live cell population (gated on total cells, singlets, live and lineage negative). (D) Frequency of transitional B cells (CD19 + CD38++CD10 + CD5+). The experiment (G and includes 9 APS-1 patients and 9 age- and sex-matched controls. (E) CD69+ cell percentage within CD19+ cells and transitional B cells (F). (G) Longitudinal sma of serum BAFF concentrations for APS-1 versus HC and (H) the levels within the different age groups. (I) Longitudinal sma of serum APRIL concentrations for APS-1 versus HC and the levels within the different age groups (J). Figures A, G, I were created in R, with rolling mean window sizes of 19 (A) and 9 (G, I). Figures B–F, H, J are created and analysed in GraphPad Prism, columns show mean ± SD. In B, H, J; percentages within each sample were analysed using Mann–Whitney *U* test followed by Holm–Šídák method for multiple testing adjustment. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. ns, Nonsignificant.

a more stable trajectory in the older age groups. Tregs have been extensively investigated in APS-1 patients, both by us and others, showing a decrease in the Treg population [10, 12–14, 23, 29]. Here we further elaborate by showing that the time point in life could impact the results and that the Treg population stabilises in the middle-aged patient population, possibly reflecting the early onset of clinical manifestations in APS-1. The decrease of Treg frequencies in patients followed by stabilisation and having a similar profile to controls in older ages might be explained by the age-related regression of thymus in activity and volume [30]. A reduced thymic function in the elderly groups of both cohorts can be speculated to cause the declining differences in Treg frequencies. Circulating TFH cells have previously been found decreased in APS-1 patients, and our results align with this in the young population [31]. This is likely to support their homing to secondary lymphoid organs during the time of active autoimmune destruction, as TFH cells play essential roles in germinal centre formation, B-cell proliferation and differentiation leading to antibody production [32]. The difference in TFH cell frequencies parallels

TABLE 2 | Mean values of frequencies of B cells, Tregs and NK cells within each age group.

		Patients (APS-1)		Healthy controls (HC)		
Cell type	**Age groups**	**Mean (%)**	**Std deviation (±)**	**Mean (%)**	**Std deviation (±)**	***p* (adjusted)**
B CELLS	0–19	7.78	3.98	21.50	9.85	0.011849
	20–29	3.53	1.81	5.61	1.71	0.042885
	30–39	2.32	1.71	4.45	2.39	0.033361
	40–49	3.62	3.04	3.81	1.42	0.259097
	50–59	1.84	1.50	3.92	1.56	0.029376
	60–69	1.65	0.64	4.14	1.98	0.029376
T REG	0–19	1.01	0.53	3.24	1.08	0.000429
	20–29	0.77	0.41	1.08	0.50	0.404815
	30–39	0.77	0.43	1.23	0.39	0.124202
	40–49	1.21	0.54	0.86	0.22	0.321789
	50–59	1.04	0.72	1.07	0.41	0.686234
	60–69	0.95	0.55	1.03	0.44	0.686234
NK	0–19	2.54	1.62	7.89	4.20	0.001809
	20–29	2.99	1.26	4.57	2.81	0.703501
	30–39	2.65	1.58	3.89	1.35	0.154644
	40–49	3.59	1.91	3.21	1.16	0.893803
	50–59	3.57	1.71	4.81	2.43	0.634187
	60–69	4.12	2.75	4.72	3.30	0.893803

that of B cells throughout life in APS-1 patients and controls, possibly as a result of TFH-B cell interaction mechanisms in the lymph nodes.

Protection against CMC is thought to be mediated mainly by IL-17 producing Th17 cells [33–37]. It has been reported that APS-1 patients have reduced levels of peripheral Th17 cells [12, 13, 31, 38], caused by impaired regulation of IL-17, IL-23 and dysregulated IL-23p19, which is required for Th17 formation [39]. The reduced frequency of Th17 cells in blood could suggest their redistribution to mucosa. However, a protective effect of Th17 cells against CMC is not observed in APS-1 patients, likely because of the high levels of neutralising autoantibodies against Th17-specific cytokines in APS-1 [33, 37]. Our findings of distinctly lower Th17 frequency in young APS-1 patients (age group 0–19), together with poor immune responses of Th17 cells against CMC, might explain why CMC is the earliest presented manifestation in APS-1.

In concordance with previous findings in B cells of APS-1 patients, including the Norwegian APS-1 cohort [7, 31, 38, 40], we found a clear decrease of B cells in all age groups except the 40–49 years group from the epigenetic quantification. To further investigate the different subtypes previously reported to be more severely affected by APS-1, we used flow cytometry analysis on PBMCs. Although we did not find an overall reduction of CD19+ B cells, we confirmed the findings that transitional B cells are reduced in APS-1 patients. BAFF and APRIL are important cytokines involved in immune homeostasis, immune cell differentiation, affecting B-cell maturation and function by regulating activation, survival and proliferation of B cells [41–45]. Elevated levels of BAFF and APRIL have been observed in various other autoimmune diseases [46–50], and increased BAFF levels have been found in APS-1 patients [11, 38]. Our data resemble what was found in the Finnish APS-1 cohort where higher APRIL levels were detected in patients, but no changes in BAFF [31]. In addition, we did not find any changes in BAFF or APRIL in relation to the age or gender of the included subjects.

In the youngest age group, CD8+T cells followed a declining pattern parallel to B cells in APS-1 patients. B cells are known to be involved in the activation and priming of CD8+T cells [51]. In multiple human and mouse studies, the effect of B cells on CD8+T cells has been shown previously in various autoimmune diseases, demonstrating that CD8+T cell count, activity and proliferation reduce after B cell depletion [51, 52]. We speculate from our findings that the decreasing frequency and the lack of activating and priming effect of B cells lead to a decreasing frequency of the CD8+T cell population in the young patients.

Interestingly, we found the innate immune cell compartment in APS-1 patients to be affected in the early stage of life, where both NK cells and ncMonocyte populations were lower in patients under age 19. A lower frequency of CD16+ monocytes has also been reported for this patient cohort previously [10]. For the NK

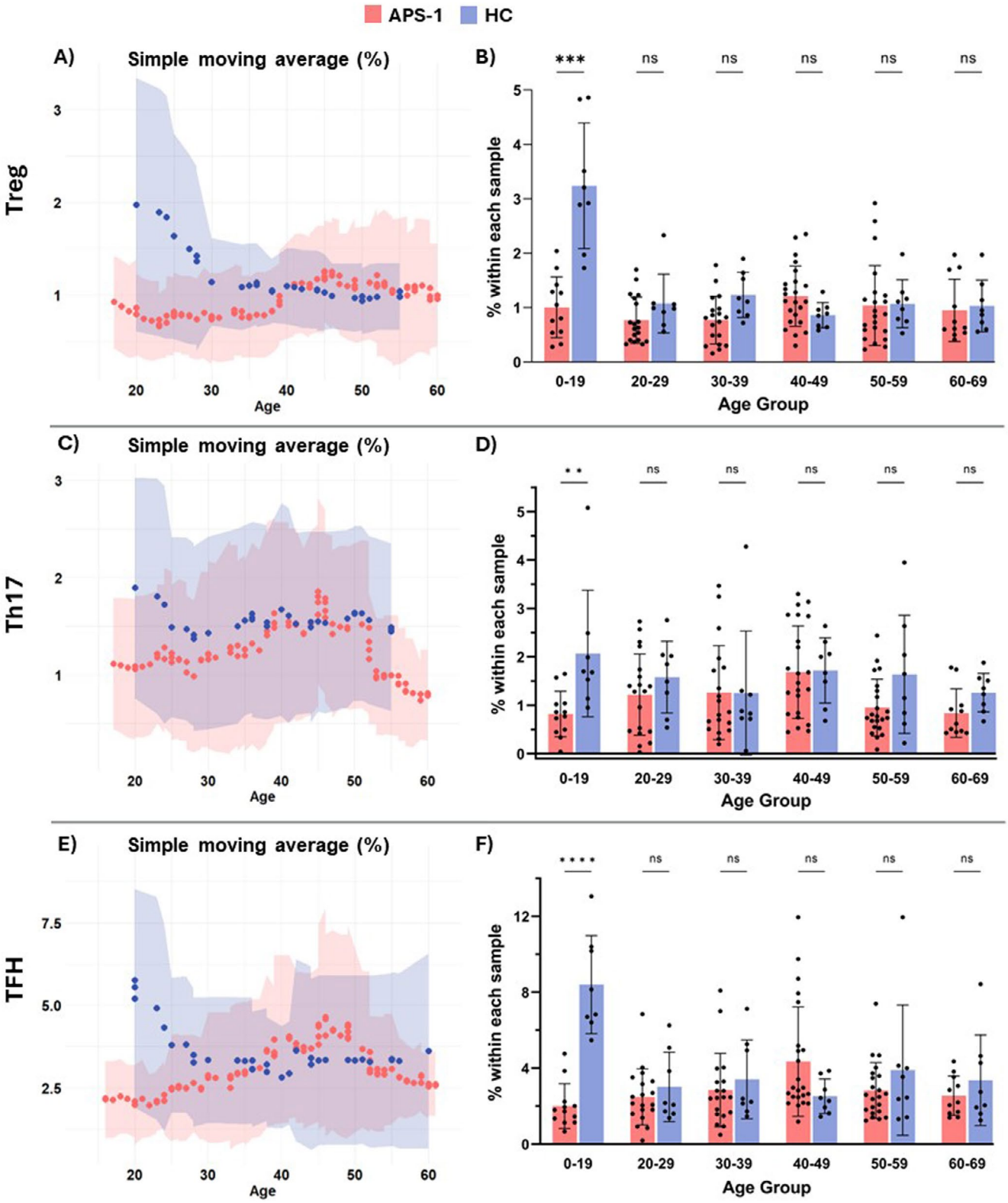

FIGURE 3 | Epigenetic quantification of T-cell subsets in APS-1 versus HC samples. (A) Longitudinal simple moving average (sma) of Treg cells frequencies (rolling mean ± SD) for APS-1 patients (red) versus controls (blue) and the frequencies within the different age groups (B). (C) Longitudinal sma of Th17 cells frequencies for APS-1 versus HC and the frequencies within the different age groups (D). (E) Longitudinal sma of regulatory TFH cells frequencies for APS-1 (red) versus HC (blue) and the frequencies within the different age groups (F). A sma window of 19 was used for Treg and Th17 cells (A,C) and window of 15 for TFH (E). In B, D, F; columns show mean ± SD. Percentages of cell types within each sample were analysed using Mann–Whitney *U* test followed by Holm–Šídák method for multiple testing. Figures A, C, E created in R. Figures B, D, F created in GraphPad prism. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

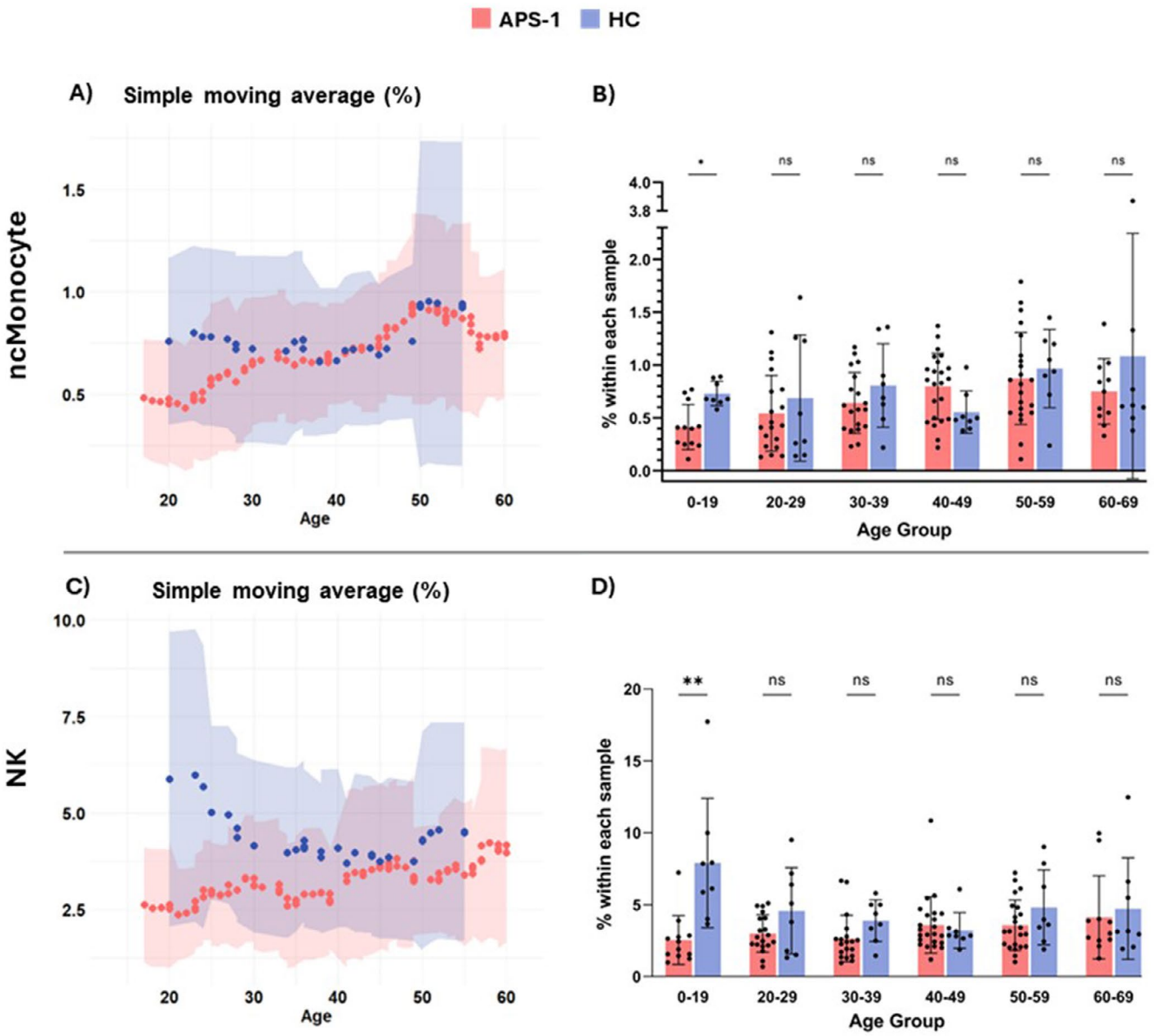

FIGURE 4 | Epigenetic quantification of innate immune cells in APS-1 versus HC samples. (A) Longitudinal simple moving average (sma) of ncMonocyte cells frequencies (rolling mean ± SD) for APS-1 patients (red) versus controls (blue) and the frequencies within the different age groups (B). (C) Longitudinal sma of NK cells frequencies for APS-1 (red) versus HC (blue) and the frequencies within the different age groups (D). A sma window of 19 was used for both cell types. In B, D; columns show mean ± SD. Percentages of cell types within each sample were analysed using Mann–Whitney *U* test followed by Holm–Šídák method for multiple testing. Figures A, C created in R. Figures B, D created in GraphPad prism. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

cells, their trend in APS-1 patients was increasing with ageing, as opposed to the decreasing frequencies in HCs over the years. NK cells have not been found to be less abundant before but are described to have both a protective and pathogenic role in autoimmune diseases [53]. In type 1 diabetes, a reduced number and decreased cytolytic activity of NK cells have been reported [54–57], but more studies in a young APS-1 cohort will be necessary to validate and functionally determine the impact of our findings.

The main limitation of this study is the rareness of APS-1 (1:100000 in the Norwegian population) which affected the size of the patient cohort in the study. We aimed to include patients having multiple samples covering a lengthy period of time to best represent the immune landscape at different ages, but the majority of samples are collected between the ages of 20–60 years, limiting our statistical power in the early and late phases of life. Our study design involved multiple samples from each patient for longitudinal representation of immune composition in blood, while each healthy control could provide only a single cross-sectional sample. This difference in collection of blood from individuals might affect the interpretation of data. To minimise this potential effect and provide a better representation of a healthy population, we included eight healthy controls in each age group. The control cohort was limited to individuals aged 18 years and above, which may impact the representation of the

0–19 age group. Future studies would benefit from including a control cohort under the age of 18 to enable a more accurate characterisation of immune profiles of the younger population.

This study investigates immune cell compositions in peripheral blood, and it is worth noting that as little as ~2% of immune cells are found in blood [58]. Therefore, the conclusions we make based on the peripheral blood data alone do not reflect the complete picture of immune activity, particularly autoimmune reactions that take place in various tissues. The decreased frequency of B cells in young APS-1 patients despite higher levels of BAFF might suggest an increase in B-cell population and activity in secondary lymphoid organs of the APS-1 patients, leading to a decreased proportion in blood.

In addition to the reduced number of B cells and early life changes of TFH cells, ncMonocytes, Tregs and NK cells in APS-1 patients, and their possible effects on onset ages of manifestations, this study highlights that variations in age can impact study findings. The potential of age to influence the outcome of immune cell studies can be so sensitive that even a 10-year age difference between cohorts may lead to noticeable changes in study findings. Therefore, it must be a key consideration for future studies when designing experiments and interpreting findings.

Author Contributions

B.E.O., I.K. and A.S.B.W. conceived and designed the project. I.K., D.I., S.B., B.E.O. and L.B. collected the data. I.K., D.I., S.B. and B.E.O. performed experiments. I.K. and B.E.O. analysed and interpreted the data. B.E.O. supervised the study. I.K. and B.E.O. wrote the paper. I.K., B.E.O., A.S.B.W., D.I., S.B., L.B. and E.S.H. reviewed and approved the final version of the manuscript.

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Ethics Statement

The authors have no conflict of interests. All patients and controls have signed informed consent, and the study is approved by the regional ethics committee. Funding sources are named in the acknowledgement.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

References

1. E. S. Husebye, M. S. Anderson, and O. Kampe, "Autoimmune Polyendocrine Syndromes," *New England Journal of Medicine* 378, no. 12 (2018): 1132–1141.

2. O. Bruserud, B. E. Oftedal, A. B. Wolff, and E. S. Husebye, "AIRE-Mutations and Autoimmune Disease," *Current Opinion in Immunology* 43 (2016): 8–15.

3. D. Payen, M. Cravat, H. Maadadi, et al., "A Longitudinal Study of Immune Cells in Severe COVID-19 Patients," *Frontiers in Immunology* 11 (2020): 580250, https://doi.org/10.3389/fimmu.2020.580250.

4. E. M. Akirav, N. H. Ruddle, and K. C. Herold, "The Role of AIRE in Human Autoimmune Disease," *Nature Reviews. Endocrinology* 7, no. 1 (2011): 25–33.

5. M. S. Anderson and M. A. Su, "Aire and T Cell Development," *Current Opinion in Immunology* 23, no. 2 (2011): 198–206.

6. O. Bruserud, B. E. Oftedal, N. Landegren, et al., "A Longitudinal Follow-Up of Autoimmune Polyendocrine Syndrome Type 1," *Journal of Clinical Endocrinology and Metabolism* 101, no. 8 (2016): 2975–2983.

7. V. Perri, E. Gianchecchi, R. Scarpa, et al., "Altered B Cell Homeostasis and Toll-Like Receptor 9-Driven Response in Patients Affected by Autoimmune Polyglandular Syndrome Type 1: Altered B Cell Phenotype and Dysregulation of the B Cell Function in APECED Patients," *Immunobiology* 222, no. 2 (2017): 372–383, https://doi.org/10.1016/j.imbio.2016.09.001.

8. A. Magnani, A. Meloni, M. Gattorno, A. Martini, and E. Traggiai, "B Cell Subsets Phenotype in Autoimmunity With Immunodeficiency: Analysis of a Large Cohort of Patients With Apeced Syndrome," *Journal of Clinical Immunology* 32 (2012): 45.

9. R. Perniola, G. Lobreglio, M. C. Rosatelli, E. Pitotti, E. Accogli, and C. De Rinaldis, "Immunophenotypic Characterisation of Peripheral Blood Lymphocytes in Autoimmune Polyglandular Syndrome Type 1: Clinical Study and Review of the Literature," *Journal of Pediatric Endocrinology & Metabolism* 18, no. 2 (2005): 155–164.

10. A. S. Wolff, B. E. Oftedal, K. Kisand, E. Ersvaer, K. Lima, and E. S. Husebye, "Flow Cytometry Study of Blood Cell Subtypes Reflects Autoimmune and Inflammatory Processes in Autoimmune Polyendocrine Syndrome Type I," *Scandinavian Journal of Immunology* 71, no. 6 (2010): 459–467.

11. E. Lindh, S. M. Lind, E. Lindmark, et al., "AIRE Regulates T-Cell-Independent B-Cell Responses Through BAFF," *Proceedings of the National Academy of Sciences of the United States of America* 105, no. 47 (2008): 18466–18471.

12. E. Kekalainen, H. Tuovinen, J. Joensuu, et al., "A Defect of Regulatory T Cells in Patients With Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy," *Journal of Immunology* 178, no. 2 (2007): 1208–1215.

13. S. M. Laakso, T. T. Laurinolli, L. H. Rossi, et al., "Regulatory T Cell Defect in APECED Patients Is Associated With Loss of Naive FOXP3(+) Precursors and Impaired Activated Population," *Journal of Autoimmunity* 35, no. 4 (2010): 351–357.

14. A. H. Berger, E. Bratland, T. Sjogren, et al., "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* 12 (2021): 722860.

15. I. Gavanescu, C. Benoist, and D. Mathis, "B Cells Are Required for Aire-Deficient Mice to Develop Multi-Organ Autoinflammation: A Therapeutic Approach for APECED Patients," *Proceedings of the National Academy of Sciences of the United States of America* 105, no. 35 (2008): 13009–13014.

16. J. J. Devoss, A. K. Shum, K. P. Johannes, et al., "Effector Mechanisms of the Autoimmune Syndrome in the Murine Model of Autoimmune

Polyglandular Syndrome Type 1," *Journal of Immunology* 181, no. 6 (2008): 4072–4079, https://doi.org/10.4049/jimmunol.181.6.4072.

17. E. M. N. Ferre, M. M. Schmitt, and M. S. Lionakis, "Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy," *Frontiers in Pediatrics* 9 (2021): 723532.

18. A. Bird, "Perceptions of Epigenetics," *Nature* 447, no. 7143 (2007): 396–398.

19. B. Samans, M. Rossello Chornet, A. Rossello Chornet, et al., "Epigenetic Immune Monitoring for COVID-19 Disease Course Prognosis," *Frontiers in Immunology* 14 (2023): 1107900.

20. U. Baron, S. Floess, G. Wieczorek, et al., "DNA Demethylation in the Human FOXP3 Locus Discriminates Regulatory T Cells From Activated FOXP3(+) Conventional T Cells," *European Journal of Immunology* 37, no. 9 (2007): 2378–2389.

21. A. S. Wolff, M. M. Erichsen, A. Meager, et al., "Autoimmune Polyendocrine Syndrome Type 1 in Norway: Phenotypic Variation, Autoantibodies, and Novel Mutations in the Autoimmune Regulator Gene," *Journal of Clinical Endocrinology and Metabolism* 92, no. 2 (2007): 595–603.

22. B. E. Oftedal, N. Delaleu, D. Dolan, A. Meager, E. S. Husebye, and A. S. B. Wolff, "Systemic Interferon Type I and B Cell Responses Are Impaired in Autoimmune Polyendocrine Syndrome Type 1," *FEBS Letters* 597, no. 9 (2023): 1261–1274.

23. T. Sjogren, S. Islam, I. Filippov, et al., "Single Cell Characterization of Blood and Expanded Regulatory T Cells in Autoimmune Polyendocrine Syndrome Type 1," *iScience* 27, no. 4 (2024): 109610.

24. U. Baron, J. Werner, K. Schildknecht, et al., "Epigenetic Immune Cell Counting in Human Blood Samples for Immunodiagnostics," *Science Translational Medicine* 10, no. 452 (2018): eaan3508.

25. G. Clavarino, N. Delouche, C. Vettier, et al., "Novel Strategy for Phenotypic Characterization of Human B Lymphocytes From Precursors to Effector Cells by Flow Cytometry," *PLoS One* 11, no. 9 (2016): e0162209.

26. E. J. Marquez, C. H. Chung, R. Marches, et al., "Sexual-Dimorphism in Human Immune System Aging," *Nature Communications* 11, no. 1 (2020): 751.

27. M. Terekhova, A. Swain, P. Bohacova, et al., "Single-Cell Atlas of Healthy Human Blood Unveils Age-Related Loss of NKG2C(+) GZMB(−)CD8(+) Memory T Cells and Accumulation of Type 2 Memory T Cells," *Immunity* 56, no. 12 (2023): 2836–2854.e9.

28. Z. Huang, B. Chen, X. Liu, et al., "Effects of Sex and Aging on the Immune Cell Landscape as Assessed by Single-Cell Transcriptomic Analysis," *Proceedings of the National Academy of Sciences of the United States of America* 118, no. 33 (2021): e2023216118.

29. K. R. Ryan, C. A. Lawson, A. R. Lorenzi, P. D. Arkwright, J. D. Isaacs, and D. Lilic, "$CD4^{+}CD25^{+}$ T-Regulatory Cells Are Decreased in Patients With Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy," *Journal of Allergy and Clinical Immunology* 116, no. 5 (2005): 1158–1159.

30. D. B. Palmer, "The Effect of Age on Thymic Function," *Frontiers in Immunology* 4 (2013): 316.

31. I. Hetemaki, J. Sarkkinen, N. Heikkila, et al., "Dysregulated Germinal Center Reaction With Expanded T Follicular Helper Cells in Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy Lymph Nodes," *Journal of Allergy and Clinical Immunology* 153, no. 5 (2024): 1445–1455.

32. G. Varricchi, J. Harker, F. Borriello, G. Marone, S. R. Durham, and M. H. Shamji, "T Follicular Helper (Tfh) Cells in Normal Immune Responses and in Allergic Disorders," *Allergy* 71, no. 8 (2016): 1086–1094.

33. K. Kisand, A. S. Boe Wolff, K. T. Podkrajsek, et al., "Chronic Mucocutaneous Candidiasis in APECED or Thymoma Patients Correlates With Autoimmunity to Th17-Associated Cytokines," *Journal of Experimental Medicine* 207, no. 2 (2010): 299–308.

34. S. LeibundGut-Landmann, O. Gross, M. J. Robinson, et al., "Syk- and CARD9-Dependent Coupling of Innate Immunity to the Induction of T Helper Cells That Produce Interleukin 17," *Nature Immunology* 8, no. 6 (2007): 630–638.

35. M. M. Curtis and S. S. Way, "Interleukin-17 in Host Defence Against Bacterial, Mycobacterial and Fungal Pathogens," *Immunology* 126, no. 2 (2009): 177–185.

36. J. Dobes, O. Ben-Nun, A. Binyamin, et al., "Extrathymic Expression of Aire Controls the Induction of Effective T(H)17 Cell-Mediated Immune Response to *Candida albicans*," *Nature Immunology* 23, no. 7 (2022): 1098–1108.

37. K. Kisand, D. Lilic, J. L. Casanova, P. Peterson, A. Meager, and N. Willcox, "Mucocutaneous Candidiasis and Autoimmunity Against Cytokines in APECED and Thymoma Patients: Clinical and Pathogenetic Implications," *European Journal of Immunology* 41, no. 6 (2011): 1517–1527.

38. J. Sng, B. Ayoglu, J. W. Chen, et al., "AIRE Expression Controls the Peripheral Selection of Autoreactive B Cells," *Science Immunology* 4, no. 34 (2019): eaav6778.

39. O. Bruserud, E. Bratland, A. Hellesen, et al., "Altered Immune Activation and IL-23 Signaling in Response to *Candida albicans* in Autoimmune Polyendocrine Syndrome Type 1," *Frontiers in Immunology* 8 (2017): 1074.

40. A. S. B. Wolff, S. Braun, E. S. Husebye, and B. E. Oftedal, "B Cells and Autoantibodies in AIRE Deficiency," *Biomedicine* 9, no. 9 (2021): 1274.

41. M. A. Ullah and F. Mackay, "The BAFF-APRIL System in Cancer," *Cancers* 15, no. 6 (2023): 1791.

42. F. Mackay and S. G. Tangye, "The Role of the BAFF/APRIL System in B Cell Homeostasis and Lymphoid Cancers," *Current Opinion in Pharmacology* 4, no. 4 (2004): 347–354.

43. F. Mackay, P. A. Silveira, and R. Brink, "B Cells and the BAFF/APRIL Axis: Fast-Forward on Autoimmunity and Signaling," *Current Opinion in Immunology* 19, no. 3 (2007): 327–336.

44. P. Schneider and J. Tschopp, "BAFF and the Regulation of B Cell Survival," *Immunology Letters* 88, no. 1 (2003): 57–62.

45. L. Baert, B. Manfroi, O. Casez, N. Sturm, and B. Huard, "The Role of APRIL - A Proliferation Inducing Ligand–In Autoimmune Diseases and Expectations From Its Targeting," *Journal of Autoimmunity* 95 (2018): 179–190.

46. S. R. Dillon, B. Harder, K. B. Lewis, et al., "B-Lymphocyte Stimulator/a Proliferation-Inducing Ligand Heterotrimers Are Elevated in the Sera of Patients With Autoimmune Disease and Are Neutralized by Atacicept and B-Cell Maturation Antigen-Immunoglobulin," *Arthritis Research & Therapy* 12, no. 2 (2010): R48.

47. M. M. Varin, L. Le Pottier, P. Youinou, D. Saulep, F. Mackay, and J. O. Pers, "B-Cell Tolerance Breakdown in Sjogren's Syndrome: Focus on BAFF," *Autoimmunity Reviews* 9, no. 9 (2010): 604–608.

48. F. B. Vincent, E. F. Morand, P. Schneider, and F. Mackay, "The BAFF/APRIL System in SLE Pathogenesis," *Nature Reviews Rheumatology* 10, no. 6 (2014): 365–373.

49. M. Thangarajh, T. Masterman, J. Hillert, S. Moerk, and R. Jonsson, "A Proliferation-Inducing Ligand (APRIL) is Expressed by Astrocytes and Is Increased in Multiple Sclerosis," *Scandinavian Journal of Immunology* 65, no. 1 (2007): 92–98.

50. M. Krumbholz, D. Theil, T. Derfuss, et al., "BAFF Is Produced by Astrocytes and Up-Regulated in Multiple Sclerosis Lesions and Primary Central Nervous System Lymphoma," *Journal of Experimental Medicine* 201, no. 2 (2005): 195–200.

51. T. Van Meerhaeghe, A. Neel, S. Brouard, and N. Degauque, "Regulation of CD8 T Cell by B-Cells: A Narrative Review," *Frontiers in Immunology* 14 (2023): 1125605.

52. G. M. Brodie, M. Wallberg, P. Santamaria, F. S. Wong, and E. A. Green, "B-Cells Promote Intra-Islet CD8+ Cytotoxic T-Cell Survival to Enhance Type 1 Diabetes," *Diabetes* 57, no. 4 (2008): 909–917.

53. M. Liu, S. Liang, and C. Zhang, "NK Cells in Autoimmune Diseases: Protective or Pathogenic?," *Frontiers in Immunology* 12 (2021): 624687.

54. M. Rodacki, B. Svoren, V. Butty, et al., "Altered Natural Killer Cells in Type 1 Diabetic Patients," *Diabetes* 56, no. 1 (2007): 177–185.

55. M. P. Nekoua, A. Bertin, F. Sane, et al., "Pancreatic Beta Cells Persistently Infected With Coxsackievirus B4 Are Targets of NK Cell-Mediated Cytolytic Activity," *Cellular and Molecular Life Sciences* 77, no. 1 (2020): 179–194.

56. Y. Zhang, H. Wang, X. Lou, et al., "Decreased Percentage of NKG2D+NK Cells in Patients With Incident Onset of Type 1 Diabetes," *Clinical and Experimental Pharmacology & Physiology* 44, no. 2 (2017): 180–190.

57. A. Oras, A. Peet, T. Giese, V. Tillmann, and R. Uibo, "A Study of 51 Subtypes of Peripheral Blood Immune Cells in Newly Diagnosed Young Type 1 Diabetes Patients," *Clinical and Experimental Immunology* 198, no. 1 (2019): 57–70.

58. R. Sender, Y. Weiss, Y. Navon, et al., "The Total Mass, Number, and Distribution of Immune Cells in the Human Body," *Proceedings of the National Academy of Sciences of the United States of America* 120, no. 44 (2023): e2308511120.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.