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Letter

Suspected ALPS with clinical and laboratory findings: Three patients—three different diagnoses

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Abstract: Autoimmune lymphoproliferative syndrome is a rare genetic disorder characterized by dysregulation of the immune system due to defective Fas mediated lymphocyte apoptosis. The clinical spectrum includes lymphoproliferative disease with lymphadenopathy, hepatomegaly, splenomegaly and an increased risk of lymphoma, as well as autoimmune disease typically involving blood cells. Definitive diagnosis is made by demonstrating infectious/non-malignant chronic lymphoproliferation for more than six months, high CD3+CD4−CD8− T Cell and defective lymphocyte apoptosis or one of the *FAS*, *FASL*, *CASP10* mutations. Since clinical and laboratory findings may overlap with other immune dysregulation or autoimmune diseases, differential diagnosis of autoimmune lymphoproliferative syndrome remains essential. Here, we present three cases of suspected autoimmune lymphoproliferative syndrome with clinical and laboratory findings, which resulted in three different diagnoses (chronic idiopathic thrombocytopenic purpura, ALPS-like and ALPS) after diagnostic evaluations. For all three cases, next-generation sequencing, flow cytometric analysis, protein expression and Fas mediated lymphocyte apoptosis with functional assays were performed.

Keywords: ALPS; ALPS-like; lymphocyte apoptosis; FAS mediated; childhood

Abbreviations: SM: splenomegaly; HM: hepatomegaly; LAP: lymphadenopathy; PIDs: primary immunodeficiencies; ALPS: autoimmune lymphoproliferative syndrome; DNT: CD4−CD8− double-negative T

1. Introduction

The altered immune or inflammatory function can be responsible for non-malignant lymphoproliferation, which can present as splenomegaly (SM), hepatomegaly (HM) or lymphadenopathy (LAP). Non-malignant lymphoproliferation can be part of the clinical spectrum of several primary immunodeficiencies (PIDs) and pose a significant diagnostic dilemma when they are the first clinical sign of immunodeficiency [1]. Autoimmune lymphoproliferative syndrome (ALPS) is a disease characterized by immune dysregulation resulting from impaired apoptosis of lymphocytes, mainly due to defective FAS-mediated apoptotic mechanism. Non-malignant lymphoproliferation and autoimmunity presenting mostly with cytopenia and an increased incidence of lymphoma are the most prominent manifestations of the clinical spectrum of ALPS [2]. Mutations in genes encoding FAS ligand (*FASL/TNFSF6*), Caspase 8 (*CAS8*), Caspase 10 (*CASP10*) and FAS-associated via death domain (*FADD*) are seen rarely. Patients with signs of ALPS who cannot be genetically identified are classified as ALPS-undetermined (ALPS-U). Conditions that may present with clinical findings that do not meet the current diagnostic criteria of ALPS and in which pathogenic variants are reported in the genes of the FAS/FASL pathway (*NRAS, KRAS, MAGT1, PIK3R1, LRBA, STAT3* GOF) are defined as ALPS-like disorders [3,4]. As a result of defects in FAS-mediated apoptosis, CD4−CD8− double-negative T (DNT) lymphocyte cells accumulate. Therefore, the functional apoptosis test can detect a defective response resulting from abnormal cell survival after FAS stimulation and is sufficient for a definitive diagnosis of ALPS, provided the criteria are met [5]. ALPS diagnostic criteria were finally revised in 2019 [6,7].

2. Case description

Since clinical and laboratory findings may overlap with other immune dysregulation or autoimmune diseases, differential diagnosis of ALPS remains essential. Here, we present three cases of suspected ALPS diagnosis with clinical and laboratory findings, which resulted in three different diagnoses after diagnostic evaluations (Table 1 and Figures 1–3). For all three cases, next-generation sequencing (NGS) was performed from genomic DNA obtained from peripheral blood mononuclear cells (PBMCs) using SOPHiA Clinical Exome Solution that covers the coding regions (±5 bp of intronic regions) of 4490 genes (target region of 12 Mb) related to rare inherited diseases including the causes of immundysregulatory syndromes.

Table 1. The demographic and clinical data of the patients.

	Case 1	Case 2	Case 3
Current age/gender	17y/F	10y/M	9y/F
Age of onset	14y	7y	8y
Consangunity	No	No	Yes
Autoimmunity	ITP	FMF	No
Lymphoproliferation	Cervical LAPs	Submandibular LAP HM	Multiple LAP in GIS Cervical LAPs
Other findings	Thrombocytopenia	Recurrent pneumonia	Elevated Vitamin B12
IgG; mg/dl	1254 (913–1884)	892 (842–1943)	1991 (842–1943)
IgM; mg/dl	236 (88–322)	101 (54–392)	61 (54–392)
IgA; mg/dl	157 (139–378)	53 (62–398)	69 (62–398)
IgE; IU/ml	53 (1–115)	88 (1–115)	202 (1–115)
Lymphocyte (µl)	2250	2600	2100
CD3+ T cells count (µl)	74.3 (64.4–85)	75.2 (57.2–86.2)	82.5 (55–86.2)
CD3+CD4+%	32.6 (31.7–57.6)	34.3 (27.3–46.7)	28 (23.4–48.7)
CD3+CD8+%	36.2 (13.9–39.1)	30.7 (16.5–39.4)	31.1 (16.8–46.5)
CD4−CD8− T lymphocytes (DNT) %	8.5 (0.5–3.9)	10.2 (0.4–3.4)	23.4 (0.2–4.5)
CD19+ %	12.8 (3.4–15.9)	14 (5.1–21.9)	9.4 (6.5–20.3)
CD16+56+ %	12.1 (5.1–24.7)	8 (1.8–26.6)	6.8 (4–29)
Gene	CASP10	PIK3R1	FAS
Mutation	c.416A > C	c.59T > A	c.795C > A
Zygosity	Heterozygous	Heterozygous	Heterozygous
Lymphocyte apoptosis	Normal	Abnormal	Abnormal
Outcome	Chronic ITP	ALPS-like	ALPS

Abbreviations: Ig: immunoglobulin; LAP: lymphadenopathy; HM: hepatomegaly; ITP: ımmune thrombocytopenic purpura; FMF: familial mediterranean fever; GIS : gastrointestinal system.

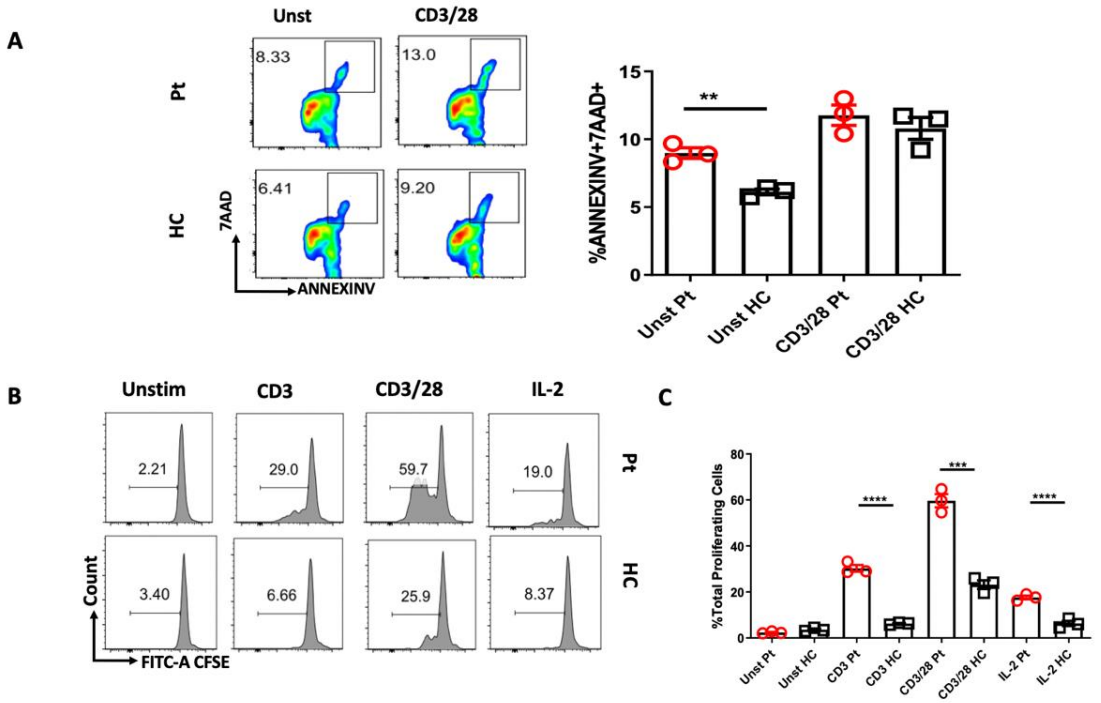

Figure 1. Lymphocyte apoptosis of the patients and healthy controls (*Case 1*): (A) Lymphocyte apoptosis is higher at the basal level compared to the control. There is no significant difference in CD3/CD28 stim condition. (B,C) Total proliferation is increased with mitogen stimulation and at baseline compared to the control.

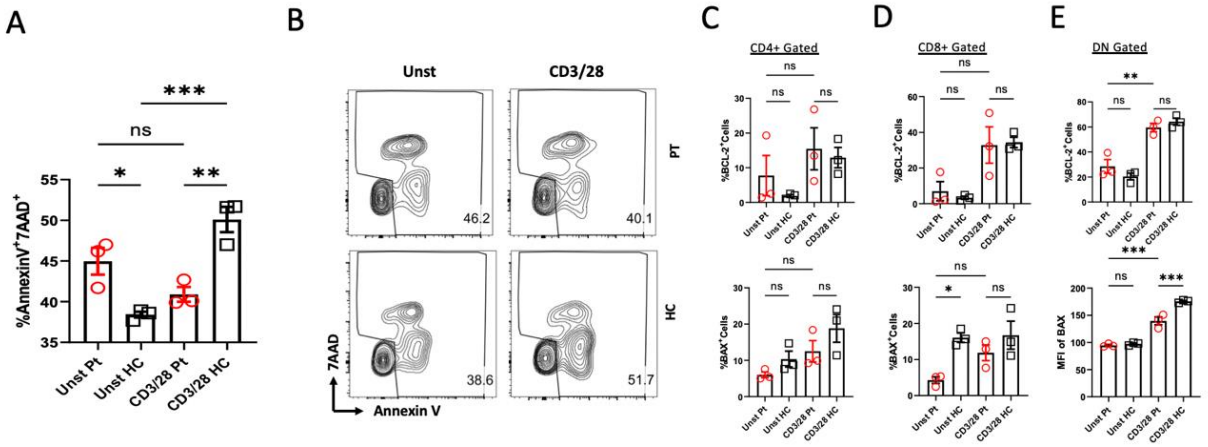

Figure 2. Lymphocyte apoptosis of the patients and healthy controls (*Case 2*): (A,B) Total apoptotic cells were reduced in the patient compared to healthy control in both unstimulated and CD3/28 stimulated conditions. (C–E) There is no difference in BCL-2

levels between the patient and healthy control, however, BAX levels were reduced in the patient's CD4+ and CD8+ T cells.

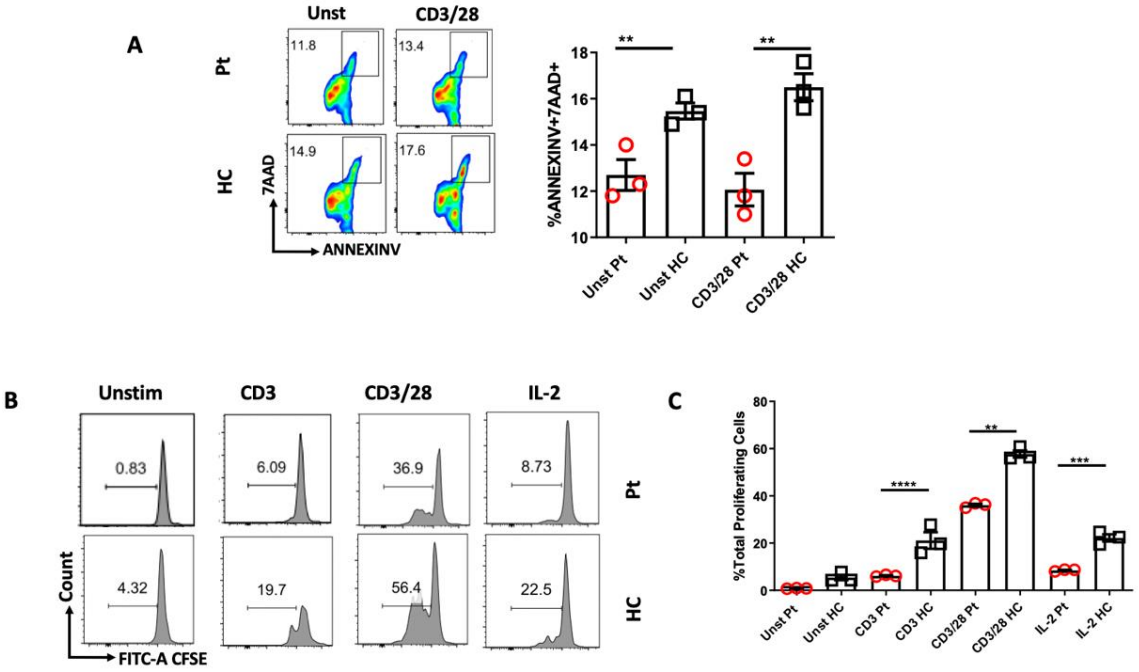

Figure 3. Lymphocyte apoptosis of the patients and healthy controls (*Case 3*): (A) Defective lymphocyte apoptosis in the patient's PBMCs with or without CD3/28 stimulation. (B,C) Total proliferations decreased with mitogen stimulation and at baseline compared to the control.

All our cases had persistent lymphoproliferation and increased DNT cells. However, *Case 1* had thrombocytopenia, *Case 2* had recurrent pneumonia and IgA deficiency and *Case 3* had elevated serum B12 and IgG levels. In these patients with suspected ALPS; *CASP10*, *PIK3R1* and *FAS* gene mutations were detected. With the results of the apoptosis tests performed for definitive diagnosis, ALPS could be excluded in *Case 1*; *Case 2* was diagnosed as ALPS-like, and *Case 3* as ALPS.

Studies describing the clinical manifestations of ALPS have shown that approximately 90% of patients have LAP, SM and HM [3,5,8]. Persistent LAP was recorded in all three cases. The authors emphasized that autoimmune cytopenias (especially hemolytic anemia) may be the first sign of the disease without lymphoproliferation [8]. The presence of chronic ITP accompanying LAP in *Case 1* led us to the conclusion that we should search for ALPS in the patient. It has also been reported that the risk of developing secondary malignancies, especially Hodgkin and Non-Hodgkin lymphoma, is approximately 9% [3]. The susceptibility to lymphoma required us to follow our patients closely.

The most important determinant laboratory finding in ALPS patients is the high presence of DNT cell lymphocyte population [9], similar to our *Case 3*. In addition, high levels of vitamin B12, IL-10, IL-18 and sFASL can commonly be encountered in patients with ALPS. Consistent with the literature, vitamin B12 and IgG levels were also high in *Case 3*. The combination of these markers has proven to be a strong predictor of *FAS* mutations in patients with the ALPS phenotype, making them the most

valuable and cost-effective biomarkers [6,7,10]. Therefore, measuring these biomarkers is important in evaluating patients with ALPS as a preliminary diagnosis.

Demonstrating a functional defect in lymphocyte apoptosis is a criterion for diagnosing ALPS. However, revealing this defect is labor-intensive and costly. The test can only be done in experienced laboratories. Demonstrating the defect in apoptosis is critical to confirm the genetic mutation compatible with the ALPS phenotype, a genetic variant of uncertain significance, or the diagnosis in patients with an unknown genetic defect [5]. Functional apoptosis studies show that ALPS patients are significantly resistant to Fas-mediated cell death compared to healthy controls. An abnormal in vitro apoptosis assay has been noted to be highly specific for patients with definite ALPS and the only biomarker showing a significant difference between definite and suspected ALPS groups. It has also been indicated that a normal in vitro apoptosis test is a marker for ruling out ALPS [9]. One of our pieces of evidence in exclusion of ALPS diagnosis in *Case 1* was normal lymphocyte apoptosis shown with functional assays (Figure 1A). To investigate apoptosis defect in our study, when Annexin V 7AAD staining was performed on cells incubated with CD3 and CD28 mitogens in a 37 ℃ incubator for 2 days, normal apoptosis tendency was observed in *Case1* (Figure 1A). When the cells from *Case 1* and *Case 3* were stimulated with CD3, CD3/CD28 and IL-2 mitogens, total proliferating cells were increased in *Case1* compared to healthy control, unlike *Case 3* (Figure 1B,C and 3B,C).

On the other hand, the abnormal apoptosis assays in *Cases 2* and *3* strongly supported the diagnosis of the patients with ALPS-like and ALPS disease, respectively (Figure 2A,B and 3A). Hafezi et al. [3] showed defects in apoptosis in 87.3% of ALPS patients and 78.3% of ALPS-like patients. Also, we investigated anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax levels on CD4+, CD8+ and DNT cells. Consistent with these results, we showed only a decrease in total apoptotic cells in *Case 2* compared to the healthy control (Figure 2C–E). Clinical immunology laboratory test revealed elevated DNT cells for the case patients (Table 1). This analysis could also be repeated on separate occasions in a research laboratory setting (Figure S1).

3. Discussion and conclusions

ALPS-FAS, the result of a germline mutation in the *FAS* gene, is seen in approximately 70% of patients. The majority of mutations affect the intracellular death domain. Mutations affecting extracellular regions of the protein usually result in loss of protein expression from one allele, and these patients have a milder clinical presentation and lower penetration. Patients with extracellular domain mutations presenting with more severe clinical manifestations are explained by somatic mutations in the second allele of FAS resulting from "two hits". Healthy relatives of ALPS-FAS patients may also carry a dominant *FAS* mutation with functional (healthy carrier T cells exhibit defective Fas-induced apoptosis) but non-clinical penetrating. *FAS* mutations that cause cellular apoptosis abnormalities alone are insufficient to cause ALPS, and additional genetic or environmental factors may play a role in the prognosis [5,9]. The mutation observed in *Case 3* was also demonstrated in the patient's mother, and the absence of clinical findings in the mother suggests this theory.

Multiple genetic defects caused by mutations outside the FAS-FASL pathway can present with the ALPS phenotype and are defined as ALPS-like syndromes. There are no clinical or laboratory guidelines for diagnosing ALPS-like patients. Heterozygous mutation in the *PIK3R1* gene is characterized by recurrent respiratory tract infections, lymphoproliferation and antibody deficiency,

which strongly overlaps with the clinical findings of our patient [4]. The detected *PIK3R1* gene mutation and ALPS-like presentation in *Case 2* led us to investigate the patient comprehensively.

Splenomegaly was the most common clinical finding, followed by autoimmune cytopenias and LAP in the systemic review evaluating ALPS and ALPS-like phenotypes [3]. However, the frequency of respiratory tract infections was significantly higher in ALPS-like patients as in patients with *PIK3R1* mutation than in ALPS [3]. Immunological analyzes showed lower serum IgA, IgG and lymphocyte counts in ALPS-like patients compared to ALPS. In addition, it was mentioned that high DNT was not pathognomonic in ALPS-like patients, and high vitamin B12, sFASL or IL-10 were detected in a limited number of patients [3]. As stated in the literature, persistent LAP, HM and recurrent pneumonia with low IgA levels in *Case 2* are consistent with the *PIK3R1* mutation and ALPS-like phenotype.

The immune dysregulation disease category is often challenging to interpret without comprehensive genetic analysis [3]. This group of diseases can be camouflaged or not considered because of the prevailing clinical features of lymphoproliferation and autoimmunity. Hence, some patients will stay undiagnosed. This risk impairs their quality of life and increases morbidity and mortality. An underlying PID should be mainly evaluated, especially in severe cases with concomitant signs of autoimmunity and unusual, recurrent, or severe infections, to initiate an appropriate treatment regimen. It is relevant to mention that immune diseases, particularly if associated with benign lymphoproliferation or autoimmunity, also exhibit an increased risk of lymphoid malignancies [1]. Patients should be briefed about the alarm symptoms of malignant neoplasms, especially lymphoma. Early diagnosis can provide better treatment options before severe organ damage occurs.

In this manuscript we reviewed the clinical pictures, laboratory features and genetic background of the patients with ALPS-like syndromes, including those characterized by lymphoproliferation, autoimmunity and immunodeficiency but with different diagnoses. Identifying many different genetic diseases with ALPS-like phenotypes has made the diagnostic approach difficult. However, flow cytometric analysis, protein expression and functional assessment of different cell subpopulations are helpful in the diagnosis.

Ethical approval

The study protocol was approved by the local ethics committee of Erciyes University (approval number 2018/388).

All methods were approved by the institutional review board. The handling of all human samples was performed following the relevant guidelines and regulations.

Use of AI tools declaration

The authors declare that they have not used artificial intelligence (AI) tools in the creation of this article.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

Author contributions

Tuba Karakurt and Nurhan Kasap conceptualized the study. Ahmet Eken and Nurhan Kasap designed and supervised the study. Kübra Aslan and Ahmet Eken performed the experiments.Tuba Karakurt, Nurhan Kasap, Kübra Aslan, and Ahmet Eken wrote the manuscript. Tuba Karakurt, Nurhan Kasap, Hayrunnisa Bozkurt, Fatma Bal Cetinkaya, Filiz Ozen, Gizem Uslu, Zafer Bıcakcı, Ozlem Cavkaytar, and Mustafa Arga cared for the patients, collected data intellectually, and contributed to the manuscript and discussions. All authors read the manuscript and contributed to the revision and discussions.

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