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Complete List of Authors:	Eisenhauer, Nina; University Hospital Erlangen, Department of Internal Medicine 5, Hematology and Oncology Miano, Maurizio; IRCCS Giannina Gaslini, Hematology Naumann-Bartsch, Nora; University Hospital Erlangen, Department of Pediatrics Leyh, Joerg; University Hospital Erlangen, Department of Pediatrics; Klinik Hallerwiese-Cnopfsche Kinderklinik, Department of Pediatric Hematology and Oncology Dell'Orso, Gianluca; IRCCS Istituto Giannina Gaslini, Hematology Unit, Department of Pediatric Hematology/Oncology Aigner, Michael; University Hospital Erlangen, Department of Internal Medicine 5, Hematology and Oncology Fecker, Gisela; University Hospital Erlangen, Department of Pediatrics and Adolescent Medicine Hinze, Claas; University Hospital Münster, Department of Pediatric Rheumatology and Immunology Wittkowski, Helmut; University Hospital Münster, Department of Pediatric Rheumatology and Immunology Bruns, Heiko; University Hospital Erlangen, Department of Internal Medicine 5, Hematology and Oncology Zierk, Jakob; University Hospital Erlangen, Department of Pediatrics and Adolescent Medicine Metzler, Markus; University Hospital Erlangen, Department of Pediatrics and Adolescent Medicine Arkwright, Peter; University of Manchester, Lydia Becker Institute of Immunology and Inflammation, Royal Manchester Children's Hospital Graw, Frederik; University Hospital Erlangen, Department of Internal Medicine 5, Hematology and Oncology Mackensen, Andreas; University Hospital Erlangen, Department of Internal Medicine 5, Hematology and Oncology Völkl, Simon; University Hospital Erlangen, Department of Internal Medicine 5, Hematology and Oncology
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Detection of signature double-negative T cells is a predictive marker to identify autoimmune lymphoproliferative syndrome associated with FAS loss of function.

Nina Eisenhauer [1*], Maurizio Miano [2*], Nora Naumann-Bartsch [3], Jörg Leyh [3,4], Gianluca Dell'Orso [2], Michael Aigner [1], Gisela Fecker [3], Claas Hinze [5], Helmut Wittkowski [5], Heiko Bruns [1], Jakob Zierk [3], Markus Metzler [3], Peter D. Arkwright [6], Frederik Graw [1], Andreas Mackensen [1], Simon Völkl [1]

[1] Department of Internal Medicine 5, Hematology and Oncology, University Hospital Erlangen, Erlangen, Germany

[2] Hematology Unit, Department of Pediatric Hematology/Oncology, IRCCS Istituto Giannina Gaslini, Genova, Italy

[3] Department of Pediatrics, University Hospital Erlangen, Erlangen, Germany

[4] Department of Pediatric Hematology and Oncology, Klinik Hallerwiese-Cnopfsche Kinderklinik, Nürnberg, Germany

[5] Department of Pediatric Rheumatology and Immunology, University Hospital Münster, Münster, Germany

[6] Lydia Becker Institute of Immunology and Inflammation, Royal Manchester Children's Hospital, University of Manchester, United Kingdom

[*]N.E. and M.M. contributed equally to this study.

Correspondence: Department of Internal Medicine 5, Hematology and Oncology, University Hospital Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany; e-mail: simon.voelkl@uk-erlangen.de; phone: +49 9131 85 43 168

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To the Editor:

The Autoimmune-lymphoproliferative syndrome (ALPS) is caused by defects in the FAS-dependent apoptosis leading to chronic lymphoproliferation, autoimmunity, chronic multilineage cytopenia an increased risk for developing B cell lymphomas and a characteristic accumulation of CD4/CD8-negative, T-cell receptor (TCR) αβ-positive T-cells (double-negative, DNT-cells).[1,2] The heterogeneous clinical phenotype of ALPS patients and overlap with similar diseases like CTLA-4 and LRBA deficiencies or STAT3 gain-of-function impedes its diagnosis. Additionally, the genetic analysis for mutations effecting the FAS-dependent apoptosis is cost and time consuming and therefore challenging, especially if uncommon alterations like deep intronic FAS mutation or copy number variants are present.

Notably, patients may not respond to steroid first-line therapy and often require specific immunosuppressive treatments such as mycophenolate mofetil (MMF) or sirolimus. Particularly, the mTOR (mammalian target of rapamycin) inhibitor sirolimus effectively and specifically reduces the clinical symptoms in ALPS patients, due to the mTOR hyperactivity of ALPS DNT-cells. Thus it should be considered as standard of treatment and provided as quickly as possible.[1] Therefore, a fast and secure tool is needed to filter patients with high probability of an ALPS related mutation to accelerate diagnosis and specific treatment.

As DNT-cells can also be increased in other diseases such as LRBA and CTLA-4 deficiencies or STAT3 gain-of-function, analyzing their percentage alone may not be sufficiently discriminatory.[3] We therefore investigated how standard flow-cytometry analysis of DNT-cells and their abnormal expression pattern is efficient to predict ALPS associated with FAS deficiency.

Effector-memory cells re-expressing CD45RA (TEMRA) can be defined by the expression of CD45RA+ and the absence of CCR7. TEMRA cells show senescence or terminal differentiation with low mitotic capacity, similar to cells expressing CD57. ALPS DNT-cells however simultaneously express CD57, the TERMA phenotype, and the proliferation marker Ki67.[2] The expression of CD38 and CD45RA was recently shown to be characteristic for the aberrant ALPS DNT phenotype.[3] Hence, we analyzed the expression of Ki67, CD57, TEMRA, CD38 and CD45RA in DNT-cells.

Patients were categorized according to the established diagnostic criteria for ALPS and ALPS related phenotypes as described in material and methods. 14 ALPS-FAS/sFAS, 12 suspected-ALPS (susp-ALPS), 22 ALPS-like cases were included in this study (suppl. Table 1). Susp-ALPS combines patients fulfilling the criteria for ALPS diagnosis but harboring another underlying mutation, ALPS-like were defined as previously reported.[4] Additionally, 16 patients with chronic autoimmune cytopenia (AC), 12 patients with chronic nonmalignant lymphoproliferation (LP) and 25 healthy donors (HD) were analyzed. Common ALPS parameters (DNT-cells, TEMRA, CD57) and novel markers (Ki67, CD38+/CD45RA+) showed significant elevation in ALPS-FAS/sFAS compared to HD, AC, and LP but several overlaps with susp-ALPS and ALPS-like (Figure 1A-E; suppl. Fig. 1). ROC curves were plotted to compare the predictive value of each marker for ALPS-FAS/sFAS diagnosis (Figure 1F, G). Using Ki67+, CD38+/CD45RA+ and TEMRA+ cells as markers resulted in a high predictive performance to distinguish ALPS-FAS/sFAS and ALPS-like with an AUC of, 1.0 (CI 1.0), 0.98 (CI 94-1.0), and TEMRA+ 0.96 (CI 0.9-1.0) respectively. DNT-cell frequency and CD57+ cells performed less well with an AUC of 0.92 (CI 0.82-1-0) and 0.86 (CI 0.73-1.0). Separating ALPS-FAS/sFAS and susp-ALPS however was more challenging with the best prediction by Ki67+ and CD38+/CD45RA+, producing an AUC of 0.88 (CI 0.73-1.0) and 0.87 (CI 0.73-1.0) respectively. DNT-cell frequency, TEMRA+ and CD57+ distinguished ALPS-FAS/sFAS and susp-ALPS with an AUC of 0.73 (CI 0.52-0.94), 0.75 (0.53-0.97), and 0.71 (CI 0.5-0.92).

Furthermore, cut-offs for each marker were calculated based on the individual ROC-curves as indicated in the Material & Methods. For simplification, ALPS-FAS/sFAS patients were compared to susp-ALPS

and ALPS-like patients combined as autoimmune-lymphoproliferative immunodeficiencies (AL-PID/ALPID-U) according to recently reported recommendations.[3] (Figure 1H, I). Patients with a DNT-cell population above 3.86% were assigned to the ALPS-FAS/sFAS cohort (F1-score: 0.72). The classification of patients with more than 31.36% Ki67+ T-cells as ALPS-FAS/sFAS lead to the highest separation quality of 0.90. Using the cut off for CD38+/CD45RA+, CD57+ or TEMRA+ DNT-cells, which were 35.33%, 43.78% and 30.76% respectively to separate ALPS-FAS/sFAS from AL-PID/ALPID-U produced an F1-score of 0.81, 0.72 and 0.72. Thus, risk identification by Ki67+ or CD38+/CD45RA+ is comparable to commonly used markers like sFASL, IL10 or vitamin B12, suggested for instance in the landmark study of Caminha et al., 2010.[5] Patients with a high probability for a FAS mutation could be quickly identified by a single 6-8 parameter flow-cytometric analysis (Suppl. Table 2) and forwarded to further genetic analysis. Moreover, patients unlikely to harbor a FAS mutation could be ruled out and provided with alternative diagnostic options. This would be much faster than performing several immunoassays to measure sFASL, IL10 or Vitamin B12 and an additional cytometric analysis of the DNT-cell frequency. Like this, time and cost might be saved by reducing the need for gene analysis and the patient can be supported with the best suiting treatment. Similarly, Palmisani et al. evaluated a flow-cytometry panel comparing the increase of four cell populations in ALPS-FAS/sFAS and ALPS-U (CD3+/TCRαβ+/CD4-/CD8-; CD3+/CD25+; CD3+/HLADR+; TCRαβ+/B220+ and CD19+/CD27+). In contrast to our study, only the concurrent elevation of all 4 populations were shown to be of value to separate ALPS-FAS/sFAS and ALPS-U.[6]

Four patients of the susp-ALPS cohort showed a CD38+/CD45RA+ and Ki67 expression in the range of ALPS-FAS/sFAS patients. Although other mutations were found by the reference laboratory, the possibility of a hidden FAS mutation cannot be definitively precluded. Therefore, patients categorized as AL-PID or ALPID-U with a high probability for a FAS mutation using the specific markers should be forwarded to additional sequencing methods to check for a somatic FAS mutation in sorted DNT-cells or copy number variants. Those sequencing methods are often not included in the standard diagnostic procedure, resulting in the possibility to overlook those hidden mutations. Unfortunately, no in vitro functional apoptosis test was performed in these four patients, which is often not feasible without specialized laboratories. Functional apoptosis defects were only detected in three ALPS-U patients, which all do not show high CD38+/CD45RA+ or Ki67 expression. This highlights the unreliability of the apoptosis assay, which is not standardized between centers and often produces false positive or negative results.

It was further assessed if a combination of the DNT-cells plus one additional marker would lead to a higher quality separation (Figure 1H). Therefore, logistic-regression was performed to predict ALPS-FAS/sFAS or AL-PID/ALPID-U based on combined marker levels. Although it did not improve separation by Ki67 and CD38/CD45RA as single markers, the combination of DNT-cell percentage plus CD57+ and TEMRA+ increased the separation quality to 0.74 and 0.77 respectively. One AL-PID patient with a high expression of CD57 and TEMRA and a DNT-cell frequency of 25.0%, even exceeding those measured in ALPS-FAS/sFAS patients, is mainly impairing the separation. This patient was diagnosed with a NFκB mutation, again underlining the sensitivity of Ki67 and CD38+/CD45RA+ as markers for ALPS-FAS/sFAS, as their expression is not increased. However it shows that analyzing the DNT phenotype might also be interesting to screen for other similar mutations beside FAS.

Concluding, we propose to perform an initial flow-cytometry staining to analyze the expression of CD38/CD45RA in DNT-cells, as it is less time and resource-intensive than the intracellular Ki67 staining. Patients with a high probability of a FAS mutation could be detected with only one standard 6-color flow-cytometry staining (Suppl. Table 2). Especially for small hospitals with no immediate access to genetic analysis, this may lead to a much faster diagnosis.

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Contribution: N.E., M.M., N.N.B., J.L., and S.V. designed the research; M.M., N.N.B., J.L., G.D., C.H., H.W., G.F., J.Z., M.M., and P.D.A. repeatedly referred patients; N.E., M.M., M.A., H.B., P.D. A., and S.V. performed experiments; N.E., M.M., F.G., A.M., and S.V. analyzed and interpreted data; N.E., M.M., N.N.B., A.R.E., A.M. and S.V. wrote the manuscript; and all of the authors edited the manuscript

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Appendix: Methods, Suppl. Table 1-3 and Suppl. Figure 1.

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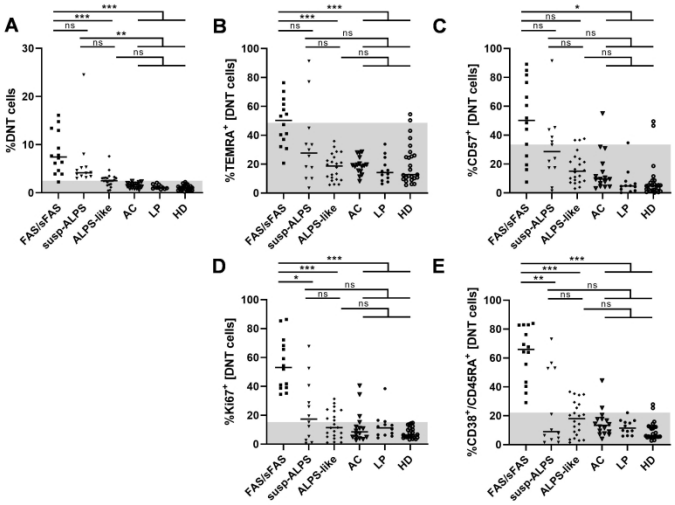
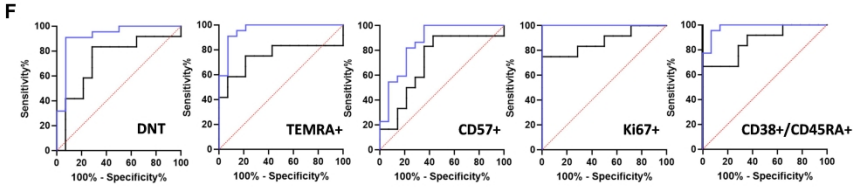
Figure legend

Figure 1: Analysis of signature double-negative T cells in ALPS and AL-PID patients.

(A-E) Patients with ALPS-FAS/sFAS, suspected-ALPS (susp-ALPS), ALPS-like, chronic autoimmune cytopenia (AC), and chronic nonmalignant lymphoproliferation (LP) patients were analyzed as described in material and methods, HD served as controls. Frequencies of DNT-cells (A) and the percentage of TEMRA+ (B), CD57+ (C), Ki67+ (D), CD38+/CD45RA+ (E) DNT-cells are shown. The grey bar depicts the diagnostic 2.5% cut-off in DNT-cell frequency (A) and a threshold represented by

mean+2*SD of the HD cohort (B-E). Each symbol represents an individual subject. ns, not significant; *p<.05, **p<.01, ***p<.001. (F-G) ROC curves and corresponding AUC values to separate ALPS-FAS/sFAS and susp-ALPS (black line) and ALPS-FAS/sFAS and ALPS-like (blue line) by each marker expression are shown. (H) Cut-offs were calculated that separate ALPS-FAS/sFAS and AL-PID as described in material and methods. The F-score for each separation using the expression of each marker or the DNT-frequency plus one additional marker is shown. (I) Cut-offs for the DNT-cell frequency plus one additional marker depicted by the intersection between red and gray area are shown. Each symbol represents an individual ALPS-FAS/sFAS (square) or an AL-PID patient (no FAS mutation; open circle).





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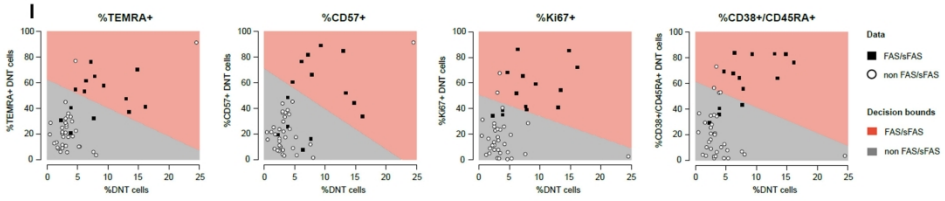
	Parameter	AUC	95% Confidence Interval	P value
ALPS-FAS/sFAS vs. susp-ALPS	%DNT cells	0.73	0.52-0.94	0.0506
	% TEMRA+	0.75	0.53-0.97	0.0308
	%CD57+	0.71	0.5-0.92	0.0718
	%CD38+/CD45RA+	0.87	0.73-1.0	0.0014
	%KI67+	0.88	0.73-1.0	0.0012
ALPS-FAS/sFAS vs. ALPS-like	%DNT cells	0.92	0.82-1.0	<0.0001
	% TEMRA+	0.96	0.9-1.0	<0.0001
	%CD57+	0.86	0.73-1.0	0.0003
	%CD38+/CD45RA+	0.98	0.94-1.0	<0.0001
	%KI67+	1.0	1.0	<0.0001

H

Parameter	F1-Score *(Individual Parameter)*	F1-Score *(%DNT+Parameter)*
%DNT cells	0.72	-
%TEMRA+	0.73	0.77
%CD57+	0.72	0.75
%Ki67+	0.90	0.81
%CD38+/CD45RA+	0.81	0.74



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Apendix: Methods, Suppl. Table 1-3, and Suppl. Figure 1.

Materials and Methods

Patient and control samples

The study was approved by the ethics committee of the University Erlangen-Nuremberg (protocol number 267_16B). Written informed consent was obtained from patients and controls. The study was conducted in accordance with the Declaration of Helsinki. The procedures were based on standard of care, and established clinical guidelines were followed. Patients were categorized according to the established protocol for ALPS diagnosis as described by the NIH in 2009 and Miano et al.[4,5] Required criteria are depicted by chronic nonmalignant lymphoproliferation with splenomegaly and/or lymphadenopathy, an elevation of DNT-cells above 2.5% in the peripheral blood. A defective FAS mediated apoptosis in-vitro and a mutation of FAS, FASLG or CASP10 are primary accessory criteria. Secondary accessory criteria include autoimmune cytopenias and increased IgG level, elevated biomarker levels (sFASL, IL10, Vitamin B12 or IL18), characteristic immunohistological findings and a family history of nonmalignant lymphoproliferation. Patients have to present with both required criteria and one primary accessory criterion for a definite ALPS diagnosis. If a FAS mutation was confirmed, patients were classified as ALPS-FAS/sFAS. Suspected-ALPS (susp-ALPS) combines patients fulfilling the criteria for ALPS diagnosis but harboring another underlying mutation. Patients were classified as ALPS-like if the required criteria without further criteria were met or if the patient presented with one required criterion and autoimmune cytopenia with or without further accessory criteria. In total 14 ALPS-FAS/sFAS (mean age 18, range 2-43), 12 susp-ALPS (mean age 13, range 1-28), and 22 ALPS-like (mean age 12, range 2-48) cases were analyzed, patient characteristics are shown in suppl. Table 1. Healthy donors (n=25; mean age 24, range 13-45), and patients with only chronic autoimmune cytopenia (n=16; mean age 13, range 1-24) or chronic nonmalignant lymphoproliferation (n=12; mean age 10, range 1-41) served as controls. The diagnostic protocol was updated according to the recently published recommendations to redefining ALPS and other lymphoproliferative immune disorders (Magerus et al., 2023).[3] Accordingly, patients harboring a FAS related mutation were categorized as ALPS-FAS/sFAS, autoimmune-lymphoproliferative immunodeficiencies with another genetic defect were re-classified as AL-PID and patients lacking genetic mutation as ALPID-U.

Flow-cytometry

Blood samples (EDTA, 200 µl per tube) were stained with fluorochrome-coupled antibodies (Suppl. Table 3), red blood cells were lysed by Lyse/Fixation kit (BD Biosciences) according to the manufacturer's protocol. Intracellular staining was performed after red blood cell lysis using the Fixation/Permeabilization Kit (BD Biosciences). Flow-cytometry data were acquired on a LSRFortessa (BD Biosciences) and cells were analyzed for indicated antibodies with FlowJo software v10 (TreeStar, Ashland, Oregon, USA) and Kaluza software v2.1 (Beckmann Coulter, Krefeld, Germany). Doublets were excluded by FSC-A/FSC-H, leukocytes were gated via CD45/SSC-A, viable lymphocytes were determined by FSC-A/SSC-A, T-cells were determined by CD3/TCRab, DNT-cells were gated for CD4-/CD8- and analyzed for additional markers (CD57, CCR7, CD45RA, CD38, Ki67). A minimum of 200,000 events were acquired per tube, and there had to be at least 20 events in the specified gates to define the cell populations. Representative expression patterns are shown in Suppl. Fig. 1.

Statistical analysis

Data were analyzed with Graphpad Prism software (GraphPad San Diego, California, USA) for results presented within Figure 1A-G, and with the R software for statistical computing (ref) version 4.1.3 for Figure 1H and I using the packages *base* and *cutpointr*. Differences in the marker expression across patient subgroups (Fig. 1A-E) were assessed by Kruskal-Wallis test followed by Dunn's multiple comparisons test to identify pairwise differences and account for multiple testing. A p-value of <0.05 was considered significant. Cut-offs for individual markers to separate ALPS-FAS/sFAS vs. AL-PID/ALPID-U were determined based on ROC-curves by maximizing the Youden-metric, which is defined by the term sensitivity + specificity -1. Subsequent performance of patient classification was assessed by calculating the F1-score. To assess the ability of classifying ALPS-FAS/sFAS and AL-PID/ALPID-U patients based on marker combinations, pairwise logistic-regression analysis was performed always combining the percentage of DNT-cells with one of the additional markers. F1-scores were calculated using the obtained confusion matrices. Decision boundaries in Figure 1I are calculated based on the prediction probabilities obtained by the logistic-regression models.

Suppl. Table 1: Patients characteristics

UPN	Category	Disease	Sex	Age	Gene	Mutation	LP	AC	DNT	Apoptosis test	soluble biomarker(s)
1	ALPS-FAS/sFAS	ALPS-FAS	M	30	FAS	p.Leu179Arg	+	-	7.8	nd	+
2	ALPS-FAS/sFAS	ALPS-FAS	F	18	FAS	c.476_489del	+	-	3.9	nd	+
3	ALPS-FAS/sFAS	ALPS-sFAS	F	13	sFAS	c.697_698dupA	+	+	4.6	-	+
4	ALPS-FAS/sFAS	ALPS-FAS	M	14	FAS	c.627_628delTC	+	-	16.1	+	+
5	ALPS-FAS/sFAS	ALPS-FAS	F	8	FAS	c.627_628delTC	+	-	14.8	+	+
6	ALPS-FAS/sFAS	ALPS-FAS	F	12	FAS	p.Gln273Hys	+	+	7.7	-	+
7	ALPS-FAS/sFAS	ALPS-FAS	F	43	FAS	c.627_628delTC	+	-	6.4	+	+
8	ALPS-FAS/sFAS	ALPS-FAS	M	18	FAS	p.Leu179Arg	+	-	2.3	nd	+
9	ALPS-FAS/sFAS	ALPS-sFAS	F	12	sFAS	c.677-1G>T	+	+	13.0	nd	+
10	ALPS-FAS/sFAS	ALPS-FAS	F	31	FAS	p.Gln273*	+	-	9.3	+	+
11	ALPS-FAS/sFAS	ALPS-FAS	F	2	FAS	p.P217fs	+		13.4	nd	+
12	ALPS-FAS/sFAS	ALPS-FAS	M	13	FAS	c.652-2A-G	+	-	6.2	+	+
13	ALPS-FAS/sFAS	ALPS-sFAS	F	28	sFAS	p.Glu202fs	+	+	3.9	-	+
14	ALPS-FAS/sFAS	ALPS-FAS	F	14	FAS	p.Cys129Arg	+	-	7.2	+	+
15	suspected-ALPS	AL-PID	M	1	LRBA CARD11	c.6036C>T c.2270-4G>A	+	+	8.0	nd	+
16	suspected-ALPS	AL-PID	F	17	KMT2D CARD11	p.Gly4999Trp*fs*6 p.Arg518Gly	+	+	3.0	nd	+
17	suspected-ALPS	AL-PID	F	9	PIK3CG	p.Gly133Ser p.Thr176Arg	+	-	3.4	nd	+
18	suspected-ALPS	AL-PID	M	19	NFκB1	c.258+2T>C	+	+	3.9	nd	+
19	suspected-ALPS	ALPID-U	F	12	NGS neg	-	+	+	3.2	+	+

20	suspected-ALPS	AL-PID	M	24	IKBKG	p.Glu125Lys	+	+	5.4	+	+
21	suspected-ALPS	ALPID-U	M	8	NGS neg	-	+	-	3.5	-	+
22	suspected-ALPS	ALPID-U	M	4	NGS neg	-	+	-	5.2	-	+
23	suspected-ALPS	AL-PID	F	28	CECR1	p.Thr145Pro	+	+	4.7	+	+
24	suspected-ALPS	AL-PID	M	13	22q11.2		+	+	24.5	nd	+
25	suspected-ALPS	ALPID-U	M	3	NGS neg	-	+	+	4.1	-	+
26	suspected-ALPS	AL-PID	M	15	TNFRSF13C	p.His159 Pro21Arg	+	+	4.2	-	+
27	ALPS-like	AL-PID	F	17	LRBA	p.Glu946* p.Cys1443Arg	-	+	7.8	-	-
28	ALPS-like	ALPID-U	M	10	NGS neg	-	+	+	2.6	nd	-
29	ALPS-like	ALPID-U	M	2	declined		+	+	0.8	nd	-
30	ALPS-like	ALPID-U	M	9	nd		-	+	3.2	nd	-
31	ALPS-like	ALPID-U	M	9	NGS neg	-	+	+	1.8	nd	-
32	ALPS-like	ALPID-U	M	2	WES neg	-	+	+	2.6	nd	+
33	ALPS-like	ALPID-U	F	16	nd		+	+	3.5	nd	-
34	ALPS-like	ALPID-U	M	8	nd		-	+	3.1	nd	-
35	ALPS-like	ALPID-U	M	16	NGS neg	-	+	+	2.7	nd	-
36	ALPS-like	ALPID-U	F	9	NGS neg	-	-	+	3.2	nd	+
37	ALPS-like	ALPID-U	F	8	NGS neg	-	-	+	4.7	-	+
38	ALPS-like	ALPID-U	M	11	WES neg	-	+	+	1.7	nd	+
39	ALPS-like	ALPID-U	M	17	NGS neg	-	+	+	1.6	-	+
40	ALPS-like	ALPID-U	M	15	WES neg	-	-	+	3.0	nd	-
41	ALPS-like	AL-PID	F	3	CARD11	p.Arg967Cys	+	+	2.5	nd	+
42	ALPS-like	ALPID-U	M	12	NGS neg	-	+	-	2.5	-	-
43	ALPS-like	ALPID-U	M	11	NGS neg	-	-	+	0.5	+	+
44	ALPS-like	ALPID-U	M	12	NGS neg	-	+	+	2.5	-	-
45	ALPS-like	ALPID-U	F	18	NGS neg	-	+	+	2.2	-	+
46	ALPS-like	ALPID-U	F	17	NGS neg	-	-	+	3.3	nd	-
47	ALPS-like	ALPID-U	M	48	NGS neg	-	+	+	1.2	nd	+
48	ALPS-like	AL-PID	M	3	TACI	p.Gln57His	-	+	3.1	-	+

AL-PID: Autoimmune-lymphoproliferative immunodeficiency; ALPID-U: AL-PID with no identified gene variant; NGS: next-generation sequencing; WES: whole-exome sequencing; LP: lymphoproliferation; AC: autoimmune cytopenia; DNT: percent of DNT-cells among $CD3^+/TCRab^+$ T cells; soluble biomarker(s): at least one positive test for soluble FASL, vitamin B12, IL-10, IL-18; nd: not determined

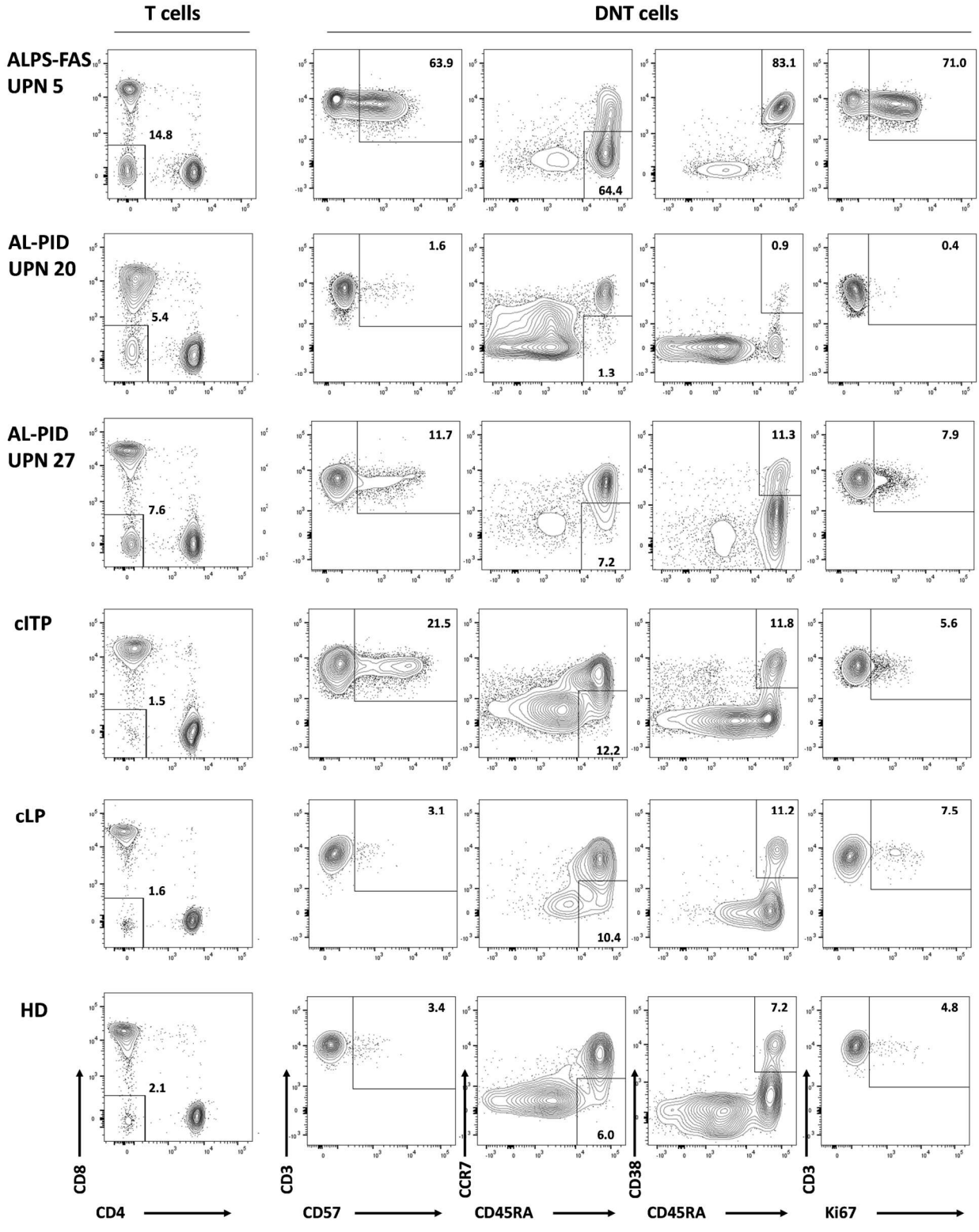
Suppl. Table 2: Recommendation for ALPS diagnostic tube

2-Laser (488 nm, 640 nm)		3-Laser (405 nm, 488 nm, 640 nm)	
Antigen	**Fluorochrome**	**Antigen**	**Fluorochrome**
CD4	FITC	Ki67	FITC
TCRαβ	PE	TCRα/β	PE
CD45	PerCP	CD4	PerCP
CD45RA	PE-Cy7	CD45RA	PE-Cy7
CD38	APC	CD38	APC
CD8	APC-Cy7	CD3	APC-Cy7/APC-H7
		CD8	BV421/Pacific Blue
		CD45	BV510/Krome Orange

Suppl. Table 3: Antibody list for multiparameter flow-cytometry.

Antigen	Fluorochrome	Clone	Company	Identifier
CCR7	AlexaFluor 647	G043H7	Biolegend	353218
CD3	APC-H7	SK7	BD Biosciences	641415
CD4	BV510	SK3	BD Biosciences	562970
CD8	BV421	RPA-T8	BD Biosciences	562428
CD8	PE-Cy7	SK1	BD Biosciences	335822
CD38	BUV737	HB7	BD Biosciences	564686
CD38	APC	HB7	BD Biosciences	345807
CD45	PerCP	2D1	BD Biosciences	345809
CD45RA	BV786	HI100	Biolegend	304140
CD45RA	PE-Cy7	L48	BD Biosciences	337186
CD57	FITC	HNK-1	BD Biosciences	333169
Ki67	FITC	B56	BD Biosciences	556026
TCRαβ	PE	BW242/412	Miltenyi Biotec	130-113-531

Suppl. Fig. 1



Suppl. Figure 1: Representative expression patterns in patients with ALPS-FAS, AL-PID, cITP, and cLP. Blood samples from different donors were stained and analyzed as described in the Methods. Contour plots show frequencies of DNT-cells among TCRab+ T cells (left) and percentages of CD57+, CCR7-/CD45RA+, CD38+/CD45RA+, and Ki67+ DNT-cells. cITP: chronic immune thrombocytopenia; cLP: chronic lymphoproliferation; HD: healthy donor.