

Brief report

FAS-L, IL-10, and double-negative CD4⁻CD8⁻ TCR α/β ⁺ T cells are reliable markers of autoimmune lymphoproliferative syndrome (ALPS) associated with FAS loss of function

Aude Magerus-Chatinet,^{1,2} Marie-Claude Stolzenberg,^{1,2} Maria S. Loffredo,^{1,2} Bénédicte Neven,¹⁻³ Catherine Schaffner,^{1,2} Nicolas Ducrot,^{1,2} Peter D. Arkwright,⁴ Brigitte Bader-Meunier,³ José Barbot,⁵ Stéphane Blanche,³ Jean-Laurent Casanova,^{2,3,6} Marianne Debré,³ Alina Ferster,⁷ Claire Fieschi,⁸ Benoit Florquin,⁹ Claire Galambun,¹⁰ Olivier Hermine,^{2-3,11} Olivier Lambotte,¹² Eric Solary,¹³ Caroline Thomas,¹⁴ Françoise Le Deist,¹⁵ Capucine Picard,^{2,3,16} Alain Fischer,¹⁻³ and Frédéric Rieux-Laucat^{1,2}

¹Inserm U768, Paris, France; ²Université Paris Descartes, Paris, France; ³Unité d'Immunologie et d'Hématologie Pédiatrique, Hôpital Necker-Enfants Malades, Assistance Publique des Hôpitaux de Paris (AP-HP), Paris, France; ⁴Department of Pediatric Immunology, Newcastle General Hospital, Newcastle upon Tyne, United Kingdom; ⁵Service d'Hématologie, Hôpital de Criaças Maria Pia, Porto, Portugal; ⁶Inserm U550, Paris, France; ⁷Hôpital Universitaire des Enfants-Reine Fabiola, Bruxelles, Belgium; ⁸Unité d'Immunopathologie, Département d'Immunologie Clinique, Hôpital Saint-Louis, Paris, France; ⁹Département de Pédiatrie, Centre Hospitalier Universitaire de Liège, Domaine Universitaire du Sart Tilman, Liège, Belgium; ¹⁰Service d'hématologie pédiatrique, Hôpital des enfants de la Timone, Marseille, France; ¹¹Centre National de la Recherche Scientifique (CNRS) UMR 8147, Service d'Hématologie et Centre de Référence des Mastocytoses, Paris, France; ¹²Service de Médecine Interne, Hôpital de Bicêtre, Le Kremlin-Bicêtre, France; ¹³Unité Mixte de Recherche (UMR) 866, Faculté de Médecine, Dijon, France; ¹⁴Service d'Oncologie Pédiatrique, Hôpital Mère Enfant, Nantes, France; ¹⁵Department of Microbiology and Immunology, Centre Hospitalier Universitaire (CHU) Sainte-Justine, University of Montréal, Montréal, QC; and ¹⁶Centre d'Etude des Déficits Immunitaires, Hôpital Necker-Enfants Malades, APHP, Paris, France

Autoimmune lymphoproliferative syndrome (ALPS) is characterized by splenomegaly, lymphadenopathy, hypergammaglobulinemia, accumulation of double-negative TCR $\alpha\beta$ ⁺ CD4⁻CD8⁻ T cells (DNT cells), and autoimmunity. Previously, DNT cell detection and a functional defect of T cells in a FAS-induced apoptosis test in

vitro had been used for ALPS diagnosis. However, a functional defect can also be detected in mutation-positive relatives (MPRs) who remain free of any ALPS-related disease. In contrast, lymphocytes from patients carrying a somatic mutation of FAS exhibit normal sensitivity to FAS-induced apoptosis in vitro. We as-

sessed the soluble FAS-L concentration in the plasma of ALPS patients carrying FAS mutations. Overall, we showed that determination of the FAS-L represents, together with the IL-10 concentration and the DNT cell percentage, a reliable tool for the diagnosis of ALPS. (Blood. 2009;113:3027-3030)

Introduction

Autoimmune lymphoproliferative syndrome (ALPS), first described in 1967 by Canale and Smith,¹ was a human disease characterized by a benign lymphoproliferative disease and autoimmune cytopenia. The molecular basis of ALPS was identified in 1995 via the demonstration of mutations in the *FAS* gene in patients with lymphocyte apoptosis defects^{2,3}; the subsequent discovery of mutations in other genes in several ALPS patients has led to the classification of the condition^{4,5} into subgroups: ALPS-0 and -Ia with homozygous and heterozygous inherited mutations of the *FAS* gene, respectively; ALPS-Ib with mutations in the gene encoding FAS-ligand (FAS-L)⁶; ALPS-IIa and -IIb with mutations in *CASPASE-10*⁷ and *CASPASE-8*⁸ genes, respectively; ALPS-III with unknown genetic defects but with a similar clinical and immunologic phenotype to mild ALPS; and finally ALPS-IV with *NRAS* mutations.⁹ A new subgroup called ALPS-Im¹⁰ includes individuals carrying somatic mosaic *FAS* mutation. The pathognomonic ALPS immunologic features are as follows: nonmalignant lymphoproliferation (splenomegaly and/or adenopathy) along with increase of double-negative CD4⁻CD8⁻ TCR $\alpha\beta$ ⁺ T-lymphocyte counts (DNT cells), hypergammaglobulinemia, and autoimmune abnormalities.

Elevated levels of IL-10 protein in the plasma and in lymphoid tissues have been reported in ALPS patients.^{11,12} In this study, we have measured the FAS-L concentrations in the plasma of ALPS to establish whether these proteins constitute useful circulating markers in supporting ALPS diagnosis.

Methods

Patients

The study protocol was approved by the local independent ethics committee at Hôpital Necker-Enfants Malades and informed consent was obtained from the patients or their families prior to study entry in accordance with the Declaration of Helsinki.

Double-negative CD4⁻CD8⁻ TCR $\alpha\beta$ ⁺ T-cell detection

The percentage of DNT cells was determined by flow cytometry as previously described.¹³

Submitted September 18, 2008; accepted December 21, 2008. Prepublished online as *Blood* First Edition paper, January 27, 2009; DOI 10.1182/blood-2008-09-179630.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

The online version of this article contains a data supplement.

© 2009 by The American Society of Hematology

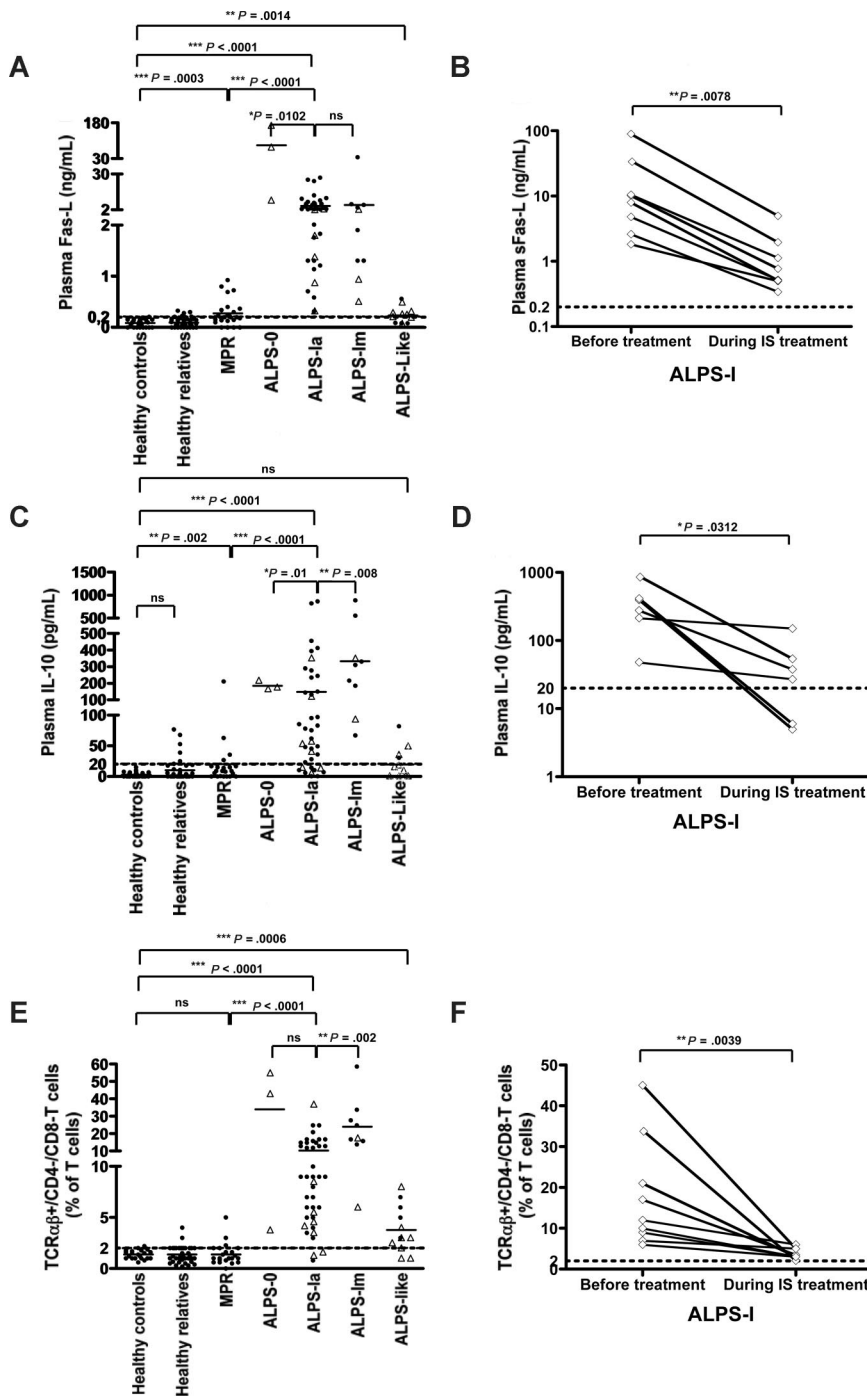


Figure 1. Plasma FAS-L and IL-10 concentrations and DNT cell percentage in untreated and treated ALPS patients. (A) The plasma FAS-L concentrations (ng/mL) are shown for 54 patients with ALPS associated with FAS mutation (3 homozygous [ALPS-0], 41 heterozygous [ALPS-Ia], and 10 somatic [ALPS-Im]), 61 of their healthy relatives with FAS mutations (MPRs, $n = 24$) or without FAS mutations (HRs, $n = 37$), 12 ALPS-like patients presenting clinical and laboratory criteria for ALPS but in whom no causative mutations have been identified and 27 unrelated, age-matched, healthy controls (HCs). Δ denotes values of patients treated with immunosuppressive drugs ($n = 26$: 3 ALPS-0; 8 ALPS-Ia; 7 ALPS-Im; and 8 ALPS-like); \bullet denotes values of untreated patients. (B) The plasma FAS-L concentrations (ng/mL) are shown for 1 ALPS-Im and 7 ALPS-Ia patients before and during immunosuppressive (IS) therapy (methotrexate [$n = 1$], pyrimethamine plus sulfadoxine [$n = 1$], azathioprine [$n = 1$], 6-mercaptopurin [$n = 2$], or azathioprine plus 6-mercaptopurin [$n = 3$]). (C, D) The plasma IL-10 concentrations (pg/mL) are shown for the same samples as in panels A and B, respectively. (E, F) The DNT cells percentages are shown for the same samples as in panels A and B, respectively. The wide horizontal bars denote the mean values for each group. P values for intergroup differences in the Mann-Whitney test are shown above the data. Dashed lines delineate the normal values (DNT cells [$N \leq 2\%$ of T cells corresponding to $N \leq 120/\text{mm}^3$ before 2 years, $N \leq 70/\text{mm}^3$ between 2 and 6 years, $N \leq 50/\text{mm}^3$ between 6 and 12 years, and $N \leq 40/\text{mm}^3$ after 18 years], plasma FAS-L [$N \leq 0.2$ ng/mL], and plasma IL-10 [$N \leq 20$ pg/mL]).

Apoptosis assay

Apoptosis assays were performed as previously described.¹³

IL-10 and FAS-ligand concentrations in plasma

Plasma IL-10 and FAS-ligand were determined in EDTA plasma samples using commercially available enzyme-linked immunosorbent assay (ELISA; R&D Systems, Lille, France, and MBL; Beckman Coulter, Paris, France) according to the manufacturers' instructions.

Statistical analysis

All analyses were performed using Prism (GraphPad Software, San Diego, CA). Populations were compared using the Mann-Whitney test.

Results and discussion

ALPS patients carrying FAS mutation exhibit elevated circulating FAS-L levels

The plasma FAS-L concentration (which is known to be elevated in *Fas*- or *Fas-L*-defective mice¹⁴) was measured in 54 patients carrying either a homozygous FAS mutation (ALPS-0; $n = 3$), a germinal heterozygous FAS mutation (ALPS-Ia; $n = 41$), or a somatic heterozygous FAS mutation (ALPS-Im; $n = 10$). We also tested samples from 12 ALPS-like patients meeting clinical and laboratory criteria for ALPS (including lymphoproliferative syndrome or pancytopenia) but in whom no

causative mutations have been identified, from 24 MPRs as well as samples from 41 healthy relatives carrying a wild-type *FAS* gene (HRs), and from 21 age-matched healthy controls (HCs). All *FAS*-mutated patients had significantly higher plasma FAS-L concentrations than controls (Figure 1A). We also observed a moderate increase of the FAS-L concentration in the plasma of some of the ALPS-like patients and some of the MPRs (Figure 1A).

Given that ALPS patients with *FAS* mutation treated with immunosuppressive drugs (open triangles in Figure 1A; n = 18: 3 ALPS-0, 8 ALPS-Ia, and 7 ALPS-Im) exhibit lower levels of FAS-L than untreated patients (dark circles), we compared the FAS-L concentration in the plasma of 8 patients before they received any treatment and then during immunosuppressive treatment (methotrexate [n = 1], pyrimethamine plus sulfadoxine [n = 1], azathioprine [n = 1], 6-mercaptopurine [n = 2], or azathioprine plus 6-mercaptopurine [n = 3]; Figure 1B). A significant drop in the plasma FAS-L level was observed in all patients following introduction of the immunosuppressive regimen. Nevertheless, these values remained above normal range. Of note, all 8 patients showed clinical improvement (substantiated by shrinking of the tumoral syndrome and remission of autoimmunity) following introduction of the immunosuppressive regimen.

Circulating IL-10 is elevated in ALPS patients

We then assessed the plasma IL-10 concentrations in the present cohort. We confirmed in this ALPS cohort that the IL-10 concentration was variably but significantly increased in the plasma of *FAS*-mutated patients as previously described.^{11,15,16} It was only moderately elevated in some ALPS-like patients (Figure 1C). We occasionally noticed slightly elevated plasma IL-10 values in a few healthy relatives. The plasma IL-10 concentration was also measured in 6 patients before or during immunosuppressive treatment (methotrexate [n = 1], pyrimethamine plus sulfadoxine [n = 1], azathioprine [n = 1], or azathioprine plus 6-mercaptopurine [n = 3]) (Figure 1D). The IL-10 concentration dropped in 5 of 6 treated ALPS-I patients showing clinical improvement.

Variable increase of the percentage of DNT cells in ALPS

A higher than normal percentage of the DNT cells is a hallmark of *FAS* deficiency in both humans and mice.¹⁷ We therefore analyzed the proportion of DNT cells in blood samples of patients of our cohort. In agreement with previous studies, the DNT cell counts were significantly higher in ALPS patients with *FAS* mutation, compared with controls (Figure 1E). However, the proportion of the DNT cells was within the normal range in 3 ALPS-Ia patients. This is probably related to the immunosuppressive treatment, since half of the treated ALPS-I patients exhibited a normal DN T-cell percentage (Figure 1F). Indeed, when the proportion of the DNT cells was measured in the same group of patients before treatment, all values were above normal values (Figure 1F). We observed significant increase in the proportion of DNT cells in 9 of 12 ALPS-like patients. We also noted a rare, moderate increase in the percentage of DNT cells in 2 HRs and 3 MPRs.

Combined analysis of the FAS-L, IL-10, and DNT cells values in ALPS

As set out in Table 1, analysis of the percentage of donors of each category exhibiting the increase in 0, 1, 2, or 3 of the studied markers (ie, the percentage of DNT cells, and the plasma IL-10 and FAS-L concentrations) showed that 100% of ALPS-0 and ALPS-Im

Table 1. Percentage of patients with elevated ALPS markers

Patients	Median age, y	No. of marker(s) with increased values			
		3	2	1	0
ALPS-0, n=3	0.08 (0.04 to 5)	100	0	0	0
ALPS-Ia, n=51	13 (0.7 to 46)	86*	14†	0	0
ALPS-Im, n=10	6.5 (1 to 18)	100‡	0	0	0
ALPS-like, n=6	6 (1 to 16)	0	33	59	8
MPRs, n=24	31.5 (6 to 56)	0	16.5	46	37.5
HRs, n=41	32 (1 to 55)	0	2.5	17	80.5

The percentage of patients exhibiting an elevation in 0, 1, 2, or 3 ALPS markers is described here: DNT cells ($N \leq 2\%$ of $TCR\alpha\beta^+$ T cells), plasma FAS-L ($N \leq 0.2$ ng/mL), and plasma IL-10 ($N \leq 20$ pg/mL) were calculated for the cohort described in Figure 1.

ALPS-0 indicates homozygous *FAS* mutation; ALPS-Ia, heterozygote germline *FAS* mutation; ALPS-Im, heterozygote somatic mosaic *FAS* mutation; ALPS-like, ALPS phenotype with no identifiable mutation; MPRs, mutation-positive relatives; and HRs, healthy relatives.

*Eight treated, 41 untreated, and 10 patients with unknown medication at time of analysis.

†Two treated and 4 untreated patients. All have increased FAS-L concentration.

‡All patients are free of treatment at time of analysis.

patients exhibited the concomitant increase in value for the 3 markers. Only 6 (14%) ALPS-Ia patients (4 untreated and 2 treated) did not exhibit an increase in IL-10 concentration. We did not observe a concomitant increase in the 3 markers in ALPS patients without *FAS* mutations.

These observations indicated that these markers are therefore more informative than the Fas-induced apoptosis assay. Indeed, the latter was unable to detect ALPS-Im, as a consequence of the death of the mutant cells *in vitro*,¹⁰ and apoptosis defect is observed in MPRs (Figure S1, available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Another important observation was the consequence of the immunosuppressive treatment on these parameters. All 3 parameter values correlated with extent of lymphadenopathy as previously described for IL-10.^{11,15} Therefore, one has to consider whether the patients were treated to interpret the results. Indeed, values close to the normal range could result from immunosuppressive treatment. Moreover, these parameters could be useful for monitoring the treatment response.

When measured on samples from ALPS-like patients, rare and moderate increases in these markers could be observed. This finding indicates that these ALPS-like cases are probably different entities and it demonstrates the specificity of increases in these parameters in *FAS*-deficient patients and thus their helpful contribution to the diagnosis of ALPS.

Overall, we show here that the percentage of DNT cells and the plasma concentrations of FAS-L and IL-10 provide useful tools for the diagnosis of ALPS, a diagnosis that becomes definitive when a *FAS* mutation is identified. In addition, they allow (1) discrimination between ALPS-Ia patients and MPRs and (2) detection of ALPS-Im patients, and may also be useful markers for monitoring treatment efficacy.

Acknowledgments

The authors acknowledge Chantal Harre, Corinne Jacques, Nathalie Lambert, and Stephanie Ndaga for technical support.

This work was supported by grants from Inserm, the Agence Nationale de la Recherche (ANR, Paris, France; grant no. 05-MRAR-017, E-rare 2007 [epinostics] and grant no. 08-GENOPATH-015), the Association pour la Recherche contre le Cancer (ARC, Paris, France), and the European STReP (Specific Targeted Re-

search Projects; autorome). A.M.-C. was supported by postdoctoral fellowship from ARC and the European STReP.

Authorship

Contribution: A.M.-C. designed and performed research, collected, analyzed, and interpreted data, and wrote the paper; M.-C.S. performed research; M.S.L. performed research and wrote the paper; B.N. collected and analyzed data and provided clinical information and samples from patients; C.S. and N.D. performed research and analyzed data;

P.D.A., B.B.-M., C.G., J.B., S.B., J.-L.C., M.D., A.F., C.F., B.F., O.H., C.G., O.L., E.S., C.T., and C.P. provided clinical information and blood sample from ALPS patients; F.L.D. designed research; A.F. provided essential clinical information from patients, designed research, interpreted data, and wrote the paper; and F.R.-L. designed research, interpreted data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Frédéric Rieux-Laucat, Inserm U768, Hôpital Necker-Enfants Malades, 149 Rue de Sèvres, 75015 Paris, France; e-mail: frederic.rioux-laucat@inserm.fr.

References

- Canale VC, Smith CH. Chronic lymphadenopathy simulating malignant lymphoma. *J Pediatr*. 1967;70:891-899.
- Rieux-Laucat F, Le Deist F, Hivroz C, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. *Science*. 1995;268:1347-1349.
- Fischer GH, Rosenberg FJ, Straus SE, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell*. 1995;81:935-946.
- Rieux-Laucat F, Le Deist F, Fischer A. Autoimmune lymphoproliferative syndromes: genetic defects of apoptosis pathways. *Cell Death Differ*. 2003;10:124-133.
- Sneller MC, Dale JK, Straus SE. Autoimmune lymphoproliferative syndrome. *Curr Opin Rheumatol*. 2003;15:417-421.
- Wu J, Wilson J, He J, Xiang L, Schur PH, Mountz JD. Fas ligand mutation in a patient with systemic lupus erythematosus and lymphoproliferative disease. *J Clin Invest*. 1996;98:1107-1113.
- Wang J, Zheng L, Lobito A, et al. Inherited human Caspase 10 mutations underlie defective lymphocyte and dendritic cell apoptosis in autoimmune lymphoproliferative syndrome type II. *Cell*. 1999;98:47-58.
- Chun HJ, Zheng L, Ahmad M, et al. Pleiotropic defects in lymphocyte activation caused by caspase-8 mutations lead to human immunodeficiency. *Nature*. 2002;419:395-399.
- Oliveira JB, Bidere N, Niemela JE, et al. NRAS mutation causes a human autoimmune lymphoproliferative syndrome. *Proc Natl Acad Sci U S A*. 2007;104:8953-8958.
- Holzelova E, Vornarbourg C, Stolzenberg MC, et al. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. *N Engl J Med*. 2004;351:1409-1418.
- Lopatin U, Williams KR, Bleesing JJH, et al. Increases in circulating and lymphoid tissue interleukin-10 in autoimmune lymphoproliferative syndrome are associated with disease expression. *Blood*. 2001;97:3161-3170.
- Ohga S, Nomura A, Takahata Y, et al. Dominant expression of interleukin 10 but not interferon gamma in CD4(-)CD8(-)alphabetaT cells of autoimmune lymphoproliferative syndrome. *Br J Haematol*. 2002;119:535-538.
- Rieux-Laucat F, Blachere S, Danielan S, et al. Lymphoproliferative syndrome with autoimmunity: a possible genetic basis for dominant expression of the clinical manifestations. *Blood*. 1999;94:2575-2582.
- Chu JL, Ramos P, Rosendorff A, et al. Massive upregulation of the Fas ligand in *lpr* and *gld* mice: implications for Fas regulation and the graft-versus-host disease-like wasting syndrome. *J Exp Med*. 1995;181:393-398.
- Fuss IJ, Strauber W, Dale JK, et al. T helper 2 T cell cytokine abnormalities in autoimmune lymphoproliferative syndrome, a syndrome marked by defective apoptosis and humoral autoimmunity. *J Immunol*. 1997;158:1912-1918.
- Rao VK, Dowdell KC, Dale JK, et al. Pyrimethamine treatment does not ameliorate lymphoproliferation or autoimmune disease in MRL/*lpr*^{-/-} mice or in patients with autoimmune lymphoproliferative syndrome. *Am J Hematol*. 2007;82:1049-1055.
- Sneller MC, Straus SE, Jaffe ES, et al. A novel lymphoproliferative/autoimmune syndrome resembling murine *lpr/gld* disease. *J Clin Invest*. 1992;90:334-341.