Published in final edited form as:

J Clin Immunol. 2013 February ; 33(2): 479–488. doi:10.1007/s10875-012-9811-z.

A rapid *ex vivo* clinical diagnostic assay for Fas receptor-induced T lymphocyte apoptosis

Bernice Lo¹, Madhu Ramaswamy², Joie Davis³, Susan Price³, V. Koneti Rao³, Richard M. Siegel², and Michael J. Lenardo¹

¹Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

²Immunoregulation Section, Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD 20892, USA

³ALPS Unit, Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Deleterious mutations in genes involved in the Fas apoptosis pathway lead to Autoimmune Lymphoproliferative Syndrome (ALPS). Demonstration of an apoptosis defect is critical for the diagnosis and study of ALPS. The traditional *in vitro* apoptosis assay, however, requires a week of experimental procedures. Here, we show that defects in Fas-induced apoptosis in PBMCs can be evaluated directly *ex vivo* using multicolor flow cytometry to analyze the apoptosis of effector memory T cells, a Fas-sensitive subset of PBMCs. This method allowed us to sensitively quantify defective apoptosis in ALPS patients within a few hours. Some ALPS patients (ALPS-sFAS) without germline mutations have somatic mutations in Fas specifically in double-negative $\alpha\beta$ T cells (DNTs), an unusual lymphocyte population that is characteristically expanded in ALPS. Since DNTs have been notoriously difficult to culture, defective apoptosis has not been previously demonstrated for ALPS-sFAS patients. Using our novel *ex vivo* apoptosis assay, we measured Fas-induced apoptosis of DNTs for the first time and found that ALPS-sFAS patients had significant apoptosis defects in these cells compared to healthy controls. Hence, this rapid apoptosis assay can expedite the diagnosis of new ALPS patients, including those with somatic mutations, and facilitate clinical and molecular investigation of these diseases.

Keywords

Apoptosis; Fas; Autoimmune Lymphoproliferative syndrome; diagnosis; effector memory T cells; double-negative T cells

Introduction

Autoimmune Lymphoproliferative Syndrome (ALPS) is a disorder of lymphocyte apoptosis, resulting in chronic lymphoproliferation and autoimmunity. The disease is characterized by chronic lymphadenopathy and/or splenomegaly, autoantibodies, autoimmune cytopenias of hematopoietic cells (i.e. neutropenia, thrombocytopenia, and anemia), and an expansion of a normally rare population of TCR $\alpha\beta$ ⁺ lymphocytes that do not express the CD4 or CD8 co-

Corresponding author: Michael J. Lenardo, Building 10, Room 11N311, 10 Center Drive, MSC 1892, Bethesda, MD 20892-1892 USA. Phone: (301) 496-6754; Fax: (301) 402-8530; lenardo@nih.gov.

Conflict of interest: The authors declare that they have no conflict of interest.

receptors (double-negative T cells, DNTs) [1, 2]. ALPS patients have defects in the Fas death receptor pathway of apoptosis, which has been shown to be critical for maintaining lymphocyte homeostasis and immune tolerance[3]. Discovery of the molecular defects in cell death pathways in different forms of ALPS has been instrumental in the understanding of normal immune homeostasis [1, 4].

The current criteria for a definitive diagnosis of ALPS requires that the patient have chronic, non-malignant, non-infectious lymphadenopathy and/or splenomegaly, elevated TCR $\alpha\beta^+$ DNTs (greater than or equal to 1.5% of total lymphocytes), and either a mutation in one of the known ALPS causative genes (*FAS*, *FASLG*, or *CASP10*) or defective lymphocyte apoptosis [5]. Therefore, the demonstration of defective apoptosis *in vitro* is essential for a conclusive diagnosis of ALPS, especially for patients in which the genetic defect is unknown. Apoptosis testing has been particularly critical for the proper identification of ALPS patients with overlapping symptoms of other disorders such as Evans syndrome, familial hemophagocytic lymphohistiocytosis (fHLH), and common variable immunodeficiency (CVID) [6, 7]. However, the conventional procedure for measuring lymphocyte apoptosis in patient specimens requires activation of T lymphocytes and *in vitro* culture for at least one week (Fig. 1). Activation and extended culture in IL-2 is thought to be required for the expansion of activated lymphocytes to enhance susceptibility to apoptosis. The expanded cells are stimulated through Fas and assessed for cell death by propidium iodide staining and flow cytometry analysis. However, with this method, inconsistencies have been observed in results between laboratories and even between different phlebotomy samples from the same patient [8]. These problems have been attributed to differences in activation or proliferation of the lymphocytes and length of time in culture. Thus a faster, more standardized apoptosis assay that is highly reproducible between different laboratories and investigators is needed. In freshly isolated peripheral blood mononuclear cells (PBMCs), there are multiple T cell subpopulations with different levels of sensitivity to Fas-induced apoptosis [9, 10]. Previous studies have shown that among PBMCs, the effector memory T cells express Fas and are the most sensitive T cell subset to Fas-mediated apoptosis [10, 9]. Potentially, multicolor flow cytometry could be used to selectively assess cell death of effector memory T cells in PBMCs, eliminating the need for activation and *in vitro* propagation. Here, we describe a novel *ex vivo* assay for assessing defects in apoptosis that is performed directly on freshly isolated PBMCs and can yield highly reproducible results within a few hours.

Methods

Antibodies and reagents

The following antibodies were used: FITC-conjugated anti-CCR7 (R&D Systems), PE-conjugated anti-CD27 (BD Pharmingen), eFluorTM450-conjugated anti-CD45RA (eBioscience), PerCP-Cy5.5 conjugated anti-CD3 (eBioscience), APC-CyTM7 conjugated anti-CD4 (BD Pharmingen), FITC-conjugated anti-TCR $\alpha\beta$ (BD Pharmingen), PE-conjugated anti-B220 (eBioscience), eFluor 605NC-conjugated anti-CD8a (eBioscience), eFluor 450-conjugated anti-CD3 (eBioscience), APC-conjugated anti-CD95 (BD Pharmingen), and APC-conjugated mouse IgG1, κ isotype control (BD Pharmingen). APC-conjugated annexin V was purchased from Invitrogen. Agonistic anti-Fas antibody (APO-1-3) was obtained from Enzo Life Sciences. Rat anti-mouse IgG3 (Southern Biotech) was used to crosslink anti-Fas antibody.

Cells

Blood samples were obtained from patients and healthy individuals with informed consent under protocols approved by the Institutional Review Boards of the National Institute of

Allergy and Infectious Diseases. PBMCs were enriched from whole blood collected in acid citrate dextrose (ACD) by Ficoll-Hypaque density gradient centrifugation. Cells were washed and resuspended at 2×10^6 cells/mL in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, and antibiotics, with or without added IL-2 (100 U/mL).

The following lists the ALPS patients and healthy mutation-positive relatives (HMPRs), included in the study, along with their corresponding Fas mutations: 121.11 (c.1073_1074delAT, p.L278DfsX2), 137.6 (c.973A>G, p.D244G), 143.16 (c.970T>C, p.I243T), 143.17 (c.970T>C, p.I243T), 320.8 (c.845+1G>T, p.V174GfsX9 or p.0), 330.1 (c.870+4A>G, p.E202MfsX4? or p.0?), 243.1 (c.498A>G, p.R86G), 161.1 (c.535A>T, p.0), 232.1 (c.535A>T, p.0), 324.11 (c.669_683del15insA, p.L143KfsX10? or p.0?), 43.1 (c.825G>T, p.E195X), 360.1 (c.913delTinsGA, p.M224RfsX7), and 276.1 (c.876 del 5; D212fs). ? means effect unknown; p.0 indicates no protein produced; p.0? means effect unknown, probably no protein produced. The mutations are listed using the classic notation for Fas mutations, in which nucleotides are numbered in relation to cDNA nucleotide positions per GenBank M67454.1 and amino acids are numbered starting at Arginine 1 of the mature Fas protein per GenBank AAA63174.1. Mutations for these individuals are also listed using the Human Genome Variation Society (HGVS) recommended nomenclature in Tables 1 and 2.

FasT Kill Assay

PBMCs (2×10^5 cells/well) from healthy blood donors and ALPS patients were plated in triplicate (unless otherwise noted) in 96-well flat bottom plates in media supplemented with 100 U/mL IL-2 and treated with varying concentrations of agonistic Fas antibody plus anti-mouse IgG3 at one-tenth the concentration of anti-Fas for crosslinking of the Fas antibody. Cells were incubated for 8 hours at 37°C 5% CO₂, then washed with Annexin-binding buffer (ABB; 10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, 0.5% BSA, pH 7.4), and stained with fluorochrome-conjugated annexin V and antibodies to CCR7, CD27, CD3, CD45RA, and CD4 to identify apoptotic T_{EM} cells. After staining, the cells were washed and resuspended in ABB then fixed by adding equal volume of 10% neutral buffered formalin (NBF). Cells were analyzed by FACS using an LSR II or LSRFortessa (BD Biosciences). At least 50,000 events per sample were acquired. Using FlowJo software (Tree Star, Inc.), we gated on CD3⁺CD4⁺CD45RA⁺CCR7⁺CD27⁺ cells (T_{EM}) and the percent annexin V⁺ fraction of T_{EM} cells was assessed. Percentage of cell loss was calculated as $100 \times (1 - [(100\% \text{ of annexin V}^+ \text{ T}_{EM} \text{ with treatment}) / (100\% \text{ of annexin V}^+ \text{ T}_{EM} \text{ without treatment})])$.

For analysis of percentage cell loss of other T cell subsets, the percent annexin V⁺ fraction of that particular subset was used with the same formula. Naïve, T_{CM}, and T_{TM} CD4⁺ T cells were defined as CD3⁺CD4⁺CD45RA⁺CCR7⁺CD27⁺, CD3⁺CD4⁺CD45RA⁺CCR7⁺CD27⁺, and CD3⁺CD4⁺CD45RA⁺CCR7⁺CD27⁺, respectively.

For the FasT Kill assay experiments in which different incubation time points (4, 6, 8, and 12 hours) were assessed, IL-2 was not added to the media as later discussed. All subsequent FasT Kill assays performed with the shorter 4 hour incubation time do not include IL-2 in the media.

For DNT apoptosis experiments, after cells were treated with Fas antibody and anti-IgG3 for 4 hours in media without IL-2, cells were washed and stained with annexin V and antibodies to CD3, TCRαβ, CD4, CD8, and B220 to identify apoptotic DNTs. DNTs were defined as CD3⁺TCRαβ⁺ CD4⁺CD8⁺B220⁺. The percentage of DNT cell loss was calculated with the above formula using the percent annexin V⁺ fraction of DNTs.

Statistics

Data in FasT Kill assays represent mean $\pm$ standard deviation for triplicate wells, unless otherwise noted. The FasT Kill assay normal control range was calculated for each dose of agonistic Fas antibody as the 10th to 90th percentiles based on the FasT Kill results obtained from 20 healthy blood donors.

Results

A multiparameter flow cytometry assay for Fas-mediated apoptosis

To develop an improved apoptosis assay, we took advantage of the inherent sensitivity of effector memory T cells to Fas-mediated apoptosis. We used multicolor flow cytometry to identify effector memory T cells among total PBMCs and assay apoptosis. We prepared whole PBMCs from a fresh blood sample by a one-step Ficoll gradient separation. We then stimulated the cells for 8 hours with APO-1-3, an agonistic anti-Fas antibody of the IgG3 isotype that was then cross-linked with anti-mouse IgG3. No *in vitro* activation or proliferation was induced. Subsequently, the cells were stained to examine the viability of different T cell subsets. We gated using CD3 and CD4 to identify CD4⁺ T cells and CD45RA to eliminate naïve cells. We also used CCR7 and CD27 to identify CD4⁺ effector memory T (T_{EM}) cells, which express CD45RO (no CD45RA) and are CCR7⁺ and CD27⁺ (Fig. 2a). As previously observed, most CD4⁺CD45RA⁺ T cells were CCR7⁺ and CD27⁺ [9, 10]. Concomitantly, we used annexin V staining to mark apoptotic cells and compared T_{EM} with or without Fas stimulation. With this method, we observed that the T_{EM} cells are clearly the most sensitive T cell subset to Fas-induced apoptosis. There was a dose-response of apoptosis to log increases in Fas stimulation and at 1 μ g/mL, >95% of T_{EM} underwent apoptosis within eight hours despite the fact that the PBMCs from which they were derived had not been stimulated with TCR agonists or IL-2 *in vitro* as in conventional assays (Fig. 2b). These results are in accordance with previous observations where a stabilized form of Fas ligand could induce the death of T_{EM} cells [10]. The sensitivity of the other populations to Fas-induced apoptosis was lower compared to T_{EM} cells, with transitional memory (T_{TM}) appearing partially sensitive and central memory (T_{CM}) cells showing a very mild induction of apoptosis. No specific killing was observed in naïve cells (Fig. 2b).

In the conventional Fas kill assay, death is quantitated in total CD3⁺ T cells and so we compared this unseparated population to the T_{EM} in the *ex vivo* kill assay and found that the percent cell death of the former was much lower, approximately 20-30%, compared to the T_{EM} cells. The latter subset showed nearly 100% cell loss at the highest dose of anti-Fas tested (Fig. 2c). Thus, using multiparameter flow cytometry to specifically quantitate death in the T_{EM} population provided a greater range in which to study cell death in patient specimens. We also observed that there was a variation in the fraction of T_{EM} cells among different individuals, which ranged between 0.5-10 percent (data not shown). A variation of this magnitude could result in major differences in the percentage of quantified cell loss when examining the total population of CD3⁺ T cells. Focusing the analysis only on the T_{EM} cells eliminates this adventitious variation in apparent cell death sensitivity between individuals. Because of the rapidity with which Fas apoptosis can be assessed in effector memory T cells we call the new assay the “FasT Kill” assay.

Evaluation of the normal control range and reproducibility of the FasT Kill assay

To validate the FasT Kill assay as a potentially useful clinical tool for ALPS diagnosis we examined the normal control range and reproducibility of the assay. In preliminary experiments on normal control donors using the FasT Kill assay, we found a modest variation in the degree of cell death of T_{EM} cells. Therefore, we compared 20 healthy individuals to define a normal control range (Fig. 3a). We also examined assay variability by

testing the PBMCs from four different individuals on three separate occasions, each representing a separate phlebotomy from the individual. We found that the FasT Kill assay is highly reproducible with minimal day-to-day variation when different phlebotomy specimens from the same individual were assayed (Fig. 3b). We hypothesized that the control range that we established would allow us to categorize patients with defective Fas-mediated apoptosis.

The FasT Kill assay robustly detected defective apoptosis in ALPS patients with Fas mutations

We defined the normal range for the FasT Kill assay as the 10th to 90th percentiles based on the 20 normal controls. We then tested five individuals with intracellular Fas mutations (three with ALPS and two healthy mutation-positive relatives (HMPRs) of ALPS patients) and four ALPS patients with extracellular Fas mutations (Table 1). Previous studies have shown that HMPRs typically have abnormal *in vitro* apoptosis responses in the traditional Fas kill assay [11, 12]. Compared to the controls, the ALPS patients and HMPRs were all demonstrably defective in Fas-mediated apoptosis, as defined by a fractional cell loss after Fas stimulation that fell below the 10th percentile of normal controls (Fig. 4a and b). Consistent with previous findings, HMPRs with deleterious Fas mutations but no clinical disease also exhibited apoptosis defects similar to ALPS patients using the new assay. Interestingly, the majority of individuals with intracellular Fas mutations displayed quantitatively greater apoptosis defects in this assay (Fig. 4a) than the patients with extracellular Fas mutations (Fig. 4b). Since previous work established that intracellular Fas mutations typically affect the “death domain” and dominantly interfere with Fas function more severely than extracellular mutations, our results indicate that this assay is sensitive enough to distinguish the severity of apoptosis impairment by different molecular variants of Fas [13-15].

The FasT Kill assay can be shortened and still identify patients with impaired apoptosis

We originally used an 8 hour stimulation in the FasT Kill assay since high levels of apoptosis were observed at this time point across a broad range of concentrations of the anti-Fas antibody used in the conventional Fas kill assay. However, to make the assay more amenable to clinical laboratory use as a diagnostic test, we tested shortening the treatment time to 4 hours. We found that 4 hours of stimulation yielded less cell loss using the lower doses of anti-Fas antibody but at 1000 ng/mL the cell loss was comparable to that observed with longer periods of stimulation (Fig. 5a). A range of higher concentrations of agonist antibody with the 4 hour stimulation resulted in a clear dose response with greater percentages of cell death (data not shown). We had previously added IL-2 to the media during the FasT Kill assay to help reduce background death (presumably due to cytokine withdrawal-driven apoptosis), however we found that in the absence of exogenously added IL-2, there was slightly more Fas-induced apoptosis (data not shown). Since the reduction in background death by IL-2 addition was negligible and IL-2 appeared to slightly suppress Fas-mediated death, we eliminated its use in all subsequent 4 hour stimulation assays. We tested ALPS patients with the FasT Kill assay using the 4 hour stimulation time and found clear differences between ALPS patients with intracellular (330.1) or extracellular (232.1) mutations and the normal controls (Fig. 5b and c and Table 1). Therefore, the shortened FasT Kill assay appears to be suitable as a clinical test that can detect a functional Fas apoptosis defect within a single working day.

A modified FasT Kill assay can reveal defective apoptosis in DNT cells

ALPS patients with somatic Fas mutations (ALPS-sFAS) have all the clinical features of ALPS (Table 2) but represent a diagnostic challenge since they have no germline Fas mutations and undergo cell death normally in PBMC apoptosis assays [16, 17]. The somatic

Fas mutations are found in the DNT cell population of these patients. We therefore asked whether we could detect impaired apoptosis in DNT cells directly *ex vivo* using a multicolor flow cytometry-based method similar to the FasT Kill assay. DNTs in ALPS patients are defined as TCR $\alpha\beta$ -positive T cells that lack the CD4 and CD8 co-receptors. However, unlike the expanded DNTs in ALPS patients, the small numbers of DNTs from normal individuals are composed of a more heterogeneous population of cells [18]. Therefore, we used the B220 marker, which appears to be uniquely expressed on the expanded population of DNTs in ALPS patients, to selectively gate on the DNT sub-population in normal controls that most likely represents the same class of DNTs found in ALPS [19]. By gating on CD3⁺TCR $\alpha\beta$ ⁺CD4⁻CD8⁻B220⁺ cells within PBMCs, we found that DNTs clearly express Fas (Fig. 6a). We first tested PBMCs from ALPS-sFAS patients along with normal control individuals for Fas-mediated apoptosis of effector memory CD4⁺ T cells using the FasT Kill assay. Consistent with our previous experience with conventional apoptosis assays, the T_{EM} cells of ALPS-sFAS patients appeared to undergo apoptosis normally (Fig. 6b). This is due to the fact that the T_{EM} cells do not harbor a germline genetic mutation in Fas and express normal Fas protein. However, when we examined the CD3⁺TCR $\alpha\beta$ ⁺CD4⁻CD8⁻B220⁺ DNT cells, we found that the DNTs from ALPS-sFAS patients were severely impaired in Fas-mediated apoptosis compared to the normal controls (Fig. 6c). A dose response in percent cell loss was observed in the DNTs from normal individuals whereas little, if any, Fas-induced cell death was detected in DNTs of ALPS-sFAS patients at any dose tested (Fig. 6c). Thus, the combined use of the FasT Kill assay and a similar apoptosis assay for DNTs is a new functional approach for the rapid diagnosis and identification of ALPS patients with somatic Fas mutations.

Discussion

The study of human disorders of apoptosis has yielded important insights about immunological disease pathogenesis and new approaches to therapy [8, 20]. In the case of ALPS, the demonstration of a lymphocyte apoptosis defect is a critical diagnostic criterion [21, 5]. Also, it has become clear that lymphocyte apoptosis defects contribute to the pathogenesis of other severe immunological disorders such as X-Linked Lymphoproliferative Syndrome [22]. A persistent problem has been the utilization of assays that require activation and extensive culture of lymphocytes, which makes it impractical for all except a few specialized centers to carry out *in vitro* tests of lymphocyte apoptosis. Furthermore, in our experience there is a great deal of variability in currently utilized protocols which diminishes confidence in assessing an abnormality even for genetic mutants that cause profound apoptosis defects [23]. Therefore, we have devised a new test in which multiparameter flow cytometry is used to assess the apoptosis response of T_{EM} and other lymphocyte subpopulations after a relatively short (~ 4 hours) stimulation through the Fas receptor.

Comparing apoptosis sensitivities between different individuals by analyzing CD4⁺ T_{EM} cells rather than whole PBMCs is advantageous for several reasons. First, different individuals will have different proportions of lymphocyte subsets with variable apoptosis susceptibility in their PBMC pool. We have observed significant differences in the proportion of memory populations in different individuals when comparing whole PBMCs or isolated pan-T cells. To some degree this correlates with age; T cells from younger subjects generally have more naïve, Fas-insensitive cells (data not shown). Gating on the CD4⁺ effector memory population and analyzing apoptosis solely in this population eliminates this variability, allowing us to better compare apoptosis sensitivity between different individuals. Second, the Fas sensitive population represents a very small fraction of total PBMCs, such that even among T cells, only a minor percentage of the cells will undergo apoptosis. By contrast, the majority of CD4⁺ effector memory cells die after

stimulation with high concentrations of anti-Fas (Fig. 2c). Third, we also observed that CCR7-CD27⁺ “transitional memory” T cells may be as sensitive as T_{EM} to Fas-mediated apoptosis but because CCR7 staining does not give clear separation between the central (T_{CM}) memory and transitional (T_{TM}) memory T cells, some of the T_{CM} cells in the transitional T cell gate may reduce the apoptosis measured. Thus, for apoptosis assessment, it is simpler to gate on CCR7-CD27⁻ effector memory cells since this provides clear separation from the other two memory T cell populations. Flow cytometry is usually available in immunological testing laboratories and medical centers and the new test was easy to perform and gave robust results even in individuals with subtle apoptosis abnormalities. Thus, this assay should be widely accessible to clinicians and researchers and provides a quick quantitative assessment of functional defects.

Based on the current criteria, apoptosis testing is essential for a definitive diagnosis of individuals displaying clinical features consistent with ALPS but lacking mutations in any of the known causative genes [5]. Those individuals that meet all ALPS diagnostic criteria but have no known genetic defects are classified as ALPS-U. Given the importance of apoptosis assays for the diagnosis of ALPS-U, we have been testing our FasT Kill assay on potential ALPS-U patients. Currently, we have found our FasT Kill assay results corroborate well with the results of the traditional Fas apoptosis assay in diagnosing ALPS-U or ALPS-like patients (Supplementary Fig. 1). With the FasT Kill method, we confirmed apoptosis defects in ALPS-U patients previously shown to have impaired death with the conventional Fas kill assay (Supplementary Fig. 1a and b). Patients with clinical features of ALPS but no defect in cell death evaluated by the traditional Fas apoptosis test, correspondingly, had no defect in apoptosis as assessed by our FasT Kill assay (Supplementary Fig. 1c and d). Thus, the FasT Kill assay may be a faster method for the diagnosis of ALPS-U patients.

In each of the FasT Kill assays we have performed, we used several different concentrations of anti-Fas antibody. Our data suggests that a single anti-Fas antibody dose at 1000 ng/mL may be sufficient to detect an apoptosis defect in most ALPS patients, especially ALPS-Fas patients with intracellular mutations. However, we recommend using at least 2-3 concentrations of anti-Fas between 100-1000 ng/mL because having more than one data point below the normal controls can generate more confidence when declaring an apoptosis defect for patients with milder defects in cell death, such as ALPS-Fas patients with extracellular mutations or ALPS-U patients.

Although a lymphocyte apoptosis defect is critical for a definitive diagnosis of ALPS-U, it is important to note that patients with clinical features resembling ALPS may still be diagnosed with “probable ALPS” without being tested for defective apoptosis [5]. For a “probable ALPS” diagnosis, the patients must have chronic lymphadenopathy and/or splenomegaly, elevated TCRαβ⁺ DNTs, and have either elevated ALPS biomarkers (i.e. high plasma levels of soluble FasL, IL-10, IL-18, or vitamin B12), immuno-histological findings characteristic of ALPS, autoimmune cytopenias along with elevated IgG levels, or a family history of ALPS-like lymphoproliferative disease. It is recommended that patients with “probable ALPS” be “treated and monitored” in the same manner as those with a definitive ALPS diagnosis [5]. However, patients with “probable ALPS” should still be tested for apoptosis defects or mutations in *FAS*, *FASLG*, or *CASP10* in order to obtain a definitive diagnosis of ALPS [5].

ALPS-sFAS patients present the clinician with a difficult diagnosis. They have the same clinical phenotype and biomarker profile as ALPS-FAS patients with germline FAS mutations, but they do not display defects in apoptosis using a traditional Fas kill assay nor genetic mutations in unfractionated peripheral blood samples [5]. This is because DNTs do not survive well in culture and are eliminated during the prolonged culture prior to the

apoptosis assay [16, 17]. The remaining conventional T cells generally do not possess the Fas mutation and therefore exhibit normal apoptosis responses. It is also difficult to establish a molecular diagnosis in such patients because they lack germline mutations in ALPS-related genes. At present, it is necessary to isolate DNT populations from suspected ALPS-sFAS patients and search for mutations in known ALPS-associated genes. Establishing a functional defect has heretofore not been possible. Using a multiparameter flow cytometric direct *ex vivo* assay, we find that B220⁺ DNTs from normal individuals, closely resembling those from ALPS patients, can undergo Fas-mediated apoptosis. However, the B220⁺ DNTs from ALPS patients were insensitive to Fas-induced apoptosis. Thus, a comparison of the results from *ex vivo* Fas killing assays between peripheral blood T cells and DNTs can yield the presumptive diagnosis of ALPS-sFAS in just a few hours. Taken together, our data show that the functional evaluation of selected lymphocyte subsets using current multiparameter flow cytometry may be more widely utilized to yield robust diagnostic assays even in immune diseases due to somatic mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the patients and healthy blood donors. We also thank Josh Milner for use of his LSRFortessa. We thank Julie Niemela for assistance in mutation nomenclature. We also thank Claire Liu for assay name recommendations, and we thank Helen Su and Chryssa Kanellopoulou for critical reading of the manuscript. This research was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

References

1. Turbyville JC, Rao VK. The autoimmune lymphoproliferative syndrome: A rare disorder providing clues about normal tolerance. *Autoimmun Rev*. 2010; 9(7):488–93. [PubMed: 20170754]
2. Rao VK, Oliveira JB. How I treat autoimmune lymphoproliferative syndrome. *Blood*. 2011; 118(22):5741–51. [PubMed: 21885601]
3. Bouillet P, O'Reilly LA. CD95, BIM and T cell homeostasis. *Nat Rev Immunol*. 2009; 9(7):514–9. [PubMed: 19543226]
4. Su HC, Lenardo MJ. Lessons from autoimmune lymphoproliferative syndrome. *Drug Discovery Today: Disease Mechanisms*. 2005; 2(4):495–502.
5. Oliveira JB, Bleesing JJ, Dianzani U, Fleisher TA, Jaffe ES, Lenardo MJ, et al. Revised diagnostic criteria and classification for the autoimmune lymphoproliferative syndrome (ALPS): report from the 2009 NIH International Workshop. *Blood*. 2010; 116(14):e35–40. [PubMed: 20538792]
6. Seif AE, Manno CS, Sheen C, Grupp SA, Teachey DT. Identifying autoimmune lymphoproliferative syndrome in children with Evans syndrome: a multi-institutional study. *Blood*. 2010; 115(11):2142–5. [PubMed: 20068224]
7. Rensing-Ehl A, Warnatz K, Fuchs S, Schlesier M, Salzer U, Draeger R, et al. Clinical and immunological overlap between autoimmune lymphoproliferative syndrome and common variable immunodeficiency. *Clin Immunol*. 2010; 137(3):357–65. [PubMed: 20832369]
8. Lenardo MJ, Oliveira JB, Zheng L, Rao VK. ALPS-ten lessons from an international workshop on a genetic disease of apoptosis. *Immunity*. 2010; 32(3):291–5. [PubMed: 20346767]
9. Riou C, Yassine-Diab B, Van grevenynghe J, Somogyi R, Grellier LD, Gagnon D, et al. Convergence of TCR and cytokine signaling leads to FOXO3a phosphorylation and drives the survival of CD4⁺ central memory T cells. *J Exp Med*. 2007; 204(1):79–91. [PubMed: 17190839]
10. Ramaswamy M, Cruz AC, Cleland SY, Deng M, Price S, Rao VK, et al. Specific elimination of effector memory CD4⁺ T cells due to enhanced Fas signaling complex formation and association with lipid raft microdomains. *Cell Death Differ*. 2011; 18(4):712–20. [PubMed: 21164519]

11. Sneller MC, Wang J, Dale JK, Strober W, Middleton LA, Choi Y, et al. Clinical, immunologic, and genetic features of an autoimmune lymphoproliferative syndrome associated with abnormal lymphocyte apoptosis. *Blood*. 1997; 89(4):1341–8. [PubMed: 9028957]
12. Bleesing JJ, Brown MR, Straus SE, Dale JK, Siegel RM, Johnson M, et al. Immunophenotypic profiles in families with autoimmune lymphoproliferative syndrome. *Blood*. 2001; 98(8):2466–73. [PubMed: 11588044]
13. Hsu AP, Dowdell KC, Davis J, Niemela JE, Anderson SM, Shaw PA, et al. Autoimmune lymphoproliferative syndrome due to FAS mutations outside the signal-transducing death domain: molecular mechanisms and clinical penetrance. *Genet Med*. 2012; 14(1):81–9. [PubMed: 22237435]
14. Jackson CE, Fischer RE, Hsu AP, Anderson SM, Choi Y, Wang J, et al. Autoimmune lymphoproliferative syndrome with defective Fas: genotype influences penetrance. *Am J Hum Genet*. 1999; 64(4):1002–14. [PubMed: 10090885]
15. Martin DA, Zheng L, Siegel RM, Huang B, Fisher GH, Wang J, et al. Defective CD95/APO-1/Fas signal complex formation in the human autoimmune lymphoproliferative syndrome, type Ia. *Proc Natl Acad Sci U S A*. 1999; 96(8):4552–7. [PubMed: 10200300]
16. Holzelova E, Vonarbourg C, Stolzenberg MC, Arkwright PD, Selz F, Prieur AM, et al. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. *N Engl J Med*. 2004; 351(14):1409–18. [PubMed: 15459302]
17. Dowdell KC, Niemela JE, Price S, Davis J, Hornung RL, Oliveira JB, et al. Somatic FAS mutations are common in patients with genetically undefined autoimmune lymphoproliferative syndrome. *Blood*. 2010; 115(25):5164–9. [PubMed: 20360470]
18. Bleesing JJ, Brown MR, Novicio C, Guarraia D, Dale JK, Straus SE, et al. A composite picture of TcR alpha/beta(+) CD4(-)CD8(-) T Cells (alpha/beta-DNTCs) in humans with autoimmune lymphoproliferative syndrome. *Clin Immunol*. 2002; 104(1):21–30. [PubMed: 12139944]
19. Bleesing JJ, Brown MR, Dale JK, Straus SE, Lenardo MJ, Puck JM, et al. TcR-alpha/beta(+) CD4(-)CD8(-) T cells in humans with the autoimmune lymphoproliferative syndrome express a novel CD45 isoform that is analogous to murine B220 and represents a marker of altered O-glycan biosynthesis. *Clin Immunol*. 2001; 100(3):314–24. [PubMed: 11513545]
20. Prabhakar M, Lenardo MJ. Human genetic approaches to diseases of lymphocyte activation. *Immunol Res*. 2009; 43(1-3):8–14. [PubMed: 18795237]
21. Fisher GH, Rosenberg FJ, Straus SE, Dale JK, Middleton LA, Lin AY, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell*. 1995; 81(6):935–46. [PubMed: 7540117]
22. Snow AL, Marsh RA, Krummey SM, Roehrs P, Young LR, Zhang K, et al. Restimulation-induced apoptosis of T cells is impaired in patients with X-linked lymphoproliferative disease caused by SAP deficiency. *J Clin Invest*. 2009; 119(10):2976–89. [PubMed: 19759517]
23. Martin D, Lenardo M. Morphological, biochemical, and flow cytometric assays of apoptosis. *Curr Protoc Immunol*. 1998;3.17.1–3.39.

Fig. 1. The FasT Kill Assay is a faster assay with fewer steps than the conventional Fas apoptosis assay

Depicted is an overview of the required steps for a conventional Fas kill assay compared to the FasT Kill assay. The FasT Kill assay can be performed within one work day whereas the traditional apoptosis assay generally requires 1.5-2 weeks to obtain results.

Fig. 2. T_{EM} cells are the most sensitive CD4⁺ T cell subset to Fas-mediated apoptosis
 PBMCs are incubated for 8 hours with varying doses of crosslinked anti-Fas (APO-1-3) antibody. The cells are then stained with fluorescently-labeled annexin V and antibodies to CD3, CD4, CD45RA, CCR7, and CD27. (a) The gating strategy for defining the CD3⁺ T cell subsets is shown. T_{CM} = central memory, T_{TM} = transitional memory, and T_{EM} = effector memory. (b) The percent cell loss was calculated at different concentrations of APO-1-3 for each T cell subset. Naïve CD4⁺ T cells are defined as CD4⁺CD45RA⁺CCR7⁺CD27⁺. (c) Fas-mediated apoptosis of T_{EM} cells compared to apoptosis of the bulk CD3⁺ PBMCs. The apoptosis assay data are shown as mean ± SD of triplicate wells.

Fig. 3. The normal control range and reproducibility of the FasT Kill assay

(a) Twenty normal controls were tested with the FasT Kill assay and the percent cell loss of TEM cells at the varying doses (25, 50, 100, 1000 ng/mL of anti-Fas) is shown. Each line represents the data from one individual in which each point is the average from triplicate wells. (b) Four normal controls (NC 1-4) were each tested with the FasT Kill assay on 3 different days with a fresh blood draw. Each line depicts the results of the FasT Kill Assay from one of the three blood draws (mean $\pm$ SD of triplicate wells).

Fig. 4. The varying degrees of apoptosis defects in ALPS-FAS patients reflect the severity of the Fas mutations

Each ALPS-FAS patient along with two normal controls were tested using the FasT Kill assay. (a) The percent cell loss at each dose is depicted for the individuals with Fas intracellular mutations. (b) The percent cell loss at each dose is plotted for the ALPS-FAS patients with Fas extracellular mutations. Each data point represents the mean $\pm$ SD of triplicate wells. The normal range is depicted in gray, representing the 10th to 90th percentiles based on the data from 20 normal individuals (shown in Fig. 3a). Patients 143.16 and 143.17 are relatives.

Fig. 5. A shorter version of the FasT Kill assay also identifies patients with impaired apoptosis (a) PBMCs from a normal control were stimulated with agonistic anti-Fas antibody for 4, 6, 8, or 12 hours in the FasT Kill assay. (b) An ALPS patient with an intracellular Fas mutation (330.1) and two normal controls (NC 1, 2) were tested with the FasT Kill assay using a stimulation period of 4 hours. (c) An ALPS patient with an extracellular Fas mutation (232.1) and two normal controls (NC 1, 2) were tested with the FasT Kill assay using the 4 hour stimulation time. All data represent the mean $\pm$ SD of triplicate wells. Percent cell losses calculated to be less than zero are plotted on the axis as zero.

Fig. 6. DNTs from patients with somatic Fas mutations (ALPS-sFAS) express Fas but have defects in Fas-mediated apoptosis

(a) PBMCs from an ALPS-sFAS patient were stained with antibodies to identify DNTs, defined as $CD3^+TCR\alpha\beta^+CD4^+CD8^-B220^+$ cells, plus an anti-Fas or an isotype control antibody. The histograms depict the expression of Fas (in red) or the isotype control staining (in gray) on the DNTs. Fas expression has also been examined and detected on the DNTs of two normal controls and two additional ALPS-sFAS patients. (b and c) In each experiment, PBMCs from two normal controls and an ALPS-sFAS patient were incubated with cross-linked anti-Fas antibody (1000, 300, 150, 50 ng/mL) for 4 hours then stained and analyzed by flow cytometry. Three ALPS-sFAS patients (43.1, 276.1, and 360.1) were tested independently each with a separate set of two normal controls (NC 1, 2). (b) Percent cell loss of the $CD4^+$ effector memory T cells (T_{EM}) from the ALPS-sFAS patients and normal controls. Data in top and bottom panels with 43.1 and 360.1 represent the mean from duplicate wells. Data in middle panel with 276.1 represent the mean from triplicate wells. (c) Percent cell loss calculated for the DNTs. Data in top and bottom panels with 43.1 and 360.1 represent the mean $\pm$ range from duplicate wells. Data in middle panel with 276.1 show the

results from individual wells. Percent cell losses calculated to be less than zero are plotted on the axis as zero.

Table 1

Fas mutations of individuals tested

ID	Mutated region	cDNA mutation ^a	Amino acid mutation ^a	Disease status	Age	Immunosuppressive therapy
121.11	Intracellular	c.879delAT	p.L294DfsX2	HMPR	30	none
137.6	Intracellular	c.779A>T	p.D260V	ALPS	17	none
320.8	Intracellular	c.651+1G>T	p.V190GfsX9 or p.0	HMPR	81	none
143.16 ^b	Intracellular	c.776T>C	p.I259T	ALPS	29	none
143.17 ^b	Intracellular	c.776T>C	p.I259T	ALPS	7	none
330.1	Intracellular	c.676+4A>G	p.E218MfsX4? or p.0?	ALPS	5	MMF
243.1	Extracellular	c.304A>G	p.R102G	ALPS	21	MMF, prednisone, Rituxan ^c
161.1	Extracellular	c.341A>T	p.G112DfsX37 or p.0 ^d	ALPS	10	MMF
232.1	Extracellular	c.341A>T	p.G112DfsX37 or p.0 ^d	ALPS	25	MMF
324.11	Extracellular	c.475_489del15insA	p.L159KfsX10? or p.0?	ALPS	4	MMF, prednisone

? indicates effect unknown; p.0, no protein produced; p.0?, effect unknown, probably no protein produced; HMPR, healthy mutation-positive relative; MMF, mycophenolate mofetil.

^aHGVS recommended nomenclature for variations (<http://www.hgvs.org/mutnomen/>) and based on NCBI reference sequence NG_009089.2.

^b143.16 and 143.17 are relatives.

^cTwo weeks post Rituxan

^dMutation effect on protein previously published. [13]

Table 2

Clinical features of the ALPS-sFAS patients

	43.1	276.1	360.1
Mutation in DNTs ^a	c.631G>T, p.E211X	c.682del5, p.D228fs	c.719delTinsGA, p.M240fsX7
Lymphadenopathy and/or splenomegaly	Yes	Yes	Yes
DNTs (% of lymphocytes) ^b	7.6	4	25.9
Autoimmune manifestation	Anemia, Neutropenia	Anemia, Neutropenia	Anemia, Neutropenia, Thrombocytopenia
sFasL (pg/mL) ^c	682	1253	2731
IL-10 (pg/mL) ^d	172	110	118
Vitamin B12 (ng/L) ^e	5656	>6000	>6000

Elevated levels of sFasL, IL-10, and B12 were used as biomarkers of ALPS.[5]

^a cDNA mutation listed followed by amino acid mutation; all mutations are heterozygous and listed using the HGVS recommended nomenclature as described in Table 1.

^b Normal range is less than 1.5%.

^c sFasL, soluble Fas ligand, levels in plasma; normal levels are below 200 pg/mL.

^d Plasma IL-10 levels; normal levels are below 20 pg/mL.

^e Serum or plasma levels of vitamin B12; normal levels are below 1500 ng/L.