Unmasking Evans Syndrome: T Cell Phenotype and Apoptotic Response Reveal Autoimmune Lymphoproliferative Syndrome (ALPS)

Authors: David T. Teacheys,1,2 Catherine S. Manno2, Kelly M. Axsom2, Timothy Andrews3, John K. Choi4, Barbara H. Greenbaum1,2, Joseph M. McMann4, Kathleen E. Sullivan3, Susan F. Travis1,2, and Stephan A. Grupp1,4.

Institutions: Divisions of 1Oncology, 2Hematology and 3Immunology in the Department of Pediatrics, and 4Department of Pathology and Laboratory Medicine, Children’s Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, PA 10104

Grant Support: Supported by the Goldman Philanthropic Partnerships and the Rockefeller Brothers Fund to D.T.T. a Charles E. Culpepper Biomedical Pilot Initiative grantee of the Goldman Philanthropic Partnerships and supported by the Sanford Chair and Weinberg Fund to S.A.G..

Corresponding Author: Stephan Grupp, MD, PhD, Division of Oncology, Children’s Hospital of Philadelphia, ARC 902, 3615 Civic Center Boulevard, Philadelphia, PA 19104. Email: grupp@email.chop.edu

Scientific Heading: Immunobiology

Running Title: High Prevalence of ALPS in Evans Syndrome

Word counts: abstract 198, body 3494
Abstract

Autoimmune Lymphoproliferative Syndrome (ALPS) is a rare disorder of disrupted lymphocyte homeostasis. Clinical manifestations of ALPS vary but typically include autoimmune cytopenias, organomegaly, lymphadenopathy, and increased risk of malignancies. A similar spectrum of symptoms may be seen in some patients with Evans Syndrome (ES), a hematologic disorder defined by autoimmune destruction of at least two hematologic cell types. We hypothesized a subset of patients diagnosed with ES may have ALPS. We screened 12 children with ES by flow cytometric analysis for CD4-/CD8- (“double negative”) T cells (DNTs) and with the definitive test for ALPS, defective in-vitro Fas-mediated apoptosis. Six of the patients had elevated DNTs suggestive of ALPS and also had defective Fas-mediated apoptosis. The other six patients displayed normal T cell apoptosis; five of whom had normal DNTs and one had a borderline result. Thus, 7/12 (58%) of ES patients had elevated DNTs suggestive of ALPS, with functional confirmation in 6/7. This suggests that analysis of DNTs may be a sensitive first line-screening test, serving as a marker of patients who should undergo definitive testing for ALPS. Our data further suggest that a number of ES patients may have ALPS, a novel finding with important therapeutic implications.
Introduction

Autoimmune Lymphoproliferative Syndrome (ALPS) is a recently described disorder of disrupted lymphocyte homeostasis. Patients with ALPS have mutations in the Fas apoptotic pathway, leading to abnormal lymphocyte survival resulting in chronic lymphoproliferation and a breakdown in immunologic tolerance. ALPS was first characterized in 1992 in a group of patients who were found to have chronic lymphoproliferation, autoimmune manifestations, and an increased number of double negative T cells (DNTs; cell phenotype CD4-/CD8-, CD3+, TCRαβ+). These patients shared similar clinical features with two mouse models of autoimmunity, lpr and gld. These mice were later found to have defective Fas-mediated apoptosis with homozygous mutations in the Fas gene and Fas ligand gene, respectively. Such patients with autoimmunity and lymphoproliferation were proposed and later confirmed to have similar genetic defects to the lpr and gld mice and were classified as having ALPS. ALPS is thought to be a rare condition and since the original report of ALPS only a few hundred cases have been reported.

Apoptotic pathways are important for maintaining lymphocyte homeostasis by eliminating excess activated, antigen-driven, and auto-reactive cells. Fas, a member of the tumor necrosis (TNF) receptor family, is normally highly expressed in activated B and T cells. Fas is activated by binding to the protein Fas ligand which is highly expressed in activated T lymphocytes. The interaction of Fas and Fas ligand leads to multiple intracellular reactions, culminating in the activation of the caspase cascade and cellular apoptosis. The Fas apoptotic pathway is crucial for the downregulation of the immune response and in its absence patients develop chronic lymphoid hyperplasia and
autoimmunity. The majority of patients with ALPS have mutations in the Fas gene, but mutations in Fas ligand, caspase 8, and caspase 10 have been found. No mutation is defined in up to 24% of patients.

Clinical manifestations in patients with ALPS vary but typically include autoimmune cytopenias, lymphoproliferation with lymphadenopathy and organomegaly, and a propensity to develop secondary neoplasms. In the largest published series of ALPS patients, all patients had lymphoproliferation and the majority but not all patients had autoimmune cytopenias. Risk of secondary malignancy is thought to approach 10%. Other infrequent manifestations include autoimmune liver and kidney disease and vasculitis. The current diagnostic criteria as proposed by the National Institutes of Health (NIH) ALPS group are listed in Table 1.

Table 1. Diagnostic Criteria for ALPS

<table>
<thead>
<tr>
<th>Required</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Chronic nonmalignant lymphoproliferation</td>
</tr>
<tr>
<td>2. Defective in vitro Fas-mediated lymphocyte apoptosis</td>
</tr>
<tr>
<td>3. 1% or greater TCR alpha/beta positive, CD3 positive, CD4 negative, CD8 negative cells (DNTs) in peripheral blood or lymphoid tissue</td>
</tr>
<tr>
<td>Supporting</td>
</tr>
<tr>
<td>1. Autoimmune antibodies</td>
</tr>
<tr>
<td>2. Mutations in Fas gene, Fas ligand gene, or caspase 8 or 10 genes</td>
</tr>
</tbody>
</table>


An overlapping constellation of clinical findings can be found in patients with ALPS and patients with Evans Syndrome (ES). In 1951, RS Evans first characterized a hematologic disorder consisting of multiple autoimmune cytopenias which was later named Evans syndrome. ES is defined as a disease in which patients have autoimmune
destruction of at least two peripheral blood cell types. ES is a chronic relapsing disease associated with significant morbidity despite therapy.\textsuperscript{17} The underlying pathophysiology of ES is unknown but is thought to be secondary to generalized immune dysregulation. ES is a diagnosis of exclusion and other confounding disorders must be ruled out before establishing the diagnosis.\textsuperscript{18} Infections, rheumatological diseases, and malignancies can present with autoimmune cytopenias and patients with these conditions do not, by definition, have ES.\textsuperscript{19} ES patients frequently have other symptoms in addition to their autoimmune manifestations, which may include lymphadenopathy, hepatomegaly, and splenomegaly. In one published series over half of patients with ES had evidence of lymphoid hyperactivity.\textsuperscript{20}

Based on this clinical overlap between ALPS and ES, we hypothesized that a subset of patients diagnosed with ES may have ALPS. This is the first report examining patients with ES for ALPS.

Methods

Patients

A review of clinical records identified 20 patients who were followed at The Children’s Hospital of Philadelphia over the period 1999-2004 with a diagnosis of ES. Patients were eligible if they 1) carried the diagnosis of ES and 2) were being actively followed (defined as at least one visit in past year). Patients were excluded from this study if autoimmune cytopenias were associated with systemic lupus erythematosis, malignancy, or treatment with immunosuppression following solid organ transplant. Similarly, patients with genetic abnormalities known to predispose to autoimmune cytopenias were excluded. Of the 20 identified ES patients, one with DiGeorge
syndrome, one with Kabukis syndrome, and one with Gauchers syndrome were excluded. Four were not available for enrollment and one had died. Thus 12 ES patients were enrolled on study. Thus research protocol was reviewed and approved by the IRB and all enrolled patients or their parent/guardian signed informed consent.

**Flow Cytometry for Double Negative T Cells**

DNT analysis was performed at the clinical immunology laboratory at CHOP. The clinical immunology laboratory has received certification for quality assurance for performing high complexity clinical testing under the Clinical Laboratory Improvement Amendments (CLIA 88). A normal control was run in tandem with each patient sample. The clinical immunology lab designed the testing protocol based on published techniques\(^1\) and evaluated 22 healthy adult controls to establish normal parameters. Normal was defined as 2 standard deviations from the mean and 2.6% CD4-/CD8-, CD3+, TCR\(\alpha\beta\)+ cells was determined to be the threshold for the normal range using the gating strategy detailed below. In addition, the clinical laboratory periodically recalculated the mean and standard deviation based on series of tested controls. Thus far, over fifty control samples have been tested with no change in mean or standard deviation.

Whole blood from each patient sample and normal control was stained in two tubes with monoclonal antibodies purchased from Beckman Coulter. The first tube was an isotype control tube and contained anti-CD3-FITC, mouse IgG1-RD1, and mouse IgG2a-PC5. The second tube contained anti-CD3-FITC, anti-CD4-RD1, anti-CD8-RD1, and anti-TCR\(\alpha\beta\)-PC5. After staining, the erythrocytes in each tube were lysed using the ImmunoPrep\(^{TM}\) reagent system (Beckman Coulter) and the Coulter TQ-Prep\(^{TM}\)
Workstation (Beckman Coulter). The white blood cells in each tube were then analyzed on a Beckman Coulter Cytomics FC-500 flow cytometer.

A histogram of CD3-FITC versus side scatter was used to gate on the T cells (CD3+/low side scatter). The events in the T-cell gate for the isotype control tube for each patient or normal control were sent to a second histogram plotting mouse IgG1-RD1 versus mouse IgG2a-PC5. Cursors were set based on the staining of the isotype control antibodies to allow for less than 2% false positives. The same cursor settings were then used for the specific antibody tube for the respective patient or normal control. The events in the T-cell gate for each specific antibody tube were sent to a second histogram plotting CD4-RD1 and CD8-RD1 versus TCRαβ−PC5. DNTs were the CD3+ cells that are CD4-/CD8- and TCRαβ+. The clinical immunology lab tested a normal control with every patient sample run. In addition, we performed DNT analysis on all of the normal controls used in evaluating patients for defective Fas-mediated apoptosis (see below) to ensure no individual in our normal control pool had elevated DNTs.

**Testing for Fas-mediated Apoptosis**

Fas-mediated apoptosis was evaluated using published techniques. All patient samples were tested in tandem with a normal control. We used a panel of ten different adult volunteers as normal controls (12 total patients; 10 total controls). Each normal control was used in 1-3 experiments. PBMCs were isolated by Ficoll gradient centrifugation and resuspended at concentration of 10^6 cells/ml in RPMI 1640 with 10% fetal calf serum plus rhIL-2 (25 U/ml, Leinco). To allow confirmatory testing, T cells were stimulated with two different mitogens. In one well, T cells were stimulated with phytohemagglutinin (PHA) (3ug/ml) and re-stimulated with PHA (1ug/ml) on day 8-14.
Apoptosis assays were performed 6 days after second stimulation. In the second well, T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (10ng/ml) and A23187 (Calcimycin) (500ng/ml) and apoptosis assays were performed in 3-5 days. The published protocols assessing Fas-mediated apoptosis in the ALPS literature are based on PHA stimulation of T cells.\textsuperscript{4,22,23} Recent work has shown PMA and A23187 is as effective in stimulating T cells for subsequently evaluating the Fas apoptotic pathway. PMA is a more potent mitogen, requiring a shorter culture period before performing assays.\textsuperscript{24} We compared the two mitogens in each sample. After stimulation, T cells were aliquoted into 24 well plates at a concentration of 50,000 cells per well. Cells were incubated with control media, anti-Fas monoclonal antibody (IgM, 10ug/ml for PMA assay and 1ug/ml for PHA assay, Upstate), steroid [either dexamethasone (10uM, Sigma) or methylprednisolone (1uM, Pharmacia)] or C2-ceramide (50uMol, Sigma) for 24-48 hr. Induction of cell death was evaluated by trypan blue exclusion and verified with FACS analysis for Forward Scatter/Side Scatter (FSC/SSC) and 7-AAD (BD Pharmigen). Trypan blue exclusion was performed by adding 11ul trypan blue dye (Cellgro) to 100 ul cell suspension and counting number of viable cells using light microscopy at 20x power. For FACS analysis, human lymphocytes both stimulated with mitogen and freshly obtained were used as isotype controls. Dead cells were defined as those displaying shrunken/hypergranular morphology on FSC/SSC and those which stained with 7-AAD. We used control stimulated T cells treated with 100% Ethanol to set parameters for dead cells on 7AAD staining. To qualify as a positive test (consistent with death with stimuli) there must be at least both a three fold and 40% increase in cell death by both FACS and trypan blue.
Results

Table 2. Clinical and Demographic Information with Laboratory Analysis

<table>
<thead>
<tr>
<th>ID</th>
<th>Age</th>
<th>Dx</th>
<th>Age At Study Entry</th>
<th>AIHA</th>
<th>ITP</th>
<th>AIN</th>
<th>Lymphadenopathy*</th>
<th>Splenomegaly</th>
<th>Hepatomegaly</th>
<th>Therapyθ</th>
<th>DNTs</th>
<th>Apoptosis Assay$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>Yes[R]</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>IVIg, Steroids</td>
<td>7.6%</td>
<td>Defective</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>18</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>Yes</td>
<td>Yes Splenectomy</td>
<td>Yes</td>
<td>IVIg, Steroids</td>
<td>3.6%</td>
<td>Defective</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>20</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>Yes</td>
<td>Yes Splenectomy</td>
<td>No</td>
<td>IVIg, Steroids</td>
<td>5.1%</td>
<td>Defective</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>13</td>
<td>13</td>
<td>Yes[R]</td>
<td>Yes[R]</td>
<td>Yes[R]</td>
<td>No</td>
<td>Yesψ</td>
<td>No</td>
<td>IVIg, Steroids</td>
<td>2.7%</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>16</td>
<td>No</td>
<td>Yes[R]</td>
<td>Yes[R]</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>None</td>
<td>2.5%</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>17</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>No</td>
<td>No</td>
<td>Yes Splenectomy</td>
<td>Yes</td>
<td>CSA Steroids</td>
<td>2.0%</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>14</td>
<td>19</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>IVIg, Steroids, CSA</td>
<td>1.1%</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>4</td>
<td>11</td>
<td>Yes[R]</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>IVIg, Steroids Fansidar</td>
<td>10.5%</td>
<td>Defective</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>6</td>
<td>19</td>
<td>Yes[R]</td>
<td>Yes[R]</td>
<td>Yes[R]</td>
<td>No</td>
<td>Yesψ</td>
<td>No</td>
<td>IVIg, Steroids</td>
<td>1.1%</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>6</td>
<td>7</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>IVIg, Steroids</td>
<td>8.9%</td>
<td>Defective</td>
<td></td>
</tr>
<tr>
<td>38</td>
<td>10</td>
<td>14</td>
<td>Yes[R]</td>
<td>Yes[C]</td>
<td>Yes[C]</td>
<td>No</td>
<td>Yes Splenectomy</td>
<td>No</td>
<td>IVIg, Steroids, Rituximab</td>
<td>7.7%</td>
<td>Defective</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>17</td>
<td>18</td>
<td>Yes[R]</td>
<td>Yes[R]</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>IVIg, Steroids</td>
<td>1.2%</td>
<td>Normal</td>
<td></td>
</tr>
</tbody>
</table>

*To qualify patient must have lymphadenopathy not associated with infection or malignancy affecting two or more nodal groups

[C] = chronic; [R] = rare; AIHA = Autoimmune Hemolytic Anemia; ITP = immune mediated thrombocytopenia; AIN = Autoimmune Neutropenia. Chronic is defined as having an exacerbation at least two times a year, requiring immunosuppressive medications. Rare indicates not chronic.

ψ|Splenomegaly present only during episodes of AIHA

θ|Treatment refers to any medication taken at any time since diagnosis with Evans. For specific information detailing medications taken at time of ALPS testing see text.

$Defective refers to defective Fas mediated apoptosis (consistent with diagnosis of ALPS); normal refers to appropriate Fas mediated apoptosis

12 patients were enrolled in the study and were tested for ALPS. A summary of demographics and clinical findings are listed in Table 2. The majority of patients (8/12)
had chronic disease with frequent exacerbations (>2 times a year requiring immunosuppressive medications). One third of the patients (4/12) had a history of lymphadenopathy (defined as non-infectious, non-malignant enlargement of at least two nodal groups). Two of these patients had persistently enlarged lymph nodes >2cm in multiple (>3) nodal regions. The other two patients had enlarged lymph nodes >2cm in multiple nodal regions; however, the enlarged nodes and the areas involved waxed and waned. One half (6/12) of the patients had a history of chronic organomegaly (enlarged greater 6 months). All four patients with a history of lymphadenopathy also had a history of chronic organomegaly. Two patients, therefore, had isolated organomegaly. Two additional patients were noted to have splenomegaly during episodes of AIHA but did not have chronic organomegaly. One third (4/12) of the patients with splenomegaly had undergone splenectomy as part of the management of their cytopenias. Prior to splenectomy, all four of these patients had spleens which were chronically enlarged at least 3-4cm. Of the other two patients with splenomegaly who did not undergo splenectomy, both had a persistently enlarged spleen at least 2cm below the costal margin. Four patients had a history of intermittent hepatomegaly. All of these patients also had a history of chronic splenomegaly. One half (6/12) of the patients had a history of clinical chronic nonmalignant lymphoproliferation (lymphadenopathy and/or organomegaly), potentially consistent with ALPS. 25% (3/12) of the patients had co-morbid conditions, including atopy, nephrotic syndrome, and diabetes mellitus. No patient had a history of malignancy or life threatening infection.

58% (7/12) of the ES patients had elevated DNTs. Examples of a positive and negative DNT test are depicted in Figure 1. All patients were then tested for Fas-
mediated apoptosis. One half (6/12) of the patients had defective apoptosis consistent with the diagnosis of ALPS. An example of a test consistent with ALPS (defective Fas-mediated apoptosis) is depicted in Figure 2.

**Figure 1. Detection of Double Negative T Cells and gating strategy.** Peripheral blood lymphocytes were analyzed by flow cytometry for DNTs (CD4-/CD8-, CD3+, TCR αβ+). A histogram of CD3-FITC versus side scatter (A and C) was used to gate on T cells (CD3+/low SS). The events in the T cell gate were sent to second histogram (B and D) of CD4 and CD8-RD1 versus TCR αβ-PC5. Double negative T cells are depicted in lower right quadrant of B and D. B represents a patient with a normal test (1.2% DNTs) and D represents a patient with an elevated test (8.9% DNTs).

**Figure 2. Detection of apoptosis.** Fas-mediated apoptosis was analyzed by testing patient sample (A) in tandem with normal control (B). T cells were stimulated with PHA. After 14-21 d culture, 10⁶ T cells were incubated with media, anti-Fas monoclonal antibody, steroid or C2-ceramide for 48 hr. (A) shows results example of defective Fas-mediated apoptosis (consistent with diagnosis of ALPS) in one patient sample with percentages indicating proportion dead as determined by FACS analysis for 7-AAD. X axis depicts % dead by 7-AAD and Y axis depicts number of cells counted. Gating strategy for dead cells determined by comparing to control stimulated T cells treated with Ethanol and stained with 7-AAD.

All patients described as having a test consistent with ALPS demonstrated apoptosis to C2 ceramide and to steroids but not to anti-Fas monoclonal antibody. All
patients described as having a negative test demonstrated apoptosis to all 3 stimuli. The anti-Fas apoptotic response was the same in all patients and controls after both PMA and PHA T cell stimulation (data not shown). All normal controls demonstrated apoptosis to all three stimuli. Figure 3 summarizes results of apoptosis testing in patients and controls.

![Figure 3](image)

**Figure 3. Summary of apoptosis results.** This figure summarizes results of all patients and controls tested. Data points show percent death as determined by trypan blue exclusion after PHA stimulation. Black diamonds represent patients with defective Fas-mediated apoptosis. Grey circles represent patients with normal Fas-mediated apoptosis. White triangles represent controls.

![Figure 4](image)

**Figure 4. DNTs predict defective Fas-mediated apoptosis.** 12 patients with Evans syndrome were screened for ALPS with DNTs by FACS and then evaluated for ALPS by *in vitro* testing for defective Fas-mediated apoptosis. Patients with defective Fas-mediated apoptosis (consistent with ALPS) are depicted with black columns and patients with normal apoptosis (not consistent with ALPS) are depicted with white columns. The ordinate depicts percent DNTs and the bold line the cutoff for an elevated result (2.6%). All patients except one (number 5) with elevated DNTs had defective Fas-mediated apoptosis. All patients with normal DNTs had normal apoptosis.

5/6 of the ES patients with defective Fas-mediated apoptosis and elevated DNTs also had a history of lymphadenopathy or organomegaly, while one did not. Of the seven patients with elevated DNTs, six had functional confirmation of defective Fas-mediated apoptosis. Thus, there was one false positive and the specificity of the DNTs test was
85.7% in this series. Of the six patients with normal DNTs, none had functional confirmation of defective Fas-mediated apoptosis. Thus, there were no false negatives and the sensitivity of the test was 100% for this series. Summary results comparing results of DNTs to apoptosis assay are depicted in Figure 4 and Table 2.

Pathology specimens were available for analysis on two patients who met diagnostic criteria for ALPS and one who did not. One of the biopsies from one of the patients with laboratory evidence of ALPS was strongly suggestive of a diagnosis of ALPS with classic features, elevated DNTs by immunohistochemical stains, paracortical expansion, and follicular hyperplasia. Another biopsy from one of the patients with laboratory evidence of ALPS was suggestive of ALPS but was not as conclusive. This biopsy was a poor specimen and was obtained from a normal sized lymph node. The node showed elevated DNTs by immunohistochemical stains and follicular hyperplasia; however, because of the quality of the specimen it was also potentially consistent with a reactive node. The biopsy from the patient who did not meet laboratory diagnostic criteria for ALPS was also not consistent with ALPS, revealing a reactive lymph node with normal DNTs by immunohistochemical stains.

Discussion

We studied a group of 12 patients with Evans syndrome for the laboratory characteristics of ALPS: elevated DNTs and defective Fas-mediated apoptosis of mitogen-stimulated T cells. Based on this testing, we found an unexpectedly high prevalence of abnormal laboratory findings highly suggestive of ALPS among patients diagnosed with ES. Our data further suggest that DNTs may serve as a sensitive marker of patients who need further definitive testing. Identifying patients who have been
diagnosed with ES who may in fact have ALPS has important prognostic and therapeutic implications. ES is poorly understood and the exact mechanism of the disease is uncharacterized. While many case reports describe an increased risk of secondary malignancies in patients with Evans syndrome, the exact risk is not known. The mode of inheritance is unknown in Evans syndrome and therapeutic options are limited to immunosuppressive therapy (IST). ALPS is better characterized as a result of a clear understanding of the pathophysiology. The lifetime incidence of secondary malignancy in ALPS approaches 10%, warranting careful observation, interval screening and family counseling. Patients with ALPS usually inherit the disease in an autosomal dominant pattern with variable penetrance. Thus, genetic counseling has a role for families of patients with ALPS. In addition, some evidence suggests an increased risk of malignancy may exist in relatives with the same Fas pathway mutation, in which the ALPS phenotype is not penetrant. Finally, while the mainstay of treatment of ALPS is similar to Evans (i.e, use of IST), newer agents are becoming available which may be specific to ALPS. Recently, pyrimethamine and sulphadoxine were shown to significantly reduce lymphoproliferation and autoimmune cytopenias in a small series of patients with ALPS. Currently, the NIH is investigating this agent in a phase I clinical trial. Understanding the mechanism behind ALPS has the potential result in more targeted therapies.

Of interest, our clinical laboratory established a different normal range for DNTs then is cited in the literature. In our laboratory, after healthy controls were tested, 2.6% was determined to be the threshold for normal range, likely as a result of our highly gated analytic strategy. The normal cutoff for DNTs varies from institution to institution and
different series publish different normal ranges.\textsuperscript{6,13} The current accepted definition of ALPS includes a result of \(>1\%\) DNTs as one of the diagnostic criterion. Thus, it is important to know the normal values of an institutional laboratory. As is described in the literature,\textsuperscript{29} healthy adults were used to establish normal parameters for DNTs since DNTs have not been found to change with age.\textsuperscript{6}

Four patients in our study were taking immunomodulating medications at time of evaluation for DNTs. Three of these patients were taking low dose (5mg or less) every other day steroids. Two of these patients had elevated DNTs and defective Fas mediated apoptosis and one of these patients had normal DNTs and normal Fas mediated apoptosis. Similar doses of steroids have not been shown to affect DNT or apoptosis analysis.\textsuperscript{30} No patient had received high dose steroids in the six months prior to testing. All three of the patients talking low dose steroids demonstrated apoptosis to steroids in vitro. One patient was treated with cyclosporine. This patient had normal DNTs and normal Fas mediated apoptosis. No data exits in the literature as to the effect of cyclosporine on either test. Thus, this patient could represent a false negative. Four other patients were periodically treated with IVIg for immune cytopenias; however, no patient received IVIg within six weeks prior to testing for ALPS, and thus should not have affected results.

One of the patients with defective Fas-mediated apoptosis and elevated DNTs had no history of lymphadenopathy or organomegaly. The lack of clinically identifiable lymphoproliferation in a patient with defective apoptosis is an unexpected finding, in apparent contradiction of the accepted definition of ALPS that lists lymphoproliferation as a mandatory diagnostic criterion. The identification of a patient with the triad of autoimmune cytopenias, elevated DNTs, and defective Fas-mediated apoptosis without
clinical lymphoproliferation, raises the question of whether the accepted criteria for
ALPS should be revised or whether this patient has a similar but different disease. Of
note, this patient is three years old and it is possible he may develop clinically identifiable
lymphoproliferation with time; nevertheless, this patient would still contradict the
published literature on ALPS which describes lymphoproliferation presenting prior to
autoimmune cytopenias. Whether ALPS is a clinical diagnosis requiring
lymphoproliferation or a genetic diagnosis causing defective Fas-mediated apoptosis is
not yet known. Evaluating more patients with Evans for DNTs and apoptosis defects may
help answer this question.

Approximately 76% percent of patients with ALPS have identifiable genetic
mutations; however, in up to 24% of patients no mutation can be found. We did not
perform genetic analysis on our patient population; however, 5 of the patients in our
series have all three mandatory diagnostic criteria for ALPS. Thus, whether or not these
patients have identifiable gene mutations would not change the results of our study.

The other five patients in our series with defective Fas-mediated apoptosis and
elevated DNTs had clinically identifiable lymphoproliferation consistent with the NIH
criteria for diagnosis of ALPS. Of the six patients with Evans and normal Fas-mediated
apoptosis, five had no history of clinically identifiable lymphoproliferation. In summary,
one of six patients (16%) with defective Fas-mediated apoptosis had no
lymphoproliferation and one of six patients (16%) without defective Fas-mediated
apoptosis had lymphoproliferation. These findings suggest that clinically identifiable
lymphoproliferation should not be used as the sole criterion for determining which
patients with ES need definitive testing for ALPS.
ALPS is presumed to be a rare diagnosis with only a few hundred reported cases. Our finding a high prevalence of ALPS in ES, a more common condition, argues that ALPS may be more common than previously thought. An assessment of the true prevalence of ALPS within patients diagnosed with ES awaits a larger prospective study. Because of the selection bias inherent in retrospectively assessing patients at a tertiary referral center, the prevalence of ALPS assessed by a prospective study could be lower than we have observed here.

In addition to ALPS, another disorder with autoimmunity and lymphoproliferation has been described, Autoimmune Lymphoproliferative Disease (ALD). This disorder is characterized by patients with clinical lymphoproliferation similar to ALPS, but with normal DNTs. These patients have defective apoptosis to both anti-Fas monoclonal antibody and ceramide, in contrast to patients with ALPS who only have defective apoptosis to anti-Fas monoclonal antibody. The patients described in our study more closely resemble ALPS than ALD since they have elevated DNTs and normal apoptosis to ceramide.

Patients with a single isolated immune cytopenias (immune thrombocytopenia purpura [ITP] or autoimmune hemolytic anemia [AIHA]) have been evaluated for elevated DNTs and/or defective Fas mediated apoptosis. No patient in any published series with ITP or AIHA have been found to have elevated DNTs and defective Fas mediated apoptosis, (i.e. they would not meet diagnostic criteria for ALPS). In one series, two patients with ITP were described with defective Fas mediated apoptosis but had normal DNTs and no lymphoproliferation. Defects in Fas mediated apoptosis have been observed in other disorders, including lupus, multiple sclerosis, and type I
diabetes mellitus. No patients in our series had a co-morbid disorder associated with defective Fas mediated apoptosis except one patient had diabetes mellitus. This patient had normal Fas mediated apoptosis and normal DNTs.

In summary, in the group of 12 ES patients tested in our study, 58% had elevated DNTs suggestive of ALPS, with functional confirmation in 6/7 tested. Our data suggest that DNTs may be a sensitive first line-screening test and may serve as a marker that identifies patients who require definitive testing. Our preliminary findings suggest a high prevalence of ALPS among ES patients, a novel finding with important implications.
References
Unmasking Evans Syndrome: T cell phenotype and apoptotic response reveal autoimmune lymphoproliferative syndrome (ALPS)

David T Teachey, Catherine S Manno, Kelly M Axsom, Timothy Andrews, John K Choi, Barbara H Greenbaum, Joseph M McMann, Kathleen E Sullivan, Susan F Travis and Stephan A Grupp