ORIGINAL ARTICLE

Autoimmune Lymphoproliferative Syndrome with Somatic *Fas* Mutations

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ABSTRACT

BACKGROUND

Impaired Fas-induced apoptosis of lymphocytes in vitro is a principal feature of the autoimmune lymphoproliferative syndrome (ALPS). We studied six children with ALPS whose lymphocytes had normal sensitivity to Fas-induced apoptosis in vitro.

METHODS

Susceptibility to Fas-mediated apoptosis and the *Fas* gene were analyzed in purified subgroups of T cells and other mononuclear cells from six patients with ALPS type III.

RESULTS

Heterozygous dominant *Fas* mutations were detected in the polyclonal double-negative T cells from all six patients. In two patients, these mutations were found in a fraction of CD4+ and CD8+ T cells, monocytes, and CD34+ hematopoietic precursors, but not in hair or mucosal epithelial cells.

CONCLUSIONS

Somatic heterozygous mutations of *Fas* can cause a sporadic form of ALPS by allowing lymphoid precursors to resist the normal process of cell death.

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Fas (also called Apo-1 and CD95) is a cell-surface receptor belonging to the tumor necrosis factor receptor (TNFR) superfamily[1] (Fas is the sixth member, TNFRSF6). Once triggered by its cognate ligand (Fas ligand), Fas initiates a cascade of events within the cell that culminates in the death of the cell (apoptosis). This process involves the formation of the death-inducing signaling complex,[2] consisting mainly of the Fas-associated death domain and the caspase 8 and caspase 10 proteins. The essential role of Fas in lymphocyte homeostasis was initially recognized in MRL *lpr/lpr* mice, which have a germ-line autosomal recessive mutation of *Fas*.[3] Subsequently, heterozygous dominant mutations of *Fas* were found in children with the autoimmune lymphoproliferative syndrome (ALPS),[4-6] which is also known as the Canale–Smith syndrome.[7] The main features of this disease are splenomegaly, lymphadenopathy, hypergammaglobulinemia (IgG and IgA), and autoimmunity.[8,9] ALPS is characterized by the accumulation of a polyclonal population of T cells called double-negative T cells. These lymphocytes display the marker common to mature T cells, CD3, and α/β T-cell–antigen receptors, but neither the CD4 nor the CD8 coreceptors (CD3+ T-cell receptor α/β+ CD4–CD8–). They normally account for less than 2 percent of peripheral α/β+ T cells[8] and are distinct from the double-negative thymocytes in the cortex of the thymus, which lack CD3 and T-cell receptors for antigen. The double-negative T cells in patients with ALPS are poorly responsive to mitogens and antigens and fail to produce growth and survival factors such as interleukin-2.[10] In Fas-deficient MRL *lpr/lpr* mice, the large population of double-negative T cells appears to originate from chronically activated CD8+ T cells that down-regulate the expression of CD8 and fail to undergo apoptosis.[3] In humans, double-negative T cells also seem to be antigen-exposed T cells that have escaped apoptosis.

ALPS is classified according to the underlying genetic defect.[11] In type 0 disease, homozygous *Fas* mutations usually cause a complete deficiency of the Fas protein and a severe form of the disease.[4,12,13] In ALPS type I, heterozygous *Fas* mutations (ALPS type Ia)[14-16] or, more rarely, heterozygous mutations in the gene for Fas ligand (ALPS type Ib)[17] are usually associated with a partial defect in apoptosis mediated by Fas and its ligand. ALPS type II, which is characterized by resistance to Fas-mediated apoptosis despite the presence of normal Fas ligand and Fas, has been found in two patients with caspase 10 mutations.[18] In ALPS type III, Fas-mediated apoptosis is also normal in vitro,[19] and the genetic defect is unclear. Patients with ALPS type III may not have all four classic features of the syndrome — lymphoproliferation, excessive numbers of double-negative T cells, hypergammaglobulinemia, and autoimmune manifestations. Many cases of ALPS type III are sporadic, precluding the use of a genetic approach to identify the molecular defect. In the present study, we obtained lymphocytes from six children with ALPS type III for an in-depth analysis.

METHODS

PATIENTS

Blood samples were obtained from the six patients, their parents, and five healthy controls. All subjects or their parents or guardians provided written informed consent, validated by the Comité Consultatif pour la Protection des Personnes en Recherche Biomédicale. Table 1 summarizes the clinical features of the six patients.

CELL CULTURE, APOPTOSIS ASSAY, AND ANALYSIS OF THE T-CELL–RECEPTOR REPERTOIRE

Peripheral-blood mononuclear cells were isolated from freshly drawn heparin-treated blood by means of Ficoll–Hypaque density gradient centrifugation. Apoptosis assays and repertoire analysis were performed on activated T cells and whole blood, respectively, as previously described.[16,20]

NUCLEIC ACID PREPARATION, AMPLIFICATION, AND DETECTION OF *FAS* MUTATIONS

Total RNA was isolated from freshly isolated peripheral-blood mononuclear cells and T cells that had been activated by nine days of in vitro exposure to phytohemagglutinin. The reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assay was performed as previously described.[4,16] DNA extracted from phytohemagglutinin-activated lymphocytes or purified double-negative T cells was amplified with oligonucleotides spanning the nine *Fas* exons with the use of PCR conditions described elsewhere,[16] except that the annealing temperatures for exons 4 and 7 were 58°C and 60°C, respectively. Leukocyte subgroups were purified by cell sorting (purification always exceeded 95 percent) with a fluorescence-activated cell sorter (FACS) (FACStar[PLUS], Becton Dickinson) from the peripheral-blood mononuclear cells as described previously.[21] DNA from these subgroups was amplified by nested PCR. Sequencing was performed directly on PCR products with the use of the Big Dye DNA Sequencing Kit (Perkin–Elmer) and an ABI PRISM 377 automated sequencer (Applied Biosystems). For quantification, PCR products corresponding to *Fas* exon 8 were ligated into the TOPO vector with the use of the TOPO-TA Cloning Instruction Manual (Invitrogen), and at least 60 clones were individually sequenced. Allele-specific PCR was performed with the successive use of two sets of primers. The first step amplified *Fas* exon 8 on both alleles; the second step amplified either the mutant or the wild-type allele. PCR products were separated on 1.5 percent agarose gel and transferred to a Hybridization Transfer Membrane (NEN Life Science Products). Oligonucleotide hybridization was performed as described previously[8] at 3°C below the melting temperature of the oligonucleotide. Descriptions of all oligonucleotide sequences and PCR conditions are available on request.

RESULTS

IDENTIFICATION OF *Fas* MUTATIONS

We studied six patients with phenotypic features of ALPS (Table 1) but with normal levels of Fas-mediated apoptosis of phytohemagglutinin-activated T-cell blasts (Table 2) and no family history of ALPS. However, because of the strong association between an excess of double-negative T cells and a defect in Fas-mediated apoptosis,[11] we looked for *Fas* gene mutations in FACS-sorted double-negative T cells from these patients and found heterozygous *Fas* mutations in all six (Table 2). Donor and acceptor splice-site mutations of exon 8 were identified in Patient 1 and Patient 6, respectively, and a donor splice-site mutation of exon 7 was identified in Patient 5 (Table 2). These mutations are predicted to lead to the splicing out of the corresponding exon on RNA. Patient 2 had a nonsense mutation in exon 8, and Patient 3 had a missense mutation in exon 9 (D244V); Patient 4 had a deletion of 8 bp in exon 9 leading to a premature stop codon at position 227 (Table 2). Identical *Fas* mutations or mutations leading to identical changes in the structure of Fas have been described in patients with ALPS type Ia (Table 2) and have been shown to be dominant.[14,15] We also looked for *Fas* mutations in sorted double-negative T cells from five healthy, age-matched controls and found none.

In contrast to the results with purified double-negative T cells, mutant *Fas* products could not be

Table 1. Clinical and Immunologic Characteristics of Six Patients with Autoimmune Lymphoproliferative Syndrome Type III.*

Patient No.	Sex/Age (yr)	Age at Presentation	LA†	Spl‡	Age at Splenectomy	Auto-immunity	Serum IgG§	Serum IgA§	Serum IgM§	Lympho-cytes¶	Lymphocyte Subgroup					
											CD3+	CD4+	CD8+	CD4–CD8–‖	CD19+	CD16+CD56+
		mo			*yr*					*mm³*	*percent*					
1	F/13	5	+++	++	6	Absent	↑↑	↑↑	N	6810	72	25	23	30	9	2
2	F/9	4	+++	++	1	A, T, UR	↑↑	↑↑	↑↑	5790	94	35	47	9	3	1
3	M/18	10	++	+++	13	UR	↑↑	↑↑	↑	4920	74	16	29	30	21	4
4	M/8	4	+	+++	—	Absent	↑↑	N	N	3400	90	24	41	20	7	2
5	M/6	24	+	++	—	A	↑↑	↑	N	1900	81	29	26	22	12	NA
6	M/13	6	+	++	—	A	↑↑	↑↑	N	1560	78	34	37	9	17	3

* A denotes autoimmune hemolytic anemia, T thrombocytopenia, UR urticarial rash, N normal serum level, and NA not applicable.

† With respect to lymphadenopathy (LA), one plus sign indicates the presence of multiple nodes less than 2 cm in diameter, two plus signs multiple nodes ranging from 2 to less than 5 cm in diameter, and three plus signs multiple nodes more than 5 cm in diameter.

‡ One plus sign indicates that splenomegaly (Spl) was palpable above the umbilicus, two plus signs that it was palpable below the umbilicus, and three plus signs that it was palpable in the iliac fossa.

§ One arrow indicates that serum levels were 2 to 4 SD above the normal range, and two arrows indicate that serum levels were more than 4 SD above the normal range.

¶ Lymphocyte counts among controls were as follows: 2000 to 4000 per cubic millimeter, with 70 to 80 percent CD3+ cells, 40 to 50 percent CD4+ cells, 20 to 30 percent CD8+ cells, 10 to 20 percent CD19+ cells, and 10 to 20 percent CD16+CD56+ cells.

‖ The normal percentage of CD4–CD8– cells in T-cell receptor α/β cells is less than 2 percent.

detected by PCR performed on DNA from T-cell blasts that had been activated in vitro for nine days by exposure to phytohemagglutinin.

EXPRESSION OF MUTANT ALLELES

The expression of the mutant alleles in T cells from Patient 1 and Patient 2 was analyzed. An aberrant product of RT-PCR amplification of *Fas* DNA, as well as the expected normal-sized product, was detected in cDNA prepared from resting peripheral-blood mononuclear cells (Fig. 1A). Sequencing of these RT-PCR products revealed a wild-type sequence and an abnormal product in which exon 8 was missing (data not shown). The omission of exon 8 in Fas messenger RNA could be the consequence of the nonsense and splice-site mutations in DNA of double-negative T cells from Patient 1 and Patient 2, respectively (Table 2). Indeed, such mutations were detected in genomic DNA from purified double-negative T cells (Fig. 1B) but not in that from phytohemagglutinin-stimulated T cells.

Moreover, the double-negative T cells of these patients, which were readily detectable among resting T cells (Fig. 1C), were undetectable after being incubated with phytohemagglutinin for nine days (Fig. 1C) or after stimulation of T cells with antibodies against T-cell receptors (data not shown). Thus, the absence of mutant cells after in vitro stimulation by phytohemagglutinin accounts for the normal Fas-mediated apoptosis in unfractionated lymphocytes from patients with ALPS type III.

Figure 1 (facing page). Analysis of the Expression of *Fas* Mutations in Activated T Cells and Purified Double-Negative T Cells from Patient 1 and Patient 2.
Panel A shows the results of RT-PCR amplification of *Fas* in double-negative T cells in resting peripheral-blood mononuclear cells and phytohemagglutinin-activated T cells. Panel B shows the sequences of exon 8 of genomic *Fas* obtained from purified double-negative T cells and phytohemagglutinin-activated T cells. Panel C shows the FACS analysis of the percentages of double-negative T cells (CD3+ T-cell receptor [TCR] α/β+CD4–CD8–) in resting peripheral-blood mononuclear cells and phytohemagglutinin-activated T cells.

Table 2. Levels of Fas-Mediated Apoptosis and Heterozygous *Fas* Mutations in Six Patients with Autoimmune Lymphoproliferative Syndrome (ALPS) Type III and Three Patients with ALPS Type Ia.

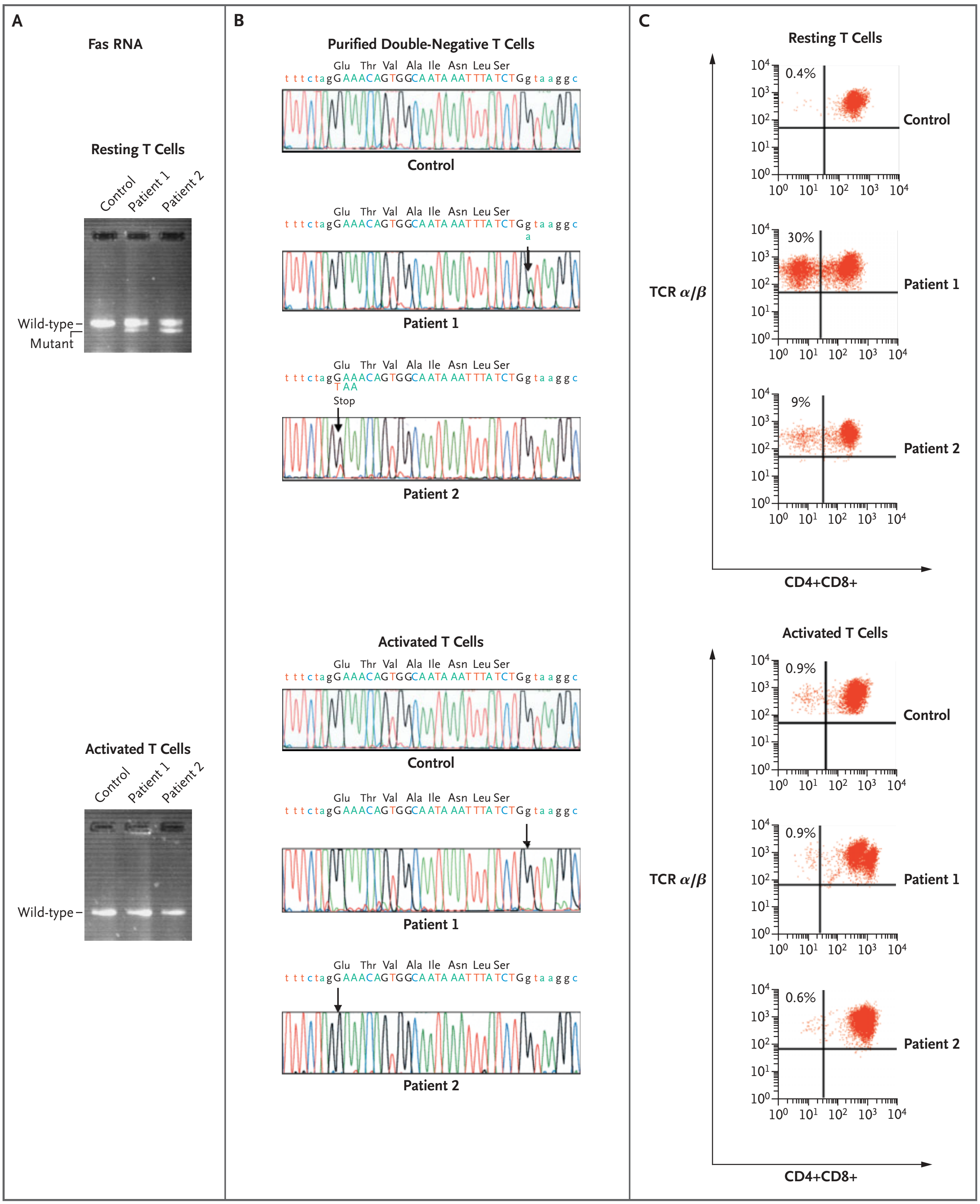
Subgroup	Fas-Mediated Apoptosis*	*Fas* Mutation		
		Type	Location	Predicted Protein
	%			
ALPS type III				
Patient 1	70	Somatic	Exon 8	P201fs (stop 204)
Patient 2	85	Somatic	Exon 8	P201fs (stop 204)
Patient 3	93	Somatic	Exon 9	D244V
Patient 4	73	Somatic	Exon 9	S214fs (stop 227)
Patient 5	86	Somatic	Exon 7	W173fs (stop 209)
Patient 6	83	Somatic	Exon 8	P201fs (stop 204)
ALPS type Ia†				
Patient 1	8	Germ line	Exon 8	P201fs (stop 204)
Patient 2	4	Germ line	Exon 9	S214fs (stop 224)
Patient 3	10	Germ line	Exon 7	K181fs (stop 199)

* The mean (±SD) value among 80 controls was 83±11 percent. Values are the percentages of phytohemagglutinin-activated T-cell blasts that underwent apoptosis in vitro.
† Data on Patients 1 and 2 are from Rieux-Laucat et al.[16] Data on Patient 3 are from Vaishnaw et al.[15]

DISTRIBUTION OF *Fas* MUTATIONS

To determine the cellular distribution of the *Fas* mutations in Patient 1 and Patient 2, we performed PCR analysis of genomic DNA from FACS-sorted peripheral-blood CD4+ or CD8+ T cells (hereafter called single-positive T cells), T-cell receptor γ/δ+ T cells, natural killer cells, B cells, monocytes, splenic CD34+ hematopoietic progenitors, hair cells, and buccal epithelial cells. To rule out cellular chimerism, the migration profiles of nine polymorphic markers on double-negative T cells and single-positive T cells were determined (data not shown) and were found to be identical.

We first used a mutation-specific PCR method[22,23] to determine which type of cell or tissue bears the *Fas* mutation, and we quantified the level of mutant cells in these populations. By means of dilution experiments, we found that heterozygous *Fas* mutations could be detected by direct sequencing of PCR products only when more than 20 percent of cells carried the mutation (data not shown). With a more sensitive analysis, which entailed cloning and sequencing PCR products, we could detect cells with mutations when more than 1 percent of cells carried the mutation, a percentage compatible with the level of purity of FACS separation techniques. The mutation in exon 8 was detected in all leukocyte subgroups from Patient 1, whichever method was used (Fig. 2). Thus, in this patient, a mutant *Fas* was present in more than 20 percent of lymphocytes and myeloid cells. The quantitative analysis


A
Fas RNA
Resting T Cells
Control
Patient 1
Patient 2
Wild-type
Mutant
Activated T Cells
Control
Patient 1
Patient 2
Wild-type
B
Purified Double-Negative T Cells
Glu Thr Val Ala Ile Asn Leu Ser
tttctagGAAACAGTGGCAATAAATTTATCTGgtaaggc
Control
Patient 1
Stop
Patient 2
Activated T Cells
Control
Patient 1
Patient 2
C
Resting T Cells
0.4%
Control
30%
Patient 1
9%
Patient 2
TCR α/β
CD4+CD8+
Activated T Cells
0.9%
Control
0.9%
Patient 1
0.6%
Patient 2
TCR α/β
CD4+CD8+

showed that 100 percent of double-negative T cells but only 20 percent of single-positive T cells and 14 percent of monocytes carried the mutation, indicating that, with the exception of double-negative T cells, these different leukocyte subgroups contained a similar proportion of mutant cells. The mutation was undetectable in hair cells and buccal epithelial cells from Patient 1 on direct sequencing.

In Patient 2, the *Fas* mutation was found in all leukocyte subgroups by means of the mutation-specific PCR method. In contrast, the mutation was detected in double-negative T cells by direct sequencing (Fig. 2) but not in other leukocyte subgroups, suggesting that it was carried by less than 20 percent of cells in the other leukocyte populations. The mutation-specific quantitative method indicated that 100 percent of double-negative T cells from the blood and spleen of Patient 2 were mutant cells, whereas only 10 percent of single-positive T cells carried the mutation.

A purified CD34+CD19–CD7– population, which was known to be enriched for hematopoietic progenitors, was obtained from a spleen sample from Patient 2. Mutation-specific PCR revealed mutant cells in this population (Fig. 2). We estimate, taking into account the purification efficiency of FACS, that less than 2 percent of these cells carried the mutation (Fig. 2). The *Fas* mutation was undetectable in nonhematopoietic cells, even when the mutation-specific PCR was used (Fig. 2). All these results suggest that in both Patient 1 and Patient 2, *Fas* mutations originated in hematopoietic stem cells or possibly earlier, in mesenchymal precursors. The specific accumulation of *Fas* mutants in the double-negative T-cell compartment underlines the essential role of Fas in the control of lymphocyte homeostasis in the periphery.

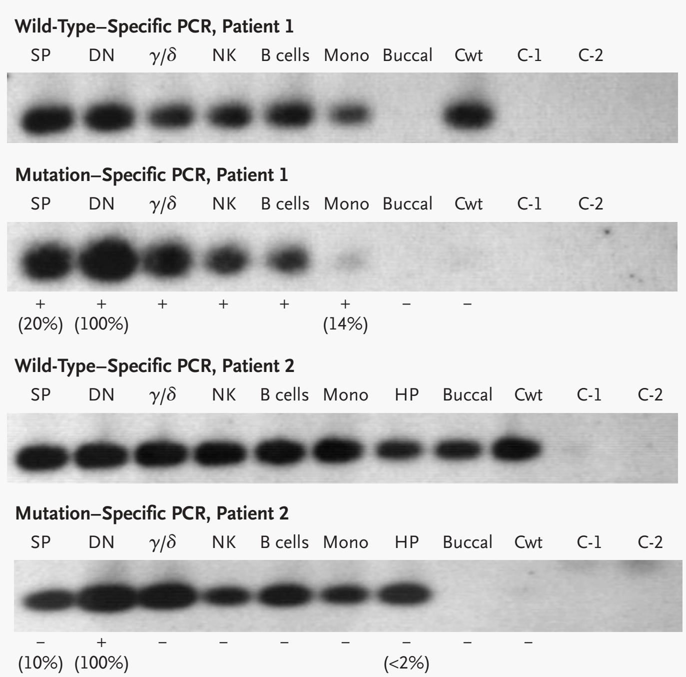


Figure 2. Hybridization of the PCR Products of *Fas* Exon 8 after Wild-Type–Specific and Mutation-Specific Amplifications.

Detection of the mutant allele after direct sequencing of PCR products is indicated by a plus sign, and the absence of the mutation by a minus sign. Numbers in parentheses are the percentages of mutant cells in a given cell population as determined by cloning and sequencing. SP denotes single-positive (CD4+ or CD8+) T cells, DN double-negative T cells, γ/δ T-cell receptor γ/δ+ T cells, NK natural killer cells, Mono monocytes, HP hematopoietic-progenitor–enriched cells, Buccal buccal epithelial cells, Cwt T cells from a wild-type control, C-1 a negative control included in the first amplification, and C-2 a negative control included in the second amplification (with the use of products of the first PCR).

T-CELL–RECEPTOR REPERTOIRE AND THE ORIGIN OF DOUBLE-NEGATIVE T CELLS

Somatic *Fas* mutations have been described in rare cases of a syndrome involving monoclonal or oligoclonal double-negative T cells.[24] We therefore analyzed the population of T-cell–receptor β chains in double-negative T cells from both patients using antibodies against the β chains and found polyclonal T-cell populations of double-negative and single-positive T cells in both patients (Fig. 3).

DISCUSSION

We found that patients with mosaicism carrying heterozygous *Fas* mutations in hematopoietic cells have an ALPS phenotype. In the light of our findings, the classification of ALPS requires revision, with patients such as ours possibly denoted as belonging to a subgroup with mosaic ALPS type I, or ALPS type Im. In support of this reclassification is the fact that the clinical phenotype associated with these somatic mutations is indistinguishable from that of ALPS type I.

Our study demonstrates that peripheral lymphocytes with a dominant somatic *Fas* mutation exhibit a selective advantage (Fig. 4). Germ-line mutations of *Fas* have been reported to impair Fas-induced apoptosis of lymphocytes in patients with ALPS type Ia.[14,15] By resisting apoptosis, the mutant cells accumulate and become double-negative T cells. This interpretation is consistent with data from Fas-deficient chimeric mice[25] and can account for the lymphadenopathy and splenomegaly in all six of our patients. Indeed, a lymph node from Patient 4 showed paracortical expansion consisting of double-negative T cells, a histologic picture indistinguishable from that seen in patients with ALPS type I (data not shown). Similarly, the relatively large proportion of mutant cells in peripheral lymphocytes from Patient 2, as compared with the smaller proportion of mutant cells among hematopoietic progenitors, suggests that *Fas* mutations provide a selective advantage (by protecting against apoptosis) during hematopoiesis, a finding consistent with observations in MRL *lpr/lpr* mice.[26]

We identified *Fas* mutations in freshly purified double-negative T cells but not in phytohemagglutinin-activated T cells. The normal in vitro response to Fas-induced apoptosis by activated T cells is consistent with the absence of *Fas* mutations in such cells. This result might be due to the high death rate of double-negative T cells in vitro. Double-negative T cells are believed to originate from activated peripheral single-positive T cells that have received a death-inducing signal but cannot die, owing to a defect in Fas signaling.[3,27,28] A similar abnormality of double-negative T cells has been described in patients with ALPS type I, suggesting that a signal required for the survival of double-negative T cells is lacking in tissue-culture medium.[10,29,30] In vivo, this signal could be provided by self-antigens, and chronic stimulation of apoptosis-resistant cells by autoantigens could account for the autoimmune manifestations and lymphoproliferation of ALPS. However, autoimmune manifestations were observed in only four of our six patients. In addition, there is no clear correlation between the numbers of double-negative T cells and autoimmunity, and whether these cells recognize and respond to self-antigens is also unknown.

A proportion of all hematopoietic cells from our patients carried *Fas* mutations, whereas they were not found in hair cells or buccal epithelial cells. Several mechanisms could account for this finding. One is chimerism, which could be the consequence of transplacental passage of maternal blood or cell fusion from an aborted dizygote twin.[31,32] This possibility was excluded, since the population of both the double-negative T cells (containing mutant cells alone) and the single-positive T cells (containing an excess of wild-type cells) had similar patterns of DNA polymorphic markers (data not shown).

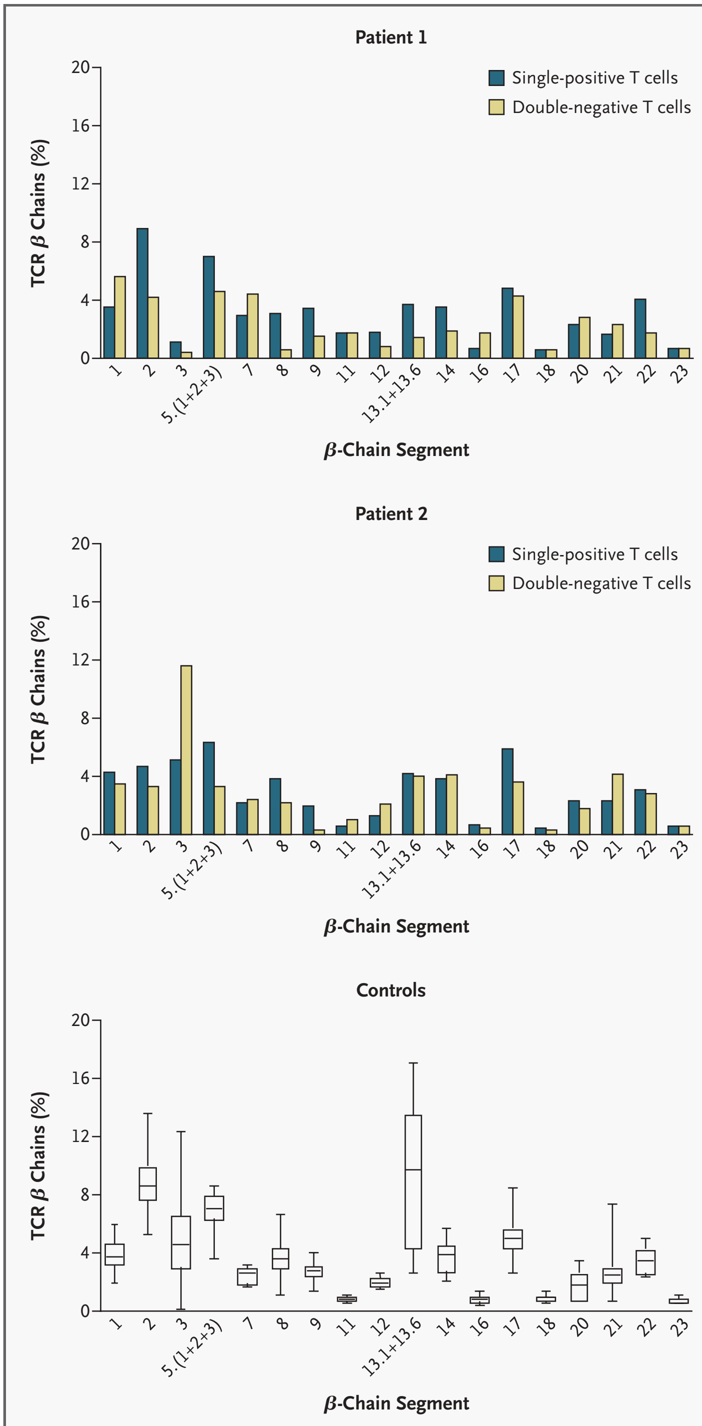


Figure 3. Analysis of the T-Cell–Receptor (TCR) Repertoire on Single-Positive (CD4+ or CD8+) T Cells and Double-Negative (CD4–CD8– TCR α/β+) T Cells from Patient 1, Patient 2, and 80 Controls.

Values in controls are expressed as the mean (horizontal line in each box), with the standard deviation (top and bottom of the box) and 95 percent confidence interval (I bar).

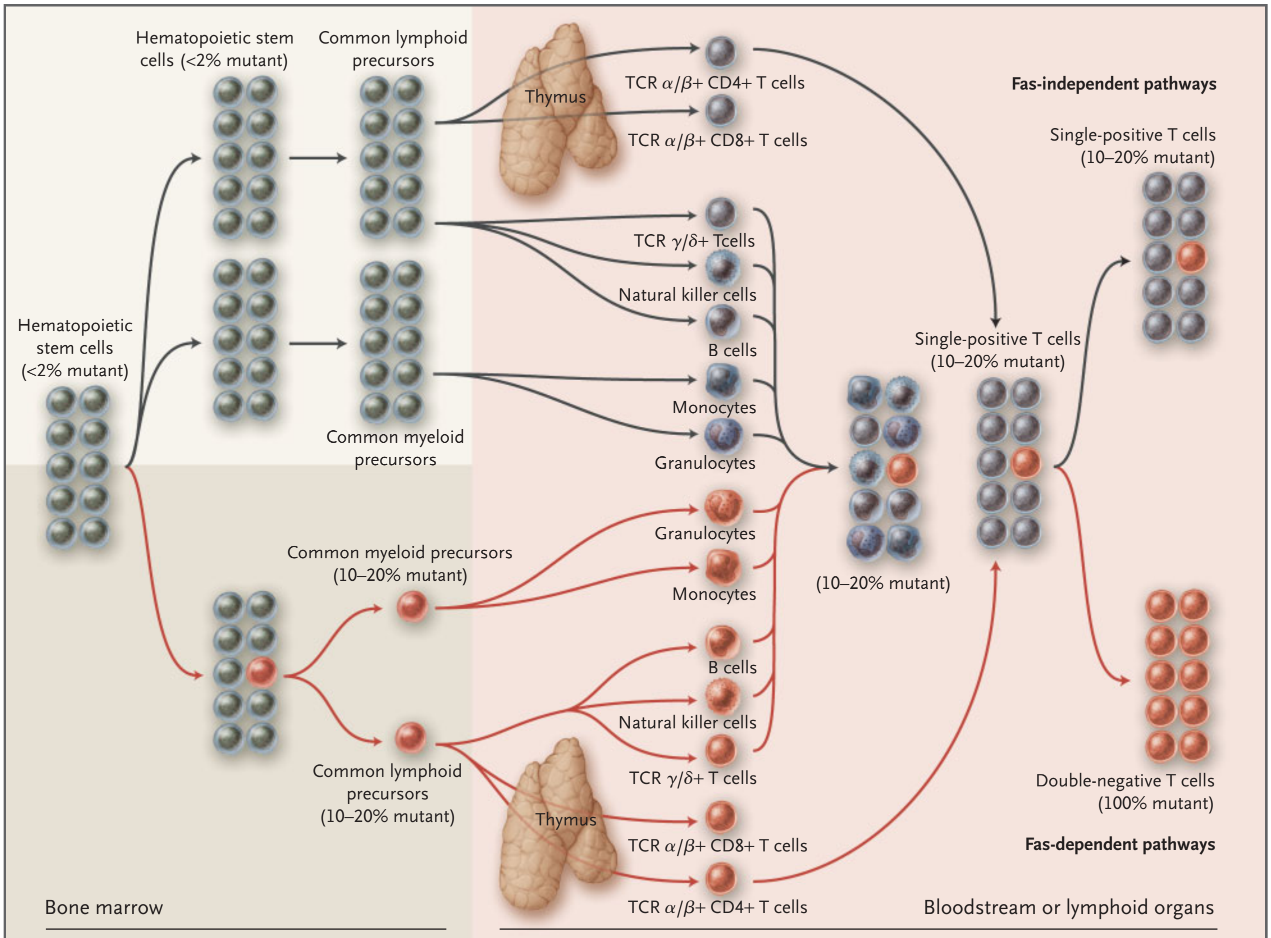


Figure 4. Hematopoietic Development and Peripheral Proliferation of Fas-Sensitive and Fas-Deficient Cells.

In the bone marrow, a mutant hematopoietic stem cell (in red), carrying a heterozygous *Fas* mutation, emerges during the course of the self-renewal or generation of these cells. It has been estimated that less than 2 percent of hematopoietic stem cells are mutated. The progeny of putative common lymphoid precursors, such as T cells, B cells, and natural killer cells, and the progeny of putative common myeloid precursors, such as monocytes and granulocytes, all contain a higher percentage of mutant cells (estimated at 10 to 20 percent). Therefore, the resistance of mutant hematopoietic stem cells to Fas-mediated apoptosis is probably the selective advantage that accounts for the increased proportion of mutant cells observed in peripheral leukocytes. In the bloodstream or lymphoid organs of patients with ALPS, some T-cell proliferations (driven by bacterial or viral antigens) are well controlled, probably by Fas-independent pathways. In contrast, other T-cell responses (perhaps driven by self-antigens), normally regulated by Fas, lead to uncontrolled proliferation and the accumulation of mutant double-negative T cells. The resistance to Fas-mediated cell death allows the proliferation or the persistence of cells that should otherwise disappear. TCR denotes T-cell receptor.

Therefore, these mutations must have resulted from a somatic mutation that occurred during embryonic or fetal development, or after birth. Although *Fas* mutations were not detected in cells from the mouth or hair (originating from ectoderm), their presence in germ cells (of endodermic origin), which would indicate a mutation early in embryogenesis, was not formally ruled out. Analyses of additional tissues would be required to narrow the timing of the mutational events.

The clinical features of our patients with ALPS type III resemble those of patients with ALPS type I who have identical or similar germ-line *Fas* mutations. In some other conditions, however, mosaicism is usually associated with a mild phenotype, because of somatic reversions to the wild type.[33] In such cases, wild-type revertant cells can have a selective advantage, enabling their expansion and the partial restoration of the wild-type phenotype.[34-36] Our findings in patients with acquired ALPS represent an example of dominant somatic mutations' conferring a selective advantage of mutant cells over normal cells. Nevertheless, a 10-year follow-up showed that the proportion of mutant lymphocytes was steady over time and that the cells did not outgrow normal cells.

Healthy relatives of patients with ALPS type I can carry an inherited dominant *Fas* mutation,[14-16] thereby illustrating the partial clinical penetrance of some *Fas* mutations. Mutations affecting the intracellular domain of Fas are associated with greater clinical penetrance than mutations affecting the extracellular domain.[15,16] Notably, all the mutations we found affected the intracellular domain. They are predicted to generate abnormal Fas molecules and have been associated with full penetrance of the disease in patients with ALPS type I.[14-16]

In conclusion, we found that some patients with ALPS type III have somatic *Fas* mutations in cells of hematopoietic lineages in the absence of any malignant condition. This situation is an example of a nonmalignant, genetically acquired disease in which a selective advantage (resistance to death) is conferred by *Fas* mutations.

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