



Onset of autoimmune lymphoproliferative syndrome (ALPS) in humans as a consequence of genetic defect accumulation

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Autoimmune diseases develop in approximately 5% of humans. They can arise when self-tolerance checkpoints of the immune system are bypassed as a consequence of inherited mutations of key genes involved in lymphocyte activation, survival, or death. For example, autoimmune lymphoproliferative syndrome (ALPS) results from defects in self-tolerance checkpoints as a consequence of mutations in the death receptor–encoding gene TNF receptor superfamily, member 6 (*TNFRSF6*; also known as *FAS*). However, some mutation carriers remain asymptomatic throughout life. We have now demonstrated in 7 ALPS patients that the disease develops as a consequence of an inherited *TNFRSF6* heterozygous mutation combined with a somatic genetic event in the second *TNFRSF6* allele. Analysis of the patients' CD4⁺CD8⁻ (double negative) T cells — accumulation of which is a hallmark of ALPS — revealed that in these cells, 3 patients had somatic mutations in their second *TNFRSF6* allele, while 4 patients had loss of heterozygosity by telomeric uniparental disomy of chromosome 10. This observation provides the molecular bases of a nonmalignant autoimmune disease development in humans and may shed light on the mechanism underlying the occurrence of other autoimmune diseases.

Introduction

Autoimmune lymphoproliferative syndrome (ALPS, also known as lymphoproliferative syndrome with autoimmunity or Canale-Smith syndrome) (1) is a human disease characterized by benign lymphoproliferation (splenomegaly and/or diffuse lymphadenopathy), and elevated serum immunoglobulins (IgG and IgA), plasma IL-10 levels (2, 3), and FAS-L levels. (4), accumulation in the blood and lymphoid organs of CD4⁺CD8⁻TCRαβ⁺ T cells (also referred to as double-negative [DN] T cells), susceptibility to malignant conditions (5), and autoimmune manifestations (6, 7).

Mutations in the TNF receptor superfamily, member 6 (*TNFRSF6*) gene (encoding FAS, also known as the CD95 receptor) are the most common cause of ALPS (8, 9). Patients carrying a homozygous *TNFRSF6* mutation have an early-onset severe phenotype, whereas subjects with heterozygous *TNFRSF6* mutations present ALPS of variable intensity. T cells from patients with homozygous or heterozygous *TNFRSF6* mutations, respectively, exhibit a complete or partial functional impairment in an in vitro FAS-induced apoptosis assay. Moreover, somatic heterozygous mutations in *TNFRSF6* in patients with a clinical ALPS phenotype were recently described (10, 11). In our experimental conditions, activated T cells showed normal sensitivity to FAS-induced apoptosis in vitro (11). The inability to detect an apoptosis defect was due to spontane-

ous in vitro apoptosis of the mutant T cells. However, more than 80% of these patients' DN T cells were mutated. These patients displayed mosaic expression of a somatic *TNFRSF6* mutation, which provided the affected cells a selective advantage and accounted for the observed lymphoproliferation and autoimmunity.

However, heterozygous germline *TNFRSF6* mutations are not always associated with clinical expression (partial clinical penetrance), since some mutation-positive relatives (MPRs) remain asymptomatic despite an in vitro FAS-induced apoptosis impairment on their T cells. This observation led us to postulate that a second event is necessary for disease expression in patients with *TNFRSF6* mutations when an incomplete penetrance is observed. Here, we report that the combination of a germline *TNFRSF6* mutation and a somatic event impairing the second *TNFRSF6* allele can account for the onset of clinical phenotype in ALPS.

Results

Biological characteristics of the 7 ALPS patients and their asymptomatic relatives. Within the cohort of ALPS patients with heterozygous *TNFRSF6* mutation studied in our institution ($n = 87$), 16% ($n = 14$) of the germline mutations affected the extracellular domain (ECD) of *TNFRSF6*. The clinical penetrance has been found to be much lower for heterozygous ECD mutations (30% of carriers developed ALPS) than for heterozygous intracellular domain (ICD) mutations (80% of carriers developed ALPS) (12, 13). This observation suggests that additional events are necessary for ALPS disease expression in

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Table 1
Clinical features of 7 ALPS-FAS patients

	P1	P2	P3	P4	P5	P6	P7
Germline mutation	Ex 5: L159fs X186	Ex4:E116G	Ex6: deletion	Ex4:R121fs X159	Ex3: C107Y	Ex3: C104G	Ex5: C157X
Age (yr)	15	18	16	28	21	22	47
Age at onset (yr)	5	0.25	2	3	10	11	24
Splenomegaly	+++	+++	+++	–	++	+++	++
Lymphadenopathy	+++	+++	++	–	++	+++	++
Autoimmunity: clinical features	None	None	Neutropenia ITP AIHA	ITP Glomerulonephritis	Arthritis	None	AIHA
Autoantibodies (anti-)	None	None	Polynuclear	Nuclear antigen platelets	Nuclear antigen	None	Erythrocytes
IgG (g/l) ^A	15.9	22.9–34.7	13.8	14.9	12.4–17.9	19.6–35	15.7
IgA (g/l) ^B	3.7	5.82–7.9	1.38	9	2.83–4.55	4.9–8.8	4.53
IgM (g/l) ^C	0.34	0.35	1.08	0.4	0.3–0.39	0.62–0.83	0.28

^AControl values, 9.2–14.8 g/l. ^BControl values, 1.42–2.62 g/l. ^CControl values, 0.88–1.84 g/l. +, enlargement of the spleen not exceeding half the distance between costal margin and umbilical line and/or a lymph node diameter of between 1 and 2 centimeters; ++, enlargement of the spleen below half distance between the costal margin and umbilicus and/or a lymph node diameter of between 3 and 5 centimeters; +++, enlargement of the spleen further down the umbilicus and/or a lymph node diameter exceeding 5 centimeter or in clumps; ITP, immune thrombocytopenia; AIHA, autoimmune hemolytic anemia.

carriers of *TNFRSF6* mutations with incomplete clinical penetrance. In order to search for these additional events, we analyzed the available T cells from 7 patients with the typical clinical symptoms of ALPS associated with monoallelic mutations affecting the ECD of *TNFRSF6* (Table 1) and compared with asymptomatic family carriers in 4 cases. We first quantified ALPS markers: the percentage of DN T cells, plasma FAS-L, and IL-10 concentrations (2, 4, 14) (Figure 1A). In strict agreement with the clinical phenotype, all 7 patients presented markedly elevated numbers of DN T cells and plasma FAS-L and IL-10 concentrations, whereas their asymptomatic MPRs carrying the same *TNFRSF6* mutations exhibited normal DN T cell percentages and plasma IL-10 concentration and little or no increase in the plasma FAS-L concentration.

For all 7 patients, FAS-mediated T cell apoptosis was lower than for controls (Figure 1A). When simultaneously tested, we found that FAS-mediated apoptosis of patients' T cells and their asymptomatic MPRs had similarly low apoptosis levels; this contrasted with the results for nonmutated relatives (Figure 1A). Since the magnitude of the FAS-mediated apoptosis defect was similar in symptomatic and asymptomatic carriers of *TNFRSF6* mutations, we postulated that any additional causative events did not directly have an impact on the FAS-induced apoptosis as assayed in vitro. This result was reminiscent of ALPS cases carrying somatic *TNFRSF6* mutations but which were associated with normal FAS-mediated T cell apoptosis in vitro (11).

Somatic mutations of TNFRSF6 in 3 patients with heterozygous germline mutations. Based on the literature description of ALPS sufferers carrying a somatic *TNFRSF6* mutation, which was predominantly found in DN T cells, we sequenced the *TNFRSF6* gene in sorted DN T cells from each of the 7 patients. In addition to the previously identified germline *TNFRSF6* mutation, we found a second heterozygous *TNFRSF6* mutation in the DN T cells from patients 1, 2, and 3 (Figure 1B). In patient 1, a base insertion in exon 9 had created a premature stop codon at position 280. In patient 2, a nonsense mutation was found in exon 2 (G34X). In patient 3, a missense mutation was found in exon 9 (A237P). These somatic heterozygous *TNFRSF6* mutations were present in the second allele, since the cloned PCR products from cDNA amplified from these patients' DN T cells exhibited only 1 of the 2 *TNFRSF6* mutations (data not shown).

The proportion of cells carrying the somatic mutation was established in several lineages for patients 1 and 2. In patient 1, the second mutation was found only in DN T cells and not in either single-positive T cells or epithelial cells (sensitivity threshold: 2.5% of cells) (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI43752SD1). It was therefore impossible to determine at which stage of lymphocyte differentiation the mutation had occurred. In patient 2, the mutation was found in 7%–35% of T cells, B cells, NK cells, and monocytes but also in buccal epithelial cells – indicating that the somatic mutation had occurred very early in embryogenesis. In the other 4 patients, *TNFRSF6* sequencing did not highlight any second heterozygous mutation but revealed only the mutated allele in DN T cells (not shown), suggesting a somatic loss of the wild-type allele.

Loss of the wild-type TNFRSF6 allele in 4 patients. In order to identify the molecular basis of the loss of the wild-type allele in DN T cells from patients 4 to 7, we checked for single-nucleotide polymorphisms at the *TNFRSF6* locus in DNA from DN T cells and from CD4⁺ T cells. In all 4 patients, DNA from the CD4⁺ T cells displayed heterozygous polymorphic microsatellite markers, whereas DNA from the DN T cells displayed homozygous markers (Figure 2A). We then used serial microsatellite typing along chromosome 10q in CD4⁺ T cell and DN T cell DNA to determine the location of the mutations. For patients 5 and 6, we honed in on a centromeric breakpoint region at about 1.3 kb upstream of the *TNFRSF6* locus (Figure 2A). Loss of heterozygosity (LOH) was observed down to the more distal telomeric marker, indicating that about 45 Mb of parental DNA was missing. The conserved heterozygosity observed in the CD4⁺ T cells suggested that very few (if any) of these cells carried the same chromosome aberration. Our data indicated that DN T cells exhibited telomeric uniparental disomy (UPD) or partial loss of the long arm of chromosome 10. The same analysis performed on the DN T cells from patient 4 and patient 7 also revealed a mosaic LOH encompassing 60 Mb or more, respectively (Figure 2A). This phenomenon was not observed in DN T cells from 3 ALPS patients carrying ICD mutations of *TNFRSF6* with incomplete clinical penetrance or from healthy carriers from families 1 to 4 (data not shown). Array-based comparative genomic hybridization in patients 4, 6, and 7 (Figure 2B) and FISH in



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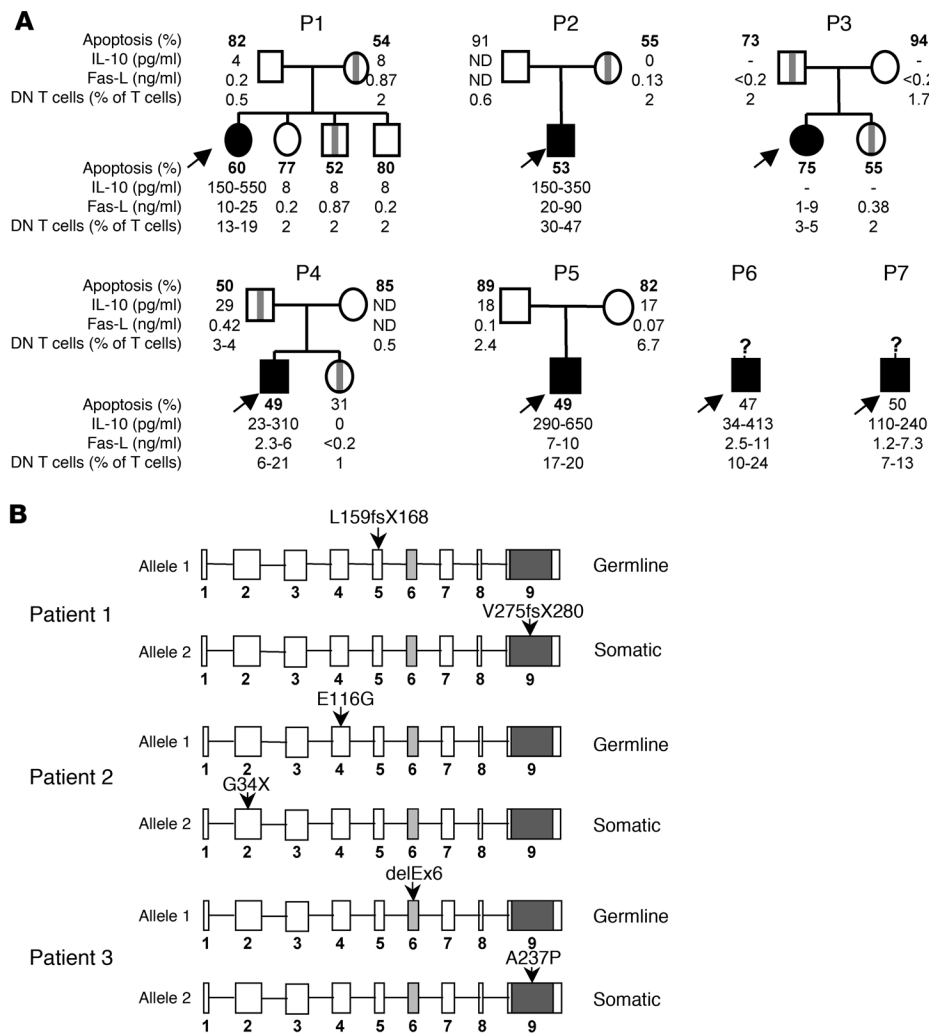


Figure 1

Family tree of 7 ALPS patients with germline heterozygous *TNFRSF6* mutations and additional somatic mutations in patients 1, 2, and 3. **(A)** Family trees of 7 ALPS patients with a germline ECD *TNFRSF6* mutation. Asymptomatic mutated relatives are indicated by vertical gray bars. Subjects with ALPS are marked in black (arrows). The range of values determined for the 4 ALPS markers, i.e., apoptosis of activated T cells (control > 75%), plasma IL-10 concentration (control < 20 pg/ml), plasma FAS-L concentration (control < 0.2 ng/ml), and the percentage of DN T cells (< 2% of TCRαβ cells) are shown for these patients and their relatives. Apoptosis values depicted in bold were obtained in an assay performed at the same time for patients' cells and relatives' cells. P1, patient 1. **(B)** Localization of the 2 mutations in the *TNFRSF6* alleles (9 exons) for patients 1, 2, and 3. The oblique gray bars in exon 6 and the dark section in exon 9 represent the transmembrane domain and the death domain, respectively.

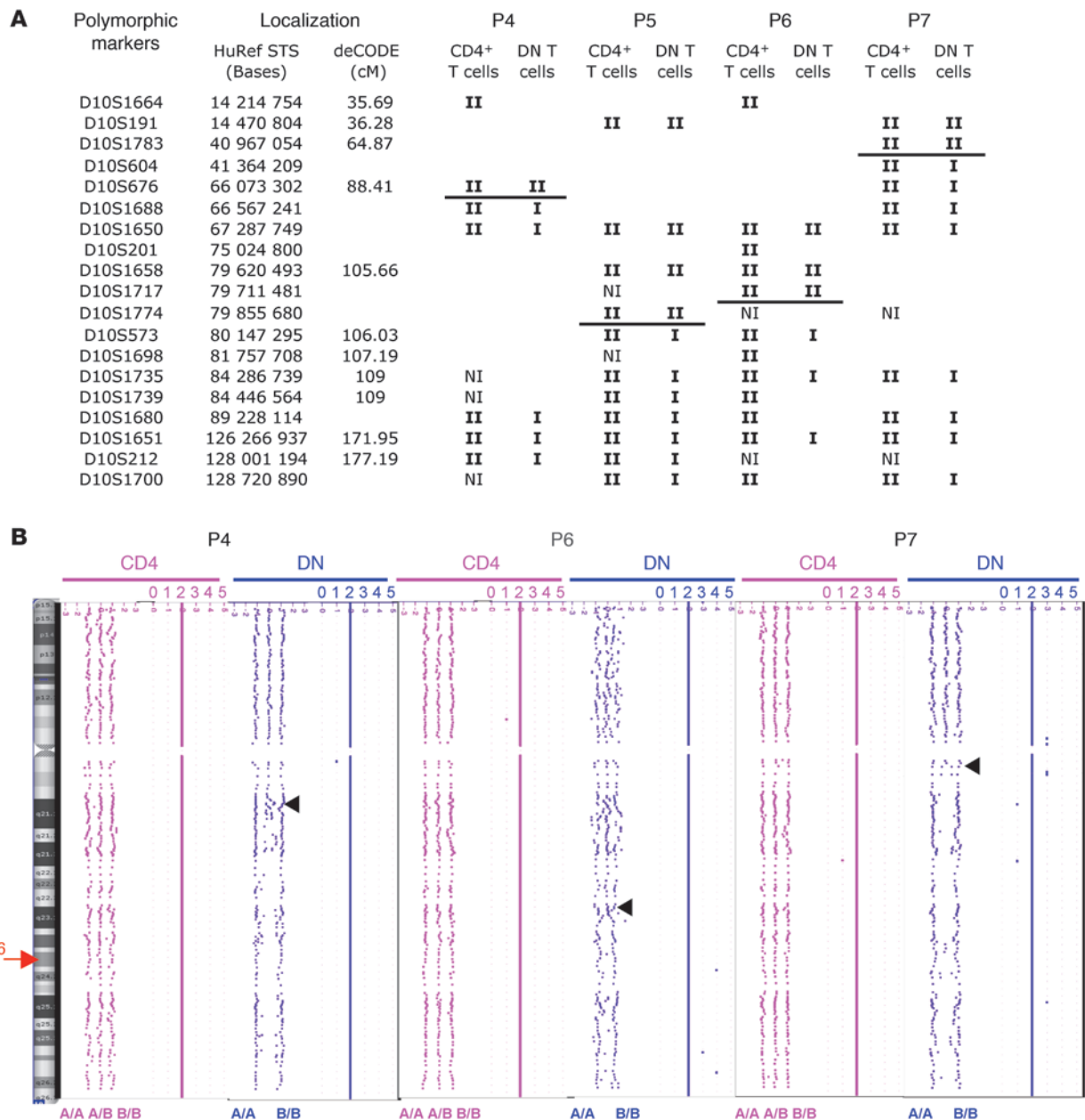
patients 6 and 7 (data not shown) confirmed the LOH and conservation of the same gene copy number. The size of the chromosomal segment affected by the LOH in patient 4 was about 75 Mb. These results demonstrate that (a) the wild-type allele had been lost and (b) the mutated allele had been duplicated from the estimated breakpoint down to the end of chromosome 10.

Measurement of FAS expression in cells with 2 disrupted TNFRSF6 alleles. In order to determine the impact of these additional *TNFRSF6* mutations or chromosome aberration, we analyzed FAS protein expression in CD4, CD8, and DN T cells from the 7 patients, 3 asymptomatic MPRs (patient 2's mother, patient 3's father, and patient 4's father), and 5 healthy controls (Figure 3). First, we found that FAS expression in single-positive (CD4 or CD8) T cells was slightly lower than control values in all patients or relatives carrying a low-penetrance ECD *TNFRSF6* mutation (except for patient 1); this suggests that the germline *TNFRSF6* mutation in allele 1 led to defective expression in patients 2 to 7. Further analysis indicated that the great majority of these patients' DN T cells did not express FAS. Interestingly, DN T cells from patients 2, 3, and 4 expressed less FAS than those from patient 2's mother, patient 3's father, and patient 4's father, respectively (Figure 3, A and B). The low FAS expression in these cells was not due to an activation defect, since they expressed high amounts of

human leukocyte antigen class II molecules (Supplemental Figure 1). Strikingly, no decrease of FAS expression was observed in 3 patients carrying germline ICD *TNFRSF6* mutations associated with low penetrance. Accordingly, no somatic mutation has been detected in these patients. These results indicated that the somatic event in DN T cells is mostly associated with reduced FAS expression via loss of the wild-type *TNFRSF6* allele.

Discussion

Severe ALPS always occurs when both *TNFRSF6* alleles are mutated in the germline in humans (9, 15, 16) or mice (17). In contrast, if the FAS defect is partial (as in the case of a heterozygous mutation, for example), the human disease develops in 70% of carriers (13). The partial clinical penetrance of heterozygous *TNFRSF6* mutations in ALPS suggests that in addition to the germline *TNFRSF6* mutation, a "second hit" is required for disease onset. Here, we identified somatic genetic events affecting the second *TNFRSF6* allele in 7 patients carrying a germline heterozygous mutation with low clinical penetrance. In these patients, a small proportion of lymphocytes lacked functional FAS — leading to uncontrolled proliferation and autoimmunity. Missense or nonsense somatic mutations were identified in 3 patients, whereas somatic UPD (duplication of the mutant allele with loss of the wild-type allele) were found in the other 4 patients.

**Figure 2**

LOH on chromosome 10 of DN T cells from 4 patients carrying germline heterozygous *TNFRSF6* mutations. **(A)** Polymorphic markers used for LOH characterization. Each allele is represented by the symbol I. Horizontal bars indicate the upstream LOH region in DN T cells. NI, not informative. **(B)** An array comparative genome hybridization profile of chromosome 10, showing the LOH (phenotype A/A or B/B but no heterozygosity A/B) and the stable allele copy number (the numbers above the schema depicted the allele copy number: 2 copies along the entire chromosome) that are characteristic of UPD (duplication of the mutant allele with loss of the wild-type allele) in DN T cells of patients 4, 6, and 7. The arrowhead indicates the upstream site of LOH.

Interestingly, we identified somatic events in 7 patients carrying germline heterozygous *TNFRSF6* mutations affecting the ECD of FAS. The latter are known to frequently induce a loss of FAS expression at the cell membrane and therefore haploinsufficiency (18). This was confirmed by analyzing cells from the MPRs, in which the only germline mutation was present. Expression of half the normal level of FAS receptor is sufficient to trigger apoptosis and might preclude the onset of ALPS and account for the low clinical

penetrance seen with ECD *TNFRSF6* mutations. The additional somatic event led to a clear, profound expression defect in a small proportion of single-positive T cells and in the majority of DN T cells from all but 1 patient. In this latter case, patient 1 carried 2 missense mutations (one germline and one somatic), which were predicted to be nonfunctional. The subsequent *in vivo* functional defect could thus be the consequence of either the lack of FAS expression at the cell membrane or the lack of apoptosis signal.



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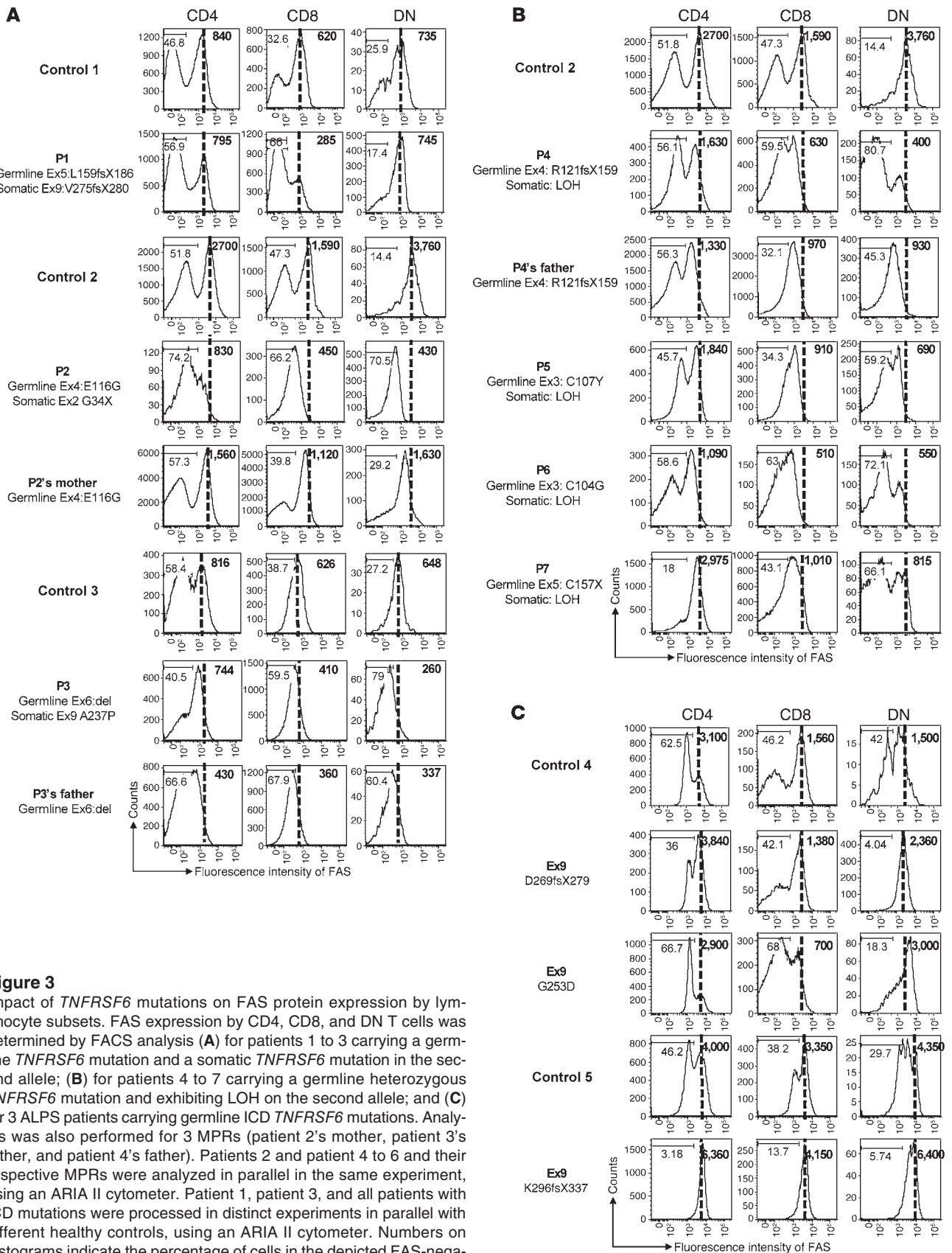


Figure 3
Impact of *TNFRSF6* mutations on FAS protein expression by lymphocyte subsets. FAS expression by CD4, CD8, and DN T cells was determined by FACS analysis (A) for patients 1 to 3 carrying a germline *TNFRSF6* mutation and a somatic *TNFRSF6* mutation in the second allele; (B) for patients 4 to 7 carrying a germline heterozygous *TNFRSF6* mutation and exhibiting LOH on the second allele; and (C) for 3 ALPS patients carrying germline ICD *TNFRSF6* mutations. Analysis was also performed for 3 MPRs (patient 2's mother, patient 3's father, and patient 4's father). Patients 2 and patient 4 to 6 and their respective MPRs were analyzed in parallel in the same experiment, using an ARIA II cytometer. Patient 1, patient 3, and all patients with ICD mutations were processed in distinct experiments in parallel with different healthy controls, using an ARIA II cytometer. Numbers on histograms indicate the percentage of cells in the depicted FAS-negative gates, and numbers in bold type indicate the mean fluorescence of FAS intensity in the whole population.



This could result from a lack of multimerization of the receptor or the lack of death-inducing signaling complex (DISC) recruitment by the residual FAS molecules. Consequently, the double-mutant lymphocytes could not be killed by FAS-L. A similar situation has been observed in patients carrying inherited homozygous or compound heterozygous mutations (9, 15). The lymphoproliferation observed in the latter patients indicated that a complete FAS defect led to uncontrolled proliferation of T and (to a lesser extent) B cells. In contrast, most missense mutations affecting the ICD of *TNFRSF6* are known to exert a transdominant negative effect, since the mutant protein is likely to trimerize with wild-type FAS. Assuming that efficient DISC formation and subsequent apoptosis triggering require wild-type FAS trimers only, it was estimated that 1 out of 8 trimers would be functional. The ensuing apoptosis defect may be sufficient to account for the onset of ALPS. Accordingly, the clinical penetrance of the ICD missense *TNFRSF6* mutation was complete in most families (19). Moreover, no additional defect in the *TNFRSF6* gene could be found in 3 tested patients with ICD mutations. It is noteworthy that previously described cases of ALPS caused by somatic heterozygous mutations (10, 11) were mostly associated with ICD *TNFRSF6* mutations. This observation suggested that somatic ICD *TNFRSF6* mutations markedly impair FAS function and provide the mutant cells with a selective advantage in vivo. Moreover, the patients carrying somatic ICD mutations developed clinical disease, despite the low proportion (10%–20%) of mutant lymphocytes. A similar picture was seen in our current cases, since the somatic events were seen in a minor fraction of single-positive T cells and in a major fraction of DN T cells. This observation supports the concept whereby the second hit (somatic mutation or UPD) together with the germline ECD-mutated *TNFRSF6* gene, profoundly impair FAS function and provide the double-mutant T cells with a selective advantage in vivo. The double-mutant cells died spontaneously in vitro and so could not be tested. Consequently, in vitro expansion of the patients' T cells led to accumulation of the germline mutants only. Accordingly, the magnitude of the FAS-induced apoptosis defect was similar in T cells from the patients and their asymptomatic mutated relatives, respectively.

The combination of germline and somatic mutations in the same gene has been reported in cancers with a hereditary predisposition, such as retinoblastoma (20). Similarly, missense mutations of *TNFRSF6* outside the death domain are associated with LOH in non-Hodgkin lymphomas (21). The LOH observed in 4 of our patients with germline *TNFRSF6* ECD mutations resulted from loss of the wild-type allele and concomitant duplication of the mutant one, i.e., telomeric UPD. This type of mechanism is usually observed in genetic disorders of development, where it can be involved in trisomy or monosomy rescue during embryonic development (22). The telomeric UPD observed in our ALPS cases might reflect a recombination hotspot on the 10q chromosome (as described in the 10q syndrome; ref. 23), but more likely reflect the selective advantage provided by the resulting homozygous *TNFRSF6* mutations. These events may occur during embryogenesis (patient 2) or later in life (since the onset of clinical manifestations was delayed in patients 5, 6, and 7). Implication of modifier genes in the onset of ALPS was previously postulated. The present findings highlight the role of somatic events affecting the second *TNFRSF6* allele as modifier in ALPS expression. However, we could not strictly exclude that these somatic FAS events result from other, yet undefined, germline mutation in the actual “modifier” genes.

Lymphocyte homeostasis and self tolerance are maintained by several checkpoints, including antigen receptor editing, modulation, energy, and central or peripheral clonal deletion of self-reactive lymphocytes (24). Variants of proteins involved in cell activation, survival, proliferation, and death may contribute to the emergence of autoimmunity (25). For instance, variants of the genes for HLA molecules (selecting for self-reactive lymphocytes), PTPN22 (regulating Antigen receptor signaling), and CTLA-4 (inhibiting T cell proliferation) are risk factors in the occurrence of various autoimmune diseases. It has been postulated that in view of the moderate functional impact of these variants, additional events are required to bypass the tolerance checkpoints. This model accounts for the relatively long time to onset of autoimmune disease in most patients. Our identification of a combined germline *TNFRSF6* mutation (with incomplete clinical penetrance) and a somatic event affecting the second *TNFRSF6* allele now demonstrates that a hit on each of the 2 alleles of the same gene can lead to lymphoproliferation and autoimmunity. On the basis of this observation, one can postulate that combinations of gene variants and somatic mutations in genes involved in cell death (such as Bim, Bid, or caspases) or in the regulation of cell proliferation (such as PD-1 or CTLA-4) could be involved in the emergence of autoimmune disease via the selection of self-reactive T and B lymphocytes. This hypothesis could be tested in models of autoimmunity in which self-reactive T and/or B cell clones can be identified. Somatic mutations occur relatively frequently (i.e., 10^{-6} per gene per cell division) (26). Consequently, in the context of the high-rate cell division seen in both lymphoid precursors and mature lymphocytes, a role for somatic mutations in triggering autoimmune disease onset should be considered.

In conclusion, our present findings validate the “2-hit” hypothesis in the setting of a nonmalignant disease and highlight the possible impact of somatic mutations on the onset of other autoimmune diseases in humans.

Methods

Study population. Seven patients presenting with typical ALPS manifestations (including autoimmune manifestations in 3 cases) and carrying germline heterozygous *TNFRSF6* mutations affecting the ECD of the protein were enrolled in this study (Table 1). Relatives of patients 1 to 5 (including 7 asymptomatic MPRs) as well as 3 patients carrying a germline heterozygous ICD *TNFRSF6* mutation (D269fsX279; G253D; K296fsX279) were also investigated as controls. The study protocol was approved by the local investigational review board (CPP ile de France II, Ministry of Research, Paris France). Informed consent was obtained from the patients or their families prior to study entry.

Immunofluorescence and cell sorting. Using flow cytometry, the percentage of DN T cells was defined as the percentage of TCR- $\alpha\beta$ -positive cells that lacked CD4 and CD8 expression. Thawed peripheral blood mononuclear cells were incubated with a cocktail of 2.5 μ l of anti-TCR PAN α/β PC5 antibody (IP26A; Immunotech; Beckman Coulter), 5 μ l of anti-CD4-APC antibody (SK3), 10 μ l of anti-CD8-V500 antibody (RPAT8), 10 μ l of anti-CD95-PE antibody (DX2), and 10 μ l of anti-HLA-DP-DQ-DR-FITC (TU39) (BD Biosciences) per 4 million cells. Acquisition and cell sorting were performed with an ARIA I or ARIA II cytometer (BD Biosciences), and data were analyzed using Flowjo software (TreeStar Inc.).

Plasma IL-10 and FAS-ligand and apoptosis assays. Plasma IL-10 and FAS-ligand levels and apoptosis were assayed as previously described (4).

Detection of *TNFRSF6* mutations. DNA was isolated by proteinase K digestion and phenol-chloroform extraction. Genomic DNA segments were amplified as previously described (11).



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DNA cloning. PCR products were cloned using TOPO TA cloning kit (Invitrogen) following the manufacturer's instructions. The sequences of at least 80 clones were then determined by capillary electrophoresis using Applied Biosystems DNA sequencing kits and a 3130xl Genetic Analyzer (Applied Biosystems).

Genotype analysis. All analyzed microsatellite markers flanked the *TNFRSF6* locus or genes located on the 10q chromosome. Primer sequences were retrieved from the Genome Database (<http://www.ncbi.nlm.nih.gov>). FISH experiments were performed with BAC RP11-399O19 (a probe spanning the *TNFRSF6* locus) as previously described (27). Analysis of copy number variations and LOH was performed using Whole-Genome Cytogenetics 2.7M DNA chips (Affymetrix). The results were visualized using the Chromosome Analysis Suite (version 1.0.1; Affymetrix).

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