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Heterogeneity Among Patients With Tumor Necrosis Factor Receptor–Associated Periodic Syndrome Phenotypes

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Objective. **To investigate the prevalence of tumor necrosis factor receptor–associated periodic syndrome (TRAPS) among outpatients presenting with recurrent fevers and clinical features consistent with TRAPS.**

Methods. **Mutational screening was performed in affected members of 18 families in which multiple members had symptoms compatible with TRAPS and in 176 consecutive subjects with sporadic (nonfamilial) "TRAPS-like" symptoms. Plasma concentrations of soluble tumor necrosis factor receptor superfamily 1A (sTNFRSF1A) were measured, and fluorescence-activated cell sorter analysis was used to measure TNFRSF1A shedding from monocytes.**

Results. **Eight novel and 3 previously reported *TNFRSF1A* missense mutations were identified, including an amino acid deletion (ΔD42) in a Northern Irish family and a C70S mutation in a Japanese family, both reported for the first time. Only 3 *TNFRSF1A* variants were found in patients with sporadic TRAPS (4 of 176 patients). Evidence for nonallelic heterogeneity in TRAPS-like conditions was found: 3 members of the "prototype familial Hibernian fever" family did not possess C33Y, present in 9 other affected members. Plasma sTNFRSF1A levels were low in TRAPS patients in whom renal amyloidosis had not developed, but also in mutation-negative symptomatic subjects in 4 families, and in 14 patients (8%) with sporadic TRAPS. Reduced shedding of TNFRSF1A from monocytes was demonstrated in vitro in patients with the T50M and T50K variants, but not in those with other variants.**

Conclusion. **The presence of TNFRSF1A shedding defects and low sTNFRSF1A levels in 3 families without**

a *TNFRSF1A* mutation indicates that the genetic basis among patients with "TRAPS-like" features is heterogeneous. *TNFRSF1A* mutations are not commonly associated with nonfamilial recurrent fevers of unknown etiology.

The tumor necrosis factor receptor–associated periodic syndrome (TRAPS; MIM no. 142680) is a dominantly inherited multisystem chronic inflammatory disorder that has a relapsing and remitting nature. The phenotype and clinical severity of TRAPS vary, but characteristic features include recurrent fevers, abdominal pain, and cutaneous and synovial inflammation (1,2), which typically last several days to weeks or longer. Other features include muscle tenderness, periorbital edema, and an increased incidence of inguinal hernia among men in some families. A proportion of patients develop systemic AA amyloidosis, which usually presents with nephropathy and is potentially life-threatening.

TRAPS was formerly known as familial Hibernian fever (FHF) and is associated with mutations in the gene on chromosome 12p13 that encodes tumor necrosis factor receptor superfamily 1A (TNFRSF1A) (2). Six different missense mutations affecting the extracellular domains of TNFRSF1A, 5 of which involved cysteine residues, were initially described in northern European families. At least 24 pathogenic *TNFRSF1A* mutations have now been identified (3), all of which are located in either the first or the second cysteine-rich N-terminal extracellular domains (CRD1 and CRD2) of *TNFRSF1A* (4–11), except for an F112I mutation in CRD3, found in affected members of a Finnish family (12). Most reported mutations involve cysteine residues, but variants that disrupt other residues do occur and may be associated with reduced penetrance (7,13).

Little epidemiologic data on TRAPS is available, but it is the most prevalent autosomal-dominant recurrent fever syndrome (ADRF) and the second most common inherited periodic fever overall, after familial Mediterranean fever (FMF; MIM no. 249100), which is a recessive disorder caused by mutations in the gene for pyrin/marenostrin (14,15). Most reported patients with TRAPS are European, many of Irish Scottish descent, but the disorder has also been reported in patients with diverse ethnic backgrounds, including those in which FMF most characteristically occurs, i.e., Ashkenazi and Sephardic Jews as well as the Maghrebian population (10), Israeli Arabs (6), Argentinian Arabs, Puerto Ricans (7), and Dutch Indonesians (9).

A single gene is responsible for 2 related autosomal-dominant periodic fevers, Muckle-Wells syndrome (MWS; MIM no. 191900) and familial cold urticaria (FCU; MIM no. 120100) (also called familial cold autoinflammatory syndrome [FCAS]); furthermore, different mutations have been reported in neonatal-onset multisystem inflammatory disease (also known as chronic infantile neurologic cutaneous and articular syndrome) (16). This protein has homology to pyrin/marenostrin, and is variously termed cryopyrin, NALP3, or PYPAF1 (17–19).

Impaired cleavage of the TNFRSF1A ectodomain upon cellular activation, with consequent reduction in the plasma concentration of soluble TNFRSF1A (sTNFRSF1A), has been proposed as a mechanism underlying the hyperinflammatory response in TRAPS (2), although this defect cannot always be demonstrated (4,7). Phorbol myristate acetate (PMA) stimulates metalloproteinase activity, which leads to cleavage of the TNF receptor; the T50M and C52F variants are associated with a marked reduction of PMA-induced TNFRSF1A shedding (2,7). Shedding of this TNF receptor from monocytes is impaired to differing extents among patients with the H22Y, C33Y, and P46L variants (7,20). In contrast, although cell surface expression of TNFRSF1A after PMA stimulation appears normal in patients with the less penetrant R92Q variant and in those with the c.193-14G>A splice mutation (7), the plasma concentration of sTNFRSF1A can nevertheless be abnormally low in these cases (2,7).

TRAPS was first described as a distinct genetic entity in 1999 (2). To further characterize this syndrome and syndromes with similar symptoms, we performed genetic, biochemical, and functional studies in a further 18 families with ADRF, as well as in 176 patients of diverse ethnic backgrounds who had features compatible with TRAPS ("sporadic," i.e., nonfamilial, cases).

PATIENTS AND METHODS

Study subjects. The study group comprised a total of 222 patients who were referred with longstanding histories of fevers and other symptoms consistent with a diagnosis of TRAPS, including 46 members from 18 apparently unrelated multiplex families and 176 unrelated patients with apparently sporadic cases. Clinical criteria included periodic attacks of fever lasting at least 1 week, abdominal pain, rashes, periorbital edema, the presence of an acute-phase response when symptomatic, and a poor response to colchicine (21). There were at least 2 patients with some features of TRAPS in each of the families, with affected members in at least 2 consecutive generations, consistent with autosomal-dominant transmission. Among the 18 unrelated multiplex families, 4 were from England (1 of Polish English ancestry), 2 each from Spain,

Table 1. *TNFRSF1A* mutation–positive TRAPS families and patients with sporadic TRAPS*

Ethnicity, family member	Mutation (nucleotide change)	Exon/intron	Soluble TNFRSF1A level, pg/ml (normal 746–1,966)	TNFRSF1A shedding, %, measured by FACS (normal ≥25%)	TNFRSF1A shedding before/after PMA stimulation, %
Spanish, sporadic	G36E (194 G→A)	3	1,761, 3,717†	–	–
Polish English					
1-I-1‡	T37I (197 C→T)	3	1,528	54	67/13
1-II-1	T37I (197 C→T)	3	680	–	–
Northern Irish					
2-II-1‡	ΔD42 (del211–213)	3	2,139	27	61/44
2-III-1	ΔD42 (del211–213)	3	390	–	–
German					
3-III-1	T50K (236 C→A)	3	672	15	91/76
3-III-2	T50K (236 C→A)	3	824†	–	–
3-II-1	T50K (236 C→A)	3	580	–	–
3-II-2	Normal	–	693	–	–
3-I-1	Normal	–	1,367	–	–
English					
4-I-1	T50M (236 C→T)	3	475	8	98/90
4-II-1	T50M (236 C→T)	3	677	–	–
4-II-2	T50M (236 C→T)	3	952†	–	–
Spanish					
5-II-8	C52R (241 T→C)	3	487	–	–
5-II-9	C52R (241 T→C)	3	320	46	75/29
5-III-2	C52R (241 T→C)	3	714	–	–
5-III-5§	C52R (241 T→C)	3	8,820	–	–
5-III-6	C52R (241 T→C)	3	477¶	–	–
5-III-12	C52R (241 T→C)	3	323, 746, 1,278†	52	75/23
5-III-16§	C52R (241 T→C)	3	1,888	–	–
5-IV-1	C52R (241 T→C)	3	539	–	–
Scottish					
6-I-1	F60L (264 C→G)	3	–	–	–
6-II-1	F60L (264 C→G)	3	599	–	–
Dutch					
7-I-1	N65I (281 A→T)	3	792†	28	90/62
7-II-1	N65I (281 A→T)	3	660	37	95/58
Japanese					
8-I-1	C70S (295 T→A)	3	–	–	–
8-II-1	C70S (295 T→A)	3	461, 1,367, 3,280†	–	–
Scottish Australian, 9-II-1	C88R (349 T→C)	4	–	–	–
English, 10-I-1	R92Q (362 G→A)	4	599	31	87/56
English, sporadic	R92Q (362 G→A)	4	2,047, 872, 2,006, 670	–	–
English, sporadic	Splice junction (c.193-14G>A)	Intron 3	1,062	–	–

* TRAPS = tumor necrosis factor receptor–associated periodic syndrome; TNFRSF1A = tumor necrosis factor receptor superfamily 1A; FACS = fluorescence-activated cell sorter; PMA = phorbol myristate acetate.
† During episode.
‡ Renal transplant amyloidosis.
§ Receiving dialysis.
¶ Asymptomatic.

Scotland, Australia, and the US, and 1 each from Germany, The Netherlands, Ireland, Northern Ireland, New Zealand, and Japan (Tables 1 and 2). A family history of disease was, by definition, absent among the 176 patients who were investigated for possible sporadic TRAPS, most of whom were English.

The study protocol was approved by the East London and City Health Authority Research Ethics Committee. Informed consent was obtained from all subjects. Venous blood was drawn from each study subject, plasma was separated, and genomic DNA was extracted using PureGene kits (Gentra Systems, Minneapolis, MN). Each patient was screened for *TNFRSF1A* mutations, and both sTNFRSF1A and sTNFRSF1B levels were measured.

Genetic analysis of TNFRSF1A. *TNFRSF1A mutation detection.* Two approaches were used for the mutational screening. Screening of exons 2–5 was performed on all 222 patients, by a combination of genomic DNA sequence analysis and denaturing high-performance liquid chromatography (DHPLC). When an altered DHPLC pattern was found, the

Table 2. *TNFRSF1A* mutation–negative TRAPS families*

Ethnicity, family member	Soluble TNFRSF1A level, pg/ml (normal 746–1,966)	Soluble TNFRSF1B level, pg/ml (normal 1,003–3,170)	TNFRSF1A shedding, %, measured by FACS (normal ≥25%)	TNFRSF1A shedding before/after PMA stimulation, %
Irish Scottish				
11-III-2	132	2,897	–	–
11-III-3	132	2,874	–	–
11-III-4	920	–	–	–
American				
12-I-1	1,209	1,943	18	78/56
12-II-1	685	1,068	16	88/72
American				
13-I-1	533	1,150	16	79/63
13-II-1	653	1,635	2	69/67
Irish				
14-III-1†	731	1,235	–	–
14-III-2	781	1,839	–	–
14-III-3†	1,140	2,162	–	–
14-III-4	650, 806	2,088, 1,433	40	62/22
14-III-5†	826	1,731	–	–
14-IV-1‡	650	1,850	–	–
14-IV-3	727	2,828	5	67/62
Spanish				
15-II-1†	944	2,160	–	–
15-II-2	856	2,219	–	–
15-II-3	809	1,658	–	–
15-II-4	1,429	2,771	–	–
15-II-5†	798	1,560	–	–
15-III-1†	815	1,777	–	–
New Zealand				
16-II-1	972	1,563	–	–
16-I-1	1,217	2,122	–	–
Australian				
17-I-1	1,398	–	–	–
17-II-1	1,095	–	–	–
English				
18-I-1§	1,246	2,003	–	–
18-II-1	1,058	1,683	–	–
18-III-1	721	1,227	–	–
18-III-2	1,410	2,119	–	–

* See Table 1 for definitions.
† Unaffected family member.
‡ Possibly affected.
§ R92Q mutation.

corresponding exons were reamplified prior to sequencing (2). Because the elution profiles and sequences of exons 2–5 were normal in the probands from 8 of the 18 families, the remaining *TNFRSF1A* coding region (i.e., exons 1 and 6–10) was then sequenced in all of these individuals. These exons were also sequenced in 7 of the patients with sporadic TRAPS. The primers used for reamplification and sequencing were as described (2), except for exon 10 (Table 3).

Analysis of TNFRSF1A promoter region. An additional 800 bp of the *TNFRSF1A* promoter was also sequenced in the probands of the 8 families that were negative for the *TNFRSF1A* coding region mutation, in the 7 sporadic cases, and in 3 of the 12 affected members from the original FHF family in whom *TNFRSF1A* mutations (specifically C33Y, which was present in the other 9 affected members) had previously been excluded (2). This 800-bp region was first amplified using the external primers TNFR1Pro/1 (forward) and TNFR1Pro/2 (reverse). The polymerase chain reaction (PCR) product was then sequenced bidirectionally using the external primers, in addition to 4 overlapping internal primers (Pro/4–7) (Table 3).

PCR. All reactions were performed under the following PCR cycling conditions: initial denaturation at 94°C for 15 minutes, 35 denaturation cycles at 94°C for 45 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute, and extension at 72°C for 10 minutes.

DNA sequencing. The PCR products were purified, sequenced by fluorescent dye primer chemistry (Amersham, Little Chalfont, UK), and run on an ABI 3700 automated sequencer. Sequence data were analyzed with Sequencher 3.0 (Gene Codes, Ann Arbor, MI).

Cloning. Since direct sequencing of the ΔD42 mutation produced an illegible electopherogram with superimposed tracefile around residues 40–44, amplified product of exons

Table 3. Primers used for analysis of *TNFRSF1A, TNFRSF1B,* and *NALP3*/*CIAS1*/*PYPAF1* loci

Region	Primer(s)*	Size, bp
TNFRSF1A exon 10	5′-TGGGGTTGCCGCCCGAGGCT-3′	396
	5′-CATCTCGCAGGACGGTCCTTAG-3′	
TNFRSF1A Pro/1	5′-CTAGGAGGCTAGTGAAGAACTCTG-3′	942
TNFRSF1A Pro/2	5′-GTGGCTGAGGTTAGGACCTG-3′	
TNFRSF1A Pro/4	5′-CTGAATTGGAACCCAGAGAAT-3′	
TNFRSF1A Pro/5	5′-ACTCCCAACCAAACACCAAG-3′	
TNFRSF1A Pro/6	5′-CTCCTCCAGCTCTTCCTGTC-3′	
TNFRSF1A Pro/7	5′-CAGTGATCTTGAACCCCAAAG-3′	
T50K	5′-CAGGCCCGGGGCAGGGTA-3′	120
	5′-CCTGTGCACACTCACCCTTTC-3′	
F60L	5′-GCCCCATTCACAGGAACCTACTTG-3′	111
	5′-TCTGAGGTGGTTTTCTGAAGCGGTTA-3′	
ΔD42	5′-CCTCTCTTGATGGTGTCTCC-3′	575
	5′-CTGACTCTCCTGCCTGTGC-3′	
TNFRSF1B exon 2	5′-GATGGCAGTCTTCCCTTCTT-3′	180
	5′-CACACGCTCCTCCAGGCAT-3′	
TNFRSF1B exon 3	5′-AGAGGCTCGCCCAGCTGAGA-3′	215
	5′-TGGAGGCAGGGGTGTAAGG-3′	
TNFRSF1B exon 4	5′-GTGACCGTTTGCGCCCTCT-3′	244
	5′-GCAAGGAGTTCTACAAAGGAG-3′	
TNFRSF1B exon 5	5′-GAGTGGTTGACAAGTTCGGA-3′	162
	5′-CTGCTCCTCCAGAACCAAAG-3′	
CIAS1 exon 3a	5′-GTTACCACTCGCTTCCGATG-3′	901
	5′-CCTCGTTCTCCTGAATCAGAC-3′	
CIAS1 exon 3b	5′-CATGTGGAGATCCTGGGTTT-3′	649
	5′-GGCCAAAGAGGAAACGTACA-3′	
CIAS1 exon 3c	5′-TTCCAGGGAGTCGTTTGAAG-3′	597
	5′-GAGATGAGAGGAGGCAGGTG-3′	

* Where only 1 primer is shown for a region, it was used for internal sequencing, which is either forward or reverse. For polymerase chain reaction amplification, 2 primers are shown; the first is the forward primer and the second the reverse primer. Underlined nucleotides were modified to create an enzyme restriction site.

2–3 of *TNFRSF1A* was directly subcloned into the pGL3 Easy vector (Promega, Madison, WI) and transfected into TOP 10 bacteria (Invitrogen, Carlsbad, CA) to circumvent this problem. Plasmid DNA samples isolated from the subcloning were prepared with the UltraClean Mini Prep Kit (Mo Bio, Solana Beach, CA) and sequenced.

Restriction endonuclease assays for TNFRSF1A mutations. Upon detection of a *TNFRSF1A* mutation in the proband of a specific family, other available family members were screened for the same mutation. DNA sequencing was generally used for this purpose. Specific restriction fragment length polymorphisms (RFLPs) were developed for mutations in the larger families (i.e., T50K, F60L, and ΔD42). The sequences of the RFLP primers are shown in Table 3. The T50K mutation abolishes an *Rsa* I site, C52R creates a *Pst* I site, F60L abolishes an *Mse* I site, and the ΔD42 deletion creates an *Mfe* I restriction site.

Study of other candidate genes in ADRF families without *TNFRSF1A* mutations. *TNFRSF1B mutation detection. TNFRSF1B* on chromosome 1p36 was selected as a candidate gene for mutational screening because of the significant structural and functional homology to *TNFRSF1A*. Sequencing of exons 2–5 (which encode the extracellular ligand-binding domain, structurally homologous to TNFRSF1A) was performed in the probands of these families, using the primers shown in Table 3.

NALP3/*CIAS1*/*PYPAF1 mutation detection*. Exon 3 of the *NALP3*/*CIAS1*/*PYPAF1* gene, encoding the NACHT domain and flanking regions (19), where all mutations identified so far have been found, was screened in a total of 18 patients. These included probands of the 8 families who were negative for *TNFRSF1A* mutations and 10 patients with sporadic TRAPS with at least 1 of the clinical features of MWS/FCU/FCAS (i.e., amyloidosis, cold-induced urticarial-appearing rash, and hearing loss). To ensure coverage of the entire 1.5-kb region, 3 pairs of overlapping primers, *CIAS1* exons 3a–3c (Table 3), were used for PCR of genomic DNA.

Microsatellite and single-nucleotide polymorphism (SNP) analysis of candidate loci. Informative microsatellite markers flanking the 4 known periodic fever loci, as well as intragenic SNPs, were used to genotype all of the available members of the *TNFRSF1A* mutation–negative families. This was done to test for linkage to the *TNFRSF1A* locus and other known candidate gene loci (*MEFV* and *MVK*) by haplotype sharing, as previously described (22).

TNFRSF1B locus (chromosome1q36). A biallelic variable-number tandem repeat (VNTR) promoter polymorphism (23) and an informative microsatellite marker from intron 4 were used (24). The VNTR promoter alleles result from the presence of either 1 (allele 1) or 2 (allele 2) repeats of a 15-bp sequence, 5′-GCCGGGCAGGTGGAG-3′; the

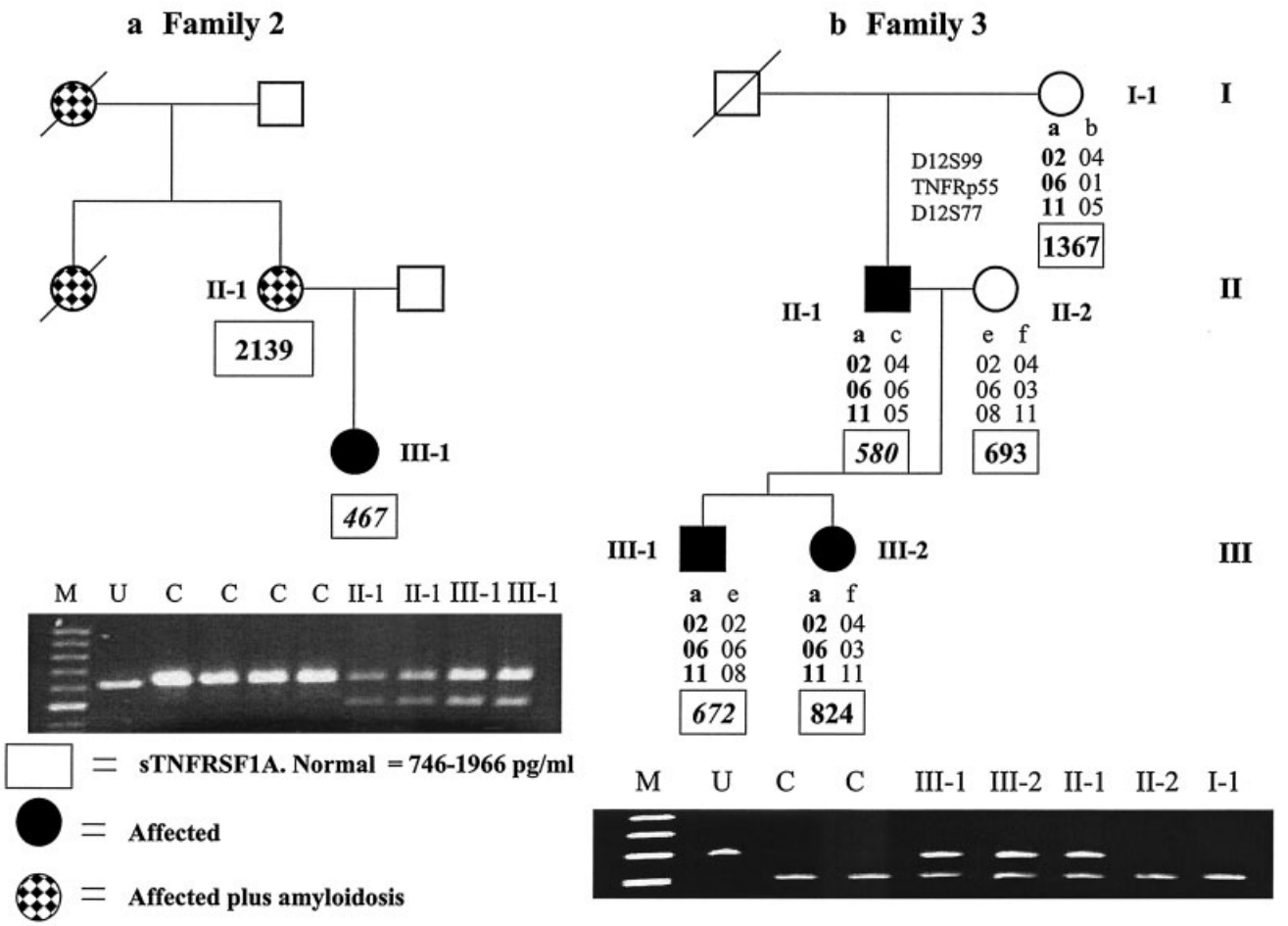

Figure 1. a, Family 2 (Northern Irish). Pedigree and findings of restriction fragment length polymorphism (RFLP) assay for the ΔD42 mutation, which creates an *Mfe* I restriction site, in the 2 affected family members. **b,** Family 3 (German). Pedigree and findings of RFLP assay, showing that the T50K mutation, carried on haplotype a, has arisen de novo, since it is not present in family member I-1. T50K abolishes an *Rsa* I site. Levels of soluble tumor necrosis factor receptor superfamily 1A (sTNFRSF1A; pg/ml) are shown in boxes, with low levels indicated by italics. M = marker; U = uncut; C = cut.

allelic frequencies observed in a Caucasian population were 0.3 (allele 1) and 0.7 (allele 2).

Intragenic SNPs. Two intragenic SNPs (exon 1 G/A and intron 7 G/A) from the *TNFRSF1A* locus were also analyzed (24,25). Allele frequencies were as reported (7,26).

Measurement of TNF receptors, C-reactive protein, and serum amyloid A levels. The concentrations of sTNFRSF1A and sTNFRSF1B in plasma stored at −80°C were measured by enzyme-linked immunosorbent assay, as described (2). Plasma C-reactive protein concentration was determined using an automated microparticle-enhanced latex turbidimetric immunoassay (COBAS MIRA; Roche Diagnostics, Mannheim, Germany) (27), and serum amyloid A protein was measured by latex nephelometry (BNII autoanalyzer; Dade Behring, Marburg, Germany) (28).

Determination of TNFRSF1A expression and shedding by fluorescence-activated cell sorter (FACS) analysis. The expression of TNFRSF1A was studied by FACS analysis on the probands and affected members of 10 of the 18 families (Tables 1 and 2). Mononuclear cells were isolated from peripheral blood using Lymphoprep (Nycomed Pharma, Oslo, Norway). Parallel cultures of 10^6 cells were maintained in RPMI 1640/10% fetal calf serum at 37°C/5% CO_2. PMA (Sigma, St. Louis, MO) was added to one culture at a concentration of 10 ng/ml for 30 minutes. A total of 10 μl of fluorescein isothiocyanate (FITC)–labeled TNFRSF1A monoclonal antibody (R&D Systems, Abingdon, UK) was added to 25-μl cell aliquots in triplicate. An isotype antibody, $\gamma 1^{FITC}$ (Becton Dickinson Immunocytometry Systems, San Jose, CA), was used as a control. To identify the monocyte population, double staining was carried out by simultaneous incubation with a phycoerythrin-labeled antibody recognizing the monocyte marker CD14 (1:10 dilution; Becton Dickinson Immunocytometry Systems). After a 45-minute incubation on ice, cells were washed and acquired into a FACSort flow cytometer (Becton Dickinson Immunocytometry Systems). To quantify

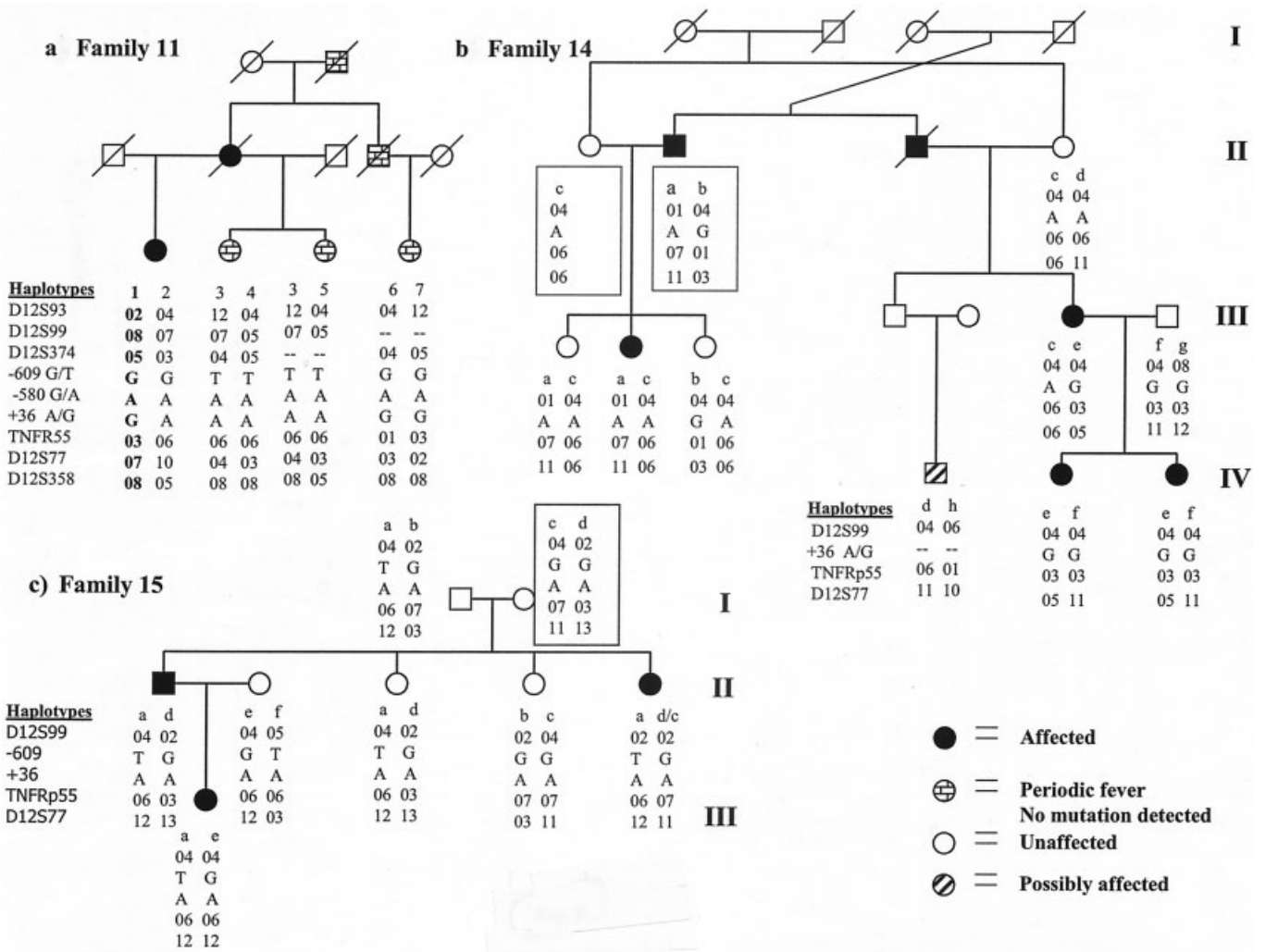

Figure 2. a, Genotyping for 6 microsatellite markers flanking the tumor necrosis factor receptor superfamily 1A (TNFRSF1A) locus, plus 3 intragenic markers, in family 11 (Irish Scottish). A C33Y mutation was found in the proband (11-III-1), as well as 8 other family members (results not shown), but not in 3 other symptomatic individuals. The C33Y carrier haplotype 1 in the proband, inferred from segregation analysis performed in the rest of the pedigree, was present in all of the other 8 affected members (results not shown). Two of the symptomatic individuals (11-III-2 and 11-III-3) are half-siblings of the proband, and they have not inherited this haplotype from the affected mother. Also, subject 11-III-4 does not share any haplotype with either 11-III-1, 11-III-2, or 11-III-3. Only subject 11-III-1 in family 11 has a TNFRSF1A mutation (indicated by a boldfaced haplotype). Alleles TA and GA in family 11 are biallelic single-nucleotide polymorphism markers. **b,** Pedigree of family 14 (Irish). There is a single recombinant in an affected cousin (14-III-6). **c,** Pedigree of family 15 (Spanish). There is a single recombinant in an asymptomatic sibling (15-II-3). Rectangles refer to inferred haplotypes; the second haplotype of subject 14-II-1 is unknown.

the FITC-positive cells, a marker was set in the fluorescence-1 histogram, using the isotype antibody as a negative control.

RESULTS

Among the 18 families studied, we found 7 novel *TNFRSF1A* mutations, T37I, ΔD42, T50K, C52R, F60L, N65I, and C70S, in the probands of families 1 (Polish English), 2 (Northern Irish), 3 (German), 5 (Spanish), 6 (Scottish), 7 (Dutch), and 8 (Japanese), respectively (Table 1). The ΔD42 mutation in family 2 is the first amino acid deletion identified in TRAPS, and was associated with AA amyloidosis in 3 individuals (Figure 1a). The C70S mutation represents the first report of TRAPS in a patient from the Far East (family 8 from Japan). In the German family with the T50K mutation (family 3), the disease was found to segregate with a 4-marker haplotype, including the TNFRSF1A locus (Figure 1b). This mutation appears to have arisen de novo in the proband (3-II-1), since it was not present in his asymptomatic mother (3-I-1), from whom the haplo-

type was inherited. It was, however, found in both of his affected children, including patient 3-III-2, who had symptoms involving the central nervous system. The T37I mutation was confirmed by direct DNA sequencing in the affected offspring (1-II-1) of the proband in the Polish English family (family 1), and RFLP assays were used to screen for mutations in all available members of families 3 and 6. These assays confirmed the presence of 2 novel mutations (T50K and F60L) in the affected members of both families (Figure 1b and results not shown).

More than 100 northern European and 20 Japanese control chromosomes were also screened by RFLP and DHPLC WAVE analysis for all of these mutations, including 10 samples from healthy Polish controls. No *TNFRSF1A* mutations were found in any of these controls.

Two previously reported *TNFRSF1A* mutations (T50M and C88R) were found in English (T50M; family 4) and Scottish Australian (C88R; family 9) families (Table 1). No *TNFRSF1A* mutations were found in the remaining 8 families (apart from R92Q in a single patient from a total of 4 affected members in family 16), despite sequencing of all 10 exons and 800 bp of the promoter region (Table 2). Family 16 was therefore considered *TNFRSF1A* mutation negative because the R92Q variant did not segregate with disease, and no other variants were identified.

Among the 176 patients with sporadic TRAPS, a de novo mutation, G36E, which was not present in the parents, was found in a Spanish patient (Table 1). The R92Q or c.193-14G>A variant was found in 2 further patients from England with sporadic TRAPS (each in 1 patient). Our mutation survey of the remaining 172 patients with TRAPS revealed no mutations in exons 2–5. The complete *TNFRSF1A* coding and promoter regions, further analyzed in 7 patients with sporadic TRAPS, were also mutation negative.

***TNFRSF1A* promoter.** In addition to the patients listed above, the 5′ promoter region of the *TNFRSF1A* gene was sequenced in 26 Caucasian controls (52 chromosomes). Two previously reported promoter SNPs, at positions −609G/T and −580A/G relative to the transcription start site, were identified (21): the −609 SNP was found in both cases and controls, and there were no differences in frequencies between the 2 groups. However the −580G SNP was only found in 1 patient from Africa, and is present in healthy African populations (21).

Haplotype analysis of other candidate loci in the *TNFRSF1A* mutation–negative families. We were unable to identify any of the other known periodic fever loci or the *TNFRSF1B* candidate locus in any of the 8

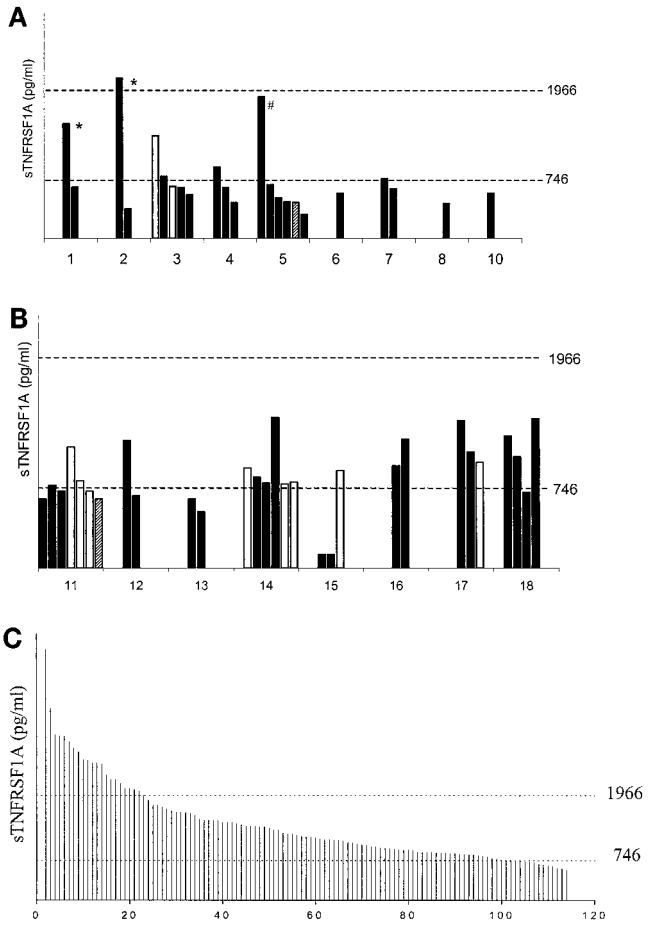

Figure 3. Soluble tumor necrosis factor receptor superfamily 1A (sTNFRSF1A) levels in **A,** patients and families with tumor necrosis factor receptor–associated periodic syndrome (TRAPS), **B,** *TNFRSF1A* mutation–negative patients and families, and **C,** patients with sporadic TRAPS. Solid columns represent patients; open columns represent asymptomatic family members; shaded columns indicate probably affected family members. Upper and lower limits of normal are shown by the horizontal broken lines. * denotes patients with renal transplant; # denotes a patient receiving dialysis.

TNFRSF1A mutation–negative families. Genotyping was performed on all available members of families 11, 14, and 15 (Figure 2). Each of these 3 families had at least 3 affected members; family 14 was composed of 13 living members spanning 3 generations, of which 5 members were affected. Recombinants at each candidate locus were found in all of these families.

Family 11 (Irish Scottish). Both microsatellite and SNP genotyping were carried out in family 11 in order to compare extended haplotypes in the proband with C33Y (family member 11-III-1) and the 3 affected relatives (11-III-2, 11-III-3, and 11-III-4) (Figure 2), who were negative for this mutation. The 2 *TNFRSF1A* mutation–

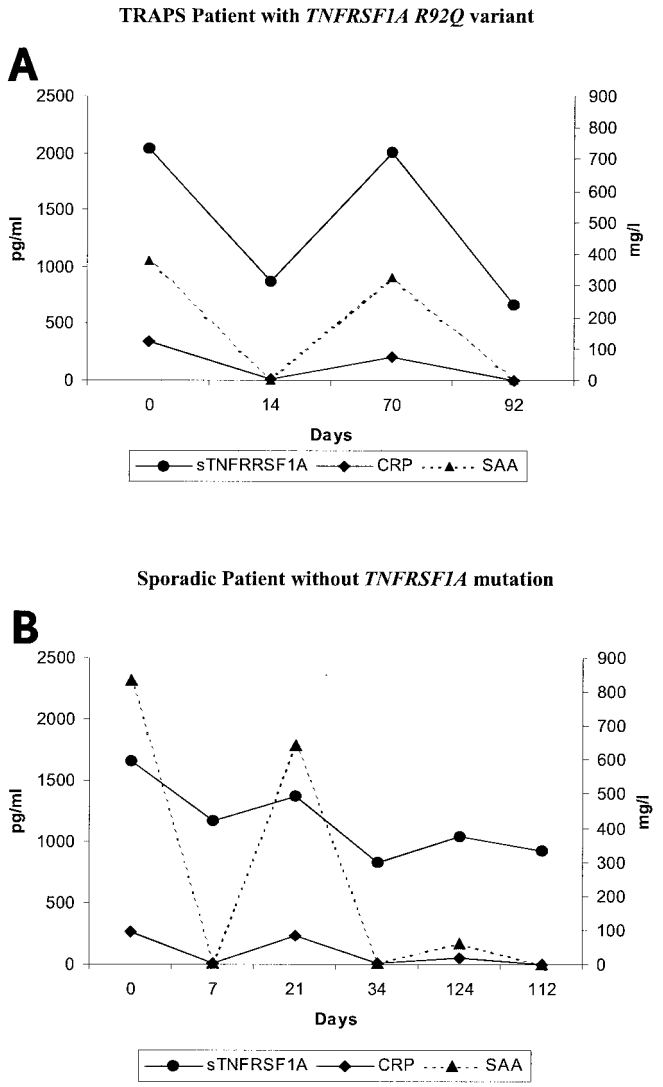

Figure 4. Serial measurements of sTNFRSF1A (in pg/ml; scale on left), C-reactive protein (CRP) (in mg/liter; scale on right), and serum amyloid protein (SAA) (in mg/liter; scale on right) in **A,** a TRAPS patient with the R92Q variant and **B,** a *TNFRSF1A* mutation–negative patient, showing that all levels tended to rise and fall simultaneously in these patients. See Figure 3 for other definitions.

negative siblings (11-III-2 and 11-III-3) inherited different haplotypes at this locus, as did their affected cousin (11-III-4). Nevertheless, the clinical presentation was markedly similar in all affected individuals in the family, regardless of the presence or absence of mutations.

Family 14 (Irish). In family 14 the intragenic marker (TNFRp55) was fully informative, and 1 recombination was observed in an affected cousin (14-III-6) (Figure 2), suggesting that *TNFRSF1A* is not the susceptibility gene. Although cold intolerance and urticaria were among the disease features in this family, no mutations were found in the NACHT domain of *NALP3*/*CIAS1*/*PYPAF1*; the *MEFV* and *MVK* loci were also excluded.

Family 15 (Spanish/Sephardic Jewish). All 3 affected members of family 15 shared a haplotype at the *TNFRSF1A* locus, but this was also present in an unaffected parent and sibling (Figure 2). The possibility of a low-penetrance *TNFRSF1A* mutation was not supported by the results of sequencing of the complete coding region and 800 bp of the promoter region of *TNFRSF1A* in the proband. Furthermore, there were recombinants at all other known periodic fever loci, as well as *TNFRSF1B* (data not shown). FMF was also excluded by direct sequencing.

Exclusion of mutations in both TNFRSF1B and NALP3/CIAS1/PYPAF1 genes. No mutations in the *TNFRSF1B* or *NALP3*/*CIAS1*/*PYPAF1* genes were identified in the 8 *TNFRSF1A* mutation–negative families.

Soluble TNFRSF1A levels in plasma. Soluble TNFRSF1A levels (normal range 746–1,966 pg/ml) were reduced in most affected members of all of the TRAPS families, regardless of the mutation (Table 1 and Figure 3A). The exceptions were 4 patients with renal insufficiency and 5 others, 2 of whom had very active inflammatory disease. Elevation of the sTNFRSF1A concentration in association with active inflammation was shown to produce pseudonormalization of the value in patient 5-III-12 with the C52R mutation and the patient

Table 4. Mean ± SD soluble TNFRSF1A and TNFRSF1B levels in TRAPS families, *TNFRSF1A* mutation–negative families, patients with sporadic TRAPS, and controls*

Subjects	No. of subjects	Soluble TNFRSF1A, pg/ml (normal 746–1,966)	Soluble TNFRSF1B, pg/ml (normal 1,003–3,170)
TRAPS families	19†	637 ± 242‡	2,181 ± 560
Mutation-negative families	21†	862 ± 349	1,894 ± 527
Sporadic TRAPS	114	1,417 ± 735§	2,530 ± 1,468
Controls	24	962 ± 228	–

* See Table 1 for definitions.
† All subjects were affected family members.
‡ $P < 0.0001$ versus controls.
§ $P < 0.0033$ versus controls.

with sporadic TRAPS and the R92Q mutation (Table 1 and Figure 4). The relationship more generally between sTNFRSF1A concentration and the acute-phase plasma protein response, determined by measurement of C-reactive protein, is shown in Figure 4.

The sTNFRSF1A concentration was also low in an asymptomatic carrier of the C52R mutation, and in 4 of 8 families in whom a *TNFRSF1A* mutation was not found (Table 2 and Figure 3B). Among 114 patients with sporadic TRAPS from whom sera were available for study, values were around the lower limit of the normal range in 34 (30%) and below this in 8 (Figure 3C). The values in the remaining 72 patients with sporadic TRAPS were either within (50 patients) or above (22 patients) the normal range. Soluble TNFRSF1A levels were significantly lower in TRAPS patients with mutations (mean ± SD 637 ± 242 pg/ml) (Table 4) than in normal control subjects (962 ± 228 pg/ml; $P < 0.0001$ by Student's 2-tailed *t*-test) (Table 4). Although the mean value among affected members of mutation-negative families (862 ± 349 pg/ml) was within the normal range, there was marked interfamilial variation, and very low levels were observed in the symptomatic members of families 11, 12, 13, 15, and 18. Repeated measurements revealed low or borderline sTNFRSF1A levels in members of some other families (Figure 3B).

Plasma sTNFRSF1A levels varied substantially among the patients with sporadic TRAPS and were elevated in 19% (22 of 118) (Figure 3C). The mean values in this group were significantly higher than in controls ($P < 0.0033$) (Table 4), a finding that would be expected in patients with active inflammatory diseases. Levels of sTNFRSF1B were similar among patients from families with TRAPS, families without mutations, and patients with sporadic TRAPS.

Determination of TNFRSF1A expression and shedding by FACS analysis. TNFRSF1A was expressed under basal conditions in most cells of the monocyte-enriched population, at an intensity >10-fold greater than in the isotype control. Preparations from 4 healthy controls showed at least 35–40% TNFRSF1A shedding following PMA stimulation (Figure 5). A value of <25% was considered to indicate impairment of the TNFRSF1A shedding mechanism, and this was demonstrated in association with the T50K variant and, as previously described (7), the T50M variant. In contrast, receptor shedding comparable with that found in healthy control preparations was demonstrated in the probands of the families with the T37I mutation (Figure 5), the ΔD42, C52R, and N65I mutations, and in a patient with sporadic TRAPS with the R92Q substitution.

A moderate-to-severe TNFRSF1A shedding deficiency associated with low sTNFRSF1A levels was present in all affected members of 2 mutation-negative families (families 12 and 13) from the US (Figure 5), and TNFRSF1A shedding was also impaired in the proband of family 14. However, defective receptor shedding was not seen in the affected mother (14-III-4), who also had low sTNFRSF1A levels.

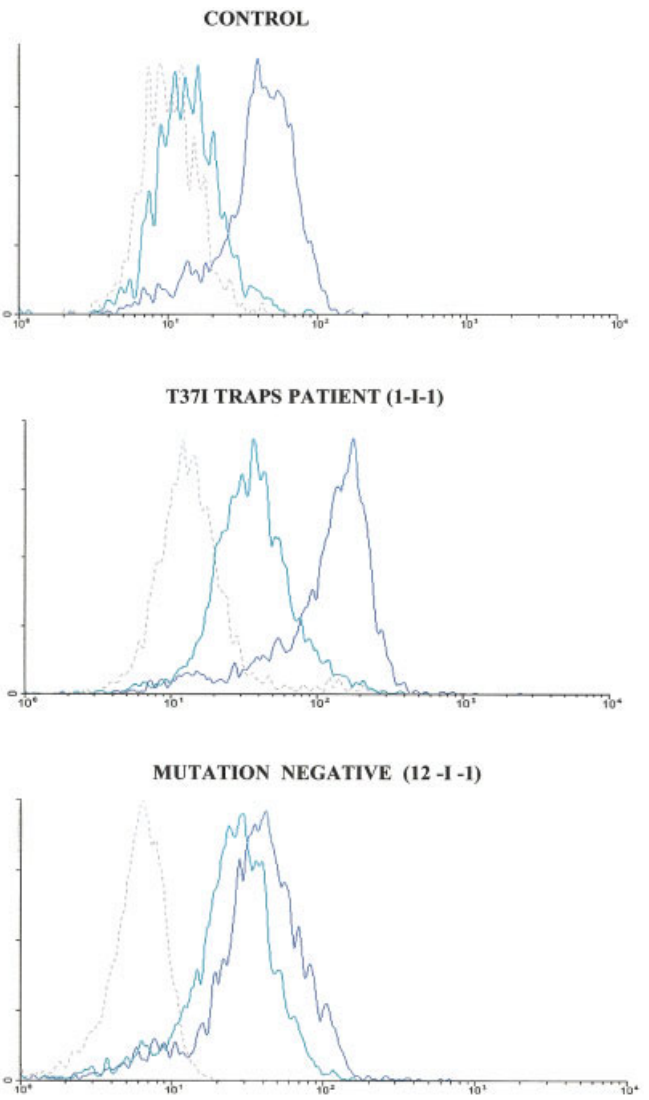

Figure 5. Examples of TNFRSF1A clearance from monocytes in TRAPS and *TNFRSF1A* mutation–negative patients. Fluorescence histograms are shown for a control subject and for the patient with the T37I mutation, as well as a mutation-negative affected parent. Monocytes were analyzed for TNFRSF1A expression before (dark blue lines) or after (light blue lines) phorbol myristate acetate activation. See Figure 3 for definitions.

Founder effect: intragenic haplotype sharing in T50M and C88R carriers. The T50M mutation, already reported in Irish and French Canadian families (2), was present in family 4 (English), and the C88R mutation

Table 5. Haplotypes constructed from flanking *TNFRSF1A* microsatellite markers and intragenic single-nucleotide polymorphisms (SNPs) in affected members of 3 families with the T50M mutation and 2 families with the C88R mutation

	Disease haplotype				
Family*	D12S99	Exon 1 SNP	p55	Intron 7 SNP	D12S77 microsatellite
T50M mutation					
English (3 patients)	01	G	03	A	10
French Canadian (2 patients)	03	G	03	A	10
Irish (6 patients)	03	G	03	A	08
C88R mutation					
Scottish Australian (3 patients)	05	G	01	G	03
Scottish Australian (13 patients)	05	G	01	G	03

* The English family with the T50M mutation is family 4 in the present report, and the first Scottish Australian family with the C88R mutation is family 9 in the present report; other families are from previously studied series (2,7).

was found in family 9 (Scottish Australian), plus a second Scottish Australian family from our initial series (2). This raised the question as to whether these mutations were due to genetic founder effects. Genotyping included the 2 intragenic *TNFRSF1A* SNPs, an intragenic microsatellite (p55), and 2 flanking microsatellites (Table 5).

A distinct common 5-marker haplotype, showing linkage disequilibrium over at least 2 cM, cosegregated with the C88R mutation (Table 5). However, in all mutation carriers in the 3 T50M families, the flanking microsatellites were divergent from a common haplotype, with convergence only at the intragenic markers, where the haplotypes were identical. A common allele (144 bp) of the TNFRp55 marker was observed in all T50M patients, and combined with 2 intragenic markers, produced a G-03-A haplotype in all 3 families (Table 5).

DISCUSSION

Our findings indicate that the genetic etiology and pathogenesis of inherited inflammatory disease among patients and families in whom there are clinical and laboratory features consistent with TRAPS are heterogeneous, in terms of both the presence or absence of mutations in the *TNFRSF1A* gene and defects in shedding of TNFRSF1A from cell surfaces. In half the ADRF families investigated no mutation in the *TNFRSF1A* gene was found to be responsible for the TRAPS-like disease, and the relationship between defects in sTNFRSF1A shedding and plasma concentration of this receptor was inconsistent. Only 2 variants, T50K and T50M, were associated with a marked shedding defect, and the presence or absence of this defect had no bearing on the severity of clinical symptoms.

It is notable that only 2 of the newly identified mutations involved a cysteine residue, as hitherto these have been associated with TRAPS in the majority of cases. Based on the crystal structure of TNFRSF1A proposed by Banner et al (29), both the F60 and T37 residues are crucial for proper domain-2 folding; F60 is a structurally conserved residue between the second and third extracellular domains, and T37 is also a conserved residue between domains 1 and 2 and resides in the beta-turn position in loop 2 of domain 1. Residue 42, deleted in one of these families, is located adjacent to a cysteine residue at position 43, and this deletion would be predicted to significantly inhibit formation of the disulfide bond with the cysteine residue at position 30. Although it has been suggested that mutations that disrupt cysteine residues confer an increased risk for development of AA amyloidosis (7), this complication evidently can occur in patients who have both noncysteine mutations and normal TNFRSF1A shedding. Duration and severity of inflammatory disease are risk factors for susceptibility to AA amyloidosis, but risk is probably influenced by other genetic and possibly environmental factors, as highlighted by the occurrence of amyloidosis in half of the members of the Northern Irish family with the ΔD42 variant, which was not associated with particularly severe inflammation.

We also screened 176 patients with possible sporadic TRAPS, the vast majority of whom were found to be negative for the *TNFRSF1A* mutation. Findings in these patients and some of the families confirm that the

TRAPS clinical phenotype has a heterogeneous etiology. Indeed it is remarkable that 3 of the 12 affected members of the prototype FHF family (2) did not actually possess the C33Y variant that segregated with an apparently identical disease phenotype in the other 9 affected members.

A moderate-to-severe TNFRSF1A shedding deficiency was present in some affected members of the 3 mutation-negative families in whom this was studied, and several members of these families had low sTNFRSF1A levels. Among the patients with sporadic TRAPS, sTNFRSF1A levels were lower than normal in 14, including the 1 patient with R92Q. These observations suggest that sTNFRSF1A levels may be influenced by other gene(s)/pathways, and we speculate that some individuals who have constitutionally low sTNFRSF1A levels might, in association with other environmental or genetic influences, be susceptible to developing clinical disease that is indistinguishable from TRAPS. It is relevant that sTNFRSF1A levels are normal in patients with MWS/FCU/FCAS or hyperimmunoglobulinemia D with periodic fever syndrome (Aganna E, et al: unpublished data) as well as those with FMF (30), and that we also found normal rates of TNFRSF1A cleavage in a selected patient from each of these disease groups.

We have thus provided some evidence for other, as-yet-unknown, susceptibility genes in *TNFRSF1A* mutation–negative families. Because there were 2 patients with sporadic TRAPS with the R92Q or c.193-14G>A splice mutations, both of which are low-penetrance (7), it might be surmised that these mutations would be more common among asymptomatic carriers and healthy controls, as well as in the sporadic cases. The increased frequency of R92Q among TRAPS patients (present in 1.4% of this group and absent in our controls), as well as the low sTNFRSF1A levels, suggest that it is indeed a low-penetrance mutation rather than a benign polymorphism. There is also accumulating evidence that the presence of the R92Q and c.193-14G>A TNFRSF1A variants in individuals who do not have features of TRAPS may augment the intensity of the nonspecific inflammatory response in other disease processes, analogous to the situation among patients who have the pyrin variant E148Q (7).

The phenotype of the Spanish/Sephardic Jewish family with ADRF (family 15) is unusual. The symptoms in this family included pericarditis and, although pericarditis can occur in FMF (31), the latter was excluded by sequencing and haplotype analysis, and our findings suggest that another gene is responsible for the disease in this family. Potential candidates include members of the ADAM gene family, involved in diverse processes including ectodomain shedding (32), and ARTS1, an aminopeptidase regulator of TNFRSF1A shedding.

Although a diversity of T50M carrier chromosomes has been reported (7), a single conserved intragenic haplotype was demonstrated in 3 T50M families in this study. This suggests that T50M is relatively ancient and is carried on a specific haplotype which, over time, has undergone meiotic recombination to generate the various identified haplotypes. Because the C88R haplotype covers 2 cM, it probably has resulted from a more recent mutational event, thus not allowing sufficient time to permit recombination. Furthermore, this haplotype may have spread from Scotland to Australia with the emigration of the eighteenth and nineteenth centuries, and the 2 families with this mutation (family 9 and the previously reported family [2]) probably share a common ancestor.

From a clinical standpoint, we have shown that plasma sTNFRSF1A concentration can vary substantially in individual patients according to the degree of inflammatory activity. Therefore, the significance of sTNFRSF1A measurements should be interpreted only with full knowledge of an individual's clinical condition and concurrent estimation of the acute-phase plasma protein response.

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