Clinical and functional characterisation of a novel TNFRSF1A c.605T>A/V173D cleavage site mutation associated with tumour necrosis factor receptor-associated periodic fever syndrome (TRAPS), cardiovascular complications and excellent response to etanercept treatment

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ABSTRACT

Objectives: To study the clinical outcome, treatment response, T-cell subsets and functional consequences of a novel tumour necrosis factor (TNF) receptor type 1 (TNFRSF1A) mutation affecting the receptor cleavage site.

Methods: Patients with symptoms suggestive of tumour necrosis factor receptor-associated periodic syndrome (TRAPS) and 22 healthy controls (HC) were screened for mutations in the *TNFRSF1A* gene. Soluble TNFRSF1A and inflammatory cytokines were measured by ELISAs. TNFRSF1A shedding was examined by stimulation of peripheral blood mononuclear cells (PBMCs) with phorbol 12-myristate 13-acetate followed by flow cytometric analysis (FACS). Apoptosis of PBMCs was studied by stimulation with TNFα in the presence of cycloheximide and annexin V staining. T cell phenotypes were monitored by FACS.

Results: *TNFRSF1A* sequencing disclosed a novel V173D/p.Val202Asp substitution encoded by exon 6 in one family, the c.194–14G>A splice variant in another and the R92Q/p.Arg121Gln substitution in two families. Cardiovascular complications (lethal heart attack and peripheral arterial thrombosis) developed in two V173D patients. Subsequent etanercept treatment of the V173D carriers was highly effective over an 18-month follow-up period. Serum TNFRSF1A levels did not differ between TRAPS patients and HC, while TNFRSF1A cleavage from monocytes was significantly reduced in V173D and R92Q patients. TNFα-induced apoptosis of PBMCs and T-cell senescence were comparable between V173D patients and HC.

Conclusions: The TNFRSF1A V173D cleavage site mutation may be associated with an increased risk for cardiovascular complications and shows a strong response to etanercept. T-cell senescence does not seem to have a pathogenetic role in affected patients.

Hereditary autoinflammatory syndromes are characterised by recurrent multisystemic inflammation with increased acute phase reactants, but absent infection, high-titre autoantibodies or antigen-specific T lymphocytes (reviewed by Stojanov and Kastner[1]). The tumour necrosis factor receptor-associated periodic syndrome (TRAPS; Mendelian inheritance in man no 142680) is the most common autosomal dominantly inherited autoinflammatory syndrome affecting patients of diverse ethnicities.[2] Characteristic clinical features are recurrent and prolonged (>5 days) episodes of fever in conjunction with severe abdominal pain, centrifugally migrating localised myalgia with associated painful erythematous skin rash as well as conjunctivitis and unilateral periorbital oedema.[3 4]

TRAPS is caused by sequence alterations in the *TNFRSF1A* gene on chromosome 12p13, which encodes the ubiquitously expressed 55 kDa TNF receptor (TNFRSF1A, CD120a).[2] More than 50 TRAPS-related mutations have been reported so far (INFEVERS database at http://fmf.igh.cnrs.fr/infevers (accessed 29 May 2008)[5]). The majority are single nucleotide missense mutations within *TNFRSF1A* exons 2, 3 and 4, affecting the first three of four extracellular cysteine-rich domains of the receptor. R92Q, a *TNFRSF1A* variant encoded by exon 4, is found in 1–2.5% of the normal population and presents with an incomplete penetrance and milder or TRAPS-atypical clinical features.[6–9] To date, only two mutations have been reported in exon 6: (*a*) I170N/p.Ile199Asn,[10] an amino acid substitution very close to the TNFRSF1A cleavage site (between p.Asn201 and p.Val202), which is adjacent to the transmembrane domain of TNFRSF1A[11 12] and (*b*) L167_G175del/p.Leu196_Gly204del,[9] the first large in-frame interstitial deletion.

The TRAPS episodes were initially considered to be the consequence of a diminished antagonistic effect of soluble TNF receptors owing to an impaired activation-induced receptor cleavage (shedding), leading to an increase of unbound TNFα in the circulation.[2] However, affected members of the first thoroughly described TRAPS family showed raised levels of interleukin 6 (IL6) and IL8, but not of TNFα,[13 14] suggesting additional pathogenetic mechanisms. Recent experiments have shown a more complex situation with mutation- and cell type-dependent TNF receptor cleavage defects,[15] TNF-independent NF-κB activation[16] and impairment of intracellular TNF receptor trafficking, TNF binding[17] and TNF-induced apoptosis,[9 18] probably caused by misfolding of the

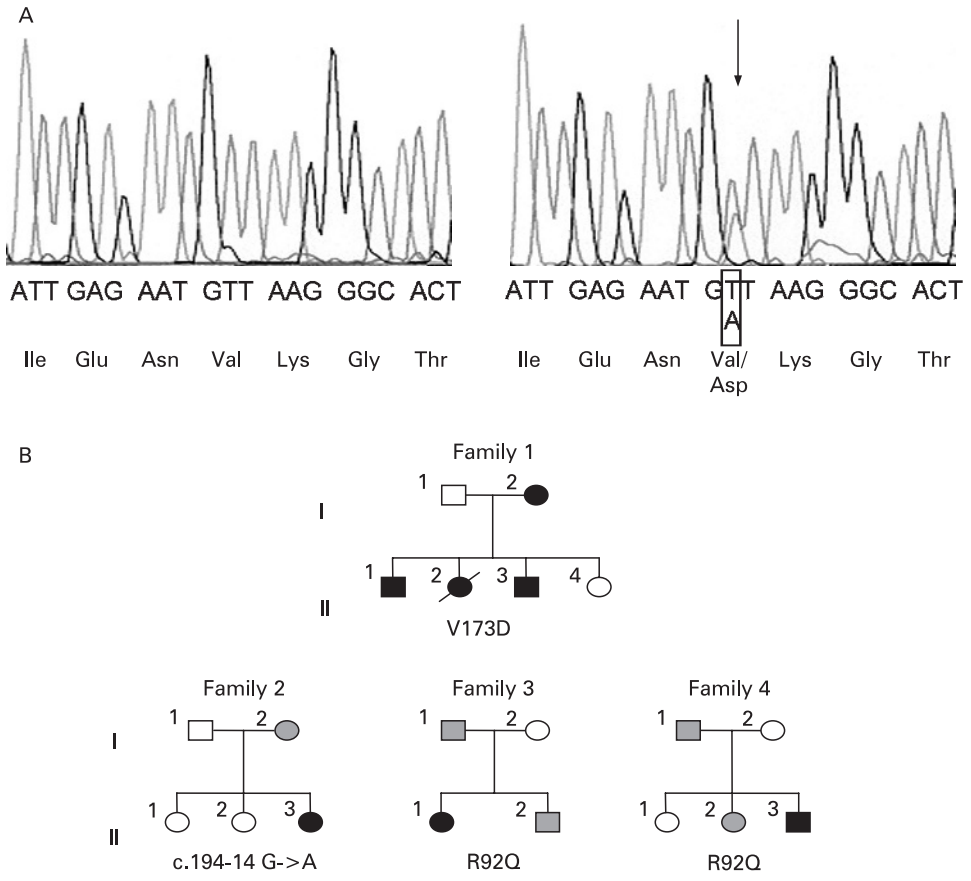

Figure 1 New *TNFRSF1A* cleavage site mutation V173D and pedigrees of the TRAPS families investigated. (A) Partial *TNFRSF1A* exon 6 sequence of a normal subject (left panel) and of a heterozygous mutation carrier (right panel). The arrow indicates the position of the T→A nucleotide substitution, resulting in a valine (GTT)-to-aspartic acid (GAT) exchange at amino acid position 173 of the mature protein. (B) Pedigrees of the four families with tumour necrosis factor receptor-associated periodic fever syndrome (TRAPS) caused by three different *TNFRSF1A* mutations, c.605T>A/V173D, c.194–14G>A and c.362G>A/R92Q. Open symbols represent healthy subjects, solid symbols affected subjects and grey symbols healthy carriers.

extracellular domain of TNFRSF1A and leading to retention of the mutated receptors in the endoplasmic reticulum.[19]

The prognosis of TRAPS is mainly dependent on the development of systemic AA amyloidosis, which occurs in up to 14% of mutation-positive patients and asymptomatic carriers,[3] therefore warranting an efficient treatment. Although corticosteroids can be quite effective in more severe episodes, patients often require escalating doses, with subsequent side effects. Lately, etanercept, a TNFα-neutralising agent, has been shown to elicit a beneficial therapeutic response in many patients (reviewed by Stojanov and McDermott[4]).

Here, we report a new *TNFRSF1A* mutation at the receptor cleavage site in an Austrian family. Two of the four affected members presented with cardiovascular complications. The V173D mutation was characterised by receptor shedding in comparison with two other *TNRSF1A* mutations and the treatment effect of etanercept was studied. The question of T-cell senescence in TRAPS patients was also examined.

PATIENTS AND METHODS

Study participants

We included the members of four families carrying one of three different TRAPS-causing *TNFRSF1A* mutations, one of which was a novel mutation affecting the receptor cleavage site. One family was of Austrian, two of German and one of German/Spanish ancestry. The probands or their parents, or both, were asked about their clinical symptoms and disease history. A family history was present in one affected family. Blood was taken from all family members and also from 22 healthy Caucasian subjects, who served as normal controls. The study was approved by the ethics committee of the Innsbruck Medical University, Austria. Written informed consent was obtained from all participants or their parents before enrolment.

DNA extraction and sequence analysis of the *TNFRSF1A*, *MVK*, *MEFV* and *NLRP3/CIAS1* genes

EDTA blood samples were collected from all participants and genomic DNA was extracted from white blood cells using the QIAamp blood mini-kit (Qiagen, Hilden, Germany). After amplification by PCR, *TNFRSF1A* exons 2, 3, 4 and 6 were sequenced. *MVK* exons 9 and 11, *MEFV* exons 2 and 10 and *NLRP3/CIAS1* exon 3 were also analysed in order to exclude common mutations resulting in other hereditary autoinflammatory fever syndromes.[20 21]

Determination of soluble TNF receptors in serum and culture supernatants

Soluble tumour necrosis factor receptor superfamily 1A (sTNFRSF1A) and 1B (sTNFRSF1B) were measured in serum samples obtained from *TNFRSF1A* mutation-positive family members between attacks and from healthy controls (HC) as well as in culture supernatants of stimulated peripheral blood mononuclear cells (PBMCs) using ELISA kits (Bender MedSystems, Vienna, Austria) according to the manufacturer's

Table 1 Clinical and laboratory characteristics of *TNFRSF1A* mutation-positive family members

	Family member											
Characteristics	**1-I.2**	**1-II.1**	**1-II.2**	**1-II.3**	**2-I.2**	**2-II.3**	**3-I.1**	**3-II.1**	**3-II.2**	**4-I.1**	**4-II.2**	**4-II.3**
Ethnicity	Austrian	Austrian	Austrian	Austrian	German	German	German	German/Spanish	German/Spanish	German	German	German
Gender	F	M	F	M	F	F	M	F	M	M	F	M
Age (years)	46	26	Deceased	23	57	15	44	8	5	53	21	12
Age at disease onset (years)	6	1	6	5	–	12	–	3	–	–	–	10
Duration of fever (days)	4–5	3–7	7	3	–	1–7	–	1–5	–	–	–	28–56
Fever-free interval (weeks)	4	4	3	4	–	4–24	–	2–12	–	–	–	24
Abdominal pain	–	+	–	+	–	–	–	++	–	–	–	–
Vomiting	+	–	+	+	–	–	–	+	–	–	–	–
Hepatomegaly	–	–	–	+	–	–	–	–	–	–	–	–
Myalgia	++	–	+	–	–	–	–	–	–	–	–	+
Rash	+	++	+	+	–	–	–	–	–	–	–	+
Conjunctivitis	–	–	–	–	–	–	–	–	–	–	–	+
Arthralgia	++	+	+	++	–	–	–	+	–	–	–	++
Arthritis	+	–	+	–	–	–	–	–	–	–	–	–
Headache	–	–	–	–	–	+	–	–	–	–	–	+
Pharyngitis	–	+	+	–	–	++	–	++	–	–	–	–
Lymphadenopathy	–	+	–	–	–	+	–	–	–	–	–	–
Amyloidosis	–	–	–	–	–	–	–	Mild proteinuria	–	–	–	–
Mutation in the *TNFRSF1A* gene	V173D	V173D	V173D	V173D	c.194–14G>A	c.194–14G>A	R92Q	R92Q	R92Q	R92Q	R92Q	R92Q
Leucocytes $\times 10^9$/l	8.8†	Normal*†	11.2*	3.8*, 8.6†	Normal	5.1†	NA	11.5*, 7.1†	8.2	NA	NA	5.8†
CRP (mg/l)	27†	590*, 121†	381‡	269*, normal†	Normal	0.5†	NA	38*, <0.5†	<0.5	NA	NA	<0.5†
SAA (mg/l)	ND	773*	ND	ND	NA	110*, <0.7†	NA	220*, 2.3†	1.4	NA	NA	5.2†
ESR (mm/1st h)	60†	90*, 72†	ND	105*, 16†	NA	7†	NA	19*	6	NA	NA	6†
Fibrinogen (g/l)	5.50†	8.85†	10.0*, 4.32†	10.59*	NA	ND	NA	ND	ND	NA	NA	ND

*Highest documented value measured during an attack; †highest documented value measured between attacks.
CRP, C-reactive protein (normal <5 mg/l); ESR, erythrocyte sedimentation rate (normal <10 mm/1st h); NA, not available; ND, not determined; SAA, serum amyloid A (normal <5 mg/l); ++, severe; +, present; −, absent.

instructions. The detection limits of the assays were <53 pg/ml for sTNFRSF1A and <99 pg/ml for sTNFRSF1B. Samples from patients (but not from controls) were analysed in duplicate and the mean of the two values was calculated. Investigators were blinded to the identity of all samples.

Cell activation and flow cytometric analysis

To study cleavage of the extracellular domain of TNFRSF1A, PBMCs were isolated from patients with TRAPS and from HC as previously described.[22] Assays using PBMCs from healthy and affected family members were performed in parallel and investigators were unaware of the results of the *TNFRSF1A* mutation analysis. Data from healthy family members were allocated to the HC group.

PBMCs were stimulated with phorbol 12-myristate 13-acetate (PMA; Sigma, Munich, Germany) for 25 min at 37°C. The optimal concentration of PMA was critical for performance of the shedding assays. In a preliminary study, concentrations ranging from 1 to 20 ng/ml induced optimal shedding in HC without destroying cells. After washing, cells were stained for 30 min at 4°C with fluorescein isothiocyanate (FITC)-conjugated anti-CD120a (TNFRSF1A) monoclonal antibody (mAb) and peridinin chlorophyll protein-conjugated anti-CD14 (monocytes) and anti-CD3 (T cells) (Becton Dickinson, San Diego, California, USA).[23] Corresponding isotype control antibodies were used.

For T-cell subtype analysis, freshly isolated PBMCs were stained with FITC-conjugated mAbs (CD4, CD25, CD28 and CD45RA), phycoerythrin-conjugated mAbs (CD28 and CD45RO) and peridinin chlorophyll protein-conjugated mAbs (CD3, CD4 or CD8), as appropriate.

Stained cells were analysed on a FACS-Calibur (Becton Dickinson). At least 10 000 events were counted for each acquisition. Data were analysed using the WinMDI software (version 2.5, Joseph Trotter, Scripps Research Institute, La Jolla, California, USA).

Study of TNF-induced apoptosis in PBMCs

Apoptosis assays were performed as previously described.[18] Briefly, freshly isolated PBMCs (1×10^6) were stimulated with 0, 1, 10 and 100 ng/ml TNFα and cycloheximide (50 μg/ml, Sigma) either alone or in combination for 6 h at 37°C. Apoptosis was analysed using FITC-conjugated annexin V staining (Becton Dickinson) and flow cytometry.

Statistical analysis

Statistical analyses were performed using the SPSS program, version 12.0. The Kolmogorov–Smirnov test was used to test for normal distribution. Parametric and non-parametric tests, including the Student *t* test, Mann–Whitney U, Wilcoxon, Kruskal–Wallis and Friedman tests, were used as appropriate. To test for shedding differences between patients and controls, ratios and differences between stimulated and unstimulated cells were calculated to adjust for differences in a priori receptor expression. p Values <0.05 were considered significant.

Table 2 Effect of etanercept treatment on serum acute phase parameters as well as tumour necrosis factor α and interleukin 6 concentrations in V173D mutation-positive family members

	1-I.2	1-II.1	1-II.3
CRP (mg/l)			
Before etanercept	26	121	268
Etanercept for 3 months	10	1	7
Etanercept for 6 months	108	3.1	1
Etanercept for 12 months	4	18.5	39
ESR (mm/1st h)			
Before etanercept	60	72	70
Etanercept for 3 months	16	4	8
Etanercept for 6 months	1	6	10
Etanercept for 12 months	8	8	14
TNFα (pg/ml)			
Before etanercept	19	15	64
Etanercept for 3 months	251	190	198
Etanercept for 6 months	111	86	96
Etanercept for 12 months	37	30	36
IL6 (pg/ml)			
Before etanercept	3	3	82
Etanercept for 3 months	3	10	3
Etanercept for 6 months	7	<3	3
Etanercept for 12 months	4	<3	5

CRP, C-reactive protein (normal <5 mg/l); ESR, erythrocyte sedimentation rate (normal <10 mm/1st h); IL6, interleukin 6 (normal <3 pg/ml); TNFα, tumour necrosis factor α (normal <20 pg/ml).

RESULTS

TNFRSF1A mutation detection

Sequence analysis of exons 2, 3, 4 and 6 of the *TNFRSF1A* gene in the index patient of family 1 revealed a novel heterozygous GTT to GAT transversion in exon 6, resulting in a valine-to-asparagine substitution at residue 173 (V173D/p.Val202Asp) (fig 1A). This amino acid exchange directly affects the major receptor cleavage site, which is located between p.Asn201 and p.Val202. This mutation was present in all family members except the healthy father and one healthy sister (fig 1B) and was not detected in more than 2000 control chromosomes. Three additional *TNFRSF1A* mutation-positive families were included, of which family 2 demonstrated heterozygosity for c.194–14G>A, a known *TNFRSF1A* splice site mutation, while families 3 and 4 were positive for the low-penetrance *TNFRSF1A* mutation R92Q (fig 1B).

The V173D mutation is associated with TRAPS: typical clinical features, atypical complications and sustained clinical response to etanercept

Table 1 summarises the main clinical signs/symptoms and laboratory findings of the 11 *TNFRSF1A* mutation-positive subjects.

The male:female ratio was 6:5. Most of the TRAPS characteristic features were associated with the fully penetrant V173D substitution in family 1. Cardiovascular complications developed in two affected family 1 members. After a severe gastroenteritis, the V173D-heterozygous sister (1-II.2) of the index patient (1-II.1) died at the age of 22 years owing to heart failure associated with myocardial infarction with subsequent ventricular thrombosis and A cerebri media infarction. Serological antibody screening for viruses was negative. At the age of 21 years, her brother (1-II.3) developed an arterial thrombosis of the left A poplitea owing to cystic degeneration of the intima, warranting local desobliteration with thrombectomy and subsequent oral anticoagulation. None of the affected patients were active smokers. A thorough analysis of the coagulation system (including screening for known hereditary coagulopathies) in all members of family 1 did not show any anomalies at five consecutive clinical visits, except for highly increased fibrinogen concentrations in all *TNFRSF1A* mutation-positive family members. Repeated assessments of antiphospholipid antibodies, including anticardiolipin and β_2-microglobulin antibodies as well as lupus anticoagulant, also yielded negative results.

All affected family 1 members were initially treated with various non-steroidal anti-inflammatory drugs and cumulative doses of glucocorticosteroids. However, reduction of the intensity of disease-associated symptoms was limited. In contrast, all clinically affected patients of families 2–4 responded to steroid treatment with shortening of the fever episodes or rapid cessation of symptoms, or both.

After diagnosis of TRAPS, TNFα-blocking treatment with 25 mg etanercept (Enbrel; Wyeth, Philadelphia, USA) twice weekly was started in all affected family 1 members. All three patients (patient 1-II.2 had already died) showed an immediate response and remained symptom-free with etanercept treatment until the end of follow-up after 18 months. Table 2 shows the measurements of acute phase reactants and proinflammatory cytokines before and after etanercept treatment.

Normal serum levels of sTNFRSF1A and sTNFRSF1B in patients with TRAPS

Median serum concentrations of sTNFRSF1A and sTNFRSF1B were 1.8 ng/ml (range 0.92–3.0) and 5.52 ng/ml (0.92–36.6) in HC. sTNFRSF1A and sTNFRSF1B levels of patients affected by the V173D (sTNFRSF1A median 1.8 ng/ml, range 1.2–2.6 and sTNFRSF1B median 8.0 ng/ml, range 4.8–13.1), the c.194–14G>A and the R92Q mutation did not differ from HC (fig 2A).

Impaired shedding of TNFRSF1A from stimulated monocytes in patients with the V173D and R92Q mutations

Stimulation with PMA induced shedding of TNFRSF1A from CD14+ monocytes in all TRAPS patients and HC (figs 2B and C). Compared with HC, however, TNFRSF1A shedding was reduced in patients with the V173D (mean (SEM) 86.2 (2.6)% vs 95.1 (2.4)%; $p<0.05$ at a dosage of 10 ng/ml PMA) and the R92Q substitution, but not in patients with the c.194–14G>A mutation. Levels of sTNFRSF1A in supernatants of PBMCs showed a comparable increase in patients and HC after stimulation with PMA (fig 2D).

Normal induction of apoptosis in TNFα-stimulated PBMCs from patients with the V173D mutation

To assess whether the V173D mutation is associated with functional alterations other than TNF receptor cleavage, TNFα-induced apoptosis was analysed in PBMCs from patients carrying the V173D mutation and from HC. After stimulation of PBMCs with 0, 1, 10 and 100 ng/ml TNFα in the presence of cycloheximide for 6 h, a similar percentage of cells were annexin V positive in V173D heterozygotes (mean (SEM) ratio 1 ng/ml vs unstimulated 17.2 (12.0); vs 10 ng/ml 16.2 (12.0) and vs 100 ng/ml 20.3 (8.7)) and in HC (11.5 (4.9), 23.2 (5.9) and 22.2 (4.5)), respectively) (fig 3).

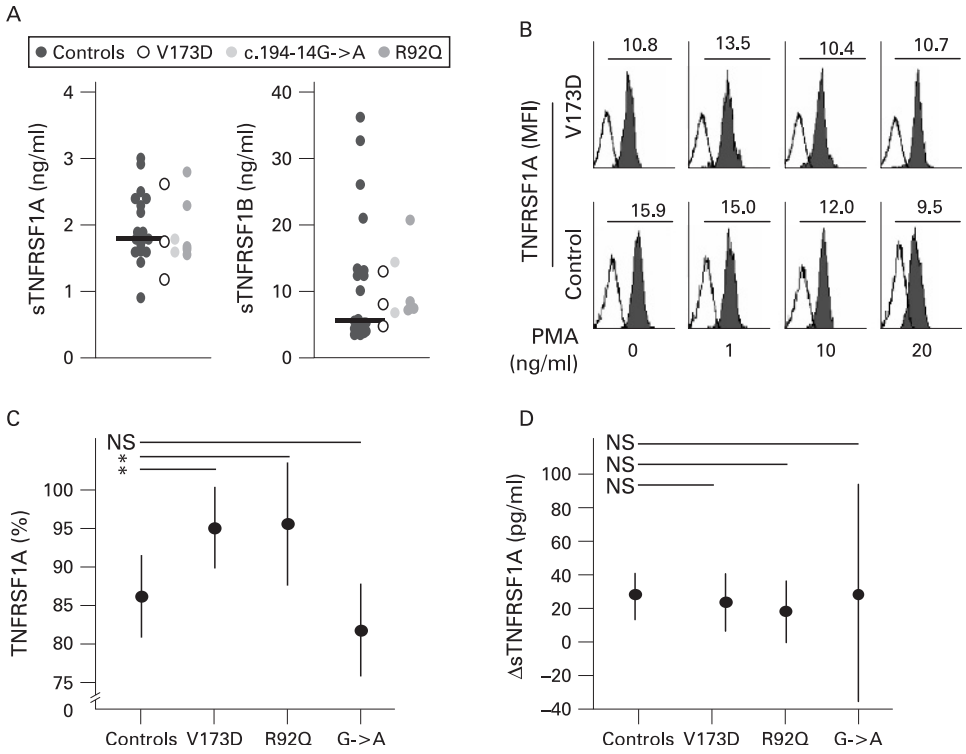

Figure 2 TNFRSF1A shedding in patients with different *TNFRSF1A* mutations and in healthy controls. (A) Normal levels of soluble TNF receptor superfamily 1A (sTNFRSF1A) and 1B (sTNFRSF1B) in patients with the V173D mutation. Levels of sTNFRSF1A and sTNFRSF1B in serum samples from patients with the *TNFRSF1A* mutations c.605T>A/V173D (n = 3), c.194–14G>A (n = 2) and c.362G>A/R92Q (n = 4) and from healthy controls (n = 21) were determined by ELISA. Each dot represents the mean of two identical aliquots from a patient, while horizontal lines indicate the median values. (B, C) Impaired shedding of TNFRSF1A from stimulated CD14+ monocytes in patients with the V173D and R92Q mutations. (B) Representative histograms show expression of TNFRSF1A (filled curve; the black line represents isotype control) on CD14+ cells from a patient with the V173D mutation and a healthy control without and after stimulation with 1, 10 and 20 ng/ml phorbol 12-myristate 13-acetate (PMA) for 25 min at 37°C. MFI, mean fluorescence intensity. (C) Error bars summarise TNFRSF1A shedding from CD14+ cells of healthy controls (n = 26) and from patients with the V173D (n = 3), the R92Q (n = 4) and the c.194–14G>A (n = 2) mutation after stimulation with 10 ng/ml PMA. Data are shown as the mean (95% confidence interval) percentage change (ratio (MFI stimulated/MFI unstimulated cells) ×100). (D) Comparable increment of levels of sTNFRSF1A in the supernatant of peripheral blood mononuclear cells from TRAPS patients and healthy controls after stimulation with 10 ng/ml PMA. Error bars indicate the mean (95% confidence interval) difference of sTNFRSF1A before and after stimulation. *p<0.05 according to the Student t test; NS, not significant.

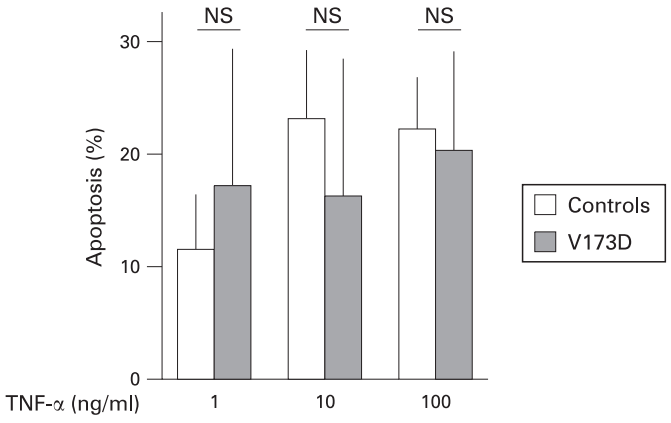

Figure 3 Tumour necrosis factor α (TNFα)-induced apoptosis is not altered in V173D heterozygotes. Freshly isolated peripheral blood mononuclear cells from patients carrying the V173D mutation (n = 3) and from healthy controls (n = 6) were incubated for 6 h at 37°C with different doses of TNFα in combination with 50 μg/ml cycloheximide as indicated. Boxes indicate the mean (SEM) increase of annexin V-positive cells as a percentage compared with cells incubated with cycloheximide only. Differences were not significant (NS) according to the Student t test.

Absence of T-cell senescence in patients with the V173D mutation

Because chronic TNFα receptor signalling is known to induce T-cell senescence,[24] we investigated whether T-cell subsets are altered in patients with the V173D mutation. Table 3 shows that the prevalences of CD4+CD25+ activated T cells, CD4+CD45RA+ and CD8+CD45RA+ naïve T cells, CD4+CD45RO+ and CD8+CD45RO+ memory T cells as well as CD4+CD28− and CD8+CD28- memory/effector T cells did not differ from HC and did not change significantly after TNFα-blocking therapy.

DISCUSSION

The novel TNFRSF1A mutation V173D/p.Val202Asp encoded by exon 6 of the *TNFRSF1A* gene is unique, since it directly affects the receptor cleavage site, which is located between p.Asn201 and p.Val202.[11 12] All V173D carriers presented with a fully penetrant TRAPS phenotype. Two of them developed cardiovascular complications in addition, leading to the death of one sister due to myocardial infarction and to the development of arterial thrombosis in one brother. None of the mutation-positive family members showed any coagulation abnormality and hyperfibrinogenaemia as the sole cause of these complications seems to be unlikely.

Table 3 T-cell subtypes in V173D carriers before and after treatment with etanercept compared with healthy controls

T-cell subtypes	V173D − etanercept (n = 3)	V173D + etanercept (n = 3)	Healthy controls (n = 17)
CD4+ T cells			
Naïve (CD28+CD45RA+)	67.0 (59.3–71.7)	29.0 (23.0–32.7)	44.7 (27.8–69.3)
Memory (CD28+CD45RO+)	41.3 (24.1–43.5)	46.8 (35.3–52.4)	45.2 (30.5–68.1)
Memory-effector (CD28–)	0.2 (0.1–0.3)	0.7 (0.2–0.9)	0.7 (0–38.0)
Activated (CD25+)	25.0 (6.8–32.4)	7.8 (6.6–9.6)	12.3 (3.0–26.4)
CD8+ T cells			
Naïve (CD28+CD45RA+)	77.8 (63.9–90.2)	70.5 (42.4–71.8)	45.9 (14.5–65.2)
Memory (CD28+CD45RO+)	11.0 (1.6–40.8)	12.4 (6.1–39.4)	19.8 (6.3–51.8)
Memory-effector (CD28–)	19.2 (5.9–25.7)	14.2 (1.9–28.2)	21.9 (7.6–77.4)
Activated (CD25+)	ND	0.9 (0.8–1.0)	2.7 (0.4–10.5)

Data are shown as the median (range) percentage of CD4+ and CD8+ T cells, respectively. ND, not done.

An increased risk of atherosclerosis has been suggested for R92Q carriers in a large European study of subjects with myocardial infarction.[25] Furthermore, six R92Q carriers with Behçet disease developed extracranial venous thrombosis.[26] Pericarditis and myocarditis have also been reported in R92Q[7] and P46L—another low-penetrance variant—heterozygotes.[27] To assess a potential association of our novel V173D mutation with atherosclerosis, young patients presenting with cardiovascular disease should be screened for this *TNFRSF1A* receptor cleavage site mutation.

The V173D/p.Val202Asp mutation may have an atherogenic effect, as TNFα is involved in the development of endothelial dysfunction and atherosclerosis, probably potentiated by the prolonged increase of acute phase parameters.[28] Another possibility is the development of amyloidosis with subsequent cardiomyopathy. However, except for the deceased patient, who was not examined for amyloidosis post mortem, none of the mutation-positive family members showed any clinical signs of amyloidosis. It has also been suggested that *TNFRSF1A* mutations interfere with the local antithrombotic effect of TNFα during inflammation by impairing the endothelial TNF receptor-mediated iNOS stimulation.[26] A link between premature T-cell ageing, early atherosclerotic damage and multivessel coronary artery disease as established for rheumatoid arthritis and unstable angina,[29–31] on the other hand, seems unlikely for patients carrying *TNFRSF1A* mutations because concentrations of CD28− T cells as a marker of early T-cell ageing were normal in our V173D patients.

Earlier in vitro experiments had demonstrated that TNFRSF1A shedding is partially impaired when the valine residue at position 202 is substituted by aspartic acid, glycine or arginine, whereas replacement with proline resulted in a marked decrease in shedding.[12] Accordingly, monocytes from our TRAPS patients with the V173D/p.Val202Asp mutation showed a partially, but significantly reduced shedding compared with HC. Levels of sTNFRSF1A, in contrast, increased to a comparable extent in PBMC supernatants after stimulation. One reason for this discrepancy may be that the sTNFRSF1A concentrations measured by ELISA were near the detection limit of the assay, causing some imprecision. Alternatively, and as already described for other TRAPS mutations, receptor shedding may differ between various cell types.[15] This hypothesis is corroborated by our results for the R92Q and c.194–14G>A mutations, which are consistent with previously published data.[6 9] Furthermore, our results suggest that the normal receptor protein synthesised from the intact allele compensates in part for the cleavage deficiency, at least in monocytes.

TNFα-induced apoptosis of PBMCs in our V173D patients did not differ from that of HC. However, as we did not analyse other cell types such as fibroblasts or neutrophils, for which apoptotic resistance has been described in TRAPS patients carrying cysteine mutations,[9 18] we cannot completely rule out such a defect in our V173D patients. Whether *TNFRSF1A* cleavage site mutations, including the V173D variant, cause functional impairments in addition to the TNFRSF1A shedding defect awaits further investigations.

Owing to the limited responsiveness of our V173D patients to non-steroidal anti-inflammatory drugs and steroids and the suggested receptor shedding defect, we started anti-TNFα therapy with etanercept. All patients responded with cessation of their clinical symptoms shortly after the start of treatment. The associated sharp rise in TNFα serum levels seen in our patients is consistent with previous findings about the "buffer" function of sTNFRSF1A which can continually release TNFα into the circulation.[14] Under sustained etanercept treatment, however, the TNFα levels declined and remained only slightly raised in all patients. The effectiveness of etanercept treatment in our patients with an obvious TRAPS phenotype again confirms the results of the in vitro experiments performed earlier by Brakebusch *et al*.[12]

In summary, we have identified a new *TNFRSF1A* mutation directly affecting the receptor cleavage site, which is associated with a severe TRAPS phenotype and cardiovascular complications. Despite an associated partial shedding defect, patients had normal serum levels of sTNFRSF1A and TNFα, but were highly responsive to etanercept treatment over the course of 1½ years.

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Clinical and functional characterisation of a novel TNFRSF1A c.605T>A/V173D cleavage site mutation associated with tumour necrosis factor receptor-associated periodic fever syndrome (TRAPS), cardiovascular complications and excellent response to etanercept treatment

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