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Abnormal Tumor Necrosis Factor Receptor I Cell Surface Expression and NF-κB Activation in Tumor Necrosis Factor Receptor–Associated Periodic Syndrome

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Objective. **Tumor necrosis factor receptor–associated periodic syndrome (TRAPS) is an autosomal-dominant autoinflammatory condition caused by mutations in the *TNFRSF1A* gene. The cellular mechanisms by which mutations in this gene trigger inflammation are currently unclear. Because NF-κB is the major intracellular signaling component inducing secretion of proinflammatory cytokines, we sought to determine whether differences in the clinical phenotype of patients with TRAPS may be attributable to variable effects of *TNFRSF1A* mutations on TNFRI expression, localization, or NF-κB activity.**

Methods. **Peripheral blood mononuclear cells were obtained from patients (following informed consent), and cellular nuclear and cytosolic fractions were generated by subcellular fractionation. Localization of IκBα and NF-κB was determined by Western blotting of the resultant fractions. NF-κB subunit activity was determined by enzyme-linked immunosorbent assay analysis and confirmed by electrophoretic mobility shift assay. Subcellular localization of TNFRI was determined by immunofluorescence confocal microscopy or by immunoblotting following affinity isolation of plasma membrane by subcellular fractionation.**

Results. **Cells from patients with the fully penetrant C73R mutation had marked activation of the proinflammatory p65 subunit of NF-κB. In contrast, cells from patients with the low-penetrant R92Q mutation displayed high levels of DNA binding by the p50 subunit, an interaction previously linked to repression of inflammation. Interestingly, although cells from patients with the C73R mutation have no TNFRI shedding defect, there was nonetheless an unusually high concentration of functional TNFRI at the plasma membrane.**

Conclusion. **High levels of TNFRI at the cell surface in patients with the C73R mutation hypersensitizes cells to stimulation by TNF, leading to increased NF-κB p65 subunit activation and an exaggerated proinflammatory response.**

Tumor necrosis factor receptor–associated periodic syndrome (TRAPS; previously known as familial Hibernian fever) is an autosomal-dominant autoinflammatory condition caused by mutations in the *TNFRSF1A* gene (1). Autoinflammatory disorders are defined by spontaneously relapsing and remitting bouts of systemic inflammation in the absence of pathogens, autoantibodies, or antigen-specific T cells. Examples include both monogenic disorders (e.g., TRAPS, familial Mediterranean fever, Muckle-Wells syndrome, and hyperimmunoglobulinemia D with periodic fever syndrome [HIDS]) and multifactorial disorders (e.g., Crohn's disease and

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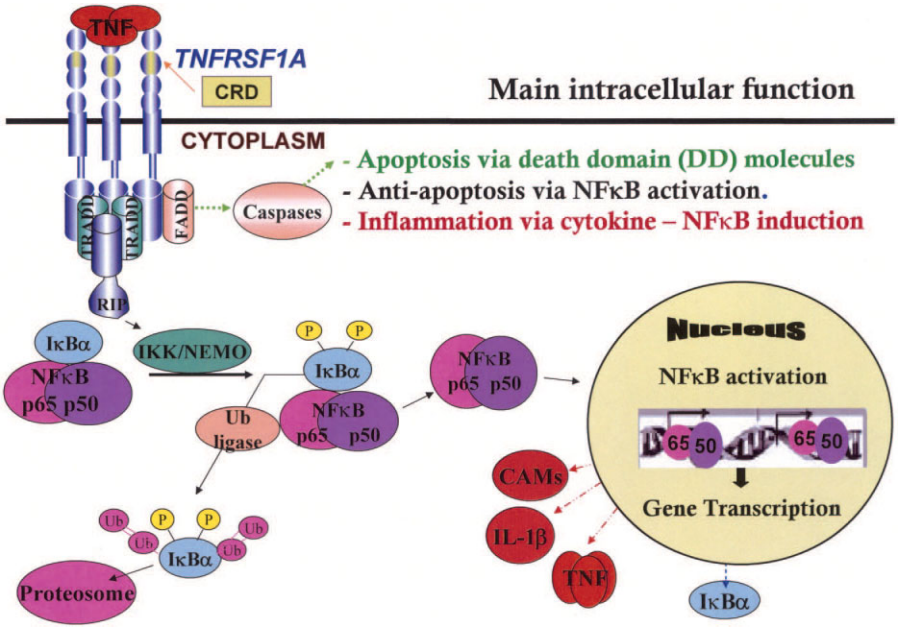

Figure 1. Cell surface signaling from tumor necrosis factor receptor I (TNFRI) leads to NF-κB activation and inflammatory response. Binding of TNFα to the trimeric form of TNFRI results in conformational changes in the intracellular region of the receptor, leading to rapid recruitment of several adaptor proteins including FADD and receptor-interacting protein (RIP). This initiates signaling cascades that lead to either caspase-mediated apoptosis or NF-κB activation and inflammatory response. NF-κB activation is characterized by dissociation from IκBα, following IKK complex–mediated phosphorylation and NF-κB–essential modulator (NEMO)–dependent ubiquitin (Ub) ligase–mediated ubiquitination, which leads to proteasomal degradation. The p65/p50 heterodimer subunits then translocate to the nucleus, where they bind to DNA target sequences of a number of cytokines and additional factors that are associated with inflammation. TRAPS-associated mutations are primarily found in the extracellular cysteine-rich domains (CRD) of TNFRI.

Behçet's disease) associated with mutations of genes involved in inflammation and/or apoptosis. TRAPS is characterized by recurrent periodic fevers, abdominal pain, skin lesions, conjunctivitis, and myalgia; AA amyloidosis develops in some patients, leading to renal problems.

Thus far, more than 50 different *TNFRSF1A* mutations have been described that are associated with TRAPS (INFEVERS database; online at http://fmf.igh.cnrs.fr/infevers) (2,3), with the majority (>90%) being single-nucleotide missense mutations within exons 2, 3, 4, and 6. Most mutations are located within the extracellular region in cysteine-rich domains, with I170N being an exception, because it is located very close to the receptor cleavage site (4). Although the majority of mutations are fully penetrant, there are also some low-penetrance variants (e.g., P46L and R92Q) in which certain family members are symptomatic whereas others may remain unaffected despite carrying the same variant (5). These low-penetrance *TNFRSF1A* variants are also observed in the background population (6), indicating a possible role in inflammation in general.

The cellular mechanisms by which *TNFRSF1A* mutations lead to clinical disease are still largely unknown. NF-κB is a ubiquitous transcription factor activated by proinflammatory stimuli (7), which in turn leads to the up-regulation of a large number of genes involved in inflammation and apoptosis, including TNFα (Figure 1). Not only does activation of NF-κB stimulate the production of TNFα, but there is a positive feedback mechanism whereby binding of TNFα to its cell surface receptor augments the activity of NF-κB (8–10). NF-κB, however, is not a single molecule but consists of complexes of different combinations of the subunits RelA (p65), RelB, c-Rel, p50, and p52, each with distinct DNA-binding characteristics (11).

TNFRI cell-signaling studies have, to date, been difficult to assess, due in part to proapoptotic conse-

quences from overexpression studies. In addition, researchers have resorted to the use of constructs with cytoplasmic truncations of TNFRI that might have a major impact on protein structure, folding, and conformation. In this study, we set out to examine NF-κB activity in C73R and R92Q TNFRI mutant cells from patients with TRAPS, and also in a P46L TNFRI mutant cell line from a patient with the HIDS form of periodic fever with additional in vitro and in vivo evidence of TNFRI dysfunction, in order to determine whether signaling abnormalities could explain the variability in disease penetrance.

After finding increased NF-κB activity in peripheral blood mononuclear cells (PBMCs) from patients with TRAPS, we then investigated whether this might be attributable to increased cell surface expression and activity of TNFRI. Until now, reports have been contradictory as to whether TRAPS mutations lead to increased or decreased TNFRI expression. Studies reliant on transfection of complementary DNA vectors into immortalized cell lines demonstrated restricted cell surface expression of mutant TNFRI (12,13). Similarly, the use of transfected mutant *TNFRI*–containing plasmids has been reported to result in abnormal disulfide-linked TNFR oligomerization, retention in the endoplasmic reticulum, and diminished NF-κB signaling (14). We used primary cells from patients with TRAPS mutations to answer this question more directly.

PATIENTS AND METHODS

Materials. Unless stated otherwise, all materials were obtained from Sigma-Aldrich, Poole, UK.

Patients. Patients harboring 3 distinct mutations of *TNFRSF1A* were studied; all had the classic TRAPS phenotype. Following informed consent, blood samples were obtained from the patients with TRAPS, all of whom were free of symptoms of inflammation at the time that blood was drawn, and were not receiving any medication. All studies were approved by the relevant ethics committees (East London and City Health Authority Research Ethics Committee, and the Internal Diseases Ethical Review Board of Helsinki and Uusimaa Joint Health Authority).

C73R. Patients with the C73R mutation included a mother and daughter from a Finnish family. Both patients had experienced intermittent episodes of abdominal pain, episodic fever, periorbital edema, episcleritis, and myalgia since childhood. In addition, the daughter had peritonitis, pleurisy, and erythema, which was located around her ankles; the episodes were alleviated by steroids but not colchicine. Her mother additionally had cellulitis and arthritis.

R92Q. Patients with the R92Q mutation included 2 unrelated adults. Both patients had classic symptoms of TRAPS, including periodic fever lasting at least 1 week, abdominal pain, rashes, and the presence of an acute-phase response during severe episodes. The first patient was a woman in her 40s, in whom infliximab treatment provoked severe attacks of 2 days' duration. She is currently being treated with etanercept, and she has a sister who is also a carrier of R92Q. The second patient is a man with no other affected relatives. Both patients had documented low levels of soluble TNFRI between attacks, which is likely indicative of a functional effect resulting from the R92Q mutation.

P46L. The proband was a 3-year-old boy who experienced high fevers beginning at 3 days of age, with recurrences every 3–5 weeks thereafter (15). A typical attack was preceded by anorexia and a musty body odor, followed by coryzal symptoms, conjunctival congestion, cervical lymphadenopathy, severe abdominal pain, and diarrhea, with a high fever lasting 5–7 days. The fever was often associated with recurrent, prolonged febrile seizures. Genetic analysis revealed that the patient had HIDS due to a heterozygous mutation (V377I/G211A) of the mevalonate kinase gene, which encodes an enzyme involved in cholesterol and nonsterol isoprenoid biosynthesis, but he also harbored a P46L mutation of the *TNFRSF1A* gene. Because this patient was shown to have a shedding defect of TNFRI and partially responded to etanercept (15), although he was not considered to have a "classic" TRAPS phenotype, the P46L mutation was thought to contribute to his clinical disease and was consistent with the effects of the low-penetrant nature of this gene mutation. The child's white mother and African Caribbean father were both healthy.

Control subjects. Blood was also obtained from 8 healthy volunteers, who had no symptoms of inflammation.

Cell separation and culture. Blood was collected into sterile tubes containing EDTA/anticoagulant and was either shipped immediately at 4°C or, in the case of control specimens, kept refrigerated at 4°C. Samples were then diluted by the addition of an equal volume of 0.9% NaCl. The resulting solution was then layered over 3 ml of Lymphoprep (Axis Shield, Dundee, UK) in a centrifuge tube and centrifuged at 800*g* for 20 minutes at room temperature. After centrifugation, mononuclear cells formed a distinct band at the medium interface. PBMCs were removed from the interface, diluted with 0.9% NaCl, and pelleted by centrifugation at 250*g* for 10 minutes. Cells were then suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Paisley, UK), 26 m*M* $NaCO_3$, and 1% penicillin–streptomycin, and incubated at 37°C for 4 hours prior to experimental manipulation, to exclude the possibility of experimental anomalies arising as a consequence of indirect effects resulting from extracellular cytokines circulating in the patient's blood.

Immunoblotting. PBMCs (2×10^6) were washed in phosphate buffered saline (PBS). In order to generate cytosolic and nuclear extracts, cells were lysed at 4°C by incubation in hypotonic cytosol extraction buffer (BioVision, Mountain View, CA) plus protease inhibitors and 1 m*M* dithiothreitol (DTT) for 10 minutes. Nuclei were pelleted by centrifugation at 16,000*g* for 5 minutes at 4°C and resuspended in nuclear extraction buffer mix. The suspension was vortexed for 15 seconds every 10 minutes over a 40-minute period of time. Insoluble nuclear material was pelleted at 16,000*g* for 10 minutes and discarded. Concentrations of both cytosolic and nuclear extract were determined with the BCA Protein Assay kit (Pierce, Rockford, IL). Protein samples (30 μg) were boiled in sodium dodecyl sulfate (SDS) dissociation buffer and sepa-

rated by SDS–polyacrylamide gel electrophoresis (PAGE), then electrotransferred to Hybond P membrane (Amersham, Little Chalfont, UK). Immunoblotting was conducted using either anti–NF-κB subunit (p65) or anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase–conjugated anti-rabbit IgG (Bio-Rad, Hemel, Hempstead, UK). Protein bands were visualized by autoradiography, using enhanced chemiluminescence detection techniques (Amersham).

Electrophoretic mobility shift assay (EMSA). DNA oligonucleotides (Invitrogen) containing an NF-κB binding site were generated as described previously (16). Nuclear extracts (1 μg total protein) were mixed with 2 μl dilution buffer (20 m*M* HEPES, pH 7.9, 60 m*M* KCl, 0.25 m*M* EDTA, 0.125 m*M* EGTA, 20% glycerol, 1 m*M* DTT, 2.5 μg/ml aprotinin, 2.5 μg/ml leupeptin, 2.5 μg/ml pepstatin, 2 m*M* benzamidine, 50 μ*M* [2-aminoethyl]benzenesulfonyl fluoride HCl) in a final volume of 7 μl. For competition assays, unlabeled double-stranded oligonucleotides were added to appropriate reactions and incubated for 20 minutes at room temperature prior to the addition of 7 μl of binding reaction mixture (10 m*M* Tris HCl, pH 7.9, 50 m*M* NaCl, 2.5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* DTT, 5 m*M* spermidine, 4% glycerol), poly(dI-dC) (0.5 μg/reaction), and radiolabeled probe (0.5 ng/reaction). For supershift analyses, antibodies (1–2 μg) were added 20 minutes after the addition of a binding reaction mixture containing the labeled DNA probe, and reactions were incubated for an additional 25 minutes at 4°C. The reaction mixtures were then subjected to electrophoresis in 6% polyacrylamide gels (AccuGel 29:1 sequencing grade; National Diagnostics, Hull, UK) prepared in Tris−borate−EDTA buffer (22.5 m*M* Tris–borate, 0.5 m*M* EDTA, pH 8.0). Following electrophoresis, the gels were dried at 80°C onto 3MM paper and exposed to x-ray films (Hyperfilm; Amersham) at −70°C for 8–48 hours.

Enzyme-linked immunosorbent assay (ELISA). TransAM NF-κB Family Kits (Active Motif, Rixensart, Belgium) were used for the study of specific NF-κB subunit pathways. Briefly, oligonucleotide containing an NF-κB consensus-binding site was immobilized onto 96-well plates. The binding of NF-κB to its consensus sequence was detected by adding 30 μl of complete binding buffer to each well, and 1 μg of nuclear extract diluted in complete lysis buffer was then added to each well. The plates were sealed and incubated for 1 hour at room temperature with mild agitation. Each well was then washed 3 times with 200 μl 1× wash buffer. Antibody directed against one of the NF-κB p50, p52, p65, c-Rel, or RelB subunits was bound to the protein–oligonucleotide complex and detected following the addition of secondary antibody conjugated to horseradish peroxidase. Developing solution was added, and the plate was incubated for 2–10 minutes at room temperature. Absorbance was then measured, using a Victor 1420 Multilabel counter spectrophotometer (PerkinElmer Wallac, Milan, Italy) at 450 nm, with a reference wavelength of 655 nm.

Immunofluorescence. PBMCs were suspended in 1% paraformaldehyde and fixed for 5 minutes at room temperature. Cells were then spun down and permeabilized with Triton X-100 (0.2% volume/volume) prior to the addition of antibody against calnexin, 4′,6-diamidino-2-phenylindole, or TNFRI (all from Santa Cruz Biotechnology). Cells were washed and then incubated with Alexa 488 $F(ab')_2$ anti-rabbit or Alexa 594 $F(ab')_2$ anti-mouse secondary antibodies (Invitrogen) for 30 minutes at room temperature. Cells were again washed in PBS, then resuspended in PBS/0.1% bovine serum albumin, mounted on slides, and dried. Images were captured using a Zeiss LSM 510 confocal microscope, with scan module LSM 510 mounted on an Axiovert 200 Inverted Microscope (Zeiss, Thornwood, NY). Images were extracted using LSM Core 2 software (Carl Zeiss Imaging Microscopy, Jena, Germany).

Plasma membrane isolation. PBMCs (1×10^7) were washed in PBS. Cells were lysed at 4°C, and a plasma membrane fraction was generated using the Qproteome Plasma Membrane Protein kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. Briefly, ligand specific for plasma membrane molecules was added to cellular postnuclear supernatant. Ligand–vesicle complexes were then precipitated using magnetic beads that bind the ligand. After washing, plasma membrane vesicles were eluted under native conditions. Fractions were then boiled in SDS–dissociation buffer, and protein was separated by SDS-PAGE, electrotransferred to Hybond P membrane (Amersham), and immunoblotted using antibodies against TNFRI protein, the Golgi marker protein GM130, the plasma membrane marker protein β1 integrin, or actin (all from Santa Cruz Biotechnology).

Statistical analysis. Analysis between 2 groups was performed using Student's *t*-test for independent samples. All experimental data were gathered from a series (≥4) of independent experiments. The results are expressed as the mean ± SEM.

RESULTS

Increased NF-κB subunit activity in PBMCs from patients with TRAPS. The p65 component of NF-κB and its associated regulatory protein IκBα were found to be largely confined to the cytoplasm of PBMCs from healthy control subjects and patients with the low-penetrant P46L or R92Q *TNFRSF1A* mutation, as would be expected under normal resting conditions (Figures 2a and b). In contrast, p65 and IκBα in cells with the fully penetrant C73R *TNFRSF1A* mutation were localized to the nucleus, indicative of an activated NF-κB status (17).

Given the differences in NF-κB subunit localization between C73R and either control or low-penetrance R92Q and P46L mutations, the activity of the entire NF-κB subunit family in these variants was determined using an NF-κB panel ELISA kit (Figure 2c). The C73R mutation resulted in a 5-fold increase in p65 relative to controls ($P < 0.001$), whereas p65 levels in other TRAPS mutations were modestly elevated but were not statistically significantly different from control levels. Interestingly, the pattern for p50 was different, with the P46L and C73R mutations showing a modest increase, whereas the R92Q mutation resulted in an almost 8-fold

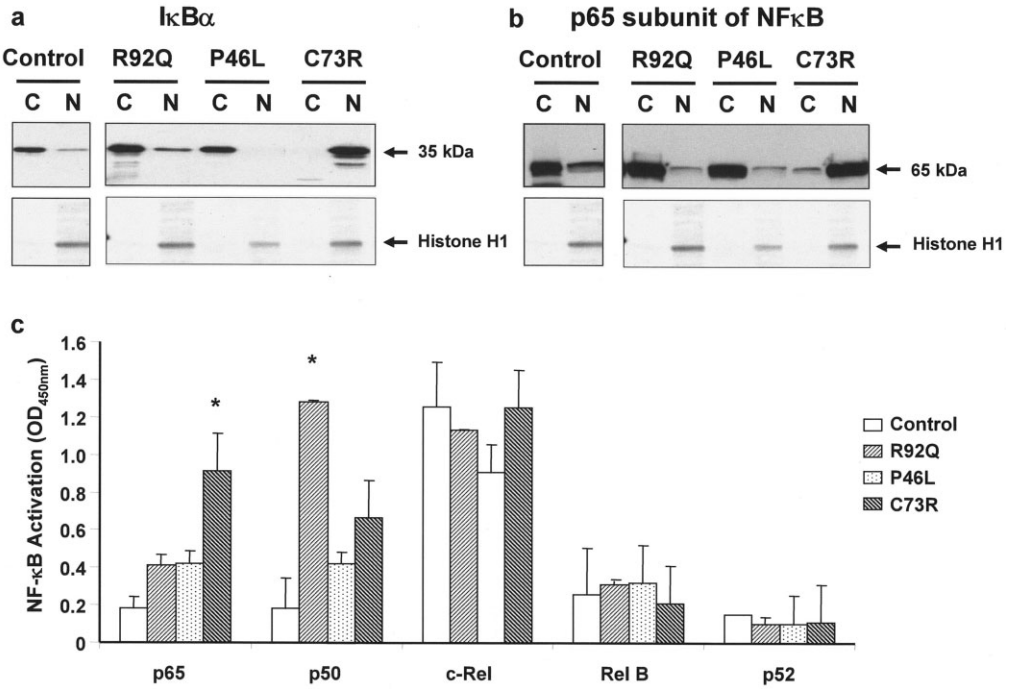

Figure 2. Cells expressing the C73R and R92Q mutations in *TNFR1A* show unusual NF-κB subunit activation. **a** and **b,** Cytosolic (C) and nuclear (N) extracts from patients with the R92Q, P46L, or C73R mutation and from healthy controls were analyzed by Western blotting, using anti-IκBα (**a**) and anti-p65 NF-κB subunit (**b**) antibodies. The subcellular localization of the IκBα and the p65 subunits are represented by a 35-kd band and a 65-kd band, respectively. Histone H1 controls indicate equal loading of nuclear fractions. **c,** Nuclear extracts (1 μg) were incubated with oligonucleotide containing NF-κB consensus binding sites immobilized onto 96-well TransAM enzyme-linked immunosorbent assay plates. NF-κB subunit activation was determined as detailed in Patients and Methods. The results are presented as the mean and SEM and are representative of 4–8 independent experiments involving 2 patients with the C73R mutation, 2 patients with the R92Q mutation, 1 patient with the P46L mutation, and 8 healthy control subjects. OD = optical density. $* = P < 0.001$ versus control.

increase relative to control ($P < 0.001$). There were no significant differences in the activity of other NF-κB subunits when comparing PBMCs from patients with TRAPS mutations and control subjects.

Increased DNA binding of the NF-κB p50 subunit in PBMCs from TRAPS patients with the low-penetrance R92Q mutation. Although subcellular localization of the p65 and IκBα subunits was normal (Figures 2a and b), we used EMSA to determine whether there was active DNA-bound NF-κB in PBMCs from patients with the low-penetrance R92Q mutation. An electrophoretic mobility or gel shift of radiolabel is observed whenever DNA-bound protein forms a complex with radiolabeled oligonucleotide, resulting in the detectable label migrating at a higher apparent molecular weight. The gel supershift assay refers to the additional increase in apparent molecular weight resulting from binding a specific antibody to the DNA-binding protein after reaction with radioactive probe. Hence, through the use of a specific antibody and a consensus oligonucleotide, the presence of a specific DNA-binding protein in any nuclear extract can be identified.

NF-κB DNA–binding patterns showed that the migration of DNA-bound protein extracted from PBMCs possessing the R92Q mutation ran at a lower molecular weight than either controls or other TRAPS mutations (Figure 3a). The specificity of NF-κB DNA–binding complexes was assessed in the presence of excess unlabeled NF-κB oligonucleotides. Almost complete displacement of the NF-κB DNA–binding complex was seen after the addition of 50 ng of cold NF-κB DNA oligonucleotides (Figure 3b). In contrast, 50 ng of unlabeled activator protein 1 DNA oligonucleotides had no effect on NF-κB DNA–binding activity. Taken together, these data indicate that the bands seen in R92Q cells were indeed NF-κB.

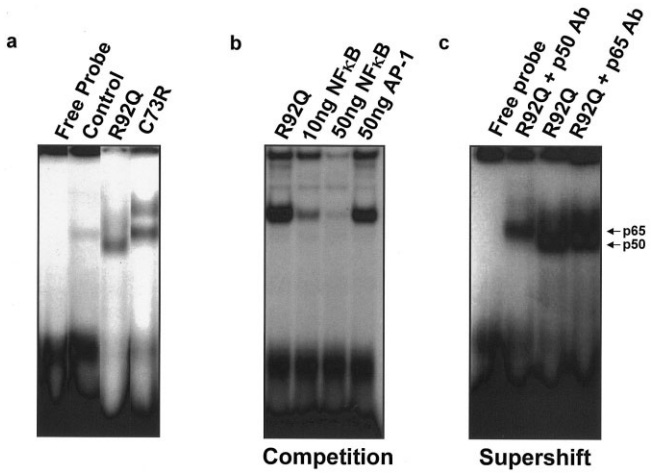

Figure 3. The p50 subunit is the major NF-κB DNA–binding subunit in R92Q cells. Nuclear extracts (1 μg) were incubated with NF-κB ^{32}P-labeled probe (1 ng), and electrophoretic mobility shift assay was performed. **a,** Banding pattern of NF-κB subunits from R92Q, C73R, and control peripheral blood mononuclear cells under resting conditions. **b,** Competition between cold NF-κB or activator protein 1 (AP-1) and endogenous NF-κB. **c,** Nuclear extracts analyzed after running reactions in the absence or presence of anti–NF-κB p50 or anti-p65 (RelA) antibodies. The positions of NF-κB subunit DNA–binding complexes are indicated by arrows. The results shown are representative of a series of 4 independent experiments involving 2 patients with the C73R mutation, 2 patients with the R92Q mutation, and 8 healthy control subjects. Ab = antibody.

The composition of NF-κB subunits associated with R92Q was investigated further, using supershift analysis with antibodies against the p50 and p65 (RelA) subunits of NF-κB (Figure 3c). The addition of anti-p65 (RelA) to the binding reaction mixture resulted in no change to the NF-κB–binding complex. However, the addition of anti-p50 resulted in abolition of the previously observed banding pattern and an upward supershift in the NF-κB DNA–binding complex. This result indicates that p50 is the subunit responsible for the unusual banding pattern observed in Figure 3a.

NF-κB activation after stimulation with TNFα in PBMCs from low-penetrance R92Q mutants. In order to determine how cells with the R92Q mutation respond to ligand activation, we incubated PBMCs from patients with the R92Q mutation and PBMCs from healthy control subjects with TNFα. There was a modest transient increase in p65 subunit activity from baseline, 5 minutes after the addition of TNFα to R92Q cells, as measured in nuclear extracts by ELISA (Figure 4a). However, PBMCs with the R92Q mutation had significantly higher baseline p50 activity compared with controls, which was not increased further after TNFα stimulation (Figure 4b).

TNFRI trafficking and cell surface expression in PBMCs from patients with TRAPS. Immunofluorescence techniques (Figure 5) were used to determine whether abnormal TNFRI localization and activity might have caused the observed increase in NF-κB activity in PBMCs from patients with TRAPS. In control cells, TNFRI was observed as distinct punctate clusters localized within the cell body, consistent with previous data showing that wild-type TNFRI resides predominantly within Golgi storage pools (18–22). Similarly, C73R cells showed no significant cofluorescence with calnexin, as would have been expected were this mutation to lead to a defect in TNFR oligomerization and endoplasmic reticulum (ER) retention. Instead, consistent with the original observations of elevated cell surface expression of TNFRI in TRAPS patients with

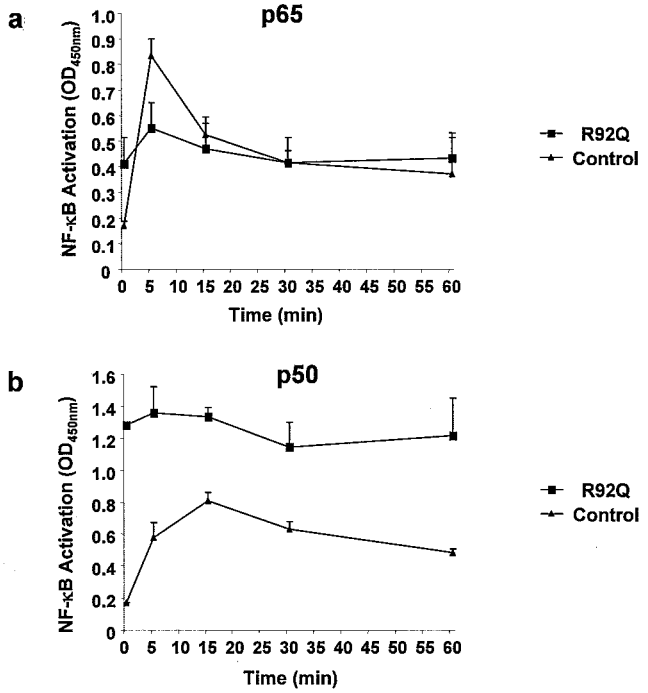

Figure 4. R92Q results in repressed activation of NF-κB in response to stimulation with tumor necrosis factor α (TNFα). Peripheral blood mononuclear cells (PBMCs) were stimulated with TNFα for 5, 15, 30, or 60 minutes. Nuclear extracts (1 μg) from R92Q and healthy control PBMCs were incubated with oligonucleotide containing NF-κB consensus binding sites immobilized onto 96-well TransAM enzyme-linked immunosorbent assay plates. NF-κB subunit binding was determined as detailed in Patients and Methods. Values are the mean and SEM results from 4 independent experiments involving 2 patients with the R92Q mutation and 8 healthy control subjects. OD = optical density.

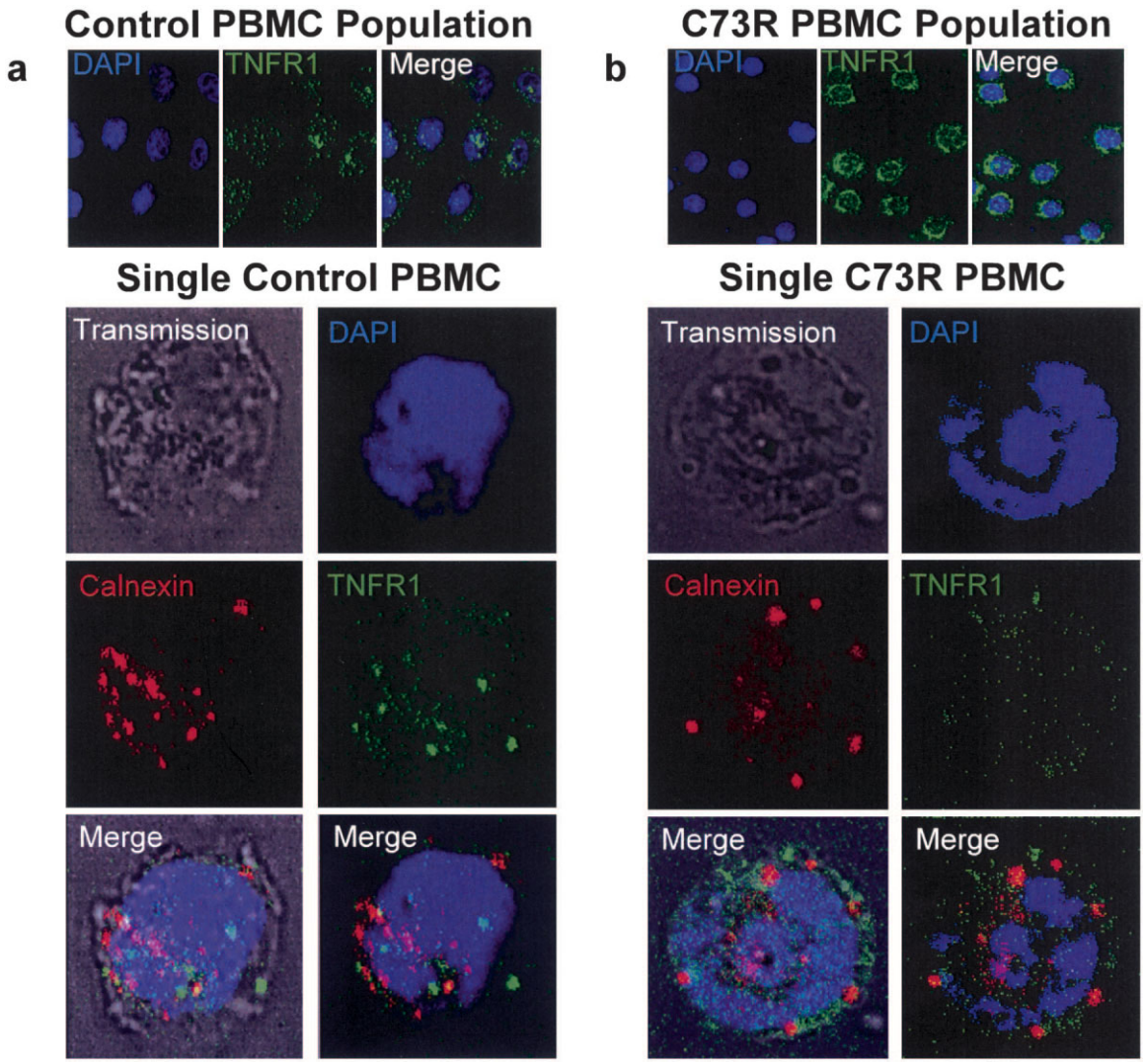

Figure 5. Elevated levels of tumor necrosis factor receptor I (TNFRI) are found at the cell surface in C73R cells. Peripheral blood mononuclear cells (PBMCs) from control and C73R cells were permeabilized and then treated with antibodies against either TNFRI (green), the endoplasmic reticulum marker calnexin (red), or the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI; blue). Images are either single-channel confocal images or merged images between channels or with transmission to define cell perimeter. The results are representative of 4 independent experiments involving 2 patients with the C73R mutation and 8 healthy control subjects.

the C52F mutation (1), we observed that the fully penetrant C73R mutation also resulted in a significant increase in cell surface TNFRI expression compared with control. However, because the polyclonal anti-TNFRI antibody we used was raised against amino acid residues 30–301 of the TNFRI extracellular domain, we cannot formally rule out the possibility that there was ER retention of the C73R mutant receptor that was not detectable with this antibody due to altered protein structure resulting from the C73R mutation. If that were indeed the case, our observations could instead be interpreted as showing a large compensatory up-regulation of wild-type receptor at the cell surface.

In order to confirm the observed differences in TNFRI cell surface expression, we isolated plasma membrane fractions from PBMCs from patients with the C73R, P46L, or R92Q mutation and healthy control subjects, using a plasma membrane ligand-specific binding and pull-down protocol. The validity of plasma membrane isolation was verified through use of antibody

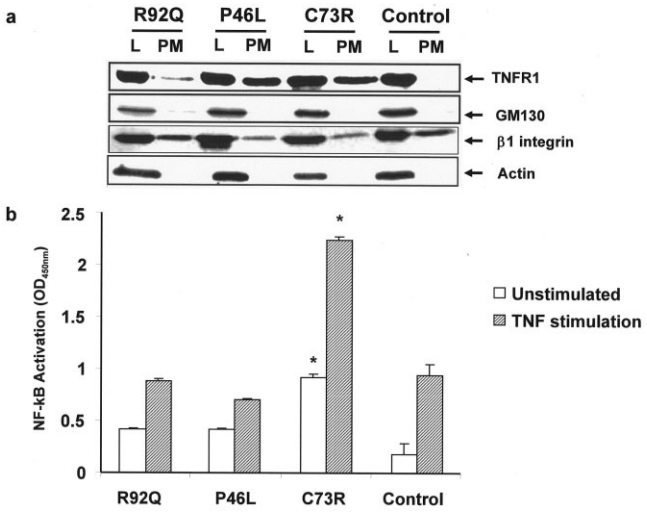

Figure 6. Elevated levels of TNFRI at the cell surface of C73R cells lead to increased NF-κB p65 subunit activation. **a,** C73R, P46L, R92Q, and control PBMCs were lysed into whole cell lysates (L), followed by isolation of plasma membrane fractions (PM), as detailed in Patients and Methods. Samples were run on sodium dodecyl sulfate gels, electrotransferred to polyvinylidene difluoride and immunoblotted with antibody against TNFRI, the Golgi marker GM130, the plasma membrane marker β1 integrin, or actin. **b,** C73R, P46L, R92Q, and control PBMCs were stimulated with TNFα for 5 minutes. Nuclear extracts (1 μg) were incubated with oligonucleotide containing NF-κB consensus-binding sites immobilized onto 96-well TransAM enzyme-linked immunosorbent assay plates, and NF-κB subunit activation was determined as detailed in Patients and Methods. The results are presented as the mean and SEM and are representative of 4 independent experiments involving 2 patients with the C73R mutation, 2 patients with the R92Q mutation, 1 patient with the P46L mutation, and 8 healthy control subjects. OD = optical density (see Figure 5 for other definitions). * = $P < 0.001$ versus control.

against the plasma membrane marker β1 integrin and the Golgi marker protein GM130, the latter excluding the possibility of nonspecific bands arising from the presence of non–plasma membrane microsomal membranes. Consistent with previous studies of patients with the R92Q mutation and healthy control subjects (6), we observed that almost all TNFRI was located intracellularly and not on the cell surface (Figure 6a). In contrast, the plasma membrane fraction of fully penetrant C73R contained a far higher percentage (62.0 ± 2.0%) of total cellular TNFRI when compared with control (5.2 ± 0.5%).

Because the P46L mutation has previously been shown to result in defective TNFRI shedding (6), it is not surprising that we also observed highly elevated levels (86.9 ± 4.5%) of TNFRI in the plasma membrane fraction of these cells (Figure 6a). However, if the presence of elevated levels of TNFRI at the cell surface was a determining factor leading to full penetrance, then one would expect patients with the P46L mutation to exhibit more aggressive TRAPS symptoms. In order to resolve this apparent discrepancy, we examined the ability of P46L, C73R, and healthy control cells to respond to TNFα stimulation (Figure 6b). C73R cells had elevated basal NF-κB activity but also showed an elevated response to TNFα stimulation, consistent with the presence of increased levels of active receptor at the cell surface. In contrast, P46L cells showed no additional increase in response to TNFα above control stimulation.

DISCUSSION

Early reports suggested that a TNFRI shedding defect was one of the principal molecular mechanisms resulting in TRAPS (1). However, it is still unclear whether impaired cytokine receptor clearance is the pathophysiologic basis for a majority or just a subset of TRAPS-associated mutations. Several recent studies using transfected cells suggested other possible mechanisms of the disease: reduced cell-surface expression of mutant receptors (13), an abnormal oligomerization with ER retention (14), decreased binding of mutant forms of receptor to TNFα (12), ligand-independent signaling (12,16), and a reduction in TNF-induced apoptosis (23,24). Although some of the findings were consistent among different groups, others were variable, most probably due to various experimental conditions (e.g., full-length versus truncated mutant constructs, different cell types used for in vitro transfections). Moreover, few studies used mutant cells from patients for ex vivo experiments, and there also has been some disagreement regarding apoptosis and shedding defects that may be explained by fibroblast versus PBMC cell type effects (25).

In addition to the above, one of the major unresolved issues is whether the NF-κB pathway is up-regulated or down-regulated in the presence of TRAPS-associated mutations. The NF-κB transcription factor consists of functional homodimers or heterodimers composed of its 5 monomeric family member subunits: RelA (p65), RelB, c-Rel, p50, and p52. TNFα is a widely studied inducer of NF-κB activity, with active NF-κB in turn modulating the expression of a range of genes associated with inflammation and immune responses (Figure 1). TNFα activity is mediated through 2 receptors, TNFRI and TNFRII, with mutations in the extracellular domains of the former being associated with the chronic inflammatory disorder, TRAPS. In the

current study, we set out to examine NF-κB activity in PBMCs from patients with 3 *TNFRSF1A* mutations (C73R, P46L, and R92Q), in order to determine whether either TNFRI subcellular localization or intracellular signaling abnormalities could explain the variability in disease penetrance associated with these mutations.

Increased expression of the p50 subunit has previously been shown to play a role in the development of tolerance to lymphotoxin (26). Indeed, in lipopolysaccharide-tolerant monocytes, it has been shown that there is a 3-fold increase in p50 mobilization to the nucleus but no change in p65 status (27). Although both p50 and p65 bind DNA through a shared Rel homology domain, p65 also contains C-terminal transcription activator domains that are absent in p50. This has led to the suggestion that p50/p50 homodimers act as transcription repressors (28). Our data here show that in R92Q cells, basal levels of p65 were modestly elevated, which might in part help explain why this mutation can sometimes lead to disease association; nonetheless, basal levels of the p50 subunit were far higher. Moreover, in R92Q cells, the level of p65 is only very modestly elevated in response to TNFα stimulation relative to control cells. This likely reflects the presence of large quantities of p50/p50 homodimers bound to DNA of R92Q cells, thereby preventing significant high-penetrance immune activation.

It should be noted, however, that in addition to the aforementioned repressor action of p50, there are additional nuclear functions of p50. The IκB family member Bcl-3 performs a role similar to that of other IκB family members in removing NF-κB subunits from DNA. However, Bcl-3 also has the ability to form activating complexes with p50 on DNA (29,30). Therefore, although p50 lacks a transcription activator domain, it might nevertheless still act as a transcription activator through Bcl-3 complex interactions. Indeed, p50 has been shown not only to interact with Bcl-3, but through this complex it can also interact with several additional transcription factors (31). Thus, the consequence of such abundant p50 localization to the nucleus in R92Q cells might be serving as a general proinflammatory activator through a number of off-target interactions, as well as acting to repress p65 NF-κB subunit signaling. Moreover, this might perhaps explain why there are low-penetrance associations between R92Q and several inflammatory diseases.

In contrast to R92Q cells, the C73R mutation in TNFRI results in significant translocation of the active p65 subunit of NF-κB. Furthermore, these data are highly reproducible, being pooled from 8 independent experiments using freshly isolated PBMCs from a mother and her daughter who possess this mutation. As is the case with C52R (32), fluorescence-activated cell sorting analysis of monocyte cell surface following proteinase activation has previously shown that there is no shedding defect in C73R cells (33). The results presented here show that cell surface localization can be influenced by mutations within the cysteine-rich domain (CRD), CRD2. This raises the possibility that there is a Golgi retention motif within this region, or in an area of the molecule that is influenced by mutations in this region, and that the C73R mutation abolishes this retention signal. Further study of other fully penetrant TRAPS mutations is, however, needed before we can accurately determine whether this is a common phenomenon of TRAPS mutations or is instead a specific characteristic of C73R.

The resulting effect of the C73R mutation is initiation of the NF-κB pathway, and this therefore links TNFRI cell surface expression to inflammation status. Interestingly, the P46L mutation would be predicted to alter bending in the protein secondary structure, which likely introduces a conformational change in loop 3 of the highly conserved CRD1 (6,34). We suggest that although P46L cells contain high levels of TNFRI at the cell surface, they fail to show an enhanced response to inflammatory stimulation above control due to inactive receptor. We cannot, however, rule out the possibility that the mutation of mevalonate kinase in this patient contributed to the current findings, and thus the results should be interpreted with some caution. Nonetheless, in a separate patient with only the P46L mutation, it was previously demonstrated that P46L results in a TNFRI shedding defect (6) that is consistent with the current observations. Further study is required to determine precisely how TRAPS-associated conformational changes in TNFRI impact receptor assembly and trimerization.

In conclusion, this study is the first to show that the R92Q mutation associated with low-penetrance TRAPS likely results from p50/p50 NF-κB homodimer repression. The P46L mutation, however, does not alter p50 signaling but instead renders TNFRI nonfunctional. Because these observations were made on cells from patients with TRAPS and either the R92Q or P46L mutation, it would be of considerable interest for future studies to investigate TNFRI functionality and NF-κB signaling in asymptomatic carriers of these mutations. In contrast, the fully penetrant C73R mutation results in persistent elevated localization of functional TNFRI at the cell surface and is associated with

increased TNFα induction of the proinflammatory intracellular NF-κB pathway. Thus, variation in NF-κB activity in PBMCs from patients with different TNFRI genotypes provides an explanation for the observed variation in clinical phenotype.

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AUTHOR CONTRIBUTIONS

Dr. Turner had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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