membranes to permeabilization, then low NF-κB activity levels might result from subunit leakage out of these cells. Conversely, failure to permeabilize sufficiently would exclude antibody from entry into the nucleus, again producing artefactually low signals. As a consequence this technique is at best highly unreliable. As such, without supporting confirmation of NF-κB activity data through established and well-characterized methods, such as electrophoretic mobility shift assay or ELISA of nuclear extracts, one must remain skeptical of the current data.

Finally, the authors speculate that their NF-κB signalling data might be a consequence of defective receptor folding and endoplasmic reticulum (ER) retention, although they present no data to support this hypothesis. The C73R variant at least shows no sign of ER retention, however, as we have previously shown using confocal microscopy that C73R cells stained heavily for TNFR1 at the cell surface, while there was very little co-fluorescence with the ER marker calnexin [3]—as would have been evident, were the molecule misfolded and retained within the ER as hypothesized. In addition, the authors cite a previous study of ours [5] (where the TNFR1 mutant studied was mainly the T50K variant) to suggest that TNF-independent signalling might be a consequence of TNFR1 misfolding in the ER, and subsequent intracellular mutant TNFR1 aggregate formation and signalling. However, the receptor aggregates observed in that study were identified through chemical cross-linking by reagents that are unable to cross the plasma membrane. As such, we can confidently state that the TNFR aggregates under study were localized entirely at the cell surface, strongly arguing against ligand-independent signalling that is linked to ER retention.

It is also important to note that different mutant TRAPS genotypes have subtle mechanistic differences, and other genes may compensate for a final phenotype in this disease. Generalizing that all genotypes have a similar mechanism of action in different cell types is insufficiently rigorous to warrant full understanding of this complex trait.

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Comment on: Low TNF-induced NF-κB and p38 phosphorylation levels in leucocytes in tumour necrosis factor receptor-associated periodic syndrome: reply

Sir, We thank Turner and Chernajovsky [1] for their interest in our study [2]. First, the authors emphasize that in their study [3] C73R cells from two Finnish tumour necrosis factor receptor-associated periodic syndrome (TRAPS) patients were handled in the laboratory concurrently and identically with the cells of healthy controls, which would exclude the possibility of artefactual Nuclear factor kappa B (NF-κB) activation, a point on which we agree. However, we emphasize that the artefactual activation may have occurred during transportation before the TRAPS blood samples arrived at the laboratory. Namely, the patient-derived fresh blood samples were both sent from Finland with a courier to London in the spring and summer of 2005. The blood sample of one of the patients was obtained in Raahe, a city 400 miles north of Helsinki and the sample of the other one in Helsinki. Both samples were collected in commercially available tubes supplemented with EDTA as instructed by the collaborating group. Such tubes, however, have been reported to be contaminated with bacterial lipopolysaccharides (LPS), which promote leucocyte activation [4]. Monocytes, like neutrophils, contain small intracellular vesicles [5], whose membranes contain CD11b/CD18 heterodimers and probably TNFR1 molecules [6]. Upon activation, the vesicles degranulate, in other words fuse with the plasma membrane thereby increasing the cell surface expression of CD11b/CD18 and other vesicle membrane components, including TNFR1, which would result, along with increased surface expression, in decreased intracellular expression of these molecules. Decreased
Fig. 1 Validation of p65 antibody in blood samples of healthy subjects using immunoblot (A, B, C, D), phospho-specific flow cytometry (E, F), EMSA (G) and ELISA-based NF-κB assay (H). Whole-blood samples from healthy donors were stimulated for 0–60 min with TNF-α (10 ng/ml) at 37°C. Cell lysates (erythrocytes removed) were prepared for immunoblot analysis and the same, but unlabelled, primary antibody against phosphorylated p65 (p65 pS529) was used as in our flow cytometric analyses. (C) Densitometric analysis of four similar immunoblots is shown below the blot. Results are given relative to total p65 and corrected with a house-keeping protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (E) Time response of TNF-α stimulated NF-κB p65 phosphorylation (p65 pS529) in monocytes of healthy subjects measured by phospho-specific flow cytometry (n = 6). (B) Stimulation of whole-blood samples with different concentrations of TNF-α for 10 min. Antibodies and analyses are the same as in (A). (D) Densitometric analysis of TNF-α dose response (n = 4) and (F) respective phospho-specific flow cytometry results (n = 6). (G) Mononuclear samples were stimulated with TNF-α (10 ng/ml) for 20 min and nuclear lysates were prepared for EMSA analysis to show interaction with κB oligonucleotide binding motif. Excess of unlabelled wild-type or mutant oligonucleotide in the binding reaction was used for validation. (H) Mononuclear samples were stimulated with TNF-α for different time periods and nuclear lysates were prepared for ELISA based NF-κB transcription factor activity assay.
intracellular expression of TNFR1 in C73R cells is exactly what Turner and Chernajovsky [1] report in their study. As stated by the authors [1], crucially, the finding was confined to C73R cells. This may reflect the long distance that the blood samples had travelled. The transit time, at the room temperature, was 24 h for the Helsinki sample and 48 h for the Raase sample. Against this background, and the fact that EDTA itself may rapidly interfere with the plasma membrane integrity [4], it is obvious that the blood samples should have been collected in LPS-free tubes supplemented with anti-coagulant, such as LPS-free acid-citrate-dextrose. Secondly, to check the effect of transportation on cell activation, blood samples from (i) two to three healthy subjects in Raase, (ii) two to three healthy subjects in Helsinki and (iii) two to three healthy subjects in London, immediately before separating leucocytes, should have been requested and included in the study. Finally, the degree of phagocyte activation could have been easily monitored using cellular markers, such as the integrin CD11b/CD18 expression level on neutrophils and monocytes [4]. As to our study, we worked with absolutely fresh blood samples, in other words 10 patients and 10 healthy controls donated their blood on site in the laboratory [2], using a protocol, which we had optimized in terms of anti-coagulant-acceptable storage before cell stimulation. Thus, in the absence of the above obligatory controls, it is not possible to decide whether the findings of the Turner group [3] represent innate features of the C73R cells or artefactual ex vivo activation of the cells.

Another important aspect raised by Turner and Chernajovsky involves phospho-specific flow cytometry, the novel method that we used in our study [2]. Membrane permeabilization differences, as suggested by Turner and Chernajovsky, were not likely since the LPS-induced NF-κB and p38 phosphorylation of the patients and the reference subjects were comparable. After the TRAPS study, we have found, using the same method, that NF-κB activation is depressed in patients with acute pancreatitis complicated by immune suppression in monocytes [7], commented in [8] and lymphocytes [9], commented in [10], but not in patients with active SpA and those with early, untreated RA [Alanänä et al., 2011, data not published]. In order to further validate our flow cytometry results and specificity of the antibodies, we have recently performed western blot analysis with TNF-α stimulated whole-blood samples from healthy donors. The immunoblot analysis of whole-cell lysates showed a dose-dependent increase in phosphorylated p65 levels with maximal activation at 0.1 ng/ml and maximal stimulation time of 5 min (Fig. 1A–E). These values are comparable with those obtained by phospho-specific flow cytometry, where the maximum stimulation was obtained with 1 ng/ml TNF-α stimulation and the maximal stimulation time was 5 min (Fig. 1C and F). We have also shown with electrophoretic mobility shift assay (EMSA) that 20 min of TNF-α (10 ng/ml) stimulation leads to nuclear activation of NF-κB and binding to NF-κB consensus sequence (Fig. 1G) and that the maximum stimulation time in binding is 20 min (Fig. 1H). We have also published an extensive validation of the method in a supplement available online [7].

In summary, the data and the comments by other authors [8, 10] give credence to the view that the phospho-specific flow cytometry data are meaningful and may not be explained by differences in cell permeabilization only. Finally, we agree that EMSA/ELISA of nuclear extracts is the golden standard and are currently working on both methods whenever possible in studies of clinical samples. Unlike stated by Turner and Chernajovsky, we have not generalized our findings, but reported low phosphorylation levels in the C88Y and F112I families studied, while the phosphorylation pattern was low in one of the two C73R patients and completely normal in the other one. Thus, as we discussed [2] and as stated by Turner and Chernajovsky [1], it is important to note that different TRAPS genotypes may show differences and that other genes and pathways may compensate for the clinical phenotype.

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Comment 1 on: Homeopathy has clinical benefits in rheumatoid arthritis patients that are attributable to the consultation process but not the homeopathic remedy: a randomized controlled trial

Sir, Brien et al. [1] conclude from the results of their randomized controlled trial that the clinical benefits of homeopathy in ‘rheumatoid arthritis patients are attributable to the consultation process but not the homeopathic remedy’ [1], a conclusion that is embraced with enthusiasm by the editor. I, however, am concerned to see such a conclusion drawn from the results of under half of the secondary outcomes (6/14), and in disregard of the results of both the primary outcomes and the majority (8/14) of secondary outcomes (Tables 5 and 6). Is this how randomized controlled trials are to be analysed in the future?

The article also states that the intervention delivered truly reflected normal clinical practice [1]. I cannot see how this can be the case, as I know of no normal clinical practice [1] where patients are told that there is a one in two or three chance that they will receive a dummy or placebo treatment. Indeed, this patient information may have been the reason for the under-recruitment to this trial.

Despite only 75% (83/110) of the patients required by the original sample size calculation being recruited, if these results are compared with the results reported with other homeopathy trials, there are some interesting results. For instance, despite blinding being secure, patients in the individualized consultation group (Group 1) reported at least 30% more adverse events than patients in any of the other groups. This is similar to a finding from a systematic review of homeopathy randomized controlled trials [2], which found that the mean incidence of adverse effects of homeopathic medicines was greater than those for placebo in controlled clinical trials (ratio 9.4/6.1).

A repeat study is clearly needed to address the researchers’ important and interesting question again. However, any repeat study must be able to recruit the required number of patients, taking into account in the design the potential impact of the information provided to patients during the recruitment and trial processes, using the standard methods in analysing and reporting the results.

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Comment 2 on: Homeopathy has clinical benefits in rheumatoid arthritis patients that are attributable to the consultation process but not the homeopathic remedy: a randomized controlled clinical trial

Sir, We read with interest the article by Brien et al. [1] published recently in this Journal. The authors conclude that homeopathic consultations, but not homeopathic remedies, are associated with clinically relevant benefits for patients with active but relatively stable RA. In the same edition, Ernst in his editorial [2] suggests that we should take these results at face value; that homeopathic remedies are ineffective and empathetic therapeutic encounters are helpful.

Brien et al. [1] sought to assess whether any benefits from adjunctive homeopathic treatment in patients with RA are due to the homeopathic consultation, homeopathic remedies or both. Participants in the trial were randomly allocated to consultation [further randomized to either individualized homeopathy (Group 1) or the complex homeopathic medicine Rheumaselect (Group 2) or placebo...