Title:

Patients with TNF Receptor Associated Periodic Syndrome (TRAPS) are hypersensitive to Toll-like receptor 9 stimulation.

Subtitle:

TLR9 hypersensitivity in TRAPS.

Authors:

1- Ola H. Negm*, School of Medicine, Queen's Medical Centre, University of Nottingham, Nottingham, NG7 2UH, United Kingdom; Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Mansoura, 35516, Egypt.

2- Sonali Singh*, Immunology, School of Life Sciences, University of Nottingham, Nottingham NG7 2UH, United Kingdom.

3- Wesam Abduljabbar, Immunology, School of Life Sciences, University of Nottingham, Nottingham NG7 2UH, United Kingdom.

4- Mohamed R Hamed, School of Medicine, Division of Primary Care, University Park, University of Nottingham, Nottingham, NG7 2RD, UK.; Medical Microbiology and Immunology Department, Faculty of Medicine, Mansoura University, Mansoura, 35516, Egypt

5- Paul Radford, Immunology, School of Life Sciences, University of Nottingham, Nottingham NG7 2UH, United Kingdom.

6- Elizabeth M. McDermott, Nottingham University Hospitals National Health Service Trust, Queen's Medical Centre Campus, Nottingham, NG7 2UH, United Kingdom.

7- Elizabeth Drewe, Nottingham University Hospitals National Health Service Trust, Queen's Medical Centre Campus, Nottingham, NG7 2UH, United Kingdom.
8- Lucy Fairclough, Immunology, School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom.
9- Ian Todd, Immunology, School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom.
10- Patrick J. Tighe#, Immunology, School of Life Sciences, University of Nottingham, Nottingham NG7 2RD, United Kingdom.

*** Joint first authors.**

 # Corresponding Author

Dr Paddy Tighe, School of Life Sciences, Life sciences Building, University Park Campus, University of Nottingham, Nottingham NG7 2RD, England, UK.

Email: paddy.tighe@nottingham.ac.uk

Summary:

Tumour necrosis factor receptor-associated periodic syndrome (TRAPS) is an hereditary autoinflammatory disorder characterised by recurrent episodes of fever and inflammation. It is associated with autosomal dominant mutations in *TNFRSF1A*, which encodes tumour necrosis factor receptor-1 (TNFR1). Our aim was to understand the influence of TRAPS mutations on the response to stimulation of the pattern recognition receptor TLR9. Peripheral blood mononuclear cells (PBMCs) and serum were isolated from TRAPS patients and healthy controls: Serum levels of fifteen pro-inflammatory cytokines were measured to assess the initial inflammatory status. IL-1β, IL-6, IL-8, IL17, IL22, TNF-α, VEGF, IFN-γ, MCP-1 and TGF-β were significantly elevated in TRAPS patients sera, consistent with constitutive inflammation. Stimulation of PBMCs with TLR9 ligand (ODN2006) triggered significantly greater upregulation of pro-inflammatory signalling intermediates (TRAF3, IRAK2, TOLLIP, TRAF6, pTAK, TAB2, pTAB2, IRF7, RIP, NF-kB p65, pNF-κB p65, and MEK1/2) in TRAPS patients' PBMCs. This upregulation of proinflammatory signalling intermediates and raised serum cytokines occurred despite concurrent anakinra treatment and no overt clinical symptoms at time of sampling. These novel findings further demonstrate the wide-ranging nature of the dysregulation of innate immune responses underlying the pathology of TRAPS and highlights the need for novel pathway-specific therapeutic treatments for this disease.

Introduction:

The rare autoinflammatory disease, Tumour Necrosis Factor Receptor Associated Periodic Syndrome (TRAPS) is caused by autosomal dominant missense mutations in the *TNFRSF1A* gene, encoding TNFR1 [1]. TRAPS patients demonstrate exaggerated innate immune responsiveness and are characterised clinically by recurrent prolonged episodes of fever, migratory rash, peritonitis, myalgia, and arthralgia, with systemic amyloidosis occurring in a proportion of patients [2]. Intracellular aggregation of mutant TNFR1 plays a major role in the pathogenesis of TRAPS through a variety of mechanisms including: misfolding and ligand-independent signalling, an unfolded protein response, induction of reactive oxygen species (ROS) and impaired autophagy [3, 4]. Both transfected cells and TRAPS patients' PBMCs expressing a prototypic TRAPS-related mutant TNFR1 (C33Y), reveal perturbations and activation of multiple pro-inflammatory signalling pathways[5]. Thus, mutant TNF receptors create an intracellular environment conducive to drive chronic inflammation and synergy between mutant and wild-type TNFR1 actions is required to generate the full TRAPS phenotype [6, 7].

An area of increasing interest is the cross-talk between mutant TNFRs and pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs) involved in innate immune activation. It has recently been proposed that cells with TRAPS-related mutant TNFR display greater sensitivity to PRR ligands such as the TLR4 ligand, bacterial lipopolysaccharide (LPS), due to excessive activation of downstream signalling through the mitogen-activated protein kinases (MAPKs), resulting in enhanced production of pro-inflammatory cytokines [8].

TLR9 is an endosomal PRR which recognises unmethylated CpG-rich DNA motifs, frequently found in bacterial, mitochondrial and viral DNA. TLR9 activation initiates innate immune responses via the adaptor molecule MyD88, and subsequently IRAK4 and TRAF6, and ultimately activation of IRF7, NFκB, JNK and P38 MAP kinases, ultimately leading to the

production of inflammatory cytokines and type 1 interferons[9].

This study investigates how the host innate immune response to TLR9-stimulation is influenced by the TRAPS-associated C33Y mutant TNFR1 ($TNFR1_{(C33Y)}$). Venous blood from patients with $TNFR1_{(C33Y)}$ and controls were examined for serum cytokine levels to establish baseline levels of inflammatory mediators, and the TLR9 agonist (ODN2006) was used to stimulate isolated peripheral blood mononuclear cells (PBMCs) from both sample groups and the expression and activation of pro-inflammatory signalling pathways was subsequently evaluated using protein microarray technologies [10].

Material and Methods:

Patients and Healthy Controls:

Four TRAPS patients carrying $TNFR1_{(C33Y)}$ [2 males, 2 females; mean age of 46.3 (range 33-66)] and five healthy controls [2 males,3 females; mean age of 43.5 (range 27-62)] were included for TLR9 signalling pathway studies in PBMCs. For cytokine assays, seven controls sera [3 males, 4 females; mean age of 48 (range 27-62)] were compared to the patients' samples. All TRAPS patients were being treated with anakinra and none had any clinical symptoms of inflammation at the time of blood sampling.

Serum collection and PBMC separation:

Blood samples were collected after ethical approval and with informed consent, into citrate phosphate dextrose (ACD; Baxter Health Care, Norfolk, UK) and processed within 2h of sampling. PBMCs were isolated by ficoll-density gradient centrifugation. PBMCs were resuspended in serum-free RPMI 1640, containing 25mM HEPES and L-glutamine, at a density of $2x10^5$ cells/ml. Cells were exposed to 5µM ODN 2006 (InvivoGen, Toulouse, FR) for 2h, cells were then washed with ice-cold PBS, pelleted and lysed with 100 µl of RIPA lysis buffer containing 5 units/ml benzonase nuclease (Sigma-Aldrich, UK) and 1X Halt™ Protease and

Phosphatase Inhibitor Cocktail (Fisher Scientific, UK) for 20m with continuous agitation on ice.

TLR9 expression in PBMCs from TRAPS and healthy controls:

Cells were stained for flow cytometry as previously described [11] to evaluate TLR9 expression. Following washing with PBA (PBS / 1% BSA / 0.05% NaN_3), the PBMCs were permeabilised using saponin buffer and stained for 2h in the dark with anti-human CD289 (TLR9) PE or appropriate isotype controls (rat IgG2a PE) (e-Bioscience). Cells were then washed twice in PBA, resuspended in 0.5% formaldehyde fixative and analysed on a Coulter FC500 flow cytometer. Analysis was performed using WEASEL software (Walter and Eliza Hall Institute, Parkville, Australia).

Reverse Phase Protein Arrays (RPPA) analysis:

RPPA was used as optimised and validated previously[5]. Briefly, PBMC lysates were diluted 3+1 with 4x SDS sample buffer (278mM Tris.Cl, Ph 6.8, 44% glycerol, 4.4% SDS, 8% 2-mercaptoethanol) and heated (95°C for 5minutes). Samples were spotted in quadruplicate onto nitrocellulose-coated glass slides (Grace Bio-labs) with a MicroGrid II robot (BioRobotics Inc., USA). The printed slides were blocked overnight at 4°C in blocking solution (0.2% I-block (Tropix, Bedford, MA, USA), 0.1% Tween-20 in PBS) at 4°C. After washing three times for 5 minutes each in PBS,+0.05% Tween-20 (PBST), the slides were incubated overnight at 4°C with the specific primary antibodies (Cell Signaling Technology, USA). Supplementary table 1S shows the details of the primary antibodies used for RPPA analysis. β-actin antibody was used as a housekeeping protein to control protein loading. After washing in PBST (3x), primary antibody detection was achieved using infrared Licor secondary antibodies (800-CW anti-rabbit antibody and 700-CW anti-mouse antibody) for 30 minutes at room temperature in the dark with shaking. After washing in PBST 3x and rinsing in water, arrays were centrifuged to dry,

and scanned with a Licor Odyssey SA scanner (LI-COR Biosciences, UK) at 20 μm resolution. Fluorescence images were processed with Axon Genepix Pro-6 Microarray Image Analysis software (Axon Instruments Inc.). Target signals were finally determined after background subtraction and normalization to the internal housekeeping targets using RPPanalyzer, a module within the R statistical language [12]. The specificity of all the primary antibodies was verified using gel electrophoresis and western blotting as described previously [13]. Only antibodies detecting single bands of the correct molecular weight target were used.

Cytokine analysis:

Cytokines were analysed in sera from TRAPS patients and controls using antibody microarray based sandwich ELISA. DuoSet paired antibody kits (R&D Systems, Minneapolis, MN) were used for the detection of 16 cytokines: eotaxin-1, eotaxin-2, IFN-γ, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17, IL-22, MCP-1, RAGE, TGF-β, TNF-α, and VEGF [10]. In brief, capture antibodies were printed at 100 μg/mL in quadruplicate as regular arrays (16 per slide) onto poly-L-lysine-coated glass slides (Thermo-Fisher, UK) using a MicroGrid II robot. The array slides were blocked with I-Block (0.2% in PBS) for 1h and washed three times with PBST. A cocktail of recombinant cytokine standards was prepared with each cytokine at the maximum recommended standard curve concentration, and then used to generate eight doubling dilutions. Standard dilutions and samples were added to independent arrays for 1h at room temperature with frequent shaking followed by three washes with PBST. A mixture of the manufacturers biotinylated detection antibodies was added at the recommended concentrations to each array for 1h with shaking. After washing with PBST as before, A 1:1:300 mixture of Genisphere Streptavidin 3DNA Oyster 650-40 : Nucleic Acid Blocker (Genisphere UltraAmp, UK): Antibody diluent (Dako, Ely, UK was added and incubated for 30 minutes at room temperature with frequent shaking in the dark. Slides were then washed three times with PBST, rinsed with ultra-pure water and dried by centrifugation. Slides were scanned at 635 nm with a Genepix

4200AL scanner (Axon Instruments) and fluorescence signals quantified using Axon Genepix Pro-6 software. The concentration of each cytokine in samples was subsequently extrapolated from the standard curve values.

Statistical analysis:

Statistical analyses were performed using GraphPad Prism 6 software. One-way ANOVA and Bonferroni's multiple comparisons test was undertaken to compare signalling molecules expression in different conditions. The statistical differences in cytokine expression and TLR9 expression between patient and control samples were determined by Mann-Whitney test.

Results:

In vivo constitutive inflammatory stimulation in TRAPS patients:

Fifteen inflammatory cytokines and chemokines was measured in the sera of TRAPS patients with $TNFR1_{(C33Y)}$ and age/sex-matched healthy controls to determine the underlying inflammatory status of each patient at the time of PBMC collection (Figure 1). Significantly higher levels of the following cytokines were observed in TRAPS patients compared to the healthy controls: IL-1β, IL-6, IL-8, IL-17, IL-22, TNF-α, VEGF, IFN-γ, MCP-1 and TGF-β (Figure 1a,c,d,f,g,h,i,m and o). Eotaxin-1, RAGE, IL-10 and IL-4 were not significantly different between the groups (Figure 1b,e,n) and eotaxin-2 was significantly higher in the controls (Figure 1k). These findings support and extend previous studies[14-17] by revealing the presence of additional pro-inflammatory serum cytokines/chemokines (IFN-γ, IL17, IL22 and MCP1) in TRAPS patients and also increased serum TGF-β and VEGF.

Deleted: [14, 15][14-17]

Ex vivo constitutive activation of inflammatory pathways in TRAPS patients:

TRAPS patients' PBMCs in the absence of external stimulation have already been shown to exhibit enhanced activation of pro-inflammatory signalling pathways [13]. In this study, we therefore investigated the influence of $TNFR1_{(C33Y)}$ on responses to TLR9 stimulation isolate PBMCs. Binding of the synthetic agonist ODN2006 to TLR9 triggers a complex series of intracellular signal transduction events[9]. Concurrent evaluation of target protein expression and activation related post-translational modifications were measured using reverse-phase protein arrays. PBMCs from TRAPS patients with $TNFR1_{(C33Y)}$ and from healthy controls were cultured with or without ODN2006, lysed and 49 signalling intermediates, including

TLR9 pathway-associated molecules were subsequently measured. β-actin abundance was measured as a normalizing protein control. Supplementary table 1S shows the list of the primary antibodies used in this study. Comparisons were then made between the $TNFR1_{(C33Y)}$ patient samples and control samples, both before and after ODN2006 stimulation (Figure 2). Table 1 (column A) shows that 36 out of the 49 signalling molecules measured revealed significantly higher baseline levels in untreated TRAPs patients' PBMCs compared to untreated PBMCs of healthy controls. Similarly, comparison of ODN2006-stimulated PBMCs revealed 42/49 signalling molecules detected at significantly higher levels in patients' PBMCs compared to healthy controls (table 1, column B). However, the most important finding derives from comparisons between ODN2006-stimulated PBMCs and unstimulated cells: for the TRAPS patients, 17/49 signalling molecules were significantly raised by stimulation with ODN2006 (table 1, column C), but only 4/49 signalling molecules were significantly raised in the healthy control PBMCs, only one of which (IRAK4) was not also up-regulated in the patients' cells (table 1, column D).

TLR 9 expression in PBMCs from TRAPS patients and healthy controls:

To establish whether the hypersensitivity of the TLR9-stimulated response seen the $TNFR1_{(C33Y)}$ patients PBMCs might be due to differential TLR9 expression, we compared TLR9 expression in PBMCs of patients and healthy controls by intracellular staining and flow cytometry. There was no significant difference in TLR9 expression between the two groups as measured by the mean fluorescence intensity (MFI) or the percentage of TLR9 positive cells (supplementary figure 1S). Thus, the hypersensitivity of the TRAPS patients' PBMCs to ODN2006 is not due to increased levels of TLR9.

Discussion:

TRAPS and the other autoinflammatory diseases reflect primary dysfunctions of innate immunity, with increasing support suggesting a complex interplay of inflammatory signals and inflammasome involvement resulting in the clinical presentations seen[18,19]. We hypothesised that the effects of the TRAPS mutant TNFR1 and any subsequent TLR activation (TLR9 used here), may synergise to exacerbate pro-inflammatory responses in TRAPS.

The TRAPS patients studied, all heterozygous for $TNFR1_{(C33Y)}$, were on anakinra (IL-1 receptor antagonist) therapy at the time of sampling and were without clinical symptoms of inflammation. These patients nevertheless demonstrate increased serum levels of multiple pro-inflammatory cytokines, including IL1-β, IL6, IL8 and TNF-α relative to healthy controls samples. The elevated levels of IL1-β seen also give further support to the role of inflammasome activation and IL1-β release in TRAPS, as with many other autoinflammatory diseases[18,20], and emphasizes the usefulness of therapeutics targeting IL1 responses, despite TRAPS being a TNFR1mutation-associated disease[21].

In addition our results also revealed increased serum levels of Interferon-γ, IL17 and IL22, suggestive of additional pro-inflammatory responses, most likely from NK and T cell populations[22] and of VEGF and the pleotrophic cytokine TGF-β, although their relevance in TRAPS disease pathology is currently unclear. The raised levels of MCP1 seen in patients may be a consequence of the elevated IL1-β, IL6 and TNF-α and may have some bearing on muscle inflammation in TRAPS[23,24]. Our findings from patient sera therefore highlight the complexity of the constitutive inflammatory status of TRAPS

patients, even when clinically well and maintained on a cytokine neutralization therapy that does not target the central disease mechanisms [16].

TLR9-ligation by appropriate DNA ligands leads to stimulation of IRF7, NFκB, JNK and P38 MAP kinase pathways, and can also prime the NLRP3 inflammasome by increasing expression of both NLRP3 and pro-IL1-β. Similar expression levels of TLR9 in PBMCs from TRAPS patients and healthy controls were observed, however heightened expression and activation of intracellular signaling molecules are seen in PBMCs of the $TNFR1_{(C33Y)}$ patients. These include, as expected, molecules involved in the TLR9 signal transduction, including key adaptors such as TRAF6 and, as we have reported before, many elements of the NFκB associated with TNFR1 signalling (figure 2, I and fig 2, II k,i,o). Phosphorylation of many of these molecules is linked with activation status. The increased levels of phosphorylated IRF7, Tak and Tab2, the IKK's and NFκB P65 (examples shown in figure 2(II) i,d,g,j and o) indicates that TLR9 stimulation can further increase activation of the associated pathways from an already above supra-normal level of activity in PBMCs of TRAPS patients. The evidence that numerous other signalling intermediates are also further stimulated suggests that the $TNFR1_{(C33Y)}$ mutation has a wide-ranging influence on cross-talk and activation of pro-inflammatory pathways.

This study therefore highlights the effect of TLR9 activation in cells expressing the $TNFR1_{(C33Y)}$ mutation. The TRAPS patients' PBMCs demonstrate a constitutive pro-inflammatory state of activation, but are also much more sensitive than those of healthy controls to activation of pro-inflammatory signalling pathways via stimulation of TLR9. This hypersensitivity, like that reported for the TLR4 ligand LPS [7] could partly explain the seemingly spontaneous inflammatory attacks seen in TRAPS, may be triggered by

subclinical infections and TLR or possibly other PRR stimulation, that would go unnoticed in normal individuals.

Despite being subject to IL1-neutralizing therapy (anakinra) prior to and at the time of sampling, patients showed considerable serum levels of multiple pro-inflammatory mediators, including IL1-β, IL6, IL8, IL17, IL22 and TNF-α indicating that the underlying effects of $TNFR1_{(C33Y)}$ mediated dysregulation are largely unchecked as a result of treatment[13]. The extensive signaling activation seen intracellularly in patients PMBCs, in both the absence and presence of TLR9 stimulation, does however suggests that suppressing such activity may require direct targeting of pathway components which lie at convergent points, such as has been suggested for TAB1&2/TAK1 complex[25], inhibition of the NLRP3 inflammasome[18,26] or further novel targets[27,28].

Acknowledgments:

This work was supported by The Jones 1986 Charitable Trust.

We would like to thank Dr Luisa Martinez-Pomares (University of Nottingham) for helpful discussions regarding the PBMC stimulations.

Conflict of interest: The authors declare that they have no conflict of interest.

References:

1. McDermott MF, Aksentijevich I, Galon J, McDermott EM, Ogunkolade BW, Centola M, et al. Germline mutations in the extracellular domains of the 55 kDa TNF receptor, TNFR1, define a family of dominantly inherited autoinflammatory syndromes. Cell 1999; 97:133-44.
2. Magnotti F, Vitale A, Rigante D, Lucherini OM, Cimaz R, Muscari I, et al. The most recent advances in pathophysiology and management of tumour necrosis factor receptor-associated periodic syndrome (TRAPS): personal experience and literature review. Clin Exp Rheumatol 2013; 31:141-9.
3. Todd I, Radford PM, Draper-Morgan KA, McIntosh R, Bainbridge S, Dickinson P, et al. Mutant forms of tumour necrosis factor receptor I that occur in TNF-receptor-associated periodic syndrome retain signalling functions but show abnormal behaviour. Immunology 2004; 113:65-79.

4. Bulua AC, Simon A, Maddipati R, Pelletier M, Park H, Kim KY, et al. Mitochondrial reactive oxygen species promote production of proinflammatory cytokines and are elevated in TNFR1-associated periodic syndrome (TRAPS). The Journal of experimental medicine 2011; 208:519-33.
5. Negm OH, Mannsperger HA, McDermott EM, Drewe E, Powell RJ, Todd I, et al. A pro-inflammatory signalome is constitutively activated by C33Y mutant TNF receptor 1 in TNF receptor-associated periodic syndrome (TRAPS). Eur J Immunol 2014; 44:2096-110.
6. Fairclough LC, Stoop AA, Negm OH, Radford PM, Tighe PJ, Todd I. Tumour necrosis factor receptor I blockade shows that TNF-dependent and TNF-independent mechanisms synergise in TNF receptor associated periodic syndrome. Eur J Immunol 2015; 45:2937-44.
7. Simon A, Park H, Maddipati R, Lobito AA, Bulua AC, Jackson AJ, et al. Concerted action of wild-type and mutant TNF receptors enhances inflammation in TNF receptor 1-associated periodic fever syndrome. Proc Natl Acad Sci U S A 2010; 107:9801-6.
8. Rigante D, Lopalco G, Vitale A, Lucherini OM, De Clemente C, Caso F, et al. Key facts and hot spots on tumor necrosis factor receptor-associated periodic syndrome. Clin Rheumatol 2014; 33:1197-207.
9. O'Neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors - redefining innate immunity. Nat Rev Immunol 2013; 13:453-60.
10. Selvarajah S, Negm OH, Hamed MR, Tubby C, Todd I, Tighe PJ, et al. Development and validation of protein microarray technology for simultaneous inflammatory mediator detection in human sera. Mediators Inflamm 2014; 2014:820304.
11. Huggins ML, Radford PM, McIntosh RS, Bainbridge SE, Dickinson P, Draper-Morgan KA, et al. Shedding of mutant tumor necrosis factor receptor superfamily 1A associated with tumor necrosis factor receptor-associated periodic syndrome: differences between cell types. Arthritis Rheum 2004; 50:2651-9.
12. Mannsperger HA, Gade S, Henjes F, Beissbarth T, Korf U. RPPanalyzer: Analysis of reverse-phase protein array data. Bioinformatics 2010; 26:2202-3.
13. Akrem A, Yousef N, Begum A, Negm A, Meyer A, Perbandt M, et al. Preliminary crystallographic analysis of a cruciferin protein from seeds of Moringa oleifera. Protein J 2014; 33:253-7.
14. Rebelo SL, Amel-Kashipaz MR, Radford PM, Bainbridge SE, Fiets R, Fang J, et al. Novel markers of inflammation identified in tumor necrosis factor receptor-associated periodic syndrome (TRAPS) by transcriptomic analysis of effects of TRAPS-associated tumor necrosis factor receptor type I mutations in an endothelial cell line. Arthritis Rheum 2009; 60:269-80.
15. La Torre F, Muratore M, Vitale A, Moramarco F, Quarta L, Cantarini L. Canakinumab efficacy and long-term tocilizumab administration in tumor necrosis factor receptor-associated periodic syndrome (TRAPS). Rheumatol Int 2015; 35:1943-7.
16. Vaitla PM, Radford PM, Tighe PJ, Powell RJ, McDermott EM, Todd I, et al. Role of interleukin-6 in a patient with tumor necrosis factor receptor-associated periodic syndrome: assessment of outcomes following treatment with the anti-interleukin-6 receptor monoclonal antibody tocilizumab. Arthritis Rheum 2011; 63:1151-5.

17. Nedjai B, Hitman GA, Church LD, Minden K, Whiteford ML, McKee S, et al. Differential cytokine secretion results from p65 and c-Rel NF-kappaB subunit signaling in peripheral blood mononuclear cells of TNF receptor-associated periodic syndrome patients. Cell Immunol 2011; 268:55-9.
18. Hoffman HM, Broderick L. The role of the inflammasome in patients with autoinflammatory diseases. J Allergy Clin Immunol 2016; 138:3-14.
19. Peckham D, Scambler T, Savic S, McDermott MF. The burgeoning field of innate immune-mediated disease and autoinflammation. J Pathol 2017; 241:123-39.
20. Carta S, Semino C, Sitia R, Rubartelli A. Dysregulated IL-1beta Secretion in Autoinflammatory Diseases: A Matter of Stress? Front Immunol 2017; 8:345.
21. Jesus AA, Goldbach-Mansky R. IL-1 blockade in autoinflammatory syndromes. Annu Rev Med 2014; 65:223-44.
22. Sabat R, Ouyang W, Wolk K. Therapeutic opportunities of the IL-22-IL-22R1 system. Nat Rev Drug Discov 2014; 13:21-38.
23. Bostrom EA, Kindstedt E, Sulniute R, Palmqvist P, Majster M, Holm CK, et al. Increased Eotaxin and MCP-1 Levels in Serum from Individuals with Periodontitis and in Human Gingival Fibroblasts Exposed to Pro-Inflammatory Cytokines. PLoS One 2015; 10:e0134608.
24. Patsouris D, Cao JJ, Vial G, Bravard A, Lefai E, Durand A, et al. Insulin resistance is associated with MCP1-mediated macrophage accumulation in skeletal muscle in mice and humans. PLoS One 2014; 9:e110653.
25. Fechtner S, Fox DA, Ahmed S. Transforming growth factor beta activated kinase 1: a potential therapeutic target for rheumatic diseases. Rheumatology (Oxford) 2016.
26. Moll M, Kuemmerle-Deschner JB. Inflammasome and cytokine blocking strategies in autoinflammatory disorders. Clinical immunology 2013; 147:242-75.
27. Marcuzzi A, Piscianz E, Valencic E, Monasta L, Vecchi Brumatti L, Tommasini A. To Extinguish the Fire from Outside the Cell or to Shutdown the Gas Valve Inside? Novel Trends in Anti-Inflammatory Therapies. Int J Mol Sci 2015; 16:21277-93.
28. Feltham R, Vince JE, Lawlor KE. Caspase-8: not so silently deadly. Clin Transl Immunology 2017; 6:e124.

Table 1: Statistical evaluation of intracellular signalling molecule expression in PBMCs from TRAPS patients and healthy controls without and with ODN2006 treatment.

Table showing statistical comparison (one-way ANOVA with Bonferroni's multiple comparison test) of patient and control group normalised signal intensities of 49 signalling molecules, measured by RPPA, with and without ODN2006 stimulation. Significant differences between the patient and control group are reported (p values <0.05). Detailed data are shown in figure 2;. **A)** Patients US (unstimulated patients' PBMCs) vs. Controls US (unstimulated healthy controls' PBMCs). **B)** Patients ODN (ODN2006-stimulated patients' PBMCs) vs. Controls ODN (ODN2006-stimulated healthy controls' PBMCs). **C)** Patients ODN vs. Patients US. **D)** Controls ODN vs. Controls US.

Signalling molecule	A) Patients US vs. Controls US	B) Patients ODN vs. Controls ODN	C) Patients ODN vs. Patients US	D) Controls ODN vs. Controls US
MyD88				
TRAF3	0.0001	< 0.0001	0.002	
IRAK-4	0.0052			0.0273
pIRAK-4				
IRAK-1	< 0.0001	< 0.0001		
IRAK-2	0.0048	< 0.0001	0.0003	0.0425
IRAK-M				
TOLLIP	< 0.0001	< 0.0001	< 0.0001	< 0.0001
TRAF6	< 0.0001	< 0.0001	0.0132	
pTAK	< 0.0001	< 0.0001	0.0115	
TAB2		0.0007	0.0336	
pTAB2	0.0011	< 0.0001	0.0150	
TBK-NAK		0.0009		
pTBK-NAK				
IRF7	0.0028	< 0.0001	0.0028	
pIRF7	0.0222	0.0054		
IRF3	0.0037	0.0002	0.0428	
pIRF3	< 0.0001	< 0.0001		
TRAF2	0.0123	0.0006		
RIP	0.0003	< 0.0001	0.0107	
pRIP		0.0106		
IKB-alpha	0.0003	< 0.0001		
pIKK-alpha	0.0001	< 0.0001		
NF-KB p65	0.0004	< 0.0001	0.0216	
pNF-κB p65	< 0.0001	< 0.0001	0.0455	
PIKK-gamma	0.0178	0.0014		
PIKK-beta	0.0006	0.0005		
NF-KB p50	0.0013	0.002		
A20		0.0105		
MEK1/2			0.0276	0.0063
pMEK1/2	< 0.0001	< 0.0001		
pP38		0.0057		
pERK1/2	0.0311			
MKK7		0.0028		
pSEK1/MKK4		0.0022		
pHSP27	< 0.0001	< 0.0001		
pROS	0.0016	< 0.0001		
pATF	0.0169	< 0.0001	0.0109	
pCRAF	0.0071	0.0015		
pAKT Thr	0.0002	< 0.0001	0.0078	
pAKT Serine	< 0.0001	< 0.0001		
pPDK1	0.0035	0.0001		
pGSK-3B	< 0.0001	< 0.0001		
PI3K110	0.0039	0.0014		
PI3KP85	0.0004	< 0.0001		
CIAP		0.0041	0.025	
ICAM	0.0014	< 0.0001	0.0174	
MMP12	< 0.0001	< 0.0001		
Caspase 8	0.0001	< 0.0001		

Figure Legends:

FIGURE 1: Serum cytokine levels in TRAPS patients and healthy controls.

The data are shown for individual serum samples (4 TRAPS patients and 7 healthy controls, with three technical replicates per sample). Horizontal bars indicate the mean values. Mann-Whitney test was performed and $p < 0.05$ was considered statistically significant.

Figure 2: Inflammatory signalling pathway intermediates measured by RPPA in PBMCs of TRAPS patients and controls. I) Heat-map columns represent control samples (C1-5) and Patient samples (P1-4) either Unstimulated (US) or ODN2006 stimulated (ODN). Individual rows represent the signalling molecules studied. Antibodies usedand working dilutions are described in full in table 1S. The white to Black heat-map colour range represents RPPA signal data normalised to represent percentages of the signal range for each molecule examined (0-100%). Letters a-r at the left-hand side refer to the scatter plots in section II of the figure. II) Scatter plots representing actin-normalized relative abundance of selected target proteins which show statistically significant differences in columns C and D of table 1. Data are shown for individual PBMC lysates (mean of four technical replicates per sample) of four patient samples and five control samples. Signals are presented as arbitrary fluorescence units after background subtraction and normalization to β-actin. The four conditions were: Unstimulated healthy controls' PBMCs ;Con (US), unstimulated patients' PBMCs; Pat (US), ODN2006-stimulated healthy controls' PBMCs; Con (ODN) and ODN2006-stimulated patients' PBMCs; Pat (US) . Significant differences between conditions for

each signalling molecule were determined by one-way ANOVA and Bonferroni's multiple comparisons test; these are presented in table 1.

Figure 3: Visual depiction of findings: the graphic represents elements of the common pathways active by both TNFR1 signalling and signalling through TLR9. Arrows indicate activation route of molecules in the pathways. Red arrows indicate those pathways which are found to be both more abundant and more activated as a result of the C33Y TRAPS mutation *and* also further stimulated by TLR9 ligation as ODN2006 as an artificial activator. Molecules depicted in grey were not studied.