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Janus kinase 1/2 inhibition for the treatment of autoinflammation associated with heterozygous *TNFAIP3* mutation

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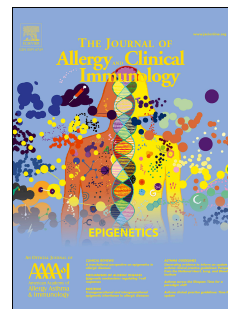
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**Capsule summary:** We report a case of autoinflammation caused by a heterozygous variant in *TNFAIP3* manifesting as progressive neuroinflammation. Mutant A20 protein failed to control interferon-dependent transcription. We demonstrate excellent therapeutic response to Janus kinase inhibition.

**Keywords:** *TNFAIP3*, interferon, janus kinase inhibition

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**To the editor,**

Heterozygous loss-of-function pathogenic variants in Tumour-Necrosis-Factor-Alpha-Induced-Protein-3 gene (*TNFAIP3*) cause autoinflammation due to haploinsufficiency of A20 protein (HA20). HA20 commonly manifests as severe orogenital ulceration and uveitis (1). Central nervous system (CNS) inflammation is rare, but is reported in HA20; and animal studies have illustrated that mice with mutant *TNFAIP3* are prone to severe neuroinflammation (2, 3). Loss of A20 function causes spontaneous cerebral inflammation, as demonstrated by robust microglial activation, reactive astrogliosis, endothelial activation, increased oxidative/nitrosative stress and expression of NF $\kappa$ B regulated pro-inflammatory soluble mediators such as IL-1 $\beta$ , TNF, IL-6 and MCP-1 in the brain (3). CNS involvement as the sole clinical manifestation of heterozygous *TNFAIP3* variants in humans has never been described. We report a case of *TNFAIP3* mediated autoinflammation manifesting as progressive neuroinflammation. We show that mutated A20 protein failed to control interferon (IFN)-dependent transcription, highlighting an entirely novel mechanism of autoinflammation in HA20. Targeted treatment with the Janus kinase inhibitor (JAKI) baricitinib led to marked clinical, radiological and immunological improvement. Methods are provided online.

The proband (III.1) was an 8 year-old female of non-consanguineous Pakistani-Indian descent (**Fig1A**) who presented with left-sided focal seizures and hemiparesis, uveitis and cognitive decline. She had choreoretinitis from the age of 6-months, considered secondary to congenital infection; no pathogen had been identified, however. There was no history of systemic symptoms; she had occasional mouth ulcers, but no genital ulcers. Magnetic resonance imaging (MRI) of her brain revealed contrast-enhancing T2-hypointense intracranial mass lesions, affecting predominantly the grey matter of the paracentral lobule and of the thalamus on the left, with surrounding oedema (**Fig1B-E**). Laboratory tests

included: negative autoantibody screen; and negative/normal: complement function studies; targeted *NOD2* genetic testing; nitroblue tetrazolium test; immunoglobulin levels, lymphocyte subsets and CSF analysis (**supplemental table 1**). She had modestly elevated erythrocyte sedimentation rate (ESR) of 25 mm/h (normal range < 5mm/h); and normal C-reactive-protein (CRP, 5 mg/L, normal range < 5mg/L). Brain histology revealed granulomatous inflammation, focal necrosis, and calcification (**Fig1F**). Tuberculosis (TB) was still considered a possible differential diagnosis, and empirical anti-TB treatment was started (isoniazid/rifampicin/pyrazinamide/moxifloxacin). Brain tissue culture, PCR-testing for mycobacteria, and Quantiferon test were all negative, however. There was progression of the mass lesion, worsening of left-sided hemiplegia, and new onset ataxia. At that point, she was considered to have an unclassified granulomatous neuroinflammatory disorder, reminiscent of neurosarcoid. Based on that, she received treatment with prednisolone 2 mg/kg/day, weaning to 0.5 mg/kg/day over 6 months; and mycophenolate mofetil (1200 mg/m<sup>2</sup>/day). There was no response to this treatment, with further increase in size of the brain lesion. Intravenous cyclophosphamide (6 doses, 500-750 mg/m<sup>2</sup>) was then given, and prednisolone continued at a higher dose (2 mg/kg/day). There was poor response to these treatments and significant steroid related side effects noted (weight gain, Cushingoid appearance, and arterial hypertension). A second brain biopsy again revealed necrotising granulomatous inflammation, and no evidence of infection. Whole-body PET-CT revealed no evidence of malignancy; brain-CT revealed intracerebral calcification (**Fig1G**). In view of the significant development of intracerebral calcification, she was considered to have an unclassified interferonopathy and was started on baricitinib (6 mg/day), an oral JAK1/JAK2 inhibitor that blocks IFN signalling (via a programme sponsored by Eli Lilly and Company). This resulted in rapid clinical and radiological improvement: 24 months later she remains stable with no further seizures and marked resolution of the intracerebral inflammatory lesion (**Fig1H-J**). Prednisolone was weaned off for the first time in 2 years. Her younger sister (III.2) suffered from arthritis, and a facial malar type rash from the age of 6 months old. II.4 had died at the age of 17 years, from early-onset systemic lupus erythematosus. II-2 had mild oral ulceration.

Whole exome sequencing (WES) revealed a heterozygous c.A1939C (NM\_001270508) p.T647P variant in *TNFAIP3*, confirmed with Sanger sequencing in III-1, also present in the symptomatic sibling (III-2), and mildly symptomatic mother (II-2; **Fig1A and K**). This variant resides in the fourth zinc finger (Znf4) domain of A20, an area that has E3 ubiquitin

ligase activity and is involved in recruiting adaptor proteins, such as Tax-1-binding-protein-1 (TAX1BP1) and the A20-binding-inhibitor of NF- $\kappa$ B (ABIN-1), to enable A20 to exert its inhibitory function (4).

PBMCs derived from heterozygotes for the p.T647P *TNFAIP3* showed increased expression of NF- $\kappa$ B phosphorylated p65 transcription factor compared to control cells,  $p=0.0124$  (**Fig1L**). Similar differences were also observed in Human dermal fibroblast cells (HDFC) from III-1 compared to control HDFC,  $p=0.0001$  (**Fig1M**), suggesting that the heterozygous p.T647P *TNFAIP3* variant impaired the ability of the A20 to regulate the canonical NF- $\kappa$ B pathway. TNF-stimulated HDFC from III-1 also showed increased molecular weight of Lys63-ubiquitinated NF-kappa-B essential modulator (NEMO) as a result of insufficient A20 deubiquitinase activity. (Fig1N).

Activated NF- $\kappa$ B subunits are known to promote the transcription of genes encoding proinflammatory cytokines (5). Levels of several proinflammatory cytokines were substantially higher in heterozygotes for p.T647P *TNFAIP3* compared to healthy controls: IL-1 $\beta$  ( $p=0.02$ ), IL-6 ( $p=0.02$ ), IL-8 ( $p=0.02$ ) and TNF- $\alpha$  ( $p=0.02$ ).

Recent studies suggest that A20 functions as a negative regulator of the NLRP3 inflammasome independently of its role in NF- $\kappa$ B regulation (1). Consistent with these data, PBMC from III-1 showed constitutive activation of the NLRP3 inflammasome, which resulted in activation of caspase-1 ( $p=0.009$ ) and increased secretion of active IL-1 $\beta$  ( $p=0.007$ ) and IL-18 ( $p=0.01$ ); supplemental figure 1.

Activation of the interferon regulatory factor-3 (IRF-3), a critical transcription factor that regulates Interferon (IFN) immune responses, is negatively regulated by A20 through interaction with the NF- $\kappa$ B-activating kinase/Traf family member-associated NF- $\kappa$ B activator-binding kinase 1 (TBK1) (6). We thus next examined if IFN immune responses were impaired. Type-1 IFN stimulated gene expression levels were upregulated in whole blood from III-1 (**Fig2A**); serum IFN- $\alpha$  and IFN- $\beta$  were also elevated compared to controls ( $p=0.04$  and  $p=0.049$  respectively). We also observed enhanced expression of phosphorylated IRF-3 in lymphocytes from all heterozygotes for p.T647P *TNFAIP3* variants ( $p=0.0007$ , **Fig2B**), and in HDFC from III-1 compared to control,  $p=0.009$  (**Fig2C**). Lymphocytes from III-1 also demonstrated increased phosphorylation of STAT-1 and STAT-3 compared to control,  $p=0.0001$  and  $p=0.0001$  respectively; similar differences in STAT-1 and STAT-3 were observed in HDFC (**Fig2D-E and supplemental figure 2 A-B**).

siRNA mediated silencing of *TNFAIP3* in HDFC resulted in upregulation of phosphorylated p65 ( $p=0.04$ ), phosphorylated IRF-3 ( $p=0.0043$ ), phosphorylated STAT-1 ( $p=0.037$ ), and phosphorylated STAT-3 ( $p=0.03$ ) expression compared to scrambled siRNA control cells (**supplemental Fig2C-G**).

We documented almost complete clinical and radiological resolution of neuroinflammation in III-1 (**Fig1H-J**), and normalisation of IFN-induced gene expression in whole blood (**Fig2F**) following treatment with baricitinib.

To establish whether the loss of A20 negative regulatory control over IRF-3 activation was secondary to the reduced binding capacity of the A20 protein to TBK1, we next employed a co-immunoprecipitation assay. In HDFC from III-1, there was no significant upregulation in binding of TBK1 to the A20 protein in response to TNF $\alpha$ , in contrast to significantly increased binding of A20/TBK1 observed in control cells (**Fig2G-H**).

We next examined whether IFN-mediated immune responses might be generally dysregulated in HA20, irrespective of neurological involvement, in a 4 year-old non-consanguineous Caucasian male with HA20, without any neurological involvement (**Fig2I**). He presented with recurrent oral inflammation and penile ulceration, and was heterozygous for the p.N98Tfs25 *TNFAIP3* variant, as was his symptomatic father who suffered similar clinical features from early childhood. We confirmed significant upregulation of p65 ( $p=0.11$ ), phosphorylated IRF-3 ( $p=0.0001$ ; **Fig2J**) and STAT-1 ( $p=0.0013$ ) and STAT-3 expression ( $p=0.009$ ; **Fig2K/L**) in lymphocytes from this boy compared to healthy controls.

We expand the spectrum of clinical presentation associated with HA20 caused by variants in *TNFAIP3*, which now includes progressive neuroinflammation. We provide insights into the mechanism for the observed immunophenotype through loss of A20-mediated negative regulatory control of IRF3 activation, and subsequent dysregulated IFN pathway signalling. *TNFAIP3* mediated autoinflammation should now be considered in the differential diagnosis of neuroinflammation, particularly in the presence of intracerebral calcification and uveitis. JAK inhibition may represent a novel therapeutic approach for autoinflammation and neuroinflammation associated with heterozygous *TNFAIP3* variants.

Neuroinflammation is rare, but has been previously reported in HA20. In a recent case series, CNS vasculitis was reported in 2/16 (13%) HA20 patients. The true frequency of CNS involvement in HA20 may be underappreciated, however, as not all patients were

systematically assessed for presence of neurological involvement (2). We therefore suggest that clinicians should consider screening for neuroinflammation in all patients with suspected HA20. Future collaborative studies may facilitate more detailed phenotype-genotype correlation and could help identify which HA20 patients might be more at risk of neuroinflammation, but currently these data do not exist. Notably, animal studies have previously suggested that heterozygous variants in *TNFAIP3* cause milder neuroinflammatory changes compared to the severe neuroinflammation observed in complete A20 KO mice (3). The heterozygous state in our patients, therefore, may contribute to the less severe phenotype observed in some of our patients. Of note, immune cells from II-2 and III-2, also heterozygotes for the p.T647P *TNFAIP3* variant, exhibited enhanced NF- $\kappa$ B activity and IRF-3 activation, but these individuals currently have a much milder phenotype, further emphasising the previously described clinical heterogeneity of HA20 even within the same kindred (1,2). Additional modifying alleles, genetic and/or environmental risk factors (such as intercurrent infection or other triggers) may play a role in modifying the phenotype and influence susceptibility to, or disease severity of HA20.

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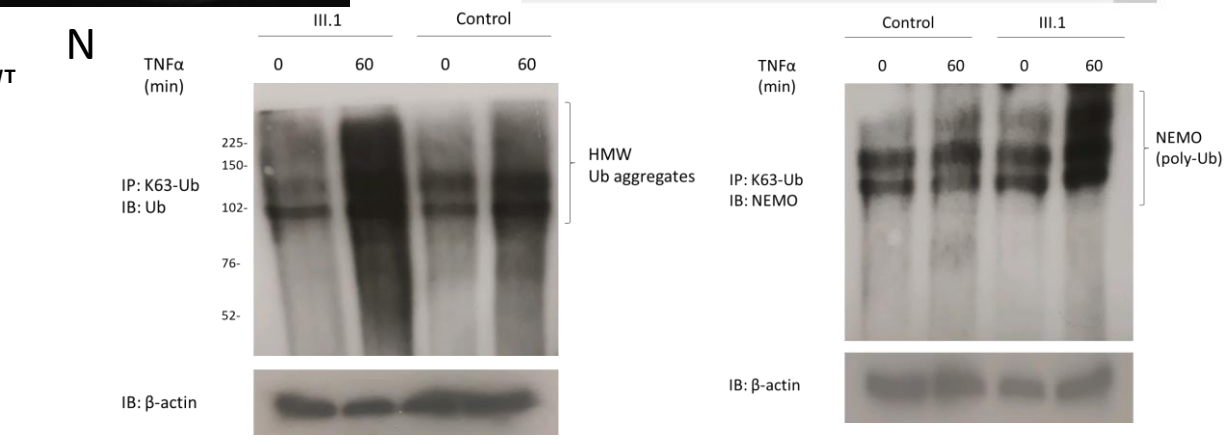
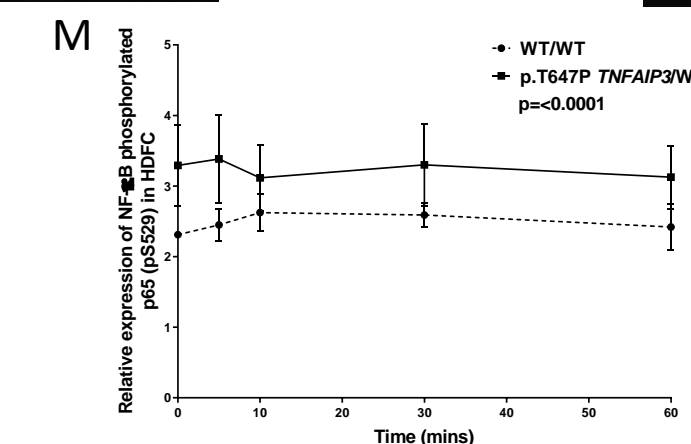
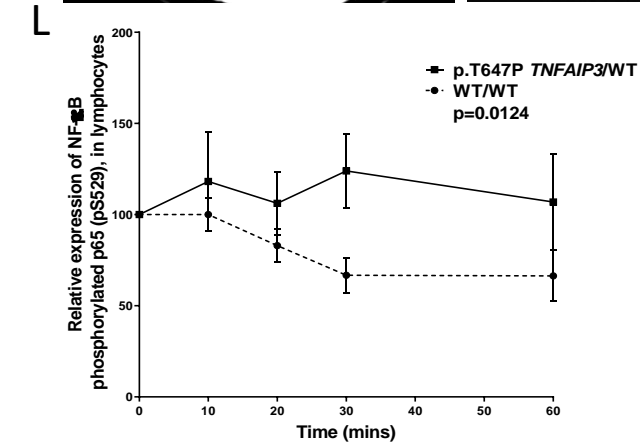
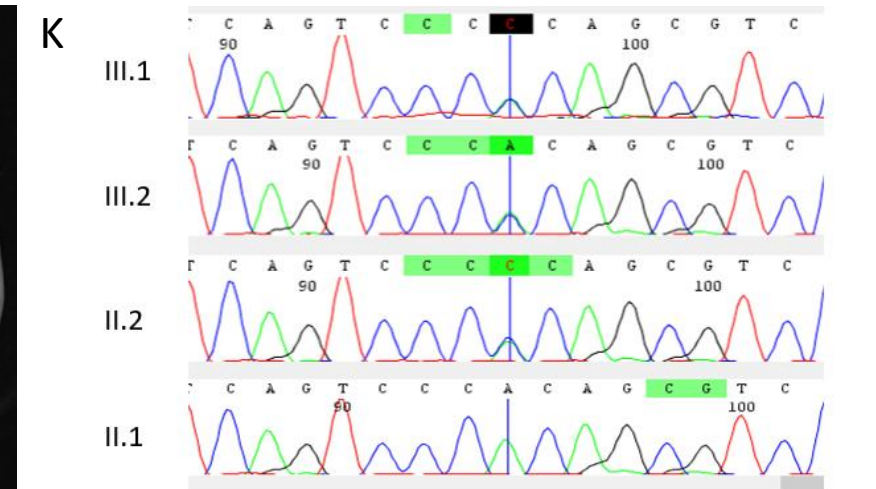
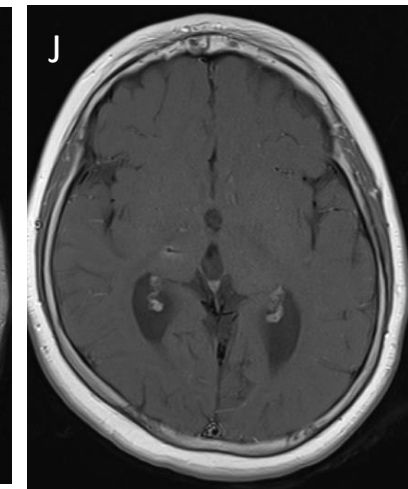
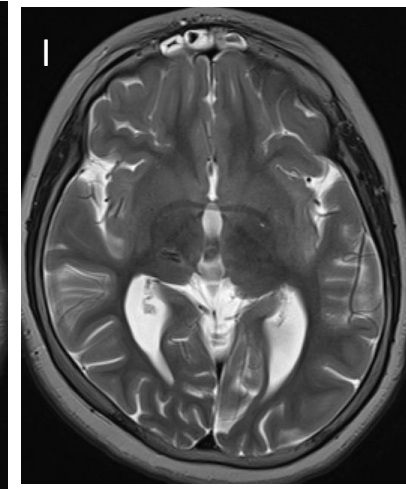
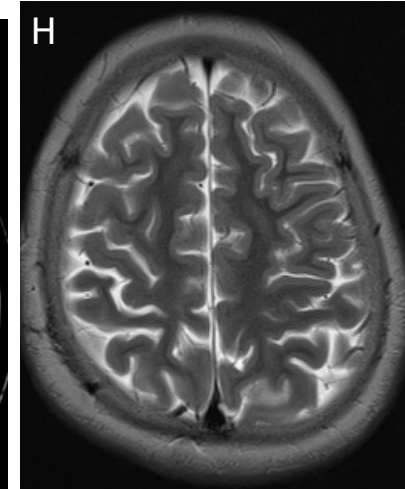
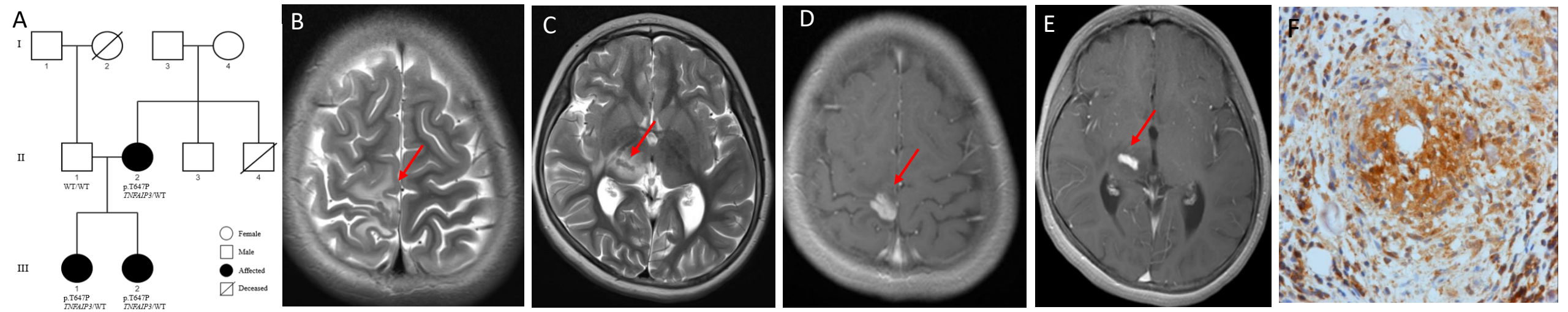
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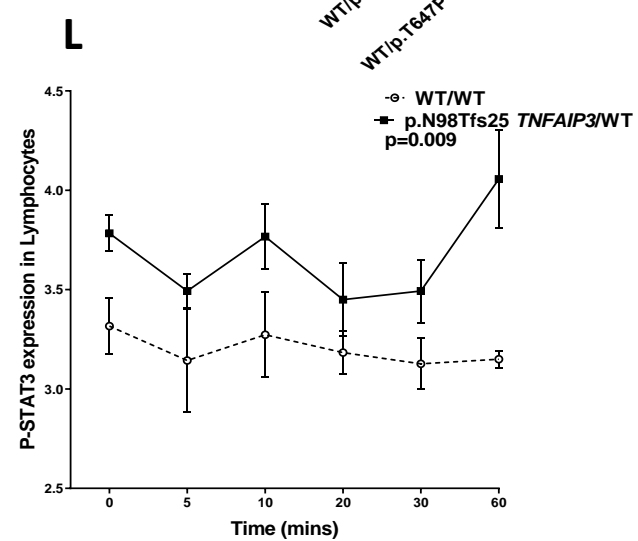
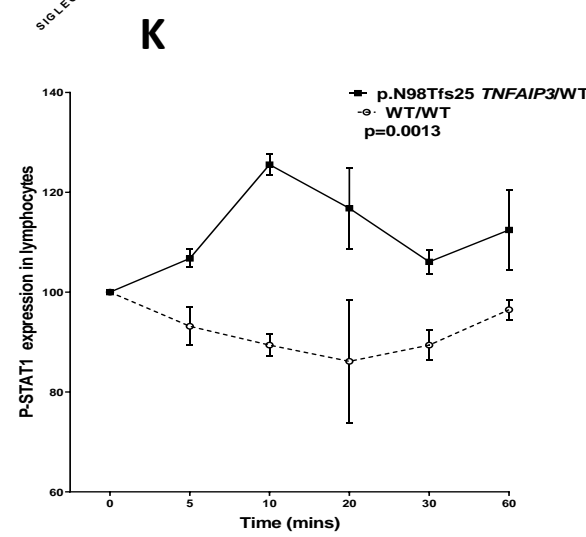
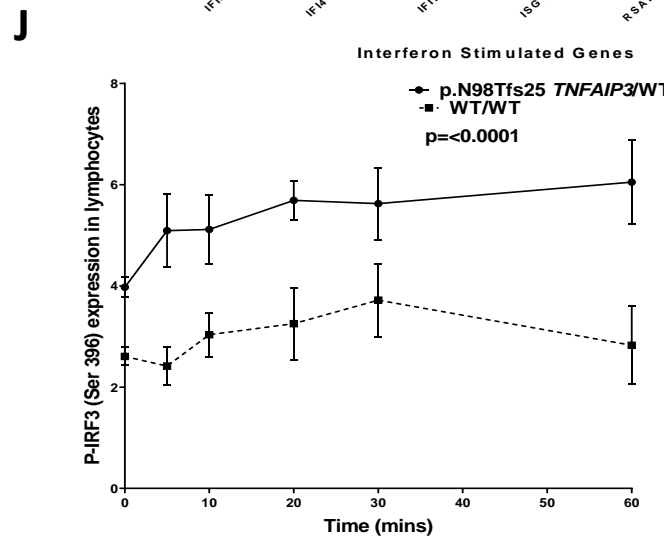
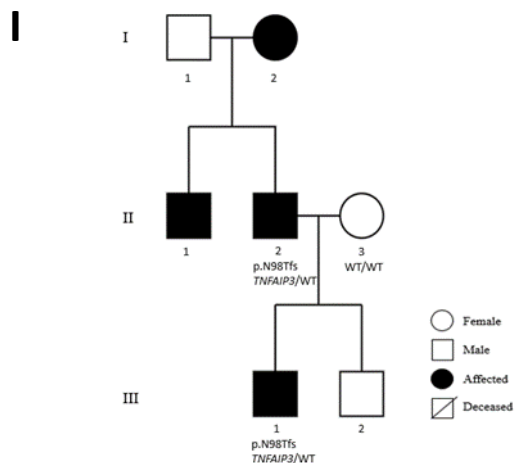
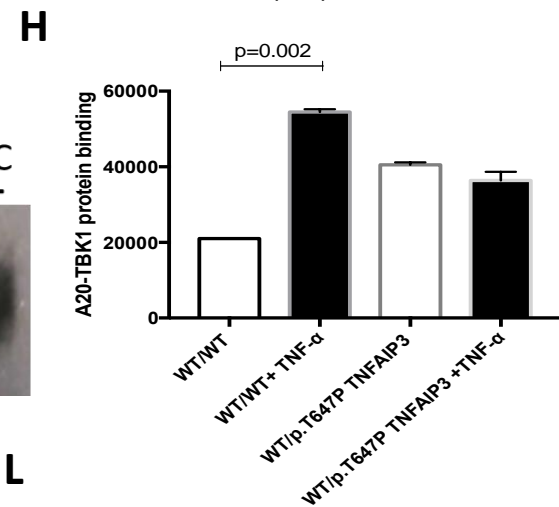
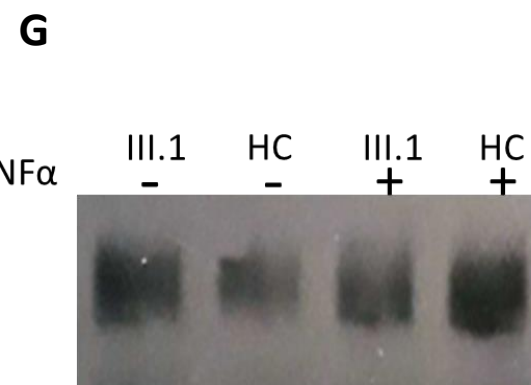
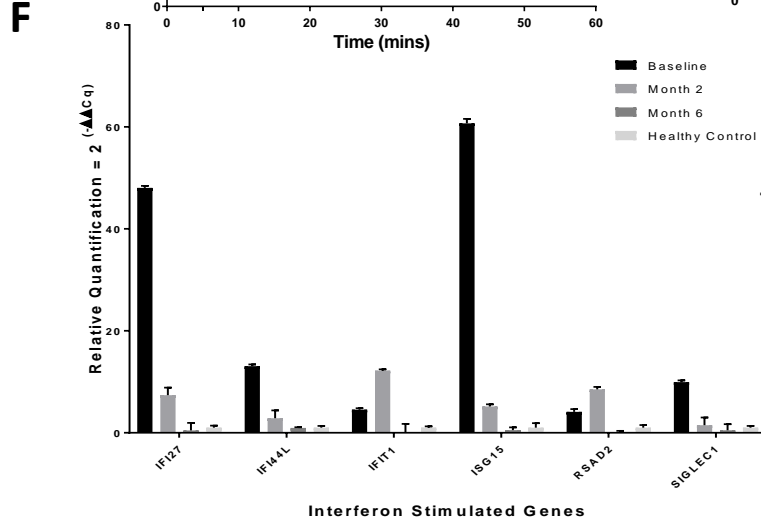
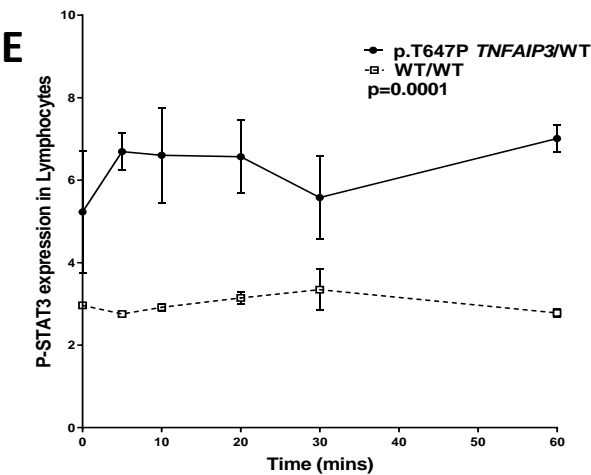
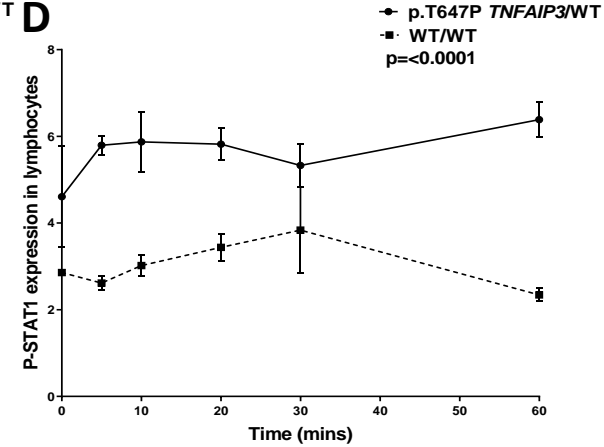
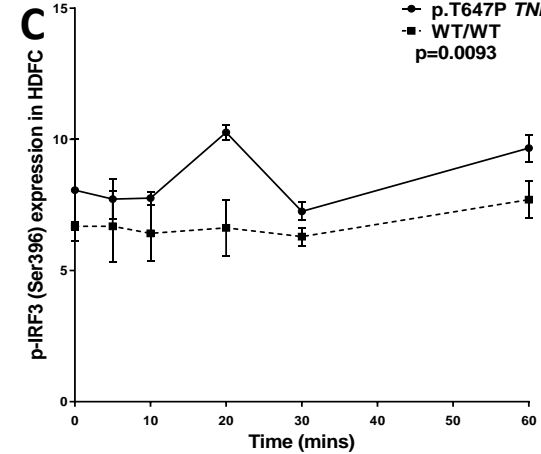
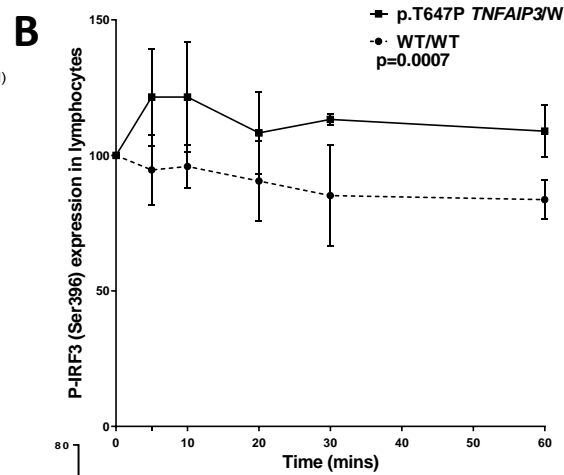
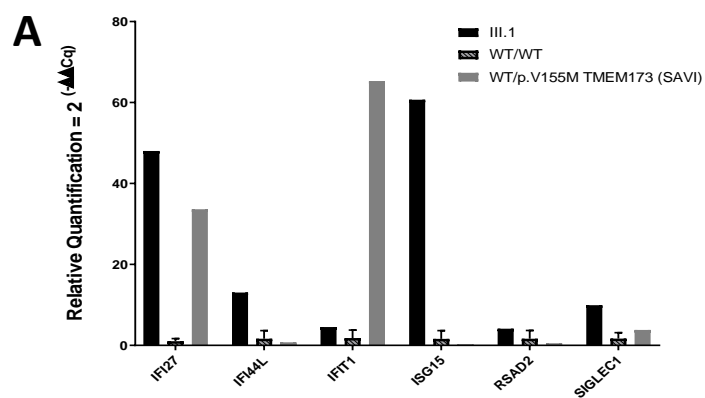
**Figure 1. Pedigree, magnetic resonance imaging (MRI) imaging features and genetic sequencing results.** (A) Pedigree shows segregation of the p.T647P heterozygous TNF alpha induced protein 3 (*TNFAIP3*) variant. (B-C) Axial T2 weighted images at the level of the peri-rolandic cortex and basal ganglia shows hypointense solid areas (arrows) surrounded by vasogenic oedema in the right paracentral lobule (B) and thalamus (C). (D-E) Axial T1 WI post contrast at the same levels shows intense enhancement of the solid lesions (arrows). (F) Brain biopsy showed necrotising granulomatous inflammation with positive p65 nuclear stain. (G) Computed tomography (CT) brain scan demonstrated foci of calcifications (arrow) in the thalamic lesion and in the periventricular white matter within the area of vasogenic oedema. (H-J) Axial T2 and T1 WI post-contrast showed almost complete resolution of the previously noted lesions. (K) Sanger sequencing chromatogram of *TNFAIP3* gene aligned to reference sequence exon 8. Line indicates a heterozygous *TNFAIP3* p.T647P variant in III.1, III.2 and II-2. (L-M) There was increased expression of phosphorylated p65 in patient lymphocytes ( $p=0.0124$ ) and patient human dermal fibroblast cells (HDFC,  $p=0.0001$ ) compared to control cells. (N) TNF-stimulated dermal fibroblasts from III.1 showed increased abundance and molecular weight of Lys63- ubiquitinated NF-kappa-B essential modulator (NEMO).

**Figure 2. Enhanced type 1 IFN (interferon) signalling in immune cells derived from patients with heterozygous p.T647P variant in *TNFAIP3*.** (A) Type 1 IFN stimulated gene expression levels were upregulated in whole blood from III-1 at comparable levels to that seen in a patient with STING vasculopathy with onset in infancy (SAVI). Control data were derived from  $n=13$  individuals. (B-C) There was increased expression of phosphorylated IRF-3 in lymphocytes from all heterozygotes for p.T647P *TNFAIP3* ( $p=0.0007$ ) and in human dermal fibroblast cells (HDFC) from III-1 ( $p=0.009$ ) compared to control cells. (D-E). Lymphocytes from p.T647P *TNFAIP3* heterozygotes demonstrated increased STAT-1 and STAT-3 expression,  $p=0.0001$ . (F) Treatment with an oral JAK 1/2 inhibitor resulted in a significant decrease in type I IFN gene expression. (G-H) In fibroblasts derived from III-1 there was impaired ability of the p.T647P A20 protein to bind to TBK1 in response to TNF- $\alpha$ . (I) Pedigree for family of patient with heterozygous p.N98Tfs25 *TNFAIP3* associated autoinflammation. (J-L) There was also increased expression of phosphorylated IRF-3 ( $p=0.0001$ ), STAT-1 ( $p=0.0013$ ) and STAT-3 ( $p=0.009$ ) in lymphocytes derived from the p.N98Tfs25 *TNFAIP3* heterozygote compared to control cells ( $p=0.0001$ ). IRF3=interferon-

regulatory-factor-3; STAT1 and STAT3=signal transducer and activator of transcription-1 and 3; STING=stimulator of interferon genes; *TNFAIP3*= TNF alpha induced protein 3.

ACCEPTED MANUSCRIPT





## Supplemental online methods

### *Patients*

This study was approved by the Bloomsbury ethics committee (ethics number 08H071382). We obtained written informed consent from all the patients, family members and healthy controls (ethics number 11/LO/0330) who participated in the study. Baricitinib was made available through the following program sponsored by Eli Lilly and Company: Compassionate Use Treatment Protocol I4V-MC-JAGA: Treatment of Conditions Expected to Benefit From JAK 1/2 Inhibition: CANDLE, CANDLE-Related Conditions, SAVI and Severe Juvenile Dermatomyositis (NCT01724580).

### *Genetic sequencing*

Whole exome sequencing (WES) was performed using the Illumina HiSeq 300 platform. Data were analysed in Galaxy platform (usegalaxy.org) and SNPs annotated using wANNOVAR. The additional case of HA20 studied as disease control, was sequenced on the Vasculitis and Inflammation Panel (VIP)(7).

The *TNFAIP3* variant was confirmed and familial segregation ascertained by PCR and Sanger sequencing using BigDye v3.1 kit (Applied Biosystems) and primers to amplify and sequence exon 8 forward: ATCTCTGTATCGGTGGGGTG and reverse: TTGTCACTGTCGGTAGAAAACG.

### *Peripheral blood mononuclear cell (PBMC) isolation and stimulation*

Peripheral Blood Mononuclear cells (PBMC) were isolated from freshly drawn heparinized blood by gradient density centrifugation using Lymphoprep™ (Stemcell™ Technology, Vancouver, Canada). Blood was diluted with the equal volume of RPM1640 medium and overlaid on an equal volume of Ficoll-Paque reagent, and then centrifuged 800 g for 10 min. The PBMC layer was taken, washed and resuspended in warm RPMI with 10% FCS. Cells were seeded at  $2 \times 10^6$  cells per well in a 6-well plate and were processed for protein extraction or stimulated with 100ng/ml TNF $\alpha$  for up to 60 minutes to induce NF- $\kappa$ B activation.

*Immunoprecipitation and immunoblotting*

Whole cell lysates were prepared using RIPA buffer (Thermo Fisher Scientific) and 1% proteinase inhibitor from isolated PBMC or dermal fibroblasts. For immunoprecipitation, lysates were mixed with antibodies (1:100) and incubated with 50  $\mu$ l of protein A Dynabeads (Thermo Fisher Scientific) overnight at 4 °C. Immunoprecipitates were boiled in 30  $\mu$ l of 2 $\times$  Laemmli's buffer with 10%  $\beta$  Mercaptoethanol, washing with PBS. For Immunoblotting, samples were subjected to SDS-polyacrylamide gel electrophoresis analysis, and electro transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked with milk and probed with primary and secondary antibodies and visualized with enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech). The following antibodies were used: A20/TNFAIP3 (#5630 CST), TBK1 (D1B4, CST), ACTN (MAB 1501R, MERCK Millipore), RIP1 (#3493, CST), K65-Ub antibody (Merck, 05-1308), Ub (sc-271289) and NEMO (sc-56919).

*Caspase-1 activity*

PBMC were seeded in a 96-well plate at a density of  $1.6 \times 10^5$  cells/well ( $8.0 \times 10^5$  cells/ml). Relevant wells were primed with 100 ng/ml LPS for 4 h, and, if required, then stimulated with 10 mM ATP for 30 min. Caspase-1 activity was measured using FLICA (ImmunoChemistry Technologies), a cell-permeable fluorescent probe (FAM-YVAD-FMK) that binds active caspase-1. Cells were incubated for 1 h with FLICA at 37°C and stained with PE-conjugated anti-CD14 (BD) to identify monocytes. Cells were subsequently analyzed for the frequency of CD14<sup>+</sup> FLICA<sup>+</sup> cells.

*Interferon (IFN) stimulated gene RNA expression from whole blood*

Whole blood was collected into PAXgene tubes and RNA extracted using the PreAnalytix RNA isolation. RNA concentration was assessed with a Nanodrop (FLUOstar Omega, Labtech). We performed quantitative reverse transcription polymerase chain reaction (qPCR) analysis using the iTaq Universal SYBR Green Supermix (172-5121, Bio-Rad), and cDNA derived from 400 ng total RNA. Using Qiagen Quantitect primers for IFI27(NM\_001130080), IFI44L(NM\_006820), IFIT1 (NM\_001001887), ISG15(NM\_005101), RSAD2(NM\_080657), SIGLEC1(NM\_023068), the relative abundance

of each of these target transcripts were normalized to the expression level of HPRT1(NM\_000194) (HS\_HPRT1\_1\_SG), assessed using the CFX Maestro software.

*Interferon regulatory factor (IRF-3), Signal transducer and activator of transcription 1 and 3 (STAT-1, STAT-3) and p65 phosphorylation assays*

PBMC were treated with 100 ng/ml TNF- $\alpha$  for up to 60 min. Cells were fixed using BD Cell Fix Buffer (10 min at 37°C) and then permeabilized using BD PermIII Buffer (30min at 4°C) prior to staining with PE-anti-NF- $\kappa$ B p65 pS529 (BD Bioscience, cat: 565447), PE-anti-IRF3 (BD Bioscience, cat: 612564) PE-anti-STAT1( BD Bioscience, cat: 61256) or Alexa-fluor-488-STAT3 (BD Bioscience, cat 557814) antibodies. Flow cytometry analysis was performed on a BD Calibre flow cytometer. Results were analysed using FlowJo v10.4.2.

*Dermal fibroblasts and siRNA silencing of TNFAIP3 experiments*

Dermal fibroblasts were explanted from III-1 and from controls without any identifiable inflammatory condition and maintained in DMEM/F12 (Dulbecco's Modified Eagle medium: Nutrient Mixture F-12) supplemented with 10% FBS and 100U/ml Penicillin and 100 $\mu$ g/ml Streptomycin (LifeTechnologies) at 37°C in 5% CO<sub>2</sub> in a humidified incubator. siRNA knockdown was performed in these fibroblast via transient transfection using with 100 nM of siRNA targeting TNFAIP3 (Santa Cruz, SC-37007) or a negative control. siRNAs were transfected via complexation with Lipofectamine RNAiMAX (Thermofisher scientific) according to the manufacturer's instructions. At 48 h post transfection knockdown efficiency was evaluated by quantitative real-time PCR. The Quantitic TNFAIP3 primers were used (Qiagen, QT00070301).

*Measurement of cytokines*

Multiple cytokines were quantified at different time points in the serum of affected patients (III-1, III-2 and II-2), family members (II-1), and healthy controls by electrochemiluminescence immunoassay using Meso Scale Discovery plates according to the manufacturer's instructions. Data were analysed with Discovery Workbench 4.0. Control sera was obtained from healthy paediatric controls (n=15, 9 female median age 14, range 12-18 years old) with local ethics approval (REC 11/LO/0330).

*Statistical analyses*

All experiments were complicated in triplicates unless otherwise specified. Results are expressed as mean and standard error of the mean or median and range where appropriate. All statistics (ANOVA, unpaired Student's t test, or Mann Whitney U test) and graphs were produced using Prism version 7 (GraphPad).  $P < 0.05$  was considered significant.

**Supplemental figure 1. NLRP3 inflammasome activation associated with p.T647P heterozygous variant in *TNFAIP3*.** **A** Peripheral blood mononuclear cells (PBMC) from III.1 constitutively expressed higher levels of FLICA (caspase-1) in response to lipopolysaccharide (LPS) (mean 16.57, SEM 2.533) compared with controls (mean 4.667, SEM 0.01,  $p=0.009$ ). **(B and C)**. There was increased release of IL-1 $\beta$  and IL-18 in supernatants from PBMC derived from III.1 following LPS stimulation compared with healthy controls ( $p=0.007$  and  $p=0.01$  respectively). Differences in IL-1 $\beta$ , IL-18 secretion and caspase 1 activation were also observed between PBMC derived from III.1 and healthy control cells after ATP addition. Results are expressed as mean and standard error of the mean.  $P < 0.05$  by unpaired t test were considered significant. NLRP3= NLR Family Pyrin Domain Containing 3; *TNFAIP3*= TNF alpha induced protein 3.

**Supplementary figure 2. siRNA mediated silencing of *TNFAIP3* enhances type I interferon (IFN) signalling.** **(A-B)**. Human dermal fibroblasts (HDFC) from III-1 also demonstrated increased phosphorylation of STAT1 and STAT3 compared to healthy control cells ( $p < 0.0001$  and  $p < 0.0001$  respectively) **(C-G)** siRNA mediated silencing of *TNFAIP3* in human dermal fibroblast cells (HDFC) resulted in enhanced expression of phosphorylated p65 (0.04), IRF-3 ( $p=0.004$ ), STAT-1 ( $p=0.03$ ), and STAT-3 ( $p=0.03$ ) compared to scrambled siRNA control cells. **(G-H)** There was increased expression of phosphorylated p65 in lymphocytes from a patient with p.N98Tfs25 *TNFAIP3* variant in comparison to healthy control cells ( $p=0.011$ ). Experiments were performed in triplicate, and results expressed as mean and standard error of the mean.  $P < 0.05$  by unpaired t test were considered significant. IRF3=interferon regulatory factor3; STAT1 and STAT3=signal transducer and activator of transcription-1 and 3; siRNA= small inhibitory ribonucleic acid; *TNFAIP3*= TNF alpha induced protein 3.

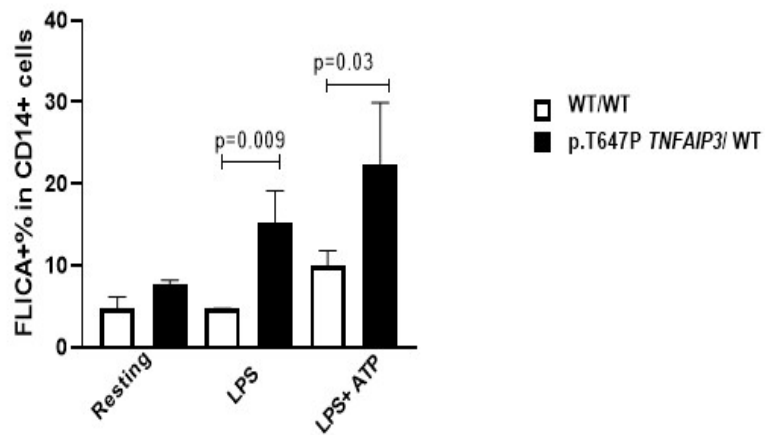


**Supplementary Table 1: Patient III-1 routine clinical laboratory investigations.**

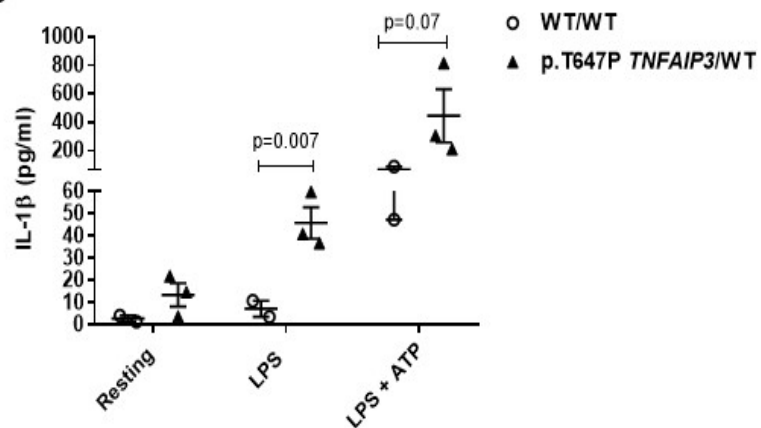
Laboratory investigations	Patient III.1 (Reference range)
Autoantibodies persistent >3 months	Absent <sup>1</sup>
Haemoglobin	10g/L <sup>2</sup> (120-160)
Platelet count	182x10 <sup>9</sup> /L <sup>3</sup> (150-450)
White blood cell count	6.25 x10 <sup>9</sup> /L <sup>4</sup> (4.0-11)
Lymphocyte subsets	Normal
Immunoglobulin G	19.9 g/L (3.1-13.8)
Immunoglobulin A	1.94 g/L (0.4-0.7)
Immunoglobulin M	2.29 g/L (0.5-2.2)
Immunoglobulin D	7 kU/L (2-100)
Adenovirus, CMV, EBV, HSV, VZV,	Negative
Parechovirus PCR	
Toxoplasma PCR	Negative
Mycoplasma antibodies	Negative
Quantiferon	Negative
Nitroblue tetrazolium test	Normal
Brucella serology	Negative
Toxocara serology	Normal
16sPCR and 18s PCR CSF	Negative
JC and BK virus	Negative
Complement C3	1.77 g/L (0.75-1.65)
Complement C4	0.24 g/L (0.14-0.54)
Liver enzymes	ALT 15 U/L (10 – 25) ALP 96 U/L (150-380)
CSF white cell count	< 1x 10 <sup>6</sup>
CSF cytospin	Negative
CSF oligoclonal bands	Negative

1. Autoantibodies tested: antinuclear antibodies, anti-neutrophil cytoplasm antibodies, rheumatoid factor, anti-tissue transglutaminase antibodies, anti-thyroid peroxidase antibodies, anti myelin oligodendrocyte antibodies, anti-yo, anti-hu, anti ri antibodies, NMDAR antibodies, rheumatoid factor antibodies, coeliac screen antibodies, b2 glycoprotein and anticardiolipin antibodies.

A



B



C

