

Somatic Mosaic *NLRP3* Mutations and Inflammasome Activation in Late-Onset Chronic Urticaria

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Chronic urticaria is a common skin disorder with heterogeneous causes. In the absence of physical triggers, chronic urticarial rash is called idiopathic or spontaneous. The objective of this study was to identify the molecular and cellular bases of a disease condition displayed by two unrelated patients aged over 60 years who presented for two decades with a chronic urticaria resistant to standard therapy that occurred in the context of systemic inflammation not triggered by cold. In both patients, a targeted sequencing approach using a next generation technology identified somatic mosaic mutations in *NLRP3*, a gene encoding a key inflammasome component. The study of several of both patients' cell types showed that, despite the late onset of the disease, *NLRP3* mutations were not found to be restricted to myelomonocytic cells. Rather, the data obtained strongly suggested that the mutational event occurred very early, during embryonic development. As shown by functional studies, the identified mutations—an in-frame deletion and a recurrent *NLRP3* missense mutation—have a gain-of-function effect on *NLRP3*-inflammasome activation. Consistently, a complete remission was obtained in both patients with anti-IL-1 receptor antagonists. This study unveils that in late-onset chronic urticaria, the search for autoinflammatory markers and somatic mosaic *NLRP3* mutations may have important diagnostic and therapeutic consequences.

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INTRODUCTION

Urticarial rash is a common dermatological disorder characterized by sudden and recurrent onset of usually pruritic cutaneous wheals with pale central swelling, surrounded by skin erythema and edema, affecting about 15–25% of individuals (Kanani et al., 2018). Hives appear over any part of

the body, and individual lesions usually last for a few hours and up to 24 hours (Criado et al., 2015). In its acute form, the urticarial rash lasts for less than 6 weeks, whereas in the chronic form, the rash extends over 6 weeks in a continuous or intermittent manner (Kaplan and Greaves, 2009). Chronic urticaria is more common in adults, with a peak age of onset between 20 and 40 years, and can be associated with significant morbidity and decreased quality of life. In the absence of physical triggers, chronic urticarial rash is called idiopathic or spontaneous (Beck et al., 2017; Zuberbier et al., 2014). Allergy and autoimmune and infectious disorders can underlie chronic urticaria, a symptom that requires extensive tests for identifying the causative factors (Beck et al., 2017). Urticarial rash can also be one of the symptoms of *NLRP3*-associated autoinflammatory disease (*NLRP3*-AID), a group of rare monogenic systemic autoinflammatory disorders previously known as cryopyrin-associated periodic syndrome (Ben-Chetrit et al., 2018; Kuemmerle-Deschner et al., 2017). *NLRP3*-AID is clinically a heterogeneous syndrome and consists of three overlapping entities with increasing severity: (i) familial cold autoinflammatory syndrome (OMIM #120100), characterized by neonatal onset of cold-induced inflammatory episodes of fever, arthralgia, and urticarial rash; (ii) Muckle-Wells syndrome (OMIM #191900), characterized by infantile-to-childhood onset of recurrent attacks of fever, urticarial skin rash, and arthritis with subsequent sensorineural hearing loss; and (iii) chronic infantile neurological, cutaneous, and articular syndrome (OMIM

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Abbreviations: EV, empty vector; *NLRP3*-AID, *NLRP3*-associated autoinflammatory disease; WT, wild-type

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#607115), characterized by infantile onset of a chronic persistent clinical disease course of ongoing fever, central nervous system involvement, continuous urticarial skin rash, and deformative joint arthritis (Kuemmerle-Deschner, 2015).

NLRP3, the disease-causing gene, is expressed mainly in myeloid blood cells but also in B and T lymphocytes (Hoffman et al., 2001a; Sutterwala et al., 2006) and keratinocytes, which could therefore account for the predisposition of patients for urticarial rash (Dai et al., 2017; Kummer et al., 2007). The encoded *NLRP3* protein (also known as cryopyrin) comprises the following domains: N-terminal pyrin, NACHT, NAD, and C-terminal leucine-rich repeats. *NLRP3* initiates the assembly of a multiprotein complex called the inflammasome (Martinon et al., 2002; Schroder and Tschopp, 2010) through the following steps: *NLRP3* interacts with the adaptor protein ASC, leading to the assembly of ASC dimers into multimers designated as ASC specks (Fernandes-Alnemri et al., 2007). ASC specks, the hallmark of inflammasome activation, form a scaffold for recruitment and subsequent activation of procaspase-1 into caspase-1. Active caspase-1 cleaves proinflammatory cytokines, mainly IL-1 β , into their mature and secreted forms, which orchestrate the systemic inflammatory response (Martinon et al., 2009).

NLRP3 mutations identified in *NLRP3*-AID patients result in a gain-of-function effect leading to *NLRP3*-inflammasome activation. Initially described in affected families as autosomal dominant disorders because of heterozygous *NLRP3* mutations (Hoffman et al., 2001a), *NLRP3*-AID is now also known as a sporadic disease because of de novo germline or somatic mutations. Primarily identified in leukocytes from pediatric patients (Aróstegui et al., 2010; Lasigliè et al., 2017; Nakagawa et al., 2015; Omyinmi et al., 2014; Saito et al., 2008, 2005; Tanaka et al., 2011), somatic mosaic *NLRP3* mutations have been found more recently in some adult patients with a typical *NLRP3*-AID phenotype (Mensa-Vilaro et al., 2016; Rowczenio et al., 2017; Zhou et al., 2015).

We describe here two unrelated elderly patients presenting for two decades with a chronic urticarial skin rash resistant to standard therapy and associated with systemic inflammation during the rashes. Two different mosaic *NLRP3* mutations were identified by means of targeted sequencing using next generation technologies. To identify the origin of these mutational events, we studied the mosaicism level in several cell types from both patients. In addition, to assess the pathogenicity of the identified variations, we studied their functional consequences using different in vitro assays.

RESULTS

Disease phenotype (Table 1)

Proband I, a 65-year-old man, had recurrent episodes of diffuse nonpruritic fleeting urticarial skin rash involving the trunk and limbs (Figure 1a). The rash was intermittent, lasted for a few hours (4–5 hours) and repeated every 2–3 days since the age of 49 years. No triggers such as cold exposure, physical exercise, stress, nutrition, or medications were noted. The urticarial lesions were accompanied with high-grade fever (39 °C), chills, and flu-like symptoms including arthralgia, fatigue, and headache. There was no vasculitis, adenopathy, organomegaly, or ophthalmological, abdominal or neurological manifestations. During the crises, C-reactive

protein was elevated (20 mg/liter). Immunological and infectious profile, serum protein electrophoresis, and kidney function tests were normal. Skin biopsy revealed urticarial lesions with a rich inflammatory infiltrate composed mostly of neutrophils with no signs of vasculitis (Figure 1c).

Proband II, a 67-year-old woman, developed the first episode of chronic urticarial skin rash at the age of 46 years. Urticarial lesions manifested as migratory transient erythematous plaques (Figure 1b) with no pruritus. Distributed over the whole body (thighs, abdomen, thorax, upper limbs, and face), the rash, which was not triggered by cold, lasted fewer than 12 hours and occurred two to three times per week. It was associated with high-grade fever (39–40 °C), chills, arthritis, myalgia, headache, adenopathy, and hepatomegaly. No other abdominal or ophthalmological manifestations were observed. During the attacks, C-reactive protein levels ranged from 70 to 120 mg/liter. At the age of 50 years, she developed bilateral sensorineural hearing loss. A skin biopsy revealed the presence of dermal neutrophilic infiltration with no alteration of capillaries and no signs of vasculitis (Figure 1d). Kidney function tests were normal, and extensive analyses of the immunological and infectious profile were negative. Serum protein electrophoresis revealed polyclonal hypergammaglobulinemia.

Neither of the patients had a family history of skin, autoimmune, or autoinflammatory disorders. In both probands, second generation H1-antihistamines (the standard therapy), oral corticosteroids, and colchicine showed no efficacy.

Molecular analyses

With the aim to identify the etiology of the chronic recurrent urticaria that occurred in a systemic inflammatory context, we collected the genomic DNA from whole blood samples of Proband I and II. We looked for systemic autoinflammatory disorders through targeted sequencing of the main genes so far involved in these conditions. In both patients, somewhat unexpectedly, somatic mosaic mutations in *NLRP3* exon 3 were identified. Proband I carried an in-frame deletion [c.926_934del p.(Gly309_Phe311del)] and Proband II, a missense variation [c.1705G>A p.(Glu569Lys)]. The mosaicism level in the whole blood DNA for c.926_934del was 17.2%, and the level was 11% for the c.1705G>A transition (Supplementary Figure S1).

The in-frame deletion of three amino acids (Gly309, Ala310, and Phe311) identified in Proband I is located within the NACHT domain of *NLRP3* (Figure 2a) in close proximity to the Walker B motif of the protein, which is a Mg²⁺-binding loop implicated in ATP hydrolysis and essential for *NLRP3*-inflammasome activation (Neven et al., 2004). The glutamic acid residue at position 569 (Glu569), involved in the mutation identified in Proband II (p.Glu569Lys), belongs to the NAD domain of the *NLRP3* protein, a domain of so far unknown function (Figure 2a). The identified mutations involved evolutionarily conserved amino acids in mammals (Figure 2b). Moreover, both the *NLRP3* transcript, including exon 3 that contains these *NLRP3* mutations (<https://ecrbrowser.dcode.org>), and the encoded regions are uniformly conserved across mammalian species (Duéñez-Guzmán and Haig, 2014; Lv et al., 2017).

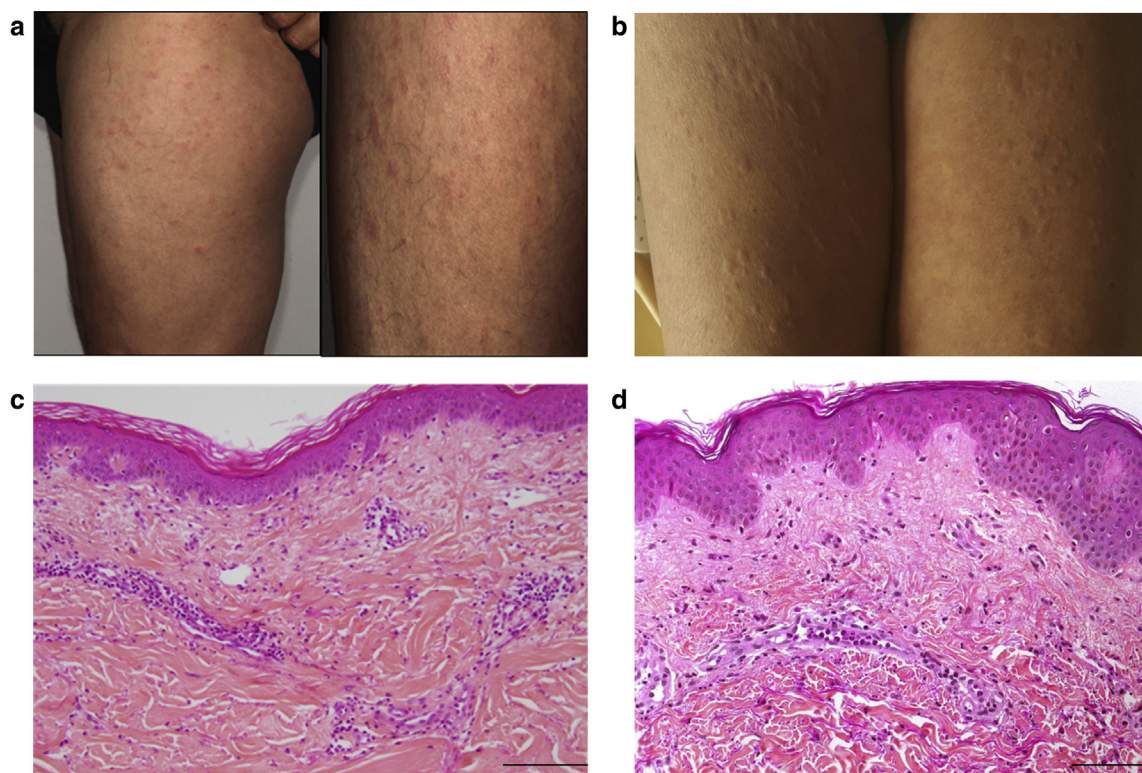


Figure 1. Urticarial lesions and skin biopsy. Cutaneous photography of (a) Proband I and (b) Proband II. Skin biopsy of the urticarial lesions of (c) Proband I (Bar = 200 μ m) and (d) Proband II (Bar = 100 μ m), showing rich dermal polymorphonuclear cells, mainly neutrophilic infiltration, using HES stain. HES, hematoxylin, eosin, and saffron.

Modelization of the *NLRP3* structure, based on a Nod2 crystal (Maekawa et al., 2016), showed that the three deleted amino acids and Glu569 are located close to the Walker B motif (Figure 2c).

Cellular distribution of *NLRP3* mosaicism

To study the cellular distribution of the *NLRP3* mosaicism, different cell types were subsequently isolated from Probands I and II. The analyzed cells included monocytes, neutrophils, B and T cells that have a mesodermal embryonic origin, urinary epithelial cells that are representative of the endodermal embryonic cells, and buccal cells that are of ectodermal origin. Targeted sequencing showed a wide distribution of the mutated alleles in the studied cells (Figure 3, lower panel and Supplementary Figure S1). We took advantage of these data to look for the two identified *NLRP3* variants by Sanger sequencing. This study revealed the presence of a superimposed sequence, mimicking a background noise, but corresponding to those mutations (Figure 3, upper panel). Mutated alleles were detected in monocytes, neutrophils, and B cells at average levels of 13% in Proband I and 12% in Proband II. The T cells showed a proportion of the mutated allele of 10% for Proband I and 5% for Proband II. In buccal cells, the proportion of the mutated allele was 3.8% for Proband I, and it was estimated around 5% (using Sanger sequencing because of the low amount of DNA available) for Proband II. In the urinary epithelial cells, the proportion of the mutated allele was estimated around 5% (using Sanger sequencing) in both probands.

Impact of *NLRP3* mutations on ASC speck formation

To assess the pathogenicity of the identified *NLRP3* mutations, we tested their effects on *NLRP3* function by studying ASC speck formation, a common readout of inflammasome activation (Man and Kanneganti, 2015). To this end, we transfected HEK293T (ASC-GFP_C1-FLAG) cells with plasmids encoding wild-type (WT) *NLRP3*, *NLRP3* carrying the deletion (p*NLRP3*-Del) or the missense variation (p*NLRP3*-Glu569Lys), or an empty vector (EV). In agreement with the known role of *NLRP3* in inflammasome assembly and ASC speck formation, cells transfected with p*NLRP3*-WT

Table 1. Phenotype and Genotype of Patients with Chronic Urticaria and a Mosaic *NLRP3* Mutation

Characteristics	Proband I	Proband II
Sex	Male	Female
Current age (years)	65	67
Age of urticarial onset (years)	49	45
Fever	+	+
Arthralgia/Arthritis	+	+
Deafness (SNHL)	– ¹	+
<i>NLRP3</i> genotype	c.926_934del p.(Gly309_Phe311del)	c.1705G>A p.(Glu569Lys) ²
Percentage of the mutated allele	17%	11%

Abbreviation: SNHL, sensorineural hearing loss.

¹SNHL, which was not reported at study inclusion, was identified by means of audiometric tests that were performed once the *NLRP3* mutation was found.

²Also known as c.1699G>A, p.E567K.

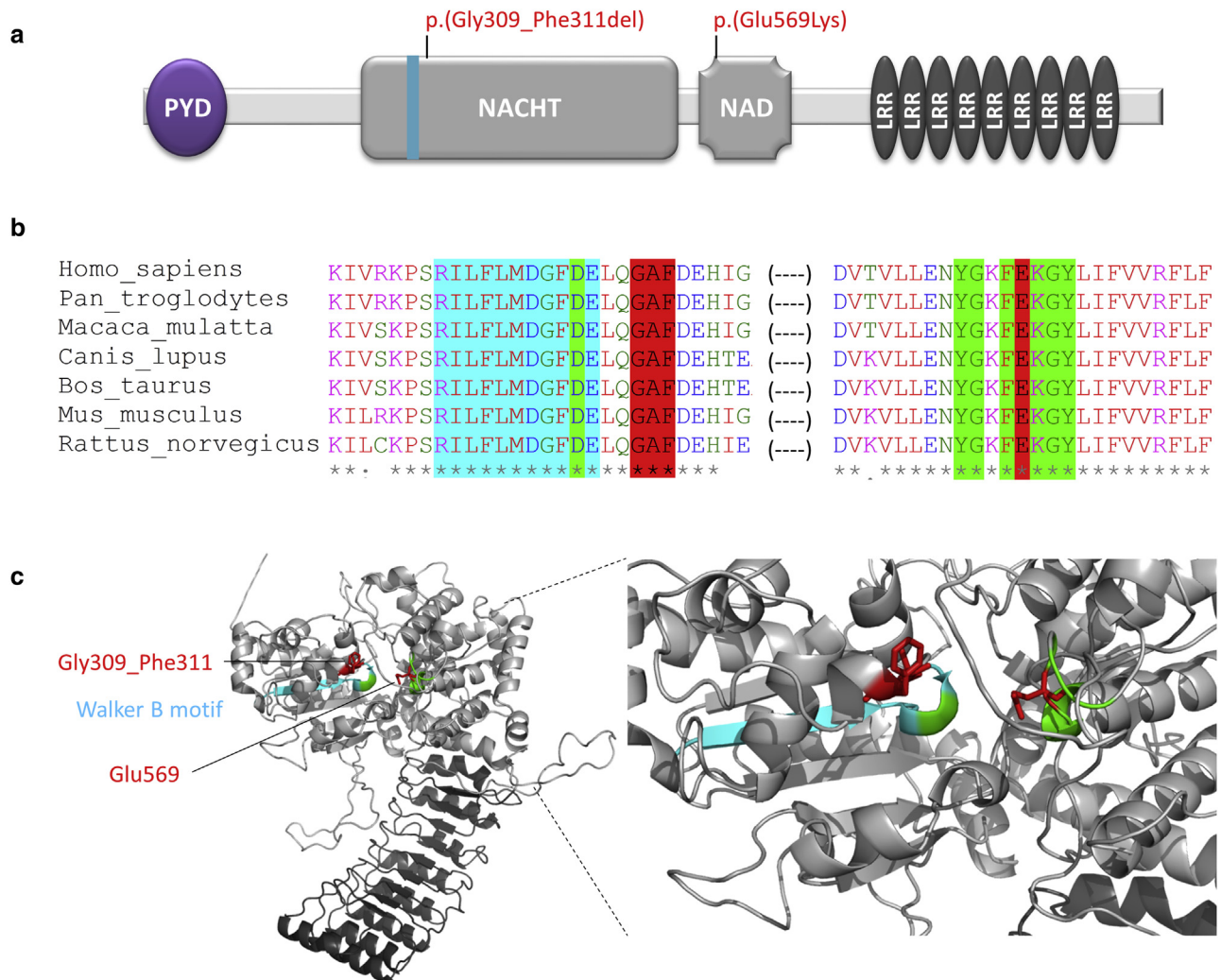


Figure 2. *NLRP3* mutations and their impact at the protein level. (a) *NLRP3* protein domain-organization diagram. Location of the c.926_934del p.(Gly309_Phe311del) and the c.1705G>A p.(Glu569Lys) mutations identified in this study in the NACHT and NAD domains, respectively. The Walker B motif is illustrated in blue. (b) Partial protein alignment of *NLRP3* in different species showing the evolutionary conservation of amino acids Gly309_Phe311, Glu569, and those involved in the previously reported mosaic *NLRP3* mutations (hot spots). (*) indicates fully conserved residue; (:) indicates strongly similar properties; (.) indicates similar properties. (c) Modelization of the *NLRP3* structure showing the location of the identified mutations, p.(Gly309_Phe311del) and p.(Glu569Lys), and of the previously reported *NLRP3* mutations. The NACHT and NAD domains are represented in light gray and the LRR domain in dark gray. The residues involved in the mutations identified in this study are in red, those previously reported are in green, and the Walker B motif is in blue. LRR, leucine-rich repeat; PYD, pyrin domain.

displayed a significantly higher percentage of ASC specks than cells transfected with the EV alone. Importantly, cells transfected with the pNLRP3-Del or pNLRP3-Glu569Lys construct showed a significantly higher percentage of ASC specks than cells transfected with pNLRP3-WT, thereby revealing a gain-of-function effect of the two identified mutations on inflammasome activation (Figure 4 and Supplementary Figure S2).

Impact of *NLRP3* mutations on IL-1 β secretion

To test the impact of the p.(Gly309_Phe311del) and p.(Glu569Lys) mutations on cytokine secretion, we used THP1 cells, which express endogenously *NLRP3*, ASC, procaspase-1, and IL-1 β . THP1 cells were first primed with phorbol 12-myristate 13-acetate to obtain an adherent macrophage-like phenotype and to induce *IL1B* gene expression. Cells were then transfected with pNLRP3-WT, pNLRP3-Del, pNLRP3-Glu569Lys, or the EV and treated with lipopolysaccharide, a

well-known activator of the *NLRP3* inflammasome. IL-1 β secretion, a hallmark of inflammasome activation, was assessed in the cell culture supernatants. IL-1 β levels were found to be significantly higher in the supernatant of cells transfected with pNLRP3-Del or pNLRP3-Glu569Lys than cells transfected either with pNLRP3-WT or the EV (Figure 5).

Treatment

Consistent with the described data showing *NLRP3*-inflammasome activation, both probands showed complete remission with the anti-IL-1 receptor antagonist anakinra (100 mg/day) administered by subcutaneous injections. One week after anakinra administration, both patients reported a significant clinical and biological improvement. A complete resolution of the urticarial skin rash, fever, and systemic inflammation (C-reactive protein levels <5 mg/liter) was observed. Proband I and II are still under treatment today, having received the treatment for the past 2 and 3 years,

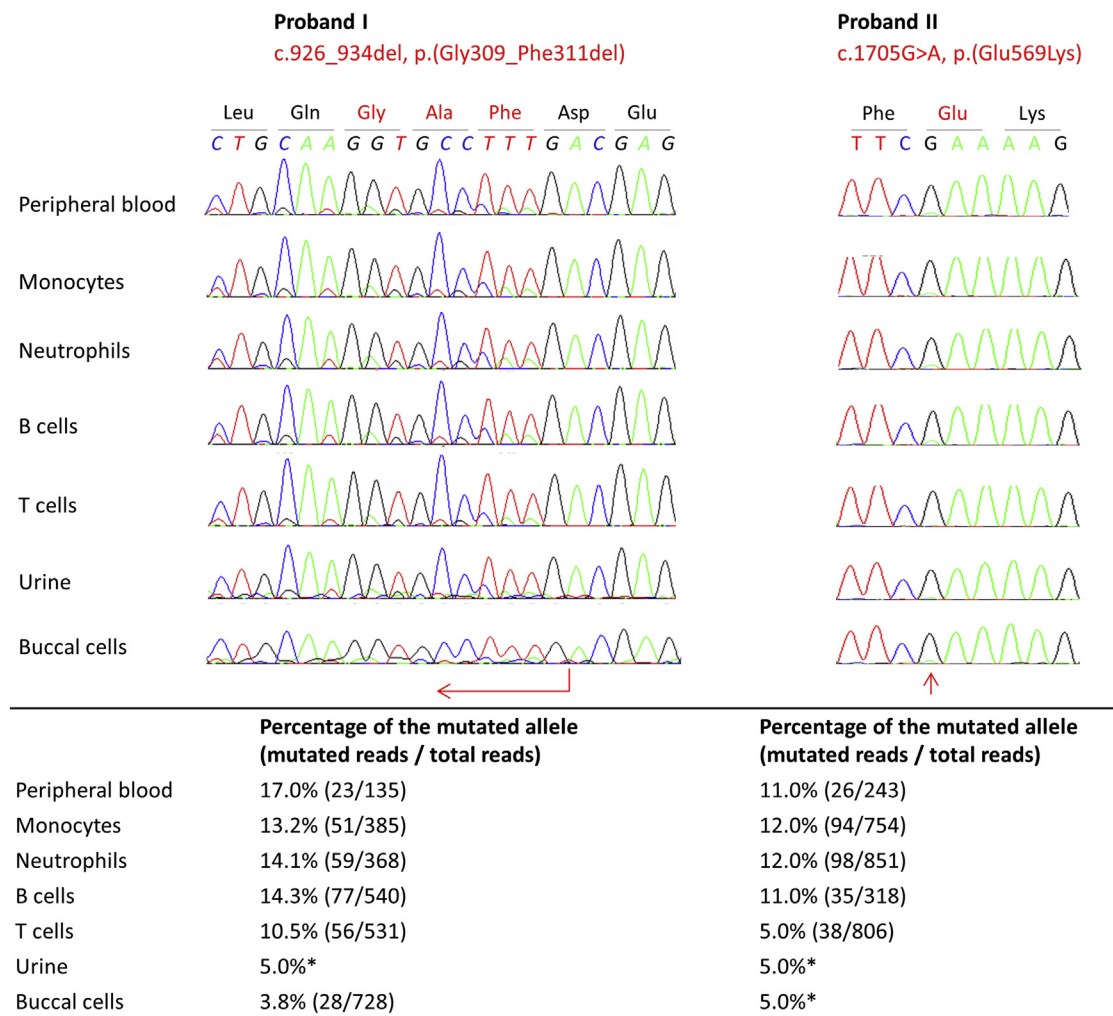


Figure 3. *NLRP3* DNA sequencing in different cell types. Upper panel: Sanger electropherograms showing the identified mosaic *NLRP3* mutations c.926_934del p.(Gly309_Phe311del) in Proband I (left) and c.1705G>A p.(Glu569Lys) in Proband II (right). The mutated amino acids are highlighted in red. The red arrows indicate the beginning of the deletion (c.926_934del) sequenced with the reverse primer in Proband I and the point mutation (c.1705G>A) identified in Proband II. Lower panel: The percentage of the mutated alleles and the ratio of the mutated reads to the total number of reads identified by targeted sequencing approach. * indicates the estimated percentage of the mutated allele based on Sanger sequencing.

respectively. During the follow-up period, kidney function tests were found to be normal in both probands. However, the treatment has so far no effect on hearing loss.

DISCUSSION

We described two unrelated patients presenting long-term chronic urticarial skin rash of late-adulthood onset. The clinical presentation of the patients is reminiscent of neutrophilic urticarial dermatosis, a condition characterized by an urticarial rash with predominate neutrophilic infiltration at skin biopsy and systemic inflammation (Gusdorf and Lipsker, 2018; Kieffer et al., 2009). For two decades, our two patients were refractory to antihistamines, steroids, and colchicine and, despite broad investigations, the underlying etiology was not identified. In both patients, the results of serum protein electrophoresis strongly argued against the diagnosis of Schnitzler syndrome, which is mainly characterized by the simultaneous occurrence of monoclonal gammopathy and chronic urticaria (Gusdorf and Lipsker, 2017). Taken together, these data prompted us to look for possible gene mutations

through the use of a targeted sequencing approach using next generation sequencing technology focusing on the main genes so far implicated in autoinflammatory disorders. Somewhat unexpectedly, this analysis identified in both patients a somatic mosaic *NLRP3* variation, raising the question of an *NLRP3*-AID diagnosis in the two patients. The diagnosis of *NLRP3*-AID is indeed challenging for several clinical and molecular reasons.

First, mostly reported in children (either in familial forms or sporadic cases), the clinical diagnosis of *NLRP3*-AID is puzzling in the absence of a contributive family history and in adult patients with late-onset chronic urticaria occurring in the context of systemic inflammation. Although the occurrence of deafness in such context should evoke the diagnosis of *NLRP3*-AID, hearing loss is common with aging. Of note, sensorineural hearing loss, which is a hallmark of Muckle-Wells syndrome, was present in both probands. It was identified at the age of 50 years for Proband II, whereas for Proband I, it was identified at the age of 65 years by means of audiometric tests that were performed once the *NLRP3* mutation was found.

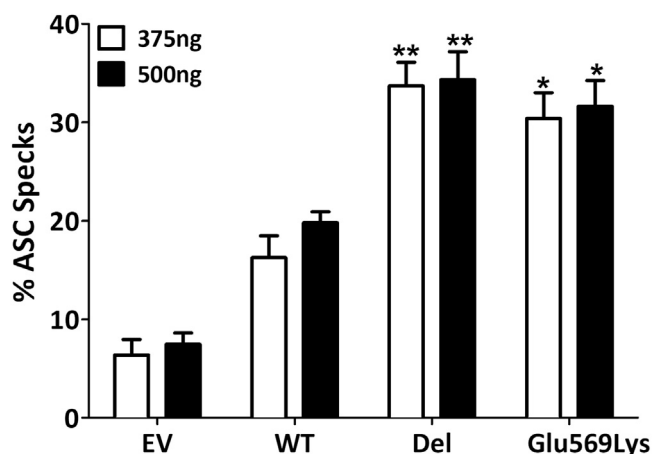


Figure 4. Impact of *NLRP3* mutations on ASC speck formation. HEK (ASC-GFP_C1-FLAG) cells were transfected with different amounts (375 ng or 500 ng) of the expression plasmids encoding the WT *NLRP3* (pNLRP3-WT), the *NLRP3* protein carrying the in-frame deletion p.(Gly309_Phe311del) called pNLRP3-Del or the missense mutation p.(Glu569Lys) called pNLRP3-Glu569Lys, or the EV. The percentage of ASC specks was calculated as described in Materials and Methods. Results represent the mean + SD from three independent experiments. *P*-values were calculated using unpaired Student's *t*-test. * indicates significant *P*-value (<0.05) compared with the wild-type (***P* < 0.01, **P* < 0.05). Del, deletion; EV, empty vector; SD, standard deviation; WT, wild-type.

Second, regarding the molecular investigations, it is important to underline that mosaic mutations are usually undetectable by Sanger sequencing. Targeted sequencing using next generation sequencing technologies increases the sensitivity of detection of such somatic mutations, and high-depth sequencing is necessary to detect low level mosaicism. To further determine the cellular distribution of the mutated alleles, we analyzed the DNA obtained from patients' cells derived from different embryonic germ layers. A wide cell-type distribution was observed for both mutations, suggesting that the mutational events arose early during human embryogenesis. Similarly, a wide tissue distribution of the mutated mosaic *NLRP3* alleles also has been reported in five patients with a late-onset typical *NLRP3*-AID (Rowczenio et al., 2017). In patients carrying mosaic *NLRP3* mutations, gonadal cells were not studied for obvious reasons. Vertical transmission to the offspring cannot be excluded. The mechanism underlying the late-disease onset in patients carrying widely distributed somatic mutations is so far not understood. Variable age of disease onset was also observed in patients with germline *NLRP3* mutations (Hoffman et al., 2001b). As suggested by different authors (Giat and Lidar, 2014; Walker et al., 2016; Yu and Leslie, 2011), external factors such as cold, stress, or exercise could trigger the disease development.

Finally, to demonstrate the pathogenicity of newly identified *NLRP3* variants and, therefore, to establish the diagnosis of *NLRP3*-AID, functional studies are mandatory. Indeed, *NLRP3* variants do not always affect the protein function. The mosaic variations identified in our patients are located in two regions considered as hot spots for *NLRP3* mosaicism (Nakagawa et al., 2015). The in-frame deletion (p.Gly309_Phe311del) is located in the region of the NACHT domain (305–309 amino acids) that contains 8 out of the 47

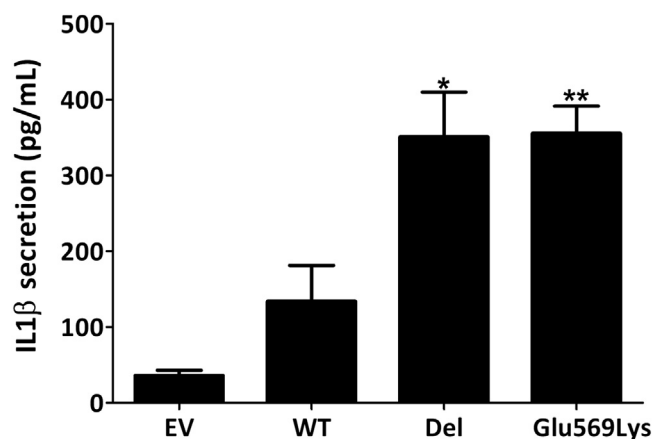


Figure 5. Impact of *NLRP3* mutations on IL-1 β secretion. IL-1 β secretion, as assessed by ELISA in cell culture supernatants of PMA-primed THP1 cells transfected with 500 ng of pNLRP3-WT, pNLRP3-Del, pNLRP3-Glu569Lys, or the EV and treated with LPS, a known stimulus of the *NLRP3* inflammasome. Results represent the mean + SD of three independent experiments performed in triplicates. *P*-values were calculated using unpaired Student's *t*-test. * indicates significant *P*-value (<0.05) as compared with the wild-type (**P* < 0.05, ***P* < 0.01). Del, deletion; EV, empty vector; PMA, phorbol 12-myristate 13-acetate; SD, standard deviation; WT, wild-type.

reported mosaic mutations. The p.(Glu569Lys) variant is located in the NAD domain (565–572 amino acids) that includes half of the reported mosaic mutations ($n = 22$ out of 47). The three-dimensional model of *NLRP3* showed that these two hot spots, including the in-frame deletion and the p.(Glu569Lys) variation, are close to the Walker B motif, thereby suggesting their pathogenicity. Using in vitro assays, we found significantly higher ASC speck formation and IL-1 β secretion in cells expressing the *NLRP3* mutants identified in our patients, thereby clearly demonstrating the pathogenicity of these mutations.

In conclusion, our data support the idea that in chronic urticaria, the presence of systemic inflammatory markers should evoke an *NLRP3*-related disorder. Beyond the search for *NLRP3* mutations, functional assays demonstrating a gain-of-function effect of those variants are essential to confirm their pathogenicity. Consistent with mutations triggering *NLRP3*-inflammasome activation, complete remission can be achieved with anti-IL-1 receptor antagonists, thereby highlighting the importance of identifying the underlying cause of chronic urticaria in those patients.

MATERIALS AND METHODS

Patients

Clinical features were recorded on a standardized form. Informed written consent for genetic studies was given by the participants. Skin punch biopsy was performed on recent urticarial lesions after local anesthesia. Skin sections were stained with hematoxylin, eosin, and saffron and examined under a light microscope. Human studies were approved by the ethics committee CPP Ile de France1 (No. 2018-nov-14970).

Molecular analyses

Genomic DNA was extracted from peripheral blood leukocytes of Proband I and II using standard procedures. Peripheral blood mononuclear cells were isolated from peripheral blood using

Pancoll density gradient. Peripheral blood mononuclear cells were separated by positive selection using immunomagnetic microbeads (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany), anti-CD14 (monocytes) followed by anti-CD15 (neutrophils), anti-CD19 (B cells), and anti-CD3 (T cells). DNA was then extracted from monocytes, neutrophils, B cells, T cells, urine, and buccal cells using proteinase K digestion followed by phenol-chloroform extraction protocol. The DNA samples were sequenced following a targeted sequencing approach using a custom targeted capture (SeqCap EZ system, Roche, Basel, Switzerland) on a NextSeq500 or MiSeq (Illumina, San Diego, CA) platform as previously described (Jeanson et al., 2016). The position of the target oligos is available on request. We used a custom targeted capture which contains the coding and the untranslated regions of the following genes: *ADA2*, *CARD14*, *IL1RN*, *IL36RN*, *LACC2*, *LPIN2*, *MEFV*, *MVK*, *NLRC4*, *NLRP12*, *NLRP3*, *NOD2*, *PLCG2*, *PSMB8*, *PSTPIP1*, *RBCK1*, *TMEM173*, *TNFAIP3*, *TNFRSF11A*, and *TNFRSF1A*. Sequence data were analyzed using an in-house double pipeline with sequence reads in fastq format aligned to the reference human genome (hg19) using BWA and Bowtie2. Duplicate sequence reads were removed (by Picard and SAMtools, respectively). Variant calling was performed using GATK and SAMtools, respectively. Additional screening of insertion-deletions was performed by the Pindel software. Variant calls in VCF format were then annotated through ANNOVAR. All results obtained by this targeted sequencing were confirmed using Sanger sequencing. *NLRP3* mutation numbering was based on the cDNA sequence (NM_004895).

Modelization of the *NLRP3* structure

The three-dimensional structure of the human *NLRP3* protein has been modeled as follows: Using the *HHpred* software, we found that the three-dimensional structure of the NACHT, NAD, and leucine-rich repeat domains of Nod2 (51RN, *Oryctolagus cuniculus*) (Maekawa et al., 2016) has been identified as the closest to human *NLRP3* protein. Using the *Modeller* software, the predicted structure of human *NLRP3* has been obtained. Using the *Pymol* software, the amino acids involved in mosaic *NLRP3* mutations have been highlighted.

Plasmid constructs

Human *NLRP3* cDNA was subcloned into the expression plasmid (pcDNA3.1/V5-His-TOPO, Invitrogen, Carlsbad, CA) to generate the WT expression vector (pNLRP3-WT) as described previously (Jéru et al., 2006). Site-directed mutagenesis was subsequently performed to generate plasmids carrying either the deletion identified in Proband I (p.Gly309_Phe311del), designated as pNLRP3-Del, or the previously reported p.(Glu569Lys) mutation that we identified in Proband II (pNLRP3-Glu569Lys), using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The resulting plasmid constructs were confirmed by Sanger sequencing.

Cell culture and transfection

HEK293T cells stably expressing FLAG-tagged procaspase-1 (procaspase-1-FLAG) and GFP-tagged ASC (designated ASC-GFP and C1-FLAG), kindly provided by Professor Emad Alnemri (Thomas Jefferson University, Philadelphia, PA), were cultured in DMEM/F-12 (Gibco, Life Technologies, Paisley, UK). HEK293T cells (ASC-GFP and C1-Flag) were transfected with 375 ng or 500 ng of either pNLRP3-WT, pNLRP3-Del, pNLRP3-Glu569Lys, or the EV using FuGENE-HD (Promega, Madison, WI) for 24 hours. THP1 cells (ATCC-TIB202) were initially primed with 100 ng/ml phorbol 12-myristate 13-acetate for 3 hours, then transfected with 500 ng of

pNLRP3-WT, pNLRP3-Del, pNLRP3-Glu569Lys, or the EV using the FF-100 program in the 4D-Nucleofector, Amaxa (Lonza, Basel, Switzerland) for THP1 cells. Following transfection, cells were treated with 100 ng/ml of lipopolysaccharide and incubated at 37 °C for 24 hours. At the indicated time, supernatants of transfected cells were collected and stored at -80 °C for IL-1 β measurement by ELISA.

Speck quantification assay

Following transfection of HEK293T cells (ASC-GFP_C1-FLAG), the formation of ASC aggregates (specks) was calculated 24 hours later. Manual counting of cells containing ASC-GFP specks was performed in five representative randomly selected fields at $\times 20$ magnification. The percentage of specks was calculated as the number of specks divided by the total number of counted cells for three independent experiments. Cells were observed using a Nikon Eclipse TS100 inverted fluorescent microscope.

Statistical analyses

Differences were analyzed statistically using the unpaired Student *t*-test and were plotted with the Prism 5, GraphPad Software, (San Diego, CA). A *P*-value < 0.05 was considered as statistically significant.

Data availability statement

All the data sets related to this study are included in the article.

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CONFLICT OF INTEREST

GG declares fees less than 5000 euros from Novartis, BMS, and SOBI. The remaining authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

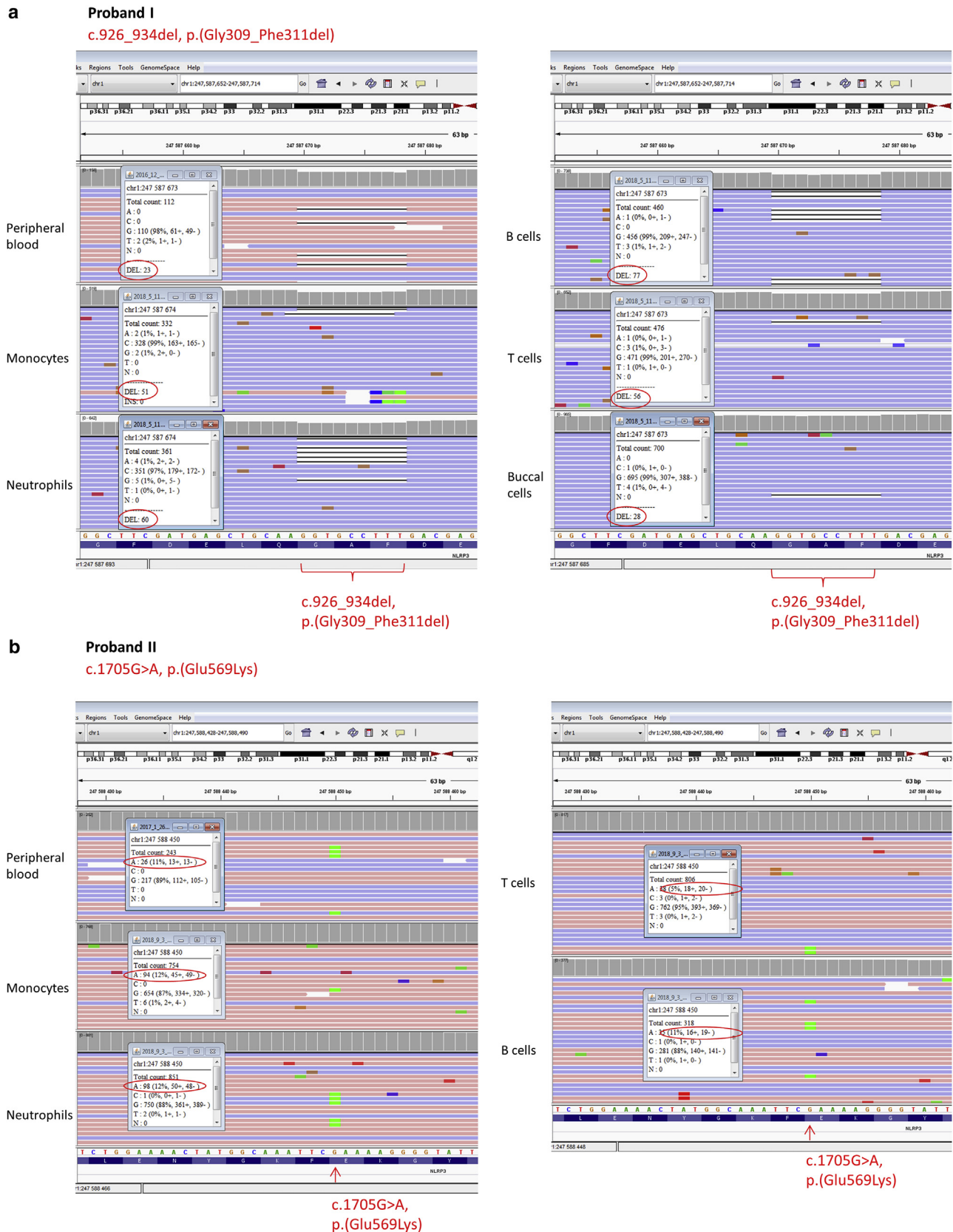
Conceptualization: IG, SA; Data Curation: EA, CLo; Formal Analysis: EA, CLo; Funding Acquisition: SGL, GG, SAK, SA, IG; Investigation: EA, PM, WP, MDVP; Methodology: FA, PD, SAK, ML; Project Administration: IG, SA; Resources: CL, SGL, JDB, FM, GG; Software: BC; Supervision: SAK, IG, SA; Validation: CJ, LC, EEK, PD; Visualization: EA, CLo, CL; Writing - Original Draft Preparation: EA, ML, SAK, IG, SA. Writing - Review and Editing: EA, CLo, CL, SGL, JDB, FA, FM, PM, MDVP, WP, CJ, LC, EEK, BC, PD, ML, GG, SAK, IG, SA.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at <https://doi.org/10.1016/j.jid.2019.06.153>.

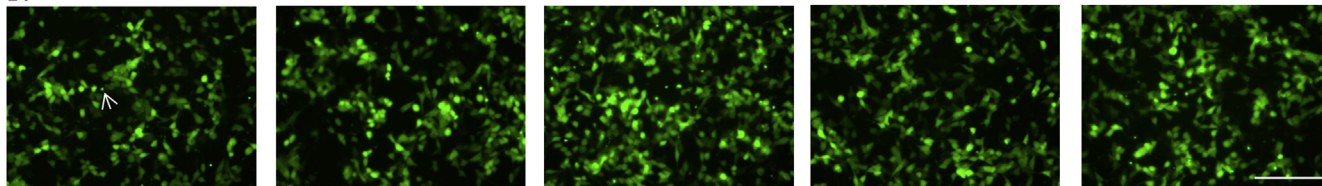
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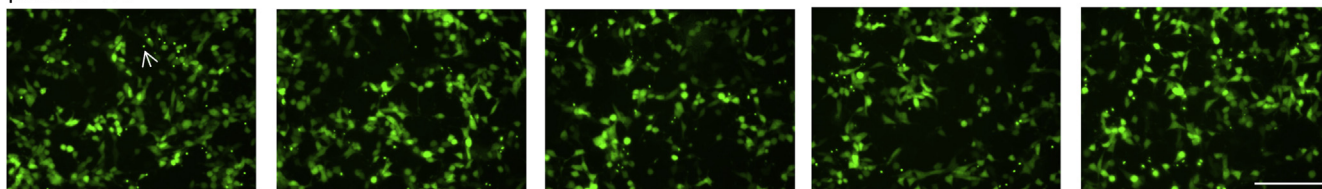


Supplementary Figure S1. Identification of somatic mosaic *NLRP3* mutations. Visualization using Integrative Genomics Viewer of the mosaic *NLRP3* mutations, c.926_934del p.(Gly309_Phe311del) and c.1705G>A p.(Glu569Lys), identified by Illumina sequencing in different cell types of (a) Proband I and (b) Proband II.

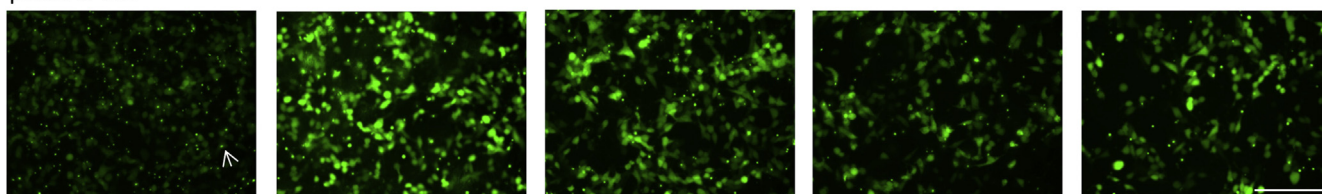
EV



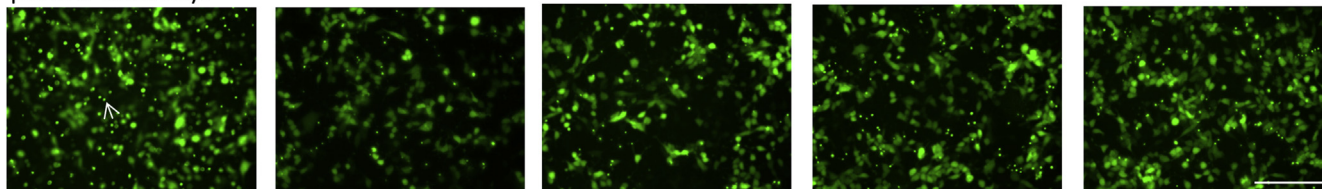
pNLRP3-WT



pNLRP3-Del



pNLRP3-Glu569Lys



Supplementary Figure S2. ASC speck formation. Examples of five randomly selected fields at $\times 20$ magnification of HEK cells containing ASC-GFP specks collected through one experiment and plotted in [Figure 4](#). White arrowheads point examples of ASC specks. EV, empty vector; WT, wild-type. Bar = 50 μm .