SHORT REPORT

Contrasting role of NLRP12 in autoinflammation: evidence from a case report and mouse models

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ABSTRACT

Objective To explore at the molecular level the phenotype of a patient suffering an autoinflammatory syndrome which was diagnosed as familial cold autoinflammatory syndrome type 2 (FCAS-2). To explore the functions of Nlrp12 in inflammation using mouse models.

Methods Whole exome sequencing and Nlrp12 targeted resequencing were performed on DNA isolated from the patient and her family members. In vivo and ex vivo models of inflammation (urate crystals-dependent acute joint inflammation and urate crystals-induced peritonitis) were analysed in Nlrp12-deficient and Nlrp12-competent mice.

Results A rare missense NLRP12 variant (c.857C>T, p.P286L) was identified in the patient and her healthy relatives. Nlrp12-deficient mice exhibit reduced systemic inflammation and neutrophil infiltration.

Conclusion Nlrp12 mediates proinflammatory functions in mice. In humans, the identification of Nlrp12 variants must be cautiously interpreted depending on clinical and paraclinical data to diagnose FCAS-2.

Key messages

What is already known about this subject?
- In humans, several rare mutations in NLRP12 have been associated to autoinflammatory syndromes.

What does this study add?
- This study reports the case of a rare NLRP12 variant in both healthy and affected family members, suggesting low penetrance/variable manifestation of the mutation.

How might this impact on clinical practice or further developments?
- Diagnosis of familial cold autoinflammatory syndrome type 2, an autoinflammatory disease exhibiting considerable phenotypic variation, requires both genetic and clinical (therapeutic response to IL-1β blockers) information.

INTRODUCTION

Systemic autoinflammatory disorders (SAIDs) are caused by defects of innate immune responses leading to excessive inflammation via many different pathways.1 Patients share recurrent flares of fever, dramatic elevation of acute phase reactants and variable clinical manifestations such as rash. Recently, a new classification (including genetic data) of these defects, also called hereditary recurrent fever syndromes, has been published.2 Among these diseases, inflammasomopathies are the most common.3 Inflammasomes are intracellular multiprotein complexes involved in sensing and responding to both extracellular and intracellular factors, leading to the maturation and release of bioactive interleukin (IL)-1β which is necessary for appropriate responses against various pathogens.4 These complexes are formed on ligand-dependent association of a sensor, the adaptor ASC (Apoptosis-associated speck like protein containing a caspase recruitment domain) (except in the case of the NLRP1 and NLRC4) and the procaspase 1 which becomes activated by proximity-induced self-cleavage.5 Many sensors have been described, belonging to either the NLRs (nucleotide-binding domain and leucine-rich repeat containing receptors), absent in melanoma 2-like receptors and pyrin families, but the most extensively studied is NALP3, which forms the NLRP3 inflammasome. Together with Pyrin (306 unique variants reported in the Leiden Open Variation Database), NALP3 (235 variants) is a frequently affected inflammasome, causing respectively cryopyrinopathies (or cryopyrin-associated period syndromes) and familial Mediterranean fever.1 6 In contrast, NLRP12 plays controversial functions, as it can assemble into an inflammasome or act as a negative regulator of inflammation.7 Its involvement in host defence appears...
undisputable, at least in mice, but whether variants in the NLRP12 gene in humans are associated with autoinflammatory syndromes is still debated. To date, 57 unique variants have been described in patients who exhibit considerable clinical heterogeneity. Nevertheless, familial, cold, autoinflammatory syndrome type 2 (FCAS-2, OMIM # 611762) is depicted as a rare condition associated with NLRP12. Since the initial description of the first two patients, 61 cases with NLRP12 variants have been reported so far. Altogether, it appears that NLRP12, which ligand remains unknown, carries a potential role in autoinflammation.

Here, we describe a rare missense variant in NLRP12 in a patient with late onset autoinflammatory syndrome that was successfully treated with IL-1 blockade. These clinical observations suggesting a potential role of NLRP12 in autoinflammation were further supported by in vivo and ex vivo investigations of mouse models. However, further genetic analyses revealed that this variant was also present in healthy family members of the proposita, indicating a possible incomplete penetrance and supporting a controversial role of NLRP12 in autoinflammation.

**METHODS**

**Genetic analyses**

For whole exome sequencing (WES), genomic DNA was isolated from patient and parents’ peripheral blood or saliva using standard protocols. Exome sequencing was performed on a NextSeq500 sequencer (Illumina, San Diego, California, USA) as described previously. We focused only on protein-altering variants (missense, nonsense, splice site variants and indels). To identify potential causal variants, we further filtered the variants based on a dominant mode of inheritance, that is, variants present in the patient and absent in her healthy daughter. For targeted resequencing, the candidate variants were confirmed by capillary Sanger sequencing on an ABI PRISM 3730x1 sequencer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and analysed on the SeqScape software (Thermo Fisher Scientific).

**Quantification of cytokines**

IL-1β (ref no 88-7261-22), IL-6 (ref no 88-7066-22) and IL-8 (ref no 88-8086-88) were quantified using dedicated ELISA according to the manufacturer’s recommendations (eBioscience, San Diego, California, USA).

**Real-time quantitative PCR**

Total RNA was prepared from joints (dissected hindpaws) as described and reverse transcribed using the cDNA synthesis kit (iScript ready-to-use cDNA supermix, Biorad, ref no 4106228). Real-time quantitative PCR (RT-qPCR) was performed in a total volume of 20µL using the Sso-advanced universal SYBR-Green supermix (Biorad, ref no 10000076382) and gene-specific primers (list available on request). After a denaturing step at 95°C for 30 s, 40 cycles were performed (95°C for 5 s and 60°C for 20 s) using a Rotor-Gene 6000 RT-PCR machine (Corbett Life Science). Results were obtained using the SDS Software (Perkin Elmer). Melting-curve analysis was performed to assess the specificity of PCR products. Relative expression was calculated using the comparative threshold cycle (Ct) method. The Ct of the gene of interest was adjusted to the average Ct of three housekeeping genes (Hprt, Actin and Gapdh) to obtain a ΔCt.

**Statistics**

Following normality tests (Kolmogorov-Smirnov and Shapiro-Wilk), data were analysed with a Mann-Whitney test or unpaired t test (two-tailed unpaired) to compare two independent groups and non-parametric (Spearman) test for correlation analysis. Statistics were calculated with GraphPad V.5.01 software. A p < 0.05 was considered significant. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

**RESULTS**

**Case presentation**

The patient is a 75-year-old French woman (born 1944 of Italian parents) who was diagnosed with rheumatoid arthritis in 2008 (with rheumatoid factor, antinuclear antibodies), a benign cutaneous mastocytosis and osteoporosis without fracture. No long-term therapy was initiated. In December 2011 (at the age of 67), she suffered periodic fever (38°C–38.8°C) occurring in the morning twice or three times per week, associated with diffuse myalgia, chronic urticaria (that lasted 4 months and was not cold-dependent), unilateral deafness and a deterioration of the general state. Joint examination revealed no synovitis, enthesis or dactylitis. Her cardiac and pulmonary examination was within normal limits. Laboratory data revealed increased inflammatory markers (neutrophilia, C reactive protein, CRP above 100 mg/L), anti-nuclear antibody positive (1/640) with dense speckled fluorescence, native-DNA antibody and rheumatic factor slightly positive, anti extractable nuclear antigen negative; analyses of complement components and Creatine Kinase (CK) were normal. Q fever serology was slightly positive (treatment with doxycycline was not contributing); other analyses (including blood cultures collected during fever peaks) for potential infectious diseases (brucellosis, borreliosis, rickettsiosis, HIV, hepatitis B, hepatitis C, leptospirosis) were negative. Positron-emission tomography, transthoracic echocardiography and bone marrow biopsy were normal. Muscular biopsy showed mild non-specific inflammatory infiltrates. Steroids and methotrexate did ameliorate her symptoms but had no effect on fever and the inflammatory syndrome persisted (CRP at 40 mg/L). An autoimmune inflammatory syndrome was evoked. She finally received IL1-blockers (anakinra first, that was stopped because of induced urticaria, followed by canakinumab, once a month, then once every 2 months) quickly resulting in a full clinical and biological recovery.
Genetic investigations

All the individuals in which genetic investigations were realised in this study gave their written informed consent to participate and for publication.

At this stage, an autoinflammatory syndrome was considered and genetic analysis involving the targeted resequencing of all the exons in a panel of 32 genes involved in autoinflammatory diseases (online supplemental table 1) was performed. This revealed the presence of a heterozygote variant (c.857C>T, p.P286L) in a conserved domain of NACHT, LRR and PYD domains-containing protein 12 (NLRP12) (online supplemental figure 1) which has not been described so far; the patient was diagnosed a NLRP12-associated periodic syndrome (NAPS12)\(^1\) with late onset. However, because the association of NLRP12 variants and inflammatory syndrome is still questioned,\(^1\) a WES was performed on the patient’s DNA and her unaffected daughter (born in 1974, aged 46 when blood was collected). We analysed all variants and found no pathogenic variants in an extended list of 55 genes related to inflammasome activation (a compilation of the genes included in the resequencing panel, of the fever panel described in \(^2\) and of additional inflammasome-related genes, online supplemental table 1). This analysis confirmed, in the patient’s exome, that the very rare (allele frequency=0.0001554 in the GnomAD database) variant of NLRP12 was the only candidate variant that could possibly explain the phenotype. However, we discovered that the unaffected daughter inherited the c.857C>T variant in NLRP12; further genetic analyses (consisting in a targeted resequencing of NLRP12, and not a WES) of additional family members revealed that this variant is also present in the proposita’s son, brother and sister (figure 1A,B), in which nor clinical items of autoinflammatory syndrome/FCAS2 (fever, rash, urticaria, arthralgia, myalgia, headache), neither biological signs of inflammation were apparent.

We interpreted these data as an additional example of possible low penetrance of NLRP12 variants, as recently discussed.\(^1^7\)

Mouse experiments

To better describe the potential role of NLRP12, we used mouse models of inflammation. For this, we compared the phenotype of C57Bl/6N (B6N) mice (here used as controls harbouring a wild-type NLRP12 gene) to that of C57Bl/6J (B6J) animals which have a spontaneous missense mutation (inducing a p.R1034K change at the protein level) that is phenocopied by the deletion of NLRP12.\(^1^8\) We first induced an acute inflammatory response in the joint, mimicking gouty arthritis, by subcutaneous injection of monosodium urate (MSU) crystals.\(^1^4\) As seen in figure 2, no difference was seen between controls (B6N) and Nlrp12 mutants (B6J) mice with regard to paw thickness (figure 2A) and the clinical score reflecting redness and oedema of the joint (figure 2B). However, we noticed that only a moderate increase in the body temperature could be recorded in C57Bl/6J (B6J) mice on MSU crystals injection, as opposed to controls (figure 2C). This observation, along with reduced seric IL-1β in B6J animal 120 hours after...
MSU injection (figure 2D), suggests that NLPR12 likely promotes systemic responses to an inflammatory trigger.

We next used a model in which peritonitis is induced by injection of MSU crystals into the peritoneal cavity. In this setting, while the total number of cells in the peritoneal fluid obtained 6 hours after MSU injection remained similar in controls (B6N) and Nlrp12 mutants (B6J, online supplemental figure 2A), we noted reduced neutrophilic infiltration in Nlrp12-deficient mice (online supplemental figure 2B,C). Quantification of inflammatory cytokines IL-6 and TNF remained similar in the peritoneal fluid of both strains (online supplemental figure 2D,E). These data suggest that Nlrp12 participates in the development of an inflammatory setting by promoting neutrophil migration.

Finally, we turned to an ex vivo model using primary thioglycolate-elicited macrophages and neutrophils harvested from C57Bl/6N and J mice to better understand Nlrp12-dependent molecular mechanisms at the cellular level. We first observed that Nlrp12 deficiency in B6j mice does not impair IL-1β secretion by stimulated macrophages or neutrophils (following Lipopolysaccharide (LPS) and MSU or LPS and Adenosine triphosphate (ATP) addition in the culture medium, figure 3A,B). To analyse the interactions between these cells, we used Boyden chambers and quantified the proportion of neutrophils migrating towards macrophages that are placed inside the well in 24 hours. As seen in figure 3C, cells harvested from B6j mice exhibit reduced migration. Of note, LPS stimulation abolished the difference between controls (B6N) and Nlrp12-deficient cells (B6j). This is in line with reduced Cxcl1 (encoding a major neutrophil chemoattractant) expression in steady-state macrophages isolated from B6j mice; again, LPS stimulation normalised gene expression compared with control B6N cells (figure 3D). Finally, we noted that Il-1β expression by resting or stimulated macrophages remained similar whether Nlpr12 is functional or not (figure 3E) and that LPS-stimulated B6j macrophages expressed reduced Il-6 (figure 3F), which may account for the low fever seen on MSU crystal-induced arthritis in these animals.

**DISCUSSION**

Our observations in mouse models indicate that Nlrp12 plays important roles in neutrophil recruitment, as noted previously. Given the prominent role of these cells in sterile inflammation, we suspect that their limited recruitment in Nlrp12-deficient C57BL/6J mice participates in the reduced inflammatory responses in vivo in these animals (figure 3 and online supplemental figure 2).

Inflammation is a complex process conferring protective immunity against a vast array of pathogens. To fulfil...
this task, inflammation requires many pathogen recognition receptors which have been selected throughout evolution, and trigger multiple signalling pathways driving innate and adaptive immunity. This response needs to be highly controlled, as evidenced in several instances of excessive inflammation driving tissue damage and chronic diseases. In the past 10 years, huge progresses have been made in the identification of genes involved in autoinflammatory diseases. With the advent of Next-Generation Sequencing technologies, the number of genes associated with autoinflammatory diseases has grown to 60. However, many of these diseases remain difficult to diagnose because of their heterogeneous clinical presentation. Furthermore, genetic analyses are difficult to interpret because a large number of variants in inflammatory genes have been described, some of which are included in genetic panels, while others, of ‘unknown significance’, are not considered. Hence, predictions of autoinflammatory syndromes based on genetic analyses remain challenging. This is particularly true for inflammamomopahies, a rare subset of SAIDs exhibiting a wide spectrum of phenotypes. Among them, NLRP12-associated autoinflammatory disease (also known as familial cold autoinflammatory syndrome 2) is a recently described entity essentially based on the presence of arthralgia, periodic fever in patients without canonical mutations in NLRP3. To date, few such patients were identified (see ref. for the most recent description) but the association between NLRP12 variants and spontaneous inflammation is not yet firmly established, nor is the molecular role of this inflammasome in the establishment of systemic inflammatory settings. In this work, we described a patient with late onset autoinflammatory syndromes which was diagnosed a likely FCAS2 based on the identification, by targeted gene resequencing, of a NLRP12 variant of unknown significance (although several bioinformatics programmes suggest potential damaging consequences of this mutation, see online supplemental table II) which has not been ascribed so far as causal for any disease. Consequently, Anakinra was prescribed and resulted in a complete remission, including fever extinction and CRP nivation. Following cutaneous reaction, Canakinumab was preferred, with similar results and better tolerance. IL-1β overproduction and inflammasome overactivation are hence likely responsible for the symptoms observed in this patient. WES performed secondarily confirmed that the NLRP12 variant was the best candidate variant to explain the phenotype but also unexpectedly showed that it was inherited by her (presently) healthy daughter. Furthermore, targeted NLRP12 resequencing showed that the son, the brother and the sister of the patient’s and her daughter’s monocytes (online supplemental figure 3B). These data should, however, be cautiously interpreted, as our gene expression quantification in mouse cells performed by RT-qPCR, may be not comparable to cytokine quantification performed by ELISA in the supernatant of culture human cells. Altogether, our work in mouse models highlights the importance of Nlrp12 in neutrophil-dependent inflammation, while evidence for a role of NLRP12 in human inflammamomopathies remains equivocal.

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Autoinflammatory disorders

NLRP3
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