Clinical Immunology 203 (2019) 45–52

Contents lists available at ScienceDirect

Clinical Immunology

journal homepage: www.elsevier.com/locate/yclim

MCC950 blocks enhanced interleukin-1β production in patients with NLRP3 low penetrance variants

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ARTICLE INFO

Keywords:
NLRP3 low penetrance mutations
IL-1β
MCC950
Autoinflammation
Autoimmunity

ABSTRACT

Objective: To determine the role of the NLRP3 inflammasome by using the selective NLRP3 inhibitor MCC950 in patients with NLRP3 low penetrance variants and clinical symptoms suggestive for an autoinflammatory syndrome including central nervous system (CNS) involvement.

Methods: Nineteen symptomatic patients with low penetrance NLRP3 variants (Q703K n = 17, V198M n = 2) recruited between 2011 and 2017 were included in this monocentric study. A functional inflammasome activation assay was performed in patients in comparison to healthy controls (HC), including the determination of interleukin-1beta (IL-1β), interleukin-6 (IL-6) and tumor-necrosis factor alpha (TNF-α) secretion in the presence of the NLRP3 selective small-molecule inhibitor MCC950. Detailed clinical features were assessed and anti-IL-1 treatment response was determined.

Results: Peripheral blood mononuclear cells (PBMC) from patients with low penetrance NLRP3 variants displayed enhanced IL-1β levels following inflammasome activation compared to HC. Furthermore, IL-1β release was NLRP3-dependent as it was blocked by MCC950. The production of IL-6 and TNF-α was also increased in patients with low penetrance NLRP3 variants. Clinically, they presented with a heterogenous spectrum of neurological manifestations, while cranial nerve inflammation was the most common feature. Overall inflammasome activation did not correlate with disease severity. Eight of ten treated patients responded to anti IL-1 treatment, however a complete response was only documented in four patients.

Conclusion: PBMC of several patients with NLRP3 low penetrance variants and CNS manifestation showed increased NLRP3-specific IL-1β release upon stimulation and elevated NLRP3-independent IL-6 and TNF-α levels as those were not suppressed by MCC950. Our data suggest that beside the possible causal involvement of the NLRP3 inflammasome additional, yet unidentified genetic or environmental factors may contribute to the multiorgan inflammation in our patients and explain the partial response to IL-1 targeting therapies.

1. Introduction

Cryopyrin-associated periodic syndromes (CAPS) comprise rare hereditary systemic autoinflammatory diseases [1] with a broad clinical spectrum of severity, from benign familial cold-induced autoinflammatory syndrome (FCAS) [2], to Muckle-Wells syndrome (MWS) [3], and to the debilitating chronic infantile neurological, cutaneous, and articular syndrome (CINCA; also known as neonatal onset multisystem inflammatory [NOMID] disease) [4]. They are caused by gain-of-function mutations in exons 3, 4, or 6 of the *NLRP3* [5–7] (also called nucleotide-binding oligomerization domain-like receptors and PYD domain containing protein 3), the gene that encodes cryopyrin. NLRP3 itself, together with the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and the inflammatory caspase-1, forms the NLRP3 inflammasome. Its activation leads to caspase-1–mediated processing of the proinflammatory mediators interleukin 1β (IL-1β) and interleukin 18 (IL-18) [8].

Common symptoms in patients with pathogenic mutation are recurrent episodes of fever, abdominal pain, myalgia, arthralgia, and cutaneous inflammation as well as ocular and central nervous system

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https://doi.org/10.1016/j.clim.2019.04.004
Received 3 December 2018; Received in revised form 5 April 2019; Accepted 5 April 2019
Available online 08 April 2019

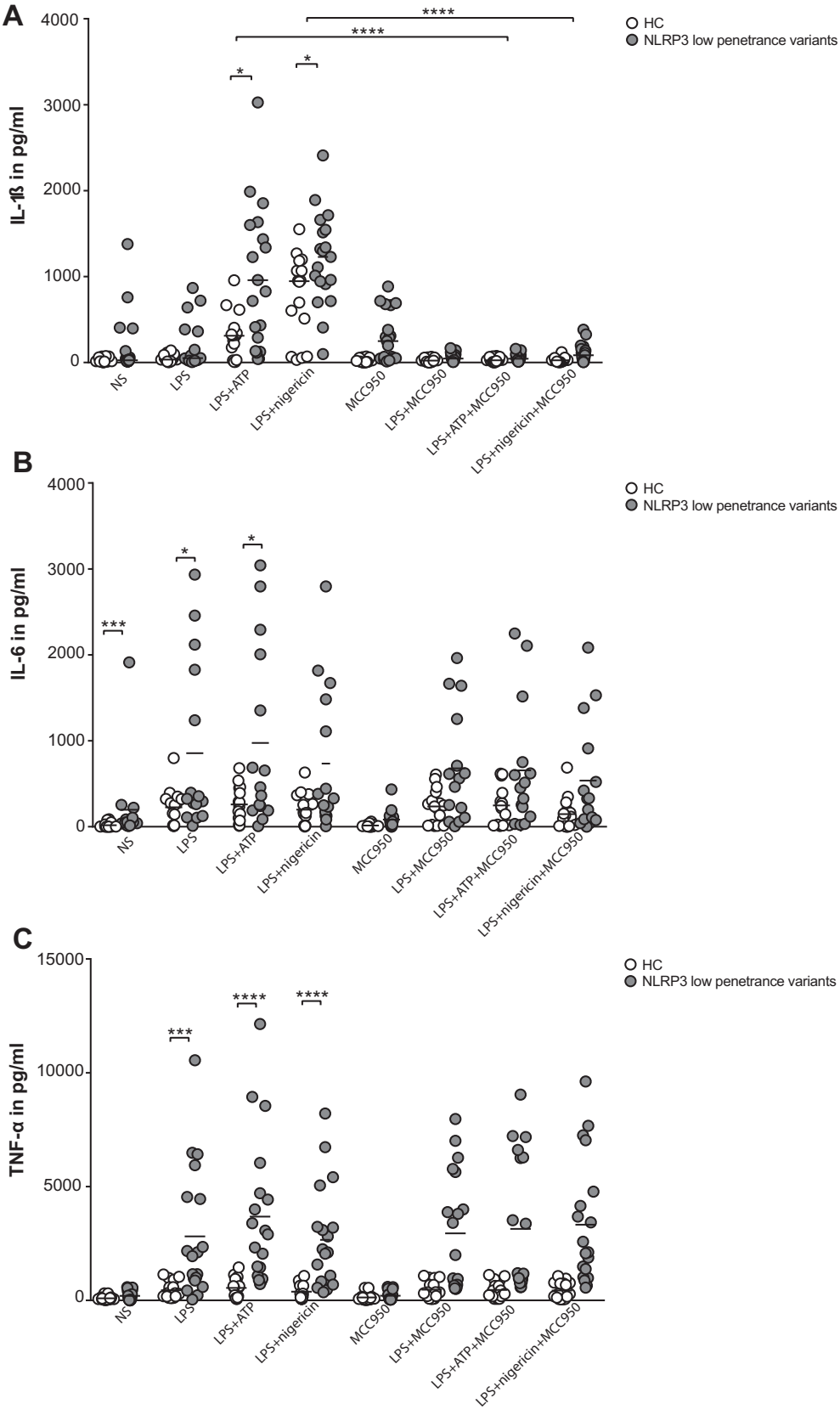

Fig. 1. Patients with low penetrance NLRP3 variants show increased NLRP3 dependent and independent inflammasome pathway activation. (A) IL-1β concentrations in the supernatant of cells left unstimulated, LPS primed, or stimulated with LPS plus ATP or nigericin and in the presence or absence of MCC950 from HC (n = 16) compared to patients (n = 19) are shown. White dots: HC, grey dots: low penetrance NLRP3 variants. (B) IL-6 (n = 15) and (C) TNF-α (n = 19) concentrations in the supernatant of cells from HC (n = 16) and low penetrance NLRP3 variants that were primed with LPS, or stimulated with LPS plus ATP or nigericin and in the presence or absence of MCC950 are depicted. White dots: HC, grey dots: low penetrance NLRP3 variants. Data with means are shown as scatter plots Mann-Whitney U test; * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001. CRP: C-reactive protein, SAA: serum amyloid-A.

(CNS) involvement [9–11]. In contrast, patients with low penetrance variants have been reported to exhibit a milder phenotype, usually lacking CNS symptoms as well as an up-regulation of the IL-1β axis [12–14]. However, more recently we have described several patients with low penetrance variants associated with severe CNS manifestations and inflammation [15,16]. Nevertheless, these low penetrance variants are observed at a similar frequency in patients and in healthy individuals and the clinical and functional significance of the low

penetrance variants V198 M and Q703K remains a matter of debate [15,17,18].

MCC950 is a potent and selective small-molecule inhibitor of the NLRP3 inflammasome [19,20]. It was previously shown that MCC950 strongly reduces neuroinflammation [19,21] and ameliorates brain injury after intracerebral hemorrhage in mice [22].

The aims of the present study were 1) to investigate the cytokine release from peripheral blood mononuclear cells (PBMC) of patients with low penetrance variants upon inflammasome activation and treatment with the NLRP3 selective, small molecule inhibitor MCC950, and 2) to assess the clinical characteristics of these patients, including the treatment response to IL-1-targeting drugs.

2. Methods

2.1. Study population

All patients were consecutively examined at our neuroimmunological outpatient clinic between 2011 and 2017 and had a clinical presentation suggestive for an autoinflammatory syndrome as well as neurological complaints. Standard gene mutation screening of the NLRP3-, MEFV- (familial Mediterranean fever and TNFRSF1A gene (tumor necrosis factor receptor–associated periodic syndrome) was performed in all patients. Patients with genetically proven low penetrance NLRP3 variants (V198M, Q703K) were included in the study. Demographic data including sex, age, ethnicity, family history, and age at the onset of symptoms and at the time of diagnosis were collected. All medical data including clinical manifestations, disease course, inflammatory markers such as C-reactive protein (CRP), serum amyloid-A (SAA) values, cerebrospinal fluid (CSF) data from lumbar puncture performed for diagnostic purpose, magnetic resonance imaging (MRI) data and treatment strategies in individual patients were collected and evaluated. At the time of blood/CSF sampling none of the patients received anti-IL-1 treatment or high dose glucocorticoid (GC) therapy. Treatment response to anti-IL-1 therapy was defined as follows: (1) Complete remission: absence of clinically active inflammation (no systemic symptoms, no inflammation on MRI and/or, CSF examination) and unremarkable inflammatory markers (including CRP, SAA); (2) Partial remisssion: improvement of clinical symptoms and signs of active inflammation and reduction in inflammatory markers; (3) No response: no change of clinical symptoms and ongoing elevation of inflammatory markers. Sixteen sex- and age-matched healthy controls (HC) were included in the study.

All human samples were collected following written informed consent according to local ethics policy guidelines of the Ludwig Maximilian University in accordance with the Declaration of Helsinki (project no: 600-15).

2.2. Collection and storage of PBMC

In parallel, PBMC from patients and HC were prepared from heparinized blood samples by Pancoll (Pan Biotech, Aidenbach, Germany) -density gradient centrifugation up to 2–4 h after blood was drawn. Cells were washed twice in RPMI 1640 medium and resuspended in 10 ml RPMI 1640 supplemented with 10% FCS, 1% L-Glutamine, 1% Penicillin/Streptomycin. 0.5% Trypan blue staining solution was used to differentiate between viable and nonviable cells and showed > 90% viability in all samples. PBMC from patients and HC were cryopreserved at a concentration of 1×10^7/ml in freezing medium containing 10% DMSO (Sigma-Aldrich, St. Louis, Missouri) diluted in FCS (Thermo scientific fisher, Waltham, Massachusetts, USA). Samples were placed in Mr. Frosty (Thermo Fisher Scientific) containers, gradually cooled to −80 °C, and transferred quickly into liquid nitrogen tanks after 72 h.

2.3. Functional inflammasome assay

PBMC from each donor were seeded at a concentration of 2×10^6 cells/ml in a 96 flat-bottom well plate. PBMC were either left untreated (baseline) or initially primed with 100 ng/ml LPS (Sigma-Aldrich) and incubated for 3 h and 30 min at 37 °C, 5% CO_2. PBMC were then treated with 5 μM of the NLRP3 selective inhibitor MCC950 for 30 min as indicated. In a final step, PBMC were activated by adding the NLRP3 inflammasome activators ATP (5 mM) or nigericin (10 μM) (both from Sigma-Aldrich) (Suppl. Fig. 1A). After stimulation, plates were centrifuged twice at 1000 *g* for 10 min each. Supernatants were directly transferred to coated ELISA plates and incubated at 4 °C overnight.

In addition the IL-1 release from fresh and frozen PBMC of 3 HC was compared and showed substantially higher IL-1 secretion in fresh PBMC (Suppl. Fig. 1B). For facilitated clinical applicability we continued to work with frozen material.

2.4. ELISA

ELISA Kits (Thermo Fisher Scientific) were used to quantify IL-1β, IL-6 and TNF-α in fresh cell culture supernatants following inflammasome stimulation. Assays were performed according to the manufacturer's recommendations.

2.5. Statistical analysis

Data are reported as means ± SEM. Differences between two unpaired groups were analyzed by Mann–Whitney *U* test, differences between paired samples were assesed by Wilcoxon ranking test and correlations are described with Pearson's correlation coefficient *r* using Prism 6.01 software (GraphPad Software Inc., San Diego, California). In all tests, p-values of < 0.05 were considered significant and are designated in the figures as follows *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001. Stimulation index were calculated as the ratio of stimulated to unstimulated cytokine release.

3. Results

3.1. Patients and genetics

19 patients (4 male, 15 female) were included in the study. All patients were caucasian and adult at the time of initial contact. The median age at study entry in the NLRP3 -penetrance variants cohort was 49 ± 11 years. The median age at symptom onset of NLRP3 low penetrance variants was 35 ± 15 years, median follow-up time comprised 5 ± 2 years. NLRP3 low penetrance variants included the Q703K in 17 (89%; 1 homozygous, 16 heterozygous) and the V198M variant in 2 (11%; all heterozygous) of all cases (Table 1). One woman (patient 6) was additionally found to carry a R92Q low penetrance variant encoded by exon 4 of the *TNFRSF1A* gene (Suppl. Table 1).

Table 1
Demographic data of patient and control groups.

	NLRP3 low penetrance variants	Healthy controls
N	19	16
Age (y)	49 ± 11	35 ± 6
Sex (m/f)	4/15	5/11
Mutation	V198M: n = 2 Q703K: n = 17	n.a.
Genotype	18 +/−, 1 +/+ [Q703K]	n.a.
Ethnicity	All Caucasian	All Caucasian

Altogether demographic data of 19 NLRP3 low penetrance variants and 16 healthy controls were assessed; n.a. = not applicable.

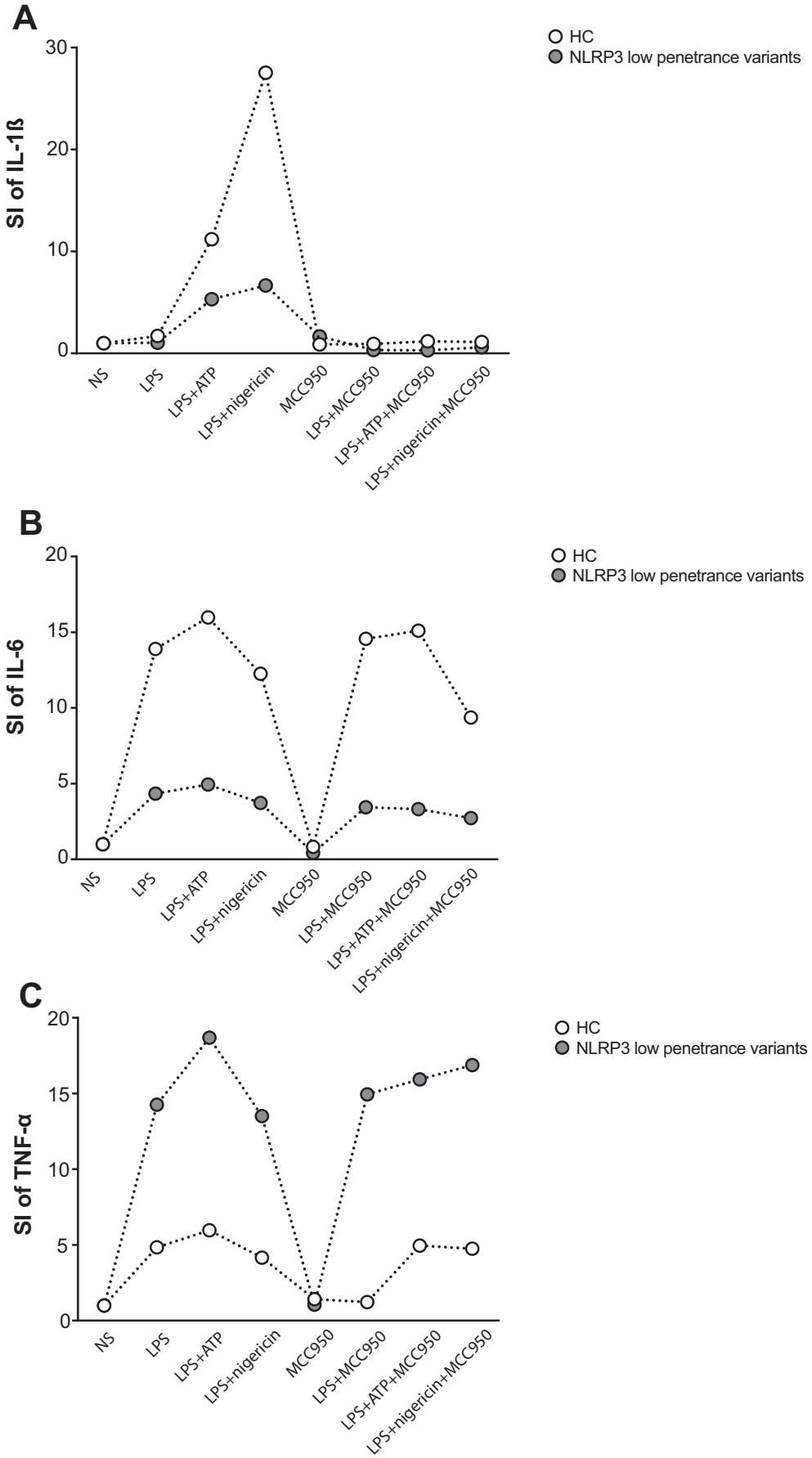

Fig. 2. Stimulation indices of patients with-penetrance NLRP3 variants (A, B, C) Stimulation indices of IL-1β, IL-6 and TNF-α were calculated as follows: stimulated value divided by the non-stimulated value. White dots: HC, grey dots: low penetrance NLRP3 variants. SI: stimulation index, NS: non-stimulated.

Table 2
Clinical features of patient cohort.

	n = 19	%
Phenotype		
FCAS	1	5
FCAS/MWS	0	
MWS	1	5
MWS/NOMID/CINCA	17	90
NOMID/CINCA	0	
Undefined autoinflammatory disease	0	
Disease course		
Recurrent	4	21%
Cronic	0	
Chronic with flares	15	79%
Age at disease onset (y)	35 ± 15	
Diagnostic latency (y)	9 (1 – 32)	
Positive family history	6	32
Systemic symptoms		
myalgias	12	63
Arthalgias	12	63
Conjunctivitis, papillitis, uveitis	11	58
Skin efflorescences	10	53
Gastrointestinal symptoms	8	42
Fever	12	63
Fatigue	12	63
Neurological manifestations		
Tension type headache	6	32
Migraine	3	16
Sensory symptoms	10	53
Cranial nerve affection	17	90
Optic nerve	6	35
Oculomotor nerve	7	41
Trochlearis	1	6
Trigeminal nerve	5	29
Abducens nerve	2	12
Facial nerve	1	6
Vestibulo-cochlear nerve	5	29
Glossopharyngeal nerve	1	6
Hypoglossal nerve	1	6
Recurrent meningoencephalitis	4	21
Cerebral vasculitis	1	5
Elevation of inflammatory markers (n = 18)		
SAA	12	67
CRP	6	33
Leucocytosis	5	29
CSF (n = 17)		
pleocytosis	10	59
OCB (CSF specific)	9	53
Elevated protein levels	4	23
Elevated opening pressure	1	6
MRI (n = 18)		
Abnormal	11	61
White matter lesions	10	56
Gadolinium enhancement	5	28
Anti-IL-1 therapy (n = 10)		
Anakinra	10	100
Canakinumab	4	40
Complete remission (anakinra/canakinumab)	4/1	40/25
Partial remission (anakinra/canakinumab)	4/2	40/50
No response (anakinra/canakinumab)	2/1	20/25

Clinical data and laboratory markers are shown in absolute numbers (n) and percentages (%).
CRP: C-reactive protein, SAA: serum amyloid a, OCB: oligoclonal bands.

3.2. Functional implications of low penetrance NLRP3 variants

3.2.1. Interleukin-1β

Resting PBMC from several patients with low penetrance NLRP3 variants spontaneously secreted higher IL-1β levels compared to cells from HC, although this effect reached no statistical significance. LPS alone did not enhance IL1-β secretion in low penetrance NLRP3 variants compared to HC. When PBMC were stimulated with inflammasome stimuli (following an LPS priming period of 3 h and 30 min), we observed a significantly higher mean increase of IL-1β in low penetrance NLRP3 variants compared to HC (LPS + ATP: HC 311 ± 65, patients 958 ± 185, $p < 0.05$; LPS + nigericin: HC 764 ± 123, patients 1199 ± 121, $p < 0.05$) (Fig. 1A). The lower stimulation index of IL-1β observed in low penetrance NLRP3 variants is explained by higher basal IL-1β release in the patients (Fig. 2A). MCC950 treatment resulted in a dramatic and significant inhibition of ATP-induced (LPS + ATP + MCC950: HC 33 ± 6 pg/ml, patients 53 ± 10 pg/ml, $p < 0.0001$) and nigericin-induced (LPS + nigericin + MCC950: HC 31 ± 7 pg/ml; patients 109 ± 24 pg/ml, $p < 0.0001$) secretion of IL-1β in both groups (Fig. 1A).

3.2.2. Interleukin-6 and TNF-α release

LPS and IL-1β can stimulate the production of other inflammasome-independent proinflammatory cytokines such as IL-6 and TNF-α. Therefore we additionally tested the PBMC supernatants of patients with low penetrance NLRP3 variants in comparison to HC for the cytokines IL-6 and TNF-α. Samples were either left unstimulated or treated with LPS alone, LPS + nigericin or LPS + ATP in the presence or absence of the small molecule inhibitor MCC950 as described above. Mean IL-6 levels from PBMC of patients with low penetrance NLRP3 variants were significantly higher compared to HC, both when PBMC were primed with LPS alone (LPS: HC 225 ± 51 pg/ml, patients 856 ± 255 pg/ml, $p < 0.05$) or after treatment with LPS + ATP (LPS + ATP: HC 260 ± 50 pg/ml, patients 975 ± 271 pg/ml, $p < 0.05$) (Fig. 1B). MCC950 treatment did not influence the amount of IL-6 released (Fig. 1B). Mean basal IL-6 levels were found to be significant higher in low penetrance NLRP3 variants compared to HC (NS: HC 18 ± 7 pg/ml, patients 197 ± 124 pg/ml, $p < 0.001$) (Fig. 1B), a fact also reflected by a lower IL-6 stimulation index (Fig. 2B). As compared to PBMC from HC, PBMC from patients showed enhanced TNF-α secretion, when treated with LPS alone or in combination with an inflammasome stimuli (LPS: HC 446 ± 94 pg/ml, patients 2811 ± 651 pg/ml, $p < 0.001$; LPS + ATP: HC 550 ± 110 pg/ml, patients 3683 ± 734 pg/ml, $p < 0.0001$; LPS + nigericin: HC 383 ± 86 pg/ml, patients 2663 ± 516 pg/ml, $p < 0.0001$) (Fig. 1C). MCC950 treatment did not influence the amount of TNF-α released (Fig. 1C). Patients with low penetrance NLRP3 variants showed comparable amounts of basal TNF-α compared to controls, explaining the higher stimulation index of TNF-α observed in the group of low penetrance NLRP3 variants (Fig. 2C).

IL-1β, IL-6 and TNF-α cytokine release of PBMC from patients did neither correlate inter- and intra-individually (data not shown) nor with acute phase reactants (Suppl. Fig. 1C). Furthermore we did not observe any difference in cytokine release among patients carrying the Q703K substitution compared to V198 M mutation variants (Suppl. Table 1).

3.2.3. Clinical phenotype

Four patients fulfilled the diagnostic criteria for CAPS [23]. The most common clinical phenotype in the low penetrance NLRP3 variant group comprised an overlapping clinical presentation of MWS/NOMID/CINCA (n = 17, 90%; 15 patients with Q703K variants and 2 with V198 M substitutions), while only two individuals displayed either typical FCAS (5%) or MWS (5%) symptoms. Most patients showed a chronic (79%) disease course with recurrent flares. Fever, myalgias, arthralgias and fatigue were present in 63% of patients with low penetrance NLRP3 variants followed by eye involvement (58%), skin efflorescences (53%) and gastrointestinal symptoms (42%).

Nervous system involvement was present in all patients with a broad spectrum of neurological manifestations. Involvement of the cranial nerves was the most common neurological feature (n = 17, 90%), followed by sensory symptoms including dysaesthesia as well as hypaesthesia (n = 10, 53%) and headache syndromes (n = 9, 48%). In 4 patients a demyelinating autoimmune disease of the CNS was suggested. Four patients had recurrent aseptic meningoencephalitis and one suffered from cerebral vasculitis with ischemic strokes (Table 2). In all patients extensive investigations were performed to exclude

important differential diagnosis such as neurosarcoidosis and other rheumatic diseases including lupus erythematosus with CNS involvement. Rheumatologist examined all patients and none of them developed a rheumatic disease on follow-up (median follow-up time 5 ± 2 years) so far.

3.2.4. Laboratory findings

Overall 15 from 18 patients (83%) showed intermittent elevated levels of acute phase reactants including SAA (n = 12, 67%), CRP (n = 6, 33%) and leukocytosis (n = 5, 29%). Lumbar puncture was performed in 18 out of 19 patients with low penetrance NLRP3 variants. CSF was abnormal in 65% (n = 11) of them and included pleocytosis (n = 10, 59%), elevated protein levels (n = 4, 22%) and positive oligoclonal bands (OCB, n = 9, 53%). In 4 patients with positive OCB, a demyelinating autoimmune diseases of the CNS was suggested, 3 patients showed severe recurrent meningoencephalitis, 1 had vasculitis and 2 had severe inflammation of the optic nerve. An elevated opening pressure was detected in one patient (Table 2).

3.2.5. MRI findings

MRI was performed and data available in 18 patients. Eleven patients showed abnormalities (61%) including cerebral white matter and spinal cord lesions as well as meningeal and/or cranial nerve gadolimium enhancement (Table 2).

3.2.6. Treatment strategies and therapy response to IL-1 inhibitors

Overall 11 patients (n = 11; 58%, 11 with Q703K variant and none with V198M) were intermittently treated with high-dose glucocorticoid (GC) therapy during episodes of severe CNS inflammation and/or systemic inflammation and all of them responded. A total of 10 patients (53%; all with Q703K variant) were treated with IL-1 inhibitors. Four patients showed a full clinical remission (3 patients were treated with anakinra, 1 patient was initially treated with anakinra and subsequently with canakinumab), while 4 patients (4 patients treated with anakinra, of whom 1 patient was treated with canakinumab later on) showed partial improvement. 2 patients did not respond to anti-IL-1 therapy at all (both were treated with anakinra, and 1 of them was also treated with canakinumab later on) and showed persisting CNS inflammation. 1 of them suffered from severe optic nerve inflammation, while the other experienced unilateral orbital pain caused by granulomatous inflammation of both cavernous sinus (Table 2 and Suppl. Table 1). Systemic symptoms including abdominal pain, skin rash, arthalgias, myalgias, conjunctivitis/uveitis and fatigue improved in 8 patients. In 5 patients anti-IL-1 therapy is continued with persistent drug response. In 1 patient, who stopped anti-IL-1 therapy, symptoms reoccurrred, thus anti-IL-1 treatment was recommenced and is continued until now. Four patients noticed a clear reduction of the treatment effect and worsening of symptoms after reduction of either dosage or elongation of intervals. There was no clear association between the method of anti-IL-1 inhibition, clinical phenotype and treatment effect. Overall, improvement in response to anti-IL-1 therapy did not completely correlate with the amount of cytokine release, however both IL-1 therapy non-responders did not show a significant IL-1β release upon inflammasome activation.

4. Discussion

This study revealed the following key findings: (I) PBMC from patients with NLRP3 low penetrance variants released more readily NLRP3-specific IL-1β, demonstrated by inhibition with the NLRP3-selective small molecule inhibitor MCC950; (II) in addition, an NLRP3-independent release of IL-6 and TNF-α could be demonstrated in these patients; and (III) patients with NLRP3 low penetrance variants may present with severe CNS manifestations and partially respond to IL-1 targeting therapies.

The NLRP3 inflammasome is a cytoplasmic danger signaling complex that is important for host protection against pathogenic microorganisms and sterile stressors. NLRP3 initiates an inflammatory cascade by triggering the maturation and release of the potent pro-inflammatory cytokine IL-1β. CAPS is caused by gain-of-function mutations within the NLRP3 gene. Dysregulated inflammasome activation and IL-1β release have a pathogenic role in this disease. However, in patients carrying low penetrance variants, the inflammasome-dependence of cytokine release remains controversial. While previous studies did not identify increased IL-1β secretion in primary human PBMC from patients carrying a low penetrance variant [14,24,25], it was shown that the Q703K and V198M substitutions, when retrovirally transduced in THP-1 cells, produced increased IL-1β and IL-18 levels compared to wild-type cells [25,26]. To shed further light on this issue, we tested the so far largest group of patients with low penetrance variants and neurological manifestations for inflammasome activation. In contrast to patients with pathogenic mutations, our data showed only a trend to higher spontaneous IL-1β secretion in PBMC from low penetrance NLRP3 variants compared to HC. By using the small molecule inhibitor MCC950, we validated that IL-1β release in our experimental system was NLRP3-specific.

The release of IL-1β in mice usually requires a two-step activation process: priming via toll-like receptor (TLR) or cytokine receptor signaling [27,28] triggers transcriptional upregulation of pro-IL-1β and NLRP3, whereas a second signal such as extracellular ATP, potassium (K^+) ionophores such as nigericin, crystals, insoluble particles, and certain pathogens trigger activation of the NLRP3 inflammasome [14,28–31]. Although, the exact mechanism of NLRP3 inflammasome activation remains elusive, the theory that K^+ efflux is the common trigger of NLRP3 inflammasome activation [32,33] was recently disproved in studies of NLRP3 activation by the small molecule imiquimod [34]. In human monocytes, the aforementioned two-step NLRP3 activation model does not hold. Specifically, LPS alone is sufficient to trigger caspase-1-dependent IL-1β maturation and secretion [35–39]. Before that, it has been suggested that in monocytes of HC, two signals (LPS and ATP) are required to achieve a rapid activation of the inflammasome, whereas only in patients carrying a pathogenic NLRP3 mutation, LPS alone is sufficient to trigger NLRP3 activation and IL-1β secretion [40]. In our experimental setting with PBMC from patients with low penetrance NLRP3 variants, two signals were required for inflammasome activation and IL-1β release. This discrepancy regarding a two-step model might be explained by our patient cohort of low penetrance mutation variants, whereas in previous studies CAPS patients with proven mutations were investigated [40]. Secondly, different experimental methodologies might have contributed to diverging results, since we used -in contrast to other groups e.g. frozen material of PBMC [24,39,40]. Our supplemental data on IL-1β release after LPS stimulation in fresh versus frozen PBMC from HC support the view that cell viability and responsiveness to exogenous stimuli might be altered after cryopreservation [41]. This point is further underscored by an overall lower IL-1β secretion and the necessity of higher LPS concentrations in our experimental setting. Nevertheless, in clinical routine the usage of frozen material in this assay might facilitate applicability.

In contrast to others [24,42,43], we also found that compared to HC, secretion of IL-6 from PBMC of low penetrance mutation variants was enhanced spontaneously in several patients, while the release of both IL-6 and TNF-α were increased after stimulation with LPS alone and after combined treatment with LPS and inflammasome stimuli. However, the secretion of IL-6 and TNF-α was NLRP3-independent, as it was not suppressed by MCC950. Overall inter-individual and intra-individual cytokine release profiles were heterogeneous in patients and did not correlate with clinical manifestation. This points to additional yet undefined genetic and/or environmental factors, which may contribute to the inflammatory phenotype in patients with low penetrance variants.

We previously reported that CNS manifestation in low penetrance mutation variants may resemble a MWS/NOMID/CINA phenotype [15,16]. The spectrum of neurological manifestations is heterogeneous

and broad, but affection and inflammation of the cranial nerves is a hallmark of these patients and severe CNS inflammation also occurs [15]. In our previous study several low penetrance mutation carriers also had a concomitant diagnosis of multiple sclerosis (MS) and in the current cohort in 4 patients a demyelinating autoimmune disease of the CNS was suggested. These findings are corroborated by Soares and colleagues, who have recently reported that the NLRP3 variant Q703K may be associated with multiple sclerosis (MS) and may serve as a risk factor for progression [15,44]. More than half of the patients in our cohort showed evidence for CNS inflammation, reflected by CSF abnormalities. OCB were positive in 9 patients, 4 of them with severe recurrent inflammation of the CNS, 1 with cerebral vasculitis and 4 with suggested demyelinating autoimmune disease. This is a novel finding since OCB have not been investigated so far in NLRP3 mutation carriers, especially with low penetrance variants. In a recent study, the CSF of 17 pediatric NOMID patients was examined and CSF cytokines, but no OCB were determined [45]. In all of our patients, other differential diagnoses were excluded by intensive diagnostic work-up. Furthermore, no other diagnoses have been set so far during the median follow-up time of up 5 years as compared to the study by Naselli and colleagues [14].

All patients responded to GC therapy but only partially to anti IL-1 therapy, which might be explained by the additional and heterogeneous up-regulation of IL-6 and TNF-α in several patients. Nevertheless, clear therapy failure to anti-IL-1 treatment was only observed in two patients, who showed no significant IL-1β release following inflammasome activation. Whether the NLRP3-independent IL-6 and TNF-α release contribute to the multi-organ inflammation in our patients, requires further investigation.

Our study is limited by the fact that we were unable to include patients with pathogenic mutations. Further and more sophisticated genetic investigations such as whole-exome sequencing and exclusion of somatic mutations would also be desirable. A further limitation is that the in vitro investigation of PBMC may not fully reflect the in vivo situation responsible for disease manifestation, especially for the development of CNS symptoms in our patients.

Undoubtedly, prompt diagnosis, awareness for neurological signs and symptoms as well as tight clinical monitoring of those patients is of great importance. If replicated and validated in larger patient cohorts, this functional inflammasome assay using MCC950 might facilitate the diagnosis and treatment of patients with NLRP3 low penetrance variants. In summary, our observations offer new insights into the pathophysiology of patients with low penetrance variants who suffer from CNS manifestations. Furthermore, our data suggest that the NLRP3 inflammasome might be one of the causally involved factors in disease pathogenesis of patients with low penetrance NLRP3 variants and CNS involvement.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2019.04.004.

Disclosures

ES, CJG, DW, MS and OG report no disclosures. T. Kuempfel has received travel expenses and speaker honoraria from Bayer Healthcare, Teva Pharma, Merck, Novartis Pharma, Sanofi-Aventis/Genzyme, CLS Behring, Roche Pharma and Biogen as well as grant support from Bayer-Schering AG, Novartis and Chugai Pharma.

Author contributions

ES: acquisition, analysis and interpretation of experimental and clinical data, patient care and evaluation, manuscript writing and editing.

CJG: development of study concept, study supervision, analysis and interpretation of data, reviewing and editing of manuscript.

DW: acquisition analysis and interpretation of clinical data.

MS: clinical sample acquisition and patient care.

OG: development of study concept, analysis and interpretation of data, reviewing and editing of manuscript.

TK: analysis and interpretation of data, patient care and evaluation, writing, reviewing and editing of manuscript.

All authors discussed the results and commented on the manuscript.

Study funding

This work was supported by the Deutsche Forschungsgemeinschaft (German Research Foundation) within the framework of the Munich Cluster for Systems Neurology (EXC 1010 SyNergy), by the European Research Council by an ERC Starting Grant (337689 to OG), by a scientific grant from Friedrich-Baur Institute as well as partially by a scientific grant from Novartis Pharma and by the German Ministry for Education and Research (BMBF, “German Competence Network Multiple Sclerosis” (KKNMS)).

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Glossary

ATP adenosine triphosphate:
CAPS cryopyrin-associated periodic syndromes:
CNS central nervous system:
CRP C-reactive protein:
CSF cerebrospinal fluid:
GC glucocorticoid:
HC healthy controls:
IL-1β interleukin-1 beta:
IL-6 interleukin-6:
IL-1RA interleukin-1 receptor antagonist:
MRI magnet resonance imaging:
MS multiple sclerosis:
LPS lipopolysaccharide:
PBMC peripheral blood mononuclear cells:
SAA serum amyloid a:
TNF-α tumor necrosis factor alpha: