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The Q705K Polymorphism in NLRP3 Is a Gain-of-Function Alteration Leading to Excessive Interleukin-1β and IL-18 Production

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Abstract

Background: The Q705K polymorphism in NLRP3 has been implicated in several chronic inflammatory diseases. In this study we determine the functional role of this commonly occurring polymorphism using an in-vitro system.

Principal Findings: NLRP3-WT and NLRP3-Q705K were retrovirally transduced into the human monocytic cell line THP-1, followed by the assessment of IL-1β and IL-18 levels in the cell culture supernatant. THP-1 cells expressing the above NLRP3 variants were sorted based upon Green Fluorescent Protein (GFP) expression. Cytokine response to alum (one of the most widely used adjuvants in vaccines) in the cells stably expressing NLRP3-WT and NLRP3-Q705K were determined. IL-1β and IL-18 levels were found to be elevated in THP-1 cells transduced with NLRP3-Q705K compared to the NLRP3-WT. Upon exposure to alum, THP-1 cells stably expressing NLRP3-Q705K displayed an increased release of IL-1β, IL-18 and TNF-α, in a caspase-1 and IL-1 receptor-dependent manner.

Conclusions: Collectively, these findings show that the Q705K polymorphism in NLRP3 is a gain-of-function alteration leading to an overactive NLRP3 inflammasome. The option of IL-1β blockade may be considered in patients with chronic inflammatory disorders that are unresponsive to conventional treatments.

Introduction

Inflammasomes are essential regulators of interleukin (IL)-1β production. Upon activation, NLRP3 (formerly known as Cryopyrin/CIASS/NALP3), associates with ASC (PYCARD) adaptor and pro-caspase-1 to form the NLRP3 inflammasome. This interaction leads to the activation of caspase-1, which proteolytically processes pro-IL-1β and pro-IL-18 to form active IL-1β and IL-18 [1]. CARD-8 has been suggested to be a binding partner of the inflammasome [2] but its role in the inflammasome is still a matter of debate. Gain-of-function mutations in the gene encoding NLRP3 can lead to its constitutive activation resulting in an uncontrolled IL-1β production. NLRP3 mutations have been implicated in hereditary inflammatory diseases and are grouped under cryopyrin-associated periodic syndromes (CAPS) or cryopyrinopathies [3]. The CAPS are regarded as monogenic disorders, comprising a trio of autoinflammatory conditions varying in severity of disease manifestation; familial cold associated syndrome (FCAS) being the mildest form, Muckle-Wells syndrome (MWS) being intermediate, and neonatal onset multisystem disorder (NOMID, also known as chronic infantile neurological cutaneous and articular syndrome; CINCA), being the most severe. Patients suffering from these syndromes typically present with fever, skin rashes and arthritis-like symptoms. IL-1β plays a central role in the pathogenesis of these disorders, which is proved by the remarkable improvement in symptoms upon IL-1β blockade [4].

We previously reported a patient with chronic inflammatory symptoms carrying the gene polymorphisms Q705K in NLRP3 (reported in the infivers database as Q703K [http://infivers.curs.fr/ISSAID/infivers/] and C10X in CARD-8 [6]. This patient had a long history of arthritis and antibiotic-resistant fever but lacked the typical signs of FCAS, MWS or NOMID. Remarkably, like in
other typical CAPS patients, IL-1 receptor (IL-1R) blockade using anakinra effectively abolished the patient’s symptoms. The abundance of this polymorphism in the general population (5–11%) [5] makes it highly relevant to study its functional significance, particularly since several studies have shown a correlation of Q705K alone or in conjunction with C10X with increased risk of chronic inflammation [7,8,9,10,11]. Our results reveal a gain-of-function phenotype of the Q705K polymorphism which, unlike the other known genetic alterations in NLRP3, is associated with only moderately increased IL-1β levels. These findings combined with above epidemiological data are indicative of an important role of this polymorphism in susceptibility to chronic inflammatory conditions. Our findings also provide insight into the requirement of effective IL-1R activation for efficient IL-1β production in cells with overactive inflammasomes, demonstrating an autocrine feedback loop for IL-1β release under sterile conditions.

Results
Enhanced IL-1β and IL-18 release in THP-1 cells retrovirally transduced with NLRP3-Q705K

To determine whether the Q705K variant of NLRP3 led to a spontaneous cytokine production, the cells were transduced with a retroviral vector expressing NLRP3-wild type (WT) or NLRP3-Q705K. The MWS-associated mutation NLRP3-R260W was used as a positive control and the empty vector (EV) was used as negative control. IL-1β, IL-18 and TNF-α levels were measured 48 h after retroviral transduction. Cells expressing NLRP3-Q705K demonstrated a five-fold increase in IL-1β levels as compared to the WT control, indicating that this variant leads to a constitutively activated inflammasome (Fig. 1A). The NLRP3-R260W variant displayed a seven-fold increase as compared to the WT control. The use of the caspase-1 inhibitor Z-YVAD-FMK with NLRP3-Q705K-expressing cells during the 48 hour time period resulted in IL-1β levels reduced to 47% (data not shown) demonstrating that this process is to a large extent dependent on caspase-1. The transduced cells were expressing GFP, the fluorescence of which was used to determine the transduction efficiency using flow cytometry (Fig. 1A inset). Correction of the IL-1β levels for the number of GFP-positive cells rendered data showing a similar trend, with the Q705K variant inducing higher levels of IL-1β than the NLRP3-WT (Fig. 1B). IL-18 levels were also found to be elevated in samples transduced with NLRP3-Q705K and NLRP3-R260W (Fig. 1C; GFP-corrected data). The TNF-α levels in all samples were below the detection level at this early time point after transduction.

NLRP3-Q705K is a gain-of-function alteration

To obtain a population of cells stably expressing the wild type and mutant variants of NLRP3, we sorted the THP-1 cells expressing GFP and expanded them in culture medium. Next, in order to determine the production of cytokines in resting and stimulated THP-1 expressing NLRP3-Q705K, PMA-differentiated cells were stimulated with alum, which is one of the most widely used adjuvants in vaccines [12] and known to trigger sterile inflammation through the NLRP3 inflammasome [13,14,15]. Alum exposure resulted in a substantial increase in IL-1β production (Fig. 2A) in THP-1 expressing NLRP3-WT and the two mutant variants, the latter ones giving a more pronounced response (Fig. 2A). The NLRP3-Q705K displayed a statistically significant two-fold increase compared to WT expressing cells (Fig. 2A inset). A similar trend was observed in IL-18 levels upon alum stimulation, where NLRP3-Q705K and -R260W-expressing...
The alum-induced IL-1β and IL-18 release by Q705K is caspase-1 and IL-1R-dependent

To confirm the specificity of our system, we tested whether the IL-1β and IL-18 production was dependent on caspase-1, which has been shown to be crucial for production of the studied cytokines in recent reports [13,14]. Figure 2 shows a remarkable decrease in cytokine levels upon treatment with the caspase-1-inhibitor Z-YVAD-FMK, indicating that alum induces a caspase-1-dependent IL-1β and IL-18 release. It is well established that TLR ligands enhance the production of pro-IL-1β via NF-κB activation, and such microbial ligands are therefore widely used in *in vitro* studies to enhance IL-1β secretion. Given the fact that TLRs and the IL-1R share the same signalling pathway for NF-κB activation [16,17], we investigated to what extent the IL-1R was required for the observed cytokine production using anakinra in our sterile setting. Anakinra is a recombinant IL-1R antagonist, which competitively binds to the IL-1R and blocks downstream activation, and such microbial ligands are therefore widely used in *in vitro* studies to enhance IL-1β secretion. These two cytokines have previously been shown to correlate with each other [18].

Generally upregulated cytokine levels in patients with mutated NLRP3 have been shown [19] and in line with this observation we could detect elevated TNF-α levels in THP-1 cells stably expressing WT and mutant NLRP3 (Fig. 2C). A substantial increase in secreted TNF-α levels in alum-stimulated transduced samples could be observed, however, there were no significant difference between alum-stimulated NLRP3-WT- and -Q705K-expressing THP-1 cells, showing that the production of both cytokines in response to a sterile stimulus like alum involves an autocrine positive feedback mechanism via the IL-1R.

In agreement with a previous report suggesting TNF-α to be a late event associated with IL-1β release [2], we could detect TNF-α in cells stably expressing the mutant variants but not in the freshly transduced cells. This lack of significantly increased TNF-α release was likely a result of pyronecrosis, which previously has been shown to be caspase-1 independent [26]. Although using the same cell line and constructs as in a previous report [24], we observed lower IL-1β levels, which could be attributed to lab variations in handling of cells and methods.

Discussion

In our previous report of a patient with recurrent inflammatory symptoms, Q705K in NLRP3 and C10X in CARD-8 were found to coincide with increased caspase-1 activity and IL-1β secretion [6]. Our and others’ earlier published epidemiological data obtained from studies of patients with rheumatoid arthritis and Crohn’s disease pointed towards a role for this polymorphism in susceptibility to chronic inflammatory conditions [9,10].

We undertook these studies with the aim of evaluating the role of Q705K in IL-1β production. The human monocytic cell line THP-1 was used, since it expresses all the components of the NLRP3 inflammasome [23,24] and does not carry the NLRP3-Q705K alteration. In addition, this cell line is heterozygous for the C10X in CARD-8, which makes it a suitable model to study the effect of Q705K with a heterozygous C10X background. The increased IL-1β and IL-18 cytokine levels obtained upon retroviral expression of the Q705K into THP-1 cells indicate that it is a gain-of-function variation leading to an overactive inflammasome. The moderately produced IL-1β levels from NLRP3-Q705K (compared to the MWS-causing NLRP3-R260W) suggest an important role of this common variant in chronic inflammatory diseases. In line with previous reports, we observed lower transduction efficiencies in both the mutant variants, reflecting the induction of inflammasome-linked cell death [24,25,26]. However, since the caspase-1 inhibitor Z-YVAD-FMK effectively reduced IL-1β levels, we can rule out the possibility that the produced IL-1β was a result of pyronecrosis, which previously has been shown to be caspase-1 independent [26].

Generally upregulated cytokine levels in patients with mutated NLRP3 have been shown [19] and in line with this observation we could detect elevated TNF-α levels in THP-1 cells stably expressing WT and mutant NLRP3 (Fig. 2C). A substantial increase in secreted TNF-α levels in alum-stimulated transduced samples could be observed, however, there were no significant difference between alum-stimulated NLRP3-WT- and -Q705K-expressing THP-1 cells, showing that the production of both cytokines in response to a sterile stimulus like alum involves an autocrine positive feedback mechanism via the IL-1R.

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As a trigger for sterile inflammation we used alum, which is one of the most widely used vaccine adjuvants and a known activator of the NLRP3 inflammasome, to investigate the release of cytokines from THP-1 cells expressing wild type and mutated NLRP3 [13,15]. Indeed, we found that mutated NLRP3 produced more cytokines in response to alum as compared to wild type NLRP3. These findings suggest that the NLRP3-Q705K variant is associated with a lower threshold for inflammasome activation, potentially implying an increased genetic susceptibility for inflammatory stimuli in individuals possessing this variant. Further confirmation of NLRP3-Q705K being a gain-of-function variant was obtained when elevated spontaneous mRNA levels of pro-IL-1β were detected in the THP-1 cells stably expressing this variant. In agreement with a previous report suggesting TNF-α to be a late event associated with IL-1β release [2], we could detect TNF-α in cells stably expressing the mutant variants but not in the freshly transduced cells. The increased TNF-α release was likely a downstream effect of IL-1β release, since anakinra could block the effect. The lack of significantly increased TNF-α release in the NLRP3-Q705K compared to the WT expressing cells could possibly be due to sub-threshold levels of IL-1β observed in this polymorphic variant compared to the NLRP3-R260W. How NLRP3-Q705K enhances IL-1β and TNF-α expression independently of the IL-1R remains elusive, but the involvement of other pathways cannot be ruled out. For instance, signalling through IL-18R has been shown to activate NF-κB [29], which could lead to...
Figure 2. Alum-induced levels of cytokine production by NLRP3-tranduced THP-1 cells. THP-1 cells expressing NLRP3-Q705K, NLRP3-R260W, NLRP3-WT were pre-treated with Z-YVAD-FMK (50 μM, 2 h) or anakinra (5 μg/mL, 1 h) and exposed to alum (130 μg/mL, 4 h). A, IL-1β, B, IL-18 and C, TNF-α secretion levels in unstimulated, alum, Z-YVAD-FMK or anakinra treated THP-1 cells are shown. Data represents one independent experiment, out of 2–5. *Inset, Fold higher A, IL-1β, B, IL-18 and C, TNF-α in NLRP3-Q705K and NLRP3-R260W compared to NLRP3- WT. Data represent mean ± SEM of 4–5 independent experiments.
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increased expression of pro-IL-1β and TNF-α [29,30,31]. Alternatively, apoptotic speck-like protein (ASC), which is another component of the inflammasome, might through activation by the NLRP3-Q705K directly up-regulate NF-κB-regulated genes such as pro-IL-1β and TNF-α independently of the IL-1R [32,33,34,35].

A number of patients displaying either classical or atypical CAPS, possessing NLRP3-Q705K alone or in combination with other polymorphisms has previously been reported [4,6,36,37] many of which have successfully been treated with anakinra [4,6,25,37]. However, as all these patients do not fit into the conventional CAPS phenotype, the interpretation of NLRP3-Q705K presents a diagnostic challenge to the clinicians. Nonetheless, IL-1β blockade has been successful in treating many of these patients [4,6,25,37]. In a recent paper we have reported four patients with inflammatory symptoms carrying the NLRP3-Q705K in combination with CARD-8-C10X, where increased IL-1β release from patient’s monocytes was observed [38].

Based upon our in vitro studies as well as earlier published epidemiological data [8,9,10,11], we suggest that these polymorphisms, in conjunction with an environmental cue such as an infection, or with other, yet unidentified genetic variations, predispose for enhanced inflammation. Using a similar THP-1 cell model, we have earlier published data showing a novel M299V mutation in NLRP3 to be functional [23]. The contribution of environmental factors rather than additional genetic alterations would explain the later-onset of symptoms in some of the patients, compared to the neonatal to early onset in most of the CAPS patients carrying the severe disease-causing mutations [4]. A similar situation has been described in FMF where the alteration MEFV-E148Q, referred to as a polymorphism due to its presence in 10% of asymptomatic individuals, is detected in patients with milder symptoms [39]. In this case, environmental factors are suggested as the responsible triggers of inflammation [40,41]. Increasing numbers of studies showing synergistic effects between polymorphisms in different genes associated to autoinflammation are emerging [36,42,43], signifying the need of a careful diagnosis of the patients possessing low-penetrance alterations.

In many reports, TLR ligands are used to drive the production of pro-IL-1β for efficient IL-1β release [14,44,45]. Here we studied the response of the inflammasome under sterile conditions, where no TLR ligands or other bacterial products were used to enhance transcription of pro-IL-1β. Our study reinforces the importance of IL-1β signalling through its receptor for effective production of the same cytokine under sterile conditions, which has previously been demonstrated in healthy individuals [46] as well as in CAPS patients [47,48]. We also show that IL-1β release precedes TNF-α release via binding to the IL-1R, and that treatment with anakinra decreases both IL-1β and TNF-α releases, which has earlier been shown to occur in vivo in mice [49]. It is possible that such a pro-inflammatory feedback loop, once established through different exogenous and endogenous stimuli, may be difficult to break in patients with alterations in the NLRP3 inflammasome.

The discovery of disease-causing mutations in NLRP3 has led to the recognition of a connection between autoinflammatory disorders and a dysregulated innate immunity. The molecular mechanism for the constitutive phenotype of disease-causing NLRP3 alterations is not known, but it is suggested that this is due to structural instability leading to unprovoked association of NLRP3 with its adaptor molecules, causing spontaneous IL-1β production [4,50]. Our present data are in agreement with previous results showing nonsense alterations in exon 3 of NLRP3 to be associated with increased IL-1β [23].

In summary, we report an increased activity of the NLRP3-Q705K polymorphism as demonstrated by the increased spontaneous and stimulated release of cytokines under sterile conditions. Our study points to the need of extending the conventional categorization of patients with non-classical CAPS, at the same time the risk for over-interpretation of this genotype can be avoided with careful diagnosis. The option of IL-1 blockade might be considered, particularly in the patients unresponsive to standard treatments.

Materials and Methods

Site-Directed Mutagenesis

Mutant forms of NLRP3 (NM_004895.3) were generated by site-specific mutagenesis of the wild type NLRP3 cloned into a retroviral vector, pHSGP, tagged with Green Fluorescent Protein.
of 5 μg/mL in the incubation medium 1 hr before the addition of stimuli.

Stimulations

THP-1 cells stably expressing WT and mutant forms of NLRP3 were grown to a density of 1.5 x 10^6/mL in cell culture flasks. 24 h before stimulations, the cells were differentiated by treatment with 0.5 μM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 3 h. The cells were then washed with PBS and seeded at a density of 1 x 10^6 cells/well in 12-well plates in standard RPMI medium. The next day, cells were washed with PBS and 1 mL RPMI medium was added. Aluminium Hydrogel (Alum; Sigma-Aldrich) was used at 130 μg/mL for 4 h to stimulate the cells.

Detection of cytokines

The cytokines IL-1β, IL-18 and TNF-α were assessed in the cell culture media using ELISA. The lower detection limit of IL-1β and IL-18 (R&D systems, Minneapolis, MN and MBL International) were 0.16 pg/mL and 12.5 pg/mL, respectively, while that for TNF-α (Abcam, Cambridge, MA) was 25 pg/mL.

Gene Expression

Total RNA was isolated from THP-1 cells using TRIzol® (Invitrogen) and reverse transcribed to cDNA with Superscript II (Invitrogen) following the recommended protocol. mRNA expression of pro-IL-1β, F-cttgctgctggtggatggt & R-acggcgaactactgaccaaatcc and TNF-α F-cagagggctcagatctc & R-gaggtt-gaccctgtgctgt, were determined in triplicates using the SYBR green PCR kit on the 7900HT sequence detection system (Applied Biosystems, Carlsbad, CA), β-actin, F-acccgacagaaatgac & R-tctgatacatctgtgtc, was used for samples normalization and Ct values were calculated using the 2^-ΔΔct method.

Statistical methods

Data are represented as mean ± SEM in figures 1, 2, 3. Statistical comparisons were performed by Student’s unpaired t test (figure 1) or two-way ANOVA with Tukey’s correction (figures 2) using the software package SPSS 18.0. * represents P values<0.05, ** represents P values<0.01 and *** represents P values<0.001.

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Author Contributions

Conceived and designed the experiments: DV ES PE JJJ ML PS. Performed the experiments: DV HA JJJ. Analyzed the data: DV HA MF PS. Wrote the paper: DV ES HA PE ML PS.


