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Identification of IL-1 β -producing monocytes that are susceptible to pyronecrotic cell death in patients with NOMID

Jehad H. Edwan, PhD¹, Raphaela Goldbach-Mansky, MD^{1,2}, and Robert A. Colbert, MD, PhD¹

¹Pediatric Translational Research Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD, USA

²Translational Autoinflammatory Disease Section, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MD, USA

Abstract

Objective—Spontaneous inflammatory responses initiated by NLRP3 mutations promote inflammasome-mediated IL-1 β processing and release, and can induce rapid necrotic cell death. The cells that produce IL-1 β in neonatal onset multisystem inflammatory disease (NOMID) have not been clearly identified, nor have the mechanisms mediating IL-1 β release and cell death been completely elucidated.

Methods—Whole blood cells were stimulated with LPS in the presence of cathepsin B and caspase-1 inhibitors, followed by ATP treatment. Supernatants were collected and incubated with IL-1 β capturing beads. Cells were fixed, permeabilized and stained for a combination of cell surface and intracellular markers, and a novel flow cytometry bead-based assay was used to measure secreted IL-1 β . LPS stimulated cells were also evaluated using immunofluorescence microscopy.

Results—Monocytes characterized by CD14^{hi} CD16^{low} expression and intracellular CD83 are increased in NOMID subjects and are responsible for the majority of IL-1 β production in response to LPS stimulation. This population of monocytes also undergoes a rapid death response with LPS alone that is temporally associated with IL-1 β and ASC release and has characteristic features of pyronecrotic but not pyroptotic cell death. Inhibition of cell death reduces IL-1 β production from NOMID patient cells. In addition, IL-1 triggers cell death in monocytes from NOMID patients.

Conclusions—These results identify monocytes as the predominant IL-1 β -producing cell population in the peripheral blood of NOMID patients. Furthermore, they suggest that IL-1 receptor blockade may work in part by preventing pyronecrotic cell death, which may be an important target in NOMID and other forms of cryopyrin-associated periodic syndromes.

Keywords

Inflammasome; monocytes; NOMID; pyronecrosis; and cathepsin B

Mutations in the gene encoding nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) pyrin domain containing 3 (*NLRP3*), a member of the NLR family that regulates innate immune function, result in a spectrum of autoinflammatory diseases known as cryopyrin-associated periodic syndromes (CAPS) (1, 2). The most severe form of CAPS, neonatal-onset multisystem inflammatory disease (NOMID), is characterized by profound systemic inflammation with recurrent episodes of rash, fever, arthritis, progressive hearing loss, and eye and central nervous system manifestations. NLRP3 interacts with apoptosis-associated speck-like protein containing a caspase-activation and recruitment domain (ASC) and procaspase-1 to nucleate a multi-protein inflammasome complex (3–5). Inflammasome activation cleaves procaspase-1 to form active caspase-1, which catalyzes processing of pro-IL-1 β and secretion of biologically active mature IL-1 β (5).

Several toll-like receptor (TLR) agonists are potent inducers of intracellular pro-IL-1 β , while a second signal such as extracellular adenosine triphosphate (ATP) is required for mature IL-1 β release. ATP activates the P2X7 receptor, which opens a potassium channel. The fall in intracellular potassium triggers assembly and activation of the NLRP3 inflammasome resulting in IL-1 β processing and release (6). Inflammasome activation can be triggered by other diverse stimuli that promote K⁺ efflux, including reactive oxygen species and lysosomal destabilization (4, 7–14). Intracellular Ca²⁺ and cyclic AMP (cAMP) directly regulate NLRP3 inflammasome activation, suggesting a unifying molecular mechanism (15). Mutations in *NLRP3* that cause CAPS eliminate the requirement for a second signal to trigger IL-1 β release (16), which may result at least in part from reduced binding of cAMP to CAPS-associated mutant NLRP3 (15).

NLRP3 has also been linked to necrosis through pyronecrosis and pyroptosis (17, 18). Unlike apoptosis, necrotic cell death is highly inflammatory due to the release of pro-inflammatory cytokines and other mediators such as high mobility group protein B1 (HMGB1) (19), and ASC (20). ASC is an adaptor that is induced by NLRP3 to polymerize (forming ASC specks), that in turn cause pro-caspase-1 to self-activate. ASC has recently been shown to accumulate in the extracellular space after pyroptosis, where it can promote maturation of IL-1 β . Interestingly, phagocytosis of extracellular ASC by macrophages can induce lysosomal damage leading to IL-1 β production from recipient cells, thus propagating inflammation in a ‘prion-like’ mechanism, reflecting the ability of ASC to seed its own formation from soluble precursors. While the pro-inflammatory consequences of pyroptosis and pyronecrosis may be similar, pyroptosis is dependent on caspase-1 whereas pyronecrosis requires cathepsin B but is independent of caspase-1 (21), indicating that the latter process occurs independently of a complete inflammasome. Since TLR4 and the IL-1 receptor share a common intracellular Toll/IL-1R (TIR) domain, many responses to IL-1 and TLR4 ligands are similar (22) including increased production of IL-1 β (23, 24). The clinical response of patients with NOMID and other forms of CAPS to IL-1 blockade underscores the importance of this cytokine in driving many inflammatory disease manifestations (25–27). However, while myeloid cells are known to be an important source of IL-1 β , the mechanism of necrotic cell death in NOMID patients has not been clearly established.

In this study we use a combination of cell surface and intracellular markers, and a novel bead-based assay to measure secreted IL-1 β , to show that the majority of this cytokine

derives from monocytes expressing high levels of CD14, low levels of CD16, and intracellular CD83 (CD14^{hi} CD16^{low} iCD83). This population of monocytes is increased in NOMID subjects, and exquisitely sensitive to pyronecrotic but not pyroptotic cell death when stimulated with LPS alone. We also show that pyronecrotic cell death is associated with ASC release. We provide evidence that inhibition of cell death further reduces IL-1 β and ASC production from NOMID patient cells harboring NLRP3 mutations. In addition, we show that IL-1 triggers cell death in monocytes from NOMID patients suggesting that IL-1 receptor blockade may work in part by preventing pyronecrotic cell death.

Patients and Methods

Patients

Blood samples were collected from NOMID patients recruited under a National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Diabetes and Digestive and Kidney Diseases (NIAMS/NIDDK) Institutional Review Board (IRB) approved protocol. Written informed consent was obtained from patients and/or their legal guardians. The clinical protocol was conducted according to principles expressed in the Declaration of Helsinki (clinicaltrials.gov: NCT00069329).

Isolation of human blood cells

Peripheral blood was collected in tubes containing sodium heparin. Erythrocytes were removed using ACK lysing buffer (8.3 g/L NH₄Cl, KHCO₃ 1 g/L, EDTA 2H₂O 0.0372 g/L) (Quality Biological, Inc. Gaithersburg, MD) in 0.01 M Tris-HCl buffer. Briefly, blood cells were pelleted and mixed with 10 ml of ACK lysing buffer for 1 min. Cells were washed and re-suspended in RPMI medium. Cell viability before culture was assessed using trypan blue (Invitrogen, Frederick MD) exclusion.

Cell culture

Cells were seeded into 24 well plates at a density of 1x10⁶ viable cells per well. Each well contained RPMI medium supplemented with 10% fetal bovine serum (FBS), 50 IU/mL penicillin, and 50 μ g/mL streptomycin (Invitrogen, Frederick MD). Cells for inhibition studies were pre-incubated with the following inhibitors for 30 minutes with 50 μ M cathepsin B inhibitor; CA-074Me ([L-3-trans-(Propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline Methyl Ester), 20 μ M pan caspase inhibitor; zVAD-FMK (carbobenzoxycarbonyl-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone), 20 μ M caspase-1 inhibitor; Ac-YVAD-CMK (N-acetyl-L-tyrosyl-L-valyl-N-[(1S)-1-(carboxymethyl)-3-chloro-2-oxo-propyl]-L-alaninamide); (all from Enzo Life Sciences, Inc. Farmingdale, NY). Cells were stimulated with 50 ng/mL LPS for 150 minutes. ATP (2 mM final concentration) (InvivoGen, San Diego, CA) was added (where indicated) at 150 minutes for an additional 30 minutes. Blood cells were collected by centrifugation, and supernatants were collected and used to measure IL-1 β . For IL-1 β and IL-1 α stimulation experiments cells were pre-incubated with 150 μ g/ml anakinra (Kineret; Biovitrum, Stockholm, Sweden) and then stimulated with 10, 30, or 100 pg/ml of either IL-1 β or IL-1 α .

Preparation of IL-1 β capture beads

Streptavidin coated polystyrene particles (3.0–3.4 μ m in diameter); (Spherotech, Inc, Lake Forest, IL) were coupled with biotin anti-human IL-1 β antibody clone JK1B-2 (Biolegend, San Diego, CA). Briefly, affinity coupling of 10 μ g biotinylated anti-human IL-1 β antibody to 100 μ l of 0.5% w/v streptavidin coated polystyrene particles was done in a 5 mL glass tube in sodium phosphate buffer, 0.1 M, pH 5.5. The particles and antibody mixture was vortexed and incubated on a rolling mixer for at least one hour at ambient temperature. The particles were then centrifuged at 3000 x g for 5 minutes, and the supernatant was carefully removed. The pellet containing the anti-IL-1 β antibody bound to polystyrene beads (IL-1 β capture beads) was re-suspended in 4 mL of 0.1 M phosphate buffer. IL-1 β capture beads were incubated with cell supernatants at 4 °C overnight and analyzed together with cells (see below).

Intracellular and IL-1 β capture bead staining and flow cytometry

For intracellular staining, cells were fixed in 4% paraformaldehyde in PBS at 4 °C for 20 minutes and permeabilized in buffer containing 0.01% saponin (Cytofix/Cytoperm™ Kit, BD Biosciences, San Jose, CA) prior to staining. The following antibodies obtained from Biolegend were used according the manufacturer's protocols: FITC-labeled anti-CD3 (clone HIT3a), PE-labeled anti-CD83 (clone HB15e), PE-Cy7-labeled anti-CD16 (clone 3G8), AF647-labeled anti-IL-1 β (clone JK1B-1), and Pacific blue-labeled anti-CD14 (clone M5E2). IL-1 β capture beads that had been incubated with the cell supernatant were added back to fixed and permeabilized cells, stained with antibodies, and analyzed using a FACSCanto instrument with FACSDiva v6.1.2 software (BD Biosciences). The anti-IL-1 β capture (JK1B-2) and detection (JK1B-1) antibodies recognize different determinants on IL-1 β , thus enabling capture and detection of the same molecule. This bead-based assay together with intracellular staining of IL-1 β enables simultaneous measurement of IL-1 β -producing cell populations, and released IL-1 β . Both antibodies react with the pro- as well as mature forms of IL-1 β and thus measure both forms of the cytokine.

Western blot analysis

Supernatants from equal numbers of cells stimulated without or with LPS and ATP were diluted in one-third the volume of 4X Laemmli sample buffer (Bio-Rad, Hercules, CA) boiled for 90 seconds, and then subjected to electrophoresis on precast 4–20% gradient SDS-polyacrylamide gels (Bio-Rad). Proteins were transferred to PVDF membranes using Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked with 5% milk buffer and probed with primary antibody against ASC, (clone E1E3I) (Cell Signaling Technology, Beverly, MA), and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody, (catalog # sc-2054) (Santa Cruz Biotechnology, Dallas, TX). The signal was developed with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA).

Confocal microscopy

CD14+ monocytes from NOMID patients or healthy donors were separated from PBMCs using anti-CD14 magnetic microbeads (Miltenyi Biotec, Auburn, CA), and an AutoMACS

sorter (Miltenyi Biotec). Sorted monocytes were then stained with Qdot 655 anti-CD14, (clone (TüK4) (Invitrogen) and cultured in Lab-Tek chambered coverglass (Thermo Scientific, Waltham, MA) in complete medium. Cells were stimulated with 50 ng/mL LPS (Sigma, St. Louis, MS) and observed with a Zeiss LSM 510 Meta microscope (Carl Zeiss Microscopy, LLC Thornwood, NY). Pictures were merged using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

Statistical analysis was performed using Student's *t*-test. *P* values less than 0.05 were considered significant.

Results

Patient demographics and clinical characteristics

We studied 19 patients whose demographic and clinical characteristics are shown in Table 1. Sixteen subjects have germline mutations in *NLRP3*, with 3 more exhibiting somatic mosaicism (28). All patients exhibited classical NOMID features before beginning treatment such as urticarial rash, CNS inflammation (including aseptic meningitis), hearing loss, visual impairment, and many had developmental delay (29). A subset of patients also presented with characteristic bony overgrowth (30). At the time of blood draw for this study, all patients were receiving biologics that inhibit IL-1; 16 patients were on anakinra and 3 patients were receiving canakinumab (Table 1).

Identification of IL-1 β -producing monocytes

To identify IL-1 β -producing cells in peripheral blood, we took advantage of the intracellular accumulation of this cytokine in response to short-term LPS stimulation. However, intracellular staining requires permeabilization, which limits the separation of monocytes from neutrophils based on forward and side scatter alone. In preliminary experiments we found that cells expressing IL-1 β also expressed intracellular CD83, in addition to traditional monocyte markers. A typical experiment using peripheral blood cells from a healthy donor identifying IL-1 β -producing cells is shown in supplemental Figure S1 and Figure 1. Peripheral blood leukocytes were treated without or with LPS for 3 hours, fixed and permeabilized, and stained with antibodies against CD14, CD16, CD83 and IL-1 β . The overall gating strategy based on forward and side scatter is shown in supplemental Figure S1A, and differences between non-permeabilized and permeabilized cells are shown in supplemental Figure S1B. Staining for CD14, CD83, and CD16 on the gated cells identified in supplemental Figure S1A (monocytes and most neutrophils) reveals a CD14^{hi}, CD16^{low}, and CD83⁺ population (Figure 1A). The abundance of CD16^{hi} cells (Figure 1A, lower left and lower middle panels) is due to neutrophils that are HLA-DR negative (supplemental Figure S1C). Since neutrophils are strongly positive for CD16, and weakly positive for CD14 (Figure 1A, lower left), but negative for intracellular CD83 (Figure 1A, lower middle), intracellular CD83 is a convenient marker for distinguishing monocytes. The monocyte/neutrophil gate is negative for CD3⁺ cells (yellow cells eliminated from the gate in supplemental Figures S1A and S1B, and data not shown).

Exposure to LPS for 3 hours shows increased intracellular IL-1 β expression in the CD83+ cells (CD14^{hi} CD16^{low}), but not the CD83-population including CD14^{low} CD16^{hi} neutrophils (Figure 1B). To further characterize CD83 expression, we compared permeabilized and non-permeabilized CD14^{hi} CD16^{low} monocytes without and with LPS treatment (Figure 1C). Unstimulated monocytes are negative for cell surface CD83, but positive upon permeabilization (Figure 1C, left panel). LPS-stimulated non-permeabilized monocytes become CD83+ (Figure 1C, right panel, red histogram), with a small increase in total CD83 staining (Figure 1C, orange histograms in right vs. left panel), but the majority of CD83 remains intracellular (Figure 1C, right panel, orange vs. red). We examined additional markers and found that the CD14^{hi} CD16^{low} intracellular CD83+ (iCD83+) monocytes also express CD62L, CD45, CD141, HLA-DR, and CD123, and were negative for CD1c, with no significant differences between healthy donors and NOMID subjects. HLA-DR, CD83 and CD1c were absent from neutrophils (unpublished observations).

We next asked whether the CD14^{hi} CD16^{low} iCD83+ monocytes were more frequent in the peripheral blood of patients with NOMID. Compared to healthy donors (HD), the CD14^{hi} CD16^{low} iCD83+ population (expressed as a percentage of CD14+/CD16+ cells) was significantly increased in NOMID patients (9.37 ± 0.53 n=19 vs. 5.65 ± 0.45 n=20; $P < 0.001$) (Figure 1D).

IL-1 β production and cell loss in NOMID

We developed a flow cytometric cell and bead-based assay to simultaneously measure intracellular as well as released IL-1 β . In the example shown in Figure 2, peripheral blood cells from a representative HD and a NOMID patient were incubated for 3 hours without or with LPS, and then cells and supernatants were collected. IL-1 β capture beads were incubated with supernatants, isolated by centrifugation, and then added back to fixed and permeabilized cells. Beads and cells were stained together, and assessed by flow cytometry to simultaneously measure intracellular and released IL-1 β .

Only the CD14^{hi} CD16^{low} iCD83+ monocytes accumulated intracellular IL-1 β (Figure 2). LPS treatment resulted in IL-1 β release from NOMID cells without the need for a second signal (i.e. ATP) and was bound by the IL-1 β capture beads (Figure 2B vs. 2A, middle and lower panels, green dots). The IL-1 β capture beads performed similarly to a bead-based luminex assay for detecting released IL-1 β (supplemental Figure S2).

Using this flow cytometric assay, we compared cells from multiple HD and NOMID subjects (Figure 3A–C). The accumulation of intracellular IL-1 β in CD14^{hi} CD16^{low} iCD83+ cells in response to LPS and LPS plus ATP was similar in HDs and individuals with NOMID (Figure 3A). IL-1 β release in response to LPS plus ATP was also comparable for HD and NOMID cells, but was also seen with LPS alone in cells from NOMID patients (Figure 3B), as reported previously (16, 31). However, we also found that the proportion of CD14^{hi} CD16^{low} iCD83+ monocytes remaining after LPS stimulation was significantly lower in NOMID patient samples compared to HDs (Figure 3C). While there was cell loss in HD samples stimulated with LPS alone (Figure 3C), this occurred without a measurable increase in IL-1 β release (Figure 3B). Furthermore, the addition of ATP to HD cells

stimulated with LPS resulted in robust IL-1 β release (Figure 3B) without any additional cell loss (Figure 3C).

CD14⁺ monocytes from NOMID patients exhibit rapid cell death

In order to investigate the fate of CD14⁺ monocytes in samples from NOMID subjects, we used immunofluorescence and confocal microscopy to visualize cells following stimulation with LPS. CD14⁺ monocytes were isolated from peripheral blood of NOMID subjects using magnetic sorting, and then stained with fluorescent-labeled anti-CD14 (red). LPS was added (Time 0), and time-lapse video microscopy images were recorded at ten-second intervals. After approximately 55 minutes of LPS stimulation, previously normal appearing cells began to flatten and lose staining intensity, followed by apparent rupture of the cell membrane and loss of a distinct nucleus; all occurring over a period of about 6–7 minutes (Supplemental Video 1; representative images shown in Figure 3D). This series of changes, including the rapid evolution, is consistent with cell death by pyronecrosis (17). Characteristics of cell death by apoptosis such as cellular contraction, membrane blebbing, and chromatin condensation, were not apparent and thus apoptosis does not appear to be contributing to the rapid loss of cells after LPS stimulation. Although there was some cell loss in HD samples stimulated with LPS, we did not see the features described above that were observed with NOMID cells (data not shown).

LPS induced loss of NOMID cells is cathepsin B-dependent

To determine pathways involved in monocyte death, we examined the effects of caspase-1 and cathepsin B inhibition. To inhibit caspase-1 the pan-caspase inhibitor, zVAD-FMK, and the more specific caspase-1 inhibitor, Ac-YVAD-CMK, were used. Cathepsin B was blocked using CA-074Me. Neither caspase nor cathepsin B inhibition reduced IL-1 β accumulation in cells stimulated with LPS (Figure 4A, B, left panel). Caspase-1 inhibition partially reduced IL-1 β release from NOMID and HD cells, whereas cathepsin B inhibition completely abrogated IL-1 β release. This effect occurred in NOMID patients and HD cells, including cells treated with ATP (Figure 4A, B, middle panel). While caspase-1 inhibitors reduced IL-1 β release by approximately 62.5%, there was no inhibition of LPS induced cell death (Figure 4A, B, right panel). In contrast, cathepsin B inhibition reduced cell death by about 60–70% in LPS and LPS plus ATP treated NOMID patient cells, respectively (Figure 4A, B, right panel). Cathepsin B inhibition blocked IL-1 β release by about 95%, in the context of even slightly greater intracellular accumulation (Figure 4A, B, left panel). Taken together, these data as well as previously published studies (32), indicate that, in contrast to HD cells, IL-1 β processing and release from NOMID patient cells was only observed in the context of significant cell death. Nevertheless, some IL-1 β release could be prevented with caspase inhibition even without significant reduction in cell death (Figure 4A, B, middle panel).

Since necrotic cell death is associated with release of pro-inflammatory mediators, we examined supernatants for the presence of ASC. NOMID cells released ASC after 3 hours of LPS or LPS plus ATP in a caspase-independent, but cathepsin B dependent process, while cells from HD only released ASC when stimulated with LPS plus ATP (Figure 4C). Interestingly, ASC was detectable in supernatants from unstimulated NOMID cells even

when CA-074Me was added, suggesting that there may be a small percentage of cells dying off without TLR stimulation that are beyond the point of rescue by cathepsin B inhibitors.

LPS induced cell death and IL-1 β release are not suppressed by IL-1 receptor antagonist

To determine the extent to which autocrine or paracrine IL-1 β might mediate the effects of LPS on NOMID patient cells, we examined the effects of LPS in the absence and presence of the IL-1 receptor antagonist anakinra. Anakinra had no effect on LPS-induced IL-1 β accumulation or release from NOMID patient cells (Figure 5A) in this short-term (3 hour) incubation. In addition, the LPS-induced loss of CD14^{hi} CD16^{low} iCD83+ cells was unaffected by IL-1 blockade with anakinra (Figure 5B, C). These data indicate that TLR4-mediated activation of the inflammasome in NOMID patients does not require autocrine or paracrine IL-1. Nevertheless, both IL-1 β and IL-1 α are capable of promoting death of NOMID patient cells, an effect that is inhibited by IL-1 blockade with anakinra (Figure 5C). This suggests that while IL-1 is not required to mediate rapid immediate effects of TLR4 agonists, it may nevertheless contribute to a positive feedback loop in affected patients that can be broken by IL-1 inhibition.

Discussion

In this report we identify a population of peripheral blood monocytes expressing high levels of CD14, low levels of CD16, and intracellular CD83 (CD14^{hi} CD16^{low} iCD83) that accumulates in NOMID patients. These cells represent the major monocyte population in peripheral blood producing IL-1 β in response to LPS in the absence of added ATP. The same cells derived from healthy donors express and produce IL-1 β , but only after maximal stimulation with LPS and LPS plus ATP, respectively.

Our results indicate that intracellular CD83 is expressed by CD14^{hi} CD16^{low} monocytes that produce IL-1 β . CD83 is expressed on the surface of dendritic cells (33) and can be immunostimulatory (34), while soluble CD83 has been reported to have immunosuppressive characteristics (35). CD83 has been shown to exist inside monocytes and macrophages, with transient surface expression induced by LPS (36), consistent with our findings (Figure 1C). The CD83 promoter contains binding sites for IRF-1, IRF-2 and IRF-5, as well as NF- κ B, and thus expression of CD83 may reflect some degree of cellular activation (37). While the functional significance of intracellular CD83 remains unclear, it provides a convenient marker, along with CD14 and CD16 for monocytes that are robust producers of IL-1 β in response to LPS. In human blood other phagocytic and pro-inflammatory monocyte subsets have been identified based on relative levels of CD16 and CD14 expression (38, 39). In our experiments cell permeabilization for intracellular staining, which was necessary to identify the IL-1 β -producing cells, limited our ability to definitively distinguish additional monocyte subsets. However, it does not appear that significant numbers of CD14^{low} cells became IL-1 β -positive with LPS stimulation (Figure 2, middle panel). In addition, the CD16^{hi} neutrophils remained IL-1 β negative.

Cell death pathways are broadly categorized by the events leading to cell disintegration. Pyronecrosis and pyroptosis are two forms of cell death (40) that are distinct from apoptosis. Both occur rapidly, and are characterized by swelling and loss of plasma membrane integrity

without the blebbing seen during apoptosis (21). There is apparent flattening of the cell without chromatin condensation seen in apoptosis. Despite similarities, pyroptosis appears to depend on caspase-1, whereas pyronecrosis is caspase-1-independent and instead requires cathepsin B (21). Interestingly, both pyroptosis and pyronecrosis are reported to occur after bacterial infection, but the pathways responsible may depend on the organism. Monocytic cells infected with *Shigella flexneri* undergo NLRP3-dependent caspase-1-independent pyronecrosis (17), while monocytic cells infected with *Salmonella typhi* undergo caspase-1-dependent pyroptosis (41, 42). Our results indicate that monocytes from NOMID patients harboring NLRP3 mutations exposed to LPS exhibit morphological features of cell death consistent with pyroptosis or pyronecrosis. However, cell death is cathepsin B-dependent and caspase-1-independent, and thus is consistent with pyronecrosis. We did not observe morphological features of pyronecrosis when HD monocytes were stimulated with LPS (unpublished observations), and there was no increase in cell loss with the addition of ATP (Figures 3C and 4B). However, we do see increased cell death with the addition of ATP to LPS-stimulated THP-1 cells (unpublished observations), and Brough et al. reported that ATP increased cell death in LPS-primed murine macrophages (43). In contrast, Ferrari et al. found that cell death was not associated with IL-1 β release after treatment of human macrophages with LPS plus ATP (44). These inconsistent results could be a consequence of differences in the cell types studied and/or experimental conditions. With regard to freshly isolated human monocytes, it is also possible that NLRP3 mutations alter additional pathways that sensitize cells to pyronecrotic cell death. Nevertheless, while IL-1 β release and cell loss in HD and NOMID cells are both cathepsin B-dependent, more work will need to be done to understand differences in cell death pathways.

Our results demonstrate that IL-1 β release in response to LPS in cells expressing mutant NLRP3, or LPS plus ATP in normal cells, is partially blocked by caspase inhibition. However, under these conditions, the death of cells harboring NLRP3 mutations is not prevented. In contrast, cathepsin B inhibitors completely block IL-1 β release and substantially inhibit death of cells expressing mutated NLRP3. Cathepsin B inhibitors have the same effect on IL-1 β production in cells from healthy donors, and similar trends are seen for cell death. Cathepsin B has been implicated in NLRP3-mediated IL-1 β release in response to silica crystals, aluminum salts, and lysosomal permeabilization, as has been reported previously (45). These findings suggest that a significant proportion of IL-1 β release from cells with NLRP3 mutations might lie downstream of cathepsin B and be tied to pyronecrosis. Necrotic cell death associated with pyronecrosis and pyroptosis elicits substantial inflammation, in part through release of the NLRP3 inflammasome in patients with active CAPS (46), and in part through damage associated molecular pattern molecules such as HMGB1 and ASC specks (19, 20), and thus may play an important role in the pathogenesis of NOMID. The adaptor protein ASC has recently been shown to have extracellular prion-like activities that propagate inflammation, including promoting activation of pro-IL-1 β (20), prompting us to look for this protein in cell supernatants. We found a clear increase in ASC when NOMID cells were stimulated with LPS (or when NOMID and HD cells were stimulated with LPS + ATP) that was inhibited by blocking cathepsin B but not caspases. These results suggest that NLRP3 mutants, by contributing to pyronecrotic cell death, may promote inflammation beyond the release of cytokines.

However, in preliminary experiments we were unable to detect ASC in stored serum from treated NOMID patients. This may be due to limited sensitivity of western blotting and/or the fact that the patients are well controlled on IL-1 blockers.

The presence and accumulation of CD14^{hi} CD16^{low} iCD83⁺ monocytes suggests a population that is readily triggered to produce IL-1 β and undergo pyronecrotic death, supporting the relevance of these cells to NOMID pathogenesis. However, whether accumulation is a direct consequence of disease is not clear, since all of the patients we studied were receiving either anakinra or canakinumab to control inflammation. Since IL-1 can promote the death of CD14^{hi} CD16^{low} iCD83⁺ cells (Figure 5), we cannot rule out the possibility that blocking IL-1 *in vivo* results in enhanced survival and accumulation. However, even in the face of effective IL-1 blockade, activation of TLR4 with LPS and probably other damage or pathogen-associated molecular patterns is sufficient to induce substantial pyronecrosis (Figure 5C) with potential pro-inflammatory consequences. Recently, elegant studies using CAPS mutant knock-in mice revealed ongoing inflammation despite the inability to make or respond to IL-18 and IL-1 (47). For example, in *Nlrp3* mutant mice genetically deficient in IL-1 and IL-18 signaling (*Il1r-/-Il18-/-*), the mice still exhibited enhanced necrosis of bone marrow-derived cells and premature death. While the cause was not clear, pyronecrotic cell death induced by *Nlrp3* mutations could be a contributing factor. Thus, the tendency of CD14^{hi} CD16^{low} iCD83⁺ monocytes and perhaps other NLRP3 mutant-expressing cells in NOMID patients to undergo pro-inflammatory pyronecrotic cell death, raises the possibility that developing ways to inhibit pyronecrosis may provide a means to better control disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Masters SL, Simon A, Aksentijevich I, Kastner DL. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease. *Annu Rev Immunol.* 2009; 27:621–68. [PubMed: 19302049]
2. Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet.* 2001; 29(3):301–5. [PubMed: 11687797]
3. Dinarello CA. Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme. *Ann N Y Acad Sci.* 1998; 856:1–11. [PubMed: 9917859]
4. Martinon F, Petrilli V, Mayor A, Tardivel A, Tschopp J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature.* 2006; 440(7081):237–41. [PubMed: 16407889]
5. Agostini L, Martinon F, Burns K, McDermott MF, Hawkins PN, Tschopp J. NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder. *Immunity.* 2004; 20(3):319–25. [PubMed: 15030775]
6. Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, et al. The P2X7 receptor: a key player in IL-1 processing and release. *J Immunol.* 2006; 176(7):3877–83. [PubMed: 16547218]

7. Mariathasan S, Weiss DS, Newton K, McBride J, O'Rourke K, Roose-Girma M, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*. 2006; 440(7081):228–32. [PubMed: 16407890]
8. Halle A, Hornung V, Petzold GC, Stewart CR, Monks BG, Reinheckel T, et al. The NALP3 inflammasome is involved in the innate immune response to amyloid-beta. *Nat Immunol*. 2008; 9(8):857–65. [PubMed: 18604209]
9. Shio MT, Eisenbarth SC, Savaria M, Vinet AF, Bellemare MJ, Harder KW, et al. Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases. *PLoS Pathog*. 2009; 5(8):e1000559. [PubMed: 19696895]
10. Walev I, Reske K, Palmer M, Valeva A, Bhakdi S. Potassium-inhibited processing of IL-1 beta in human monocytes. *EMBO J*. 1995; 14(8):1607–14. [PubMed: 7737113]
11. Perregaux D, Gabel CA. Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. *J Biol Chem*. 1994; 269(21):15195–203. [PubMed: 8195155]
12. Perregaux D, Barberia J, Lanzetti AJ, Geoghegan KF, Carty TJ, Gabel CA. IL-1 beta maturation: evidence that mature cytokine formation can be induced specifically by nigericin. *J Immunol*. 1992; 149(4):1294–303. [PubMed: 1500719]
13. Hogquist KA, Nett MA, Unanue ER, Chaplin DD. Interleukin 1 is processed and released during apoptosis. *Proc Natl Acad Sci U S A*. 1991; 88(19):8485–9. [PubMed: 1924307]
14. Davis BK, Wen H, Ting JP. The inflammasome NLRs in immunity, inflammation, and associated diseases. *Annu Rev Immunol*. 2011; 29:707–35. [PubMed: 21219188]
15. Lee GS, Subramanian N, Kim AI, Aksentjevich I, Goldbach-Mansky R, Sacks DB, et al. The calcium-sensing receptor regulates the NLRP3 inflammasome through Ca²⁺ and cAMP. *Nature*. 2012; 492(7427):123–7. [PubMed: 23143333]
16. Gattorno M, Tassi S, Carta S, Delfino L, Ferlito F, Pelagatti MA, et al. Pattern of interleukin-1beta secretion in response to lipopolysaccharide and ATP before and after interleukin-1 blockade in patients with CIAS1 mutations. *Arthritis Rheum*. 2007; 56(9):3138–48. [PubMed: 17763411]
17. Willingham SB, Bergstralh DT, O'Connor W, Morrison AC, Taxman DJ, Duncan JA, et al. Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. *Cell Host Microbe*. 2007; 2(3):147–59. [PubMed: 18005730]
18. Saito M, Nishikomori R, Kambe N, Fujisawa A, Tanizaki H, Takeichi K, et al. Disease-associated CIAS1 mutations induce monocyte death, revealing low-level mosaicism in mutation-negative cryopyrin-associated periodic syndrome patients. *Blood*. 2008; 111(4):2132–41. [PubMed: 18063752]
19. Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, Washburn NR, et al. The grateful dead: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. *Immunol Rev*. 2007; 220:60–81. [PubMed: 17979840]
20. Franklin BS, Bossaller L, De Nardo D, Ratter JM, Stutz A, Engels G, et al. The adaptor ASC has extracellular and 'prionoid' activities that propagate inflammation. *Nat Immunol*. 2014; 15(8):727–37. [PubMed: 24952505]
21. Ting JP, Willingham SB, Bergstralh DT. NLRs at the intersection of cell death and immunity. *Nat Rev Immunol*. 2008; 8(5):372–9. [PubMed: 18362948]
22. Palsson-McDermott EM, O'Neill LA. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology*. 2004; 113(2):153–62. [PubMed: 15379975]
23. Warner SJ, Auger KR, Libby P. Interleukin 1 induces interleukin 1. II. Recombinant human interleukin 1 induces interleukin 1 production by adult human vascular endothelial cells. *J Immunol*. 1987; 139(6):1911–7. [PubMed: 3497983]
24. Dinarello CA, Ikejima T, Warner SJ, Orencole SF, Lonnemann G, Cannon JG, et al. Interleukin 1 induces interleukin 1. I. Induction of circulating interleukin 1 in rabbits in vivo and in human mononuclear cells in vitro. *J Immunol*. 1987; 139(6):1902–10. [PubMed: 3497982]
25. Goldbach-Mansky R, Dailey NJ, Canna SW, Gelabert A, Jones J, Rubin BI, et al. Neonatal-onset multisystem inflammatory disease responsive to interleukin-1beta inhibition. *N Engl J Med*. 2006; 355(6):581–92. [PubMed: 16899778]

26. Leslie KS, Lachmann HJ, Bruning E, McGrath JA, Bybee A, Gallimore JR, et al. Phenotype, genotype, and sustained response to anakinra in 22 patients with autoinflammatory disease associated with CIAS-1/NALP3 mutations. *Arch Dermatol.* 2006; 142(12):1591–7. [PubMed: 17178985]
27. Hoffman HM, Throne ML, Amar NJ, Sebai M, Kivitz AJ, Kavanaugh A, et al. Efficacy and safety of riloncept (interleukin-1 Trap) in patients with cryopyrin-associated periodic syndromes: results from two sequential placebo-controlled studies. *Arthritis Rheum.* 2008; 58(8):2443–52. [PubMed: 18668535]
28. Tanaka N, Izawa K, Saito MK, Sakuma M, Oshima K, Ohara O, et al. High incidence of NLRP3 somatic mosaicism in patients with chronic infantile neurologic, cutaneous, articular syndrome: results of an International Multicenter Collaborative Study. *Arthritis Rheum.* 2011; 63(11):3625–32. [PubMed: 21702021]
29. Sibley CH, Plass N, Snow J, Wiggs EA, Brewer CC, King KA, et al. Sustained response and prevention of damage progression in patients with neonatal-onset multisystem inflammatory disease treated with anakinra: a cohort study to determine three- and five-year outcomes. *Arthritis Rheum.* 2012; 64(7):2375–86. [PubMed: 22294344]
30. Hill SC, Namde M, Dwyer A, Poznanski A, Canna S, Goldbach-Mansky R. Arthropathy of neonatal onset multisystem inflammatory disease (NOMID/CINCA). *Pediatr Radiol.* 2007; 37(2): 145–52. [PubMed: 17136361]
31. Dowds TA, Masumoto J, Zhu L, Inohara N, Nunez G. Cryopyrin-induced interleukin 1beta secretion in monocytic cells: enhanced activity of disease-associated mutants and requirement for ASC. *J Biol Chem.* 2004; 279(21):21924–8. [PubMed: 15020601]
32. Chae JJ, Cho YH, Lee GS, Cheng J, Liu PP, Feigenbaum L, et al. Gain-of-function Pyrin mutations induce NLRP3 protein-independent interleukin-1beta activation and severe autoinflammation in mice. *Immunity.* 2011; 34(5):755–68. [PubMed: 21600797]
33. Zhou LJ, Tedder TF. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J Immunol.* 1995; 154(8):3821–35. [PubMed: 7706722]
34. Prechtel AT, Turza NM, Theodoridis AA, Steinkasserer A. CD83 knockdown in monocyte-derived dendritic cells by small interfering RNA leads to a diminished T cell stimulation. *J Immunol.* 2007; 178(9):5454–64. [PubMed: 17442926]
35. Scholler N, Hayden-Ledbetter M, Dahlin A, Hellstrom I, Hellstrom KE, Ledbetter JA. Cutting edge: CD83 regulates the development of cellular immunity. *J Immunol.* 2002; 168(6):2599–602. [PubMed: 11884422]
36. Cao W, Lee SH, Lu J. CD83 is preformed inside monocytes, macrophages and dendritic cells, but it is only stably expressed on activated dendritic cells. *Biochem J.* 2005; 385(Pt 1):85–93. [PubMed: 15320871]
37. Stein MF, Lang S, Winkler TH, Deinzer A, Erber S, Nettelbeck DM, et al. Multiple interferon regulatory factor and NF-kappaB sites cooperate in mediating cell-type- and maturation-specific activation of the human CD83 promoter in dendritic cells. *Mol Cell Biol.* 2013; 33(7):1331–44. [PubMed: 23339870]
38. Ziegler-Heitbrock L. The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol.* 2007; 81(3):584–92. [PubMed: 17135573]
39. Belge KU, Dayyani F, Horelt A, Siedlar M, Frankenberger M, Frankenberger B, et al. The proinflammatory CD14+CD16+DR++ monocytes are a major source of TNF. *J Immunol.* 2002; 168(7):3536–42. [PubMed: 11907116]
40. Fernandes-Alnemri T, Wu J, Yu JW, Datta P, Miller B, Jankowski W, et al. The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. *Cell Death Differ.* 2007; 14(9):1590–604. [PubMed: 17599095]
41. Mariathasan S, Newton K, Monack DM, Vucic D, French DM, Lee WP, et al. Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf. *Nature.* 2004; 430(6996): 213–8. [PubMed: 15190255]
42. Brennan MA, Cookson BT. Salmonella induces macrophage death by caspase-1-dependent necrosis. *Mol Microbiol.* 2000; 38(1):31–40. [PubMed: 11029688]

43. Brough D, Rothwell NJ. Caspase-1-dependent processing of pro-interleukin-1beta is cytosolic and precedes cell death. *J Cell Sci.* 2007; 120(Pt 5):772–81. [PubMed: 17284521]
44. Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Melchiorri L, Baricordi OR, et al. Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. *J Immunol.* 1997; 159(3):1451–8. [PubMed: 9233643]
45. Hornung V, Bauernfeind F, Halle A, Samstad EO, Kono H, Rock KL, et al. Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization. *Nat Immunol.* 2008; 9(8):847–56. [PubMed: 18604214]
46. Baroja-Mazo A, Martin-Sanchez F, Gomez AI, Martinez CM, Amores-Iniesta J, Compan V, et al. The NLRP3 inflammasome is released as a particulate danger signal that amplifies the inflammatory response. *Nat Immunol.* 2014; 15(8):738–48. [PubMed: 24952504]
47. Brydges SD, Broderick L, McGeough MD, Pena CA, Mueller JL, Hoffman HM. Divergence of IL-1, IL-18, and cell death in NLRP3 inflammasomopathies. *J Clin Invest.* 2013; 123(11):4695–705. [PubMed: 24084736]

Figure 1. Identification of IL-1 β -producing monocytes in peripheral whole blood

(A–C) Peripheral blood leukocytes from a healthy donor were incubated without (unstimulated) or with LPS (50 ng/ml) for 3 hours, fixed and permeabilized, or left intact, and stained with isotype control or the antibodies indicated. Initial gating was based on size and granularity as described in results and supplemental Figure S1A. (A) Density plot showing expression of CD14, CD16, and CD83 in unstimulated monocytes, with isotype control in the upper panel. (B) Density plot showing IL-1 β expression in CD83+ (CD14^{hi}, CD16^{low}) peripheral blood monocytes after treatment with LPS for 3 hours (lower plot) or unstimulated (upper plot). (C) Histogram comparing surface and total (intracellular + cell surface) expression of CD83 in unstimulated and LPS-stimulated (3 hours, 50 ng/ml) CD14^{hi} CD16^{low} cells. (D) Frequency of CD14^{hi} CD16^{low} iCD83+ cells as a percentage of total CD14⁺/CD16⁺ cells. For healthy donors (HD) n=20, and for NOMID subjects n=19.

Figure 2. Flow cytometric bead-based detection of IL-1 β production

Peripheral blood cells from (A) a healthy donor (HD) and (B) a subject with NOMID were cultured without (unstimulated) or with LPS-stimulated (50 ng/ml) for 3 hours. Supernatants were incubated with IL-1 β capture beads, while cells were fixed and permeabilized. Beads and cells were mixed and stained with the antibodies indicated. Density plots of unstimulated and LPS-stimulated HD and NOMID cells are shown. Purple dots represent CD14^{low} CD16^{hi} CD83⁻ cells, blue dots represent CD14^{hi} CD16^{low} CD83⁺ cells, and green dots are IL-1 β capture beads.

Figure 3. CD14^{hi} CD16^{low} monocytes from NOMID subjects exhibit rapid cell death and IL-1 β release

Peripheral blood from healthy donors (HD) (n=7) and NOMID subjects (n=17) was processed as described in the legend to Figure 2. (A) Intracellular IL-1 β in CD14^{hi} CD16^{low} iCD83+ monocytes after 3 hours of LPS, with ATP added for the last 30 minutes (MFI of intracellular staining). (B) Released IL-1 β captured on anti-IL-1 β beads after LPS without or with the addition of ATP for the last 30 minutes. (C) CD14^{hi} CD16^{low} iCD83+ cells as a percentage of total CD14+/CD16+ cells remaining after 3 hours of LPS without or with ATP (** = P<0.01). (D) CD14+ monocytes were isolated from NOMID patients using magnetic

selection and labeled with Qdot-655 anti-human CD14 (red). Cells were stimulated with LPS (50 ng/ml), and time-lapse video microscopy images were recorded at ten-second intervals. The 6 frames span about 7 minutes, from 54 minutes 24 seconds after LPS was added (upper left), to 60 minutes 48 seconds (lower right), and show a monocyte undergoing flattening, loss of staining intensity, and apparent rupture of the cell membrane with eventual disappearance of the distinct nucleus. The images shown are representative of many cells observed in 3 independent experiments from NOMID patients.

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Figure 4. Cathepsin B inhibition abrogates LPS induced killing and IL-1 β release from CD14^{hi} CD16^{low} monocytes

(A–C) Cells were pretreated with the specified inhibitors for 30 minutes, followed by three hours stimulation with LPS (50 ng/ml), with ATP added for the last 30 minutes. Intracellular IL-1 β levels in CD14^{hi} CD16^{low} iCD83+ monocytes in NOMID subjects (A, left panel) and healthy donors (HD) (B, left panel). IL-1 β production from NOMID (A, middle panel) and HD cells (B, middle panel). CD14^{hi} CD16^{low} iCD83+ cells remaining as a percentage of total CD14+/CD16+ monocytes in cultures from NOMID (A, right panel) and HD (B, right panel). For NOMID subjects n=17; for HD n=7. (C) Cell culture supernatants from a NOMID subject (top) and a HD (bottom) were subjected to SDS-PAGE and blotted for ASC. Molecular weight standards were run (not shown), with the approximate molecular weights of ASC bands indicated.

(** P<0.01; *** P<0.001)

Figure 5. Cell death in CD14^{hi} CD16^{low} iCD83⁺ monocytes can be induced via IL-1 receptor signaling

Cells from NOMID patients were stimulated with LPS (50 ng/ml) in the absence or presence of 150, 300 or 600 μg/mL anakinra for 3 hours. (A) Released IL-1β detected with IL-1β capture beads (green dots). (B) CD14^{hi} CD16^{low} iCD83⁺ monocytes remaining after LPS treatment in the absence or presence of anakinra (AK) at the concentrations indicated. Numbers indicate the CD14^{hi} CD16^{low} iCD83⁺ monocytes as the percentage of CD14⁺/CD16⁺ cells. (C) Cells from NOMID patients were stimulated with IL-1β or IL-1α at the concentrations indicated in the absence or presence of 150 μg/mL anakinra for 3 hours. Bars represent remaining CD14^{hi} CD16^{low} iCD83⁺ monocytes as a percentage of CD14⁺/CD16⁺ cells. Data shown are representative of three independent experiments. (* P<0.05; ** P<0.01; *** P<0.001)

Table 1

Age*	Sex	CRP at time of Blood Draw (mg/dL)	Mutation	Other NOMID features	IL-1 inhibitor dose at time of Blood Draw	Prednisone equivalent steroid dose (mg/kg/day)
16	F	0.411	A374N	papilledema, hearing loss, aseptic meningitis, severe developmental delay	Anakinra 5.2mg/kg/dy	0
19	M	0.107	D303N	papilledema, aseptic meningitis, bony overgrowth, growth retardation, cochlear enhancement, hearing loss	Canakinumab 450mg	0.29
48	M	0.466	D303N	papilledema, arachnoid adhesions	Anakinra 3.5mg/kg/dy	0.55
16	F	0.12	D303N	hearing loss, visual impairment, aseptic meningitis,	Anakinra 2.8mg/kg/dy	0
3	F	0.076	G307E	uveitis	Anakinra 3.5mg/kg/dy	0.44
16	F	0.182	G569R	papilledema, bony overgrowth, hearing loss, aseptic meningitis	Canakinumab (no dose documented)	1.04
6	M	0.11	G569R	papilledema, mild cerebral atrophy	Anakinra 4.8mg/kg/dy	0.83
5	M	0.137	G569R	aseptic meningitis, papilledema, hearing loss	Anakinra 4.1mg/kg/dy	0.16
7	F	0.43	G755A	aseptic meningitis, papilledema	Anakinra 2.7mg/kg/dy	0
7	F	0.017	I334F	aseptic meningitis, papilledema, arachnoid adhesions	Anakinra 4.5mg/kg/dy	0
7	F	0.339	L264F	papilledema	Anakinra 3.2mg/kg/dy	0
15	F	<0.016	L264F	papilledema, bony overgrowth, hearing loss, aseptic meningitis, mild developmental delay	Anakinra 5.9mg/kg/dy	0.08
1	F	0.372	N479K	bone lesions, rash	Anakinra 4.1mg/kg/dy	0.97
27	M	0.138	Q600P	hearing loss, vision loss, aseptic meningitis, developmental delay	Anakinra 5.6mg/kg/dy	0.11
18	M	0.628	Mosaic E567K (16.2%)	papilledema, bony overgrowth, hearing loss, aseptic meningitis, developmental delay	Canakinumab 9.2mg/kg	0.09
3	F	0.901	Mosaic F302L (8.4%)	Rash, enlarged knees	Anakinra 3.4mg/kg/dy	0
27	F	0.291	Mosaic K355N (20.1%)	papilledema, bony overgrowth, hearing loss, developmental delay, arachnoid adhesions	Anakinra 6.5mg/kg/dy	0
19	F	0.244	V351L	papilledema, hearing loss	Anakinra 3.6mg/kg/dy	0
8	F	0.44	Y441H	papilledema, hearing loss	Anakinra 4mg/kg/dy	0
16	F	0.411	A374N	papilledema, hearing loss, aseptic meningitis, severe developmental delay	Anakinra 5.2mg/kg/dy	0
19	M	0.107	D303N	papilledema, aseptic meningitis, bony overgrowth, growth retardation, cochlear enhancement, hearing loss	Canakinumab 450mg	0.29
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16	F	0.12	D303N	hearing loss, visual impairment, aseptic meningitis,	Anakinra 2.8mg/kg/dy	0
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7	F	0.017	I334F	aspeptic meningitis, papilledema, arachnoid adhesions	Anakinra 4.5mg/kg/dy	0
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* age at time of blood draw