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ORIGINAL RESEARCH

Inflammasome Genetic Variants, Macrophage Function, and Clinical Outcomes in Cystic Fibrosis

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Abstract

Cystic fibrosis (CF) is characterized by chronic airway infection, inflammation, and tissue damage that lead to progressive respiratory failure. NLRP3 and NLRC4 are cytoplasmic pattern recognition receptors that activate the inflammasome, initiating a caspase-1–mediated response. We hypothesized that gain-of-function inflammasome responses are associated with worse outcomes in children with CF. We genotyped nonsynonymous variants in *NLRP3* and the *NLRC4* pathway from individuals in the EPIC (Early *Pseudomonas* Infection Control) Observational Study cohort and tested for association with CF outcomes. We generated knockouts of *NLRP3* and *NLRC4* in human macrophage–like cells and rescued knockouts with wild-type or variant forms of *NLRP3* and *NLRC4*. We identified a SNP in *NLRP3*, p.(Q705K), that was associated with a higher rate of *P. aeruginosa* colonization ($N = 609$; $P = 0.01$; hazard ratio, 2.3 [Cox model]) and worsened lung function over time as measured by forced expiratory volume in 1 second ($N = 445$; $P = 0.001$ [generalized estimating equation]). We identified a SNP in *NLRC4*, p.(A929S), that was associated with a lower rate of *P. aeruginosa* colonization as part of a composite of rare variants ($N = 405$; $P = 0.045$; hazard ratio, 0.68 [Cox model]) and that was individually associated with protection from lung function decline ($P < 0.001$ [generalized estimating equation]). Rescue of the *NLRP3* knockout with the p.(Q705K) variant produced significantly more IL-1β in response to NLRP3 stimulation than rescue with the wild type ($P = 0.020$ [Student's t test]). We identified a subset of children with CF at higher risk of early lung disease progression. Knowledge of these genetic modifiers could guide therapies targeting inflammasome pathways.

Keywords: NLRP3; NLRC4; inflammasome; cystic fibrosis

Clinical Relevance

This research identifies a subset of children with cystic fibrosis who are at higher risk of early lung disease progression. Knowledge of these genetic modifiers could guide therapies targeting inflammasome pathways.

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The cystic fibrosis (CF) lung is susceptible to organisms that produce chronic infection, but the ensuing inflammatory response is often unable to eliminate the infection and is responsible for much of the pathologic damage (1–4). To date, there is no widely adopted antiinflammatory therapy for people with CF (5). Host-directed therapies, including corticosteroids (e.g., for maintenance and for pulmonary exacerbations) and nonsteroidal antiinflammatory drugs (e.g., high-dose ibuprofen for maintenance care), have been used to treat CF lung disease with some success, but overall progress in mitigating the inflammatory damage has been modest (6). Understanding which inflammatory processes are beneficial and which are harmful is critical to improving the efficacy of host-directed therapeutics in patients with CF lung disease.

Inflammasomes are cytoplasmic protein complexes that sense microbial patterns and oligomerize to recruit and cleave procaspase 1. Active caspase-1 initiates a downstream inflammatory response that includes the secretion of IL-1β, IL-18, and inflammatory eicosanoids as well as the formation of gasdermin pores in the cell membrane, resulting in a form of programmed cell death called pyroptosis and possibly leading to gasdermin-mediated bacterial killing (7–9). Activation of the canonical inflammasome pathway requires two signals (10). Signal 1 is usually mediated by TLR (Toll-like receptor)/NF-κB signaling and upregulates expression of pro–IL-1β. Signal 2 is inflammasome specific and is produced by a variety of pathogen-associated molecular patterns and damage-associated molecular patterns (11). The NLRC4 inflammasome recognizes intracellular flagellin and components of the bacterial type 3 secretion system (12, 13). The NLRP3 inflammasome is activated by a variety of mechanisms, including potassium efflux and mitochondrial reactive oxygen species generation (11, 14). *Pseudomonas* species can activate both the NLRC4 and the NLRP3 inflammasome, although the process is complex because bacterial exotoxins can also inhibit inflammasome activation (15, 16). Recent evidence suggests that in neutrophils, inflammasomes are constitutively active and require only activation of the TLR/NF-κB pathway (17). Although inflammasome activation can be protective, the inflammatory response can also be harmful to host tissues. Understanding this balance in CF is crucial to the understanding risks and benefits of potential therapeutic agents.

Inflammasome-mediated inflammation leads to lung injury in several mouse models (8, 18, 19). In mice with CF, inflammasome activity is dysregulated, and IL-1β contributes to lung pathology (20). The use of a small molecule inhibitor of NLRP3 in mice significantly reduced levels of IL-1β in the lung and improved clearance of *P. aeruginosa* (17). In a mouse model of *P. aeruginosa* pneumonia, macrophage depletion or deletion of *NLRC4* enhanced bacterial clearance and reduced pathologic damage to the lung (21). *Aspergillus* species are commonly isolated from the lungs of patients with CF and may be associated with worsened outcomes (22). *Aspergillus* species induce inflammasome activation in mice (20), and the suppression of NLRP3 reduces lung damage in a mouse model of invasive pulmonary aspergillosis (23).

In humans, genetic variants in inflammasomes, particularly in *NLRP3*, are associated with a range of autoinflammatory conditions collectively referred to as the cryopyrin-associated periodic syndromes (CAPSs) (24). The CAPS disorders are caused by gain-of-function mutations in *NLRP3* that display variable penetrance and are characterized primarily by symptoms of an innate immune response, including fever, rash, and systemic inflammation, without evidence of autoantibodies or antigen-specific T cells (25). Less is known about inflammasome activity in humans with CF. BAL samples from people with CF have higher levels of proinflammatory cytokines, including IL-1β, than BAL samples from age-matched healthy control subjects (26), and higher IL-1β levels are associated with worse clinical outcomes, including those for lung function (17). In human monocytes and airway epithelia, CF-associated mutations result in dysregulation of epithelial sodium channels, leading to NLRP3-mediated inflammation (27). In a case series of CF lung explants, *P. aeruginosa* from segments of the lung with radiographic evidence of increased lung damage were found to hypersecrete the type 3 secretion system components (28). *P. aeruginosa* colonization is common in children with CF and is associated with poor outcomes (29, 30), although whether chronic *P. aeruginosa* clinical isolates are able to activate the inflammasome in adults is not clear (31).

In the following study, we examine candidate genetic variants in the *NLRP3* and *NLRC4* inflammasome pathway for association with chronic *P. aeruginosa* infection and lung function in a large cohort of children with CF. We identify two variants in *NLRP3* and one variant in *NLRC4* with significant associations with clinical outcomes in this cohort. We then study the mechanistic impact of these variants in human macrophage–like cells to test our hypothesis that hyperinflammatory inflammasome variants are harmful in CF and that hypoinflammatory variants are protective.

Methods

EPIC Observational Study Cohort

The EPIC (Early *Pseudomonas* Infection Control) Observational Cohort consists of 1,794 children with CF enrolled at 59 recruitment centers in the United States (32). Two *P. aeruginosa* entry criteria were used: criterion 1 includes children who had never had a *P. aeruginosa*–positive respiratory culture result ("never *P. aeruginosa*" group). Criterion 2 includes children who had had a positive *P. aeruginosa* respiratory culture result in the past but who had had negative respiratory culture results for the two years preceding enrollment ("past *P. aeruginosa*" group) (32).

Statistical Analysis

Outcomes included age of onset of chronic *P. aeruginosa* (33, 34) and longitudinal forced expiratory volume in 1 second (FEV_1) based on Global Health Initiative prediction equations. We performed association tests for those with the wild type (WT) versus those who were variant carriers by using the Cox model for the age of chronic *P. aeruginosa* onset and by using generalized estimating equations for differences in longitudinal FEV_1. Analyses were performed separately by using enrollment criteria because of the high potential for criteria to differentially select for underlying genetic factors and were performed by using both groups combined when no difference between groups was found.

Genotyping and SNP Selection

Genotyping was performed for EPIC individuals in two phases; whole exome sequencing ($n = 189$) and exome chip sequencing ($N = 989$) were performed during the Exome Sequencing Project (34), which were followed by a second phase of targeted sequencing using single-molecule molecular inversion probes for candidate genes ($n = 1{,}201$ with complete data). The *NLRP3* variants p.(V198M) and p.(Q705K) were selected on the basis of existing data suggesting their association with the autoinflammatory condition CAPS (24, 35). For *NLRC4*, pathway analysis using phase 1 data was performed with a variable comprising the presence or absence of the uncommon allele of any of 14 variants in the NLRC4 pathway (*NAIP*, *PYCARD*, *NLRC4*, and *CASP1* [13]; *see* Table E1 in the data supplement).

Cell Lines, Genetic Manipulation, and Inflammasome Assays

We used CRISPR/Cas9 to generate *NLRP3* and *NLRC4* knockouts in the human monocyte–like cell lines THP-1 and U937. Gene rescue ("knockin") was performed by reintroducing either the WT or variant version of the knocked-out gene. Western blotting was performed to quantify protein expression (Figure E1). For inflammasome assays, cell lines were plated in PMA (50 ng/ul) for 48 hours, washed with Hanks' balanced salt solution, and rested overnight in media (RPMI with 10% FBS). For NLRP3-specific stimulation, cells were incubated with nigericin (10 μm) for 4 hours after 2-hour prestimulation with LPS (0.1 ng/ml). For NLRC4-specific stimulation, a fusion of *Burkholderia thailandensis* needle protein with the *Bacillus anthracis* lethal factor was cytoplasmically delivered via the *B. anthracis* protective antigen (gifts of Russel Vance, University of California Berkeley) for 4 hours. Supernatant cytokines were measured by using a sandwich ELISA (R&D Systems).

***P.aeruginosa* macrophage assays.** The *P. aeruginosa* laboratory strain O1 with a multiplicity of infection of 5 (PAO1) (gift of Matthew Parsek, University of Washington) or a clinical isolate (gift of Pradeep Singh, University of Washington) was picked from a single colony on a freshly streaked Luria broth plate and grown overnight in Luria broth media. The overnight culture was back-diluted to an optical density of 0.01 and grown to log-phase, at which point cell lines were exposed to bacteria at the indicated multiplicity of infection.

For further details, see the data supplement.

Results

Association of NLRP3 Variants with CF Outcomes in the EPIC Cohort

To examine whether inflammasome variants are associated with clinical outcomes in CF, we studied genetic variants in *NLRP3* and the *NLRC4* pathway for association with clinical outcomes in the EPIC cohort. Enrolled children either had never had a an airway (e.g., oropharyngeal or sputum) culture positive for *P. aeruginosa* (never *P. aeruginosa* group) or had had an airway culture positive for *P. aeruginosa* in the past but had cleared their cultures for the two years preceding enrollment (past *P. aeruginosa* group). We used a hypothesis-driven approach to determine whether SNPs with suspected hyperinflammatory phenotypes influenced clinical outcomes in CF. Of the CAPS-associated rare variants we included in our analysis (Table E2), two were present in the EPIC cohort: p.(Q705K) and p.(V198M). The p.(Q705K) (rs35829419) variant was associated with a higher rate of chronic *P. aeruginosa* infection ($N = 609$; $P = 0.01$; hazard ratio [HR], 2.3 [at age 10, Cox model]) in children from the past *P. aeruginosa* enrollment group (Figure 1A). The p.(Q705K) variant was also associated with worsened lung function over time as measured by the FEV_1 ($N = 445$; $P = 0.001$ [generalized estimating equation]) in enrollees from the past *P. aeruginosa* group (Figure 1B). A second nonsynonymous rare variant in *NLRP3*, p.(V198M) (rs121908147), was not associated with rate of *P. aeruginosa* colonization but showed a trend toward a protective effect ($N = 1{,}348$; $P = 0.07$; HR, 0.50 [Cox model]) in the combined enrollment group averaged over all ages (Figure 1C). The p.(V198M) SNP was not associated with lung function over time in the EPIC cohort (data not shown).

Association of an NLRC4 Rare Variant with CF Outcomes in the EPIC Cohort

To further understand the relationship between the inflammasome and CF outcomes, we investigated a group of variants in the *NLRC4* pathway. Most variants were synonymous and had a minor allele frequency less than 5% in the EPIC cohort (Table E1); they included polymorphisms in *CASP1*, *NAIP*, *NLRC4*, and *ASC*. To increase our statistical power for the initial analysis, we collapsed all variants into a single variable on the basis of the presence of any single variant. The presence of any of 14 rare variants in the *NLRC4* pathway was associated with a delayed time to chronic *P. aeruginosa* infection ($P = 0.045$; HR, 0.68 [Cox model]; $N = 405$; adjusted for age at enrollment and CFTR residual function; Figure 2A) among individuals with a high-risk *CAV2* genotype (WT at locus rs8940). The sample size was large enough for individual variant testing of the FEV_1 outcome for the A929S *NLRC4* variant (rs61754192). We examined the *NLRC4* A929S variant for association with lung function among EPIC enrollees from the never *P. aeruginosa* group and found that the presence of this variant was associated with higher FEV_1 percentiles (difference $= +8.2\%$; $P < 0.001$ [generalize estimating equation]) than those of CF children without a pathway variant (Figure 2B).

Generation of Inflammasome Knockouts Using CRISPR/Cas9

To study the impact of inflammasome genetic variants on macrophage inflammatory responses, we first generated *NLRP3* and *NLRC4* knockouts in human macrophage–like cells (36, 37) (U937 for the *NLRP3* knockout; U937 and THP-1 for the *NLRC4* knockout). A restriction fragment-length polymorphism was used to select for successful targeting of *NLRP3* (Figure 3A) and *NLRC4* (Figure 3B) (38). After stimulation with nigericin, an NLRP3-specific potassium ionophore (39), the *NLRP3*-knockout cell line showed a dramatic reduction in IL-1β production relative to the WT cell line or to the *NLRC4*-knockout cell line (Figure 3C). Similarly, *NLRC4*-knockout U937 cells (data shown) and THP-1 cells (data not shown) showed a dramatic reduction in the IL-1β response to needle protein (Figure 3C). IL-1β was also reduced in the *NLRP3*-knockout cells relative to the WT cells after stimulation with the *NLRC4*-specific *B. thailandensis* needle protein, but this was not shown to the same degree as with the NLRP3-specific stimulus. Levels of IL-6 production in response to LPS were not significantly

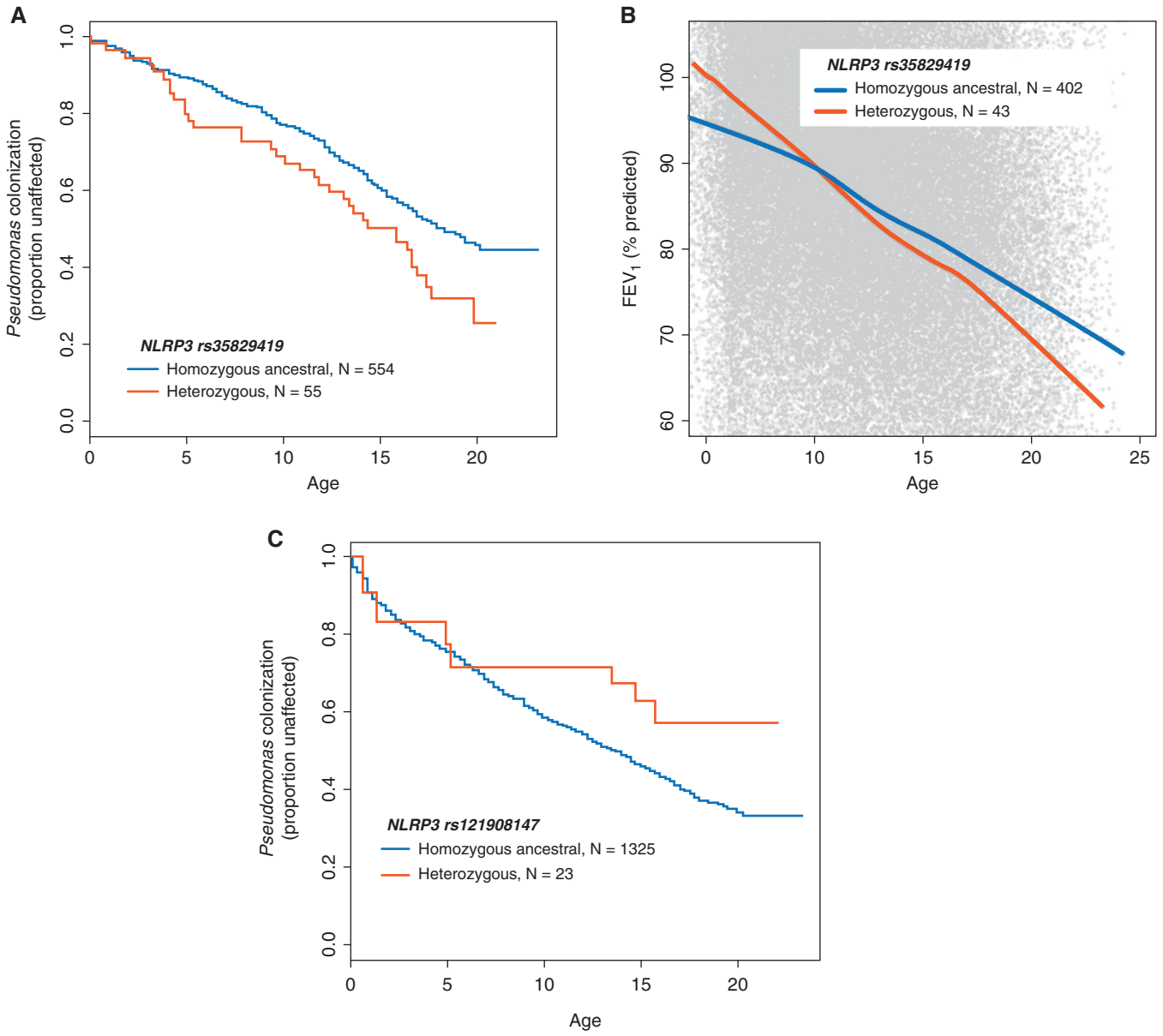

Figure 1. The p.(Q705K) and p.(V198M) *NLRP3* variants and clinical outcomes in the EPIC (Early *Pseudomonas* Infection Control) cohort. (*A*) Proportion of individuals in the EPIC cohort without *P. aeruginosa* in the sputum by age based on the rs35829419 [p.(Q705K)] genotype (N = 609; P = 0.01; hazard ratio [HR], 2.3 [at age 10, Cox model, "past *P. aeruginosa*" enrollees only]). (*B*) The forced expiratory volume in 1 second (FEV_1) percent predicted relative to the Global Lung Initiative standard by age based on the rs35829419 genotype (N = 445; P = 0.001 [generalized estimating equation, past *P. aeruginosa* enrollees only]). (*C*) Proportion of individuals in the EPIC cohort without *P. aeruginosa* in the sputum by age based on the rs121908147 [p.(V198M)] genotype (N = 1,348; P = 0.07; HR, 0.50 [Cox model]). The model was adjusted for age at enrollment, CFTR residual function, and *CAV2* rs8940 status. *CAV2* = caveolin 2; CFTR = cystic fibrosis transmembrane conductance regulator; *NLRP3* = NLR family pyrin domain containing 3; *P. aeruginosa* = *Pseudomonas aeruginosa*.

different in the knockout lines relative to the WT U937 line (Figure 3D).

NLRP3 Rare Variants and Inflammatory Responses in Human Macrophage–like Cell Lines

To understand the mechanism by which rare *NLRP3* variants influence CF outcomes, we generated a gene rescue of either WT *NLRP3* or the p.(V198M) or p.(Q705K) *NLRP3* variants in the U937 *NLRP3*-knockout cells. IL-1β and IL-6 production increased relative to that in the knockout cells after rescue with WT *NLRP3* (Figure 4A, 4B). Relative to WT rescue, rescue with the p.(Q705K) variant resulted in a mildly greater (but significant) level of IL-1β production after stimulation with NLRP3-specific nigericin (P = 0.004), but this was not shown after NLRC4-specific stimulation or infection with PAO1 (Figure 4C). IL-6 levels were similarly increased after nigericin stimulation (P = 0.04) but were not

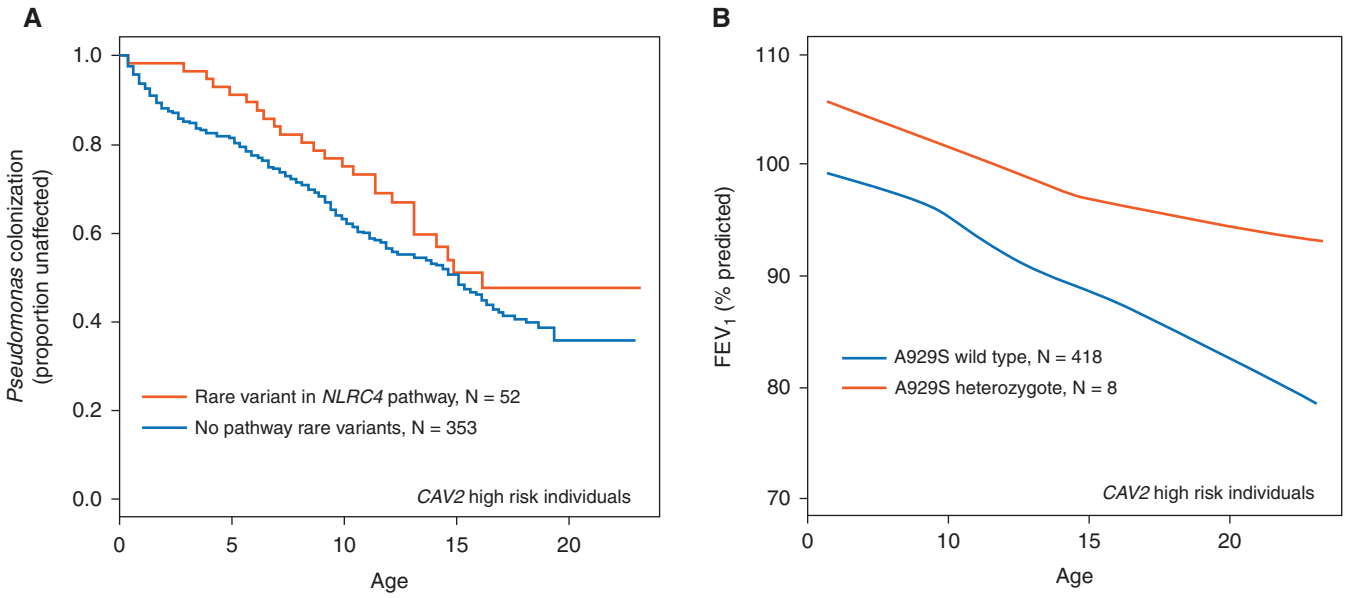

Figure 2. The p.(A929S) *NLRC4* variant and clinical outcomes in the EPIC cohort. (*A*) Proportion of individuals without *P. aeruginosa* in the sputum by age based on a yes/no composite for the presence of any one of 14 rare nonsynonymous variants in *NLRC4*, *NAIP*, *CASP1*, and *ASC* ($N = 405$; $P = 0.045$; HR, 0.68 [Cox model]). Individuals were from the "never *P. aeruginosa*" enrollment group and had the high-risk *CAV2* genotype (rs8940, wild type [WT]). (*B*) The FEV_1 percent predicted relative to the Global Lung Initiative standard by age based on the rs61754192 [p.(A929S)] genotype (difference = +8.2%; $P < 0.001$ [generalized estimating equation]). *ASC* = apoptosis-associated speck-like protein containing a CARD; *NAIP* = NLR family apoptosis inhibitory protein; *NLRC4* = NLR family CARD domain containing 4.

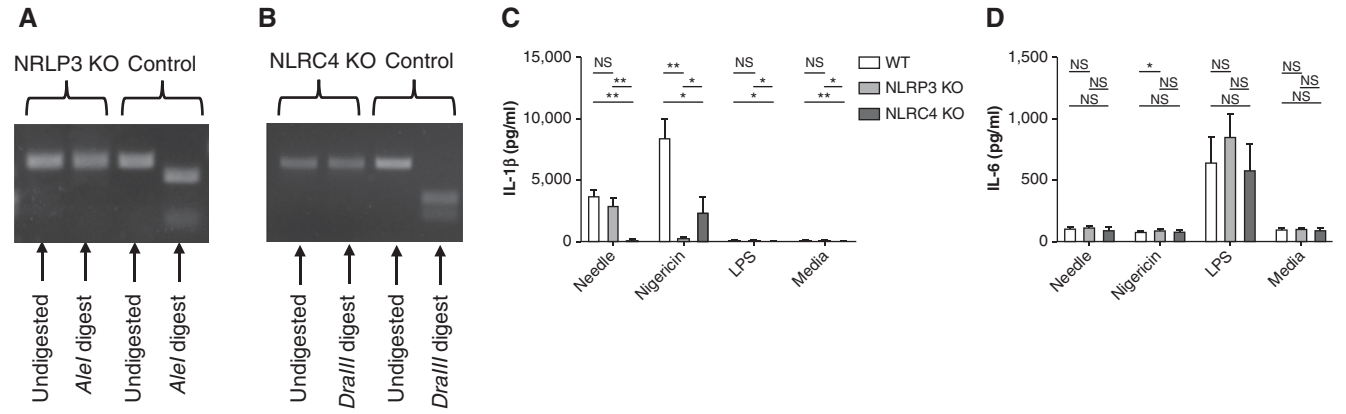

Figure 3. Inflammasome KOs in human monocyte-like cells. The restriction fragment-length polymorphism of CRISPR/Cas9 targets in (*A*) *NLRP3* and (*B*) *NLRC4* is shown. Control lines were generated by using a nontargeting guide RNA but otherwise were exposed to the same CRISPR/Cas9 delivery system. A short region of DNA containing the target site was PCR amplified and exposed to the indicated restriction endonuclease. The (*C*) IL-1β and (*D*) IL-6 responses to *Burkholderia thailandensis* needle protein (Needle), nigericin (10 μM), LPS (50 ng/ml), or Media. The WT and KO variants were generated in U937 cells. Needle was administered at 8 ng/ml in conjunction with 16 ng/ml *Bacillus anthracis* protective antigen. $^*P < 0.05$ and $^{**}P < 0.005$ (Student's *t* test). KO = knockout; Media = media control (RPMI + 10% FBS); NS = not significant.

increased in other conditions (Figure 4D). The association with nigericin-induced IL-1β was reproducible across three experiments, each with six replicates, as was the lack of association in needle protein– or PAO1-induced IL-1β. In addition to evaluating responses to PAO1, we also evaluated cytokine responses to a *P. aeruginosa* clinical isolate (28). The response elicited by the clinical isolate was similar to that elicited by PAO1 (Figures E2A and E2B). In the experiment shown, the response elicited by both strains of *P. aeruginosa* was significantly greater in the p.(Q705K) variant–rescue cell line than in the WT-rescue cell line (Figure E2A). However, this difference between the WT and p.(Q705K) cell lines was not consistently observed with repeat experiments.

To determine whether the observed effect on IL-1β between the WT cell line and the p.(Q705K)-variant cell line was due to a difference in protein expression or protein

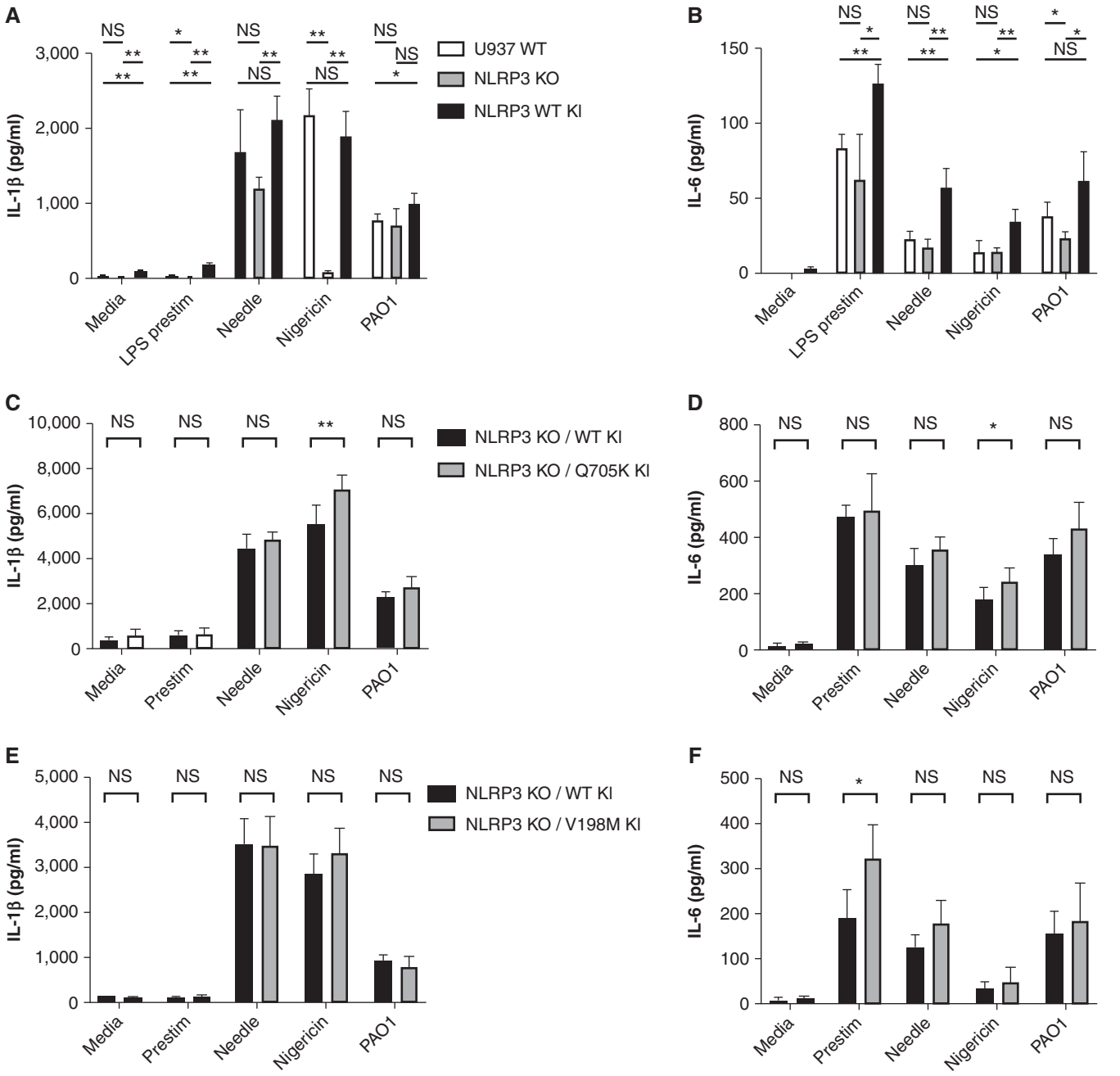

Figure 4. *NLRP3* gene rescue and rare-variant inflammatory responses to inflammasome stimuli. The (*A*) IL-1β and (*B*) IL-6 responses to stimuli in U937 WT cells, NLRP3-KO cells, and NLRP3-KO cells after stable replacement of the knocked-out gene with WT human *NLRP3* (NLRP3 WT knockin [KI]). (*B*) IL-6 concentrations were below detectable limits for the WT and NLRP3-KO lines. The (*C*) IL-1β and (*D*) IL-6 responses to stimuli in NLRP3-KO cells rescued with either the WT or the p.(Q705K) variant of human *NLRP3* (NLRP3 KO/Q705K KI). The (*E*) IL-1β and (*F*) IL-6 responses to stimuli in NLRP3-KO cells rescued with either WT or the p.(V198M) variant of human *NLRP3* (NLRP3 KO/V198M KI). All stimulation conditions other than the media control received LPS prestim. Nigericin at a 10uM concentration was used. Needle was administered at 8 ng/ml in conjunction with 16 ng/ml of *B. anthracis* protective antigen. $^{*}P < 0.05$ and $^{**}P < 0.005$ (Student's *t* test). LPS prestim = prestimulation with LPS at 0.1 ng/ml; PAO1 = *P. aeruginosa* laboratory strain O1 with a multiplicity of infection of 5.

function, we compared the relative expression of NLRP3 in the knockin lines by using Western blotting (Figures E1A and E1B). The WT-knockin cell line had relatively more expression than either variant-knockin cell line, suggesting that the increase in IL-1β in the p.(Q705K) variant was due to the change in protein function rather than degree of expression. The normalization of IL-1β concentration to protein expression increased the difference between nigericin-mediated IL-1β in the

WT-rescue cell line compared with the p.(Q705K)-variant cell line ($P = 0.00003$) and also showed increased IL-1β in the variant relative to the WT cell line after stimulation with media ($P = 0.04$), needle protein ($P = 0.0002$), and PAO1 ($P = 0.002$) for the experiment shown in Figure 4C (normalized data not shown). IL-6 was also significantly greater in the p.(Q705K)-variant cell line than in the WT cell line after stimulation with needle protein ($P = 0.002$), nigericin ($P = 0.003$), and PAO1 ($P = 0.003$) for the experiment shown in Figure 4D after normalization (normalized data not shown).

Gene rescue with the p.(V198M) variant did not yield a consistent association with the various stimulation conditions and IL-1β production relative to the WT (Figure 4E). Although IL-6 production was significantly greater with low-dose LPS in the experiment shown (Figure 4F), this association was not reproducible across multiple experiments. We also compared cytokine responses between the WT- and p.(V198M)-rescue lines by using a *P. aeruginosa* clinical isolate (Figures E2C and E2D). In these experiments, neither the clinical isolate nor PAO1 elicited a different IL-1β response between the WT- and p.(V198M)-rescue lines.

When normalized for protein expression based on Western blotting (Figures E1A and E1B), IL-1β production was greater with the p.(V198M) variant than with the WT rescue for media alone ($P = 0.0003$), LPS prestimulation ($P = 0.4$), needle protein ($P = 0.009$), and nigericin ($P = 0.0005$) but was not greater with PAO1 in the experiment shown in Figure 4E (normalized data not shown). IL-6 production remained significantly greater with low-dose LPS and became significant for needle protein stimulation ($P = 0.002$) for the experiment shown in Figure 4F (normalized data not shown).

The Rare NLRC4 A929S Variant Was Not Associated with Cytokine Responses in Human Macrophage–like Cell Lines

We took a similar approach to study the mechanism by which the *NLRC4* p.(A929S) variant influences the time to *Pseudomonas* infection acquisition and lung function in children with CF. We introduced either the WT or the p.(A929S) variant into *NLRC4*-knockout macrophage-like cell lines. Replacement of the *NLRC4* knockout with either WT or p.(A929S) *NRLC4* increased IL-1β production and, to a lesser extent, IL-6, in response to the intracellular needle protein (Figure 5A, 5B). However, we did not observe a significant difference in the cytokine response between the WT and the p.(A929S) variant across a range of needle protein doses, nor did we observe significant differences in response to PAO1 (Figure 5C) or a *P. aeruginosa* clinical isolate (Figures E2E and E2F). Although the media control showed greater IL-1β in the p.(A929S)-variant cell line (Figure E2E), this was not consistent across repeat experiments. Western blotting of NLRC4 expression between the two knockin lines showed a minimal difference in protein expression between the WT and p.(A929S)-variant cell lines (Figures E1C and E1D).

In summary, we identified three inflammasome nonsynonymous variants with relevance to the clinical outcome in the EPIC cohort; of these, the p.(Q705K) *NLRP3* variant showed a consistent hyperinflammatory phenotype relative to WT *NLRP3* in human macrophage–like cells.

Discussion

We identified a gain-of-function variant, p.(Q705K), in *NLRP3* that was associated both with earlier onset of chronic *P. aeruginosa* in the airway secretions of children with CF and with an increased rate of lung function decline. The p.(Q705K) variant was also associated with increased levels of IL-1β production in macrophage-like cell lines after stimulation with the NLRP3-specific nigericin, but not after stimulation with NLRC4-specific needle protein, and has previously been reported to underlie the autoinflammatory NLRP3-mediated condition CAPS. The p.(V198M) *NLRP3* variant, although also associated with CAPSs, showed a trend toward protection from *P. aeruginosa* acquisition in children with CF and was not associated with an inflammatory phenotype in our macrophage-like cells lines. Lastly, the p.(A929S) *NLRC4* variant, which to our knowledge does not have a previously described functional consequence, was protective against chronic *P. aeruginosa* infection with a composite of other variants in our cohort and was protective against lung function decline as an individual variant in the EPIC cohort but was not associated with inflammatory phenotype in our macrophage-like cell lines.

Previous studies have implied that increased lung damage and worse outcomes are due to higher IL-1β–mediated responses (17, 19, 20). We used genetic models with well-defined and clinically important criteria to study the associations between inflammasome genetics and outcomes in children. To our knowledge, this is the largest analysis of inflammasome genetics and clinical outcomes in CF to date. The use of a pediatric cohort was particularly relevant because an earlier age of *P. aeruginosa* onset has been shown to be strongly associated with the likelihood of severe lung disease later in life (30). The p.(Q705K) variant has been identified as relevant in other diseases aside from autoinflammatory conditions and has now been identified in CF. One recent study found an association between the *NLRP3* p.(Q705K) variant and poor outcomes in Ethiopian patients treated for tuberculosis (40). Another study evaluated the functional consequences of the p.(Q705K) *NLRP3* variant in macrophage-like cells (35). The authors transiently transfected THP-1 WT cells with either WT *NLRP3* or the p.(Q705K) variant and measured IL-1β output; they found a small but significant increase in IL-1β concentration in the p.(Q705K)-variant cells at baseline. Our molecular data support this result yet show that the phenotypic difference occurred after exposure to an *NLRP3*-specific stimulus rather than at baseline, which is an important and potentially clinically relevant distinction when considering the use of host-directed therapeutics targeting inflammatory pathways.

Our results with the p.(Q705K) variant support the hypothesis that inflammasome-mediated inflammation is harmful in CF and suggest that macrophages are an important mediator of this inflammation. The results of both our genetic and molecular studies with the p.(V198M) *NLRP3* variant, which is also associated with autoinflammatory CAPSs, are not consistent with the p.(Q705K) result. The genetic analysis shows a trend toward a protective effect, whereas the molecular analysis does not show a consistent hyper- or hypoinflammatory response in macrophages with the p.(V198M) variant. There are a few potential explanations for this inconsistency. Multiple SNPs in *NLRP3* are associated with CAPSs, many of which display variable penetrance (24). It is possible that the p.(V198M) mutation does not produce the same degree of inflammatory response in

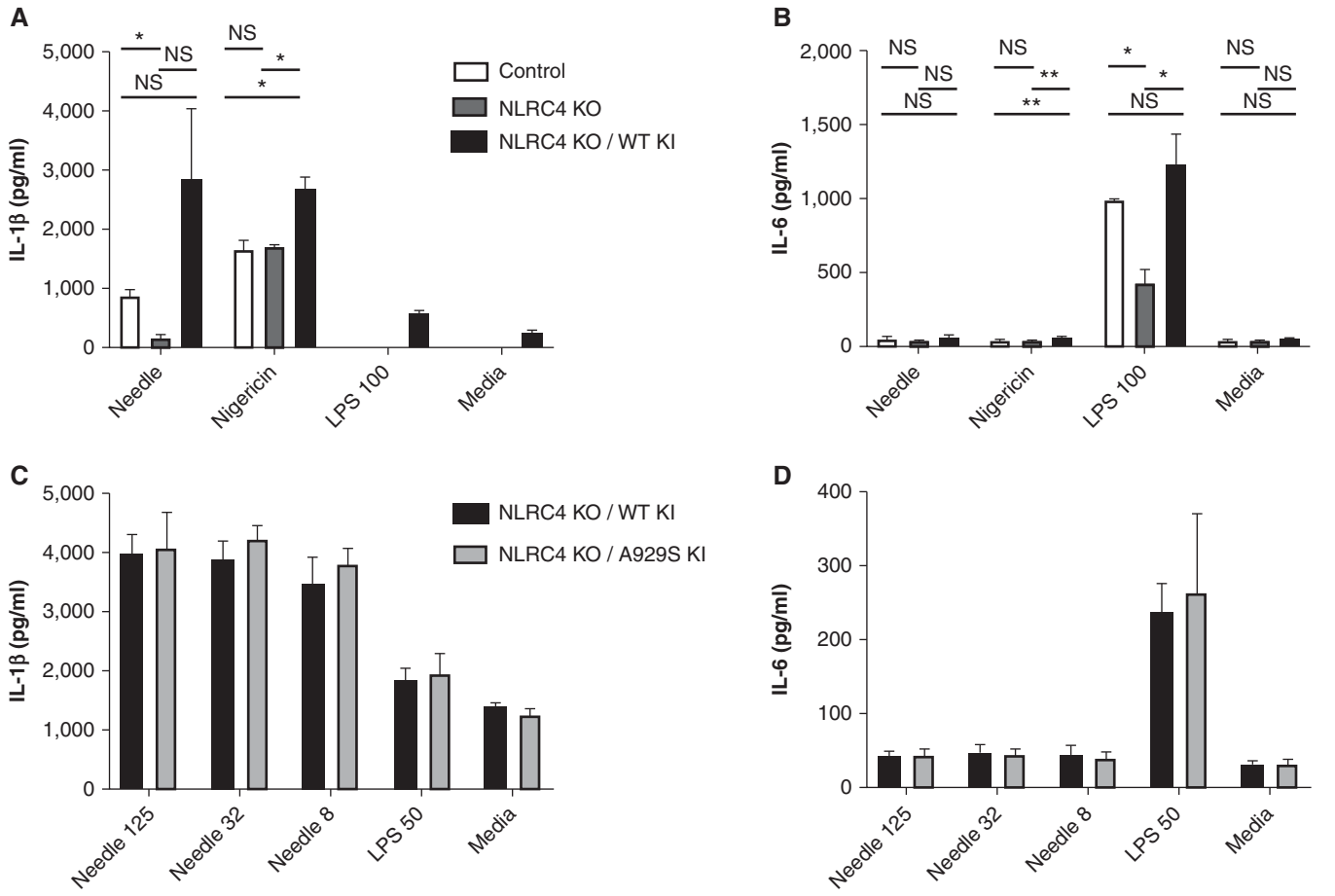

Figure 5. *NLRC4* gene rescue and rare-variant inflammatory responses to inflammasome stimuli. The (*A*) IL-1β and (*B*) IL-6 responses to stimuli in U937 CRISPR/Cas9 control cells, NLRC4-KO cells, and NLRC4-KO cells after stable replacement of the knocked-out gene with WT human *NLRC4* (NLRC4 WT KI). (*A*) The difference in IL-1β between the NLRC4-KO and NLRC4 WT–KI cells after Needle stimulation was borderline significant, with a *P* value of 0.052. The control and NLRC4-KO lines did not produce measurable IL-1β after stimulation with 100 ng/ml of LPS or Media. The (*C*) IL-1β and (*D*) IL-6 responses to stimuli in NLRC4-KO cells rescued with either WT or the p.(A929S) variant of human *NLRC4* (NLRC4 KO/A929S KI). No differences between the WT- and p.(A929S)-rescue cell lines were significant in *C* or *D*. LPS was provided at 50 ng/ml, Needle was administered in doses of 125, 32, or 8 ng/ml in conjunction with *B. anthracis* protective antigen administered at 250, 64, or 16 ng/ml, respectively. $^{*}P < 0.05$ and $^{**}P < 0.005$ (Student's *t* test). *B. anthracis* = *Bacillus anthracis*.

this population. Given the protective effect of the *NLRC4* p.(A929S) variant in the EPIC cohort, we predicted a hypoinflammatory response in macrophages, which we did not observe in our macrophage-like cells. It is possible that such a phenotype does exist in macrophages but that it exists under different conditions; we tested a range of NLRC4-specific conditions as well as broader inflammasome stimulation conditions but still did not observe an effect. It may also be the case that such a phenotype only becomes evident in the absence of a functioning CFTR. Exposure to neither a laboratory strain nor a clinical isolate of *P. aeruginosa* consistently produced a significant difference in IL-1β production between the WT and *NLRP3*-variant cell lines. In this context, the clinical significance of our significant finding with nigericin is less clear. It is similarly possible that such a phenotype requires the absence of a functioning CFTR or that overexpression of the inflammasome variants in our rescue lines obscured a difference that might be seen with endogenous levels of protein expression.

We focused primarily on the role of inflammasome genetic variants in macrophage-like cells. IL-1β secretion has been best characterized in monocytes and macrophages (41–44), and blood monocytes may be the primary producers of IL-1β (45). In CF, BAL samples show the expansion of a small macrophage population with surface markers that are more consistent with newly migrated monocytes than with alveolar macrophages (46). However, it is possible that macrophages are not the most important cell type responsible for our observations in the EPIC cohort. CF is primarily an epithelial-cell disorder, and the airway epithelia themselves could be an important mediator of inflammasome responses; NLRP3-specific inflammation in both monocytes and epithelial cells was shown to be enhanced by CF-mediated alterations in epithelial sodium channels (27). In a murine CF model, neutrophils can produce IL-1β in the absence of signal 2, and NLRP3 inhibition reduces airway inflammation (although the NLRP3

inhibition was not specific to only neutrophils) (17). Taken together, our results suggest an important role for inflammasomes in the CF lung acting via mechanisms that we are only starting to understand.

Our study has several limitations. Sequencing was limited to exons and then to a subset of SNPs within inflammasome genes of interest. Some of our variants of interest are rare, which limited our statistical power to identify associations with clinical outcomes. For this reason, we used a composite of *NLRC4* nonsynonymous variants. However, it is possible that variants could have opposing hyper- or hypoinflammatory phenotypes that we would miss by grouping them together. The use of two enrollment criteria, never *P. aeruginosa* and past *P. aeruginosa*, in the EPIC cohort has subtle but important implications for the generalizability of our results. When evaluating for genetic variants that influence the early acquisition of *P. aeruginosa*, enrolling older children who have never had *P. aeruginosa* by definition biases the cohort away from containing these variants. Such a bias could explain why we only saw the effect of the p.(Q705K) *NLRP3* variant in children from the past *P. aeruginosa* enrollment group. For *NLRC4*, our genetic analysis found an association only in the subgroup of children without a protective variant in *CAV2*, rs8940. The variant rs8940 is common in European Americans (minor allele frequency = 0.19) and was found by our group to be a disease modifier in children with CF (34). *CAV2* is clustered with *CAV1* on a 300-kb region on chromosome 7, with the function of the latter being far better characterized. In a rat bleomycin-induced idiopathic pulmonary fibrosis model, caveolin-1 overexpression was protective against IL-1β–mediated fibrogenesis of the lungs, suggesting a role for caveolin-1 in inflammasome regulation (47). Both caveolin-1 and caveolin-2 seem to be structural components of lipid rafts that are important for bacterial endocytosis, and caveolin-2 may act as a caveolin-1 antagonist (48); in mouse lung epithelial cells, caveolin-2 was found to be necessary for invasion by *P. aeruginosa* (49). The importance of the *CAV2* genotype for *NLRC4*-variant outcomes suggests the possibility of a mechanistic relationship between the two molecules that will be important for further study.

Both Food and Drug Administration–approved and experimental medications are currently available that alter inflammasome pathways and could provide benefit in the CF lung under specific circumstances of chronic airway infection and inflammation. These therapies include IL-1β antagonism (anakinra) (20), IL-18 neutralization (50), and eicosanoid pathway inhibition (ibuprofen) (6). Our work provides evidence that inflammasome genetics influences inflammatory responses in the CF lung in humans and specifically supports the hypothesis that hyperinflammatory variants are harmful to the CF lung. We also characterized a genetic variant whose carriers are likely at a higher risk of lung damage and who may benefit the most from host-directed therapies. The recognition of host genetic variants outside of *CFTR* that alter inflammatory responses allows for the tailoring of host-directed therapies for subpopulations of individuals with CF who will receive the most benefit from the treatment. ■

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