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The NLRP3 Inflammasome Is Differentially Activated by Pneumolysin Variants and Contributes to Host Defense in Pneumococcal Pneumonia

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Streptococcus pneumoniae is a leading cause of pneumonia, meningitis, and sepsis. Pneumococci can be divided into >90 serotypes that show differences in the pathogenicity and invasiveness. We tested the hypotheses that the innate immune inflammasome pathway is involved in fighting pneumococcal pneumonia and that some invasive pneumococcal types are not recognized by this pathway. We show that human and murine mononuclear cells responded to S. pneumoniae expressing hemolytic pneumolysin by producing IL-1β. This IL-1β production depended on the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome. Some serotype 1, serotype 8, and serotype 7F bacteria, which have previously been associated with increased invasiveness and with production of toxins with reduced hemolytic activity, or bacterial mutants lacking pneumolysin did not stimulate notable IL-1β production. We further found that NLRP3 was beneficial for mice during pneumonia caused by pneumococci expressing hemolytic pneumolysin and was involved in cytokine production and maintenance of the pulmonary microvascular barrier. Overall, the inflammasome pathway is protective in pneumonia caused by pneumococci expressing hemolytic toxin but is not activated by clinically important pneumococcal sequence types causing invasive disease. The study indicates that a virulence factor polymorphism may substantially affect the recognition of bacteria by the innate immune system. The Journal of Immunology, 2011, 187: 000–000.

S. pneumoniae is both a frequent colonizer of the upper respiratory tract and a leading cause of life-threatening infections such as pneumonia, meningitis, and sepsis. Pneumococci can be divided into >90 serotypes and even more sequence types. Serotypes 3, 6A, 6B, 19F, 23F, and others are associated with nasopharyngeal colonization, infections in patients with underlying diseases, and higher mortality when causing pneumonia. Serotypes 1, 7F, and 8 are frequently found in invasive diseases that, however, show lower case-fatality rates (1–4). Whereas the mechanistic basis for the varying pathogenicity of different pneumococcal types is poorly understood (5), the virulence of pneumococci in general depends on virulence factors such as the polysaccharide capsule and the cytolsin pneumolysin (PLY). PLY of most pneumococci forms large pores in membranes and is therefore cytotoxic (hemolytic) to mammalian cells (6, 7). Its critical role in causing disease has been demonstrated in various mouse models of pneumococcal pneumonia (8, 9). Hemolytic PLY is also a key factor in S. pneumoniae-induced lung injury (10, 11). Notably, PLY exists in 16 different protein variants. Some of these variants show reduced or abrogated hemolytic activities, including allele 5 PLY, which is frequently expressed in specific strains of serotypes 1 and 8 pneumococci (12–14).

The innate immune system recognizes microbial infections through, for example, the transmembrane TLRs and the cytosolic NOD-like receptors (NLRs) (15). After recognition of bacterial components, different TLRs activate signaling via the adapter MyD88 leading to expression of NF-κB-dependent proinflammatory genes including IL-1β. The production of IL-1β and related cytokines such as IL-18, however, additionally requires a second signal provided by inflammasomes (16, 17). Inflammasomes are multiprotein complexes composed of NLRs, including NLR family, pyrin domain containing 3 (NLRP3), or by the...
HIN200 protein AIM2, and additionally contain the adapter molecule ASC as well as caspase-1 for cleavage of pro–IL-1β and pro–IL-18. The NLRP3 inflammasome is activated by bacteria expressing pore-forming toxins or secretion systems (18–23). Moreover, it can be stimulated by necrotic cells and endogenous danger-associated molecules such as uric acid crystals and ATP, as well as by silica crystals, asbestos, and aluminum salts (21, 24–30).

TLR2, TLR4, TLR9, and NOD2 have been implicated in innate immune recognition of S. pneumoniae (31–35). However, whereas TLR2-, TLR4-, and TLR9-deficient mice showed no or only a moderately increased susceptibility, MyD88 knockout mice were highly susceptible toward pneumococcal infections (36). These different susceptibilities might be explained by TLR redundancies and by functional defects in signaling of the IL-1 and IL-18 receptors that also engage MyD88. Considering that inflammasomes are required for production of functional IL-1β and IL-18, we hypothesized that these multiprotein complexes are critically involved in innate immune responses to S. pneumoniae infection. We demonstrate that the NLRP3 inflammasome is differentially activated by pneumococcal sequence types expressing PLY variants and contributes to host protection in pneumococcal pneumonia.

Materials and Methods

Bacterial strains and pneumolysin

S. pneumoniae D39, D39Δply, D39 complementation mutant expressing domain 1–3 PLY, domain 4 PLY, or full-length PLY (domains 1–4) were used (see Table I). A version of D39 in which toxin production was interrupted by introduction of a translational stop codon at the 5’ end of the gene was made by first inserting an extra T base after base 6 in the ply gene by site-directed mutagenesis. The altered gene was then introduced into the chromosome as described above using Janus mutagenesis. Full-length or truncation variants of PLY were constructed by transformation with the shuttle vector pALY1 (37) containing the appropriate DNA insert. Hemolytic assay was performed as described previously (38). The PLY gene was amplified by PCR from the appropriate strain and cloned into PET33b (Novagen), which results in addition of poly-His tag to the N terminus of the protein. The resulting construct was transformed into Escherichia coli BL21 for expression following the manufacturer’s instructions. Cell extracts were prepared by sonication of cell pellets followed by clarification by centrifugation. PLY was purified by metal affinity purification of the His-tagged protein following the manufacturer’s instructions. Purity was confirmed by Coomassie blue staining of proteins separated by SDS-PAGE. Serotype 1 strains ST306 and ST217, serotype 7F strain ST191, and serotype 8 strain ST53 were from the National Reference Center for Streptococci (Aachen, Germany).

Bacterial infection

Bone marrow-derived macrophages (BMMs) were prepared from wild-type, NLRP3−/− (30), and ASC−/− mice on C57BL/6 backgrounds (B6) (39), as well as from wild-type, TLR2−/−, and TLR4−/− mice on a C57BL/10 background (B10) (40). BMMs were transfected with small interfering RNAs (siRNAs) as described previously (41). BMMs and human PBMCs were infected for 6 h (mRNA expression) or 16 h (protein production). mRNA expression was analyzed as previously described (22). Concentrations of IL-1β, IL-8, murine IL-1β, or murine TNF-α in cell supernatants were quantified by ELISA (BD Biosciences, eBioscience).

Murine pneumonia

Animal procedures were approved by local and institutional authorities (LAgGeSo Berlin, Charité Berlin). NLRP3-deficient female C57BL/6 mice and wild-type littermates were anesthetized by i.p. ketamine (1.6 mg) and xylazine (0.5 mg) and transanally inoculated with 7.5 × 107 or 5 × 106 CFU S. pneumoniae serotype 3 (PN36; NCTC7978) in 20 μl PBS as described previously (11, 42). Survival was recorded every 12 h for 240 h.

Cytokine and cell analysis from bronchoalveolar lavage

Bronchoalveolar lavage (BAL) samples were harvested after S. pneumoniae infection from ventilated lungs after flushing via the pulmonary artery (for 5 min with 37˚C sterile saline), and cytokines were quantified according to the manufacturer’s instructions (Biolplex; BioRad, Hercules, CA). BAL cells were counted by hemocytometer and differentiated by flow cytometry (FACSCalibur; BD, Heidelberg, Germany) analyzing forward and side scatter characteristics and expression of surface Ags CD45, Gr-1, and F4-80.

Determination of lung microvascular leakage

HSA was intravenously infused 1 h before BAL. HSA concentration in BAL and serum was measured by ELISA, and the HSA BAL/serum ratio was calculated (11).

Determination of bacterial load

Anesthetized (3.2 mg ketamine, 1.5 mg xylazine) mice were heparinized, tracheostomized, and ventilated in a closed chamber and perfused via the pulmonary artery with 37˚C sterile saline for 5 min. Lungs were homogenized at 4˚C. Serial dilutions of lung samples were plated on blood agar and incubated at 37˚C for 16 h before colonies were counted.

Data analysis

Data are expressed as mean ± SEM. Differences between two groups were analyzed by Mann–Whitney U test. Multiple-group comparisons were performed by one-way ANOVA, followed by Newman–Keuls post hoc test, and p values <0.05 were considered significant.

Results

Production of IL-1β in S. pneumoniae-infected cells

To test the hypothesis that inflammasomes are involved in sensing S. pneumoniae in host cells, we analyzed IL-1β production, which is known to depend on inflammasome activation. Murine BMMs were infected with S. pneumoniae serotype 2 expressing a pore-forming PLY (D39) or with S. pneumoniae mutants lacking PLY (D39Δply). D39 pneumococci were used because of the availability of different PLY mutants, which are not available for, for example, serotype 3 bacteria. Strong IL-1β mRNA expression was observed in BMMs infected with PLY-positive and PLY-negative pneumococci (Fig. 1A). In contrast, release of IL-1β protein was induced by wild-type S. pneumoniae but not by PLY-deficient pneumococci (Fig. 1B), as shown before in experiments with peritoneal macrophages (43). Similarly, IL-1β production depended on PLY expression also in human PBMCs and THP-1 monocytes when infected with the unencapsulated serotype 2 pneumococci RBS2 (Fig. 1C, 1D). These data suggest that S. pneumoniae activates an inflammasome-mediated IL-1β production in murine and human host cells depending on bacterial PLY.

Expression of full-length, pore-forming PLY is required to induce IL-1β production in S. pneumoniae-infected cells

Next, we further investigated the involvement of PLY in inducing IL-1β production in S. pneumoniae-infected host cells. First, we incubated PBMCs with PLY from serotype 2 bacteria or with different truncation variants of PLY, consisting of either domains 1–3 or domain 4. We found that only full-length, hemolytic PLY was capable of stimulating IL-1β production in the cells examined (Fig. 1E, 1F). Moreover, serotype 2 pneumococcal revertants expressing nonhemolytic, truncated PLY did not activate IL-1β release, whereas the related full-length PLY revertant bacteria stimulated a strong production of this cytokine (Fig. 1G, 1H). All pneumococcal mutants were equally capable of stimulating TNF-α production (data not shown). Thus, full-length PLY is required to activate IL-1β production in murine and human host cells.

Serotype 1 ST306 or serotype 8 ST53 pneumococci expressing a nonhemolytic PLY do not stimulate IL-1β production

S. pneumoniae serotype 1, sequence type (ST) 306 is one of the most prevalent invasive pneumococci (1). Recent studies indicated
that serotype 1 (ST306, ST228, ST617, and others) as well as some serotype 8 pneumococci (ST53, ST578, ST835, ST1110, ST1722) express an atypical allele 5 PLY, which lacks hemolytic activity (12). In agreement with the recent study, serotype 1 ST306 pneumococci as well as purified allele 5 PLY showed little hemolytic activity in our assays (Fig. 2A, 2B). We found that human PBMCs infected with S. pneumoniae serotype 1 ST306 did not produce IL-1β, whereas serotype 2 (D39) pneumococci evoked strong IL-1β production as well as murine IL-1β mRNA expression were measured. H. PBMCs were treated with allele 1 PLY (PLY A1) or allele 5 PLY (PLY A5). After 16 h, IL-1β production was measured by ELISA. Data shown are representatives of at least three independent experiments carried out in triplicate. n.d., not detectable.

PBMCs infected with S. pneumoniae serotype 1 ST306 did not produce IL-1β, whereas serotype 2 (D39) pneumococci evoked strong IL-1β release (Fig. 2C, Table I). Both serotypes 1 and 2 pneumococci stimulated a similar release of the inflammasome-independent chemokine IL-8 (Fig. 2D). Moreover, murine PBMCs infected with ST306 or ST53 pneumococci did not release IL-1β (Fig. 2E, 2F). ST191 serotype 7F bacteria that are known to express PLY with reduced hemolytic activity compared with D39 PLY (12) activated a strongly diminished but not blunted IL-1β production, whereas ST217 [serotype 1 bacteria that express a hemolytic PLY (12)] or D39 pneumococci were able to induce an intermediate or strong IL-1β secretion, respectively (Fig. 2E, 2F). All pneumococcal strains were capable of inducing IL-1β mRNA expression (Fig. 2G). Moreover, purified allele 5 PLY, in contrast to allele 1 PLY expressed in serotype 2 pneumococci D39, failed to activate production of IL-1β in PBMCs (Fig. 2H). Taken together, murine and human cells produced IL-1β when infected with pneumococci expressing hemolytic allele 1 PLY but not after infection with bacteria expressing allele 5 PLY such as S. pneumoniae serotype 1 ST306.
IL-1β production in S. pneumoniae-infected cells is dependent on TLR2 and on an NLRP3 inflammasome

To examine which host cell molecules are involved in IL-1β production in cells infected with S. pneumoniae expressing a hemolytic PLY, we made use of BMMs of different knockout mice. We found that release of IL-1β protein was partly reduced in TLR2−/− BMMs (Fig. 3A). Moreover, production of IL-1β was dependent on the inflammasome adapter ASC (Fig. 3B). BMMs lacking NLRP3 were partly defective in producing IL-1β but were fully capable of producing the inflammasome-independent cytokine TNF-α after pneumococcal infection (Fig. 3C–E). Similarly, siRNA-mediated knock-down of NLRP3 in human PBMCs reduced S. pneumoniae-stimulated IL-1β release (Fig. 3F, 3G). Activation of the NLRP3 inflammasome in unrelated inflammatory conditions has been shown to depend on K+ efflux (44). We found that inhibition of K+ efflux diminished the release of IL-1β after pneumococcal infection of human PBMCs (data not shown). As expected, caspase-1 inhibition also strongly reduced the S. pneumoniae-induced IL-1β production in the cells examined (data not shown). Collectively, these data suggest that stimulation of IL-1β production in S. pneumoniae-infected murine and human cells involves signals dependent on 1) TLR2 and on 2) the NLRP3 inflammasome.

NLRP3 contributes to host defense in pneumococcal pneumonia in mice

Next, we examined the role of NLRP3 in pneumococcal pneumonia. Because infection with S. pneumoniae serotype 2 (D39) leads to a sepsis-like disease in mice, we used serotype 3 pneumococci, which are known to cause pneumonia in mice and also express hemolytic PLY (45). Mortality of NLRP3-deficient mice with pneumococcal pneumonia was 70% 72 h postinfection (Fig. 4A–D). Similarly, siRNA-mediated knock-down of NLRP3 in human PBMCs reduced S. pneumoniae-stimulated IL-1β release (Fig. 3G). Production of IL-1β was measured by ELISA. Data shown are representatives of at least three independent experiments carried out in triplicate.
In contrast, mortality of wild-type mice was only 20% after 72 h. At this time point, a significant disadvantage in survival for the NLRP3-deficient mice was identified. Moreover, decline of body weight (Fig. 4B) and body temperature (Fig. 4C) in the first 48 h postinfection was aggravated in NLRP3-deficient compared with wild-type mice. However, mortality of wild-type mice increased in the following course of the disease, reaching 70% 5 d postinfection.

In line with inferior clinical outcome, bacterial clearance was moderately compromised in NLRP3-deficient mice compared with wild-type mice (Fig. 4D). Whole-lung bacterial counts were determined 48 h postinfection with $5 \times 10^6$ S. pneumoniae. Although not being significantly altered, lung bacterial loads were increased by trend in NLRP3-deficient mice compared with wild-type mice ($p = 0.0535; n = 10$).

Next, we analyzed the course of less severe pneumonia post-infection with LD 20% in wild-type mice (Fig. 4E). Albeit not being statistically significant, mortality was increased by trend (to 55%) in NLRP3-deficient mice (Fig. 4E). To address further the role of bacterial invasiveness and bacteremia for mortality in the current model, blood was drawn from the caudal vein 48 h post-infection while survival was monitored. More NLRP3$^{-/-}$ mice (3 of 9) had bacteremia compared with wild-type mice (1 of 10) 48 h postinfection (Fig. 4F). Notably, all NLRP3-deficient mice that were bacteremic 48 h postinfection died within further 48 h (Fig. 4E, 4F). Thus, bacteremia was directly associated with mortality and preceded death in NLRP3-deficient mice with pneumococcal pneumonia in the currently used model.

Serotype 1 ST306 bacteria did not cause pneumonia in our infection model. These bacteria were detectable 1 h postinfection in lungs of wild-type mice ($6 \times 10^5$ CFU) but not 48 h postinfection, suggesting complete elimination (data not shown).

Taken together, our observations suggest that NLRP3 is beneficial in the early course of pneumonia induced by hemolytic PLY-producing pneumococci.

Lung barrier integrity and lung inflammation in pneumococcal pneumonia

In wild-type mice infected with S. pneumoniae, HSA BAL/serum ratio was significantly elevated 24 h postinfection indicating increased lung permeability (Fig. 5). We further observed several-fold higher permeability 48 h postinfection than 24 h postinfection in wild-type mice (Fig. 5). Of note, lung hyperpermeability was increased in pneumonic NLRP3-deficient mice compared with pneumonic wild-type mice 24 h postinfection but not 48 h postinfection. These data demonstrate that NLRP3 deficiency was disadvantageous with respect to pulmonary microvascular barrier integrity in the early course of pneumococcal pneumonia in the currently used mouse model.

S. pneumoniae infection increased numbers of leukocytes in BAL fluid 48 h postinfection compared with uninfected mice (Fig. 6A), including neutrophils and macrophages (data not shown). NLRP3 deficiency did not significantly alter leukocyte numbers (Fig. 6A) or relative proportions of neutrophils and macrophages in BAL fluid (data not shown).

Also, pulmonary liberation of inflammatory cytokines within 24 h was evoked by transnasal infection with pneumococci (Fig. 6B). A reduction by trend of IL-1β and IL-18 levels as well as of KC and INF-γ in the lungs of NLRP3-deficient mice compared with wild-type mice was observed 24 h postinfection, which subsided during the later course of the disease. No differences in IL-6 or IL-10 levels (Fig. 6B) were observed 24 h postinfection.

To analyze direct correlations of bacterial load, clinical course, inflammatory parameters, and lung integrity 24 h postinfection in pneumonia in greater detail, we performed another experiment, infecting mice transnasally with LD 100%. Bacterial load was increased and body temperature was reduced in NLRP3$^{-/-}$ mice compared with infected wild-type mice (Supplemental Fig. 1A, 1B). Furthermore, dynamic lung compliance was reduced, reflecting increased lung edema in NLRP3$^{-/-}$ mice (Supplemental Fig. 1C). In accordance with the previous experiment (Fig. 5), cell count (Supplemental Fig. 1D) as well as IL-1β and KC levels (Supplemental Fig. 1E) in BAL fluid were not significantly altered by NLRP3 deficiency in mice with pneumonia.

![FIGURE 5](http://www.jimmunol.org/)  
NLRP3 deficiency increased lung permeability in pneumococcal pneumonia. Mice were intranasally infected with LD 70% of S. pneumoniae, and lung permeability was quantified by determining the HSA BAL/serum ratio after i.v. injection of HSA as described in Materials and Methods. Values are given as mean ± SEM; $n = 7–12$. *$p < 0.05$.

![FIGURE 6](http://www.jimmunol.org/)  
Lung permeability, leukocyte recruitment, and cytokine liberation in pneumococcal pneumonia. Mice were intranasally infected with S. pneumoniae. In BAL fluid, leukocyte recruitment (A) and cytokine liberation (B) were analyzed. Values are given as mean ± SEM; $n = 7–12$ (A) or $n = 7$ (B).
Collectively, these findings point toward an only moderate contribution of NLRP3 to the inflammatory cytokine and immune cell response in pneumococcal pneumonia in C57BL/6 mice, whereas NLRP3 contributed to bacterial defense and lung integrity.

Discussion

Our study identifies the NLRP3 inflammasome as an important mediator of innate immune responses to infections with *S. pneumoniae* expressing hemolytic PLY. The data show that NLRP3, ASC, and caspase-1 are involved in the IL-1β production of *S. pneumoniae*-infected cells. These findings are consistent with a report published during the period when our manuscript for this article was in the review process (46). However, NLRP3−/− cells were still capable of producing some IL-1β, whereas ASC−/− cells hardly released any IL-1β after pneumococcal infection. These data suggest that additional ASC-containing inflammasomes, possibly those involving AIM2 or other NLR proteins, contribute to production of IL-1 family cytokines in infections with *S. pneumoniae*.

Recent observations indicated a constitutive inflammasome activation in human PBMCs (47). In our experiments, however, only pneumococci expressing hemolytic PLY substantially activated release of mature IL-1β, whereas all pneumococci examined were capable of stimulating IL-1β mRNA. This suggests that PBMCs, in addition to their constitutive inflammasome activity, possess caspase-1 activity that is stimulated by pore-forming toxins.

NLRP3−/− mice demonstrated increased susceptibility to pneumococcal pneumonia compared with wild-type mice. NLRP3−/− mice showed a trend toward a reduced production of IL-1β and IL-18, and—possibly as a consequence—of KC and IFN-γ. We did, however, note a defect in leukocyte recruitment to the lungs of NLRP3−/− mice infected with *S. pneumoniae*, which is in line with findings obtained in a model of pneumococcal pneumonia caused by *Klebsiella pneumoniae* (48). NLRP3−/− mice, in contrast, showed enhanced permeability of the pulmonary endothelial–epithelial barrier, resulting in increased lung edema as reflected by reduction of dynamic lung compliance. In previous studies, we provided evidence that hemolytic PLY centrally contributes to lung permeability in the early course of pneumococcal pneumonia (11). As lung permeability is an important characteristic of life-threatening acute lung injury, the permeability increase may have been contributing to the survival reduction currently observed in NLRP3−/− mice in the early phase of pneumonia. Although the underlying mechanism of the protective effect of NLRP3 on pulmonary microvascular integrity needs further examination, it might at least partly depend on a direct inflammasome-regulated membrane repair function (49) and/or on IL-1β and IL-18, which have been implicated in the maintenance and repair of the lung endothelial–epithelial and gut epithelial barriers, respectively (50–53).

Our results add PLY to the list of bacterial toxins including listeriolysin O, streptolysin O, and α-hemolysin (19–23) that are known to activate the NLRP3 inflammasome. Importantly, our data additionally indicate that the inflammasome-stimulating activities of these toxins can differ within a bacterial species. This is, to our knowledge, the first demonstration indicating that a virulence factor polymorphism affects the recognition of a bacterium by the innate immune system. The nonhemolytic allele 5 PLY expressed in the majority of serotype 1 (e.g., ST306, ST228, ST617) and serotype 8 (e.g., ST53, ST758, ST835, ST1110, ST1722) isolates in Europe (12) did not stimulate IL-1β production. Moreover, certain sequence types of serotype 2 (ST74), 7F (ST191), 23F (ST40), and 27 (ST751) pneumococci have been indicated to express PLY variants that exhibit reduced pore-forming activities compared with those of allele 1 PLY expressed in, for example, the serotype 2 strain D39 and the serotype 3 strain PN36 (12–14). One might speculate that these bacteria are also less efficiently recognized by inflammasomes, an assumption that is supported by our data showing diminished IL-1β secretion after ST191 infection.

We think that the pore-forming activity of PLY is a “double-edged sword” for the bacteria providing capacity to damage host cells and tissues and to evade certain defense mechanisms but also triggering production of IL-1β and IL-18, which are key cytokines of the immune system (16, 52, 54). Pneumococci expressing nonhemolytic PLY might be less damaging and harmful for the host and are therefore associated with lower case-fatality rates. In contrast, they appear to be less efficiently recognized by the innate immune system, which might contribute to their capability to invade sterile compartments. However, allele 5 PLY might still possess some of the other toxin activities (e.g., complement activation) that help the bacteria to cause pulmonary disease in humans.

Serotype 1 ST306 pneumococci are among the most prevalent invasive pneumococci and are known for their lack of colonization of the nasopharynx, their association with parapneumonic effusion and empyema, and their association with disease outbreaks in small or closed communities (55). While these characteristics might partly be related to the expression of a non–pore-forming PLY, the overall characteristic of a given pneumococcal type (colonizer/cause of invasive disease) is certainly the sum of different features and clearly depends on variations of other bacterial factors including the capsular polysaccharides (56, 57).

Collectively, the NLRP3 inflammasome is critically involved in the immune response to *S. pneumoniae* expressing hemolytic PLY. This defense system, however, is less efficient in recognizing certain clinically important pneumococcal types that express nonhemolytic PLY. Although conclusive studies are warranted to increase the evidence, we speculate that the specific responsiveness of the NLRP3 inflammasome to PLY variants may contribute to differences in invasiveness and pathogenicity of different pneumococcal serotypes.

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Disclosures

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