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BRIEF REVIEWS

COPs and POPs: Modulators of Inflammasome Activity[1]

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***Inflammasomes represent[1,2] molecular platforms for the activation of inflammatory caspases and are essential for processing and secretion of the inflammatory cytokines IL-1β and IL-18. Multiple key proteins of inflammasomes contain caspase recruitment domains (CARDs) or pyrin domains (PYDs). Dissecting CARD- and PYD-mediated interactions substantially improved our understanding of the mechanisms by which these protein platforms are activated and emphasized their essential role during the inflammatory cytokine response. However, their precise regulation is still poorly understood. A family of small proteins that are composed of either a CARD or a PYD only emerged as important inflammasome regulators. These CARD-only proteins (COPs) and PYD-only proteins (POPs) function as endogenous dominant negative proteins that modulate the activity of inflammasomes in response to pathogen infection and tissue destruction. In this review we will summarize the most recent advances in the regulation of inflammasomes and highlight their importance for immunity and inflammatory disease. The Journal of Immunology,* 2007, 179: 7993–7998.**

Pathogens carry diverse virulence factors to support host colonization, replication, and spreading. However, eukaryotic hosts evolved mechanisms to rapidly and efficiently counter their destructive function. Germline-encoded pattern recognition receptors (PRRs)[3] provide a first line of defense. PRRs recognize damage-associated molecular patterns (DAMPs), which are either from pathogens (pathogen-associated molecular patterns or PAMPs) or are host-derived stress signals (stress or danger-associated molecular patterns or SAMPs) (1, 2). TLRs are well-established transmembrane PRRs that initiate inflammatory signals during host defense to aid pathogen clearance (3). Recognition of DAMPs by TLRs initiates a signaling cascade leading to the activation of MAPKs and proinflammatory transcription factors such as NF-κB and IRF-3/7. A complementary cytosolic PRR system is based on Nod-like receptors (NLRs) (also known as PAN, NALP, PYPAF, Nod, and Caterpiller), which are essential for the activation of inflammatory caspases and subsequent processing of their cytokine substrates (4–9). NLRs sense DAMPs with their leucine-rich region (LRR), resulting in receptor oligomerization followed by the recruitment of adaptor proteins that subsequently leads to the activation of the proinflammatory caspase-1. The inflammatory cytokines IL-1β, IL-18, and potentially IL-33 are synthesized as precursors, which can be processed by caspase-1 into the bioactive forms, although IL-33 is still controversial. This process occurs in specialized protein platforms referred to as inflammasomes (10).

Mechanisms regulating IL-1β and IL-18 function

IL-1β is a potent pyrogen that exerts its effect at the low pg/ml range, while higher concentrations can be lethal. Synthesis, activation, secretion, and activity of IL-1β and IL-18 are highly regulated (11). First, IL-1β transcripts are inducible and require an additional activation step to promote splicing of the pre-mRNA to prevent its degradation. In response to inflammatory signals, the transcripts are stabilized and translation is augmented. Second, IL-1β is synthesized as a precursor of 31 kDa and its activity depends on posttranslational processing to produce bioactive, secreted 17-kDa IL-1β. Third, two receptors exist for IL-1: IL-1 type I receptor (IL-1RI) and type II receptor (IL-1RII), the latter being a decoy receptor. IL-1 receptor antagonist is readily secreted due to its signal sequence and competes for receptor binding. Once IL-1β is bound to IL-1RI, a high-affinity ternary complex is formed with the IL-1R accessory protein to initiate signal transduction and activation of proinflammatory mediators. Fourth, processing of pro-IL-1β requires caspase-1, which is also regulated and will be discussed later.

Pro-IL-18 is constitutively expressed and requires caspase-1 for posttranslational processing to the active 18-kDa form. The IL-18 receptor complex is similar to the IL-1RI complex and requires IL-18R accessory protein signaling. Interaction with

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[3] Abbreviations used in this paper: PRR, pattern recognition receptor; CARD, caspase recruitment domain; COP, CARD-only protein; DAMP, damage-associated molecular pattern; FMF, familial Mediterranean fever; LRR, leucine-rich region; MDP, muramyl dipeptide; NLR, Nod-like receptor; NLRC, NLR containing a CARD; POP, PYD-only protein; PYD, pyrin domain; RA, rheumatoid arthritis.

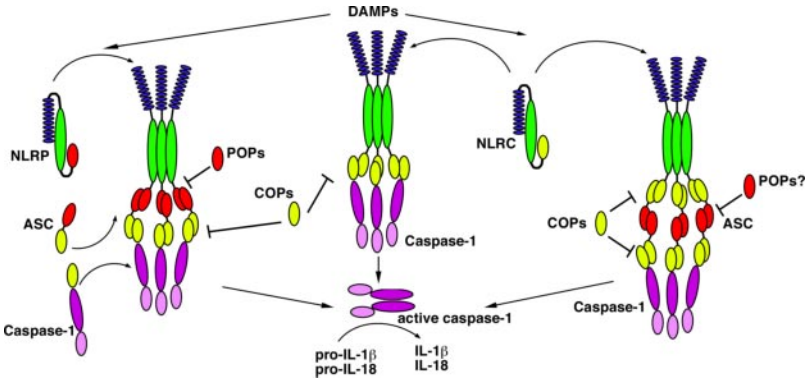

FIGURE 1. Inflammasomes. In response to pathogen or cellular stress, NLRs are activated followed by NTP-mediated oligomerization and recruitment of the caspase-1 adaptor ASC (for NLRP) or Rip2 (for NLRCs). Some NLRCs, such as IPAF, can directly recruit caspase-1. Because the assembly of inflammasomes is based upon CARD and PYD interactions, small single domain proteins (COPs and POPs) function to modulate these interactions. COPs impair CARD interactions of procaspase-1 with ASC and Rip2 and prevent the recruitment of procaspase-1 into inflammasomes. POPs modulate the interaction between ASC and NLRPs, thereby hindering the recruitment of ASC. Likely, POPs might also affect caspase-1 activation downstream of NLRCs that interact with ASC (64). ASC also self-associates, and enforced oligomerization of ASC is sufficient to cause activation of caspase-1 (65, 66). Presumably, the free CARD of a second ASC protein could then interact with procaspase-1.

the IL-18 binding protein prevents receptor activation and subsequent MAPK and NF-κB signaling (11).

Inflammasomes: molecular platforms for the activation of inflammatory caspases

The best-studied inflammatory caspase is caspase-1, while caspase-4 and caspase-5 are less well understood. Inflammatory stimuli induce activation of caspase-1, which is required for the processing and subsequent secretion of IL-1β and IL-18 (12–14). Several other proteases are also able to process pro-IL-1β; however, their physiological significance is poorly understood (11). Human inflammasomes cause activation of inflammatory caspases as well as activation of NF-κB, whereas murine inflammasomes appear to be specific for inflammatory caspases (15). For more than a decade activated monocytes and macrophages from caspase-1$^{-/-}$ mice have been known to be deficient in pro-IL-1β secretion (12). Nevertheless, the molecular mechanisms leading to the activation of this protease have been elucidated only recently. Caspase-1 activation is initiated by NTP-mediated oligomerization of NLR protein family members, as described for NALP1 (NLRP1) and NALP3 (cryopyrin, NLRP3) (16, 17). Oligomerization of NLRPs (NLRs containing a pyrin domain or PYD) appears to be a prerequisite for the recruitment of the adaptor protein ASC (TMS1, CARD5, PyCard) by PYD interactions. However, ASC can also be recruited to some NLRCs (NLRs containing a caspase recruitment domain or CARD) by CARD interactions. ASC recruitment of procaspase-1 by CARD interactions generates a protein complex known as an inflammasome. Subsequently, caspase-1 is activated by the induced proximity mechanism, similar to other apical caspases (Fig. 1) (18). Therefore, inflammasomes function analogously to other caspase-activating protein complexes, such as the caspase-2-activating PIDDosome, the caspase-8- and caspase-10 activating DISC, or the caspase-9-activating apoptosome. The essential role of ASC in inflammasome function has been emphasized in ASC$^{-/-}$ mice, which are deficient in caspase-1 activation and IL-1β and IL-18 secretion in response to Gram-negative and Gram-positive pathogens (15, 19). However, deficiency of NALP3 results in impaired caspase-1 activation only in response to Gram-positive bacteria, suggesting that the pathogenic stimulus is specific for a certain NLR (Fig. 2). Caspase-mediated cytokine processing and the subsequent initiation and amplification of the inflammatory host response are rigorously controlled. Caspase-1 activation can be regulated directly or within the context of inflammasomes through PYD and CARD interactions.

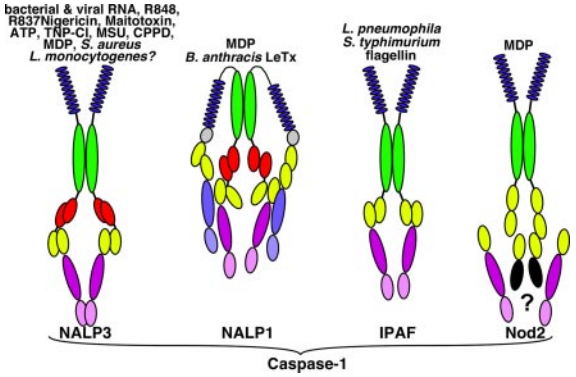

FIGURE 2. Activators of inflammasomes. A number of ligands for NALP3 have been identified, although it is elusive whether it directly interacts with ligands. Inducers for NALP3 include bacterial and viral RNA, the antiviral compounds R848 and R837, potassium ionophores (nigericin and maitotoxin), extracellular ATP in context with $P2X_7$, the contact hypersensitivity-inducing trinitrophenylchloride (TNP-Cl), the uric acid crystals monosodium urate (MSU) and calcium pyrophosphate dihydrate (CPPD) (which are deposited in the joints of patients with gout and pseudogout, respectively), muramyl peptide, and Gram-positive bacteria (*Staphyloccocus aureus* and potentially *Listeria monocytogenes*, although the latter one is still controversial, as a recent study failed to a find evidence for NALP3 involvement) (67–72). Nalp1b-containing inflammasomes respond to *Bacillus anthracis* lethal toxin (LeTx), while human NALP1 senses MDP (17, 73). NALP1 is unique and encodes also a FIIND-CARD, which recruits caspase-5 (shown in light blue) into the complex. IPAF directly binds caspase-1 in response to flagellin (15, 74, 75). Nod2, which is also unique by encoding a tandem CARD, recognizes MDP and is well established to recruit the adaptor Rip2 to activate NF-κB. Whether Rip2 is also required for caspase-1 activation still needs to be investigated. Yellow, CARD; red, PYD; green, NACHT; blue, LRRs; gray, FIIND; pink, caspase-1 domain; light blue, caspase-5 domain; black, Rip2 kinase domain.

CARD-only proteins (COPs) as modifiers of inflammasomes

To date, five proteins qualify to belong to the COP protein family: Iceberg, COP1/Pseudo-ICE, INCA, caspase-12s, and Nod2-S. With the initial characterization of Iceberg, a protein composed of only a CARD, a first glimpse into the possible mechanisms involved in inflammasome regulation was revealed (20, 21). Iceberg is highly similar to the CARD of caspase-1 (53% protein identity) and functions as a decoy protein by sequestering caspase-1 via CARD interaction, which prevents binding to activating adaptors. Expression of Iceberg in monocytes abrogates the secretion of IL-1β in response to LPS challenge. Because expression of Iceberg is elevated during inflammation, it might function as a negative feedback regulator to prevent systemic inflammation. The complexity of caspase-1 regulation was further emphasized with the identification of additional COPs that share a high degree of similarity to the CARD of caspase-1. COP1/Pseudo-ICE is 92% identical with the CARD of caspase-1 (21, 22). Similar to Iceberg, COP1/Pseudo-ICE interacts with the CARD of caspase-1 to prevent its activation. Recently INCA, which shares 81% protein identity with the CARD of caspase-1 and blocks its activation, has been discovered (23). Caspase-12 was recently identified as a negative regulator of the inflammatory cytokine response by binding to and inhibiting caspase-1 (24, 25). The pro-domain (a CARD) is sufficient for causing reduced cytokine secretion. Significantly, in the majority of the human population a single nucleotide polymorphism causes a premature stop that results in the expression of only the CARD, comprising essentially a COP (25). Nod2, an NLRC protein, functions as an activating adaptor for NF-κB and caspase-1 in response to muramyl dipeptide (MDP) recognition, although its precise role in the activation of caspase-1 is still controversial (26–28). A short variant of Nod2, Nod2-S, was identified, which encodes only the first CARD and functions similar to other COPs (29). Nod2-S does not interact with caspase-1 but with its adaptor Rip2 and competes with Nod2 for Rip2 binding, resulting in impaired caspase-1 activation. Consistently, Nod2-S expression is elevated in response to the anti-inflammatory cytokine IL-10 but is decreased in response to the proinflammatory cytokines TNF-α or IFN-γ (29).

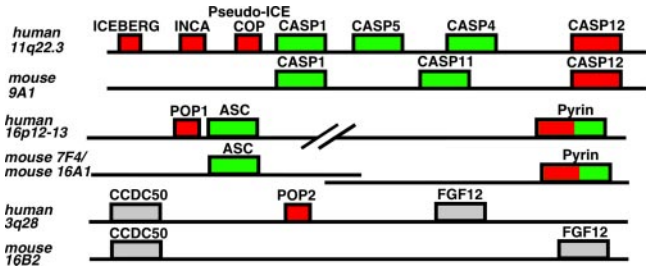

FIGURE 3. Chromosomal organization of COPs and POPs. Most COPs and POPs appear to have originated by gene duplication and cluster in close chromosomal proximity. COPs, including caspase-12 but with the exception of Nod2-S, cluster on chromosome 1q22.3 with caspase-1, caspase-4, and caspase-5. Caspase-1, caspase-11, and caspase-12 are similarly clustered on the syntenic mouse chromosome 9A1. The lack of COP-encoding genes in the mouse genome is evident. POP1 localizes in close proximity to ASC and pyrin on chromosome 16p12.1. ASC is found on the syntenic mouse chromosome 7F4 and pyrin is found on the syntenic chromosome 16A1. Striking is also the lack of POPs in the mouse genome. Potential activators and inhibitors are labeled in green and red, respectively.

COPs, except for Nod2-S, cluster with inflammatory caspases on chromosome 11q22.3 and originated by gene duplication from a common ancestor (Fig. 3). Caspase-1, caspase-11 (the murine ortholog of caspase-4), and caspase-12 are similarly clustered on the syntenic mouse chromosome 9A1. Significantly, there are no known COP-encoding genes in the mouse genome, suggesting an increased complexity in the regulation of inflammasomes in humans. COPs vary in their ability to bind to other CARD proteins. Only COP/Pseudo-ICE and Nod2-S interact with the CARD of the NF-κB-activating protein Rip2 and, as a consequence, these COPs can also modulate NF-κB activation in response to NLRC activation. Some COPs might also associate with NLRCs that interact with caspase-1 either directly or via Rip2. For NLRP activation, this type of regulation is indeed in place. Recruitment of the adaptor protein ASC to activated NLRP proteins via PYD interaction is disrupted by PYD-only proteins (POPs).

POPs as modifiers of inflammasomes

POP1 (ASC2, ASCI, ASCL, PYDC1) was identified in silico by its homology to the PYD of ASC (30, 31). POP1 shows 64% identity to the PYD of ASC and interacts with ASC in a PYD-dependent manner to displace other ASC-interacting proteins, thereby preventing ASC recruitment to NLRPs (30). A second POP (POP2) is also encoded in humans (32, 33). POP2 shows a lesser degree of homology to the PYD of ASC but is very similar to the PYD of several NLRPs, in particular NALP2 (NLRP2) and NALP7 (NLRP7), where it shows 69 and 50% protein similarity, respectively. POP2 binds to the PYD of NALP2 and prevents the recruitment of ASC and subsequently caspase-1 activation (32). Because POP2 can interact with the PYD of several NLRPs, it will likely also modulate caspase-1 activation downstream of other NLRPs. Contrary to POP1, POP2 interacts only weakly with the PYD of ASC, suggesting that different POPs modulate inflammasome formation in response to different pathological stimuli. POP1 and POP2 also prevent NF-κB activation (30, 33). POP1 originated from gene duplication of exon 1 of ASC on chromosome 16p12.1. This chromosomal locus also encodes pyrin, another ASC-binding protein (Fig. 3). POP2 is located on chromosome 3q28 with no recognizable PYD protein in close proximity. Reminiscent of COPs, neither POP1 nor POP2 is encoded in the mouse genome, although ASC and pyrin are both present on the syntenic mouse chromosomes 7F4 and 16A1, respectively. The genes flanking POP2 on human chromosome 3q28 are encoded on the syntenic mouse chromosome 16B2, indicating that POP-mediated regulation of inflammation also evolved into a more complex network in humans.

Caspase-8 activation is modulated by the DED-only proteins PEA-15 and FLIP, the latter being first identified in viruses. Viral FLIPs interfere with the binding of caspase-8 to its adaptor FADD and prevent virus-infected cells from undergoing apoptosis. Significantly, several poxvirus strains may also prevent the cellular inflammatory response to virus infection by modulating inflammasomes with viral POPs (34, 35). Viral POPs were identified in the myxoma virus, the Shope fibroma virus, the swine poxvirus, the Yaba-like disease virus, and the mule deer poxvirus. Poxviruses are well known to suppress the host immune response for efficient propagation by using immune evasive proteins targeting crucial pathways of the cellular and humoral host defense (36). The observation that poxviruses also

target inflammasomes further emphasizes the crucial importance of these complexes for host defense.

Other inflammasome modulators

Although pyrin does not share common domain architecture with NLRs, it has been shown to interact with ASC to form caspase-1-activating inflammasomes (37–40). However, contrary to being an inflammasome activator, pyrin has also been suggested to function as a negative regulator of inflammasomes, because targeted disruption of pyrin in mice resulted in enhanced secretion of IL-1β (41). Pyrin disrupts PYD interactions between NALP3 and ASC and is capable of interacting directly with other inflammasome components, resulting in caspase-1 inhibition (42–44). The SPRY domain of pyrin can bind to the caspase domain of caspase-1, to pro-IL-1β, and to the NACHT domains of several NLRPs. Hereditary mutations in the SPRY domain are linked to familial Mediterranean fever (FMF), the prototype of periodic fever disorders (45, 46). The heightened inflammatory response in FMF patients suggests that the mutation of pyrin impairs its negative regulator function of inflammasomes. However, if pyrin is indeed capable of forming an inflammasome with ASC, mutations in pyrin could render it constitutively active as has been proposed for NALP3 (40, 47). The precise role of pyrin in the regulation of inflammasomes is still controversial but could be influenced by the type of activation of inflammasomes.

The NLRP protein PYNOD (NLRP10) interacts with ASC but lacks the ligand-sensing LRRs (48). Although removal of the LRR from NLRs usually renders them constitutively active, PYNOD associates with ASC without recruiting and activating caspase-1. The NACHT domain mediates homotypic and heterotypic protein oligomerization (49). NTPase activity of NALP1 and NALP3 is essential for oligomerization, and mutation of the nucleotide-binding domain even disabled the disease-associated, constitutively active NALP3 mutations, suggesting that NLR oligomerization is crucial for inflammasome initiation (16, 17).

One of the first identified caspase-1 inhibitors was cytokine response modifier A (CrmA) from cowpox virus, which forms a complex with the catalytic center of caspase-1 (50). CrmA also inhibits several other proteases, including caspase-8 and granzyme B (51). Another serpin, PI-9 (proteinase inhibitor 9), functions as a caspase-1 inhibitor, and recently Bcl-2 and Bcl-x_L were also suggested to influence NALP1 inflammasomes (52).

Clinical relevance of inflammasome inhibition

Inflammatory reactions in response to infections are highly coordinated. The recruitment of leukocytes to sites of infection is orchestrated by a complex array of soluble mediators and is beneficial for efficient immune responses. In contrast, uncontrolled production of these cytokines is associated with disease. Most prominent are the periodic fever syndromes, which are directly linked to the inappropriate production of IL-1β or TNF-α (45, 46). Genetic studies revealed that hereditary mutations in inflammasome components are linked to a number of disorders. Mutations in pyrin are linked to FMF and indirectly to the pyogenic sterile arthritis, pyoderma gangrenosum, and acne (PAPA) syndrome, while mutations in NALP3 are linked to cryopyrin-associated periodic syndromes (CAPS) (45, 46). Based upon these observations, cytokine traps such as rIL-Ra (anakinra) that neutralize the effects of IL-1β were used in clinical trials and proved effective in several autoinflammatory disorders (53–55). Other IL-1β inhibitors were evaluated, including IL-1β and IL-1R-specific Abs and caspase-1 inhibitors (56). In autoimmune disorders such as rheumatoid arthritis (RA), IL-1β is not initiating the disease but contributes to the symptoms; hence, anti-IL-1β therapy showed only minor effects. Nevertheless, mutations in pyrin are linked to more severe RA, and expression of NALP3 and pyrin is elevated in the RA synovium (57, 58). The recent observation that inflammasomes sense the uric acid crystals calcium pyrophosphate dihydrate and monosodium urate, which are deposited in patients with pseudogout and gout, respectively, resulted in the first effective trial for acute gouty arthritis patients with anti-IL-1β therapies (59, 60).

Conclusions

Although it is desirable to enhance immune responses for effective pathogen clearance, it is of great importance to develop strategies for limiting inappropriate inflammation. Inflammasomes are becoming an increasingly complex hot spot for the regulation of inflammatory caspase activation and the processing and secretion of their cytokine substrates. These cytokines play pivotal roles in many human autoinflammatory and autoimmune disorders and their inhibition has led to significant results in patients, which positions inflammasomes as a promising drug target. Current therapies are solely based on neutralizing cytokines that are already in circulation, but direct targeting of the inflammasome could prevent their generation and provide a novel approach to clinical intervention. Blocking NTP binding of NLRs, the further refinement of direct caspase-1 inhibitors, and the use of small endogenous inhibitors such as COPs and POPs are only few of the possible targets. To date, functional characterization of COPs and POPs has relied on protein overexpression and in vitro assays, but ongoing studies will help to better characterize their role in inflammasome regulation in

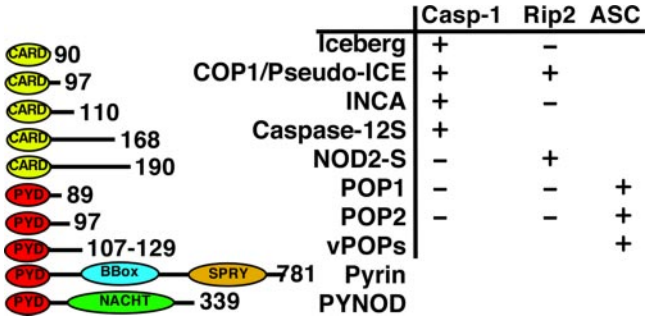

FIGURE 4. PYD and CARD-containing inhibitors of the inflammasomes. Five COPs regulate procaspase-1 interaction with the adaptors ASC, Rip2, IPAF, and Nod2. The majority of humans carry caspase-12 short (Caspase-12S) as depicted (25). Nod2-S is a CARD-only splice variant of Nod2 (29). Two POPs are encoded in humans and are also present in several poxviruses. In addition, pyrin and PYNOD function as potential modulators of inflammasomes. PYNOD lacks the LRRs that are usually encoded by NLRs and might compete for PYD-mediated ASC binding as well as heterotypic NACHT interactions with other NLRs (49). These modulators compete for essential protein interactions with inflammasome proteins. The known targets for POPs and COPs are indicated by a plus sign (+), showing that COPs compete with crucial adaptor proteins for binding to procaspase-1. POPs bind to ASC and prevent its interaction with NLRPs and potentially NLRCs to hinder the recruitment of procaspase-1 into inflammasomes. Yellow, CARD; red, PYD; blue, BBox; orange, SPRY; green, NACHT.

vivo (Fig. 4). Unfortunately, human COPs and POPs lack mouse orthologs and are therefore not part of murine inflammasome regulation, which prevents gene ablation studies. Less than 1% of human genes are estimated to lack a mouse ortholog and have likely originated from gene duplication. These genes appear particularly interesting, because they may be responsible for species-specific functions (61). There is evidence that the regulation of inflammatory cytokine production in humans differs from that in mice. Although TNF-α-neutralizing Abs can prevent sepsis in mice, similar efforts in humans failed (62, 63). A more complex regulation of key proinflammatory signaling pathways in humans could allow for a more precise fine tuning of inflammatory responses. Therefore, a better understanding of the molecular biology of these endogenous inhibitors is necessary.

Disclosures

The authors have no financial conflict of interest.

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