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Tripping the wire: sensing of viral protease activity by CARD8 and NLRP1 inflammasomes

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Abstract

Host innate immune sensors are vital for the initial detection of pathogen infection. Such sensors thus need to constantly adapt in escalating evolutionary arms races with pathogens. Recently, two inflammasome-forming proteins, CARD8 and NLRP1, have emerged as innate immune sensors for the enzymatic activity of virus-encoded proteases. When cleaved within a rapidly evolving 'tripwire' region, CARD8 and NLRP1 assemble into inflammasomes that initiate pyroptotic cell death and pro-inflammatory cytokine release as a form of effector-triggered immunity (ETI). Short motifs in the CARD8 and NLRP1 tripwires mimic the protease-specific cleavage sites of picornaviruses, coronaviruses, and HIV-1, providing virus-specific sensing that can rapidly change between closely related hosts and within the human population. Recent work highlights the evolutionary arms races between viral proteases and NLRP1 and CARD8, including insights into the mechanisms of inflammasome activation, host diversity of viral sensing, and means that viruses have evolved to avoid tripping the wire.

Introduction

Viruses and their hosts are locked in molecular arms races that lead to rapid evolution at host-virus interfaces [1–3]. Such evolutionary conflicts drive innovations in host antiviral mechanisms and select for adaptations that evade viral counterstrategies. Importantly, the diversity that results from these host-virus arms races shapes host-specific immune responses, the ability of viruses to 'jump' species, and the susceptibility of humans to emerging zoonotic viruses [4,5]. Even within humans, variability in innate immune-associated genes impacts viral susceptibility and pathogenesis, indicating that human polymorphism is an important but understudied determinant of severity of infectious diseases [6].

A central function of the innate immune system is the ability to sense and respond to viral infection. Extensive research has focused on the roles of innate immune sensing through the recognition of highly conserved pathogen-associated molecular patterns (PAMPs) such as double-stranded RNA (dsRNA) [7]. However, there is increasing appreciation of the role of mammalian effector-triggered immunity (ETI), which can sense the activity of pathogen-encoded effectors, such as toxins and proteases [8–11]. Although less broadly conserved

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than PAMPs (e.g. all RNA viruses generate dsRNA), pathogen-encoded activities sensed by ETIs are often important for pathogen fitness and are therefore evolutionary constrained in a manner that allows ETI sensors to be effective at detecting a wide diversity of pathogens [8].

Recently, two inflammasome proteins, NLRP1 and CARD8, have emerged as ETI sensors of the enzymatic activities of diverse viral proteases. Proteases are encoded by many human viruses, including +ssRNA viruses in *Coronaviridae* (e.g. SARS-CoV-2), *Picornaviridae* (e.g. enteroviruses, rhinovirus), and *Flaviviridae* (e.g. dengue virus, hepatitis C virus) families, as well as members of *Retroviridae* (e.g. HIV-1). These proteases are critical to viral propagation, as they both liberate functional proteins from virally-encoded polyproteins and cleave host factors to further facilitate viral replication [12–14]. Importantly, viral protease specificity is constrained to cleave the viral polyprotein at multiple sequence-specific locations, limiting protease evolvability [12]. Taking advantage of this evolutionary constraint on viral protease substrate specificity, NLRP1 and CARD8 have evolved N-terminal disordered ‘tripwire’ sequences that mimic the preferred cleavage sequences of several virus-encoded proteases. Cleavage of NLRP1 and CARD8 at these sites results in immune activation and cell death in a virus- and host-specific manner [15–21] (Table 1). Here we discuss the mechanism and outcomes of tripwire ETI sensing of protease activity by NLRP1 and CARD8, the consequences of host and viral evolution on inflammasome sensing of protease activity, and additional possibilities for ETI sensing of viral proteases.

NLRP1 and CARD8 are innate immune ETI sensors activated by viral protease cleavage

NLRP1 and CARD8 are homologous proteins that each assemble into an inflammasome, a cytoplasmic immune complex whose activation triggers pyroptotic cell death and release of pro-inflammatory cytokines, including IL-1β and IL-18 [22–26] (Figure 1A). The first indication that NLRP1/CARD8 homologs may contribute to ETI sensing of pathogen infection was observed in mice. Mouse NLRP1B is cleaved in a disordered N-terminal region, which we refer to as the ‘tripwire’, by the secreted Lethal Factor (LF) protease from *Bacillus anthracis*, resulting in protective immune response against bacterial challenge [27,28]. The mechanism by which LF protease cleavage leads to inflammasome activation has been termed ‘functional degradation’ [29,30] (Figure 1B). Important for this mechanism of ETI sensing is the unusual domain architecture of NLRP1 and CARD8, in which the C-terminal end comprises a function-to-find domain (FIIND) followed by a caspase activation and recruitment domain (CARD) [24,26]. A constitutive self-cleavage event occurs in the FIIND, separating the protein into an N-terminal ‘sensing’ region and a C-terminal CARD-containing region that remain non-covalently associated [31–33]. Following cleavage by pathogen-encoded proteases like LF, the new N-terminus serves as an N-degron for ubiquitylation and subsequent proteasomal degradation by the ‘N-end rule’ pathway, thus liberating the CARD-containing C-terminus to self-oligomerize and recruit and activate Caspase-1 (CASP1) [29,30]. CASP1 initiates pyroptotic cell death through cleavage of gasdermin D (GSDMD) and inflammatory cytokine signaling through pro-inflammatory cytokine (IL-1β and IL-18) processing [24,26,29,30]. Thus, functional degradation of the N-terminus following tripwire cleavage is mechanistically tied to the release of a CARD-containing C-terminus and activation of pyroptosis and inflammation (Figure 1B). Notably, NLRP1/CARD8 homologs differ in their N-terminal domains (Figure 1A), which may be

important for homolog-specific functions outside of ETI sensing, and human NLRP1, but not mouse NLRP1 or human CARD8, requires ASC for the recruitment and activation of CASP1[18,34–38]. Irrespective of these differences, all homologs have a conserved C-terminal FIIND-CARD architecture that enables ETI sensing by functional degradation.

This system of activation by functional degradation suggested that NLRP1 and CARD8 may have the capacity to sense a variety of pathogen-encoded proteases beyond LF. Indeed, NLRP1 and CARD8 can sense cleavage by proteases encoded by diverse viruses from at least three distinct families: *Picornaviridae*, *Coronaviridae*, and *Retroviridae* [15–21] (Table 1). For instance, the disordered human NLRP1 tripwire region, analogous to the LF-sensing tripwire region in mouse NLRP1B, contains a mimic of the preferred cleavage sequence of the 3C protease ($3C^{pro}$) encoded by enteroviruses such as human rhinovirus (HRV) and coxsackievirus B3 (CVB3) within *Picornaviridae* [15,16] (Figure 2A). Viral protease-mediated cleavage within this human NLRP1 tripwire activates the inflammasome and results in pro-inflammatory cytokine release and pyroptotic cell death during HRV infection of primary human airway epithelial cells [15] or CVB3 infection of human keratinocytes [16]. NLRP1 also contains a cleavage site within the NACHT domain for the 3CL protease ($3CL^{pro}$) of coronaviruses, resulting in cell death upon SARS-CoV-2 infection, which induces a non-canonical pathway involving CASP3, CASP8, and GSDME [17]. Similarly, CARD8 can sense a diverse array of viral proteases through protease site mimics in a disordered N-terminal tripwire region (Figure 2B). For instance, HIV-1 protease cleavage of CARD8 results in pyroptotic cell death and pro-inflammatory cytokine release in HIV-1 infected immune cells, which can both eliminate latently infected cells [18,39] and prevent HIV-1 infection [20]. CARD8 also contains a site mimic for both picornavirus $3C^{pro}$ and coronavirus $3CL^{pro}$, resulting in inflammasome-mediated death of SARS-CoV-2 infected human monocytes [19] and CVB3-infected cardiomyocytes [21]. These results establish the importance of human NLRP1 and CARD8 at ETI sensors of diverse viral infections. However, as described below, the molecular arms race between inflammasome sensors and viral proteases is ongoing, and both host and virus evolution have the capacity to turn the tables in the arms race.

Rapid NLRP1 and CARD8 tripwire evolution results in host-specific sensing of viral infection.

The NLRP1/CARD8 mechanism for recognition of viral proteases relies on the presence of viral polyprotein cleavage site mimics in the disordered tripwire region, suggesting such sequences may evolve rapidly and recurrently. Indeed, both NLRP1 and CARD8 are evolving under strong positive (diversifying) selection in their respective disordered tripwire regions [19,34]. This suggests that evolution of the tripwire region is driven by host-virus arms races in which host evolution of short linear motifs (SLiMs), specifically viral protease cleavage site mimics, allows hosts to exploit the evolutionarily constrained viral protease to gain a selective advantage [12]. For instance, phylogenetic analyses of NLRP1 revealed that the $3C^{pro}$ cleavage site mimic in NLRP1 evolved in the primate lineage, but is lacking in other mammals [16] (Figure 2C). Importantly, genetic and functional differences in the tripwire region are observed among closely related primate species, and even a single-nucleotide polymorphism (SNP) within the human population can prevent cleavage

and inflammasome activation by enterovirus $3C^{pros}$ [16] (Figure 2C). Human NLRP1 also contains sites elsewhere in the tripwire that can be cleaved by $3C^{pros}$ from non-enterovirus picornaviruses, suggestive of independent acquisition of novel virus sensing functions. Similarly, NLRP1 homologs in other species, such as mice, have convergently evolved site mimics for $3C^{pros}$ [16]. Together, these data suggest that novel SLiMs can be easily evolved in protein sequences [40] and that ETI tripwires are a robust and highly evolvable system that underlies host- and virus-specific protease recognition. While the divergent pathogen specificity of NLRP1 sensing between different host species may appear paradoxical at first, rapid sequence evolution and changes in pathogen specificity are hallmarks of innate immune sensors that are engaged in arms races with pathogens [1,2,5,8], providing further evidence for the importance of NLRP1 in ETI sensing.

The tripwire region of CARD8 is also rapidly evolving under positive selection and is cleaved and activated by viral proteases in a virus- and host-specific manner [19]. Proteases from endemic and pandemic human coronaviruses, including SARS-CoV-2, cleave and activate human CARD8, but the site mimic is lacking in other publicly available primate CARD8 sequences, and many species, including rodents, lack CARD8 altogether [19] (Figure 2D). Intriguingly, bats are one group of species that lack CARD8-mediated $3CL^{pro}$ sensing. Like rodents, microbats lack a CARD8 ortholog, and CARD8 from the megabat, *Rousettus aegypticus*, is not activated by $3CL^{pros}$, suggesting one mechanism of viral tolerance in this important reservoir of coronaviruses [19]. Additionally, a SNP found at >20% frequency in some human populations attenuates cleavage and activation of CARD8 by coronavirus $3CL^{pro}$ [19]. Strikingly, this same SNP potentiates the ability of CARD8 to sense $3C^{pros}$ from some picornaviruses, including enteroviruses such as HRV. Thus, a single amino acid substitution within the tripwire region in humans toggles CARD8 sensing of protease activity from one family of respiratory viruses, coronaviruses, to another, enteroviruses [19]. Finally, human CARD8 contains a site unique among known primate sequences that confers the ability to sense proteases from HIV-1 and a related simian immunodeficiency virus from chimpanzees (SIVcpz) (Figure 2D) [20]. Although the selective pressure that drove this change in human CARD8 is unknown, these data suggest that the functional consequences of CARD8 evolution may be a potential mechanism for differential pathogenesis of lentiviruses among primates. Together, the evolutionary dynamics of NLRP1 and CARD8 across species and within humans reveals that ETI sensing is influenced by host genetic diversity, which can in turn possibly impact host susceptibility to viral infection and viral pathogenesis.

The virus strikes back: protease evolution leads to evasion or antagonism of inflammasome sensing

While deeply constrained by the need to retain cleavage of multiple independent polyprotein sites, viral proteases do evolve in a manner that affects their sequence specificity [12] and these changes impact NLRP1 and CARD8-mediated sensing. For instance, divergence of picornavirus $3C^{pros}$ results in differential ability to be sensed by human NLRP1 and mouse NLRP1B [16] (Figure 3A). This has the functional consequence of human NLRP1 inflammasome sensing and activation in response to infection by one picornavirus, CVB3, but not by another picornavirus, EMCV [16]. Intriguingly, protease specificity

evolution can not only evade sensing by NLRP1 but can also actively antagonize NLRP1 inflammasome function. As one example, several non-enterovirus $3C^{pros}$ can attenuate NLRP1 function when it is activated by a heterologous protease. Although the mechanism for this antagonism is unknown, these proteases do cleave NLRP1 near or within the FIIND-CARD C-terminus [16]. In addition, both $3C^{pro}$ and $3CL^{pro}$ can cleave and inactivate GSDMD, thus antagonizing inflammasome-mediated pyroptosis [17,41]. These data suggest that protease evolution can not only result in lack of tripwire cleavage, but also in cleavage of novel sites that may antagonize inflammasome function (Figure 3A). Similar antagonistic effects of viral proteases are seen with megabat CARD8 by coronavirus $3CL^{pros}$. In this case, the site of $3CL^{pro}$ cleavage within the C-terminal region of megabat CARD8 is specific to the megabat lineage [19], providing another example of host-specific inflammasome antagonism by viral proteases. The need to evade or antagonize ETI sensors may therefore be one driving force for diversification of viral proteases that are otherwise expected to evolve very slowly [12].

Just the beginning: outstanding questions and additional innate immune sensors of viral proteases

Much remains to be explored at the intersection of the ongoing molecular arms race between viral proteases and host ETI sensors. For instance, expression of NLRP1 and CARD8 varies across tissues and cell types [23,42,43], and other host factors such as DPP8/9 impact responsiveness of NLRP1 and CARD8 [44–46]. This indicates that the inflammasome response may be an important but underexplored determinant of tissue tropism of viral replication and pathogenesis. Likewise, the consequences of viral protease-mediated activation of NLRP1 and CARD8 on systemic viral replication and immunopathology in humans remains to be elucidated. Development of animal models for NLRP1/CARD8 inflammasome sensing of viral infection, possibly taking advantage of the one known example of mouse NLRP1B sensing of viral proteases [19], will be critical for assessing the *in vivo* contribution of ETI inflammasome activation on viral control and pathogenesis. Additionally, human polymorphism of NLRP1 contributes to autoinflammatory diseases [46–50], but how this human genetic variation impacts protease or pathogen sensing is not known. Finally, although ETI sensing appears to be the unifying function of both CARD8 and NLRP1, other sensing functions have been described for these inflammasome proteins, including activation of NLRP1 by dsRNA and ribotoxic stress during infection [51–53]. Whether there is a common mechanism relating these functions and protease sensing, or whether they are independent, remains to be determined.

As the role of ETI in animal innate immunity is increasingly appreciated, it is tempting to speculate that host evolution of protease cleavage site mimics might be a broadly employed strategy in host-pathogen arms races (Figure 3B). For instance, within pyroptotic cell death pathways, both IL-1β and GSDMA can be directly cleaved and activated by bacterial proteases to provide antibacterial immunity [54–56]. Similarly, GSDMD can be directly cleaved near its natural site of CASP1-mediated maturation by Zika virus protease to initiate pyroptotic cell death [57]. Whether other inflammasome sensors from the NLR superfamily also contain a tripwire region sensitive to virally-encoded protease cleavage is unknown. However, other NLR inflammasomes sensors do not share the unique domain

architecture seen in NLRP1 and CARD8, where the CARD is at the C-terminus and separated from the rest of the protein by the self-cleaving FIIND (Figure 1A). In fact, many NLR inflammasome sensors contain a CARD at the N-terminus or not at all [58]. Thus, it is unlikely that cleavage of these sensors would result in activation by functional degradation, although cleavage of these sensors could result in activation via an unknown mechanism. More broadly, a variety of innate immune proteins have been described as 'guards' that could represent ETI protease sensors, similar to what has been observed with ETI sensing in plants [8,9,59]. For instance, CVB3 $3C^{pro}$ cleaves an inhibitor of NF-κB signaling, IκBα, resulting in increased apoptosis and decreased viral replication [60]. These examples suggest that NLRP1 and CARD8 are likely just the first of many innate immune sensors that mimic viral protease cleavage sites to promote an immune response. We envision that further studies will not only uncover novel mechanisms of effector-triggered immunity in humans but also strategies employed by viruses to escape them. Understanding how such ETI-protease interactions evolve across species will shed further light on virus-specific and host-specific determinants of host range, tissue tropism, pathogenesis, and chance of virus spillover into humans.

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** - of outstanding interest

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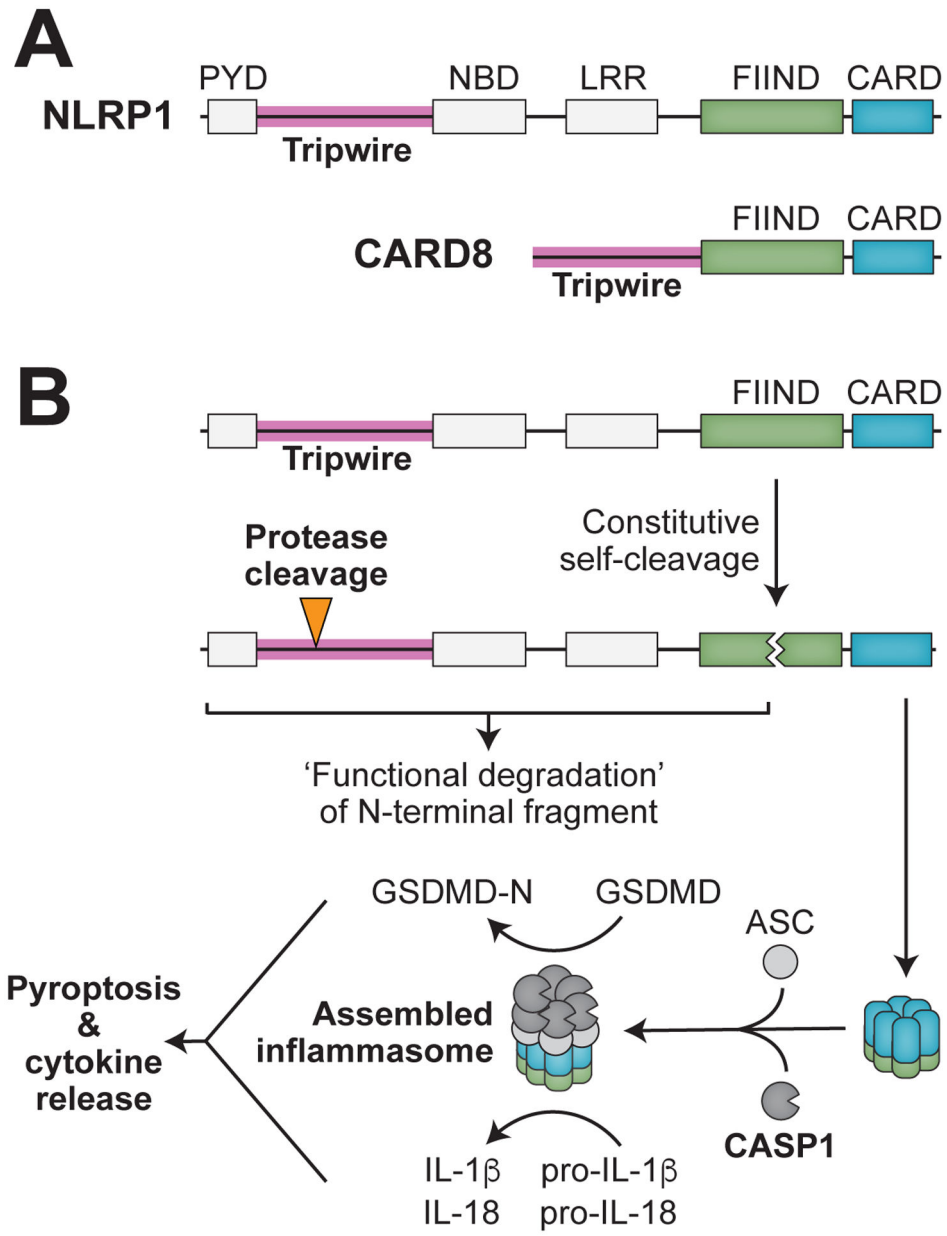

Figure 1. NLRP1 and CARD8 inflammasomes are effector-triggered immune sensors that are activated by 'functional degradation'.

(A) Schematic of the domain structure of human NLRP1 and CARD8. Both NLRP1 and CARD8 contain a rapidly evolving disordered region in their N-terminus that we refer to as the 'tripwire' (pink shading) for its ability to sense cleavage by diverse pathogen-encoded proteases. (B) 'Functional degradation' model for NLRP1 inflammasome activation. The FIIND, shared by both NLRP1 and CARD8, undergoes constitutive self-cleavage, resulting in a CARD-containing C-terminus being non-covalently bound to the N-terminus of the respective proteins. Cleavage by a pathogen-encoded protease in a rapidly evolving and disordered 'tripwire' region exposes an N-degron, which subsequently results in proteasomal degradation of the N-terminal domains. The bioactive CARD-containing C-terminus is liberated allowing for recruitment and activation of CASP1, requiring ASC in the case of

human NLRP1 but not CARD8. Inflammasome activation results in maturation and release of pro-inflammatory cytokines, IL-1β and IL-18, and initiation of pyroptotic cell death via GSDMD.

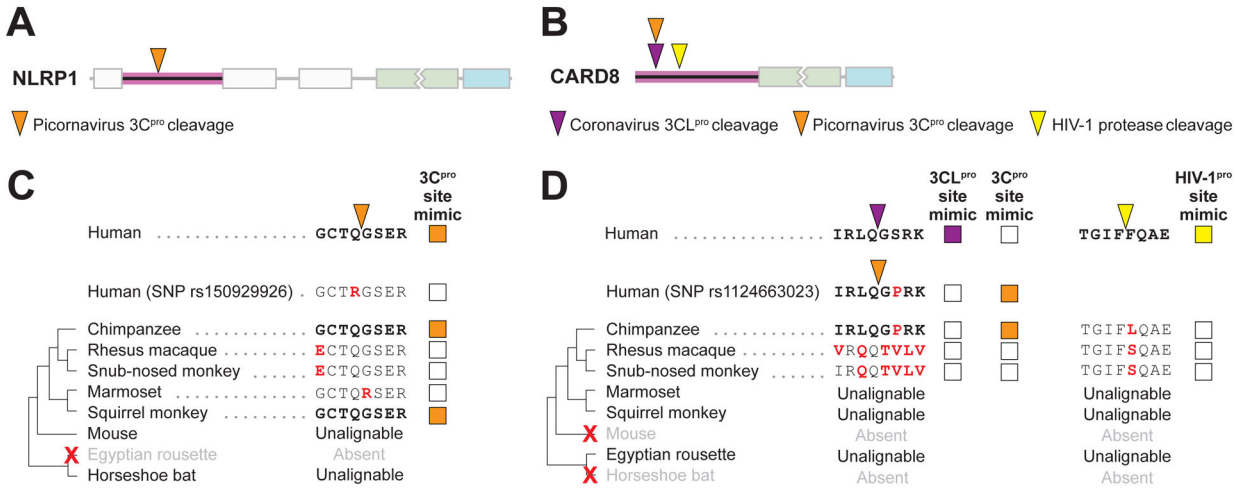

Figure 2. Host- and virus-specific activation of NLRP1 and CARD8 inflammasomes by virus-encoded proteases.

(A) Site of picornavirus 3C protease (3C^{pro}) cleavage (orange triangle) within the rapidly evolving tripwire region (pink shading) of human NLRP1 [16,19]. (B) Sites of coronavirus 3CL protease (3CL^{pro}) (purple triangle) [19], picornavirus 3Cpro (orange triangle) [19,21], and HIV-1 protease (HIV-1^{pro}) (yellow triangle) [18] cleavage within the tripwire region (pink shading) of human CARD8. (C) Sequence within human NLRP1 that mimics the preferred enteroviral 3C^{pro} cleavage site is shown at top. Below are NLRP1 sequences from other human individuals or non-human mammalian species that align to this region. NLRP1 homologs from mouse and microbat (represented by the horseshoe bat) are unalignable to this region of human NLRP1. Megabats (represented by the Egyptian rousette), have lost NLRP1 altogether, as indicated by a red X on the phylogenetic tree to the left. Amino acid changes relative to the human NLRP1 reference sequence are highlighted in red. NLRP1 sequences that are cleaved and activated by 3C^{pro} are in bold and are marked with a filled orange box. Data from [16]. (D) Sequence within human CARD8 that mimics the preferred cleavage sites of the indicated proteases are shown. Megabat CARD8 lack any sequence that is alignable to this region of primate CARD8, and mice and microbats lack a CARD8 ortholog altogether. Amino acid changes relative to the human CARD8 reference sequence are highlighted in red. CARD8 sequences that are cleaved and activated by viral proteases are in bold and are marked with filled boxes of the appropriate color. Data on 3CL^{pro} and 3C^{pro} from [19], data on HIV-1^{pro} from [20].

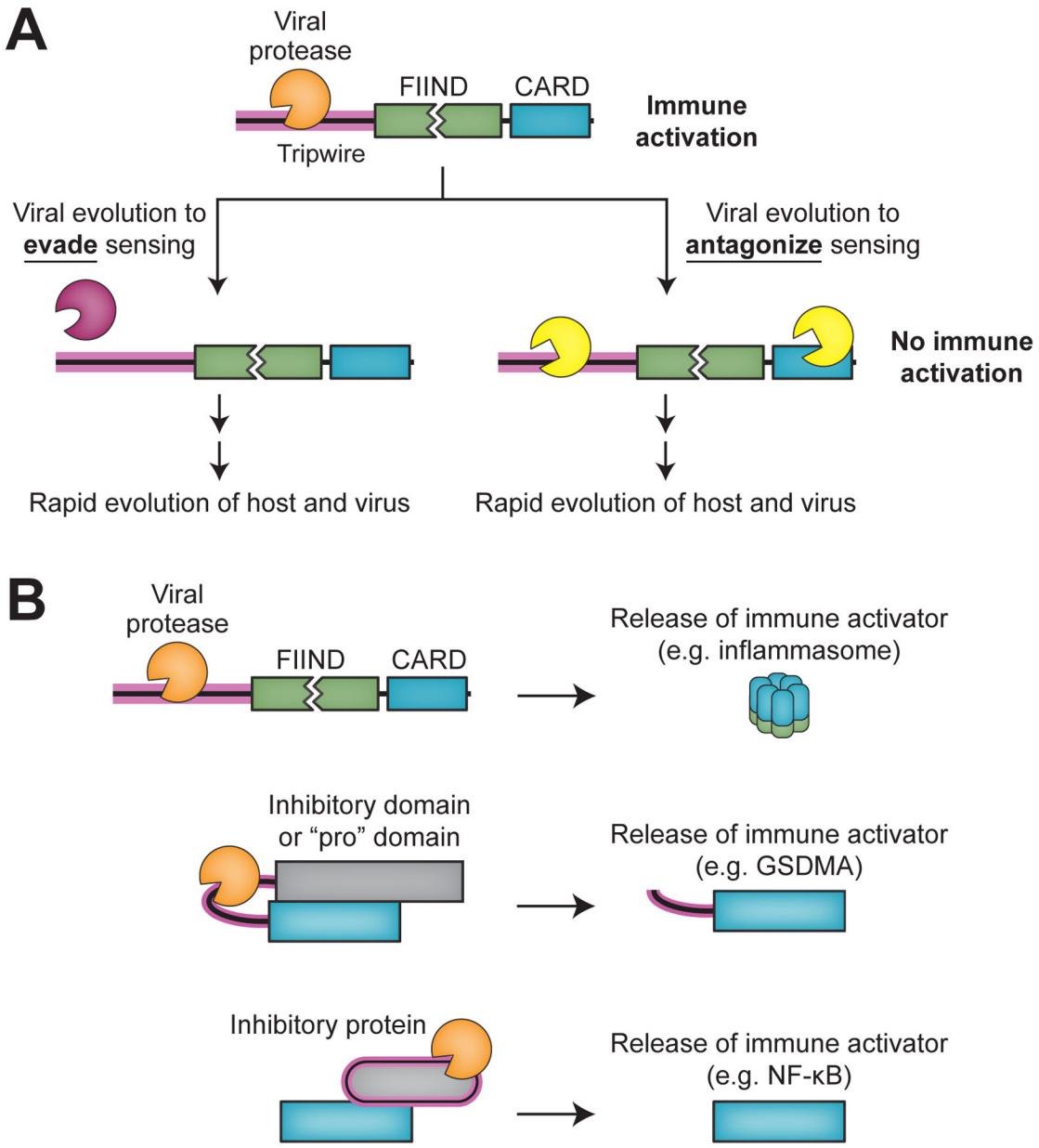

Figure 3. Potential mechanisms of innate immune sensing of viral proteases and routes of viral escape.

(A) Two possible routes for how viral protease evolution can evade inflammasome sensing and immune activation. On the left, viral evolution may result in a protease that no longer cleaves the tripwire region of the inflammasome sensor. On the right, viral evolution may result in a protease that cleaves the tripwire region of the inflammasome sensor but also cleaves in another position that antagonizes inflammasome activation. (B) Proposed models for diverse mechanisms for ETI sensing of viral protease activity. At the top is the inflammasome-mediated mechanism characterized by CARD8 and NLRP1. Other potential mechanisms of innate immune activation as a result of viral protease sensing include viral protease-mediated cleavage to release inhibitory or 'pro' domains that attenuate immune activation (middle) or cleavage of inhibitory proteins that attenuate immune activation

(bottom). In all cases, rapidly evolving sequences (pink shading) may allow host adaptation to specific viral proteases.

Table 1.

Viral proteases sensed by NLRP1 and CARD8 inflammasomes.

Host protein	Virus	Protease	Reference	Notes
NLRP1	HRV	3C	Robinson et al, 2020 [15]	Activation in primary airway epithelial cells.
	CVB3, others	3C	Tsu et al, 2021 [16]	Host- and virus-specific activation described.
	SARS-CoV-2	3CL	Planes et al, 2022 [17]	Cleaves outside tripwire. Activation via caspase-3 and GSDME.
CARD8	HIV-1	PR	Wang et al, 2021 [18]	Inflammasome-dependent death of latently infected immune cells.
	HIV-1, SIVcpz	PR	Kulsuptrakul et al, 2022 [20]	Host- and virus-specific activation described.
	SARS-CoV-2 HRV, others	3CL 3C	Tsu et al, 2023 [19]	Host- and virus-specific activation described. Activation sensitive to human SNP.
	CVB3	3C	Nadkarni et al, 2022 [21]	Activation in endothelial cells and cardiomyocytes.

Viral abbreviations: human rhinovirus (HRV), coxsackievirus B3 (CVB3), chimpanzee SIV (SIVcpz).