

## REVIEW

# Post-translational regulation of inflammasomes

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Inflammasomes play essential roles in immune protection against microbial infections. However, excessive inflammation is implicated in various human diseases, including autoinflammatory syndromes, diabetes, multiple sclerosis, cardiovascular disorders and neurodegenerative diseases. Therefore, precise regulation of inflammasome activities is critical for adequate immune protection while limiting collateral tissue damage. In this review, we focus on the emerging roles of post-translational modifications (PTMs) that regulate activation of the NLRP3, NLRP1, NLRC4, AIM2 and IFI16 inflammasomes. We anticipate that these types of PTMs will be identified in other types of and less well-characterized inflammasomes. Because these highly diverse and versatile PTMs shape distinct inflammatory responses in response to infections and tissue damage, targeting the enzymes involved in these PTMs will undoubtedly offer opportunities for precise modulation of inflammasome activities under various pathophysiological conditions.

*Cellular & Molecular Immunology* (2017) **14**, 65–79; doi:10.1038/cmi.2016.29; published online 27 June 2016

**Keywords:** inflammasome; phosphorylation; post-translational modifications; ubiquitination

## INTRODUCTION

The innate immune system relies on pattern recognition receptors (PRRs) to sense microbial infections and tissue damage and to initiate inflammatory responses.<sup>1–3</sup> Inflammasomes constitute a group of PRRs that have essential roles in immune protection against bacterial, viral, fungal and parasitic infections.<sup>4–11</sup> Dysregulation of inflammasomes is implicated in various human diseases, including autoinflammatory disorders,<sup>12</sup> metabolic disorders<sup>13,14</sup> and cancer.<sup>15,16</sup> Each type of inflammasome is characterized by a particular sensor or receptor molecule from the nucleotide-binding domain (NOD), leucine-rich repeat (LRR)-containing protein (NLR) family, or pyrin and HIN domain-containing protein (PYHIN) family. These include the NLRP1,<sup>4</sup> NLRP2,<sup>17</sup> NLRP3,<sup>18</sup> NLRC4,<sup>19–22</sup> NLRP6,<sup>23,24</sup> NLRP7,<sup>25</sup> NLRP12,<sup>26</sup> AIM2<sup>27–30</sup> and IFI16<sup>31,32</sup> inflammasomes. NLRP proteins contain a pyrin domain (PYD) at their N-termini, whereas NLRC4 contains a caspase activation and recruitment domain (CARD).<sup>33</sup> The AIM2 and IFI16 proteins are DNA sensors that contain N-terminal PYD domains and C-terminal DNA-binding HIN domains. The adapter molecule apoptosis-associated speck-like protein containing a caspase-recruitment domain (ASC) consists of N-terminal PYD and a C-terminal CARD domains that facilitate its interaction with both

upstream sensor molecules through its PYD domain and downstream caspase-1 through its CARD domain. Assembly of the canonical inflammasome complexes containing caspase-1, as well as the noncanonical inflammasomes containing caspases-4, -5 and -11 that are bound to intracellular LPS,<sup>34–36</sup> leads to activation of these caspases, which promotes maturation and secretion of proinflammatory cytokines, such as IL-1 $\beta$ , IL-18 and IL-1 $\alpha$ .<sup>9,11</sup> Activated caspase-1 and caspase-11 also cleave gasdermin D (GSDMD) and other substrates that induce pyroptotic cell death through as-yet unknown mechanisms.<sup>37–39</sup> Not all cell types undergo pyroptosis upon inflammasome activation. For example, activation of the NLRC4 inflammasome in neutrophils infected by *Salmonella* leads to the production of IL-1 $\beta$  without apparent pyroptosis, thus facilitating clearance of invading pathogens by the neutrophils.<sup>40–42</sup> Furthermore, the AIM2 and NLRP3 inflammasomes are recruited to autophagosomes for degradation, which serves as a mechanism to eliminate activated inflammasomes and terminate inflammatory signaling.<sup>43</sup> It is unclear whether other inflammasomes can also be degraded through similar mechanisms.

Because of the highly proinflammatory consequences of inflammasome activation, this process is tightly regulated to limit collateral damage. Prior to the activation of inflammasomes by diverse stimuli or ligands, a priming step, which can

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Received: 10 March 2016; Revised: 5 May 2016; Accepted: 5 May 2016

be provided by ligands for the Toll-like receptors (TLRs), is necessary. Priming leads to the activation of NF- $\kappa$ B and expression of inflammasome components, such as sensor molecules and the pro-forms of inflammatory cytokines, such as pro-IL1 $\beta$  and pro-IL18.<sup>44</sup> Priming also modulates the post-translational modifications (PTMs) of NLRP3 and ASC to facilitate assembly of inflammasome complexes.<sup>45–47</sup> In fact, the PTMs of inflammasome components have emerged as a major regulatory mechanism for inflammatory signaling. Virtually all cellular processes are regulated by a combinatorial post-translational modification ‘code’ that changes the surface features of proteins to endow them with diverse functionalities. Of these modifications, phosphorylation and ubiquitination are among the best characterized.<sup>48–51</sup> PTMs can covalently modify 15 of the 20 amino acids or cause proteolytic cleavage at specific residues to regulate the size, conformation, location, turnover and interaction of target proteins. These PTMs are catalyzed by hundreds of enzymes that are themselves modulated by binding partners or small molecule inhibitors. As a result, targeting these enzymes may offer opportunities for fine-tuning inflammatory signaling processes for therapeutic benefits in infectious diseases and metabolic, autoimmune and autoinflammatory disorders.<sup>52,53</sup>

In this review, we focus on recent developments in post-translational modification of inflammasome components, such as sensor/receptor molecules and the adapter ASC, through phosphorylation, ubiquitination, proteolytic processing and other mechanisms (Table 1 and Figure 1). Convergence of these post-translational modifications contributes to comprehensive and balanced immune surveillance mechanisms. We anticipate that knowledge regarding PTMs will expand from these examples to other PTM types and less well-characterized inflammasomes. Because highly diverse and versatile PTMs shape distinct inflammatory responses toward infections and resulting from tissue damage, the role of post-translational modifications as a regulatory mechanism for inflammasome activation will remain an exciting area of research for the foreseeable future. Other mechanisms of inflammasome regulation relevant to NLRP3 were recently reviewed by Jo *et al*.<sup>54</sup>

## PHOSPHORYLATION

Phosphorylation is the most common post-translational modification and involves hundreds of kinases and phosphatases that dynamically modulate the proteome. It is well established that several inflammasome sensor molecules and the adapter ASC are regulated by distinct phosphorylation events, some of which have been mapped to specific residues of relevant inflammasome components. In most cases, the exact mechanisms by which phosphorylation of these proteins affects their structure and function remain to be clarified.

### Phosphorylation of ASC modulates the activation of multiple inflammasomes

ASC is an essential adapter molecule for most inflammasome sensors.<sup>55</sup> Phosphorylation of ASC was reported soon after

identification of the inflammasome,<sup>56</sup> but the role of its phosphorylation in inflammasome activation remained unclear. More than a decade later, it was discovered that in macrophages, ASC phosphorylation was required for activation of the NLRP3 and AIM2 inflammasomes, but not for that of NLRC4.<sup>57,58</sup> Specifically, Syk and Jnk kinase phosphorylate Tyr144 within the CARD domain of murine ASC<sup>57</sup> or Tyr146 and Tyr187 in human ASC.<sup>58</sup> This phosphorylation event facilitates formation of the ASC speck in the perinuclear area. Interestingly, inhibition of Syk and Jnk kinase activities does not disrupt the interaction between ASC and NLRP3, which suggests that phosphorylation of ASC may impact downstream events such as its ability to migrate to the perinuclear region, self-associate or recruit procaspases-1 through CARD–CARD interactions. This may also explain the fact that the NLRC4 inflammasome is not affected by the ASC–CARD phosphorylation because NLRC4 contains a CARD that can directly engage procaspase-1 in the absence of ASC.<sup>20,21,59</sup> In contrast to the above studies in macrophages, phosphorylation of ASC does not appear to be important for inflammasome activation in bone marrow-derived dendritic cells.<sup>57</sup> The mechanisms for such cell-type specificity and inflammasome selectivity remain to be determined. Furthermore, another study reported that Syk deficiency in macrophages did not reduce activation of the NLRP3 inflammasome.<sup>60</sup> The reason for this discrepancy is not clear, but may be related to the different mouse strains used in the studies.

Bruton’s tyrosine kinase (BTK) was shown to interact with both ASC and NLRP3 through its kinase domain and, possibly, to phosphorylate ASC, which promotes activation of the NLRP3 inflammasome, but not the AIM2 inflammasome.<sup>61</sup> Importantly, treatment with an FDA-approved BTK inhibitor has a neuroprotective effect in a brain ischemia model in mice, which suggests potential roles for BTK and inflammasomes in brain ischemia.

In addition to these examples of ASC phosphorylation that enhances inflammasome activation, there are also phosphorylation events that suppress inflammasome activation. Loss of I $\kappa$ B kinase  $\alpha$  (IKK $\alpha$ ) kinase activity is associated with spontaneous inflammation and lung cancer.<sup>62,63</sup> This prompted an investigation of the role of IKK $\alpha$  in inflammasome activation.<sup>64</sup> It turned out that IKK $\alpha$  functions as a negative regulator of the NLRP3, AIM2 and NLRC4 inflammasomes through its interaction with the adapter ASC, by which it reduces the translocation of ASC from the nucleus to the cytoplasm.<sup>64</sup> The residues Ser193 in the CARD and Ser16 in the PYD domain of ASC are phosphorylated by IKK $\alpha$ , which is important for the ASC–IKK $\alpha$  association in the nucleus that prevents its translocation to the cytoplasm for inflammasome activation. Mutations of either residue result in enhanced activation of both the NLRP3 and AIM2 inflammasomes. In agreement with the role of IKK $\alpha$  as a negative regulator of inflammasomes, stimulation of the NLRP3 inflammasome with ATP or nigericin suppresses the kinase activity of IKK $\alpha$  and its association with ASC, perhaps through dephosphorylation of IKK $\alpha$  by the serine/threonine phosphatase PP2A.

**Table 1 Regulation of inflammasome activities through post-translational modifications**

<i>Post-translational modifications</i>	<i>Modification sites/types</i>	<i>Enzymes/triggers</i>	<i>Effects on inflammasome activation</i>	<i>References</i>
<i>Phosphorylation</i>				
ASC	Tyr144 (mouse) Tyr146, Tyr187 (human)	Syk/Jnk	↑NLRP3 ↑AIM2 -NLRC4	Hara <i>et al.</i> , <sup>57</sup> Lin <i>et al.</i> <sup>58</sup>
	?	BTK	↑NLRP3 -AIM2	Ito <i>et al.</i> <sup>61</sup>
	Ser19 and Ser193	IKK $\alpha$	↓NLRP3 ↓AIM2 ↓NLRC4	Martin <i>et al.</i> <sup>64</sup>
	Ser58	IKKi	↑NLRP3 ↑AIM2	Martin <i>et al.</i> <sup>64</sup>
NLRP3	?	Syk, DAPK, TAK1, ERK1	↑NLRP3	Gross <i>et al.</i> , <sup>66</sup> Said-Sadier <i>et al.</i> , <sup>67</sup> Shio <i>et al.</i> , <sup>68</sup> Wong <i>et al.</i> , <sup>69</sup> Chuang <i>et al.</i> , <sup>70</sup> Gong <i>et al.</i> , <sup>71</sup> Ghonime <i>et al.</i> <sup>72</sup>
	?	PKR	?NLRP3 ?NLRP1 ?NLRC4 ?AIM2	Lu <i>et al.</i> , <sup>73</sup> He <i>et al.</i> , <sup>76</sup> Hett <i>et al.</i> , <sup>75</sup> Yim <i>et al.</i> , <sup>77</sup> Boriushkin <i>et al.</i> <sup>74</sup>
	?	IRAK1, IRAK4	↑NLRP3	Fernandes-Alnemri <i>et al.</i> <sup>78</sup>
	?	NEK7	↑NLRP3 -AIM2 -NLRC4	Shi <i>et al.</i> , <sup>79</sup> Schmid-Burgk <i>et al.</i> , <sup>80</sup> He <i>et al.</i> <sup>81</sup>
NLRC4	Ser533	PKC $\delta$	↑NLRC4	Qu <i>et al.</i> , <sup>100</sup> Yang <i>et al.</i> , <sup>93</sup> Hu <i>et al.</i> , <sup>98</sup> Suzuki <i>et al.</i> , <sup>94</sup> Matusiak <i>et al.</i> <sup>101</sup>
<i>Ubiquitination</i>				
ASC	?/K63-linked poly-Ub	?	↓NLRP3 ↓AIM2	Shi <i>et al.</i> <sup>43</sup>
	?/linear poly-Ub	LUBAC	↑NLRP3 ↑AIM2 -NLRC4	Rodgers <i>et al.</i> <sup>47</sup>
	Lys174/K63-linked poly-Ub	TRAF3	↑NLRP3 -AIM2 -NLRC4	Guan <i>et al.</i> <sup>109</sup>
NLRP3	Lys689/K48-linked poly-Ub? ?/K48-linked poly-Ub	FBXL2 MARCH7	↓NLRP3 ↓NLRP3 -AIM2 -NLRC4	Han <i>et al.</i> <sup>112</sup> Yan <i>et al.</i> <sup>113</sup>
	?	XIAP	↓NLRP3	Yabal <i>et al.</i> <sup>119</sup>
NLRC4	?	Sug1	↑NLRC4	Kumar <i>et al.</i> <sup>129</sup>
IFI16	?	ICP0 (HSV-1)	↓IFI16 -AIM2 -NLRC4	Orzalli <i>et al.</i> , <sup>143</sup> Johnson <i>et al.</i> , <sup>144</sup> Kalamvoki <i>et al.</i> , <sup>145</sup> Cuchet-Lourenco <i>et al.</i> <sup>146</sup>
Caspase-1	?	?	↓NLRP3 ↓AIM2 ↓NLRC4	Van Opdenbosch <i>et al.</i> <sup>147</sup>
<i>Deubiquitination</i>				
NLRP3	LRR domain/K63-linked poly-Ub ? (May target pro-IL1 $\beta$ )	BRCC3 (mouse)/BRCC36 (human) A20	↑NLRP3 ↓NLRP3	Py <i>et al.</i> , <sup>46</sup> Lopez-Castejon <i>et al.</i> <sup>148</sup> Vande Walle <i>et al.</i> , <sup>126</sup> Duong <i>et al.</i> <sup>127</sup>

**Table 1 (Continued)**

Post-translational modifications	Modification sites/types	Enzymes/triggers	Effects on inflammasome activation	References
<i>Proteolytic processing</i>				
NLRP1(b) (murine)	Lys44 (mouse)/Pro44 (rat)	LF (anthrax)	↑NLRP1(b)	Chavarria-Smith <i>et al.</i> , <sup>152</sup> Levinsohn <i>et al.</i> , <sup>154</sup> Hellmich <i>et al.</i> , <sup>155</sup> Boyden <i>et al.</i> <sup>153</sup>
NLRP3	Gly493, Gln225 ?	2A and 3C (EV71) Zmp1 (mycobacteria)	↓NLRP3 ↓NLRP3 ↓NLRC4	Wang <i>et al.</i> <sup>161</sup> Master <i>et al.</i> , <sup>180</sup> Wong <i>et al.</i> <sup>69</sup>
<i>S-nitrosylation</i>				
Caspase-1	Cys284	Nitric oxide	↓Inflammasome	Kim <i>et al.</i> <sup>185</sup>
NLRP3	?	Nitric oxide/SNAP	↓NLRP3	Mishra <i>et al.</i> , <sup>186</sup> Hernandez-Cuellar <i>et al.</i> , <sup>187</sup> Guarda <i>et al.</i> , <sup>188</sup> Mao <i>et al.</i> <sup>189</sup>
<i>ADP-ribosylation</i>				
NLRP3	?	CARDS toxin (mycoplasma)	↑NLRP3 -NLRP1	Bose <i>et al.</i> <sup>194</sup>

Note: ↑, stimulate inflammasome activation; ↓, suppress inflammasome activation; -, no effects on inflammasome activation; ?, not determined or conflicting reports on inflammasome regulation.

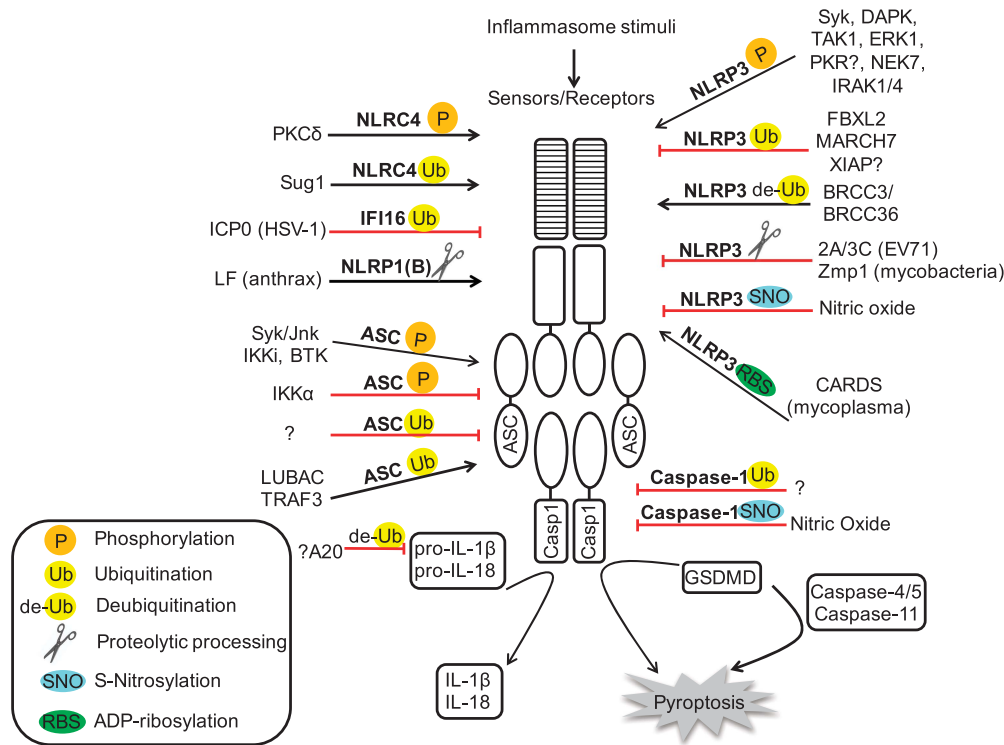
This dephosphorylation event appears to be unique to NLRP3 because activation of the AIM2 inflammasome is not affected by the PP2A knockdown.<sup>64</sup> Therefore, activation of the AIM2 inflammasome may be preceded by other mechanisms that inactivate IKK $\alpha$ . In strong contrast to IKK $\alpha$ , the IKK-related kinase (IKKi) phosphorylates ASC at Ser58, which facilitates its nucleus-to-cytoplasm translocation and promotes inflammasome activation.<sup>64</sup> Deficiency of IKKi results in reduced activation of both the NLRP3 and AIM2 inflammasomes. How these two related kinases modulate inflammasome activation in completely different manners remains to be determined but may be related to their participation in distinct signaling pathways: a main difference between the functions of IKK $\alpha$  and IKKi is that the former is primarily involved in activation of the NF- $\kappa$ B pathway, whereas the latter is implicated in activation of the interferon response factor (IRF) 3 and 7.<sup>65</sup>

#### Multiple kinases regulate the NLRP3 inflammasome

The NLRP3 inflammasome responds to structurally and chemically diverse stimuli from infections and tissue damage.<sup>6,55</sup> Similar to ASC, NLRP3 has been reported to be phosphorylated by Syk, which is essential for the activation of the NLRP3 inflammasome upon infection by fungal, malarial and mycobacterial pathogens. For example, activation of the NLRP3 inflammasome by the fungal pathogens *Candida albicans* or *Aspergillus fumigatus*, but not by *Salmonella* infection or nigericin treatment, was shown to be dependent on Syk kinase, which induces ROS production and potassium efflux.<sup>66,67</sup> Similarly, Syk was phosphorylated and activated by Src kinases upon exposure to hemozoin during *Plasmodium* infection, perhaps with the effect of initiating immune

responses through the NLRP3 inflammasome.<sup>68</sup> In addition, activation of the NLRP3 inflammasome by the ESX-1 secretion system and its substrate ESAT-6 from *Mycobacterium tuberculosis* was reported to be dependent on Syk kinase activity.<sup>69</sup> It is not clear from these studies whether Syk directly phosphorylates NLRP3, although conceivably the phosphorylation of ASC by Syk could be one of the mechanisms promoting inflammasome activation, as discussed above.<sup>57,58</sup>

Similar to Syk, DAPK kinase was shown to associate with NLRP3 and contribute to NLRP3 inflammasome assembly.<sup>70</sup> However, macrophages deficient in DAPK still retained residual caspase-1 activation and IL-1 $\beta$  maturation in response to stimulation by monosodium urate (MSU) and ATP. Because the kinase-inactive DAPK mutant was also capable of enhancing IL-1 $\beta$  secretion, it appears that the role of DAPK in the NLRP3 inflammasome activation is independent of its kinase activity. In agreement with this concept, the NLRP3 LRR was shown to co-IP with the ankyrin repeats, death domain or cytoskeleton-binding region of DAPK rather than with its kinase domain.<sup>70</sup> Another kinase implicated in NLRP3 regulation is TAK1. Through screening of natural products and testing of kinase inhibitors, Gong *et al.*<sup>71</sup> identified TAK1 as a kinase that promotes NLRP3 inflammasome activation in a manner independent of its role in the activation of gene expression. It was suggested that TAK1 may regulate the NLRP3 inflammasome through phosphorylation of NLRP3 or other components of the inflammasome complex. Similarly, the ERK1 kinase-mediated phosphorylation events were reported to be essential for priming of the NLRP3 inflammasome.<sup>72</sup> However, whether NLRP3 is phosphorylated by these kinases was not addressed in the studies described above.



**Figure 1** Combinatorial post-translational modifications regulate inflammasomes. Phosphorylation, ubiquitination, de-ubiquitination, proteolytic processing, S-nitrosylation and ADP-ribosylation are indicated according to the keys in the lower left corner. The PTMs that promote activation of inflammasomes are shown as black arrows, whereas those that suppress inflammasome activation are shown as red blocks.

PKR is a dsRNA-dependent protein kinase. It undergoes autophosphorylation upon inflammasome activation and is broadly implicated in promoting activation of the NLRP3, NLRP1, NLRC4 and AIM2 inflammasomes, but not the NOD1, NLRP12 or NLRX1 inflammasomes.<sup>73–75</sup> PKR is physically associated with NLRP3 in human THP-1 cells, and co-immunoprecipitation experiments in which PKR and NLRP3 were overexpressed in HEK293 cells showed that the PYD, NOD and LRR domains of NLRP3 were associated with PKR.<sup>73</sup> By comparing the wild type with the mutant PKR, Lu *et al.* showed that the kinase activity of PKR is required for the activation of the NLRP3 inflammasome. In contrast, Hett *et al.*<sup>75</sup> demonstrated that known PKR inhibitors did not reduce the activation of NLRP3 or NLRP1 inflammasomes. In agreement with these results, He *et al.*<sup>76</sup> demonstrated that although PKR enhanced the generation of iNOS and bacteria-killing by macrophages, it was not required for activation of the NLRP3, NLRC4 and AIM2 inflammasomes in terms of caspase-1 activation and IL-1 $\beta$ /IL-18 processing. Furthermore, Yim *et al.*<sup>77</sup> showed that the kinase activity of PKR suppressed NLRP3 inflammasome activation by inhibiting the expression of NLRP3 and pro-IL-1 $\beta$ . It was proposed that these apparent discrepancies may be due to the use of different macrophage cells or cell lines, as well as different mouse strains, and to the multiple effects of PKR on protein expression and signaling.<sup>77</sup> Clearly, the mechanisms of PKR regulation of inflammasomes remain to be further clarified.

The IL-1R-associated kinases IRAK1 and IRAK4 function downstream of the TLRs in response to priming signals that enhance the transcription of inflammasome components.<sup>44</sup> Fernandes-Alnemri *et al.*<sup>78</sup> showed that these two kinases were also implicated in the rapid activation of NLRP3 by *Listeria monocytogenes* within 45 min of infection. They further demonstrated that this rapid NLRP3 activation was independent of transcription but required generation of reactive oxygen species. Although co-immunoprecipitation experiments have shown that IRAK1 associates with NLRP3, it is not clear whether its kinase activity is required for this rapid NLRP3 activation or whether any of the NLRP3 inflammasome components are phosphorylated by IRAK1.<sup>78</sup>

Recently, the serine–threonine kinase NEK7 was identified by three independent groups as being essential for activation of the NLRP3 inflammasome, but not the AIM2 or NLRC4 inflammasomes.<sup>79–81</sup> Diverse screening approaches were used by these groups, including forward genetic screening for *N*-ethyl-*N*-nitrosourea-induced mutations,<sup>79</sup> genome-wide CRISPR (clustered regularly interspaced short palindromic repeats) screening in macrophages<sup>80</sup> and proteomic approaches using a tagged NLRP3 protein.<sup>81</sup> The interaction of NEK7 with NLRP3 is dependent on the LRR and perhaps the NOD domain of NLRP3 and the kinase domain of NEK7. However, mutation of the NEK7 catalytic residues K63, K64 or G43 did not impair IL-1 $\beta$  secretion or the NEK7–NLRP3 interaction.<sup>79,81</sup> The NEK7–NLRP3 association is necessary

for the recruitment of ASC by NLRP3 and the formation of ASC oligomers. In addition, activation of the NLRP3 inflammasome is blocked during mitosis, which suggests that NEK7 acts as a switch between inflammasome activation and cell division. Its kinase activity is dispensable for the former but required for the latter.<sup>79,82–84</sup>

### The role of phosphorylation in NLRC4 inflammasome activation

NLRC4 is one of the key inflammasome sensors that is responsible for the immune response to bacterial infections.<sup>20–22,85</sup> Gain-of-function mutations in NLRC4 also lead to autoinflammatory disorders in patients.<sup>86–88</sup> Members of the family of NLR apoptosis-inhibitory proteins (NAIPs) are direct receptors for bacterial flagellin and type III secretion systems.<sup>89–95</sup> Ligand-bound NAIPs induce a wheel-like assembly of the NAIP-NLRC4 inflammasomes that may catalyze the activation of oligomerized caspase-1.<sup>96–99</sup> An important regulatory mechanism for NLRC4 was revealed through the discovery that mouse NLRC4 in macrophages is phosphorylated following infection with *Salmonella typhimurium*.<sup>100</sup> The phosphorylation site Ser533 is conserved among the NLRC4 proteins from different species, which suggests that it is potentially important for the structure or function of NLRC4. Biochemical characterization of kinase activities, screening of kinase inhibitors and *in vitro* phosphorylation assays identified PKC $\delta$  as the kinase that phosphorylates the Ser533 residue in mouse NLRC4.<sup>100</sup> Importantly, the S533A mutant was not able to activate the NLRC4 inflammasome, whereas the phosphomimetic NLRC4 S533D mutant induced rapid pyroptosis in macrophages in the absence of infection. However, PKC $\delta$ -deficient macrophages retained residual inflammasome activation upon *S. typhimurium* infection, which suggests that other kinases provided compensatory mechanisms. Matusiak *et al.*<sup>101</sup> further demonstrated that phosphorylation of NLRC4 primed the inflammasome for activation. However, the detection of bacteria by NAIP5 was independent of NLRC4 phosphorylation, although both required the D0 domain of the bacterial flagellin. In contrast to the studies described above, reconstitution of the NAIP–NLRC4 complexes in the presence of bacterial flagellin or needle proteins demonstrated that the phosphorylation of NLRC4 is not required for assembly of the inflammasome signaling complex.<sup>93</sup> In addition, crystallization of an apparently inactive form of NLRC4 in which Ser533 was already phosphorylated has been achieved.<sup>98</sup> In a third study, PKC $\delta$  did not appear to play any role in NLRC4 activation upon *Shigella* or *Salmonella* infection.<sup>94</sup> The reasons for these apparent contradictory results are not clear, and further studies are required to clarify the mechanisms of NLRC4 phosphorylation and the role of PKC $\delta$  in inflammasome activation.

### UBIQUITINATION

Protein ubiquitination and deubiquitination are versatile and dynamic processes that regulate the degradation, trafficking and signaling properties of the proteome in numerous aspects of the biological processes.<sup>102–105</sup> Conjugation of the ubiquitin

chains is mediated by an E1–E2–E3 cascade involving ~40 E2 and 600 E3 enzymes, whereas ~100 deubiquitinating enzymes (DUBs) catalyze the removal or editing of ubiquitin chains. The distinct surfaces of the various ubiquitin chain types act as three-dimensional ‘codes’ that are ‘read’ by more than 20 ubiquitin-binding domains to facilitate the assembly of diverse protein interaction networks.<sup>106,107</sup> Thus, the ubiquitination, deubiquitination and ubiquitin recognition events involving inflammasome components constitute highly diverse processes that significantly expand the complexity of inflammatory signaling.

### ASC ubiquitination modulates inflammasome activation

In addition to being phosphorylated at multiple sites, ASC is also ubiquitinated. Shi *et al.*<sup>43</sup> reported that activation of the AIM2 inflammasome with poly(dA:dT) resulted in K63-linked polyubiquitination of ASC in macrophages, which led to its recruitment to the autophagy pathway that degrades the inflammasome. This was proposed to be a mechanism to prevent excessive inflammation by eliminating activated inflammasomes. In contrast, Rodgers *et al.*<sup>47</sup> showed that the linear ubiquitin assembly complex (LUBAC) promotes ASC-dependent inflammasome activation through linear ubiquitination of ASC. LUBAC is an ubiquitin ligase complex composed of HOIL-11, HOIP and SHARPIN. HOIL-11-deficient bone marrow-derived macrophages demonstrated reduced production of mature IL-1 $\beta$  upon stimulation of NLRP3 and AIM2, but NF- $\kappa$ B activation was not affected. Multiple domains of HOIL-11, including the HOIP-binding, ubiquitin-binding and ubiquitination ligase domains, are required for robust ubiquitination of ASC, formation of ASC speck, and promotion of NLRP3 inflammasome activation. Of note, the suppressive effects on NLRP3 were more severe than those on AIM2, and the IL-1 $\beta$  secretion resulting from NLRC4 stimulation was not affected by the HOIL-11 deficiency. These results suggest a specific role for HOIL-11 in NLRP3 inflammasome regulation. However, *in vitro* ubiquitination experiments demonstrated that ASC is directly linear ubiquitinated by LUBAC, whereas NLRP3 was not. It is likely that LUBAC also regulates other factors specific for the NLRP3 pathway to modulate its activation.

The apparent contradictions between the studies by Shi and Rodgers regarding the roles (suppressive versus stimulatory) of ASC ubiquitination in inflammasome regulation have not been resolved, although it was suggested that the apparent differences in the ubiquitination of ASC (linear vs K63-linked) between these studies may be the result of cross-detection of linear ubiquitination by K63-ubiquitin antibodies.<sup>108</sup> It remains to be determined how NLRP3-specific stimuli trigger linear ubiquitination of ASC to promote inflammasome activation. Importantly, the *in vivo* role of LUBAC in inflammasome activation was demonstrated through the observation that HOIL-11 was essential for IL-1 $\beta$  secretion and neutrophil recruitment in an MDP-induced peritonitis model, as well as in an LPS-induced lethal inflammation model that is dependent on the NLRP3 inflammasome.<sup>47</sup>

Another E3 ubiquitin ligase, TNFR-associated factor 3 (TRAF3), was suggested to target ASC for K63-linked ubiquitination upon infection with RNA viruses, such as vesicular stomatitis virus (VSV) or influenza virus.<sup>109</sup> The mitochondrial antiviral signaling protein (MAVS) serves as a platform that recruits both TRAF3 and ASC to promote TRAF3-mediated K63-linked ubiquitination of ASC at residue Lys174. In fact, MAVS also colocalizes with NLRP3 within 2 h of VSV infection, although the MAVS–NLRP3 interaction appears to be independent of the MAVS–TRAF3 association. MAVS-mediated ubiquitination of ASC enhances activation of the NLRP3 inflammasome in response to VSV infection, but not following exposure to crystalline materials, such as MSU crystals. Furthermore, MAVS does not enhance activation of the AIM2 or NLRC4 inflammasomes. Clearly, infections by RNA viruses that activate the RIG-I pathway may stimulate MAVS-mediated ubiquitination of ASC and activation of the NLRP3 inflammasome. Whether RIG-I has a role in this ubiquitination event or whether production of interferon by the RIG-I pathway promotes NLRP3 activation remains to be determined.

#### Ubiquitination of NLRP3 negatively regulates inflammasome activation

The potential role of ubiquitination in inflammasome regulation was inferred from the observation that the ubiquitin ligase-associated protein SGT1 is associated with a number of NLR proteins, including NLRP2, NLRP3, NLRP4, NLRP12, NLRC4, NOD1 and NOD2.<sup>110</sup> In particular, binding of SGT1 to the LRR domain of NLRP3 was essential for maintaining its inactive but signaling-competent state.<sup>110</sup> This is analogous to the essential role of plant SGT1 in activation of the nucleotide-binding site/leucine-rich repeat (NBS–LRR) proteins for host protection.<sup>111</sup> However, the specific roles of ubiquitination in the functions of any of the NLR/NBS–LRR proteins were not demonstrated in the above study.

Recently, one of the E3 ubiquitin ligases for NLRP3 was identified as the Skp–Cullin–F box (SCF) family member, F-box L2 (FBXL2), which mediates the ubiquitination and proteasomal degradation of NLRP3.<sup>112</sup> FBXL2 is in turn regulated by another F-box protein, F-box O3 (FBXO3), through its own ubiquitination and degradation. A small molecule FBXO3 inhibitor, BC-1215, was shown to release the inhibition of FBXL2 and reduce NLRP3-mediated inflammation. FBXL2 binds Trp73 in the PYD domain and Lys689 in the LRR domain of NLRP3, the latter of which is a ubiquitination site. The authors argue that rather than enhancing the expression of NLRP3, the role of LPS priming is to reduce the ubiquitin-mediated proteasomal degradation of NLRP3 via the elevated expression of FBXO3.

Another ubiquitination-mediated process in the regulation of the NLRP3 inflammasome was identified through the dopamine D1 receptor DRD1 pathway.<sup>113</sup> The neurotransmitter dopamine activates the DRD1 signaling pathway to produce the second messenger cyclic adenosine monophosphate (cAMP). Binding of cAMP to NLRP3, perhaps in conjunction

with activation of other pathways by cAMP, promotes Lys48-linked polyubiquitination of NLRP3 by the E3 ubiquitin ligase MARCH7. The ubiquitinated NLRP3 is subsequently degraded through the autophagy pathway. This ubiquitination-mediated regulation by dopamine and its receptor is specific for the NLRP3 inflammasome and does not affect the AIM2 or NLRC4 inflammasomes. Furthermore, *in vivo* experiments demonstrated that the dopamine signaling pathway suppressed neurotoxin-induced inflammation (a model of Parkinson's disease), lipopolysaccharide-induced systemic inflammation and MSU-induced peritonitis.<sup>113</sup> This suggests that DRD1 agonists may have therapeutic value in NLRP3-driven inflammatory diseases. The role of ubiquitination in regulating the NLRP3 degradation was further demonstrated through the study of the autophagy chaperone p62. P62 was shown to colocalize with a detergent-insoluble polyubiquitinated protein aggregates containing NLRP3 and to promote the degradation of NLRP3.<sup>114</sup> P62 also promotes the degradation of damaged mitochondria through mitophagy, thus reducing the release of NLRP3-activating stimuli from mitochondria.<sup>115</sup> In agreement with these results, p62 deficiency in mice results in an increased atherosclerotic plaque burden<sup>114</sup> and elevated inflammation in alum-induced peritonitis and fulminant hepatitis.<sup>115</sup>

In addition to FBXL2 and MARCH7, another E3 ubiquitin ligase, X-linked inhibitor of apoptosis protein (XIAP), has been implicated in regulation of the NLRP3 inflammasome. XIAP is a member of the IAP family that inhibits caspases and suppress apoptotic cell death.<sup>116</sup> The C-terminal RING domain of XIAP harbors an E3 ubiquitin ligase activity that is required for NOD2 signaling.<sup>117,118</sup> It was reported that the loss of XIAP or its RING domain resulted in excessive cell death or elevated secretion of IL-1 $\beta$ , although the cell death appeared to be independent of caspase-1, -11 or -8.<sup>119</sup> Currently, it is not clear whether XIAP directly ubiquitinates NLRP3 or whether its RING domain mediates ubiquitination of any other inflammasome components.

Similar to the Guan study of the MAVS–ASC interaction, MAVS was reported to promote NLRP3 inflammasome activation through the association of MAVS with NLRP3.<sup>120</sup> The residues 2–7 (KMASTR) within the PYD domain of NLRP3 are essential for this interaction. The recruitment of NLRP3 to the mitochondria by MAVS may promote formation of the ASC speck,<sup>120</sup> oligomerization of NLRP3 and/or stimulation of the mitochondrial formation of reactive oxygen species.<sup>121</sup> The role of ubiquitination was not explored in this study, although, presumably, TRAF3 may be recruited by MAVS, as shown in the Guan study,<sup>109</sup> in which TRAF3 was found to be located in close proximity to NLRP3, which harbors multiple potential ubiquitination sites. Similar to the Guan study,<sup>109</sup> MAVS is required for optimal stimulation of the NLRP3 inflammasome by poly (I:C)<sup>120</sup> or Sendai virus infection,<sup>121</sup> but not by crystalline substances, such as alum or MSU.<sup>120</sup> These similarities suggest potential cross-regulation of the RIG-I and inflammasome pathways. In agreement with these results, association of MAVS with NLRP3 suppressed the

MAVS-induced type I interferon response to Sendai virus infection.<sup>121</sup>

A20 is an ubiquitin-editing enzyme that is involved in negative feedback regulation of NF- $\kappa$ B signaling.<sup>122–125</sup> Independent of its role in NF- $\kappa$ B regulation, A20 was recently reported to be an inhibitor of the NLRP3, but not NLRC4 or AIM2, inflammasome.<sup>126,127</sup> The significance of this inflammasome regulatory function is borne out by the observations that A20 deficiency in mice leads to pathological inflammation that resembles rheumatoid arthritis<sup>126</sup> and that heterozygous germline mutations of A20 in humans lead to early onset systemic inflammation.<sup>128</sup> Mechanistically, the suppression of NLRP3 by A20 was dependent on TRIF and RIPK3. Furthermore, A20 may reduce the ubiquitination of pro-IL-1 $\beta$  at its conserved residue K133, thus impacting its maturation by caspases or secretion through the autophagy pathway.<sup>127</sup>

#### Ubiquitination of NLRC4 activates the inflammasome

Sug1 is a regulatory protein with ubiquitin ligase activity that is associated with the 26S proteasome and has been identified as a binding partner for NLRC4 through yeast two-hybrid assays.<sup>129</sup> Sug1 binds residues 91–253 of NLRC4, which include its CARD-NOD linker region and NOD domain. The Sug1–NLRC4 interaction releases its autoinhibited conformation, followed by ubiquitination and aggregation of NLRC4 that in turn leads to recruitment and activation of caspase-8. However, the site and type of ubiquitination of NLRC4 have not yet been characterized. In addition, it is not clear how the ubiquitinated NLRC4 recruits caspase-8, although a potential adapter molecule that bridges the ubiquitinated NLRC4 and caspase-8 has been proposed.<sup>129</sup>

#### Ubiquitination and degradation of IFI16 mediated by viral E3-ubiquitin ligase

IFI16 belongs to the p200 family of interferon-inducible proteins and the PYHIN family.<sup>130,131</sup> As a PRR, it functions as a DNA sensor in the cytosol and nucleus that induces interferon production. It also induces the assembly of inflammasome complexes in response to DNA viruses, such as herpes simplex virus 1 (HSV-1),<sup>31</sup> Kaposi's sarcoma-associated herpesvirus (KSHV)<sup>32</sup> and Epstein–Barr virus,<sup>132</sup> as well as retroviruses such as HIV-1.<sup>133,134</sup> Interferon production downstream of IFI16 is dependent on the endoplasmic reticulum adapter molecule stimulator of interferon genes (STRING), which is also known as mediator of IRF3 activation (MITA), endoplasmic reticulum IFN stimulator (ERIS) or met-pro-tyr-ser (MPYS),<sup>135–138</sup> and an essential transcription factor IRF-3.<sup>139</sup> IFI16 is known to form an inflammasome complex with the adapter ASC in the nucleus and translocate to the cytoplasm to activate caspase-1.<sup>32</sup> In addition to directly binding to DNA, IFI16 and its murine ortholog p204 were recently shown to cooperate with another DNA sensor, cyclic GMP–AMP synthase,<sup>140</sup> to recognize DNA from HSV-1 and *Francisella* infection in human foreskin fibroblasts and macrophages, respectively.<sup>141,142</sup> To counter IFI16-mediated immune responses, HSV-1 expresses an immediate-early protein, infected

cell protein 0 (ICP0), which contains a RING finger domain with E3-ubiquitin ligase activity. Within a few hours after HSV-1 infection, ICP0 inhibits the nuclear localization of activated IRF-3 and promotes the ubiquitination and proteasome-dependent degradation of IFI16 and p204.<sup>143–145</sup> The specific types of IFI16 ubiquitination have not been characterized.<sup>144</sup> ICP0-mediated degradation is specific for IFI16 on the basis that the NLRP3 and AIM2 inflammasomes are not affected.<sup>144</sup> In contrast to the studies described above, another report did not observe IFI16 degradation upon expression of ICP0 in the absence of HSV-1 infection,<sup>146</sup> which suggests that factors other than ICP0 may be responsible for IFI16 regulation following HSV-1 infection. The reasons for such seemingly contradictory results are not clear but may be attributed to differential degradation of IFI16 in various cell types.

#### Ubiquitination of caspase-1 upon inflammasome activation

While investigating activation of the Nlrp1b inflammasome by the anthrax lethal toxin, Lamkanfi and colleagues noticed the presence of high-molecular-weight species in immunoblots of macrophage cell lysates for caspase-1.<sup>147</sup> This was interpreted as indicative of caspase-1 post-translational modifications, which was confirmed by immunoblotting for ubiquitin. Importantly, high-molecular-weight caspase-1 species were also present following activation of the NLRP3 inflammasome by ATP or nigericin, the AIM2 inflammasome by *Francisella* infection and the NLRC4 inflammasome by *Salmonella* infection. The apparent post-translational modification of caspase-1 required its enzymatic activity on the basis that treatment with the caspase-1 inhibitor YVAD-cmk significantly reduced the formation of these high-molecular-weight species. These results also suggest that caspase-1 ubiquitination occurs downstream of caspase-1 activation. The authors proposed that caspase-1 ubiquitination may represent a negative feedback mechanism that dampens inflammatory signaling by targeting activated caspase-1 for degradation. However, it is currently not clear whether ubiquitinated caspase-1 is destined for degradation because treatment with the proteasome inhibitor MG132 suppressed rather than enhanced caspase-1 activation.<sup>147</sup> Future studies are required to clarify the fate of ubiquitinated caspase-1.

#### Deubiquitination of NLRP3 promotes inflammasome activation

Observation of NLRP3 ubiquitination suggested that deubiquitination was a likely mechanism for inflammasome regulation. Lopez-Castejon *et al.*<sup>148</sup> identified a DUB inhibitor b-AP15 that inhibited activation of the NLRP3 inflammasome and partially inhibited activation of the AIM2 inflammasome. The authors concluded that multiple as-yet uncharacterized DUBs may be required for IL-1 $\beta$  processing and release. Employing an NLRP3 ubiquitination assay, Yuan and colleagues identified a DUB/isopeptidase inhibitor, G5, that suppressed IL-1 $\beta$  secretion downstream of the NLRP3 inflammasome in macrophages, but not downstream of the AIM2 or NLRC4



inflammasomes.<sup>46</sup> G5 treatment enhanced polyubiquitination of the NLRP3 NOD and LRR domains, but not the PYD domain, and suppressed the formation of ASC oligomers. Subsequently, the deubiquitination enzyme BRCC3 (mouse)/BRCC36 (human) was identified, which coimmunoprecipitated with the NOD and LRR domains of NLRP3 and deubiquitinated its LRR.<sup>46</sup> BRCC3 is a JAMM domain-containing Zn<sup>2+</sup> metalloprotease that specifically cleaves Lys63-linked, but not Lys48-linked, polyubiquitin chains.<sup>149</sup> Currently, it is unclear how NLRP3 stimuli activate BRCC3. Nonetheless, deubiquitination-dependent activation of NLRP3 may be a potential target for therapeutic modulation of inflammatory pathogenesis that involve NLRP3.

### PROTEOLYTIC PROCESSING OF INFLAMMASOME COMPONENTS

In addition to phosphorylation and ubiquitination, cleavage of inflammasome sensor proteins by proteases from bacteria or the host and complex interactions among proteases, their substrates and protease inhibitors have crucial roles in regulating inflammasome activation. In fact, serine protease inhibitors were shown to suppress activation of both NLRC4 and NLRP3 inflammasomes. These results contrast with those for pan-cathepsin inhibitors, which were ineffective, suggesting that proteolytic processing is an integral component of inflammasome regulation.<sup>150,151</sup> In contrast to phosphorylation and ubiquitination, proteolytic processing is an irreversible PTM process that generates new protein isoforms.

#### Anthrax protease activates the Nlrp1b inflammasome

Both mouse Nlrp1b and rat Nlrp1 were shown to be cleaved by the anthrax lethal factor (LF), a zinc metalloprotease that is part of the anthrax lethal toxin.<sup>152–155</sup> The cleavage site is located in the N-terminal region of these proteins, outside of the predicted NOD domain. This cleavage event was sufficient for Nlrp1b activation, implying that Nlrp1b can serve as a sensor for other proteases from diverse pathogens or even host cells. Mechanistically, protease cleavage may release an auto-inhibited conformation of Nlrp1b to facilitate assembly of the inflammasome complex.<sup>156</sup> Alternatively, the cleaved Nlrp1b may recruit unknown binding partners that may in turn promote inflammasome activation. Whether such mechanisms apply to human NLRP1 protein remains to be determined. Compared with murine Nlrp1 proteins, human NLRP1 contains an additional PYD domain at its N terminus that may endow it with distinct activation mechanisms. In addition, the LF cleavage sites of murine Nlrp1 proteins have poor sequence conservation: murine Nlrp1b is cleaved at a Lys-Leu sequence, whereas rat Nlrp1 is cleaved at a Pro-Leu sequence. Therefore, it remains to be determined whether the human NLRP1 protein is also cleaved by the anthrax LF protease or other microbial proteases. It is interesting to note that such mammalian immune responses to proteolytic events are analogous to the recognition of modifications of host proteins by plant resistance (R) proteins, the majority of which are also NLR

proteins. This suggests that recognition of proteolytic events is an ancient mechanism of immune protection.<sup>157–159</sup>

#### Enterovirus proteases suppress the NLRP3 inflammasome

The enterovirus 71 (EV71) is responsible for many epidemics in the world and is manifested by hand, foot and mouth disease, and neurological complications in children.<sup>160</sup> Following-up on the observation that EV71-infected patients, particularly those with neurological complications, showed elevated levels of IL-1 $\beta$ , Wang *et al.*<sup>161</sup> identified NLRP3 as the inflammasome that is activated upon EV71 infection. Conversely, EV71 encodes two proteases, 2A and 3C, that specifically cleave NLRP3 at G493-L494 and Q225-G226, respectively, thus antagonizing the NLRP3 inflammasome, but not other inflammasomes. Both cleavage sites are located within the predicted NOD domain of NLRP3, suggesting that this cleavage may prevent NLRP3 oligomerization and/or assembly of the inflammasome complex. In fact, both of the EV71 proteases have been shown to antagonize innate immune responses through cleavage of antiviral signaling proteins, such as MAVS<sup>162</sup> and IRF7.<sup>163</sup>

#### Mycobacterial Zmp1 protease reduces inflammasome activation

Infection of macrophages by *Mycobacterium tuberculosis* (Mtb) leads to formation of granulomas, in which the bacteria become latent, often for the lifetimes of infected individuals.<sup>164</sup> Reactivation of latent infections can be triggered under immunosuppressive conditions, and under these conditions, active tuberculosis ensues. IL-1 and the NLRP3<sup>165–173</sup> and AIM2<sup>174–177</sup> inflammasomes have been shown to have important roles in the host response to mycobacterial infection. However, there is controversy regarding the protective role of the NLRP3 inflammasome.<sup>69,167,170,178,179</sup> Mycobacterial species encode a zinc metalloprotease, Zmp1, that was shown to inhibit activation of the NLRP3 and NLRC4 inflammasomes on the basis that compared with the wild type, an *M bovis* BCG strain deficient in Zmp1 induced significantly higher levels of IL-1 $\beta$  and phagolysosome biogenesis in murine macrophages.<sup>180</sup> However, whether Zmp1 cleaves components of the inflammasomes was not clarified. In addition, suppression of the inflammasomes was not reproduced upon infection of human macrophages with *Mycobacterium tuberculosis* strain H37Rv.<sup>69</sup> It is possible that the proteases encoded by different mycobacterial strains may antagonize inflammasomes through distinct mechanisms.

### OTHER POST-TRANSLATIONAL MODIFICATIONS OF NLRP3

#### S-nitrosylation suppresses the NLRP3 inflammasome

Nitric oxide (NO) was shown to inhibit caspases-1, 2, 3, 4, 6, 7 and 8 through S-nitrosylation of the caspase catalytic cysteine residue.<sup>181–184</sup> As a result, NO or its reaction products suppressed caspase-1 activity and inhibited the release of IL-1 $\beta$  and IL-18 from activated macrophages.<sup>185</sup> In addition, the NO donor S-nitroso-N-acetylpenicillamine (SNAP) inhibited

caspace-1 activity *in vitro*, and treatment with the inducible NO synthase (iNOS) inhibitor  $N^G$ -monomethyl-L-arginine significantly enhanced IL-1 $\beta$  secretion from wild type, but not iNOS-deficient, macrophages and mice. Using purified recombinant caspace protein, inhibition of caspace-1 activity was shown to be reversible by treatment with dithiothreitol (DTT), which suggested that the caspace catalytic cysteine residue is S-nitrosylated by NO. However, DTT did not completely reverse the effects of NO treatment when macrophage cell lysates were used. This suggests that modification of the caspace catalytic cysteine residue may not be the sole mechanism underlying the inflammasome regulation mediated by NO.<sup>185</sup>

In this context, a recent study confirmed that IFN- $\gamma$ -stimulated NO production mediated the suppression of IL-1 $\beta$  secretion by *Mycobacterium tuberculosis*-infected macrophages.<sup>186</sup> This was proposed as a regulatory mechanism that would reduce the destructive innate inflammatory responses elicited during chronic mycobacterial infection. Mechanistically, NO or SNAP treatment leads to S-nitrosylation of NLRP3, but not AIM2. The resulting S-nitrosylation results in suppression of ASC oligomerization and IL-1 $\beta$  processing. Related studies reported that secretion of mature IL-1 $\beta$  and IL-18 by ATP or nigericin-stimulated macrophages was significantly reduced upon treatment with SNAP, whereas only partial reduction was observed upon stimulation of the AIM2 or NLRC4 inflammasomes.<sup>187–189</sup> SNAP treatment for 1 h leads to S-nitrosylation of both caspace-1 and NLRP3,<sup>187</sup> which implicates direct modification of NLRP3 by S-nitrosylation as the main regulatory mechanism induced by NO. NLRP3 is extremely rich in cysteine residues. Exactly which cysteine residues are the functional targets of S-nitrosylation remains to be determined. Interestingly, a group of naturally occurring compounds, ginsenosides, were shown to reduce S-nitrosylation of NLRP3 by reducing iNOS expression.<sup>190</sup> This was proposed as a mechanism by which ginsenosides can protect against LPS-induced endotoxic shock.

Activation of inflammasomes has been shown to promote NO production, perhaps as a negative feedback mechanism. Both activation of the NLRP3 inflammasome upon *Leishmania* infection<sup>191</sup> and stimulation of the NLRC4 inflammasome by intracellular flagellin<sup>192</sup> were shown to activate iNOS and the potential S-nitrosylation of inflammasome components. This mitigate the detrimental effects of uncontrolled inflammation.

#### ADP-ribosylation of NLRP3 triggers robust inflammation and cytopathology

*Mycoplasma pneumoniae* is a common airway pathogen. Although infection by mycoplasma rarely results in death, the hyperinflammatory conditions that manifest in response to mycoplasma infection have been associated with chronic pulmonary dysfunction, such as bronchial asthma, chronic obstructive pulmonary disease and cystic fibrosis.<sup>193</sup> Therefore, immunomodulatory therapeutics in combination with conventional antimicrobial therapeutics are recommended in the management of mycoplasma pneumonia. A mycoplasma toxin observed in community-acquired respiratory distress syndrome

(CARDS) is an ADP-ribosyltransferase and was shown to colocalize with NLRP3 and to catalyze its ADP-ribosylation.<sup>194</sup> This post-translational modification is essential for NLRP3 activation upon mycoplasma infection of human and mouse macrophages and may account for the hyperinflammatory lung pathologies manifested in the clinic. Importantly, the role of the CARDS toxin as an inflammation-promoting soluble factor is consistent with its ability to trigger allergic reactions in the airway long after the primary infection by mycoplasma subsides.<sup>195</sup> As such, the CARDS toxin represents an important therapeutic target for modulating lung inflammation upon mycoplasma infection. The suppressive effect of the CARDS toxin was specific for the NLRP3, but not NLRP1, inflammasome and was dependent on both the N-terminal catalytic domain of the CARDS toxin and its C-terminal domain, which is responsible for its attachment to host cells and subsequent internalization. Although the specific domain of NLRP3 that is modified by the CARDS toxin was not defined, it was suggested that the CARDS toxin may alter the conformation of NLRP3 or its interaction with other partner proteins during assembly of the inflammasome.<sup>194</sup> Detailed mechanisms regarding the CARDS toxin–NLRP3 interaction still await future investigations.

#### DISCUSSION

Here we have focused on our evolving understanding of how diverse yet highly specific post-translational modifications regulate inflammasome activation. Because these post-translational modifications are dependent on enzymatic activities, they represent attractive therapeutic targets for modulation of inflammasome activities in a broad range of inflammatory pathologies. It is clear that a repertoire of post-translational modifications is involved in the precise regulation of inflammasomes. These combinatorial modifications regulate inflammasomes by changing the conformations of inflammasome proteins and by controlling their turnover, distribution and location, as well as by regulating the intricate protein–protein interactions essential for inflammasome assembly.

Various post-translational modifications in inflammasome signaling can be cross-regulated by each other, perhaps within the context of different cell types. The most prominent crosstalk is between phosphorylation and ubiquitination, which can positively or negatively regulate each other.<sup>51,196</sup> Phosphorylation and ubiquitination are among the most abundant PTMs involving hundreds of enzymes that catalyze both the attachment and the removal of phosphate/ubiquitin. This highly complex network of inflammasome regulation may contribute to the ability of inflammasomes to respond to various stimuli. How these multiple PTMs spatially and temporarily coordinate during the process of specific inflammasome activation in a particular cell type will be an important topic for further studies. For example, the phosphorylation of ASC by Syk kinase is required for ASC speck formation and caspace-1 activation in macrophages, but not in bone marrow-derived dendritic cells.<sup>57,58</sup> In addition, proteolytic cleavage of GSDMD and the subsequent pyroptotic cell death of macrophages was

not observed in neutrophils upon activation of the NLR4 inflammasome, although mature IL-1 $\beta$  was produced.<sup>40–42</sup> It is clear that to appreciate the full scope of inflammasome regulation, studies of cell-type-specific PTMs and exploration of additional PTMs are important future areas of investigation.

Understanding the mechanisms of PTMs in inflammasome regulation may offer tremendous opportunities for the development of therapeutic agents to target inflammatory conditions, such as autoinflammatory disorders, metabolic disorders, neurodegenerative diseases and cardiovascular disorders. For example, some of the specific kinase inhibitors that are developed for cancer treatment can be repurposed as agents to treat inflammatory disorders. The dopamine signaling pathway, which triggers ubiquitination and inhibition of the NLRP3 inflammasome, ameliorated neuro-inflammation in a mouse model of Parkinson's disease and systemic inflammation in a mouse model of sepsis.<sup>113</sup> This suggests that an agonist of the dopamine D1 receptor or the downstream E3 ubiquitin ligase MARCH7 may provide therapeutic benefit in the above diseases. Similarly, the G5 and b-AP15 inhibitors<sup>46,148</sup> of DUBs that have previously been shown to block caspase-1 activation and IL-1 $\beta$  maturation may be developed as agents to combat diseases that involve excessive activation of the NLRP3 inflammasome, such as cardiovascular disorders.<sup>197</sup>

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

The work was supported by the Case Research Institute and University Hospitals Case Medical Center fund and the National Institutes of Health (AR069908 to T.S.X.). We thank Dongni Ma for assistance with preparing Figure 1.

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