INFLAMMASOMES AND IL-1 FAMILY CYTOKINES IN SARS-COV-2 INFECTION:

from prognostic marker to therapeutic agent

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ABSTRACT

Despite global vaccination programs, infections with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) continue to cause severe disease with significant morbidity and mortality. Severe coronavirus disease 20219 (COVID-19) is characterized by an exuberant inflammatory response in the lung leading to acute lung injury and consequent gas exchange problems. Complete insights in this hyperinflammatory response are still lacking. However, a thorough understanding of immunopathogenesis of severe COVID-19 is needed to not only develop personalized targeted therapies, but also to identify biomarkers that predict disease outcome and therapeutic responses. Here we review the current evidence that SARS-CoV-2 activates the inflammasome, which is an intracellular multiprotein complex that leads to the activation and secretion of the interleukin (IL)-1 family cytokines, IL-1 and IL-18, and to a lytic form of cell death, called pyroptosis. Further we discuss the contribution of inflammasomes and IL-1 family cytokines to the immunopathogenesis of COVID-19 and its clinical implications.

Introduction

The potential of respiratory RNA viruses, such as influenza viruses and coronaviruses, to adapt and mediate human-to-human transmission and to consequently cause a pandemic, poses a constant and realistic threat to global health. This is illustrated by the current severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic, as well as by previous pathogenic coronavirus and influenza virus outbreaks. To date, SARS-CoV-2 continues to cause morbidity and mortality. Despite a massive global effort to understand the virus and the response of the host to it, there is still an unmet need for more effective therapies to treat the most severe COVID-19 patients. A thorough understanding of the immunopathogenesis of severe coronavirus infections is key for identifying targeted therapies and might also be important for possible future pandemics caused by respiratory RNA viruses.

Severe coronavirus disease-2019 (COVID-19) traditionally follows a biphasic course with an early viral response phase followed by a second hyperinflammatory phase, that usually occurs around 8-10 days after onset of the first symptoms, when viral replication is waning[1,2]. This hyperinflammatory phase is characterized by exuberant production of inflammatory cytokines and chemokines in the lungs, leading to the recruitment of pro-inflammatory cells, further amplifying the inflammation and causing lung injury. The triggers and drivers of the hyperinflammatory response in COVID-19 are currently incompletely understood, but parallels to other respiratory viral infections can be important pointers. It is widely recognized that inflammasomes are activated during viral infections, and these inflammasomes are involved in the activation of the interleukin (IL)-1 family cytokines, particularly IL-1β and IL-18 that depend on caspase-1 activity for full biological activity[3]. Dysregulated activation of inflammasomes could be a trigger for the hyperinflammation seen in severe COVID-19, a hypothesis that was introduced already early after the first appearance of the virus[4]. Here, we review the current evidence for inflammasome activation during SARS-CoV-2 infection and its role in the immunopathogenesis of COVID-19.

Inflammasomes and type 1 family cytokines

Inflammasomes are intracellular multiprotein complexes that respond to intracellular and extracellular danger-associated molecular patterns, thereby contributing to innate immunity. Inflammasome activation is regulated at the transcriptional, as well as the post-translational level. A first or priming signal is initiated by toll-like receptors (TLR), RIG-I-like receptors (RLR) or other protein receptor engagement and induces the NF-κB-dependent transcription of NLRP3, pro-caspase-1, pro-IL-1β and pro-IL-18[5,6]. Once all components are available, a second signal leads to inflammasome assembly, a process that is initiated by a sensor protein. Of note, circulating monocytes might release processed IL-1β after only one stimulation by TLR-ligands, resulting from constitutively expressed caspase-1 and release of endogenous ATP[7]. The nature of the sensing protein differs from one to the other named inflammasome complex. The NLR family pyrin domain containing 3 (NLRP3) inflammasome is the most extensively studied one and also the most promiscuous. NLRP3 inflammasome assembly can be induced by different endogenous (such as ATP, uric acid crystals, etc) and exogenous (such as bacterial products, viruses, etc) triggers. Unlike TLRs and RLRs, which detect specific agonists, NLRP3 rather senses cellular damage and distress. Several mechanisms of NLRP3 activation have been proposed, including ROS production, ion flux and lysosomal damage, yet the exact mechanism remains to be elucidated[8]. Upon activation, NLRP3 multimerizes and recruits the apoptosis-associated speck-like protein containing a CARD (ASC) adaptor protein. In a very similar stepwise approach like the NLRP3 inflammasome, the RIG-I and AIM2 sensing proteins can interact with ASC to form the so-called RIG-I and AIM2 inflammasomes during viral infection[9]. The ASC adaptor protein recruits pro-caspase-1 and activates it. Once caspase-1 is active, the inflammasome complex cleaves the precursor cytokines pro-IL-1β and pro-IL-18 into their active forms IL-1β and IL-18 respectively. In addition, the inflammasome complex cleaves the pore-forming protein gasdermin D (GSDMD), resulting in the release of GSDMD N-terminal fragments that are essential for its

pore formation in cell membranes. GSDMD pore formation leads to inflammatory cell death or pyroptosis, but also to the release of the processed cytokines IL-1β and IL-18[5,6]. IL-1β and IL-18 are pleiotropic proinflammatory cytokines that play crucial roles in innate immune responses, in addition to instructing adaptive immune responses [10–13]. However, aberrant expression of these cytokines might induce tissue damage, and elevated IL-1 and/or IL-18 have been involved in the pathogenesis of severe pneumonia, sepsis and shock[14]. IL-1β also enhances the production of TNF, and IL-6 is stimulated by both cytokines providing an integrated amplified inflammatory response.

Evidence of inflammasome activation by SARS-CoV-2

Extensive immune profiling of serum from COVID-19 patients revealed high concentrations of inflammatory markers, such as CRP and ferritin, and pro-inflammatory cytokines, chemokines, and complement activation products, although concentrations of these were lower than in classical cytokine release syndromes[15,16]. CRP, ferritin and complement can be induced by the inflammasome derived cytokines IL-1β and IL-18[17]. Serum levels of the inflammasome derived cytokine IL-18 are indeed consistently increased in COVID-19 patients compared to healthy controls, with the highest levels observed in the most severe patients[18–21]. The pro-inflammatory cytokine IL-6, which might be induced by IL-1β and is a potent inducer of CRP, is also consistently increased in serum of COVID-19 patients and highly predictive for poor outcomes[22,23]. However, most studies could not detect increased serum levels of IL-1β in COVID-19 patients[16,21,24–28], which might be due to the extremely short half-life of IL-1β[28]. Accordingly, in many trials in rheumatology and sepsis, it has been very difficult to detect serum or plasma levels of the cytokine, and there has been a big interest in finding alternative biomarkers that could identify patients with high IL-1 bioactivity. As an example, the soluble IL-1 receptor antagonist (IL-1RA) is induced by IL-1 and its serum concentration can be measured as a surrogate of IL-1 biological activity, without however discriminating between

IL-1α and IL-1β. IL-1RA levels are increased in the serum of COVID-19 patients[16,24–26,29,30] and correlate with disease severity[29,30]. Recently, the soluble urokinase type plasminogen activator receptor (suPAR) also emerged as an early biomarker for hyperinflammation in COVID-19 patients, at least identifying patients where IL-1 blockade might be beneficial[31]. Another explanation for the normal IL-1β serum levels in COVID-19 patients, even in the most severe, might be a more localized production of IL-1β in the lungs. This is supported by the observation that serum cytokine levels often do not correlate with their whole blood RNA levels[27,32], while single cell RNA sequencing of BAL fluid cells did show increased expression of pro-inflammatory cytokines and chemokines locally in the lung[33,34]. Accordingly, IL-1β levels were significantly increased in the bronchoalveolar lavage (BAL) fluid of COVID-19 patients compared to healthy controls, and correlated also with disease severity[35,36]. Moreover, immunohistochemical staining of the lungs for IL-1β and IL-18 revealed higher production of these cytokines by macrophages in COVID-19 patients compared to healthy donors, supporting the idea that cytokine production might be highly compartmentalized to the lungs.

Localized production of the inflammasome-dependent cytokines and their downstream target cytokines, strongly suggests that there is activation of (potentially several) inflammasomes in the lungs of COVID-19 patients. Accordingly, several groups reported the presence of NLRP3 and ASC specks in lung biopsies from COVID-19 patients[18,37]. These inflammasome specks were higher in COVID-19 samples compared to control subjects that died from cardiopulmonary arrest. ASC specks have also been observed in SARS-CoV-2 infected peripheral blood monocytes from COVID-19 patients[18,28]. Rodrigues and colleagues found NLRP3 puncta in monocytes from COVID-19 patients[18], while Junqueira et al found, next to NLRP3, also AIM2 puncta[28]. The activation of the AIM2 sensor upon SARS-CoV-2 infection is unexpected, as AIM2 senses cytosolic DNA[38]. However, AIM2 activation was also observed during experimental IAV infection in mice[39]. Possibly, AIM2 is activated during SARS-CoV-2 infection by a bacterial surinfection or by host genomic DNA or mitochondrial DNA, released

through ruptured membranes of dying cells. In vitro experiments provide further evidence for inflammasome activation by SARS-CoV-2[18,19,28,40]. In vitro SARS-CoV-2 infection of human monocytes induced the secretion of cleaved IL-1β, LDH and active caspase-1 and these were diminished when NLRP3 or caspase-1 specific inhibitors were added, suggestive for inflammasome activation[18,19]. In addition, the direct presence of ASC and/or NLRP3 puncta in these in vitro infected monocytes were shown[18,28]. Interestingly, in the presence of a NLRP3 selective inhibitor (MCC950) ASC-specks were still formed, suggesting that SARS-CoV-2 can activate multiple inflammasomes[18].

Also in mouse model of SARS-CoV-2 infection using humanized K18-hACE2 mice, NLRP3 inflammasome priming, activation of caspase-1 and maturation of IL-1β were established in the lungs of infected mice[41]. Nevertheless, the presence of NLRP3 and ASC was not assessed as direct evidence of inflammasome activation.

Next to high levels of pro-inflammatory cytokines and chemokines, the cell lysis marker LDH is increased in the serum of COVID-19 patients, and high LDH is a strong indicator of severe disease and poor clinical outcome[28,42–44]. LDH might be a sign of pyroptosis, as it is released into the extracellular space when plasma membrane integrity is disrupted. Observations of increased cleaved caspase-1 and GSDMD in the serum of COVID-19 patients support the hypothesis that the increased LDH concentrations are due to inflammasome induced pyroptosis[28,45]. In addition, GSDMD was also found to be increased in the lung tissue of COVID-19 patients[45].

Activation of the inflammasome by SARS-CoV-2

In vivo and *in vitro* data thus support that SARS-CoV-2 induces NLRP3 inflammasome assembly. However, the molecular mechanisms by which NLRP3 inflammasome assembly is induced upon SARS-CoV-2 infection, and more broadly upon viral RNA infection, are still incompletely understood. Several mechanisms have been proposed (***figure 1***).

Indirect inflammasome activation by ion flux It is increasingly evident that NLRP3 senses viral infections by cellular damage or distress induced by viroporins. Viroporins are transmembrane pore-forming viral proteins that enhance viral shedding from infected cells, but also mediate ion in- and efflux. The envelope (E) protein of both SARS-CoV-1 and SARS-CoV-2 has been shown to form a K^+ permeable ion channel[46,47], suggesting that these proteins might contribute to inflammasome activation. Indeed, mice infected with a mutant SARS-CoV-1 virus that has suppressed ion conductivity of the E protein, exhibit the same amount of pro-IL-1β, but lower levels of cleaved active IL-1β in the lungs compared to the mice infected with the wild type SARS-CoV-1 virus[46]. During SARS-CoV-2 infection, inhibition of the E channel similarly limits pulmonary inflammation, but it has not been formally investigated whether this observation is due to reduced inflammasome activation[47].

Seemingly at odds with the above-described findings, are the observations of decreased inflammasome priming in bone marrow derived macrophages transduced with E protein lentivirus compared to those transfected with control lentivirus[48]. The same observations were made in vivo when mice received E protein or control lentivirus and were next challenged with poly(I:C) to mimic the effects of viral RNA, yet this should be validated in SARS-CoV-2 infection mouse models. In contrast, when the authors primed the bone marrow derived macrophages with LPS and poly(I:C), transduction with E protein lentivirus enhanced NLRP3 inflammasome activation, maybe suggesting that during the later stages of infection, when the NLRP3 inflammasome is primed by other triggers, the E protein can contribute to NLRP3 inflammasome activation. However, this should be investigated in in vivo models of SARS-CoV-2 infection. In addition, the ORF3a viroporin of SARS-CoV-1 has been shown to activate the NLRP3 inflammasome by disrupting intracellular K^+ concentrations and causing mitochondrial ROS production[12]. There is a high conservation of the ORF3a protein across coronavirus genomes and indeed, the SARS-CoV-2 viroporin ORF3a is also able to promote NLRP3 inflammasome assembly through the induction of K+ efflux, a well-known trigger of the NLRP3 inflammasome[49]. Moreover, ORF3a of both SARS-CoV-1 and SARS-CoV-2 also

primes the inflammasome (signal 1) by activating the NF-kB pathway and consequent expression of pro-IL-1β[49,50]. Ion efflux might also be mediated by other mechanisms than viroporins. Da Costa and colleagues reported that RNA viral replication induces lytic cell death and K+ efflux, leading to NLRP3 inflammasome activation[51]. Many of these findings rely on in vitro overexpression of viroporins in cell lines, and consequently these findings need to be validated in *in vivo* models of SARS-CoV-2 infection.

Direct interaction with inflammasome sensing proteins. It has been reported that coronavirus derived proteins can activate the inflammasome by direct interaction with inflammasome proteins. Siu et al. found that the SARS-CoV-1 ORF3a protein activates the NLRP3 inflammasome also independently of its ion channel activity[50]. Instead, they proposed a mechanism by which ORF3a directly interacts with TRAF3, thus promoting the ubiquitination of ASC, with consequent NLRP3 inflammasome activation. This has not been described for the ORF3a protein of SARS-CoV-2 yet. The ORF8b protein of SARS-CoV-1 promotes inflammasome assembly by the formation of insoluble intracellular aggregates that directly interact with NLRP3[52]. Aggregates of ORF8b induce lysosomal stress, which is a well-recognized trigger for NLRP3 inflammasome assembly[53,54]. Despite the ability of the SARS-CoV-2 ORF8 protein to also form intracellular aggregates[55], it has not been reported to be involved in NLRP3 inflammasome activation, potentially due to the substantial amino acid differences between the SARS-CoV-1 and SARS-CoV-2 ORF8 proteins[56]. Nevertheless, the N protein of SARS-CoV-2 has been shown to directly interact with the NLRP3 protein in vivo, leading to inflammasome assembly and consequent secretion of IL-1β and pyroptosis[57].

Inflammasome sensing of viral RNA It is widely accepted that NLRP3 assembly is also induced by viral RNA, but the exact underlying mechanism remains a matter of debate[58–62]. GU-rich single-stranded (ss) RNA of SARS-CoV-2 was shown to elicit the expression and maturation of IL-1β from human macrophages through NLRP3 inflammasome activation, yet in the absence of pyroptosis[62]. NLRP3 inflammasome activation was dependent on TLR8 activation, with K^+ efflux acting as a second signal. In addition, it was suggested that viral RNA

225 or RNA cleavage products bind with the DexD/H-box RNA helicase family member DHX33,
226 which consequently directly interacts with NLRP3 to induce inflammasome assembly[59,63].
227 However, others could not find a major role for DHX33 in RNA virus induced NLRP3
228 activation[51,60]. Whether this pathway is involved during SARS-CoV-2 infection remains to be
229 elucidated. Finally, it is described that viral dsRNA can trigger inflammasome activation by
230 activating the RIP1-RIP3-DRP1 pathway which promotes mitochondrial damage, an important
231 stimulus for NLRP3 assembly[60]. The RIP1-RIP3 pathway is involved in necroptosis, a lytic
232 form of cell death. However, inflammasome activation by this pathway was independently of
233 MLKL, an essential downstream effector of RIP1-RIP3-dependent necroptosis. Whether RIP1-
234 RIP3-DRP1 dependent inflammasome activation also applies in the context of SARS-CoV-2
235 infection remains to be elucidated. Viral infections can also indirectly activate inflammasomes
236 as they induce tissue damage[8]. Cell death releases a series of DAMPS, such as ATP,
237 hyaluronan, uric acid, etc, that also induce inflammasome assembly with consequent cytokine
238 release and pyroptosis. Indeed, necroptosis and inflammasome induced pyroptosis lead to
239 additional inflammasome activation by the release of DAMPS, resulting in a positive feedback
240 loop.

241 In addition to the above-mentioned mechanisms, Kucia et al showed that interaction of the
242 Spike (S) protein with ACE2 and TLR4 receptors on hematopoietic stem/progenitor cells and
243 endothelial progenitor cells induced inflammasome activation and pyroptosis, as was
244 assessed by increased levels of active caspase-1 and LDH in the culture supernatant[40]. When
245 MCC950 was added, caspase-1 activity and LDH levels significantly decreased, suggesting
246 the involvement of the NLRP3 inflammasome. Yet this possible inflammasome assembly
247 induced by the direct interaction of the SARS-CoV-2 S protein with its receptor needs further
248 validation in in vivo models.

249 Other hypotheses of inflammasome activation during SARS-CoV-2 infection have been
250 postulated, but need experimental validation[4]. Binding of Angiotensin II to its AT1 receptor can
251 activate the NLRP3 inflammasome, and consequently as the ACE2 receptor is internalized

after SARS-CoV-2 binding, this might reduce the conversion of angiotensin 2, leading to increased triggering of the renin-angiotensin-aldosterone system. Moreover, it has been shown that SARS-CoV-2 activates all three arms of the complement pathway[64]. Complement activation might influence inflammasome activation, in both an activating (C5b-9 complex, C3a and C5a) and an inhibiting way (C1q)[65]. Yet the interaction between the complement pathway and inflammasome activation needs to be explored in the context of SARS-CoV-2 infection.

Taken together, inflammasomes might be activated by multiple possible mechanisms during SARS-CoV-2 infection. However, many pathways still need to be investigated specifically in the context of SARS-CoV-2, and most of the findings specific to SARS-CoV-2 need validation in *in vivo* models and in humans. Understanding the mechanisms by which SARS-CoV-2 induces inflammasome assembly is important in order to develop novel therapeutic strategies to target this pathway.

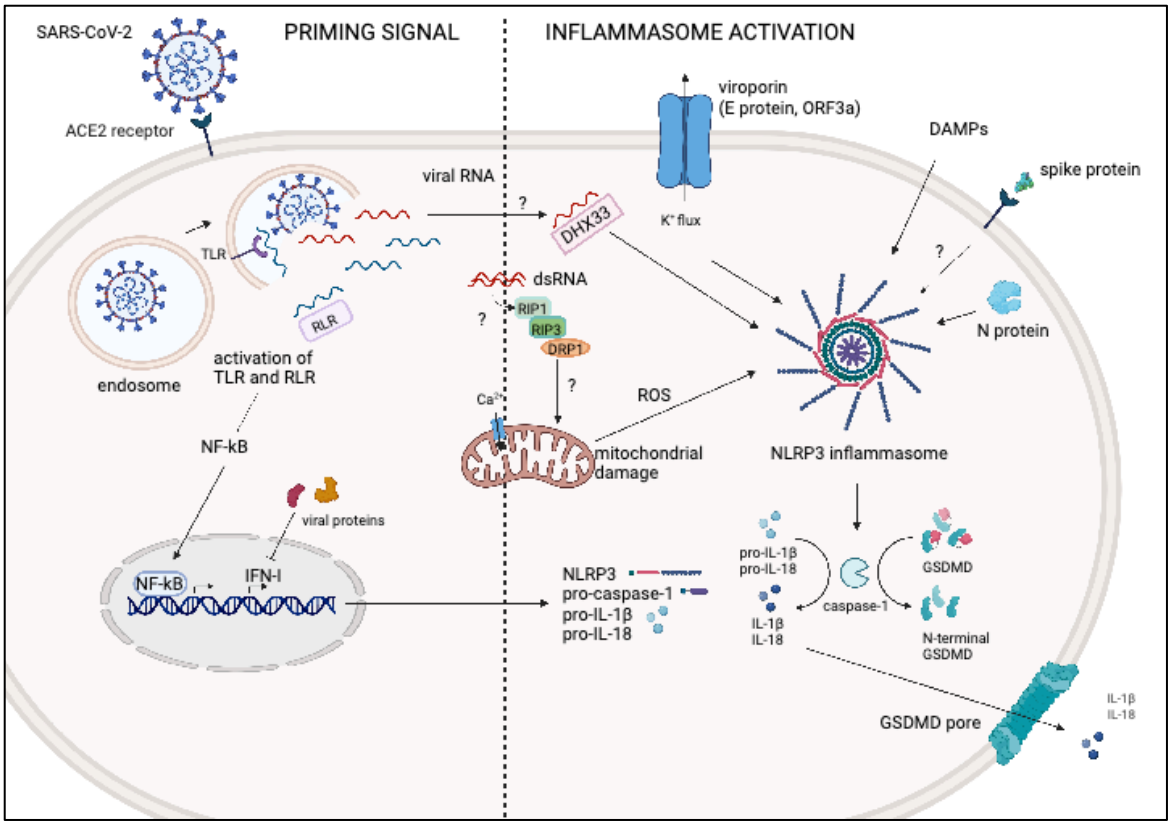

Figure 1: Possible mechanisms of inflammasome activation by SARS-CoV-2. *SARS-CoV-2 infection triggers the activation of toll-like receptors (TLR) and RIG-I-like receptors (RLR) with consequent priming of the inflammasome by inducing the NF-κB dependent transcription of NLRP3, pro-caspase-1, pro-interleukin (IL)-1β and pro-IL-18. Next, SARS-CoV-2 viroporins (ORF3a and the envelope (E) protein), might activate the NLRP3 inflammasome by the induction of ion flux. In addition, the N protein of SARS-CoV-2 was shown to directly interact with NLRP3 to activate its assembly. Viral RNA can activate the NLRP3 inflammasome by binding through DHX33, which directly interacts with NLRP3, or by activating the RIP1-RIP3-DRP1 pathway, which induces mitochondrial damage and consequent NLRP3 activation. Of note, these latter 2 pathways remain to be investigated in the context of SARS-CoV-2 infection. Binding of the S protein to the ACE2 receptor was shown to inflammasome activation in vitro, but the exact mechanism remains to be elucidated. Finally, SARS-CoV-2 induces tissue damage with the release of danger associated molecular patterns (DAMP), also leading to inflammasome activation. NLRP3 activation leads to the assembly of the inflammasome complex with consequent cleavage of pro-caspase-1 into active caspase-1. Caspase-1 cleaves pro-IL-1β and pro-IL-18 into their active forms. In addition, it cleaves gasdermin D (GSDMD) of which the N-terminal fragments form a transmembrane pore. GSDMD pore formation leads to the release of cytokines and*

lytic cell death or pyroptosis. ROS: reactive oxygen species; dsRNA: double-stranded RNA; ACE2 receptor: angiotensin converting enzyme-2 receptor. Created with BioRender.com.

Inflammasome and its downstream cytokines: contribution to pathogenesis

A temporal role for inflammasome activation during SARS-CoV-2 infection?

As described above, serum and BAL fluid levels of the inflammasome derived cytokines IL-18 and IL-1β, and its downstream cytokines IL-6 and surrogate biomarkers IL-1RA and suPAR, are significantly correlated with severe COVID-19, suggestive for inflammasomes to be drivers of an exuberant host response. Accordingly, GSDMD, NLRC4 and NLRP3 eQTLs linked to increased blood expression are significantly associated with severe COVID-19[28]. Moreover, it was reported that lung injury and cytokine production induced by the SARS-CoV-2 N protein were reversed in mice treated with the NLRP3 inhibitor MCC950 and in *Nlrp3*$^{-/-}$ mice[57], further suggesting that NLRP3 activation contributes to severe disease.

However, several lines of evidence from other mouse models of viral RNA infections suggest that inflammasomes and their downstream cytokines might also be protective against severe disease, especially early during the infection[11,58,66]. A lot of this knowledge stems from influenza A virus (IAV) models, where a temporal role for inflammasomes and its downstream cytokines applies. While mice carrying a gain-of-function mutation in the *Nlrp3* gene are strongly resistant to IAV infection due to IL-1β mediated neutrophil recruitment[11], mice defective for NLRP3 or caspase-1 were more susceptible to IAV infection due to a decreased neutrophil and monocyte recruitment and increased lung damage early during infection[58,66]. In accordance with these observations, administration of the NLRP3 specific inhibitor MCC950 directly after IAV infection increased disease severity[67]. However, when MCC950 was given later in the disease course, when symptoms were present, mice were protected from severe IAV infection. In consistency with NLRP3 contributing to early disease control, mice lacking the IL-1-receptor exhibit increased mortality with reduced inflammatory lung pathology upon

IAV infection, suggesting that IL-1 signaling, by both IL-1α and IL-1β, limits virus induced damage, potentially by affecting viral titers[68]. In contrast, treatment with anti-IL-1β from day 3 post IAV infection ameliorated the hyperinflammation and increased survival[69]. When anti-IL-1β treatment was initiated earlier, increased survival was still observed, although to a lesser extent compared to treatment initiated at day 3. These observations in IAV infection, suggest that inflammasome activation and consequent IL-1 signaling is needed to limit initial virus induced disease, while exuberant IL-1β release might contribute to hyperinflammation driving severe disease. In a SARS-CoV-2 infection model using humanized K18-hACE2 mice, treatment with IL-1RA, starting 1 day after infection, ameliorated survival, weight loss and lung inflammation, while slightly increasing viral load[41]. This is consistent with what is described in IAV infection. However, whether the temporal role of IL-1 signaling observed in IAV models, also applies to SARS-CoV-2 infection, needs to be investigated by using timed IL-1 inhibition and IL-1R$^{-/-}$ mice.

Also accordingly to observations from IAV mouse models, Pan and colleagues reported that lung injury and cytokine production induced by the SARS-CoV-2 N protein were reversed in mice treated with the NLRP3 inhibitor MCC950 and in NLRP3$^{-/-}$ mice[57], suggesting that NLRP3 activation contributes to severe disease. They could not assess if early inflammasome activation limits viral replication and virus induced lung injury, as they only investigated the role of SARS-CoV-2 N protein. Consequently, further exploration of these findings is needed in more physiologic models of SARS-CoV-2 infection. Recently, a not yet peer reviewed report described therapeutic benefits of caspase-1 and NLRP3 blockade in a humanized COVID-19 mouse model that uses AAV to deliver ACE2 to the lungs of humanized MISTRG-6 mice[70]. While observing higher viral loads, caspase-1 blockade starting 6 days post-infection reduced the inflammatory profile in the lungs of infected mice, reversing the immune-pathological state of the lung, measured by scoring of lung histology. However, the effects of earlier caspase-1 blockade were not studied. Accordingly to the previous 2 studies, Zeng et al observed ameliorated pulmonary inflammation and lung injury in NLRP3$^{-/-}$ mice compared to wild type

controls in a mouse model of SARS-CoV-2 infection[71]. In contrast to other studies, they also observed a reduced viral load in the absence of NLRP3 signaling. The same observations were made when hACE2 transgenic mice were treated with MCC950 starting at the day of infection.

Next to IL-1β, the other inflammasome derived cytokine IL-18 is also important for initial control of virus induced damage. Mostly in combination with IL-12, IL-18 activates T and NK cells to proliferate and produce IFNγ, which is a crucial element for defense against infections[72]. Accordingly, upon IAV infection, mice lacking IL-18 exhibit increased mortality with pronounced virus growth and massive inflammatory cell influx[73]. Upon murine hepatitis coronavirus infection, IL-18R$^{-/-}$ mice were also more vulnerable, with poor survival and elevated viral replication compared to wild-type mice[74]. The same observation was made in mice lacking all inflammasome signaling (Casp-1/11 $^{-/-}$). However, mice lacking IL-1 signaling exhibited similar survival upon infection with murine hepatitis coronavirus compared to their wild-type littermate controls, although viral replication was increased in the IL-1R$^{-/-}$ mice[74]. In contrast, when produced in excessive amounts, IL-18 might be detrimental by inducing hyperinflammation-related injury[72]. IL-18 has also been shown to play a role in hemophagocytic lymphohistiocytosis syndromes. Taken together, IL-18 is protective in the early phase of viral infection driving an appropriate response against the pathogen, while it can become detrimental in later phases. Whether IL-18 effectively contributes to hyperinflammation in later stages of viral infection remains to be elucidated, as timed IL-18 antagonism has not been investigated. Moreover, the possible protective and detrimental roles of IL-18 in COVID-19 need to be validated in mouse models of this disease.

The exact functional role of inflammasome activation during SARS-CoV-2 infection remains to be elucidated by using accurate mouse models of COVID-19, but based on the data described above, it is clear that tight regulation of inflammasome activation during viral

infection is crucial. Once activated, inflammasomes can amplify the inflammatory response in a paracrine manner, as their activation induces pyroptosis with the release of a second series of inflammasome agonists (e.g. ATP, hyaluronan, etc)[8]. In addition, IL-1β and IL-18 contribute to the recruitment of additional effector populations[8]. Moreover, binding of IL-1β to its receptor results in the transcription of pro-IL-1β, increasing the availability of substrate for activated inflammasomes [75]. The precise role of IL-18 binding protein (IL-18BP) also deserves more study, since it is a major antagonist of the biological activity of IL-18[76]. Lack of IL-18BP might be related to the hyperactivation of macrophages seen in COVID-19 patients.

Inflammasomes and adaptive immune responses

Despite the report of preserved adaptive immune responses in *Nlrp3*$^{-/-}$ and *Casp1*$^{-/-}$ mice during IAV infection[66], other studies suggest that inflammasome activation is needed for optimal adaptive immune responses. ASC and caspase-1 are required for effective CD4 and CD8 T cell responses, as well as for mucosal IgA secretion and systemic IgG responses during IAV infection[77]. However, NLRP3 was not required, suggesting that also other inflammasomes are activated during IAV infection which contribute to the initiation of effective adaptive immune responses. IL-1 signaling was shown to be necessary during IAV for effective CD4 T cell activation and IgM production, while the activation of CD8 T cells, virus killing, IgG and IgA levels were intact in *Il1r1*$^{-/-}$ mice[68]. In *Il18*$^{-/-}$ mice, antibody production and generation of CD8 T lymphocytes was preserved during IAV infection, yet the specific CD8 T cells produced less IFNγ, TNFα and IL-2[68,73]. This might provide an additional explanation for the previously described reduced viral clearance in IL-18 deficient animals. To date, no data on inflammasomes and adaptive immunity in SARS-CoV-2 infection are available. Whether inflammasome activation is needed to initiate effective adaptive immune responses during SARS-CoV-2 infection needs to be investigated.

Crosstalk between inflammasomes and type 1 interferons

Type 1 interferon (IFN) is crucial as it provides an immediate suppression of viral replication [78] and inhibition of inflammasome activation[79]. Besides that, it is also required for protective T cell responses[80]. A characteristic feature of SARS-CoV and MERS-CoV viruses is their ability to inhibit and delay the induction of type I IFN by infected cells[81,82]. SARS-CoV-2 is also able to inhibit the type I IFN responses in infected cells, leading to delayed or overall suppressed type I IFN responses[83,84]. This mechanism might be a virulence factor of SARS-CoV-2, thereby escaping from the host innate immune response.

The suppressed type I IFN response might be a driver of severe COVID-19, as inborn errors in the type I IFN pathway or the presence of neutralizing auto-antibodies to type I IFN are strongly over-represented among individuals who developed life-threatening COVID-19 [85,86]. In contrast to these findings, it has initially been suggested that the type I IFN response contributes to the hyperinflammatory response seen in severe COVID-19 patients[87]. However, all other reports consistently show a decreased type 1 IFN response in severe COVID-19, along with an exacerbated pro-inflammatory response[24,32,88]. Again, timing is everything to explain the effects of type I interferons in COVID-19. In a mouse model of SARS-CoV-1, delayed type 1 IFN signaling was accompanied by the recruitment of inflammatory monocyte-macrophages that produce the inflammasome derived cytokine IL-1β, along with TNFα and IL-6[89]. This population of cytokine producing inflammatory monocyte-macrophages has also been identified in the BAL fluid of patients infected with SARS-CoV-2 by RNA sequencing[35,36]. The recruitment of these monocyte-macrophages was also reduced upon abrogation of endogenous type 1 interferon signaling in a mouse model of SARS-CoV-2[90], suggesting that the delayed type 1 interferon response also contributes to disease pathogenesis in COVID-19. However, this needs further investigation in models of SARS-CoV-2.

In addition, in a mouse model of MERS infection, delayed type 1 IFN responses are also associated with reduced virus clearance, increased pro-inflammatory cytokines and poor outcomes[91]. Type 1 IFN signaling was shown to inhibit inflammasome activation in a STAT-1 dependent manner[79]. In addition, the suppressed type 1 IFN response might enhance SARS-CoV-2 replication and consequent tissue damage, both leading to increased inflammasome activation.

Taken together, severe COVID-19 patients are characterized by a defective or delayed type 1 interferon response and a concomitant exuberant inflammatory cytokine production[24]. This raises the question whether COVID-19 is a disease driven by immunosuppression or hyperinflammation, as the reduced type 1 IFN response might be the driver of exuberant inflammasome activation. Moreover, despite high levels of pro-inflammatory cytokines in serum, ex vivo stimulation of peripheral blood mononuclear cells (PBMC) of COVID-19 patients led to decreased cytokine production compared to healthy controls, septic patients and critically ill non-septic patients[25,32].

Risk factors for severe COVID-19 are associated with increased inflammasome activation and pro-inflammatory cytokines

Various host intrinsic risk factors for severe COVID-19 are correlated with increased inflammasome activation. Obesity and type 2 diabetes are both predictors of increased morbidity and mortality during SARS-CoV-2 infection. Both conditions are characterized by chronic low grade inflammation and inflammasome activation[5]. This pre-existing inflammasome priming might enhance SARS-CoV-2 induced inflammasome activation, as different positive feedback loops for inflammasome activation have been described[8].

Male sex is also an independent risk factor for increased morbidity and mortality from COVID-19[92,93]. The male immune response is characterized by a lower type 1 IFN response and consequently higher susceptibility to viral infections compared to females[94]. Accordingly, in the early phase of COVID-19, type 1 IFN is lower in males compared to females, whereas IL-8 and IL-18 levels are higher in the plasma of males[95], suggestive for increased inflammasome

activation in male patients. In a mouse model of SARS-CoV-1 infection, the higher mortality of male mice was attributed to the protective roles of the female sex hormone estrogen [92,96]. Estrogens have been shown to dampen the exuberant production of pro-inflammatory cytokines and chemokines[92], providing an explanation for females to be at reduced risk of severe COVID-19.

Finally, older age is associated with severe SARS-CoV-2 infection. Aging is accompanied by a decreased type 1 IFN response and elevated innate proinflammatory cytokines and chemokines upon viral infection[97], suggesting that older individuals are more prone to exuberant inflammasome activation during SARS-CoV-2 infection.

Clinical implications

Increasing evidence suggests that NLRP3 inflammasome activation with consequent release of IL-1β and IL-18, and downstream IL-6 and TNF production, contributes to the hyperinflammation, characteristic for severe COVID-19. Several randomized controlled trials (RCTs) with repurposed drugs targeting the inflammasome and its downstream cytokines, have been conducted in COVID-19 patients (**Table 1**). Many RCTs with IL-6 or IL-6R blockade have been published, yet mixed results were observed across different trials[98]. Two large platform trials showed improved outcomes with IL-6 blockade: the RECOVERY trial observed an increased survival rate in patients with respiratory failure and increased serum CRP concentration with tocilizumab, and the REMAP-CAP trial showed an increased number of organ-support free days at day 21 in ventilated patients or patients with cardiovascular organ support with tocilizumab or sarilumab[99,100]. Other trials with IL-6 or IL-6R blockade could not observe improved outcomes in COVID-19 patients[101–112].

Trials targeting the more upstream cytokine IL-1 also had mixed results. An RCT employing canakinumab, an anti-IL-1β antibody, in non-ventilated COVID-19 patients with hypoxia and systemic inflammation failed to significantly increase the likelihood of survival without invasive mechanical ventilation[113]. Another RCT employing the IL-1 receptor antagonist anakinra,

which targets both IL-1β and IL-1α, was prematurely terminated for absence of effect[114]. In accordance, the COV-AID trial that was conducted by us in Belgian centers could not observe therapeutic benefits for anakinra in COVID-19 patients with signs of systemic cytokine release, even when the subgroup with the highest concentrations of serum IL-1RA or IL-6 were analyzed separately in a post-hoc analysis of the data[109]. In marked contrast, another RCT (SAVE-MORE) reported an impressive and much more favorable outcome of anakinra treatment on day 28 survival compared to standard of care in patients selected on the basis of high concentration of the biomarker soluble urokinase plasminogen activator receptor (SuPAR)[31]. Possibly, the clinical severity of these patients was milder compared with the ones in the COV-AID trial. A recent systematic review of RCTs targeting IL-1 signaling in COVID-19 patients could not find evidence for an important beneficial effect of IL-1 blocking agents[115]. The mixed success of trials targeting single cytokines might be explained by the redundancy of inflammatory cytokine pathways able to drive the hyperinflammatory response along many paths. Consequently, direct targeting of the inflammasome might be more effective. Several trials targeting the NLRP3 inflammasome with colchicine or metformin have been initiated. RCTs with colchicine in COVID-19 patients showed different results, and a recent meta-analysis of those RCTs could not identify a benefit of colchicine in COVID-19 patients[116–120]. Currently, 3 RCT with metformin are ongoing (NCT04604678, NCT04625985, NCT04510194) and 1 one was prematurely stopped (NCT04626089). We need to await analysis of these trials, before firm conclusions can be made.

Targeting of GSDMD pore formation has also been proposed as a treatment for COVID-19 patients, as this could prevent the release of IL-1β and DAMPs. Disulfiram, a drug approved for alcohol dependence, inhibits GSDMD pore formation, and RCTs investigating its effect in COVID-19 patients have been initiated, but no results have been published yet (NCT04485130 and NCT04594343)[121]. Dimethyl fumarate and fumarate have also been shown to inhibit GSDMD[122]. To date, the RECOVERY trial investigates the safety and efficacy of dimethyl

fumarate in patients hospitalised with COVID-19, but results are not available yet (NCT04381936).

Direct targeting of IL-18 has not yet been investigated in COVID-19 patients. To date, no IL-18 blocking drugs are approved, but a monoclonal anti-IL-18 antibody, as wells as recombinant human IL-18BP have been tested in type 2 diabetes, rheumatic diseases and hemophagocytic lymphohistiocytosis syndrome[72]. Of note, IL-18BP in high concentrations also binds IL-37, preventing IL-37 to suppress inflammation[123]. IL-37 acts as an anti-inflammatory cytokine, suppressing both innate and adaptive immunity. Consequently, administration of recombinant IL-18BP could lead to sequestration of IL-37, thus possibly making the hyperinflammation worse.

Table 1: Overview of targeting strategies with repurposed drugs to inhibit the inflammasome and its downstream effectors in COVID-19.

Target	Repurposed drug	Published RCTs with primary outcome	Evidence summary
1. Direct inhibition	Colchicine	GRECCO-19: improved time to clinical deterioration (ref 117) RECOVERY: no effect on mortality (ref 118) COLCORONA: no effect on mortality or hospital admission in community treated patients, but decreased mortality and hospital admission in the subgroup with PCR-confirmed COVID-19 (ref 119) Lopes et al: reduced length of supplemental oxygen therapy and hospitalisation (ref 120)	No reduced risk of mortality, need for ventilatory support, ICU admission or length of hospital stay (ref 103)
	Metformine	No published trials (NCT04626089: prematurely stopped; other registered: NCT04604678, NCT04625985, NCT04510194)	Not available
2. IL-1 signaling	Anakinra	CORIMUNO-ANA: no effect on mortality, need for ventilation or survival without ventilation (ref 113) COV-AID: no effect on time to clinical improvement (ref 107) SAVE-MORE: increased clinical status at day 28 (ref 31)	Meta-analysis of RCTs: little or no increase in clinical improvement at day 28 (ref)
	Canakinumab	CAN-COVID: no effect on survival without mechanical ventilation (ref 113)	
3. IL-6 signaling	Sarilumab	CORIMUNO-SARI-1: no effect on survival or need for ventilation (ref 101) REMAP-CAP: increased number of organ-support free days (ref 100) SANOFI: no effect on time to clinical improvement (ref 102) SARICOR: no effect on evolution ARDS (ref 103) SARTRE: no effect on progression to severe respiratory faillure (ref 104)	Meta-analysis of RCTs: Lower 28-day all-cause mortality (ref 99)
	Tocilizumab	BACC-BAY: no effect on preventing intubation or death (ref 105) CORIMUNO-TOCI-1: no effect on survival or need for ventilation (ref 106) COV-AID: no effect on time to clinical improvement (ref 107) COVACTA: no effect on clinical status or mortality (ref 108) COVIDSTORM: better clinical recovery and shorter duration of hospitalisation (ref 109) COVINTOC: no effect on time to clinical improvement (ref 110) EMPACTA: reduced progression to mechanical ventilation or death, no effect on survival (ref 111) RECOVERY: increased survival (ref 99) REMAP-CAP: increased number of organ-support free days (ref 100) REMDACTA: no effect on time to hospital discharge (ref 112)	
	Siltuximab	COV-AID: no effect on time to clinical improvement (ref 107)	
4. Gasdermin D	Disulfiram	No published trials (registered: NCT04485130, NCT04594343)	Not available
	Dimethyl fumarate	No published trials (registered: NCT04381936)	Not available

ICU: intensive care unit ; RCT: randomised controlled trial.

Taken together, a deeper understanding of the immunopathogenesis of COVID-19 might help us to explain the inconsistent results of trials in COVID-19 patients with cytokine and inflammasome blockade, and to identify additional therapeutic targets, as well as biomarkers that predict outcome and treatment responses. So, research in *in vivo* models of SARS-CoV-2 infection are urgently needed to unravel the immunopathogenesis of severe COVID-19. A thorough understanding of the immunopathogenesis of severe coronavirus infections, might not only be important to reduce morbidity and mortality of the current COVID-19 pandemic, but also of future coronavirus outbreaks[46].

Conclusion

Taken together, reduced type 1 interferon responses, together with excessive inflammatory cytokine and chemokine production, might be the drivers of severe COVID-19. Inflammasomes, especially the NLRP3 inflammasome, are contributing to the exuberant cytokine production, yet in early stages of SARS-CoV-2 infection they might be crucial to limit viral replication and consequent tissue damage. However, these findings need to be validated in *in vivo* models of SARS-CoV-2 infection. The most recent results of the SAVE-MORE trial call for optimism of anakinra blockade in COVID-19, yet results from COV-AID temper this optimism, suggesting that IL-1 and inflammasome inhibition will not be the wonder drug for all patients with severe COVID-19. A thorough understanding of the immunopathogenesis of severe COVID-19 is key to not only develop personalized targeted therapies, but also to identify biomarkers that predict disease outcomes and identify the correct time window when these therapies might be most beneficial.

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