Mechanism of inflammasome activation by SARS coronavirus 3a protein: abridged secondary publication

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KEY MESSAGES

1. SARS-CoV and its ORF3a protein sufficiently activate pro-IL-1β transcription and IL-1β secretion, which are the two signals required for full activation of NLRP3 inflammasomes.
2. Activation of pro-IL-1β transcription by ORF3a is mediated through NF-κB, and it requires ubiquitin ligase TRAF3.
3. ORF3a interacts with TRAF3 and an adaptor protein ASC required for NLRP3 inflammasome activation.
4. ORF3a activates NLRP3 inflammasomes by promoting TRAF3-mediated ubiquitination of ASC.

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Introduction

SARS-CoV and MERS-CoV are more pathogenic than other human coronaviruses, as they are capable of inducing a very potent pro-inflammatory response.[1] Interleukin-1β (IL-1β) is a key pro-inflammatory cytokine induced during early infection of SARS-CoV. Its production and secretion require the action of multiprotein complexes called inflammasomes. Inflammasomes can assemble around NLRP3 and AIM2 proteins. When activated, NLRP3 recruits ASC adaptor protein ASC to interact with and activate caspase 1, which catalyses pro-IL-1β processing. NLRP3 inflammasomes are activated only when two signals that stimulate pro-IL-1β transcription and promote IL-1β cleavage are in place.

The first viral activator of NLRP3 inflammasomes identified is influenza M2 ion channel protein.[2] All currently known viral inflammasome activators including M2 can only induce IL-1β maturation when signal 1 that stimulates pro-IL-1β transcription is activated by lipopolysaccharide. Multiple ion channel proteins are also encoded by SARS-CoV. Among them, E protein has been shown to be a virulence factor activating NLRP3 inflammasomes.[3] Based on these findings, we extended our analysis to ORF3a ion channel protein, also termed X1. ORF3a coding region is located between S and E. ORF3a is a lineage-specific accessory protein and contains two transmembrane domains. Similar to other SARS-CoV proteins, ORF3a is localised to the Golgi apparatus. In a recombinant ORF3a-deficient virus, ORF3a is non-essential but still contributes to pathogenesis. ORF3a is multifunctional, particularly it induces the production of chemokines such as IL-8 through NF-κB. Because the pro-IL1β promoter is also activated by NF-κB, it is of great interest to clarify whether ORF3a is a viral modulator of NLRP3 inflammasomes that can simultaneously activate the two signals required for inflammasome activation.

Methods

We performed gain-of-function and loss-of-function experiments to characterise the inflammasome-activating property of SARS-CoV and its ORF3a protein in transfected and infected cells as described.[4] In addition, we delineated the molecular mechanism, molecular determinants, and biological significance for ORF3a induction of NLRP3 inflammasome activation in the context of SARS-CoV infection.

Results

NLRP3 inflammasome activation by ORF3a and SARS-CoV

Because ORF3a is both an NF-κB activator and an ion channel protein resembling M2, we hypothesised that ORF3a activates both pro-IL-1β transcription and IL-1β secretion. We tested this hypothesis using quantitative RT-PCR analysis of pro-IL-1β transcript in THP-1 monocytic cells and western blot analysis of IL-1β in the conditioned media of HEK293 cells reconstituted for NLRP3 inflammasome activation. Indeed, the level of pro-IL-1β transcript increased eight-fold in ORF3a-expressing THP-1 cells. The amount of mature IL-1β detected in the conditioned media was also more pronounced. Both pro-IL-1β

transcription and IL-1β secretion, which are the two signals required for optimal activation of NLRP3 inflammasomes, were activated by ORF3a.

To validate our finding in the context of SARS-CoV infection, we made use of an infectious clone of SARS-CoV in bacterial artificial chromosome and constructed an ORF3a-deficient SARS-CoV, SARS-CoVΔ3a. We then compared the mutant virus with wild-type SARS-CoV. Consistent with other study,[3] infection of THP-1 monocytic cells with wild-type SARS-CoV boosted the transcription of pro-IL-1β and the maturation followed by secretion of IL-1β. Whereas viral replication or infection was not affected by the loss of ORF3a, SARS-CoVΔ3a could not elevate pro-IL-1β transcription or the accumulation of mature IL-1β in the conditioned media. This phenotype was reversed when ORF3a was re-expressed in THP-1 cells. Therefore, ORF3a is absolutely required for SARS-CoV activation of NLRP3 inflammasomes.

ORF3a promotes p50 maturation leading to NF-κB activation

An IκBα super-repressor, ie the dominant active S32A S36A mutant of IκBα, was used to verify the requirement of NF-κB activation for ORF3a induction of pro-IL-1β transcription. Indeed, the pro-IL-1β-inducing activity of ORF3a was lost when the IκBα super-repressor was expressed. Thus, ORF3a induction of pro-IL-1β is mediated through NF-κB. We used the reporter construct driven by the IL-8 promoter as a surrogate marker for our subsequent mechanistic analysis of the activation of NF-κB by ORF3a.

A systematic analysis of different NF-κB and IκB subunit proteins in HEK293 cells overexpressing ORF3a indicated that NF-κB subunit p50 was abundantly accumulated in the nucleus. Among the different forms of NF-κB, p65-p50 is most common and also best characterised. p50 is derived from its cytoplasmic precursor p105 through proteolytic processing. Our working model is that ORF3a facilitates p105 cleavage boosting the canonical NF-κB signalling pathway for NF-κB activation.

Definition of a TRAF-binding motif in ORF3a

In addition to its previously characterised ion channel and caveolin-binding domains, ORF3a possesses a putative TRAF-binding domain very similar to those found in Epstein-Barr virus LMP1 oncoprotein and other proteins know to bind with TRAF proteins, all of which contain the consensus sequence PxQx(T/S/D), where x could be any residue. Amino acids 36-40 of ORF3a (PLQAS) are compatible with this TRAF-binding motif. To determine which domain of ORF3a is absolutely required for its ability to activate NF-κB and NLRP3 inflammasomes, we constructed three point mutants: M-T, M-I, and M-C, in which the TRAF-binding, ion channel, and caveolin-binding domains had been individually inactivated. Whereas M-C and M-I were fully capable of activating NF-κB and NLRP3 inflammasomes, M-T was unable to activate either IL8-Luc or IL-1β secretion. To our surprise, caveolin-binding or ion channel activity was dispensable for activation of NF-κB and NLRP3 inflammasomes by ORF3a. In contrast, TRAF-binding was absolutely essential for both activities of ORF3a.

We next conducted co-immunoprecipitation assays to assess the interaction of ORF3a with three representative TRAF proteins, which are important in NF-κB activation. The ability of ORF3a to interact with TRAF2/3/6 was validated. In contrast, the M-T mutant of ORF3a had no TRAF-binding activity. In addition, our immunoprecipitation results also indicated the interaction of ORF3a with ASC plausibly through a separate ASC-binding domain. In keeping with these results, substantial co-localisation of ORF3a with TRAF3 and ASC to discrete cytoplasmic punctate structures was found in A549 cells.

ORF3a activation of NF-κB requires TRAF3

Three deubiquitinases (DUBA, A20, and CYLD), which are able to remove ubiquitin from TRAF proteins specifically, were used to sort out which TRAF protein(s) might mediate ORF3a activation of NF-κB. Because ORF3a was unable to activate NF-κB when it was coexpressed with DUBA or A20, but this activity was intact in the presence of CYLD, TRAF2 was not required for ORF3a activation of NF-κB, whereas TRAF3 was likely required. The results on TRAF6 were inconclusive.

To clarify the role of TRAF3 and TRAF6, CRISPR/Cas9 technology was used to construct $TRAF3^{-/-}$ and $TRAF6^{-/-}$ HEK293 cells for additional loss-of-function experiments. Because NF-κB activation was intact in $TRAF6^{-/-}$ HEK293 cells but not observed in $TRAF3^{-/-}$ HEK293 cells, we concluded that only TRAF3 was essential for ORF3a activation of NF-κB.

ORF3a promotes TRAF3-induced ubiquitination of ASC to facilitate NLRP3 inflammasome activation

ORF3a interacts with TRAF3 ubiquitin ligase and ASC adaptor protein. Because TRAF3 catalyses polyubiquitination of ASC,[5] we hypothesised that ORF3a might promote TRAF3-mediated ubiquitination of ASC. We performed *in vivo* polyubiquitination assay to test this idea. Because a pronounced polyubiquitination smear of ASC was observed when ORF3a was expressed, and this was less prominent in the case of M-T, ORF3a indeed promoted ASC ubiquitination and TRAF-binding is

indispensable for this activity. K48 and K63 mutants of ubiquitin were then used to demonstrate that ORF3a interacts with TRAF3 and ASC to promote TRAF3-mediated K63-linked ubiquitination of ASC. Finally, *in vivo* polyubiquitination assay was performed in TRAF6$^{-/-}$ and TRAF3$^{-/-}$ HEK293 cells to verify the importance of TRAF3. In keeping with the above results, ASC ubiquitination was only slightly affected in TRAF6$^{-/-}$ cells but completely lost in TRAF3$^{-/-}$ cells. Hence, TRAF3, but not TRAF6, was absolutely required for ORF3a-faciliated ubiquitination of ASC.

Discussion

One critical event in SARS-CoV pathogenesis is the hyperactivation of pro-inflammatory response.[1] Clues for therapeutic intervention might be revealed from the mechanistic details of this process. We provided the first example of a viral activator of NLRP3 inflammasomes, which is fully capable of activating both signals that mediate pro-IL-1β transcription and IL-1β proteolytic processing. All previously characterised viral activators of inflammasomes including influenza M2 and PB1-F2 can only activate the second signal, and their operation requires priming by lipopolysaccharide, which activates pro-IL-1β transcription.

Our study also provides the first example for a viral ion channel protein to promote inflammasome activation through an ion channel-independent mechanism. All previously known viral activators of NLRP3 inflammasomes (except PB1-F2) are ion channel proteins. They are thought to activate NLRP3 inflammasomes in an ion channel-dependent manner. However, the activation of neither NF-κB nor NLRP3 inflammasomes is affected by the disruption of ion channel domain in ORF3a.

We delineated a new mechanism for viral activation of NLRP3 inflammasomes in which ORF3a stimulates NF-κB and TRAF3-mediated K63-linked polyubiquitination of ASC, which is a prerequisite for the assembly of NLRP3 inflammasomes.[5] A similar strategy might be used by other viruses. Our findings have important implications in disease intervention.

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Disclosure

The results of this research have been previously published in:

1. Siu KL, Yuen KS, Castaño-Rodriguez C, et al. Severe acute respiratory syndrome coronavirus ORF3a protein activates NLRP3 inflammasomes by promoting TRAF3-dependent ubiquitination of ASC. FASEB J 2019;33:8865-77.

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