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INSIGHTS

CARD8 makes coxsackievirus more "heartbreaking"

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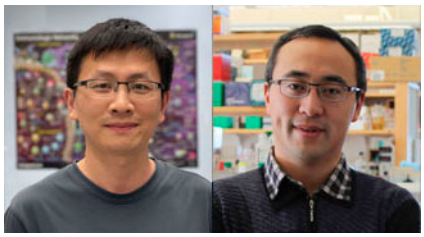
In this issue of *JEM*, Nadkarni et al. (2022. *J. Exp. Med.* https://doi.org/10.1084/jem.20212117) identify CARD8 as an innate sensor triggered by coxsackievirus B3 proteases to drive pyroptosis of aortic endothelial cells and cardiac myocytes, fueling viral replication and heart inflammation.

Enteroviruses, most commonly coxsackie B viruses, account for most cases of acute myocarditis and inflammatory cardiomyopathy (Dominguez et al., 2016). The cardiotropic coxsackievirus B3 (CVB3) infects aortic endothelial cells (AECs) and cardiomyocytes (CMs), and can thereby induce myocardial injury (Bozym et al., 2010; Tschope et al., 2021). As a cytolytic virus, CVB3 lyses its target cells and releases IL-1β and damage-associated molecular patterns (DAMPs), which recruit inflammatory leukocytes to the myocardium, leading to antithetical outcomes including cardiac viral clearance and chronic inflammation uncoupled from the initial viral injury (Cooper, 2009; Garmaroudi et al., 2015). Therefore, understanding the molecular mechanisms of CVB3 cytopathogenesis may help find novel strategies to reduce CVB3-induced myocarditis. CVB3 encodes two proteases named 2A (2Apro) and 3C (3Cpro), both of which can induce direct myocardial injury through proteolytic cleavage of essential host proteins and perturb cellular RNA and protein synthesis (Yajima and Knowlton, 2009). In this issue of *JEM*, Nadkarni et al. (2022) discover a novel mechanism with which the CVB3 proteases directly cause heart tissue damage. In this study, the authors find that 2Apro and 3Cpro both trigger CARD8 inflammasome activation and pyroptosis of infected AECs and CMs, an inflammatory form of cell death.

To detect microbial protease activities, the human genome encodes two proteins named NLRP1 (NACHT, LRR, and PYD domains-containing protein 1) and CARD8 (caspase recruitment domain-containing protein 8) that serve as tripwire sensors to set off inflammatory cell death upon proteolytic cleavage (Tsu et al., 2021). Nadkarni et al. (2022) find that CARD8 is the predominant sensor in AECs and CMs as determined by gene expression levels. To date, studies on the CARD8 inflammasome are limited to the cells of hematopoietic origin such as T cells, B cells, NK cells, and macrophages (Johnson et al., 2020; Linder et al., 2020). CARD8 can be activated through proteolytic cleavage by the HIV protease, resulting in proteasome degradation of the neo-N-terminus and liberation of the bioactive C-terminal fragment, which in turn induces caspase 1–mediated pyroptosis (Wang et al., 2021). However, the function of CARD8 in non-hematopoietic cells such as AECs and CMs is unclear. In this study, Nadkarni et al. (2022) set out to examine the functionality of CARD8 and NLRP1 in AECs. They find that treatment of AECs with Val-boroPro (VbP), an activator for both sensors (Johnson et al., 2018; Zhong et al., 2018), results in the cleavage of caspase 1 and GSDMD into the p10/12 fragment and the p30 form, respectively. VbP also induced the secretion of IL-18 from AECs, which is another readout of inflammasome activity. VbP-induced cell death was partially blocked in *CARD8*- and *NLRP1*-KO cells and was completely abrogated in cells with *CARD8* and *NLRP1* double knockout. These observations suggest that CARD8 and NLRP1 can form functional inflammasomes in AECs and can be activated by VbP simultaneously.



Insights from Qiankun Wang and Liang Shan.

Next, the authors asked whether CVB3 could activate the NLRP1 or CARD8 inflammasome in AECs and CMs through its 2A and 3C proteases. They first infected AECs with CVB3 and observed virus-induced pyroptosis by measuring GSDMD cleavage, SYTOX green uptake, and release of lactate dehydrogenase (LDH), which are indicative of lytic cell death. Interestingly, unlike VbP treatment, CVB3-induced cell death was solely dependent on CARD8, while *NLRP1*-KO AECs were killed as efficiently as control cells upon CVB3 infection. To evaluate the CARD8 inflammasome in CMs and the links to myocarditis, the authors generated human pluripotent stem cell (hPSC)-derived CMs and showed that CVB3-infected CMs also underwent CARD8-mediated pyroptosis, similar to the observations in AECs. Furthermore, they demonstrated that CVB3 infection-induced CARD8 activation was due to degradation

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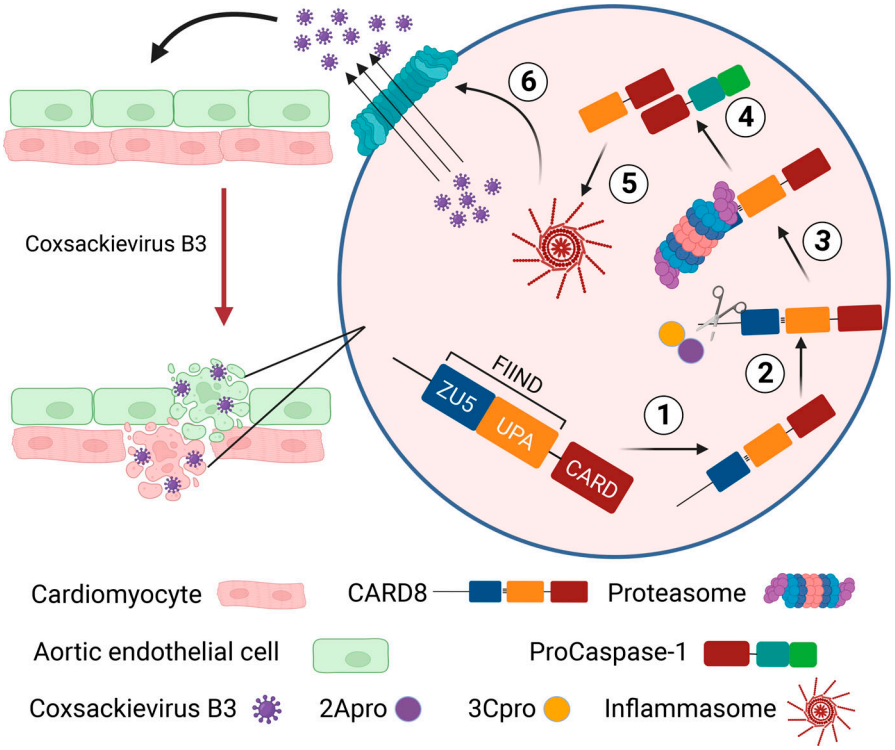

Coxsackievirus B3 proteases 2Apro and 3Cpro activate the CARD8 inflammasome. CVB3 infects aortic endothelial cells and cardiomyocytes. In CVB3-infected cells, CARD8 undergoes autoprocessing to generate N-terminal and C-terminal fragments associated by a noncovalent bond (1); CVB3 2Apro and 3Cpro cleave CARD8 N-terminus (2); the cleaved CARD8 neo-N-terminus is degraded by proteosome through the N-degron pathways (3); the bioactive UPA-CARD domain is released after proteosome degradation (4); the CARD8 UPA-CARD domain interacts with and activates caspase 1 (5); CARD8 and caspase 1 interaction leads to inflammasome assembly and pyroptosis, which promotes viral release and spread to neighboring target cells (6). Created with BioRender.

of the CARD8 N-terminus by the CVB3 2Apro and 3Cpro, as evidenced by the loss of N-terminus of CARD8 (CARD8-NT) in infected cells (see Fig. 1). Transient overexpression of CVB3 2Apro or 3Cpro in HEK293T cells expressing CARD8 also led to degradation of CARD8-NT and the appearance of N-terminal cleavage products, suggesting that either 2Apro or 3Cpro alone is sufficient to activate the CARD8 inflammasome. The authors then convincingly showed that the 2Apro inhibitor SNAP and the 3Cpro inhibitor Rupintrivir inhibited CARD8 cleavage and prevented pyroptosis of infected AECs. Notably, the two proteases do not appear to cleave CARD8-NT at the same site because the cleavage products did not have the same sizes. The authors were able to identify the cleavage site for 3Cpro, which was at the G38 position. Although both proteases can activate CARD8, the authors speculated that 2Apro would trigger CARD8-dependent pyroptosis before 3Cpro because of earlier expression of 2Apro, which is encoded 5′ to the 3Cpro in the CVB3 genome. Since the cleavage site for 2Apro is unknown, it is unclear whether the 3Cpro cleavage site would be removed by 2Apro.

Lastly, Nadkarni et al. (2022) examined the role of CARD8 inflammasome in CVB3 replication and dissemination. The authors performed elegant co-culture experiments in which AECs and CMs were separated by a 0.4-μM transwell membrane, sufficient for the passage of virions. With the CVB3-infected AECs maintained on the apical side, the authors were able to examine viral spread to the underlying CMs. Interestingly, the underlying CMs were protected from apical CVB3 infection when the donor AECs were CARD8-deficient, as evidenced by reduced CVB3 genome copy number and viral protein expression in the CMs co-cultured with *CARD8*-KO AECs, suggesting that CARD8 potentiates CVB3 replication and propagation. Since myocarditis is characterized by recruitment of leukocytes to the myocardium, the authors asked whether CARD8 affected CVB3-induced leukocyte adhesion. They found that *CARD8*-KO significantly reduced expression of ICAM-1 in AECs, a ligand for LFA-1 that stabilizes interactions between leukocytes and the endothelium. As expected, *CARD8*-KO AECs recruited fewer leukocytes upon CVB3 infection. Furthermore, CARD8-KO CMs had reduced expression of pro-inflammatory and interferon-responsive genes, indicating that CARD8 directly contributes to the CVB3-induced heart inflammation, although the underlying mechanism is unclear.

Overall, these results clearly demonstrate that coxsackie B3 virus activates the CARD8 inflammasome in human aortic endothelial cells and cardiac myocytes, which provide critical insights into how the CARD8 inflammasome modulates CVB3 infection and pathogenesis. However, beyond direct damage to the AECs and CMs by CVB3, gaps remain in our understanding of the physiological role of the CARD8 inflammasome in CVB3-induced inflammation in the heart. Pharmacologic inhibition of the CARD8 inflammasome should be tested in animal models to investigate CVB3-induced myocarditis and inflammatory cardiomyopathy. In addition, further studies are warranted to understand how CARD8 facilitates CVB3 infection and spread and promotes production of pro-inflammatory cytokines in the cardiovascular system.

The study by Nadkarni et al. (2022) also raises questions that will influence future studies on the CARD8 and NLRP1 inflammasomes. Before this study, the function of CARD8 inflammasome has only been explored in immune cells, and oddly, cells with functional CARD8 do not have detectable NLRP1 activity. Since human endothelial cells (ECs) are the first cell type reported to have CARD8 and NLRP1 both functional, these cells will allow us to examine the interplay between NLRP1 and CARD8, such as whether and how one exerts an antagonistic effect on the other. In this study, the authors showed that overexpression of NLRP1 abrogated CVB3-induced CARD8 activation, whereas NLRP1 knockout enhanced CVB3-induced pyroptosis of ECs. These results raise important questions on how the functions of these two inflammasome sensors are regulated to orchestrate host protection.

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