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Trends in Immunology

Spotlight

Ragulator-Rag and ROS TORment gasdermin D pore formation

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Upon cleavage, the Gasdermin D (GSDMD) N-terminal fragment assembles into pores on the plasma membrane to orchestrate the lytic cell death known as pyroptosis. In a recent article, Evavold *et al.* showed that the Ragulator-Rag-mTORC1-ROS pathway controls the transition from cleavage and membrane localization to oligomerization and pore formation.

Identification of GSDMD as a bona fide substrate of inflammatory caspases (caspase-1, 4, 5, and 11) upon inflammasome activation and a central executioner of pyroptosis has reignited interest in understanding the mechanistic basis of cell death [1–4]. Extensive efforts have led to elucidation of several mammalian factors both upstream (other GSDMD-cleavage enzymes) and downstream of GSDMD (ESCRT-dependent pore removal) [4]. However, the factors, if any, that regulate the cleaved N-terminal fragments of GSDMD (GSDMD-NT) to be organized into functional pores on the plasma membrane has remained unkown.

Evavold *et al.* [5] established an elegant platform to specifically search for such factors independently of inflammasomes by engineering doxycycline (Dox)-inducible expression of GSDMD-NT and full-length GSDMD in Tet3G transactivator-expressing immortalized bone marrow-derived macrophages (iBMDMs) [5]. The I105N GSDMD-NT mutant, initially isolated by ethyl-*N*-nitrosourea mutagenesis in mice as partially defective [2], was used in the study because it was more readily detectable compared with wild-type (WT) [5], likely due to lesser toxicity. Both GSDMD-NT and GSDMD constructs harbored a *Blue* Fluorescent Protein (BFP) tag at their C terminus. Furthermore, Dox-induction of GSDMD-NT resulted in pore formation at the plasma membrane and cell death; the former was detected by plasma membrane-localized BFP signals under confocal microscopy, as well as via propidium iodide (PI) uptake using flow cytometry. Lastly, cell death was assessed by the release of lactate dehydrogenase (LDH), a marker of cell lysis.

The authors performed a forward genetic screen by lentivirally transducing GSDMD-NT-expressing iBMDMs with a genome-wide single-guide RNA (sgRNA) library and isolating cells that were BFP positive but did not show PI negativity. The top 15% of these cells were processed for genomic DNA isolation, PCR amplifications of sgRNA sequences, and next-generation sequencing. Moreover, hypergeometric and gene ontology analysis revealed positive regulation of GSDND-NT pore formation by mTOR signaling, which was surprising because no such link had been suspected before. Of the genes with the highest positive log-fold change in expression and the most differentially significant *P* values between mutants and WT, many were part of the Ragulator-Rag complex, which is best known for its role in mTOR regulation, namely, genes encoding GTPases RagA and RagC, GTPase-activating protein (GAP) FLCN, and Lamtor1 to Lamtor4 (Figure 1).

To confirm these findings, the authors used the synthetic toxin system FlaTox to activate the NLRC4 inflammasome in unprimed WT and *RagA*$^{-/-}$ primary BMDMs, generated by chimeric immune editing [6]. Similar levels of GSDMD cleavage were observed in WT and *RagA*$^{-/-}$ macrophages, suggesting that RagA did not affect inflammasome formation. However, RagA ablation showed a marked reduction in GSDMD-NT pore formation relative to WT. The effects were also recapitulated in WT and RagA-deficient primary splenocytes. Additionally, Evavold and colleagues reconstituted RagA/B double-knockout (DKO) 293T cells with either human GSDMD-NT or a destabilized human full-length variant L290D, and showed attenuated PI uptake and LDH release in DKO cells compared with WT.

Did GSDMD pore formation involve mTOR, a signaling partner of Ragulator-Rag (Figure 1)? To address this, the authors showed that the mTOR activator MHY1485 promoted GSDMD-NT pore formation and that the mTOR inhibitor Torin-1 hampered GSDMD-NT pore formation [5]; this occurred to a similar extent as observed using the GSDMD inhibitor disulfiram [7]. To determine the roles of mTOR complex 1 (mTORC1) versus mTOR complex 2 (mTORC2), the Raptor component in mTORC1 and the Rictor component in mTORC2 were targeted in iBMDMs [5]. Upon priming by interferon-β (IFN-β) or lipopolysaccharide (LPS) to induce caspase-11 expression, these cells were electroporated with LPS to activate caspase-11. PI uptake and LDH release were normal in WT and *Rictor*$^{-/-}$ iBMDMs, but reduced in *Raptor*$^{-/-}$ iBMDMs, with comparable GSDMD cleavage in all cells. In a more physiological setting *in vivo* using *Salmonella enterica* serovar Typhimurium infection in mice, inflammasome activation was shown to be compromised in Raptor-deficient iBMDMs. These data attest to the role of mTORC1 downstream of Ragulator-Rag in GSDMD pore formation.

Of note, in RagA- or RagC-deficient cells, as in WT cells, GSDMD-NT displayed similar membrane or cell surface localization. GSDMD-NT pores run as an oligomeric band on nonreducing SDS-PAGE [3], and Evavold and coworkers used this method to show that GSDMD-NT was mainly monomeric in RagA- and RagC-deficient cells, but oligomeric in WT cells [5]. Thus, the authors concluded that GSDMD pore oligomerization was promoted by RagA and RagC.

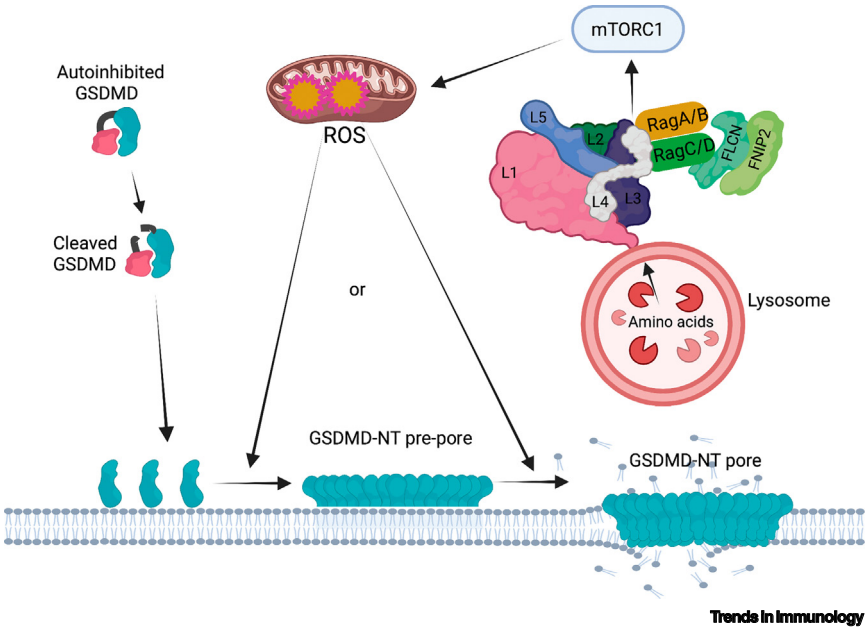

Figure 1. Ragulator-Rag, reactive oxygen species (ROS), and gasdermin D (GSDMD) pore formation in pyroptotic cell death. L1-L5, FLCN, and FNIP2 are additional subunits of the Ragulator-Rag complex. The two potential steps requiring ROS are organization of the N-terminal fragments of GSDMD (GSDMD-NT) pre-pore from its monomeric subunits or transition of assembled pre-pores into membrane-inserted pores. Figure created with BioRender (Biorender.com).

Furthermore, previous reports have shown that the Ragulator-Rag complex and mTORC1 are linked to mitochondrial dysfunction [8]. Evavold *et al.* reported that the amount of basal reactive oxygen species (ROS) in resting WT cells was higher than in RagA- or RagC-deficient cells; exogenous treatment of the cells with hydrogen peroxide partially rescued the defect in the formation of GSDMD pore oligomers in RagA- or RagC-deficient cells [5]. Moreover, consistent with the role of ROS in GSDMD pore formation, and to generate the production of ROS, the authors inhibited Mitochondrial Respiratory Complex I or III in the cells using rotenone and antimycin A, respectively, which completely rescued GSDMD-NT pore forming activity in RagA- or RagC-deficient cells.

These novel findings by Evavold *et al.* [5] set the stage for future studies (Figure 1). How ROS impact GSDMD pore formation but not GSDMD plasma membrane localization is a question that remains to be addressed. Structural studies on GSDMD have revealed that binding of GSDMD-NT to acidic lipids in membranes is important for the formation of both transmembrane pores and pre-pores with oligomerized GSDMD-NT before membrane insertion [9]. Thus, it is unclear whether the Ragulator-Rag complex regulates oligomerization or insertion. The requirement for ROS also raises the question of whether any GSDMD residue that is sensitive to oxidation is a mediator of this effect. In this context, alanine mutation of human Cys191 or the equivalent mouse Cys192 in GSDMD-NT caused partial defects in its oligomerization upon expression in HEK293T cells [3,10]. Of note, Cys191 is the target site for the GSDMD inhibitors disulfiram and dimethyl fumarate [7,11], which supports its potential importance in sensing ROS.

Several questions also arise from a recent publication by Zheng *et al.* on the role of the Ragulator-Rag complex in RIPK1- and caspase-8-mediated pyroptosis upon *Yersinia* sp. infection in mice [12]. In this study, Ragulator-Rag was shown to act as a platform for recruiting and activating a FADD-RIPK1-caspase-8 complex in response to LPS-mediated Toll-like receptor (TLR) ligation and TAK1 inhibition by a *Yersinia* sp. effector [12]. However, this study also showed that Ragulator-Rag was not essential for canonical or noncanonical inflammasome driven pyroptosis, which, at a first glance, appears to contradict the Evavold *et al.* study [5]. One possible explanation is that Zheng *et al.* used LPS priming in their inflammasome assays, and TLR4 signaling stimulates mTORC1 and ROS directly [12]. By contrast, Evavold *et al.* did not need to use priming for either GSDMD-NT expression-induced, or NLRC4-induced pyroptosis [5]. The latter only used priming to assess the role of Raptor in fostering GSDMD pore formation, which is also required for TLR4-induced ROS generation. Thus, further studies are required to validate these and other potential explanations and mechanisms that control GSDMD pore formation.

In summary, Ragulator-Rag, which operates on lysosomes to sense amino acid availability and tunes mTOR activity, has been revealed as a novel factor for GSDMD pore formation, juxtaposing pyroptotic signaling with metabolic signaling via ROS generation [5]. Evavold *et al.* have revealed that GSDMD cleavage and GSDMD-NT membrane localization do not ensure pore formation on the plasma membrane on their own, and that additional players are needed to regulate pyroptosis at this downstream step of the pathway. Hence, elucidating the underlying mechanisms of pore formation and pyroptosis in more depth would help provide a rationale as to why and how nutrients regulate inflammation. In addition, such future endeavors can enhance our understanding of candidate druggable modules that might be targeted in this complex inflammatory cell death pathway for potential therapeutic interventions in inflammation-associated pathologies.

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Declaration of interests

None declared by authors.

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