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AUTOPHAGIC PUNCTUM

RIPosomes are targets of IRGM-SQSTM1-dependent autophagy

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ABSTRACT

The NOD1-NOD2-RIPK2-NFKB/NF-κB pro-inflammatory axis plays a significant role in regulating the immune response to bacterial infection. However, an excess of NFKB-dependent cytokine response can be detrimental and, thus, should be kept under control to maintain the innate immune balance. In our recent study, first, we showed that bacterial infection induces the biogenesis of RIPK2 oligomers (RIPosomes) that are recruited around the bacteria to enhance an NFKB-dependent pro-inflammatory response. Next, we showed that SQSTM1- and IRGM-dependent selective macroautophagy/autophagy degrades RIPosomes and their components to limit NOD1-NOD2-RIPK2-NFKB pro-inflammatory signaling. Consistently, depletion of IRGM results in an augmented RIPK2-dependent pro-inflammatory cytokine response induced by *Shigella flexneri* and *Salmonella typhimurium*. Further, bacterial infection- and DSS-induced gut inflammation in *irgm1*^{KO} mice is dampened upon therapeutic inhibition of RIPK2. Taken together, we showed that autophagy selectively degrades RIPosomes to suppress inflammation and maintain innate immune homeostasis.

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The cytosolic pattern recognition receptors (PRRs) play a critical role in restricting pathogen invasion and replication. Among cytosolic PRRs, NOD1 and NOD2 sense bacteria and pathogen-associated molecular patterns/PAMPs to activate adaptor protein RIPK2 resulting in the induction of an NFKB-dependent pro-inflammatory cytokine response. Structural and overexpression studies showed that detergent-insoluble higher-order oligomeric structures of RIPK2 structures (RIPosomes) are critical for NODs-dependent NFKB signaling. However, it was not clear whether endogenous RIPK2 forms RIPosomes in physiologically relevant conditions.

In our recent study [1], we showed that infection of *Shigella flexneri, Salmonella typhimurium, Mycobacterium tuberculosis* (*M.tb*, H37Rv), and Crohn disease-associated adherent-invasive *Escherichia coli*/AIEC strain LF82 is able to induce endogenous RIPosomes formation. Interestingly, the RIPosomes are recruited around these bacteria as observed in different cell lines, primary cells, and the mouse colon (Figure 1). We also found that pathogenic bacteria (*M. tb* and adherent-invasive *Escherichia coli*) are more efficient in triggering RIPosomes formation as compared to nonpathogenic family members (*Mycobacterium smegmatis, E. coli* DH5α).

Several of the innate immune signaling proteins such as NLRP3 and MAVS form a detergent-insoluble oligomeric structure that is important for signal amplification and transduction. Next, we investigated the oligomerization capacity of NODs and RIPK2. To our surprise, NOD1 and NOD2 are not able to oligomerize when overexpressed in the cells, whereas RIPK2 forms large detergent-insoluble oligomers. The CARD domain of RIPK2 forms large filamentous structures in cells, whereas NODs CARD domains fail to do so suggesting that only RIPK2 tends to self-oligomerize. Although NODs do not form oligomers upon overexpression in HEK293T cells, *Shigella* infection induces oligomerization of endogenous NODs in THP-1 cells. We tested whether this difference can be due to the presence of RIPK2 in THP-1 (but its absence in HEK293T) that prompts the oligomerization of NODs. Indeed, depleting RIPK2 in THP-1 cells reduces levels of oligomeric NODs, and overexpression of RIPK2 in HEK293T induces NODs oligomerization. Thus, NODs oligomerization is triggered by the presence of self-oligomerizing RIPosomes. We also noted that NODs presence augments the self-assembling capacity of RIPK2 as well. Both proteins colocalize in cells to form large structures, which we named NODo-RIPosomes.

Next, we tested whether RIPosomes are inert structures or if they have a role in the activation of NFKB signaling. We employed a HeLa cell line that stably expresses doxycycline-inducible human GFP-RIPK2 to understand this. The doxycycline treatment can induce GFP-RIPK2 expression in this cell line, however, RIPosomes are only formed when these cells are infected with *Shigella*. We found NFKB RELA/p65 is not translocated to the nucleus (a signature of NFKB activation) in cells treated with just doxycycline, whereas *Shigella* infection that prompts RIPosomes formation results in nuclear accumulation of NFKB RELA/p65. Interestingly, we noted that within the population of *Shigella*-infected cells, only the cells that form RIPosomes have nuclear NFKB RELA/p65, suggesting that the formation of RIPosomes is important for the activation of NFKB. Additionally, we found purified RIPosomes can induce an NFKB response. Thus, our results showed that RIPosomes are active complexes that are critical for NFKB response.

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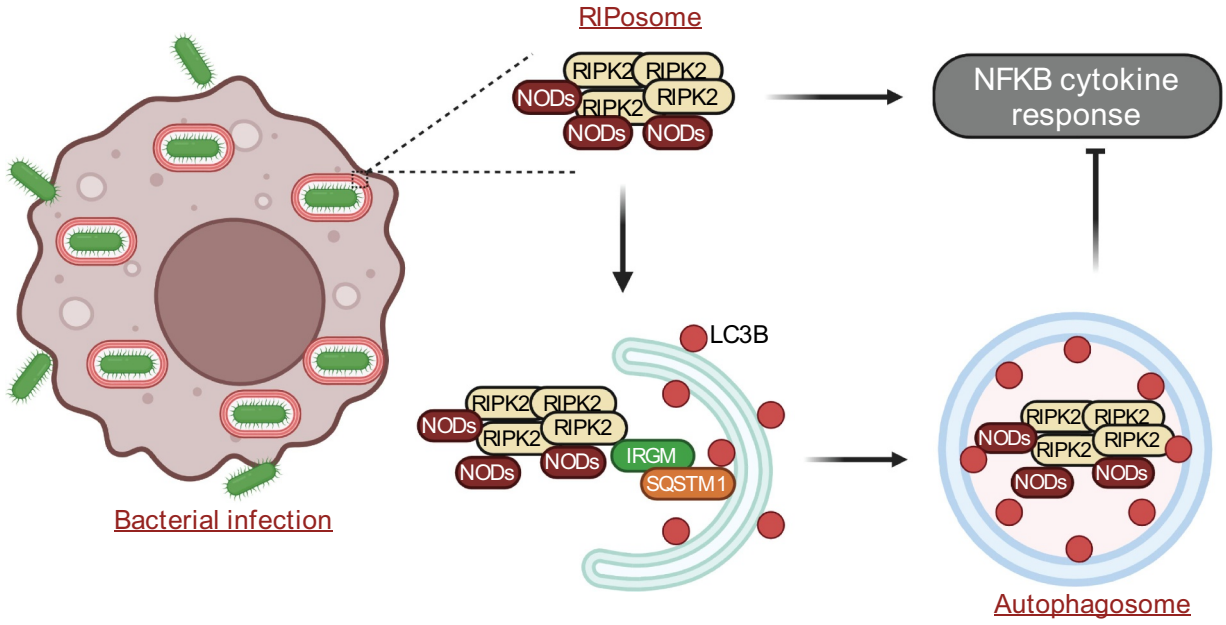

Figure 1. The schematic summarizes the results of our recent work [1]. We found that infection of pathogenic bacteria induces RIPK2 oligomerization (RIPosomes). RIPosomes coat intracellular bacteria. The oligomerization of RIPK2 to form RIPosomes is important for NFKB activation and cytokine response. Selective autophagy of RIPosomes mediated by SQSTM1 and IRGM limits the NFKB-dependent pro-inflammatory response and hence innate immune homeostasis during pathogen invasion. The schematic is made using Biorender.com.

The activation of the NODs-RIPK2-NFKB pro-inflammatory axis is important for restricting the invading bacteria, however, overactivation of this pathway can lead to an autoinflammatory state and tissue damage. Thus, it is critical to keep pro-inflammatory NFKB under check. Autophagy suppresses several pro-inflammatory pathways, however, it was unclear whether it regulates the NODs-RIPK2 axis. We found that the autophagy inhibitor bafilomycin A_1 and depletion of ATG5, an essential autophagy protein, increase the levels of NODs, RIPK2, and RIPosomes suggesting that autophagy mediates their degradation (Figure 1). RIPK2 directly interacts with the autophagy receptor SQSTM1/p62 as well as other autophagy proteins including ULK1, BECN1, and ATG16L1. Also, ubiquitinated RIPosomes colocalize with SQSTM1, LC3B, and several other autophagy proteins (Figure 1). Further knockdown of SQSTM1 increases levels of NODs, RIPK2, and RIPosomes suggesting that SQSTM1 acts as an autophagy receptor for the degradation of these proteins and complexes. In agreement with these data, the depletion of ATG5 or SQSTM1 significantly enhances NOD1-RIPK2-dependent NFKB activity and *Shigella*-induced pro-inflammatory cytokine response (TNF/TNFα, IL1B/IL-1β, and IL1A/IL-1α).

Previously, we found that IRGM protein scaffolds the interaction of autophagy machinery and several PRRs including NLRP3, RIGI, and CGAS to mediate their degradation resulting in the suppression of pro-inflammatory signaling pathways. We examined and found that NODs and RIPK2 directly interact and colocalize with IRGM. Endogenous IRGM is recruited around the cytosolic *Shigella* along with RIPosomes. Silencing IRGM increases the levels of NODs, RIPK2, and RIPosomes, whereas overexpression of IRGM dramatically reduces them. Further, IRGM overexpression reduces the NODs agonists (MDP and iE-DAP)-induced NFKB promoter activity, and its depletion robustly induces the RIPK2-dependent pro-inflammatory cytokine response. Thus, IRGM suppresses the NODs-RIPK2-NFKB pro-inflammatory axis by degrading RIPosomes and their components. Mechanistically, we showed that IRGM cooperates with SQSTM1 to conduct selective autophagy of NODs, RIPK2, and RIPosomes. Thus, IRGM could suppress the *Shigella*-induced NODs-RIPK2-NFKB response by mediating the degradation of NODs, RIPK2, and RIPosomes.

To understand the extent by which IRGM suppresses bacteria-induced inflammation in a NOD-RIPK2-dependent manner, we performed RNA-sequencing analysis with *Shigella*-infected control, IRGM knockdown, and IRGM RIPK2 double-knockdown cells. We found a very large number of interferons, interleukins, chemokines, and other cytokines that are induced by bacterial infection in a RIPK2-dependent manner are kept under check by IRGM.

Several population-wise studies linked single nucleotide polymorphisms/SNPs in *IRGM* to increased susceptibility to sepsis, bacterial infections, and gut inflammatory diseases. Consistently, previous studies including from our lab showed that *irgm1*$^{-/-}$ mice are more susceptible to DSS-induced colitis. Here, we tested whether therapeutic inhibition of RIPK2 using GSK583 can suppress shigellosis and DSS-induced gut inflammation in *irgm1*$^{-/-}$ mice. Indeed, we found that GSK583 ameliorates symptoms, pathology, immune cell invasion and pro-inflammatory cytokine response in *irgm1*$^{-/-}$ mice.

Altogether, in our study we determined the role of RIPosomes in the bacterial-induced NODs-RIPK2-NFKB cytokine response. Next, we delineated mechanisms by which autophagy keeps this pro-inflammatory axis under check. We showed that both RIPosomes and autophagy machinery are docked around the

bacteria (Figure 1). Where RIPosomes formation induces NFKB pro-inflammatory response, autophagy curtails the inflammation to maintain innate immune homeostasis.

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Disclosure statement

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Reference

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