Post-Graduate Course: Lung innate immunity

Cytosolic immune receptors and the inflammasome

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Proper regulation of mitophagy for mitochondrial homeostasis is important in various inflammatory diseases. However, the precise mechanisms by which mitophagy is activated to regulate inflammatory responses remain largely unknown. The NLRP3 (NLR family, pyrin domain containing 3) inflammasome serves as a platform that triggers the activation of CASP1 (caspase 1) and secretion of proinflammatory cytokines. Here, we demonstrate that SESN2 (sestrin 2), known as stress-inducible protein, suppresses prolonged NLRP3 inflammasome activation by clearance of damaged mitochondria through inducing mitophagy in macrophages. SESN2 plays a dual role in inducing mitophagy in response to inflammasome activation. First, SESN2 induces "mitochondrial priming" by marking mitochondria for recognition by the autophagic machinery. For mitochondrial preparing, SESN2 facilitates the perinuclear-clustering of mitochondria by mediating aggregation of SQSTM1 (sequestosome 1) and its binding to lysine 63 (Lys63)-linked ubiquitins on the mitochondrial surface. Second, SESN2 activates the specific autophagic machinery for degradation of primed mitochondria via an increase of ULK1 (unc-51 like kinase 1) protein levels. Moreover, increased SESN2 expression by extended LPS (lipopolysaccharide) stimulation is mediated by NOS2 (nitric oxide synthase 2, inducible)-mediated NO (nitric oxide) in macrophages. Thus, *Sesn2*-deficient mice displayed defective mitophagy, which resulted in hyperactivation of inflammasomes and increased mortality in 2 different sepsis models. Our findings define a unique regulatory mechanism of mitophagy activation for immunological homeostasis that protects the host from sepsis.

Introduction

Mitophagy, a selective autophagic process that specifically removes damaged or excess mitochondria, is critical for maintaining the mitochondrial population and cellular homeostasis.[1-4] Failure of mitophagy regulation results in abnormal cellular function caused by the accumulation of damaged mitochondria, leading to many pathophysiological states.[5-8] Accumulating data suggests that mitophagy has an essential role in the regulation of the innate immune response.[9-12] When mitophagy is impaired, the increase of damaged mitochondria caused by immune stimulators results in the generation of mitochondrial ROS (reactive oxygen species) and release of mitochondrial DNA, which induces hyperactivation of the NLRP3 inflammasome, and in turn leads to over-inflammation, tissue injury and increased mortality in the host.[9,10,12,13]

Although a number of mitophagy-related factors have been identified, detailed mechanisms by which they take action are still largely unknown. Recent studies on mitophagy in mammalian cell studies reveal that signal-dependent removal of damaged mitochondria by mitophagy requires 2 steps. The first is preparing damaged mitochondria and the second is activating specific autophagy machinery for the degradation of primed mitochondria.[5] Mitochondrial priming is initiated by PINK1 (PTEN induced putative kinase 1) stabilization and the E3 ubiquitin ligase PARK2/PARKIN (Parkinson disease [autosomal recessive, juvenile] 2, parkin) recruitment to damaged mitochondria.[7,14,15] Activated PARK2 promotes ubiquitination of outer membrane proteins on the mitochondria, which in turn triggers translocation of the ubiquitin-binding receptor SQSTM1 or NBR1 (neighbor of Brca1 gene 1) to mitochondria, thus completing mitochondrial priming.[6,16-19] Among the components of the autophagy machinery required for mitophagy, ULK1 and BNIP3L (BCL2/adenovirus E1B 19kDa interacting protein 3-like), are critical for clearance of mitochondria during erythroid cell maturation.[2,20,21] However, it remains unclear how these 2 steps are connected so that the mitophagy process can be completed under inflammatory conditions.

SESNs (sestrins) are highly conserved proteins that protect cells exposed to a variety of environmental stresses, including oxidative stress and DNA damage.[22,23] Apart from their antioxidant function, SESNs maintain metabolic homeostasis through regulation of AMPK (protein kinase, AMP-activated) and MTOR (mechanistic target of rapamycin [serine/threonine kinase]) signaling.[23-26] SESN2 is also able to induce autophagy through activation of AMPK and inhibition of MTOR under conditions of genotoxic stress and in cancer cell lines.[24,27,28] In addition, SESN2 can induce autophagic degradation of KEAP1 (kelch-like ECH-associated protein 1) through association with SQSTM1 triggered by the acute lipogenic stimulus.[29] Furthermore, despite significant progress in understanding the function of SESN2 in metabolic pathways and diseases, the regulatory roles of SESN2 in immune responses and the mechanisms involved therein have not yet been revealed. Here, we demonstrate that bone marrow-derived macrophages (BMDMs) from *sesn2*$^{-/-}$ mice displayed defective mitophagy upon immune stimulation, which resulted in hyperactivation of the NLRP3 inflammasome and increased mortality from sepsis. We showed that in order to induce mitophagy,

SESN2 facilitates mitochondrial priming by mediating the aggregation of SQSTM1 and its binding to ubiquitinated mitochondria, and also activates specific autophagy machinery for degradation of primed mitochondria via increase of ULK1 protein levels. Our results highlight previously unknown mechanisms of 2 different phases of mitophagy activation regulated by SESN2, leading to the suppression of hyperactivation of the NLRP3 inflammasome.

Results

sesn2$^{-/-}$ macrophages display increased CASP1 activation in response to LPS and ATP, after extended (12 h) LPS priming.

To investigate the involvement of SESN2 in inflammasome activation in macrophages, we isolated BMDMs from *Sesn2*$^{+/+}$ and *sesn2*$^{-/-}$ mice, primed the cells with LPS for 0, 6, and 12 hours (h), and then stimulated them with ATP for 30 minutes (min). ATP-driven activation of CASP1 in LPS-primed macrophages is a well-established model for NLRP3 inflammasome-mediated activation of CASP1 *in vitro*, which involves signaling pathways mediated by TLR4 (toll-like receptor 4) and **P2RX7** (purinergic receptor P2X, ligand-gated channel, 7). SESN2 suppresses NLRP3-dependent CASP1 activation and the secretion of IL1B and IL18 in macrophages primed with LPS for 12 h, rather than 6 h, followed by stimulation with ATP.

SESN2 is required for maintenance of mitochondrial homeostasis in response to LPS and ATP stimulation

Treatment with LPS and ATP produces damaged mitochondria, followed by mitochondrial ROS generation, leading to NLRP3 inflammasome activation in macrophages. We thus investigated whether SESN2 can suppress NLRP3 activation through regulation of mitochondrial homeostasis. We first examined mitochondrial superoxide production using MitoSOX (a mitochondrial superoxide indicator) at 12 h after priming with LPS. SESN2 suppresses prolonged activation of inflammasomes in response to LPS and ATP through maintenance of mitochondrial integrity.

SESN2 induces mitochondrial priming by facilitating perinuclear clustering of damaged mitochondria

We sought to uncover the regulatory mechanism whereby SESN2 prevents the over-production of mitochondrial ROS and the aggravation of mitochondrial permeability transition caused by damaged mitochondria. SESN2 induces mitochondrial priming through facilitating perinuclear clustering of damaged mitochondria by mediating SQSTM1 aggregation and its recruitment to Lys63-linked ubiquitins on the mitochondrial surface.

SESN2 induces autophagosome formation and increases mitophagic activity

In addition to the critical role of SESN2 in mitochondrial priming, we hypothesized that SESN2 may also activate specific autophagy machinery for the degradation of damaged mitochondria upon stimulation. We first examined the level of autophagosome formation by counting the green fluorescent protein-microtubule-associated protein 1 light chain 3 (GFP-MAP1LC3) puncta in transgenic mice with either *Sesn2*$^{+/+}$ (*Sesn2*$^{+/+}$ GFP-MAP1LC3) or *sesn2*$^{-/-}$ (*sesn2*$^{-/-}$ GFP-MAP1LC3) genetic backgrounds. SESN2 is required for mitophagic activity via the induction of autophagosome formation and autophagic activity in a stimulation-dependent manner.

SESN2 induces autophagic activity for mitophagy by increase of ULK1 protein level

We wondered how SESN2 can initiate autophagosome formation in response to LPS and ATP. We first examined whether SESN2 regulates the activity of AMPK and MTOR signaling in BMDMs upon stimulation, since SESN2 regulates the activity of AMPK and MTOR in response to genotoxic stress, and AMPK and MTOR are closely involved in the initiation of autophagy in response to nutrient depletion. SESN2 induces autophagic activity for mitophagy by increase of ULK1 protein levels.

SESN2 plays a protective role in 2 different sepsis mouse models

To examine the physiological role of SESN2 in the inflammatory response to septic shock, we employed the CLP (cecal ligation and puncture) technique, a clinically relevant murine model of polymicrobial sepsis. SESN2 is an essential factor in protection against septic shock, as shown in 2 different mouse models.

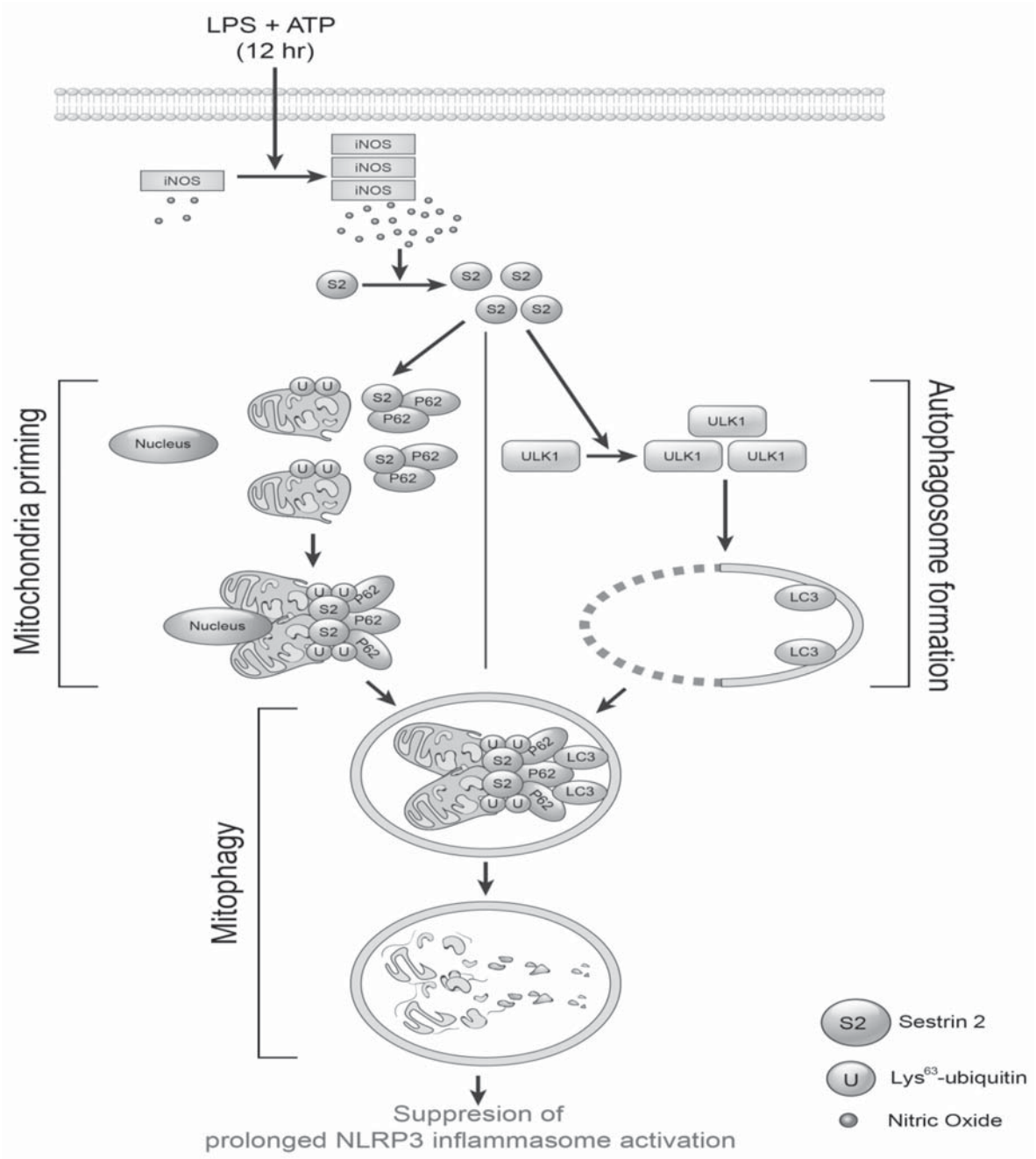
Protein levels of SESN2 are increased in monocytes from the sepsis mouse model

Since signal-dependent SESN2 protein, which was increased at specific time points suppressed inflammasome activation in macrophages, and the presence of SESN2 played a protective role in 2 septic shock mouse models, we wondered if the SESN2 protein levels were regulated in blood monocytes under septic conditions. SESN2 plays a protective role against systemic inflammatory conditions such as sepsis via increasing its expression in monocytes

Conclusion

In this study, SESN2 protein was increased by NO generated by increased NOS2 12 h after stimulation with LPS and ATP in macrophages. This increased SESN2 induced mitophagy activation through regulation of 2 synchronized procedures in a cooperative manner. First, SESN2 induced mitochondrial priming by mediating the aggregation of SQSTM1 and its binding to Lys63-ubiquitinated mitochondria. Second, SESN2

activated specific autophagic machinery for the degradation of primed mitochondria via maintenance of ULK1 protein levels. Altogether, we found that mitophagy was accomplished by SESN2- and ULK1-mediated selective autophagy of perinuclear-clustered mitochondria primed by SESN2-SQSTM1, leading to the suppression of prolonged NLRP3 inflammasome activation.



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