Transient Aggregation of Ubiquitinated Proteins Is a Cytosolic Unfolded Protein Response to Inflammation and Endoplasmic Reticulum Stress

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Background: ALIS are transient aggregation of ubiquitinated proteins.

Results: p62, NF-κB and mTOR were required for ALIS formation. Lysosomal activity is responsible for ALIS clearance.

Conclusion: There is a cross talk between UPR in the ER and cytosolic ALIS.

Significance: There is an adaptive mechanism for cellular responses against inflammation and ER stress.

Failure to maintain protein homeostasis (proteostasis) leads to accumulation of unfolded proteins and contributes to the pathogenesis of many human diseases. Accumulation of unfolded proteins in the endoplasmic reticulum (ER) elicits unfolded protein response (UPR) that serves to attenuate protein translation, and increase protein refolding or degradation. In contrast to UPR in the ER, the regulatory molecules operative in cytosolic responses and their potential relation to ER stress are not well elucidated. Aggresome-like induced structures (ALIS) have been described as transient aggregation of ubiquitinated proteins in the cytosol. In this study, we show that cells respond to inflammation, infection or ER stress by cytosolic formation of ALIS, indicating that ALIS formation represents an early event in cellular adjustment to altered proteostasis that occurs under these conditions. This response was aided by rapid transcriptional up-regulation of polyubiquitin-binding protein p62. NF-κB and mTOR activation were also required for ALIS formation. Importantly, we show a cross talk between UPR in the ER and cytosolic ALIS. Down-regulation of ER UPR in XBP1 deficient cells increases cytosolic ALIS formation. Furthermore, lysosomal activity but not macroautophagy is responsible for ALIS clearance. This study reveals the underlying regulatory mechanisms of ALIS formation and clearance, and provides a previously unrecognized common adaptive mechanism for cellular responses against inflammation and ER stress.

Protein biogenesis is an error-prone process and newly synthesized proteins are constantly subjected to misfolding. Cells have developed a network of pathways aimed at maintaining protein homeostasis (proteostasis) (1). These include protein refolding systems, degradation pathways and sequestration strategies. Failure to maintain proteostasis leads to accumulation of unfolded proteins and contribute to the pathogenesis of many human diseases (2). Understanding the cellular responses to unfolded proteins is critical for elucidating the pathophysiology of these diseases.

In eukaryotic cells, the endoplasmic reticulum (ER) (2) is the major site for protein folding and trafficking and is central to many cellular functions. When the ER becomes stressed due to the accumulation of unfolded proteins, this organelle elicits a classic adaptive response known as unfolded protein response (UPR) (2–4).

In the cytosol, responses to unfolded proteins include chaperon refolding, aggresome sequestration, and protein degradation (2, 5, 6). In contrast to UPR in the ER, the regulatory molecules operative in cytosolic responses and their potential relation to ER stress are not well elucidated. Aggresome-like induced structures (ALIS) have been described as transient aggregation of ubiquitinated proteins that occur in response to LPS stimulation or puromycin treatment (7–10). Although initially described in dendritic cells during LPS-induced maturation, they were later observed in other cells including macrophages and epithelial cells (7, 8, 11, 12). The mechanism of ALIS formation in response to LPS is thought to occur due to sudden increase in protein synthesis, associated with increased ribosomal defective products. Puromycin induces ALIS by prematurely ending translation leading to production of truncated misfolded peptides. ALIS have been shown to form rapidly within 2–4 h of LPS treatment and resolve spontaneously within 24–48 h (9, 10). Thus, ALIS represent an early event in cellular adjustment to altered proteostasis. Because of these unique features, we reasoned that understanding the underlying regulatory mechanisms of ALIS formation and clearance would reveal important information about how cells regulate protein homeostasis in response to stress.

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This article contains supplemental Figs. S1–S9.

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2 The abbreviations used are: ER, the endoplasmic reticulum; proteostasis, protein homeostasis; UPR, unfolded protein response; ALIS, Aggresome-like induced structures; LPS, lipopolysaccharide; BCG, Mycobacterium bovis; Bacille Calmette-Guérin; BMMs, bone marrow-derived macrophages; CHX, cycloheximide.
Ubiquitinated Protein Aggregation and ER Stress

In this study, we show that cells respond to inflammation, infection or ER stress by cytosolic formation of ALIS. ALIS formation was aided by rapid transcriptional up-regulation of polyubiquitin-binding protein p62. Both NF-κB and p62 were required for mounting a cytosolic unfolded protein response through the formation of ALIS. Importantly, we showed a cross talk between UPR in the ER and cytosolic ALIS. Furthermore, lysosomal activity but not macroautophagy is responsible for ALIS clearance.

EXPERIMENTAL PROCEDURES

Mice—We purchased C57BL/6, p50 knock-out mice, LC3B knock-out mice, and LysM-Cre transgenic mice from Jackson Laboratory. TIRAP knock-out mice and TRIF knock-out mice were a kind gift from Ruslan Medzhitov and Shizuo Akira, respectively. Atg7flox mice and p62 knock-out mice were previously described (13). Mice were housed within a biosafety level 3 laboratory. TIRAP knock-out mice and TRIF knock-out mice were infected with BCG at MOI of 2 for 1 h. After three washes with PBS, cells were continued to culture in DMEM for another 7 h, if not otherwise indicated.

Cell Culture—RAW264.7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum. Bone marrow cells from mice of 4–8 weeks old were cultured in DMEM containing 32 ng/ml mouse macrophage colony-stimulating factor (M-CSF) for 7 days to differentiate into macrophages (BMMs).

Antibodies and Reagents—LC3B antibody was prepared according to Aoki et al. (16). Briefly, rabbits were immunized with a synthetic peptide corresponding to the NH2-terminal 14 amino acids of the human LC3 isoform B and an additional cysteine (PSEKTFKQRRTFEQC), and hyperimmune sera from rabbits were purified by affinity purification (Bethyl Laboratories, Inc.). Ubiquitin antibody (U5379) and γ-tubulin antibody (GTU88) were from Sigma-Aldrich. p62 C-terminal specific antibody was from American Research Products, Inc (03-GP62-C-1). ATG7 antibody was from Rockland Immunochemicals, Inc. (600-401-487). iNOS antibody was from Research & Diagnostic Antibodies (MC-5200). β-Actin antibody was from Ambion (AM4302). p65 antibody (F-6, sc-8008) and p62 knock-out mice were previously described (13). Mice were housed within a biosafety level 2 vivarium.

Bacterial Culture and Infection—BCG was grown in Middlebrook 7H9 broth medium supplemented with 0.2% glycerol and 0.25% Tween 80. BCG-expressing RFP was grown in the same medium containing 25 µg/ml kanamycin. BCG infection was carried out as described (14, 15). BMMs or RAW264.7 cells were infected with BCG at MOI of 2 for 1 h. After three washes with PBS, cells were continued to culture in DMEM for another 7 h, if not otherwise indicated.

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Transfection and Establishment of Stable Cell Lines—siRNAs or plasmids were transfected with Lipofectamine 2000 (Invitrogen) as we previously described (14). To establish stable cell lines expressing empty plasmid pcDNA 3.1, I-κB dominant negative mutant or LC3-GFP, RAW 264.7 cells were selected and maintained in medium containing 320 µg/ml G418. To establish stable cell lines expressing control shRNA (Sigma, SHC002 MISSION® Non-Target shRNA), mouse XBP1 shRNA (Sigma, SHVRSC-TRCN0000232018) or mouse ATF6 shRNA (Sigma, SHVRSC-TRCN0000321327), RAW 264.7 cells were transduced with lentivirus particles containing control shRNA or p62 shRNA, selected and maintained in medium containing 2 µg/ml puromycin.

Immunofluorescence and Image Quantification—BMMs were grown on collagen-precotted glass coverslips directly in six-well plates. Cells were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100, blocked with 10% normal goat serum, incubated with indicated primary antibodies and subsequently corresponding Alexa Fluor-labeled secondary antibodies, mounted using the Prolong Gold Antibide reagent with DAPI (Invitrogen), and viewed using a Zeiss Axiovert 200 M microscope (14). For the quantification of the percentage cell with ALIS or p62 aggregates, at least 300 cells from 5 different fields were counted for each independent experiment.

Cell Lysis and Immunoblot Analysis—For immunoblot analysis, cells or liver tissues were lysed on ice for 30 min in RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS) in the presence of protease inhibitor mixture (BD Biosciences Pharmingen). Cell lysate were heated at 95 °C for 5 min in Laemmli sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (Invitrogen). Proteins were transferred to nitrocellulose membranes using semidry transfer cell (Bio-Rad). Membranes were incubated with primary antibody and subsequently secondary antibodies. Immunoreactive bands were acquired with Odyssey Imaging System (LI-COR Bioscience).

RNA Isolation, RT-PCR, and Real-Time PCR—Total RNAs from RAW264.7 cells, BMMs or liver tissue were isolated and purified using RNAeasy Mini Kit (Qiagen), and converted to cDNA using cDNA Reverse Transcription kit (Applied Biosystems). As described by Martinon et al. (17), XBP1 expression and splicing were measured using RT-PCR, and PCR products were separated by electrophoresis on a 2.5% agarose gel. GAPDH was used as a control. Primer sequences for RT-PCR: XBP1, 5′-ACACGCCGTTGGAAATGGACAC-3′ and 5′-CCAT-GGGAGATGTTGTGGG-3′; GAPDH, 5′-GAAGGTGAAG-GTCCGAGTCAC-3′ and 5′-CCGCAAAAGTTGGAGGAGTG-3′; p62 and Sec61α expression was measured using a real-time PCR detection system (Applied Biosystems StepOnePlusTM) in 96-well optical plates using SYBR GREEN Universal PCR Master Mix (Applied Biosystems). GAPDH was used as an endogenous control for normalization. Primer sequences for real-time PCR: GAPDH, 5′-CATGGCCTCCGTGTTCTCCT- A-3′ and 5′-CCCCGACGTCAGTCCA-3′; p62, 5′-AGGGA-
**RESULTS**

**Inflammation and ER Stress Up-regulate p62 and Induce ALIS Formation**—p62 is a cytosolic protein that expresses a ubiquitin-binding domain (9–11). p62 has been implicated in binding and transfer of polyubiquitinated proteins to proteasomes (18), aggresomes (19), and autophagosomes (7, 8, 20), and in ALIS formation (7, 8, 20). These findings suggest that p62 might have a proliferic role in proteostasis. We speculated that p62 might be a cellular regulator involved in control of cellular burden of unfolded proteins. To test this hypothesis, we examined the response of p62 to various stimuli of inflammation and ER stress, known to induce cellular accumulation of unfolded proteins either in the cytosol or in the ER. Inflammation was induced by treatment with lipopolysaccharide (LPS), a cell wall constituent of Gram-negative bacteria, or infection with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). ER stress was induced by treatment with thapsigargin or tunicamycin, a cell wall constituent of Gram-negative bacteria, or infection with *Mycobacterium bovis* Bacille Calmette-Guérin (BCG). ER stress was induced by treatment with thapsigargin or tunicamycin, resulted in the cytosolic formation of ALIS, suggesting a cross-talk between cytosolic responses to unfolded proteins and ER stress. These results indicate that cytosolic ALIS formation provides a common mechanism for cellular responses against inflammation and ER stress.

**Chaperons Co-localize with ALIS**—It has been reported that LPS-induced ALIS are not aggresomes, since they do not localize to the microtubule-organizing center (MTOC). Here we find that ER stress also induced ALIS which are structurally indistinguishable to those induced by inflammation. To characterize ER stress-induced ALIS formation, we found that ALIS formed in thapsigargin-treated RAW264.7 cells did not localize to MTOC that was identified by the presence of γ-tubulin (supplemental Fig. S1A). Importantly, we observed that ALIS, indicated by p62 aggregates, colocalized with the chaperones HSP40 and HSP90 (supplemental Fig. S1, B and C), suggesting a role in protein re-folding. Consistent with this hypothesis, we found that the mere treatment of macrophages with 17-AAG, an HSP90 inhibitor (21) induced ALIS formation and was synergistic when combined with LPS or thapsigargin (supplemental Fig. S1D). These results indicate that the accumulation of unfolded proteins triggers ALIS formation and that chaperones are recruited to ALIS in attempt to refold protein. Thus ALIS formation, as a cytosolic unfolded protein response, entails attempts to re-fold proteins as well as to enhance their degradation. These responses are very similar to those implicated in UPR in the ER.
**Ubiquitinated Protein Aggregation and ER Stress**

Pathogen-related TLR Activation Induces ALIS Formation—LPS is recognized by TLR4, and BCG is recognized by TLR2 and TLR4 on the cell surface, and its DNA can be recognized by TLR9 in the cytosol (22). MyD88-adaptor-like protein (Tirap), and Toll-interleukin-1 receptor domain-containing adaptor-inducing interferon-β (Trif) are two important adaptor proteins involved in TLR signaling pathways. Tirap is used by TLR2 and TLR4, Trif is used by TLR4 but not TLR2, while neither of them is used by TLR9 (23, 24). Fujita et al. have reported that MyD88 and Trif were required for LPS-induced ALIS formation (20). To determine the specific roles of TLR and adaptor proteins in BCG-induced ALIS formation, we performed the experiments using TLR4−/−, Tirap−/−, and Trif-deficient BMM cells. We observed that TLR4 deficiency abolished LPS- but not BCG-induced ALIS formation (supplemental Fig. S2). Further, BCG-induced ALIS formation was TIRAP-dependent but TRIF-independent, evidenced by reduction of ALIS formation in BMMs from TIRAP but not TRIF knock-out mice (supplemental Fig. S3), indicating that BCG induced ALIS formation through a different pathway used by LPS. Since TIRAP is a TLR adaptor for TLR2 and TLR4, these data suggested that BCG-induced ALIS was dependent on TLR2 signaling. We then investigated if BCG internalization was required for ALIS formation. We found that heat-killed BCG still induced ALIS formation. We observed that heat-killed BCG still induced ALIS formation (supplemental Fig. S3), indicating that BCG induced ALIS formation through a different pathway used by LPS. Since TIRAP is a TLR adaptor for TLR2 and TLR4, these data suggested that BCG-induced ALIS was dependent on TLR2 signaling. We then investigated if BCG internalization was required for ALIS formation. We found that heat-killed BCG still induced ALIS formation. Cytochalasin D, an endocytosis inhibitor (25), inhibited BCG-RFP internalization but not ALIS formation (BCG-RFP/CCD) (supplemental Fig. S4). These results suggest that BCG-induced ALIS formation was mediated by TLR2 activation, and did not require internalization of BCG. Consistent with above results, TLR2 activation by other ligands was found to induce ALIS formation. TLR9 activation by various ligands also resulted in ALIS formation.

p62 IsRequired for ALIS Formation—p62 interacts with ubiquitinated protein aggregation via its UBA domain. It has been reported that p62 is required for ALIS formation in puromycin-treated Hela cells (7), and LPS-treated RAW cells (20). Our data indicated close association between p62 up-regulation and ALIS formation in response to LPS treatment, BCG infection and ER stress. To determine if p62 was also required for ALIS formation under these conditions, we compared ALIS formation in BMMs from wild-type or p62 knock-out mice. In p62 deficient BMMs, ALIS formation was absent in response to LPS treatment, BCG infection or ER stress induction by thapsigargin or tunicamycin (Fig. 2). ALIS formation can be induced by treatment of cells with puromycin (7), an agent known to prematurely stop translation and thus lead to generation of truncated peptides. We tested if p62 was required for puromycin-induced ALIS. Puromycin treatment of wild type BMMs resulted in a significant increase in ALIS formation. In contrast, there was virtually no ALIS formation in BMMs from p62 knock-out mice (Fig. 2B). These results indicate that p62 is required for ALIS formation in response to inflammation, ER stress, or abnormal protein synthesis.

NF-κB Signaling Pathway Is Required for ALIS Formation—Inflammation, through TLR activation, and ER stress are known to activate NF-κB (3, 22, 26). Therefore, we studied ALIS formation in BMMs from mice deficient in NF-κB activity due to knock-out of p50, a component of NF-κB heterodimer (26). In contrast to wild-type cells, p50 knock-out BMMs did not form ALIS in response to inflammation or ER stress stimuli, or to puromycin treatment (Fig. 3A). These results indicated that NF-κB activity was required for ALIS formation. To further confirm the role of NF-κB signaling in ALIS regulation, we established RAW264.7 stable cell lines expressing either empty vector or plasmid containing dominant negative I-κB mutant (S32/36A). Because this I-κB mutant cannot be phosphorylated and degraded, it prevents the release and activation of NF-κB complex (26, 27). Its inhibitory effect on NF-κB signaling was confirmed by the attenuation of inducible nitric oxide synthase induction by LPS or BCG (supplemental Fig. S5A). Dominant negative I-κB mutant blocked inflammation- and ER stress-induced ALIS (supplemental Fig. S5B). Taken together, NF-κB signaling pathway, an important mediator of UPR in the ER, is also required for cytosolic UPR through ALIS formation.

NF-κB binding site has been found in the 5′ flanking region of p62 gene (28). To address the mechanisms by which NF-κB signaling regulates ALIS formation, we examined p62 expression in p50-deficient BMMs. At baseline, there was no significant difference in p62 mRNA or protein between BMMs from wild-type and those from p50 knock-out mice (Fig. 3, B and C). However, there was significant attenuation in induction of p62 mRNA by LPS and thapsigargin in p50 knock-out BMMs (Fig. 3B). During writing up of this study, Ling et al. reported that IKKβ activation is required for p62 expression during the development of pancreatic ductal adenocarcinoma (29). Further, there was attenuated mTOR activation caused by p50 deficiency. LPS- and thapsigargin-induced S6 phosphorylation was largely inhibited in the absence of p50 (Fig. 3C). These results suggest that NF-κB regulates ALIS formation in response to inflammation and ER stress by up-regulating p62 expression and mTOR activity.

mTOR Activation Is Required for ALIS Formation—The formation and maintenance of ALIS require continuous protein translation (10). The mammalian target of rapamycin (mTOR) is a major sensor of nutrient and energy availability in the cell and regulates a variety of cellular process, including growth, proliferation and metabolism. mTOR forms two complexes called mTOR complex 1 (mTORC1) and mTORC2 with Raptor and Rictor, respectively. mTORC1 regulates numerous steps involved in protein synthesis, including initiation, elongation and the biogenesis of ribosomes and other translational components (30). We suspected that mTOR mediated protein translation is involved in ALIS formation. We observed that LPS or thapsigargin activated mTORC1 signaling pathway, as indicated by the phosphorylation of its downstream substrate ribosomal protein S6 at Ser235/236 (supplemental Fig. S6A). However, LPS but not thapsigargin activated mTORC2 as indicated by the phosphorylation of AKT at Ser473 (supplemental Fig. S6A). More importantly, we found that the mTOR inhibitors, pp242 (31) or Ku0063794 (32), decreased mTOR activity and ALIS formation (supplemental Fig. S6, B and C). These results indicate that mTOR signaling is required for ALIS formation.

Crosstalk between ER-UPR and Cytosolic ALIS Formation—We described above that p62-mediated cytosolic ALIS formation is sensitive to ER stress, suggesting a cross talk between...
ALIS formation in the cytosol and UPR in the ER. To this end, we hypothesized that, under condition of ER stress, cells that are made deficient in ER UPR would exhibit enhanced cytosolic ALIS formation. IRE1-XBP1 branch of the classic UPR in the ER is the most conserved response during ER stress (3). To study p62 expression and ALIS formation in cells deficient in ER-UPR, we generated macrophage RAW264.7 stable cell line deficient in XBP1 by stable expression of shRNA. The real-time PCR results confirmed shRNA knockdown was effective in reducing XBP1 levels (Fig. 4A). Consistent with this result, RT PCR data showed that both XBP1u and XBP1s were reduced in RAW264.7 cells stably expressing XBP1 shRNA (Fig. 4B). To further confirm that XBP1 knockdown resulted in functional reduction of ER-UPR, we measured the induction of Sec61a, an important downstream mediator in XBP1s-transcribed proteins (4). Cells expressing control shRNA had marked increase in Sec61a expression in response to thapsigargin-induced ER stress, whereas cells with XBP1 knockdown had attenuated response (Fig. 4C). Importantly, thapsigargin-induced ER stress resulted in exaggerated up-regulation of p62 in cells deficient in XBP1 (Fig. 4, D–F). Consistent with these data, there was significant increase in ALIS formation in response to thapsigargin-induced ER stress in cells deficient in XBP1, compared with cells in which ER-UPR was intact (Fig. 4G). These results suggest that there is a cross talk between the IRE1-XBP1 branch of ER-UPR and cytosolic ALIS formation. We then conducted similar analysis in cells deficient in another branch of ER-UPR, namely ATF6. In contrast to the data obtained in XBP1 deficient cells, knockdown of ATF6 did not have significant effect on p62 levels or on ALIS formation (supplemental Fig. S7), suggesting that the cross talk between cytosolic ALIS formation and ER-UPR was specific for the IRE1-XBP1 branch.

ALIS Formation and Clearance Are Independent of Autophagy—Autophagy is known to be involved in lysosome-dependent turnover of large cellular structures including protein aggregates. Macroautophagy (hereafter autophagy) has been suggested to be responsible for clearance of ALIS (7, 8, 20). This role has been supported by the finding that LC3-GFP co-local-
autophagy deficiency. Autophagy deficiency in Atg7F/FCre increase in LC3B-II in response to BCG infection, confirming known autophagy substrate (7, 8, 33). Further, there was no induction in response to BCG was transcriptional. Importantly, induction by BCG infection (Fig. 5

BMMs increased the basal level of p62 but did not affect p62 protein 1 light chain 3B (LC3-B). We also examined ALIS for-protein 1 light chain 3B (LC3-B) instead of ALIS disappearance systems. In this study, we investigated the role of autophagy in both ALIS formation and ALIS clearance using genetically modified autophagy-deficient primary cells. We used primary BMMs from mice deficient in autophagy-related gene 7 (Atg7) and microtubule-associated protein 1 light chain 3B (LC3-B). We also examined ALIS formation in murine macrophage cell line RAW264.7 with knock-down of Beclin1 or Rab9.

To generate myeloid-specific Atg7 knock-out mice, we crossed mice bearing an Atg7floxed allele with LysM-Cre transgenic mice, which express Cre recombinase in a myeloid specific manner, including macrophages and neutrophils. Consistent with conditional Atg7 knock-out in macrophages, BMMs from Atg7floxed/Cre+ exhibited dramatic reduction of Atg7 (Fig. 5A) and a corresponding increase in baseline level of p62, a known autophagy substrate (7, 8, 33). Further, there was no increase in LC3B-II in response to BCG infection, confirming autophagy deficiency. Autophagy deficiency in Atg7floxed/Cre+ BMMs increased the basal level of p62 but did not affect p62 induction by BCG infection (Fig. 5A), confirming that p62 induction in response to BCG was transcriptional. Importantly, there was no significant difference in percentage of cells expressing ALIS between Atg7floxed/Cre+ and Atg7floxed/Cre- BMMs, evaluated at peak levels at 8 h after BCG infection (taken as an index of ALIS formation) and at 36 h (considered as an index for ALIS clearance) (Fig. 5, B and C). Similar results were obtained when LPS or thapsigargin were used to induce ALIS (supplemental Fig. S8). Clearance of ALIS is best evaluated under conditions in which new ALIS formation is prevented using protein synthesis inhibitor cycloheximide (CHX) (9, 10). Addition of CHX for 4 h resulted in clearance of BCG-induced ALIS, to similar extent, in both wild-type and autophagy-deficient BMMs (Fig. 5, B and C). Interestingly, the lysosomal inhibitor bafilomycin (34), delayed ALIS clearance to a similar extent in both wild type and Atg7-deficient BMMs. In these experiments, CHX was added to prevent de novo formation of ALIS. Collectively, these data indicate that ALIS clearance is dependent on lysosomal activity but is independent of autophagy.

Although the data above did not support a role for autophagy in ALIS clearance, we found that LC3-GFP colocalized with ALIS, induced by BCG, LPS, tunicamycin or thapsigargin in RAW264.7 cells stably expressing human LC3A-GFP (supplemental Fig. S9). Co-localization of ALIS with LC3-GFP is consistent with prior reports (7, 8, 35). We tested the possibility that LC3 might be involved in ALIS biology, independent of autophagy. We, therefore, compared ALIS formation and clearance between BMMs from wild type and LC3B knock-out mice. In BMMs from LC3B knock-out mice, in response to BCG infection, p62 was induced to similar extent, as in BMMs from wild type mice (Fig. 6A). LC3B deficiency did not affect ALIS formation or clearance (Fig. 6B), indicating that these processes are independent of LC3B.

Recently, an Atg5/Atg7-independent non-canonical autophagy pathway has been shown to require Beclin1 and Rab9 (36). We, therefore, tested the possibility that ALIS clearance is mediated by the non-canonical autophagy pathway. We utilized siRNA-mediated knockdown of Beclin1 or Rab9 in murine macrophage cell line RAW264.7 and studied ALIS formation in response to BCG infection or thapsigargin treatment. Deficiency of Beclin1, but not Rab9, resulted in increased base-
line levels of p62 (Fig. 6C). Because Beclin1 is required for both classic and non-canonical autophagy, whereas Rab9 is only involved in non-canonical autophagy (36), these data confirmed that classic autophagy is the primary pathway for p62 degradation. However, the extent of ALIS presence in response to either BCG or thapsigargin was not affected by knocking down Beclin1 or Rab9 (Fig. 6D), indicating that the non-canonical autophagy pathway is not involved in ALIS clearance. The above results collectively indicate that although lysosomes are critical for ALIS clearance, neither classic nor alternative autophagy pathways is involved in ALIS clearance.

DISCUSSION

In this study, we show that p62 is an important regulator of proteostasis, and it mediates ALIS formation as cytosolic response that links inflammation and ER stress together (Fig. 7). There is clearly a cross talk between UPR in the ER and ALIS formation in the cytosol. There are several novel findings of this study. First, the study shows that cytosolic ALIS formation is effectively a cytosolic unfolded protein response, similar to that previously characterized for the ER, and various stimuli of inflammation and ER stress converge upon common pathway in handling the associated production of unfolded proteins.

Second, Both p62 and NF-κB are required for ALIS formation. Third, there is clearly a cross talk between UPR in the ER and ALIS formation in the cytosol. Fourth, lysosomal activity but not autophagy is needed for clearance of ALIS.

Prior work has demonstrated that p62 interacts with both polyubiquitinated proteins and autophagy protein LC3 and that p62 itself is a substrate for autophagy (7, 8, 33). It has been also shown that p62 can be induced by oxidative stress under the control of Nrf2 (37, 38). Although some studies demonstrated p62 induction by proteasomal inhibitors or overexpression of polyQ-expanded proteins (19, 38), little information is available on p62 general role in proteostasis. Our study shows that inflammation and ER stress, both conditions known to be associated with accumulation of unfolded proteins, lead to rapid up-regulation of p62. This finding places p62 as an additional qualification in this context. Our data lend evidence to the notion that p62 is a...
regulator of proteostasis by mediating the transient aggregation of ubiquitinated proteins, i.e. ALIS formation, under stress conditions.

Initial reports have speculated that, by forming ALIS, cells can delay or prevent excessive or aberrant major histocompatibility complex (MHC) class I antigen presentation (9). However, experimental data elucidating the exact nature and function of ALIS formation have been lacking. By transiently aggregating proteins in concentrated cellular locations, ALIS essentially sequester these proteins from the remaining cellular pool of proteins. The transient nature of ALIS, compared with bona fide aggresomes or inclusion bodies, suggest that ALIS represent “overflow stations” critical for proteostasis. Our studies provide evidence that these “overflow stations” become operative under various conditions of inflammation and ER stress, which lead to increased cellular load of unfolded proteins.

FIGURE 5. ALIS formation and clearance are independent of ATG7. BMMs from wild type (ATG7+/+) or ATG7 knock-out (ATG7F/F Cre- ) mice were infected with BCG for 1 h followed by culture in regular medium for 7 h (BCG8h) or 35 h (BCG36h). In some experiments, BMMs were infected with BCG for 1 h followed by culture in regular medium for 7 h and then treated for 4 h with 25 μM cycloheximide either with or without 200 nM of bafilomycin A. Cell lysates were subjected to immunoblot analysis using antibodies against ATG7, p62, LC3B, or α-actin (A). Cells were examined by immunofluorescence microscopy using ubiquitin antibody to demonstrate ALIS (green) or p62 antibody (B). Quantitation of the percentage of cells expressing ALIS is shown (C). Data represent mean ± S.D., n = 5. **, p < 0.001. Scale bar, 5 μm.
Some recent studies have suggested interactions between TLR and ER stress. Woo et al. (39) reported that TRIF-mediated TLR signaling could serve to prevent prolonged UPR in the ER by suppression of CHOP to protect the cells from apoptosis. Martinon et al. showed that TLR2 or TLR4 activation in macrophages resulted in a non-canonical activation of the ER stress sensor kinase IRE1α and its downstream target spliced XBP1 transcription factor. Spliced XBP1 then activated inflammatory cytokines in a response that is distinct from its role in activating UPR stress genes (17). These studies suggested that a link between TLR activation and ER stress.

Our studies provide novel mechanisms for such link by revealing that both TLR activation and ER stress induced mTOR-mediated protein translation, and p62-mediated ALIS formation. It has been previously shown that p62 can homopolymerize in vitro and that p62 could form aggregates in cultured cells, which co-localized with ubiquitin and GFP-LC3 (7, 8, 13, 20). Our study confirms that the p62 aggregates are essentially ALIS and that ALIS formation requires p62. p62 is a polyubiquitin-binding protein, which binds ubiquitinated proteins through its UBA domain (33). p62 is able to polymerize via its N-terminal Phox and Bem1p (PB1) domain and to bind ubiquitin and polyubiquitin chains via its C-terminal UBA domain (33). UBA domain is not required for p62 oligomerization in vitro but it is required for p62 aggregation in cells (7). Our study confirms that the p62 aggregates are essentially ALIS and that ALIS formation requires p62. p62 is a polyubiquitin-binding protein, which binds ubiquitinated proteins through its UBA domain (33). p62 is able to polymerize via its N-terminal Phox and Bem1p (PB1) domain and to bind ubiquitin and polyubiquitin chains via its C-terminal UBA domain (33). UBA domain is not required for p62 oligomerization in vitro but it is required for p62 aggregation in cells (7).

FIGURE 6. ALIS are independent of LC3B, Beclin 1, or Rab9. A and B, BMMs from wild type or LC3B knock-out mice were infected with BCG for 1 h followed by culture in regular medium for 7 h (BCG8h) or 48 h (BCG48h). Cell lysates were subjected to immunoblot analysis using antibodies against LC3B, p62, or β-actin (A). Quantitation of the percentage of cells expressing ALIS by immunofluorescence microscopy (B). C and D, RAW264.7 cells were transfected for 48 h with control siRNA, Beclin1 siRNA, or Rab9 siRNA. Cells were incubated for 12 h with thapsigargin (TG, 5 μM) or infected with BCG for 1 h followed by culture in regular medium for 7 h. Cell lysates were subjected to immunoblot analysis using antibodies against Beclin1, Rab9, p62, or β-actin (C). Quantitation of the percentage of cells expressing ALIS by immunofluorescence microscopy using ubiquitin (D). Data represent mean ± S.D., n = 5. **, p < 0.001, compared with control cells.

FIGURE 7. Working model: p62 and NF-κB mediate ALIS in response to inflammation and ER stress. TLR activation initiated by pathogen infection and/or inflammation and ER stress initiated by accumulation of unfolded proteins activate mTOR signaling to increased protein synthesis, which is unavoidsly associated with production of unfolded proteins in the cytosol. Inflammation is also associated with ER stress and UPR in the ER serves to reduce protein translation and increase protein refolding and degradation. ER stress is also associated with “leakage” of unfolded proteins to the cytosol. p62 recruits unfolded proteins in the cytosol to form ALIS as an adaptive mechanism to altered proteostasis. There is a reciprocal regulation between p62 and NF-κB. p62 is important for sustained NF-κB activation, and NF-κB is required for p62 mRNA transcription induced by ER stress or inflammation.

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According to this hypothesis, p62 requirements for ALIS could be explained by the need for p62 to bind ubiquitinated proteins and then aggregates to form ALIS. However, since detecting p62 oligomerization in cells is dependent on these oligomers reaching the threshold size of immunofluorescence detection, it is plausible that p62 oligomerization might be the initial event. According to this hypothesis, p62 oligomerization will form a nidus for ubiquitinated and perhaps non-ubiquitinated proteins to accumulate to form ALIS. We favor the later hypothesis for several reasons. Although p62 has
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UBA, its affinity to either ubiquitin or ubiquitin chains is relatively low (40, 41). Although, ALIS are defined as ”transient aggregation of ubiquitinated proteins,” there is no strong evidence to indicate that all ALIS proteins are ubiquitinated. The rapid dynamics of ALIS formation and their transient nature would suggest that they might contain both ubiquitinated and non-ubiquitinated proteins. Regardless of which hypothesis is correct, p62 oligomerization property seems to contribute to p62 requirement for ALIS formation. Given that our study identifies p62 as an important regulator of proteostasis, modulating p62 oligomerization might represent a therapeutic target for diseases characterized by altered proteostasis.

Previous work has shown that NF-κB is required for ER-UPR (3, 4), and our study reveals that cytosolic ALIS formation also requires NF-κB. The requirement of NF-κB for ALIS formation was confirmed in p50 knock-out mice and using IκB dominant negative mutant. Given the position of NF-κB downstream of TLRs, diminished NF-κB activity will likely result in marked reduction in protein synthesis in inflammation. Further, our data suggest that NF-κB is required for p62 up-regulation and mTOR activation in response LPS and thapsigargin. Thus, NF-κB regulates ALIS formation by multiple inter-related pathways. These data further establish NF-κB as a common regulator required for responses to altered proteostasis, both in the ER and in the cytosol.

Hitherto, chaperone refolding and proteasomal degradation of proteins were considered to be the cytosolic equivalents of UPR in the ER. Our study describes that ALIS formation is a novel cytosolic response to ER stress. Evidence of cross talk between ER-UPR and cytosolic ALIS formation was demonstrated. ER stress led to rapid up-regulation of p62 and ALIS formation. Interference with UPR in the ER, led to exaggerated up-regulation of p62 and increased ALIS formation. One question that could arise from these observations is that how the reduction in protein translation mediated by the ER UPR (via PERK-mediated phosphorylation of eIF2a) can be reconciled with ALIS formation, which is canonically thought to be a byproduct of increased protein synthesis. We believe that the reduction in translation of UPR is not sufficient to prevent accumulation of unfolded proteins. This situation is exacerbated in XBP1-deficient cells in which there is reduction in ERAD or refolding of unfolded proteins. Previous studies have shown that ERAD by the ubiquitin-proteasome system requires protein polyubiquitination and dislocation from the ER to the cytosol. ER stress, caused by accumulation of misfolded proteins in the ER, is also associated with “leakage” of misfolded proteins to the cytosol (42, 43). Therefore, the coordination between ER-UPR and cytosolic ALIS formation is needed to maintain proteostasis under such conditions. Our study provides novel molecular mechanisms for such coordination.

In addition to its ability to interact with ubiquitinated proteins, p62 has a LC3 binding domain that mediates its interactions with LC3 (8, 13). Therefore, autophagy was suggested as a logical candidate to mediate clearance of p62-ubiquitin aggregates (7). Additional studies have shown that p62 itself is a substrate for autophagy and that autophagy-deficient cells had higher levels of p62 (13, 44, 45). More importantly, the deficiency of constitutive autophagy increases ubiquitin-contain-
and distinct roles of each Atg8 isoforms. Additional studies are needed to elucidate the roles of these isoforms in autophagy and their autophagy-independent roles. Finally, we showed that HSP90 and HSP40 are recruited to ALIS, indicating that ALIS may initially form to rescue unfolded proteins.

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