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Endoplasmic Reticulum Stress Regulates the Innate Immunity Critical Transcription Factor IRF3

Yi-Ping Liu,* Ling Zeng,* Austin Tian,* Ashley Bomkamp,* Daniel Rivera,* Delia Gutman,† Glen N. Barber,‡ Julie K. Olson,‡ and Judith A. Smith*  

IFN regulatory factor 3 (IRF3) regulates early type I IFNs and other genes involved in innate immunity. We have previously shown that cells undergoing an endoplasmic reticulum (ER) stress response called the unfolded protein response produce synergistically augmented IFN-β when stimulated with pattern recognition receptor agonists such as LPS. Concomitant ER stress and LPS stimulation resulted in greater recruitment of the IRF3 transcription factor to ifnb1 gene regulatory elements. In this study, we used murine cells to demonstrate that both oxygen–glucose deprivation and pharmacologic unfolded protein response inducers trigger phosphorylation and nuclear translocation of IRF3, even in the absence of exogenous LPS. Different ER stressors used distinct mechanisms to activate IRF3: IRF3 phosphorylation due to calcium-mobilizing ER stress (thapsigargin treatment, oxygen–glucose deprivation) critically depended upon stimulator of IFN gene, an ER-resident nucleic acid-responsive molecule. However, calcium mobilization alone by ionomycin was insufficient for IRF3 phosphorylation. In contrast, other forms of ER stress (e.g., tunicamycin treatment) promote IRF3 phosphorylation independently of stimulator of IFN gene and TANK-binding kinase 1. Rather, IRF3 activation by tunicamycin and 2-deoxyglucose was inhibited by 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, a serine protease inhibitor that blocks activating transcription factor 6 processing. Interfering with ER stress-induced IRF3 activation abrogated IFN-β synergy. Together, these data suggest ER stress primes cells to respond to innate immune stimuli by activating the IRF3 transcription factor. Our results also suggest certain types of ER stress accomplish IRF3 phosphorylation by co-opting existing innate immune pathogen response pathways. These data have implications for diseases involving ER stress and type I IFN.  

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Abbreviations used in this article: AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; ATF, activating transcription factor; CBP, CREB-binding protein; 2DG, 2-deoxyglucose; ER, endoplasmic reticulum; F, forward; HA, hemagglutinin; IKK, Ikβ kinase; IRF, IFN regulatory factor; MEF, murine embryonic fibroblast; OGD, oxygen–glucose deprivation; PERK, protein kinase receptor-like endoplasmic reticulum kinase; PRR, pattern recognition receptor; R, reverse; RNAi, RNA interference; TBK1, TANK-binding kinase 1; Tg, thiop sagarin; Tm, tunicamycin; UPR, unfolded protein response; WT, wild-type; XBP1, X-box binding protein 1.

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including oxygen and nutrient deprivation, calcium dysregulation, misfolded proteins, and N-linked glycosylation inhibition (26). The three major signaling cascades of the UPR stem from activation of ER-resident molecules: protein kinase receptor-like ER kinase (PERK), the proto-transcription factor activating transcription factor 6 (ATF6), and inositol-requiring enzyme-1 (IRE-1). IRE-1 is both a kinase and endonuclease that cleaves 26 bp from the X-box binding protein 1 (XBPI) transcription factor mRNA. This atypical splicing eliminates a premature stop codon and thus enables translation of full-length active XBPI (26). XBPI is essential for synergistic type 1 IFN responses to PRR agonists (22). We have shown that XBPI binds an enhancer element 6 kb downstream of the ifnb1 gene (23). Interestingly, during concomitant UPR and LPS stimulation, IRF3 recruitment increased at both the ifnb1 promoter and enhancer (23). IRF3 binding was only observed in the presence of LPS (23). Although XBPI, a member of the CREB family of transcription factors, is known to hetero-dimerize with other transcription factors, IRF3 is not a known binding partner (27, 28).

Investigations of gene expression in macrophages from rats overexpressing the misfolding protein HLA-B27 reveal both UPR and IFN signature, including several IFR3-regulated genes (IFN-β, OAS, and IP-10) (29). However, the relationship between ER stress and IFR3 activation was unclear. A previous study had demonstrated activation of IFR3, evident by phosphorylation, nuclear translocation, and CREB-binding protein (CBP) association in response to DNA-damaging agents such as doxorubicin in HeLa cells (30). However, a later study employing a wider range of stressors failed to validate these findings in HEK293 cells (31). For LPS stimulations, both the 7 macrophages were treated with LPS and bone marrow macrophages and MEFS treated with 10 ng/ml for 3 h unless otherwise indicated. For cytokine and chemokine protein assessment, IFN-α and IFN-β ELISA kits were from PBL InterferonSource and Rantes (RANTES). For Tbk1/iKbα kinase (Ikk) e inhibition, cells were pretreated with 2 μM MRT67307 (generous gift from Dr. Philip Cohen, University of Dundee, Dundee, Scotland) (34). The serine protease inhibitor AEBSF was from Sigma-Aldrich.

In this study, we sought to determine whether ER stress exerts a direct effect on IFR3 activation. We found that multiple pharmacologic ER stress inducers and oxygen-glucose deprivation (OGD) triggered activation of IFR3, as indicated by IFR3 phosphorylation and nuclear translocation. Mechanism of IFR3 activation varied by type of ER stressor: some forms of ER stress, in particular those that also involve calcium mobilization, require Tbk1 and STING for phosphorylation and nuclear translocation of IFR3. Indeed, STING was essential for synergistic IFR3 phosphorylation and optimal IFN-β induction by concurrent Tg and LPS. These data suggest intracellular stress responses may use innate immune pathogenesis sensing pathways to augment IFR3-regulated cytokine and chemokine production. In contrast, other ER stress inducers activated IFR3 by a pathway inhibited by 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), a serine protease inhibitor that prevents UPR-dependent processing of ATF6. Thus, at least two distinct pathways link ER stress to IFR3 activation.

**Materials and Methods**

**Cells, reagents, and stimulations**

The RAW264.7 macrophage cell line (American Type Culture Collection) was maintained in DMEM/high glucose (Mediatech) with 10% FBS (Hyclone) and 1× antibiotic-antimycotic solution (Mediatech). Murine bone marrow macrophages were isolated from C57BL/6 femurs with Histopaque 1083 (Sigma-Aldrich) and plated 3 d in non–tissue-culture petri dishes in DMEM (as above) supplemented with 5% M-CSF–containing conditioned supernatant from CMG-14-12 cells (23, 32). Adherent cells were detached by 10 mM EDTA and replated in tissue-culture dishes with CMG-14-12 supernatant 3 more d. IRF3−/− mice were previously described (13). Wild-type (WT) and STING−/− murine embryonic fibroblasts (MEFs) were maintained in DMEM supplemented with 10% FBS (not heat-inactivated; Invitrogen) and 1× antibiotic-antimycotic solution (4). XBPI−/− MEFs were kindly provided by Lime Huang (Harvard University, Boston, MA). For RNA interference (RNAi) studies, RAW 264.7 cells were transiently transfected with STING or control RNAi (Dharmacon) using AMAXA (Lonza).

To induce ER stress, cells were treated with 10 μg/ml tunicamycin (Tm), 20 μM 2-deoxyglucose (2DG), 10 μM A23187, or 1 μM Tg (all from Sigma-Aldrich). For OGD, cells were washed three times with a glucose-free isotonic salt solution (OGD buffer [pH 7.4]: 20 mM NaHCO3, 120 mM NaCl, 5.36 mM KCl, 0.33 NaHPO4 34, 4.04 mM KH2PO4, 127 mM CaCl2, and 0.81 mM MgSO4) and then incubated in OGD buffer in hypoxic conditions containing 94% N2, 5% CO2, and 1% O2 (33). Following OGD, cells were provided with growth media with or without LPS and transferred to a standard 5% CO2 incubator. For LPS stimulations, RAW 264.7 macrophages were treated with 10 ng/ml LPS for 3 h unless otherwise indicated. For cytokine and chemokine protein assessment, IFN-α and IFN-β ELISA kits were from PBL InterferonSource and Rantes (RANTES). For Tbk1/IκB kinase (Ikk) e inhibition, cells were pretreated with 2 μM MRT67307 (generous gift from Dr. Philip Cohen, University of Dundee, Dundee, Scotland) (34). The serine protease inhibitor AEBSF was from Sigma-Aldrich.

**Immunofluorescence microscopy**

Cells were plated on coverslips in 60-mm dishes for 24 h prior to treatment. For STING–TBK1 colocalization, MEFs were transiently transfected with MPYS-HA (generous gift of John Cambier, University of Colorado, Denver, CO) using Fugene HD (Roche) 24 h prior to stimulation (5). After treatment, cells were washed (3 × 5 min PBS) and then fixed in 4% paraformaldehyde 30 min at room temperature. Cells were then washed with PBS, Tris A buffer (0.1 M [pH 7.6] Tris and 0.1% Triton X-100), and Tris B buffer (0.1 M [pH 7.6] Tris, 0.1% Triton X-100, and 0.2% BSA) 3 × 5 min each and incubated with 10% goat serum in Tris B buffer 1 h. Primary Abs were added in Tris B buffer and cells incubated at 4˚C overnight. After washing the cells with Tris A 3 × 5 min, secondary fluorescence-conjugated Ab was added and samples incubated 1 h at room temperature. Cells were then washed with PBS 3 × 5 min and the coverslips mounted on slides with Poly-L-Lys (dag) antifade reagent with DAPI nuclear stain (Invitrogen). For negative controls, the same concentration of primary mouse IgG (Sigma-Aldrich) or rabbit IgG (Sigma-Aldrich) was added. Images were acquired on a Nikon Eclipse 50l fluorescence microscope (Nikon). Scale bars in images are in 50 μm.

**Quantitative PCR**

Cells were lysed with TRizol (Invitrogen) and processed according to the manufacturer’s instructions. Briefly, RNA was extracted with chloroform and precipitated with isopropanol. Total RNA was purified with and precipitated with isopropanol and treated with DNase I (Invitrogen) prior to reverse transcription using random and 18S rRNA housekeeping primers (Promega). Gene expression level in cDNA was quantitated by Relative mRNA expression was normalized to 18S RNA housekeeping gene. Primers were designed using Beacon design software (Premier Biosoft): 18S rRNA, forward (F) 5′-GGA CAC GGA GAC GAT TGA CAG-3′ and reverse (R) 5′-ATC OCT CCA CCA CAA ACT AAG AAC G-3′; β-actin, F 5′-ACT AGA GAA AAA GCA AGA GGA AAG-3′ and R 5′-GCT GAT CCA TAC CAC TGA-3′; ifit2, F 5′-TGT TGG TTA TCC ACC TTC TC-3′ and R 5′-GGA CAC GGA CAG GAT TGA CAG-3′; BiP, F 5′-AGG TGA AGA-3′ and R 5′-GCT GAT CCA TAC CCA ACC AGG CAG-3′; STING, F 5′-GCG TAT AGG TGG TAC-3′ and R 5′-GGA CAC GGA CAG GAT TGA CAG-3′; IP-10, F 5′-TGT TGG TTA TCC ACC TTC TC-3′ and R 5′-GGA CAC GGA CAG GAT TGA CAG-3′; ifn-α, F 5′-CAG AAT GGC TAG CCT CTG-3′ and R 5′-TGT TGG TTA TCC ACC TTC TC-3′; IL-6, F 5′-CTT CCA TAC AGT TGT CTT C-3′ and R 5′-ATT TCC AGG ATT CCT C-3′; IFN-β, F 5′-CAG AAT GGC TAG CCT CTG-3′ and R 5′-TGT TGG TTA TCC ACC TTC TC-3′; Il-1β, F 5′-TGT TGG TTA TCC ACC TTC TC-3′ and R 5′-GGA CAC GGA CAG GAT TGA CAG-3′; β-Actin, F 5′-GGA CAC GGA CAG GAT TGA CAG-3′ and R 5′-GGA CAC GGA CAG GAT TGA CAG-3′; STING, F 5′-GCG TAT AGG TGG TAC-3′ and R 5′-GGA CAC GGA CAG GAT TGA CAG-3′; USF2 rabbit polyclonal Ab, and Aβ rabbit mAb from Promega; Gene expression level in cDNA was quantitated by SYBR Green (Bio-Rad) fluorescence, using an iCycler or MyQ (Bio-Rad). Relative mRNA expression was normalized to 18S RNA housekeeping gene.
inducers of the UPR, or an overexpressed misfolding MHC allele. In previous studies, we determined that multiple pharmacologic UPR inducers. In vitro OGD has been shown to synergistically augments LPS-induced IFN-β production. MEFs were subjected to OGD for various times and then stimulated with LPS for 3 more h (Fig. 1A). OGD alone did not induce IFN-β mRNA; however, OGD dramatically augmented LPS-induced IFN-β, with maximal enhancement of a log-fold occurring after 1 h of OGD pretreatment. Thus, in vitro ischemia augmented IFN-β mRNA levels to a similar degree as the pharmacologic UPR inducers. In vitro OGD has been shown to induce early UPR events within 15 min (35). We confirmed BiP induction and increase in spliced XBP1 mRNA during the culture frame (Fig. 1B). OGD also induced synergistic IFN-β and BiP in RAW 264.7 macrophages (data not shown).

In dissecting the mechanism behind UPR–TLR4 synergistic IFN-β production, we observed increased IRF3 recruitment to both the ifnb1 promoter and a newly described enhancer in the context of ER stress (23). Although ER stress alone was not sufficient for recruitment of IRF3 to DNA, these findings raised the possibility that ER stress might contribute to the activation of IRF3. In unstimulated cells, IRF3 resides in the cytoplasm as monomers. Upon stimulation through PRRs, serine-threonine kinases such as TBK1 and IKKe/IKKi phosphorylate IRF3 at multiple sites, resulting in dimerization of IRF3 and nuclear translocation (10). We tested whether OGD would affect IRF3 localization in MEFs in the absence of exogenous LPS (Fig. 1C, 1D). After 1 h of OGD, IRF3 resided in the cytoplasm. However, after 2 h of reoxygenation, IRF3 appeared predominantly nuclear, colocalizing with DAPI by immunofluorescence microscopy. Nuclear translocation was also evident as assessed by Western blot (Fig. 1D).

ER stress results in the phosphorylation and nuclear translocation of IRF3

OGD induces various stress responses including the UPR (35, 36). To determine if pharmacologic UPR induction stimulates the nuclear translocation of IRF3, MEFs were treated with Tg, a sarcoplasmic/ER Ca2+-ATPase pump inhibitor frequently used to study the UPR in vitro (37). By 2 h of Tg treatment, IRF3 levels increased in nuclear extracts as detected by Western blot (Fig. 2A). Differentially migrating forms of IRF3 have been reported in unstimulated cells (referred to as forms I and II) and stimulated cells (forms III and IV); these forms may represent variably phosphorylated or alternatively spliced forms of IRF3 detected by polyclonal Ab preparations (31, 38, 39). Similar results were observed in RAW 264.7 cells (data not shown). The kinetics observed by immunofluorescence microscopy correlated well with these findings (Fig. 2B). Previous studies had identified a critical role for the UPR-regulated transcription factor XBP1 in synergistic IFN induction (22, 23, 25). To determine if XBP1 was required for IRF3 activation, nuclear translocation was examined in XBP1–/– MEFs (Fig. 2C). Tg-induced nuclear translocation of IRF3 was intact, suggesting that the UPR activates IRF3 independently of the XBP1 pathway. Similar results were obtained with other ER stressors (data not shown).

Nuclear translocation of IRF3 theoretically implies preceding phosphorylation. To directly test if the UPR elicits IRF3 phosphorylation, MEFs were stimulated with multiple commonly used pharmacologic UPR inducers, including Tg, Tm (N-linked glycosylation inhibitor), 2DG (glucose deprivation simulator), and OGD, with LPS as a positive control (Fig. 2C). Treatment times were based upon mechanism of action, time required to induce XBP1 splicing (data not shown), and required pretreatment times for synergistic IFN-β induction (23). All UPR agents induced detectable IRF3 phosphorylation at S386, comparable with LPS

3’; and RANTES, F 5’-GAA TAC ATC AAC TAT TTG GAG AT-3’ and R 5’-TAG AGC CAA CAA TGA CAG-3’.

Western blotting and coimmunoprecipitations

Cells were lysed with RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich) and whole-cell lysates or cytoplasmic and nuclear fractions (Nuclear extraction kit; Sigma-Aldrich) resolved by 10% SDS-PAGE. Samples were transferred to polyvinylidene difluoride membrane (Amersham Biosciences) and immunoblotted with primary Ab followed by HRP-conjugated secondary Ab (Bio-Rad). Samples were rotated at 4°C overnight. Beads were washed two times with PBS, added to loading buffer, and then samples were denatured and resolved by SDS-PAGE.

Statistics

Statistical significance between different groups of data were determined by two-tailed Student t test. In all figures, error bars represent SEM of the mean from combined experiments or SD within a representative experiment.

Results

OGD synergistically augments LPS-induced IFN-β and activates IRF3

In previous studies, we determined that multiple pharmacologic inducers of the UPR, or an overexpressed misfolding HLA allele, greatly augment LPS-dependent IFN-β mRNA and protein production (22, 23). This robust synergy was observed in primary macrophages, macrophage cell lines, MEFs, and LPS receptor-transfected HEK293 cells (22, 23). However, it was unclear if more physiologic stress, such as the oxygen and nutrient deprivation that might occur with ischemia, would enhance IFN-β production. MEFs were subjected to OGD for various times and then stimulated with LPS for 3 more h (Fig. 1A). OGD alone did not induce IFN-β mRNA; however, OGD dramatically augmented LPS-induced IFN-β, with maximal enhancement of a log-fold occurring after 1 h of OGD pretreatment. Thus, in vitro ischemia augmented IFN-β mRNA levels to a similar degree as the pharmacologic UPR inducers. In vitro OGD has been shown to induce early UPR events within 15 min (35). We confirmed BiP induction and increase in spliced XBP1 mRNA during the culture frame (Fig. 1B). OGD also induced synergistic IFN-β and BiP in RAW 264.7 macrophages (data not shown).

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treatment (Fig. 2D). IRF3 phosphorylation was not detected in IRF32/2 primary macrophages, confirming immunofluorescence specificity (Fig. 2E). Synergistic IRF3 phosphorylation with dual Tg and LPS treatment was not evident by immunofluorescence (data not shown) in comparison with Western blot (see below). The pattern of phospho-IRF3 localization differed between LPS and UPR inducers; LPS treatment resulted in qualitatively higher order clustering (dots arranged in small circular clusters, compare OGD versus LPS).

ER stress does not augment all IRF3-regulated genes

Our previous studies examining UPR–TLR synergy had focused on IFN-b production (23). However, the activation of IRF3 by ER stress raised the possibility that ER stress might augment the production of other known IRF3-regulated genes. Martinon et al. (24) reported log-fold synergistic induction of the IRF3 regulated gene ISG15 in Tm plus LPS-stimulated cells. IFN-a4 transcription is an early type I IFN, for which expression is highly dependent upon IRF3 (15, 40). In addition to increased IFN-b production, primary bone marrow-derived mouse macrophages treated concurrently with Tg and LPS produced increased levels of IFN-a4 (Fig. 3). Thus, the effect of ER stress on IRF3 activation has consequences for other antiviral and inflammatory mediators besides IFN-b. As previously described, synergistic IFN-b mRNA induction correlated well with protein production (23). IFN-a was produced at such a low level; only Tg plus LPS stimulated cytokine was present above the limit of detection (data not shown). However, synergistic induction of IRF3-regulated genes was not universal: neither Iit2/ISG54 nor RANTES (as noted by others) were significantly augmented by UPR induction (24).

Activation of IRF3 by Tg requires STING and TBK1

To begin elucidating the mechanism of UPR-induced IRF3 phosphorylation, we sought to determine the relevant IRF3 kinase. TBK1 is an upstream serine/threonine kinase that serves as the final point of convergence for multiple innate immune-sensing pathways culminating in the phosphorylation of IRF3 (8, 9, 16). Within the past few years, STING has been identified as an ER-resident transmembrane protein that plays a critical role in the induction of IRF3 and thus IFN-b by cytoplasmic nucleic acids.
acids (4). The direct nucleic acid-binding molecule(s) upstream of STING have not yet been well defined, but may include the helicase DDX41 (41). Upon activation by nucleic acids, STING associates with and phosphorylates IRF3 via TBK1 (4, 6).

The ER-resident location of STING raised the possibility that ER stress-induced activation of IRF3 might proceed through this pathway. However, it was not clear if ER stress-induced IRF3 phosphorylation required TBK1 or STING or induced TBK1–STING association. To determine the requirement for TBK1, MEFs were pretreated with MRT67307, a TBK1/IKKe family kinase inhibitor, prior to Tg stimulation (34). MRT67307 abrogated Tg-dependent IRF3 phosphorylation (Fig. 4A). To determine if ER stress-induced association between TBK1 and STING, coimmunoprecipitation was performed. TBK1 and STING associated even before stimulation, and the association continued with Tg treatment (4). Similar results were obtained in primary macrophages (data not shown). However, by immunofluorescence microscopy, a striking ER stress-induced relocation of STING and TBK1 was evident, with association into larger order clusters around the nucleus (Fig. 4C). Together, these data suggested that Tg-induced IRF3 phosphorylation requires TBK1 and that Tg mobilizes STING and TBK1. However, it was not clear if STING was required for Tg-induced IRF3 phosphorylation via TBK1.

To determine if STING was specifically required for ER stress-induced IRF3 phosphorylation, STING−/− MEFs were stimulated with Tg and assessed by immunofluorescence for IRF3 phosphorylation (4). Neither IRF3 phosphorylation nor nuclear translocation was evident in STING−/− MEFs (Fig. 4D, IRF3 immunofluorescence not shown). By immunoblot, in WT MEFs, p-IRF3 (S396) is detectable in LPS-stimulated nuclear lysates, although we did not detect Tg-induced p-IRF3 nuclear phosphorylation. In the WT cells, Tg and LPS cotreatment increased levels of p-IRF3 over that observed with LPS alone (Fig. 4E). These results are consistent with those observed by Hu et al. (25), in which Tg augmented polyinosinic-polycytidylic acid-dependent IRF3 phosphorylation but did not appear to induce IRF3 on its own. However, in the STING−/− MEFs, no p-IRF3 was observed by immunoblot. The lack of p-IRF3 was not because of IRF3 deficiency or generally defective LPS signaling in the MEFs, as LPS-induced NF-κB nuclear translocation was intact in STING−/− MEFs. Together, these results support the idea that Tg uses STING and downstream TBK1 to phosphorylate IRF3 and that synergistic p-IRF3 induction by LPS and Tg requires STING.

**Optimal synergistic induction of IFN-β by Tg and LPS requires STING**

STING appeared to be required for phosphorylation of IRF3 during ER stress and synergistically induced p-IRF3 during Tg and LPS stimulation. However, the consequences for an IRF3-regulated gene, such as IFN-β, were not clear. Thus, synergistic IFN-β induction was evaluated in RNAi-transfected RAW 264.7 macrophages and STING−/− bone marrow-derived macrophages. Even moderate knockdown of STING with RNAi (Fig. 5A, 5B) decreased IFN-β induction by combined Tg and LPS stimulation. There was no significant effect of STING knockdown on LPS-induced IFN-β alone or the induction of two IRF3-independent cytokines, IL-1β and IL-6, by any of the stimuli. The effect of STING deficiency was more evident in the primary STING−/− bone marrow macrophages in comparison with the RNAi knockdown (Fig. 5C). Again, IL-6 induction was intact. Synergy was not completely abrogated in the STING−/− macrophages or in STING−/− MEFs (data not shown). However, the magnitude of synergistic IFN-β expression was decreased by about a log-fold in primary macrophages.
FIGURE 5. STING is required for optimal synergistic induction of IFN-β by Tg and LPS. (A) RAW264.7 macrophages were transfected with 100–200 nM control or STING-specific RNAi. After 24 h, cells were treated with 1 μM Tg followed by 100 ng/ml LPS for 3 h. Relative expression of STING, IFN-β, IL-1β, and IL-6 was determined by quantitative PCR with normalization to 18S RNA. Results were combined from three (STING and IL-1β) or four (IFN-β and IL-6) independent experiments with error bars representing the SEM. *p ≤ 0.032, **p = 0.011. (B) RAW264.7 macrophages were transfected with control or STING-specific RNAi and lysed at 24, 48, or 72 h. Lysates were resolved by SDS-PAGE and immunoblotted with anti-STING. Western blot was performed twice. (C) WT or STING−/− bone marrow-derived macrophages were treated with 1 μM Tg, 10 ng/ml LPS, or TPG+LPS for 3 h. Cells were processed as in (A) by quantitative PCR to assess IFN-β and IL-6 mRNA expression. Results were combined from three (WT) and five (STING−/−) independent experiments, with error bars representing the SEM. **p = 6.1e-6.

Not all ER stressors require TBK1 and STING for IRF3 phosphorylation; ER stressors mobilizing calcium depend upon STING for IRF3 phosphorylation

Although all of the frequently used pharmacologic ER stress-inducing agents activate the three primary signaling pathways encompassed by the UPR and have final common end points, their mechanisms of action vary. Ultimately, the quality and magnitude of all the various UPR stress pathways induced by these agents are likely to differ as well. To determine if the dependence on STING was a general property of UPR induction, STING+/+ and /− MEFs were stimulated with Tm, an agent that induces the UPR independently of calcium (Fig. 6A). There were no evident differences in Tm-induced IRF3 phosphorylation in the presence or absence of STING. Likewise, 2DG-induced IRF3 phosphorylation occurred in the absence of STING (data not shown). Consistent with these findings, Tm-induced IRF3 phosphorylation also did not require TBK1 family kinases, because MRT67307 did not prevent Tm-induced IRF3 phosphorylation (Fig. 6A). Thus, although Tg and Tm treatment may lead to similar common end points, the ER stress responses are qualitatively different. These data suggest that different UPR inducers activate IRF3 through a variety of signaling pathways.

One profound difference between Tg and Tm is that Tg induces the UPR by inhibiting the sarcoplasmic/ER Ca2+-ATPase pump, severely depleting ER calcium stores (37). Thus, it was possible that mobilization of STING reflected altered calcium metabolism and not the UPR at all. To test this hypothesis, cells were stimulated with ionomycin, a calcium ionophore that mobilizes calcium by influx, but does not trigger the UPR at 1 μM (data not shown and 42, 43). Even at higher doses, ionomycin is much less effective at upregulating the UPR gene BiP as compared with another ionophore A23187 (44). In contrast to Tg, ionomycin did not induce significant IRF3 phosphorylation (Fig. 6B). However, A23187, an agent widely used to induce the UPR, did induce IRF3 phosphorylation (45). These results indicate that collapsing the calcium gradient is not sufficient for IRF3 phosphorylation and that some component of the ER stress response, or ER calcium depletion, is required as well. Furthermore, A23187 also required STING for IRF3 phosphorylation. Thus, Tg is not the only UPR inducer dependent upon STING for IRF3 activation. It was not clear if the STING–TBK1 pathway would be involved in a more physiologic setting. During ischemia-reperfusion injury, ATP depletion disrupts sequestration of calcium in the ER, resulting in excess cytosolic calcium, mitochondrial calcium uptake, and ER dysfunction (46, 47). Intracellular disruptions in calcium handling are reproduced by in vitro OGD (48). As seen in Fig. 6C, OGD-dependent nuclear IRF3 translocation was impaired in STING−/− cells. Together, these data suggest that ER stressors that dysregulate calcium metabolism phosphorylate IRF3 through the STING–TBK1 pathway.

A site 1 protease inhibitor of ATF6 processing prevents Tm-induced activation of IRF3

We sought to determine which of the three major UPR pathways, stemming from IRE1/XBP1, PERK, and ATF6, might be playing a role in IRF3 activation in response to ER stressors. As mentioned above (Fig. 2), XBP1 is not absolutely required for nuclear translocation. PERK was not obligatory for synergistic IFN-β mRNA induction by either Tg or Tm (22). In response to ER stress, ATF6 transits to the Golgi, where it is cleaved to an active transcription factor by site 1- and site 2-specific proteases. AEBSF, a serine protease inhibitor, has been shown to block this processing event by inhibiting the site 1 protease (49). We confirmed this activity (Fig. 7). AEBSF prevented Tm- and 2DG- but not Tg-dependent IRF3 phosphorylation, as visualized by immunofluorescence (Fig. 7A). By Western blot, the presence of AEBSF prevented synergistic p-IRF3 in dually treated LPS+Tm cells, reducing p-IRF3 to the level of LPS alone (Fig. 7B); however, AEBSF did not prevent LPS+Tg-enhanced p-IRF3. Consistent with these results, AEBSF significantly diminished Tm, but not Tg-dependent IFN-β mRNA synergy (Fig. 7C). The lack of effect on Tg-induced synergy argues against nonspecific toxicity. Together, these results indicate that AEBSF sensitivity distinguishes between IRF3 activation by Tg and Tm/2DG and is consistent with a possible role for ATF6 in the latter. Furthermore, ER stressors activate IRF3, leading to synergistic IFN-β mRNA induction, by at least two distinguishable pathways.

Discussion

This study describes the novel observation that ER stress, even in absence of PRR stimulation, activates IRF3. Different forms of ER stress accomplish this through at least two distinct pathways, requiring either TBK1/STING or AEBSF-sensitive signaling. The synergistic induction of several IRF3-regulated inflammatory...
mediators by concurrent UPR and LPS stimulation suggest the activation of IRF3 by ER stress may have a wider impact in innate immunity, beyond augmenting IFN-β production. Even though ER stress alone is not sufficient to trigger the induction of an IRF3-regulated gene, activation of IRF3 by ER stress is necessary for the dramatic IFN-β synergism observed with LPS. In evidence of this requirement, interfering with Tg-dependent IRF3 phosphorylation through the modulation of STING severely impacts the magnitude of Tg-induced synergy. Similarly, disrupting Tm-dependent IRF3 phosphorylation with AEBSF significantly di-

**FIGURE 6.** STING-dependent IRF3 phosphorylation requires both calcium mobilization and ER stress. (A) WT or STING⁻/⁻ MEFs were pretreated with 2 μM MRT67307 for 30 min then 10 μg/ml Tm for 6 h as indicated. Cells were incubated with anti–p-IRF3 (S386) followed by Alexa Fluor 488 secondary Ab and visualized by immunofluorescence microscopy. Results are representative of four independent experiments. (B) WT or STING⁻/⁻ MEFs were untreated (NT) or treated with 1 μM ionomycin or 2 μM A23187 as indicated. Fixed cells were incubated with anti–p-IRF3 followed by Alexa Fluor 488 secondary Ab and visualized by immunofluorescence microscopy. Results are representative of three independent experiments. (C) WT or STING⁻/⁻ MEFs were subjected to OGD (as in Fig. 1) for 1 h followed by 2 h reoxygenation. Cells were fixed (C) or lysed (D) and IRF3 nuclear translocation detected by immunofluorescence or Western blot, respectively. Scale bars, 50 μM.

**FIGURE 7.** AEBSF, a site 1 protease inhibitor, blocks Tm- and 2DG-induced IRF3 phosphorylation and Tm-dependent synergistic IFN-β induction. (A) RAW cells were pretreated with 300 μM AEBSF for 1 h, then stimulated with 1 μM Tg for 2 h, 20 mM 2DG for 5 h, or 10 μg/ml Tm for 5 h. Cells were fixed and then stained with anti–p-IRF3 (S386) plus secondary anti-rabbit Alexa Fluor 488. Results are representative of two independent experiments. (B) RAW cells were pretreated with AEBSF as in (A) and then untreated (NT), stimulated with Tg 1 h or Tm 5 h, and followed by an additional 3 h media or LPS as indicated. Whole-cell lysates were resolved by SDS-PAGE and immunoblotted with anti–p-IRF3 (S396), IRF-3, or actin. Results are representative of two independent experiments. (C) RAW cells were pretreated with AEBSF as in (A) and then stimulated with 1 h Tg or 5 h Tm followed by an additional 3 h LPS as indicated. Relative IFN-β mRNA was quantified by quantitative PCR with normalization to 18S rRNA. Results were combined from four to five independent experiments, and error bars represent the SEM. *p < 0.007. (D) Cells were stimulated as in (B). Whole-cell lysates were resolved by SDS-PAGE and immunoblotted for precursor (P) and mature (M) cleaved forms of ATF6. Results are representative of three independent experiments. Scale bar, 50 μM.
minimized synergistic IFN-β expression. Residual IFN-β induction in the LPS+Tg-treated STING−/− MEFs may relate to IRF3 phosphorylation below our limits of detection or compensation by another IRF3 serine (e.g., S339) for which we did not assay (50).

This study supports the novel concept that intracellular stress responses may co-opt innate immune-signaling pathways previously thought to be dedicated to pathogen sensing. The proximity of STING to the mitochondria-associated membrane a site of interorganelle calcium transport and regulation may suggest why UPR inducers that affect calcium also mobilize STING (4, 51). Our findings suggest that, at least in MEFs and macrophages, STING and TBK1 associate even prior to stimulation. Upon ER stress induction, STING and TBK1 dramatically reorganize into larger macroscopic collections. This mobilization may be a result of reorganization of the ER membranes themselves (containing STING), translocation to another organelle (e.g., Golgi), or association with other unidentified molecules in a multimolecular complex. The augmentation of type I IFN responses by ER stress/calciun dysregulation and STING may be of particular relevance to viral infections, such as hepatitis C, that induce ER stress and cause calcium leak (52). Our results obtained with in vitro OGD (Fig. 6) have direct implications for in vivo ischemia-reperfusion injury: they suggest the dysregulation of calcium, ER stress, and type I IFN-dependent inflammatory injury may be critically interrelated (20, 53–57).

The UPR has been shown to activate both NF-κB and AP-1 family member transcription factors (58). Thus, it is unclear why UPR-induced IRF3 phosphorylation is not sufficient to induce IFN-β expression in vitro. Even though the UPR induces nuclear translocation of IRF3, translocation does not automatically confer transcriptional activity; dissociation between translocation and transcriptional activity has been noted in multiple models of viral IRF3 inhibition (59–62). IRF3 has two activation clusters comprising seven potentially phosphorylated serines and threonines (S385, S386, S396, S398, S402, S405, and T404). Some controversy remains regarding serine phosphorylation requirements for IRF3 activity: phosphorylation of S396 has been proposed as an essential minimal acceptor site for responses to Sendai virus and threonine (S385, S386, S396, S398, S402, S405, and T404). Some controversy remains regarding serine phosphorylation requirements for IRF3 activity: phosphorylation of S396 has been proposed as an essential minimal acceptor site for responses to Sendai virus and may be critical for homodimerization (50, 63). More recently, S396 has been shown to promote higher-order oligomerization (64). However, others have identified S386 as the critical site for homodimerization and nuclear translocation (65, 66). Unfortunately, it is not yet clear exactly which sites on IRF3 correspond to optimal transcriptional activity in response to LPS and other specific pathogens. Ultimately, both may act cooperatively to bind CBP/p300 with higher affinity (64). Thus, one possibility is that even though ER stress induces S386 phosphorylation, ER stress in isolation does not induce strong enough phosphorylation at S396, as suggested by Western blot (e.g., Fig. 4E); the UPR may only induce partial phosphorylation of IRF3 and LPS remains necessary for additional phosphorylation at other serines/threonines. Alternatively, as suggested by our Western blot data, UPR-induced phosphorylation at S386 may facilitate or enhance LPS-dependent S396 phosphorylation. The requirement for multiple-site IRF3 phosphorylation to promote oligomerization may explain the qualitative differences in immunofluorescence between LPS and ER stressors such as OGD (Fig. 2) (64). Our data would suggest that ultimate phosphorylation at S396 correlates best with IRF3 DNA binding by chromatin immunoprecipitation and transcriptional activation of IFN-β (23).

Apart from suboptimal IRF3 activation, there are other possible explanations: IRF3 alone is not sufficient for IFN gene transcription; the enhanceosome also contains NF-κB and AP-1 transcription factors. Transcriptional activation following enhanceosome formation requires binding of multiple elements including critical scaffolding molecules (HMGA1) and histone acetyltransferases (e.g., CBP/p300) (11). LPS stimulation may be required to recruit these other molecules. Another possibility is that a stronger NF-κB signal may be required than that generated during ER stress alone. Finally, there could be a cell-type issue, because our studies are conducted in macrophages and MEFs. When mice are treated in vivo with Tm alone, we observed detectable serum IFN-β (preliminary data not shown), suggesting that an unidentified cell type is capable of producing IFN during a UPR.

In this study and others, ER stress has been noted to augment transcription of select IFN-β-regulated genes (e.g., IFN-β but not RANTES) (24). IRF3 binds similar DNA sequences within gene promoters designated as IFN-stimulated response elements or positive regulatory domains (PRD I and III in the IFN-β promoter) (67). The selectivity in synergism may relate to promoter complexity and requirement for multiple transcription factors, as mentioned above. Constitutively activated IRF3 (an aspartate containing phosphomimetic) is sufficient to activate only a small subset of IFN-stimulated response elements containing genes, including ifit2/ISG54, ISG56, ISG60, CIG5, and PMA inducible protein 1 (68). However, we did not detect robust activation of ISG54 by Tg alone. This failure may reflect suboptimal IRF3 activation at specific serines. Alternatively, given the independence of XBP1 and IRF3 translocation (Fig. 2) and the discovery of XBP1 binding sites in cytokine promoters and enhancers, significant synergy might require DNA binding sites for both IRF3 and UPR-dependent transcription factors (22–24). The experience with IFN-β would favor this multi-hit hypothesis.

It is not clear which aspects of the UPR are necessary for IRF3 phosphorylation and nuclear translocation. The answer may differ depending upon type of ER stress. Our studies would suggest that XBP1 is not required for ER stress-induced IRF3 nuclear translocation. PERK is not necessary for synergistic IFN induction (22 and data not shown). AEBSF, a protease inhibitor that prevents ATF6 processing, blocked Tm but not Tg-dependent IRF3 phosphorylation and synergy (Fig. 7) (22). Tg may use an IRE1 kinase-mediated pathway to activate IRF3. Alternatively, Tg and A23187 could mobilize a nonclassical UPR ER stress pathway related to calcium flux that has not been described. Another possibility is that IRF3 activation resulting from profound ER calcium depletion and the UPR are independent outcomes of treatment with these stressors.

Our results are consistent with the hypothesis that Tm and 2DG-induced IRF3 phosphorylation proceed through ATF6 or a related protein. ATF6 belongs to the OASIS family of transcription factors that is processed by the site 1 proteases. However, protein distribution of these other family members is much more restricted than ATF6 (69). AEBSF also inhibits reactive oxygen species generation by NADPH (70). Tg, Tm, and ER stress related to cholesterol loading have all been found to induce oxidative stress via NADPH (71–73). Reactive oxygen species potentiate IRF3 activation (74). However, NADPH oxidase inhibition by AEBSF would not explain the divergent effects on Tg and Tm, particularly given the involvement of calcium in ER stress-mediated NADPH oxidase activation (71). The specific AEBSF-sensitive signaling event remains to be confirmed.

Another outstanding question is the identity of the kinase activated by non-Tg UPR inducers that is responsible for phosphorylating IRF3. In this study, Tm led to IRF3 phosphorylation in the presence of a TBK1/IKKe inhibitor. The kinase cascade involving NF-κB–inducing kinase and IKKe has been reported to phosphorylate IRF3 independently of TBK1 (75). The MAPK cascade initiated by IRE-1 that includes p38 may also play a role...
Disclosures

The authors have no financial conflicts of interest.

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