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Inhibition of x-box binding protein 1 reduces tunicamycin-induced apoptosis in aged murine macrophages

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Summary

Endoplasmic reticulum (ER) stress is induced by the accumulation of unfolded and misfolded proteins in the ER. Although apoptosis induced by ER stress has been implicated in several aging-associated diseases, such as atherosclerosis, it is unclear how aging modifies ER stress response in macrophages. To decipher this relationship, we assessed apoptosis in macrophages isolated from young (1.5–2 months) and aged (16–18 months) mice and exposed the cells to the ER stress inducer tunicamycin. We found that aged macrophages exhibited more apoptosis than young macrophages, which was accompanied by reduced activation of phosphorylated inositol-requiring enzyme-1 (p-IRE1α), one of the three key ER stress signal transducers. Reduced gene expression of x-box binding protein 1 (XBP1), a downstream effector of IRE1α, enhanced p-IRE1α levels and reduced apoptosis in aged, but not young macrophages treated with tunicamycin. These findings delineate a novel, age-dependent interaction by which macrophages undergo apoptosis upon ER stress, and suggest an important protective role of IRE1α in aging-associated ER stress-induced apoptosis. This novel pathway may not only be important in our understanding of longevity, but may also have important implications for pathogenesis and potential treatment of aging-associated diseases in general.

Key words: aging; macrophages; endoplasmic reticulum stress; apoptosis.

Introduction

Macrophages are critical for the longevity of an organism. Indeed, aberrant function of macrophages contributes to several age-associated diseases such as atherosclerosis (Tabas, 2010). When macrophages engulf debris or pathogens to maintain tissue integrity and prevent inflammation, endoplasmic reticulum (ER) stress is induced in these cells. ER stress activates three transducers located on the ER membrane: inositol-requiring enzyme-1 (IRE1), activating transcription factor-6 (ATF6), and protein kinase RNA (PKR)-like ER kinase (PERK). Each of these transducers triggers a series of cellular responses, which either relieve ER stress or induce cellular apoptosis. Mounting the appropriate response to ER stress is critical for cell survival.

Aging may induce ER stress via multiple mechanisms including alterations in oxidative stress levels and accumulation of environmental toxins and misfolded/unfolded proteins (Salminen & Kaarniranta, 2010). However, the exact molecular mechanisms by which aging affects ER stress remain unclear, especially in macrophages.

Cellular apoptosis is one of the main consequences of prolonged and severe ER stress. If the imbalance between unfolded and folded proteins is not restored, apoptosis ensues (Ron & Walter, 2007). Two signaling mediators in the ER stress response have been suggested to play major roles in mediating apoptosis (Tabas & Ron, 2011). One is CHOP (C/EBP-homologous protein), which is a PERK downstream effector that induces apoptosis by changing the Bcl2-associated X protein (Bax) vs. B-cell CLL/lymphoma 2 ratio (Bcl-2) (Oyadomari & Mori, 2004). The other one is IRE1, an ER stress transducer that induces apoptosis through tumor necrosis factor receptor associated factor (TRAF) and Jun amino terminal kinase (JNK) (Xia *et al.*, 1995; Li *et al.*, 2011). IRE1 may also induce apoptosis through IRE1 dependent Decay (RIDD) (Hollien & Weissman, 2006; Hollien *et al.*, 2009), which is dependent on the ribonucleolytic function of IRE1. Normally, IRE1 targets specific mRNA, such as x-box binding protein 1 (XBP1) to exert its ribonuleolytic function and create splicing of XBP1 (XBP1s) (Nekrutenko & He, 2006). However, prolonged ER stress induces RIDD and leads to indiscriminate degrading of membrane-associated mRNA regardless of their sequences (Han *et al.*, 2009).

Here, we investigated how aging affects ER apoptosis in murine macrophages in response to tunicamycin (TM), a known ER stress inducer. We measured apoptosis in peritoneal macrophages isolated from young (1.5–2 months) and aged (16–18 months) mice *in vitro* using positive Annexin V staining by fluorescent microscopy and cleaved caspase-3 measurement. Our findings indicate that aged macrophages are more susceptible to TM-induced apoptosis than young macrophages and that aged macrophages express less phosphorylated IRE1α (p-IRE1α) than young macrophages after ER stress induction. Knocking down XBP1 using si-XBP1 (small interference RNA targeted XBP1) increased protein levels of p-IRE1α and reduced apoptosis in aged, but not in young, macrophages. Moreover, concurrently knocking down gene expression of both IRE1α and XBP1 abrogated the apoptosis-reducing effects of si-XBP1 in aged macrophages. These results suggest an important role of the IRE1α-XBP1 axis in age-associated apoptosis induced by ER stress and identify a novel interaction by which aging enhances ER stress-induced apoptosis in macrophages. Our findings may have important implications for the pathogenesis and potential treatment of aging-associated diseases, in which macrophage apoptosis plays a role.

Results

Aging increases macrophage susceptibility to ER stress-induced apoptosis

To evaluate whether aging modifies macrophage sensitivity to ER stress-induced apoptosis, we treated thioglycollate-elicited peritoneal macrophages with TM, a known inducer of ER stress. We assessed cell

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apoptosis using positive Annexin V staining by fluorescent microscopy, an established approach in the field (Devries-Seimon *et al.*, 2005; Pechous *et al.*, 2006; Timmins *et al.*, 2009; Seimon *et al.*, 2010a,b) and also by cleaved caspase-3 measurement. Upon TM stimulation, peritoneal macrophages isolated from aged (16–18 months of age) mice exhibited significantly higher levels of apoptosis and cleaved capsase-3 than macrophages from young mice (1.5–2 months of age). This difference was dose dependent (Fig. 1A–C). Similar results were observed in resident peritoneal macrophages from young and aged mice (Fig. S1).

To determine whether this aging-associated effect was only restricted to TM, we performed the experiments using other ER stress inducers: free cholesterol and 7-ketocholesterol (Fig. S2). Free cholesterol induces ER stress by depletion of stored calcium within the ER (Zhang & Kaufman, 2003), and 7-ketocholesterol triggers an ER stress response via induction of nicotinamide adenine dinucleotide phosphate reduced oxidase (NOX) (Pedruzzi *et al.*, 2004). Similar to TM, both free cholesterol and 7-ketocholesterol induced more apoptosis in aged macrophages than in young macrophages, indicating this aging-associated effect is not restricted in TM. Altogether, these results indicate that aging increases macrophage sensitivity to ER stress-induced apoptosis.

Aging increases BiP levels and reduces IRE1α activation in macrophages during ER stress

To examine the mechanisms by which aging increases macrophage sensitivity to ER stress-induced apoptosis, we measured the ER stress chaperon BiP (also known as GRP78), which is increased with accumulation of unfolded proteins within the ER, and assessed the three branches of ER stress: IREα, PERK, and ATF6. We found that BiP levels

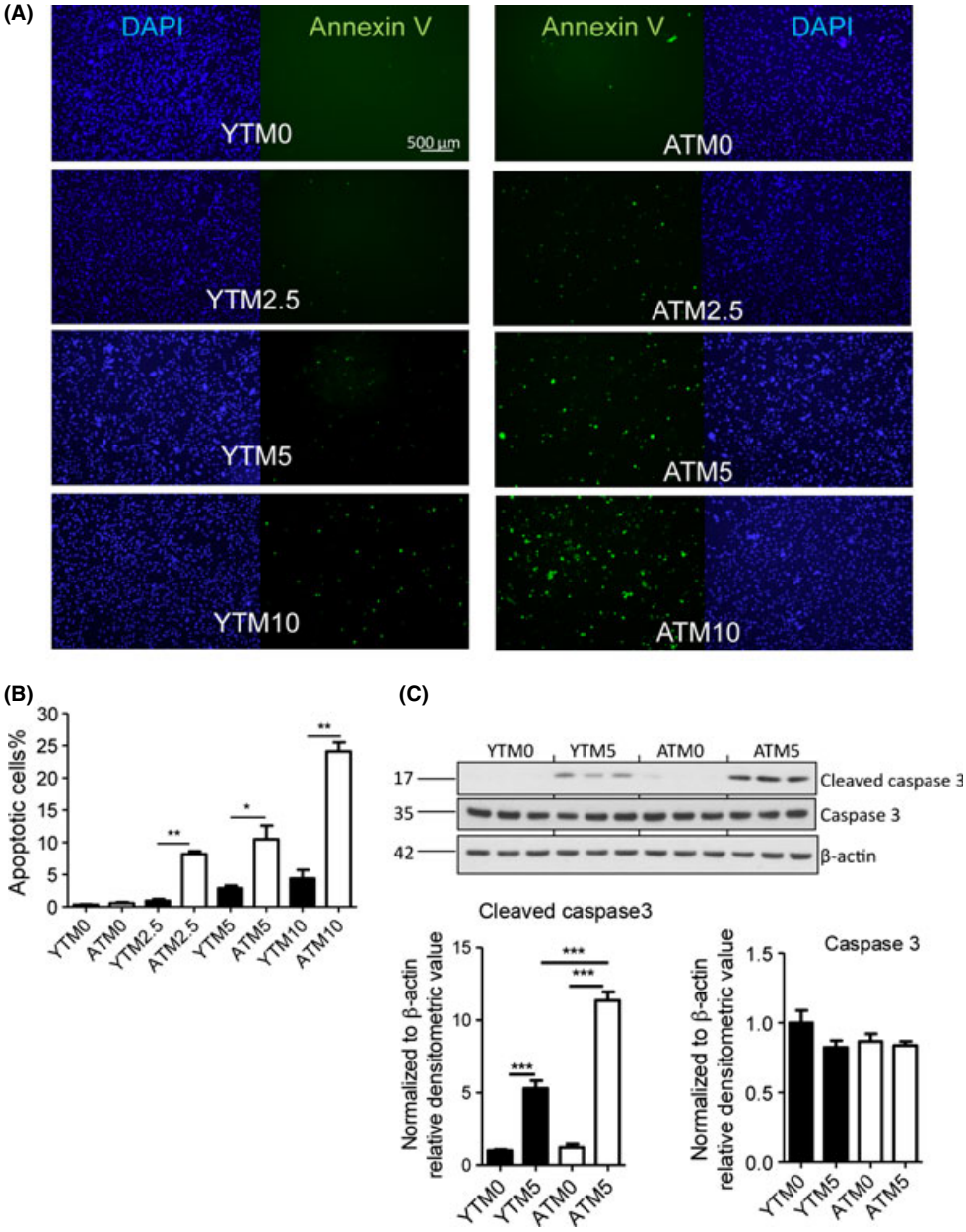

Fig. 1 Aged macrophages are more susceptible to TM-induced apoptosis than young macrophages. Aged (16–18 months) and young (1.5–2 months) peritoneal macrophages were cultured with different concentrations of TM for 4 h, then in TM-free medium for another 16 h. Apoptosis was measured by Annexin V positive staining by fluorescent microscopy. Apoptotic cells, Annexin V positive staining (green); nuclei, DAPI staining (blue). Representative images are shown in (A) and quantification of apoptotic cells is shown in (B). Total and cleaved caspase 3 was measured by Western blot, and representative images are shown in (C). Experiments were repeated three times. For each experiment, three mice per group were used as a source of cells. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t*-test). Error bars = standard error of mean (SEM). TM0 = 0 μg mL^{-1} TM; TM2.5 = 2.5 μg mL^{-1} TM; TM5 = 5 μg mL^{-1} TM; TM10 = 10 μg mL^{-1} TM10. Y, young; A, aged.

were higher in aged macrophages as compared to young macrophages upon ER stress induction (Fig. 2A,B).

We then measured two molecules that play pivotal roles in promoting ER stress-induced apoptosis p-IRE1α and CHOP, a component of the PERK ER stress pathway (Tabas & Ron, 2011). Both p-IRE1α and CHOP expressions were induced by TM in aged and young macrophages as expected (Fig. 2A,B). However, the induction of p-IRE1α was significantly lower in aged macrophages than in young macrophages (Fig. 2A,B, Fig. S3), whereas CHOP levels remained similar for aged and young macrophages (Fig 2A,B). Total IRE1α levels were similar between young and aged macrophages (Fig. 2). Aging also impaired p-IRE1α levels in macrophages exposed to free cholesterol or 7-ketocholesterol (Fig. S2B). Further interrogation of the PERK branch found a small reduction in p-PERK in aged macrophages as compared to young macrophages after TM treatment, but there were no differences in other signaling proteins in the PERK branch including GADD34, and p-ELF2α (Fig. S4A), compatible with our findings with CHOP (Fig. 2). Interrogation of the ATF6 branch did not reveal alterations in macrophages at baseline or upon ER stress with aging (Fig. S4B). Overall, these results suggest that upon ER stress induction, aging impairs the activation of IRE1α, but besides a small impairment in activation of PERK, does not impact other branches of the ER stress response, in macrophages.

Small interference RNA knockdown of XBP1 reduces apoptosis in aged, but not young, macrophages

To examine the mechanism by which aging increases macrophage sensitivity to ER stress in more detail, we evaluated the IRE1α pathway in aged macrophages. Upon ER stress induction, IRE1α undergoes *trans*-autophosphorylation activation, which uncovers an endoribonucleolytic function, leading to unconventional splicing of XBP1 mRNA (XBP1s) (Han *et al.*, 2009). Prior work has shown that the spliced form of XBP1 mRNA is translated into a multifunctional transcription factor, denoted as XBP1s, which is known to activate genes to reduce ER stress (Yoshida *et al.*, 2001). As a direct downstream target of IRE1α, XBP1 reduction or inhibition may regulate the activation of IRE1α. Interestingly, a prior study has found that inducible XBP1-deficient mice exhibit constitutive activation of IRE1α in the liver (Hur *et al.*, 2012). Therefore, to restore the reduced activation of IRE1α in aged macrophages, we indirectly enhanced p-IRE1α by knocking down XBP1 gene expression in macrophages.

For this purpose, we tested whether siRNA targeting XBP1 (denoted as si-XBP1) reduced XBP1 gene and protein expression. Transfecting young macrophages with si-XBP1 for 48 h reduced XBP1 mRNA by 78% (Fig. 3A). Downstream target genes of XBP1s, including ERdj4 and p58, were also reduced by si-XBP1 in young macrophages treated with TM (Fig. 3A), indicating that the functional effects of XBP1s were also inhibited by si-XBP1. In addition, we evaluated whether si-XBP1 reduced XBP1 protein levels. We measured both XBP1s and unspliced XBP1 (denoted as XBP1u), but did not detect significant levels of XBP1u in macrophages stimulated with and without TM (data not shown). However, si-XBP1 reduced XBP1s protein expression in TM-treated young macrophages, but not in TM-treated macrophages cultured with scrambled control (Fig. 3B). Altogether, these results suggest that si-XBP1 transfection effectively inhibits the induction of XBP1s in macrophages during ER stress.

We also assessed whether p-IRE1α levels were enhanced by si-XBP1. We found that in young macrophages transfected with si-XBP1 and treated with TM, p-IRE1α levels were significantly increased (Fig. 3C), and this increase was associated with a reduction in XBP1 (Fig. 3D). Consistent with current findings, bone marrow-derived macrophages from young XBP1 knockout mice also exhibited enhanced IRE1α activation unlike young wild-type cells during TM treatment (Fig. S5). Altogether, these results indicate that reducing XBP1 expression enhances the activation of IRE1α in macrophages during ER stress and increases the levels of p-IRE1α.

To evaluate whether enhanced IRE1α activation affects aging-associated apoptosis in macrophages with ER stress, we transfected both aged and young macrophages with si-XBP1. For this purpose, 48 h after si-XBP1 or scramble control transfection, aged, and young macrophages were subjected to 4 h of TM treatment followed by 16 h or culture. Annexin V staining showed that apoptosis was reduced in aged macrophages transfected with si-XBP1, but not in cells transfected with scramble control (Fig. 4A,B). This reduction was also not observed in young macrophages (Fig. 4A,B). Importantly, si-XBP1 transfection reduced apoptosis in aged macrophages to the levels found

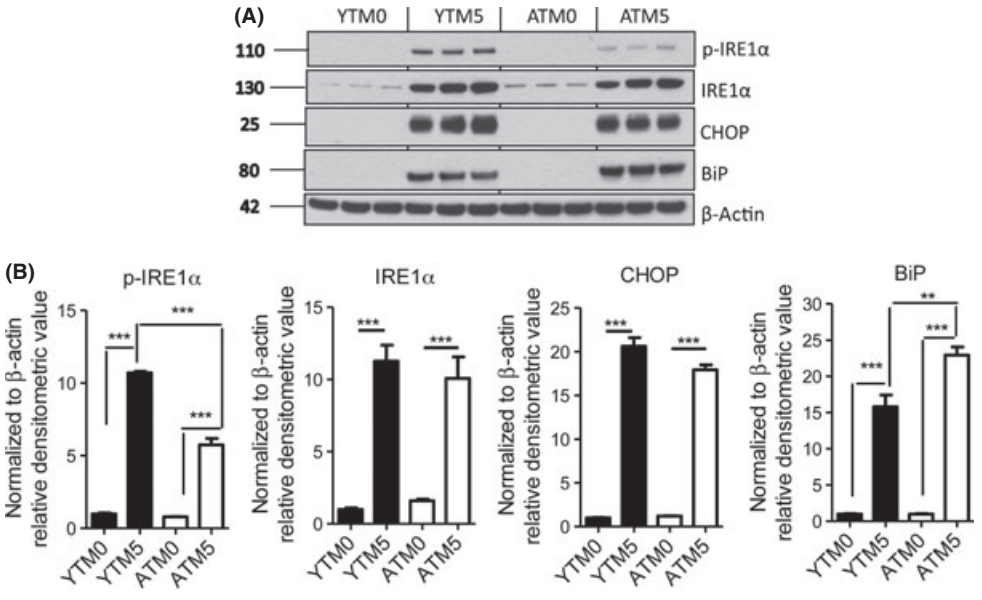

Fig. 2 Aged macrophages exhibit lower p-IRE1α and higher BiP protein levels than young macrophages after tunicamycin (TM) treatment. Aged (16–18 months) and young (1.5–2 months) macrophages were treated with 5 μg mL^{-1} TM for 4 h, then switched to TM-free medium. Cell lysates were obtained 16 h later. IRE1α, p-IRE1α, BiP and CHOP levels along with that of the loading control (β-actin) were determined by Western blot. Representative blots are shown in (A) and quantification based on densitometry relative to loading control is shown in (B). Experiments were repeated three times. For each experiment, four mice per group were used as a source of cells. Each lane in the Western blot represents a biological replicate. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t*-test). Error bars = SEM. TM0 = 0 μg mL^{-1} TM; TM5 = 5 μg mL^{-1} TM. Y, young; A, aged.

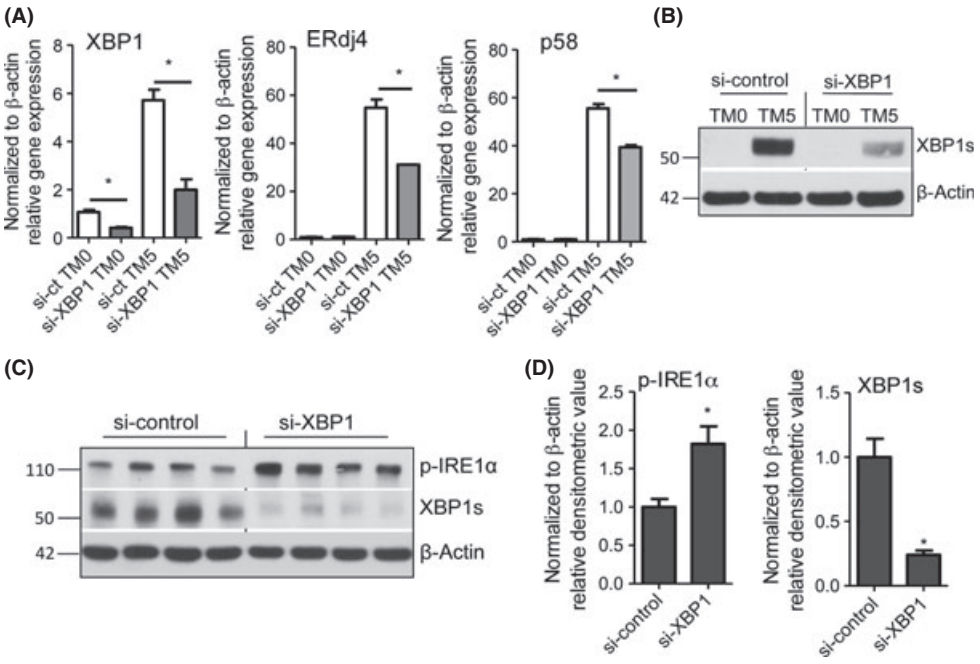

Fig. 3 Knocking down XBP1 mRNA enhances p-IRE1α protein levels in young macrophages. Young macrophages were treated with either scrambled control (denoted as si-ct or si-control) or small interference RNA to XBP1 (denoted as si-XBP1) for 48 h, and then treated with 5 μg mL^{-1} TM for 4 h followed by TM-free medium for another 16 h. Total RNA and cell lysates were collected for further analysis. TM0 = 0 μg mL^{-1} TM; TM5 = 5 μg mL^{-1} TM. Y, young; A, aged. (A) Gene expression of XBP1 and its direct downstream effectors, ERdj4 and p58, were measured by real -time RT–PCR. Error bars = SEM. (B) As per A but XBP1 protein levels were determined by Western blot analysis. (C–D) As per A but p-IREα and XBP1 protein levels were determined by Western blot analysis. Quantification of densitometry relative to loading control is shown in (D). *$P < 0.05$ (Student's *t*-test). Error bars = SEM. For A–D, 3 mice per group/experiment were used as a source of cells. Experiments were repeated independently three times.

in young si-XBP1-transfected macrophages (Fig. 4B, compare si-XBP1 ATM5 vs. si-XBP1 YTM5). The reduction of apoptosis by si-XBP1 was associated with enhanced p-IRE1α levels (Fig. 4C,D). The fold increase of p-IRE1α induced by si-XBP1 was significantly higher in aged macrophages (4.1-fold increase) than in young macrophages (1.9-fold increase) (Fig. 4E).

When we increased the culture time after 4 h of TM stimulation from 16 to 44 h, the level of apoptosis was increased in both young and aged microphages, and there was still a significant difference in apoptosis between the groups (Fig. 4F). However, si-XBP1 treatment only reduced apoptosis in aged macrophages (Fig. 4F), indicating that its apoptosis-reducing effects of si-XBP1 are age-specific and not solely related to higher apoptosis levels. si-XBP1 treatment did not impact other components of the ER stress response, such as CHOP (Fig. S6).

To confirm that the apoptosis-reducing effect of si-XBP1 is dependent on IRE1α, we knocked down IRE1α gene expression concurrently with that of XBP1. As a result, the ability of si-XBP1 to enhance IRE1α in aged macrophages was abrogated when IRE1 was inhibited (Fig. S7). We found that si-IRE1α significantly ($P < 0.05$) diminished the apoptosis-reducing effects of si-XBP1 in aged macrophages (Fig. 5A,B). Altogether, these results indicate that enhancing the activation of IRE1α in aged macrophages through knockdown XBP1 effectively reduces ER stress-induced apoptosis.

Discussion

In the current study, we demonstrate that aged macrophages exhibit increased apoptosis in response to several ER stressors. On a molecular level, aging decreases activation of IRE1α within macrophages during ER stress. Inhibiting XBP1 enhances IRE1α activation and ameliorates aging-associated apoptosis in macrophages in an IRE1α-dependent fashion. Furthermore, our study provides evidence that the apoptotic-reducing effects of XBP1 inhibition are age-specific and not merely due to higher apoptosis levels (Fig. 4F). Overall, our study has uncovered a novel interaction by which aging enhances apoptosis in macrophages during ER stress.

Although the exact mechanism by which aging enhances apoptosis during ER stress is not fully determined by our study, it is possible that aging may increase ER stress-associated apoptosis through a two-hit model. The first hit may be comprised of increased generation of ER stress. Specifically, aging may impair the cell machinery for protein folding, and increase the production of unfolded and misfolded proteins, which results in ER stress (Naidoo, 2009). This is supported by our study, which found elevated BiP levels in aged macrophages during ER stress (Fig. 2). Aging may also perturb calcium homeostasis in the ER (Smaili *et al.*, 2009), or impair autophagy, which could both lead to increased protein unfolding within the ER, and enhance ER stress with aging.

The second hit may consist of a reduced ability of aged cells to tolerate ER stress. Rodent studies have observed that several ER stress response signaling molecules, including, GADD34 and p-JNK, are reduced with aging (Paz Gavilan *et al.*, 2006; Hussain & Ramaiah, 2007). Our study also discovered a reduced activation of IRE1α in aged macrophages (Fig. 2). Together, these results indicate that aging enhances ER stress and alters ER stress signaling to induce apoptosis in macrophages.

Accumulating evidence suggests that CHOP and IRE1 are two pivotal molecules that mediate ER stress-induced apoptosis (Oyadomari & Mori, 2004; Tabas & Ron, 2011). However, we detected reduced activation of only IRE1α but not of CHOP, a component of the PERK pathway of ER stress, in aged macrophages (Fig. 2B). Besides a minor impairment in p-PERK upregulation, we did not find any other alterations in the other UPR branches that transduce ER stress, namely the PERK and ATF6 branches with aging. Moreover, enhancing IRE1α activation by inhibiting XBP1 gene expression diminished the aging-associated apoptosis induced by ER stress, an effect that is IRE1α-dependent (Figs 4 and 5). A prior study also found that inhibiting XBP1 gene expression led to feedback activation of IRE1α in young mice (Hur *et al.*, 2012). This effect protected the young mice from acetaminophen-induced liver toxicity (Hur *et al.*, 2012). The exact molecular interactions by which XBP1 inhibition leads to activation of IRE1α will require future study. As autophagy and apoptosis are connected, it is possible that XBP1 inhibition alters autophagy, which is dysfunctional with aging (Hubbard *et al.*, 2012), to increase activation of IRE1α, a pathway that will require

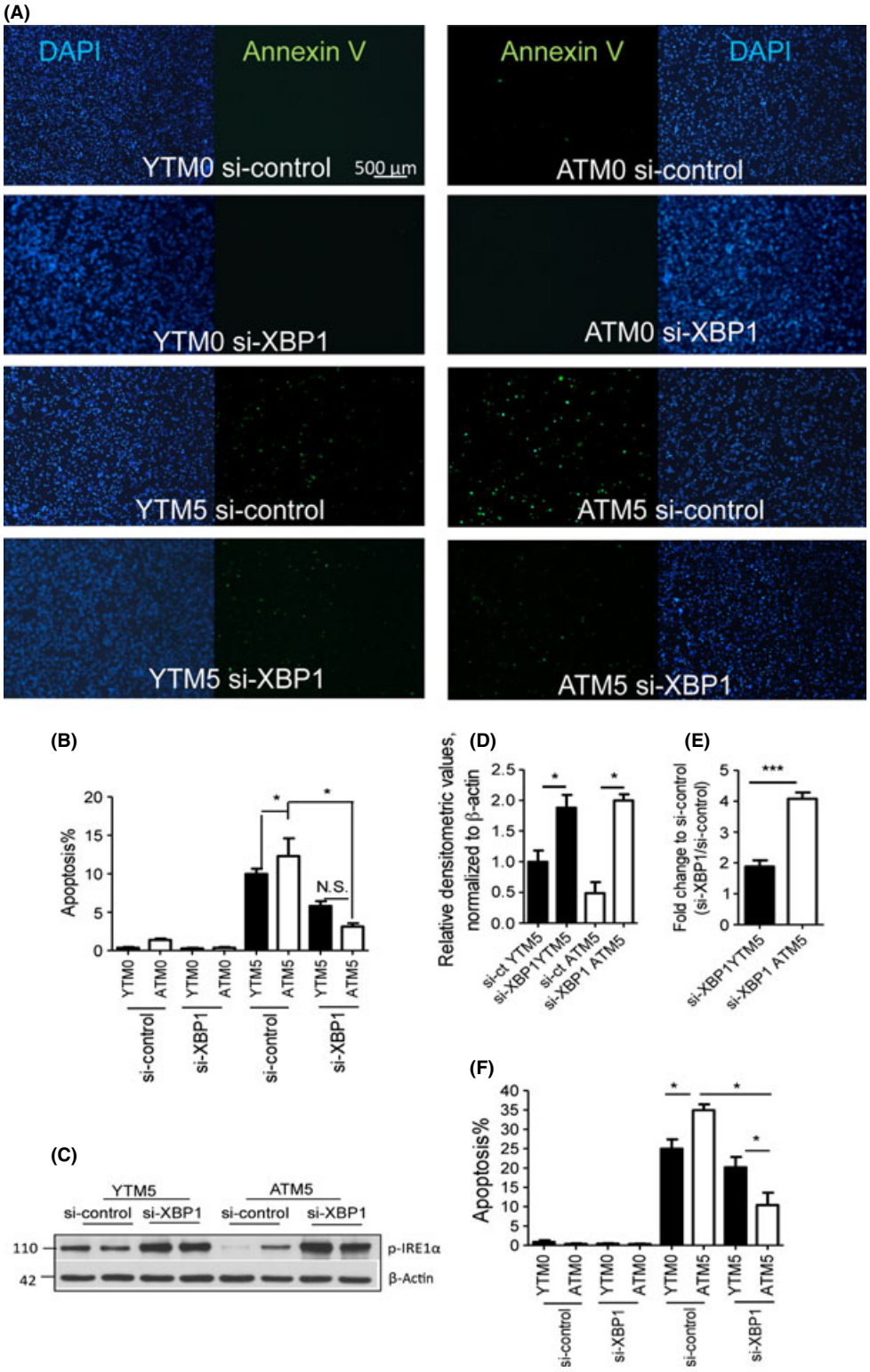

Fig. 4 XBP1 gene silencing reduces apoptosis in aged but not in young macrophages during treatment with TM. Peritoneal macrophages were obtained from aged (16–18 months) and young (1.5–2 months) mice. Cells were treated with either scrambled control (denoted as si-control) or small interference RNA to XBP1 (denoted as si-XBP1) for 48 h, and then treated with 5 μg mL^{-1} TM for 4 h followed by TM-free medium for another 16 h. The degree of apoptosis was assessed by Annexin V staining (green), and nuclei are shown by DAPI staining (blue) through fluorescent microscopy. Representative images are shown in (A) and quantification of apoptotic cells is shown in (B). Experiments were repeated three times. For each experiment, three mice per group were used as a source of cells. *$P < 0.05$ (Student's *t*-test). Error bars = SEM. TM0 = 0 μg mL^{-1} TM; TM5 = 5 μg mL^{-1} TM. Y; young; A; aged. (C–F) Cell lysates from (A–B) above were obtained and p-IRE1α protein levels in young and aged macrophages were determined by Western blot analysis. Representative blots (C) and quantification based on densitometry relative to loading control (D) are shown. Fold-increases of p-IRE1α in si-XBP1 group compared to si-control groups are shown in (E). Experiments were repeated three times. (F) Peritoneal macrophages were obtained from aged and young mice. Cells were treated with either si-control or si-XBP1 for 48 h, and then treated with 5 μg mL^{-1} TM for 4 h followed by TM-free medium for another 44 h. For each experiment, three mice per group were used as a source of cells. *$P < 0.05$, ***$P < 0.001$ (Student's *t*-test). Error bars = standard error of mean (SEM).

future investigation. Given that IRE1 promotes longevity in insulin/IGF1 pathway mutants in *Caenorhabditis elegans* (Henis-Korenblit *et al.*, 2010) and that IRE1 is required for dietary restriction-mediated lifespan extension (Chen *et al.*, 2009), our studies suggest an important protective role of IRE1α in aging-associated ER stress-induced apoptosis. It is also possible that this molecule promotes longevity.

Our results imply that increased sensitivity to ER stress with aging may contribute to the pathogenesis of several age-associated diseases, in which macrophages play a role in disease progression. Macrophage apoptosis contributes to the pathogenesis of multiple aging-associated diseases, such as diabetes (O'Brien *et al.*, 2002) and atherosclerosis (Tabas, 2005). Macrophages may promote these diseases by defective

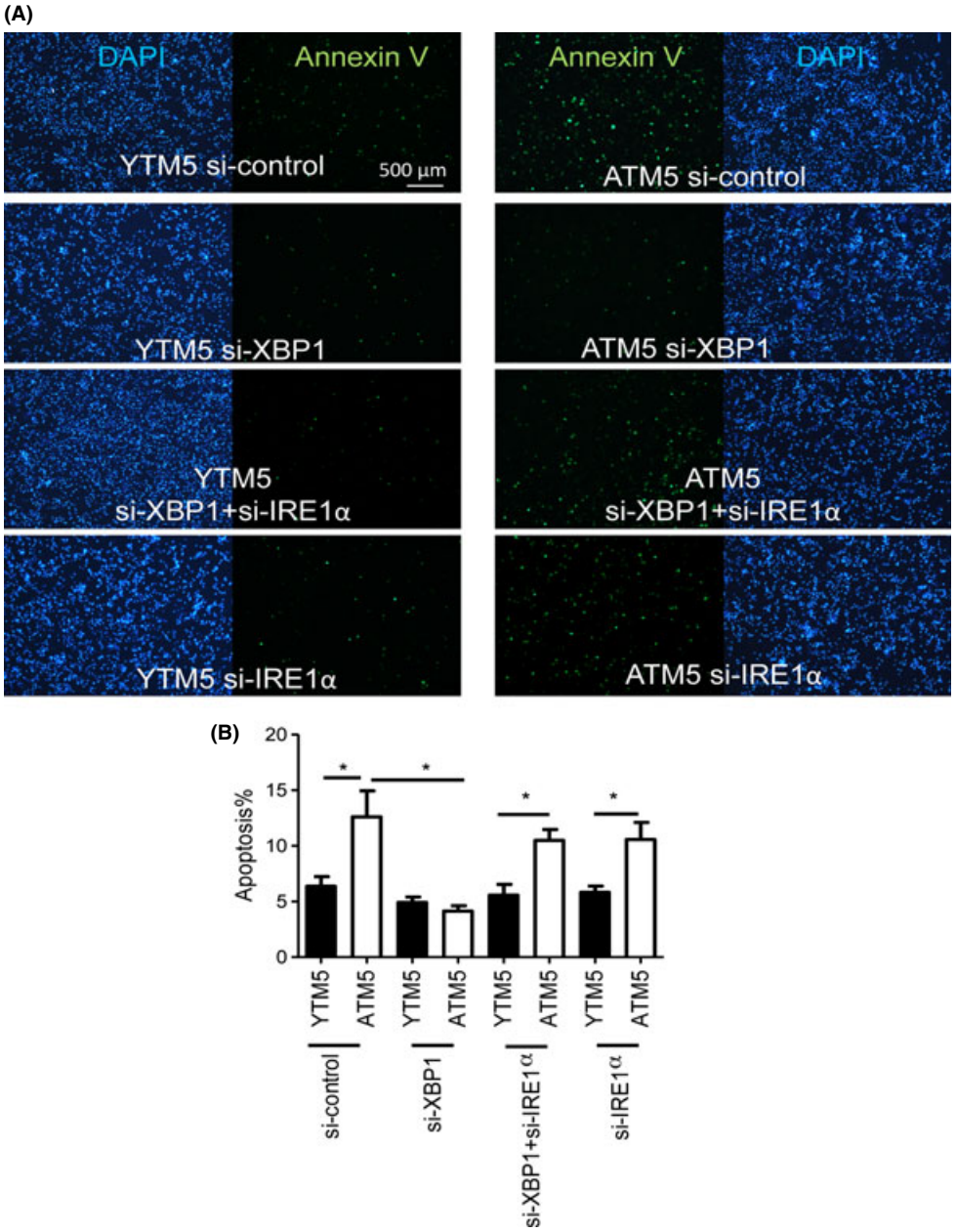

Fig. 5 Reduction of apoptosis by si-XBP1 in aged macrophages undergoing endoplasmic reticulum (ER) stress is IRE1α dependent. Peritoneal macrophages were obtained from aged (16–18 months) and young (1.5–2 months) mice. Cells were treated with either: siRNA to XBP1 (si-XBP1); siRNA to IRE1α (siIRE1α); si-XBP1 plus si-IRE1α; or scrambled control sequences for 48 h, and then treated with 5 μg mL^{-1} TM for 4 h followed by TM-free medium for another 16 h. The degree of apoptosis was assessed by Annexin V staining (green), and nuclei are shown by DAPI staining (blue) through fluorescent microscopy. Representative images are shown in (A) and quantification of apoptotic cells is shown in (B). Experiments were repeated three times. For each experiment, three mice per group were used as a source of cells. $*P < 0.05$ (Student's *t*-test). Error bars = SEM. TM0 = 0 μg mL^{-1} TM; TM5 = 5 μg mL^{-1} TM. Y, young; A, aged.

phagocytosis of cellular debris and apoptotic cells. Debris engulfed by macrophages, including ox-LDL and oxysterols, induces ER stress (Tabas, 2005; Fan *et al.*, 2007; Joyce *et al.*, 2009; Seimon *et al.*, 2010a,b). The capacity to tolerate ER stress and maintain ER homeostasis is crucial for optimal function of macrophages. Our study shows that aging enhances macrophage apoptosis during ER stress. Future studies will be required to determine if other cells of the immune system, for example, dendritic cells are more susceptible to ER stress-induced apoptosis with aging, and if so, the pathways that are altered with aging. A prior study has shown murine hepatocytes undergo more apoptosis with aging and that this was associated with elevated CHOP levels (Li & Holbrook, 2004). Finally, ER stress contributes to chronic diseases such as atherosclerosis and obesity. Whether aging-associated reductions in p-IRE1α, or other ER stress pathways, contribute to these diseases will require future investigations and relevant *in vivo* disease models.

In conclusion, our study has uncovered a new pathway by which aging promotes ER stress-induced apoptosis in macrophages. This novel pathway occurs via alterations in the XBP1-IRE1α-signaling pathway, and may not only be important in our understanding of mammalian lifespan, but may also have important implications for the pathogenesis and potential treatment of aging-associated diseases.

Experimental procedures

Animals

Wild-type female young (1.5–2 months) and aged (16–18 months) C57BL/6 mice were purchased from the National Institute of Aging (NIA) rodent facility. Mice were not included in this study if they exhibited signs of illness (reduced feeding, mobility, or grooming, or evidence of skin disease or lymphadenopathy). Bones from Xbp1 fl/fl Mx1-Cre mice in which XBP1

can be inducibly deleted were kindly provided by Drs. Glimcher and Lee (Harvard School of Public Health) (Hur *et al.*, 2012). Mice were euthanized by isoflurane administration. The institutional animal care and use committee at Yale University approved the use of animals in this study.

Cell culture

Macrophages were isolated from the peritoneum of young and aged mice 4 days after the i.p. injection of 1 mL 3% Brewer thioglycollate medium. Thioglycollate treatment recruits macrophages into the peritoneum, allowing a sufficient yield for *in vitro* culture. This treatment did not induce Annexin V positive staining or induction of ER stress markers such as CHOP in peritoneal macrophages cultured in control media for 24 h (data not shown). Resident peritoneal macrophages were harvested after i.p. lavage with sterile PBS from mice that were not treated with thioglycollate. Isolated macrophages were cultured in macrophage complete medium (DMEM supplemented with 10% FBS, 100 unit mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) overnight before further treatment. Macrophages derived from bone marrow cells were isolated according to prior work (Zhang *et al.*, 2008). Briefly, bone marrow cells were isolated and expanded in macrophage medium for 7 days before further experiments. Cells were cultured in a humidified atmosphere containing 5% CO2 at 37°C.

siRNA transfection

Mouse XBP1 siRNA (Gene ID 22433) and ERN1 siRNA (encoding IRE1α, Gene ID78943) were purchased from Dharmacon (Lafayette, CO, USA). Control scramble siRNAs (Gene ID 4390816) were purchased from Invitrogen (Carlsbad, CA, USA). *Trans*IT-TKO transfection reagent was purchased from Mirus (Madison, WI, USA). Peritoneal macrophages were seeded in 24 well plates at 6×10^5 cells per well 24 h before transfection according to manufacturer's instructions. Briefly, for each well, 1.9 μL of 4 μM siRNA stock solution (25 nM final concentration per well) was mixed with 2.5 μL TransIT-TKO transfection medium and 50 μL serum-free medium (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and incubated at room temperature for 30 min. Then, the mixtures were added to wells containing 0.25 mL macrophage complete medium. After 48 h, cells were treated with ER stress inducers as described below.

ER stress induction

Tunicamycin (TM) induces ER stress by inhibiting protein glycosylation (Lizard *et al.*, 2000). Free cholesterol and 7-ketocholesterol are also known to induce ER stress response in macrophages via independent mechanisms (Lizard *et al.*, 2000; Yao & Tabas, 2000). To induce ER stress, peritoneal macrophages, treated with or without specified siRNAs, were incubated for 4 h in macrophage medium with 5 μg mL^{-1} (0.06 μM, or otherwise indicated dose) of TM (Sigma, St. Louis, MO, USA), or 80 μg mL^{-1} 7-ketocholesterol, or 40 μg mL^{-1} Methyl-β-cyclodextrin: Cholesterol (Sigma) plus 10 μg mL^{-1} acyl-CoA/cholesterol acyltransferase (ACAT) inhibitor (compound 58035, Sigma) for 4 h; then, cells were cultured in fresh medium for another 16 h or 44 h and harvested for further analysis. Control cells were treated with PBS.

Cell apoptosis measured by Annexin V staining

Cell apoptosis was measured by Annexin V: FITC Fluorescence Microscopy Kit (BD Pharmagen, San Jose, CA, USA) according to manufacturer's instructions. Briefly, peritoneal macrophages cultured in Lab-Tek II glass 4-chamber slides were stained with Annexin V-FITC diluted in 1× Annexin V Binding Buffer for 15 min at room temperature, then washed with Binding Buffer once. After removing the chamber wall, ProLong® Gold Antifade Reagent with DAPI was applied directly to the cells, and a coverslip was added. Slides were stored at 4°C until imaging by immunofluorescence microscopy. Images were obtained with an immunofluorescence microscope system (Olympus BX61, Olympus, Central Valley, PA, USA) equipped with QCAPTURE 2.68.6 software (QImaging, Surrey, BC, Canada) . Three fields of approximately 4000 cells/field were imaged for each experimental condition. Images were taken with the software setting of exposure 600 ms, gain 1 and offset 0. Cells stained with bright green fluorescence against the background were identified as Annexin V positive cells according to prior publications (Fang *et al.*, 2013). The proportion of Annexin V^+ cells was calculated by dividing the counted Annexin V^+ by the total number of cells/field.

Western blot analysis

Western blot analysis was performed as previously described (Song *et al.*, 2010). Antibodies against XBP1 (Santa Cruz), CHOP (Santa Cruz), p-IRE1α (Novus Biologicals, Littleton, CO, USA), IRE1α (Cell Signaling, Danvers, MA, USA), p-PERK (Santa Cruz), BiP (Santa Cruz), p-eIF2α (Invitrogen), GADD34 (Santa Cruz), Caspase 3 (Cell Signaling), and ATF-6 (Novus Biologicals) were used at 1:1000 dilution, original concentration = 0.2 μg mL^{-1}. Anti-β-actin antibody (1:1000; Invitrogen) was used as a loading control. All antibodies were prepared in 2% bovine serum albumin/PBS. Secondary antibodies of anti-mouse, anti-rat, and anti-rabbit IgG were purchased from Santa Cruz (1:5000 dilutions). Densitometry analysis was performed using IMAGE J (NIH, Bethesda, MD, USA). Components of Western blots images demarcated within a black box indicate that these components were derived from different gels.

Quantitative real-time PCR analysis

Total RNA was isolated from macrophages using a NucleoSpin RNA II Kit (Macherey-Nagel, Bethlehem, PA, USA). First-strand cDNA was reverse transcribed from isolated RNA using RNA to cDNA EcoDry Premix (Clotech, Mountain View, CA, USA). Real-time PCRs were performed using SYBR® Advantage® qPCR Premix (Clotech) and CFX96™ Real-Time PCR Detection System and Automation System (Biorad, Hercules, CA, USA). Gene expression was quantified using comparative Ct method with β-actin as a reference gene. The primer set for the gene measured are, forward primer 5′-CCGCCCTAGGCACCAGGGTG-3′, and reverse primer 5′-GGCTGGGGTGTTGAAGGTCTCAAA-3′ for β-actin, forward primer 5′-AAACAGAGTAGCAGCTCAGACTGC-3′ and reverse primer 5′-TCCTTCTGGGTAGACCTCTGGGA-3′ for XBP1s, forward primer 5′-TTTACTGCCGCAAGACTACAG-3′, and reverse primer 5′-CTGGGGT TAGATTTGAGCACTT-3′ for p58, forward primer 5′- ATAAAAGCCCT GATGCTGAAGC-3′, and reverse primer 5′- GCCATTGGTAAAAGCA CTGTGT-3′ for ERdj4.

Statistical analysis

All data are presented as means ± standard error of mean (SEM) as indicated in the figure legends. Differences between groups were analyzed using Student's *t*-test or one-way ANOVA, with Bonferroni's *post hoc* test when appropriate. Statistics was performed using GRAPHPAD (Graphpad Software, La Jolla, CA, USA) prism software. Differences were considered significant at $P < 0.05$. Error bars = SEM.

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Author contributions

YS: Designed and conducted the experiments, analyzed data and wrote the paper. HS + WD: provided technical assistance with experiments. DRG: Designed experiments and wrote the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Resident macrophages from aged mice are more susceptible to TM-induced apoptosis than young macrophages.

Fig. S2 Aged macrophages are more susceptible to 7-ketocholesterol and free cholesterol induced apoptosis than young macrophages.

Fig. S3 Aged macrophages exhibit lower p-IRE1α protein levels than young macrophages after different doses of TM treatment.

Fig. S4 The impact of aging on the PERK and ATF6 branches in macrophages before and after treatment with TM.

Fig. S5 IRE1α activation is enhanced in macrophages from XBP1-inducible knockout mice.

Fig. S6 Si-XBP1 does not impact CHOP levels in macrophages.

Fig. S7 IRE1α activation is enhanced in macrophages treated with si-XBP1, and reduced in macrophages treated with si-IRE1α.