

1 **Aggregated and hyperstable damage-associated molecular patterns**
2 **are released during ER stress to modulate immune function**

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22

23 **Running title: ER stress and DAMP secretion**

24 **Abstract**

25 Chronic ER stress occurs when protein misfolding in the ER lumen remains unresolved despite
26 activation of the unfolded protein response. We have shown that traumatic injury such as a
27 severe burn leads to chronic ER stress *in vivo* leading to systemic inflammation which can last
28 for more than a year. The mechanisms linking chronic ER stress to systemic inflammatory
29 responses is not clear. Here we show that induction of chronic ER stress leads to the release of
30 known and novel damage-associated molecular patterns (DAMPs). The secreted DAMPs are
31 aggregated and markedly protease resistant. ER stress-derived DAMPs activate dendritic cells
32 which are then capable of polarizing naïve T cells. Our findings indicate that induction of
33 chronic ER stress may lead to the release of hyperstable DAMPs into the circulation resulting in
34 persistent systemic inflammation and adverse outcomes.

35

36 **Introduction**

37 The endoplasmic reticulum (ER) is the site of secretory and membrane-bound protein
38 synthesis. Under conditions where ER protein synthesis rates exceed the folding capacity of the
39 ER, unfolded or misfolded proteins accumulate in the ER lumen or membrane. The presence of
40 an excess of misfolded proteins in the ER results in the activation of ER stress signaling
41 pathways to restore homeostasis [1]. For example, ER chaperone content is increased while
42 global protein synthesis rates are decreased in an effort to resolve the folding stress. If ER
43 luminal protein folding stress cannot be resolved, pro-apoptotic pathways are activated resulting
44 in cell death [2, 3]. Chronic ER stress is characterized by activation of this pathway without
45 significant cell death resulting in cellular and organ dysfunction over extended time periods [4].
46 Inflammatory stimuli can lead to chronic ER stress in multiple cells and tissues [5]. For example,

47 activation of the acute phase response results in dramatic upregulation in the synthesis of
48 secretory proteins such as C-reactive protein resulting in hepatic ER stress [6, 7]. We previously
49 found that chronic ER stress is prominent post-burn injury and persists for an extended period
50 after the initial insult [8-17]. How chronic ER stress mechanistically contributes to post-burn
51 inflammation and metabolic dysfunction is still unclear.

52 NLR Family Pyrin Domain Containing 3 (NLRP3) plays a central role in regulating
53 inflammatory signaling transmitted by damage-associated molecular pattern molecules (DAMPs)
54 derived from stressed or damaged cells [18, 19]. Inflammatory DAMPs include intracellular
55 proteins such as high mobility group box 1 (HMBG1) and non-protein DAMPs such as nucleic
56 acids, both of which are released from dying/damaged cells. Inflammasome activation by
57 DAMPs leads to caspase 1 activation, resulting in maturation and secretion of IL-1 β and
58 downstream inflammatory responses [20, 21]. DAMP molecules are known to significantly
59 contribute to systemic inflammation and adverse outcomes in trauma [22, 23]. We have
60 previously shown that NLRP3 activation is central to post-burn responses including the induction
61 of ER stress and systemic inflammation [17, 24, 25]. Pro-inflammatory cytokines such as IL-6
62 and IL-1 β can lead to ER stress highlighting a positive feedback loop promoting inflammatory
63 signaling [26-28]. Thus, DAMPs may contribute to systemic inflammation and long-lasting
64 metabolic dysfunction after burn injury.

65 ER stress is known to induce the release of DAMPs. For example, chemotherapeutics can
66 induce ER stress leading to the release of DAMPs and “immunogenic cell death” of cancer cells
67 [29, 30]. It has also been shown that ER stress can lead to the release of DAMPs within secreted
68 extracellular vesicles [31]. Here we show that inducing chronic ER stress in hepatoma cells leads
69 to the release of non-vesicular DAMPs that are aggregated and hyperstable as determined by

70 protease sensitivity. DAMP release was most likely associated with amphisome-mediated
71 secretion *versus* apoptotic/necrotic cell permeabilization. The released DAMPs potentially
72 stimulated dendritic cell activation and the production of inflammatory mediators. Our results
73 link chronic ER stress with the long-lasting inflammation and hypermetabolism found in trauma
74 patients with significant therapeutic implications.

75

76 **Results**

77 *ER stress leads to the secretion of intracellular proteins into the extracellular space*

78 We previously demonstrated that ER calcium store depletion is a central mediator of post-burn
79 hepatic ER stress [10]. To model this *in vitro* we depleted ER calcium stores with the SERCA
80 pump inhibitor thapsigargin (TG) in HepG2 hepatoma cells, a well-characterized polarized
81 human hepatocyte cell line model [32, 33]. We hypothesized that ER stress may lead to the
82 release of aggregated proteins and/or extracellular vesicles into the media which could function
83 as DAMPs. HepG2 cells were treated with TG for 24 hours and the media was subjected to
84 differential centrifugation as in Figure 1A. There was a notable increase in the size of the cell-
85 free high speed pellet in cells subjected to ER stress (Figure 1B). When the 40,000 *xg*
86 supernatant and pellet fractions were run on SDS-PAGE, a large number of additional proteins
87 were apparent in the pellet fraction of ER-stressed cells (Figure 1C). Identification of the bands
88 by mass spectroscopy analysis revealed established DAMPs, such as histones, among other
89 proteins which have not yet been established as *bone fide* DAMPs.

90

91 *Protein released during ER stress are non-vesicular and protease resistant*

92 Recent evidence indicates that DAMPs may be secreted as extracellular vesicles during ER stress
93 [31, 34]. To test whether the secreted proteins identified in Figure 1 are present within lipid
94 vesicles, we subjected the 40,000 *xg* pellet to an additional purification step through a sucrose
95 cushion. Using this protocol, vesicular components will remain in the sucrose cushion whereas
96 high molecular weight non-vesicular (NV) components such as aggregated proteins will pellet
97 through the cushion (Figure 2A; [35-37]). Using this fractionation protocol, there was a large
98 prominent pellet present only in ER stressed cells (Figure 2B). When run on SDS-PAGE
99 followed by Coomassie staining, the pellet fraction from ER stressed cells had an abundant
100 number of proteins, some of which were also prominent in the 40,000 *xg* fraction (Figure 2C).
101 Many of these proteins were also isolated in the NV fraction from another recent study which
102 provided evidence that they are secreted in an amphisome-dependent manner [38]. Dilution of
103 the sucrose cushion fraction and re-centrifugation to isolate extracellular vesicles resulted in no
104 visible protein by Coomassie staining, indicating that extracellular vesicles are secreted at low
105 levels in HepG2 under the conditions used in this study. Some of the proteins secreted from ER
106 stressed cells are established DAMPs, such as histones, actin, and HMGB1. Western blotting
107 confirmed the presence of these proteins in the NV fraction derived from the media of ER
108 stressed cells (Figure 2D). We confirmed by Western blotting the presence of several other
109 proteins which are not classically characterized as DAMPs such profilin-1 and enolase-1 (Figure
110 2D). ER stress leads to the production of misfolded and aggregated proteins which would be
111 predicted to have increased resistance to protease digestion. To test whether proteins derived
112 from the NV fractions were aggregated, we subjected these fractions to limited trypsin digestion.
113 As a control we utilized total protein HepG2 Triton-X100 lysates. As shown in Figure 2E, almost
114 all proteins present in HepG2 Triton-X100 lysates were digested within 15 minutes by *in vitro*

115 trypsin digestion (Figure 2E). In sharp contrast, most proteins in the NV preps from ER stressed
116 cells were detectable for the entire 180 minute time course (Figure 2F). Thus, ER stress leads to
117 the secretion of known, and potentially novel, highly protease-resistant DAMPs which are not
118 present within extracellular vesicles.

119

120 *Activation of apoptotic/necroptotic programs during ER stress*

121 Release of NV proteins may either be through lysis of the plasma membrane or regulated release
122 through other mechanisms such as the classical secretory pathway or non-canonical pathways
123 such as the release of amphisome contents after fusion with the plasma membrane. To test these
124 possibilities, we examined the activation of apoptotic and necrotic pathways in ER stressed
125 HepG2 cells (Figure 3A). Thapsigargin dose-dependently induced the expression of BiP at all
126 concentrations of TG after 24 hours of treatment, indicating activation of the ER stress program
127 (Figure 3B). The broad spectrum kinase inhibitor staurosporine (STS), a positive control for the
128 induction of apoptosis, did not induce BiP expression (Figure 3B). Light microscopy revealed
129 significant cell loss only at TG concentration higher than 1 μ M (Supplementary Figure 1).

130 Consistent with this observation, significant cleavage of the caspase and calpain substrate α -
131 fodrin was only obvious at TG concentrations of 1 μ M and above (Figure 3C). To more
132 quantitatively assess apoptosis induction, we measured enzymatic caspase-3 activities in lysates
133 from HepG2 cells treated with either TG or STS. Concentrations of TG between 100 nM and 10
134 μ M significantly activated caspase-3, however at much lower levels than the classic apoptosis
135 inducer STS (Figure 3D). Propidium iodide (PI) is a cell-impermeant DNA dye commonly used
136 to evaluate cell membrane permeabilization in apoptotic/necrotic models. Surprisingly, the
137 number of PI positive cells in TG treated cells was higher than in STS treated cells at all

138 concentrations except 10 nM (Figure 3E). One possible interpretation is that TG activated
139 necroptotic signaling resulting in membrane permeabilization. However, there was no evidence
140 of necroptosis activation as determined by phospho-MLKL Western blotting (Figure 3F-I).
141 Single cell imaging of PI stained cells revealed TG treated cells did not have canonical nuclear
142 staining, but rather display peripherally associated DNA staining (Supplementary Figure 2). We
143 interpret this finding to indicate the amphisome-mediated secretion of free nucleic acids as seen
144 by others [38] which are then stained by extracellular propidium iodide. Future work will be
145 needed to confirm this interpretation. Regardless, it is possible to readily purify biochemically
146 characterizable NV fractions from less than 100 mls of media of HepG2 cells treated with doses
147 of TG as low as 100 nM. This concentration of TG leads to minimal activation of
148 apoptotic/necroptotic signaling pathways, suggesting a secretion-based mechanism for NV
149 protein release.

150

151 *ER stress-derived DAMPs regulate the expression of costimulatory molecules and cytokine*
152 *production in dendritic cells*

153 The NV fractions purified from ER stressed HepG2 cells contain well-characterized DAMPs
154 (Figures 1-2). To test whether the NV fraction from ER stressed cells functions as a *bone fide*
155 immune modulator, we tested whether this fraction could dose-dependently promote the
156 maturation and activation of dendritic cells (DCs) [39]. During the development from bone
157 marrow derived monocytes to DCs, there is a loss of macrophage marker F4/80 and increased
158 expression of CD11b and CD11c (Figure 4A-B) [40]. To determine whether the putative NV-
159 derived DAMPs shape DC phenotypes, we stimulated immature DCs with increasing
160 concentrations of DAMPs purified from HepG2 cells treated with 100 nM TG. As a positive

161 control we utilized 100 ng/mL lipopolysaccharide (LPS) and as a negative control we utilized
162 PBS (vehicle). About 50% of DAMP-treated DCs were MHCII+, a marker suggesting they were
163 ready to present antigens (data not shown). As shown in Figure 4B, DCs had increased
164 expression of activation marker CD40 and CD86 as a function of DAMP concentration. Vehicle
165 (PBS) treated DCs had no expression of CD40 or CD86. LPS is a well-known activator of DCs
166 through TLR4. LPS treated DCs had strong expression of both CD40 and CD86. We next
167 examined cytokine production in DAMP treated DCs. Both IL-6 and TNF- α production
168 increased corresponding to DAMP concentration but in different manners. IL-6 production
169 reached a plateau around 1 ng/mL when treated with 100 ng/mL DAMP. In contrast, TNF- α
170 production kept rising as DAMP concentration was increased (Figure 4E-H). Although LPS
171 induced stronger expression of CD40 and CD86, DAMP treatment induced similar levels of
172 cytokine production when compared to LPS. These data demonstrate that the NV fraction
173 purified from ER stressed HepG2 cells functions as a potent DAMP leading to the maturation of
174 DCs and cytokine production.

175

176 *Dendritic cells matured by NV-derived DAMPs polarize naïve CD4+ T cells into a Th2*
177 *phenotype*

178 Maturation of DCs through danger signals can be translated into the promotion of an
179 inflammatory T-cell response. To further evaluate how NV-derived DAMPs modulate immune
180 responses, DAMP-matured DCs were co-cultured with naïve CD4+ murine T cells for 5 days
181 without additional DAMP stimulation. The co-culture supernatant was collected for cytokine
182 analysis. After co-culture with DAMP-matured DCs, naïve CD4+ T cells produced a high
183 amount of the Th2 cytokine IL-6 in a dose-dependent manner (Figure 5A). Furthermore, DCs

184 treated with a relatively high concentration of DAMPs (10 μ g/mL) induced T cells to produce
185 another Th2 cytokine, IL-13 (Figure 5B). We also tested cytokines of other T helper cell
186 phenotypes, however, no INF- γ or IL-17 production was detectable suggesting that the T cells
187 were only differentiated into the Th2 phenotype. Thus, DCs were competent to present NV-
188 derived DAMPs as a pro-inflammatory signal to T cells and could successfully induce a Th2
189 reaction. This has significant implications for systemic inflammatory responses in diseases
190 associated with ER stress such as diabetes, cardiovascular disease, and trauma.

191

192 **Discussion**

193 In this report we show that inducing low levels of ER stress in HepG2 cells leads to the
194 release of DAMPs in the context of minimal cell death. In our experimental model, the DAMPs
195 released are not encapsulated with lipids in contrast to other models [31]. In a recent study on the
196 secretion of intracellular components, it was found that many intracellular proteins and enzymes
197 are secreted in an amphisome-dependent manner including cytosolic enzymes and nucleic acids
198 [38]. The non-vesicular amphisome-secreted fraction isolated in this study has a protein
199 composition remarkably similar to that observed in our study (Figures 1-2). Thus, we conclude
200 that a similar mechanism mediates the release of intracellular components as DAMPs during ER
201 stress. Future work will examine mechanistically whether TG-induced release of DAMPs
202 requires amphisome formation and fusion with the plasma membrane.

203 Many studies have demonstrated the link between ER stress and the production of
204 DAMPs. Cellular stress induced by chemotherapeutics cause the release of DAMPs and so-
205 called “immunogenic cell death” or ICD [29, 30]. How ER stress couples to the release of
206 DAMPs is still unknown, however it is thought to require cell membrane permeabilization. We

207 found robust production of DAMPs which could be purified in biochemically characterizable
208 amounts in cells stressed with low doses of TG. Under these conditions we found minimal
209 caspase activation and cell permeabilization. Thus, we conclude there is active release/secretion
210 of DAMPs during chronic ER stress. It is well established that ER stress activates autophagic
211 pathways to rid the cell of excess unfolded/misfolded polypeptide chains [41-43]. We propose a
212 similar model wherein chronic ER stress leads to activation of autophagic pathways to rid the
213 cell of misfolded proteins. Autophagic vesicles then form amphisomes which fuse with the
214 plasma membrane to release the excess of misfolded proteins in an effort to restore proteostasis.
215 This is consistent with other recent studies showing the release/secretion of misfolded proteins in
216 chronically stressed cells *in vitro* and *in vivo* [44, 45]. The released proteins then activate
217 immune responses as DAMPs.

218 We have previously shown that burn injury leads to chronic systemic ER stress in
219 multiple tissues, and in particular the liver [8-11, 13, 14, 17]. This contributes to metabolic
220 syndrome leading to adverse outcomes. Mechanistically, burn injury leads to calcium store
221 depletion *via* IP₃R calcium channels leading to ER calcium store depletion and chronic hepatic
222 ER stress [10]. The IP₃R channel is a well-known central regulator of both ER stress and
223 autophagic pathways [12, 46]. Thus, the IP₃R calcium channel may provide the molecular link
224 between burn injury, ER stress, and amphisome-mediated release of DAMPs. Future work will
225 examine whether targeting this calcium channel is a potential therapeutic target to limit systemic
226 inflammation and metabolic syndrome after a severe burn injury.

227

228 **Materials and Methods**

229 *Animals*

230 This study was carried out in accordance with the recommendations of the National Institutes
231 of Health Guidelines for the Use of Laboratory Animals. The protocol was approved by the
232 Animal Care and Use Committee of the University of Texas Health Science Center. Female
233 C57BL/6 mice were bred at the animal facility at the University of Texas and used at the age of
234 8–12 weeks.

235 *Cell culture and treatment*

236 HepG2 cells were purchased from ATCC and maintained in DMEM supplemented with 10%
237 FBS, 1% penicillin/streptomycin, and 1% L-Glutamine. The cells were incubated at a constant
238 temperature of 37°C and 5% CO₂. When the cells had grown to 80%-90% confluency,
239 thapsigargin (TG) purchased from Sigma Aldrich was added to fresh media at a final
240 concentration indicated in the text/figures. Thapsigargin added at 100 nM and 1 μM for 24 hours
241 produced similar amounts of NV DAMPs. Only DAMPs produced with 100 nM TG were used
242 for biochemical characterization (Figure D-F) and DAMP functional analysis (Figures 4-5). An
243 equal volume of DMSO was used as a control condition. A typical DAMP preparation required
244 starting material from 60 mls media isolated from 2 x150 mm plates for each condition. This led
245 to an average yield of approximately 50 μg protein at a concentration of 0.5 mg/ml.

246 *DAMP Isolation*

247 The media collected from TG and DMSO treated cells were spun down at 1000 *xg* to remove
248 dead cells and debris. The supernatants from this spin were transferred to round-bottomed
249 centrifuge tubes and spun down at 40,000 *xg* at 4°C for 2 hours using an SS-34 rotor in a super-
250 centrifuge. The pellets from the 40,000 *xg* spin were resuspended in an equal volume of PBS and
251 10 μL of each condition was removed for analysis by SDS-PAGE. The remainder of the
252 resuspended pellets was diluted into PBS for density-gradient centrifugation. A 4 ml sucrose

253 cushion consisting of 1M sucrose and 0.2M Tris base, pH 7.4 was transferred to centrifuge tubes
254 and the resuspended pellets were carefully layered on top. The samples were placed in a
255 Beckman SW-28 rotor and centrifuged at 100,000 xg at 4°C for 75 minutes in an ultracentrifuge.
256 The pellets from this final centrifugation were resuspended in TTB buffer (120 mM KCl, 50 mM
257 Tris/HCl pH 8.0, 1 mM EDTA, 1 mM DTT, 1% Triton-X100) or PBS and passed through a 25½
258 gauge needle before 10 µL of each condition was removed for analysis by SDS-PAGE.

259 *Proteomic Analysis*

260 Protein bands on Coomassie-stained SDS-PAGE gels were excised with a razor and subjected to
261 mass spectrometry-based protein identification by the Clinical and Translational Proteomics
262 Service Center, The Brown Foundation Institute of Molecular Medicine, The University of Texas
263 Health Science Center at Houston. In some cases, bands identified by mass-spectroscopy were
264 confirmed by Western blotting.

265 *Trypsin Digestion*

266 Untreated HepG2 cells were cultured for 48 hours, rinsed with PBS, and lysed with TTB buffer.
267 The whole cell lysate was then diluted to the concentration of the DAMP isolation so that
268 equivalent levels of protein were used. The whole cell lysate and DAMP isolation were then
269 exposed to a trypsin solution (10 µg/mL trypsin, 30 µg/mL chymostatin, 100 µg/mL tosyl
270 phenylalanyl chlormethyl ketone) in TTB for 0 min, 15 min, 30 min, 60 min, 120 min, 180 min
271 at 37°C. At the end of the exposure time, the samples were quenched and run on separate
272 gradient gels for each condition and Coomassie stained.

273 *In Vitro Generation of DCs and DC-naïve CD4+ T cell co-culture*

274 Dendritic cells (DCs) were generated *in vitro* as previously described [1]. Briefly, tibias and
275 femurs of C57BL/6 mice were removed under sterile conditions. Both ends of the bone were cut

276 off, and the needle of a 1-mL syringe was inserted into the bone cavity to rinse the bone marrow
277 out of the cavity. The cells were resuspended with Tris-NH₄Cl red blood cell lysis buffer to
278 remove the red blood cells. Bone marrow cells were then cultured in DMEM with 10% FBS,
279 glutamine, nonessential amino acids, sodium pyruvate, HEPES, and penicillin/streptomycin
280 (complete medium) for 2 hours. Floating cells were discarded and adherent cells were kept in
281 complete medium with granulocyte-macrophage colony-stimulating factor (GM-CSF) (20 ng/ml)
282 for 5 days. Complete medium and GM-CSF were renewed every 2 days. Immature DCs were
283 treated with different concentration of DAMPs for 2 days and the supernatant was collected for
284 cytokines analysis. In some experiments, stimulated DCs were processed for flow cytometry.
285 Alternatively, stimulated DCs were re-plated in 96-well flat-bottom plates alone (3 x 10⁵
286 cells/0.2 ml well volume) or with autologous naïve CD4⁺ T cells at a ratio of 1:10 for 5 days.
287 Naïve CD4⁺ T cells were collected from the spleen and lymph nodes of WT mice using naïve
288 CD4⁺ T cell isolation kit (Miltenyi Biotech). Co-culture supernatant was collected for cytokine
289 analysis at the end of day 5.

290

291 *Assessment of the cytokine profile*

292 Concentrations of IFN- γ , IL-13, IL-6, IL-17 (R&D Systems) and TNF- α (Thermo Fisher) in
293 DCs or DC/T-cell co-culture supernatants were measured by ELISA, according to the
294 manufacturer's recommendations, and expressed in picograms per milliliter. Results were
295 expressed as mean \pm standard deviation.

296

297 *Phenotype analysis*

298 DC phenotype was evaluated by flow cytometry using a standardized protocol [2]. Cells were
299 kept on ice during all the procedures. For the extracellular markers, cells were stained with
300 CD11c-AF700, F4/80-PE-Cy5, CD11b-APC-eF780, MHCII (I-Ab)-APC, CD40-Ef450 and
301 CD86-FITC (ThermoFisher). Detection of cell surface markers was conducted using a Beckman-
302 Coulter Gallios Flow Cytometer (BD Biosciences, San Jose, CA, USA) and data were analyzed
303 by Kaluza Analysis Software. Results were shown as mean \pm standard deviation.

304

305 **Conflict of Interest**

306 The authors declare that the research was conducted in the absence of any commercial or
307 financial relationships that could be construed as a potential conflict of interest.

308

309 **Author Contributions**

310 A.A, M.I.G, Y.F., M.C.T. and A.A. performed experiments. A.A., M.I.G., Y.F., M.C.T., A.A.
311 and D.B. analyzed data and prepared figures. M.G.J. and D.B. conceived of the project. All
312 authors contributed intellectually, wrote, and approved of the final manuscript.

313

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318 Health Science Center at Houston.

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320

321 **Figure Legends**

322 **Figure 1. ER stress leads to the release of intracellular proteins.** (A) Schematic of the
323 treatment and purification protocol. (B) Image of the 40,000 *xg* pellets from a representative
324 experiment. Pellet margins are highlighted. (C) Coomassie staining of the 1000 *xg* and 40,000 *xg*
325 fractions. Fractions were run on a 4-20% SDS-PAGE gradient gel. The broad band running
326 between the 50 and 70 kDa markers is bovine serum albumin from the media. Unique proteins
327 identified by mass spectroscopy in ER stressed cells are indicated by gene name.

328

329 **Figure 2. Proteins released from ER stressed cells are non-vesicular and protease resistant.**

330 (A) Schematic of the purification scheme. (B) Image of the 100,000 *xg* pellets from a
331 representative experiment. Pellet margins are highlighted. Control (DMSO) treated cells do not
332 have a visible pellet. (C) Unique proteins identified by mass spectroscopy in ER stressed cells
333 are indicated by gene name. Known DAMPs are indicated in bold. (D) SDS-PAGE and Western
334 blotting of a preparation as in (C) and identification with antibodies to the indicated proteins. (E)
335 Trypsin digestion of HepG2 total cell lysate for the indicated times followed by Coomassie
336 staining. (F) Trypsin digestion of the 100,000 *xg* pellet isolated from the media of ER stressed
337 cells for the indicated times followed by Coomassie staining.

338

339 **Figure 3. Activation of apoptotic and necroptotic pathways in TG treated HepG2 cells.** (A)

340 Schematic of apoptotic and necroptotic signaling pathways. (B) Western blotting of HepG2
341 lysates with BiP/GRP78, an ER stress marker, after the indicated treatments for 24 hours. TG,
342 thapsigargin; STS, staurosporine. (C) Western blotting of the same membrane in (B) with α -
343 fodrin, a marker of caspase and calpain activation. (D) Caspase-3 enzymatic activity in cells

344 treated with the indicated compounds. Data is the mean +/- s.e.m. from three separate
345 determinations. P values are indicated above the bars. (E) Propidium iodide (PI) positive cells
346 treated with the indicated compounds. Data is the mean +/- s.e.m. from three separate blinded
347 determinations. See text for additional information regarding scoring. (F-H) Western blotting of
348 HepG2 lysates with MLKL and phospho-MLKL, a necroptosis marker, after the indicated
349 treatments for 24 hours. (I) Same blot as in (F-H) probed with GAPDH as a loading control.

350

351 **Figure 4. The non-vesicular (NV) fraction isolated from ER stressed cells activate dendritic**
352 **cells as *bone fide* DAMPs.** (A) Schematic of the *in vitro* bone marrow-derived dendritic cell
353 (BMDC or simply DC) maturation protocol. (B) Macrophage and dendritic cell markers in
354 control and DAMP treated BMDCs. Lipopolysaccharide (LPS) was used as a positive control.
355 (C-D) Dendritic cell markers CD40 and CD86 after treatment with indicated compounds. Data
356 are the mean +/- stdv from three separate determinations. (E-F) TNF- α cytokine production after
357 treatment with the indicated compounds. (G-H) IL-6 production after treatment with the
358 indicated compounds.

359

360 **Figure 5. DAMP-differentiated dendritic cells are competent to differentiate naïve T cells**
361 **into a Th2 phenotype.** (A) Production of IL-6 in CD4⁺ T cells co-cultured with DAMP-
362 stimulated DCs. The data represent the mean +/- stdv from three determinations. (B) Production
363 of IL-13 in CD4⁺ T cells co-cultured with DAMP-stimulated DCs. The data represent the mean
364 +/- stdv from three determinations. See methods for details.

365

366

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Figure 1, Andersohn et. al.

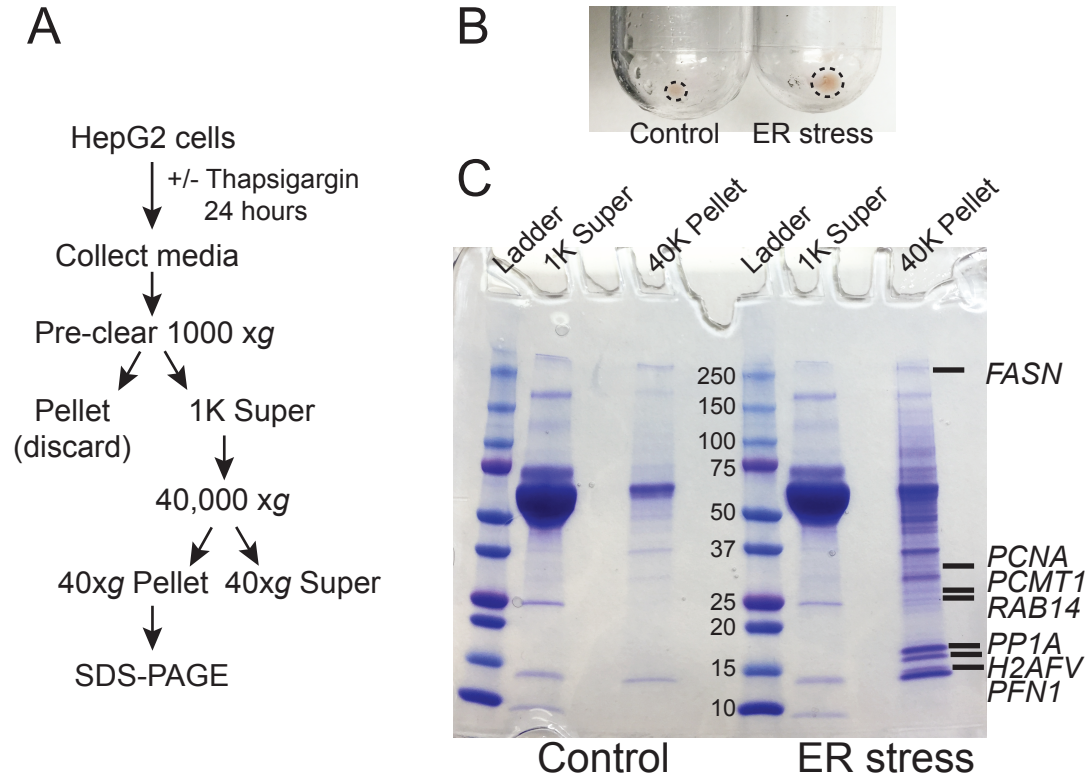


Figure 2, Andersohn et. al.

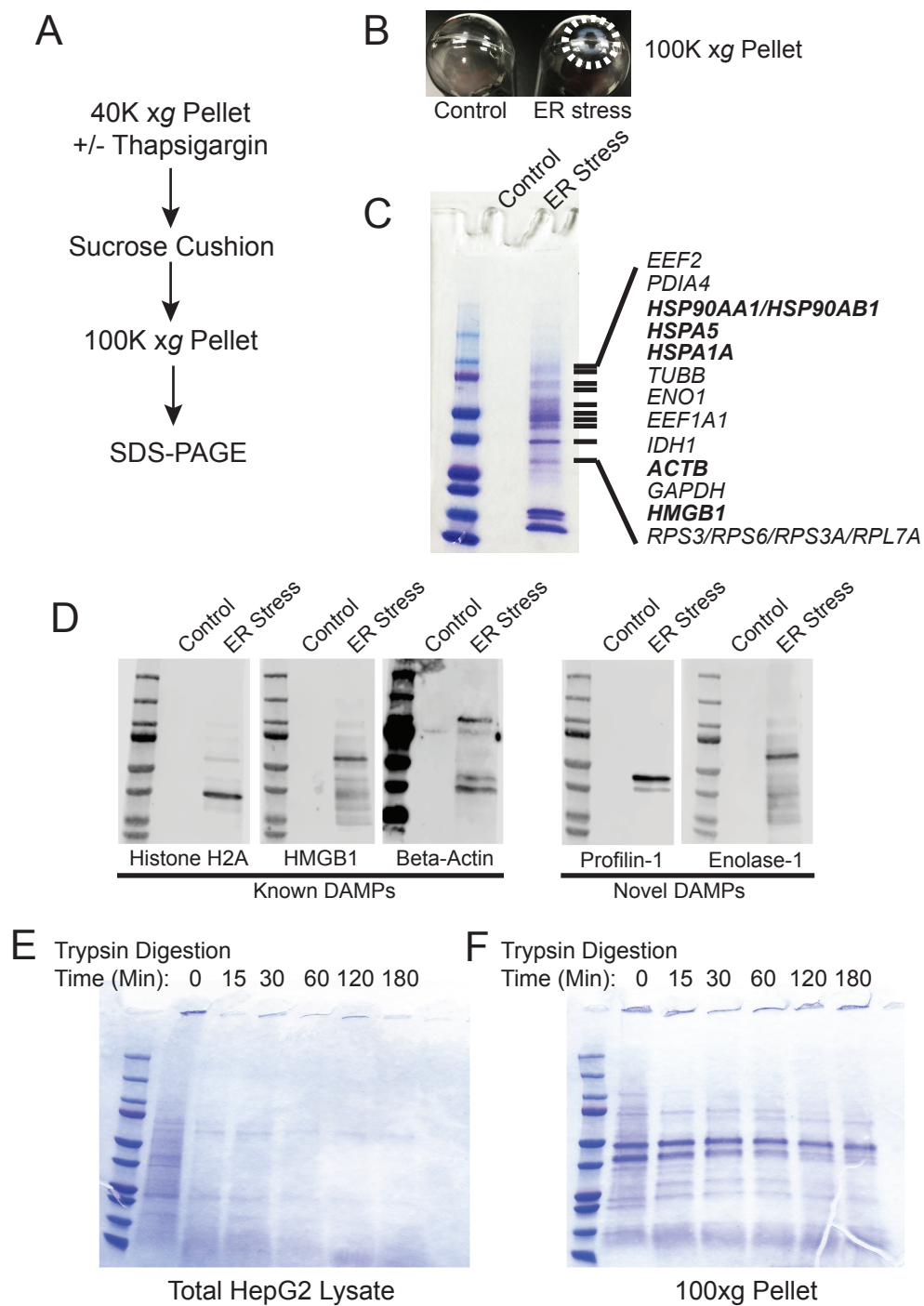


Figure 3, Andersohn et. al.

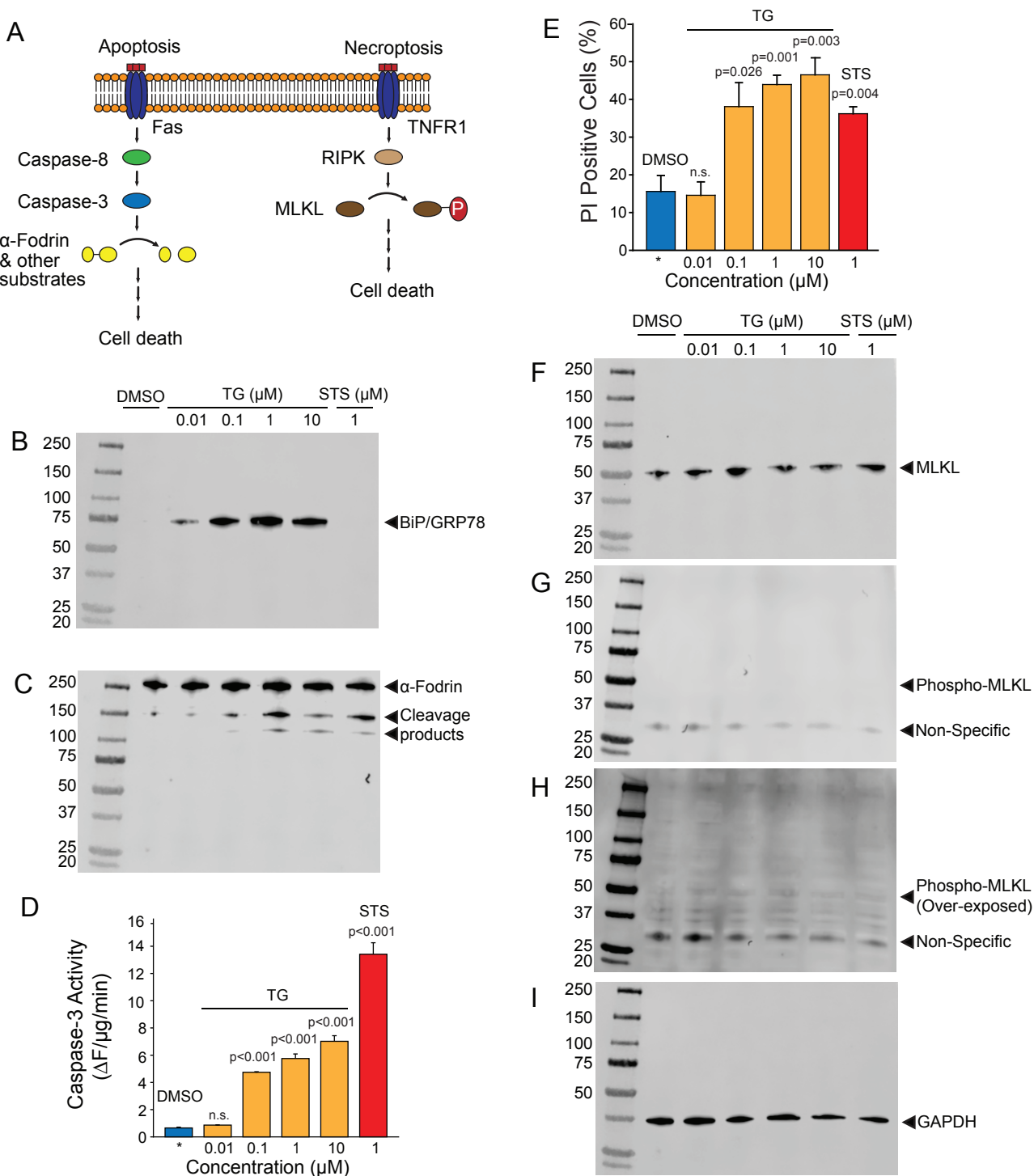
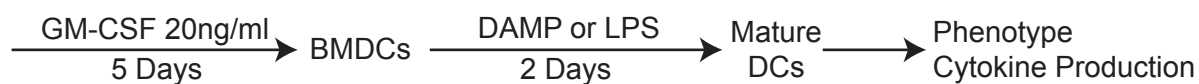
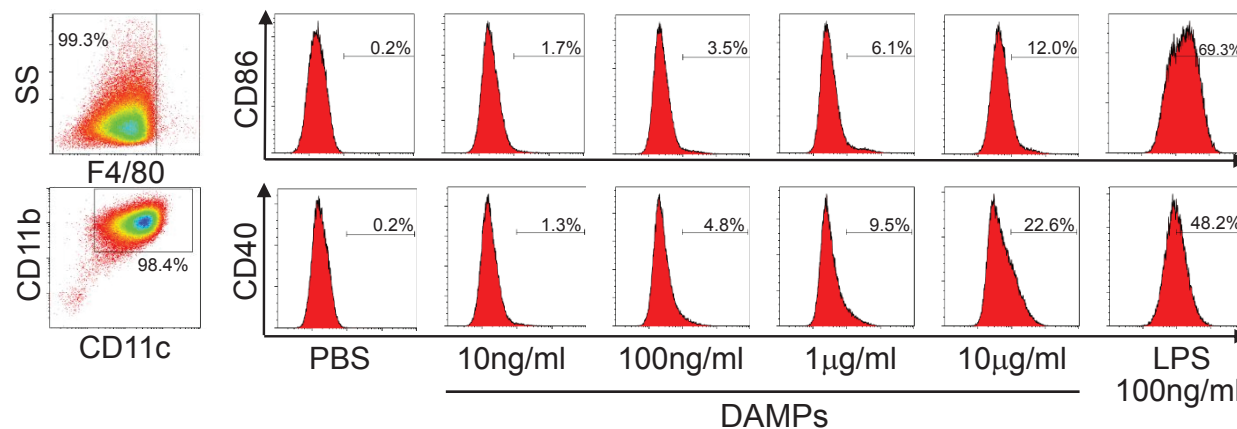


Figure 4, Andersohn et. al.

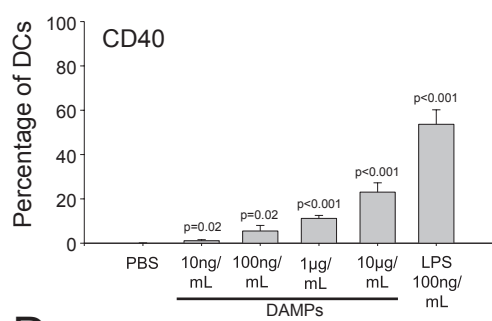
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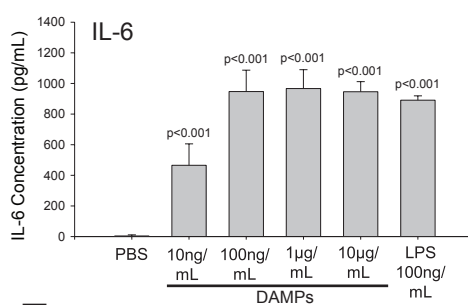
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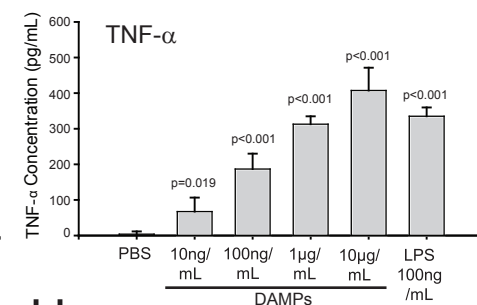
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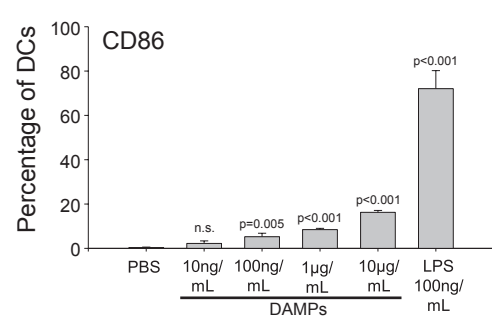
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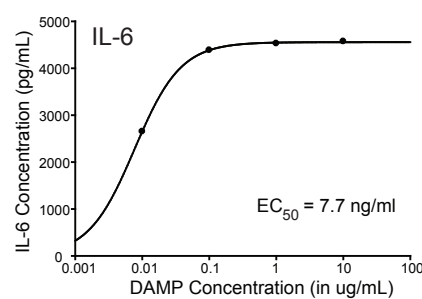
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D



F



H

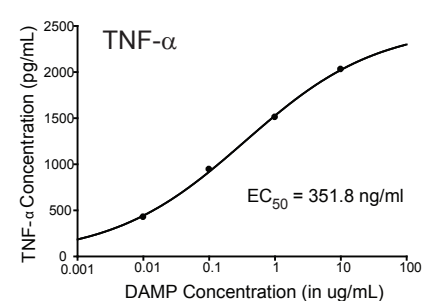
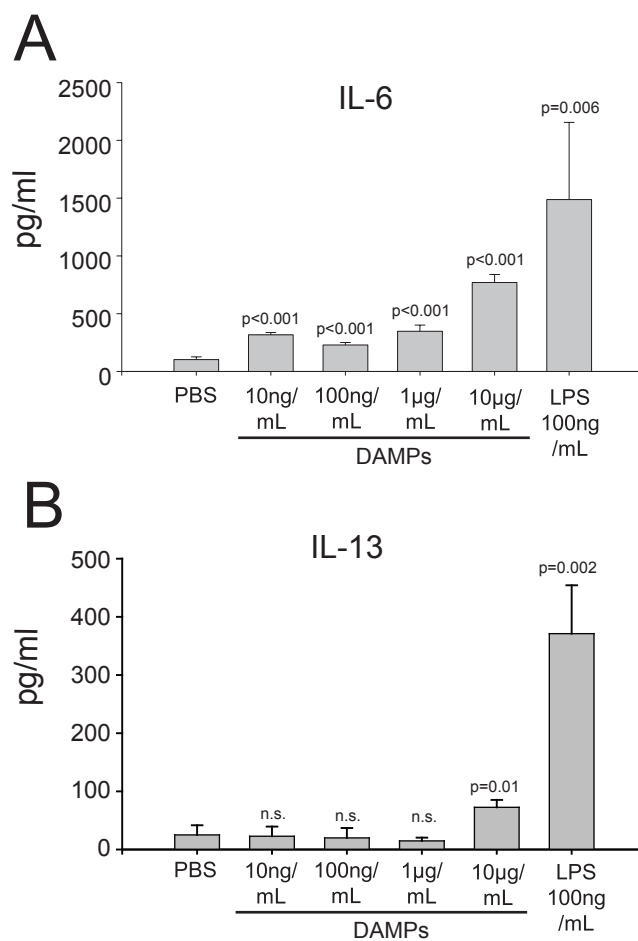
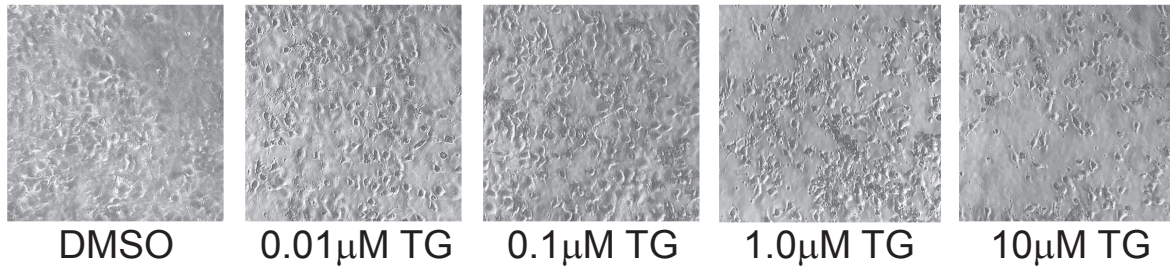


Figure 5, Andersohn et. al.

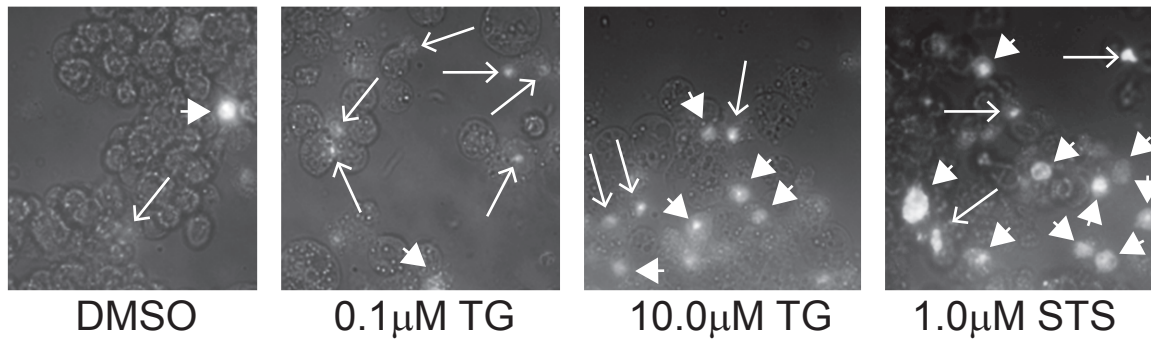


Supplemental Figure 1, Andersohn et. al.



Supplementary figure 1. Phase contrast microscopy of TG treated HepG2 cells. Cells were treated with the indicated concentration of thapsigargin (TG) for 24 hours. Images are a representative field from at least three separate determinations.

Supplemental Figure 2, Andersohn et. al.



▶ Nuclear PI staining
→ Peripheral/Non-nuclear PI staining

Supplementary figure 2. Propidium iodide (PI) staining of TG treated HepG2 cells. Cells were treated with the indicated concentration of thapsigargin (TG) for 24 hours. Images are a representative field from at least three separate determinations. Big arrow heads represent nuclear staining; narrow arrow indicate non-nuclear or peripheral DNA staining. Note staurosporine (STS) treated cells have abundant stained nuclei whereas ER stressed cells have more peripheral/non-nuclear staining. See text for details.