Streptolysin-induced endoplasmic reticulum stress promotes group A streptococcal *in vivo* biofilm formation and necrotizing fasciitis

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Supplementary Methods:

Bacterial strains and growth conditions. GAS was cultured in Todd-Hewitt medium (Sigma Aldrich, Missouri, USA) supplemented with 0.2% yeast extract (Becton, Dickinson, San Jose, USA) (THY media). The bacterial cultures were statically incubated at 37^{o}C in 50 mL Falcon tubes for 18 h in 5% CO_2. Bacto^{TM} Agar (Becton Dickinson, San Jose, USA) was added to a final concentration of 1.4% for solid media. Antibiotics were added at the following concentrations where appropriate: 250 µg/ml kanamycin (Kan), 1 µg/ml erythromycin (Erm), 50 µg/ml spectinomycin (Spec).

Construction of bacterial mutants. *hasA* mutant construction in JS95 was performed as previously described for GAS strain JRS4 (1). The *speB*$^{-}$ mutant was constructed similarly as follows: a PCR fragment of *speA* containing almost the entire *speB* gene was generated, using the primers pSpeBF (5'GATCAAAACTTTGCTCGTAACG3') and pSpeBR (5'CTAAGGTTTGATGCCTACAACAG3'). The PCR product was digested with *HindIII* and *PstI* and cloned into the temperature-sensitive *E. coli*-streptococcal shuttle vector pJRS233 (2), and digested with the same enzymes. The resulting plasmid, pJRS233*speB*, was introduced into JS95 by electroporation. Erythromycin-resistant transformants were isolated at the permissive temperature (30°C). Growth of transformants at the non-permissive temperature (37°C) resulted in the integration of the plasmid at *speB* homologous DNA sequences found on both the plasmid and the chromosomal DNA resulting in the formation of

an inactive *speB* allele. Proper integration of the *speB* fragment was confirmed by PCR analyses.

Biofilm quantitation. Following biofilm growth, media from the wells was removed, the plates were washed 2 times with 1X phosphate-buffered saline (PBS), and adherent bacteria were stained with 0.1% crystal violet (CV) for 15 min and washed twice with PBS to remove the excess stain. To extract CV from the underlying MEFs, 1 mL ethanol:acetone (1:1) decolorizer was then added to the wells and removed immediately. Wells containing MEFs without bacteria or media alone served as controls. The CV from the bacterial biofilms was eluted with 500 ml of 96% ethanol and the density of crystal violet stain was measured by absorbance at 590 nm using a M200 Microplate reader (Tecan AG, Männedorf, Switzerland). For quantification of viable bacteria in GAS biofilms, media was aspirated from biofilms formed either on plastic or on MEFs, and the biofilms were gently washed with 1X PBS. The biofilms were then gently scraped from the respective surfaces, resuspended in 1X PBS, and quantified for CFU by dilution plating on THY agar plates.

Immunofluorescence and microscopy of *in vitro* biofilms. Biofilms grown for the indicated timepoints were washed with 1 X PBS twice, fixed with 4% paraformaldehyde (PFA; Sigma Aldrich, Missouri, USA) in PBS for 10 mins, and permeabilized for 10 mins with 0.1% saponin (Sigma Aldrich, Missouri, USA) in PBS. Next, non-specific sites were blocked using 2% bovine serum albumin (BSA; Sigma Aldrich, Missouri, USA). Biofilms were next incubated with goat polyclonal anti-*S. pyogenes* Group A Carbohydrate (1:100, Abcam, Cambridge, UK) primary antibody at 37^{o}C for 3 h, followed by incubation with rabbit anti-goat Alexa Fluor 488 (1:500, Life Technologies, California, USA) secondary antibody for 45 mins. MEFs were stained either with phalloidin Alexa Fluor 350 conjugate (1:40, Life Technologies, California, USA) or ConcanavalinA Alexa Fluor 594 conjugate for 15 min (working concentration 50µg/ml; Life Technologies, California, USA). Excess dye was

removed by washing with 1 X PBS, and the dishes were air dried and mounted with Vectashield mounting medium (Vector Laboratories, California, USA).

Scanning electron microscopy (SEM) analysis. Biofilm samples were fixed using 2.5% glutaraldehyde (prepared in 0.1 M PBS; pH 7.4; Sigma-Aldrich, Missouri, USA) for 48 h at 4°C and then washed thrice with 0.1 M PBS. Samples were then post fixed in 0.2% osmium tetroxide (prepared in 0.1 M PBS; pH 7.4; Electron Microscopy Services, Pennsylvania, USA) for 2 h at room temperature and washed thrice with 0.1 M PBS. Fixed samples were then dehydrated with a graded ethanol series in distilled water (once with 30%, 50%, 70%, 80%, 90% and thrice with 100% ethanol for 15 minutes at each step) together with gentle agitation. Samples were then treated with a 1:2 EtOH:hexamethyldisilane (Sigma-Aldrich, Missouri, USA) for 20 minutes, then with a 2:1 EtOH:hexamethyldisilane for a further 20 minutes with gentle agitation. Samples were then treated with 100 % hexamethyldisilane twice for 20 minutes before air-drying in a fume hood overnight. Following chemical drying, samples were then deposited onto the SEM specimen stubs using one side of a double-sided adhesive NEM Tape (Nisshin Em. Co. Ltd, Tokyo, Japan). Samples were then sputter coated with gold using a Bal-Tec SCD 005 sputter coater (Bal-Tec AG, Balzers, Liechtenstein). Samples were viewed using a JSM-6360LV (JEOL, Tokyo, Japan) scanning electron microscope.

Antibiotic susceptibility testing and activity against bacterial biofilms. Minimum inhibitory concentration (MIC) for ampicillin against GAS was determined by the microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations (3). For antibiotic susceptibility of pre-formed biofilms, culture medium was removed from 24, 48 and 72 h old biofilms and immediately replaced by fresh media (control) or media containing ampicillin at increasing concentrations (0.5 to 10-fold the MIC)

and incubated for 24 h at 37°C. Biofilms were then disrupted by pipetting and viable CFU enumerated by plating on THY agar plates.

ER stress, apoptosis, and supernatant complementation experiments. MEFs were incubated for indicated times with 1.0 µM thapsigargin (TG) (Sigma-Aldrich, Missouri, USA), 10 µM etoposide (Sigma-Aldrich, Missouri, USA), or 0.01% DMSO (Sigma-Aldrich, Missouri, USA) as the buffer control. Cell-free supernatants filtered through Acrodisc® Syringe Filters with Supor® Membrane (0.22µm) (PALL, New York, USA), referred to as conditioned media (CM), were collected and used as culture media for Δ*slo,sagΓ* biofilm assays on MEFs. The cytotoxicity of TG or ET on GAS was assessed by incubating 5 x 10^5 CFU planktonic GAS with the indicated concentration of drugs for 24 h, with subsequent enumeration on THY agar plates. Bacteria-free CM were also collected from WT or Δ*slo,sagΓ* mutant GAS strains grown on plastic or MEFs for the indicated timepoints, and used as culture media for Δ*slo,sagΓ* mutant biofilm assays. For the ASN supplementation experiments, the culture media was supplemented at a final concentration of 15, 60 or 100 mg/L of asparagine (Sigma-Aldrich, Missouri, USA).

LDH assay. MEFs were infected with GAS strains in DMEM lacking phenol red (Gibco, New York, USA) with 5% FBS in 24-well plates. At the indicated timepoints, cell culture plates were centrifuged at 400 x g for 10 mins and 100 µl of the cell free supernatant (filtered through a 0.22µm Millex® syringe filter) was collected from each well and used for the assay. The LDH assay was performed using the LDH cytotoxicity determination kit (Clonetech, California, USA) according the manufacturer's directions. For negative controls, supernatants from uninfected MEFs and for positive controls, supernatants from MEFs treated with 0.2% Triton X-100 (Sigma Aldrich, Missouri, USA) for complete cell lysis were used. Additional controls included the assay medium alone to assess basal LDH levels in the culture media and GAS bacterial suspension containing 5 X 10^5 CFU in DMEM with 5% FBS in the absence of

MEFs. The assay plate was read at 490 nm using a M200 Microplate reader (Tecan AG, Männedorf, Switzerland).

Animal experiments. The ability of GAS to form *in vivo* microcolonies was assessed by injecting GAS WT and Δ*slo,sagI* mutant strains in a murine model of soft-tissue infection as described previously (4-6). A 100 µl of GAS inoculum at mid log phase containing $1x10^8$ CFU was injected subcutaneously in the rear flank of female BALB/c mice (InVivos Pte. Ltd., Singapore) aged 3-4 weeks weighing 10-12 g. For pre-induction of ER stress or non-ER stress mediated apoptosis, respectively, 2 mg/kg body weight of thapsigargin (TG; Sigma Aldrich, Missouri, USA) and 320 mg/kg body weight of etoposide (ET; Sigma Aldrich, Missouri, USA) in 100 µl of 1 X PBS was injected subcutaneously in shaved mouse flanks 12 h prior to GAS injection at the same site. Eight mm punch biopsies (Robbins Instruments. Inc, New Jersey, USA) of tissue sections from the site of injection were excised 12 h after infection with GAS strains. The excised biopsies were incubated in 4% PFA for 4 h at room temperature followed by 30% sucrose at 4^oC for 24 h. Samples were next frozen in Optimal Cutting Temperature (OCT) embedding media (Sakura, California, USA), using liquid nitrogen. 12 µm sections were then obtained with a Leica CM1860 UV cryostat (Leica Biosystems, Ernst-Leitz Strasse, Germany) and stained for immunological analysis using antibody specific to GAS (goat polyclonal anti-*S. pyogenes* Group A Carbohydrate (1:100, Abcam, Cambridge, UK), and counterstained with DAPI and phalloidin Alexa Fluor 568 (1:20; Life Technologies, California, USA) to visualize the surrounding tissue. To ascertain that the drugs as well as GAS induced ER stress or apoptosis, tissue sections were co-immunostained with rabbit polyclonal anti-GADD153 (CHOP) antibody (1:100, Santa Cruz, California, USA) followed by donkey anti-rabbit IgG Alexa Fluor 594 secondary antibody (1:500, Life Technologies, California, USA) and the TUNEL Assay Kit (Roche, Indiana, USA) respectively. For CFU quantification of GAS *in vivo*, the tissue biopsies were

homogenized in sterile 1X PBS and plated on THY plates. CLSM-acquired images at x20 magnification spanning the transverse plane of the tissue, including the epidermis, dermis, fascia and muscle were tiled to observe the degree of transverse spread of GAS microcolonies. To quantify the degree of lateral spread of the microcolonies beyond the site of bacterial infection, the mean fluorescence intensity (MFI) values from CLSM images of immunostained (described above) biopsies from 40 serial sections per tissue biopsy (spaced 36 μm apart) were quantified by ImageJ and plotted as a distribution plot for each mouse.

Table S1: Bacterial strains used in this study, related to the experimental procedures

SN	GAS strains	Description	Antibiotic resistance	Ref
1	JS95 WT	GAS clinical isolate from PPBS in Israel	-	(4)
2	Δ*emm14*	*emm* deletion mutant of JS95	Kan 250	(5)
3	*speB*$^{-}$	*speB* insertion-inactivated derivative of JS95	Erm 1	this work
4	*hasA*$^{-}$	*hasA* insertion-inactivated derivative of JS95	Erm 1	this work
5	Δ*slo*	*slo* deletion mutant of JS95	Kan 250	(7)
6	*sagI*$^{-}$	*SagI* insertion-inactivated derivative of JS95	Erm 1	(7)
7	Δ*slo sagI*$^{-}$	*slo* deletion and *sagI* insertion-inactivated mutant of JS95	Erm1 Kan 250	(7)
8	Δ*slo,sagI,*-pLZ*sagI*	*sagI* complementation mutant of *Δslo,sagI-*	Erm1 Kan 250 Spec 50	(7)
9	Δ*slo,sagI,*-pLZ*slo*	*slo* complementation mutant of *Δslo,sagI-*	Erm1 Kan 250 Spec 50	(7)
10	MGAS5005	Representative of GAS MITI serotype, highly invasive clinical strain	-	(8)
11	JRS4	GAS M6 serotype, streptomycin-resistant derivative of strain D471	-	(9)

Supplementary Figures:

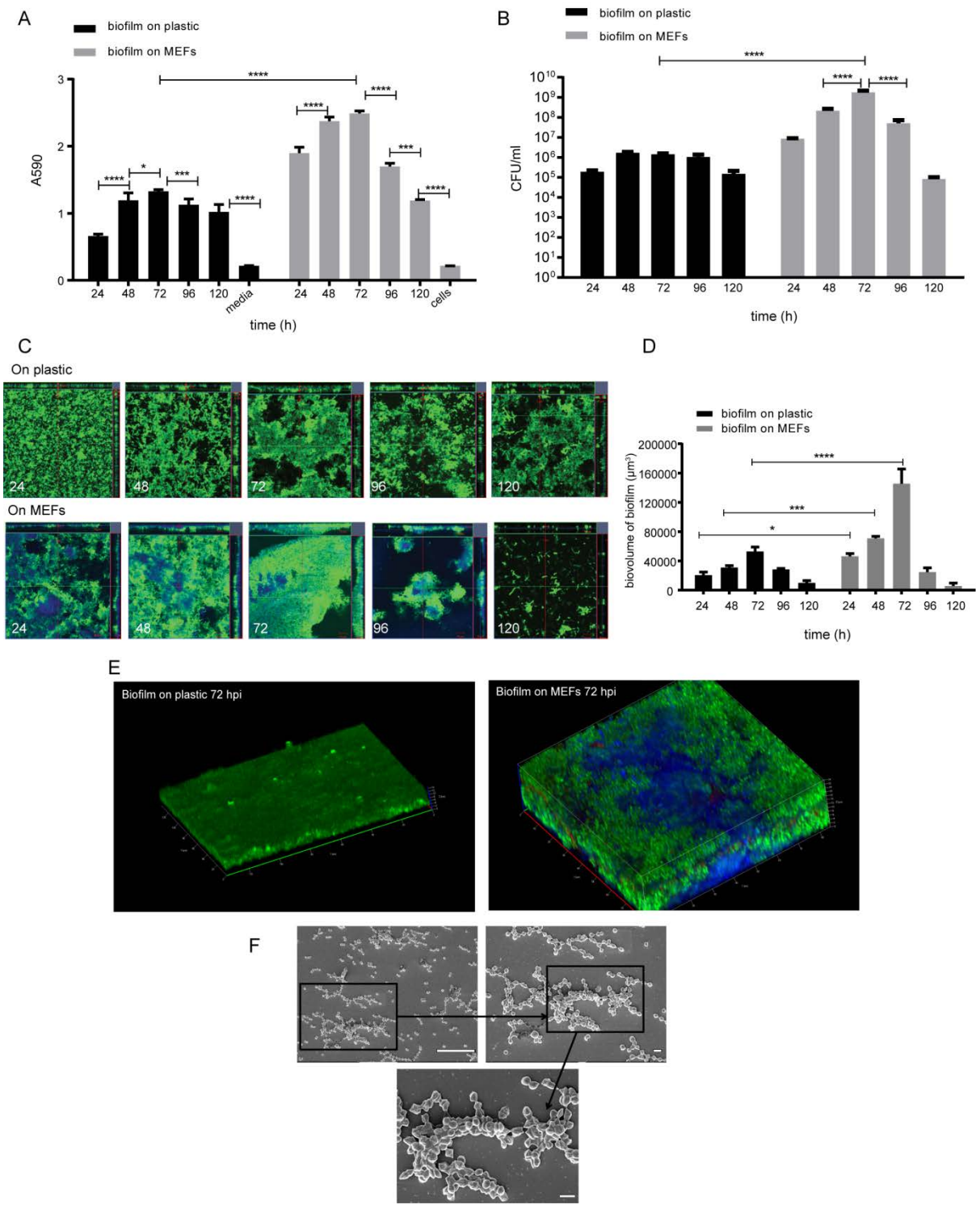

Fig. S1. GAS biofilms on MEFs have distinct properties from those on plastic. Time course of biofilm development by GAS JS95 on MEFs was quantified by: *(A)* CV staining or *(B)* CFU enumeration at the indicated timepoints (hours). Controls represent media only (for

plastic) or cells only (for MEFs). Data represent the mean ± SEM values; N=3. Statistical significance was calculated by using multiple unpaired t tests. *(C)* CLSM performed on GAS biofilm grown on either plastic or MEFs after the indicated time points (hours). Bacteria were stained with anti-GAS antibody (green) and MEFs with phalloidin AF350 (blue). Orthogonal CLSM images were acquired at x63. *(D)* Bio-volume quantification of x63 CLSM orthogonal images of Z stacks of biofilms comparing GAS biofilms grown on plastic vs MEFs for indicated hours by Imaris software. Data represent the mean ± SEM values; N=5. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test. *(E)* 3D representation of GAS biofilms grown on plastic vs MEFs at peak time point of 72 hpi from CLSM orthogonal images of Z stacks of biofilms acquired at x63 magnification. Biofilms on MEFs were observed to be 3x times thicker than biofilms on plastic (based on scale). *(F)* SEM images of GAS biofilms grown on glass at magnifications of x2500 (upper left), x5000 (upper right), and x10,000 (bottom panel). Scale bars indicate 10 µm, 1 µm, and 1 µm, respectively. Biofilm on MEFs data in **S1*A*-*C*** are the same as shown in **Fig. 1*A*, 1*B*,** and **2*A*** in the main text, and are reproduced here for direct comparison with biofilm on plastic.

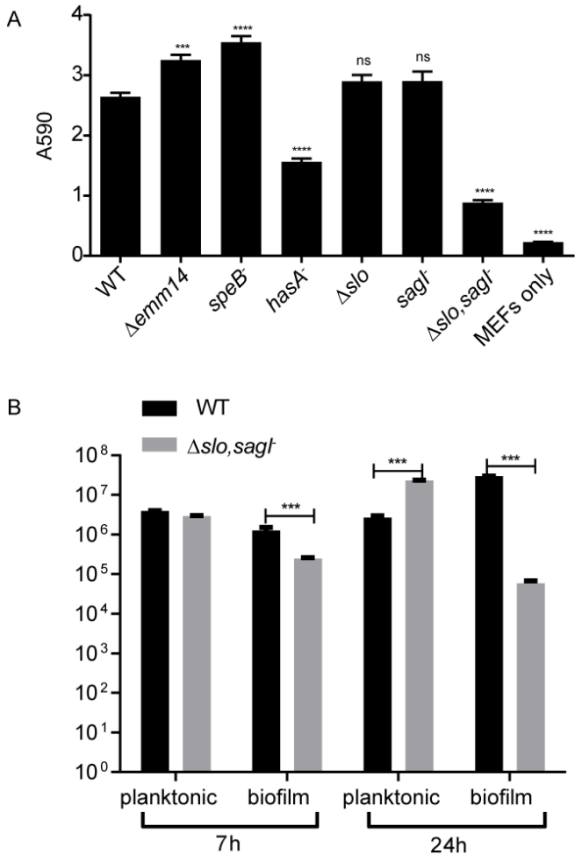

Fig. S2. GAS streptolysins (SLS or SLO) contribute to biofilm formation on mammalian cells. *(A)* Biofilm biomass of GAS wild type (WT) or isogenic mutants deficient for M protein, cysteine proteinase SpeB, hyaluronic capsule, streptolysin O, streptolysin S and both streptolysins (*Δslo,sagI-)* were quantified by CV assay. Data show the mean ± SEM; N=3. Statistical significance was calculated against WT biofilm biomass by one-way Anova using the Tukey's multiple comparisons test. *(B)* CFU quantification of WT and *Δslo,sagI-* strains grown on MEFs for 7 and 24 h in either the planktonic (supernatant) fraction or biofilm (MEF-associated) fraction. Data show the mean ± SEM values; N=2. Statistical significance was calculated by two-way Anova using the uncorrected Fisher's LSD test.

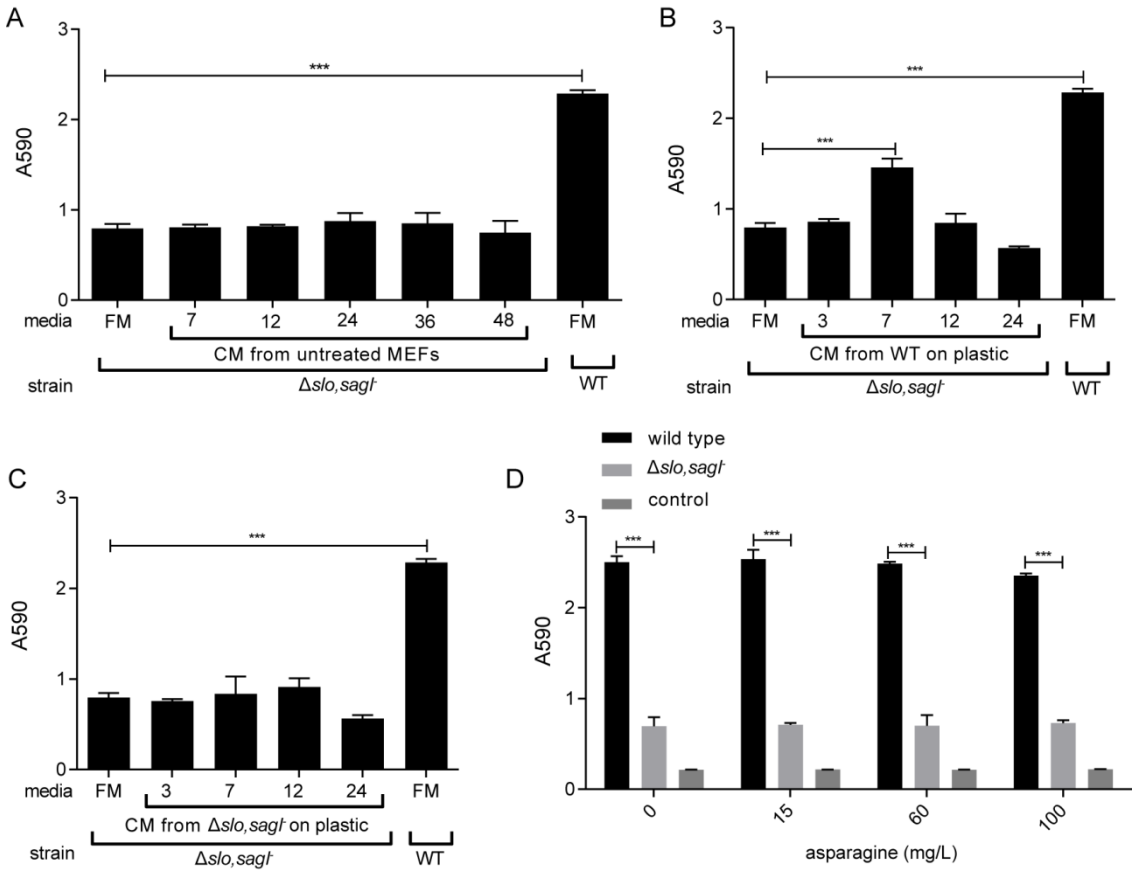

Fig. S3. An ER-stress induced host-associated factor in addition to asparagine is required for biofilm formation on mammalian cells. (*A*) Conditioned media (CM) from uninfected MEFs treated with drug-free (untreated) fresh media was collected at the indicated timepoints (numbers denote hours of treatment). Formation of biofilm by WT GAS on top of MEFs in fresh media (FM) was used as a positive control and FM alone served as negative control. Similarly, CM generated by growing either WT GAS (*B*) or *Δslo,sagI (C)* on polystyrene plastic plates were collected at the indicated times. *Δslo,sagI* biofilm biomass was quantified in their presence after 24 hpi. Positive and negative controls are the same as above. *(D)* Biofilm biomass quantification of WT GAS and *Δslo,sagI* on MEFs supplemented with increasing concentrations of asparagine. Data show the mean ± SEM values; N=3. Statistical significance was calculated by one-way Anova using Tukey's multiple comparison test (*A*-*C*) or two-way Anova using the uncorrected Fisher's LSD test (*D*).

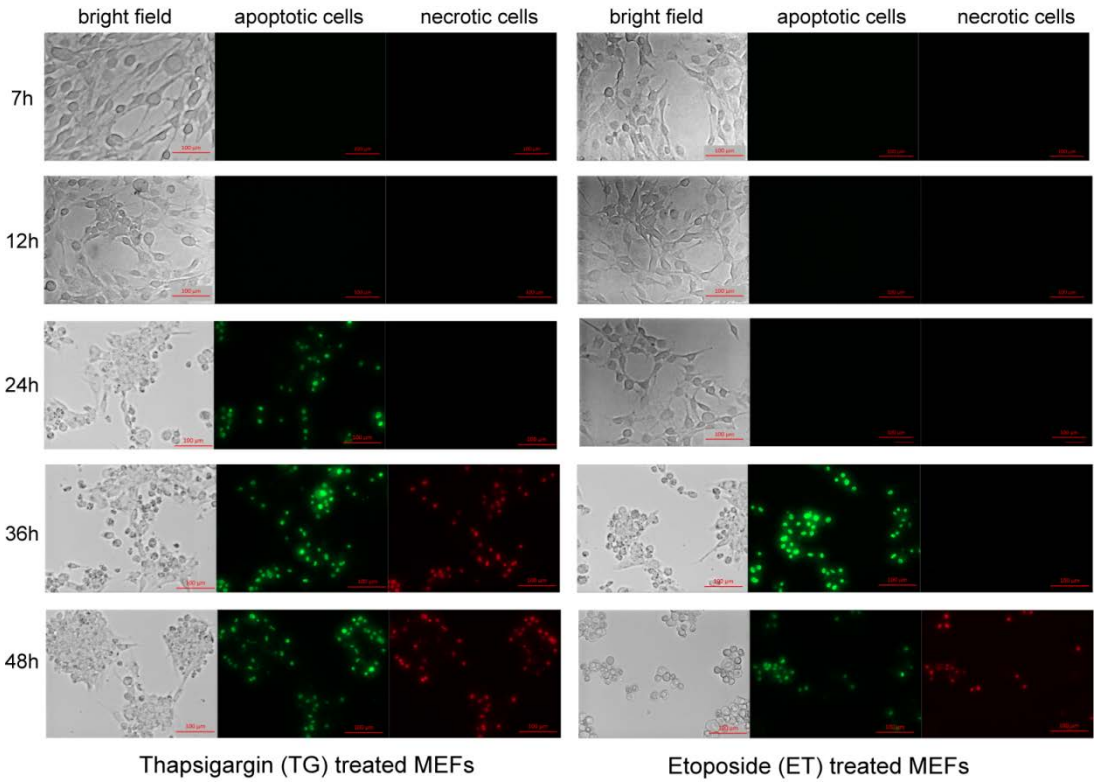

Fig. S4. TG and ET treatment induces apoptotic cell death in mammalian cells. Representative epifluorescence images (x20) of MEFs treated with TG (thapsigargin; 1.0 µM) or ET (etoposide; 10.0 µM) for the indicated time points and subsequently stained with the NucView 488 and RedDot 2 Apoptosis & Necrosis Kit. Green indicates cleavage of the caspase 3 substrate in apoptotic MEFs. The Red Dot 2 nucleic acid dye stains the DNA of necrotic cells with compromised membranes in red. Scale bars represent 100 µm.

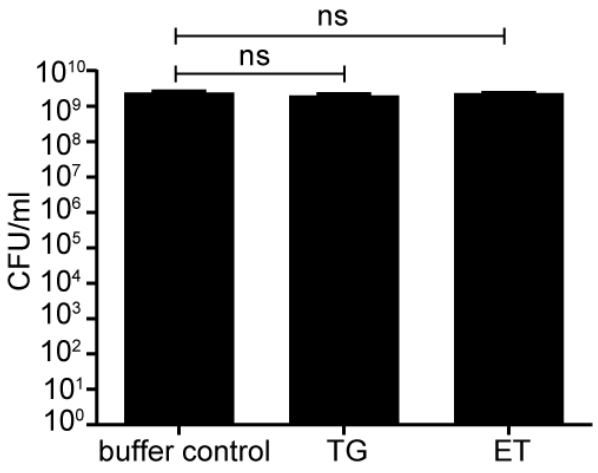

Fig. S5. Drug treatment does not affect GAS planktonic growth. CFU enumeration after treatment of GAS with TG (thapsigargin) and ET (etoposide) for 24 h, compared to buffer control (0.01% DMSO). Data show the mean ± SEM values; N=2. Statistical significance was calculated by one-way Anova using Tukey's multiple comparison test.

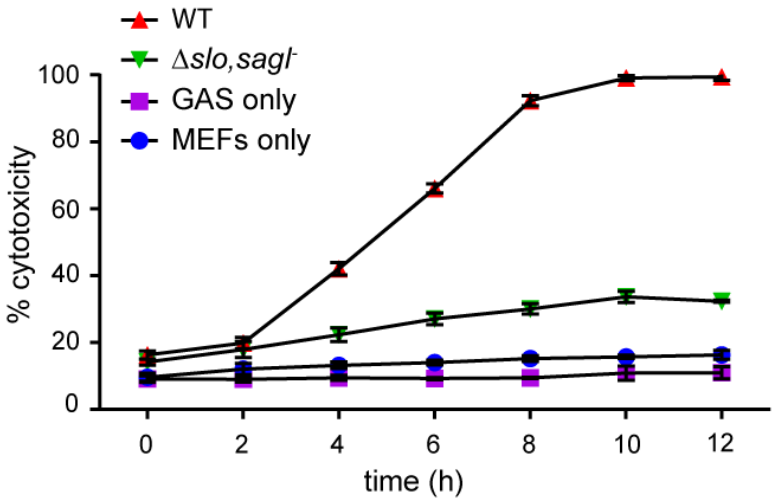

Fig. S6. GAS streptolysins induce progressive cell death in MEFs. GAS-mediated cytotoxicity of MEFs quantified by release of LDH into the media, compared to LDH release by GAS only or uninfected MEFs only. Cytotoxicity is represented as a percentage of total LDH release from cells lysed with Triton X-100. Data show the mean ± SEM values; N=3.

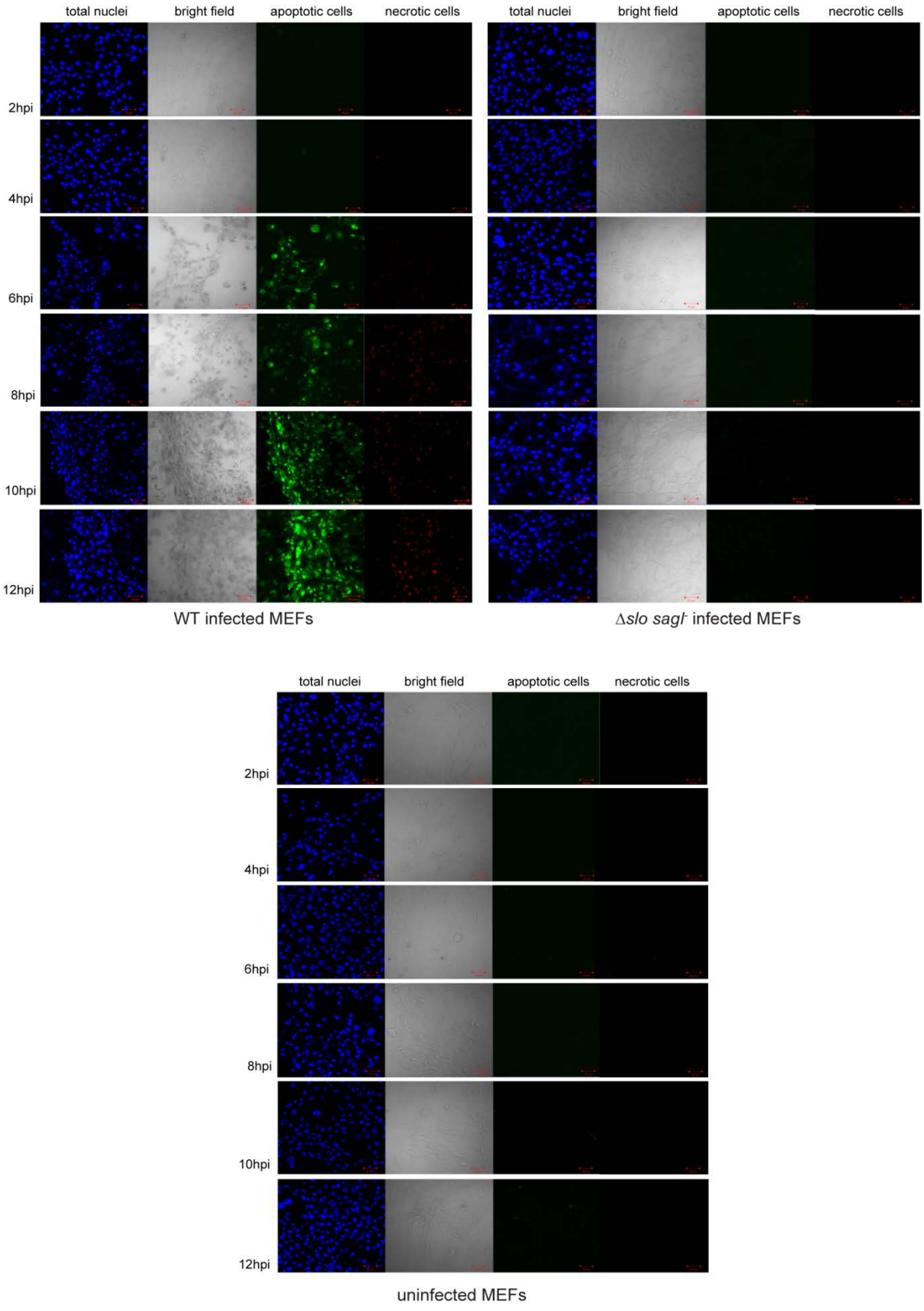

Fig. S7. GAS induces streptolysin-mediated apoptosis on MEFs. CLSM images (x20) of MEFs either infected with GAS WT, *Δslo,sagI*, or untreated and subsequently stained with

the NucView 488 and RedDot 2 Apoptosis & Necrosis Kit at indicated time points. Green indicates cleavage of the Caspase3 substrate in apoptotic MEFs. The Red Dot2 nucleic acid dye stains the DNA of necrotic cells with compromised membranes red. Scale bars represent 50 µm.

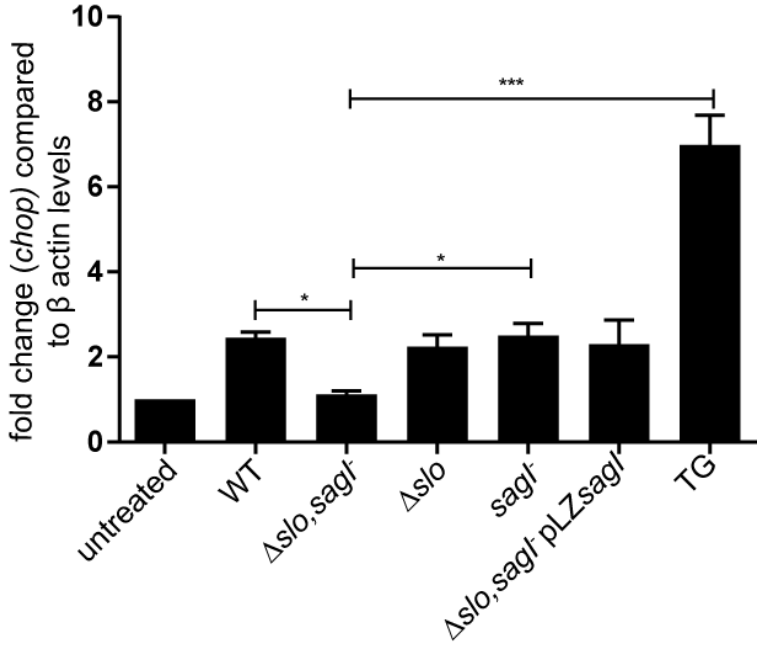

Fig. S8. GAS induces streptolysin-mediated ER stress on MEFs. MEFs were infected with the indicated strains for 7 h or treated with 1.0 µM TG for 12 h. RNA was extracted from the MEFS and the fold change in mRNA for the ER stress marker *chop* as compared to the expression of β actin was quantified by RT-PCR. Data show the mean ± SEM values; N=2, n=3. Statistical significance was calculated by unpaired multiple t tests.

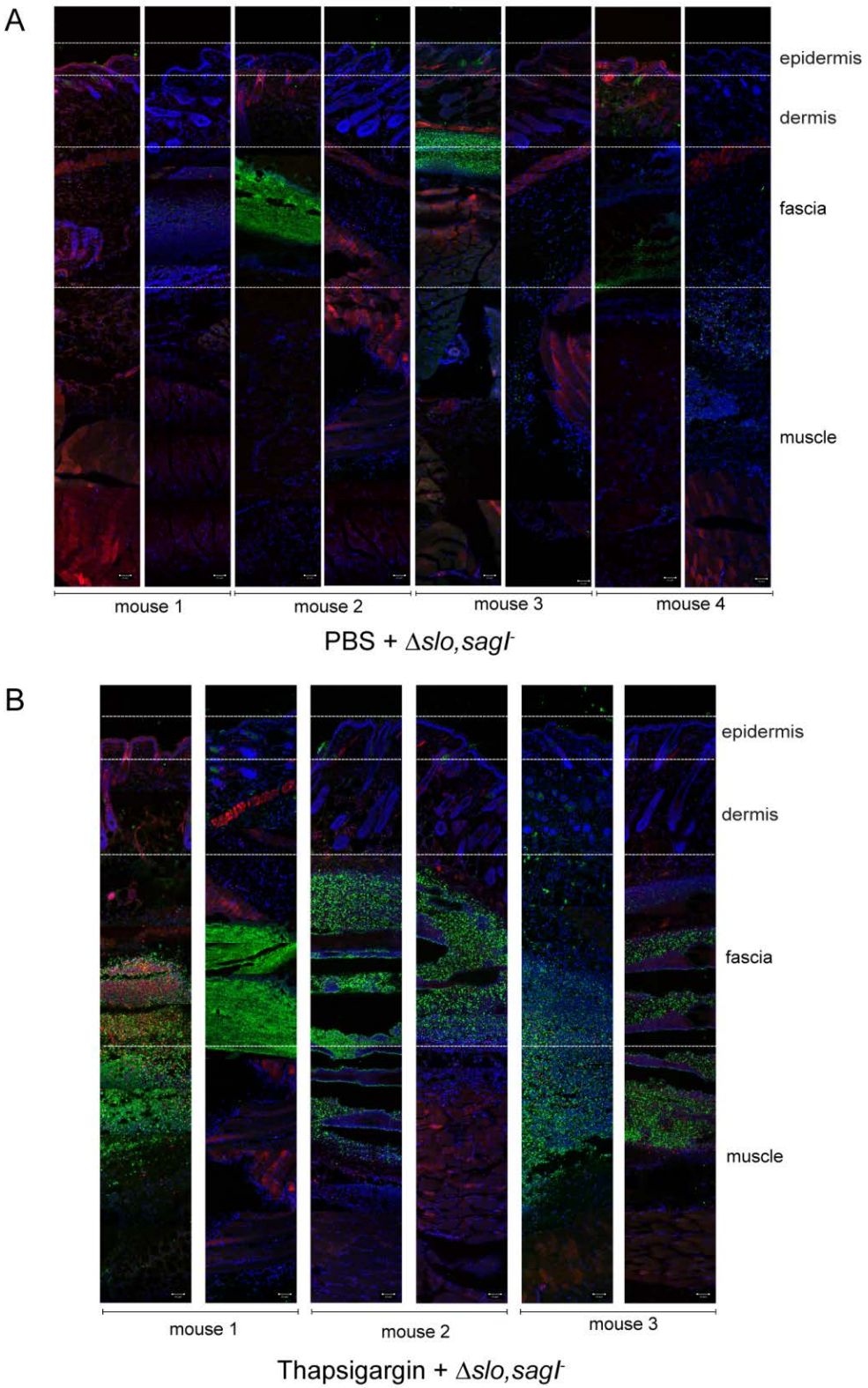

Fig S9. Pre-induction of ER stress restores *Δslo,sagI-* microcolony spread. Mice were pretreated with PBS or TG for 12 h and subsequently injected with Δ*slo,sagI-*. Tissue was harvested at 12 hpi. (*A*, *B*) Δ*slo,sagI-* microcolony distribution across the lateral plane of the

tissue biopsy is represented by tiled x20 CLSM images of representative tissue sections immunostained with anti-GAS antibody (green), phalloidin AF 568 (red) and DAPI (blue); N=4 (*A*), N=3 (*B*), and two representative sections per biopsy (per mouse) are shown. One mouse of each group (mouse 3 for PBS-treated, mouse 2 for TG-treated) is also shown in **Fig. 5*C***, and included in the panel here with the remainder of the mice for comparison. Scale bars represent 50 μm.

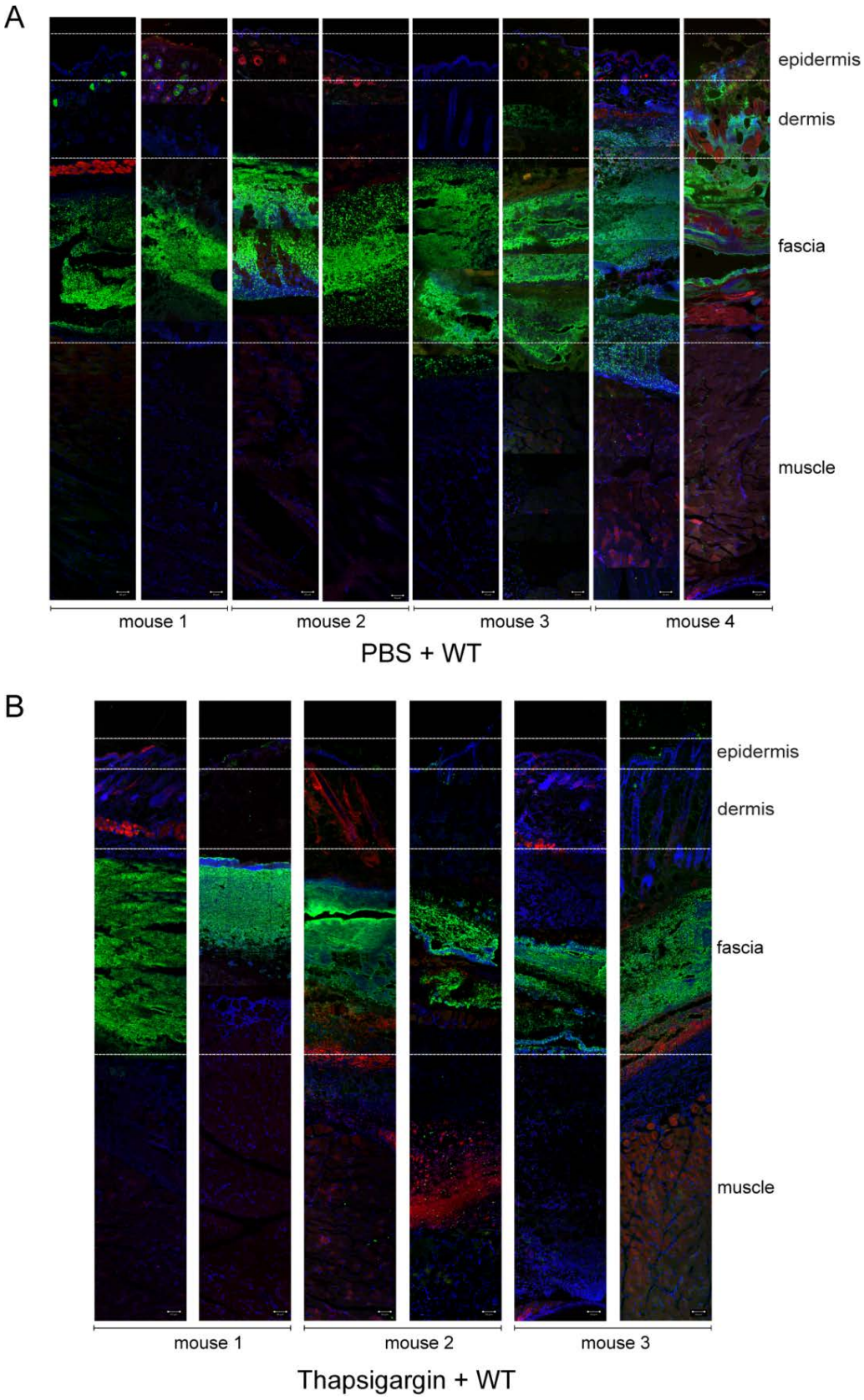

Fig S10. Pre-induction of ER stress does not significantly alter WT microcolony spread.

Mice were pretreated with PBS or TG for 12 h and subsequently injected with the WT GAS.

Tissue was harvested at 12 hpi. (*A*, *B*) WT GAS microcolony distribution across the lateral plane of the tissue biopsy is represented by tiled x20 CLSM images of representative tissue sections immunostained with anti-GAS antibody (green), phalloidin AF 568 (red) and DAPI (blue); N=4 (*A*), N=3 (*B*), and two representative sections per biopsy (per mouse) are shown. One mouse of each group (mouse 4 for PBS-treated, mouse 2 for TG-treated) is also shown in **Fig. 6A**, which is included in the panel here with the remainder of the mice for comparison. Scale bars represent 50 μm.

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