Chemically based transmissible ER stress protocols are unsuitable to study cell-to-cell UPR transmission

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Abstract

Renal epithelial cells regulate the destructive activity of macrophages and participate in the progression of kidney diseases. Critically, the Unfolded Protein Response (UPR), which is activated in renal epithelial cells in the course of kidney injury, is required for the optimal differentiation and activation of macrophages. Given that macrophages are key regulators of renal inflammation and fibrosis, we suppose that the identification of mediators that are released by renal epithelial cells under Endoplasmic Reticulum (ER) stress and transmitted to macrophages is a critical issue to address. Signals leading to a paracrine transmission of ER stress (TERS) from a donor cell to a recipient cells could be of paramount importance to understand how ER-stressed cells shape the immune microenvironment. Critically, the vast majority of studies that have examined TERS used thaspigargin as an inducer of ER stress in donor cells in cellular models. By using multiple sources of ER stress, we evaluated if human renal epithelial cells undergoing ER stress can transmit the UPR to human monocyte-derived macrophages and if such TERS can modulate the inflammatory profiles of these cells. Our results indicate that carry-over of thapsigargin is a confounding factor in chemically based TERS protocols classically used to induce ER Stress in donor cells. Hence, such protocols are not suitable to study the TERS phenomenon and to identify its mediators. In addition, the absence of TERS transmission in more physiological models of ER stress indicates that cell-to-cell UPR transmission is not a universal feature in cultured cells.

Introduction

Adaptive responses to stress regulate both intrinsic and extrinsic cell functions [1]. Cellular extrinsic responses are involved in paracrine communication and produce signals that alert neighboring cells of the presence of a stressful condition. These adaptive responses participate in building communication networks that shape the stressed cell microenvironment, generally in a paracrine manner, leading to the activation of preemptive responses in cells that have not yet been subjected to the stress and to the production of alarm signals, including danger associated molecular pattern signals (DAMPs), proinflammatory cytokines and chemokines, that will activate innate immune cells such as macrophages and dendritic cells [1-3]. These processes are particularly relevant in the pathophysiology of kidney diseases given the strong interplay between injured resident renal cells, such as tubular epithelial cells, and peritubular cells. The communication network between tubular epithelial cells, fibroblasts and macrophages promotes fibrosis and inflammation, which are critical for kidney structural deterioration and loss of function [4, 5]. Hence, adaptive stress responses pathways, as they shape the endogenous repair and scarring equilibrium in tissues, support kidney tissue remodeling and significantly impact the functional outcomes of kidney injuries ultimately leading to chronic kidney disease.

Endoplasmic reticulum (ER) stress often occurs in glomerular or renal epithelial cells in an injured microenvironment, and is involved in the pathophysiology of various renal diseases [6-9]. The injured kidney microenvironment predominantly fuels ER stress via oxygen and nutrient deprivation or exposition to nephrotoxins, though hypernutrition can also contribute during obesity. Upon ER stress, the unfolded protein response (UPR) is activated, engaging transcriptional, post-transcriptional and translational programs to reduce the amount of nascent proteins translocated in the ER and increase ER protein folding capacity. In addition to cell-autonomous functions, the UPR controls non-cell autonomous responses, including angiogenesis [10, 11], tissue remodeling and inflammation [12, 13], which are critical mediators of human renal diseases progression. An intriguing component of this intercellular crosstalk activated by the UPR is the possibility of ER Stress transmission from a donor to a recipient cell, through yet unknown mechanisms and signals. This process is sometimes referred to as Transmissible ER stress (TERS). To date, the molecular nature of the signal leading to TERS is not known. TERS could be mediated by a simple change in the extracellular concentration of metabolites, a protein, a lipid or by a complex TERS signal consisting of several molecules individually active or assembled in extracellular complexes. However, TERS could be, if it really occurs, of paramount importance to understand how ER-stressed cells (for example cancer cells) shape the tumor immune microenvironment. Indeed, the UPR modulate critical aspects of the phenotype of almost all types of immune

cells, including macrophages, dendritic cells, neutrophils, CD8 T cells, NK cells, B cells and may have profound effects on their activation profile, depending on the cell type and the microenvironment [14, 15].

Among the recently identified crucial mechanisms that are involved in the pathogenesis of acute kidney injury and chronic kidney disease, myeloid cells, most notably macrophages, which regulate the inflammatory response to acute injury and the repair and progression phases, appear to be critical mediators of chronic histological changes [16]. Tubular epithelial cells regulate the destructive activity of macrophages through the alteration of their activation, proliferation, polarization, and migration. Critically, the UPR, and more specifically, the IRE1α-XBP1 signaling, is required for the optimal differentiation and activation of macrophages [17] [18]. Given that macrophages are key regulators of renal inflammation and fibrosis, we supposed that the identification of mediators that are generated by stressed renal epithelial cells and that are required for macrophage survival, proliferation and activation is a critical issue to address. By combining multiple sources of ER stress in cultured cells, we evaluated if ER stress of human renal epithelial cells (HREC) was transmissible to macrophages and if such TERS can modulate the inflammatory profiles of these cells.

Materiel and methods

Cell culture

Human Renal Epithelial Cells (HREC) of proximal origin immortalized with the HPF 16 E6/E7 genes (HK-2 cells) and Human monocytes derived from the peripheral blood of a childhood case of acute monocytic leukemia (THP-1 cells) were purchased from American Type Culture Collection (ATCC CRL-2190 lot #710257641 and ATCC TIB-202, respectively).

HREC were grown at 37°C in an atmosphere containing 5 % CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, 41965-039 Gibco, MD, USA) supplemented with 1 % fetal bovine serum (HyClone, SV30160.03 GE lifesciences), 0.5 µg/ml hydrocortisone (Sigma-Aldrich, Saint-Quentin-Fallavier, France), 1X Insulin Transferrin Selenium mix (Sigma-Aldrich), 10 ng/mL Epithelial Growth Factor (Sigma-Aldrich), 6.5 ng/mL Triiodothyronine (Sigma-Aldrich), and 1X of Penicillin-Streptomycin mix (Gibco). Sub-confluent HREC were trypsinized using 0.25 % Trypsine-EDTA (Gibco) and sub-cultured at a density of 35.10^3 cells/cm² in 12 or 6-wells plates containing 1 or 2 ml of complete medium for the indicated times under the indicated conditions. Monocytes were cultured at 37°C under a 5 % CO_2 atmosphere in Roswell Park Memorial Institute's medium (RPMI-1640, 10491-01 Gibco) supplemented with 10 % fetal bovine serum (HyClone, SV30160.03 GE lifesciences) at a density of $3\text{-}8.10^5$ cells/ml. Differentiation of these monocytes into human monocyte-derived Macrophages was performed in 12-wells plates at a density of 115.10^3 cells/cm², through a 3 days-long

incubation with 1 ml of complete medium supplemented with 200 nM phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich) followed by a further 5 days of incubation in 2 ml of fresh complete medium devoid of PMA, as previously described [38]. All experiments were performed using Mycoplasma-free cells (Mycoalert Mycoplasma Detection Kit, Lonza) sub-cultured less than 6 times.

Conditioned Medium

ER stress-conditioned media (c.m.) from HREC "donor cells" used for TERS experiments were generated as follows. ER stress was induced in HRECs through an 8 hours incubation in complete medium containing either 0.25 µM Thapsigargin (Tg, T9033 Sigma-Aldrich), 50 ng/ml Tunicamycin (Tun, T7765 Sigma-Aldrich) or 1 mM Dithiothreitol (DTT, 43815 Sigma-Aldrich). To generate c.m. from apoptotic cells, HREC were incubated with 100 µM Etoposide (Eto, E1383 Sigma-Aldrich). In all experiments, control cells were incubated with an equal volume of drug's vehicle (DMSO 41639, Sigma Aldrich). HRECs were then washed 3 times with warm PBS and incubated with complete medium for a further 40 hours. For glucose deprivation experiments, HRECs were incubated 48 hours in a complete culture medium in which high-glucose DMEM was replaced by a glucose-deprived DMEM (11966-025, Gibco), supplemented with 4.5 g/L glucose (2494001, Gibco) for control cells. To generate c.m. from cells undergoing intrinsic ER stress, we harvested the culture supernatant of previously described mTAL cells displaying stable expression of wild-type (WT) or mutant (C150S) Uromodulin (UMOD) through lentivirus transduction [24]. Finally, to generate c.m. from cell-free (CF) dishes, we treated 12-wells plates devoid of cells exactly as for Tg-mediated TERS experiment. All culture supernatants were centrifuged at 4°C for 5 min at 5000g and filtered through 0.22-µm filters (Millipore) to remove cellular debris. Unless specified, resulting c.m. were supplemented with glucose to reach 4.5 g/L and frozen before use. HRECs or macrophage "recipient cells" were finally incubated in those c.m. for 8 hours or 24 hours.

Viability assay

HRECs were seeded at a density of 35.10^3 cells/cm² in 96-well plates with 200 µl of complete medium then treated as for c.m. production. At different specified time points of the process the relative number of living cells per well was determined on the basis of mitochondrial integrity by assay with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega, Charbonnieres, France), according to the manufacturer's instructions.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using the RNeasy Mini Kit® (Qiagen) according to the manufacturer's protocol. The yield and purity of RNA were measured using a NanoDrop ND-1000® spectrophotometer (Nanodrop Technologies). Reverse transcription was performed on 1 µg of RNA with oligo(dT) primer and random hexamers, using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's protocol. Transcripts expression levels were quantified through SYBR green RT-qPCR using an ABI PRISM 7900 sequence detector system (Applied Biosystems). The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta CT}$ method and Ribosomal Protein L13A (RPL13A) as housekeeping gene. Samples of vehicle-treated donor cells or samples of cells treated with c.m. from vehicle-treated donor cells were used as references. Sequences of primers used are listed in the **Supplementary Table 1**.

Protein extraction and Western blot analysis

Cells were washed in ice-cold PBS, lysed in M-PER buffer (Thermo-Scientific, Waltham, MA, USA) supplemented with a mixture of phosphatases and proteases inhibitors (Roche, Mannheim, Germany). Extracts were centrifuged at 14.000 g for 5 min and protein concentration in supernatants were measured using the bicinchoninic acid method (Pierce, Rockford, IL, USA). 25 µg of protein extracts were resolved by electrophoresis in 10 % SDS-PAGe (Thermo-Scientific) and transferred to nitrocellulose membranes (iBlot, Thermo-Scientific). Membranes were blocked with Tris-Buffered Saline supplemented with 0.2 % Tween20 (TBS-Tween) and 5 % of proteins from non-fat milk for 1 h at room temperature and then incubated overnight at 4 °C with primary antibody diluted in blocking buffer. Primary antibodies were a goat anti-bip N-20 (sc-1050, Santa cruz, TX, USA) and a mouse anti-Tubulin (T6793, Sigma-Aldrich). After washings in TBS-Tween buffer, membranes were incubated with Horseradish peroxidase(HRP)-conjugated polyclonal secondary antibodies at room temperature in blocking buffer. Indirect HRP detection was performed by chemiluminescence using the Pierce ECL Western Blotting Substrate (Pierce) and an ImageQuant LAS 4000 series camera (GE Healthcare, IL, USA). The Bip protein signal was quantified using the ImageJ freeware and normalized on the Tubulin signal.

Enzyme-linked immunosorbent assays (ELISA) and Dot blot Cytokine micro-arrays

Soluble Angiogenin (ANG), Interleukin-1β (IL-1β) and Macrophage Inflammatory Protein 1β (MIP-1β) were quantified in c.m. and culture supernatants using Human Angiogenin/MIP-1β/IL-1β Quantikine ELISA Kits (R&D Systems, MN, USA) and a TECAN microplates reader (TECAN, Mannedorf, Switzerland), according to the manufacturer's protocol.

Cytokine relative expression was evaluated in cell culture supernatants using the Dot blot RayBio® Human Inflammation Antibody Array C3 kit (AAH-IFN-3, RayBiotech, GA, USA) according to the manufacturer's protocol. The signal intensities were quantified by densitometry using the NIH freeware ImageJ (Bethesda, MN, USA) after background subtraction and positive controls were used to normalize the results from the different membranes being compared. Evaluations of the relative cytokine expression levels were made by comparing the signal intensities between the different conditions.

Chemical analyses

LDH, Ca^{2+}, K^{+} and Glucose measurements in conditioned medium were performed at the Clinical Chemistry Department of the European Georges Pompidou Hospital using a Beckman Coulter AU680 analyser.

HPLC-MS analysis

Thapsigargin in culture medium was quantified by mass spectrometry after sample clean-up by solid phase extraction using acetonitrile to precipitate proteins. Tg was isolated by reverse phase using Acquity UPLC CSH C18 1,7µm x 2,1mm 150mm columns on an UltiMate™ 3000 UHPLC System (Dionex). The injection volume was 5 µL, column temperature was 40°C and flow rate 0,2 mL/min. Solvant A was 0,1% formic acid in water. Solvant B was methanol with the following elution gradient : 0 min, 60 % ; 9 min, 90 % ; 11 min, 90 % and 12 min 60 %. Samples were analyzed by mass spectrometric on a LTQ-Orbitrap XL (Thermofisher Scientific) using electrospray ionization in positive mode. Source parameters were: Sheath Gas Flow Rate (arb) : 20; Aux Gas Flow Rate (arb) :15; Sweep Gas Flow Rate (arb) : 0; I Spray Voltage (kV) : 4.20; Spray Current (µA): 100; Capillary Temp (°C) : 275; Capillary Voltage (V) : 35; Tube Lens (V) : 100. Detection: Full scan (m/z 70-1500). Quantitation was based on peak area generated by the protonated molecular ion parent (theoretical m/z 673.3199). Data were analyzed using the Xcalibur software (Thermo Fisher Scientific).

Statistical analysis

All data are represented as individual values and means ± standard error of the mean of at least two independent experiments in duplicates or triplicates, unless otherwise specified. Graphs were generated using GraphPad Prism 7 Software (GraphPad Software, Inc.). According to current discussion and criticisms on the performance and interpretation of statistical tests in experimental settings, and in particular the misuse and misinterpretation of *p* values [39-41], we choose not to systematically perform comparison of biological data using statistic tests to compute *p* values for significance.

Results

Validation of multiple models of ER stress

We derived a model from the original study demonstrating that TERS exists in vitro [19] to explore the influence of ER-stressed HRECs on myeloid cells in a system in which ER stress-conditioned medium is transferred to human macrophages. We produced a 48 h HRECs conditioned medium (c.m.) in incubating HRECs with ER stressors for 8 h followed by three washings with PBS and then incubation in fresh medium for 40 h. Recipient cells were then incubated with this ER stress c.m. for 8h or 24 h (**Supplementary Figure 1**).

ER stressors were thapsigargin (Tg), a sesquiterpene lactone that inhibits the sarco/endoplasmic reticulum Ca^{2+}-ATPase [20], tunicamycin (Tn), a nucleoside antibiotic that inhibits GlcNAc phosphotransferase, which catalyzes the first step of protein N-glycosylation [21], and Dithiotreitol (DTT) a reducing agent that prevents disulfide bonds formation between cysteine residues of proteins [22]. We also used more physiologically relevant models of ER stress (**Supplementary Figure 1**). For this, HRECs were cultured for 48 h in medium lacking glucose, as glucose starvation is a condition demonstrated to elicit ER stress in ischemic tissues [23]. We also produced c.m. from immortalized renal epithelial cells lining the thick ascending limb of Henle's loop (mTAL) expressing the Uromodulin (UMOD) under its wild-type (WT) or a mutant isoform which accumulates in the ER lumen and produces ER stress (C150S) [24]. Mutations in UMOD, the gene encoding uromodulin, cause autosomal dominant tubulointerstitial kidney disease uromodulin-related (ADTKD-UMOD) and ER stress participates in the pathophysiology of this renal disease [25].

As expected, a robust ER stress transcriptional signature (upregulation of BiP, CHOP, ERDJ4, sXBP1, GADD34 transcripts) was observed in HREC donor cells after incubation with chemicals or after 48 h of glucose starvation (**Figure 1A-D**). The expression of these transcripts had various rates between 8 h of incubation and the end of the 40 h recovery period in normal culture medium according to the chemical used. At this moment, the chaperon Bip was strongly up regulated (**Figure 1E and Supplementary Figure 2A**) in all conditions, indicating that the ER stress response is still active at the time of c.m. harvesting. In addition, a significant increase in proinflammatory cytokines and chemokines transcripts that usually accompanies ER stress [26] was measured after incubation with glucose-deprived medium, Tg, DTT or Tn (**Supplementary Figure 2B, C and D)**.

Extracellular consequences of ER stress

The reduction of cell viability was 50 % in average in all conditions at the time of c.m. collection (**Figure 2A**). Etoposide (Eto), a topoisomerase inhibitor that induces HREC apoptosis without ER stress [27] served as a positive control. In line with an increase in cell

mortality, LDH **(Figure 2B)** and potassium **(Figure 2C)** concentrations increased in the culture medium of ER-stressed cells, indicating that plasma membranes are permeabilized (neither LDH nor potassium were released after DTT washout for reasons that remain to be determined). The medium composition in Ca^{2+} was not significantly altered (**Figure 2D**) and glucose concentrations increased after incubation with ER stressors (possibly due to a reduced number of cells) and did not significantly differ between ER stress inducers, except upon glucose starvation (**Figure 2E**). Angiogenin (ANG), which is produced by HRECs when the IRE1-sXBP1 axis is activated [27], was secreted in the culture medium, indicating that conventionally secreted proteins with potent biological function on recipient cells accumulate in the extracellular medium upon ER stress (**Figure 2F**). Finally, we did not observe the induction of Golgi protein 73 (GP73), a recently reported TERS mediator [28] (**Figure 2G)**. Together, these results indicate that upon ER stress, dying cells secrete and release intracellular components, including K^+, which can inhibit glucose uptake in surrounding T cells [26]. In our experimental conditions, ER stress did not deplete extracellular glucose nor increased GP73 expression, two potent candidate mediators for TERS.

Transmission of ER stress from HRECs to macrophages

We next tested whether c.m. could induce an ER stress response in recipient cells. In order to do this, we incubated human macrophages derived from THP-1 monocytes in c.m. for 8 h and 24 h. In line with previous data, human macrophages incubated for 8h or 24h with medium conditioned with Tg (Tg.c.m.) elicited a transcriptional signature characteristic of the UPR as compared with control c.m. (Ctrl.c.m.) (**Figure 3A and 3B**). The expression levels of the transcripts after 24 h of incubation with Tg.c.m. was, albeit lower, in the same range of values than in cells incubated in control c.m. directly supplemented with Tg (Ctrl.c.m.+Tg). This effect is likely not due to cell death and the potent paracrine effects of intracellular components released during plasma membrane permeabilization because culture medium conditioned with Etoposide (Eto.c.m.), which contains intracellular compounds released after cell permeabilization, did not activate the UPR in recipient macrophages **(Supplementary Figure 3A)**. Tg.c.m. also produced TERS in recipient HRECs instead of macrophages (**Supplementary Figure 3B-E).** Medium conditioned with Tn (Tn.c.m.) did not induce ER stress in recipient macrophages after 8h and 24h of incubation **(Figure 3C and 3D)**. Medium conditioned with DDT (DTT.c.m.) induced CHOP expression in recipient macrophages after 8h of incubation, but not after 24h of incubation, and the other UPR markers were not affected or with a very low magnitude **(Figure 3E and 3F)**. Macrophages incubated for 8 h or 24 h with medium conditioned by glucose deprivation and supplemented with glucose before incubation to avoid the confounding biological effect of glucose deprivation in recipient cells (NoGluc.c.m.) did not produce ER stress (**Figure 3G and 3H)**. Similarly, NoGluc.c.m. did not

produce any effect when recipient cells were HRECs instead of macrophages (**Supplementary Figure 3F and 3G).** Notably, macrophages incubated for 24 hours with NoGluc.c.m not resupplemented with glucose or directly incubated with culture medium without glucose did not elicit ER stress (**Supplementary Figure 2C**), suggesting that, unlike HRECs, they have biochemical characteristics that enable them to support their energetic metabolism from nutrients sources other that glucose. Finally, macrophages incubated with UMOD-C150S.c.m. did not express any UPR marker (**Figure 3I and 3J)**. Together, these results indicate that Tg.c.m. promotes ER stress in recipient macrophages, but that other chemical or physiological ER stresses do not. Thus, the effects observed after incubation with Tg.c.m. might not be generalizable to all ER stress inducers.

Effect of TERS on macrophages activation phenotype

We then determined the inflammatory profile induced by TERS in macrophages. In line with previous reports [19], Tg.c.m. triggered a robust inflammatory response characterized by the production of cytokines and chemokines, at the transcript level, but also the secretion of a large range of these mediators in the culture medium (**Figure 4 A-C**). Interestingly, CCL2 and 8 fold change in secretion was much higher in Tg.c.m. compared to Ctrl.c.m.+Tg**.**. This could indicate that a factor present in the Tg.c.m is inducing CCL2 production in recipient cells to a much higher extent that the medium containing Tg. As it was the case with the UPR transcripts, the level of expression of transcripts (**Figure 4B**) and concentration of secreted chemokines (**Figure 4C**) was roughly comparable between macrophages incubated with Tg.c.m. and macrophages stimulated with Ctrl.c.m.+Tg**.** However, Gluc.c.m. and UMOD-C150S.c.m., which do not produce TERS, did not promote the expression and inflammation-related transcripts nor the secretion of MIP-1β, one of the most robust marker of the Tg.c.m.-induced inflammatory response in macrophages observed in our models (**Figure 4D-F**). Finally, DTT.c.m and Tn.c.m. did not induce an inflammatory response in recipient macrophages after 8h and 24h of incubation **(Supplementary figure 4)**. Thus, the impact of c.m. on macrophages inflammatory profile was similar to that observed with the UPR markers, suggesting a specific effect of Tg.

Exploration of the possibility of a carry-over of Tg.

Given the major differences of effects between Tg.c.m. and the other conditioned media on macrophages, we asked whether the effects of Tg.c.m. on recipient macrophages could be related to a carry-over of Tg. To address this issue, we used two approaches. First, we produced 48h cell-free (CF) c.m. (incubation of dishes devoid of cells with or without Tg for 8 h followed by three washings with PBS and then incubation in fresh medium for 40 h). HRECs recipient cells incubated with the CF-Tg.c.m for 8h or 24 h activated a robust ER

stress response, suggesting that TERS produced by Tg.c.m. does not require donor cells, and that the TERS mediator is not produced by donor cells (**Figure 5A and 5B**).

We next attempted to detect Tg by mass spectrometry (MS). The observed m/z of the protonated molecular ion parent is 673.3178 for a theoretical m/z of 673,3199 (**Figure 6A**). We measured a limit of detection of Tg in dimethyl-sulfoxide of ≈0.05 μM after direct infusion. To detect and quantify Tg in the culture medium, we had to couple MS to high-performance liquid chromatography (HPLC) after sample clean-up by solid phase extraction. Tg was not directly visible on the chromatogram and the Ion Extraction Chromatogram led to measure the retention time of Tg at 9.1 minutes (**Figure 6B)**. The precipitation of the solid phase dramatically decreased the apparent concentration of Tg (**Table 1 and Figure 6C)**. Indeed, the peak area at m/z 673,318 was ≈5.500.000 when Tg was diluted in dimethyl sulfoxide at a final concentration of 0.2 μM, whereas the peak area was measured at ≈850.000 when Tg was diluted at the same concentration in culture medium and contained in a glass tube. The tube in which Tg is contained has indeed an importance because at a final concentration of 0.2 μM in culture medium, Tg was no more detectable when collected within a polystyrene tube (**Table 1**). Tg was not detected by this method in any samples of Tg.c.m. that we used for TERS. We performed a concentration-effect curve of Tg in HRECs and measured UPR markers after 24 hours, and we observed that Tg elicited the UPR starting at 0.025 μM, with a robust activation at 0.10 μM, concentrations that are all below the limit of detection of the MS method used to analyze culture medium (**Supplementary Figure 5A-D**).

These results indicate that Tg-induced TERS is cell-independent, and that Tg, which is adsorbed by polystyrene, promotes ER stress at concentrations that are below the limit of detection in c.m. of the HPLC-MS method. Consequently, a carry-over of Tg from the donor dish to recipient cells is the likely explanation of the effects of Tg.c.m. on macrophages.

Discussion

Assuming that TERS exists, our results indicate that the cellular models classically used to study this phenomenon, and to identify the mediators, are likely not suitable when they use chemicals to induce ER stress in donor cells. Critically, the vast majority of studies used Tg as an inducer of ER stress in donor cells in cellular models derived from the princeps study [19, 28-33] and did not resolve the issue of a carry-over of Tg. Our results provide keys arguments supporting the possibility of a carry-over of Tg in the conditioned medium. Tg, probably due to its hydrophobicity [34], is adsorbed on polystyrene, which can explain that Tg.c.m. produced in polystyrene dishes devoid of cells still transfer ER stress to recipient cells, a finding that has recently been observed in another model [35]. Indeed, Tg can be released from the polystyrene dish in the culture medium after PBS washing, and be present

in the Tg.c.m. at concentrations that can still elicit ER stress. In addition, the HPLC-MS study suggests that Tg can attach to proteins and/or lipids contained in fetal calf serum (FCS) and be carried out in culture medium without being detectable. Indeed, sample clean-up prior to the chromatography is supposed to precipitate the molecules to which Tg is fixed, thus eliminating a high proportion of Tg from the sample to be analyzed by MS. In line with a possible role for FCS components to carry Tg, FCS-free Tg.c.m. failed to promote ER stress in recipient cells and supplementation in albumin restored ER stress in a model of astrocytes to neurons TERS [32]. In addition, in this model, depletion of lipids from Tg.c.m. abrogated TERS. Thus, an absence of detection of Tg by HPLC-MS in Tg.c.m. does not eliminate the possibility that Tg is present in c.m. at a biologically relevant concentration to produce ER stress in recipient cells. Thus, the binding of Tg to polystyrene tubes can actually have two consequences. First, it can reduce the quantity of molecules in the culture medium contained in the polystyrene tube that are available for mass spectrometry analyses, leading to falsely negative results (ie. the carry over contained in the culture medium cannot be detected). Second, Tg can bind to the bottom of the culture dishes because experiments are not performed in confluent cells, and can be released after washing.

More physiologically relevant models of ER stress are thus required to demonstrate that TERS occurs and to identify the potent mediator(s). However, results are ambiguous in these models too. Culture medium conditioned by glucose starvation is relevant to mimic tissue ischemia and does not pose the problem of carry-over. Our results indicate that NoGluc.c.m. does not elicit TERS, which is in contradiction with the results of the initial study by Mahadevan and coll. [19]. A possible explanation for this discrepancy is that in this study, unlike in ours, NoGluc.c.m. was not supplemented in glucose before incubation with recipient cells, which constitutes a cofounding factor inducing ER stress. In addition, intrinsic ER stresses, such as those caused UMOD mutations or the overexpression of heavy chains of immunoglobulins[35], failed to promote TERS. However, there are also clear examples supporting the occurrence of TERS in vitro. For example, prostate apoptosis response 4 (Par-4) is secreted by ER-stressed prostate cancer cells in culture and extracellular Par-4 induces apoptosis by binding to GRP78 expressed at the surface of cancer cells. The interaction of extracellular Par-4 and cell surface GRP78 led to apoptosis via ER stress [36]. In addition, acute myeloid leukemia cells proliferation results in an intrinsic UPR that is transferred to stromal cells (mesenchymal stem cells and osteoblastic progenitor cells) in part by an increased production of extracellular vesicles containing bone morphogenic protein 2 [37]. These examples indicate that TERS occurs in vitro, but is not a universal mechanism that systematically occurs in all condition associated with ER stress. Rather,

TERS probably occurs in very specific situations, with mediators depending on the donor and recipient cell type and the nature of cellular stress.

In conclusion, carry-over is a confounding factor in chemically based TERS protocols that are therefore unsuitable to study cell-to-cell UPR transmission. In addition, the absence of TERS transmission in some physiological models of ER stress indicates that cell-to-cell UPR transmission is not a universal feature of ER stress in cultured cells.

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Conflict of interest statement

The authors have no conflict of interest to disclose

Author contributions

Yohan Bignon and Virgine Poindessous: collection and assembly of data, data analysis and interpretation and final approval of the manuscript; Luca Rampoldi: material support; Violette Haldys: mass spectrometry analysis; Nicolas Pallet: conception and design, financial support, collection and assembly of data, data analysis and interpretation, manuscript writing.

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Figure legends

Fig. 1: Validation of multiple models of ER stress in HRECs donor cells. (A-C) RT-qPCR analysis of UPR-related genes expression in HRECs submitted to 8 hours of treatment with 0.25 µM thapsigargin **(A)**, 50 ng/ml tunicamycin **(B)**, 1mM dithiothreitol **(C)** followed by three PBS washings and incubation in complete medium for a further 40 hours (8h Chem. / 40h Wo) or not (8h Chem.). **(D)** RT-qPCR analysis of UPR-related genes expression in HRECs submitted to 8 hours or 48 hours of glucose deprivation (NoGluc). **(E)** Expression level of Bip protein in cells treated for 8h / 40h Wo or 48h as in **(A-D)** determined by semi-quantitative analysis of Western blots depicted in **Supplemental Figure 1A**. Results are represented as fold change of expression to be compared with control cells treated similarly with vehicle or in high-glucose medium (dotted lines).

Fig. 2: Extracellular consequences of ER stress in HRECs donor cells. (A) Histogram showing the relative number of living HRECs donor cells determined by MTS-assay after 8 or 48 hours of glucose deprivation (NoGluc) and 8 hours of treatment with 0.25 µM thapsigargin (Tg), 50 ng/ml tunicamycin (Tn), 1mM dithiothreitol (DTT) or 100 nM etoposide (Eto) followed by three PBS washings and incubation in complete medium for a further 40 hours of washout (8h / 40h Wo) or not (8h). Results are expressed in percentage of living cells as compared from seeding (0h). **(B-E)** Concentrations of LDH **(B)**, K^+ **(C)**, Ca^{2+} **(D)** or glucose **(E)** in conditioned media (c.m.) of HREC donor cells submitted to glucose deprivation (NoGluc.c.m.), transient treatment with thapsigargin (Tg.c.m.), tunicamycin (Tn.c.m.), dithiothreitol (DTT.c.m.), or etoposide (Eto.c.m.). Mean concentrations (dotted lines) in the c.m. of vehicle-treated HRECs (Ctrl.c.m.) and initial concentrations in complete culture medium (DMEM) are shown for comparison. **(F)** Angiogenin secretion by HRECs donor cells in their c.m. following transient treatment with thapsigargin (Tg), tunicamycin (Tn), dithiothreitol (DTT), or glucose deprivation (NoGluc) to be compared with the mean secretion (dotted line) of vehicle-treated cells (Ctrl). **(G)** RT-qPCR analysis of GP73 mRNA relative expression in HRECs treated for 24 hours with 0.25 µM thapsigargin (Tg), 2.5 µg/ml tunicamycin (Tn) or vehicle (Ctrl) and in HRECs submitted or not to 48 hours of glucose (NoGluc) deprivation.

Fig. 3: Transmission of ER stress from HRECs to macrophages. (A-J) RT-qPCR analysis of UPR-related genes expression in macrophages recipient cells incubated during 8 or 24 hours in conditioned media (c.m.) of HRECs donor cells transiently treated with thapsigargin **(A-B)**, tunicamycin **(C-D)** or dithiothreitol **(E-F)**, in the conditioned medium of glucose-deprived HRECs donor cells (**G-H**) or in the conditioned medium of mTAL donor cells expressing a wild-type (WT) or mutant (C150S) Uromodulin (UMOD) **(I-J)**. Gene

expression in macrophages incubated in control c.m. from vehicle-treated HRECs and from HRECs cultured under normal glucose concentration (Ctrl.c.m.) or from mTAL cells expressing WT UMOD (UMOD-WT.c.m.) have been used as references. Control c.m. supplemented with the respective chemical ER stressor before use (Ctrl.c.m.+Chem.) have been used as internal positive controls for UPR in recipient macrophages in each experiment.

Fig. 4: Effect of TERS on macrophages phenotype. (A) Histogram representing the relative expression level of inflammation-related proteins measured by dot-blot micro-array in the culture supernatant of recipient macrophages incubated for 24 hours in the c.m. of HRECs transiently treated with 0.25 µM thapsigargin (Tg.c.m.), in control c.m. from vehicle-treated HRECs donor cells (Ctrl.c.m., dotted line) or in control c.m. of HRECs supplemented with 0.25 µM thapsigargin (Ctrl.c.m.+Tg). Inset depicts representative images of chemiluminescence detection from dot-blot membranes incubated with the culture supernatant of macrophages submitted to Ctrl.c.m. or Tg.c.m. for 24 hours. **(B)** RT-qPCR analysis of inflammation-related genes expression in recipient macrophages incubated during 8 hours (upper panel) or 24 hours (lower panel) in the c.m. of HRECs donor cells transiently treated with 0.25 µM thapsigargin (Tg.c.m.) or in the c.m. of vehicle-treated HRECs donor cells supplemented with 0.25 µM thapsigargin before use (Ctrl.c.m.+Tg). Gene expression in macrophages incubated in the c.m. of vehicle-treated HRECs donor cells (Ctrl.c.m.) has been used as reference. **(C)** MIP-1β concentration measured by ELISA in the culture supernatant of macrophages recipient cells incubated during 24 hours in the c.m. of HRECs donor cells transiently treated with thapsigargin (Tg.c.m.) or vehicle-treated (Ctrl.c.m.) and in the c.m. of vehicle-treated HRECs donor cells supplemented with 0.25 µM thapsigargin before use (Ctrl.c.m.+Tg) to serve as internal positive control. **(D)** RT-qPCR analysis of inflammation-related genes expression in recipient macrophages incubated during 8 hours (upper panel) or 24 hours (lower panel) in the c.m. of HRECs donor cells submitted to glucose deprivation (NoGluc.c.m.). To avoid the confounding biological effect of glucose deprivation in recipient cells, the c.m. of glucose deprived donor cells has been supplemented with glucose before use. Gene expression in macrophages incubated in the c.m. of HRECs cultured under normal glucose concentration (Ctrl.c.m.) has been used as reference. **(E)** MIP-1β concentration measured by ELISA in the culture supernatant of macrophages recipient cells incubated during 24 hours in c.m. from HRECs donor cells submitted or not to glucose deprivation (NoGluc) and in c.m. of mTAL donor cells expressing wild-type (WT) or ER-retained mutant (C150S) Uromodulin (UMOD). **(F)** RT-qPCR analysis of inflammation-related genes expression in recipient macrophages incubated during 8 hours (left panel) or 24 hours (right panel) in the c.m. of mTAL donor cells expressing the

Uromodulin (UMOD) protein under its wild-type (WT) isoform (UMOD-WT.c.m.) or an ER-retained mutant (C150S) isoform (UMOD-C150S.c.m.). Gene expression in macrophages incubated in the UMOD-WT.c.m. has been used as reference.

Fig. 5: Thapsigargin-mediated TERS phenomenon in the absence of donor cells. (A-B) RT-qPCR analysis of UPR-related genes expression in HRECs recipient cells incubated during 8 hours **(A)** or 24 hours **(B)** in cell-free conditioned medium (CF-c.m.). CF-c.m. have been collected from polystyrene dishes devoid of cells incubated for 8 hours with 0.25 µM thapsigargin (CF-Tg.c.m.) or an equivalent concentration of vehicle (CF-Ctrl.c.m.), washed three times with PBS and then incubated with complete medium for a further 40 hours. Gene expression in HRECs incubated in CF-Ctrl.c.m. has been used as reference while gene expression in HRECs incubated in CF-Ctrl.c.m. supplemented with 0.25 µM thapsigargin before use (CF-Ctrl.c.m.+Tg) has been used as internal positive control for UPR in recipient HRECs.

Fig. 6: Exploration of the possibility of a carry-over of Tg using Mass-spectrometry. (A) Mass spectrum of Thapsigargin diluted at 1 µM in dimethyl sulfoxide after direct infusion in Acetonitrile/Water (60%/40%). (**B**) Chromatogram spectrum (Upper panel) and Ion Extracted Chromatogram (Lower panel) of thapsigargin diluted at 0.5 µM in culture medium after solid phase extraction. (**C**) Mass spectrum of thapsigargin diluted at 0.5 µM in complete culture medium after solid phase extraction.

Table 1. Peak area of thapsigargin at m/z 673.318

Values of peak area of thapsigargin at m/z 673.318 according to solvants, concentrations and tubes.

Sample	**Peak area at m/z 673.318** (Retention time 9.1)
CH_3CN/H_2O	0
Tg. 1 μM (DMSO)	30 422 004
Tg. 0.2 μM (DMSO)	5 660 025
Tg. 0.1 μM (DMSO)	2 716 694
Tg. 0.2 μM (culture medium/glass tube)	868 851
Tg. 0.2 μM (culture medium/plastic tube)	Not detected

Figure 1

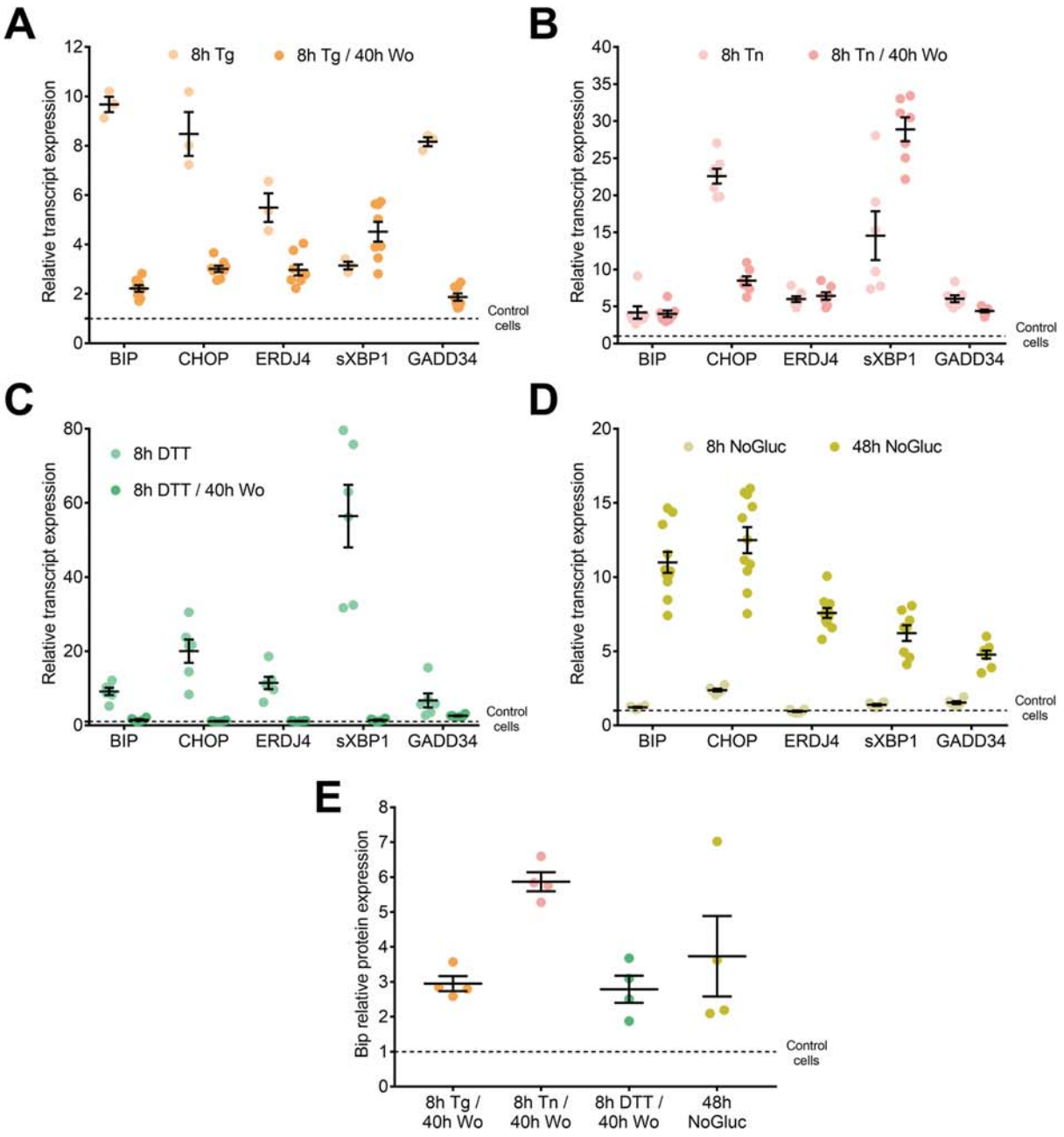


Figure 2

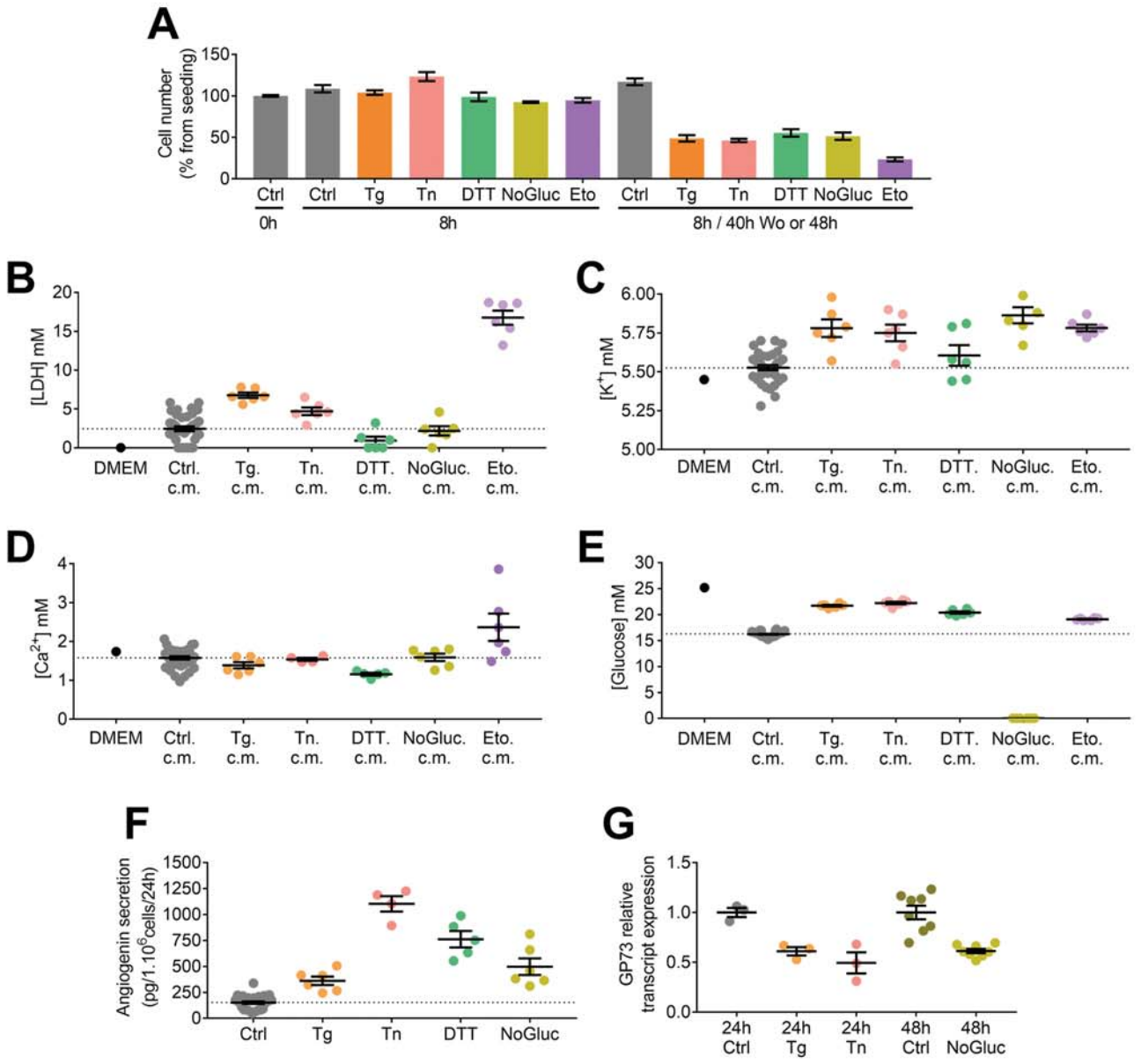


Figure 3

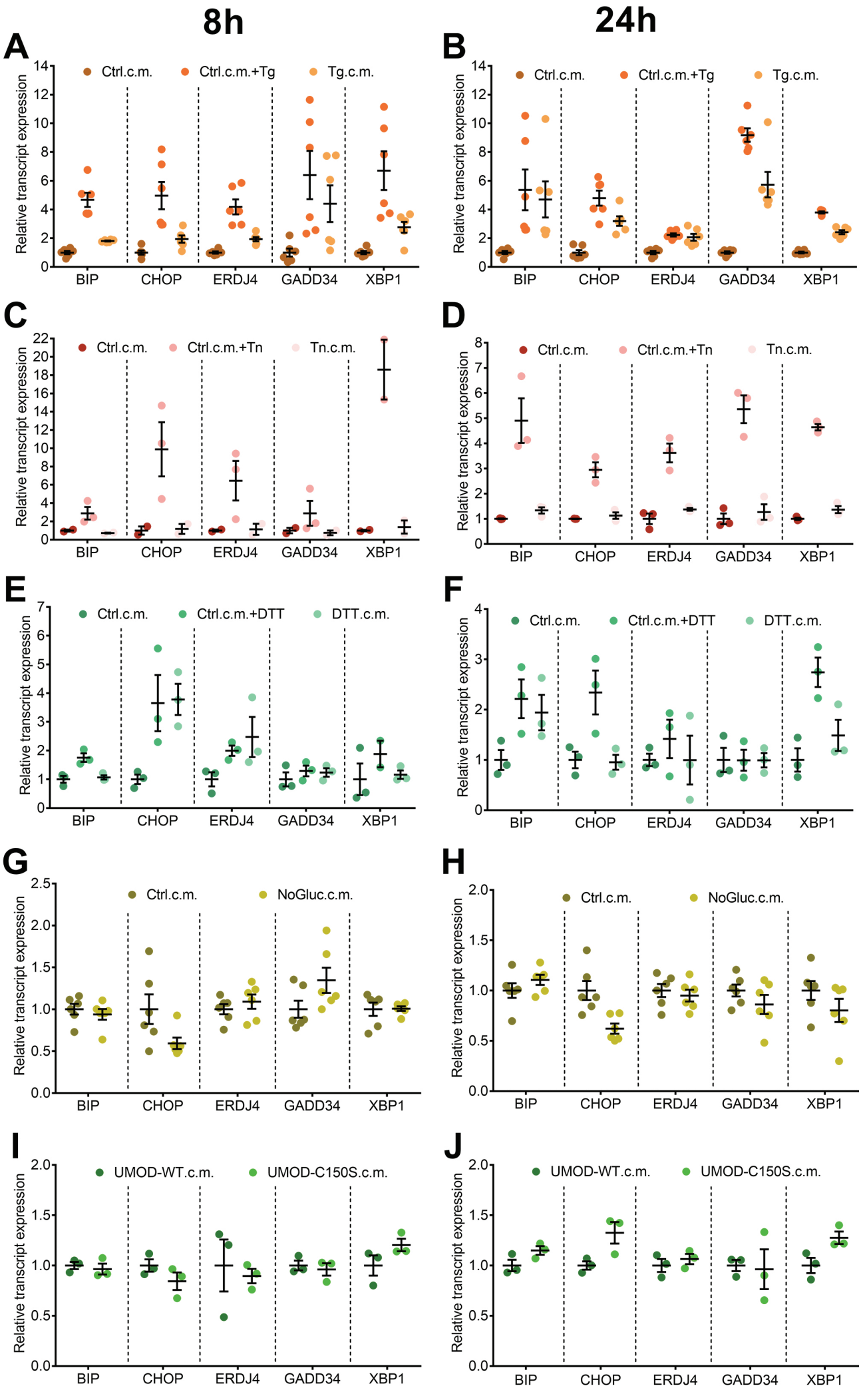
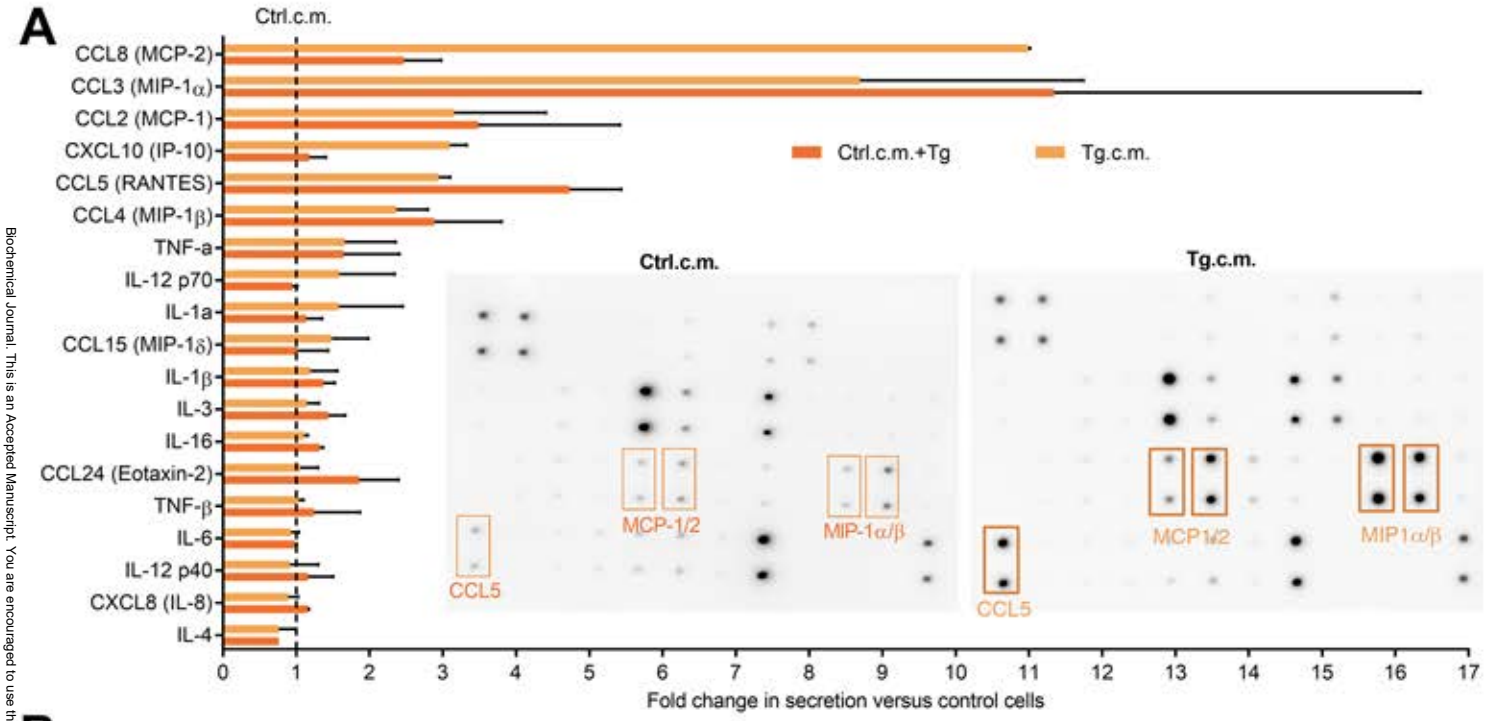
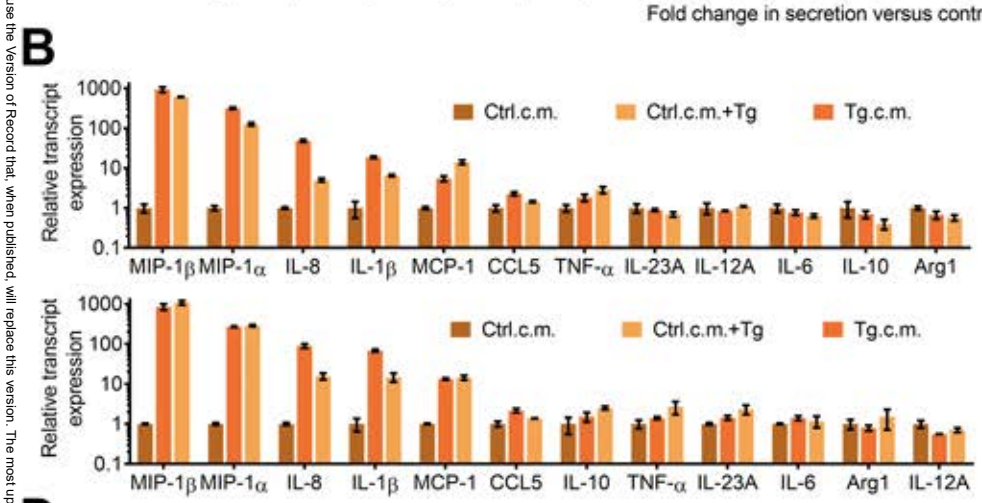
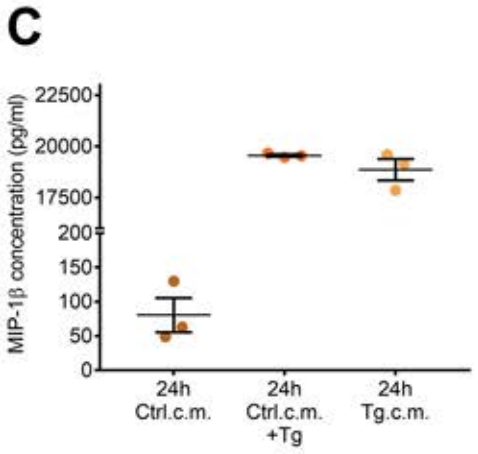
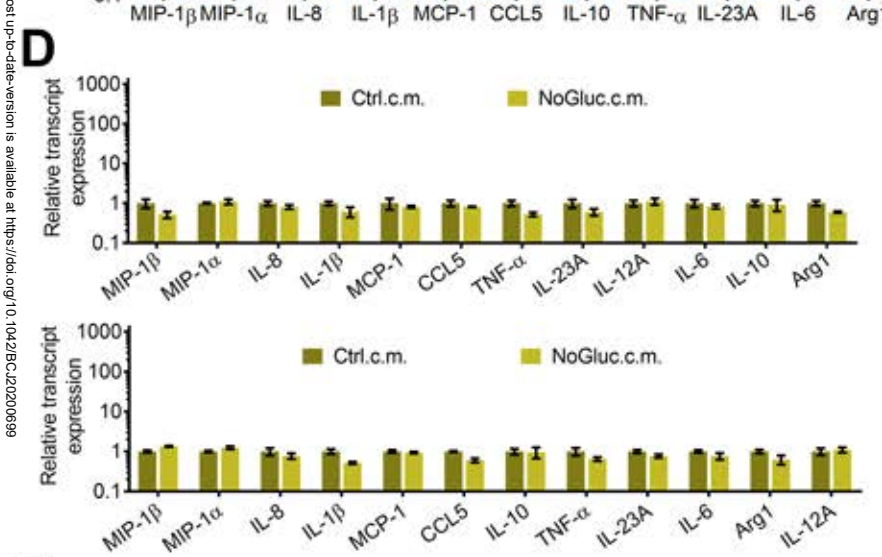


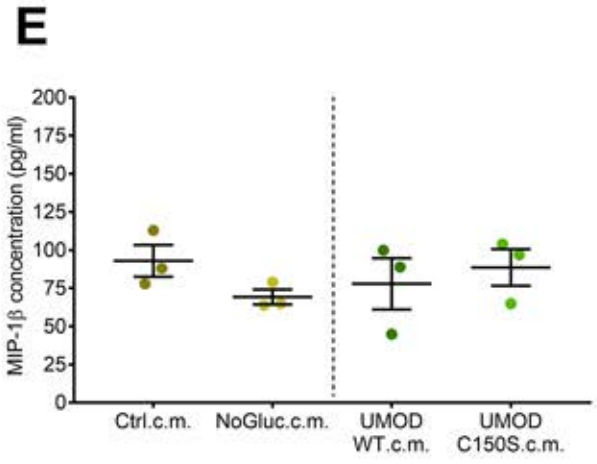
Figure 4











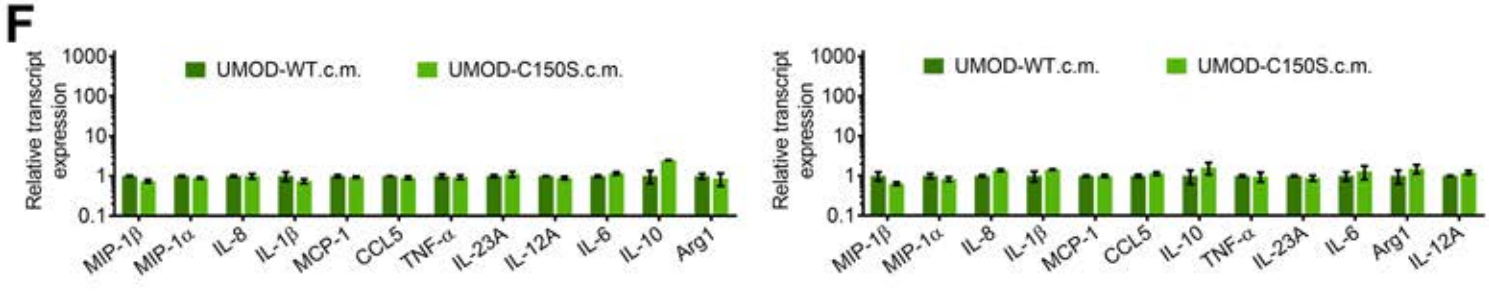


Figure 5

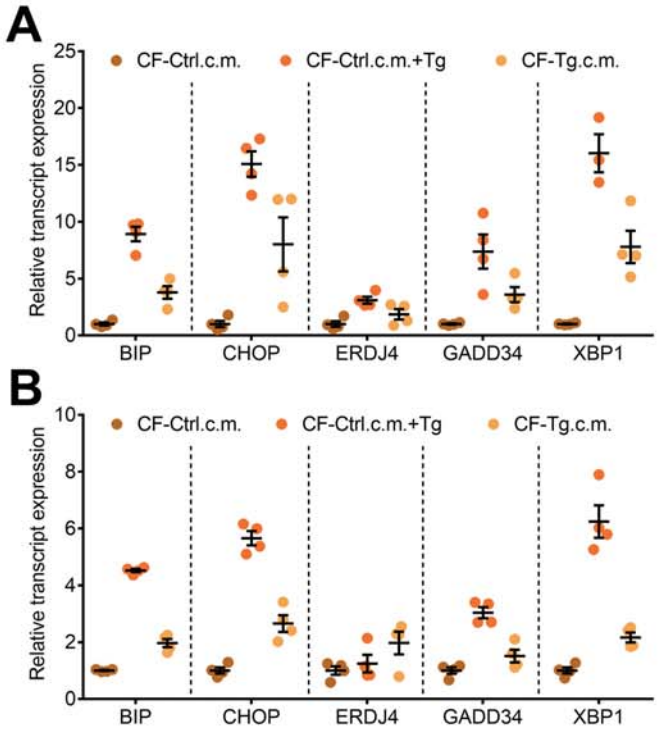


Figure 6