

Tumor-derived endoplasmic reticulum stress reprograms immune cells toward a regulatory phenotype in renal cell carcinoma

Barbora Vavrušáková

Masaryk Memorial Cancer Institute

Lenka Krejčí

Masaryk Memorial Cancer Institute

Lukáš Pečinka

Masaryk Memorial Cancer Institute

Kateřina Vašíčková

Masaryk University

Renata Bartošová

Masaryk Memorial Cancer Institute

Naděžda Vaškovcová

Masaryk University

Kateřina Šumberová

Masaryk Memorial Cancer Institute

Michal Uher

Masaryk Memorial Cancer Institute

Tomáš Kazda

Masaryk Memorial Cancer Institute

Lukáš Moráň

lukas.moran@mou.cz

Masaryk Memorial Cancer Institute

Marek Svoboda

Masaryk Memorial Cancer Institute

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Abstract

Background

Renal cell carcinoma (RCC) is characterized by a paradoxically immunosuppressive tumor-immune microenvironment despite dense immune infiltration, and resistance to immune checkpoint inhibitor (ICI) therapy remains a major clinical challenge. Endoplasmic reticulum stress (ERS) and the unfolded protein response (UPR) are persistently activated in RCC and have been implicated in tumor survival and immune dysfunction, yet whether tumor-derived ERS actively reprograms infiltrating immune cells remains undefined.

Methods

Human RCC cell lines (Caki-2 and TK-10) were cultured alone or co-cultured with peripheral blood mononuclear cells (PBMCs) and exposed to the ERS inducer tunicamycin (Tun) and the chemical chaperone tauroursodeoxycholic acid (TUDCA). Cell viability, migration, UPR, and immune marker expression were assessed by MTT assay, scratch wound healing, RT-qPCR, western blot, and 2D/3D immunofluorescence. Ultrastructural analysis was performed by scanning and transmission electron microscopy. To test cell-non-autonomous ERS transmission, naïve PBMCs were exposed to conditioned media (CM) collected from drug-pretreated RCC cells following complete media replacement to eliminate residual compounds; CM from unstressed RCC cells served as control.

Results

Tun induced robust UPR activation — confirmed across qPCR, western blot, and immunofluorescence by *HSPA5*/BiP and *DDIT3*/CHOP induction — in both RCC lines and in directly treated PBMCs. At the sublethal dose used, PBMC viability was preserved, yet Tun drove coordinated downregulation of CD3, CD4, CD8, PD-1, PD-L1, and FoxP3 in co-culture, indicating non-lethal immune reprogramming rather than cytotoxicity. In 3D spheroid co-cultures, Tun excluded CD3⁺ T cells from the tumor mass while TUDCA permitted immune infiltration, spatially confirming ERS-dependent immune exclusion. CM from Tun-treated — but not unstressed — RCC cells activated UPR markers (*HSPA5*/BiP and *EIF2AK*/PERK) and induced FoxP3 and CTLA-4 in naïve PBMCs across both donor lines, establishing that ER-stressed tumor cells transmit an immunosuppressive signal via their secretome independently of direct drug exposure. Prior ERS relief in donor cells with TUDCA attenuated this transmissible effect.

Conclusions

Tumor-derived ERS non-lethally remodels the RCC-immune interface and transmits a UPR-activating, FoxP3-associated immunosuppressive program to bystander immune cells via the tumor secretome.

These findings reframe ERS not as a passive metabolic correlate but as an active, transmissible regulator of immune dysfunction in RCC, with direct implications for understanding ICI resistance and for rational combination of ERS-targeting strategies with immunotherapy.

Introduction

Renal cell carcinoma (RCC), particularly its predominant clear cell subtype (ccRCC), remains a therapeutic challenge due to its intrinsic resistance to conventional treatments and its complex, immunologically active tumor immune microenvironment (TIME). The RCC TIME is a dynamic and heterogeneous ecosystem composed of immune cells, cytokines, stromal components, and metabolic and stress-response pathways that collectively shape tumor progression and therapeutic response [1]. Among the diverse immune populations within this niche, cytotoxic CD8⁺ T cells (T_c) play a central role in antitumor immunity through recognition and elimination of malignant cells [2–4]. Despite substantial immune infiltration—one of the highest observed across solid malignancies—RCC frequently exhibits immune dysfunction that undermines effective tumor clearance and limits responses to immunotherapy [5].

A defining feature of the RCC TIME is its combination of dense immune infiltration and pervasive immunosuppression. RCC tumors recruit regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) and secrete immunosuppressive cytokines such as IL-10 and TGF- β , which inhibit effector T cell activity and foster regulatory phenotypes [6–8]. High expression of immune checkpoint molecules, including PD-L1, CTLA-4, and LAG-3, further attenuates T cell cytotoxicity, while hypoxia-driven production of metabolites such as adenosine and kynurenine impairs T cell proliferation and dendritic cell (DC) function. Although ccRCC generally demonstrates a pro-inflammatory immune signature—with elevated infiltration of CD8⁺ T cells, Th1 cells, DCs, and neutrophils—T cell dysfunction and exhaustion emerge as stronger predictors of outcome than the magnitude of immune infiltration alone [9]. Recent studies highlight significant heterogeneity within ccRCC, revealing distinct angiogenic, metabolic and immune infiltration programs that influence clinical behavior and therapeutic responsiveness. Single-cell transcriptomic analyses have further refined our understanding of immune-tumor interactions in RCC. Tumor epithelial cells actively shape immune infiltration, helping explain why RCC responds to immune checkpoint inhibitors (ICIs) despite its low neoantigen burden [9, 10]. In ccRCC, CD8⁺ T cell dysfunction and exhaustion are stronger predictors of outcome than immune infiltration magnitude alone, and specific immunosuppressive programs — including Treg expansion and checkpoint upregulation — emerge as key determinants of ICI resistance [11, 12].

Within this complex immunological landscape, emerging evidence points to **endoplasmic reticulum stress** (ERS) and the **unfolded protein response** (UPR) as critical regulators of RCC biology and its immunosuppressive TIME [13]. ERS occurs when the ER's folding capacity is overwhelmed by metabolic perturbations, hypoxia, oxidative stress, and nutrient fluctuations, leading to the accumulation of misfolded proteins and UPR activation. Beyond its signaling functions, ERS produces characteristic subcellular morphological changes — including dilation of ER cisternae, disruption of ER-mitochondria

contact sites, and altered cristae architecture — detectable by transmission electron microscopy and reflective of the severity and duration of proteostatic disruption [14]. In response, the UPR, mediated by PERK, IRE1 α , and ATF6, acts to restore proteostasis or initiate apoptosis if stress is severe or unresolved [15]. In cancer, however, chronic low-level UPR activation frequently promotes tumor survival, metabolic reprogramming, immune evasion, and resistance to therapy [16]. Although activating mutations in canonical UPR mediators are rare in RCC [16], ccRCC exhibits persistent activation of UPR pathways, which supports tumor growth under hypoxic and nutrient-poor conditions, enables adaptation to oxidative stress, promotes autophagy, and inhibits apoptosis [17–24]. Mild but sustained ERS in RCC has been implicated in resistance to therapy. Treatment-induced ERS can upregulate BiP and activate PERK/eIF2 α and IRE1 α signaling, enhancing proliferation and conferring resistance to apoptosis [25]. Sunitinib, a multitarget tyrosine kinase inhibitor (TKI) used for the treatment of RCC, was described to activate the IRE1 α and the PERK branches of the UPR *in vitro* in 786-O RCC cells, therefore increasing the expression of NF- κ B [26]. These pathways also induce pro-inflammatory cytokines (IL-6, IL-8, TNF α), linking ERS to tumor-driven inflammation [26]. BiP, a major ER chaperone and UPR mediator, is upregulated in RCC tissues and serum and correlates with tumor stage, indicating its potential as a prognostic biomarker [27]. Beyond its canonical ER role, BiP can localize to the cell surface or nucleus, where it promotes PI3K/AKT signaling and inhibits DNA damage-induced apoptosis, further enhancing tumor survival [28].

ERS also exerts profound effects on immune cell function within the TIME [15]. In immune cells, ERS can induce dysfunction, exhaustion, or apoptosis, thereby weakening antitumor responses [29]. Conversely, tumor-cell ERS can influence immune surveillance by modulating cytokine secretion, antigen presentation, and immunogenic cell death (ICD). ERS-mediated exposure of calreticulin [30], release of HMGB1 and ATP, and activation of DCs and CD8 $^{+}$ T cells are key hallmarks of ICD, yet tumor-associated chronic ERS can subvert these processes [31]. Moreover, ERS propagates from tumor cells to infiltrating immune cells, activating their UPR pathways and impairing effector functions. Recent findings demonstrate that specific ERS-driven tumor–immune interactions—such as altered DC recruitment, suppressed IL-1 β production, disrupted chemokine signaling, and enhanced T cell exhaustion—may critically limit ICI efficacy in RCC.

While immunotherapies such as ICIs have transformed RCC management, only a subset of patients derive durable benefit. Understanding how ERS shapes immune cell dysfunction and modulates responsiveness to immunotherapy is therefore essential for developing more effective, rationally designed therapeutic strategies. Targeting ERS–UPR pathways, either alone or in combination with ICIs, TKIs, or metabolic inhibitors, represents a promising frontier for overcoming immune resistance and improving clinical outcomes in RCC.

Whether pharmacological modulation of ERS can reshape the reciprocal interaction between RCC cells and infiltrating immune cells remains poorly defined, and most evidence to date is correlative or transcriptomic rather than functional. To address this, we established an *in vitro* co-culture model of human RCC cell lines (Caki-2 and TK-10) with PBMCs and bidirectionally perturbed ERS —inducing it with

tunicamycin (Tun) and mitigating it with the chemical chaperone tauroursodeoxycholic acid (TUDCA). This induce-and-rescue design allowed us to test whether immune and tumor phenotypes track causally with ERS rather than with off-target drug effects. We assessed tumor and immune cell viability, profiled the expression of UPR and immune-related genes, and validated key targets at the protein level by western blotting and 2D/3D immunofluorescence (IF). To determine whether ER-stressed RCC cells transmit UPR-activating signals to immune cells independently of direct drug exposure, naïve PBMCs were exposed to conditioned media (CM) collected from drug-pretreated RCC cells following complete media replacement to eliminate residual compounds. To resolve the morphological and subcellular correlates of ERS within the tumor–immune interface, we further examined co-cultures by scanning (SEM) and transmission electron microscopy (TEM). We show that ERS induction reprograms the RCC–immune interface and alters PBMC effector marker expression, identifying ERS as an important node governing the RCC–immune interaction.

Materials & Methods

In vitro cell culture

Human RC cells Caki-2 and TK-10 were obtained from the American Type Culture Collection (ATTC). Prior to use, the cell lines were authenticated and the cell identity was confirmed (STR loci amplification PowerPlex® 16 HS System, Promega; fragment analysis was done on an ABI3730xl, Life Technologies; data were analyzed with GeneMarker HID Software, Softgenetics). Caki-2 cells were maintained in Dulbecco's Modified Eagle Medium High Glucose (Biosera - cat. no. LM-D1111/500) supplemented with 10% FBS (Gibco - cat. no. A1048501), 1% L-glutamine (Biosera - cat. no. XC-T1715/100), and 1% Penicillin-Streptomycin (Biosera - cat. no. XC-A4122/100) at 37°C and 5% CO₂. TK-10 cells were maintained in RPMI-1640 culture medium (Biosera – cat. no. LM-R1639/500) supplemented with 10% FBS, 1% L-glutamine, and 1% Penicillin-Streptomycin at 37°C and 5% CO₂.

Peripheral blood mononuclear cells (PBMCs) were isolated from a healthy donor's buffy coat (Transfusion and Tissue Department University Hospital Brno) using Histopaque-1077 (Merck - cat. no. 10771) according to the manufacturer's manual. Isolated PBMCs were resuspended in complete OpTmizer media (OpTmizer culture medium; Gibco - cat. no. A1048501) supplemented with 10% FBS, 1% L-glutamine, 1% Penicillin-Streptomycin, and 2 ng/mL IL-2 (Merck - cat. no. SRP3085-50UG) at concentration 5×10^6 cells/mL at 37°C in a humidified atmosphere with 5% CO₂. All donor buffy coats underwent standard quality control and biochemical screening mandatory for all blood donations at the university hospital provider to ensure physiological baseline stability. Detailed table of donor-derived buffy coats is included in *Supplementary Information (Suppl. Table 2)*.

Co-culture experiments

For *co-culture experiments* human RC cells Caki-2 and TK-10 cells were seeded and cultured for 24h until fully adhered. Activated PBMCs (4 µg/mL Phytohemagglutinin (Merck - cat. no. L2646); 24h) were added

to cancer cell culture in ratio 2:1. Cell co-cultures were treated with ERS modulators: tunicamycin (Tun; 0.5µM working concentration; Merck – cat. no. T7765), tauroursodeoxycholic acid (TUDCA; 1mM working concentration; Merck - cat. no. 580549-5GM), or their combination (T + T). Treatments were maintained in co-cultures for 24h (RT-qPCR), 48h (SDS-PAGE and WB, Flow cytometry, IF staining, SEM, and TEM), or 72h (3D spheroids).

ERS transmission experiments

For *ERS transmission experiments* human RC cells Caki-2 and TK-10 were seeded and cultured for 24h until fully adhered. Cells were treated with ERS modulators TUDCA [1mM] or Tun [0.5µM]. Treatments were maintained in co-cultures for 24h followed with 3× wash with PBS and fresh media replacement. Conditioned media (CM) was collected from Caki-2 and TK-10 cells. CM was filtered using 0.22 µm PES Membrane Filter (Millipore – cat. no. GPWP14250) to remove cellular components and mixed with complete OpTmizer medium at a 1:1 ratio and added to PBMC cultures for 24h (RT-qPCR) or 48h (SDS-PAGE and WB).

Viability assay (MTT)

Cell metabolic activity was assessed using colorimetric MTT assay. Cancer cells were seeded at 1500 cells/well in technical triplicates in 96-well plates and cultured for 24h until fully adhered. Subsequently, activated PBMC were added in a final volume of 200 µL. Cell co-cultures were treated as listed in the *Co-culture experiments* section. Viability/metabolic activity assessment was performed using MTT Reagent 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (Merck - cat. no. M2128-1G) at concentration of 0.5 mg/mL for 3h. The formazan was then solubilized using DMSO (Serva - cat. no. 2038502) and its concentration was determined by absorbance measurement at 595 nm using a microplate reader Infinite M1000 PRO (Tecan – cat. no. 30064852).

RT-qPCR

Total RNA was isolated using phenol-chloroform isolation (RNA blue, TopBio - cat. no. R013; 1-Bromo-3-Chloropropane, Merck - cat. no. B9673; isopropanol, 75% ethanol, PCR H₂O). RNA samples were transcribed into cDNA using the High Capacity cDNA Reverse transcription Kit (Thermo Fisher Scientific - cat. no. 4368814). The amplification reactions were carried out in a final volume of 10 µL in a reaction mixture containing Luna Universal qPCR Master Mix (New England Biolabs - cat. no. M3003X), primers, H₂O, and cDNA. All reactions were performed in technical triplicates and relative gene expression was quantified using the comparative threshold cycle $2^{-\Delta\Delta CT}$ method with *ACTB* as a housekeeping gene. The amplifications were performed on LightCycler Instrument QuantStudio 5 (Applied Biosystems, USA). All primers were synthesized by Generi Biotech.

SDS-PAGE and Western blotting

For western blot analysis, cell lysates were harvested into 100 µL of NET buffer (150M NaCl, 1% NP-40, 50 mM Tris pH 8.0, 50 mM NaF, 5 mM EDTA pH 8.0, in 100 mL H₂O). Following protein quantification using Bradford Assay (Bio-Rad - cat. no. 5000006), lysates were stabilized in CSB medium (glycerol, 1 M Tris pH 6.8, bromphenol blue, 1 M Tris pH 6.8, SDS, β-merkaptoethanol) and incubated at 95°C for 10 min. 10 µg of proteins were loaded to 10% polyacrylamide gel, and resolved by SDS-PAGE, following electrotransfer onto PVDF membrane Immobilon-P (Merck - cat. no. IPVH00010). Membranes were blocked with 5% BSA (Serva – cat. no. 11930.04) and incubated with primary antibodies (Ab) overnight (*Suppl. Table 1*). Protein containing membranes were washed with 0.1% Tween-20 solution, incubated with HRP-conjugated secondary Ab (*Suppl. Table 1*), washed, and ECL reagent was applied to enhance signal visualization. Signal was detected using G:BOX (Syngene). β-Actin was used as a loading control.

Scratch wound healing assay

Cell migration was assessed using a scratch wound assay. Cancer cells were seeded in an IncuCyte Imagemock 96-Well Microplate (Sartorius - cat. no. BA-04857) (Caki-2: 50,000 cells/well; TK-10: 60,000 cells/well) in technical hexaplicates and allowed to grow to 90% confluence. PBMCs were stained with CellTracker Deep Red Dye (ThermoFisher Scientific - cat. no. C34565) according to the manufacturer's instructions prior to adding them to the cancer cell culture.

A scratch was performed using the IncuCyte 96-Well Woundmaker Tool (Sartorius - cat. no. BA-04858). After scratching, the wells were washed with PBS, the cells were treated as listed in the *Co-culture experiments*, and pre-stained PBMCs were added. Cell co-cultures were placed in a live-cell imaging system IncuCyte (Sartorius, Germany) and captured using 4 × magnification once an hour for 24h.

Immunofluorescence staining of in vitro co-cultures

For immunofluorescence staining, cells were seeded in an 8 Well Chamber removable (ibidi - cat. no. 80841) and treated as listed in the *Co-culture experiments* section. Following the experiment, the cells were fixed with 4% paraformaldehyde (PFA; Roth – cat. no. R67334). Triton 0.1% solution was used to permeabilize cells, and 3% BSA solution for saturating excess protein-binding sites. Cells were stained with primary and secondary Abs (*Suppl. Table 1*). Cell nuclei were counterstained with DAPI (4',6-Diamidino-2-phenylindole dihydrochloride; Merck- cat. no. MBD0015). The stained cells were mounted using Roti-Mount FluorCare (Carl Roth - cat. no. R.HP19.1). Images were acquired using an Eclipse Ti-E microscope (Nikon) at 20× magnification. Images containing separate channels for individual signals were exported as 16-bit .jpeg files and processed for image analysis.

Image Analysis

Data were analyzed using images containing separate channels for individual signals. To identify specific signals, noise was removed from each channel by applying a Gaussian filter, and the image was segmented into useful and background signals using the Otsu method for automatic thresholding. Two main attributes were used to characterize the images **(1) Occurrence of the observed signal**: This represents the proportion of pixel counts with the detected signal relative to all signals within the same frame. Additionally, the occurrence of a specific signal in a particular environment (e.g., immune cells) was calculated as the proportion of pixel counts limited to the area where that specific signal was detected. **(2) Relative intensity of the observed signal**: This refers to the ratio of the average intensity values of the signal under evaluation to the intensity of the reference signal (e.g., cell nuclei). From each sample, 20 images were acquired and combined into a single composite image object, which was then analyzed using standard methods. The Mann–Whitney test and Fisher’s exact test were used to assess the statistical significance of differences between groups. All data processing and statistical analyses were conducted using R statistical software version 4.3.1 (R Core Team, 2023). Specifically, functions from the *EImage* library [32] were utilized for image data analysis and the calculation of derived attributes.

Cancer-Immune 3D spheroid co-culture

Spheroid formation was induced by single cell suspension culture in the Nunclon™ Sphera™ 96-Well, Nunclon Sphera-Treated, U-Shaped-Bottom Microplate (Thermo Scientific – cat. no. 174925) using 10,000 cells/well. Spheroid formation was allowed for 48h prior to adding PBMCs and treatments (see *Co-culture experiments*). Spheroids were collected by gentle centrifugation, washed with PBS and fixed using 4% PFA. Tissue clearing was performed using CUBIC prior to immunofluorescence staining to reduce light scattering and improve optical transparency. For IF staining, spheroids were washed with 3% BSA and 0.1% Triton X-100 and incubated with primary Abs (*Suppl. Table 1*), at 4°C overnight. IF signals were obtained by staining with conjugated secondary Abs (*Suppl. Table 1*). Spheroid images were acquired using ZEISS Lightsheet 7 (Carl Zeiss AG, Germany) with a 10× magnification. Data processing was performed in ZEN Blue and IMARIS software.

Scanning electron microscopy (SEM)

Cells (see *Co-culture experiments*) were harvested and fixed in 3% glutaraldehyde (Sigma – cat. no. 108790) with 1% tannin (Sigma – cat. no. W304204) in 0.1 M cacodylate buffer (Sigma – cat. no 97068). After washing with 0.1 M cacodylate buffer, the samples were dehydrated by increasing ethanol concentrations (30%, 50%, 70%, 80%, 90%, 96%, and absolute ethanol). The samples were dried in a Critical Point Dryer (Bal-Tec CPD 030, LEICA, Wetzlar, Germany). After that, samples were fixed on aluminum pin stubs (Micro to Nano) and sputtered with gold in SCD 040 sputter coater (BALZERS UNION). The samples were examined using SEM (Vega, Tescan Orsay Holding, Brno). SEM micrographs were obtained using a secondary electron detector at a voltage of 15 kV using 5000 × magnification.

Transmission electron microscopy (TEM)

Cells were harvested and fixed in 3% glutaraldehyde with 1% tannin in 0.1 M cacodylate buffer and post-fixed in 1% OsO₄ (Sigma – cat. no. 201030) in 0.1 M cacodylate buffer. Cell suspensions were dehydrated in increasing concentrations of ethanol (50%, 70%, 96%, and 100%), treated with 100% acetone, and embedded in Durcupan (Sigma – cat. no. 44610) resin. Ultrathin sections were prepared using a Leica EM UC6 ultramicrotome, stained with uranyl acetate and Reynold's lead citrate (Penta Chemicals), and examined using a Morgagni 268D TEM (ThermoFisher Scientific). Images were captured using a Veleta CCD Camera (Olympus, Prague, Czech Republic). One hundred cells from each experimental group were examined in two independent experiments.

Statistical Analysis

The experimental data were statistically processed using R statistical software version 4.3.1 (R Core Team, 2023) (<https://www.r-project.org>). Significance threshold was set as follow: (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). P-values are reported both with (☒) and without (Δ) False Discovery Rate (FDR) correction due to the exploratory nature of the study, the limited sample size, and to allow balanced interpretation of statistical and biological relevance. All experiments were conducted in at least three biological replicates.

Cell viability assay, Scratch wound healing assay, and RT-qPCR

Prior to statistical analyses, outliers were detected using a robust z-score method (threshold $|z| > 3.5$) and excluded from further comparisons. Pairwise comparisons between groups were performed using Dunn's test. Raw p-values were subsequently adjusted for multiple comparisons using the FDR method (Benjamini–Hochberg procedure).

Results

Cellular viability

In Caki-2 cultures, the addition of activated immune cells shifted the growth kinetics downward in all conditions compared to the monocultures (Fig. 1A-B). Tun did not significantly affect Caki-2 viability at either timepoint, confirming sublethal dosing in this line.

In TK-10 cultures, the experimental groups clearly segregated into two distinct clusters based solely on the presence of Tun treatment (Fig. 1C-D). The non-Tun groups exhibited a continuous, uniform increase in normalized log absorbance up to 96h. Tun significantly reduced viability at both 72h (Fig. 1C) and 96h (Fig. 1D), with co-culture with PBMCs further modifying this response.

Scratch wound healing

To determine whether and how ERS modulation affects cellular motility of Caki-2 and TK-10 cells, we performed scratch wound healing assay. Wound closure of Caki-2 cells showed no significant difference among treatment groups 12h after scratch (Fig. 1E). Wound closure of TK-10 cells 12h after scratch

showed reduced cellular motility in the Tun- and T + T-treated monoculture compared to TUDCA-treated condition ($p < 0.05$; Fig. 1F-G). The effect is modest in magnitude but directionally consistent – ERS induction impairs TK-10 motility. Interestingly, no statistically significant difference was observed among treatment groups in co-culture.

ERS modulation drives gene and protein expression changes

To link our initial observations with underlying molecular mechanisms, we evaluated alterations in ERS and immune marker expression to elucidate cellular responses to ERS modulation. We observed slight differences between the two RCC cell lines, reflecting their distinct molecular characteristics (Fig. 1H-I).

In TK-10 cells, Tun robustly induced *HSPA5* and *DDIT3* in both monoculture and co-culture conditions ($p < 0.01-0.05$), with T + T producing a partial attenuation of the Tun-induced signal (Fig. 1H; *Suppl. Figure 1A*). *ATF6* and *ERN1* showed modest, variably significant elevations under Tun and co-culture conditions (Δ). Among immune transcripts, *CD8* was significantly elevated in PBMC co-culture under T + T treatment (Δ), while *CD3*, *CD4*, *IL2RA*, and *ENTPD1* did not reach significance across conditions.

In Caki-2 cells, *HSPA5* and *DDIT3* were significantly induced by Tun in monoculture (Δ), with attenuation observed under T + T and in co-culture conditions (Fig. 1I; *Suppl. Figure 1B*). *EIF2AK*, *ATF6*, and *ERN1* showed high variability and no consistent significant differences. Immune transcripts *CD3*, *CD4*, *IL2RA*, and *ENTPD1* were reduced under Tun and T + T co-culture conditions (Δ), while *CD8* showed a trend toward elevation under co-culture that did not reach corrected significance.

In PBMCs, Tun significantly induced *HSPA5* ($p < 0.001-0.01$), *DDIT3* ($p < 0.001-0.01$), *EIF2AK* ($p < 0.05$), and *ERN1* ($p < 0.01$) relative to untreated control, confirming UPR activation in the immune compartment under ERS modulation (Fig. 1J; *Suppl. Figure 1C*). *ATF6* showed a non-significant trend toward elevation. Among immune transcripts, *CD4* was significantly elevated under Tun treatment ($p < 0.01-0.05$), while *CD3*, *CD8*, *IL2RA*, and *ENTPD1* did not differ significantly across conditions. PBMCs from different donors were used in each biological replicate to reflect the variability of the population (*Suppl. Table 2*). Consequently, the transcriptional levels of immune-related genes varied depending on the PBMC donor.

To investigate the molecular changes, protein expression profiles of key ERS sensors, downstream UPR targets, and immune-related markers were evaluated by Western blot. Analysis of the ERS sensors and downstream effectors revealed a coordinated upregulation across both lines upon ERS induction. Exposure to Tun triggered a prominent induction of BiP, CHOP, and XBP1s in both RCC lines, and IRE1 α in Caki-2 cells (Fig. 1K). Combination with TUDCA did not attenuate the activation of these canonical UPR branches. In PBMC monoculture, only BiP and CHOP were upregulated upon Tun treatment. This effect on BiP was attenuated with TUDCA.

Within the co-culture systems, robust bands for the core T-lymphocyte markers *CD3*, *CD4*, and *CD8* were observed under baseline conditions. Following Tun exposure, a downregulation in the signal intensity of

CD3, CD4, PD-1, PD-L1 and FoxP3 was detected in both the TK-10 and Caki-2 co-cultures, more visible Caki-2 lysates. The PBMC monocultures showed no alteration in immune markers upon treatment across biological replicates.

TUSC3, a subunit of the oligosaccharyltransferase complex responsible for N-linked glycosylation, was assessed as a marker of glycosylation machinery integrity. Its expression remained stable across all treatment conditions and cell types, confirming that Tun inhibitory effect operates at the substrate level rather than through transcriptional suppression of the transferase complex.

Tumor-derived ERS transmission to immune cells

To determine whether ER-stressed RCC cells transmit UPR-activating signals to immune cells independently of direct drug exposure, naïve PBMCs were treated with CM collected from Tun- or TUDCA-pretreated Caki-2 and TK-10 cells following media replacement to eliminate residual drug. CM from unstressed RCC cells served as control (Fig. 2).

UPR activation in Caki-2 and TK-10 cells was determined on gene expression level via RT-qPCR and protein level via Western blot. In Caki-2, Tun significantly induced *HSPA5* ($p < 0.05$) and *ATF6* ($p < 0.05$) relative to untreated control, confirming pharmacological ERS activation in Caki-2 donor cells prior to CM collection (Fig. 2A, *Suppl. Figure 2A*). *ERN1*, *EIF2AK*, and *DDIT3* showed trends toward elevation that did not reach corrected significance, consistent with the modest ERS sensitivity of Caki-2 relative to TK-10.

CM from Tun-treated Caki-2 cells (CM-Tun) significantly induced *HSPA5* ($p < 0.05$) and *EIF2AK* ($p < 0.05$) in naïve PBMCs relative to untreated control, with *ATF6* showing a trend toward elevation (Δ ; Fig. 2B, *Suppl. Figure 2B*). These findings confirm transmission of a UPR-activating signal via the Caki-2 secretome independently of direct drug exposure. Among immune transcripts, *FOXP3* was significantly elevated in PBMCs receiving CM-control relative to control ($p < 0.05$), suggesting baseline tumor-secretome-driven immunosuppressive priming. *IFNG* was significantly elevated under CM-Tun ($p < 0.05$). *IL2* showed the largest fold-change elevation under CM-control and CM-Tun conditions, with CM-control reaching significance versus control ($p < 0.05$). *CD274* and *CTLA4* did not reach significance across CM conditions.

In TK-10 cells, Tun robustly induced all five UPR transcripts: *HSPA5* ($p < 0.05$), *ERN1* ($p < 0.001$), *EIF2AK* ($p < 0.05$), *ATF6* ($p < 0.05$), and *DDIT3* ($p < 0.001$), confirming strong ERS activation in TK-10 donor cells (Fig. 2C, *Suppl. Figure 2C*). The magnitude of induction was substantially higher than in Caki-2, consistent with greater Tun sensitivity in this line (Fig. 1C–D).

CM from Tun-treated TK-10 cells significantly induced *HSPA5* ($p < 0.05$), *EIF2AK* ($p < 0.05$), and *ATF6* ($p < 0.05$) in naïve PBMCs, replicating the transmissible UPR signal observed with Caki-2-derived CM (Fig. 2D, *Suppl. Figure 2D*). *DDIT3* showed a trend toward elevation (Δ). Among immune transcripts, *IFNG* showed a trend toward elevation under CM-TUDCA (Δ). *IL2*, *CD274*, *CTLA4*, and *FOXP3* did not reach significance across TK-10-derived CM conditions, representing a divergence from the Caki-2-derived CM pattern that warrants further investigation.

The magnitude of PBMC UPR induction was comparable between Caki-2- and TK-10-derived CM despite the larger fold-change in TK-10 donor cells, suggesting a saturable or buffered transmission mechanism.

Western blot data further confirmed ERS induction in Caki-2 (Fig. 2E). BiP and CHOP were strongly induced by Tun. XBP1s is detectable under Tun. PBMC protein response to Caki-2-derived CM showed BiP and PERK detectable across CM conditions. PD-L1 and PD-1 were present in CM-exposed PBMCs. FoxP3 was detectable across CM conditions with apparent elevation under CM from Tun-treated cells. Similarly, CTLA-4 showed strongest signal under under CM from Tun-treated condition, consistent with induction of an immunosuppressive/exhaustion-associated phenotype.

In TK-10 cells, BiP was strongly induced by Tun, CHOP showed a distinct band under Tun treatment, and XBP1s was detectable (Fig. 2F). In response to TK-10-derived CM, PBMCs showed elevated BiP and PERK under CM conditions. FoxP3 was detectable across CM-exposed conditions. CTLA-4 signal was present under CM from Tun-treated condition, replicating the pattern observed in Fig. 2E. PD-L1 and PD-1 were detectable in CM-exposed PBMCs.

Immunofluorescence analysis of ERS markers in RCC

Further, we performed immunofluorescence quantification of BiP and CHOP in mono- and co-cultures to confirm ERS induction at the single-cell spatial level in a compartment-dependent manner (Fig. 3).

In Caki-2 monoculture, Tun induced a highly significant increase in both BiP and CHOP total signal intensity compared to untreated controls ($p < 0.001$; Fig. 3B-C). In co-culture conditions, BiP signal was broadly attenuated relative to monoculture across all treatment groups, whereas CHOP remained significantly elevated under Tun and T + T treatment ($p < 0.001$). Co-culture with PBMCs significantly increased BiP relative intensity under control, Tun, and T + T conditions compared to monoculture ($p < 0.05$), while CHOP relative intensity was reduced in co-culture versus monoculture across the same conditions ($p < 0.05$).

In TK-10 monocultures, Tun robustly elevated both BiP and CHOP total signal relative to baseline ($p < 0.01$), with effects further amplified in co-culture ($p < 0.001$; Fig. 3D-E). TUDCA co-treatment attenuated the Tun-induced BiP rise in monoculture, whereas in co-culture, TUDCA alone was sufficient to upregulate both markers above control levels ($p < 0.01$). Tun and T + T significantly elevated relative signal intensity of BiP and CHOP in monoculture ($p < 0.001$) and by TUDCA, Tun, and T + T in co-culture ($p < 0.001-0.01$). As in Caki-2, co-culture with PBMCs was associated with a modest but significant decrease in CHOP relative intensity compared to monoculture ($p < 0.05$).

Immunofluorescence analysis of cancer-immune spheroids

To recapitulate features of the solid tumor microenvironment – including secretion of soluble mediators, uneven nutrient distribution, and immune infiltration – Caki-2 cells were co-cultured with PBMCs in a 3D spheroid model and analyzed by IF (BiP, CHOP, and CD3) and 3D microscopy (Fig. 4). Under control

conditions, both BiP and CHOP signal were detected throughout the spheroid. TUDCA treatment restricted BiP signal to cells deep within the spheroid structure, whereas Tun elevated BiP at the spheroid surface — a spatial pattern consistent with stress propagating inward under pharmacological ERS induction.

ERS modulation profoundly affected immune infiltration, assessed by CD3⁺ T-lymphocyte staining. TUDCA-treated spheroids exhibited the highest CD3⁺ infiltration, with CD3 signal notably colocalizing with elevated CHOP signal. However, whether this reflects ERS in tumor cells within immune-infiltrated regions or stress responses within the infiltrating T-lymphocytes themselves remains to be determined. By contrast, Tun-treated spheroids showed elevated surface BiP but markedly reduced immune cell infiltration, indicating that pharmacological ERS induction suppresses rather than promotes T cell entry into the tumor mass.

Changes in cellular morphology

To evaluate cell surface architecture and topological changes induced by ERS modulation, scanning electron microscopy (SEM) was performed on Caki-2 (Fig. 5A), TK-10 (Fig. 5B), and PBMC (Fig. 5C) cells, cultured as monocultures or in co-culture with PBMCs.

In Caki-2 monocultures, control cells formed a characteristic flattened, epithelial-like monolayer with extensive surface attachment and continuous, intact plasma membranes. TUDCA alone did not alter this phenotype, with cells maintaining cellular spreading, membrane integrity, and intercellular contacts comparable to untreated controls. Tun exposure triggered profound morphological remodeling, characterized by loss of the flattened epithelial architecture, prominent membrane blebbing, the formation of multicellular aggregates, and the accumulation of cellular debris — changes consistent with ERS-induced cytoskeletal disruption at the population level despite preserved bulk viability at this dose (Fig. 1A, B). T + T co-treatment produced partial morphological rescue; although cell spreading remained restricted relative to controls, membrane disruption and the frequency of rounded, detached cells were substantially reduced compared to Tun-treated cultures. In co-cultures, control conditions showed well-spread adherent Caki-2 cells with scattered PBMCs attached to their surfaces. TUDCA alone did not alter cell topology or tumor–PBMC interactions. Severely disrupted Caki-2 surface architecture, producing loss of adhesion, membrane collapse, cellular contraction, and rounded cell cluster formation, with numerous PBMCs remaining clustered around damaged tumor cells. T + T co-treatment partially attenuated these effects, with improved surface adherence and reduced membrane disruption relative to Tun alone.

In TK-10 monocultures, control cells displayed a well-spread, spindle-shaped morphology with extensive surface anchoring and numerous thin cytoplasmic protrusions. TUDCA alone induced no discernible changes. Tun caused severe architectural disruption, marked by loss of surface attachment, cell rounding, extensive membrane blebbing, and formation of compact spherical aggregates surrounded by fragmented cellular material. T + T co-treatment partially preserved the adherent phenotype; although some rounded cells and membrane irregularities persisted, detached aggregate frequency was reduced

and a larger proportion of cells retained their elongated morphology. In TK-10 co-cultures, control conditions mirrored the monoculture phenotype, with adherent tumor cells and occasional PBMCs attached to their surface. TUDCA alone had no impact on cell morphology or immune-tumor interactions. Tun induced widespread surface remodeling, including tumor cell contraction, partial detachment, and membrane blebbing. In T + T-treated co-cultures, PBMCs remained closely associated with tumor cells while retaining their structurally intact, spherical morphology. This phenotypic preservation of immune cells was accompanied by a marked reduction in surrounding cellular debris compared to Tun-alone conditions, underscoring the protective effect of TUDCA on bystander immune cells within the stressed microenvironment.

Ultrastructural changes

To resolve the intracellular correlates of ERS-induced morphological remodeling, transmission electron microscopy (TEM) was performed on TK-10 cells in mono- and co-culture (Fig. 5D). Under control conditions, cells exhibited a well-preserved ultrastructure with elongated profiles, intact nuclei with evenly distributed chromatin, and an organized cytoplasm containing elongated mitochondria with distinct cristae. TUDCA alone induced no detectable ultrastructural abnormalities. Tun treatment induced severe intracellular lesions. Cells displayed irregular contours accompanied by extensive cytoplasmic vacuolization, marked dilation of intracellular membranous compartments (consistent with ER expansion), and altered mitochondrial profiles, including swelling and cristae disruption. Localized cytoplasmic rarefaction and general loss of organelle organization were frequently observed, contributing to marked cellular heterogeneity. T + T co-treatment was associated with clear preservation of ultrastructural integrity relative to the Tun-treated group, the cytoplasm remained more compact, mitochondrial architecture was better preserved, and extensive vacuolization occurred less frequently, though minor intracellular alterations remained detectable.

In co-cultures, TEM confirmed the preserved ultrastructure of control TK-10 cells in close proximity to PBMCs, which maintained intact nuclei, continuous plasma membranes, and well-organized organelles. TUDCA alone did not alter these baseline ultrastructural interactions. Tun exposure produced severe intracellular disorganization within the tumor cells – marked by vacuolization, pronounced ER dilation, and mitochondrial damage – frequently observed adjacent to attached PBMCs and cellular debris field. Strikingly, ultrastructural analysis by TEM confirmed that TUDCA exerted a potent cytoprotective effect on immune cells; PBMCs within the T + T-treated group retained ultrastructural integrity, featuring well-defined nuclear envelopes, continuous plasma membranes, and intact cytoplasmic organelles. This stood in a contrast to the Tun- treated co-cultures, where extensive tumor cell lysis and the resulting cytotoxic debris field correlated with ultrastructural deterioration of the adjacent PBMCs. These findings suggest that TUDCA-mediated ERS mitigation is associated with preserved ultrastructural integrity of neighboring immune cells, consistent with a protective effect on bystander immune cells within the stressed TIME.

Discussion

RCC remains a therapeutic challenge due to its intrinsic resistance to conventional treatment and its complex, immunologically active microenvironment, comprising immune cells, cytokines, stromal components, and stress-response pathways that shape tumor progression and therapeutic response [1].

The present study demonstrates that pharmacological ERS modulation non-lethally remodels the tumor–immune protein landscape in RCC co-cultures and that a UPR-activating, immunosuppressive signal is transmissible from ER-stressed tumor cells to naïve immune cells via the secretome – independently of direct drug exposure. Taken together, these findings position tumor-derived ERS as an active, modifiable regulator of immune cell phenotype in RCC rather than a passive correlate of metabolic dysfunction.

ERS induction is robust and cross-validated, but cell-line specific

Tun consistently induced *HSPA5*/BiP and *DDIT3*/CHOP across qPCR, western blot, and IF in both Caki-2 and TK-10 cells, providing strong methodological convergence for an *in vitro* study – three independent readouts agreeing on the same targets. However, the two lines are not interchangeable models. Caki-2 tolerated 0.5 μ M Tun without significant viability loss at either timepoint, confirming sublethal ERS induction. TK-10 exhibited significant viability reduction at both 72h and 96h under the same dose, operating under cytotoxic rather than purely proteostatic stress. This difference likely reflects the distinct molecular backgrounds of the two lines – Caki-2 is a metastatic RCC line with high baseline stress tolerance, while TK-10 carries characteristics of papillary RCC with different glycoprotein dependency [33].

TUDCA rescue is selective, not global

TUDCA consistently attenuated CHOP induction but did not reliably reverse BiP elevation. This selectivity is mechanistically coherent: CHOP is the terminal apoptotic effector of the PERK branch and is more pharmacologically accessible to chemical chaperones that reduce misfolded protein load, whereas BiP is a transcriptional target of multiple UPR branches including ATF6 and IRE1 α , whose activation may persist despite partial proteostatic relief. The implication is that the UPR arms are differentially regulable – chemical chaperoning closes the apoptotic gate without fully restoring ER homeostasis. This has direct therapeutic relevance: strategies targeting CHOP-mediated apoptosis may be separable from those targeting the adaptive UPR, and combination approaches may be required for complete ERS modulation in RCC.

ERS non-lethally reprograms the immune compartment in co-culture

The coordinated decline of CD3, CD4, CD8, PD-1, PD-L1, and FoxP3 under Tun in co-culture, in the context of preserved PBMC viability (data not shown) at the sublethal dose used, cannot be attributed to immune cell death and is likely explained by functional reprogramming of the immune compartment. The

convergence of reduced cytotoxic T cell markers with the spatial finding of reduced CD3⁺ infiltration in Tun-treated 3D spheroids strengthens this interpretation – immune exclusion and phenotypic suppression appear to operate in concert under conditions of ERS induction. The mechanism may involve ERS-driven alterations in tumor-cell surface presentation, adhesion molecule expression, or cytokine secretion that collectively reduce immune cell recruitment and activation, though the present data do not resolve which of these operates dominantly.

Tumor-derived ERS transmits an immunosuppressive signal

The co-culture experiments established that ERS modulation reshapes immune phenotype in PBMCs, but could not exclude a direct pharmacological effect of Tun on immune cells independent of tumor-cell signaling. Although the sublethal dose preserved PBMC viability (data not shown), Tun's broad inhibition of N-glycosylation could in principle alter surface receptor stability and protein expression in PBMCs without tumor involvement. The CM experiments address this ambiguity directly. Because residual drug was eliminated prior to conditioning, any effect on naïve PBMCs is attributable to the tumor secretome rather than the inducer itself. The unstressed-CM control further isolates the contribution of the stress state specifically, distinguishing it from constitutive RCC secretory activity.

CM from Tun-treated RCC cells activated UPR markers (*HSPA5*/BiP and *EIF2AK*/PERK) and induced FoxP3 and CTLA-4 in naïve PBMCs that had never contacted the drug, with residual Tun eliminated by media replacement prior to conditioning. The replication of this pattern across two independent donor lines – Caki-2 and TK-10 – with unstressed-CM controls confirming stress-state specificity for UPR transmission establishes that ER-stressed tumor cells secrete factors sufficient to activate the UPR and skew immune phenotype in bystander immune cells. This finding extends prior correlative evidence for transmissible ERS [34] by demonstrating cell-non-autonomous UPR activation in a tumor-immune co-culture model with pharmacological controls. To our knowledge, direct pharmacologically controlled evidence for tumor-to-immune UPR transmission has not been previously demonstrated.

However, FoxP3 elevation in PBMCs receiving CM from unstressed Caki-2 cells indicates that baseline tumor secretome-driven immunosuppressive priming occurs. This indicates that immunosuppression signal is in Caki-2 partially constitutive rather than purely ERS-dependent. The most plausible interpretation is that ERS amplifies an immunosuppressive program already initiated by the constitutive RCC secretome, rather than initiating it *de novo*. This is consistent with the broader literature showing that RCC tumors maintain baseline immunosuppressive tone through multiple overlapping mechanisms, of which ERS is one modulator rather than a singular driver [1, 5].

PD-L1 falls under ERS

PD-L1 was reduced rather than elevated under Tun treatment in both cell lines. This may appear contrary to the assumption that tumor ERS upregulates checkpoint molecules to evade immunity. The most mechanistically coherent explanation is that PD-L1 is an N-glycoprotein whose surface stability and

trafficking depend on intact glycosylation. Tun, as a global N-glycosylation inhibitor, destabilizes and misdirects PD-L1 independently of its transcriptional regulation. The stability of TUSC3 expression across all treatment conditions confirms that N-glycosylation inhibition in this model reflects substrate-level blockade rather than loss of transferase machinery, consistent with Tun's known mechanism of action and supporting a glycoprotein-destabilization rather than translational-suppression explanation for PD-L1 reduction. Consistent with this, deglycosylated PD-L1 is known to undergo accelerated proteasomal degradation [35, 36].

IL-2 elevation in CM-exposed PBMCs

IL-2 elevation in PBMCs exposed to tumor CM may represent a compensatory proliferative or survival response to the incoming stress signal — a known feature of T cell responses to proteotoxic cues — or alternatively reflect activation of a regulatory IL-2 circuit associated with FoxP3⁺ Treg maintenance, since IL-2 is both produced by and required for Treg survival and expansion [37]. Thus, IL-2 and FoxP3 co-elevation in CM-exposed PBMCs may represent coordinated Treg priming.

Spheroid immune infiltration is ERS-dependent and spatially resolved

The 3D spheroid model provided spatial resolution unavailable in 2D co-culture, revealing that TUDCA treatment permitted substantially greater CD3⁺ T cell infiltration into the tumor mass while Tun restricted immune access to the surface. This spatial pattern — immune exclusion under ERS induction, immune permissiveness under ERS relief — is consistent with the 2D co-culture immune marker data and suggests that ERS state governs not only immune cell phenotype but also the physical accessibility of the tumor to immune surveillance. The colocalization of CD3 signal with elevated CHOP in TUDCA-treated spheroids cannot be directionally interpreted from static IF alone: it may reflect ERS in tumor cells within immune-infiltrated regions, UPR activation within the infiltrating T-lymphocytes themselves in the nutrient-poor spheroid core, or cytotoxicity-induced proteotoxic stress in tumor targets. Resolving this would require spatial transcriptomics and represents a clear priority for future work.

Electron microscopy reveals distinct surface and ultrastructural remodeling upon ER stress modulation

SEM and TEM profiling provided direct structural evidence of the cellular remodeling occurring at the RCC-immune interface under ERS modulation. Our ultrastructural analysis captured the severe architectural damage inflicted by unmitigated ERS on Caki-2 and TK-10 cells. Tun exposure triggered a complete loss of the typical flattened, adherent epithelial phenotype, inducing prominent membrane blebbing and the formation of detached multicellular aggregates. At the intracellular level, TEM directly visualized the physical toll of this proteotoxic strain, manifested as massive cytoplasmic vacuolization and dilation of the ER network. Furthermore, the observed mitochondrial swelling and cristae disruption highlight the tight inter-organelle crosstalk between the ER and mitochondria under prolonged stress,

leading to bioenergetic collapse and localized cytoplasmic rarefaction. The most critical insight, however, emerges from the co-culture models and the potent protective capacity of the chemical chaperone TUDCA. While RCC is heavily infiltrated by immune cells, the TIME typically enforces profound immune dysfunction. Our functional data revealed that Tun exposure drives a coordinated, non-lethal downregulation of key immune markers (CD3, CD4, CD8, PD-1, PD-L1, and FoxP3) and excludes T cells from 3D tumor masses. Electron microscopy provided the structural correlation for this phenomenon. In Tun-treated co-cultures, adjacent PBMCs were exposed to a hostile environment filled with tumor-derived cytotoxic debris, resulting in noticeable structural deterioration of the immune cells. Strikingly, mitigating tumor ERS with TUDCA effectively shielded these bystander immune cells. Under T + T co-treatment, TEM and SEM confirmed that PBMCs fully retained their healthy, spherical, microvilli-rich morphology and ultrastructural integrity, featuring intact nuclear envelopes and well-organized cytoplasmic organelles. This visual preservation strongly aligns with our assays, where prior ERS relief with TUDCA attenuated the capacity of RCC CM to transmit an immunosuppressive program to naïve PBMCs.

Taken together, these findings demonstrate that tumor-derived ERS is not merely a passive metabolic byproduct, but an active, transmissible regulator of the RCC immune landscape. By structurally safeguarding both the tumor cell architecture and the adjacent PBMCs population, TUDCA highlights the therapeutic potential of targeting the UPR.

Limitations and future directions

Several limitations bound the conclusions of this study. First, the differential cytotoxicity of Tun across cell lines prevents a fully unified mechanistic interpretation. Second, TUDCA's rescue was selective rather than global, precluding clean bidirectional causal claims. Third, the IFN- γ data derive from a single biological replicate and are hypothesis-generating only. Fourth, the identity of the secreted factor(s) mediating ERS transmission remains unknown — candidates include misfolded protein aggregates, exosome-packaged UPR components, or inflammatory cytokines such as IL-6 and IL-8 previously linked to ERS-driven tumor inflammation [28]. Addressing these limitations through flow cytometry, exosome fractionation, and cytokine profiling in future studies would strengthen the mechanistic framework proposed here.

Conclusion

This study demonstrates that ERS non-lethally remodels the tumor–immune protein landscape in RCC and that this remodeling extends beyond the co-culture niche — ER-stressed tumor cells transmit an UPR-activating, immunosuppressive signal to naïve immune cells via their secretome, independently of direct drug exposure. Pharmacological ERS induction with Tun drove coordinated downregulation of cytotoxic T cell markers in co-culture without compromising PBMC viability, while CM from stressed RCC cells was sufficient to activate UPR signaling and induce FoxP3 expression in naïve PBMCs — an effect replicated across two independent donor lines and attenuated by prior ERS relief with TUDCA. Three-dimensional spheroid modeling provided spatial confirmation: ERS induction excluded CD3⁺ T cells from

the tumor mass, while ERS relief with TUDCA permitted immune infiltration. This spatial rescue was corroborated at the single-cell level by electron microscopy, which revealed that TUDCA completely shielded bystander immune cells from stress-induced degradation. While Tun-mediated tumor disruption created a hostile, cytotoxic debris field, co-treatment with TUDCA visually preserved the healthy surface topology and intracellular organelle ultrastructure of the adjacent PBMCs. Collectively, these findings reframe tumor-derived ERS not as a passive correlate of metabolic dysfunction but as an active, transmissible regulator of immune cell phenotype in RCC.

The differential Tun sensitivity between Caki-2 and TK-10, the selective rather than global rescue by TUDCA, and the partial stress-independence of FoxP3 induction under unstressed CM each define boundaries on the conclusions and identify priorities for future investigation. Specifically, cell-type-resolved profiling of CM-exposed PBMC subpopulations, exosome fractionation to identify the transmissible stress signal, and *in vivo* validation in RCC tumor models are required to translate these findings toward therapeutic application. The convergence of ERS modulation with immune checkpoint biology suggests that ERS pathway targeting may have previously unrecognized consequences. Combining ERS-relieving strategies with immunotherapy represents a rational approach to dismantle the immunosuppressive barriers of the tumor secretome and overcome ICI resistance in RCC.

Abbreviations

Ab Antibody

ATF6 Activation Transcription Factor 6

BiP Binding Immunoglobulin Protein

CD Cluster of Differentiation

CHOP C/EBP Homologous Protein

CM Conditioned Media

CTLA-4 Cytotoxic T lymphocyte-associated protein-4

DCs Dendritic Cells

ERS Endoplasmic Reticulum Stress

FDR False Discovery Rate

FoxP3 Forkhead Box P3

ICI Immune Checkpoint Inhibitor

IFN- γ Interferon Gamma

IRE1 α Inositol-Requiring Enzyme 1 α

MDSCs Myeloid-Derived Suppressor Cells

PBMC Peripheral Blood Mononuclear Cells

PD-1 Programmed Death 1

PD-L1 Programmed Death-Ligand 1

PERK Protein Kinase R-like ER Kinase

SD Standard Deviation

TILs Tumor Infiltrating Lymphocytes

TIME Tumor Immune Microenvironment

TKI Tyrosine Kinase Inhibitor

TL T-Lymphocyte

Tregs Regulatory T cells

TUDCA Tauroursodeoxycholic Acid

Tun Tunicamycin

UPR Unfolded Protein Response

XPB1 X-box Binding Protein 1

Declarations

Ethics Approval

This study was approved by the Ethics Committee of Masaryk Memorial Cancer Institute (2020/1515/MOU). All methods were carried out in accordance with the relevant guidelines and regulations. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy coat samples. Written informed consent was obtained from all patients and/or their legal guardians prior to sample collection.

Consent for Publication

Not applicable.

Declaration of interests

The authors declare no conflict of interest.

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Author Contribution

Conceptualization, LM, BV, KV; methodology, LM, LP, BV, KV, MU, KŠ; validation, LP; investigation, BV, LK, RB, NV; resources, MS, TK, LM; data curation, BV, LK; writing—original draft preparation, BV; writing—review and editing, BV, LM; visualization, BV, LK, MU, KŠ; supervision, MS, TK; project administration, LM; funding acquisition, MS, TK, LM. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The datasets used/and or analyzed during the present study are available from the corresponding author on reasonable request.

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Figures

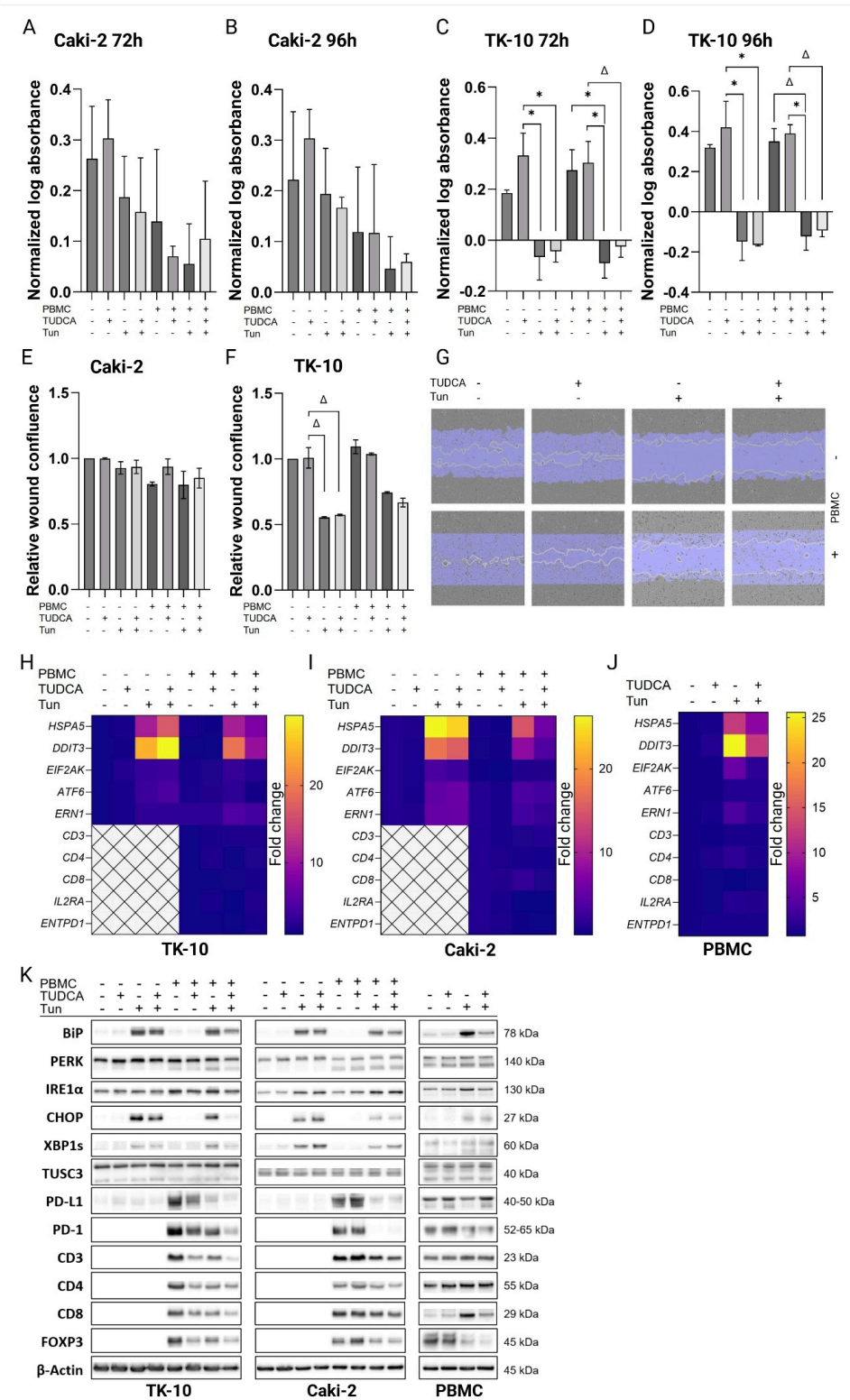


Figure 1

Effects of Tun, TUDCA, and their combination on RCC cells (Caki-2, TK-10) in mono- and co-culture with PBMCs *in vitro*.

(A-D) Cell viability (MTT assay, 72h and 96h). Data represent normalized, background-subtracted mean absorbance $\pm$ SD. **(A)** No significant differences in viability across treatment groups or PBMC co-culture conditions in Caki-2 after 72h. **(B)** Viability trends downward across all conditions by 96h but no significant differences are marked. Sublethal dosing confirmed in Caki-2 at both timepoints. **(C)** TK-10 cellular viability was significantly affected by Tun treatment after 72h. **(D)** TK-10 viability was further suppressed at 96h.

(E-G) Migration (scratch assay, 12h wound closure). **(E)** Wound closure of Caki-2 cells showed no significant differences in wound closure across any condition. ERS modulation does not affect Caki-2 migration at 12h. Notably flat across all groups including co-culture.

(F) Wound closure of TK-10 cells 12h after scratch showed reduced cellular motility in the Tun- and T+T-treated monoculture compared to TUDCA-treated condition ($p < 0.05$). Tun and T+T reduce wound closure compared to TUDCA alone in monoculture ($\Delta p < 0.05$). Co-culture conditions show intermediate values without reaching significance.

(G) Representative image of TK-10 wound closure 12h after scratch. Upper row monoculture, lower row PBMC co-culture. Blue marks original scratch, yellow marks migrated cells. Tun-treated wells visually show less yellow coverage than control or TUDCA, consistent with panel F. The co-culture images appear denser, reflecting PBMC presence in the scratch zone

(H-J) Gene expression (heatmap). Gene expression was assessed by RT-qPCR and expressed as fold change relative to untreated control. **(H)** Tun robustly induced *HSPA5* and *DDIT3* in both TK-10 monoculture and co-culture conditions, with T+T producing a partial attenuation of the Tun-induced signal. *ATF6* and *ERN1* showed modest, variably significant elevations under Tun and co-culture conditions. Among immune transcripts, *CD8* was significantly elevated in PBMC co-culture under T+T treatment, while *CD3*, *CD4*, *IL2RA*, and *ENTPD1* did not reach significance across conditions.

(I) *HSPA5* and *DDIT3* were significantly induced by Tun in Caki-2 monoculture, with attenuation observed under T+T and in co-culture conditions. *EIF2AK*, *ATF6*, and *ERN1* showed high variability and no consistent significant differences. Immune transcripts *CD3*, *CD4*, *IL2RA*, and *ENTPD1* were reduced under Tun and T+T co-culture conditions, while *CD8* showed a trend toward elevation under co-culture that did not reach corrected significance.

(J) In PBMCs, Tun significantly induced *HSPA5*, *DDIT3*, *EIF2AK*, and *ERN1* relative to untreated control, confirming UPR activation in the immune compartment under ERS modulation. *ATF6* showed a non-significant trend toward elevation. Among immune transcripts, *CD4* was significantly elevated under Tun treatment, while *CD3*, *CD8*, *IL2RA*, and *ENTPD1* did not differ significantly across conditions.

(K) Protein expression (Western blot, 48h).

Western blot analysis of UPR and immune markers at 48h in TK-10, Caki-2, and PBMC fractions. Tun induced BiP and CHOP across all three cell contexts. XBP1s showed Tun-dependent induction in tumor

lines. PD-L1 signal was most prominent in co-culture conditions and was reduced under Tun treatment. FoxP3 was detected in PBMC fractions under co-culture conditions. CD3/CD4/CD8 were present in co-cultures, reduced under Tun – consistent with non-lethal immune reprogramming. β -Actin served as loading control.

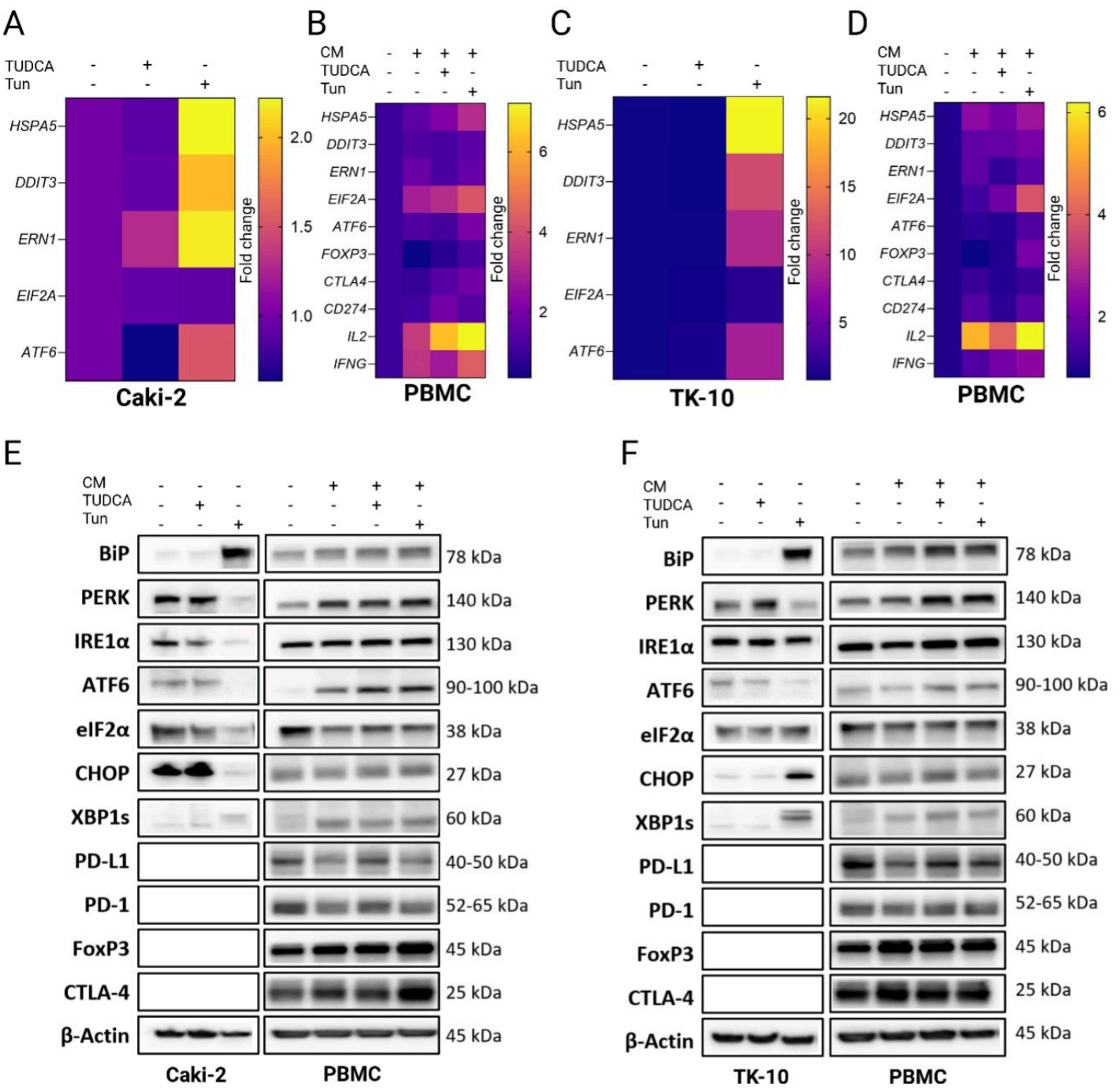


Figure 2

Tumor-derived ERS transmission to immune cells: gene expression and protein analysis. Conditioned media (CM) was collected from Caki-2 or TK-10 cells following Tun or TUDCA treatment and media

replacement to eliminate residual drug. Naïve PBMCs were subsequently exposed to CM for 24h. Gene expression was assessed by RT-qPCR and expressed as fold change relative to untreated control. Protein expression was assessed by western blot at 48h. In all PBMC conditions, cells were exposed to CM only; Tun and TUDCA were not added directly to PBMC cultures. Statistical comparisons were performed using Dunn's test; significance indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$; $\Delta p < 0.05$ without FDR correction.

(A-D) Gene expression (heatmap). Gene expression was assessed by RT-qPCR and expressed as fold change relative to untreated control.

(A) Caki-2 – Tun induced robust upregulation of *HSPA5* and *DDIT3* relative to untreated control, confirming pharmacological ERS induction in the donor tumor line.

(B) CM from Tun-treated Caki-2 cells induced *HSPA5* and *DDIT3* in naïve PBMCs, confirming transmission of a UPR-activating signal via the tumor secretome. *FOXP3* was elevated in PBMCs receiving CM from Tun-treated cells, consistent with induction of an immunosuppressive phenotype. *IL2* showed the highest fold-change signal under Tun-CM conditions. *IFNG* was modestly elevated under CM from TUDCA-treated cells. *CTLA4* and *CD274* showed low but detectable signal across CM conditions.

(C) Tun induced substantially higher fold-change elevations of *HSPA5* and *DDIT3* in TK-10 than in Caki-2 (scale reaching ~20-fold versus ~2-fold), consistent with greater Tun sensitivity. *ERN1*, *EIF2A*, and *ATF6* showed low-level induction. These data confirm robust ERS in TK-10 donor cells prior to media collection.

(D) CM from Tun-treated TK-10 cells induced *HSPA5*, *DDIT3*, and *ERN1* in naïve PBMCs, replicating the transmissible UPR signal observed with Caki-2-derived CM. *FOXP3* was elevated under CM from Tun-treated TK-10 cells. *IL2* showed prominent induction. *IFNG* and *CTLA4* signals were low.

(E-F) Protein expression (Western blot).

(E) Left panel confirms ERS induction in Caki-2: BiP and CHOP are strongly induced by Tun. XBP1s is detectable under Tun. Right panel shows PBMC protein responses to Caki-2-derived CM: BiP and PERK are detectable across CM conditions. PD-L1 and PD-1 are present in CM-exposed PBMCs. FoxP3 is detectable across CM conditions with apparent elevation under CM from Tun-treated cells. CTLA-4 shows strongest signal under CM from Tun-treated condition, consistent with induction of an immunosuppressive/exhaustion-associated phenotype.

(F) Left panel confirms ERS induction in TK-10: BiP is strongly induced by Tun; CHOP shows a distinct band under Tun treatment; XBP1s is detectable. TUDCA alone induces BiP without CHOP, consistent with panel E. Right panel shows PBMC responses to TK-10-derived CM: BiP and PERK are elevated under CM conditions. FoxP3 is detectable across CM-exposed conditions. CTLA-4 signal is present under CM from Tun-treated condition, replicating the pattern observed in panel E. PD-L1 and PD-1 are detectable in CM-exposed PBMCs. β -Actin confirms equal loading across all lanes.

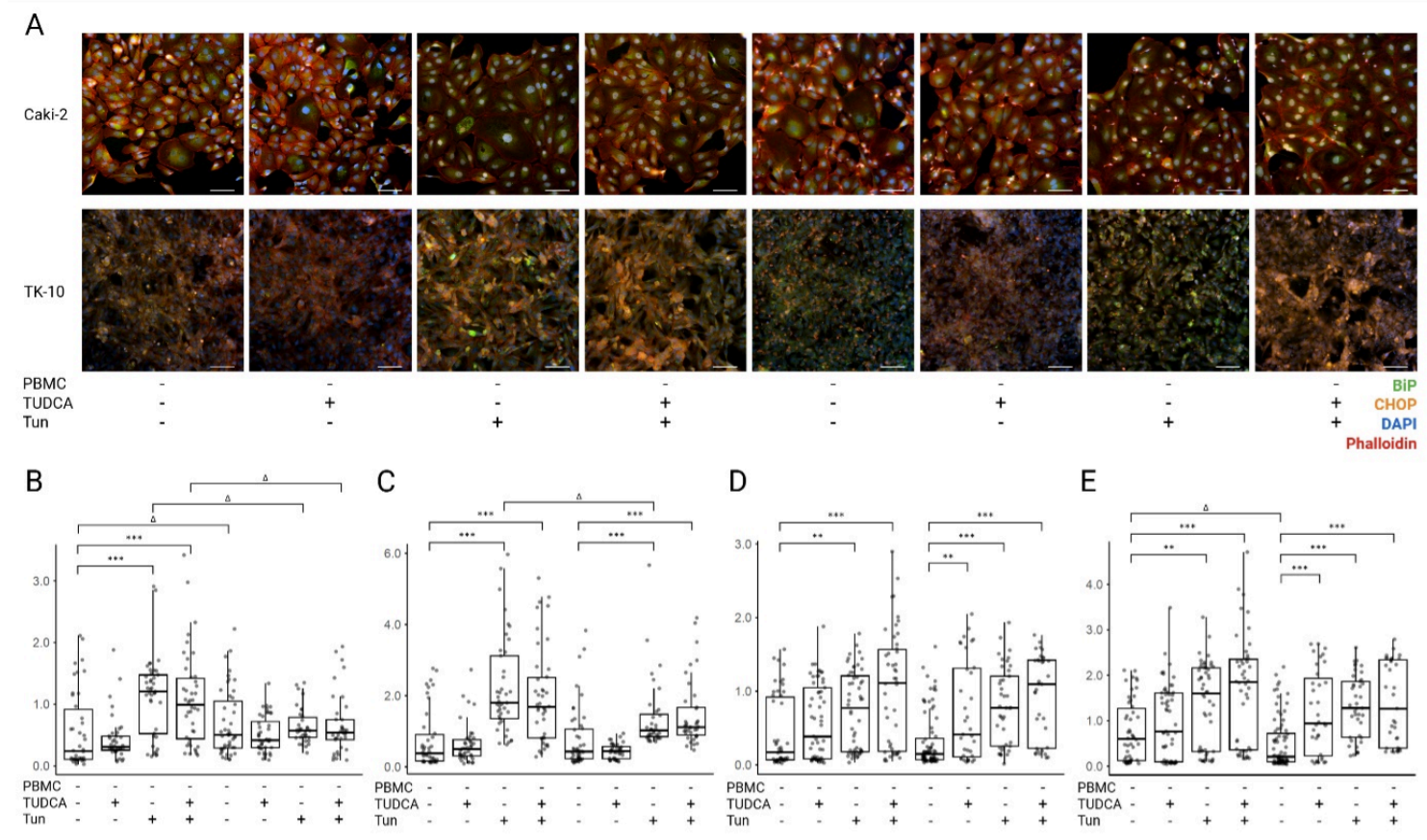


Figure 3

Immunofluorescence analysis of ERS markers in RCC mono- and co-cultures.

(A) Representative immunofluorescence images. UPR markers BiP (green), CHOP (orange), cell nuclei-DAPI (blue), and actin filaments-Phalloidin (red). Scale bar 100 μ m. **(B-E)** Image analysis of RCC cells showing the relative signal intensities of BiP and CHOP in Caki-2 and TK-10 cell cultures.

(B-E) Quantification of BiP and CHOP fluorescence intensity.

(B) Tun treatment affected the relative intensity of BiP signal in both Tun- ($p < 0.001$) and T+T-treated Caki-2 monocultures ($p < 0.001$). A significant increase in BiP signal intensity was observed when co-cultured with PBMCs compared to monocultured cells in control, Tun, and T+T conditions ($p < 0.05$).

(C) The relative signal intensity of CHOP was elevated in Tun- and T+T-treated monoculture ($p < 0.001$) and co-culture with PBMC ($p < 0.001$). A significant decrease in CHOP signal intensity was observed when co-cultured with PBMCs compared to monocultured cells ($p < 0.05$).

(D) Tun treatment affected the relative intensity of BiP signal in both Tun- ($p < 0.01$) and T+T-treated TK-10 monocultures ($p < 0.001$). BiP was significantly elevated in TK-10 co-cultures treated with TUDCA ($p < 0.01$), Tun ($p < 0.001$), and T+T ($p < 0.001$).

(E) Tun treatment affected the relative intensity of CHOP signal in both Tun- ($p < 0.01$) and T+T-treated TK-10 monocultures ($p < 0.001$). CHOP was significantly elevated in TK-10 co-cultures treated with TUDCA ($p < 0.001$), Tun ($p < 0.001$), and T+T ($p < 0.001$). A significant decrease in CHOP signal intensity was observed when co-cultured with PBMCs compared to monocultured cells ($p < 0.05$).

Statistical comparisons performed using Mann-Whitney test without FDR correction (Δ).

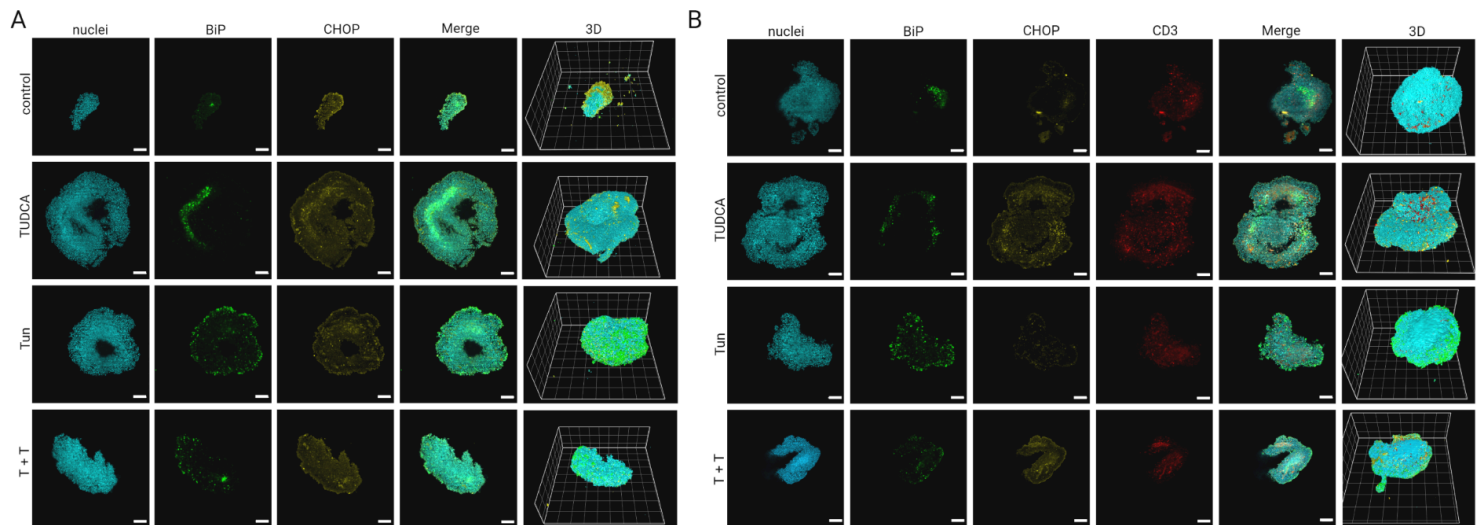


Figure 4

Immunofluorescence (IF) staining and analysis of *in vitro* cancer-immune spheroids.

(A-B) Cancer-immune spheroids of Caki-2 cells and PBMCs were stained for IF analysis of BiP, CHOP, and CD3. Scale bar 500 μ m.

(A) BiP and CHOP were detected in Caki-2 spheroids under control conditions. In TUDCA-treated spheroids, BiP signal was detected solely in cells deep within the spheroid structure. Tun-treated spheroids show elevated surface BiP signal.

(B) In co-culture with PBMCs, high immune cell infiltration—detected by CD3 staining—was observed in TUDCA treated spheroids. High CD3 signal was colocalized with high CHOP signal. In TUDCA-treated spheroids, BiP signal was detected solely in cells deep within the spheroid structure. Tun-treated spheroids showed elevated surface BiP signal and lower immune cell infiltration.

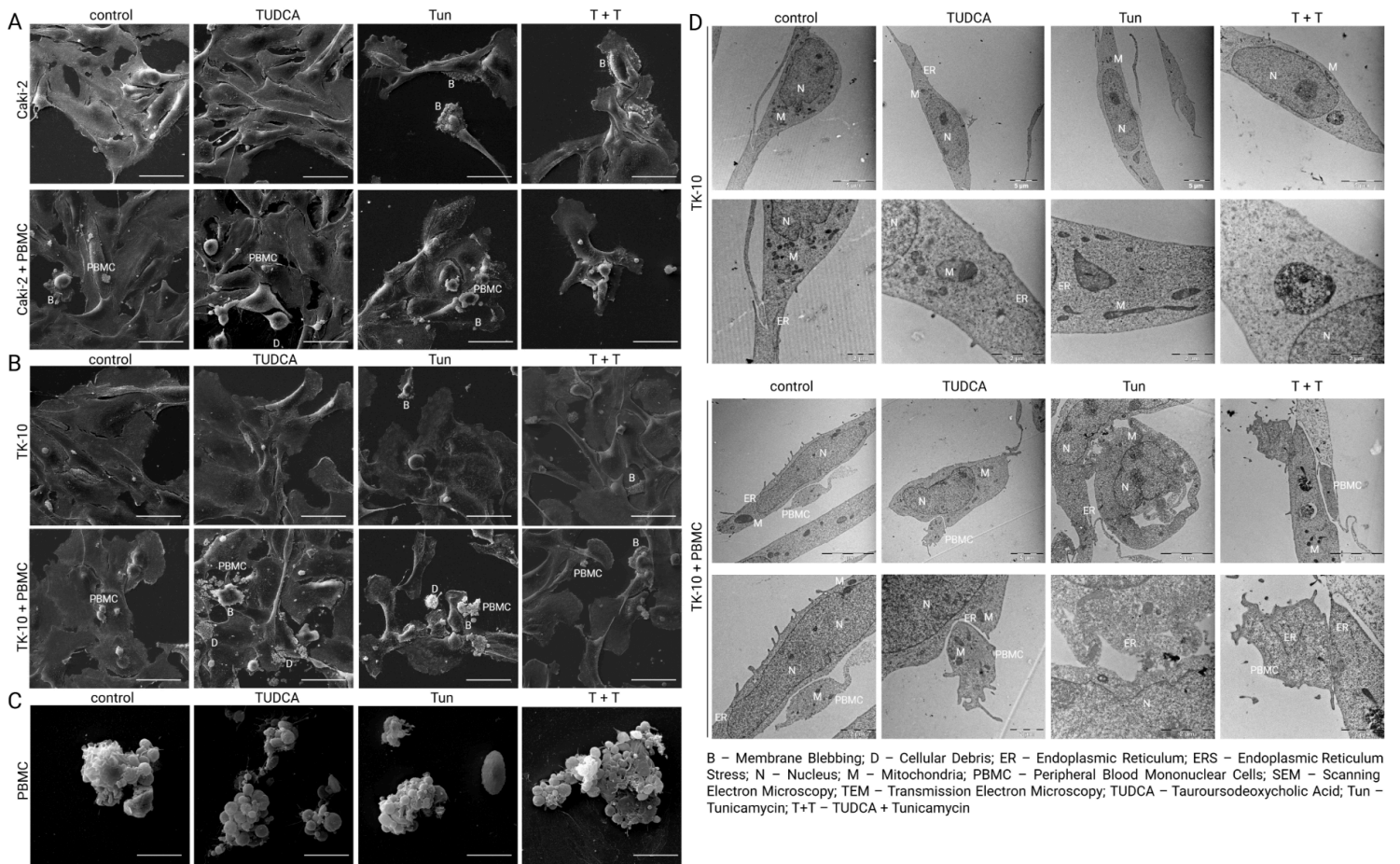


Figure 5

ERS modulation and immune co-culture induce severe morphological and ultrastructural alterations in RCC cells *in vitro*.

(**A-C**) Representative **SEM** micrographs of (**A**) Caki-2 cells in mono- and co-culture with PBMCs (scale bar: 50 μ m); (**B**) TK-10 cells mono- and co-culture with PBMCs (scale bar: 50 μ m); and (**C**) PBMCs monoculture (scale bar: 10 μ m). Control and TUDCA groups show typical flattened, adherent epithelial (Caki-2) or spindle-shaped (TK-10) morphologies with continuous plasma membranes and preserved surface attachment. Tun treatment induces severe surface remodeling characterized by cell contraction, rounding, membrane blebbing, loss of surface adherence, and accumulation of abundant cytotoxic debris. In co-cultures, rounded and clustered PBMCs remain associated with highly damaged tumor cells. TUDCA co-treatment demonstrates partial morphological rescue, with preserved substrate attachment, decreased membrane disruption, and reduced accumulation of extracellular debris. Associated PBMCs maintain a healthy, intact spherical shape with preserved surface microvilli.

(**D**) Representative **TEM** micrographs of TK-10 cells mono- and co-culture with PBMCs, scale bar: 5 μ m (top), 2 μ m (bottom). Lower rows in each section show high-magnification views of intracellular organelles and cellular interfaces. Control and TUDCA groups display intact nuclei with evenly distributed

chromatin and well-organized cytoplasm rich in elongated mitochondria with distinct cristae. In co-culture, healthy physical contact is observed between adherent tumor cells and PBMCs. Tun treatment triggers severe intracellular lesions, including massive cytoplasmic vacuolization and marked dilation of membranous compartments consistent with ER expansion. Mitochondria exhibit swelling and cristae disruption alongside localized cytoplasmic rarefaction. In combined treatment, TEM reveals significant preservation of cytoplasmic compactness and mitochondrial architecture. In co-cultures, TUDCA exerts a cytoprotective effect; PBMCs adjacent to tumor cells retain ultrastructural integrity, featuring intact nuclear envelopes, continuous plasma membranes, and well-organized cytoplasmic organelles.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [AllWBRawdata.pdf](#)
- [SupplementaryInformationRCCERS20260717.docx](#)