

ORIGINAL ARTICLE

Basic and Translational Allergy Immunology

IRE1 and PERK signaling regulates inflammatory responses in a murine model of contact hypersensitivity

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Abstract

Background: Contact sensitizers may interfere with correct protein folding. Generation of un-/misfolded proteins can activate the IRE-1 or PERK signaling pathways initiating the unfolded protein response (UPR) and thereby determine inflammatory immune responses. We have analyzed the effect of sensitizers with different potencies on the induction of UPR activation/inhibition and the subsequent generation of a pro-inflammatory microenvironment in vitro as well as the effect of UPR modulation on the inflammatory response in the murine contact hypersensitivity (CHS) in vivo.

Methods: Semi-quantitative and quantitative PCR, fluorescence microscopy, ELISA, NF-κB activation and translocation assays, DC/keratinocyte co-culture assay, FACS, and in vivo CHS experiments were performed.

Results: Sensitizers and irritants activate IRE-1 and PERK in murine and human keratinocytes. Synergistic effects occur after combination of different weak sensitizers / addition of irritants. Moreover, tolerogenic dinitrothiocyanobenzene can be converted into a strong sensitizer by pre-activation of the UPR. Blocking UPR signaling results in decreased NF-κB activation and cytokine production in keratinocytes and in activation marker downregulation in a HaCaT/THP-1 co-culture. Interestingly, not only systemic but also topical application of UPR inhibitors abrogates CHS responses in vivo.

Conclusion: These observations highlight an important role of the UPR in determination of the inflammatory response in vitro and in vivo further underlining the importance of tissue stress and damage responses in the development of ACD and provide mechanistically based concepts as a basis for the development of new therapeutic approaches to treat allergic contact dermatitis.

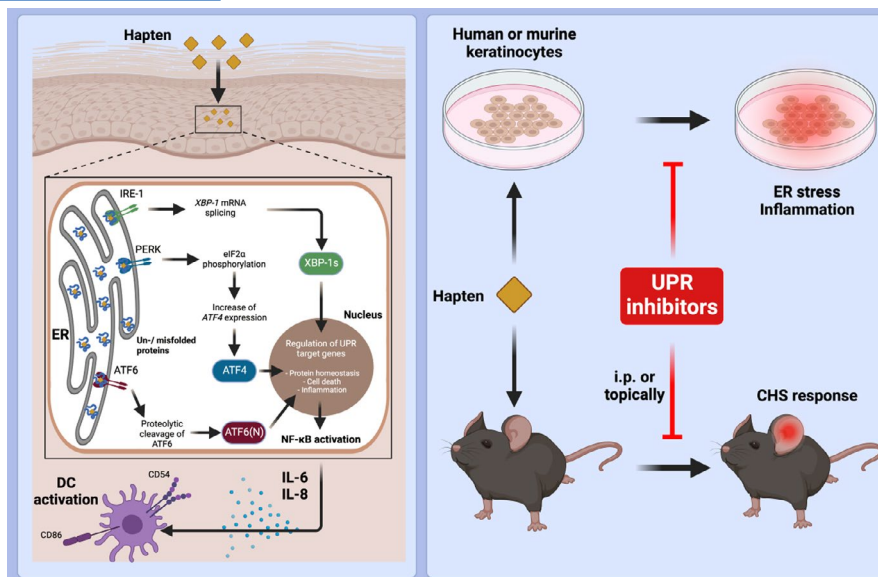
KEYWORDS

allergic contact dermatitis, contact hypersensitivity, inflammation, unfolded protein response

Abbreviations: ACD, allergic contact dermatitis; ATF6, activating transcription factor 6; CHS, contact hypersensitivity; DC, dendritic cell; DNTB, 2,4-dinitrothiocyanobenzene; IRE1, inositol-requiring transmembrane kinase/endonuclease 1 alpha; PERK, protein kinase RNA-like ER kinase; TNCB, 2,4,6-trinitrochlorobenzene; UPR, unfolded protein response; Xbp1, X-box binding protein 1.

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GRAPHICAL ABSTRACT

UPR in murine and human keratinocytes is activated by contact sensitizers and irritants; combining weak sensitizers results in synergistic effects. UPR inhibition reduces NF-κB activation and pro-inflammatory cytokine production thereby preventing ER stress-mediated DC activation in a keratinocyte/DC co-culture assay *in vitro*. Systemic or topical inhibitor application prevents CHS; UPR pre-activation enhances CHS to the weak allergen/tolerogen DNTB.

1 | INTRODUCTION

Allergic contact dermatitis (ACD) is a chemical-induced, T cell-mediated inflammatory skin disease. It results in local to systemic erythema and eczema formation.¹ In Europe, ACD has a high socio-economic impact^{2–4} with a prevalence of ~20% of the population.^{2–4} It is caused by contact with one of >4350⁵ electrophilic, low molecular weight (up to ~2.2 kDa), organic chemical or metal sensitizers.⁶ The lack of causative treatments for ACD often necessitates long-term therapy with immunosuppressive drugs like corticosteroids with significant side effects.^{7,8}

ACD is often caused by mixtures and formulations like cosmetic products containing combinations of weak sensitizers and irritants resulting in additive or synergistic effects with respect to the innate immune and stress responses and thereby enable or facilitate the development of ACD.^{9–12}

Therefore, a better understanding of mechanisms determining the strength of the sensitizing potential of the chemicals is necessary to allow for better risk assessment. Despite recent efforts to understand the underlying mechanisms^{13,14} and the acknowledgement that sensitizers have adjuvant effects, the nature of tissue stress and tissue damage responses in the development of ACD remains ill-defined.

To exert their immunogenic potential, sensitizers need to bind covalently or non-covalently to proteins.¹⁵ Keratinocytes are the first cells to encounter exogenous chemicals. Malmberg et al¹⁶ showed that penetration of sensitizers into the deeper skin layers might be even limited/restricted to the upper epidermis, indicating that keratinocytes might be the major cell type coming into contact with sensitizers in their protein reactive form. Therefore, keratinocytes

act as sensor cells for potential danger, contribute critically to the cutaneous cytokine milieu,¹⁷ and are paramount for the full activation of dendritic cells (DCs) and finally sensitization in ACD. The clinically inapparent sensitization phase in ACD involves activation of both innate and adaptive immune responses after the first encounter of the skin with a sensitizer. Importantly, to facilitate priming and activation of naïve T cells, DCs need to initially receive a “tissue stress and tissue damage” signal provided by the sensitizers’ chemical reactivity.^{1,13,14,18,19} Renewed contact with the same sensitizer initiates the elicitation phase, leading to T cell-mediated killing of hapten-modified keratinocytes and subsequent clinically relevant erythema and eczema formation.

Here, we hypothesized that direct binding of sensitizers to proteins (haptization process) and sensitizer-mediated reactive oxygen species (ROS) generation^{20,21} would lead to changes in cellular protein structures, generating and accumulating misfolded proteins in the endoplasmic reticulum (ER). This may lead to the activation of an ER stress response, the unfolded protein response (UPR), which has been linked to the generation of pro-inflammatory conditions.²²

Several pathologic conditions result in UPR activation.^{23,24} Under both physiological^{25–27} and pathological conditions, newly synthesized proteins are processed within the ER. Accumulation of un- or misfolded proteins activates the UPR to restore correct protein folding. Three distinct stress sensors in the ER membrane are described: inositol-requiring transmembrane kinase/endonuclease 1 alpha (IRE1α protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6).²⁸ Activation of IRE1α leads to an unconventional splicing of a 26-nucleotide fragment from the unspliced X-box binding protein 1 (uXbp1) mRNA to the spliced sXbp1 mRNA.²⁹ The resulting protein Xbp1 acts as a potent

transcription factor.³⁰ Activation of the PERK branch by trans-autophosphorylation ends in subsequent translational arrest of most proteins and cell cycle arrest. Activation of ATF6 exposes a Golgi localization sequence and allows translocation of ATF6 into the Golgi, where it is sequentially cleaved. The ATF6 fragment translocates to the nucleus where it acts as a transcription factor activating several UPR genes including *Xbp1*.²⁹

Here, we analyzed the impact of different sensitizers and irritants on the activation of the UPR in keratinocytes and the subsequent effect of the UPR on the sensitization and elicitation phase in CHS. Importantly, we show that sensitizers activate all three UPR branches and modulate the generation of a pro-inflammatory milieu in vitro. This is in line with previous work by Luis et al. showing that the extreme sensitizer DNFB activates the ATF-4 pathway in the monocytic cell line THP-1.³¹ Interestingly, in our system combinations of weak sensitizers result in synergistic UPR activation. Concomitant activation of the UPR by tunicamycin application before sensitization enhances the CHS response. Using ER stress reporter mice,³² we show that small molecule inhibitors can suppress the UPR when applied topically. In addition, modulation of the UPR by systemic or topical application of small molecule inhibitors for IRE1 α or PERK abrogates CHS responses in vivo.

This indicates that the UPR is a crucial element of the inflammatory response to sensitizers and is involved in the modulation of their sensitizing potency. This provides mechanistically based concepts for the development of novel causative therapies.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Experimental procedures were in accordance with institutional, state, and federal guidelines on animal welfare, approved by the Regierungspräsidium Freiburg and supervised by the Animal Protection Representatives of the CEMT Freiburg. Primary human keratinocytes were obtained from biopsies after informed donor consent as approved by the local Ethics committee and in compliance with the declaration of Helsinki.

2.2 | Mice

Female C57BL/6 mice were purchased (Charles River Laboratories) or provided by the breeding facility of the CEMT in Freiburg, Germany. ERAI mice³² were provided by Prof. R. Zeiser from a breeding stock at CEMT Freiburg. Mice were used at the age of 6–10 weeks.

2.3 | Statistical analysis

Unless noted otherwise, all experiments were performed at least three times and data show results from all three independent

experiments. Animal experiments used 3–5 mice per group and experiment; again, results from all three experiments are shown. Analyses were done using Graph Pad Prism 5.0 and one-way ANOVA with Dunnett's post-test (untreated set as controls) or unpaired Student's t test unless indicated otherwise. Error bars represent standard deviation (SD) as indicated. $p < .05$ was considered to be statistically significant.

All procedures used in this study are fully described in the Methods section in this article's Supplementary Material and Methods S1.

3 | RESULTS

3.1 | Sensitizers and the irritant SDS induce *Xbp1* splicing, differentially induce PERK phosphorylation, and modulate *Atf6* expression

Pam212 cells were treated with the UPR activator tunicamycin, the extreme sensitizer oxazolone, the strong sensitizer 2,4,6-trinitrochlorobenzene (TNCB), or the irritant sodium dodecyl sulfate (SDS). All chemicals increase spliced *Xbp1* (s*Xbp1*) expression in a dose (Figure S1a–d) and time (Figure 1A–E)-dependent manner. Splicing started 2–4 h after tunicamycin treatment (Figure 1A + B) while oxazolone (Figure 1C) and SDS (Figure 1E) treatment induced splicing already 1 h after stimulation with a peak after 6 h. For TNCB, significant splicing was observed after 16 h.

Additionally, IRE1 α -mediated *Xbp1* splicing is also activated in primary human keratinocytes (Figure S2a), indicating that the effect is not limited to murine keratinocytes.

To confirm that *Xbp1* splicing was mediated by IRE1 α activation, cells were pre-treated with the specific small molecule IRE1 α inhibitor II. Blocking IRE1 α activity led to complete inhibition of *Xbp1* splicing 6 h after sensitizer/irritant treatment and a strong reduction of *Xbp1* splicing after tunicamycin treatment (Figure 1F).

Next, we addressed the phosphorylation of PERK (Figure 1G + H). Treatment with tunicamycin and thapsigargin induced a strong PERK phosphorylation, while oxazolone showed only a faint phosphorylation compared to its solvent control ethanol. In contrast, TNCB and SDS (acetone/untreated as solvent control) induced strong phosphorylation.

Since we detected differences in the activation of IRE1 α and PERK between oxazolone and TNCB, we controlled for ATF6 activation (Figure 1I). We observed a strong increase in gene expression levels after stimulation with tunicamycin (~4.16-fold) and SDS (~3.86-fold), while oxazolone showed a weaker (~2.94-fold) and TNCB just a weak (~1.89-fold) upregulation of *Atf6* (Figure 1I), resembling the results observed for *Xbp1* splicing (Figure 1A–F). To test whether the effect with the irritant was SDS-specific, we stimulated cells with the strong irritant crotonoil. As shown, also crotonoil stimulation resulted in an upregulation of *Atf6* expression (~2.86-fold) (Figure 1I).

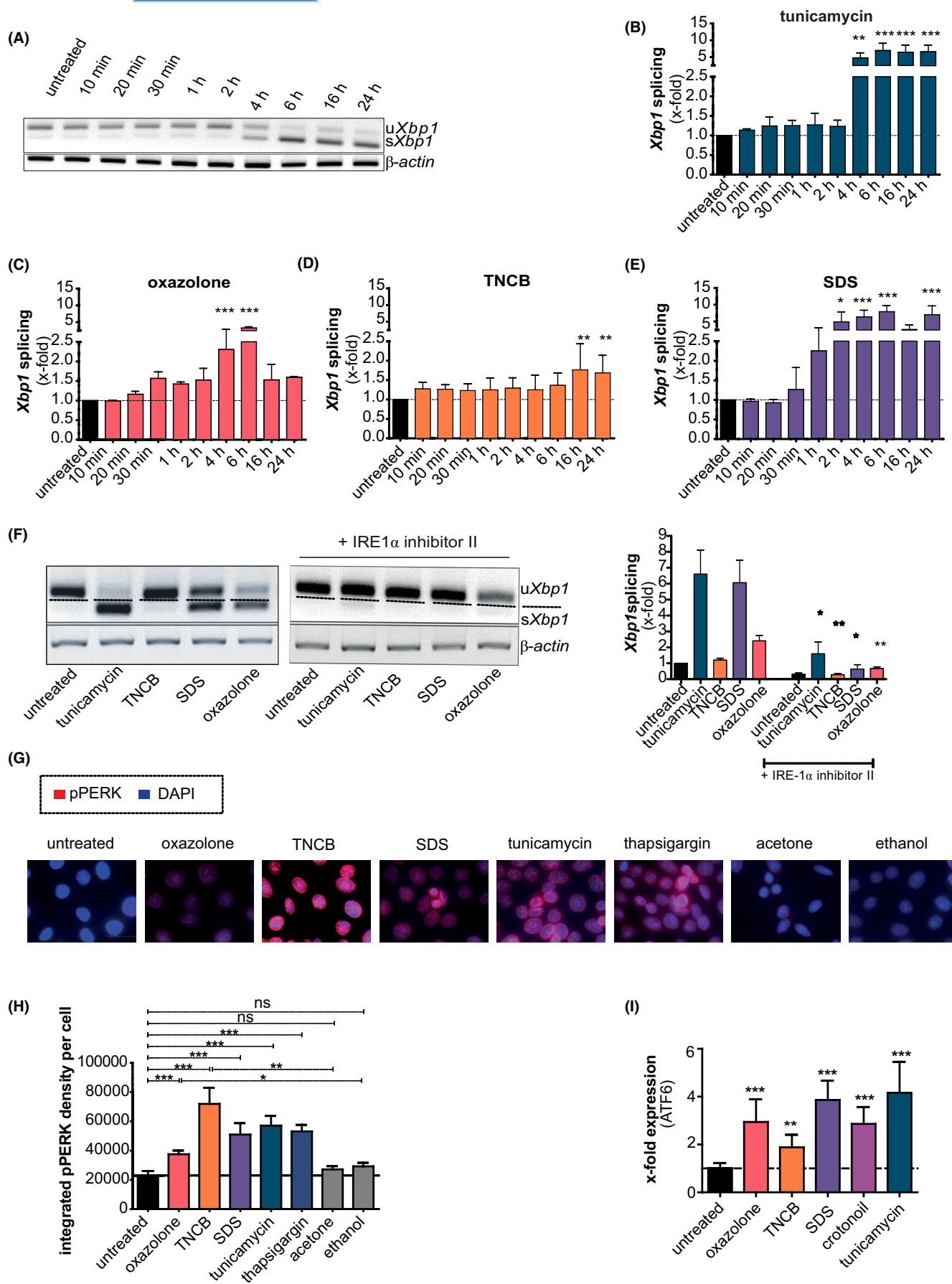


FIGURE 1 IRE1 α , PERK, and ATF-6 signaling is activated by sensitizers and irritants in Pam212 keratinocytes. (A–F) Cells were treated with (A, B) tunicamycin (10 μ g/mL), (C) oxazolone (625 μ M), (D) TNCB (30 μ M), or (E) SDS (260 μ M) for the indicated time. *Xbp1* splicing was determined by semi-quantitative PCR. (A) Gel-electrophoresis: unspliced (u*Xbp1*, 171 bp) and lower spliced (s*Xbp1*, 145 bp) band; beta-actin as loading control. (B–E) X-fold splicing $\pm$ SD from $n = 3$ (B, C), $n = 4$ (E), or $n = 6$ (D) independent experiments. One-way ANOVA, *** $p < .0001$. (F) Left panel: left side analysis of *Xbp1*-splicing after 6 h, right side: pre-treatment with 25 μ M IRE1 α inhibitor 1 h before stimulation. One of three independent experiments. For better visualization, the upper region containing the unspliced band was separated by a dashed line from the lower region containing the spliced band. Right panel: quantification of data from three independent experiments. One-way ANOVA, *** $p < .0001$ (G) Cells were stained after 6 h of chemical stimulation with oxazolone (625 μ M), TNCB (30 μ M), SDS (260 μ M), thapsigargin (300 nM), or tunicamycin (10 μ g/mL) with an anti-phospho-PERK (pPERK) antibody (red). Nuclei were visualized by DAPI staining (blue). Scale bar = 50 μ m. One of three independent experiments. (H) shows the corresponding quantification of integrated density of the PERK fluorescence from three independent experiments. t test, *** $p < .0001$. (I) ATF6 mRNA expression levels were determined by RT-qPCR after stimulation for 6 h as before with an additional crotonoil-treated sample (2000 μ g/mL). Results are shown as fold change ($2^{-\Delta\Delta C_t}$) in mRNA expression levels $\pm$ SD of $n = 6$ (tunicamycin $n = 4$) independent experiments. t test, *** $p < .0001$

Taken together, sensitizers and irritants activate all three branches of the UPR. Interestingly, a difference in the strength of activation of the different UPR pathways between the extreme sensitizer oxazolone and the strong sensitizer TNCB is observed.

3.2 | IRE1 α and PERK inhibition blocks chemical-induced NF- κ B nuclear translocation and activation

To address the influence of UPR inhibition on the activation of pro-inflammatory NF- κ B signaling, we pre-treated Pam212 cells with IRE1 α and PERK inhibitors (Figure 2A,B). Sensitizer and irritant treatment resulted in significant upregulation of p65 translocation and enhanced NF- κ B activation. Pretreatment with IRE1 α or PERK inhibitor led to significant reductions in upregulated NF- κ B translocation and activation. The inhibitors significantly reduced even the extreme NF- κ B activation by crotonoil treatment (containing PMA).

These results indicate that sensitizer- and irritant-induced activation of the UPR results in activation of the NF- κ B signaling pathway.

3.3 | Synergistic effects on IRE1 α signaling and NF- κ B activation by combination of different weak sensitizers or a weak sensitizer with the irritant SDS

Combination of different weak sensitizers or of weak sensitizers and irritants results in a CHS reaction that resembles one of a strong sensitizer.¹² This led us to investigate the effect of the combination of weak sensitizers (resorcinol, cinnamic aldehyde (CA), DNTB, TNCB, and the irritant SDS on UPR activation and NF- κ B translocation in Pam212 (Figure 3A,B). Sensitizers induced only weak *Xbp1* splicing (Figure 3A, dark black bars). SDS treatment resulted in enhanced *Xbp1* splicing. Next, we calculated the hypothetical splicing that would occur when the weak sensitizer combined with SDS would show an additive effect (Figure 3A, dark gray bars). However, for all tested combinations, experimental data (light gray bars) showed an increase in *Xbp1* splicing that was higher than a purely additive effect (Figure 3A).

These results were reflected in the analysis of NF- κ B translocation (Figure 3B), suggesting synergistic effects of combinations of weak sensitizers with irritants on the UPR activation.

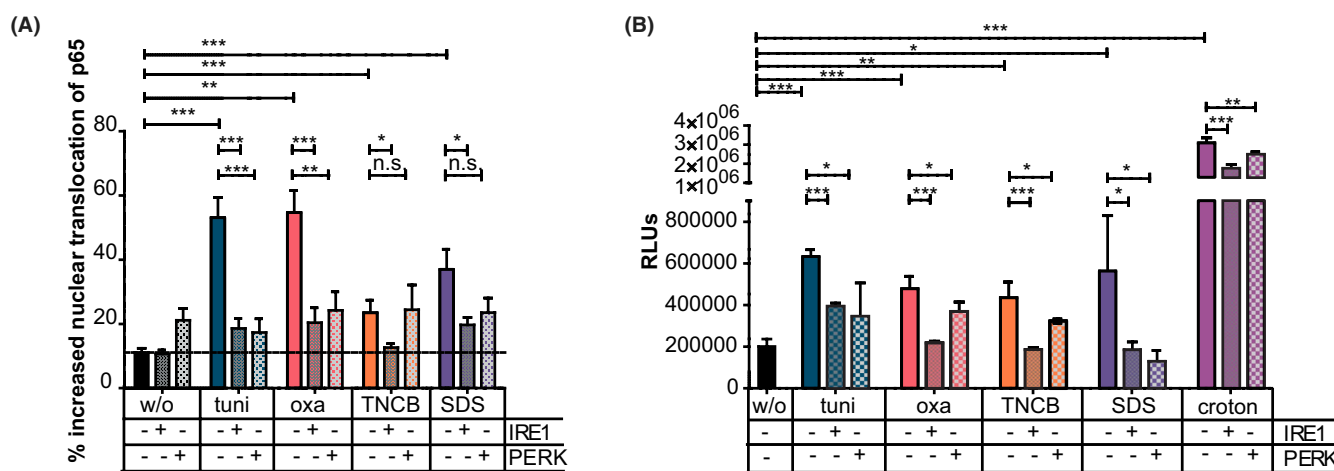


FIGURE 2 IRE1 α and PERK inhibition blocks chemical-induced NF- κ B translocation and activation. Pam212 cells were treated with tunicamycin (10 μ g/mL), oxazolone (625 μ M), TNCB (30 μ M), SDS (260 μ M), or crotonoil (20 μ g/mL) for 1 h with or without 1 h pre-incubation with IRE1 α inhibitor (25 μ M) or PERK inhibitor (10 μ M). (A) NF- κ B translocation was assessed by staining for increased nuclear p65+ cells. Results show data from four independent experiments $\pm$ SD. t test, *** $p < .0001$. (B) Activation of NF- κ B was analyzed using stably transfected Pam212 reporter cells. Cells were stimulated as in (A) and relative luminescence units determined after 6 h. Graphs show results from three independent experiments $\pm$ SD. t test, *** $p < .0001$

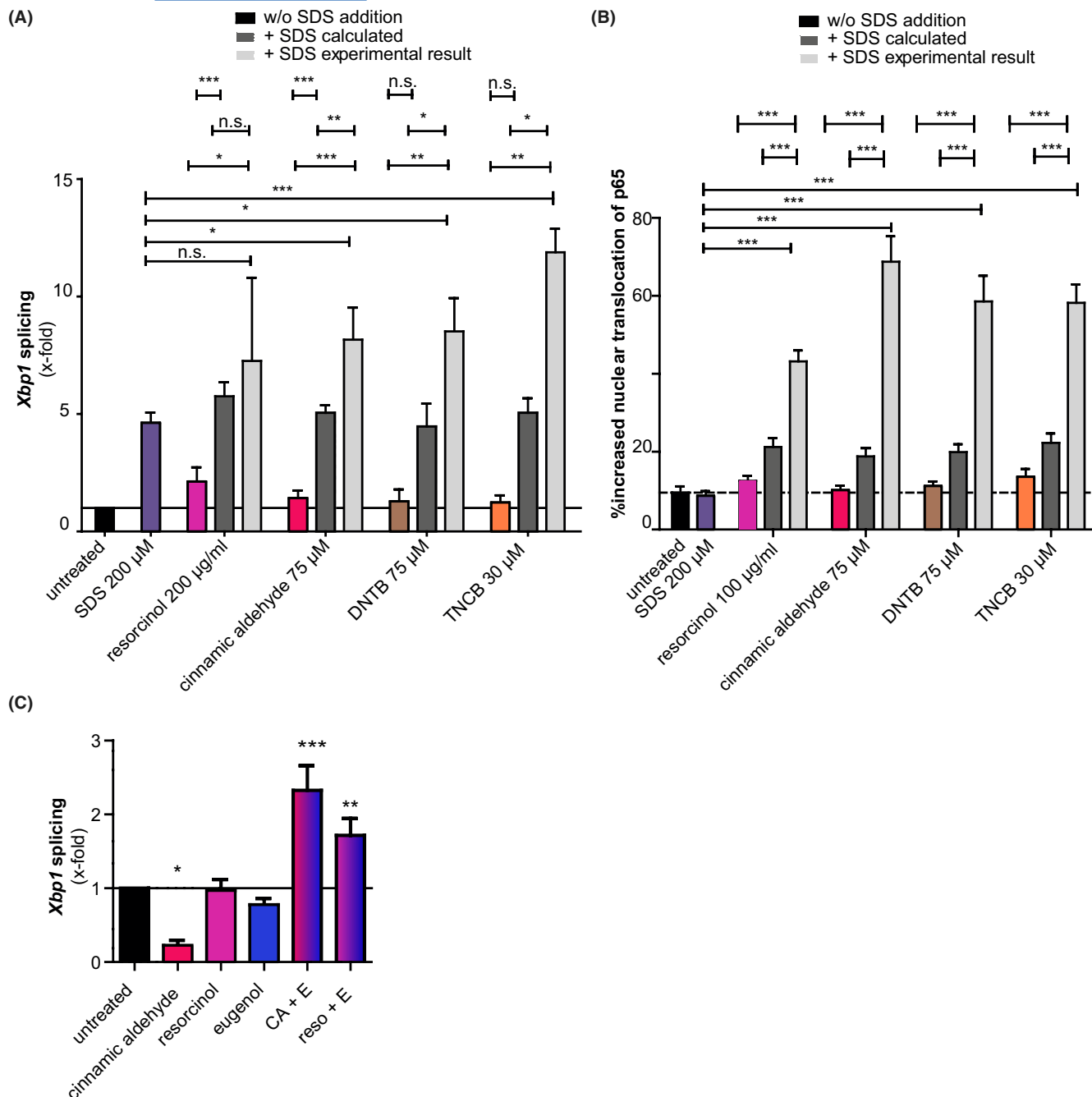


FIGURE 3 Synergistic UPR activation and NF- κ B translocation after combination of weak sensitizers with the irritant SDS. (A) Pam212 cells were treated as indicated for 6 h, and *Xbp1* splicing was analyzed by semi-quantitative PCR. Data show x-fold *Xbp1* mRNA splicing from 4 independent experiments $\pm$ SD. *t* test, ****p* < .0001 (A + B) dark black bars = experimental results obtained from untreated or contact sensitizer only stimulated cells (ie, w/o further SDS addition), dark gray bars = calculated values (hypothetical values obtained by addition of sensitizer results + results from SDS only treatment), and light gray bars = experimental results (SDS treatment only or sensitizer + SDS treatment) (B) Pam212 cells were treated as indicated for 1 h, and NF- κ B translocation was analyzed. Data show increased nuclear translocation of p65 from three independent experiments $\pm$ SD. *t* test, ****p* < .0001. (C) Pam212 cells were treated with cinnamic aldehyde (75 μ M), resorcinol (100 μ g/mL), eugenol (200 μ g/mL) or combinations of cinnamic aldehyde or resorcinol with eugenol for 6 h and *Xbp1* splicing was analyzed by semi-quantitative PCR. Data show x-fold *Xbp1* mRNA splicing from 3 independent experiments $\pm$ SD. One-way ANOVA, ****p* < .0001

Next, we assessed the effect of mixing different weak sensitizers on the activation of IRE1 α . Neither CA, resorcinol, nor eugenol treatment resulted in an upregulated *Xbp1* splicing, with CA leading

to a reduction in *Xbp1* splicing (Figure 3C). However, when CA or resorcinol are combined with eugenol, *Xbp1* splicing is significantly enhanced (Figure 3C).

Taken together, combination of two weak sensitizers and also the combination of sensitizers with an irritant not only leads to a synergistic activation of the UPR but also leads to a synergistic activation of the pro-inflammatory NF- κ B signaling pathway in keratinocytes.

3.4 | UPR inhibitors reduce cytokine production in human keratinocytes and DC activation marker expression in a keratinocyte/dendritic cell co-culture assay

Cell culture supernatants of the human keratinocyte cell line HaCaT were analyzed for the release of the pro-inflammatory cytokine IL-6³³ (Figure 4A–C). Oxazolone, TNCB, or SDS dose dependently enhanced cytokine production, while treatment with either IRE1 α or PERK inhibitor reduced cytokine production. The effect of the PERK inhibitor was comparable to the effect of the IRE1 α inhibitor. This is in line with the activation of *Xbp1* splicing (Figure 4D) and PERK phosphorylation in HaCaT cells (Figure 4E).

To analyze whether this reduced activation and cytokine production by keratinocytes would suffice to provide reduced maturation signals for immature DCs, we performed an adapted CoCaT-assay protocol.³⁴ The expression of activation marker CD86 by the human monocytic DC-like cell line THP-1 was reduced in HaCaT cells pre-treated with UPR inhibitor (Figure 4F). This indicates that UPR inhibition can prevent the provision of a pro-inflammatory signal from keratinocytes to dendritic cells and thus prevents DC maturation.

Taken together, inhibition of UPR signaling results both in murine and in human keratinocytes in a reduced activation by sensitizers and irritants. This activation is necessary to produce pro-inflammatory cytokines like IL-6 and subsequently for the efficient activation of immature DCs.

3.5 | Modulation of UPR influences sensitizer potency and CHS responses in vivo

Several animal studies have shown that the endogenous amphiphilic bile acid tauroursodeoxycholic acid (TUDCA) can act as a chemical chaperone, thereby inhibiting UPR dysfunction and ameliorating ER stress. Clinical trials were performed (eg, NCT 01855360, NCT 01857284, NCT 02218619, <https://clinicaltrials.gov>). Reduction of ER stress by TUDCA prior to sensitization significantly reduced TNCB-induced ear swelling responses upon challenge (Figure 5A). Responses obtained were like those in mice receiving vehicle control during sensitization, and thus most likely represent the irritative response to TNCB applied for elicitation. Interestingly, increasing UPR activity before sensitization by pretreating mice with the UPR activator tunicamycin resulted in a significant increase in ear thickness after elicitation compared to the group that received TNCB sensitization and elicitation only (Figure 5A).

Next, we wanted to address the UPR influence on the CHS elicitation phase as a clinically more relevant setting. In addition, we decided to stress the system using the extreme sensitizer oxazolone. We also asked if specific inhibition of either the IRE1 α or PERK pathway would suffice to modulate CHS responses. Therefore, we applied the inhibitors to already oxazolone-sensitized mice 1 h before elicitation. Sensitization and elicitation with oxazolone resulted in a significant increase in ear thickness compared to mice lacking either sensitization or elicitation (Figure 5B). However, pretreatment with either IRE1 α - or PERK inhibitor resulted in significant reduction in ear swelling responses independent of the vehicle control (DMSO) (Figure 5B). This indicates that also interference with the UPR activation during the elicitation phase results in an inhibition of the inflammatory CHS response.

Taken together, reduction of the UPR results in a reduction of the CHS response, while enhanced UPR activation during sensitization resulted in an enhanced CHS response.

3.6 | Induction of CHS to the weak sensitizer/tolerogen DNTB by UPR activation

Combination of SDS with DNTB was shown to turn the weak sensitizing/tolerogen DNTB into a strong sensitizer,³⁵ potentially caused by enhancing DNTB penetration into the skin³⁶ or increased tissue stress response and production of danger signals to the irritant.^{9,19} To test our hypothesis, that enhanced UPR activation converts weak or non-sensitizers into potent ones, we treated mice with a known non-sensitizing dose of DNTB (3%), followed 5d later by an elicitation with 1% DNTB applied to the ears (Figure 5C). This setup resulted in very weak inflammation ($21.1 \pm 13.23 \mu\text{m}$), while the irritation control, that is, mice treated with ethanol for sensitization and DNTB 1% for elicitation resulted just in background inflammation ($5 \pm 8.36 \mu\text{m}$, data not shown). However, pretreatment of mice with tunicamycin before sensitization resulted in a significant increase in ear thickness after elicitation ($78.89 \pm 17.79 \mu\text{m}$) with values resembling the response obtained after sensitization and challenge with similar concentrations of the strong sensitizer TNCB (Figure 5A + C).

These results show that modulation of the UPR response can modulate the sensitizer potency.

3.7 | Topical inhibitor application inhibits IRE1 α activation ex vivo and inhibits elicitation of oxazolone induced CHS responses

To address a more clinically relevant setting, we applied the inhibitors topically. Figure 6A + B shows that ears of ERAI (ER stress-activated indicator (ERAI) mice),³² exhibit background UPR activity owing to constant protein renewal, while activation of the UPR with tunicamycin results in a significant increase in *Xbp1* splicing. The same effect is seen in oxazolone-treated ears. However, topical pretreatment with the IRE1 α inhibitor in combination with oxazolone treatment reduced the fluorescence emission to the background level (Figure 6A,B).

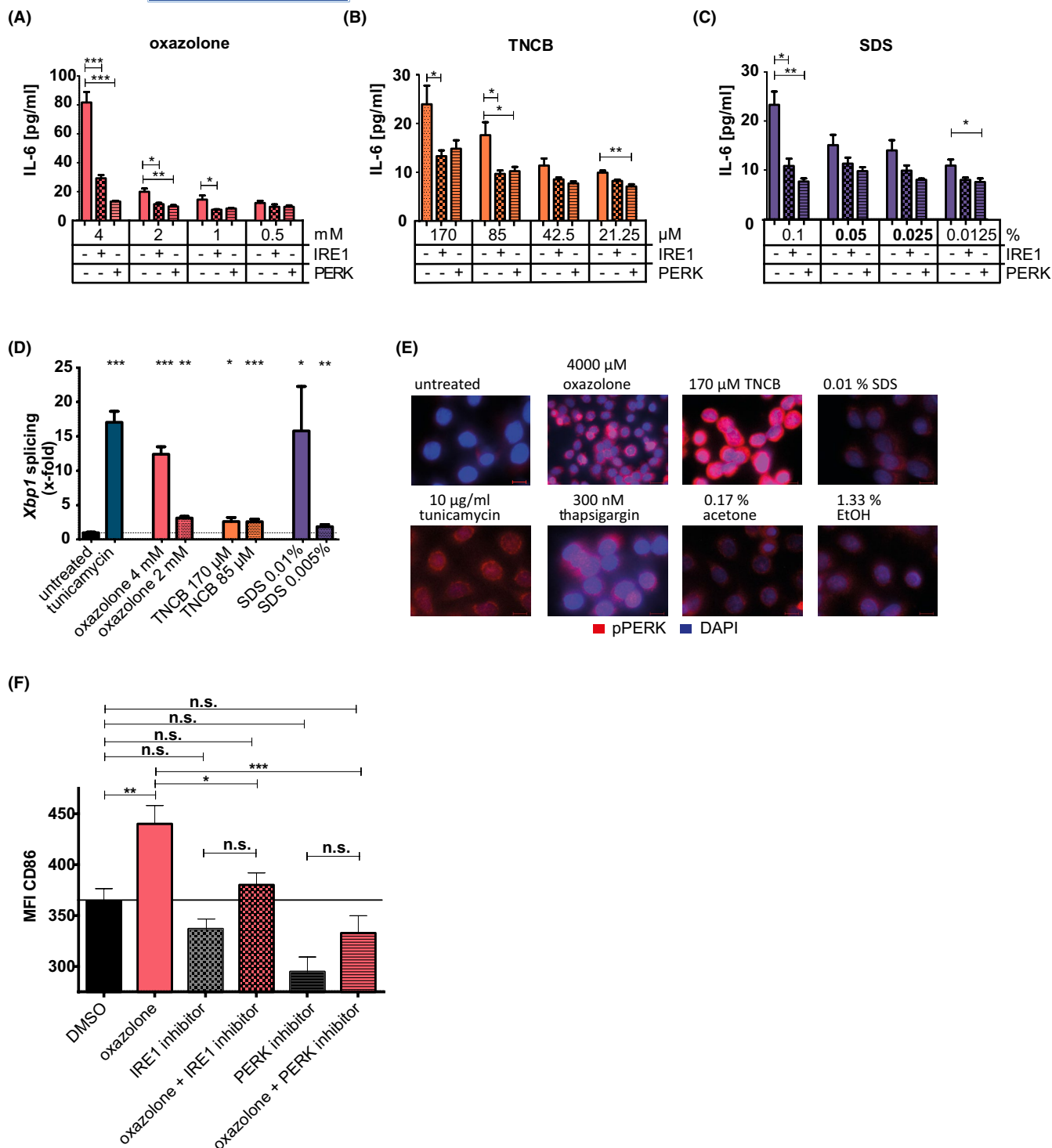
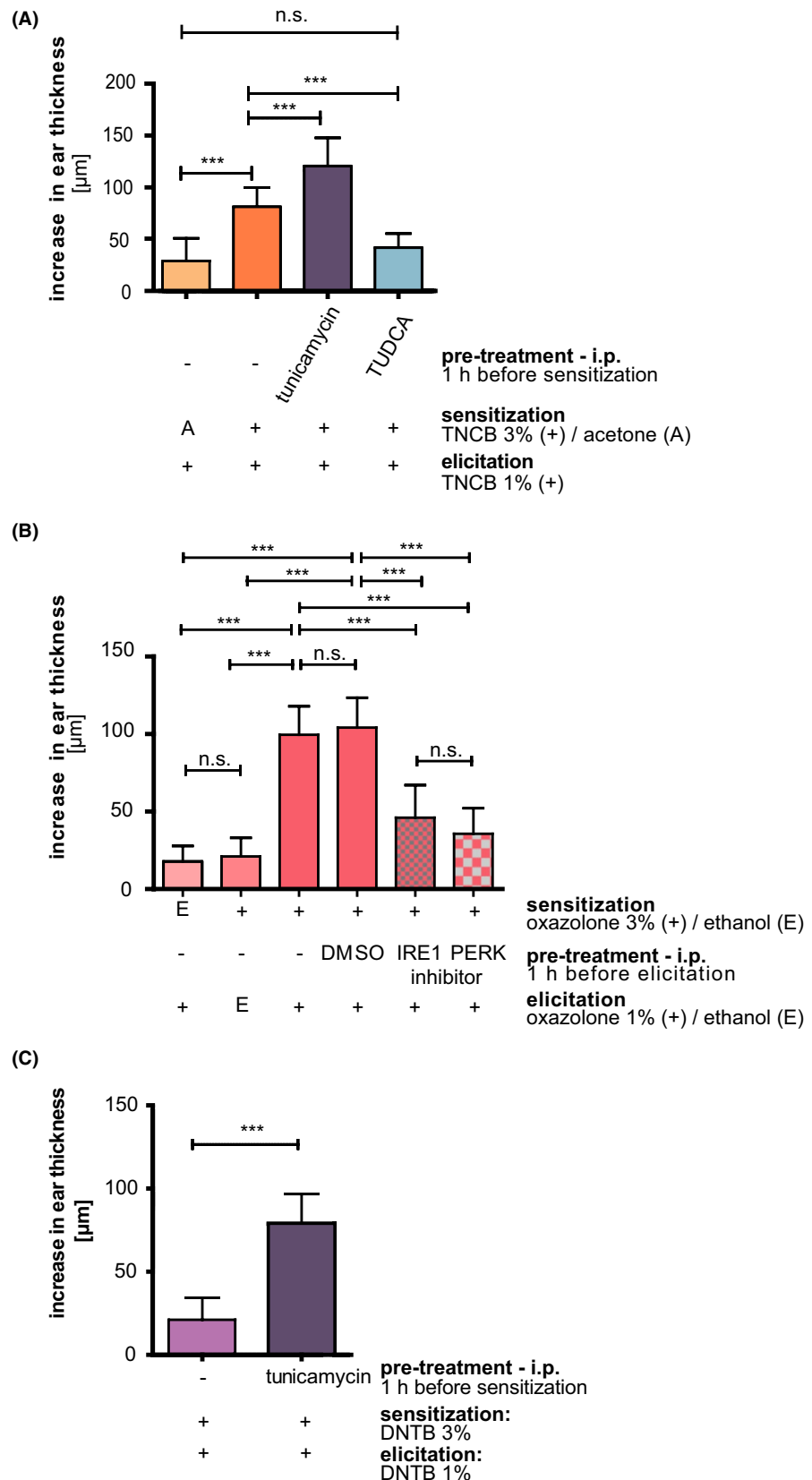


FIGURE 4 Influence of UPR modulation on human keratinocyte cytokine production and activation marker expression in a human keratinocyte/DC co-culture assay. (A–C) Levels of IL-6 production by the human keratinocyte cell line HaCaT were analyzed by ELISA after stimulation of cells for 24 h with chemical concentrations as described with or without pretreatment for 1 h using IRE1α (25 μM) or PERK (10 μM) inhibitor. IL-6 levels in pg/mL from three independent experiments are shown ± SD. *t* test, ****p* < .0001. (D) HaCaT cells were treated as indicated for 6 h, and *Xbp1* splicing was analyzed by semi-quantitative PCR. Data show x-fold *Xbp1* mRNA splicing from 3 independent experiments ± SD. *t* test, ****p* < .0001. (E) HaCaT cells were treated as indicated and stained after 6 h of chemical stimulation with oxazolone (4 mM), TNCB (170 μM), SDS (0.01%), thapsigargin (300 nM), or tunicamycin (10 μg/mL) with an anti-phospho-PERK (pPERK) antibody (red). Nuclei were visualized by DAPI staining (blue). Scale bar = 50 μm. Pictures from one representative of two independent experiments are shown. (F) Expression of activation marker CD86 mean fluorescence intensity (MFI) levels on THP-1 cells after co-culture with chemical-treated HaCaT cells as determined by FACS-analysis. MFI levels from 10 independent experiments are shown ± SD. *t* test, ****p* < .0001

FIGURE 5 Modulation of CHS responses by activation or inhibition of the UPR. (A) Mice were pre-treated by i.p. injection with tunicamycin (20 μ g) or TUDCA (5 mg/mL) 1 h before sensitization with 3% TNCB or mock-sensitization with acetone on the shaved abdomen. 5 d later, mice were challenged on the ears. (b) Mice were sensitized on the abdomen with 3% oxazolone or treated with the solvent ethanol. 5 d later, mice were pre-treated by i.p. injection of DMSO (solvent) or the IRE1 α (25 μ M) or PERK (10 μ M) inhibitor 1 h prior to challenge with 1% oxazolone on the ears. (A, B) Ear thickness was measured before and 24 h after challenge. Data show increase in ear thickness [μ m] $\pm$ SD of three (two for DMSO control group) independent experiments with 4 mice per group and experiment (ie, total number 12 (8 for DMSO) mice/condition). (C) Mice received i.p. injection of DMSO (-) or tunicamycin 1 h before abdominal sensitization with 3% DNTB. 5 d later, mice were challenged by application of 1% DNTB to the ears. Ear thickness was measured before and 24 h after challenge. Data show increase in ear thickness [μ m] $\pm$ SD of three independent experiments with 3 mice per group and experiment (ie, total number of 9 mice/condition)



Importantly, Figure 6C shows that topical ear pretreatment of mice sensitized to oxazolone with either inhibitor results in a significant reduction of ear swelling responses.

This experiment indicates that topical application of the small molecule inhibitors is not only sufficient to block UPR activation but also to prevent the CHS response in a clinically relevant setting of already sensitized mice.

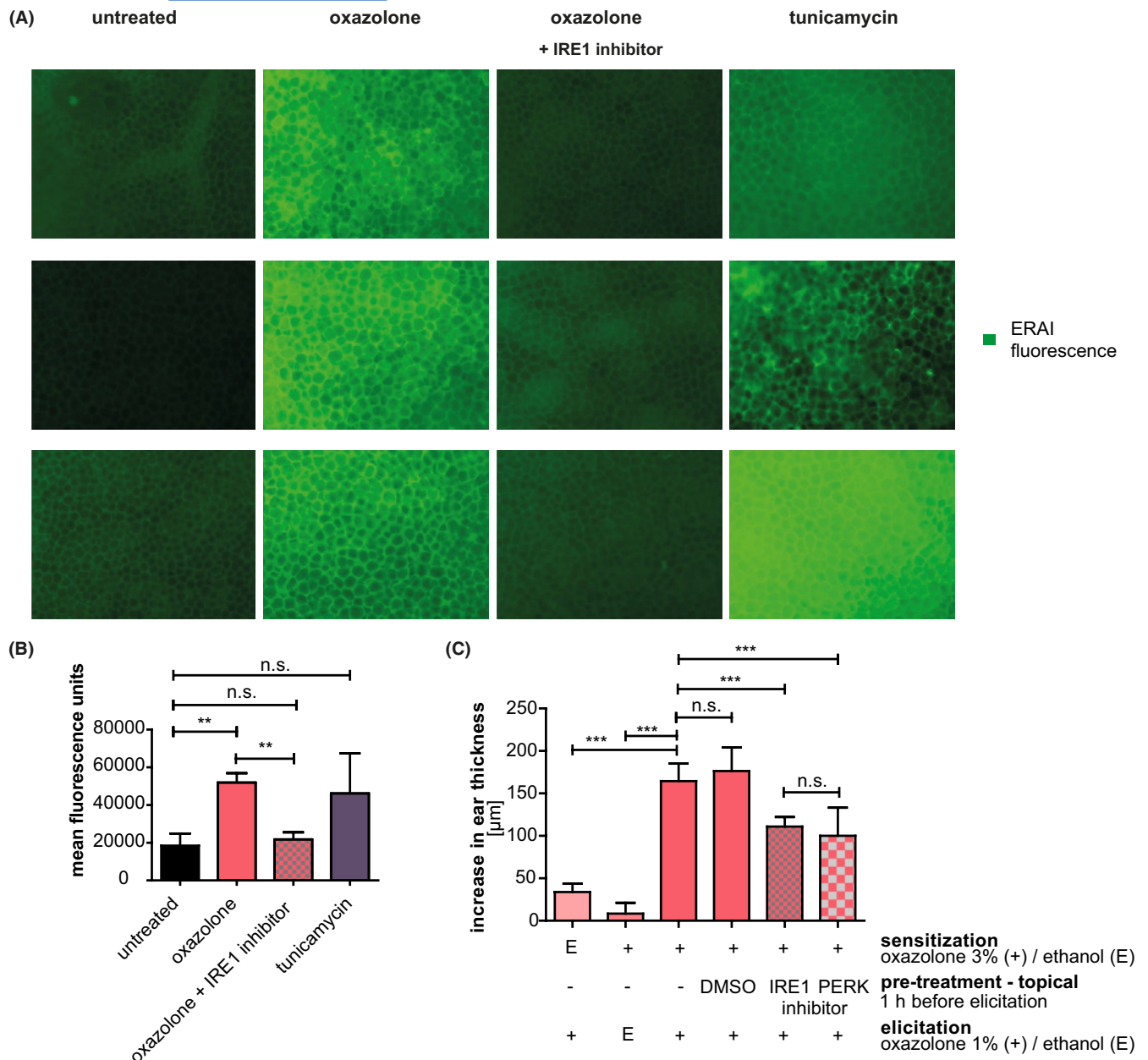


FIGURE 6 Topical IRE1 α inhibitor application reduces IRE1 α pathway activation and CHS response to oxazolone. (A) Ears from ERAI mice were left untreated or treated topically as indicated with tunicamycin (10 μ g/mL) or oxazolone (3%) for 6 h with or without 1 h pretreatment with IRE1 α inhibitor. Pictures show ear sheets from three independent experiments (top to bottom) for each condition. Activation of *Xbp1* splicing is revealed by green fluorescence. (B) shows the quantification of the results from (A) as mean fluorescence units $\pm$ SD. (c) Mice were sensitized on the abdomen with 3% oxazolone or treated with the solvent ethanol. 5 d later, mice were pre-treated by topical application of DMSO (solvent) or the IRE1 α - (25 μ M) inhibitor 1 h prior to challenge with 1% oxazolone on the ears. Ear thickness was measured before and 24 h after challenge. Data show increase in ear thickness [μ m] $\pm$ SD of two independent experiments with 8 to 12 mice/condition (numbers below bars)

4 | DISCUSSION

Chemical-induced skin inflammation is the key event of ACD and requires activation of the innate immune system and tissue stress and damage responses.¹⁹

Here, we show that oxazolone and TNCB activate all three UPR branches in keratinocytes. This is in line with previous results in the monocytic THP-1 cell line showing that the extreme sensitizer

2,4-dinitrofluorobenzene (DNFB) activated the PERK-eIF2 α signaling pathway.³¹

Strikingly, our data show that TNCB treatment resulted in the weakest induction of the IRE1 α pathway as compared to oxazolone but induced a strong phosphorylation of PERK, indicating that not all sensitizers activate the same UPR branches to the same extent. If this discrepancy between the strength of activation of the different UPR signaling pathways by different

sensitizers reflects their intrinsic potential for sensitization *in vivo* or not remains to be shown, the same weak activation by TNCB was observed regarding ATF-6 expression. Whether or not this is due to a direct link between ATF-6 and IRE-1 activation remains to be shown. While Yoshida et al. propose that ATF-6 activation increases XBP-1 splicing³⁷ the opposite effect was reported by Walter et al.³⁸, showing that silencing of ATF-6 results in increased XBP-1. Nevertheless, using a chemical chaperone-like TUDCA or small molecule inhibitors for IRE-1 or PERK *in vivo*, we were able to abrogate the sensitization to TNCB or elicitation of oxazolone CHS, respectively. This suggests that inhibition of either pathway is sufficient to reduce the tissue stress response and, most importantly, CHS.

HaCaT cells were used to address the question, whether or not the modulation of UPR activation might also play a role in the activation of human cells. Both inhibitors reduced the chemical-induced cytokine production. This is in line with the observation that the UPR enhances TLR signaling and promotes activation of pro-inflammatory signaling pathways.^{22,39} Thus, as shown for the innate immune response to sensitizers, all UPR branches operate in a non-redundant manner and are all needed to overcome a critical activation threshold.⁴⁰ Further studies will have to address whether the differential activation of the UPR branches by TNCB and oxazolone is due to a general difference between the two sensitizers.

Interestingly, we also observed a strong activation of the UPR with the irritants SDS and crotonoil, indicating that besides the direct protein binding and modification by sensitizers the so-called toxic effect of irritants and their intrinsic ability to modify protein structures plays an important role in the activation of the UPR. SDS denatures proteins thus most likely driving unfolded protein cargo accumulation. The active compound in crotonoil is phorbol 12-myristate 13-acetate (PMA), a potent activator of protein kinase C (PKC). While PKC- β plays a role in contact dermatitis, for example, in the activation of DCs,⁴¹ the exact mechanism of UPR induction by crotonoil remains to be determined.

Combinations of weak skin sensitizers or sensitizers with irritants result in synergistic or additive effects on the efficiency of sensitization.^{10,42} Addition of SDS to nickel in the murine system⁴³ compensated for the inability of nickel to bind to and activate murine TLR4. Direct binding of nickel to human TLR4 is essential for the innate inflammatory response.⁴⁴ Likewise, the addition of the irritant SDS to the weak contact allergen/tolerogen DNTB resulted in a full sensitization to DNTB.³⁵ To address the ability of the UPR to provide such heterologous pro-inflammatory signals,^{9,40} we used 2,4-dinitrothiocyanobenzene (DNTB). Both DNTB and the structurally related 2,4-dinitrochlorobenzene (DNCB) form dinitrophenyl group coupled proteins. However, while DNCB is a strong sensitizer, DNTB is only a weak sensitizer or even tolerogenic. Remarkably, as described for SDS, UPR activation also enabled a sensitization to DNTB and enhanced the CHS response to the strong sensitizer TNCB. In combination with our observation that SDS rapidly activated the UPR branches and induced cytokine production in a

UPR-dependent manner, this enhanced sensitization as a result of UPR activation by tunicamycin might explain the described effects of SDS treatment on the weak sensitizing potencies of sensitizers such as DNTB. Whether or not the efficiency to activate the UPR correlates with the sensitizing potency of sensitizers remains to be addressed. Interestingly, we have already shown that mice deficient for nuclear-related factor2 (Nrf2), which is involved in internal redox homeostasis, show an increased sensitization potential toward weak sensitizers.⁴⁵

Our data firmly establish the UPR as an important mechanism involved in the orchestration of sensitizer-induced skin inflammation. This tissue stress response in concert with the innate immune response is needed to drive CHS responses. The importance of the UPR in immune responses is being recognized, and mechanistic parallels to pro-inflammatory signaling such as NF- κ B activation have been shown.^{22,39} The cross-talk between the UPR and the innate immune response will be investigated in future studies also including potential cross-talks between other innate immune cells playing essential roles in the CHS induction like neutrophils,⁴⁶ basophils, and mast cells.⁴⁷ It remains to be shown, whether the modulation of the UPR activation can be also observed in lesional vs nonlesional skin from patients. Ideally, control subjects or patients with a known allergy to a specific substance would be recruited, the substance applied, 6 h later punch biopsies taken and the epidermis analyzed for the influence of existing sensitization on the strength of UPR activation.

Importantly, as shown here, the small molecule UPR inhibitors can also be applied topically, potentially allowing for the formulation of a cream as basis for a localized topical CHS treatment. However, the reduction in ear swelling response after topical application of the inhibitors was lower than after *i.p.* injection, necessitating further work to address the optimal dose. Interestingly, the chemical chaperone TUDCA has already been included in several human phase 1–3 trials for the treatment of different diseases. While ACD is usually not a life-threatening disease and would not warrant severe side effects as they might occur due to a prolonged systemic UPR inhibition, localized and topical application might be beneficial compared to corticosteroid treatment.

In addition, for therapeutic applications, the time kinetic allowing for an effective treatment in patients remains to be addressed in further studies as well as potential benefits or increases in unwanted side effects from blocking different branches of the UPR at the same time remain to be shown. Last but not least, if the inhibitors can indeed be applied in a formulation for topical application in a cream that is stable enough for storage and can be used to prevent CHS in mice and eventually reduce ACD reactions in sensitized humans remains an interesting question.

In conclusion, our work shows the ability of sensitizers and irritants to activate all three branches of the UPR and indicates that modulation of the UPR results in a modulation of the CHS response. This not only provides new mechanistic insight into the processes underlying ACD but also provides novel therapeutic targets for the treatment of ACD.

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CONFLICT OF INTEREST

All authors declare no conflict of interest directly related to the work. Dr. Apostolova reports personal fees from Novartis Pharma, non-financial support from Takeda, outside the submitted work. Dr. Zeiser reports personal fees from Novartis, personal fees from Incyte, personal fees from Mallinckrodt, outside the submitted work. T. Jakob reports grants, personal fees, and non-financial support from Novartis, grants, personal fees, and non-financial support from ALK-Abello, personal fees and non-financial support from Allergy Therapeutics/ Bencard, personal fees from Allergopharma, personal fees from Thermo Fisher, outside the submitted work. All other authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

PRE and SFM developed the overall concept and designed research. FG, LV, MF, and PRE conducted the experiments, FG and PRE analyzed data. PA, RZ, and TJ provided reagents and/or conceptual input. FG and PRE drafted the manuscript. PRE and SFM provided funding and supervision. All authors discussed the results and contributed to the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Supplementary Material and Methods S1:

Chemicals

Crotonoil, N-acetyl-cysteine (NAC), oxazolone, sodium dodecyl sulfate (SDS), tunicamycin, 1-chloro-2,4,6-trinitrochlorobenzene (TNCB) were from Sigma Aldrich, Taufkirchen, Germany. IRE1 α inhibitor II, PERK inhibitor GSK2606414, tauroursodeoxycholic acid (TUDCA) were from Calbiochem, Darmstadt, Germany. 2,4-dinitrothiocyanobenzene (DNTB) was from Alfa Aesar, Karlsruhe, Germany, CM-H₂DCFDA was from Invitrogen/Thermo Scientific Dreieich, Germany, and hydrogen peroxide (H₂O₂) was from J.T.Baker/VWR Darmstadt, Germany.

Optimal doses used for *in vitro* experiments were chosen based on incomplete splicing of *Xbp1* and according to a lactate dehydrogenase (LDH) cytotoxicity analysis resulting in 10–20% toxicity after 6 h. This toxicity range is required for the efficient activation of innate immune cells without masking the chemical specific effects by unspecific cell death (1,2). For tunicamycin as a positive control for UPR activation, 10 μ g/ml were used throughout the study.

Cell culture

The murine keratinocyte cell line Pam212 cells was maintained in RP10 medium (RPMI1640 (Invitrogen/Thermo Scientific Dreieich, Germany) supplemented with 2 mM L-glutamine, 12.5 μ M beta-mercaptoethanol, 25 mM HEPES and 10% FCS (Lonza, Cologne, Germany). The human keratinocyte cell line HaCaT was maintained in DMEM (Gibco, Thermo Scientific Dreieich, Germany) supplemented with 10% FCS and 2 mM L-glutamine. All cells were kept at 37°C in a humidified 5% CO₂ incubator. For co-culture experiments, THP-1 cells (donor: Cell line services, Eppenheim, Germany, source: BIOS, Freiburg, Germany) were kept in RPMI1640 supplemented with 4 mM L-glutamine, 50 μ M beta-mercaptoethanol, 25 mM HEPES and 10% FCS. HaCaT-THP-1 co-culture was performed in THP-1 medium.

CHS experiments

CHS was induced as described (3). In brief, in case of UPR modulator application these or respective solvents were injected (i.p.) 1 h before sensitization or challenge as indicated. For topical application, 20 μ l of UPR modulators or solvents were applied to the ear skin as indicated. Mice were sensitized by application of 100 μ l oxazolone or TNCB or DNTB (all 3% w/v) or respective volumes of solvent controls on the shaved abdomen (3–5 mice/group/experiment). Ear thickness was measured on day 5 with a pocket micrometer gauge (Mitutoyo, Japan) and mice were challenged by application of 20 μ l sensitizer (1%) or respective solvent control to the ears. 24 h later ear thickness was measured again and increase in ear thickness was calculated. Shown are results from at least three independent experiments.

Co-culture assay and FACS analysis of activation marker expression

To test the ability of keratinocytes to activate innate immune cells, especially DCs, a modified version of the COCAT assay(4,5) was used. In brief, HaCaT cells were seeded (96-well flat bottom plates at 1.25x10⁵ cells/200 μ l) and cultured for 48 h before pretreatment with

inhibitors or DMSO as solvent control for 1 h. Afterwards, oxazolone or solvent control DMSO was added for 6 h. To specifically assess the effect of UPR modulation on the ability of keratinocytes to activate DCs, medium with oxazolone was removed and HaCaT cells rinsed twice with THP-1 medium. After washing away oxazolone/DMSO and inhibitors, THP-1 cells at 8×10^4 cells/180 μ l were added and co-cultured for 18 h. CD86 expression as activation marker was analyzed on THP-1 cells using flow cytometry (mouse anti-human CD86 FITC (BD Bioscience, Heidelberg, Germany) 1:25 in PBS/10% FCS; 30 min, 4°C). DAPI was added right before analysis (final conc. 500 ng/ml) to allow exclusion of dead cells. Raw data was collected using a FACSCanto II from BD and analyzed using FlowJo.

Ear sheets for fluorescence microscopic analysis of ERAI ear skin

After incubation of ears from ER-stress activated indicator (ERAI)(6) mice (ubiquitously expressing a genetic reporter construct that activates GFP expression after splicing of *Xbp1*) with the respective chemicals (1 h pretreatment for inhibitors, followed by 6 h for Oxazolone) ears were split into an upper and a lower part by mechanical disruption using two tweezers. After mounting of the upper part on a microscope slide, the previously inner side was analyzed using a Zeiss Fluorescence microscope. Pictures were taken using the same settings for all conditions. Mean fluorescence units were determined using ImageJ software.

ELISA

Determination of IL-6 release into the cell culture supernatant was performed using a Becton-Dickinson OptEia ELISA Kit. In brief, 7500 HaCaT cells were seeded in 96-Well-Plates in 200 μ l DMEM/10%FCS and incubated for 48 h at 37°C, 5%CO₂. Next, medium was removed and 200 μ l fresh medium containing inhibitors or solvents were added. After 1 h pre-incubation, cells were stimulated by addition of the respective sensitizers or irritants. After 24 h, cell culture supernatant was removed and IL-6 release in 100 μ l determined.

Immunofluorescence staining for PERK-phosphorylation

Cells were seeded into 8-well chamber slides (2×10^5 /400 μ l) and incubated o.n. After stimulation for 6 h, cells were washed with PBS and fixed by addition of 200 μ l ice cold acetone for 20 min at -20°C. Acetone was aspirated and cells rinsed 3x with PBS before blocking for 1 h at RT with PBS/ 5% BSA /0.3% triton-X100. Phospho-PERK was stained with a specific antibody (Biorbyt Ltd, Cambridge, UK) at a 1:500 dilution o.n. in a humidified box at 4°C. Cells were washed with PBS and labeled with a fluorescent secondary antibody (goat anti rabbit IgG, alexa-fluor488, Thermo Scientific, Schwerte, Germany) at a 1:1000 dilution for 2 h at RT. After rinsing cells three times, nuclei were stained by addition of DAPI (200 μ l of 100 ng/ml stock) for 30 s. Stained cells were washed again with PBS and covered with fluorescence mounting medium (Dako, Hamburg, Germany) and a cover slip. Pictures were taken using the same settings for all stimulations.

NF- κ B activation assay

Pam212 cells stably transfected with a Luciferase reporter plasmid (pGL4.32[luc2P/NF- κ B-RE/Hygro]) for NF- κ B activation (Promega). Cells were seeded at 2.5×10^4 cells per 96-well in 200 μ l RP10 medium. After incubation o.n., medium was removed and fresh medium containing inhibitors or solvent was added. Cells were pre-treated for 1 h before addition of stimulating chemicals. NF- κ B activation was determined by removal of supernatant 6 h later

and luminescence was detected in a Tekan ELISA-Reader after addition of 100 µl BrightGlo (Promega) per well.

p65 nuclear translocation assay

Pam212 cells were seeded on rat collagen I coated coverslips in 24-well plates. After o.n. culture, cells were stimulated for 1 h. Medium was removed and cells washed once with 200 µl 1x PBS for 5 min. To fix cells, 200 µl of 4% paraformaldehyde (PFA) in PBS was added for 10 min. The PFA was aspirated and the cells washed three times with PBS for 5 min. Cells were permeabilized with 0.25 % Triton X-100 (Sigma Aldrich, Germany) in PBS for 10 min at RT. The cells were washed three times and 200 µl of the primary antibody (mouse anti-mouse NF-κB (p65), clone F-6, Santa Cruz Inc., Germany) diluted in antibody diluent (DAKO, Germany) (final concentration of 4 µg/ml) was added before o.n. incubation at 4°C in a humid chamber. Cells were washed three times with PBS before addition of 200 µl fluorophore labeled secondary antibody (goat anti-mouse IgG Alexa Fluor 555, Thermo Fisher, Germany) diluted in antibody diluent (final concentration of 10 µg/ml) and 2 h incubation at RT. Cells were rinsed three times with PBS and nuclei were stained with DAPI (200 µl of 100 ng/ml DAPI in PBS). Glass slides were mounted using Mowiol mounting medium before analysis using a Zeiss fluorescence microscope. The nuclear translocation of p65 was assessed using the method described by (7). Pictures of three random fields per sample were taken and the total cell numbers as well as the number of cells with increased nuclear translocation of p65 were counted. Increased translocation was defined as the lack of a shadow in the place of the nucleus. Finally, the ratio of cells with increased nuclear translocation was calculated.

Semi-quantitative PCR protocol (*Xbp1* splicing)

Cells were seeded in 24-well plates (2x10⁵/400 µl) and incubated o.n. 1 µg of Trizol (Invitrogen, Germany) isolated RNA was transcribed into cDNA with the iScript reverse transcription-kit (BioRad, München, Germany) and PCR was performed with the Maxima Polymerase (Thermo Fisher Scientific, Germany) and *Xbp1* specific primers (see Supplementary Table 1) (8). PCR program used: 95°C for 5 min; followed by 34 cycles 95°C for 1 min, 58°C for 30 s and 72°C for 30 s; final 72°C for 5 min. 100 ng of PCR products were loaded on a 3% agarose gel containing ethidium bromide and analyzed after electrophoresis at 70 V for 2 h in 1x TAE buffer. Quantification of spliced *Xbp1* mRNA as a percentage of total *Xbp1* was done using ImageJ (9) software.

Quantitative real time PCR (RT-qPCR)

cDNA was prepared as described for semi-quantitative-PCR. For RT-qPCR, 12.5 ng of cDNA was used. Quantitative PCR using Maxima HotStart SybrGreen Mix (Thermo Fisher) was carried out in a LightCycler 1.5 (Roche) at 95°C for 10 min; followed by 45 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and subsequent melting curve analysis. Beta-actin was used as the control for calculation of fold change in expression ($2^{-\Delta\Delta Ct}$) by subtracting the Ct values of the house-keeping gene beta-actin minus the Ct value of the gene of interest (ATF-6) of the chemical treated samples divided by the values derived from subtraction of Ct values from the house keeping gene beta actin minus the CT values of ATF-6 of the untreated control as described by Schmittgen and Livak (10).

Table 1: Primer sequences

RT-Primer name:	
Murine <i>Xbp1</i> fwd.	5'- ACACGCTTGGGAATGGACAC-3'
Murine <i>Xbp1</i> rev.	5'- CCATGGGAAGATGTTCTGGG-3'
Human <i>Xbp1</i> fwd.	5'-TTACGAGAGAAAACATCATGGC-3'
Human <i>Xbp1</i> rev.	5'-GGGTCCAAGTTGTCCAGAATGC-3'
RT-qPCR Primer name:	
ATF6 fwd.	5'-GAGAGGTGTCTGTTTCGGGG-3'
ATF6 rev.	5'-CAACGTCGACTCCCAGTCTT-3'

Supplementary references:

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Supplementary Figure legends:

Supplemental Figure 1: Dose kinetics of XBP-1 splicing in Pam212 cells (a-d) Pam212 cells were treated for 6 h with **(a)** tunicamycin **(b)** oxazolone **(c)** TNCB or **(d)** SDS at indicated concentrations and *Xbp1* splicing was determined. **(a-d)** Left panel shows results of the quantitative analysis of x-fold splicing of one experiment. The right panel shows the result for each chemical with an upper unspliced (*uXbp1*, 171 bp) and a lower spliced (*sXbp1*, 145 bp) band.

Supplemental Figure 2: XBP-1 splicing in primary human keratinocytes (a) Primary human keratinocytes were treated for 6 h with indicated concentrations of TNCB, SDS, oxazolone, tunicamycin or the solvent controls acetone and ethanol and *Xbp1* splicing was determined. Left panel shows results of the quantitative analysis of x-fold splicing of three independent experiment (for acetone and ethanol from one experiment). The right panel shows the result for each chemical with an upper unspliced (*uXbp1*, 171 bp) and a lower spliced (*sXbp1*, 145 bp) band from one experiment.