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Gliadin-dependent UPR induction directly triggers the expression of TG2 and pro-inflammatory cytokines, dysregulates intestinal permeability, and reduces CFTR expression in intestinal epithelial cells of celiac disease patients

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Abstract

Background Celiac disease (CD) is an autoimmune disorder that primarily affects the gut of genetically predisposed individuals and is triggered by gliadin peptides (PT) produced by the digestion of gluten. Although inappropriate activation of the immune system is thought to be the main trigger of CD, the interaction between PT and intestinal epithelial cells (IECs) remains a key step. Recently, the possible involvement of ER stress in the pathogenesis of CD has been pointed out, although its role is still largely unclear. Therefore, discovering the molecular mechanism(s) activated in IECs exposed to PT represents a unique opportunity to better understand the disease and define new potential therapeutic targets.

Methods In this study we used three different experimental set-ups: intestinal biopsies from CD patients and non-CD control subjects, an in vitro model, based on human CaCo-2 cells, and an ex vivo model, based on our recently described mouse gut-ex-vivo system (GEVS), with the latter two systems were studied after stimulation with gliadin peptides (PT). To understand the signaling pathways involved we monitor the expression of a number of proteins by qPCR, Western blotting, IF, ELISA or a combination of tests. Specifically, we have analyzed the level of CD, ER stress, tissue permeability, and inflammation markers.

Results Indeed, our study demonstrated a prompt induction of the transcription factors ATF4, ATF6 and XBP1 in IECs upon PT exposure. Thus, the upregulation of TG2 and downregulation of CFTR were prevented by ER stress inhibition/

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buffering by a pharmacological chaperone, also leading to restored physiological expression of OCL, CLD-2 and CLD-15, while preventing the expression of IFNγ, IL-15 and IL-17 A.

Conclusion Overall, our analysis has highlighted the key role of ER stress in the pathogenesis of CD and identified the chemical chaperones as a new potential valuable therapeutic treatment for CD patients.

Keywords CD, Chemical chaperone, UPR, Endoplasmic reticulum stress, IEC

Introduction

Celiac disease (CD) is a complex autoimmune disease characterized by an increased immune response to gluten, a protein found in wheat, barley, rye, and their derivatives [1]. It is a multifaceted disease in which both genetic and environmental components influence the pathogenesis. While the association between gluten consumption and the development of CD has long been known, the molecular mechanisms underlying its pathogenesis are still the subject of intense research [2].

Genetic predisposition plays a central role in CD, with most affected individuals expressing human leukocyte antigen (HLA) class II molecules, particularly HLA-DQ2 or HLA-DQ8 alleles. These genetic factors are crucial for triggering the immune response to gluten. The adaptive immune system plays a central role in the development of celiac disease, with gluten peptides, which are produced during the digestion of gluten in the gastrointestinal tract, that are presented to T cells by antigen-presenting cells, leading to T cell activation [2].

The intestinal mucosa, especially the small intestine, is the primary site of manifestation of CD. The activation of gluten-specific T cells triggers an inflammatory reaction in the intestinal mucosa, which leads to the production of proinflammatory cytokines such as interleukin-15 (IL-15), interleukin-17 A (IL-17 A) and interferon-gamma (IFNγ). This cytokine milieu stimulates the recruitment and activation of immune cells, enhances the immune response, and perpetuates the inflammatory cascade, with damage to the intestinal villi being one of the main hallmarks of CD pathology. The persistent inflammation and immune-mediated destruction of the villous structure leads to villous atrophy, resulting in malabsorption of essential nutrients and clinical manifestations such as diarrhea, weight loss and nutrient deficiency [3].

Although the role of the immune system has been extensively studied, the role of intestinal epithelial cells (IECs) is still unclear. In fact, IECs– the main component of the intestinal epithelium– are the key component of the intestinal barrier and the primary point of contact between the body's tissues and food, the microbiota, and all other components of the intestinal "soup".

In the complicated landscape of CD pathogenesis, intracellular transglutaminase 2 (TGM2 or TG2) plays a key role, particularly in IECs. TG2, an enzyme with multiple functions, plays a central role in the modification of gluten peptides and contributes to the immunogenicity that triggers the autoimmune response characteristic of CD [4].

Recently, has been increasingly recognized that CFTR (cystic fibrosis transmembrane conductance regulator), a chloride ion channel primarily known for its role in cystic fibrosis, maybe involved in the pathogenesis of CD. Emerging evidence suggests that CFTR, which is expressed by IECs, may contribute to the dysregulation of ion transport and immune responses associated with CD. Gliadin peptides are known to bind the extracellular domains of CFTR, promoting its degradation [5]. The resulting altered ion transport could affect the luminal environment and influence the solubility and presentation of gliadin peptides to the immune system.

We have observed signs in a mouse model of CD that may indicate the induction of ER stress during disease pathogenesis, although the role of this stress-related signaling pathway is still unclear [6, 7].

The endoplasmic reticulum (ER) is a multifunctional organelle involved in cellular homeostasis and in the synthesis, folding and trafficking of proteins. When there is an imbalance between the load of unfolded or misfolded proteins and the folding capacity of the ER, the cell is exposed to endoplasmic reticulum stress (ER stress) [8]. Various cellular insults such as nutrient deprivation, oxidative stress and changes in calcium homeostasis can trigger ER stress and activate a complex signaling network known as the Unfolded Protein Response (UPR) orchestrated by three transmembrane ER proteins: IRE1, PERK and ATF6. Once activated, these sensors initiate a series of adaptive responses aimed at restoring ER homeostasis, mediated by the major transcription factors (TFs) XBP1, ATF4 and ATF6, respectively. The UPR promotes protein folding by upregulating chaperone proteins, enhances ER-associated degradation (ERAD) to eliminate misfolded proteins, and attenuates global protein synthesis to reduce ER workload. However, prolonged or severe ER stress can lead to apoptotic cell death, emphasizing the dual role of the UPR in cell survival and death. The consequences of unresolved ER stress have been implicated in various pathological conditions, including neurodegenerative diseases, metabolic disorders, and inflammation-related autoimmune diseases [8, 9]. An important mediator of the interaction between ER stress and inflammation is the transcription

factor Nuclear Factor Kappa B (NF-kB), a central regulator of the immune response that controls the expression of several pro-inflammatory genes [10], and also regulates the expression of TG2 [11].

Since the only currently available treatment for CD patients is a lifelong gluten-free diet (GFD), which is not 100% effective and whose strict adherence is a major challenge, understanding the molecular mechanisms underlying the development of CD could provide new insights for the development of new effective therapies.

In this context, we show here that ER stress is induced in IECs of CD patients and in both in vitro and ex vivo CD models immediately after gliadin exposure and, importantly, is directly responsible for the occurrence of key hallmarks of CD, such as inflammation, TG2 and CFTR dysregulation and altered intestinal permeability, mainly through the activation of NF-kB. We show that administration of chemical chaperons may represent a novel valuable approach to attenuate the deleterious effects of gliadin peptides on the intestinal tissue of CD patients, and may also be effective in active CD.

Results

In this study we used intestinal biopsies from non-CD and CD patients, and two CD models: an in vitro model, based on CaCo-2 cells, and an ex vivo model, based on our recently described gut-ex-vivo system (GEVS) [7, 12, 13], stimulated with gliadin peptides (PT).

ER stress induction in intestinal tissues from CD patients

Recently, the possible involvement of ER stress has been demonstrated in both in vitro and in vivo models of CD [6], although its induction in patients has not yet been demonstrated.

Therefore, we decided to test this hypothesis using intestinal biopsies from pediatric CD patients. For this purpose, biopsies from 8 non-celiac disease (NCD) and 9 CD patients (Supplementary Table 1) were used, and the presence of CD markers, such as the expression of TG2, C-X-C motif chemokine receptor 3 (CXCR3) and tight junction (TJ), were analyzed by qPCR, together with the expression of CFTR. The data presented in Fig. 1 clearly show increased expression of TG2 (Fig. 1A) and CXCR3 (Fig. 1B left panel), together with dysregulated expression of CFTR (Fig. 1B, right panel), in the CD group compared to controls (NCD). In addition, dysregulated expression of TJ proteins was observed, indicating impaired tissue permeability, which is typical for CD (Fig. 1C). CD-dependent tissue inflammation was also observed, as evidenced by increased expression of the pro-inflammatory cytokines IFNγ, IL-15 and IL-17 A (Fig. 1D). Of note, in the same samples an increased expression of the three ER stress markers ATF4, ATF6 and XBP1s was also

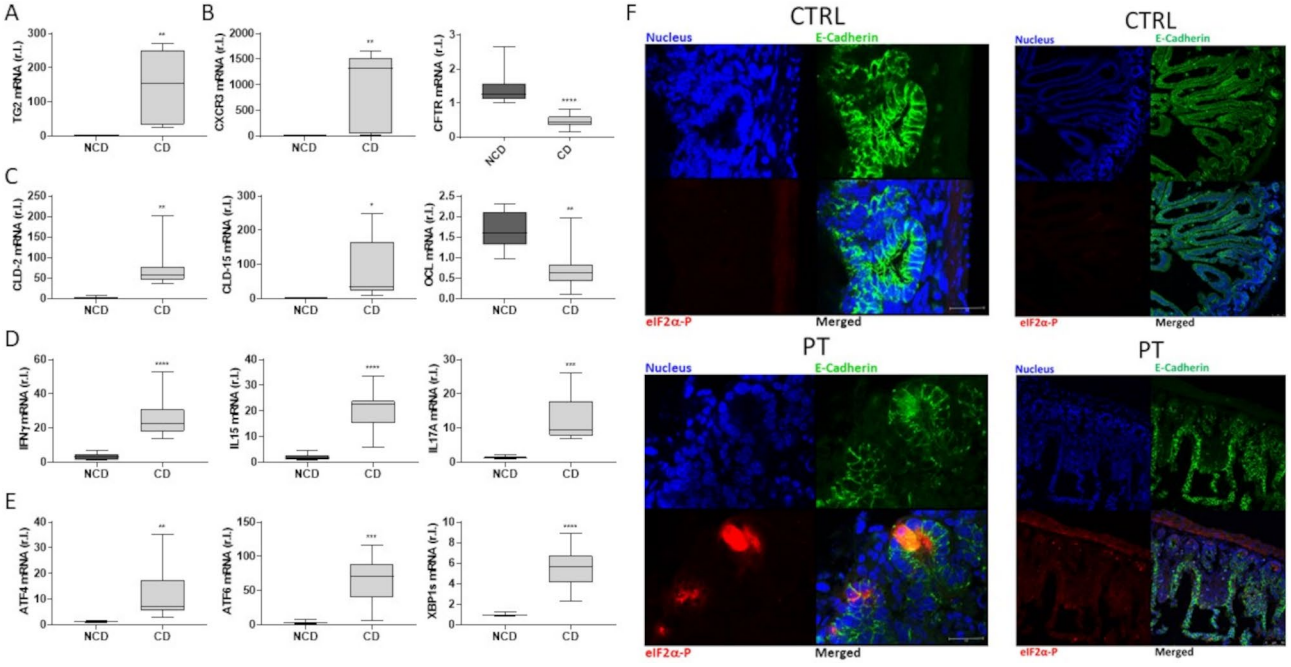

Fig. 1 PT stimulated ER stress induction in CD patients and mice gut tissue. Gut biopsies from 8 non-CD (NCD) and 9 CD pediatric patients were collected and the expression of TG2 (**A**), CXCR3 (B, left panel), CXCR3 (**B**, right panel) CLD-2, CLD-15 and OCL (**C**), IFNγ, IL15, IL17A (**D**), ATF4, ATF6, and XBP1s (**E**) was evaluated by qPCR and reported in the corresponding box plot

S.i. from GF mice was untreated or exposed to PT (5 mg/mL) for 9 h, in GEVS. Tissue distribution of the ER stress marker eIF2α-P was evaluated (red) in IEC evidenced by the anti-E-Cadherin (green), while nuclei were stained by DAPI (blue; F). Representative images of experiment performed in triplicate; scale bar = 10 µm

detected (Fig. 1E), suggesting that this stress-induced signaling pathway is also activated in CD patients.

To support the concept that IECs are the primary site of gliadin peptides activity, driving ER stress induction, we examined the expression of the ER stress marker eIF2α-P, in the small intestine of our mouse model of CD, consisting of Balb/c mice feed with a gluten-free diet, for at least three generations, than exposed to PT and cultivated in our gut-ex-vivo system (GEVS) [7, 12, 13]. To this end, the tissues were exposed or not to PT (2.5 mg/ml, 3 h), and then stained with an anti-eIF2α-P (red) antibody. IECs were stained by using an anti-E-Cadherin (green) antibody. The data shown in Fig. 1F (and Suppl. S1) clearly demonstrate a prompt and PT-dependent induction of ER stress in IECs exposed to PT, compared to the untreated control.

Taken together, these data confirm that gliadin exposure stimulates the induction of ER stress in intestinal tissues of CD patients.

PT-induced UPR regulates the main signs of CD

To investigate the role of ER stress in the pathogenesis of CD we next used our GEVS as a CD model, in which intestinal tissue from GF mice was exposed to PT (2.5 mg/ml) for 16 h, with the well-known ER stress inducer thapsigargin (TG; 5 μg/ml) serving as a positive control. The data reported in Fig. 2A clearly show that both PT and TG increase the expression of the ER stress markers ATF4, ATF6 and XBP1s.

However, regardless of which model is used, it is still unclear whether ER stress is an epiphenomenon, a side effect, or an upstream or downstream effect of exposure of intestinal epithelial cells to PT, which is the first site of contact. To answer this question, the induction of CD-related markers was investigated under the same experimental conditions described above. PT was found to be able to consistently increase the expression of TG2 while decreasing that of CFTR (Fig. 2B), dysregulates tissue permeability (Fig. 2C-D and Suppl. S2), and induces the production of the pro-inflammatory cytokines, IFNγ, IL-17 A and IL-15 (Fig. 2E). All the observed effects were similar to that of the control treatment with TG. These data suggest that ER stress per se may stimulate the appearance of typical CD-associated signs, thus placing this signaling pathway early upstream, upon PT exposure. To support this conclusion, PT- (2.5 mg/ml) or TG- (5 μg/ml) induced ER stress was inhibited by the simultaneous administration of the chemical chaperone 4-phenylbutyric acid (4PBA; 3 μM), in the GEVS [14, 15]. The data presented in Fig. 2A-E show that inhibition of PT- (and TG-) induced ER stress (Fig. 2A) completely prevents: TG2 upregulation and CFTR downregulation (Fig. 2B), tissue permeability dysregulation (Fig. 2C-D and Suppl. S2) [13] and tissue inflammation (Fig. 2.E). As expected 4PBA only partially restored physiological levels of CFTR protein after PT exposure, since it was previously demonstrated the ability of gliadin peptides to interact with plasma membrane CFTR, thus driving its degradation [5]. Next, we test whether inhibition of ER stress could effectively restore gut physiology in a model mimicking active CD and rule out a possible interference by 4PBA due to non-specific interaction with PT in the co-administration protocol. To this end we repeated the latter experiment postponing 4PBA treatment, relative to PT or TG. For this purpose, the small intestines were exposed to PT or TG, and after 4 h 4PBA or vehicle was added (Fig. 3A). Tissues were recovered after a total of 16 h and CD marker analysis was performed. The data presented in Fig. 3 are in perfect agreement with the data presented in Fig. 2, thus ruling out a possible non-specific interaction between 4PBA and PT or TG that would reduce the bioavailability of these compounds.

Overall, these data demonstrate that ER stress is an early event that occurs in IECs exposed to gliadin peptides and triggers the main CD-associated intestinal changes. In addition, our data also suggest that chemical chaperones can potentially block most of the direct deleterious effect of gliadin in active CD.

UPR-related ATF6 activation downregulates the expression of CFTR

To uncover the molecular mechanism linking PT-stimulated ER stress to the key feature of CD, we first investigated the link between the UPR [8, 16] and CFTR expression. To this end, we tested the ability of 4PBA to inhibit the induction of ER stress in CaCo-2 cells exposed to PT. Thus, cells were left untreated or treated with PT (1 mg/ml) alone or in combination with 4BPA (5 μM), and the expression of the UPR markers ATF4, ATF6, and XBP1s was determined by qPCR after 9 h. The data presented in Fig. 4A show that the PT-dependent expression of the three ER stress markers was completely abolished by the presence of the chemical chaperone. In addition, 4PBA also abolished the PT-dependent deregulated expression of CFTR at the mRNA level (Fig. 4B). To determine which branch of the UPR pathway is involved in the transcriptional repression of CFTR, we inhibited the ATF6, PERK, or IRE1α pathway individually with AEBSF, GSK2606414, and STF-083010, respectively (Fig. 4C; Suppl. S3). Thus, cells were exposed to 1 mg/ml PT alone or in combination with 500 μM AEBSF, 5 μM GSK2606414, or 60 μM STF-083010 for 9 h, and their impact on ATF4, ATF6, or XBP1s was verified by qPCR (Fig. 4D). We then measured the expression of CFTR under the same experimental conditions and found that only inhibition of ATF6 activation completely abolished its down-regulation (Fig. 4E). These data are consistent with the findings of Bartoszewski and coworkers who

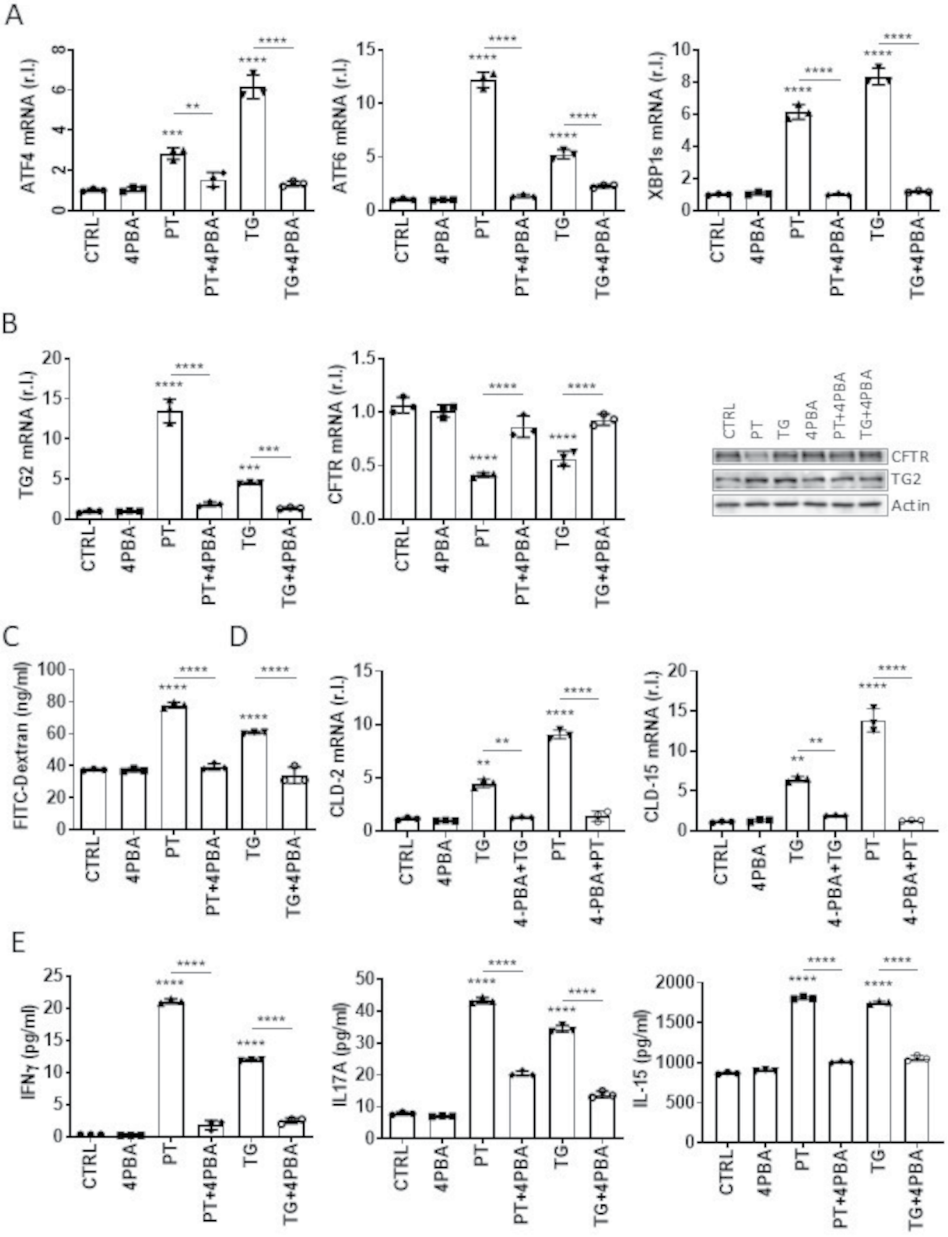

Fig. 2 Pivotal role of ER stress in the pathogenesis of PT. The small intestine from GF mice were cultivated in the GEVS and unexposed (CTRL) or exposed to 4PBA (3 μM), PT (2.5 mg/mL), or TG (5 ug/mL) alone or in combination for 16 h, and ER stress markers (**A**), TG2 and CFTR (**B**) was evaluated by qPCR. TG2 and CFTR protein levels were evaluated by western blotting analysis (**B**, right panel), in the same experimental conditions. Tissue permeability was assessed using the FITC-Dextran test (**C**), or by qPCR analysis (**D**) of the tight junction proteins claudin-2 (CLD-2) and claudin-15 (CLD15), while the production of pro-inflammatory cytokines was evaluated by ELISA (**E**)

previously demonstrated the ability of ATF6 to transcriptionally repress CFTR expression under ER stress conditions [17], as well as with our previous data in which we demonstrated the transcriptional repression activity of ATF6 [18].

Taken together, these data show that CFTR is transcribed under the control of the ATF6 branch of the UPR, independent of the stimulus. As shown previously, inhibition of ATF6 only partially restored physiological CFTR protein levels (Fig. 4F), further emphasizing the dual control over CFTR expression: ATF6-dependent transcriptional repression and gliadin-dependent protein degradation.

UPR-dependent activation of NF-kB regulates the expression of TG2

We then focused on the ER stress-dependent modulation of TG2 expression. First, we demonstrated that

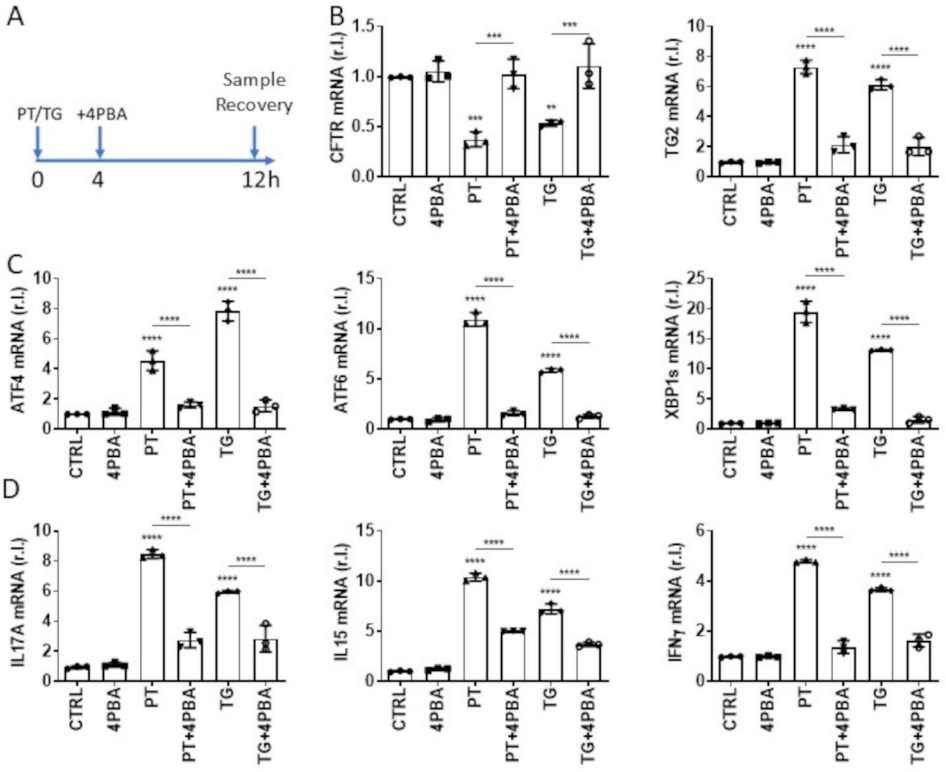

Fig. 3 4PBA does not interfere with IEC-PT/TG interaction. The small intestine by GF mice were cultivated in the GEVS and stimulated with PT (2.5 mg/ml) or TG (5 µg/ml). The 4PBA (3µM) or the vehicle were added 4 h post-treatment, and tissues grown for an additional 12 h (for a total of 16 h; **A**). Tissues were thus recovered and the expression of CFTR (**B**), TG2 (**C**), the indicated UPR markers (**D**), and the indicated inflammatory cytokines (**E**) was evaluated by qPCR. Data are representative of three independent experiments performed in triplicate. Histograms represent mean ± s.d.; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$

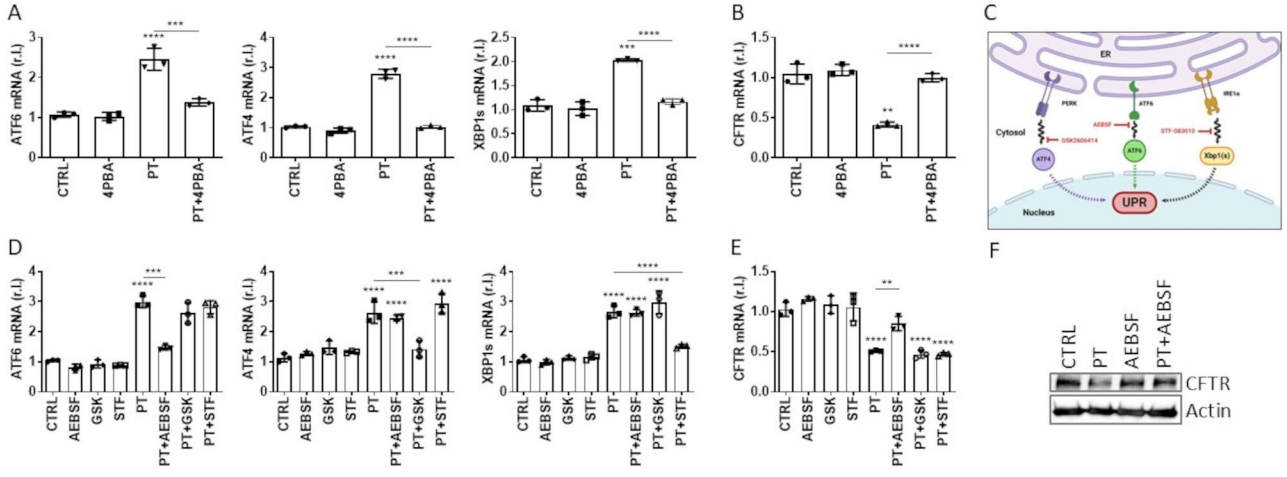

Fig. 4 Molecular mechanisms linking UPR and CFTR dysregulation. CaCo-2 cells were unexposed (CTRL) or exposed 9 h to 4PBA (5 µM) or PT (1 mg/mL) alone or in combination, and the expression of ATF6, ATF4, XBP1s (**A**), and CFTR (**B**) was evaluated by qPCR. (**C**) Schematic representation of the three branches of ER stress, with the 3 inhibitors used in this study highlighted in red. Cells were unexposed (CTRL) or exposed 9 h to PT (1 mg/mL), AEBSF (500 µM), GSK2606414 (GSK, 5 µM), or STF-083010 (STF, 60 µM) alone or in combination, and the expression of ER stress markers (**D**), and CFTR (**E**) was evaluated by qPCR. CFTR protein levels were also evaluated by western blotting analysis (**F**)

co-administration of 4PBA during PT-dependent ER stress induction could inhibit TG2 upregulation in CaCo-2 cells (Fig. 5A). Next, we examined the mRNA levels of TG2 in cells in which the activity of each of the three UPR branches was inhibited, as described above for CFTR, and found that TG2 expression levels were only partially inhibited (Fig. 5B). We then asked whether more than one UPR axis is involved in the regulation of TG2

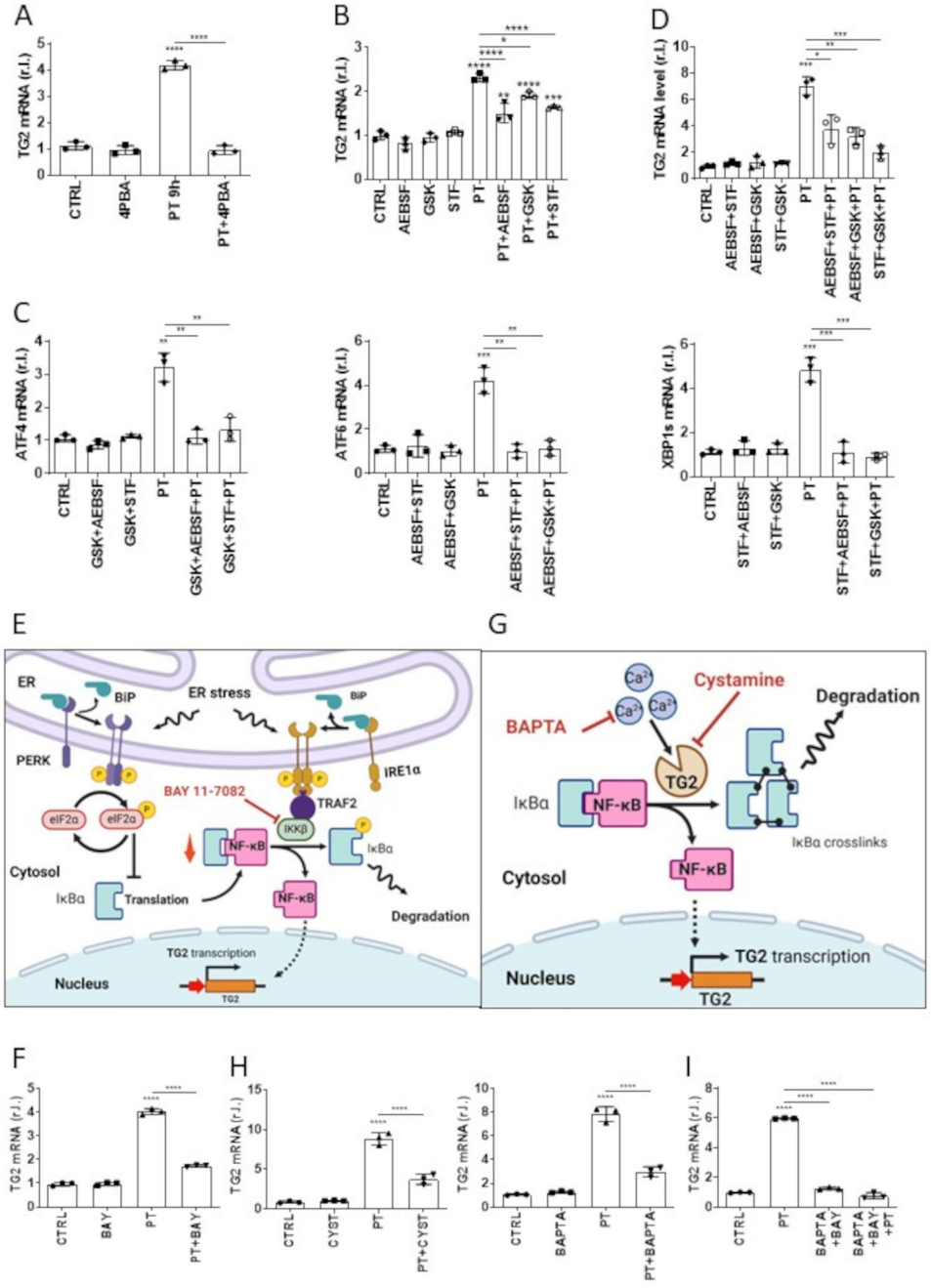

Fig. 5 Molecular mechanisms linking UPR and TG2 dysregulation. CaCo-2 cells were unexposed or exposed 9 h to 4PBA (5 μM) or PT (1 mg/mL) alone or in combination, and the expression of TG2 was evaluated by qPCR (**A**). Cells were unexposed or exposed 9 h to PT (1 mg/mL), AEBSF (500 μM), GSK2606414 (GSK, 5 μM), or STF-083010 (STF, 60 μM) alone or in combination, and the expression of TG2 was evaluated by qPCR (**B**). Cells were untreated or treated 9 h with PT (1 mg/mL) alone or with a combination of the indicated inhibitors, and the expression of ER stress markers (**C**) or TG2 (**D**) was evaluated by qPCR. Schematic representation of the canonical (**E**) or non-canonical (G) pathway linking ER stress, NF-kB and TG2. CaCo-2 cells were unexposed or exposed 9 h to PT (1 mg/mL) alone or in combination with BAY11-7082 (BAY, 5 μM; 1 h pretreatment) (**F**), Cystamine (CYST, 250 μM; 1 h pretreatment) (**H**, left panel), or BAPTA-AM (BAPTA, 10μM; 1 h pretreatment) (**H**, right panel), and the expression of TG2 was evaluated by qPCR. Finally, cells were unexposed or exposed 9 h to PT (1 mg/mL) alone or in combination BAPTA-AM (BAPTA, 10 μM; 1 h pretreatment) and BAY11-7082 (BAY, 5 μM; 1 h pretreatment, and the expression of TG2 was evaluated by qPCR (I)

expression. To this end, we simultaneously and alternatively inhibited two of the three UPR branches (Fig. 5C) and observed that PT-stimulated and ER stress-dependent TG2 upregulation was particularly attenuated when both PERK and IRE1α signaling pathways were simultaneously inhibited (Fig. 5D).

The literature indicates that the transcription factor (TF) NF-kB is the master regulator of TG2 transcription [11], and that its activation relies on a "canonical" (and IKK-dependent) and/or on a "non-canonical" (and IKK-independent) signaling pathways [19]. Interestingly, the IKK-dependent signaling pathway is under the control of the PERK/IRE1α/TRAF2/IKKβ axis of the UPR, which is responsible for IkBα phosphorylation and NF-kB activation. At the same time, inhibition of general Cap-mediated translation due to eIF2α phosphorylation by activated PERK prevents de novo synthesis of IkBα, thereby maintaining NF-kB activity (Fig. 5E). To test whether this signaling pathway is involved in PT-stimulated and ER stress-dependent upregulation of TG2, we inhibited the activity of IKKβ with BAY11-7082. For this purpose, CaCo-2 cells were exposed to PT (1 mg/ml; 9 h) alone or in combination with BAY11-7082 (BAY, 5 μM; 1 h pretreatment), and the expression of TG2 was assessed by qPCR. As shown in Fig. 5F, inhibition of IKKβ reduced the expression of TG2 in cells exposed to PT. We then also investigated the possible involvement of the non-canonical pathway of NF-kB activation. This pathway relies on the calcium-dependent activation of TG2, which cross-links IkBα, to release active NF-kB (Fig. 5G) [19]. It is important to note that Ca^{2+}-dependent activation of TG2 can also be associated with the induction of ER stress. A progressive passive release of calcium from this organelle is a consequence of the accumulation of unfolded proteins in the lumen of the ER under ER stress conditions [20]. On the other hand, active release of Ca^{2+} from the ER leads directly to ER stress, disrupting the redox homeostasis of the organelle that controls protein folding activities [21]. To test this hypothesis, we exposed CaCo-2 cells to PT (1 mg/ml; 9 h) in the presence or absence of the TG2 inhibitor cystamine (CYST, 250 μM; 1 h pretreatment), and the expression of TG2 was assessed by qPCR. The data in Fig. 5H (left panel) clearly show a decrease in TG2 expression when the activity of the enzyme is inhibited. To further substantiate the involvement of active TG2 in its own transcriptional regulation, we also treated CaCo-2 cells with PT (1 mg/ml; 9 h) alone or in combination with the cytosolic calcium chelator BAPTA-AM (BAPTA, 10 μM; 1 h pretreatment). The results of this assay exactly matched those of cystamine, suggesting that IKK-independent activation of NF-kB is also involved (Fig. 5H, right panel). Finally, we simultaneously inhibited both the canonical and non-canonical signaling pathways. For this purpose, CaCo-2 cells were exposed to PT (1 mg/ml; 9 h) alone or in combination with BAPTA-AM (BAPTA, 10 μM; 1 h pretreatment) and BAY11-7082 (BAY, 5 μM; 1 h pretreatment), and the expression of TG2 was determined by qPCR. The data shown in Fig. 5I clearly demonstrate a complete abrogation of PT-stimulated TG2 upregulation when both IKK-dependent and IKK-independent signaling pathways were inhibited.

Taken together, these data indicate that the increased expression of TG2 in cells and tissues exposed to PT is under the control of ER stress.

PT-stimulated active NF-kB also regulates the expression of both pro-inflammatory cytokines and TJ proteins

PT-induced and UPR-mediated activation of NF-kB may also provide a link to explain the induction of pro-inflammatory cytokines in IECs exposed to gliadin peptides. NF-kB is well-known to be a central mediator of inflammatory responses, regulating the expression of several pro-inflammatory genes, such as those encoding cytokines and chemokines, and also playing a role in the regulation of the inflammasome [10]. To validate the role of this TF in regulating the pro-inflammatory cytokines observed in IECs exposed to gliadin peptides, we inhibited the activation of NF-kB in CaCo-2 cells exposed to PT (1 mg/ml; 9 h), by using a combination of BAPTA-AM (BAPTA, 10 μM; 1 h pretreatment) and BAY11-7082 (BAY, 5 μM; 1 h pretreatment). Our results clearly show that inhibition of PT-induced and UPR-mediated activation of NF-kB leads to almost complete abrogation of the expression of IFNγ, IL15 and IL17A (Fig. 6A).

It has been previously shown that epithelial NF-kB activation plays an active role in opening paracellular spaces, affecting TJ proteins function/expression [22, 23]. Therefore, we investigate the effect of NF-kB inhibition on the PT-induced TJ proteins dysregulation, in CaCo-2 cells. To this end, we examined the expression of occludin, CLD-2 and CLD-15 in cells treated as described above. Interestingly, we found that NF-kB inhibition restored physiologic expression of the above TJs (Fig. 6B), possibly preventing PT-induced dysregulated intestinal epithelial permeability.

Altogether, these results suggest that downstream activation of NF-kB, after PT exposure of IECs, also regulates the expression of pro-inflammatory cytokines and TJ proteins (Fig. 6C).

Discussion

Although celiac disease is widely considered to be a CD4 T cell-based disease triggered by gliadin peptides per se and/or deamidated and TG2-linked gliadin peptides (TG2-PT) presented to CD4 T cells by APCs, nowadays the involvement of other immune cells such as mucosal dendritic cells (DCs), macrophages and B cells is evident

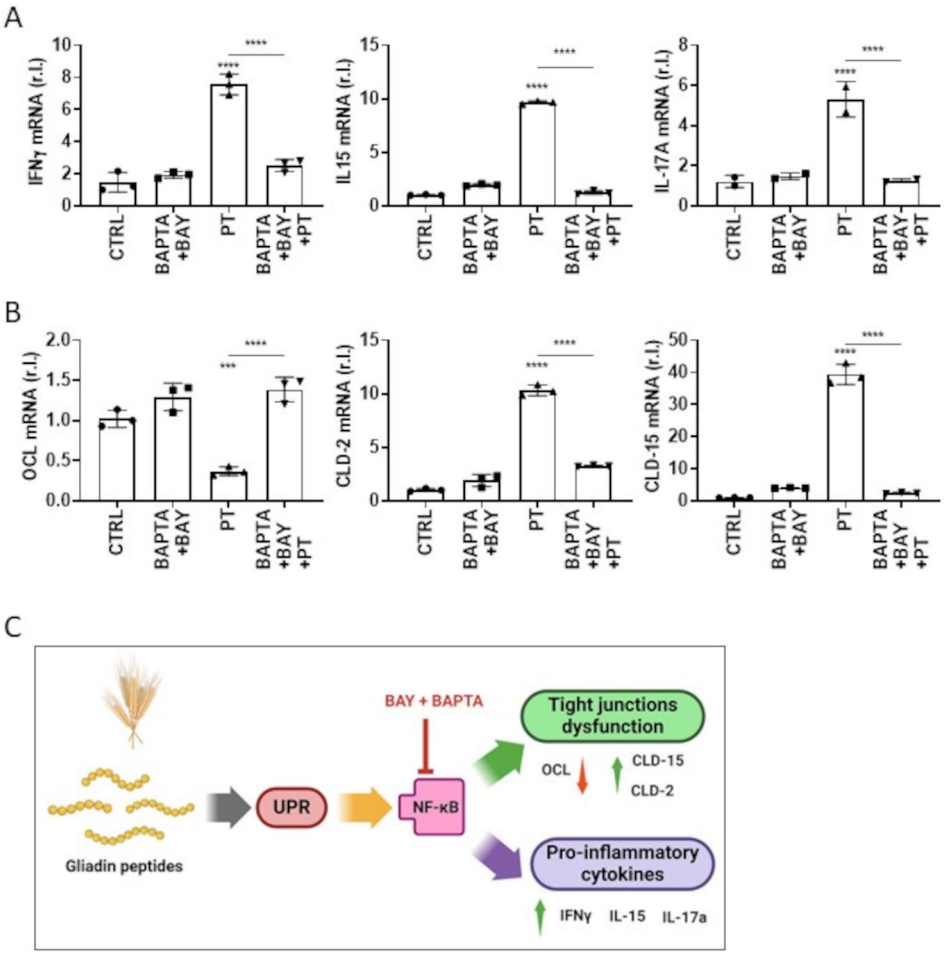

Fig. 6 PT-induced UPR also regulates inflammation and gut permeability mainly through NF-*k*B. CaCo-2 cells were unexposed or exposed 9 h to PT (1 mg/mL) alone or in combination with BAPTA-AM (BAPTA, 10 μM; 1 h pretreatment) and BAY11-7082 (BAY, 5 μM; 1 h pretreatment), and the expression of the indicated pro-inflammatory cytokines (**A**) or tight junction proteins (**B**) was evaluated by qPCR. (**C**) A schematic representation of the signaling pathway by which PT stimulates the activation of the transcription factor NF-kB and the downstream inflammatory cytokines expression/production and TJs function/expression, in IECs

[2]. In addition, the natural high affinity of proline- and glutamine-rich motifs of gliadin peptides, resulting from incomplete digestion of dietary gluten in the intestine, for HLA-DQ2 and HLA-DQ8 clefts is further enhanced by TG2-dependent peptide deamidation [24, 25]. The TG2-PT complexes act as autoantigens and promote the production of autoantibodies against both TG2, one of the most important biomarkers for CD, and deamidated PT [26]. The subsequent massive production of pro-inflammatory cytokines eventually leads to tissue damage and villous atrophy responsible for disease manifestations such as abdominal pain, bloating, nutrient malabsorption, and diarrhea [27].

On the other hand, previous reports indicated the possible involvement of ER stress in IECs exposed to gliadin peptides. However, the role of PT-dependent ER stress and thus the promotion of UPR is still unclear. Here we demonstrated, for the first time, the upregulation of UPR markers in intestinal biopsies from CD patients and showed that this is not merely a consequence of PT-IECs interaction but may represents a crucial step in the pathogenesis of CD. Indeed, we found a clear upregulation of ER stress markers in the group of CD patients compared to the group of non-CD patients. Importantly, in contrast to the control group, we found a heterogeneous expression of UPR markers in the CD group, which could be explained by individual sensitivity to gliadin and/or the degree of activation of the disease itself. Further studies are needed to investigate this point in more detail. Moreover, by buffering PT-stimulated ER stress, we were able to completely prevent the occurrence of important biomarkers of CD such as upregulation of TG2, dysregulation of intestinal permeability and production of pro-inflammatory cytokines.

When analyzing the molecular events linking the induction of ER stress and the appearance of key CD

markers, we also found a dual effect of PT on CFTR expression/activity in IECs. In addition to interaction-mediated degradation, we also observed PT-dependent transcriptional downregulation of CFTR mRNA mediated by ER stress-dependent activation of ATF6. Moreover, we observed the involvement of both canonical and non-canonical ER stress-dependent activation of NF-kB, which is responsible for the PT-dependent upregulation of TG2, dysregulation of TJ proteins expression, and production of pro-inflammatory cytokines.

In this context, the potential key role played by IECs in the pathogenesis of CD is further emphasized by the known active production of the chemokine CXCL10, under the control of NF-kB [28]. Indeed, it has been suggested that initially, activation of innate immunity triggers CXCL10 expression (by the IECs), which can be further enhanced by the presence of IFNγ in the chronic phase. In addition, key cell populations infiltrating the small intestine mucosa in CD patients (mainly $CD3^+$ and $CD4^+$ lymphocytes, and $CD138^+$ plasma cells) highly express CXCR3, the natural receptor of CXCL10 [29]. As a result, the CXCR3/CXCL10 axis not only plays a role in the recruitment of critical cells involved in mucosal damage in active CD, such as Th1 cells and IELs, but may also contribute to the initiation and maintenance of the inflammatory process [29].

Therefore, active induction of ER stress in IECs stimulated by luminal gliadin peptides is crucial for the initiation of CD. Buffering of the UPR by a chemical (or pharmacological) chaperone (such as 4PBA) [14, 15] completely abrogates the deleterious effects of PT on the intestinal epithelium.

This idea opens up a valuable new option for the treatment of CD patients based on oral administration of chemical chaperones, potentially avoiding the expensive, psychologically unfavorable, and lifelong gluten-free diet, and offering the possibility of effectively treating active CD. Our results also suggest that inhibition of gliadin peptides-dependent UPR induction by chemical chaperones may also have a beneficial effect on active CD.

It is also important to note that the prevalence of fully diagnosed celiac disease (CD) has increased approximately threefold among patients with cystic fibrosis (CF). CF, a common lethal genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, primarily affects respiratory function but also leads to intestinal issues due to CFTR's widespread expression in the intestine. CFTR not only functions as an anion channel but also helps regulate cellular stress responses. Malfunction of CFTR leads to epithelial stress, which shares similarities with the effects of gliadin in celiac disease [5]. Therefore, the dual activity of gliadin peptides on CFTR expression, involving binding-dependent protein degradation and UPR/ATF6-dependent gene expression repression, highlights potential clinical implications for using CFTR potentiators in combination with UPR inhibitors to mitigate the negative effects of gliadin.

Conclusions

Overall, we can speculate that ER stress markers could represent a new valuable biomarker for the onset and progression of CD, which together with currently available biomarkers could help physicians to better diagnose CD.

Finally, one can speculate that our results may shed light on the still widely debated issue of 'potential' celiac disease patients. Detection of ER stress markers in biopsies from patients with celiac disease autoimmunity but little histological damage could increase the likelihood of CD positivity and untangle ambiguous histologic images often associated with patchy duodenal mucosal involvement.

Materials and methods

Cells

CaCo-2 cells were maintained in DMEM (Merck) supplemented with 10% FBS (EuroClone), 2 mM glutamine (Merck), and 1% penicillin/streptomycin (Merck). Treatments are reported in Supplementary Table 2 [30].

Gut-ex-vivo system (GEVS)

Small intestine (s.i.) from 13 days old Balb/c mice, feed with a gluten-free diet for at least three-generation [6, 31, 32], were freshly resected and cultivated in a silicone-based Gut-Ex-Vivo System (GEVS), as previously described [7, 12, 13, 33].

All procedures were approved by the local Ethics Committee for Animal Welfare (DB064.N.TMC) and conformed to the European Community regulations for animal use in research (2010/63 UE).

Tissue cultures and treatments

Each intestine was infused with serum-free tissue culture medium (IMDM), supplemented with 20% KnockOut serum replacement (Gibco), 2% B-27 and 1% of N-2 supplements (Gibco), 1% L-glutamine, 1% NEAA (Gibco), 1% HEPES (EuroClone) and stimulated as described in Supplementary Table 3 [12].

Gliadin peptides (PT) Preparation

Gliadin from wheat (Sigma) was prepared as previously described, ad stored at -20 °C [34].

Quantitative PCR (qPCR)

Trizol reagent (Thermo Fisher Scientific) was used to extract total RNA [35]. ExcelRT™ Reverse Transcription Kit (Smobio) was used to produce cDNA, by using 2 µg

of RNA, while the ExcelTaq™2XFastQ-PCR Master Mix (Smobio) was used to produce fluorescently labeled PCR products [36]. Primer sequences are reported in Supplementary Table 4. Mouse Gapdh or human L34 were used as reference [37].

Western blotting analysis

Tissues/cells were lysates by using the Cell Lytic buffer (Merk) supplemented with a protease inhibitors cocktail (Merk) plus Na_3VO_4 1 mM and NaF 10 mM (Merck). Proteins (20 µg/sample) were separated through SDS-PAGE and electroblotted onto nitrocellulose (Bio-Rad) membranes. Antibodies were diluted in 5% non-fat dry milk (Santa Cruz) in PBS plus 0.1% Tween20 and incubated overnight at 4 °C (Supplementary Table 5) [35].

Tissue viability

Tissue viability was evaluated through AlamarBlue staining (Thermo Fisher Scientific Fisher), as previously described [7].

Tissue permeability

0.1 mg/ml FITC-dextran (FITC D4000, Merck) was supplemented to infusing medium, and fluorescence was evaluated in 100 ml outer medium from each sample, by TECAN (SPARK). FITC-dextran concentration was calculated by using a standard curve (0–100 µg/ml).

ELISA

ELISA test was performed on tissue samples lysates using commercially available ELISA kits (R&D Systems) for mouse IL-15, IL-17 A, IFN-γ (R&D System), as previously described [7].

Biopsies

Duodenal biopsies were obtained from pediatric patients (< 18-year-old) at Maggiore della Carità University Hospital (Novara, Italy) via upper gastrointestinal endoscopy. Parents or guardians and patients, where appropriate, provided written informed consent, and the local ethics committee (Comitato Etico Interaziendale Novara, CE 402/23) approved the study protocol.

Endoscopies were performed, according to current ESPGHAN guidelines [38], to confirm the suspicion of CD in patients with anti-transglutaminase (TG2) IgA antibodies levels lower than 10 times the upper level of normal (ULN), or in the case of total IgA deficiency, or when a biopsy-sparing diagnosis was refused by the patient's family, even if in the presence of diagnostic elevation of anti-TG2 IgA above 10 times the ULN. If the histologic examination confirmed the suspicion of CD (Marsh grade 2 or higher), the patient is diagnosed with CD. As control subjects, pediatric patients undergoing upper gastrointestinal endoscopy for different complaints with other endoscopic and histologic diagnosis (gastritis, eosinophilic esophagitis, etc.) were enrolled (Suppl. Table 5). The samples were frozen at -80 °C immediately after resection and stored until analysis. Total RNA was extracted and qPCR was performed as described above.

Immunofluorescence analysis

Tissues were collected and processed for immunofluorescence analysis as previously described [13].

Primary antibodies are reported in Supplementary Table 6.

Images were acquired by using an SP8 LIGHTNING Confocal Microscope (Leica) and images analyzed by LAS X Life Science (Leica) software [13].

Statistical analysis

All experiments were performed in triplicate, repeated at least three times, and statistical analysis was performed using GraphPad_Prism_6. The student-t test or ANOVA was used to determine statistical significance. A p-value equal to or less than 0.05 was considered significant. Histograms represent mean ± SD; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (vs. controls); • $p < 0.05$; •• $p < 0.01$; ••• $p < 0.001$; •••• $p < 0.0001$.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13062-025-00644-9.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Image acquisition and analyses were performed at the Advanced Microscopy Facility of the Center for Translational Research on Autoimmune and Allergic Disease (CAAD), University of Piemonte Orientale, Novara, Italy.We now included: 'Parents or guardians and patients, where appropriate, provided written informed consent, and the local ethics committee (Comitato Etico Interaziendale Novara, CE 402/23) approved the study protocol.' in the ETHICS DECLARATION section in the meìain text file.

Author contributions

Investigation and data acquisition: R.M., M.G., V.S., N.C., F.N., S.P., D.L., S.S., N.P., L.DL., E.F.; Supervision: M.C., D.S.; Conceptualization: M.C., D.S., A.M., I.R.; Writing M.C., D.S.; Review & Editing: M.C., D.S., A.M.; Project administration: M.C.; Funding acquisition: M.C.; All authors reviewed the manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
All procedures were approved by the local Ethics Committee for Animal Welfare (DB064.N.TMC) and conformed to the European Community regulations for animal use in research (2010/63 UE). Parents or guardians and patients, where appropriate, provided written informed consent, and the local ethics committee (Comitato Etico Interaziendale Novara, CE 402/23) approved the study protocol.

Conflict of interest
The authors declare no conflict of interests.

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References

1. Singh P, et al. Global prevalence of Celiac disease: systematic review and Meta-analysis. Clin Gastroenterol Hepatol. 2018;16(6):823–36. e2.
2. Besser HA, Khosla C. Celiac disease: mechanisms and emerging therapeutics. Trends Pharmacol Sci. 2023;44(12):949–62.
3. Discepolo V, et al. How future Pharmacologic therapies for Celiac disease will complement the Gluten-Free diet. Gastroenterology. 2024;167(1):90–103.
4. Khosla C. Celiac disease: lessons for and from chemical biology. ACS Chem Biol. 2017;12(6):1455–9.
5. Villella VR et al. A pathogenic role for cystic fibrosis transmembrane conductance regulator in Celiac disease. EMBO J. 2019;38(2).
6. Ferrari E et al. Probiotics supplements reduce ER stress and gut inflammation associated with Gliadin intake in a mouse model of gluten sensitivity. Nutrients. 2021;13(4).
7. Gagliardi M, et al. Gut-Ex-Vivo system as a model to study gluten response in Celiac disease. Cell Death Discov. 2021;7(1):45.
8. Corazzari M, et al. Endoplasmic reticulum stress, unfolded protein response, and cancer cell fate. Front Oncol. 2017;7:78.
9. Yuan S, et al. Endoplasmic reticulum stress and therapeutic strategies in metabolic, neurodegenerative diseases and cancer. Mol Med. 2024;30(1):40.
10. Liu T, et al. NF-kappaB signaling in inflammation. Signal Transduct Target Ther. 2017;2:17023.
11. Brown KD. Transglutaminase 2 and NF-kappaB: an odd couple that shapes breast cancer phenotype. Breast Cancer Res Treat. 2013;137(2):329–36.
12. Gagliardi M et al. A Gut-Ex-Vivo System to Study Gut Inflammation Associated to Inflammatory Bowel Disease (IBD). Biology (Basel). 2021;10(7).
13. Monzani R et al. The Gut-Ex-Vivo system (GEVS) is a dynamic and versatile tool for the study of DNBS-Induced IBD in BALB/C and C57BL/6 mice, highlighting the protective role of probiotics. Biology (Basel). 2022;11(11).
14. Corazzari M, et al. Oncogenic BRAF induces chronic ER stress condition resulting in increased basal autophagy and apoptotic resistance of cutaneous melanoma. Cell Death Differ. 2015;22(6):946–58.
15. Villani S et al. Quantification of the chemical chaperone 4-Phenylbutyric acid (4-PBA) in cell culture media via LC-HRMS: applications in fields of neurodegeneration and cancer. Pharmaceuticals (Basel). 2023;16(2).
16. Lim D, et al. The Endoplasmic reticulum stress and unfolded protein response in Alzheimer's disease: A calcium dyshomeostasis perspective. Ageing Res Rev. 2023;87:101914.
17. Bartoszewski R, et al. The mechanism of cystic fibrosis transmembrane conductance regulator transcriptional repression during the unfolded protein response. J Biol Chem. 2008;283(18):12154–65.
18. Pagliarini V, et al. Downregulation of E2F1 during ER stress is required to induce apoptosis. J Cell Sci. 2015;128(6):1166–79.
19. Yu H, et al. Targeting NF-kappaB pathway for the therapy of diseases: mechanism and clinical study. Signal Transduct Target Ther. 2020;5(1):209.
20. Deniaud A, et al. Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis. Oncogene. 2008;27(3):285–99.
21. Mekahli D et al. Endoplasmic-reticulum calcium depletion and disease. Cold Spring Harb Perspect Biol. 2011;3(6).
22. Tang Y, et al. Epithelial NF-kappaB enhances transmucosal fluid movement by altering tight junction protein composition after T cell activation. Am J Pathol. 2010;176(1):158–67.
23. Al-Sadi R, et al. TNF-alpha modulation of intestinal tight junction permeability is mediated by NIK/IKK-alpha axis activation of the canonical NF-kappaB pathway. Am J Pathol. 2016;186(5):1151–65.
24. Kim CY, et al. Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in Celiac disease. Proc Natl Acad Sci U S A. 2004;101(12):4175–9.
25. Raki M, et al. A unique dendritic cell subset accumulates in the Celiac lesion and efficiently activates gluten-reactive T cells. Gastroenterology. 2006;131(2):428–38.
26. Loppinet E, et al. LRP-1 links post-translational modifications to efficient presentation of Celiac disease-specific T cell antigens. Cell Chem Biol. 2023;30(1):55–68. e10.
27. Lebwohl B, Rubio-Tapia A. Epidemiology, presentation, and diagnosis of Celiac disease. Gastroenterology. 2021;160(1):63–75.
28. Yeruva S, Ramadori G, Raddatz D. NF-kappaB-dependent synergistic regulation of CXCL10 gene expression by IL-1beta and IFN-gamma in human intestinal epithelial cell lines. Int J Colorectal Dis. 2008;23(3):305–17.
29. Bondar C, et al. Role of CXCR3/CXCL10 axis in immune cell recruitment into the small intestine in Celiac disease. PLoS ONE. 2014;9(2):e89068.
30. Ferrari E, et al. Cysteamine re-establishes the clearance of Pseudomonas aeruginosa by macrophages bearing the cystic fibrosis-relevant F508del-CFTR mutation. Cell Death Dis. 2017;8(1):e2544.
31. Papista C, et al. Gluten induces coeliac-like disease in sensitised mice involving IgA, CD71 and transglutaminase 2 interactions that are prevented by probiotics. Lab Invest. 2012;92(4):625–35.
32. Marietta EV, Murray JA. Animal models to study gluten sensitivity. Semin Immunopathol. 2012;34(4):497–511.
33. Yissachar N, et al. An intestinal organ culture system uncovers a role for the nervous system in Microbe-Immune crosstalk. Cell. 2017;168(6):1135–e114812.
34. Gujral N, Suh JW, Sunwoo HH. Effect of anti-gliadin IgY antibody on epithelial intestinal integrity and inflammatory response induced by Gliadin. BMC Immunol. 2015;16:41.
35. Gagliardi M, et al. Transglutaminase 2 and ferroptosis: a new liaison? Cell Death Discov. 2023;9(1):88.
36. Cosgarea I, et al. Melanoma secretion of transforming growth factor-beta2 leads to loss of epidermal AMBRA1 threatening epidermal integrity and facilitating tumour ulceration. Br J Dermatol. 2022;186(4):694–704.
37. Saverio V, et al. AKRs confer oligodendrocytes resistance to differentiation-stimulated ferroptosis. Redox Biol. 2024;79:103463.
38. Husby S, et al. European society paediatric gastroenterology, hepatology and nutrition guidelines for diagnosing coeliac disease 2020. J Pediatr Gastroenterol Nutr. 2020;70(1):141–56.

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