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Activation of epithelial cells by the major kiwifruit allergen Act d 1 in human and mouse-derived intestinal model

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ABSTRACT

In this study, two intestinal models were employed to assess the modulatory potential of a major kiwifruit allergen on the innate immunity of epithelial cells. Effects of Act d 1 were analyzed in terms of gene expression and structural changes of tight junction (TJ) proteins, as well as up-regulation of pro-inflammatory cytokines in Caco-2 cells and, for the first time, in mouse-derived intestinal 2-dimensional (2D) organoids.

Biologically active Act d 1 induced up-regulation of TJ genes for CLDN-2, CLDN-3, CLDN-4, ZO-1, and on the protein level induced release of pro-inflammatory cytokines IL-1β, TNFα and IL-33 in both employed model systems. In 2D-organoids, active Act d 1 impaired the TJ protein networks of E-cadherin, claudin-3, and ZO-1.

2D-organoids generated from mouse intestine are a promising new model system for the assessment of allergen-induced intestinal cell responses and a useful tool for mitigation of risks associated with novel food proteins.

1. Introduction

Food allergy is a chronic inflammatory disease that may cause serious complications and significantly affects the quality of life (Sampson et al., 2018). The prevalence of food allergy has increased in the last decades (up to 10% of the world population is affected) (Sicherer & Sampson, 2018). Understanding the molecular mechanisms which play a role in the development of food allergy should help in the design of novel therapeutic options, but it could also enable us to anticipate the possible risks of introducing novel foods into the human diet.

Oral tolerance is the physiological response to ingested food proteins, and breakdown of this tolerance results in sensitization to food antigens (Sampson et al., 2018). Overall homeostasis of oral tolerance encompasses suppression of Th2 cells and effector T-cells by generation of Treg cells, decreased IgE and increased IgA and IgG4 production by plasma cells, induction of IL-10-producing dendritic cells (DCs), and suppression of eosinophils, basophils and mast cells activation (Yu, Freeland, & Nadeau, 2016). The initiation of food allergy starts with the sensitization phase when a food allergen passes the mucosal barrier of the gastrointestinal tract (GIT). During this process, the allergen may induce the activation of intestinal epithelial cells, which then secrete cytokines and chemokines and induce activation of dendritic cells, which migrate to the mesenteric lymph nodes. After allergen processing and presentation to naïve T lymphocytes, the final outcome is the activation and differentiation of B lymphocytes into allergen-specific IgE secreting plasma cells (Divekar & Kita, 2015; Yu et al., 2016). Intestinal epithelial cells play an important role in this sensitization phase as they are involved in sensing intestinal stress and consequently regulate innate and adaptive immunity by producing cytokines, chemokines and danger signals. The elicitation of allergic symptoms occurs on re-exposure to the allergen.

A number of *in vitro* cellular models have been used to study mechanisms of sensitization to food allergens in order to overcome the difficulties associated with usage of animal models (Qian et al., 2018).

Abbreviations: Act d 1, actinidin; IgE, immunoglobulin E; TJ, tight junction; GIT, gastrointestinal tract; CLDN, claudin; OCLN, occludin; ZO, zonula occludens; IL-1β, interleukin 1β; TNFα, tumor necrosis factor α; IL-33, interleukin 33; IL-25, interleukin 25; TSLP, thymic stromal lymphopoietin; TEER, transepithelial electrical resistance; FCS, fetal calf serum; BLG, β-Lactoglobulin; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ACTB, β-actin

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For instance, human intestinal epithelial cell lines (IECs) such as Caco-2, T84, and HCT-8, which form highly differentiated polarized monolayers with apical microvilli, functional tight junctions, and expression of both apical and basolateral specific transporters, have been widely used to study the human intestinal barrier (Borchardt, 2011; Cubells-Baeza et al., 2015; Hurley et al., 2016). The limitation of these *in vitro* intestinal models is that they do not recapitulate the physiological microenvironment of the intestine, and contain only one cell-type. (Alemany, Laparra, Barberá, & Alegría, 2012; Alghazeer, Gao, & Howell, 2008; Gerloff, Fenoglio, Carella, Kolling, Albrecht, Boots, & Schins, 2012; Okada, Narai, Matsunaga, Fusetani, & Shimizu, 2000). 2-dimensional (2D) organoids are self-sustained mini-guts which derive from adult leucine-rich repeat-containing G protein-coupled receptor 5-positive stem cells and, upon differentiation into enterocytes, form the intercellular seals of the epithelial layer tight junctions (TJs) and adherent junctions. There are several advantages of the gut organoid system, such as a broad range of both available host species (mouse, rat, pig, bovine, human) and gastrointestinal (GI) compartments (stomach, ileum, colon), as well as gene manipulation possibilities (Koo et al., 2011). Intestinal organoids have found various applications, including analysis of endogenous stem cell characteristics and gene function, as well as disease modeling (Cubells-Baeza et al., 2015). Human organoids, which are derived from either human pluripotent stem cells or primary human donor tissue, have been mainly exploited to address questions related to stem cell biology, human development, and organ regeneration. In spite of the remarkable progress that has been made in culturing human organoid model systems, there are still challenges which should be resolved for their improvement in terms of their complexity (presence of nervous system, immune cell lineages and vasculature) and maturation into an adult-like tissue (Holloway, Capeling, & Spence, 2019). For instance, organization of exogenously added cell types (endothelial cells, immune cells, and neurons) often does not resemble or does not function as the mature *in vivo* tissue, and transplantation into murine hosts is needed to enhance organization within the organoid (Holloway et al., 2019). There is no literature data on the use of intestinal organoids in food allergy studies. However, *in vitro* model systems could prove to be complementary to animal models and may provide novel information on the mechanisms of sensitization.

Allergy to kiwifruit is regarded as one of the most significant fruit allergies and can develop either by monosensitization or associated with either pollen (birch and/or grass pollen allergy) or latex allergy (Ballmer-Weber & Hoffmann-Sommergruber, 2011). Clinical manifestations range from mild symptoms to anaphylactic reactions, and allergen sensitization profiles differ across Europe (Le et al., 2013). According to the International Union of Immunological Societies Allergen Nomenclature Subcommittee (www.allergen.org), 13 kiwifruit allergens have been identified to date. Act d 1 (actinidin, a cysteine protease), the most abundant protein (50%) in kiwifruit extract, is well established as a major kiwifruit allergen and is frequently recognized by kiwifruit monosensitized patients.

The primary objective of our study was to investigate the effects of Act d 1 protease on epithelial cells by using mouse intestinal 2D organoids as a tool. For this purpose, we explored whether Act d 1, a major kiwifruit allergen, could trigger any significant and reproducible cell responses in this model system, and how these responses would compare to the already well-established Caco-2 model. Although mouse-derived intestinal 2D organoids are not considered as representative of physiological conditions as the human-derived intestinal organoids are, they are still a suitable option to garner insights into the molecular interactions of food allergens with epithelial cells. The results of this study could help in the development of model systems suitable for probing the potential allergenic hazard of different proteins.

2. Materials and methods

2.1. Act d 1 preparation

Cysteine proteinase Act d 1 was isolated from kiwifruit (*Actinidia deliciosa*) by two successive ion-exchange chromatographic separations as previously described (Grozdanovic et al., 2016, 2012, 2014). A total of 80 mg of Act d 1 was isolated from 400 g of kiwifruit. Purity of the obtained Act d 1 preparation was confirmed by SDS-PAGE and by mass spectrometry (data not shown). Proteolytic activity of purified Act d 1 (0.98 IU/mL) was determined by using a previously described procedure (Cavic et al., 2012; Drapeau, 1976; Grozdanovic et al., 2016). Act d 1 was activated by resuspension in cell culture medium and incubation for 1 h at 37 °C. Inactivated enzyme was prepared by adding an equimolar amount of cysteine protease inhibitor E-64 (*Sigma–Aldrich*, St. Louis, USA) into the Act d 1 sample.

2.2. Caco-2 cell culture and exposure to Act d 1

Human epithelial colorectal adenocarcinoma (Caco-2) cells (*ATCC*, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 4.5 g/L glucose (*Gibco*, Invitrogen, Carlsbad, USA), and supplemented with 10% fetal calf serum (FCS), 1% (v/v) 200 mM L-glutamine (*Gibco*, Invitrogen, Carlsbad, USA), and 1% Pen/Strep. Cultures were maintained in an atmosphere of 5% CO_2 at 37 °C. Cells at passage of 20–25 were used in experiments for 21 days.

Caco-2 cells at a density of 0.3×10^5 cells/insert were grown on polycarbonate trans-well inserts (0.4 μm diameter pores, *Corning Transwell,* Costar, NY, USA) placed in a 24-well plate. The cells were treated with activated (350 μL) (1 mg/mL and 0.1 mg/mL) and E64-inactivated Act d 1 (1 mg/mL). The experiment was designed according to a previously published protocol (Akbari et al., 2014). The Act d 1 preparation was applied onto the apical side of the cell monolayer and transepithelial electrical resistance (TEER) was measured after 4, 8, and 24 h of the treatment using a Millicell-ERS VoltOhmmeter (*Millipore*, Amsterdam, the Netherlands). Measurements were corrected for the background resistance of membranes without cells and are expressed as percentage of initial monolayer resistance (100%). The results represent mean ± standard error (SEM) of three independent experiments. Untreated Caco-2 cell monolayers were used as control.

B-lactoglobulin (BLG) was prepared in PBS (20 mg/mL) and 5 μL of this preparation was loaded onto the apical side of Caco-2 monolayers after 23 h of Act d 1 treatment. Aliquots of 100 μL were withdrawn from the receiver compartment after 30 and 60 min. Transported BLG was detected by commercially available ELISA kit (*Bethyl Laboratories*, Inc., Montgomery) according to the manufacturer's instructions.

2.3. Cytotoxicity assays

To assess the viability of Caco-2 epithelial cells after Act d 1 treatment, Cell Counting Kit-8 (WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, Sigma-Aldrich, St. Louis, Mo, USA) was employed as previously described (Grozdanovic et al., 2016). In brief, Caco-2 cells were seeded in flat-bottom 96-well plates (Nunc™, Reskilde, Denmark) at a cell density of 0.1×10^5 cells/well. After 15–16 days, confluent and differentiated Caco-2 cells were exposed for 6 h to 0.1 mg/mL and 1 mg/mL of active Act d 1 as well as to 1 mg/mL of E-64 inactivated Act d 1. Cell Counting Kit-8 (10 μL) was added to the cultures and incubated for 4 h at 37 °C. Absorbance was recorded at 460 nm using a microplate reader (LKB 5060-006 Micro Plate Reader, Vienna, Austria).

2.4. 2D organoid culture and exposure to Act d 1

Mouse small intestine was dissected and cells were isolated and suspended in 2D organoid medium as previously described (van der

Wielen et al., 2016). Organoids were seeded into plates coated with Matrigel (1.5 mL per 24-wellplate) for 3–4 days in order to obtain well-defined 2D differentiated organoids. Organoids were exposed for 6 h to activated Act d 1 (1 mg/mL) or to E-64-inactivated Act d 1 (1 mg/mL). Untreated organoids were used as control.

2.5. RNA isolation and gene expression analysis

After 6 h of treatment with Act d 1, medium was collected and RNA from Caco-2 cells and 2D organoids was isolated using *RNaesy* Mini Kit (*Qiagen*, Hilden, Germany). Extracted total RNA (1 μg) was reverse transcribed using random primers and the MultiScribe™ Reverse Transcriptase from the High-Capacity cDNA Reverse Transcription kit (50 U/μL, *Applied Biosystems*, Carlsbad, USA). Following reverse transcription, cDNA was analyzed by PCR. The reaction mixture, comprised of 300 ng diluted cDNA mixed with MasterMix (*Applied Biosystems,* Carlsbad, US), forward and reverse primers (final concentration 300 nM for each primer), and sterile deionized water was prepared according to the manufacturer's instructions. PCR cycle parameters were as follows: denaturation at 95 °C for 3 min. (1 cycle), followed by 40 cycles of 95 °C for 20 s, annealing temperature (AT) for 30 s, and elongation at 72 °C for 30 s. Gene-specific primers for murine and human *CLDN-1*, *CLDN-2*, *CLDN-3*, *CLDN-4*, *OCLN*, *ZO protein 1* (ZO-1), *E-cadherin* and *IL-33* are given in Table 1. The primers were derived from GenBank (U.S. National Center for Biotechnology Information) and were purchased from Invitrogen (Paisley, Scotland, UK).

The presence, size and quantity of the amplified PCR products was analyzed on 2% agarose/SimlySafe™ dye (*EURx*, Gdansk, Poland) gels, while running molecular weight markers (O' Gene Ruler 100 bp DNA Ladder, *Fermentas*, Vilnius, Lithuania). Quantification of PCR band intensities was performed on samples obtained from three independent experiments, after normalization to the respective reference genes, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB), using Image Studio Lite v5.2 (*LICOR Biosciences*, Nebraska, US), and presented as mean ± SEM. mRNA levels measured upon treatment were compared to the corresponding non-treated samples (set to a value of 1), and the graph was prepared using GraphPad Prism v6.07 (*GraphPad Software,* California, US).

2.6. ELISA detection of pro-inflammatory cytokines

Supernatants were collected after 6 h of Act d 1 treatment of Caco-2 cells and 2D organoids, and ELISA capture was performed for detection of IL-1β, TNFα and IL-33 according to the manufacturer's instructions (*e-Bioscience*, San Diego, CA). Detection of cytokines in cell culture supernatants was performed in ELISA Maxisorp immunoplates (Nunc™, Reskilde, Denmark) as previously described (Nikolić et al., 2017).

2.7. Immunofluorescence staining of TJ proteins

Cellular localization of TJ proteins in 2D organoids was detected by immunofluorescence. The organoids were treated either with activated or E-64-inactivated Act d 1 (1 mg/mL), previously dissolved in the organoid medium. Untreated 2D organoids or Caco-2 monolayers incubated in medium were used as control. Immunofluorescence staining of TJs was performed as previously described (Akbari et al., 2014). Stained organoids and Caco-2 monolayers were examined by OLYMPUS BX60 fluorescence microscope. Images were acquired with a 40x objective and assembled using Leica LAS-AF software (*Leica Microsystems*, Wetlzar, Germany). The final images were obtained using Adobe Photoshop CS5 (*Adobe Systems Incorporated*, CA, USA).

2.8. Statistical analysis

The results are presented as mean ± SEM. Analyses were performed by using GraphPad Prism v6.01 (GraphPad, La Jolla, CA, USA).

Table 1
Primer sequences used for PCR analysis.

Target gene (reference)	Human primer sequence (5′–3′)	AT (°C)
CLDN-1 (NM_021101)	For: AGCTGGCTGAGACACTGAAGA Rev: GAGAGGAAGGCACTGAACCA	63
CLDN-2 (NM_020384.3)	For: GCCTCTGGATGGAATGTGCC Rev: GCTACCGCCACTCTGTCTTTG	63
CLDN-3 (NM_001306)	For: CTGCTCTGCTGCTCGTGTC Rev: CGTAGTCCTTGCGGTCGTAG	62.5
CLDN-4 (NM_001305)	For: GTCTGCCTGCATCTCCTCTGT Rev: CCTCTAAACCCGTCCATCCA	62.5
OCLN (NM_002538)	For: TTGGATAAAGAATTGGATGACT Rev: ACTGCTTGCAATGATTCTTCT	57
ZO1 (NT_010194.17)	For: GAATGATGGTTGGTATGGTGCG Rev: TCAGAAGTGTGTCTACTGTCCG	55.8
E-cadherin (NM_004360.5)	For: CTGAGAACGAGGCTAACG Rev: GGAGCAGCAGGATCAGAATC	64.3
IL-33 (NM_001164724.1)	For: GGTGTGGATGGGAAGAAGCTG Rev: GAGGACTTTTTGTGAAGGACG	61
GAPDH (NM_002046.5)	For: AGCAATGCCTCCTGCACCACCAAC Rev: CCGGAGGGGCCATCCACAGTCT	65
Target gene (Reference)	Murine primer sequence (5′–3′)	AT (°C)
CLDN-1 (NM_016674.4)	For: TCTACGAGGGACTGTGGATG Rev: TCAGATTCAGCTAGGAGTCG	57
CLDN-2 (NM_016675.4)	For: GGCTGTTAGGCTCATCCAT Rev: TGGCACCAACATAGGAACTC	55
CLDN-3 (NM_009902.4)	For: AAGCCGAATGGACAAAGAA Rev: CTGGCAAGTAGCTGCAGTG	58.7
CLDN-4 (NM_009903.2)	For: CGCTACTCTTGCCATTACG Rev: ACTCAGCACACCATGACTTG	55
OCLN (NM_008756.2)	For: ATGTCCGGCCGATGCTCTC Rev: TTTGGCTGCTCTTGGGTCTGTAT	61.2
ZO1 (NM_009386.2)	For: CGAGGCATCATCCCAAATAAGAAC Rev: TCCAGAAGTCTGCCCGATCAC	58.7
E-cadherin (NM_009864.2)	For: ACTGTGAAGGGACGGTCAAC Rev: GGAGCAGCAGGATCAGAATC	64.3
IL-33 (NM_001164724.1)	For: GGTGTGGATGGGAAGAAGCTG Rev: GAGGACTTTTTGTGAAGGACG	61
ACTB (NM_007393.3)	For: ATGCTCCCCGGGCTGTAT Rev: CATAGGAGTCCTTCTGACCCATTC	61

Differences between sample means were statistically examined by using two-way analysis of variance (ANOVA) and the Sidak post-test was used to adjust the *P*-value for multiple comparisons. Differences were considered significant if $P < 0.05$.

3. Results

3.1. Biologically active Act d 1 increases permeability of Caco-2 monolayers

A MTT assay revealed that Act d 1 isolated and prepared according to the employed protocol does not influence the viability of Caco-2 cells. No significant difference between the control cells and cells treated with active Act d 1 (0.1 mg/mL and 1 mg/mL), or cells treated with E-64-inactivated Act d 1 (1 mg/mL), was observed in viability.

Transepithelial electrical resistance (TEER) is a validated and broadly used technique for measuring the integrity of tight junction dynamics in cell culture models of epithelial monolayers. A TEER value indicates the integrity of the cellular barriers. Proteolytically active Act d 1 changed the permeability of the Caco-2 monolayer, measured as a reduction of TEER, and increased the transfer of BLG. Control Caco-2

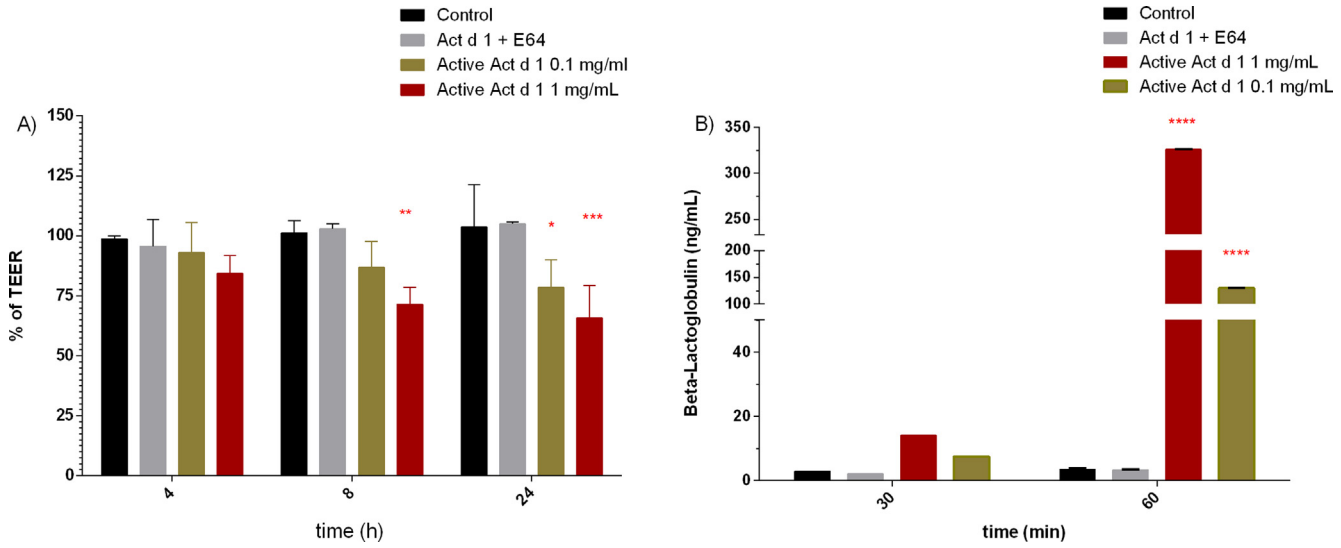

Fig. 1. (A) Relative TEER of Caco-2 cell monolayers upon 4, 8, and 24 h of Act d 1 treatment. Values are expressed as % of the pretreatment monolayer resistance (intact untreated transepithelial monolayers have been assigned a value of 100% TEER) $*P \leq 0.05$; $**P \leq 0.01$, $***P \leq 0.0\ 01$, $****P \leq 0.0001$ *vs* control B) β-Lactoglobulin (BLG) leakage across the Caco-2 monolayer upon Act d 1 treatment. Values are expressed as concentration of BLG in basolateral medium at 30 and 60 min.

cells maintained basal TEER levels (100%) over the tested 4 h period (Fig. 1A), while cells treated with 1 mg/mL of Act d 1 showed a reduction of TEER down to 84% of the control value. This effect was more pronounced after 8 h and 24 h of treatment, with TEER values decreasing to 71% and 65%, respectively, as compared to the control. E-64-inactivated Act d 1 did not change the TEER of monolayers over the measured time period, indicating that the observed effect was protease-dependent. A lower concentration of Act d 1 (0.1 mg/mL) also caused significant decrease of TEER 24 h upon treatment (78% as compared to control, $*P \leq 0.05$).

The epithelial integrity upon Act d 1 treatment was measured by assessing the transport of BLG, the major whey protein of cow milk. BLG was applied to the apical compartment 23 h upon Act d 1 treatment. After 30 min of incubation, the measured level of BLG in the basolateral compartment was relatively low considering the employed Act d 1 concentrations (0.1 and 1 mg/mL); However, after 1 h both concentrations of Act d 1 induced BLG leakage when compared to the control (Fig. 1B). E-64-inactivated Act d 1 did not change levels of BLG in the basolateral compartment.

3.2. Act d 1 treatment leads to up-regulation of TJ protein genes in 2D organoids and Caco-2 cells

We compared IL-33 and TJ protein gene expression in 2D organoids and in the Caco-2 model after 6 h of treatment with both activated and inactivated Act d 1. Analysis of relative gene expression levels in Act d 1 treated mouse-derived intestinal 2D organoids are shown in Fig. 2A, and the levels found in Caco-2 cells are shown in Fig. 2B. Total RNA from cells was isolated, transcribed to cDNA and gene expression levels were analyzed by means of PCR. Act d 1 induced a significant increase in gene expression levels of tight junction proteins (occludin, claudin-1-4, and ZO-1) in 2D organoids and Caco-2 cells when compared to untreated controls or E-64-inactivated Act d 1.

3.3. 2D organoids and Caco-2 cells release pro-inflammatory cytokines in response to Act d 1 treatment

We next evaluated whether the interaction of Act d 1 with intestinal epithelial cells induced a production of pro-inflammatory cytokines in

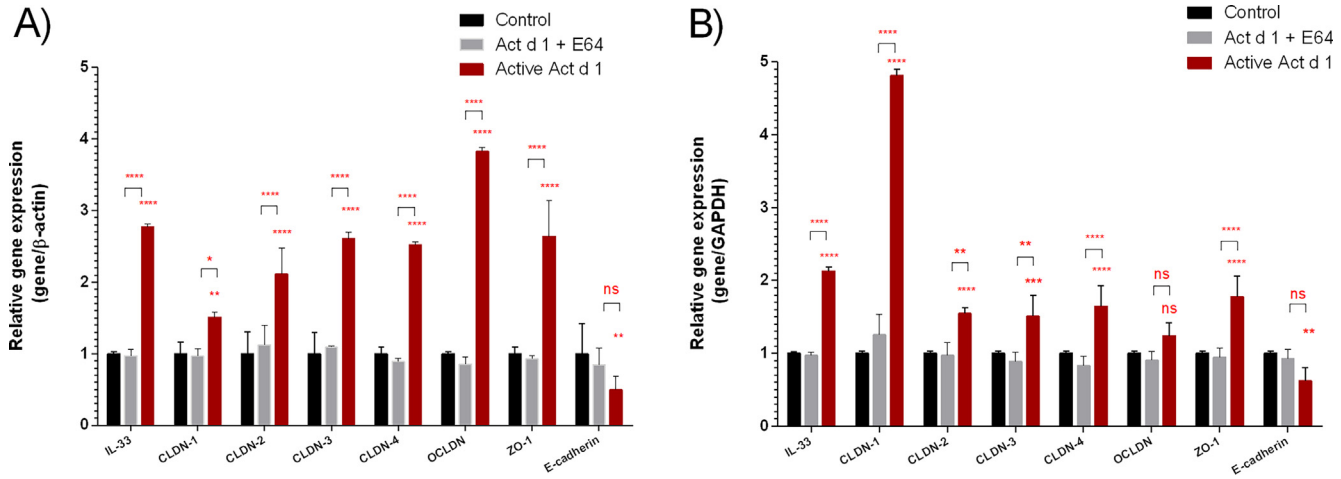

Fig. 2. Analysis of relative gene expression levels for IL-33 and TJ proteins in Act d 1 treated (A) mouse-derived intestinal 2D organoids and (B) Caco-2 cells. Cells were treated for 6 h with Act d 1 (activated or E-64-inactivated). Total RNA from cells was isolated, transcribed to cDNA, and gene expression levels were analyzed by means of PCR. $**P \leq 0.01$; $***P \leq 0.001$; $****P \leq 0.0001$ *vs* control.

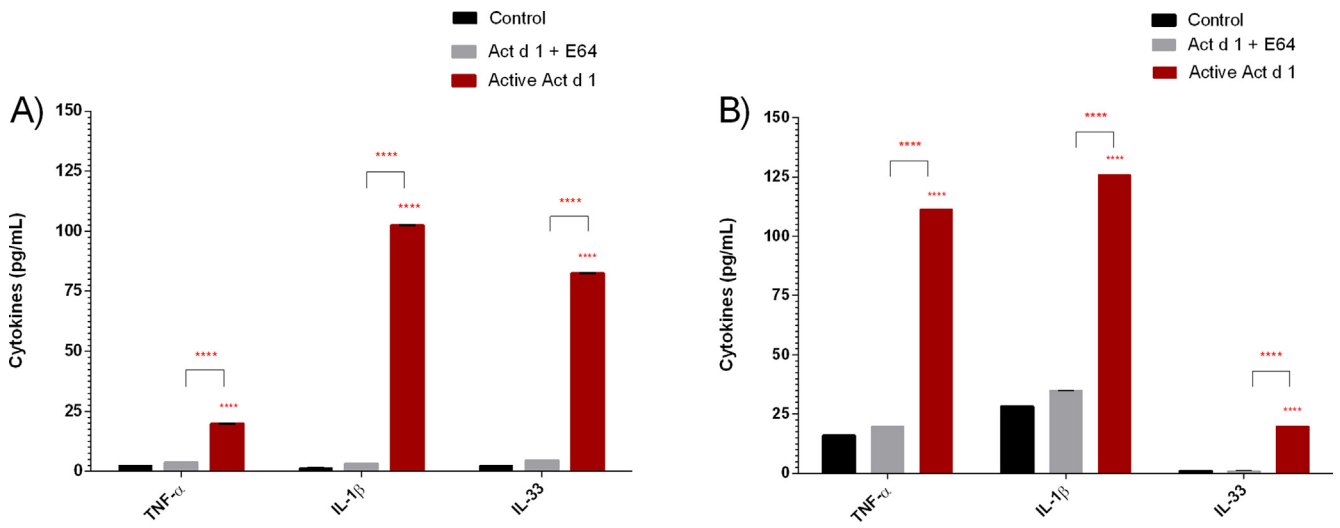

Fig. 3. Act d 1 induces cytokine release from (A) mouse-derived intestinal 2D organoids and (B) Caco-2 cells. Activated (1 mg/mL) or E-64-inactivated Act d 1 (1 mg/mL) were added to organoids/Caco-2 cells for 6 h. Supernatants were collected and TNFα, IL-1β, and IL-33 levels were quantified by ELISA. ****$P \leq 0.0001$ *vs* control.

2D organoids or Caco-2 cells. The treatment induced a strong release of TNFα, IL-1β, and IL-33 in 2D organoids (Fig. 3A). Similarly, treatment of Caco-2 cells with Act d 1 also induced a release of TNFα, IL-1β, and IL-33 cytokines (Fig. 3B).

3.4. Act d 1 disrupts continuity of TJ proteins

Effects of Act d 1 protease activity on the tight junction proteins *in situ* was analyzed by immunofluorescence staining of 2D organoids. The control samples of organoids showed membrane-associated localization of Claudin-3, E-cadherin and ZO-1, which appeared as continuous rings encircling adjacent cells (Fig. 4, A, B, C). Act d 1-exposed organoids exhibited irregular structures of the stained proteins, suggesting disruption of the TJ continuity. These altered distribution patterns were observed for CLDN-3, E-cadherin and ZO-1. A similar finding was obtained for immunofluorescence staining of Caco-2 cell monolayers after Act d 1 treatment. Continuous rings appeared after ZO-1 protein staining with AlexaFluor 488 conjugate of anti-human ZO-1 antibody in untreated cells (Fig. S1, Supplementary material). In Caco-2 cell monolayers treated with activated Act d 1 (1 mg/mL) a disruption in the continuity of ZO-1 was found, along with a difference in intensity of staining when compared to the non-treated monolayer (control) and Caco-2 monolayer treated with E-64 inactivated Act d 1 (Fig. S1).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jff.2019.103556.

4. Discussion

In this study, the effect of the cysteine protease allergen Act d 1 on the function and integrity of intestinal epithelial cells was analyzed by employing mouse-derived intestinal 2D organoids and the human intestinal Caco-2 cell line. Epithelial cells of the gastrointestinal tract act as sensors of environmental insults and are involved in modulation of the innate and adaptive immune response by producing cytokines, chemokines and danger signals (Rescigno, 2014). In addition, disruption of the epithelial barrier is one of the key events in the initiation of the inflammation process, which in the presence of chronic stimuli leads to the development of chronic inflammation (Van Bilsen et al., 2017). We have previously shown that Act d 1 can interrupt the epithelial barrier function through its protease activity and by degradation of the tight junction protein occludin (Cavic et al., 2012, 2014; Grozdanovic et al., 2016). To get a more detailed insight into the effect of Act d 1 on the activation of intestinal epithelial cells in the sensitization phase of food allergy, we employed Caco-2 cells as a frequently used *in vitro* model system, but also expanded our observations to include mouse-derived intestinal 2D organoids, a novel approach which has not been used before for these kind of studies. The existing methodology for culturing organoids provided us with a 2D model that harbored different epithelial cell types, including intestinal enterocytes and enteroendocrine and mucin-producing goblet cells (van der Wielen et al., 2016).

Active Act d 1 decreased the TEER of the Caco-2 monolayer indicating alterations in tight junction proteins that may lead to increased paracellular transport. Leakage of the Caco-2 cell monolayer after Act d 1 treatment was confirmed by detection of BLG in the basolateral compartment. This effect was found to be more pronounced with time and was in accordance with the noticed changes in TEER.

We tested the suitability of mouse-derived intestinal 2D organoids for the assessment of food allergen interactions and for evaluation of epithelial cells activation, as components of the intestinal barrier and innate immunity. For this purpose, we analyzed the effects of Act d 1 on TJ proteins and on the release of pro-inflammatory cytokines. ZO is considered to be important for maintenance of the TJs' network through its interaction with the actin cytoskeleton (S. H. Lee, 2015). The TJs maintain the polarity of intestinal epithelial cells, and regulate permeability of water, ions, and nutrients. Experiments in animal models, as well as in clinical studies, emphasized compromised epithelial barrier in food allergies with enhanced permeability toward food compounds and with uptake of intact antigens through the disrupted intestinal barrier (Samadi, Klems, & Untersmayr, 2018). Our findings show that Act d 1 treatment of 2D organoids and Caco-2 cells induced an up-regulation of TJ proteins gene expression (occludin, claudins 1–4, and ZO-1). Although a direct comparison between the *in vitro* findings in human Caco-2 cells with those obtained in 2D mouse organoids should take into consideration possible species differences, a number of interesting similarities in the response to Act d 1 were observed. The mRNA expression levels of analyzed TJ proteins, such as CLDN-1, CLDN-2, CLDN-3, CLDN-4, ZO-1 and OCLN were increased upon treatment with Act d 1 both in Caco-2 cells and 2D organoids. The up-regulation of mRNA levels for TJ proteins might be a compensatory effect for the degradation of the TJ proteins (Akbari et al., 2014); however, further experimental evidence should be provided to elucidate the molecular mechanisms behind these events. Of interest, E-cadherin was previously reported to be down-regulated in patients with allergic rhinitis (Lee et al., 2016; van Oosterhout, Nawijn, Heijink, Hackett, & Postma, 2011). It seems that active Act d 1 could affect E-

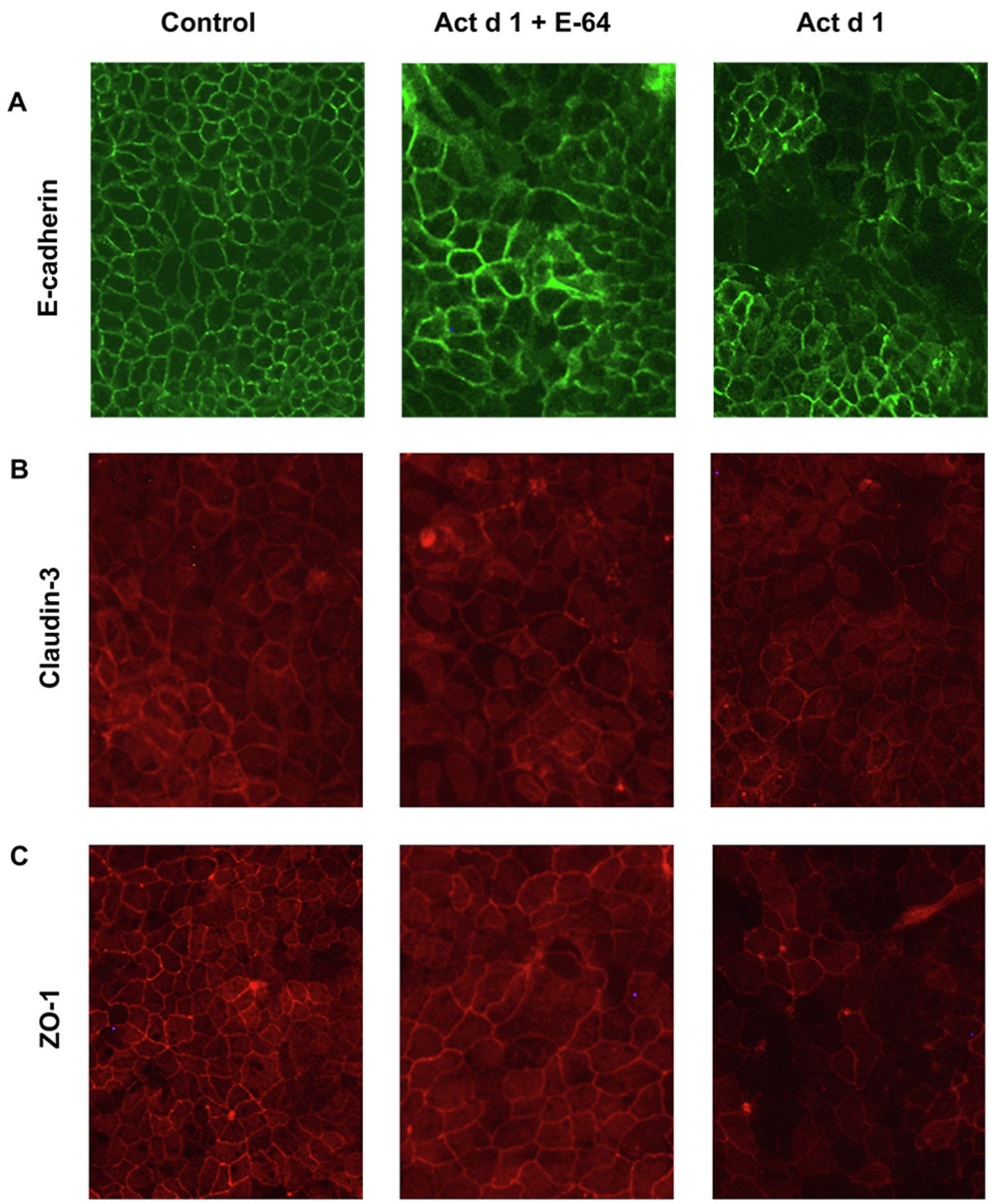

Fig. 4. Immunofluorescence staining of mouse-derived 2D cultured organoids. Organoids were exposed to Act d 1 for 24 h and detection was performed for E-cadherin (A), claudin-3 (B) and ZO-1 (C).

cadherin levels, and down regulation of E-cadherin could lead to increased intestinal permeability (Kauffman et al., 2014; Lee et al., 2016; Tunggal et al., 2005). It has been previously reported that papain, the cysteine protease from papaya, is able to alter TJ networks via degradation of ZO-1, claudin-4 and occludin in keratinocytes *in vitro*. E-64 inhibited papain does not show alterations in tight junctions (Annaházi et al., 2013; Furuse, Fujimoto, Sato, Hirase, & Tsukita, 1996). Similarly, Act d 1 exerts its proteolytic activity on occludin and disrupts the TJ integrity of human T84 cell monolayer (Cavic et al., 2014), and it was also shown to fragment recombinant human occludin *in vitro* (Grozdanovic et al., 2016). In this study, by utilizing immunofluorescent staining of 2D differentiated organoids that contain mucin-producing goblet cells, we were able to detect Act d 1- induced disruption of the TJ continuity of claudine-3, E-cadherin and ZO-1 as well.

Allergens may interact with epithelial cells directly, hereby promoting Th2 responses (Salazar & Ghaemmaghami, 2013). It has been shown that airway epithelial cells can directly recognize various allergens (Der p 1, Der p 2, Pen c 13, etc.) by transmembrane receptors (PARs, TLRs or CLRs) and, through NF-κB signaling, can induce the production of a range of cytokines (TSLP, GM-CSF, IL-33, IL-25, IL-1β), chemokine ligands (CCL2, CCL20), and danger signals (ATP, uric acid) (Salazar & Ghaemmaghami, 2013; Van Bilsen et al., 2017). IL-33 is a pro-allergenic cytokine, also denoted as an endogenous danger signal that is involved in Th2-type of immune response. Active Act d 1 induced an increase in IL-33 gene expression and IL-33 cytokine release in both model systems. This effect was not detected for inactivated Act d 1, indicating that these cell responses are induced by proteolytic activity. In addition to IL-33, Act d 1 induced secretion of TNFα and IL-1β in both Caco-2 cells and 2D organoids. Several food allergens, including Ara h 2 from peanut, Pru p 3 from peach, and Gal d 2 from egg white, have been shown to induce the up-regulation of pro-inflammatory cytokines (such as IL-1β, IL-6, TNFα, IL-25, IL-33, and TSLP) in epithelial cells (Nikolić et al., 2017; Starkl et al., 2012; Tordesillas et al., 2013).

In this study, a novel model system was introduced for evaluating the activation potential of food allergens in the sensitization process of food allergy. Upon treatment with Act d 1, 2D organoids cultured from mouse intestine and composed of enterocytes, enteroendocrine cells, and mucin secreting goblet cells, released Th2 response related

cytokines and exhibited effects on tight-junctions' proteins on gene and protein level. 2D organoids can provide insight into the sensitization potential of other food allergens and could be a useful tool in risk assessment determination of novel food proteins. The results of this study may provide the first step towards the establishment of a novel model system that could, if not replace, then at least reduce the usage of laboratory animals in food allergen testing.

The ethics statement

In vivo experimental protocol was approved by the local Ethics Committee for Animal Experiments and was performed in compliance with national and international guidelines on animal experimentation.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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