Validating Enteroid-Derived Monolayers From Murine Gut Organoids for Toxicological Testing of Inorganic Particles: Proof-of-Concept With Food Grade Titanium Dioxide

Yann Malaisé , Eva Casale , Aurélie Pettes-Duler , Christel Cartier , Eric Gaultier , Natalia Martins Breyner , Eric Houdeau * , Lauris Evariste , Bruno Lamas *

Posted Date: 18 January 2024

doi: 10.20944/preprints202401.1383.v1

Keywords: intestinal organoids; toxicity testing; food toxicology; inorganic particles; food additive titanium dioxide

Article

Validating Enteroid-Derived Monolayers from Murine Gut Organoids for Toxicological Testing of Inorganic Particles: Proof-of-Concept with Food Grade Titanium Dioxide

Yann Malaisé †, Eva Casale †, Aurélie Pettes-Duler, Christel Cartier, Eric Gaultier, Natalia Martins Breyner, Eric Houdeau *, Lauris Evariste and Bruno Lamas *

Toxalim UMR1331 (Research Centre in Food Toxicology), Toulouse University, INRAE, ENVT, INP-Purpan, UPS, Toulouse, France

* Correspondence: E.H. eric.houdeau@inrae.fr; B.L. bruno.lamas@inrae.fr

† Co-first authors who contributed equally to this study.

Abstract: Human exposure to foodborne inorganic nanoparticles (NPs) is a growing concern. However, identifying potential hazards linked to NP ingestion often requires long-term exposure in animals. Owing these constraints, intestinal organoids are a promising alternative to *in vivo* experiments, as such an *in vitro* approach should enable rapid and reliable assessment of the effects on the gut of ingested chemicals. However, this remains to be validated for inorganic substances. In our study, a transcriptomic analysis and immunofluorescence staining were performed to compare the effects of food-grade TiO_2 (*fg*-TiO_2) on enteroid-derived monolayers (EDMs) from murine intestinal organoids to known impacts of TiO_2 on intestinal epithelium. After validating their ability to dose-dependently respond to a pro-inflammatory cytokine cocktail, EDMs were exposed to 0.1, 1 or 10 µg *fg*-TiO_2/mL for 24h. In line with known data, a dose-related increase of *muc2*, *vilin 1* and *chromogranin A* gene markers of cell differentiation was observed. In addition, *fg*-TiO_2 induced apoptosis and dose-dependent genotoxicity, while a decreased expression of genes encoding for antimicrobial peptides, and of genes related to tight junction function was observed. These results validated the use of EDMs as a reliable model for toxicity testing of foodborne NPs likely to affect the intestinal barrier.

Keywords: intestinal organoids; toxicity testing; food toxicology; inorganic particles; food additive titanium dioxide

1. Introduction

Manufactured inorganic particles (metals and minerals) are abundant in daily life products (e.g., cosmetics, textiles, building materials), including foodstuffs as food additives for their colouring or anti-caking properties [1,2], or as antimicrobial agents or oxygen scavenger in food packaging [2,3]. Given their chronic ingestion on a daily basis, health agencies are constantly re-evaluating the potential health risks for humans [4,5]. This requires *in vitro* models or time-consuming *in vivo* experiments in rodents. However, with regards to the intestine as first target organ, mono or co-culture of intestinal cell lines are not relevant enough in term of self-organization (polarization and 3D structure), and do not represent the variety of cell types and functions found in the gut epithelium, i.e., absorptive enterocytes, secreting Paneth cells, enteroendocrine and goblet (mucus-producing) cells, chemosensory tuft cells [6], all of them have to be present *in vitro* to mimic *in vivo* conditions. Recent technical advances in stem cells and three-dimensional cultures have allowed the use of intestinal organoids that closely recapitulate the architecture and cellular composition of the intestinal epithelium [7–9]. They indeed represent a good alternative model to classical *in vitro* cultures as well as to *in vivo* experiments according to the animal ethic principle of Replacement, Reduction and Refinement. For example, gut organoids have been used in several studies for modelling diseases such as inflammatory bowel disease or for exploring the interactions between pathogens and the epithelium as well as the mechanisms of action and transport of drugs, among

 doi:10.20944/preprints202401.1383.v1

others applications [10–12]. As the closed 3D geometry of gut organoids prevents direct access to the apical region of the epithelium, these applications require technically challenging methods such as organoid microinjection, which limits the routine use of organoids. Alternatively, open-up 3D gut organoids to obtain an enteroid-derived monolayer (EDM) model, whose resemble the physiologic gut lining in cell variety, have been used for functional tissue barrier assays [13–15]. However, the interest of EDMs for toxicological testing of inorganic substances in the intestine, from their potential impacts on cell proliferation to cell functions and responses, has been poorly addressed. Experimental validation is required through a comparative analysis of EDM responses to an inorganic compound with the already reported toxicity data for the same substance in the intestine.

Among these particle models, food-grade (*fg*) titanium dioxide (TiO_2, referred to as E171 in EU) may be viewed as a referent substance due to the numerous toxicity studies performed during the last decade [16–24]. Commonly used as a whitening and brightening agent in confectionary, processed food, white sauces and icing [25], as well as coating agent on pharmaceutical tablets [26,27], *fg*-TiO_2 is one of the most produced worldwide food additive [28,29]. Noteworthy, due to a mixed composition of micro- and nanoparticles (NPs), *fg*-TiO_2 is also representative of manufactured nanomaterials that expose the general population to NPs through the diet [30]. Although the use of *fg*-TiO_2 is still authorised outside the EU, the precautionary principle has led the public policies to ban the use of the *fg*-TiO_2 in Europe in 2022 [4,31] based on its capacity to induce oxidative stress [23] and genotoxicity [20,32], with a potential of developmental impacts when *in utero* exposure occurs from the mother diet [18,19,24,33,34]. In this context, many studies have reported a wide range of effects on the gut barrier integrity when TiO_2-NPs accumulate in the intestine, from growth inhibition of epithelial cells [35], altered nutrient absorption [36,37] and epithelial permeability defect [18,34,38], as well as increased reactive oxygen species (ROS) production [23,39] and proinflammatory signalling [18,21,34,40,41] after acute and/or chronic exposure of intestinal cells to TiO_2, both *in vivo* and *in vitro* [4,16,42]. Studies also demonstrated the ability of foodborne TiO_2 particles to cross both the small intestine and colon barrier [21,43,44], and to induce genotoxic effects [23,39,45–47] while promoting development of precancerous colorectal lesions in colon [21,48].

Overall, these reports make *fg*-TiO_2 a relevant particle model for benchmarking EDM use in toxicological studies and regulatory purposes. In the current study, we first evaluated the cellular response of a murine EDM model to a common pro-inflammatory stimulus using a cytokine cocktail, in order to validate the EDM ability to physiologically and dose-dependently react to an environmental stimulus. Second and consistent with most reported toxicity data in the literature, we showed that the integrity of the gut (EDM) barrier in terms of cell proliferation/differentiation/apoptosis, genotoxicity, epithelial innate (anti-microbial) defences and tight junction (TJ) function was found altered after exposure to *fg*-TiO_2 for 24h. Altogether, these results validated the use of EDM prepared from murine intestinal organoids as a reliable alternative to conventional *in vivo* experiments for screening the effects of inorganic food additives on the gut epithelium, including NPs.

2. Results

2.1. EDM model is functional and competent to respond to an inflammatory stress

Before assessing the impact of fg-TiO_2 on EDMs, we first evaluated their ability to dose-dependently respond to an IFN-γ/TNF-α cocktail used to mimic a pro-inflammatory stimulus (Figure 1). Compared to controls, no difference in LDH secretion was observed following IFN-γ/TNF-α exposure for 24h, concluding on the absence of cytotoxicity for cytokine doses and duration of treatment herein used (Figure 1a). The expression of genes known to be regulated by IFN-γ and/or TNF-α, such as Chemokine (C-C motif) ligand 5 (ccl5), toll like receptor 4 (tlr4), ki67 (mki67), nuclear factor-κB (nfkb2) and its p65 subunit (rela) were next evaluated. A dose-dependent up-regulation of ccl5 and mki67 was observed after 24h-exposure of EDMs to the IFN-γ/TNF-α cocktail (Figure 1b,c). At the highest dose of 10 ng/mL, these proinflammatory cytokines also induced an increase in the expression of nfkb2, rela and tlr4 genes compared to control EDMs (Figure 1d–f). These results

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 January 2024 doi:10.20944/preprints202401.1383.v1

highlighted that EDMs are functional and competent to respond to inflammatory stresses during 24h of culture, and that this model can be used to assess the direct impact of foodborne inorganic particles on the gut epithelial barrier, such as fg-TiO_2.

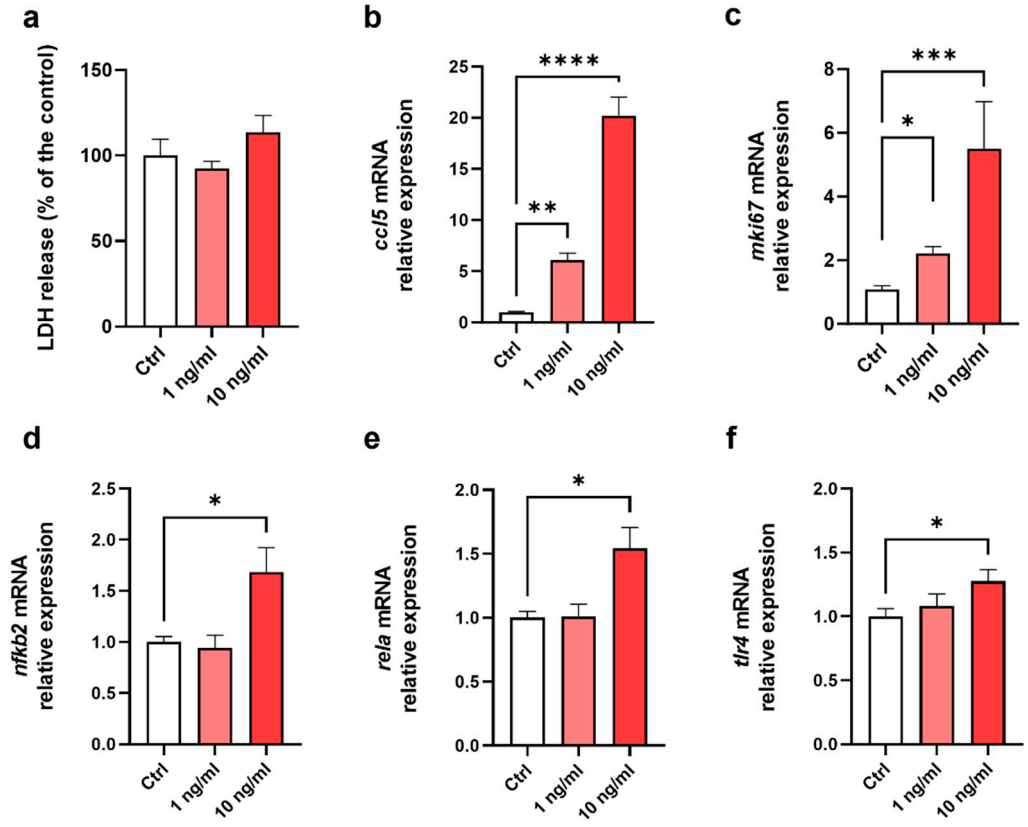

Figure 1. EDMs response to a pro-inflammatory cytokine cocktail. LDH release **(a)** and mRNA relative expressions of ccl5 **(b)**, mki67 **(c)**, nfkb2 **(d)**, rela **(e)** and tlr4 **(f)** genes in EDMs exposed to 1 and 10 ng/ml of IFN-γ/TNFα cocktail for 24h. Data are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ by Kruskal wallis test followed by post hoc Dunn's multiple comparison test.

2.2. Fg-TiO_2 dose-dependently modulated expression of genes regulating intestinal barrier function

To investigate the effects of fg-TiO_2 on gut barrier integrity and function, EDMs were exposed to the food additive at 0.1, 1 or 10 µg/mL for 24h, and the expression of forty-one genes participating in the maintenance of the gut barrier was evaluated (Figure 2a). A dose-dependent increase in the number of genes differentially expressed was observed (Figure 2b–d). At 0.1 µg/mL, 5 out of 41 genes were significantly down-regulated while none of the others showed altered expression compared to control EDMs (Figure 2b).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 January 2024 doi:10.20944/preprints202401.1383.v1

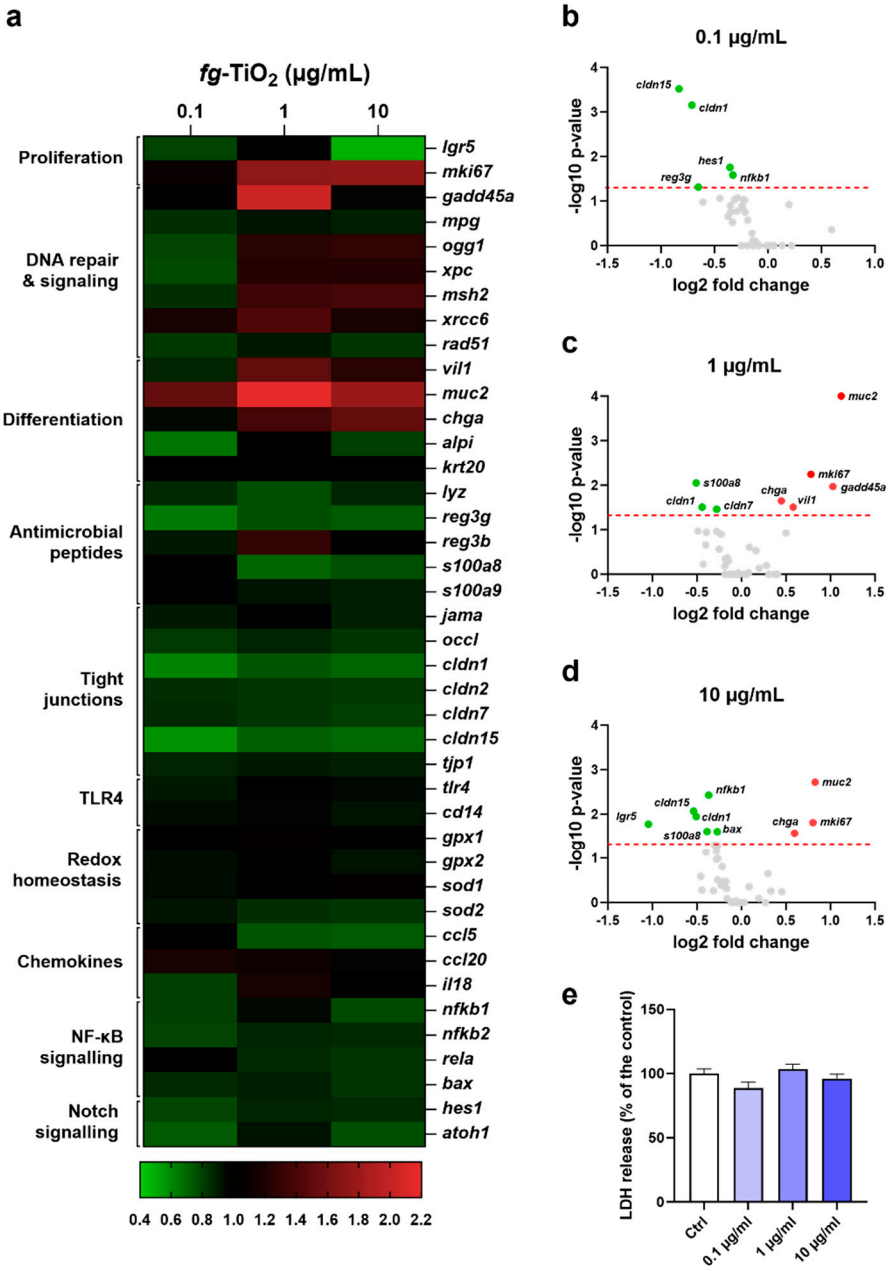

Figure 2. Differential expression of genes involved in the maintenance of the gut barrier after exposure of EDMs to fg-TiO2. **(a)** Heat-map showing differential gene expression in EDMs exposed for 24h to fg-TiO2 at 0.1, 1 and 10 µg/ml compared to control. Red and green shadings represent higher and lower relative expression levels, respectively. **(b-c)** Volcano plot illustrating significantly different genes expressed in EDMs exposed for 24h to fg-TiO2 at 0.1 **(b)**, 1 **(c)** and 10 **(d)** µg/ml. Red and green dots represent higher and lower relative expression levels, respectively. **(e)** LDH release of EDMs exposed to fg-TiO2 at 0.1, 1 and 10 µg/ml.

Next, the expression of 8 and 9 genes were significantly impacted following fg-TiO2 treatment at 1 and 10 µg/mL, respectively (Figure 2c,d). At these doses, the most differentially expressed and induced gene was the goblet cells marker mucin 2 (muc2) that encodes a mucin contributing to the mucus barrier of the intestine (Figure 2c,d). Furthermore, no difference in the level of LDH release was observed, regardless of the treatment dose, showing that EDMs remained all viable and that the transcriptomic effects herein observed consistently resulted from their exposure to the food additive only (Figure 2e). Overall, these results showed that a 24h-exposure of murine EDMs to fg-TiO2

 doi:10.20944/preprints202401.1383.v1

induced a dose-dependent modulation of genes known as key players in the intestinal barrier function.

2.3. Fg-TiO_2 altered gene expression involved in secretory cell differentiation, innate defences and epithelial TJ function of EDMs

A deeper analysis of the fg-TiO_2-mediated transcriptomic effects revealed decreased gene expression of the stem cell marker, leucine-rich repeat-containing G-protein coupled receptor 5 (lgr5), at the dose of 10 μg/mL (Figure 3a). In contrast, a dose-dependent increase of muc2 expression occurred, while a significant up-regulation of the enterocyte and enteroendocrine cells markers, Vilin (vil)1 and chromogranin (chg)A were observed at the highest doses of fg-TiO_2 only (Figure 3b). On the other hand, no significant change was reported for the Paneth cells marker lysozyme (lyz) regardless of the dose (Figure 3b). Moreover, the gene expression of regenerating islet-derived 3γ (reg3γ), encoding a C-type lectin with bactericidal activity, and S100 calcium binding protein A8 (s100a8), encoding another antimicrobial peptide in the gut, and produced by epithelial cells or Paneth cells [49–51], was found decreased in fg-TiO_2-treated EDMs (Figure 3c). For reg3γ mRNA, these effects were significant at the dose of 0.1 μg/mL only, while significant down-regulation of s100a8 occurred at the two highest concentrations of the food additive. In addition, claudin (cldn)1, cldn7 and cldn15 mRNAs encoding tight junction (TJ) proteins regulating epithelial (paracellular) permeability were decreased after fg-TiO_2 treatment, with significance at 0.1 and 10 μg of fg-TiO_2 /mL for cldn1 and cldn15, and at the highest dose only for cldn7 expression (Figure 3d). In contrast, no change was noted for junctional adhesion molecule a (jama), occludin (occl), tight junction protein 1 (tjp1) and cldn2 genes (Figure S1). As claudin-1 regulated also the Notch pathway [52], involved in signalling cell fate and homeostasis in the gut, we investigated the gene expression of atoh1 (also named math-1) and its repressor hes1, both involved in Notch-signalling. EDM exposure to fg-TiO_2 induced a reduced hes1 expression at 0.1 μg/mL, while a slight but not significant drop in expression occurred for atoh1 (Figure 3e). Altogether, these results showed that a 24h exposure of EDMs to fg-TiO_2 downregulated genes involved in innate defences and epithelial TJ permeability while gene expression of secretory cell markers was increased, suggesting a remodelling of the epithelium towards secretory lineages.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 January 2024 doi:10.20944/preprints202401.1383.v1

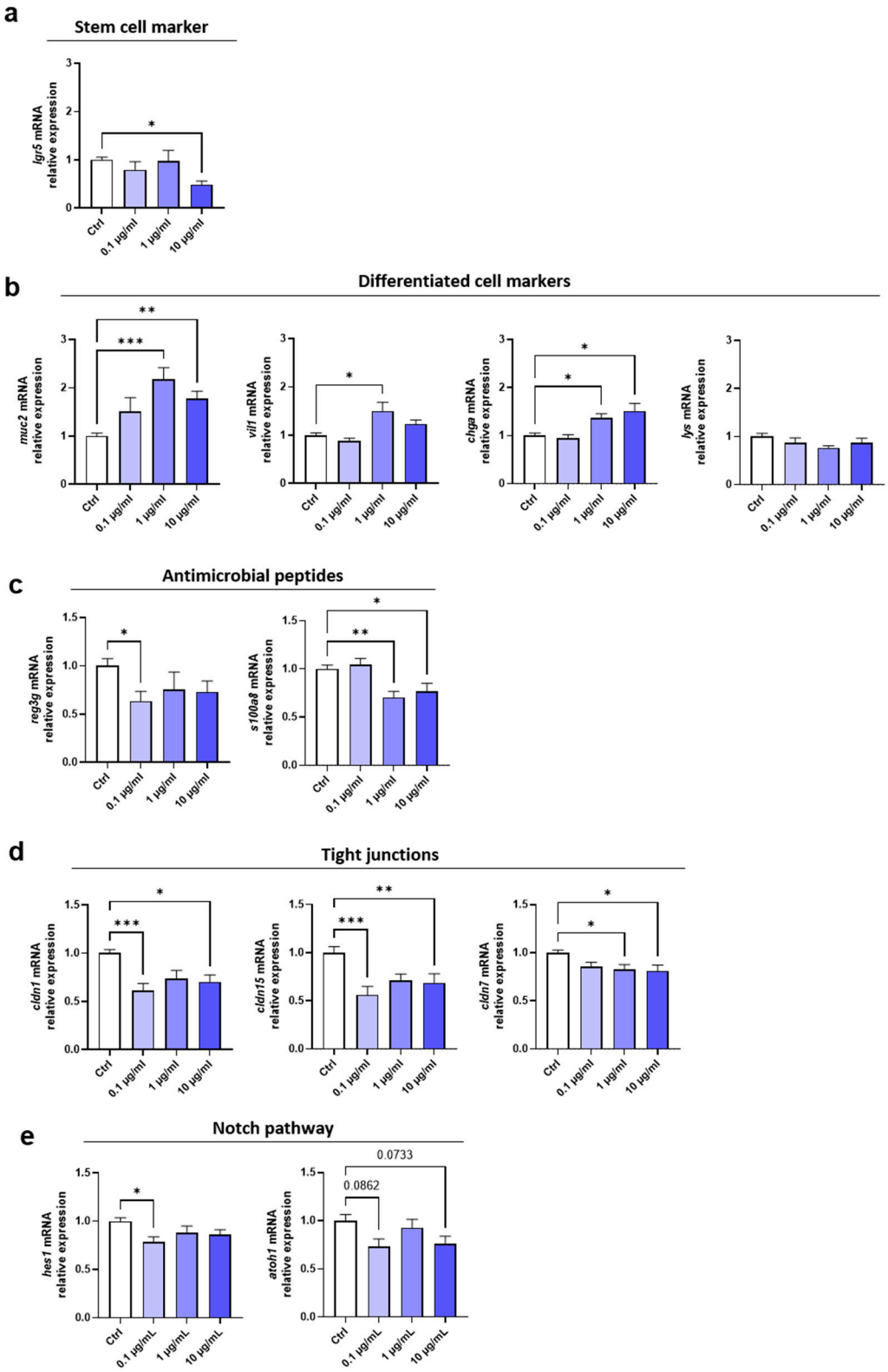

Figure 3. Effects of fg-TiO_2 exposure on EDM gene markers of secretory cell, innate defences and epithelial TJ. Relative expression of genes from stem cells (**a**), differentiated cells (**b**), antimicrobial peptides **(c)**, tight junctions **(d)**, and notch pathway **(e)** in EDMs exposed to fg-TiO_2 at 0.1, 1 and 10 µg/ml for 24h. Data are expressed as mean ± SEM. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ by one-way ANOVA followed by post hoc Dunnett's multiple comparison test or nonparametric Kruskal wallis test followed by post hoc Dunn's multiple comparison test.

2.4. Fg-TiO_2 increased apoptosis and induced genotoxicity

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 January 2024 doi:10.20944/preprints202401.1383.v1

Rodent studies as well as in vitro data using intestinal cell lines commonly reported TiO_2-NPs inducing genotoxicity in the gut [21,23,45–47] known to impact cell proliferation and apoptosis. Therefore, expression of markers involved in cell proliferation, apoptosis and DNA damage were evaluated in EDMs treated with fg-TiO_2. An increased mRNA expression of the proliferation marker mki67 was observed at 1 and 10 µg/mL of the food additive (Figure 4a).

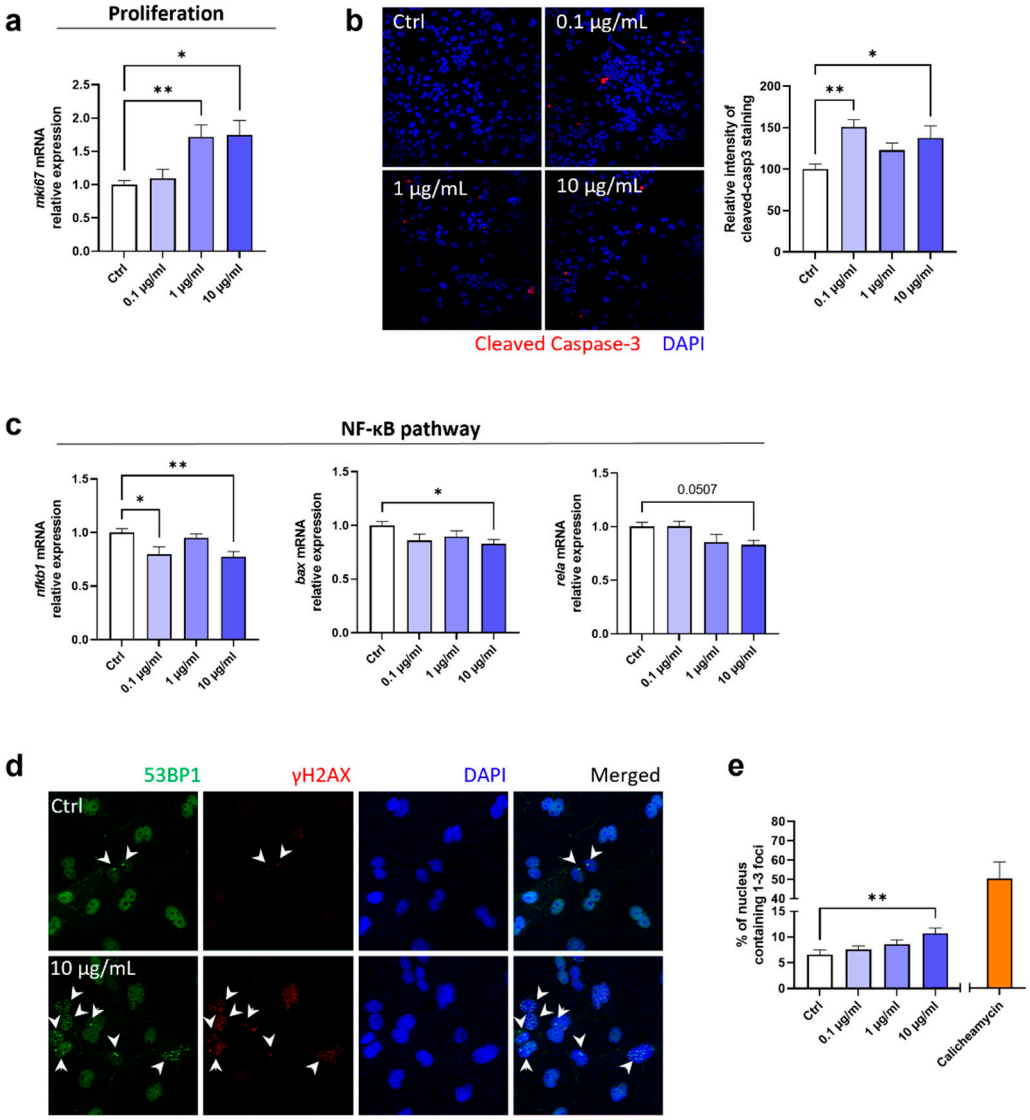

Figure 4. Effect of fg-TiO_2 exposure on cell proliferation, apoptosis and genotoxicity in EDMs exposed to fg-TiO_2 at 0.1, 1 and 10 µg/ml for 24h. **(a)** Relative expression of mki67 gene. **(b)** Immunofluorescence staining of cleaved caspase-3 and the histogram showing the relative intensity staining compared to control. **(c)** Relative expressions of genes involved in NF-κB pathway. **(d)** Immunofluorescence staining of γH2AX and 53BP1 (white arrows pointing foci of γH2AX and 53BP1). **(e)** Percentage of nucleus containing 1-3 foci of γH2AX and 53BP1. EDMs exposed to calicheamycin γ-1 was used as positive control. Data are represented as mean ± SEM. * $p<0.05$ and ** $p<0.01$ by one-way ANOVA followed by post hoc Dunnett's multiple comparison test or nonparametric Kruskal wallis test followed by post hoc Dunn's multiple comparison test.

Immunofluorescence analysis showed significant increase in protein production of cleaved caspase-3 (a marker of apoptosis) when EDMs were treated with 0.1 and 10 µg/mL of fg-TiO_2 compared to control (Figure 4b). Accordingly, a down-regulation of the anti-apoptotic NF-κB pathway [53] occurred following the same treatments, characterized by a decreased mRNA expression of nfkb1 as well as of the subunits rela and bax (Figure 4c). Next, the genotoxic potential

 doi:10.20944/preprints202401.1383.v1

of fg-TiO_2 was assessed by immunofluorescence analysis of EDMs using antibodies directed against 53BP1 and γH2AX, two well-established DNA damage biomarkers [20]. In basal conditions, the 53BP1 staining appeared as a diffuse nuclear protein with a localization pattern as large nuclear speckles in cells (Figure 4d), named 53BP1 nuclear bodies [54]. In the presence of DNA double-strand breaks, 53BP1 is recruited to the damaged site and forms compact foci, and 53BP1 foci were systematically increased in number in EDMs exposed for 24h to the highest dose of fg-TiO_2 compared to unexposed controls (Figure 4d). We next evaluated the phosphorylation of γH2AX, which occurs at DNA double-strand breaks, also forming nuclei foci. As shown in the Figure 4d, while only a few cells presented a γH2AX staining in control conditions, EDMs exposed to the fg-TiO_2 exhibited accumulated γH2AX foci in the nuclei. A quantitative analysis of merged staining showed a dose-dependent increase of the percentage of nuclei showing colocalization of γH2AX and 53BP1 foci in EDMs exposed to fg-TiO_2 (Figure 4e). When we focused on the DNA damage repair pathways genes (mpg, ogg1, xpc, msh2, xrcc4, rad51), no significant change was reported despite a tendency to increase at the two highest doses of the food additive (Figure S2a). Furthermore, for redox homeostasis genes (gpx1, gpx2, sod1, sod2), no change was reported regardless of the fg-TiO_2 concentration (Figure S2b). Taking together, these results showed that exposure of EDMs to fg-TiO_2 induced DNA damage together with increased apoptosis and proliferation of epithelial cells.

3. Discussion

Given the increased evidence of the general population exposure to inorganic particles from various environmental sources (atmospheric ultrafine particles, livestock contamination by ground and feed, food additives, processing aids, personal care products, pharmaceuticals) and routes of exposure (airways, oral, dermal), assessing the variety of toxicological impacts of these inorganic substances as part of the human exposome represents a complex challenge. For example, studies on food-related inorganic particles such as certain food additives (mainly colouring and anti-caking agents) have highlighted the need for permanent re-evaluation of their safety for human health with regards to a number of recent studies depicting new potential hazards, part of them being due to the presence of NPs in their composition [55–57]. This often requires long-term exposure performed *in vivo* using rodent models, and cell lines of various organs including those making barriers between the body and the environment, such as the intestine. Although animal studies continue to be central to answer these questions, the regulatory environment encourages alternative methods aimed at replace animal models [58]. However, *in vitro* experiments using intestinal cell lines lacks of cell diversity and specific cell functions to decipher the variety of effects of these food contaminants. In the current study, we showed that enteroid-derived monolayers (EDMs) prepared from murine intestinal organoids, and exposed at the apical-luminal surface for 24h to an inorganic particle model commonly found in the human diet, namely the white pigment *fg*-TiO_2, recapitulate most of the known effects of this food additive in the gut from long-term *in vivo* studies.

In order to validate the ability of this EDM model to react physiologically and dose-dependently to an environmental stimulus as a potential hazard signal, their response to a pro-inflammatory stimulus was first tested using a cocktail of IFN-γ/TNF-α cytokines. During pathogenic infection in the gut, innate and adaptive immune cells act together through IFN-γ and TNF-α secretion, to enhance TLR4 mRNA and its protein expression by epithelial cells to induce pathogen recognition and innate immunity activation [59]. This TLR4 signalling can be MyD88-dependent or -independent pathway, involving the MAPK and the NF-κB pathway activations that lead to proinflammatory cytokine release [60–62]. The production of CCL5 by the intestinal epithelial cells was also regulated by TNF-α and IFN-γ and lead to inflammation maintenance, and migration of cells to the inflammatory site [63,64]. Finally, TNF-α mediated epithelial proliferation in intestinal inflamed tissues characterized notably by an increased number of Ki-67$^+$ cells per crypt [65]. In our study, the EDM exposure to this cocktail consistently induced a dose-dependent up-regulation of *nfκb2* and of its subunit *rela* as well as of *ccl5*, *mki67* and *tlr4*. These results confirmed that our EDM model is able to respond to inflammatory stresses, and supported their use to evaluate the direct impact of *fg*-TiO_2 on the gut epithelial barrier.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 January 2024 doi:10.20944/preprints202401.1383.v1

Following this validation step, the current study focused on a list of 41 genes of which expression then protein products are involved in the regulation of the intestinal barrier [66–69]. In the presence of *fg*-TiO_2, a dose-dependent modulation was observed, with 5, 8 and 9 of the 41 genes whose expression significantly differed from controls in EDMs exposed to 0.1, 1 and 10 µg/mL of this food additive, respectively. Among these genes, the expression of the stem cell marker *lgr5* was decreased at the highest dose of *fg*-TiO_2. Zhang et al. showed similar down-regulation of *lgr5* expression in mice and human gut organoids when exposed to 50µg/mL of non-food TiO_2-NPs (time of exposure not detailed), which is consistent with data in the mouse small intestine after a two-months exposure to the same dose of TiO_2-NPs by drinking water [70]. This effect could explain previous *in vivo* studies showing modification of intestinal epithelium morphology exposed to *fg*-TiO_2, and characterized notably by a reduction in the length of the crypts [71,72] which contain stem cells. Furthermore, an increased expression of goblet cells (*muc2*), enterocytes (*vil1*) and enteroendocrine (*chga*) cell markers was also observed in our study, while no change in the gene expression of the Paneth cell marker (*lyz*) occurred in *fg*-TiO_2-exposed EDMs. The effects of TiO_2 on intestinal expression of *vil1*, *chga* and *lyz* have been rarely or not evaluated, while studies exploring the impact of TiO_2 on *muc2* expression or mucus production often reported contradictory results. Indeed, co-culture of Caco-2 and HT29-MTX cells forming a regular mucus-secreting epithelium *in vitro* showed an increase in mucus secretion with no change in *muc2* gene expression after 21 days of exposure to 10, 50 or 100µg/mL of *fg*-TiO_2 [73]. Moreover, *in vivo* studies reported both decreased or increased *muc2* gene expression or goblet cell population after TiO_2 exposure, depending on time of exposure, the dose and the vehicle for treatment (*i.e.*, gavage, drinking water or incorporated into food pellets) [45,71,72,74,75]. Altogether, our study showed that all epithelial cell types are present and reactive to an inorganic agent in our EDM model, and that a 24-hour exposure to *fg*-TiO_2 altered the stem cell homeostasis (*lgr5*) in a dose-dependent manner while promoting the differentiation of secretory cells such as goblet (*muc2*) and enteroendocrine (*chga*) cells, suggesting a remodelling/restructuring of the intestinal epithelium mainly towards secretory lineages.

Given the importance of gut permeability in systemic toxicity of chemicals, investigating the impact of xenobiotics on gut permeability is particularly relevant. Indeed, an alteration of this barrier after ingestion of a xenobiotic can increase its passage into the bloodstream, as well as that of other environmental factors such as pathogenic substances or opportunistic bacteria, with potential health consequences. Paracellular permeability along the gut epithelium is controlled by TJ protein complexes sealing cells between them, and composed of transmembrane proteins of the claudin family, occludin, and junctional adhesion molecules that are essentials to the function of the physical gut barrier [76]. Of note, some discrepancies have been found in the literature regarding the ability of TiO_2 to influence intestinal permeability, which could be related to data obtained from the small or large intestine *in vitro* (i.e., by using Ussing chambers) or from the total gut *in vivo* (oral macromolecules), as well as the period for TiO_2 exposure, i.e., including perinatal life or not. For instance, an increased *in vivo* intestinal permeability associated with a decreased expression in the jejunum of various genes related to intercellular junctions, such as *occl* and *cldn15*, was observed in male mice perinatally exposed to *fg*-TiO_2 [18], while no permeability change occurred in this intestinal segment when exposure was limited to adulthood [43]. Another study also showed an increased permeability in colon of mice perinatally exposed to *fg*-TiO_2 [34], still not observed in adulthood [21]. In the ileum of adult mice, a down-regulation of *tjp1*, *tjp2*, *cldn2*, *cldn3* and *occl* has been reported after a single oral gavage of TiO_2-NPs at 12.5 mg/kg bw [44]. Similarly, a decreased mRNA expression of *tjp1* occurred in colon of adult rats exposed to *fg*-TiO_2 at 500 mg/kg bw/week for 10 weeks [77]. In our study, EDMs prepared with organoids obtained from small intestine stem cells of adult mice also exhibited a decreased expression of genes encoding TJ proteins, mainly *cldn1*, *cldn7* and *cldn15* after 24h of exposure to *fg*-TiO_2. However, genes encoding for occludin and JAM-A were found unaltered, both are key transmembrane proteins controlling intercellular spaces along the intestine [78,79]. This observation is consistent with limited or no alteration of intestinal permeability when the adult gut is directly exposed to the food additive, in contrast to a perinatal treatment. In addition to its role as a TJ protein sealing adjacent cells, claudin-1 also regulates gut homeostasis through the regulation of

 doi:10.20944/preprints202401.1383.v1

Notch-signalling. Interestingly, using a villin-claudin-1 transgenic (Cl-1Tg) mouse model, authors showed that overexpression of claudin 1 led to Notch-signalling activation, which in turn downregulated *muc2* expression and inhibited the goblet cell differentiation [52]. Therefore, one may hypothesize that the increase in *muc2* expression observed in *fg*-TiO_2-treated EDMs could be partly due to the observed decrease in *cldn1* expression. This hypothesis is herein supported by the absence of Notch pathway activation in EDMs exposed to the food additive, which is consistent with another study showing no modulation of Notch signalling target gene *hes1*, in mouse and human intestinal organoids exposed to 50µg/mL of non-food TiO_2-NPs [70].

In vitro, we also showed a decreased expression of the antimicrobial peptides *reg3γ* and *s100a8* genes after *fg*-TiO_2 treatment of EDMs. A down-regulation of *reg3γ* has been reported in the colon of juvenile mice treated with TiO_2 NPs at 10 and 40 mg /kg bw/day for 28 days [80]. The authors postulated that such effect may result from a direct alteration of the gut microbiota (namely gut dysbiosis) induced by sustained exposure to non-absorbed NPs in the gut lumen affecting gut microbiota–host co-metabolites leading to intestinal barrier damage [80]. However, in the *in vitro* model of EDMs that we used, i.e., in the absence of gut bacteria, the *fg*-TiO_2-evoked decrease in *reg3γ* expression clearly suggested a microbiota-independent pathway for such regulation. This impact of the food additive could be linked to the increased expression of *muc2* herein observed because, *in vivo*, mucin deficiency in Muc2 knock out mice enhanced expression of *reg3γ* in the small intestine and colon [81]. Taken together, these *in vivo* data and our results using EDM model suggested that in addition to a direct impact of *fg*-TiO_2 on intestinal bacteria [22,82,83], the food additive could also indirectly induced gut dysbiosis *via* a reduction in the secretion of antimicrobial peptides by epithelial cells associated with an increase in mucus production in the intestine.

We further investigated whether the genotoxic potential of TiO_2 previously reported *in vivo* and *in vitro* [21,23,45–47] is also observed in EDMs. Accordingly, the two markers of DNA double-stand breaks, γH2AX and 53BP1 [84], were found accumulated and formed foci in EDMs exposed to *fg*-TiO_2. Some studies have reported that TiO_2-related genotoxicity mainly resulted from oxidative stress [39,85]. However, we do not observe changes in the expression of genes encoding for antioxidant enzymes, such as the glutathione peroxidase 1 and 2 (*gpx1* and *gpx2*) and the superoxide dismutase 1 and 2 (*sod1* and *sod2*). Consequently, it seems that some of the *fg*-TiO_2-induced DNA damage in the intestine could not result from induction of oxidative stress, at least at a transcriptomic level, which is concordant with main conclusions in an *in vitro* study using Caco-2 and HT29-MTX co-culture model [86]. Whatever the origin for DNA lesions, DNA damage may interfere with the cell cycle and have consequences for cell proliferation and apoptosis [87,88]. Consistently, we report an increased expression of *mki67* and cleaved caspase 3 in EDMs exposed to *fg*-TiO_2, suggesting a pro-proliferative and pro-apoptotic effect of the food additive. In human colon organoids, significant increased expression of apoptotic genes and proteins was also showed after a 48h exposure to TiO_2-NPs [89]. Furthermore, NF-κB pathway markers such as *nfkb1, rela* and *bax*, were found down-regulated in the current study. Interestingly, NF-κB signalling pathway is activated in numerous cancers, leading to decreased apoptosis in malignant cells [90,91], and one may hypothesize in our study that the pro-apoptotic effect of the *fg*-TiO_2 could be partly due to the inhibition of the NF-κB signalling pathway. Overall, these results support the genotoxic potential of *fg*-TiO_2 using an EDM model, with DNA damage appearing independently of oxidative stress, while leading to increased apoptosis, probably *via* inhibition of the NF-κB pathway.

In conclusion, the effects of *fg*-TiO_2 described in our study using an EDM model for toxicological testing are in concordance with the already reported data on intestinal effects of TiO_2 (including NPs) when used as food additive. Indeed, we showed that the integrity of the gut barrier in terms of cell proliferation/differentiation, genotoxicity, innate defences and epithelial TJs is altered in murine EDMs exposed for 24h to *fg*-TiO_2. As our food-grade form of TiO_2 (commercial E171 in EU) is representative of manufactured inorganic nanomaterials exposing the general population through the diet, this study suggests that EDMs, which recapitulate the complex cellular composition of the gut epithelium, could constitute a reliable tool for rapid toxicological screening of inorganic foodborne chemicals.

 doi:10.20944/preprints202401.1383.v1

4. Materials and Methods

4.1. Murine intestinal organoids

Intestinal crypts were collected from 5 to 8-week-old C57BL/6J male mice (Janvier Labs, France) (n= 3 mice). Small intestine was collected, longitudinally opened and cut into 3 fragments in a petri dish containing cold PBS. After removing all feces and intestinal content, the small intestine fragments were cut into 0.5 cm pieces, transferred to a 50-mL tube, washed by up and down pipetting and supernatant removed. This step was repeated 20-30 times. Small intestine pieces were then digested with Gentle Cell Dissociation (GCD) solution (STEMCELL, #100-0485) for 20 minutes at room temperature (RT) with gentle agitation. Supernatant was removed and small intestine pieces were resuspended with cold PBS 0.1% BSA. After up and down pipetting, the supernatant was filtered with a 70 µm filter. This step was repeated to collect 7 fractions of cell suspension. All fractions were then centrifugated at 290g for 5 minutes at 4°C and supernatants were discarded. Pellets were washed with cold PBS 0.1% BSA and centrifugated again. All pellets were then resuspended in DMEM/F12 medium (STEMCELL, #36254) and the crypts were counted. Fractions containing at least 1500 crypts/mL were used for organoid culture. After a centrifugation at 290g for 5 minutes at 4°C, pellets were resuspended with cold Intesticult OGM mouse medium (STEMCELL, #6005) supplemented with 1% penicillin/streptomycin (Fischer Scientific, #11548876) (referred here as complete mouse medium), and ice-cold Matrigel (STEMCELL, #) at 1:1 ratio and each crypts/Matrigel suspension were seeded in pre-warmed 24-well plate at density of 2500 crypts/50µL. After incubation for 10 minutes at 37°C, 5% CO_2 for polymerization, organoids were cultured with complete mouse medium. Media were replaced every 2-3 days and organoids were passed every week. Briefly, organoids were mechanically broken by pipetting in GCD solution and centrifuged at 290g for 5 minutes at 4°C. Pellets were washed and re-seeded with a dilution ratio 1:4 and cultured as described above.

4.2. Preparation of enteroid-derived monolayers (EDMs) and treatments

After 4 passages, organoids were proceeded for EDM culture and treatments. To obtain EDMs, organoids were first cultured in Intesticult OGM Human medium (STEMCELL, #6010) supplemented with 10µM Y2763 (STEMCELL, #72302) and 1% penicillin /streptomycin (referred here as complete Human medium) for 24h. Organoids were then disrupted, centrifuged and washed as described above. Pellets were resuspended with TrypLExpress (GIBCO, #12604-013), incubated at 37°C for 10 minutes for a total dissociation of the organoids and the reaction was stopped by adding a volume of DMEM/F12. 2.10^5 cells were seeded in a 2% Matrigel precoated 24 well-plate with or without coverslip and cultured for 5 days at 37°C 5% CO_2, prior to treatment.

The *fg*-TiO_2 was purchased as powder from the website of a French commercial supplier of food colouring agents, and was already characterized in previous studies as a representative E171 sample in the anatase crystal form that has been placed on the EU market [18–21,24,43]. Briefly, to obtain a stable dispersion of TiO_2 particles, the *fg*-TiO_2 stock suspension (10mg/mL) was sonicated in an ice bath for 16 min at 30% amplitude with a VCX 750-230 V (Sonics Materials), then stocked at 4°C during 15 days maximum before use. In a first experiment, EDMs were exposed to a cytokine cocktail of interferon (IFN)-γ (VWR, #PRSI92-539) and tumor necrosis factor (TNF)-α (VWR, #SHBT200-31) at 1 or 10 ng/mL for 24h as a control for dose-dependent responses to a proinflammatory stimulus. In a second series, EDMs were exposed to *fg*-TiO_2 at 0.1, 1 or 10 µg/mL for 24 hours, then prepared for cytotoxicity assay, gene expression analysis and immunofluorescence. Calicheamicin γ1 (200nM for 1 hour) was used as a positive genotoxic control for γH2AX/53BP1 immunostaining.

4.3. Measurement of lactate dehydrogenase (LDH) release

LDH release was measured using the CytoTox96 Non-Radiocative Cytotoxicity Assay kit (Promega, #G1781) according to the manufacturer's instructions. Briefly, culture media of EDMs exposed to *fg*-TiO_2 or cytokine cocktail for 24h were collected, and the CytoTox reagent was added to

 doi:10.20944/preprints202401.1383.v1

each sample and incubated for 30 minutes at room temperature. The reaction was stopped using Stop Solution and the absorbance at 490nm was recorded with the Spark microplate reader (TECAN). LDH release was depicted as percent of the control.

4.4. Gene expression analysis

Total RNA was extracted from EDM culture lysed in RLT buffer from the RNeasy Mini Kit (QIAGEN, 74106), according to the manufacturer's specification. A DNAse I digestion step was included in the purification protocol. The concentration and purity of total mRNA were measured by the N60 nanospectrophotometer (Implen). 400 ng of total RNA was used for cDNA synthesis using supermix iScript RT (BioRad, #1708841) following manufacturer's protocol. cDNA was diluted 1:25 in nuclease-free water. Real-time PCR was performed using the ROX SYBR Mastermix blue dTTP (Takyon, #UF-RSMFB0701) and specific primers (Table S1) on a Viaa7 Real-Time PCR System (Applied Biosystems). Each assay was performed in duplicate and the specificity of PCR products was verified by melting curve analysis. The relative expression level of the target genes was calculated by the ΔCt method ($2^{-\Delta Ct}$) and was normalized to the gene expression of the Ribosomal Protein L19 (RPL19). All expressions were relative to the untreated control (fold change).

4.5. Immunofluorescence

EDM cultures on coverslips were collected and fixed with 4% formalin for 30 minutes. All steps were performed at room temperature. EDMs were then permeabilised with PBS 0.2X Triton for 30 minutes and treated with NH_4Cl for 30 minutes to remove residual formalin. EDMs were incubated in PBS 1% BSA for 1 hour, followed by overnight incubation at 4°C with a rabbit anti-mouse cleaved caspase-3 antibody (Cell Signaling, #9661), or a mix of mouse anti-vertebrate γH2AX (Merk Millipore, #05-636) and rabbit anti-mouse 53BP1 (Novus Biologicals, #NB100-304) or rhodamine Phalloidin (Invitrogen, #R415). EDMs were then incubated for 2 hours with a mix of Alexa Fluor 680 goat anti-mouse IgG (H+L) (Invitrogen, #A21058) and Alexa Fluor 488 chicken anti-rabbit IgG (H+L) (Invitrogen, #A21441) or with an Alexa Fluor Plus 680 Donkey anti-rabbit IgG (H+L) (Invitrogen, #A32802). EDMs were mounted in Prolong gold antifade mounting medium with DAPI (Invitrogen, #P10144) and examined under a Leica SP8 confocal microscope. Number of 53BP1 and γH2AX foci, and cleaved caspase-3 fluorescence intensity were quantified with the ImageJ/Fiji software.

4.6. Statistical analysis

All results represented a mean of 4 to 6 technical replicates from 3 biological replicates. Statistical analysis was performed using GraphPad Prism (version 9.3.1) and data presented as the mean ± SEM. Normal distribution was determined by Kolmogorov-Smirnov test with Dallal-Wilkinson-Lillie correction. For datasets that failed normality tests, nonparametric tests were used to analyse significant differences. Multiple comparisons were evaluated by one-way ANOVA followed by Dunnett's multiple comparisons tests or by nonparametric Kruskal-Wallis tests followed by Dunn's multiple comparisons tests. Volcano plots were realised by plotting log2 of the fold change to -log10 of the p-value. Differences corresponding to $p < 0.05$ were considered significant.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: tight junction expression in EDMs exposed to *fg*-TiO_2; Figure S2: DNA repair pathway and redox homeostasis in EDMs exposed to *fg*-TiO2; Table S1: list of the primers used for the q-PCR

Author Contributions: Conceptualization, Y.M., E.C., E.H., L.E. and B.L.; methodology, Y.M., E.C., A.P-D., C.C., E.G., N.M.B., and L.E.; validation, E.H. and B.L.; formal analysis, Y.M., E.C., A.P-D., C.C., E.G., N.M.B., and L.E.; writing—original draft preparation, Y.M., E.C., L.E., E.H. and B.L.; writing—review and editing, Y.M., E.C., L.E., E.H. and B.L.; supervision, E.H. and B.L.; funding acquisition, E.H. and B.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work has received fundings from the European Union's Horizon 2020 Research and Innovation Program under Grant agreement no. 720964 [npSCOPE], and from the French National Research Agency, number ANR-19-CE34-0015-01 (grant TitADiet) and number ANR-20-CE34-0011-01 (grant DevADDIRisk).

Institutional Review Board Statement: Ethical review and approval were waived for this study due to the use of post-mortem tissues of animals that did not get any clinical invasive procedures.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

Acknowledgments: We acknowledge Dr. Julien Vignard for his generous gift of Calicheamicin γ1, as well as for his expertise and extensive advice on DNA damage and DNA repair signaling analysis. We also acknowledge Dr Martin Beaumont for his expertise and advice on intestinal organoid culture and maintenance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chaudhry, Q.; Scotter, M.; Blackburn, J.; Ross, B.; Boxall, A.; Castle, L.; Aitken, R.; Watkins, R. Applications and Implications of Nanotechnologies for the Food Sector. *Food Addit. Contam. Part A* **2008**, *25*, 241–258, doi:10.1080/02652030701744538.
2. Sahani, S.; Sharma, Y.C. Advancements in Applications of Nanotechnology in Global Food Industry. *Food Chem.* **2021**, *342*, 128318, doi:10.1016/j.foodchem.2020.128318.
3. Garcia, C.V.; Shin, G.H.; Kim, J.T. Metal Oxide-Based Nanocomposites in Food Packaging: Applications, Migration, and Regulations. *Trends Food Sci. Technol.* **2018**, *82*, 21–31, doi:10.1016/j.tifs.2018.09.021.
4. EFSA Panel on Food Additives and Flavourings (FAF); Younes, M.; Aquilina, G.; Castle, L.; Engel, K.-H.; Fowler, P.; Frutos Fernandez, M.J.; Fürst, P.; Gundert-Remy, U.; Gürtler, R.; et al. Safety Assessment of Titanium Dioxide (E171) as a Food Additive. *EFSA J.* **2021**, *19*, e06585, doi:10.2903/j.efsa.2021.6585.
5. EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS); Younes, M.; Aggett, P.; Aguilar, F.; Crebelli, R.; Dusemund, B.; Filipič, M.; Frutos, M.J.; Galtier, P.; Gott, D.; et al. Re-Evaluation of Silicon Dioxide (E 551) as a Food Additive. *EFSA J.* **2018**, *16*, e05088, doi:10.2903/j.efsa.2018.5088.
6. Costa, J.; Ahluwalia, A. Advances and Current Challenges in Intestinal in Vitro Model Engineering: A Digest. *Front. Bioeng. Biotechnol.* **2019**, *7*.
7. Min, S.; Kim, S.; Cho, S.-W. Gastrointestinal Tract Modeling Using Organoids Engineered with Cellular and Microbiota Niches. *Exp. Mol. Med.* **2020**, *52*, 227–237, doi:10.1038/s12276-020-0386-0.
8. Wu, X.; Su, J.; Wei, J.; Jiang, N.; Ge, X. Recent Advances in Three-Dimensional Stem Cell Culture Systems and Applications. *Stem Cells Int.* **2021**, *2021*, 9477332, doi:10.1155/2021/9477332.
9. Rahmani, S.; Breyner, N.M.; Su, H.-M.; Verdu, E.F.; Didar, T.F. Intestinal Organoids: A New Paradigm for Engineering Intestinal Epithelium in Vitro. *Biomaterials* **2019**, *194*, 195–214, doi:10.1016/j.biomaterials.2018.12.006.
10. Leslie, J.L.; Young, V.B. A Whole New Ball Game: Stem Cell-Derived Epithelia in the Study of Host–Microbe Interactions. *Anaerobe* **2016**, *37*, 25–28, doi:10.1016/j.anaerobe.2015.10.016.
11. d'Aldebert, E.; Quaranta, M.; Sébert, M.; Bonnet, D.; Kirzin, S.; Portier, G.; Duffas, J.-P.; Chabot, S.; Lluel, P.; Allart, S.; et al. Characterization of Human Colon Organoids From Inflammatory Bowel Disease Patients. *Front. Cell Dev. Biol.* **2020**, *8*.
12. Zietek, T.; Giesbertz, P.; Ewers, M.; Reichart, F.; Weinmüller, M.; Urbauer, E.; Haller, D.; Demir, I.E.; Ceyhan, G.O.; Kessler, H.; et al. Organoids to Study Intestinal Nutrient Transport, Drug Uptake and Metabolism – Update to the Human Model and Expansion of Applications. *Front. Bioeng. Biotechnol.* **2020**, *8*.
13. Takahashi, Y.; Noguchi, M.; Inoue, Y.; Sato, S.; Shimizu, M.; Kojima, H.; Okabe, T.; Kiyono, H.; Yamauchi, Y.; Sato, R. Organoid-Derived Intestinal Epithelial Cells Are a Suitable Model for Preclinical Toxicology and Pharmacokinetic Studies. *iScience* **2022**, *25*, 104542, doi:10.1016/j.isci.2022.104542.
14. Sharma, A.; Lee, J.; Fonseca, A.G.; Moshensky, A.; Kothari, T.; Sayed, I.M.; Ibeawuchi, S.-R.; Pranadinata, R.F.; Ear, J.; Sahoo, D.; et al. E-Cigarettes Compromise the Gut Barrier and Trigger Inflammation. *iScience* **2021**, *24*, 102035, doi:10.1016/j.isci.2021.102035.

15. Altay, G.; Larrañaga, E.; Tosi, S.; Barriga, F.M.; Batlle, E.; Fernández-Majada, V.; Martínez, E. Self-Organized Intestinal Epithelial Monolayers in Crypt and Villus-like Domains Show Effective Barrier Function. *Sci. Rep.* **2019**, *9*, 10140, doi:10.1038/s41598-019-46497-x.
16. Bischoff, N.S.; de Kok, T.M.; Sijm, D.T.H.M.; van Breda, S.G.; Briedé, J.J.; Castenmiller, J.J.M.; Opperhuizen, A.; Chirino, Y.I.; Dirven, H.; Gott, D.; et al. Possible Adverse Effects of Food Additive E171 (Titanium Dioxide) Related to Particle Specific Human Toxicity, Including the Immune System. *Int. J. Mol. Sci.* **2020**, *22*, 207, doi:10.3390/ijms22010207.
17. Bischoff, N.S.; Proquin, H.; Jetten, M.J.; Schrooders, Y.; Jonkhout, M.C.M.; Briedé, J.J.; van Breda, S.G.; Jennen, D.G.J.; Medina-Reyes, E.I.; Delgado-Buenrostro, N.L.; et al. The Effects of the Food Additive Titanium Dioxide (E171) on Tumor Formation and Gene Expression in the Colon of a Transgenic Mouse Model for Colorectal Cancer. *Nanomater. Basel Switz.* **2022**, *12*, 1256, doi:10.3390/nano12081256.
18. Issa, M.; Michaudel, C.; Guinot, M.; Grauso-Culetto, M.; Guillon, B.; Lecardonnel, J.; Jouneau, L.; Chapuis, C.; Bernard, H.; Hazebrouck, S.; et al. Long-Term Exposure from Perinatal Life to Food-Grade TiO2 Alters Intestinal Homeostasis and Predisposes to Food Allergy in Young Mice. *Allergy* **2023**, doi:10.1111/all.15960.
19. Lamas, B.; Chevalier, L.; Gaultier, E.; Cartier, C.; Weingarten, L.; Blanc, X.; Fisicaro, P.; Oster, C.; Noireaux, J.; Evariste, L.; et al. The Food Additive Titanium Dioxide Hinders Intestinal Production of TGF-β and IL-10 in Mice, and Long-Term Exposure in Adults or from Perinatal Life Blocks Oral Tolerance to Ovalbumin. *Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc.* **2023**, *179*, 113974, doi:10.1016/j.fct.2023.113974.
20. Vignard, J.; Pettes-Duler, A.; Gaultier, E.; Cartier, C.; Weingarten, L.; Biesemeier, A.; Taubitz, T.; Pinton, P.; Bebeacua, C.; Devoille, L.; et al. Food-Grade Titanium Dioxide Translocates across the Buccal Mucosa in Pigs and Induces Genotoxicity in an in Vitro Model of Human Oral Epithelium. *Nanotoxicology* **2023**, *17*, 289–309, doi:10.1080/17435390.2023.2210664.
21. Bettini, S.; Boutet-Robinet, E.; Cartier, C.; Coméra, C.; Gaultier, E.; Dupuy, J.; Naud, N.; Taché, S.; Grysan, P.; Reguer, S.; et al. Food-Grade TiO2 Impairs Intestinal and Systemic Immune Homeostasis, Initiates Preneoplastic Lesions and Promotes Aberrant Crypt Development in the Rat Colon. *Sci. Rep.* **2017**, *7*, 40373, doi:10.1038/srep40373.
22. Radziwill-Bienkowska, J.M.; Talbot, P.; Kamphuis, J.B.J.; Robert, V.; Cartier, C.; Fourquaux, I.; Lentzen, E.; Audinot, J.-N.; Jamme, F.; Réfrégiers, M.; et al. Toxicity of Food-Grade TiO2 to Commensal Intestinal and Transient Food-Borne Bacteria: New Insights Using Nano-SIMS and Synchrotron UV Fluorescence Imaging. *Front. Microbiol.* **2018**, *9*, 794, doi:10.3389/fmicb.2018.00794.
23. Dorier, M.; Béal, D.; Marie-Desvergne, C.; Dubosson, M.; Barreau, F.; Houdeau, E.; Herlin-Boime, N.; Carriere, M. Continuous in Vitro Exposure of Intestinal Epithelial Cells to E171 Food Additive Causes Oxidative Stress, Inducing Oxidation of DNA Bases but No Endoplasmic Reticulum Stress. *Nanotoxicology* **2017**, *11*, 751–761, doi:10.1080/17435390.2017.1349203.
24. Guillard, A.; Gaultier, E.; Cartier, C.; Devoille, L.; Noireaux, J.; Chevalier, L.; Morin, M.; Grandin, F.; Lacroix, M.Z.; Coméra, C.; et al. Basal Ti Level in the Human Placenta and Meconium and Evidence of a Materno-Foetal Transfer of Food-Grade TiO2 Nanoparticles in an Ex Vivo Placental Perfusion Model. *Part. Fibre Toxicol.* **2020**, *17*, 51, doi:10.1186/s12989-020-00381-z.
25. Weir, A.; Westerhoff, P.; Fabricius, L.; Hristovski, K.; von Goetz, N. Titanium Dioxide Nanoparticles in Food and Personal Care Products. *Environ. Sci. Technol.* **2012**, *46*, 2242–2250, doi:10.1021/es204168d.
26. Radtke, J.; Wiedey, R.; Kleinebudde, P. Alternatives to Titanium Dioxide in Tablet Coating. *Pharm. Dev. Technol.* **2021**, *26*, 989–999, doi:10.1080/10837450.2021.1968900.
27. Béchard, S.R.; Quraishi, O.; Kwong, E. Film Coating: Effect of Titanium Dioxide Concentration and Film Thickness on the Photostability of Nifedipine. *Int. J. Pharm.* **1992**, *87*, 133–139, doi:10.1016/0378-5173(92)90236-U.
28. Grande, F.; Tucci, P. Titanium Dioxide Nanoparticles: A Risk for Human Health? *Mini Rev. Med. Chem.* **2016**, *16*, 762–769, doi:10.2174/1389557516666160321114341.
29. Skocaj, M.; Filipic, M.; Petkovic, J.; Novak, S. Titanium Dioxide in Our Everyday Life; Is It Safe? *Radiol. Oncol.* **2011**, *45*, 227–247, doi:10.2478/v10019-011-0037-0.
30. Peters, R.J.B.; van Bemmel, G.; Herrera-Rivera, Z.; Helsper, H.P.F.G.; Marvin, H.J.P.; Weigel, S.; Tromp, P.C.; Oomen, A.G.; Rietveld, A.G.; Bouwmeester, H. Characterization of Titanium Dioxide Nanoparticles in Food Products: Analytical Methods To Define Nanoparticles. *J. Agric. Food Chem.* **2014**, *62*, 6285–6293, doi:10.1021/jf5011885.

31. COMMISSION REGULATION (EU) 2022/63 of 14 January 2022 Amending Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council as Regards the Food Additive Titanium Dioxide (E 171) 2022.
32. Charles, S.; Jomini, S.; Fessard, V.; Bigorgne-Vizade, E.; Rousselle, C.; Michel, C. Assessment of the in Vitro Genotoxicity of TiO2 Nanoparticles in a Regulatory Context. *Nanotoxicology* **2018**, *12*, 357–374, doi:10.1080/17435390.2018.1451567.
33. Takeda, K.; Suzuki, K.; Ishihara, A.; Kubo-Irie, M.; Fujimoto, R.; Tabata, M.; Oshio, S.; Nihei, Y.; Ihara, T.; Sugamata, M. Nanoparticles Transferred from Pregnant Mice to Their Offspring Can Damage the Genital and Cranial Nerve Systems. *J. Health Sci.* **2009**, *55*, 95–102, doi:10.1248/jhs.55.95.
34. Carlé, C.; Boucher, D.; Morelli, L.; Larue, C.; Ovtchinnikova, E.; Battut, L.; Boumessid, K.; Airaud, M.; Quaranta-Nicaise, M.; Ravanat, J.-L.; et al. Perinatal Foodborne Titanium Dioxide Exposure-Mediated Dysbiosis Predisposes Mice to Develop Colitis through Life. *Part. Fibre Toxicol.* **2023**, *20*, 45, doi:10.1186/s12989-023-00555-5.
35. Li, X.; Song, L.; Hu, X.; Liu, C.; Shi, J.; Wang, H.; Zhan, L.; Song, H. Inhibition of Epithelial-Mesenchymal Transition and Tissue Regeneration by Waterborne Titanium Dioxide Nanoparticles. *ACS Appl. Mater. Interfaces* **2018**, *10*, 3449–3458, doi:10.1021/acsami.7b18986.
36. Guo, Z.; Martucci, N.J.; Moreno-Olivas, F.; Tako, E.; Mahler, G.J. Titanium Dioxide Nanoparticle Ingestion Alters Nutrient Absorption in an In Vitro Model of the Small Intestine. *NanoImpact* **2017**, *5*, 70–82, doi:10.1016/j.impact.2017.01.002.
37. Gao, Y.; Ye, Y.; Wang, J.; Zhang, H.; Wu, Y.; Wang, Y.; Yan, L.; Zhang, Y.; Duan, S.; Lv, L.; et al. Effects of Titanium Dioxide Nanoparticles on Nutrient Absorption and Metabolism in Rats: Distinguishing the Susceptibility of Amino Acids, Metal Elements, and Glucose. *Nanotoxicology* **2020**, *14*, 1301–1323, doi:10.1080/17435390.2020.1817597.
38. Pedata, P.; Ricci, G.; Malorni, L.; Venezia, A.; Cammarota, M.; Volpe, M.G.; Iannaccone, N.; Guida, V.; Schiraldi, C.; Romano, M.; et al. In Vitro Intestinal Epithelium Responses to Titanium Dioxide Nanoparticles. *Food Res. Int. Ott. Ont* **2019**, *119*, 634–642, doi:10.1016/j.foodres.2018.10.041.
39. Proquin, H.; Rodríguez-Ibarra, C.; Moonen, C.G.J.; Urrutia Ortega, I.M.; Briedé, J.J.; de Kok, T.M.; van Loveren, H.; Chirino, Y.I. Titanium Dioxide Food Additive (E171) Induces ROS Formation and Genotoxicity: Contribution of Micro and Nano-Sized Fractions. *Mutagenesis* **2017**, *32*, 139–149, doi:10.1093/mutage/gew051.
40. Ruiz, P.A.; Morón, B.; Becker, H.M.; Lang, S.; Atrott, K.; Spalinger, M.R.; Scharl, M.; Wojtal, K.A.; Fischbeck-Terhalle, A.; Frey-Wagner, I.; et al. Titanium Dioxide Nanoparticles Exacerbate DSS-Induced Colitis: Role of the NLRP3 Inflammasome. *Gut* **2017**, *66*, 1216–1224, doi:10.1136/gutjnl-2015-310297.
41. Carolina Maciel Nogueira, W.M. de A.; rgio Hiroshi Toma, A.; Zonetti de Arruda Leite, M.L.L.; cia Ortiz-Agostinho, M.I.S.D. Titanium Dioxide Induced Inflammation in the Small Intestine. *World J. Gastroenterol.* **2012**, *18*, 4729–4735, doi:10.3748/wjg.v18.i34.4729.
42. Racovita, A.D. Titanium Dioxide: Structure, Impact, and Toxicity. *Int. J. Environ. Res. Public. Health* **2022**, *19*, 5681, doi:10.3390/ijerph19095681.
43. Coméra, C.; Cartier, C.; Gaultier, E.; Catrice, O.; Panouille, Q.; El Hamdi, S.; Tirez, K.; Nelissen, I.; Théodorou, V.; Houdeau, E. Jejunal Villus Absorption and Paracellular Tight Junction Permeability Are Major Routes for Early Intestinal Uptake of Food-Grade TiO2 Particles: An in Vivo and Ex Vivo Study in Mice. *Part. Fibre Toxicol.* **2020**, *17*, 26, doi:10.1186/s12989-020-00357-z.
44. Brun, E.; Barreau, F.; Veronesi, G.; Fayard, B.; Sorieul, S.; Chanéac, C.; Carapito, C.; Rabilloud, T.; Mabondzo, A.; Herlin-Boime, N.; et al. Titanium Dioxide Nanoparticle Impact and Translocation through Ex Vivo, in Vivo and in Vitro Gut Epithelia. *Part. Fibre Toxicol.* **2014**, *11*, 13, doi:10.1186/1743-8977-11-13.
45. Urrutia-Ortega, I.M.; Garduño-Balderas, L.G.; Delgado-Buenrostro, N.L.; Freyre-Fonseca, V.; Flores-Flores, J.O.; González-Robles, A.; Pedraza-Chaverri, J.; Hernández-Pando, R.; Rodríguez-Sosa, M.; León-Cabrera, S.; et al. Food-Grade Titanium Dioxide Exposure Exacerbates Tumor Formation in Colitis Associated Cancer Model. *Food Chem. Toxicol.* **2016**, *93*, 20–31, doi:10.1016/j.fct.2016.04.014.
46. Vieira, A.; Vital, N.; Rolo, D.; Roque, R.; Gonçalves, L.M.; Bettencourt, A.; Silva, M.J.; Louro, H. Investigation of the Genotoxicity of Digested Titanium Dioxide Nanomaterials in Human Intestinal Cells. *Food Chem. Toxicol.* **2022**, *161*, 112841, doi:10.1016/j.fct.2022.112841.
47. Zijno, A.; De Angelis, I.; De Berardis, B.; Andreoli, C.; Russo, M.T.; Pietraforte, D.; Scorza, G.; Degan, P.; Ponti, J.; Rossi, F.; et al. Different Mechanisms Are Involved in Oxidative DNA Damage and Genotoxicity

Induction by ZnO and TiO2 Nanoparticles in Human Colon Carcinoma Cells. *Toxicol. In Vitro* **2015**, *29*, 1503–1512, doi:10.1016/j.tiv.2015.06.009.

48. Chakrabarti, S.; Goyary, D.; Karmakar, S.; Chattopadhyay, P. Exploration of Cytotoxic and Genotoxic Endpoints Following Sub-Chronic Oral Exposure to Titanium Dioxide Nanoparticles. *Toxicol. Ind. Health* **2019**, *35*, 577–592, doi:10.1177/0748233719879611.
49. Fujita, Y.; Khateb, A.; Li, Y.; Tinoco, R.; Zhang, T.; Bar-Yoseph, H.; Tam, M.A.; Chowers, Y.; Sabo, E.; Gerassy-Vainberg, S.; et al. Regulation of S100A8 Stability by RNF5 in Intestinal Epithelial Cells Determines Intestinal Inflammation and Severity of Colitis. *Cell Rep.* **2018**, *24*, 3296-3311.e6, doi:10.1016/j.celrep.2018.08.057.
50. Sonnenberg, G.F.; Fouser, L.A.; Artis, D. Border Patrol: Regulation of Immunity, Inflammation and Tissue Homeostasis at Barrier Surfaces by IL-22. *Nat. Immunol.* **2011**, *12*, 383–390, doi:10.1038/ni.2025.
51. Cash, H.L.; Whitham, C.V.; Behrendt, C.L.; Hooper, L.V. Symbiotic Bacteria Direct Expression of an Intestinal Bactericidal Lectin. *Science* **2006**, *313*, 1126–1130, doi:10.1126/science.1127119.
52. Pope, J.L.; Bhat, Ajaz.A.; Sharma, A.; Ahmad, R.; Krishnan, M.; Washington, M.K.; Beauchamp, R.D.; Singh, A.B.; Dhawan, P. Claudin-1 Regulates Intestinal Epithelial Homeostasis through the Modulation of Notch Signaling. *Gut* **2014**, *63*, 622–634, doi:10.1136/gutjnl-2012-304241.
53. Bernal-Mizrachi, L.; Lovly, C.M.; Ratner, L. The Role of NF-KB-1 and NF-KB-2-Mediated Resistance to Apoptosis in Lymphomas. *Proc. Natl. Acad. Sci.* **2006**, *103*, 9220–9225, doi:10.1073/pnas.0507809103.
54. Fernandez-Vidal, A.; Vignard, J.; Mirey, G. Around and beyond 53BP1 Nuclear Bodies. *Int. J. Mol. Sci.* **2017**, *18*, 2611, doi:10.3390/ijms18122611.
55. Smolkova, B.; El Yamani, N.; Collins, A.R.; Gutleb, A.C.; Dusinska, M. Nanoparticles in Food. Epigenetic Changes Induced by Nanomaterials and Possible Impact on Health. *Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc.* **2015**, *77*, 64–73, doi:10.1016/j.fct.2014.12.015.
56. Medina-Reyes, E.I.; Rodríguez-Ibarra, C.; Déciga-Alcaraz, A.; Díaz-Urbina, D.; Chirino, Y.I.; Pedraza-Chaverri, J. Food Additives Containing Nanoparticles Induce Gastrotoxicity, Hepatotoxicity and Alterations in Animal Behavior: The Unknown Role of Oxidative Stress. *Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc.* **2020**, *146*, 111814, doi:10.1016/j.fct.2020.111814.
57. ANSES- French Agency for Food, Environmental and Occupational Health & Safety, France; Anastasi, E.; Riviere, G.; Teste, B. Nanomaterials in Food - Prioritisation & Assessment. *EFSA J. Eur. Food Saf. Auth.* **2019**, *17*, e170909, doi:10.2903/j.efsa.2019.e170909.
58. Robinson, N.B.; Krieger, K.; Khan, F.M.; Huffman, W.; Chang, M.; Naik, A.; Yongle, R.; Hameed, I.; Krieger, K.; Girardi, L.N.; et al. The Current State of Animal Models in Research: A Review. *Int. J. Surg.* **2019**, *72*, 9–13, doi:10.1016/j.ijsu.2019.10.015.
59. Abreu, M.T.; Arnold, E.T.; Thomas, L.S.; Gonsky, R.; Zhou, Y.; Hu, B.; Arditi, M. TLR4 and MD-2 Expression Is Regulated by Immune-Mediated Signals in Human Intestinal Epithelial Cells. *J. Biol. Chem.* **2002**, *277*, 20431–20437, doi:10.1074/jbc.M110333200.
60. Vaure, C.; Liu, Y. A Comparative Review of Toll-Like Receptor 4 Expression and Functionality in Different Animal Species. *Front. Immunol.* **2014**, *5*, 316, doi:10.3389/fimmu.2014.00316.
61. Shang, L.; Fukata, M.; Thirunarayanan, N.; Martin, A.P.; Arnaboldi, P.; Maussang, D.; Berin, C.; Unkeless, J.C.; Mayer, L.; Abreu, M.T.; et al. Toll-Like Receptor Signaling in Small Intestinal Epithelium Promotes B-Cell Recruitment and IgA Production in Lamina Propria. *Gastroenterology* **2008**, *135*, 529-538.e1, doi:10.1053/j.gastro.2008.04.020.
62. Daghero, H.; Doffe, F.; Varela, B.; Yozzi, V.; Verdes, J.M.; Crispo, M.; Bollati-Fogolín, M.; Pagotto, R. Jejunum-Derived NF-KB Reporter Organoids as 3D Models for the Study of TNF-Alpha-Induced Inflammation. *Sci. Rep.* **2022**, *12*, 14425, doi:10.1038/s41598-022-18556-3.
63. Lee, J.W.; Wang, P.; Kattah, M.G.; Youssef, S.; Steinman, L.; DeFea, K.; Straus, D.S. Differential Regulation of Chemokines by IL-17 in Colonic Epithelial Cells1. *J. Immunol.* **2008**, *181*, 6536–6545, doi:10.4049/jimmunol.181.9.6536.
64. Warhurst, A.C.; Hopkins, S.J.; Warhurst, G. Interferon γ Induces Differential Upregulation of α and β Chemokine Secretion in Colonic Epithelial Cell Lines. *Gut* **1998**, *42*, 208–213, doi:10.1136/gut.42.2.208.
65. Bradford, E.M.; Ryu, S.H.; Singh, A.P.; Lee, G.; Goretsky, T.; Sinh, P.; Williams, D.B.; Cloud, A.L.; Gounaris, E.; Patel, V.; et al. Epithelial TNF Receptor Signaling Promotes Mucosal Repair in Inflammatory Bowel Disease. *J. Immunol.* **2017**, *199*, 1886–1897, doi:10.4049/jimmunol.1601066.

66. Vancamelbeke, M.; Vanuytsel, T.; Farré, R.; Verstockt, S.; Ferrante, M.; Van Assche, G.; Rutgeerts, P.; Schuit, F.; Vermeire, S.; Arijs, I.; et al. Genetic and Transcriptomic Basis of Intestinal Epithelial Barrier Dysfunction in Inflammatory Bowel Disease. *Inflamm. Bowel Dis.* **2017**, *23*, 1718–1729, doi:10.1097/MIB.0000000000001246.
67. Burgueño, J.F.; Abreu, M.T. Epithelial Toll-like Receptors and Their Role in Gut Homeostasis and Disease. *Nat. Rev. Gastroenterol. Hepatol.* **2020**, *17*, 263–278, doi:10.1038/s41575-019-0261-4.
68. Beaumont, M.; Lencina, C.; Painteaux, L.; Viémon-Desplanque, J.; Phornlaphat, O.; Lambert, W.; Chalvon-Demersay, T. A Mix of Functional Amino Acids and Grape Polyphenols Promotes the Growth of Piglets, Modulates the Gut Microbiota in Vivo and Regulates Epithelial Homeostasis in Intestinal Organoids. *Amino Acids* **2022**, *54*, 1357–1369, doi:10.1007/s00726-021-03082-9.
69. Noah, T.K.; Donahue, B.; Shroyer, N.F. Intestinal Development and Differentiation. *Exp. Cell Res.* **2011**, *317*, 2702–2710, doi:10.1016/j.yexcr.2011.09.006.
70. Zhang, L.; He, Y.; Dong, L.; Liu, C.; Su, L.; Guo, R.; Luo, Q.; Gan, B.; Cao, F.; Wang, Y.; et al. Perturbation of Intestinal Stem Cell Homeostasis and Radiation Enteritis Recovery via Dietary Titanium Dioxide Nanoparticles. *Cell Prolif. n/a*, e13427, doi:10.1111/cpr.13427.
71. Medina-Reyes, E.I.; Delgado-Buenrostro, N.L.; Díaz-Urbina, D.; Rodríguez-Ibarra, C.; Déciga-Alcaraz, A.; González, M.I.; Reyes, J.L.; Villamar-Duque, T.E.; Flores-Sánchez, M.LO.; Hernández-Pando, R.; et al. Food-Grade Titanium Dioxide (E171) Induces Anxiety, Adenomas in Colon and Goblet Cells Hyperplasia in a Regular Diet Model and Microvesicular Steatosis in a High Fat Diet Model. *Food Chem. Toxicol.* **2020**, *146*, 111786, doi:10.1016/j.fct.2020.111786.
72. Pinget, G.; Tan, J.; Janac, B.; Kaakoush, N.O.; Angelatos, A.S.; O'Sullivan, J.; Koay, Y.C.; Sierro, F.; Davis, J.; Divakarla, S.K.; et al. Impact of the Food Additive Titanium Dioxide (E171) on Gut Microbiota-Host Interaction. *Front. Nutr.* **2019**, *6*, 57, doi:10.3389/fnut.2019.00057.
73. Dorier, M.; Béal, D.; Tisseyre, C.; Marie-Desvergne, C.; Dubosson, M.; Barreau, F.; Houdeau, E.; Herlin-Boime, N.; Rabilloud, T.; Carriere, M. The Food Additive E171 and Titanium Dioxide Nanoparticles Indirectly Alter the Homeostasis of Human Intestinal Epithelial Cells in Vitro. *Environ. Sci. Nano* **2019**, *6*, 1549–1561, doi:10.1039/C8EN01188E.
74. Yan, J.; Wang, D.; Li, K.; Chen, Q.; Lai, W.; Tian, L.; Lin, B.; Tan, Y.; Liu, X.; Xi, Z. Toxic Effects of the Food Additives Titanium Dioxide and Silica on the Murine Intestinal Tract: Mechanisms Related to Intestinal Barrier Dysfunction Involved by Gut Microbiota. *Environ. Toxicol. Pharmacol.* **2020**, *80*, 103485, doi:10.1016/j.etap.2020.103485.
75. Zhu, X.; Zhao, L.; Liu, Z.; Zhou, Q.; Zhu, Y.; Zhao, Y.; Yang, X. Long-Term Exposure to Titanium Dioxide Nanoparticles Promotes Diet-Induced Obesity through Exacerbating Intestinal Mucus Layer Damage and Microbiota Dysbiosis. *Nano Res.* **2021**, *14*, 1512–1522, doi:10.1007/s12274-020-3210-1.
76. Horowitz, A.; Chanez-Paredes, S.D.; Haest, X.; Turner, J.R. Paracellular Permeability and Tight Junction Regulation in Gut Health and Disease. *Nat. Rev. Gastroenterol. Hepatol.* **2023**, *20*, 417–432, doi:10.1038/s41575-023-00766-3.
77. Jensen, D.M.; Løhr, M.; Sheykhzade, M.; Lykkesfeldt, J.; Wils, R.S.; Loft, S.; Møller, P. Telomere Length and Genotoxicity in the Lung of Rats Following Intragastric Exposure to Food-Grade Titanium Dioxide and Vegetable Carbon Particles. *Mutagenesis* **2019**, *34*, 203–214, doi:10.1093/mutage/gez003.
78. Braniste, V.; Leveque, M.; Buisson-Brenac, C.; Bueno, L.; Fioramonti, J.; Houdeau, E. Oestradiol Decreases Colonic Permeability through Oestrogen Receptor β-Mediated up-Regulation of Occludin and Junctional Adhesion Molecule-A in Epithelial Cells. *J. Physiol.* **2009**, *587*, 3317–3328, doi:10.1113/jphysiol.2009.169300.
79. Braniste, V.; Jouault, A.; Gaultier, E.; Polizzi, A.; Buisson-Brenac, C.; Leveque, M.; Martin, P.G.; Theodorou, V.; Fioramonti, J.; Houdeau, E. Impact of Oral Bisphenol A at Reference Doses on Intestinal Barrier Function and Sex Differences after Perinatal Exposure in Rats. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 448–453, doi:10.1073/pnas.0907697107.
80. Yan, J.; Chen, Q.; Tian, L.; Li, K.; Lai, W.; Bian, L.; Han, J.; Jia, R.; Liu, X.; Xi, Z. Intestinal Toxicity of Micro- and Nano-Particles of Foodborne Titanium Dioxide in Juvenile Mice: Disorders of Gut Microbiota–Host Co-Metabolites and Intestinal Barrier Damage. *Sci. Total Environ.* **2022**, *821*, 153279, doi:10.1016/j.scitotenv.2022.153279.
81. Paassen, N.B.; Loonen, L.M.P.; Witte-Bouma, J.; Male, A.M.K.; Bruijn, A.C.J.M. de; Sluis, M. van der; Lu, P.; Goudoever, J.B.V.; Wells, J.M.; Dekker, J.; et al. Mucin Muc2 Deficiency and Weaning Influences the

Expression of the Innate Defense Genes Reg3β, Reg3γ and Angiogenin-4. *PLOS ONE* **2012**, *7*, e38798, doi:10.1371/journal.pone.0038798.

82. Dudefoi, W.; Moniz, K.; Allen-Vercoe, E.; Ropers, M.-H.; Walker, V.K. Impact of Food Grade and Nano-TiO2 Particles on a Human Intestinal Community. *Food Chem. Toxicol.* **2017**, *106*, 242–249, doi:10.1016/j.fct.2017.05.050.
83. Lamas, B.; Martins Breyner, N.; Houdeau, E. Impacts of Foodborne Inorganic Nanoparticles on the Gut Microbiota-Immune Axis: Potential Consequences for Host Health. *Part. Fibre Toxicol.* **2020**, *17*, 19, doi:10.1186/s12989-020-00349-z.
84. Chatterjee, N.; Walker, G.C. Mechanisms of DNA Damage, Repair and Mutagenesis. *Environ. Mol. Mutagen.* **2017**, *58*, 235–263, doi:10.1002/em.22087.
85. Shukla, R.K.; Sharma, V.; Pandey, A.K.; Singh, S.; Sultana, S.; Dhawan, A. ROS-Mediated Genotoxicity Induced by Titanium Dioxide Nanoparticles in Human Epidermal Cells. *Toxicol. In Vitro* **2011**, *25*, 231–241, doi:10.1016/j.tiv.2010.11.008.
86. Dorier, M.; Tisseyre, C.; Dussert, F.; Béal, D.; Arnal, M.-E.; Douki, T.; Valdiglesias, V.; Laffon, B.; Fraga, S.; Brandão, F.; et al. Toxicological Impact of Acute Exposure to E171 Food Additive and TiO2 Nanoparticles on a Co-Culture of Caco-2 and HT29-MTX Intestinal Cells. *Mutat. Res. Toxicol. Environ. Mutagen.* **2019**, *845*, 402980, doi:10.1016/j.mrgentox.2018.11.004.
87. Kaina, B. DNA Damage-Triggered Apoptosis: Critical Role of DNA Repair, Double-Strand Breaks, Cell Proliferation and Signaling. *Biochem. Pharmacol.* **2003**, *66*, 1547–1554, doi:10.1016/S0006-2952(03)00510-0.
88. Ruiz-Losada, M.; González, R.; Peropadre, A.; Gil-Gálvez, A.; Tena, J.J.; Baonza, A.; Estella, C. Coordination between Cell Proliferation and Apoptosis after DNA Damage in Drosophila. *Cell Death Differ.* **2022**, *29*, 832–845, doi:10.1038/s41418-021-00898-6.
89. Park, S.B.; Jung, W.H.; Kim, K.Y.; Koh, B. Toxicity Assessment of SiO2 and TiO2 in Normal Colon Cells, In Vivo and in Human Colon Organoids. *Molecules* **2020**, *25*, 3594, doi:10.3390/molecules25163594.
90. Dolcet, X.; Llobet, D.; Pallares, J.; Matias-Guiu, X. NF-KB in Development and Progression of Human Cancer. *Virchows Arch.* **2005**, *446*, 475–482, doi:10.1007/s00428-005-1264-9.
91. Xia, L.; Tan, S.; Zhou, Y.; Lin, J.; Wang, H.; Oyang, L.; Tian, Y.; Liu, L.; Su, M.; Wang, H.; et al. Role of the NFκB-Signaling Pathway in Cancer. *OncoTargets Ther.* **2018**, *11*, 2063–2073, doi:10.2147/OTT.S161109.