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Effects of Emulsifiers on an In vitro Model of Intestinal Epithelial Tight Junctions and the Transport of Food Allergens

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Abstract

Scope

Certain food emulsifiers may interfere with gut barrier function in ways correlating to increased exposure to allergens. Understanding the consequences of interactions between these food ingredients and the intestinal epithelium is important for evaluating allergen dose exposure characteristics.

Methods and Results

This study challenged Caco-2 cell monolayers, an in vitro model of human intestinal epithelial tight junctions with synthetic polysorbate-80 or natural lecithin alone, or in combination with known allergens (egg proteins: ovalbumin, ovomucoid, and ovotransferrin; and a synthetic form of galactose- α -1,3-galactose [α -gal], an allergen of increasing concern). For most doses of individual emulsifiers and allergens, >90% cell viability and <15% cytotoxicity are observed; however, toxicity increased at a 0.5% concentration of emulsifiers. At low cytotoxic concentration (0.2%), only polysorbate-80 treatment reduced monolayer integrity ($\approx$ 20%) with increased lucifer yellow passage. Dose-related differences in expression of tight junction-associated genes and occludin protein are observed with emulsifier treatments. The transport of all tested allergens across the cell monolayers, excluding ovotransferrin, nearly doubled in the presence of 0.2% polysorbate-80 compared to lecithin and untreated control.

Conclusion

By modulating paracellular permeability, polysorbate-80 may enhance absorption of allergens in a size-dependent manner.

1 Introduction

The permeability of the gut epithelium allows efficient passage of nutrients into the body and restricts the entry of harmful larger molecules and foreign antigens. In humans, the gut epithelium consists of a single layer of epithelial cells with dynamic structures called tight junction (TJ) in between each cell. If TJ integrity is disrupted, substances that are normally kept inside the gut lumen may pass through the epithelial barrier, thereby enhancing, rather than restricting, the absorption of those substances.^[1] Although this might be desirable when epithelial permeability is deliberately altered to enhance the absorption of medications,^[2] if increased permeability leads to enhanced exposure to toxins^[3] or food allergens, susceptible individuals may be at risk for harmful outcomes.^[4-7]

A distinctive feature of many food allergens is their resistance to gastric digestion,^[8] consequently, the epitopes of these substances are still antigenic when they reach the intestine. However, not all antigenic epitopes are proteins; among these are carbohydrates, such as galactose-alpha-1,3-galactose (alpha-gal),^[9] or micro-molecules, such as sulphites.^[10, 11] Development of alpha-gal mediated meat allergy is strongly linked to certain tick bites^[12-14] and certain drugs.^[15, 16] Antigens provoking allergic reactions are usually transported across the gut barrier by the transcellular route, but depending on the health of the individual, it is possible for exposures to occur via paracellular transport or through both routes.^[17] The size, polarity, shape, aggregation status, and 3-dimensional structure of a particular antigen can also influence its transport. Systematic studies are still needed to determine whether absorption of antigens via the paracellular route is a consequence of allergic reaction or vice versa.^[18, 19] Nevertheless, it is well established that the amount of antigen absorbed plays a key role in the development of food allergic reactions. Therefore, understanding which substances might increase the absorption of antigens is important to mitigate the adverse effects of exposure.

Food ingredients in conjunction with allergenic materials can play a role in the development of allergic reactions in susceptible individuals.^[20, 21] Among these, several emulsifiers (also called surfactants or detergents) have already been shown to increase TJ permeability;^[3, 7, 22-24] this suggests there might be a relationship between epithelial barrier integrity or function, and absorption of allergens from foods.^[17, 19] Emulsifiers are amphipathic molecules, having both hydrophobic and hydrophilic regions that allow these molecules to interact with both oily and aqueous substances. The usefulness of emulsifiers (e.g., polysorbates, polyglycerol polyricinoleate, sorbitans, etc.) in processed foods to maintain desirable functional characteristics (e.g., stabilizing the oil and water for mayonnaise, ice cream, etc.), has resulted in broad consumer exposure to these ingredients in foods and pharmaceuticals.^[19, 25, 26]

Previous studies have observed paracellular uptake of the egg white allergen ovomucoid^[4] and soy allergens,^[27] while certain allergens are transported transcellularly.^[28-30] The transcellular route is important for absorption of proteins in non-sensitized individuals,^[18, 31-33] whereas in sensitized individuals proteins can also be transported via the paracellular route.^[34] Importantly, increased TJ permeability persists in food allergic or intolerant children and adults, even if these people adhere to an allergen-free diet.^[35-37] Different food emulsifiers might affect one route of allergen transport through the epithelium more than another route.

The effects of two well-known emulsifiers, polysorbate-80 (P-80) and lecithin (LE), on epithelial permeability were evaluated in this study. Research has already shown that the chemical structures and physicochemical properties of a given emulsifier can lead to intestinal cell cytotoxicity; with some polysorbates, even in small amounts can exert such effects.^[38, 39] However, whether LE has similar effects has not yet been explored. Three major allergenic proteins from egg, ovomucoid (OM; 28 kD), ovalbumin (OA; 43 kD), and ovotransferrin (OT; 78 kD), which represents a range of molecular weights and structure-functions,^[40] and alpha-gal,^[41] a sugar molecule responsible for human allergic reactions to red meat were used to examine their transport across gut epithelium. The intact, or non-degraded, form of each egg allergen was used to investigate the effect of molecular size on their transport. The effects of P-80 and LE separately and in combination with allergens were examined utilizing the cultured Caco-2 intestinal epithelial cells on Transwell inserts, which form highly differentiated polarized monolayers exhibiting functional TJs. This model is often used to evaluate potential risks to epithelial barriers posed by various substances or microbes.^[42-45] The cell viability and the responsiveness of TJ proteins, including their gene expression to emulsifiers were also assessed. This research can help provide evidence on the influence of emulsifiers on TJ permeability and the resultant change in allergen dose exposure.

2 Results and Discussion

2.1 Establishing Experimental Doses of Selected Emulsifiers and Allergens

The changes in epithelial resistance and associated cytotoxicity caused by exposure to increasing concentrations of emulsifiers and allergens were determined. We examined two biomarkers: 1) lactate dehydrogenase (LDH) release indicating damage to the plasma membrane or cytotoxicity^[46] and 2) enzymatic reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to formazan for evaluating metabolically active viable cells.^[47] Treatment of cells with OA, OM, and OT at a broad range of concentrations (0.01%, 0.1%, and 1%) showed minimal ($\leq 5\%$) to no effect on the relative LDH release (**Figure 1A**). Based on these data, we selected an allergen concentration of 0.05% for subsequent quantitative apical to basolateral transport experiments. No cytotoxicity was determined examining cells treated with 0.125% s-alpha-gal (**Figure 1C**). At the lower exposure levels

(0.01%, 0.05%, 0.1%, and 0.2% concentrations of P-80 and LE), we detected no remarkable release of LDH (<10%), and no differences across exposure conditions (Figure 1B). However, as the concentration of emulsifiers increased, we saw increased release of LDH and remarkable differences between the cytotoxic properties of P-80 and LE (Figure 1B). This suggested that 0.2% solutions of emulsifiers should be the maximum used in subsequent experiments. Figure 1C–E illustrates the combined cytotoxic effects of allergens with various concentrations (up to 0.2%) of emulsifiers. The observed effects with combinations were comparable to the emulsifiers alone treatments at all tested concentrations, which was expected, since our data (Figure 1A) indicated that none of the tested allergens produced cytotoxic effects.

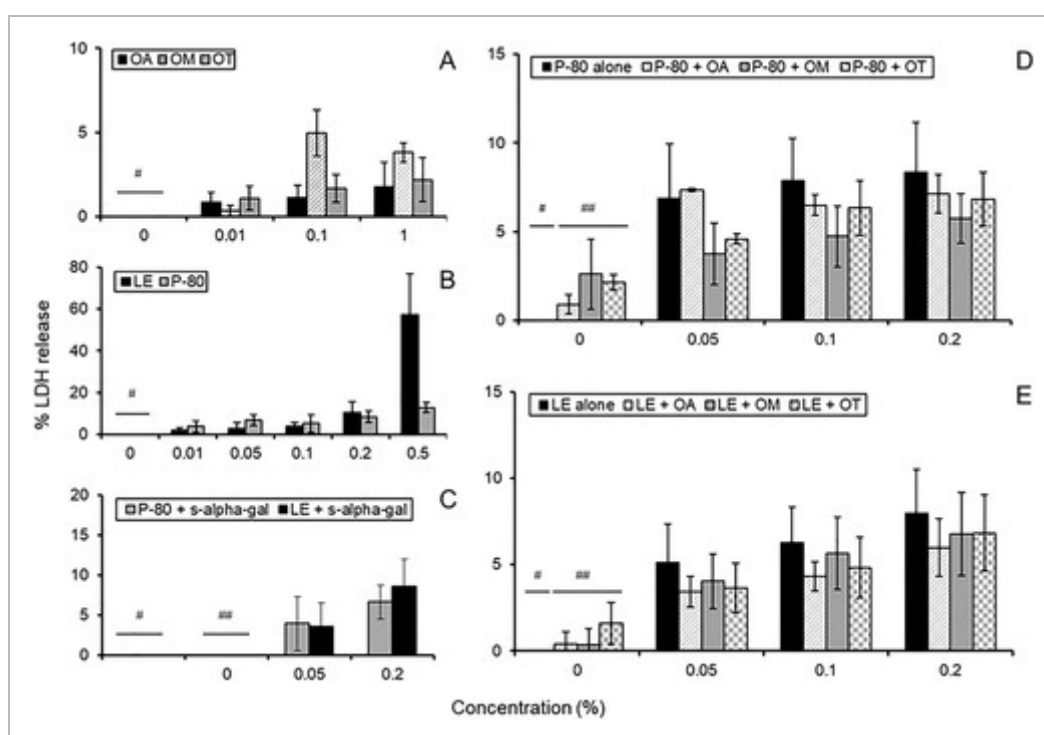


Figure 1

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LDH assays to determine cytotoxic effects of emulsifiers, allergens, and combinations. Cells were treated with increasing concentrations of A) egg proteins: ovalbumin (OA), ovomucoid (OM), and ovotransferrin (OT); B) lecithin (LE) and polysorbate-80 (P-80); C) 0.125% synthetic α 1-3, β 1-4 galactotriose (s-alpha-gal) with P-80 or LE; D) 0.05% egg proteins with P-80; and E) 0.05% egg proteins with LE. Values expressed as mean $\pm$ SEM. # HBSS control; ## allergen alone.

To perform MTT assays, cells were incubated overnight with assay reagents and fresh culture medium after treatment removal. Results from MTT assays (Figure 2A) confirmed that neither OA, OM, nor OT altered the viability of cells at any of our treatment concentrations; a similar effect was observed with s-alpha-gal treatment (Figure 2C). Interestingly, treatment with P-80 seemed to increase cell viability in a dose-dependent manner, while increasing concentrations of LE decreased the percentage of viable cells

(Figure 2B). It had been assumed that both emulsifiers would damage the cell membrane in concentration-dependent manner, since previous research indicated that higher concentrations of emulsifiers can interfere with cell viability.^[38, 39]

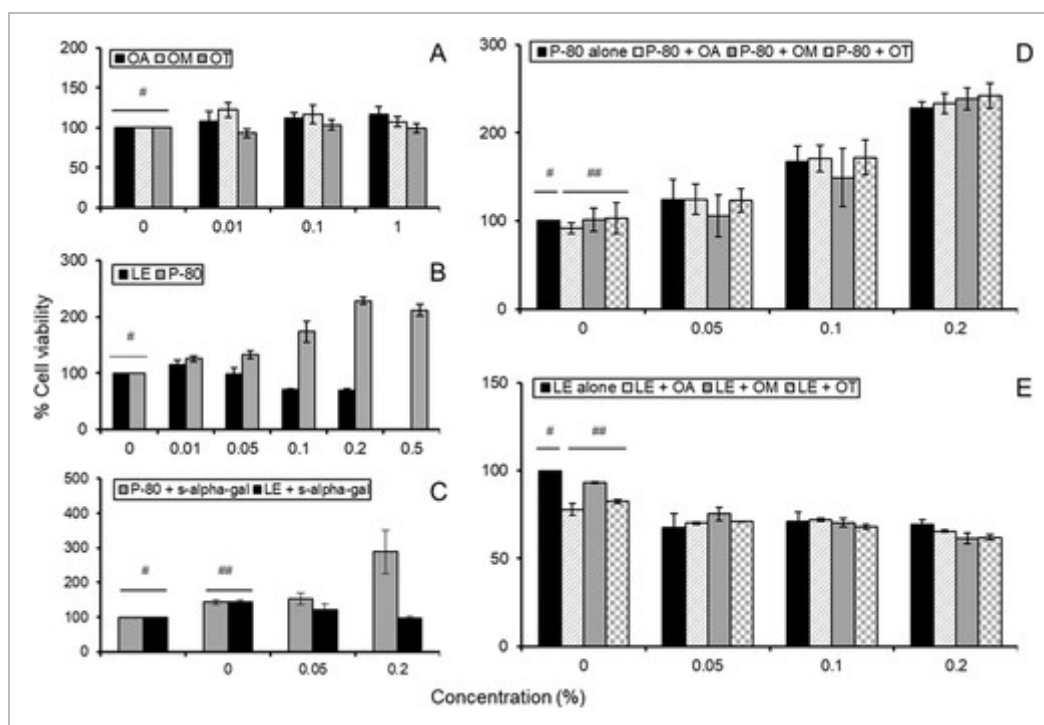


Figure 2

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Viability (MTT) assays for cells treated with increasing concentrations of allergens and emulsifiers: A) egg proteins: ovalbumin (OA), ovomucoid (OM), and ovotransferrin (OT); B) lecithin (LE) and polysorbate-80 (P-80); C) 0.125% synthetic α 1-3, β 1-4 galactotriose (s-alpha-gal) with P-80 or LE; D) 0.05% egg proteins with P-80; and E) 0.05% egg proteins with LE. Values expressed as mean $\pm$ SEM. # HBSS control; ## allergen alone.

The effects of foreign molecules are considered irreversible if exposure is toxic to the cells, and reversible if only the TJ functions are interrupted by exposure, and normal protein interactions can eventually be restored after removal of the triggers.^[39] Similarly in our experiments, alterations by P-80 (up to 0.2%) were reversible: the cell viability increased in a dose-dependent manner upon treatment removal and re-incubation with growth media. However, treatment with doses of LE appeared to cause irreversible cell damage: we observed a dose-dependent decrease of cell viability. Figure 2C–E illustrates the combined effects of allergens and emulsifiers on cell viability. As expected, these results were similar to the treatments that only used emulsifiers (Figure 2B); the addition of allergens did not alter the response. Our observations from this in vitro system fit well with previous investigations showing that these proteins exhibited no cytotoxic effects on their own. ^[44, 48, 49]

2.2 Impact of Emulsifiers and Allergens on TJs Integrity

2.2.1 Transepithelial Electrical Resistance (TEER) of Cell Monolayers

Our experiments showed that the tested emulsifiers exhibited different cytotoxicity properties, but the effect of allergens was identical. However, in vitro cytotoxicity data alone cannot simply be extrapolated to represent in vivo barrier function; instead, results of in vitro epithelial permeability can help predict in vivo responses.^[50] Therefore, these next experiments were designed to determine whether emulsifiers, allergens, or their combinations could trigger any significant and reproducible responses in an in vitro epithelial model at low-cytotoxic concentrations. Our initial TEER measurements were carried out using low-cytotoxic concentrations (0.05%, 0.1%, and 0.2%) of P-80 or LE that could be added to the cell culture system without inducing severe toxic effects. Exposure to P-80 resulted in about 20% ($p < 0.05$) TEER reduction at 0.2% concentration (**Figure 3A**), and LE had no effect on TEER at any of the concentrations we tested (**Figure 4A**). Our observations show that different emulsifiers at specific concentration may have different effects on TJ integrity.

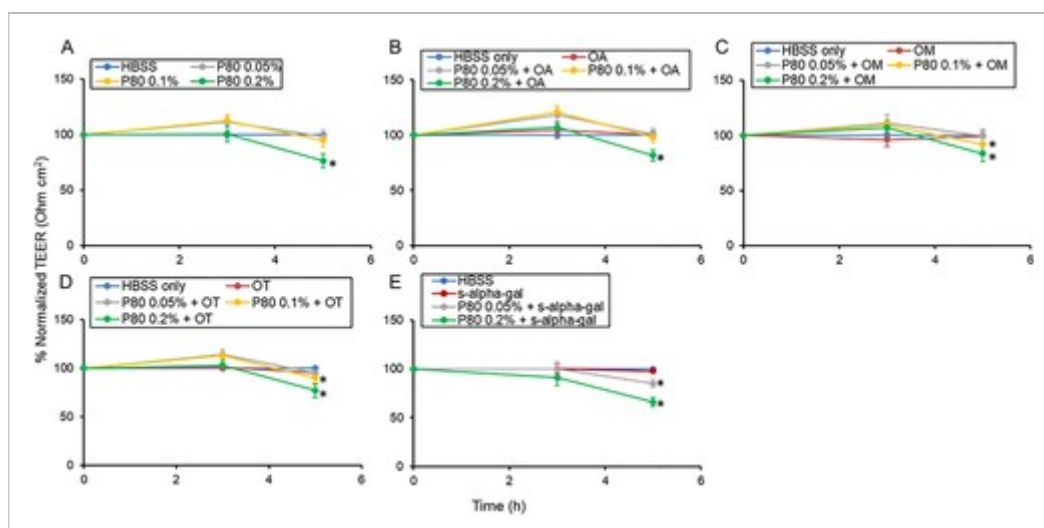
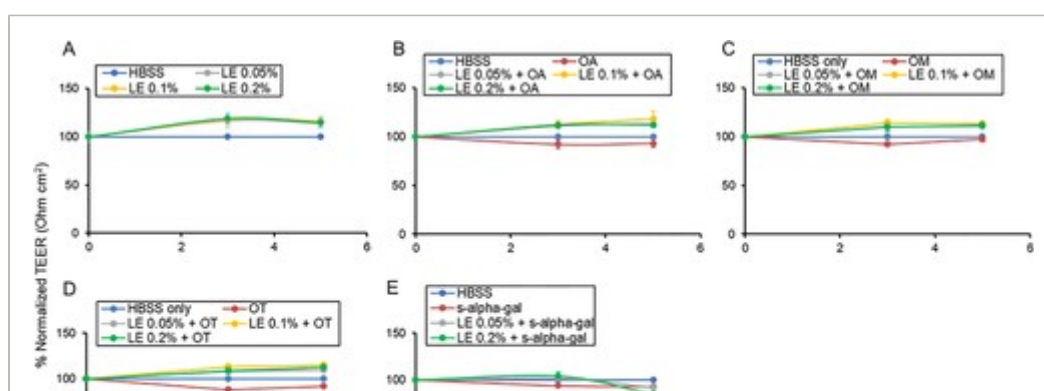


Figure 3

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Relative transepithelial electrical resistance (TEER) of cell monolayers treated with A) polysorbate-80 (P-80), alone, and in combination with allergens, B) ovalbumin (OA), C) ovomucoid (OM), D) ovotransferrin (OT), or E) synthetic α 1-3, β 1-4 galactotriose (s-alpha-gal) at increasing concentrations over 5 h. Values are expressed as mean $\pm$ SEM (* $p < 0.05$).



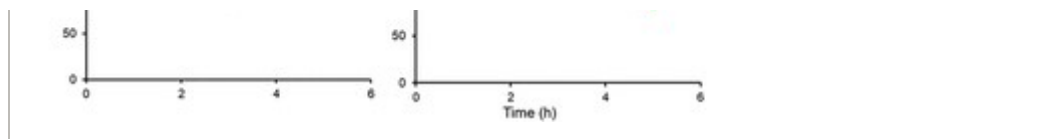


Figure 4

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Relative transepithelial electrical resistance (TEER) of cell monolayers treated with A) lecithin (LE) alone, and in combination with allergens, B) ovalbumin (OA), C) ovomucoid (OM), D) ovotransferrin (OT), or E) synthetic α 1-3, β 1-4 galactotriose (s- α -gal) at increasing concentrations over 5 h. Values are expressed as mean $\pm$ SEM.

Treatments using individual allergens alone: OA (Figures 3, 4), OM (Figures 3, 4), OT (Figures 3, 4), and s- α -gal (Figures 3, 4), did not interfere with cell monolayers, which contrasts with a previous report in which peanut allergens significantly decreased TEER. Combination treatments seemed to follow the pattern observed in our cell monolayer experiments: P-80 in combinations with OA (Figure 3B), OM (Figure 3C), OT (Figure 3D), and s- α -gal (Figure 3E) affected the TEER in the same manner and magnitude as observed with P-80 alone. Though P-80 at 0.05% and 0.1% concentrations showed significant reduction in TEER ($p < 0.05$) for certain treatments, it was not consistent for all combination treatments as observed with P-80 at 0.2% concentration. Combinations of LE with OA (Figure 4B), OM (Figure 4C), OT (Figure 4D), or s- α -gal (Figure 4E) did not show any effect on TEER. Previous studies have shown that while emulsifiers do have the potential to modify cell membrane integrity, those effects depend on the nature of the specific emulsifier.^[3, 39, 51, 52] Our findings fit that pattern, where P-80 interacted with cell monolayers, decreasing TEER, while LE did not. The specific chemical structure and properties of each emulsifier may determine how it interacts with the intestinal epithelium and whether there are irreversible changes in barrier functions.^[38, 39]

2.2.2 Assessment of Lucifer Yellow (LY) Passage

Molecules cross the intestinal epithelium by four major pathways: 1) passive/transcellular diffusion (mainly lipophilic compounds), 2) transporter mediated transcellular/active transport (ions, glucose, amino acids, di- and tri-peptides), 3) transcytosis (high-molecular-weight substances such as proteins), and 4) paracellular transport (water-soluble low molecular-substances).^[53] LY is a small, hydrophilic molecule that has been utilized as a marker to assess paracellular permeability.^[39, 50, 54] Transcellular passage of LY can be considered negligible because LY is too small to bind to transporters for active transcellular passage, and it is also unlikely to rely on the transcellular diffusion, which is mainly applicable to lipophilic molecules.^[55] Paracellular diffusion may also occur, however, the diffusion rate would not change significantly unless there were changes in the cell monolayers integrity by the treatments. Also, our cytotoxicity and cell viability data indicated that the latter was unlikely. **Figure 5** plots the % fluorescence intensity as a measure of LY passage, relative to untreated controls. Exposure to P-80 at 0.2% concentration, alone or in combination with

allergens, increased LY passage remarkably. However, that effect was mainly attributed to P-80. In contrast, we did not detect alterations in LY passage quantity in the presence of LE, indicating no changes in monolayers integrity (data not shown). Nevertheless, our results do not exclude the possibility of LY passage to the basal side via a transcellular pathway. Although LY passage is mainly through paracellular route,^[52] it can also passage via transcytosis.^[48] Our results showed that the baseline absorbances of untreated controls, representing transcellular passage, were negligible and the data of treated samples represented in Figure 5 were normalized with the obtained values from untreated controls, indicating that the application of P-80 mainly activated additional paracellular pathways. The reductions observed in TEER, compared to controls, also suggests there is increased paracellular passage of LY. This is consistent with articles reporting that emulsifiers can act as passage or absorption enhancers.^[39, 56]

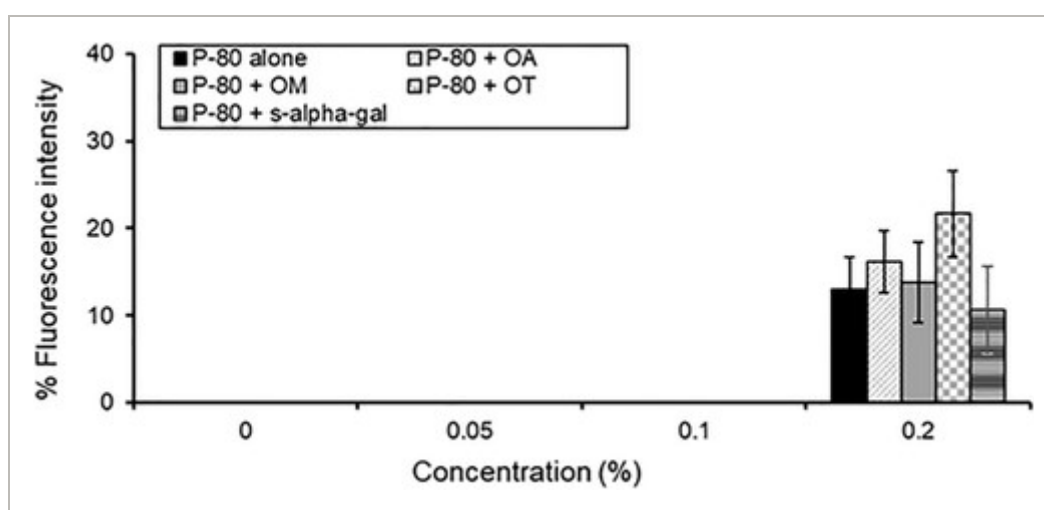


Figure 5

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Fluorescence intensity as a measure of lucifer yellow passage demonstrates the integrity or disruption of cell monolayers after exposure to treatments for 5 h. Values are expressed as mean $\pm$ SEM. Polysorbate-80 (P-80); OA, ovalbumin; OM, ovomucoid; OT, ovotransferrin; s-alpha-gal, synthetic α 1-3, β 1-4 galactotriose.

2.3 TJ Protein Levels and Gene Expression

To determine whether exposure to either P-80 or LE could affect TJ-related gene expression, we performed microarray analyses to compare transcription levels of TJ-related proteins in cells treated with 0.2% and 0.5% concentrations of each emulsifier. The “gate and fence function,” of TJ relies on complexes of over 50 proteins that act in concert to seal the paracellular space between cells and strictly control which molecules of food or environmental components are permitted across the epithelial barrier.^[23, 57-59] These proteins include several transmembrane and cytosolic proteins, as well as OCLN, claudins (CLDN), zonula occludens (ZO), tricellulin, cingulin, and junctional adhesion molecules which all interact with one other; these also form a complex architecture with the cytoskeleton of

each cell.^[60] Any alteration or damage to these proteins will be detrimental to membrane integrity.^[61]

One way to measure alterations in protein transcription is to examine fold changes, which represent transcription results in a biologically meaningful way. As shown in the heatmap (Figure 6A–D), Table 1, and Table S1–4, Supporting Information, we determined the amount of fold change by dividing the normalized gene expression in the test sample by the normalized gene expression in the control sample. Fold-change values greater than one indicates a positive- or an up-regulation, and less than one indicates a negative or down-regulation. The colors used in Figure 6A–D map the different levels of expression for genes that encode TJ proteins, observed after exposure to different concentrations of P-80 or LE. We observed a slight reduction of gene expression for the predominant claudins (CLDN1; B1 position in heatmap, and CLDN5; C2 position in heatmap), OCLN (F3 position in heatmap), and ZO-1 (G7 position in heatmap), all crucial for the maintenance of epithelial barrier integrity, in cells treated with 0.2% P-80. In contrast, the expression of these genes was mostly unchanged or upregulated in cells treated with 0.2% LE (Table 1). Overall, there was a slight difference in expression of these genes between cells treated with 0.2% and 0.5% P-80, but the values in Table 1 show a big difference in expression of the indicated genes including CLDN4 (C1 position in heatmap and supplementary Tables, Supporting Information) occurring between the cells treated with 0.2% LE versus those treated with 0.5% LE.

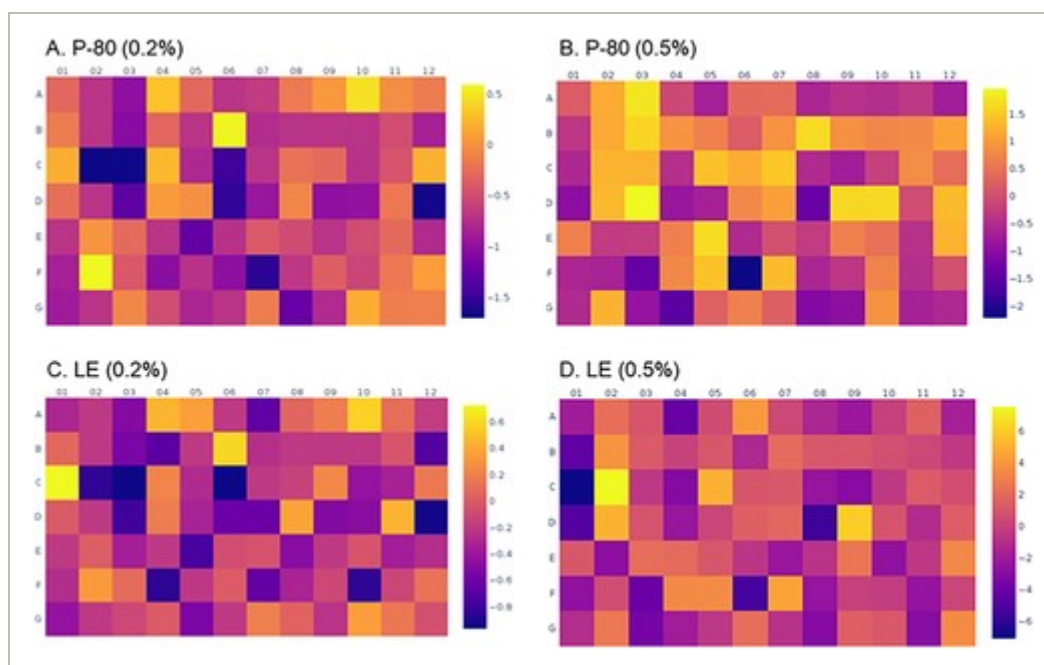


Figure 6

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Visualization of fold change in mRNA expression of tight junction-associated genes from cell monolayers treated with A) 0.2% of polysorbate-80 (P-80), B) 0.5% of P-80, C) 0.2% of lecithin (LE), and D) 0.5% of LE for 5 h.

Table 1. Fold change ($n = 3$) of mRNA expression of indicated tight junctions-associated genes of cell monolayers treated with 0.2%, and 0.5% polysorbate-80 (P-80) or lecithin (LE) for 5 h

Human TJ-associated genes	Fold change			
	P-80		LE	
	0.2%	0.5%	0.2%	0.5%
CLDN1 (Claudin 1)	−1.11 to −1.36	−1.28 to −1.56	1.03 to 1.2	−1.29 to −8.45
CLDN4 (Claudin 4)	−1.03 to 1.26	−1.12 to −1.48	1.44 to −2.59	−39.8 to −50.4
CLDN5 (Claudin 5)	−1.05 to −3.25	1.27 to 2.39	2.1 to 2.5	51.4 to 70.6
OCN (Occludin)	−1.24 to −1.44	−2.25 to −2.65	1.07 to 1.19	−5.9 to −7.1
TJP1 (TJ protein 1 (zona occludens 1))	−1.95 to −2.38	−2.14 to −2.71	1.03 to 1.21	−1.45 to −8.72

Notably, two genes showed distinct responses to the two emulsifiers. Expression of CLDN4 decreased remarkably in cells treated with 0.5% LE compared to 0.5% P-80 treated cells. Whereas, there was a marked increase of CLDN5 expression in 0.5% LE treated cells compared to 0.5% P-80 treated cells. Although differential expressions and biological functions of claudins are still not completely understood,^[62] all claudins are barrier- and/or pore-forming proteins. The behaviors of overexpressed or knocked-down claudins on transepithelial resistance differ depending on the TJ protein background and trigger mechanisms within the host cell line.^[60, 63] In our heatmaps, the effects of treatment with 0.5% LE were stronger for various other genes such as YBX3 (Cold shock domain protein A), F11R (F11 receptor), PARD6B (Par-6 partitioning defective 6 homolog beta^[1]), VAPA (VAMP [vesicle-associated membrane protein]-associated protein A, 33 kDa), etc. than what was observed in cells treated with 0.5% P-80 (Tables [S3–S4](#), Supporting Information). This might explain why the effects on TJ functions can vary as a consequence of exposure to P-80 and LE.

Immunohistochemistry experiments visualized TJs using OCLN labeling (**Figure 7**). In the control condition, the intact cell monolayers displayed bright OCLN signals localized to the plasma membrane (Figure [7A](#)). Neither emulsifier visibly disrupted the cell monolayer at treated concentrations up to 0.2% during the 5-h incubation. However, P-80 and LE treatments did decrease immunostaining for OCLN in concentration-dependent manner. At the 0.2% concentrations, the staining intensities seen after P-80 treatment were slightly lower compared to control, but the changes were remarkable when concentrations increased to 0.5% for both P-80 and LE. These changes might be linked to impaired

expression of mRNA (Table 1) resulting in reduced monolayer integrity in 0.2% P-80 treated cells (Figure 3A). The observed weaker intensities in 0.2% LE treated cells could be related to interference of the opaque LE emulsion (vs clear P-80 solutions) on the fluorescent signal detection of OCLN labeling, because mRNA expression level was nearly upregulated/unchanged (Table 1). In our model system, treatments with P-80 and LE did not produce major effects on the localization of OCLN. Therefore, P-80 has the potential to cause paracellular damage affecting OCLN protein expression level, but without altering where this protein is distributed.

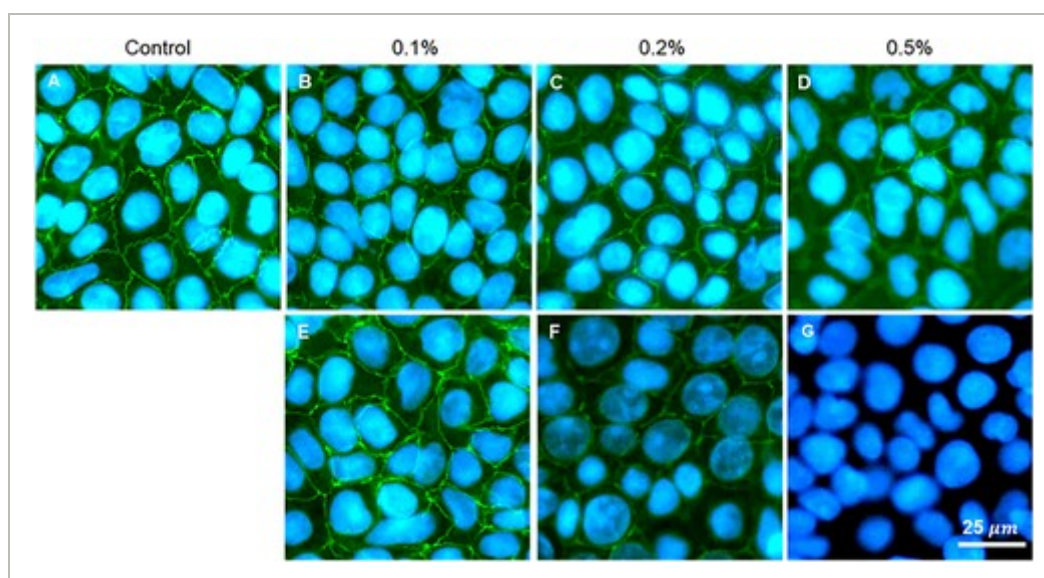


Figure 7

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Green fluorescence shows expression of occludin protein in cell monolayers exposed to A) HBSS control; B) 0.1% polysorbate-80 (P-80), C) 0.2 % P-80, D) 0.5% P-80, E) 0.1% lecithin (LE), F) 0.2% LE, and G) 0.5% LE for 5 h. Cell nuclei (blue) were counterstained with 4', 6-diamidino-2-phenylindole.

2.4 Impact of Emulsifiers on the Transport of Allergens across Cell Barrier

The transcellular route for transport of food antigens is more prominent than paracellular route, involving a direct pathway without degradation or a lysosomal degradative pathway.^[18] The transcellular pathway has been previously reported in the transport of intact allergens from apical to basal side in Caco-2 monolayers without altering permeability of the membranes.^[28, 64-66] As the allergens we tested on their own neither decreased TEER nor increased TJ permeability, any allergens which transported across the monolayers in the “allergens alone” conditions were probably absorbed by the transcellular route. We used enzyme-linked immunosorbent assay (ELISA) to estimate the apical to basolateral flux across the cell monolayers of OA, OM, OT, and s-alpha-gal in the presence of P-80 or LE (**Figure 8A–D**). The allergens recovered from the basolateral side confirmed the transport of the allergens across the cell monolayers. The transport rate of OA, OM, and s-alpha-gal across

monolayers increased significantly ($p < 0.05$) in the presence of P-80 and was dependent on the concentration of P-80. However, the transport rate of the allergen with the highest molecular weight in our tests, OT (78 kD) (Figure 8B), did not change in the presence of P-80. These results suggest that while P-80 can enhance the absorption of allergens via the paracellular route, this occurs in a size-dependent manner. In contrast, LE did not significantly alter the function of the cell monolayers or the transport of tested egg proteins. Transport of s-alpha-gal in the presence of LE was moderate in comparison to the transport achieved in the presence of P-80 (Figure 8D). It is possible that the low molecular weight of s-alpha-gal allows paracellular transport in the presence of LE, which appeared to make only minor modifications to the cell monolayers. However, it should be noted that in vivo mechanisms are more complex and allergens can be digested into smaller peptides with intact epitopes and thus may be absorbed via the paracellular route as well. The high molecular weight allergens which are not transported in the intact form across the Caco-2 cell monolayer may be able to pass in peptide form upon digestion by gastric and intestinal enzymes. Further studies are necessary to evaluate the effect of allergen in vitro digestion on the transport of allergenic epitopes in the presence of food components such as an emulsifier. We speculate that extensive use of some emulsifiers in foods may be enhancing exposure to food allergens including alpha-gal, although at this time there is no direct experimental evidence.^[67, 68]

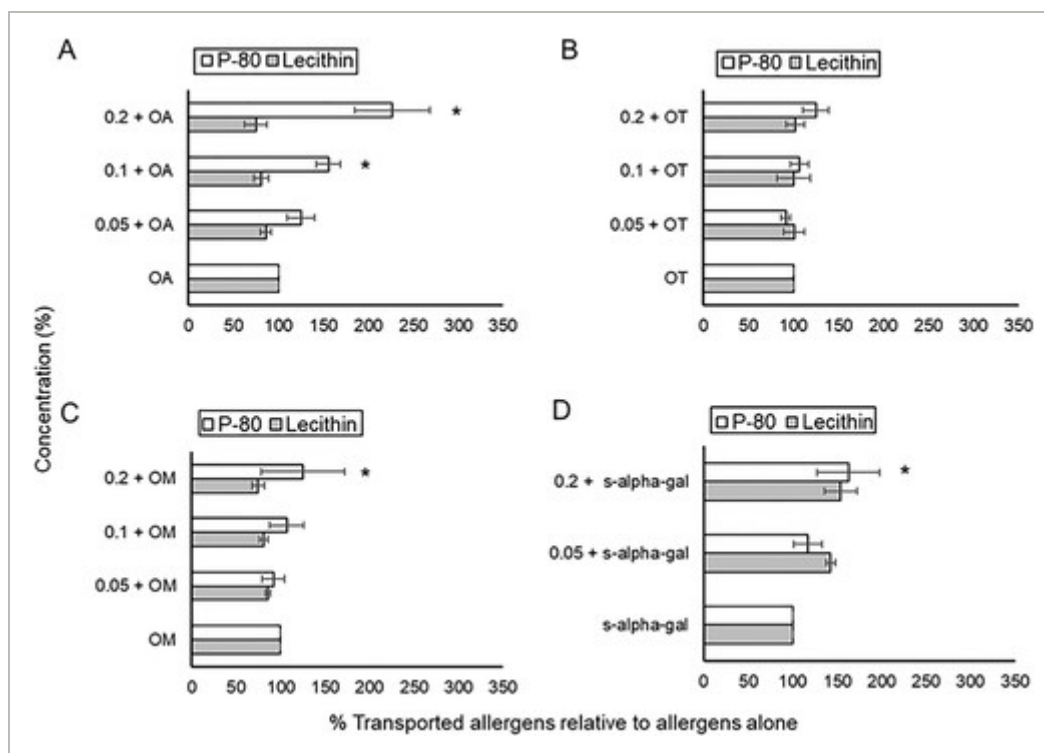


Figure 8

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Dose-dependent effects of polysorbate-80 (P-80) and lecithin (LE) on quantity of A) ovalbumin (OA), B) ovomucoid (OM), C) ovotransferrin (OT), and D) synthetic α 1-3, β 1-4 galactotriose (s-alpha-gal) transported after exposure to treatments for 5 h. Values are expressed as mean $\pm$ SEM (* $p < 0.05$).

The interaction in this study is specific between the emulsifier and allergens without any other interference, but in reality many processed foods are complex with multiple ingredients that can have the potential to change the assay results including mechanisms of absorption, which is not known. Though transcytosis mechanism is prominent,^[53] other transcellular mechanisms can occur but their contributions are likely negligible when compared with the overall allergen passage through the cells. Further investigations can characterize possible transcellular route of allergen transport. Basolateral to apical transport, combined with apical to basolateral transport experiment can provide bi-directional permeability and indicate any active efflux. Manipulation of the extracellular buffer (the types of ions, the ionic strength, incubation temperature, and pH), and use of a specific inhibitor can rule out passages of allergens by transporter proteins.^[69, 70] However the identity of the transporter(s) in allergen transports across the Caco-2 monolayer is less known, and therefore detailed inhibitor screening is needed.

Various issues need to be considered to demonstrate the physiological relevance of food components such as emulsifiers and others on promoting the pathogenesis of diseases.^[71] The major issues include, 1) the relationship of concentrations exposed to cells in vitro in experimental studies to actual exposure, 2) effects (additive, synergistic, or antagonistic) of multiple emulsifiers in processed foods, and 3) the behavior of the emulsifiers exposed to cells except in the gastrointestinal tract or in the context of food. Thus, translating phenomena from an in vitro experimental model to human clinical conditions should be treated with caution and further investigations on the relevance of such effects are warranted.

3 Concluding remarks

This study helps in understanding the influence of emulsifiers on the transport of allergens across the epithelial cell barrier in the gut. The Caco-2 cell monolayers in Transwells provide an useful in vitro model of TJs to examine paracellular and transcellular transport, however, this system cannot replicate the full complexity of the gut environment.^[69] It is possible that P-80 differs from LE in disrupting paracellular permeability, altering the function of TJ at levels associated with low cytotoxicity based on current experiments. Factors influencing gut permeability on the development of food allergies in predisposed subjects is poorly understood.^[36] Further investigations are necessary to identify which emulsifiers are more potent disruptors of epithelial integrity, and which of those are most likely to promote the transport of food allergens. Our research model has the potential to further characterize the biological responses of allergen dose exposures.

4 Experimental Section

LDH Release Assay

Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in DMEM supplemented with 20% fetal bovine serum (FBS), 10% Glutamax, and 50 U mL⁻¹ Penicillin-

Streptomycin and incubated at 37 °C in a humidified 5% CO₂ incubator. Cells between 8 and 12 passages were seeded at a density of 6000 cells per well into flat bottom 96-well plates and cultured for 6 days. After two hanks' balanced salt solution (HBSS) washes, cells were equilibrated with fresh HBSS at 37 °C for 30 min. Prewarmed (37 °C) HBSS was used throughout the experiments. For treatments, emulsifiers [P-80 (v/v) or LE (w/v) (Sigma Aldrich, St. Louis, MO, USA)] at concentrations 0%, 0.01%, 0.05%, 0.1%, 0.2%, 0.5% or purified allergens (OA, OM, or OT [Sigma]) at 0, 0.01, 0.1, 1 w/v% concentrations were added in triplicate wells. Due to the limited availability, treatments with synthetic α1-3, β1-4 galactotriose (s-alpha-gal) (Dextra, Reading, UK) were limited to a single concentration of 0.125 w/v%. The cells were treated for 5 h.

For combination treatments, cells were incubated with an emulsifier for 3 h and then 0.05% of egg proteins (OA, OM, OT) or 0.125% of s-alpha-gal were added to the appropriate wells followed by another 2 h incubation. LDH activity was determined following instructions of the Pierce LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Rockville, MD, USA) and considering HBSS alone treatment as the spontaneous/negative (0%) and lysis buffer treatment as the positive (100%) controls. Absorbances (490 and 680 nm) from a SpectraMax M5 spectrophotometer reader (Molecular Devices Corp., Sunnyvale, CA, USA) were used for calculation. Data represent triplicate wells in three independent experiments ($n = 9$).

$$\% \text{ LDH release} = \frac{\text{Treatment specific} - \text{Spontaneous (HBSS)}}{\text{Maximum (lysed)} - \text{Spontaneous (HBSS)}} \times 100$$

(1)

Cell Viability Assay

After collecting the supernatants for the LDH cytotoxicity assay, HBSS containing 20% FBS was added to each well of the plates and subsequent steps were performed following the protocol of the Vybrant MTT Cell Proliferation Assay kit (Thermo Fisher Scientific). The conversion of water soluble tetrazole (MTT:3-[4,5-di methyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide) to an insoluble formazan was determined using the absorbances (540 nm) from a SpectraMax M5 spectrophotometer reader. The percentage (%) cell viability was calculated using the HBSS alone treatment as positive (100%) and the lysis buffer treatment as the negative (0%) controls. These treatments and measurements in triplicates were repeated in two other independent experiments.

$$\% \text{ Cell viability} = \frac{\text{Treatment specific} - \text{lysis control}}{\text{HBSS control} - \text{lysis control}} \times 100$$

(2)

Paracellular Transport Studies

Cells between 8 and 12 passages were seeded at a density of 36000 cells per well into a high throughput screening (HTS) 24-well Transwell plate (Costar 3378) and grown for 21 days. Fresh media replacements were made every 3 days for the first 2 weeks, then every other day. On the transport experiment day (≈ 21 days post seeding), medium was removed, the cell monolayers were washed twice with HBSS, and then equilibrated with fresh HBSS for 30 min at 37 °C in a humidified CO₂ (5%) incubator. The integrity of each cell layer was evaluated by TEER measurements with an EVOM epithelial volt-Ohm meter (World Precision Instruments, Sarasota, FL, USA), equipped with the STX100 electrode, designed specifically for use with HTS plates. The plates were cooled to room temperature (RT) for 15 min before TEER measurement. Only wells with cell monolayers showing TEER values higher than 500 ± 50 Ohm cm² were used for further experimental exposures to emulsifiers and/or allergens.^[72, 48] For the first step of treatments, HBSS containing LY (40 µg mL⁻¹; w/v) (Sigma-Aldrich) was added to controls and treatment-specific wells (either P-80 or LE ; 0, 0.05, 0.1, and 0.2%; v/v) on the apical side. Then, plates were incubated for 3 h, followed by TEER measurements, and 0.05% (w/v) OA, OM, OT or 0.125% (w/v) s-alpha-gal in HBSS were added to the appropriate experimental wells. Plates were incubated for another 2 h. Thereafter, final TEER measurements were taken, and the basal supernatants and the cell monolayers were retained for further analyses.

Measuring the Integrity of Cell Monolayers

The permeability of the treated cell monolayers (section Paracellular Transport Studies) was determined in two ways: (1) changes in barrier resistance measured by TEER and (2) the transport of LY from the apical to the basal side. The resistance for each well was estimated by multiplying the measured TEER value with the membrane area (0.33 cm²). Thereafter, a no-cell control was used as blank, and its mean resistance was subtracted from all experimental wells of each plate. Percent changes in TEER values in response to exposure to the emulsifiers and allergens were calculated using following formulas. First, TEER values from all treatment groups were normalized with those from the HBSS control at respective experimental time point (Equation 3), then the obtained values were normalized against $t = 0$ within each treatment group (Equation 4).

$$\text{Buffer normalized TEER}(\%) = \frac{\text{TEER at } t_i}{\text{TEER of HBSS at } t_i} \times 100 \quad (3)$$

$$\begin{aligned} &\text{Time normalized TEER} (\%) \\ &= \frac{\text{Buffer normalized TEER at } t_i}{\text{Buffer normalized TEER at } t = 0} \times 100 \end{aligned} \quad (4)$$

where TEER is in ($\Omega \text{ cm}^2$) and $t_i = 0, 3$, and 5 h.

As described above, LY was added to the apical compartment of each Transwell at the beginning of the experiment ($t = 0$), and its transport to the basal compartment was examined at the end of the experiment ($t = 5$ h). Fluorescence intensity (FI) of basal supernatant of each experimental well was measured at excitation and emission wavelengths of 428 and 536 nm respectively using a SpectraMax M5 spectrophotometer. The FI relative to that exhibited by the HBSS control wells was expressed in percentage, to assess the effects of each experimental condition on the passage of LY.

$$\% \text{ FI} = \frac{\text{FI of treatment} - \text{FI of HBSS control}}{\text{FI of HBSS control}} \times 100 \quad (5)$$

Gene Expression Analysis of TJ Proteins

Cell monolayers treated for 5 h as described in section Paracellular Transport Studies were harvested from the Transwell inserts and washed three times with HBSS. The total RNA was extracted using the RNeasy mini kit (Qiagen, Germantown, MD, USA) following the manufacturer's instructions. A NanoDrop spectrophotometer (Thermo Fisher Scientific) was used to determine RNA concentrations and equal amounts of RNA from all samples were

reverse-transcribed to cDNA using the RT² First Strand Kit (Qiagen). The RT² Profiler PCR Array Human TJs in combination with RT² SYBR Green Mastermixes (Qiagen) was used to examine the expression of genes related to TJ functions on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., PA, USA). The C_T values from all wells were used to analyze gene expression using the web-based Qiagen GeneGlobe Data Analysis Center.

Immunofluorescence Microscopy

Cells between 8 and 12 passages were seeded at a density of 1×10^5 cells per well into four-well glass chamber slides and allowed to grow for 6 days. Confluent cell layers were treated with emulsifiers as described in section LDH Release Assay. After three washes with PBS pH 7.4, cells were fixed using a 1:1 methanol–acetone solution for 10 min. Then, cells were blocked with 3% BSA/PBS and incubated with an Alexa Fluor 488 conjugated monoclonal antibody against occludin (OCLN) (1:100 in blocking buffer) (Invitrogen, CA, USA) overnight at 4 °C. Cell nuclei were then stained with 4', 6-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific) (1:1000 in PBS) by incubating for 5 min at RT. Between incubations, cells were washed three times with PBS. After the final wash, chamber slides with stained cells were mounted to coverslips with proLong Gold Antifade Mountant with DAPI (Thermo Fisher Scientific) following the manufacturer's instructions.

Additionally, emulsifier and allergen-treated (as described in section Paracellular Transport Studies) cell monolayers in HTS Transwell plates were stained in the same way as the cells grown on chamber slides, after removing treatment supernatants and three PBS washes. Then the stained membranes from the Transwell inserts were collected using scalpels, and mounted, cell side up, between a glass slide and coverslip containing antifade mountant with DAPI. Images of stained cell layers of chamber slides and Transwell inserts were captured by a Nanozoomer (Hamamatsu Phototonics K.K., Japan) and analyzed using NDP-view-2 software (Hamamatsu).

Determination of Allergen Transport Using ELISA

Egg proteins were quantified using the in-house established quantitative sandwich ELISAs.^[73] For these ELISAs, the capture antibodies were anti-raw egg white to quantify OA or OT or an OM-specific polyclonal antibody. Purified OA, or OM at a range of concentration (0–1000 ng mL⁻¹), or OT at a range of concentration (1–5000 ng mL⁻¹) were used as standards. The detection antibodies were HRP-conjugated anti-raw egg white or OM-specific antibody.

To quantify transported s-alpha-gal in basal supernatants, a competitive ELISA was developed. Briefly, gala1-3gal-BSA (14 atom spacer) (Dextra)^[74] in carbonate buffer (pH 9.6), was used to coat Polysorb Nunc plates (Thermo Fisher Scientific) overnight at 4 °C. After three washes with PBS, the plates were blocked with PBS/1% fish gelatin/0.1% T-20 for 30 min at RT. Basal supernatants and s-alpha-gal (0–250 µg mL⁻¹) as standards were incubated

with anti-alpha-gal monoclonal antibody (Enzo Life Sciences Inc, NY, USA)^[74] (1:600 in HBSS), for 30 min at RT in a separate low-binding 96-well plate to form antigen-antibody complexes. After two washes with PBS/0.2% T-20, blocked plates were incubated with previously-prepared mixtures of antigen-antibody complexes for 30 min at RT. Then the plates were incubated with HRP-conjugated goat anti-mouse IgM (Jackson ImmunoResearch Inc., PA, USA) (diluted 1:5000 in blocking buffer) for 30 min at RT and later with (3,3',5,5'-Tetramethylbenzidine) substrate for 10 min in the dark at RT. The reactions were terminated with 1M HCl, the absorbances were read at 450 nm, and four-parameter curves of the s-alpha-gal standards were generated using Softmax Pro 5.3 software (Molecular Devices Corp., CA, USA).

The concentrations derived from the standard curves were used for the estimation of transported allergens through treated cell monolayers using the following equation.

$$\begin{aligned} & \% \text{ Transported allergens} \\ &= \frac{\text{Treatment specific} - \text{HBSS control}}{\text{Allergen alone treatment} - \text{HBSS control}} \times 100 \end{aligned}$$

(6)

Statistical Analysis

All data are presented as the mean $\pm$ standard error of the mean (SEM) by analyzing at least three subsamples from three independent experiments in triplicate. Statistical significance for TEER and ELISA data was determined using Wilcoxon Ranked Sign test and unpaired t test respectively. Values of $p < 0.05$ were considered significant.

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Conflict of Interest

The authors declare no conflict of interest.

Open Research



Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Supporting Information



Filename	Description
mnfr4125-sup-0001-tableS1-S4.pdf 98 KB	Supporting Information

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Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	ACTN1 /- 1.23	ACTN2 /- 1.63	ACTN3 /- 2.02	ACTN4 / 1.23	AMOTL1 /-1.23	ARHGEF2 /-1.63	ASH1L /- 1.56	CASK /- 1.12	CD99 / 1.03	CDC42 /1.38	CDK4 /- 1.02	CGN /- 1.11
B	CLDN1 /- 1.11	CLDN10 /- 1.63	CLDN11 /- 2.08	CLDN12 /- 1.24	CLDN14 /- 1.63	CLDN15 / 1.49	CLDN16 /- 1.69	CLDN17 /- 1.63	CLDN18 /- 1.63	CLDN19 /- 1.63	CLDN2 /- 1.41	CLDN3 /- 1.81
C	CLDN4 / 1.13	CLDN5 /- 3.25	CLDN6 /- 3.23	CLDN7 / 1.18	CLDN8 /- 1.73	CLDN9 /- 2.78	CRB1 /- 1.63	CRB3 /- 1.16	YBX3 /- 1.22	CSNK2A1 /-1.65	CSNK2A2 /-1.36	CSNK2B /1.14
D	CTNNA1 /-1.18	CTNNA2 /-1.63	CTNNA3 /-2.46	CTNNB1 /1.04	CTTN /- 1.01	EPB41 /- 2.87	ESAM /- 1.93	F11R /- 1.05	GNAI1 /- 2.02	HCLS1 /- 1.99	ICAM1 /- 1.13	ICAM2 /- 3.17
E	IGSF5 /- 1.63	ILK / 1.01	INADL /- 1.21	JAM2 /- 1.63	JAM3 /- 2.42	LLGL1 /- 1.65	LLGL2 /- 1.32	MAGI1 /- 1.42	MAGI2 /- 1.63	MARK2 /- 1.42	MLLT4 /- 1.22	MPDZ /- 1.71
F	MPP5 /- 1.82	MPP6 / 1.51	OCN /- 1.34	PARD3 /- 2.06	PARD6A /-1.64	PARD6B /-2.03	PECAM1 /-2.96	PRKCI /- 1.59	PRKCZ /- 1.29	PTEN /- 1.47	RAC1 /- 1.13	RHOA / 1.05
G	SMURF1 /-1.87	SPTA1 /- 1.63	SPTAN1 /- 1.05	SPTB /- 1.41	SYMPK /- 1.77	TIAM1 /- 1.63	TJAP1 /- 1.1	TJP1 /- 2.38	TJP2 /- 1.72	TJP3 / 1.13	VAPA /- 1.09	ZAK /- 1.09

Supplemental Table 1. Value in each square represents the fold change in mRNA expression of specific tight junction-associated gene of cell monolayers treated with 0.2% polysorbate-80 for 5 h. ACTN, Actinin; AMOTL1, Angiomotin like 1; ARHGEF2, Rho/rac guanine nucleotide exchange factor (GEF) 2; ASH1L, Ash1 (absent, small, or homeotic)-like (Drosophila); CASK, Calcium/calmodulin-dependent serine protein kinase (MAGUK family); CD99, CD99 molecule; CDC42, Cell division cycle 42 (GTP binding protein, 25kDa); CDK4, Cyclin-dependent kinase 4; CGN, Cingulin; CLDN, Claudin 1; CRB1, Crumbs homolog 1 (Drosophila); CRB3, Crumbs homolog 3 (Drosophila); YBX3, Cold shock domain protein A; CSNK2A1 Casein kinase 2; CTNN, Catenin (cadherin-associated protein); CTTN, Cortactin; EPB41, Erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked), ESAM, Endothelial cell adhesion molecule; F11R, F11 receptor; GNAI1, Guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1; HCLS1, Hematopoietic cell-specific Lyn substrate 1; ICAM, Intercellular adhesion molecule; IGSF5, Immunoglobulin superfamily, member 5; ILK, Integrin-linked kinase; INADL, InaD-like (Drosophila); JAM; Junctional adhesion molecule, LLGL, Lethal giant larvae (Drosophila); MAGI; Membrane associated guanylate kinase, WW and PDZ domain containing, MARK2; MAP/microtubule affinity-regulating kinase 2; MLLT4, Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4; MPP, Membrane protein, palmitoylated (MAGUK p55 subfamily member); OCLN, Occludin; PARD, Par- partitioning defective homolog (C. elegans); PECAM1, Platelet/endothelial cell adhesion molecule; PRKC, Protein kinase C; PTEN, Phosphatase and tensin homolog; RAC1, Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1); RHOA, Ras homolog gene family, member A; RHOA, Ras homolog gene family, member A; SMURF1, SMAD specific E3 ubiquitin protein ligase 1; 1SPTA1, Spectrin, alpha, erythrocytic 1 (elliptocytosis 2); SPTAN, Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin); SPTB,,Spectrin, beta, erythrocytic; SYMPK, Symplekin; TIAM1, T-cell lymphoma invasion and metastasis 1; TJAP1, Tight junction associated protein 1 (peripheral); TJP, Tight junction protein (zona occludens); VAPA, VAMP (vesicle-associated membrane protein)-associated protein A, 33kDa; ZAK, Sterile alpha motif and leucine zipper containing kinase AZK; ACTB, Actin, beta; B2M, Beta-2-microglobulin.

Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	ACTN1 / - 1.24	ACTN2 / - 1.15	ACTN3 / - 1.42	ACTN4 / 1.36	AMOTL1 / 1.28	ARHGEF2 /-1.15	ASH1L / - 1.58	CASK / 1.04	CD99 / 1.14	CDC42 / 1.49	CDK4 / 1.07	CGN / - 1.13
B	CLDN1 / 1.05	CLDN10 / - 1.15	CLDN11 / - 1.47	CLDN12 / - 1.61	CLDN14 / - 1.15	CLDN15 / 1.51	CLDN16 / - 1.19	CLDN17 / - 1.15	CLDN18 / - 1.15	CLDN19 / - 1.15	CLDN2 / - 1.02	CLDN3 / - 1.64
C	CLDN4 / 1.66	CLDN5 / - 1.8	CLDN6 / - 1.95	CLDN7 / 1.17	CLDN8 / - 1.22	CLDN9 / - 1.95	CRB1 / - 1.15/ C	CRB3 / - 1.1	YBX3 / 1.18	CSNK2A1 /-1.35	CSNK2A2 /-1.26	CSNK2B /1.12
D	CTNNA1 /-1	CTNNA2 /-1.15	CTNNA3 /-1.73	CTNNB1 / 1.13	CTTN / - 1.26	EPB41 / - 1.53	ESAM / - 1.53	F11R / 1.29	GNAI1 / - 1.42	HCLS1 / - 1.4	ICAM1 / 1.36	ICAM2 / - 1.92
E	IGSF5 / - 1.15	ILK / 1.01	INADL / - 1.28	JAM2 / - 1.15	JAM3 / - 1.7	LLGL1 / - 1.05	LLGL2 / - 1.03	MAGI1 / - 1.4	MAGI2 / - 1.15	MARK2 / - 1.03	MLLT4 / - 1.29	MPDZ / - 1.2
F	MPP5 / - 1.21	MPP6 / 1.26	OCLN / 1.07	PARD3 / - 1.81	PARD6A /-1.16	PARD6B / 1.0	PECAM1 /-1.56	PRKCI / - 1.25	PRKCZ / - 1.08	PTEN / - 1.8	RAC1 / - 1.08	RHOA / 1.1
G	SMURF1 /-1.35	SPTA1 / - 1.15	SPTAN1 / - 1.08	SPTB / - 1.0	SYMPK / - 1.46	TIAM1 / - 1.15	TJAP1 / 1.15	TJP1 / 1.03	TJP2 / - 1.18	TJP3 / 1.27	VAPA / 1.11	ZAK / - 1.04

Supplemental Table 2. Value in each square represents the fold change in mRNA expression of specific tight junction-associated gene of cell monolayers treated with 0.2% lecithin for 5 h. Supplement Table 1 describes acronyms of the listed genes in this Table.

Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	ACTN1 /1.14	ACTN2 /2.25	ACTN3 /3.43	ACTN4 /- 1.07	AMOTL1 /-1.61	ARHGEF2 /1.29	ASH1L /1.31	CASK / - 1.52	CD99 / - 1.35	CDC42 /- 1.41	CDK4 /- 1.26	CGN / - 1.66
B	CLDN1 /- 1.28	CLDN10 /2.22	CLDN11 /3.04	CLDN12 /1.86	CLDN14 /1.53	CLDN15 / 1.14	CLDN16 /1.85	CLDN17 /3.18	CLDN18 /1.82	CLDN19 /1.66	CLDN2 /1.72	CLDN3 /2.11
C	CLDN4 / - 1.48	CLDN5 /2.39	CLDN6 /2.37	CLDN7 /- 1.38	CLDN8 /2.66	CLDN9 /2.27	CRB1 / 2.68	CRB3 / - 1.45	YBX3 / - 1.66	CSNK2A1 /-1.15	CSNK2A2 /1.76	CSNK2B /1.34
D	CTNNA1 /-2.0	CTNNA2 /2.49	CTNNA3 /3.9	CTNNB1 /-1.8	CTTN / - 1.51	EPB41 /1.71	ESAM / 2.05	F11R / - 2.61	GNAI1 /3.1	HCLS1 /3.11	ICAM1 /- 1.02	ICAM2 /2.51
E	IGSF5 /1.59	ILK / -1.22	INADL /- 1.2	JAM2 / 1.59	JAM3 / 3.2	LLGL1 /- 1.45	LLGL2 /1.02	MAGI1 /- 1.22	MAGI2 /1.59	MARK2 /1.37	MLLT4 /- 1.35	MPDZ /2.37
F	MPP5 / - 1.52	MPP6 / - 1.57	OCLN /- 2.65	PARD3 /1.7	PARD6A /2.66	PARD6B /-4.64	PECAM1 /2.53	PRKCI /- 1.52	PRKCZ /- 1.28	PTEN / 1.62	RAC1 / - 1.38	RHOA / 1.01
G	SMURF1 /-1.45	SPTA1 /2.36	SPTAN1 /- 1.86	SPTB / - 2.86	SYMPK /1.19	TIAM1 /1.59	TJAP1 / 1.17	TJP1 / - 2.14	TJP2 / - 1.96	TJP3 / 1.185	VAPA / - 1.59	ZAK / - 1.48

Supplemental Table 3. Value in each square represents the fold change in mRNA expression of specific tight junction-associated gene of cell monolayers treated with 0.5% polysorbate-80 for 5 h. Supplement Table 1 describes acronyms of the listed genes in this Table.

Layout	1	2	3	4	5	6	7	8	9	10	11	12
A	ACTN1 / - 3.85	ACTN2 /4.55	ACTN3/ 1.93	ACTN4 / - 18.77	AMOTL1 / 1.41	ARHGEF2 /16.12	ASH1L /1.24	CASK / - 2.68	CD99 / - 4.65	CDC42 / - 1.06	CDK4 / 2.87	CGN / - 3.37
B	CLDN1 / - 22.5	CLDN10 /14.1	CLDN11 /2.29	CLDN12 / - 1.01	CLDN14 /2.08	CLDN15 / -2.5	CLDN16 /3.95	CLDN17 /2.08	CLDN18 /2.08	CLDN19 /1.61	CLDN2 /1.24	CLDN3 / - 1.4
C	CLDN4 / - 50.09	CLDN5 /60.02	CLDN6 / - 1.42	CLDN7 / - 8.56	CLDN8 / - 1.22	CLDN9 / - 1.95	CRB1 / 2.08	CRB3 / - 5.32	YBX3 / - 8.06	CSNK2A1 /-1.37	CSNK2A2 /2.4	CSNK2B /1.51
D	CTNNA1 /-29.23	CTNNA2 /32.39	CTNNA3 /1.83	CTNNB1 / -5.0	CTTN / 1.15	EPB41 /2.77	ESAM / 3.49	F11R / - 50.05	GNAI1 /64.77	HCLS1 / 1.85	ICAM1 / - 2.23	ICAM2 /2.61
E	IGSF5 /2.08	ILK / -6.98	INADL /4.37	JAM2 / 3.94	JAM3 / 2.08	LLGL1 / - 1.56	LLGL2 / - 4.97	MAGI1 / - 1.73	MAGI2 /5.73	MARK2 / - 6.08	MLLT4 / - 1.44	MPDZ / 9.7
F	MPP5 / - 6.31	MPP6 / 1.48	OCLN / - 16.98	PARD3 /10.61	PARD6A /10.91	PARD6B / -42.98	PECAM1 /21.32	PRKCI / - 5.51	PRKCZ / - 1.03	PTEN / - 1.18	RAC1 / - 5.52	RHOA / 1.16
G	SMURF1 /-2.1	SPTA1 /6.01	SPTAN1 / - 12.49	SPTB / - 3.85	SYMPK / - 1.43	TIAM1 /4.4	TJAP1 / - 1.81	TJP1 / - 8.72	TJP2 /2.77	TJP3 /2.13	VAPA / - 7.88	ZAK / 11.19

Supplemental Table 4. Value in each square represents the fold change in mRNA expression of specific tight junction-associated gene of cell monolayers treated with 0.5% lecithin for 5 h. Supplement Table 1 describes acronyms of the listed genes in this Table.