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ORIGINAL CONTRIBUTION

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Intricate Crosstalk Between Food Allergens, Phages, Bacteria, and Eukaryotic Host Cells of the Gut-skin Axis

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Bacterial and food allergens are associated with immune-mediated food allergies via the gut-skin axis. However, there has been no data on the potential use of phages to rescue this pathological process. A human triple cell co-culture model incorporating colonocytes (T84 cells), macrophages (THP-1 cells), and hepatocytes (Huh7 cells) was established and infected with *Pseudomonas aeruginosa* PAO1 (P.a PAO1) in the absence or presence of its KPP22 phage in Dulbecco's Modified Eagle's Medium (DMEM), DMEM+ ovalbumin (OVA), or DMEM+β-casein media. The physiological health of cells was verified by assessing cell viability and Transepithelial electrical resistance (TEER) across the T84 monolayer. The immune response of cells was investigated by determining the secretions of IL-1β, IL-8, IL-22, and IL-25. The ability of P.a PAO1 to adhere to and invade T84 cells was evaluated. The addition of either OVA or β-casein potentiated the P.a PAO1-elicited secretion of cytokines. The viability and TEER of the T84 monolayer were lower in the P.a PAO1+OVA group compared to the P.a PAO1 alone and PAO1+β-casein groups. OVA and β-casein significantly increased the adherence and invasion of P.a PAO1 to T84 cells. In the presence of the KPP22 phage, these disruptive effects were abolished. These results imply that: (1) food allergens and bacterial toxic effector molecules exacerbate each other's disruptive effects; (2) food allergen and bacterial signaling at the gut-skin mucosal surface axis depend on a network of bacteria-phage-eukaryotic host interactions; and (3) phages are complementary for the evaluation of pathobiological processes that occur at the interface between bacteria, host cellular milieu, and food antigens because phages intervene in P.a PAO1-, OVA-, and β-casein-derived inflammation.

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Abbreviations: APCs, antigen-presenting cells; DMEM, Dulbecco's Modified Eagle's Medium; FASTER Act of 2021, Food Allergy Safety, Treatment, Education, and Research Act of 2021 (Public Law 117-11 of the US Government Publishing Office); Huh7, human liver cell line-7; IL-1β, Interleukin 1 beta; IL-8, Interleukin 8; IL-22, Interleukin 22; IL-25, referred also as IL-17E and IL-17F, Interleukin 25; LB, Luria-Bertani; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; NFκB, Nuclear factor kappa B; OVA, ovalbumin; P.a PAO1, *Pseudomonas aeruginosa* PAO1; PMA, phorbol 12-myristate 13-acetate; rpm, revolutions per minute; PRR, Pattern recognition receptors; SCFAs, short chain fatty acids; SM, salt magnesium; TLRs, Toll-like receptors.

Keywords: Bacteriophages, food allergens, inflammation, cytokine, infection

INTRODUCTION

The biological evolution of allergies has been elucidated by Blackley [1], Preston [2], Rook [3], and Strachan [4], highlighting how they evolved due to human environmental changes and modern lifestyles. They emphasize the intricate interplay between genetics, the environment, and immune responses.

Gut-derived food and bacterial allergens cause heterogeneous inflammatory pathologies such as type 2 immune responses and allergic inflammation, leading to respiratory, skin, and food allergies [5,6]. For example, atopic disease and asthma are caused by intestinal, lung, and skin barrier dysfunction, which has been set explicitly in the diet-microbiota-mucus-food allergy axis [7]. Among food allergens, some food sweeteners and food additives are shown to break down oral tolerance when administered orally to mice together with ovalbumin (OVA) [7]. There is a strong link between mucin-degrading pathogenic bacteria growth, dietary components, and food allergies [7]. Within these complex sets of binary interactions, food additive-enteric apathogenic bacteria interaction influences the (patho) biological nature of both the host eukaryotic cells and bacteria, including their cellular uptake into host eukaryotic cells, pathogenic signaling pathways, and cell toxicity [8]. Further, food additive-derived foodborne nanoparticles influence bacterial pathobiology and fate and their host eukaryotic cells [9].

Pseudomonas aeruginosa is a highly opportunistic human pathogen with virulence factors, biofilm-forming capability, and antimicrobial resistance. It is associated with hospital infections and contamination of food groups. Antibiotic treatment-driven dysbiosis facilitates *P. aeruginosa* colonization, threatening public health and food safety. Recent studies suggest that *P. aeruginosa* stimulates neutrophils to secrete high levels of histamine, leading to inflammation and asthma. It also secretes a protein causing Type 2 immune responses and allergic inflammation [5,10-12].

At the food level, according to the FASTER Act of 2021, Public Law 117-11 of the US Government Publishing Office, cow's milk proteins and eggs are the top two causes of allergies among the eight major food allergens. Cow's milk proteins are divided into two main classes: caseins (α1-, α2-, β-, and κ-casein) and whey proteins (α-lactalbumin and β-lactoglobulin), and all can be potential allergens [13]. The hydrolysis yields of cow's milk proteins are immunomodulatory proteins and peptides. The effects of these proteins on the immune system depend on their type of protein and degree of hydrolysis. Consequently, their biological activity and immunomodulatory properties are also quite different. For example, intact whey and casein are capable of inducing the pro-inflammatory cytokines IL-8 and TNF-α, while extensively hydrolyzed casein does not induce TLR activation [14]. Four different casein proteins are recognized as individual allergens (αs1-casein, αs2-casein, β-casein, κ-casein, Bos d 9–12). Among these, the sensitization of cow's milk αS1-casein damaged the intestinal barrier of mice, caused the leakage of lipopolysaccharides (LPS), and activated the TLR4-NFκB pathway [15]. In addition to that, consumption of milk containing A1 β-casein was also associated with increased gastrointestinal inflammation [16], increased abundances of various bacterial species related to diabetes, such as *Streptococcus pyogenes* [17], and elicited TLR4-mediated inflammatory responses *in vivo* [18]. On the other hand, the role of gut microbes in mediating food allergen-associated responses has been well reviewed [19-21]. In this context, enteric dysbiosis is a risk factor for dietary protein-related intestinal alterations, which develop food allergies and gastrointestinal disorders. For example, a dysbiotic-like milieu provoked by LPS facilitates the sensitization to a luminal antigen, OVA, *in vivo* [22]. On the other hand, OVA causes intestinal cell morphological damage, including epithelial detachment, villous atrophy, mucosal atrophy, and edema, and increases the abundance of enteric pathogenic bacteria, including Proteobacteria, *Epsilonbacteraeota*, *Alloprevotella*, *Bacteroides*, and *Parabacteroides* [23]. Additionally, OVA-derived food allergies disrupt the function of the intestinal epithelial barrier by increasing enteric pathogenic bacteria [24,25].

Within the gastrointestinal tract (GIT) and at the cellular level, different cell types (eg, enterocytes, macrophages, and hepatocytes) and their secreted mediators play a pivotal role in healthy immune responses to food allergens and influence the balance between tolerance and sensitization, ensuring the maintenance of tolerance. Intrinsic activities of food components on innate cells and exposure to bacterial toxins are important signals that initiate sensitization. In this context, dysbiosis of inflammatory and anti-inflammatory gut bacteria and defective barrier functions in either the skin or intestine have been shown to facilitate sensitization to food allergens [26-28]. These data imply that food allergens (dietary antigens) and gut microbiota are potential factors contributing to the pathophysiology of inflammatory diseases of the GIT.

Within the GIT, phages are another key biologically active player. It has been shown that phages adhere to and are subsequently internalized by eukaryotic host cell lines, including endothelial, epithelial, macrophage, and fibroblast [29]. The therapeutic application of bacteriophages has long been investigated, with many anecdotal reports of success suggesting that it could improve the efficacy of currently available antibiotics. Recent clinical successes using personalized phage cocktails have rekindled interest in phage therapy, and multiple clinical trials are currently in progress (reviewed by reference [30]).

The fate, activity, and impact of ingested lytic phages within the human gut microbiota and the protective immunomodulatory activity of human gut phages have also been well reviewed [31,32]. However, no study has been conducted to investigate their ability to interfere with inappropriate immune responses mediated by bacterial and food antigens. In this context, essentially, there has been no information on the potential use of bacteriophages to rescue food allergen-induced gut-skin axis microbiota dysbiosis. We hypothesized that immune responses to food allergens could preferentially benefit from phages. Hence, the aim of the present study was to further explore the impact of host-bacteria-phage states of the GIT on the generation of abnormal responses to food allergens and if phages function as allergic immune modulators to prevent microbial and food allergies. For this purpose, we established a triple cell co-culture model incorporating monolayers of intestinal cells, macrophages, and hepatocytes, immersed in three different media (DMEM, DMEM + OVA, and DMEM + casein), and treated them with P.a PAO1 in the absence or presence of its KPP22 phage. The potential facilitatory effects of OVA and A1 β-casein on P.a PAO1-infectious inflamed milieus were studied by assessing colonic monolayer barrier functionality and the cellular pathogenesis of P.a PAO1 complicated by the presence of the antigens (OVA or casein). To better understand the potential role of phages in the functional alterations observed, changes in bacterial adherence were determined. Finally, to gain insight into the immune nature of host-bacteria-phage-mediated responses related to food allergens, we evaluated changes in cytokine secretion, the potential reciprocal involvement of phages and food allergens, and the dynamics of colonic, liver, and skin functions. We established a human triple cell co-culture model and an OVA- or β-casein-stimulated food-allergic cell culture model under an infectious-inflamed milieu. This model consists of colonocytes (T84), macrophages (THP-1), and hepatocytes (Huh7). The rationale for this model is that: (i) intestinal epithelial cells are absorptive, immunological, and metabolic cells, and without their appropriate function, the skin organ has no sense; (ii) macrophages play a pivotal role in skin homeostasis; and (iii) the liver and the skin are deeply intertwined, and because of their immunological and metabolic functions and the skin-liver connection, the skin mirrors the health of the liver. Consequently, liver disease of any origin, if acute or chronic, can appear on the skin.

MATERIALS AND METHODS

Media, Reagents, Kits

The reference allergen chicken egg ovalbumin protein (OVA cat # A5503-1G; grade V, ≥98% purity) and cow's milk β-casein (cat # C6905-250MG) were obtained from Sigma Chemical (Sigma-Aldrich, Taufkirchen, Germany). The test proteins were dissolved in sterile PBS. Twenty-four mm Transwell with 0.4 µm Pore Polyester Membrane Insert, Sterile (cat # 3450) and 75 cm² U-Shaped Canted Neck Cell Culture Flask with Vent Cap (cat # 430641U), DMEM, (cat # 11960044), Minimum Essential Medium (MEM) nonessential amino acids (cat # 11140035), DPBS without calcium and magnesium (cat # 14190144), FBS (cat # 16000044), Pierce Chromogenic Endotoxin Quant Kit (cat # A39552), TPP cell spatula (cat # 99010), and Corning Small Cell Scraper (cat # 3010) were purchased from Thermo Fisher Scientific (Schwerte, Germany). A 0.22 µm membrane syringe filter was purchased from Sarstedt (Nümbrecht, Germany). Human ELISA kits (IL-1β, IL-8, IL-22, and IL-25) were obtained from Abcam (Berlin, Germany).

Bacterial Strain and Propagation Conditions

The *P. aeruginosa* strain PAO1 (P.a PAO1) was grown as previously described [33]. Briefly, P.a PAO1 was plated from a -80°C glycerol stock on LB agar and grown aerobically at 37°C for 24 h. Next, a single colony was picked up, transferred to 9 ml of LB broth, and grown overnight at 37°C with shaking at 182 rpm. After overnight growth, bacterial cultures were harvested by centrifugation and washed twice with PBS. The obtained pellets were then resuspended in 5 ml of PBS, and 100 µl of the bacterial cell pellet resuspended in PBS, with approximately 10^8 cells, was inoculated in 100 ml of fresh LB broth medium. The culture was incubated overnight at 37°C with shaking at 182 rpm, and in the morning, the cell growth was measured according to the optical density at 600 nm (OD600) using a Hach Lange DR2800 spectral photometer. For the enumeration of P.a PAO1 and to ensure viability before co-culturing with eukaryotic host cells, bacterial counts were obtained by serially diluting the strain in PBS, plating on LB agar, and incubating at 37°C overnight.

Phage KPP22 Supernatant Preparation

The phage KPP22, which has been characterized before by Uchiyama et al. [33], was prepared according to the previously published method [33]. The phage titration, concentration, and removal of endotoxin were essentially based on previously published processes [33-36]. Briefly, 10 ml of LB broth was inoculated with 100 µL of an overnight culture of P.a PAO1. Following 4 h shaking of the incubation at 182 rpm at 37°C, the phage was added with a final MOI of 1. The culture was incubated for a further 4 h at 37°C or until lysis before centrifuging it at 5000× g. The supernatant (phage lysates) was then sterilized through a 0.22 µm membrane syringe filter, which

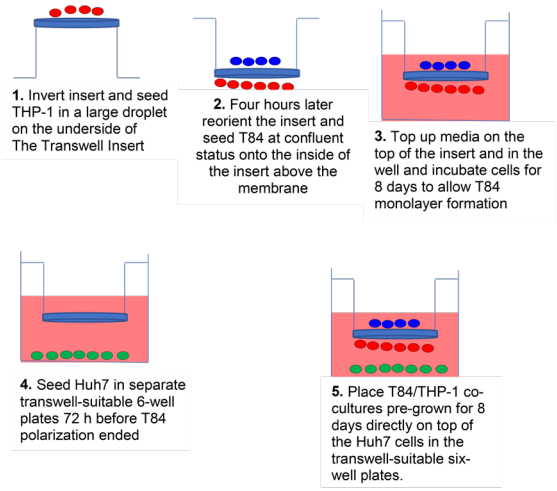

Figure 1. **Schematic steps of the procedure for the infectious-inflamed triple cell co-culture preparation**. THP-1 cells were cultured on the underside of transwell inserts by inverting the insert (**1**). After 4 hours, T84 cells were cultured on the upper side (**2**). This T84/THP-1 co-culture was then incubated for 8 days to allow T84 monolayer formation (**3**). Huh7 cells were seeded in the bottom of separate transwell-suitable 6-well plates 72 h before T84 polarization ended (**4**). To start the infectious-inflamed triple co-culture, T84/THP-1 co-cultures pre-grown for 8 days, were used to set up the triple co-culture with Huh7 cells. For this, the T84/THP-1 co-culture (the upper chamber containing the T84/THP-1 cells) was then placed directly on top of the Huh7 cells in the transwell-suitable six-well plates (**5**). Afterward, P.a PAO1 and/or KPP22 phages were added to the apical surfaces of T84 cell monolayers. Then, the assembled triple cell co-cultures were nutritionally immersed in either DMEM, DMEM + OVA, or DMEM + β-casein media and incubated for 6 h at 37°C in a humidified atmosphere of 5% CO_2.

does not cause titer loss. For the enumeration of the phage KPP22, SM buffer was used for serial dilutions as previously described [33,36]. Briefly, 10 µl aliquots of 10^{-4}, 10^{-5}, and 10^{-6} phage dilutions were incubated with 100 µl of an overnight culture of P.a PAO1 as host bacterium and then plated on 8cm LB agar plates. After overnight incubation at 37°C, plaques appeared on each plate, were counted to determine the titer of the original phage solution and expressed as plaque-forming units per microliter (pfu/µl). Because cross- and endotoxin-contaminations are key constant risk factors for immune-related adverse events, the endotoxin contamination value in the phage preparations was assessed using the *Limulus* amoebocyte lysate method (Pierce Chromogenic Endotoxin Quant Kit, cat# A39552, Thermofisher). The level of endotoxins in phage lysates complied with the recommendations of the GMP and ISO:13485 best practice guidelines for cell cultures and did not exceed the acceptable level (less than 10.U.E./ml). The KPP22 phage was concentrated by differential centrifugation and was not contaminated with cellular debris or bacterial proteins. The lysate was then titrated and stored at 4°C. Stock preparations of KPP22 phage (10^9 PFU/ml) were diluted with DMEM, B. Braun isotonic NaCl 0.9% solution and DPBS solution just before the cell culture experiments. NaCl 0.9% and DPBS were used to compare the stability of the KPP22 phage. Then, for running all the subsequent assays, from a 10^9 phage KPP22 per ml suspension, we carefully added 15 µl into the upper compartment of the inserts (10^7 PFU/ml) so that all inserts ended up with 1.5 ml of medium, which will be described hereafter. In our preliminary experiments (data not shown) to determine the implication of KPP22's role in the interactions between different participants, we included three additional conditions in the series: KPP22 alone, KPP22+OVA and KPP22+β-casein. Although KPP22 phage alone exhibited inhibitory effects on reduced TEER and viability of uninfected control cells treated with OVA or β-casein, it did not have significant reproducible effects (in our hands). Therefore, we did not include KPP22 phage alone for further experiments. We have presented the results of experiments with P.a PAO1+ KPP22 phage. This is important because in order to use phages therapeutically, they should: 1) preferably be lytic; and 2) efficiently kill the bacterial host [37].

CELL LINES, CULTURE CONDITIONS, AND TREATMENTS

Cell Lines: T84 cells and the human monocyte cell line THP-1 (frozen cell stocks in liquid nitrogen) were from the American Type Culture Collection (ATCC, LGC, Wesel, Germany). Huh7 cells (our frozen cell stock

in liquid nitrogen) were from the Japanese Collection of Research Biosources Cell Bank (Tebubio GmbH, Offenbach, Germany). T84, THP-1, and Huh7 were maintained in standard DMEM medium supplemented with 5% FBS and 1% MEM non-essential amino acids. Cells were routinely split at~ 80% confluence and used at passages 23-31 after thawing for experiments. The cells were kept in a humidified incubator at 37°C in a 5% CO_2 atmosphere. Cells were trypsinized with 0.25% trypsin. All cell lines were tested for Mycoplasma with the MycoStrip Mycoplasma Detection Kit (InvivoGen, Toulouse, France) according to the manufacturer's protocols.

Setup of Triple-cell Co-cultures

To mimic the distributions of the three types of cells in the gut-liver-skin axis, 6-well transwell inserts with semi-permeable polyester membrane supports with a 24 mm diameter and 0.4 μm pore size from Corning were used, and triple cell co-cultures were carried out in a three- step process as depicted in Figure 1. First, THP-1 cells were maintained, centrifuged, resuspended, differentiated, and seeded on the underside of inverted transwell inserts as described in detail previously [38,39]. Briefly, one day prior to seeding for the co-cultures, THP-1 cells were stimulated to differentiate into macrophage-like cells by the addition of PMA. Afterward, PMA differentiated THP-1 macrophages (100 μl= 10^5 cells per insert) were seeded on the underside of the inverted transwell inserts and left to incubate for a period of 4 h. After this time, the transwell inserts were placed back inside their respective wells. Subsequently, T84 cells were added to the apical side of the transwell at a seeding density of 7.5 x 10^4 cells/insert in DMEM (1.5 ml) as described previously [40,41]. The apical and basolateral compartments of the co-culture were refreshed every 48 h with DMEM. The co-culture was maintained for 8 days to obtain tight T84 monolayers, until confluent monolayers of the polarized columnar epithelium with a TEER of ≥1000 Ohm/cm^2 were obtained [40]. Three days prior to seeding for the triple co-cultures, Huh7 cells (3 x 10^5 cells per well) cells were seeded at the bottom of separate transwell-suitable 6-well plates. To start the infectiously inflamed triple co-culture, T84/THP-1 co-cultures pre-grown for 8 days were used to set up the triple co-culture with Huh7 cells. For this, the T84/THP-1 co-culture (the upper chamber containing the T84/THP-1 cells) was then placed directly on top of the Huh7 cells in transwell-suitable six-well plates to initiate triple co-culture. After 24 hours of triple co-culture, triple co-cultured cells were divided into nine experimental groups as follows: 1) DMEM with uninfected cells (control); 2) DMEM with P.a PAO1-infected cells; 3) DMEM with P.a PAO1-infected cells and with KPP22 phage; 4) OVA-supplemented DMEM with uninfected cells; 5) OVA-supplemented DMEM with P.a PAO1-infected cells; 6) OVA-supplemented DMEM with P.a PAO1-infected cells and with KPP22 phage; 7) β-casein-supplemented DMEM with uninfected cells; 8) β-casein-supplemented DMEM with P.a PAO1-infected cells; and 9) β- casein-supplemented DMEM with P.a PAO1-infected cells and with KPP22 phage.

DMEM medium was supplemented with 100 μg/ml of β-casein because β-casein alone, at 100 μg/ml, activates macrophages and leads to the production of pro-inflammatory cytokines such as IL-1β and TNF-α [42]. The concentration of OVA used in this model was 80 μg/ml, which was used previously [43].

The Experimental Setup of the Infectious-inflamed Triple Cell Co-cultures

For the infectious-inflamed triple cell co-cultures, P.a PAO1 and/or KPP22 phages were applied to the apical surfaces of T84 cell monolayers grown on transwell insert membranes, as simply summarized as follows:

One day prior to infection or experiment, P.a PAO1 was grown in LB overnight at 37°C, washed twice with DPBS and diluted 1:10 into fresh antibiotic-free DMEM for 1 hour (37°C in a 5% CO_2 atmosphere) before adjusting the optical density of OD600 to 0.5 (~1×10^8 bacteria/ml). Once the triple co-cultures were ready to be treated (Section "Setup of Triple-cell Co-cultures," above), the medium was removed and cells were washed with DPBS before adding fresh antibiotic-free medium containing 10^7 CFU/ml P.a PAO1 and/or 10^7 PFU/ml phage KPP22. Following the cell washing steps, 10^7 CFU/ml P.a PAO1 in a volume of 150 μl was added first on top of the designated T84 cell monolayers to treat triple co-cultured cells. Then, from a 10^9 phage KPP22 per ml suspension (section "Phage KPP22 Supernatant Preparation," above), we dispensed directly 15 μl into the upper compartment of the inserts (10^7 PFU/ml) so that all inserts ended up with 1.5 ml of medium.

After this point, the transwell inserts were immersed in three different types of antibiotic-free culture media: DMEM, DMEM with OVA, and DMEM with β-casein. The final culture medium volumes in the Transwell inserts were 1.5 ml (apical) and 2.6 ml (basolateral). Subsequently, the resulting host-bacteria-phage co-cultures were maintained for 6 h at 37°C and 5% CO_2 under humidified conditions. Control cells and cells exposed to P.a PAO1 without phage treatment were also run in parallel and subjected to the same changes in medium and washes. After 6 h, cells were visually inspected, and the physiological and structural functions of cells (TEER) as well as their secreted signal molecules were evaluated in the following sections. Cell-free supernatants were collected, centrifuged (14000 rpm, 5 min, 4°C), filter sterilized (0.2 μm), and frozen and stored at -80°C until further analysis. Cells were then trypsinized or scrapped, washed, and

counted as detailed hereinafter.

TEER Measurements

The TEER values of the T84 monolayers were assessed with the epithelial voltohmmeter EVOM2 (World Precision Instruments, USA) together with the STX2 Chopstick Electrode Set (World Precision Instruments, USA). Briefly, before each measurement, the electrodes were first cleaned with 70% isopropanol, washed once with 1 x PBS, dried, and then placed in the apical or basolateral compartment, respectively. Then, at the end of the experiment, semi-permeable membrane transwell inserts containing polarized T84 membrane layers in the transwell (apical) compartment were carefully removed from co-culture. After the removal of apical medium, the T84 cell mucosal layer was washed twice with DPBS. A volume of 1.5 ml of DPBS was added to the transwell, and 2.6 ml was added to the basolateral compartment outside the transwell insert. TEER was recorded across the cellular barrier in the insert and adjusted for the transwell surface area of 4.67 cm^2 to present as Ω cm^2. All readings of TEER were repeated across duplicate sample transwells.

Physiological Health of Triple Co-cultured Cells in Response to Stimulants

In separate experiments, the impact of treatments on the viability of the triple-cell co-cultures was evaluated. When T84 cells were confirmed to have reached confluence using TEER $\geq 1000\,\Omega/cm^2$, the T84 cell monolayers were washed twice with 500 µl DMEM without antibiotics or supplements, and cell viability assays were performed by adding the bacterial inoculum of P.a PAO1 (approximately 1×10^7 CFU/ml) without and with KPP22 phage (approximately 1×10^7 PFU/ml) in the apical chamber. This ratio was chosen because the phage-to-bacteria ratio in the gut is 1:1 or lower [44]. The cell cultures were incubated for 6 h at 37°C in a humidified atmosphere of 5% CO_2. After being incubated for 6 h, the T84/THP-1/Huh7 cells were collected separately by scraping the entire surface of the culture area with small cell scrapers. Collected cells were resuspended in DPBS, and the viability of treated cells was evaluated using Trypan blue dye exclusion. The results were compared with the viability of the cells in the control wells, where the cells were not exposed to any stimulants.

Measurement of Cytokines

After 6 h of infection and subsequently after measuring the TEER values and cell viability, the supernatants from triple co-cultured cells were collected, and the levels of IL-1β, IL-8, IL-22, and IL-25 cytokines were assessed using Abcam ELISA kits, according to the manufacturer's protocols. In addition, the pH values of the supernatants were measured. Before the start of the measurements, the pH meter (Mettler-Toledo Micro pH Electrode, Gießen, Germany) was calibrated using buffer solutions at pH 4.0, 7.0, and 9.21 (Fisher Scientific).

Bacterium's Adhesion to and Invasion into Eukaryotic Host Cells

The cell adhesion and invasion assays were carried out in separate experiments as described previously [45-47]. Briefly, one day before starting the experiments, the overnight cultures of the P.a PAO1 strain were centrifuged (5000 rpm, 10 min), washed with sterile PBS, and centrifuged again until the culture medium was completely removed. Next, the bacterial pellets were mixed with DMEM without antibiotics. Simultaneously, once the triple co-cultures were ready to be treated (see "Set-up of Triple Cell Co-cultures," above), the medium was aspirated from the triple co-cultured cells within 6-well transwell inserts, and the cells were washed with DPBS. After washing two times, nine treatments were examined: T84 cell monolayers on the upper membrane surface were infected with P.a PAO1 (10^7 CFU/ml) in the presence or absence of KPP22 phage (10^7 PFU/ml), and then the transwell inserts containing the T84/THP-1/Huh7 cells were immersed in three different media: antibiotic-free DMEM alone, DMEM supplemented with 80 µg/ml OVA, or DMEM supplemented with 100 µg/ml β-casein. The cells were incubated for 2 hours at 37°C in 5% CO_2 and a humidity of >95%. After 2 h, the non-adhered P.a PAO1 was removed by washing with PBS, and 250 µL of DMEM containing 150 µg/mL gentamicin was added to kill extracellular P.a PAO1 for 45 min. In the next step, adhered P.a PAO1 with T84 cells on the upper membrane surface (inside the transwell insert) were scraped off carefully using a mini sterile cell scraper, transferred into sterile barcoded 5 ml tubes using a pipette, and centrifuged (5000 rpm, 10 min). In order to disrupt the T84 cells, 0.1% Triton X-100 was added (5 min, room temperature). Serial dilutions of the disrupted cell suspension were then spread on LB Agar plates for viable enumeration of adherent bacteria after overnight incubation at 37°C. To evaluate the number of invaded P.a PAO1, the same method was used but without the gentamicin treatment. Adhesion and invasion rates were calculated as percentages of adhered or invaded P.a PAO1 to the initially applied numbers. For the adhesion and invasion assays in DMEM with OVA or β-casein (each condition applied in duplicate), 3 independent experiments were performed using different T84/THP-1/Huh7 passages each (n= 3).

Among triple co-cultured cells, we assessed adhesion to and invasion into T84 cell monolayers because the 0.4-µm membrane inserts allow only the diffusion of media and molecules secreted by the bacteria and do not allow

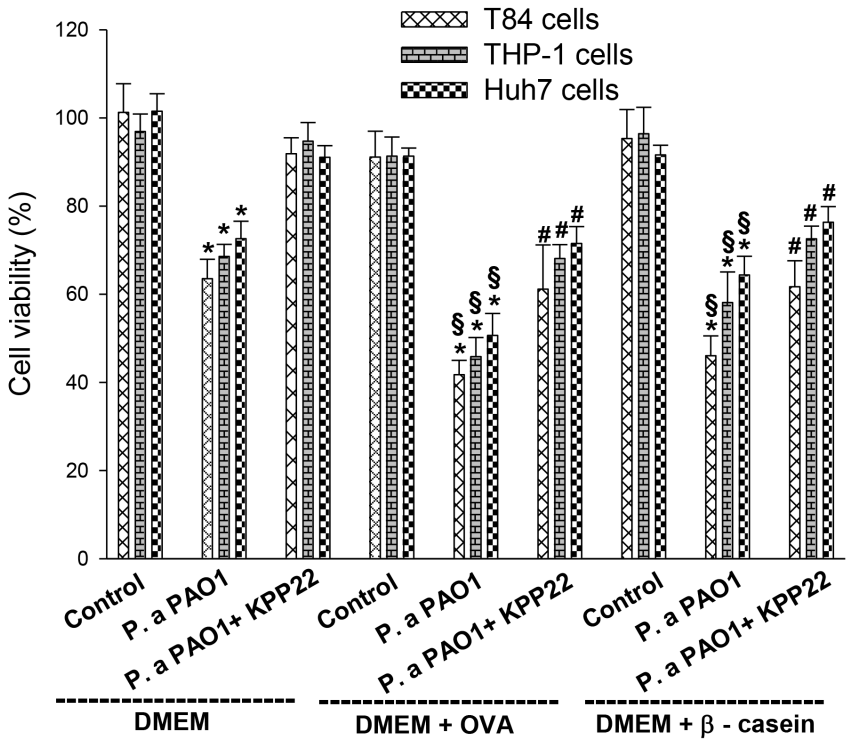

Figure 2. **Effect of food allergens on the viability of P.a PAO1-infected triple co-cultured cells**. The cytotoxic effect of P.a PAO1, in the absence or presence of KPP22 phage, OVA, or β-casein, on triple co-cultured cells was determined by direct trypan blue viable cell counting after 6 h of treatment. Results were calculated as the mean values (± SEM) of three independent experiments, and $p < 0.05$ was considered statistically significant. * $p < 0.05$ versus control uninfected cells, # $p < 0.05$ versus P.a PAO1 alone, and § $p < 0.05$ versus DMEM alone, namely significant difference between DMEM alone and DMEM + OVA or β-casein.

bacterial passage through the pores, retaining the bacteria and thus direct contact of whole bacteria with underlying THP-1/Huh7 cells. Transwells with a 0.4 μm membrane insert remove micron-sized particles, like bacteria, yeast cells, colloids, and smoke particles, from suspensions.

Statistical Analysis

All data were analyzed using STATGRAPHICS Plus statistical software version 4.1 using one- way ANOVA. Data were presented as the mean ± SEM of three independent experiments run in duplicate. The results were compared at a 95% confidence level. The intergroup comparison was carried out using the Kruskal–Wallis test and the intragroup comparison using the Student-t test, and p values of ≤ 0.05 were considered of statistically significant differences. The normality of data and statistical significance were assessed by Shapiro-Wilk.

RESULTS

Effect of Food Allergens on the Viability of P.a PAO1-infected Triple Co-cultured Cells

Since OVA and bovine β-casein are known to reduce cell viability *in vitro* [48,49], the cytotoxic effect of P.a PAO1, in the absence or presence of KPP22 phage, OVA, or casein, on triple co-cultured cells was determined by direct trypan blue viable cell counting (Figure 2). The results show that P.a PAO1 alone exhibited cytotoxic activity, reaching 64.4 ± 0.2% of cell death after 6 h of infection. The cytotoxic activity of P.a PAO1 plus OVA was higher, with 88.1 ± 0.3% of T84 cell death after 24 h of infection. P.a PAO1 plus β-casein exhibited cytotoxic activity, reaching 67.2 ± 0.1% of cell death. However, these toxic side effects were diminished by the presence of KPP22 phages. Similar results were obtained in the cases of underlying THP-1 and Huh7 cells, although with less intensity. These results imply that the toxic effector molecule produced by the bacteria (in the apical chamber) was freely secreted in sufficient concentrations, and the secreted form was cytotoxic. The reason is that a 0.4-μm membrane efficiently removes micron-sized particles, like bacteria, yeast cells, colloids, and particles,

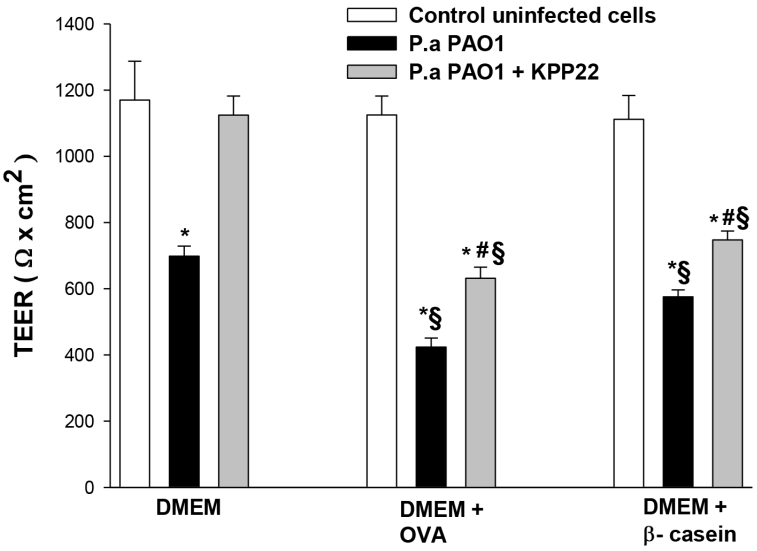

Figure 3. **Effect of food allergens on the barrier integrity of P.a PAO1-infected T84 cell monolayers**. Triple T84, THP-1, and Huh7 co-cultured cells were treated for 6 h. The TEER was expressed as percentages of the control TEER measured across T84 cell monolayers. Results are the mean values (± SEM) of three independent experiments, and $p < 0.05$ was considered statistically significant. * $p < 0.05$ versus control uninfected cells, # $p < 0.05$ versus P.a PAO1 alone, and § $p < 0.05$ versus DMEM alone, namely significant difference between DMEM alone and DMEM + OVA or β-casein. Values (Ω * cm^2) of TEER relative to the average TEER value of uninfected control cells (control, 100%). TEER values compare the mean measurements of duplicate measurements (wells).

from suspensions, whereby it allows only the diffusion of media and molecules secreted by the bacteria, does not allow bacterial passage through the pore, retains the bacteria, and thus directs contact between whole bacteria.

Effect of Food Allergens on the Barrier Integrity of P.a PAO1-infected T84 Cell Monolayers

The effect of the bacteria, in the absence or presence of KPP22 phage, OVA, or β-casein, on epithelial barrier integrity was assessed by measuring the TEER across polarized T84 cell monolayers. As shown in Figure 3, the decrease of TEER induced by P.a PAO1 alone was 41.4 ± 2.1% compared to uninfected control cells, whereas P.a PAO1 plus OVA led to a deeper decrease of TEER, reaching 57.9 ± 1.2%, and P.a PAO1 plus β-casein led to a decrease of the TEER value, reaching 49.8 ± 2.2%. The decrease in TEER cannot be caused by acidification of the medium because the pH remained constant during the experiments. However, these disruptive side effects were diminished by the presence of KPP22 phages. Meanwhile, the KPP22 phage significantly reduced the fall in TEER caused by P.a PAO1, OVA, and β-casein. The results showed that the cell viability profile was reflected by the TEER profile. Since cell viability is reduced, the ability of epithelial monolayers to maintain their barrier function is compromised. This is also in agreement with previous studies showing that P.a PAO1 and its secreted peptides reduce the viability and barrier integrity of intestinal cells [46,47,50,51].

Measurement of IL-1β, IL-8, IL-22, and IL-25 Secretion

At the gut level, the secretion of cytokines is a key biological process in modulating host immune responses to infection- and inflammation-driven dysbiosis. To evaluate the functional role of combined bacterial and food allergens on cytokine secretions, triple co-cultured cells were cultivated for 6 h in the absence or presence of P.a PAO1 in DMEM supplemented with or without 80 µg/ml OVA and/or 100 µg/ml β-casein. Additionally, we used the KPP22 phage as a natural bacteria-lysing phage for cell stimulation to evaluate the capacity of cells to respond to the stimulus and to identify whether there is a differential response between groups. The bacterial proinflammatory effect of P.a PAO1 was assessed by measuring IL-1β, IL-8, IL-22, and IL-25 secretion in triple co-cultured cells after 6 h of infection. As shown in Figures 4A-D, P.a PAO1 alone induced significant stimulation of IL-1β (Figure 4A), IL-8 (Figure 4B), IL-22 (Figure 4C), and IL-25 (Figure 4D) secretion in triple co-cultured cells compared to uninfected control cells. However, when triple co-cultured cells were incubated in the presence of OVA or β-casein, marked potentiation of release was observed in both groups, although β-casein was less potent

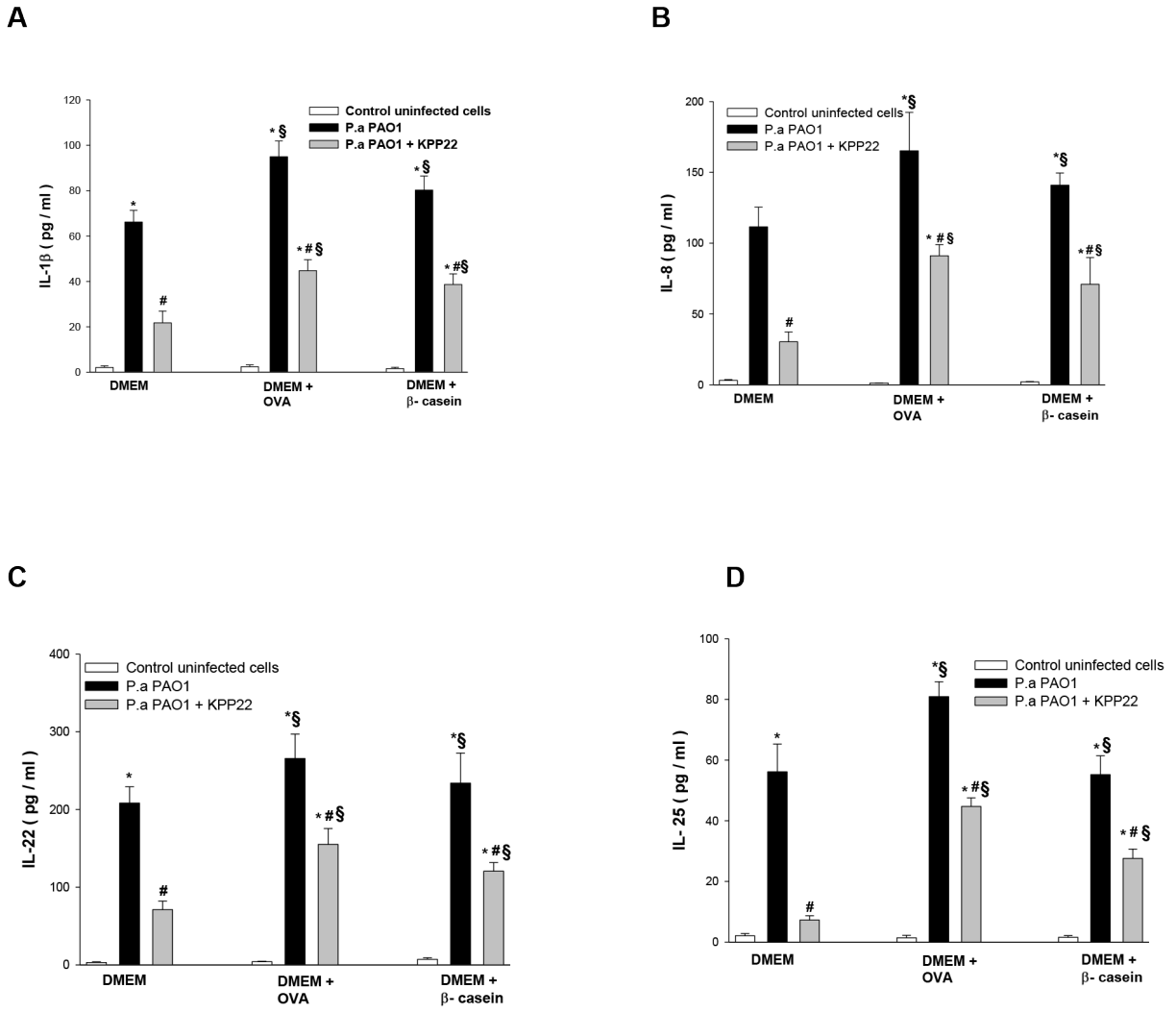

Figure 4. **Proinflammatory effects of food allergens on P.a PAO1-infected triple co-cultured cells**. IL-1β, IL-8, IL-22, and IL-25 cytokines were measured in triple co-cultured T84/THP-1/Huh7 cell supernatant after 6 h of treatment. Results were calculated as the mean values (± SEM) of three independent experiments, and $p < 0.05$ was considered statistically significant. * $p < 0.05$ versus control uninfected cells, # $p < 0.05$ versus P.a PAO1 alone, and § $p < 0.05$ versus DMEM alone, namely significant difference between DMEM alone and DMEM + OVA or β-casein.

than OVA. These results illustrate their pro-inflammatory effects; whether they have possible clinical effects is to be investigated. On the contrary, IL-1β, IL-8, IL-22, and IL-25 secretions were strongly abolished following treatment with KPP22 phages. These data suggest that phages play an important role in the production of cytokines triggered by P.a PAO1. Overall, these data suggest that at the eukaryotic host cell level, food allergens and bacterial toxic effector molecules exacerbate each other's disruptive effects, secreting pro-inflammatory mediators, and phages intervene in this patho(bio)logical process.

Effect of Food Allergens on Proliferative and Invasive Ability of P.a PAO1 in T84 Cells

Adhesion and invasion are two key pathogenicity indices in bacterial pathogens. Therefore, to evaluate whether the bacterial proinflammatory effect of P.a PAO1 triggered by OVA or β-casein could be correlated with adhesion and invasion, the ability of P.a PAO1 to adhere to and invade T84 cell monolayer cells was manifested using the gentamicin exclusion test (Figure 5). We observed that β-casein and particularly OVA enhance the proliferative and invasive abilities of P.a PAO1 in T84

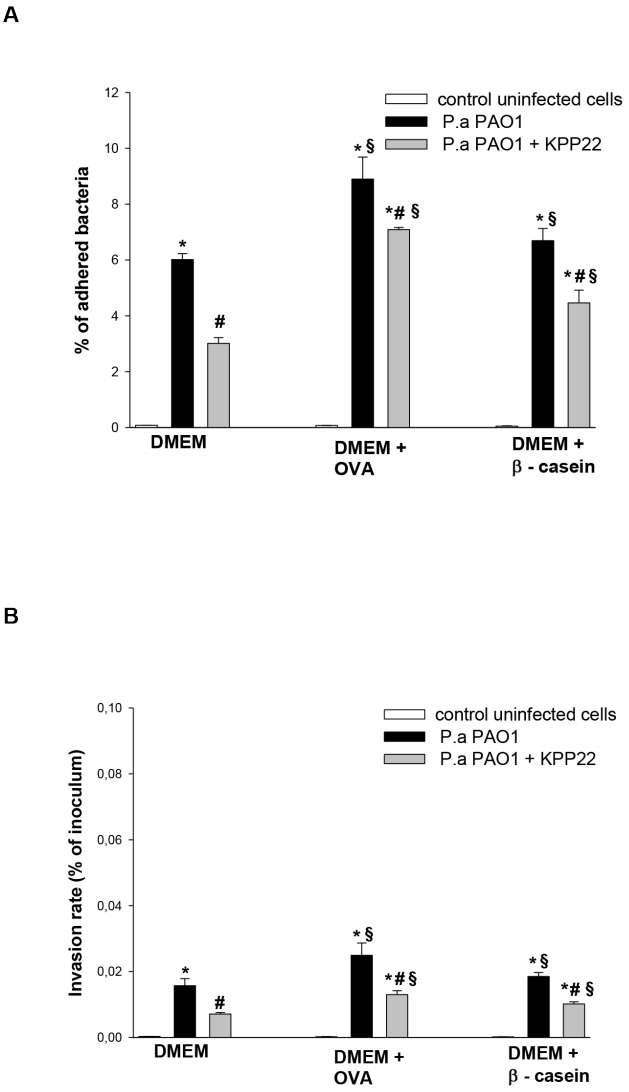

Figure 5. **Effect of food allergens on P.a PAO1's adhesion to and invasion into eukaryotic host cells**. **A)** Adhesion to the surface of the T84 host cells. **B)** Bacterial invasion into T84 cell monolayers. Nine treatments were examined: T84 cell monolayers on the upper membrane surface were infected with P.a PAO1 (10^7 CFU/ml) in the presence or absence of KPP22 phage (10^7 PFU/ml), and then the transwell inserts containing the T84/THP-1/Huh7 cells were immersed in three different media: antibiotic-free DMEM alone, DMEM supplemented with 80 µg/ml OVA, or DMEM supplemented with 100 µg/ml β-casein. Two hours after infection of triple co-cultured T84/THP-1/Huh7 cells with P.a PAO1, extracellular bacteria were killed by gentamicin. T84 cell monolayers were harvested and disrupted at 2 h after treatments. The adhesion and invasion assay using P.a PAO1 alone serves as a reference. The percentage of adhered bacteria was calculated as the percentage of adhered bacteria relative to the initial number of bacteria inoculated in the well of the transwell insert. Invasion was calculated as the percentage of invaded bacteria relative to the initial number of bacteria inoculated in the well of the transwell insert. The data were calculated as the mean values (± SEM) of three independent experiments, and $p < 0.05$ was considered statistically significant. * $p < 0.05$ versus control uninfected cells, # $p < 0.05$ versus P.a PAO1 alone, and § $p < 0.05$ versus DMEM alone, namely significant difference between DMEM alone and DMEM + OVA or β-casein.

cell monolayers, suggesting that this process might play an important role in disease induction, immune escape, and chronic infection associated with bacterial and food antigens. The results show that P.a PAO1 alone can have an invasive behavior with $1.4 \pm 0.1 \times 10^5$ CFUs/ml that entered T84 cells. In the presence of OVA or β-casein, the invasive capacity of P.a PAO1 was significantly higher, with $2.1 \pm 0.2 \times 10^5$ CFU/ml and $1.6 \pm 0.1 \times 10^5$ CFU/ml, respectively. The KPP22 phage mitigated the invasion ability of P.a PAO1 in the presence of OVA or β-casein.

DISCUSSION

We investigated the immunomodulatory role of bacterial and food allergens and phages in the homeostatic and inflammatory milieu of the intestinal mucosal lining, in which the immune process occurs. To recreate the gut epithelial–macrophage–microbial-liver interface in an *in vitro* setting, a triple cell co-culture model was established by assembling the intrinsic intestinal barrier-antigen-presenting cells-detox axis, made up of enterocytes, macrophages, and hepatocytes. Therefore, a triple cell co-culture model consisting of enterocytes (T84 epithelial cells), immune cell type (macrophages-like THP-1), and hepatocyte cell type (Huh7 cells), which offers the possibility to study the interaction of bacterial and food allergens with those cells, was developed. We investigated the capacity of OVA and β-casein to induce the production of the major proinflammatory and immunoregulatory cytokines: IL-1β, IL-8, IL-22, and IL-25, which are among the first cytokines produced in response to pathogenic bacteria. At the gut level, IL-1β and IL-8, IL-22, and IL-25 play a crucial role in modulating host immune responses to inflammation-driven dysbiosis. IL-22, with a double-edged role, and IL-25 have complex and pleiotropic functions [52-55]. Overall, it can be observed that both OVA and β-casein significantly enhance the release of IL-1β, IL-8, IL-22, and IL-25 in the P.a PAO1-treated triple co-cultured cells. These results illustrate their pro-inflammatory effects; whether they have possible clinical effects is to be investigated. Additionally, we used the KPP22 phage as a naturally occurring bacteria-lysing phage for cell stimulation to evaluate the capacity of cells to respond to the stimulus and to identify whether there is a differential response between groups. However, when triple co-cultured cells were incubated in the presence of KPP22 phages, a marked reduction in release was observed. These results provide a new paradigm for molecular mimicry between bacterial antigens and food allergen-augmented inflammation etiology, whereby an epithelial integrity gatekeeper is modulated by changes in apical surface phage exposure.

To clarify the cellular basis of these inflammatory signal molecules, we investigated the structure and physiologic characteristics of cells. Among these characteristics, the viability of cells and the integrity and impermeability of the formed cell monolayer are important. Regarding the first indicator, the P.a PAO1 bacterium alone had an adverse effect on the viability of all three types of co-cultured cells. However, this effect was more on intestinal cells (T84 cells) than the other two cells and less on immune cells (THP-1 cells). When OVA or β-casein was added to the culture medium, the effect of P.a PAO1 on the cells intensified. These imply that T84 cells grown on transwell inserts were more sensitive to the adverse effects of the treatments than their THP-1 and Huh7 cell counterparts. Moreover, Huh7 cells are more responsive to effects on cell viability when compared to THP-1 cells, although the paired comparisons of the data are not statistically different. These effects on cell life spans were reflected in the cell architecture. As for the second index, cell viability studies with monolayers of T84 cells grown above the insert indicated that there is a high correlation between cell viability and TEER for six exposure times. In other words, a decrease in TEER was inversely proportional to an increase in cell viability and inflammatory cytokine markers. For example, P.a PAO1 alone led to a decrease in the TEER value, an indicator of junctional integrity, and had a cytotoxic effect on T84 cell monolayers and underlying cells after 6 h of incubation compared to the uninfected control cells. This is in agreement with the previous study [47]. OVA and β-casein, albeit to a lesser extent, promoted P.a PAO1-induced decrease of TEER in T84 cell monolayers. The fall in TEER cannot be attributed to damages provoked by the acidification of the medium since the pH of the medium remained constant over the studies. This provides evidence that OVA and β-casein exposure lead to P.a PAO1-mediated cytotoxicity in the intestinal epithelium and facilitate the pathogen-to-host interaction. However, the decrease in Huh7 cell viability during 6 h of infection suggests that, in the triple-cell co-culture model, the hepatoma cells could sense the P.a PAO1-secreted inflammatory mediators. In fact, the liver is an important site of infectious diseases, and its APCs exhibit a spectrum of abilities to capture, process, and present antigens to immune effector cells [56]. Overall, cell viability in THP-1 and Huh7 cells was higher than that of T84 cells. In this aspect, it has been shown that 1) milk proteins regulate the immune system at the cell level; 2) inflammation of the intestine, such as that seen in milk protein intolerance, may be due to the activation of macrophages; and 3) there are interactions between milk proteins and macrophages [42].

Similar to the cytokine pattern secretions, we observed that P.a PAO1-induced cytotoxicity in OVA or β-casein-treated cells is reduced by the presence of a bacteriophage (KPP22) on an apical surface. These imply a pathogenic nexus between bacterial toxic effector molecules, food allergens, and the pathophysiology of intestinal permeability, whereby an epithelial integrity gatekeeper is modulated by changes in apical surface phage load and modulation. Additionally, the KPP22 phage, as a natural bacteria-lysing phage, significantly reduced the secretion of inflammatory signals and resulted in a 61% increase in TEER. TEER is a sensitive measure of tight junctional barrier function and reflects the condition of tight junction formation, which is responsible for translocating an arsenal of cytotoxic effectors into either extracellular space or target cells

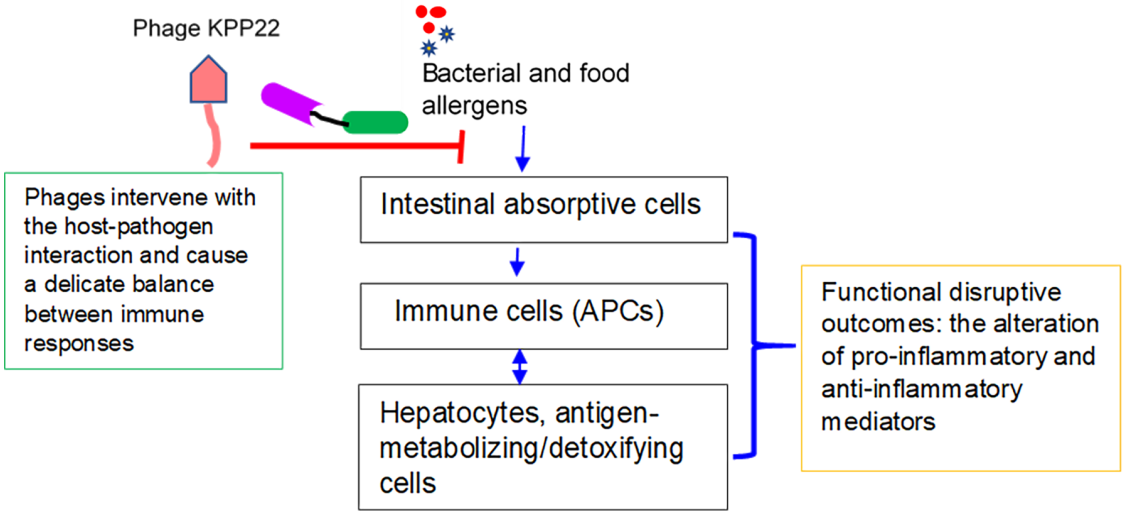

Figure 6. **A schematic overview of how phages intervene in all molecular and cellular areas of eukaryotic host cells that sense and process ingested bacterial and food allergens**. There are three conceivable ways for the direct and indirect ameliorative effects of KPP22 phages: direct clearance of bacterial pathogens from eukaryotic host cells; protein-protein interaction between phage proteins and OVA or β-casein proteins; and direct interactions between phages and eukaryotic host cells.

upon cellular contact. Overall, our findings suggest that since the epithelial barrier becomes highly polarized, it becomes sensitive to P.a PAO1 infection and/or KPP22 phages. However, triple-cell co-cultured cells sense the presence of apically dispersing KPP22 phages, and they may do so through a different group of surface proteins and/or downstream signaling pathways. There are three conceivable ways for the inhibitory effect of KPP22 phage on inflammatory mediators. One is that the KPP22 phage directly destroys the P.a PAO1, hence preventing its side effects. The second is that a protein-protein interaction occurs, namely that KPP22 phage-derived proteins interact with OVA or β-casein proteins. Protein-protein interactions exhibit a wide range of biological processes, including cell-to-cell interactions and metabolic control [57]. The other (third) is that the KPP22 phage directly acts on triple co-cultured cells. In fact, intestinal mucosal surfaces are the interphase between exogenous ingested phage-derived epitopes, bacterial and food antigens, and our body [58,59]. Bacteriophages move beyond the mucosal environments they inhabit and have been found in the bloodstream, spleen, kidney, liver, and brain. Because bacteriophages are microorganisms, they possess a number of epitopes that would be recognized by the PRR of the innate immune system and have been shown to stimulate anti-phage antibody production [58].

Next, we wanted to know how these measurements relate to the ability of P.a PAO1 to adhere to and invade the T84 cell monolayers grown on the transwell inserts. The adhesion rate of P.a PAO1 to T84 cells was significantly increased by the P.a PAO1 plus OVA or β-casein treatments compared to the untreated group. The invasion rates of P.a PAO1 to T84 cells were significantly ($p < 0.05$) increased in the P.a PAO1 plus OVA and P.a PAO1 plus β-casein groups compared with the results of the untreated group. These results can be explained by the effects of inflammatory mediators released by cells during exposure to OVA and β-casein, which could alter the epithelium, allowing P.a PAO1 to adhere better, leading to an augmented response. This is in line with other previous studies showing that OVA and β-casein challenges *in vivo* induce the abundance of pro-inflammatory pathogenic bacteria, and allergic inflammation increases the infection and density of *S. pneumoniae* growth [24,25,59-62].

From the perspective of disease pathogenesis and the functional role of combined bacterial and food allergens, while destructive antigens have to elicit a rapid immune response to eliminate invading pathogens and mitigate re-infection, immunological tolerance and homeostasis have to be mounted against self- and undigested food antigens, including diet and microbiota [63]. For example, recent studies have shown that IL-25 exhibits pleiotropic roles in gut microbiota dysbiosis, inflammation-related diseases, epithelial barrier dysfunction, and pathogenic infection-related allergic diseases, depending on the site of expression, cell type, type of bacteria, and type of allergen [64-68]. On the one hand, bacterial LPS induces maximal expression of IL-25 in epithelial cells

and leads to activation of the IL-25/ILC2/IL-5 axis and the production of IgM, which exhibits a protective effect against bacterial infection and clearance of pathogens. On the other hand, IL-25 is a driver of multiple allergic diseases by promoting type 2 immunity via activation of macrophages and is closely associated with inflammation-related diseases. IL-25 also has positive effects. For example, commensal-dependent expression of IL-25 by intestinal epithelial cells alleviates TLRs-induced intestine inflammation.

These data imply that: (i) at the eukaryotic host cell levels, food allergens and bacterial toxic effector molecules exacerbate each other's disruptive effects, triggering pro-inflammatory mediators and causing infection and inflammation-driven dysbiosis (ii), phages are complementary for the evaluation of pathobiological processes that occur at the interface between bacteria, the host cellular milieu, and food antigens because phages intervene in P.a PAO1-, OVA-, and β-casein-derived inflammation.

In brief, we demonstrated that our model efficiently recapitulated the pathophysiology of bacterial and food allergens (the inflammatory milieu of the intestinal mucosal lining) *in vitro*, and phages deeply affected various aspects of interactions that occur between bacterial and food allergens and the host immune system, from antigen sensing to antigen biological processing, effectively rerouting allergens within and between cells in order to mitigate bacterial propagation. A schematic overview of how phages intervene in all molecular and cellular areas of eukaryotic host cells that sense and process ingested bacterial and food allergens is shown in Figure 6. In this way, both OVA and β-casein act as factors that increase the growth of pathogenic bacteria *in vitro* and *in vivo* [23,24,69,70]. On the other hand, KPP22 phage impacts directly and indirectly on both P.a PAO1 and eukaryotic host cells, as aforementioned. The proposed molecular mechanisms involved in the disruptive effects of OVA, β-casein, and P.a PAO1 include, at least in part, aberrant SCFA levels, elevated systemic intestinal-derived bacterial LPS, and the MAPK/NF-kB pathways, which integrate microbial molecules through PRRs and, in turn, orchestrate the innate immune responses of the gut-skin axis [71,72]. Evidently, our model does not fully mimic the *in vivo* complexity of the natural structure of the allergen-exposed mucosal organs due to differences in molecular, immunological, and cellular responses. Further studies, at both cellular and molecular levels, are warranted to elucidate the active center of phages regulation of immune sensing of bacterial and food allergens and the mechanisms by which the observed regulation occurs. To predict how these experiments could be replicated *in vivo*, obtain more applicable and realistic results, and extrapolate these data to humans, further research (eg, mouse models) is needed.

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Informed Consent Statement: Not applicable.

Animal Study Statements: Not applicable. This study has been done only in *in vitro* cell culture models.

Data Availability Statement: All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author upon reasonable request.

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