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# Biomarkers Induced by the Immunomodulatory Bacterial Extract OM-85: Unique Roles for Peyer's Patches and Intestinal Epithelial Cells

Vania Manolova[1], Anna Flace[1], Patricia Jeandet[2], Wolfgang C Bessler[3] and Christian Pasquali[2]*

[1]Vifor (International) AG, Rechenstrasse 37, St. Gallen, Switzerland
[2]OM Pharma SA Geneva, Rue du Bois-du-Lan 22, 1217 Meyrin, Geneva, Switzerland
[3]Institute for Molecular Medicine and Cell Research, University Clinic, Freiburg, Germany

## Abstract

**Objective:** The orally administered bacterial lysate OM-85 (Broncho-Vaxom®, Broncho-Munal®, Ommunal®, Paxoral®, Vaxoral®) is known to protect against recurrent respiratory tract infections. Despite mechanistic investigations performed during clinical and pre-clinical studies, little is known regarding the initial immune response to OM-85 following its passage through the stomach. To better understand the primary steps in the OM-85 gut-mediated immune-response cascade, we investigated its effects on two candidate cell types: intestinal epithelial cells (IECs) and Peyer's patch (PP) leukocytes and confirmed its sustained immune effect in a reconstituted gastric buffer.

**Methods:** In order to confirm OM-85's continued activity following gastric transit, THP-1 cells were stimulated with OM-85 following its incubation in reconstituted gastric buffer (pH 1.7, 8 pM Pepsin). The ability of OM-85 to stimulate IECs was tested by incubating epithelial-cell lines (Caco-2 and HT-29) and freshly isolated mouse IEC aggregates with OM-85 or standard pattern-recognition receptor (PRR) ligands (Pam$_3$CSK$_4$, LPS, flagellin, or PGN). To test the ability of OM-85 to stimulate mucosal immune cells, PP cells isolated from mouse intestine were incubated with OM-85 or PRR ligands.

**Results:** THP-1 cells released macrophage inflammatory protein-3 alpha (MIP-3α), both when OM-85 was pre-incubated in gastric buffer and when it was left untreated. In the presence of OM-85, functional PP cells freshly isolated from the intestine solely and dose-dependently released MIP-1α, a chemokine produced by myeloid cells which is involved in the recruitment and activation of various immune effector cells. Surprisingly, neither established epithelial-cell lines nor primary IECs of human or mouse origin released any of the tested cytokines in the presence of OM-85 or standard purified toll-like receptor (TLR)/nucleotide-binding oligomerisation domain (NOD)-like receptor ligands.

**Conclusion:** These data suggested that primary mucosal PPs, but not IECs, are activated by ligands from the bacterial lysate OM-85. The secretion of MIP-1α from PPs might be a trigger signal inducing tonic stimulation of mucosal tissues to prepare host immune defense towards invading pathogens.

## Introduction

Respiratory tract infections (RTIs) constitute a major healthcare burden throughout the world and are the leading cause of morbidity and mortality in children. Epidemiological data on RTIs are variable depending on the specific year and setting studied. In general, while mortality rates in children within developed countries are relatively low, over 2 million deaths occur per year in developing countries [1]. RTIs can also have severe impacts in adults, particularly in vulnerable populations, for example through acute exacerbations during chronic bronchitis or chronic obstructive pulmonary disease. Furthermore, recurrent and acute RTIs are often associated with inappropriate prescription of antibiotics, which is an important driver of antibiotic resistance [2].

The bacterial extract Broncho-Vaxom (OM-85), an orally administered bacterial lysate of 21 known respiratory pathogenic strains from eight bacterial species, was originally developed more than 30 years ago. OM-85 was designed to protect against recurrent RTIs through broad immunization against bacterial antigens, mimicking natural environmental exposure to microbes. This exposure driven prophylaxis works *via* various mechanisms that involve activation of the innate and adaptive immune system [3]. *In vivo* experimental studies have demonstrated the beneficial use of bacterial extracts and in particular, the effect of OM-85-induced prophylaxis against various

RTIs by triggering of the innate immune system and subsequent protective adaptive immune responses against these pathogens [4-7]. Cell interplay between the lymphoid and lung tissue allows orally administered immunomodulators, like OM-85, to attenuate and in some cases prevent RTIs following immune-cell migration from the gut-associated lymphoid tissue (GALT) to the lung mucosa-associated lymphoid tissue (MALT) [6,8,9].

*In vivo* and *in vitro* studies suggested that OM-85 administered orally stimulates antigen presenting cells following activation of various pattern recognition receptors (PRR) [10-12]. The results from these studies support a model in which effector cells activated by OM-85 in GALT migrate to MALT in the respiratory tract and mediate immune protection [4,10]. In spite of these important mechanistic findings, the initial triggering signal induced by OM-85 in the GALT is not fully elucidated.

The main part of the gut surface is covered with a single layer of intestinal epithelial cells (IECs) that form a barrier between host connective tissues and environmental/dietary antigens, as well as numerous commensal bacteria. Therapeutic bacterial extracts interact with this epithelial-cell barrier after passing through the stomach and recent papers have shown that Peyer's patch (PP) cells might be the initial point of interaction with the immune system [5,13]. Alternatively, IECs may respond to dangerous signals such as intact pathogens and trigger inflammatory immune responses [14]. These cells play a critical role in maintaining the gut homeostasis not only as physical barrier but also by influencing innate and adaptive immune response in GALT [15]. Hence, IECs are crucial mediators of intestinal homeostasis that enable the establishment of an immunological environment permissive to colonization by commensal bacteria.

Therefore, both IECs and PP cells represent potential targets for the initial steps of immune modulation induced by the orally administered bacterial extract OM-85. The aim of this study was to investigate which cell types in the GALT (IECs or PP cells) are activated by OM-85 and respond with the release of cytokines/chemokines.

## Materials and Methods

### Stimulation of THP-1 cells with OM-85 following incubation in reconstituted gastric buffer

Gastric buffer was freshly prepared according to pharmacopeia directions (USP 38 and Eur. Ph. 1039900). Heat pre-incubated gastric solution (pH 1.7, 8 pM Pepsin [Sigma-Aldrich, P0525000]) and OM-85 (pH 7.1) or control (Batch 1611070, OM Pharma) were mixed at a ratio of 3:2 and left for 10, 20, 40, or 60 minutes at room temperature. Following an additional incubation of 30 minutes at 37°C the reaction was stopped by neutralization with NaOH.

Subsequent cell stimulation by OM-85 was performed using THP-1 differentiated macrophages (Sigma-Aldrich, 88081201). Macrophage cell differentiation was performed in 96 well plates after seeding $1 \times 10^6$ cell/mL for 72 h in RPMI 1640 (PAA, E15-039) complete medium (L-glutamine (2 mM/L) with penicillin (100 U/mL) and streptomycin (100 µg/mL, [Sigma-Aldrich, G6784]); 10% Fetal Bovine Serum (FBS, [Pan Biotech, P40-37500]); HEPES (10 mM); 2-mercaptoethanol (50 µM, [Sigma-Aldrich, M3148]); and Sodium pyruvate (1 mM, [Sigma-Aldrich, S8636]) complemented with PMA (100 ng/mL, [Sigma-Aldrich, P1585]).

MIP-3α release and cell viability were performed in parallel to ensure that cytokine release was not induced by cell damage. THP-1 cells were incubated with gastric-buffer exposed OM-85 and positive (unexposed OM-85) and negative controls (culture medium) for 24 h. Assessment of subsequent CCL20/MIP-3α cytokine release was performed using ELISA (DuoSet ELISA R&D Systems, DY360) according to manufacturer's instructions except for sample dilution (1/30) and medium supernatant (1/20).

### Stimulation of peripheral blood mononuclear cells (PBMC) with OM-85

Healthy blood from donors was provided courtesy of the Centre de Transfusion Sanguine (CTS from Geneva University Hospital, Switzerland). PBMCs were isolated from buffy coats by Ficoll gradient (Ficoll-Paque™ Plus, GE Healthcare) as previously described [16] with the following modifications: 20 mL of whole blood was layered over 10 ml of Ficoll-Paque Plus at 1.077 g/mL (Pharmacia Biotech) and centrifuged at 1200 g for 25 minutes without centrifuge break.

Rings containing mononuclear cells from three whole blood samples were diluted to 50 mL with cell medium. Following platelet removal, freshly isolated PBMC pellets were washed twice, resuspended in complete medium (Thermo Fisher Scientific, RPMI-1640), FBS 10%, β-Mercaptoethanol (50 mM) and counted in a Neubauer chamber.

PBMC pre-activation was performed by resuspending $1 \times 10^6$ cells/well in 24 well microplates (Costar 3524) in complete medium for 30 minutes. To assess cytokine release, cells were incubated for 18 h in complete medium with increasing concentrations of OM-85 (0.008-2 mg/g), lipopolysaccharide (LPS) from *E. coli* K12 or Pam3cyc as positive control, or bovine serum albumin (BSA) as a negative control.

### Stimulation of IECs with OM-85

Immortalized human epithelial cells, primary mouse IECs, and primary human epithelial cells were used to investigate response to OM-85. The human colon adenocarcinoma cell line HT-29 (Sigma, 91072201) was cultured in McCoy's 5A (Sigma) + 2 mM Glutamine + 10% FBS. The human colon carcinoma cell line Caco-2 was cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% heat-inactivated FBS and 2 mM Glutamine. The cells were grown in 5% $CO_2$ at 37°C. For stimulation, cells were plated in 96-well flat-bottom collagen-coated plates at 25,000 cells/well (Greiner, Cell Coat) and allowed to adhere overnight, followed by 18 hours of incubation with selected ligands. Supernatants were harvested and cytokines were measured as described below.

The protocol for the isolation of primary mouse IECs was based on a protocol described by Campbell [17]. Briefly, 10-12 week old female C57BL/c mice (Janvier, France) were sacrificed. Tissue running from the duodenum to 1 cm above the cecum was excised and collected in cold isolation medium (PBS, 1 % Glucose, Penicillin/Streptomycin and 3 % FBS). The gut lumen was rinsed twice with cold isolation medium, and the intestine was opened longitudinally with sterile scissors. To avoid contamination, the intestine received four washes with 20 mL cold isolation medium in a fresh Petri dish before being cut into 1-3 cm pieces. The tissue was washed again by centrifuging with 50 ml of cold isolation medium for 3 minutes at 50 rpm and cut once more into pieces approximately 0.5 cm in length. To release the epithelial layer, intestinal tissue was incubated with 50 ml of 1 mg/ml Collagenase D (Roche Diagnostics, 11 088 882 001) and 0.3 mg/ml Dispase (Gibco, 17105-041) in DMEM containing 1% Glucose, Penicillin/Streptomycin and 3% FBS for 25 minutes at 25°C with slow shaking. The tissue pieces were then agitated by pipet for 3 minutes and any large fragments were left to settle for 1 minute under gravity. The supernatant was collected, diluted in 30 ml of DMEM, and centrifuged at 300 rpm for 3 minutes in 15 ml Falcon tubes. After discarding the supernatant the pellet containing the IECs was washed four times with DMEM without supplements before resuspending cells in 10 ml DMEM containing insulin 5 µg/ml (Sigma), heparin 50 µg/mL (Sigma), 1 % Glucose, penicillin/streptomycin and 3 % FBS. The identity of the primary mouse IECs was confirmed by light and fluorescent microscopy. For fluorescence microscopy, paraformaldehyde-fixed primary mouse epithelial cells were stained in 96-well plates with the antibodies against cytokeratine (Abcam, ab9377) and vimentin (Abcam, ab8978). Anti-rabbit IgG conjugated to Alexa488 (Thermo Fisher Scientific) was used as the secondary, reagent and nuclei were stained with Draq5 (Biostatus). The isolated primary mouse IECs were plated in 96-well collagen-coated plates (Greiner, Cell Coat) and incubated for 16 hours with selected ligands (see below) for chemokine assessment. IECs are cell aggregates and counting is not possible. The standard procedure of IECs isolation yielded a pellet resuspended in 1 ml DMEM per mouse intestine which was plated at 100 µl/well for stimulation with ligands.

Primary human IECs were provided by Lonza (CC-2931, frozen vial of 800,000 cells) and were grown in SmGM™-2 BulletKit™ (CC-3182) with supplements (CC-4149), all from Lonza. Cells were plated in 96-well flat-bottom collagen-coated plates at 10,000 cells/well (Greiner, Cell Coat) and incubated with selected ligands for 18 hours. Cytokine release was assessed in supernatants as described below.

The following ligands were used to investigate IECs responses: pro-inflammatory cytokines (IL-1β and IL-1α [R&D System], positive controls), TLR ligands (Pam$_3$CSK$_4$, LPS, flagellin from *S. typhimurium* [InVivoGen]), NOD1 and NOD2 ligands (Peptidoglycan [PGN]), OM-85, or BLANK (used as negative control). Soluble biomarkers (Table 1) released following IECs were assessed either by cytometric bead array (Table 1: TNF to IL-9) or flow cytometry/ELISA (Table 1: TSLP, CXCL5 and beta-defensin). Bulk splenocytes were harvested by passing spleen pieces through a 70 μM cell strainer. Splenocytes were plated as described for PP cells and were used to confirm the activity of LPS and PGN (not shown).

### Isolation of primary mouse PP cells

The procedure for PP-cell isolation was adapted from a protocol by Lefrançois et al. [25]. Briefly, PPs were excised from the gut under illuminated magnification glass and kept on ice in isolation medium. Excess connective tissue was removed and PPs were cut into small pieces and incubated with Collagenase D (1 mg/mL) and DNAse (40 μg/mL, Sigma) in RPMI supplemented with 5% FBS for 30 min at 37°C with agitation. Single cell suspension was obtained by passing PPs through 70 μM cell strainer (BD Biosciences). The lymphocytes were collected and washed once in cold RPMI medium (Invitrogen). PP leucocytes were separated from epithelial cells and connective tissue cells by Percoll gradient centrifugation in 50 mL Falcon tube (P1644 SIGMA, Sigma-Aldrich). The Percoll gradient was prepared as follows: 20 ml of 30% Percoll was under laid with 5 mL of 100 % Percoll (90 % Percoll + 10xPBS) using needle and syringe. PP cells were resuspended in 5 ml of PBS containing 5% FBS and were passed once gently through a 21 gauge needle. The cell suspension in 5 mL of PBS and 5% FBS was layered onto the gradient and spun down at 650 g for 30 minutes with acceleration set at minimum and the brake off. The PP leukocytes were harvested from the interface between 30 and 100% Percoll using a 10 ml needle and syringe. Cells were washed in a large volume of RPMI

containing 10% FBS and immediately plated at 25–50 × 10$^4$ cells/well for stimulation with OM-85 or ligands. Ligands used, biomarkers, and screening methods were as described above for IECs analyses (Table 1).

### ELISA, flow cytometry and cytokine bead arrays (CBA)

Commercially available kits or antibody sets were used to measure proteins in supernatants from stimulated cells, according to manufacturer instructions. Mouse defensin β1 was measured with ELISA Kit provided by CUsabio (ABIN578278). Mouse CXCL5-ENA78/LIX and human CXCL5 were measured by ELISA duo set (R&D Systems, DY443 and DY254, resp.). Mouse TSLP was detected by ELISA using the R&D Systems duo set (DY555). Human CCL20-MIP-3α (DY360) and CXCL1-GRO-α (DY275) were from R&D. The following CBA flex set beads (all from BD) were used to measure multiple cytokines/chemokines in a single aliquot of supernatant: mouse TNF-α (558299), mouse IL-10 (558300), mouse IFN-γ (558296), mouse IL-12/IL-23p40 (560151), mouse IL-6 (558301), mouse CCL3-MIP-1α (558449), mouse IL-5 (558302), mouse KC (558340), mouse CCL2-MCP-1 (558342), mouse IL-1β (560232), mouse IL-9 (558348), human CCL3-MIP-1α (558325), human CXCL8-IL-8 (558277), human IL-1β (558279).

### Statistical analysis

Unpaired t-tests were used to compare MIP-1α release induced by corresponding concentrations of either OM-85 or BLANK.

## Results

### OM-85 retains its cell activation capacity following gastric-buffer incubation

To investigate the resistance OM-85 to the digestive environment in the stomach we incubated the bacterial extract in a buffer mimicking gastric fluid before stimulation of THP-1 macrophage cell line. THP-1 cells released MIP-3α regardless of whether OM-85 was pre-incubated in gastric buffer or left untreated (Figure 1). Treatment of cells with these preparations of OM-85 did not affect cell viability (Supplementary Figure A1). These results suggest that OM-85 is resistant to gastric digestion and retains its capacity to activate cells, confirming previous data (data on file).

| Biomarker | Receptor | Expression | Function |
|---|---|---|---|
| TNFα | TNF-R1 and TNF-R2 | Macrophages | Systemic inflammation |
| IL-10 | IL-10R | Macrophages, DCs, T reg | Regulation |
| INFγ | INFγR1 and INFγR2 | NK, NKT, Th1, macrophages | Th1 response, macrophage activation |
| IL-12/IL-23p40 | IL-12R-β1 and IL-12R-β2 | DCs, macrophages | Th1 priming |
| MIP-1α | CCR1 and CCR5 | Macrophages and dendritic cells | Chemotaxis of macrophages, B cells, T cells |
| IL-6 | IL-6 R | Macrophages, DCs, B cells | Acute phase response |
| IL-5 | IL-5 R | Th2 cells | Th2 response, IgG1 switch |
| CXCL1-GRO-α (mouse KC) | CXCR2 | Macrophages, epithelial cells | Neutrophil chemoattractant |
| MCP-1 | CCR2 and CCR4 | Monocytes, macrophages, DC | Macrophages, DC, T cell recruitment |
| IL-1β | IL-1βR, IL-1βRA | DCs, Mph, epithelial cells | Inflammation |
| IL-9 | IL-9R | T cells | IgE class switch |
| β-defensin | Bacterial wall | Epithelial cells | Chemotaxis of DCs, macrophage phagocytosis |
| TSLP | TSLP R | Epithelial cells, fibroblasts | Maturation of DCs |
| CXCL5-ENA78/LIX | CXCR2 | Epithelial cells, eosinophils | Chemotaxis of neutrophils |
| CCL20-MIP-3α | CCR6 | Monocytes, macrophages, dendritic cells, epithelial cells | Chemotaxis of lymphocytes and dendritic cells towards epithelial cells |
| CXCL8-IL-8 | CXCR1 and CXCR2 | Macrophages, epithelial cells | Chemotaxis of neutrophils |

**Table 1:** List of the biomarkers screened with their associated receptor, cell-type expressing the biomarker, and function [18-24]. The biomarkers listed in the table were measured either by flow cytometry bead array or by ELISA in experimental replicates (n=2 to 4). At least two independent experiments have been performed.
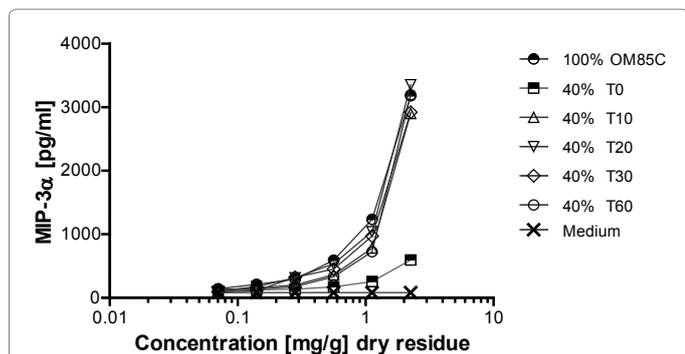
**Figure 1:** OM-85-induced MIP-3α release from THP-1 macrophages following exposure to gastric buffer for 10-60 minutes. MIP-3α was measured by ELISA. The figure shows representative data from a single experiment performed once as confirmation of the multiple previous experiments performed to test many variable conditions. To avoid misinterpretation of cytokine release from damaged cells, viability studies were systematically performed for each experimental setting and only those with >90% cell viability were considered in the results.

## OM-85-induced release of chemokines from human PBMCs

To identify potential biomarkers of cell activation we incubated human PBMCs with OM-85. The bacterial extract OM-85 induced dose-dependent release of epithelial-derived neutrophil-activating peptide 78 (ENA78), a chemokine also known as CXCL5. As expected, there were some numerical differences in response intensity between donors (Figure 2A). Incubation with OM-85, and positive controls LPS and Pam3cyc, resulted in dose-dependent release of myeloid-cell-related chemokines IL-8 (Figure 2B), GRO-α (Figure 2C), and MIP-1α (Figure 2D). These data suggested that OM-85 contains ligands able to induce activation of primary human cells and provided biomarkers for investigating the response of intestinal tissue to the bacterial extract.

## IECs are not activated by ligands from OM-85

The intestine is covered by epithelial cells that contribute to immune homeostasis by providing tolerogenic or activating signals to cells in the underlying lamina propria, PPs, or mesenteric lymph nodes. To investigate the cytokine response of cells with intestinal epithelial origin we stimulated Caco-2 and HT-29 lines with either OM-85 or blank control. In contrast to PBMCs, Caco-2 and HT-29 did not secrete ENA78/CXCL5 when stimulated with OM-85 (Figure 3A). However, incubation with recombinant IL-1β dose-dependently induced ENA78/CXCL5 release from both Caco-2 and HT-29 cell lines, demonstrating the capacity of these IECs to produce ENA78/CXCL5 (Figure 3B). In order to investigate whether Caco-2 and HT-29 express the cognate PRRs necessary to respond to activating ligands from OM-85, cells were stimulated with one of the following ligands: PGN (Nod-1 and Nod-2 ligand), $Pam_3CSK_4$-synthetic triacylated lipoprotein (TLR1/TLR2 ligand), LPS (TLR4 ligand) and flagellin (TLR5 ligand). None of the tested agonists triggered ENA78/CXCL5 release from Caco-2 or HT-29 cells (data not shown). The activity of LPS and $Pam_3CSK_4$ but not of PGN and flagellin was confirmed using bulk splenocytes (data not shown).

To test the activity of OM-85 using more physiologically relevant cells, primary IECs were freshly isolated from mouse intestinal tissue. The IEC isolation procedure yielded classical epithelial-cell aggregates, also called epithelial organoids (Figure 4). The isolated primary cells expressed cytokeratin (epithelial-cell marker) but stained negatively for the fibroblast-specific molecule vimentin. In agreement with data
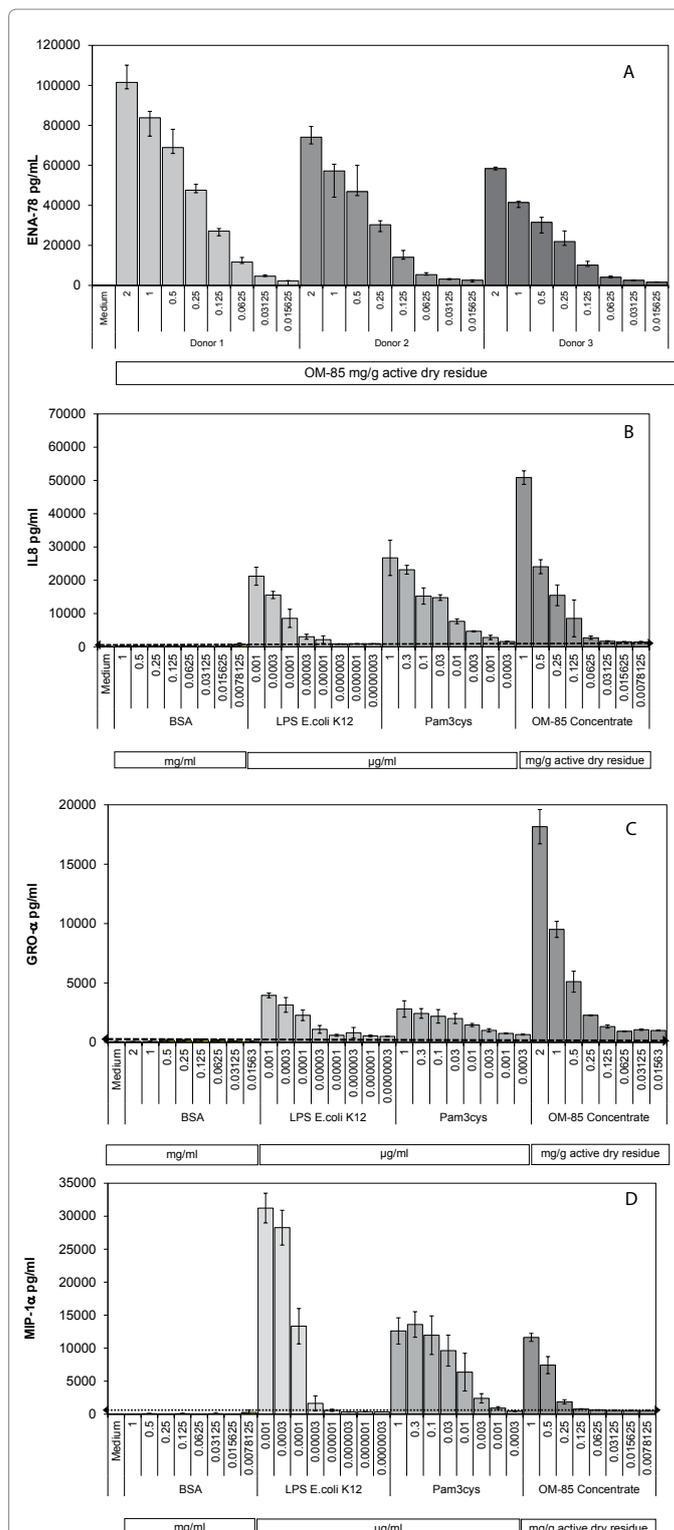


**Figure 2:** ENA78/CXCL5 release from PBMCs derived from three donors following incubation with increasing concentrations of OM-85 or control medium **(A)**, and IL-8 **(B)**, GRO-α **(C)**, and MIP-1α **(D)** release in response to incubation of PBMCs derived from a single donor with OM-85, LPS, Pam3cyc, or BSA. The biomarkers were measured by ELISA. The figure shows mean and standard deviations from experimental triplicates.

OM-85 mg/g of active dry residue correspond to the active ingredient from OM-85 liquid form following 18 hours incubation.

**Figure 3:** ENA78/CXCL5 release from CaCo-2 and HT-29 cells following incubation with OM-85 **(A)** or IL-β **(B)** ENA78/CXCL5 was measured by ELISA and data represent mean and standard deviations from experimental triplicates. The figure shows representative data from four experiments.



**Figure 4:** Light microscopic picture of primary mouse intestinal cell aggregates **(A)** and immunofluorescent staining of freshly isolated mouse intestinal cell aggregates with epithelial-cell marker (rabbit anti-cytokeratin antibody) or fibroblast cell marker (mouse anti-vimentin antibody), nuclei were counterstained with Draq5 **(B)**.
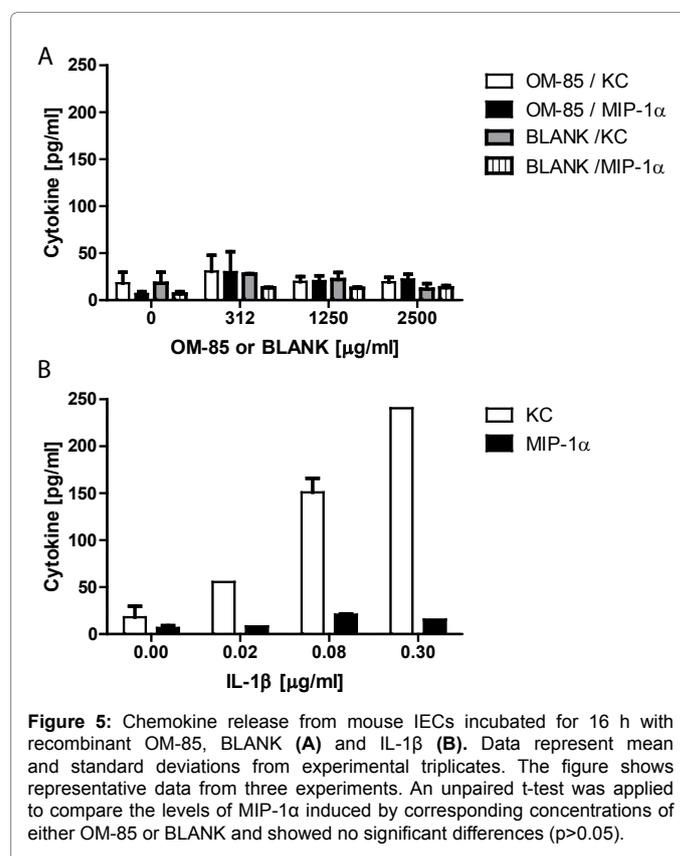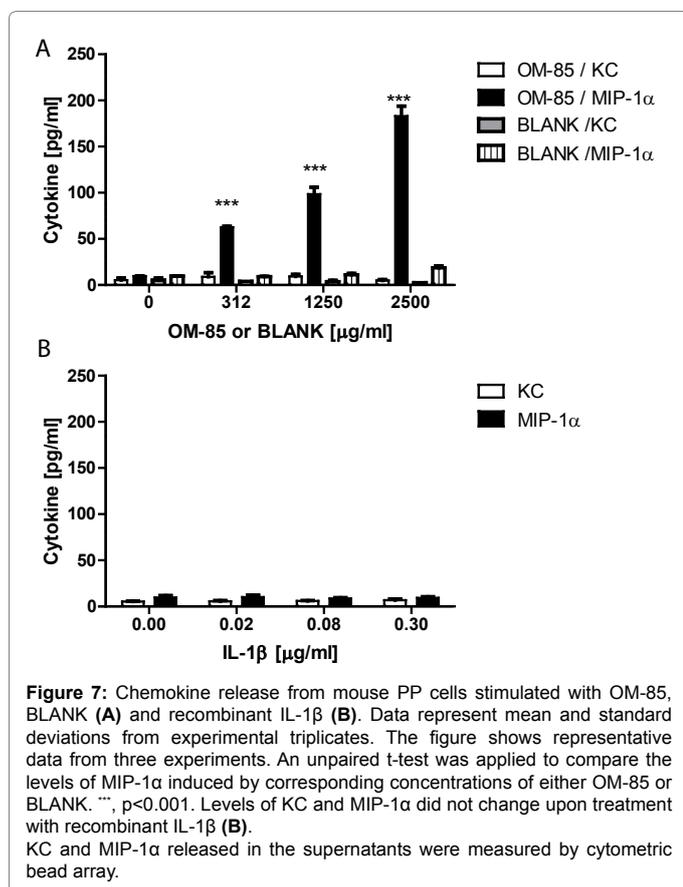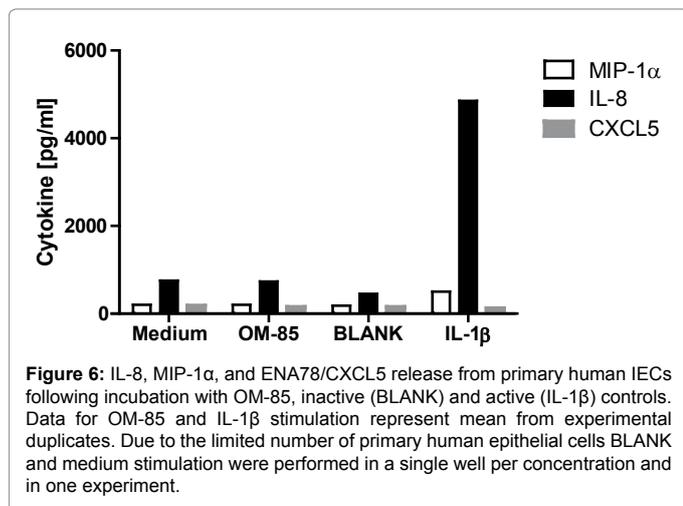
obtained using immortalized epithelial cells, OM-85 did not induce release of KC (mouse counterpart of human IL-8) from primary mouse IECs (p>0.05 OM-85 *vs.* BLANK) (Figure 5A). However, primary mouse IECs stimulated with IL-1α (not shown) and IL-1β (Figure 5B) dose-dependently responded to the stimuli as shown by the release of KC, demonstrating that IECs cells were functional during the incubation period. However, IECs secreted none of the other tested chemokines in response to OM-85, IL-1α/β, or PRR ligands (data not shown).

The fact that primary mouse IECs did not release ENA78/CXCL5 in response to IL-1β, in contrast to the results obtained with Caco-2 and HT-29 cells, may be due to species-related differences or alternatively because of inherent changes in immortalized cells. In order to address the species specificity of ENA78/CXCL5 secretion we stimulated primary human IECs. These cells did not secrete any of the 14 soluble biomarkers (Table 1) in the presence of either OM-85 (Figure 6) or standard PRR ligands (data not shown; $Pam_3CSK_4$, LPS, flagellin, or PGN). Similarly to primary mouse IECs, human primary IECs released IL-8 following incubation with IL-1α (not shown) or IL-1β but did not release ENA78/CXCL5 or MIP-1α (Figure 6). The inability of IECs to secrete KC or MIP-1α upon stimulation with OM-85 was confirmed in three independent experiments and suggested that epithelial cells do not respond to PRR ligands from OM-85.

### OM-85 activated mouse PP cells

Orally delivered OM-85 may cross the epithelial layer and interact with mucosal leucocytes from lamina propria, interepithelial lymphocytes, or PP cells. To investigate the effect of OM-85 on mucosal immune cells, PP cells isolated from mouse intestine were stimulated with OM-85, BLANK or the same collection of ligands used for IECs



**Figure 5:** Chemokine release from mouse IECs incubated for 16 h with recombinant OM-85, BLANK **(A)** and IL-1β **(B)**. Data represent mean and standard deviations from experimental triplicates. The figure shows representative data from three experiments. An unpaired t-test was applied to compare the levels of MIP-1α induced by corresponding concentrations of either OM-85 or BLANK and showed no significant differences (p>0.05).

**Figure 6:** IL-8, MIP-1α, and ENA78/CXCL5 release from primary human IECs following incubation with OM-85, inactive (BLANK) and active (IL-1β) controls. Data for OM-85 and IL-1β stimulation represent mean from experimental duplicates. Due to the limited number of primary human epithelial cells BLANK and medium stimulation were performed in a single well per concentration and in one experiment.



**Figure 7:** Chemokine release from mouse PP cells stimulated with OM-85, BLANK **(A)** and recombinant IL-1β **(B)**. Data represent mean and standard deviations from experimental triplicates. The figure shows representative data from three experiments. An unpaired t-test was applied to compare the levels of MIP-1α induced by corresponding concentrations of either OM-85 or BLANK. ***, p<0.001. Levels of KC and MIP-1α did not change upon treatment with recombinant IL-1β **(B)**.
KC and MIP-1α released in the supernatants were measured by cytometric bead array.

## Discussion and Conclusion

In the present study, we demonstrated that OM-85 remained active following exposure to conditions mimicking transit through the gastrointestinal tract. In addition, we investigated the cytokine and chemokine response of epithelial and PP cells to OM-85 in an attempt to elucidate the primary cellular target(s) of the orally delivered bacterial extract. Surprisingly, our data demonstrated that only PP cells and not IECs are activated by the constituents of OM-85. In addition, IECs did not respond to any of a collection of pathogen-associated molecular patterns (PAMPs) which were tested.

The assumption that orally administered OM-85 retains the capacity to stimulate cells from the intestine after passage through the stomach was confirmed using THP-1 macrophages in a validated quality assurance bioassay. To mimic biochemical gastric digestion, we used a reconstituted gastric buffer containing pepsin. This enzyme is present in the stomach and preferentially hydrolyses proteins in acidic conditions. The resistance of the bacterial extract to gastric buffer supports numerous studies showing evidence of immune modulation of the respiratory tract following oral administration of OM-85 [4,6,16,26-29].

Relatively high concentrations of OM-85 were used to induce secretion of the biomarkers identified in this study. The reason might be that OM-85 is a complex mixture of twenty one bacterial strains with multiple active pharmaceutical ingredients (APIs) activating various receptors expressed on different cell types. It is therefore plausible, that the concentration of the individual ligand(s) activating the cell types tested in this study is low, which imposes using high concentration of bacterial lysates *in vitro*. The lower efficacious doses used *in vivo* might reflect the crosstalk of various cell types in the body activated by multiple APIs in OM-85.

Chemokines play a central role in initiating immune responses by attracting and activating effector cells. Based on studies with human PBMCs, we selected several chemokines as potential biomarkers of OM-85-mediated cell activation. Among these the CXC type chemokine ENA78 (CXCL5) is preferentially expressed in intestinal epithelium, while IL-8, GRO-α and the CC chemokine MIP-1α are also secreted by myeloid cells [16,30-33]. Human IECs express an array of CXC chemokines including but not limited to IL-8, GRO-α, ENA78, and, to a lesser extent, CC chemokines, such as MIP-1α. These chemokines are chemotactic for polymorphonuclear leukocytes, monocytes, and subsets of T lymphocytes [34]. Accordingly, gut pathogens activate IECs which release ENA78, IL-8, GRO-α, and recruit neutrophils into the intestine [35,36]. We therefore considered these four chemokines as potential surrogate markers induced by OM-85 from both myeloid cells and IECs.

OM-85 and a collection of PRR ligands did not induce ENA78 secretion following stimulation of immortalized or primary IECs tested in this study. However, Caco-2 and HT-29 released ENA78 following stimulation with IL-1β. Previous data have shown that IECs are able to respond to IL-1β at concentrations known to occur in the inflamed mucosa [37]. The sole stimuli that triggered release of the neutrophil attracting chemokine IL-8 from Caco-2 from primary human IECs was recombinant human IL-1β. These data indicated that human IECs do not respond directly to the investigated PRR ligands but need to receive a signal from another cell type which is activated by the bacterial ligands and produce IL-1β in response to them. In agreement with the data obtained using human IECs, OM-85 did not induce release of any of the tested biomarkers from primary mouse IECs. Stimulation of primary

activation studies, and release of the same biomarkers (Table 1) was assessed. The cell composition of PP cells corresponded to the published data, with B cells being the large majority (~70%), T cells (~25%), macrophages and DC ~5% (data not shown). PP cells incubated with OM-85 but not with IL-1β, or BLANK dose-dependently produced MIP-1α (p<0.0001 OM-85 *vs*. BLANK) (Figure 7). In a control experiment OM-85 also induced release of MIP-1α from mouse bulk splenocytes, thus confirming the activity of OM-85 on primary leucocytes (data not shown). These data suggested that primary mucosal leucocytes from PP but not intestinal epithelial cells are activated by OM-85 and release the chemokine MIP-1α.

mouse IEC with recombinant mouse IL-1β induced dose-dependently the neutrophil attracting chemokine KC, a mouse orthologue of IL-8, thus confirming the findings with human IECs.

The finding of a lack of IEC response against pathogen-derived molecules may be a protective mechanism to avoid excessive inflammation induced by commensal microorganisms, thereby aiding the maintenance of intestinal homeostasis [38-40]. Indeed, IECs are recognized as central mediators of immune homeostasis in the intestine by maintaining a commensal bacteria permissive environment while responding to excessive inflammation induced by pathogens [41].

More importantly, OM-85 induced dose-dependent secretion of the macrophage inflammatory protein-1 alpha (MIP-1α or CCL3) from mouse PPs, demonstrating the effect of this bacterial extract on primary mucosal immune cells. PPs are considered to be the immune sensors of the intestine [42] and orally acquired antigens are continuously sampled by PP-resident cells. Accordingly, orally delivered radioactively labeled glycoprotein extract from *Klebsiella pneumoniae* has been found largely distributed in rat's PP [43].

MIP-1α is produced by myeloid cells and is known to attract monocytes/macrophages, T and B cells as well as NK cells through interaction with CCR1 or CCR5. Further experiments are needed to identify the cell type(s) secreting MIP-1α following stimulation with OM-85. Based on published data, it is very likely that antigen presenting cells, such as macrophages and dendritic cells from PPs recognize antigens from OM-85 and release MIP-1α. Accordingly, MIP-1α has been detected in supernatants from OM-85-activated human monocyte-derived dendritic cells [16], confirming the myeloid origin of the cells secreting this chemokine in response to bacterial lysates. Interestingly, published data showed that children with cow's milk protein allergy have decreased concentrations of MIP-1α and MCP-1 in sera [44]. The serum concentrations of MCP-1 and MIP-1α increased significantly after antigen specific desensitization therapy, which could reflect an ongoing subclinical response to food substances and differential effector cell recruitment in response to specific immunotherapy. The capacity of OM-85 to induce MIP-1α secretion from PP cells indicates that PP-resident leucocytes might be the primary target cells that respond to local bacterial ligands and initiate distal mucosal cell activation, resulting in the clinically established beneficial immune modulation of OM-85.

The data from this study suggest that bacterial lysates stimulate gut lymphoid tissues but not IECs. The unresponsiveness of IECs to OM-85 antigens is consistent with the clinically proven gastro-intestinal tolerability of the product. The initial recognition of the bacterial antigens appears to take place in the gut associated lymphoid tissues, where professional antigen presenting cells fully armed with pattern recognition receptors can mount an appropriate immune response. Further investigation using appropriate animal models are needed to address the physiological importance of MIP-1α release for the protective immune response triggered by OM-85, as well as to identify the PP cells and receptors involved.

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## Disclosure of Potential Conflicts of Interest

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## Informed Consent

Informed consent was obtained from all individual participants included in the study.

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