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Fish-derived low molecular weight components modify bronchial epithelial barrier properties and release of pro-inflammatory cytokines

Tanja Kalic[a], Isabella Ellinger[a], Sandip D. Kamath[b], Chiara Palladino[a], Vanessa Mayr[a], Angelika Tscheppe[a], Thimo Ruethers[b], Eva E. Waltl[c], Verena Niederberger[c], Nina Lengger[a], Christian Radauer[a], Christine Hafner[d,e], Andreas L. Lopata[b], Merima Bublin[a], Heimo Breiteneder[a,*]

[a] Institute of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria
[b] College of Public Health, Medical and Veterinary Sciences, Australian Institute of Tropical Health and Medicine, Molecular Allergy Research Laboratory, James Cook University, Townsville, Queensland, Australia
[c] Department of Otorhinolaryngology, Medical University of Vienna, Vienna, Austria
[d] Department of Dermatology, University Hospital St. Pölten, Karl Landsteiner University of Health Sciences, St. Pölten, Austria
[e] Karl Landsteiner Institute for Dermatological Research, St Pölten, Austria

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ABSTRACT

The prevalence of fish allergy among fish-processing workers is higher than in the general population, possibly due to sensitization via inhalation and higher exposure. However, the response of the bronchial epithelium to fish allergens has never been explored. Parvalbumins (PVs) from bony fish are major sensitizers in fish allergy, while cartilaginous fish and their PVs are considered less allergenic. Increasing evidence demonstrates that components other than proteins from the allergen source, such as low molecular weight components smaller than 3 kDa (LMC) from pollen, may act as adjuvants during allergic sensitization.

We investigated the response of bronchial epithelial cells to PVs and to LMC from Atlantic cod, a bony fish, and gummy shark, a cartilaginous fish. Polarized monolayers of the bronchial epithelial cell line 16HBE14o- were stimulated apically with fish PVs and/-or the corresponding fish LMC. Barrier integrity, transport of PVs across the monolayers and release of mediators were monitored.

Intact PVs from both the bony and the cartilaginous fish were rapidly internalized by the cells and transported to the basolateral side of the monolayers. The PVs did not disrupt the epithelial barrier integrity nor did they modify the release of proinflammatory cytokines. In contrast, LMC from both fish species modified the physical and immunological properties of the epithelial barrier and the responses differed between bony and cartilaginous fish. While the barrier integrity was lowered by cod LMC 24 h after cell stimulation, it was increased by up to 2.3-fold by shark LMC. Furthermore, LMC from both fish species increased basolateral and apical release of IL-6 and IL-8, while CCL2 release was increased by cod but not by shark LMC.

In summary, our study demonstrated the rapid transport of PVs across the epithelium which may result in their availability to antigen presenting cells required for allergic sensitization. Moreover, different cell responses to LMC derived from bony versus cartilaginous fish were observed, which may play a role in different allergenic potentials of these two fish classes.

1. Introduction

Fish allergy is a potentially life-threatening disease. Its prevalence has increased worldwide over the past decade, most likely due to increased fish processing, trade and consumption (Sharp and Lopata, 2014). The current prevalence of fish allergy worldwide ranges from

Abbreviations: BSA, bovine serum albumin; CD, circular dichroism; CBB, Coomassie brilliant blue; DPBS, Dulbecco's phosphate-buffered saline; FBS, fetal bovine serum; FD10, FITC-Dextran 10; LAMP2, lysosome-associated membrane protein 2; LMC, low molecular weight components; PV, parvalbumin; SDS, sodium dodecyl sulfate; TBST, Tris-buffered saline with Tween 20; TEER, trans-epithelial electrical resistance

* Corresponding author at: Division of Medical Biotechnology, Institute of Pathophysiology and Allergy Research, Medical University of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria.
E-mail address: heimo.breiteneder@muv.ac.at (H. Breiteneder).

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0.2% to 3% in the general population, whereas in fish-processing workers it is much higher and reaches up to 8% (Sharp and Lopata, 2014; Tjalvin et al., 2018). The major fish allergens are parvalbumins (PVs) which are proteins of 11–13 kDa and belong to the EF-hand protein superfamily (Arif, 2009). Several PV isoforms can be present in the muscle tissue of a fish (Saptarshi et al., 2014). Fish PVs belong to one of two evolutionary lineages, alpha or beta. Beta PVs are abundant in bony fish and are the primary sensitizers in 75–95% of patients with fish allergy, whereas alpha PVs are abundant in cartilaginous fish and higher vertebrates, and are considered less allergenic (Kuehn et al., 2014; Ruethers et al., 2018b).

The mechanisms underlying allergic sensitization to fish are not well understood. Allergic sensitization and reactions may occur due to exposure to fish allergens via ingestion. However, in an occupational setting, inhalation of or skin contact with fish allergens may contribute to the higher prevalence of fish allergy (James and Crespo, 2007; Jeebhay and Lopata, 2012). In allergic sensitization via inhalation, airway epithelia are exposed to air-borne fish allergens released from fish during food processing (Jeebhay et al., 2005). It has been estimated that the amount of fish allergens present in aerosols ranges from 0.001 to 5 μg/m^3 (Jeebhay et al., 2001). Currently, it is not known why there is such a high prevalence of allergic sensitization to fish among fish-processing workers (exposed to fish allergens mainly by inhalation and skin contact) as compared to ingestion-induced fish allergy. Food processing and different routes of exposure may impact the sensitizing capacity, as well as the number of retained IgE epitopes of the allergenic food proteins (Bogh and Madsen, 2016; Kuehn and Hilger, 2015).

To be able to induce allergic sensitization, allergens need to cross epithelial barriers in an immunologically active state before being presented to the immune system. Airway epithelial cells do not just represent a passive barrier stopping allergens from penetration into the body, but they initiate an innate response by secretion of mediators needed for sensitization (Lambrecht and Hammad, 2014). However, the mechanisms of interaction between fish PVs and airway epithelial cells have not been explored. Increasing evidence points towards a role of the food matrix, which contains lipids and other low molecular weight components (LMC), as potential adjuvants during allergic sensitization (Blume et al., 2015; Palladino et al., 2018). LMC (< 3 kDa) from pollen increased the barrier integrity of bronchial epithelial cells and induced production of proinflammatory cytokines by these cells (Blume et al., 2015). In addition, LMC enhanced the allergen-specific immune response *in vivo* (Gilles-Stein et al., 2016).

We sought to understand the mechanisms of the allergic sensitization to fish via inhalation. We thus explored the interaction of two fish PVs with 16HBE14o- cells, a widely used model for investigating the barrier function of bronchial epithelium (Georas and Rezaee, 2014). The PVs used in this study were isolated from Atlantic cod (*Gadus morhua*), a highly allergenic bony fish, and from gummy shark (*Mustelus antarcticus*), a cartilaginous fish. We hypothesized that the LMC present in the fish-derived organic matter might play a role in the immune response leading to allergic sensitization via inhalation. Thus, we investigated the epithelial barrier integrity, the transport of the PVs across the cell monolayers and the release of mediators upon exposure to PVs as well as LMC from both bony and cartilaginous fish. For the first time, we demonstrated that fish PVs are internalized by bronchial epithelial cells and rapidly transported across the tight epithelial monolayers in an intact and immunologically active form. Furthermore, fish-derived LMC modified the physical barrier properties and the release of pro-inflammatory cytokines in a fish-source dependent pattern. Our data suggest a role of fish LMC as possible adjuvants in allergic sensitization to fish via inhalation.

2. Methods

2.1. Parvalbumins and low molecular weight components (LMC) from fish

Natural beta-PV from Atlantic cod (*Gadus morhua*) and alpha-PV from gummy shark (*Mustelus antarcticus*) were purified from extracts of muscle tissue of the respective fish species according to the previously described protocols (Griesmeier et al., 2010; Ruethers et al., 2018a). Purified PVs were visualized on 15% SDS-PAGE gels by Coomassie brilliant blue (CBB) staining (Supplementary Fig. S1A). Protein identity was confirmed by Western blotting using anti-beta PV antibody (Swant, Switzerland, cat. number 235) and anti-alpha PV antibody (Abcam, UK, cat. number ab11427) (Supplementary Fig. S1B). Presence of the alpha helical secondary structure in the purified PVs was determined by circular dichroism (CD) spectroscopy using a Jasco J-810 spectropolarimeter (Jasco International Co., Hachioji, Tokyo). The spectra were measured from 190 to 250 nm at 25 °C using a 1 mm path length quartz cell and represent the average of 5 accumulations (Supplementary Fig. S1C). The concentration of the PVs was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). MALDI-TOF mass spectrometry was used for mass determination of intact parvalbumins. Proteins were mixed with matrix (α-cyano-4-hydroxycinnamic acid) in 1:1 ratio and spotted onto a ground steel target plate. The mass was measured using a MALDI-TOF mass spectrometer (Microflex, Bruker Daltonics, Bremen, Germany). For each parvalbumin, two isoforms were detected (data not shown). Endotoxins were removed from the purified protein preparations using MACS endotoxin removal beads (Miltenyi Biotec, Germany). The absence of measurable endotoxins was demonstrated using the EndoZyme recombinant Factor C endotoxin detection assay (Hyglos GmbH, Germany) according to the protocols recommended by the manufacturer. The endotoxin levels in the PV preparations were below the detection limit of the assay (0.005 EU/mL).

For the preparation of fish LMC, muscle tissues of Atlantic cod or gummy shark were cut into pieces of 1 cm^3, frozen in liquid nitrogen and ground to a fine powder. Samples were resuspended in 3 volumes of minimum essential medium (MEM) without supplements (Gibco, cat. number 11095080), extracted by gentle shaking for 3 h at 4 °C and centrifuged (17,000 × *g*, 4 °C, 45 min). The supernatant was collected and sterile filtered. To obtain the allergen-free LMC, extracts were ultrafiltrated using 3 kDa cut-off filters (Merck Millipore, Germany). Absence of low molecular weight peptides in LMC which might bind IgE was demonstrated by CBB staining of a 16.5% Tris-Tricine gel (Bio-Rad, cat. number 456–3064) (Supplementary Fig. S2A). Furthermore, a dot blot using serum of a patient with sensitization to cod PV was performed with cod LMC. Cod PV (positive control), cod LMC or bovine serum albumin (BSA, negative control) were spotted onto a nitrocellulose membrane. The membrane was blocked using 3% BSA (Carl Roth GmbH, Germany) in Tris-buffered saline with Tween 20 (TBST) and incubated with the serum of the patient 1 diluted 1:10 in TBST with 1% BSA. Detection of the IgE bound to the membrane was performed using an alkaline phosphatase-conjugated mouse anti-human IgE monoclonal antibody (BD Biosciences, cat. number 555859) and the BCIP/NBT substrate system (BCIP, 5-Brom-4-chlor-3-indoxylphosphat, Biomol; NBT, Nitroblue Tetrazolium chloride, Biomol, Austria). No IgE-binding to the LMC was detected, confirming the absence of allergen degradation products in LMC preparations (Supplementary Fig. S2B).

2.2. Bronchial epithelial cell culture

The human bronchial epithelial cell line 16HBE14o- was used as a model for bronchial epithelium. Cells were cultured as previously described (Waltl et al., 2018). Briefly, cells were cultured in collagen-fibronectin coated tissue culture flasks in MEM supplemented with 10% fetal bovine serum (FBS) (Gibco, 10270-106), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C in a humidified

atmosphere containing 5% CO_2. Cells were passaged when they reached 80–90% confluence. All experiments were performed with cells within four passages.

If not indicated otherwise, cells were seeded on permeable filter supports (0.4 µm, Corning, 3460) at a density of 4×10^5 cells per mL in MEM supplemented with FBS, penicillin and streptomycin as described above. Formation of polarized monolayers was monitored by measurement of the transepithelial electrical resistance (TEER) as well as the permeability for the tracer FITC-dextran (FD10; Sigma-Aldrich). TEER was determined using an EVOM2 Epithelial Voltohmmeter (World Precision Instruments, Sarasota, Florida). Permeability of monolayers for FD10 was measured 24 h, 36 h and 48 h after seeding. FD10 solution (3 mg/mL) in MEM supplemented with 1.5% FBS was added apically to the cells for 4 h at 37 °C. Basolateral medium was collected, and the fluorescence measured using the Spark multimode microplate reader (Tecan, Switzerland). The excitation and emission wavelengths were 490 nm and 520 nm, respectively. The amount of transported FD10 was calculated from a standard curve.

All subsequent experiments were performed only after the formation of polarized monolayers with a TEER exceeding 1500 $\Omega \cdot cm^2$ and a permeability for FD10 of less than 1% compared to the empty transwell filters.

2.3. Patients' sera

Serum samples from three patients with clinical histories of IgE-mediated type 1 fish allergy were used. Sensitization to fish was verified by determination of fish-specific IgE using ImmunoCAP (Thermo-Fisher Diagnostics, Vienna, Austria). Additionally, levels of PV-specific IgE were determined for each patient. For patients 1 and 2, levels of IgE specific to Cyp c 1 (parvalbumin from European carp) were determined by radioallergosorbent testing and were 15.8 kU/L and 1.4 kU/L, respectively. For patient 3, the level of IgE to Gad c 1 (parvalbumin from Baltic cod) was measured by ImmunoCAP ISAC and was 19.7 ISAC standardized units.

The study was approved by the Ethics Committee of Lower Austria (GS4-EK-4/242–2013).

2.4. Detection and quantification of transepithelial parvalbumin transport

Cod and shark PVs (1 mg/mL) were labelled with Alexa Fluor 488 5-TFP (Alexa Fluor 488 Carboxylic Acid, 2,3,5,6-Tetrafluorophenyl Ester), 5-isomer (Thermo Fisher Scientific) using the protocol recommended by the manufacturer. Labelled proteins were extensively dialyzed against Dulbecco's Phosphate-Buffered Saline (DPBS; Gibco, Thermo Fisher Scientific) and sterile filtered. Labelled proteins were visualized by CBB staining of 15% SDS-PAGE gels, and by fluorescence detection after blotting to a nitrocellulose membrane using a VersaDoc Imaging System (Bio-Rad Laboratories, Hercules, CA, USA).

Polarized cell monolayers were treated apically with fluorescence-labelled PVs (80 µg/mL or 160 µg/mL, 350 µl per well) diluted in MEM supplemented with 1.5% FBS at 37 °C. The volume of basolateral medium was 1.5 mL per well. Samples of basolateral medium were collected after 1 h and 24 h and subjected to SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, and fluorescence was detected using a VersaDoc Imaging System. To quantify the amounts of PVs transported to the basolateral side, aliquots of the supernatants were taken, and the fluorescence intensity was measured by a Spark multimode microplate reader (Tecan, Switzerland). The concentration of the PVs was calculated from a standard curve using a third-degree polynomial curve fitting. The experiments were performed in triplicates.

To explore whether the PVs transported across the epithelial monolayers were able to bind patients' IgE, monolayers were treated apically with cod PV (160 µg/mL) for 24 h, the basolateral cell culture medium was collected, separated by 15% SDS-PAGE and proteins were transferred to nitrocellulose membrane. The membrane was blocked using 5% BSA in TBST and incubated with sera of three fish-allergic patients diluted 1:10 in TBST with 1% BSA. Detection of bound IgE was performed using an alkaline phosphatase-conjugated mouse anti-human IgE monoclonal antibody (BD Biosciences, cat. number 555859) and the BCIP/NBT substrate system.

2.5. Evaluation of the barrier integrity upon stimulation with PVs and LMC

Confluent monolayers were stimulated apically with the purified fish PVs (80 µg/mL or 160 µg/mL) or with fish LMC diluted in MEM supplemented with 1.5% FBS. MEM to LMC ratios were as follows: Dose I – 1:6, Dose II – 1:3, Dose III – 1:2. In control wells, only MEM containing 1.5% FBS was used (untreated cells). The calcium chelator EGTA (5 mM) was used as a control to increase paracellular permeability and decrease TEER (Tomita et al., 1996). TEER was measured at several time points up to 24 h after stimulation. Data represent results of three independent experiments, each performed in duplicate. Furthermore, the permeability of the monolayers for FD10 and the PVs after 24 h of stimulation with fish LMC was measured as indicated above.

2.6. Cell viability analysis

Cell viability after apical exposure to different stimuli was assessed by an MTT assay. Cells were cultured for 55 h (average time needed to form tight, polarized monolayers as determined by TEER measurements in separate experiments) in 96-well plates coated with collagen and fibronectin as described before (Gangl et al., 2015). Confluent cell monolayers were stimulated for 24 h with PVs, fish LMC, or 1% SDS (sodium dodecyl sulfate) as a control for cell toxicity. The supernatant was removed and replaced by thiazolyl blue tetrazolium bromide (MTT) dissolved at 5 mg/ml in Tyrode's salt solution with 0.1% BSA. After incubation for 45 min at 37 °C, the supernatant was removed, and the MTT formazan was solubilized by adding a 1:6 solution of 10 mM glycine in DMSO (dimethyl sulfoxide). Absorbance at 565 nm was measured and the percentage of viable cells was calculated using untreated cells as a reference.

2.7. Confocal microscopy

Cells were seeded (3.4×10^5 cells/mL) on sterilized round 12 mm coverslips coated with collagen and fibronectin and grown for 55 h. Monolayers were washed with DPBS and incubated in serum-free MEM for 30 min at 37 °C to remove serum components from cellular receptors. Alexa Fluor 488-labelled PVs (100 µg/mL) were added and cells were incubated for 1 h at 37 °C. Cells were washed twice with cold DPBS and fixed using 4% formaldehyde solution in DPBS. After washing, the cells were blocked and permeabilized (0.05% saponin, 1% BSA in DPBS) for 1 h at room temperature. Monolayers were incubated with a primary antibody against the late endosome/lysosome marker lysosomal-associated membrane protein-2 (LAMP 2; BD555803, BD Biosciences) diluted 1:50 in blocking buffer, followed by washing with DPBS and incubation with Alexa Fluor 568-conjugated secondary antibody (A-11004, Thermo Fisher Scientific), diluted 1:2000 in blocking buffer). Both antibody incubations were performed for 1 h at room temperature. Nuclei were stained with DRAQ5 (Abcam, UK) and coverslips were mounted onto microscope glass slides using Fluoromount-G (Southern Biotech, Birmingham, AL, USA). Intracellular localization of PVs and cellular markers was explored by confocal microscopy, using an UltraVIEW ERS Rapid Confocal Imager (PerkinElmer, Waltham, MA, USA) connected to a Zeiss Axiovert 200 microscope fitted with a 63x/1.4 oil objective lens (Plan-Apochromat, Zeiss). Pictures were digitalized and processed by the Volocity software (Version 6.1 PerkinElmer).

2.8. Quantification of cytokine release

Concentrations of cytokines released to apical and basolateral cell culture supernatants upon stimulation with fish PVs or LMC were measured 24 h after apical cell stimulation. Quantification of the cytokines was performed using the xMAP Technology reagents and the Luminex 100 System supported by the xPONENT software, according to the protocols recommended by the manufacturer (Merck Millipore, Massachusetts, USA). IL-6, IL-8 and CCL2 were measured using the HCYTOMAG-60K kit. IL-25 and IL-33 were measured by the HTH17MAG-14K kit, and TSLP by the HCYP2MAG-62K kit (all three kits from Merck Millipore). Stimulation of the cells with TNFα (50 ng/mL) and IL-1β (20 ng/mL) was used as a positive control for release of proinflammatory cytokines (Ozaki et al., 1996; Tanaka et al., 2014; Tobe et al., 2002). Cytokines were measured in supernatants derived from 3 independent experiments performed in duplicates. The selection of the cytokines was made based on the published literature on the importance of these cytokines in epithelial cell damage response and allergic sensitization (Chow et al., 2010; Kosaka et al., 2011; Lee et al., 2015; Yi et al., 2017).

2.9. Statistical analysis

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Significant differences in TEER and cytokine release upon treatments with PVs and LMC compared to untreated cells were determined by the Friedman test with Dunn's post-test. Significant differences in permeability for FITC-Dextran of monolayers stimulated with cod LMC, shark LMC or without stimulation, as well as differences between permeability for PVs in presence or absence of LMC were determined by One-way ANOVA, followed Tukey's and Sidak's test, respectively. P values below 0.05 were regarded as significant (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

3. Results

3.1. Fish PVs cross the bronchial epithelial monolayers and remain intact

PVs from Atlantic cod, a bony fish, and gummy shark, a cartilaginous fish, were fluorescence-labelled using Alexa Fluor 488 (Fig. 1A). To explore whether the PVs have the capability to cross the epithelial barrier, 16HBE14o- bronchial epithelial cells were cultured on permeable filter supports. PVs were added to the apical compartment of polarized cell monolayers, and the basolateral cell culture medium was collected after 24 h. Labelled PVs of the expected molecular weight (11–13 kDa) were detected in the medium (Fig. 1B). Furthermore, the PVs transported across the epithelial monolayers into the basolateral medium were quantified after 1 h and 24 h of stimulation (Fig. 1C). Already after 1 h of apical exposure to the PVs, we were able to detect PVs transported to basolateral cell culture medium (Fig. 1C). After 24 h of apical stimulation with 80 μg/mL of cod or shark PV, the basolateral PV concentration was ˜200 ng/mL, while the stimulation with 160 μg/mL resulted in basolateral PV concentration of around 400 ng/mL (Fig. 1C). Thus, when the volumes of apical and basolateral media were taken into account, the calculated amount of transported PVs was approximately 1%. The amount of the transported PVs was concentration-dependent and no significant difference in the amount of cod or shark PV transported to basolateral medium was observed. Importantly, the PVs which crossed the epithelial barrier remained intact and were able to bind patients' IgE, as demonstrated for cod PV by IgE immunoblots using sera of three fish-allergic individuals sensitized to cod (Fig. 1D).

3.2. PVs do not interfere with physical barrier integrity and viability of bronchial epithelial cells

Transepithelial transport is the result of transcellular as well as paracellular pathways. Paracellular transport of molecules is controlled by tight junctions. To test whether the PVs interfered with the barrier properties of 16HBE14o- cells, the TEER of the monolayers exposed apically to two different concentrations of the PVs was monitored. Although a higher concentration (160 μg/mL) of both PVs induced an initial moderate reduction of TEER (5–10%), the barrier fully recovered after 16 h of treatment and this effect was not statistically significant (Fig. 2A and B). Interestingly, no difference was observed between the influence of cod PV (highly allergenic) or shark PV (considered to be less allergenic) on the physical barrier integrity (Fig. 2A). The calcium chelator EGTA, used as a control for increase of paracellular permeability, drastically reduced TEER (Fig. 2C). Furthermore, the PVs did not affect cell viability (Fig. 2D). Thus, fish PVs do not change the paracellular permeability of 16HBE14o- cell monolayers.

3.3. PVs are rapidly taken up by bronchial epithelial cells

Transcellular transport of proteins occurs by transcytosis, which is a unique endocytic route of polarized cells (Garcia-Castillo et al., 2017). To explore whether the PVs were internalized by the cells, cell monolayers were exposed apically to fluorescently labelled PVs and incubated at 37 °C. After one hour of exposure, the cells were fixed and analyzed by confocal microscopy. Both cod and shark PV were internalized by the cells and localized in a perinuclear area (Fig. 3). The PVs did not colocalize with LAMP2, a marker of late endosomes/lysosomes, indicating that the majority of PVs did not enter the degradative pathway (Fig. 3). There was no apparent difference in internalization patterns between cod and shark PVs (Fig. 3).

3.4. PVs do not influence the release of proinflammatory cytokines

To explore the possible influence of PVs on the release of mediators from the cells, aliquots of basolateral and apical media were taken after 24 h of apical stimulation of the cells with two different concentrations of PVs. None of the PVs induced significant changes in release of IL-6, IL-8 and CCL2, neither from the basolateral nor the apical side (Fig. 4A and B). TSLP, IL-25, IL-33, and IL-10 were below the detection limit of the assay in all samples (data not shown). Stimulation with TNFα and IL-1β as a positive control increased basolateral and apical release of IL-6, IL-8 and CCL-2 to 180–250% compared to untreated cells (100%) (Supplementary Fig. S3).

3.5. LMC from fish modify the physical barrier properties

As we did not observe an effect of PVs on the physical and immunological properties of the epithelial barrier, we next explored the influence of LMCs from bony and cartilaginous fish on 16HBE14o- cell monolayers. Apical stimulation with the LMC from both bony (cod) and cartilaginous fish (shark) resulted in an initial significant increase in TEER which was dose-dependent and persisted for both fish species for up to 8 h (Fig. 5A). Thereafter, monolayers treated with cod LMC demonstrated a significant drop in TEER to values below those of untreated monolayers (Fig. 5A). Interestingly, shark LMC showed the opposite effect from cod after a prolonged exposure, by continuously increasing the resistance of the monolayers for up to 24 h (Fig. 5A).

To investigate whether these changes in TEER reflected alterations of the physical barrier integrity, the permeability of the monolayers for FD10 was determined. The permeability for FD10 was significantly higher ($p < 0.05$) for monolayers treated with cod LMC (Fig. 5B), which was in line with the decreased resistance of these monolayers after 24 h. Also in line with the results from the TEER measurements, shark LMC significantly reduced ($p < 0.01$) the permeability for FD10 in 16HBE14o- cell monolayers (Fig. 5B). As expected, the calcium chelator EGTA drastically increased permeability for FD10 which was 10-fold higher compared to untreated monolayers (data not shown).

To elucidate whether the exposure to LMC interferes with the

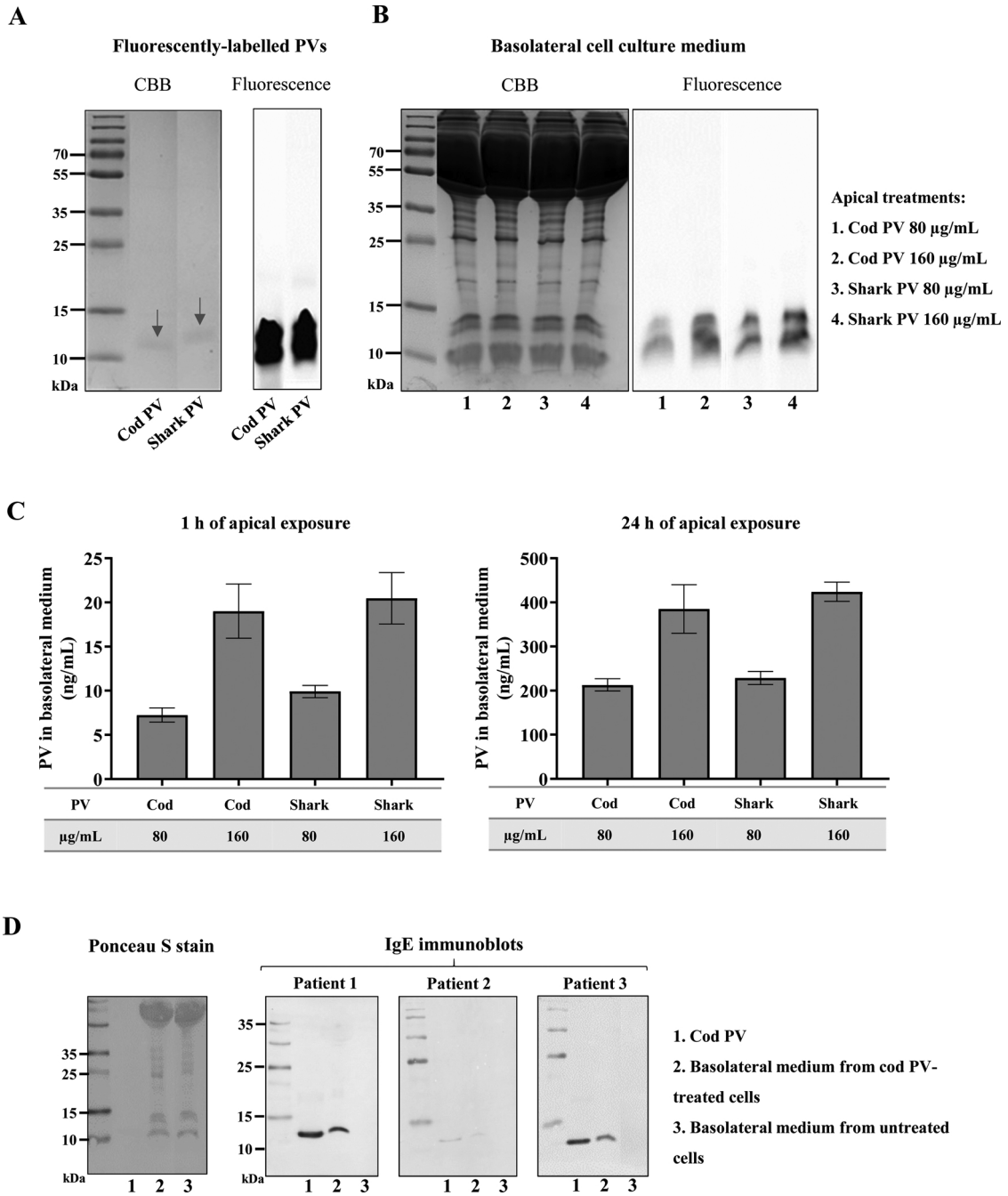

Fig. 1. PV transport across epithelial monolayers. A. Detection of 0.1 µg of PVs conjugated to Alexa Fluor 488 by CBB staining of an SDS-PAGE gel and fluorescence detection on a nitrocellulose membrane. B. CBB-stained gel and fluorescence detection of proteins in the basolateral medium from monolayers treated apically with 80 µg/mL or 160 µg/mL of labelled PVs for 24 h. C. Quantification of PVs in the basolateral medium after 1 h and 24 h of apical stimulation (n = 3, data shown as mean ± SD). D. Fish allergic patients' IgE binding to cod PV in basolateral medium. Purified cod PV (0.2 µg) was used as a positive control.

transport of PVs across the epithelium, cells were co-incubated with PVs and corresponding LMC for 24 h and the amount of PVs transported to the basolateral medium was quantified (Fig. 5C). When cells were treated with the combination of PV and LMC from the corresponding fish, the PVs were still transported to basolateral medium. However, their concentrations were significantly lower compared to the concentrations measured after exposure to PVs in absence of fish LMC (Fig. 5C). No difference between the amounts of cod versus shark PV was observed.

Athough they influenced TEER of the monolayers (Fig. 5A), the fish LMC did not reduce viability of the cells, as determined by MTT assay 24 h after exposure of the cells to different concentrations of LMC (Fig. 5D).

3.6. Fish LMC increase release of proinflammatory cytokines

In contrast to the PVs which did not influence the release of IL-6, IL-8 and CCL2 from the cells, notable differences in release of these cytokines were observed upon stimulation of the cells with fish LMC. Basolateral and apical release of IL-6 and IL-8 was increased by exposure to both cod and shark LMC (Fig. 6A and B). This effect was clearly dose-dependent for cod LMC, reaching an increase of up to 2.2-fold in IL-6 release and up to 50% in IL-8 release after exposure to Dose III (Fig. 6B). The influence of shark LMC on cytokine release was not

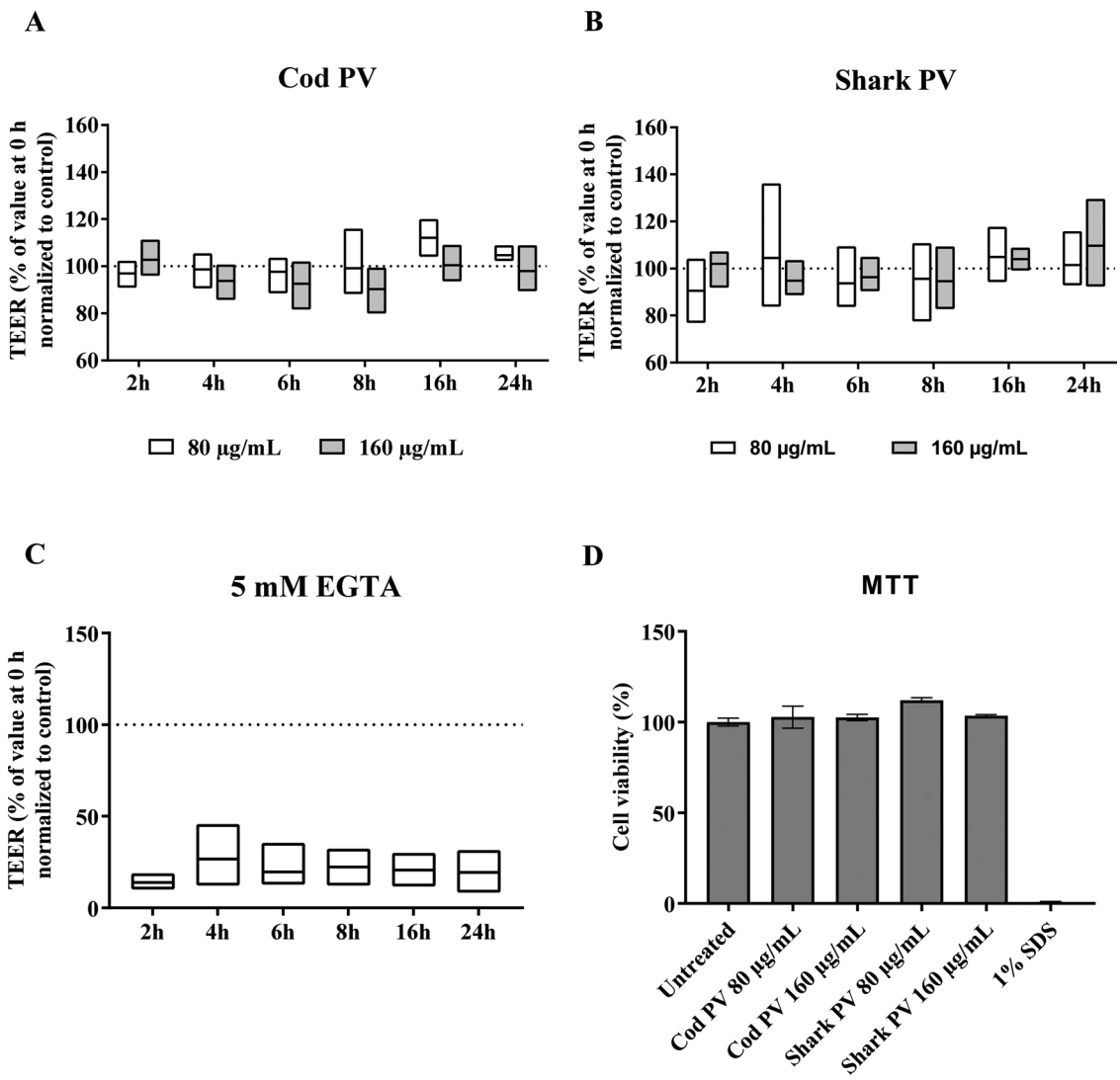

Fig. 2. PVs do not significantly influence epithelial barrier integrity and the viability of bronchial epithelial cells. Polarized 16HBE14o- cell monolayers were exposed apically to (A) cod PV, (B) shark PV (80 or 160 µg/mL) or to (C) 5 mM EGTA and TEER was monitored for 24 h. Untreated monolayers were used as controls. Data are obtained from three independent experiments performed in duplicates and show means with ranges (min to max), normalized to control monolayers. D. Cell viability analyzed by an MTT assay 24 h after apical exposure to PVs. 1% SDS was used as a control for toxicity. Data show mean ± SD (n = 3).

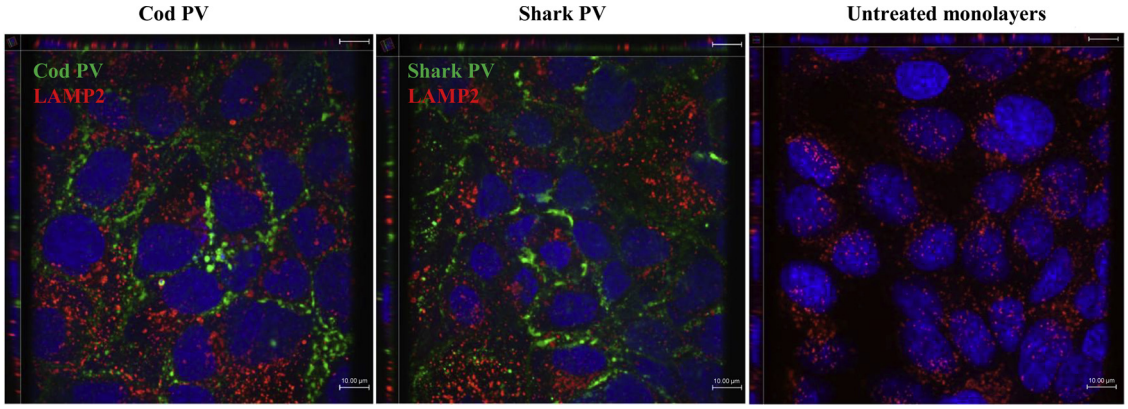

Fig. 3. PVs are internalized by 16HBE14o- cells and do not co-localize with LAMP-2-positive late endosomes/lysosomes. The figure shows results of confocal microscopy of 16HBE14o- cell monolayers after apical exposure to cod or shark PV (100 µg/mL) for 1 h at 37 °C, or monolayers without treatment with PVs. Alexa Fluor 488-labelled PVs (green), late endosome/lysosome marker LAMP2 detected by a specific primary and Alexa Fluor 568-labelled secondary antibody (red), DRAQ5-labelled nuclei (blue). Bar = 10 µm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

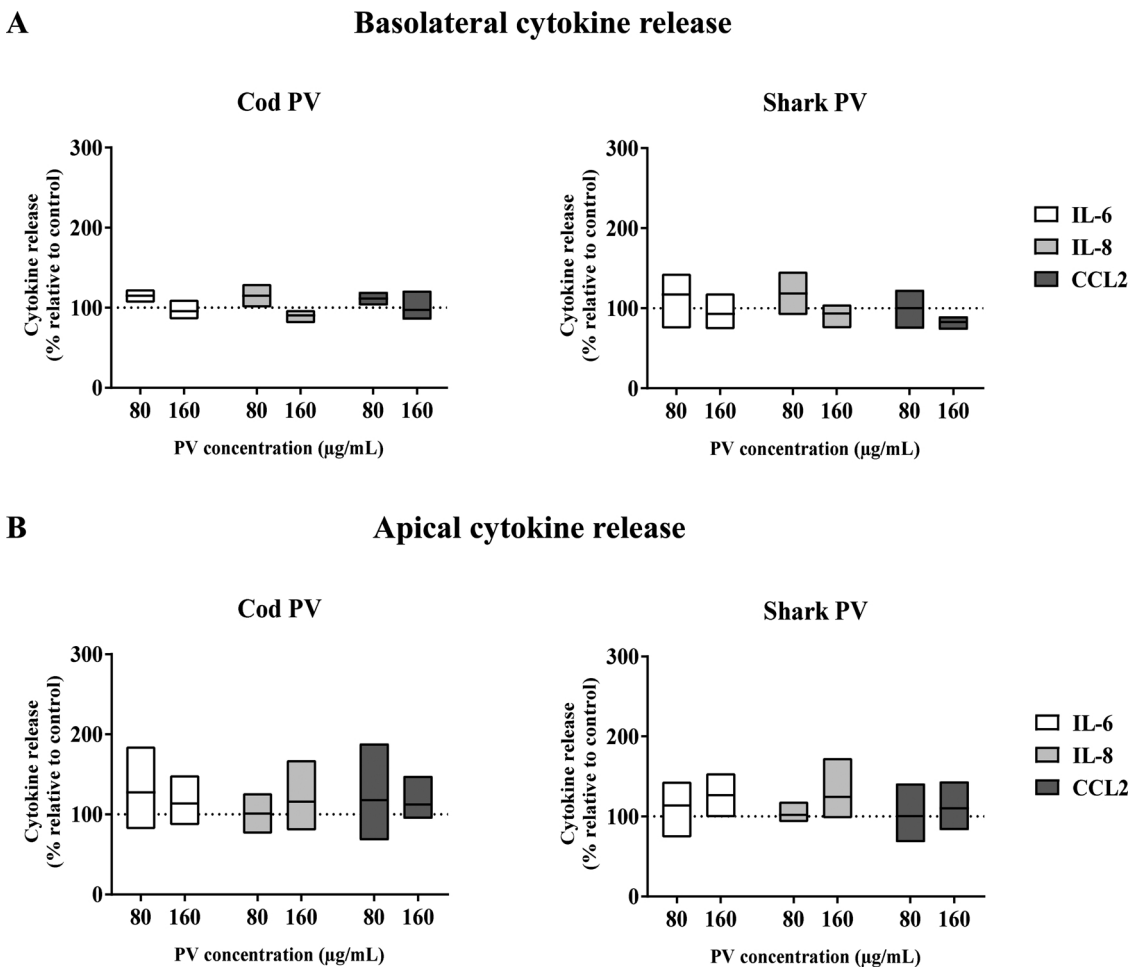

Fig. 4. PVs do not modify the release of IL-6, IL-8 and CCL2 by 16HBE14o- cells. Epithelial cell monolayers were stimulated apically with cod or shark PV (80 or 160 µg/mL) and the concentrations of the cytokines released to the basolateral (A) or apical (B) media were measured after 24 h of stimulation. Cytokine concentrations were normalized to the concentrations measured for untreated monolayers. Data are representative of three independent experiments performed in duplicates and depict means with ranges (min to max).

dose-dependent and was statistically significant only for basolateral release of IL-6 (Fig. 6A). Furthermore, basolateral and apical release of CCL2 was increased by exposure to cod (up to 50%), but not to shark LMC (Fig. 6A and B).

IL-10, IL-25, IL-33 and TSLP were below the detection limit for all treatments (data not shown).

4. Discussion

The prevalence of fish allergy has been increasing worldwide and is especially high in fish processing workers who are mainly exposed to fish allergens via inhalation (Jeebhay and Lopata, 2012; Moonesinghe et al., 2016). However, the mechanisms of the interaction between the major fish allergen PV and the bronchial epithelial cells, as well as the possible role of the food matrix in allergic sensitization to fish have not been explored before. Our study is the first to demonstrate that PVs from both a highly allergenic bony and a less allergenic cartilaginous fish are internalized by bronchial epithelial cells and rapidly cross the epithelial barrier in an intact form. Furthermore, LMC from fish exerted an effect on the barrier integrity of the cell monolayers and on mediator release. This activity differed between bony and cartilaginous fish. Therefore, LMC possibly play a role as adjuvants in allergic sensitization to fish via inhalation.

The respiratory epithelium is a physical, chemical and immunological barrier which plays an active role in detecting local changes of the environment to protect the body from possibly harmful molecules such as allergens (McClain et al., 2014; Vroling et al., 2008). Besides the need for the allergens to pass the epithelium and be presented to the immune system to induce allergic sensitization, the role of the food matrix as an adjuvant has emerged in recent years (McClain et al., 2014). Components other than allergens found in allergen sources may increase the stability of the allergens, act as danger signals or drive the immune system towards Th2 response (Aglas et al., 2018; Palladino et al., 2018; van Wijk et al., 2005; Wavrin et al., 2015).

The major fish allergens belong to the beta lineage of PVs and are abundant in bony fish. Cartilaginous fish contain mainly PVs of the alpha lineage and their allergenic potential is not well understood (Kuehn et al., 2014). Our recent study demonstrated the low reactivity to Thornback ray, a cartilaginous fish, in a cohort of patients sensitized to bony fish (Kalic et al., 2019). Possible explanations for the low allergenicity of cartilaginous fish may include the low sensitizing capacity of fish alpha PVs or different properties of their fish matrices, which could differently interact with epithelial barrier cells and the immune system resulting in different responses.

Epithelial cells are able to endocytose antigens, however most of the endocytosed material is sorted into lysosomal compartments and degraded thus preventing transcytosis of potentially antigenic proteins to the luminal side (Vroling et al., 2008). Moreover, the tight junction complexes between epithelial cells exclude the paracellular transport of most of the proteins (Georas and Rezaee, 2014). It is therefore being extensively investigated which mechanisms allergens use to cross the epithelial barrier. While several allergenic proteins use a paracellular

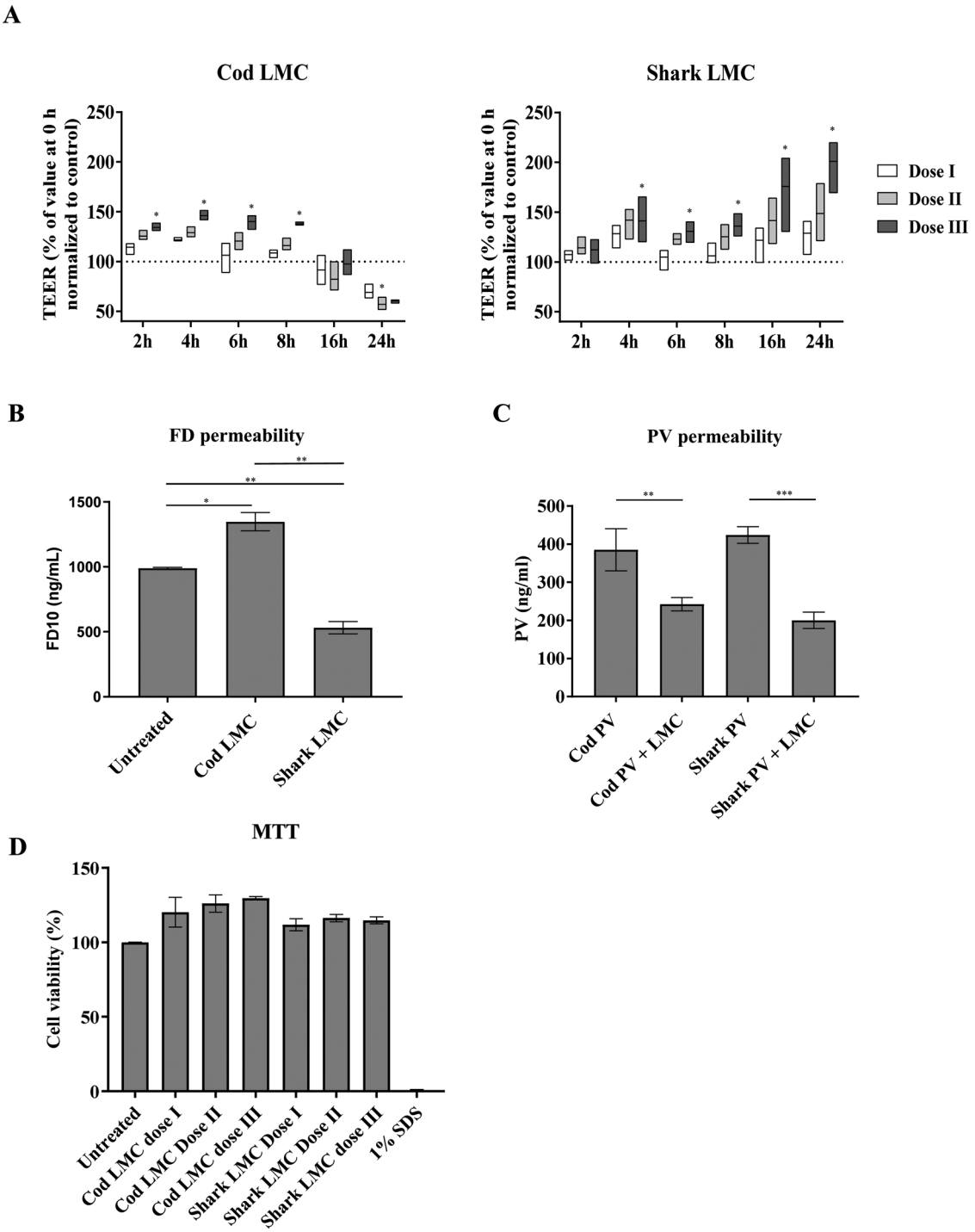

Fig. 5. Fish LMC modify the epithelial barrier properties. A. 16HBE14o- cell monolayers were exposed apically to three doses of cod or shark LMC and TEER measured over time. TEER was normalized to untreated monolayers for each time point. Data show means with ranges (min to max) from three independent experiments performed in duplicates. B. Polarized monolayers were treated for 24 h with fish LMC (Dose III) and the permeability for FD10 was explored. Untreated cells were used as reference. n = 2, data show mean ± SD. C. Quantification of the PVs in basolateral cell culture medium after 24 h of apical stimulation in presence or absence of LMC. n = 3, data show mean ± SD. D. Cell viability analyzed by MTT assay 24 h after apical exposure to different doses of LMC. n = 3, data show mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

epithelial transport by interfering with the epithelial barrier integrity (by direct protease activity or by inducing an inflammatory epithelial response) (Georas and Rezaee, 2014), we demonstrated the rapid internalization of PVs and their subsequent transport to the basolateral side without their degradation in endo-lysosomal compartments, suggesting a transcellular transport route. On the basolateral side, these allergens are accessible to antigen presenting cells possibly leading to allergic sensitization. Furthermore, the rapid transport of PVs across the epithelium may be an explanation for the rapid onset of allergic reactions to fish in already sensitized individuals, when exposed to minute amounts of fish allergens found in aerosols or due to ingestion of trace amounts of fish (Crespo et al., 1995; Untersmayr et al., 2007).

In our study, PVs did not change the viability of the cells, nor did they significantly influence the release of the proinflammatory

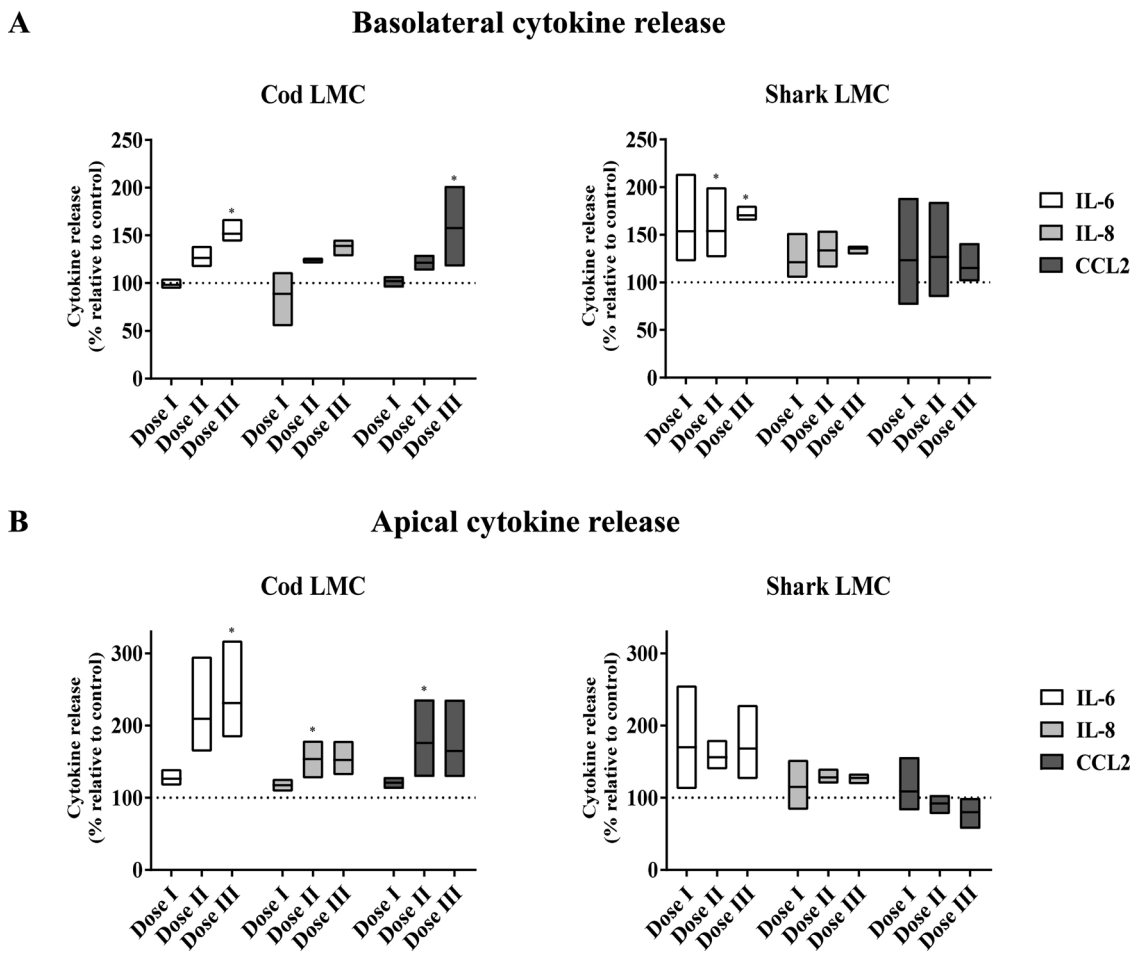

Fig. 6. Fish LMC modify proinflammatory cytokine release. Protein levels of IL-6, IL-8 and CCL2 in basolateral (A) and apical (B) media were quantified 24 h after apical stimulation of the cells. Concentrations are normalized to concentrations of cytokines released from untreated cells. Data are representative of three independent experiments performed in duplicates and depict means with ranges (min to max). *$p < 0.05$.

cytokines IL-6, IL-8 and CCL2. Interestingly, no difference between the interaction of cod versus shark PV with the cells was observed. Therefore, although being an important step in allergic sensitization, the ability of PVs to cross a tight epithelial barrier did not explain the different sensitizing capacities of bony versus cartilaginous fish. It is, however, important to note that our study used a cell line and that future studies on primary cells will be required to understand the mechanisms of allergic sensitization via inhalation in more detail.

Besides the allergens themselves, multiple external and internal factors contribute to polarization of the immune system towards allergic sensitization or tolerance when exposed to allergenic proteins (Curotto de Lafaille et al., 2010).

To further explore the differences in the response of epithelial cells to bony versus cartilaginous fish, we next investigated the possible influence of food matrix components as potential adjuvants in allergic sensitization. The role of LMC from the allergen source as an adjuvant had already been demonstrated for pollen (Blume et al., 2015). Birch pollen extract but not the major birch pollen allergen Bet v 1 was able to efficiently activate dendritic cells and induce a Th2 polarization (Aglas et al., 2018). We used fish LMC instead of whole fish extract to study the role of components other than proteins which are small enough to be aerosolized and to act as potential adjuvants in allergic sensitization via inhalation. Interestingly, the response of epithelial cells to cod or shark LMC was different, these differences possibly being the reason for their different allergenic potential. After 24 h of exposure to fish LMC, the TEER decreased below that of the control monolayers in case of cod, while it became higher in case of shark LMC, showing a potential protective effect of shark LMC on the epithelial barrier. Quantities of transported cod and shark PV after 24 h of cell stimulation were lower in presence than in absence of LMC from the corresponding fish. However, the amounts of transported cod versus shark PV were similar, although the TEER was different upon treatment with cod versus shark LMC. A possible explanation is that transcellular transport of PVs may be altered in the presence of LMC but this effect did not depend on the fish species. The mechanism of PV transport across the epithelium in the presence or absence of LMC will be the subject of further studies.

Furthermore, fish LMC modified the proinflammatory cytokine release from the cells which differed between cod and shark and may play a role in differential recruitment of other immune cells or further release of mediators, subsequently contributing to the different allergenicity of these two fish classes.

IL-6 and IL-8 are classic proinflammatory cytokines which influence the function of the bronchial epithelium and their polarized secretion by 16HBE14o- cells in response to the potential cell damage was demonstrated by the study of Chow et al. (2010). In our study, basolateral and apical release of IL-6 and IL-8 was increased by exposure to LMC from both species, while CCL2 release was increased only by cod LMC. The induction of CCL2 release by airway epithelial cells had been demonstrated for several allergens such as house dust mite proteins and chitin (Plantinga et al., 2013; Roy et al., 2012). It was demonstrated that CCL2 can induce differentiation of monocytes into inflammatory $CD11b^{+}$ dendritic cells, which are one of the crucial players in induction of allergic sensitization, biasing the differentiation of naïve T cells

towards a Th2 phenotype (Lee et al., 2015; van Ree et al., 2014). Furthermore, the role of neutrophil recruitment by IL-8 was implicated in allergic sensitization in several studies (Hosoki et al., 2016; Lambrecht and Hammad, 2014). In our study, while LMC from both cod and shark increased the release of IL-8 from the cells, the increased release of CCL2 was observed only upon exposure to LMC from cod, possibly playing a role in the higher allergenic potential of this bony fish.

The role of IL-6 in allergic sensitization to fish is not fully understood as it can elicit both Th1 and Th2 responses based on the signaling pathways activated in the cells, as well as the microenvironment which may include different cell types and the presence or absence of other cytokines (Lambrecht and Hammad, 2014; Tanaka et al., 2014). In our study, IL-6 release was increased after exposure to both cod and shark LMC. Possible differences in signaling pathways activated in the cells upon exposure to LMC from different fish species, as well as the composition of the LMC and the molecules responsible for the observed effects need to be elucidated in future studies. Furthermore, a larger study including multiple species of bony and cartilaginous fish classes is required to further explore the possibility of fish LMC as a contributing factor to the lower sensitizing capacity of cartilaginous compared to bony fish.

The importance of our study is the demonstration of the rapid transport of PVs in an immunologically active form across the epithelium, resulting in their availability to antigen presenting cells which is required for allergic sensitization. Furthermore, LMC from fish modified the barrier properties and the cytokine response which differed between bony and cartilaginous fish, possibly contributing to the different allergenic potential of these two fish classes.

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Conflicts of interest

All authors declare no conflicts of interest regarding the work presented in this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.04.029.

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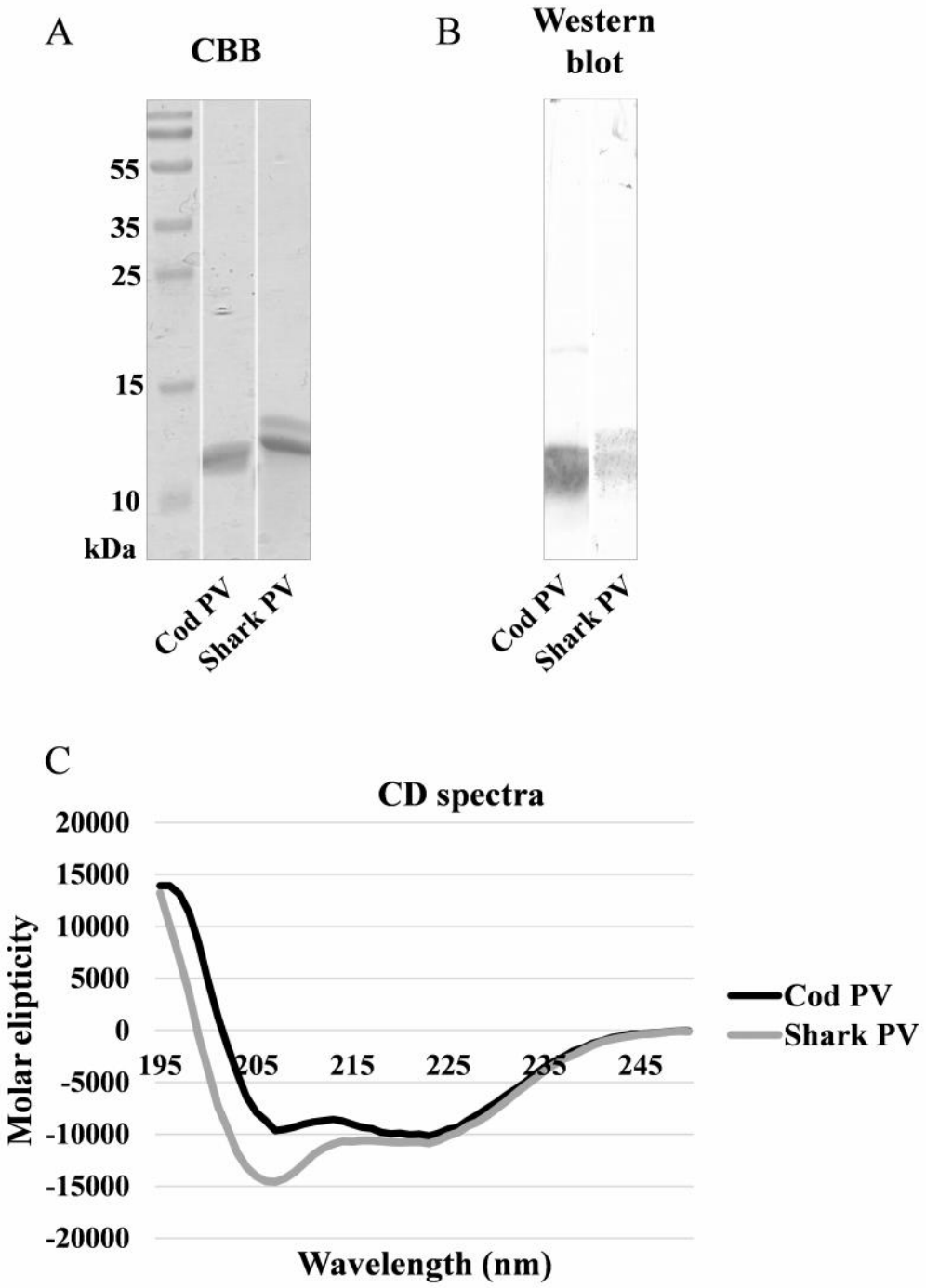

Figure S1. Characterization of the study parvalbumins. A: Coomassie brilliant blue staining of an SDS-PAGE gel demonstrating purity of the proteins. B: Western blot using anti-parvalbumin antibodies, confirming the protein identity. C: Circular dichroism (CD) spectroscopy, showing the expected alpha helical secondary structure of the proteins.

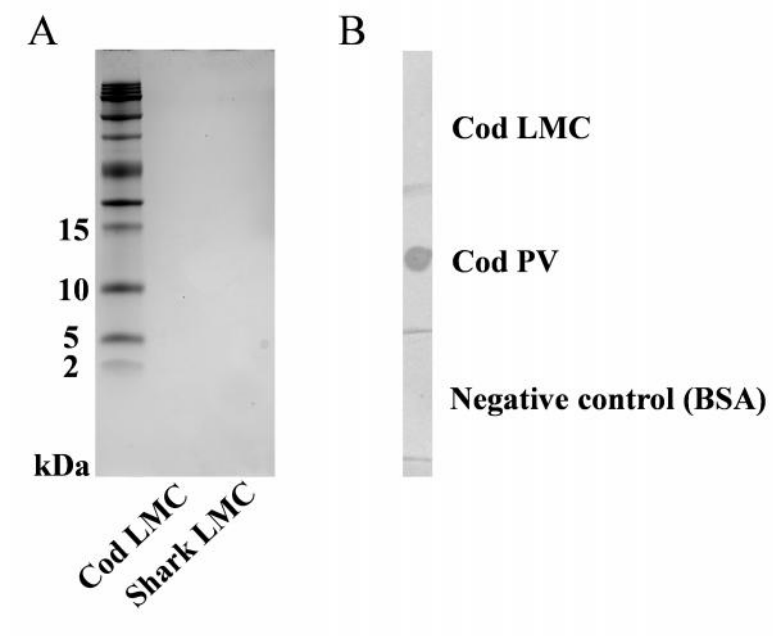

Figure S2. A: Absence of low molecular weight peptides in cod and shark LMC demonstrated by Coomassie Brilliant Blue staining of 16.5 % Tris-Tricine gel. B: Absence of IgE-binding to cod LMC demonstrated by dot blot using serum of Patient 1.

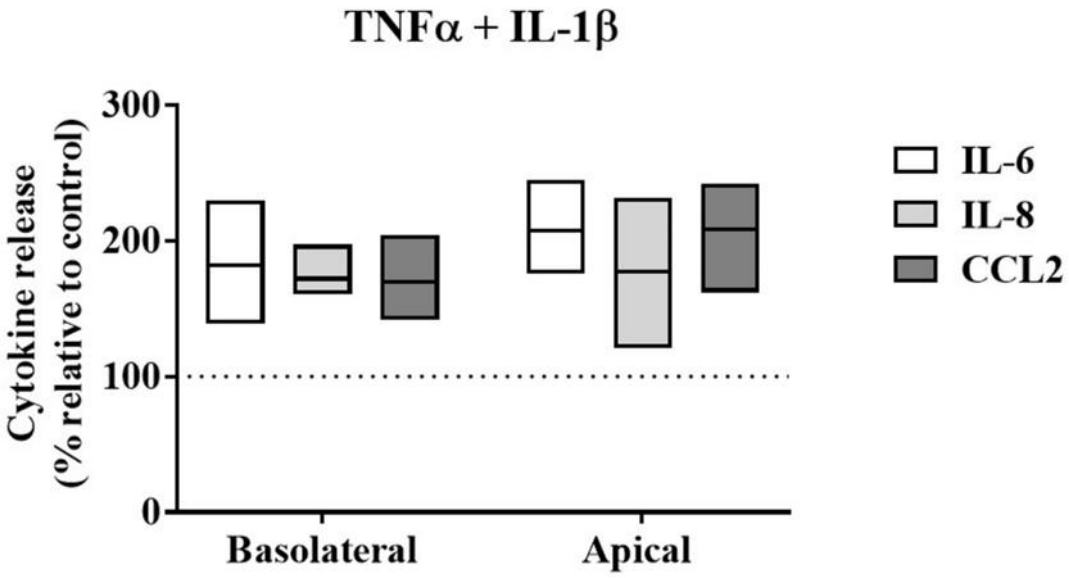

Figure S3. Basolateral and apical cytokine release after apical stimulation of 16HBE14o- cells with a mixture of TNFa and IL-1b as a positive control for induction of inflammation. Data show means with ranges obtained from three independent experiments performed in duplicates and represent cytokine release relative to release from untreated cells.