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Atopic donor status does not influence the uptake of the major grass pollen allergen, Phl p 5, by dendritic cells

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ABSTRACT

Dendritic cells (DCs) are sentinels of the immune system for antigen recognition and uptake, as well as presentation to naïve T cells for stimulation or priming. Internalization and endocytic degradation of allergens by DCs are important steps required for T cell priming.

In the current study we investigated binding and internalization of purified recombinant non-glycosylated grass pollen allergen, Phl p 5, and natural non-specific lipid transfer protein from sunflower, SF-nsLTP to human monocyte derived dendritic cells (MoDCs). Colocalization of Phl p 5 with low affinity (CD23) or high affinity receptor (FcεRI) was investigated by immunofluorescence staining. Likewise, localization of the allergens in early (EE) and late endosomes (LE) was detected by co-staining for early endosome antigen (EEA1) and lysosomal-associated membrane protein 1 (LAMP1).

In our experimental setting we could demonstrate that Phl p 5 as well as SF-nsLTP bound to MoDCs from both, grass pollen allergic and non-allergic individuals. Competitive allergen uptake experiments demonstrated non-preferential and simultaneous uptake of Phl p 5 and SF-nsLTP by MoDCs. No overlap of signals from Phl p 5 and CD23 or FcεRI was detectable, excluding IgE-mediated uptake for this allergen. Both allergens, Phl p 5 and SF-nsLTP, were localized in early and late endosomes.

The present study applied a set of methods to assess the allergen uptake by MoDCs in an in vitro model. No qualitative and quantitative differences in the allergen uptake of both, Phl p 5 and SF-nsLTP were detected in single and competitive assays.

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1. Introduction

1.1. Dendritic cells and allergic immune response

Allergic diseases represent a worldwide health problem that has dramatically increased in the past 2 decades (Pawankar et al., 2013). During the allergic reaction, dendritic cells (DCs) as professional antigen-presenting cells occupy a central position during the immune responses followed by a series of events that eventually result in IgE antibody production and mast cell sensitization (Froidure and Pilette, 2015).

DCs recognize, internalize and present antigens, including allergens. In allergic individuals, subsequent presentation of the resulting allergenic peptides to uncommitted CD4+ Th cells, expression of costimulatory molecules and an altered cytokine expression pattern lead to differentiation into a Th2 phenotype. This Th2-biased immune response finally culminates in allergen-specific IgE production by B cells. In contrast, the response to allergens in non-allergic individuals is characterized by Th0/Th1 responses and regulatory T (Treg) cells.

Abbreviations: APC, allophycocyanin; CLR, C-type lectin receptor; DAPI, diamidino-2-phenylindole; DC, dendritic cell; DC-SIGN, dendritic cell-specific intracellular adhesion molecule 3-grabbing non-integrin; EE, early endosome; EEA1, early endosome antigen 1; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; LAMP1, lysosomal-associated membrane protein 1; LE, late endosome/lysosome; MoDC, monocyte derived dendritic cell; MR, mannose receptor; NA, non-allergic healthy donors; PBMC, peripheral blood mononuclear cells; PCC, Pearson correlation coefficient; Phl p 5, *Phleum pratense* (Timothy grass pollen) allergen 5; SF-nsLTP, sunflower non-specific lipid transfer protein; SPT, skin prick test; Th cell, T helper cell; TLR, Toll-like receptor.

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The initial triggers of DCs leading to the manifestation of an allergic disease are still insufficiently resolved and are probably diverse. For example, different agonists of Toll-like receptors (TLRs) can cause divergent T cell differentiation (Deifl et al., 2014). Moreover, a robust Th2 skewing following an exposure of DCs to allergens seems to depend on the nature of the allergens and whether DCs interacting with the allergen are derived from allergic or healthy donors. A Th2 polarization has been shown for Bet v 1 isoforms in DCs from birch pollen allergic patients versus samples from healthy non-atopic individuals (Smole et al., 2010, 2015; Kitzmuller et al., 2012; Ashjaei et al., 2015).

Three mechanisms for allergen acquisition and processing are currently known (Roche and Furuta, 2015). One mechanism is receptor-mediated endocytosis involving interaction of allergens with various plasma membrane located receptors. These receptors include C-type lectin carbohydrate receptors (CLRs; e.g. DC-SIGN), Mannose receptor (MR), langerin, BDCA-2, and others (Jiang et al., 1995; Sallusto et al., 1995; Figdor et al., 2002), Fcγ-receptors (CD32, CD64) or Fcε-receptors (FcεRI) (Sallusto and Lanzavecchia, 1994).

DC-SIGN and MR have been identified as receptors for glycoallergens from house dust mite cockroach, dog saliva and peanut extract (Royer et al., 2010; Salazar and Ghaemmaghami, 2013; Sharquie et al., 2013). Numerous studies have also emphasized the role of high and low affinity IgE receptors for internalization of allergens (Maurer et al., 1998; Turcanu et al., 2010; Sallmann et al., 2011; Galli and Tsai, 2012). The presence of IgE significantly increases Der p 1 uptake to level and above that mediated by MR (Sharquie et al., 2013). Uptake of allergens via receptor-mediated endocytosis can enhance the efficiency of priming of naïve T cells by 1000-fold in comparison to macropinocytosis. Macropinocytosis is a second non-specific uptake mechanism, which does not require plasma membrane recognition of the cargo (de Baey and Lanzavecchia, 2000). In immature DCs this is a constitutive process and the main entry mechanism for several types of virus particles (e.g. herpes simplex virus). Macropinocytosis has been suggested for e.g. Bet v 1 uptake (Noirey et al., 2000), (Smole et al., 2015). Finally, DCs are also able to phagocyte particulate antigens such as bacteria, viruses and latex beads (Svensson et al., 1997; Banchereau et al., 2000).

Upon allergen uptake by DCs, the antigen is transported via mildly acidic early endosomes (EEs, EEA1 +) to the more acidic late endosomes (LEs, LAMP1 +). Low pH-dependent proteolytic enzymes in these endosomes degrade the allergen, with higher activity of proteases as observed in LEs. Degradation allows for loading of allergen-derived peptides onto Class II MHC molecules in LEs. Regulation of proteolysis in endosomes and lysosomes is important for MHC-restricted antigen processing and presentation. Interestingly, enhanced protease activity, which leads to increased allergen proteolysis, may result in increased destruction of T cell epitopes. Increased proteolysis has been observed in activated DCs due to increased acidification of antigen-processing compartments (Roche and Furuta, 2015). Class II MHC molecules loaded with processed peptides are then targeted to the cell surface (Villadangos and Schnorrer, 2007; Platt et al., 2010; Compeer et al., 2012, 2014). Subsequently, naïve T cells are activated into Th2 cells in allergic individuals (Kapsenberg, 2003; MacDonald and Maizels, 2008; Smole et al., 2009; Pulendran et al., 2010; Salazar and Ghaemmaghami, 2013; Ashjaei et al., 2015).

Overall, early events at the interface of allergens and DCs play a key role in downstream events leading to allergic reactions. The circumstances of internalization of a particular allergen may significantly affect intracellular routing and thereby the extent of allergen processing (Kitzmuller et al., 2012). So far, few studies compared the internalization of allergens by human DCs derived from allergic individuals versus samples from non-allergic persons applying the causative allergen and a non-related allergen. Furthermore, these studies were mostly performed by flow cytometry and rarely involved microscopy to more precisely explore subcellular events in DCs.

1.2. Aim of this study

This study focused on the visualization of allergen uptake by human MoDCs from grass pollen allergic and non-allergic individuals. Applying fluorescence microscopy and FACS analyses cell surface binding and internalization of allergens were investigated. Recombinant purified major grass pollen allergen, Phl p 5, and purified natural SF-nsLTP, a food allergen, were used, both well characterized non-glycosylated proteins low in LPS content. We mainly aimed to investigate whether the atopic status of donors had an impact on allergen uptake and intracellular routing by their autologous MoDCs.

2. Materials and methods

2.1. Materials

Recombinant Phl p 5, a 33.5 kDa non-glycosylated protein was purchased from Biomay AG (www.biomay.com). Natural sunflower LTP (SF-nsLTP; non-glycosylated, 9 kDa) was purified from sunflower seeds as previously described (Burnett et al., 2002). Fluorescein isothiocyanate (FITC)-transferrin was purchased from Invitrogen (Molecular Probes®, www.lifetechnologies.com) and FITC-dextran from Sigma-Aldrich (www.sigmaaldrich.com). The influenza virus hemagglutinin HA (H1-Cal09-wt) was produced from viral strain A/California/2009 using the baculovirus insect cell expression system as described previously (Palmberger et al., 2014). A modified variant of the protein carrying higher mannose type N-glycans (H1-Cal09-man9) was produced by addition of 25 μM kifunensine to the cell culture medium. The endotoxin content of the proteins was <3 EU/mg as determined using Recombinant Factor C Endotoxin Detection Assay (EndoZyme®, www.hyglos.de). Cy3 Mono-Reactive (GE Healthcare, www.gelifesciences.com) and AlexaFluor® 488 Dye (Invitrogen) were used for labeling of the proteins according to the manufacturer's instructions. Concentrations of the labeled proteins were determined by a spectrophotometer U-1800, Hitachi, (www.hitachi.com) according to the manufacturer's instructions prior to use. The following conjugated antibodies were used: anti-IgE-FITC, anti-FcεRIα-APC, and anti-CD23 (FcεRII)-APC (all from Miltenyi Biotec GmbH, www.miltenyibiotec.com). Anti-EEA1 (BD Biosciences, www.bdbiosciences.com) and anti-LAMP1 (DSHB, University LOWA) were used as primary antibodies and detected by incubation with goat anti-mouse IgG labeled with Alexa Fluor® 647 (Life Technologies, www.lifetechnologies.com). Diamidino-2-phenylindole (DAPI) dye (Sigma-Aldrich) was used for staining of cell nuclei.

2.2. Donors

Four individuals suffering from inhalant grass pollen allergy were involved in this study. Grass pollen allergy was diagnosed by convincing case history and either positive skin prick test (SPT) or positive in vitro detection of grass pollen specific IgE (ImmunoCAP). Serum IgE specific for Phl p 5, the major allergen from grass pollen, and SF-nsLTP was determined by ELISA as described previously (Suzuki et al., 2010). Optical density (OD) values were counted positive if they exceeded the mean OD of the negative controls by more than three standard deviations. Blood samples from 4 non-allergic healthy donors (NA) without any allergic symptoms and allergen-specific IgE antibodies were used as the control group. Donor's characteristics are shown in Table 1. The study was authorized by the Ethical Committee of the Medical University of Vienna (ethics committee approval number: 038/2009).

2.3. Cell isolation and culture

Heparinized blood samples obtained from donors were used for preparation of peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation (1000 ×g, 30 min) using Ficoll-Paque (GE

Table 1
Subjects' characteristics.

Patient (P)/ control (C)	Age (Y)	Sex (M/F)	Allergy history	Diagnostic test	Symptoms	sp IgE Phl p 5
P 1	33	F	BP, GP	CAP	A, C, R	+
P 2	44	F	BP, GP	CAP	C, R, U	+
P 3	23	F	GP	SPT	C, R	+
P 4	28	F	GP	SPT, CAP	C, R	+
C 1	28	M	No	ND	No	−
C 2	52	F	No	ND	No	−
C 3	29	F	No	ND	No	−
C 4	30	F	No	ND	No	−

BP, birch pollen; GP, grass pollen; A, asthma; C, conjunctivitis; R, rhinitis; U, urticaria; SPT, skin prick test; CAP, ImmunoCAP;
sp IgE, allergen-specific IgE tested by ELISA: −, negative; +, positive.

Healthcare). Interphase cells were washed in free Hanks Balanced Salt Solution with Phenol Red (HBSS; BioWhittaker, www.lonza.com) supplemented with 2% fetal calf serum (FCS; Invitrogen). Monocytes and peripheral blood lymphocytes were isolated from purified PBMCs by Percoll (GE Healthcare) density gradient centrifugation (2100 ×g, 45 min). Immature MoDCs were generated by culturing monocytes in Iscove's modified Dulbecco medium (IMDM; BioWhittaker, Lonza) containing 86 μg/ml gentamycin (Lactan, www.lactan.at) and 10% FCS using 24-well plates (Thermo Fisher Scientific, www.thermofisher.com). For differentiation, the medium was supplemented with GM-CSF (500 U/ml) and IL-4 (250 U/ml; both R&D Systems, www.rndsystems.com) and cells were incubated for 6 days at 37 °C in a humidified 5% CO_2 incubator (Smole et al., 2009).

2.4. In vitro binding and uptake of allergens

The cells were seeded on sterilized (with 95% ethanol and flamed) round 12 mm coverslips (Thermo Fisher Scientific) placed inside 24-well plates at a density of 4×10^5 cells/well. MoDCs grown on glass coverslips were washed with PBS (Dulbecco's Phosphate Buffered Saline; Thermo Fisher Scientific) and incubated in serum free IMDM medium for 30 min at 37 °C to remove serum components from cellular receptors. Fluorescence-labeled allergens Cy3-Phl p 5 and AlexaFluor488-SF-nsLTP and all other markers used were added to the cells at concentrations and times indicated in the result section. To investigate surface/plasma membrane binding of molecules, the cells were kept at 4 °C and for internalization the plates were incubated at 37 °C. In competitive assays, MoDCs were simultaneously treated with AlexaFluor488-SF-nsLTP and Cy3-Phl p 5 at 4 °C for surface binding and at 37 °C for internalization, respectively. In all experiments, untreated MoDCs were used as a negative control. Treated MoDCs were washed with cold PBS and fixed with a 4% formaldehyde solution in PBS. The fixed cells were incubated with DAPI in PBS and then the coverslips were mounted onto microscope glass slides using Fluoromount-G (Southern Biotech, www.southernbiotech.com).

2.5. Detection of IgE, Fcε-receptors and markers of endosome subcompartments by immunocytochemistry

To study the role of IgE and Fcε-receptors in Phl p 5 uptake, Cy3-Phl p 5 was either bound at 4 °C or internalized at 37 °C for 1 h in MoDCs. Cells were washed with cold PBS, fixed and permeabilized using 1%BSA in PBS + 0.5% saponin (Sigma). Cells were then incubated with anti-IgE-FITC and anti-FcεRIα-APC and anti-IgE-FITC and anti-CD23-APC (1:11 diluted in 1%BSA in PBS + 0.5% saponin) at room temperature, respectively.

In order to evaluate arrival of allergens in endosomal subcompartments, two sets of MoDCs were prepared and incubated with either Cy3-Phl p 5 or AlexaFluor488-SF-nsLTP or both allergens at 37 °C for 15 min to follow trafficking of allergens to EEs and for 30–45 min to follow trafficking of allergens to LEs. After fixation and permeabilization, the cells were stained with unconjugated primary antibodies anti-EEA1 (1/200) or anti-LAMP1 (1/500) diluted in 1% BSA in PBS + 0.5% saponin, incubated for 1 h at room temperature. Subsequently, cells were incubated with fluorescence-conjugated secondary antibodies (1/1000, diluted in 1% BSA in PBS + 0.5% saponin) for another hour at room temperature. Finally, all cells were incubated with DAPI in PBS and the coverslips were mounted onto microscope glass slides.

2.6. Fluorescence microscopic analysis

A wide-field fluorescence microscope (Imager Z1/Zeiss, www.zeiss.com) in combination with TissueFAXS software (TissueGnostic GmbH, www.tissuegnostic.com) was used to view overall cell performance after labeling of cells. Binding and uptake of markers as well as colocalization of proteins in single cells was investigated using a Zeiss Axiovert 200 confocal microscope equipped with a 63× oil immersion lens (Zeiss). Pictures were digitalized using Volocity software (Perkin Elmer, www.perkinelmer.com). Averages of 60–100 cells per experiment were evaluated. The Pearson correlation coefficient value (PCC), *r*, was used as a statistic measure to show colocalization of two proteins. The values for *r* were considered as very weak (0.0–0.19), weak (0.20–0.39), moderate (0.40–0.59), strong (0.60–0.79) and very strong (0.80–1.0) (Williams, 1996; Dunn et al., 2011). Images were processed using Image J and Adobe Photoshop software.

2.7. Flow cytometry

MoDCs were harvested by scraping, washed, counted and dispensed at 2.5×10^5 cells per tube into 5 ml polystyrene Round-Bottom FACS (Fluorescence Activated Cell Sorting) tubes (BD Biosciences). Then, cells were treated with 10 μg/ml Cy3-Phl p 5 or AlexaFluor488-SF-nsLTP or both labeled allergens. Alternatively, cells were incubated with 10 μg/ml FITC-transferrin or 500 μg/ml FITC-dextran, to investigate receptor-mediated and fluid-phase endocytosis or were used without any treatment. The cells were incubated at 37 °C for 5, 10, 30, 60 and 120 min. In another set of experiments, MoDCs were treated with 0.5, 1 and 2 μg/ml AlexaFluor488-labeled wild type or highly mannosidic HA antigens for 1, 5, and 15 min at 37 °C. The cells were then washed with serum free IMDM and the cell pellet was dissolved in FACS buffer consisting of PBS, 1% BSA, 0.1% sodium azide (Merck, www.merck.de), 2% FCS and 1% human serum (Lonza). The samples were analyzed with a minimum of 10,000 events on a FACS Canto flow cytometer using the BD FACS Diva software (BD Biosciences).

3. Results and discussion

We applied confocal laser microscopy and FACS to compare allergen uptake and intracellular routing by MoDCs from grass pollen allergic individuals with MoDCs derived from healthy donors. We studied the uptake of the major grass pollen allergen, Phl p 5, in comparison to an unrelated food allergen, sunflower LTP. The uptake of these non-glycosylated proteins was compared to FITC-transferrin and FITC-dextran. FITC-transferrin was used as a classical marker for receptor-mediated uptake (Sallusto et al., 1995; Doherty and McMahon, 2009). In the majority of cell types, FITC-dextran is a standard marker for fluid-phase endocytosis/macropinocytosis (Shurety et al., 1998; Khan et al., 2011). However, in DCs, it can also be also internalized by MR (Sallusto et al., 1995). Mannose receptor is a major endocytic receptor for influenza virus antigens (Reading et al., 2000). AlexaFluor488-labeled influenza virus wild type HA or highly mannosylated HA antigens were used as examples for ligands of C-type lectins (CLR) receptors.

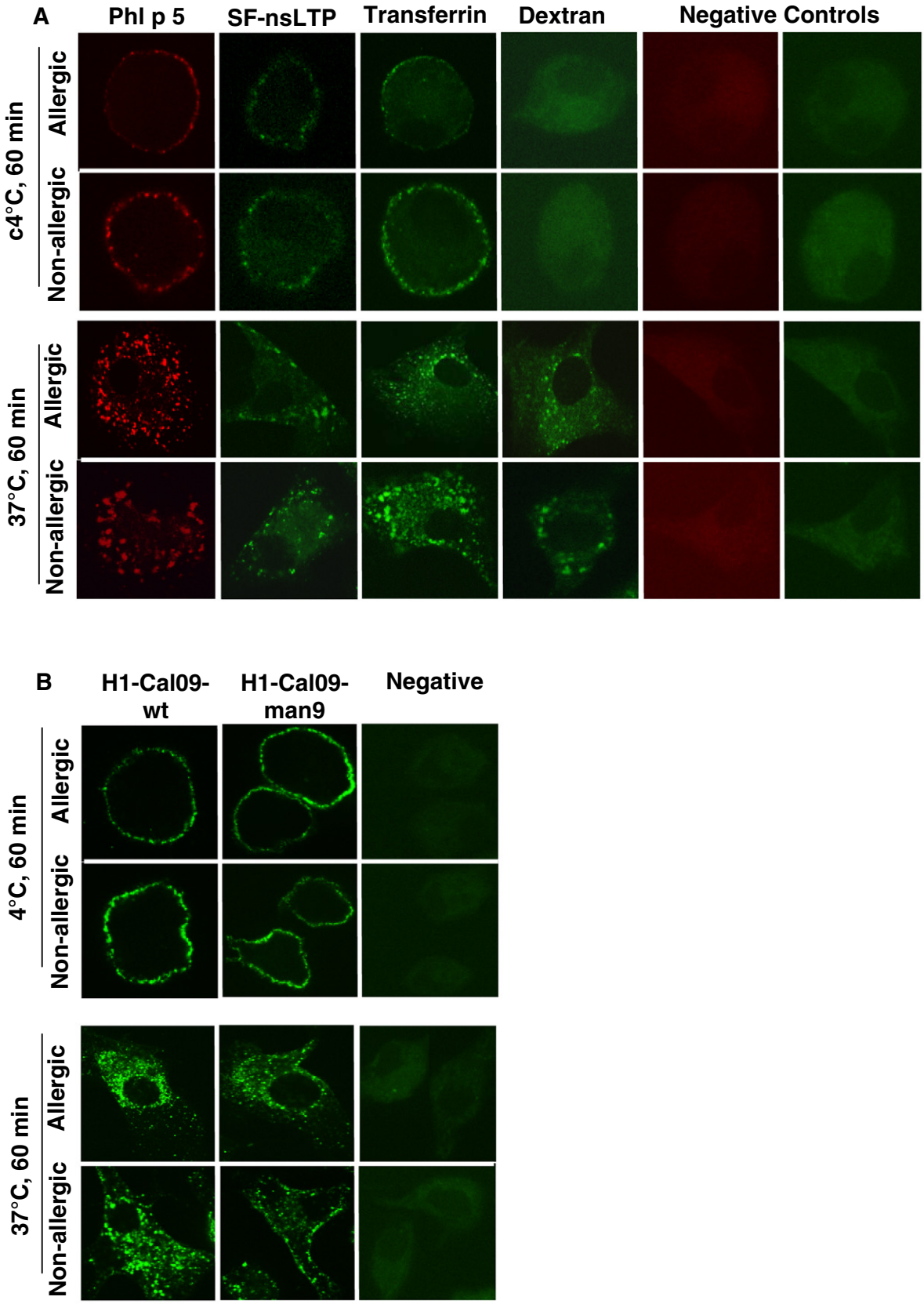

Fig. 1. Confocal microscopy of monocyte derived dendritic cells (MoDCs) from grass pollen allergic and non-allergic donors to visualize binding and internalization of Phl p 5 and SF-nsLTP. Cells were incubated for 1 h at 4 °C for binding and at 37 °C for internalization with either purified and labeled allergens (10 μg/ml), Cy3-Phl p 5 (red), Alexa-Fluor488-SF-nsLTP (green), FITC-transferrin (10 μg/ml, green) and FITC-dextran (500 μg/ml; green) (A). Wild type (H1-Cal09-wt) and N-glycosylation pattern (H1-Cal09-man9) of influenza HA antigen labeled with AlexaFluor488 (green) were applied at a concentration of 10 μg/ml (B). Untreated MoDCs were used as negative controls. Analysis was performed by laser scanning confocal microscopy (488 nm (green) and 568 nm (red)).

3.1. No difference in allergen uptake by MoDCs from allergic and non-allergic donors

Immature MoDCs (day 6 of culture) obtained from grass pollen allergic and non-allergic healthy donors were treated with 10 μg/ml labeled allergens of either Cy3-Phl p 5 or AlexaFluor-SF-nsLTP at 4 °C or 37 °C for 1 h to visualize binding or internalization, respectively. The allergens were taken up by the majority (72–88%) of MoDCs (data not shown) as analyzed by wide field fluorescence microscopy. To distinguish between bound and internalized allergens, single cells were analyzed by confocal microscopy. With this approach, binding (4 °C) and internalization (37 °C) of Phl p 5 and SF-nsLTP by MoDCs from both, allergic and non-allergic individuals was clearly detected (Fig. 1A). MoDCs were also incubated with FITC-transferrin and FITC-dextran as controls. Binding and internalization of FITC-transferrin was clearly visualized (10 μg/ml protein at 4 °C and at 37 °C for 60 min), (Fig. 1A). The lowest concentration of FITC-dextran, which could be detected by microscopy, was 500 μg/ml. At that concentration, internalization of FITC-dextran at 37 °C was demonstrated, but no binding of the marker to the plasma membrane occurred at 4 °C arguing against a (MR)-receptor-mediated internalization as observed by others (Sallusto et al., 1995; Kato et al., 2000). Fig. 1B shows a representative picture of the receptor-mediated binding (4 °C, 1 h) and internalization (37 °C, 1 h) of AlexaFluor488-labeled influenza virus HA (H1-Cal09-wt) and its modified variant carrying higher mannose type N-glycans (H1-Cal09-man9) by MoDCs from both study groups.

Taken together these findings suggest that the non-glycosylated allergens Phl p 5 and SF-nsLTP were taken up by MoDCs, in both, cells from allergic and non-allergic donors in a receptor-mediated process, which leads to the delivery of allergens to intracellular compartments. In contrast to recently published results from the uptake of Bet v 1 and its homologue, Api g 1, (Smole et al., 2015) we observed no major differences between internalization of our allergens.

3.2. Simultaneous uptake of Phl p 5 and SF-nsLTP by MoDCs from allergic and non-allergic donors

Next, we performed allergen binding (4 °C) and uptake (37 °C) experiments applying Cy3-Phl p 5 and AlexaFluor488-SF-nsLTP, simultaneously. As illustrated in Fig. 2A our results demonstrate that the majority of MoDCs recognized and internalized both allergens and colocalization of Phl p 5 and SF-nsLTP during binding and internalization was detected and estimated by statistical analysis (PCC, r). The PCC values for surface binding in samples from grass pollen allergic and non-allergic individuals were $r = 0.68$, $r = 0.58$, and the values for internalization were $r = 0.67$, $r = 0.80$ respectively (Fig. 2A showing a representative sector of the image), which is classified to be strong (Williams, 1996; Dunn et al., 2011). The significant overlap between these two allergens suggests common uptake/sorting mechanisms and/or potentially shared receptors for these allergens. In a competitive quantitative assay we next tested whether the simultaneous uptake of Cy3-Phl p 5 (10 μg/ml) and AlexaFluor488-SF-nsLTP (10 μg/ml) was quantitatively influenced by a fivefold increase in the concentration of Phl p 5 (50 μg/ml). The increase in Cy3-Phl p 5 concentration resulted in higher number of cells positive for Phl p 5 but did not influence the uptake of AlexaFluor488-SF-nsLTP as shown in Fig. 4D, suggesting that these non-glycosylated allergens do not share the same receptor.

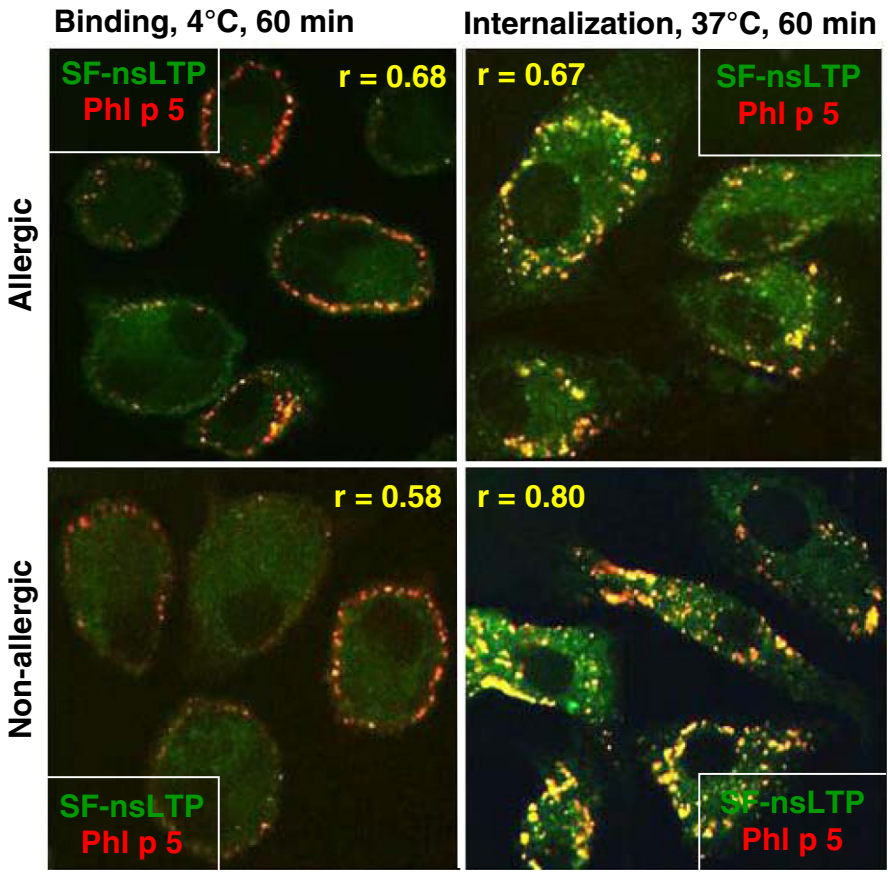

Fig. 2. Simultaneous binding or internalization of Phl p 5 and SF-nsLTP by MoDCs from grass pollen allergic and non-allergic donors. Human MoDCs from grass pollen allergic and non-allergic donors were incubated with Cy3-Phl p 5 (red) and Alexa-Fluor 488-SF-nsLTP (green) simultaneously, for 1 h at 4 °C for binding and at 37 °C for internalization, respectively. Analysis was performed by laser scanning confocal microscopy (488-nm (green) and 568-nm (red) wavelengths). As shown in a representative sector of the image yellow (green + red) color indicates overlap of two markers. Pearson's coefficient (r) shows a quantitative assessment of colocalized allergens.

3.3. Cellular uptake of Phl p 5 by MoDCs in the absence of specific IgE

Binding of Phl p 5 to the plasma membrane of MoDCs as demonstrated in Fig. 1 suggests a receptor-mediated uptake of the allergen. As the allergen is not glycosylated, it is unlikely that a CLR mediates the uptake of Phl p 5 (Salazar and Ghaemmaghami, 2013). Studies on allergen uptake have provided evidence that IgE and its high affinity receptor, FcεRI, play an important role in allergen recognition and uptake by human DCs (Turcanu et al., 2010; Sharquie et al., 2013). By confocal microscopy we therefore investigated the role of IgE, FcεRI and CD23 receptors in the uptake of Phl p 5 by immature MoDCs. Following binding (4 °C, 1 h) or internalization (37 °C, 1 h) of Cy3-Phl p 5 in MoDCs, cells were fixed and IgE, FcεRI and CD23 were detected with anti-IgE-FITC, anti-FcεRI-APC, and anti-CD23-APC antibodies, respectively. The results revealed high CD23 expression levels in MoDCs from grass pollen and non-allergic donors. Nevertheless, the PCC values for Phl p 5 and CD23 were weak in both study groups ($r = 0.24$ and $r = 0.28$, at 4 °C and 37 °C; Fig. 3A). MoDCs expressed FcεRI at low levels and colocalization with Phl p 5 was rarely detectable without any difference between samples from allergic and non-allergic donors, at 4 °C and 37 °C, respectively (PCC values: $r = 0.037$, $r = 0.027$; Fig. 3B). These findings are in agreement with a previous study that showed expression of IgE receptors on antigen presenting cells and monitored the kinetics of FcεRI and CD23 expression during the generation of MoDCs. Sharquie, I. et al. demonstrated a modest percentage (43%) of FcεRI expression and high expression of CD23 (91%) on immature MoDCs during in vitro generation from peripheral blood monocytes (Sharquie et al., 2013). The relationship between levels of serum

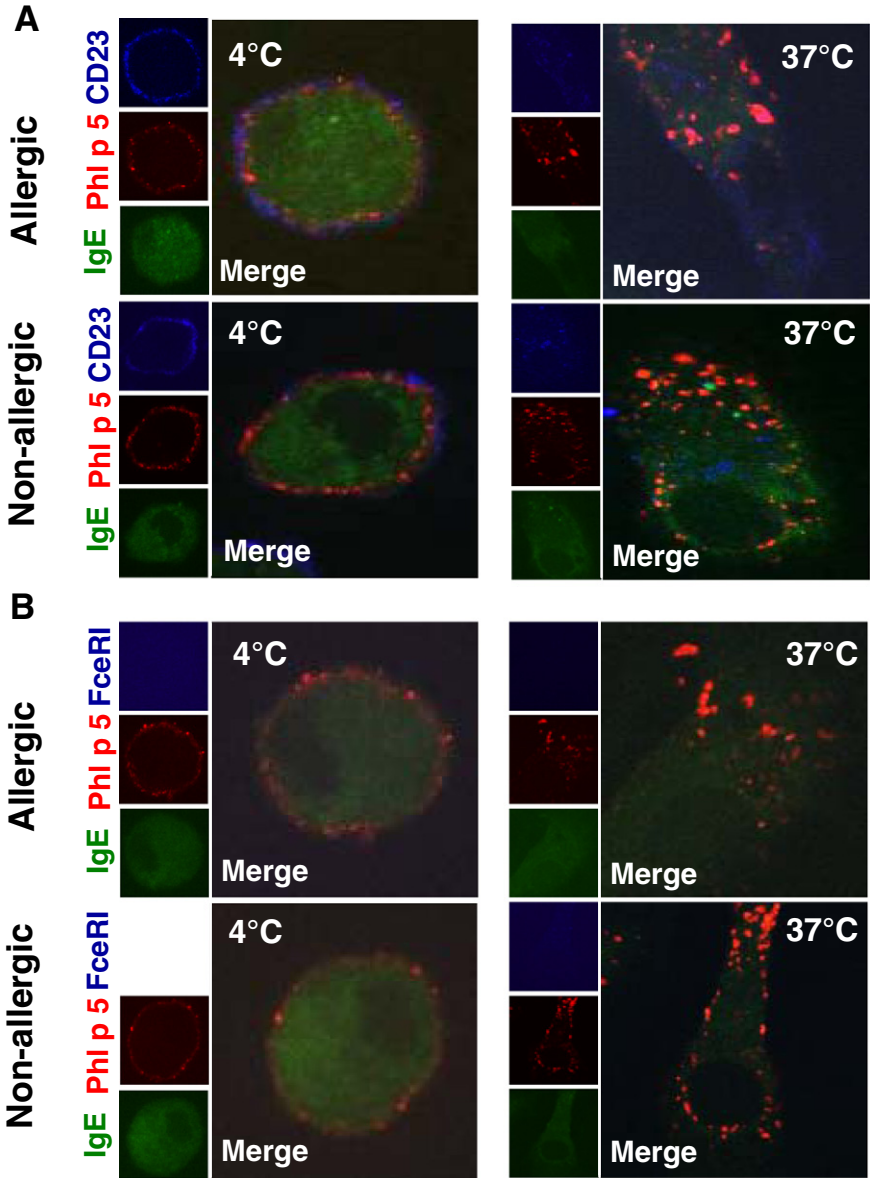

Fig. 3. High and low affinity IgE receptors and IgE detection after Phl p 5 binding and internalization in MoDCs from grass pollen and non-allergic donors. MoDCs derived from allergic and non-allergic donors were incubated with Phl p 5 at 4 °C for binding and at 37 °C for internalization. Cy3-Phl p 5 (red) stimulated MoDCs were fixed, permeabilized and immunostained using anti-IgE (green), anti-CD23 (A, blue) and anti-FcεRIα antibodies (B, blue). Three different fluorescence signals were detected by laser scanning confocal microscopy at 488 nm (green), 568 nm (red) and 633 nm (blue) wavelengths. White color indicates a triple overlap of all three markers (IgE, Phl p 5 and CD23 or FcεRIα), whereas yellow (green + red), pink (red + blue) and light blue (green + blue) indicate overlap of two markers. Pearson's coefficients were calculated to quantitatively assess colocalization. Pearson's coefficient values for Phl p 5 and CD23 were weak ($r = 0.24$, $r = 0.28$) in allergic and non-allergic donors, respectively, these values for Phl p 5 and FcεRIα were very weak ($r = 0.037$, $r = 0.027$).

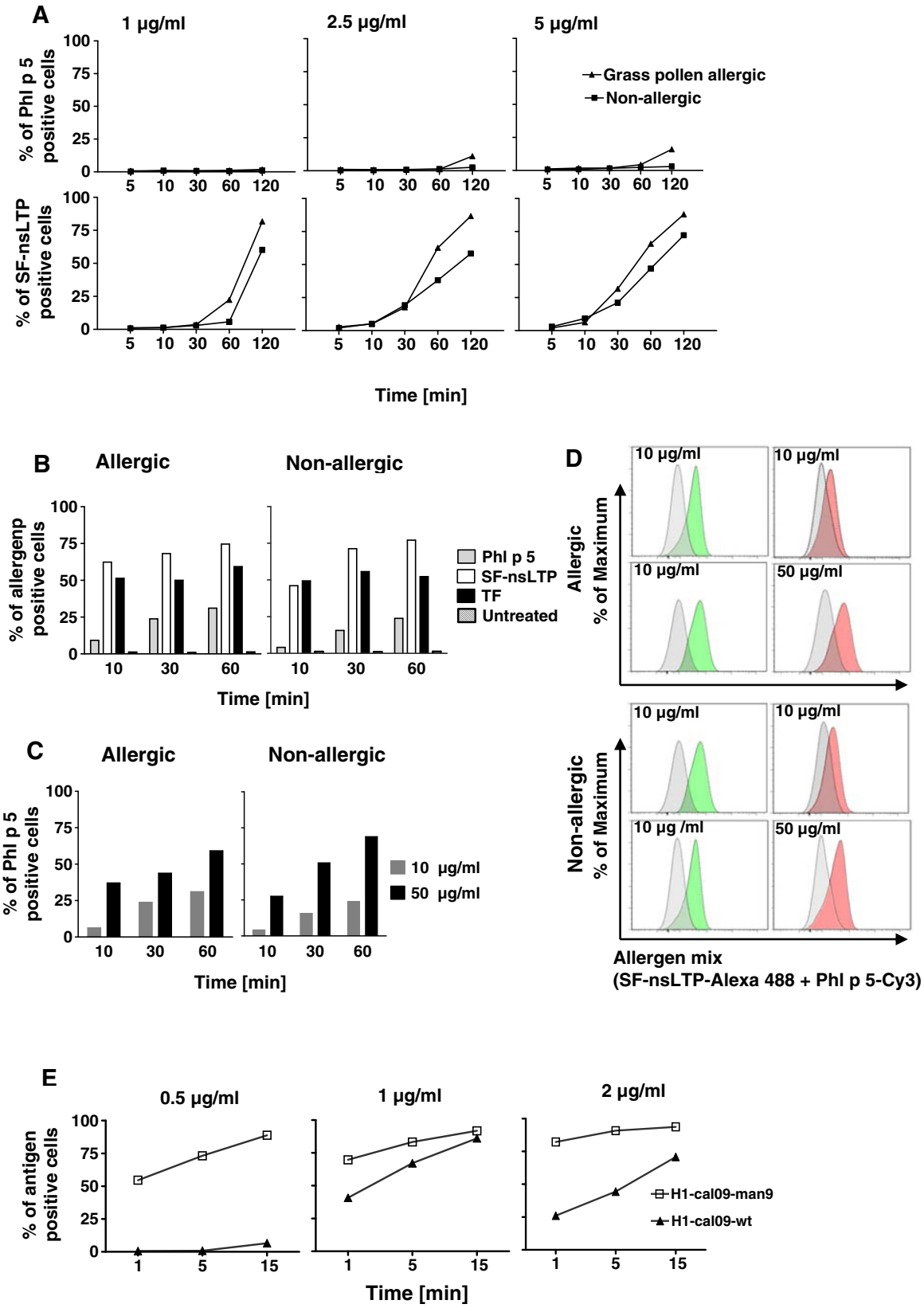

Fig. 4. Flow cytometry analyses of Phl p 5 and SF-nsLTP internalization by MoDCs: Immature MoDCs were incubated with four different concentrations: 1, 2.5, 5 µg/ml (A) and 10 µg/ml (B) of Cy3-Phl p 5, Alexa-Fluor 488-SF-nsLTP at 37 °C. In further experiments different concentrations of Cy3-Phl p 5 (10 and 50 µg/ml) were tested (C). Two different concentrations of Phl p 5 were tested in a competitive assay with mixed allergens (10 µg/ml Alexa-Fluor 488-SF-nsLTP + 10 µg/ml or + 50 µg/ml Cy3-Phl p 5) (D). Flow cytometry uptake assay of H1-Cal09-wt and H1-Cal09-man9 labeled with Alexa-Fluor 488 by MoDCs. Uptake assessments were performed with three different concentrations (0.5, 1, and 2 µg/ml) and at three different time periods (1, 5, 15 min) (E). Uptake was measured by flow cytometry as mean percentage of allergen positive cells. FITC-transferrin (10 µg/ml) was included as another example for receptor-mediated endocytosis.

IgE, cell-bound IgE and IgE receptors has been shown in previous studies (Novak, 2006; Lundberg et al., 2008; Dehlink et al., 2010; Sharquie et al., 2013). In our study, we used serum free medium to generate MoDCs and were unable to detect specific IgE in supernatants from immature MoDCs on day 7 (data not shown). Due to channel limitations we could not quantify PCC values for three components (Phl p 5, IgE and CD23 or FcεRI), simultaneously. However, a representative quantitative analysis of these three components using TissueQuest Software is given in Supplementary Fig. S1. These findings are consistent with a previous study showing that Bet v 1 uptake was equally efficient in both, IgE-positive and IgE-negative cells (Smole et al., 2015). Smole et al. also identified comparable mRNA levels for CD23 and FcεRI in MoDCs from birch pollen allergic and non-allergic donors (Smole et al., 2015). Taken together, our results show that Phl p 5 uptake is not facilitated by FcεRI and CD23 receptor engagement of Phl p 5 specific IgE.

3.4. Antigen/allergen uptake by human MoDCs is time- and concentration-dependent

For quantitative analysis of allergen uptake by MoDCs, we next performed flow cytometry experiments. A previous study showed that Phl p 5 binding in human oral mucosal Langerhans cells is dose- and time-dependent (Allam et al., 2010). In our study immature MoDCs were incubated with 1, 2.5, 5, and 10 μg/ml Cy3-Phl p 5 or AlexaFluor-SF-nsLTP for 5, 10, 30, 60, and 120 min at 37 °C and 10 μg/ml FITC-transferrin for 10, 30, and 60 min at 37 °C. Our results showed a dose- and time-dependent uptake of SF-nsLTP and Phl p 5 by MoDCs

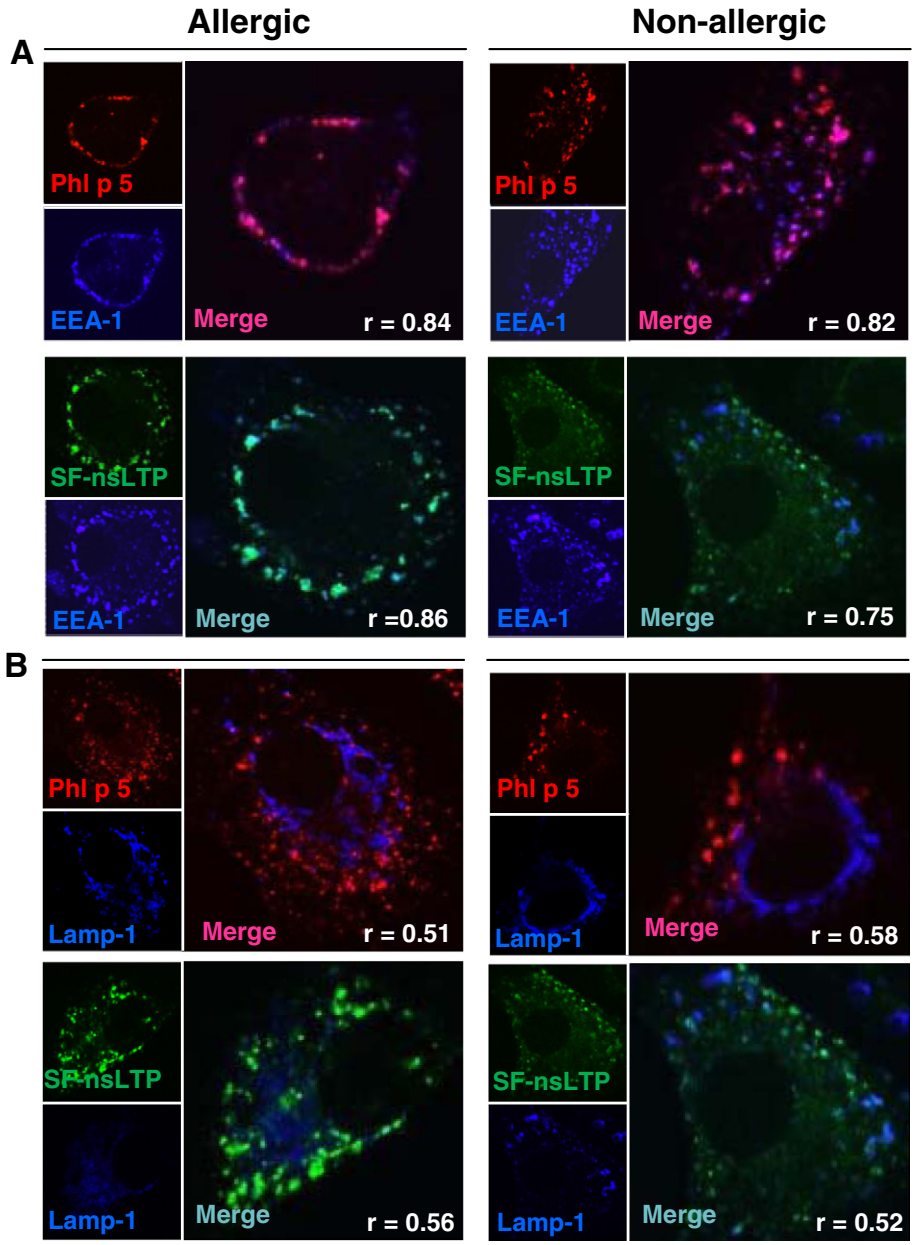

Fig. 5. Localization of allergens in early and late endosomes in MoDCs from grass pollen allergic and non-allergic. MoDCs obtained from allergic and non-allergic donors were incubated with Cy3-Phl p 5 (red) or Alexa-Fluor 488-SF-nsLTP (green) at 37 °C for 15 min to detect EEs and 30–45 min to identify LEs. The cells were fixed, permeabilized and immunostained using antibodies raised against EEA1 (A) or LAMP1 (B) and the associated fluorescence was detected by laser scanning confocal microscopy at 488 nm (green), 568 nm (red) and 633 nm (blue) wavelength. Pearson's coefficient (r) was calculated for a quantitative assessment of colocalization. In competitive assay Phl p 5 + SF-nsLTP, were used simultaneously to follow localization of the allergens using antibodies against EEA1 and LAMP1. (C). Pink (red + blue) and light blue colors (green + blue) indicate overlap of two markers Phl p 5 or SF-nsLTP with EEA1 or LAMP1 whereas, white color indicates a triple overlay of all three markers, Phl p 5, SF-nsLTP and EEA-1 or LAMP1 in competitive assays.

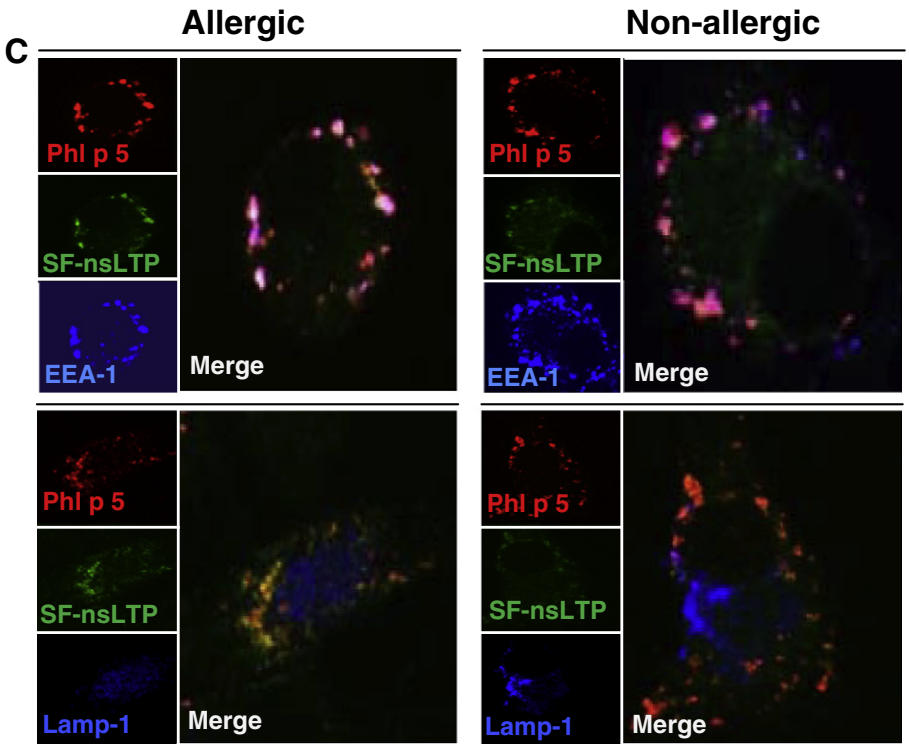

Fig. 5 (*continued*).

(Fig. 4A). At concentrations lower than 10 μg/ml for Phl p 5, almost no allergen uptake could be detected. Internalization of Phl p 5 at 10 μg/ml was still lower than SF-LTP or transferrin (Fig. 4B). A further increase of Phl p 5 concentrations from 10 to 50 μg/ml increased uptake of Phl p 5 at 10, 30, and 60 min at a comparable rate in both allergic and healthy donors (Fig. 4C). Uptake of Phl p 5 after 1 h of incubation at 37 °C increased in both, allergic (from 31% to 62%) and non-allergic (from 23% to 68%) donors (Fig. 4D), again with no significant differences between the two donor groups (Fig. 4A).

In line with the effect of the post-translational modifications used by eukaryotic cells to diversify their protein functions and signaling networks (Wang et al., 2014), uptake analysis of the wild type and the highly mannosidic H1-Cal09 at different time points (1, 5, and 15 min) and concentrations (0.5, 1, and 2 μg/ml) (Fig. 4E) were analyzed by a flow cytometer. At the lowest concentration, 0.5 μg/ml, even after 1 min 54.6% of MoDCs were able to take up H1-Cal09-man9. This percentage was increased to 88.9% after 15 min incubation. In contrast, at the same time points, only 0.6% and 6.6%, H1-Cal09-wt positive cells were detected.

Our analysis indicated that carbohydrate moieties on the influenza HA antigen play a vital role in the recognition and uptake by MoDCs and involvement of specific N-glycosylation patterns can facilitate antigen uptake by MoDCs quickly even at low concentrations (0.5 μg) (Fig. 4E). Such mechanism involving CLR can be ruled out for Phl p 5 and SF-nsLTP, since they are non-glycosylated allergens.

3.5. Transport of Phl p 5 and SF-nsLTP to endosomal compartments in MoDCs from allergic and non-allergic donors

Typically, when eukaryotic cells internalize cell surface proteins or materials from their environment by endocytosis, the material is transferred to EEA1-positive EE. Many ligands dissociate from their receptors at the mildly acidic internal pH of the EE and are delivered for degradation to more acidic, LAMP1-positive LE (Deneka and van der Sluijs, 2002; Vander Lugt et al., 2014).

We performed a set of experiments to visualize Phl p 5 and/or SF-nsLTP localization in EE and LE of MoDCs. MoDCs were incubated with Cy3-Phl p 5, AlexaFluor488-SF-nsLTP or a mixture of both for either 15 min or 45 min to follow trafficking of allergens to early and late endosomal compartments respectively. After fixation, cells were stained with anti-EEA1 and anti-LAMP1 antibodies to visualize early and late endosomal compartments, respectively. MoDCs from allergic as well as non-allergic donors displayed very strong colocalization of Phl p 5 and EEA1 ($r = 0.84$, $r = 0.82$) upon internalization for 15 min. A similar strong colocalization was obtained for SF-nsLTP and EEA1 in MoDCs from allergic and non-allergic donors ($r = 0.86$, $r = 0.75$) (Fig. 5A). Colocalization of Phl p 5 or SF-nsLTP and LAMP1 continuous internalization was considerably lower as compared to EEA1, most likely because a significant fraction of the marker was still localized in EEs. PCC values for colocalization of Phl p 5 and LAMP1 were therefore moderate (allergic: $r = 0.51$, non-allergic: $r = 0.58$), but very similar in MoDCs from allergic as well as non-allergic donors. A similar result was obtained for SF-LTP and LAMP1 colocalization (allergic: $r = 0.56$, non-allergic: $r = 0.52$) (Fig. 5B). Fig. 3C shows the result for the simultaneous uptake of the two allergens and their colocalization with either EEA1 after 15 min or LAMP1 after 30 min, where we likewise could not observe differences between allergic versus non-allergic donors. These results are in line with the study that showed a clear colocalization of Bet v 1 and EEA1 in MoDCs from birch allergic and non-allergic individuals (Kitzmuller et al., 2012). Furthermore, we found very similar colocalization of allergens, Phl p 5 and SF-nsLTP with EEA1 and with LAMP1 in MoDCs obtained from allergic and non-allergic donors (Fig. 5C). In line with other studies these results suggest a common route of allergen trafficking following internalization by Phl p 5 and SF-nsLTP in MoDCs respectively, with no obvious difference between allergic and healthy individuals.

4. Conclusions

For the first time, the simultaneous uptake of an inhalant allergen and a food allergen by MoDCs from grass pollen allergic and non-allergic

individuals has been visualized by immunofluorescence microscopy. The most important finding of this work is an, uptake mechanism of both, Phl p 5 and SF-nsLTP by MoDCs, which does not display significant differences between grass pollen allergic and non-allergic individuals. This in vitro cellular model suggests a receptor-mediated uptake, which is independent from IgE facilitated processes for Phl p 5 and similar intracellular trafficking of Phl p 5 and SF-nsLTP in DCs. The receptors involved in the uptake of the allergens, remain to be identified. Taken together, current data provide evidence for allergen uptake by MoDCs that occurs independently of the allergic status of the donors.

Methods to study the allergen uptake on a molecular level may facilitate the identification of relevant pathways for antigen processing and presentation to T cells. This in turn can provide information to counter-regulate Th2 responses in a therapeutic approach for allergic diseases.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jim.2015.06.001.

Conflicts of interest

The authors declared no conflict of interest.

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