Human Epithelial Cells of the Respiratory Tract and the Skin Differentially Internalize Grass Pollen Allergens

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Epithelial cells of both the respiratory tract and the skin form a tight barrier against environmental harm. They represent the site of first contact for airborne allergen carriers. Consequently, in this study, we analyzed the uptake of grass pollen allergens by epithelial cells: Phl p 1 was selected as a glycosylated allergen containing disulfide bridges whereas Phl p 6 lacks post-translational modifications. Allergen uptake by the respiratory epithelial cell line A549 reached a plateau at 2 hours, and both allergens were localized intracellularly in non-acidic vesicles. In addition, in A549 cells allergens were exocytosed, suggesting a transcytosis mechanism in the passage of allergens over the respiratory epithelial barrier. In contrast, allergens were predominately localized in lysosomes in keratinocytes, and allergen uptake did not reach a plateau up to 24 hours. Notably, keratinocytes from atopic patients showed a significantly increased uptake of Phl p 1 as compared with healthy donors. Preincubation of epithelial cells with IL-4 and/or IFN-γ to simulate inflammatory status led to an increased allergen uptake only in keratinocytes. This higher engulfment of allergens by inflammatory-type keratinocytes suggests a higher susceptibility of inflamed skin for the uptake of allergens and consequently a potentially higher risk for sensitization under natural exposure conditions, such as chronic atopic eczema.

INTRODUCTION

The allergic reaction process begins when a relevant antigen penetrates the boundary surface of susceptible individuals and reaches an antigen-presenting cell that then instructs T cells in a Th2-dominated, proallergic immune response. Airborne antigens such as pollen-derived allergens are mainly exposed to two types of epithelial surfaces: the skin and the respiratory tract. In the skin, the epidermis acts as a physical, chemical, and immunologic barrier. Especially, the stratum corneum comprised of keratin-filled keratinocytes is a potent barrier against antigens (Elias, 2005). Epithelia of the respiratory tract differ significantly from the skin in both morphology and function. The barrier of the respiratory system is composed of the mucociliary system, the surfactant film, airway macrophages, and tight junctions between epithelial cells (Knight and Holgate, 2003). Absorption of allergens across the airway epithelium has been seen in many studies (Greiff *et al.*, 2002; Passalacqua *et al.*, 2005; Reisinger *et al.*, 2005), but only little is known about demonstrating a direct allergen uptake by epithelial cells of the respiratory tract. Equally, there is no data available showing an uptake of allergens by the skin, especially by keratinocytes.

Recent studies support the hypothesis that atopic eczema (AE) is linked to epidermal barrier dysfunctions caused by genetic as well as environmental factors (Cork *et al.*, 2006) leading to increased allergen penetration followed by the recruitment and activation of immune cells and ending in allergen sensitization (Strid *et al.*, 2004). To date, many genes linked with AE have been described (Morar *et al.*, 2006), and most of these genes are involved in the development of physical, chemical, or immunological epithelial barrier functions. The most popular candidate gene for causing AE is the filaggrin gene, which is involved in flattening of keratinocytes into the compact layer of the stratum corneum (Weidinger *et al.*, 2006). As keratinocytes are the outermost cells of the skin and are the first cells encountered by environmental antigens, they are an important factor in triggering immune responses. Alongside toll-like receptors (Mempel *et al.*, 2003), keratinocytes also express major histocompatibility complex (MHC) class II molecules after

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Abbreviations: AE, atopic eczema; AR, allergic rhinoconjunctivitis; CLSM, confocal laser scanning microscopy; HSA, human serum albumin; HRP, horseradish peroxidase; MFI, mean fluorescence intensity; MHC, major histocompatibility complex

activation by inflammatory stimuli (Albanesi *et al.*, 1998). A precondition for keratinocyte presentation of antigens through MHC class II is that the antigens are first internalized. Until now, no MHC class II-dependent activation of immune cells by keratinocytes has been clearly demonstrated.

In this study, we therefore compared the uptake of well-characterized major allergens of timothy grass pollen Phl p 1 and Phl p 6 (Petersen *et al.*, 1995; Blume *et al.*, 2004; Wicklein *et al.*, 2004) by different epithelial cell types. The major allergen Phl p 1 is a 30- to 32 kDa glycoprotein and exists as a variety of isoforms with differing post-translational modifications (Petersen *et al.*, 1995). Besides disulfide bonds, Phl p 1 has both an *N*- and an *O*-glycosylation site (Wicklein *et al.*, 2004). In contrast, the 12 kDa Phl p 6 lacks post-translational modifications, but several degradation products have been described (Blume *et al.*, 2004). As a model of respiratory epithelial cells, the type II resembling human A549 cell line was used (Lieber *et al.*, 1976; Shapiro *et al.*, 1978). To mimic bronchial epithelium, NCI-H727 cells with endocrine properties (Hegedus *et al.*, 1987) and Calu-3 cells isolated from a submucosal adenocarcinoma of conducting airways (Florea *et al.*, 2003) were used. To investigate the allergen uptake in skin epithelial cells, cells from the cell line HaCat and primary keratinocytes from healthy (non-atopic) subjects and patients with allergic rhinoconjunctivitis (AR) or AE were used.

Our data reveal clear differences in allergen uptake between keratinocytes and the respiratory epithelial cell lines. The results suggest transcytosis of allergens by respiratory epithelial cells, whereas in keratinocytes, allergens accumulate in lysosomes—a prerequisite for internal processing. Indeed, a scarce intracellular co-localization of allergen and MHC II could be observed. Inflammatory conditions significantly enhanced allergen uptake in keratinocytes—not in A549 cells—suggesting a higher allergen uptake in inflamed skin. In conclusion, these data point to a disparate assimilation of allergens by respiratory and skin epithelial cells. The transcytosis of allergens by respiratory epithelial cells may enable allergens to come into contact with subepithelially localized antigen-presenting cells.

RESULTS

Time-dependent uptake of allergens

Epithelial cells of the lung are the first cells to get into contact with inhaled pollen allergens. In this study, we analyzed allergen uptake by different airway epithelial cell lines: the respiratory epithelial cell line A549 and the bronchial epithelial cell lines NCI-H727 and Calu-3. The two structurally highly divergent grass pollen allergens Phl p 1 and Phl p 6 as well as the non-allergenic proteins human serum albumin (HSA) and horseradish peroxidase (HRP) were internalized by A549 cells as seen by flow cytometry (Figure 1a). A plateau in fluorescence intensity was reached after an incubation period of 2 hours, and no further significant changes of the mean fluorescence intensity (MFI) were observed over a period of 24 hours. The allergen-uptake kinetics were similar to those of the non-allergenic proteins HSA and HRP (plateau after 6 hours). The bronchial epithelial

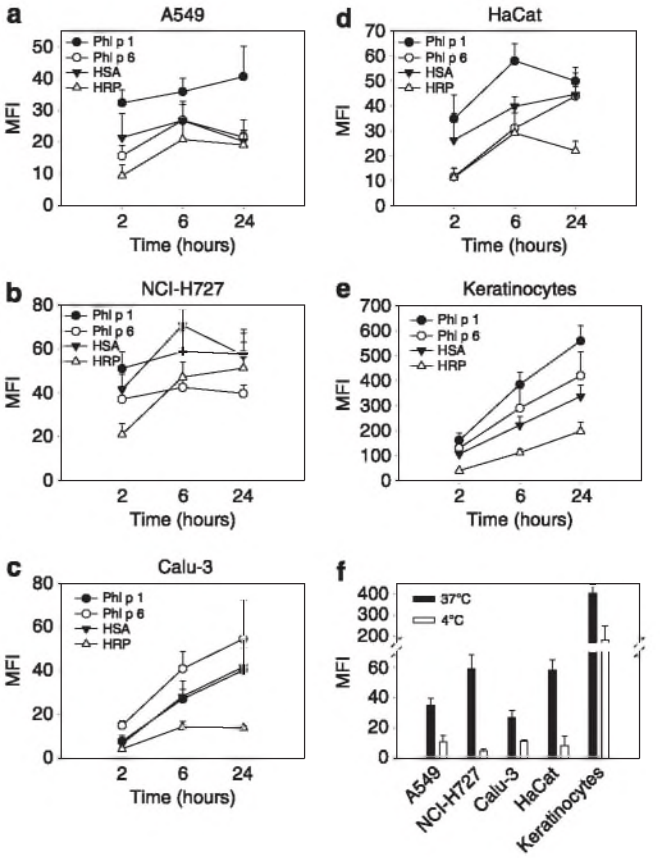

Figure 1. Time-dependent uptake of allergens by epithelial cells. Internalization of allergens by the cell lines A549 (**a**), NCI-H727 (**b**), Calu-3 (**c**), HaCat (**d**), and primary keratinocytes (**e**). Cells were pulsed with 20 μg ml^{-1} of FITC-labeled Phl p 1, Phl p 6, HSA, or HRP for the indicated time periods and mean fluorescence intensity was analyzed by flow cytometry. (**f**) Internalization of Phl p 1-FITC by epithelial cells for 6 hours at 37°C compared with incubation at 4°C. Data are shown as mean MFI ± SEM ($n = 3$–5).

cell lines NCI-H727 and Calu-3 also internalized allergenic and non-allergenic proteins (Figure 1b and c). Whereas the uptake of proteins by NCI-H727 was comparable with that by A549 cells (compare Figure 1b and a), we observed differences in the time-dependent uptake of proteins by Calu-3 cells. As shown in Figure 1c, the uptake of proteins by Calu-3 cells increased over time and a plateau was not observed except for HRP.

Besides the respiratory tract, the skin is also an important contact surface for pollen-derived allergens. Therefore, in analogy to our experiments with airway epithelial cell lines, time-dependent allergen uptake by the keratinocyte cell line HaCat and primary human keratinocytes was analyzed by flow cytometry. In HaCat cells, the maximal MFI was reached after 6 hours and was constant over 24 hours (Figure 1d). In contrast to HaCat cells, the uptake of Phl p 1 and Phl p 6 as well as HSA and HRP by primary human keratinocytes increased over time (Figure 1e). A plateau in allergen uptake was not observed. Notably, the overall level of allergen uptake was significantly higher for keratinocytes than for all

of the other cell lines tested. Furthermore, all cells tested gave a higher MFI for Phl p 1 as compared with Phl p 6, HSA, and HRP, an effect that is caused by different FITC-labeling efficiencies of the proteins. Labeling efficiency depends on the 3D structure and the number of lysine residues present in the proteins. Phl p 1 contains 28 lysine residues, whereas Phl p 6 contains only 9.

To exclude an unspecific adsorption of fluorescently labeled proteins, we incubated the cells with fluorescently labeled proteins at 4°C. After 6 hours, the mean fluorescence intensities of all cells incubated at 4°C with FITC-labeled Phl p 1 were significantly lower than those after incubation at 37°C (Figure 1f). As these effects were also observed for Phl p 6 and the non-allergenic proteins HSA and HRP (data not shown), we can exclude unspecific adsorption of unlabeled dye and conclude that the proteins were internalized by an active process in the epithelial cells tested.

Intracellular localization of allergens

Intracellular localization of the allergens was analyzed through confocal laser scanning microscopy (CLSM). By counter-staining internalized fluorescently labeled allergens with monoclonal antibodies, we observed a high degree of co-localization, indicating the uptake of allergens and not unlabeled dye (data not shown). Staining the membrane of A549 cells with an anti-CD29 antibody revealed that only a small amount of the allergens was detected at the plasma membrane. The major amount of allergens was localized in cytoplasmatic vesicles (data not shown). Cellular vesicles were further analyzed by staining with the pH-sensitive dye LysoTrackerRed, which exclusively labels the acidic lysosomal compartment. This staining revealed that in A549 cells, Phl p 1 is not localized in lysosomes (Figure 2a). The same distribution was observed for Phl p 6 (data not shown). These results indicate that in A549 cells allergens remain in non-acidic vesicles.

In bronchial epithelial cell lines, NCI-H727 and Calu-3, the allergens are also localized in vesicles. In contrast to A549 cells, a small amount of allergens is localized in acidic vesicles, indicating that allergens are delivered into lysosomes in NCI-H727 and Calu-3 cells (Figure 2b and c). However, most of the allergens are localized in non-acidic vesicles as observed in A549 cells.

In keratinocytes, a different intracellular distribution of allergens was observed. In HaCat cells as well as in primary keratinocytes, Phl p 1 was primarily localized in cytoplasmatic vesicles, and these vesicles were characterized as lysosomes by staining with the dye LysoTrackerRed and CLSM (Figure 2d and e). In keratinocytes, the intracellular localization of Phl p 6 was comparable with that of Phl p 1 (data not shown). The localization of allergens in lysosomes might indicate a degradation by lysosomal proteases. As lysosomal proteins can be presented by MHC class II and keratinocytes showed an expression of HLA-DR after IFN-γ stimulation, we additionally stained the allergen-pulsed keratinocytes intracellularly with an anti-HLA-DR antibody. As shown in Figure 2f, a considerable amount of Phl p 1-positive vesicles also express HLA-DR.

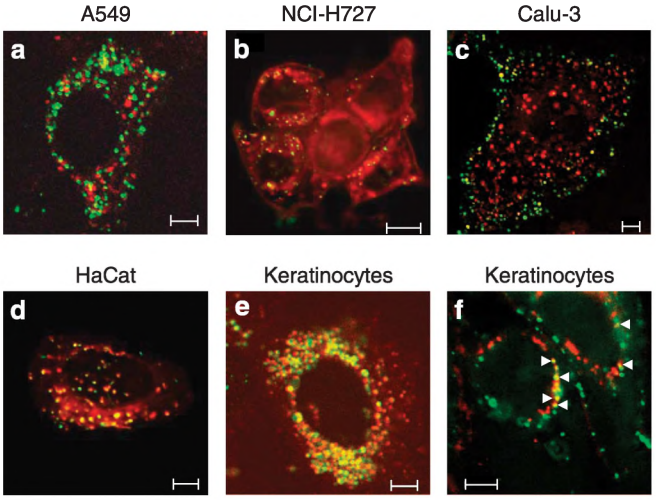

Figure 2. Characterization of allergen-bearing vesicles in epithelial cells. After incubation of the cells with 20 μg ml^{-1} of fluorescently labeled Phl p 1 for 24 hours, lysosomes were stained with LysoTrackerRed (**a**–**e**) or anti-HLA-DR antibody (**f**) and the cells analyzed by confocal laser scanning microscopy. (**a**–**e**) Lysosomal localization of Phl p 1-Alexa488 in A549 cells (**a**), NCI-H727 cells (**b**), Calu-3 (**c**), HaCat (**d**), and primary keratinocytes (**e**). Red staining indicates lysosomes, green staining represents the fluorescence of Phl p 1-Alexa488, and yellow staining displays a co-localization. (**f**) Staining of primary keratinocytes pulsed with Phl p 1-Alexa488 (green) for 24 hours with anti-HLA-DR antibody (red). Co-localization (yellow staining) is indicated by arrows. Results are representative of one experiment repeated three times. Scale bar = 5 μm.

Pulse-chase experiments

After internalization, allergens may be released by exocytosis. To investigate this mechanism, we performed pulse-chase experiments. A549 cells were pulsed with allergens or non-allergenic proteins for 2 hours, washed, and incubated for different time intervals in fresh medium. Directly after the 2-hour pulse, the MFI was defined as 100% uptake (equivalent to MFI ± SEM Phl p 1: 46.16 ± 9.44; Phl p 6: 21.92 ± 5.62; HSA: 16.23 ± 3.33; and HRP: 9.23 ± 3.22). After a 2-hour chase period, only 50–70% of the initially internalized proteins remained inside the cells and after a 6 hour chase period about 40% (Figure 3a). After a chase period of 24 hours, only background levels of proteins were observed inside A549 cells. Furthermore, the incubation of A549 cells at 4°C after the pulse with fluorescently labeled proteins resulted in no decrease of fluorescence (Figure 3b), indicating an active process of protein delivery. These data suggest that A549 cells release the internalized proteins by exocytosis.

In primary keratinocytes pulsed for 2 hours with Phl p 1 (MFI 191.38 ± 23.30), Phl p 6 (MFI 176.07 ± 32.83), HSA (MFI 89.22 ± 17.36), or HRP (MFI 26.01 ± 5.16), constant levels of fluorescence were observed (Figure 3c). Only after 24 hours a slight decrease in fluorescence intensity was observed. Differences between the proteins were not seen. This indicates that, in contrast to A549 cells, keratinocytes retain the proteins and do not release them by exocytosis. In addition, the analysis of released Phl p 1 in the supernatant through ELISA showed that A549 cells released higher

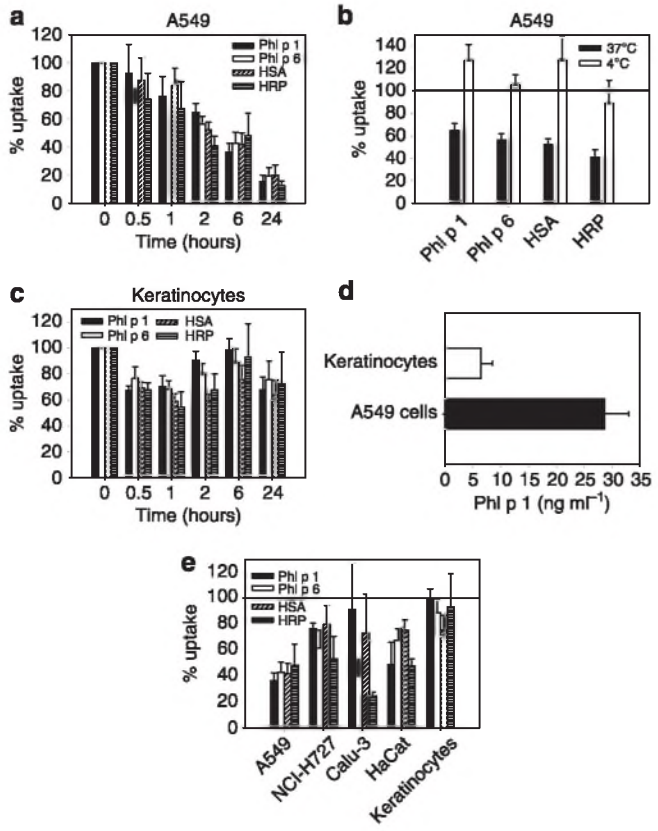

Figure 3. Exocytosis of proteins by epithelial cells. Pulse-chase experiments of protein uptake by epithelial cells. Cells were incubated for 2 hours with 20 μg ml^{-1} of fluorescently labeled proteins and washed extensively. Fresh medium without labeled proteins was added and the mean fluorescence intensity of the cells was analyzed by flow cytometry at indicated time points. The mean fluorescence intensity at time point 0 was defined as 100% uptake. (**a**) Pulse-chase experiments with proteins in A549 cells. (**b**) After the 2-hour pulse with the proteins, A549 cells were incubated in fresh medium for 2 hours at 37 and 4°C. (**c**) Pulse-chase experiments of proteins by primary keratinocytes. (**d**) Detection of Phl p 1 in the supernatant of A549 cells and primary keratinocytes by ELISA after a chase period of 24 hours. (**e**) Comparison of retained proteins by different epithelial cells after a chase period of 6 hours. Data are shown as mean ± SEM ($n = 3$).

amounts of Phl p 1 after 24 hours as compared with keratinocytes (Figure 3d).

Bronchial epithelial cell lines NCI-H727 and Calu-3 also showed a release of proteins, but these effects were delayed as compared with A549 cells. Again, the MFI after the 2-hour pulse was defined as 100% uptake in NCI-H727 cells (Phl p 1: 36.24 ± 1.38; Phl p 6: 32.54 ± 0.09; HSA: 27.17 ± 5.27; and HRP: 14.88 ± 2.47) and Calu-3 cells (Phl p 1: 7.87 ± 1.71; Phl p 6: 14.72 ± 2.19; HSA: 7.62 ± 2.18; and HRP: 11.99 ± 1.29). After a chase period of 6 hours, 60–80% of the proteins remained in the cells (Figure 3e), whereas A549 cells retain only 50% of the proteins. A delayed decrease in fluorescence intensity was also observed in the keratinocyte cell line HaCat after a pulse of 2 hours with fluorescently labeled proteins compared with A549 cells. After a chase period of 6 hours, about 50–80% of initially internalized proteins are retained in HaCat cells (100% uptake equivalent to MFI ± SEM: Phl p 1 46.01 ± 25.47; Phl p 6 16.42 ± 6.39; HSA 30.18 ± 15.01; HRP 12.99 ± 5.63), a percentage that is comparable with primary human keratinocytes.

Impact of an inflammatory microenvironment on allergen uptake

As inflammatory stimuli are potent mediators of allergic immune reactions, we investigated the effect of IFN-γ and IL-4 on the internalization of allergens and non-allergenic proteins by airway epithelial cells. Activation of A549 cells by IFN-γ stimulation was mirrored by an enhanced ICAM-1 expression (Figure 4b), which was further increased by an additional stimulation with IL-4. However, the expression of HLA-DR was not induced in A549 cells by IFN-γ stimulation. In A549 cells, the uptake of Phl p 1 and Phl p 6 as well as HSA and HRP was not affected by stimulation with IFN-γ and/or IL-4 (Figure 4a). In addition, the uptake of proteins by the bronchial epithelial cell lines NCI-H727 and Calu-3 was also unaffected by IFN-γ and IL-4 stimulation (Table S1). In NCI-H727 cells, IFN-γ stimulation resulted in no expression of HLA-DR, whereas the expression of ICAM-1 was increased as shown for A549 cells (data not shown). In contrast, Calu-3 cells showed an increased expression of HLA-DR and ICAM-1 after stimulation with IFN-γ (data not shown), but internalization of proteins was not affected.

In primary keratinocytes, the stimulation with IFN-γ as well as combined stimulation with IFN-γ and IL-4 resulted in an increased expression of ICAM-1 and HLA-DR (Figure 4d). In contrast to A549 cells, the uptake of Phl p 1 was significantly increased in primary keratinocytes stimulated with IFN-γ (Figure 4c). IFN-γ-stimulated primary keratinocytes also showed a significantly enhanced uptake of Phl p 6. The uptake of HSA and HRP by primary keratinocytes was also increased after IFN-γ stimulation. Interestingly, IL-4 stimulation alone had no effect on allergenic or non-allergenic protein internalization, but a combined stimulation of primary keratinocytes with IFN-γ and IL-4 resulted in an enhanced uptake of Phl p 1 and Phl p 6 as compared with untreated primary keratinocytes. In contrast to keratinocytes stimulated with IFN-γ only, the uptake of Phl p 1 was also enhanced in keratinocytes stimulated with both IL-4 and IFN-γ. This effect was also observed after incubation of keratinocytes with allergenic and non-allergenic proteins for a time period 24 hours (Table S1). However, this effect was only statistically significant for keratinocytes incubated with Phl p 1. In HaCat cells, IFN-γ stimulation also induced the expression of ICAM-1 and HLA-DR (data not shown), and the uptake of proteins by HaCat cells was also increased after IFN-γ stimulation (Table S1).

Uptake of allergens by keratinocytes from different donors

To detect differences in the internalization of allergens by keratinocytes from donors with or without atopic diseases, we analyzed the uptake of Phl p 1 and Phl p 6 as well as the non-allergenic proteins HSA and HRP by primary keratinocytes from non-atopic and atopic donors, including AE patients. The group of AR patients showed a significantly

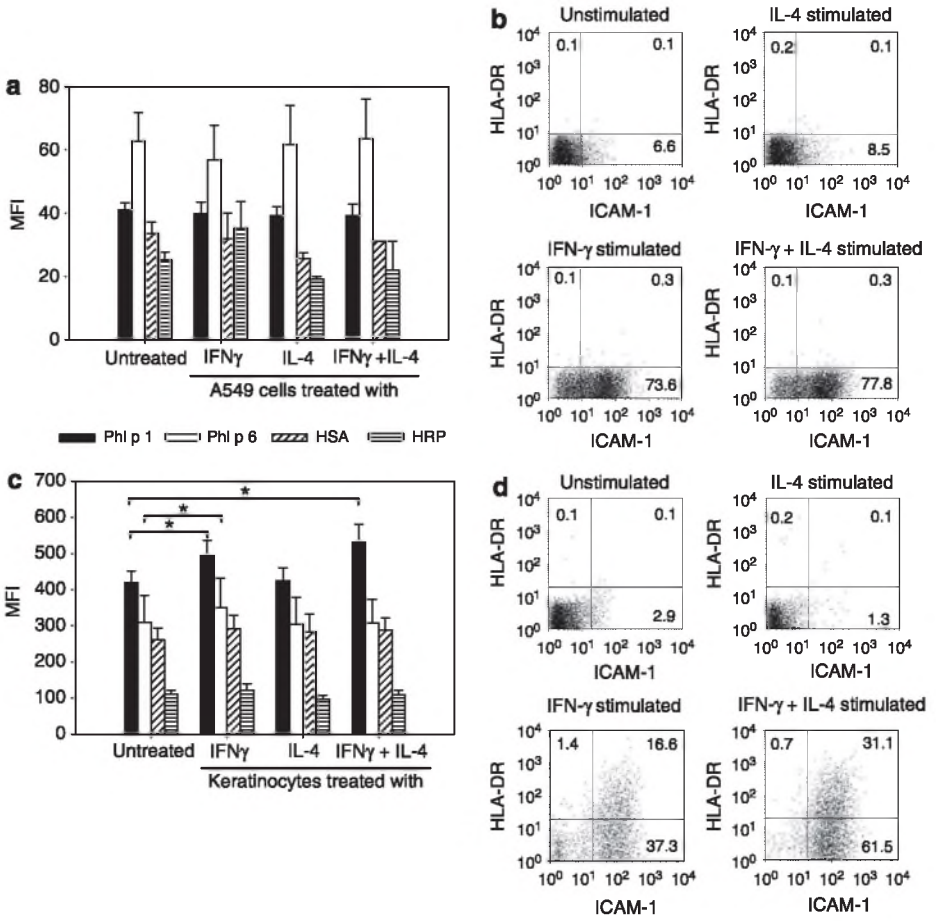

Figure 4. Uptake of allergens by epithelial cells under inflammatory conditions. Effect of IFN-γ and IL-4 stimulation on protein uptake by A549 cells (**a**) and keratinocytes (**c**). Before allergen pulse, cells were stimulated for 24 hours with IFN-γ, IL-4, or IFN-γ in combination with IL-4. Stimulated cells were pulsed for 6 hours with 20 μg ml^{-1} of FITC-labeled Phl p 1, Phl p 6, HSA, or HRP and MFI was analyzed by flow cytometry. Data are shown as mean MFI ± SEM (Phl p 1 and Phl p 6: A549 cells $n=7$, keratinocytes $n=9$; HSA and HRP: A549 cells $n=3$, keratinocytes $n=6$; *$P<0.05$ versus untreated). (**b** and **d**) Expression of ICAM-1 and HLA-DR by A549 cells (**b**) and keratinocytes (**d**) after IFN-γ and IL-4 stimulation. Results are representative of one experiment repeated five times.

increased uptake of Phl p 1 after 24 hours as compared with non-atopic donors (Figure 5a). An increased uptake of Phl p 1 was also observed after 2 and 6 hours (data not shown). A difference in allergen uptake between non-atopic donors and AE patients was not observed. The uptake of Phl p 6, HSA, and HRP was also enhanced in keratinocytes from AR patients, but significant levels were not achieved (Phl p 6: $P=1$; HSA: $P=0.157$; HRP: $P=0.355$). To exclude differences in the differentiation status of keratinocytes from different donors, levels of cytokeratin 14 (marker of undifferentiated keratinocytes) and involucrin (upregulated in differentiated keratinocytes) expression in keratinocytes from non-atopic donors, AR and AE patients were analyzed by flow cytometry. Over all, keratinocytes of all groups tested exhibited a relatively high expression of cytokeratin 14, whereas involucrin was low—thus keratinocytes were not differentiated. Furthermore, no significant difference in cytokeratin 14 expression levels between the keratinocytes of the three groups tested could be observed (Figure 5b). Concerning the involucrin expression only keratinocytes from AE patients showed a significant increase compared with non-atopic donors (Figure 5c). However, to explore any correlation between allergen uptake and the differentiation status of keratinocytes, keratinocytes were differentiated *in vitro* by Ca^{2+} for 3 days. Differentiated keratinocytes showed a slight decrease in allergen internalization (Figure 5d), without reaching statistical significance (Phl p 1: $P=0.289$; Phl p 6: $P=0.275$).

DISCUSSION

The epithelium is the site of first contact with environmental substances such as microbes, noxious chemicals, or allergen carriers. Pollen are the most frequent source of airborne allergens, and allergens are released within seconds after hydration, in a process influenced by air pollutants (Behrendt and Becker, 2001). In particular, the skin and respiratory tract epithelia are exposed to pollen allergens. After hydration, the pollen grains release starch granules, which are covered by allergens and have a diameter less than 7.5 μm (Schäppi *et al.*, 1999). In addition, fine dust particles, such as diesel exhaust particles, can adsorb allergens (Knox *et al.*, 1997). These fine particles presumably help transport allergens to the lower airways and thus can induce allergic immune responses. It is accepted that sensitization toward airborne

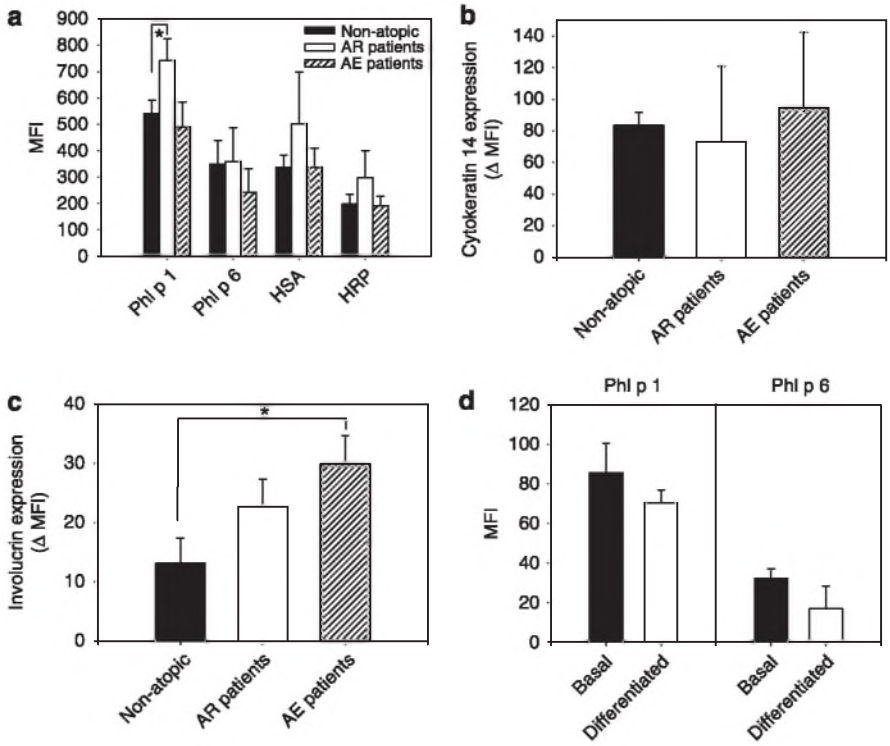

Figure 5. Allergen uptake of primary keratinocytes depending on the atopy status of the donor. Protein uptake and differentiation of primary keratinocytes from non-atopic donors, AR and AE patients. (**a**) Keratinocytes from non-atopic donors ($n=9$) and AR as well as AE patients ($n=8$) were incubated with 20 μg ml^{-1} of FITC-labeled proteins for 24 hours and MFI was analyzed by flow cytometry (*$P<0.05$ vs non-atopic). (**b** and **c**) Expression levels of cytokeratin 14 (**b**) and involucrin (**c**) were determined by intracellular staining and flow cytometry. (**d**) Uptake of Phl p 1 and Phl p 6 for 6 hours by undifferentiated (basal) and differentiated keratinocytes ($n=5$). Data are shown as mean MFI ± SEM.

allergens occurs primarily through the respiratory tract. We investigated whether this effect is due to a different handling of allergens/proteins by skin- and respiratory tract-derived epithelial cells, which are highly different in both morphology and physiology. Thus, we compared allergen internalization by keratinocytes and airway epithelial cells. As a model for respiratory epithelial cells, we used A549 cells, a cell line related to type II alveolar epithelial cells (Lieber *et al.*, 1976). The uptake of allergens by bronchial epithelial cells was modeled using NCI-H727 and Calu-3 cells.

Interestingly, we observed clear differences between airway epithelial cells and keratinocytes in their allergen uptake and processing. The respiratory epithelial cell line A549 showed a constant level of allergen uptake over time and allergens were localized in intracellular vesicles. As these vesicles were not characterized as lysosomes, it is most likely that the allergens were not cleaved by lysosomal proteases. In addition, allergens were released rapidly by exocytosis. These results suggest the transcytosis of intact allergens across the respiratory epithelium where they can come into contact with subepithelial dendritic cells, which in turn may favour sensitization in susceptible individuals. Unidirectional transport of an exogenous protein, the HRP, across the airway epithelium was shown by Matsukawa *et al.* (1996), and a receptor-mediated apical-to-basolateral transcytosis of albumin across the alveolar epithelium resulted in the release of over 90% intact albumin (Kim *et al.*, 2003). These data are consistent with the rapid allergen release and the weak localization of allergens in lysosomes observed in this study for the respiratory epithelial cell line A549. Transcytosis of allergens by respiratory epithelial cells may also explain higher serum concentrations of proteins after lower airway absorption than after nasal absorption observed in a mouse model (Hens *et al.*, 2007).

Allergens and non-allergenic proteins were also internalized by the bronchial epithelial cell lines NCI-H727 and Calu-3. As shown for A549 cells, NCI-H727 cells exhibit a constant level of protein uptake, and the internalized proteins were released by exocytosis. However, this process was delayed with respect to A549 cells. In contrast, Calu-3 cells showed an increase in protein uptake over time. Pulse-chase experiments showed that Calu-3 cells release internalized proteins by exocytosis, even though also delayed compared with A549 cells. In addition, in bronchial epithelial cell lines, but not A549 cells, a small amount of allergen is localized in lysosomes. These differences in protein uptake between A549, NCI-H727, and Calu-3 cells may be due to their different origins. A549 cells are related to type II alveolar epithelial cells, whereas Calu-3 cells were isolated from a submucosal adenocarcinoma of conducting airways (Florea *et al.*, 2003) and NCI-H727 cells are bronchial epithelial cells with endocrine properties (Hegedus *et al.*, 1987).

Airway epithelial cells are discussed to act as non-professional antigen-presenting cells. A prerequisite for

antigen presentation is the expression of MHC class II and co-stimulating molecules, as well as antigen-processing proteases like cathepsins. Salik *et al.* (1999) and Oei *et al.* (2004) showed an expression of co-stimulating molecules, cathepsins as well as HLA-DR in A549 cells and primary respiratory epithelial cells. In our study, we did not detect any HLA-DR expression by flow cytometry in either A549 or NCI-H727 cells even after IFN-γ stimulation, in contrast to Calu-3 cells, in which HLA-DR expression was observed. In terms of allergen uptake, we observed no differences between IFN-γ-stimulated and resting A549, NCI-H727, and Calu-3 cells. Together with the obvious lack of localization of allergens in the lysosomes of A549 cells and the absence of HLA-DR in A549 and NCI-H727 cells, a processing and presentation of allergens by A549 and NCI-H727 cells seems unlikely. As allergens in Calu-3 cells are localized in the lysosomes and Calu-3 cells express HLA-DR, a presentation of antigens is possible.

To date, the mechanisms of allergen entry at the site of first contact, such as the skin and the bronchial tract, and subsequent sensitization are unclear. Many recent studies support the hypothesis that apart from the respiratory tract, the skin also plays a key role in sensitization against allergens, especially through barrier dysfunctions caused by genetic and environmental factors (reviewed by Cork *et al.* (2006)). Keratinocytes are as outermost skin cells, the primary contact site for environmental agents. The stratum corneum forms both a mechanical and chemical barrier through defensins and proteases cleaving proteins, thus preventing protein/allergen contact with living keratinocytes (Cork *et al.*, 2006). By analyzing the keratinocyte handling of different allergens and proteins we observed that in contrast to respiratory epithelial cells, primary keratinocytes showed a continuous increase of allergen uptake over time and the highest allergen/protein uptake of all cells investigated. As seen in pulse-chase experiments, allergens remained inside the cells and were not released by exocytosis. The observed slight decrease in the fluorescence intensity of primary keratinocytes in the pulse-chase experiments after 24 hours may be caused by keratinocyte proliferation. Compared with A549 cells, the uptake of Phl p 1 by primary keratinocytes after a 2-hour pulse was fivefold higher; however, after a 24-hour chase period, the amount of released Phl p 1 in the supernatant was fourfold lower. Furthermore, the intracellular localization of allergens in primary keratinocytes differed from the localization in A549 cells. In primary keratinocytes, Phl p 1 and Phl p 6 were localized in lysosomes indicating a potential cleavage of the allergens by lysosomal proteases. Under inflammatory conditions as simulated by IFN-γ stimulation, keratinocytes exhibited an increased allergen uptake. It has been shown that in keratinocytes, IFN-γ also induces the expression of cathepsin S (Schwarz *et al.*, 2002), which is essential for the cleavage of the invariant chain of MHC class II complexes (Riese *et al.*, 1996) and is also involved in the generation of peptide epitopes from exogenous proteins (Hsieh *et al.*, 2002). Consequently, keratinocytes should have the potential to process and present allergens to T cells. In a model for contact allergy, MHC-dependent presentation of nickel by keratinocytes results in an enhanced apoptosis of keratinocytes induced by antigen-specific T cells (Traidl *et al.*, 2000). However, antigen presentation by keratinocytes does not lead to induced proliferation of T cells, but rather to the inhibition of T-cell proliferation and the release of T-cell-derived cytokines (Foerster *et al.*, 2007). Stimulation of keratinocytes with IFN-γ and IL-4 resulted in a much higher internalization of allergens compared with keratinocytes stimulated with IFN-γ alone. This is in line with data from Albanesi *et al.* (2000) showing the additive effects of IL-4 and IFN-γ on keratinocytes with respect to chemokine release. Furthermore, our data substantiate the atopic march hypothesis, in which inflamed tissue such as chronic AE is more prone to allergen uptake and subsequent sensitization against environmental allergens.

As keratinocytes can modulate immune responses, they may also play a central role in the sensitization to allergens and in the manifestation of atopic diseases. Therefore, it is important to further analyze the processing and presentation of allergens by keratinocytes and to elucidate the differences in the uptake, processing, and presentation of allergens between atopic and non-atopic individuals. In this study, we show a significantly increased uptake of Phl p 1 by keratinocytes from AR patients and a lack of enhancement in AE patients. The possibility that the increase in Phl p 1 uptake by keratinocytes from AR patients is caused by an altered differentiation of the keratinocytes is unlikely, as the expression levels of involucrin and cytokeratin 14 in non-atopic donors' and AR patients' keratinocytes were comparable and the uptake of Phl p 6 is the same in all three groups. The decreased uptake of Phl p 1 by keratinocytes from AE patients compared with AR patients might be due to an increased differentiation status of keratinocytes from AE patients as the expression level of involucrin is significantly increased in keratinocytes from AE patients. A further hint that the differences in keratinocyte differentiation status do account for the altered allergen uptake observed in AE patients is given by the fact that differentiated keratinocytes showed a decreased uptake of Phl p 1 and Phl p 6.

The observed differences between the uptake of these two allergens may reflect their highly different structures. Phl p 6 is only 12 kDa and lacks post-translational modifications (Blume *et al.*, 2004), whereas Phl p 1 is a 30- to 32 kDa glycoprotein with disulfide bonds (Petersen *et al.*, 1995; Wicklein *et al.*, 2004). Especially, the glycosylation of Phl p 1 may account for the increased uptake, possibly by Phl p 1 binding to lectins expressed by keratinocytes (Cerdan *et al.*, 1991; Szolnoky *et al.*, 2001). Consistent with this, the expression of these receptors is increased by cytokines, such as tumor necrosis factor. These data are supported by the increased uptake of the glycosylated proteins HSA and HRP by keratinocytes of AR patients. In summary, this study shows that allergen uptake by epithelial cells is dependent not only on the structure of the allergen, but also on the atopic background of the donor and on the nature of the epithelial cell: respiratory or skin derived. The increased uptake of allergens by keratinocytes in inflammatory status suggests an

increased uptake of allergens in inflamed skin and possibly a higher risk for sensitization under natural exposure in conditions such as chronic AE.

MATERIALS AND METHODS

Patients and control subjects

Keratinocytes were obtained from non-atopic individuals ($n=9$) and from patients with AR ($n=8$) as well as from AE patients ($n=8$). Non-atopic individuals were defined by a normal total IgE titer below 20 kU l^{-1} in the serum, a negative radio-allergen-sorbent-test (RAST) against eight common environmental allergens, and the absence of atopic diseases in history. Patients with AR showed an increase in grass pollen-specific IgE (RAST class $\geqslant 2$) accompanied by allergic symptoms such as AR. Patients with AE diagnosed according to the criteria of Hanifin and Rajka (1980) (Ring *et al.*, 2006) showed also symptoms of AR and exhibited IgE against grass pollen. The study was performed in adherence to the Declaration of Helsinki Guidelines. Patients were enrolled in the study after written informed consent, and the study was approved by the medical ethical committee of Technische Universität Munich.

Isolation of grass pollen allergens

The native allergens Phl p 1 and Phl p 6 were isolated from timothy grass pollen by chromatography as described earlier (Suck *et al.*, 1999, 2003). Briefly, proteins were extracted from *Phleum pratense* pollen (ARTU Biologicals, Lelystad, The Netherlands) by incubation for 30 minutes in 20 mM Tris and 1 mM EDTA (pH 8.0) at 37°C. Phl p 1 was isolated from the pollen extract by hydrophobic interaction chromatography and size exclusion chromatography using Phenyl Sepharose High Performance and Superdex 75 (GE Healthcare Life Sciences, Uppsala, Sweden). Phl p 6 was isolated by a combination of metal chelate chromatography using a chelating sepharose charged with Ni^{2+} ions (GE Healthcare Life Sciences), followed by hydrophobic interaction chromatography using Phenyl Sepharose High Performance and size exclusion using Superdex 75.

Fluorescent labeling of proteins

Allergens and control proteins, human serum albumin fraction V (HSA; Sigma, Taufkirchen, Germany), and inactivated HRP (Sigma) were dissolved in 0.2 M $NaHCO_3$, pH 8.3, at a concentration of 5 mg ml^{-1}. A FITC solution (10 mg ml^{-1} in dimethyl sulfoxide; Sigma) was added to achieve a molar ratio of 60:1 and Alexa Fluor 488 carboxylic acid succinimidyl ester (Alexa488; Molecular Probes, Eugene, OR) a ratio of 100 µg per 1 mg protein. After incubation for 2 hours at room temperature under stirring, unbound dye was removed by chromography using a HiTrap Desalting column (GE Healthcare Life Sciences).

Culture of cell lines

The human respiratory epithelial cell line A549, the bronchial epithelial cell lines NCI-H727 and Calu-3, and the keratinocyte cell line HaCat were obtained from American Type Culture Collection (Manassas, VA). A549 and NCI-H727 cells were cultured in DMEM supplemented with 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (PAA Laboratories, Cölbe, Germany), and 10% fetal calf serum (Biochrom, Berlin, Germany) and Calu-3 cells in 50% RPMI, 50% F-12 Nutrient Mixture supplemented with penicillin–streptomycin (GIBCO, Karlsruhe, Germany), and 10% fetal calf serum (Perbio Science, Bonn, Germany) at 37°C and 95% humidity. HaCat cells were cultured in RPMI supplemented with 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin (GIBCO), and 10% fetal calf serum (Perbio Science). Cells were cultured to 80% confluence, harvested using trypsin-EDTA, and seeded on sterile 24-well culture plates for experiments. After 2 days in culture, cells reached 70% confluence and the experiments were conducted.

Isolation and culture of keratinocytes

Primary human keratinocytes were isolated from the epidermis by suction blister as described earlier (Traidl *et al.*, 2000). Briefly, single-cell suspensions of epidermal cells from suction blisters' roofs were prepared by trypsinization of the blister roof and seeded on a feeder layer of 3T3/J2 fibroblasts treated with 10 µg ml^{-1} mitomycin (Sigma) for 2 hours. First-passage keratinocytes were cryopreserved in liquid nitrogen. Experiments were performed in sterile 48-well culture plates with second-passage keratinocytes grown in keratinocyte growth medium (PromoCell, Heidelberg, Germany) to 70% confluence 5 days after seeding. Differentiation of primary keratinocytes was induced by supplementing the culture medium with 1.2 mM $CaCl_2$ for 3 days.

Internalization of allergens

At 70% confluence, epithelial cells were washed once with phosphate-buffered saline (PBS) (GIBCO), resuspended in fresh medium, and pulsed with 20 µg ml^{-1} of fluorescently labeled allergens and proteins. After different incubation periods, cells were washed extensively with PBS and harvested with trypsin-EDTA. The MFI was analyzed by flow cytometry using Epics XL-MCL (Beckman Coulter, Krefeld, Germany) or FACS Calibur (Becton Dickinson, Heidelberg, Germany).

Pulse-chase experiments

For the pulse-chase experiments, cells were pulsed for 2 hours with 20 µg ml^{-1} of FITC-labeled allergens or proteins. After washing the cells extensively with PBS, fresh medium was added. Mean fluorescence of the cells was analyzed by flow cytometry at the time points 0, 2, 6, and 24 hours after adding fresh medium. MFI at time point 0 was defined as 100%. Alternatively, after 24 hours, supernatants were taken and the content of Phl p 1 released by the cells was analyzed by ELISA.

Quantification of Phl p 1 by sandwich ELISA

MaxiSorp ELISA Plates (Nunc, Wiesbaden, Germany) were coated with the Phl p 1-specific monoclonal antibody HB 7 (Petersen *et al.*, 2007) (4 µg per well in 60 µl of 65 mM Na_2CO_3, pH 11, overnight at 4°C). All further steps were performed at room temperature in a humidity chamber. Tris-buffered saline containing 0.05% (v/v) Tween 20 (TTBS) was used for washing and antibodies were diluted in TTBS containing 2.5% (w/v) skimmed milk powder. Plates were washed seven times with TTBS and then blocked for 2 hours with TTBS containing 5% (w/v) skimmed milk powder. After recurrent washing, 60 µl per well of Phl p 1 standard solution ranging from 1 ng ml^{-1} to 1 µg ml^{-1} or 60 µl per well of supernatants were added in duplicates. Supernatants of A549 cells were concentrated fourfold and supernatants of keratinocytes were concentrated eightfold in a SpeedVac. After 2 hours of incubation, plates were extensively

washed and incubated for 2 hours with a rabbit hyperimmune serum directed against Phl p 1 (diluted 1:1,000). Subsequently, 60 µl per well of alkaline phosphatase-labeled anti-rabbit IgG (diluted 1:5,000; Dianova, Hamburg, Germany) was applied for 2 hours. After extensive washing, a substrate solution (1 mg ml^{-1} *p*-nitrophenylphosphate; Sigma) was added in the dark for 1 hour in 100 mM diethanolamine (Sigma) at a pH of 9.5. The color reaction was stopped by adding 50 µl of 1 M NaOH and the absorbance was measured at 405 nm.

IFN-γ and IL-4 stimulation

Inflammatory conditions were simulated by IFN-γ and IL-4 stimulation of the cells before allergen uptake. Cells were treated for 6 hours with 300 U ml^{-1} IFN-γ (R&D Systems, Minneapolis, MN) or 50 U ml^{-1} IL-4 (Immunotools, Friesoythe, Germany) in medium without hydrocortisone as well as a combination of IFN-γ and IL-4. After extensively washing with PBS, cells were cultured in fresh medium without hydrocortisone for an additional 18 hours. To confirm the inflammatory phenotype, cells were stained with an FITC-labeled mouse anti-human ICAM-1 antibody (R&D Systems) or an APC-labeled mouse anti-human HLA-DR antibody (BD Pharmingen, Heidelberg, Germany) and analyzed by flow cytometry (FACS Calibur).

Confocal laser scanning microscopy

For CLSM, cells were grown on cover glasses or chambered cover glasses and incubated with 20 µg ml^{-1} of fluorescently labeled allergens for the indicated time intervals. Cells were then washed extensively with PBS and fixed for 30 minutes in PBS containing 2% paraformaldehyde. For intracellular staining of allergens with monoclonal antibodies, cells were permeabilized with 0.25% triton X-100 in PBS for 10 minutes. After blocking for 30 minutes with 10% BSA in PBS, cover glasses were incubated with primary mouse anti-human CD29 antibodies (Immunotech, Marseille, France), Bo14 (anti-Phl p 1), Bo12 (anti-Phl p 6), or anti-HLA-DR (BD biosciences) at 4°C for at least 12 hours. Cover glasses were then washed with PBS and incubated with the secondary goat anti-mouse antibodies labeled with Alexa Fluor 568 (Molecular Probes) for at least 2 hours at room temperature. After washing, the fixation step was repeated and cover glasses were mounted on glass slides with 2.5% 1,4-diaza-bi-cyclo-[2,2,2]-octane (Sigma) in 80% glycerol (Johnson *et al.*, 1982). Cells were analyzed with CLSM using Leica TCS SP (Leica, Bensheim, Germany) or Zeiss LSM 510 (Zeiss, Göttingen, Germany). To visualize lysosomes, the cells were incubated for 30 minutes with 50 nM LysoTrackerRed DND-99 (Molecular Probes) after pulsing with the fluorescent allergens. The cells were then washed with PBS, fixed, and analyzed immediately by CLSM.

Differentiation status of primary keratinocytes

After 5 days of culture, the expression of involucrin and cytokeratin 14 was analyzed by intracellular staining and flow cytometry. After detachment, the cells were fixed with 2% paraformaldehyde and permeabilized with 1% saponine (Sigma) at room temperature. Involucrin and cytokeratin 14 expression was detected with mouse anti-human antibodies (Sigma) following incubation with FITC-labeled goat anti-mouse IgG antibody (Southern Biotech, Birmingham, AL) or goat anti-mouse IgM antibody (Beckman Coulter) and subsequent flow cytometry. Expression levels were calculated by difference in MFI to isotype controls.

Statistical analysis

Statistical evaluation was performed using SPSS (SPSS Inc., Chicago, IL). The non-parametric Mann–Whitney *U* test was used for independent samples and the Wilcoxon test for related samples. Differences were regarded as significant when $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Table S1. Effect of IFN-γ and IL-4 stimulation on protein uptake by epithelial cells.

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