

Proteolytic antigens interfere with endosome/lysosome fusion in epithelial cells

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Abstract: The airway epithelial barrier function is important in maintaining the homeostasis in the body. A number of airway disorders are associated with the epithelial barrier dysfunction. The present study aims to elucidate a possible mechanism by which the proteolytic allergens compromise the epithelial barrier function. The airway epithelial cell line, RPMI 2650 cells (Rp cells) and kidney epithelial cell line, MDCK cells, were cultured to be monolayers and used as an *in vitro* epithelial barrier model. House dust mite antigen, Der P1 (Der) was used as an antigen that has the proteolytic property. The epithelial barrier permeability and transepithelial resistance (TER) were used as the indicators of epithelial barrier function. Both epithelial cell lines could endocytose Der in the culture. Some of the Der was transported across the epithelial barrier to the basal chambers of the Transwells without affecting the TER. The endocytic Der could suppress the expression of ubiquitin E3 lase A20 and further interfered with the fusion of endosome/lysosome in the epithelial cells. Mite antigen, Der, can interfere with the fusion of endosome/lysosome in epithelial cells to induce the epithelial barrier dysfunction.

Key words: airway, epithelial cell, barrier function, antigen, ubiquitination.

Résumé : La fonction de la barrière épithéliale des voies respiratoires est importante au maintien de l'homéostasie du corps. Plusieurs maladies des voies respiratoires sont associées à un dysfonctionnement de la barrière épithéliale. La présente étude vise à élucider le mécanisme par lequel des allergènes protéolytiques compromettent la fonction de la barrière épithéliale. La lignée cellulaire épithéliale dérivée des voies respiratoires RPMI 2650 (cellules Rp) et la lignée épithéliale rénale MDCK ont été cultivées en monocouches et utilisées comme modèle de barrière épithéliale *in vitro*. L'antigène d'acarien Der P1 (Der) a été utilisé comme antigène protéolytique. La perméabilité de la barrière épithéliale et la résistance trans-épithéliale (TER) ont été utilisées comme indicateurs de la fonction de la barrière épithéliale. Les deux lignées cellulaires pouvaient capter l'antigène Der en culture par endocytose. Une partie de Der était transportée à travers la barrière épithéliale pour atteindre les chambres basales d'inserts Transwell® sans affecter la TER. L'antigène Der capté par endocytose pouvait supprimer l'expression de A20 et interférer avec la fusion endosome/lysosome dans les cellules épithéliales. Nous concluons que l'antigène d'acarien Der peut interférer avec la fusion endosome/lysosome dans les cellules épithéliales pour induire un dysfonctionnement de la barrière épithéliale. [Traduit par la Rédaction]

Mots-clés : voies respiratoires, cellule épithéliale, fonction de barrière, antigène ubiquitination.

Introduction

The surface of the airway is covered by a layer of epithelial cells. The epithelial cell bodies and the tight junctions form the epithelial barrier (Hammad and Lambrecht, 2011). The healthy epithelial barrier restricts noxious substances, such as antigens, to be absorbed into the deep region of tissues in the airway (Tam et al. 2011). However, under a given environment, antigens can be absorbed into the airway mucosa, a condition designated the epithelial barrier dysfunction (Liu et al. 2012), which can initiate skewed immune reactions and induce inflammations, such as allergic asthma (Post et al. 2012). The aetiology of the epithelial barrier dysfunction is largely unknown.

Published data indicate that the airway epithelial cells can endocytose some antigens (Yang et al. 1999). The endocytic antigens may be degraded within the epithelial cells. After endocytosis, the antigens can be wrapped by the plasma membrane to form endosomes. The endosomes fuse with lysosomes; the antigens then may be degraded by the acetic hydrolases of lysosomes (O'Leary et al. 2011). Thus, the process of fusion of endosome/lysosome is important in the antigen degradation in epithelial cells, yet what

factors interfere with the fusion of endosome/lysosome in epithelial cells remain to be further elucidated.

Ubiquitination is an enzymatic, protein post-translational modification process; it is involved in the antigen processing (Katkere et al. 2012). The ubiquitin E3 ligase A20 (A20) is proposed as a critical molecule in the maintenance of the homeostasis in the body (Kool et al. 2011). Deficiency of A20 is associated with a number of diseases in the body (Hammer et al. 2011). A20 is also involved in the fusion of endosome/lysosome in phagocytes (Li et al. 2009). Whether A20 is associated with the endocytic antigen processing in epithelial cells is unclear. In this study, we observed that an airborne antigen, Der P1 (Der, in short), after being endocytosed by epithelial cells, inhibited the expression of A20 and escaped the degradation in epithelial cells to be transported across the epithelial barrier.

Materials and methods

Reagents

The Der P1 (Der) and anti-Der antibody were a gift from Dr. Zhijiang Tian at Nanjing University (Nanjing, China). The Der P1 ELISA kit was purchased from Citeq biologics (Groningen, The

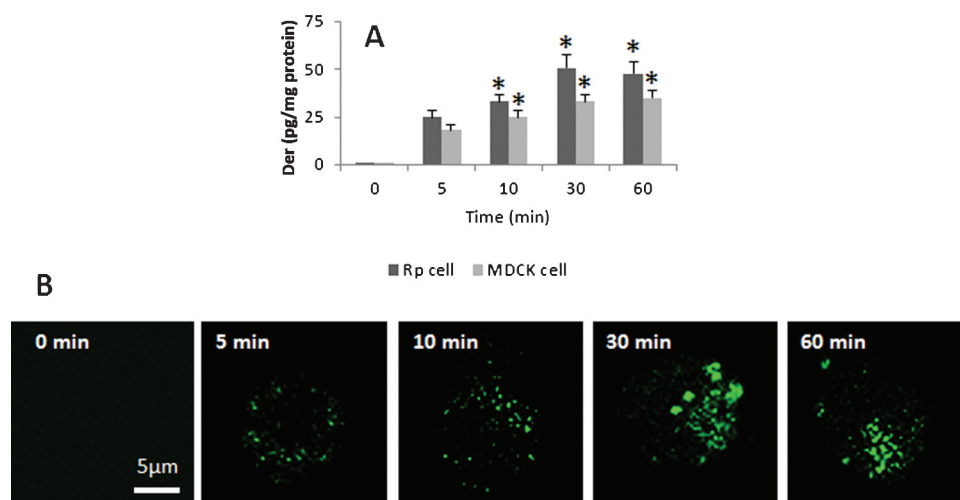
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Fig. 1. Epithelial cells endocytose Der. The RPMI 2650 (Rp) cell line and MDCK cell line were cultured into monolayers in the Transwell systems. Der P1 (Der) was added to the culture medium. The monolayers were collected at indicated time points (X-axis). (A) Cell extracts were analyzed by ELISA. The bars indicate the Der contents of the cell extracts. The data were presented as mean $\pm$ SD. * $p < 0.05$, compared with time “5 min”. (B) FITC-labeled Der was added to the cell culture. The cells were collected at the indicated time points and observed by confocal microscopy. The representative confocal images show the endocytic Der (in green) in epithelial cells. The experiments were repeated 3 times.



Netherlands). The antibodies of EEA1, LAMP2, and A20 were purchased from Santa Cruz Biotech (Shanghai, China). The FITC-labeling kit was purchased from Thermo Fisher Scientific China (Beijing, China).

Mice and ethic statement

BALB/c mice were purchased from Maosheng Animal Institute (Shanghai, China). The mice were maintained in cages equipped with filter lids and accessed food and water freely. The animal experimental procedures were approved by the ethics committee at China Medical University.

Cell culture

The human airway epithelial cell line (RPMI 2650 cell, Rp cell) and kidney epithelial cell line (MDCK cell) were purchased from the China Cell Line (Shanghai, China). The cells were cultured with DMEM medium supplemented with 10% fetal bovine serum, 1% l-glutamine, and 1% penicillin/streptomycin at 37 °C in 5% CO₂.

Labeling Der with FITC

Der was labeled with FITC with a commercial reagent kit following the manufacturer's instruction.

Endocytosis of antigen by epithelial cells

The epithelial cells were cultured at 10⁶ cell/ml in the presence of Der (10 μ g/ml). The cells were collected at time points of 0, 5, 10, 30, and 60 min respectively after the addition of Der. The cells were processed for extraction of protein to determine the contents of Der by ELISA. In separate experiments, the cells were exposed to FITC-labeled Der and collected at the same time points. The cells were smeared on a slide and observed under a confocal microscope.

Assessment of transepithelial resistance (TER)

The epithelial cells (10⁶ cells/ml) were seeded on the inserts of the Transwell system. About 5 to 7 days culture, the cells formed into a confluent monolayer. The TER was measured with an ohmmeter following published procedures (Feng et al. 2009).

Assessment of epithelial barrier permeability

The permeability to a macromolecular protein antigen (Der) was determined by a flux assay referring to published procedures (Berin et al. 1997). Briefly, Der was added to the apical chamber of

Transwells at a concentration of 10 μ g/ml. Two hours later, the culture medium samples were taken from the basal chambers of the Transwell system. The samples were treated with ammonium sulfate precipitation following published procedures (Li et al. 2012). The contents of Der in the samples were assessed with enzyme-linked immunoassay (ELISA).

ELISA

The contents of Der and A20 were assessed by ELISA with commercial reagent kits following the manufacturer's instruction.

Immunofluorescence

A portion of the culturing epithelial cells was processed by immunofluorescence with published procedures (Chen et al. 2010). Briefly, cells were removed from the inserts of Transwells using trypsin-EDTA and fixed with 2% paraformaldehyde for 2 h at room temperature. After washing with phosphate buffered saline (PBS) 3 times, the cells were blocked with 5% skim milk for 30 min and sequentially incubated with the first antibodies (1 μ g/mL) and secondary antibodies (0.5 μ g/mL) at room temperature. Washing with PBS was performed between each step. The stained cells were smeared onto a glass slide, covered with cover slips, and observed with confocal microscopy.

Confocal microscopy

The LSM510 confocal microscope was employed to observe the fusion of endosome and lysosome in the epithelial cells. Under the $\times 630$ objective, the positively stained cells were localized. The immune positive endosomes and lysosomes within the positive cells were observed by the enlarging system of the LSM510 software; 30 pictures were taken from each experimental group. The positive staining particles (endosome or lysosome) were counted blindly.

Flow cytometry

BALB/c mice were sensitized to Der following the published procedures (HuangFu et al. 2006). The spleen cells were prepared from the sensitized mice and naïve mice; the CD4⁺ CD25⁻ T cells and dendritic cells (DC) were isolated from the spleen cells by the magnetic cell sorting (MACS) with reagent kits following the manufacturer's instruction. The T cells (labeled with Carboxyfluorescein succinimidyl ester; CFSE), and DCs were cultured at a ratio of 10:1 in the presence

Fig. 2. Der in the basal chambers of Transwells. The culture medium samples were taken from the basal chambers of the Transwells in Fig. 1. (A) The bars indicate the Der contents in the culture medium, which were assessed by ELISA. The data were presented as mean $\pm$ SD. * $p < 0.01$, compared with the time point of “30 min”. The experiments were repeated 3 times. (B) A Transwell system.

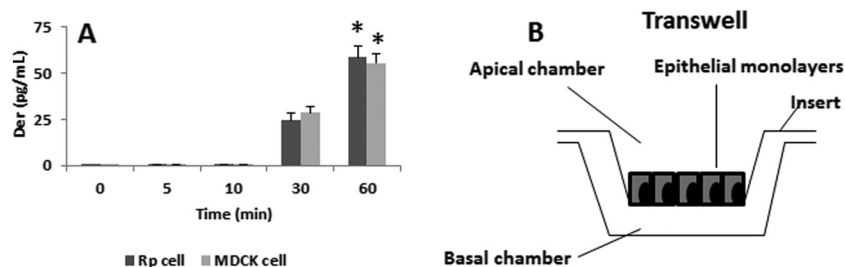
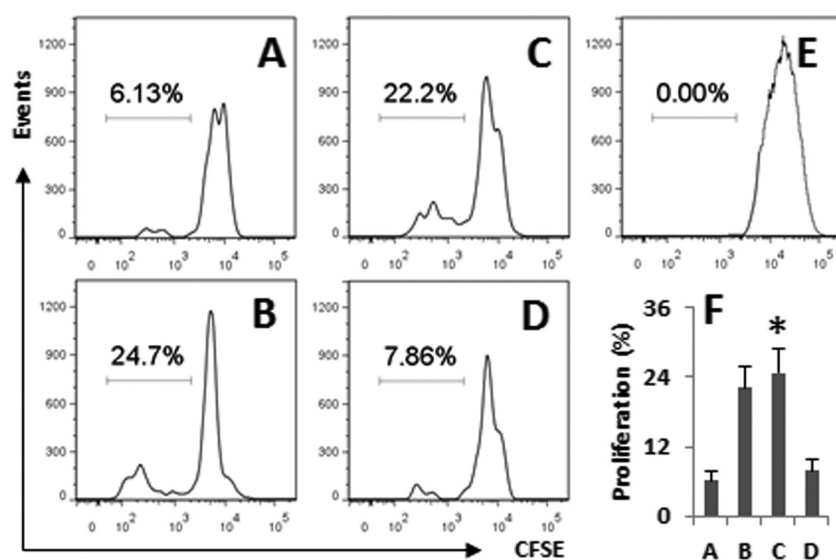


Fig. 3. T cell proliferation. The spleen cells were isolated from mice sensitized to Der, labelled with CFSE and cultured in the presence of saline (A), Der from Rp group (B), Der from MDCK group (C), or OVA (D; used as a control) for 4 days. The histograms indicate the proliferation rate of T cells. (E) CFSE staining control (negative control). (F) The bars indicate the summarized data (mean $\pm$ SD) of A–D. The data were from 3 separate experiments. * $p < 0.01$, compared with group A.



of Der (1 μ g/mL) or OVA (using as a control antigen) for 4 days. The T cell proliferation was assessed by flow cytometry (the CFSE-dilution assay).

Sensitization of mice

BALB/c mice were subcutaneously injected with ovalbumine (OVA, 0.1 mg/mouse) mixed with 0.1 mL alum on day 1 and day 3. The mice were challenged by nasal drop of OVA (10 μ g in 20 μ L saline) daily from day 9 to day 13. The mice were sacrificed on day 14; the spleen was used for further experiments.

Statistics

The data were expressed as mean $\pm$ SD. The difference between groups was assessed by the Student's *t* test or χ^2 test. The Pearson correlation assay was used to analyze the correlation between two groups. $p < 0.05$ was set as the significant criterion.

Results

Airway epithelial cells endocytose proteolytic allergen, Der p1

Airway epithelial cell lines, RPMI 2650 cell (Rp cell, in short) and kidney epithelial cells (MDCK cell, in short), were cultured to a monolayer in the Transwell system. An airborne allergen, Der was added to the apical chamber at a concentration of 10 μ g/mL. The epithelial monolayer was collected at time points of 0, 5, 10, 30, and 60 min respectively after the addition of Der. As shown by

Fig. 4. TER of epithelial monolayer. After culturing to confluence, epithelial monolayers were exposed to Der in the culture for 24 h. The TER was recorded at 0 and 24 h, respectively. The bars indicate the TER at 0 and 24 h time points, respectively. The data were expressed as mean $\pm$ SD. * $p < 0.05$, compared with 0 h of the same group. The data represent 3 separate experiments.

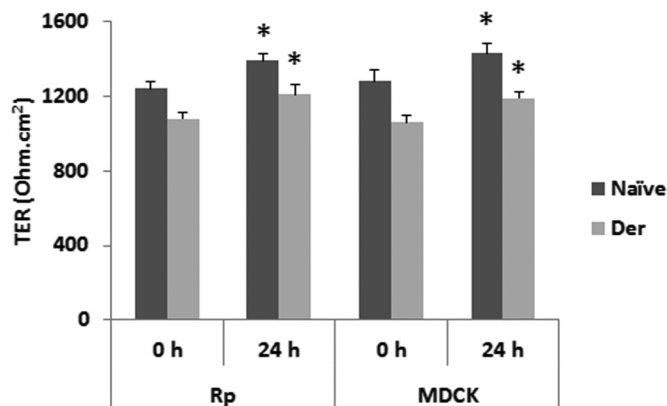
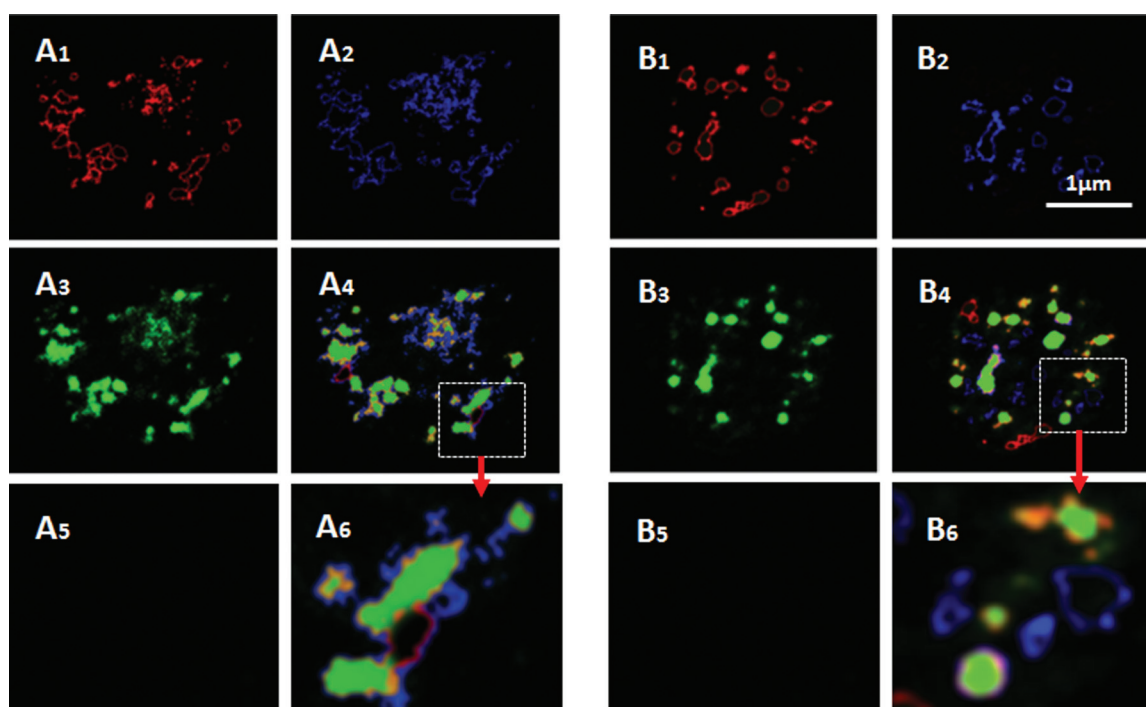


Fig. 5. The fusion of endosome/lysosome in epithelial cells. Rp cells were cultured in the presence of saline (A) or Der (B) for 48 h; the cells were stained with antibodies of LAMP2 (in blue; a lysosome marker), EEA1 (in red; an endosome marker), and Der (in green); the cells were observed under a confocal microscope. A5 and B5 are isotype control images. A6 and B6 are enlarged image spots of A4 and B4 (arrows), respectively. Each panel shows the immune positive particles of one cell. The data represent 3 separate experiments. The colour figure may be viewed in the online version of this article.



ELISA, the Der was endocytosed by the epithelial cells; the contents of Der in the extracts of epithelial cells were in an incubation time-dependent manner (Fig. 1). The results indicate that the airway cells can endocytose the airborne allergen, Der.

Der passes across the epithelial barrier

We next observed if the endocytic Der by epithelial cells could be transported across the epithelial barrier. We collected the culture medium sample from the basal chambers of the Transwells of the experimental setting of Fig. 1. The samples were analyzed by ELISA. The results showed that the Der was detected in the basal chambers; it was also in a cultural time-dependent manner (Fig. 2).

Transported Ag conserves antigenicity

We concentrated the protein (containing Der) from the basal chambers of the experiments of Fig. 1. Spleen cells were isolated from mice sensitized to Der; the cells were labelled with CFSE and cultured for 4 days in the presence of Der. As shown by the results from flow cytometry, significant T cell proliferation was detected in the cells stimulated by the protein collected from the basal chambers (Fig. 3). The results indicate that the epithelial cell transported Der still conserved the antigenicity.

Der passes through the epithelial barrier without disturbing the paracellular pathway

The data in Figs. 2–3 indicate that Der can pass through the epithelial barrier. We next observed if Der affected the paracellular pathway by measuring the epithelial monolayer TER before and after the exposure to Der. The results showed that the exposure to Der did not decrease the TER; instead, the TER was increased about 10% above the baseline after 24 h culture in the presence of Der (Fig. 4). The results indicate that Der does not affect the paracellular pathway of the epithelial monolayer.

Der interferes with the fusion of endosome/lysosome

The data in Figs. 2 and 4 imply that the Der pass across the epithelial barrier via the transcellular pathway rather than the paracellular pathway. In general, the endocytic cargo, such as protein, can be degraded by the lysosome system within the epithelial cells, in which the fusion of endosome/lysosome is a critical step. Thus, we next observed if the endocytosis of Der affected the fusion of endosome/lysosome in epithelial cells. The epithelial cells were exposed to Der for 48 h and observed by immunofluorescence. As shown by Fig. 5, more than 90% endosomes were fused with lysosomes in saline group, but less than 10% of Der⁺ endosomes fused with lysosomes in Der group (Fig. 5). The results imply that the endocytic Der prevents the fusion of endosome/lysosome in epithelial cells.

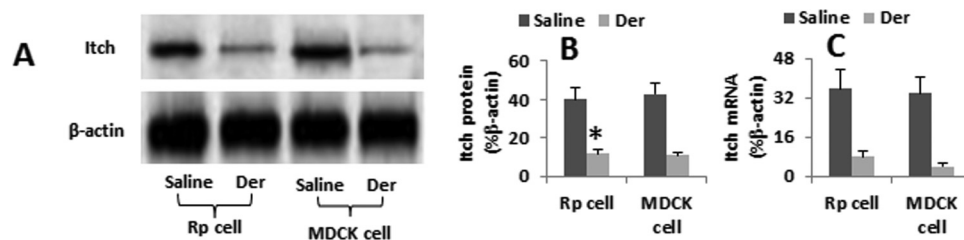
Der suppress the expression of A20 in epithelial cells

It is reported that A20 plays a role in tethering endosome to lysosome in cells. Der has a proteolytic property. We then observed if the endocytic Der affected the expression of A20 in epithelial cells. The epithelial cells were cultured in the presence of Der (10 μg/mL) for 48 h. The epithelial cells were collected, and the cell extracts were analysed by Western blotting. The results showed that the exposure to Der did suppress the levels of A20 in the epithelial cells (Fig. 6).

Discussion

Previous studies indicate that the proteolytic antigen, Der, can cleave CD23 to be soluble CD23 (Hewitt et al. 1995), which further promotes the production of IgE and exacerbates the existing allergic diseases (Bowles et al. 2011). The present study adds further understanding to the nature of Der; although it can be endocytosed by epithelial cells, it can interfere with the fusion of endosome/lysosome to escape from the degradation in epithelial cells.

Fig. 6. Endocytic Der suppress A20 in epithelial cells. Epithelial cells were treated with saline or Der in the culture for 48 h. The cells were collected. The cell extracts were analyzed by Western blotting and qRT-PCR. (A) The immune blots indicate the levels of A20 in the cell protein extracts. (B) The bars indicate the summarized integrated density of the immune blots. (C) The bars indicate the mRNA levels of A20 in the epithelial cells. The data were expressed as mean $\pm$ SD. * $p < 0.01$, compared with saline group. The data represent 3 separate experiments.



Epithelial cells directly face the external environment. The air-borne antigens mixing in the air are naturally inhaled into the airway to contact the epithelial cells. In line with previous reports (Yang et al. 1999) that depict the internalization of antigens by rat airway epithelial cells, our results also show the absorption of one of the common airborne antigen, Der, by airway epithelial cell line. Two major parameters, the permeability and TER, are associated with the epithelial barrier function. The TER is the major indicator of the paracellular status, which was not affected by the endocytosis of Der; the fact implies that Der does not open the paracellular space. However, increases in the transcellular permeability of the epithelial monolayers were observed in the present study, which implies that Der was transported across the epithelial barrier via the transcellular pathway. Our data are somewhat different from previous reports. Wan et al. (1999) reported that Der P1 disrupted tight junction of epithelial cells within 7 h by augmenting permeability and facilitating transepithelial allergen delivery. Roche et al. (2000) also reported that the cysteine protease activity of crude Dermatophagoides pteronyssinus extract was able to increase the epithelial paracellular conductance of rat tracheal tissues. On the other hand, the data are in line with some other published data, such as Berin et al. (1997), who observed the transcellular antigen transportation in rat jejunal epithelial cells; the authors indicated that this pathway was even a "fast" pathway. The subsequent reports indicated that the IgE low affinity receptor, CD23, was involved in the transcellular antigen transport across the epithelial barrier under allergic environment (Yang et al. 2000).

The fusion of endosome/lysosome in phagocytes and epithelial cells is the major process degrading the endocytic cargo, which has been investigated extensively (Schirmbeck and Reimann 2002; Stahl and Barbieri, 2002). Our data are in line with previous studies. The fusion of endosome/lysosome was commonly observed in epithelial cells. After exposure to Der in culture, Der-containing endosomes were observed in epithelial cells, but few of them fused with lysosomes. The data imply that the endocytic Der interferes with the fusion of endosome/lysosome. The increase in the Der transport across the epithelial barrier was subsequently observed. Der has the proteolytic property; it cleaves CD23 on the surface of B cells to promote the generation of soluble CD23. Our data add novel information to this common antigen, that it also can interfere with the fusion of endosome/lysosome in epithelial cells. A20 has the tethering function that facilitates the degradation of other proteins such as TNF receptor-associated factor 2 (Li et al. 2009) and facilitates the fusion of endosome/lysosome (Li et al. 2009; An et al. 2012; Song et al. 2012). Our data are in line with these reports by showing that reduction of A20 in epithelial cells disturbed the fusion of endosome and lysosome. As a consequence of the A20 reduction, large quantities of non-degraded antigens were transported across the epithelial barrier and induced the antigen-specific T cell proliferation, as shown by the present data (Fig. 3).

A20 is an E3 ligase of ubiquitin. A number of studies indicate that A20 is a critical molecule in maintaining the homeostasis in the body (Hammer et al. 2011; Kool et al. 2011; Shembade and Harhaj, 2010). The deficiency of A20 results in immune inflammation, such as colitis (Vereecke et al. 2010) and autoimmune disorders (Chu et al. 2011) as well as in the airway epithelium (Li et al. 2013). The etiology of the deficiency of A20 is not understood yet. Our data indicate that A20 can be suppressed by the endocytic antigen Der, which results in antigens with the functional antigenicity to be transported across the epithelial barrier. The antigens have the potential to induce skew immune reactions.

In summary, the present study observed that the airway epithelial cells endocytosed Der; the Der was not degraded in the epithelial cells, but was transported across the epithelial barrier. The endocytic Der interfered with the fusion of endosome/lysosome and suppressed the expression of A20 in epithelial cells.

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