

# Antigen-specific activities of CD8<sup>+</sup> T cells in the nasal mucosa of patients with nasal allergy

Shuqi Qiu,<sup>1,2,3</sup> Xiaobei Duan,<sup>1</sup> Xiaorui Geng,<sup>1</sup> Jianxiong Xie<sup>2</sup> and Han Gao<sup>1,2,3</sup>

## Summary

**Background:** The prevalence of chronic rhinitis is increasing rapidly. Its pathogenesis is not fully understood but immune inflammation is one plausible causative factor. Antigen specific CD8<sup>+</sup> T cells play a critical role in the induction of chronic inflammation. This study aims to investigate the role of antigen specific CD8<sup>+</sup> T cells in the pathogenesis of chronic AR.

**Methods:** Nasal mucosal epithelial samples obtained by the surface of the nasal mucosa of patients with AR complicated with inferior turbinate hypertrophy. Exosomes were purified from the scratching samples and examined by immune gold electron microscopy. Cell culture models were employed to evaluate the effect of exosomes on modulating CD8<sup>+</sup> T cell activity.

**Results:** Exosomes purified from patients with chronic AR carried microbial products, *Staphylococcal* enterotoxin B (SEB), and airborne antigen, Derp1. Dendritic cells pulsed by SEB/Derp1-carrying exosomes showed high levels of CD80, CD86 and the major histocompatibility class I (MHC I). Exosome-pulsed dendritic cells could induce naïve CD3<sup>+</sup> T cells to differentiate into CD8<sup>+</sup> T cells. Upon exposure to a specific antigen, the CD8<sup>+</sup> T cells released granzyme B and perforin and more than 30% antigen specific CD8<sup>+</sup> T cells proliferated.

**Conclusions:** Antigen specific CD8<sup>+</sup> T cells play an important role in the pathogenesis of chronic AR complicated with inferior turbinate hypertrophy. (*Asian Pac J Allergy Immunol* 2012;30:107-13)

**Key words:** rhinitis, CD8 T lymphocytes, epithelium, antigen specific response

## Introduction

The nasal obstruction is a clinical symptom that substantially affects the life quality of human beings. The symptom mostly appears in patients who suffer from chronic idiopathic rhinitis or chronic AR; many of them try multiple therapeutic remedies to ameliorate their nasal obstruction and they may ask for surgical intervention to remove a portion of the hypertrophic nasal mucosa to improve the airway in the nasal cavity.<sup>1-2</sup>

The pathogenesis of chronic obstructive rhinitis are not fully understood.<sup>3</sup> It can result from AR, vasomotor rhinitis, viral infection or bacterial infection.<sup>4</sup> Non-specific eosinophilia also plays a role in the pathogenesis of a subgroup of chronic rhinitis cases.<sup>5</sup> Cytotoxic CD8<sup>+</sup> T cells can release a number of cytotoxic molecules, such as granzyme B and perforin, to the microenvironment and these interfere with cell activities. The mediators from CD8<sup>+</sup> T cells can activate the apoptotic pathway to induce cell death or cell injury. Published data indicate that antigen specific CD8<sup>+</sup> T cells also play a role in chronic inflammation, such as inflammatory bowel disease,<sup>6</sup> dermatitis<sup>7</sup> and diabetes.<sup>8</sup> In AR, investigators have noted that CD8<sup>+</sup> cells release interleukin (IL)-4 which is involved in the pathogenesis of the disease. It is not clear whether CD8<sup>+</sup> T cells play a role in the chronic AR.

In clinical practice, we have noted a subgroup of patients with chronic AR and inferior turbinate hypertrophy as well as chronic nasal obstruction. How the nasal mucosa hypertrophy occurs in patients with AR is not fully understood. Therefore, we recruited a group of patients with chronic nasal obstruction and AR to participate in the present study. The results indicate that most of these patients have a high frequency of allergen specific CD8<sup>+</sup> T cells in their nasal mucosa. Exposure to specific antigens can trigger these CD8<sup>+</sup> T cells to release inflammatory molecules.

From the 1, Shenzhen ENT Institute,  
2, Shenzhen Longgang Central Hospital,  
3, ENT Hospital, Shenzheng, China.

Corresponding author: Shuqi Qiu

E-mail: qiuqi66858@163.com

Submitted date: 2/11/2011

Accepted date: 22/3/2012



## Methods

### Reagents

Antibodies of beta 6, CD80, CD86, MHCI, porforn and granzyme B were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Derp1 was obtained from BioWorld (Shanghai, China). Anti-Derp1 antibody was purchased from QCBioLtc. (Shanghai, China). Staphylococcal enterotoxin B (SEB) was purchased from Sigma Aldrich (Shanghai, China).

### Recruitment of human subjects

Patients with perennial AR were diagnosed based on our routine procedures including: (1) clinical symptoms; (2) antigen skin scratch test and (3) serum antigen specific IgE and IgG. AR patients with or without chronic nasal obstruction (ob, in short) were recruited for this study. Patients with acute airway infection within the previous month were excluded. None of the recruited subjects had a history of nasal surgery. In addition, a group of patient with chronic rhinitis without AR (nAR group) was also recruited. The use of human tissue in this study was approved by the Research Ethics Committee at the Longgang Center Hospital. Informed consent was obtained from each human subject. Patients with inferior turbinate hypertrophy and sinusitis were treated with partial turbinectomy and endoscopic sinus surgery. The demographic data of the patients are presented in Table 1.

**Table 1.** Demographic data.

|                         | ARob | ARnob | Control |
|-------------------------|------|-------|---------|
| Male                    | 7    | 8     | 5       |
| Female                  | 8    | 7     | 5       |
| Age                     | 43.5 | 40.8  | 35.8    |
| Disease history (years) | 8.6  | 7.6   | 6.5     |
| Sinusitis (patients)    | 15   | 0     | 10      |
| ITH (patients)          | 9    | 4     | 7       |
| NSD (patients)          | 6    | 3     | 6       |
| sIgE                    | 22.6 | 25.8  | BD      |
| sIgG                    | 24.3 | 31.5  | BD      |

ITH: Inferior turbinate hypertrophy.

NSD: Nasal septic deviation.

ARob: Allergic rhinitis with chronic nasal obstruction.

ARnob: Allergic rhinitis without chronic nasal obstruction.

sIgE: Serum specific IgE (ng/ml)

sIgG: Serum specific IgG (ng/ml).

BD: Below detectable levels.

### Collection of nasal epithelial samples

Which was used to The nasal epithelia were sampled with a plastic spoon (4 mm in diameter) scratch the surface of the nasal mucosa 2 or 3 times. The samples were put in 0.5 ml phosphate buffered saline (PBS) and passed through a gauge 18 syringe needle 10 times. The samples were then processed for exosome purification (see below).

### Cell culture

Human nasal epithelial cells from the cell line RPMI2650 (ATCC, CCL-30), were seeded into 75 cm<sup>2</sup> flasks in RPMI1640 medium containing 10% FCS, 4 mM glutamine, 50 µg/ml penicillin /streptomycin. On day 3, when the cells reached 60% confluence, the mite antigen, Derp1, with or without SEB was added to the cultures. Culture media were collected and the exosomes were purified by gradient centrifugation.

### Exosome preparation

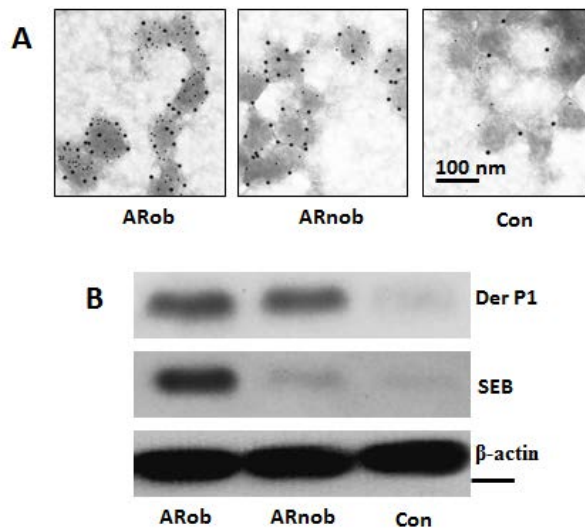
Cells from the nasal epithelial cell line, RPMI2650, were incubated with Derp1 or/and SEB for 24 h; the supernatants were collected and centrifuged at 300 × g (10 min), 1200 × g (20 min), and 10,000 × g (30 min) to remove cell debris. Exosomes were pelleted at 100,000 × g for 1 h and resuspended in PBS for further experiments. The protein in exosomes was quantified using a Bradford protein assay.

### Electron microscopy

Exosomes were prepared following our previously established procedures.<sup>9</sup> The purified exosomes were fixed with fixatives that were added to the culture supernatant for 2 h before ultracentrifugation at final concentrations of 0.75% (glutaraldehyde) and 2% (paraformaldehyde). The samples were dropped onto grids that were pre-coated with formva and dried overnight, stained with anti-Derp1 or SEB antibodies followed by a gold particle labeled second antibody (12 nm for Derp1; 6 nm for SEB). The grids were then stained with a negative contrast solution. Samples were observed with a JEOL JEM-1200 EX transmission electron microscope.

### Flow cytometry

Cells were collected and incubated with primary antibodies on ice for 30 min (For the intracellular staining, cells were fixed with 1% paraformaldehyde on ice for 30 min and incubated with permeabilization reagents for 30 min on ice). The stained cells were analyzed using a FACSarray (BD Bioscience, San Jose, CA). Data were analyzed with software FlowJo.



**Figure 1.** Exosomes carrying microbial products and protein antigens are localized in the nasal mucosa. The nasal epithelial layer was sampled from AR patients with chronic nasal obstruction (ob) or without chronic nasal obstruction (nob) as described in the text. The representative EM pictures (A) show exosomes stained with antibodies of SEB (smaller particles) and Derp1 (larger particles). B: the gels show the immune blots of Derp1 and SEB in the protein extracts of collected exosomes. The blots of beta-actin were used as internal loading controls. The data represent 3 experiments.

### Statistics

All values were expressed as the means  $\pm$  SD of at least three separate experiments. The values were analyzed using the two-tailed unpaired Student's t-test when the data consisted of two groups or by ANOVA when three or more groups were compared.  $P < 0.05$  was accepted as statistically significant.

### Results

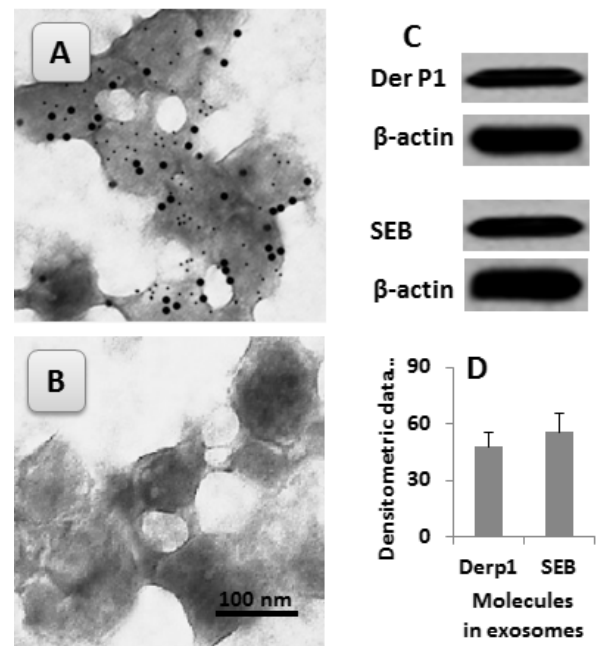
#### *Exosomes carrying the proteins Ag and SEB are identified in the human nasal mucosa*

The nasal epithelial layer samples were collected from AR patients with or without chronic nasal obstruction. The patients had typical nasal obstruction, inferior turbinate hypertrophy, and skin scratch test showed positive responses to one or more airborne antigens. Nasal epithelial samples were obtained from each patient. Exosomes were purified from the samples with our established procedures.<sup>9</sup> The exosomes were stained with antibodies SEB and Derp1 and processed for

electron microscopy (EM). As shown by the EM pictures, (Figure 1) exosomes were successfully purified from the obtained samples from the nasal epithelium. The gold labels for SEB (smaller particles) and Derp1 (larger particles) were seen on most exosomes from the ARob group. In the samples from the ARnob group, Derp1 staining was observed in most exosomes, but SEB staining was rarely observed. In the normal control group (Con), much less staining of both Derp1 or SEB was observed. That exosomes were carrying Derp1 and SEB was confirmed by Western blotting (B).

#### *Nasal epithelial cell-derived exosome generation and purification*

To further investigate the significance of the nasal mucosa-derived exosomes carrying microbial products and protein antigens as shown in Figure 1,

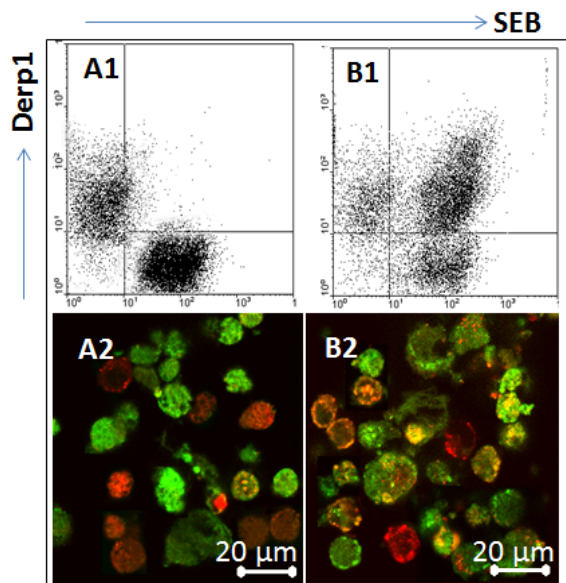


**Figure 2.** Generation of nasal mucosal cell-derived exosomes. A-B, the representative EM images show the nasal epithelial cell-derived exosomes carrying Derp1 and SEB (A). Panel B is an isotype control. C: proteins were extracted from purified exosomes and analyzed by Western blotting. The gel graphs show the immune blots of Derp1 and SEB. The  $\beta$ -actin immune blots were used as internal controls. D: bars indicate the summarized integrated density data of panel C. The data are presented as mean  $\pm$  SD (from 3 experiments). Magnification of A and B:  $\times 30,000$ .

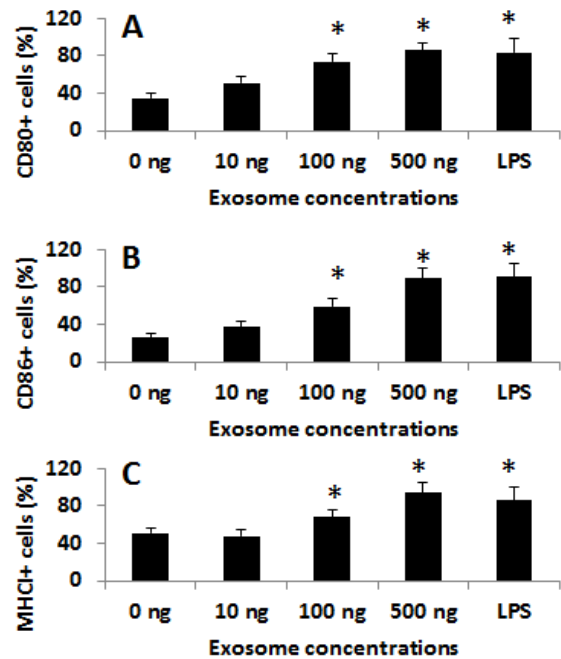
we performed a cell culture study. Human nasal epithelial cells from (RPMI2650 cells) were cultured in the presence of a mite antigen, Derp1, and SEB overnight. The supernatant was collected and processed for purification of exosomes with our established procedures.<sup>9</sup> As observed by immune gold EM, exosomes were observed that contained Derp1 and SEB (Figure 2A-C). The data from Western blotting confirmed the Derp1 and SEB were entrapped by exosomes (Figure 2D).

#### *Dendritic cells (DCs) capture nasal epithelial cell-derived exosomes*

To determine if DCs could capture the epithelial cell-derived exosomes, we generated DCs from peripheral blood mononuclear cells (PBMC). The DCs were exposed to purified exosomes that were carrying both SEB and Derp1. As examined by flow cytometry and immunocytochemistry, DCs only captured either SEB or Derp1 if they were added to the culture separately (Figure 3A), while DCs captured both SEB and Derp1 if SEB/Derp1-carrying exosomes were added to the culture (Figure 3B).



**Figure 3.** DCs can capture the nasal epithelial cell-derived exosomes. DCs were generated from isolated PBMC and cultured in RPMI1640 medium. SEB, or Derp1, or SEB/Derp1-carrying exosomes were added to the culture. The cells were collected 6 h later and analyzed by flow cytometry and immunocytochemistry respectively. A1-B1: the flow cytometry dot plots show SEB+ or Derp1+ DCs. A2-B2, the representative confocal images show SEB+ staining (in green) and Derp1+ cells (in red). The yellow color was merged by green and red colors. The data represent 3 experiments.

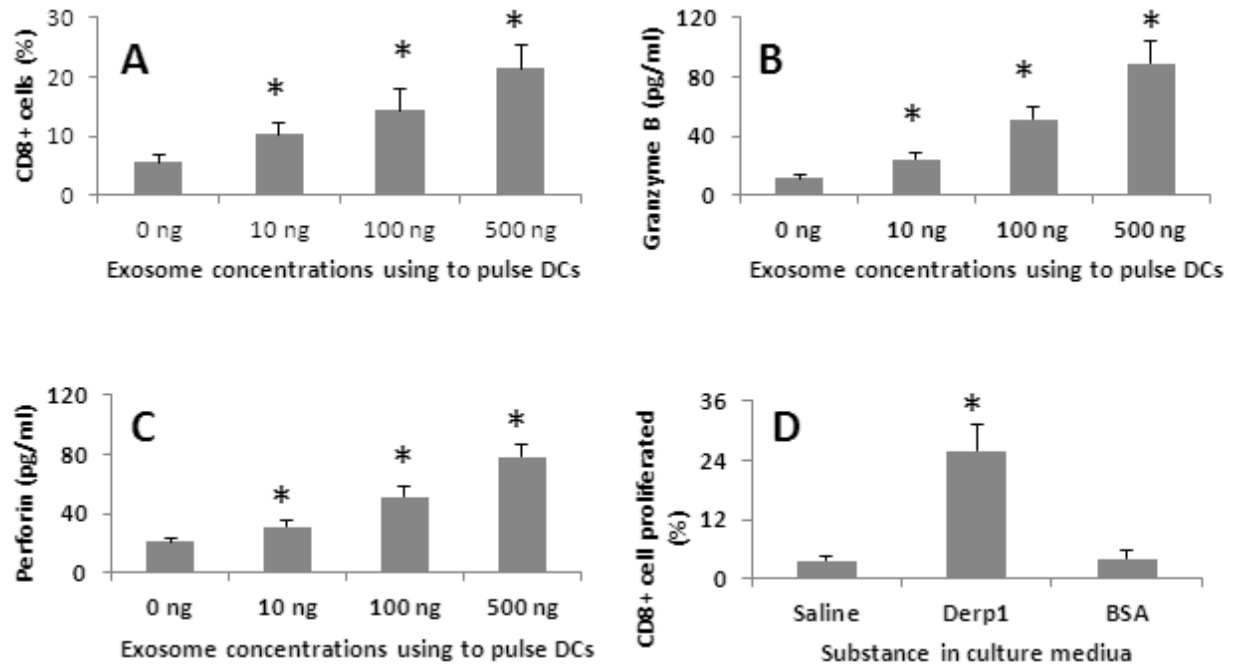


**Figure 4.** Nasal epithelial cell-derived exosomes modulate the properties of DCs. Human peripheral DCs were cultured in the presence of nasal epithelial cell-derived exosomes at the indicated concentrations for 5 days. The cells were analyzed by flow cytometry. The bars indicate the percentage of cells of CD80 (A), CD86 (B) and MHCI (C). \*,  $p < 0.05$ , compared with the “0 ng” group. The data were presented as mean  $\pm$  SD from 3 experiments. LPS: DCs were stimulated by LPS at 50 ng/ml of culture.

#### *Derp1/SEB-carrying exosomes regulate the properties of DC*

Since DCs disperse immediately under the epithelial layer, they certainly encounter epithelial cell-released exosomes. How the exosomes regulate the properties of DCs needs to be further understood. We therefore generated exosomes as described in Figure 2. DCs were prepared with isolated human PBMC, as we reported previously.<sup>10</sup> The DCs were cultured in the presence of exosomes at graded concentrations for 5 days. The DCs were collected at the end of the culture and analyzed by flow cytometry. The data showed that the stimulation of exosomes significantly increased the levels of CD80, CD86 and the major histocompatibility complex I (MHCI). These data indicate that the exposure to SEB/Derp1-carrying exosomes can activate naïve DCs to become type I DCs.





**Figure 5.** Exosome-pulsed DCs facilitate antigen specific CD8+ T cell differentiation. A: bars indicate the frequency of CD8+ T cells. B-C: bars indicate the levels of granzyme B (B) and perforin (C) in culture medium. D: The cell culture procedures in panel D were the same as that in A-C except the CD3+ CD8+ T cells were labeled with CFSE prior to the culture. The specific antigen, Derp1, was added to the culture in the last culture round. The cells were collected at the end of culture and analyzed by CFSE-dilution assay. The bars indicate the frequency of proliferated CD8+ T cells. The data were presented as mean  $\pm$  SD from 3 experiments. \*,  $p < 0.05$ , compared with the “0 ng” group in panels A-C and the saline group in panel D.

#### ***Nasal epithelial cell-derived exosomes from AR patients with chronic nasal obstruction promote the antigen specific CD8+ cell differentiation***

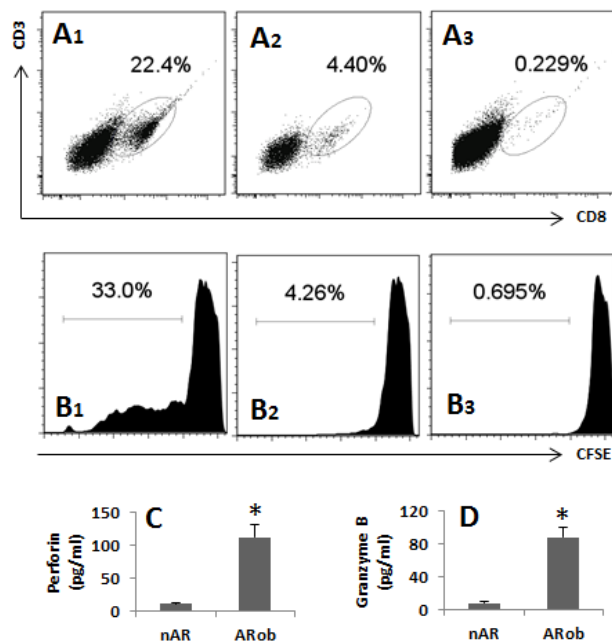
The finding that exosome-pulsed DCs expressed high levels of MHCI indicates that the DCs can facilitate the development of CD8+ T cells. To test this hypothesis, we isolated the CD3+ CD25- T cells from human PBMC; the cells were cultured with exosome-pulsed DCs for 3 rounds (each round consisted of 3 days). The cells were analyzed by flow cytometry. As shown by Figure 5, abundant CD8+CD25+ cells were generated. The cells were activated upon exposure to a specific antigen, Derp1, stimulating an increase in perforin and granzyme B in the culture supernatant.

#### ***CD8+ T cells are increased in the nasal mucosa of AR patients with chronic nasal obstruction***

The data in Figure 5 prompted us to observe the CD8+ T cells in the nasal mucosa. We collected surgically removed nasal mucosal specimens from 15 ARob patients. These patients had several skin test positive responses for one or more antigens;

all of them were sensitized to mite Derp1. Single cells were prepared from the specimens of nasal mucosa and analyzed for the frequency of CD8+ T cells by flow cytometry. The data showed that the frequency of CD8+ T cells was  $22.8\% \pm 5.6\%$  in the ARob group while only  $4.4\% \pm 3.2\%$  ( $p < 0.01$ , compared with the ARob group) in nasal mucosa collected from 10 patients with chronic rhinitis without AR. To clarify if the CD8+ T cells were antigen specific, a portion of the isolated single cells were labeled with carboxyfluoresceinsuccinimidyl ester (CFSE) and cultured in the presence of the specific antigen, Derp1, for 3 days. As shown by the CFSE-dilution assay,  $33.3\% \pm 11.5\%$  CD8+ T cells proliferated. With the gating technique,  $88.6\% \pm 15.4\%$  proliferated cells were found to be CD3+ CD8+ T cells. The levels of perforin and granzyme B were significantly increased in the supernatants of the ARob group than those in the ARnob group (Figure 6). The results indicate that there are abundant antigen-specific CD8+ T cells in the nasal mucosa of ARob patients.





**Figure 6.** Antigen-specific CD8<sup>+</sup> T cells in AR nasal mucosa. Mononuclear cells were isolated from the surgically removed nasal mucosa of 15 ARob patients (A1, B1) and 10 nAR patients (A2, B2). The cells were analyzed by flow cytometry. A: the dot plots show the frequency of CD3<sup>+</sup> CD8<sup>+</sup> T cells. B: a portion isolated CD8<sup>+</sup> T cells were labeled with CFSE and cultured in the presence of DC and specific antigen Derp1 for 3 days. The histograms show the frequency of proliferated CD8<sup>+</sup> T cells. C-D: the bars indicate the levels of perforin (C) and granzyme B (D) (mean  $\pm$  SD. \*,  $p < 0.05$ , compared with nAR group).

## Discussion

The present study reports a set of novel data about a subgroup of AR patients with chronic nasal obstruction. These patients have detectable but low levels of antigen specific IgE and relatively high levels of antigen specific IgG in the sera. The major clinical symptom of these patients is the chronic nasal obstruction caused by the inferior turbinate hypertrophy. Based on our previous studies, SEB plays a critical role in AR<sup>10</sup> and allergic disorders in other organs.<sup>11</sup> We detected the absorption of SEB by the nasal mucosa and an exosome system was found to carry a microbial product, SEB, in the subepithelial region. The purified exosomes or generated exosomes can regulate the properties of DCs and pulsed DCs express high levels of CD80, CD86 and MHCI. These DCs can induce antigen specific CD8<sup>+</sup> T cell differentiation. The antigen

specific CD8<sup>+</sup> T cells can release inflammatory mediators upon exposure to specific antigens.

The pathogenesis of chronic hypertrophic rhinitis is incompletely understood. Viral or bacterial infection is suggested as the primary causal factor and pus secretions can be another. Chronic nasal inflammation is usually observed in patients with persistent AR,<sup>12</sup> idiopathic rhinitis and in long-standing septal deviation. Immune inflammation can be one of the factors in the initiation and perpetuation of chronic rhinitis. Our results are in line with previous studies which showed that the *S. aureus*-derived SEB plays a role in chronic rhinitis.<sup>13-14</sup> Our study also revealed a previously undescribed mechanism in the pathogenesis of chronic hypertrophic rhinitis; that is that the skewed antigen specific CD8<sup>+</sup> T cell response may play a role.

DCs are antigen presenting cells which capture antigens to process and transport the antigen information to T cells. One of the features of DC capturing antigens is that once they have taken in a substance, they mature quickly and do not capture another antigen in the future. Our results also confirmed this notion. The present study unraveled a novel aspect of DC capturing of antigens; DCs could capture exosomes. The exosomes released from the nasal epithelial cells entrap microbial products and antigens, which enables a DC to capture more than one substance. Since the absorbed microbial products, such as SEB in the present study, promote the expression of MHCI and high levels of MHCI/antigen information may be passed to CD3<sup>+</sup> T cells. The subsequent experimental data have supported this deduction; a large number of antigen specific CD8<sup>+</sup> T cells were generated.

The CD8<sup>+</sup> T cells are also called cytotoxic T cells. Upon activation, the CD8<sup>+</sup> T cells release cytotoxic molecules, such as granzyme B and perforin, to the microenvironment. These molecules can cause other cells or invading microbes to undergo apoptosis in a non-specific way, resulting in cell death or injury. The epithelial barrier function can be compromised in this way.<sup>15</sup> Since the epithelial barriers play a critical role in maintaining homeostasis in the tissues, the dysfunction of epithelial barriers can be one of the causes of chronic inflammation.<sup>16</sup>

In summary, the present data indicate that nasal epithelial cell-derived exosomes contain microbial products and protein antigens; such exosomes can be detected in the patients with chronic AR.

Exosomes carrying microbial products and airborne antigens can promote DC maturation and expression of high levels of MHCI; the DCs can induce antigen specific CD8+ T cell differentiation. Re-exposure to specific antigens trigger the antigen specific CD8+ T cells to release cytotoxic molecules, such as granzyme B and perforin, which have the potential to initiate and perpetuate inflammation in the nasal mucosa.

### Acknowledgements

This study was supported by grants from the Shenzhen-Hong Kong Innovation circle of Shenzhen Technology Program (SG200810220171A).

### Conflicts of interest

All authors have neither conflicts of interest nor financial support from the drug companies.

### References

1. Suh JD, Kennedy DW. Treatment Options for Chronic Rhinosinusitis. *Proc Am Thorac Soc.* 2011;8:132-40.
2. Timperley D, Schlosser RJ, Harvey RJ. Chronic rhinosinusitis: An education and treatment model. *Otolaryngol Head Neck Surg.* 2010;143:S3-S8.
3. Tomassen P, Zele TV, Zhang N, Perez-Novo C, Bruaene NV, Gevaert P, et al. Pathophysiology of chronic rhinosinusitis. *Proc Am Thorac Soc.* 2011;8:115-20.
4. Greiner AN, Meltzer EO. Overview of the treatment of allergic rhinitis and nonallergic rhinopathy. *Proc Am Thorac Soc.* 2011;8:121-31.
5. Sok J, Ferguson B. Differential diagnosis of eosinophilic chronic rhinosinusitis. *Curr Allergy Asthma Rep.* 2006;6:203-14.
6. Liu Z, Yadav PK, Xu X, Su J, Chen C, Tang M, et al. The increased expression of IL-23 in inflammatory bowel disease promotes intraepithelial and lamina propria lymphocyte inflammatory responses and cytotoxicity. *J Leukoc Biol.* 2011;89:597-606.
7. Hennino A, Jean-Decoster C, Giordano-Labadie F, Debeer S, Vanbervliet B, Rozieres A, et al. CD8+ T cells are recruited early to allergen exposure sites in atopy patch test reactions in human atopic dermatitis. *J Allergy Clin Immunol.* 2011;127:1064-7.
8. Ye Z, Ye Z, Ahmed KA, Ye Z, Ahmed KA, Hao S, et al. Active CD4+ helper T cells directly stimulate CD8+ cytotoxic T lymphocyte responses in wild-type and MHC II gene knockout C57BL/6 mice and transgenic RIP-mOVA mice expressing islet-cell ovalbumin antigen leading to diabetes. *Autoimmunity.* 2008;41:501-11.
9. Luketic L, Delanghe J, Sobol PT, Yang P, Frotten E, Mossman KL, et al. Antigen presentation by exosomes released from peptide-pulsed dendritic cells is not suppressed by the presence of active CTL. *J Immunol.* 2007;179:5024-32.
10. Liu T, He SH, Zheng PY, Zhang TY, Wang BQ, Yang PC. *Staphylococcal* enterotoxin B increases TIM4 expression in human dendritic cells that drives naive CD4 T cells to differentiate into Th2 cells. *Mol Immunol.* 2007;44:3580-7.
11. Yang PC, Xing Z, Berin CM, Soderholm JD, Feng BS, Wu L, et al. TIM-4 expressed by mucosal dendritic cells plays a critical role in food antigen-specific Th2 differentiation and intestinal allergy. *Gastroenterology* 2007;133:1522-33.
12. Broide DH. Allergic rhinitis: Pathophysiology. *Allergy Asthma Proc.* 2010;31:370-4.
13. Liu T, Song CH, Liu AM, Xie C, Zhao F, Chen X, et al. Forkhead box P3+ T cells express interleukin-17 in nasal mucosa of patients with both allergic rhinitis and polyposis. *Clin Exp Immunol.* 2011;163:59-64.
14. Zhang N, Holtappels G, Gevaert P, Patou J, Dhaliwal B, Gould H, et al. Mucosal tissue polyclonal IgE is functional in response to allergen and SEB. *Allergy.* 2011;66:141-8.
15. Epple HJr, Allers K, Troger H, Knhl A, Erben U, Fromm M, et al. Acute HIV infection induces mucosal infiltration with CD4+ and CD8+ T cells, epithelial apoptosis, and a mucosal barrier defect. *Gastroenterology* 2010;139:1289-300.
16. Salim SY, Soderholm JD. Importance of disrupted intestinal barrier in inflammatory bowel diseases. *Inflamm Bowel Dis.* 2011;17:362-81.