

Original article

CD69 expression on airway eosinophils and airway inflammation in a murine model of asthma

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Keywords: *asthma; eosinophils; CD69; airway inflammation; murine model*

Background Asthma is a chronic airway disease with inflammation characterized by physiological changes (airway hyper-responsiveness, AHR) and pathological changes (inflammatory cells infiltration and mucus production). Eosinophils play a key role in the allergic inflammation. But the causative relationship between eosinophils and airway inflammation is hard to prove. One of the reasons is lack of activation marker of murine eosinophils. We investigated the expression of CD69 on murine eosinophils *in vitro*, the relationship between the expression of CD69 on eosinophils from peripheral blood and bronchoalveolar lavage fluid and on airway inflammation in asthmatic mice.

Methods Eosinophils from peripheral blood of IL-5 transgenic mice (NJ.1638) were purified. Mice were divided into five groups: wild type mice sensitized and challenged with saline (WS group), wild type mice sensitized and challenged with ovalbumin (WO group), IL-5^{-/-} mice sensitized and challenged with saline and transferred with purified eosinophils (ISE group), IL-5^{-/-} mice sensitized and challenged with OVA and transferred with purified eosinophils (IOE group), IL-5^{-/-} mice sensitized and challenged with OVA and transferred with purified eosinophils, pretreated with anti CD4 monoclonal antibody (IOE+antiCD4mAb group). IL-5^{-/-} mice were sensitized with OVA at day 0 and day 14, then challenged with OVA aerosol. On days 24, 25, 26 and 27 purified eosinophils were transferred intratracheally to IL-5^{-/-} mice. On day 28, blood and BALF were collected and CD69 expression on eosinophils measured by flowcytometry.

Results Purified eosinophils did not express CD69. But eosinophils cultured with PMA+MA, IFN- γ , IL-5 or GM-CSF expressed CD69 strongly. Eosinophils from blood of WO, WS group did not express CD69 at all. The numbers of eosinophils in BALF of WO group, IOE group, ISE group and IOE+antiCD4mAb group were significantly higher than in mice of WS group which did not have eosinophils at all. CD69 expression on eosinophils in BALF of IOE and WO groups was strong. Eosinophils in BALF of ISE and IOE+antiCDmAb groups did not express CD69. The mucus production result was similar to CD69 expression. There were eosinophils infiltration in lung slides of all groups except WS group.

Conclusion Activation in airway of eosinophils could directly lead to airway inflammation.

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Asthma is a chronic airway disease with inflammation characterized by physiological changes (airway hyperresponsiveness, AHR) and pathological changes (inflammatory cells infiltration, mucus production).¹ Among these inflammatory cells, eosinophils are considered the most important cells that play a key role in allergic airway inflammation.² The infiltration of eosinophils in lung is a hallmark of asthma³ and their degree of accumulation and activation in the airway correlates with the clinical severity of asthma and development of AHR.⁴ In murine models of asthma, many kinds of different gene knockout mice such as interleukin IL-5 deficient (IL-5^{-/-}) mice and IL-4 deficient (IL-4^{-/-}) mice, show

that there is no mucus production and AHR without eosinophils infiltration in airway after allergen

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sensitization and challenge.⁵⁻⁷

Though the importance of eosinophils in asthma was widely recognized, the causative role of eosinophils in airway pathological and physiological changes was still debated. There were also numerous incongruent experimental results. For example, some studies showed that CD4⁺ T cells, not eosinophils, were responsible for the airway mucus production.⁸ Other studies using different antibodies blocking airway eosinophil infiltration still showed airway histopathological changes and AHR.^{9,10} Birrell et al¹¹ found that a small dose of dexamethasone could block the infiltration of eosinophils in airway without impact on AHR; however, increasing the dose could block AHR. In addition, Xie and his colleagues¹² demonstrated antigen presenting function of ovalbumin (OVA) exposed airway eosinophils both *in vivo* and *in vitro* to promote secretion of Th2 cytokines from lung lymphocytes. Thus, the role of eosinophils in asthma is not clear yet. The confounding results might be related to the study models. All these studies focused on the link between the infiltration of eosinophils in airway and airway pathology/physiology. There were no studies on whether eosinophils were activated whilst in the airway because there was no accepted activation marker of murine eosinophils. So a causative relationship between airway and eosinophil could not be proved.

In our previous work, we found IL-5^{-/-} mice provided model systems that were nearly devoid of eosinophils.¹³ In these IL-5^{-/-} mice, the loss of eosinophils appeared to be linked to the disappearance of airway pathological changes. Using adoptive transfer technique, we restored the pulmonary eosinophils via intratracheal transfer (IT) and found that IL-5^{-/-} mice ovalbumin sensitized and challenged showed eosinophils in lung and AHR and mucus production, but pretreatment with anti-CD4 antibody could block the mucus production and AHR without impact of eosinophils in lung. Without OVA sensitization, eosinophils only challenged in lung could not induce mucus production and AHR. These results provide a new model for studying the activation status of eosinophils in lung with or without airway AHR and mucus production, but raise the question of the

cause that separates the eosinophils infiltration in lung from AHR and mucus production.

Recently, we found a possible very early activation marker for mouse eosinophils, CD69. It could express on wild cells though there is no report about its expression on murine eosinophils.¹⁴ Our previous work showed that it could appear on murine eosinophils *in vitro* after phorbol 12-myristate 13-acetate (PMA) stimulation. Here we used CD69 as an activation marker of murine eosinophils, and used our previous model to explain the relationship of activated eosinophils and airway inflammation.

METHODS

Animals

IL-5^{-/-} mice on a C57BL/6J background were a kind gift of M. Kopf (The Basel Institute of Immunology, Switzerland). Since receiving these animals the mice have been continuously backcrossed with C57BL/6J mice (>10 generations) purchased from the Jackson Laboratory (Bar Harbor, USA). Genotypes of mice derived from these crosses were determined by PCR of tail DNA, detecting the presence of the mutant IL-5 allele. Control C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor) and all procedures were conducted on mice 8–12 weeks of age maintained in ventilated microisolator cages housed in a pathogen free animal facility.

IL-5 transgenic mice express this cytokine exclusively from mature T cells using an enhancer/promoter construct derived from the mouse CD3 gene (transgenic line: NJ.1638). NJ.1638 mice are maintained on a C57BL/6J genetic background by continuous backcross (>12 generations) with mice purchased from the Jackson Laboratory (Bar Harbor). The average circulating eosinophils counts in 4–7 month old animals are $>100 \times 10^9/L$ eosinophils of blood, representing 50% of total white blood cells.¹⁵

Purification of eosinophils

Peripheral blood (tail bleeds) from NJ.1638 mice was collected into PBS containing 20 U/ml heparin, layered onto a single step, Percoll gradient and centrifuged (45 minutes, $2000 \times g$, 4°C). The buffy coat, containing almost exclusively lymphocytes and eosinophils, was free of contaminating red blood

cells by hypotonic lysis. Eosinophils were isolated by removing B cells and T cells with Ab conjugated magnetic beads specific for CD45-R (B220) and CD90 (Thy 1.2) (Miltenyi Biotec, USA), respectively, using Magnetic Activated Cell Sorter (MACS, Miltenyi Biotec, USA). The remaining cells were in PBS at 4°C before use. The purity of the recovered eosinophils was determined by visual examination of Diff-Quick stain (Baxter Healthcare, USA) stained cytopsin preparations and was typically >99%. Eosinophil viability was determined by trypan blue exclusion and was always >99%.

Stimulation of purified eosinophils

Purified eosinophils were suspended at 1×10^6 /ml in RPMI, 24-well plates at 37°C 5% CO₂. PMA plus ionomycin (Sigma, USA) at 10⁻⁷ mol/L and cytokines interferon gamma (IFN- γ), IL-5, granulocyte macrophage colony stimulating factor (GM-CSF) at 0.1 ng/ml were added separately for 18 hours. Then the expression of CD69 was assayed by flow cytometry (FACs).

Groups of mice

Mice were divided into five groups: wild type mice sensitized and challenged with saline (WS group), wild type mice sensitized and challenged with chicken OVA (Sigma-Aldrich, USA) (WO group), IL-5^{-/-} mice sensitized and challenged with saline and transferred with purified eosinophils (ISE group), IL-5^{-/-} mice sensitized and challenged with OVA and transferred with purified eosinophils (IOE group), IL-5^{-/-} mice sensitized and challenged with OVA and transferred with purified eosinophils, pretreated with anti CD4 monoclonal antibody anti-CD4mAb (GK1.5) (R&D Systems, USA) (IOE+antiCD4mAb group). Ten mice were in each group.

OVA sensitization/aerosol challenge and intra tracheal transfer of eosinophils

Mice were sensitized and challenged with chicken OVA (Fig. 1). Briefly, mice were sensitized by an i.p. injection (100 μ l) of 20 μ g chicken OVA emulsified in inject alum (2.25 mg Al(OH)₃, 2 mg Mg(OH)₂) (Pierce, USA) on days 0 and 14. Mice were subsequently challenged with an aerosol generated from 1% OVA in saline, or saline alone, for 20 minutes by ultrasonic nebulization on days 24, 25 and 26. The mice were sacrificed on day 28 and bronchoalveolar lavage fluid (BALF) was collected. Lungs were taken out for

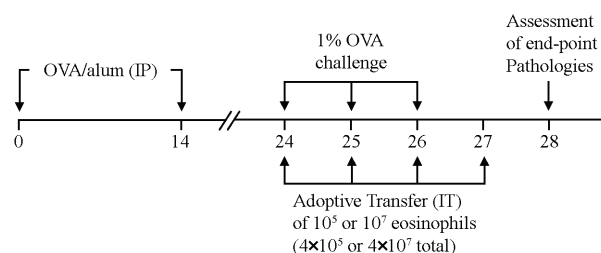


Fig. 1. Time line representation of the ovalbumin (OVA) protocol used and the intratracheal transfer (IT) adoptive transfer of eosinophils.

histological study. Adoptive transfer was accomplished in lightly anaesthetized intratracheally mice 4 to 6 hours after OVA aerosol challenging on day 24 to day 27. Eosinophils were suspended, 10^7 /20 μ l PBS. On days 21 and 24, GK1.5 was administered to the IOE+antiCD4mAb group via the peritoneal cavity (0.5 mg/100 μ l).

Assessment of airway inflammation

Eosinophils in BALF were counted. Periodic acid Schiff (PAS) and major basic protein (MBP) staining on lung slides were performed. The mucus content of airways (both proximal and distal) were assessed by bright field microscopy ($n=7$ mice per group) using image analysis software (Image ProPlus, USA) to derive an airway mucus index that is reflective of both the amount of mucus per airway and the number of airways affected.¹⁶

Assays of CD69 expression on eosinophils

Freshly purified eosinophils and eosinophils cultured with PMA+MA and different cytokines were centrifuged; the deposit added into 100 μ l PBS and incubated with block antibody Fc 2 μ l at 4°C 10 minutes. After two washes, the pellet was added to 100 μ l PBS and incubated with anti-CD69 fluorescein isothiocyanate (FITC, BD Biosciences, USA) and propidium iodide (PI, BD Biosciences, USA) for 30 minutes. Then those cells were loaded on FACS to measure geometric mean fluorescence intensity (GMFI) which represents CD69 expression of PI negative cells. Fresh unstained eosinophils were set as negative control. Eosinophils from blood or BALF of mice from five groups were stained with FITC-antiCCR3Ab and phycoerythrin (PE)-anti-CD69Ab (BD Biosciences). After loading on FACScan (Bluelion Biotech, USA), GMFI was assayed on gated FITC positive cells. Again, fresh unstained eosinophils were set as negative control.

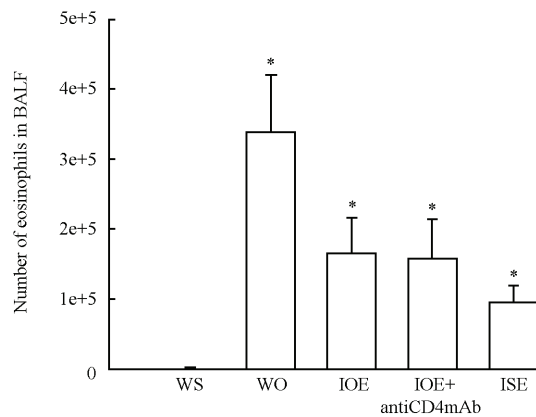


Fig. 2. Counts of eosinophils in BALF. * $P < 0.05$, compared with WS group.

Statistical analysis

Data presented are the means $\pm$ standard error (SE). Statistical analysis was performed on parametric data using one way analysis of variance (ANOVA). If the data did not follow Gaussian distribution, one way ANOVA on RANK was used. A $P < 0.05$ was considered statistically significant.

RESULTS

Eosinophils count in BALF

Eosinophils were hardly seen in BALF of WS group (129 ± 73). However, eosinophils in BALF of WO group increased significantly ($3.39 \pm 0.82 \times 10^6$) ($P < 0.05$). ISE, IOE and IOE+antiCD4mAb groups had obvious increase of eosinophils in BALF [GMFI ($0.95 \pm 0.24 \times 10^6$, $(1.60 \pm 0.50) \times 10^6$, $(1.6 \pm 0.55) \times 10^6$ respectively]. Though eosinophils in ISE group were less than those in other groups, the three groups had significant difference from WS group ($P < 0.05$, Fig. 2).

CD69 expression on eosinophils

Compared with unstained negative control, fresh purified eosinophils did not express CD69 (3.13 ± 0.14). After culture with PMA+MA, IFN- γ or GM-CSF for 18 hours, the expression of CD69 had clearly increased (GMFI 21.2 ± 0.94 , 14.4 ± 0.78 , 12.8 ± 0.61 respectively). Eosinophils in media and IL-5 also had increased CD69 expression without statistical significance (Fig. 3).

Mice of ISE, IOE and IOE+antiCD4mAb groups did not have eosinophils in peripheral blood. Fig. 4 shows that eosinophils from blood of WS and WO group and IL-5

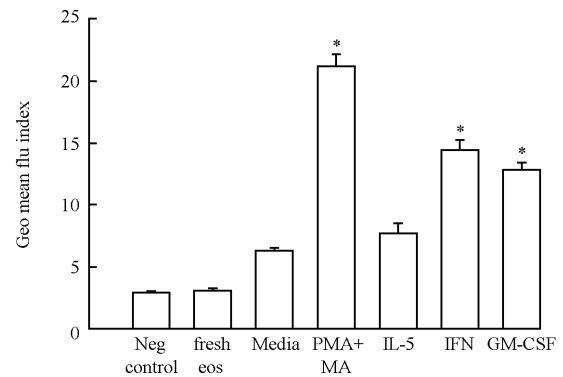


Fig. 3. Flu intensity of CD69 on eosinophils *in vitro*. * $P < 0.05$, compared with fresh eosinophils.

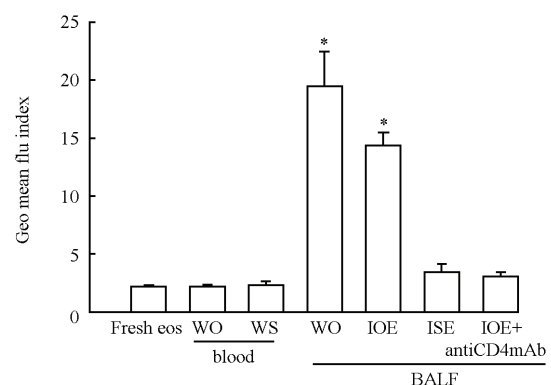


Fig. 4. CD69 expression on eosinophils from blood or BALF. * $P < 0.05$, compared with WS group.

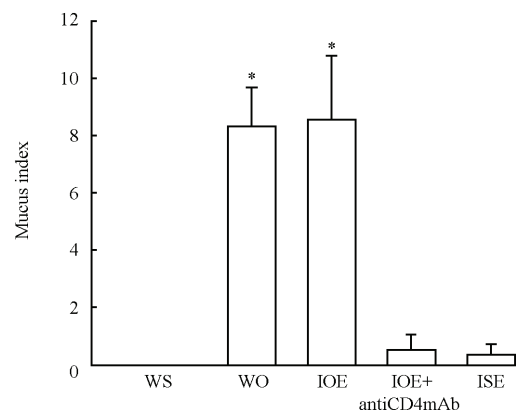


Fig. 5. Measurement of mucus production in lung. * $P < 0.05$, compared with WS group.

transgenic mice did not express CD69. BALF of WS group had no eosinophils. Eosinophils from BALF of WO and IOE groups had significantly higher expression of CD69 (GMFI 19.51 ± 2.97 , 14.4 ± 1.11 respectively) than other groups. There was no CD69 expression on eosinophils in BALF of ISE or IOE+antiCD4Ab groups (GMFI 3.46 ± 0.71 and

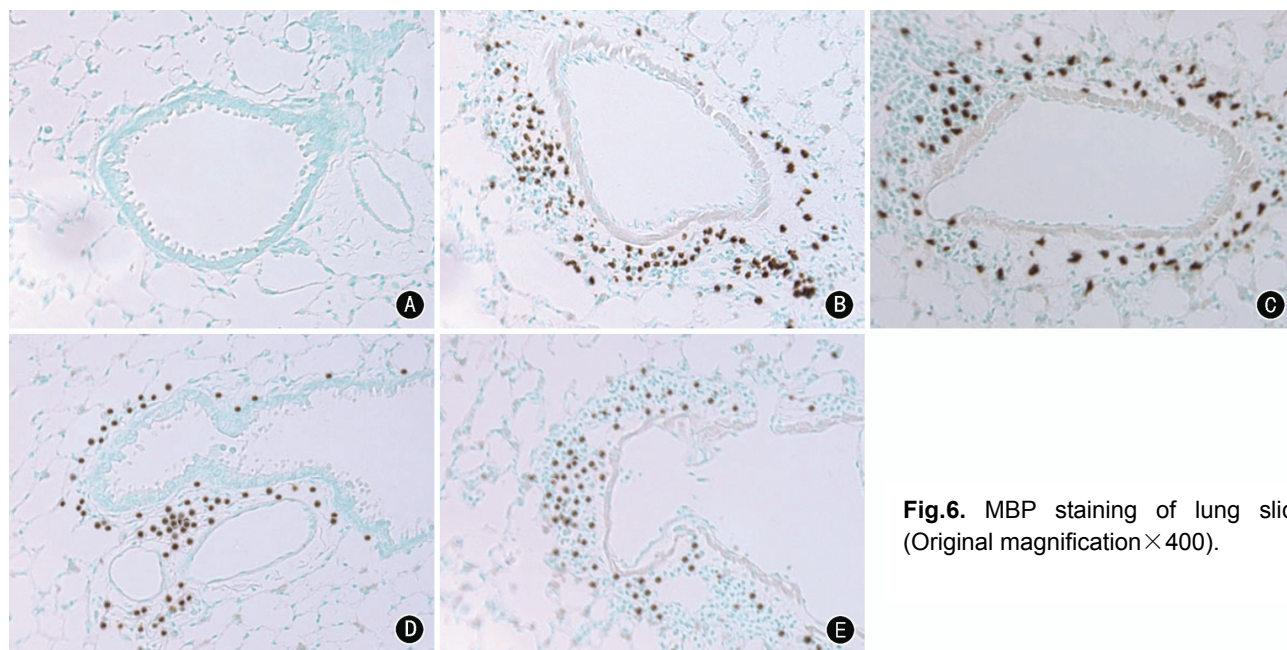


Fig.6. MBP staining of lung slides (Original magnification $\times 400$).

3.07 ± 0.27 respectively, $P < 0.05$).

Assessment of mucus production

Fig. 5 shows that little mucus was produced in WS group. ISE and IOE+antiCD4mAb groups had a little mucus around bronchioles, which was not different significantly from WS group. One could see a large amount of mucus around bronchioles of WO group and IOE group different from WS group ($P < 0.05$).

Infiltration of eosinophils in airway

There were few eosinophils in the lung of WS group. However, WO, IOE, ISE and IOE+antiCD4mAb groups had MBP positive cells infiltrated in airways (Fig. 6).

DISCUSSION

Murine models have made big contributions to the study of allergic inflammation. In studies on mechanism of asthma, different murine models have their unique roles. Compared to clinical trials, animal models have the advantages of short experimental cycles, convenient specimen collection and easy control of different conditions. Most importantly, mature transgenic mouse models allow investigation of biochemistry of single cytokines. Compared to other animal models, murine models are the closest to human. Different mature mice asthma models have been used in laboratories all over the world.¹⁷ Among all models, chicken OVA is the major allergen.

Mice sensitized by OVA first and then challenged by OVA aerosol have physiological and pathological characters of asthma such as AHR and production of excessive mucus, T lymphocytes and eosinophils infiltration in airway. Researchers have tested the important functions of eosinophils in asthma through murine asthma models. Eosinophil infiltration in airway of asthmatic mice has tight relation with AHR, mucus production and airway reconstruction. AHR and airway inflammation cannot be induced in eosinophil deficient mice and IL-4 or IL-5 deficient mice. Eosinophils play the key role in the mechanism of asthma. Yet there have been few studies on the activation mechanism of eosinophils in airways until now because of lack of eosinophilic activation marker. Unlike humans, eosinophils of asthmatic mice do not degranulate to release MBP and ECP during the inflammatory response.¹⁸ Free MBP did not show up in either eosinophilic culture *in vitro* or mice lung slides. MBP deficient mice made a more convincing improvement. Denzler et al^{19,20} found in his studies that MBP^{-/-} mice had AHR and excessive production of mucus in airway after OVA sensitization and challenging as did Epo^{-/-} mice. This made a big limitation to the study about activation of eosinophils, which could now only be speculated from the changes of airway function and pathology. Whether eosinophils' activation could result in airway inflammation directly is not clear yet. This study is the first to use CD69, a very early activation marker, as the surface activation marker of

eosinophils. CD69 is a type II transmembrane glucoprotein and expresses on cell surface in phosphorylation.²¹ CD69 gene is a member of natural killer cell, signal transducing gene, complex family. It was discovered in 1981 and named as AIM and EA1 amongst other names and was once considered as a T lymphocytic activation marker. CD69 appears an hour after T cells are activated, and lasts for 72 hours, so it may enhance activation and proliferation/differentiation of T cells.²² However, it expresses on almost all kinds of myeloid cells. One of its most important characteristics is that, except for mononuclear cells having active expression, CD69 does not show up on resting cells. Once activated, these cells will express CD69 quickly. The studies of CD69 on eosinophils are rare and mainly focus on human eosinophils. Hartnell et al²³ found that eosinophils from BALF of asthmatic patients expressed CD69, so did eosinophils from peripheral blood after GM-CSF stimulation. In 1995 Mastsumoto and his associates²⁴ used 473 kinds of human leukocyte antibodies provided by the Fifth International Workshop to study the phenotype of human eosinophils. They found that resting eosinophils from peripheral blood did not express CD69, but those in BALF from asthmatic patients or cultured with GM-CSF, IL-5 *in vitro* did express CD69. However, whether CD69 could express on murine eosinophils and be an activation marker was unclear. The low number of murine eosinophils, which makes them difficult to isolate might be responsible.

In this study, we took advantage of IL-5 transgenic mice to collect large numbers of eosinophils from peripheral blood. Fresh purified eosinophils from negative selection of MACS do not have CD69 expression, which indicated that the separating system did not affect the expression of surface antigen. After stimulation by PAM+MA, GM-CSF, IL-5 or IFN- γ , CD69 was expressed on cell surface. PMA+MA could activate eosinophils strongly, which made the highest GMFI of CD69. The role of GM-CSF was comparatively weaker, but it could maintain the viability of cells longer, comparable with the studies of human eosinophils. IFN- γ was considered to inhibit the activation of eosinophils, morphological change and migration of eosinophils through inhibiting the activation of ERK *in vitro*.^{25,26} However, in our study, GM-CSF induced the expression of CD69 on eosinophils proving that

mouse eosinophils had IFN- γ receptor and IFN- γ could direct the activation of eosinophils agreeing with the conclusion made by Lopez et al.²⁷ The interesting observation that cultured eosinophils spontaneously expressed CD69 may be due to the culturing.

IL-5^{-/-} mice did not have eosinophils in peripheral blood, thus eosinophils infiltration in airway, airway inflammation and AHR could not be induced after OVA sensitization and challenging.²⁸ Based on previous work, this study we focused on activation status of eosinophils in BALF using the technique of adoptive transfer to transport eosinophils from NJ.1638 mice to IL-5^{-/-} mice via IT. The number of eosinophils transferred to ISE, IOE and IOE+AntiCD4mAb groups was equal, so eosinophils could be found in BALF from three groups. Nevertheless, measured eosinophils in ISE group were fewer than the other two groups. This was related to the effects of different cytokines and chemokines. Eosinophils are a type of mature differentiated cells that will undergo apoptosis automatically *in vitro*. However, high level of cytokines such as IL-5, IL-3 or GM-CSF in lumen induced by OVA sensitization and challenging can activate eosinophils and maintain their viability.²⁹⁻³¹ Besides, activated epithelial cells also could activate eosinophils directly to prolong the lives of eosinophils. Though initially lacking IL-5 in IOE group, there were many other cells and cytokines to keep the viability of eosinophils and activated eosinophils could release IL-5 and GM-CSF by autocrine.³² For the same reason, IOE+AntiCD4mAb group still had the airway cytokine network provoked by OVA to activate eosinophils after blocking the function of CD4⁺ T cells. However, ISE group had fewer eosinophils through lack of all types of cytokines. In three groups, eosinophils infiltration in airway on lung slides could be seen, which proved that eosinophils could not only migrate from peripheral blood to airway under the function of different chemokines, but also transmembrane of epithelial cells from lumen to lung tissue. This matches the findings of Shi et al¹³ that eosinophils could migrate from lumen to lung lymph nodes and is consistent with the previous results we obtained. This may be explained by the autodefensive function of eosinophils. Airway epithelial cells can release neutrophil to prolong the lives of eosinophils,³³ and

express ICAM-1 after activation that can react with CD18 on eosinophils to enhance the adhesion of these two cells and lead to migration. However, in ISE group, without chemotactic factors, eosinophils also could immigrate transmembrane to lung tissue. This indicated that eosinophils had automatic adhesion with epithelial cells that directed migration of eosinophils to different areas.

Purified eosinophils did not express CD69, nor did eosinophils from peripheral blood of WO group and WS group. Eosinophils from BALF from IOE group and WO group had strong expression of CD69. This illustrated that OVA sensitization and challenging could not activate eosinophils in peripheral blood. However, migrating to lung did not activate eosinophils. Under the impacts of different cells and cytokines, eosinophils in lung were activated and had changes of surface receptors. Though IL-5^{-/-} mice lacked IL-5, extrinsic transferred eosinophils still could be activated and express CD69. But once the roles of CD4⁺ T cells had been blocked, eosinophils could not be activated. There are some reports about human eosinophils being activated by different cytokines and undergoing degranulation *in vitro*, but in murine eosinophils' activation, CD4⁺ T cells are essential. This could be related to the priming and controlling function of CD4⁺ T cells in asthmatic inflammation.³⁴ Moreover, CD4⁺ T cells can activate eosinophils directly through intercellular adhesive molecules.³⁵

CD69 expression on eosinophils is related closely to airway inflammation. Though BALF and lung slides of ISE group and IOE+AntiCD4mAb group had eosinophilic infiltration, there was no expression of CD69 on eosinophils. This hinted that eosinophils were inactivated and the two groups had no mucus production in airway, whereas WO and IOE groups had strong expression of CD69 on eosinophils in BALF accompanied with a lot of mucus production. These results confirmed that airway inflammation of asthma was induced directly by activated eosinophils. There was no inflammation without activated eosinophils.

In conclusion, eosinophils in peripheral blood from asthmatic mice are inactive, but eosinophils in airway become activated by different factors in the microenvironment and express CD69. CD4⁺ T cells

are essential for the activation of eosinophils. Asthmatic inflammation of airway is the direct result of eosinophils activation.

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REFERENCES

1. Sugita M, Kuribayashi K, Nakagomi T, Miyata S, Matsuyama T, Kitada O. Allergic bronchial asthma: airway inflammation and hyper-responsiveness. *Intern Med* 2003; 42: 636-643.
2. Shen HH. Eosinophil: central mediator of allergic asthma? *Chin Med J* 2005; 118: 4-5.
3. Busse WW, Lemanske RF J. Asthma. *N Engl J Med* 2001; 344: 350-362.
4. Busse WW, Calhoun WF, Sedgwick JD. Mechanism of airway inflammation in asthma. *Am Rev Respir Dis* 1993; 147 (6 Pt 2): S20-S24.
5. Leigh R, Ellis R, Wattie JN, Hirota JA, Matthaei KI, Foster PS, et al. Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice. *Am J Respir Crit Care Med* 2004; 169: 860-867.
6. Trifilieff A, Fujitani Y, Coyle AJ, Kopf M, Bertrand C. IL-5 deficiency abolishes aspects of airway remodelling in a murine model of lung inflammation. *Clin Exp Allergy* 2001; 31: 934-942.
7. Hamelmann E, Gelfand EW. Role of IL-5 in the development of allergen-induced airway hyperresponsiveness. *Int Arch Allergy Immunol* 1999; 120: 8-16.
8. Cohn LR, Homer J, MacLeod H, Mohrs M, Brombacher F, Bottomly K. Th2-induced airway mucus production is dependent on IL-4R α , but not on eosinophils. *J Immunol* 1999; 162: 6178-6183.
9. Hogan SP, Matthaei KI, Young JM, Koskinen A, Young IG, Foster PS. A novel T cell-regulated mechanism modulating allergen induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. *J Immunol* 1998; 161: 1501-1509.
10. Coyle AJ, Kohler G, Tsuyuki S, Brombacher F, Kopf M. Eosinophils are not required to induce airway hyperresponsiveness after nematode infection. *Eur J Immunol* 1998; 28: 2640-2647.
11. Birrell MA, Battram CH, Woodman P, McCluskie K, Belvisi MG. Dissociation by steroids of eosinophilic inflammation from airway hyperresponsiveness in murine airways. *Respir Res* 2003; 4:1-7.
12. Xie ZF, Shi HZ, Qin XJ, Kang LF, Huang CP, Chen YQ. Effects of antigen presentation of eosinophils on lung Th1/ Th2 imbalance. *Chin Med J* 2005; 118: 6-11.
13. Shen HH, Ochkur SI, McGarry MP, Crosby JR, Hines

- EM, Borchers MT, et al. A causative relationship exists between eosinophils and the development of allergic pulmonary pathologies in the mouse. *J Immunol* 2003; 170: 3296-3305.
14. Lensmar C, Katchar K, Eklund A, Grunewald J, Wahlstrom J. Phenotypic analysis of alveolar macrophages and lymphocytes following allergen inhalation by atopic subjects with mild asthma. *Respir Med* 2006; 100:918-925.
15. Lee NA, McGarry MP, Larson KA, Horton MA, Kristensen AB, Lee JJ. Expression of IL-5 in thymocytes/T cells leads to the development of a massive eosinophilia, extramedullary eosinophilopoiesis, and unique histopathologies. *J Immunol* 1997; 158: 1332-1344.
16. Justice JP, Crosby J, Borchers MT, Tomkinson A, Lee JJ, Lee NA. CD4⁺ T cell-dependent airway mucus production occurs in response to IL-5 expression in lung. *Am J Physiol Lung Cell Mol Physiol* 2002; 282: L1066-L1074.
17. Pabst R. Animal models for asthma: controversial aspects and unsolved problems. *Pathobiology* 2002-2003; 70: 252-254.
18. Malm-Erfjelt M, Persson CG, Erfjelt JS. Degranulation status of airway tissue eosinophils in mouse models of allergic airway inflammation. *Am J Respir Cell Mol Biol* 2001; 24: 352-359.
19. Denzler KL, Borchers MT, Crosby JR, Cieslewicz G, Hines EM, Justice JP, et al. Extensive eosinophils degranulation and peroxidase-mediated oxidation of airway proteins do not occur in a mouse ovalbumin-challenge model of pulmonary inflammation. *J Immunol* 2001; 167: 1672-1682.
20. Denzler KL, Farmer SC, Crosby JR, Borchers M, Cieslewicz G, Larson KA, et al. Eosinophil major basic protein-1 does not contribute to allergen-induced airway pathologies in mouse models of asthma. *J Immunol* 2000; 165: 5509-5517.
21. Vance BA, Harley PH, Backlund PS, Ward Y, Phelps TL, Gress RE. Human CD69 associates with an N-terminal fragment of calreticulin at the cell surface. *Arch Biochem Biophys* 2005; 438:11-20.
22. Lewkowich IP, Rempel JD, HayGlass KT. Prevention of allergen-specific, Th2-biased immune responses *in vivo*: role of increased IL-12 and IL-18 responsiveness. *J Immunol* 2005; 175: 4956-4962.
23. Hartnell A, Robinson DS, Kay AB, Wardlaw AJ. CD69 is expressed by human eosinophils activated *in vivo* in asthma and *in vitro* by cytokines. *Immunology* 1993; 80: 281-286.
24. Matsumoto K, Appiah-Pippim J, Schleimer RP, Bickel CA, Beck LA, Bochner BS. CD44 and CD69 represent different types of cell-surface activation markers for human eosinophils. *Am J Respir Cell Mol Biol* 1998; 18: 860-866.
25. Park CS, Choi EN, Kim JS, Choi YS, Rhim TY, Chang HS, et al. Interferon-gamma inhibits *in vitro* mobilization of eosinophils by interleukin-5. *Int Arch Allergy Immunol* 2005; 136: 295-302.
26. Ishihara C, Ochiai K, Kagami M, Takashahi H, Matsuyama G, Yoshida S, et al. Human peripheral eosinophils express functional interferon-gamma receptors (IFN-gammaR). *Clin Exp Immunol* 1997; 110: 524-529.
27. Lopez JA, Newburger PE, Condino-Neto A. The effect of IFN-gamma and TNF-alpha on the eosinophilic differentiation and NADPH oxidase activation of human HL-60 clone 15 cells. *J Interferon Cytokine Res* 2003; 23: 737-744.
28. Trifilieff A, Fujitani Y, Coyle AJ, Kopf M, Bertrand C. IL-5 deficiency abolishes aspects of airway remodelling in a murine model of lung inflammation. *Clin Exp Allergy* 2001; 31: 934-942.
29. Ip WK, Wong CK, Wang CB, Tian YP, Lam CW. Interleukin-3, -5, and granulocyte macrophage colony-stimulating factor induce adhesion and chemotaxis of human eosinophils via p38 mitogen-activated protein kinase and nuclear factor kappaB. *Immunopharmacol Immunotoxicol* 2005; 27: 371-393.
30. Vassina EM, Yousefi S, Simon D, Zwicky C, Conus S, Simon HU. cIAP-2 and survivin contribute to cytokine-mediated delayed eosinophil apoptosis. *Eur J Immunol* 2006 36: 1975-1984.
31. Stout BA, Bates ME, Liu LY, Farrington NN, Bertics PJ. IL-5 and granulocyte-macrophage colony-stimulating factor activate STAT3 and STAT5 and promote Pim-1 and cyclin D3 protein expression in human eosinophils. *J Immunol* 2004; 173: 6409-6417.
32. Yamamoto H, Sedgwick JB, Vrtis RF, Busse WW. The effect of transendothelial migration on eosinophils function. *Am J Respir Cell Mol Biol* 2000; 23:379-388.
33. Hahn C, Islamian AP, Renz H, Nockher WA. Airway epithelial cells produce neurotrophins and promote the survival of eosinophils during allergic airway inflammation. *J Allergy Clin Immunol* 2006; 117: 787-794.
34. Shinkai K, Mohrs M, Locksley RM. Helper T cells regulate type-2 innate immunity *in vivo*. *Nature* 2002; 420: 825-829.
35. Douglas IS, Leff AR, Sperling. CD4⁺ T cell and eosinophil adhesion is mediated by specific ICAM-3 ligation and results in eosinophil activation. *J Immunol* 2000; 164: 3385-3391.

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