

Original article

Effects of antigen presentation of eosinophils on lung Th1/Th2 imbalance

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Keywords: Eosinophil · Th1/Th2 cell · antigen-presenting cell · lung

Background Antigen-loaded eosinophils (EOSs) instilled intratracheally into mice were capable of inducing Th2-type cytokine production in the draining lymph nodes. The aim of the present study was to evaluate whether EOSs within the tracheobronchial lumen can stimulate Th2 cell expansion in the lung tissues.

Methods Airway EOSs were recovered from ovalbumin-sensitized and -challenged BALB/c mice, these EOSs were then cocultured with CD4⁺ cells isolated from sensitized mice in the absence or presence of anti-CD80 or/and -CD86 monoclonal antibodies. Airway EOSs were instilled into the trachea of sensitized mice. At the day 3 thereafter, the lung tissues were removed and prepared into cell suspensions for culture. Cell-free culture supernatants were collected for detection of cytokines.

Results Airway EOSs functioned as CD80⁻ and CD86⁻ dependent antigen-presenting cells to stimulate lung CD4⁺ lymphocytes to produce interleukin-4, interleukin-5 and interleukin-13, but not interferon- γ in *in vitro* assay. When instilled intratracheally in sensitized recipient mice, airway EOSs primed lung Th2 cells *in vivo* for interleukin-4, interleukin-5 and interleukin-13, but not interferon- γ , production during the *in vitro* culture that was also CD80⁻ and CD86⁻ dependent.

Conclusion EOSs within the lumina of airways could process inhaled antigen and function *in vitro* and *in vivo* as antigen-presenting cells to promote expansion of Th2 cells in the lungs.

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At sites of allergic disease and parasitic infection, eosinophil (EOS) is thought to occupy key roles in pathogenesis and worm expulsion through the release of proinflammatory molecules and granular proteins.^{1,2} Although the EOSs are classically thought to act as an effector cells, there is emerging *in vivo* evidence that these leukocytes may also have discrete afferent immunomodulatory roles.³ Allergen-induced recruitment of EOSs into lung tissues is correlated with roles of CD4⁺ T cells, presumably Th2 cells, and cytokines released by such T cells.⁴⁻⁶ In humans, interleukin-4 (IL-4) and IL-5 are functionally important in causing EOS infiltration into airway and its activation as well as airway hyperresponsiveness to methacholine.^{7,8} The accumulation of EOSs in tissues, as in chronic asthma or following acute challenges in the lungs, correlates with measures of local T cell activation. Thus, there has been an increasing recognition that EOS accumulation and enhanced effector functions at tissue sites of allergic reactions may be intimately related to lymphocyte activation,

especially by Th2-like lymphocytes elaborating cytokines, including IL-5 and granulocyte/macrophage colony-stimulating factor (GM-CSF), that prolong the viability and enhance the effector responses of mature EOSs.

Recently, we have shown that airway EOSs functioned as CD80⁻ and CD86⁻ dependent antigen-presenting cells (APC) to stimulate sensitized CD4⁺ lymphocytes from

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lymph nodes to produce IL-4, IL-5 and IL-13, but not interferon- γ (IFN- γ) in *in vitro* assay. When instilled intratracheally in sensitized recipient mice, airway EOSs migrated into draining paratracheal lymph nodes and primed Th2 cells *in vivo* for IL-4, IL-5 and IL-13, but not IFN- γ , production that was also CD80- and CD86-dependent.⁹ In the present investigation, we extended the above findings and demonstrated that ovalbumin (OVA) exposed airway EOSs function both *in vitro* and *in vivo* as APCs to promote secretion of Th2 cytokines from lung lymphocytes.

METHODS

Mice

Six-eight weeks old female BALB/c mice were provided by Experimental Animal Center of Guangxi Medical University, Nanning, China. All mice were maintained under specific pathogen free conditions in the animal facility in our hospital.

EOS purification

Mice were sensitized intraperitoneally three times with 10 μ g of OVA (Sigma Chemical Co., St. Louis, MO, USA) plus 1 mg Al(OH)₃ in 0.2 ml phosphate buffered saline (PBS) on days 1, 7 and 14. On days 21, 22, 23 and 24, sensitized mice underwent inhalational challenges within a plastic chamber with OVA by daily exposures to aerosolized antigen (5% in PBS) delivered by a nebulizer driven by compressed air at 5 L/min for 30 minutes. Twenty four hours after the final challenge, mice were given a lethal dose of pentobarbital (60 mg/kg intraperitoneally), and the lungs were lavaged six times with 0.8 ml aliquots of PBS containing 4 mmol/L EDTA and 2% normal mouse serum (PBS/serum) to obtain bronchoalveolar lavage fluid (BALF) for EOS purification. Mouse EOSs in BALF were purified by centrifugation on discontinuous Percoll density gradients followed by further immunomagnetic bead purifications. Percoll was adjusted to isotonicity by adding one part 10 \times PBS to nine parts Percoll, and four different densities (1.085, 1.080, 1.075, and 1.070 g/ml) of Percoll were prepared with PBS. Five ml of each decreasing density were sequentially layered in 50 ml plastic tubes and then BALF cells from 5 to 6 mice in 5 ml PBS/serum was placed on top of the gradient. After centrifugation for 25 minutes at 1500 \times g at room temperature, EOSs were between the 1.075 and 1.070 g/ml layers, with the majority of macrophages on top of the gradients. EOSs were collected and washed in PBS/serum, and after 1 hour culture on plastic petri dishes in RPMI-1640 supplemented with penicillin (100 U/ml),

streptomycin (100 μ g/ml), L-glutamine (2 mmol/L), HEPES (10 mmol/L), and 5% normal mouse serum to eliminate remaining adherent macrophages, EOSs were further purified by negative immunomagnetic selection, using a magnetically activated cell separator system (Miltenyi Biotec, Auburn, CA, USA). To remove contaminating lymphocytes, anti-CD90- and anti-CD19-coated micromagnetic beads were added to the EOS suspension. By negative selection, highly purified mouse EOSs (>99.5%) were routinely obtained, as assessed by microscopy and flow cytometric analyses. Viability determined by trypan blue exclusion of purified EOSs was >97%.

Cytokine production from sensitized lung CD4⁺ T cells induced by EOSs *in vitro*

To obtain OVA-sensitized lung CD4⁺ cells, BALB/c mice were immunized intraperitoneally with 10 μ g of OVA plus 1 mg Al(OH)₃. A cell preparation enriched for interstitial lymphocytes was prepared by first lavaging mice as above and then perfusing the lungs with heparinized saline via right heart puncture until the great pulmonary vessels were grossly clear. Lungs were then excised at the pleural surface, minced finely with scissors, and incubated in RPMI 1640 containing 30 μ g/ml DNase-1 and 150 U/ml collagenase (Sigma, USA) for 35 minutes at 37 $^{\circ}$ C with constant gentle swirling. Next, the lung fragments were forced through a SS80 mesh and washed twice in calcium/magnesium-free PBS. Portions of the resulting cell preparation were depleted of accessory cells by passage over a Sephadex G-10 column (Sigma), and then lung cell suspensions were incubated with anti-CD4-coated micromagnetic beads as described above. By positive selection with Miltenyi Biotec magnetically activated cell separator system, highly purified (>97%), OVA-primed CD4⁺ cells were routinely obtained with viabilities >98%. Microscopic examination of several hundred, and at times of 700 to 1000, cytocentrifuged CD4⁺ cells demonstrated no macrophages among lymphocytes. Purified CD4⁺ cells (1 \times 10⁶ cells/ml) were incubated with or without purified airway EOSs from BALB/c mice (5 \times 10⁵ cells/ml) in supplemented RPMI-1640 medium in the absence or presence of 200 μ g/ml OVA in 48-well plates. As controls, EOSs alone were also cultured. EOSs were cultured with 5 ng/ml recombinant mouse GM-CSF (BioSource, Camarillo, CA, USA) to sustain their viability. In some experiments, anti-CD80 (16-10A1; 10 μ g/ml) or/and anti-CD86 (GL1; 10 μ g/ml) mAb (PharMingen, San Diego, CA, USA), or control rat IgG_{2a} (10 μ g/ml) were added. Cultures were incubated for 96 hours at

37 in a 5% CO₂ humidified atmosphere. Cell-free culture supernatants were collected and stored in aliquots at -70 before analysis of IL-4, IL-5, IL-13, and IFN-.

Cytokine production from lung lymphocytes primed in vivo by EOSs

Mice were immunized intraperitoneally with 10 µg of OVA plus 1 mg Al(OH)₃. Two weeks later, mice were lightly anesthetized with avertin (250 µg/kg), and their tracheas were surgically exposed. 1 ×10⁶ purified OVA-exposed EOSs were instilled slowly in a volume of 50 µl PBS into the trachea via a 25-gauge needle. In some experiments, OVA-sensitized mice were injected intravenously with anti-CD80 (100 µg), anti-CD86 (100 µg), a combination of both anti-CD80 and anti-CD86 mAbs (total of 200 µg), or control rat IgG_{2a} (100 µg) immediately before, and 1 and 2 days after they received endotracheal instillations of EOSs. At 3 days thereafter, lung interstitial lymphocyte suspension was prepared using abovementioned methods. Lung lymphocytes (2 ×10⁶/ml) were cultured in 48-well plates for 48 hours in the absence or presence of 200 µg/ml OVA. Cell-free culture supernatants were collected and stored in aliquots at -70 before analysis of IL-4, IL-5, IL-13, and IFN-.

Cytokine analysis

IL-4, IL-5, IFN- (all from PharMingen), and IL-13 (R&D Systems, Minneapolis, MN, USA) concentrations in the supernatants were determined by ELISA according to the manufacturer's protocols.

Statistics

Values are presented as mean ±standard error of mean (SEM). Analysis of variance (ANOVA) or paired *t* test was used to test the statistical significance of the results. *P* values of less than 0.05 were considered significant.

RESULTS

Cytokine production from sensitized lung CD4⁺ T cells in vitro

In the absence of EOSs as APCs during 4-day *in vitro* culture, lung CD4⁺ cells produced none of IL-4, IL-5, IL-13, or IFN- even when incubated with 200 µg/ml of exogenous antigen (Table 1). The addition of airway EOSs yielded a significant cytokine production of IL-4, IL-5 and IL-13; although IFN- in the supernatants was also detectable, its concentration was quite low. Incubation of EOSs alone in medium resulted in no

appreciable production of detected cytokines. The addition of exogenous OVA to these EOS-CD4⁺ cell co-cultures yielded even greater productions of IL-4, IL-5 and IL-13, but not IFN-, indicating that EOSs *in vitro* were further capable of processing antigen and serving as APCs.

To further evaluate whether EOSs were providing requisite B7 costimulatory signals for their APC function to induce cytokine production by Th2 cells, we cultured 1 ×10⁶ cells/ml of OVA-sensitized lung CD4⁺ cells and 5 ×10⁵/ml of OVA-exposed EOSs in the presence or absence of inhibitory concentrations of anti-CD80 mAb, anti-CD86 mAb, or a combination of both. Anti-CD80 and anti-CD86 mAb alone partially blocked productions of IL-4, IL-5 and IL-13 (Table 2). A combination of both anti-CD80 and anti-CD86 blocked mAbs yielded even greater inhibition of EOS-elicited Th2 type cytokine production.

Table 1. EOSs stimulate Th2-type cytokine productions from lung CD4⁺ cells in vitro (ng/L)

Cytokines	EOSs only		EOSs + CD4 ⁺ cells	
	OVA (-)	OVA (+)	OVA (-)	OVA (+)
IL-4	5.2 ±1.2	5.6 ±1.1	90.2 ±14.2	325.3 ±40.2 *
IL-5	12.2 ±2.4	14.5 ±3.2	376.3 ±74.2	895.9 ±90.5 *
IL-13	7.7 ±1.3	11.1 ±1.0	157.9 ±34.4	449.5 ±44.2 *
IFN-	6.8 ±0.9	4.9 ±1.0	25.3 ±10.1	32.5 ±9.4

**P* < 0.01, compared with values for no exogenous OVA, by paired *t* test.

Table 2. The role of B7 molecules on Th2-type cytokine productions elicited by EOSs in vitro (ng/L)

Cytokines	Control	Anti-CD80	Anti-CD86	Anti-CD80 + anti-CD86
IL-4	95.2 ±13.2	61.6 ±11.2 *	44.9 ±11.6 *	15.3 ±8.5 *
IL-5		186.5 ±42.1 *		42.1 ±15.2 *
IL-13		108.2 ±19.5 *	79.5 ±20.1 *	21.5 ±9.5 *

**P* < 0.05, compared with nonimmune IgG_{2a} controls, respectively, by ANOVA.

Cytokine production from lung cells primed in vivo by EOSs

We noted that like the *in vitro* experiments, lung lymphocytes yielded mainly cytokine production of IL-4, IL-5 and IL-13 (Table 3). The addition of exogenous OVA to these lung lymphocytes yielded even greater Th2 type cytokine production indicative that lung Th2 cells were primed *in vivo* by antigen presentation of EOSs and ready for cytokine production. As expected, the endotracheal instillation of fixed, nonviable EOSs induced no significant productions of Th2 type cytokines.

Having established the APC functional activity of airway EOSs *in vivo* for expansion of Th2 cells, we then analyzed the role of B7/CD28/CTLA-4 pathway in antigen presentation by EOSs to lung Th2 cells *in vivo* by blocking CD80, CD86 or both. Treatment of OVA-sensitized mice with either anti-CD80 or anti-CD86 mAb alone partially suppressed the Th2 type cytokine production elicited *in vivo* by tracheal instillation of OVA-exposed EOSs (Table 4). Treatments with the combination of anti-CD80 and anti-CD86 mAbs resulted in a greater inhibition for production of each of IL-4, IL-5 and IL-13.

Table 3. EOSs prime lung Th2 cells *in vivo* for Th2-type cytokine productions (ng/L)

Cytokines	Fixed EOSs		Viable EOSs	
	OVA (-)	OVA (+)	OVA (-)	OVA (+)
IL-4	10.2 ±2.1	9.7 ±2.5	48.4 ±9.1	123.4 ±20.4 *
IL-5	12.0 ±2.9	13.7 ±2.3	132.6 ±24.6	388.6 ±49.3 *
IL-13	8.8 ±1.4	7.2 ±1.1	57.6 ±16.5	160.7 ±24.7 *
IFN-	9.7 ±0.9	6.4 ±0.8	12.6 ±4.3	13.2 ±3.4

* P < 0.01, compared with values for no exogenous OVA, by paired t test.

Table 4. The role of B7 molecules on Th2-type cytokine productions primed by EOSs *in vivo* (ng/L)

Cytokines	Control	Anti-CD80	Anti-CD86	Anti-CD80 + anti-CD86
IL-4	45.3 ±7.5	34.6 ±5.9 *	27.8 ±5.6 *	9.2 ±3.3 *
IL-5	145.3 ±22.2	102.2 ±21.5 *	79.6 ±18.7	21.8 ±8.7 *
IL-13	60.5 ±9.6	38.6 ±8.8 *	33.5 ±7.2 *	9.2 ±4.5 *

* P < 0.05, compared with nonimmune IgG_{2a} controls, by ANOVA.

DISCUSSION

Currently, EOSs are primarily thought to act as effector cells, inducing pathological changes through the release of granular proteins and proinflammatory mediators.¹⁰ However, under normal physiological conditions, tissue EOSs do not induce diseases and their numbers and activation status are tightly regulated. The presence of EOSs in mucosal surfaces at baseline, in association with their ability to release cytokines and express molecules that engage T cells, suggests that these cells may also have immunomodulatory functions. Indeed, limited *in vivo* investigations suggest that EOSs and mast cells have the potential to direct immune responses associated with allergic inflammation and parasitic infestation.^{11,12}

For several years, it has been described that EOSs are able to express major histocompatibility complex II complex and costimulatory molecules and to function as APCs.³

Although the presence of EOSs within airways

secretions is characteristic of allergic diseases of the airways, including asthma and rhinitis,^{13,14} functional roles for EOSs within the lumina of the airways are not known. Many cell types have the capacity to serve as APCs. Thus for EOSs, a critical test was to ascertain whether EOSs within the airways, after encountering inhaled antigens, could function to present antigens and elicit T cell responses *in vivo*. Our previous studies have established not only that endotracheal EOSs homed to T cell rich regions of draining peritracheal lymph nodes, but also that these antigen-exposed, airway-derived EOSs, when instilled into the airways of OVA-sensitized mice, were capable of stimulating T cell proliferative responses *in vivo* within draining lymph nodes.¹⁵ These EOS-mediated *in vivo* T cell proliferative responses were antigen-specific, CD80/CD86-dependent and limited to CD4⁺ T cells.¹⁵ It should be noted that the work of van Rijt and colleagues¹⁶ do not support any role for airway EOSs as APCs to naive T cells, despite their migration to the draining lymph nodes at times of allergen exposure and antigen presentation to sensitized T cells.

It is well known that Th2 cells secrete IL-4, IL-5, IL-13, etc., that promote allergic inflammation and stimulate B cells to produce IgE and other antibodies. In contrast, Th1 cells produce IFN- and IL-2, which initiate the killing of viruses and other intracellular organisms by activating macrophages and cytotoxic T cells.¹⁷ These two subgroups of helper T cells arise in response to different immunogenic stimuli and cytokines, and they constitute an immunoregulatory loop: cytokines from Th1 cells inhibit Th2 cells, and *vice versa*. An imbalance in this reciprocal arrangement may be the key to asthma.¹⁸

Since EOSs can express the molecules associated with antigen presentation, stimulate CD4⁺ T cell proliferation, and localize to T cell-rich regions of draining lymph nodes,^{15,19} it is necessary to explore the effects of antigen presentation of EOSs on Th1/Th2 balance. MacKenzie and colleagues¹⁹ have established that purified EOSs from IL-5-transgenic mice were able to induce IL-4, IL-5, and IL-13 production in Th2 cells in an antigen-specific manner. These cells did not produce IFN- on stimulation. Thus, EOSs are able to induce proinflammatory cytokine secretion from CD4⁺ Th2 cells polarized *in vitro*. In a more recent study,⁹ we further showed that EOSs isolated and purified from the airways of mice challenged with aerosolized OVA were capable of stimulating Th2 type cytokine production indicative of Th2 cell expansion by

presenting antigen in *in vitro* assay. These results were consistent with the previous findings reported by Mackenzie and colleagues.¹⁹ In addition, we also showed that the expansion of lymph node Th2 cells caused by airway EOSs was CD80- and CD86-dependent, because the production of IL-4, IL-5 and IL-13 could be inhibited by the blockade of B7/CD28/CTLA-4 costimulatory pathway by using anti-CD80 or/and -CD86 mAbs. In that study,⁹ EOSs from OVA-sensitized and -challenged mice were instilled into tracheas of OVA-sensitized mice, draining paratracheal lymph nodes were taken 3 days thereafter and tested for cytokine production. We noted that like the *in vitro* experiments, lymph node cells yielded mainly cytokine production of IL-4, IL-5 and IL-13, but not of IFN-. The addition of exogenous OVA to these lymph node cells yielded even greater Th2 type cytokine production indicative that Th2 cells were primed *in vivo* by antigen presentation of EOSs and ready for cytokine production. As expected, the endotracheal instillation of fixed, nonviable EOSs induced no significant productions of Th2 type cytokines.

In the present study, we extended the above findings and showed that EOSs isolated and purified from the airways of mice challenged with aerosolized OVA were capable of stimulating T cells isolated from tissue to produce Th2 type cytokines by presenting antigen in *in vitro* assay. We noted that the expansion of lung Th2 cells observed in this study caused by airway EOSs was CD80- and CD86-dependent, because the production of IL-4, IL-5 and IL-13 could be inhibited by the blockade of B7/CD28/CTLA-4 costimulatory pathway by using anti-CD80 or/and -CD86 mAbs. Moreover, our present study established that these OVA-exposed, airway-derived EOSs, when instilled into the airways of OVA-sensitized mice were capable of priming Th2 cell expansion *in vivo* in the lung tissues. These EOS APC-mediated *in vivo* Th2 type responses were also B7/CD28/CTLA-4-dependent. These investigations highlight the potential of EOSs to not only act as terminal effector cells but also to actively amplify allergic responses by promoting Th2 cell immunity.

In conclusion, we have shown that OVA-sensitized lung CD4⁺ cells may directly engage EOSs pulsed with cognate antigen to promote the production of cytokines (IL-4, IL-5, and IL-13) that are intimately involved in allergic responses. Thus, EOSs have the potential to activate lung Th2 cells to release disease-modulating cytokines.

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