

Original article

Endobronchial eosinophils preferentially stimulate T helper cell type 2 responses

Background: Antigen-loaded eosinophils instilled intratracheally into mice were capable of migrating into local lymph nodes and localize to the T cell-rich paracortical zones where they stimulated antigen-specific proliferation of CD4⁺ T cells. The aim of the present study was to evaluate whether eosinophils within the tracheobronchial lumen can stimulate Th2 cell expansion by presenting antigen both *in vitro* and *in vivo*.

Methods: Airway eosinophils were recovered from ovalbumin-sensitized and -challenged BALB/c mice, these eosinophils were then co-cultured with sensitized CD4⁺ cells in the absence or presence of anti-CD80 or/and -CD86 monoclonal antibodies. Airway eosinophils were instilled into the trachea of sensitized mice. At 3 days thereafter, the draining paratracheal lymph nodes were removed and teased into cell suspensions for culture. Cell-free culture supernatants were collected for detection of cytokines.

Results: Our data showed that airway eosinophils functioned as CD80- and CD86-dependent antigen-presenting cells (APCs) to stimulate sensitized CD4⁺ lymphocytes to produce interleukin (IL)-4, IL-5, and IL-13, but not interferon (IFN)- γ in *in vitro* assay. When instilled intratracheally in sensitized recipient mice, airway eosinophils migrated into draining paratracheal lymph nodes primed Th2 cells *in vivo* for IL-4, IL-5, and IL-13, but not IFN- γ , production during the *in vitro* culture that was also CD80- and CD86-dependent.

Conclusion: Eosinophils within the lumina of airways could process inhaled antigen function *in vitro* and *in vivo* as APCs to promote expansion of Th2 cells. This investigation highlights the potential of eosinophils to not only act as terminal effector cells but also to actively modulate immune responses by amplifying Th2 cell responses.

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Large numbers of eosinophils are recruited into tissues involved in allergic diseases that affect lungs (bronchial asthma) and upper airway (allergic rhinitis) (1). Although eosinophils are classically thought to act as effector cells, there is emerging *in vivo* evidence that these leukocytes may also have discrete afferent immunomodulatory roles. In rodent models, allergen-induced recruitment of eosinophils into lung tissues is correlated with roles of CD4⁺ T cells and cytokines released by these T cells (2–4). Likewise in humans, the accumulation of eosinophils in tissues, as in chronic asthma or following acute antigen challenges in the airways, correlates with local T cell activation. For instance, increases in activated T lymphocytes, eosinophils, and cytokine mRNA expression for interleukin (IL)-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) have been documented in bronchial biopsies after inhalational allergen challenges in atopic asthmatics (5). Thus, eosinophil 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 that enhance the effector responses of eosinophils.

Eosinophils express major histocompatibility complex (MHC) class II molecules (6–10) and the costimulatory molecules CD28, CD40, CD80 (B7-1), and CD86 (B7-2) (11–13), suggesting that these cells can directly communicate with helper T cells to regulate immune responses. In addition, eosinophils also secrete a range of cytokines that are not only proinflammatory but also function as growth factors, stimulants, and chemoattractants including GM-CSF, transforming growth factor- β_1 (TGF- β_1), tumor necrosis factor- α (TNF- α), IL-1 α , IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-11, IL-12, IL-13, interferon- γ (IFN- γ), IL-16, and RANTES (regulated upon activation, normal T cell expressed and secreted) for T cells

(14). Notably, IL-4 and IFN- γ are critical factors for the polarization of Th lymphocytes to the Th2 and Th1 phenotypes, respectively (15, 16). Eosinophils have been shown to function as antigen-presenting cells (APCs) and thus to stimulate the proliferation of cultured antigen-specific T cells (13, 17, 18). Recently, Shi and coworkers have shown that antigen-loaded eosinophils instilled intratracheally into mice were capable of migrating into local lymph nodes and localize to the T cell-rich paracortical zones where they stimulated antigen-specific expansion of CD4⁺ T cells (19). In the present investigation, we extended the above findings and demonstrated that antigen exposed airway eosinophils function both *in vitro* and *in vivo* as APCs to promote secretion of Th2 cytokines.

Materials and methods

Mice

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

Preparation eosinophils

Mice were sensitized intraperitoneally three times with 10 μ g of ovalbumin (OVA, Sigma Chemical Co., St Louis, MO) 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 DeVilbiss 646 nebulizer (DeVilbiss Corp., Somerset, PA) driven by compressed air at 5 l/min for 30 min. 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 mM ethylenediamine-tetraacetic acid (EDTA) and 2% normal mouse serum (PBS/serum) to obtain bronchoalveolar lavage fluid (BALF) for eosinophil purification. Mouse eosinophils 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 five to six mice in 5 ml PBS/serum was placed on top of the gradient. After centrifugation for 25 min at 1500 *g* at room temperature, eosinophils were between the 1.075 and 1.070 g/ml layers, with the majority of macrophages on top of the gradients. Eosinophils were collected and washed in PBS/serum, and after 1 h culture on plastic petri dishes in RPMI-1640 supplemented with penicillin (100 U/ml), streptomycin (100 μ g/ml), L-glutamine (2 mM), HEPES (10 mM), and 5% normal mouse serum to eliminate remaining adherent macrophages, eosinophils were further purified by negative immunomagnetic selection, using a magnetically activated cell separator system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). To remove contaminating lymphocytes, anti-CD90- and anti-CD19-coated micromagnetic beads were added to the eosinophil suspension. By negative

selection, highly purified mouse eosinophils (>99.5%) were routinely obtained, as assessed by microscopy and flow cytometric analyses. Viability determined by trypan blue exclusion of purified eosinophils was >97%.

Cytokine production from sensitized CD4⁺ T cells induced by antigen-loaded eosinophils *in vitro*

To obtain antigen-sensitized CD4⁺ cells, BALB/c mice were immunized intraperitoneally with 10 μ g of OVA plus 1 mg Al(OH)₃. Spleens, taken aseptically 2 weeks later, were pressed through wire mesh screens to separate cells. Hypotonic saline (0.2%) was added to spleen cell suspensions to lyse erythrocytes and then an equal volume of 1.5% saline was added to restore isotonicity. After passage over Sephadex G-10 columns to remove accessory cells, spleen 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 700–1000, cytocentrifuged CD4⁺ cells demonstrated no macrophages among lymphocytes. Purified CD4⁺ cells (1 $\times$ 10⁶ cells/ml) that were incubated with or without purified BALF eosinophils 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, CD4⁺ cells or eosinophils alone were also cultured. Eosinophils were cultured with 5 ng/ml recombinant mouse GM-CSF (BioSource, Camarillo, CA) 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), or control rat IgG_{2a} (10 μ g/ml) were added. Cultures were incubated for 96 h at 37°C in a 5% CO₂ humidified atmosphere. Cell-free culture supernatants were collected and stored in aliquots at –70°C before analysis of IL-4, IL-5, IL-13, and IFN- γ .

Cytokine production from draining lymph node cells primed *in vivo* by eosinophils

Mice were immunized intraperitoneally with 10 μ g of OVA plus 1 mg Al(OH)₃. Two weeks later, mice were lightly anesthetized with avertin (250 μ g/kg), and their tracheas were surgically exposed. Purified OVA-exposed eosinophils (1 $\times$ 10⁶) were instilled slowly in a volume of 50 μ l PBS into the trachea via a 25-gauge needle. In some experiments, OVA-sensitized mice were injected intravenously with anti-CD80 (100 μ g), anti-CD86 (100 μ g), a combination of both anti-CD80 and anti-CD86 mAbs (total of 200 μ g), or control rat IgG_{2a} (100 μ g) immediately before, and 1 and 2 days after they received endotracheal instillations of eosinophils. At 3 days thereafter, the draining paratracheal lymph nodes were removed and teased into cell suspensions. Lymph node cells (2 $\times$ 10⁶/ml), cultured in 48-well plates for 48 h in the absence or presence of 200 μ g/ml OVA. Cell-free culture supernatants were collected and stored in aliquots at –70°C before analysis of IL-4, IL-5, IL-13, and IFN- γ .

Cytokine analysis

Interleukin-4, IL-5, IFN- γ (all from PharMingen) and IL-13 (R & D Systems, Minneapolis, MN) concentrations in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol.

Results

Cytokine production from sensitized CD4⁺ T cells induced by antigen-loaded eosinophils *in vitro*

In the absence of eosinophils as APCs during 4-day *in vitro* culture, CD4⁺ cells produced no IL-4, IL-5, IL-13, or IFN- γ even when incubated with 200 μ g/ml of exogenous antigen (Fig. 1). The addition of airway eosinophils yielded a significant cytokine production of IL-4, IL-5, and IL-13; although IFN- γ in the supernatants was also detectable, its concentration was quite low. Incubation of eosinophils alone in medium resulted in no appreciable production of detected cytokines. The addition of exogenous antigen to these eosinophil-CD4⁺ cell co-cultures yielded even greater productions of IL-4, IL-5, and IL-13, but not IFN- γ , indicative that eosinophils *in vitro* were further capable of processing antigen and serving as APCs. These results were compatible with eosinophils presenting processed OVA peptides to the sensitized CD4⁺ cells and drove these T cells to secrete Th2 type cytokines.

It has been previously shown that airway eosinophils recovered from OVA-sensitized and -challenged BALB/c

mice not only expressed high levels of MHC class II molecules, but also expressed high levels of CD80 and CD86 (19), two proteins with important roles as costimulatory signals for T cell responses (20). To further evaluate whether eosinophils were providing requisite B7 costimulatory signals for their APC function to induce cytokine production by Th2 cells, we cultured 1×10^6 cells/ml of OVA-sensitized CD4⁺ cells and 5×10^5 /ml of OVA-exposed eosinophils in the presence or absence of inhibitory concentrations of anti-CD80 mAb, anti-CD86 mAb, a combination of both, Anti-CD80 and anti-CD86 mAb alone partially blocked productions of IL-4, IL-5, and IL-13 (Fig. 2). A combination of both anti-CD80 and anti-CD86 blocking mAbs yielded even greater inhibition of eosinophil-elicited Th2 type cytokine production.

Cytokine production from draining lymph node cells primed *in vivo* by eosinophils

Shi and coworkers have also shown that eosinophils exposed to inhaled antigen within the airways of donor

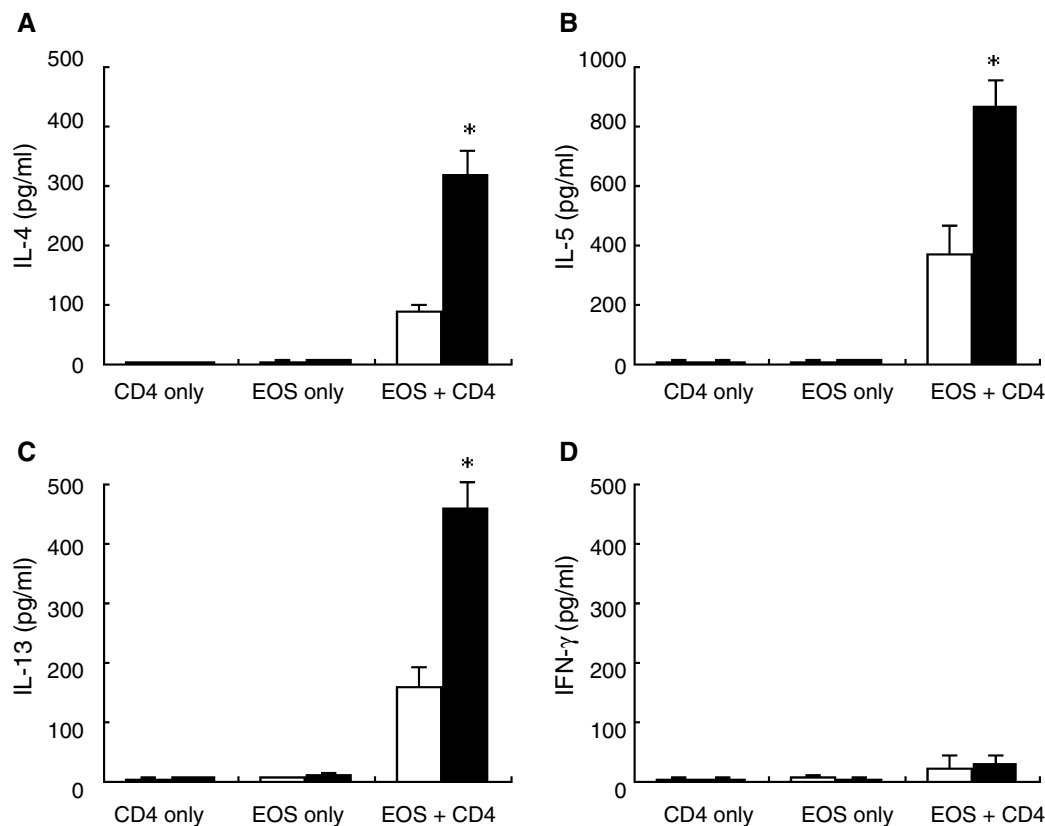


Figure 1. Eosinophils stimulate Th2-type cytokine productions from sensitized CD4⁺ cells *in vitro*. Purified BALF eosinophils (5×10^5 cells/ml) from OVA-sensitized and OVA-challenged BALB/c mice were incubated with OVA-sensitized CD4⁺ cells (1×10^6 cells/ml) in the absence (open bars) or presence (closed bars) of 200 μ g/ml exogenous OVA. As controls, CD4⁺ cells or eosinophils alone were also cultured. After 96 h, cell-free culture supernatants were collected and stored in aliquots at -70°C before analysis of IL-4 (A), IL-5 (B), IL-13 (C), and IFN- γ (D) determined by ELISA. Results are the mean values $\pm$ SD of six independent experiments. * $P < 0.01$, compared with values for no exogenous OVA, by paired t test. EOS = eosinophil, CD4 = CD4⁺ cell.

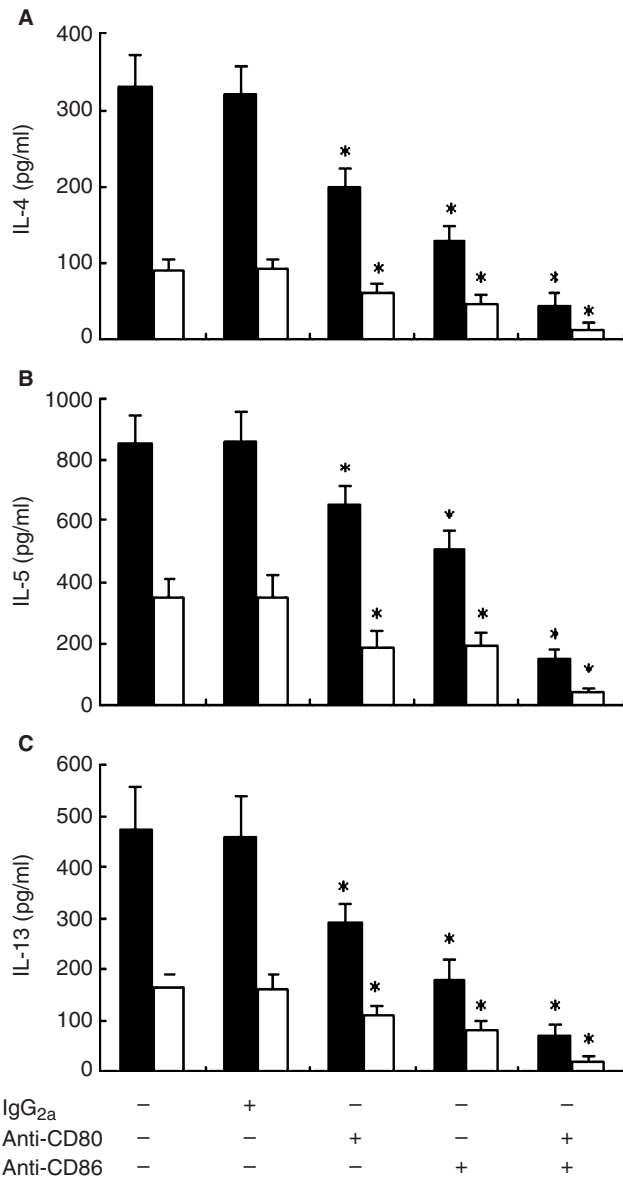


Figure 2. The role of B7 costimulatory molecules on Th2-type cytokine productions elicited by eosinophils *in vitro*. OVA-sensitized CD4⁺ cells (1×10^6 cells/ml) were incubated with purified BALF eosinophils (5×10^5 cells/ml) from OVA-sensitized and OVA-challenged BALB/c mice in the absence (open bars) or presence (closed bars) of 200 μ g/ml exogenous OVA. Anti-CD80 (10 μ g/ml) or/and anti-CD86 (10 μ g/ml) mAb, or control rat IgG_{2a} (10 μ g/ml) were added into the co-cultures. After 96 h, cell-free culture supernatants were collected and stored in aliquots at -70°C before analysis of IL-4 (A), IL-5 (B), and IL-13 (C) determined by ELISA. Results are presented as mean $\pm$ SD of six independent experiments. * $P < 0.05$, compared with non-immune IgG_{2a} controls, respectively, by ANOVA.

mice, when instilled in the airways of sensitized recipient mice, migrate from the airways and home to draining lymph nodes where they serve as antigen-specific, CD80- and CD86-dependent APCs to elicit the proliferation of

nodal CD4⁺ T cells. In OVA-sensitized mice that received intratracheal instillation of OVA-exposed eosinophils, *in vivo* T cell proliferative responses in the paratracheal nodes developed within 1 day, peaked at day 3, and then declined over 1 week (19). In the present study, 1×10^6 eosinophils 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 (Fig. 3). The addition of exogenous antigen to these lymph node cells yielded even greater Th2 type cytokine production indicative that Th2 cells were primed *in vivo* by antigen presentation of eosinophils and ready for cytokine production. As expected, the endotracheal instillation of fixed, nonviable eosinophils induced no significant productions of Th2 type cytokines.

Having established the APC functional activity of airway eosinophils *in vivo* for expansion of Th2 cells, we then analyzed the role of B7/CD28/CTLA-4 pathway in antigen presentation by eosinophils to 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 eosinophils (Fig. 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.

Discussion

The infiltration of eosinophils into the airways is a hallmark of allergic airway disease (21). Currently, these cells are primarily thought to act as effector cells, inducing pathological changes through the release of granular proteins and proinflammatory mediators. However, under normal physiological conditions, tissue eosinophils do not induce diseases and their numbers and activation status are tightly regulated. The presence of eosinophils 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. Handzel and colleagues have reported that eosinophils participate in rhinovirus-induced immune responses through antigen presentation and T cell activation (22). Moreover, eosinophils within the lumina of airways can process inhaled antigens, traffic to regional lymph nodes and function *in vivo* as APCs to stimulate responses of CD4⁺ T cells (19). Our present studies aimed specifically at evaluating the capabilities of airway eosinophils to function as APCs to regulate Th1/Th2 cell differentiation both *in vitro* and *in vivo*.

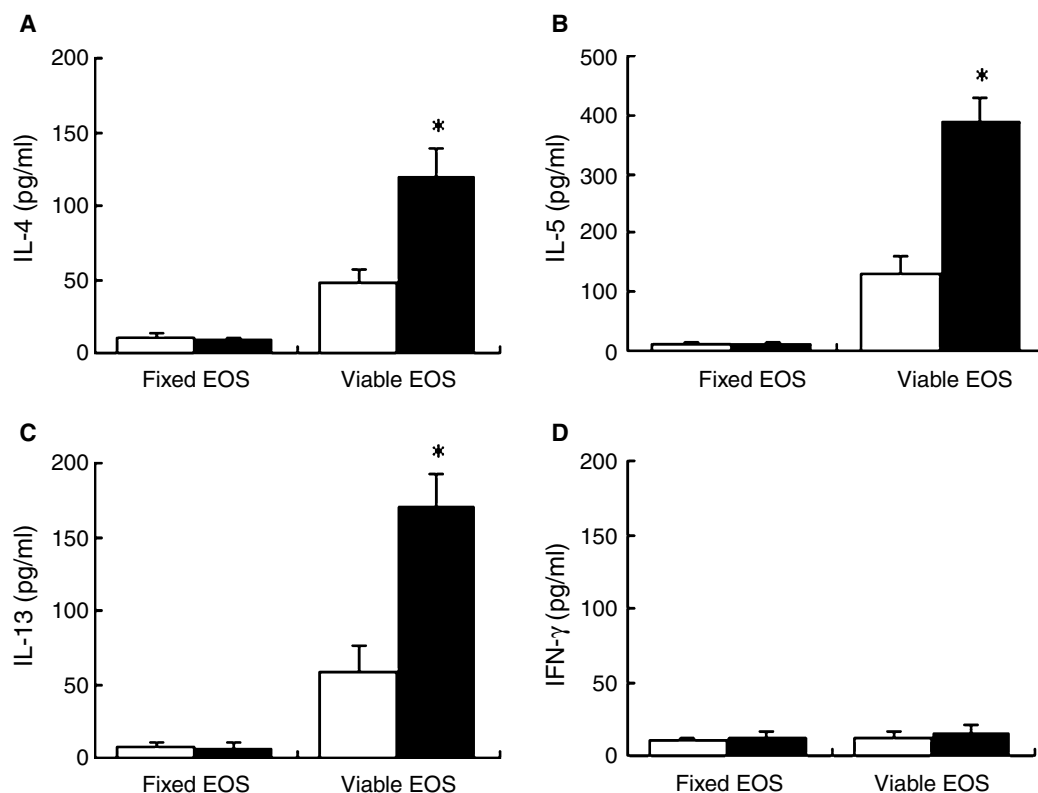


Figure 3. Eosinophils prime Th2 cells *in vivo* for Th2-type cytokine productions. Eosinophils (1×10^6) from OVA-sensitized and OVA-challenged mice were instilled into tracheas of OVA-sensitized mice, draining paratracheal lymph nodes were taken 3 days thereafter and tested for cytokine production of IL-4 (A), IL-5 (B), IL-13 (C), and IFN- γ (D). Lymph node cells (2×10^6 cells/ml) were incubated at 37°C in the absence (open bars) or presence (closed bars) of 200 μ g/ml exogenous OVA for 48 h, and then cell-free culture supernatants were collected and stored in aliquots at -70°C before analysis of cytokines. Results are presented as mean $\pm$ SD of four independent experiments. * $P < 0.01$, compared with values for no exogenous OVA, by paired *t* test. EOS = eosinophil.

Shi and coworkers have previously demonstrated that murine eosinophils from the antigen-challenged airways of sensitized BALB/c mice, like human airways eosinophils (23–25), express class II MHC proteins (19). Also, the inhalational exposures of airway eosinophils to antigen enable these murine airway eosinophils to express B7-1 and B7-2 (19), again comparable to human eosinophils that express at least B7-2 (11). Furthermore, eosinophils recruited into the luminal airways by aerosol antigen challenge possess the capacity to process OVA to which they are exposed within the airways, such inhalational exposures to OVA is shown to be sufficient for these airway eosinophils to present OVA to T cells and thus induced T cell proliferation *in vitro* (19). Our present studies extended the above findings and showed that eosinophils 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 (26). Moreover, our data further demonstrated that the expansion of Th2 cells observed in this study caused by

airway eosinophils 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.

Korsgren and colleagues in an analogous murine model of asthma demonstrated that OVA-sensitized and OVA-airways challenged mice exhibited eosinophil accumulations in the airways, paratracheal tissues and especially into paratracheal lymph nodes (27). Whereas the lymph node localization of eosinophils in that study was in the context of endogenous eosinophil accumulations in the entire airways tissues following inhalational challenge in sensitized mice, our previous study demonstrated that exogenous eosinophils introduced specifically into the tracheobronchial lumina of normal mice exhibit homing to paratracheal lymph nodes where they serve as antigen-specific, CD80- and CD86-dependent APCs to elicit the proliferation of nodal CD4⁺ T cells (19). Our present studies established that these antigen-exposed, airway-derived eosinophils, when instilled into the airways of antigen-sensitized mice were capable of priming Th2 cell expansion *in vivo* within draining

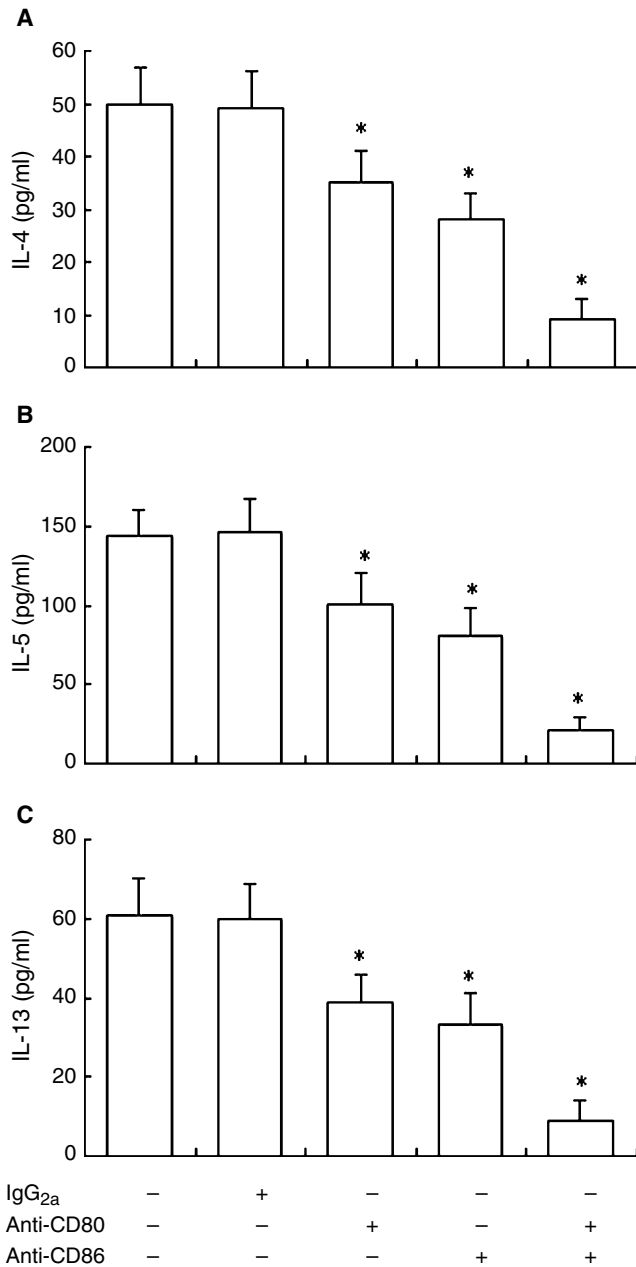


Figure 4. The role of B7 costimulatory molecules on Th2-type cytokine productions primed by eosinophils *in vivo*. OVA-sensitized mice were injected intravenously with anti-CD80, anti-CD86, both anti-CD80 and anti-CD86 mAbs, or control IgG_{2a}, immediately before, 1 and 2 days after they received intratracheal instillations of 1×10^6 OVA-elicited airway eosinophils. Draining paratracheal lymph nodes were taken 3 days after eosinophil instillation and evaluated for Th2-type cytokine productions of IL-4 (A), IL-5 (B) and IL-13 (C). Lymph node cells (2×10^6 cells/ml) were incubated 48 h at 37°C in the absence of exogenous OVA, and then cell-free culture supernatants were collected and stored in aliquots at -70°C before analysis of cytokines. Results are presented as mean $\pm$ SD of four independent experiments. * $P < 0.05$, compared with nonimmune IgG_{2a} controls, by ANOVA.

paratracheal lymph nodes. These eosinophil APC-mediated *in vivo* Th2 type responses were also B7/CD28/CTLA-4-dependent.

In comparison with other cell types of APCs such as dendritic cells, the APC function of eosinophils is presumably marginal, however, several properties of eosinophils likely combine to endow them with distinct roles as APCs. The first property would be their tissue localization. The normal localization of eosinophils within mucosal tissues of the respiratory, gastrointestinal, and lower genitourinary tracts (28) would position them to encounter foreign antigens at these mucosal surfaces. Moreover, in allergic airway diseases eosinophils are characteristically found directly within the lumen and secretions of airways (29, 30) and could directly interact with inhaled allergens within the airways. Second, eosinophils have the capacity to transmigrate from the luminal surface of the mucosa into regional lymph nodes (19). Both alveolar macrophages (31) and dendritic cells (32, 33) can also migrate from the airways into tissues, but *in vivo* antigen-pulsed macrophages do not transfer processed peptides to dendritic cells (34) and alveolar macrophages do not function as APCs *in vivo* (35, 36). A third feature of eosinophils that might be important in their role as APCs at airway surfaces is their capacity to interact with particulate antigens. While dendritic cells and B cells very effectively present soluble protein antigens, they are unable to handle particulate antigens (37). In the respiratory tract, inhaled allergens are particulate (38). The principal cells recognized to ingest particulate antigens are phagocytic macrophages (39); but alveolar macrophages are not effective APCs and even antagonize APC function of dendritic cells (35, 36, 40). Alternatively, eosinophils would be well suited to handle particulate antigens since eosinophils are phagocytic, characteristically engage large, even nonphagocytosable multicellular targets, and accumulate early at tissue sites of particulate antigens (41).

In conclusion, we have shown that antigen-sensitized CD4⁺ cells may directly engage eosinophils 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, eosinophils have the potential to activate Th2 cells to release disease-modulating cytokines. These investigations highlight the potential of eosinophils to not only act as terminal effector cells but also to actively amplify allergic responses by promoting Th2 cell immunity.

Acknowledgments

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