

A simple preparation method for mouse eosinophils and their responses to anti-allergic drugs

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Abstract. *Objective and design:* A simple method for preparing mouse eosinophils was established, and the characteristics of the eosinophils were assessed including their responses to anti-allergic drugs.

Materials or subjects: Mouse eosinophils were prepared from peritoneal exudate cells of BALB/c mice primed and boosted with antigen ovalbumin (OVA).

Methods: Surface phenotypes, migration activities and leukotriene C₄ (LTC₄) production abilities of these eosinophils were examined. In addition, the effects of anti-allergic drugs, oxatomide and tranilast, on generation of LTC₄ from mouse eosinophils were examined.

Results: Eosinophils of mice boosted with OVA were phenotypically and functionally identical with human eosinophils. Around 1×10^7 eosinophils were obtained from mouse peritoneal exudate. It was found that these mouse eosinophils enabled to migrate in response to eotaxin as well as platelet-activating factor (PAF), and generated LTC₄ by IL-5 stimulation. Moreover, it was revealed that clinically used anti-allergic drugs inhibited LTC₄-production dose-dependently.

Conclusions: The present study provides a convenient method to obtain fully functional mouse eosinophils that are useful for drug screening and for evaluating implications of eosinophils in allergic responses.

Key words: Mouse eosinophil – Drug screening – LTC₄ – IL-5 – Anti-allergic drug

Introduction

Eosinophils have been devoted much attention recently, since they may play crucial roles in pathophysiology of allergic diseases, especially on abnormal remodeling process in bronchial asthma and atopic dermatitis [1, 2]. When activated by various stimuli, eosinophils generate several inflammatory mediators, such as cytokines, growth factors,

superoxide anions, toxic cationic granule proteins and lipid mediators, caused to damage of tissue and exacerbation of inflammatory responses. Recently, accumulated findings have demonstrated that eosinophils are implicated in the progression of airway abnormal remodeling, resulted in airway hyper-responsiveness in asthma [3–5]. In the case of atopic dermatitis, it has also been reported that eosinophils and the generated mediators exacerbate the symptoms and skin inflammation [6]. These recent findings have led us to attempt to discover a novel drug able to control eosinophil functions.

In IL-5 transgenic (TG) mice, high numbers of eosinophils have been detected in various tissues such as bone marrow, peripheral blood and spleen [7]. Based on that, IL-5 TG mice have been utilized as a source of mouse eosinophils for investigating the functions and responses to drugs [8–13]. In allergic patients and mice induced by allergic antigens, on the other hand, not only IL-5 but also other cytokines and differentiation factors seem to induce the differentiation and activation of eosinophils, suggesting that eosinophils of IL-5 TG mouse might not fully reflect on those generated in pathological condition [7]. To utilize mouse eosinophils for drug screening and investigation in allergic responses, the preparation of mouse eosinophils in allergic condition is important. While a couple of preparation methods such as the use of horse-sera and Sephadex beads have been reported [14, 15], these eosinophil-inducing agents are not the stimuli that cause to allergic condition. In experimental allergic models, different kinds of allergens have been used. Among them, a defined protein antigen ovalbumin (OVA) has widely been used to induce various experimental allergic models [16, 17], since the sensitization of OVA causes to eosinophilia in either the peripheral blood or the tissues. In previous report, Zuany-Amorim et al. have been used OVA for sensitizing BALB/c mice, and investigated antigen-induced eosinophil infiltration into the peritoneal cavity [18].

For drug screening *in vitro*, a large number of purified intact eosinophils should be needed, since there is not an appropriate eosinophilic cell line sustaining the eosinophil functions. In this report, we have tried to purify eosinophils from the peritoneal cavity of mice primed and

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boosted with OVA, by one step negative selection using a magnetic cell sorter (MACS). Around 1×10^7 cells, whose purity were 90 % to 98 %, were obtained from a sensitized mouse, and were applied for *in vitro* assays to clarify characteristics as eosinophils. The functions and surface phenotypes of these eosinophils were similar with those of human eosinophils. Moreover, the defined response and sensitivity of these eosinophils towards anti-allergic drugs will be discussed.

Materials and methods

Animals

Female BALB/c mice (6–10 weeks) were obtained from SLC (Shizuoka, Japan) and maintained under specific pathogen-free conditions at a room temperature of $23 \pm 3^\circ\text{C}$ and air humidity of $55 \pm 15\%$ in a 12-h light/dark cycle environment. This experiment complied with the guidelines of our laboratories on animal experimentation.

Reagents

Mouse IgE was obtained from Seikagaku corporation (Tokyo, Japan). For immunostaining, we used the following monoclonal antibodies (mAbs): Fluorescein isothiocyanate (FITC)- or Phycoerythrin (PE)-conjugated anti-CCR3 (83101) from R&D Systems (Minneapolis, MN); anti-Gr-1 (RB6-8C5), anti-CD16/32 (2.4G2), anti-CD23 (B3B4), and anti-IgE (R35-72) from BD Biosciences Pharmingen (San Diego, CA). Recombinant mouse IL-5, eotaxin, and regulated upon activation, normal T cells expressed and secreted (RANTES) were purchased from R&D Systems, and were dissolved in phosphate-buffered salines (PBS, containing 0.1 % bovine serum albumin (BSA)) to concentrations of 5 $\mu\text{g}/\text{ml}$, 20 $\mu\text{g}/\text{ml}$, and 25 $\mu\text{g}/\text{ml}$ respectively. Platelet-activating factor (PAF), phorbol 12-myristate 13-acetate (PMA), ionomycin, oxatomide, and tranilast were obtained from Sigma-Aldrich (St. Louis, MO). These reagents except for PAF were dissolved at 10 mM in dimethylsulfoxide (DMSO, Nacalai Tesque, Kyoto, Japan). PAF was dissolved at 10 mM in PBS containing 0.1 % BSA.

Purification of eosinophils from peritoneal exudate

Peritoneal eosinophils were purified from BALB/c mice as previously described [18] with some modifications. Briefly, mice were sensitized by subcutaneous (s.c.) injection of 0.4 ml saline containing 100 μg OVA adsorbed to 1.6 mg aluminum hydrate (alum) on days 1 and 8. On day 15, the animals were boosted with an intraperitoneal (i.p.) injection of 0.4 ml of 2.5 $\mu\text{g}/\text{ml}$ OVA in PBS. After 48 h, the mice were peritoneally lavaged and the cells were collected. Eosinophils were then purified by depletion of contaminating other lineage cells using magnetic cell sorting (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Peritoneal exudates cells (PECs) were stained with microbeads-conjugated mAbs specific to CD90 (Thy1.2), CD45R (B220), or MHC class II, then they were applied for MACS to purify mouse eosinophils.

Flow cytometric analysis

Cell surface staining was carried out in accordance with the standard protocol, using appropriate mAbs. In the case of analysis regarding the expression of Fc ϵ RI, cells were cultured in the presence of 0.1 $\mu\text{g}/\text{ml}$ mouse IgE for 24 h, and detected with FITC conjugated anti-mouse IgE. Flow cytometric analysis was performed with a Cytomics FC500 Flow Cytometer (Beckman Coulter, Fullerton, CA) using Cytomics RXP

(Beckman Coulter) and FlowJo (Tree Star, Ashland, OR) software. Propidium iodide-positive cells were eliminated from the analysis as dead cells.

Migration assays

The migration analysis of purified mouse eosinophils *in vitro* was performed using Transwell polycarbonate membrane inserts (6.5 mm-diameter, 5 μm pore size; Corning Costar, Cambridge, MA). Migration medium (RPMI1640; 540 μl , containing 0.1 % BSA) was added to the lower wells, and the Transwell inserts were then placed in the wells and pre-incubated with the medium at 37°C for 1 h. Medium containing stimulators such as eotaxin, RANTES or PAF (60 μl) was added to the lower chambers, and 100 μl of the eosinophils suspension at 1×10^7 cells/ml was added to the upper chambers. The plates were incubated at 37°C for 4 h. Cells that migrated from the upper to the lower chambers were harvested and the living cells were counted by a Cytomics FC500 using Flow-Count Fluorospheres (Beckman Coulter).

Analysis of LTC₄ generation

To analyze leukotriene C₄ (LTC₄) generation *in vitro*, purified mouse eosinophils were suspended in phenol red-free Dulbecco's modified eagle medium (DMEM) at 2×10^7 cells/ml as described previously [19]. Cells (100 μl) were placed in 96-well flat-bottomed plates (Becton Dickinson, Franklin Lakes, NJ), to which 100 μl of various stimulators, such as IL-5, eotaxin or PMA plus ionomycin, in the same medium, were added in advance. In other experiments, cell suspension of eosinophils was pre-incubated with various amounts of anti-allergic drugs, oxatomide and tranilast, for 5 min at 37°C . Then, cells were stimulated with mouse IL-5 as described above. The culture supernatant was harvested at each time point, and LTC₄ in the supernatant was measured by ELISA (Cayman Chemical, Ann Arbor, MI) in accordance with the manufacturer's instructions. EC₅₀ values were calculated as the concentration of the drugs required to display 50 % inhibition of LTC₄ production.

Statistical analysis

The data were expressed as means $\pm$ SD from the indicated number of samples. Multiple comparisons were assessed by 1-way ANOVA followed by Dannett test (StatLight, Yukms, Tokyo, Japan). Differences were considered as significant at $p < 0.05$.

Results

Separation of eosinophils from mouse primed and boosted with OVA

The simple protocol as shown in Figure 1A were established by the modification of previously reported method [18], based on the below results of phenotypical analysis of cells infiltrated into peritoneal cavity. After the boost of OVA to BALB/c mouse primed with OVA plus alum, the isolated cell population from the peritoneum was consisted of 50–60 % CCR3-positive eosinophils, less than 5 % neutrophils, 20 % antigen presenting cells (APCs) and 15–20 % lymphocytes. As the population was contained only few neutrophils, the simple negative sort of T cells (Thy1.2⁺ cells), B cells (B220⁺ cells) and APCs (MHC class II⁺ cells) with MACS could enrich CCR3⁺ cells with the purity of

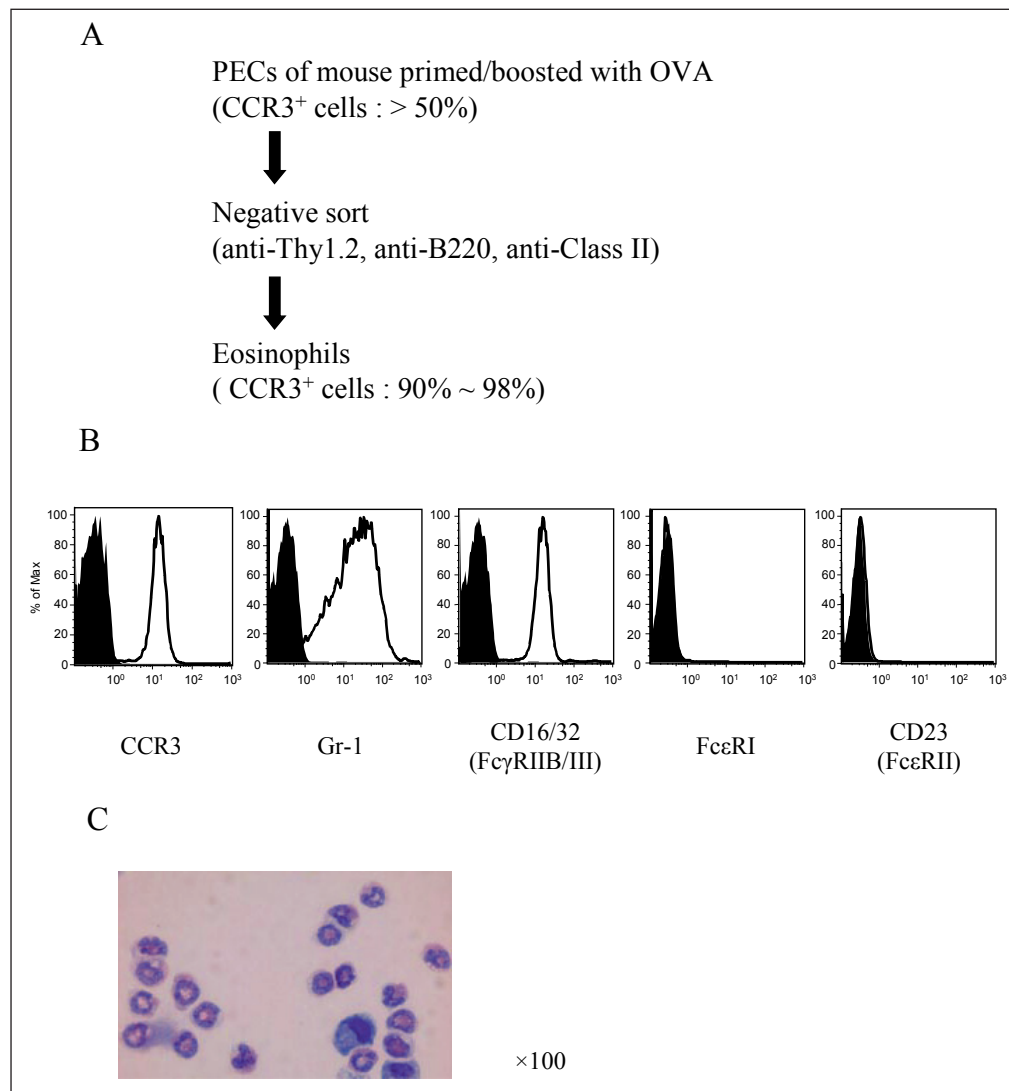


Fig. 1. Preparation protocol of mouse eosinophils and their phenotypical characteristics. (A) Scheme of preparing eosinophils from mouse primed and boosted with OVA. (B) Flow cytometric analysis for CCR3, Gr-1, FcγRIIB/III, FcεRI, and FcεRII expression on mouse eosinophils. Open histograms indicate binding of specific antibodies, and isotype matched control antibodies or secondary reagents are represented by closed histograms. Results are representative of at least three independent experiments. Y-axis is % of Max, the percentage of maximum value of cell count. (C) Light microscopic morphology of mouse eosinophils after Diff-Quik staining. Original magnification, ×100. Almost all of cells are colored by the acidic stain, and eosinophils with basic granules.

90 % to 98 % (Fig. 1A). The number of eosinophils separated by one step preparation was around 1×10^7 cells and more a mouse.

Analysis of purified mouse eosinophils

To assess if purified cells from mouse peritoneal cavity sustained phenotypical features of eosinophils, cell surface antigens were profiled by flow cytometer. As shown in Figure 1B, these cells expressed CCR3, which is a receptor for eotaxin, a granulocyte marker Gr-1 and FcγRIIB/III CD16/32. On the other hand, they were negative for both FcεRI and FcεRII/CD23. These results were consistent with previous findings on eosinophils from parasite-infected mice [20–22], and similar to those of human eosinophils [23, 24]. To confirm these cells as eosinophils, cyto-spin samples were stained with various reagents. As shown in Figure 1C, more than 95 % of these cells were colored by the acidic stain, and positive for the granules that contain basic proteins.

Migration of mouse eosinophils in response to eotaxin and PAF but not to RANTES

We next examined migration ability of mouse eosinophils *in vitro* with a couple of chemokines. In parallel, PAF was set as a positive control [8]. As shown in Figure 2, mouse eosinophils migrated by the addition of eotaxin in a dose-dependent manner. On the other hand, RANTES failed to elicit eosinophil migration. These results indicate that mouse eosinophils prepared by this method are functionally comparable to previously reported mouse eosinophils in terms of migration activity [25], and that at least CCR3 and PAF receptors expressed on these eosinophils were functional.

LTC₄ generation from mouse eosinophils in response to IL-5

In most of the *in vitro* analysis reported previously, the responses to IL-5 of mouse eosinophils were not investigated because the eosinophils derived from IL-5 TG mice were used. To clarify the response to IL-5 of mouse eosinophils

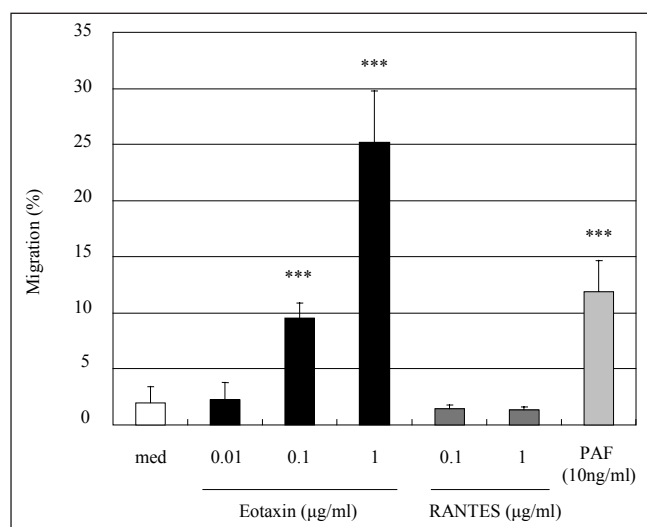


Fig. 2. Chemotactic migration of mouse eosinophils by eotaxin and platelet-activating factor (PAF). The eosinophil migration in response to eotaxin and PAF were assessed using a Transwell insert system as described in Materials and methods, and the data are shown as percentage of migrated cells to plating cells. Data are shown as means $\pm$ SD ($n = 6$). *** $p < 0.001$ compared with the medium control group by Dannett test. "med", medium control.

prepared by this method, the LTC_4 generation was examined. As shown in Figure 3A and B, it was found that these mouse eosinophils produced LTC_4 by IL-5 stimulation in a dose-dependent manner, at the condition that PMA plus ionomycin also induced LTC_4 generation. These results indicated that IL-5 as eosinophil differentiation and activation factor stimulated mouse eosinophils to produce LTC_4 .

Next, we examined the effect of anti-allergic drugs on the production of LTC_4 from mouse eosinophils by IL-5 stimulation *in vitro*. As oxatamide and tranilast have been reported to inhibit the release of chemical mediators from basophils and mast cells [26, 27], and also to be effective in airway allergic responses [28, 29]. It was also reported that the inhibition of LTC_4 production by oxatamide was observed in human eosinophils with the EC_{50} values of 5×10^{-6} M [30]. As shown in Figure 3C, both drugs inhibited LTC_4 production from mouse eosinophils in a dose-dependent manner. The EC_{50} values were around 1×10^{-7} M in oxatamide and 5×10^{-6} M in tranilast. These results suggested that anti-allergic drugs such as oxatamide inhibits LTC_4 generation from mouse eosinophils, like as the case of human eosinophils.

Discussion

IL-5 TG mice provide a powerful experimental tool for studying eosinophil function, because eosinophils from these animals have been reported to be functionally intact [8]. However, it is also suggested that prolonged exposure to high concentration of IL-5 could alter the functional charac-

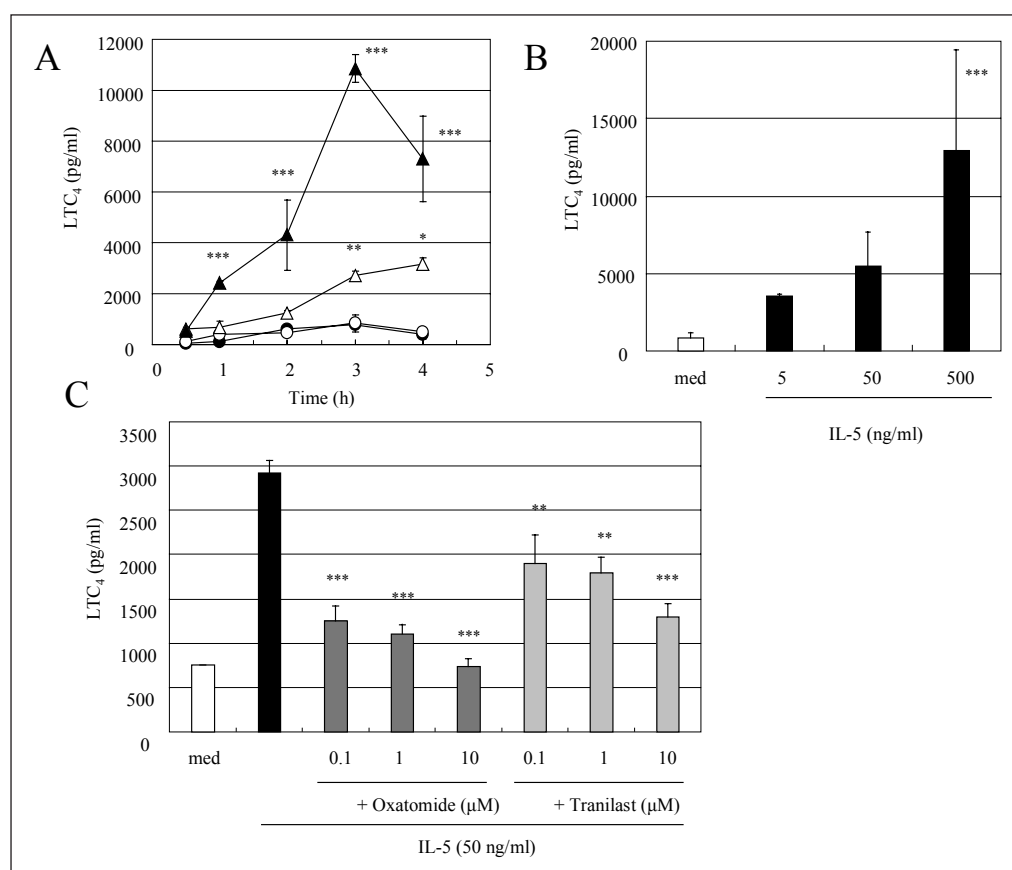


Fig. 3. Leukotriene C_4 (LTC_4) release from mouse eosinophils. (A) Kinetics of LTC_4 generation from eosinophils induced by medium (open circles), $1 \mu\text{g/mL}$ eotaxin (closed circles), 50 ng/mL IL-5 (open triangles), or 10 ng/mL phorbol 12-myristate 13-acetate (PMA) plus 500 ng/mL ionomycin (closed triangles). Data are shown as means $\pm$ SD ($n = 3$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with the medium control group by Dannett test. (B) Dose-dependent stimulation of IL-5 on LTC_4 production from the mouse eosinophils. Data are shown as means $\pm$ SD ($n = 3$). *** $p < 0.001$ compared with the medium control group by Dannett test. (C) Effect of oxatamide and tranilast on IL-5-induced LTC_4 generation. Eosinophils were pre-incubated with each drug or 0.2% dimethylsulfoxide (DMSO) for 5 min at 37°C before IL-5 stimulation (50 ng/mL) for 3 h. The samples were tested at each drug concentration. Data are shown as means $\pm$ SD ($n = 3$). "med", medium control. ** $p < 0.01$ and *** $p < 0.001$ compared with the IL-5 stimulated group by Dannett test.

teristics of eosinophils. The cells from parasite-infected mice are also used in *in vitro* study, since eosinophils had been considered as the cells terminally differentiated and involved in host protection against parasites [19, 25, 31]. Many lines of recent findings, however, have changed this hypothesis to one that eosinophils are pleiotropic multifunctional leukocytes involved in the initiation and propagation of diverse allergic inflammatory responses [1]. Therefore, eosinophils from parasite-infected animals might be functionally different from those of allergic mice. In this study, we have established a simple preparation method of eosinophils from allergic mice induced by prime and boost of a well-defined allergen OVA, which is usually used to induce various experimental allergic models. As shown in Figure 1B, almost all of the cells purified mouse eosinophils expressed CCR3, which has been used as eosinophil markers in peripheral blood cells from IL-5 TG mice [4]. The confirmation of eosinophil functions indicated that these cells were comparable to *Schistosoma mansoni*-infected mice derived [25] or human [1] eosinophils, in terms of eotaxin-induced chemotaxis. In addition, RANTES, which can bind to CCR1, CCR3 and CCR5 [32], had no significant effect on the migration of mouse eosinophils *in vitro* (Fig. 2). These results are also supported by an *in vivo* study, which showed intradermal injection of eotaxin, but not RANTES, recruits eosinophils selectively in mouse skin [33]. RANTES acts as a chemoattractant of human eosinophils which express CCR3 on their surface [34], but it was also reported that mouse RANTES is unable to displace ¹²⁵I-eotaxin binding on mouse CCR3-expressing cells [12]. Therefore, it is likely that the lack of RANTES-responsiveness of mouse eosinophils is due to the unbinding of RANTES to CCR3 on their cell surface.

Eosinophils have the capability to generate lipid mediators, including LTC₄, after activation of the lipoxygenase pathway [35]. LTC₄ has been considered as a potent bronchoconstrictor, and shown to increase mucous production and vascular permeability [36]. Significantly, mouse eosinophils also produced LTC₄ in response to IL-5 (Fig. 3A and B), as well as in human [37]. The LTC₄ generation was not observed on eosinophils from IL-5 TG mice [13]. At least on this point, the mouse eosinophils obtained in this protocol seem to have an advantage to those of IL-5 TG mouse for anticipating drug activity in human eosinophils. It is well known that IL-5 is produced by some effector cells such as Th2 cells and mast cells, and is implicated in the pathophysiology of allergic responses [38]. The data presented here also demonstrates that IL-5 is also a stimulator for LTC₄ elaboration from mouse eosinophils. Furthermore, we found that oxatomide and tranilast also inhibited LTC₄ production from mouse eosinophils at a lower dose compared with basophils or mast cells [26, 27]. These results may indicate that the two drugs exert ameliorative effects on allergic responses partly by the inhibition of eosinophil functions such as mediator generation in addition to the effect on mast cells, though further detailed analysis should be done to clarify cellular targets of these drugs.

Taken with data presented here and reported observations together, the mouse eosinophils obtained by this simple one step preparation provide the features of eosinophils, which resemble human eosinophils in their features, allowing us to use them for drug screening and evaluating implications of eosinophils in allergic responses.

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