

The effects of montelukast on tissue inflammatory and bone marrow responses in murine experimental allergic rhinitis: interaction with interleukin-5 deficiency

Johanna Roa,¹ Hiroshi Morikawa,¹
Lynn Crawford,¹ Adrian Baatjes,¹
Mylinh Duong² and Judah A.
Denburg¹

¹Division of Clinical Immunology & Allergy,
and ²Division of Respiriology, Department of
Medicine, McMaster University, Hamilton,
ON, Canada

Summary

The cysteinyl leukotrienes (cysLTs) are potent lipid mediators in allergic disease, acting through the receptors, cysLT1R and cysLTR2, and are produced by eosinophils derived from eosinophil/basophil (Eo/B) bone marrow (BM) progenitors. We have demonstrated the suppressive effects of either interleukin-5 (IL-5) deficiency or montelukast on eosinophil recruitment in murine allergic rhinitis, but neither of them fully abrogated the symptoms caused by residual inflammation and cytokine redundancy in eliciting BM Eo/B responses. We hypothesized that IL-5 deficiency and montelukast act synergistically to suppress tissue inflammatory and BM responses. Our objective was to investigate the effects of the cysLT1R antagonist, montelukast, on *in vivo* tissue inflammatory and BM responses in murine experimental allergic rhinitis with or without IL-5 deficiency. Three groups of age-matched BALB/c mice with or without IL-5 deficiency were tested: controls (ovalbumin sensitization and challenge, placebo treatment) and two montelukast-treated groups (2.5 mg/kg or 5 mg/kg). Nasal symptoms, BM and nasal mucosal eosinophils, basophils, and BM Eo/B colony-forming units (CFU) were evaluated. Montelukast decreased nasal symptoms in a dose-dependent manner, and significantly decreased the number of eosinophils in both BM and nasal tissue in IL-5-replete mice compared to controls. In IL-5-deficient mice, in which eosinophilia was absent, montelukast significantly decreased both nasal symptoms and basophils in BM and nasal mucosal tissue, and lowered IL-5-responsive Eo/B-CFU *ex vivo*, compared to controls. The addition of cysLT1R blockade to IL-5 deficiency more fully attenuates symptoms and upper airway inflammation than either factor alone, providing evidence of systemic, BM mechanisms in allergic rhinitis.

Keywords: allergic rhinitis; cytokines; haemopoietic progenitors; leukotriene receptor antagonist

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Correspondence: Dr Judah A. Denburg,
McMaster University, HSC-3V46, 1200 Main
Street West, Hamilton, ON L8N 3Z5,
Canada. Email: denburg@mcmaster.ca
Senior author: Dr Judah Denburg

Introduction

Allergic rhinitis is an inflammatory disease of the nasal mucosa resulting from a specific immunoglobulin E (IgE) response to allergen, and characterized clinically in part by the expression of nasal symptoms such as itch, rhinorrhoea and obstruction.¹ The initiation and regulation of

allergic inflammation are influenced by many factors, including cell type, membrane receptors and mediators generated – relevant to allergic rhinitis, this includes the recruitment and activation of mast cells, basophils and eosinophils.^{2,3} Of these, eosinophils have been shown to play a central role in the pathogenesis and clinical expression of the disease.^{4,5} Eosinophils, derived from

Abbreviations: ANOVA, analysis of variance; BM, bone marrow; CFU, colony-forming units; cysLT, cysteinyl leukotriene; GM-CSF, granulocyte-macrophage colony-stimulating factor; Eo/B, eosinophil/basophil; ML, montelukast; OVA, ovalbumin; PBS, phosphate-buffered saline; rm, recombinant murine.

a common eosinophil/basophil (Eo/B) progenitor, are released from the bone marrow (BM) in response to haemopoietic and chemotactic signals – principally interleukin-5 (IL-5) and eotaxin – and traffic via the peripheral circulation to the tissues.⁶ We have previously shown that BM cells, specifically lineage-committed Eo/B progenitors, play a significant role in the systemic contribution to the allergic airways inflammatory response.^{6–11}

Upon activation, eosinophils provide a rich cellular source of cysteinyl leukotrienes (cysLTs),¹² including leukotriene C₄ (LTC₄), LTD₄ and LTE₄, lipid mediators derived from arachidonic acid; cysLTs are also produced by a variety of other cell types, such as monocytes, basophils and mast cells. They play important roles in the pathogenesis of allergic airway inflammation, because they can act both as direct bronchoconstrictors and pro-inflammatory mediators.^{13–17}

Until now, two receptor subtypes for cysLTs have been cloned, namely cysLT1R and cysLT2R.^{18,19} It has been previously shown that mature eosinophils and basophils express both receptors, while the less mature haemopoietic progenitors until recently have been demonstrated to express only cysLT1R.^{20–22} The effects mediated via cysLT binding and concomitant signalling through the cysLTR include airway smooth muscle contraction and proliferation, vascular leakage, increased mucus secretion, decreased mucociliary clearance, and the recruitment of inflammatory effector cells, such as eosinophils, to tissue sites.^{23–26}

One means of blocking the action of leukotrienes is to inhibit cysLT1R using montelukast (ML), a selective antagonist.^{27,28} Previous studies by us in murine models have indicated that a key determinant of tissue eosinophilic and basophilic inflammatory responses is BM Eo/B differentiation.^{29,30} Moreover, our more recent studies utilizing this murine model of allergic rhinitis have revealed that ML suppresses nasal tissue inflammatory responses, as well as BM Eo/B differentiation and maturation in the presence of IL-5.^{29,30} However, in IL-5^{-/-} animals, although no eosinophilic response was detectable in the nasal mucosa, basophils were still residually present, and nasal symptomatology was only delayed, not abrogated.²⁹ In the present study, we therefore focused on the combined effects of ML and IL-5 deficiency on *in vivo* tissue and marrow responses in this model.

Materials and methods

Experimental animals, ovalbumin sensitization and treatments

IL-5^{+/+} (BALB/c) mice were purchased from Harlan (Indianapolis, IN) while IL-5^{-/-} (BALB/c) mice were kindly donated by Dr Klaus Matthaei (Canberra, ACT, Australia). Both strains were bred over a period of 5 years in the same environment at the McMaster Animal Control Facility and housed in pathogen-free conditions for 1 week before use. Eight to 12-week-old IL-5^{+/+} and IL-5^{-/-} BALB/c mice, under pathogen-free conditions, were sensitized with ovalbumin (OVA) using a previously described protocol (Fig. 1).³⁰ In short, OVA sensitization was achieved by administering 40 µg/kg OVA (Sigma, St Louis, MO), diluted in sterile normal saline containing alum adjuvant (40 mg/kg), to animals four times by intraperitoneal injection on days 1, 5, 14 and 21. This was followed by daily intranasal challenge to non-anaesthetized animals with OVA, diluted in sterile normal saline (20 µl of 25 mg/ml OVA per mouse) from day 22 to day 35 (Fig. 1). Each strain was divided into three groups of 10 mice, as follows. (1) The placebo group was OVA-sensitized, then challenged daily, for the next 14 days with intranasal OVA. Sterile water was administered by gavage, using an oral tube (Animal feeding tube, Popper®; Fisher Scientific, Nepean, ON, Canada) 1 hr before each challenge. (2) The ML 2.5 mg/kg group was OVA sensitized similar to the placebo group. Before daily intranasal challenge with OVA, 2.5 mg/kg ML diluted in 150 µl sterile water was administered orally by gavage for a 2-week treatment period, 1 hr before each challenge. (3) The ML 5.0 mg/kg group was OVA-sensitized similarly to the ML 2.5 mg/kg group, but given 5.0 mg/kg ML sodium instead of 2.5 mg/kg.

Clinical symptoms

Nasal symptoms were evaluated for each mouse in each group on day 35 by counting the number of sneezes and nasal itching (nasal rubs) for 10 min after OVA intranasal challenge. Mice were then killed for removal of nasal tissue and femoral BM.

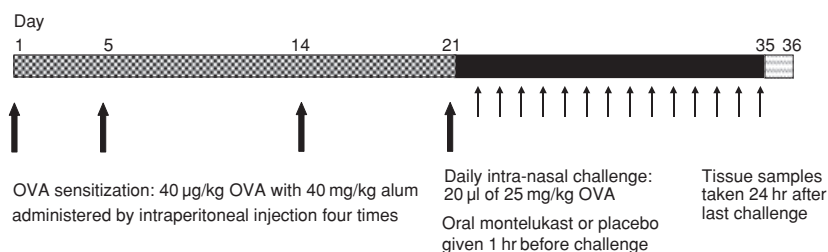


Figure 1. Sensitization and challenge protocol. Schematic representation of the murine experimental allergic rhinitis model. Mice were killed on day 36 and replicate bone marrow and nasal tissue samples were taken for analysis. OVA, ovalbumin. (modified from *Immunology* 2004;113:246–52).

Tissue samples and preparation

Mice were killed by administration of a ketamine hydrochloride/xylazine cocktail to induce deep anaesthesia followed by cardiac puncture. The trachea was exposed and cannulated using a blunted needle inserted into the trachea and tied in place. With the mouse head tilted back, a syringe was inserted into the needle and phosphate-buffered saline (PBS) was gently washed through the trachea into the nasal cavity and collected in a tube. Nasal tissue was collected and fixed in 10% formalin and embedded in paraffin wax blocks and cut into sections for histological analysis after staining with haematoxylin & eosin. The total numbers of eosinophils and basophilic cells were counted in the lamina propria of the nasal mucosa. Basophils were identified as basophilic stained polymorphonuclear leucocytes. BM cells were obtained from one femur, removed and flushed with McCoy's 3 + culture medium, as previously described,³¹ and cell numbers were counted in cell pellets.

BM methylcellulose cultures and cell differentiation analyses

Mononuclear cells from femoral BM were separated using Accuprep, and adherent cells were removed by incubating for 2 hr at 37° in 5% CO₂. Methylcellulose cultures were set up (0.9% methylcellulose, 20% fetal bovine serum and Iscove's 2+) and incubated with an optimal dose of recombinant murine (rm) IL-5. After 6 days, colonies of more than 40 cells were counted using an inverted microscope; Eo/B colony-forming units (CFU) were classified by previously published criteria.^{29,30}

Statistical analyses

For all cell counts, stained slides were read randomly and in a blinded manner. All statistical calculations were performed using SPSS software (Version 10, SPSS Inc., Chicago, IL). All summary statistics were expressed as the mean ± SEM. Group mean comparisons were performed on absolute numbers using repeated measures analysis of variance (ANOVA). After significant group effects, Newman-Keuls post-hoc analysis was used for specific comparisons. All comparisons were two-tailed and $P < 0.05$ was considered statistically significant for all analyses.

Results

Nasal symptoms

In both mouse strains, treatment with ML significantly reduced the observed nasal rubbing and sneezing in both ML dosage groups, compared with controls. The higher dose of ML resulted in fewer symptoms than the lower

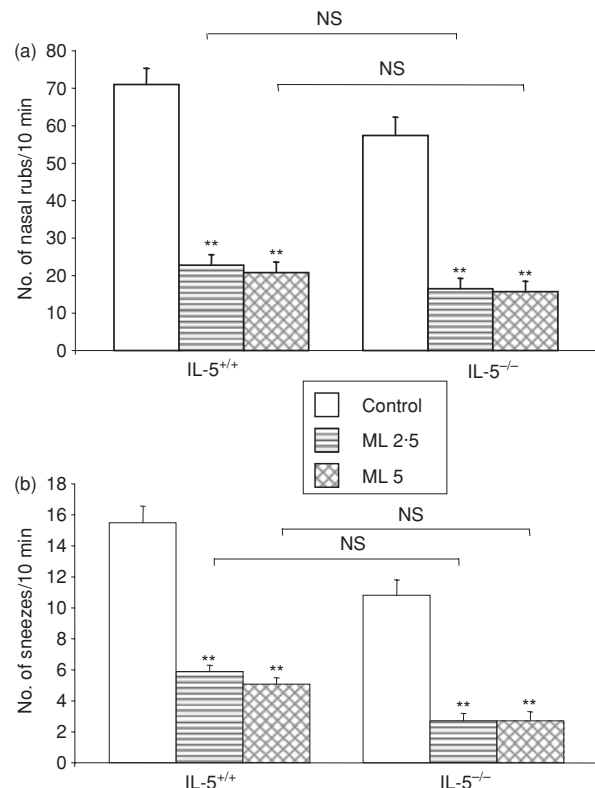


Figure 2. Allergic nasal symptoms at day 35 (2 weeks) of the sensitization protocol, (a) nasal rubs, (b) sneezes. ML reduced the observed symptoms in both treatment groups compared with controls. SEM shown. ** $P < 0.001$; statistical analysis performed within groups, comparing treatment to control. Between groups statistics compared the differences between treatment and control groups.

dose, except in the case of sneezing in the IL-5-deficient mice, where the values were the same (Fig. 2).

Pathology of BM and nasal mucosa

In both mouse strains, the percentages of basophils and eosinophils found in BM were significantly lower in both ML groups, compared to controls. The higher dose of ML resulted in lower percentages of basophils and eosinophils than the lower dose in all conditions (Fig. 3).

Absolute numbers of basophils in the nasal mucosa were significantly decreased in the ML groups in both mouse strains, compared to controls; again, the higher dose of ML resulted in lower numbers of basophils (Fig. 4). In addition, ML at both doses resulted in a greater reduction in nasal mucosal basophils in the IL-5^{-/-} group compared to the wild-type. When eosinophils in the nasal mucosa were counted, there were significantly fewer found in the treatment groups than in the controls in the IL-5^{+/+} strain, with the higher-dose group slightly lower than the low-dose group. As

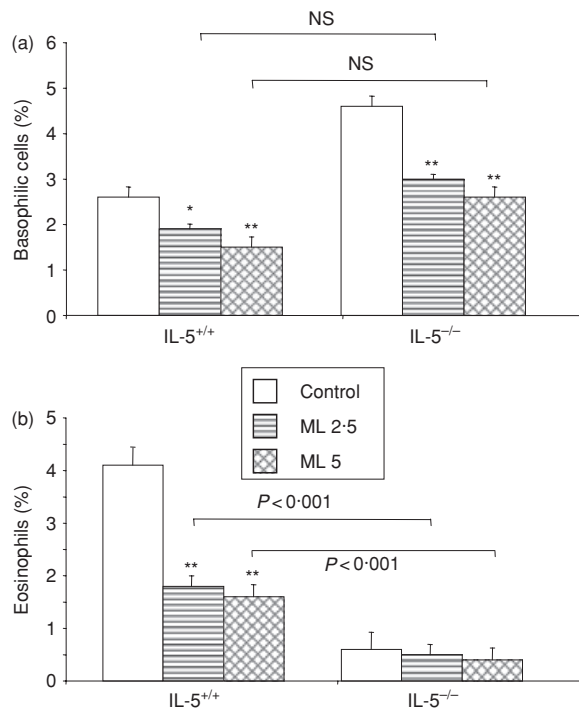


Figure 3. Bone marrow changes. ML reduced the percentages of total cells for (a) basophilic cells and (b) eosinophils in both treatment groups when compared to controls. SEM shown. * $P < 0.05$, ** $P < 0.001$; statistical analysis performed within groups, comparing treatment to control. Between groups statistics compared the differences between treatment and control groups.

previously observed,²⁹ there were no eosinophils evident in the IL-5-deficient mice (Fig. 4).

BM methylcellulose cultures

After 6 days of incubation, Eo/B-CFU were enumerated. ML-treated groups produced significantly fewer colonies than controls in both mouse strains. The higher dose of ML resulted in fewer colonies than the low dose in both strains, but the differences between results were not significant (Fig. 5). All results are summarized semiquantitatively in Table 1.

Discussion

It well known that cysLTs play critical roles in airway inflammation, both in animal models³² and human studies.^{26,33,34} CysLT receptor antagonists have been shown to be effective in attenuating eosinophilic inflammation in the airways of asthmatic subjects and in the nasal mucosa of allergic subjects.^{27,35–37} Recent studies in murine allergic rhinitis have demonstrated the suppressive effects of either IL-5 deficiency or ML on tissue eosinophil recruitment.^{29,30} Neither IL-5 deficiency nor ML alone, however, was sufficient to fully abrogate inflammation or

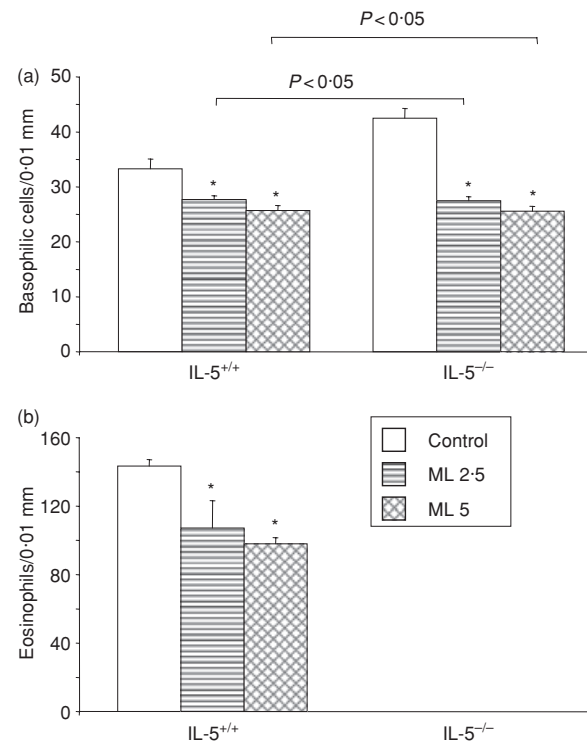


Figure 4. Nasal mucosa. The absolute numbers of (a) basophilic cells and (b) eosinophilic cells counted per 0.01 mm² of lamina propria are shown. ML reduced the numbers of eosinophils and basophils in both treatment groups, except for IL-5-deficient mice, in which no eosinophils were present. SEM shown. * $P < 0.05$, ** $P < 0.001$; statistical analysis performed within groups, comparing treatment to control. Between groups statistics compared the differences between treatment and control groups.

symptoms, because of basophilia and cytokine [IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF)] redundancy, the latter as the result of a shared, common β -receptor subunit with IL-5 in eliciting BM responses.

In this study, we examined the combined effects of ML, a cysLT1 receptor antagonist administered *in vivo*, and IL-5 deficiency on *in vivo* tissue inflammatory and BM responses in murine experimental allergic rhinitis. Since neither IL-5 deficiency nor ML alone could fully abrogate inflammation or clinical symptoms, we hypothesized that the combined effects of both IL-5 deficiency and ML would be more effective.

The study demonstrated that in IL-5-deficient mice compared to controls, pretreatment with ML significantly suppressed IL-5-responsive BM Eo/B-CFU cultured *ex vivo* in the presence of IL-5. Although the role of cysLTs in the BM is still not fully elucidated, the finding that cysLTs are produced in human BM supports their potential role in the haemopoietic microenvironment.³⁸ Previous reports support a direct connection between cysLT actions and cytokine-driven haemopoiesis,³⁹ including a

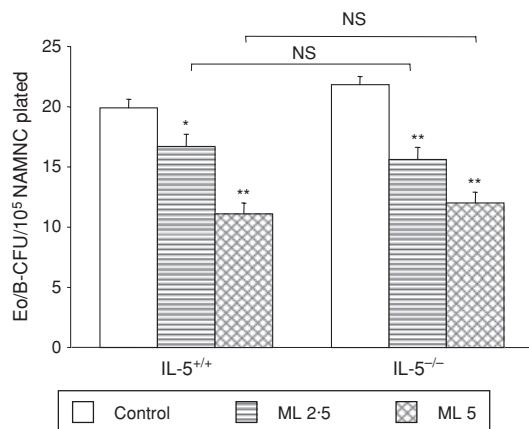


Figure 5. Eo/B-CFU counts. The results of methylcellulose colony assays in the presence of rmIL-5 are shown. ML treatment groups produced fewer colonies than controls. SEM shown. * $P < 0.05$; statistical analysis performed within groups, comparing treatment to control. Between groups statistics compared the differences between treatment and control groups.

Table 1. Summary of results

		IL-5 ^{+/+}		IL-5 ^{-/-}	
		ML (2.5 mg/kg)	ML (5.0 mg/kg)	ML (2.5 mg/kg)	ML (5.0 mg/kg)
Bone marrow	Eo/B CFU	↓	↓↓↓↓	↓↓↓↓	↓↓↓↓
Bone marrow	Eo	↓↓↓↓	↓↓↓↓	NS	NS
Bone marrow	Baso	↓	↓↓↓↓	↓↓↓↓	↓↓↓↓
Nasal mucosa	Eo	↓	↓	N/A	N/A
Nasal mucosa	Baso	↓	↓	↓	↓
Nasal symptoms	Rubbing	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓
Nasal symptoms	Sneezing	↓↓↓↓	↓↓↓↓	↓↓↓↓	↓↓↓↓

Eo, eosinophils; Baso, basophils; ↓ $P < 0.05$; ↓↓ $P < 0.01$; ↓↓↓ $P < 0.005$; ↓↓↓↓ $P < 0.001$; N/A not available; NS not significant.

study performed on normal human cells which demonstrated that peripheral blood Eo/B CFU grown in the presence of GM-CSF, and BM Eo/B CFU grown in the presence of IL-5, were potentiated by the addition of LTD₄ to either cytokine, respectively. This suggests that LTD₄ can stimulate the proliferation of haemopoietic progenitor cells in the presence of eosinophilopoietic cytokines.³⁹ The *ex vivo* suppressive effect in these experiments by ML further demonstrated that this is a cysLT1 receptor-mediated effect. Therefore, the cysLTs behave as cofactors for the growth of Eo/B CFUs, or could induce autocrine production of haemopoietic growth factors.

In the current studies, we also showed that wild-type mice responded to ML treatment by exhibiting highly significant decreases in numbers of BM mature basophils and eosinophils compared to placebo, corroborating our previous reports.³⁰ In contrast, IL-5-deficient mice had

very low numbers of BM eosinophils present, showing no significant change in numbers after ML treatment. However, ML treatment significantly decreased the number of nasal mucosal tissue basophil cells present. One of the mechanisms for the decrease in the number of BM and tissue eosinophils and basophils could be abrogation of the BM Eo/B progenitors.

The results further show that in IL-5-deficient mice, where there was no evidence of eosinophilia in the nasal mucosa, basophils were present and decreased significantly after ML treatment, corresponding with the attenuation of nasal symptoms (Table 1, Fig. 2); this suggests that basophils were also contributing to the residual symptoms in IL-5-deficient mice. Interestingly, ML at both doses showed a greater reduction in basophils in the nasal mucosa of IL-5-deficient mice compared to wild-type. However, this differential effect did not translate to the comparison in the reduction in symptoms with ML between the two mouse strains.

We and others have previously shown that the suppression of the IL-5-eosinophil pathway, via blockade of IL-5 using monoclonal antibodies *in vivo* in humans, does not fully abrogate clinical symptoms or airway hyperresponsiveness; among the reasons for this, we have argued the possible existence of other cytokine-progenitor pathways that may induce and maintain the presence of other inflammatory cell populations.^{29,40–42} This contrasts with animal models in which almost complete abrogation of eosinophilic responses has been shown after anti-IL-5 therapy alone.^{43,44} Until recently, profiling basophils and defining their functional characteristics have been difficult. However, there is steadily increasing evidence of the participation of these cells in airway diseases.⁴⁵

Basophils have been shown to accumulate in the airways of asthmatic subjects after inhalation of LTE₄,²⁶ suggesting that cysLTs might play a role in the accumulation of basophils in allergic tissue. Recently, Gauvreau et al. demonstrated the expression of functional cysLT1 receptors on basophils, suggesting the presence of an autocrine signalling pathway involving cysLTs in these cells or higher expression of cysLT receptors on basophils of asthmatic subjects.²² Thus, a cysLT1R antagonist could have direct effects on basophils through cysLTR.

The exact mechanisms for the effects of ML on basophils could be similar to those described previously by our group regarding changes in eosinophil accumulation *in vivo*, as well as *ex vivo* effects on eosinophilo- (and basophilo-) poiesis,³⁰ which include down-regulation of relevant cytokine receptors or alterations in cytokine generation and responsiveness within maturing eosinophils and basophils, linked to effects of ML on cysLT1R. Equally important are possible interactions between IL-5 receptor and cysLTR on eosinophils/basophils and their progenitors,⁴⁶ an area which still requires further exploration.

In summary, we have demonstrated that *cys*LTR antagonism, in the context of existing IL-5 deficiency, provides added therapeutic benefit in an animal model of allergic rhinitis. The results have clinical implications for the optimization of therapy in allergic rhinitis and asthma.

Acknowledgements

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