

Petasites Extract Ze 339 (PET) Inhibits Allergen-induced Th2 Responses, Airway Inflammation and Airway Hyperreactivity in Mice

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Background: The herbal *Petasites hybridus* (butterbur) extract (Ze 339, PET) is known to have leukotriene inhibiting properties, and therefore might inhibit allergic diseases.

Methods: The effect of PET was investigated in ovalbumin (OVA) immunized BALB/c mice given intranasally together with antigen challenge in the murine model of allergic airway disease (asthma) with the analysis of the inflammatory and immune parameters in the lung.

Results: PET given with the antigen challenge inhibited the allergic response. PET inhibited airway hyperresponsiveness (AHR) and eosinophil recruitment into the bronchoalveolar lavage (BAL) fluid upon allergen challenge, but had no effect in the saline control mice. Eosinophil recruitment was further assessed in the lung by eosinophil peroxidase (EPO) activity at a concentration of 100 µg PET. Microscopic investigations revealed less inflammation, eosinophil recruitment and mucus hyperproduction in the lung with 100 µg PET. Diminution of AHR and inflammation was associated with reduced IL-4, IL-5 and RANTES production in the BAL fluid with 30 µg PET, while OVA specific IgE and eotaxin serum levels remained unchanged.

Conclusion: PET, which has been reported to inhibit leukotriene activity, reduced allergic airway inflammation and AHR by inhibiting the production of the Th2 cytokines IL-4 and IL-5, and RANTES. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: Petasites extract PET; leukotriene synthesis inhibitor; murine asthma model; allergic airway inflammation; airway hyperreactivity, immune response reduction.

INTRODUCTION

Allergic airway diseases such as rhinitis and asthma affect up to 15% of the population and their prevalence is increasing. There is general consensus that allergic inflammation is driven by the activation of T-helper (Th) type 2 cells. They are known to produce IL-4 and IL-13 contributing to IgE production, mucus hypersecretion, airway hyperresponsiveness (AHR) and IL-5 promoted eosinophilic inflammation (Aher and Robinson, 2005; Robinson, 2000). Therefore, several attempts are being made to reduce the inappropriate Th2 response to reduce allergic airway diseases. Therapeutic concepts include Th2 cytokine inhibitors, neutralizing antibodies directed towards B-cell IgE, histamine and leukotriene blockers, as well as other targets (Barnes, 2008; Holgate and Polosa, 2008; Wills-Karp, 1999).

The PET extract from leaves of *Petasites hybridus* L. acts as a leukotriene inhibitor (Thomet *et al.*, 2001a, 2001b) and has been tested successfully in intermittent allergic rhinitis (Schapowal, 2005; Thomet *et al.*, 2002). There is substantial evidence that leukotrienes play an

important role in allergic diseases, as the response to allergens is reduced with inhibitors of leukotriene synthesis and receptor antagonists (Cao *et al.*, 2008; Hicks *et al.*, 2007; Ohnishi *et al.*, 2008; Spahr and Krawiec, 2004; Yokomizo *et al.*, 2000). Moreover, lipoxygenase deficient mice, which are genetically unable to produce leukotrienes have a poor allergic response (Andersson *et al.*, 2008).

The aim of the present investigation was to test the ability of the PET extract to inhibit Th2 response. Murine models can be used for such investigations, since systemic responses as well as airway responses are well defined (Kips *et al.*, 2003). It was demonstrated previously that intranasally applied allergens are equally distributed within the upper and lower airway systems, and that repeated allergen challenges induced airway hyperresponsiveness (AHR) (Wang and McCusker, 2005).

Therefore, Th2 activation, allergic tissue reactions, inflammatory cell recruitment in the bronchoalveolar lavage (BAL) and airway hyperresponsiveness to methacholine were studied in the OVA antigen-induced experimental model in mice, and then compared with negative and positive controls. It is reported that PET, which reportedly inhibits leukotriene production, effectively inhibits an established Th2 response in the lung.

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METHODS

Animals and immunization. Balb/c mice, aged 6–8 weeks, were immunized twice subcutaneously in groups of six mice at weekly intervals with a 0.4 mL saline solution containing 1 µg OVA and 1.6 mg alums. One week after the second immunization, on day 14, intranasal challenge was performed under light i.v. ketamine anaesthesia by applying 50 µL OVA in alum-free saline solution (10 µg) or saline alone as a control. The intranasal challenges were given on 3 consecutive days (Couillin *et al.*, 2004). All experimental studies were approved by the local ethical animal research committee.

Drug administration. Petasites extract PET was given intranasally in concentrations of 10 µg, 30 µg and 100 µg (in 40 µL) before saline or OVA challenge in OVA sensitized Balb/c mice under light ketamine anaesthesia (Fig. 1). The PET extract was manufactured using liquid carbon dioxide with leaves of the cultivated Petasites variety PETZELL. The identical extract had already been used in clinical trials (Schapowal, 2002, 2004, 2005; Thomet *et al.*, 2002).

Airway hyperresponsiveness (AHR). Airway resistance was evaluated by whole-body plethysmography as described before (Couillin *et al.*, 2004). Bronchial hyper-reactivity to aerosolized methacholine was investigated 24 h after the last saline or OVA challenge in OVA sensitized mice as described before (Couillin *et al.*, 2004). Unrestrained conscious mice were placed in whole-body plethysmography chambers (Buxco Electronic, Sharon, CO, USA). Methacholine at 100 mm was aerosolized for 1 min and mean airway bronchoconstriction readings, as assessed by enhanced respiratory pause (PenH), were obtained over 15 min periods. PenH can be conceptualized as the phase shift of the thoracic flow and the nasal flow curves; where an increased phase shift correlates with increased respiratory system resistance. PenH is calculated by the formula

$$\text{PenH} = (T_e/RT - 1) \times PEF/PIF$$

where T_e is the expiratory time, RT the relaxation time, PEF the peak expiratory flow and PIF the peak inspiratory flow.

Bronchoalveolar lavage (BAL). BAL was performed 24 h after the last antigen challenge by cannulating the trachea under ketamine anaesthesia and washing with 0.5 mL of ice-cold phosphate buffered saline (PBS)

(Couillin *et al.*, 2004). The first 500 µL of the lavage fluid was centrifuged and the supernatant frozen for subsequent cytokine determinations and the trachea was further lavaged with 3×0.5 mL of PBS and centrifuged. The cell pellet was resuspended in PBS, counted by a haemocytometer chamber and cytospin preparations were made using a Shandon cytocentrifuge. The cells were analysed after differential staining with May–Gruenwald–Giemsa.

Lung histology. After bronchoalveolar lavage, the mice were killed 24 h after the last saline or OVA challenge. The whole lung was removed and fixed in 4% buffered formaldehyde for standard microscopic analysis with haematoxylin and eosin (H&E) and periodic acid Schiff reagent (PAS) staining. Peribronchial inflammation, eosinophilic recruitment and mucus hypersecretion was assessed using a semi-quantitative score (a scale from 0 to 5, absent; mild, slight, moderate, distinct, severe) by two independent observers as described before (Couillin *et al.*, 2004).

Eosinophil peroxidase (EPO) activity. Fresh lung tissues were frozen and homogenized in buffer and substrate was added to assess EPO activity as described before (Couillin *et al.*, 2004).

Cytokines in BAL and plasma. IL-4, IL-5, IL-10, IL-12, IL-13, IFN γ and the chemokines RANTES and eotaxin in the first volume of 500 µL of the BAL fluid, and eotaxin in plasma were evaluated by enzyme-linked immunosorbent assays (ELISA) from Pharmingen and R&D following the instructions of the manufacturer.

Serum IgE levels. Blood was taken 24 h after the last saline or OVA challenge and plasma was prepared. Total and specific IgE levels were measured in plasma by ELISA as described before (Couillin *et al.*, 2004).

Statistical analysis. The Student's *t*-test was used to compare the differences between experimental groups ($p < 0.05$).

RESULTS

Petasites extract reduces airway hyperresponsiveness (AHR)

PET was given intranasally in doses of 10 µg, 30 µg and 100 µg immediately before each antigen challenge in

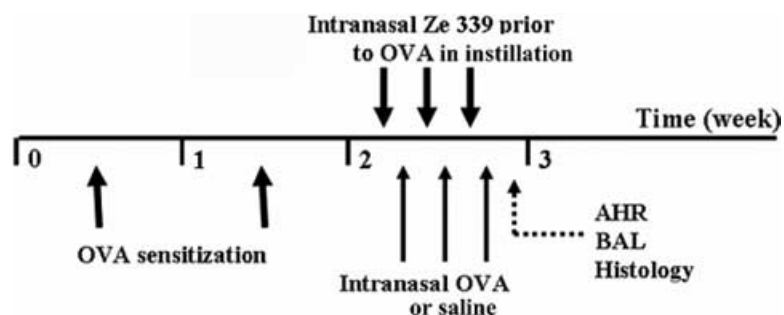


Figure 1. Scheme of allergen OVA sensitization (day 0 and 7) and intranasal instillation of PET followed by OVA or saline challenge (days 14, 15, 16) and analysis on day 17 (AHR, BAL, cytokines, IgE and histology).

OVA sensitized Balb/c mice. PET inhibited partially methacholine induced AHR, which was assessed by whole body plethysmography. PenH is an indirect measure of airway resistance and reflects inflammatory changes in the airway system (Couillin *et al.*, 2004). The PenH values after methacholine exposure are shown over a period of 15 min (Fig. 2A). The inhibitory effect was more prominent in the later phase of the response which is after 5 min. The dose effect was calculated by the area under the curve (AUC) of PenH over the 15 min time period as shown in Fig. 2B. PET caused a significant reduction of PenH at 30 μg ($p < 0.05$) and 100 μg ($p < 0.01$). Importantly, PET had no effect on PenH in saline challenged, sensitized mice. Therefore, the data suggest that the Petasites extract may inhibit AHR as reported for leukotriene inhibitors.

Petasites extract reduces eosinophil recruitment.

A hallmark of allergic asthma is the recruitment of eosinophils into the lung. Therefore, the study investigated the BAL fluid obtained 24 h after the last antigen challenge, counted the cells and prepared cytospin preparations for the differential cell counts. PET at the 100 μg dose inhibited eosinophil recruitment into the BAL fluid significantly ($p < 0.05$), while the 30 μg dose showed only a trend (Fig. 3A). The recruitment of eosinophils was further evaluated in lung tissue homogenate followed by the determination of eosinophil peroxidase (EPO) activity expressed as optical absorbance (OD) as described (Couillin *et al.*, 2004). PET

inhibited EPO activity at the dose of 100 μg and 30 μg ($p < 0.05$, Fig. 3B), while PET at 10 μg had no effect. Therefore, Petasites extract given together with the antigen challenge reduces eosinophil recruitment in the lung.

Petasites extract reduces IL-4, IL-5 and RANTES production in BAL fluid

Since eosinophilic inflammation was reduced, the question was asked whether typical mediators of a Th2 response are affected by Petasites. The antigen challenge induced a significant increase in IL-4, IL-5, and RANTES levels in the BAL fluid in immunized mice compared with saline controls. The levels of IL-4, IL-5 and RANTES were reduced in the BAL fluid from mice treated with 30 μg PET ($p < 0.05$, Fig. 4), the other doses of PET were not tested.

Furthermore, IL-10 levels were measured in the BAL. IL-10 was detectable upon OVA challenge, but was not modified by PET administration at the 100 μg dose. IFN γ , eotaxin or IL-13 in the BAL fluid were not detected in any experimental group (data not shown). Furthermore, plasma eotaxin levels were determined as an indicator of the systemic allergic response. OVA immunization induced an increase in eotaxin plasma levels which, however, at a 100 μg dose of PET was not

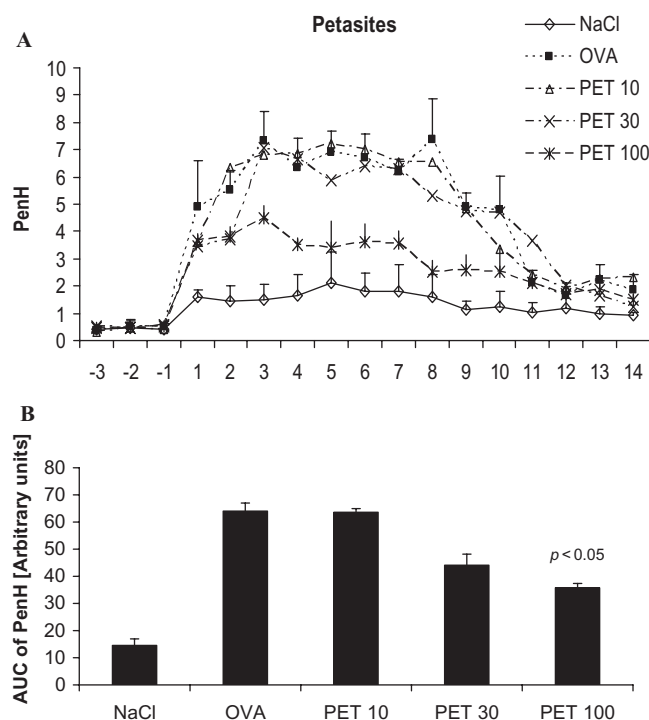


Figure 2. Methacholine induced airway hyperresponsiveness (AHR) at 24 h after the last antigen challenge (day 17). (A) Methacholine (100 μM) induced AHR in saline controls (NaCl), OVA (OVA) in the absence or presence of PET in OVA sensitized mice. PET was given at 10 μg (PET 10), 30 μg (PET 30) and 100 μg (PET 100) each to both, saline and OVA challenged mice (Figure OVA data only). Mean $\pm$ SD of PenH values and SD are shown ($n = 4$ mice per group). (B) Dose response effect of PET (10–100 μg) on PenH values expressed as mean $\pm$ SD of the area under curve, (AUC; $n = 4$ mice per group).

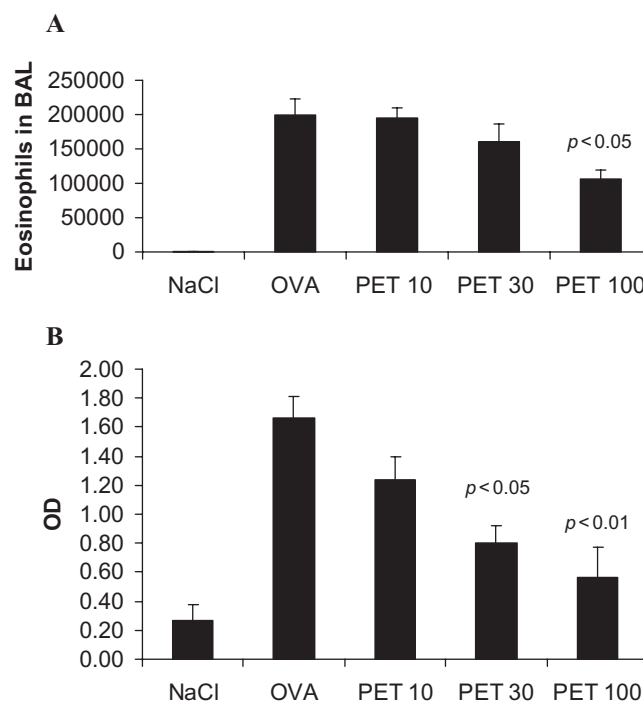


Figure 3. Reduced recruitment of eosinophils in BAL and lung at 24 h after antigen challenge (day 17). (A) Effect of PET doses (10–100 μg) on eosinophil recruitment in BAL (day 17). Total count of eosinophils in BAL of saline controls (NaCl), OVA challenged mice in the absence of PET (OVA) and OVA challenged mice with PET at doses of 10 μg (PET 10), 30 μg (PET 30) and 100 μg (PET 100). (B) Effect of PET on lung eosinophil peroxidase (EPO) activity in the homogenized lung tissue after BAL lavage (day 17). OD values of EPO in homogenized lung tissue after BAL lavage of saline controls (NaCl), OVA challenged mice in the absence of PET (OVA) and OVA challenge mice with PET at doses of 10 μg (PET 10), 30 μg (PET 30) and 100 μg (PET 100). Graph shows mean values $\pm$ SD and indicated p values ($n = 6$ mice per group).

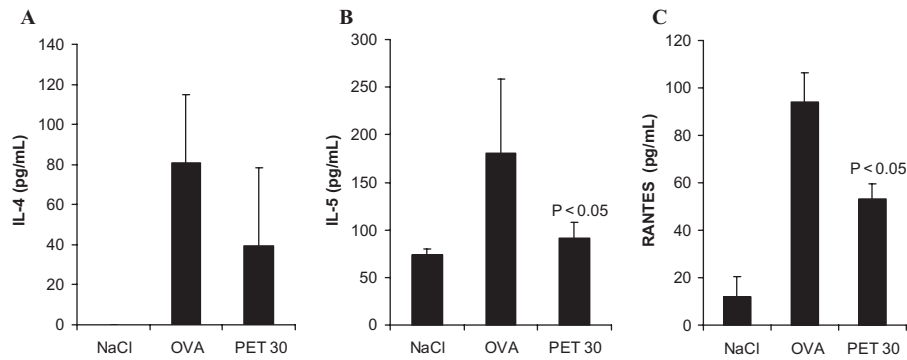


Figure 4. Inhibition of cytokine secretion in BAL fluid at 24 h after last antigen challenge as analysed on day 17. Determination by ELISA of IL-4 (A), IL-5 (B) and RANTES (C) concentrations in the first 500 μ L BAL fluid of saline controls (NaCl), OVA challenged mice in the absence of PET (OVA) and OVA challenged mice with PET at doses of 30 μ g (PET 30). Graphs show mean $\pm$ SD and indicated *p* values (*n* = 4–6 mice per group).

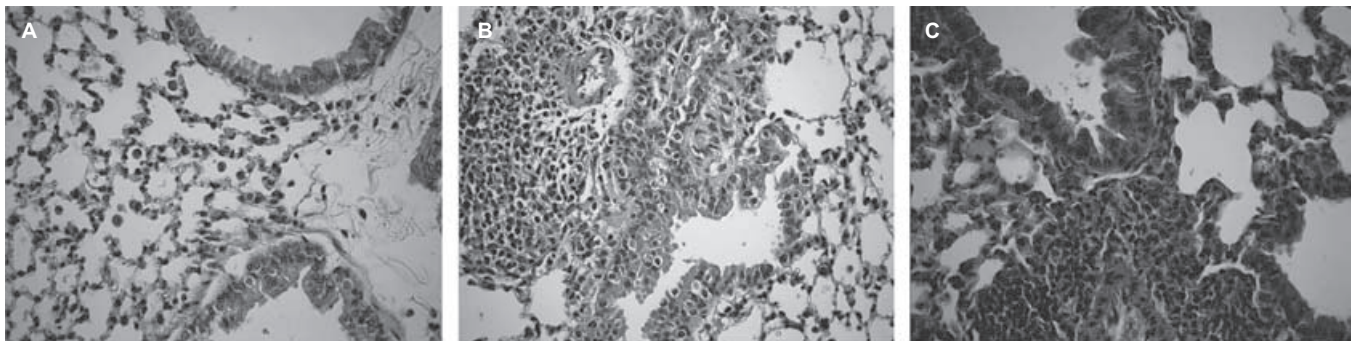


Figure 5. Representative microscopic plates of lung tissue demonstrating reduced infiltration by inflammatory cells, eosinophil recruitment and mucus hypersecretion 1 day after the last OVA challenge and 30 μ g PET administration (day 17). (A) Saline controls, (B) OVA challenged in the absence of PET, (C) OVA challenged with 30 μ g PET.

significantly different from the saline group (data not shown).

Finally, total and OVA specific IgE levels were determined in the serum at 24 h after the last saline or OVA challenge. OVA administration had a slight effect on total IgE and OVA specific IgE levels. PET given at a dose of 100 μ g had no effect on IgE levels (data not shown).

Therefore, *Petasites* inhibits the production of IL-4, IL-5 and RANTES, which are critical mediators of the allergic response.

Reduced inflammation and mucus hypersecretion

Microscopic investigations were performed after the antigen challenge to investigate lung morphology. Typical representative microscopic pictures are shown in Fig. 5. While the antigen-challenged immunized mice displayed impressive inflammation, eosinophilic infiltration and mucus production (B), saline-challenged immunized mice had normal lung architecture (A). PET treated mice (C) showed a distinct reduction of inflammation and mucus production.

The lung sections were further analysed semi-quantitatively by a severity score (0–5). The extent of peribronchial inflammation, infiltration by eosinophils and mucus hyperproduction were recorded. It was found that PET inhibited the recruitment of eosinophils and the production of mucus at concentrations of 100 μ g (Table 1). Therefore, the morphological findings corroborated the functional data and in particular the reduced eosinophil recruitment in the BAL.

DISCUSSION

The PET extract prepared from leaves of *Petasites hybridus* L. showed activities in the murine model of allergic asthma using OVA challenge of OVA sensitized BALB/c mice. The typical Th2 cytokine profile response to an allergen challenge was seen in the present investigation, which consisted of a prevailing IL-4 and IL-5 production and relatively low or undetectable levels of IFN- γ and IL-10 (Ohkawara *et al.*, 1997; Wegmann *et al.*, 2005). This cytokine profile reflects an allergen-induced release by activation of Th2 cells and therefore may be related to extrinsic eosinophilic disorders (Simon and Simon, 2007). There was no evidence that Th1 cells are activated to balance Th2 stimulation, since neither IFN- γ nor IL-12 were found in BAL. The absence of IL-13 in BAL could be due to the limited sensitivity of the ELISA detection system, as IL-13 clearly participates in the allergic response and neutralizing IL-13 antibody diminishes allergy (Wang and McCusker, 2005).

The Th2 cytokines IL-4 and IL-5 were significantly reduced in response to PET when given just before intranasal OVA challenge, but had no effect in saline challenged mice. The dose related reduction of AHR, as well as mucus hypersecretion, may be due to a reduced IL-4 production by PET. The reduced activity of EPO within lung tissue indicates a diminished recruitment of eosinophils into the lung parenchyma. Moreover, accumulation of eosinophils into lung tissue and migration into BAL fluid were significantly inhibited. On the one hand, this observation may be linked to the

Table 1. Quantification of the effects of PET on allergic inflammation, eosinophil infiltration and mucus hypersecretion in the lung (day 17)

Group	#	Inflammation	EOS infiltration	Mucus hyperproduction
NaCl	1	1	0	0
	2	0	0	0
	3	0	0	0
	4	0	0	0
	5	0	0	0
Mean $\pm$ SD		0.2 $\pm$ 0.4	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
OVA	11	3	3	4
	12	3	4	2
	13	4	3	4
	14	2	3	3
	15	3	4	3
Mean $\pm$ SD		3.0 $\pm$ 0.7	3.4 $\pm$ 0.5	3.2 $\pm$ 0.8
Petasites 10	51	2	4	3
	52	3	3	3
	53	3	4	4
	54	4	3	3
	55	3	2	4
Mean $\pm$ SD		3.0 $\pm$ 0.7	3.2 $\pm$ 0.8	3.4 $\pm$ 0.5
Petasites 30	61	4	2	3
	62	2	4	4
	63	5	4	3
	64	3	3	2
	65	4	4	3
Mean $\pm$ SD		3.6 $\pm$ 1.1	3.4 $\pm$ 0.9	3.0 $\pm$ 0.7
Petasites 100	71	2	2	2
	72	2	2	2
	73	2	2	1
	74	3	3	2
	75	4	2	2
Mean $\pm$ SD		2.6 $\pm$ 0.9	2.2 $\pm$ 0.4	1.8 $\pm$ 0.4

Microscopic evaluation of lung tissue sections stained with haematoxylin and eosin and PAS (mucus) was performed by a semi-quantitative score (0 = no change, 1–5 with increasing severity as described in Methods. Mean scores $\pm$ SD are given ($n = 5$ mice per group).

reduced IL-5 secretion in BAL. On the other hand, leukotriene B4 may play a pivotal role in mediating the selective recruitment of eosinophils in a model of chronic eosinophilic inflammation driven by allergen challenge of CD4⁺ cells (Cherain *et al.*, 2008).

Leukotriene B4 has been shown to exert its proinflammatory activity by binding to a specific G protein-coupled receptor BLT1, widely expressed on leukocytes (Yokomizo *et al.*, 1997). BLT 2, a second, highly homologous receptor with lower affinity is a pharmacologically distinct receptor for leukotriene B4 (Yokomizo *et al.*, 2000). The colocalization of the gene of BLT1 and BLT2 suggests shared transcriptional regulation and their expression in tissues other than leukocytes, for example vascular endothelial cells. These cells are known to mediate localised infiltration of eosinophils (Moser *et al.*, 1992a, 1992b; Qiu *et al.*, 2006).

Total and OVA specific IgE levels were augmented upon allergen challenge. It has been shown that IL-4-induced IgE production by normal PBMCs was enhanced by leukotriene B4, whereas by itself leukotriene B4 was not effective for IgE production (Yamaoka *et al.*, 1994). However, the data demonstrate that intra-

nasal PET administration during the antigen challenge does not reduce IgE production.

Reduced airway inflammation and hyperreactivity are thought to be mediated by petasins contained in the PET extract, which block leukotriene synthesis (Thomet *et al.*, 2001a, 2001b). Cysteinyl leukotrienes contribute markedly to the airway reactions in an OVA sensitized murine asthma model, since the cysteinyl leukotriene receptor antagonist Montelukast significantly reduces eosinophil infiltration and mucus production in the lung (Henderson *et al.*, 2002). Whether other components within the extract, e.g. antiinflammatory (Fiebich *et al.*, 2005) or mast cell stabilizing agents (Shimoda *et al.*, 2006), additionally contribute to the observed reactions can not be excluded.

In conclusion, when given during an antigen challenge, PET extract effectively inhibits allergen-induced inflammation and airway hyperreactivity by reducing the Th2 immune response in a murine model of asthma.

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