

ORIGINAL ARTICLE

Over-expression of the LTC₄ synthase gene in mice reproduces human aspirin-induced asthmaH. Hirata¹, M. Arima^{1,2}, Y. Fukushima¹, K. Honda¹, K. Sugiyama¹, T. Tokuhisa² and T. Fukuda¹¹Department of Pulmonary Medicine and Clinical Immunology, Dokkyo University School of Medicine, Tochigi, Japan and ²Department of Developmental Genetics, Chiba University Graduate School of Medicine, Chiba, Japan

Clinical & Experimental Allergy

Summary

Background The pathogenesis of aspirin-induced asthma (AIA) is presumed to involve the aspirin/non-steroidal anti-inflammatory drug (NSAID)-induced abnormal metabolism of arachidonic acid, resulting in an increase in 5-lipoxygenase (5-LO) metabolites, particularly leukotriene C₄ (LTC₄). However, the role of LTC₄ in the development of AIA has yet to be conclusively demonstrated.

Objective The aim of this study was to evaluate the contribution of the lipid product LTC₄ secreted by the 5-LO pathway to the pathogenesis of AIA.

Methods To evaluate antigen-induced airway inflammation, the concentrations of T-helper type 2 cytokine in bronchoalveolar lavage fluid (BALF) obtained from LTC₄ synthase-transgenic (Tg) and wild-type (WT) mice after challenge with ovalbumin were measured. Subsequently, the *ex vivo* and *in vivo* effects of the NSAID sulpyrine were investigated in these Tg and WT mice by measuring the secretion of LTC₄ from sulpyrine-treated BAL cells and the levels of LTC₄ in BALF following challenge with sulpyrine. Finally, the sulpyrine-induced airway response by the administration of pranlukast, an antagonist of the cysteinyl (cs)-LT1 receptor, was analysed.

Results The concentrations of IL-4, -5, and -13 in BALF from Tg mice were significantly higher than those in WT mice. In addition, sulpyrine augmented the secretion of LTC₄ in BALF and by BAL cells in Tg mice, but not in WT mice. Additionally, the increased airway resistance induced by sulpyrine could be reduced by treatment with pranlukast. Furthermore, the secretion of LTC₄ from mast cells, eosinophils, and macrophages was increased in the allergen-stimulated LTC₄ synthase gene Tg mice, even in the absence of sulpyrine, as well as in BAL cells after sulpyrine.

Conclusion and clinical relevance The over-expression of the LTC₄ synthase in a mouse asthma model also replicates the key features of AIA. And our study supports that cys-LTs play a major role in the pathogenesis of AIA in patients with chronic asthma.

Keywords aspirin-induced asthma (AIA), cysteinyl (cys)-LT1 receptor antagonist, 5-lipoxygenase pathway, LTC₄ synthase gene, sulpyrine, Th2 cytokine, transgenic mice

Submitted 27 October 2010; revised 12 January 2011; accepted 17 January 2011.

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Introduction

The arachidonic acid (AA) cascade is divided into two major pathways, the cyclooxygenase (COX) pathway, which yields prostanoids [prostaglandins (PGs) and thromboxane A₂], and the lipoxygenase pathway, which produces leukotrienes (LTs). The sequential catalytic action of 5-lipoxygenase (5-LO), which is expressed in eosinophils, basophils, mast cells, macrophages, platelets, and endothelial cells, on AA generates LTA₄, which is either hydroxylated to LTB₄ or metabolized into the first

of the cysteinyl (cys) LTs, leukotriene C₄ (LTC₄), by the action of LTC₄ synthase (LTC₄S) [1–5]. LTC₄ is then converted to LTD₄ in the extracellular space where it is subsequently cleaved to form LTE₄ [6, 7]. Cys-LTs play important roles in the pathogenesis of asthma [8], such as airway remodelling that is characterized by contraction and hyperplasia of bronchial smooth muscle, enhancement of secretion from bronchial glands, increased vascular permeability, and recruitment of eosinophils [9–12].

Recently, the relevance of cys-LTs in the development of aspirin-induced asthma (AIA) has emerged from

clinical studies [13, 14]. AIA affects 5–10% of adults with chronic asthma, in whom acute bronchoconstriction is induced by the ingestion of aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) [15, 16]. The pathogenesis of AIA is associated with chronic overproduction of cys-LTs in AIA patients even in the absence of NSAID ingestion, and the acute overproduction of cys-LTs triggered by exposure to NSAIDs. The acute triggering mechanism may depend on inhibition of PGE₂, which can have an anti-inflammatory effect, by NSAID or the shunting of arachidonate from the COX pathway [17–19]. In patients with AIA, an increased activity of LTC₄S in bronchial mucosa has been observed, which is supported by a study that suggested a biallelic polymorphism in the promoter region of the LTC₄S gene enhanced its transcription in these patients [20, 21]. Although several underlying genetic mechanisms of AIA have been proposed based on the identification of candidate genes other than LTC₄S, to date, it appears that genetic influences on AIA are subtle [22–25]. Nevertheless, LT-modifying drugs [26] are effective in blocking the bronchoconstriction provoked by aspirin and are typically used in the treatment of AIA [27]. Therefore, aspirin/NSAIDs may shift the balance of the eicosanoid pathways to augment airway inflammation and obstruction by the over-production of cys-LTs, including LTC₄. Despite this speculation, the role of cys-LTs in the pathogenesis of AIA in humans is unclear. Because LTC₄S is the critical terminal enzyme for cys-LT biosynthesis in the 5-LO/LTC₄S pathway, we generated LTC₄S (*Ltc₄s*) gene transgenic (LTC₄S-Tg) mice to examine the functions of this synthase *in vivo*. In this study, we have clearly demonstrated for the first time that the over-expression of the LTC₄ synthase in a mouse asthma model also replicates the key features of AIA.

Materials and methods

Reagents and antibodies

Magnetic microbeads labelled with anti-CD90 (Thy1.2), -CD45R (B220), -CD11b, -CD11c, or -PE antibodies (abs) were purchased from Miltenyi Biotec (Gladbach, Germany). PE-conjugated anti-CD3 ϵ chain, APC-conjugated anti-CD11b, APC- and PE-conjugated anti-Gr-1(Ly-6G), and APC-conjugated anti-c-kit (CD117) abs were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). PE-conjugated anti-Fc ϵ RI α was purchased from eBioscience (San Diego, CA, USA). PE-conjugated anti-chemokine receptor (CCR)3 was purchased from R&D Systems (Minneapolis, MN, USA).

Generation of LTC₄S-Tg mice

To generate transgenic mice containing the *Ltc₄s* gene, we amplified murine cDNA encoding the full-length LTC₄S protein and placed it under the control of a CMV enhancer/chicken β -actin promoter in the transgenic vector

Pcaggs [28], which has been shown to facilitate widespread transgene expression in mice. The DNA fragment coding LTC₄S was generated by RT-PCR from mRNA obtained from splenocytes stimulated with phorbol myristate acetate (PMA) and ionomycin. Full-length LTC₄S cDNA (540 bp) was PCR-amplified with *Eco*RI-anchored sense (5'-CCGCTCGAG CTGGCAACATGAAGGACGAAG TG-3') and antisense primers (5'-ACGCCTCGAGAAAAA CTGGGATTCGGGGGC-3') (*Eco*RI sites underlined), and cloned into the *Eco*RI site of pCAGGS to make pCAGGS-LTC₄S. Transgenic mice (LTC₄S-Tg mice) were generated using pCAGGS-LTC₄S by standard procedures, genotyped by PCR, and then backcrossed to BALB/c mice (Charles River Laboratories, Atsugi, Japan) for 10 generations. We successfully generated four Tg lines (#22, 33, 65, and 90) of LTC₄S-Tg mice, with each line having different copy numbers of the *Ltc₄s* transgene. To evaluate their capacity for LTC₄ production, we measured LTC₄ in the culture supernatants of splenic cells isolated from the four Tg lines after stimulation with PMA/ionomycin (described below). As splenocytes from mouse LTC₄S-Tg line #90 produced the largest amount of LTC₄ (data not shown), this mouse line was used for subsequent experiments. All mice were maintained under specific pathogen-free conditions in the animal centre at Dokkyo University School of Medicine. This study using experimental animals was approved by the Ethics Committee.

Sensitization and antigen challenge, sulpyrine administration, and treatment with mouse cys-LTs receptor antagonist

An outline of the protocols used in this study is shown in Fig. 1. To investigate the roles of LTC₄S in the development of AIA, we first established an antigen-dependent murine model of asthma. Briefly, LTC₄S-Tg and wild-type (WT) mice were intraperitoneally injected on days 0 and 5 with ovalbumin (OVA) (Sigma-Aldrich, St Louis, MO, USA) (8 μ g/mouse) adsorbed in aluminium hydroxide (alum) (Wako Pure Chemical Industries, Osaka, Japan) for sensitization, or aluminium hydroxide alone as a control. To induce airway inflammation, the sensitized and control mice were challenged with aerosolized 1% OVA or saline, respectively, for 60 min on day 12. On day 13, the mice were analysed for airway hyperresponsiveness (AHR). Subsequently, on day 14, a second analysis for AHR was performed, which was followed by bronchoalveolar lavage (BAL) and splenectomy for cellular analysis. For the establishment of the AIA model, 48 h after the OVA challenge, mice were bronchoprovoked with aerosol inhalation of either sulpyrine (1, 10, or 50 mg/mL) or saline for 30 min. After 2 h, airway resistance was measured, and BAL was then performed. In addition, to assess the effect of a Cys-LT1 receptor antagonist on the development of AIA, pranlukast hydrate (ONO Pharmaceutical Co., Osaka, Japan) or lactose (Sigma-Aldrich) as the control was

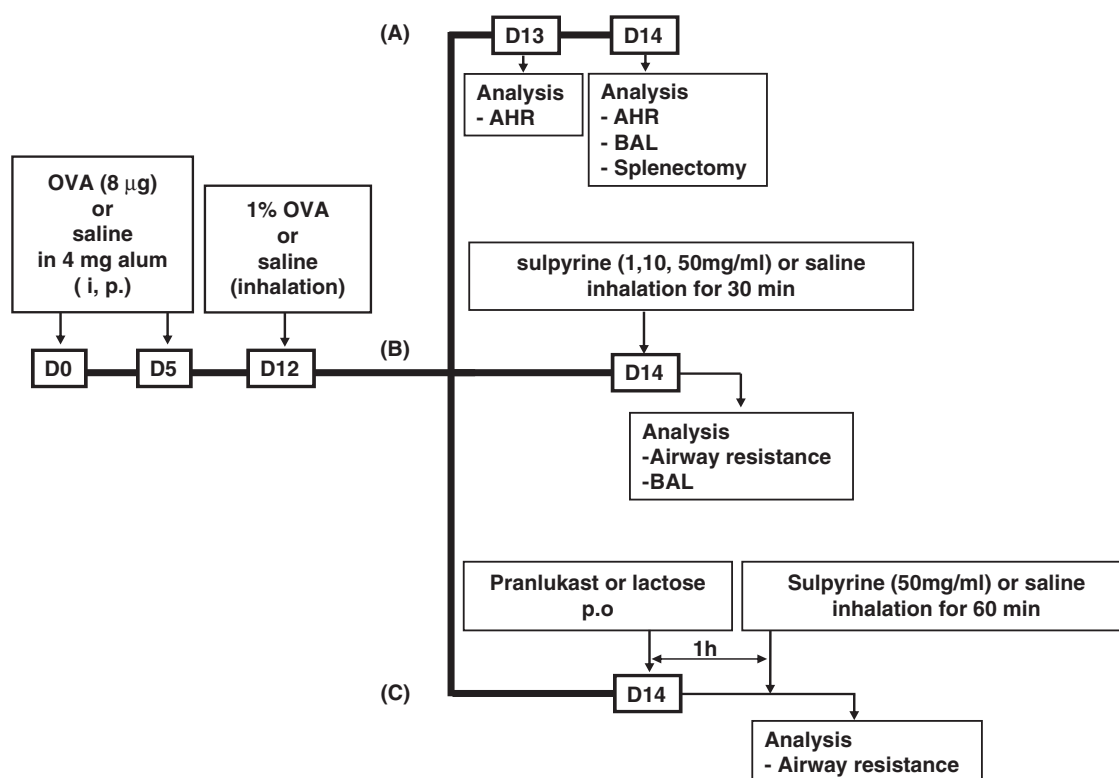


Fig. 1. An outline of the protocols used in this study. Mice were first subjected to twice intraperitoneal immunization with ovalbumin (OVA)/alum or saline/alum on day (D) 0 and 5, followed by challenge with 1% OVA or saline on D12. After the challenge, mice were separated into three groups (A–C). In group A, the role of over-expression of LTC₄ on antigen-induced asthmatic response was evaluated. AHR, airway resistance to inhaled methacholine (D13 and D14). BAL, measurement for amounts of cytokines and LTC₄, examination of cell population, isolation of each inflammatory cells (D14). Splenectomy, isolation of mast cells (D14). In group B, the effect of sulpyrine on airway inflammation was examined. Airway resistance was analysed immediately after the administration of inhaled sulpyrine (1, 10, or 50 mg/mL) or saline (D14). In group C, the effects of pranlukast hydrate on sulpyrine-induced airway obstruction was examined. Airway resistance in mice 2 h after inhalation of sulpyrine (50 mg/mL) or saline at 1-h post-treatment with pranlukast hydrate or lactose (25 mg/kg body weight) was measured (D14). i.p., intraperitoneally; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; LTC₄, leukotriene C₄.

dissolved in dimethylsulphoxide and given to mice by intragastric administration (25 mg/kg body weight) 1 h before sulpyrine (50 mg/mL) inhalation.

Measurement of airway resistance

To estimate AHR to inhaled methacholine or AIA, airway resistance in conscious, spontaneously breathing mice was measured by barometric whole-body plethysmography (Buxco Electronics, Sharon, CT, USA) as described previously [29, 30]. Briefly, saline or increasing concentrations of methacholine (6–24 mg/mL) or sulpyrine (1–50 mg/mL) were aerosolized through an inlet of the main chamber of the plethysmograph. Mice were challenged with methacholine or sulpyrine for 3 or 30 min, respectively, and readings were recorded and averaged 3 min after each challenge. Airway resistance was expressed as enhanced pause (Penh) values for each tested concentration.

Bronchoalveolar lavage

BAL was performed immediately after the last aerosol provocation with OVA, methacholine or sulpyrine. At the

time of lavage, mice were killed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg body weight). After the trachea was exposed and cannulated with polyethylene tubing, three successive volumes of saline (0.75 mL) were instilled, gently aspirated, and pooled. Each BAL fluid (BALF) sample was centrifuged at 400 g for 10 min, and the supernatants were stored at -80°C until needed for analysis. Total cell numbers in the resulting pellets were counted using a haemocytometer. Cytospin preparations of BAL cells were stained with Diff-Quik Solution (Dade Behring Inc., Newark, DE, USA) to determine the differential cell count, which was evaluated by counting at least 500 leucocytes. The remaining cells in BALF were harvested and analysed in subsequent experiments.

Cell preparation

Macrophages, T cells, neutrophils, and eosinophils were obtained from BAL cells, while mast cells were harvested from splenocytes. To obtain macrophages, BAL cells in 5 mL RPMI 1640 medium containing 10% fetal calf serum (FCS) (RPMI 1640/10% FCS) were transferred into an

MSP-P monocyte-specific culture plate (Jimro, Takasaki, Japan) and incubated at 37 °C in a CO₂ incubator for 3 h to allow macrophages to attach. After incubation, the medium was decanted to remove the non-adherent cells, which were then resuspended in RPMI 1640 supplemented with 2% FCS and saved for further treatment. The culture plate was then rinsed five times with cold RPMI 1640/10% FCS to completely remove the non-adherent cells. After washing, all adherent cells were then removed from the plate by incubation at 4 °C with PBS containing 0.2% EDTA. The purity of macrophages (>90%) was estimated by the number of CD11b⁺ cells detected using flow cytometry with a FACS Caliber (Becton Dickinson, Franklin Lakes, NJ, USA) and CellQuestPro software. To obtain PanT cells, CD90.2 MicroBeads (Miltenyi Biotec) were added to the collected non-adherent cells to isolate CD90.2 (Thy1.2)⁺ T cells by positive selection. The purity of T cells (>90%) was estimated by the number of CD3⁺ cells detected using flow cytometry. In addition, eosinophils and neutrophils were enriched from the remaining non-adherent cells by positive and negative selection, respectively, with PE-labelled anti-CCR3 abs using anti-PE MicroBeads (Miltenyi Biotec). The purity of eosinophils (Ly-6G⁺CCR3⁺) (>90%) and neutrophils (Ly-6G⁺CCR3⁻) (>90%) was estimated by cytological confirmation and flow cytometry. Mast cells were enriched from splenocytes by negative selection using a cocktail of MicroBeads specific for CD90, CD45R/B220, CD11b, and CD11c (Miltenyi Biotec). The purity of the mast cells (FcεRI⁺c-kit⁺) (>80%) was examined by flow cytometry.

Cell culture to evaluate LTC production

For the measurement of LTC₄ production, the isolated neutrophils, eosinophils, T cells, macrophages, and mast cells (2 × 10⁵/mL) were cultured in RPMI 1640/10% FCS in the presence or absence of both phorbol myristate acetate (PMA) (25 ng/mL) (Sigma-Aldrich) and ionomycin (1 µg/mL) (Sigma-Aldrich). After a 1-h incubation, the amount of LTC₄ in the culture supernatant was measured using an enzyme immunoassay (EIA). To examine the effects of sulpyrine on LTC₄ secretion, BAL cells (2 × 10⁵/well) were treated with sulpyrine (1 µg/mL) for 1 h before the activation by PMA/ionomycin in RPMI 1640/10% FCS medium in 96-well plates, which were incubated at 37 °C in a 5% CO₂ environment.

Measurement of chemical mediator and cytokines

Concentrations of LTB₄, LTC₄, PGD₂ (Cayman, Ann Arbor, MI, USA) itself, PGE₂ (Oxford Biomedical Research, Oxford, MI, USA), and histamine (SPI Bio, Montigny le Bretonneux, France) in BALF or culture supernatants were assessed by EIA, which had sensitivities >3.9, 7.8, and 2.0 pg/mL, 0.1 ng/mL, and 0.39 nM, respectively. Concen-

trations of IL-4, IL-5, IL-13, and IFN-γ in BALF were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA). The sensitivities of the assays exceeded 7.8, 15.6, 7.8, and 9.4 pg/mL, respectively. The harvested BALF and culture supernatants were stored at -80 °C until subjected to analyses.

Statistical analysis

Data are expressed as the mean ± SD. The statistical significance of differences between groups was examined by one-way analysis of variance with Bonferroni's test. *P*-values of <0.05 were considered to indicate statistical significance.

Results

OVA antigen-induced AHR in LTC₄S-Tg and WT mice

To investigate the role of LTC₄S on the development of antigen-dependent asthma, AHR to inhaled methacholine in LTC₄S-Tg and WT mice 24 h (day 13) and 48 h (day 14) after immunization with OVA/alum was first measured (Fig. 2). On day 13, increases in AHR were observed in both LTC₄S-Tg and WT mice compared with the saline inhalation controls. AHR was clearly augmented in the Tg mice (Penh = 7.8 ± 0.5) compared with the WT mice (Penh = 4.0 ± 0.5) immunized with OVA (*P* < 0.01); however, by day 14, the Penh value of Tg mice had reduced to a level similar to WT. At both measurement time points, the OVA-immunized LTC₄S-Tg and WT mice displayed higher Penh values than the saline-immunized controls.

Cell number of subpopulations in BALF after OVA challenge

To analyse allergic inflammation in airways by OVA challenge, we determined the cell number and types

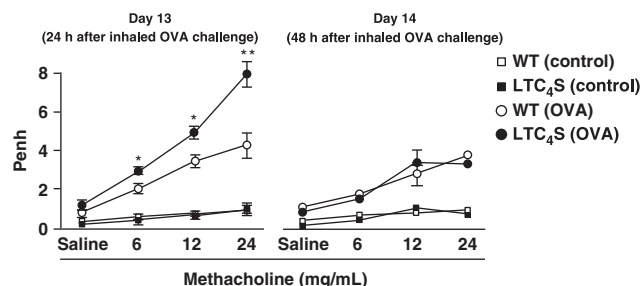


Fig. 2. AHR to inhaled methacholine in LTC₄S-Tg and WT mice after antigen challenge. The AHR in mice immunized with OVA/alum was estimated 24 h (day 13) and 48 h (day 14) after antigen (Tg, closed circle; WT, open circle) or saline as control (Tg, closed square; WT, open square) exposure. Airway resistance was expressed as enhanced pause (Penh) values for each concentration of methacholine (6, 12, and 24 mg/mL) or saline (controls). Data are expressed as the mean ± SD for each group (*n* = 7). **P* < 0.05, ***P* < 0.01 as compared with immunized WT mice after antigen challenge. AHR, airway hyperresponsiveness; WT, wild-type; OVA, ovalbumin; Tg, transgenic.

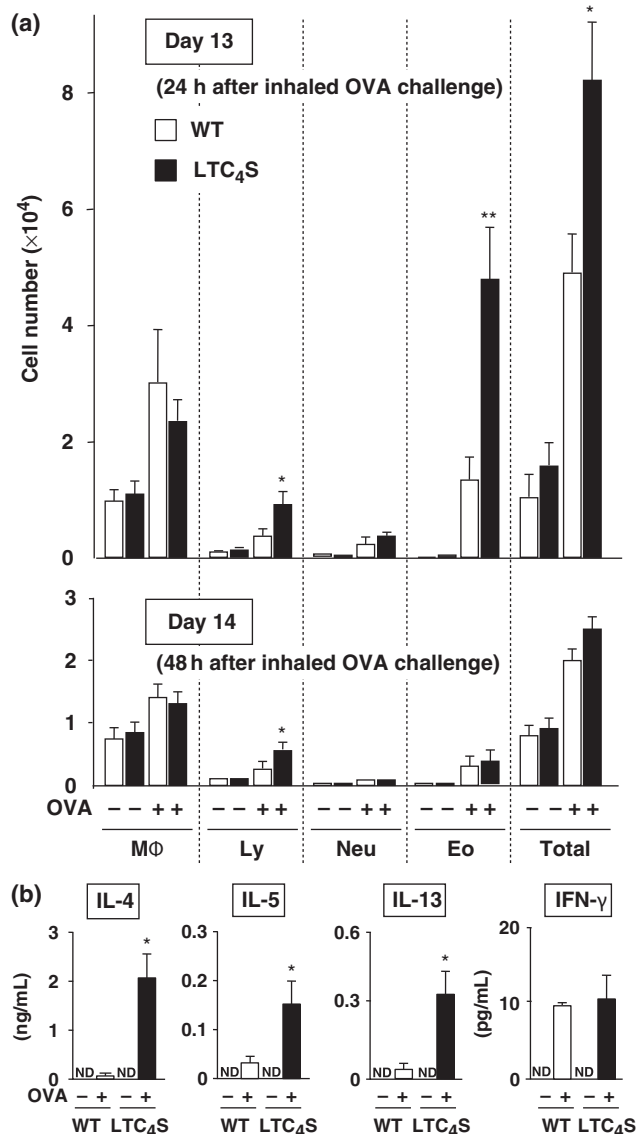


Fig. 3. Cell number and types and concentration of cytokines in BALF samples from LTC₄-Tg and WT mice after antigen challenge. (a) The number of cells were assessed 24 and 48 h after antigen (OVA+) or saline (OVA-) (Tg, closed bar; WT, open bar) exposure. Data are expressed as the mean±SD for each group ($n=7$). * $P<0.05$, ** $P<0.01$ as compared with immunized WT mice after antigen challenge. MΦ, macrophages; Ly, lymphocytes; Neu, neutrophils; Eo, eosinophils. (b) The concentration of Th2 cytokines (IL-4, IL-5, and IL-13) and Th1 cytokine (IFN-γ) in samples were measured 24 and 48 h after mice were immunized with either antigen (OVA+) or saline (OVA-) (Tg, closed bar; WT, open bar). The concentration of cytokines in the supernatants was determined by ELISA. Data are expressed as the mean±SD for each group ($n=7$). * $P<0.05$ as compared with immunized WT mice after antigen challenge. ND, not detected; WT, wild-type; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; Tg, transgenic.

present in the subpopulations in BALF obtained from the Tg and WT mice (Fig. 3a). Twenty-four hours after challenge, the total cell number in BALF was greater in Tg ($8.1\pm0.9\times10^4$) than in WT mice ($4.9\pm0.8\times10^4$)

($P=0.042$). Significantly, the numbers of lymphocytes ($P<0.05$) and eosinophils ($P<0.01$) in Tg mice were approximately two- and threefold greater, respectively, than those in WT mice. The population distribution of other cell types was similar between both types of mice. However, 48 h after the OVA challenge, only the number of lymphocytes in Tg mice ($0.6\pm0.2\times10^4$) was significantly ($P<0.05$) higher than that in WT mice ($0.3\pm0.1\times10^4$). The total cell number in both groups of mice had decreased compared with those 24 h after the OVA challenge, although the cell number in Tg mice ($0.26\pm0.02\times10^5$) remained greater than that in WT mice ($0.21\pm0.01\times10^5$).

Antigen-induced Th2 cytokine production in airways

Because an enhancement of lymphocytosis and eosinophilia was observed in the Tg mice, we next analysed T-helper type 2 (Th2) cytokine production in BALF samples 48 h after OVA challenge (Fig. 3b). Although no differences between the Tg and WT mice were observed in the absence of OVA stimulation, the production of Th2 cytokines (IL-4, -5, and -13) in Tg mice was greater ($P<0.05$) than that in WT mice 48 h after exposure to OVA. The level of IFN-γ production was similar between both groups of mice. These results indicated that LTC₄ was involved in the enhancement of Th2 responses.

LTC₄ secretion by different cell types

To investigate LTC₄ secretion in culture supernatants by neutrophils, eosinophils, T cells, and macrophages

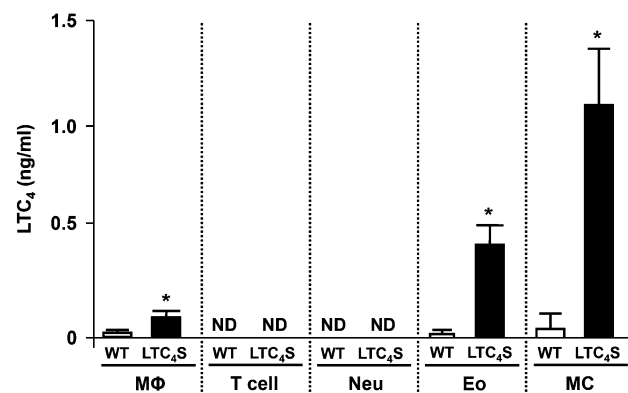


Fig. 4. LTC₄ secretion by inflammatory cells from mice after antigen challenge. Macrophages, T cells, neutrophils, and eosinophils were isolated from BALF, and mast cells were isolated from spleens of mice 48 h after antigen challenge. LTC₄ in culture supernatants of each cell type was measured 1 h after stimulation with PMA and ionomycin. The concentration of LTC₄ in the supernatants was quantified by EIA. Data are expressed as the mean±SD for each group ($n=10$). * $P<0.05$ as compared with WT mice. MΦ, macrophages; Neu, neutrophils; Eo, eosinophils; MC, mast cells; LTC₄, leukotriene C₄; BALF, bronchoalveolar lavage fluid; PMA, phorbol myristate acetate; EIA, enzyme immunoassay.

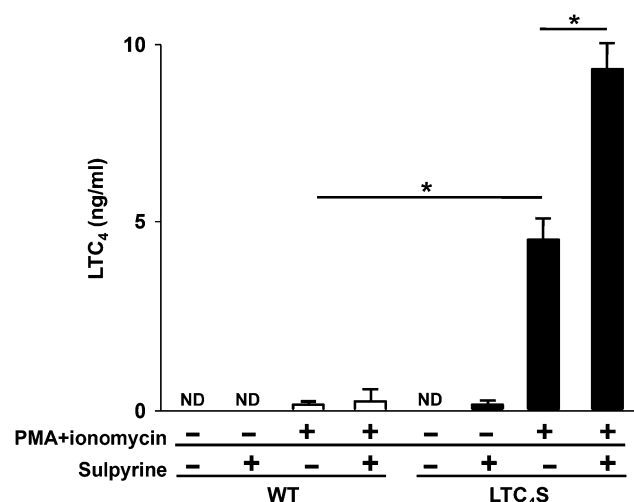


Fig. 5. Effects of sulpyrine on LTC₄ secretion by BAL cells from mice 48 h after antigen challenge. LTC₄ in the culture supernatants of total BAL cells from Tg (closed bar) and WT (open bar) mice was measured after a 1-h stimulation with PMA and ionomycin in the presence or the absence of sulpyrine. Data are expressed as the mean $\pm$ SD for each group ($n=8$). * $P<0.05$ as compared with WT mice or Tg mice in the absence of sulpyrine. LTC₄, leukotriene C₄; BAL, bronchoalveolar lavage; WT, wild-type; PMA, phorbol myristate acetate; Tg, transgenic.

obtained from BALF, in addition to mast cells collected from spleens, each cell type was isolated 48 h after OVA challenge. After the isolated cells were seeded in culture dishes, they were stimulated with PMA and ionomycin for 1 h, and the levels of LTC₄ were then quantified (Fig. 4). The amounts of LTC₄ secreted by mast cells, eosinophils, and macrophages from Tg and WT were 1.12 ± 0.31 vs. 0.11 ± 0.15 ng/mL ($P<0.05$), 0.42 ± 0.12 vs. 0.02 ± 0.03 ng/mL ($P<0.05$), and 0.11 ± 0.02 vs. 0.02 ± 0.01 ng/mL ($P<0.05$), respectively. In contrast, no LTC₄ secretion by neutrophils and T cells was observed in either Tg or WT mice. In addition, LTC₄ secretion was not detected in the culture supernatants of any cell types from both groups of mice without OVA challenge (data not shown).

Effects of sulpyrine on LTC₄ secretion from BAL cells

We also examined the effects of sulpyrine on LTC₄ secretion in culture supernatants by BAL cells isolated from Tg and WT mice 48 h after OVA challenge (Fig. 5). When cells were incubated with sulpyrine alone, a small amount of LTC₄ was only produced by BAL cells from LTC₄S-Tg mice. In contrast, BAL cells stimulated with PMA and ionomycin produced much higher levels of LTC₄, which was secreted by Tg cells (4.5 ± 0.6 ng/mL) at significantly ($P<0.05$) greater quantities than those by WT cells (0.2 ± 0.1 ng/mL). Furthermore, sulpyrine significantly ($P<0.05$) augmented the secretion of LTC₄ by PMA- and ionomycin-stimulated Tg cells (9.1 ± 1.0 ng/mL), but not WT cells (0.3 ± 0.2 ng/mL).

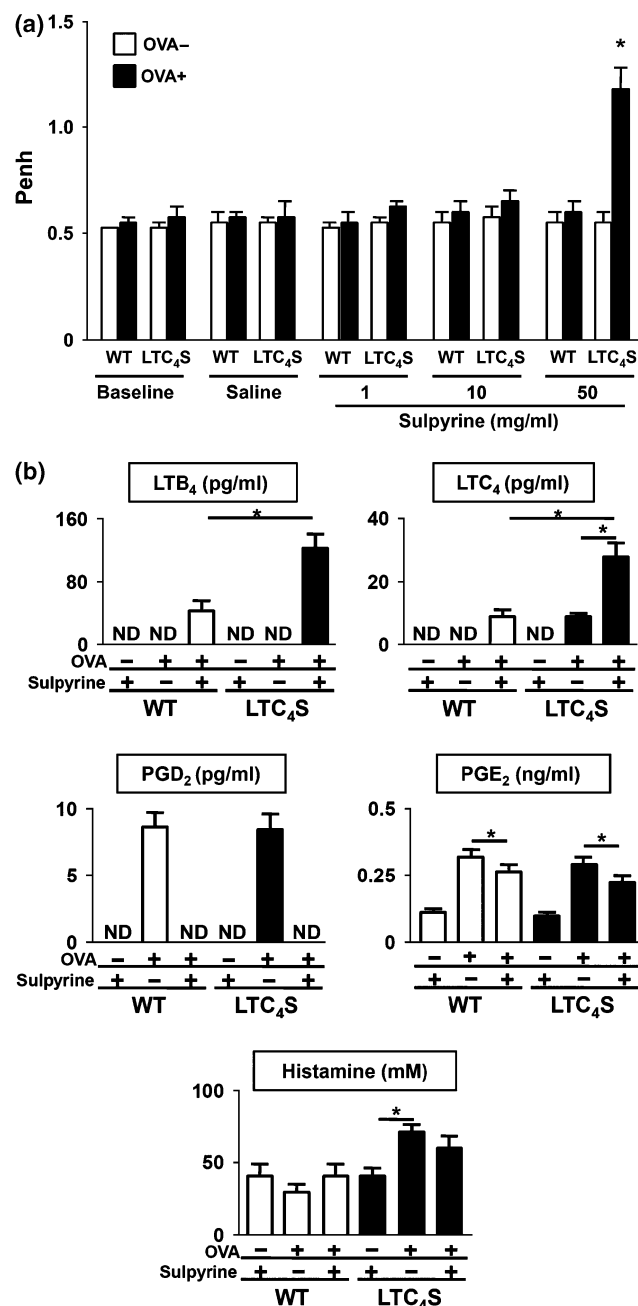


Fig. 6. Effect of sulpyrine on airway response and secretion of chemical mediators in BALF. (a) Airway resistance in Tg and WT mice with or without OVA challenge (OVA+ or OVA-, respectively) was analysed immediately after administration of sulpyrine (1, 10, or 50 mg/mL) or saline. Airway reactivity was expressed as enhanced pause (Penh) values for each concentration of sulpyrine, the saline controls, and baseline. Data are expressed as the mean $\pm$ SD for each group ($n=6$). * $P<0.05$ as compared with WT mice (OVA+). (b) Concentrations of LTB₄, LTC₄, PGD₂, PGE₂, and histamine in BAL fluid were quantified by EIA. BAL fluid was obtained immediately after inhalation of sulpyrine or saline in Tg (closed bar) and WT (open bar) mice 48 h after antigen (OVA+) or saline (OVA-) exposure. Data are expressed as the mean $\pm$ SD for each group ($n=7$). * $P<0.05$ as compared with immunized WT mice in the presence of sulpyrine, or immunized Tg mice in the absence of sulpyrine. BAL, bronchoalveolar lavage; WT, wild-type; OVA, ovalbumin; Tg, transgenic; EIA, enzyme immunoassay.

Effect of sulpyrine on airway response and secretion of chemical mediators in BALF

To investigate the effect of COX pathway inhibition on the development of AIA, we measured airway resistance in Tg and WT mice immediately after inhalation of sulpyrine 48 h post-OVA challenge (Fig. 6a). Airway resistance in Tg and WT mice without OVA challenge (control) was unchanged by exposure to sulpyrine, whereas only OVA-challenged Tg mice demonstrated a significant ($P < 0.05$) increase in airway resistance, from 0.52 ± 0.01 to 1.21 ± 0.22 (Penh values), by sulpyrine treatment at a concentration of 50 mg/mL. Subsequently, we analysed the secretion of LTC₄ and several other chemical mediators, such as LTB₄, LTC₄, PGD₂, PGE₂, and histamine in BALF obtained from Tg and WT mice immediately after the last inhalation of sulpyrine (Fig. 6b). Tg and WT mice showed increases in PGD₂ and PGE₂ after challenge with OVA, which diminished or decreased significantly ($P < 0.05$) when mice were pre-treated with sulpyrine or not challenged with OVA. LTB₄ was not detected in Tg and WT mice after provocation with sulpyrine or OVA, whereas sulpyrine-induced LTB₄ secretion in both Tg (125.4 ± 25.3 pg/mL) and WT (40.4 ± 20.3 pg/mL) mice post-OVA challenge ($P < 0.05$). LTC₄ secretion was detected in WT mice (8.5 ± 2.3 pg/mL) only when they were administered sulpyrine after OVA provocation. In contrast, Tg mice demonstrated OVA-induced LTC₄ secretion (8.4 ± 1.3 pg/mL), which was further ($P < 0.05$) augmented by threefold (27.5 ± 3.3 pg/mL) after the administration of sulpyrine, although LTC₄ was not detected by sulpyrine treatment alone. The levels of LTB₄ ($P < 0.05$) and LTC₄ ($P < 0.05$) after sulpyrine administration in Tg mice were both threefold higher than those in WT mice. We also observed an increase ($P < 0.05$) in histamine in Tg, but not WT, mice after challenge with OVA; however, sulpyrine had no effect on histamine secretion in Tg mice.

Effect of antagonist to the cys-LT1 receptor on sulpyrine-induced airway response

Finally, to evaluate the role of cys-LTs in AIA, we examined the effects of pranlukast hydrate, an antagonist of the cys-LT1 receptor, on the sulpyrine-induced airway response in Tg and WT mice 48 h post-OVA challenge (Fig. 7). Although exposure to pranlukast hydrate had no effect on airway resistance in WT mice, regardless of the administration of sulpyrine, the AIA response was ameliorated in Tg mice, as shown by the reduction of airway resistance from 1.21 ± 0.22 to 0.62 ± 0.18 (Penh values) ($P < 0.05$).

Discussion

We demonstrated that in LTC₄S-Tg mice, mast cells, eosinophils, and macrophages, but not neutrophils or T

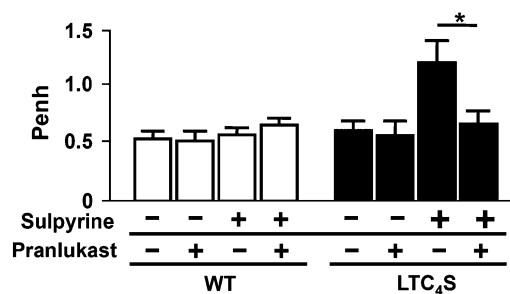


Fig. 7. Effect of antagonist to the cys-LT1 receptor on sulpyrine-induced airway responses. Forty-eight hours after antigen challenge, Tg (closed bar) and WT (open bar) mice were orally administered pranlukast hydrate (25 mg/kg body weight) or lactose (control), and 1 h later, were administered either sulpyrine (50 mg/mL) or saline using a nebulizer for 30 min. Airway resistance was measured immediately after the final inhalation. Airway reactivity was expressed as enhanced pause (Penh) values for the dosage of pranlukast hydrate or lactose with or without the administration of sulpyrine. Data are expressed as the mean $\pm$ SD for each group ($n = 6$). * $P < 0.05$ as compared with LTC₄S-Tg administered sulpyrine in the absence of pranlukast hydrate. WT, wild-type; cys-LT1; cysteinyl-leukotrienes 1; Tg, transgenic.

cells, produced excess amounts of LTC₄ in immunized mice after antigen exposure, albeit under control of the CMV enhancer/chicken β -actin promoter in the transgenic vector pCAGGS. Although the former three cell types are known to synthesize cys-LTCs, including LTC₄ [31], the over-expression of LTC₄ in LTC₄S-Tg mice make them a useful model for the study of AIA. However, these Tg cells did not secrete LTC₄ in immunized Tg mice without antigen-challenge (data not shown), which indicates that LTC₄ secretion requires not only sufficient levels of LTC₄S but also activation of a certain cell-specific pathway for its biosynthesis and subsequent export. Although we did not investigate LTC₄ production by pulmonary endothelial cells, which are also a source of LTC₄, these cells in Tg mice likely produce a large amount of cys-LTs [32, 33]. The key immunopathological features involving eicosanoid pathways in human AIA are firstly, elevated LTC₄ production, even in the absence of exposure to NSAIDs; secondly, NSAIDs inhibit prostanoids in all subjects, but an increase in LTC₄ production is only observed in AIA subjects; thirdly, cys-LT antagonists block most of the bronchoconstriction that results in the AIA subjects; and fourthly, excess eosinophils and eosinophilopoietic cytokines. The study by Cowburn et al. [20] postulated that these features may be explained by the increased LTC₄S expression in AIA bronchial biopsies, which was associated with elevated LTC₄ production, correlated with airway NSAID responsiveness, and accompanied by eosinophilia and IL-5 expression. The results shown in this study replicate these findings clearly in LTC₄S transgenic mice, and suggest that an over-expression of LTC₄S, perhaps driven by a genetic anomaly and exacerbated by positive feedback from Th2 cytokines, is the primary aetiological factor in human AIA.

LTC₄ functions to accelerate development of the Th2 response and it has been suggested that dendritic cell/T cell interaction is modulated bifunctionally by cys-LTs, including LTC₄ [34–38]. In addition, AIA is associated with an increased expression of the cys-LT1 receptor [39]. And the intracrine cys-LT receptor plays a role in the Th2 response by mediating the signalling of eosinophil vesicular transport-mediated IL-4 secretion [40]. Here, we observed that Th2-type airway inflammation, which was characterized by eosinophilia, profound Th2 cytokine production, and increased AHR, was augmented in immunized LTC₄S-Tg mice 24 h after antigen exposure. Although the detailed mechanism is unclear, it is known that Th2-type cytokines, such as IL-4 and IL-5, potentiate LTC₄ synthesis by basophils, eosinophils, and mast cells [41–43]. Thus, in the Tg mice, the high levels of LTC₄ might serve to accelerate Th2 cytokine production, which in turn augments the biosynthesis and secretion of LTC₄ by the expression of the endogenous and/or exogenous LTC₄S gene after immunization and challenge with antigen.

As most patients with AIA are non-atopic, our murine model, which is associated with antigen-dependent inflammation, appears to be inappropriate for the examination of the development of AIA. However, chronic and persistent inflammation with increased numbers of mast cells and eosinophils has been observed in the bronchial mucosa of AIA patients with concomitant increases in nasal and oral exhaled IL-4 [44–47]. In addition, the bronchial mucosa of patients with AIA also exhibits elevated expression and enhanced local production of IL-5 [48], which is the key regulator of eosinophil maturation and function. Therefore, the murine AIA model developed here is suitable for analysing AIA with respect to eosinophilic inflammation associated with increased Th2 cytokine production, regardless of its induction mechanism. The enhancement of LTC₄ secretion in Tg mice was also demonstrated upon treatment with sulpyrine, which was further induced in airways (*in vivo*) by pre-challenge with antigen and in BAL cells (*ex vivo*) by stimulation with PMA/ionomycin. These results indicate that LTC₄ biosynthesis in activated cells is basically dependent on the level of LTC₄S, which might be amplified by allergic inflammatory factors, such as Th2 cytokines, and a sulpyrine-induced balance shift towards the 5-LO pathway in the AA metabolic cascade.

Interestingly, in addition to LTC₄, the amplified secretion of LTs was also demonstrated for LTB₄ synthesis in mice, particularly the LTC₄S-Tg mice. Recently, it has been reported that LTB₄ secretion is also increased in patients with AIA [49]. Although the detailed mechanisms in patients with AIA and Tg mice have yet to be clarified, we speculate that the induction of LTA₄ hydrolyase by IL-4 and IL-13 may augment the secretion of LTB₄ [50].

Notably, the AIA response in Tg mice was observed by sulpyrine inhalation 48 h after antigen exposure when it was confirmed before the sulpyrine treatment that AHR and cell population distribution, including eosinophils, in Tg mice had reduced to the identical or similar level to those in WT mice. Therefore, the AIA response that developed in Tg mice might not be related to the preceding OVA-induced airway response, although LTC₄-producing cells require priming with specific factors, such as Th2 cytokines. Taking into consideration the emergence of an AIA airway response that is distinct from the antigen-induced response, the pathogenesis of the AIA mouse model appears to mimic that of human AIA, which represents a type of non-allergic asthma. In LTC₄S-Tg mice, the sulpyrine-induced airway response was completely abolished by an antagonist to the cys-LT1 receptor. Thus, this result supports our speculation that LTC₄ is a major player in AIA, and clearly demonstrates that the observed balance shift towards the 5-LO pathway in metabolism of AA contributes to the pathogenesis of AIA. However, as LTC₄ was also secreted *in vivo* by WT mice following administration of sulpyrine after antigen exposure, albeit in lower amounts than that found in Tg mice, it appears that the level of LTC₄ must exceed a certain threshold level for the development of AIA. This speculation is supported by a report that LTs were present in high concentrations in urine at baseline, and were further increased after an ingestion of aspirin in patients with AIA [13, 51]. It has been reported that the number of activated eosinophils that are immunoreactive for LTC₄S are elevated in the bronchial mucosa of AIA patients compared with that from ATA patients [20]. Furthermore, the over-expression of LTC₄S is strongly correlated with elevated levels of cys-LTs in BALF from AIA patients in a stable state and with AHR to inhaled aspirin. In addition, over-expression of LTC₄S has also been described in the nasal airways of aspirin-intolerant patients, suggesting a systemic anomaly of LTC₄S expression associated with NSAIDs intolerance [20, 52]. Taken together, these reports suggest that the increase of LTC₄S in inflammatory cells of human airways in a stable state may be a hallmark of AIA in that cys-LTs is chronically over-produced, even in the absence of NSAID ingestion, as observed in BAL fluid, sputum, and urine in the patients. In conclusion, by generating and examining LTC₄S-Tg mice, we demonstrated for the first time that AIA can be induced in the presence of elevated levels of cys-LTs. This induction resulted from persistent airway inflammation characterized by eosinophilic inflammation associated with an environment enriched in Th2 cytokines, independently of the presence of antigen-induced airway obstruction. Their data showed that increased LTC₄S is itself sufficient to drive increased LTC₄ production chronically, and also acutely after NSAIDs exposure. This is the first study to show that LTC₄ synthase transgenic mice replicate the key features of human AIA.

Clinical relevance

By generating and examining LTC₄ synthase-transgenic mice, we demonstrated for the first time that AIA can be induced in the presence of elevated levels of cys-LTs owing to persistent airway inflammation characterized by eosinophilic inflammation associated with an environment enriched in Th2 cytokines, independently of the presence of antigen-induced airway obstruction. Our study supports that cys-LTs play a major

role in the pathogenesis of AIA in patients with chronic asthma.

Acknowledgements

We thank Mrs Kazumi Okazaki, Mrs Takako Takako, Miss Yuko Kotemori, Mr Takashi Namatame, Mr Masahide Oyama, and Mr Hiromichi Kaneko for skillful technical assistance.

References

- Cowburn AS, Holgate ST, Sampson AP. IL-5 increases expression of 5-lipoxygenase-activating protein and translocates 5-lipoxygenase to the nucleus in human blood eosinophils. *J Immunol* 1999; **163**:456–65.
- Sjöström M, Jakobsson PJ, Juremalm M *et al.* Human mast cells express two leukotriene C₄ synthase isoenzymes and the CysLT₁ receptor. *Biochim Biophys Acta* 2002; **1583**:53–62.
- Voelkel NF, Tudor RM, Wade K *et al.* Inhibition of 5-lipoxygenase-activating protein (FLAP) reduces pulmonary vascular reactivity and pulmonary hypertension in hypoxic rats. *J Clin Invest* 1996; **97**:2491–8.
- Ford-Hutchinson AW, Gresser M, Young RN. 5-Lipoxygenase. *Annu Rev Biochem* 1994; **63**:383–417.
- Martinez Molina D, Wetterholm A, Kohl A *et al.* Structural basis for synthesis of inflammatory mediators by human leukotriene C₄ synthase. *Nature* 2007; **448**:613–6.
- Maekawa A, Kanaoka Y, Xing W *et al.* Functional recognition of a distinct receptor preferential for leukotriene E₄ in mice lacking the cysteinyl leukotriene. *Proc Natl Acad Sci USA* 2008; **105**:16695–700.
- Montuschi P, Sala A, Dahlén SE *et al.* Pharmacological modulation of the leukotriene pathway in allergic airway disease. *Drug Discov Today* 2007; **12**:404–12.
- Montuschi P. Leukotrienes, antileukotrienes and asthma. *Mini Rev Med Chem* 2008; **8**:647–56.
- Lee KS, Kim SR, Park HS *et al.* Cysteinyl leukotriene upregulates IL-11 expression in allergic airway disease of mice. *J Allergy Clin Immunol* 2007; **119**:141–9.
- Holgate ST, Peters-Golden M., Panettieri RA *et al.* Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling. *J Allergy Clin Immunol* 2003; **111** (Suppl.): S18–34; discussion S34–6.
- Busse WW. Leukotrienes and inflammation. *Am J Respir Crit Care Med* 1998; **157** (Part 2):S210–3; discussion S247–8.
- Lee KS, Kim SR, Park HS *et al.* Cysteinyl leukotriene receptor antagonist regulates vascular permeability by reducing vascular endothelial growth factor expression. *J Allergy Clin Immunol* 2004; **114**:1093–9.
- Szczeklik A. Mechanism of aspirin-induced asthma. *Allergy* 1997; **52**:613–9.
- Dahlén B, Nizankowska E, Szczeklik A *et al.* Benefits from adding the 5-lipoxygenase inhibitor zileuton to conventional therapy in aspirin-intolerant asthmatics. *Am J Respir Crit Care Med* 1998; **157** (Part 1):1187–94.
- Szczeklik A, Stevenson DD. Aspirin-induced asthma: advances in pathogenesis and management. *J Allergy Clin Immunol* 1999; **104**:5–13.
- Samter M, Beers RF Jr Intolerance to aspirin. Clinical studies and consideration of its pathogenesis. *Ann Intern Med* 1968; **68**:975–83.
- Kumlin M, Dahlén B, Björck T *et al.* Urinary excretion of leukotriene E₄ and 11-dehydro-thromboxane B₂ in response to bronchial provocations with allergen, aspirin, leukotriene D₄, and histamine in asthmatics. *Am Rev Respir Dis* 1992; **146**:96–103.
- Ortolani C, Mirone C, Fontana A *et al.* Study of mediators of anaphylaxis in nasal wash fluids after aspirin and sodium metabisulfite nasal provocation in intolerant rhinitic patients. *Ann Allergy* 1987; **59** (Part 2):106–12.
- Kowalski ML, Sliwiska-Kowalska M, Igarashi Y *et al.* Nasal secretions in response to acetylsalicylic acid. *J Allergy Clin Immunol* 1993; **91**:580–98.
- Cowburn AS, Sladek K, Soja J *et al.* Overexpression of leukotriene C₄ synthase in bronchial biopsies from patients with aspirin-intolerant asthma. *J Clin Invest* 1998; **101**:834–46.
- Sanak M, Pierzchalska M, Bazan-Socha S *et al.* Enhanced expression of the leukotriene C₄ synthase due to overactive transcription of an allelic variant associated with aspirin-intolerant asthma. *Am J Respir Cell Mol Biol* 2000; **23**:290–6.
- Jinnai N, Sakagami T, Sekigawa T *et al.* Polymorphisms in the prostaglandin E₂ receptor subtype 2 gene confer susceptibility to aspirin-intolerant asthma: a candidate gene approach. *Hum Mol Genet* 2004; **13**:3203–17.
- Sakagami T, Jinnai N, Nakajima T *et al.* ADAM33 polymorphisms are associated with aspirin-intolerant asthma in the Japanese population. *J Hum Genet* 2007; **52**:66–72.
- Palikhe NS, Kim SH, Cho BY *et al.* Association of three sets of high-affinity IgE receptor (FcεR1) polymorphisms with aspirin-intolerant asthma. *Respir Med* 2008; **102**:1132–9.
- Palikhe NS, Kim SH, Cho BY *et al.* IL-13 Gene polymorphisms are associated with rhinosinusitis and eosinophilic inflammation in aspirin intolerant asthma. *Allergy Asthma Immunol Res* 2010; **2**:134–40.
- Montuschi P, Peters-Golden ML. Leukotriene modifiers for asthma treatment. *Clin Exp Allergy* 2010; **40**:1732–41.
- Hamad AM, Sutcliffe AM, Knox AJ. Aspirin-induced asthma: clinical aspects, pathogenesis and management. *Drugs* 2004; **64**:2417–32.
- Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991; **108**:193–9.
- Honda K, Arima M, Cheng G *et al.* Prostaglandin D₂ reinforces Th2 type inflammatory responses of airways to low-dose antigen through bronchial ex-

- pression of macrophage-derived chemokine. *J Exp Med* 2003; **198**:533–43.
- 30 Cheng G, Arima M, Honda K *et al*. Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. *Am J Respir Crit Care Med* 2002; **166**:409–16.
 - 31 Peters-Golden M, Henderson WR Jr Leukotrienes. *N Engl J Med* 2007; **357**: 1841–54.
 - 32 Folco G, Murphy RC. Eicosanoid transcellular biosynthesis: from cell-cell interactions to in vivo tissue responses. *Pharmacol Rev* 2006; **58**:375–88.
 - 33 Feinmark SJ, Cannon PJ. Endothelial cell leukotriene C4 synthesis results from intercellular transfer of leukotriene A4 synthesized by polymorphonuclear leukocytes. *J Biol Chem* 1986; **261**:16466–72.
 - 34 Machida I, Matsuse H, Kondo Y *et al*. Cysteinyl leukotrienes regulate dendritic cell functions in a murine model of asthma. *J Immunol* 2004; **172**:1833–8.
 - 35 Kim DC, Hsu FI, Barrett NA *et al*. Cysteinyl leukotrienes regulate Th2 cell-dependent pulmonary inflammation. *J Immunol* 2006; **176**:4440–8.
 - 36 Robbani DF, Finch RA, Jäger D *et al*. The leukotriene C4 transporter MRP1 regulates CCL19 (MIP-3b, ELC)-dependent mobilization of dendritic cells to lymph nodes. *Cell* 2000; **103**:757–68.
 - 37 Okunishi K., Dohi M., Nakagome K *et al*. A novel role of cysteinyl leukotrienes to promote dendritic cell activation in the antigen-induced immune responses in the lung. *J Immunol* 2004; **173**:6393–402.
 - 38 Parameswaran K, Liang H, Fanat A *et al*. Role for cysteinyl leukotrienes in allergen induced change in circulating dendritic cell number in asthma. *J Allergy Clin Immunol* 2004; **114**:73–9.
 - 39 Sousa AR, Parikh A, Scadding G *et al*. Leukotriene-receptor expression on nasal mucosal inflammatory cells in aspirin-sensitive rhinosinusitis. *N Engl J Med* 2002; **347**:1493–9.
 - 40 Bandeira-Melo C, Woods LJ, Phoofolo M *et al*. Intracrine cysteinyl leukotriene receptor-mediated signaling of eosinophil vesicular transport-mediated interleukin-4 secretion. *J Exp Med* 2002; **196**:841–50.
 - 41 Muñoz NM, van Seventer GA, Semnani RT *et al*. Augmentation of LTC₄ synthesis in human eosinophils caused by CD3-stimulated Th2-like cells in vitro. *Am J Physiol Lung Cell Mol Physiol* 2000; **278**:L1172–9.
 - 42 Sarmiento EU, Espiritu BR., Gleich GJ *et al*. IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor potentiate basophil mediator release stimulated by eosinophil granule major basic protein. *J Immunol* 1995; **155**:2211–21.
 - 43 Hsieh FH, Lam BK, Penrose JF *et al*. T helper cell type 2 cytokines coordinately regulate immunoglobulin E-dependent cysteinyl leukotriene production by human cord blood-derived mast cells profound induction of leukotriene C₄ synthase expression by interleukin 4. *J Exp Med* 2001; **193**:123–34.
 - 44 Babu KS, Salvi SS. Aspirin and Asthma. *CHEST* 2000; **118**:1470–6.
 - 45 Vaszar LT, Stevenson DD. Aspirin-induced asthma. *Clin Rev Allergy Immunol* 2001; **21**:71–87.
 - 46 Bochenek G., Bánska K, Szabó Z *et al*. Diagnosis, prevention and treatment of aspirin-induced asthma and rhinitis. *Curr Drug Targets Inflamm Allergy* 2002; **1**:1–11.
 - 47 Carpagnano GE, Resta O, Gelardi M *et al*. Exhaled inflammatory markers in aspirin-induced asthma syndrome. *Am J Rhinol* 2007; **21**:542–7.
 - 48 Higashi N, Mita H, Ono E *et al*. Profile of eicosanoid generation in aspirin-intolerant asthma and anaphylaxis assessed by new biomarkers. *J Allergy Clin Immunol* 2010; **125**:1084–91.
 - 49 Mita H, Higashi N, Taniguchi M *et al*. Increase in urinary leukotriene B₄ glucuronide concentration in patients with aspirin-intolerant asthma after intravenous aspirin challenge. *Clin Exp Allergy* 2004; **34**:1262–9.
 - 50 Zaitis M, Hamasaki Y, Matsuo M *et al*. New induction of leukotriene A₄ hydroxylase by interleukin-4 and interleukin-13 in human polymorphonuclear leukocytes. *Blood* 2000; **96**:601–9.
 - 51 Christie PE, Tagari P, Ford-Hutchinson AW *et al*. Urinary leukotriene E₄ concentrations increase after aspirin challenge in aspirin-sensitive asthmatic subjects. *Am Rev Respir Dis* 1991; **143** (Part 1): 1025–9.
 - 52 Adamjee J, Suh YJ, Park HS *et al*. Expression of 5-lipoxygenase and cyclooxygenase pathway enzymes in nasal polyps of patients with aspirin-intolerant asthma. *J Pathol* 2006; **209**:392–9.