Overexpression of the leukotriene C_4 synthase gene in mice reproduces human aspirin-induced asthma

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Abstract

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-lopoxygenase (5-LO) metabolites, particularly leukotriene C_4 (LTC_4) which is a highly potent bronchial constrictor. However, the role of LTC_4 in the development of AIA has yet to be conclusively demonstrated. To elucidate the effect of LTC4 on the development of AIA, we generated LTC_4 synthase (*Ltc4s*) gene transgenic (LTC_4S-Tg) mice. In contrast to wild-type (WT) mice, LTC_4S-Tg mice displayed NSAID-induced airway obstruction that was associated with increases of LTC_4 and Th2 cytokines in lung tissue. This is the first study to demonstrate clearly that a balance shift towards the 5-LO pathway contributes to the pathogenesis of AIA in the presence of elevated levels of LTC_4S.

The arachidonic acid (AA) cascade is divided into two major pathways: the cyclooxygenase (COX) pathway, which yields prostanoids (prostagrandins (PGs) and thromboxane A_2), and the lipoxygenase (LO) pathway, which produces leukotrienes (LTs). The sequential catalytic action of 5-lipoxygenase (LO), which is only expressed in eosinophils, basophils, mast cells, macrophages, platelets, and endothelial cells, on AA generates LTA_4, which is either hydroxylated to LTB_4 or metabolized into the first of the cysteinyl (cys) LTs, LTC_4, by the action of LTC_4 synthase (LTC_4S)[1-5]. LTC_4 is further converted to LTD_4 in the extracellular space, which is in turn cleaved to form LTE_4[6]. It is known that cys-LTs play important roles in the pathogenesis of asthma, such as airway remodeling that is characterized by contraction and hyperplasia of bronchial smooth muscle, enhancement of secretion from the bronchial gland, increased vascular permeability, and the recruitment of eosinophils[7-10].

Recently, the relevance of cys-LTs in the development of aspirin-induced asthma (AIA) has emerged from clinical studies[11,12]. 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)[13,14]. The pathogenesis of AIA is associated with alterations in arachidonate metabolic pathways[15-17]. Through the inhibition of the COX pathway, aspirin/NSAIDs divert AA metabolites to the LO pathway, resulting in a decrease in PGE_2, anti-inflammatory PG, and an increase in the biosynthesis of cys-LTs[15-17]. In patients with AIA, an increased activity of LTC_4S in bronchial mucosa has been observed, which is supported by a study that suggested a biallelic polymorphism in the promoter region of the LTC_4S gene enhanced its

transcription[18,19]. Although several underlying genetic mechanisms of AIA have been proposed based on the identification of candidate genes other than LTC_4S, to date, it appears that genetic influences on AIA are subtle[20-23]. Nevertheless, LT-modifying drugs are effective in blocking the bronchoconstriction provoked by aspirin and are typically used in the treatment of AIA[24]. Therefore, aspirin/NSAIDs may shift the balance of the 5-LO pathway to augment airway inflammation and obstruction by the over-production of cys-LTs, including LTC_4. However, it remains unclear in humans how cys-LTs are involved in the pathogenesis of AIA. Since LTC_4S is the critical terminal enzyme for cys-LT biosynthesis in the 5-LO/LTC_4S pathway, we generated LTC_4S (*Ltc4s*) gene transgenic (LTC_4S-Tg) mice to examine the functions of this synthase in vivo. In this study, we have clearly demonstrated for the first time that a shift in the balance towards the 5-LO pathway contributes to the pathogenesis of AIA in the presence of excess amounts of LTC_4S.

RESULTS

OVA antigen-induced AHR in LTC_4S-Tg and WT mice

To investigate a role of LTC_4S on the development of antigen-dependent asthma, the airway hyper-reactivity (AHR) to inhaled methacholine in LTC_4S-Tg and WT mice 24 h (day 13) and 48 h (day 14) after immunization with OVA/alum was first measured (**Fig. 1**). On day 13, increases in AHR were observed in both LTC_4S-Tg and WT mice compared to the saline inhalation controls. The AHR was clearly augmented in the Tg

mice (Penh = 7.8 ± 0.5) than the WT mice (Penh = 4.0 ± 0.5) immunized with OVA; 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 LTC_4S-Tg and WT mice displayed higher Penh values than the saline-immunized controls.

Cell number of subpopulations in BALF after OVA challenge

To analyze allergic inflammation in airways by OVA challenge, we determined the cell number and types present in the subpopulations in BALF obtained from the Tg and WT mice (**Fig. 2a**). Twenty-four hours after the OVA challenge, the total cell number in BALF was greater in Tg ($8.1 \pm 0.9 \times 10^4$) than in WT mice ($4.9 \pm 0.8 \times 10^4$). Significantly, the cell numbers of lymphocytes and eosinophils in the Tg mice were approximately 2- and 3-fold 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 the Tg mice ($0.6 \pm 0.2 \times 10^4$) was significantly higher than that in WT mice ($0.3 \pm 0.1 \times 10^4$). The total cell number in both groups of mice has decreased compared with those 24 h after the OVA challenge, although the cell number in the Tg mice ($0.26 \pm 0.02 \times 10^5$) remained greater than that in WT mice ($0.21 \pm 0.01 \times 10^5$).

Antigen-induced Th2 cytokine production in airways

Since an enhancement of lympocytosis and eosinophilia was observed in the Tg mice, we next analyzed Th2 cytokine production in the BALF samples 48 h after OVA

challenge (**Fig. 2b**). Although no differences between the Tg and WT mice were observed, the production of Th2 cytokines (IL-4, -5, -13) in Tg mice was greater than that in WT mice. The level of IFN-γ production was similar between both groups of mice. These results indicated that LTC_4 was involved in the enhancement of Th2 responses.

LTC_4 secretion by different cell types

To investigate LTC_4 secretion in culture supernatants by neutrophils, eosinophils, T cells, and macrophages from BALF, in addition to mast cells 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 LTC_4 were quantified (**Fig. 3**). The amounts of LTC_4 secreted by mast cells, eosinophils, and macrophages from Tg and WT were 1.12 ± 0.31 vs 0.11 ± 0.15 ng/ml, 0.42 ± 0.12 vs 0.02 ± 0.03 ng/ml, and 0.11 ± 0.02 vs 0.02 ±0.01 ng/ml, respectively. In contrast, no LTC_4 secretion by neutrophils and T cells was observed in either Tg or WT mice. In addition, LTC_4 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 LTC_4 secretion from BAL cells

We also examined the effects of sulpyrine on LTC_4 secretion in culture supernatants by the total BAL cells isolated from Tg and WT mice 48 h after OVA challenge (**Fig. 4**). When cells were incubated with sulpyrine alone, a small amount of LTC_4 was only

produced by BAL cells from LTC_4S-Tg mice. In contrast, BAL cells stimulated with PMA and ionomycin produced much higher levels of LTC_4, which was secreted by Tg cells (4.5 ± 0.6 ng/ml) at significantly greater quantities than those by WT cells (0.2 ± 0.1 ng/ml). Furthermore, sulpyrine significantly augmented the secretion of LTC_4 by PMA- and ionomycin-stimulated Tg cells (9.1 ± 1.0 ng/ml), but not WT cells (0.3 ± 0.2 ng/ml).

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. 5a**). 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 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 analyzed the secretion of LTC_4 and several other chemical mediators, such as LTB_4, LTC_4, PGD_2, PGE_2, and histamine in BALF obtained from Tg and WT mice immediately after the last inhalation of sulpyrine (**Fig. 5b**). Tg and WT mice showed increases in PGD_2 and PGE_2 after challenge with OVA, which diminished or decreased significantly when mice were pre-treated with sulpyrine or not challenged with OVA. LTB_4 was not detected in Tg and WT mice after provocation with sulpyrine or OVA, whereas sulpyrine induced LTB_4 secretion in both Tg (125.4 ± 25.3 pg/ml) and

WT (40.4 ± 20.3 pg/ml) mice post-OVA challenge. LTC_4 secretion was detected in WT mice (8.5 ± 2.3 pg/ml) only when they were administrated with sulpyrine after OVA provocation. In contrast, Tg mice demonstrated OVA-induced LTC_4 secretion (8.4 ± 1.3 pg/ml), which was further augmented by three-fold (27.5 ± 3.3 pg/ml) after the administration of sulpyrine, although LTC_4 was not detected by sulpyrine treatment alone. The levels of LTB_4 and LTC_4 after sulpyrine administration in Tg mice were both 3-fold higher than those in WT mice. We also observed an increase in histamine in Tg, but not WT, mice after challenge with OVA; however, sulpyrine had no effect on the histamine secretion in Tg mice.

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

To investigate 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. 6**). 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).

DISCUSSION

We demonstrated that in LTC_4S-Tg mice, mast cells, eosinophils, and macrophages, but not neutrophils or T cells, produced excess amounts of LTC_4 in immunized mice after antigen exposure, albeit under control of the CMV enhancer/chicken ß-actin promoter in the transgenic vector pCAGGS. Although the former three cell-types are known to synthesize cys-LTCs, including LTC_4[25], the overexpression of LTC_4 in LTC_4S-Tg mice make them a useful model for the study of AIA. However, these Tg cells did not secrete LTC_4 in the immunized Tg mice without antigen-challenge (data not shown), which indicates that LTC_4 secretion requires not only sufficient levels of LTC_4S, but also activation of a certain cell-specific pathway for its biosynthesis and subsequent export. Although we did not investigate LTC_4 production by pulmonary endothelial cells, which are also a source of LTC_4, these cells in Tg mice likely produce a large amount of cys-LTs[26,27]. LTC_4 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 LTC_4[28-32]. In addition, the intracrine cys-LT receptor plays a role in the Th2 response by mediating the signaling of eosinophil vesicular transport–mediated IL-4 secretion[33]. Here, we observed that Th2-type airway inflammation, which was characterized by eosinophilia, profound Th2 cytokine production, and increased AHR, was augmented in immunized LTC_4S-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 LTC_4 synthesis by basophils, eosinophils, and mast cells[34-36].

Thus, in the Tg mice, the high levels of LTC_4 might serve to accelerate Th2 cytokine production, which in turn augments the biosynthesis and secretion of LTC_4 by expression of the endogenous and/or exogenous LTC_4S gene after immunization and challenge with antigen. Th2-type cytokines stimulate LTC_4 synthesis, LTC_4 might serve to accelerate Th2 cytokine production, and Th2 cytokines then augment the biosynthesis and secretion of LTC_4.

As most patients with AIA are non-atopic, in terms of antigen-dependent inflammation for the development of AIA, our murine model which is associated with antigen-dependent inflammation appears inappropriate for the examination of the development of AIA. However, chronic and persistent inflammation with increased numbers of mast cells and eosinophils have been observed in the bronchial mucosa of AIA patients with concomitant increases in nasal and oral exhaled IL-4[37-40]. In addition, the bronchial mucosa of patients with AIA also exhibits elevated expression and enhanced local production of IL-5[41], which is the key regulator of eosinophil maturation and function. Therefore, the murine AIA model developed here is suitable for analyzing AIA with respected to eosinophilic inflammation associated with increased Th2 cytokine production, regardless of its induction mechanism. The enhancement of LTC_4 secretion in the 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 LTC_4 biosynthesis in activated cells is basically dependent on the level of LTC_4S, 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. The occurrence of such a balance shift was supported by findings that the synthesis of PGD_2 and PGE_2 was down-regulated in vivo after the treatment with sulpyrine. Decreased PGE_2 levels are also typically observed in patients with AIA, suggesting that this anti-inflammatory PG may be involved in the pathogenesis of AIA. In contrast, there is relatively little evidence for the role of PGD_2 in this disorder; however, Higashi et al have reported that the level of PGD_2 increases in patients with AIA as compared to patients with aspirin-tolerant asthma (ATA), although other studies have not identified differences in PGD_2 between AIA and ATA patients[42]. The decrease in PGD_2 levels observed in this study may reflect the pharmacological action of sulpyrine on the AA metabolic pathway, which is independent of the actual pathogenesis of human AIA, in which the role of PGD_2 remains uncertain. Nevertheless, the results presented here suggest that the action of LTC_4 on airway obstruction is robust in the AIA model even though PGD_2, which is potent bronco-constrictor, was down-regulated. Interestingly, in addition to LTC_4, the amplified secretion of LTs was also demonstrated for LTB_4 synthesis in mice, particularly the LTC_4S-Tg mice. Recently, it has been reported that LTB_4 secretion is also increased in patients with AIA[43]. Although the detailed mechanisms in patients with AIA and the Tg mice have yet to be clarified, we speculate that the induction of LTA_4 hydrolase by IL-4 and IL-13 may augment the secretion of LTB_4[44].

Notably, the AIA response in the Tg mice was observed by sulpyrine inhalation 48 h

after antigen exposure when it was confirmed prior to 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 the Tg mice might not be related to the preceding OVA-induced airway response, although LTC_4-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 LTC_4S-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 LTC_4 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 LTC_4 was also secreted in vivo by WT mice following administration of sulpyrine after antigen exposure, although in lower amounts than that found in Tg mice, it appears that the level of LTC_4 must exceed a certain threshold level for the development of AIA. This speculation is supported by a report that LTs were found in urine in high concentrations at baseline, but after the ingestion of aspirin, large amounts of LTs were formed and detectable in the urine[45,46]. Furthermore, it has been reported that the number of activated eosinophils that are immunoreactive for LTC_4S are elevated in the bronchial mucosa of AIA patient compared to that from ATA patients[47]. In addition, the overexpression of LTC_4S is strongly correlated with elevated levels of cys-LTs in BALF from AIA patients in a stable state and with AHR to inhaled aspirin[47].

Taken together, these reports suggest that the increase of LTC_4S in inflammatory cells of human airways in a stable state may be a hallmark of AIA.

In conclusion, by generating and examining LTC_4S-Tg mice, we demonstrated for the first time that AIA can be induced in the presence of elevated levels of cyst-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 strongly suggests that cys-LTs play a major role in the pathogenesis of AIA in patients with chronic asthma.

METHODS

Reagents and antibodies. Magnetic microbeads labeled with anti-CD90 (Thy1.2), -CD45R (B220), -CD11b, -CD11c, or -PE antibodies (abs) were purchased from Miltenyi Biotec (Gladbach, Germany). PE-conjugated anti-CD3ε 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 RIα 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 LTC_4S-Tg mice. To generate transgenic mice containing the *Ltc₄s* gene, we amplified murine cDNA encoding the full-length LTC_4S protein and placed it under the control of a CMV enhancer/chicken ß-actin promoter in the transgenic vector

pCAGGS[48], which has been shown to facilitate widespread transgene expression in mice. Detailed methodology is described in the **Supplementary Methods.** All mice were maintained under specific pathogen-free conditions in the animal center at Dokkyo University School of Medicine. This study was approved by the Ethics Committee for Animal.

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 the **Supplementary Fig. 1**. To investigate the roles of LTC_4S in the development of AIA, we first established an antigen-dependent murine model of asthma. Briefly, LTC_4S-Tg and wild-type (WT) mice were intraperitoneally injected on days 0 and 5 with ovalbumin (OVA) (Sigma-Aldrich) (8 μg/mouse) adsorbed in aluminum hydroxide (alum) (Wako Pure Chemical Industries) for sensitization, or aluminum 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 analyzed for airway hyper-reactivity (AHR). Subsequently, on day 14, a second analysis for AHR was performed, which was followed by bronchial-alveolar lavage (BAL) and splenectomy for cellular analysis. For the establishment of the AIA model, 48 h after the OVA challenge, mice were provocated with aerosol inhalation of sulpyrine (1, 10, or 50 mg/ml) or saline for 30 min. After 2 h, airway resistance was measured, and then BAL was performed. In addition, to access the effect of a LTC_4 receptor antagonist on the development of AIA, Pranlukast

hydrate (ONO Pharmaceutical Co., Osaka, Japan) or lactose (Sigma-Aldrich, St.Louis, MO, USA) as the control were dissolved in dimethylsulfoxide and given to mice by intragastric administration (25 mg/kg body weight)1 h prior to 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) as described previously [49,50]. 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. Methacholine or sulpyrine was provocated 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.

Bronchial-alveolar lavage. BAL was performed immediately after the last aerosol provocation with OVA, methacholine or sulpyrine (**Supplementary Methods**).

Cells preparation. Macrophages, T cells, neutrophils and eosinophils, or mast cells were obtained from BAL cells, or splenocytes, respectively (**Supplementary Methods**).

Cell culture for LTC_4 measurement. For the measurement of LTC_4 production, the isolated neutrophils, eosinophils, T cells, macrophages, and mast cells (2×10^5/ml) were cultured in RPMI 1640/10% FCS in the presence or absence of both phorbol myristate

acetate (PMA) (25 ng/ml) (Sigma, St. Louis, Mo, USA) and ionomycin (1 μg/ml) (Sigma, St. Louis, Mo, USA). After an 1-h incubation, the amount of LTC_4 in the culture supernatant was measured using an enzyme-immunoassay (EIA). To examine effects of sulpyrine on LTC_4 secretion, the BAL cells (2 x 10^5/well) were treated with sulpyrine (1 μg/ml) for 1 h prior to the activation by PMA/ionomycin in RPMI 1640/10% FCS medium in 96-well plates, which were incubated at 37 °C in a 5% CO_2 environment.

Measurement of chemical mediator and cytokines. Concentrations of LTB_4, LTC_4, prostaglandin (PG)D_2 (Cayman, Ann Arbor, MI, USA), PGE_2 (Oxford Biomedical Research, Oxford, MI, USA), histamine (SPI Bio, Montigny le Bretonneux, France) in BALF or culture supernatants were assessed by enzyme immune assay (EIA) (**Supplementary Methods**).

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 less than 0.05 were considered to indicate statistical significance.

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AUTHOR CONTRIBUTIONS

H.H. performed initial experiments and participated in the writing of the paper; Y.F. and K.H. designed the study and analyzed the data; M.A., T.T., and T.F. designed the study and wrote paper.

COMPETING FINANCIAL INTEREST

The authors declare that they have no competing financial interests.

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Figure legends

Figure 1 AHR to inhaled mathacholine in LTC_4S-Tg and WT mice after an 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 to immunized WT mice after antigen challenge.

Figure 2 Cell number, types and concentration of cytokines in BALF samples from LTC_4S-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 to immunized WT mice after antigen challenge. MΦ, macrophages; Ly, lymphocytes; Neu, neutrophils; Eo, eosinophils. (**b**) The concentration of Th2 cytokines (IL-4, IL-5, 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 to immunized WT mice after antigen challenge. ND, not detected.

Figure 3 LTC_4 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. LTC_4 in culture supernatants of each cell type was measured 1 h after stimulation with PMA and ionomycin. The concentration of LTC_4 in the supernatants was quantified by EIA. Data are expressed as the mean ± SD for each group (n=10). **P*<0.05 as compared to WT mice. MΦ, macrophages; Neu, neutrophils; Eo, eosinophils; MC, mast cells.

Figure 4 Effects of sulpyrine on LTC_4 secretion by BAL cells from mice 48 h after antigen challenge. LTC_4 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 ± SD for each group (n=8). *P<0.05 as compared to WT mice or Tg mice in the absence of sulpyrine.

Figure 5 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 analyzed 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 ± SD for each group (n=6). *P<0.05 as compared to WT mice (OVA+). (**b**) Concentrations of LTB_4, LTC_4, PGD_2, PGE_2, 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 ± SD for each group (n=7). *P<0.05 as compared to immunized WT mice in the presence of sulpyrine, or immunized Tg mice in the absence of sulpyrine.

Figure 6 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 sulyprine. Data are expressed as the mean ± SD for each group (n=6). *P<0.05 as compared to LTC_4S-Tg administered sulpyrine in the absence of Pranlukast hydrate.

Figure 1

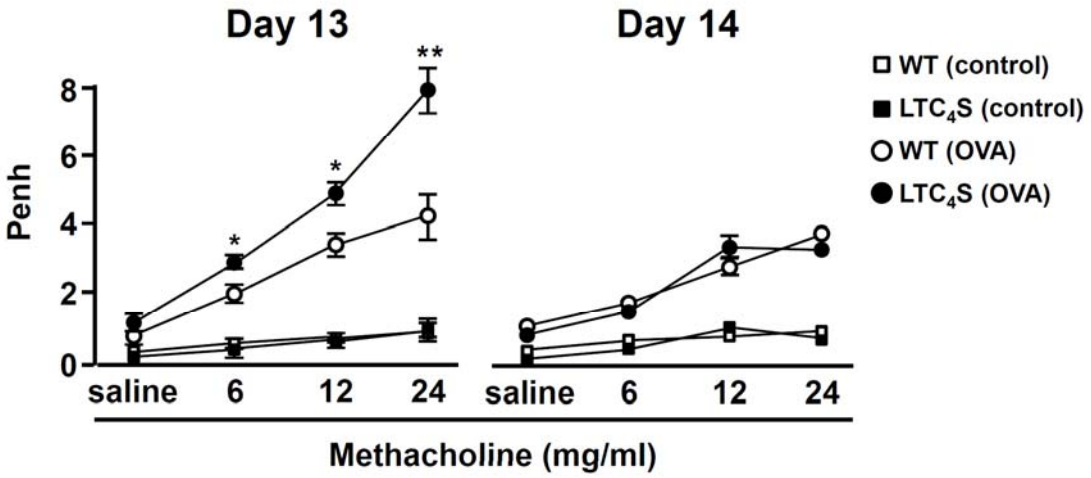

Figure 2

a

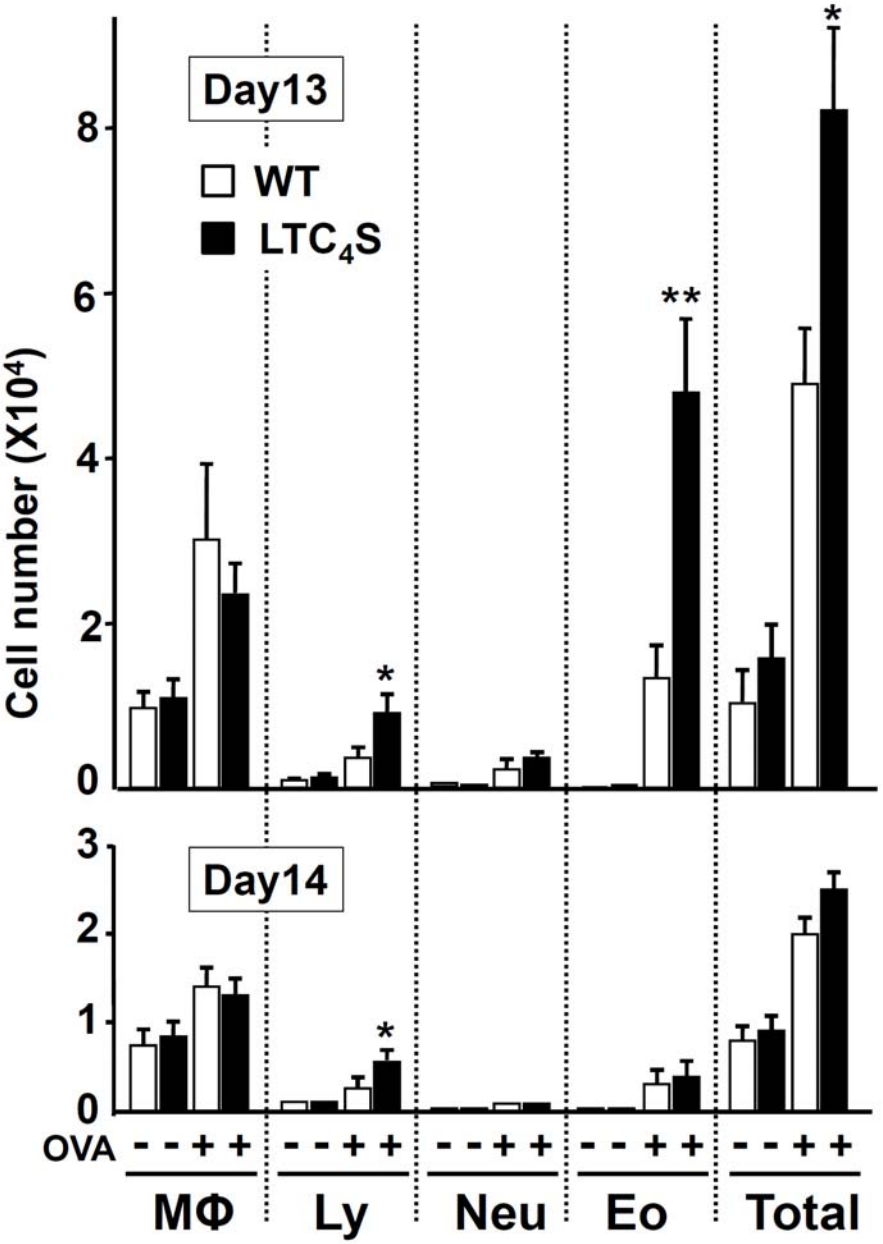

Figure 2

b

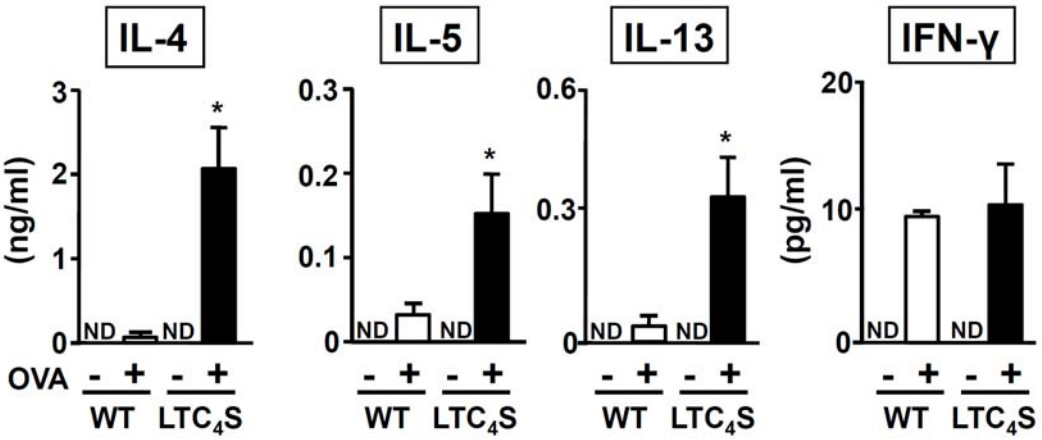

Figure 3

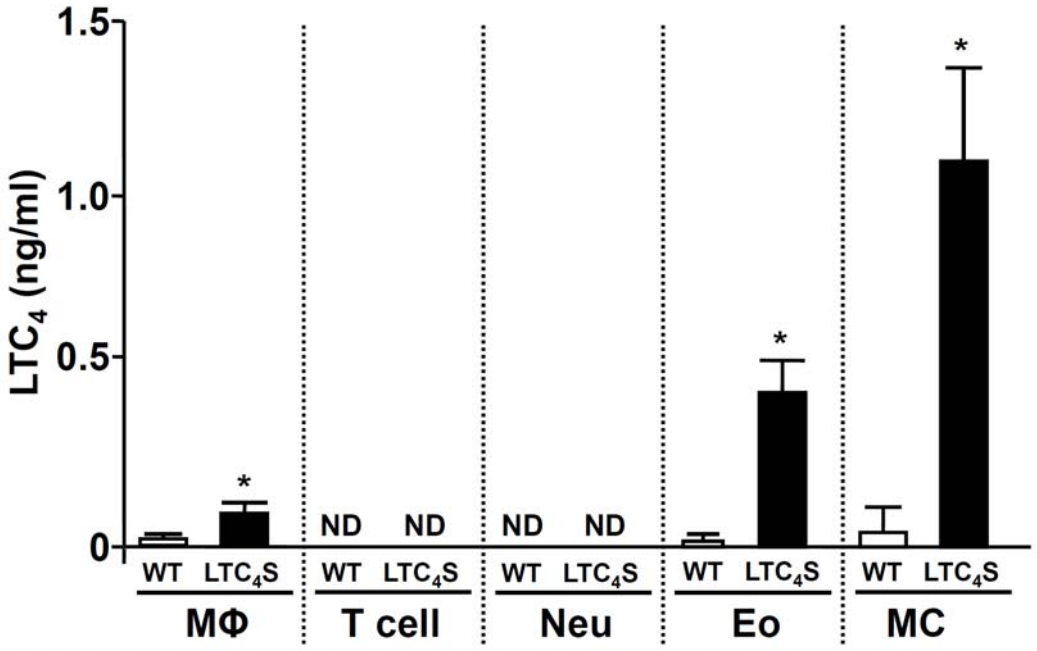

Figure 4

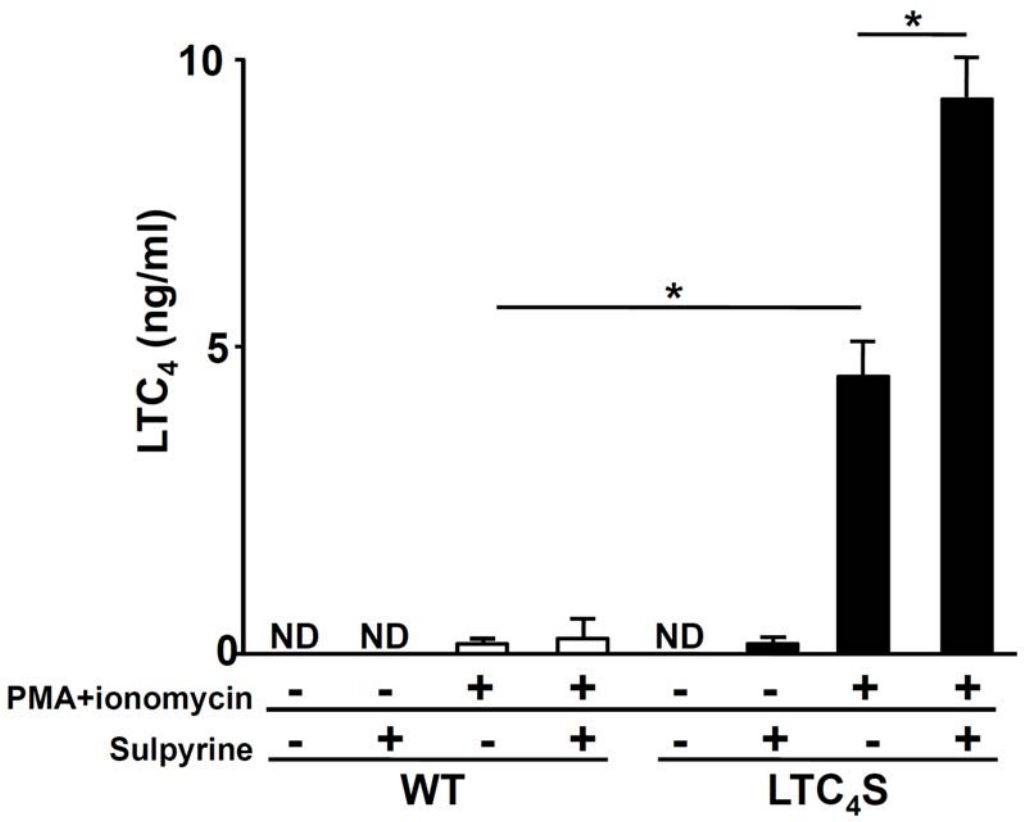

Figure 5

a

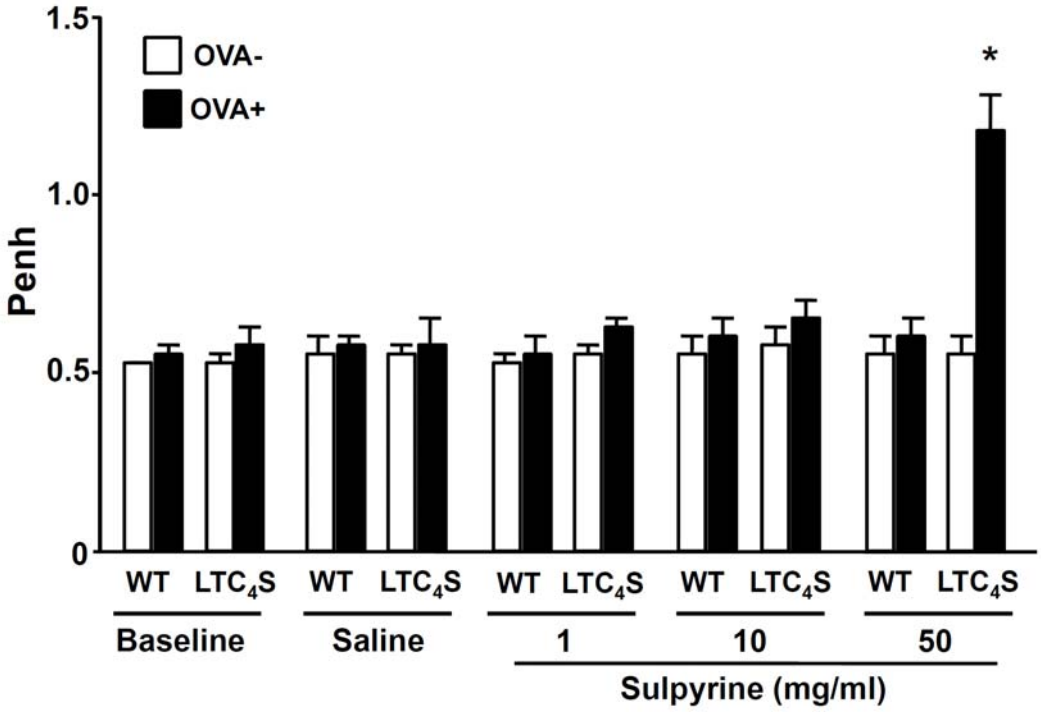

Figure 5

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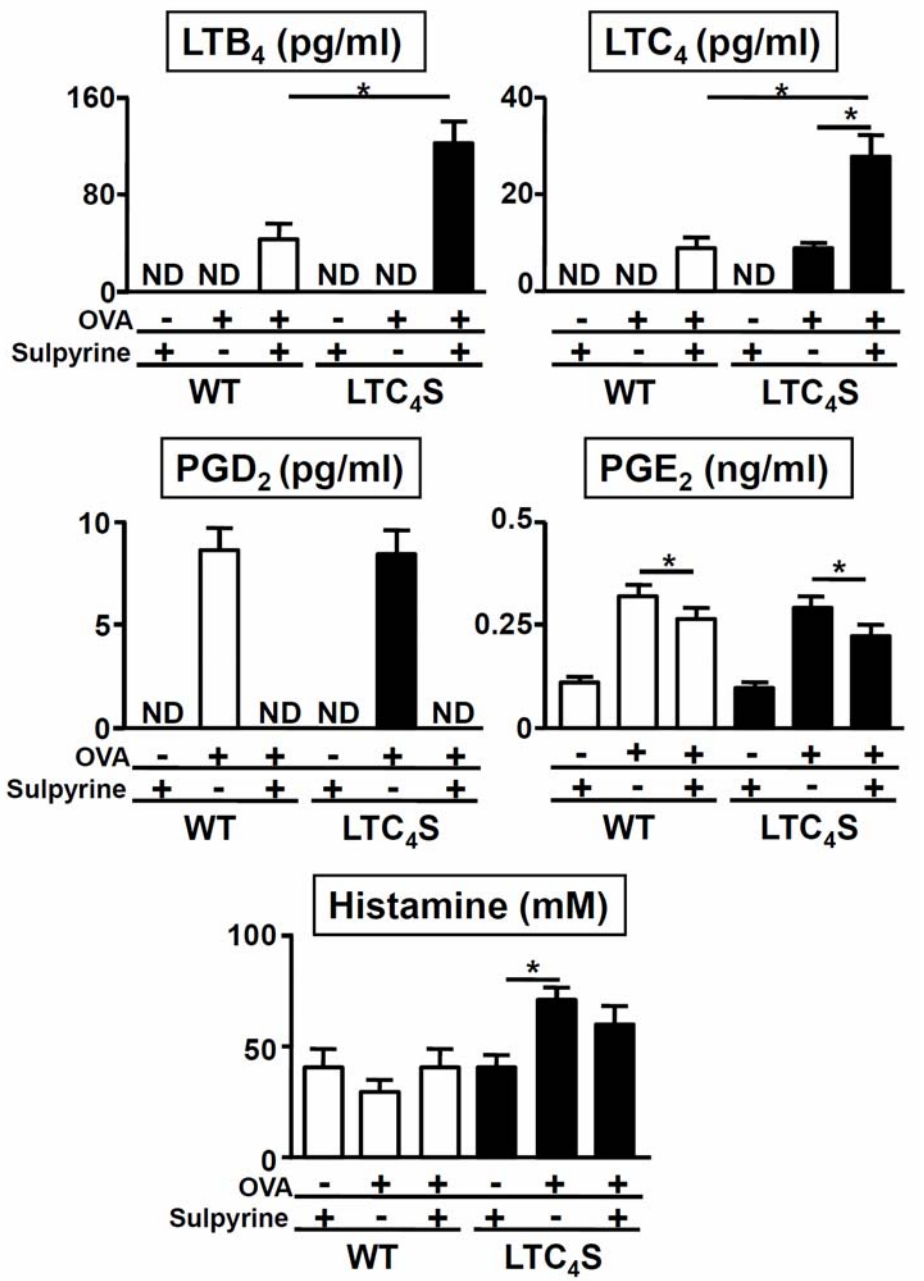

Figure 6