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SUPPLEMENTAL MATERIALS AND METHODS

Analysis of BAL fluid eicosanoids by high performance liquid chromatography (HPLC)–tandem mass spectrometry. After collection of 0.9–1.4 ml of BAL fluid, it was immediately centrifuged at 1,200 rpm for 5 min at 4°C in a swinging bucket rotor at room temperature to pellet BAL fluid cells. A 0.5-ml portion of supernatant was immediately transferred to a labeled, amber glass vial containing 2 ml methanol/0.2% formic acid (HPLC-grade methanol and reagent-grade formic acid) and 10 μl of internal standard mixture. The deuterated internal standards were as follows: 50 pg/μl each of 5(*S*)-HETE-d8, LTB_4-d4, PGE_2-d4, PGD_2-d4, 6-keto-$PGF_{1\alpha}$-d4, and TxB_2-d4; 100 pg/μl PAF-C_{16}-d4; and 20 pg/μl each of LTC_4-d4 and LTE_4-d4, (all obtained from Cayman Chemical except the labeled LTC_4 and LTE_4, which were prepared by in-house synthesis and stored as stock solution in methanol/0.2% formic acid at −80°C under argon). The sample in the vial was mixed briefly on a vortexer, and the vial was capped with a Teflon-line septum screw cap and placed on dry ice and then into a −80°C freezer.

After BAL collection, samples in the vials were thawed on ice, and the liquid was transferred to a 16 × 125–mm glass tube. Samples were brought to ~15% methanol by addition of Milli-Q (Millipore) purified water/0.03% formic acid. Samples were loaded using a Pasteur pipet onto an Oasis HLB column (10-mg column bed; Waters Corporation) that was previously equilibrated with 1 ml methanol/0.2% formic acid, followed by 2 × 1 ml water/0.03% formic acid. A 1-ml portion of water/0.03% formic acid was added to the empty culture tube, and the sample was mixed briefly on a vortexer. The liquid was transferred to the Oasis HLB cartridge. The columns were processed in a vacuum manifold (Waters Corporation) connected to a water aspirator. The receiver tubes in the manifold were replaced with amber autosampler vials without inserts (Agilent Technologies). The Oasis cartridge was removed from the manifold so that traces of water in the column tip could be pushed out of the column by applying a positive pressure of N_2 to the top of the column using Tygon tubing. N_2 was also used to clear any water remaining in the transfer tube adaptor attached to the column tip in the manifold. Eicosanoids were eluted from the cartridge into the autosampler vial with 100 μl methanol/0.2% formic acid, followed by 2 × 200 μl methanol/0.2% formic acid. The volume in each autosampler vial was reduced to 200–250 μl with a gentle stream of N_2. The liquid was transferred to an autosampler insert (Agilent Technologies) with a Pasteur pipet. The volume in the insert was reduced to ~50 μl with N_2, the vial was capped with a PTFE/silicone septum cap (Agilent Technologies), and the vial was purged with argon and stored at −80°C until submitted to HPLC–tandem mass spectrometry (within 1–2 d).

Combined HPLC–tandem mass spectrometry was performed using a triple-quadrupole mass spectrometer with an electrospray ionization source (API-4000; Applied Biosystems). Mass spectrometer parameters were as described previously by Kita et al. (1). The HPLC column was a Shiseido Capcell PAK C18 MGII S3 column (1 id × 75 mm; ESA Biosciences, Inc.). The column temperature was 46°C, and the flow rate was 0.12 ml/min. The column was preequilibrated with 70% solvent A (water)/30% solvent B (CH_3CN/0.1% formic acid) for 12 min. After injection of 5 μl of sample, the solvent program was as follows: 3 min, 70% A; 3–10 min, 35% A; and 10–13 min, 0% A. Ion peaks corresponding to each analyte and internal standard were integrated, and the amount of each analyte was obtained from the area ratio and the amount of internal standard added to each sample. It was assumed that LTD_4 and LTE_4 give the same response in the mass spectrometer as does LTC_4 (only an internal standard for LTC_4 was present). The analyte amounts were corrected for the amount of BAL fluid obtained from each mouse to obtain the amount of eicosanoid per mouse lung.

Analysis of the BAL fluids PGE_2, LTB_4, and cysLT by EIA. A 0.25-ml aliquot of the BAL fluid supernatant (after centrifugation of the collected BAL fluid; see Analysis of BAL fluid...) was mixed with 0.5 ml methanol/0.2% formic acid (internal standards were omitted). The sample was processed using an Oasis HLB cartridge as described in Analysis of BAL fluid... After elution of eicosanoids, the 500-μl sample was placed in a centrifugal vacuum concentrator (Savant SpeedVac Concentrator; Fisher Scientific) under vacuum for 30 min at room temperature to remove all traces of methanol. The residue was resuspended in 20 μl EIA buffer (Cayman Chemical), and portions were used for EIA analysis (i.e., PGE_2 kit–monoclonal, PGE_2 metabolite EIA kit, LTB_4 EIA kit, and cysLT EIA kit from Cayman Chemical; see Materials and methods) according to the manufacturer's instructions.

Analysis of the BAL fluid PGD_2 by EIA. A 0.25-ml aliquot of the BAL fluid supernatant (after centrifugation of the collected BAL fluid; see Analysis of BAL fluid...) was mixed with 0.25 ml of ice-cold, reagent-grade acetone in a glass vial. After incubation on ice for 5 min, the sample was centrifuged at 3,000 *g* for 10 min at 4°C. The supernatant was transferred to a new glass vial on ice. After resuspension of the pellet in 0.5 ml of ice-cold acetone, the sample was briefly mixed on a vortexer and centrifuged as in Analysis of BAL fluid... After combining the supernatants, the sample was stored on dry ice for up to 4–5 h. The sample was concentrated to dryness in a Savant SpeedVac concentrator at room temperature, and 100 μl EIA buffer from the PGD_2-MOX EIA kit (Cayman Chemical) was added to resuspend the dried sample. The sample was submit-

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ted to methoximation as indicated in the Cayman instructions. Two volumes of methanol/0.2% formic acid were added, and the pH was checked by spotting an ~1-ml aliquot onto pH paper to ensure that the pH was <4. The sample was diluted with water/0.03% formic acid to give 15% methanol. The sample was submitted to purification using an Oasis HLB cartridge as in Analysis of BAL fluid... The methanol eluant was dried in the Savant SpeedVac concentrator under vacuum for 30 min at room temperature to ensure that all methanol was removed. The residue was dissolved in 20 μl of Cayman EIA buffer before EIA analysis according to the manufacturer's instructions.

Noninvasive plethysmography. For noninvasive plethysmography, mice were challenged with increasing doses of aerosolized methacholine (0, 5, and 20 mg/ml in normal saline) generated by an ultrasonic nebulizer (DeVilbiss Health Care, Inc.) for 2 min. The degree of bronchoconstriction was expressed as enhanced pause (Penh), a calculated dimensionless value that correlates with measurement of airway resistance, impedance, and intrapleural pressure (2, 3). *Penh* is calculated as follows: $Penh = [(T_e/T_r - 1) \times (PEF/PIF)]$, where T_e is expiration time, T_r is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow. Tr is the time it takes for a user-defined percentage of the expired volume (computed from the box-flow waveform) to be exhaled, starting at end expiration. The endpoint of Tr is chosen to be when 40% of the volume remains to be exhaled (4). Penh is primarily independent of functional residual capacity, tidal volume, and respiratory rate, because the ratio of measurements is obtained during the same breath and has a strong correlation with both airway resistance (Raw) measured directly in anesthetized, tracheotomized, and mechanically ventilated mice (2, 3, 5) and the intensity of the allergen-induced airway eosinophil infiltration in the mouse asthma model (6).

REFERENCES

1. Kita, Y., T. Takahashi, N. Uozumi, and T. Shimizu. 2005. A multiplex quantitation method for eicosanoids and platelet-activating factor using column-switching reversed-phase liquid chromatography-tandem mass spectrometry. *Anal. Biochem.* 342:134–143.
2. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G.L. Larsen, C.G. Irvin, and E.W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156:766–775.
3. Henderson, W.R., Jr., L.-O. Tang, S.-J. Chu, S.-M. Tsao, G.K.S. Chiang, F. Jones, M. Jonas, C. Pae, H. Wang, and E.Y. Chi. 2002. A role for cysteinyl leukotrienes in airway remodeling in a mouse asthma model. *Am. J. Respir. Crit. Care Med.* 165:108–116.
4. Lomask, M. 2006. Further exploration of the Penh parameter. *Exp. Toxicol. Pathol.* 57(Suppl. 2):13–20.
5. Justice, J.P., Y. Shibata, S. Sur, J. Mustafa, M. Fan, and M.R. van Scott. 2001. IL-10 gene knockout attenuates allergen-induced airway hyperresponsiveness in C57BL/6 mice. *Am. J. Physiol. Lung Cell Mol. Physiol.* 280:L363–L368.
6. Dohi, M., S. Tsukamoto, T. Nagahori, K. Shinagawa, K. Saitoh, Y. Tanaka, S. Kobayashi, R. Tanaka, Y. To, and K. Yamamoto. 1999. Noninvasive system for evaluating the allergen-specific airway response in a murine model of asthma. *Lab Invest.* 79:1559–1571.