Leukotriene B4 mediates macrophage influx and pulmonary hypertension in bleomycin-induced chronic neonatal lung injury

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[1]*Program in Physiology and Experimental Medicine, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada;* [2]*Division of Neonatology, Department of Pediatrics, University of Toronto, Toronto, Ontario, Canada;* [3]*Department of Physiology, University of Toronto, Toronto, Ontario, Canada;* [4]*Heart and Stroke Richard Lewar Centre of Excellence in Cardiovascular Research, University of Toronto, Toronto, Ontario, Canada; and* [5]*Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada*

Ee MT, Kantores C, Ivanovska J, Wong MJ, Jain A, Jankov RP. Leukotriene B4 mediates macrophage influx and pulmonary hypertension in bleomycin-induced chronic neonatal lung injury. *Am J Physiol Lung Cell Mol Physiol* 311: L292–L302, 2016. First published June 17, 2016; doi:10.1152/ajplung.00120.2016.—Systemically-administered bleomycin causes inflammation, arrested lung growth, and pulmonary hypertension (PHT) in the neonatal rat, similar to human infants with severe bronchopulmonary dysplasia (BPD). Leukotrienes (LTs) are inflammatory lipid mediators produced by multiple cell types in the lung. The major LTs, LTB4 and cysteinyl LTs, are suggested to contribute to BPD, but their specific roles remain largely unexplored in experimental models. We hypothesized that LTs are increased in bleomycin-induced BPD-like injury, and that inhibition of LT production would prevent inflammatory cell influx and thereby ameliorate lung injury. Rat pups were exposed to bleomycin (1 mg·kg^{-1}·day^{-1} ip) or vehicle (control) from postnatal *days 1–14* and were treated with either zileuton (5-lipoxygenase inhibitor), montelukast (cysteinyl LT1 receptor antagonist), or SC57461A (LTA4 hydrolase inhibitor) 10 mg·kg^{-1}·day^{-1} ip. Bleomycin led to increased lung content of LTB4, but not cysteinyl LTs. Bleomycin-induced increases in tissue neutrophils and macrophages and lung contents of LTB4 and tumor necrosis factor-α were all prevented by treatment with zileuton. Treatment with zileuton or SC57461A also prevented the hemodynamic and structural markers of chronic PHT, including raised pulmonary vascular resistance, increased Fulton index, and arterial wall remodeling. However, neither treatment prevented impaired alveolarization or vascular hypoplasia secondary to bleomycin. Treatment with montelukast had no effect on macrophage influx, PHT, or on abnormal lung structure. We conclude that LTB4 plays a crucial role in lung inflammation and PHT in experimental BPD. Agents targeting LTB4 or LTB4-mediated signaling may have utility in infants at risk of developing BPD-associated PHT.

rat; newborn; inflammation; lung injury

THE SURVIVAL OF EXTREMELY low-birth-weight infants has improved over recent decades, but at the cost of a high risk of developing chronic lung injury, known as bronchopulmonary dysplasia (BPD) (3). Chronic pulmonary hypertension (PHT) is common in infants with severe BPD, heralding a greatly increased morbidity and mortality (11, 31, 34, 47). The pathogenesis of BPD is multifactorial, with upregulation of inflammatory mediators leading to, or caused by, infiltration of inflammatory cells playing a major role (38, 43, 46). However, the specific mediators contributing to inflammatory neonatal lung injury remain unclear, and there are presently no effective treatments.

Leukotrienes (LTs) are potent lipid mediators, first described in 1979 by Borgeat and Samuelsson (5) as a new lineage of arachidonic acid-derived metabolites. LTs are produced by, recruit, and activate immune cells, thus initiating, augmenting, and sustaining tissue inflammation. The concerted action of 5-lipoxygenase (5-LPO) and 5-LPO-activating protein (FLAP) on arachidonic acid produces LTA4, which is converted either by LTA4 hydrolase (LTA4H) to LTB4 or is conjugated with reduced glutathione by LTC4 synthase to produce cysteinyl (cys) LTs (LTC4, LTD4, and LTE4) (32) (illustrated in Fig. 1). 5-LPO activity requires perinuclear translocation from the cytosol (9) and association with FLAP homodimer (7, 27, 39) and is increased by phosphorylation at serine 271 (55). LTB4 and cysLTs exert their actions by binding to distinct G protein-coupled receptors: BLT1 and BLT2 for LTB4, and cysLT 1 and 2 receptors (cysLT$_1$R and cysLT$_2$R) for cysLTs (Fig. 1). LTB4 is a particularly potent chemoattractant for multiple inflammatory cell types, especially neutrophils and macrophages. Due to the wide-ranging pathological effects of LTs, including increased production of matrix proteins, increased smooth muscle contractility and proliferation, and enhanced cell survival, critical roles have been proposed for a number of lung disorders, including asthma, pulmonary fibrosis, and chronic PHT (36, 50).

A critical role for LTs has been established in adult rat models of chronic PHT (49–51, 54) and is suggested by observational studies in adult humans with primary PHT (50, 51, 56) and in newborn infants with evolving or established BPD (10, 16, 20, 45). In neonatal animals, LTB4 receptor blockade prevented acute hyperoxic lung injury in preterm guinea pigs (37), and a 5-LPO inhibitor decreased lung inflammation and edema induced by saline lavage in newborn piglets (1). Inhibitors of 5-LPO or FLAP prevented inhibited alveolar development secondary to severe hyperoxia (6), whereas montelukast (a cysLT$_1$R antagonist) had no effect on chronic neonatal lung injury secondary to moderate hyperoxia (21) in neonatal rats. In premature human infants with evolving BPD, LTB4 and LTE4 were increased in tracheal aspirate fluid (16) and urine (20, 45), respectively. Ex-premature infants with severe BPD also had increased urinary LTE4 (10). Montelukast given as preventive therapy in at-risk preterm infants did not reduce the incidence of moderate-severe BPD (25); however,

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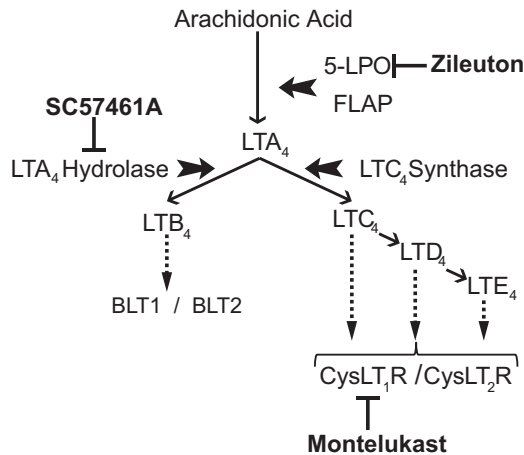
Fig. 1. Illustration of the arachidonic acid-leukotriene (LT) pathway and drug targets. The concerted action of 5-lipoxygenase (5-LPO) and 5-LPO-activating protein (FLAP) on arachidonic acid produces LTA$_4$, which either is converted by LTA$_4$ hydrolase to LTB$_4$, or is conjugated by LTC$_4$ synthase to produce cysteinyl (cys) LTs (LTC$_4$, LTD$_4$, and LTE$_4$). Attenuated production of LTB$_4$ by inhibition of 5-LPO with zileuton or by inhibition of LTA$_4$ hydrolase with SC57461A prevented bleomycin-induced pulmonary hypertension. Antagonism of the cysLT1 receptor (cysLT$_1$R), for which LTs C$_4$, D$_4$, and E$_4$ act as ligands, had no effect on bleomycin-induced pulmonary hypertension.

when employed as rescue therapy for severe late-stage BPD, it conferred a survival advantage (42). Increased cysLTs have also been reported in tracheal aspirates of infants with persistent PHT of the newborn, an acute form of PHT (48). However, the specific role of various LTs in the pathogenesis of chronic neonatal PHT, particularly in the context of BPD or experimental BPD-like lung injury, has not been previously studied.

Bleomycin sulfate is a chemotherapeutic agent that produces a dose-dependent pulmonary inflammatory and fibrotic response when administered systemically or intratracheally (4, 24). In neonatal rats, our laboratory and others have reported that repeated systemic administration of bleomycin leads to a selective decrease in lung growth, along with arrested alveolarization, vascular hypoplasia, and chronic PHT (15, 30, 44, 52), similar to the lung pathology observed in human infants with severe BPD. We have employed this model to demonstrate a critical role for macrophage influx and macrophage-derived tumor necrosis factor (TNF)-α in the pathogenesis of chronic PHT (26, 44). In the present study, we hypothesized that LTs are upregulated in bleomycin-induced lung injury, and that inhibition of LT biosynthesis or signaling would attenuate inflammation and consequent chronic PHT. Our observations reported herein suggest a critical role for LTB$_4$, but not for cysLTs, in experimental chronic neonatal PHT.

MATERIALS AND METHODS

Materials. Bleomycin sulfate was purchased from Calbiochem (San Diego, CA). Zileuton, montelukast, and C18 solid-phase extraction cartridges (catalog no. 400020) were purchased from Cayman Chemical (Ann Arbor, MI). SC57461A was purchased from Tocris Biosciences (Bristol, UK). Acids, alcohols, organic solvents, paraformaldehyde, Permount, and Superfrost/Plus microscope slides were from Fisher Scientific (Whitby, ON, Canada). Weigert's resorcin-fuchsin stain was from Rowley Biochemical (Danvers, MA). Anti-chemokine (C-C motif) ligand 4 (CCL4) (catalog no. sc-393441), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; catalog

no. sc-25778), and anti-FLAP (catalog no. sc-28815) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cluster of differentiation (CD) 68 (catalog no. MCA341R) was from Serotec (Raleigh, NC). Anti-TNF-α (catalog no. HP8001) was from Hycult Biotech (Uden, The Netherlands). Anti-5-LPO (catalog no. 3289), anti-phospho-serine 271 5-LPO (catalog. no. 3748), and goat anti-rabbit IgG-peroxidase antibody were from Cell Signaling Technology (Beverly, MA). Anti-LTA4H (catalog no. NBP1-96835) was purchased from Novus Biologicals (Littleton, CO). Anti-myeloperoxidase (MPO; catalog. no. A0398) was from DAKO Agilent (Mississauga, Ontario, Canada). ELISA kits for LTB$_4$ (catalog no. ADI-900-068) and cysLTs (catalog no. ADI-900-070) were from Enzo Life Sciences (Farmingdale, NY). Tris-glycine precast gels and polyvinylidene difluoride (PVDF) membranes were from Thermo Scientific (Rockford, IL). Unless otherwise specified, all other chemicals and reagents were from Bioshop Canada (Burlington, Ontario, Canada).

Animal exposures and interventions. All procedures involving animals were performed in accordance with the standards of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Hospital for Sick Children Research Institute. Timed-pregnant Sprague-Dawley dams were purchased from Taconic Farms (Germantown, NY). Commencing on the day after birth, pups received 1 mg/kg bleomycin sulfate (0.2 mg/ml suspended in 0.9% saline + 20% DMSO; 5 μl/g body wt by 27G needle in the right iliac fossa) or 0.9% saline + 20% DMSO (control) daily intraperitoneally for 14 days, as previously described (30), with or without zileuton (5-LPO inhibitor), montelukast (cysLT$_1$R antagonist), or SC57461A (LTA4H inhibitor; all at 2 mg/ml = 10 mg/kg) (see Fig. 1). For zileuton, dose-response studies (5, 10, or 20 mg/kg ip daily) were conducted, demonstrating maximal inhibitory effect on lung macrophage influx at 10 mg/kg (data not shown). For both montelukast and SC57461A, which are orally active, the intraperitoneal route of delivery was necessitated by an inability to safely perform daily gavage in newborn rats. For montelukast, dose-response studies (1, 5, or 10 mg/kg ip daily) were conducted, with the lowest dose based on a previous negative study in neonatal rats (1 mg/kg ip daily) (21). These studies demonstrated maximal inhibitory effect on lung CCL4 content at 10 mg/kg (data not shown). For SC57461A, dose-response studies were conducted using a range of doses surrounding 10 mg/kg (5, 10, and 20 mg/kg ip daily) based on equivalence of IC$_{50}$ with zileuton and similar oral bioavailability (2). These studies demonstrated maximal inhibitory effect of SC57461A on lung LTB$_4$ content at 10 mg/kg (data not shown), the same (orally administered) dose and dose interval reported to have inhibitory effects on inflammatory injury in adult rodents (22). Each litter was maintained at $n = 10$–12 pups to control for nutritional effects. At the end of each 7- or 14-day exposure period, pups either were killed by pentobarbital overdose, or were exsanguinated after anesthesia.

Cardiac ventricular weights. Right ventricular (RV) hypertrophy was quantified by measuring the RV-to-left ventricle and septum dry weight ratio (Fulton index), as previously described (19).

Two-dimensional echocardiography-derived measurements of pulmonary hemodynamics. Pulmonary vascular resistance (PVR) was evaluated noninvasively using two-dimensional echocardiography and Doppler ultrasound (Vivid 7 cardiac ultrasound system and I13L linear probe; GE Medical Systems, Milwaukee, WI), as previously described (23, 30). Briefly, following induction of anesthesia with 5% (vol/vol) isoflurane, the animal was laid supine while spontaneously breathing 2–3% (vol/vol) isoflurane through a modified face mask. A short-axis view of the heart was acquired at the level of the aortic valve, and the pulmonary artery was identified by color flow Doppler. The pulsed Doppler gate was placed proximal to the pulmonary valve leaflets and aligned with an insonation angle of <20° to obtain a Doppler profile. The pulmonary artery acceleration time (PAAT) was measured as the time from the onset of systolic flow to peak velocity and the RV ejection time (RVET) as the time from onset to completion of systolic flow. PVR index was derived using the formula,

Table 1. *Rat primer sequences for quantitative PCR*

Gene	RefSeq Accession No.	Common Name	Forward 5′-3′	Reverse 5′-3′
Alox5	NM_012822	5-lipoxygenase	CTTCCTGGCATGACTTTGCT	CTAGGCTGCACTCCACCATT
Alox5ap	NM_017260	5-lipoxygenase activating protein (FLAP)	GGGTCTACACTGCCAACCAG	CTCCCAGATAGCCGACAAAG
Ltc4s	NM_053639	Leukotriene C4 synthase	AGCTCTTCTGGCTACCGTCA	ATTTACCTGGGCTCGGAAGA
Lta4h	NM_001030031	Leukotriene A4 hydrolase	AGCATCGAAGACCTGAAGGA	GCCGTAACCATCTGAATCGT
Ltb4r	NM_021656	Leukotriene B4 receptor	GGCATGTCCCTGTCTCTGTT	CCGAGCCAGAAAGTGTAGGA
Ltb4r2	NM_053640	Leukotriene B4 receptor 2	CGTCTTTACTGCGGGTGATT	TGCCCTGACCCACTACTTTC
Cyslt1r	NM_053641	Cysteinyl leukotriene receptor 1	TGGAGCTGAAAATATGACAGCA	GGAAGGCTGATTTCTCATGG

RVET/PAAT. All values were averaged from three Doppler traces per animal using an offline analysis system (EchoPAC, GE Medical Systems).

Histological studies. Lungs from six animals from each group (3 from each of 2 separate litters) were air-inflated and perfusion-fixed at constant pressure, embedded in paraffin, sectioned, and immunostained for CD68 (to identify macrophages), or were stained with hematoxylin-eosin, or for elastin, as previously described (23, 29, 33, 35). For all analyses, measurements were carried out on four noncontiguous left lung sections per animal by an observer blinded to group identity. For assessment of percentage of arterial medial wall area, pulmonary arteries were identified by the presence of both inner and outer elastic lamina using Hart's elastin stain, as previously described in detail (14, 26). Analyses of tissue macrophage (CD68-positive) cell numbers, mean linear intercept (using hematoxylin and eosin-stained sections), and counts of peripheral arteries (identified as vessels of external diameter between 20 and 65 μm with both internal and external elastic laminae visible on elastin staining) were conducted as previously described in detail (28, 29, 33) from 10 random, nonoverlapping, low-power fields captured from each section. Paraformaldehyde-fixed cardiac ventricular tissues were also embedded in paraffin oriented in the short axis and stained with hematoxylin-eosin. For measurement of tissue neutrophils, immunostaining for MPO was performed as previously described (26), and low-power images (10 per section, 2 sections per slide, and 2 slides per animal) were digitally captured using identical magnification, white balance settings, and exposure times. A tissue neutrophil index was derived representing the percentage of tissue area reaching a preset staining intensity threshold (for MPO-positive cells, normalized to total tissue area), using ImmunoRatio online software (http://153.1.200.58:8080/immunoratio).

Quantitative PCR. RNA was extracted and reverse transcribed, and quantitative PCR was performed as previously described (44). Primer sequences of genes of interest are listed in Table 1. A standard curve for each primer set was run to ensure that reaction efficiency was equivalent to primers for the housekeeping genes, β-actin and GAPDH, the expressions of which were determined in preliminary experiments to be unaffected by exposure to bleomycin. Samples were run in duplicate, and fold or fraction change in expression relative to control samples was calculated by the $2^{-\Delta ct}$ method.

ELISA. Lung lysates from six animals per group (3 from each of 2 separate litters) were purified and analyzed according to the manufacturer's instructions.

Western blot analyses. Lung tissues from six animals per group (3 from each of 2 separate litters) were lysed in RIPA buffer containing protease and phosphatase inhibitors. Samples were fractionated by SDS-PAGE, transferred to PVDF membranes, and blotted, and band densities were measured as previously described (18). Differences in protein loading were compensated for by reblotting for GAPDH, the expression of which was found, in preliminary studies, to be unaffected by chronic exposure to bleomycin. Dilutions of primary antisera were 1:100 for FLAP, 1:200 for CCL4 and TNF-α, 1:500 for MPO, 1:1,000 for 5-LPO and phospho-serine 271 5-LPO, 1:5,000 for GAPDH, and 1:10,000 for LTA4H and secondary antisera. Protein bands were identified using enhanced chemiluminescent substrate (SuperSignal West Dura, Thermo Fisher Scientific), and images were digitally captured using a MicroChemi chemiluminescent system (DNR Bio-imaging Systems, Jerusalem, Israel). Bands were quantified by digital densitometry of nonsaturated images with background density removed (ImageJ, NIH, Bethesda, MD).

Data presentation and analysis. All values are expressed as means ± SE. Statistical analyses were performed using Sigma Plot 12.5 (Systat Software, San Jose, CA). Where three or more groups were compared, statistical significance ($P < 0.05$) was determined by one-way ANOVA, followed by pairwise multiple comparisons using the Tukey test. Where two groups were compared, the Student *t*-test was used.

RESULTS

Temporal changes in effects of bleomycin on lung mRNA expression and protein content of key enzymes and receptors mediating LT signaling and on inflammatory cell influx. As shown in Table 2, relative changes in mRNA expression were examined after 7 or 14 days of bleomycin (or vehicle) exposure. Relative to controls, exposure to bleomycin for 7 (but not 14) days led to significantly increased Alox5, Alox5ap, Ltc4s, Ltb4r, and Ltb4r2 mRNA expression. As shown in Fig. 2, lung content of 5-LPO, FLAP, and LTA4H were also examined after 7 (Fig. 2*A*) or 14 days (Fig. 2*B*) of bleomycin (or vehicle) exposure. Relative to vehicle, exposure to bleomycin for 7 days led to significantly increased lung content of 5-LPO and FLAP and no change in LTA4H (Fig. 2*A*). In contrast, 5-LPO and FLAP content in bleomycin-exposed lungs was no longer significantly increased after 14 days, whereas there was a small, but significant, increase in lung LTA4H (Fig. 2*B*). Lung content of phospho-serine 271 5-LPO did not differ between vehicle- and bleomycin-exposed animals at either time point (data not shown). Numbers of lung tissue (CD68-positive) macrophages (normalized to tissue fraction) secondary to bleomycin were increased by *day 7* of exposure (Fig. 2*C*), to a similar extent as at *day 14*. As shown in Fig. 2*D*, tissue

Table 2. *Relative changes in mRNA expression secondary to bleomycin exposure*

Gene	Day 7	Day 14
Alox5	1.53 ± 0.16*	0.88 ± 0.27
Alox5ap	7.72 ± 2.24*	0.98 ± 0.13
Ltc4s	2.65 ± 0.49*	0.76 ± 0.15
Lta4h	1.18 ± 0.08	0.74 ± 0.6
Ltb4r	70.16 ± 23.8*	0.95 ± 0.08
Ltb4r2	2.03 ± 0.27*	0.74 ± 0.07*
Cysltr1	0.97 ± 0.17	0.90 ± 0.1

Values are means ± SE of 4 samples per group relative to control (vehicle-treated) group, which was assigned a value of 1. *$P < 0.05$, by *t*-test, compared with control.
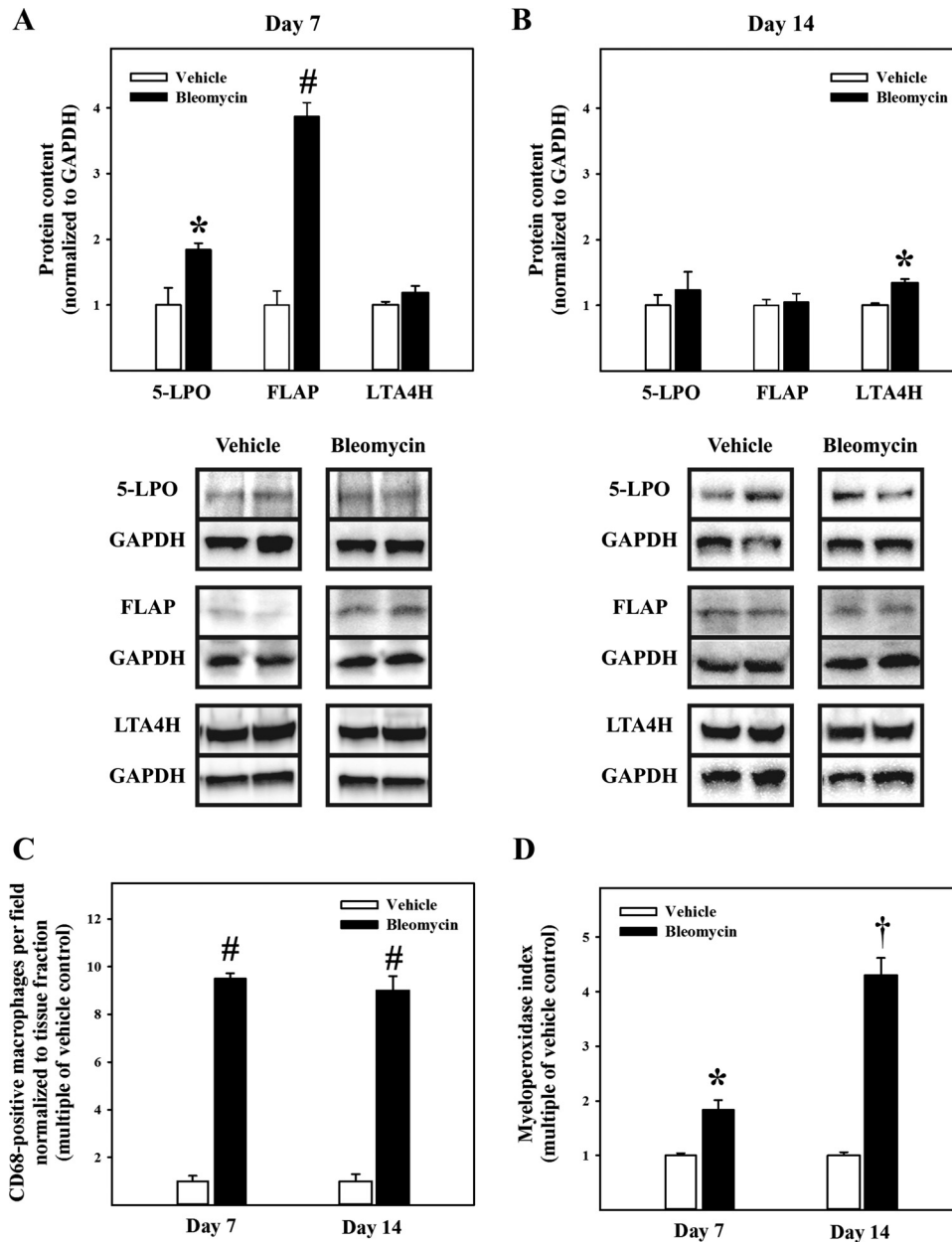
Fig. 2. Exposure to bleomycin increases lung contents of 5-lipoxygenase (5-LPO), 5-LPO activating protein (FLAP), and leukotriene A$_4$ hydrolase (LTA4H), and numbers of tissue macrophages and neutrophils. Pups were exposed to daily intraperitoneal bleomycin (1 mg/kg) or 20% DMSO in 0.9% saline vehicle from postnatal *day 1*. Western blot analyses are shown of 5-LPO (78 kDa), FLAP (18 kDa), or LTA4H (70 kDa) on *day 7* (*A*) or *day 14* (*B*) of bleomycin exposure. Representative immunoblots are shown *below* each graph, with noncontiguous gel lanes demarcated by black lines. $n = 5$–6 Samples/group. *C*: tissue macrophage counts per field normalized to tissue fraction on *days 7* or *14* of vehicle or bleomycin exposure. $n = 4$ Animals/group. *D*: tissue neutrophil index on *days 7* or *14* of vehicle or bleomycin exposure. $n = 4$ Animals/group. Values are means ± SE. *$P < 0.05$, by *t*-test, compared with vehicle-treated group. #$P < 0.01$, by *t*-test, compared with vehicle-treated group. †$P < 0.001$, by ANOVA, compared with all other groups.

neutrophils were also increased in the bleomycin-exposed lung by 7 days, with a further significant increase at 14 days.

Effects of bleomycin exposure and zileuton (5-LPO inhibitor) treatment on lung LTB$_4$ and CysLT content. Exposure to bleomycin for 7 days had no effect on LTB$_4$ content (data not shown); however, LTB$_4$ content was significantly increased after 14 days of bleomycin exposure (Fig. 3*A*). The bleomycin-induced increase in LTB$_4$ at 14 days was completely prevented by treatment with zileuton (Fig. 3*A*). CysLT (LTC$_4$, LTD$_4$, and LTE$_4$) contents were unaltered by bleomycin exposure or by zileuton treatment, both at 7 (data not shown) and at 14 days (Fig. 3*B*).

Effects of zileuton on bleomycin-induced PHT. As previously reported (30, 44), exposure to bleomycin for 14 days led to significantly increased PVR index (Fig. 4*A*), Fulton index (Fig. 4*B*), and percentage of medial wall area in pulmonary resistance arteries (Fig. 4*C*), relative to vehicle-treated controls. Treatment of

bleomycin-exposed animals with zileuton restored these parameters to values comparable to controls (Fig. 4).

Effects of montelukast (CysLT$_1$R antagonist) or SC57461A (LTA4H inhibitor) on bleomycin-induced PHT. Treatment with montelukast did not prevent bleomycin-induced PHT, as evidenced by a lack of effect on PVR (Fig. 5*A*) and Fulton indexes (Fig. 5*B*) or on percentage of medial wall area (Fig. 5*C*). In contrast, treatment with SC57461A completely normalized PVR index (Fig. 5*A*), Fulton index (Fig. 5*B*), and percentage of medial wall area (Fig. 5*C*). As shown in Fig. 5*E*, treatment with SC57461A completely prevented the bleomycin-induced increase in lung LTB$_4$, which was unaffected by montelukast. To determine whether a lack of effect of montelukast on CysLT$_1$R signaling was responsible for a failure to prevent bleomycin-induced PHT, we examined lung content of CCL4 (also known as macrophage inflammatory protein-1β), a chemokine known

Fig. 3. Zileuton prevented bleomycin-induced increase in leukotriene (LT) B$_4$ content. Pups were exposed to daily intraperitoneal bleomycin (1 mg/kg) or 0.9% saline in 20% DMSO vehicle and treated with daily intraperitoneal zileuton (10 mg·kg^{-1}·day^{-1}) or vehicle (control) from postnatal *days 1–14*. Lung content of LTB$_4$ (*A*) or cysteinyl LTs (*B*) was quantified by ELISA. Values are means ± SE for *n* = 5–6 samples per group. *P < 0.05, by ANOVA, compared with all other groups.
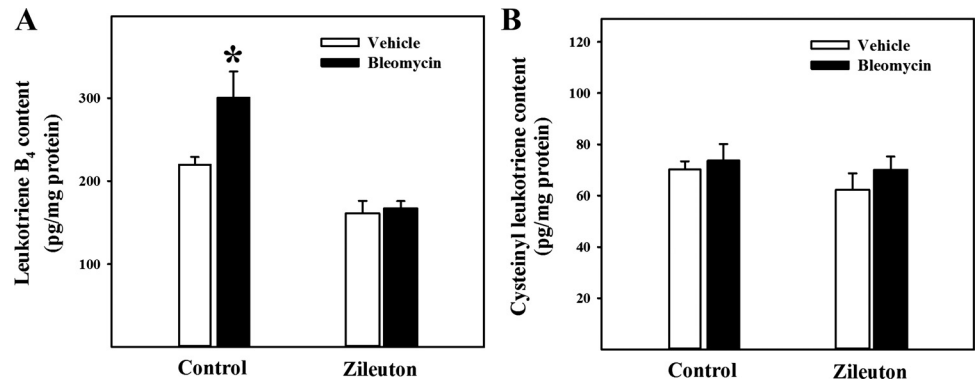
to be stimulated by CysLT$_1$R activation (13). Despite there being no increase in lung CysLT content secondary to bleomycin exposure (Fig. 3*B*), CCL4 was found to be significantly increased in the bleomycin-exposed lung (Fig. 5*F*). Increased lung CCL4 secondary to bleomycin was completely normalized by treatment with montelukast (Fig. 5*F*), in keeping with effective blockade of the cysLT$_1$R.

Effects of zileuton, montelukast, or SC57461A on bleomycin-induced lung inflammation. The lungs of animals exposed to bleomycin had greatly increased CD68-positive cell numbers

Fig. 4. Zileuton prevented bleomycin-induced pulmonary hypertension. Pups were exposed to daily intraperitoneal bleomycin (1 mg/kg) or 0.9% saline in 20% DMSO vehicle and treated with daily intraperitoneal zileuton (10 mg·kg^{-1}·day^{-1}) or vehicle (control) from postnatal *days 1–14*. *A*: pulmonary vascular resistance (PVR) index, as estimated by the right ventricular (RV) ejection time (RVET)-to-pulmonary arterial acceleration time (PAAT) ratio. *n* = 8–11 Animals/group. *B*: RV-to-left ventricle + septum (LV+S) dry weight ratios (Fulton index) as a marker of RV hypertrophy. *n* = 8–12 Animals/group. Tiled low-power photomicrographs of hematoxylin and eosin-stained cardiac sections, oriented in the short axis below the atrioventricular valves (RV cavity = RV), are shown to demonstrate differences in RV wall thickness between groups. *C*: percentage of arterial medial wall area. Values (for *n* = 4 animals/group) are a marker of pulmonary arterial remodeling. Representative photomicrographs of elastin staining (dark brown inner and outer elastic laminae delineating the medial vascular wall; scale bar = 50 µm) are shown demonstrating medial wall thickening in bleomycin-exposed animals (bleomycin control), which was largely prevented by concurrent treatment with zileuton (bleomycin zileuton). Values are means ± SE. *P < 0.05, by ANOVA, compared with all other groups. #P < 0.05, by ANOVA, compared with vehicle groups.
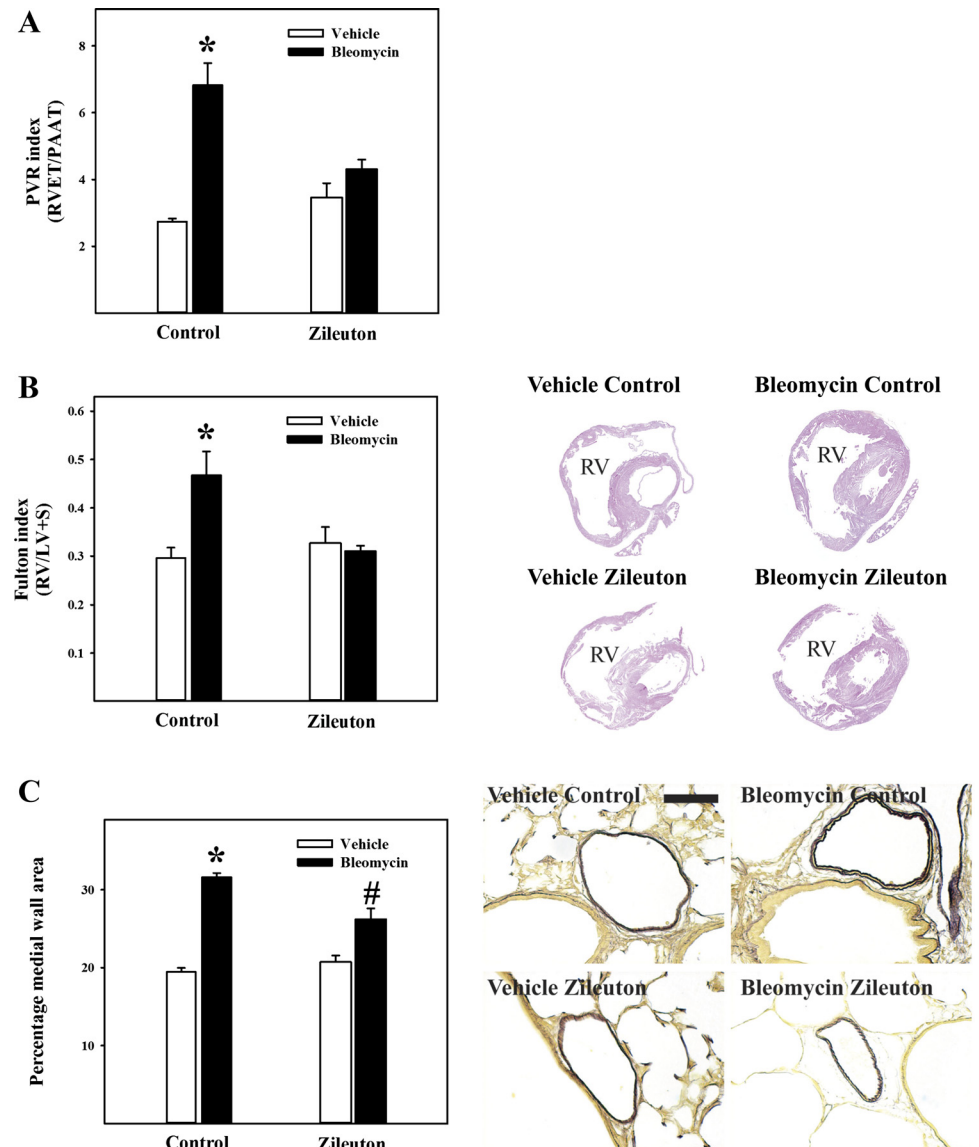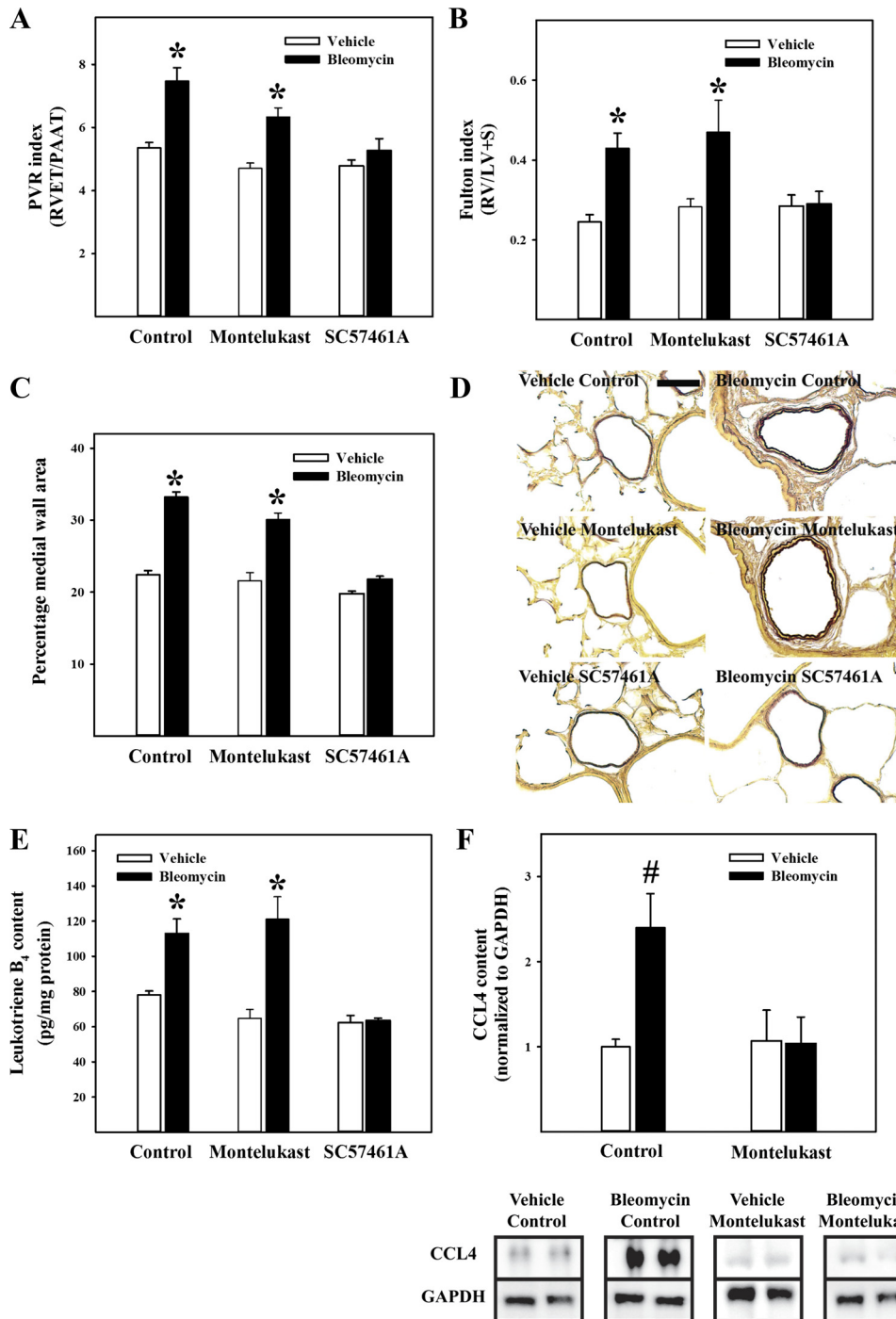
Fig. 5. Bleomycin-induced pulmonary hypertension was prevented by SC57461A, a leukotriene (LT) A$_4$ hydrolase inhibitor, but not by montelukast, a cysteinyl LT receptor antagonist. Pups were exposed to daily intraperitoneal bleomycin (1 mg/kg) or 0.9% saline in 20% DMSO vehicle and treated with daily intraperitoneal montelukast (10 mg·kg^{-1}·day^{-1}) or SC57461A (10 mg·kg^{-1}·day^{-1}) from postnatal *days 1–14*. *A*: pulmonary vascular resistance (PVR) index, as estimated by the right ventricular (RV) ejection time (RVET)-to-pulmonary arterial acceleration time (PAAT) ratio. $n = 6$–10 Animals/group. *B*: RV-to-left ventricle + septum (LV+S) dry weight ratios (Fulton index) as a marker of RV hypertrophy. $n = 4$–6 Animals/group. *C*: percentage of arterial medial wall area ($n = 4$ animals/group) as a marker of pulmonary arterial remodeling. *D*: representative photomicrographs of elastin staining (dark brown inner and outer elastic laminae delineating the medial vascular wall). Scale bar = 50 μm. *E*: lung content of LTB$_4$, quantified by ELISA. $n = 6$ Samples/group. *F*: Western blot analyses of CCL4 (17 kDa). Representative immunoblots are shown *below* the graph with noncontiguous gel lanes demarcated by black lines. $n = 4$ Samples/group. Values are means ± SE. *$P < 0.01$, by *t*-test, compared with vehicle group. #$P < 0.05$, by ANOVA, compared with all other groups.

(Fig. 6*A*) and increased lung TNF-α content (Fig. 6*B*). Treatment of bleomycin-exposed animals with zileuton or SC57461A significantly decreased tissue macrophage numbers (Fig. 6*A*), while montelukast had no effect (Fig. 6*A*). Treatment of bleomycin-exposed animals with zileuton also normalized lung TNF-α content (Fig. 6*B*), in keeping with previous findings indicating that TNF-α is predominantly macrophage derived (44). Treatment with zileuton also completely prevented neutrophil influx in the bleomycin-exposed lung (Fig. 6*C*).

Effects of zileuton, montelukast, or SC57461A on bleomycin-induced changes in lung morphology. As demonstrated by representative low-power elastin-stained sections (Fig. 7*A*), the lung structure of bleomycin-exposed animals was characterized by septal thinning, arrested alveolarization [manifesting as "emphysematous" distal air spaces, quantified as increased mean chord length (Fig. 7*B*)], and vascular hypoplasia (Fig. 7*C*). Treatment with zileuton, montelukat, or SC57461A had no effects on these parameters (Fig. 7).

DISCUSSION

In human preterm infants with respiratory distress, early increases in lung macrophages are known to persist in infants who develop severe BPD, while declining in those who do not (8). The
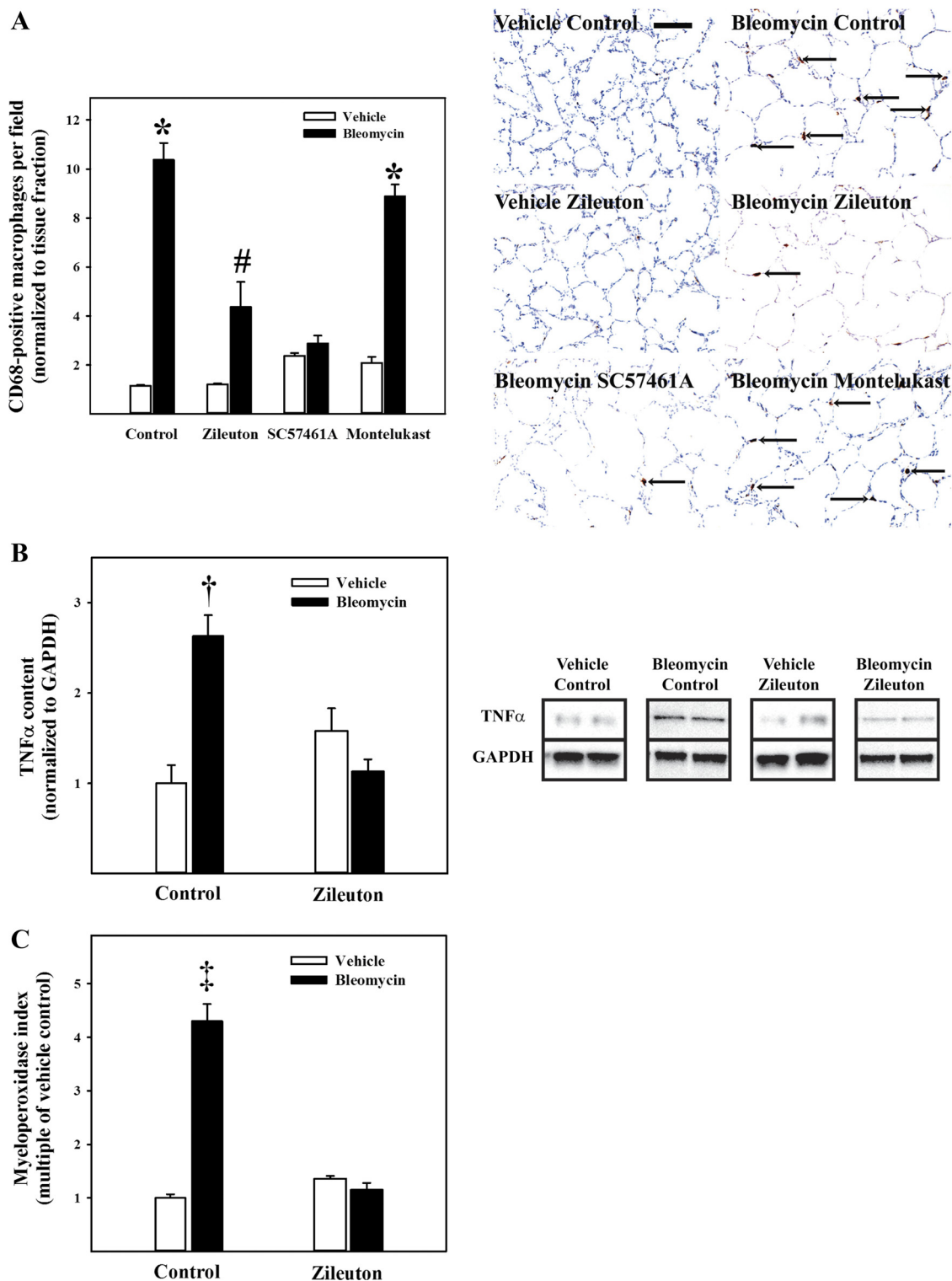
Fig. 6. Effects on inflammation in the bleomycin-exposed lung. Pups were exposed to daily intraperitoneal bleomycin (1 mg/kg) or 0.9% saline in 20% DMSO vehicle and treated with daily intraperitoneal zileuton (10 mg·kg^{-1}·day^{-1}), montelukast (10 mg·kg^{-1}·day^{-1}), SC57461A (10 mg·kg^{-1}·day^{-1}), or vehicle (control) from postnatal *days 1–14*. *A*: tissue macrophage counts per field normalized to tissue fraction. Representative medium-power photomicrographs are shown of CD68 immunostaining demonstrating increased numbers of tissue macrophages (large dark brown cells, some highlighted by arrows) in bleomycin-exposed animals (bleomycin control), which was prevented by treatment with zileuton (bleomycin zileuton) or SC57461A (bleomycin SC57461A), but not by montelukast (bleomycin montelukast). Scale bar = 100 μm. *B*: Western blot analyses of lung TNF-α (17 kDa) content. Representative immunoblots are shown adjacent to the graph with noncontiguous gel lanes demarcated by black lines. *C*: tissue neutrophil index. All values are means ± SE for 4 animals or samples per group. *$P < 0.001$, by *t*-test, compared with vehicle group. #$P < 0.05$, by *t*-test, compared with vehicle group. †$P < 0.05$, by ANOVA, compared with all other groups. ‡$P < 0.001$, by ANOVA, compared with all other groups.
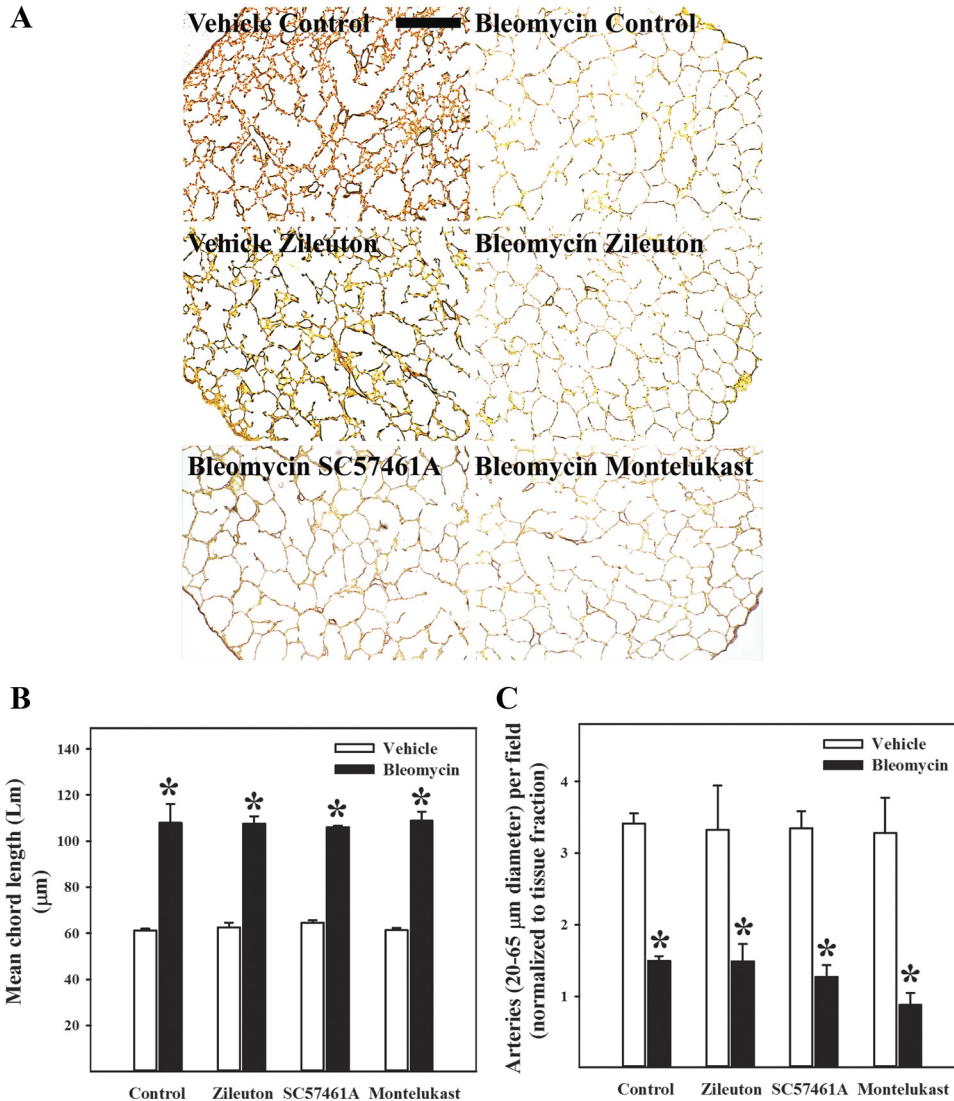
Fig. 7. Targeting leukotriene pathways did not prevent abnormal lung morphology induced by bleomycin. Pups were exposed to daily intraperitoneal bleomycin (1 mg/kg) or 0.9% saline vehicle and treated with daily intraperitoneal zileuton (10 mg·kg^{-1}·day^{-1}), montelukast (10 mg·kg^{-1}·day^{-1}), SC57461A (10 mg·kg^{-1}·day^{-1}), or 20% DMSO in 0.9% saline (control) from postnatal *days 1–14*. *A*: representative low-power photomicrographs of elastin-stained sections demonstrating marked distal airway simplification, septal thinning, and decreased numbers of small peripheral arteries (outlined by dark brown stain for elastin) in bleomycin-exposed (bleomycin control) animals, which were not improved by treatment with zileuton (bleomycin zileuton), montelukast (bleomycin montelukast), or SC57461A (bleomycin SC57461A). Scale bar = 250 µm. Morphometric analyses of mean chord length (Lm; *B*) and peripheral arteries per field (*C*), corrected for tissue fraction, are shown. Values are means ± SE for *n* = 4 animals/group. *$P < 0.001$, by *t*-test, compared with vehicle group.

potential role of LTs in this inflammatory process has received limited attention. We report in bleomycin-exposed neonatal rat pups, a model with similarities to human BPD, that treatment with zileuton, a 5-LPO inhibitor, or SC57461A, a LTA4H inhibitor, prevented upregulation of LTB$_4$, inflammatory cell influx, and the hemodynamic and structural changes of chronic PHT. Our laboratory has previously shown that macrophage-derived TNF-α is critical to bleomycin-induced chronic PHT (44). In the present study, increased TNF-α was also attenuated by 5-LPO inhibition. Treatment with montelukast, a cysLT$_1$R inhibitor, had no effect on macrophage influx or on markers of chronic PHT. These findings are in agreement with studies in adult experimental animals of inflammatory injury, which favor a dominant role for LTB$_4$, rather than for cysLTs (49–51).

We did not observe any effects of LT pathway inhibition on arrested alveolarization or vascular hypoplasia. This divergence is consistent with our laboratory's own previous observations employing alternative interventions [e.g., therapeutic hypercapnia, arginase inhibition (15, 44)] that were also efficacious in preventing inflammation and vascular remodeling, but not in preventing vascular hypoplasia or emphysematous lung structure. It appears, therefore, at least in bleomycin-exposed neonatal rats, that inflammation and vascular remodeling are regulated by pathways distinct from those that regulate angiogenesis and alveologenesis. Whether this also holds true in humans remains unclear.

We observed increased mRNA expression of 5-LPO, FLAP, BLT1, and BLT2, which was confirmed at the protein level for 5-LPO and FLAP in the lungs of bleomycin-exposed animals. We did not observe increased lung content of phospho-serine 271 5-LPO, which increases 5-LPO activity and favors LTB$_4$ synthesis (41, 55). Nonetheless, these changes were accompanied by increased lung LTB$_4$ in the bleomycin-exposed lung. The reasons for our observations regarding temporal changes in 5-LPO and FLAP (increased at *day 7*, but not at *day 14*) are unclear, but could represent a negative feedback effect of increased LTB$_4$, which was only elevated at *day 14*. The degree of increase in LTB$_4$ secondary to bleomycin exposure was small, which we speculate may have reflected a dilutional effect inherent to measurement in whole tissue.

LTB$_4$ has been previously described to be predominantly produced by inflammatory cells, particularly activated macrophages and neutrophils (32, 50), which are both present in

abundance in the bleomycin-exposed lung (15, 26, 30, 44). In the present study, we were unable to determine the cellular sources of LTB$_4$, due to a lack of commercially available antibodies suitable for in situ immunostaining. However, it appears unlikely that macrophages are a major source of LTB$_4$ in the lungs of bleomycin-exposed neonatal rats, given our present findings that tissue macrophage numbers plateaued by 7 days of bleomycin exposure when LTB$_4$ was not increased. Increased LTB$_4$ production by pulmonary artery endothelial cells has also been described (32, 50). As nonmyeloid cells are poor in 5-LPO, LT production in such cells is believed to be facilitated by intercellular transfer of LTA$_4$ (12, 32, 50). The pattern of temporal changes in inflammatory cell number and the observation that treatment with zileuton decreased both macrophage and neutrophil influx opens the possibility that one inflammatory cell type may recruit the other, particularly that macrophages may recruit neutrophils; however, our laboratory's previous work in the present model suggests that this is not the case. For example, an intervention (therapeutic hypercapnia) that prevented macrophage influx had no effect on tissue neutrophils (44). Conversely, a CXCR2 antagonist intervention that prevented neutrophil influx had no effect on increased tissue macrophages or on the development of PHT (26).

Our present results are consistent with previous work by Tian and colleagues (51), in which blockade of LTA4H-expressing macrophages prevented endothelial injury and reversed PHT in sugen-exposed, athymic rats. Based on in vitro observations, the suggested mechanism of LTB$_4$-mediated injury was endothelial cell apoptosis via inhibited expression of sphingosine-1-phosphate and endothelial nitric oxide synthase (51). Whether changes in sphingosine-1-phosphate and endothelial nitric oxide synthase represented a direct effect of LTB$_4$ or the result of limiting macrophage influx and thereby other macrophage-derived products was unclear. LTB$_4$ has also been reported to stimulate pulmonary artery smooth muscle cell proliferation and migration (17) and to activate adventitial fibroblasts (40), thus potentially playing a direct role in vascular remodeling.

There are several limitations to this study. First, a major feature of bleomycin-induced lung injury is collagen deposition, which is not a consistent feature of modern BPD. Our use of this model was predicated on a striking degree of arrested lung growth and development, which is disproportionate to the effects on other organs, despite systemic administration (53). Second, our intervention studies demonstrating an effect in preventing chronic PHT were limited to inhibition of 5-LPO and LTA4H. Confirmation of the marked upregulation of BLT1 mRNA in the bleomycin-exposed lung at the protein level, localization of BLT1-expressing cells in the bleomycin-exposed lung, and further intervention studies employing specific BLT1 antagonists (17, 40, 51) are a focus of ongoing and future work. The cellular localization of LT pathway mediators upregulated in the bleomycin-exposed lung and whether LTB$_4$ plays a direct role in the pathogenesis of vascular remodeling, in addition to an indirect role via macrophages, are also issues worthy of future consideration. Finally, our early preventive strategy in the present study was to treat throughout the 14-day period of injury. As macrophage influx in this model plateaued by 7 days of life, it is possible that a shorter duration of therapy may be sufficient to confer a protective effect.

In conclusion, LTB$_4$, but not cysLTs, appears to play a crucial role in lung inflammation and PHT in experimental BPD-like injury secondary to systemic bleomycin. Agents targeting LTB$_4$ or LTB$_4$-mediated signaling may have utility in human infants at risk of developing BPD-associated PHT.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

M.T.E. and R.P.J. conception and design of research; M.T.E., C.K., J.I., M.J.W., A.J., and R.P.J. performed experiments; M.T.E., C.K., J.I., M.J.W., A.J., and R.P.J. analyzed data; M.T.E. and R.P.J. interpreted results of experiments; M.T.E. and R.P.J. prepared figures; M.T.E. and R.P.J. drafted manuscript; M.T.E., C.K., J.I., M.J.W., A.J., and R.P.J. edited and revised manuscript; M.T.E., C.K., J.I., M.J.W., A.J., and R.P.J. approved final version of manuscript.

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