Improved Survival and Reduced Vascular Permeability by Eliminating or Blocking 12/15-Lipoxygenase in Mouse Models of Acute Lung Injury (ALI)[1]

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Acute lung injury (ALI) is a prevalent disease associated with high mortality. 12/15-lipoxygenase (12/15-LO) is an enzyme producing 12-hydroxyeicosatetraenoic acid (HETE) and 15-HETE from arachidonic acid. To test whether 12/15-LO is involved in increasing vascular permeability in the lung, we investigated the role of 12/15-LO in murine models of LPS-induced pulmonary inflammation and clinically relevant acid-induced ALI. The vascular permeability increase upon LPS inhalation was abolished in *Alox15*$^{-/-}$ mice lacking 12/15-LO and in wild-type mice after pharmacological blockade of 12/15-LO. *Alox15*$^{-/-}$ mice also showed improved gas exchange, reduced permeability increase, and prolonged survival in the acid-induced ALI model. Bone marrow chimeras and reconstitution experiments revealed that 12-HETE produced by hematopoietic cells regulates vascular permeability through a CXCR2-dependent mechanism. Our findings suggest that 12/15-LO-derived 12-HETE is a key mediator of vascular permeability in acute lung injury. *The Journal of Immunology,* 2009, 183: 4715–4722.

Acute lung injury (ALI)[3] is a common disease with an incidence of 79 per 100,000 person-years in the United States (1). Despite improved treatment, this disease is associated with a high mortality of up to 38%. The 3.6 million hospital days per year associated with ALI have a large impact on the health system (1). ALI has intrapulmonary causes such as pneumonia and aspiration of gastric content, and extrapulmonary causes such as sepsis and massive transfusion. In ALI, the alveolar capillary and epithelial membranes are damaged by infiltration of polymorphonuclear neutrophils and monocytes, leading to leakage of protein-rich edema fluid into the alveolar space, formation of hyaline membranes, and impaired gas exchange (2). Currently available treatments are only marginally effective (3).

Tissue injury of many etiologies changes vascular barrier function and consequently leads to fluid loss, edema, and organ dysfunction (4–7). The vascular endothelium presents the predominant barrier to prevent movement of molecules across the blood vessel wall (5, 8). Under physiological conditions, vascular permeability is tightly regulated. In response to different inflammatory stimuli, the increase of endothelial permeability is regulated by several receptors, including the chemokine receptor CXCR2, the adenosine receptor A2B, and the thromboxane A_2 receptor (7, 9, 10). Sphingosine 1-phosphate, a biologically active lipid, also regulates endothelial cell activation and vascular permeability by binding to several G protein-coupled receptors (11, 12).

In addition to chemokines, lipid mediators play a role in pulmonary inflammation (13–15). Lipoxygenases incorporate oxygen into unsaturated fatty acids and are named according to the position of the carbon double bonds they oxidize (16). Humans and rabbits express 15-lipoxygenase (15-LO) (17, 18), whereas pigs, rats, and mice express "leukocyte-type" 12-LO (19) with some 15-LO activity. Mouse leukocyte 12/15-LO is highly related to 15-LO in humans and probably represents the mouse ortholog of human 15-LO (20, 21). 12/15-LO catalysis of arachidonic acid yields short-lived peroxidized products, which are reduced or enzymatically converted to 12-hydroxyeicosatetraenoic acid (12-HETE), lipoxins, hepoxilins, and others (22). 12/15-LO mRNA expression is highest in monocytes and macrophages (23), and some is found in endothelial cells (24) and other cells. 12-HETE is a proinflammatory chemoattractant for neutrophils (25), regulates endothelial cell cytoskeleton rearrangement (26), induces cytokine production (27) and expression of adhesion molecules on endothelial cells (28), and is involved in chronic inflammatory processes (23). *Alox15*$^{-/-}$ mice were found to be protected from the development of allergic sensitization and airway inflammation (29). However, leukotrienes and other lipoxygenases, such as the 5-LO, are also involved in modulating inflammation. Elimination of 5-LO preserves gas exchange and increases survival in a ventilator-induced lung injury model (30).

The present study was designed to investigate the role of 12/15-LO and 12-HETE on vascular permeability in murine models of LPS-induced pulmonary inflammation and acid-induced lung

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Received for publication August 6, 2008. Accepted for publication August 3, 2009.

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[1] This study was supported by Else Kröner-Fresenius-Stiftung (Grant A69/07 to A.Z.), by the German Research Foundation (ZA428/2-1 to A.Z., VI508/1-1 to S.v.V.), by National Institutes of Health Grant P01 HL 73361 to Joel Linden (project 2 to K.L.), and by National Institutes of Health Grant P01 HL 55798 to J.N. (project 4 to K.L.).

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[3] Abbreviations used in this paper: ALI, acute lung injury; BAL, bronchoalveolar lavage; CDC, cinnamyl-3,4-dihydroxy-α-cyanocinnamate; FiO_2, fraction of inspiratory oxygen; HETE, hydroxyeicosatetraenoic acid; LO, lipoxygenase; PaO_2, partial pressure of arterial oxygen; WT, wild type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802592

injury. To investigate the main source of 12/15-LO relevant to vascular permeability, chimeric mice were generated by bone marrow transplantation that expressed 12/15-LO in hematopoietic cells, nonhematopoietic cells, both, or neither. To address the mechanism by which 12/15-LO products influence vascular permeability in ALI, we tested the interaction between 12-HETE and the CXCL1-CXCR2 axis of inflammatory chemokine signaling.

Materials and Methods

Animals

We used 8- to 12-wk-old C57BL/6 mice (The Jackson Laboratory) and *Alox15*-deficient mice (31) backcrossed to C57BL/6 for at least 10 generations (UVA colony) (32). Furthermore, *Cxcr2*$^{-/-}$ mice (The Jackson Laboratory) on the BALB/c background and BALB/c controls were used. Mice were housed in a barrier facility under specific pathogen-free conditions. The Animal Care and Use Committee of the University of Virginia (Charlottesville) approved all animal experiments.

Murine model of LPS-induced pulmonary inflammation and acid-induced ALI

LPS from *Salmonella enteritidis* (500 μg/ml; Sigma-Aldrich) was nebulized to induce pulmonary inflammation as previously described (33). Briefly, mice were exposed to 30 min of aerosolized LPS or saline aerosol as a control.

In some experiments 12/15-LO activity was pharmacologically inhibited by cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC; BIOMOL International). Mice were injected 1 h (8 mg/kg, i.p.) before induction of the pulmonary inflammation.

Acid-induced ALI was induced by injection of 2 μl/g HCl (pH 1.5) intratracheally, followed by a bolus of air (30 μl/g) as previously described (7). Following a tracheotomy, mice were ventilated with a respirator (MiniVent, type 845; Hugo Sachs Elektronik) for 2 h (tidal volume, 10 μl/g; respiration rate, 140/min; fraction of inspiratory oxygen (FiO_2), 0.21). Control animals received saline instead of HCl in the same manner.

Pulmonary microvascular permeability

We determined pulmonary microvascular permeability in wild-type (WT), *Alox15*$^{-/-}$, and chimeric mice using the Evans blue dye extravasation technique as described previously (10). Briefly, Evans blue (20 mg/kg; Sigma-Aldrich) was injected i.v. 30 min before euthanasia. Lungs were perfused, removed, and Evans blue was extracted. The absorption of Evans blue was measured and calculated. The results were corrected for hemoglobin. Extravasated Evans blue was determined in the different animal groups 6 h after LPS or saline inhalation and expressed as micrograms per gram of lung. Vascular permeability in the acid-induced lung injury model was determined by measuring protein concentration in the supernatant of the bronchoalveolar lavage (BAL) (Lowry's method). In some experiments CXCL1 was neutralized by a polyclonal Ab (R&D Systems). Mice were injected either with the Ab or a preimmune control 6 h (25 mg/kg, i.p.) before induction of the pulmonary inflammation.

To measure pulmonary blood volume, we injected Evans blue 5 min before the end of the experiment. After this time, mice were killed, the pulmonary vessels ligated, the vessels transected proximal to the ligation, and the lungs removed. The weight of the blood-filled lungs and the Evans blue concentration in lungs and blood were measured. The period between the injection of the Evans blue and the end of the experiment was too short for the Evans blue to leave the intravascular compartment, and we calculated blood volume based on indicator dilution: $V_{pul} = V_B \times M_{EB}$ (in lung)/C_{EB} (in blood), where V_{pul} is the blood volume in the lung, M_{EB} is the concentration of EB in whole lung tissue including the trapped blood, C_{EB} is the concentration of EB in the blood, and V_B is the blood volume measured.

In some experiments, the lungs were removed, wet weight was measured, and the lungs were dried in an incubator set at 70°C. The dry weight was obtained after 72 h of incubation and the ratio of wet-to-dry weight was calculated.

Pulmonary function: oxygenation

Blood was obtained from an arterial catheter, and standard arterial blood gas analyses were accomplished 2 h after the induction of the acid-induced ALI (Rapidlab 800 System; Bayer HealthCare). Partial pressure of arterial oxygen (PaO_2) was normalized to FiO_2 to yield the oxygenation index (PaO_2/FiO_2).

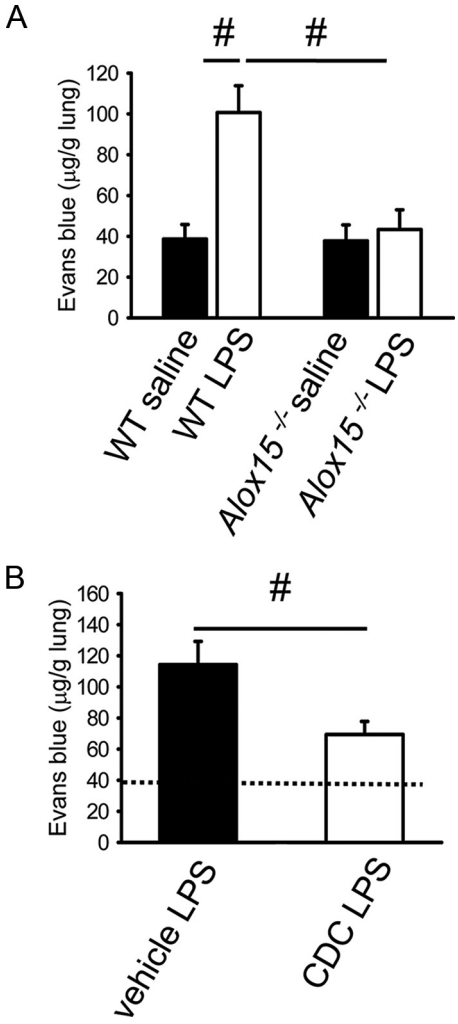

FIGURE 1. 12/15-LO is involved in LPS-induced regulation of vascular permeability. *A*, Vascular permeability of WT mice and *Alox15*$^{-/-}$ mice was measured 6 h after LPS inhalation by the Evans blue method ($n = 6$–7). *B*, After pharmacological blockade of 12/15-LO by the inhibitor CDC (open bars) or in vehicle control mice (filled bars), vascular permeability (6 h after LPS) was measured by the Evans blue method ($n = 4$ each). Dotted line indicates Evans blue in saline-treated WT mice for comparison. #, $p < 0.05$.

In vitro endothelial and epithelial cell assay

To assess the distribution of F-actin upon 12-HETE stimulation (Sigma-Aldrich), human pulmonary microvascular endothelial cells (American Type Culture Collection) or human alveolar epithelial cells (American Type Culture Collection) were cultured on glass coverslips. After stimulation, cells were fixed, permeabilized, and stained with FITC-phalloidin (Invitrogen) as previously described (7). Coverslips were mounted on glass slides, and microscopy was accomplished on a Nikon Diaphot inverted fluorescence microscope.

Generation of 12/15-LO chimeric mice

To distinguish the role of non- and hematopoietic 12/15-LO in pulmonary inflammation, chimeric mice were generated following a previously described protocol (7). C57BL/6 (on CD45.2 and CD45.1 background) and *Alox15*-deficient mice (CD45.2) were used as donors and/or recipients. Recipient mice were lethally irradiated in two doses of 600 rad each (separated by 4 h). Bone marrow was isolated from donor mice under sterile conditions, and ~5×10^6 cells were injected i.v. into recipient mice. Experiments were performed 6 wk after bone marrow transplantation.

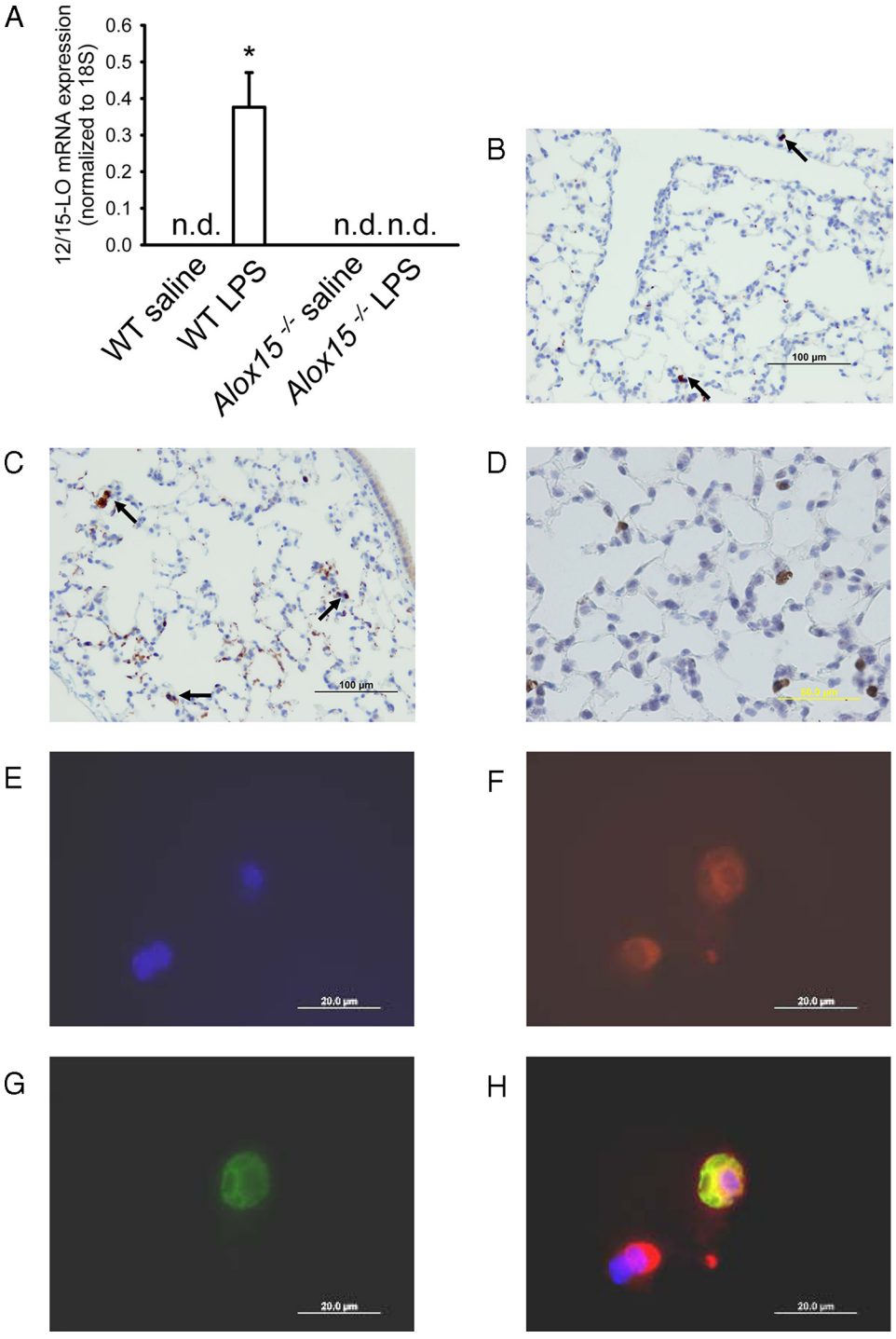

FIGURE 2. 12/15-LO produced in lung macrophages is up-regulated following LPS inhalation. *A*, 12/15-LO mRNA expression in lungs of unstimulated and stimulated WT mice and *Alox15*-deficient mice was investigated by quantitative RT-PCR (n.d., not detectable). 12/15-LO protein expression shown by immunohistochemistry in lungs from untreated WT mice (*B*) and WT mice 3 h after LPS inhalation (*C* and *D*). *E–H*, Fluorescence microscopy images of monocytes/macrophages in the lung upon LPS stimulation. Nuclei (blue, DAPI, *E*), Mac-2 (red, clone M3/38, *F*), and 12/15-LO (green, polyclonal rabbit anti-porcine, *G*). *H*, The merged image demonstrates colocalization (yellow) of Mac-2 and 12/15-LO in one of the two monocytes/macrophages. Scale bar equals 20 μm (*E–H*).

Quantitative real-time RT-PCR

Total RNA from whole lung tissue was extracted using TRIzol (Invitrogen). Reverse transcription and PCR steps were performed using QuantiTect SYBR Green RT-PCR kit (Qiagen) on an iCycler iQ real-time detection system (Qiagen), and sequence-specific primers were designed on Beacon Designer 2.06 software. Samples used to construct the standard curve consisted of WT LPS-stimulated mesenteric peritoneal macrophages using 20, 6.3, and 2 ng of RNA. One and a half micrograms of total RNA was used for all lung samples. Values were determined using iCycler iQ real-time detection system software v3.0 (Qiagen). The corresponding values were normalized to 18S mRNA.

The primers for CXCR-2 (forward, 5′-ATGCCCTCTATTCTGCC AGAT-3′, reverse, 5′-GTGCTCCGGTTGTATAAGATGAC-3′) were selected from the National Center for Biotechnology Information Primer Bank.

Chemokine measurements

CXCL1 in the BAL fluid were measured in triplicates using ELISA kits, following the procedures supplied by the manufacturer (R&D Systems). Chemokines were determined in control mice (saline) and LPS-treated mice.

Histology

To visualize morphological changes during LPS-induced lung injury, paraffin-embedded lung sections (5 μm) were stained for 12/15-LO (polyclonal rabbit anti-porcine Ab) (34) using the avidin-biotin technique (Vector Laboratories) as described previously (35).

For immunofluorescence labeling, biotin-labeled Mac-2 (clone M3/38; Accurate Chemicals) was visualized with streptavidin Alexa Fluor 555 (Molecular Probes), 12/15-LO was visualized by using anti-rabbit-Alexa

Fluor 488 (Molecular Probes), and nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories).

Statistics

Statistical analysis was performed with SPSS (version 9.0) and included one-way ANOVA, a Student-Newman-Keuls test, or a *t* test where appropriate. The Kaplan-Meier method was used for analyzing survival rate. All data are presented as means ± SEM. A value of $p < 0.05$ was considered significant.

Results

12/15-LO is involved in LPS-induced regulation of vascular permeability

To investigate the role of 12/15-LO in pulmonary inflammation, mice were exposed to aerosolized LPS for 30 min and vascular permeability was determined. Vascular permeability as measured by the Evans blue method (10) significantly increased in WT mice 6 h after LPS stimulation compared with saline control mice (Fig. 1*A*). Baseline vascular permeability of *Alox15*$^{-/-}$ mice (31) was similar to WT mice, but *Alox15*$^{-/-}$ mice were completely protected from permeability increase upon LPS inhalation (Fig. 1*A*).

To investigate whether the lack of 12/15-LO had an effect on the lung vascular surface area, we measured pulmonary blood volume in WT mice and *Alox15*$^{-/-}$ mice. Pulmonary blood volume was similar in WT mice and *Alox15*$^{-/-}$ mice (WT, 141 ± 13 μl; *Alox15*$^{-/-}$, 153 ± 21 μl; $n = 4$, NS). There was no difference in lung wet-to-dry weight ratio (data not shown). These data suggest that the lack of 12/15-LO does not influence lung vascular surface area.

Alox15$^{-/-}$ mice lack the *Alox15* gene and all its products from conception. To investigate whether acute blockade of 12/15-LO by a pharmacological inhibitor also reduces vascular permeability, mice were injected with the 12/15-LO inhibitor CDC 1 h before LPS exposure. A single injection of 8 mg/kg CDC was previously shown to significantly reduce 12/15-LO activity, as measured by urinary 12-HETE concentration (36). Similar to the observation in *Alox15*$^{-/-}$ mice, acute pharmacological inhibition of 12/15-LO also significantly reduced vascular permeability (Fig. 1*B*).

LPS inhalation induces 12/15-LO expression in Mac-2^{+} *cells in the lung*

To determine 12/15-LO expression in lungs, we measured 12/15-LO mRNA levels by quantitative RT-PCR. Without stimulation, 12/15-LO mRNA expression was not detectable in lungs from WT mice, but significantly increased 3 h after LPS exposure (Fig. 2*A*). As expected, 12/15-LO mRNA was undetectable in *Alox15*$^{-/-}$ mice. 12/15-LO protein expression in the lung was visualized by immunohistochemistry with and without LPS stimulation. Rare 12/15-LO-expressing cells were detectable in lungs of untreated mice (Fig. 2*B*). After LPS inhalation, more 12/15-LO-expressing cells were found (Fig. 2, *C* and *D*). To investigate whether lung macrophages are the main source of 12/15-LO, we used immunofluorescence microscopy of the lung. LPS-treated lungs were stained for both 12/15-LO and Mac-2, a macrophage marker. The colocalization (Fig. 2*H*) of the nucleus (Fig. 2*E*), Mac-2 (Fig. 2*F*), and 12/15-LO (Fig. 2*G*) showed that some but not all Mac-2^{+} cells express 12/15-LO in the lung after LPS exposure. In untreated lungs from WT mice, only 0.75% of macrophages were positive for 12/15-LO. Three hours following LPS exposure the percentage increased to 4% ($n = 3$, $p < 0.05$).

Hematopoietic 12/15-LO is responsible for regulation of vascular permeability following LPS inhalation

To determine whether 12/15-LO expression in bone marrow-derived cells is important in lung inflammation, we generated chimeric mice using *Alox15*$^{-/-}$ mice (CD45.2) and CD45.1 WT mice. CD45.1 and CD45.2 are functionally equivalent alleles of the CD45 Ag expressed by all leukocytes. Six weeks after the bone marrow transplantation, >90% of all macrophages/monocytes in the lung were reconstituted with cells from donor mice (Fig. 3*A*).

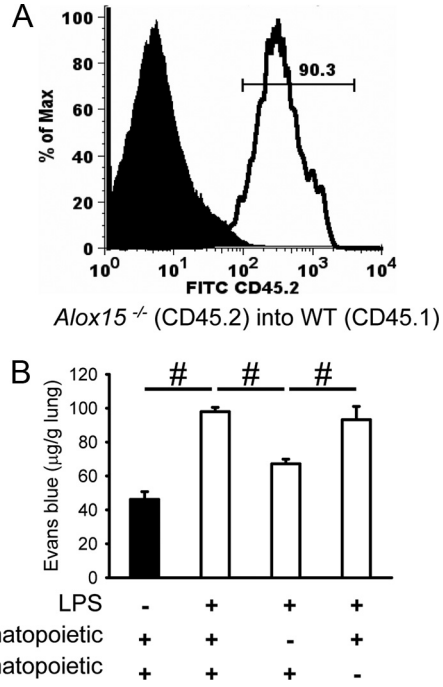

FIGURE 3. Hematopoietic 12/15-LO is responsible for regulation of vascular permeability following LPS inhalation. *Alox15*$^{-/-}$ mice on a CD45.2 background and CD45.1 WT mice were used in bone marrow transplant experiments. *A*, Monocytes and macrophages in single cell lung preparations were identified by gating on CD45^{+}F4/80^{+} cells, and the percentage of reconstituted cells are shown in the histogram by CD45.2 expression (donor-derived). *B*, Chimeric mice lacking hematopoietic *Alox15* are protected from increased vascular permeability as measured by Evans blue extravasation upon LPS inhalation ($n \geq 4$). #, $p < 0.05$.

Mice lacking hematopoietic *Alox15* were almost completely protected from increased vascular permeability after LPS inhalation (Fig. 3*B*). Chimeric mice lacking nonhematopoietic *Alox15* showed a similar increase of vascular permeability after LPS exposure as control mice (Fig. 3*B*). No 12/15-LO mRNA was detectable in blood leukocytes (data not shown), suggesting that monocyte-derived resident lung macrophages are the main source of 12/15-LO.

12-HETE regulates vascular permeability

12/15-LO generates a number of oxidized lipids from various substrates (22). The main arachidonic acid product is 12-HETE. Therefore, we investigated 12-HETE concentration in BAL fluid of WT mice and *Alox15*$^{-/-}$ mice 3 h after LPS inhalation. In WT mice, the concentration of 12-HETE was 42 ± 4 pg/ml, but 12-HETE was undetectable in *Alox15*$^{-/-}$ mice. Cultured human pulmonary endothelial cells and human alveolar epithelial cells stimulated with 0.1 nM (32 pg/ml) 12-HETE, a concentration similar to that found in the BAL fluid of WT mice after LPS inhalation, showed a striking increase in F-actin. F-actin rings appeared at the edge of the cells and caused retraction. These changes reached a peak at 15 min (Fig. 4, *A* and *B*, and data not shown), suggesting

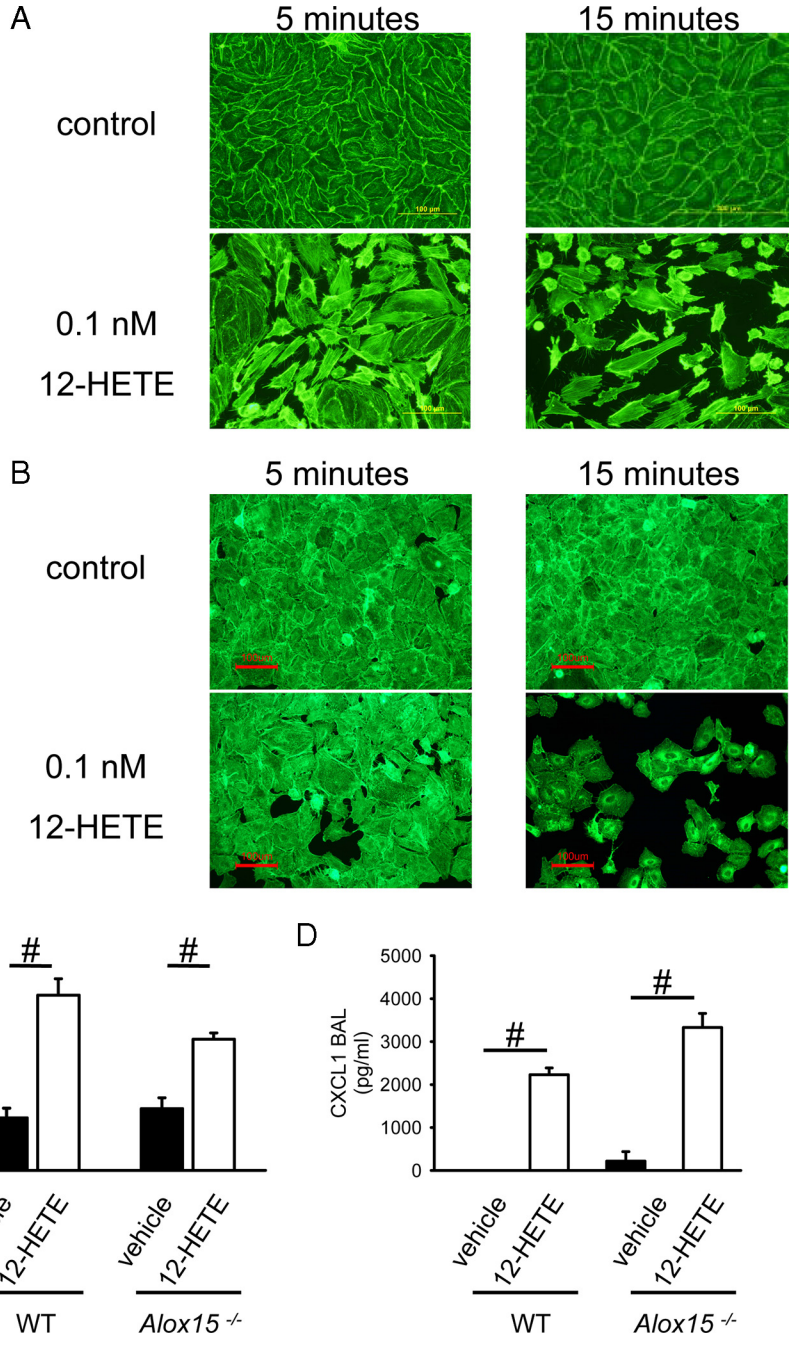

FIGURE 4. 12-HETE regulates endothelial and epithelial integrity and vascular permeability. Endothelial (*A*) and alveolar (*B*) cell response to 12-HETE activation as reflected by F-actin localization. Human pulmonary endothelial (*A*) and alveolar (*B*) cells were treated with 0.1 nM 12-HETE, and F-actin was localized by phalloidin staining. Images are representative of three experiments with similar results. *C*, Vascular permeability was measured by the Evans blue method 6 h after intratracheal injection of 12-HETE or vehicle into WT or *Alox15*$^{-/-}$ mice ($n = 4$). #, $p < 0.05$. *D*, CXCL1 levels in the lung of WT and *Alox15*$^{-/-}$ mice after 12-HETE application.

that 12-HETE is a major regulator of vascular permeability following LPS inhalation. Consistent with these in vitro data, intratracheal 12-HETE application of 5 pg (100 μl of 50 pg/ml solution) induced increased vascular permeability in WT and *Alox15*$^{-/-}$ mice (Fig. 4*C*). CXCL1 is an important ligand for CXCR2, a receptor known to be involved in regulating lung endothelial permeability (10). CXCL1 levels measured by ELISA increased in response to intratracheal injection of 12-HETE in WT mice and *Alox15*$^{-/-}$ mice ($p < 0.05$; Fig. 4*D*). The levels of CXCL1 in *Alox15*$^{-/-}$ mice and WT mice were similar.

Role of CXCR2 and CXCL1 in 12-HETE-induced permeability

To address the mechanism through which 12-HETE induces vascular permeability of the lung, we investigated the role of the chemokine receptor CXCR2 and one of its ligands, CXCL1. CXCR2 expressed by endothelial and epithelial cells plays an important role in regulating vascular permeability following LPS exposure of the lung (10). To investigate the role of the CXCL1/CXCR2 axis in WT mice and *Alox15*$^{-/-}$ mice, we determined the CXCL1 levels in the BAL fluid and mRNA expression of CXCR2 in the lung following LPS inhalation. CXCL1 levels in the BAL fluid of WT mice and *Alox15*$^{-/-}$ mice were similar under baseline conditions (Fig. 5*A*). The levels of CXCL1 in the BAL fluid significantly increased in WT and *Alox15*$^{-/-}$ after LPS exposure with a more pronounced increase in *Alox15*$^{-/-}$ mice compared with WT mice (Fig. 5*A*). CXCR2 mRNA expression in WT mice and *Alox15*$^{-/-}$ mice significantly increased following LPS stimulation (Fig. 5*B*). It appears that CXCR2 has a special role among chemoattractant receptors in that it seems to control neutrophil access to the lung (37). Since vascular permeability in the lung is almost completely dependent on CXCR2 after LPS inhalation (10), but also on 12/15-LO (present data), we reasoned that CXCR2 may also be

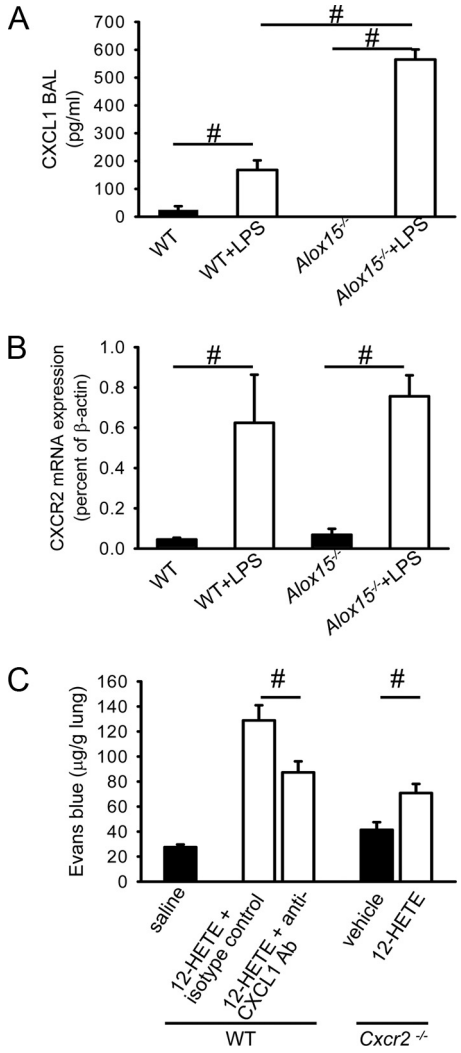

FIGURE 5. 12-HETE-induced vascular leakage requires CXCL1 and CXCR2. CXCL1 levels in the BAL fluid (*A*) and CXCR2 mRNA expression in the lung of WT mice and *Alox15*$^{-/-}$ mice (*B*). Vascular permeability was measured by the Evans blue method 6 h after intratracheal injection of 12-HETE (open bars) or vehicle (filled bars) into WT or *Cxcr2*$^{-/-}$ mice ($n = 4$) (*C*). Neutralization of CXCL1 by Ab ($n = 3$–4) reduced the vascular permeability increase in response to 12-HETE, similar to the level seen in *Cxcr2*$^{-/-}$ mice.

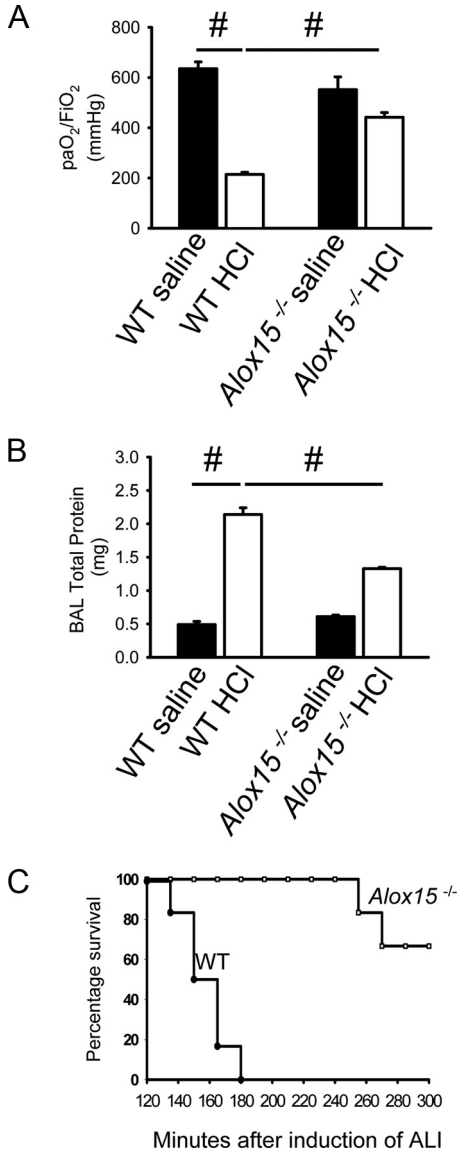

FIGURE 6. Absence of 12/15-LO improves acid-induced acute lung injury. Two hours after initiation of acid-induced ALI, gas exchange was measured in *Alox15*$^{-/-}$ and wild-type mice before and after HCl instillation (*A*). Permeability measured by BAL protein contents (*B*) compared with control mice ($n \geq 3$ mice). #, $p < 0.05$. *C*, *Alox15*$^{-/-}$ mice showed significantly prolonged survival after induction of acid-induced ALI compared with HCl-treated WT mice ($n = 6$/group); $p < 0.001$ by log rank test.

involved in 12-HETE-dependent permeability. To test this hypothesis, 12-HETE (100 μl of 50 pg/ml) was injected intratracheally into CXCR2-deficient mice, and vascular permeability was measured by Evans blue after 6 h. Following 12-HETE instillation, vascular permeability slightly increased in CXCR2-deficient mice compared with vehicle-treated mice (Fig. 5*C*), but the increase was not as pronounced as in WT mice reconstituted with 12-HETE (Fig. 5*C*). Ab blockade of CXCL1, a CXCR2 ligand, for 6 h before induction of ALI, reduced vascular permeability in WT mice (Fig. 5). These data suggest that 12-HETE regulates vascular permeability through a largely CXCR2- and CXCL1-dependent mechanism.

12/15-LO determines outcome of acid-induced acute lung injury

Based on our finding that the absence of 12/15-LO reduces vascular permeability in response to aerosolized LPS, we hypothesized that this enzyme and its products may also determine disease outcome in acid-induced ALI (7), which mimics aspiration of gastric contents that can occur in anesthetized or unconscious patients. This is a much more severe model than LPS-induced lung injury, leading to 100% lethality within 3 h (7). Two hours after induction of ALI, WT mice showed significantly reduced gas exchange (Fig. 6*A*) and increased accumulation of proteins in the alveolar compartment (Fig. 6*B*). Protein accumulation, as a marker of vascular permeability, was blunted in *Alox15*$^{-/-}$ mice. This was associated with a dramatically improved lung function as reflected by PaO_2/F_iO_2. These protective effects resulted in a significant survival benefit to *Alox15*$^{-/-}$ mice after acid-induced ALI (Fig. 6*C*).

Discussion

The results of our study suggest that the enzyme 12/15-LO plays a crucial role in the regulation of vascular permeability in LPS-induced pulmonary inflammation and acid-induced ALI. Absence of 12/15-LO greatly improves survival in an acid-induced model

of ALI that mimics aspiration of gastric contents. Elimination of hematopoietic *Alox15* by bone marrow reconstitution experiments demonstrates a dominant role of myeloid-derived 12-HETE in the regulation of vascular permeability in a largely CXCR2-dependent manner.

12/15-LO has been implicated in a variety of chronic inflammatory diseases including atherosclerosis (23, 38, 39) and β cell destruction in diabetes (40). Furthermore, two LO products, 5-HETE and 12-HETE, have been proposed to be involved in modulating the vascular permeability during pulmonary inflammation caused by Gram-negative endotoxemia (41). In most studies, 12/15-LO had deleterious effects, and eliminating 12/15-LO by gene targeting improved the disease outcome. Inhibiting 12/15-LO has been proposed as a viable target for antiinflammatory therapy (42, 43). In analyzing different studies investigating the role of *Alox15*, it is important to recognize that the products of this enzyme are different in mice (mostly 12-HETE) and humans (mostly 15-HETE). Here, we show that 12/15-LO also plays a deleterious role in acute lung inflammation in mice.

Previous studies have demonstrated that macrophage-derived 12/15-LO plays a key role in atherosclerosis development in the $Apoe^{-/-}$ model (23). Here, we show that 12/15-LO expression in hematopoietic-derived cells plays a dominant role in regulating vascular permeability in acute lung injury. This is consistent with a role for the 12/15-LO product 12-HETE in regulating endothelial and epithelial cell contraction and permeability. Although the receptor for 12-HETE is unknown, its signaling is pertussis toxin-sensitive, indicating that it is coupled through members of the $G\alpha_i$ family of G proteins (44). Our studies suggest that 12-HETE induces increased lung vascular permeability through a CXCR2-dependent mechanism, but absence of *Alox15* does not reduce CXCL1 secretion into the BAL fluid. Further research will be needed to determine the exact relationship between the 12-HETE and CXCR2 pathways. It has been shown that 12-HETE causes pulmonary vasoconstriction, an effect that may contribute to protein leakage independent of lung vascular permeability (45). In this in vivo study, we did not directly measure the pulmonary surface area and, therefore, we cannot fully rule out that the vascular permeability may also be modulated by the vasoconstrictor properties of 12-HETE.

Since CXCR2 on endothelial cells is known to regulate vascular permeability following LPS exposure (10), we reasoned that CXCR2 may be involved in 12-HETE-induced permeability. We show that eliminating CXCR2 (in $Cxcr2^{-/-}$ mice) or blocking one of its major ligands, CXCL1, indeed curbs 12-HETE-induced permeability. However, the response to 12-HETE in $Cxcr2^{-/-}$ mice is not completely abolished, suggesting that direct effects of 12-HETE or other chemokines and chemokine receptors are involved in regulating vascular permeability. Our data suggest that LPS activates 12/15-LO in lung macrophages and leads to the release of 12-HETE with the subsequent production and release of CXCL1. CXCL1 then binds to endothelial CXCR2 in a paracrine manner and regulates vascular permeability. Translating this finding to humans may be complicated by the expression of an additional chemokine, CXCL8 (46).

Stimulation of cells with 12-HETE activates a cascade of downstream targets, such as p38 MAPK (47), c-Jun amino terminal kinase (48), PI3K, and p21-activated kinase (PAK) (49). Although the details of the signaling cascade downstream of 12-HETE are not known, the observation that PAK is involved in regulating vascular permeability (50) provides a possible mechanism for the endothelial effects of 12-HETE.

The biology of 12/15-LO is complicated, because products of this enzyme have pro- and antiinflammatory effects (51). In addition to producing 12-HETE and 13-hydroxyoctadecadienoic acid, this enzyme is involved in the biosynthesis of lipoxins (50). Although it is well established that the mouse enzyme encoded by *Alox15* has more 12-LO activity than does human *ALOX15* (52), both enzymes produce pro- and antiinflammatory mediators. 15-LO products such as 15-HETE and lipoxins have antiinflammatory activities (53, 54). Incorporation of 15-HETE into phosphatidylinositol can modulate intracellular signaling that subsequently leads to reduced activation and recruitment of inflammatory cells and altered adhesion molecule expression (55, 56). The published data suggest that certain stimuli may induce mainly the 12-LO activity and subsequently the production of inflammatory mediators, whereas other stimuli predominantly activate the antiinflammatory pathway (15-HETE and lipoxins). However, further studies will be necessary to address this hypothesis. Furthermore, specific inhibitors for the (largely unknown) receptors of its products will be needed to fully dissect the functional role of 12/15-LO and to determine whether this enzyme is a valid target for pharmacological intervention in human disease.

Our study suggests that blocking or eliminating 12-HETE production improved arterial oxygen partial pressure and resulted in a survival benefit in acute lung injury as shown by *Alox15*-deficient mice. CDC, the 12/15-LO inhibitor used in our study, was effective when given as a pretreatment. However, high doses of CDC are required to inhibit 12/15-LO (57–59). More specific and effective pharmacological inhibitors of 12/15-LO may be valuable in the treatment of ALI.

Acknowledgments

The authors thank Tracy Burcin for technical support.

Disclosures

The authors have no financial conflicts of interest.

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