Lysosomal acid lipase deficiency causes respiratory inflammation and destruction in the lung

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Lian, Xuemei, Cong Yan, Li Yang, Yan Xu, and Hong Du. Lysosomal acid lipase deficiency causes respiratory inflammation and destruction in the lung. Am J Physiol Lung Cell Mol Physiol 286: L801-L807, 2004.—The functional roles of neutral lipids are poorly understood in the lung. Blocking cholesteryl ester and triglyceride metabolism in lysosomal acid lipase gene knockout mice (lal−/−) resulted in a high level of neutrophil influx in the lungs as early as 2 mo of age. Bronchoalveolar macrophages appeared foamy and gradually increased in number with age progression. Affymetrix GeneChip array analysis of lung mRNA showed increased levels of proinflammatory cytokine (including IL-1β, IL-6, and TNF-α) and matrix metalloproteinase (including MMP-8, MMP-9, and MMP-12) expression in lal−/− mice. With age progression, some areas of lal−/− mice developed severe abnormal cell proliferation and alveolar remodeling. In other areas, alveolar destruction (i.e., emphysema) was observed. In addition, Clara cell hypertrophy and hyperplasia developed in conducting airways. The pathophysiological phenotypes in the lal−/− mouse lungs became more severe with increasing age. The studies support the concept that neutral lipid metabolites play essential roles in pulmonary homeostasis, inflammatory responses, remodeling, and injury repair.

neutral lipid; remodeling; emphysema

PULMONARY SURFACTANT consists of 90–95% lipids and 5–10% surfactant proteins (SP). The majority (~80%) of surfactant lipids are phospholipids. Disaturated phosphatidylcholine (PC), principally dipalmitoyl-PC, is the major phospholipid component of pulmonary surfactant; it protects the lung from collapse by lowering tension at the air-liquid interface during the respiratory cycle (28). Pulmonary surfactant consists of ~10% neutral lipids, the functional roles of which are less clear in the lung. Many neutral lipid metabolites serve as ligands for nuclear receptors (7, 16), which are potent transcription factors, and control gene expression of cytokines/chemokines, proteinases, and structural proteins, which are essential for maintaining normal lung function in various physiological conditions and host defenses.

Cholesteryl ester (CE) and triglycerides (TG) are important components in neutral lipids that can be hydrolyzed by lysosomal acid lipase (LAL) in the lysosome of cells to generate free cholesterol (FC) and free fatty acids (FFA) (3, 3a). LAL is a lysosomal hydrolase that is synthesized in the rough endoplasmic reticulum and is cotranslationally glycosylated as it emerges in the endoplasmic reticulum lumen. After cleavage of the leader sequence, the enzyme is modified during transit through the Golgi apparatus. The oligosaccharides on LAL are modified to mannose 6-phosphate, which serves as a targeting signal for lysosomal sorting. The newly synthesized LAL is delivered to the lysosome by the mannose 6-phosphate receptor system. LAL plays a central role in the modulation of cholesterol metabolism in all cells. The low-density lipoprotein receptor or other receptors on the plasma membranes of various cells can deliver low-density lipoprotein-bound CE and TG to the lysosome.

In the human, LAL deficiency produces two phenotypes, Wolman’s disease and CE storage disease. The major manifestations of Wolman’s disease are hepatosplenomegaly, adrenalc calcification, and malabsorption of nutrients, which lead to death within the first year of life. The major symptom of CE storage disease is hepatomegaly. The lal−/− knockout mouse model was previously generated by gene targeting (3, 3a). The lal−/− mice appeared normal at birth, survived into adulthood, and were fertile. Massive storage of TG and CE was observed in adult liver, spleen, adrenal glands, and small intestine. Homozygous lal−/− mice died at ~9 mo of age. The lal−/− mice developed a single large mesenteric lymph node that was full of stored lipids. At 6–8 mo of age, lal−/− mice had no inguinal, interscapular, and retroperitoneal white adipose tissue. Brown adipose tissue was progressively lost. The plasma FFA levels were significantly higher in lal−/− mice than in age-matched wild-type (WT) mice. Plasma insulin levels were elevated on glucose challenge. Energy intake was higher in lal−/− mice. Early in the disease course, hepatocytes were the main storage cell in the liver. By 3–8 mo of age, the lipid-storage Kupffer cells (i.e., liver macrophages) progressively filled the liver. In a gene therapy study (adenovirus-mediated expression of human LAL) and an enzyme therapy study (human LAL purified from Pichia pastoris) in lal−/− mice, human LAL protein and mRNA were detected, TG and CE levels were reduced, and normalization of histopathology was observed (4, 5). Little is known about lal−/− pathophysiological phenotypes in the lung.

Pulmonary inflammation is a physiological, protective response to tissue injury and infection. It can facilitate repair after injury. However, an overexuberant inflammatory response can cause further lung injury and dysfunction. The inflammation starts with stimulation of vascular endothelial cells by proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6 (32). The

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binding of proinflammatory cytokines to their cognate plasma membrane receptors results in increased expression of adhesion molecules, which facilitates migration and adhesion of neutrophils to the vascular endothelium adjacent to the site of inflammation. Neutrophils migrate through the vessel wall, entering the inflamed tissues. The activated neutrophils produce free radicals (e.g., superoxide) and proteinases [e.g., neutrophil elastase and matrix metalloproteinases (MMPs)], which work in concert to kill invading fungi or bacteria but may also cause tissue damage. Later in the chronic phase of inflammation, other cell types (e.g., macrophages and lymphocytes) will be recruited to the site of inflammation. Phagocytic cells (i.e., neutrophils and macrophages) and intrinsic lung cells release MMPs on proinflammatory cytokine stimulation. The MMPs are a group of zinc-dependent endopeptidases, including collagenases, gelatinases, and stromelysins (14). By degrading components of the extracellular matrix, these molecules have a profound impact on lung structure and function (24). Although these proteinases are important for lung development and repair by modulating the extracellular matrix, excessive proteinase activities can cause unwanted extracellular matrix remodeling and lung injury, leading to emphysema (1, 23).

Because the functional roles of neutral lipids are poorly understood in the lung, lal−/− mice were utilized to assess physiological consequences after the CE and TG metabolic pathways in the lung were blocked. These studies demonstrated that blocking CE and TG metabolism results in respiratory inflammation, unwanted remodeling of the alveolar structure, pulmonary emphysema, and Clara cell hypertrophy and hyperplasia. These phenotypes are associated with neutrophil infiltration, an increase in the number of foamy macrophages, and an increase in proinflammatory cytokines/chemokines and MMPs. The studies support the concept that neutral lipids play critical roles in lung inflammation, remodeling, and injury repair.

MATERIALS AND METHODS

Animal care. All scientific protocols involving the use of animals have been approved by the Cincinnati Children’s Hospital Institutional Animal Care and Use Committee and follow guidelines established by the Panel on Euthanasia of the American Veterinary Medical Association. Protocols involving the use of recombinant DNA or biohazardous materials have been reviewed by the Cincinnati Children’s Hospital Biosafety Committee and follow guidelines established by the National Institutes of Health. Animals were housed under Institutional Animal Care and Use Committee-approved conditions in a secured animal facility at Cincinnati Children’s Hospital Research Foundation. Animals were regularly screened for common respiratory pathogens and murine viral hepatitis. Experiments involving animal death utilize CO2 narcosis to minimize animal discomfort.

Lung histology and morphometric analysis. The study followed a procedure published previously (17). Briefly, WT and lal−/− mice were anesthetized, and the lungs were inflation fixed with 4% paraformaldehyde in PBS overnight at 4°C. Lungs were washed with PBS, dehydrated in series in ethanol, and embedded in paraffin. Sections (5 μm) were loaded onto slides for staining with hematoxylin and eosin. The morphometric measurements were made following a previously published procedure (34). Three lal−/− mice of various ages (1, 2, and 6 mo) and three age-matched WT mice were used for analysis. Briefly, the overall proportion (i.e., percent fractional area) of the respiratory parenchyma and the air space was determined by using a point-counting method. Measurements were performed on sections taken throughout various lobes. Images were transferred by video camera to a computer screen using Metamorph imaging software. A computer-generated, 121-point lattice grid was superimposed on each field, and the number of intersections (i.e., points) falling over respiratory parenchyma (i.e., alveoli and alveolar ducts) or air space were counted. Points falling over bronchioles, large vessels, and smaller arterioles and venules were excluded from the study.

Oil red O staining. Frozen tissue sections were prepared from the lungs of WT and lal−/− mice following a standard cryostat procedure. Tissue section slides were stained with oil red O solution (0.5% in propylene glycol) in a 60°C oven for 10 min and placed in 85% propylene glycol for 1 min. The slides were counterstained in hematoxylin.

Immunohistochemistry. The study followed a procedure published previously (17). Tissue slides were incubated overnight at 4°C with primary Ly6G antibody (1:500; BD Biosciences Clontech), Mac3 antibody (1:500; BD Biosciences Clontech), pro-SP-C (1:200; Santa Cruz Biotechnology), and Clara cell secretory protein (CCSP) antibody (1:4,000; a kind gift from Dr. J. A. Whitsett). The tissue sections were washed and treated with biotinylated secondary antibodies. The interactions were detected with a Vectastain Elite ABC kit to visualize the signals following a procedure recommended by the manufacturer. For cell counting, measurements were performed on sections taken from various lobes. Images were transferred by video camera to a computer screen using Metamorph imaging software. A computer-generated, 121-point lattice grid was superimposed on each field, and the number of the cells positively stained by Ly6G or Mac3 antibodies were counted. In general, three fields per animal were chosen randomly for cell counting. The average numbers of three fields from three animals were used for statistical analysis. Differences between various samples were analyzed by ANOVA.

RNA microarray and data analysis. Lungs were isolated from four lal−/− or WT mice at 4 mo of age. To eliminate sample differences generated by individual mice, the lung tissues were combined in each group and homogenized for RNA isolation. The total lung RNAs were purified using the Qiagen total RNA purification kit as recommended by the manufacturer. The Affymetrix GeneChip assay was performed in duplicate by the Affymetrix Core Facility at the Cincinnati Children’s Hospital Medical Center Research Foundation. Briefly, the same amounts (10 μg) of total RNAs from the lungs of lal−/− and WT mice were subjected to reverse transcription using oligo(dT) with T7 promoter sequences attached and then to second-strand cDNA synthesis. Antisense cRNA was amplified and biotinylated using T7 RNA polymerase before hybridization to the mouse 430A GeneChip (Affymetrix) using the Affymetrix-recommended protocol. Affymetrix MicroArray Suite version 5.0 was used to scan and quantitate the chips using default scan settings. Intensity data were collected from each chip and scaled to a target intensity of 1,500, and the results were analyzed using GeneSpring 5.0 (Silicon Genetics) and JMP4 (SAS Institute). Hybridization data were sequentially subjected to normalization, transformation, filtration, and functional classification as previously described (33). Genes differentially expressed between lal−/− and WT mice were identified by Student’s t-test at P ≤ 0.05 and fold change ≥ 2. To evaluate data consistency and reproducibility, coefficients of variation among replicates were calculated with the maximal cutoff of 50%. In addition, Affymetrix difference call from all possible combinations of comparison between lal−/− and control samples was used as an additional filter to select genes with consistent changes.

RESULTS

Neutral lipid accumulation in the lung of lal−/− mice. LAL plays a central role in modulating neutral lipid metabolism in cells by cleavage of CE and TG to generate FC and FFA in the
lysosome. To determine whether neutral lipids accumulate in the lungs of lal−/− mice, frozen sections of inflated lung of 1-, 2-, and 6-mo-old lal−/− mice were stained with oil red O. Age-matched WT mice were used as controls. Our results showed very little oil red O staining in the WT mouse lungs at all ages. On the other hand, a faint oil red O staining started to appear in the lungs of 1-mo-old lal−/− mice (data not shown). At ≥2 mo of age, oil red O staining was significantly enhanced. The massive neutral lipid accumulation was predominantly detected in bronchoalveolar macrophages and alveolar type II epithelial cells (Fig. 1). No oil red O staining was observed in bronchiolar epithelial cells along the conducting airways (data not shown).

Neutrophil infiltration in the alveolar region of lal−/− mice. The overall alveolar structure and the conducting airways appeared normal in <2-mo-old mice. Although a low degree of neutral lipid accumulation was observed in 1-mo-old lal−/− mice, no neutrophil infiltration was observed at this age as determined by Ly6G antibody staining (data not shown). Significant neutrophil infiltration was detected at 2 mo of age by immunohistochemical staining with Ly6G antibody (Fig. 2A). A more quantitative analysis indicated that the neutrophil numbers were consistently high in the lungs of lal−/− mice with age progression (Fig. 2B). In contrast, relatively few neutrophils were detected in the lungs of WT mice. This observation strongly indicates that the lungs in lal−/− mice are in the proinflammatory state, even at an early age.

Foamy macrophage malformation and increase in the lungs of lal−/− mice. Compared with WT mice, bronchoalveolar macrophages were foamy in lal−/− mice. Some alveolar macrophages aggregated to form small patches and filled the alveolar lumen (Fig. 3A). With Mac3 antibody as an immunohistochemical marker, bronchoalveolar macrophages were determined and counted. In contrast to neutrophils, there were

Fig. 1. Neutral lipid accumulation in lungs of lysosomal acid lipase gene knockout (lal−/−) mice. Frozen sections of lungs from 6-mo-old wild-type (WT; A) and lal−/− (B,C) mice were stained with oil red O for determination of neutral lipid accumulation. Massive neutral lipid accumulation (red areas) was detected primarily in alveolar macrophages and alveolar type II epithelial cells in lungs of lal−/− mice. In A, WT lungs showed very little oil red O staining. In B, arrows represent oil red O-stained alveolar type II epithelial cells. In C, arrows represent alveolar macrophages.

Fig. 2. Neutrophil infiltration in lungs of lal−/− mice. A: paraffin-embedded lung sections of 2-mo-old WT (a) and lal−/− (b) mice immunostained with Ly6G antibody. Small black dots represent stained neutrophils. B: counts of Ly6G-stained cells (neutrophils) from various microscopic fields of paraffin-embedded lung sections of 6-mo-old WT, 2-mo-old (2m) lal−/−, and 6-mo-old (6m) lal−/− mice. Differences between WT and lal−/− samples were analyzed by ANOVA (P < 0.05). Values are means ± SD of 3 mice.
fewer bronchoalveolar macrophages at 2 mo of age (Fig. 3B). With age progression, the number of foamy bronchoalveolar macrophages steadily increased at 6 mo of age (Fig. 3B), an indication of chronic inflammation in the lung.

Remodeling of the alveolar structure in lal−/− mice. At 2 mo of age, some small alveolar areas of lal−/− mice exhibited minor structural remodeling (data not shown). With age progression, severe alveolar remodeling developed and spread to larger areas of lal−/− mice at 6 mo of age (Fig. 4B). Cells in these areas showed robust proliferation as determined by immunohistochemical staining with the cell proliferation marker PCNA antibody (Fig. 4D). Interestingly, with pro-SP-C antibody as a marker, the distinct alveolar type II epithelial cells could be identified in these remodeling areas (Fig. 4F). Compared with WT mice, the numbers of alveolar type II epithelial cells were also increased in these areas of lal−/− mice (Fig. 4, E and F).

Pulmonary emphysema in lal−/− mice. Another major abnormality observed in lal−/− mouse lungs was heterogeneous pulmonary emphysema. At 6 mo of age, severe emphysema could be observed in many lal−/− lung areas (Fig. 5A). Morphometric analysis showed increased air space and diminished alveolar surface in some areas of lal−/− lungs with age progression (Fig. 5B). This finding is consistent with the massive bronchoalveolar macrophage and neutrophil presence in lal−/− lungs. Neutrophils, activated macrophages, and intrinsic lung cells produce and secrete elastase and MMPs (1, 13, 23). These enzymes can degrade and damage the structure of the extracellular matrix membrane through digestion of

![Fig. 3](http://ajplung.physiology.org/)  
![Fig. 4](http://ajplung.physiology.org/)
structural proteins, including elastin, collagen, fibronectin, and laminin, leading to emphysema.

Clara cell hypertrophy and hyperplasia in lal−/− mice. In the conducting airways, the most striking morphological change is Clara cell hypertrophy and hyperplasia. The size and numbers of Clara cells were dramatically increased along the surface of the conducting airways compared with WT mice. In addition, expression of CCSP was significantly augmented in lal−/− mice compared with WT mice, as determined by immunohistochemical staining with CCSP antibody (Fig. 6). This abnormality probably represents a Clara cell response to inflammation in lal−/− mice. It has been reported previously that CCSP has an anti-inflammatory function in the lung (8).

Affymetrix GeneChip analysis of cytokines, chemokines, and MMPs in lal−/− and WT mice. Because pulmonary remodeling, destruction, and Clara cell metaplasia are generally considered results of inflammation caused by abnormal expression of proinflammatory cytokines and chemokines, a gene expression profile study was performed to determine which genes are altered in the lal−/− lungs. As shown in Table 1, a subset of cytokines/chemokines and their receptors were upregulated in lal−/− mice. Among them, mRNA expression of the hallmark proinflammatory cytokines/chemokines, including IL-1β, IL-6, and TNF-α, was significantly increased, ranging from 2- to 11-fold in the lal−/− lungs compared with the WT lungs. The expression level of CXCR2 was also increased 10-fold in the lal−/− lungs. Higher levels of CXCL1 and CXCL2, ligands for CXCR2 with neutrophil chemotactic function (15), were also detected in lal−/− mice. In addition, expression of macrophage receptor with collagenous structure (MARCO) was upregulated to 11-fold. MARCO has been shown to play a role in the binding and removal of bacteria (12). Consistent with
neutrophil infiltration and bronchoalveolar macrophage proliferation, mRNA expression levels of several MMP family members were significantly higher in the lal−/− lungs. These include MMP-8 (6-fold), MMP-9 (27-fold), and MMP-12 (6-fold). These enzymes can digest various components of the extracellular membrane matrix, causing emphysema and alveolar structure remodeling (29).

**DISCUSSION**

The conducting airways and the alveoli are essential for gas exchange between the circulating blood and the environment. Various cells and molecules in these systems play important roles in maintaining the architectural and functional integrity of the airway and alveolar epithelia. Neutral lipids, including CE and TG, are important components of pulmonary surfactant.

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Taken together, these studies support the concept that neutral lipid metabolites play critical roles in controlling pulmonary inflammatory responses, remodeling, and injury repair. Therefore, the lal−/− mouse model provides a unique system to study the molecular mechanism and therapeutic treatment for pulmonary inflammatory diseases. The observations strongly suggest that nuclear receptors may play critical roles in pulmonary inflammation, remodeling, destruction, and injury repair in the lung. Nuclear receptors are ligand-dependent transcription factors. It is well known that metabolites from FC and FFA serve as ligands for many nuclear receptors. These ligands are lipophilic hormones, which are readily diffusible through cell membranes. After coupling of a specific hormonal ligand, nuclear receptor family members translocate into the cell nucleus, bind to hormone responsive elements on target genes, and recruit nuclear receptor coactivators [including p160 family members (SRC-1, ACTR, and TIF2) and CBP/p300] (2, 10, 18, 27, 30). Nuclear receptor coactivators possess intrinsic histone

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<th>Gene</th>
<th>Description</th>
<th>Ratio</th>
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<tr>
<td>CCL3</td>
<td>Chemokine (C-C motif) ligand 3</td>
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<td>Macrophage receptor with collagenous structure</td>
<td>15.58</td>
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<tr>
<td>Ikbke</td>
<td>Inhibitor of κB kinase-ε</td>
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<td>IL-1β</td>
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<td>CXCR2</td>
<td>CXCL1 and CXCL2 receptor</td>
<td>10.24</td>
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<td>IL-17b</td>
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**MMP**

| MMP9    | MMP-9                        | 27.95|
| MMP8    | MMP-8                        | 6.49 |
| MMP12   | MMP-12                       | 5.75 |

Ratio represents comparison between average values of 2 Affymetrix GeneChip assays from wild-type (WT) and lysosomal acid lipase gene knock-out (lal−/−) mice. MMP, matrix metalloproteinase; MIP, macrophage inflammatory protein; NF-κB, nuclear factor-κB. The conducting airways and the alveoli are essential for gas exchange between the circulating blood and the environment. Various cells and molecules in these systems play important roles in maintaining the architectural and functional integrity of the airway and alveolar epithelia. Neutral lipids, including CE and TG, are important components of pulmonary surfactant. Their functional roles are poorly understood in the lung. Surprisingly, blocking the CE and TG metabolic pathway in lal−/− mice caused unwanted pulmonary inflammation, remodeling, and destruction as reported here. In lal−/− mice, neutral lipids primarily accumulated in bronchoalveolar macrophages and alveolar epithelial cells (Fig. 1), suggesting that pulmonary abnormalities originate in these cells. In early adulthood (2 mo of age), pulmonary neutrophil infiltration was prominent in the lungs and remained high throughout the lifetime of lal−/− mice (Fig. 2). Bronchoalveolar macrophages appeared foamy (Fig. 3A). With age progression, the number of bronchoalveolar macrophages increased steadily (Fig. 3B), consistent with chronic inflammatory responses. The proinflammatory state of lal−/− mice was also indicated by Affymetrix GeneChip analysis, which showed dramatically increased expression levels of major proinflammatory cytokines and their receptors (Table 1). At 6 mo of age, unwanted alveolar remodeling and cell proliferation were evident in lungs of lal−/− mice as determined by the cell proliferation marker PCNA and the alveolar type II epithelial cell marker pro-SP-C (Fig. 4). With age progression, lal−/− mice developed heterogeneous emphysema as determined by morphometric and statistical analysis (Fig. 5). Consistent with these observations, mRNA levels of the destructive proteinases MMP-8, MMP-9, and MMP-12 were elevated in lungs of lal−/− mice. These enzymes are produced and secreted by inflammatory cells (i.e., neutrophils and macrophages) and intrinsic lung cells (i.e., epithelial cells). Excessive MMP and elastase activities degrade and damage the structure of the extracellular matrix through digestion of structural proteins, including elastin, collagen, fibronectin, and laminin, leading to pulmonary emphysema (1, 13, 23).

Clara cells are nonmucous, nonserous, cuboidal secretory cells lining the bronchial tree. Clara cells act as progenitor cells for the bronchial epithelium and facilitate regeneration of the epithelium after injury. Proteins with anti-inflammatory functions are secreted by Clara cells (25). CCSP is an abundant inflammatory molecule and is involved in modulating lung inflammation during viral infection and host defense. Lung inflammation was markedly increased in CCSP−/− mice in association with an increased number of polymorphonuclear cell infiltrates and epithelial cell injury (8). In the lungs of lal−/− mice that are in the highly inflammatory state, hyperplasia and hypertrophy of Clara cells were obvious compared with WT mice in association with robust expression of CCSP in lal−/− mice (Fig. 6A). It seems that, to compensate unwanted inflammation in the lungs, lal−/− mice over-secrete anti-inflammatory molecules to balance the effect of proinflammatory molecules. In addition to secreting CCSP, Clara cells also produce an acid-stable 12-kDa protein that inhibits neutrophil elastase and cathepsin G (20–22, 25).
acetylation activity, which leads to histone hyperacetylation, chro-
matin remodeling, and target gene activation.

It is well documented that some nuclear receptors regulate
ctokine/chemokine gene expression and have anti-inflammato-
y functions in vivo. Therefore, identification of these ligands
and nuclear receptors is the key to understanding the molecular
mechanisms for development of pulmonary inflammation.
Such studies will greatly facilitate discovery of drugs for
clinical treatment of patients with lung inflammatory diseases.
For example, FFA derivative compounds, hydroxyeicosatetra-
enoic acids, hydroxycyclooctadecenoic acids, and 15-deoxy-
prostaglandin J1 serve as ligands for peroxisome proliferator-
activated receptor (PPAR)-γ, which has anti-inflammatory
functions in various systems (11, 19, 26, 31). PPAR-γ sup-
presses proinflammatory cytokine IL-1β, IL-6 gene, and
TNF-α gene expression (9). It is highly possible that upregu-
lation of these proinflammatory cytokines in lal/−/− mice is
due to the lack of PPAR-γ ligand, which leads to inactivation
of PPAR-γ function. PPAR-γ also suppresses SP-B gene
expression, which is stimulated by IL-6 in respiratory epithelial
cells (35). In lal/−/− mice, a buildup of CE and TG may cause
cell toxicity. Therefore, the observed phenotype could be a
response to damage of type II cells and bronchoalveolar macro-
rophages.

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