Molecular Pathogenesis of Genetic and Inherited Diseases

Neutral Lipids and Peroxisome Proliferator-Activated Receptor- γ Control Pulmonary Gene Expression and Inflammation-Triggered Pathogenesis in Lysosomal Acid Lipase Knockout Mice

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The functional roles of neutral lipids in the lung are poorly understood. However, blocking cholesteryl ester and triglyceride metabolism in lysosomal acid lipase gene knockout mice (*lal*^{-/-}) results in severe pathogenic phenotypes in the lung, including massive neutrophil infiltration, foamy macrophage accumulation, unwanted cell growth, and emphysema. To elucidate the mechanism underlining these pathologies, we performed Affymetrix GeneChip microarray analysis of 1-, 3-, and 6-month-old mice and identified aberrant gene expression that progressed with age. Among changed genes, matrix metalloproteinase (MMP)-12, apoptosis inhibitor 6 (Api-6), erythroblast transformation-specific domain (Ets) transcription factor family member Spi-C, and oncogene MafB were increased 100-, 70-, 40-, and 10-fold, respectively, in *lal*^{-/-} lungs versus the wild-type lungs. The pathogenic increases of these molecules occurred primarily in alveolar type II epithelial cells. Transcriptional activities of the MMP-12 and Api-6 promoters were stimulated by Spi-C or MafB in respiratory epithelial cells. Treatment with 9-hydroxyoctadecanoic acids and ciglitazone significantly rescued *lal*^{-/-} pulmonary inflammation and aberrant gene expression. In addition, both compounds as well as peroxisome proliferator-activated receptor gamma inhibited MMP-12 and Api-6 promoter activities. These data suggest that inflammation-triggered cell growth and emphysema during lysosomal acid lipase deficiency are partially caused by peroxisome proliferator-activated receptor- γ inactivation. (*Am J Pathol* 2005, 167:813–821)

Neutral lipids account for 10% composition of pulmonary surfactant that protects alveoli from collapse during respiratory cycles.¹ Cholesteryl ester and triglycerides are important components in neutral lipids, which can be hydrolyzed by lysosomal acid lipase (LAL) in the lysosome of cells to generate free cholesterol and free fatty acids. After LAL cleaves these lipids, they exit the lysosome and enter the cytosol. In the LAL knockout (*lal*^{-/-}) mouse model, we previously reported unwanted cell proliferation (tumorigenesis) and emphysema in the lung in association with pulmonary inflammation (including massive neutrophil influx and foamy macrophage accumulation).² The observation suggests that neutral lipids are required for balancing pro- and anti-inflammation and remodeling in the lung.

Pathogenic phenotypes are often caused by aberrant gene expression. Previously, we reported that genes related to inflammation (cytokines, chemokines) or tissue remodeling (matrix metalloproteinases, or MMPs) are significantly changed in the *lal*^{-/-} lung at 4 month of age.² Because this is only a single-point study, it is not clear if these gene changes correlate with pathogenic changes in an age-dependent manner. Gene profile changes at different stages of pulmonary pathogenesis need to be determined. It is possible that these genes can be used as markers for diagnostic purpose after determination of their functional roles in pulmonary diseases. In some aspects, the *lal*^{-/-} animal model resembles the smoking model in the human. In the smoking population, some patients develop chronic obstructive pulmonary disease (COPD) and lung cancer. Both diseases are tightly associated with pulmonary inflammation. The major clinical

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characteristic of COPD is pulmonary emphysema.³ COPD is the fourth leading death disease in the United States.⁴ Smokers with COPD are at high risk to develop lung cancer. In the United States, lung cancer accounts for 28% of all cancer deaths, more than colon, breast, prostate, and pancreatic cancer combined.⁵ In the world, there were an estimated 1.2 million new cases (12.6% of all new cancers) and 1.1 million deaths (17.8% of cancer deaths) each year.⁶ The genes involved in pathogenic progression of COPD and lung cancer are not fully understood yet.

Although LAL deficiency causes striking pulmonary malformation, the molecular mechanism underlining the *lal*^{-/-} phenotypes and gene expression is not clear. It is known that many metabolic derivatives of free cholesterol and free fatty acids serve as hormonal ligands for nuclear receptors that have profound and diverse functions in gene regulation, cell proliferation, differentiation, and apoptosis. We reason that nuclear receptor members that control tissue inflammation may participate in *lal*^{-/-} pathogenesis. Particularly, peroxisome proliferator-activated receptor gamma (PPAR- γ) is of high interest. LAL downstream free fatty acid derivative compounds serve as ligands for PPAR- γ . On binding to the ligands, PPAR- γ interacts with the retinoid X receptor (RXR) to form the PPAR- γ /RXR dimer on target genes. PPAR- γ plays an important role in anti-inflammation in various tissues.⁷⁻⁹ It has been shown that PPAR- γ agonists suppress gene expression of inflammatory cytokines tumor necrosis factor- α , interleukin (IL)-1 β , and IL-6.⁷ In the *lal*^{-/-} lung, these proinflammatory cytokines are all up-regulated.² Therefore, we hypothesize that LAL deficiency causes inactivation of PPAR- γ by depleting ligand production, which in turn promotes pulmonary inflammation and pathogenesis.

To further identify the genes that are responsible for *lal*^{-/-} pathogenesis in the lung and the molecular mechanism underneath, Affymetrix GeneChip microarray analysis was performed at different pathogenic development stages. The gene profile changes correlate well with pulmonary pathogenic progression in the *lal*^{-/-} lung in an age-dependent manner. Molecules involved in apoptosis inhibition and MMPs are highly induced in respiratory epithelial cells. Intranasal treatment of *lal*^{-/-} mice with natural occurring and synthetic PPAR- γ ligands significantly reduced neutrophil infiltration, macrophage proliferation, and aberrant gene expression. PPAR- γ and its ligands showed a direct inhibition on Api-6 and MMP-12 promoter activities in respiratory epithelial cells.

Materials and Methods

Animal Care

All scientific protocols involving the use of animals in this study have been approved by the Cincinnati Children's Hospital Institution Animal Care and Usage Committee and follow guidelines established by the Panel on Euthanasia of the American Veterinary Medical Association. Protocols involving the use of recombinant DNA or bio-

hazardous 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 Institution Animal Care and Usage 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 sacrifice use CO₂ narcosis to minimize animal discomfort.

Affymetrix GeneChip Microarray and GeneSpring Analyses

The lungs were isolated from five individual *lal*^{-/-} or wild-type (WT) mice at 1, 3, and 6 months 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 (Qiagen, Valencia, CA). The Affymetrix analysis was performed by the Affymetrix Core Facility at the Cincinnati Children's Hospital Medical Center, Research Foundation. Three Mouse 430 GeneChips (Affymetrix Inc., Santa Clara, CA) were used for each sample to assure accuracy. The same amounts (10 μ g) of total RNA from various samples were subject to reverse transcription using oligo dT with T7 promoter sequences attached, followed by second-strand cDNA synthesis. Anti-sense cRNA was amplified and biotinylated using T7 RNA polymerase, before hybridization to the Mouse 430 GeneChip (Affymetrix Inc.) using the Affymetrix recommended protocol. Affymetrix MicroArray Suite was used to scan and quantitate the Genechips. Intensity data were collected from each chip, scaled to a target intensity of 1500, and the results were analyzed using GeneSpring 6.1 (Silicon Genetics, Inc., Redwood City, CA). Hybridization data were sequentially subjected to normalization, transformation, filtering, and functional classification. Genes differentially expressed in *lal*^{-/-} mice versus WT mice were identified at a *P* value ≤ 0.05 and fold change ≥ 2 by two-way analysis of variance. Co-efficiency of variation among replicates was calculated with the maximal cutoff line as 98%. The Benjamini and Hochberg false discovery rate correction for multiple testing has been used.

AT II Cell Purification

The study was performed essentially the same as previously described.¹⁰ Four-month-old *lal*^{-/-} and WT mice were anesthetized by intraperitoneal injection. The abdominal cavity was opened and the mouse was exsanguinated by severing the inferior vena cava and the left renal artery. The trachea was isolated and cannulated with a 20-gauge luer stub adapter. Using a 21-gauge needle fitted on a 10-ml syringe, lungs were perfused with 10 to 20 ml of 0.9% saline via the pulmonary artery. Three ml of dispase was rapidly instilled through the cannula in the trachea followed by 0.5 ml of agarose (45°C). Lungs were immediately covered with ice for 2

minutes to solidify the agarose. After this incubation, lungs were removed from the animals and incubated in 1 ml of dispase for 45 minutes (25°C). Lungs were subsequently transferred to a 60-mm culture dish containing 7 ml of HEPES-buffered Dulbecco's modified Eagle's medium and 100 U/ml DNase I and lung tissues were gently teased from the bronchi. The cell suspension was filtered through progressively smaller cell strainers (100 μ m, 40 μ m) and nylon gauze (20 μ m). Cells were collected by centrifugation at $130 \times g$ for 8 minutes (4°C). More than 98% of the cells were stained positively using surfactant protein B antibody (an AT II cell marker) by immunocytochemical staining.

Bronchoalveolar Lavage (BAL) Cell Purification

BAL fluids were collected by perfusing the lung with 1-ml aliquots of 0.9% sodium chloride and withdrawing back fluids for three times. BAL fluids were combined and centrifuged for 5 minutes at 1000 rpm and 4°C to collect cell pellets. Approximately 95% of the cells were stained positively using Mac3 antibody (a macrophage marker) by immunocytochemical staining.

Isolation of Total mRNAs from Whole Lung Tissues, BAL Cells, and AT II Cells for Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) Assay

Whole lung tissues were dissected out from 1-, 3-, and 6-month-old *lal*^{-/-} and WT mice after being anesthetized by intraperitoneal injection. Whole lung tissues were homogenized in RLT lysis buffer for total RNA purification. AT II cells and BAL cells were purified from 4-month-old *lal*^{-/-} and WT mice. AT II cells and BAL cells were lysed in RLT lysis buffer. Total RNA from whole lung tissues or cells was purified using the Qiagen total RNA purification kit as recommended by the manufacturer (Qiagen).

For semiquantitative RT-PCR assay, 0.1-1 μ g of total RNAs was used to detect mRNA expression by SuperScript One-Step RT-PCR kit (Invitrogen, Carlsbad, CA). The RT reaction was performed at 45°C for 30 minutes. PCR cycles (25 to 35) include 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Primers for amplification of MMP-12, MMP-9, *Api6*, *Spi-C*, *MafB*, *SP-B*, and GAPDH genes are listed as follows: MMP-12: upstream primer, 5'-CAGTGAAACCCCATCCTTGAC-3' and downstream primer: 5'-GAAGTAATGTTGGTGGCTGACTC-3'; MMP-9: upstream primer: 5'-GATTCCTTGTAACCCAGGAAG-3' and downstream primer: 5'-CAGTGAGTGAGAGAGTCCCAAAG-3'; *Api6*: upstream primer: 5'-TTGGAG-AACAACGTATCCCATGGC-3' and downstream primer: 5'-AGGCTGAGGGAAAGGTGTCTAAAG-3'; *Spi-C*: upstream primer: 5'-GCAAACATTTCAAGACAGC-3' and downstream primer: 5'-CTGTACGGATTGGTGGAAAGC-3'; *MafB*: upstream primer: 5'-CAAACGCTACCCAC-TAGCCA-3' and downstream primer: 5'-GGCGAGTT-TCTCGCACTTGA-3'; *SP-B*: upstream primer: 5'-TGCTGTGGAGCCTCTGATAGAAG-3' and downstream primer:

5'-CATAGCCTGTTC-ACTGGTGTTC-3'; GAPDH: upstream primer: 5'-CAGAAGACTGTGGATGGCCCC-3' and downstream primer: 5'-GTCCACCACCCTGTTGCTGTAGCC-3'.

Western Blot

AT II epithelial cells were isolated as mentioned above. Protein extracts were prepared in RIPA buffer and fractionated on a 10% polyacrylamide gel. After transferring to a nitrocellulose membrane, MMP-12 antibody (1:50) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used to detect MMP-12 expression. Protein bands were visualized with Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA).

Immunohistochemistry and Immunofluorescent Assay

The study followed a procedure published previously.¹¹ Briefly, WT and *lal*^{-/-} mice were anesthetized and the lungs were inflation-fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. Lungs were washed with PBS and dehydrated through a series of ethanol followed by paraffin embedding. Five- μ m sections were incubated with rat anti-mouse Ly6G antibody (1:500; BD Biosciences Clontech, San Diego, CA), rat anti-mouse Mac3 antibody (1:500; BD Biosciences Clontech, San Diego, CA), and rabbit anti-mouse MMP-9 antibody (1:1000, a kindly gift from Dr. Robert M. Senior, Washington University School of Medicine, St. Louis, MO) as the primary antibody. 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. At least three slides per animal were counted. Twenty fields were recorded per slide. Images were transferred by video camera to a computer screen using the Metamorph imaging software. A computer-generated, 121-point lattice grid was superimposed on each field and numbers of the positively stained cells by antibodies in immunohistochemistry were counted. The average numbers from three animals were used for statistic analysis. Differences between various samples were analyzed by analysis of variance. For immunofluorescent detection, Ly6G antibody (BD Biosciences) and MMP-9 antibody were used as primary antibodies. A Cy2-conjugated donkey anti-rabbit IgG and a Cy3-conjugated donkey anti rat IgG (Jackson Immunoresearch, West Grove, PA) at a dilution of 1:200 were used as the secondary antibodies. Slides were examined under a Nikon fluorescent microscope (Nikon, Melville, NY) and analyzed for red (Cy3) and green (Cy2) fluorescence.

Plasmid and Transient Transfection Assays

The MMP-12 1.5-kb promoter was amplified from mouse tail genomic DNAs using an upstream primer with the

Figure 1. Gene profile changes in the *lal*^{-/-} lungs by Affymetrix GeneChip microarray analysis. Red and orange colors represent genes whose expression profiles were increased in the *lal*^{-/-} lungs versus the WT lungs at 1, 3, and 6 months. Blue or gray colors represent genes whose expression profiles were decreased in the *lal*^{-/-} lungs. Values are means of differentially expressed gene numbers.

*Mlu*I site (5'-CTAGACGCGTCATTCCCTTTTGAACCC-3') and a downstream primer with the *Bgl*II site (5'-GAA-GATCTTGTGCAGACTCCTTTCAGC-3') by PCR. The *Api6* 2.0-kb promoter was amplified from mouse tail genomic DNAs using an upstream primer with the *Mlu*I site (5'-CTAGACGCGTAAATAATGTCCCTGCTGACTG-3') and a downstream primer with the *Bgl*II site (5'-GAA-GATCTTTGAACACTAGAATAAGGAAG-3') by PCR. The italicized sequences are restriction enzyme sites. The PCR products were digested with restriction enzymes and subcloned into the pGL2-B luciferase reporter gene vector to generate MMP-12 1.5-kb or *Api6* 2.0-kb luciferase reporter gene construct.

The Spi-C (GenBank NM011461) cDNA was amplified from total RNA purified from the 6-month-old *lal*^{-/-} lung by RT-PCR using an upstream primer with the *Bam*HI site (5'-CGCGGATCCGCCACCATGACTTGTGTATTGAT-3') and a downstream primer with the *Not*I site (5'-AAGGA-AAAAGCGGCCGCTTATCACTTGTTCATCGTCGTCCTTG-TAGTCGCTCTGGTAAGTCCGTCG-3'). The MafB (GenBank L36435) cDNA was amplified using an upstream primer with the *Bam*HI site (5'-CGCGGATCCGCCACCATGCGCGGAGCTGAGCATG-3') and a downstream primer with the *Not*I site (5'-AAGGAAAAAGC-GGCCGCTTATCACTTGTTCATCGTCGTCCTTGTCATGTCAGAAAGAACTCAGGAGAGGA-3'). In each construct, a Flag sequence was placed at the C-terminal end of molecules. The RT-PCR products were digested with restriction enzymes and subcloned into the PCR3.0 mammalian expression vector. The PPAR- γ expression vector was obtained from Dr. Krishna K. Chatterjee (University of Cambridge, Cambridge, UK). The RXR- α expression vector was obtained from Dr. Pierre Chambon (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France).

For transient transfection assay, H441 cells were cultured in RPMI supplemented with 10% fetal calf serum, glutamine, and penicillin/streptomycin. H441 cells were

seeded at a density of 2×10^5 cells per well in six-well plates 1 day before transfection. The MMP-12 1.5-kb luciferase reporter gene construct (0.25 μ g) or *Api6* 2.0-kb luciferase reporter construct (0.25 μ g) and pCMV- β gal plasmid (0.5 μ g) were co-transfected with various concentrations of Spi-C, MafB, or PPAR- γ /RXR- α constructs into H441 cells. The CMV- β -galactosidase plasmid was included for normalization. Luciferase assays were performed using the luciferase assay system (Promega, Madison, WI). The light units were assayed by luminometry (Mololight 3010; BD Pharmingen, San Diego, CA). Triplicate transfections were performed for each experiment, and the mean values were used for data presentation.

PPAR- γ Ligand Treatment

One-month-old *lal*^{-/-} mice were treated with 30 μ l of vehicle (50% ethanol), ciglitazone (100 μ g/ml; Cayman Chemical Co., Ann Arbor, MI) or 9-HODE (100 μ g/ml, Cayman Chemical Co.) by intranasal administration twice a week. WT animals were also treated as comparison. The lungs were harvested after 3 months treatment for analysis.

Results

Gene Profile Analysis in the *lal*^{-/-} Lung versus the WT Lung by Affymetrix GeneChip Microarray

As reported previously, progression of pulmonary inflammation, unwanted cell proliferation, and emphysema in the *lal*^{-/-} lung is age-dependent.² At 1 to 2 months of age no obvious structural difference was observed in the alveolar region between the WT and *lal*^{-/-} lung. But a high level of neutrophil influx into the alveolar region was readily detectable. Macrophages were foamy and moderately increased at this stage. At 6 months of age both neutrophil and macrophage numbers remained high. Some *lal*^{-/-} mice developed unwanted cell proliferation and emphysema. Clara cell hypertrophy and hyperplasia were also obvious at this stage. It seems that the time period between 3 to 6 months is critical for pathogenic progression in the *lal*^{-/-} lung. It is generally believed that pathogenic phenotypes are caused by aberrant gene expression. To identify correlation between aberrant gene expression and phenotypes at different pathogenic stages, the gene expression microarray study was performed using total RNAs isolated from the *lal*^{-/-} mouse lungs at 1, 3, and 6 months of ages. The age-matched WT lungs were used for comparison. Figure 1 summarizes the results from the analysis. At 1 month of age there were 275 genes whose expression profiles were changed. At 3 months of age, the changed genes were increased to 1064. At 6 months of age the changed genes were increased to 1209. This study clearly demonstrates that the numbers of genes differentially expressed in the *lal*^{-/-} lung are gradually increased in an

age-dependent manner, which correlates well with pathogenic phenotype progression.

Overexpression of MMP-12, *Api-6*, *Spi-C*, and *MafB* in the *lal*^{-/-} Lung

Many genes showed increased expression profiles in the *lal*^{-/-} lung by the Affymetrix GeneChip microarray analysis (see Supplementary Materials at <http://ajp.amjpathol.org>). These genes are potentially responsible for *lal*^{-/-} pulmonary pathogenic phenotypes, including inflammation and tissue remodeling. Among them, matrix metalloproteinase 12 (MMP-12), apoptosis inhibitor 6 (*Api-6*, also termed as AIM, AAC 11, Sp α , Pdp 1/6) and erythroblast transformation-specific domain (Ets) transcription factor family member *Spi-C* had the highest increases in the *lal*^{-/-} lung (Figure 2A). Importantly, expression of proto-oncogene *MafB* (also termed as Kreisler or kr) was also highly increased in the *lal*^{-/-} lung versus the WT lung. This observation has been confirmed by semiquantitative RT-PCR analysis of the RNA samples extracted from the WT and *lal*^{-/-} whole lungs (Figure 2B).

Overexpression of MMP-12, *Api-6*, *Spi-C*, and *MafB* in *lal*^{-/-} BAL Cells and AT II Cells

Previously, we have shown that neutral lipid accumulation occurs primarily in alveolar macrophages and AT II cells,² suggesting that these two cell types are the major sites for causing pathogenesis during LAL deficiency in the lung. To assess gene profile changes of MMP-12, *Api-6*, *Spi-C*, and *MafB* in these two cell types, total RNAs were purified from BAL cells (more than 95% macrophages) and AT II cells that were isolated from the WT and *lal*^{-/-} lungs (4 to 6 months of age) for RT-PCR analysis. Figure 3A shows that the pathogenic increases of *Api-6*, *Spi-C*, and *MafB* gene expression occurred primarily in *lal*^{-/-} AT II cells, while MMP-12 gene expression was increased in both *lal*^{-/-} BAL cells and AT II cells. In control, the AT II cell-specific SP-B gene showed decreased expression in *lal*^{-/-} AT II cells. The housekeeping GAPDH gene remained unchanged in both cell types. Previously, MMP-12 expression has been reported in macrophages.⁴ To confirm MMP-12 expression in AT II cells, a Western blot analysis has been performed using an anti-MMP-12 antibody. Figure 3B showed an increased expression level of MMP-12 protein in *lal*^{-/-} AT II cells, consistent with the observation made by RT-PCR analysis in Figure 3A. This study clearly supports a concept that respiratory epithelial cells play a major role in pulmonary pathogenic progression during LAL deficiency.

Transcriptional Regulation of the MMP-12 and *Api-6* Genes in Respiratory Epithelial Cells

Because elevation of *Spi-C* gene expression is higher than that of MMP-12 gene expression in early adulthood (1 month) (Figure 2A) and is co-elevated in *lal*^{-/-} AT II cells (Figure 3A), it is possible that *Spi-C* controls MMP-12 gene expression in epithelial cells. To test this assumption, the

Figure 2. Overexpression of MMP-12, *Api-6*, *Spi-C*, and *MafB* in the *lal*^{-/-} lungs. **A:** Affymetrix GeneChip microarray analysis of MMP-12, *Api-6*, *Spi-C*, and *MafB*. The numbers on the bottom of the figure represent fold changes of mRNA expression in the *lal*^{-/-} lungs versus the WT lungs at 1, 3, and 6 months. Values are means $\pm$ SD of expression fold changes, $n = 3$. **B:** RT-PCR analysis of MMP-12, *Api-6*, *Spi-C*, *MafB*, MMP-9, and GAPDH in RNA samples extracted from the *lal*^{-/-} versus the WT whole lungs at 1, 3, and 6 months.

MMP-12 1.5-kb luciferase reporter gene was subcloned and co-transfected with the *Spi-C* expression construct. As demonstrated in Figure 4A, *Spi-C* stimulated transcription of

Figure 3. Overexpression of MMP-12, Api-6, Spi-C, and MafB in *lal*^{-/-} BAL cells and AT II cells. **A:** RT-PCR analysis of MMP-12, Api-6, Spi-C, MafB, MMP-9, SP-B, and GAPDH expression in BAL cells and AT II cells that were isolated from mice at 4 to 6 months. **B:** Western blot analysis of MMP-12 expression in AT II cells.

the MMP-12 1.5-kb luciferase reporter gene in a dose-dependent manner in respiratory epithelial H441 cells. On the other hand, MafB showed significant stimulation of the Api6 2.0-kb luciferase reporter gene in a dose-dependent manner in H441 cells (Figure 4B). Therefore, Spi-C and MafB play differential roles in controlling aberrant gene expression in the *lal*^{-/-} lung.

*Up-Regulation of MMP-9 Is Associated with Neutrophil Infiltration in *lal*^{-/-} Mice*

Another important proteinase MMP-9 (gelatinase B)¹² showed a significant mRNA increase in the *lal*^{-/-} lung by Affymetrix GeneChip analysis and RT-PCR using total lung RNAs. But the increase was observed in neither *lal*^{-/-} BAL cells or AT II cells (Figure 3A). Therefore, another mechanism is involved in MMP-9 increase in the *lal*^{-/-} lung. To identify the mechanism by which MMP-9 is up-regulated in the *lal*^{-/-} lung, immunohistochemical staining was performed to localize cells that express MMP-9 using antibody against MMP-9. In the WT control, there were a few MMP-9-positive cells in the alveolar region. As a comparison, MMP-9-positive cells were dramatically increased in the *lal*^{-/-} lung with age progression (Figure 5A). Because the morphology and the location of the MMP-9-positive cells resemble neutrophils, a co-localization double-immunofluorescent staining was performed to confirm this assumption using antibodies against MMP-9 and Ly6G (a neutrophil-specific marker). The overlay of MMP-9- and Ly6G-positive cells were almost 100% identical in the *lal*^{-/-} lung (Figure 5B). As a control, the WT lung showed no immunofluorescent stain-

Figure 4. Stimulation of the MMP-12 1.5-kb or Api-6 2.0-kb luciferase reporter gene by Spi-C or MafB in H441 cells. **A:** H441 cells were co-transfected with the MMP-12 1.5-kb luciferase reporter gene construct (0.25 μg/well) and the Spi-C expression vector at various concentrations. **B:** H441 cells were co-transfected with the Api6 2.0-kb luciferase reporter gene construct (0.25 μg/well) and the MafB expression vector at various concentrations. Luciferase activities were measured 72 hours later after each transfection. Luciferase activity of the MMP-12 or Api6 luciferase reporter gene alone was defined as 1. Values are means ± SD, *n* = 3.

ing by both antibodies (data not shown). This is a clear indication that the increase of MMP-9 comes primarily from neutrophil influx into the *lal*^{-/-} lung.

PPAR-γ Ligand Treatment Reduced Pulmonary Pathogenesis and Aberrant Gene Expression during LAL Deficiency

It is known that metabolic derivatives of free cholesterol and free fatty acid serve as hormonal ligands for PPAR-γ. Linoleic acid derivative compound 9-hydroxyoctadecanoic acid (9-HODE) and synthetic compound ciglita-

Figure 5. Neutrophil-associated MMP-9 increase in the $lal^{-/-}$ lung. **A:** Immunohistochemical staining of MMP-9-positive cells in the $lal^{-/-}$ lung with age progression. **B:** Immunofluorescent staining of MMP-9 (green) and Ly6G (red) in the $lal^{-/-}$ lung. The overlay staining image of two antibodies is represented as orange color. **Arrows** point to neutrophils.

zone (both are ligands for PPAR- γ) were given to $lal^{-/-}$ mice through intranasal administration twice a week for 3 months, the critical moment for aberrant gene expression and pathogenic development in the $lal^{-/-}$ lung. In comparison with vehicle-treated $lal^{-/-}$ mice, ligand treatment significantly reduced neutrophil infiltration (judged by Ly6G antibody staining) and alveolar macrophage proliferation (judged by Mac3 antibody staining) (Figure 6, A and B). In addition, aberrant gene expression of molecular pathogenic hallmarks MMP-12, A β 6, Spi-C, and MafB for the $lal^{-/-}$ lung was significantly reduced after PPAR- γ ligand treatment (Figure 7).

PPAR- γ /RXR- α Directly Inhibited Transcriptional Activities of the MMP-12 and A β 6 Promoters in Respiratory Epithelial Cells

Down-regulation of aberrant gene expression by PPAR- γ ligands can be a direct effect at the gene transcriptional level through PPAR- γ . To test this hypothesis, MMP-12 and A β 6 2.0 promoters (two mostly up-regulated genes in the $lal^{-/-}$ lung) were chosen as model systems to test this assumption. MMP-12 1.5-kb or A β 6 2.0-kb luciferase reporter genes were transiently transfected into H441 cells. Treatment of 9-HODE and ciglitazone significantly reduced transcriptional activities of both reporter genes in H441 cells (Figure 8, A and B). Because PPAR- γ mediates the 9-HODE action, PPAR- γ /RXR- α expression vectors were co-transfected with the reporter genes into H441 cells. In co-transfection studies, PPAR- γ /RXR- α directly inhibited transcriptional activities of both promoters (Figure 8, A and B). This study implicates that inactivation of the ligands/PPAR- γ axis elevates MMP-12 and A β 6

Figure 6. Treatment of PPAR- γ ligands reduced pulmonary inflammation in the $lal^{-/-}$ lung. Paraffin-embedded lung sections of ligand-treated or untreated $lal^{-/-}$ mice were immunostained with Ly6G (**A**) or Mac3 (**B**) antibody. The positively stained cells from 20 microscopic fields of each sample were counted. The significant differences between samples were analyzed by analysis of variance. $P < 0.05$. Values are mean $\pm$ SD, $n = 5$ mice.

expression in the $lal^{-/-}$ lung. It is interesting to know that PPAR- γ mRNA expression is increased approximately twofold in the $lal^{-/-}$ lung versus the WT lung (data not shown), indicating that the body tries to compensate the loss of ligand production. Due to the absence of ligands, the PPAR- γ molecules remain inactivated.

Discussion

The $lal^{-/-}$ animal model demonstrates that neutral lipid homeostasis plays an essential role in balancing pro- and anti-inflammatory responses, cell proliferation, tissue re-

Figure 7. Treatment of PPAR- γ ligands reduced aberrant gene expression in the *lal*^{-/-} lung. RT-PCR analysis of MMP-12, Api-6, Spi-C, MafB, and GAPDH mRNAs in PPAR- γ ligand-treated or untreated *lal*^{-/-} mice in duplicate.

modeling, and gene expression in the lung. Blockage of cholesteryl ester and triglyceride metabolism results in exuberant inflammation, unwanted cell growth, and emphysema in the lung. These phenotypes are caused by aberrant gene expression during LAL deficiency. To identify these genes, Affymetrix GeneChip microarray analysis was performed. More than 1000 genes were changed in an age-dependent manner in the *lal*^{-/-} lung (Figure 1). These genes include cytokines, chemokines, endopeptidases, apoptosis inhibitors, oncogenes, and transcription factors. In this report, we specifically investigated expression of a set of genes that showed the highest increase in the *lal*^{-/-}

lung, including MMP-12, Api6, Spi-C, and MafB. The expression of these genes is tightly associated with the pathogenic phenotypes during LAL deficiency.

Interstitial structure degradation is an essential and necessary step for lung tissue remodeling during tumorigenesis and emphysema. In the *lal*^{-/-} lung, MMP-12 was induced to more than 103-fold (Figure 2A). MMP-12 has long been considered as a macrophage-specific elastase.^{4,13} In the *lal*^{-/-} lung, MMP-12 overexpression was detected in both macrophages and AT II cells, indicating that both migrating and residential cells are responsible for MMP-12 increase. Gelatin-degrading enzyme MMP-9 was also dramatically increased in the *lal*^{-/-} lung. Immunohistochemical and double-immunofluorescent staining showed MMP-9 increase that was primarily associated with neutrophil influx into the *lal*^{-/-} lung (Figure 5).

Apoptosis is a biological event of programmed cell death triggered by signal cascades in a variety of physiological conditions. Both positive and negative regulation of apoptosis influence the initiation and the progression of tissue remodeling and damage. Unwanted cell proliferation (tumorigenesis) in the *lal*^{-/-} lung implies inhibition of the normal apoptosis event. In agreement with this observation, Api-6 expression was induced to 70-fold in the *lal*^{-/-} lung, second to MMP-12 expression. Until recently, the functional role of Api6 has been reported in the immune compartment. Api6 is a secreted protein with molecular weight of 37 kd (331 amino acids) and belongs to the scavenger receptor cysteine-rich (SRCR) superfamily.^{14,15} Api6 mRNAs are expressed in lymphoid tissues, spleen, liver, and weakly in the lung.¹⁴ Api6 functions as an apoptotic inhibitor. In the immune system, Api6 inhibits apoptosis of T cells, NKT cells, and double-positive thymocytes awaiting maturation in the thymus.^{15,16} In this study, pathogenic overexpression of Api6 was dramatically induced in *lal*^{-/-} AT II cells, but not in inflammatory cells (Figure 3A).

The above studies identified AT II cells as a primary source for aberrant gene expression in the *lal*^{-/-} lung. In addition to MMP-12 and Api6, transcription factor Spi-C and MafB were highly induced in the *lal*^{-/-} AT II cells. Transient-transfection studies showed that Spi-C stimulated MMP-12 gene transcription, whereas MafB stimulated Api6 gene transcription (Figure 4). In both cases, Spi-C and MafB showed synergism with oncogenic Ras to stimulate downstream MMP-12 or Api6 gene expression (data not shown). Spi-C belongs to Ets family of transcription factors.¹⁷ The Ets proteins comprise a large family of transcription factors involved in a variety of cellular processes, including myeloid development.¹⁸ The proteins share a conserved DNA-binding ETS domain and bind purine-rich GGAA/T DNA motifs.¹⁹ Although all Ets family members have similar DNA-binding specificities and overlapping expression patterns, gene-targeting experiments have suggested that they have exclusive roles in gene regulation.¹⁷ MafB belongs to maf proto-oncogene family.²⁰ The MafB gene is expressed in a wide variety of tissues and encodes for a protein of 311 amino acids containing a typical bZip motif in its carboxy-terminal region.²¹ In the bZip domain, MafB shares extensive homology with other Maf-related proteins.²⁰ MafB forms a homodimer through its leucine

Figure 8. Inhibition of MMP-12 or Api6 promoters by PPAR- γ ligands or PPAR- γ /RXR- α in H441 cells. **A:** H441 cells were transfected with the MMP-12 1.5-kb luciferase reporter gene construct (0.25 μ g/well) and treated with 9-HODE or ciglitazone next day at concentrations as indicated. In a separate experiment, H441 cells were co-transfected with PPAR- γ /RXR- α (equal concentrations were used as indicated) and the MMP-12 1.5-kb luciferase reporter gene construct (0.25 μ g/well). **B:** H441 cells were transfected with the Api6 2.0-kb luciferase reporter gene construct (0.25 μ g/well) and treated with 9-HODE or ciglitazone next day at concentrations as indicated. In a separate experiment, H441 cells were co-transfected with PPAR- γ /RXR- α (equal concentrations were used as indicated) and the Api6 2.0-kb luciferase reporter gene construct (0.25 μ g/well). Luciferase activity was measured 72 hours later after transfection. Luciferase activity of the MMP-12 1.5-kb or Api6 2.0-kb luciferase reporter gene alone was defined as 1. Values are means $\pm$ SD, $n = 3$.

repeat structure and specifically binds Maf-recognition elements (MAREs). In addition, MafB forms heterodimers with v-Maf and Fos through its zipper structure.²¹ The synergism of Spi-C and MafB with Ras to regulate downstream target genes support a concept that interaction and coordination between these transcription factors and oncogenic signaling pathways are pivotal to pathogenic development during LAL deficiency.

To prove that depletion of free fatty acid metabolites initiates pulmonary inflammation and pathogenesis in the *lal*^{-/-} lung, a LAL downstream metabolic derivative 9-HODE (a natural occurring ligand for PPAR- γ) and a synthetic ligand compound ciglitazone for PPAR- γ were used to treat *lal*^{-/-} mice. During a 3-month trial, both compounds significantly improved pathogenesis of the *lal*^{-/-} lung. Abnormal neutrophil infiltration and macrophage proliferation were significantly reduced (Figure 6, A and B). Aberrant overexpression of characteristic marker genes MMP-12, MMP-9, Api6, Spi-C, and MafB in the *lal*^{-/-} lung was inhibited accordingly (Figure 7). The inhibition apparently occurred at the gene transcriptional level. Both compounds directly inhibited the promoter activity in respiratory epithelial cell line (Figure 8). Importantly, a downstream transcription factor of these compounds PPAR- γ also inhibited transcriptional expression of the MMP-12 and Api6 promoters (Figure 8). Therefore, the ligands/PPAR- γ axis plays an important role in controlling inflammation-triggered aberrant gene expression in the *lal*^{-/-} lung. It is worthy to mention that during the same trial, pathogenesis of other organs (eg, the liver) was also improved (data not shown), suggesting that some common mechanisms are shared by pathogenic development in different organs of *lal*^{-/-} mice.

The formation of tumor and emphysema in the lung is a complex process, which involves multiple molecules and mechanisms. Our study identified a novel mechanism by which neutral lipids contribute significantly to control pro- and anti-inflammation and tissue remodeling in the lung. Other events (eg, smoking) that interfere with this pathway may lead to the same pathogenesis in the lung. Thorough studies of the *lal*^{-/-} mouse model will facilitate us to better understand molecular mechanisms for the formation of COPD and lung cancer. New strategies and approaches for pharmacological and clinical applications will be designed based on these studies.

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