ABSTRACT

THE ROLE OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR-GAMMA IN SURFACTANT CATABOLISM IN THE ALVEOLAR MACROPHAGE

by

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Pulmonary alveolar proteinosis (PAP) is a lung disease characterized by surfactant accumulation in the alveolar spaces and alveolar macrophages. Although PAP is rare, surfactant abnormalities occur in many lung diseases including acute respiratory distress syndrome, sarcoidosis, and asthma. Studies have shown that surfactant accumulation in PAP patients results from insufficient catabolism by alveolar macrophages. Research in PAP patients and granulocyte-macrophage colony-stimulating factor knockout (GM-CSF KO) mice revealed deficiencies in the transcription factor peroxisome proliferator-activated

receptor-gamma (PPARγ) and downstream cholesterol transporter ATP-binding cassette G1 (ABCG1). PPARγ regulates lipid metabolism in macrophages and is a prominent target of research in the fields of atherosclerosis and diabetes.

This study tested the hypothesis that PPARγ promotes catabolism of surfactant in alveolar macrophages through the transcriptional regulation of ABCG1. Alveolar macrophages from macrophage-specific PPARγ knockout (PPARγ KO) mice accumulate surfactant and exhibit reduced expression of ABCG1 and reduced ABCG1-mediated cholesterol efflux. These results directly link PPARγ-deficiency to surfactant accumulation and demonstrate that PPARγ regulates cholesterol efflux in alveolar macrophages. We next investigated the expression of genes involved in the uptake and biosynthesis of cholesterol in PPARγ KO alveolar macrophages. Expression of key cholesterol biosynthesis genes was suppressed, and cholesterol influx genes (scavenger receptors) were up-regulated. These results suggested PPARγ regulates cholesterol metabolism in alveolar macrophages.

We next investigated the up-regulation of PPARγ in the GM-CSF KO alveolar macrophages by instilling mice with a Lentivirus vector containing PPARγ (Lenti-PPARγ). Reconstitution of PPARγ promoted ABCG1 expression and ABCG1-mediated cholesterol efflux in the alveolar macrophages of GM-CSF KO instilled with Lenti-PPARγ.

Taken together, these observations support the hypothesis that PPARγ-mediated transcriptional regulation of ABCG1 is critical to cholesterol metabolism and the maintenance of surfactant homeostasis overall. Understanding the role of PPARγ in normal surfactant homeostasis provides insight into the pathophysiology of PAP and identifies a potential therapeutic target.

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LIST OF SYMBOLS AND ABBREVIATIONS

27-OH: 27-hydroxycholesterol

ABC: ATP-binding cassette

ApoA-I: apolipoprotein A-I

ApoE: apolipoprotein E

BAL: bronchoalveolar lavage

BLSD: blasticidin

C: Celsius

CD: cluster of differentiation

cDNA: complementary deoxyribonucleic acid

CE: cholesteryl ester

Cholestenoic acid: 3ß-hydroxy-5-cholestenoic acid

Ci: curie

CMV: cytomegalovirus

CO_2: carbon dioxide

CoA: Coenzyme A

CT: critical threshold

CYP27A1: cytochrome P450 sterol 27-hydroxylase

DMEM: Dulbecco’s modified Eagle’s medium

DNA: deoxyribonucleic acid

DPPC: dipalmitoylphosphatidylcholine

eGFP: enhanced Green Fluorescent Protein

EMSA: electrophoretic mobility shift assay

FBS: fetal bovine serum

FC: free cholesterol

GAPDH: glyceraldehyde 3 phosphate dehydrogenase

GM-CSF: granulocyte-macrophage colony-stimulating factor

GM-CSF KO: granulocyte-macrophage colony-stimulating factor knockout

HDL: high-density lipoprotein

HMGCR: 3-hydroxy-3methyl-gllytaryl-Coenzyme A reductase

IFNγ: interferon-gamma

KO: knockout

LDL: low-density lipoprotein

LDL-R: low-density lipoprotein receptor

LPS: lipopolysaccharide

LTR: long terminal repeat

LXR: liver X receptor

LXRE: liver X receptor response element

M: molar

MCS: multiple-cloning site

mL: milliliter

MOPS: 3-morpholinopropanesulfonic acid

mRNA: messenger ribonucleic acid

ng: nanogram

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

nm: nanometers

ox: oxidized

PAP: pulmonary alveolar proteinosis

PBS: phosphate buffered saline

PHS: public health service

PPAR: peroxisome proliferator-activated receptor

PPARγ KO: peroxisome proliferator-activated receptor-gamma knockout

PPRE: peroxisome proliferator-activated receptor response element

RDS: respiratory distress syndrome

RNA: ribonucleic acid

rpm: revolutions per minute

RRE: rev response element

RT-PCR: real time-polymerase chain reaction

RXR: retinoid acid receptor

SA: splice acceptor

SD: splice donor

SDS: sodium dodecyl sulfate

SEM: standard error mean

SIN: self-inactivating

SP: surfactant-associated protein

SRA-I: scavenger receptor A-I

SRE: sterol response element

SREBP: sterol-regulatory binding element

SV40: simian virus 40

Th-1: Type 1 T helper cell

μg: microgram

μL: microliter

WT: wild type

CHAPTER 1

INTRODUCTION

Pulmonary alveolar proteinosis (PAP) is an autoimmune lung disease caused by neutralizing auto-antibodies to granulocyte-macrophage colony stimulating factor (GM-CSF) (1). The functional loss of GM-CSF manifests disease only in the lung, resulting in the filling of the respiratory tract of PAP patients with the lipoproteinaceous material called surfactant (2). Inhibition of GM-CSF signaling impairs catabolism of surfactant in alveolar macrophages (3). Although PAP is a rare lung disorder, surfactant abnormalities are problematic in many inflammatory lung diseases, including acute respiratory distress syndrome, sarcoidosis, and asthma (4).

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a ligand-activated nuclear transcription factor and a key regulator of lipid metabolism [reviewed by Tontonoz and Spiegelman (5)]. PPARγ is up-regulated by GM-CSF (6,7) and constitutively expressed in the alveolar macrophages of healthy individuals (6). Neutralization of GM-CSF by auto-GM-CSF antibodies results in significantly reduced levels of PPARγ in the alveolar macrophages of PAP patients (6). Alveolar macrophages of PAP patients have decreased expression of the lipid transporter ATP-binding cassette G1 (ABCG1). Although the role of PPARγ in maintaining surfactant homeostasis is unknown, this data suggest that

PPARγ controls surfactant catabolism in alveolar macrophages via regulation of ABCG1.

The work presented in this dissertation addresses the role of PPARγ in surfactant catabolism in alveolar macrophages. We hypothesized that (1) PPARγ promotes surfactant catabolism by the regulation of the lipid transporter ABCG1; and (2) up-regulation of PPARγ will increase surfactant catabolism in PPARγ-deficient alveolar macrophages and reduce the presence of lipid-engorged alveolar macrophages in the lung. The following sections in this chapter will review the current literature on surfactant metabolism, alveolar macrophages, and the regulatory functions of PPARγ with a specific emphasis on lipid metabolism.

Lung Health and Immunity

Air drawn into the human body travels through the nose and mouth, trachea, bronchi, and bronchioles terminating in the alveoli where exchange of oxygen and carbon dioxide occurs. Alveoli require a lipid-rich material called surfactant to reduce surface tension and to stay open. Type I epithelial cells occupy 90% of the alveolar surface area and are the primary site of gas exchange (8). Type II epithelial cells are nearly equal in number to type I cells and are responsible for surfactant production. Alveolar macrophages are also located in the alveolar space and regulate inflammation and the catabolism of surfactant.

The lungs contain many layers of defense including the mucociliary clearance system, innate immune system, and adaptive immune system to control inflammation and infection from continuous exposure to foreign debris, pathogens, and bacteria from the environment. The mucociliary clearance system cleans the lining of the lung by the movement of the cilia in the respiratory epithelium pushing debris towards the pharynx and into the digestive system. If a particular antigen or particle is not cleared by the mucociliary system and reaches the epithelial surface of the lung, the innate immune system responds through the activation of epithelial cells, neutrophils, dendritic cells, and monocytes and macrophages (9). The adaptive immune system in the lung is complex and depends greatly on the targeted pathogen. Three major players are

generally involved: dendritic cells, T cells, and B cells (10). The adaptive immune system promotes clonal expansion of antigen-specific effector cells in response to a challenge.

The term “macrophage” refers to cells differentiated from monocyte precursors arising from hematopoietic stems cells in the bone marrow. Macrophages are located throughout the body and are distributed in most tissues. Alveolar macrophages are terminally differentiated macrophages located in the alveolar space and are continually replaced by circulating monocytes. Because of their location, alveolar macrophages are among the first responders for foreign debris and pathogens. Alveolar macrophages play a major role in the health and immunity of the lung classically through the clearance of debris and initiation of inflammatory cascades. However, the emerging role of alveolar macrophages in the maintenance of lipid homeostasis in the lung through the clearance of surfactant is the focus of this investigation.

Function, Composition, and Metabolism of Surfactant

Surfactant is a lipoproteinacious material found at the air-fluid interface in the alveoli that serves to reduce pulmonary surface tension to increase the efficiency of breathing. It is a mixture of 80% phospholipid, up to 10% neutral lipid (predominantly cholesterol), 10% protein, and less than 1% carbohydrate (11). Phosphatidylcholine makes up the majority of the phospholipids in surfactant of which nearly half is dipalmitoylphosphatidylcholine (DPPC), the main surface-active lipid in surfactant. Phosphatidylcholine is associated with four surfactant apoproteins. Surfactant-associated proteins B (SP-B) and C (SP-C) are small, extremely hydrophobic proteins that contribute to the surface tension-lowering properties of surfactant (12). Surfactant-associated proteins A (SP-A) and D (SP-D) are involved in the innate immunity of the lung (13). They are members of the collectin family and contain carbohydrate recognition domains that bind oligosaccharides on the surface of microorganisms (14).

While the complex organization and structure of surfactant remains unclear, the use of genetically modified mice has greatly advanced our understanding of the roles of surfactant-associated proteins. SP-A and SP-D deficient mice exhibit increased susceptibility to infections (15-17). SP-B knockout mice develop lethal respiratory distress (18). Additionally, these mice exhibit deficient mature forms of SP-C, confirming the importance of SP-B in surfactant (18,19).

Surfactant is continuously produced and degraded in healthy lungs through a highly regulated system. Surfactant pool size is regulated by the net synthesis, uptake, recycling, and catabolism of surfactant. Alveolar type II epithelial cells (pneumocytes) produce, partially assemble, and secrete surfactant [reviewed by Serrano and Pérez-Gil (20)]. Upon secretion, lipid and protein transfer to the interfacial surfactant film in response to surface tension. Components are also assembled into tubular myelin and large and small aggregates in the alveolar space. Surfactant synthesis is better defined than the clearance of surfactant for which two pathways have been described [reviewed by Hawgood and Poulain (21)]. Type II cells endocytose surfactant lipids and complexes and recycle them into new surfactant. Alveolar macrophages phagocytose and degrade surfactant and are therefore identified as the primary site of surfactant catabolism.

Surfactant Abnormalities in Human Disease

Although it is known that type II cells produce surfactant and alveolar macrophages catabolize surfactant, the complexities of surfactant metabolism and its role in human disease are not fully understood. Surfactant abnormalities are problematic in many inflammatory lung diseases, including PAP, acute respiratory distress syndrome, sarcoidosis, and asthma (4). Excesses or shortages in pulmonary surfactant disrupt normal lung function. Conversely, the surfactant pool size and composition are affected by disease states (22-24).

Infant respiratory distress syndrome (RDS) is a common example that emphasizes the importance of the surface tension-reducing properties of surfactant. RDS occurs in premature infants whom have not yet produced surfactant. Intra-alveolar surface tension is too great without surfactant and the alveolar airspace collapses.

At the other end of the spectrum, patients with the lung disease PAP have a build-up of surfactant in the alveolar spaces and alveolar macrophages. Given that PAP patients produce normal levels of surfactant and no type II cell abnormalities have been observed, the accumulation of surfactant has been linked to reduced surfactant clearance by the alveolar macrophages (25-28). Investigation of the GM-CSF knockout (GM-CSF KO) mouse, which provided the initial evidence for GM-CSF involvement in surfactant homeostasis, has greatly contributed to the understanding of PAP pathogenesis (29,30). Although PAP is

a rare lung disease, it provides a unique opportunity to investigate the regulation of surfactant with regard specifically to surfactant catabolism.

Overview of PAP

PAP is a lung disease characterized by the pathogenic accumulation of surfactant in the lungs. There are three forms of PAP: congenital, secondary, and idiopathic. Congenital PAP is a genetic disorder resulting from defects in either the SP-B or GM-CSF receptors (31,32). Secondary PAP is typically a result of inflammatory systemic diseases or hematological cancers and is generally resolved upon treatment of the primary disease (33-35). Idiopathic (acquired) PAP, the focus of this thesis, is the most common form of PAP, accounting for 90% of the documented cases (36-39). It is an autoimmune disease in which the patients produce neutralizing auto-antibodies to GM-CSF (1) with an estimated prevalence of 3.7 cases per million (40). Interruption of GM-CSF signaling results in insufficient surfactant catabolism characterized by the accumulation of surfactant aggregates, the formation of foamy alveolar macrophages, and ultimately diminished air space within the alveoli (3). The loss of biologically active GM-CSF manifests disease only in the lung, as GM-CSF is required for terminal differentiation of alveolar macrophages but not other tissue macrophages (41). The resulting accumulation of surfactant in the lungs is consistent with a direct role of GM-CSF in surfactant catabolism (26).

Pathophysiology of PAP

Many reports following the initial description of the lungs of PAP patients (2) have detailed the biochemical composition of the material accumulating in the alveoli (27,42). All authors reported increased levels of phospholipids and protein. The SP-A to phospholipid ratio is significantly increased in the sera and bronchoalveolar lavage (BAL) fluid from patients (43,44). SP-D is also found at elevated levels in sera and BAL fluid of patients and represents a possible biomarker for disease severity in PAP (45). Total amounts of SP-B and SP-C are found at increased levels in the lungs of patients with PAP (46). However, the gene expression of all four proteins is unchanged supporting an impairment in turnover and clearance in pathogenesis (29). Cholesterol is a major lipid component in surfactant and has been found at significantly elevated levels in the lungs of PAP patients (47-49).

Since the accumulation of surfactant in PAP patients and GM-CSF KO mice has been linked to reduced surfactant catabolism (25-28), the emphasis of research has been on the alveolar macrophage. Alveolar macrophages from PAP patients and GM-CSF KO mice have an activated phenotype resembling foam cells and are engorged with neutral lipid (50,51). Microarray studies, confirmed by real time RT-PCR and immunocytochemistry, from our laboratory revealed that the nuclear transcription factor PPARγ was highly expressed in the alveolar macrophages of healthy individuals but deficient in PAP patients (6).

The role of PPARγ in regulating inflammation and lipid metabolism combined with the fact that PPARγ is up-regulated by GM-CSF makes it a prime target in the investigation of disease pathogenesis in PAP (6,7).

Diagnosis and Treatment of PAP

Excess surfactant in the lungs of PAP patients causes shortness of breath, fatigue, and overall reduced pulmonary fitness. Diagnosis of PAP is commonly confirmed by open lung biopsy. A growing number of non-invasive alternatives have emerged, notably a serum anti-GM-CSF titer (52). Over 70% of PAP patient mortality is due to respiratory failure (37).

Standard therapy for PAP patients is a bilateral whole-lung lavage which removes excess surfactant but it does not to treat the underlying cause of pathogenesis and involves the risk of general anesthesia. Treatment with exogenous GM-CSF targets the cause of the PAP and is currently in clinical trials. The results of GM-CSF therapy are varied with resolution of disease in approximately 50% of the patients (53). Non-responding patients have very high titers of neutralizing auto-antibodies to GM-CSF—perhaps too high for the exogenous GM-CSF treatments to overcome (54). Depletion of B cells using monoclonal antibodies is being explored as a treatment for PAP. Understanding the mechanisms involved in surfactant metabolism may lead to improved therapy for PAP and many other pulmonary diseases.

Overview of PPARγ

The PPARs are a family of lipid-binding nuclear transcription factors that regulate inflammation, adipocyte differentiation, and glucose and lipid metabolism. Three isotypes of PPAR (alpha, delta/beta, and gamma) vary in tissue distribution and ligand affinity. PPARγ is constitutively expressed at high levels in activated macrophages, adipose tissue, and the intestines [reviewed by Tontonoz and Spiegelman (5)]. The remaining discussion will be focused on the expression of PPARγ specifically in macrophages.

Three isoforms of PPARγ (γ1, γ2, and γ3) are produced by alternative splicing events, PPARγ2 differing in length by an additional 30 amino acids at the 5' end. While PPARγ2 is less abundant overall than PPARγ1 (55), it is found at higher levels in macrophages and foam cells (56).

Prostaglandin 15-d-PGJ2 and fatty acids are the major natural ligands for PPARγ (57,58). Rosiglitazone, a thiazolidinedione, is a synthetic PPARγ agonist. Upon ligand binding, PPARγ heterodimerizes with retinoid X receptor (RXR) in the nucleus, forming a complex with co-activators which then bind to PPAR response elements (PPRE) in the DNA to promote transcription of downstream genes involved in lipid metabolism.

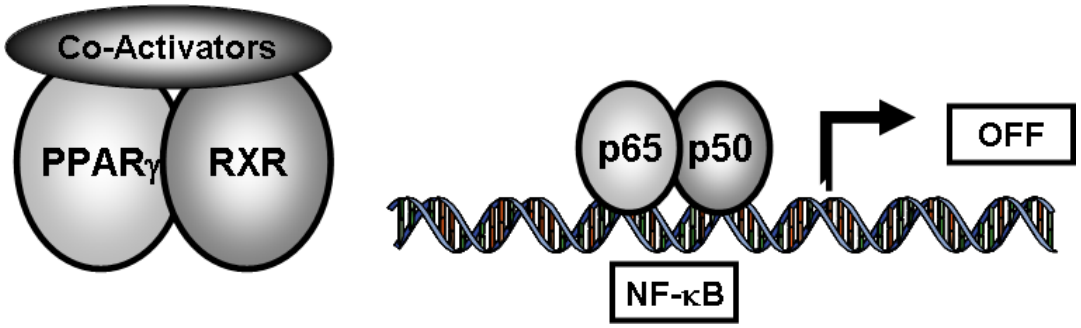
PPARγ has also been shown to negatively regulate inflammation. Although the mechanisms are not fully understood, PPARγ exerts anti-

inflammatory responses by antagonizing pro-inflammatory factors such as NF-κB and activator protein-1 (AP-1) [reviewed by Ricote and Glass (59)]. PPARγ reduces the DNA binding of NF-κB through three major mechanisms of transrepression including competitive binding of NF-κB co-activator complexes, direct binding of the p65 subunit of NF-κB, and promotion of Inhibitor of κBα (IκBα) (Figure 1.1). PPARγ also transrepresses the pro-inflammatory actions of AP-1 by inhibiting mitogen-activated protein kinase (MAPK) activity. Several models have suggested that the transrepression by PPARγ is gene and cell type-specific. More studies are needed to fully clarify the mechanisms of PPARγ transrepression.

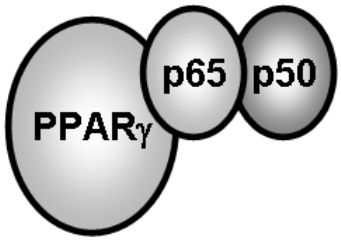
PPARγ is expressed in a variety of macrophages, such as thioglycollate-elicited peritoneal macrophages, foam cells in atherosclerotic lesions, and alveolar macrophages from healthy individuals (6,60,61). The data available on PPARγ is primarily focused on lipid and glucose metabolism and the promotion of foam cell formation in atherosclerotic lesions (7,62). PPARγ is considered a potential therapeutic target for both diabetes and atherosclerosis due to its role in regulating lipid influx and efflux in macrophages (63).

Figure 1.1 Mechanisms of PPARγ-mediated transrepression of NF-κB. PPARγ antagonizes NF-κB transcriptional activity by (A) competitively binding NF-κB co-activator complexes, (B) direct binding of the p65 subunit of NF-κB, and (C) promotion of IκBα. Image was adapted from Ricote and Glass (59).

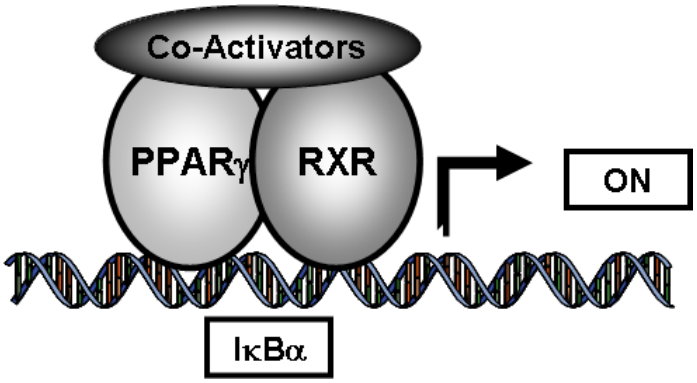
A.


Co-Activators
PPARγ
RXR
p65
p50
OFF
NF-κB

B.


PPARγ
p65
p50

C.


Co-Activators
PPARγ
RXR
ON
IκBα

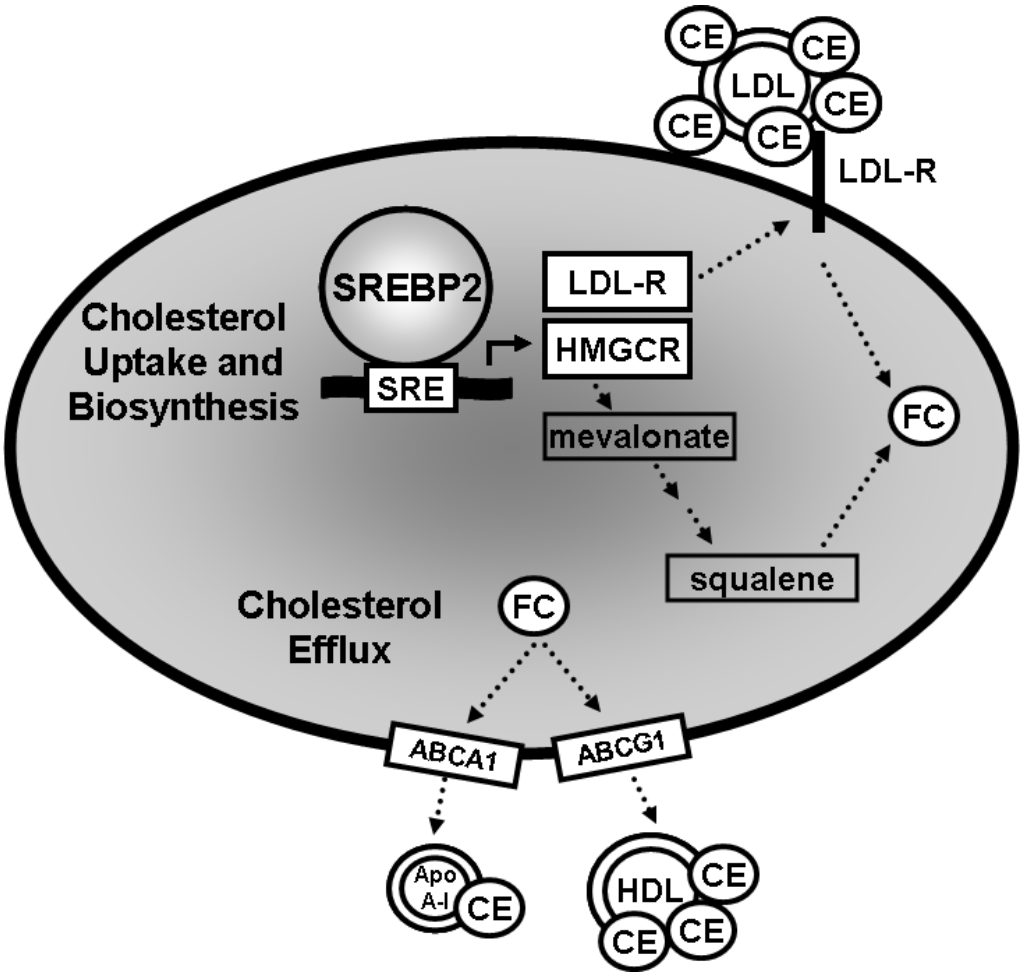
Cholesterol Biosynthesis, Uptake, and Efflux in Macrophages

Cholesterol is essential for membrane structure and organization, lipid rafts and cell-signaling, and endocytosis [reviewed by Maxfield and Tabas (64)]. Excess cholesterol, on the other hand, can disrupt cell-signaling, cause membrane rigidity, and stimulate pro-apoptotic cascades. Cells maintain cholesterol levels through the uptake of cholesterol from circulation and biosynthesis of cholesterol de novo (Figure 1.2). The intracellular level of cholesterol is a critical part of the regulation of these processes [reviewed by Brown and Goldstein (65)].

Cholesterol is synthesized in the cell from acetyl-CoenzymeA through the mevalonate pathway. In response to limited cholesterol, the nuclear transcription factor sterol response element-binding protein 2 (SREBP2) undergoes proteolytic cleavage, enters the nucleus, and promotes the transcription of all the cholesterol biosynthesis pathway enzymes including the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) (66,67). HMGCR is the first committed step in the biosynthesis of cholesterol and a major site of feedback regulation (68,69).

Cholesterol obtained from the diet is carried in circulation by the low-density lipoprotein (LDL). In addition to cholesterol biosynthesis enzymes, in response to low sterol levels SREBP2 also promotes transcription of the LDL receptor (LDL-R) (70,71). As cell cholesterol levels increase, LDL-R expression

Figure 1.2 Cholesterol biosynthesis, uptake, and efflux in macrophages. In response to limited cholesterol, SREBP2 promotes the transcription of important cholesterol biosynthesis enzyme HMGCR and cholesterol receptor LDL-R. Free cholesterol is actively transported from macrophages by ABCA1 and ABCG1 to acceptor molecules Apo-AI and HDL, respectively. CE, cholesteryl ester; FC, free cholesterol; SRE, sterol response element.


CE
CE
LDL
CE
CE
CE
LDL-R
SREBP2
SRE
Cholesterol
Uptake and
Biosynthesis
LDL-R
HMGCR
mevalonate
FC
squalene
Cholesterol
Efflux
FC
ABCA1
ABCG1
Apo
A-I
CE
HDL
CE
CE
CE

is reduced (72). Further, LDL-derived cholesterol negatively regulates transcription of LDL-R and HMGCR through the SREBP2 pathway (71). HMGCR is also subject to degradation in the presence of sterols through a sterol-sensing domain (73).

In addition to LDL-R, macrophages express several scavenger receptors that bind and internalize excess cholesterol in tissues. Contrasting with the LDL-R, scavenger receptor A-I (SRA-I) and CD36 are not inhibited by high intracellular cholesterol levels but rather are up-regulated in the presence of extracellular cholesterol bound to modified-LDL particles (74). SRA-I and CD36 are expressed on extrahepatic macrophages, such as those found in the vascular tissue. The removal of cholesterol from macrophages is mediated by lipid transporters ABCG1 and ABCA1 which transfer cholesterol to the acceptor molecules high-density lipoprotein (HDL) and apolipoprotein A-I (Apo A-I), respectively. Cholesterol is then recycled to the liver to make bile through a process called reverse cholesterol transport. The accumulation of cholesterol in macrophages promotes foam cell formation and pro-inflammatory responses (75). In fact, it has been demonstrated in models of atherosclerosis that “unrestricted” uptake of cholesterol by the scavenger receptors (due to high substrate levels) in cholesterol-rich tissues can result in intracellular cholesterol overload and foam cell formation when the efflux of cholesterol is insufficient or dysregulated (75).

Cholesterol is primarily delivered to the lungs by lipoproteins (76). Although cholesterol is a relatively minor component, pulmonary surfactant is a major pool of cholesterol in the body. Cholesterol is a functionally diverse lipid capable of influencing cell structure and signaling.

It has been shown that cholesterol levels are increased in the lungs of PAP patients while no difference was measured in the sera (47). Cholesterol metabolites 3ß-hydroxy-5-cholestenoic acid (cholestenoic acid) and 27-hydroxycholesterol (27-OH), however, were significantly elevated in the lungs and sera of PAP patients (47). It was suggested that the increases were likely due to increased substrate supply rather than altered hepatic metabolism. These results indicated that cholesterol metabolites could be a biomarker of pulmonary cholesterol homeostasis. Combined with the presence of foam cells in the lungs of patients, these results also provided evidence that homeostasis of cholesterol catabolism may be disrupted in PAP. Interestingly, the contribution of impaired cholesterol catabolism to the overall disruption of surfactant catabolism in the pathogenesis of PAP has not been specifically addressed.

While little is known of regulatory role of PPARγ in the lipid metabolism of alveolar macrophages specifically, data from other tissue macrophages have shown that macrophages are critical in maintaining the balance of cholesterol in circulation and in many tissues. Cholesterol metabolism in turn is transcriptionally regulated by PPARγ through the expression of lipid transporters and scavenger receptors, described in the next section.

Regulation of Lipid Receptors, Lipid Transporters, and Transcription Factors in Macrophages by PPARγ

Little is known of the regulatory role of PPARγ in the lipid metabolism of alveolar macrophages. Most of the research regarding PPARγ and lipid metabolism in macrophages has been done on other tissue macrophages such as peritoneal macrophages. This work has shown that PPARγ transcriptionally promotes the efflux of cholesterol via transactivation of sterol-sensing transcription factor liver X receptor-alpha (LXRα) and lipid transporters ABCG1 and ABCA1 (Figure 1.3) (77-79). In a macrophage, cholesterol is metabolized into PPARγ ligands (oxidized fatty acids) (58) and LXR ligands (oxysterols) (80-82) which transcriptionally activate both transcription factors and in turn, drives the removal of lipids from the macrophage via ABCG1 and ABCA1. As described in the previous section, free cholesterol is transported by ABCG1 and ABCA1 to acceptor molecules HDL and ApoA-I and then on to the liver via reverse cholesterol transport (83,84). The PPARγ-LXR-ABC cascade is critical to maintaining cholesterol efflux (85) as it is regarded as the rate-limiting step in reverse cholesterol transport.

Studies from knockout models have shown that the ABC transporters work synergistically and appear to have compensatory mechanisms. When one ABC transporter is deficient, PPARγ and LXR ligands accumulate and induce expression of the other transporter (86,87). ABCA1 deficient macrophages

Figure 1.3 PPARγ and LXR regulate cholesterol efflux. Upon ligand binding, PPARγ and LXRα/β heterodimerizes with RXR and promotes the transcription of downstream genes ABCG1, ABCA1, ApoE, and CYP27A1. The PPARγ-LXR cascade is critical to the maintenance of cholesterol efflux in macrophages. This image was adapted from Argmann, et al. (88).

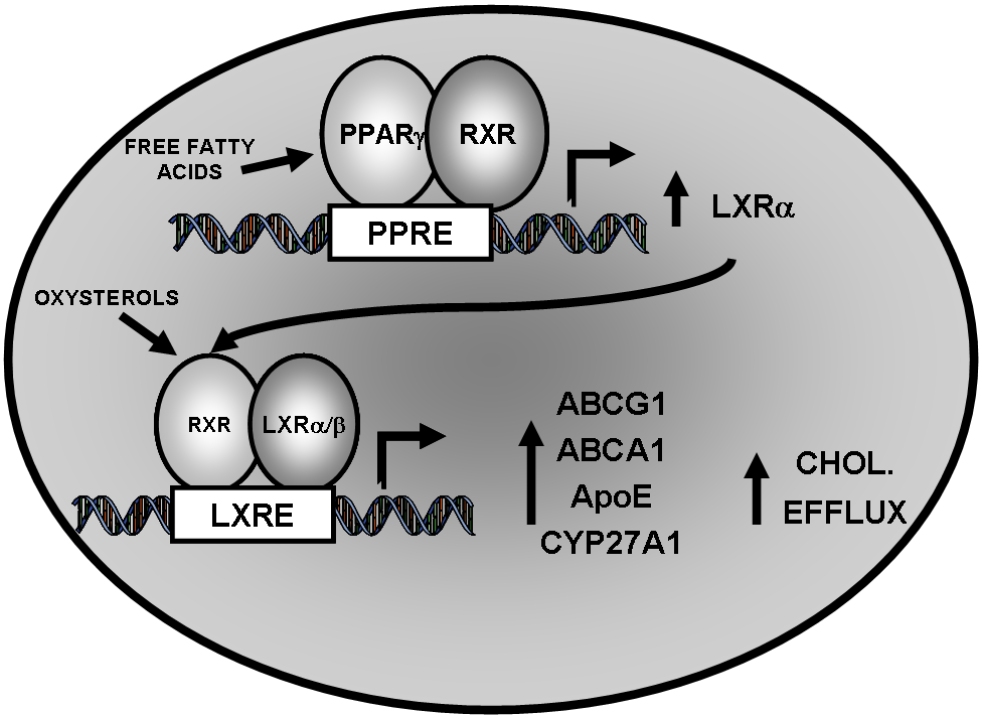
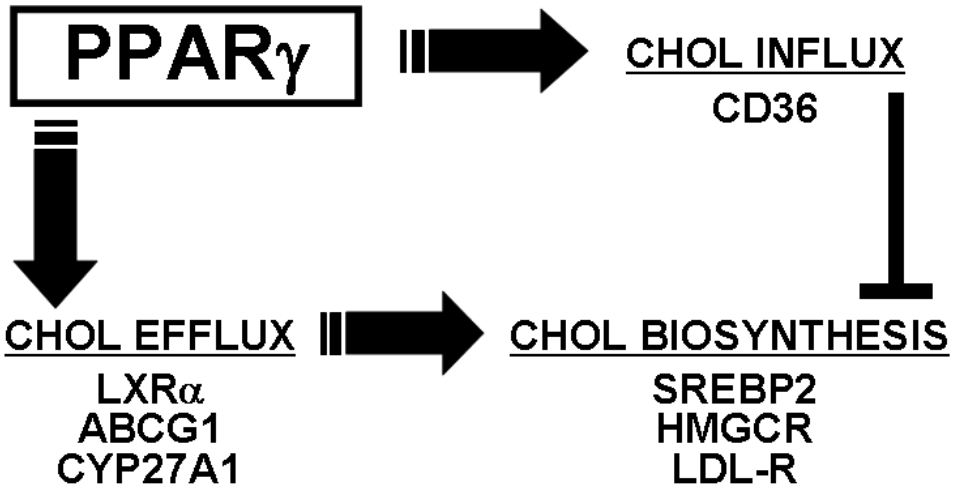

FREE FATTY ACIDS
PPARγ
RXR
PPRE
LXRα
OXYSTEROLS
RXR
LXRα/β
LXRE
ABCG1
ABCA1
ApoE
CYP27A1
CHOL.
EFFLUX

exhibit increased ABCG1-mediated cholesterol efflux and ABCG1 deficient macrophages have increased ABCA1-mediated cholesterol efflux (86). These studies also demonstrated that ABCA1 is less able to compensate for loss of ABCG1 as ABCG1-deficient mice develop more severe lipid accumulation in the lung (86,89,90).

Although scavenger receptor-mediated uptake of modified-LDL particles is complex and is not fully understood, it is known to be regulated in part by PPARγ: CD36 is directly up-regulated by PPARγ (61) while SRA-I is negatively regulated by PPARγ (60). Cholesterol influx and efflux contribute to the overall cholesterol level in macrophages which in turn regulates the biosynthesis of cholesterol. As PPARγ directly regulates cholesterol influx (via scavenger receptors) and efflux (via ABC transporters), it indirectly affects the biosynthesis of cholesterol in macrophages (Figure 1.4).

Although PPARγ has been implicated as a critical mediator in cholesterol metabolism in various tissue macrophages, PPARγ may hold particular importance in cholesterol catabolism in alveolar macrophages given the amount of cholesterol in the lung.

Figure 1.4 PPARγ regulates cholesterol influx, efflux, and biosynthesis. PPARγ directly promotes transcription of genes involved in the movement of cholesterol in and out of the cell, including scavenger receptor CD36 and genes involved in cholesterol efflux LXRα, ABCG1, and CYP27A1, which influence the intracellular cholesterol level. In turn, cholesterol levels regulate the biosynthesis of cholesterol through the expression of transcription factor SREBP2 and downstream targets HMGCR and LDL-R. Therefore, PPARγ directly and indirectly regulates the influx, efflux, and biosynthesis of cholesterol in macrophages.


PPARγ
CHOL INFLUX
CD36
CHOL EFFLUX
LXRα
ABCG1
CYP27A1
CHOL BIOSYNTHESIS
SREBP2
HMGCR
LDL-R

Statement of the Problem

The expression of PPARγ is deficient in the alveolar macrophages of PAP patients and GM-CSF KO mice, which exhibit PAP-like pulmonary pathology and accumulate pulmonary cholesterol (6). However, the accumulation of surfactant has not been directly linked to the deficiency of PPARγ. Further work in the laboratory on the alveolar macrophages of PAP patients and GM-CSF KO mice revealed that the expression of the lipid transporter ABCG1 was also deficient (51). We therefore hypothesized that PPARγ promotes surfactant catabolism through regulation of ABCG1. To address the hypothesis, we utilized a macrophage-specific PPARγ knockout (PPARγ KO) mouse model.

Chapter 2 addresses the hypothesis by utilizing PPARγ KO mice. The specific objectives of Chapter 2 were to: (1) analyze the lipids accumulating in the alveolar spaces and alveolar macrophages of PPARγ KO mice, and (2) evaluate the expression of downstream PPARγ genes, including ABCG1, which may be involved in the catabolism of surfactant.

Chapter 3 describes the generation of a Lentivirus vector containing the PPARγ sequence (Lenti-PPARγ). We utilized Lenti-PPARγ to up-regulate PPARγ in primary murine and human alveolar macrophages in vitro. Methods detailed in this chapter were critical to the in vivo experiments in Chapters 4 and 5.

Chapter 4 discusses experiments that evaluate the effects of replacing PPARγ in vivo using Lenti-PPARγ on the regulation of cholesterol metabolism

genes. The specific objectives of Chapter 4 were to: (1) up-regulate deficient expression of LXRα and ABCG1, and (2) investigate the expression of genes involved in cholesterol influx and synthesis in the alveolar macrophages of PPARγ KO mice.

Chapter 5 investigates the reconstitution of PPARγ in PPARγ-deficient alveolar macrophages of GM-CSF KO mice. The specific purpose of Chapter 5 was to investigate PPARγ-mediated changes on cholesterol efflux gene expression and clearance of surfactant in GM-CSF KO. We hypothesized that surfactant catabolism is regulated by a PPARγ-ABCG1 pathway and that up-regulation of PPARγ will increase surfactant catabolism and reduce the presence of lipid-engorged alveolar macrophages in the lung.

CHAPTER 2

TARGETED DELETION OF PPARγ IN ALVEOLAR MACROPHAGES DISRUPTS SURFACTANT CATABOLISM

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Abstract

Surfactant accumulates in the alveolar macrophages of granulocyte-macrophage colony-stimulating factor knockout (GM-CSF KO) mice and pulmonary alveolar proteinosis (PAP) patients with a functional loss of GM-CSF resulting from neutralizing anti-GM-CSF antibody. Alveolar macrophages from PAP patients and GM-CSF KO mice are deficient in peroxisome proliferator-activated receptor-gamma (PPARγ) and ATP-binding cassette (ABC) lipid transporter ABCG1. Previous studies have demonstrated that GM-CSF induces PPARγ. We therefore hypothesized that PPARγ promotes surfactant catabolism through regulation of ABCG1. To address this hypothesis macrophage-specific PPARγ knockout (PPARγ KO) mice were utilized. PPARγ KO mice develop foamy, lipid-engorged Oil Red O positive alveolar macrophages. Lipid analyses revealed significant increases in the cholesterol and phospholipid contents of PPARγ KO alveolar macrophages and extracellular bronchoalveolar lavage (BAL)–derived fluids. Increased surfactant proteins A and D were detected in BAL fluid. PPARγ KO alveolar macrophages showed decreased expression of ABCG1 and a deficiency in ABCG1-mediated cholesterol efflux to HDL. Lipid metabolism may also be regulated by liver X receptor (LXR)—ABCA1 pathways. Interestingly, ABCA1 and LXRβ expression were elevated indicating that this pathway is not sufficient to prevent surfactant accumulation in alveolar macrophages. These

results suggest that PPARγ mediates a critical role in surfactant homeostasis through the regulation of ABCG1.

Introduction

Pulmonary alveolar proteinosis (PAP) is a rare autoimmune lung disease characterized by neutralizing auto-antibodies to granulocyte-macrophage colony-stimulating factor (GM-CSF) (1,91). This loss of functional GM-CSF results in a filling of the alveolar spaces of the lungs with the lipoproteinaceous material called surfactant. While PAP is a rare lung disorder, surfactant abnormalities are problematic in many lung diseases, including acute respiratory distress syndrome (ARDS), sarcoidosis, and asthma [reviewed by Sorensen (4)].

Pulmonary surfactant is comprised of 90% lipid, 10% protein, and less than 1% carbohydrate. Phospholipids are the major lipid in surfactant and are associated with four surfactant-associated proteins (SP-A, -B, -C, and –D). SP-B and SP-C contribute to the surface tension lowering properties of surfactant and SP-A and SP-D are actively involved in the innate immunity of the lung [reviewed by Trapnell and Whitsett (92)]. Other lipids associated with surfactant include cholesterol, triglycerides, and free fatty acids. Cholesterol is the major neutral lipid (up to 90%) in pulmonary surfactant (93). Surfactant is produced by type II pneumocytes and two pathways have been described in the clearance of surfactant [reviewed by Hawgood and Poulain (21)]. Type II cells endocytose surfactant lipids and complexes and recycle them into new surfactant. Alveolar macrophages phagocytose and degrade surfactant and are considered to be the primary cell involved in the clearance and catabolism of surfactant (3).

PAP patients produce normal levels of surfactant (25). The accumulation of surfactant in the lungs of PAP patients is due to insufficient surfactant catabolism by alveolar macrophages (25,26,28). Alveolar macrophages from PAP patients have an activated phenotype resembling foam cells and are engorged with neutral lipid, as evidenced by positive Oil Red O staining (50). The nuclear transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ) is constitutively expressed in the alveolar macrophages of healthy controls and is up-regulated by GM-CSF (6,7). Our previous studies have shown that the alveolar macrophages of PAP patients and the GM-CSF knockout (GM-CSF KO) mouse model of PAP are deficient in PPARγ (6,51).

While the role of PPARγ in surfactant catabolism in the lung remains unclear, PPARγ is known to directly and indirectly regulate many genes involved in cholesterol metabolism and transport including the nuclear transcription factor liver X receptor alpha (LXRα) and ATP-binding cassette (ABC) lipid transporters, ABCG1 and ABCA1 (51,77,78,94). Studies have suggested that PPARγ deficiencies result in decreased expression of ABCG1 (51,77). The deletion of ABCG1 in mice (ABCG1 KO) results in severe pulmonary lipidosis (89). Cholesterol and phospholipid accumulate and foam cell formation occurs in the macrophages of ABCG1 KO (84,86,89). Moreover, ABCG1 KO macrophages display reduced capacities to efflux cholesterol and phospholipid (83-85,95). We therefore hypothesized that PPARγ may promote surfactant catabolism through regulation of the lipid transporter ABCG1. To test this hypothesis, we

investigated the alveolar macrophages from macrophage-specific PPARγ knockout (PPARγ KO) mice.

Materials and Methods

Mice. Animal studies were conducted in conformity with Public Health Service policy on the humane care and use of laboratory animals and were approved by the Institutional Animal Care Committee. C57Bl/6 wild type (WT) mice were obtained from Jackson Laboratory (Bar Harbor, ME). Macrophage-specific PPARγ KO mice have been previously described (96). Bronchoalveolar lavage (BAL) cells were obtained as described earlier from 8–12 week old PPARγ KO mice and age- and gender-matched wild type C57Bl/6 controls (96). Briefly, the thoracic cavity was opened and the lungs were exposed. After cannulating the trachea, a tube was inserted and BAL was carried out with warmed (37℃) PBS in 1 mL aliquots x 5. Except where indicated, sample number (n) refers sets of BAL cells pooled from 3-5 mice while BAL fluid was analyzed from individual mice. Following previously established guidelines for analysis of acellular components of BAL fluid (97), analysis BAL fluid protein and lipid utilized samples with similar volumes recovered [ranging from 4.25-5.0mL for wild type and 4.1-4.8mL for PPARγ KO]. Cell viability was measured by trypan blue exclusion. BAL cell differentials from all animals used in the experiments were stained with a Wright-Giemsa stain and revealed >90% macrophages. Cytospins of BAL cells were stained with Oil Red O to detect intracellular neutral lipids. BAL cells were fixed in 4% paraformaldehyde, stained with Gill's hematoxylin (Sigma, St. Louis, MI), and incubated in Oil Red O solution (Rowley Biochemical Inc.,

Danvers, MA) overnight. BAL cells were washed in 85% propylene glycol and mounted in Mount-Quick aqueous mounting medium (Daido Sangyo Co., Tokyo, Japan). Oil Red O positivity was quantified by counting 100 cells on each cytospin slide from C57Bl/6 and PPARγ KO mice.

RNA purification and analysis. Total RNA was extracted from the cells by the RNeasy protocol (Qiagen, Valencia, CA). Expression of mRNA was determined by real-time RT-PCR analysis using the ABI Prism 7300 Detection System (TaqMan; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. RNA specimens were analyzed in duplicate using primer sets for mouse LXRα (Mm00443454), LXRβ (Mm00437262), ABCA1 (Mm00442646), ABCG1 (Mm00437390), CYP27A1 (Mm00470430), and APOE (Mm00437573) (Applied Biosystems). Relative gene expression was quantified as described (98). Briefly, the control group (C57Bl/6) values were calculated by subtracting the raw cycle (CT) data for the housekeeping gene (GAPDH, 4352339E) from the cycle data for the gene of interest. The ensuing values (ΔCT) were averaged and normalized to 1.0. Data from PPARγ KO were quantified in a similar fashion and expressed as fold change in gene expression relative to wild type. For these experiments, BAL cells were isolated from individual PPARγ KO mice and compared to pooled samples of C57Bl/6 BAL cells.

Cholesterol efflux assay. Pooled BAL cells ($3.5x10^5$/well) were plated in 48-well cell culture plates in complete DMEM media (Invitrogen, Carlsbad, CA) and maintained at 37℃ and 5% CO_2. Non-adherent cells were removed after one hour. Cells were incubated for 24 hours in 2 μCi/mL of [1,2-^{3}H(N)]-cholesterol (NEN, Perkin Elmer, Waltham, MA), equilibrated in serum free media for 24 hours, and incubated in the presence of 10% fetal bovine serum (FBS), apolipoprotein A-I (ApoA-I) (25 μg/mL) (Sigma), or HDL (25 μg/mL) (Intracel, Frederick, MD) for 24 hours. Supernatant fluids were harvested and centrifuged at 1800rpm for 5 minutes to remove cellular debris. Cells were washed with PBS and lysed in 0.2 M sodium hydroxide (NaOH) with 0.1% SDS for 1 hour at room temperature. Supernatant and cell-associated radioactivity was measured by liquid scintillation. Cholesterol efflux was expressed as the percentage of radioactivity in the supernatant divided by the total radioactivity of the cells and supernatant. Each assay was performed in duplicate and results from three independent assays were used to calculate percent efflux.

Immunoblotting. Equal volumes of BAL fluid were used for analysis of surfactant protein. For analysis of BAL cell protein, samples were loaded based on equal total protein determined using a modified Lowry assay (Dc Protein Assay, Bio-Rad Laboratories, Hercules, CA). Gels were eletrophoresed under reducing conditions using a 10% Bis-Tris gel (Bio-Rad) with MOPS buffer (Invitrogen). The following primary antibodies and dilutions were used: 1:500

ABCG1 (sc-11150) and 1:500 ABCA1 (sc-5491, Santa Cruz Biotechnology, Inc., Santa Cruz, CA); 1:5000 SP-A (AB3420) and 1:2000 SP-D (AB3434, Millipore, Billerica, MA). Bands corresponding to ABCG1 were normalized to β-actin as the loading control and the intensity of the protein bands were quantified using ImageQuant TL (GE Healthcare, Little Chalfont, England). Bands corresponding to ABCA1 were analyzed in the same manner using ImageJ.

Lipid extraction. Lipid analysis was carried out on sets of pooled BAL cells and 1mL aliquots of BAL fluids with similar effluent volumes. Total lipids were extracted using a modified method of Bligh and Dyer in HPLC-grade chloroform/methanol/1M sodium chloride (NaCl) (2/1/1.25, v/v/v) (Sigma) (99). The organic phase was obtained by centrifugation at 1500rpm. Lipids were dried under a gentle stream of nitrogen gas. The Phospholipids C kit (Wako Pure Chemicals, Osaka, Japan) was analyzed according to manufacturer's instructions. Phospholipid content was expressed as mg phospholipid per mg total protein.

Cholesterol content analysis. Sets of pooled BAL cells and BAL fluid samples with similar effluent recovered were analyzed for cholesterol content using the Amplex Red Cholesterol Assay (Invitrogen) according to the manufacturer's protocol. Cells and aliquots of cell-free BAL fluid were assayed in serial dilution

in 96-well plates. Cholesterol content was expressed as μg cholesterol per mg total protein.

Statistical analysis. Data were analyzed by Student's *t*-test using Prism software (GraphPad, Inc., San Diego, CA). Significance was defined as $p \leq 0.05$.

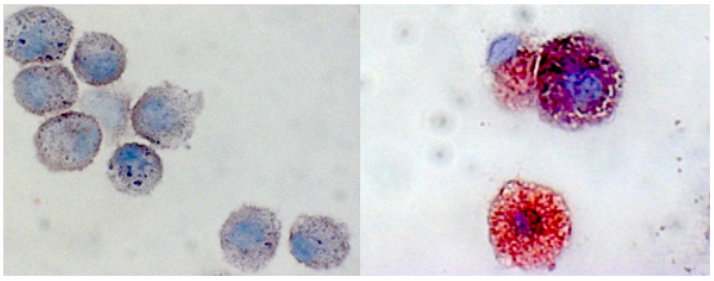
Results

PPARγ deficiency results in lipid accumulation and dysregulation of lipid transporters in alveolar macrophages. Wright-Giemsa staining revealed large foamy alveolar macrophages and Oil Red O staining showed that 88.8±1.7% of PPARγ KO alveolar macrophages stained positive, compared to 2.4±1.0% of wild type, indicating neutral lipid accumulation in the PPARγ KO ($p<0.0001$) (Figure 2.1A). Due to the lipid accumulation, we evaluated mRNA expression of the lipid transporters ABCG1 and ABCA1 which are known to be involved in lipid metabolism in macrophages and are downstream targets of PPARγ. (100). ABCG1 mRNA was decreased 1.5-fold and in contrast ABCA1 was increased 5.9-fold (Figure 2.1B). Decreased ABCG1 and increased ABCA1 protein expression were confirmed at the protein level by immunoblotting (Figure 2.1C-D).

Surfactant lipids and proteins accumulate in the lungs of PPARγ KO mice. The composition of the lipid accumulating in the lungs of the PPARγ KO was determined by measuring both cholesterol and phospholipid levels in the alveolar macrophages and BAL fluids. Compared to wild type mice, cellular content of free cholesterol was significantly increased in PPARγ KO mice (0.39±0.07 versus 5.80±1.69 μg/mg protein) while the cholesteryl ester content was not significantly different (0.12±0.01 versus 0.58±0.29 μg/mg protein) (Figure 2.2A). Free

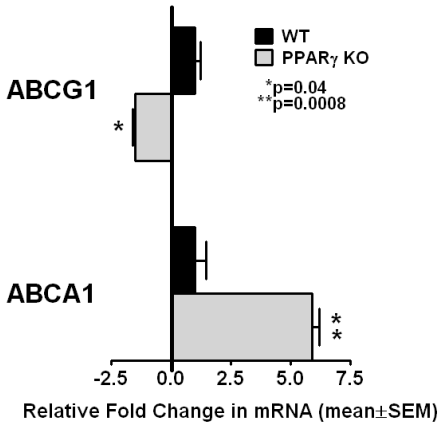
Figure 2.1 PPARγ deficiency results in dysregulation of lipid metabolism in alveolar macrophages. (A) Marked Oil Red O staining of alveolar macrophages from PPARγ KO indicate neutral lipid accumulation compared to wild type (n=3). (B) ABCG1 is decreased whereas ABCA1 expression is enhanced in PPARγ KO compared to wild type as measured by RT-PCR (n=6). (C) ABCG1 protein is decreased and (D) ABCA1 protein is increased in PPARγ KO alveolar macrophages as shown in a representative immunoblot from one of two experiments. The numbers above the bands refer to sets of pooled BAL cells from each genotype. The intensity ratios of the ABCG1 and ABCA1 protein bands to actin are indicated.

A.

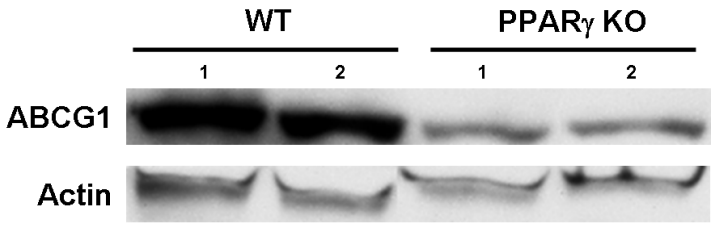


WT PPARγ KO

B.

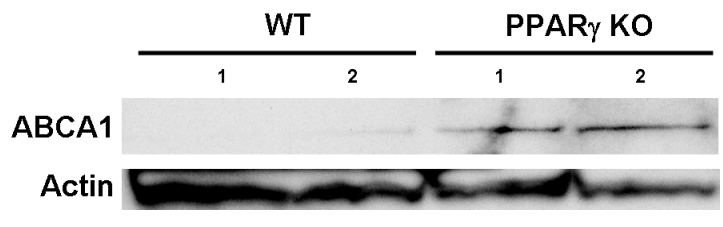


C.



ABCG1 Relative to Actin 2.0 3.1 1.1 0.7

D.

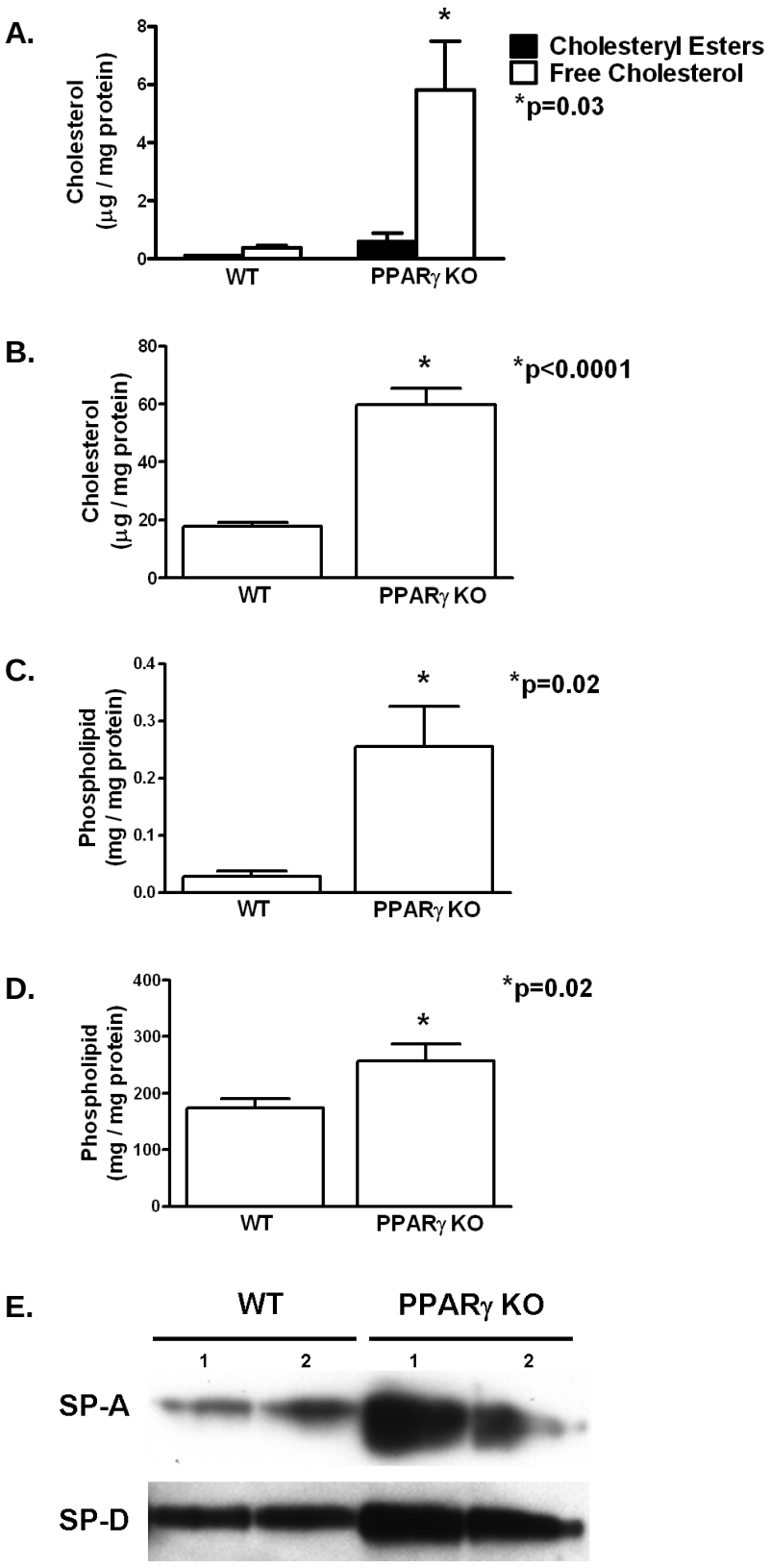


ABCA1 Relative to Actin 0.01 0.1 1.5 3.9

cholesterol was also elevated in the BAL fluid of PPARγ KO mice (59.6±5.7 μg/mg protein) compared to wild type (17.8±1.3 μg/mg protein) (Figure 2.2B). Cholesteryl esters were not detected in the BAL fluid of wild type or PPARγ KO mice. The cellular phospholipid content in PPARγ KO alveolar macrophages was significantly increased over wild type (0.03±0.01 versus 0.26±0.07 mg/mg protein) (Figure 2.2C). Extracellular phospholipids were elevated in the BAL fluid of PPARγ KO mice (257.5±28.9 mg/mg protein) compared to wild type (174.2±16.0 mg/ mg protein) (Figure 2.2D). Surfactant-associated proteins A (SP-A) and D (SP-D) were increased in the BAL fluid of PPARγ KO mice as shown by immunoblotting (Figure 2.2E).

PPARγ deficiency results in decreased cholesterol efflux to HDL from alveolar macrophages. The accumulation of cholesterol in the lungs and alveolar macrophages of the PPARγ KO and decreased expression of key cholesterol efflux mediators led us to evaluate the cholesterol efflux system. Baseline cholesterol efflux (no acceptor) was increased in the PPARγ KO alveolar macrophages (8.3±0.8%) compared to wild type (4.5±0.3%), and the overall cholesterol efflux to media supplemented with FBS was decreased in the PPARγ KO (59.5±1.7%) relative to wild type (70.5±3.5%) (Figure 2.3). We next measured the efflux of cholesterol to acceptor molecules HDL and ApoA-I. Cholesterol efflux to ApoA-I in PPARγ KO (25.7±1.7%) was significantly

Figure 2.2 Surfactant lipids and proteins accumulate in the lungs of PPARγ KO mice. (A) Cholesterol levels of PPARγ KO alveolar macrophages (n=3 sets) and (B) BAL fluid (n=5) are increased. Total and free cholesterol were measured and cholesteryl ester was determined by subtraction (90). (C) The phospholipid content of PPARγ KO alveolar macrophages (n=4 sets) and (D) BAL fluid (n=11 mice) are increased. (E) Surfactant associated protein A (SP-A) and surfactant associated protein D (SP-D) were found at increased levels in the BAL fluid of PPARγ KO mice as shown by a representative immunoblot from one of three experiments. The numbers above the bands refer to individual mice. Following previously established guidelines for analysis of acellular components of BAL fluid (97), analysis BAL fluid protein and lipid utilized samples with similar volumes recovered.


A.
Cholesterol (μg / mg protein)
8
6
4
2
0
*
WT
PPARγ KO
Cholesteryl Esters
Free Cholesterol
*p=0.03
B.
Cholesterol (μg / mg protein)
80
60
40
20
0
*
WT
PPARγ KO
*p<0.0001
C.
Phospholipid (mg / mg protein)
0.4
0.3
0.2
0.1
0.0
*
WT
PPARγ KO
*p=0.02
D.
Phospholipid (mg / mg protein)
400
300
200
100
0
*
WT
PPARγ KO
*p=0.02
E.
WT
PPARγ KO
1
2
1
2
SP-A
SP-D

increased over wild type (17.3±1.5%) and efflux to HDL was significantly decreased in PPARγ KO (46.2±1.5%) compared to wild type (56.7±3.6%). These results suggest impairment of ABCG1-mediated cholesterol efflux.

PPARγ deficiency results in dysregulated LXRα and LXRβ expression. Given the increased expression of ABCA1 in PPARγ KO alveolar macrophages, we next investigated the expression of the LXR transcription factors, which may regulate cholesterol metabolism in macrophages in part by mediating transcription of the ABC transporters (101). RT-PCR analysis revealed a 1.7-fold decrease in LXRα mRNA and a 2.1-fold increase in LXRβ mRNA expression in PPARγ KO alveolar macrophages (Figure 2.4A). RT-PCR analysis also revealed increased expression in apolipoprotein E (ApoE) (34-fold) and sterol 27-hydroxylase (CYP27A1) (2.3-fold) mRNA in PPARγ KO alveolar macrophages, indicating that the LXR pathway is enhanced (Figure 2.4B).

Figure 2.3 PPARγ deficiency results in decreased cholesterol efflux to HDL from alveolar macrophages. The efflux of ^{3}H labeled cholesterol was measured in PPARγ KO alveolar macrophages and compared to wild type (n=3).

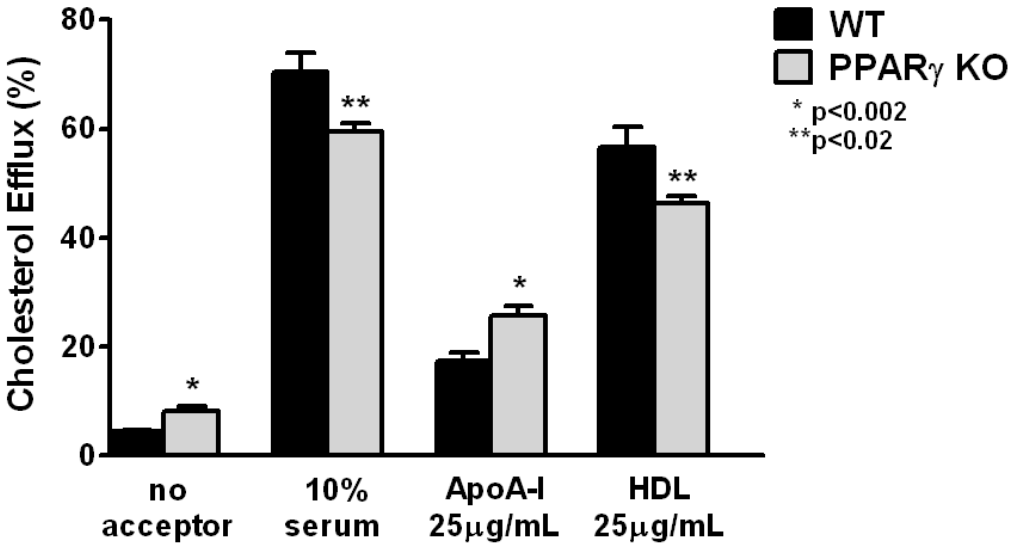
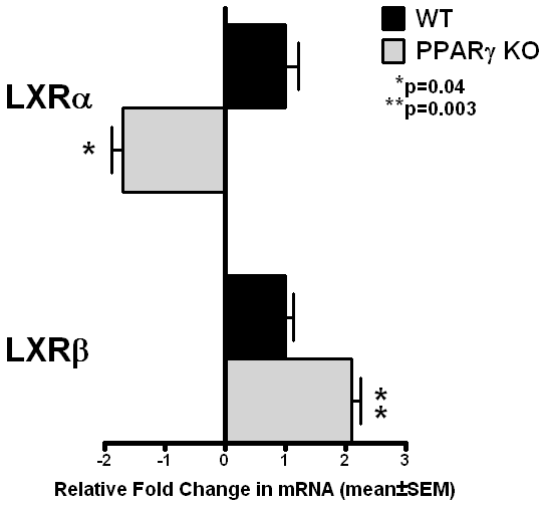
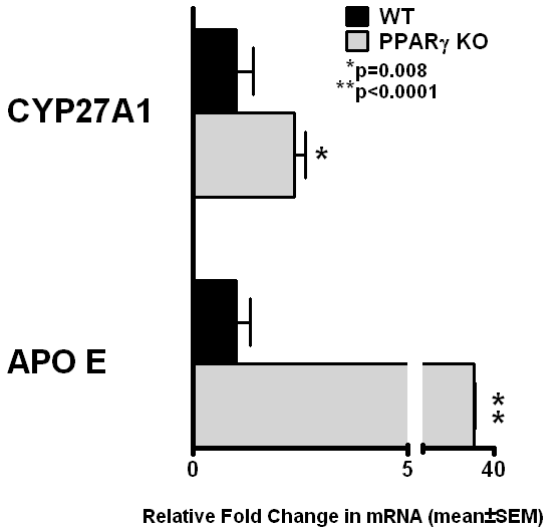

Cholesterol Efflux (%)
80
60
40
20
0
*
**
*
**
no acceptor
10% serum
ApoA-I 25μg/mL
HDL 25μg/mL
WT
PPARγ KO
* p<0.002
**p<0.02

Figure 2.4 PPARγ deficiency results in dysregulated LXRα and LXRβ expression. (A) Gene expression of LXRα and LXRβ in BAL cells from wild type (n=4) PPARγ KO mice (n=6) were analyzed by RT-PCR. LXRα mRNA expression is decreased in PPARγ KO alveolar macrophages in contrast LXRβ is increased. (B) ApoE and CYP27A1 mRNA expression are increased in PPARγ KO alveolar macrophages (n=6) compared to wild type (n=5).

A.


WT
PPARγ KO
*p=0.04
**p=0.003
LXRα
*
LXRβ
**
-2
-1
0
1
2
3
Relative Fold Change in mRNA (mean±SEM)

B.


WT
PPARγ KO
*p=0.008
**p<0.0001
CYP27A1
*
APO E
**
0
5
40
Relative Fold Change in mRNA (mean±SEM)

Discussion

In the present study we show that the targeted knockout of PPARγ in macrophages results in the accumulation of surfactant-like material in the alveolar spaces of the lung and within the alveolar macrophages. This is the first report directly linking the deficiency of PPARγ to lipid accumulation in the lung. PPARγ KO alveolar macrophages phenotypically resemble those of PAP patients in that they are foamy and Oil Red O positive for neutral lipid accumulation (50). Additionally, surfactant components (surfactant-associated proteins, phospholipids, and cholesterol) are increased within extracellular BAL fluids. Finally, the alveolar macrophages of PPARγ KO mice have reduced expression of ABCG1 and exhibit reduced ABCG1-mediated cholesterol efflux to HDL. Our results support the hypothesis that PPARγ-mediated regulation of ABCG1 expression is critical for surfactant catabolism in alveolar macrophages.

Previous studies have suggested that PPARγ is a key mediator of surfactant clearance and catabolism by alveolar macrophages (6,51). Surfactant accumulates in alveolar macrophages of PAP patients. PPARγ is deficient in the alveolar macrophages of these patients and is associated with the presence of neutralizing auto-antibodies against the hematopoietic growth factor GM-CSF (6). PPARγ deficiencies were also demonstrated in the GM-CSF KO mouse model of the disease (51).

GM-CSF also promotes cell survival, proliferation, and differentiation of alveolar macrophages and promotes the transcription of PPARγ in macrophages (7,29,102). The biological loss of GM-CSF has been reported to impair the differentiation of alveolar macrophages through dysregulation of the transcription factor PU.1 [reviewed by Trapnell and Whitsett (92)]. It was further demonstrated that PU.1 is deficient in the alveolar macrophages of PAP patients and GM-CSF KO mice (103,104); however no deficiency in GM-CSF or PU.1 expression was observed in the alveolar macrophages of the PPARγ KO mice. GM-CSF was up-regulated 2.7±0.25-fold (n=5, p=0.02) while PU.1 expression was not different from wild type mice (n=3). These data suggest that maturation of the PPARγ KO alveolar macrophages is not disrupted as it is in PAP patients and GM-CSF KO mice (92). This is consistent with current literature suggesting that although PPARγ is not necessary for the differentiation of monocytes, a variation in the expression levels of PPARγ may modulate differentiation (105-107).

Consistent with the findings in PAP patients and GM-CSF KO mice (47,49,108-110), the PPARγ KO mice exhibit elevated levels of the major components of surfactant including cholesterol, phospholipid, and surfactant-associated proteins in the BAL fluid and alveolar macrophages. The alveolar macrophages from the PPARγ KO mice exhibited significantly increased cholesterol content comprised predominantly of free cholesterol. Cellular deposition of free cholesterol is considered to be a pivotal step in foam cell formation (111) and is consistent with the foamy phenotype of the PPARγ KO

alveolar macrophages. The pattern of lipid accumulation both in the alveolar space and alveolar macrophages of the lungs of PPARγ KO mice suggests deficient or incomplete surfactant catabolism by the alveolar macrophages.

RT-PCR analysis of the PPARγ KO alveolar macrophages revealed similar gene expression patterns of downstream PPARγ targets to those previously reported from PAP patients and GM-CSF KO mice with decreased expression of ABCG1 mRNA (1.5-fold) and increased expression in ABCA1 mRNA (5-fold) (6,51). These results are consistent with several studies indicating deficiency of one ABC transporter is compensated by the other transporter and is mediated by the sterol-sensing nuclear transcription factor LXR in response to the buildup of oxysterol ligands (87) or the oxidation of cholesterol metabolites by CYP27A1 in foamy macrophages (112). The two isoforms of LXR, LXR alpha (LXRα) and LXR beta (LXRβ), have overlapping roles in promoting cellular cholesterol export through regulation of the ABC transporters and ApoE (101).

In contrast to increased LXRα expression reported in PAP and GM-CSF KO (51), LXRα was down-regulated nearly 2-fold in the PPARγ KO mice. The differential expression of LXRα, which is regulated in part by PPARγ (77), may be explained by the varying levels of PPARγ in these systems: PPARγ is deficient in PAP and GM-CSF KO and is absent in PPARγ KO. This is supported by the finding that LXRβ expression, which is regulated independently of PPARγ (77), is

increased nearly 2-fold in the PPARγ KO alveolar macrophages. The expression of LXRβ has not been reported in PAP or GM-CSF KO alveolar macrophages.

While more study is needed to elucidate the possible mechanisms and differential regulation of the LXRs, it has been shown that the function and expression of the LXR isoforms are tissue-dependent (101,113,114). LXRβ is expressed at higher levels than LXRα in macrophages and is more effective than LXRα at up-regulating ABCA1 in response to sterol ligands (115). While the contributions of the individual LXR isoforms are unknown, as specific gene targets have yet to be identified, the LXR pathway overall is enhanced in the PPARγ KO, as evidenced by increased expression of downstream targets ABCA1 and ApoE. We show that increased expression of the LXR pathway is not sufficient to maintain surfactant catabolism in the absence of PPARγ.

The accumulation of cholesterol in the lungs and alveolar macrophages of PPARγ KO mice and the dysregulation of several cholesterol transport genes led us to investigate the efflux of cholesterol in PPARγ KO alveolar macrophages in vitro. PPARγ promotes lipid influx and efflux in macrophages through transcriptional regulation of ABC transporters and LXRs. ABCG1 mediates transport of cholesterol to extracellular acceptor HDL and ABCA1 transports cholesterol to lipid-free ApoA-I (83-85,116,117). In the present study, ABCG1-mediated cholesterol efflux to HDL was reduced and ABCA1-mediated efflux to ApoA-I was increased. Overall cholesterol efflux to FBS (10% serum) was decreased compared to wild type, consistent with the high levels of cholesterol in

the macrophages in vivo. These findings indicate that the reduction in cholesterol efflux in the PPARγ KO alveolar macrophages may be due to deficient transporter mediated cholesterol efflux pathways, specifically transport mediated by ABCG1.

Interestingly, similar patterns of cholesterol and phospholipid accumulation and altered lipid efflux have been reported in ABCG1 KO models (86,89). A reduction in total cholesterol efflux and a specific reduction in efflux to HDL were noted in ABCG1 KO peritoneal macrophages (86). Importantly, the authors also noted significantly increased efflux to ApoA-I in ABCG1 KO indicating compensation by ABCA1. Taken together, deficiencies in ABCG1 may result in dysregulated or insufficient cholesterol efflux and therefore cholesterol accumulation in the lung.

A summary of the differential gene expression of various lipid regulators and transporters in the alveolar macrophages from PPARγ KO mice, GM-CSF KO mice, and PAP patients is presented in Table 2.1. Comparison of the data supports the hypothesis that PPARγ-mediated regulation of ABCG1 is necessary to prevent the accumulation of surfactant. Additionally, the LXR pathway is enhanced in all of the groups, as evidenced by increased expression in ABCA1. An interesting difference, however, is the expression of LXRα which is increased in PAP and GM-CSF KO (PPARγ-deficient) alveolar macrophages and decreased in the PPARγ KO model. We speculate that lipid accumulation

Table 2.1

Summary table of expression levels of key lipid regulator and transporter genes in the alveolar macrophages from PPARγ KO mice, GM-CSF KO mice, and PAP patients.

Lipid Regulators	PPARγ KO MICE	GM-CSF KO MICE	PAP PATIENTS
GM-CSF	Increased	Not expressed (29)	Decreased* (118)
PPARγ	Decreased	Decreased (51)	Decreased (6)
ABCG1	Decreased	Decreased (51)	Decreased (51)
ABCA1	Increased	Increased (51)	Increased (51)
LXRα	Decreased	Increased (51)	Increased (51)
LXRβ	Increased	Not reported	Not reported

**GM-CSF mRNA is increased (118). However, protein is functionally reduced due to neutralizing antibodies against GM-CSF (1).*

activates the LXR-ABCA1 pathway as a compensation mechanism, and that in the absence of PPARγ, LXRβ is the predominant isoform driving the up-regulation of ABCA1 and ApoE.

In the PPARγ KO mouse model, the absence of PPARγ results in reduced expression levels of ABCG1 and LXRα. Despite increased expression of LXRα and ABCA1 and increased ABCA1-mediated cholesterol efflux, surfactant components accumulate in the alveolar macrophages and BAL fluid of PPARγ KO mice. Our results indicate that as part of surfactant catabolism, ABCG1-mediated cholesterol efflux to HDL may be the major pathway for cholesterol efflux in alveolar macrophages. Thus PPARγ-mediated regulation of ABCG1 expression may be critical to the maintenance of surfactant homeostasis. This is the first report directly linking PPARγ deficiency in alveolar macrophages to lipid accumulation in the lungs. Understanding the role of PPARγ in normal surfactant homeostasis contributes to our knowledge of the pathophysiology of PAP and identifies a potential target for therapy.

Acknowledgments

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CHAPTER 3

GENERATION OF A LENTIVIRUS EXPRESSION SYSTEM TO STUDY THE ROLE OF PPARγ IN ALVEOLAR MACROPHAGES

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Running Title: Generation of a Lentivirus-PPARγ Vector

Keywords: surfactant, pulmonary alveolar proteinosis, GM-CSF

Abstract

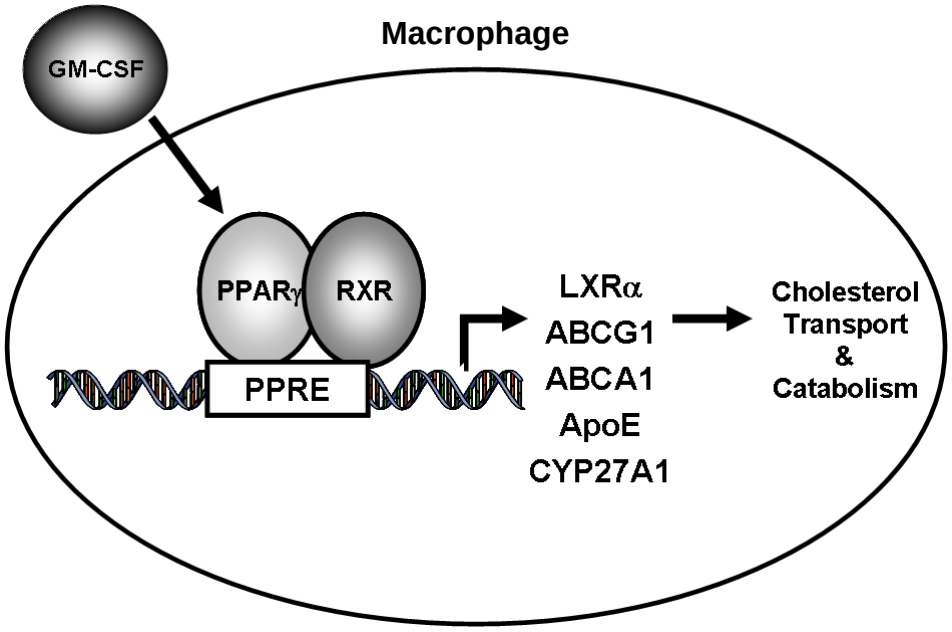
The use of the Lentivirus expression system for gene delivery has gained significant interest due to its rapid, stable and long-term gene expression into a wide variety of cell types. Herein, we have utilized this system to successfully generate a Lentivirus, (Lenti-PPARγ), which expresses human PPARγ, a transcription factor of the nuclear hormone receptor superfamily which is involved in lipid metabolism in macrophages. Transduction of Lenti-PPARγ into several cell types including cell lines and primary murine and human alveolar macrophages isolated from bronchoalveolar lavage led to the efficient expression of PPARγ as confirmed by RT-PCR and immunoblotting. Moreover, RT-PCR analysis revealed expression was dose-dependent. These results demonstrate that this system provides a reliable approach to study gene expression.

Introduction

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a lipid-binding nuclear transcription factor involved in lipid and glucose metabolism in macrophages. Alveolar macrophages constitutively express high levels of PPARγ (6). The alveolar macrophages from patients with the autoimmune lung disease pulmonary alveolar proteinosis (PAP) are deficient in PPARγ (6). The biological loss of granulocyte-macrophage colony-stimulating factor (GM-CSF) due to a neutralizing auto-antibody results in the accumulation of surfactant in the lungs and alveolar macrophages of PAP patients (1,91). GM-CSF has been shown to up-regulate PPARγ (7). Therefore, we hypothesized that the GM-CSF-PPARγ pathway is involved in the regulation of surfactant catabolism (Figure 3.1). In order to test this hypothesis, we have utilized a Lentivirus expression system to restore expression of PPARγ in the PPARγ-deficient alveolar macrophages of GM-CSF knockout (GM-CSF KO) mice, a well-established model for studying PAP (29).

The most common mediators of gene delivery are DNA plasmids, liposome vectors, and recombinant viral vectors. While DNA plasmids are unstable and liposome vectors can evoke inflammatory responses (119), recombinant viral vectors are robust and can be adapted to be non-disruptive to host cells.

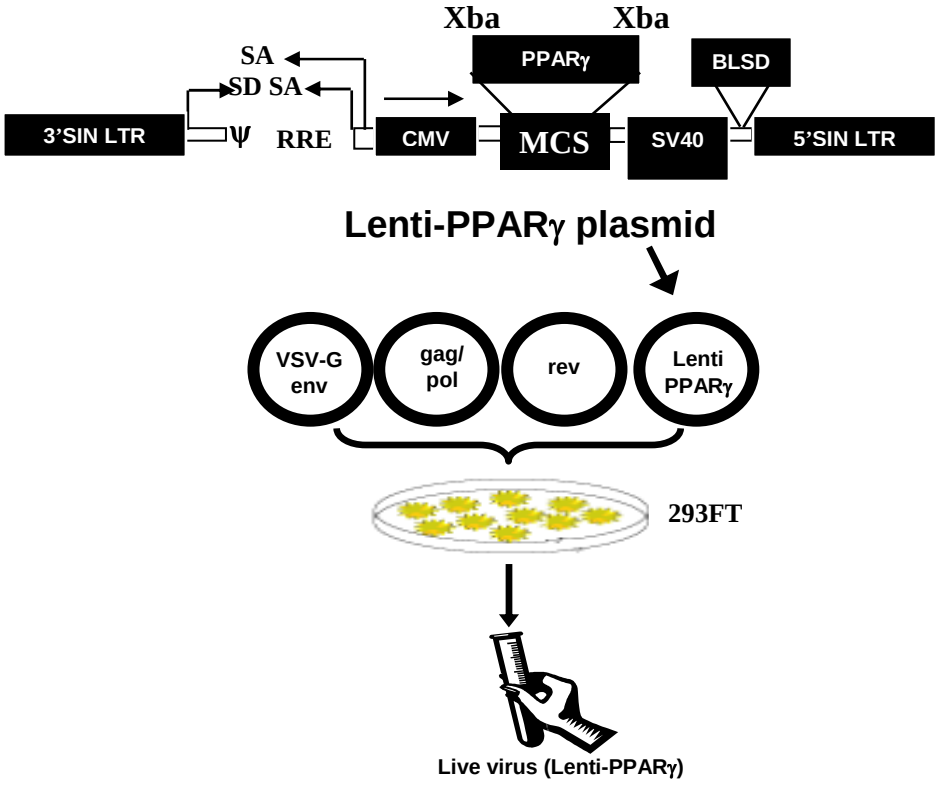
Figure 3.1 Proposed pathway of interest. The GM-CSF-PPARγ pathway in alveolar macrophages promotes the clearance of surfactant, particularly the cholesterol component, through the regulation of liver X receptor-alpha (LXRα), ABC transporters (ABC) ABCG1 and ABCA1, apolipoprotein E (ApoE), and 27-hydroxylase (CYP27A1). PPRE, PPARγ response element; RXR, retinoid X receptor.


Macrophage
GM-CSF
PPARγ
RXR
PPRE
LXRα
ABCG1
ABCA1
ApoE
CYP27A1
Cholesterol
Transport
&
Catabolism

The Lentivirus expression system offers many advantages. In contrast to adenoviral-based vectors, recombinant retroviral vectors offer the unique capacity of delivering, integrating, and expressing genetic material in the host cell without undergoing lytic, destructive cycles of replication, making them ideal for targeting non-dividing cells (120). Lentivirus has been adapted for gene therapy by the removal of essential wild type replication genes and the addition of self-inactivating long terminal repeats (Figure 3.2). Lentivirus vectors may be engineered to target specific cell types by pseudotyping the viral protein coat. Lentivirus-mediated integration is stable and heritable, unlike the adenoviral vectors which offer poor efficiency and transient expression of the delivered gene. Additionally, Lentivirus is capable of transducing non-dividing cells, such as terminally differentiated alveolar macrophages.

Herein, we have transduced primary murine macrophages resulting in the up-regulation of PPARγ, in vitro. Additionally, preliminary experiments on human alveolar macrophages demonstrated the effectiveness of the Lentivirus system on primary human cells. These results demonstrate an efficient, reliable technique for the up-regulation of PPARγ in primary macrophages for the further study of PPARγ in surfactant catabolism.

Figure 3.2 Production of live Lenti-PPARγ virus. The Lentivirus plasmid containing the PPARγ sequence along with plasmids encoding for *env*, *gag/pol*, and *rev* genes were transfected into 293FT cells to produce live Lenti-PPARγ virus. Image adapted from Malur, et al. (121). SIN, self-inactivating; LTR, long terminal repeat; RRE, Rev response element; SA, splice acceptor; SD, splice donor; CMV, cytomegalovirus promoter, MCS, multiple-cloning site; SV40, Simian virus 40 promoter; BLSD, blasticidin.


Xba
Xba
SA
SD SA
PPARγ
BLSD
3'SIN LTR
Ψ
RRE
CMV
MCS
SV40
5'SIN LTR
Lenti-PPARγ plasmid
VSV-G env
gag/ pol
rev
Lenti PPARγ
293FT
Live virus (Lenti-PPARγ)

Materials and Methods

Mice. Animal studies were conducted in conformity with Public Health Service (PHS) Policy on humane care and use of laboratory animals and were approved by the institutional animal care committee. C57Bl/6 wild type (WT) mice were obtained from Jackson Laboratory (Bar Harbor, ME). The GM-CSF KO mice were generated by Dr. Glenn Dranoff (29). The mice have been backcrossed eight generations to C57Bl/6.

Bronchoalveolar lavage (BAL). Macrophages from 3-5 animals were pooled for each set of experiments. Mice received ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally. The thoracic cavity was opened and the lungs were exposed. After cannulating the trachea a tube was inserted and bronchoalveolar lavage was carried out with warmed (37^{o}C) PBS in 1ml aliquots. Cytospins of BAL cells were stained with a modified Wright–Giemsa stain for differentials. Viability was >95% as determined by trypan blue for all cell preparations.

Peritoneal exudate cells. To obtain peritoneal macrophages, mice were euthanized with Isofluorane. Five mL of warmed PBS was injected intraperitoneally using a 23 gauge needle. After exposing the peritoneal cavity, a small incision was made in the peritoneum and a transfer pipet was used to draw out the fluid. Cells and fluid were pooled and processed as above.

Healthy control cell collection. This protocol was approved by the Institutional Review Board, and written informed consent was obtained from all subjects. Healthy control individuals had no history of lung disease and were not on medication (n=3). Alveolar macrophages were derived from BAL fluid obtained by fiber-optic bronchoscopy as described previously (122). Differential cell counts were obtained from cytospins stained with a modified Wright's stain. Differential cell counts from BAL fluid indicated that >95% of healthy control cells were alveolar macrophages. The mean viability of lavage cells was >95% as determined by trypan blue dye exclusion.

Lentivirus construction and transduction. A self-inactivating Lentivirus expression vector that was previously utilized in the generation of a stable cell line expressing the human parainfluenza virus type 3 C protein was used for these experiments (51,121). cDNA corresponding to the human PPARγ sequence was cloned into the multiple cloning sites downstream of a CMV promoter using standard techniques as described (121). The recombinant Lentiviral plasmid thus obtained was transfected into 293FT cells along with plasmids encoding the *gag*, *pol* and *rev* genes and a plasmid possessing the vesicular stomatitis glycoprotein (G) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 72 hrs post transfection, cell culture supernatant containing the Lentivirus-PPARγ (Lenti-PPARγ) was collected and then purified by centrifugation at 27,000 rpm at 4^0C for 3.5 hrs. The Lenti-PPARγ virus pellet was

resuspended in PBS and aliquots of 100μl were stored at -70^{0}C. The concentration of Lenti-PPARγ virus was determined by a p24 ELISA (Cell Biolabs, San Diego, CA). A Lentivirus expressing the enhanced Green Fluorescent Protein (Lenti-eGFP) was obtained using a similar protocol and was utilized as a control in experiments for the determination of transduction efficiency. Cells expressing eGFP were observed by fluorescent microscopy. For in vitro analyses, BAL and peritoneal macrophages were transduced in culture with Lenti-PPARγ or Lenti-eGFP for 24 hours in Opti-MEM (Invitrogen) media and then incubated 24 hours in complete DMEM media (Invitrogen) prior to collection.

RNA purification and analysis. Total RNA was extracted from cells by RNeasy protocol (Qiagen, Valencia, CA). Expression of mRNA was determined by real time RT-PCR using the ABI Prism 7300 Detection System (TaqMan, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. RNA specimens were analyzed in duplicate using primer/probe sets for mouse PPARγ (Mm0040945) and human PPARγ (Hs00234592) (ABI) as previously described (51). Primer/probe sequences for eGFP have been described (123). Threshold cycle (CT) values for genes of interest were normalized to a housekeeping gene [glyceraldehyde 3 phosphate dehydrogenase, (GAPDH)] and used to calculate the relative quantity of mRNA expression. Data were expressed as a fold change in mRNA expression relative to control values (98).

Protein analysis. Whole cell lysates were prepared using a modified Nonidet P-40 lysis buffer as described previously (51). 10% SDS-PAGE gels were loaded based on equal protein obtained by the bicinchoninic acid assay (Pierce, Rockford, IL). Primary antibody to PPARγ (H-100, Santa Cruz Biotechnology, Santa Cruz, CA) was diluted to 1:000 and blots were visualized by chemiluminescence.

Statistical analysis. Data were analyzed by Student's t-test using Prism software (GraphPad, Inc., San Diego, CA). Significance was defined as $p \leq 0.05$.

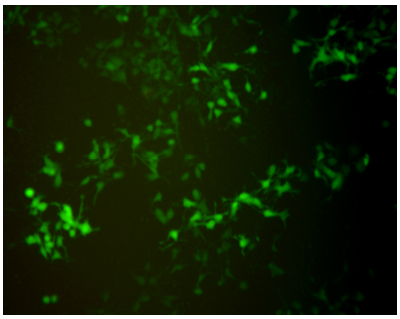
Results

Lenti-PPARγ mediates stable expression of PPARγ in human and mouse cell lines. Preliminary experiments demonstrating the stable expression of eGFP and PPARγ were carried out on HeLa and MH-S cell lines. HeLa and MH-S cells were transduced with Lenti-eGFP. After 72 hours, cells were challenged with blasticidin (10 μg/uL). After several passages, cells were collected for analysis. HeLa cells exhibited eGFP positive cells (Figure 3.3A). Experiments testing the Lenti-PPARγ virus were carried out in a similar manner. Neither HeLa nor MH-S cells express detectable amounts of PPARγ. RT-PCR analysis of HeLa and MH-S cells transduced with Lenti-PPARγ, however, exhibit increased expression of PPARγ mRNA (Figure 3.3B). Transduction with serial dilutions of Lenti-PPARγ virus resulted in up-regulation of PPARγ protein in HeLa cells (Figure 3.3C).

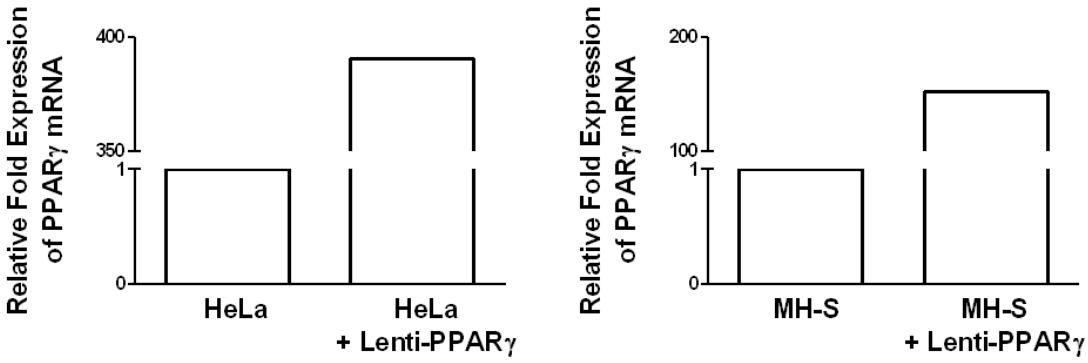
Lenti-PPARγ mediates efficient up-regulation of PPARγ in primary murine macrophages. To determine the transduction efficiency of the Lenti-PPARγ virus on primary cells, we harvested peritoneal macrophages from wild type and GM-CSF KO mice which do not express detectable levels of PPARγ and treated the cells with Lenti-PPARγ in vitro. Analysis by RT-PCR demonstrated increased levels of PPARγ in a dose-dependent manner in wild type and GM-CSF KO cells

Figure 3.3 Lenti-PPARγ mediates expression of PPARγ in human and mouse cell lines. (A) HeLa cells expressed eGFP after transduction with Lenti-eGFP. (B) RT-PCR analysis of HeLa and MH-S cell lines transduced with Lenti-PPARγ exhibited increased expression of PPARγ mRNA. (C) Transduction with serial dilutions of Lenti-PPARγ virus resulted in the up-regulation of PPARγ protein in HeLa cells.

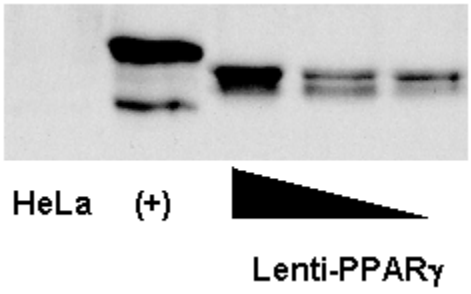
A.



B.


Relative Fold Expression of PPARγ mRNA
400
350
1
0
HeLa
HeLa + Lenti-PPARγ
Relative Fold Expression of PPARγ mRNA
200
100
1
0
MH-S
MH-S + Lenti-PPARγ

C.

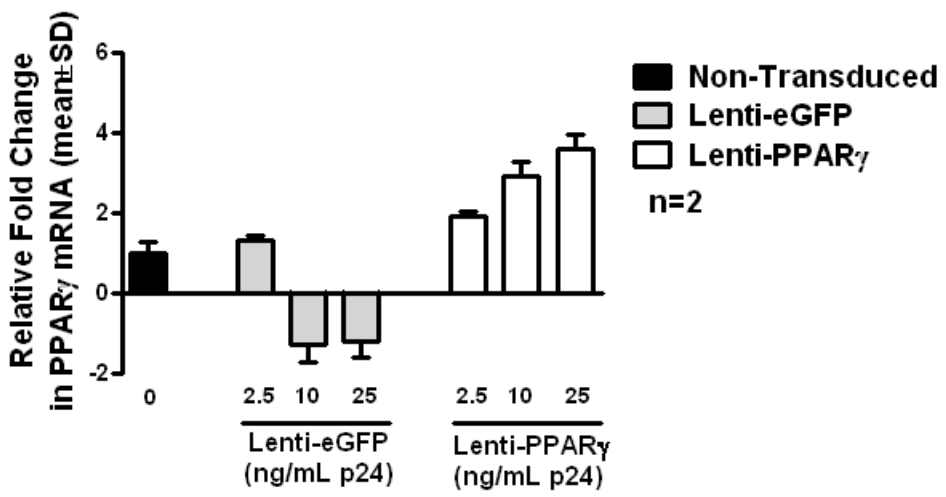

HeLa (+)
Lenti-PPARγ

compared to non-transduced controls (Figure 3.4A-B). Further, we transduced alveolar macrophages of GM-CSF KO and noted increased expression of PPARγ mRNA (Figure 3.4C). Transduction with control Lenti-eGFP virus did not result in increases in PPARγ mRNA in any experiment. These results demonstrated efficient up-regulation of PPARγ in peritoneal macrophages that do not constitutively express PPARγ and alveolar macrophages deficient in PPARγ.

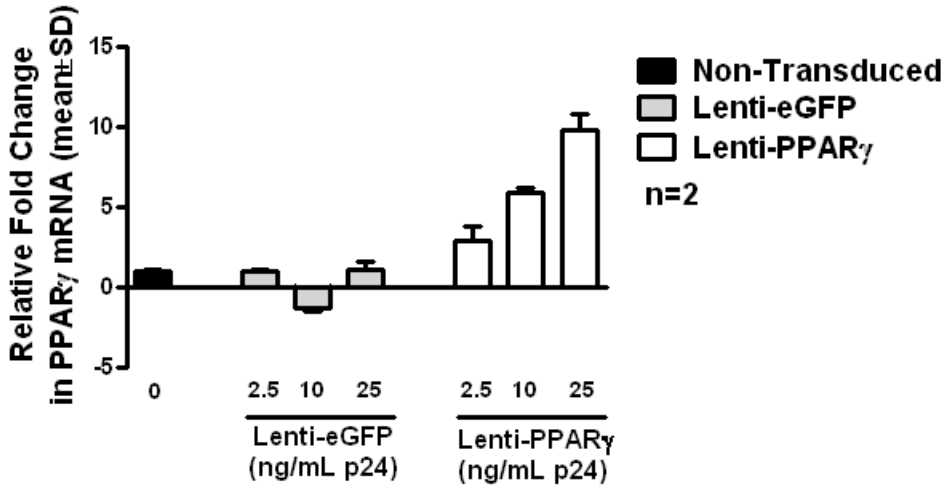
Lentivirus mediates up-regulation of eGFP and PPARγ in alveolar macrophages from healthy human controls. In order to evaluate the Lentivirus expression system in primary human macrophages, we transduced alveolar macrophages from healthy controls with increasing titers of Lenti-eGFP and measured eGFP mRNA by RT-PCR (Figure 3.5A). Transduction efficiency was also determined by counting eGFP-positive cells [data previously published (51)]. Additionally, a preliminary experiment demonstrated a 60-fold increase in PPARγ mRNA expression in alveolar macrophages from a healthy individual upon transduction with Lenti-PPARγ (Figure 3.5B). Taken together, these results provide evidence that Lenti-PPARγ is an effective investigative tool to study the up-regulation of PPARγ in primary alveolar macrophages.

Figure 3.4 Lenti-PPARγ mediates efficient up-regulation of PPARγ in primary murine macrophages. (A) RT-PCR analysis demonstrated dose-dependent up-regulation of PPARγ mRNA in WT and (B) GM-CSF KO peritoneal macrophages treated with Lenti-PPARγ virus compared to non-transduced cells (0 ng/mL p24). Expression of PPARγ mRNA in macrophages treated with control virus (Lenti-eGFP) was not different from non-treated cells. (C) Up-regulation of PPARγ was also detected by RT-PCR in GM-CSF alveolar macrophages upon treatment with Lenti-PPARγ (25 ng/mL p24).

A.


Relative Fold Change in PPARγ mRNA (mean±SD)
6
4
2
0
-2
Non-Transduced
Lenti-eGFP
Lenti-PPARγ
n=2
0
2.5 10 25
Lenti-eGFP (ng/mL p24)
2.5 10 25
Lenti-PPARγ (ng/mL p24)

B.


Relative Fold Change in PPARγ mRNA (mean±SD)
15
10
5
0
-5
Non-Transduced
Lenti-eGFP
Lenti-PPARγ
n=2
0
2.5 10 25
Lenti-eGFP (ng/mL p24)
2.5 10 25
Lenti-PPARγ (ng/mL p24)

C.

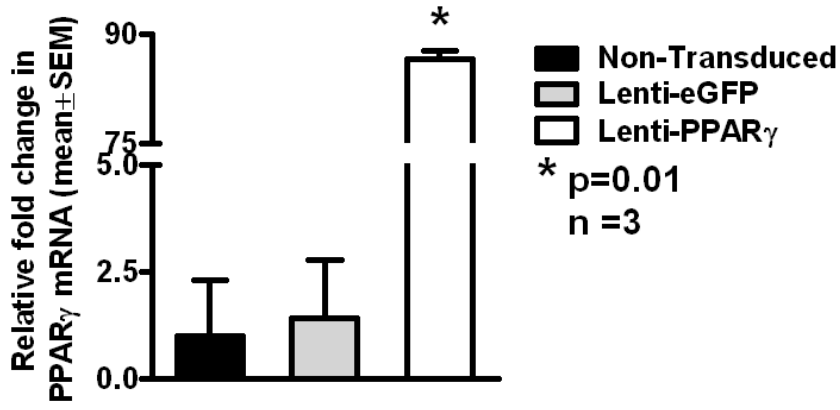
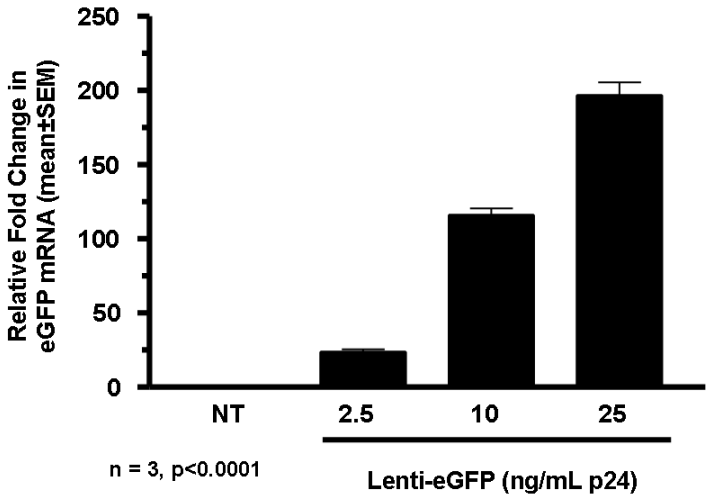
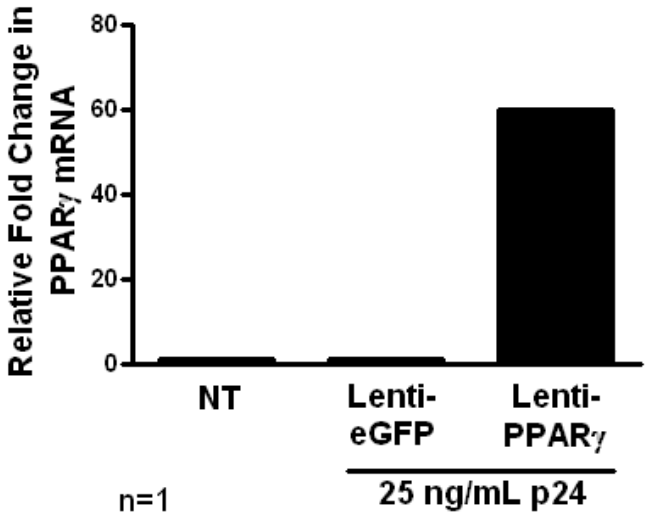

Relative fold change in PPARγ mRNA (mean±SEM)
90
75
5.0
2.5
0.0
*
Non-Transduced
Lenti-eGFP
Lenti-PPARγ
* p=0.01
n =3

Figure 3.5 Lentivirus mediates up-regulation of eGFP and PPARγ in alveolar macrophages from healthy human controls. (A) Human alveolar macrophages transduced with Lenti-eGFP for 24 hours up-regulated eGFP mRNA in a dose-dependent manner. (B) Alveolar macrophages from a healthy control exhibited increased PPARγ mRNA expression upon transduction with Lenti-PPARγ.

A.


Relative Fold Change in eGFP mRNA (mean±SEM)
250
200
150
100
50
0
NT
2.5
10
25
n = 3, p<0.0001
Lenti-eGFP (ng/mL p24)

B.


Relative Fold Change in PPARγ mRNA
80
60
40
20
0
NT
Lenti-eGFP
Lenti-PPARγ
n=1
25 ng/mL p24

Discussion

PPARγ is deficient in the alveolar macrophages of PAP patients and GM-CSF KO mice (6,51). Previous studies from our laboratory have implicated PPARγ in the promotion of surfactant catabolism in alveolar macrophages (unpublished data). PPARγ regulates several genes involved in the transcription and transport of lipids in macrophages. In order to investigate our hypothesis that the up-regulation of PPARγ will increase surfactant catabolism and reduce the presence of lipid-engorged alveolar macrophages in the lung, we required an efficient system to up-regulate PPARγ in primary macrophages.

Lentivirus vectors are capable of delivering and expressing a gene of interest without being disruptive to the host cell (120). Secondly, the Lentivirus expression system is an advantageous therapeutic gene delivery system and has the potential for delivery of genetic material in vivo. In the present study, we utilized the Lentivirus system to transduce primary murine and human alveolar macrophages in vitro.

In initial experiments, the Lenti-eGFP virus was used to measure transduction efficiency. Additionally, macrophages transduced with Lenti-eGFP were used as a control in RT-PCR analysis as these samples did not exhibit significant changes in PPARγ mRNA compared to non-transduced cells. Thus, the up-regulation in PPARγ is due to the specific Lenti-PPARγ construct and not produced in response to foreign DNA or macrophage activation upon the

introduction of virus. Additional preliminary work entailed determining optimal Lentivirus transduction concentrations. Dose responses ranging from 2.5-25 ng/mL p24 were carried out on cultured peritoneal macrophages from wild type and GM-CSF KO mice. Alveolar macrophages from GM-CSF KO mice were transduced with 25 ng/mL p24. We next investigated the use of the Lentivirus constructs on alveolar macrophages from healthy volunteers. All doses induced transcription of PPARγ supporting the efficiency of the Lentivirus expression system.

These experiments demonstrate successful up-regulation of the proteins of interest, eGFP and PPARγ, and provide a foundation to pursue in vivo replacement of PPARγ in GM-CSF KO alveolar macrophages using Lenti-PPARγ. Unlike adenoviral vectors which offer poor efficiency and transient expression of the delivered gene, Lenti-PPARγ provides efficient up-regulation of PPARγ and may used in a variety of experiments to investigate the transcriptional regulation of PPARγ. This system provides a reliable approach to study the role of PPARγ gene expression in surfactant catabolism and homeostasis in alveolar macrophages. Understanding surfactant homeostasis is fundamental to the treatment of human pulmonary disease.

CHAPTER 4

PPARγ REGULATES THE EXPRESSION OF CHOLESTEROL METABOLISM GENES IN ALVEOLAR MACROPHAGES

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Running Title: PPARγ and Cholesterol Metabolism in Alveolar Macrophages

Keywords: ABCG1, LXR, surfactant, pulmonary alveolar proteinosis, GM-CSF

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Abstract

Peroxisome proliferator-activated receptor-gamma (PPARγ) is a nuclear transcription factor involved in lipid metabolism that is constitutively expressed in the alveolar macrophages of healthy individuals. While the mechanism remains unclear, PPARγ has recently been implicated in the catabolism of surfactant by alveolar macrophages, specifically the cholesterol component of surfactant. Studies from other tissue macrophages have shown that PPARγ transcriptionally regulates genes involved in cholesterol metabolism. PPARγ promotes the expression of the liver X receptor-alpha (LXRα) and ATP-binding cassette G1 (ABCG1) which are involved in cholesterol efflux and CD36 which is involved in the uptake of cholesterol. We have recently shown that macrophage-specific PPARγ knockout (PPARγ KO) mice accumulate cholesterol-laden alveolar macrophages that exhibit decreased expression of LXRα and ABCG1 and reduced cholesterol efflux. We hypothesized that in addition to dysregulated cholesterol efflux, cholesterol biosynthesis and uptake genes were also dysregulated and that replacement of PPARγ would induce expression of these genes. To investigate this hypothesis, we have utilized a Lentivirus expression system (Lenti-PPARγ) to restore PPARγ expression in the alveolar macrophages of PPARγ KO mice. Our results show that the alveolar macrophages of PPARγ KO mice have decreased expression of key cholesterol biosynthesis genes and increased expression of cholesterol receptors CD36 and scavenger receptor A-I

(SRA-I). The replacement of PPARγ induced transcription of LXRα and ABCG1; (2) corrected suppressed expression of cholesterol biosynthesis genes; and (3) enhanced the expression of CD36. These results suggest that PPARγ regulates cholesterol metabolism in alveolar macrophages.

Introduction

Pulmonary alveolar proteinosis (PAP) is an autoimmune lung disease characterized by the accumulation of surfactant (1). Pulmonary surfactant is comprised of 90% lipid, 10% protein, and less than 1% carbohydrate. The lipid component is comprised of phospholipids and neutral lipids, cholesterol being the major neutral lipid (93). Alveolar macrophages catabolize and recycle cholesterol from surfactant. However, the alveolar macrophages of PAP patients do not catabolize surfactant sufficiently and become engorged with lipid (25,26,28,50). It has been shown that cholesterol levels are increased in the lungs of PAP patients (47). Combined with the presence of foam cells in the lungs of patients, these results provided evidence that homeostasis of cholesterol catabolism may be disrupted in PAP. Interestingly, the contribution of impaired cholesterol catabolism to the overall disruption of surfactant catabolism in the pathogenesis of PAP has not been specifically addressed.

Our laboratory was the first to determine that the alveolar macrophages of PAP patients were deficient in the nuclear transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ) (6,51). Although the role of PPARγ in the lung is relatively unexplored, PPARγ has been implicated as a critical mediator in cholesterol metabolism in various tissue macrophages. Given the amount of cholesterol present in lung surfactant, PPARγ may hold particular

importance in promotion of cholesterol catabolism in alveolar macrophages and deficient cholesterol catabolism could impair the catabolism of surfactant overall.

Studies on other tissue macrophages have shown that PPARγ regulates many genes involved in cholesterol transport (influx and efflux) and metabolism. PPARγ transcriptionally promotes the sterol-sensing nuclear transcription factor liver X receptor-alpha (LXRα) and cholesterol transporter ATP-binding cassette G1 (ABCG1) (51,77,94). The PPARγ-LXR cascade is critical to maintaining cholesterol efflux in macrophages (85). CYP27A1 is an important enzyme that converts cholesterol into LXR ligands thereby promoting cholesterol transport from extrahepatic macrophages (112). Additionally, alveolar macrophages have been shown to readily secrete the more polar hydroxycholesterol produced by CYP27A1 in response to cholesterol-loading (124,125).

While deficiencies in cholesterol efflux could lead to cholesterol accumulation (75), the uptake of cholesterol via scavenger receptors has also been strongly associated with the accumulation of cholesterol (126,127). Scavenger receptors are up-regulated in the presence of substrate yielding macrophages with the unique capacity for cholesterol uptake regardless of intracellular cholesterol levels. The scavenger receptors CD36 and scavenger receptor A-I (SRA-I) internalize cholesterol bound to oxidized (ox)-LDL [reviewed by Glass and Witztum (74)]. Macrophages from mice lacking CD36 and SRA-I exhibit a 90% reduction in ox-LDL uptake (126). Although scavenger receptor-mediated cholesterol uptake is complex and is not fully understood, it is known to

be regulated in part by PPARγ: CD36 is directly up-regulated by PPARγ (61) while SRA-I is negatively regulated by PPARγ (60).

PPARγ regulates both the cholesterol influx (CD36 and SRA-I) and efflux genes (LXRα and ABCG1) and therefore influences the level of intracellular cholesterol present in macrophages. In turn, cholesterol levels regulate the de novo synthesis of cholesterol. In response to limited cholesterol, the nuclear transcription factor sterol response element-binding protein 2 (SREBP2) promotes cholesterol synthesis and uptake through the up-regulation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) and low-density lipoprotein receptor (LDL-R) [reviewed by Goldstein, et al. (67)]. Alternatively, intracellular cholesterol and oxysterols negatively regulate cholesterol synthesis in part by reduced transcriptional activity of SREBP2.

To investigate PPARγ and the promotion of cholesterol catabolism in alveolar macrophages, we have utilized macrophage-specific PPARγ knockout (PPARγ KO) mice which develop a PAP-like lung pathology with the accumulation of cholesterol and foamy, cholesterol-laden alveolar macrophages (unpublished data). The alveolar macrophages of PPARγ KO mice exhibit decreased expression of LXRα and ABCG1 and reduced cholesterol efflux.

These results suggest PPARγ is a key mediator of cholesterol catabolism in alveolar macrophages. Thus, we hypothesized that in addition to dysregulated cholesterol efflux genes, the expression of cholesterol synthesis and influx genes

were also dysregulated in the alveolar macrophages of PPARγ KO mice. To test this hypothesis we investigated the expression cholesterol metabolism genes and the effects of in vivo replacement of PPARγ using a Lentivirus expression system (Lenti-PPARγ).

Materials and Methods

***Mice*.** Animal studies were conducted in conformity with Public Health Service policy on the humane care and use of laboratory animals and were approved by the Institutional Animal Care Committee. C57Bl/6 wild type mice were obtained from Jackson Laboratory (Bar Harbor, ME). Macrophage-specific PPARγ KO mice have been previously described (96). BAL cells were obtained as described earlier from 8–12 week old PPARγ KO mice and age- and gender-matched wild type C57Bl/6 controls (96). For experiments, a minimum of 4 individual PPARγ KO mice were used except where indicated. For wild type mice, a minimum of 3 sets of pooled cells from 3-5 mice were used in all experiments. Briefly, the thoracic cavity was opened and the lungs were exposed. After cannulating the trachea, a tube was inserted and BAL was carried out with warmed (37℃) PBS in 1 mL aliquots. Cell via bility was measured by trypan blue exclusion. BAL cell differentials from all animals used in the experiments were stained with a Wright-Giemsa stain and revealed >90% macrophages.

Lentivirus plasmid and instillation. A self-inactivating Lentivirus expression vector containing cDNA corresponding to the human PPARγ (Lenti-PPARγ) was cloned into the multiple cloning sites downstream of a CMV promoter using standard techniques as described (96,121). A Lentivirus expressing the

enhanced Green Fluorescent Protein (Lenti-eGFP) was obtained using a similar protocol and was utilized as a control. For experiments utilizing Lenti-eGFP and Lenti-PPARγ, mice were intra-tracheally instilled 30 days prior to BAL, as described previously (96).

RNA purification and analysis. Total RNA was extracted from the cells by the RNeasy protocol (Qiagen, Valencia, CA). Expression of mRNA was determined by real-time RT-PCR analysis using the ABI Prism 7300 Detection System (TaqMan; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. RNA specimens were analyzed in duplicate using primer sets for mouse ABCA1 (Mm00442646), ABCG1 (Mm00437390), CD36 (Mm00432403), HMGCR (Mm01282494), LDL-R (Mm00440169), LXRα (Mm00443454), LXRβ (Mm00437262), SRA-I (Mm00446214), and SREBP2 (Mm01306300) (Applied Biosystems). Threshold cycle values for genes of interest were normalized to a housekeeping gene (GAPDH, 4352339E) (Applied Biosystems) and used to calculate the relative quantity of mRNA expression in PPARγ KO samples compared with wild type murine controls. For the Lentivirus experiments, Lenti-eGFP samples were used as the controls for Lenti-PPARγ samples. Data are expressed as fold change in mRNA expression relative to control values (98).

Statistical analysis. Data were analyzed by Student's *t*-test using Prism software (GraphPad, Inc., San Diego, CA). Significance was defined as $p \leq 0.05$.

Results and Discussion

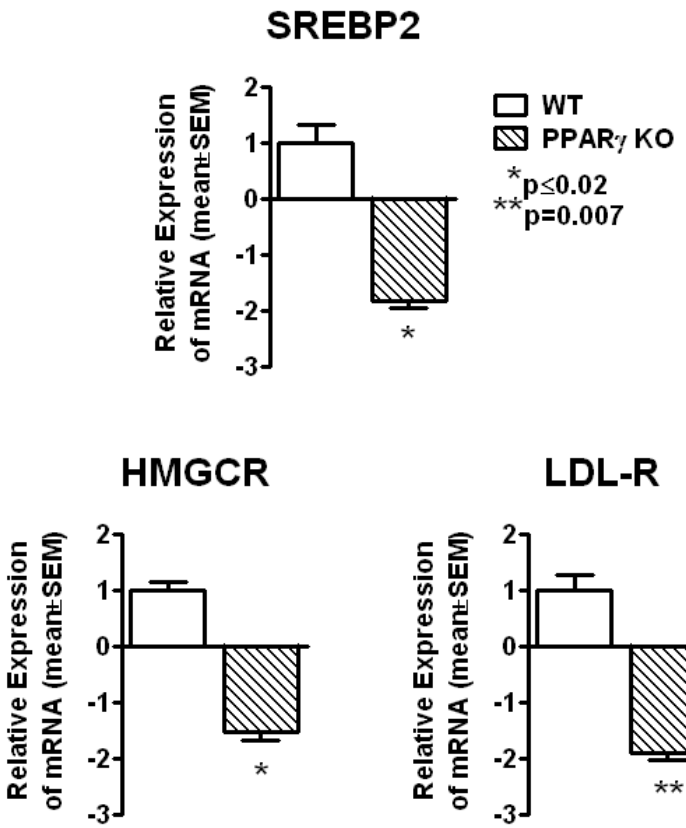
Cholesterol metabolism genes are dysregulated in PPARγ KO alveolar macrophages. We have previously shown that the alveolar macrophages of PPARγ KO mice exhibit reduced cholesterol efflux and are loaded with cholesterol (unpublished data). The lungs of PPARγ KO mice also have elevated levels of cholesterol. Therefore, we investigated the expression of cholesterol metabolism and biosynthesis genes that are regulated by intracellular and extracellular cholesterol in the alveolar macrophages of PPARγ KO mice.

RT-PCR analysis of SREBP2 and downstream targets HMGCR and LDL-R in the alveolar macrophages of PPARγ KO mice demonstrated that the expression of SREBP2, HMGCR, and LDL-R was significantly decreased (1.8-fold, 1.5-fold, and 1.9-fold respectively) compared to wild type (Figure 4.1A). Alternatively, the expression of scavenger receptors CD36 and SRA-I which are positively regulated by extracellular cholesterol content, specifically cholesterol bound to ox-LDL (74), was up-regulated 1.5-fold and 10.1-fold, respectively (Figure 4.1B).

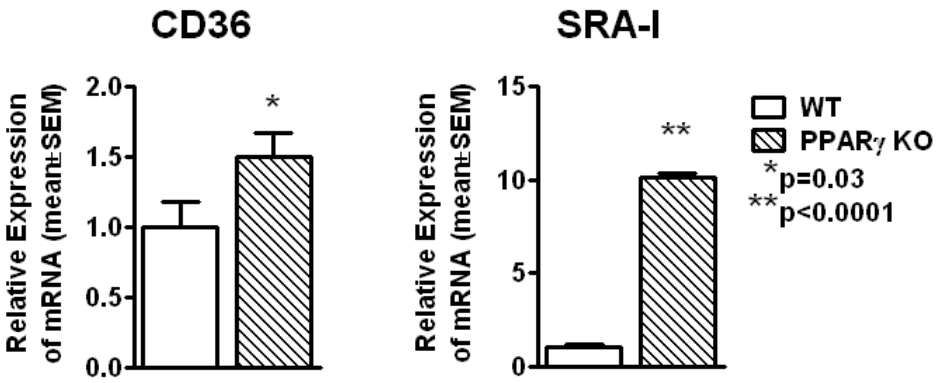
While the regulation of cholesterol synthesis genes is complex, cholesterol has been shown to negatively regulate cholesterol synthesis at the transcriptional level (128). Wang, et al. demonstrated that the accumulation of free cholesterol in ABCG1-deficient macrophages inhibited the expression of HMGCR and LDL-R (129). THP1-derived macrophages loaded with ox-LDL displayed reduced

Figure 4.1 Cholesterol metabolism genes are dysregulated in PPARγ KO alveolar macrophages. (A) RT-PCR analysis demonstrated that genes involved in the biosynthesis and uptake of cholesterol SREBP2, HMGCR, and LDL-R are down-regulated in the PPARγ KO compared to wild type. (B) Expression of scavenger receptors CD36 and SRA-I are increased in the PPARγ KO compared to wild type.

A.


SREBP2
Relative Expression of mRNA (mean±SEM)
2
1
0
-1
-2
-3
*
WT
PPARγ KO
*p≤0.02
**p=0.007
HMGCR
Relative Expression of mRNA (mean±SEM)
*
LDL-R
Relative Expression of mRNA (mean±SEM)
**

B.


CD36
Relative Expression of mRNA (mean±SEM)
2.0
1.5
1.0
0.5
0.0
*
SRA-I
Relative Expression of mRNA (mean±SEM)
15
10
5
0
**
WT
PPARγ KO
*p=0.03
**p<0.0001

HMGCR mRNA (130). Moreover, incubation of murine macrophages with modified-LDL decreased HMGCR enzymatic activity (131). Decreased expression of cholesterol synthesis genes in the cholesterol-laden alveolar macrophages of PPARγ KO mice indicates that the suppression of cholesterol synthesis is intact, in contrast to sterol-insensitive cells which continue to produce cholesterol when loaded (132).

While reduced cholesterol efflux, such as that observed in the PPARγ KO, can contribute to cholesterol-overload, enhanced ox-LDL uptake via scavenger receptors is also strongly linked to the accumulation of cholesterol. PPARγ has been implicated in the regulation of cholesterol uptake by CD36 and SRA-I. Exposure of THP-1 macrophages with ox-LDL induces expression of PPARγ, CD36, and SRA-I (58). While PPARγ directly promotes expression of CD36 (61), SRA-I can be negatively regulated by PPARγ and independently of PPARγ by the presence of ox-LDL (105,106). In the present study, PPARγ KO alveolar macrophages exhibited highly up-regulated SRA-I. Therefore, elevated expression of SRA-I may reflect the combination of substrate availability and the absence of PPARγ.

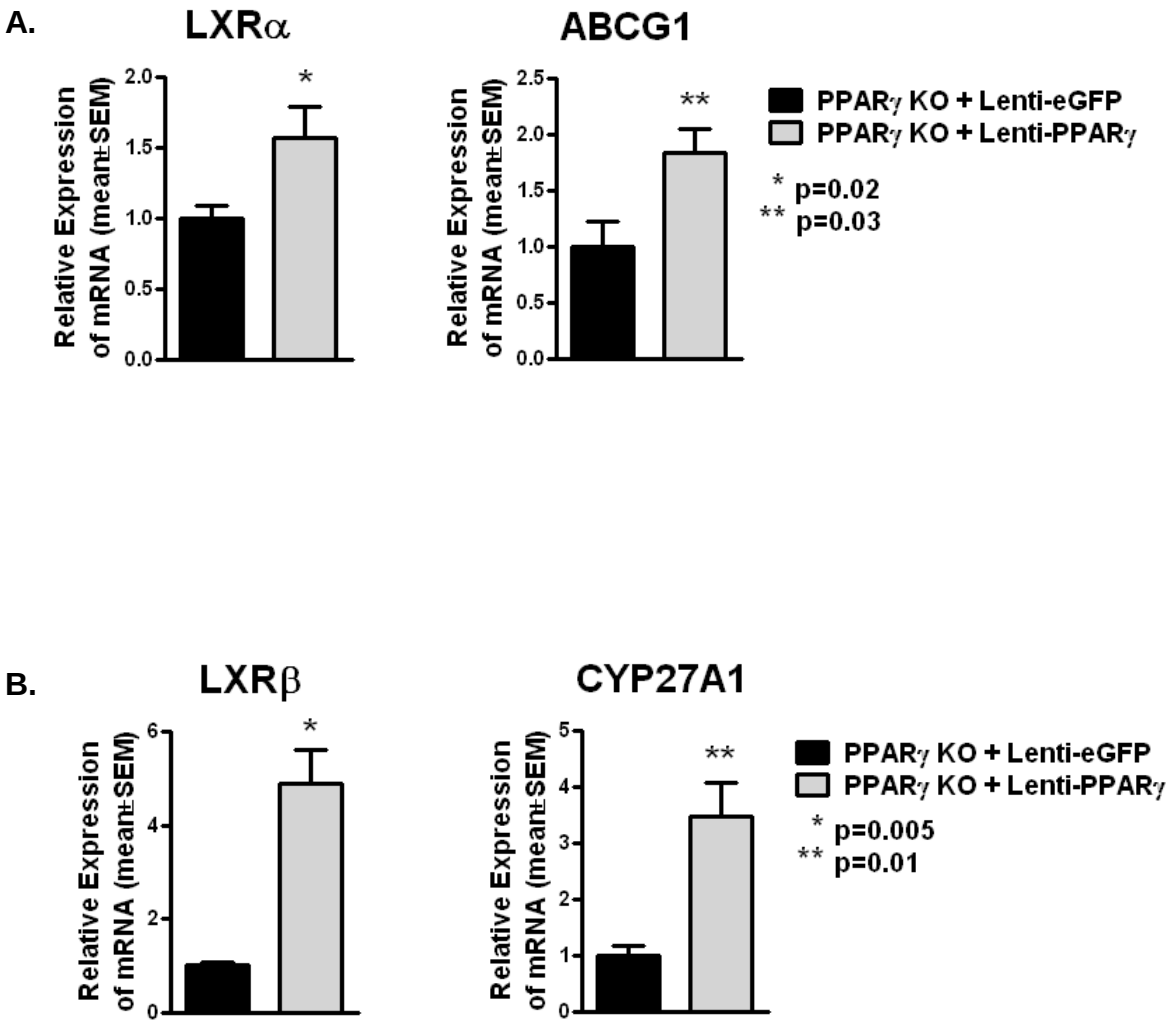
In addition to cholesterol, we have also shown that other surfactant lipids accumulate in the alveolar macrophages of PPARγ KO mice (unpublished data). Analysis of phospholipid, fatty acid, and triglyceride synthesis genes (lipin1, fatty acid synthase, SREBP1c, diacylglycerol acyltransferase, and glycerol-3-phosphate acyltransferase) by RT-PCR revealed no differences in expression

compared to wild type mice (data not shown). This data indicates (1) it is unlikely that the intracellular accumulation of lipids is due to de novo synthesis, and (2) PPARγ is particularly critical to the regulation of cholesterol.

We demonstrate in the present study that the cholesterol synthesis pathway is suppressed in PPARγ KO alveolar macrophages, as evidenced by decreased expression of SREBP2, HMGCR, and LDL-R while the uptake of ox-LDL may be enhanced given that the expression of CD36 and SRA-I is increased. These results demonstrate that both systems are responsive to the accumulation of cholesterol in the lungs and alveolar macrophages of PPARγ KO mice.

Up-regulation of PPARγ in vivo promotes the expression of cholesterol efflux genes. We have determined that the expression of PPARγ-regulated genes LXRα and ABCG1 was reduced in the alveolar macrophages of PPARγ KO mice (unpublished data). Additionally, we demonstrated that LXRβ and CYP27A1 were up-regulated. As a continuation of this work, we investigated the expression of these genes upon up-regulation of PPARγ in vivo. RT-PCR analysis demonstrated ABCG1 and LXRα mRNA was up-regulated (1.8-fold and 1.6-fold, respectively) in PPARγ KO mice instilled with Lenti-PPARγ compared to mice instilled with Lenti-eGFP (Figure 4.2A). LXRβ and CYP27A1 were also up-

Figure 4.2 Up-regulation of PPARγ in vivo promotes the expression of cholesterol efflux genes. (A) RT-PCR analysis revealed ABCG1 and LXRα expression in the alveolar macrophages of PPARγ KO mice instilled with Lenti-PPARγ were up-regulated compared to controls. (B) The expression of LXRβ and CYP27A1 was increased in Lenti-PPARγ treated animals (n=3) compared to Lenti-eGFP controls.


A.
LXRα
ABCG1
Relative Expression of mRNA (mean±SEM)
2.0
1.5
1.0
0.5
0.0
2.5
*
**
PPARγ KO + Lenti-eGFP
PPARγ KO + Lenti-PPARγ
* p=0.02
** p=0.03
B.
LXRβ
CYP27A1
6
4
2
0
5
3
1
* p=0.005
** p=0.01

regulated 4.9-fold and 3.5-fold, respectively, in PPARγ KO instilled with Lenti-PPARγ (Figure 4.2B).

While the restoration of ABCG1 mRNA confirms previous results directly linking PPARγ to ABCG1 expression (51), perhaps the most significant finding in this report is the induction of LXRα in treated PPARγ KO mice. Peritoneal macrophages from PPARγ KO mice have been shown to exhibit decreased expression of LXRα which did not increase upon treatment with ligand due to the absence of PPARγ (100). Consistent with findings from other tissue macrophages in which upstream PPARγ signaling was essential to the promotion of cholesterol efflux by LXRα (77), PPARγ appears to be essential to the promotion of cholesterol efflux by LXRα in alveolar macrophages. These results indicate that PPARγ is a key regulator of cholesterol efflux.

Increased expression of CYP27A1 in the alveolar macrophages of PPARγ KO mice instilled with Lenti-PPARγ may have contributed to increased LXRβ expression through the production of LXR ligand (112). Llaverias, et al. have reported that the loading of macrophages with cholesterol results in increased CYP27A1 expression (133). While LXRβ has been shown to be regulated independently of PPARγ (77), CYP27A1 can be regulated by PPARγ. At least two reports have demonstrated that the transcription of CYP27A1 is inducible by PPARγ ligands, independent of LXR or cellular cholesterol content (134,135). Therefore, increased expression of CYP27A1 in untreated PPARγ KO may result

from intracellular cholesterol content, while in treated mice PPARγ may directly contribute to the expression of CYP27A1 to promote the removal of cholesterol from alveolar macrophages.

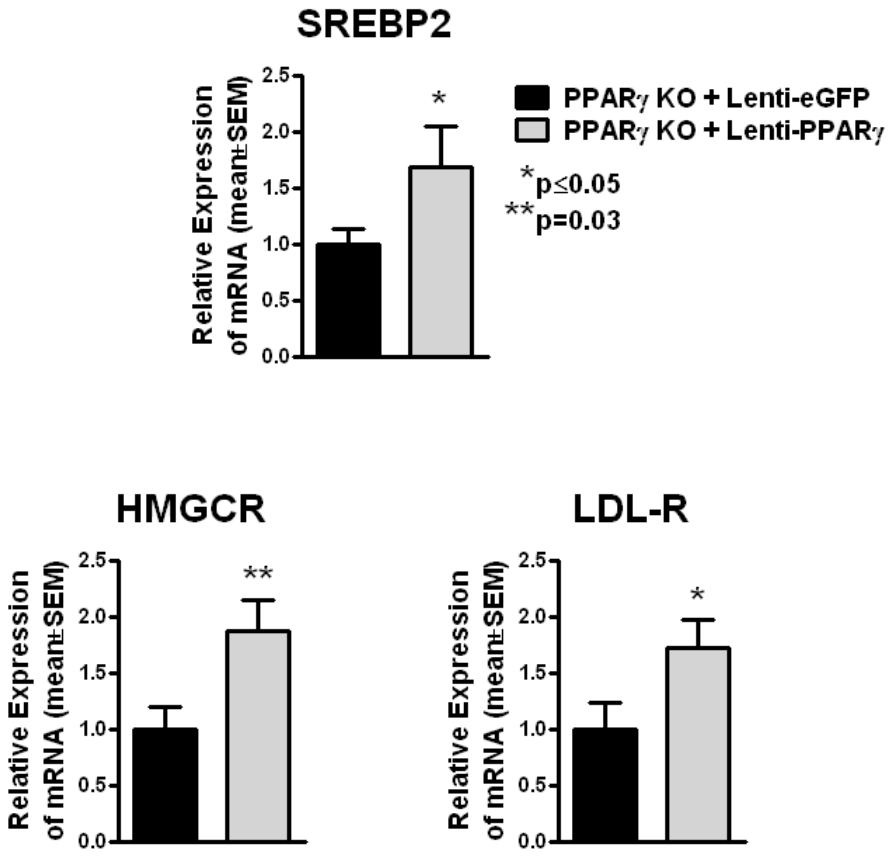
Taken together, these data indicate that the replacement of PPARγ in cholesterol-laden macrophages promotes the expression of cholesterol efflux through transcription of LXRα, ABCG1, and CYP27A1.

Up-regulation of PPARγ in vivo increases expression of cholesterol metabolism genes. In contrast to decreased expression of cholesterol synthesis genes in PPARγ KO alveolar macrophages, restoration of PPARγ in vivo resulted in the up-regulation of SREBP2, HMGCR, and LDL-R mRNA compared to Lenti-eGFP controls (1.7-fold, 1.9-fold, and 1.7-fold, respectively) (Figure 4.3A). Replacement of PPARγ also resulted in increased expression of CD36 (2.1-fold) while no significant changes in SRA-I expression were observed (Figure 4.3B).

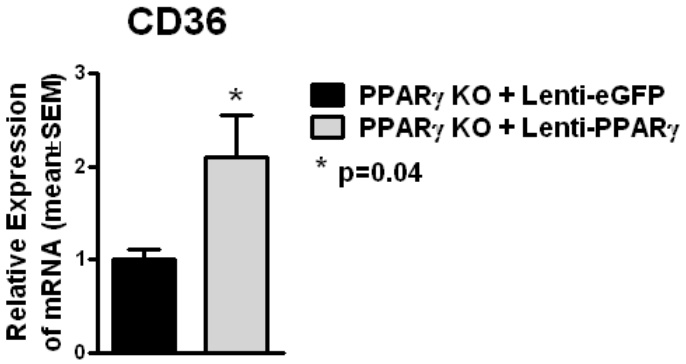
Overexpression of SREBP-2 has been shown to increase the mRNA expression of all cholesterol synthesis enzymes, with the highest induction of HMGCR (136). Although predominantly recognized to be transcriptionally regulated by SREBP2 (137), PPARγ has been also implicated in the transcription of HMGCR. Both the mRNA and enzyme activity of HMGCR was increased THP-1 cells treated with PPARγ agonists (138). Increased expression of cholesterol biosynthesis genes SREBP-2 and HMGCR upon up-regulation of

Figure 4.3 Up-regulation of PPARγ in vivo increases expression of cholesterol metabolism genes. (A) RT-PCR analysis of alveolar macrophages revealed increased expression of SREBP2, HMGCR, and LDL-R in PPARγ KO instilled with Lenti-PPARγ (n=3) compared to controls. (B) CD36 mRNA was up-regulated after instillation with Lenti-PPARγ compared to Lenti-eGFP controls.

A.


SREBP2
Relative Expression of mRNA (mean±SEM)
2.5
2.0
1.5
1.0
0.5
0.0
*
PPARγ KO + Lenti-eGFP
PPARγ KO + Lenti-PPARγ
*p≤0.05
**p=0.03
HMGCR
**
LDL-R
*

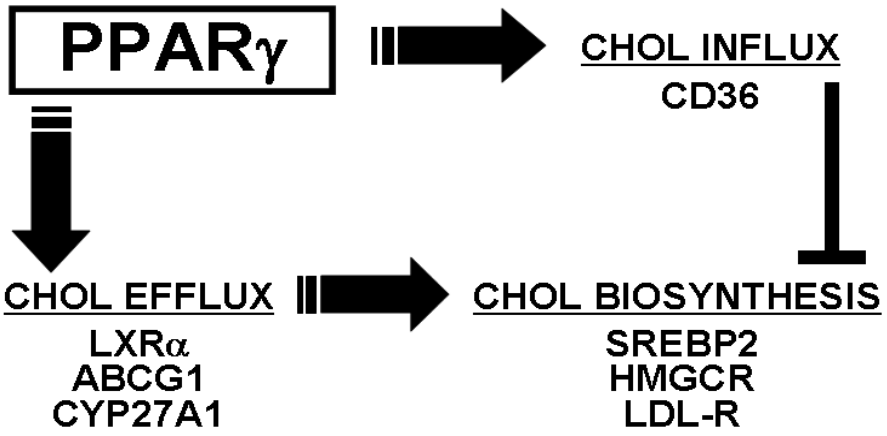
B.


CD36
Relative Expression of mRNA (mean±SEM)
3
2
1
0
*
PPARγ KO + Lenti-eGFP
PPARγ KO + Lenti-PPARγ
* p=0.04

PPARγ suggests an indirect relationship between PPARγ and the synthesis of cholesterol, probably mediated by intracellular sterol levels. These results provide evidence that the up-regulation of PPARγ and downstream genes involved in cholesterol efflux including LXRα, ABCG1, and CYP27A1 relieved inhibition of the cholesterol biosynthesis genes (Figure 4.4).

The induction of CD36 mRNA upon the restoration of PPARγ is consistent with previous studies demonstrating that CD36 is transcriptionally promoted by PPARγ (58). Given that PPARγ can negatively regulate SRA-I (105,106), it was somewhat unexpected to find that the expression of SRA-I was unchanged in PPARγ KO mice upon up-regulation of PPARγ. However, ox-LDL can induce expression of SRA-I independently of PPARγ (139). Our results suggest that increased expression of SRA-I occurs in the absence of PPARγ, and that continued expression SRA-I after PPARγ replacement may be attributed to substrate availability. Further, elevated expression of CD36 and SRA-I suggest that the uptake of cholesterol is enhanced in the alveolar macrophages of PPARγ KO mice.

Figure 4.4 Hypothetical pathway by which PPARγ regulates cholesterol influx, efflux, and biosynthesis in alveolar macrophages. PPARγ directly promotes transcription of cholesterol influx and efflux genes which determine the intracellular cholesterol level. In turn, cholesterol levels regulate the de novo synthesis of cholesterol.


PPARγ
CHOL INFLUX
CD36
CHOL EFFLUX
LXRα
ABCG1
CYP27A1
CHOL BIOSYNTHESIS
SREBP2
HMGCR
LDL-R

Conclusions

Alveolar macrophages of PPARγ KO mice exhibit decreased expression cholesterol influx, efflux, and biosynthesis genes. The replacement of PPARγ in vivo increased expression of cholesterol efflux genes ABCG1 and LXRα, cholesterol biosynthesis genes SREBP2 and HMGCR, and receptors LDL-R and CD36. These results suggest that PPARγ regulates cholesterol metabolism in alveolar macrophages and promotes the catabolism and transport of cholesterol in the lung. Our findings provide evidence that the catabolism of cholesterol affects the catabolism of surfactant overall and may have implications in the study of PPARγ and human lung diseases associated with the accumulation of surfactant.

Acknowledgments

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CHAPTER 5

RESTORATION OF PPARγ REVERSES LIPID ACCUMULATION IN ALVEOLAR MACROPHAGES OF GM-CSF KNOCKOUT MICE

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Running Title: Lentivirus-PPARγ in Alveolar Macrophages

Keywords: alveolar macrophage, Lentivirus, PPARγ, ABCG1, foam cells

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The results presented in this chapter are part of a manuscript that is in final preparations for submission to a peer-reviewed journal.

Abstract

Pulmonary alveolar proteinosis (PAP) is a lung disease characterized by neutralizing auto-antibodies to granulocyte-macrophage colony-stimulating factor (GM-CSF). The loss of functional GM-CSF results in the filling of alveolar spaces and alveolar macrophages of the lungs with surfactant. The nuclear transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ), a key regulator of lipid metabolism, is constitutively expressed in the alveolar macrophages of healthy individuals and is up-regulated by GM-CSF. We have previously demonstrated decreased levels of PPARγ and ATP-binding cassette transporter G1 (ABCG1) in the alveolar macrophages from both PAP patients and GM-CSF knockout (GM-CSF KO) mice, suggesting the involvement of PPARγ and ABCG1 in surfactant catabolism. We hypothesized that up-regulation of PPARγ would lead to an increase in ABCG1 and decrease lipid accumulation in alveolar macrophages. The up-regulation of PPARγ was achieved using a Lentivirus expression system in vivo. GM-CSF KO mice received instillation of Lentivirus (Lenti)-PPARγ or control Lenti-eGFP (enhanced Green Fluorescence Protein) via the intra-tracheal route. Alveolar macrophages were harvested 10 and 30 days post-instillation. Fluorescence microscopy indicated that 79 and 54% of alveolar macrophages instilled with Lenti-eGFP contained eGFP at 10 and 30 days post-instillation, respectively. We demonstrated significant increases in PPARγ and ABCG1 gene expression in

alveolar macrophages of mice instilled with Lenti-PPARγ, while PPARγ and ABCG1 levels remained unchanged in control groups instilled with Lenti-eGFP or PBS. Oil Red O positivity was reduced within the alveolar macrophages of mice treated with Lenti-PPARγ at 10 days post-instillation. Finally, we measured increased ABCG1-mediated cholesterol efflux in alveolar macrophages from Lenti-PPARγ treated mice at 10 days. Results with Lenti-PPARγ instillations demonstrate: (1) efficient in vivo transduction of alveolar macrophages; (2) up-regulation of ABCG1; (3) a reduction in macrophage lipid accumulation; and (4) increased ABCG1-mediated cholesterol efflux. These studies suggest that the expression PPARγ and ABCG1 in alveolar macrophages promotes the catabolism of surfactant.

Introduction

Pulmonary alveolar proteinosis (PAP) is a rare autoimmune disease characterized by neutralizing auto-antibodies to granulocyte-macrophage colony-stimulating factor (GM-CSF) (1,91). Genetically engineered mice which are homozygous for a disrupted GM-CSF gene develop a PAP-like lung disease with the accumulation of excess surfactant (29,30,140). These studies have revealed a previously unknown role for GM-CSF in normal lung homeostasis, as pathogenesis in PAP patients and mouse models are confined to the lung.

The alveolar macrophages in PAP patients and GM-CSF knockout (GM-CSF KO) mice are foamy and laden with lipid, as evidenced by positive Oil Red O staining (50,51). The accumulation of surfactant has been linked decreased clearance by the alveolar macrophages of PAP and GM-CSF KO rather than increased production of surfactant (25,26,28). Interestingly, GM-CSF up-regulates the nuclear transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ) expression in alveolar macrophages suggesting the involvement of PPARγ in surfactant clearance (6). PPARγ is constitutively expressed in the alveolar macrophages of healthy individuals but deficient in the alveolar macrophages of PAP patients and GM-CSF KO mice (6).

A critical role for PPARγ has been reported in regulation of genes involved in lipid metabolism as well as in inflammation (141). PPARγ is one of the key proteins involved in regulating macrophage cholesterol efflux and this regulation

may involve expression of the ATP-binding cassette (ABC) transporters ABCG1 and ABCA1 (58,61,77,100,142,143). Our laboratory has recently shown that the expression of ABCG1 is decreased in the alveolar macrophages of PAP patients and GM-CSF KO mice (51). The studies of PAP and GM-CSF KO mice have shown that GM-CSF is a critical mediator of surfactant clearance. We hypothesized that GM-CSF mediates surfactant catabolism in alveolar macrophages through a PPARγ-ABCG1 pathway and that up-regulation of PPARγ in would lead to an increase in ABCG1 and decrease the lipid accumulating in the alveolar macrophages of GM-CSF KO mice. To address this hypothesis we utilized a Lentivirus expression system in vivo to up-regulate PPARγ.

Materials and Methods

Mice. Animal studies were conducted in conformity with Public Health Service (PHS) Policy on humane care and use of laboratory animals and were approved by the institutional animal care committee. The GM-CSF KO mice were generated by Dr. Glenn Dranoff (29). The mice have been backcrossed eight generations to C57Bl/6.

Bronchoalveolar lavage (BAL). Post lenti-instillation (10 or 30 days) 5-7 animals per group were lavaged. Mice received ketamine (90 mg/kg) and xylazine (10 mg/kg) intraperitoneally. The thoracic cavity was opened and the lungs were exposed. After cannulating the trachea a tube was inserted and bronchoalveolar lavage was carried out with warmed (37^{o}C) PBS in 1ml aliquots. Cytospins of BAL cells were stained with a modified Wright–Giemsa stain for differentials or Oil Red O stain to detect intracellular neutral lipids, and counterstained with Gill's hematoxylin stain. Viability was >95% as determined by trypan blue for all cell preparations.

Lentivirus construction and transduction. A self-inactivating Lentivirus expression vector that was previously utilized in the generation of a stable cell line expressing the human parainfluenza virus type 3 C protein was used for these experiments (51,121). cDNA corresponding to the human PPARγ

sequence was cloned into the multiple cloning sites downstream of a CMV promoter using standard techniques as described (121). The recombinant Lentiviral plasmid thus obtained was transfected into 293FT cells along with plasmids encoding the *gag*, *pol* and *rev* genes and a plasmid possessing the vesicular stomatitis glycoprotein (G) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 72 hrs post transfection, cell culture supernatant containing the Lentivirus-PPARγ (Lenti-PPARγ) was collected and then purified by centrifugation at 27,000 rpm at 4^0C for 3.5 hrs. The Lenti-PPARγ virus pellet was resuspended in PBS and aliquots of 100μl were stored at -70^0C. The concentration of Lenti-PPARγ virus was determined by a p24 ELISA (Cell Biolabs, San Diego, CA). A Lentivirus expressing the enhanced Green Fluorescent Protein (Lenti-eGFP) was obtained using a similar protocol and was utilized as a control in experiments for the determination of transduction efficiency. For in vivo analyses, GM-CSF KO received 50 ng/mL p24 of Lenti-PPARγ in 50 uL of PBS, Lenti-eGFP or PBS alone (sham) by intra-tracheal instillation.

RNA purification and analysis. Total RNA was extracted from cells by RNeasy protocol (Qiagen, Valencia, CA). Expression of mRNA was determined by real time RT-PCR using the ABI Prism 7300 Detection System (TaqMan, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. RNA specimens were analyzed in duplicate using primer/probe sets for mouse PPARγ,

ABCA1 and ABCG1 (ABI) as previously described (51). Threshold cycle (CT) values for genes of interest were normalized to a housekeeping gene [glyceraldehyde 3 phosphate dehydrogenase, (GAPDH)] and used to calculate the relative quantity of mRNA expression. Data were expressed as a fold change in mRNA expression relative to control values (98).

Cholesterol efflux assay. BAL cells were plated in 48 well cell culture plates ($3.5x10^5$ per well) in complete media and maintained at 37℃ and 5% CO_2. Cells were incubated for 24 hours in 2 μCi/mL [1,2-^{3}H(N)]-cholesterol (NEN, Perkin Elmer, Waltham, MA), washed and equilibrated in serum free media for 24 hours. Cells were then treated with no acceptors or in the presence of high density lipoprotein (HDL) (25μg/uL) (Intracel, Frederick, MD) for an additional 24 hours. Supernatants were harvested and cell debris removed by a 5 minute centrifugation at 1800 rpm. Cells were washed with 1XPBS and dissolved in 0.2 M NaOH with 0.1% SDS for 1 hour at room temperature. Aliquots of cell lysates and supernatants were counted by liquid scintillation. Cholesterol efflux was expressed as the percentage of radioactivity in the supernatant divided by the total radioactivity of the cells and supernatant. Each assay was run in duplicate.

Cholesterol content analysis. Cholesterol was measured in BAL fluid using the Amplex Red Cholesterol Assay (Molecular Probes, Invitrogen, Eugene, OR) according to the manufacturer's protocol. Aliquots of cell-free BAL fluid were

assayed in serial dilution in 96-well plates. Data is expressed as μg of cholesterol per mL of BAL fluid.

Statistical analysis. Data were analyzed by Student's t-test using Prism software (GraphPad, Inc., San Diego, CA). Significance was defined as $p \leq 0.05$.

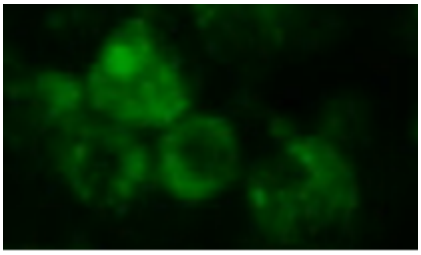
Results

Lentivirus efficiently transduces alveolar macrophages in vivo. To monitor the transduction efficiency of Lentivirus vectors in alveolar macrophages of GM-CSF KO mice, we utilized Lenti-eGFP and quantified the number of eGFP positive alveolar macrophages (Figure 5.1). The transduction efficiency was 79% at 10 days and 54% at 30 days. Based on these results, the remaining experiments were carried only to 10 days post-instillation.

PPARγ and cholesterol transporters ABCG1 and ABCA1 are up-regulated. To evaluate the effect of Lenti-PPARγ transduction on alveolar macrophages, RNA was extracted from freshly isolated BAL cells at 10 days post-instillation and compared to sham treated GM-CSF KO mice (Figure 5.2). PPARγ mRNA was up-regulated 2-fold. We next evaluated the effect of Lenti-PPARγ on the PPARγ-regulated transporters ABCG1 and ABCA1, and determined that their mRNA expression was also elevated. PPARγ, ABCG1, and ABCA1 gene expression in alveolar macrophages from Lenti-eGFP instilled animals were not different from sham. Increased expression of PPARγ, ABCG1, and ABCA1 protein was also determined by immunofluorescence (data not shown).

Figure 5.1 Lentivirus efficiently transduces alveolar macrophages in vivo. GM-CSF KO instilled with Lenti-eGFP in vivo exhibit eGFP positive cells at 10 and 30 days post-instillation. (A) Representative image of eGFP positive GM-CSF KO alveolar macrophages. (B) Cytospin preparations of BAL cells were counted for eGFP positivity.

A.

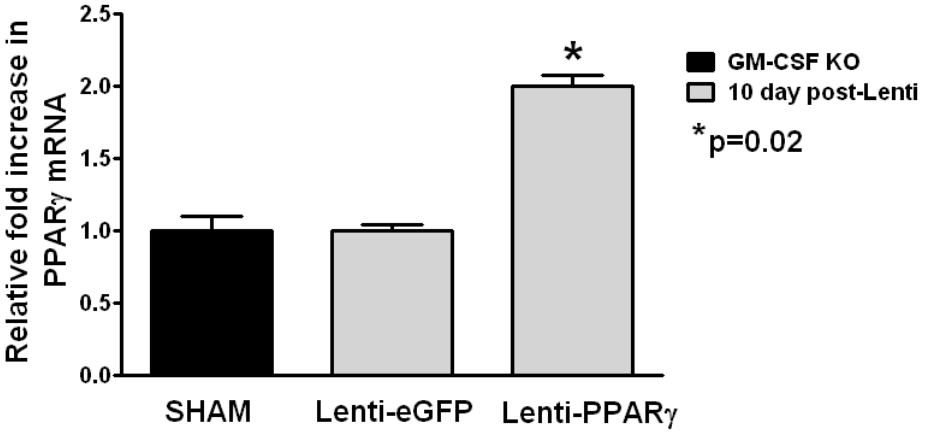


B.

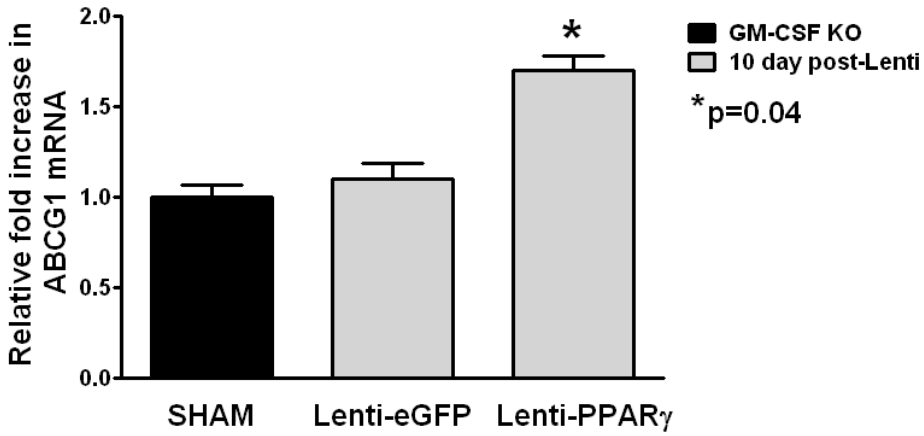
Days Post-Instillation	% eGFP Positive
10	79 ± 3 (n=3)
30	54 ± 6 (n=3)

Figure 5.2 PPARγ and cholesterol transporters ABCG1 and ABCA1 are up-regulated. (A) RT-PCR analysis demonstrated up-regulation of PPARγ, (B) ABCG1, and (C) ABCA1 mRNA in the alveolar macrophages of GM-CSF KO mice instilled with Lenti-PPARγ virus at 10 days post-instillation, compared to untreated animals. RT-PCR values for GM-CSF KO mice instilled with control virus Lenti-eGFP were not different from untreated animals.

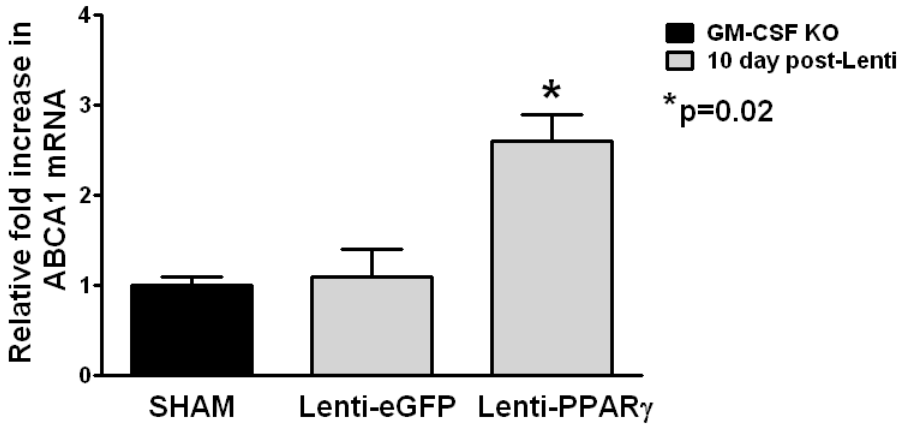
A.


Relative fold increase in PPARγ mRNA
2.5
2.0
1.5
1.0
0.5
0.0
*
GM-CSF KO
10 day post-Lenti
*p=0.02
SHAM
Lenti-eGFP
Lenti-PPARγ

B.


Relative fold increase in ABCG1 mRNA
2.0
1.5
1.0
0.5
0.0
*
GM-CSF KO
10 day post-Lenti
*p=0.04
SHAM
Lenti-eGFP
Lenti-PPARγ

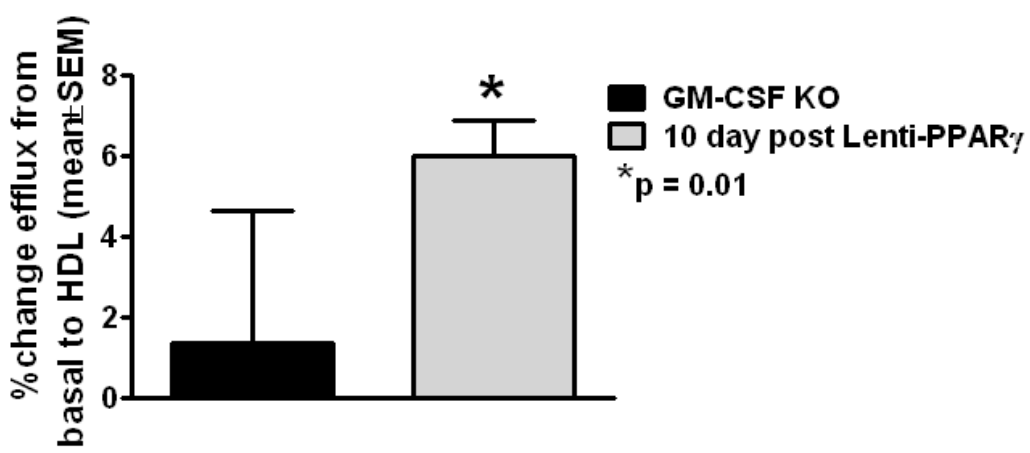
C.


Relative fold increase in ABCA1 mRNA
4
3
2
1
0
*
GM-CSF KO
10 day post-Lenti
*p=0.02
SHAM
Lenti-eGFP
Lenti-PPARγ

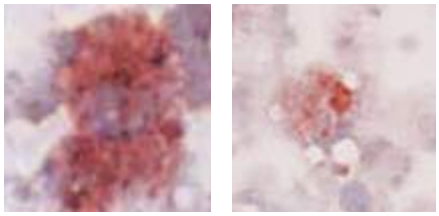
Cholesterol clearance from alveolar macrophages is increased. The previously reported accumulation of cholesterol in the alveolar macrophages and BAL fluids of GM-CSF KO mice (110) together with our observations of decreased PPARγ and ABCG1 (51) suggested that cholesterol efflux to HDL might be impaired. Having demonstrated the up-regulation of the cholesterol transporter ABCG1, we next investigated the cholesterol efflux in the alveolar macrophages of Lenti-PPARγ treated GM-CSF KO mice compared to macrophages from untreated animals. The mean cholesterol efflux to HDL compared to basal efflux (no acceptor) was 1.4±3.3% in untreated GM-CSF macrophages whereas efflux in alveolar macrophages 10 days post-instillation was 6.0±0.9% (Figure 5.3A). To further evaluate the clearance of cholesterol, we measured the intracellular neutral lipid accumulation at 10 days post-instillation with Lenti-PPARγ. The intensity of Oil Red O staining was decreased in treated mice (Figure 5.3B). Cell size of Lenti-PPARγ treated GM-CSF KO mice was also significantly reduced (data not shown). Given enhanced cholesterol efflux and decreased cellular lipid accumulation, we speculated that extracellular cholesterol would be increased in the BAL fluid. As expected, the extracellular cholesterol was increased (27±3 μg/mL) as compared to BAL fluid from untreated GM-CSF KO mice (12±0.2 μg/mL) (Figure 5.3C). These results suggest PPARγ promotes cholesterol catabolism in alveolar macrophages through ABCG1.

Figure 5.3 Cholesterol clearance from alveolar macrophages is increased. (A) ABCG1-mediated cholesterol efflux to HDL is increased in the alveolar macrophages of GM-CSF KO mice instilled with Lenti-PPARγ. (B) Representative images from alveolar macrophages stained with Oil Red O to detect neutral lipid accumulation show decreased intensity of Oil Red O staining in the alveolar macrophages treated with Lenti-PPARγ at 10 days post-instillation. (C) Cholesterol accumulates in the BAL fluid of GM-CSF KO mice instilled with Lenti-PPARγ.

A.



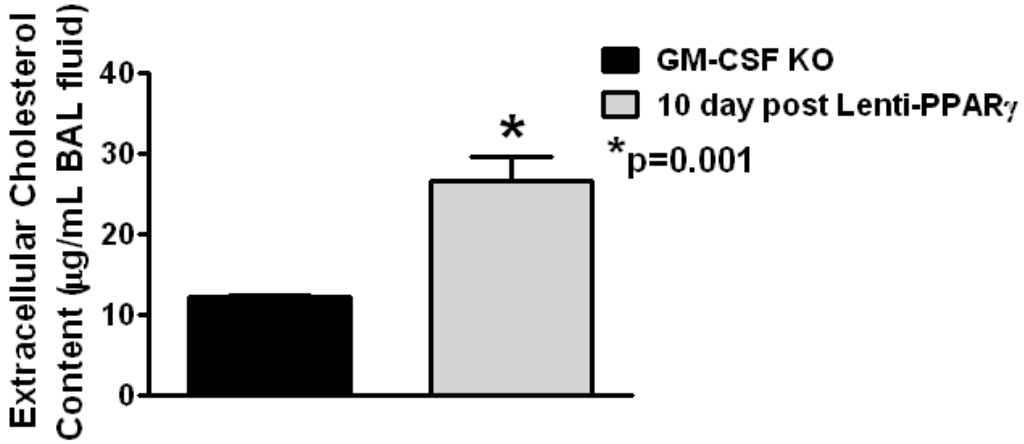
B.



SHAM

10 day Lenti-PPARγ

C.



Discussion

Alternative therapies for PAP are needed, as less than 50% of patients treated with recombinant GM-CSF exhibit symptomatic and radiographic improvement (53,144,145). Previous studies have suggested that the clearance of surfactant by alveolar macrophages is mediated by the GM-CSF-PPARγ pathway (6,7). Our findings confirm PPARγ transcriptionally regulates surfactant catabolism in alveolar macrophages and provide evidence that PPARγ is a valid therapeutic target.

To address the role of PPARγ in surfactant catabolism, we have up-regulated PPARγ in PPARγ-deficient alveolar macrophages of GM-CSF KO mice in vivo. We have demonstrated increased expression of PPARγ and downstream targets ABCG1 and ABCA1. Up-regulation of PPARγ and ABCG1 resulted in increased cholesterol efflux via the ABCG1-HDL pathway. Neutral lipid accumulation was reduced in treated alveolar macrophages, as evidenced by Oil Red O. Finally, free cholesterol was increased in the BAL fluid of treated GM-CSF KO mice. Taken together, these studies suggest that the expression PPARγ and ABCG1 in alveolar macrophages promotes the catabolism of surfactant, specifically the cholesterol component of surfactant.

PPARγ regulates the catabolism and transport of cholesterol in other tissue macrophages via the lipid transporters ABCG1 and ABCA1 (77,78). The expression of PPARγ, ABCG1, and ABCA1 were increased in the alveolar

macrophages of GM-CSF KO mice treated with Lenti-PPARγ at 10 days post-instillation. These results are an extension of our previous results in which PPARγ and ABCG1 were deficient in GM-CSF KO alveolar macrophages. Replacement of PPARγ corrects these deficiencies and further links the PPARγ-ABCG1 pathway in the catabolism of surfactant.

Cholesterol is the major neutral lipid in surfactant. Therefore, dysregulation of cholesterol catabolism in alveolar macrophages may have impacts on the clearance of surfactant overall. We have shown that the alveolar macrophages of GM-CSF KO mice accumulate neutral lipids and are deficient in PPARγ (47). We show that by restoring PPARγ in this system, there is a reduction in neutral lipid accumulation which we speculated was due to increased cholesterol efflux from the macrophages. This was supported both by the increased ABCG1-HDL cholesterol efflux and elevated levels of extracellular cholesterol in Lenti-PPARγ treated GM-CSF KO mice.

Utilizing a Lenti-PPARγ virus, we restored expression of PPARγ in the PPARγ-deficient alveolar macrophages of GM-CSF KO mice. Replacement of PPARγ resulted in increased expression of the cholesterol transporter ABCG1 and decreased intracellular lipid accumulation, confirming our hypothesis. Taken together, these studies directly link the expression of PPARγ and ABCG1 in the promotion of surfactant catabolism and may serve as potential therapeutic targets in the treatment of PAP.

CHAPTER 6

SUMMARY

The overall purpose of the studies described in Chapters 2-5 was to investigate the role of the nuclear transcription factor PPARγ in surfactant catabolism in alveolar macrophages. This dissertation investigated the hypothesis that surfactant catabolism is regulated by the PPARγ-ABCG1 pathway. Chapters 2 and 4 detailed experiments which utilized a macrophage-specific PPARγ KO mouse model and demonstrated the following:

(1) PPARγ KO mice exhibit a PAP-like lung pathology with the accumulation of surfactant and the presence of foamy alveolar macrophages.

(2) The deletion of PPARγ resulted in the dysregulation of ABC transporters and LXR transcription factors and reduced ABCG1-mediated cholesterol efflux.

(3) In addition to dysregulated cholesterol efflux genes LXRα and ABCG1, the alveolar macrophages of PPARγ KO exhibited decreased expression of cholesterol biosynthesis gene SREBP2 and downstream targets HMGCR and LDL-R and increased expression of cholesterol scavenger receptors CD36 and SRA-I.

(4) Replacement of PPARγ in vivo in PPARγ KO mice resulted in up-regulation of cholesterol efflux genes LXRα and ABCG1, cholesterol

biosynthesis genes SREBP2 and HMGCR, and receptors LDL-R and CD36 in alveolar macrophages.

Results from these studies indicate that PPARγ regulates surfactant catabolism through the regulation of LXR transcription factors and ABC lipid transporters in alveolar macrophages.

Chapter 5 relates experiments in which PPARγ expression was restored in vivo in the PPARγ-deficient alveolar macrophages of GM-CSF KO mice, the mouse model for the human lung disease PAP. These experiments demonstrated the following:

(1) GM-CSF KO mice instilled in Lenti-PPARγ in vivo efficiently up-regulated PPARγ and ABCG1.

(2) Expression of the PPARγ-ABCG1 pathway resulted in increased cholesterol efflux from GM-CSF KO alveolar macrophages.

(3) Expression of PPARγ resulted in reduction of lipid-laden alveolar macrophages and increased extracellular cholesterol in the lungs of GM-CSF mice.

Macrophage-specific Deletion of PPARγ

Previous findings in our laboratory demonstrated that the alveolar macrophages of PAP patients and GM-CSF KO mice were deficient in PPARγ (6,51). We utilized a macrophage-specific PPARγ KO mouse to investigate the role of PPARγ in alveolar macrophages and surfactant catabolism. Interestingly, PPARγ KO mice developed pulmonary disease similar to that of PAP patients and GM-CSF KO mice with the accumulation of surfactant phospholipids, cholesterol, and associated proteins. Additionally, the foamy, lipid-laden alveolar macrophages of PPARγ KO mice phenotypically resembled those of PAP patients and GM-CSF KO mice. This is the first report directly linking the deficiency of PPARγ in alveolar macrophages to the accumulation of surfactant.

Consistent with PAP patients and GM-CSF KO mice (51), the expression of ABCG1 was decreased and ABCA1 increased in alveolar macrophages of PPARγ KO mice. Functional analyses of the transporters corresponded to gene expression in that PPARγ KO alveolar macrophages exhibited decreased ABCG1-mediated cholesterol efflux and increased ABCA1-mediated efflux. These results may reflect a compensatory mechanism in which the deficiency of one ABC transporter results in PPARγ and LXR ligand accumulation leading to the induction of the other transporter (86,87). However, it has been suggested, and our results provide further evidence, that the induction of ABCA1 is less efficient at compensating for the loss of ABCG1, as ABCG1-deficient mice

develop more severe lipid accumulation in the lung than ABCA1 knockout models (86,89,90). Our results indicate that ABCG1-mediated cholesterol efflux to HDL may be the major pathway for cholesterol efflux in alveolar macrophages.

An important, unexpected difference emerged from the study of PPARγ KO alveolar macrophages from what has been published previously on PAP patients and GM-CSF KO mice: LXRα expression was increased in PAP and GM-CSF KO (51) but decreased in PPARγ KO. We postulate that this difference is due to the fact that PPARγ is not completely absent in PAP patients and GM-CSF KO mice. Indeed, upon restoration of PPARγ in PPARγ KO mice, LXRα expression was induced. Although regulated by additional independent mechanisms, LXRα is directly regulated by PPARγ. Chawla, et al. concluded that PPARγ is an essential regulator of cholesterol metabolism in arterial macrophages (146) and our findings indicate the same is true in alveolar macrophages.

Although the expression of LXRβ has not been reported in PAP or GM-CSF KO, we demonstrated that LXRβ was up-regulated in PPARγ KO alveolar macrophages. While LXRα is typically regarded as the predominant isoform in the cholesterol efflux pathway in macrophages, both LXRα and LXRβ have been reported to transcriptionally promote genes involved in cholesterol efflux. The transcriptional efficiency and tissue-specificity of the individual isoforms remain in question (101,113-115,147). Importantly, LXRβ has been shown to be regulated

independently of PPARγ and thereby represents a PPARγ-independent pathway in the transcriptional promotion of cholesterol efflux from macrophages (146).

Interest in differentiating the LXR isoforms has increased due to the potential therapeutic application for atherosclerosis. For example, ligand activation of LXRβ reduced atherosclerosis in the vascular tissue of mice lacking LXRα (148). However, unlike LXRα, specific activation of LXRβ did not result in increased triglyceride production (149,150). The contribution of the individual LXR isoforms in surfactant catabolism is unknown and warrants further investigation.

While the LXR pathway is enhanced, as evidenced by increased expression of LXRβ, ABCA1, ApoE, and CYP27A1, surfactant accumulates in PPARγ KO mice suggesting this pathway is not sufficient to maintain surfactant catabolism in the absence of PPARγ. Additionally, this data indicates LXRβ is responsive to intracellular oxysterol build-up and may also reflect the presence of LXRβ-specific ligand. Increased basal expression of LXRβ was unexpected and to our knowledge has not been observed in other tissues that express both isoforms. For example, the hepatic tissues of LXRα KO mice did not exhibit compensatory LXRβ expression (147). Increased expression of LXRβ in the alveolar macrophages of PPARγ KO mice may demonstrate site and/or cell specific differences. The deficiencies of transcription factors PPARγ and LXRα and cholesterol transporter ABCG1 and the resulting accumulation of various

surfactant components in PPARγ KO mice emphasize the importance of cholesterol catabolism in the overall clearance of surfactant.

Several comprehensive reviews have suggested that the PPARγ-LXR pathway is the link between lipid metabolism and inflammation in macrophages (151,152). Studies presented in this thesis demonstrate that PPARγ regulates lipid metabolism in alveolar macrophages. We have also recently reported that the alveolar macrophages of PPARγ KO mice exhibit increased expression of pro-inflammatory Th-1 associated cytokines (96). Although the presence of modified-LDL in the lungs of PPARγ KO mice has not been determined, research in the field of atherosclerosis has shown that foam cell phenotype, the accumulation of cholesterol, and a pro-inflammatory state are indicative of increased uptake of modified-LDL (7). We have presented indirect evidence supporting the presence of modified-LDL in the lungs of PPARγ KO mice. First, enhanced expression of scavenger receptors CD36 and SRA-I indicate the increased presence of substrate (modified-LDL). Secondly, the accumulation of predominantly free cholesterol in the alveolar macrophages of PPARγ KO mirrors the late atherosclerotic event in which free cholesterol deposition results from insufficient cholesterol hydrolysis in lysosomes due to oxidized-LDL particles (64). Finally, data from Yvan-Charvet and colleagues have suggested ABCG1 has a specific role of protecting the lungs from oxidized sterol accumulation (86,153). ABCA1-deficient macrophages treated with oxidized-LDL rapidly underwent apoptosis but the addition of HDL significantly decreased the

percentage of cells undergoing apoptosis. Therefore, the reduced expression of ABCG1 in PPARγ KO alveolar macrophages may have deleterious effects on the accumulation of oxidized sterols.

Our findings indicate that a combination of decreased cholesterol efflux and increased uptake of modified-LDL by scavenger receptors contributed to cholesterol overload and foam cell formation in the alveolar macrophages of PPARγ KO mice. We speculate that insufficient catabolism of cholesterol derived from modified-LDL impairs the overall catabolism of surfactant in alveolar macrophages. Similar to other tissue macrophages, the influx and efflux of cholesterol in alveolar macrophages appears to be regulated by PPARγ. However, the lung is a cholesterol-rich environment and the maintenance of cholesterol catabolism is critical. While the changes in the lungs of PPARγ KO mice cannot be attributed to a single mechanism, the abundance of cholesterol indicates cholesterol may contribute to the dysregulated state of surfactant catabolism.

Restoration of PPARγ in GM-CSF KO Mice

To further investigate the PPARγ-ABCG1 pathway in the catabolism of surfactant, we next reconstituted PPARγ expression in GM-CSF KO mice in vivo using the retroviral vector Lenti-PPARγ. Ten days post-instillation of Lenti-PPARγ, we measured increased expression of PPARγ and ABCG1 in the GM-CSF KO alveolar macrophages. Correlating to the up-regulation of the cholesterol transporter ABCG1, ABCG1-mediated cholesterol efflux was increased. These results corresponded to decreased intracellular neutral lipid accumulation and increased extracellular cholesterol levels to suggest that cholesterol catabolism was enhanced in vivo in treated alveolar macrophages. These results provide further evidence that PPARγ transcriptionally regulates ABCG1 and thereby promotes cholesterol catabolism in alveolar macrophages.

Extrapolating from the GM-CSF KO model, our results suggest that foam cell formation and the accumulation of cholesterol in the lungs of PAP patients resulted from deficiencies in PPARγ and ABCG1 and perhaps contributed to the accumulation of surfactant overall. This data extends our previous results and indicates that the GM-CSF-PPARγ pathway regulates surfactant clearance in alveolar macrophages through the ABCG1 lipid transporter.

It has been clearly demonstrated that GM-CSF is a critical mediator of surfactant homeostasis in alveolar macrophages. Our work has continued this pathway to include PPARγ and ABCG1. Alternative therapies are needed, as

treatment with recombinant GM-CSF results in symptomatic or radiographic improvement in approximately 50% of PAP patients (53,144,145). A follow-up study demonstrated that non-responding patients have high titers of neutralizing GM-CSF auto-antibodies which may be too high for exogenous GM-CSF treatments to overcome (54). In clinical trials, PAP patients were treated with higher doses of GM-CSF than current dose recommendations to reduce the duration and degree of neutropenia in certain cancer patients undergoing chemotherapy (53). While there is no evidence to suggest that PAP patients increase production of neutralizing antibodies in response to GM-CSF treatment (53), the possibility remains that increasing doses of antigen could stimulate antibody production. Taken together, this suggests PPARγ may be a more suitable therapeutic target than GM-CSF for PAP.

PPARγ agonist rosiglitazone may confer additional anti-inflammatory properties through the activation of PPARγ. In fact, PPARγ agonists have been shown to diminish inflammatory responses in murine models of asthma (154) and improve lung function in corticosteroid-resistant asthma patients (155). Decreased levels of PPARγ protein in the alveolar macrophages of PAP patients may attenuate the effects of PPARγ agonists. However, recent in vitro models have demonstrated that the presence of synthetic and natural PPARγ ligands up-regulates the expression of PPARγ likely through downstream positive feedback mechanisms (156). While currently only conjecture, PPARγ may prove to be a more suitable target for the treatment of PAP. Our work, however, confirms

PPARγ regulates surfactant catabolism in alveolar macrophages and provides evidence that PPARγ is a valid therapeutic target.

Future Directions

Several questions arise from the work reported in this thesis. First, one major difference between PPARγ KO and GM-CSF KO mice is the presence of GM-CSF. GM-CSF is a hematopoietic growth factor that promotes cell survival and proliferation and has a specific role in the differentiation of alveolar macrophages (7,29,102). One explanation for why GM-CSF deficiency only disrupts the terminal differentiation of alveolar macrophages is the expression of the transcription factor PU.1 (41). PU.1 promotes differentiation in several hematopoetic lineages including alveolar macrophages, unlike other tissue macrophages (104,157).

The alveolar macrophages of PAP patients and GM-CSF KO mice have significantly reduced levels of PU.1 (103,104). The alveolar macrophages from GM-CSF KO mice do not express differentiation marker CD11c (158,159), and PU.1-dependent terminal differentiation markers CD32, mannose receptor (MR), and macrophage colony-stimulating factor receptor (MCSFR) are reduced in the alveolar macrophages of PAP patients and GM-CSF KO mice (103,104). Replacement of PU.1 in GM-CSF KO mice corrected surfactant catabolism (3).

While induced upon differentiation into macrophages (160), PPARγ is not required for the differentiation of macrophages (100). Further, the expression of GM-CSF and PU.1 is not deficient in the alveolar macrophages of PPARγ KO mice. This suggests the differentiation and maturation of the PPARγ KO alveolar

macrophages is not affected as with GM-CSF KO mice. Several cell-surface markers of macrophage maturation and differentiation, such as those mentioned above, could be analyzed by flow cytometry to conclusively address the differentiation of PPARγ KO alveolar macrophages.

Another area of interest stemming from this work is the relationship between lipid accumulation and inflammation in PPARγ KO mice. Several comprehensive reviews have suggested that the PPARγ-LXR pathway is the link between lipid metabolism and inflammation in macrophages (151,152). Herein we have demonstrated that PPARγ regulates lipid metabolism in alveolar macrophages. We have also recently reported that the alveolar macrophages of PPARγ KO mice exhibit increased expression of Th-1 associated pro-inflammatory cytokines (96). It is unclear whether the deficiency of PPARγ resulted in lipid accumulation which lead to inflammation or if it was the production of inflammatory mediators that lead to lipid accumulation.

To address this question, we would first investigate the presence of pro-inflammatory cytokines and surfactant accumulation in younger PPARγ KO mice than the 8-12 week old mice utilized in the present studies. As GM-CSF KO mice develop pulmonary disease by 3 weeks of age (30), studying 3-6 week old PPARγ KO mice may clarify the onset of surfactant accumulation and inflammatory responses. Since it is unlikely that the results from these experiments would be definitive, additional in vitro experiments would be needed. For example, we could compare the inflammatory response elicited from

cholesterol-depleted wild type and PPARγ KO alveolar macrophages, by treatment with methyl-beta-cyclodextrin, cultured in the presence surfactant and an inflammatory agent such as lipopolysaccharide (161,162). Understanding the complex progression of lipid accumulation and inflammation in PPARγ KO mice is critical to the study of the regulation of surfactant metabolism in a broader context.

We have demonstrated that the alveolar macrophages of PPARγ KO mice develop cholesterol-laden alveolar macrophages that exhibit a foam cell phenotype. Free cholesterol comprised the bulk of the intracellular cholesterol accumulation. Importantly, the source of accumulating cholesterol (ie, LDL-derived or modified-LDL-derived) is a major factor in the storage and metabolism of the cholesterol. Cholesterol derived from modified-LDL is poorly catabolized and can result in the accumulation of free cholesterol which can disrupt cell signaling, cause plasma membrane rigidity, and induce pro-apoptotic cascades (163). The presence of modified-LDL can be indirectly determined utilizing several protocols to detect the presence of modified-LDL antibodies in sera (164,165). Alternatively, antibodies against modified-LDL could be used to measure the protein directly (166,167).

We propose that insufficient catabolism of cholesterol derived from modified-LDL impairs the overall catabolism of surfactant. Phospholipid efflux studies on the alveolar macrophages of PPARγ KO mice, if found to be similar to wild type, could confirm specific defects in the regulation of cholesterol. These

findings would have implications in the study of PPARγ and human lung diseases associated with surfactant accumulation.

Finally, the maintenance of cholesterol efflux is a crucial part of cholesterol homeostasis and is essential in the prevention of foam cell formation (77,168). The results presented in this thesis suggest that ABCG1-mediated cholesterol efflux is especially critical to the catabolism of surfactant in the alveolar macrophage. We have demonstrated that PPARγ KO alveolar macrophages exhibit reduced ABCG1 and ABCG1 cholesterol efflux. Replacement of PPARγ in GM-CSF KO mice induced expression of ABCG1 and increased ABCG1-mediated cholesterol efflux. The next step in developing the PPARγ-ABCG1 pathway would be to silence ABCG1 expression using small interfering RNA (siRNA) in wild type alveolar macrophages and measure the efflux of cholesterol to serum as a measure of maximum cholesterol efflux potential and to specific ABCG1-mediated efflux HDL. If reduced, this would indicate ABCG1 is a major contributor to cholesterol efflux from alveolar macrophages. LXR agonists could be used in conjunction with ABCG1 siRNA to induce ABCA1 expression to reflect conditions in PPARγ KO alveolar macrophages.

Conclusions

The work presented in this dissertation addresses the role of PPARγ in surfactant catabolism in alveolar macrophages. We hypothesized that PPARγ promotes surfactant catabolism by the regulation of ABCG1 and that the up-regulation of PPARγ will increase surfactant catabolism in PPARγ-deficient alveolar macrophages and reduce the presence of lipid-engorged alveolar macrophages in the lung.

We show that the targeted knockout of PPARγ in macrophages resulted in the accumulation of surfactant in the alveolar spaces of the lung and within the alveolar macrophages, reduced expression of ABCG1, and reduced ABCG1-mediated cholesterol efflux. These results support the hypothesis and directly link the deficiency of PPARγ to lipid accumulation in the lung.

To study the effects of PPARγ replacement on surfactant catabolism in PPARγ-deficient alveolar macrophages in vivo, we developed an efficient Lentivirus expression system to up-regulate PPARγ. Replacement of PPARγ in GM-CSF KO mice resulted in increased expression of ABCG1, enhanced ABCG1-mediated cholesterol efflux, and reduced intracellular lipid accumulation. Results from all sets of experiments suggest that PPARγ mediates a critical role in surfactant homeostasis through the regulation of ABCG1. These findings have potential implications in lung diseases such as PAP in which surfactant accumulates and PPARγ and ABCG1 are deficient in alveolar macrophages.

Understanding the role of PPARγ in normal surfactant homeostasis provides insight into the pathophysiology of PAP and identifies a potential therapeutic target.

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APPENDIX A

The Brody School of Medicine
Office of Prospective Health
East Carolina University
188 Warren Life Science Building • Greenville, NC 27834
252-744-2070 office • 252-744-2417 fax

Occupational Medicine
Employee Health
Radiation Safety
Infection Control
Biological Safety

TO: Dr. Mary Jane Thomassen
Department of Internal Medicine - Pulmonary

FROM: Eddie Johnson/John Williams
Biological Safety Officers

RE: Registration **Final** Approval

DATE: December 30, 2008

Your Biological Safety Protocol Thomassen, MJ, 08-01 *"Macrophages and Surfactant Homeostasis"* has received **final approval** by the ECU Biological Safety Committee.

This approval is effective for a period of 3 years and may be renewed with an updated registration if needed.

Please do not hesitate to contact Biological Safety at 744-2070 if you have any questions, concerns, or need any additional information. Best wishes on your research.

cc: Dr. Jeff Smith, Chair, Biosafety Committee
Dr. Paul Bolin, Jr, Chair
Janine Davenport, IACUC

APPENDIX B

Animal Care and Use Committee
East Carolina University
212 Ed Warren Life Sciences Building
Greenville, NC 27834
252-744-2436 office • 252-744-2355 fax

October 23, 2006

Mary Jane Thomassen, Ph.D.
Department of Medicine
Brody 3E-149
ECU Brody School of Medicine

Dear Dr. Thomassen:

The Amendment to your Animal Use Protocol entitled, "Macrophages and Surfactant Homeostasis," (AUP #J185) was reviewed by this institution's Animal Care and Use Committee on 10/18/06. The following action was taken by the Committee:

"Approved as amended"

A copy of the Amendment is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

APPENDIX C

The Brody School of Medicine
Office of Prospective Health
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Occupational Medicine
Employee Health
252-744-2070

Radiation Safety
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Infection Control
252-744-3202

Biological Safety
252-744-3437

TO: Dr. Malur

FROM Mercus Jeannette, MSEH, RSO

DATE: November 20, 2009

SUBJECT: Radisotope Approval

The Radiation Safety Committee approved the 3-H radiation source application on October 30, 2008. Prior to this date I granted you conditional approval.

The Radiation Safety Committee approves radiation source application Indefinitely unless there is a major change in protocol! If you have any additional question please call me at 744-2070.