ABCG1 is deficient in alveolar macrophages of GM-CSF knockout mice and patients with pulmonary alveolar proteinosis

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Abstract **Patients with pulmonary alveolar proteinosis (PAP) display impaired surfactant clearance, foamy, lipid-filled alveolar macrophages, and increased cholesterol metabolites within the lung. Neutralizing autoantibodies to granulocyte-macrophage colony-stimulating factor (GM-CSF) are also present, resulting in virtual GM-CSF deficiency. We investigated ABCG1 and ABCA1 expression in alveolar macrophages of PAP patients and GM-CSF knockout (KO) mice, which exhibit PAP-like pulmonary pathology and increased pulmonary cholesterol. Alveolar macrophages from both sources displayed a striking similarity in transporter gene dysregulation, consisting of deficient ABCG1 accompanied by highly increased ABCA1. Peroxisome proliferator-activated receptor γ (PPARγ), a known regulator of both transporters, was deficient, as reported previously. In contrast, the liver X receptor α, which also upregulates both transporters, was highly increased. GM-CSF treatment increased ABCG1 expression in macrophages in vitro and in PAP patients in vivo. Overexpression of PPARγ by lentivirus-PPARγ transduction of primary alveolar macrophages, or activation by rosiglitazone, also increased ABCG1 expression. These results suggest that ABCG1 deficiency in PAP and GM-CSF KO alveolar macrophages is attributable to the absence of a GM-CSF-mediated PPARγ pathway. These findings document the existence of ABCG1 deficiency in human lung disease and highlight a critical role for ABCG1 in surfactant homeostasis.**—Thomassen, M. J., B. P. Barna, A. G. Malur, T. L. Bonfield, C. F. Farver, A. Malur, H. Dalrymple, M. S. Kavuru, and M. Febbraio. **ABCG1 is deficient in alveolar macrophages of GM-CSF knockout mice and patients with pulmonary alveolar proteinosis.** ***J. Lipid Res.*** **2007.** 48: **2762–2768.**

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Pulmonary alveolar proteinosis (PAP) is a rare autoimmune disease in which surfactant clearance is defective and granulocyte-macrophage colony-stimulating factor (GM-CSF) is deficient as a result of neutralizing autoantibodies (1). In patients with PAP, the lungs are filled with excess lipoproteinaceous material and alveolar macrophages are engorged with lipid, resulting in a foamy appearance (2). We have shown that alveolar macrophages from PAP patients are also severely deficient in peroxisome proliferator-activated receptor γ (PPARγ), a key regulator of lipid metabolism (3). This deficiency is correctable by GM-CSF therapy (3). Subsequently, we reported that cholesterol and the cholesterol metabolites, cholestenoic acid and 27-hydroxycholesterol, are highly increased in PAP bronchoalveolar lavage (BAL) fluid (4). Interestingly, a PAP-like pulmonary histopathology is displayed by GM-CSF knockout (KO) mice (5, 6). Exogenous or local overexpression of GM-CSF reverses this pathology (7, 8). Pulmonary cholesterol is also increased in the GM-CSF KO mouse lung (9), and GM-CSF KO macrophages are deficient in PPARγ (10, 11).

Surfactant, which is produced by type II alveolar pneumocytes, is composed of 90% lipid, 10% protein, and a small amount of carbohydrate. Approximately 80–90% of surfactant lipid is phospholipid; the other lipids, in decreasing order, are cholesterol, triacylglycerol, and free fatty acids (12). The processes mediating and regulating surfactant clearance, recycling, and catabolism are not completely defined (13). Two primary surfactant catabolic pathways have been described (reviewed in Ref. 14). The first pathway is a recycling process requiring type II pneumocytes. The second pathway is a clearance pathway in which surfactant is phagocytosed and degraded by alveolar macrophages. Although a small amount of surfactant is degraded by type II pneumocytes, recent findings em-

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phasize the critical role of the alveolar macrophage in surfactant catabolism (15).

The ABC transporters ABCA1 and ABCG1 are members of a group of transmembrane proteins that transport a wide variety of substrates across membranes (16–19). Macrophages are a rich source of both ABCA1 and ABCG1, which can be regulated by a number of pathways, including PPARγ activation and the activation of liver X receptor α (LXRα) via the uptake of cholesterol or oxysterols (20–22). Cholesterol is esterified by macrophages, and accumulated cholesteryl esters are stored within the cell, giving it a foamy appearance (reviewed in Ref. 18). ABCA1 and ABCG1 play critical roles in mediating the cellular efflux of both cholesterol and phospholipids to lipoproteins (18). Recent findings have implicated these transporters in surfactant homeostasis (16, 23, 24). ABCA1 KO mice exhibit morphologic abnormalities in the lungs, including a massive accumulation of lipid-laden macrophages and type II pneumocytes (24). Similarly, lungs from ABCG1 KO mice show abnormal accumulation of lipid deposits in both alveolar macrophages and type II pneumocytes (16).

Although the phenotype of GM-CSF KO mice appears to resemble that of PAP, the status of genes involved in alveolar macrophage lipid efflux has not been investigated in either case. We hypothesized that the ABC transporters might be dysregulated in PAP and in GM-CSF KO mice. Our results indicate that alveolar macrophages from PAP patients and GM-CSF KO mice display a striking similarity of transporter dysregulation characterized by deficient ABCG1 and increased ABCA1.

MATERIALS AND METHODS

Study population

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 = 6). The diagnosis of idiopathic PAP was established by histopathological examination of material from open lung or transbronchial biopsies showing the characteristic filling of the alveoli with eosinophilic amorphous material with preserved lung architecture, the absence of inflammation, and the exclusion of secondary etiologies by negative lung cultures or occupational history (25). All PAP patients were symptomatic with dyspnea, were hypoxemic on room air, and had typical alveolar infiltrates on radiographs. Six PAP patients participated in a prospective clinical trial of recombinant human GM-CSF (Leukine; Berlex, Seattle, WA) as described previously (26). Treatment consisted of 250 μg/day by subcutaneous administration, with increased dosage every 2 weeks and maximum daily dosage of 18 μg/kg/day by 8 weeks. Patients were evaluated at baseline before the initiation of GM-CSF therapy and after 6 months of therapy. Clinical improvement or response to therapy was defined a priori as ⩾10 mm Hg improvement in partial pressure of arterial oxygen from baseline.

Cell collection

Alveolar macrophages were derived from BAL fluid obtained by fiber-optic bronchoscopy as described previously (27). Differential cell counts were obtained from cytospins stained with a modified Wright's stain. Differential cell counts from BAL fluid indicated that 93 ± 3% of PAP cells and 98 ± 0.3% of healthy control cells were alveolar macrophages. The mean viability of lavage cells was >95% as determined by trypan blue dye exclusion.

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. The GM-CSF KO mice were generated by Dr. Glenn Dranoff (5). The mice have been backcrossed eight generations to C57Bl/6. C57Bl/6 wild-type mice were obtained from Jackson Laboratory (Bar Harbor, ME) for controls. BAL and peritoneal cells were obtained from 8–12 week old GM-CSF KO mice and age- and gender-matched wild-type C57Bl/6 controls. For BAL cell harvest, mice received ketamine (80 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 BAL fluid was carried out with warmed (37°C) PBS in 1 ml aliquots. To obtain elicited peritoneal macrophages, mice were injected intraperitoneally with 0.5 ml of sterile 4% Brewer's thioglycollate medium (Sigma). Four days later, mice were euthanized and cells were recovered by lavage with 5 ml of sterile PBS using a 23 gauge needle. BAL and peritoneal cell differentials from all animals used in the experiments revealed >90% macrophages. For all experiments, three sets of pooled BAL or peritoneal cells from three to five mice were used. Cytospins of BAL or peritoneal cells were stained with Oil Red O to detect intracellular neutral lipids and counterstained with Gill's hematoxylin stain.

Lentivirus plasmid and transduction

Primary human alveolar macrophages were transduced with a self-inactivating lentivirus expression vector that was used previously in the generation of a stable cell line expressing the human parainfluenza virus type 3 C protein (28). cDNA corresponding to the human PPARγ was cloned into the multiple cloning sites downstream of a cytomegalovirus promoter using standard techniques as described (28). The recombinant lentiviral plasmid thus obtained was then transfected into 293FT cells along with plasmids encoding the *gag*, *pol*, and *rev* genes and a plasmid possessing the vesicular stomatitis glycoprotein using Lipofectamine 2000. At 48 h after transfection, cell culture supernatant containing the Lentivirus-PPARγ (Lenti-PPARγ) was purified by centrifugation at 27,000 rpm at 4°C for 3.5 h. The Lenti-PPARγ virus pellet was resuspended in tris EDTA buffer, and aliquots of 100 μl were stored at −80°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 used as a control in experiments for the determination of transduction efficiency. The percentage efficiency of in vitro transduction as determined by the examination of Lenti-eGFP incorporation under fluorescence microscopy was 87 ± 1 (n = 3) in human alveolar macrophages.

RNA purification and analysis

Total RNA was extracted from human and mouse cells by the RNAeasy protocol (Qiagen, Valencia, CA). Expression of mRNA was determined by real-time RT-PCR using the ABI Prism 7000 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 or human PPARγ, LXRα, ABCA1, and ABCG1 (Applied Biosystems). Threshold cycle values for genes of interest were normalized to a housekeeping gene (GAPDH) and used to calculate the relative quantity of mRNA expression in PAP or GM-CSF KO samples compared with healthy human or wild-type murine controls. Data were expressed as fold change in mRNA expression relative to control values (29).

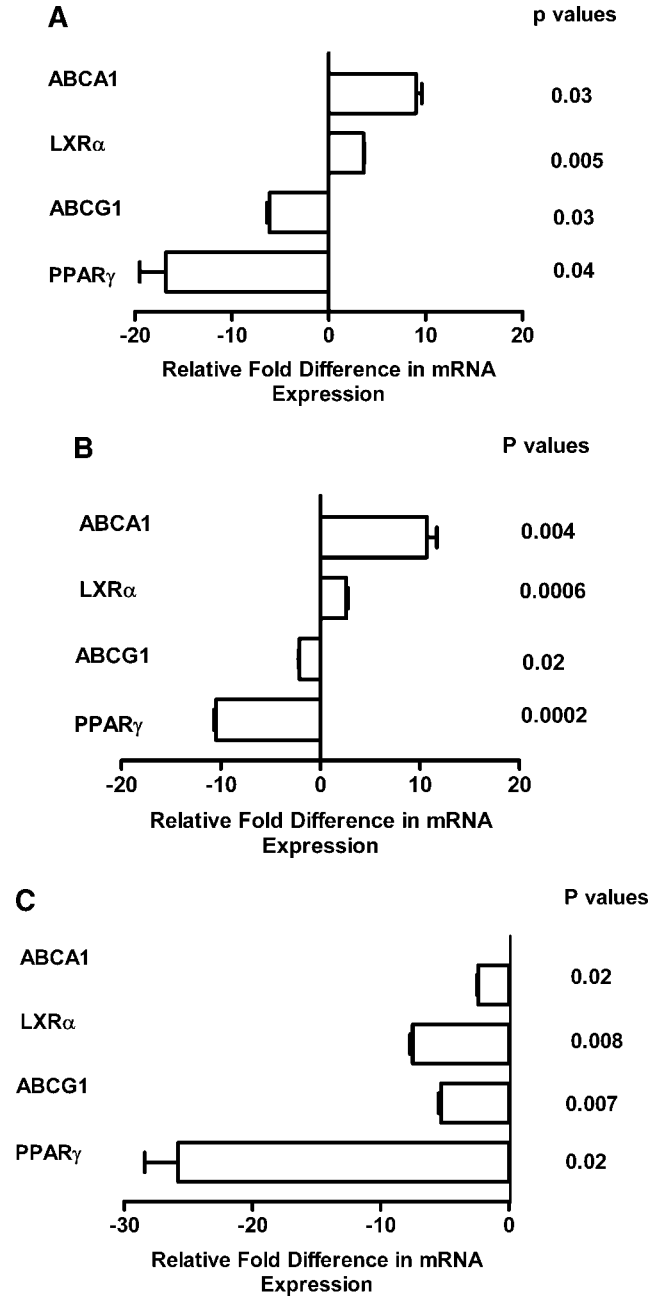

Fig. 1. Dysregulation of lipid-regulatory genes in macrophages of pulmonary alveolar proteinosis (PAP) patients and granulocyte-macrophage colony-stimulating factor (GM-CSF) knockout (KO) mice. Expression of mRNA was determined by quantitative PCR relative to controls. Data shown are from freshly obtained uncultured human alveolar macrophages (A), murine alveolar macrophages (B), and murine peritoneal macrophages (C). Human macrophage data were compiled from groups of three PAP patients and three healthy controls; murine data were obtained from three pooled sets of macrophages (three to five animals each) from GM-CSF KO and wild-type C57Bl/6 mice. LXRα, liver X receptor α; PPARγ, peroxisome proliferator-activated receptor γ. Error bars indicate ± SEM.

Immunoblotting

Cultured human alveolar macrophages or freshly obtained BAL cells from five animals per group of GM-CSF KO and wild-type C57Bl/6 mice were washed in PBS and lysed in a modified Nonidet P-40 lysis buffer as described previously (30). An equivalent amount of protein (25 μg) from each sample group was analyzed by 7.5% SDS-gel electrophoresis and transferred onto nitrocellulose membranes. Primary antibodies to ABCG1 (E-20; Santa Cruz Biotechnology) were diluted 1:500 and those to β-actin (Chemicon) were diluted 1:5,000 before incubation with membranes. Proteins were visualized by chemiluminescence.

Statistics

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

ABC transporter gene expression is dysregulated in PAP and GM-CSF KO alveolar macrophages

Endogenous mRNA expression of lipid transporter genes was determined in uncultured preparations of BAL-derived cells that consisted of >90% alveolar macrophages (see Materials and Methods). ABCG1 mRNA level was significantly ($P = 0.03$) decreased in PAP alveolar macrophages compared with healthy controls (**Fig. 1A**) and in GM-CSF KO alveolar macrophages ($P = 0.02$) compared with wild-type mice (Fig. 1B). In contrast, ABCA1 mRNA level was increased in both cases ($P = 0.03$ for PAP patients, $P = 0.004$ for GM-CSF KO mice) (Fig. 1A, B). Because transporter expression may be governed by both LXRα (17, 31) and PPARγ (32), we investigated the status of these regulatory genes in alveolar macrophages. LXRα mRNA level was increased in both PAP patients ($P = 0.005$) and GM-CSF KO mice ($P = 0.0006$), whereas PPARγ mRNA was decreased ($P = 0.04$ for PAP patients, $P = 0.0002$ for GM-CSF KO mice), as reported previously (3, 10) (Fig. 1A, B). Apolipoprotein E (APOE), another

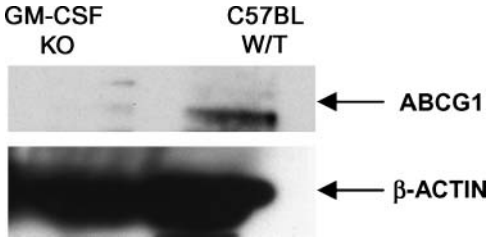

Fig. 2. ABCG1 is deficient in GM-CSF KO alveolar macrophages. Lysates (25 μg of protein) from freshly obtained uncultured GM-CSF KO and wild-type (W/T) C57Bl/6 alveolar macrophages (five animals per group) were analyzed for ABCG1 and β-actin expression by immunoblot.

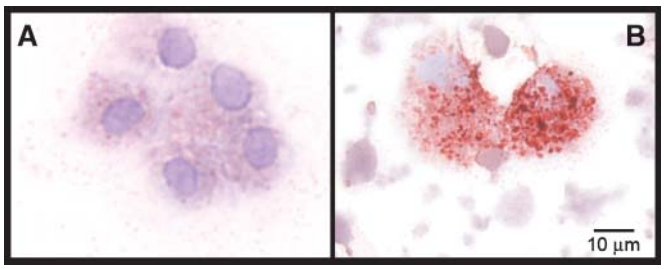

Fig. 3. GM-CSF KO alveolar macrophages are filled with neutral lipids. Cytospin preparations of uncultured bronchoalveolar lavage (BAL) cells from C57Bl/6 wild-type (A) and GM-CSF KO (B) mice were stained with Oil Red O.

gene subject to LXR regulation, was also investigated and found to be highly increased in alveolar macrophages from both PAP patients (7.1 ± 1.9-fold increase; $P = 0.03$) and GM-CSF KO mice (618 ± 1.4-fold; $P < 0.0001$) compared with controls.

ABCG1 protein expression is reduced in GM-CSF KO alveolar macrophages

Immunoblotting revealed no detectable ABCG1 protein in BAL cell lysates from GM-CSF KO mice (**Fig. 2**). Protein bands of ~110 kDa corresponding to ABCG1 were visible, however, in BAL cell lysates from wild-type C57Bl/6 mice (Fig. 2).

GM-CSF KO and PAP alveolar macrophages are morphologically similar

Oil Red O staining highlighted the foamy cytopathology of GM-CSF KO alveolar macrophages, which strongly resembled that reported previously in PAP (33). Compared with wild-type C57Bl/6 mice (**Fig. 3A**), GM-CSF KO alveolar macrophages were filled with cytoplasmic deposits of Oil Red O-positive (neutral) lipids and large lipid vacuoles (Fig. 3B). The majority (97.6 ± 1.0%) of wild-type alveolar macrophages (n = 5) were Oil Red O-negative, with only 2.4 ± 1.0% positive cells. In contrast, 92.4 ± 4.3% of GM-CSF KO macrophages were Oil Red O-positive (n = 5, $P = 0.001$). Oil Red O staining was unremarkable in GM-CSF KO peritoneal macrophages, which resembled those from wild-type mice (data not shown), although PPARγ and ABCA1 gene expression were decreased (Fig. 1C), as noted by Ditiatkovski et al. (11). ABCG1 was also decreased in peritoneal macrophages, but in contrast to alveolar macrophages, LXRα was not increased (Fig. 1C). Furthermore, as expected because LXR was not increased, APOE expression was also not increased in peritoneal cells.

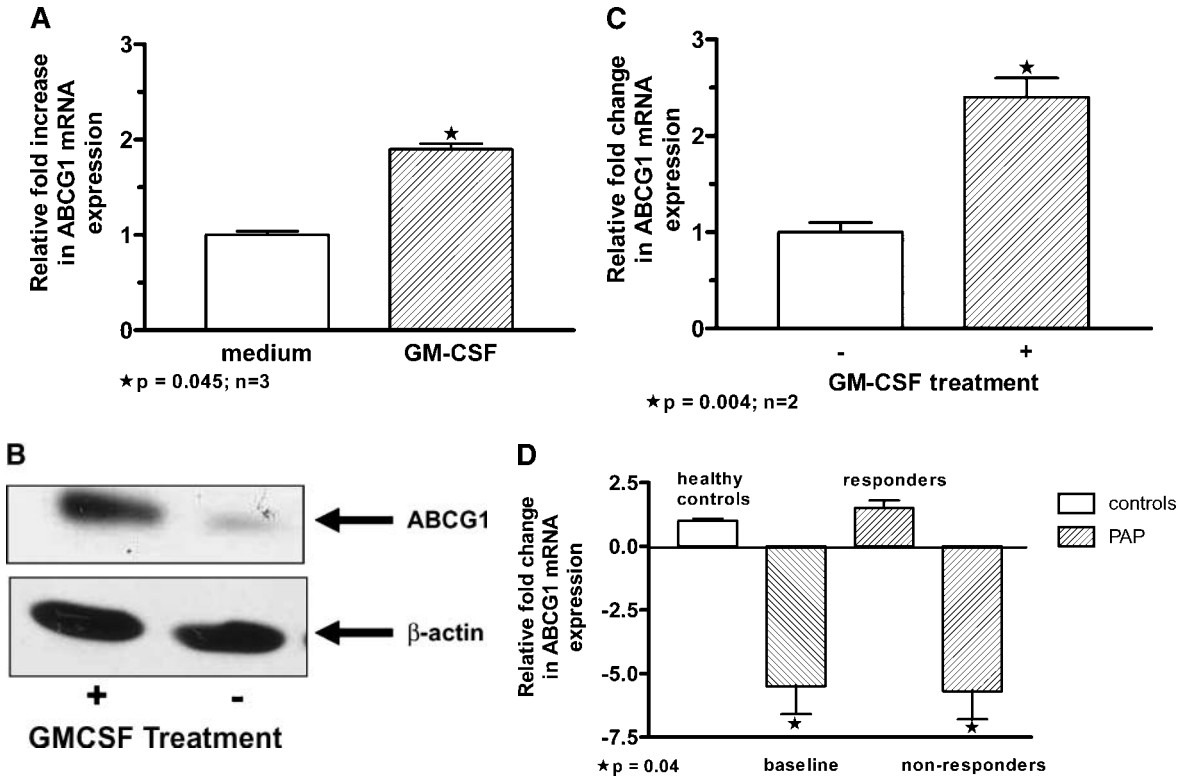

Fig. 4. GM-CSF increases ABCG1 expression in human and murine macrophages. Expression of ABCG1 was determined by quantitative PCR or immunoblot compared with controls. For in vitro studies, macrophages were cultured with or without GM-CSF (100 ng/ml) for 24–48 h. A: ABCG1 mRNA expression in cultured human alveolar macrophages from healthy controls (n = 3). B: Immunoblot of lysate from cultured human alveolar macrophages showing increased ABCG1 protein. C: ABCG1 mRNA expression in cultured GM-CSF KO macrophages. D: ABCG1 mRNA expression in freshly obtained BAL cells from PAP patients (n = 6) before (baseline) and after 6 months of recombinant GM-CSF therapy compared with healthy controls (n = 6). ABCG1 levels in BAL cells from clinical nonresponders (n = 4) remained significantly below control levels ($P = 0.04$), whereas in clinical responders (n = 2), levels were restored to the control range.

GM-CSF treatment upregulates ABCG1 expression

To determine whether GM-CSF modified ABCG1 levels, human and murine macrophages were treated with GM-CSF (100 ng/ml) in vitro. GM-CSF exposure significantly increased ABCG1 mRNA ($P = 0.04$, n = 3) and protein expression in alveolar macrophages from healthy controls compared with untreated macrophages (**Fig. 4A, B**). Alveolar macrophages from a PAP patient also responded to GM-CSF culture with a 3.1-fold increase in ABCG1 mRNA level compared with culture medium (data not shown). Similarly, GM-CSF KO macrophages cultured in the presence of GM-CSF displayed increased ABCG1 mRNA ($P = 0.004$) (Fig. 4C). ABCG1 mRNA expression was also examined in BAL cells from PAP patients (n = 6) who had received GM-CSF therapy in vivo (Fig. 4D). ABCG1 mRNA levels were significantly deficient ($P = 0.04$) before therapy (baseline) compared with healthy controls (n = 6) and remained so in clinical nonresponders (n = 4). In two clinically responsive PAP patients after GM-CSF therapy, however, ABCG1 mRNA levels were restored to the control range (Fig. 4D).

PPARγ overexpression or activation increases ABCG1 expression

Although PPARγ pathways are cited as regulating ABCG1 in experimental models, no data are available for primary human alveolar macrophages. Lenti-PPARγ and, as control, Lenti-eGFP were used to transduce primary human alveolar macrophages in vitro. Compared with Lenti-eGFP, Lenti-PPARγ significantly increased ABCG1 mRNA ($P = 0.03$, n = 3), and as anticipated, PPARγ mRNA was also increased ($P = 0.02$) (**Fig. 5A**). As an additional control, PPARα mRNA was also evaluated and Lenti-PPARγ transduction had no effect on PPARα expression. PPARγ and ABCG1 mRNA levels were not affected in Lenti-eGFP-transduced alveolar macrophages compared with untreated macrophages (data not shown). Ligand activation of PPARγ via rosiglitazone (10 μM) treatment of human alveolar macrophages in vitro also produced brisk upregulation in ABCG1 mRNA levels (Fig. 5B).

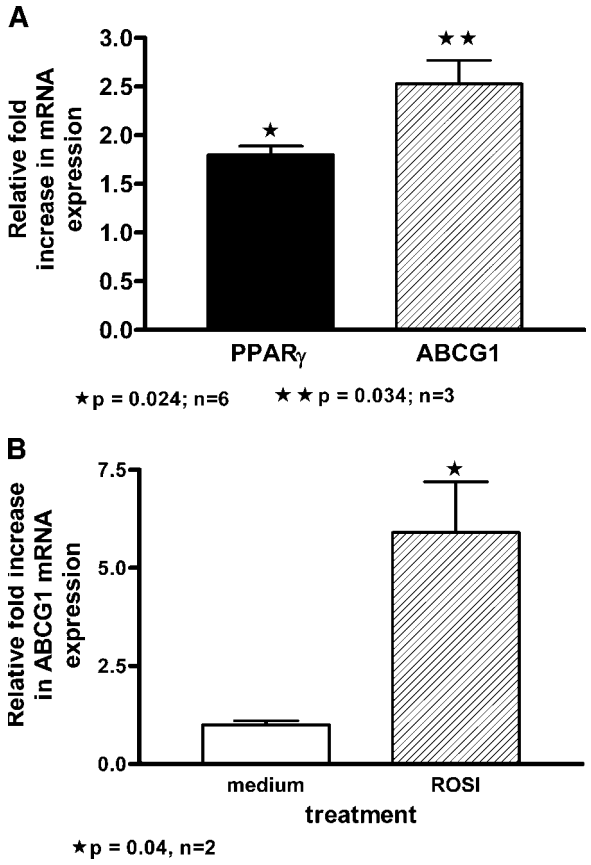

Fig. 5. PPARγ overexpression or activation increases ABCG1 expression in primary alveolar macrophages. Human alveolar macrophages were transduced with lentivirus-enhanced Green Fluorescent Protein (control; ABCG1) or lentivirus-enhanced PPARγ vectors followed by 48 h of culture (A) or cultured with or without rosiglitazone (ROSI; 10 μM) for 24 h (B). Expression of mRNA expression was quantified by PCR.

DISCUSSION

This report demonstrates similar phenotypes and profiles of lipid transport genes in alveolar macrophages from PAP patients and GM-CSF KO mice, thus supporting the role of GM-CSF in the etiology of this disease and identifying potential downstream target genes. Alveolar macrophages from both PAP patients and GM-CSF KO mice present a foamy appearance and contain excess Oil Red O-positive neutral lipids. Peritoneal macrophages from GM-CSF KO mice, in contrast, do not exhibit lipid accumulation, suggesting that a portion of the etiology of the disease is specific to the demands of the macrophage environment. We previously reported decreased expression of PPARγ in PAP alveolar macrophages (3) and now show this to be a characteristic of both alveolar and peritoneal macrophages of GM-CSF KO mice. Ditiatkovski et al. (11) also reported similar findings in GM-CSF KO peritoneal macrophages. Such data indicate that PPARγ is downsteam of GM-CSF.

In both PAP patients and GM-CSF KO mice, LXRα expression was increased in alveolar macrophages. This seems counter to the hypothesis put forth by Ricote, Valledor, and Glass (34), which suggested that LXRα is regulated by PPAR/retinoid X receptor (RXR) heterodimers. Accordingly, one would expect a downregulation of LXRα in GM-CSF KO alveolar macrophages when PPARγ is absent. In fact, such downregulation in LXRα mRNA level is observed in peritoneal macrophages. Our previous report indicated increased cholesterol and oxysterol levels in PAP lung (4). Thus, the increased LXRα expression found in alveolar macrophages in the current study is consistent with the localized increase in cholesterol/cholesterol metabolites that would directly influence LXRα expression (20). Baldan et al. (16), however, also reported increased LXRα and ABCA1 in alveolar macrophages of ABCG1 KO mice.

Interestingly, these ABCG1 KO mice also accumulate lipid in the lung.

A novel observation in this study is the demonstration of an ABCG1 transporter deficiency in human pulmonary disease. Previously, ABCA1 deficiency was recognized in Tangier disease (35). The accumulation of surfactant lipid despite increased ABCA1 and LXRα in PAP and GM-CSF KO alveolar macrophages suggests that the expression of these genes is not sufficient to maintain surfactant homeostasis and prevent foam cell formation. Such findings may indirectly implicate ABCG1 as the primary transporter for the efflux of accumulated surfactant phospholipid. Alternatively, the dysregulation of these (and potentially other) genes may result in aberrant compartmentalization of surfactant such that it is unavailable for processing or efflux. Our data further suggest that ABCA1 expression may be regulated by RXR/LXR dimers, as suggested by Ricote, Valledor, and Glass (34), and in the case of LXRα may be altered as a consequence of lipid accumulation. ABCG1 may also be regulated by RXR/LXR heterodimers (34), but we found decreased ABCG1 expression in both peritoneal and alveolar macrophages. Recent studies have shown that PPARγ activation may induce ABCG1 expression in an LXR-independent manner (32), and a deficiency of this pathway may be responsible for the decreased ABCG1 we observed in PAP and GM-CSF KO mice.

The extremely large increase in APOE mRNA expression in GM-CSF KO mice compared with human alveolar macrophages is likely related to species-specific differences in lipoprotein metabolism. Mice lack cholesteryl ester transfer protein; therefore, there is a difference in the manner by which cholesterol is transferred between lipoproteins and dispersed (36, 37). Mice may be more dependent upon liver-mediated mechanisms for cholesterol excretion than are humans, and the exaggerated increase in APOE found in GM-CSF KO mice may reflect the importance of a murine salvage pathway.

In summary, the results of the current studies support the hypothesis that GM-CSF promotes surfactant catabolism by upregulating ABCG1 via PPARγ pathways. First, the deficiency of alveolar macrophage ABCG1 reported in both PAP patients and GM-CSF KO mice parallels the deficiency of PPARγ. Second, GM-CSF increased ABCG1 expression in both in vitro experiments and a clinical trial of recombinant GM-CSF therapy in PAP patients. In the latter case, PAP patients who demonstrated an excellent clinical response with almost complete resolution of disease also displayed upregulated ABCG1 mRNA levels in BAL cells, whereas ABCG1 did not increase in BAL cells of clinically unresponsive patients. Such findings with ABCG1 parallel those we reported previously showing PPARγ restoration in clinically responsive PAP patients receiving recombinant GM-CSF (3). Finally, the increase in alveolar macrophage ABCG1 expression after either Lenti-PPARγ overexpression or ligand activation of PPARγ demonstrates the functional link between PPARγ and ABCG1 in alveolar macrophages.

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