A Critical Role for ABCG1 in Macrophage Inflammation and Lung Homeostasis

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A Critical Role for ABCG1 in Macrophage Inflammation and Lung Homeostasis

Allison J. Wojcik,*† Marcus D. Skaflen,‡ Suseela Srinivasan,† and Catherine C. Hedrick2*†‡

ATP-binding cassette transporter G1 (ABCG1) effluxes cholesterol from macrophages and plays an important role in pulmonary lipid homeostasis. We hypothesize that macrophages from Abcg1−/− mice have increased inflammatory activity, thereby promoting acceleration of pulmonary disease. We herein demonstrate increased numbers of inflammatory cytokines and infiltrating neutrophils, eosinophils, dendritic cells, T cells, and B cells into lungs of Abcg1−/− mice before the onset of severe lipidosis. We further investigated the role of macrophages in causing pulmonary disease by performing bone marrow transplantations using B6 and Abcg1−/− bone marrow. We found that it was the macrophage, and not pneumocyte type II cells or other nonhematopoietic cells in the lung, that appeared to be the primary cell type involved in the onset of both pulmonary lipidosis and inflammation in the Abcg1−/− mice. Additionally, our results demonstrate that Abcg1−/− macrophages had elevated proinflammatory cytokine production, increased apoptotic cell clearance, and were themselves more prone to apoptosis and necrosis. However, they were quickly repopulated by monocytes that were recruited to Abcg1−/− lungs. In conclusion, we have shown that ABCG1 deletion in macrophages causes a striking inflammatory phenotype and initiates onset of pulmonary lipidosis in mice. Thus, our studies reveal a critical role for macrophage ABCG1 in lung inflammation and homeostasis. The Journal of Immunology, 2008, 180: 4273–4282.

Macrophages are involved in maintaining cholesterol homeostasis and regulating inflammation in the lung. There are different populations of macrophages that play distinct roles in the lung. Alveolar macrophages are localized in the alveolar space, and tissue macrophages are located within the alveolar and airway walls (1, 2). Alveolar macrophages release more proinflammatory mediators and demonstrate increased phagocytosis, cytotoxicity, and release of reactive oxygen species compared with tissue macrophages (3). Tissue macrophages are more specialized in immune responses and immunoregulation, as seen by their increased Ag presentation and secretion of IL-1 and IL-6 (4–6).

Macrophages play a crucial role in innate and acquired immunity, defense against pathogens, and in the clearance of inhaled particles. Alveolar macrophages are also important in initiating the inflammatory response in the lung. In response to danger, alveolar macrophages produce various proinflammatory mediators to orchestrate the inflammatory response (3, 7). This leads to the recruitment of numerous inflammatory cells, including monocytes, dendritic cells, eosinophils, neutrophils, B cells, and T cells (3, 7, 8). These cells contribute to the immune response and eventually apoptose once the inflammation is resolved. The alveolar macrophages clear the apoptotic cells and produce antiinflammatory mediators (9, 10).

Alveolar pulmonary surfactant is composed of phospholipids and surfactant proteins (SP) and is essential for maintenance of normal lung function (11). Surfactant associated with SP-B or SP-C functions to maintain a low surface tension for alveolar inflation and normal gas exchange in the lung (12). Surfactant associated with SP-A or SP-D is involved in innate immunity and the clearance of apoptotic cells (13, 14). Saturated phosphatidylcholine, the most abundant phospholipid in surfactant, is synthesized and secreted by type II cells. Surfactant is taken up equally by both alveolar macrophages and type II cells. Alveolar macrophages catalyze all surfactant, but type II cells both degrade and recycle surfactant (15). There are many pulmonary diseases associated with altered surfactant homeostasis and ATP-binding cassette transporters may play a role in some of them (16).

ATP-binding cassette transporter G1 (ABCG1) is a transmembrane transporter that mediates cholesterol efflux from macrophages (17–23). Recently, a role for ABCG1 in pulmonary function was discovered (24). The lung phenotype of older (8 mo of age) Abcg1−/− mice is similar to that observed for Abca1−/− mice (25) in the present study, we tested whether ABCG1 deficiency in macrophages was causal in the onset of pulmonary inflammation and lipidosis in these mice. Surprisingly, we found that ABCG1 expression in macrophages is an important regulator of pulmonary inflammation. ABCG1-deficient macrophages are highly proinflammatory and cause increased trafficking of inflammatory cells to the lungs. Abcg1−/− macrophages have enhanced ability to engulf apoptotic cells, accumulate lipid, and become apoptotic. Thus, we show a novel role for ABCG1 in the inflammatory response.

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Abbreviations used in this paper: SP, surfactant protein; ABCG1, ATP-binding cassette transporter G1; BAL, bronchoalveolar lavage; ER, endoplasmic reticulum; TAAAD, 7-aminoadenomycin D; Siglec-F, siaic acid-binding Ig-like lectin F.

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Materials and Methods

Reagents

Oil Red O and collagenase D were purchased from Sigma-Aldrich. ABCG1 Ab was purchased from Novus Biologicals. HRP-conjugated anti-mouse and anti-rabbit secondary Abs came from Amersham Biosciences. Fluorochrome-labeled Abs were purchased from BD Pharmingen or eBioscience and used according to the manufacturers’ protocols. NuPAGE 4–12% denaturing gels and TAMRA dye came from Invitrogen. The mouse 23-plex suspension bead arrays were purchased from Bio-Rad.

Mice

C57BL/6J (stock no. 000664) were purchased from The Jackson Laboratory, and Abcg1<sup>−/−</sup>/lacZ knock-in mice on a C57BL/6J background were purchased from Deltagen. Cx3cr1<sup>GFP</sup> mice on a C57BL/6J background were a gift from Dr. Steffen Jung (The Weizmann Institute of Science, Rehovot, Israel). Mice were fed a standard rodent chow diet and housed in microisolator cages in a pathogen-free facility. All experiments followed University of Virginia Animal Care and Use Committee guidelines, and approval for use of rodents was obtained from the University of Virginia.

Histology

Mice were euthanized with isoflurane, and the tracheas were exposed and cannulated. For frozen lung sections, OCT compound (Sakura Finetek USA) was injected into the lungs, the trachea was tied off, and the lungs were removed and frozen on dry ice. Lungs were sectioned into 10-μm sections and stained with Oil Red O for lipid accumulation. Images were taken at ×40 and ×200. For morphometric and lung structure analysis, lungs were fixed with paraformaldehyde at 30 cm inflation pressure and paraffin-embedded. The tissue sections were stained with H&E and images were taken at ×200. To measure alveolarization, five random tissue sections were taken from age-matched mice. An alveolarization scoring system was used to calculate mean linear intercept, as previously described (26).

Bronchoalveolar lavage (BAL)

Mice at 7 wk of age were euthanized with isoflurane, and the tracheas were exposed and cannulated. The lungs were flushed three times with 0.5 ml aliquots of BAL buffer (TBS and 10 mM EDTA). The aliquots were combined and centrifuged (200 × g, 5 min) to separate the supernatant (surfactant and soluble proteins) and cells. The cellular fraction was used for flow cytometry or plated to purify the macrophage population. The supernatant was kept at −80°C until used for cytokine or surfactant measurements.

Cytokine measurements

Cytokines and chemokines were measured in BAL, fluid, plasma, and alveolar macrophage culture media using a Bio-Plex Mouse 23-plex suspension bead array (Bio-Rad) according to the instructions of the manufacturer. IL-12p40, IL-12p70, IL-13, IL-6, G-CSF, GM-CSF, IFN-γ, MIP-1α, RANTES, IL-1α, IL-1β, TNF-α, IL-2, IL-3, IL-5, IL-6, and IL-10 were detected using high-sensitivity settings.

Lung tissue digestion

Mice were euthanized with isoflurane. The heart was exposed, the aorta was severed, and the lungs were perfused gently with 5 ml of cold PBS through the right ventricle using a syringe fitted with a 25-gauge needle. The lungs were excised, minced, and digested for 30 min at 37°C with collagenase D (200 U/ml). The suspensions were passed through 70-μm cell strainers (BD Biosciences). The cells were washed with TBS, and RBCs were depleted with ammonium-chloride lysing buffer. Samples were used for flow cytometry experiments as described below.

Flow cytometry

Single-cell suspensions of BAL cells, lung tissue digest, or blood were surface-stained for 20 min at 4°C with the following Abs: anti-CD8 clone 53-6.7, anti-CD4 clone RM4-5, anti-CD3 (clone 145-2C11), anti-CD19, anti-CD11b clone M1/70, anti-7/4 (clone MCA771FA), anti-CD11c (clone N418), anti-sialic acid-binding Ig-like lectin F (Siglec-F) (clone E50-2440), MHC class II (clone M5/114.15.2), anti-CD115 (clone AF598), and anti-Nk1.1 (clone PK136). After 20 min, cells were washed and analyzed at the University of Virginia Flow Cytometry Core using a BD FACSCalibur instrument (BD Biosciences), collecting data on the entire sample. Analysis was performed using FlowJo software. Calculations of percentages were based on live cells as determined by FSC/SSC analysis. To determine total cell counts, flow cytometry counting beads (Invitrogen) were used and the actual number of cells was calculated based on the number of beads in the sample and the number of beads counted by the flow cytometer.

Cytokine measurements

Lungs were perfused and frozen for histology. Sections were stained for lipid using Oil Red O. Representative images at ×200 are shown: (A) 3-wk-old B6. (B) 7-wk-old B6. (C) 8-mo-old B6. (D) 3-wk-old Abcg1<sup>−/−</sup>. (E) 7-wk-old Abcg1<sup>−/−</sup>, and (F) 8-mo-old Abcg1<sup>−/−</sup>. G–L, Lungs were fixed with paraformaldehyde and paraffin-embedded. Sections were stained with H&E and images were taken at ×200; (G) 3-wk-old B6. (H) 7-wk-old B6. (I) 8-mo-old B6. (J) 3-wk-old Abcg1<sup>−/−</sup>. (K) 7-wk-old Abcg1<sup>−/−</sup>, and (L) 8-mo-old Abcg1<sup>−/−</sup>. M. Number of alveolar wall intercepts (n = 1 mouse, five slides per mouse, and four random measurements per slide). Data were analyzed by two-way ANOVA with Bonferroni posttest. F test for age and interaction were significant as *, p < 0.05.

Bone marrow transplantation

B6 or Abcg1<sup>−/−</sup> mice were lethally irradiated (650 rad, 2 repetitions) and injected with either B6 or Abcg1<sup>−/−</sup> bone marrow (4 × 10<sup>6</sup> cells). The mice were allowed to recover for 9 wk to allow for repopulation of resident alveolar macrophages with donor cells (27). To test for reconstitution, bone marrow-derived macrophages were tested for ABCG1 expression by immunoblotting.

Monocyte trafficking studies

Bone marrow was harvested from tubias of Cx3cr1<sup>GFP</sup> mice. The cells were washed and RBGs were depleted with ammonium chloride lysing buffer. The bone marrow cells were injected into the tail veins of B6 or Abcg1<sup>−/−</sup> mice. After 48 h, the lungs and spleen were removed for flow cytometry analysis.

Annexin V and 7-aminoactinomycin D (7AAD) staining

BAL cells were isolated and stained with annexin V (Invitrogen Life Technologies) and 7AAD (BD Biosciences) according to the manufacturers’ protocols.

TUNEL staining

BAL macrophages were isolated, plated for 1 h, washed to remove nonadherent cells, and stained for DNA strand breaks with TUNEL according to the instructions of the manufacturer (Roche). Cells were visualized by fluorescence microscopy, and images were taken at ×100.

Apoptotic cell clearance assay

Jurkat cells were made apoptotic by UV cross-linking at 100 mJoules. Apoptotic Jurkat cells were labeled with TAMRA dye (Invitrogen Life Technologies) and 7ADD (BD Biosciences) according to the manufacturers’ protocols.
Technologies), incubated for 2 h at 37°C, and washed extensively. BAL macrophages and apoptotic Jurkat cells were then incubated together for 45 min at 37°C to allow engulfment of apoptotic cells by the macrophages to occur. At the end of the incubation period, macrophages were washed extensively to eliminate any unengulfed cells, and engulfment was quantified using flow cytometry following the methods of Kiss et al. (28). Results are shown as the percentage of macrophages that engulfed apoptotic Jurkat cells.

**Statistical analysis**

Data for all experiments were analyzed by unpaired Student's t test (2 groups) or ANOVA (>2 groups) using the Statview 6.0 software program (SAS Institute). Data are graphically represented as means ± SEM. Comparisons between groups and tests of interactions were made assuming a two-factor analysis, with the interaction term testing each main effect and with the residual error testing the interaction. All comparisons were made using Fisher's least significant difference procedure, so that multiple comparisons were made at the 0.05 level only if the overall F test from the ANOVA was significant at p < 0.05.

**Results**

**Lipid accumulates in the lungs of weanling Abcg1<sup>−/−</sup> mice**

To determine how early a disruption in lipid homeostasis occurs in Abcg1<sup>−/−</sup> mice, we examined Oil Red O staining of lung tissue in

**FIGURE 2.** Increased expression of proinflammatory cytokines in BAL fluid and alveolar macrophages of Abcg1<sup>−/−</sup> mice. Cytokine and chemokine protein expression was measured using a cytokine bead array (Bio-Rad). Values shown are means ± SEM. Significantly higher than control by unpaired t test: *, p < 0.05; $, p < 0.005; and #, p < 0.0001. A, BAL fluid was analyzed from B6 (n = 6) and Abcg1<sup>−/−</sup> 7-wk-old (n = 6) mice. B, Alveolar macrophages were isolated from B6 (n = 10) and Abcg1<sup>−/−</sup> (n = 10) mice and plated for 24 h. The supernatant was collected and analyzed. C, Plasma was analyzed from B6 (n = 9) and Abcg1<sup>−/−</sup> (n = 8) mice.

**FIGURE 3.** Increased infiltration of inflammatory cells into the alveolar space of Abcg1<sup>−/−</sup> mice. BAL cells from B6 (n = 4) and Abcg1<sup>−/−</sup> mice (n = 4) were collected, washed, and stained with fluorophore-conjugated Abs. Samples were run on a FACSCalibur flow cytometer and analyzed using FlowJo software. Representative plots are shown in A–F: (A) T cells were identified as CD3<sup>+</sup> and CD8<sup>+</sup> or (B) CD3<sup>+</sup> and CD4<sup>+</sup>, (C) B cells were identified as CD19<sup>+</sup>, (D) neutrophils were identified as CD11b<sup>+</sup>Gr-1<sup>+</sup>7/4<sup>+</sup> cells, (E) eosinophils were identified as Siglec-F<sup>+</sup>CD11b<sup>+</sup>CD11clow cells, and (F) dendritic cells were identified as Siglec-F<sup>+</sup>CD11b<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup> cells. G, Plot of average number of cells per group from three experiments. Values shown are means ± SEM. Significantly higher than control by unpaired t test: *, p < 0.05; $, p < 0.005; and #, p < 0.0001.
Increased inflammation in the lungs of Abcg1<sup>−/−</sup> mice

To investigate the inflammatory state of young Abcg1<sup>−/−</sup> mice before onset of pulmonary lipodiosis, we measured cytokine and chemokine levels in the BAL fluid, plasma, and alveolar macrophages of 7-wk-old B6 and Abcg1<sup>−/−</sup> mice. Abcg1<sup>−/−</sup> mice had significant increases in levels of cytokines in BAL fluid compared with B6 mice (Fig. 2A). Because we had hypothesized that the macrophage is the inflammatory cell promoting disease in the lungs of Abcg1<sup>−/−</sup> mice, we measured cytokine levels in isolated alveolar macrophage cell supernatants. We found a significant elevation in the inflammatory cytokines IL-6, IL-1β, IL-1α, IL-12, and KC and a decrease in antiinflammatory IL-10 (Fig. 2B). Surprisingly, chemokine and cytokine levels in Abcg1<sup>−/−</sup> plasma were normal, except for the chemokine KC (Fig. 2C).

3-wk-old, 7-wk-old, and 8-mo-old C57BL/6J (B6) and Abcg1<sup>−/−</sup> mice. We found that 3-wk-old weanling Abcg1<sup>−/−</sup> mice accumulated lipid at the edge of the lung lobe (Fig. 1D). This was not observed in 3-wk-old B6 mice (Fig. 1A). At 7 wk of age, there was no additional increase in lipid accumulation in Abcg1<sup>−/−</sup> mice (Fig. 1, B and E). However, at 8 mo of age, as previously reported by Edwards and colleagues (24), Abcg1<sup>−/−</sup> mice had severe lipid accumulation in all parts of the lung that was not observed in B6 mice (Fig. 1, C and F). Moreover, we found significant alveolar enlargement in 8-mo-old Abcg1<sup>−/−</sup> mice compared with B6 mice (Fig. 1, I, L, and M). However, there was no change in lung structure or alveolar space in 3-wk-old and 7-wk-old Abcg1<sup>−/−</sup> mice (Fig. 1, J and K), with lung structure being similar to that of age-matched B6 control mice (Fig. 1, G and H). Thus, lipid accumulation in Abcg1<sup>−/−</sup> mice begins at a very early age and becomes progressively more severe.

Next, we evaluated the type of pulmonary surfactant that was accumulating in the lungs of Abcg1<sup>−/−</sup> mice. We chose to study 7-wk-old mice for all remaining experiments. SP-A, SP-B, SP-C, and SP-D were measured in the lung tissue of 7-wk-old B6 and Abcg1<sup>−/−</sup> mice. SP-A, SP-B, and SP-C appeared normal in Abcg1<sup>−/−</sup> mice at 7 wk of age, yet SP-D was greatly increased in 7-wk-old Abcg1<sup>−/−</sup> mice (data not shown). SP-D is involved in regulating the immune response in the lung (13, 14), and therefore these data suggest that lungs of Abcg1<sup>−/−</sup> mice are inflamed.

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Significance was calculated using ANOVA: \( p \), \( \# \), and \( \ast \). Siglec-F\(^{\text{high}}\)CD11b\(^{\text{high}}\)CD11c\(^{\text{low}}\) cells, and \( \ast \), and reconstituted with B6 or Abcg1\(^{-/-}\) bone marrow (BM) \( (n = 5/\text{group}) \). Cytokine and chemokine protein expression was measured in BAL fluid from bone marrow transplant mice using a cytokine bead array (Bio-Rad). Values shown are means \( \pm \) SEM. Significance was calculated using ANOVA: \( \ast \), \( p < 0.05 \); \( S \), \( p < 0.005 \); and \( \# \), \( p < 0.0001 \).

Given the change in lung cytokine levels, we sought to determine which inflammatory cells were infiltrating the lungs. Inflammatory cells were identified in the alveolar space and lung tissue of 7-wk-old B6 and Abcg1\(^{-/-}\) mice by flow cytometry. T cells were identified as CD3\(^{\text{high}}\) and CD8\(^{\text{high}}\) or CD4\(^{\text{high}}\) (Fig. 3A, A–D). Neutrophils were defined as CD11b\(^{\text{high}}\)Gr-1\(^{\text{high}}\)7/4\(^{\text{high}}\) (Fig. 3B, E). Eosinophils were identified as CD11b\(^{\text{high}}\)Gr-1\(^{\text{high}}\)CD11c\(^{\text{low}}\) (Fig. 3E). Dendritic cells were identified as Siglec-F\(^{\text{high}}\)CD11c\(^{\text{high}}\) (Fig. 3F). First, we measured inflammatory cells recruited into the alveolar space of the lung by analyzing the BAL. In normal, noninflamed lungs, these cells are very low in number. However, in the Abcg1\(^{-/-}\) mice at 7 wk of age, we found dramatic increases in populations of CD8\(^{+}\) and CD4\(^{+}\) T cells, B cells, eosinophils, neutrophils, and dendritic cells in the BAL fluid (Fig. 3G).

Next, we examined whether more inflammatory cells resided in the lung tissue as well. In contrast to the alveolar space, there were resident neutrophils, eosinophils, dendritic cells, T cells, and B cells in the tissue of noninflamed lungs (data not shown). Inflammatory cells were identified in the digested lung tissue of 7-wk-old B6 and Abcg1\(^{-/-}\) mice using the same cell-surface markers mentioned above. Abcg1\(^{-/-}\) mice had a 1.3-fold increase in CD8\(^{+}\) T cells (Abcg1\(^{-/-}\) vs B6 1.46 \( \pm 0.07 \times 10^{5} \) vs B6 1.06 \( \pm 0.11 \times 10^{5} \) cells), a 1.5-fold increase in CD4\(^{+}\) T cells (Abcg1\(^{-/-}\) vs B6 2.13 \( \pm 0.14 \times 10^{5} \) vs B6 1.44 \( \pm 0.14 \times 10^{5} \) cells), a 1.7-fold increase in B cells (Abcg1\(^{-/-}\) vs B6 7.89 \( \pm 1.1 \times 10^{5} \) vs B6 4.59 \( \pm 0.54 \times 10^{5} \) cells), a 2.1-fold increase in neutrophils (Abcg1\(^{-/-}\) vs B6 3.56 \( \pm 0.11 \times 10^{5} \) vs B6 1.63 \( \pm 0.29 \times 10^{5} \) cells), and a 1.7-fold increase in eosinophils (Abcg1\(^{-/-}\) vs B6 4.59 \( \pm 0.54 \times 10^{5} \) vs B6 2.67 \( \pm 0.29 \times 10^{5} \) cells).
(Abcg1−/− 3.26 ± 0.59 × 10^4 vs B6 0.26 ± 0.15 × 10^4 cells), and a 2.6-fold increase in dendritic cells (Abcg1−/− 4.24 ± 0.58 × 10^5 vs B6 1.63 ± 0.41 × 10^5 cells) compared with B6 mice. Thus, there is a dramatic infiltration of inflammatory cells to both the alveolar space and lung tissue of young Abcg1−/− mice before onset of severe lipidosis.

Abcg1−/− mice show no change in macrophage content of lungs

We next investigated the alveolar and tissue macrophage content in the lungs of 7-wk-old B6 and Abcg1−/− mice using flow cytometry. We used both forward vs side scatter and 7AAD staining to gate on live cells (Fig. 4A). Macrophages were defined as Siglec-F^high^CD11c^high^ (Fig. 4B). Surprisingly, in contrast to other inflammatory cell populations, there were no significant differences in the total numbers of alveolar macrophages or tissue macrophages between the two mouse groups (see Fig. 4C). However, there was a striking difference in the appearance of the macrophages when evaluating forward vs side scatter. Both tissue and alveolar Abcg1−/− macrophages had a higher degree of side scatter, which is indicative of granularity (Fig. 4B). Lipid accumulation in macrophages would make them appear more granular, as seen in Fig. 4B. Indeed, we found that alveolar macrophages from Abcg1−/− mice were larger in size and had severe lipid accumulation, whereas the B6 alveolar macrophages were lipid-free (Fig. 4D).

ABC1 expression in macrophages regulates development of pulmonary lipidosis and inflammation

To determine the role of macrophages in causing pulmonary lipidosis in this mouse model, we performed bone marrow transplantations using B6 and Abcg1−/− bone marrow. After 9 wk of reconstitution, lungs from mice were harvested to examine inflammation and onset of pulmonary lipidosis. Surprisingly, we found that bone marrow-derived cells appeared to be solely responsible for onset of lipidosis (Fig. 5). Moreover, the presence of ABCG1 on bone marrow-derived cells prevented onset of lipidosis in Abcg1−/− mice (Fig. 5B), while absence of ABCG1 expression in bone marrow-derived cells caused onset of lipidosis in wild-type B6 mice (Fig. 5C). Because ABCG1 is highly expressed on macrophages and plays a significant role in reverse cholesterol transport (22), we anticipate the macrophage to be the bone marrow-derived cell causing pulmonary disease. Thus, these data strongly suggest that the macrophage, and not hematopoietic cells in the lung, appears to be the primary cell type involved in onset of pulmonary lipidosis in Abcg1−/− mice.

We next examined inflammatory cytokine levels in the BAL fluid of the bone marrow-transplanted mice, and noted some interesting findings. First, B6 mice reconstituted with Abcg1−/− bone marrow had significantly higher levels of cytokines compared with B6 mice reconstituted with B6 bone marrow (Fig. 6). Furthermore, Abcg1−/− mice reconstituted with B6 bone marrow had significantly lower levels of IL-12p40 and KC, with no detectable TNF-α production, compared with Abcg1−/− mice reconstituted with Abcg1−/− bone marrow (Fig. 6). We unexpectedly found that transplantation of Abcg1−/− bone marrow into B6 mice increased cytokine levels more strongly than did transplantation of Abcg1−/− bone marrow into Abcg1−/− mice. We are unsure as to why this occurs; however, one likely possibility is that the Abcg1−/− recipient mice have adapted to their global loss of ABCG1 and are somewhat sensitized to the chronic cytokine elevation that occurs when Abcg1−/− marrow is introduced. The B6 control recipients have not, of course, encountered the chronic cytokine elevation that occurs in the global ABCG1-deficient mouse, so the nonhematopoietic cells in the B6 recipient mice may have a heightened inflammatory response after transplantation with Abcg1−/− bone marrow. Overall, inflammatory cytokine production in the lung was drastically higher in mice with macrophages lacking ABCG1 expression, again supporting the notion that ABCG1 expression in macrophages regulates lung inflammation.

We also found that macrophage accumulation in the BAL was significantly lower in B6 mice receiving Abcg1−/− bone marrow, but we found a significant increase in alveolar macrophage accumulation in Abcg1−/− mice reconstituted with B6 bone marrow (Fig. 7A). There was no significant difference in the number of macrophages in B6 mice reconstituted with B6 marrow and Abcg1−/− mice reconstituted with Abcg1−/− marrow (Fig. 7E). The results of our bone marrow transplantation studies suggest that both the environment of the lung and macrophage ABCG1 expression affect accumulation of macrophages in the lung.
The absence of ABCG1 in bone marrow-derived cells also significantly contributed to the accumulation of neutrophils, eosinophils, and dendritic cells in the alveolar space (Fig. 7, B–E). Both B6 and Abcg1−/− recipient mice reconstituted with B6 bone marrow had few neutrophils (Fig. 7B) and eosinophils (Fig. 7C) in the alveolar space, as expected in mice with noninflamed lungs. However, both B6 and Abcg1−/− recipient mice reconstituted with Abcg1−/− bone marrow had significant infiltration of neutrophils (Fig. 7B) and eosinophils (Fig. 7C) into the alveolar space. There was also approximately a 2-fold increase in the number of dendritic cells in the alveolar space of B6 mice reconstituted with Abcg1−/− bone marrow compared with B6 mice reconstituted with B6 bone marrow (Fig. 7D). We also observed a trend toward a reduction in dendritic cell infiltration when Abcg1−/− mice were reconstituted with B6 bone marrow (Fig. 7D). Thus, these results demonstrate that the absence of ABCG1 in bone marrow-derived cells leads to inflammation and recruitment of inflammatory cells into the alveolar space.

**Increased monocyte trafficking to the lungs of Abcg1−/− mice**

We were struck by the fact that there appeared to be no change in the number of macrophages in the lungs of Abcg1−/− mice, yet the macrophages appeared lipid-filled and appeared to be of primary importance in disease onset in the Abcg1−/− mice. These findings suggest that there is either reduced trafficking of macrophages to the lung in Abcg1−/− mice or increased macrophage apoptosis occurring in the lungs of Abcg1−/− mice. To examine monocyte trafficking, we first quantified the number of monocytes in the blood of 7-wk-old B6 and Abcg1−/− mice. Monocytes were identified as CD11bhighCD115highCD11bhigh cells by flow cytometry (Fig. 8A). Surprisingly, Abcg1−/− mice had a 2-fold increase in the number of circulating monocytes compared with B6 mice (Fig. 8B). This result suggests that inflammation in the lung caused increased circulating monocytes in Abcg1−/− mice. We next used mice expressing GFP in monocytes to measure the number of newly recruited monocytes to the lung. Cx3cr1GFP/− mice are heterozygous mice that express GFP only in Cx3cr1-expressing cells (29). Specifically, monocytes, dendritic cells, and NK cells are Cx3cr1high and have been shown to traffic normally in heterozygous Cx3cr1GFP/− mice (29). For these experiments, we injected bone marrow from Cx3cr1GFP/− mice on a B6 background into 7-wk-old B6 and Abcg1−/− recipient mice. After 48 h, the lungs and spleen were analyzed for the presence of newly recruited monocytes and newly differentiated macrophages. The spleen was used as a control to determine whether the monocyte recruitment was specific to the lung. Newly recruited monocytes were identified as GFPhigh CD11bhighNK1.1high and newly differentiated macrophages were identified as GFPhighCD11bhighNK1.1highSiglec-FhighCD11chigh (Fig. 8C). Abcg1−/− mice had a 3.4-fold increase in the number of newly recruited monocytes to the lung (Fig. 8D). Furthermore, there was a 45-fold increase in the number of newly differentiated macrophages in the lungs of Abcg1−/− mice (Fig. 8D). However, there was no change in the number of monocytes recruited to the spleen (Fig. 8D). The results from this experiment demonstrate increased monocyte recruitment and increased macrophage differentiation to the lungs in Abcg1−/− mice.
phages were isolated from the alveolar space of 7-wk-old B6 and Abcg1−/− mice, incubated with fluorescently-labeled apoptotic Jurkat cells, and measured by flow cytometry for engulfment of apoptotic Jurkat cells. Abcg1−/− alveolar macrophages had a 2-fold increase in the percentage of macrophages that engulfed apoptotic cells compared with B6 macrophages (Fig. 10A). Moreover, we found that Abcg1−/− macrophages were activated, having increased expression of the activation markers CD11b and MHC class II (Fig. 10B). Taken together, our studies indicate that Abcg1−/− macrophages are activated and show increased ability to engulf apoptotic cells. However, these macrophages also become apoptotic, and subsequently necrotic, most likely due to increased lipid accumulation in response to lack of ABCG1. These dying macrophages appear to be quickly repopulated in the Abcg1−/− lung by newly recruited blood monocytes, as we observed in our monocyte trafficking studies.

Discussion

In this study, we have demonstrated that ABCG1 expression in macrophages plays a critical role in pulmonary inflammation and lipidosis. Abcg1−/− mice had increased proinflammatory cytokine levels in their lungs, leading to the recruitment of neutrophils, eosinophils, B cells, T cells, and dendritic cells (Figs. 2 and 3). Furthermore, we show for the first time that the absence of ABCG1 expression in the macrophages was responsible for the lung inflammation and lipid accumulation (Figs. 5–7). Our results reveal a novel role for ABCG1 in regulating macrophage inflammation and lung homeostasis.

Our most exciting finding was the role of the macrophage in the development of pulmonary disease. We performed bone marrow transplant studies to look at the contribution of bone marrow-derived cells vs non-bone marrow-derived cells to the development of pulmonary disease. We anticipated that the macrophage is the bone marrow-derived cell responsible for pulmonary disease, because macrophages both initiate the immune response in the lung and are involved in reverse cholesterol transport (3, 7, 22). We showed that both B6 and Abcg1−/− mice lacking ABCG1 expression in macrophages accumulated lipid in their lungs (Fig. 5) and had massive lung inflammation (Figs. 6 and 7). Moreover, the presence of ABCG1 on macrophages in Abcg1−/− mice was able to rescue both the lipidosis (Fig. 5) and the inflammatory phenotype (Figs. 6 and 7). Therefore, we rescued the pulmonary disease in Abcg1−/− mice by reconstituting mice with wild-type B6 bone marrow, thereby restoring ABCG1 expression in macrophages (Figs. 6 and 7). One question that remains is which process occurs first, macrophage lipid accumulation or inflammation. It will be of interest to determine whether the accumulation of lipid inside Abcg1−/− macrophages causes the macrophage to become inflamed, or whether the absence of ABCG1 in macrophages triggers inflammation, leading to the excess production of surfactant and pulmonary lipidosis. This question will be addressed in future studies.

The lipid phenotype in the lungs of Abcg1−/− mice has been described by others and shown to resemble various respiratory syndromes. Abcg1−/− lungs at >6 mo of age displayed cellular accumulation, macrophage and pneumocyte type II cell hypertrophy, increased levels of surfactant, and massive lipid accumulation (24). There are several pulmonary diseases that are associated with a disruption in lipid homeostasis and inflammation, including alveolar proteinosis, respiratory distress syndrome, and Niemann-Pick disease (30–32). Niemann-Pick disease is a lipid storage disease that results in lipid accumulation in various organs, such as spleen, liver, lung, and brain, similar to that observed in Abcg1−/− mice. Progressive lung disease is one manifestation of the disease.
characterized by the presence of “foamy” macrophages leading to chronic inflammation (33). Pulmonary alveolar proteinosis is a disease that is also similar to the lung disease found in Abcg1−/− mice. Patients with pulmonary alveolar proteinosis are deficient in the macrophage growth factor GM-CSF, due to the presence of circulating GM-CSF-neutralizing Abs (34). Pulmonary alveolar proteinosis is characterized by impaired surfactant clearance, lipid accumulation in alveolar macrophages, and increased cholesterol metabolites in the lung (35, 36). Recently, patients and mice with pulmonary alveolar proteinosis have been shown to lack ABCG1 in alveolar macrophages (37), due to the absence of GM-CSF signaling, thus illustrating a critical link between ABCG1 and lung homeostasis.

ABCG1 has been suggested to play a critical role in maintaining lipid homeostasis in a variety of organs and is highly expressed in the ileum, liver, lung, spleen, and kidney (17). Abcg1−/− mice accumulate lipid in their liver and lungs, but not the spleen, kidney, or ileum, when fed a high-fat/high-cholesterol diet (17). Surprisingly, Abcg1−/− mice have reduced adipose cell size, and the mice are protected against diet-induced obesity, suggesting a role for ABCG1 in regulating energy balance (38). Furthermore, ABCG1, along with ABCG4, has been demonstrated to regulate cholesterol transport in the brain (39). Edwards and colleagues have shown that ABCG1 and ABCG4 mediate intracellular vesicular transport of sterols within both neurons and astrocytes (39). Thus, there is still much to be revealed about the function of ABCG1 in these organs.

In our studies, a surprising observation was that there was no change in macrophage content in Abcg1−/− mice, even though the lungs were noticeably inflamed and accumulating lipid (Fig. 4). We hypothesized that more macrophages would be present in the lungs of Abcg1−/− mice to engulf apoptotic cells and clear surfactant lipids. In the bone marrow transplant studies, we found that B6 mice given Abcg1−/− bone marrow had fewer macrophages in their lungs, despite the increased inflammation and lipid accumulation (Fig. 7). These data suggest that Abcg1−/− monocytes are not properly trafficking to the lung and differentiating into macrophages. Further studies are needed to determine how the absence of ABCG1 in monocytes affects their ability to traffic to inflamed tissue.

Our data clearly demonstrate that macrophages contribute to pulmonary disease, the mechanism by which, to some extent, is explained after measuring macrophage inflammation, apoptosis, and apoptotic cell clearance. Abcg1−/− alveolar macrophages are clearly more activated, having increased production of proinflammatory cytokines and decreased IL-10 production (Fig. 2). Our studies also show that Abcg1−/− alveolar macrophages are more prone to apoptosis (Fig. 9). These data are in concordance with what was previously published by Edwards and colleagues, who showed that Abcg1−/− peritoneal macrophages were more apoptotic (40). Oxyesters that are formed within the macrophages as a consequence of cholesterol accumulation may cause the macrophages to be more prone to apoptosis (41). When free cholesterol accumulates in the macrophage, the ratio of free cholesterol to phospholipids is disturbed in the endoplasmic reticulum (ER) membrane (42). The “stiffening” of the ER membrane bilayer leads to ER protein dysfunction, ER stress, and the unfolded protein response, resulting in apoptosis of the cell (42). Furthermore, ABCG1 has been shown to protect against 7-ketocholesterol- and 7β-hydroxycholesterol-induced cell death by effluxing these oxyesters from macrophages to prevent oxyester-induced cytotoxicity (43, 44). Therefore, one possibility is that Abcg1−/− alveolar macrophages are more prone to apoptosis due to the toxic effects of lipid accumulation. Another explanation is that Abcg1−/− macrophages engulf many apoptotic cells (Fig. 10), yet cannot properly efflux the cholesterol derived from these apoptotic cells, despite up-regulation of ABCA1 (24). Furthermore, the apoptosis of a cell is considered an anti-inflammatory process, but necrosis is proinflammatory. We measured increases in both apoptosis and necrosis by Abcg1−/− alveolar macrophages (Fig. 9). There may be different factors leading to the apoptotic and necrotic pathways in Abcg1−/− macrophages, which could contribute to or dampen pulmonary inflammation.

The effect of macrophage ABCG1 expression on atherosclerotic lesions is highly controversial. Because ABCG1 is important in macrophage reverse cholesterol transport and inflammation, we would expect that the absence of ABCG1 would increase atherosclerosis development. However, two groups have found a slight decrease in atherosclerotic lesion size in mice lacking ABCG1 in bone marrow-derived cells (40, 45). Yet, Van Eck and colleagues have shown that both macrophage-specific deletion of ABCG1 and total body deletion of ABCG1 moderately increase atherosclerotic lesion size (46, 47). Thus, despite the pulmonary inflammation and macrophage activation observed in Abcg1−/− mice, the role of macrophage ABCG1 in atherosclerosis is not clear. One hypothesis is that the increased engulfment of apoptotic cells by Abcg1−/− macrophages may be important in the aortic wall for atherosclerosis (48). Another possible explanation is that only organs containing an abundance of macrophages, such as the lung, spleen, and liver, are affected by the lack of ABCG1. The environment of the lung in particular appears to be easily disrupted by changes in ABCG1 expression. This could be due to increased surfactant lipid or the abundance of resident inflammatory cells in the lung tissue that can quickly respond to inflammation. All factors considered, it is still puzzling that the lack of ABCG1 in macrophages does not significantly increase inflammation in the aortic wall and in turn affect atherosclerotic lesion development.

In conclusion, we propose that ABCG1 plays a critical role in maintaining lung homeostasis. We show that ABCG1 deletion in macrophages causes a striking inflammatory phenotype and contributes to onset of pulmonary lipidosis in mice. These studies suggest a novel role for ABCG1 in inflammation; understanding this role may be important for developing therapeutic approaches for lipid and inflammatory diseases.

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References


