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ABCG1 Is Required for Pulmonary B-1 B Cell and Natural Antibody Homeostasis

Angel Baldan,*‡ Ayelit Gonen,† Christina Choung,† Xuchu Que,† Tyler J. Marquart,† Irene Hernandez,§⅓ Ingemar Bjorkhem,‖ David A. Ford,† Joseph L. Witztum,‡ and Elizabeth J. Tarling*

Many metabolic diseases, including atherosclerosis, type 2 diabetes, pulmonary alveolar proteinosis, and obesity, have a chronic inflammatory component involving both innate and adaptive immunity. Mice lacking the ATP-binding cassette transporter G1 (ABCG1) develop chronic inflammation in the lungs, which is associated with the lipid accumulation (cholesterol, cholesterol ester, and phospholipid) and cholesterol crystal deposition that are characteristic of atherosclerotic lesions and pulmonary alveolar proteinosis. In this article, we demonstrate that specific lipids, likely oxidized phospholipids and/or sterols, elicit a lung-specific immune response in Abcg1−/− mice. Loss of ABCG1 results in increased levels of specific oxysterols, phosphatidylcholines, and oxidized phospholipids, including 1-palmitoyl-2-(5'-oxovaleroyl)-sn-glycero-3-phosphocholine, in the lungs. Further, we identify a niche-specific increase in natural Ab (NAb)-secreting B-1 B cells in response to this lipid accumulation that is paralleled by increased titers of IgM, IgA, and IgG against oxidation-specific epitopes, such as those on oxidized low-density lipoprotein and malondialdehyde-modified low-density lipoprotein. Finally, we identify a cytokine/chemokine signature that is reflective of increased B cell activation, Ab secretion, and homing. Collectively, these data demonstrate that the accumulation of lipids in Abcg1−/− mice induces the specific expansion and localization of B-1 B cells, which secrete NABs that may help to protect against the development of atherosclerosis. Indeed, despite chronic lipid accumulation and inflammation, hyperlipidemic mice lacking ABCG1 develop smaller atherosclerotic lesions compared with controls. These data also suggest that Abcg1−/− mice may represent a new model in which to study the protective functions of B-1 B cells/NABs and suggest novel targets for pharmacologic intervention and treatment of disease. The Journal of Immunology, 2014, 193: 000–000.

B-2 B cells are hallmark effectors of the adaptive immune response and are characterized by their production of specific Abs (1–4). However, not all Ab production is triggered by a prior exposure and immune response. B-1 B cells, the innate-immune component of the B cell lineage, are the source of natural Abs (NABs) that are produced in the absence of exposure to an Ag (2, 5). B-1 B cells are primarily localized to the peritoneal cavity (PerC) and pleural space (5, 6). B-1 B cells were shown to affect the progression of multiple autoimmune diseases, human B cell leukemias, and inflammatory disorders, such as atherosclerosis (7–9). Despite the importance of B cell homeostasis in human disease, factors that regulate B cell movement into specific compartments are not well understood.

The maintenance of tissue homeostasis and defense against mucosal pathogens is highly dependent on B-1 B cells and NAB production due to a BCR repertoire that is enriched for cross-reactive receptors that bind to both self and nonself (e.g., microbial) Ags (9–11). This selection for self-reactivity is restricted to B-1 B cells and yet contradicts the current paradigm of lymphocyte selection wherein self-reactive cells undergo deletion to avoid autoimmunity. The presence of such potentially autoreactive B cells dictates the requirement for tightly regulated mechanisms to control their activation. Increasing evidence suggests that loss of cellular lipid homeostasis plays an essential role in regulating lymphocyte and hematopoietic cell proliferation, lymphocyte movement within the follicular regions of the spleen, and immune responses (12–17).

Cellular cholesterol homeostasis is influenced by a number of proteins, including the sterol ATP-binding cassette transporter G1 (ABCG1) (18–21). Expression of ABCG1 in intracellular vesicles stimulates activity of the transcription factor SREBP-2 via the redistribution of sterols out of the endoplasmic reticulum (20, 22) and eventually results in the removal of cholesterol from the cell to extracellular acceptors, such as high-density lipoprotein (18, 23–25). Analysis of the alveolar macrophages and/or brains
of mice lacking ABCG1 demonstrated that loss of ABCG1 was associated with a marked accumulation of free and esterified cholesterol, as well as 24-, 25-, and/or 27-hydroxycholesterols, which are synthesized enzymatically by Cyp24a1, Ch25h, and Cyp27a1, respectively (26–28). In addition, the levels of 7-ketocholesterol, a product of nonenzymatic autodissociation of cholesterol, are increased in both peritoneal macrophages and the aortas of Abcg1−/− mice (29, 30). Despite recent progress in understanding how lipid homeostasis impacts lymphocyte function, little is known about how lipid metabolism impacts B cell–specific responses.

In this article, we demonstrate that loss of ABCG1 results in the accumulation of specific oxidized sterols and phospholipids, eliciting a lung-specific immune response. We show a niche-specific accumulation of B-1 B cells in the pleural cavity and lungs of Abcg1−/− mice, which is accompanied by increased IgM, IgA, and IgG titers to oxidized lipid epitopes in both plasma and whole lung. Additionally, macrophage oxytetracycline production drives homing of B-1 B cells specifically to the lungs and pleural cavity. Our data suggest that ABCG1-dependent control of intracellular lipid homeostasis represents a previously unrecognized mechanism for the regulation of B-1 B cell movement and homing.

Materials and Methods

Animals

All animals were bred and maintained at the University of California Los Angeles in temperature-controlled, pathogen-free conditions under a 12-h light/dark cycle. Abcg1−/− LacZ knock-in mice (Deltagen) (18, 31) were backcrossed ≥10 times onto a C57BL/6 background. Control C57BL/6 mice (originally purchased from The Jackson Laboratory) were generated from C57BL/6 × 129/SvJ crosses. Mice were fed a chow diet or a Western diet (Research Diets #D12079B, containing 21% fat and 0.2% cholesterol), as indicated. Mice expressing the GFP C57BL/6-Tg(CAG-EGFP)1Osb/J were purchased from the Jackson Laboratory (strain #003291). The Institutional Animal Care and Research Advisory Committee at the University of California Los Angeles approved all experimental protocols.

Adoptive transfer

Cells were isolated with Ab-tagged magnetic beads and AutoMACS (Miltenyi Biotec). Peritoneal CD19+CD23+ B-1 B cells were isolated from C57BL/6-Tg(CAG-EGFP)1Osb/J mice by negative selection on a CD23+ column, followed by positive selection of CD19+ cells. Cell purity (≥98%) was confirmed by FACS analysis using fluorochrome-labeled CD19, CD23, and CD5 Abs (eBioscience). Cell viability (>97%) was assessed by trypan blue exclusion. To obtain 10 × 10^6 B-1 B cells, a pool of peritoneal cells from 20 donor mice was used, and 1 × 10^6 B-1 B cells were adoptively transferred into 6- to 8-wk-old chow-fed wild-type and Abcg1−/− mice.

Surfactant isolation

Pulmonary surfactant was isolated from 6- to 8-wk-old wild-type and Abcg1−/− mice by bronchoalveolar lavage, as previously described (31). Briefly, tracheas were exposed and cannulated before the lungs were flushed three times with 1-ml aliquots of bronchoalveolar lavage buffer (10 mmol/l Tris, 100 mmol/l NaCl, 0.2 mmol/l EDTA [pH 7.2]). The aliquots were combined and centrifuged (200 × g, 5 min) to separate surfactant and cells.

Lipid analyses

Cells, bronchoalveolar lavage fluid, or lung tissue was snap-frozen in liquid nitrogen. Lung tissue was homogenized on ice in PBS. Cell suspensions, bronchoalveolar lavage fluid, or lung homogenates were subsequently subjected to a modified Bligh-Dyer lipid extraction (32) in the presence of lipid class internal standards, including eicosanoic acid, 1-0-heptadecanoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphocholine, and 1,2-ditetradecanoyl-sn-glycero-3-phosphoethanolamine (33). Fatty acids were converted to their pentfluorobenzyl esters and subsequently quantified using gas chromatography–mass spectrometry (GC-MS) with negative-ion chemical ionization using methane as the reactant gas (34). For phospholipids, lipid extracts were diluted in methanol/chloroform (4/1, v/v), and molecular species were quantified by electrospray ionization–tandem mass spectrometry (ESI-MS/MS) on a triple quadrupole instrument (Thermo Fisher Quantum Ultra) using shotgun lipidomics methodologies (35). Phosphatidylcholine molecular species were quantified as lithiated adducts in the positive-ion mode using neutral loss scanning for 59.1 amu (collision energy = −28 eV). Individual molecular species were quantified by comparing the ion intensities of the individual molecular species to that of the lipid class internal standard, with additional corrections for type I and type II [13C] isotope effects (35).

Flow cytometry

Single-cell suspensions from lungs and spleen were depleted of RBCs using hypotonic lysis. Cells were resuspended in PBS with 0.2% BSA and 0.1% sodium azide (FACS buffer). Single-cell suspensions were incubated for 15 min with anti-CD16/32 (Fc block) and stained for 30 min at 4°C. DAPI, anti-mouse CD3 (17A2), CD19 (1D3), CD11b (M1/70), CD5 (53-7.3), and IgM (II/41) were purchased from eBioscience. Cells were analyzed on an LSR II (Becton Dickinson). More than 0.5 × 10^7 cells were analyzed per sample, with dead cells excluded by DAPI staining. Surface marker analysis was performed using FlowJo software (TreeStar). B cells (CD19+), B-1 B cells (CD19+, IgM+, CD11b−), B-1a B cells (CD19+, IgM+, CD11b+, CD5−), and B-1b B cells (CD19+, IgM+, CD11b−, CD5+) were identified with the appropriate gating. Gating strategy and controls are shown in Fig. 1.

RNA isolation and analysis

Total RNA was isolated from 50 mg lung tissue (tissue weight determined after blotting of excess buffer) using QIAzol (QIAGEN). Gene expression profiling of inflammatory markers was determined using the GEArray mouse inflammatory cytokines and receptors microarray system (SA Biosciences), as previously described (36). Total RNA (0.5 μg) was reverse transcribed with random hexamers using the TaqMan Reverse Transcription Reagents Kit (Applied Biosystems). TaqMan quantitative real-time PCR (RT-qPCR) assays were performed using an Applied Biosystems 7000HT sequence detector, and RT-qPCR assays were performed on a LightCycler 480 (Roche). Results are the averages of triplicate experiments normalized to GAPDH (TaqMan) or 36B4 (RT-qPCR). Primer sequences are available on request.

Measurement of Ab titers

Total and specific Ab titers were determined by chemiluminescent enzyme immunoassays, as previously described (37). In brief, capture Abs were coated on plates at 5 μg/ml in PBS overnight at 4°C (AB-12, copper oxidized low-density lipoprotein [Cu-OxLDL], IgM [goat anti-mouse IgM; Sigma-Aldrich], IgG [goat anti-mouse IgG; Sigma-Aldrich], IgA [rat anti-mouse IgA; Sigma-Aldrich], and malondialdehyde-modified low-density lipoprotein [MDA-LDL]). Plates were blocked with 1% BSA in TBS, and serially diluted antisera from individual mice was added. Plates were incubated for 1.5 h at room temperature. Bound plasma Ig isotype levels were assessed using various anti-mouse Ig isotype-specific alkaline phosphatase (AP) conjugates using Lumiphos 530 (Lumigen, Southfield, MI) solution and a Dynex luminometer (Dynex Technologies, Chantilly, VA). Several secondary Abs were used at dilutions of 1:3,000. These included AP-labeled goat anti-mouse IgM (μ-chain specific), goat anti-mouse IgG (γ-chain specific), and goat anti-mouse IgA (α-chain specific; all from Sigma-Aldrich). Specific controls were used for each specific Ab, and formal Ab-dilution curves were determined in an initial study to identify the linearity range of each Ab. The total and specific Ab titers of each dilution curve that plasma samples could be optimally measured at 1:100 dilutions and lung samples could be optimally measured at 1:10 dilutions to yield concentrations within the linear detection range for each assay.

Where indicated, Abs were extracted from lung tissue using a method modified from Yiai-Hertuala et al. (38). Lungs were perfused extensively with physiological saline to ensure removal of all blood prior to analysis. Briefly, a small piece of lung (50 mg) was homogenized (Polytron homogenizer), on ice, in 0.5 ml PBS [pH 7.2] containing the following preservatives: 2.7 mmol/l EDTA, 2 mmol/l benzamidine, 1 mmol/l PMSF, 40 μmol/l elastatin, 10 μmol/l procubulin, 0.01% aprotinin, 0.008% chloramphenicol, 0.008% gentamicin, and Protease Inhibitor Cocktail (1:100; Sigma-Aldrich PS830). Samples were incubated overnight at 4°C. The extract was collected by low-speed centrifugation at 4°C (30 min at 3000 rpm) and recentrifuged for 15 min at 4000 rpm at 4°C prior to assay.

Protein-lipid overlay analysis

Total-lung lipids were isolated by Folch extraction, as previously described (31), from 25 mg tissue. Total-lung lipid extracts were spotted onto nitrocellulose membrane and allowed to dry. Membranes were either
incubated in the presence of total-lung protein extracts (15 mg tissue) or E06-IgM Ab (1:500) for 16 h at 4°C. Extracts containing Abs were removed by extensive washing in PBS, and cross-reactivity was detected using HRP-conjugated anti-mouse IgM (Invitrogen).

**Immunohistochemistry**

Frozen-embedded sections (lungs) from wild-type and *Abcg1*−/− mice were fixed in 4% paraformaldehyde, blocked with 5% goat serum, stained with HRP-conjugated anti-mouse IgM, IgG, or IgA, and detected with ECL. A VECTASTAIN ABC-AP Kit (Vector Laboratories) was used to visualize Ab staining. Where indicated, slides were counterstained with Harris Hematoxylin (Fisher Scientific). Frozen tissue sections of lungs from wild-type and *Abcg1*−/− mice also were stained with Abs that recognize CXCL13 (GeneTex), oxidized phospholipid (OxPL; E06), B220 (B cell marker; clone RA3-6B2; BD Biosciences), or proliferating cell nuclear Ag (PCNA; proliferative marker; GeneTex), followed by anti-mouse IgM Alexa Fluor 488, anti-rat Alexa Fluor 594, or anti-rabbit Alexa Fluor 488 (PCNA; proliferative marker; GeneTex), and secondary Abs (Molecular Probes, Life Sciences). Immunostaining of adjacent sections in the absence of primary Ab was used as a negative control.

**TUNEL staining**

The presence of apoptotic cells was assessed by TUNEL assay of frozen-embedded tissue sections or primary alveolar macrophages, as previously described (39).

**Statistics**

Lipid parameters (cholesterol, oxysterols, phosphatidylcholine, OxPLs) were analyzed by two-way ANOVA, with genotype as one factor and lipid species as another. Where there was an effect of either genotype or lipid species with no apparent interaction, absolute cell numbers (determined using flow cytometry) were analyzed by the unpaired Student *t* test. Ab titers were analyzed by two-way ANOVA, with genotype as one factor and Ag (MDA-LDL, Cu-OxLDL, E06/T15) as another. If there was an effect of either genotype or lipid species with no apparent interaction, data were analyzed further by a post hoc Bonferroni test to determine differential effects. Absolute cell numbers (determined using flow cytometry) were analyzed by the unpaired Student *t* test.

**Results**

**ABCG1 regulates pulmonary B cell homeostasis**

To investigate the role of ABCG1 in B cell homeostasis and innate immunity, we examined specific immunological properties of 6-mo-old *Abcg1*−/− mice. Flow cytometric analysis did not reveal any significant difference in the number of B cells (Supplemental Fig. 1A) or T cells (Supplemental Fig. 1B) recovered from the spleens of wild-type and *Abcg1*−/− mice. We previously observed gross lymphocytic infiltrates consisting predominantly of B cells in the lungs of *Abcg1*−/− mice (36). Consistent with these observations, FACS analysis revealed a significant increase in the B cell population (defined as CD19+) in the lungs of chow-fed *Abcg1*−/− mice (Figs. 1, 2A). In contrast, there was no significant difference in the number of T cells (defined as CD3+) in the lung (Fig. 2B).

B cells can be subdivided into B-1 and B-2 B cells. B-1 B cells are primarily localized to the PerC and pleural space, whereas B-2 cells are localized to the spleen (1, 4). FACS analysis of cells recovered from the lung or pleural cavity demonstrated that B-1 B cells (CD19+, IgM+, CD11b+) were increased in *Abcg1*−/− mice compared with wild-type mice (Fig. 2C, 2D). In contrast, there was no difference in the number of B-1 B cells present in the spleen or PerC (Fig. 2E). Taken together, these data support the conclusion that a global loss of ABCG1 results in a niche-specific increase in B-1 B cells in the lungs and pleural space. Interestingly, there also was a marked increase in the number of conventional B-2 cells in the lungs, but not the spleens, of *Abcg1*−/− mice (Fig. 2F). Because B-2 and B-1 cells are generally considered mediators of adaptive immunity (40) and innate immunity (6, 41), respectively, these data demonstrate that loss of ABCG1 has a broad impact on pulmonary B cell biology in mice.

Innate B-1 B cells can be divided into B-1a cells (slgM+, CD11b+, CD5+) and B-1b cells (slgM+, CD11b+, CD5−). In the absence of infection, B-1a cells are the source of most serum IgM, are present in serous cavities, and are known to generate NAbS that, for example, specifically bind to phosphocholine (PC)-containing OxPLs (42). FACS analysis of cells recovered from the pleural cavity and lungs of wild-type and *Abcg1*−/− mice shows that there is a significant increase in B-1a cells recovered from *Abcg1*−/− mice (Fig. 2G, 2H). In contrast to B1-a cells, B-1b cells are thought to be the primary source of T cell–independent Ab production, and they provide delayed, but long-term, protection against infectious pathogens (1). The finding that B-1b cells also are increased in both the lungs and pleural cavities of *Abcg1*−/− mice compared with wild-type mice (Fig. 2I) provides further support for the proposal that loss of ABCG1 affects multiple arcs of immunity. These effects are niche/lung-specific because there was no significant difference in the numbers of B-1a cells (Supplemental Fig. 1C) or B-1b cells (Supplemental Fig. 1D) in the spleen or PerC of wild-type and *Abcg1*−/− mice.

![FIGURE 1.](#) Gating strategy for the isolation of B-1 B cells. (A) Flow cytometry gating strategy to identify B cell subsets in lung, PerC, pleural cavity, and spleen. Single-cell suspensions were stained with different fluorophore-conjugated Abs and analyzed by flow cytometry. Among single cells, the live cells were selected for further analysis to identify B cells (CD19+), B-1 B cells (CD19+IgM−CD11b+), B-1a B cells (CD19+IgM−CD11b−CD5+), and B-1b B cells (CD19+IgM+CD11b+CD5−). (B) Isotype controls for lung B-1 B cells. Single-cell suspensions were stained with Ag-specific fluorophore-conjugated Abs and control fluorophore-conjugated Abs and analyzed by flow cytometry.

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Lipid-driven expansion of B-1 B cells in the lungs of Abcg1−/− mice

To test the hypothesis that the lung-specific expansion of B cells is dependent upon lipid accumulation, we analyzed cells isolated from lungs, spleens, PerCs, and pleural cavities of chow-fed 12-wk-old wild-type and Abcg1−/− mice. Importantly, there was little or no lipid accumulation in the lungs of these young Abcg1−/− mice (18). Consistent with our hypothesis, in the absence of pulmonary lipid accumulation, there was no significant difference in the number of B-1 B cells in the tissues of wild-type and Abcg1−/− mice (Fig. 3A). We demonstrated previously that the pulmonary lipidosis in Abcg1−/− mice can be accelerated by feeding mice a Western diet (18). Consequently, 4-wk-old wild-type and Abcg1−/− mice were fed a Western diet for 8 wk to promote lipid deposition. FACS analysis of cells recovered from the pleural cavity and lungs showed a significant increase in the number of B-1 B cells recovered from Abcg1−/− mice, with no change observed in spleen and PerC (Fig. 3B, 3C).

It is possible that there are off-target effects from feeding a Western diet. Further, it is known that Abcg1−/− mice older than 12 wk of age exhibit increasing pulmonary lipidosis on a chow diet (18). Therefore, to test the requirement of pulmonary lipidosis for B-1 B cell expansion in Abcg1−/− mice, we isolated B-1 B cells from 12-wk-old transgenic mice expressing GFP under control of the chicken β-actin promoter. These GFP+ B-1 B cells were injected (1 × 10^6 cells/mouse) i.p. into 6-mo-old chow-fed wild-type and Abcg1−/− mice. Ten weeks after reconstitution, FACS analysis of cells recovered from lung, spleen, pleural cavity, and PerC showed significantly increased numbers of GFP+ cells specifically in the lungs and pleural cavities of Abcg1−/− mice compared with wild-type mice (Fig. 3D, 3E). In contrast, there was no difference in the number of GFP+ cells in either the spleens or PerCs of wild-type and Abcg1−/− mice (Fig. 3E).

Accumulation of oxysterols and OxPLs in the lungs of Abcg1−/− mice

To better define the lipids that accumulate in the lungs of Abcg1−/− mice, we used GC-MS and ESI-MS/MS to analyze lipids extracted under conditions that minimize autoxidation. Fig. 4A–F show for the first time, to our knowledge, that both the lungs and surfactant from 6-mo-old chow-fed Abcg1−/− mice contain significantly increased levels of a number of enzymatically synthesized oxysterols.
suspensions were analyzed for expression of CD19, CD11b, and IgM as markers of B-1 B cells. (The GFP transgene under the control of the chicken β-actin promoter. (Fig. 3) Flow cytometric analysis of GFP+ B-1 B cells recovered from the lungs, spleens, PerC, and pleural cavity of 6-mo-old chow-fed wild-type and Abcg1−/− mice. Single-cell suspensions were analyzed for expression of CD19, CD11b, and IgM as markers of B-1 B cells. (D) Representative flow cytometry graph of GFP+ cells obtained from the pleural cavity of wild-type (WT) and Abcg1−/− (KO) mice injected with GFP+ B-1 B cells from wild-type mice expressing the GFP transgene under the control of the chicken β-actin promoter. (E) Flow cytometric analysis of GFP+ B-1 B cells recovered from the lungs, spleens, PerC, and pleural cavity of 6-mo-old chow-fed wild-type and Abcg1−/− mice. Data are mean absolute cell number ± SEM; n = 4–6 mice/gene/ genotype. ***p < 0.001.

FIGURE 3. Lipid-driven B cell expansion in lungs and pleural cavities of Abcg1−/− mice. Flow cytometric analysis of cells isolated (as in Fig. 1) from the lungs, spleens, PerC, and pleural cavity of 12-wk-old Western diet–fed (B and C) wild-type and Abcg1−/− mice. Single-cell suspensions were analyzed for expression of CD19, CD11b, and IgM as markers of B-1 B cells. (D) Representative flow cytometry graph of GFP+ cells obtained from the pleural cavity of wild-type (WT) and Abcg1−/− (KO) mice injected with GFP+ B-1 B cells from wild-type mice expressing the GFP transgene under the control of the chicken β-actin promoter. (E) Flow cytometric analysis of GFP+ B-1 B cells recovered from the lungs, spleens, PerC, and pleural cavity of 6-mo-old chow-fed wild-type and Abcg1−/− mice. Data are mean absolute cell number ± SEM; n = 4–6 mice/ genotype. ***p < 0.001.

(24-, 25-, and 27-hydroxycholesterol) and autoxidation derivatives of cholesterol (7α-hydroxycholesterol, 7β-hydroxycholesterol, 3,5,6-tri-o-hydroxycholesterol, and 7-ketocholesterol), in addition to total cholesterol. Further, we demonstrate that the lungs of Abcg1−/− mice contain 3–6-fold higher levels of numerous phosphatidylcholine species that differ in their content of fatty acids at the sn-1 and/or sn-2 positions (Fig. 4G). Phosphatidylcholines, especially dipalmitoyl phosphatidylcholine, represent the major phospholipids in the mammalian lung (43, 44). Thus, whole-body loss of ABCG1 leads to a generalized increase in all lung phosphatidylcholines, rather than affecting the metabolism of specific species. Given the increase in autoxidation products of cholesterol in the lungs and surfactant of Abcg1−/− mice (Fig. 4A, 4D), we used ESI-MS/MS to identify PC-containing OxPLs, possible oxidation products of phosphatidylcholine. These analyses identified two OxPLs—1-palmitoyl-2-(5′-oxovaleroyl)sn-glycero-3-PC (POVPC) and 1-palmitoyl-2,9′-oxonanoyl)sn-glycero-3-PC (PoxnoPC)—that were increased 2–15-fold in the lungs of Abcg1−/− mice (Fig. 4H). Interestingly, we reported previously that POVPC and PoxnoPC are able to serve as Ags for B-1 cells (42), a cell type that has a critical role in innate immunity (1, 4).

ABCG1 regulates B-1 B cell homing

Despite chronic inflammation in the lungs of Abcg1−/− mice (18, 36), pathway analysis of inflammatory gene expression identified a signature of chemokines and cytokines, present in Abcg1−/− mice compared with wild-type animals, which is reflective of B cell activation and homing (Table I). Importantly, it is now widely accepted that lipids act as both signaling molecules and mediators produced locally in response to various stimuli (45). Indeed, 25-hydroxycholesterol is a metabolite of cholesterol that is produced and secreted by macrophages and has potent and diverse effects on the immune system (46). Catabolism of 25-hydroxycholesterol by the enzyme CYP7B1 results in the formation of 7α,25-dihydroxycholesterol, a ligand for the G protein-coupled receptor GPR183/EBI2, which is expressed on the surface of B cells (47). 25-hydroxycholesterol levels are significantly increased in the lungs and alveolar macrophages of Abcg1−/− mice, respectively, suggesting that there are enhanced B cell homing signals in the lungs of Abcg1−/− mice (Fig. 4B) (27). Indeed, mRNA levels of the enzymes Ch25h and Cyp7b1 are significantly increased in the lungs of Abcg1−/− mice (Fig. 5A).

Naïve B cells expressing the CXCL13 receptor CXCR5 migrate in response to the chemokine CXCL13, and in mice lacking CXCL13 B cells fail to migrate into the PerC and pleural cavity (48). The data in Fig. 5B (left panel) show that there is no change in Cxcl13 mRNA expression in young 12-wk-old mice that lack significant lung lipidosis. In contrast, Cxcl13 mRNA levels were increased in Abcg1−/− mice fed a Western diet for 8 wk to induce lipidosis (Fig. 5B, right panel). Six-month-old chow-fed Abcg1−/− mice also exhibit pulmonary lipidosis (18, 31). Compared with wild-type mice, the lungs of these older, chow-fed Abcg1−/− mice also have elevated levels of Cxcl13 mRNA (Fig. 5C). Additionally, B-1 B cells isolated from the pleural cavities of Abcg1−/− mice expressed significantly increased levels of Gpr183/EBI2 and Cxcr5 mRNA compared with cells from wild-type mice (Supplemental Fig. 2A), which is in agreement with increased homing of B-1 B cells to the lungs and pleural cavity. Finally, immunohistochemical analysis of the lungs of 6-mo-old chow-fed mice demonstrates significantly increased levels of CXCL13 in the lungs of Abcg1−/− mice compared with wild-type animals (Fig. 5D).

Increased Igs in the lungs of Abcg1−/− mice

The data in Figs. 2–5 suggest that the lungs of Abcg1−/− mice may contain increased titers of Abs that function in innate (IgM, IgA, or IgG3 from B-1 cells) and adaptive (IgG from B-2 cells) immunity. Consistent with this hypothesis, immunohistochemical analysis of the lungs and spleens of wild-type and Abcg1−/− mice demonstrates that the lungs, but not spleens, of the knockout mice contain elevated levels of IgM, IgA, and IgG, with the most positive staining observed adjacent to alveolar sacs (Fig. 6A,
We next performed a modification of the protein-lipid overlay technique (49) to determine whether the lungs of Abcg1<sup>−/−</sup> mice contained increased levels of lipid Ags and corresponding Igs. We also noted markedly increased titers of IgA to the classic B-1 cell Ag 1,3-dextran in the lungs of Abcg1<sup>−/−</sup> mice. The latter finding suggests a generalized increase in B-1 cell–derived IgA Abs, which are typically mucosal, but a preferential increase in IgM NAb s to OSEs. Further, the lungs of Abcg1<sup>−/−</sup> mice exhibited a 2-fold increase in total IgM mRNA but a 6-fold increase in E06-IgM–specific mRNA (Fig. 7D), consistent with the idea that there are increased levels of E06-secreting B-1a B cells in Abcg1<sup>−/−</sup> lungs. These data indicate that the lungs of Abcg1<sup>−/−</sup> mice contain increased levels of multiple Igs that function in both innate and adaptive immunity.

Increased Ab titers to oxidation-specific epitopes in the lungs of Abcg1<sup>−/−</sup> mice

The increase in specific oxidized sterols and phospholipids (Fig. 4) in the lungs of Abcg1<sup>−/−</sup> mice suggested an enhanced oxidative environment and the generation of oxidation-specific epitopes (OSEs). Consequently, we performed chemiluminescent ELISAs, using various Ags, to specifically determine whether the lungs of Abcg1<sup>−/−</sup> mice contained Abs binding to OSEs. Fig. 7A–C show that titers of IgM, IgA, and IgG to MDA-LDL and Cu-OxLDL were increased 2.5–6-fold in the lungs of knockout mice. In addition, using the anti-idiotypic Ab AB1-2, which binds specifically to the E06/T15 idiotype that binds OxPL (50), we measured titers of the specific E06 IgM and T15 IgA NAb s. There were markedly increased titers of both E06 IgM (Fig. 7B) and T15 IgA (Fig. 7C) in the lungs of Abcg1<sup>−/−</sup> mice. We also noted markedly increased titers of IgA to the classic B-1 cell Ag α1,3-dextran in the lungs of Abcg1<sup>−/−</sup> mice. The latter finding suggests a generalized increase in B-1 cell–derived IgA Abs, which are typically mucosal, but a preferential increase in IgM NAb s to OSEs. Further, the lungs of Abcg1<sup>−/−</sup> mice exhibited a 2-fold increase in total IgM mRNA but a 6-fold increase in E06-IgM–specific mRNA (Fig. 7D), consistent with the idea that there are increased levels of E06-secreting B-1a B cells in Abcg1<sup>−/−</sup> lungs. These data indicate that the lungs of Abcg1<sup>−/−</sup> mice contain increased levels of B-1a B cells (Figs. 2, 3), oxidized lipid Ags (Fig. 4H), and IgA and IgM NAb s (Figs. 6, 7) that are part of innate immunity, as well as IgGs that are likely part of adaptive immunity (Figs. 6, 7).

**Loss of Abcg1 results in pulmonary accumulation of lipid Ags and specific IgM Abs**

We next performed a modification of the protein-lipid overlay technique (49) to determine whether the lungs of Abcg1<sup>−/−</sup> mice contained increased levels of lipid Ags and corresponding Igs. Total lipids were isolated from perfused lungs of 6-mo-old chow-fed wild-type and Abcg1<sup>−/−</sup> mice. Total phosphatidylcholine phospholipid (6-mo-old chow-fed wild-type and knockout mice) were determined in surfactant isolated by bronchoalveolar lavage, as described (31), from 6-mo-old chow-fed wild-type and Abcg1<sup>−/−</sup> mice. Data are total sterol/ml surfactant volume recovered. Total phosphatidylcholine phospholipid (G) and OxPL (H) species were determined in whole lung of 6-mo-old chow-fed wild-type and Abcg1<sup>−/−</sup> mice by GC-MS and ESI-MS/MS, respectively. Data are mean ± SEM; n = 5 mice/genotype. ***p < 0.001.

**FIGURE 4.** Accumulation of cholesterol, phosphatidylcholine, and specific oxidized sterol derivatives and phospholipids in lungs and surfactant of Abcg1<sup>−/−</sup> mice. Lipids were extracted from lung or surfactant as previously described (31). Cholesterol and oxidized cholesterol derivatives were quantified using MS/MS. Autoxidation (A) and enzymatic oxidation (B) products of cholesterol, as well as total cholesterol (C), were determined in whole lung of 6-mo-old chow-fed wild-type and Abcg1<sup>−/−</sup> mice. Autoxidation (D) and enzymatic oxidation (E) products of cholesterol, as well as total cholesterol (F), were determined in surfactant isolated by bronchoalveolar lavage, as described (31), from 6-mo-old chow-fed wild-type and Abcg1<sup>−/−</sup> mice. Data are total sterol/ml surfactant volume recovered. Total phosphatidylcholine phospholipid (G) and OxPL (H) species were determined in whole lung of 6-mo-old chow-fed wild-type and Abcg1<sup>−/−</sup> mice by GC-MS and ESI-MS/MS, respectively. Data are mean ± SEM; n = 5 mice/genotype. ***p < 0.001.

**Arrows.** Western blot analysis confirmed that the levels of IgM and IgA were markedly increased, and IgG was slightly increased, in the lungs of the knockout mice (Fig. 6B). Further, titers of total IgM and IgA determined by ELISA also were increased in the lungs of Abcg1<sup>−/−</sup> mice (Fig. 6C). Thus, loss of Abcg1 results in increased levels of multiple Igs that function in both innate and adaptive immunity.
B cells in the lungs of Selective stimulation and increased proliferation of B-1a plasma of Abcg1 of ABCG1 results in increased titers of NAbs in the lungs and Together, the data in Figs. 2–7 and Table II demonstrate that loss from Abcg1 titers to oxidized low-density lipoprotein (OxLDL) and increased plasma titers of IgA to MDA-LDL in 

ingly, although IgA are predominantly serosal Abs, there also were counterstained with DAPI. Original magnification ×20. **p < 0.01.

Elevated plasma titers of natural and adaptive Abs in Abcg1−/− mice To assess whether loss of ABCG1 leads to a more global expansion of Abs involved in innate and/or adaptive immunity, we used chemiluminescent ELISAs to measure total and OSE Ab titers in the lung tissue sections from wild-type and Abcg1−/− mice that were injected with GFP+ B-1 B cells (as in Fig. 3). First, consistent with the data in Fig. 2 and our previous results (36), we observed increased staining of B-1 B cells in the lungs of Abcg1−/− mice compared with wild-type mice, using GFP expression levels (Fig. 8B, left panels). We also observed increased proliferation of B-1 B cells, as indicated by the increased staining and colocalization of the proliferative marker PCNA with GFP (Fig. 8B, far right panels [merged images]). Finally, we also observed that cells expressing PCNA also express IgM, a marker of B-1 B cells (Supplemental Fig. 2B). Taken together, these data demonstrate that loss of ABCG1 and tissue sterol homeostasis profoundly affect both adaptive and innate immune responses (Fig. 9).

Selective stimulation and increased proliferation of B-1a B cells in the lungs of Abcg1−/− mice Together, the data in Figs. 2–7 and Table II demonstrate that loss of ABCG1 results in increased titers of NAbs in the lungs and plasma of Abcg1−/− mice, as well as a niche-specific pulmonary expansion of B cells, especially B-1 B cells. However, we cannot rule out the possibility that, in addition to the increased homing of B-1 B cells to the lungs and pleural cavity (Fig. 5, Table I), there may be local expansion of B-1 B cells. To directly test whether a localized increase in B-1a B cells might be related to a selective stimulation of these clones with their respective Ags (e.g., the presence of OxPL in the lungs, leading to enhanced secretion of IgM E06 and IgA T15), we performed immunohistochemical analyses of lung sections to determine the presence of OxPLs that are recognized and bound by E06. In agreement with the increased titers of E06 NAbs (Fig. 7) and increased OxPL Ags (Fig. 4H), we observed positive E06 staining in the lungs of 6-mo-old chow-fed Abcg1−/− mice compared with wild-type mice (Fig. 8A). We also determined whether there was increased proliferation of B cells in situ in the lung using proliferative and B cell marker costaining on lung tissue sections from wild-type and Abcg1−/− mice that were injected with GFP+ B-1 B cells (as in Fig. 3). First, consistent with the data in Fig. 2 and our previous results (36), we observed increased staining of B-1 B cells in the lungs of Abcg1−/− mice compared with wild-type mice, using GFP expression levels (Fig. 8B, left panels). We also observed increased proliferation of B-1 B cells, as indicated by the increased staining and colocalization of the proliferative marker PCNA with GFP (Fig. 8B, far right panels [merged images]). Finally, we also observed that cells expressing PCNA also express IgM, a marker of B-1 B cells (Supplemental Fig. 2B). Taken together, these data demonstrate that loss of ABCG1 and tissue sterol homeostasis profoundly affect both adaptive and innate immune responses (Fig. 9).

**FIGURE 5.** ABCG1 regulates B-1 B cell homing. Total RNA was extracted from lungs (25 mg) of 6-mo-old chow-fed (A and C), 12-wk-old chow-fed (B), or 12-wk-old Western diet-fed (D) wild-type and Abcg1−/− mice prior to mRNA quantification of Ch25h, Cyp7b1, and Ccxl13 by RT-qPCR. Values were normalized to 36B4. Data are mean ± SEM; *n = 5 mice/genotype. (D) Immunohistochemical analysis of lung from 6-mo-old chow-fed wild-type and Abcg1−/− mice. Frozen tissue sections (10 μm) were incubated with an Ab to mouse CXCL13. A goat anti-mouse Alexa Fluor 488-conjugated secondary Ab was used for detection. Sections were counterstained with DAPI. Original magnification ×20. **p < 0.01.
animal is selected to bind to evolutionarily important epitopes. We demonstrated previously that OSEs constitute a significantly large portion of these self-Ags and are a major target of innate NAbs in both mice and humans (50, 55).

ABCG1 is highly expressed in many cell types, including macrophages, lymphocytes (T and B cells), and type II pneumocytes that play essential roles in maintaining normal lung homeostasis (18, 31, 36). Consistent with important roles for ABCG1 in these cells, we (31, 36) and other investigators (56) reported that the lungs of Abcg1/−/− mice contain increased numbers of lipid-filled, Oil Red O+ macrophage foam cells, abnormal lamellae (phospholipid)-loaded type II pneumocytes, increased lymphocytic infiltrates, and elevated cytokines. Studies in Abcg1/−/− mice demonstrated that whole-body deletion of ABCG1 leads to cell-specific phenotypes that include increased lymphocyte proliferation (12, 13), leukocytosis (15), increased macrophage apoptosis

FIGURE 6. Increased Igs in lungs and plasma of Abcg1/−/− mice. (A) Immunohistochemical analysis of lung and spleen from 6-mo-old chow-fed wild-type and Abcg1/−/− mice. Frozen tissue sections (10 μm) were stained with secondary HRP-conjugated Abs for IgA, IgG, and IgM. Arrows indicate positive staining. Original magnification ×10. (B) Lungs of 6-mo-old chow-fed wild-type and Abcg1/−/− mice were perfused extensively with PBS to remove blood contaminants prior to homogenization and isolation of total lung proteins. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane prior to incubation with Abs to IgA (α-chain, 55 kDa), IgG (γ-chain, 53 kDa), IgM (μ chain, 65 kDa), and β-actin (42 kDa). (C) Abs were extracted from lung tissue (50 mg) following a protocol adapted from Yla¨-Herttuala et al. (38). Lung Abs were diluted 1:10, and the quantity of total IgA, IgG, and IgM was determined using ELISA techniques, as described in Materials and Methods. Data (ng/ml) are expressed as mean Ab titer over wild-type levels at a 1:10 dilution. Bars represent mean ± SEM of triplicate determinations of individual mice, comparing total Ig in Abcg1/−/− versus wild-type mice (n = 4–6 mice/genotype). (D) Lung lipid extracts (from 25 mg tissue) from 6-mo-old chow-fed wild-type and Abcg1/−/− mice were spotted onto nitrocellulose membrane and incubated in the presence of total lung protein extract (from 15 mg tissue) from 6-mo-old chow-fed wild-type or Abcg1/−/− mice. Secondary HRP-conjugated goat anti-mouse Abs to IgA, IgG, or IgM were used to detect the specific Ab isotype. ***p < 0.001.

FIGURE 7. Specific lipid Ags and Abs are present in the lungs of Abcg1/−/− mice. (A–C) Abs were extracted from lung tissue, as in Fig. 5, diluted 1:10, and tested for binding to the indicated Ags. HRP-conjugated IgG (A), IgM (B), or IgA (C) was used for detection. Data are mean Ab titer (ng/ml) ± SEM comparing Abcg1/−/− and wild-type mice (n = 4 mice/genotype). (D) Total RNA was extracted from lungs (25 mg tissue) of wild-type and Abcg1/−/− mice prior to mRNA quantification of total IgM and E06-IgM by TaqMan RT-PCR. Values were normalized to GAPDH. Data are expressed as fold change ± SEM, Abcg1/−/− versus wild-type mice (n = 4 mice/genotype). ***p < 0.001. α-1,3-Dex, α-1,3-dextran.
altered signaling of endothelial cells (30), and reduced insulin secretion from β cells (21). These data suggest that ABCG1 is required for the normal function of many cell types. Whether all of these changes are a result of altered control of intracellular sterol homeostasis (20, 27) remains unknown.

In this study, we demonstrate for the first time, to our knowledge, that there is a niche-specific increase in B-1 B cells in the lungs and pleural cavities of Abcg1<sup>2/2</sup> mice; this is accompanied by parallel increases in IgM and E06-specific IgM mRNA, consistent with increased NAb generation (Fig. 2). In addition, we demonstrate that the lungs and pleural cavities of these knockout mice contain increased levels of B-1a B cells, a subset of B-1 B cells that is known to specifically secrete IgM and IgA NAbs and to be involved in innate immunity. Consistent with this latter finding, we also noted increased pulmonary levels of IgM and IgA NAbs. These changes are also cell specific, because the levels of T cells were unaltered. Collectively, these data are in agreement with the hypothesis that loss of ABCG1 leads to changes in innate immunity. Because we also show that there are elevated levels of B-2 cells and IgG Ab titers in the lungs of these knockout mice, we propose that loss of ABCG1 is associated with increases in both innate and adaptive immunity.

What are the Ags that lead to the increase in B-1/B-1a B cell number and to the increase in Nabs, which include E06 IgM and T15 IgA, in the lungs and/or plasma of Abcg1<sup>2/2</sup> mice? Despite the critical importance of B-1 B cells, little is known about how these cells enter or accumulate in specific body cavities. It is well established that chemokines and chemokine receptors play an essential role in the homing of lymphocytes. Homing of B cells to the lymphoid follicles in the spleen is regulated by the chemokine CXCL13, and mice lacking CXCL13 or its receptor CXCR5 fail to generate lymphoid follicles (17, 48). CXCL13 is constitutively expressed by cells in both the PerC and pleural cavity, including macrophages, and mice lacking CXCL13 have a severe deficiency of B cells in the PerC and pleural cavity (48). Consistent with the role of CXCL13 in B-1 B cell homing, we showed increased levels of CXCL13 in the lungs of Abcg1<sup>2/2</sup> mice compared with wild-type mice (Fig. 5).

The CXCL13-mediated accumulation of B-1 B cells has significant consequences for NAb production and local immunity.

**Table II.** Ab titers in plasma from 6-mo-old chow-fed wild-type and Abcg1<sup>2/2</sup> mice.

<table>
<thead>
<tr>
<th>Ab Specificity</th>
<th>Ab Class</th>
<th>Wild-Type (ng/ml)</th>
<th>Abcg1&lt;sup&gt;2/2&lt;/sup&gt; (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>IgA</td>
<td>325,000 ± 37,571</td>
<td>305,432 ± 136,999</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>343,513 ± 36,994</td>
<td>441,131 ± 52,656*</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>372,777 ± 50,347</td>
<td>581,597 ± 70,206*</td>
</tr>
<tr>
<td>MDA-LDL</td>
<td>IgA</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>79,518 ± 6,608</td>
<td>135,757 ± 8,440**</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>251,904 ± 78,329</td>
<td>306,314 ± 58,127</td>
</tr>
<tr>
<td>Cu-OxLDL</td>
<td>IgA</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>59,390 ± 619</td>
<td>64,395 ± 2,626</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>74,639 ± 2,7931</td>
<td>212,358 ± 25,990**</td>
</tr>
<tr>
<td>E06/T15</td>
<td>IgA</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>87,647 ± 14,887</td>
<td>298,786 ± 39,055**</td>
</tr>
<tr>
<td>α-1,3-Dextran</td>
<td>IgA</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>IgG</td>
<td>n/d</td>
<td>n/d</td>
</tr>
<tr>
<td></td>
<td>IgM</td>
<td>31,609 ± 11,698</td>
<td>45,252 ± 28,432</td>
</tr>
</tbody>
</table>

Plasma from 6-mo-old chow-fed wild-type and Abcg1<sup>2/2</sup> mice was diluted 1:100 and tested for binding to IgA, IgG, and IgM, as well as for binding to the indicated Ags. HRP-conjugated Abs were used for detection. Data are mean Ab titer (ng/ml) ± SEM (n = 4 mice/genotype).

* p < 0.05, ** p < 0.01, versus wild-type mice.

n/a, not measured; n.d., not detected.

**FIGURE 8.** Oxidized lipid Ags are present in lungs and atherosclerotic lesions of Abcg1<sup>2/2</sup> mice. (A) Frozen lung tissue sections (10 μm) from 6-mo-old chow-fed wild-type and Abcg1<sup>2/2</sup> mice were incubated in the presence of mouse E06 Abs that recognize and bind to OxPLs. Secondary anti-mouse Alexa Fluor 488–conjugated IgM was used for detection. Secondary anti-rat Alexa Fluor 594 was used for detection. All tissue sections were counterstained with DAPI. Original magnification ×20.

(B) Frozen lung tissue sections from 6-mo-old chow-fed wild-type and Abcg1<sup>2/2</sup> mice that were injected with GFP<sup>+</sup> cells were incubated in the presence of Abs that recognize PCNA (proliferative marker) and analyzed for GFP expression. Secondary anti-rat Alexa Fluor 594 was used for detection. All tissue sections were counterstained with DAPI. Original magnification ×20.
Our data suggest that changes in the lipid specific expansion of B-1 cells in response to the accumulation of B-1 cells, mostly to bacterial Ags (e.g., LPS) (59–62). However, to determine whether an oxidized Ag could be responsible for the positive selection of a B-1 cell clone (64). Indeed, E06 was initially identified for its ability to bind to OxPLs; only after sequencing of its variable heavy/light genes was it discovered that E06 was identical to the previously described IgA NAb named T15 (50). T15 was well known because it provides optimal protection to mice against lethal infection with pathogens, such as S. pneumoniae. IgM E06 and IgAT15 bind to the same PC moiety, present either as the PC headgroup of OxPLs or as the PC covalently linked to a carbohydrate on the cell wall polysaccharide of pathogens (65). Remarkably, E06/T15 do not bind to the same PCs present on unoxidized PCs containing phospholipids. It was further shown that E06 bound strongly to apoptotic cells and apoptotic debris, which have increased content of such OxPLs (66). In contrast, E06 did not bind to viable cells that lack OxPLs. We suggested previously that the need to provide homeostasis against apoptotic cells, which are proinflammatory and immunogenic if not promptly removed, has provided a selective pressure for the expansion of E06 and related NAbs to OxPLs (66, 67). In this context, it will be important to determine whether the IgM Abs secreted by IRA B-1 cells in the study by Weber and colleagues (63) noted above include E06 and related IgMs that also bind to OxPLs.

Consistent with the idea that endogenously produced oxidized lipids could drive selection and/or expansion of specific B cell subsets, we showed that PC-containing OxPLs were increased in the lungs of Abcg1−/− mice (Fig. 4H). Furthermore, we also showed that titers of Abs to specific oxidized lipid Ags were increased in the lungs and plasma of Abcg1−/− mice compared with wild-type mice (Figs. 6, 7, Table II). We reported previously that POVP and PoxnoPC can serve as Ags for B-1 B cells, resulting in increased secretion of E06 IgM (42). Indeed, we now demonstrate that IgM-E06-specific mRNA was increased 6-fold in the lungs of Abcg1−/− mice compared with a 2-fold increase in total IgM mRNA, suggesting that there has been a specific, localized expansion of E06-secreting B-1 B cells, possibly from a selective increase in specific oxidized lipids. In turn, this B-1 cell expansion appears to be due to both homing of B-1 cells and localized proliferation. Consequently, our data are in agreement with a model in which loss of ABCG1 results in accumulation of both sterols and phospholipids; some of these lipids, once oxidized, act as homing signals for B-1 B cell movement into the lungs and pleural cavity, and OSEs drive expansion of B-1 cells and increased secretion of NAb (Fig. 9).

In addition to changes in IgM NAbs, we observed significant increases in IgG Abs to OSEs in Abcg1−/− mice. These changes in adaptive immune IgG responses are not unexpected, given the significant changes in the lungs, and are reflected in the IgG increases in both the plasma and lung of Abcg1−/− mice. Most likely, these changes represent IgG generation from sites outside the lung. Collectively, these data suggest that loss of ABCG1 has broad consequences for innate and adaptive immunity. Draper et al. (68) demonstrated that Abcg1−/− mice display altered IL-17 signaling and a reduced Th2 response, suggesting a role for ABCG1
in the adaptive immune response. Furthermore, Bensinger et al. (13) reported that the availability of intracellular sterols is an essential check point for T cell activation and is dependent on ABCG1. The rapid proliferation of T cells during activation is an important characteristic of the adaptive immune response to an Ag challenge, further implicating a significant role for ABCG1 in adaptive immunity. It was also reported that, consistent with the data from this study, Abcg1−/− mice show no differences in T cell number compared to wild-type animals; however, increased T cell cycling was reported in Abcg1−/− mice (12). It is conceivable that the increased T cell cycling could underlie an increased T cell activation, which may account for the increased B-2 B cells and IgG Abs. Nevertheless, our findings provide strong evidence for the hypothesis that changes in intracellular sterols/lipids have profound effects on the immune system (12–15, 69–71).

We reported previously that atherosclerotic lesions of mice lacking ABCG1 exhibit increased numbers of apoptotic macrophages obtained by bronchoalveolar lavage from neodeterminants on apoptotic cells, as well as those present on changes in intracellular sterols/lipids have profound effects on the immune system (12–15, 69–71).


SUPPLEMENTAL MATERIALS

**Figure S1. (A-B)** Flow cytometric analysis of cells isolated from spleens of 6 month old chow-fed wildtype and Abcg1<sup>−/−</sup> mice. Single cell suspensions were analyzed for expression of CD19 and CD3 as markers for B (A) and T (B) cells, respectively. **(C-D)** Flow cytometric analysis of cells isolated from the spleen and peritoneal cavity (PerC) of 6 month old chow-fed wildtype and Abcg1<sup>−/−</sup> mice. Single cell suspensions were analyzed for expression of CD19, CD11b, IgM, and CD5 as markers for B-1a (A; CD19<sup>+</sup>, CD11b<sup>+</sup>, sIgM<sup>+</sup>, CD5<sup>+</sup>) and B-1b (B; CD19<sup>+</sup>, CD11b<sup>+</sup>, sIgM<sup>+</sup>, CD5<sup>−</sup>) cells, respectively. Data are expressed as mean ±SEM; n = 4 mice/genotype.
**Figure S2.** (A) Total RNA was extracted from lungs (25 mg tissue) of wildtype and Abcg1−/− mice prior to mRNA quantification of Gpr183 (EBI2) and Cxcr5 by RT-PCR. Values were normalized to 36B4. Data are expressed as fold change ±SEM comparing Abcg1−/− vs. wildtype mice; n = 4 mice/genotype; * p<0.05, ** p<0.01. (B) Frozen lung tissue sections (10 µm) from 6 month old chow-fed wildtype and Abcg1−/− mice that were injected with 1x10^6 GFP+ B-1 B cells were incubated in the presence of antibodies that recognize IgM (B-1 B cell marker) and PCNA (proliferative marker). Secondary anti-mouse Alexafluor 488 and anti-rat Alexafluor 594 were used for detection. Tissue sections were counter-stained with DAPI.
Figure S3. (A-D) Alveolar macrophages were isolated from 6 month old chow-fed wildtype and Abcg1−/− mice by broncho-alveolar lavage as previously described [13]. Cells were stained for apoptosis using TUNEL-staining, and counter-stained with DAPI, as previously described [58]. Percent of TUNEL-positive cells (green) from multiple analyses are shown. (E-F) Lung sections (10 µM) from 6-month old chow-fed wildtype and Abcg1−/− mice were stained for the presence of apoptotic cells by TUNEL stained as previously described [58], and were counter-stained with DAPI.