ATP-Binding Cassette Transporter 1 Attenuates Ovalbumin-Induced Neutrophilic Airway Inflammation

Cuilian Dai^{1*}, Xianglan Yao^{1*}, Boris Vaisman¹, Todd Brenner¹, Katharine S. Meyer¹, Meixia Gao¹, Karen J. Keeran², Gayle Z. Nugent², Xuan Qu³, Zu-Xi Yu³, Pradeep K. Dagur⁴, J. Philip McCoy⁴, Alan T. Remaley¹, and Stewart J. Levine¹

¹Cardiovascular and Pulmonary Branch, ²Animal Surgery and Resources Core Facility, ³Pathology Core Facility, and ⁴Flow Cytometry Core Facility, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland

Abstract

Apolipoprotein A-I (apoA-I) is an important component of high-density lipoprotein particles that mediates reverse cholesterol transport out of cells by interacting with the ATP-binding cassette transporter 1 (ABCA1). apoA-I has also been shown to attenuate neutrophilic airway inflammation in experimental ovalbumin (OVA)-induced asthma by reducing the expression of granulocyte colony-stimulating factor (G-CSF). Here, we hypothesized that overexpression of the ABCA1 transporter might similarly attenuate OVA-induced neutrophilic airway inflammation. Tie2-human ABCA1 (*hABCA1*) mice expressing human ABCA1 under the control of the Tie2 promoter, which is primarily expressed by vascular endothelial cells, but can also be expressed by macrophages, received daily intranasal OVA challenges, 5 d/wk for 5 weeks. OVA-challenged Tie2-*hABCA1* mice had significant reductions in total bronchoalveolar lavage fluid (BALF) cells that reflected a decrease in neutrophils, as well as reductions in peribronchial inflammation, OVA-specific IgE levels, and airway epithelial thickness. The reduced airway neutrophilia in OVA-challenged Tie2-*hABCA1* mice was associated with significant decreases in G-CSF protein levels in pulmonary vascular endothelial cells, alveolar macrophages, and BALF. Intranasal administration of recombinant murine G-CSF to OVA-challenged Tie2-*hABCA1* mice for 5 days increased BALF neutrophils to a level comparable to that of OVA-challenged wild-type mice. We conclude that ABCA1 suppresses OVA-induced airway neutrophilia by reducing G-CSF production by vascular

endothelial cells and alveolar macrophages. These findings suggest that ABCA1 expressed by vascular endothelial cells and alveolar macrophages may play important roles in attenuating the severity of neutrophilic airway inflammation in asthma.

Keywords: vascular endothelial cells; airway inflammation; ovalbumin; neutrophil; ATP-binding cassette transporter 1

Clinical Relevance

Apolipoprotein A-I (apoA-I) is a key component of high-density lipoprotein particles that mediates reverse cholesterol transport out of cells by interacting with the ATP-binding cassette transporter 1 (ABCA1). apoA-I also suppresses neutrophilic airway inflammation in experimental ovalbumin (OVA)-induced asthma by reducing the expression of granulocyte colony-stimulating factor (G-CSF). This study reports that mice overexpressing human ABCA1 under the control of the Tie2 promoter in vascular endothelial cells and alveolar macrophages have attenuated OVA-induced neutrophilic airway inflammation that is in part mediated by the reduced expression of G-CSF. These findings support the concept that an apoA-I/ABCA1 pathway negatively regulates OVA-induced neutrophilic airway inflammation by suppressing G-CSF expression.

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*These co-first authors contributed equally to this work.

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Correspondence and requests for reprints should be addressed to Stewart J. Levine, M.D., Cardiovascular and Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, Room 6D03, MSC 1590, Bethesda, MD 20892-1590. E-mail: levines@nhlbi.nih.gov

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Asthma has a complex pathogenesis that is mediated by a variety of immune and inflammatory cells in the lung, such as dendritic cells, T and B lymphocytes, mast cells, and eosinophils (1). It has increasingly been recognized, however, that neutrophils also participate in the pathogenesis of asthmatic airway inflammation, particularly in patients with severe disease (2). For example, a cluster analysis of severe asthma phenotypes identified a subgroup of patients with low lung function, less atopy, frequent sinopulmonary infections, obesity, and an increase in sputum neutrophils (3). Individuals with asthma with increased numbers of sputum neutrophils have more severe airflow obstruction, whereas increased numbers of neutrophils have been recovered in tracheal aspirates from subjects with severe asthma with acute exacerbations associated with respiratory failure and mechanical ventilation (4, 5). The neutrophilic inflammatory asthmatic phenotype has also recently been correlated with increased levels of systemic inflammation (6). Neutrophil-predominant subjects with asthma have increased sputum levels of IL-8 (CXCL8), which is a key chemokine that mediates neutrophil recruitment (7), whereas clarithromycin treatment has been reported to significantly reduce both sputum neutrophil numbers and IL-8 concentrations (8). Neutrophilic asthma has also been associated with increased sputum mRNA levels of IL-1 β , Toll-like receptor (TLR) 2, TLR4, CD14, and surfactant protein A, as well as higher amounts of endotoxin (9).

Our laboratory has recently shown that neutrophilic airway inflammation in ovalbumin (OVA)-challenged mice is attenuated by apolipoprotein A-I (apoA-I) (10).

apoA-I is expressed by alveolar epithelial cells, and the amount of apoA-I protein in the lung is reduced by multiple OVA challenges. OVA-challenged *apoA-I*^{-/-} mice have significant increases in airway neutrophils as compared with wild-type (WT) mice, which can be reversed by site-directed administration of an apoA-I mimetic peptide to the airway. Although multiple pathways promoted neutrophilic inflammation in OVA-challenged *apoA-I*^{-/-} mice, including the up-regulated expression of proinflammatory cytokines (IL-17A, TNF- α), NF- κ B signaling, CXC chemokines (CXCL5), vascular adhesion molecules (vascular cell adhesion molecule [VCAM]-1),

and granulocyte colony-stimulating factor (G-CSF), the ability of apoA-I to suppress OVA-induced airway inflammation was primarily mediated by a reduction in G-CSF expression. These results show that apoA-I in the lung might play an important role in modulating the pathogenesis and severity of neutrophilic airway inflammation in asthma.

apoA-I is a key component of high-density lipoprotein (HDL) particles that mediates reverse cholesterol transport out of cells and thereby protects against atherosclerosis (11, 12). Cholesterol is transported out of cells by interactions between HDL and the ATP-binding cassette (ABC), subfamily A, member 1 (ABCA1), as well as other transporters, such as ABCG1 and SR-BI (12). Furthermore, the ability of endothelial cells to bind, internalize, and transcytose lipid-free apoA-I is mediated by ABCA1 (13). Based upon our prior finding that apoA-I attenuates OVA-induced neutrophilic airway inflammation, we hypothesized that the vascular endothelial-specific expression of ABCA1 in mice might similarly reduce OVA-mediated neutrophilic airway inflammation. Here, we show that OVA-challenged transgenic mice that overexpress human ABCA1 (hABCA1) under the control of the Tie2 promoter, which is primarily expressed by vascular endothelial cells, but can also be expressed by macrophages in the setting of inflammation, had significantly reduced neutrophilic airway inflammation (14–16). Furthermore, overexpression of hABCA1 reduced OVA-induced neutrophilic airway inflammation by a mechanism that involved the attenuated expression of G-CSF by vascular endothelial cells and alveolar macrophages. These findings are consistent with the conclusion that an ABCA1 transporter-dependent mechanism suppresses neutrophilic airway inflammation in asthma by attenuating G-CSF production by vascular endothelial cells and alveolar macrophages.

Materials and Methods

See the online supplement for additional MATERIALS AND METHODS.

Murine Models

Tie2-hABCA1 transgenic mice that express the full-length hABCA1 under the control

of the mouse Tie2 promoter have been previously described (14). WT C57BL/6 mice (6–8 wk old) were purchased from the Jackson Laboratory (Bar Harbor, ME). On Days 1 and 8, mice were sensitized with an intraperitoneal injection of 50 μ g OVA (grade V) plus 0.4 mg of aluminum hydroxide (Sigma-Aldrich, St. Louis, MO). Mice received daily intranasal challenges with 150 μ g OVA in 10 μ l saline, 5 d/wk, on Days 15–42. Control mice were sensitized and challenged with saline. Endpoints were analyzed 24 hours after the final challenge. For neutrophil migration experiments, mice received 2.5 μ g of recombinant, carrier-free, murine G-CSF (rmG-CSF; Biolegend, San Diego, CA) without OVA or saline challenges, and bronchoalveolar lavage fluid (BALF) cells were collected after 24 hours. For G-CSF rescue experiments, saline- and OVA-challenged mice received 2 μ g of rmG-CSF or vehicle (saline) via intranasal administration, daily on Days 38–42, and BALF was collected on Day 43. All experiments were approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute.

Pulmonary Vascular Endothelial Cell Isolation

Murine pulmonary vascular endothelial cells (PVECs) were isolated as previously described with modifications (17). Mouse lungs were mechanically minced, incubated in 2 mg/ml of collagenase in 25 ml of Dulbecco's modified Eagle's medium (Sigma-Aldrich) for 30 minutes at 37°C with gentle agitation every 5 minutes, and passed through a 70- μ m disposable cell strainer into 15 ml of high-glucose Dulbecco's modified Eagle's medium containing 20% FBS. Cells were pelleted by centrifugation at 300 $\times$ g for 8 minutes at 4°C and resuspended in cold PBS with 0.1% BSA. Anti-intercellular adhesion molecule (ICAM)-2 beads were prepared by mixing a rat anti-mouse, FITC-conjugated CD102 antibody (BD Pharmingen, Franklin Lakes, NJ) with sheep anti-rat IgG magnetic beads (Invitrogen, Carlsbad, CA). Cells were incubated with anti-ICAM-2 antibody-conjugated magnetic beads for 12 minutes at room temperature on a rotating platform. PVECs were isolated using a Magnetic Particle Concentrator (DynaMag-2; Invitrogen) and resuspended in PBS with 0.1% BSA. Five rounds of purification were performed, and isolated

cells from both WT and Tie2-hABCA1 mice were characterized by flow cytometry using an FITC-conjugated anti-CD102 antibody and 7-AAD (BD Pharmingen), and shown to have greater than 95% purity and 85% viability.

Statistical Analysis

Results are presented as mean ($\pm$ SEM). A one-way ANOVA with the Bonferroni multiple comparison test was used to analyze results, except for the neutrophil migration experiment, which was analyzed using a Mann-Whitney test, and the airway hyperresponsiveness (AHR) experiment, which was analyzed using a two-way ANOVA with Bonferroni's post test (GraphPad Prism, version 5.0a; GraphPad Software, Inc., La Jolla, CA). A *P* value less than 0.05 was considered significant.

Results

ABCA1 Is Expressed by PVECs and Alveolar Macrophages

Confocal immunofluorescence microscopy was performed to assess whether ABCA1 is expressed by PVECs and alveolar macrophages in WT mice under basal conditions. As shown in Figure 1A, ABCA1 was detected in PVECs that express von Willebrand factor, as well as by alveolar macrophages that express F4/80. Next, PVECs were isolated from WT mice, as well as from mice that overexpressed human ABCA1 under the control of the Tie2 promoter (Tie2-hABCA1). To quantify total ABCA1 protein expression in PVECs, Western blots were performed using an anti-ABCA1 antibody that reacts with ABCA1 of both mouse and human origin (see Figure E1 in the online supplement). As shown in Figure 1B, the total quantity of mouse and hABCA1 protein present in PVECs from Tie2-hABCA1 mice that had or had not been challenged with OVA was significantly increased as compared with similarly treated cells from WT mice, which only express murine ABCA1, by 35.9 and 46.6%, respectively.

Airway Neutrophilia Is Reduced in OVA-Challenged Tie2-hABCA1 Mice

Experiments were next performed to assess whether the increased expression of hABCA1 under the Tie2 promoter modified airway inflammation and remodeling responses to OVA. As shown in Figure 2A,

Figure 1. Expression of ATP-binding cassette transporter 1 (ABCA1) in the lung. (A) Confocal immunofluorescence microscopy was performed on sections of lung tissue from wild-type (WT) mice that were reacted with antibodies against ABCA1, von Willebrand factor (vWF), or F4/80. Secondary antibodies were conjugated with Alexa Fluor 488 (green) or Alexa Fluor 568 (red) and nuclei were stained with 4',6-diamidino-2-phenylindole (blue). (B) Western blots of proteins present in pulmonary vascular endothelial cells (PVECs) from WT and Tie2-human ABCA1 (hABCA1) mice were reacted with an antibody that recognizes both hABCA1 and mouse ABCA1. Membranes were then stripped and reacted with an antibody that recognizes β -actin. The ratio of ABCA1 to β -actin protein was quantified by densitometry ($n = 10$ mice). **P* < 0.05, WT + saline versus Tie2-hABCA1 + saline and WT + ovalbumin (OVA) versus Tie2-hABCA1 + OVA. Data presented are means ($\pm$ SEM).

OVA-challenged Tie2-hABCA1 mice had significant reductions in the total number of BALF cells, which reflected a specific decrease in the number of neutrophils, whereas the numbers of BALF macrophages, lymphocytes, and eosinophils were unchanged as compared with OVA-challenged WT mice. Consistent with this, lung histologic sections showed a marked reduction in peribronchial inflammatory cell infiltrates in OVA-challenged Tie2-hABCA1 mice (Figure 2B). Additional experiments were performed to assess whether the OVA-induced neutrophilia

reflected an adaptive immune response to OVA, rather than an innate immune response to LPS. As shown in Figure E2, only WT mice that were sensitized and challenged with OVA developed a significant increase in BALF neutrophils, whereas mice that were sham sensitized with saline did not manifest a significant increase in BALF neutrophils in response to subsequent OVA challenges. This is consistent with the conclusion that the increases in BALF neutrophilia in our model primarily represented an adaptive immune response to OVA.

Figure 2. Neutrophilic airway inflammation is attenuated in OVA-challenged Tie2-hABCA1 mice. (A) Number of total bronchoalveolar lavage fluid (BALF) inflammatory cells and inflammatory cell types ($n = 20$ – 21 mice). * $P < 0.0001$, WT + OVA versus Tie2-hABCA1 + OVA. (B) Histologic sections of lungs from WT and Tie2-hABCA1 mice that had been challenged with saline or OVA and stained with hematoxylin and eosin (H&E) ($\times 200$) or periodic acid–Schiff (PAS; $\times 200$ and $\times 1,000$). These images are representative of 10 mice per group. Data presented are means ($\pm$ SEM). NS, not significant.

Next, we assessed whether airway remodeling responses and AHR were attenuated in Tie2-hABCA1 mice. As shown in Figures 3A and 3B, airway epithelial cell thickness was significantly reduced in OVA-challenged Tie2-hABCA1 mice, whereas there was no difference in the thickness of the lamina reticularis. There was also no difference in the extent of mucous cell metaplasia between OVA-challenged Tie2-hABCA1 mice and WT mice (Figures 2B and 3C). Consistent with our prior findings using this OVA-challenge model, neither WT nor Tie2-hABCA1 mice demonstrated AHR in response to OVA challenge, which likely reflects the reduced susceptibility of mice on a C57BL/6 genetic background to develop AHR (Figure E3) (10, 18). Furthermore, there were no differences in

airway resistance between OVA-challenged WT and Tie2-hABCA1 mice.

We next investigated further the effect of the Tie2-mediated overexpression of hABCA1 on airway inflammatory responses. As shown in Figure 3D, there was a significant reduction in serum levels of OVA-specific IgE in OVA-challenged Tie2-hABCA1 mice as compared with WT mice. However, consistent with the finding that the number of BALF lymphocytes was not modified in OVA-challenged Tie2-hABCA1 mice, we found that lung mRNA levels of T helper (Th) 1 (IFN- γ and TNF), Th2 (IL4 and IL-13), and Th17 (IL-17A) cytokines were not different in OVA-challenged Tie2-hABCA1 mice as compared with OVA-challenged WT mice (Figure 4). Similarly, the amounts of CXCL1 and CXCL2 present in BALF

(Figures 5A and 5B) and CXCL5 present in BALF or PVECs from OVA-challenged Tie2-hABCA1 mice were not different as compared with OVA-challenged WT mice (Figures 5C and 5D). Therefore, we did not find evidence that the reduction in airway neutrophilia in OVA-challenged Tie2-hABCA1 mice was mediated at the level of IL-17A, TNF, CXCL1, CXCL2, or CXCL5.

Adhesion Molecule Expression by PVECs Is Not Modified in OVA-Challenged Tie2-hABCA1 Mice

We next hypothesized that a potential mechanism by which airway neutrophilia is attenuated in OVA-challenged Tie2-hABCA1 mice might reflect the reduced expression of adhesion molecules that mediate leukocyte recruitment to the lung by PVECs. Therefore, Western blots were performed to assess whether VCAM-1, ICAM-1, or E-selectin expression were modified in PVECs isolated from OVA-challenged Tie2-hABCA1 mice. As shown in Figure 5E, expression of VCAM-1 and E-selectin in PVECs was increased in response to OVA challenges, whereas expression of ICAM-1 was not. However, the amount of VCAM-1, ICAM-1, or E-selectin proteins were not different in PVECs isolated from OVA-challenged Tie2-hABCA1 mice as compared with those from OVA-challenged WT mice. Thus, we did not find evidence that the reduction in BALF neutrophilia in OVA-challenged Tie2-hABCA1 mice was mediated by the reduced expression of adhesion molecules by PVECs.

The Mechanism by which Neutrophilic Airway Inflammation Is Attenuated in OVA-Challenged Tie2-hABCA1 Mice Involves the Reduced Expression of G-CSF by Vascular Endothelial Cells and Alveolar Macrophages

We next assessed whether the reduction in OVA-induced airway neutrophilia in Tie2-hABCA1 mice involved the attenuated expression of G-CSF. As shown in Figures 6A–6C, G-CSF mRNA levels in PVECs and G-CSF protein quantities in PVECs and BALF were significantly increased in OVA-challenged WT mice as compared with saline-challenged WT mice. In contrast, G-CSF mRNA levels in PVECs and G-CSF protein quantities in PVECs and BALF were significantly reduced in OVA-challenged Tie2-hABCA1 mice as compared with OVA-challenged WT

Figure 3. Airway epithelial thickness and IgE production are reduced in OVA-challenged Tie2-hABCA1 mice. The thicknesses of the airway epithelium (A) and lamina reticularis (B) were obtained by analyzing five images per airway from four to eight airways per mouse and dividing the area by the length of the corresponding basement membrane ($n = 5-7$ mice). $*P < 0.001$, WT + OVA versus Tie2-hABCA1 + OVA. (C) Mucous cell metaplasia is presented as the percentage of airways on lung histological sections that contained periodic acid-Schiff-positive cells ($n = 10-11$ mice). $P = NS$, WT + OVA versus Tie2-hABCA1 + OVA. A total of $37.2 (\pm 1.2)$ airways were counted in each animal. (D) Quantification of serum OVA-specific IgE ($n = 19-20$ mice). $*P < 0.0001$, WT + OVA versus Tie2-hABCA1 + OVA. Data presented are means ($\pm$ SEM).

mice. Collectively, this demonstrates a concordant relationship between the induction of OVA-induced BALF neutrophilia and the OVA-induced increases in G-CSF by PVECs and BALF from WT and Tie2-hABCA1 mice. Furthermore, the reductions in G-CSF by PVECs and BALF from OVA-challenged Tie2-hABCA1 mice were concordant with the reductions in BALF neutrophils.

Next, we investigated if the reductions in BALF neutrophils in OVA-challenged Tie2-hABCA1 mice might have been mediated by changes in serum G-CSF levels and the subsequent mobilization of neutrophils from the bone marrow compartment or extramedullary sites of hematopoiesis to the circulation. As shown in Figures 6D and 6E, although serum G-CSF levels were significantly reduced in OVA-challenged Tie2-hABCA1 mice as compared with OVA-challenged WT mice, there was no difference in blood neutrophil counts, which suggests that the decreases in

BALF neutrophils in OVA-challenged Tie2-hABCA1 mice were not mediated by the reduced recruitment of neutrophils into the circulation. Finally, we assessed whether migration from the circulation to the lung was reduced in Tie2-hABCA1 mice as a mechanism by which airway neutrophilia is attenuated in response to OVA challenges. As shown in Figure 6F, intratracheal administration of rmG-CSF resulted in increased neutrophil influx into BALF at 24 hours in Tie2-hABCA1 mice as compared with WT mice, which suggests that neutrophil migration to the lung was not impaired in Tie2-hABCA1 mice.

Because inflammatory stimuli can activate the Tie2 promoter in macrophages, we also assessed whether hABCA1 was expressed by alveolar macrophages from OVA-challenged Tie2-hABCA1, and whether this modified G-CSF expression (15, 19). As shown in Figure 7A, expression of hABCA1 mRNA was induced in alveolar macrophages present in BALF isolated

from OVA-challenged Tie2-hABCA1 mice, whereas hABCA1 mRNA was not detected in alveolar macrophages from saline-challenged Tie2-hABCA1 mice or WT mice. Next, the mean fluorescence intensity of ABCA1 expression by $CD45^{+}/\text{side scatter intensity (SSC)}^{\text{hi}}/CD11c^{+}/\text{sialic acid-binding immunoglobulin-type lectins (Siglec)} F^{+}/CD64^{+}$ alveolar macrophages was assessed by flow cytometry using an antibody that was raised against a recombinant protein corresponding to amino acids 1,248–1,350 of hABCA1 (Figure 7B) (20–22). This region of hABCA1 shares 97% identity with the amino acid sequence of murine ABCA1. Consistent with the high degree of homology between human and murine ABCA1, the antibody reacted with murine ABCA1 expressed by alveolar macrophages from WT mice (Figure 7C). This also showed that the antibody could detect total (human and murine) ABCA1 protein expression by alveolar macrophages from Tie2-hABCA1 mice. Next, flow cytometry demonstrated that alveolar macrophages present in BALF from OVA-challenged Tie2-hABCA1 mice had a significantly higher mean fluorescence intensity of ABCA1 expression as compared with alveolar macrophages from OVA-challenged WT mice (Figure 7C). Taken together, these results show that the Tie2 promoter is activated in alveolar macrophages when OVA-induced neutrophilic airway inflammation is present. This was associated with a 47% increase in total ABCA1 protein. Furthermore, G-CSF expression at the mRNA and protein levels were significantly reduced in alveolar macrophages present in BALF from OVA-challenged Tie2-hABCA1 mice as compared with alveolar macrophages from OVA-challenged WT mice (Figures 7D and 7E). Collectively, these results show that G-CSF expression by both alveolar macrophages and PVECs are reduced in OVA-challenged Tie2-hABCA1 mice.

Next, experiments were performed to demonstrate a causal relationship between decreased G-CSF expression and attenuated neutrophilic airway inflammation in Tie2-hABCA1 mice. rmG-CSF was administered via intranasal instillation to OVA-challenged Tie2-hABCA1 mice for 5 days to assess whether the decreases in neutrophilic airway inflammation could be reversed. As shown in Figure 7F, OVA-challenged Tie2-hABCA1 mice that received intranasal rmG-CSF had

Figure 4. T helper (Th) 1, Th2, and Th17 cytokines are not modified in the lungs from OVA-challenged Tie2-hABCA1 mice. Quantification of lung mRNA levels for Th1 (IFN- γ , TNF), Th2 (IL-4 and IL-13), and Th17 (IL-17A) cytokines was performed using quantitative RT-PCR and presented as relative mRNA expression ($n = 14-16$ mice). $P = NS$, WT + OVA versus Tie2-hABCA1 + OVA. Data presented are means ($\pm$ SEM).

a significant increase in the number of BALF neutrophils as compared with mice that received intranasal vehicle (saline) as a control, which reached levels similar to those in OVA-challenged WT mice. In contrast, administration of intranasal rmG-CSF to OVA-challenged WT mice did not result in a further increase in BALF neutrophilia, which suggests that the OVA-induced increases in endogenous G-CSF were sufficient to mediate maximal BALF neutrophilia in WT mice. Collectively, these results are

consistent with the conclusion that neutrophilic airway inflammation is attenuated in OVA-challenged Tie2-hABCA1 mice by a mechanism that involves the reduced expression of G-CSF by PVECs and alveolar macrophages.

Discussion

ABCA1 is a membrane protein that plays a key role in cholesterol efflux from cells via the reverse cholesterol transport pathway

(11). In the liver and intestine, where most HDL is synthesized, ABCA1 transfers lipids to lipid-poor apoA-I, which results in the formation of nascent HDL particles (14, 23). Nascent HDL has atheroprotective effects via the removal of excess cholesterol from macrophage foam cells by ABCA1 and other lipid transporters (11, 24). ABCA1 also functions as an anti-inflammatory receptor for apoA-I in macrophages via a mechanism that involves the activation of Janus kinase 2 and signal transducer and activator of transcription 3 signaling pathways (24, 25). Conversely, macrophages from *Abca1*^{-/-} mice have an increase in both cholesterol content and lipid raft microdomains that activate proinflammatory signaling pathways and up-regulate the expression of cytokines, chemokines, growth factors, scavenger receptors, and TLRs (24, 26–28). ABCA1 is also expressed by type I and type II alveolar epithelial cells in the lung, where it plays a major role in surfactant metabolism and lipid homeostasis (29, 30). Mice with a targeted disruption of the *Abca1* gene have a complete lack of circulating HDL, as well as an accumulation of lipid-laden macrophages and type II pneumocytes in the lung (31). Furthermore, *Abca1*^{-/-} mice have a phenotype of alveolar proteinosis and respiratory distress that is associated with cholesterol accumulation in tissue, surfactant, and macrophages (32).

Because apoA-I has been demonstrated to attenuate OVA-induced neutrophilic airway inflammation (10), we hypothesized that transgenic mice that overexpress hABCA1 might attenuate OVA-induced neutrophilic airway inflammation. First, we showed that PVECs and alveolar macrophages endogenously express ABCA1 in WT mice. Next, we used transgenic mice that express the h*Abca1* gene under the control of a 2.1-kb mouse Tie2 promoter and a 1.6-kb Tie2 enhancer to assess whether increased expression of the ABCA1 protein can attenuate OVA-induced neutrophilic airway inflammation (14). Tie2 is a receptor-like tyrosine kinase that is primarily expressed by endothelial cells and plays a key role in vascular endothelial development during embryogenesis (16). Consistent with its key role in the vascular endothelium, expression of hABCA1 mRNA in Tie2-hABCA1 mice under basal conditions has previously been shown to be 80-fold higher in lung endothelial cells than in peritoneal

Figure 5. Expression of CXC chemokines and adhesion molecules are not modified in the lungs from OVA-challenged Tie2-hABCA1 mice. Quantification of CXCL1 (A) and CXCL2 (B) protein levels in BALF ($n = 16$ –20 mice). $P = NS$, WT + OVA versus Tie2-hABCA1 + OVA. Quantification of CXCL5 protein levels in (C) BALF ($n = 19$ –20) and (D) isolated PVECs presented as pg of CXCL5 per μ g of cellular protein ($n = 14$ –16 mice; $P = NS$, WT + OVA versus Tie2-hABCA1 + OVA) were performed by ELISA. (E) Western blots of proteins from PVECs were reacted with antibodies against vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1, or E-selectin. The amount of β -actin present is shown as a control for equivalency of protein loading. A representative blot is shown from six to eight replicate experiments. Data presented are means ($\pm$ SEM).

macrophages (14). Furthermore, primary cultures of isolated aortic endothelial cells from Tie2-hABCA1 mice have been shown to have a 70% increase in ABCA1 protein as compared with those from control mice, which were associated with a 2.6-fold increase in cholesterol efflux to apoA-I (14). This increase in vascular endothelial cell ABCA1 expression is biologically relevant, as consumption of a high-fat, high-cholesterol diet by Tie2-hABCA1 mice resulted in a 40% increase in plasma HDL-C levels and a 40% decrease in aortic atherosclerotic lesions. This demonstrates that vascular endothelial expression of hABCA1 at this level is sufficient to protect mice against diet-induced atherosclerosis. Expression of Tie2, however, is not exclusive to vascular endothelial cells, as Tie2 can also be produced by early hematopoietic cells and proangiogenic monocytes (16, 33). Furthermore, Tie2 can be expressed by macrophages in the setting of inflammation, such as in rheumatoid

arthritis, and Tie2 expression by human monocyte-derived macrophages has been shown to be up-regulated by cytokines, such as IFN- γ and IL-10 (15, 19). Consistent with this, we show that Tie2-mediated expression of hABCA1 was induced in alveolar macrophages in the setting of OVA-induced neutrophilic airway inflammation.

Here, we demonstrate that OVA-challenged Tie2-hABCA1 mice had a significant reduction in neutrophilic airway inflammation that was associated with significant decreases in serum levels of OVA-specific IgE and airway epithelial thickness. Next, we investigated the mechanism by which increased expression of hABCA1 under the control of the Tie2 promoter attenuates OVA-induced neutrophilic airway inflammation. We did not find reduced expression of IL-17A or TNF, which are cytokines that act synergistically to induce neutrophilic airway inflammation (34), or CXC

chemokines (CXCL1, CXCL2, or CXCL5) that mediate neutrophil chemotaxis via the CXCR2 receptor (35, 36). We also did not find reduced expression of adhesion molecules that either slow leukocyte rolling, such as E-selectin, or mediate leukocyte activation and arrest, such as VCAM-1 and ICAM-1, by PVECs (37). However, because analyses were performed at the conclusion of the study, it cannot be excluded that differences in expression might have occurred at earlier time points. Instead, we show that the mechanism mediating the attenuated airway neutrophilia in OVA-challenged Tie2-hABCA1 mice involved the reduced expression of G-CSF by vascular endothelial cells and alveolar macrophages, which were associated with decreases in BALF levels of G-CSF. This is consistent with the increases in ABCA1 protein levels in vascular endothelial cells and alveolar macrophages from Tie2-hABCA1 mice. We cannot, however, exclude the possibility that G-CSF production might have also

Figure 6. Granulocyte colony-stimulating factor (G-CSF) levels are reduced in PVECs and BALF from OVA-challenged Tie2-hABCA1 mice. (A) Quantification of G-CSF mRNA levels in PVECs ($n = 10$ mice; $*P < 0.05$). (B) Quantification of G-CSF protein presented as picograms of G-CSF per microgram of PVEC lysate ($n = 13$ –16 mice). $*P < 0.004$, WT + OVA versus Tie2-hABCA1 + OVA. (C) Quantification of G-CSF protein levels presented as picograms of G-CSF per milliliter of BALF ($n = 18$ –20 mice). $*P < 0.0001$, WT + OVA versus Tie2-hABCA1 + OVA. (D) Quantification of G-CSF protein levels in serum ($n = 19$ –21 mice; $*P < 0.05$). (E) Quantification of blood neutrophils ($10^3/\mu\text{l}$; $n = 11$ –12 mice). (F) Quantification of BALF neutrophils 24 hours after the intratracheal administration of $2.5 \mu\text{g}$ of recombinant murine G-CSF (rmG-CSF) to WT and Tie2-hABCA1 mice ($n = 5$ mice; $*P = 0.0079$, Mann-Whitney test). Data presented are means ($\pm$ SEM).

been reduced in additional cell types. Furthermore, in reconstitution experiments, rmG-CSF, which is known to promote tissue neutrophilia via its ability to reduce cellular apoptosis and thereby prolong neutrophil survival, was administered to the lungs of OVA-challenged Tie2-hABCA1 mice, and restored BALF neutrophil numbers to levels comparable with those found in WT mice (38). These results are consistent with our previous study, which showed that airway neutrophilia is increased in OVA-challenged apoA-I^{-/-} mice by a mechanism that is primarily mediated by the enhanced expression of G-CSF (10). Collectively, our findings support the concept that an apoA-I/ABCA1 pathway negatively regulates OVA-induced neutrophilic airway inflammation by reducing G-CSF expression.

In addition to stimulating the generation of granulocytic precursors and

enhancing their terminal differentiation within the bone marrow, G-CSF is an essential regulator of neutrophil mobilization to the peripheral blood (39). Furthermore, *Abca1*^{-/-} *Abcg1*^{-/-} double-knockout mice, which have defects in cholesterol efflux from cells due to the deletion of ABC transporters, have increased mobilization of hematopoietic stem cells and extramedullary hematopoiesis driven by elevated serum G-CSF levels, due to increased production of IL-17 and IL-23 by splenic macrophages and dendritic cells (40). Similarly, *Ldlr*^{-/-} recipients of bone marrow cells with macrophage-specific double deletions of *Abca1*^{-/-} and *Abcg1*^{-/-} displayed increased bone marrow neutrophil production driven by increased plasma levels of G-CSF (41). Therefore, we also assessed whether the decreased neutrophilic airway inflammation in OVA-challenged Tie2-hABCA1 mice might be regulated by

reductions in systemic levels of IL-17, IL-23, or G-CSF. In our model, serum levels of IL-23 and IL-17 were below the limit of detection in both WT and OVA-challenged Tie2-hABCA1 mice. Although serum G-CSF levels were lower in OVA-challenged Tie2-hABCA1 mice than in WT mice, blood neutrophil counts were not different between the two groups. Furthermore, serum G-CSF levels were decreased in OVA-challenged WT mice as compared with saline-challenged WT mice, which is discordant with the OVA-induced increases in BALF neutrophils. Therefore, we believe that it is unlikely that systemic G-CSF levels played a primary role in the OVA-induced recruitment of neutrophils to the lung in our model. Instead, our data suggest that modulation of G-CSF levels in the lung, as evidenced by the concordant changes in BALF neutrophils and G-CSF levels in BALF, PVEC lysates, and alveolar macrophages,

Figure 7. Alveolar macrophages from OVA-challenged Tie2-hABCA1 mice have reduced G-CSF production. (A) Quantification of hABCA1 mRNA levels in alveolar macrophages isolated from BALF ($n = 5$ mice). * $P < 0.0001$, WT + OVA versus Tie2-hABCA1 + OVA. (B) The gating strategy used to analyze alveolar macrophages present in BALF from saline- and OVA-challenged WT and Tie2-hABCA1 mice is presented. Cellular debris and doublets were excluded using physical scatter properties of the cells. Single CD45⁺/side scatter intensity (SSC)^{hi}/CD11c⁺/sialic acid-binding immunoglobulin-type lectins (Siglec) F⁺/CD64⁺ cells were gated as alveolar macrophages, as shown in the image of flow-sorted BALF cells from an OVA-challenged Tie2-hABCA1 mouse. (C) The mean fluorescence intensity (MFI) of total (human and murine) ABCA1 expression by alveolar macrophages present in BALF was quantified by flow cytometry ($n = 9$ –11 mice). * $P < 0.01$, WT + OVA versus Tie2-hABCA1 + OVA. (D) Quantification of G-CSF mRNA levels in alveolar macrophages isolated from BALF ($n = 5$ mice). * $P < 0.0001$, WT + OVA versus Tie2-hABCA1 + OVA. (E) The MFI of murine G-CSF expression by alveolar macrophages present in BALF was quantified by flow cytometry ($n = 9$ –11 mice). * $P < 0.0001$, WT + OVA versus Tie2-hABCA1 + OVA. (F) WT and Tie2-hABCA1 mice were challenged with saline or OVA concurrent with intranasal administration of either rmG-CSF (2 $\mu\text{g}/\text{d}$) or vehicle control (saline) during Days 38–42, and BALF was collected on Day 43. The numbers of BALF neutrophils are shown ($n = 10$ –15 mice). * $P < 0.0001$, Tie2-hABCA1 + OVA + vehicle versus Tie2-hABCA1 + OVA + rmG-CSF. Data were pooled from two independent experiments. Data presented are means ($\pm$ SEM).

played a predominant role in regulating OVA-induced BALF neutrophilia.

It is also interesting to consider whether a causal relationship might exist between the reductions in neutrophilic airway inflammation and IgE levels. Neutrophils from patients with atopic eczema/dermatitis syndrome have been reported to enhance IgE production by B cells via galectin-3, which is consistent with the concordant reductions in BAL neutrophils and antigen-specific IgE levels in OVA-challenged Tie2-hABCA1 mice that were present in our model (42). We also cannot exclude the possibility that the decreases in IgE levels may have contributed to the reduction in BALF neutrophils. Activation of the high-affinity IgE receptor (FcεRI) on human neutrophils has been reported to induce the release of CXCL8 (IL-8), a human CXC chemokine with chemotactic activity primarily toward neutrophils (43). However, mice do not express CXCL8, and we did not find decreases in expression of other CXC chemokines that could mediate neutrophil chemotaxis in OVA-challenged Tie2-hABCA1 mice (44). IgE has also been shown to reduce neutrophil apoptosis, which may contribute to increased neutrophilic inflammation in subjects with allergic asthma (45). Therefore, we cannot exclude that the reductions in OVA-specific IgE levels in

OVA-challenged Tie2-hABCA1 mice may have contributed to enhanced neutrophil apoptosis with consequent decreases in BALF neutrophils.

Our results provide further evidence regarding the role of cholesterol metabolism in regulating the pathogenesis of inflammatory lung diseases, such as neutrophil-predominant asthma, and are consistent with an anti-inflammatory pathway that promotes cholesterol efflux via the ABCA1 transporter to apoA-I (14, 46). The question of whether there is an association between serum cholesterol levels and asthma in human populations has been investigated by several studies that have produced conflicting results, which may, in part, represent differences in methodology, age, genetic backgrounds, and the heterogeneity of HDL composition and subspecies (47). Future studies that focus on the lung microenvironment, where the apoA-I/ABCA1 pathway is likely to modulate the pathogenesis of asthma, may provide additional insights regarding the association between apoA-I or HDL and asthma. Consistent with this, a recent study has shown that apoA-I levels in BALF from subjects with asthma were reduced when compared with normal volunteers (48).

In conclusion, we have shown that overexpression of ABCA1 under the control of the Tie2 promoter significantly reduces

OVA-induced airway neutrophilia, IgE production, and airway epithelial remodeling. The mechanism by which the increased expression of ABCA1 by vascular endothelial cells and alveolar macrophages attenuated OVA-induced neutrophilic airway inflammation involved a significant decrease in G-CSF production. Furthermore, our results suggest that ABCA1 expression by vascular endothelial cells and alveolar macrophages may play an important role in down-regulating neutrophilic airway inflammatory responses in asthma. This provides additional evidence to support the concept of developing new therapies that target the apoA-I/ABCA1 pathway for the treatment of neutrophil-predominant asthma. ■

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