Apolipoprotein A-I Attenuates Ovalbumin-Induced Neutrophilic Airway Inflammation via a Granulocyte Colony–Stimulating Factor–Dependent Mechanism

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Apolipoprotein A-I (apoA-I) is a key component of high-density lipoproteins that mediates reverse cholesterol transport from cells and reduces vascular inflammation. We investigated whether endogenous apoA-I modulates ovalbumin (OVA)–induced airway inflammation in mice. We found that apoA-I expression was significantly reduced in the lungs of OVA-challenged, compared with saline-challenged, wild-type (WT) mice. Next, to investigate the role of endogenous apoA-I in the pathogenesis of OVA-induced airway inflammation, WT and *apoA-I*$^{-/-}$ mice were sensitized by intraperitoneal injections of OVA and aluminum hydroxide, followed by multiple nasal OVA challenges for 4 weeks. OVA-challenged *apoA-I*$^{-/-}$ mice exhibited a phenotype of increased airway neutrophils compared with WT mice, which could be rescued by an administration of a 5A apoA-I mimetic peptide. Multiple pathways promoted neutrophilic inflammation in OVA-challenged *apoA-I*$^{-/-}$ mice, including the upregulated expression of (*1*) proinflammatory cytokines (IL-17A and TNF-α), (*2*) CXC chemokines (CXCL5), (*3*) vascular adhesion molecules (i.e., vascular cell adhesion molecule–1), and (*4*) granulocyte colony–stimulating factors (G-CSF). Because concentrations of G-CSF in bronchoalveolar lavage fluid (BALF) were markedly increased in OVA-challenged *apoA-I*$^{-/-}$ mice, we hypothesized that enhanced G-CSF expression may represent the predominant pathway mediating increased neutrophilic inflammation. This was confirmed by the intranasal administration of a neutralizing anti–G-CSF antibody, which significantly reduced BALF neutrophilia by 72% in OVA-challenged *apoA-I*$^{-/-}$ mice, compared with mice that received a control antibody. We conclude that endogenous apoA-I negatively regulates OVA-induced neutrophilic airway inflammation, primarily via a G-CSF–dependent mechanism. Furthermore, these findings suggest that apoA-I may play an important role in modulating the severity of neutrophilic airway inflammation in asthma.

Keywords: airway inflammation; ovalbumin; neutrophil; apolipoprotein A-I; G-CSF

CLINICAL RELEVANCE

This study shows that endogenous apolipoprotein A-I negatively regulates ovalbumin-induced neutrophilic airway inflammation primarily via a granulocyte colony–stimulating factor–dependent mechanism. These findings suggest that apolipoprotein A-I may play an important role in modulating the severity of neutrophilic airway inflammation in asthma.

Asthma is a common disorder that affects approximately 300 million individuals worldwide (1). Asthma, which is manifested by airway inflammation, airway remodeling, and airway hyperreactivity, has a complex and heterogeneous pathogenesis that involves both innate and adaptive immune responses (2). Multiple cell types are involved in the pathogenesis of asthma, including dendritic cells, T cells, epithelial cells, mast cells, basophils, macrophages, and neutrophils. Furthermore, distinct inflammatory asthma phenotypes have been identified and are characterized by a predominance of eosinophils and neutrophils or the absence inflammatory cells (i.e., pauci-granulocytic) (3).

Apolipoproteins are key constituents of lipoprotein particles that primarily mediate lipid transport. Recent studies, however, suggest that apolipoprotein E (apoE) and apolipoprotein A-I (apoA-I) may also modulate the pathogenesis of asthma. For example, apoE, which is expressed by alveolar macrophages in the lung, was shown to negatively regulate airway hyperreactivity (AHR) and goblet-cell hyperplasia in a murine model of house dust mite (HDM)–induced asthma via an interaction with low-density lipoprotein (LDL) receptors that are expressed by ciliated airway epithelial cells (4). Furthermore, administration of an apoE mimetic peptide that corresponded to the LDL receptor–binding domain of the holo-apoE protein attenuated the induction of HDM-induced airway inflammation, AHR, and goblet-cell hyperplasia. Apolipoprotein A-I is the major protein constituent of high-density lipoproteins (HDLs) that promotes reverse cholesterol transport out of cells via binding to the ATP-binding cassette (ABC) transporter A1 (ABCA1) (5, 6). Based on the anti-inflammatory properties of apoA-I in vascular inflammation, we previously hypothesized that apoA-I might be used as a pharmacologic agent to attenuate the induction of asthma (7). We showed that the systemic administration of a 5A apoA-I mimetic peptide reduced airway inflammation, airway remodeling, and airway hyperreactivity in a murine model of HDM-induced asthma (8). In particular, HDM-challenged mice that received the 5A apoA-I mimetic peptide demonstrated significant reductions in the number of bronchoalveolar lavage fluid neutrophils, lymphocytes, and eosinophils. Similarly, the intranasal administration of D-4F, another apoA-I mimetic

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peptide, was also shown to reduce airway eosinophilia and airway resistance in ovalbumin (OVA)–challenged mice (9).

Although these studies provide evidence to suggest that the pharmacologic administration of apoA-I mimetic peptides may represent a novel treatment approach that attenuates the manifestations of asthma, the role of endogenous apoA-I in the pathogenesis of asthma is not known. Here, we show that apoA-I is expressed in the lung, where it functions as a negative regulator of neutrophilic airway inflammation in OVA-challenged mice via the suppression of multiple pathways that include proinflammatory cytokines (IL-17A and TNF-α), NF-κB signaling, chemokines (i.e., C-X-C motif ligand [CXCL]5), vascular adhesion molecules (i.e., vascular cell adhesion molecule–1; VCAM-1), and granulocyte colony–stimulating factor (G-CSF). Furthermore, we show that the ability of apoA-I to suppress OVA-induced airway inflammation is mediated primarily via a G-CSF–dependent mechanism. These results suggest that apoA-I in the lung may play an important role in modulating the pathogenesis and severity of neutrophilic airway inflammation in asthma.

MARTERIALS AND METHODS

More detailed methods are provided in the online supplement.

Model of OVA Challenge

Six-to-8-week-old C57BL/6 mice and *apoA-I*$^{-/-}$ mice were obtained from Jackson Laboratory (Bar Harbor, ME). On Days 1 and 8, mice were sensitized with an intraperitoneal injection of 50 μg OVA (Grade V) plus 0.4 mg of aluminum hydroxide (alum) (Sigma-Aldrich, St. Louis, MO). Mice received daily intranasal challenges with 150 μg OVA in 10 μl saline, 5 days per week, on Days 15–42. Control mice were sensitized and challenged with saline. For apoA-I reconstitution experiments, 10 mg/kg of the 5A apoA-I mimetic peptide or an irrelevant scrambled apolipoprotein E control peptide were nasally administered daily, 5 days per week on Days 15–42, 30 minutes after the OVA challenge (4, 8). For G-CSF inhibition experiments, 5 μg of a neutralizing, anti-mouse G-CSF antibody or control antibody (R&D Systems, Minneapolis, MN) were nasally administered 3 days per week during Days 15–42. Endpoints were analyzed 24 hours after the final challenge. For the single OVA challenge experiment, mice that had not been sensitized to OVA + alum received one intranasal administration of 150 μg OVA in 10 μl saline, and bronchoalveolar lavage (BAL) was performed 24 hours later. Experiments were approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute.

BAL and Lung Histopathologic Examination

BAL cell counts were performed using Diff-Quik–stained cytospin slides (Siemens Healthcare Diagnostics, Deerfield, IL) (4, 8). Lungs were inflated to 25 cm H_2O, fixed in formalin, dehydrated through gradient ethanol, and embedded in paraffin before the cutting of 5-μm sagittal sections. The quantification of goblet-cell hyperplasia was performed as previously described (4, 8).

Quantitative RT-PCR

Total RNA was isolated using the mirVana kit from lungs frozen in RNAlater (Ambion, Austin, TX). DNase-treated RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit and amplified using the TaqMan Universal PCR Master Mix, FAM dye-labeled TaqMan MGB probes, and a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA). Gene expression was quantified relative to 18S ribosomal RNA, using the control sample as a calibrator to calculate the difference in cycle threshold values.

Western Blotting

Western blots were performed as previously described (10). Lung proteins (12.5 μg) or BAL fluid (BALF) (30 μl) were separated by SDS-PAGE, using 10% Bis-Tris Nupage gels (Invitrogen, Carlsbad, CA). The antibodies against apoA-I, VCAM-1, and albumin were obtained from Abcam (Cambridge, MA), the anti-IκBα and anti–phospho-IκBα antibodies were obtained from Cell Signaling (Danvers, MA), and the anti–β-actin antibody was obtained from Sigma-Aldrich. Membranes were stripped using a Re-Blot recycling kit (Chemicon International, Temecula, CA). Densitometry was performed using NIH ImageJ software (National Institutes of Health, Bethesda, MD).

ELISA

The quantity of protein in BALF was measured using kits from R&D Systems. The lower limits of detection of the assays were 5.4 pg/ml for IL-17A, 5.9 pg/ml for TNF-α, 4.7 pg/ml for IFN-γ, 15.6 pg/ml for CXCL5, and 7 pg/ml for G-CSF.

Statistical Analysis

Results are presented as means ± SEM. One-way ANOVA with the Bonferroni multiple comparison test (GraphPad Prism, version 5.0a; GraphPad Software, Inc., La Jolla, CA) was used. $P < 0.05$ was considered significant.

RESULTS

Ovalbumin Challenge Down-Regulates Lung Apolipoprotein A-I Expression

Wild-type (WT) C57BL/6 mice and *apoA-I*$^{-/-}$ mice were sensitized to OVA (50 μg) complexed with alum (0.4 mg) by intraperitoneal injection on Days 1 and 8, followed by nasal OVA (150 μg) challenges, 5 days per week for 4 weeks, beginning on Day 15 and ending on Day 42. As shown in Figure 1, the quantity of apoA-I protein present in BALF from OVA-challenged WT mice was significantly reduced, compared with that in saline-challenged WT mice. Lung mRNA concentrations of apoA-I were also significantly reduced in OVA-challenged WT mice, whereas neither apoA-I mRNA nor protein was detected in *apoA-I*$^{-/-}$ mice.

Endogenous Apolipoprotein A-I Negatively Regulates Neutrophilic Airway Inflammation in OVA-Challenged Mice

Experiments were then performed to characterize the role of endogenous apoA-I in the modulation of airway inflammation, IgE production, goblet-cell hyperplasia, and AHR, which are key pathogenic manifestations of allergic asthma. As shown in Figure 2A, both the total number of BALF inflammatory cells and the number of BALF neutrophils were increased in OVA-challenged *apoA-I*$^{-/-}$ mice, compared with OVA-challenged WT mice. Although the numbers of BALF eosinophils, lymphocytes, and macrophages were increased after OVA challenge, no differences were evident between *apoA-I*$^{-/-}$ and WT mice. Consistent with the results of the BALF cell counts, lung histologic sections (Figure 2B) demonstrated an increase in peribronchial inflammatory cells, which primarily comprised neutrophils located

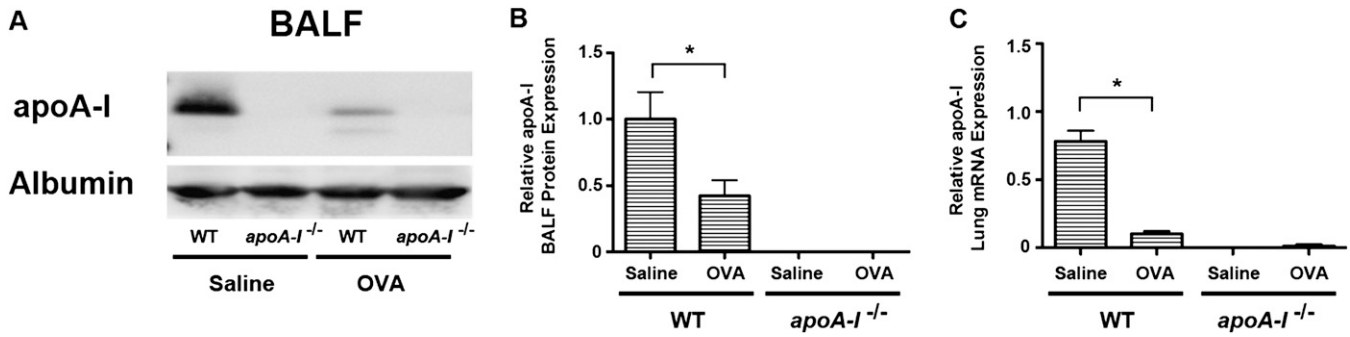

Figure 1. Apolipoprotein A-I (apoA-I) expression is attenuated in the lungs of ovalbumin-challenged mice. (*A*) The amount of apoA-I protein present in bronchoalveolar lavage fluid (BALF) from wild-type (WT) C57BL/6 mice and *apoA-I*$^{-/-}$ mice that had been challenged with nasal saline or ovalbumin (OVA) was assessed by Western blotting. Thirty microliters of BALF were loaded per lane. The amount of albumin present in BALF is shown as a control for the equivalency of protein loading. A representative blot is shown from six replicate experiments. (*B*) The relative expression of apoA-I compared with that of albumin was quantified by densitometry (*$P < 0.01$, $n = 6$, WT + saline versus WT + OVA). (*C*) The quantification of lung mRNA concentrations for apoA-I using quantitative RT-PCR is presented as relative mRNA expression (*$P < 0.001$, $n = 6$, WT + saline versus WT + OVA).

in the submucosa (Figure 2C). These submucosal inflammatory cells reacted with an anti–Gr-1 (Ly-6G) antibody, as shown by confocal immunofluorescence microscopy in Figure 2D, thus confirming the identity of the infiltrating inflammatory cells as neutrophils (11).

Next, experiments were performed to demonstrate that the increase in BAL neutrophils did not reflect an isolated innate immune response to the low concentrations of LPS present in the OVA, but instead reflected an adaptive immune response to the OVA. The quantity of LPS present in the OVA preparation was measured by a limulus amebocyte assay, and was found to correspond to 0.006 μg of LPS per challenge. This is a low amount that was previously associated with the development of Th2 responses in experimental models of murine asthma, characterized by increased numbers of BALF macrophages, lymphocytes, neutrophils, and eosinophils (12, 13). Furthermore, the administration of higher doses of LPS to the lung was previously demonstrated to result in a significant influx of neutrophils at 24 hours (14–16). Therefore, WT and *apoA-I*$^{-/-}$ mice received a single intranasal challenge of OVA or saline, and BALF was collected after 24 hours to assess whether the small amount of LPS present in the OVA preparation could induce pulmonary neutrophilia. As shown in Figure 3A, no significant increase was evident in the total number of BALF cells or the numbers of neutrophils, macrophages, eosinophils, or lymphocytes in either OVA-challenged WT or *apoA-I*$^{-/-}$ mice, compared with mice that received a saline challenge.

Apolipoprotein A-I reconstitution experiments were performed to confirm that the phenotype of increased neutrophilic airway inflammation in the OVA-challenged *apoA-I*$^{-/-}$ mice was causally mediated by the absence of apoA-I expression. To address this question, *apoA-I*$^{-/-}$ mice received either the 5A apoA-I mimetic peptide or an irrelevant control peptide via an intranasal route daily, 5 days per week for 4 weeks, 30 minutes after daily OVA challenges. As shown in Figure 3B, the number of total BALF cells and BALF neutrophils recovered from OVA-challenged *apoA-I*$^{-/-}$ mice that received the 5A apoA-I mimetic peptide was reduced, compared with those that received the scrambled control peptide. Furthermore, total BALF cells and BALF neutrophils were increased to the same extent in OVA-challenged *apoA-I*$^{-/-}$ mice that received the 5A apoA-I mimetic peptide as OVA-challenged WT mice. This demonstrates that administration of the 5A apoA-I mimetic peptide can rescue the phenotype of increased neutrophilic airway inflammation in OVA-challenged *apoA-I*$^{-/-}$ mice. This finding is consistent with the conclusion that endogenous apoA-I functions to attenuate excessive OVA-induced airway neutrophilia in WT mice.

To further confirm that multiple OVA challenges induced an adaptive rather than an innate immune response, serum concentrations of OVA-specific IgE were measured. As shown in Figure 4A, increases in OVA-specific serum IgE concentrations were observed in OVA-challenged, but not saline-challenged, WT and *apoA-I*$^{-/-}$ mice. Furthermore, serum concentrations of OVA-specific IgE were significantly reduced in OVA-challenged *apoA-I*$^{-/-}$ mice, compared with OVA-challenged WT mice. However, no difference was evident in goblet-cell hyperplasia or mRNA concentrations of mucin 5AC or chloride channel calcium activated 3 between OVA-challenged *apoA-I*$^{-/-}$ mice and WT mice (Figures 4B–4D). Neither WT nor *apoA-I*$^{-/-}$ mice demonstrated AHR in response to OVA challenge (data not shown), which may reflect the C57BL/6 genetic background that has been associated with a reduced susceptibility to the development of AHR (17). Taken together, these data demonstrate that *apoA-I*$^{-/-}$ mice have a phenotype of enhanced neutrophilic airway inflammation and reduced serum IgE concentrations in response to OVA challenge.

OVA-Induced IL-17A and TNF-α Expression and NF-κB Activation Are Increased in the Lungs of *apoA-I*$^{-/-}$ Mice

Next, we investigated the mechanisms mediating the enhanced neutrophilic airway inflammation in OVA-challenged *apoA-I*$^{-/-}$ mice. First, we examined whether the expression of Th1, Th2, and Th17 cytokines were modified. As shown in Figure 5, lung mRNA concentrations of IFN-γ and IL-17A were significantly increased in OVA-challenged *apoA-I*$^{-/-}$ mice, compared with OVA-challenged wild-type mice, whereas mRNA concentrations of IL-4, IL-5, and IL-13 were not increased. BALF IL-17A protein concentrations were also significantly increased in OVA-challenged *apoA-I*$^{-/-}$ mice, whereas BALF concentrations of IFN-γ were not detectable. In addition, both the percentage and mean fluorescence intensity of $CD3^+/CD4^+/IL\text{-}17A^+$ T cells present in BALF were increased in OVA-challenged *apoA-I*$^{-/-}$ mice, compared with OVA-challenged WT mice. Taken together, these results are consistent with the conclusion that that apoA-I suppresses OVA-induced increases in IL-17A expression by Th17 T cells as part of the mechanism by which neutrophilic airway inflammation may be attenuated.

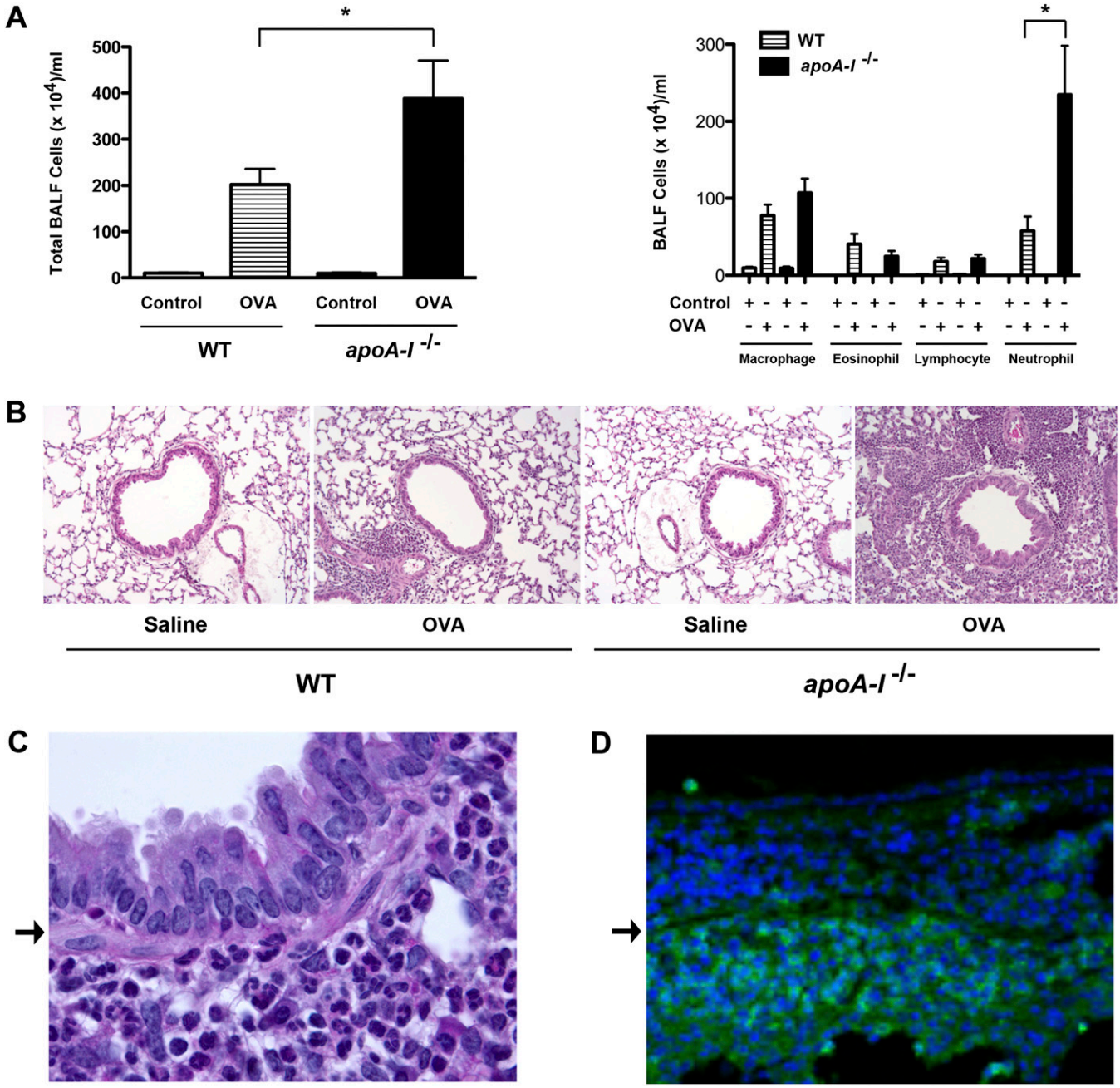

Figure 2. Neutrophilic airway inflammation is exacerbated in OVA-challenged *apoA-I*$^{-/-}$ mice. (*A*) Number of total BALF inflammatory cells (*left*) and inflammatory cell types (*right*) (n = 9–10 mice, *$P < 0.01$, *apoA-I*$^{-/-}$ + OVA versus WT + OVA). Data shown are representative of two experiments that produced similar results. (*B*) Representative histologic sections of lungs from WT and *apoA-I*$^{-/-}$ mice that had been challenged with saline or OVA, and were stained with hematoxylin and eosin (×200). (*C*) A representative higher magnification image (×1,000) of a hematoxylin and eosin–stained histologic section of a bronchus from an OVA-challenged *apoA-I*$^{-/-}$ mouse that demonstrated a submucosal infiltration comprised primarily of neutrophils. (*D*) Confocal immunofluorescence microscopy image (×400) of a bronchus from an OVA-challenged *apoA-I*$^{-/-}$ mouse that was reacted with an anti–Gr-1 antibody and a secondary antibody conjugated with Alexa Fluor 488 (*green*) that demonstrated a submucosal infiltration comprised primarily of neutrophils. The bronchus in *D* has a similar orientation to that of the bronchus in *C*, with the airway lumen located at the top of the image and the basement membrane denoted by an *arrow*.

IL-17A and TNF-α were recently shown to mediate airway neutrophilia synergistically via the enhanced expression of CXCL5 (18). Furthermore, tumor necrosis factor mediates its proinflammatory effects in part via the enhanced expression of VCAM-1 and G-CSF, which are TNF-α–responsive genes (19). Therefore, we investigated whether TNF-α expression might be up-regulated in the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice and thereby participate in the mechanism by which airway neutrophilia is increased. Compared with OVA-challenged WT mice, lung mRNA concentrations of TNF-α were significantly increased in OVA-challenged *apoA-I*$^{-/-}$ mice (Figure 6A). Similarly, TNF-α protein concentrations in BALF were increased in OVA-challenged *apoA-I*$^{-/-}$ mice, compared with OVA-challenged WT mice (Figure 6B). Furthermore, phospho-IκBα concentrations were significantly increased in OVA-challenged *apoA-I*$^{-/-}$ mice, which is consistent with an enhanced

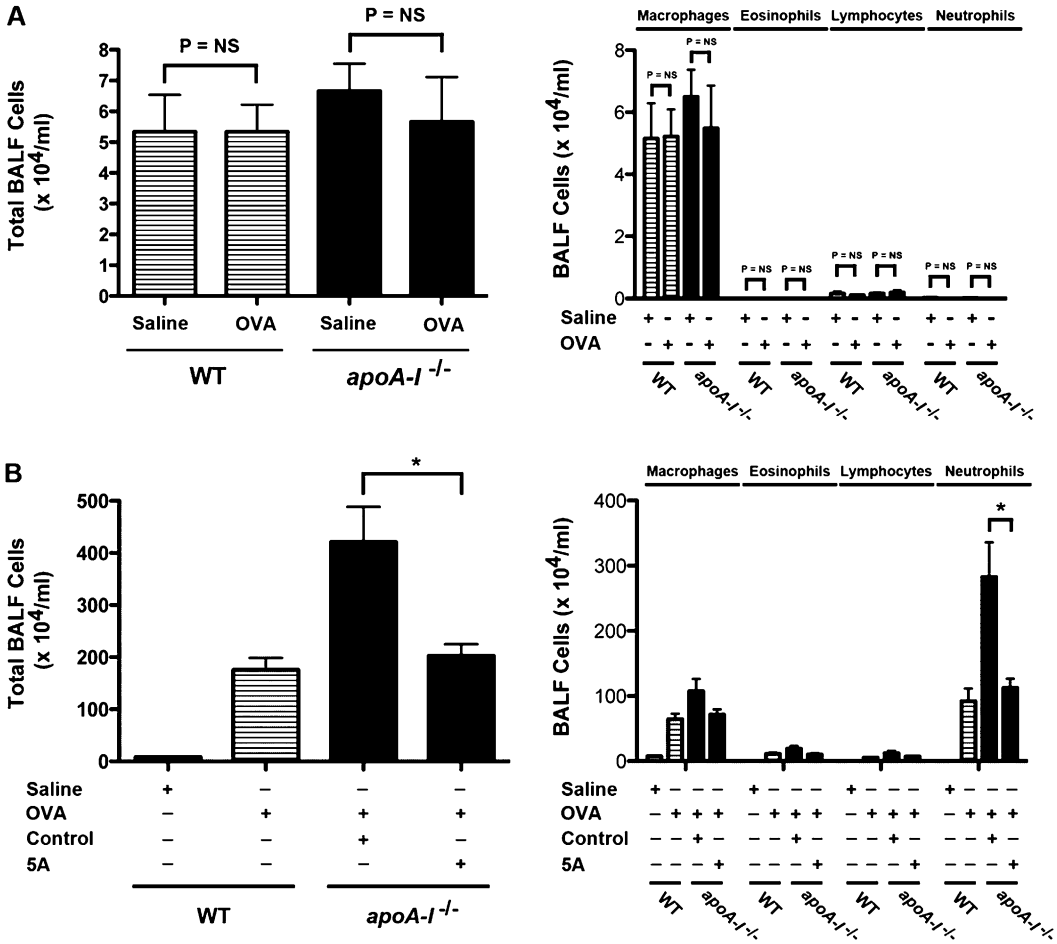

Figure 3. Modulation of BALF neutrophilia by a single OVA challenge and administration of the 5A apoA-I mimetic peptide in OVA-challenged *apoA-I*$^{-/-}$ mice. (*A*) Number of total inflammatory cells and inflammatory cell types in BALF 24 hours after a single OVA challenge ($n = 3$ mice). (*B*) Number of total inflammatory cells and inflammatory cell types in BALF from mice that had received an intranasal administration of either the 5A apolipoprotein A-I mimetic peptide (5A) or an irrelevant control peptide (control), concurrent with multiple daily saline or OVA challenges, 5 days per week for 4 weeks, after intraperitoneal sensitization with OVA plus aluminum hydroxide ($n = 10$ mice, $^*P < 0.01$). NS, no significance.

activation of NF-κB signaling pathways (Figures 6C and 6D). This suggests that increases in TNF-α expression and NF-κB signaling may participate in the mechanism by which airway neutrophilia is exacerbated in OVA-challenged *apoA-I*$^{-/-}$ mice.

Up-Regulated Expression of CXCL5, VCAM-1, and G-CSF in the Lungs of OVA-Challenged *apoA-I*$^{-/-}$ Mice

Experiments were then performed to investigate whether pathways that mediate airway neutrophilia downstream of IL-17A and TNF are attenuated by apoA-I. First, the expressions of CXC chemokines that mediate neutrophil chemotaxis were assessed. As shown in Figures 6E and 6F, lung mRNA and BALF protein concentrations of CXCL5, a chemokine that promotes neutrophil chemotaxis in the mouse, were increased in OVA-challenged *apoA-I*$^{-/-}$ mice, compared with OVA-challenged WT mice. In contrast, BALF protein concentrations of CXCL1 and CXCL2 were no different between OVA-challenged *apoA-I*$^{-/-}$ mice and OVA-challenged WT mice (Figure E1 in the online supplement). Taken together, this shows that endogenous apoA-I negatively regulates OVA-induced increases in expression of a chemokine pathway involving CXCL5 that is known to promote neutrophil chemotaxis.

Next, we examined the effects of apoA-I on cell surface adhesion molecules that mediate leukocyte–endothelial cell interactions. Lung mRNA concentrations of VCAM-1 were significantly increased in OVA-challenged *apoA-I*$^{-/-}$ mice compared with OVA-challenged WT mice, whereas lung mRNA concentrations of intercellular adhesion molecule–1 (ICAM-1) were not altered (Figure E2). As shown in Figures 7A and 7B, the quantity of VCAM-1 protein present in the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice was significantly increased. This suggests that OVA-induced increases in VCAM-1 protein expression may contribute to enhanced neutrophil transendothelial migration within the lung vasculature.

Because the pathways that mediate neutrophil chemotaxis and vascular adhesion are up-regulated in the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice, we assessed whether factors that promote the survival of recruited neutrophils are modified by apoA-I. After neutrophils have been recruited to the lung, survival factors, such as G-CSF, promote cell survival and thereby attenuate clearance from sites of inflammation (20). We found that lung mRNA concentrations of G-CSF (Figure 7C), but not of granulocyte–macrophage colony–stimulating factor (data not shown), were elevated in the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice compared with OVA-challenged WT mice. Similarly, the quantity of G-CSF protein present in BALF was significantly increased in OVA-challenged *apoA-I*$^{-/-}$ mice (Figure 7D).

Neutralization of G-CSF Attenuates OVA-Induced Increases in BALF Neutrophilia in *apoA-I*$^{-/-}$ Mice

Lastly, we hypothesized that G-CSF might play a key functional role in mediating the enhanced airway neutrophilia in *apoA-I*$^{-/-}$ mice, based on the marked increase in G-CSF protein concentrations in BALF from OVA-challenged *apoA-I*$^{-/-}$ mice compared with OVA-challenged WT mice. To investigate the role of G-CSF in mediating enhanced airway neutrophilia in *apoA-I*$^{-/-}$ mice, a neutralizing anti–G-CSF antibody or control antibody

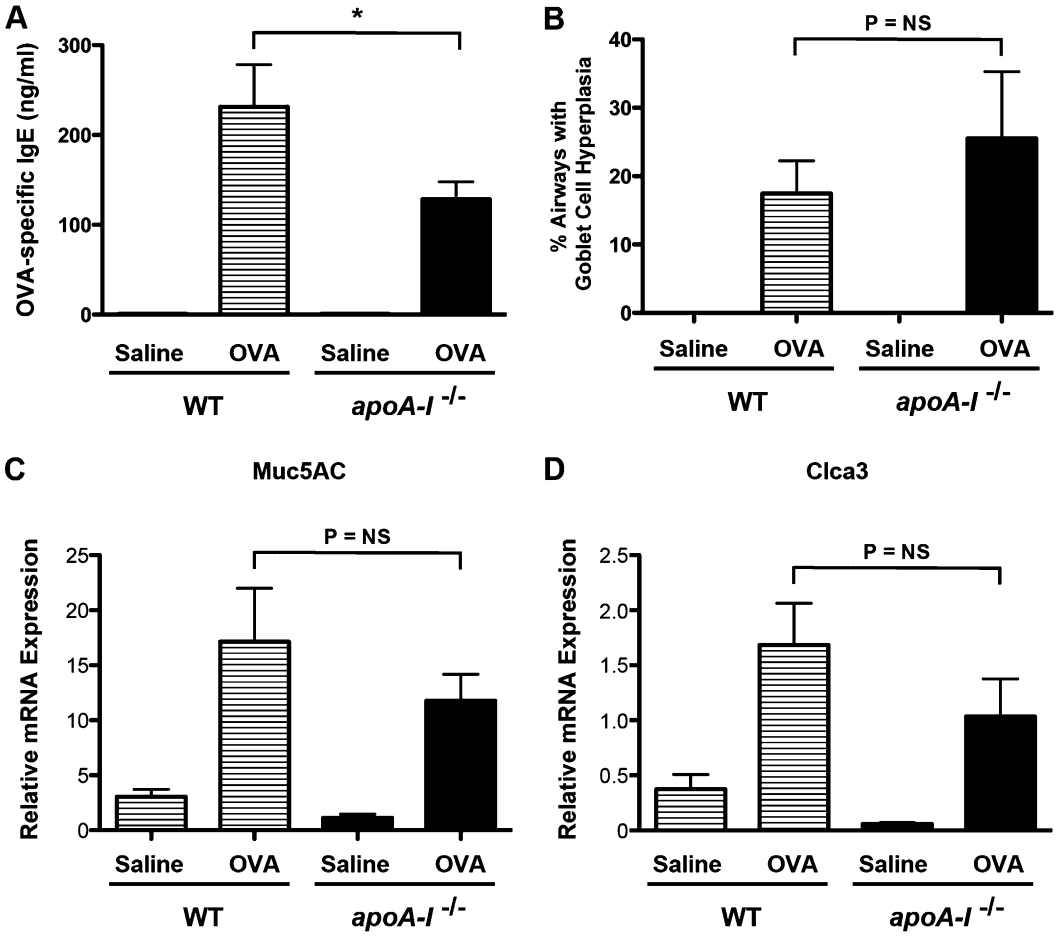

Figure 4. Attenuation of OVA-specific IgE in OVA-challenged *apoA-I*$^{-/-}$ mice. (*A*) Quantification of serum OVA-specific IgE (n = 9–10 mice, *$P < 0.01$, *apoA-I*$^{-/-}$ + OVA versus WT + OVA). (*B*) Goblet-cell hyperplasia is presented as the percentage of airways containing periodic acid–Schiff–positive cells (n = 5 mice, *P = NS, *apoA-I*$^{-/-}$ + OVA versus WT + OVA). We inspected 33.7 ± 1.9 airways in each mouse. Pooled data from two independent experiments are shown. (*C* and *D*) Quantification of lung mRNA concentrations for mucin 5AC and chloride channel calcium activated 3 (n = 6 mice, *P = NS, *apoA-I*$^{-/-}$ + OVA versus WT + OVA).

was nasally administered concurrent with the multiple nasal OVA challenges. As shown in Figure 7E, the number of neutrophils recovered in BALF was significantly reduced by 72% in OVA-challenged *apoA-I*$^{-/-}$ mice that received the neutralizing anti–G-CSF antibody, compared with OVA-challenged *apoA-I*$^{-/-}$ mice that received the control antibody. This demonstrates that

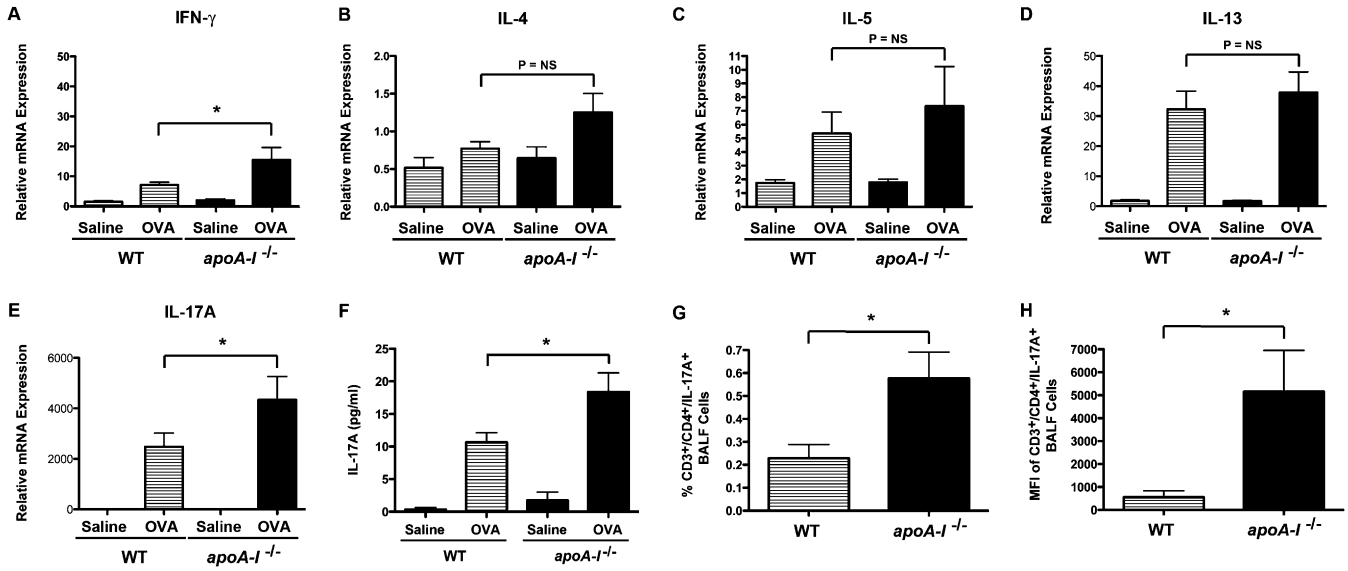

Figure 5. Enhanced expression of mRNA encoding IL-17A in the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice. (*A–E*) The quantification of lung mRNA concentrations for Th1 (*A*, IFN-γ), Th2 (*B*, IL-4; *C*, IL-5; and *D*, IL-13), and Th17 (*E*, IL-17A) cytokines was performed using quantitative RT-PCR, and is presented as relative mRNA expression (n = 6 mice, *$P < 0.05$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). (*F*) The quantification of IL-17A protein concentrations in BALF was performed by ELISA (n = 9 mice, *$P < 0.01$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). Data are representative of two experiments that produced similar results. (*G* and *H*) The percentage (*G*) and mean fluorescence intensity (MFI) (*H*) of CD3$^+$/CD4$^+$/IL-17A$^+$ cells present in BALF from OVA-challenged WT and *apoA-I*$^{-/-}$ mice (n = 6 mice, *$P < 0.05$).

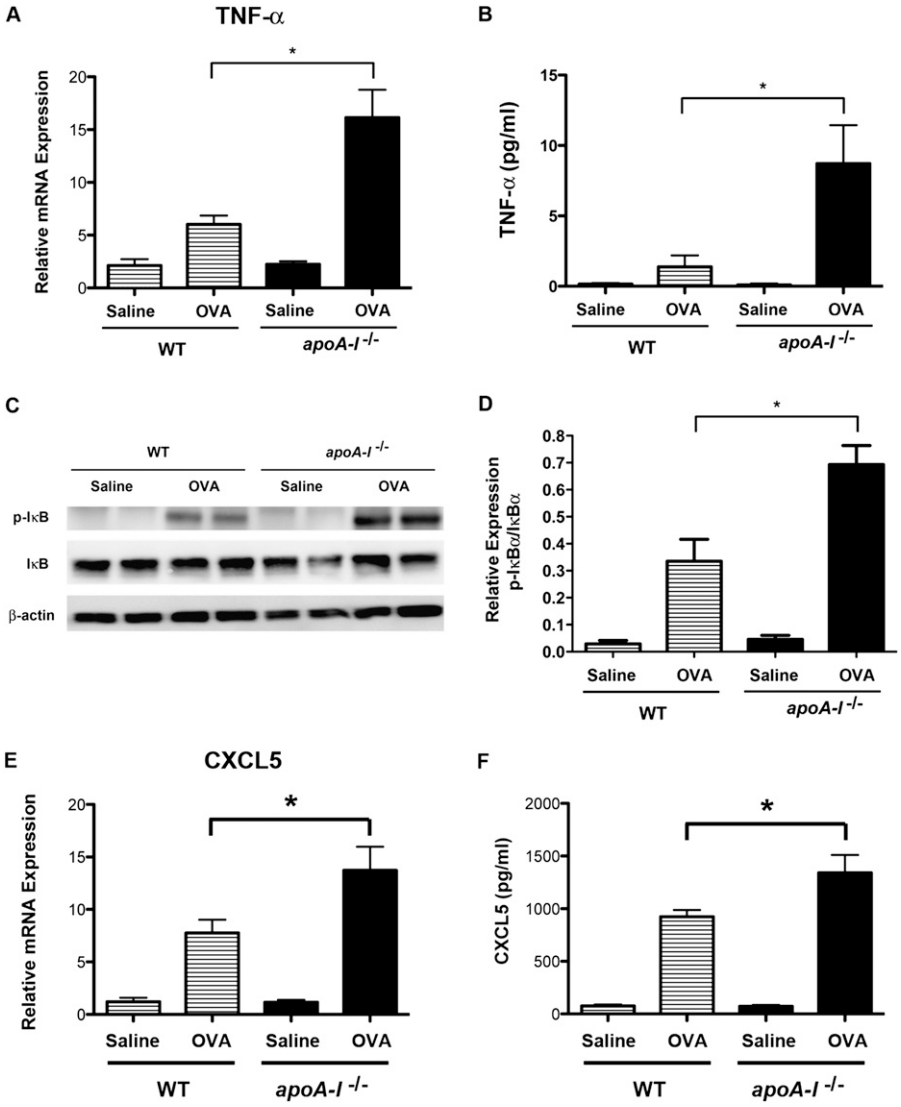

Figure 6. Enhanced expression of TNF-α and C-X-C motif ligand (CXCL)5 and increased activation of NF-κB in the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice. (*A*) The quantification of lung mRNA concentrations for TNF-α was performed using quantitative RT-PCR, and is presented as relative mRNA expression ($n = 6$ mice, $*P < 0.001$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). (*B*) The quantification of TNF-α protein concentrations in BALF was performed by ELISA ($n = 8$ mice, $*P < 0.01$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). (*C*) Western blots of lung proteins were reacted with antibodies directed against phospho-IκBα, IκBα, or β-actin. Lung proteins from two individual mice are shown. The amount of β-actin present is shown as a control for the equivalency of protein loading. A representative blot is shown from four replicate experiments. (*D*) The ratio of phospho-IκBα to IκBα protein was quantified by densitometry ($*P < 0.01$, $n = 4$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). (*E*) The quantification of lung mRNA concentrations for CXCL5 was performed using quantitative RT-PCR, and is presented as relative mRNA expression ($n = 6$ mice, $*P < 0.05$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). (*F*) The quantification of CXCL5 protein concentrations in BALF was performed by ELISA ($n = 9$–10 mice, $*P < 0.01$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). Data are representative of two experiments that produced similar results.

the ability of apoA-I to negatively regulate OVA-induced airway neutrophilia occurs primarily via a G-CSF–dependent mechanism.

DISCUSSION

Apolipoprotein A-I is a major component of HDLs that mediate reverse cholesterol transport out of cells and also have anti-inflammatory, antioxidant, antithrombotic, and antifibrotic properties (5, 6). Consistent with this, the administration of 5A and D-4F apoA-I mimetic peptides was shown to inhibit airway inflammation, AHR, and goblet-cell hyperplasia in experimental murine models of asthma (8, 9). Furthermore, the delivery of apoA-I or apoA-I mimetic peptides was found to exert beneficial effects in other experimental models of lung disease. For example, the administration of purified human apoA-I decreased the number of macrophages, neutrophils, and lymphocytes recovered from BALF in a murine model of bleomycin-induced pulmonary lung inflammation and fibrosis, and also abrogated bleomycin-induced collagen deposition (21). The administration of purified human apoA-I was reported to diminish the acute lung injury caused by LPS or lipoteichoic acid (22, 23). Similarly, the administration of D-4F apoA-I mimetic peptide in a murine model of influenza infection reduced lung viral titers and prevented arterial macrophage trafficking (24). Experiments using type II pneumocytes infected with influenza A suggested that the effect of D-4F was mediated by a reduction in proinflammatory oxidized phospholipid secretion, caspase-3 and caspase-9 activation, IL-6 production, and viral replication (25).

Although the pharmacologic administration of apoA-I mimetic peptides can be used to attenuate the manifestations of asthma in murine models, the role of endogenous apoA-I in asthma was not known (8, 9). Here, we investigated whether manifestations of experimental asthma are modified in *apoA-I*$^{-/-}$ mice. We used a murine model of chronic allergen exposure that induced increases in BALF eosinophils, lymphocytes, and neutrophils, as well as in goblet-cell hyperplasia and OVA-specific IgE. OVA-challenged WT mice demonstrated a decrease in expression of apoA-I in the lung at both the mRNA and protein levels that correlated with a decrease in apoA-I protein in BALF. Interestingly, a recent proteomic analysis of BALF proteins also found that concentrations of apoA-I were decreased in patients with asthma compared with patients without asthma (26). Furthermore, apoA-I was reported to be expressed in the lung by epithelial cells and macrophages (21). We confirmed by confocal immunofluorescence microscopy that apoA-I is expressed by alveolar epithelial cells. However, apoA-I did not colocalize with CD68^{+} alveolar macrophages (Figure E3).

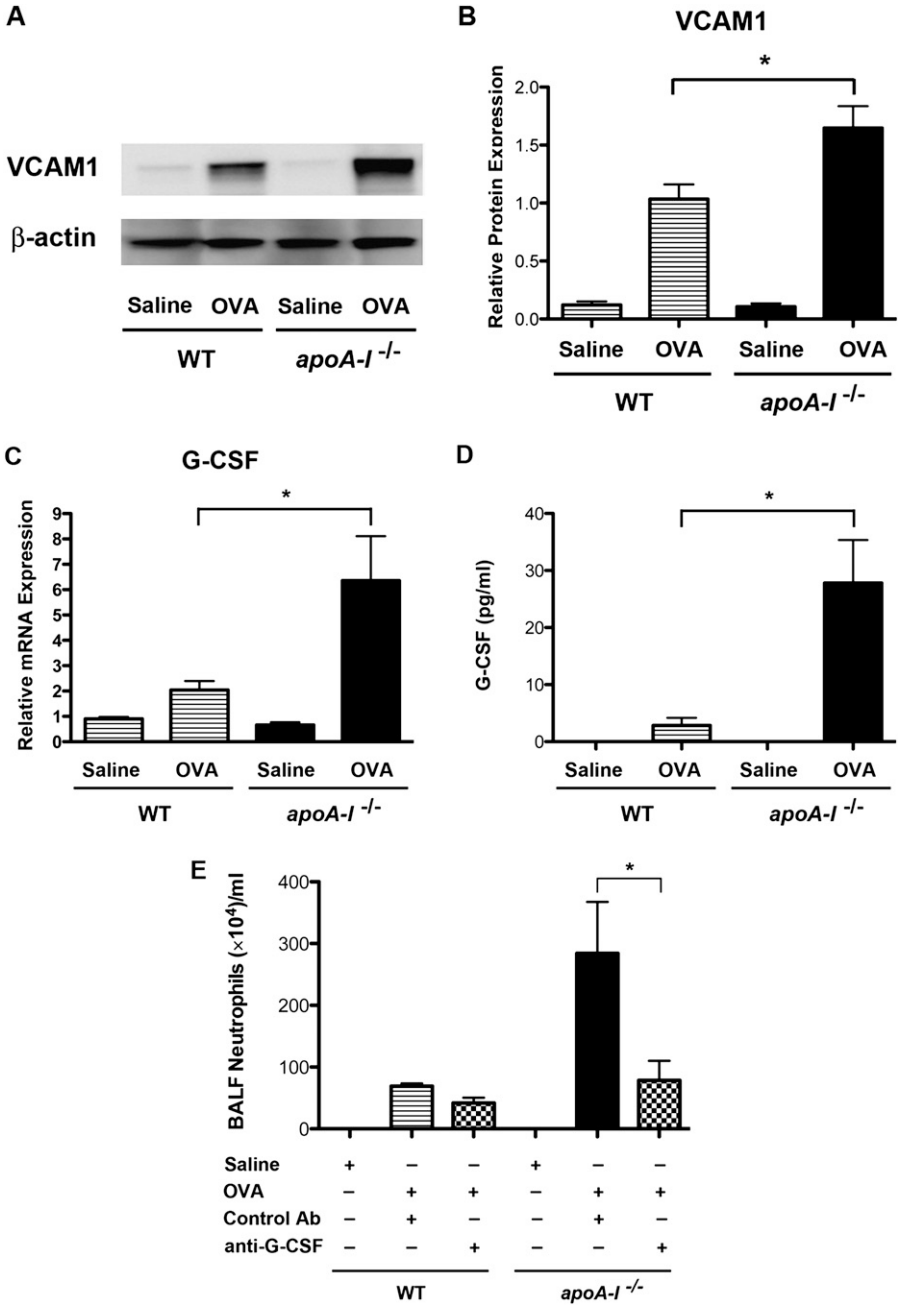

Figure 7. The role of vascular cell adhesion molecule–1 (VCAM-1) and granulocyte colony–stimulating factor (G-CSF) in OVA-induced BALF neutrophilia in *apoA-I*$^{-/-}$ mice. (*A*) Western blots of lung proteins were reacted with antibodies directed against VCAM-1 or β-actin. We loaded 12.5 μg of protein per lane. The amount of β-actin present is shown as a control for the equivalency of protein loading. A representative blot is shown from four replicate experiments. (*B*) The relative expression of VCAM-1 compared with that of albumin was quantified by densitometry (*$P < 0.01$, $n = 4$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). (*C*) The quantification of lung mRNA concentrations for G-CSF was performed, using quantitative RT-PCR presented as relative mRNA expression ($n = 6$ mice, *$P < 0.001$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). (*D*) The quantification of G-CSF concentrations in BALF was performed ($n = 9$–10 mice, *$P < 0.01$, OVA-challenged *apoA-I*$^{-/-}$ mice versus OVA-challenged WT mice). (*E*) WT and *apoA-I*$^{-/-}$ mice were challenged with saline or OVA, concurrent with a nasal administration of either a neutralizing anti–G-CSF antibody or a control immunoglobulin. Numbers of BALF neutrophils are shown ($n = 4$–5 mice, *$P < 0.001$). Data are representative of two experiments that produced similar results.

It is also important to point out that plasma may represent an additional source of apoA-I in the lung, especially in the setting of inflammatory lung diseases, which can be associated with microvascular injury and the leakage of plasma proteins into the lung.

Next, we assessed whether the manifestations of asthma were modulated by apoA-I. We found that the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice displayed a marked increase in the number of BALF neutrophils compared with wild-type mice, which suggests that endogenous apoA-I plays an important role in suppressing neutrophilic airway inflammation in asthma. Consistent with this conclusion, we showed that the phenotype of increased neutrophilic airway inflammation in OVA-challenged *apoA-I*$^{-/-}$ mice could be rescued by the administration of the 5A apoA-I mimetic peptide (8). Airway neutrophilia was shown to correlate with the severity of airflow obstruction in asthma, as well as with disease exacerbations, thus suggesting a causal relationship (27–32). Furthermore, patients with neutrophil-predominant asthma phenotypes demonstrate limited responsiveness to treatment with inhaled corticosteroids and would benefit from the development of alternative treatment approaches (33). Therefore, the identification of new mechanisms that modulate the pathogenesis of neutrophilic airway inflammation could represent a significant advance for patients with neutrophil-predominant forms of asthma.

We showed that endogenous apoA-I attenuated multiple pathways that participate in neutrophilic airway inflammation in OVA-challenged mice. First, mRNA and BALF protein concentrations of IL-17A were increased in the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice. The expression of IL-17 is associated with neutrophilia, AHR, and disease severity in patients with asthma (2, 34–37). IL-17 induces neutrophilic airway inflammation via the generation of CXC chemokines (CXCL1, CXCL2, and CXCL5) and granulocyte colony-stimulating factors (G-CSF). Furthermore, OVA was shown to induce neutrophilic airway inflammation via an IL-17–dependent pathway involving CXCL5

(epithelial cell–derived neutrophil-activating peptide–78; ENA-78) and CXCR2 (38). It was recently demonstrated, however, that the intratracheal instillation of IL-17A alone is not sufficient to induce increases in BALF concentrations of CXCL5, G-CSF, or BAL neutrophils, but instead requires a synergistic effect with TNF-α (18). Consistent with this, we found that OVA-challenged *apoA-I*$^{-/-}$ mice also demonstrated significant increases in lung mRNA and BALF concentrations of TNF-α, as well as NF-κB activation.

The role of CXC chemokines and their receptors in mediating the increase in airway neutrophilia in OVA-challenged *apoA-I*$^{-/-}$ mice was also investigated. Patients with severe exacerbations of asthma exhibited increases in bronchial mucosal neutrophils, CXCL5, CXCL8, CXCR1, and CXCR2 (39). Although CXCL8 (IL-8) is a key chemokine mediating neutrophil chemotaxis in humans, it is not present in mice (40). Therefore, other chemokines, such as CXCL1 (growth-related oncogene α and keratinocyte-derived chemokine), CXCL2 (macrophage inflammatory protein [MIP]2α), CXCL3 (MIP2β), and CXCL5 (ENA-78), were shown to mediate neutrophil chemotaxis in mice (38, 40). We found that BALF concentrations of CXCL5, but not of CXCL1 or CXCL2, were increased in OVA-challenged *apoA-I*$^{-/-}$ mice compared with OVA-challenged WT mice. Taken together, these results indicate that endogenous apoA-I functions to attenuate OVA-induced neutrophilic airway inflammation by the down-regulation of multiple pathways that recruit neutrophils to the lung, which include proinflammatory cytokines (IL-17 and TNF-α) and chemokines (CXCL5) (40).

The transendothelial migration of neutrophils that are recruited to sites of inflammation requires cellular adhesion to activated vascular endothelial cells (41). VCAM-1 plays an important role in both the adhesion and transendothelial migration of neutrophils via interactions with integrins such as α9β1, expressed in human neutrophils, and α4β1, expressed in murine and rat neutrophils (41–44). Furthermore, IL-17A and TNF-α, which are up-regulated in OVA-challenged *apoA-I*$^{-/-}$ mice, are important stimuli for the expression of both VCAM-1 and G-CSF (41, 44). In addition, VCAM-1 expression in vascular endothelial cells in the setting of OVA challenge was shown to be CXCR2-dependent (45). Here, we show that VCAM-1 expression was significantly increased in the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice that demonstrated enhanced expression of IL-17A and TNF-α. The expression of G-CSF was also significantly up-regulated in the lungs of OVA-challenged *apoA-I*$^{-/-}$ mice. Although G-CSF contributes to granulocytopoiesis in bone marrow, the extent of tissue neutrophilia is regulated by the rate of cellular apoptosis (41). G-CSF can enhance tissue neutrophilia by preventing apoptosis and thereby prolonging neutrophil survival in the lung (46). Taken together, these data suggest that the mechanism by which apoA-I attenuates OVA-induced neutrophilic airway inflammation also involves the down-regulation of VCAM-1 and G-CSF expression in the lung.

Although multiple pathways that mediate OVA-induced airway neutrophilia were up-regulated in *apoA-I*$^{-/-}$ mice, the most marked increase appeared to occur in G-CSF protein concentrations in BALF. Therefore, we hypothesized that the attenuation of G-CSF expression may represent the primary mechanism by which apoA-I negatively modulates OVA-induced neutrophilic airway inflammation. Consistent with this hypothesis, OVA-challenged *apoA-I*$^{-/-}$ mice that received an intranasal administration of a neutralizing anti–G-CSF antibody demonstrated a significant reduction in airway neutrophilia, compared with OVA-challenged *apoA-I*$^{-/-}$ mice that received a control antibody. This demonstrates that endogenous apoA-I primarily attenuates OVA-induced airway neutrophilia by suppressing the expression of G-CSF.

Further studies are ongoing to identify the relevant target cells that mediate the effects of apoA-I in the lung. Apolipoprotein A-I and the 5A apoA-I mimetic peptide both induce reverse cholesterol transport out of cells via interactions with the ATP-binding cassette transporter, ABCA1 (5, 7, 47). Thus, it is reasonable to speculate that lung cells that express ABCA1, such as alveolar macrophages, type II pneumocytes, vascular endothelial cells, and the lining epithelium of small bronchioles, mediate the ability of apoA-I to attenuate OVA-induced neutrophilic airway inflammation (48–50). Moreover, apoA-I was reported to bind LPS and directly inactivate its biological activity (51). This raises the additional possibility that the anti-inflammatory mechanism by which apoA-I attenuates neutrophilic airway inflammation could involve the direct binding and sequestration of key proinflammatory molecules or proteins.

In conclusion, we have shown that apoA-I in the lung functions as an endogenous negative regulator of multiple pathways that mediate neutrophilic airway inflammation in OVA-challenged mice. These pathways include the up-regulated expression of (*1*) proinflammatory cytokines (IL-17A and TNF-α), (*2*) NF-κB signaling, (*3*) CXC chemokines that mediate neutrophil recruitment (CXCL5), (*4*) vascular adhesion molecules (VCAM-1), and (*5*) colony-stimulating factors (G-CSF). Furthermore, we have shown that the ability of apoA-I to attenuate OVA-induced neutrophilic airway inflammation is mediated primarily via a G-CSF–dependent mechanism. Taken together, these data suggest that endogenous apoA-I may be an important negative modulator of neutrophilic airway inflammation in asthma.

Author disclosures are available with the text of this article at www.atsjournals.org.

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