

ORIGINAL ARTICLE

Rhinitis, Sinusitis, and Upper Airway Disease



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Apolipoprotein A-IV acts as an endogenous anti-inflammatory protein and is reduced in treatment-naïve allergic patients and allergen-challenged mice

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Abstract

Background: Recent studies pointed to a crucial role for apolipoproteins in the pathogenesis of inflammatory diseases. However, the role of apolipoprotein-IV (ApoA-IV) in allergic inflammation has not been addressed thoroughly thus far.

Objective: Here, we explored the anti-inflammatory effects and underlying signaling pathways of ApoA-IV on eosinophil effector function in vitro and in vivo.

Methods: Migratory responsiveness, Ca²⁺-flux and apoptosis of human peripheral blood eosinophils were assessed in vitro. Allergen-driven airway inflammation was assessed in a mouse model of acute house dust mite-induced asthma. ApoA-IV serum levels were determined by ELISA.

Results: Recombinant ApoA-IV potently inhibited eosinophil responsiveness in vitro as measured by Ca²⁺-flux, shape change, integrin (CD11b) expression, and chemotaxis. The underlying molecular mechanism involved the activation of Rev-ErbA- α and induced a PI3K/PDK1/PKA-dependent signaling cascade. Systemic application of ApoA-IV prevented airway hyperresponsiveness (AHR) and airway eosinophilia in mice following allergen challenge. ApoA-IV levels were decreased in serum from allergic patients compared to healthy controls.

Conclusion: Our data suggest that ApoA-IV is an endogenous anti-inflammatory protein that potently suppresses effector cell functions in eosinophils. Thus, exogenously applied ApoA-IV may represent a novel pharmacological approach for the treatment of allergic inflammation and other eosinophil-driven disorders.

KEYWORDS

allergic inflammation, apolipoprotein A-IV, chronic rhinosinusitis, eosinophils, house dust mite-induced asthma model, PI3-kinase, Rev-Erb- α

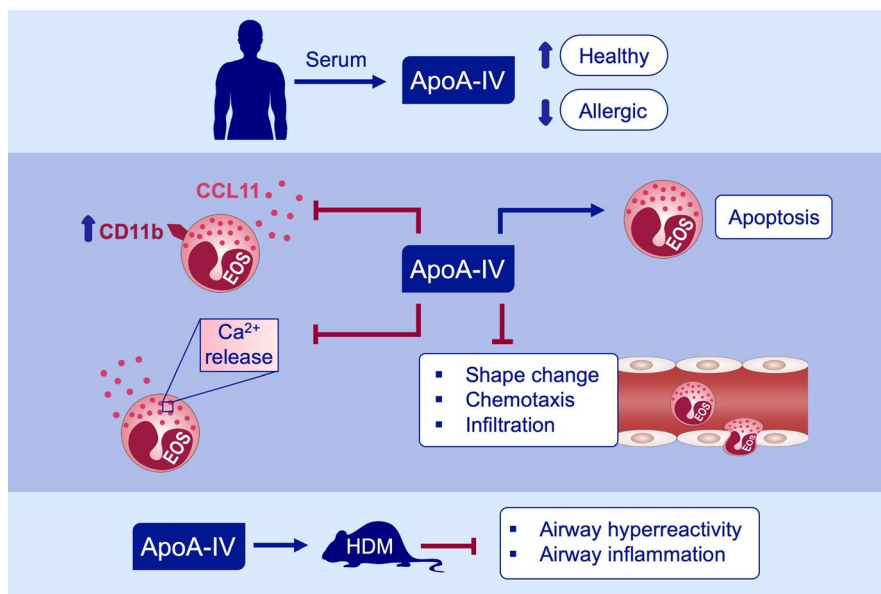
1 | INTRODUCTION

Eosinophils make up to 5% of circulating human blood leukocytes in healthy individuals and normally survive up to two days in blood.

However, this period may be extended by eosinophil-activating cytokines under inflammatory conditions such as infectious and allergic diseases.¹ Activated eosinophils are a major source of reactive oxygen species (ROS), cytotoxic proteins, and proinflammatory

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GRAPHICAL ABSTRACT

ApoA-IV is decreased in serum from untreated allergic patients compared to healthy controls. ApoA-IV potently inhibits eosinophil function in vitro. Systemic application of ApoA-IV reduces airway hyperreactivity and airway eosinophilia in a murine model of allergic asthma. ApoA-IV, apolipoprotein A-IV; HDM, house dust mite

cytokines,² and thereby modulate the immune microenvironment and promote several immunoregulatory functions. Eosinophils are involved in antigen-presentation³ and T-cell activation,⁴ interact with and activate other immunocompetent cells such as dendritic cells,⁵ mast cells,⁶ macrophages,⁷ and neutrophils.⁸ Moreover, activated eosinophils signal to and activate resident tissue cells such as epithelial cells,⁹ endothelial cells,¹⁰ goblet cells,¹¹ smooth muscle cells,¹² fibroblasts,¹³ and neurons,¹⁴ overall leading to the progression of inflammation, mucus secretion, tissue remodeling, and angiogenesis.¹⁵ Thus, eosinophils are potent effectors and modulators of various diseases ranging from bronchial asthma¹⁶ and atopic dermatitis¹⁷ to eosinophilic esophagitis,¹⁸ colitis ulcerosa¹⁹ and hyper-eosinophilic syndrome.²⁰ In asthmatics, levels of eosinophil granule proteins such as eosinophil cationic protein (ECP) or eosinophil peroxidase (EPO) largely correlate with disease severity.²¹ Moreover, patients who receive treatment based on eosinophil counts in sputum have significantly fewer exacerbations than patients treated according to standard therapy.²² Of note, eosinophilic inflammation of the upper airways may also occur independent of allergy as observed in chronic rhinosinusitis (CRS) patients.²³ Similar to allergies, CRS causes not only physical suffering, but also impacts psychological well-being and daily functioning. Patients with eosinophilic CRS represent a unique subtype and remain largely resistant to medical and surgical interventions. Thus, therapies that specifically target eosinophilic expansion and effector functions are urgently needed.

The apolipoprotein ApoA-IV is—to some extent—found on chylomicrons and HDL in plasma; however, its lipid-free form is predominant in circulation,²⁴ where it is presumed to play anti-inflammatory roles. In fact, the expression of human ApoA-IV in ApoE^{-/-} mice protected them from oxidative stress, decreased the secretion of proinflammatory cytokines after LPS administration and reduced

the formation of atherosclerotic lesions.^{25,26} Furthermore, in an experimental model of DSS-induced colitis, ApoA-IV inhibited leukocyte and platelet adhesive interactions and suppressed the upregulation of P-selectin on colonic endothelium.²⁷ In humans, ApoA-IV was found to inhibit histamine release from basophils in vitro,²⁸ and, interestingly, ApoA-IV levels increased in the blood of allergic rhinitis patients following sublingual immunotherapy and were inversely correlated with symptom-medication scores.²⁸

In this study, we set out to explore whether the anti-inflammatory properties of ApoA-IV also extend to eosinophil effector function in vitro and in a mouse model of allergen-induced pulmonary inflammation. Moreover, we assessed ApoA-IV levels in patients with eosinophil-driven diseases such as allergy and chronic rhinosinusitis. Our data clearly indicate that ApoA-IV is a potential resolution factor in eosinophilic inflammation and might have beneficial effects on eosinophil-driven diseases.

2 | METHODS

Detailed description of patient cohorts, ethical permits, materials, and procedures is provided in the Methods section in this article's Online Repository (Appendix S1).

2.1 | Isolation peripheral blood eosinophils

Human peripheral blood eosinophils were isolated from citrated whole blood from allergic or healthy donors. In brief, erythrocytes were removed by dextran sedimentation and polymorphonuclear leukocytes (pellet) were separated from mononuclear cells (buffy coat) by density gradient centrifugation using Histopaque 1077. Eosinophils were

separated from neutrophils in the polymorphonuclear leukocyte fraction by negative magnetic selection using the MACS cell separation system (Eosinophil Isolation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany) with a resulting purity of typically $\geq 98\%$.²⁹

2.2 | Shape change assay

Eosinophil shape change was assessed in polymorphonuclear leukocyte (PMNL) preparations and monitored by flow cytometry as an increase in the forward scatter signal.³⁰

2.3 | CD11b-upregulation

To assess CD11b upregulation, PMNL samples or citrated whole blood were stained with PE-Cy5-anti-CD16 and PE-anti-CD11b Abs and measured by flow cytometry.³¹

2.4 | Calcium flux

Intracellular Ca^{2+} release from purified human eosinophils was detected by flow cytometry using the Ca^{2+} sensitive dye Fluo 3-AM.²⁹

2.5 | Chemotaxis

Eosinophil chemotaxis experiments were done with purified eosinophils, whereas neutrophil chemotaxis was performed in separate experiments with PMNL preparations. Chemotaxis assays were performed in a 48-well micro chemotaxis chamber using PVP-free polycarbonate filters with a pore size of 5 μm . Migrated cells were enumerated by flow cytometry.³² Therefore, eosinophils and neutrophils were gated by their forward and side scatter properties and by autofluorescence.

2.6 | Cholesterol-rich microdomain (lipid raft) assessment

Lipid raft abundance was quantified by flow cytometry in purified eosinophils stained with FITC-cholera toxin B.³³

2.7 | CCR3 staining

CCR3 expression was evaluated by flow cytometry in PE-anti-CD193 (CCR3) stained purified eosinophils.

2.8 | Apoptosis assay

Purified eosinophils were stained with FITC-annexin-V/PI and analyzed by flow cytometry.

2.9 | House dust mite-induced allergic lung inflammation

The HDM model was performed as described by Plantinga et al³⁴ In brief, Balb/c mice were sensitized i.n. with 1 μg HDM extract on day

1 and were challenged intranasally with 10 μg HDM per day from day 7 to day 11. On day 15, lung function testing was performed or BAL fluid, bone marrow, and spleens were taken. Leukocytes were analyzed by flow cytometry.

2.10 | Statistical analysis

Data are shown as mean + or $\pm$ SEM for n observations, where n denotes independent experiments with cells from different donors. Comparisons of groups were performed as appropriate using Student's t test or Mann-Whitney test, 1-way ANOVA followed by Dunnett's or Tukey's post hoc test or 2-way ANOVA for repeated measurements followed by Bonferroni's post hoc test to determine the levels of significance for each group. Probability values of $P < .05$ were considered as statistically significant.

3 | RESULTS

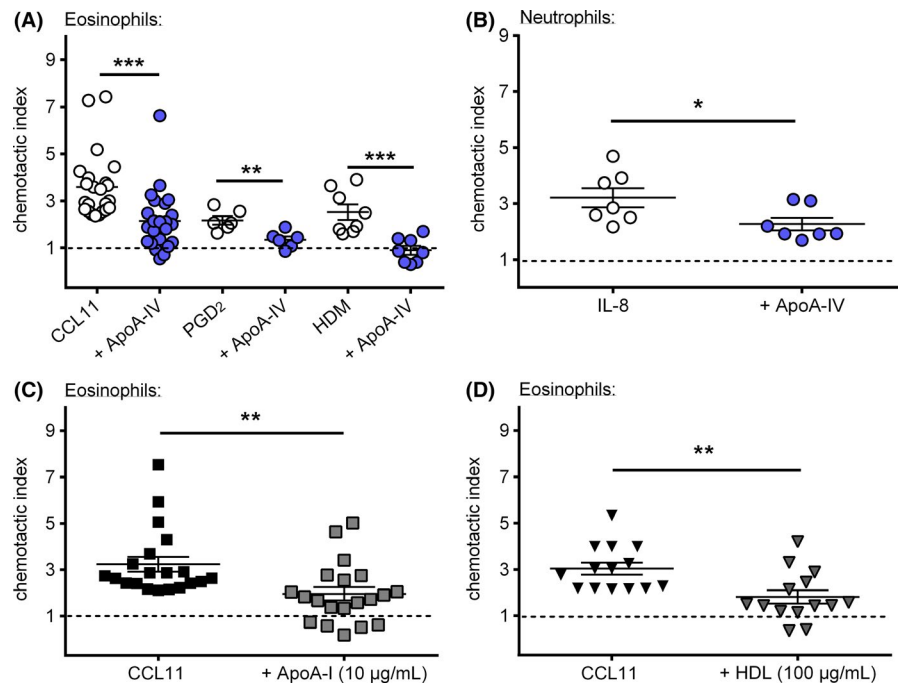
3.1 | Preliminary experiments

To define the working dose of ApoA-IV as well as ApoA-I and HDL for following assays, preparatory chemotaxis experiments were performed. As shown in Figure S1, ApoA-IV (1-10 $\mu\text{g}/\text{mL}$), ApoA-I (1-30 $\mu\text{g}/\text{mL}$), and HDL (10-100 $\mu\text{g}/\text{mL}$) concentration-dependently impaired eosinophil migration toward CCL11 (3 nmol/L). Based on these results, 1-3 $\mu\text{g}/\text{mL}$ ApoA-IV, 10 $\mu\text{g}/\text{mL}$ ApoA-I, and 100 $\mu\text{g}/\text{mL}$ HDL were used in subsequent experiments.

3.2 | ApoA-IV impairs eosinophil responsiveness

Since the effects of ApoA-IV on eosinophils have not been deciphered so far, we first explored the anti-inflammatory capacity of ApoA-IV in assays of eosinophil shape change, integrin upregulation, and intracellular Ca^{2+} mobilization. When encountering a chemotactic factor, such as CCL11, eosinophils immediately prepare for diapedesis through the endothelium by rearranging their cytoskeleton. Such morphological changes can be detected by flow cytometry as increases in the forward scatter properties of the cells. We studied the effects of ApoA-IV on eosinophil shape change in PMNL samples from healthy nonallergic donors. We pretreated samples with recombinant ApoA-IV or vehicle for 30 minutes, followed by stimulation with serial dilutions of CCL11, and shape change was monitored by flow cytometry. Of particular interest, already very low concentrations of ApoA-IV (1 $\mu\text{g}/\text{mL}$) led to a statistically significant decrease of eosinophil shape change, as the responsiveness to CCL11 was decreased by 50% (Figure S2A). Besides shape change, upregulation of adhesion molecules such as $\alpha_{\text{m}}\beta_2$ integrins (CD11b/CD18; Mac-1) is another precondition for eosinophil migration. To measure the impact of ApoA-IV on integrin mobilization, we pretreated human eosinophils in PMNL fractions with ApoA-IV (1 $\mu\text{g}/\text{mL}$) or vehicle and stimulated again with CCL11. Coinciding with the effect on shape change, ApoA-IV clearly reduced the presence of CD11b molecules on the cell surfaces by 30% (Figure S2B). Beside

FIGURE 1 ApoA-IV potently inhibits eosinophil chemotaxis. (A) Incubation with ApoA-IV (1 $\mu\text{g/mL}$) for 30 min reduced eosinophil chemotaxis toward CCL11 (3 nmol/L), PGD₂ (30 nmol/L) and house dust mite extract (HDM, 100 $\mu\text{g/mL}$) (n = 7–23) as well as (B) neutrophil chemotaxis toward IL-8 (10 nmol/L) (n = 7). Treatment with (C) ApoA-I (10 $\mu\text{g/mL}$) (n = 20) and (D) HDL (100 $\mu\text{g/mL}$) (n = 14) for 30 min similarly decreased eosinophil chemotaxis toward CCL11 (3 nmol/L). Chemotaxis of purified eosinophils and neutrophils in PMNL fractions was performed in duplicates in a 48-well microchemotaxis chamber. Migrated cells were enumerated by flow cytometry. Data are shown as mean $\pm$ SEM; * $P < .05$, ** $P < .01$, *** $P < .01$; Student's *t* test



morphological changes and integrin upregulation, CCL11 induces a rapid and transient rise in intracellular Ca^{2+} ions. Similar to shape change and CD11b, we found that ApoA-IV reduced this CCL11-induced Ca^{2+} mobilization in a concentration-dependent fashion. As presented in Figure S2C, Ca^{2+} flux was diminished by 30% and 45% in the presence of 1 and 3 $\mu\text{g/mL}$ of ApoA-IV, respectively.

3.3 | ApoA-IV inhibits eosinophil chemotaxis

Having established that ApoA-IV affects cellular responsiveness of eosinophils, we next investigated the direct impact of ApoA-IV—in comparison to ApoA-I or isolated HDL—on eosinophil migration. Chemotaxis assays were carried out in a modified Boyden chamber using a 48-well microchemotaxis assembly. As displayed in Figure 1A, ApoA-IV (1 $\mu\text{g/mL}$) not only inhibited eosinophil chemotaxis toward CCL11 (3 nmol/L), but also toward prostaglandin D₂ (30 nmol/L) by 56% and 70%, respectively. Similarly, ApoA-IV even abolished migration toward house dust mite extract (HDM; 100 $\mu\text{g/mL}$) which has been shown to directly activate and mobilize eosinophils.³⁵ For neutrophil chemotaxis toward IL-8, a reduction of 41% from CI = 3.2 to CI = 2.3 was observed (Figure 2C). Of note, ApoA-IV (1 $\mu\text{g/mL}$) (Figure 1A), ApoA-I (10 $\mu\text{g/mL}$) (Figure 1C) and HDL (100 $\mu\text{g/mL}$) (Figure 1D) impaired eosinophil chemotaxis toward CCL11 (3 nmol/L) to a similar extent.

3.4 | ApoA-IV acts NR1D1-dependently and signals via PI3K/PDK1 and PKA

It is assumed that ApoA-I attenuates neutrophil function via the ATP-binding cassette transporter AI (ABCAI), whereas anti-inflammatory effects of HDL are mediated via scavenger receptor BI (SRBI).³⁶ Thus, we next scrutinized whether ApoA-IV also signals via ABCAI or SRBI binding. As shown in Figure 2, ABCAI blocking averted the effect of ApoA-I

(Figure 2A) and the SRBI antibody impeded the HDL-mediated decrease of eosinophil chemotaxis (Figure 2B). Notably, neither ABCAI nor SRBI blocking could prevent the inhibitory effect of ApoA-IV (Figure 2C). Thus, ApoA-IV appears to inhibit eosinophil function through a mechanism different from ApoA-I and HDL. Recently, the nuclear receptor NR1D1 (Rev-Erba- α) has been identified as a putative ApoA-IV-binding protein in hepatocytes.^{37,38} Of note, the Rev-Erba- α antagonist SR8278 (1 μM) partially reversed the ApoA-IV-induced inhibition of eosinophil chemotaxis (Figure 2D) and the selective Rev-Erba- α agonist GSK4112 (SR6452) mimicked the impeding effect of ApoA-IV (Figure 2E).

To further elucidate the downstream components of the ApoA-IV pathway in eosinophils, cells were incubated with protein kinase inhibitors. As illustrated in Figure 3A, blocking PI3K and PDK1 prevented the ApoA-IV induced inhibition of eosinophil chemotaxis. ApoA-IV alone reduced eosinophil chemotaxis to ~15% of the CCL11 response, whereas pretreatment with the PI3K inhibitor LY294002 (10 $\mu\text{mol/L}$) and the PDK1 inhibitor BX912 (300 nmol/L) reverted the CCL11-induced chemotaxis to 77% of the control response. Moreover, eosinophils that were pretreated with the PKA inhibitor H89 (1 $\mu\text{mol/L}$) even reached 112% of the CCL11-induced chemotaxis (Figure 3B). In contrast, the adenylyl cyclase inhibitor SQ22536 (10 $\mu\text{mol/L}$) showed no significant effect. Hence, the anti-inflammatory activity of ApoA-IV seems to require PI3K and PDK1 as well as cAMP-independent activation of PKA. Consistently, PI3K activation has already been associated with other responses to ApoA-IV.^{39,40}

3.5 | ApoA-IV neither disrupts lipid rafts nor affects CCR3 surface expression

Given that cholesterol-rich membrane (lipid) rafts play an important role in leukocyte activation, we investigated whether ApoA-IV alters lipid raft abundance in eosinophils. For that purpose, cholera toxin

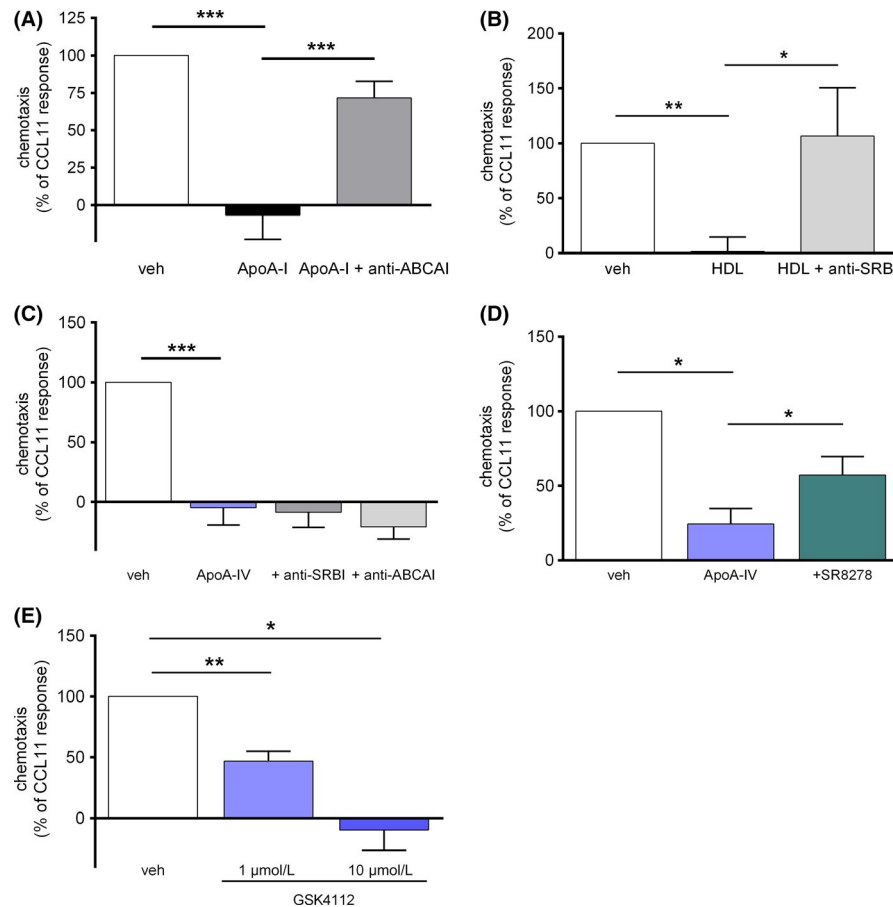


FIGURE 2 ApoA-IV acts independently from SRBI and ABCAI but requires the nuclear receptor Rev-Erba- α . (A) Purified eosinophils were pretreated with an anti-ABCAI blocking antibody for 30 min, incubated with ApoA-I (10 μ g/mL) for 30 min and chemotaxis was stimulated with CCL11 (3 nmol/L) ($n = 5$). (B) Purified eosinophils were pretreated with an anti-SRBI blocking antibody for 30 min, incubated with HDL (100 μ g/mL) for 30 min and chemotaxis was stimulated with CCL11 (3 nmol/L) ($n = 7$). (C) Eosinophils were pretreated either with an anti-ABCAI or anti-SRBI blocking antibody, incubated with ApoA-IV (3 μ g/mL) for 30 min and chemotaxis was stimulated with CCL11 (3 nmol/L) ($n = 4$). (D) Eosinophils were pretreated with the Rev-Erba- α antagonist SR8278 (1 μ mol/L) for 30 min, incubated with ApoA-IV (3 μ g/mL) for 30 min and chemotaxis was stimulated with CCL11 (3 nmol/L) ($n = 5$). (E) Eosinophils were incubated with the Rev-Erba- α agonist GSK4112 (1 and 10 μ mol/L) for 30 min and chemotaxis was stimulated with CCL11 (3 nmol/L) ($n = 5$). (A-E) Purified eosinophils were pretreated as indicated and chemotaxis to CCL11 (3 nmol/L) was determined in duplicates in a 48-well microchemotaxis chamber. Migrated cells were enumerated by flow cytometry. Data are shown as mean $\pm$ SEM; * $P < .05$, ** $P < .01$, *** $P < .001$; 1-way ANOVA

B-FITC that interacts with the raft component ganglioside GM1, was used to quantify lipid rafts by flow cytometry. However, lipid raft integrity was affected neither by ApoA-IV (3 μ g/mL) nor by ApoA-I (10 μ g/mL) treatment for 60 minutes (Figure S3A).

Since the ApoA-IV-related apolipoprotein ApoE was found to modulate the expression of proinflammatory molecules such as the CCL11 receptor CCR3 on activated microglia,⁴¹ we investigated whether eosinophil CCR3 surface expression is altered in response to ApoA-IV (3 μ g/mL). As depicted in Figure S3B, ApoA-IV did not reduce CCR3 staining after a 60-minutes treatment.

3.6 | ApoA-IV enhances apoptosis in eosinophils from allergic subjects

As apoptotic cell death plays an important role in the resolution of inflammatory reactions, we next examined the ability of ApoA-IV to modulate apoptosis in eosinophils. Purified cells were incubated for an

18-hours period with ApoA-IV (3 μ g/mL) or ApoA-I (10 μ g/mL) and apoptosis was assessed by flow cytometry using annexin-V/PI staining. Annexin-V/PI dual-negative cells were considered live cells, annexin-V-positive cells were considered apoptotic cells, whereby annexin-V-positive/PI-negative cells were considered early apoptotic and annexin-V/PI dual-positive cells were considered late apoptotic cells. PI-positive cells were considered necrotic cells. Interestingly, ApoA-IV accelerated eosinophil apoptosis only in allergic donors (Figure S4). After 18 hours of incubation, the percentage of live cells (annexin-V/PI dual-negative) decreased from 51.1 ± 3.9 (vehicle treatment) to $34.8 \pm 4.6\%$ in ApoA-IV-treated eosinophils from allergic donors (Figure S4A). Moreover, ApoA-IV specifically increased the percentage of apoptotic (PI-negative and positive) cells from $43 \pm 5.2\%$ (vehicle treatment) to $60 \pm 2.6\%$ in allergic donors, while ApoA-I was less effective (increase of apoptotic cells to $48 \pm 5.6\%$) (Figure S4B). No significant differences were observed for early apoptotic (Figure S4C), late apoptotic (Figure S4D), and necrotic cells (Figure S4E).

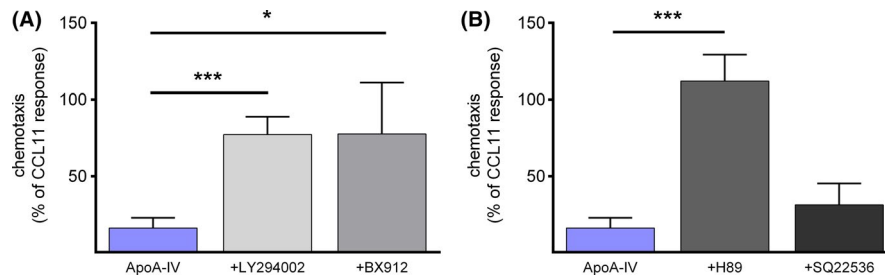


FIGURE 3 ApoA-IV involves PI3K/PDK1/PKA activity. (A) Purified eosinophils were pretreated with the PI3K inhibitor LY294002 (20 μ mol/L) ($n = 6$) or the PDK1 inhibitor BX912 (300 nmol/L) ($n = 4$) for 30 min, incubated with ApoA-IV (3 μ g/mL) for 30 min and chemotaxis was induced by CCL11 (3 nmol/L). (B) Purified eosinophils were pretreated with the PKA inhibitor H89 (1 μ mol/L) or the adenylyl cyclase inhibitor SQ22536 (10 μ mol/L) for 30 min, incubated with ApoA-IV (3 μ g/mL) for 30 min and chemotaxis was induced by CCL11 (3 nmol/L) ($n = 5-6$). (A-B) Purified eosinophils were pretreated as indicated and chemotaxis was induced by CCL11 (3 nmol/L) and performed in duplicates in a 48-well microchemotaxis chamber. Migrated cells were enumerated by flow cytometry. Data are shown as mean $\pm$ SEM; * $P < .05$, *** $P < .001$; 1-way ANOVA

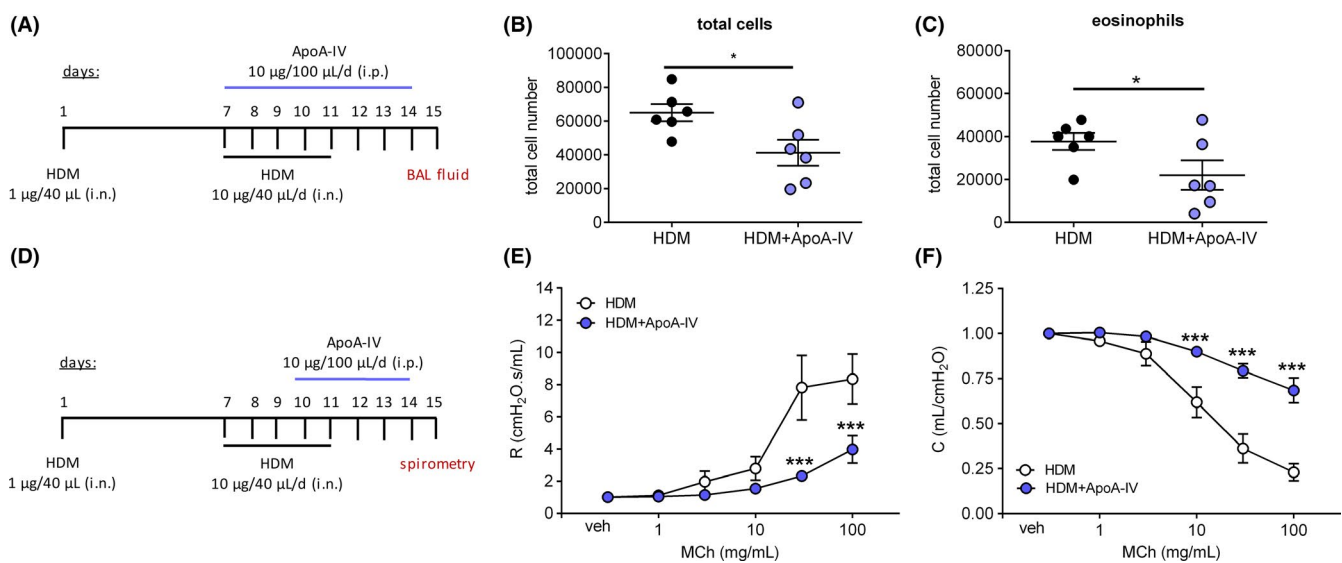


FIGURE 4 ApoA-IV reduces HDM-induced eosinophilia in BAL fluid and improves lung function in HDM-sensitized mice. (A) On day 1, eight-week-old female Balb/c mice were sensitized intranasally with 1 μ g HDM and challenged by intranasal application of 10 μ g HDM per day from day 7 to 11. From day 7 to 14 mice were daily injected with 10 μ g ApoA-IV or vehicle. On day 15 BAL fluid was taken from six mice per group and cellular contents were analyzed by flow cytometry. For (B) total cell count and for (C) eosinophils in the BAL fluid are shown. (D) On day 1, eight-week-old female Balb/c mice were sensitized intranasally with 1 μ g HDM and challenged by intranasal application of 10 μ g HDM per day from day 7 to 11. From day 10 to 14 mice were daily injected with 10 μ g ApoA-IV or vehicle. On day 15 lung function was assessed while applying increasing doses of methacholine (MCH) by a FlexiVent system and (E) airway resistance and (F) compliance were measured. (B, C) Data are shown as mean $\pm$ SEM, * $P < .05$; Student's t test; (E, F) data are shown as mean $\pm$ SEM, *** $P < .001$; 2-way ANOVA; $n = 5-6$

3.7 | Systemic application of ApoA-IV alleviates allergen-induced airway eosinophilia and airway hyperresponsiveness (AHR) in mice

Having confirmed the anti-inflammatory properties of ApoA-IV in vitro, we hypothesized that ApoA-IV might be effective at inhibiting airway inflammation and hyperresponsiveness in a murine model of house dust mite extract (HDM)-induced asthma. In brief, eight-week-old female Balb/c mice were intranasally immunized to HDM or treated with vehicle (PBS) on day 1 and challenged intranasally with HDM or vehicle from day 7 to day 11. All analyses were performed on day 15.

First, we compared ApoA-IV serum levels of HDM-sensitized/challenged mice with the vehicle-treated control group. As determined by ELISA, ApoA-IV serum levels of HDM-treated mice were significantly decreased by 34% compared to the vehicle-treated group (Figure S5). Since infiltrating immune cells are main driving forces of allergic airway inflammation, we examined these cells in the BAL fluid of ApoA-IV-treated and untreated asthmatic mice. Therefore, HDM-exposed mice received a daily i.p. injection of ApoA-IV (10 μ g/100 μ L) or vehicle (A. dest.) from day 7 to day 14 (Figure 4A). As depicted in Figure 4B-C, ApoA-IV strongly repressed the infiltration of immune cells, mainly eosinophils, into the bronchoalveolar space. Treatment with ApoA-IV for 8 days protected mice from airway eosinophilia

as reflected by a ~42% reduction in eosinophil counts in the BAL fluid of ApoA-IV-treated mice compared to vehicle-treated controls (Figure 4C). ApoA-IV also tended to reduce the numbers of alveolar macrophages in the BAL fluid; however, this difference did not reach significance (Figure S6A). Similarly, counts of lymphocytes, monocytes, and neutrophils in the BAL fluid remained unchanged (Figure S6B-D). Of note, ApoA-IV supplementation also protected from systemic eosinophilia as reflected by a ~60% reduction of eosinophil counts in spleen tissue (Figure S7A) and in bone marrow (Figure S7B).

Allergic airway inflammation causes various symptoms of asthma such as airflow obstruction, which is usually associated with an increased airway responsiveness to a variety of stimuli. Having shown that ApoA-IV serum levels decrease during allergic inflammation and that supplementation with ApoA-IV prevents the influx of eosinophils into the airways, we further assessed the impact of ApoA-IV on airway hyperresponsiveness. Therefore, HDM-challenged/sensitized mice were treated daily i.p. with recombinant ApoA-IV (10 µg/100 µL) or vehicle (A. dest.) from day 10 to day 14 (Figure 4D). On day 15, airway hyperresponsiveness to methacholine was recorded by spirometric measurements using a FlexiVent system. Strikingly, systemic application of ApoA-IV to HDM-treated mice diminished the methacholine-induced increases in airway resistance by 70% (Figure 4E) and enhanced airway compliance up to 300% (Figure 4F) compared to the HDM control group. Thus, our results show unequivocally that systemic ApoA-IV directly counteracts airway allergy in mice by inhibiting eosinophil recruitment and airway hyperresponsiveness.

3.8 | ApoA-IV is decreased in serum of allergic patients and accumulates in mucus during chronic rhinosinusitis

ApoA-IV levels have been shown to increase under immunotherapy in patients with allergic rhinitis.²⁸ However, ApoA-IV serum

levels in allergic patients and healthy controls have not been compared yet. Hence, in this study we evaluated ApoA-IV serum levels in 17 nonallergic healthy subjects and 49 untreated patients with respiratory allergic symptoms to aeroallergens (mainly grass pollen) (for further details please refer to Appendix S1, table E1). As presented in Figure 5A, ELISA analysis revealed consistently reduced ApoA-IV concentrations in serum of allergic patients, with a mean value of 428.8 ± 31.02 µg/mL, whereas serum ApoA-IV was 810.7 ± 120.1 µg/mL in healthy nonallergic controls. Albeit, no correlation between ApoA-IV serum levels and laboratory parameters such as sIgE was found (data not shown).

Similar to allergy, chronic rhinosinusitis (CRS) is characterized by a pronounced eosinophilic inflammation of the lining of the nose and paranasal sinuses. Thus, we assessed ApoA-IV levels in the mucus of CRS patients (for further details please refer to Appendix S1, table E2). Interestingly, ApoA-IV levels correlated with their histology scores: Low mean ApoA-IV levels of 0.47 ± 0.1 µg/mL and 0.55 ± 0.2 µg/mL were found in healthy controls and patients with low or medium clinical scores, respectively, whereas a mean mucus level of 5.28 ± 1.8 µg/mL was observed in patients with high histology scores or patients suffering from nasal polyps (CRSwNP) (Figure 5B). Moreover, a significant positive correlation was found between ApoA-IV mucus levels and radiologic Lund-Mackay⁴² scores ($r = 0.5039$; $P = .033$) (Figure 5C).

Thus, our data indicate that ApoA-IV expression and/or metabolism is altered in allergic patients. Moreover, ApoA-IV associates with the severity of inflammation in mucus of CRS patients.

4 | DISCUSSION

In the present study, we demonstrate through several lines of evidence that apolipoprotein A-IV bears potent anti-allergic properties and thereby reveal a hitherto unknown anti-inflammatory

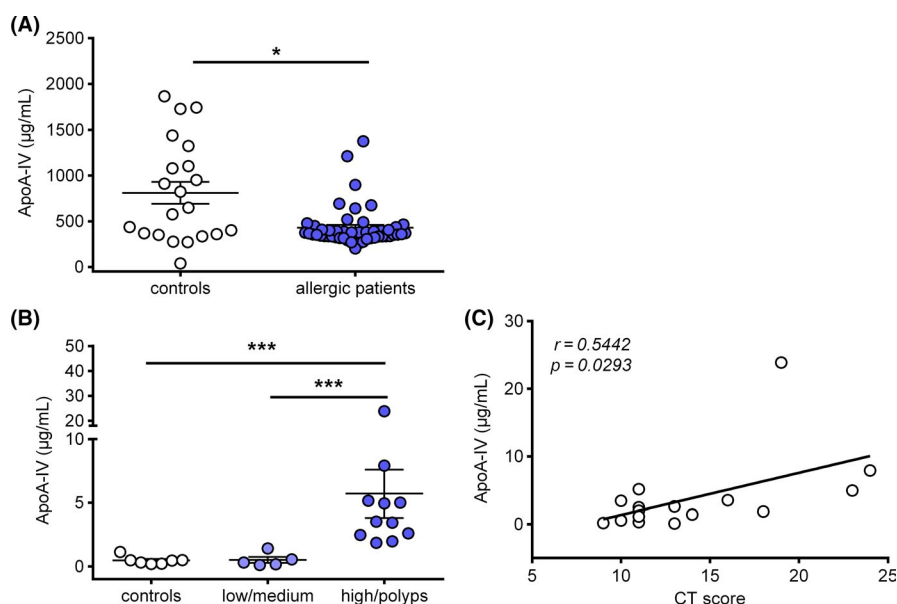


FIGURE 5 ApoA-IV serum levels are decreased in allergic patients and locally increased during CRS. (A) Serum ApoA-IV concentrations were measured with ELISA in serum from 49 allergic patients and 17 healthy controls. (B) ApoA-IV was detected by ELISA in mucus of 16 patients with rhinosinusitis and 7 healthy controls. (C) Pearson correlation of ApoA-IV mucus levels and CT scores was statistically significant. Data are shown as individual values and in A and B as mean $\pm$ SEM; * $P < .05$, *** $P < .001$, Student's *t* test or 1-way ANOVA

mechanism: First, we found that recombinant ApoA-IV inhibits eosinophil responses to chemoattractants in assays of Ca^{2+} mobilization, shape change, integrin (CD11b) surface upregulation, and chemotaxis. The underlying molecular mechanism appears distinct from ApoA-I and HDL-induced signaling cascades as it occurs independently from ABCA1 and SRBI binding, but is mediated through a novel pathway involving nuclear receptor NR1D1 (Rev-ErbA- α) and the protein kinases PI3K, PDK1, and PKA. Second, we established that ApoA-IV specifically enhances apoptosis in eosinophils from allergic individuals but not healthy volunteers. Third, and in line with these *in vitro* data, systemic administration of ApoA-IV prevented pulmonary eosinophilia and markedly improved airway hyperresponsiveness in a mouse model of HDM-induced airway inflammation. And finally, we found that therapy-naïve allergic patients have noticeably lower ApoA-IV serum levels compared to healthy individuals. Moreover, we show that ApoA-IV is present in mucus from CRS patients, where it might act in an anti-inflammatory manner.

Up to now, low ApoA-IV levels have been associated with serious conditions such as cardiovascular disorders⁴³⁻⁴⁵ and cancer.⁴⁶⁻⁴⁸ For instance, ApoA-IV has been identified as a reliable biomarker in ovarian cancer.^{49,50} Moreover, down-regulated gene expression of ApoA-IV has been demonstrated in patients with eosinophil-driven ulcerative colitis⁵¹ and blunted ApoA-IV responses to active lipid absorption after chronic high-fat diet have been implicated in obesity and metabolic disorders.^{52,53} However, the role of apolipoproteins in allergic conditions is still unclear. ApoA-I, the main protein constituent of HDL, promotes cholesterol efflux from immune cells, such as neutrophils,³⁶ monocytes,⁵⁴ and macrophages,^{55,56} and thereby attenuates their function. Recent findings from experimental mouse models suggest that ApoA-I and ApoA-I mimetic peptides might have therapeutic potential for allergic diseases.^{57,58} Similarly, treatment with ApoE mimetic peptide alleviated eosinophilic airway inflammation and hyperresponsiveness in a mouse model of house dust mite-induced asthma.⁶⁰ Recent studies revealed that ApoA-I is decreased in postanaphylactic patients,⁶¹ and serum levels of ApoA-I were found to be positively correlated with FEV1 in subjects with allergic asthma.⁶² Conversely, a study in schoolchildren showed that high ApoA-I is associated with the manifestation of asthma and atopy.⁶³

Of interest, increased serum levels of ApoA-IV were previously reported in patients with allergic rhinitis under allergen-specific immunotherapy.²⁸ We made the surprising observation that ApoA-IV serum levels are noticeably reduced in therapy-naïve allergic patients. However, the reasons for decreased ApoA-IV levels under inflammatory and allergic conditions are a matter of speculation. Of note, Li et al reported that linoleic acid induces inflammatory cytokines such as TNF- α and IL-6, which in turn are able to reduce ApoA-IV mRNA expression in hepatocytes⁶⁴ and ApoA-IV protein production in CaCo2 cells *in vitro*.⁶⁵ Moreover, IL-6 and TNF- α are released in allergic responses and elevated levels of these cytokines have been demonstrated in bronchoalveolar fluid of asthmatic subjects.^{66,67} However, the molecular mechanisms responsible for this

association are still largely unexplored, but it is conceivable that there exists a link between chronic inflammation, ApoA-IV levels and disease outcome in allergic patients.

Previous studies revealed an association of APOA-IV gene variants with ApoA-IV levels and increased risks for certain diseases such as coronary heart disease,⁶⁸ renal diseases,⁶⁹ depression,⁷⁰ and obesity.⁷¹ For instance, Ser347 homozygotes have clearly lower ApoA-IV plasma levels compared with carriers of the Thr347 allele and show a significantly increased risk of coronary heart diseases.⁶⁸ Moreover, it was demonstrated that individuals who are homozygous for the Ser347 allele have higher BMI and percentage body fat compared with individuals homozygous for Thr347.⁷¹ Up to-date no data are available whether APOA-IV gene variants are also associated with a higher risk for chronic atopic diseases such as allergic asthma or rhinitis. In nonallergic patients with chronic rhinosinusitis, we provide evidence that anti-inflammatory ApoA-IV is not only present in nasal mucus but it is also correlated with the extent of inflammation. We assume that ApoA-IV accumulates in the paranasal sinuses due to increased vascular permeability. However, it has been proposed that monocytes⁷² and dendritic cells⁷³ are able to express ApoA-IV, thus we cannot exclude that ApoA-IV is also released locally by infiltrating inflammatory cells.

In our present work, we observed that ApoA-IV potentially affected effector cells of allergic inflammation such as eosinophils and neutrophils. Pretreatment of eosinophils with recombinant ApoA-IV decreased their responses to chemoattractants by means of Ca^{2+} flux, shape change and integrin surface expression. Moreover, ApoA-IV reduced eosinophil migration to baseline levels involving a signaling cascade mediated by Rev-ErbA- α , the NR1D1 (nuclear receptor subfamily 1, group D, member 1) gene product, which is a dominant transcriptional silencer that represses the expression of genes involved in numerous physiological functions, including circadian rhythm and metabolism,⁷⁴ and plays a crucial role in maintaining immune functions.^{75,76} For instance, inflammatory stimuli were shown to promote Rev-ErbA- α degradation in mice, and complete lack of Rev-ErbA- α further enhanced inflammation in the lungs following inflammatory challenge.⁷⁶ In macrophages, Rev-ErbA- α decreased integrin expression and adhesion.⁷⁷ In addition, it was recently shown that pharmacological activation of Rev-ErbA- α reduced lipopolysaccharide (LPS)-induced neuro-inflammation in mouse microglia *in vitro* and *in vivo*.⁷⁸

In further experiments, we demonstrated that the ApoA-IV-induced signaling cascade involves the activity of PI3K, PDK1, and PKA. PI3Ks are a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, and migration.⁷⁹ In numerous cell types, PI3K acts in a heterodimeric form consisting of one 85-kDa regulatory and one 110-kDa catalytic subunit. In previous work, we already elucidated the critical role for the PI3K/PDK1 cascade in transducing inhibitory signals on eosinophil effector function mediated by the prostaglandin E_2 receptor EP4.⁸⁰ In another study, ApoA-IV has been identified to regulate food intake by acting as a satiation factor, which is released by, and is acting in, the hypothalamus.³⁹ In this context, ApoA-IV triggered the

activation of the PI3K cascade in cultured primary hypothalamic neurons, and inhibition of PI3K signaling in rat brain noticeably decreased the potency of ApoA-IV to reduce food intake.³⁹ Moreover, cell culture experiments showed that ApoA-IV improved glucose uptake in adipocytes by upregulating GLUT4 translocation in a PI3K-dependent manner.⁴⁰ These results further support our observation that ApoA-IV engages with the PI3K signaling pathway to promote its anti-inflammatory actions.

To confirm the *in vivo* relevance of the observed anti-inflammatory activities of ApoA-IV, we performed a well-established mouse model of HDM-induced airway inflammation. First, we revealed that HDM-induced allergic inflammation in mice is accompanied by a significant drop in ApoA-IV serum levels compared to healthy control mice. However, whether this effect is due to reduced ApoA-IV synthesis from epithelial cells in the small intestine or due to increased ApoA-IV degradation by the kidneys needs to be clarified in further studies. Of note, we could show that daily systemic treatment with ApoA-IV for several days not only improved lung parameters, but also reduced eosinophil counts in the airways, spleen, and bone marrow of HDM-challenged mice, suggesting that the ability of ApoA-IV to inhibit airway inflammation is mediated by mechanisms that may include the attenuated expression of eosinophil survival factors such as IL-5 and chemoattractants such as CCL11. Accordingly, Yao et al have shown that continuous application of the 5A ApoA-I mimetic peptide inhibited the expression of IL-4, -5, -10, -13 and -17 as well as the CC-chemokines CCL7, -11, -17 and -24 in HDM-challenged mice.⁵⁸ This allows the conclusion that the therapeutic efficacy of apolipoproteins may be comparable with other anti-eosinophilic drugs such as the monoclonal IL-5 antibodies mepolizumab⁸¹ and reslizumab⁸² that provide significant and clinically relevant improvements in exacerbation rate and lead to a reduced use of oral corticosteroids in patients with severe eosinophilic asthma.

Moreover, previous work provided further evidence that ApoA-IV is capable of inhibiting eosinophil-driven inflammatory processes other than asthma. ApoA-IV knockout mice exhibited a significantly greater inflammatory response in DSS-induced colitis than did their wild type littermates. This greater susceptibility to DSS-induced inflammation was reversed upon exogenous administration of ApoA-IV. The authors proposed that ApoA-IV is an endogenous anti-inflammatory protein that acts via inhibition of P-selectin-mediated leukocyte and platelet adhesive interactions.²⁷

In conclusion, our results unequivocally demonstrate the anti-inflammatory properties of ApoA-IV on effector cells of allergic inflammation. Further, we provide novel evidence that systemic elevation of ApoA-IV protects against airway hyperresponsiveness, leukocyte infiltration into the airways and reduces eosinophil count in the circulation. Moreover, ApoA-IV serum levels are significantly decreased in allergic patients and in HDM-exposed mice. Thus, the present data collectively suggest that ApoA-IV has promising diagnostic and therapeutic potential for allergic and inflammatory conditions, particularly those involving eosinophil effector functions.

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CONFLICTS OF INTEREST

AH received consultancy fees from AstraZeneca. All other authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

DR designed and performed experiments, analyzed data, interpreted the results and wrote the manuscript. AT, PL performed experiments and analyzed data. GJS, PVT and GM provided key material and interpreted the results. EMS and AH interpreted the results, supervised the study and edited the manuscript.

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REFERENCES

- Park YM, Bochner BS. Eosinophil survival and apoptosis in health and disease. *Allergy Asthma Immunol Res*. 2010;2:87-101.
- Woerly G, Roger N, Loiseau S, Capron M. Expression of Th1 and Th2 immunoregulatory cytokines by human eosinophils. *Int Arch Allergy Immunol*. 1999;118:95-97.
- Shi H-Z, Xiao C-Q, Li C-Q, et al. Endobronchial eosinophils preferentially stimulate T helper cell type 2 responses. *Allergy*. 2004;59:428-435.
- MacKenzie JR, Mattes J, Dent LA, Foster PS. Eosinophils promote allergic disease of the lung by regulating CD4(+) Th2 lymphocyte function. *J Immunol*. 2001;167:3146-3155.
- Yang D, Rosenberg HF, Chen Q, Dyer KD, Kurosaka K, Oppenheim JJ. Eosinophil-derived neurotoxin (EDN), an antimicrobial protein with chemotactic activities for dendritic cells. *Blood*. 2003;102:3396-3403.
- Shakoori B, Fitzgerald SM, Lee SA, Chi DS, Krishnaswamy G. The role of human mast cell-derived cytokines in eosinophil biology. *J Interferon Cytokine Res*. 2004;24:271-281.
- Gordon S. The role of the macrophage in immune regulation. *Res Immunol*. 1998;149:685-688.
- Watt AP, Schock BC, Ennis M. Neutrophils and eosinophils: clinical implications of their appearance, presence and disappearance in asthma and COPD. *Curr Drug Targets Inflamm Allergy*. 2005;4:415-423.
- Walsh GM. Eosinophil-epithelial cell interactions: a special relationship? *Clin Exp Allergy*. 2001;31:351-354.

10. Konya V, Philipose S, Bálint Z, et al. Interaction of eosinophils with endothelial cells is modulated by prostaglandin EP4 receptors. *Eur J Immunol*. 2011;41:2379-2389.
11. Grünig G, Warnock M, Wakil AE, et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science*. 1998;282:2261-2263.
12. Raines EW, Ferri N. Thematic review series: The immune system and atherogenesis. Cytokines affecting endothelial and smooth muscle cells in vascular disease. *J Lipid Res*. 2005;46:1081-1092.
13. Spoelstra FM, Postma DS, Kauffman HF. Mutual activation of pulmonary fibroblasts and eosinophils, and modulation by drugs in relation to asthma. *Clin Exp Allergy*. 2001;31:808-816.
14. Fryer AD, Stein LH, Nie Z, et al. Neuronal eotaxin and the effects of CCR14 antagonist on airway hyperreactivity and M2 receptor dysfunction. *J Clin Invest*. 2006;116:228-236.
15. Hogan SP, Rosenberg HF, Moqbel R, et al. Eosinophils: biological properties and role in health and disease. *Clin Exp Allergy*. 2008;38:709-750.
16. Hogan SP. Recent advances in eosinophil biology. *Int Arch Allergy Immunol*. 2007;143(Suppl 1):3-14.
17. Simon D, Braathen LR, Simon HU. Eosinophils and atopic dermatitis. *Allergy*. 2004;59:561-570.
18. Straumann A, Simon HU. Eosinophilic esophagitis: escalating epidemiology? *J Allergy Clin Immunol*. 2005;115:418-419.
19. Wedemeyer J, Vosskuhl K. Role of gastrointestinal eosinophils in inflammatory bowel disease and intestinal tumours. *Best Pract Res Clin Gastroenterol*. 2008;22:537-549.
20. Simon D, Simon HU. Eosinophilic disorders. *J Allergy Clin Immunol*. 2007;119(6):1291-1300.
21. Parra A, Sanz ML, Vila L, Prieto I, Dieguez I, Oehling AK. Eosinophil soluble protein levels, eosinophil peroxidase and eosinophil cationic protein in asthmatic patients. *J Invest Allergol Clin Immunol*. 1999;9:27-34.
22. Green RH, Brightling CE, McKenna S, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet*. 2002;360:1715-1721.
23. Hutcheson PS, Schubert MS, Slavin RG. Distinctions between allergic fungal rhinosinusitis and chronic rhinosinusitis. *Am J Rhinol Allergy*. 2010;24:405-408.
24. Ghiselli G, Krishnan S, Beigel Y, Gotto AM Jr. Plasma metabolism of apolipoprotein A-IV in humans. *J Lipid Res*. 1986;27:813-827.
25. Ostos MA, Conconi M, Vergnes L, et al. Antioxidative and antiatherosclerotic effects of human apolipoprotein A-IV in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol*. 2001;21:1023-1028.
26. Recalde D, Ostos MA, Badell E, et al. Human apolipoprotein A-IV reduces secretion of proinflammatory cytokines and atherosclerotic effects of a chronic infection mimicked by lipopolysaccharide. *Arterioscler Thromb Vasc Biol*. 2004;24:756-761.
27. Vowinkel T, Mori M, Krieglstein CF, et al. Apolipoprotein A-IV inhibits experimental colitis. *J Clin Invest*. 2004;114:260-269.
28. Makino Y, Noguchi E, Takahashi N, et al. Apolipoprotein A-IV is a candidate target molecule for the treatment of seasonal allergic rhinitis. *J Allergy Clin Immunol*. 2010;126(6):1163-1169.e5.
29. Frei RB, Luschig P, Parzmair GP, et al. Cannabinoid receptor 2 augments eosinophil responsiveness and aggravates allergen-induced pulmonary inflammation in mice. *Allergy*. 2016;71:944-956.
30. Schratl P, Sturm E, Royer J, et al. Hierarchy of eosinophil chemoattractants: role of p38 mitogen-activated protein kinase. *Eur J Immunol*. 2006;36:2401-2409.
31. Konya V, Ullen A, Kampitsch N, et al. Endothelial E-type prostanoid 4 receptors promote barrier function and inhibit neutrophil trafficking. *J Allergy Clin Immunol*. 2013;131(2):532-540.e2.
32. Sturm EM, Schratl P, Schuligoi R, et al. Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. *J Immunol*. 2008;181:7273-7283.
33. Curcic S, Holzer M, Frei R, et al. Neutrophil effector responses are suppressed by secretory phospholipase A2 modified HDL. *Biochim Biophys Acta*. 2015;1851:184-193.
34. Plantinga M, Guillems M, Vanheerswynghels M, et al. Conventional and monocyte-derived CD11b(+) dendritic cells initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen. *Immunity*. 2013;38:322-335.
35. Svensson L, Rudin A, Wenneras C. Allergen extracts directly mobilize and activate human eosinophils. *Eur J Immunol*. 2004;34:1744-1751.
36. Murphy AJ, Woollard KJ, Suhartoyo A, et al. Neutrophil activation is attenuated by high-density lipoprotein and apolipoprotein A-I in vitro and in vivo models of inflammation. *Arterioscler Thromb Vasc Biol*. 2011;31:1333-1341.
37. Zhang Y, He J, Zhao J, et al. Effect of ApoA4 on SERPINA3 mediated by nuclear receptors NR4A1 and NR1D1 in hepatocytes. *Biochem Biophys Res Commun*. 2017;487:327-332.
38. Li X, Xu M, Wang F, et al. Apolipoprotein A-IV reduces hepatic gluconeogenesis through nuclear receptor NR1D1. *J Biol Chem*. 2014;289:2396-2404.
39. Shen L, Lo CC, Woollett LA, Liu M. Apolipoprotein A-IV exerts its anorectic action through a PI3K/Akt signaling pathway in the hypothalamus. *Biochem Biophys Res Commun*. 2017;494:152-157.
40. Li X, Wang F, Xu M, Howles P, Tso P. ApoA-IV improves insulin sensitivity and glucose uptake in mouse adipocytes via PI3K-Akt Signaling. *Sci Rep*. 2017;7:41289.
41. Duan R, Chen Z, Dou Y, et al. Apolipoprotein E deficiency increased microglial activation/CCR41 expression and hippocampal damage in kainic acid exposed mice. *Exp Neurol*. 2006;202:373-380.
42. Lund VJ, Mackay IS. Staging in rhinosinusitis. *Rhinology*. 1993;31:183-184.
43. Richardson D, Pearson RG, Kurian N, et al. Characterisation of the cannabinoid receptor system in synovial tissue and fluid in patients with osteoarthritis and rheumatoid arthritis. *Arthritis Res Ther*. 2008;10:R43.
44. Feldmann M, Brennan FM, Maini RN. Role of cytokines in rheumatoid arthritis. *Annu Rev Immunol*. 1996;14:397-440.
45. Steiner G, Tohidast-Akrad M, Witzmann G, et al. Cytokine production by synovial T cells in rheumatoid arthritis. *Rheumatology*. 1999;38:202-213.
46. Liagre B, Vergne P, Rigaud M, Beneytout JL. Expression of arachidonate platelet-type 12-lipoxygenase in human rheumatoid arthritis type B synoviocytes. *FEBS Lett*. 1997;414:159-164.
47. Bondeson J. Effects of tenidap on intracellular signal transduction and the induction of proinflammatory cytokines: a review. *Gen Pharmacol*. 1996;27:943-956.
48. Kojima F, Kapoor M, Kawai S, Crofford LJ. New insights into eicosanoid biosynthetic pathways: implications for arthritis. *Expert Rev Clin Immunol*. 2006;2:277-291.
49. Rauniyar N, Peng G, Lam TT, Zhao H, Mor G, Williams KR. Data-independent acquisition and parallel reaction monitoring mass spectrometry identification of serum biomarkers for ovarian cancer. *Biomark Insights*. 2017;12:1177271917710948.
50. Dieplinger H, Ankerst DP, Burges A, et al. Afamin and apolipoprotein A-IV: novel protein markers for ovarian cancer. *Cancer Epidemiol Biomarkers Prev*. 2009;18:1127-1133.
51. Kim M, Lee S, Yang SK, Song K, Lee I. Differential expression in histologically normal crypts of ulcerative colitis suggests primary crypt disorder. *Oncol Rep*. 2006;16:663-670.
52. Liu M, Shen L, Liu Y, et al. Obesity induced by a high-fat diet down-regulates apolipoprotein A-IV gene expression in rat hypothalamus. *Am J Physiol Endocrinol Metab*. 2004;287:366-370.
53. Tso P, Liu M, Kalogeris TJ. The role of apolipoprotein A-IV in food intake regulation. *J Nutr*. 1999;129:1503-1506.

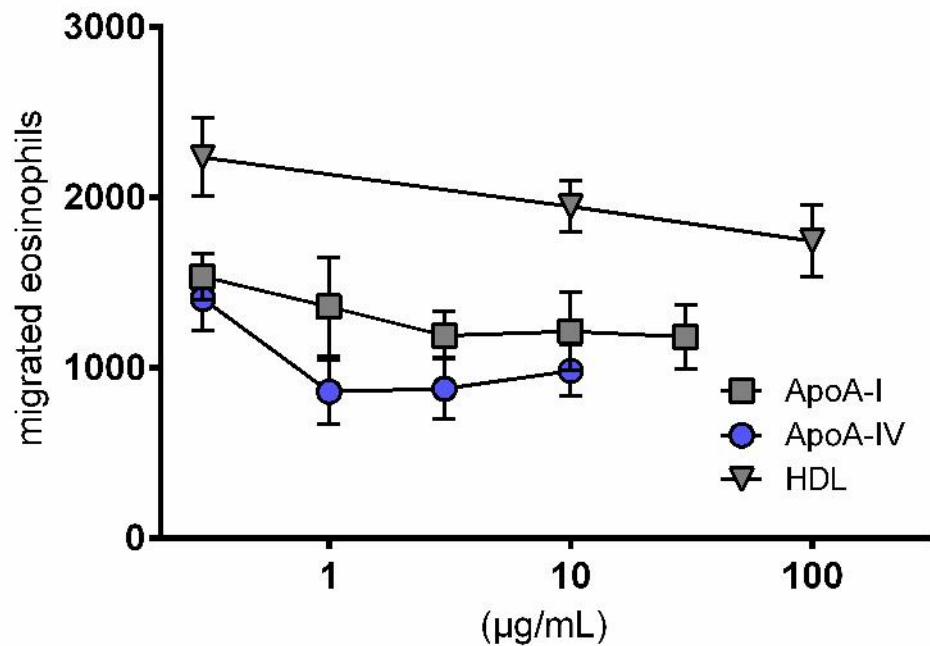
54. Murphy AJ, Woollard KJ, Hoang A, et al. High-density lipoprotein reduces the human monocyte inflammatory response. *Arterioscler Thromb Vasc Biol.* 2008;28:2071-2077.
55. Sun YU, Ishibashi M, Seimon T, et al. Free cholesterol accumulation in macrophage membranes activates Toll-like receptors and p38 mitogen-activated protein kinase and induces cathepsin K. *Circ Res.* 2009;104:455-465.
56. Yvan-Charvet L, Welch C, Pagler TA, et al. Increased inflammatory gene expression in ABC transporter-deficient macrophages: free cholesterol accumulation, increased signaling via toll-like receptors, and neutrophil infiltration of atherosclerotic lesions. *Circulation.* 2008;118:1837-1847.
57. Park S-W, Lee EH, Lee E-J, et al. Apolipoprotein A1 potentiates lipoxin A4 synthesis and recovery of allergen-induced disrupted tight junctions in the airway epithelium. *Clin Exp Allergy.* 2013;43:914-927.
58. Yao X, Dai C, Fredriksson K, et al. 5A, an apolipoprotein A-I mimetic peptide, attenuates the induction of house dust mite-induced asthma. *J Immunol.* 2011;186:576-583.
59. Nandedkar SD, Weihrauch D, Xu H, et al. D-4F, an apoA-1 mimetic, decreases airway hyperresponsiveness, inflammation, and oxidative stress in a murine model of asthma. *J Lipid Res.* 2011;52:499-508.
60. Yao X, Fredriksson K, Yu Z-X, et al. Apolipoprotein E negatively regulates house dust mite-induced asthma via a low-density lipoprotein receptor-mediated pathway. *Am J Respir Crit Care Med.* 2010;182:1228-1238.
61. Wittenberg M, Nassiri M, Francuzik W, Lehmann K, Babina M, Worm M. Serum levels of 9alpha,11beta-PGF2 and apolipoprotein A1 achieve high predictive power as biomarkers of anaphylaxis. *Allergy.* 2017;72:1801-1805.
62. Barochia AV, Kaler M, Cuento RA, et al. Serum apolipoprotein A-I and large high-density lipoprotein particles are positively correlated with FEV1 in atopic asthma. *Am J Respir Crit Care Med.* 2015;191:990-1000.
63. Nagel G, Weiland SK, Rapp K, Link B, Zoellner I, Koenig W. Association of apolipoproteins with symptoms of asthma and atopy among schoolchildren. *Int Arch Allergy Immunol.* 2009;149:259-266.
64. Navarro MA, Carpintero R, Acín S, et al. Immune-regulation of the apolipoprotein A-I/C-III/A-IV gene cluster in experimental inflammation. *Cytokine.* 2005;31:52-63.
65. Li X, Xu M, Liu M, Ji Y, Li Z. TNF-alpha and IL-6 inhibit apolipoprotein A-IV production induced by linoleic acid in human intestinal Caco2 cells. *J Inflamm (Lond).* 2015;12:22.
66. Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI. Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol.* 1992;89:958-967.
67. Tillie-leblond I, Pugin J, Marquette C-H, et al. Balance between proinflammatory cytokines and their inhibitors in bronchial lavage from patients with status asthmaticus. *Am J Respir Crit Care Med.* 1999;159:487-494.
68. Wong W-M, Hawe E, Li LK, et al. Apolipoprotein AIV gene variant S347 is associated with increased risk of coronary heart disease and lower plasma apolipoprotein AIV levels. *Circ Res.* 2003;92:969-975.
69. Lamina C, Friedel S, Coassin S, et al. A genome-wide association meta-analysis on apolipoprotein A-IV concentrations. *Hum Mol Genet.* 2016;25:3635-3646.
70. Ota VK, Chen ES, Ejchel TF, et al. APOA4 polymorphism as a risk factor for unfavorable lipid serum profile and depression: a cross-sectional study. *J Investig Med.* 2011;59:966-970.
71. Lefevre M, Lovejoy JC, DeFelice SM, et al. Common apolipoprotein A-IV variants are associated with differences in body mass index levels and percentage body fat. *Int J Obes Relat Metab Disord.* 2000;24:945-953.
72. Dai Y, Hu C, Huang Y, Huang H, Liu J, Lv T. A proteomic study of peripheral blood mononuclear cells in systemic lupus erythematosus. *Lupus.* 2008;17:799-804.
73. Sanecka A, Ansems M, van Hout-Kuijer MA, et al. Analysis of genes regulated by the transcription factor LUMAN identifies ApoA4 as a target gene in dendritic cells. *Mol Immunol.* 2012;50:66-73.
74. Duez H, Staels B. Rev-erb-alpha: an integrator of circadian rhythms and metabolism. *J Appl Physiol.* 1985;2009(107):1972-1980.
75. Gibbs JE, Blaikley J, Beesley S, et al. The nuclear receptor REV-ERBalpha mediates circadian regulation of innate immunity through selective regulation of inflammatory cytokines. *Proc Natl Acad Sci U S A.* 2012;109:582-587.
76. Pariollaud M, Gibbs JE, Hopwood TW, et al. Circadian clock component REV-ERBalpha controls homeostatic regulation of pulmonary inflammation. *J Clin Invest.* 2018;128:2281-2296.
77. Sato S, Sakurai T, Ogasawara J, et al. A circadian clock gene, Rev-erbalpha, modulates the inflammatory function of macrophages through the negative regulation of Ccl2 expression. *J Immunol.* 2014;192:407-417.
78. Guo DK, Zhu Y, Sun HY, et al. Pharmacological activation of REV-ERBalpha represses LPS-induced microglial activation through the NF-kappaB pathway. *Acta Pharmacol Sin.* 2018;40:26-34.
79. Cantley LC. The phosphoinositide 3-kinase pathway. *Science.* 2002;296:1655-1657.
80. Sturm EM, Parzmair GP, Radnai B, et al. Phosphoinositide-dependent protein kinase 1 (PDK1) mediates potent inhibitory effects on eosinophils. *Eur J Immunol.* 2015;45:1548-1559.
81. Nair P, Pizzichini M, Kjarsgaard M, et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med.* 2009;360:985-993.
82. Castro M, Zangrilli J, Wechsler ME, et al. Reslizumab for inadequately controlled asthma with elevated blood eosinophil counts: results from two multicentre, parallel, double-blind, randomised, placebo-controlled, phase 3 trials. *Lancet Respir Med.* 2015;3:355-366.

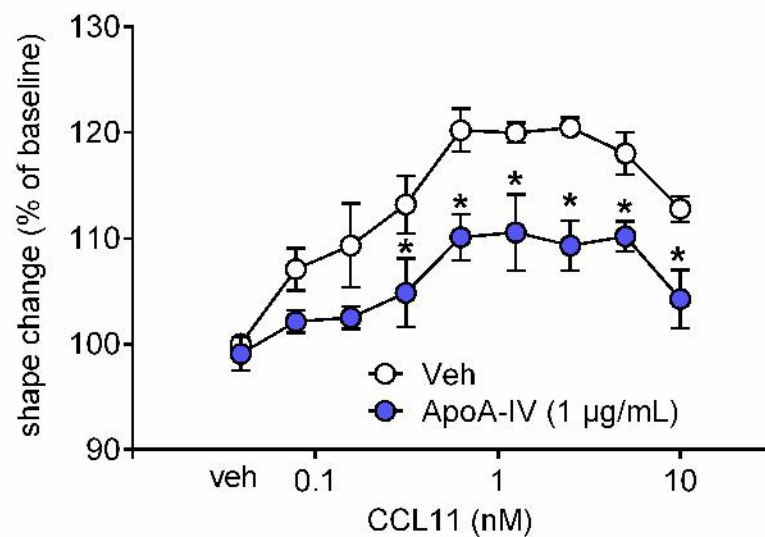
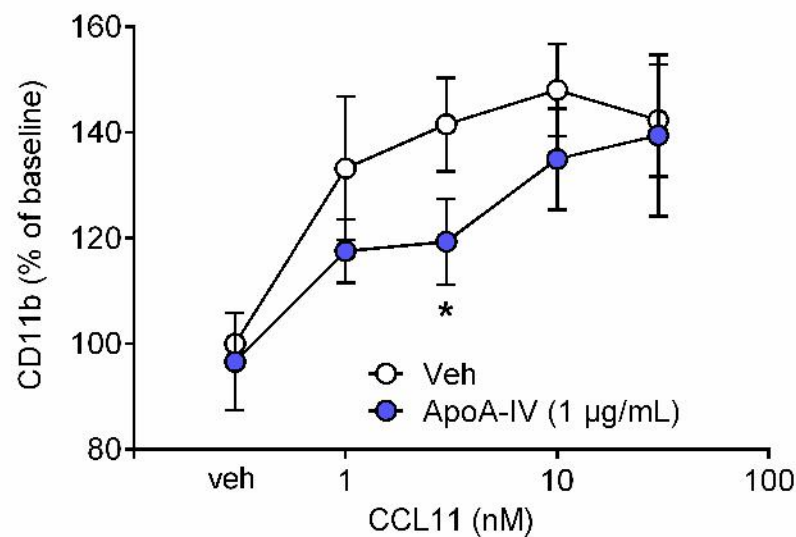
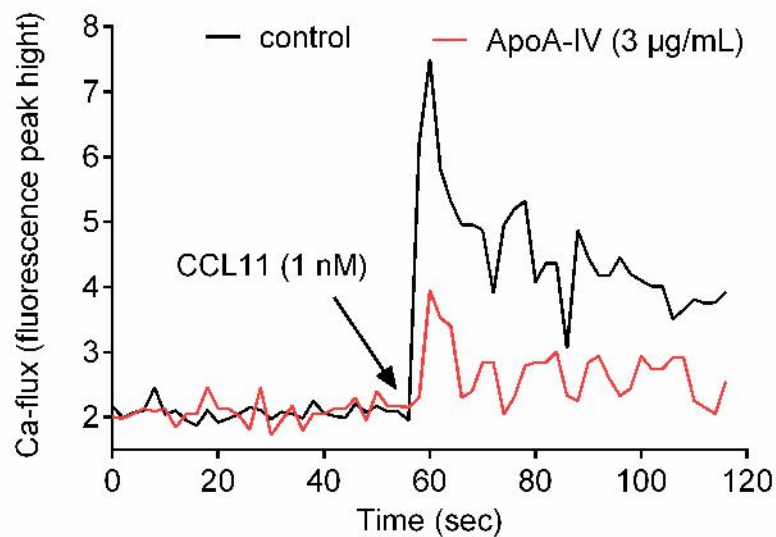
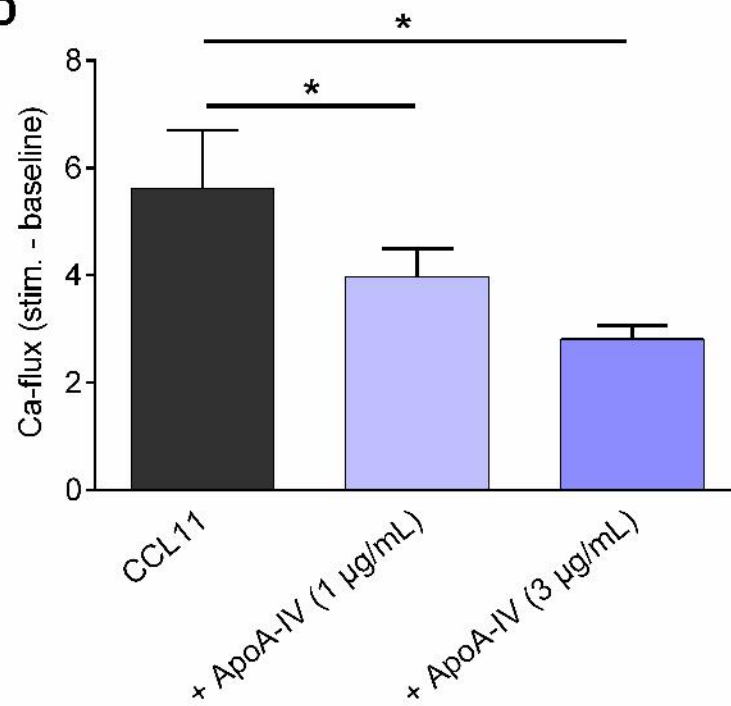
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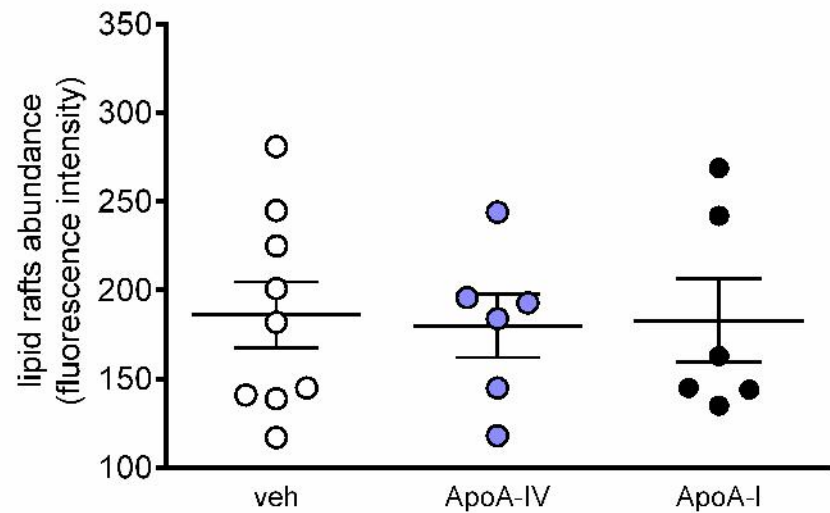
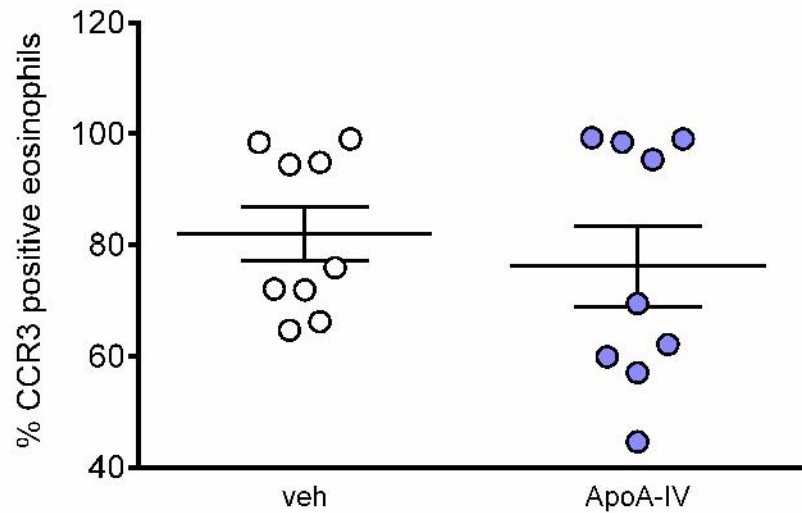
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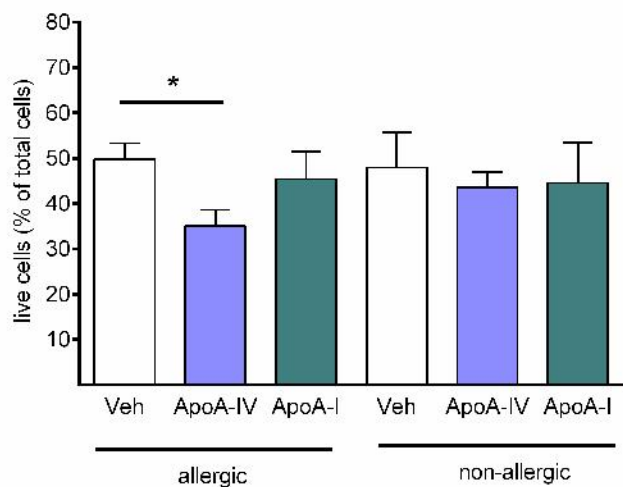
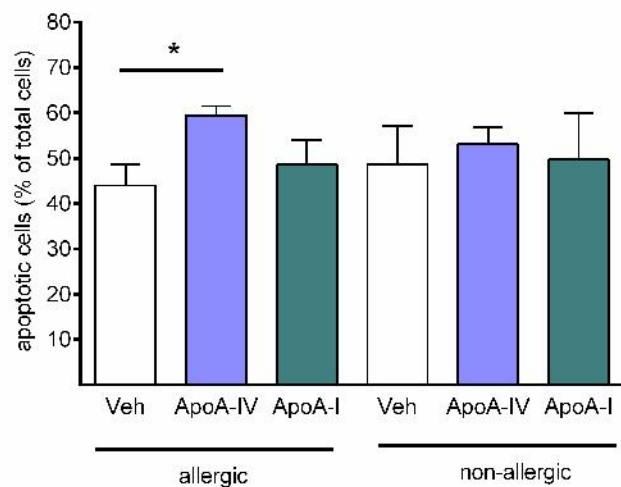
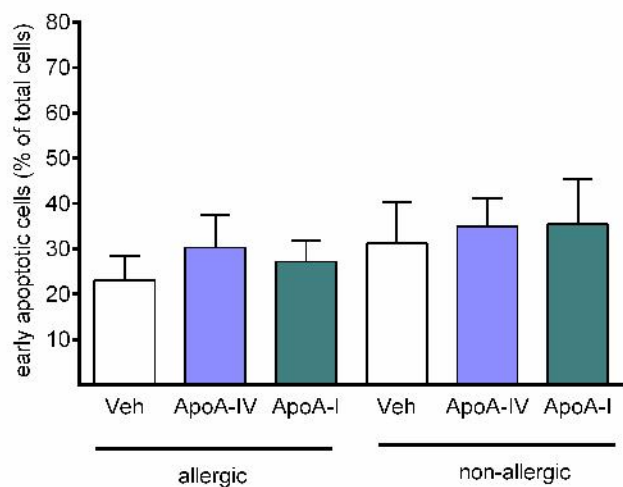
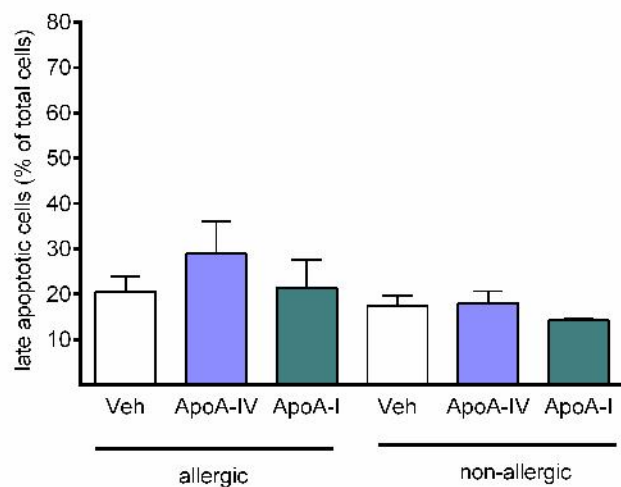
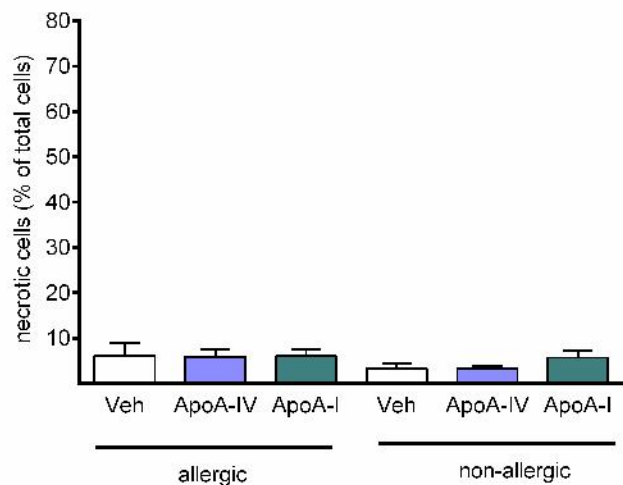
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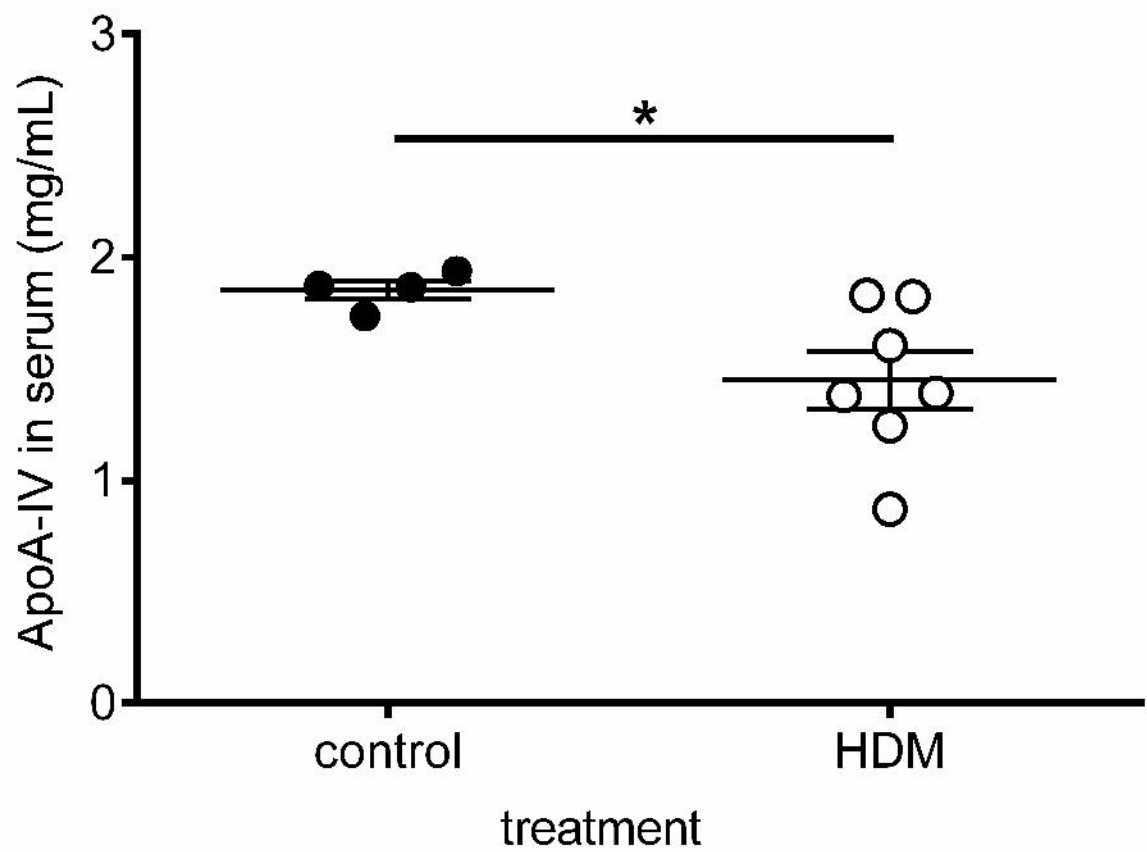
Apolipoprotein A-IV acts as an endogenous anti-inflammatory protein and is reduced in treatment-naïve allergic patients and allergen-challenged mice. *Allergy.* 2020;75:392-402. <https://doi.org/10.1111/all.14022>

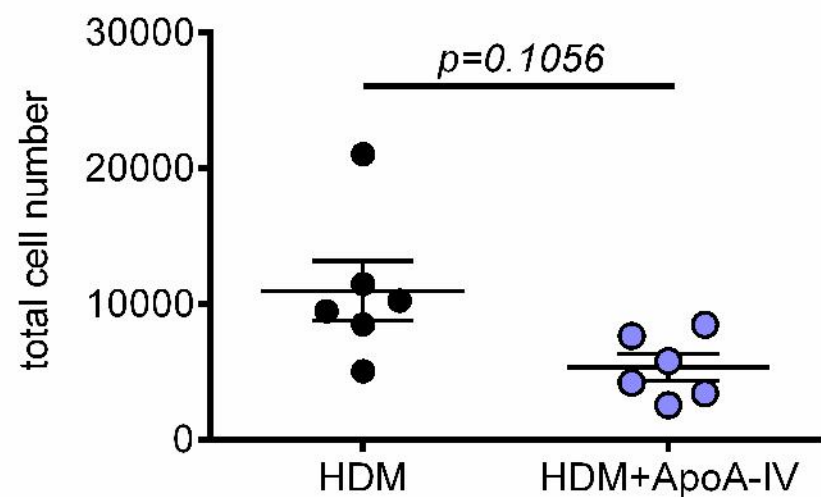
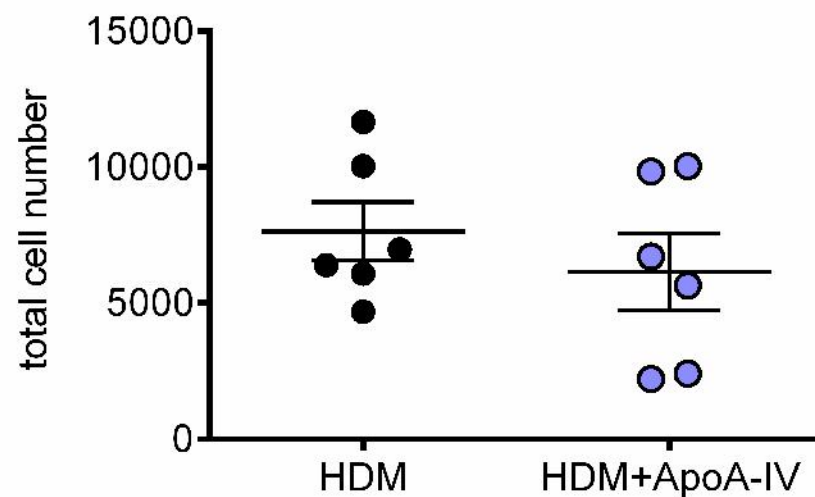
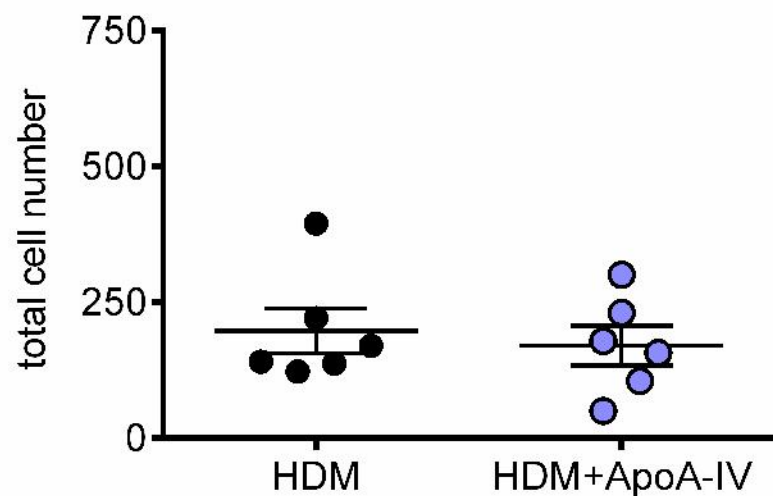
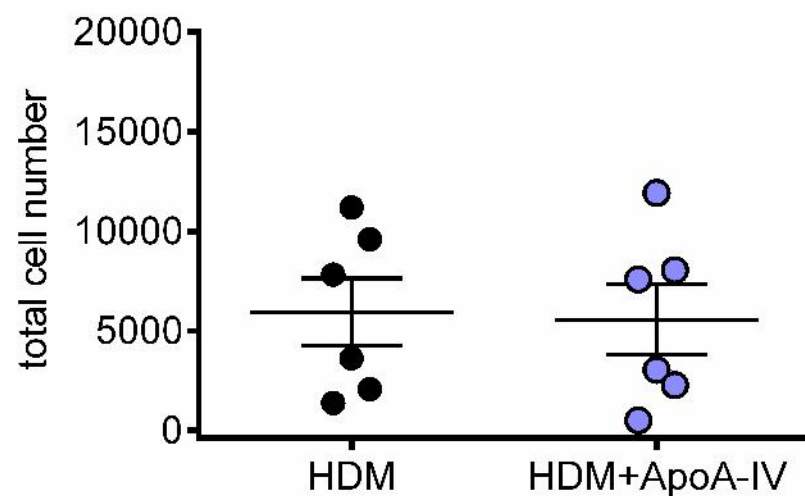


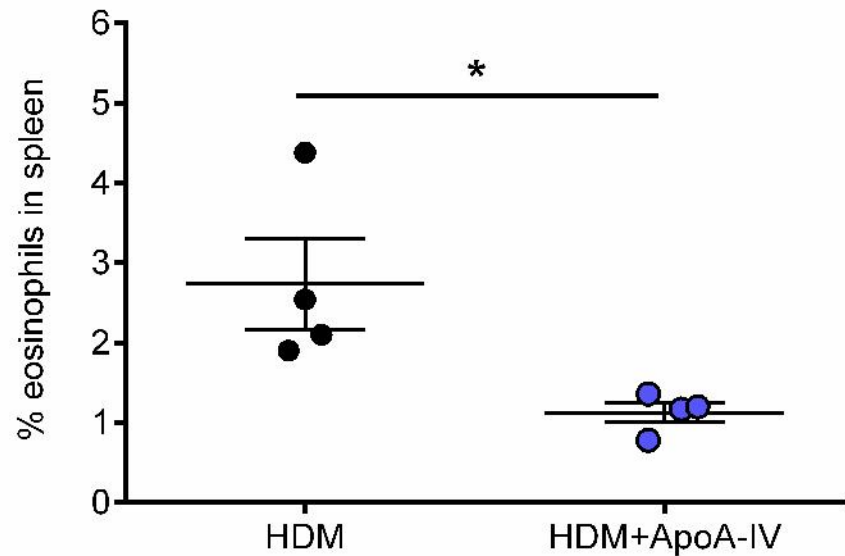
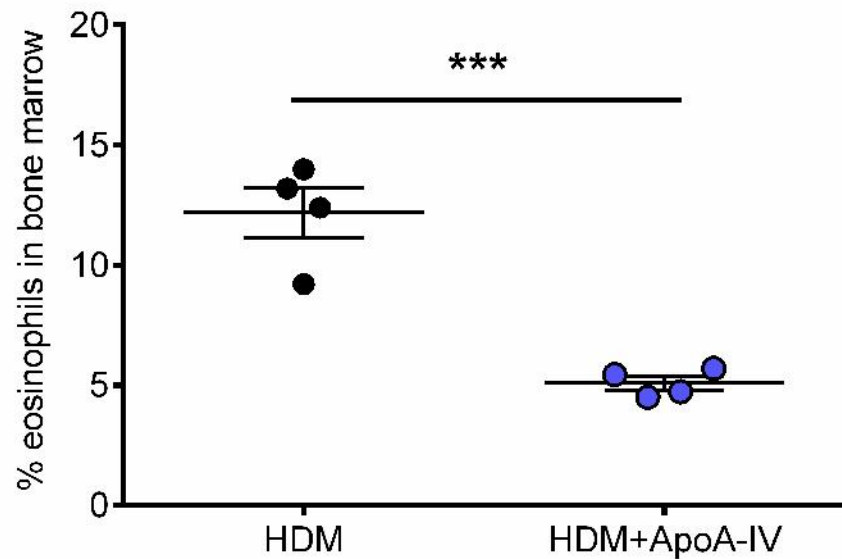
A**B****C****D**

A**B**

A**B****C****D****E**



A**alveolar macrophages****B****lymphocytes****C****monocytes****D****neutrophils**

A**B**

Material

All reagents were from Sigma (Vienna, Austria), unless specified. Assay buffer was made from Dulbecco's modified PBS (with 0.9 mmol/L Ca^{2+} and 0.5 mmol/L Mg^{2+} ; Pan Biotech, Aidenbach, Germany), 0.1% BSA, 10 mmol/L HEPES, and 10 mmol/L glucose, pH 7.4. Human recombinant ApoA-IV was from Novoprotein (Summit, USA) and ApoA-I isolated from human plasma HDL. Human ApoA-IV ELISA was from Abcam (Cambridge, UK) and mouse ApoA-IV ELISA was from Cusabio (Houston, USA). Human eotaxin/CCL11 and IL-8 were from ImmunoTools (Friesoythe, Germany). FITC Annexin V Apoptosis Detection Kit I, anti-CD16-PE-Cy5, anti-CD193-PE and anti-CD11b-PE (ICRF44) were from Becton Dickinson (Vienna, Austria). Anti-CD14 BV421 and all anti-mouse antibodies were from BioLegend (San Diego, USA). The PKA inhibitor H-89 was from Cayman Chemical (Tallinn, Estonia), the PI3K inhibitor LY294002 was supplied by Biomol (Hamburg, Germany) and the PDK1 inhibitor BX-912 was from Axon Medchem (Groningen, The Netherlands. House dust mite extract was from Stallergenes Greer (Lenoir, USA). CellFix and FACS-Flow were from Becton Dickinson (Vienna, Austria). PVP-free polycarbonate filters were from Neuro Probe (Gaithersburg, USA). Fluo-3-AM was supplied from Life Sciences (Vienna, Austria).

Fixative solution was prepared by adding 9 ml of distilled water and 30 ml of FACS-Flow to 1 ml of CellFix.

Patients and healthy controls

Blood and serum were collected according to a protocol approved by the Ethics Committee of the Medical University of Graz (27-528 ex 14/15).

For functional assays, blood was collected on a daily basis from healthy and, for apoptosis assays, also from self-reported allergic volunteers.

Serum for ApoA-IV measurements was collected from 17 diagnosed healthy non-allergic and 49 diagnosed and untreated allergic volunteers. Allergic patients, who had been admitted

to the outpatient clinic of the Department of Dermatology and Venereology of the Medical University of Graz because of allergic symptoms to aeroallergens, mainly grass pollen, were screened. Their personal history was taken and the current standard diagnostic procedures (intradermal tests, IgE determination by ImmunoCAP) were performed (Table 1). Specific and total IgE antibody levels in the patients' sera were measured by using ImmunoCAP 250 (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. Specific IgE values greater than 0.35 kU/L were considered positive.

Mucus was collected according to a protocol approved by the Ethics Committee of the Medical University of Graz (23-481 ex 10/11). Mucus was collected from 16 patients with chronic rhinosinusitis (CRS) and 7 healthy controls at the Department of Otorhinolaryngology of the Medical University of Graz. CRS was diagnosed according to the EPOS guidelines¹. All CRS patients underwent a detailed history and physical examination, which included an allergy evaluation. All 16 patients had imaging computed tomography of their sinuses (Table 2). For grading CT scans the Lund- Mackay classification was used, which relies on a score of 0–2 dependent upon the absence, partial or complete opacification of each sinus system and the ostiomeatal complex, deriving a maximum score of 12 per side².

Preparation of human peripheral blood leukocytes

Human leukocytes were isolated from blood samples of volunteers according to a protocol approved by the Institutional Review Board of the Medical University of Graz. In brief, erythrocytes were removed from citrated whole blood by dextran sedimentation for 30 min at RT. Polymorphonuclear leukocytes (pellet) were separated from mononuclear cells (buffy coat) by density gradient centrifugation (Histopaque 1077, Sigma–Aldrich) for 20 min at RT at 400 x g. Eosinophils were separated from neutrophils in the polymorphonuclear leukocyte fraction by negative magnetic selection using the MACS cell separation system (Eosinophil Isolation Kit, Miltenyi Biotech, Bergisch Gladbach, Germany). Therefore, non-eosinophils

were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies against CD2, CD14, CD16, CD19, CD56, CD123, and CD235a, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labeling reagent. The magnetically labeled non-eosinophils were depleted by retaining them on a MACS® Column in the magnetic field of a Separator, while unlabeled eosinophils passed through the column with a resulting purity of typically $\geq 98\%$.

Mucus Collection

An appropriate amount of mucus (minimum 0.2 mL) was harvested from each patient using a special mucus collection device (Sinus Secretion Collector, Medtronic-Xomed Inc., Jacksonville, FL) without doing any preoperative decongestion or irrigation. The mucus was collected under endoscopic guidance from the middle meatus and processed as described previously³. The mass of each mucus specimen was weighed, and a threefold excess of normal saline (0.15 M NaCl) was added. After vortexing, the mucus suspension was centrifuged, and the resulting supernatant fluid was frozen at -70°C .

HDL isolation

HDL was isolated from plasma of healthy volunteers by density gradient ultracentrifugation as described⁴. Plasma density was adjusted with potassium bromide to 1.24 g/mL and a two-step density gradient was generated in centrifuge tubes (16 × 76 mm, Beckman) by layering the density-adjusted plasma (1.24 g/mL) underneath a NaCl-density solution (1.063 g/mL). Tubes were sealed and centrifuged at 65,000 rpm for 6 hours at 15°C in a 90Ti fixed angle rotor (Beckman Instruments, Krefeld, Germany). After centrifugation, the HDL-containing band was collected and desalted via PD10 columns (GE Healthcare, Vienna, Austria) and immediately used for experiments.

Shape change assay

PMNL were pretreated with ApoA-IV or vehicle for 30 min and stimulated with serial dilutions of CCL11 for 20 min at 37 °C. Shape change was monitored by flow cytometry as the increase of forward scatter (FSC) and was expressed as percent of the vehicle response. Eosinophils distinguished from neutrophils due to their SSC properties and autofluorescence⁵.

CD11b- upregulation in eosinophils

PMNL samples were pretreated with ApoA-IV or vehicle for 30 min and incubated with serial dilutions of CCL11 for 30 min at 37°C. Samples were stained with anti- CD16- PE- Cy5 and anti- CD11b- PE (ICRF44) Ab. Eosinophils were identified as CD16 negative cells. CD11b upregulation was analyzed by flow cytometry as previously described⁶.

Calcium flux

Isolated human eosinophils were loaded with the calcium sensitive dye Fluo- 3- AM in the presence of 0.02% pluronic F- 127, pretreated with ApoA-IV or vehicle and stimulated with CCL11. Changes in intracellular Ca^{2+} were detected as fluorescence increase in the FL1- (530/30 nm) channel⁷.

Chemotaxis

Purified eosinophils were used to study eosinophil chemotaxis, whereas neutrophil chemotaxis was performed separately with PMNL fractions. Cells were pretreated with ApoA-I, -IV, HDL or vehicle as indicated, placed into the top wells of a 48-well micro chemotaxis chamber and were allowed to migrate towards the respective chemoattractant for 1 hour at 37 °C⁸. PVP-free polycarbonate filter membranes with a pore size of 5 µm were used. Migrated eosinophils or neutrophils in the bottom wells were collected and then

enumerated by flow cytometry as described previously⁷. Therefore, eosinophils and neutrophils were gated by their forward and side scatter properties and by autofluorescence.

In some experiments, eosinophils were treated additionally with specific blocking antibodies or protein kinase inhibitors for 30 min at 37 °C, cells were then exposed to ApoA-IV, ApoA-I, HDL or vehicle and chemotaxis was induced with CCL11. Concentrations and treatment conditions were chosen based on literature^{6, 7, 9}.

Cholesterol-rich microdomain (lipid raft) assessment

Isolated eosinophils were incubated with ApoA-IV, ApoA-I or vehicle for 60 min at 37°C; subsequently cells were washed with PBS and incubated with 1 µg/ml FITC-cholera toxin B for 60 min at room temperature. Eosinophils were fixed and lipid raft abundance was measured by flow cytometry¹⁰.

CCR3 staining

Isolated eosinophils were pretreated with ApoA-IV for 60 min at 37°C. Following stimulation cells were stained with PE-anti-CD193 (CCR3) antibodies or isotype control for 30 min at 4°C. Surface expression of CCR3 was evaluated by flow cytometry and expressed as % of CCR3 positive cells.

Apoptosis assay

Apoptosis of eosinophils was determined as described previously¹¹. Purified eosinophils were kept in RPMI (ThermoFisher Scientific) supplemented with IL-5 (50 pM), 1% FBS and PenStrep (Sigma-Aldrich) in the absence or presence of ApoA-IV (3 µg/mL). At various time points aliquots were removed and washed twice in PBS, and the cells were resuspended in binding buffer. Eosinophils were then stained with annexin V-FITC (1/100) and propidium iodide (PI) (1/50) in the dark for 10 min at room temperature according to the manufacturer's

protocol (Annexin V-FITC Apoptosis Detection Kit I, BD Pharmingen) and immediately analyzed by flow cytometry. Each sample was acquired for 1 min, and the total number of eosinophils gated on a forward scatter/side scatter plot and the percentage of non-apoptotic cells (annexin V^{neg}/propidium iodide^{neg}), early apoptotic cells (annexin V^{pos}/propidium iodide^{neg}), late apoptotic cells (annexin V^{pos}/propidium iodide^{pos}) and necrotic cells (annexin V^{neg}/propidium iodide^{pos}) was recorded.

House dust mite (HDM)-induced allergic lung inflammation

The HDM model was performed as previously described by Plantinga et al.¹². Eight-week-old female Balb/c mice were used in this study. Mice were housed in individually ventilated cages (5 per cage) under controlled conditions of temperature (21 °C), air humidity (50%) and a 12 h light/dark cycle (lights on at 6:00 a.m.). Standard chow (altromin FORTI, Altromin, Lage, Germany) and water were provided *ad libitum*. The experimental procedure used in this study was approved by the Austrian Federal Ministry of Science, Research and Economy (protocol number: BMWFW-66.010/0020-WF/V/3b/2015) conform to Directive 2010/63/EU, and was performed in accordance with national and international guidelines. Mice were randomly assigned to the different treatments. Three cohorts of mice were investigated:

1. On day 1 the first group of mice was anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection and sensitized intranasally with 1 µg HDM protein in 40 µL PBS. Mice were then anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection and challenged by intranasal application of 10 µg HDM protein in 40 µL PBS from day 7 to day 11. On day 15, whole blood was collected by cardiac puncture and serum was prepared by centrifugation. ApoA-IV serum levels in untreated and HDM sensitized and challenged mice were determined by ELISA.

2. The second group of mice was sensitized and challenged as described above but received a daily i.p. injection of ApoA-IV (10 µg/100 µL) or vehicle (Aqua dest.) from day 7 to day 14. On day 15, mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection, the trachea was exposed and a total 3 mL of BAL buffer (PBS containing 1 mM EDTA) divided into three parts was instilled into the lung and BAL fluid was collected and stored on ice. BAL fluid was centrifuged (400 x g, 4°C for 7 min) and collected cell pellets were subsequently subjected to a staining protocol. In brief, erythrocytes were lysed using an ammoniochloride solution. After a washing step, non-specific binding sites were masked using TruStain fcX™ antibody (anti-mouse CD16/32; BioLegend, San Diego, CA 1:100) for 15 min on ice, followed by incubation with specific anti-mouse antibodies: Siglec F (1:100), CD11b (1:200), CD11c (1:200) (all from BD Pharmingen), Ly6G (1:500) and MHC-II (1:200) (all from BioLegend) for 30 min at 4°C. Cells were washed, fixed and analyzed by a BD FACS Canto II flow cytometer. In detail, after doublet cells had been excluded, lymphocytes were identified as FSC/SSC^{low} CD11b^{neg}/CD11c^{neg} cells. Remaining cells were further gated as CD11c^{neg}/Siglec F^{pos} eosinophils and CD11c^{pos}/Siglec F^{pos} alveolar macrophages (AM). CD11c/Siglec F double-negative cells were characterized as CD11b^{pos}/Ly6G^{pos} neutrophils and Ly6G^{neg}/Ly6C^{pos} monocytes.

3. The third group of mice was treated in an analogous manner as the second group, but received a daily i.p. injection of ApoA-IV (10 µg/100 µL) or vehicle (Aqua dest.) from day 10 to day 14. On day 15, these mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection and airway hyperresponsiveness (AHR) to methacholine was recorded with the FlexiVent system (Scireq, Montreal, CA).

Variable	Controls (n=17) mean or % (SD)	Patients (n=49) mean or % (SD)
Demographics		
Age, years	23.9 (2.3)	25.8 (11.6)
Gender (% females)	53 %	39 %
Sensitization frequency (%)		
Grass	-	100
Birch	-	51
Ash	-	12.2
Ragweed	-	8.2
Mugwort	-	18.4
<i>Alternaria alternata</i>	-	8.2
House dust mite	-	16.3
Cat	-	2
Hazelnut	-	2
IgE reactivity, mean kU/L (SD)		
Total IgE	30.4 (36.8)	197.9 (378.8)
Grass	-	29.2 (34.7)
Birch	-	20.9 (26.9)
Ash	-	1.4 (0.5)
Ragweed	-	7.3 (5.0)
Mugwort	-	3.5 (7.2)
<i>Alternaria alternata</i>	-	4.9 (5.3)
House dust mite	-	4.1 (3.9)
Cat	-	1.5 (0.0)
Hazelnut	-	2.6 (0.0)

Table E1: Allergic patients and controls enrolled for ApoA-IV serum measurements.

Variable	Controls (n=7) mean or % (SD)	Patients (n=16) mean or % (SD)
Demographics		
Age, years	35 (7.6)	46 (10.7)
Diagnosis (%)		
CRSsP		31
		69
CRSwP		
Therapy (%)		
Topical steroids (TS)		50
Systemic steroids (SS)		6.25
TS+SS		18.75
no		25
Histology (%)		
Minimal sinusitis		6.25
Low grade		25
Medium grade		6.25
High grade		6.25
Polyps		56.25
Eosinophilia (%)		
No		18.75
Low		12.5
Middle		12.5
High		56.25
Scores		
CT score (Lund- Mackay Score)		14 (4.7)
Total symptoms score (VAS)		4 (2.2)

Table E2: Rhinosinusitis patients and controls enrolled for ApoA-IV mucus measurements.

Figure E1. Defining the working dose of ApoA-IV, ApoA-I and HDL. Purified eosinophils were pretreated with ApoA-IV (1-10 $\mu\text{g/mL}$) ($n=12$), ApoA-I (1-30 $\mu\text{g/mL}$) ($n=11$), HDL (10-100 $\mu\text{g/mL}$) ($n=14$) or vehicle for 30 min at 37 °C, placed into the top-wells of a 48-well microchemotaxis chamber and were allowed to migrate towards CCL11 (3 nM) for 60 min at 37 °C. Migrated cells were enumerated by flow cytometry. Chemotaxis is expressed as migrated eosinophils and shown as mean $\pm$ SEM.

Figure E2. ApoA-IV potently inhibits eosinophil responsiveness. (A) PMNL were pretreated with ApoA-IV (1 $\mu\text{g/mL}$) and stimulated with serial dilutions of CCL11 for 20 min. Shape change of eosinophils was recorded by flow cytometry. Eosinophils were distinguished from neutrophils by their autofluorescence as well as forward scatter and side scatter properties ($n=5$), (B) PMNL were pretreated with ApoA-IV (1 $\mu\text{g/mL}$) and stimulated with serial dilutions of CCL11 for 30 min. Samples were stained with anti- CD16- PE- Cy5 and anti- CD11b- PE (ICRF44) Ab and CD11b upregulation was detected by flow cytometry ($n=6$). Eosinophils were identified as CD16-negative cells. (C, D) Purified eosinophils were pretreated with ApoA-IV (1 and 3 $\mu\text{g/mL}$) for 30 min and Ca^{2+} flux was induced by CCL11 (3 nM) and analyzed by flow cytometry. ($n=6$). (D) Data are calculated as stimulated – baseline difference. All data are shown as mean $\pm$ SEM; * $p<0.05$; two-way/one-way ANOVA.

Figure E3. ApoA-IV does neither affect lipid raft abundance nor CCR3 expression in eosinophils. (A) Lipid rafts were assessed in purified eosinophils using cholera toxin B-FITC and measured by flow cytometry ($n=6-9$). (B) CCR3 surface expression was determined in purified eosinophils by flow cytometry and expressed as % of CCR3 positive cells ($n=9$). Data are shown as mean $\pm$ SEM, (A) one-way ANOVA, (B) Student's t-test.

Figure E4. ApoA-IV differentially regulates apoptosis in eosinophils from allergic and non-allergic donors. Purified eosinophils from allergic and non-allergic donors were resuspended in assay media, treated with ApoA-IV (3 µg/mL), ApoA-I (10 µg/mL) or vehicle and cultured for 18 h. After incubation, apoptosis was assessed by flow cytometry using annexin-V/PI staining. (A) Annexin-V-negative/PI-negative cells were considered live cells, (B) Annexin-V-positive cells were considered apoptotic cells, (C) Annexin-V-positive/PI-negative cells were considered early apoptotic cells, (D) Annexin-V-positive/PI-positive cells were considered late apoptotic cells and (E) Annexin-V-negative/PI-positive cells were considered necrotic cells. Data are expressed as % of total cells ($n=5-7$) and are shown as mean + SEM, $*p<0.05$; one-way ANOVA.

Figure E5. ApoA-IV serum levels are significantly reduced in HDM-sensitized and challenged mice. On day 1, eight- week- old female Balb/c mice were sensitized intranasally with 1 µg HDM and challenged by intranasal application of 10 µg HDM per day from day 7 to 11. On day 15 whole blood was collected by cardiac puncture and serum was gained by centrifugation. ApoA-IV serum levels were determined by ELISA ($n=5-7$). Data are shown as mean ± SEM, $*p<0.05$; Student's t-test.

Figure E6. Treatment with ApoA-IV does not affect numbers of macrophages, lymphocytes, monocytes and neutrophils in BAL fluid of mice in HDM-induced airway inflammation. On day 1, eight- week- old female Balb/c mice were sensitized intranasally with 1 µg HDM and challenged by intranasal application of 10 µg HDM per day from day 7 to 11. From day 7 to 14 mice were daily injected with 10 µg ApoA-IV or vehicle. On day 15 BAL fluid was taken from six mice per group and cellular contents were analyzed by flow cytometric staining. Data are shown as mean ± SEM; Student's t-test.

231 **Figure E7. ApoA-IV protects mice from HDM-induced systemic eosinophilia.** On day 1,
232 eight- week- old female Balb/c mice were sensitized intranasally with 1 μ g HDM and
233 challenged by intranasal application of 10 μ g HDM per day from day 7 to 11. From day 7 to
234 14 mice were daily injected with 10 μ g ApoA-IV or vehicle. On day 15 (A) splenocytes were
235 prepared and (B) bone marrow cells were isolated from femur and tibia, and cell counts were
236 analyzed by flow cytometric staining ($n=4$ per treatment group). Data are shown as mean $\pm$
237 SEM; * $p<0.05$, *** $p<0.001$; Student's t-test.

References

1. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. European Position Paper on Rhinosinusitis and Nasal Polyps 2012. *Rhinol Suppl* 2012; 23:3 p preceding table of contents, 1-298.
2. Lund VJ, Mackay IS. Staging in rhinosinusitis. *Rhinology* 1993; 31:183-4.
3. Schmid C, Habermann W, Braun H, Gugatschka M, Oriel BS, Smietana JA, et al. Released intranasal eosinophilic major basic protein as a diagnostic marker for polypoid chronic rhinosinusitis. *Otolaryngol Head Neck Surg* 2010; 143:386-91.
4. Holzer M, Kern S, Trieb M, Trakaki A, Marsche G. HDL structure and function is profoundly affected when stored frozen in the absence of cryoprotectants. *J Lipid Res* 2017; 58:2220-8.
5. Schratl P, Sturm EM, Royer JF, Sturm GJ, Lippe IT, Peskar BA, et al. Hierarchy of eosinophil chemoattractants: role of p38 mitogen-activated protein kinase. *Eur J Immunol* 2006; 36:2401-9.
6. Konya V, Ullen A, Kampitsch N, Theiler A, Philipose S, Parzmair GP, et al. Endothelial E-type prostanoid 4 receptors promote barrier function and inhibit neutrophil trafficking. *J Allergy Clin Immunol* 2013; 131:532-40 e1-2.
7. Frei RB, Luschnig P, Parzmair GP, Peinhaupt M, Schranz S, Fauland A, et al. Cannabinoid receptor 2 augments eosinophil responsiveness and aggravates allergen-induced pulmonary inflammation in mice. *Allergy* 2016; 71:944-56.
8. Sturm EM, Schratl P, Schuligoi R, Konya V, Sturm GJ, Lippe IT, et al. Prostaglandin E2 inhibits eosinophil trafficking through E-prostanoid 2 receptors. *J Immunol* 2008; 181:7273-83.
9. Sturm EM, Parzmair GP, Radnai B, Frei RB, Sturm GJ, Hammer A, et al. Phosphoinositide-dependent protein kinase 1 (PDK1) mediates potent inhibitory effects on eosinophils. *Eur J Immunol* 2015; 45:1548-59.

- 264 10. Curcic S, Holzer M, Frei R, Pasterk L, Schicho R, Heinemann A, et al. Neutrophil
265 effector responses are suppressed by secretory phospholipase A2 modified HDL.
266 Biochim Biophys Acta 2015; 1851:184-93.
- 267 11. Hartnell A, Heinemann A, Conroy DM, Wait R, Sturm GJ, Caversaccio M, et al.
268 Identification of selective basophil chemoattractants in human nasal polyps as insulin-
269 like growth factor-1 and insulin-like growth factor-2. J Immunol 2004; 173:6448-57.
- 270 12. Plantinga M, Guilliams M, Vanheerswynghe M, Deswarte K, Branco-Madeira F,
271 Toussaint W, et al. Conventional and monocyte-derived CD11b(+) dendritic cells
272 initiate and maintain T helper 2 cell-mediated immunity to house dust mite allergen.
273 Immunity 2013; 38:322-35.