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Genetic Variation in Surfactant Protein-A2 Delays Resolution of Eosinophilia in Asthma

Alane Blythe C. Dy,^{*,†} Muhammad Z. Arif,[‡] Kenneth J. Addison,^{†,§} Loretta G. Que,[¶] Scott Boitano,^{†,||} Monica Kraft,^{†,‡} and Julie G. Ledford^{†,§}

Surfactant protein-A (SP-A) is an important mediator of pulmonary immunity. A specific genetic variation in *SP-A2*, corresponding to a glutamine (Q) to lysine (K) amino acid substitution at position 223 of the lectin domain, was shown to alter the ability of SP-A to inhibit eosinophil degranulation. Because a large subgroup of asthmatics have associated eosinophilia, often accompanied by inflammation associated with delayed clearance, our goal was to define how SP-A mediates eosinophil resolution in allergic airways and whether genetic variation affects this activity. Wild-type, SP-A knockout (SP-A KO) and humanized (SP-A2 223Q/Q, SP-A2 223K/K) C57BL/6 mice were challenged in an allergic OVA model, and parameters of inflammation were examined. Peripheral blood eosinophils were isolated to assess the effect of SP-A genetic variation on apoptosis and chemotaxis. Five days postchallenge, SP-A KO and humanized SP-A2 223K/K mice had persistent eosinophilia in bronchoalveolar lavage fluid compared with wild-type and SP-A2 223Q/Q mice, suggesting an impairment in eosinophil resolution. In vitro, human SP-A containing either the 223Q or the 223K allele was chemoattractant for eosinophils whereas only 223Q resulted in decreased eosinophil viability. Our results suggest that SP-A aids in the resolution of allergic airway inflammation by promoting eosinophil clearance from lung tissue through chemotaxis, independent of SP-A2 Q223K, and by inducing apoptosis of eosinophils, which is altered by the polymorphism. *The Journal of Immunology*, 2019, 203: 000–000.

The four surfactant proteins, A, B, C, and D, are best characterized for their roles in pulmonary surfactant. Surfactant protein-A (SP-A) is the most abundant protein of the four types and is a member of the collectin superfamily, which are known participants in innate immune defense (1). Previous studies have shown that SP-A has various roles in the host response against inhaled insults and is most well known as an opsonin to enhance phagocytic uptake of pathogens (1). Additionally, SP-A has been shown to bind to *Mycoplasma pneumoniae*, which is frequently associated with asthma exacerbations (2), resulting in inhibition of its growth in vitro (3, 4). Pastva et al. (5) found that mice lacking SP-A had enhanced Th2 associated indices of inflammation 24 h after challenge as compared with wild-type (WT) mice in the OVA model. Along this line of evidence, previous studies have shown that SP-A isolated from asthmatics

is dysfunctional in attenuating IL-8 and Muc5AC production in response to *M. pneumoniae* infection as compared with SP-A isolated from nonasthmatic individuals (2).

Humans have two functional SP-A genes, *SP-A1* and *SP-A2*, that together organize into a complex octadecamer (6, 7). Several allelic variations of the SP-A genes have been identified and similarly linked to varying responses to pulmonary infections and control of inflammation (8). In particular, others have shown that the presence of a lysine (K) at position 223 was associated with higher rates of respiratory syncytial virus infection among infants (9), whereas a glutamine (Q) at this position was associated with protection against respiratory distress syndrome (10). Although we are not aware of any studies that specifically evaluate the possible alterations to the native full-length SP-A oligomeric structure due to *SP-A2* genetic variation, it has been shown that stably transfected cell lines expressing single gene SP-A variants form oligomers that are of similar patterns and orders of magnitude (7). Likewise, patterns of oligomerization were not different between SP-A purified from normal individuals when compared with those purified from asthmatic individuals (2). This suggests that the association of the polymorphism at position 223 and altered function is not likely due to changes in SP-A structure but may be due to altered functionality in the context of asthma.

Eosinophilia is the increased presence of eosinophils in the airway and peripheral blood (11) and is a well-documented phenotype in the lungs of a large subgroup of asthmatics (12). Release of preformed granules from eosinophils leads to damage of the airway mucosa and remodeling (13). Therapies that aim to minimize eosinophilic inflammation aid in the reduction of symptoms associated with allergic airway disease (14). In addition, studies have shown that obese asthmatics have more severe tissue eosinophilia (15, 16) and that they also have decreased levels of SP-A compared with lean normal and lean asthmatic individuals (17). Moreover, in a mouse model of allergic airway inflammation, administration of exogenous SP-A promoted the resolution of tissue eosinophilia (17). We have also demonstrated that SP-A

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Abbreviations used in this article: AUC, area under the curve; BALF, bronchoalveolar lavage fluid; EAR, eosinophil-associated RNase; K, lysine; KO, knockout; PI, propidium iodide; Q, glutamine; SP-A, surfactant protein-A; WT, wild-type.

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inhibits degranulation of eosinophils (18) and that this ability is altered by the genetic variation in SP-A2 position 223 (6), thus suggesting a differential role in the modulation of eosinophilic inflammation for SP-A2.

In this study, we set out to determine if genetic variation in human SP-A2, Q223K, would alter the resolution of allergic airways disease by specifically mediating eosinophil activities. Using a combination of isolated human SP-A, eosinophils and mouse models that express human SP-A2, our studies suggest, to our knowledge, two novel functions for SP-A: 1) as a chemoattractant for eosinophils, which is not dependent on genetic variation at position 223; and 2) as an inducer of eosinophil apoptosis, which is dependent on position 223. Taken together, we show that SP-A is an important contributing factor leading to the resolution of eosinophilia in allergen-challenged mice, and specific genetic variation (rs1965708) that is present in the human population alters this activity.

Materials and Methods

Human SP-A extraction

SP-A was isolated and purified from the bronchoalveolar lavage fluid (BALF) of patients with alveolar proteinosis by butanol extraction methods as previously described (17, 19). The final concentration of SP-A was determined as 1.2 mg/ml, and endotoxin levels were <0.01 pg/ml SP-A (Pierce LAL Chromogenic Endotoxin Quantitation Kit; ThermoFisher Scientific, Rockfield, IL). Human SP-A extracted by our group is available and is currently being shipped to research laboratories both nationally and internationally.

Mouse models

All experiments were done in accordance with University of Arizona on Institutional Animal Care and Use Committee–approved animal protocols. Humanized mice transgenic for SP-A2 were generated as previously described (20). SP-A knockout (KO) mice were generated as previously described (21), back-crossed 14 generations onto the C57BL/6 background, and bred in house. WT mice were purchased from Jackson Laboratories (Bar Harbor, ME) and bred in house for experiments. Depending on the timing of induction of allergic inflammation in female mice using the OVA model, it has been reported that estrogen has dual effects (22). To eliminate this factor, we therefore used male age-matched (6–8 wk, weighing 20–25 g) WT, SP-A KO, SP-A2 223Q/Q, and SP-A2 223K/K mice on a C57BL/6J background. IL-5 transgenic mice were generously provided by the late Dr. J. J. Lee (23) and were bred in house for isolation of eosinophils for our *in vitro* studies. All mice were housed under 12 h light–dark cycle with free access to standard chow and water.

Induction of allergic airways by use of OVA in mice

For the time point experiments, sensitization was accomplished by *i.p.* injections of OVA (50 µg per mouse; Sigma-Aldrich, St. Louis, MO) in 150 µl of alum (40 mg/ml; ThermoFisher Scientific) on days 0 and 7. Challenge was given by three consecutive intranasal administrations of 100 µg OVA in PBS on days 14, 15 and 16. For the rescue experiments, the protocol was slightly modified to accommodate optimal delivery of replacement SP-A as previously described (17). Briefly, mice were given *i.p.* injections of 30 µg OVA in alum on days 0 and 14 and challenged with 1% OVA aerosol on days 21, 22, and 23 via a Novag Ultrasonic 2000 Nebulizer (Novag, Goldach, Switzerland). Subsequently, on day 24, SP-A was given oropharyngeally at 25 µg in 50 µl saline per mouse. Control mice for the rescue experiment were sensitized and challenged with OVA but received vehicle (saline) in place of SP-A.

BALF and lung tissue

One, three, and five days after the terminal intranasal challenge, mice were euthanized by anesthetic overdose (urethane, 250 mg/ml, 1.5 g/kg; Sigma-Aldrich). The trachea of each mouse was exposed and cannulated with a 19-gauge catheter. Airways and lungs were washed with 1.5 ml of PBS (100 µM EDTA). Lungs were excised and removed. The left lung was fixed in 10% buffered formalin and transferred to 70% ethanol after 3 d for histologic analysis. The right lung was snap frozen in liquid nitrogen and kept at –80°C until assayed. Total cell counts were quantified using a Countess II FL Automated Cell Counter (Life Technologies, Carlsbad, CA), and differential leukocyte counts were assessed on cytocentrifuged

slides at a seeding density of 200,000 cells per slide stained with the Easy III Rapid Differential Staining Kit (Azer Scientific, Morgantown, PA) using standard morphological identification of cell types.

Histological analysis

Upon necropsy, the left lung lobe from each mouse was fixed in 10% formalin and embedded in paraffin. Midsagittal lung sections (4 µm thick) were stained with Sirius Red stain. Briefly, paraffin-embedded lungs were deparaffinized using xylene and ethanol. Slides were then counterstained in hematoxylin for 3 min, rinsed in water and 100% ethanol, and incubated in Sirius Red for 1 h following a previously described protocol (24). Histological slides at 40× magnification were photographed and scored by at least two blinded individuals. The average of these measurements were graphed for each mouse for statistical analysis.

Gene expression by real-time quantitative RT-PCR

To evaluate mRNA expression of cyclophilin (internal control; sense: 5'-AGC ACT GGA GAG AAA GGA TTT GG-3', antisense: 5'-TCT TCT TGC TGG TCT TGC CAT T-3') and eosinophil-associated RNase (EAR) (sense: 5'-CGA CTT TGT CTC CTG CTG-3', antisense: 5'-TGT CCC ATC CAA GTG AAC-3'), real-time quantitative RT-PCR was performed. Total RNA was extracted using TRIzol reagent (Invitrogen, San Diego, CA) and reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Targets were amplified using their respective primers and SYBR Green Supermix in the Bio-Rad CFX system (Bio-Rad Laboratories). Relative cycle threshold, or CT, was used to compare gene expression levels using $2^{-\Delta\Delta CT}$ method.

Determination of proteins by ELISA

Human SP-A (BioVendor, Brno, Czech Republic), mouse eotaxin-1 (CCL11; eBioscience, Vienna, Austria), and eotaxin-2 (CCL24; R&D Systems, Minneapolis, MN) assays were performed on BALF according to manufacturers' protocols. Samples for CCL11 and CCL24 were diluted 1:2 and samples for human SP-A were assayed undiluted. Briefly, BALF samples were incubated on a 96-well plate coated with the capture Ab and detected with a Streptavidin–HRP/biotin-conjugated secondary Ab complex on a plate reader (BioTek Instruments, Winooski, VT).

Eosinophil and leukocyte isolation

IL-5 transgenic mice were euthanized, and blood was collected by cardiac puncture through the left ventricle. Spleens were excised and subsequently minced. RBCs from blood or spleens were lysed and eosinophils were isolated by negative selection as previously described (18). Purity of each preparation was verified by cytochrome and an Easy III Rapid Differential Staining kit (Azer Scientific) to be >95%. Leukocytes were isolated from 20 ml of blood taken from asthmatic volunteers on an institutional review board–approved protocol at the University of Arizona. Volunteers consisted of three female, non-Hispanic, white asthmatics, between the ages of 30–59, and were either not on medication or on combination albuterol/ipratropium or albuterol/Symbicort therapies. All blood samples were placed into tubes containing anticoagulant, kept at 4°C, and processed within 6 h of patients' blood draw to avoid loss of viability. After density gradient centrifugation, RBCs were lysed, and total leukocytes were used for flow cytometry experiments detailed below.

Determination of proteins by western blotting

Purified eosinophils from blood or spleen were incubated with SP-A in RPMI 1640 at 37°C and 5% CO₂ for 16 h. For collection, 200 µl of radioimmunoprecipitation assay (RIPA) buffer (Teknova, Hollister, CA) with protease inhibitors (Roche, Basel, Switzerland) was used for cell lysis and protein extraction. Proteins from cell lysates were quantified using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific) and equal amounts of lysate were loaded onto Mini-Protein TGX precast gels (Bio-Rad Laboratories). Abs for detecting cleaved caspase-3 and GAPDH were used according to manufacturer's recommendations (Cell Signaling Technology, Danvers, MA). Western blots were imaged using a ChemiDoc Imaging System, and densitometry was quantified using Image Lab Software (Bio-Rad Laboratories).

Chemotaxis by transmigration plate assay

The migration of purified eosinophils in response to SP-A *in vitro* was assessed using 5 µm polycarbonate membrane inserts in 24-well tissue culture plates (Costar, Corning, NY) as previously described (25) with minor modifications. Briefly, inserts were incubated in RPMI 1640 (10% FBS) for 1 h, after which media was removed and eosinophils (1×10^6 cells per well with 30 ng/ml rIL-5 in 200 µl) were added onto the inserts.

Eosinophils were allowed to migrate for 90 min. Migrated cells (i.e., cells that reached the bottom chamber and on the underside of the inserts) were collected and counted using a Countess II FL Automated Cell Counter (Life Technologies). Migration index was calculated as fold of total cells migrated in test well over vehicle control. Eotaxin-2 (PeproTech, Pittsburgh, PA) was used as a positive chemoattractant control.

Assessment of eosinophil viability

Viability by trypan blue. Uptake of trypan blue by eosinophils was performed by mixing equal volumes of the dye and cell suspension. Live and dead cells were evaluated and counted using a Countess II FL Automated Cell Counter (Life Technologies).

Cytotoxicity assay by real-time impedance tracing. Real-time monitoring of eosinophil detachment and cell death were assessed by measuring electrical impedance using the xCELLigence Real-Time Cell Analyzer (ACEA Biosciences, San Diego, CA) as previously described (26, 27). Briefly, media were placed in 96-well gold electrode-coated plates (E-plates; ACEA Biosciences) and allowed to equilibrate, and a background reading was obtained. Eosinophils were then seeded at 1×10^6 cells/100 μ l and allowed to settle for ~ 5 h. SP-A was added at various concentrations, and changes in electrical impedance were measured over time. Impedance measurements, presented as a normalized "cell index," are calculated as detailed (26, 27). Under these conditions, a loss of cell index is associated with eosinophil detachment and cytotoxicity. Individual traces of cytotoxicity over time were averages of two to three technical replicates; SD were eliminated for clarity. Quantification of cytotoxicity was accomplished by measuring the area under the curve (AUC) after normalization of cell index. Graphical AUC measurements include baseline correction for untreated cells to best display changes.

Flow cytometry analysis. For the direct treatment of cells with SP-A, leukocytes from human blood were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich). After 16 h incubation with SP-A, human leukocytes were labeled with Siglec-8-PE (BioLegend, San Diego, CA). For the *in vivo* rescue mouse experiments, mouse BALF cells were collected and incubated with the following fluorescent Abs: CD11b-PE-Cy7 (BD Biosciences, San Diego, CA) and CCR3-allophycocyanin (BioLegend). Apoptotic cells were labeled using a FITC Annexin V Apoptosis Detection Kit (BD Biosciences). Flow cytometry was performed on an Attune NxT Flow Cytometer (ThermoFisher Scientific). Apoptotic eosinophils were identified as Siglec-8⁺, Annexin V⁺, and propidium iodide (PI)⁺ (human) or CD11b⁺, CCR3⁺, Annexin V⁺, and PI⁺ (mouse). Data were processed and analyzed using FlowJo 10.5.3.

Statistical analysis

All statistical analyses were done using Graphpad Prism software. Because there were four genetically distinct groups of mice, one-way ANOVA was used to assess global differences between groups, followed by multiple *t* tests with Bonferroni correction for multiple comparisons.

Results

Genetic variation in SP-A2 alters eosinophil resolution in allergic airways

To determine whether the genetic variation at position 223 of SP-A2 plays a role in mediating the immune response to allergen challenge, we used the OVA sensitization and challenge protocol (Fig. 1A). At 24 h after terminal challenge, an increased presence of eosinophils was observed in the BALF in all groups, with mice harboring the 223Q allele (223Q mice) exhibiting the most robust influx of eosinophils (Fig. 1B, Supplemental Fig. 1C). However, at 5 d after terminal challenge, mice deficient in SP-A (KO mice) and those with the 223K allele (223K mice) remained in a state of significantly enhanced eosinophilia (Fig. 1B, Supplemental Fig. 1C). When eosinophil recruitment and resolution were quantified over time, the 223Q mice had the highest frequency of eosinophils immediately after OVA challenge, but they also had the largest net decrease in eosinophils compared with all other groups by day 5 (Fig. 1C, Supplemental Fig. 1C). In contrast, eosinophil numbers in the BALF of 223K mice remained relatively unchanged from 24 h to 5 d (Fig. 1C, Supplemental Fig. 1C).

To assess eosinophil infiltration in mouse lung tissue, histochemical staining was performed with Sirius Red stain. Focal points

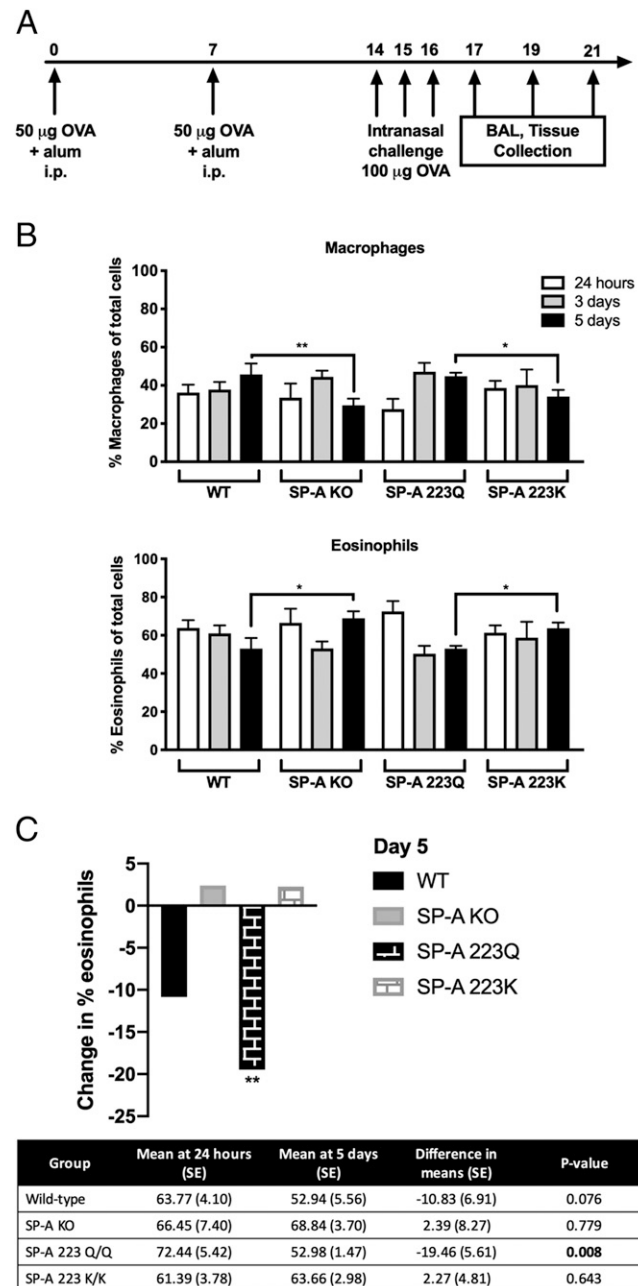


FIGURE 1. Assessment of BALF eosinophilia over time. (A) OVA model of allergic airways. (B) Cell distribution in BALF at 24 h, 3 d, and 5 d after terminal challenge. (C) Net change in eosinophil frequencies over time. Table shows difference in means at 24 h and 5 d, unpaired Student *t* test. One-way ANOVA with Bonferroni correction for multiple comparisons, data (mean $\pm$ SEM) are from at least two independent experiments with *n* = 3–5 mice per group. **p* < 0.05, ***p* < 0.01.

of accumulated peribronchiolar and perivascular eosinophils were identified and quantified (Fig. 2A). There was a notable increase in the mean perivascular eosinophil counts compared with peribronchial eosinophil counts in OVA-challenged 223K mice and an increased trend in OVA-challenged KO mice (*p* = 0.06) (Fig. 2B), which could suggest an altered communication between these two compartments. Additionally, there was an increased trend in the perivascular eosinophil counts in the KO mice compared with the WT mice (*p* = 0.06) and a significant increase compared with the 223Q mice. Although overall eosinophil numbers in the lung tissue between groups were not different (Fig. 2A, 2B), mice deficient in SP-A had persistent tissue eosinophilia at 5 d postchallenge,

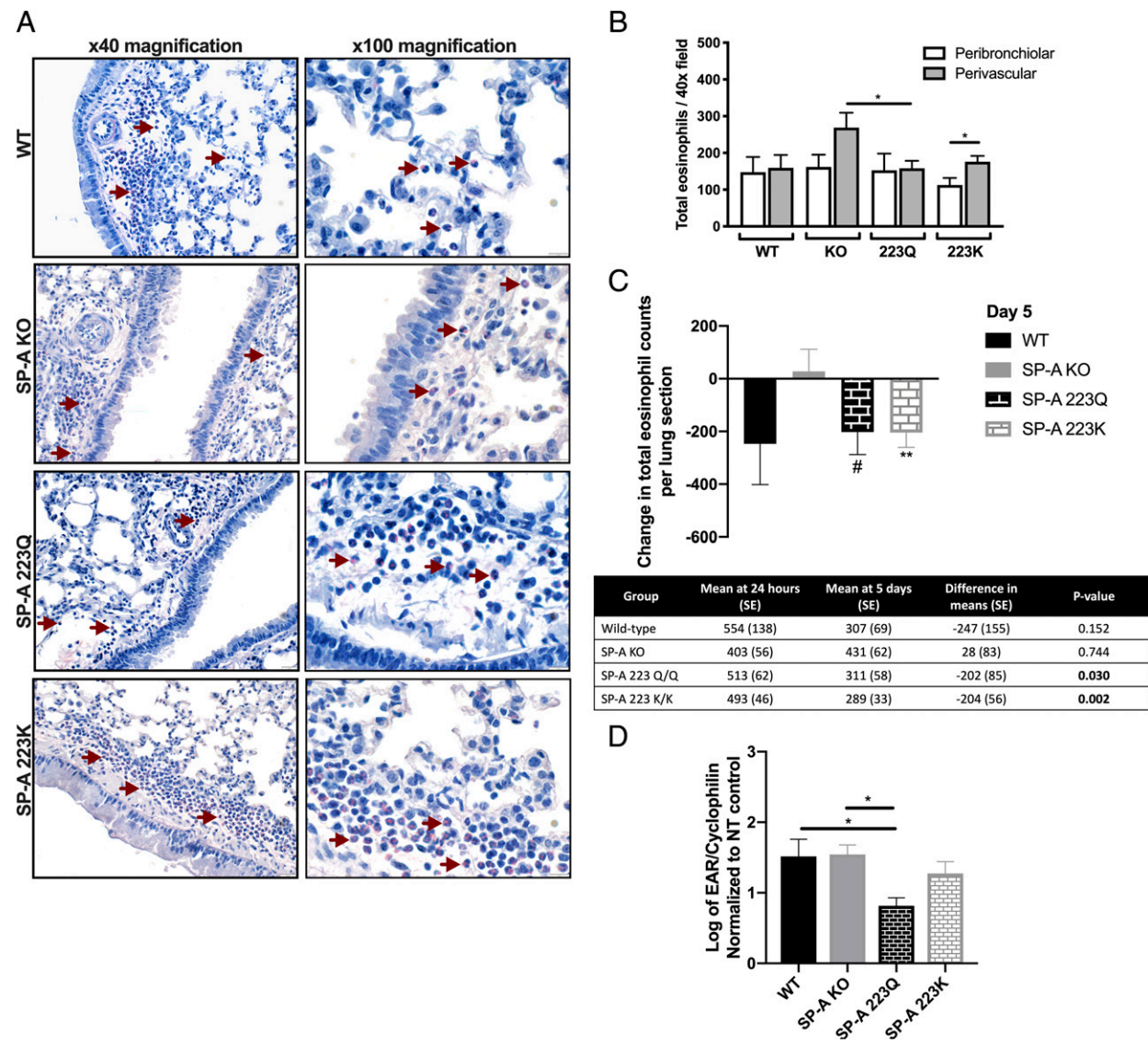


FIGURE 2. Assessment of tissue eosinophilia over time. **(A)** Representative bright field images of eosinophils (red arrows indicate representative eosinophils with bright pink-stained cytoplasm) in lung tissue by Sirius Red staining (left panel, original magnification $\times 40$; right panel, original magnification $\times 100$) and **(B)** quantification of eosinophil counts at day 5. $*p < 0.05$. **(C)** Net change in eosinophil frequencies over time. Table shows difference in means at 24 h and 5 d, unpaired Student *t* test, $\#p < 0.05$, $**p < 0.01$. **(D)** EAR mRNA in lung tissue at 5 d after terminal challenge. One-way ANOVA with Bonferroni correction for multiple comparisons. Data (mean $\pm$ SEM) are from at least two independent experiments with $n = 3$ –5 mice per group. $*p < 0.05$.

whereas both the 223Q and 223K mice had significantly decreased tissue eosinophil counts, in comparison with their respective eosinophil counts at 24 h (Fig. 2C). An examination of *EAR* mRNA as a marker of lung eosinophil activation showed that the 223Q mice had the lowest expression of *EAR* among the four groups (Fig. 2D).

Genetic variation in SP-A2 does not alter eotaxin expression

Because the presence of SP-A was associated with a decline in eosinophil counts from 24 h to 5 d in the lung tissue (Fig. 2), we next sought to determine whether SP-A played a role in regulating the production of eotaxins. Eotaxins are potent inducers of eosinophil movement. Both eotaxin-1 and eotaxin-2 have been previously shown to be elevated in the OVA model: eotaxin-2 was significantly higher in BALF and tissue, whereas eotaxin-1 was significantly increased only in tissue, over their respective controls (28). We therefore investigated the levels of these two eotaxins in the BALF 24 h, 3 and 5 d after OVA challenge. Similar to what

others have found, eotaxin-1 was not significantly elevated in OVA-treated mice over their respective untreated controls (Fig. 3A). Additionally, there were no differences in eotaxin-1 observed between OVA-challenged groups during the time course of the study (Fig. 3A). Although eotaxin-2 levels in BALF were significantly elevated at 24 h over untreated controls, these levels were quickly diminished by days 3 and 5 in all groups (Fig. 3B).

SP-A is a chemoattractant for eosinophils

As there were no differences based on SP-A genotype in the levels of eotaxins in the BALF of OVA-challenged mice, we next sought to examine whether SP-A contributes to eosinophil movement by acting as a chemoattractant. Indeed, in an *in vitro* transmigration assay, SP-A, used at physiologic concentrations typically found in the lung, acted as a chemoattractant for eosinophils (Fig. 4A). Regardless of the polymorphism at position 223, this chemoattractant ability was comparable to the positive control, eotaxin-2.

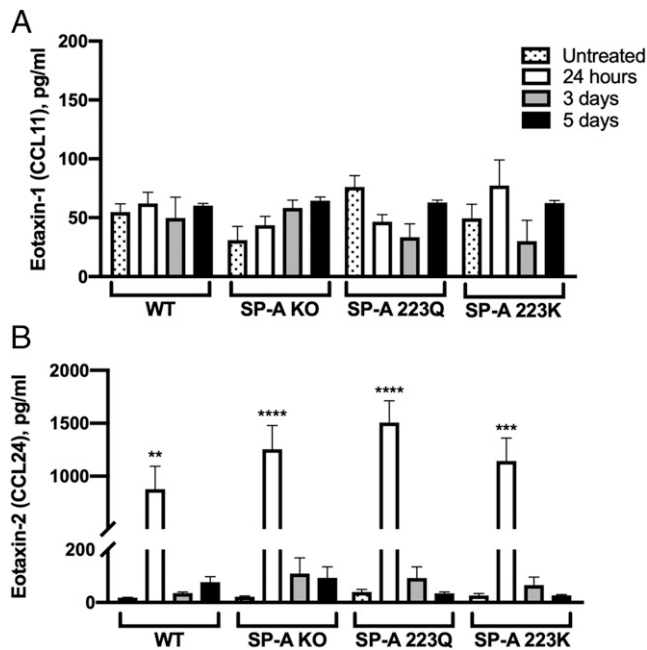


FIGURE 3. Examination of eotaxins in BALF after OVA challenge. Analysis of protein concentrations by ELISA of (A) eotaxin-1 or CCL11 and (B) eotaxin-2 or CCL24. One-way ANOVA with Bonferroni correction for multiple comparisons. Data (mean $\pm$ SEM) are from at least two independent experiments with $n = 3$ –5 mice per group. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to untreated samples within each group.

It has been previously shown that surfactant protein levels in allergen-challenged WT mice were unchanged in the BALF compared with untreated controls (18). It has also been shown that both strains of the naive humanized SP-A2 transgenic mice have similar SP-A levels in the BALF (20). To determine whether SP-A concentration after OVA challenge was altered and thus could be a contributing factor in eosinophil movement into the lung lumen, we examined the BALF of OVA-challenged 223Q and 223K mice for overall SP-A concentration. SP-A levels were similar in the BALF of 223Q and 223K mice at 24 h postterminal OVA challenge, both of which were not significantly different from untreated levels (20) (Fig. 4B). However, at day 5, 223K mice had significantly higher levels of SP-A compared with 223Q mice (Fig. 4B).

Genetic variation in SP-A2 alters the ability of SP-A to reduce eosinophil viability

It is known that SP-A can bind to eosinophils in a dose-dependent manner and that this binding is partially mediated by CD16/32 (Fc γ RIII/Fc γ RII) (18). Thus, we hypothesized that SP-A may be binding to eosinophils to initiate apoptosis and clearance. Here we examined whether the difference in eosinophil resolution was due to modulation of eosinophil cell death by SP-A. In fact, direct stimulation of purified eosinophils from IL-5 transgenic mice by isolated human SP-A homozygous for the Q allele resulted in significantly greater eosinophil death over a 24 h period compared with heterozygous SP-A (Q/K) (Fig. 5A). To more directly assess the ability of SP-A to induce cytotoxicity in eosinophils, we evaluated SP-A addition using xCELLigence Real-Time Cell Analyzer (Fig. 5B). Addition of SP-A >3 μ g/ml resulted in concentration-dependent cytotoxicity that developed in minutes and persisted for up to 48 h of measurement. To evaluate whether human eosinophils would respond similarly, leukocytes from the peripheral blood of asthmatic patients were stained for apoptosis after incubation with human SP-A. Flow cytometric analysis of

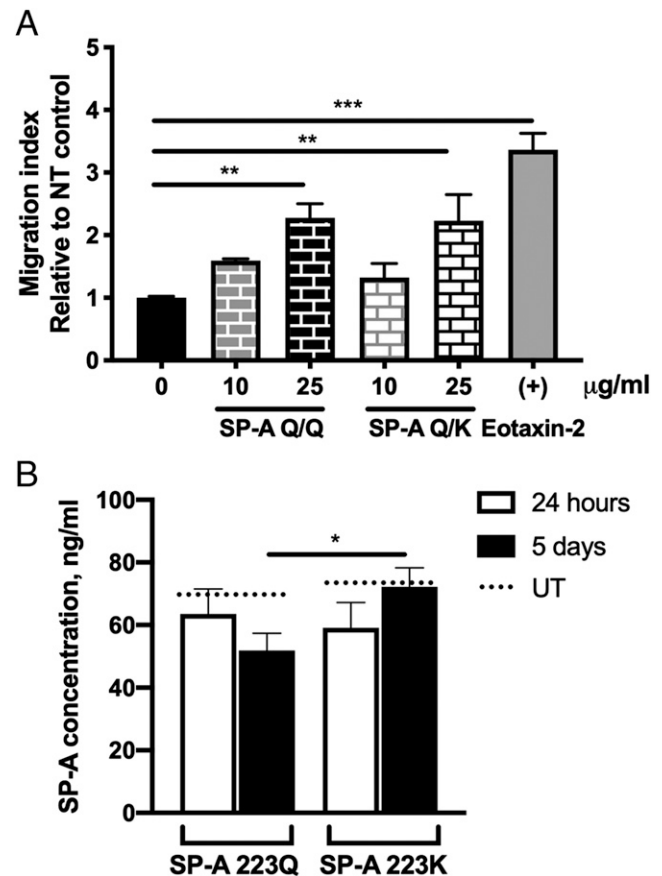


FIGURE 4. Evaluation of the ability of SP-A to induce eosinophil migration in vitro. (A) Migration of mouse eosinophils was measured by a plate-based assay. Migration index calculated as number of live eosinophils in the bottom chamber over control. (B) SP-A concentrations in BALF of OVA-challenged mice. One-way ANOVA with Bonferroni correction for multiple comparisons. Data (mean $\pm$ SEM) are from at least two independent experiments, with $n = 2$ –3 replicates per treatment or 3–5 mice per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. UT, untreated.

these cells allowed us to identify the eosinophil (Siglec-8⁺) population and, further, detect a shift from live (Annexin V[−], PI[−]) to early apoptotic (Annexin V⁺, PI[−]) within the gated eosinophil population (Fig. 5C). This phenomenon appears to be unique to eosinophils, as SP-A did not have the same effect on neutrophils, another important inflammatory cell in airway inflammation and asthma (Supplemental Fig. 2). In addition, caspase-3 cleavage was also increased in SP-A–treated eosinophils compared with vehicle; however, this increase was not significantly abrogated by blocking the CD16/32 (Fc γ RIII/Fc γ RII) (Fig. 5D).

Treatment with exogenous SP-A enhances eosinophil apoptosis in allergic airways

In Fig. 5, we showed that either the lack of SP-A or presence of an altered isoform of SP-A led to prolonged eosinophil survival, which could explain the persistent eosinophilia in the OVA-challenged KO and 223K mice. We sought to determine whether replacement of SP-A in SP-A KO mice would rescue this effect. SP-A KO mice were given OVA and subsequently treated with exogenous human SP-A homozygous for the Q allele at position 223 1 d after postterminal aerosol challenge (Fig. 6A). BALF and lung tissue were collected 5 d after aerosol challenge (Fig. 6A). Indeed, SP-A KO mice given exogenous SP-A had more apoptotic eosinophils compared with those given saline (Fig. 6B). Conversely, total live eosinophils in the BALF were decreased in the

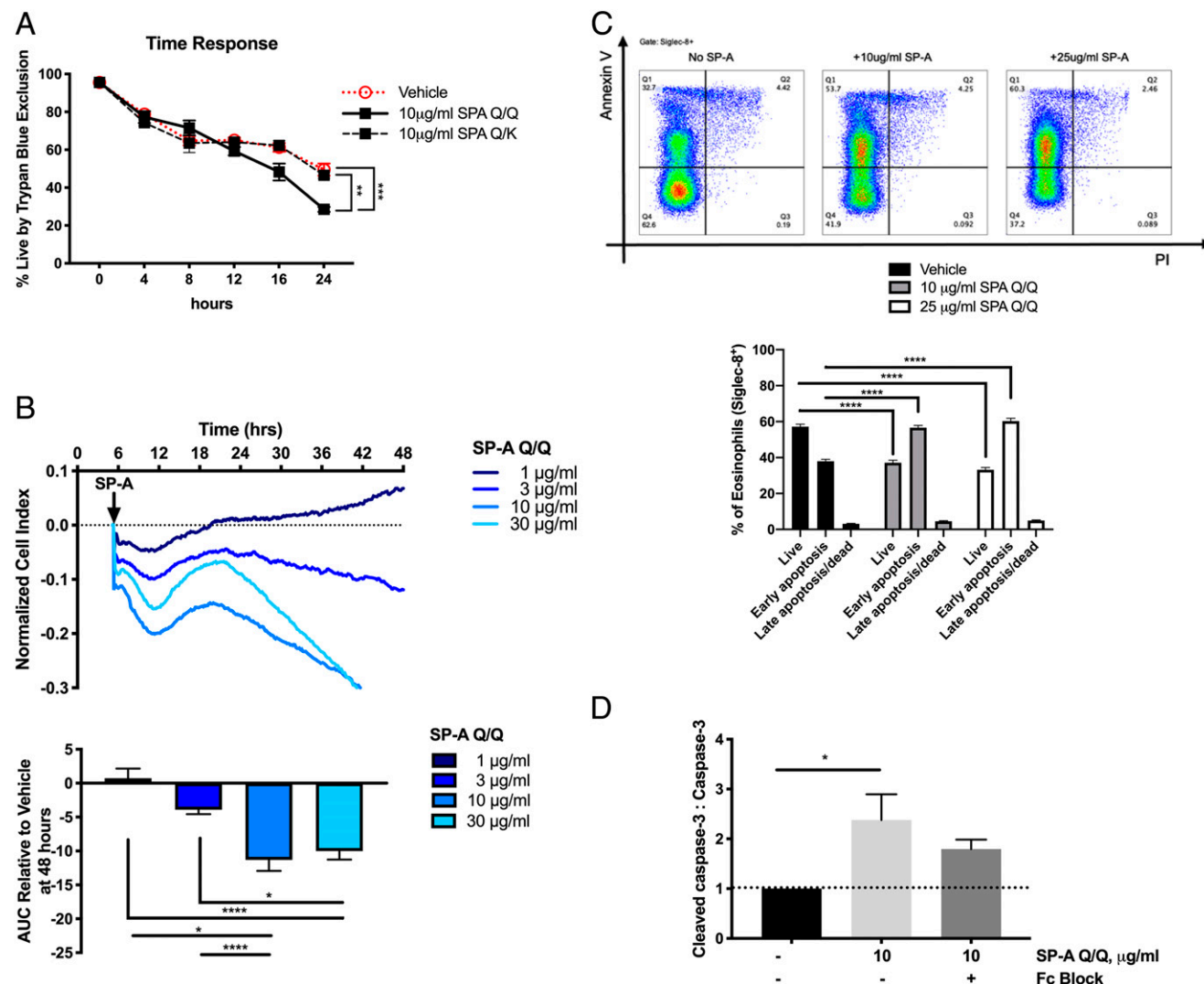


FIGURE 5. Evaluation of the ability of SP-A to induce eosinophil apoptosis in mouse and human eosinophils in vitro. **(A)** Time course of viability assessed by trypan blue and **(B)** Real-Time Cell Analyzer tracing and dose response of in vitro stimulation of mouse eosinophils by SP-A. **(C)** Representative flow diagrams of human eosinophil apoptosis and cell death by Annexin V and PI and quantification after 16 h incubation with SP-A. Live, Annexin V⁻, PI⁻; early apoptosis, Annexin V⁺, PI⁻; late apoptosis/dead, Annexin V⁺, PI⁺. **(D)** Densitometry of caspase-3 by Western blot of mouse eosinophils standardized to nontreated control. ANOVA with correction for multiple comparisons. Data (mean ± SEM) are from at least two independent experiments, with $n = 2-3$ replicates per treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

SP-A KO mice given the rescue treatment (Fig. 6C). Although not statistically significant, WT mice given exogenous SP-A also had a trend toward increased apoptotic eosinophils and decreased live eosinophils in the BALF compared with their vehicle controls (Fig. 6B, 6C).

Discussion

In this study, we present, to our knowledge, novel findings from our investigations regarding the role of SP-A in mediating the resolution of eosinophilia using a well-established model of allergic airway disease. Not only did we discover that SP-A is chemoattractant for eosinophils at physiologic concentrations, we also found that SP-A can induce eosinophil apoptosis. In humans, SP-A is comprised of products from two SP-A genes, both of which have known polymorphisms within the human population. Of those polymorphisms associated with disease, variation at position 223, which changes a Q to a K, was of special interest to us given the findings that recombinant SP-A containing the 223K was less active against eosinophil degranulation as 223Q (6).

Using SP-A humanized mice that represent the genetic variation of interest, amino acid substitution of a Q for a K at position 223 in the CRD of SP-A2, we were able to discern differences with respect to their resolution of eosinophilia in an allergic model. We further show that SP-A isolated from humans containing either Q/Q or Q/K at position 223 was chemoattractant for eosinophils at relatively the same rate. In contrast, only SP-A containing Q/Q was able to effectively induce eosinophil apoptosis in vitro, and mice expressing the Q/Q had significantly enhanced resolution of eosinophilia as compared with the K/K mice in vivo. Therefore, in some individuals, SP-A has the capacity to play dual roles during the resolution of allergic airway inflammation through mediation of eosinophil clearance from the lung tissue by directly promoting chemotaxis, which is independent of position SP-A2 Q223K, and aiding in apoptosis of eosinophils in the lumen, which is affected by the polymorphism at position SP-A2 Q223K.

Compared with 223Q mice, 223K mice had significantly more eosinophils in the BALF at 5 d postterminal OVA challenge, whereas the overall eosinophil numbers in the lung tissue were not different between groups. Although IL-5 plays a primary role in the

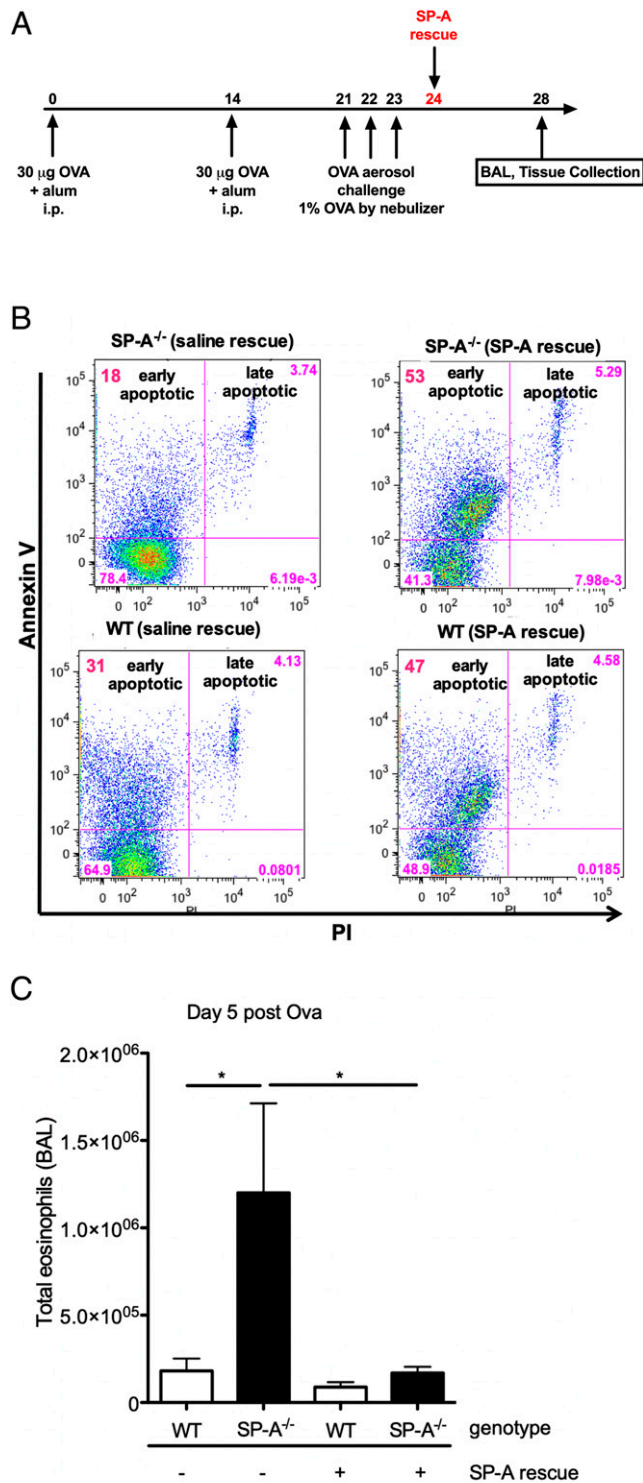


FIGURE 6. Evaluation of the effect of exogenous SP-A administration on eosinophils in SP-A-deficient mice after OVA challenge. **(A)** Schematic of OVA challenge and SP-A rescue. **(B)** Representative flow diagrams of eosinophil apoptosis and cell death by Annexin V and PI. **(C)** Total live eosinophil counts in BALF 5 d postterminal challenge. Data (mean $\pm$ SEM) are representative of two independent experiments with $n = 5$ mice per group. $*p < 0.05$.

expansion of the eosinophil cellular pool in the bone marrow and peripheral blood (29), eosinophils traffic from the lung tissue into the lung lumen predominantly by chemotaxis mechanisms during allergen-induced eosinophilia (30). Upon examination of chemotactic factors that could influence eosinophil movement from lung tissue

into the bronchoalveolar compartment, eotaxin levels were not different between the different genotypes of mice. This eliminates the likelihood that the eosinophil differences detected between the groups were attributable to eotaxin production.

We next tested the ability of SP-A to be a chemoattractant for eosinophils. Human SP-D, which, like SP-A, has sequence homology to its murine counterpart, is known to inhibit eotaxin-induced migration of eosinophils (31). However, little is known about the role of SP-A in eosinophil chemotaxis. We discovered that SP-A was chemoattractant for eosinophils regardless of the polymorphism at position 223 of SP-A2. This further highlights the significant contribution of SP-A to the net decrease in eosinophil counts in the lung tissue of 223Q and 223K mice by day 5 after OVA challenge. We assessed SP-A levels in the humanized mice to rule out the possibility that the humanized 223Q mice had more SP-A available and thus had a better resolution of eosinophilia. On the contrary, we discovered that SP-A was significantly increased in the BALF of 223K mice compared with 223Q mice at day 5 during resolution. Therefore, we cannot rule out that the increased levels of SP-A detected in the BALF of 223K mice contributes to the increased presence of eosinophils in those mice at day 5 during resolution. However, KO mice, which are completely deficient in SP-A, had similar persistent eosinophilia as the 223K mice at 5 d after allergen challenge, which supports the possibility of a separate nonchemotactic contributing mechanism to this phenomenon.

In this vein, we next examined the ability of SP-A to induce eosinophil apoptosis. It is well recognized that eosinophilia has critical contributions to the pathophysiology of asthma and airway inflammation (32–34). It has been shown that collagen deposition and increased thickness of airway smooth muscle were absent in eosinophil-deficient (Δ dbl GATA), allergen-challenged mice (32), which underlies the importance of the timely resolution and clearance of eosinophils during inflammation. In fact, numerous therapies targeting eosinophils, eosinophil-derived products, or its trafficking mechanisms are currently available or are being investigated in clinical trials (34–36). We found that SP-A harboring the SP-A2 223Q allele has the ability to induce eosinophil apoptosis, whereas the presence of at least one 223K allele diminished this activity to the levels of the vehicle control. Although the influence of SP-A as a chemoattractant may play a small role by marginally increasing the migration of eosinophils into the lung lumen, our data indicate that SP-A as an inducer of apoptosis is the foremost contributing mechanism to the resolution of eosinophilia in the allergic airways of OVA-challenged mice. More importantly, SP-A was able to induce apoptosis in human eosinophils, attesting to the translational nature and the clinical relevance of these findings.

This work was further strengthened by our studies in which allergic mice were given “rescue” SP-A. By flow cytometry, SP-A KO mice had a greater proportion of live (Annexin V⁻, PI⁺) eosinophils as compared with WT mice on day 5 during the resolution phase. When treated with exogenous SP-A, both WT and SP-A KO BALF samples had a shift from live (Annexin V⁻, PI⁺) to early apoptotic (Annexin V⁺, PI⁻). This resulted in a significant clearance of eosinophilia in KO mice as compared with their vehicle controls.

We have shown that SP-A has the ability to mediate release of eosinophil peroxidase upon stimulation with a pulmonary pathogen, *M. pneumoniae* (18). And whereas in vitro stimulation of eosinophils with *M. pneumoniae* in the presence of recombinant SP-A2 with Q present at position 223 significantly inhibited eosinophil peroxidase release, SP-A2 with K present at position 223 was less effective (6). The significantly decreased EAR expression

in the lungs of the OVA-treated 223Q mice, but not in the 223K mice, would suggest a potentially similar mediation by SP-A in limiting eosinophil activation. Importantly, the induction of apoptosis by SP-A, with 223Q being more active than SP-A with 223K, parallels our previous findings that SP-A 223Q is more protective against eosinophil degranulation (6). Overall, we believe the mechanism of action for SP-A 223Q is to bind to eosinophils and limit their degranulation while promoting their apoptosis and clearance from the lung.

One important limitation in our study is that the prevalence of SP-A homozygous for the K allele is <8% in the general population (37). As a result, we have had difficulty recruiting for this particular genotype from which to isolate human SP-A 223K/K. Therefore, there is a possibility that the chemoattractant capability will be altered when both alleles contain a K at position 223. Another important limitation is our inability to test our results observed in the OVA allergic model on another equally relevant allergic model, house dust mite. Two common house dust mite species, *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f), both possess cysteine protease activity and have been shown to cleave native human SP-A purified from BALF (38). This occurred in a time- and dose-dependent manner, affecting several biological functions of SP-A, thus making it difficult to ascertain the activity differences due to degradation within the animal model.

Our results show that SP-A aids in the resolution of allergic airways by inducing eosinophil apoptosis. We have reason to believe this induction of apoptosis may be specific for eosinophils. We did not observe an induction of apoptosis by SP-A for isolated neutrophils (Supplemental Fig. 2). In fact, SP-A has been shown to protect against lung epithelial cell apoptosis in bleomycin-induced acute lung injury (39), further supporting the notion that induction of apoptosis may be limited to eosinophils. Additionally, although binding of SP-A to eosinophils was previously shown to be partially mediated by CD16/32 (FcγRIII/FcγRII) (18), the induction of apoptosis on eosinophils does not seem to be facilitated through this receptor. Thus, further studies, which are beyond the scope of this manuscript, will involve the identification of the receptor(s) and downstream signaling processes responsible for this event.

Our findings raise the potential merit in the use of derivatives of specific SP-A variants (i.e., SP-A2 223Q/Q) as replacement therapy. It also warrants consideration of the utility of SP-A genotyping in future precision medicine initiatives for better treatment of lung diseases. Further studies with patient cohorts are needed to investigate which specific asthma endotypes would benefit from this type of therapeutic intervention. Although the presence of at least one K allele is relatively low (20–25%) in the general population, it is enriched (35%) within the African American subgroup (37). Of note, the prevalence of SP-A homozygous for the K allele is over 12% in the African American subgroup, compared with <8% in the general population (37). According to the Office of Minority Health of the United States Department of Health and Human Services, nearly 2.6 million non-Hispanic black individuals are reported to have asthma, and African Americans were three times more likely to die of causes related to asthma than white individuals. Although other factors, such as socioeconomic status, play a role in these disparities, this also suggests that there is value in the consideration of specific subgroups who are at increased risk in future investigations for SP-A differences in association with eosinophilic asthma.

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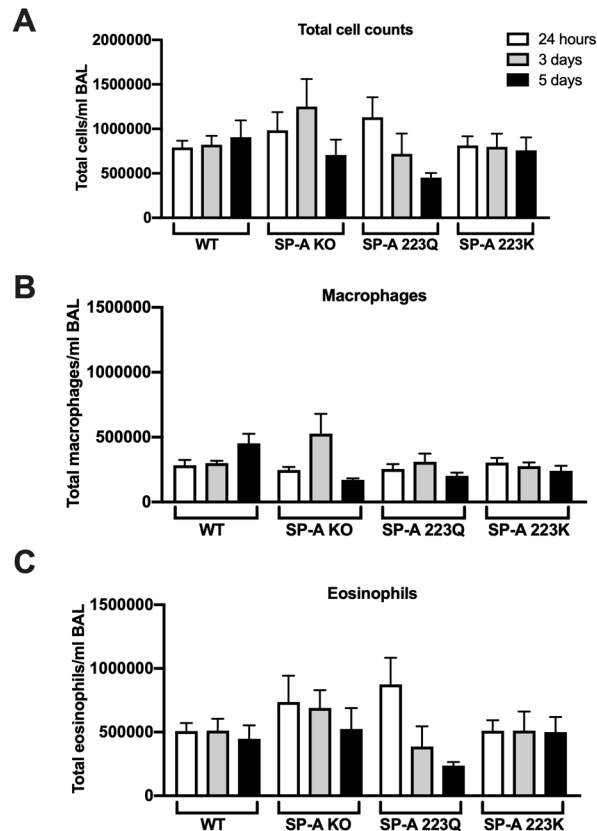
Disclosures

The authors have no financial conflicts of interest.

References

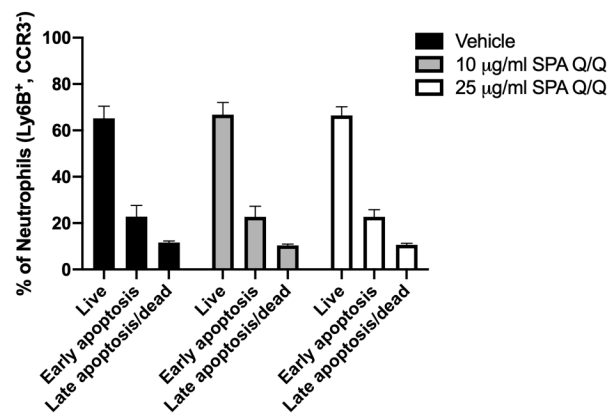
- Wright, J. R. 2005. Immunoregulatory functions of surfactant proteins. *Nat. Rev. Immunol.* 5: 58–68.
- Wang, Y., D. R. Voelker, N. L. Lugogo, G. Wang, J. Floros, J. L. Ingram, H. W. Chu, T. D. Church, P. Kandasamy, D. Fertel, et al. 2011. Surfactant protein A is defective in abrogating inflammation in asthma. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 301: L598–L606.
- Kannan, T. R., D. Provenzano, J. R. Wright, and J. B. Baseman. 2005. Identification and characterization of human surfactant protein A binding protein of *Mycoplasma pneumoniae*. *Infect. Immun.* 73: 2828–2834.
- Piboonpocanun, S., H. Chiba, H. Mitsuizawa, W. Martin, R. C. Murphy, R. J. Harbeck, and D. R. Voelker. 2005. Surfactant protein A binds *Mycoplasma pneumoniae* with high affinity and attenuates its growth by recognition of disaturated phosphatidylglycerols. *J. Biol. Chem.* 280: 9–17.
- Pastva, A. M., S. Mukherjee, C. Giamberardino, B. Hsia, B. Lo, G. D. Sempowski, and J. R. Wright. 2011. Lung effector memory and activated CD4+ T cells display enhanced proliferation in surfactant protein A-deficient mice during allergen-mediated inflammation. *J. Immunol.* 186: 2842–2849.
- Dy, A. B. C., S. Tanyaratrisakul, D. R. Voelker, and J. G. Ledford. 2018. The emerging roles of surfactant protein-A in asthma. *J. Clin. Cell. Immunol.* 9.
- Wang, G., S. R. Bates-Kenney, J. Q. Tao, D. S. Phelps, and J. Floros. 2004. Differences in biochemical properties and in biological function between human SP-A1 and SP-A2 variants, and the impact of ozone-induced oxidation. *Biochemistry* 43: 4227–4239.
- Pastva, A. M., J. R. Wright, and K. L. Williams. 2007. Immunomodulatory roles of surfactant proteins A and D: implications in lung disease. *Proc. Am. Thorac. Soc.* 4: 252–257.
- Löfgren, J., M. Rämet, M. Renko, R. Marttila, and M. Hallman. 2002. Association between surfactant protein A gene locus and severe respiratory syncytial virus infection in infants. *J. Infect. Dis.* 185: 283–289.
- Marttila, R., R. Haataja, M. Rämet, M. L. Pokela, O. Tammela, and M. Hallman. 2003. Surfactant protein A gene locus and respiratory distress syndrome in Finnish premature twin pairs. *Ann. Med.* 35: 344–352.
- Fahy, J. V. 2009. Eosinophilic and neutrophilic inflammation in asthma: insights from clinical studies. *Proc. Am. Thorac. Soc.* 6: 256–259.
- Jacobsen, E. A., N. A. Lee, and J. J. Lee. 2014. Re-defining the unique roles for eosinophils in allergic respiratory inflammation. *Clin. Exp. Allergy* 44: 1119–1136.
- Park, Y. M., and B. S. Bochner. 2010. Eosinophil survival and apoptosis in health and disease. *Allergy Asthma Immunol. Res.* 2: 87–101.
- Green, R. H., C. E. Brightling, S. McKenna, B. Hargadon, D. Parker, P. Bradding, A. J. Wardlaw, and I. D. Pavord. 2002. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 360: 1715–1721.
- van der Wiel, E., N. H. Ten Hacken, M. van den Berge, W. Timens, H. K. Reddel, and D. S. Postma. 2014. Eosinophilic inflammation in subjects with mild-to-moderate asthma with and without obesity: disparity between sputum and biopsies. *Am. J. Respir. Crit. Care Med.* 189: 1281–1284.
- Desai, D., C. Newby, F. A. Symon, P. Haldar, S. Shah, S. Gupta, M. Bafadhel, A. Singapuri, S. Siddiqui, J. Woods, et al. 2013. Elevated sputum interleukin-5 and submucosal eosinophilia in obese individuals with severe asthma. *Am. J. Respir. Crit. Care Med.* 188: 657–663.
- Lugogo, N., D. Francisco, K. J. Addison, A. Manne, W. Pederson, J. L. Ingram, C. L. Green, B. T. Suratt, J. J. Lee, M. E. Sunday, et al. 2018. Obese asthmatic patients have decreased surfactant protein A levels: mechanisms and implications. *J. Allergy Clin. Immunol.* 141: 918–926.
- Ledford, J. G., S. Mukherjee, M. M. Kislán, J. L. Nugent, J. W. Hollingsworth, and J. R. Wright. 2012. Surfactant protein-A suppresses eosinophil-mediated killing of *Mycoplasma pneumoniae* in allergic lungs. *PLoS One* 7: e32436.
- McIntosh, J. C., S. Mervin-Blake, E. Conner, and J. R. Wright. 1996. Surfactant protein A protects growing cells and reduces TNF-α activity from LPS-stimulated macrophages. *Am. J. Physiol.* 271: L310–L319.
- Ledford, J. G., D. R. Voelker, K. J. Addison, Y. Wang, V. S. Nikam, S. Degan, P. Kandasamy, S. Tanyaratrisakul, B. M. Fischer, M. Kraft, and J. W. Hollingsworth. 2015. Genetic variation in SP-A2 leads to differential binding to *Mycoplasma pneumoniae* membranes and regulation of host responses. *J. Immunol.* 194: 6123–6132.
- Korfhagen, T. R., M. D. Bruno, G. F. Ross, K. M. Huelsman, M. Ikegami, A. H. Jobe, S. E. Wert, B. R. Stripp, R. E. Morris, S. W. Glasser, et al. 1996. Altered surfactant function and structure in SP-A gene targeted mice. *Proc. Natl. Acad. Sci. USA* 93: 9594–9599.
- Riffo-Vasquez, Y., A. P. Ligeiro de Oliveira, C. P. Page, D. Spina, and W. Tavares-de-Lima. 2007. Role of sex hormones in allergic inflammation in mice. *Clin. Exp. Allergy* 37: 459–470.
- Lee, N. A., M. P. McGarry, K. A. Larson, M. A. Horton, A. B. Kristensen, and J. J. Lee. 1997. Expression of IL-5 in thymocytes/T cells leads to the development of a massive eosinophilia, extramedullary eosinophilopoiesis, and unique histopathologies. *J. Immunol.* 158: 1332–1344.

24. Meyerholz, D. K., M. A. Griffin, E. M. Castilow, and S. M. Varga. 2009. Comparison of histochemical methods for murine eosinophil detection in an RSV vaccine-enhanced inflammation model. *Toxicol. Pathol.* 37: 249–255.
25. Borchers, M. T., T. Ansay, R. DeSalle, B. L. Daugherty, H. Shen, M. Metzger, N. A. Lee, and J. J. Lee. 2002. In vitro assessment of chemokine receptor-ligand interactions mediating mouse eosinophil migration. *J. Leukoc. Biol.* 71: 1033–1041.
26. Flynn, A. N., J. Hoffman, D. V. Tillu, C. L. Sherwood, Z. Zhang, R. Patek, M. N. Asiedu, J. Vagner, T. J. Price, and S. Boitano. 2013. Development of highly potent protease-activated receptor 2 agonists via synthetic lipid tethering. *FASEB J.* 27: 1498–1510.
27. Zeng, C., C. Nguyen, S. Boitano, J. A. Field, F. Shadman, and R. Sierra-Alvarez. 2018. Cerium dioxide (CeO₂) nanoparticles decrease arsenite (As(III)) cytotoxicity to 16HBE14o- human bronchial epithelial cells. *Environ. Res.* 164: 452–458.
28. Ben-Yehuda, C., R. Bader, I. Puxeddu, F. Levi-Schaffer, R. Breuer, and N. Berkman. 2008. Airway eosinophil accumulation and eotaxin-2/CCL24 expression following allergen challenge in BALB/c mice. *Exp. Lung Res.* 34: 467–479.
29. Foster, P. S., A. W. Mould, M. Yang, J. Mackenzie, J. Mattes, S. P. Hogan, S. Mahalingam, A. N. Mckenzie, M. E. Rothenberg, I. G. Young, et al. 2001. Elemental signals regulating eosinophil accumulation in the lung. *Immunol. Rev.* 179: 173–181.
30. Pope, S. M., N. Zimmermann, K. F. Stringer, M. L. Karow, and M. E. Rothenberg. 2005. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. *J. Immunol.* 175: 5341–5350.
31. von Bredow, C., D. Hartl, K. Schmid, F. Schabaz, E. Brack, D. Reinhardt, and M. Griese. 2006. Surfactant protein D regulates chemotaxis and degranulation of human eosinophils. *Clin. Exp. Allergy* 36: 1566–1574.
32. Humbles, A. A., C. M. Lloyd, S. J. McMillan, D. S. Friend, G. Xanthou, E. E. McKenna, S. Ghiran, N. P. Gerard, C. Yu, S. H. Orkin, and C. Gerard. 2004. A critical role for eosinophils in allergic airways remodeling. *Science* 305: 1776–1779.
33. Adamko, D., P. Lacy, and R. Moqbel. 2004. Eosinophil function in allergic inflammation: from bone marrow to tissue response. *Curr. Allergy Asthma Rep.* 4: 149–158.
34. Rothenberg, M. E., and S. P. Hogan. 2006. The eosinophil. *Annu. Rev. Immunol.* 24: 147–174.
35. Flood-Page, P., A. Menzies-Gow, S. Phipps, S. Ying, A. Wangoo, M. S. Ludwig, N. Barnes, D. Robinson, and A. B. Kay. 2003. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J. Clin. Invest.* 112: 1029–1036.
36. Bel, E. H., and A. Ten Brinke. 2017. New anti-eosinophil drugs for asthma and COPD: targeting the trait! *Chest* 152: 1276–1282.
37. National Center for Biotechnology Information. Database of single nucleotide polymorphisms (dbSNP). National Library of Medicine, Bethesda, MD. Available at: <https://www.ncbi.nlm.nih.gov/snp/rs1965708>. Accessed: January 11, 2019.
38. Deb, R., F. Shakib, K. Reid, and H. Clark. 2007. Major house dust mite allergens Dermatophagoides pteronyssinus 1 and Dermatophagoides farinae 1 degrade and inactivate lung surfactant proteins A and D. *J. Biol. Chem.* 282: 36808–36819.
39. Goto, H., J. G. Ledford, S. Mukherjee, P. W. Noble, K. L. Williams, and J. R. Wright. 2010. The role of surfactant protein A in bleomycin-induced acute lung injury. *Am. J. Respir. Crit. Care Med.* 181: 1336–1344.



Group	Mean of total eosinophil counts at 24 hours	Mean of total eosinophil counts at 5 days	Difference in means (SE)	P-value
Wild-type	507889	446338	-60551 (122664)	0.637
SP-A KO	736502	524947	-211555 (263995)	0.437
SP-A 223 Q/Q	874885	236628	-638257 (210322)	0.018
SP-A 223 K/K	510498	499999	-10498 (144779)	0.943

Supplemental Figure 1. Assessment of BALF eosinophilia over time. A) Total cells, B) total macrophages and C) total eosinophils per milliliter BALF. Table shows difference in means at 24 hours and 5 days, unpaired Student's t-test.



Supplemental Figure 2. Evaluation of the effect of SP-A on neutrophil survival *in vitro*. Neutrophils were stimulated with SP-A overnight and assessed for apoptosis by flow cytometry. Live: Annexin V⁻, PI⁻; Early apoptotic: Annexin V⁺, PI⁻; Late apoptotic/dead: Annexin V⁺, PI⁺.