Supporting Information

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SI Methods

There were three separate patient cohorts involving a total of 91 patients. The studies were approved by the ethics committee of the participating institutions and informed written consent was obtained. The patient demographics of the COPD cohorts are summarized in Table S3.

SAA Expression in the Bronchoalveolar Lavage Compartment (Cohort 1). Fiberoptic bronchoscopy was performed on subjects with stable chronic obstructive pulmonary disease (COPD) [n = 41: Global Initiative for Obstructive Lung Disease (GOLD) I (n = 4), GOLD II (n = 15), GOLD III (n = 14), and GOLD IV (n = 8)]. Warmed saline (2 × 60 mL) was instilled into the airways to enable recovery of bronchoalveolar lavage (BAL) cells and BAL fluid (BALF). BAL cells were collected by centrifugation (400 × g for 5 min) and the BALF was archived at −80 °C for future analysis. Measurement of SAA in BALF and serum were performed by quantitative ELISA (Anogen) as previously described (1). Measurement of neutrophil elastase (NE) activity in BALF as a marker for neutrophil activity was determined using the specific NE substrate methoxysuccinyl-alanyl-alanyl-prolyn-valylpapnitroanalide (MEOSAAPVNA; Sigma). Neat BALF (5 μL) was diluted to 200 μL NE Buffer containing final concentrations of 0.2 mM MEOSAAPVNA, 50 mM Tris.HCL (pH 7.6), 150 mM sodium chloride, 5 mM calcium chloride, and 0.01% sodium azide. Absorbance at 405 nM was measured using microplate reader (MultiScan Ascent; ThermoScientific). IL-8 and IL-6 levels in BALF were also determined by quantitative ELISA (Peprotech). BAL cells cytospots were prepared on poly-lysine-coated slides and fixed in 4% paraformaldehyde for 10 min at room temperature, subjected to antigen retrieval in 0.2% Triton-X100 in PBS for 10 min, and stained for serum amyloid A (SAA) using the Dako Envision staining kit, as detailed above. For analysis of SAA mRNA in BAL, macrophages (2×10^6 cells) were resuspended in RLTplus buffer (Qiagen) and archived at −80 °C before RNA extraction.

Acute Exacerbation of COPD Systemic Measurements (Cohort 2). Subjects who presented with an acute exacerbation of COPD (AECOPD) to the Royal Melbourne Hospital with moderate/severe COPD [n = 10 (3 female:7 male), GOLD II (1 subject), III (6 subjects), IV (3 subjects)] defined according to American Thoracic Society (ATS)/European Respiratory Society (ERS) consensus criteria (Anthonison Grade I and II) were prospectively recruited after obtaining informed consent. The mean age was 75 ± 3 y, with a mean smoking history of 57 ± 23 pack years. Peak exacerbation severity was graded using the ATS/ERS consensus criteria as follows: requiring hospitalization (level II, n = 8) and acute respiratory failure (level III, n = 2). White blood cell count, neutrophil blood counts, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were significantly elevated during an exacerbation (Table S4). Resolution of an AECOPD was defined as the completion of treatment with increased steroids and antibiotics and return of symptoms to baseline for at least 48 h as previously detailed (1). Blood was obtained at (i) AECOPD peak and (ii) at Resolution from the same individual and standard blood markers were assessed (1). Blood Lipoxin A_4 (LXA_4) and leukotriene B_4 (LTB_4) were measured as previously described (2). Measurement of annexin A1 (ANXA1) in plasma was performed by quantitative ELISA (USCN Life Science). In addition, blood was obtained from healthy volunteers with normal lung function (7 male:3 female) with a mean age of 58 ± 7 y.

Immunohistochemical Analysis (Cohort 3). Lung tissue from resection surgery for treatment of a solitary peripheral carcinoma was collected from subjects with no airflow obstruction [n = 8 (3 male:5 female), mean age 64 ± 8, including three nonsmokers and four ex smokers] and mild/moderate COPD [n = 22 (16 male: 6 female), GOLD stage I-II]. The mean age of the COPD subjects was 70 ± 5 y, with a mean smoking history of 49 ± 25 pack years. Tissue blocks from the subpleural parenchyma avoiding areas involved by tumor were fixed in 10% neutral buffered formalin, embedded in paraffin, and 5-μm sections were prepared for immunohistochemistical analysis. Mouse monoclonal antibodies against SAA (Novus Biologicals), CD68 (Dako), ALX/FPRL1 (R&D Systems), and negative control mouse IgG (Dako) were used. Antigen retrieval was achieved by microwave heat treatment (98 °C for 20 min) in Tris-EDTA Buffer (10 mM Tris, 1 mM EDTA, 0.05% Tween 20, pH 9.0) for all stains except ALX/FPR2 that did not require retrieval. Bound antibody was developed with daiminobenzidine using a Dako Envision staining kit (K4065) according to the manufacturer's instructions. Stained sections were graded by two independent observers according to the following criteria: absent, moderate, and intense.

Cell Culture. The human macrophage THP-1 cell line (ATCC) was grown in complete media [RPMI 1640 media supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 10 mM Hepes, 10% FCS (Invitrogen), and 5 μg/mL gentamycin] in a humidified incubator at 37 °C with 5% CO_2. Cells (2×10^6) were seeded in six-well plates and maintained overnight in complete media. Cells were next treated with 1 μg/mL LPS (serotype 026:B6; Sigma) in complete media with 1% FCS for 24 h, spun down (400 × g for 5 min), and archived in RLTplus buffer before real-time PCR analysis. A concentration response curve for the GC, Dexamethasone (DEX; Sigma) was generated by pretreating the cells with DEX (10^{-8} to 10^{-4} M) for 30 min before LPS treatment.

Human lung type II alveolar A549 epithelial cells, which do not express the LXA_4 receptor (ALX/FPR2), were transfected to constitutively express full-length recombinant human ALX/FPR2 (hALX/FPR2) receptors, as previously described (3). Subconfluent A549 cells expressing human ALX/FPR2 receptors were serum-starved for 24 h and exposed to LXA_4 (10^{-8} or 10^{-7} M), 15-epi-LXA_4 (10^{-7} M), or vehicle (0.05% vol/vol ethanol, EtOH) (Calbiochem) for 30 min (37 °C, 5% CO_2) before the addition of recombinant human SAA (10^{-10} M to 10^{-7} M, 24 h, 37 °C, 5% CO_2) (Peprotech). Resulting supernatants were harvested and analyzed by an IL-8 ELISA (R&D Systems) and multiplex immunoassay (Aushon BioSystems).

Primary Human Lung Cell Preparation and Flow Cytometry. A human small airway sample was dissected from the normal lung tissue of a 70-y-old male ex-smoker following left pneumonectomy for squamous cell carcinoma and stored in DMEM/F12 media overnight at 4 °C. A single-cell suspension was then prepared by collagenase (3 mg/mL in 4 mL/cm^3 of tissue) digestion for 1 h at 37 °C with constant agitation, with subsequent filtering through a 40-μm filter. Sample was then washed in PBS-2%FBS and stained with a mixture of antibodies (Biolegend), including anti–CD45-647(1/200), anti–CD31-647(1/200), anti–EpCAM-PE (1/200), and anti–CD24-488(1/100). Labeled cells were washed in

PBS-2%FBS and viable $CD45^{neg}CD31^{neg}EpCAM^{pos}CD24^{neg}$ primary epithelial cells were sorted using a BD Aria cell sorter. Sorted primary epithelial cells were seeded on tissue culture flasks coated with 5% Matrigel (BD) in DMEM/F12 media supplemented with insulin, transferrin, selenium, penicillin, streptomycin, glutamate, heparin, FGF-10 (100 ng/mL), and hepatocyte growth factor (30 ng/mL). Cells were passaged every second week and media changed three times per week. For SAA stimulation, 100,000 cells from passage 2 were seeded in a 24-well tissue-culture plate. After reaching confluency (day 3), cells were stimulated with vehicle or SAA (1 μg/mL) for 3 h and subsequently harvested for TaqMan quantitative PCR (qPCR) analysis of inflammatory markers [IL-8, monocyte chemoattractant protein-1 (MCP)-1, GM-CSF].

SAA Challenge Protocol. Specific pathogen-free male Balb/C mice aged 7 wk and weighing ~20 g were obtained from the Animal Resource Centre (Perth, Australia). The animals were housed at 20 °C on a 12-h day/night cycle in sterile microisolators and fed a standard sterile diet. The experiments were approved and conducted in compliance with the National Health and Medical Research Council of Australia. Recombinant SAA (2 μg in 50 μL saline) was instilled into the lungs of mice using the intranasal method as previously described (4). In the indicated protocols, 4 μg 15-epi-LXA_4 was dried under nitrogen gas, diluted in the working SAA solution (2 μg in 50 μL saline) immediately before intranasal delivery. In a separate experiment, mice were treated with DEX (0.5 mg/kg) via intraperitoneal injection 2 h before intranasal treatment with SAA (1 μg) or LPS (1 μg). At the specified times, BAL was performed via tracheotomy (SP30 Duran polyethylene tubing) proximal to the larynx and total/differential BAL cell counts were performed as previously described (4). Cell-free BALF was archived at −80 °C before analysis of mediator release (CXCL1 and CXCL2; R&D Systems) and secreted SAA (Invitrogen) by quantitative ELISA. Following lavage, lungs were perfused free of blood via right ventricular perfusion with 5 mL warmed saline, rapidly excised en bloc, blotted and snap-frozen in liquid nitrogen, ground to a fine powder, and resuspended in RLTplus buffer before real-time PCR analysis.

Real-Time PCR. Total RNA was isolated using the RNeasy Plus Kit (Qiagen) and cDNA was synthesized with SuperScript III (Invitrogen), as previously described (4). TaqMan primer/probe chemistry was used to quantify mRNA levels by qPCR with the ABI PRISM 7900HT sequence detection system (Applied Biosystems). Threshold cycle values (Ct) were normalized to 18S rRNA (ΔCt = target Ct − 18S Ct) and the ΔΔCt relative expression method was used to generate fold-increase above the control group. For the in vivo analysis of mRNA expression, tissue was pooled from treatment groups before analysis. Because the SAA isoforms (SAA1 and SAA2) are 95% identical, they cannot be distinguished using current commercial reagents to quantify SAA expression by ELISA, TaqMan qPCR or immunohistochemistry.

In Vivo Studies. For the in vivo measurement of de novo SAA synthesis in lung tissue, BALB/c mice were either infected with influenza, exposed to LPS, or exposed to smoke. For influenza A infection, mice were anesthetized by penthrane inhalation and infected intranasally with 1×10^4 pfu X31 (H3N2) in a 35-μL volume, diluted in PBS. For LPS challenge, mice were anesthetized by penthrane inhalation and treated intranasally with 3 μg or 10 μg LPS diluted in 50 μL PBS (4). Additionally, mice were therapeutically treated with budesonide (10 μg) via intranasal administration 6 h following LPS challenge. For the subchronic smoke-exposure protocols, mice were placed in an 18-L perspex chamber in a class II biosafety cabinet and exposed to six cigarettes per day over 4 d, delivered three times per day spaced over 1 h, respectively. Sham-exposed mice were placed in an 18-L perspex chamber but did not receive cigarette smoke.

Statistical Analysis. Normally distributed data were expressed as mean ± SE and skewed data were log-transformed to obtain a normal distribution where appropriate. For the systemic clinical samples Mann–Whitney *U* test was performed, with the exception of matched-paired samples (AECOPD vs. Resolution) using Wilcoxon-matched paired *t* test. Pearson correlation was used to assess the relationship between secreted SAA vs. NE activity in BALF and SAA mRNA in BAL cells, and the association between systemic SAA, LXA_4, and LTB_4. For the animal studies, significance between groups was tested using an unpaired *t* test or one-way ANOVA followed by Dunnett's comparison test (ANOVA, $P < 0.05$).

Concentration response curves are presented as a percentage of the maximal response and individual curves were fitted to a logistic equation using GraphPad Prism (GraphPad Software) to calculate the EC_{50} values and Hill slope parameters. For Schild analysis, the ratio of SAA EC_{50} values in the presence and absence of LXA_4 was calculated (dose ratio, *dr*) and the logarithm of $dr - 1$ was plotted against the logarithm of LXA_4 concentrations. The pA_2 and Schild slope values for LXA_4 were determined by linear regression.

1. Bozinovski S, et al. (2008) Serum amyloid a is a biomarker of acute exacerbations of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 177:269–278.
2. Levy BD, et al. (2007) Lipoxin A4 stable analogs reduce allergic airway responses via mechanisms distinct from CysLT1 receptor antagonism. *FASEB J* 21:3877–3884.
3. Bonnans C, Fukunaga K, Levy MA, Levy BD (2006) Lipoxin A(4) regulates bronchial epithelial cell responses to acid injury. *Am J Pathol* 168:1064–1072.
4. Bozinovski S, et al. (2004) Innate immune responses to LPS in mouse lung are suppressed and reversed by neutralization of GM-CSF via repression of TLR-4. *Am J Physiol Lung Cell Mol Physiol* 286:L877–L885.

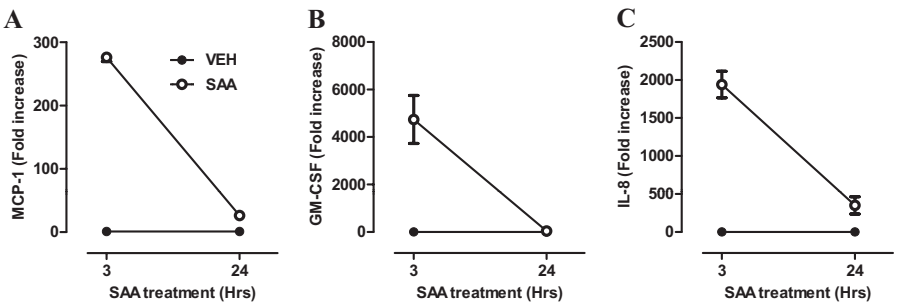

Fig. S1. BEAS-2B cells derived from noncancerous human bronchial epithelial cells were treated with Vehicle (closed circle) or SAA (1μg/mL, open circle) and the expression of MCP-1 (*A*), GM-CSF (*B*), and IL-8 (*C*) were determined by qPCR, as detailed in *SI Methods*.

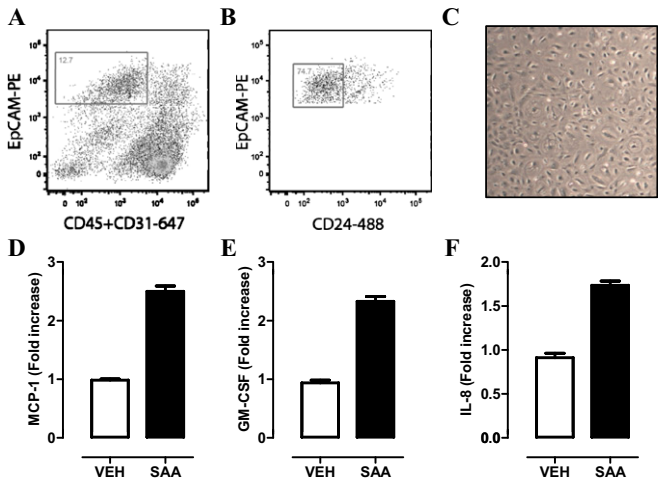

Fig. S2. Viable (Propidium Iodide^{neg}), primary human lung epithelial cells were sorted on the basis of (*A*) CD45^{neg}CD31^{neg}EpCAM^{pos} expression and further subset on (*B*) CD24^{neg} expression as described in *SI Methods*. (*C*) Phase-contrast image of cobble stone morphology of passage 2 primary human lung epithelial cells. Cells were stimulate with vehicle or SAA (1 μg/mL) for 3 h and subsequently harvested for Taqman qPCR analysis of (*D*) IL-8, (*E*) GM-CSF, and (*F*) MCP-1.

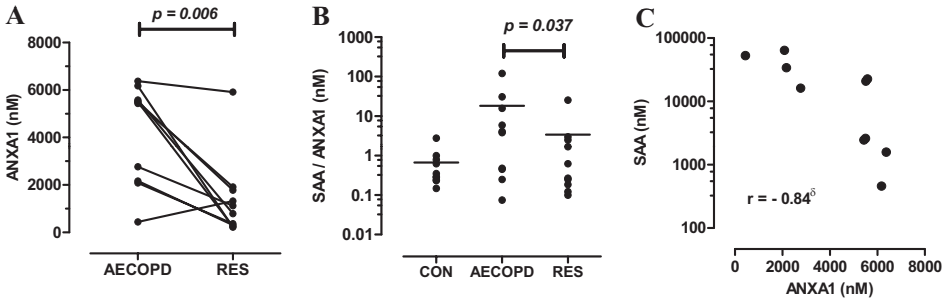

Fig. S3. (*A*) ANXA1 concentration (nM) in matching blood samples from COPD subjects during (*i*) the AECOPD and at (*ii*) clinical recovery (Resolution). (*B*) The relative ratio of SAA/LXA$_4$ was determined for the matching AECOPD and recovery samples and a control cohort (n = 10, by Wilcoxon-matched paired t test). The association of ANAX1 with (*C*) SAA was determined during the AECOPD-phase demonstrating a significant inverse correlation (P = 0.002, r = −0.84 Pearson correlation).

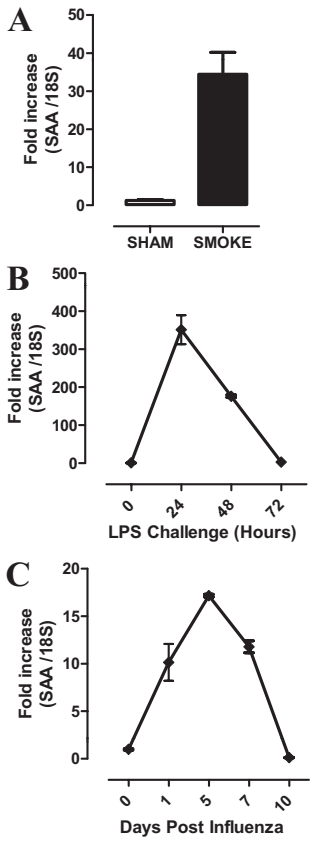

Fig. S4. SAA is directly synthesized in lung tissue in response to cigarette smoke, LPS challenge, and Influenza A infection. (*A*) Mice were exposed to cigarette smoke over 4 d and SAA mRNA expression in lung tissue was determined by qPCR, demonstrating a 34-fold increase. (*B*) LPS (10 μg) was intranasally administered to mice and SAA expression in lung tissue was determined at the specified time points, demonstrating a peak induction of 350-fold at 24 h postchallenge. (*C*) Mice were infected with 10^4 pfu Influenza A (H3N2) and at the specified time points, SAA was measured by qPCR and demonstrated a peak of 17-fold at day 5.

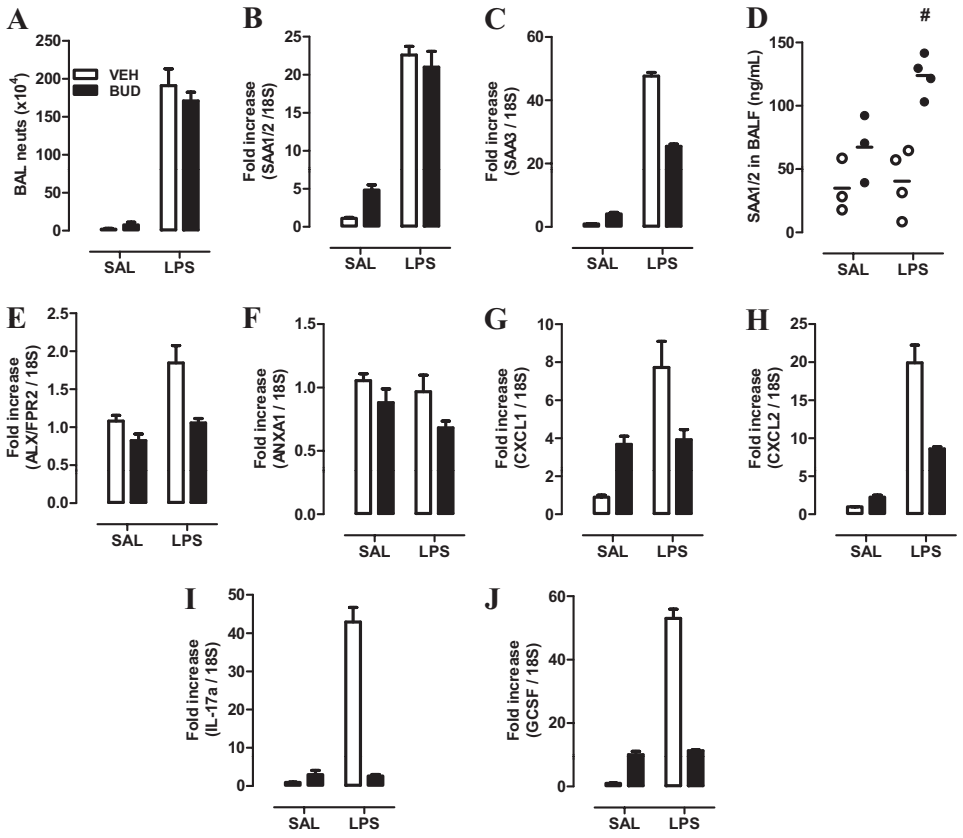

Fig. S5. SAA1/2 but not SAA3 are directly synthesized in lung in response to LPS in a GC refractory manner. Budesonide (10 μg, i.n.) was therapeutically administered 6 h following LPS challenge (3 μg, i.n.) and BALF and lungs were harvested 24 h after LPS. (*A*) Neutrophils in BAL were quantified and (*B*) SAA1/2 and (*C*) SAA3 mRNA expression in lung tissue was determined by TaqMan qPCR. (*D*) Secreted SAA in BALF was determined by quantitative ELISA. Additionally, expression of (*E*) ALX/FPR2, (*F*) ANXA1, (*G*) CXCL1, (*H*) CXCL2, (*I*) IL-17a, and (*J*) G-CSF were determined by TaqMan qPCR. Results are expressed as mean ± SEM (n = 5–7) where $^{\#}P < 0.05$ by ANOVA.

Table S1. Airway SAA and its association with neutrophil activation

	Correlation analysis		
BALF levels (ng/mL)	*n*	*r*	*P*
vs. NE activity			
SAA	41	0.72	0.0001
IL8	41	0.47	0.002
IL6	41	0.25	0.03
vs. IL8 (ng/mL)			
SAA	41	0.35	0.02
vs. SAA mRNA (ΔCt)			
SAA	8	0.83	0.01

Table S2. SAA imunostaining characteristics

SAA staining	Control (n = 8)	COPD (n = 22)
Absent	7 (88%)	9 (41%)
Moderate	1 (12%)	7 (32%)
Intense	0	6 (27%)

Table S3. Patient demographics of the COPD cohorts used to explore the relationship between SAA and disease

	Cohort 1 (airway analysis)	Cohort 2 (systemic analysis)	Cohort 3 (tissue analysis)
Number (*n*)	41	10	22
Sex (M:F)	32:9	7:3	16:6
Age (± SE)	69 ± 5	75 ± 3	64 ± 8
Pack years	46 ± 21	57 ± 23	49 ± 25
GOLD stage (*n*)			
I	4	0	13
II	15	1	9
III	14	6	0
IV	8	3	0

Table S4. AECOPD characteristics

Characteristic	AECOPD	Resolution
WCC × 10^9/L (SD)	10.4 (3)*	8.4 (2.3)
NEUTS × 10^9/L (SD)	9 (2.8)*	5.5 (2.3)
CRP mg/L (SD)	38.5 (41.5)*	2 (16.3)
ESR s (SD)	22 (10.4)*	16 (6.4)

CRP, C-reactive protein; ESR, erythrocyte sedimentation; NEUTS, neutrophil blood count; WCC, white blood cell count.
*Wilcoxon matched paired *t* test (stable vs. AECOPD).