

an effect of EROS deficiency that is independent of its effects on the NADPH oxidase.

The high sequence similarity between mouse and human EROS and the loss of *gp91phox* expression and the phagocyte respiratory burst that accompanies its absence suggest that EROS plays an almost identical role in human and murine immunity. In summary, we have shown that the function of EROS is fully conserved between human and mouse, and that homozygous mutations in EROS underlie a novel sixth cause of CGD.

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REFERENCES

1. Segal AW. How neutrophils kill microbes. *Ann Rev Immunol* 2005;23:197-223.
2. van den Berg JM, van Koppen E, Ahlin A, Belohradsky BH, Bernatowska E, Corbeel L, et al. Chronic granulomatous disease: the European experience. *PLoS One* 2009;4:e5234.
3. Thomas DC, Clare S, Sowerby JM, Pardo M, Juss JK, Goulding DA, et al. Eros is a novel transmembrane protein that controls the phagocyte respiratory burst and is essential for innate immunity. *J Exp Med* 2017;214:1111-28.
4. Monies D, Abouelhoda M, AlSayed M, Alhassan Z, Alotaibi M, Kayyali H, et al. The landscape of genetic diseases in Saudi Arabia based on the first 1000 diagnostic panels and exomes. *Hum Genet* 2017;136:921-39.
5. Conti F, Lugo-Reyes SO, Blancas Galicia L, He J, Aksu G, Borges de Oliveira E Jr, et al. Mycobacterial disease in patients with chronic granulomatous

disease: a retrospective analysis of 71 cases. *J Allergy Clin Immunol* 2016;138:241-8.e3.

6. Parekh C, Hofstra T, Church JA, Coates TD. Hemophagocytic lymphohistiocytosis in children with chronic granulomatous disease. *Pediatric Blood Cancer* 2011;56:460-2.

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CSF3R/CD114 mediates infection-dependent transition to severe asthma



To the Editor:

The major 17q12-21 asthma susceptibility and exacerbation locus¹ has been identified as the only genetic locus that is also reproducibly associated with total white blood cell count.² However, it is not known whether there is a common gene within this locus that links these phenotypic traits. The colony-stimulating factor-3 (CSF3) gene, alternatively known as *G-CSF*, resides within this locus. CSF3 binds exclusively to CSF3 receptor (CSF3R, or CD114/G-CSFR), which is highly expressed on mature neutrophils and to a lesser extent on mononuclear cells, platelets, and lung interstitial stromal cells. Although CSF3R/CD114 signaling can dictate the intensity of the host defense inflammatory response during bacterial infection by regulating neutrophil granulopoiesis and trafficking, its role in the infection-dependent transition to persistent, severe asthma has not been investigated. Neonatal colonization of the nasopharynx by potentially pathogenic bacteria including *Streptococcus pneumoniae* is also a risk factor for asthma development.³ The Childhood Asthma Study found that children with atopy and chronic wheeze at age 5 years were twice as likely to have been colonized with *S pneumoniae* as neonates.⁴ The authors suggest that transient incursions of nasopharyngeal bacteria into the lower airways triggered by a fever-causing viral respiratory infection (respiratory syncytial virus or influenza virus) increases the risk of developing persistent asthma in atopic children. However, a plausible mechanism linking these cofactors is yet to be identified. In this study, we tested the hypothesis that CSF3-CSF3R signaling dictates the severity of infection-dependent asthma at a cellular and molecular level.

To test this hypothesis, we adapted our previously published mouse model of neonatal nasopharyngeal pneumococcal colonization and acute viral infection,⁵ and superimposed subsequent house-dust mite (HDM) aeroallergen exposure into adulthood. *S pneumoniae* (EF3030) colonization of the nasopharynx was not altered by influenza A virus or HDM (see Fig E1, A, in this article's Online Repository at www.jacionline.org). In contrast, pneumococcal loads in the lower airways were significantly increased in coinfecting neonates chronically challenged with HDM (Fig E1, B). Histological assessment of lung pathology demonstrated that HDM increased the area positive for mucus staining in small airways (Fig E1, C and D). HDM-induced mucin levels were not further increased by inoculation with a single respiratory pathogen, whereas coinfection significantly increased the mucus area surrounding the small airways (Fig E1, D). Consistent with this finding,

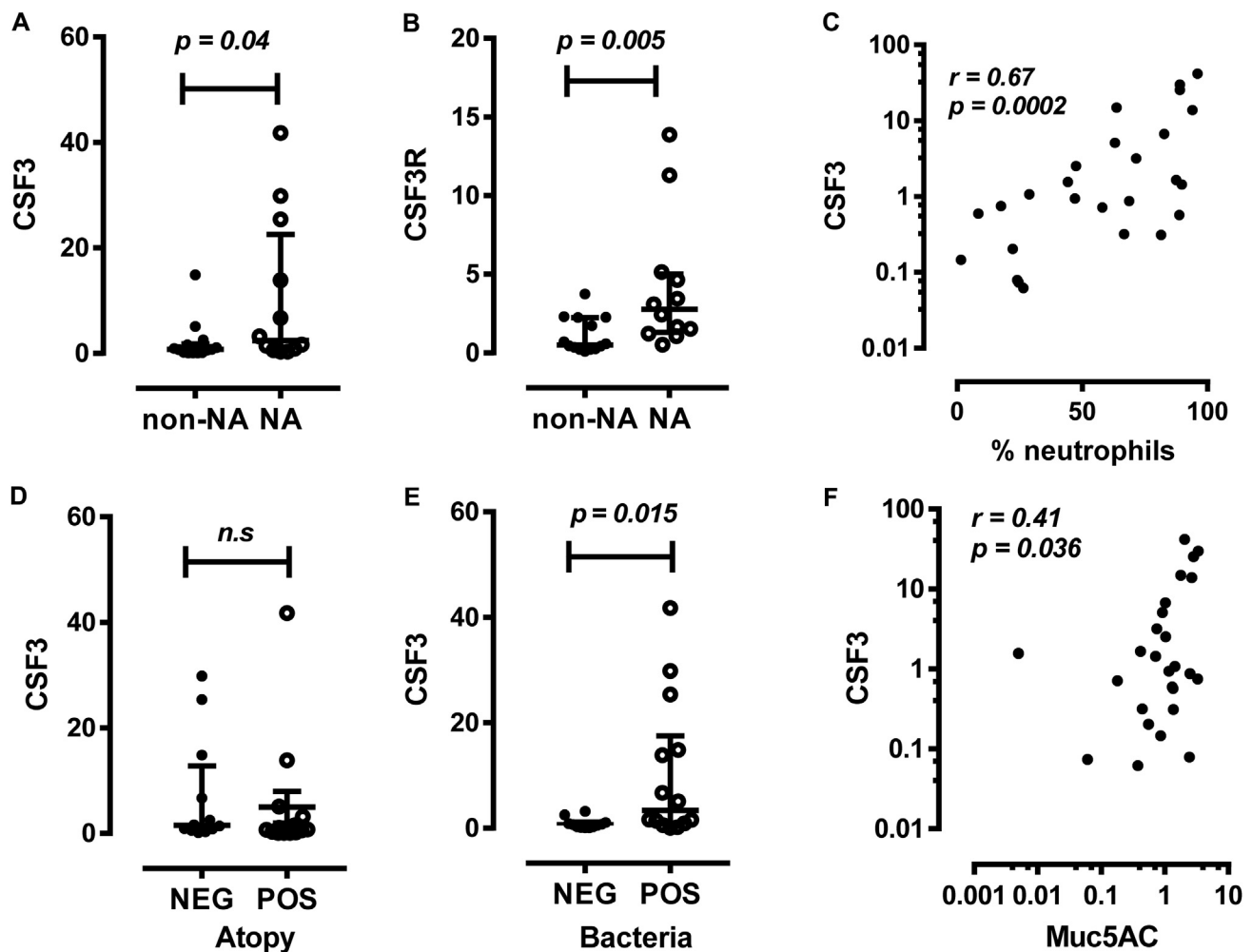


FIG 1. CSF3 expression is increased in those with poorly controlled asthma. (A) CSF3 and (B) CSF3R gene expression in bronchial biopsies was analyzed by RT-quantitative PCR from subjects with asthma ($n = 26$) grouped according to their inflammatory phenotype (non-NA vs NA). C, The percentages of neutrophils from matching BAL samples were compared with CSF3 expression. CSF3 expression levels were presented according to (D) atopy status and (E) bacterial culture status. F, In addition, CSF3 was compared with Muc5AC expression. NEG, Negative; POS, positive. Analysis includes nonparametric Mann-Whitney T test and Spearman correlation, and data are expressed as median $\pm$ interquartile range.

Muc5AC transcript levels were only significantly increased in coinfecting mice challenged with HDM (Fig E1, E). HDM challenge also elicited eosinophilic inflammation in the bronchoalveolar (BAL) compartment, and this response was not significantly altered by coinfection (Fig E1, F). In contrast, HDM-induced neutrophilic inflammation was only significantly increased in mice coinfecting as neonates (Fig E1, G). HDM challenge did not significantly alter CSF3 transcript levels, whereas the combination of neonatal coinfection and HDM significantly increased CSF3 gene expression in the lung (Fig E1, H).

To assess the translational relevance of our findings, we examined CSF3 transcript expression in bronchial biopsies obtained from adults with asthma grouped into inflammatory phenotypes: neutrophilic asthma (NA) and nonneutrophilic asthma (non-NA; consisting of paucigranulocytic and eosinophilic asthma, as summarized in Table E1 in this article's Online Repository at www.jacionline.org). NA had poor control of their asthma symptoms based on Asthma Control Questionnaire scores and significantly lower lung function (median FEV₁%, NA 72% vs

non-NA 85%; $P < .04$). CSF3 and CSF3R transcript levels were significantly higher in NA compared with non-NA (Fig 1, A and B), and CSF3 expression was positively associated with BAL neutrophil numbers across the entire asthma cohort (Fig 1, C). Atopy status did not affect CSF3 transcript levels in asthma, but rather presence of positive bacterial BAL cultures resulted in higher CSF3 expression (Fig 1, D and E). In addition, CSF3 and Muc5AC transcript expression was positively associated in asthmatic bronchial biopsies (Fig 1, F). We also analyzed a transcriptomic sputum data set from the U-BIOPRED cohort,⁶ where CSF3R expression levels were significantly increased in those with severe asthma compared with healthy volunteers (1.9-fold increase, $P < .005$; see Fig E3 in this article's Online Repository at www.jacionline.org). Although CSF3 was not significantly increased (1.2-fold increase, $P = .09$), 33 of 84 (39%) expressed high CSF3 levels (defined as above the 95th percentile of healthy control distribution), confirming that there is a subset of those with asthma who are CSF3^{high}.

We next therapeutically targeted CSF3R signaling by administering an anti-CSF3R mAb (α -CSF3R) in our mouse

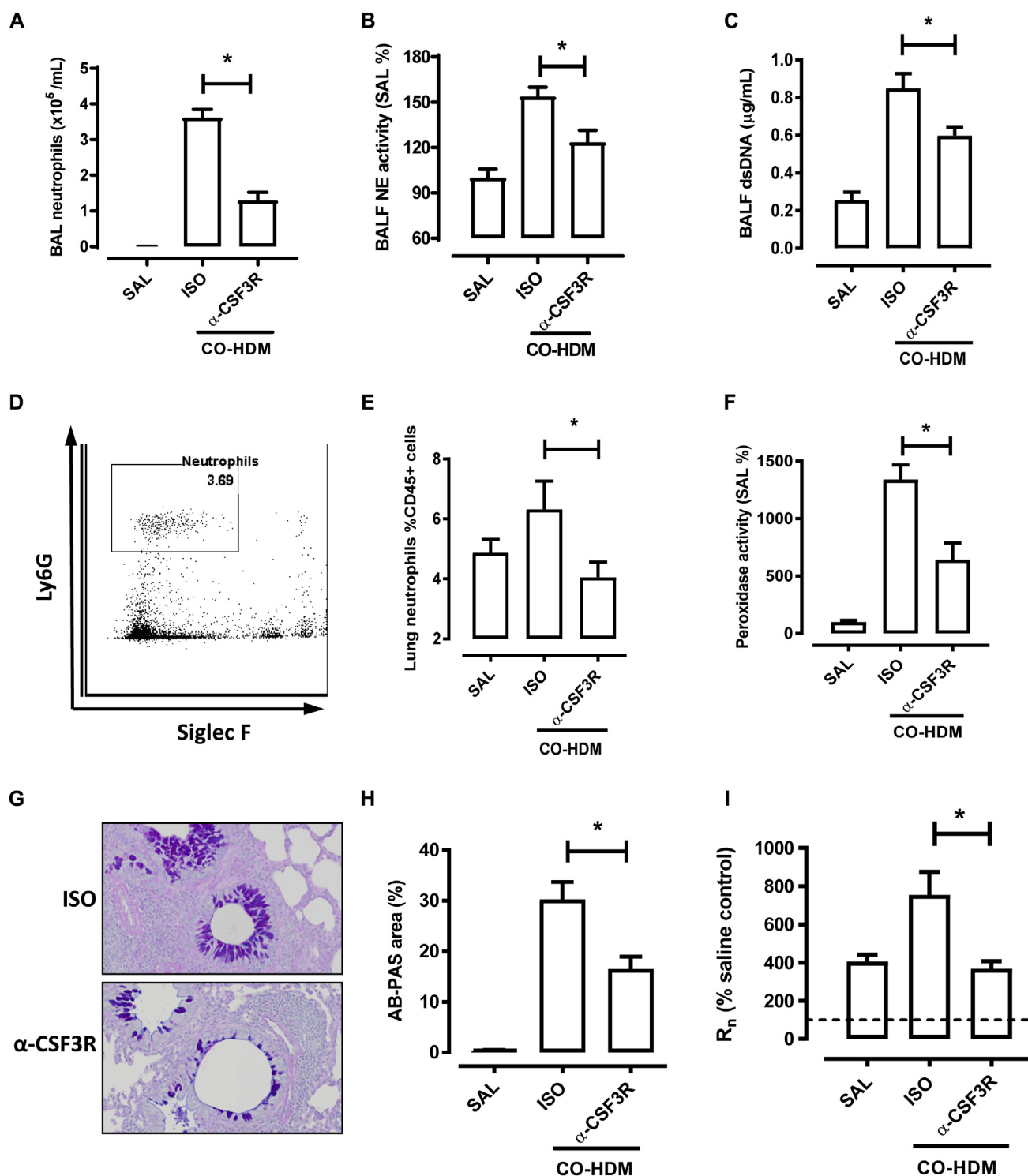


FIG 2. Neutralizing CSF3R reduced mucus production and airway hyperreactivity. **(A)** BAL neutrophil numbers, **(B)** neutrophil elastase (NE) activity, and **(C)** dsDNA levels were measured in control (SAL) and coinfectd/HDM-exposed mice (CO-HDM) treated with anti-CSF3R or isotype (ISO) control antibody. **(D)** Single-cell suspensions from homogenized lungs were analyzed by flow cytometry where neutrophils were gated (Siglec F⁺, Ly6G⁺) and **(E)** presented as a percentage of the total CD45⁺ cells. **F**, Peroxidase activity from lung tissue was determined and presented as a percentage of the SAL control group. **(G)** Mucus was stained by Alcian blue-periodic acid Schiff (AB-PAS) and quantified **(H)** as detailed in the Methods section. **I**, Newtonian resistance (R_n, central airway resistance) was measured *in vivo* using Flexivent. dsDNA, Double-stranded DNA; SAL, saline alone. Data are expressed as mean $\pm$ SEM. n = 6-10 per group, *P < .05, 1-way ANOVA with Tukey post hoc test compared with CO-HDM/ISO.

model (see Fig E2, A, in this article's Online Repository at www.jacionline.org). α -CSF3R administration significantly reduced BAL neutrophil numbers (Fig 2, A) and markers for NETosis including neutrophil elastase activity and double-stranded DNA in coinfecting mice challenged with HDM allergen (Fig 2, B and C). Lung neutrophil numbers determined by flow cytometry (Ly6G⁺, Siglec F⁺) were significantly reduced by α -CSF3R treatment (Fig 2, D and E). Total peroxidase activity in the lung tissue was also markedly increased in coinfecting mice challenged with HDM, and this response was significantly reduced by 50% with α -CSF3R treatment (Fig 2, F). Because inhibition of neutrophilic inflammation may compromise bacterial clearance, we also evaluated pneumococcal load in the nasopharynx, BAL, and lung tissue, which was not significantly altered by α -CSF3R treatment (see Fig E2, B-D). We also assessed whether α -CSF3R treatment reduced mucus levels surrounding the small airways (Fig 2, G, representative images). Elevated mucus production observed in coinfecting mice challenged with HDM was significantly reduced with α -CSF3R treatment by approximately 50% (Fig 2, H). Consistent with excessive mucus obstructing the airways, methacholine-induced airway hyperreactivity (Rn) was completely reversed with α -CSF3R therapy in coinfecting mice challenged with HDM (Fig 2, I).

In this study, we demonstrate that the combination of HDM allergen and an acute viral infection cooperate to induce the dispersion of *S pneumoniae* into the lower airways, which markedly increased CSF3-dependent neutrophilic inflammation. CSF3 cooperates with a number of factors including IL-6, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), and reactive oxygen species (ROS) to stimulate neutrophil granulopoiesis in the bone marrow to restore neutrophil homeostasis during infection. CSF3 also promotes neutrophil trafficking by modulating chemokine and adhesion receptors (CXCR2 and CD62L) on neutrophils, and in a murine arthritis model, blocking CSF3R prevented trafficking into the arthritic joint without inducing peripheral neutropenia.⁷ Consistent with this, blocking CSF3R did not completely deplete neutrophils, but rather selectively reduced excessive neutrophil trafficking into the lungs of coinfecting/HDM-exposed mice without compromising bacterial clearance. By blocking CSF3R signaling, we also show that CSF3 promotes infection-dependent production of mucus in allergic airways. Mucin overproduction is prominent in fatal asthma because it directly contributes to severe airflow obstruction. Obstruction of the airways is exacerbated by the actions of eosinophil peroxidase, which catalyzes the generation of oxidants that cross-link mucus to form hydrogels.⁸ Because the same pathological process is also mediated by neutrophil-derived myeloperoxidase,⁹ we identify CSF3R as a novel target to suppress pathological peroxidase activity in severe asthma. In summary, we identify CSF3 as a major effector molecule that drives infection-dependent transition to more persistent and severe asthma. Antagonising CSF3 production through targeted inhibition of CSF3R signaling represents a novel strategy to prevent this pathological inflammation and remodeling.

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REFERENCES

1. Bisgaard H, Bonnelykke K, Sleiman PM, Brasholt M, Chawes B, Kreiner-Moller E, et al. Chromosome 17q21 gene variants are associated with asthma and exacerbations but not atopy in early childhood. *Am J Respir Crit Care Med* 2009;179:179-85.
2. Soranzo N, Spector TD, Mangino M, Kuhnel B, Rendon A, Teumer A, et al. A genome-wide meta-analysis identifies 22 loci associated with eight hematological parameters in the HaemGen consortium. *Nat Genet* 2009;41:1182-90.
3. Bisgaard H, Hermansen MN, Buchvald F, Loland L, Halkjaer LB, Bonnelykke K, et al. Childhood asthma after bacterial colonization of the airway in neonates. *N Engl J Med* 2007;357:1487-95.
4. Teo SM, Mok D, Pham K, Kusel M, Serralha M, Troy N, et al. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* 2015;17:704-15.
5. FitzPatrick M, Royce SG, Langenbach S, McQualter J, Reading PC, Wijburg O, et al. Neonatal pneumococcal colonisation caused by Influenza A infection alters lung function in adult mice. *Sci Rep* 2016;6:22751.
6. Rossios C, Pavlidis S, Hoda U, Kuo CH, Wiegman C, Russell K, et al. Sputum transcriptomics reveal upregulation of IL-1 receptor family members in patients with severe asthma. *J Allergy Clin Immunol* 2018;141:560-70.
7. Campbell IK, Leong D, Edwards KM, Rayzman V, Ng M, Goldberg GL, et al. Therapeutic targeting of the G-CSF receptor reduces neutrophil trafficking and joint inflammation in antibody-mediated inflammatory arthritis. *J Immunol* 2016;197:4392-402.
8. Dunican EM, Elicker BM, Gierada DS, Nagle SK, Schiebeler ML, Newell JD, et al. Mucus plugs in patients with asthma linked to eosinophilia and airflow obstruction. *J Clin Invest* 2018;128:997-1009.
9. Yuan S, Hollinger M, Lachowicz-Scroggins ME, Kerr SC, Dunican EM, Daniel BM, et al. Oxidation increases mucin polymer cross-links to stiffen airway mucus gels. *Science Transl Med* 2015;7:276ra27.

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Real-life treatment of cholinergic urticaria with omalizumab

To the Editor:



METHODS

Ethics statement

All human procedures were approved by The University of Newcastle Human Ethics Committee. All animal procedures were approved by the Animal Ethics Committees of the University of Melbourne and RMIT University and complied with the National Health and Medical Research Council Australian Code of Practice for the care and use of animals for scientific purposes (8th edition, 2013).

Asthma cohort

Twenty-six adult participants were recruited on the basis of doctor diagnosis of asthma and demonstrated variable airflow obstruction, with a 15% or greater than 200 mL change in FEV₁ following bronchodilator or airway hyperresponsiveness to mannitol challenge test. All subjects were nonsmokers with less than 5 packet-year smoking history. Participants were prescribed inhaled corticosteroids or combination inhaled corticosteroid/long-acting bronchodilator therapy and were assessed during a stable phase of disease with no change in asthma medications over the previous 4 weeks. Patient characteristics including age, sex, atopy, FEV₁%, Asthma Control Questionnaire scores, and presence of bacterial cultures in BAL are presented in Table E1. The patients were grouped on the basis of inflammatory phenotypes defined using BAL differential cell count cutoff values as described below. All subjects underwent a fiberoptic bronchoscopy in accordance with standard guidelines.^{E1} To minimize variability, the bronchoscope was inserted into the third- or fourth-generation airway of the subject where sterile saline was instilled and collected. Cytospins were prepared from the recovered BAL, and NA (n = 12) was defined as greater than or equal to 65% neutrophils of total cell count, eosinophilic asthma (n = 8) as greater than or equal to 3.5% BAL eosinophils of total cell count, and paucigranulocytic asthma (n = 6) as less than 65% neutrophils and less than 3.5% eosinophils of total cell count. The presence of bacteria in the BAL was determined by qualitative and quantitative microbiology.^{E2} Bronchial biopsies were then obtained from the same areas using biopsy forceps applied under direct vision and were used for RT-quantitative PCR analysis.

U-BIOPRED sputum analysis

We analyzed a subset of the U-BIOPRED adult baseline transcriptomics data. The U-BIOPRED cohort comprises nonsmoker severe asthmatic (n = 84) and nonasthmatic healthy control subjects (n = 16). Study participants had undergone detailed phenotypic characterization using established standard operating procedures, as described previously,^{E3} and details of sputum transcriptomics methodology are detailed in Kuo et al.^{E4}

Animal model of asthma with neonatal coinfection and HDM sensitization

Advanced pregnant BALB/c dams (Animal Resources Centre, Western Australia, Australia) were housed separately and monitored for birth with minimal disruption. Upon birth, dams were housed with their litters until weaning at age 3 weeks. Mice were housed at 22°C under normal 12-hour: 12-hour light: dark cycle, and given free access to a normal diet and water. To control for potential variability caused by sex and litter size, only female mice born to litter sizes of 5 to 7 pups were used for the experimental protocol. Neonatal mice were infected with *S pneumoniae* and/or influenza A virus as previously described^{E5} with the following minor modifications. At age 8 days, BALB/c infant mice were inoculated intranasally without anesthesia with *S pneumoniae* (serotype 19F strain EF3030, 2 × 10³ CFU) in a volume of 3 μL sterile saline or saline alone. At age 15 days, mice were inoculated by the intranasal route with influenza A virus (strain HKx31, H3N2, 500 PFU) in a volume of 3 μL sterile saline or vehicle. HDM extract (*Dermaphagoides pteronyssinus*) was obtained from Greer Laboratories (Charlotte, NC). At age 20 to 21 days, weaned female mice were sensitized intranasally with HDM aeroallergen (10 μg in 10 μL saline) or vehicle (10 μL saline alone, VEH) under isoflurane anesthesia for 5 consecutive days per week over 3 weeks. All outcomes were assessed 24 hours after the final HDM treatment (or VEH) when mice were approximately aged 6 weeks. In a separate

experiment, 100 μg of anti-colony-stimulating factor-3 receptor (α-CSF3R) antibody or isotype control antibody (CSL Limited, Parkville, Victoria, Australia) was administered to coinfecting/HDM-exposed mice via intraperitoneal injection every second day during the last week of HDM sensitization. Uninfected mice that received VEH only during the HDM exposure were used as control (saline alone). All outcomes were assessed 24 hours after the final HDM/antibody treatment when mice were approximately aged 6 weeks.

Lung function

In vivo airway reactivity was measured using Flexivent (SCIREQ, Montreal, Quebec, Canada) as previously published.^{E5} Briefly, mice were anesthetized with ketamine (125 mg/kg) and xylazine (25 mg/kg) before tracheotomy was performed and a cannula was inserted. *In vivo* airway responsiveness was assessed in response to nebulized PBS and methacholine (MCh, 100 mg/mL).

Tissue collection, quantification of *S pneumoniae*, and RT-quantitative PCR

BAL was performed and total and differential BAL cell counts were determined as previously published.^{E6} Nasopharyngeal tissue was collected and homogenized. Serial dilutions of BAL fluid and nasopharyngeal tissue homogenates were cultured on horse blood agar plates with gentamycin (5 μg/mL) as previously described.^{E5} RT-quantitative PCR was used to quantify *S pneumoniae* in lung tissue using a commercial kit from Qiagen, Hilden, Germany. Briefly, bacterial DNA was isolated by homogenizing lung tissue in Trizol using TissueLyser (Qiagen), and a 2-step PCR cycle as per manufacturer's instructions (Qiagen) was used to quantify pneumococci from a standard curve. RNA was purified from snap-frozen lung tissue using RNeasy kit as per manufacturer's instructions and cDNA was prepared as previously described.^{E7} RT-quantitative PCR was performed using bioinformatically validated Taqman primer/probes. All threshold cycle values (Ct) were normalized to a reference gene (glyceraldehyde phosphate dehydrogenase) and the relative fold change determined by the ΔΔCt method as previously published.^{E7}

Flow cytometric assessment of immune cells in lung

Antibodies were purchased from BD Biosciences, San Jose, Calif (fluorescein isothiocyanate-conjugated CD45, phycoerythrin-conjugated Siglec F, and allophycocyanin/Cy7-conjugated Ly6G). Flow cytometric analysis was performed on single-cell suspension preparation from lung homogenates using cell-specific surface markers (CD45, Ly6G, and Siglec F, as previously published^{E8}). Neutrophils were gated as intermediate or high forward scatter-area and side scatter-area, further classified as CD45^{Hi}, Siglec F⁺, and Ly6G⁺. All cells were permeabilized and fixed using a Cell Fixation & Cell Permeabilization Kit (Life Technologies, Carlsbad, Calif) before being analyzed on a BD FACSARIA III flow cytometer. Data were then analyzed with FlowJo v7.6.5 (Tree Star, Ashland, Ore).

Histology and immunohistochemistry

The left lobe of lung was removed *post mortem* and immediately fixed in 10% w/v neutral-buffered formalin. Tissues were paraffin-embedded and cut at a thickness of 4 μm. Sections were stained with Alcian blue-periodic acid Schiff for assessment of goblet cell transdifferentiation. Whole-lung sections were scanned using a slide scanner (Olympus VS-120, Tokyo, Japan), and morphometric analysis was performed using CellSens Dimensions software from Olympus. Morphometric evaluation of lung tissue sections was analyzed on a minimum of 4 bronchi per mouse section selected according to size (100-350 μm luminal diameter). Briefly, a 30 μm band was selected around the subepithelial layer of randomly selected bronchioles per section and the percent positive stain areas analyzed from Alcian blue-periodic acid Schiff-stained slides using standardized threshold values.

Peroxidase, neutrophil elastase, and double-stranded DNA assays

Peroxidases from neutrophils and eosinophils were extracted by homogenizing ground lung tissue (20 mg) in 0.4 mL extraction buffer (50 mM potassium phosphate monobasic pH 6.0, 0.5% w/v hexa-decyl-trimethyl ammonium bromide and 10 mM EDTA). Following centrifugation, 10 μ L lung lysate was incubated with 90 μ L reaction buffer (50 mM potassium phosphate pH 6.0, 0.167 mg/mL o-Dianisidine [Fast Blue B, Sigma], and 0.005% H_2O_2). The change in absorbance (A_{460}) (between 2-minute and 3-minute postincubation) resulted from the peroxidase-mediated decomposition of H_2O_2 , and oxidation of o-Dianisidine was measured using a Clariostar plate reader. Neutrophil elastase activity was measured on BAL fluid with an EnzChek Elastase Assay Kit according to manufacturer's instructions (Life Technologies). Content of double-stranded DNA in the BAL fluid was also measured using Quant-iT PicoGreen double-stranded DNA reagent (Life Technologies), according to the manufacturer's instructions.

Data analysis

Data from the experimental model was presented as the mean $\pm$ SEM with normal distribution or median $\pm$ interquartile range with nonnormal distribution. All data were statistically analyzed using GraphPad Prism 7.0 (GraphPad, San Diego, Calif). Where detailed and appropriate, 1-way or 2-way ANOVA with Tukey post hoc tests were performed. P less than .05 was considered to be statistically significant. Nonparametric data from the clinical cohort were reported as the median and interquartile range and by Kruskal-Wallis test followed by Dunns correction or the Mann-Whitney U test or Spearman correlation.

REFERENCES

- E1. Sukkar MB, Wood LG, Tooze M, Simpson JL, McDonald VM, Gibson PG, et al. Soluble RAGE is deficient in neutrophilic asthma and COPD. *Eur Respir J* 2012; 39:721-9.
- E2. Wilkinson TM, Donaldson GC, Johnston SL, Openshaw PJ, Wedzicha JA. Respiratory syncytial virus, airway inflammation, and FEV1 decline in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2006;173:871-6.
- E3. Shaw DE, Sousa AR, Fowler SJ, Fleming LJ, Roberts G, Corfield J, et al. Clinical and inflammatory characteristics of the European U-BIOPRED adult severe asthma cohort. *Eur Respir J* 2015;46:1308-21.
- E4. Kuo CS, Pavlidis S, Loza M, Baribaud F, Rowe A, Pandis I, et al. T-helper cell type 2 (Th2) and non-Th2 molecular phenotypes of asthma using sputum transcriptomics in U-BIOPRED. *Eur Respir J* 2017;49. pii: 1602135. <https://doi.org/10.1183/13993003.02135-2016>.
- E5. FitzPatrick M, Royce SG, Langenbach S, McQualter J, Reading PC, Wijburg O, et al. Neonatal pneumococcal colonisation caused by influenza A infection alters lung function in adult mice. *Sci Rep* 2016;6:22751.
- E6. Anthony D, Seow HJ, Uddin M, Thompson M, Dousha L, Vlahos R, et al. Serum amyloid A promotes lung neutrophilia by increasing IL-17A levels in the mucosa and gammadelta T cells. *Am J Respir Crit Care Med* 2013;188: 179-86.
- E7. Bozinovski S, Vlahos R, Zhang Y, Lah LC, Seow HJ, Mansell A, et al. Carbonylation caused by cigarette smoke extract is associated with defective macrophage immunity. *Am J Respir Cell Mol Biol* 2011;45: 229-36.
- E8. Wang H, Anthony D, Yatmaz S, Wijburg O, Satzke C, Levy B, et al. Aspirin-triggered resolvin D1 reduces pneumococcal lung infection and inflammation in a viral and bacterial coinfection pneumonia model. *Clin Sci (Lond)* 2017;131:2347-62.

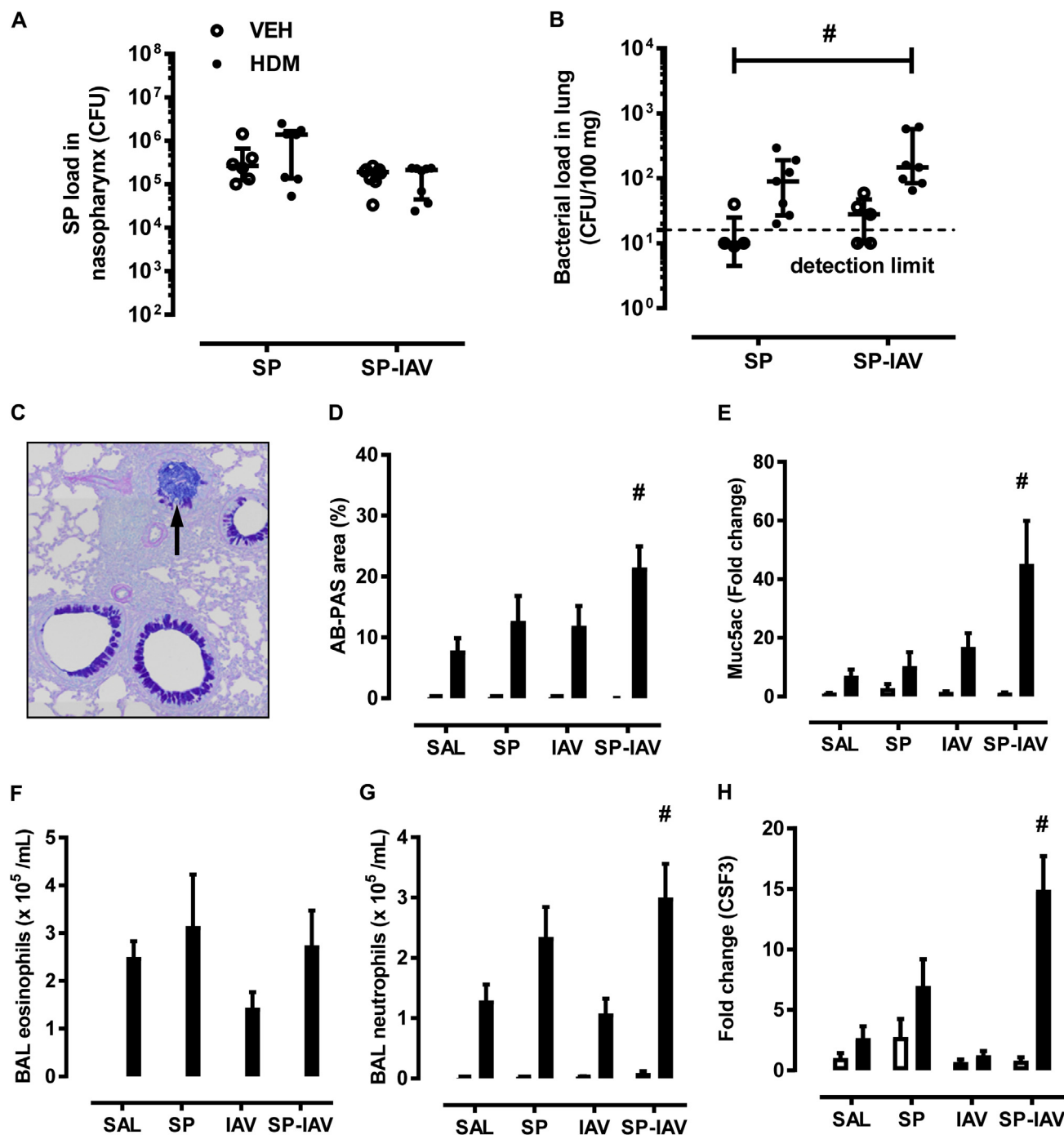


FIG E1. Neonatal coinfection and HDM challenge promotes lung infection and mucus. Twenty-four hours after final HDM challenge (age 40-42 days), *S pneumoniae* load in the (A) nasopharynx and (B) lung tissue was determined. (C) AB-PAS was used to stain airway mucus (purple) (D) and was quantified as detailed in the Methods section. E, Expression of the mucin gene Muc5AC in lung tissue was analyzed by RT-quantitative PCR. (F) Eosinophil and (G) neutrophils BAL numbers are presented across treatment groups, and (Fig E1, F) RT-quantitative PCR was performed on lung tissue for assessment of CSF3 using GAPDH as a reference gene. $n = 4-8$ per group. # $P < .05$, 2-way ANOVA analysis with Tukey post hoc test compared with SP/HDM. AB-PAS, Alcian blue-periodic acid Schiff; CFU, colony forming unit; GAPDH, glyceraldehyde phosphate dehydrogenase; IAV, influenza A virus; SP, *Streptococcus pneumoniae*. Data are expressed as median $\pm$ interquartile range in Fig E1 (A and B) or mean $\pm$ SEM in Fig E1 (D to H).

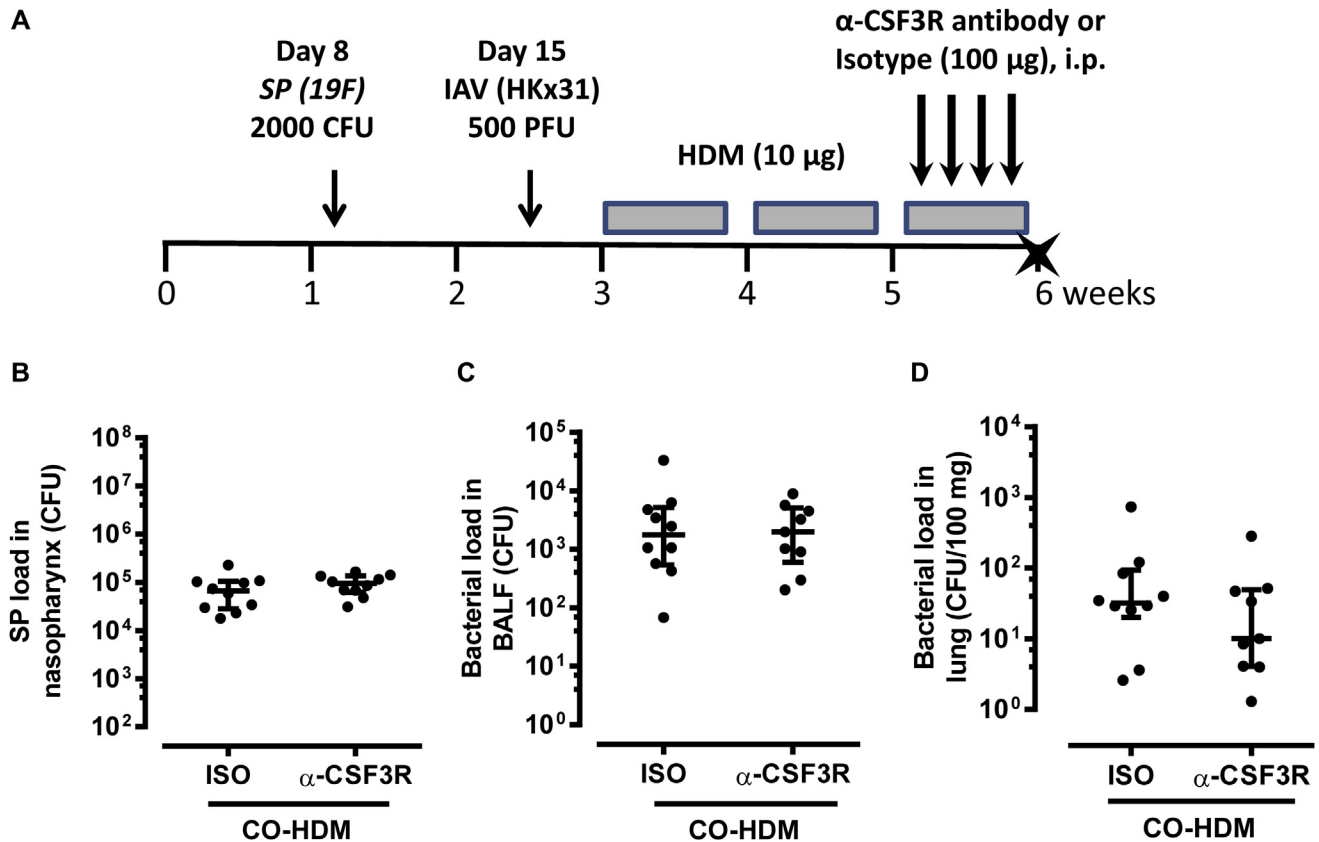
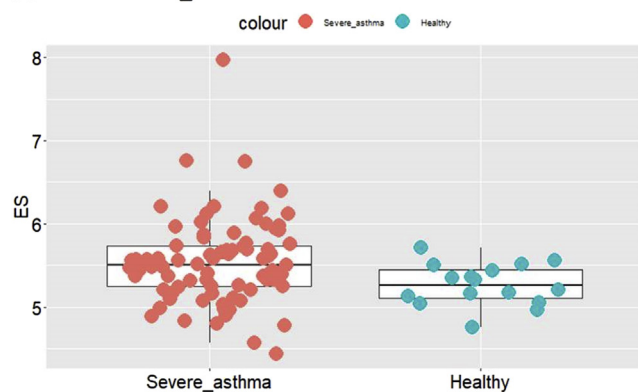


FIG E2. Neutralizing CSF3R did not affect pneumococcal nasal or lung load. **A**, In the last week of HDM challenge, mice were treated with 100 µg anti-CSF3R antibody (α-CSF3R) or isotype control antibody (ISO) by intraperitoneal injection every second day. Twenty-four hours after final HDM/antibody administration, *S pneumoniae* load in the **(B)** nasopharynx, **(C)** BAL fluid, and **(D)** lung tissue was determined ($n = 9-10$ per group). IAV, Influenza A virus; CFU, colony forming unit; PFU, plaque forming unit; *SP*, *Streptococcus pneumoniae*. Data are expressed as median $\pm$ interquartile range.

A CSF3.207442_at



B CSF3R.203591_s_at

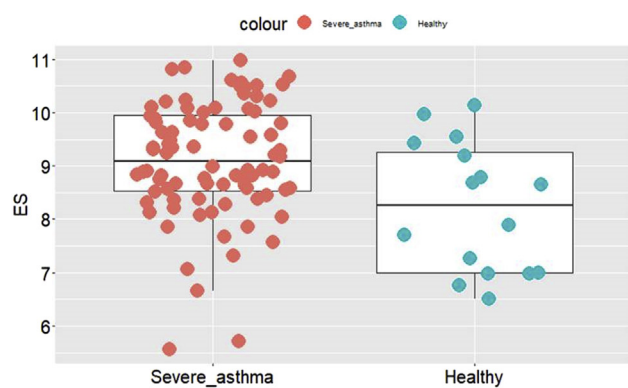


FIG E3. CSF3 and CSF3R expression in the U-BIOPRED cohort. Baseline transcript expression of **(A)** CSF3 and **(B)** CSF3R in sputum cells analyzed by microarray profiling of those with severe asthma (nonsmokers) and healthy controls enrolled within the U-BIOPRED cohort. *ES*, Enrichment Score.

TABLE E1. Patient characteristics

Characteristic	NA (n = 12)	Non-NA (n = 14)	
		Paucigranulocytic (n = 6)	Eosinophilic (n = 8)
Age (y), median (range)	65.5 (42-78)	55.5 (38-57)	61 (42-76)
Sex (M:F)	4:8	4:2	3:5
Atopy, n (%)	4 of 12 (33)	3 of 6 (50)	7 of 8 (88)
Median FEV ₁ % (range)	72 (59-97)	84 (71-104)	86 (58-108)
Median ACQ (range)	2 (1-3.3)	0.65 (0-1.3)	2.15 (1.1-3.5)
+ Bacterial culture, n (%)	8 of 12 (67)	1 of 6 (17)	5 of 8 (63)
%Neutrophil (range)	88 (67-96)	38 (1.5-58)	26 (18-64)
%Eosinophils (range)	1.1 (0.3-2.8)	1.4 (0.5-3.3)	10.5 (3.8-47.3)

ACQ, Asthma Control Questionnaire.