

Diminished airway macrophage expression of the Axl receptor tyrosine kinase is associated with defective efferocytosis in asthma



To the Editor:

Up to 15% of the 300 million people affected by asthma worldwide suffer from disease refractory to available medications and associated with significant morbidity, particularly due to frequent exacerbations secondary to respiratory viral infections. Airway macrophages from patients with severe asthma are known to be defective in apoptotic cell clearance.¹⁻³ Because phagocytosis of apoptotic cells, called efferocytosis, prevents secondary necrosis and the release of proinflammatory necrotic cell debris, defects in apoptotic cell uptake contribute to perpetuation of lung inflammation.⁴ Although several processes have been implicated in impaired efferocytosis in asthma, the underlying mechanisms and the links between apoptotic cell clearance, respiratory infections, and disease exacerbations remain unknown.

TAM (Tyro3, Axl, MerTK) receptor tyrosine kinases mediate apoptotic cell uptake by phagocytes through the bridging molecules Protein S or growth arrest specific 6 (Gas6), and are critical for lung immune homeostasis.⁴ We and others have shown that Axl is specifically expressed on mouse airway macrophages, is constitutively bound to its ligand Gas6, and is critical for efficient efferocytosis.^{5,6} Axl-deficient mice develop exaggerated lung inflammation upon influenza infection associated with accumulation of apoptotic cells in the airways,⁵ indicating a critical role for Axl in apoptotic cell clearance from the inflamed lung. Here, we sought to define the TAM receptor expression profile in human airway macrophages, characterize the local factors driving their expression, and identify potential defects in TAM receptors contributing to defective efferocytosis in asthma.

We first performed mRNA expression profiling of TAM receptors in peripheral blood monocytes, monocyte-derived macrophages (MDMs), and airway macrophages isolated from sputum samples obtained from 12 healthy donors (HDs) (for details of experimental procedures and study cohort characteristics, see this article's Online Repository at www.jacionline.org). TAM receptors displayed a strong cell type-specific expression pattern: MerTK was preferentially expressed in MDMs, but not monocytes or airway macrophages, whereas Axl expression was restricted to airway macrophages (Fig 1, A). Using macrophages from the healthy margins of lung resections (which contain both airway and alveolar macrophages), we confirmed that only airway/alveolar macrophages constitutively expressed high levels of Axl protein, while MDMs were strongly MerTK-dominant (Fig 1, B and C). Apoptotic cell recognition by Axl requires the bridging molecule Gas6 and, similar to mouse models,⁵ we detected Gas6 on most airway/alveolar macrophages (see Fig E1 in this article's Online Repository at www.jacionline.org). These data reveal selective Axl expression on human airway/alveolar macrophages and constitutive binding of Gas6 rendering these cells primed for apoptotic cell recognition through Axl.

We next investigated why Axl is exclusively expressed on airway/alveolar macrophages. Axl mRNA and protein expression was significantly reduced during *ex vivo* culture of HD airway/alveolar macrophages from lung resections (Fig 1, D and E), suggesting that these cells may require the airway microenvironment to maintain their unique TAM receptor expression profile. To identify factors that drive Axl expression, we stimulated airway/alveolar macrophages with factors known to affect their activation during infection and chronic inflammation. Strikingly, only IFNs significantly restored Axl mRNA expression during *ex vivo* culture (Fig 1, F). Restoration of Axl expression by IFNs, in particular IFN- α and IFN- λ , was also evident at the protein level (Fig 1, G). These findings indicate that homeostatic levels of IFNs or their sustained production by bronchial epithelial cells following previous viral infections drive selective Axl expression and regulate the efferocytic function of human airway/alveolar macrophages.

Previous studies have shown clear defects in efferocytosis in severe asthma.¹⁻³ On the basis of our observations, we hypothesized that alterations in Axl may explain these defects. The extracellular domain of Axl is cleaved upon engagement, and alterations in soluble Axl (sAxl) levels have been reported in inflammatory conditions.⁷ We compared the levels of sAxl in serum and sputum from HDs and patients with moderate-to-severe asthma (British Thoracic Society steps 3-5). Although no difference in sAxl was detected in serum samples between the 2 groups (Fig 2, A), the levels of sAxl in sputum samples were significantly elevated in patients with asthma compared with controls (Fig 2, B), suggesting increased Axl shedding at the site of inflammation.

Next, we compared Axl and MerTK mRNA expression in peripheral blood monocytes, MDMs, and airway macrophages in sputum from patients with moderate-to-severe asthma and HDs. Similar levels of MerTK were expressed in both groups in all analyzed cell types (Fig 2, C), whereas Axl expression was significantly reduced in airway macrophages from patients with asthma ($P < .001$). The observed difference was restricted to the site of inflammation as monocytes and MDMs from asthma patients and HDs expressed comparable, albeit low, levels of Axl (Fig 2, D). To test the functional significance of reduced Axl expression in asthma, we determined the amounts of DNA-histone complexes (nucleosomes), a hallmark of necrosis and secondary necrosis of apoptotic cells,⁵ in the airways of the same patients. Extracellular nucleosome levels were elevated in sputum from patients with moderate-to-severe asthma compared with HDs (Fig 2, E), and negatively correlated with airway macrophage Axl expression (Fig 2, F; $R = -0.71$; $P < .0001$). These results indicate that Axl is the main apoptotic cell recognition receptor expressed on human airway macrophages and reduced Axl expression and/or increased shedding might perpetuate chronic inflammation in asthma.

In conclusion, we report for the first time that (1) the apoptotic cell recognition receptor Axl is exclusively expressed by human airway/alveolar macrophages, (2) Axl expression depends on the presence of type I/III IFNs, (3) increased shedding of sAxl occurs in sputum from patients with moderate-to-severe asthma, and (4) Axl mRNA expression in airway macrophages isolated from sputum is reduced in patients with moderate-to-severe asthma. These findings have important implications for those patients at

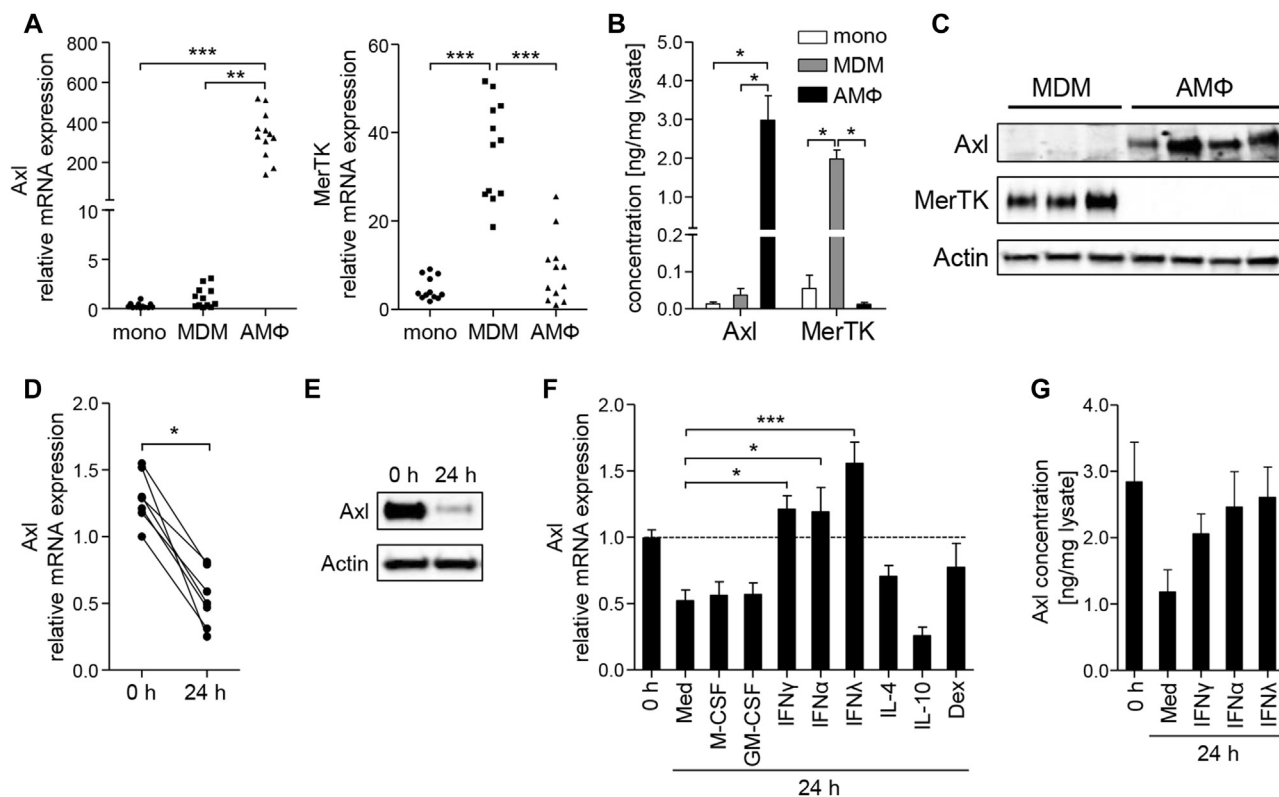


FIG 1. Axl is exclusively expressed on human airway/alveolar macrophages, is downregulated *ex vivo* and restored by interferons. **A**, Relative Axl and MerTK mRNA expression in HD monocytes (mono), MDMs, and airway macrophages (AMΦ) (n = 12). **B**, Axl and MerTK protein expression in mono, MDMs, and AMΦ from HDs (n = 5). **C**, Immunoblotting of Axl, MerTK, and actin in MDMs (n = 3) and AMΦ (n = 4) from HDs. Axl relative mRNA (n = 7) (**D**) and protein expression in HD AMΦ at 0 hour and 24 hours in culture (**E**). **F**, Axl relative mRNA expression in HD AMΦ at isolation (0 hour) and after 24-hour culture with or without M-CSF, GM-CSF, IFN-γ, IFN-α, IFN-λ, IL-4, IL-10, or dexamethasone (n = 5). **G**, Axl protein expression in AMΦ at 0 hour and 24 hours in culture with or without IFN-γ, IFN-α, or IFN-λ (n = 3). **P* < .05, ***P* < .01, and ****P* < .001.

the most difficult-to-treat end of the asthma disease spectrum, particularly in light of recent reports demonstrating the role of altered IFN responses during respiratory infections in asthma.⁸ Our results suggest that strategies aimed at restoring or activating Axl could be beneficial in patients with asthma by facilitating apoptotic cell clearance. This idea is supported by a recent clinical trial where inhaled type I IFN prevented viral infection–induced exacerbation of disease symptoms only in patients with severe asthma.⁹ In light of our observation that IFNs restore Axl expression on airway macrophages, the beneficial effects of type I IFN might be at least partly attributed to improved efferocytic function and prevention of secondary necrosis. Collectively, data from this study provide evidence that Axl is an important regulator of lung immune homeostasis and identify a novel pathway that could be targeted to improve defective efferocytosis in asthma.

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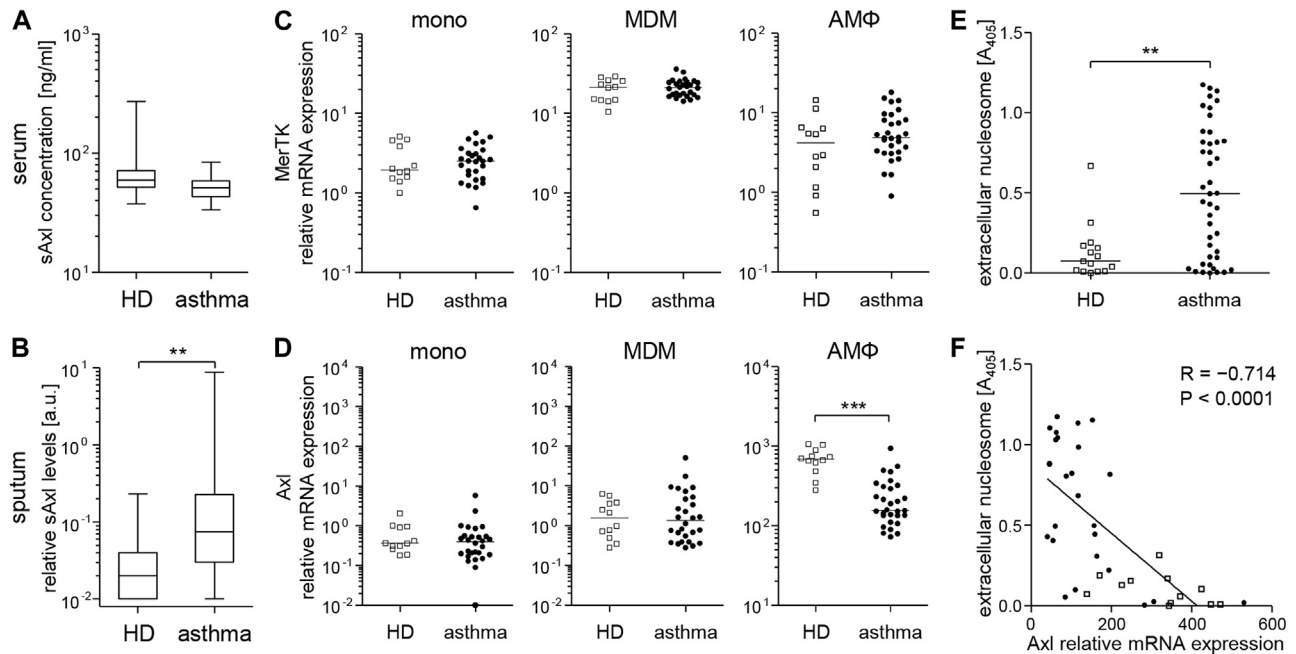


FIG 2. Airway macrophage Axl expression and shedding are altered in patients with moderate-to-severe asthma and are associated with accumulation of necrotic cell debris in the airways. **A**, Levels of sAxl in serum samples from HDs ($n = 15$) and patients with asthma ($n = 43$). **B**, Analysis of sAxl in sputum samples from HDs ($n = 15$) and patients with asthma ($n = 40$). **C** and **D**, MerTK and Axl relative mRNA expression in monocytes (mono), MDMs, and airway macrophages (AM Φ) from HDs ($n = 12$) and patients with asthma ($n = 30$). **E**, Amount of nucleosomes in sputum samples from HDs ($n = 15$) and patients with asthma ($n = 43$). **F**, Correlation analysis of AM Φ Axl mRNA expression and sputum nucleosome levels. Symbols represent values from individual HDs (open squares) and asthma patients (closed circles) and the lines denote the median. ** $P < .01$ and *** $P < .001$.

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Water-soluble chitosan inhibits nerve growth factor and attenuates allergic inflammation in mite allergen-induced allergic rhinitis



To the Editor:

Allergic rhinitis (AR), which affects approximately 10% to 15% of the population worldwide, is a chronic inflammatory disease of the nasal mucosa involving nasal airway hyper-responsiveness (AHR), an inappropriate activity of the nervous

METHODS

Study population and sample collection

Paired peripheral blood and sputum samples (spontaneous or induced by using hypertonic saline) were collected from 15 healthy individuals and 46 patients with moderate-to-severe asthma fulfilling the British Thoracic Society criteria (disease steps 3-5).^{E1} Clinical characteristics of patients are presented in Table E1. For some experiments, marginal noncancerous lung tissue was collected from patients undergoing surgery for suspected or confirmed cancer (n = 15). Patients included in this study did not have a history of any chronic inflammatory lung disease and had normal lung function (median [interquartile range] FEV₁/forced vital capacity ratio, 0.74 [0.70-0.79]). All samples were collected at the University Hospital of South Manchester/North West Lung Centre under the framework of the Manchester Allergy, Respiratory and Thoracic Surgery Biobank (study nos. M2014-17 and M2015-32) with ethical approval of the National Research Ethics Service (REC reference 10/H1010/7). All patients provided written informed consent before participation in the study. Peripheral blood from healthy individuals (leukocyte cones) was also obtained from the National Blood Transfusion Service (Manchester, UK) with ethical approval by the University of Manchester ethics committee.

Cell isolation and culture

Lung tissue was perfused with PBS. The retrieved cell suspension was centrifuged at 400g for 10 minutes, and the cell pellet was resuspended in PBS and subjected to Ficoll-Paque (GE Healthcare, Buckinghamshire, United Kingdom) density gradient centrifugation to remove granulocytes and erythrocytes. The interface of mononuclear cells, containing predominantly airway/alveolar macrophages, was collected and washed in PBS, and viable macrophages were counted using trypan blue exclusion and plated at 1×10^6 cells/mL in RPMI 1640 supplemented with 10% FCS, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 2 mM L-glutamine (complete medium) for 1 hour at 37°C for adherence. Nonadherent cells were removed by extensive washing and airway/alveolar macrophages were either lysed immediately or stimulated with M-CSF (50 ng/mL), GM-CSF (50 ng/mL), IL-4 (20 ng/mL), IL-10 (20 ng/mL), IFN- γ (50 ng/mL), IFN- λ (100 ng/mL) (all from Peprotech, Rocky Hill, NJ), IFN- α (1000 U/mL) (R&D Systems, Abingdon, United Kingdom), or dexamethasone (Dex, 1 μ M) (Sigma-Aldrich, Dorset, United Kingdom) for 24 hours. A 90% or more purity of airway macrophages was routinely achieved with this isolation method.^{E2} To purify airway macrophages from sputum, samples were processed as previously described.^{E3} Supernatants were stored at -80°C and cells were incubated with 50 μ g/mL DNase (Roche Diagnostics, Mannheim, Germany) for 30 minutes at 37°C, washed with PBS, and plated in 24-well plates in complete medium. After 1-h adherence, cells were extensively washed to remove nonadherent cells and airway macrophages were lysed in RLT buffer (Qiagen, Manchester, United Kingdom) before RNA extraction.

PBMCs were isolated by Ficoll-Paque density gradient centrifugation. Monocytes were then purified by magnetic-activated cell sorting using human CD14 MicroBeads (Miltenyi Biotec, Surrey, United Kingdom), plated at 5×10^5 cells/mL in complete medium containing 50 ng/mL M-CSF, and cultured for 6 days before further processing.

RNA extraction and quantitative PCR

Total RNA was isolated using RNeasy micro kit (Qiagen), quantified with a Nanodrop spectrophotometer (Thermo Scientific, Waltham, Mass) and equivalent amounts of RNA were reverse transcribed using High-Capacity RNA-to-cDNA Kit (Life Technologies, Paisley, United Kingdom). Quantitative PCR reactions were performed using TaqMan expression assays and TaqMan Fast Universal PCR Master Mix on a QuantStudio 12K Flex PCR system and the obtained data were analyzed using QuantStudio 12K Flex Software (all from Life Technologies). Average expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and RPLP0 was used to calculate relative mRNA expression of Axl and MerTK.

Immunoblotting and ELISA of cell lysates

Protein lysates were prepared in lysis buffer containing 1% Igepal CA-630, 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Scientific) and equivalent amounts of protein were resolved by electrophoresis on 4% to 15% mini-Protean TGX precast gels (BIO-RAD, Hercules, Calif). Proteins were then transferred to nitrocellulose membranes (BIO-RAD) using a semi-dry Trans-Blot Turbo transfer system (BIO-RAD). Membranes were washed in PBS containing 0.05% Tween-20 (Sigma-Aldrich) (PBS/T), blocked in 2% milk (BIO-RAD) in PBS/T, and incubated o/n at 4°C with antibodies recognizing MerTK (D21F11, Cell Signaling Technology, Beverly, Mass), Axl (M20), or actin (I19-R) (both from Santa Cruz Biotechnology, Santa Cruz, Calif) diluted in PBS/T containing 1% BSA. After washing in PBS/T, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit, antimouse, or antigoat immunoglobulin antibodies (Dako, Cambridge, United Kingdom), developed using a Clarity Western ECL Substrate (BIO-RAD) and visualized with a ChemiDoc MP Imaging System (BIO-RAD). Alternatively, Axl and MerTK protein levels were analyzed in cell lysates using total Axl and MerTK ELISA kits (R&D Systems) as per the manufacturer's instructions and the data were presented as ng/mg total protein in the lysate.

Flow cytometric analyses

Cells were stained with Zombie UV Flexible Viability dye (BioLegend, San Diego, Calif) for 20 minutes followed by incubation with Human TruStain FcX Fc receptor blocking solution (BioLegend) for 10 minutes. Cells were then washed with FACS buffer (PBS containing 1% FCS and 2 mM EDTA) and stained with PE/Cy7-conjugated anti-CD64 (clone 10.1, BioLegend) and biotinylated anti-Gas6 (BAF885) or control biotinylated goat IgG (BAF108) antibodies (all from R&D Systems) for 20 minutes at 4°C. After washing with FACS buffer, cells were incubated with BV421-conjugated streptavidin (BioLegend) for 20 minutes at 4°C, washed, and fixed by 10-min incubation with 2% paraformaldehyde in PBS. Cells were acquired on a BD LSRFortessa (BD Biosciences, San Jose, Calif) and data were analyzed using FlowJo software (TreeStar, Ashland, Ore). Airway/alveolar macrophages were defined as FSC^{hi}SSC^{hi}CD64⁺ and highly autofluorescent. The strategy using autofluorescence to define airway/alveolar macrophages was described before.^{E4,E5}

Detection of extracellular nucleosomes and soluble Axl

Levels of nucleosomes in sputum samples were determined using the Cell Death Detection ELISA Plus (Roche) according to the manufacturer's instructions.

To measure sAxl in serum samples, Maxisorp plates (Nunc, Roskilde, Denmark) were coated o/n with 500 ng/mL goat anti-Axl antibody (AF154, R&D Systems) in PBS. Plates were washed with wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20) and blocked in 3% fish gelatin (Norland Products Inc, Cranbury, NJ) diluted in wash buffer for 2 hours at room temperature (RT). Serum was diluted 1:10 in blocking buffer and incubated on the plates for 2 hours at 37°C. After washing, plates were incubated with polyclonal rabbit anti-Axl antibody (in-house, Ab042) diluted in blocking buffer, followed by incubation with goat antirabbit horseradish peroxidase conjugate (P0448, Dako) for 1 hour at RT. Plates were developed using an OPD-substrate (Dako, S2045) and the absorbance was measured using an Infinite 200 microplate reader (Tecan, Männedorf, Switzerland).

To detect sAxl in sputum, samples from patients and controls (600 μ L) or an equal volume of standard solution containing 1, 5, or 10 ng/mL sAxl was mixed with 1 mL PBS. sAxl standard was expressed and purified as described previously.^{E6} The samples were precleared with 30 μ L Protein G sepharose (Life Technologies) for 30 minutes at 4°C after which the beads were removed by centrifugation at 2000g for 5 minutes. One microgram

anti-Axl antibody (AF154, R&D Systems) was added together with 30 μ L Protein G sepharose to the supernatant and samples were incubated *o/n* at 4°C under rotation. The beads were then washed twice with PBS and incubated for 5 minutes in reducing Laemmli buffer at 95°C. Supernatants were separated on a 4% to 15% SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane, and probed against Axl (in-house, Ab042). Blots were analyzed using the ImageLab software and the intensities of the bands corresponding to sAxl were quantified. Normalization was performed on each blot by setting the signal of the 5 ng/mL sAxl standard solution IP reaction to 1. In all cases, samples from patients and controls were run in parallel on each blot to minimize interblot variation.

Statistical analyses

Data are presented as mean \pm SEM (Fig 1, B, F, and G), as boxplots with the line within the box denoting the median, boxes representing 25th and 75th percentiles, and the lines outside the box marking the minimum and maximum values (Fig 2, A and B), or as symbols representing data from individual donors. Nonparametric tests were used for comparisons between patient groups or cell types and for *in vitro* studies of airway macrophages: Kruskal-Wallis test followed by Dunn's multiple comparison test (Fig 1, A and B), Wilcoxon

signed rank test (Fig 1, D), Friedman test followed by Dunn's multiple comparison test (Fig 1, F), or Mann-Whitney *U* test (Fig 2, A-E). The Spearman correlation coefficient was used for correlation analyses. *P* values of less than .05 were considered statistically significant.

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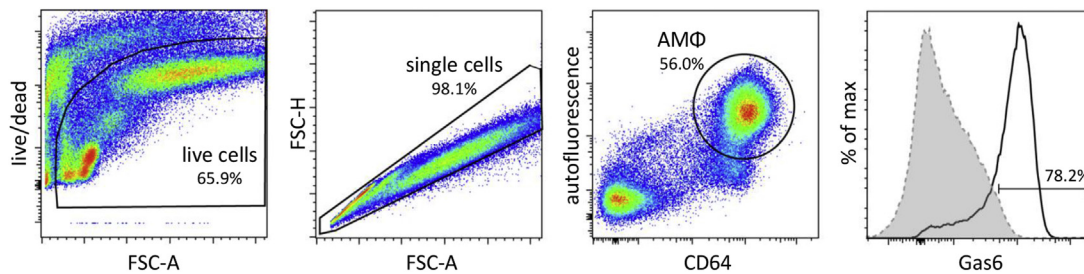


FIG E1. Gas6 is constitutively bound to the surface of human airway/alveolar macrophages (AM Φ). Flow cytometric analysis of Gas6 on AM Φ isolated from healthy lung tissue. Live single cells were selected and AM Φ were defined as FSC^{hi}SSC^{hi}CD45⁺ (gates not shown) CD64⁺ and highly autofluorescent. The histogram of Gas6 surface expression is a representative of 3 analyzed donors. Solid line/open: Gas6 staining; dotted line/shaded: isotype control. *FSC-A*, Forward scatter-area; *SSC-H*, side scatter-height.

TABLE E1. Clinical features of patients with severe asthma and healthy subjects included in the study

Feature	Moderate-to-severe asthma (n = 46)	Healthy control (n = 15)
Sex: male/female	19/27	4/11
Age (y)	55 (45-63)	37.2 (30-46)
Smoking history		
Never	21	11
Ex	18	4
Current	7	0
Asthma severity		
BTS step 3	8	
BTS step 4	12	
BTS step 5	26	
FEV ₁ (L)	2.14 (1.63-2.73)	NA
FEV ₁ % predicted	77 (64-94)	NA
FVC (L)	3.10 (2.63-3.69)	NA
FVC % predicted	93 (83-109)	NA
FEV ₁ /FVC ratio	0.7 (0.59-0.79)	NA

The values are expressed as counts or median (interquartile range).

BTS, British Thoracic Society; FVC, forced vital capacity; NA, not applicable/available.