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Semaphorin 7A is expressed on airway eosinophils and upregulated by IL-5 family cytokines

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Abstract

Semaphorin 7A (Sema7A) plays a major role in TGF-β1-induced lung fibrosis. Based on the accumulating evidence that eosinophils contribute to fibrosis/remodeling in the airway, we hypothesized that airway eosinophils may be a significant source of sema7A. *In vivo*, sema7A was expressed on the surface of circulating eosinophils and upregulated on bronchoalveolar lavage eosinophils obtained after segmental bronchoprovocation with allergen. Based on mRNA levels in unfractionated and isolated bronchoalveolar cells, eosinophils are the predominant source of sema7A. *In vitro*, among the members of the IL-5-family cytokines, sema7A protein on the surface of blood eosinophils was increased more by IL-3 than by GM-CSF or IL-5. Cytokine-induced expression of cell surface sema7A required translation of newly synthetized protein. Finally, a recombinant sema7A induced alpha-smooth muscle actin production in human bronchial fibroblasts. Semaphorin 7A is a potentially important modulator of eosinophil profibrotic functions in the airway remodeling of patients with chronic asthma.

Keywords

Eosinophil; semaphorin 7A; IL-3; fibrosis; translation

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Conflict of Interest Statement
None of the authors has conflicts of interest with the present study.

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1. INTRODUCTION

The semaphorins are a family of soluble and membrane-associated proteins characterized by a conserved “sema” domain [1]. Semaphorins were initially identified as axonal growth and guidance proteins [2]. More than 20 types of semaphorins have been described that have a variety of functions including angiogenesis, vasculogenesis, and immune regulation. Unlike the other semaphorins, which are integral membrane proteins, semaphorin 7A (sema7A) is associated with the membrane by a glycophosphatidylinositol (GPI)-linked sequence [3]. While some sema7A functions, such as axon growth and monocyte activation, are mediated through β1-integrins [1], the transmembrane protein plexin C1 binds exclusively to sema7A [4]. Sema7A is highly expressed on activated T lymphocytes [3] and its localization in the immunological synapse amplifies pro-inflammatory cytokine expression by antigen-presenting cells [5]. Recently, sema7A was found to play a critical function as an inducer of pulmonary fibrosis and liver fibrogenesis [6–8]. These observations suggest a role for sema7A in fibrotic lung diseases and potentially asthma where increase subepithelial fibrosis is well described [9].

Eosinophils (EOS) are a hallmark of allergic asthma. Although the role of airway EOS has been controversial, recent trials with anti-EOS therapies such as mepolizumab and reslizumab (both anti-IL-5 monoclonal antibodies) demonstrated reduction in asthma exacerbations [10–13]. EOS are capable of releasing cytokines that direct the T lymphocyte response [14, 15], and enhance airway remodeling [16]. Genetic ablation of EOS attenuated submucosal matrix deposition and reduced airway smooth muscle hyperplasia [17, 18]. Using genome-wide expression analyses, we recently reported that sema7A mRNA was among the transcripts that were increased in parallel with the increase of EOS in bronchoalveolar lavages (BAL) and sputum of mild allergic asthmatics following an *in vivo* allergen challenge [19]. While airway remodeling has been linked at least in part to the presence of EOS, their expression of sema7A has not yet been reported. Our present study aims to examine the expression of the profibrotic factor sema7A on airway EOS and to determine its regulation.

2. MATERIALS AND METHODS

2.1 Subjects and cell preparations

The study protocol was approved by the University of Wisconsin-Madison Health Sciences Institutional Review Board. Informed written consent was obtained from subjects prior to participation. All subjects were atopic, with at least one positive skin prick test. For the bronchoscopy study to obtain airway EOS, subjects had a history of mild asthma with airway reversibility to albuterol. None of the subjects were using inhaled or oral corticosteroids.

Detailed methods for bronchoscopy, segmental antigen challenge (SBP-Ag), and BAL cell preparation have previously been described [20]. Blood EOS were purified by negative selection as previously described [14]. More details are provided in the online supplement.

2.2 Real-time PCR

Total RNA preparation, real-time quantitative PCR (RT-qPCR) using SYBR Green Master Mix, and calculation of mRNA fold change using the comparative cycle threshold (ΔΔCT) method, have been described previously [15], and are described in the online supplement.

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2.3 ELISA

Unlabeled and biotinylated anti-IL-3 mAbs and corresponding recombinant protein standards for ELISA were from BD Biosciences. Unlabeled and biotinylated anti-GM-CSF mAb and recombinant protein standard for ELISA were from R&D Systems. To measure cytokine concentrations, BAL fluid was concentrated 20-fold at 4°C using a low protein-binding Centriprep centrifugal filter unit (Millipore) with a molecular mass cutoff limit of 3 kDa. A sensitive two-step sandwich ELISA was used as described [21]. BAL fluid was diluted in LowCross-Buffer (Boca Scientific, Boca Raton, FL, USA) to reduce non-specific low-affinity binding. The assay sensitivities were below 3 pg/ml for GM-CSF, and 12 pg/ml for IL-3.

2.4 Immunocytochemistry

EOS in suspension were fixed with 3.7% paraformaldehyde, incubated with 0.1 M glycine for 10 min and washed with PBS. Cells were onto poly-L-lysine-coated coverslips by cytocentrifugation and permeabilized with 0.5% SDS in PBS for 15 min at room temperature. Cells were washed three times with PBS and blocked with 10% BSA in PBS for 1 h at room temperature. Cells were incubated overnight at 4°C with 5 μg/ml of goat anti-human Sema7A antibody or control IgG (both from R&D Systems, Minneapolis, MN) diluted in PBS containing 2% BSA and 0.1% SDS. After 3 washes with PBS, cells were stained with 1:100 FITC-rabbit anti-goat antibody at room temperature for 1 h. Cells were washed 3 times with PBS. Nuclei were stained with diamidino-2-phenylindole (DAPI) and the coverslip was mounted to a slide. Pictures were taken using a Nikon A1R confocal microscope (Nikon Instruments INC, Melville, NY), and 100x/1.40 oil immersion objective lens with 1 or 1.2 AU pinhole and 0.15–1 μm z stack thickness. Images were obtained and exported by NIS Elements Advanced Research software (Nikon Instruments INC).

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2.5 Flow cytometry

For the *in vivo* study, unfractionated EDTA-treated blood (100 μl) or BAL cells (250,000) were analyzed by flow cytometric analysis as previously described [20]. Cells were stained with PE-conjugated anti-CD108 (BD Pharmingen™, BD Biosciences, San Jose, CA) along with a cocktail of FITC-conjugated anti-CD16 and anti-CD14 to discriminate EOS from neutrophils and monocytes, respectively. RBCs were lysed (BD-lysing solution) and 5–10,000 gated events (EOS) were acquired on a FACSCalibur (BD Biosciences). EOS were identified by forward and side scatter and then histograms were based on $CD16^{-}$ $CD14^{-}$ populations within the scatter gate. As a positive control for sema7A, $CD108^{+}$ $CD4^{+}$ T cells were assessed in unfractionated EDTA-treated blood or BAL cells. An isotype control antibody was used to set the negative gate to yield 1% positive cells thus allowing analysis of the percentage of positive cells. Data were analyzed with FlowJo (TreeStar Inc, Ashland, OR, USA) and expressed as the percentage of positive cells within the $CD4^{+}$ lymphocyte population.

For *in vitro* analyses of cultured purified blood EOS, cells were stained with PE-conjugated anti-CD108 (sema7A), anti-CD125 (IL-5Rα), or anti-CD123 (IL-3Rα) or appropriate isotype control antibodies (all purchased from BD Biosciences, San Jose, CA). Data for all EOS experiments are expressed as geometric mean of specific stain minus that of the isotype control antibody.

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2.6 Polyribosome preparation

Blood EOS were activated with either IL-3 or GM-CSF (2ng/ml) for 14 h. Cells (6×10^{6}) were washed with PBS and polyribosomes were prepared as previously described [22] with some changes. All steps of the protocol were performed on ice or at 4°C. Briefly, cell pellets

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were suspended in a lysis buffer composed of 10 mM Tris hydrochloride, pH 8.0, 1 mM potassium acetate, 1.5 mM magnesium acetate, 2 mM dithiothreitol, 0.5 % Triton X100, 0.05 % NP-40, 100 μg/ml of cycloheximide (Calbiochem,), 800 U/ml of RNasin® (Promega, Madison, WI) and a cocktail of protease inhibitor for mammalian cells (Sigma, St. Louis, MO). Cells were briefly sonicated to resuspend the pellet, and were incubated for 30 min on ice with 15 passages through a 29-gauge needle. After centrifugation to remove the nuclei and cell debris, the supernatant was layered on 30% sucrose and centrifuged at 130,000 x g for 2 h. The polyribosome pellet was then washed once with the lysis buffer without detergent and then lysed in TRIzol® (Sigma, St. Louis, MO) for RNA extraction. RT-qPCR for sema7A was performed as described in the supplemental information (Real-time PCR). Ribosomes and polyribosomes were quantified by the 18S RT-qPCR using the TaqMan human 18S rRNA endogenous control primers (reference sequence: X03205.1) and hydrolysis probe (VICR/MGB probe, Life Technologies).

2.7 EOS adhesion assay

EOS adhesion was assessed using the EOS peroxidase (EPO) assay as previously described [23]. A 96-well Immulon™ plate was coated overnight with 100 μl of plexin C1 (R&D Systems) or VCAM-7d both at 10 μg/ml in bicarbonate buffer (pH 9.6) and then wells were aspirated and blocked with 0.1% gelatin in HBSS for 1.5 hours at 37°C. After an overnight culture of the EOS at 37°C with IL-3 or IL-5 (2 ng/ml) or in complete medium only, EOS were suspended (1×10^5 cells/ml) in HBSS containing 0.2% BSA and 100 μl per well was added to the precoated plates. EOS were incubated at 37°C in a CO_2 incubator for 60 minutes and then the plate was washed three times with 200 μL of HBSS (pH of 8.0) to remove non-adherent cells. One hundred μl of the original EOS suspension were used as a measure of total EPO activity (maximum absorbance at 490 nm). 100 μl of substrate reagent (1mM o-phenylenediamine in 55 mM Tris buffer at pH 8 containing 1mM H_2O_2 and 0.1% Triton X100) was then added to the wells. After a 30 min incubation at room temperature, 50 μl of 4 M H_2S0_4 was added to stop the reaction. Absorbance was measured at 490 nm in a plate reader (Biotec, Winoski, VT, USA). Adherence was calculated as follows: % adherence = [(experimental absorbance at 490 nm/ max absorbance at 490 nm) x 100]. Each condition was performed in quadruplicate.

2.8 Expression of α-smooth muscle actin (α-SMA) by primary human bronchial fibroblasts

The preparation of primary fibroblasts has been previously described [24]. Bronchial fibroblasts were propagated in FBM (Clonetics, Walkersville, MD) supplemented with 10% FCS. Recombinant human sema7A (R&D Systems, Minneapolis, MN USA) was coated in PBS onto a 48-well plate (0.7 μg per well). Trypsinized fibroblasts were seeded on sema7A for 7 h in medium containing 10 % FCS before a 3.5 day starvation using DMEM F12, 0.1% FBS. Anti-α-SMA and anti-tubulin antibodies (Sigma, St Louis, MO USA) were used for western-blot analysis performed as previously described [25].

2.9 Statistical analyses

To compare expression of genes in total BAL cells and purified BAL EOS by RT-qPCR, data were analyzed using a Student's paired t-test. In the *in vitro* studies of purified EOS, a one way ANOVA or Student's paired *t* test was used to compare levels of sema7A mRNA, geometric means of flow cytometric analyses and α-SMA production. Log transformation was performed if data were not normally distributed. Statistical analyses were performed using SigmaPlot 11.0 software package.

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3. RESULTS

3.1 Airway EOS express sema7A mRNA

Sema7A was assessed by RT-qPCR of total unfractionated BAL cells before and 48 h after SBP-Ag and BAL EOS purified 48 h after SBP-Ag (Table 1). Sema7A mRNA level increased 163-fold in BAL cells after SBP-Ag compared to BAL cells obtained from the same subjects at baseline (before allergen challenge). The main cell population (76 % of the total) recruited into the airway 48 h after SBP-Ag was EOS (Table 1). Sema7A mRNA level was ~10 fold higher in purified BAL EOS compared to unfractionated BAL cells (Table 1). Therefore, the elevated levels of sema7A mRNA in BAL cells after antigen challenge are likely attributable to infiltrating EOS.

3.2 Airway EOS display high levels of sema7A on their membrane

Cell-surface sema7A was determined by flow cytometric analysis of gated EOS in whole blood and unfractionated BAL. Blood and BAL EOS expressed sema7A (Fig. 1A, left and central panels). The fluorescence intensity on BAL EOS (geometric mean fluorescence intensity ± SE of 62 ± 10, Fig. 1A, right panel) was significantly greater than that of blood EOS either before (D0, 38 ± 10) or after challenge (D2, 42 ± 5).

Unlike EOS that were uniformly positive, $CD4^+$ lymphocytes fell into distinct sema7A negative and sema7A positive subpopulations (Fig. 1B, left panel). The percentage of blood $CD4^+$ lymphocytes that expressed sema7A was 19 ± 9 (mean ± SD) before and 31 ± 10 after allergen challenge (p=0.08) (Figure 1B, right panel). In marked contrast to BAL EOS, the percentage of $sema7A^+$ $CD4^+$ BAL lymphocytes either before or after challenge (Fig. 1B, central and right panels) was low to undetectable.

3.3 Regulation of EOS Sema7A mRNA by IL-5 family cytokines

IL-5 family cytokines that signal through receptors comprising a specific α chain (IL-5Rα, GM-CSFRα, and IL-3Rα) and a common β chain are important for EOS survival and function. These cytokines are elevated in BAL fluid obtained after allergen challenge [26]. Therefore, we evaluated the effect of these cytokines on sema7A mRNA expression by blood EOS *in vitro*. All three cytokines equally induced sema7A mRNA expression compared to medium alone (Fig. 2A).

3.4 EOS cell surface sema7A protein is increased by IL-5 family cytokines

After 20 h of stimulation with IL-5 family cytokines, the expression of sema7A protein by blood EOS was significantly upregulated with IL-3>GM-CSF>IL-5 (Fig. 2BI and II). Fig. 2BIII and IV show that IL-3 induced sema7A/CD108 in a dose dependent manner. Kinetic studies demonstrated the presence of sema7A on the surface of blood EOS before culture (T0). IL-3 (2 ng/ml) increased the level of sema7A on the cell surface after 20 h incubation compared to T0 (Fig. S1A) while IL-5 had little effect compared to the level of sema7A observed at T0. Differential response to IL-3 vs IL-5 may reflect loss of IL-5Rα on IL-5-activated EOS (Fig. S1B) [27] and the increase in IL-3Rα on IL-3-activated EOS (Fig S1b) [28].

The biological relevance of IL-3 in allergic asthma is demonstrated by an increased amount of IL-3 in BAL fluid 48 h after SBP-Ag (84 pg/ml; Fig. 3). This amount is likely an underestimated of the concentration in bronchial epithelial lining fluid due to the dilution factor of the lavages [29].

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3.5 IL-3 induces EOS synthesis of sema7A protein

Fig. 4AI shows that inhibition of translation with cycloheximide (10^{-6} M) significantly attenuated (>80%) IL-3-induced increases of sema7A on the surface of EOS. Conversely, sema7A mRNA levels were increased (Fig. 4AII), consistent with a cessation of mRNA turnover due to the absence of translation.

In addition, Fig. 4B shows enhanced presence of sema7A mRNA with polyribosomes in IL-3- versus GM-CSF-activated EOS (Fig. 4BIV). However, we noted a similar increase in the amount of polyribosomes in IL-3-activated EOS compared to GM-CSF (Fig. 4BII). These results suggest that IL-3 may increase the general translation rate and presumably upregulates the production of additional proteins.

Confocal immunofluorescence microscopy was performed to corroborate further *de novo* synthesis of sema7A protein. Substantial increase of total sema7A protein (membrane and cytoplasmic) was appreciated after EOS stimulation with IL-3 (Fig. 4C).

3.6 IL-3-activated EOS are strongly adherent to plexin C1

To test the potential functionality of sema7A, we assessed the ability of IL-3-activated EOS to bind to plexin C1. Of note, sema7A is the only ligand known to bind plexin C1 [4, 30]. The adherence of IL-3-activated EOS to plate-bound plexin C1 compared to unactivated cells was doubled from ~30% to ~60% (Fig. 5), which indicates that the cytokine-induced sema7A protein expressed on the EOS cell surface is likely functional.

3.7 Sema7A increases production of α-SMA in primary human bronchial fibroblasts

Finally, we asked if sema7A could induce intracellular signaling into fibroblasts. To start answering this question and to avoid confounding variables due to lack of blocking antibodies against sema7A, human fibroblasts were cultured on recombinant sema7A rather than in co-culture with EOS. Consistent with productive signaling, α-SMA was elevated by sema7A (Fig. 6).

4. DISCUSSION

Our study localizes expression of sema7A mRNA to airway EOS in total BAL cells obtained from atopic subjects with mild asthma after an *in vivo* allergen challenge. Previous studies have implicated sema7A in immunity via its expression on T lymphocytes [5, 8]. In our model, we confirmed that $CD4^+$ blood lymphocytes express surface sema7A but airway T lymphocytes that are considered to have an activated phenotype, had little or no detectable sema7A on their cell surface. Thus, surface sema7A on human airway T lymphocytes is either lost during transit from blood into the airway lumen or the sema7A-expressing blood T lymphocytes are specifically not recruited into the airway. In contrast to $CD4^+$ T lymphocytes, membrane sema7A levels were increased on BAL compared to blood EOS. While other BAL cells, such as alveolar macrophages may express sema7A, our observation that the amount of sema7A mRNA in purified EOS was greater than that in the total cell population indicates that in our model of SBP-Ag, EOS are a predominant source of sema7A. Expression of sema7A on other hematopoietic BAL cells and lung parenchymal cells merit future investigations.

Little is known about regulation of sema7A at the protein and gene level. In an animal model, expression of sema7A in lung tissue was dependent on TGF-β1 [6], but the identification of the direct cell activators was not reported. Based on the observation that the spontaneous level of sema7A tended to decrease over time *in vitro*, the TGF-β1 present in the fetal bovine serum used in cell culture medium in our study does not appear to increase

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sema7A on EOS. At the transcriptional level, the hypoxia-inducible factor (HIF1A) increases sema7A mRNA through its hypoxia-response element (HRE) in endothelial cells [31]. We found here that *in vitro*, sema7A mRNA and protein are upregulated by the IL-5-family cytokines, particularly IL-3. IL-3-mediated augmentation of sema7A protein on the surface of EOS was preceded by mRNA accumulation. However, the incongruence between the relatively small increase of sema7A mRNA expression by all three IL-5 family cytokines and the great induction of membrane sema7A protein by IL-3 suggests translational regulation of sema7A protein.

Translational regulation of EOS sema7A was further implicated by the low level of sema7A protein and high level of sema7A mRNA in resting EOS. The use of a translational inhibitor (cycloheximide) and visualization of EOS sema7A by confocal microscopy demonstrated that IL-3 induced *de novo* synthesis of sema7A protein. Additional support for translational regulation was provided by the observation that IL-3 led to further recruitment of sema7A mRNA into the translational machinery (polyribosomes) compared to GM-CSF. Although specific enrichment of sema7A mRNA into the polyribosomes in IL-3- versus GM-CSF-activated cells was not observed, the presence of more polyribosomes in IL-3-activated cells suggested greater general translatability of mRNAs.

Upstream of the translational events, the differences in sema7A protein induction among IL-5, GM-CSF, and IL-3 could originate from differential regulation of their respective receptors. We, and others, have previously shown [27, 28] that IL-5 activation leads to a rapid loss of membrane IL-5Rα while GM-CSF induced no change in membrane GM-CSFRα over a period of 24 h. Conversely, as previously described [28], we confirm here that IL-3 strongly induces its own receptor, IL-3Rα. It is possible that EOS expression of GPI-coupled proteins such as sema7A, and as recently described by Munitz et al [32], CD48, are particularly responsive to IL-3 compared to IL-5 or GM-CSF.

The mediators responsible for the augmentation of sema7A *in vivo* on airway EOS after an allergen challenge are unknown. Possible candidates are IL-3 and/or GM-CSF. We have confirmed our earlier finding that airway allergen challenge leads to increased levels of IL-3 in BAL fluid [26]. In support for a role of IL-3 in asthma, serum IL-3 levels are higher in poorly controlled asthmatics [33]. Moreover, IL-3-positive cells are more abundant in BAL cells from subjects with asthma compared to control subjects, and their numbers escalate with asthma symptoms [34]. Finally, IL-3 levels are heightened in *ex vivo*-activated T lymphocytes from severe asthmatic subjects [35].

EOS expression of sema7A is yet another avenue by which EOS may drive fibroblasts toward a more profibrotic phenotype. We have showed here that fibroblasts in contact with sema7A produce more alpha-smooth muscle actin. However, a deeper analysis of EOS function on fibroblasts via sema7A would require co-cultures, and the use of blocking anti-sema7A or anti-plexin C1 antibodies, which are to date unavailable. Sema7A is considered a requirement for TGF-β1-induced fibrosis and accumulation of extracellular matrix proteins (ECM) in the lung [6, 36]. Importantly, EOS themselves have been reported to be the main source of TGF-β1 in the airways of asthmatic subjects [37], and both airway eosinophilia and TGF-β1 correlate with asthma severity [38]. In addition, the presence of EOS in the airway associates with deposition of ECM in both human and mouse models of allergen-induced airway inflammation models [16–18].

Finally, the interaction of EOS with plexin C1 and the potential interaction with β1 integrins by sema7A will bring new insight toward understanding EOS biology. We showed here that IL-3-activated EOS strongly bind to plexin C1. Through the sema7A/plexin C1 interaction, EOS may affect the function of other hematopoietic cell types. For instance, sema7A

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interaction with plexin C1 expressed on dendritic cells is known to impair their migration [30]. Also, sema7A should allow functional interaction of EOS with other β1 integrin-expressing cells such as epithelial cells, endothelial cells, or fibroblasts.

In conclusion, our study describes, for the first time, expression of sema7A on EOS. Furthermore, we demonstrate that IL-3 is a strong activator of sema7A protein production. *In vivo*, IL-3 is upregulated after an allergen challenge, and airway EOS display high levels of sema7A, a protein that is known to enhance pro-inflammatory cytokine production and fibrosis/remodeling in lung diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BAL	bronchoalveolar lavage
SBP-Ag	segmental bronchoprovocation with allergen
EOS	eosinophils
RT-qPCR	real-time quantitative PCR
sema7A	semaphorin 7A

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Highlights

- Semaphorin 7A is known to be critical for the induction of lung remodeling.
- We show that human airway eosinophils are a major carrier of semaphorin 7A.
- IL-3 is a strong inducer of *de novo* semaphorin 7A protein on blood eosinophils.
- Semaphorin 7A directly induces alpha-SMA in human bronchial fibroblasts.
- We propose that eosinophils can increase tissue remodeling via semaphorin 7A.

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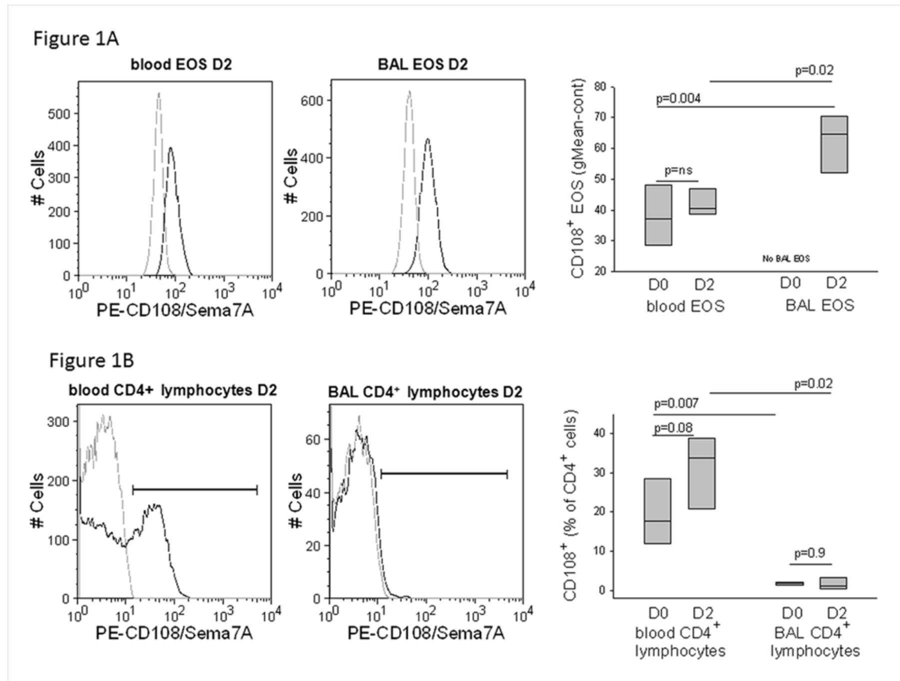

Figure 1.

Sema7A expression in blood and airway cells. A/ Representative flow cytometric histograms showing staining of Sema7A protein (solid black line) on EOS in blood or BAL fluid 48 h after a SBP-Ag (D2). The isotype control is indicated by the gray line. On the right panel, box plots are composite data from 4 subjects showing Sema7A surface staining intensity of blood and BAL EOS before (D0) and 48 h after (D2) SBP-Ag. BAL EOS were not analyzed before SBP-Ag due to their low percentage in BAL cells. B/ Representative flow cytometric histograms showing staining of Sema7A protein (solid black line) on CD4^{+} lymphocytes and EOS in blood or BAL fluid 48 h after a SBP-Ag (D2). The isotype control is indicated by the gray line. Box plots depict the median and the interquartile range between the 25th and 75th percentiles for 4 subjects. P values are indicated on the graphs.

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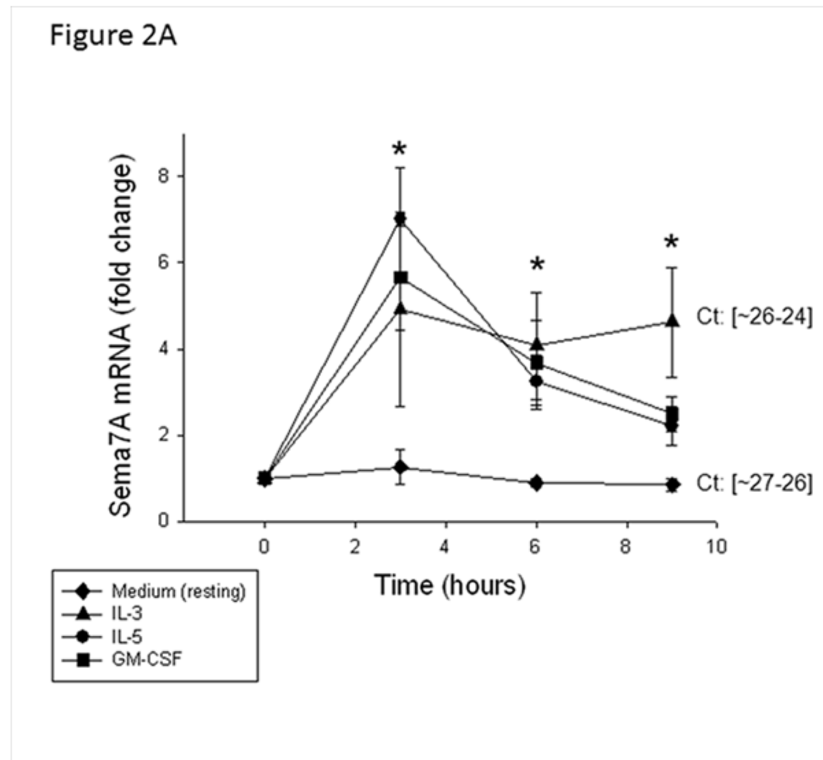

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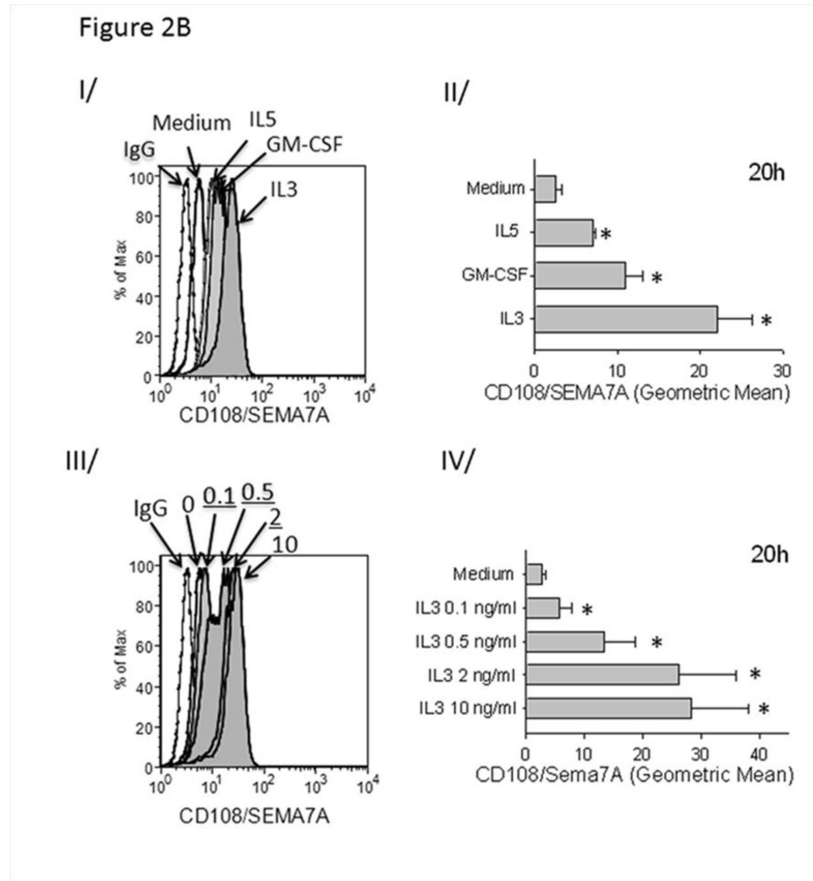

Figure 2.

Effect of IL-5 family cytokines on sema7A in EOS. A/ EOS were activated with IL-5, GM-CSF, or IL-3 (2 ng/ml) for the indicated times. Data are calculated as mRNA fold changes compared to before culture (Time = 0) are shown and represent the average ± SD of EOS from 3 different donors. * indicates that all three cytokines induced greater levels of sema7A mRNA compared to medium alone (resting). The range of cycle thresholds (Ct) for sema7A obtained with resting or IL-3-activated EOS at 9 h is shown and indicates resting EOS expressed abundant amount of sema7A (similar Ct as for the housekeeping transcript, GUSB). B/ Sema7A (CD108) was analyzed by flow cytometry; geometric means were calculated by subtracting values for isotype control. I/ and II/ EOS were incubated for 20 h with IL-5, GM-CSF or IL-3 (2 ng/ml). A representative histogram for sema7A is shown in panel I with the mean ± SD for EOS from 3 different donors depicted in panel II. III/ and IV/ EOS were activated for 20 h with increasing doses of IL-3 from 0.1 to 10 ng/ml as indicated. A representative histogram is shown in panel III with the mean ± SD for EOS from 4 different donors depicted in panel IV. *$p<0.05$ compared to resting EOS.

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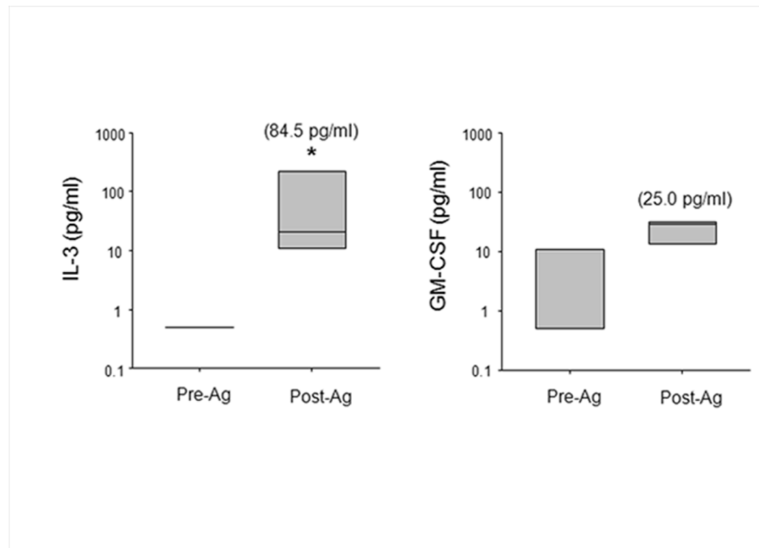

Figure 3.

Accumulation of IL-3 protein the BAL fluid 48 h after SBP-Ag. IL-3 and GM-CSF protein levels were measured by ELISA before (Pre-Ag) and after (Post-Ag) SBP-Ag. Box plots depict the median and the interquartile range between the 25th and 75th percentiles for 3 subjects. Means are presented in parentheses. *$p<0.05$ indicates statistical significant differences between groups.

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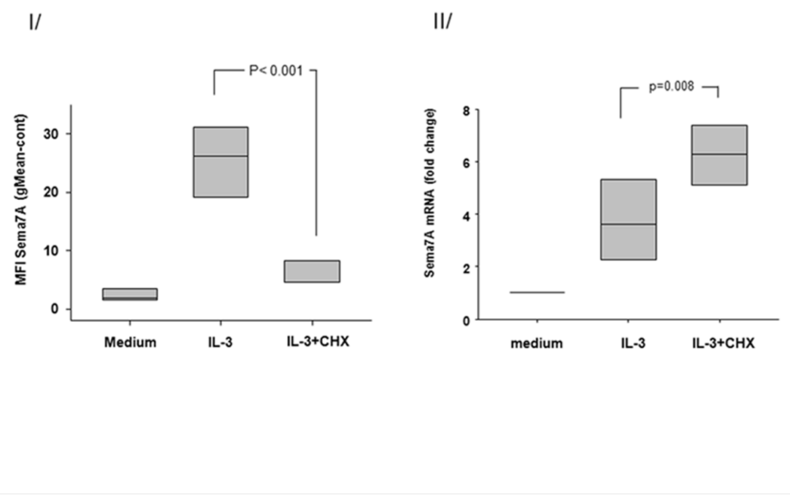

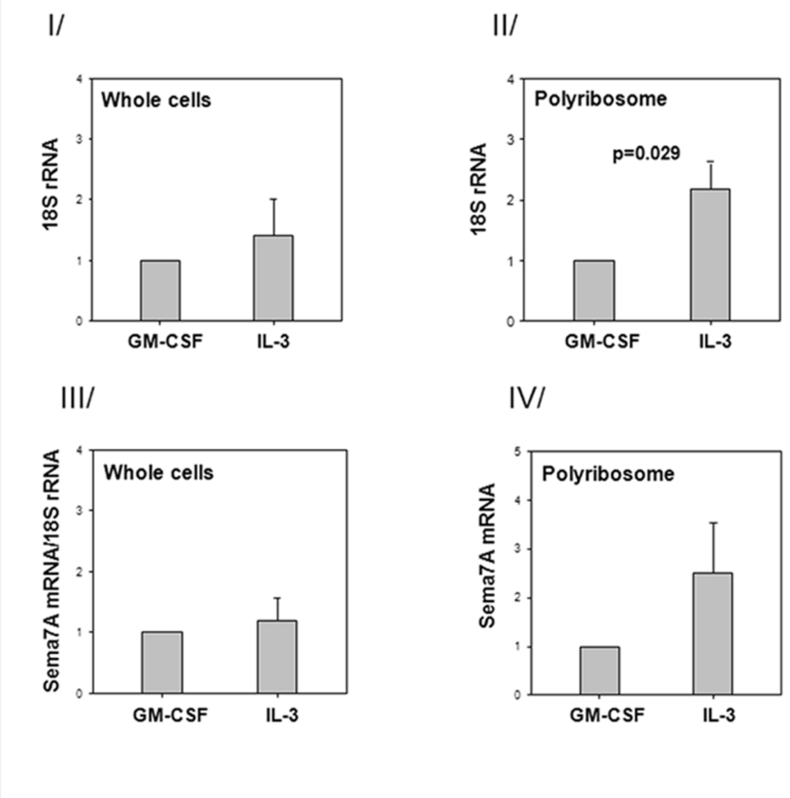

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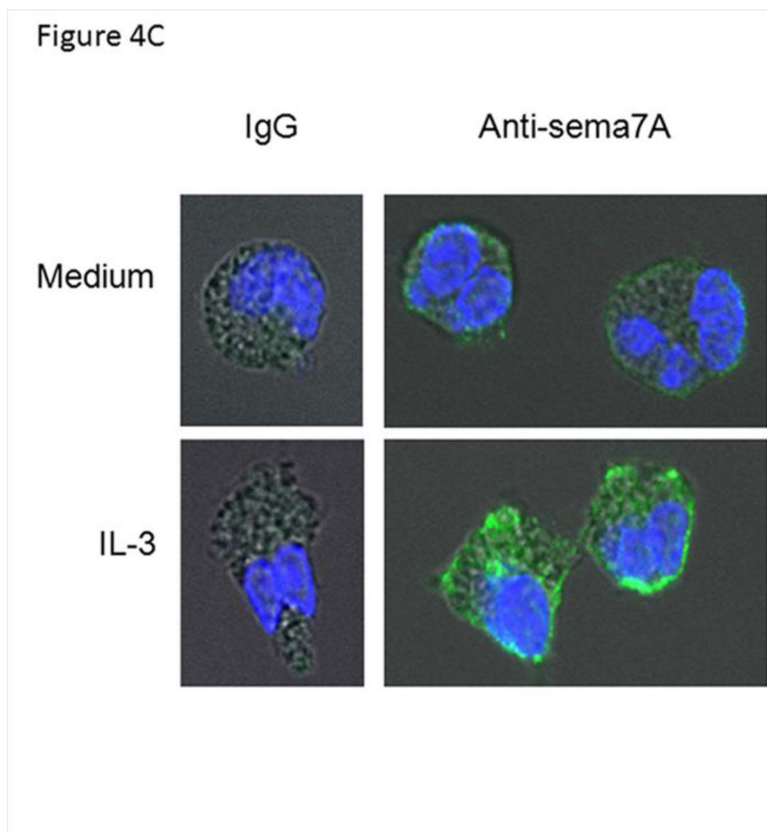

Figure 4.

IL-3 induces newly translated sema7A. A/ Effect of a protein translation inhibitor on sema7A cell surface protein of IL-3-activated EOS. EOS were treated simultaneously with IL-3 (2 ng/ml) and cyclohexamide (10^{-6} M). I/ mean fluorescence intensities (MFI) were determined by flow cytometry 20 h after culture of resting, IL-3-activated or IL-3-activated cells treated with cyclohexamide (IL-3+CHX). II/ at 4 h, sema7A mRNA levels were measured by RT-qPCR. Box plots depict the median and the interquartile range between the 25th and 75th percentiles for 5 subjects. $p<0.05$ indicates statistical significant differences between groups. B/ Amount of polyribosomes in IL-3- versus GM-CSF-stimulated EOS. EOS were cultured with IL-3 or GM-CSF (2 ng/ml) for 14 h. The amount of ribosome was determined by quantifying 18S rRNA using RT-qPCR. I/ Steady state level of 18S in whole cells indicate similar ribosome amounts in IL-3- and GM-CSF-activated cells. II/ RT-qPCR for 18S using the polyribosome preparations from IL-3- or GM-CSF-activated EOS. III/ Total sema7A mRNA in unfractionated IL-3- and GM-CSF-activated EOS normalized to total levels of 18S rRNA. IV/ RT-qPCR for sema7A using the polyribosome preparations from IL-3- or GM-CSF-activated EOS. Graphs shown are an average ± SE using EOS from 4 different donors, and $p<0.05$ indicates statistical significant differences. C/ Localization of Sema7A in IL-3-activated and resting EOS. EOS cultured in medium (resting) or treated with IL-3 (2 ng/ml) for 20 h, and were permeabilized and stained with an anti-sema7A antibody or its IgG control. Pictures using a confocal microscope show an increase of green fluorescence for the presence of sema7A protein in IL-3-activated EOS.

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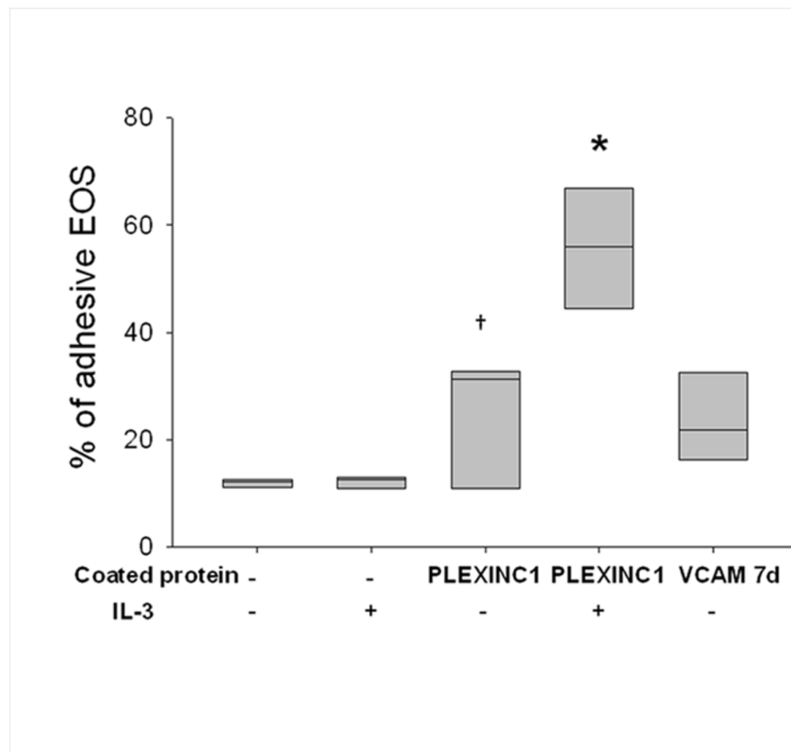

Figure 5.

Effect of IL-3 on EOS binding to plexin C1. EOS were pre-activated or not with IL-3 (2 ng/ml) for 20 h. EOS were then seeded on the indicated protein, plexin C1 or VCAM 7d (positive control), coated at 10 μg/ml and blocked with gelatin. Percentage of EOS bound to coated protein was determined with the EPO assay. *indicates % adhesive EOS is higher than either EOS on gelatin alone after treatment with IL-3, or untreated cells on plexin C1 ($P<0.05$, one way analysis of variance, n=3). †indicates adhesion of untreated EOS to plexin C1 is higher than adhesion to gelatin ($P<0.05$, paired t test, n=3).

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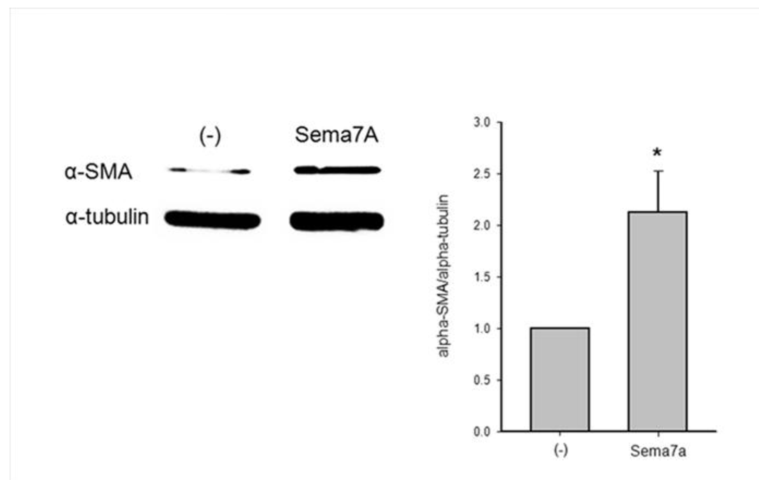

Figure 6.
Effect of recombinant human sema7A on α-SMA production in human bronchial fibroblasts. Fibroblasts were seeded on coated sema7A or plastic (−) for 7 h in medium containing 10 % FCS and for 3.5 days in starvation medium. Cellular α-SMA was quantified by western-blot. Tubulin was used as a loading control. A representative western-blot is shown on the left panel. The ratios sema7A/tubulin were calculated and the right panel shows mean ± SD for 3 experiments (*P<0.05, *t* test, n=3).

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Table 1

Eosinophils are a major source of sema7A mRNA in BAL cells after an allergen challenge*

	Total cells × 10^6	Mono/Mac (%)	Neut (%)	Lymph (%)	Eos (%)	Sema7A mRNA
Unfractionated BAL cells before SBP-Ag	11±2	92.5±2.9	0.8±0.4	6.3±2.6	0.5±0.1	1
Unfractionated BAL cells after SBP-Ag	238±43	16.3±5.1	1.9±0.8	6.4±3.7	75.7±3.7	163±84†
Purified BAL EOS after SBP-Ag	not applicable	<1	<1	<1	>99	1617±865‡

*BAL cells were obtained before and 48 h after SBP-Ag. Total cell counts and differential cell counts for monocytes (Mono), macrophages (Macs), neutrophils (Neuts), and EOS are expressed as mean ±SD. Sema7A mRNA was analyzed by qPCR in unfractionated BAL cells and purified BAL EOS. Sema7A mRNA was normalized to the housekeeping gene (GUSB) and fold change was calculated compared to sema7A mRNA level in unfractioned BAL cells before SBP-Ag. Sema7A mRNA fold change was log transformed and a student's *t* test was performed. Data are expressed as mean ± SE,

†indicates statistical difference (p<0.05) compared to BAL cells before SBP-Ag, and

‡indicates difference (p<0.05) compared to total BAL cells after SBP-Ag, n=4.