Supplemental Materials and Methods :

Reagents:

Human granulocyte/macrophage colony-stimulating factor (GM-CSF) was purchased from Novartis Pharma GmbH (Nürnberg, Germany). Mouse GM-CSF and human IL-5 and goat anti-human/mouse polyclonal anti-SP-D antibody were purchased from R&D Systems (Abingdon, UK). Human and mouse C5a were from Hycult Biotech (Uden, The Netherlands). German glass coverslips (#1 thickness, 12 mm diameter) were obtained from Karl Hecht GmbH & Co. KG "Assistent", Sondheim/Rhön, Germany. Black, glass-bottom 96-well plates were from Greiner Bio-One GmbH (Frickenhausen, Germany). DNase I was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Quant-iT^{TM}PicoGreen$^{®}$dsDNA Assay Kit, MitoSOX Red, propidium iodide (PI), Prolong Gold mounting media and Hoechst 33342 and anti-His-tag antibody (clone HIS.H8) and Alexa 488 Goat anti-mouse secondary antibody were from ThermoFisher Scientific (distributed by LuBioScience GmbH, Lucerne, Switzerland). X-VIVO™ 15 medium and Medium 199, containing L-glutamine, HEPES and 1.4 g/L $NaHCO_3$, were from Lonza (MD, USA). Polyvalent human IgG was a kind gift from CSL Behring (Bern, Switzerland). Normal goat serum was from Santa Cruz Biotechnology, Inc. (Heidelberg, Germany). ChromPure human IgG was obtained from Milan Analytica AG (Rheinfelden, Switzerland).

Mice: To increase number of eosinophils in functional *in vitro* assays, we used IL5^{tg} mice (C57BL/6J-Tg(Il5)1638Jlee) (kindly provided by Dr. J.J. Lee). C57BL/6 (wild type, WT) and SP-D$^{-/-}$ mice, bred in our in-house colonies (originally from Jackson Laboratories and the laboratory of Dr. Samuel Hawgood). O_3 and allergen exposure experiments were performed in Balb/c and C57BL/6 mice.

Peripheral blood donors and purification of human eosinophils: Written, informed consent was obtained from all blood donors. The Ethics Committee of the Canton of Bern approved this study. For eosinophil isolation, white blood cells were isolated from Lithium-heparinized blood of healthy donors by Ficoll (Biocoll, Biochrom AG) density gradient centrifugation and magnetic beads as previously described [1]. Blood was diluted with PBS, layered onto Ficoll and the peripheral blood mononuclear cell (PBMC) were separated from granulocytes/erythrocytes by centrifugation at 800 x g for 20 min. Erythrocytes were lysed with lysis solution (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.3) to obtain granulocytes. Eosinophils were isolated with kit from StemCell Technologies. Purity of eosinophils was assessed by Diff-Quik staining and light microscopy and was > 97%.

Purification of mouse eosinophils: Mouse eosinophils were isolated from *IL5^{tg}* mice, aged between 6 and 8 weeks. For eosinophils isolation, bone marrow cells were collected by flushing

the femurs and tibia with medium (2% FCS in PBS), using a 26-gauge needle, and filtering through a sterile 70-µM nylon cell strainer. Erythrocytes were lysed for 5 min by adding 3 ml of lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, pH 7.3). T cells, B cells, macrophages and neutrophils were depleted with antibodies against CD8α, CD19, CD90.2 and Ly-6G (Miltenyi Biotec, Bergisch Gladbach, Germany), using an EasySep Mouse PE Positive Selection Kit (Stemcell Technologies). Mouse eosinophil purity was higher than 95% as assessed by staining with the Hematocolor Set (Merck Millipore) followed by light microscopic analysis [1].

Combined O_3 and allergen exposure in mice was studied as we previously described [2-4]: Balb/c mice were sensitized and challenged with *Aspergillus fumigatus* (*Af*). Unless otherwise indicated, four days later mice were exposed to O_3 (3.0 ppm for 2 h). 12 hours post O_3 exposure, lung function (Flexivent) measurements were performed. Airways hyperresponsiveness was assessed to increasing concentrations of methacholine nebulized into the airways. Bronchoalveolar lavage (BAL) was collected (to assess inflammatory cells and for SP-D measurements) and lung tissue was preserved in RNA later. The BAL cell and supernatant fractions were assessed for cellular and molecular inflammatory changes, respectively. Differential cell count was performed on stained cytospins (Siemens Diff Quik Stain, Thermo), under light microscopy (x1000, oil) and counting of total live cells (Countess).

Lung function measurements

Airway hyperresponsiveness to aerosolized acetyl-β-methylcholine chloride (methacholine) inhalation was assessed by using the flexiVent system (SCIREQ, Montreal, Quebec, Canada), as described previously[4, 5]. Briefly, lung mechanics were studied in tracheostomized mice after achievement of anesthesia by means of intraperitoneal injection of ketamine and xylazine. Mice were ventilated with a tidal volume of 8 mL/kg at a rate of 150 breaths/min and a positive end-expiratory pressure of 2cm H_2O by using a computerized flexiVent setup. After mechanical ventilation for 2 minutes, a sinusoidal 1-Hz oscillation was applied to the tracheal tube. The single-compartment model was fitted to these parameters by using multiple linear regression to calculate dynamic resistance, compliance, and tissue damping of the airway. Baseline measurements and responses to aerosolized saline were followed by measurements of responses to increasing doses of 0.625 to 25 mg/mL of aerosolized methacholine (Sigma-Aldrich, St Louis, Mo). Recorded values were averaged for each dose and used to obtain dose-response curves for each mouse.

Total BAL SP-D was measured by sandwich ELISA using our in-house generated monoclonal and polyclonal antibodies. SP-D structural changes were assessed by native gel electrophoresis on the BAL cell-free lysate as previously described [6]. Optical Density was semi-quantified by ImageJ analysis.

Lung gene expression studies: To study mRNA expression in the mouse lung total RNA was extracted and processed for transcriptomic profiling by an Affymetrix Genechip assay (Affymetrix Inc). Data were standardized to GAPDH and expressed as arbitrary units (% change from wild type control).

SNO-SP-D was generated with slight modifications of the technique used by Guo et al. [7]. Recombinant mouse and human SP-D (10µg/ml) was incubated with 200 nM of GSNO (Sigma) for 30 min in dark at RT, and excess GNOS was removed by Microcon column centrifuge (MW= 30 K cut off, centrifugation was done at RT, 13'000 g, 12 min, trying not to dry the filter). Recombinant SNO-SP-D was washed 4X with PBS, adjusted to the original volume (in order to have approximately 10 µg/ ml concentration), and aliquoted and frozen until it was used. Western blot was used to assess recovery of recombinant native and GSNO-treated SP-D. 250 ng of recombinant SP-D and SNO-SP-D in presence of DTT were analyzed for SP-D protein.

Biotin-Switch Assay for Detection of SNO-SP-D was performed as described elsewhere [7]. Briefly the biotin switch method by Jaffrey et al. [8] was used. In mouse BAL or culture supernatant nitrosylated cysteines were converted to biotinylated cysteines in a biotin switch assay using biotin-HPDP. The biotinylated peptides were pulled down with Streptavidin-agarose beads. BAL (30 µg total protein) in HEN buffer (25 mM Hepes, pH 7.7/0.1 mM EDTA/0.01 mM neocuproine) and 20 µM N-ethylmaleimide (NEM) at 37°C for 30 min to block free thiols. Excess NEM was removed by protein precipitation using cold acetone. Protein pellets were resuspended in HENS buffer (HEN 1% SDS), SNO bonds were decomposed by adding 20 mM sodium ascorbate. The newly formed thiols were then linked with the sulphydryl-specific biotinylating reagent N-[6-biotinamido)-hexyl]-1-(2-pyridyldithio) propionamide (Pierce). Biotinylated proteins were precipitated with Streptavidin agarose beads and Western blot analysis was performed to detect the amount of SP-D remaining in the samples.

Western blotting: Protein expression was analyzed by Western blotting. Protein concentrations of recombinant mouse SP-D and oxidized SP-D (SNO-SP-D) were determined by BCA protein assay (ThermoFisher Scientific) and 250 ng of recombinant SP-D and SNO-SP-D in presence of DTT were analyzed for SP-D protein. Immunoblotting for SP-D was performed using an equal protein 5 µg of BAL fluid per lane. Results are representative of at least three independent experiments. Native protein samples or denatured in reducing Laemmle buffer prior were separated by 10% SDS/PAGE gel electrophoresis. Proteins were blotted on PVDF transfer membrane (Merck Millipore, MA, USA) and subsequently probed with goat anti-mouse SP-D antibody (1:2000; R&D Systems, catalogue number # Cat# AF1920). Secondary antibody coupled to horseradish peroxidase donkey anti-goat (1:10,000; GE Healthcare Life Sciences, distributed by VWR International GmbH, Dietikon, Switzerland) and signals detected by

enhanced chemiluminescence (ECL Western blotting substrate, ThermoFisher Scientific) on photosensitive film (ECL Hyperfilm, GE Healthcare Life Sciences).

S-nitrosylation of recombinant SP-D with NO obtained by iNOS mRNA translation in HEK cells *in vitro:* HEK 293 cells were transfected with iNOS mRNA. After 4 hrs, 3μg recombinant SP-D was added. After overnight incubation, cell culture supernatants were processed for biotin switch assay.

In-gel digestion and mass spectrometry of recombinant mouse SP-D Bands were extracted from BAL run on SDS-PAGE gels, digested with trypsin and analyzed using Nano LC/MS/MS with Sequest and Scaffold software. In additional experiments recombinant SP-D (3μg) was treated with air or O_3 (3.0 ppm for 30 min), denatured, reduced and alkylated before in-solution or in-gel trypsin digestion with or without PNGase F de-glycosylation and analyzed using TSQ Vantage and LTQ Orbitrap [9, 10].

Cell activation and confocal laser scanning microscopy: Isolated eosinophils were resuspended in X-VIVO™ 15 medium (2.5 x 10^6 /ml) and pre-incubated for 30 min in presence or absence of recombinant SP-D (10 μg/ ml). For neutralization experiments, recombinant SP-D (10 μg/ ml) was pre-incubated with either glucose (10 mM) or goat anti-SP-D antibody (4 μg/ ml) for 30 min at 37°C before addition to the cells. Cells were primed with 25 ng/ml mouse GM-CSF or 50 ng/ml human IL-5 for 20 min on untreated glass coverslips, which had previously been washed with acetone, ethanol, ddH_2O, and baked in a high temperature (200°C) oven for 1 h. Cells were subsequently stimulated with 10^{-8} M C5a. Cells were then fixed with 4% paraformaldehyde for 5 min, subsequently washed three times in PBS (pH 7.4), stained with 1 μg/ ml Hoechst 33342 and mounted in ProLong Gold mounting medium. Extracellular DNA was stained with MitoSox Red (5 μM), in live cells prior to fixation.

SP-D binding to the cell surface was analyzed by indirect immunofluorescence as previously described [11]. Briefly, mouse eosinophils pre-incubated with either mouse SP-D, or mouse recombinant oxidized SP-D (SNO-SP-D), both His-Tagged (5 μg/ml), and also in absence of SP-D for 30 min at 37°C in eppendorf tubes. Cells were washed 3x to remove excess unbound SP-D and seeded on glass coverslip for 15 min at 37°C to gently adhere. Cells were then fixed with 4% paraformaldehyde without permeabilization, and bound-SP-D or SNO-SP-D were detected by monoclonal anti-His-Tag antibody 1:100 (clone HIS.H8, ThermoFisher Scientific) by incubation over-night at 4°C, and after several washes, labeled with Alexa Fluor® 488 conjugated goat anti-mouse secondary antibody 1:400 (ThermoFisher Scientific). Arrows indicated mouse eosinophils having rec-SP-D or rec-SNO-SP-D attached to their surfaces. Slides were examined and images acquired by LSM 700 (Carl Zeiss Micro Imaging, Jena, Germany) using 63x /1.40 Oil DIC objective and followed by analysis with Imaris software (Bitplane AG, Zurich, Switzerland) as previously reported [11].

Quantification of released dsDNA in culture supernatants: Released dsDNA was quantified as previously described [11]. Briefly, 2 x 10^6 isolated eosinophils in 500µl of medium (X-VIVO™ 15) were stimulated as described above. At the end of the incubation time, a low concentration of DNase I (2.5 U/ ml; Worthington) and proteinase K (0.2 mg/ ml; Roche) was added for an additional 10 min, at the same time. Reactions were stopped by addition of 2.5 mM EDTA, pH 8.0. Cells were centrifuged at 500 x *g* for 5 min. 100 µl supernatant was transferred to black, glass-bottom 96-well plates (Greiner Bio-One GmbH) and the fluorescent activity of PicoGreen dye bound to dsDNA was excited at 502 nm and the fluorescence emission intensity was measured at 523 nm using a spectrofluorimeter (SpectraMax M2, Molecular Devices, Biberach an der Riß, Germany), according to the instructions described in the Quant-iT™PicoGreen® Assay Kit.

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