Ceramide 1-phosphate inhibits cigarette smoke-induced airway inflammation

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ONLINE DATA SUPPLEMENT

MATERIALS AND METHODS

Mouse model of cigarette smoke induced acute lung inflammation

According to the protocol of a three-day acute smoke model, male C57BL/6 mice (6-8 weeks old) received an intraperitonial (i.p.) injection of ketamine (10 mg/kg; CP-Pharma Handelsgesellschaft mbH, Burgdorf, Germany) and xylazin (150 mg/kg; Bayer, Leverkusen, Germany). Then, they were treated intratracheally (i.t.) with the indicated concentration of C1P or vehicle in a volume of 80 µl. After 30 minutes, mice were exposed to smoke (5 cigarettes) for 20 min each day as previously described [1]. In addition, to assess the effect of C1P on ongoing smoke-induced lung inflammation, mice were exposed to CS for either 3 or 6 days. After 3 days, at days

4-6 C1P or vehicle was instilled i.t. prior to smoke treatment or room air exposure. Mice were sacrificed 1 h after the last challenge.

Mouse model of cigarette smoke induced chronic lung inflammation and emphysema

We followed a previously described protocol [1]. Briefly, during a period of 7 months mice were exposed for 5 days per week for 12 min to the smoke of three cigarettes or to air. In the last 3 months, C1P or vehicle was administered per os, 30 min prior to smoke exposure. At the end of the protocol, bronchoalveolar lavage fluid (BALF) was collected and inflammatory cells were counted. Lungs were used for histological analysis, nSMase activity assay, and gene expression analysis.

Morphometrical assessment of murine lung emphysema

Lungs were fixed under 25 cm formalin distending pressure and embedded in paraffin. Lung sections (2-4 μm) were stained with haematoxylin and eosin (HE). The mean linear intercept (Lm) and internal surface area of the lung (ISA) were calculated. For the determination of Lm, for each pair of lungs 40 histological fields were evaluated both vertically and horizontally. ISA was calculated from the volume and Lm of the lung (for detailed protocol see Reference 1).

Immunohistochemistry for determination of inducible bronchus associated lymphoid tissue (iBALT)

Sections obtained from Formalin-fixed, paraffin-embedded lung lobes were subjected to the following immunohistological staining sequences: blocking reagent, primary antibody and detection with Vectastain Elite Goat IgG, Vectastain Elite Rabbit IgG (Vector Laboratories) or biotinylated goat anti-hamster IgG (H+L) (Vector Laboratories) and diaminobenzidine substrate (DakoCytomation). Sections were

counterstained with hematoxylin. For detection of lymphoid aggregates we stained with biotin armenian hamster anti-mouse CD3ε antibody (BioLegend) and rabbit anti-mouse CD20 (Pierce Biotechnology). Additional B cell-attracting chemokine CXCL13 in pulmonary lymphoid follicles was stained with goat anti-mouse CXCL13 (R&D Systems) and for his C-X-C chemokine receptor type 5 (CXCR5) on mature B cells we used rabbit anti-mouse CXCR5 as primary antibody.

Bronchoalveolar lavage fluid (BALF) and Flow cytometry

Total BAL cell numbers were counted and differential cell counts were assessed by flow cytometry (FacsCalibur BD Bioscience; San Diego, CA, USA) and analyzed (FlowJo version 10; TreeStar Inc.; Ashland, OR, USA) as previously described [2]. For FACS staining, anti-mouse CD11c APC (macrophages, dendritic cells); anti-mouse Ly-6G (Gr-1) FITC (neutrophils); anti-mouse F4/80 antigen PE (macrophages); anti-mouse CD3e, and anti-mouse CD45R (B220) PE-Cyanine7 (lymphocytes) were used. All antibodies were purchased from eBioscience (Frankfurt, Germany).

BALF cytokine concentrations were measured by ELISA (R&D, Minneapolis, USA) according to the manufacturer's recommendations.

Subjects

Twenty-nine adults with COPD, fifteen control never smoking and eight control ex-smoking subjects participated in this cross-sectional study. Patients were enrolled at the outpatient clinic during regular visits and control subjects were recruited among the medical or research staff at the Department Pulmonology, University Hospital Freiburg, Germany. Subject characteristics are shown in Table S1.

Patients had a diagnosis of COPD as previously established by a respiratory specialist [3] and were in stable clinical condition as judged by the physician at the outpatient clinic. Inclusion criteria for patients were current dyspnoea, symptoms of chronic cough or sputum production, and post bronchodilator FEV_1/FVC <0.70 without reversibility (FEV_1 increase <200 ml and <12%) as measured previously. Exclusion criteria for patients were the diagnosis of an acute airway or lung disease; current systemic infection; fever, use of antibiotics for any reason 4 weeks prior to the study; COPD exacerbation or systemic corticosteroid use in the past 4 weeks; current cancer or pulmonary malignancy in the past; and other chronic pulmonary disease or airway allergies.

Control subjects were either never smokers or ex-smokers (stopped smoking >6 months ago) with ≥10 pack years. All control subjects showed spirometric values in the normal range without past bronchial reversibility (if applicable). Exclusion criteria were symptoms of upper or lower airway infections, fever or antibiotic use for any reason in the past 4 weeks; current cancer or pulmonary malignancy in the past; any chronic pulmonary disease or current acute pulmonary disease; current systemic infection; and airway allergy in the past.

The protocol was approved by the local medical ethics committee of Freiburg and all subjects gave a written informed consent. The study was executed in accordance with the principles embodied in the Declaration of Helsinki.

Examinations and measurements

Forty millilitres of anti-coagulated peripheral venous blood was drawn from all subjects for the isolation of granulocytes. Cells were seeded into 24-well plates ($1x10^6$/well) in RPMI1640 culture and treated with C1P or vehicle 1 hour prior to 5% CSE for 18 hours. Supernatants were collected for cytokine measurements.

Nine millilitres of serum was additionally taken from a subgroup of subjects (control non-smoker: n = 6, control ex-smoker: n = 5, COPD: n = 11) and stored at -20°C for later measurement of neutral sphingomyelinase activity.

Furthermore blood was collected from arterialized capillaries for the measurement of blood gas parameters from patients. Spirometry was performed for control subjects, and patients underwent plethysmography measurements according to current guidelines [4, 5].

Stimulation of A549 and BEAS-2B cells with C1P and CSE

In vitro experiments were performed in human lung adenocarcinoma type-II alveolar epithelial cells A549 (DSMZ - German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany) and human bronchial epithelial BEAS-2B cells (purchased from Department of Environmental Health Science, Freiburg, Germany). They were cultured at 37ºC with 5% CO_2 in humidified atmosphere in RPMI 1640 supplemented with 10% FBS and 5% penicillin and streptomycin (P/S).

5 x 10^5 cells/well were seeded into 24-well plates and left overnight to reach confluence. Then, the medium was changed and cells were stimulated with 5% CSE and/or C1P. After 6 h, the supernatant was collected. Cell viability was assessed by light microscopy and trypan blue exclusion. For measurement of NF-κB activation,

2 x 10^6 cells per well were grown until confluence and then were starved for at least

4 h. Cells were pre-incubated with C1P for 1 h and then stimulated with 5% CSE for 10 min. For EMSA analysis, cells were washed with cold PBS and proteins were extracted with RIPA buffer.

Production of reactive oxygen species (ROS)

10^5 cells of A549 and Beas-2B were seeded into 96-well plates. After they reached confluence, cells were incubated with the indicated C1P concentration for 30 minutes. Then, 20% of CSE, PMA 200 ng/ml and Lucigenin (2mg/ml) was added.

After 10 minutes incubation chemiluminescence (CL) was monitored for 30-40 min in a Tecan infinite M200 (Männedorf, Switzerland) luminometer with temperature control set at 37°C to determine ROS signals.

Measurement of NF-κB DNA binding by the electrophoretic mobility shift assay (EMSA)

Lung tissue or cells were lysed with RIPA buffer, containing PMSF, sodium orthovanadate, phosphatase inhibitor cocktail B, and protease inhibitor cocktail (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The protein content was quantified by the Quick Start Bradford Protein Assay (BioRad GmbH, Munich, Germany). NF-κB DNA-binding was measured using EMSA as previously described [6].

Sphingomyelinase fluorometric assay

Neutral SMase activity was measured in murine lung tissue and in human sera with the SMase fluorometric assay kit (Cayman Chemical, Ann Arbor, MI, USA). The assay detection limit is 3.6 nmol/hour/ml as established by the manufacturer. Samples with activity enzyme activity below the detection limit were assigned a value correspond to half of the detection limit.

RNA isolation, cDNA synthesis, and qPCR

Total RNA was isolated with Qiazol (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Complementary DNA synthesis was carried out using the First Strand cDNA synthesis kit (Thermo-Fisher Scientific). QPCR was performed with absolute qPCR SYBR Green mix (Thermo-Fisher Scientific) on a Lightcycler 480 (Roche, Mannheim, Germany). *ß$_2$-MICROGLOBULIN (ß$_2$-m)* and *GAPDH* were used

as reference genes. For all reactions the annealing temperature was 60°C. Primer design and relative quantifications were done as previously described [7]; primer sequences are shown in Table S2 primer specification.

References

1. Cicko S, Lucattelli M, Muller T, Lommatzsch M, De Cunto G, Cardini S, Sundas W, Grimm M, Zeiser R, Durk T, Zissel G, Boeynaems JM, Sorichter S, Ferrari D, Di Virgilio F, Virchow JC, Lungarella G, Idzko M. Purinergic receptor inhibition prevents the development of smoke-induced lung injury and emphysema. J Immunol 2010;185:688-697.

2. Lucattelli M, Cicko S, Muller T, Lommatzsch M, De Cunto G, Cardini S, Sundas W, Grimm M, Zeiser R, Durk T, Zissel G, Sorichter S, Ferrari D, Di Virgilio F, Virchow JC, Lungarella G, Idzko M. P2X7 receptor signaling in the pathogenesis of smoke-induced lung inflammation and emphysema. Am J Respir Cell Mol Biol 2011;44:423-429.

3. Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA, Calverley P, Fukuchi Y, Jenkins C, Rodriguez-Roisin R, van Weel C, Zielinski J. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. Am J Respir Crit Care Med 2007;176:532-555.

4. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R, Johnson DC, MacIntyre N, McKay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G, Wanger J. Standardisation of spirometry. Eur Respir J 2005;26:319-338.

5. Wanger J, Clausen JL, Coates A, Pedersen OF, Brusasco V, Burgos F, Casaburi R, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Hankinson J, Jensen R, Johnson D, Macintyre N, McKay R, Miller MR, Navajas D, Pellegrino R, Viegi G. Standardisation of the measurement of lung volumes. Eur Respir J 2005;26:511-522.

6. Seidel P, Merfort I, Tamm M, Roth M. Inhibition of NF-kappaB and AP-1 by dimethylfumarate correlates with down-regulated IL-6 secretion and proliferation in human lung fibroblasts. Swiss Med Wkly 2010;140:w13132.

7. Jeong HJ, Chung HS, Lee BR, Kim SJ, Yoo SJ, Hong SH, Kim HM. Expression of proinflammatory cytokines via HIF-1alpha and NF-kappaB activation on desferrioxamine-stimulated HMC-1 cells. Biochem Biophys Res Commun 2003;306:805-811.

Supplementary tables

Table S1 Characteristics of subjects

	Control subjects		COPD patients according to GOLD stages			
	Non-smoker	**Ex-smoker**	**I**	**II**	**III**	**IV**
Number of subjects	15	8	6	8	6	9
Age, years	30 (5)	45 (12)	66 (6)	65 (13)	63 (11)	63 (6)
Male/Female, n	9/6	4/4	4/2	7/1	3/3	4/5
BMI, kg/m^2	22.8 (2.7)	26.9 (8.5)	27.1 (6.2)	28.9 (5.0)	26.3 (5.6)	21.8 (2.8)
Current/Ex-smoker	0/0	0/8	2/4	3/5	0/6	0/9
Pack-years	NA	18 (7)	49 (25)	48 (15)	39 (11)	40 (19)
ICS use, yes/no	NA	NA	0/6	4/4	5/1	8/1
ICS, budesonide equivalent, mcg	NA	NA	NA	890 (754)	1520 (438)	1240 (505)
LABA, yes/no	NA	NA	3/3	7/1	5/1	9/0
LAMA, yes/no	NA	NA	2/4	5/3	3/3	5/4
Roflumilast, yes/no	NA	NA	0/6	1/7	1/5	0/9
Theophylline, yes/no	NA	NA	0/6	0/8	0/6	3/6
pH	NA	NA	7.41 (0.02)	7.44 (0.04)	7.41 (0.02)	7.42 (0.03)
$PaCO_2$, mm Hg	NA	NA	38.6 (5.1)	37.6 (4.4)	40.3 (2.9)	41.6 (7.0)
PaO_2, mm Hg	NA	NA	65.4 (8.9)	70.0 (7.6)	67.3 (5.0)	63.9 (10.2)
SaO_2, %	NA	NA	95 (2)	95 (1)	95 (1)	94 (1)
FVC, % predicted	110 (10)	103 (18)	109 (16)	84 (14)	74 (15)	60 (26)
FEV_1, % predicted	107 (13)	106 (14)	83 (5)	60 (10)	42 (8)	27 (3)
FEV_1/FVC	0.82 (0.06)	0.84 (0.06)	0.65 (0.05)	0.60 (0.05)	0.51 (0.12)	0.42 (0.08)
RV, % predicted	NA	NA	142 (8)	146 (41)	189 (44)	203 (105)
RV/TLC, %	NA	NA	43 (3)	47 (8)	55 (5)	62 (11)

Data are presented as mean (standard deviation). Post-bronchodilator lung function values (recorded 15 minutes after the inhalation of 400mcg of salbutamol) are shown. NA, not applicable; BMI, body mass index; ICS, inhaled corticosteroids; LABA; long-acting beta2-agonist; LAMA, long-acting muscarinergic antagonist; pCO_2, partial pressure of arterial carbon dioxide; pO_2, partial pressure of arterial oxygen; SaO_2, saturation of oxygen; FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 second; RV, residual volume; TLC, total lung capacity.

Table S2 primer specification

Gene	Primer sequences	size of the amplicon	reference gene
m-NF-kB1	Forward: CCTGCTTCTGGAGGGTGATG Reverse:GTATGTCAAATACCTGCCAGTTGG	254bp	*GAPDH*
h-NF-kB1	Forward: GGGACGGTCTGAATGCCAT Reverse: GATCTGCTCCTGCTGCTTTG	288bp	*GAPDH*
h-SMPD2	Forward:TGCTGGAGGAGGTGTGGAGTG Reverse:TTCGGCATGGAGATGGGTCACA	284bp	*ß$_{2}$-MICROGLOBULIN*
m-Mmp-9	Forward: CGTGTCTGGAGATTCGACTTG Reverse: AGGCTTTGTCTTGGTACTGGAA	124bp	*GAPDH*
m-Myd88	Forward: AGCAGCAGAACCAGGAGTCC Reverse:TTCTGTTGGACACCTGGAGACA	394bp	*GAPDH*
m-Traf2	Forward:TGTTCAGAGATGGTGGAGACTGA Reverse:CTGTTGCACCTTGTTACTCAGGG	303bp	*GAPDH*
m-CXCL13	Forward: TCTCCAGGCCACGGTATTCTG Reverse:GACAGACTTTTGCTTTGGACATGT	239bp	*GAPDH*
m-MMP-12	Forward:ACCTAGCAGTCCACCATCAACT Reverse:GGGATAGTGTGGCTCTGGTACT	277bp	*GAPDH*
m- ß$_{2}$-m	Forward:GCTATCCAGAAAACCCCTCAAA Reverse: CGTTCTTCAGCATTTGGATTTC	130bp	
h- ß$_{2}$-m	Forward:CTATCCAGCGTACTCCAAAGAT Reverse:CATGTCTCGATCCCACTTAACTA	299bp	
m- GAPDH	Forward:CCGTAGACAAAATGGTGAAGGT Reverse: GTCAATGAAGGGGTCGTTGAT	122bp	
h- GAPDH	Forward: GAGTCAACGGATTTGGTCGTA Reverse:TTGGAACATGTAAACCATGTAGTTG	125bp	

SUPPLEMENTARY FIGURES

Figure S1. mRNA expression of NF-κB target genes in chronic cigarette smoke-induced lung inflammation

C57BL/6 mice were exposed to air or cigarette smoke for 7 months. After 4 months, mice were treated orally for 3 months with C1P (10µM) or vehicle. Directly after the last CS challenge the animals were sacrificed. QPCR was performed for matrix metalloproteinase 9 (*Mmp-9*) (a), Myeloid differentiation primary response gene 88 (*Myd88*) (b) and TNF receptor-associated factor 2 (*Traf2*) (c). *GAPDH* was used as reference gene. Data are presented as mean ± SEM. n= 4-7 mice in each group. $^{\#}P < 0.01$ Smoke/Vehicle versus Air/Vehicle. *P < 0.05, **P < 0.01, ***P < 0.001, Smoke/C1P versus Smoke//Vehicle.

Figure S2. Effect of C1P on CSE-induced ROS release of HAEC on CL.

10^5 cells of A549 (a,c) and Beas-2B (b,d) were seeded into 96-well plates. After they reached confluence, cells were incubated with the indicated C8-C1P (a,b) and C16-C1P (c,d) concentration for 30 minutes. Then, 20% of CSE, PMA 200 ng/ml and Lucigenin (2mg/ml) was added. After 10 minutes incubation chemiluminescence (CL) was monitored for 30-40 min in a Tecan luminometer with temperature control set at 37°C to determine ROS signals. The data are representative of results from 3 independent experiments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#}P < 0.001$ vehicle/CSE versus vehicle. *P <0.05, **P <0.01, ***P <0.001, C1P/CSE versus vehicle/CSE.

Figure S3. Correlation between human serum nSMase levels and post-bronchodilator FEV_1 percent predicted

We analysed nSMase activity in the serum of patients with COPD (n=22), control ex-smoker (n=8) and control non-smoker (n=11). Negative correlation between increased nSMase activity in the serum of patients with COPD (a) and COPD

patients compared to corresponding controls (b), which were negatively correlated to FEV_1% predicted, is shown.