ESTABLISHING AND CHARACTERISING RODENT MODELS OF PULMONARY AND GASTROINTESTINAL INFLAMMATION FOR THE INVESTIGATION OF SENSORY-IMMUNE INTERACTIONS

Doctoral (PhD) Thesis

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1 INTRODUCTION, BACKGROUND

1.1 Transient receptor potential Vanilloid 1 (TPV1) and Ankyrin 1 (TRPA1) and their role in neurogenic inflammation

Transient receptor potential (TRP) ion channels comprise 28 structurally related ion channels, divided into the TRPC (Canonical), the TRPV (Vanilloid), the TRPM (Melastatin), the TRPP (Polycystin), the TRPML (Mucolipin), the TRPA (Ankyrin) and the TRPN (NOMPC) subfamilies based on their sequence homology (1). Most of them are non-selective cation channels, however, they exhibit differences in permeability and selectivity. These ion channels are tetramers composed of six transmembrane domains, with a pore formed by the hydrophobic region between the fifth and sixth segments. They can assemble as homo- or heterotetramers to form functional units (2).

The most investigated members of the family in relation to gastrointestinal and airway inflammation include vanilloid 1 (TRPV1), ankyrin 1 (TRPA1). They are located predominantly on the capsaicin-sensitive sensory neurons, and often co-expressed mainly on these sensory fibers, but several non-neural expressions have recently been described that drew great attention to this research area (3). In general, they are activated by a variety of exogenous chemicals and endogenous mediators making them important regulatory structures in inflammatory and pain processes.

TRPV1 and TRPA1 are polymodal nociceptors playing an important role in thermo- mechanical- and chemo-sensation and play a complex role in hyperalgesia and neurogenic inflammation (4). Upon their activation on the capsaicin-sensitive afferents pro-inflammatory sensory neuropeptides, e.g., substance P (SP) and calcitonin gene-related peptide (CGRP) are released leading to neurogenic inflammation (vasodilatation, plasma protein extravasation and inflammatory cell activation) (4,5). Simultaneously with these pro-inflammatory mediators, anti-inflammatory neuropeptides, most importantly somatostatin and pituitary adenylate cyclase activating polypeptide (PACAP) are also released from the same terminals, counteracting the inflammatory process and tissue damage both locally and systemically (6,7).

The endogenous activators of TRPV1 and TRPA1 are often produced during inflammation, e.g., lipoxygenase products, reactive oxygen species (ROS), bradykinin, prostanoids and the acidified pH of the inflamed tissue (8). The gastrointestinal mucosa is frequently exposed to the exogenous agonists of TRPV1, such as capsaicin and piperine (the pungent agent of chili pepper and black pepper, respectively), as well as activators of TRPA1, such as cinnamaldehyde, allyl isothiocyanate

(mustard oil), allicin (garlic), menthol etc. ingested by food (8,9). The airways on the other hand can be exposed to various TRPA1 agonists, such as bacterial endotoxin and environmental irritants like acrolein, crotonaldehyde, isocyanates and nicotine found in cigarette smoke, wood smoke, diesel exhaust and tear gas (10).

TRPV1 and TRPA1 are capable of functional interaction, such as heterologous desensitization, since the majority of TRPA1 expressing nerve fibers co-express TRPV1 (11). Both ion channels can be sensitized by a variety of other mechanisms, such as prostaglandins, bradykinin and proteases (12).

TRPV1 is also present on several non-neuronal structures in the gastrointestinal tract, such as the gastrin-secreting parietal and gastric epithelial cells, as well as the oesophageal, small intestinal and colonic epithelial cells (13,14). TRPA1 is less extensively studied in the gastrointestinal tract, but its expression was described in isolated crypts and epithelial cells of the colon, as well as small intestinal neuroendocrine cells (14,15). In the airways, TRPA1 is expressed on fibroblasts, tracheal, bronchial and alveolar epithelial cells, bronchial smooth muscle cells, as well as lymphocytes (16). Moreover, both receptors were reported on CD4+ T cells emphasizing their role in sensory-immune interactions (17,18).

Therefore, these ion channels were identified as novel anti-inflammatory, analgesic and gastroprotective targets (10).

1.2 The role of TRPA1 in the respiratory system

Due to its polymodal chemosensor function and its wide expression pattern, TRPA1 has been addressed as having a key role in physiological and pathophysiological processes, particularly in neuro-immune interactions (10). It is suggested to be a particularly important chemical sensor in the respiratory system, playing a role in physiological (protective reflexes, cough and sneeze) and pathophysiological responses (inflammation, bronchial hyperreactivity) (8). Although increasing evidence suggests TRPA1 involvement in the pathogenesis of chronic obstructive pulmonary disease (COPD), asthma, chronic cough, cystic fibrosis etc., pointing to the important therapeutic potential of TRPA1 in the pharmacological treatment of chronic pulmonary diseases (16,19), there are few *in vivo* data concerning its function in airway inflammation. Therefore, the results are far from being conclusive and more information is needed to determine the significance of TRPA1 as a possible pharmacological target in inflammatory lung disease, pneumonitis and COPD.

1.3 Chronic obstructive pulmonary disease (COPD)

COPD is a major global health problem characterized by persistent respiratory symptoms and airflow limitation due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases. It is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung. Functional respiratory disorders result from chronic obstructive bronchiolitis narrowing the small airways and emphysema due to lung parenchymal destruction. Cigarette smoking is the most common cause of COPD accounting for approximately 95% of cases besides air pollutants and occupational exposure (20).

There is no curative treatment, the available therapy is restricted to corticosteroids, adrenergic β2 receptor agonists and acetylcholine muscarinic receptor antagonists that can only slow down the progression and alleviate the symptoms (20). Therefore, there is an urgent need to find novel therapeutic targets in COPD.

Due to the extensive interest in this area of research, our knowledge of the underlying mechanisms has remarkably expanded. Cigarette smoke and other airway irritants induce an abnormal inflammatory response involving CD8+ lymphocytes, neutrophils and macrophages. These immune cells release chemotactic factors, colony stimulating factors and proinflammatory cytokines, thus sustain and enhance inflammation and immune cell recruitment. Furthermore, proteases like neutrophil elastase, cathepsins and matrix metalloproteinases (MMPs) are responsible for elastin destruction resulting in emphysema formation (21). However, the complex pathophysiological mechanism, the inflammatory cascades and the role of the immune cells, sensory nerves and neuro-immune interactions, as well as the key mediators need to be determined to identify potential novel therapeutic targets.

1.4 Animal models of chronic airway inflammation

Besides human studies to analyse tissue samples, translational animal models are particularly important to define the pathophysiological processes underlying the molecular pathways.

Several studies focus on the protease-antiprotease imbalance and use only short-lasting models of various types of elastases, such as pancreatic elastase, neutrophil elastase, proteinase-3 (22), or lipopolysaccharides (LPS) and inorganic dusts to investigate their role in the development of emphysema. These models have been proved to be useful, however, they focus only on one factor

that is an intermediate player of the pathophysiological cascade. Meanwhile, cigarette smoke (CS), which is the most common initial triggering stimulus in the human disease, switches on a variety of other pathways and mechanisms that are upstream mediators (23). To investigate the potential involvement of TRPA1 and the whole complexity of the chronic persistent inflammatory process, the only authentic translational model for COPD is the chronic cigarette smoke exposure (CSE) (22). This model has been used by several groups so far, but their broad conclusive potential is limited by the facts that they 1) applied different protocols, experimental paradigms, exposure durations and intensities, 2) did not have a longitudinal self-control follow-up design, 3) did not aim to use an integrative methodological approach to investigate the complexity of the disease, only focused on certain specific parameters and 4) used different strains. Since C57Bl/6 mice are the most widely used one for genetic manipulations, and it is very sensitive to cigarette smoke, our goal was to set up, characterise and optimise a translational model in this strain.

1.5 Expression of TRPV1 and TRPA1 in the gastrointestinal tract

In the gastrointestinal tract the expression and role of TRPV1 and TRPA1 were most extensively studied in animal models of colitis. The density of TRPV1 positive fibers increase from proximal to distal regions of the colon in mice (24). Furthermore, during dextrane sulfate sodium (DSS) colitis the proportion of DRG neurons expressing TRPV1, and their relative *Trpv1* mRNA levels increase with a subsequently elevated release of sensory neuropeptides, such as CGRP and SP (25). There is growing evidence on the expression of TRPV1 and TRPA1 in intrinsic sensory neurons of the myenteric and submucosal plexuses, as well as on the surface epithelial cells of colonic mucosa (14,15). The importance of sensory-immune interactions in colonic inflammation is also supported by the expression of TRPV1 and TRPA1 on inflammatory cells like mucosal macrophages, as well as CD4+ T cells (14,17,18).

In the stomach the gastroprotective effect of capsaicin-sensitive peptidergic sensory neurons innervating the gastric mucosa has long been investigated by our group (26). However, in contrast to TRPV1, little is known about the expression changes and role of TRPA1 in the stomach (27), therefore, we aimed to elucidate the potential role of TRPA1 in an animal model of gastric injury.

1.6 Animal models of gastric mucosal injury

Gastric mucosal injury can be exhibited by various forms of macroscopic and histopathological alterations, such as diffuse hyperemia, inflammation, erosion, or even hemorrhagic ulcerations.

The gold standard treatment is often limited to acid secretion inhibitors, such as proton pump inhibitors or histamine H2 receptor antagonists, since enhancing cytoprotective mechanisms is challenging (28).

Animal models are important for the molecular investigation of gastric injury, since these models may reveal very early biochemical and molecular alterations, much before microscopic or macroscopic lesions can be seen. Good models should have translational relevance. However, in virtually all animal models of gastric injury (e.g., NSAID-, stress-induced) the lesions are well circumscribed (i.e., superficial erosions and/or deep ulcers). Gastritis in humans, on the other hand, is a diffuse inflammatory damage involving all or most parts of the stomach (29).

Iodoacetamide (IAA) is a water-soluble sulfhydryl alkylating chemical, which, by depleting sulfhydryl groups, including the protective antioxidant glutathione (GSH) in the gastric mucosa, allows ROS production and oxidative tissue damage (30). The reduced GSH plays an essential role in maintaining mucosal integrity (31). ROS react with various cell components including cell membrane, mitochondria and DNA, potentially leading to cell death/necrosis, which triggers neutrophil recruitment (32).

2 PRIMARY AIMS

The primary aims of my work was to establish and characterise rodent models of pulmonary and gastrointestinal inflammation for the investigation of sensory-immune interactions, as well as TRPA1 expressions and potential roles.

1) **Establishing and characterising mouse model of chronic airway inflammation**

There is no effective curative treatment for COPD, the complex pathophysiological mechanisms, inflammatory cascades and the role of the immune cells, therefore, sensory nerves and neuro-immune interactions, as well as the key mediators need to be determined to identify novel therapeutic interventions. Cigarette smoke-triggered inflammatory cascades and consequent tissue damage are the main causes of COPD, therefore, chronic CSE is the only translationally relevant animal model. We aimed to establish a translational mouse model for complex functional,

morphological, immunological, and biochemical investigation of chronic pulmonary pathophysiological changes characteristic to COPD. This helps to analyse the mechanisms in different stages of the disease and identify key targets for pharmacological research.

2) **Investigating the role of TRPA1 in CSE-induced COPD mouse model**

Although increasing evidence suggests TRPA1 involvement in the pathogenesis of COPD, asthma, chronic cough, cystic fibrosis pointing to the important therapeutic potential of TRPA1 in the pharmacological treatment of chronic pulmonary diseases, there are few and conflicting *in vivo* data concerning its function in airway inflammation. We investigated the potential role of TRPA1 *in vivo* in a CSE-induced COPD mouse model using *Trpa1* gene-deficient mice in comparison with their wildtypes.

3) **Investigating the expression of TRPA1 and TRPV1 in iodoacetamide (IAA)-induced diffuse gastric injury model**

Although the gastroprotective effect of capsaicin-sensitive peptidergic sensory neurons innervating the gastric mucosa has long been investigated, in contrast to TRPV1, little is known about the expression changes and role of TRPA1 in the stomach. Therefore, our aim was to characterise a translationally relevant gastritis model using the irreversible sulfhydryl-group blocker IAA and to investigate the expression changes with special focus on TRPA1.

3 EXPERIMENTAL MODELS AND INVESTIGATIONAL METHODS

3.1 CIGARETTE SMOKE-INDUCED CHRONIC AIRWAY INFLAMMATION MODEL

3.1.1 Animals and research ethics

Experiments were performed on 8-10-week-old C57Bl/6 mice, as well as on TRPA1 wildtype (*Trpa1*$^{+/+}$) and gene-deficient (*Trpa1*$^{-/-}$) counterparts weighing 20-25 g at the beginning of the study; each group consisted of 6 mice. Studies were approved and a license was given by the Ethics Committee on Animal Research of University of Pécs, Pécs, Hungary according to the Ethical Codex of Animal Experiments (licence No.: BA02/2000-5/2011, BA02/2000-35/2016).

3.1.2. Experimental design for the characterisation of cigarette smoke-induced COPD model

Male C57Bl/6 mice were exposed to cigarette smoke in a whole-body smoke exposure chamber twice daily, 10 times/week for 6 months. Age-matched non-smoking mice kept under the same circumstances served as controls. Airway responsiveness was determined by unrestrained whole-body plethysmography (WBP), while pulmonary structural changes were imaged by a microtomograph at the end of each month. Before the treatment period and at the end of each month 6 smoking and 6 intact animals were sacrificed after ketamine and xylazine anesthesia. Total cell count and the ratio of lymphocytes, monocytes and granulocytes of the bronchoalveolar lavage fluids (BALF) were analysed with flow cytometer. MMP-2/-9 activity measurement was performed by gelatin zymography at the end of the 1st and 6th months, while cytokine concentrations were analysed with the Mouse Cytokine Array Panel A from lung homogenates at the end of each month. One part of the lung tissue was placed in 6% formaldehyde solution for quantitative histological evaluation of emphysema and semiquantitative assessment of pulmonary inflammation. At the end of the 6th month restrained WBP was performed by invasive methodology in anaesthetised, tracheotomised and ventilated mice and blood samples were collected.

3.1.3 Experimental design for investigating the involvement of TRPA1 in the cigarette smoke-induced COPD mouse model

Based on the results of the 6-month-long CSE mouse model we investigated the potential involvement of TRPA1 in chronic airway inflammation in a 4-month-long experimental design focusing on the peak of airway inflammation. *Trpa1*$^{-/-}$ and their *Trpa1*$^{+/+}$ counterparts were exposed to CSE twice daily, 10 times/week for 4 months. Age-matched non-smoking mice kept under the same circumstances served as controls. Airway function was determined by unrestrained WBP at the end of each month, while pulmonary structural and morphological changes were imaged by a microtomograph and histopathological assessment after 2 and 4 months. BALF was analysed by flow cytometry at the end of the 2nd and 3rd months.

3.1.4 Cigarette smoke (CS)-induced chronic airway inflammation

Chronic bronchitis was elicited by whole body CS (3R4F Kentucky Research Cigarette; University of Kentucky, USA) in a two-port TE-2 whole body smoke exposure chamber (Teague Enterprise, USA) twice daily, 10 times/week for 6 or 4 months depending on the experimental paradigm.

3.1.5 Respiratory function measurement with unrestrained whole body plethysmography

Airway responsiveness was determined by unrestrained WBP with Buxco instrument (PLY3211, Buxco Europe Ltd., Winchester, UK) in conscious, spontaneously breathing animals in a follow-up, self-controlled manner. We determined the relaxation time (RT), breathing frequency (f), tidal volume (TV), minute ventilation (MV), inspiratory time (Ti), expiratory time (Te), peak inspiratory flow (PIF), peak expiratory flow (PEF) and Penh (enhanced pause) value correlating with airway reactivity (33).

3.1.6 Invasive respiratory function measurement with restrained WBP

At the end of the 6-month protocol C57Bl/6 mice were anaesthetised and restrained WBP was performed by invasive methodology. Mice were tracheotomised and placed in a whole body plethysmograph (PLY4111, Buxco Europe Ltd., Winchester, UK) for measuring invasive resistance and compliance. All respiratory parameters, such as airway resistance (Rl), end-expiratory work (EEW), tidal mid-expiratory flow (EF50), end-expiratory pause (EEP), Te and Ti characteristic of COPD/emphysema were measured every 10 seconds and averaged during a 10-minute baseline reading period.

3.1.7 *In vivo* micro-computed tomography (micro-CT) analysis for emphysema quantification

Structural changes of the lungs were imaged by breath-gated tomography on a Skyscan 1176 high resolution microtomograph (Skyscan, Kontich, Belgium) in a self-controlled manner. Mice were anaesthetised with i.p. pentobarbital (70 mg/kg) and placed in supine position on the bed of the scanner, a piece of paper was placed onto the chest with a high-contrast sign to enable the video gating of the breathing movements to eliminate motion artefacts. Emphysema was calculated by the ratio of low attenuation area (LAA, from -750 to -1000 Hounsfield unit, representing the air-filled regions) and total lung volume (TLV) and was expressed in percentage (34).

3.1.8 Bronchoalveolar lavage fluid (BALF)

Mice were anaesthetised with ketamine and xylazine (100 mg/kg and 5 mg/kg, s.c., respectively), lungs were flushed with cold PBS (5x1 mL) with the help of a trachea cannula and BALFs were collected. They were centrifuged at 1000 rpm for 5 minutes at room temperature, then stained and

fixed for flow cytometry. Cell preparations were analysed with CyFlow Space flow cytometer (Sysmex Partec, Münster, Germany). Total cell count and the ratio of lymphocytes, monocytes and granulocytes were calculated regarding their forward/side scatter (FSC/SSC) feature (35).

3.1.9 Histopathological evaluation of pulmonary inflammation and emphysema

Semiquantitative histopathological analysis of lung sections was performed to evaluate perivascular/peribronchial oedema, acute and chronic inflammation, interstitial acute and chronic inflammation, epithelial damage and goblet cells. The mean linear intercept (chord) length (L_m) was measured to evaluate the size of the acinar air space complex related to emphysema (36) by using the Case Viewer software (3DHISTECH Ltd., Budapest, Hungary).

3.1.10. Detection of MMP-2 and MMP-9 activities by gelatin zymography in the lung

Gelatin zymography was performed to measure the activities of the major isoforms of pulmonary MMP-2 (64 and 72 kDa) and MMP-9 (86 and 92 kDa) (37). Lung samples were incubated for 20 h and at 37°C in zymogram development buffer (Bio-Rad Laboratories, Hercules, CA). Gels were then stained with 0.05% Coomassie brilliant blue and destained in aqueous 4% methanol-8% acetic acid (vol/vol). Band intensities were quantified, expressed as the ratio to the internal standard, and presented in arbitrary units.

3.1.11 Cytokine profile analysis

Excised lung tissues were thawed, weighed, and immediately homogenised in PBS containing 1% protease inhibitor phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich, Budapest, Hungary), and centrifuged at 10,000 g for 5 min to remove cell debris. Triton X-100 was added to a final concentration of 1%. Blood samples from 6 months smoking and intact animals were left to clot in room temperature for 30 min, then centrifuged for 20 min at 2000g. Total protein content of the homogenates and serum samples were determined prior to cytokine measurement with BioRad DC protein assay kit. Forty different inflammatory cytokines were determined simultaneously with Mouse Cytokine Array Panel A (R&D Systems) according to the manufacturer's instruction. Cytokine heat map was generated by Matrix2png 1.2.1 online freeware.

3.1.12 Statistical analysis

Values for all measurements were expressed as the mean ±SEM of n=6 mice in each group except for restrained WBP (n=5 per group) and cytokine determination of lung homogenates (n=3 per group). Evaluation of the unrestrained WBP, and micro-CT results have been performed by repeated measures ANOVA followed by Bonferroni's modified t-test to see statistical differences between different data sets and then between the respective data points. Data collected from BALF flow cytometry and mean linear intercept length (80-100 measurements/slides) were analysed with two-way ANOVA followed by Sidak's multiple comparison test. Expression level of forty different cytokines was statistically compared to intact values with two-way ANOVA followed by Bonferroni's post-test. Restrained WBP results were analysed by Student's t-test for unpaired comparison. Evaluation of the semiquantitative histopathological scoring was analysed by Kruskal-Wallis followed by Dunn's multiple comparison test to observe intragroup differences by time, while Mann-Whitney test was performed to analyse intergroup differences at given time points.

3.2 CHRONIC GASTRITIS MODEL

3.2.1 Animals and ethics

Experiments were performed on 8-week-old male CD1 and C57Bl/6 mice weighing 18–25 g and Wistar rats weighing 180–220 g at the beginning of the study; each group consisted of 6 animals. All procedures were approved by the Ethics Committee on Animal Research of University of Pécs according to the Ethical Codex of Animal Experiments (license no.: BA02/2000-20/2019, June, 2019).

3.2.2 Iodoacetamide (IAA)-induced gastritis model

IAA-containing drinking water was prepared freshly every day by dissolving 0.1, 0.2, 0.4, 0.6 or 1 g IAA in 200 mL tap water (0.05%, 0.1%, 0.2%, 0.3% and 0.5% concentration, respectively) for 7 or 14 days consecutively, depending on the experimental paradigm. Fluid intake and body weight were measured daily in each study. At the end of the study, animals were euthanised under ketamine (120 mg/kg ip.; Calypsol, Gedeon Richter Plc., Budapest, Hungary) and xylazine (6 mg/kg ip.; Sedaxylan, Eurovet Animal Health B.V., Bladel, Netherlands) anesthesia. The stomach was excised, opened along the greater curvature and rinsed with room temperature saline. After photo documentation of the macroscopic lesions, the stomach was cut in four sections: antrum and

corpus specimens were fixed in 6% formaline and 5 μm sections were prepared for histopathologic and immunohistochemical evaluation. Other antral and corpus samples were snap-frozen for molecular biological assessments.

3.2.3 Experimental design for IAA-induced gastric injury model in rats

Rats were randomized into 8 groups of 6 animals in each, and received 0.05%, 0.1% or 0.2% IAA solution in the drinking water for 7 or 14 days consecutively. Littermates drinking IAA-free tap water served as control animals.

3.2.4 Experimental design for IAA-induced gastric injury model in mice

In the first mouse study, CD1 mice were randomized into 4 groups: mice receiving 0.1% IAA for 7 and 14 days, with the respective control groups. Based on the negative results of this study, CD1 mice were randomized in 3 groups receiving 0.3% and 0.5% IAA for 7 days; the control group drank tap water. To investigate interstrain differences, C57Bl/6 mice were randomized into 4 groups receiving 1) 0.3% IAA-containing drinking water, 2) 0.3% IAA-containing drinking water, which also contained 2% sucrose, 3) a control group drinking tap water, and 4) a second control group receiving 2% sucrose dissolved in tap water.

3.2.5 Semiquantitative macroscopic and qualitative histopathological evaluation of gastritis

The extent and severity of the macroscopic lesions were evaluated by a semiquantitative scoring system based on the extent of hyperemia and the number of erosions/ulcerations. Excised gastric samples were paraformaldehyde-fixed (4%) and embedded in paraffin, 5 μm sections were cut and stained with HE for further qualitative histological analysis.

3.2.6 Total glutathione concentration measurement

Total GSH (tGSH) and total γ-glutamyl-cystein (tGluCys) were measured in gastric mucosa specimens by a specific high power liquid chromatography (HPLC) method with RP 18 NUCLEOSHELL HPLC columns (Macherey-Nagel, Düren, Germany) after incubation with *N*-acetylcysteine ethyl ester.

3.2.7 TRPV1 and TRPA1 immunohistochemistry and scoring

For antigen recovery, the paraformaldehyde-fixed and paraffin-embedded tissue samples were deparaffinised, rehydrated and incubated in acidic citrate buffer (pH 6) in a microwave oven.

Endogenous peroxidase activity was quenched 3% hydrogen peroxide. The sections were washed and incubated in blocking solution, then treated with a 1:1000 dilution of rabbit polyclonal anti-TRPA1 (ab68848; Abcam, Cambridge, UK) and anti-TRPV1 (GP14100; Neuromics, Edina, MN, USA) antibodies. Slides were incubated with anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) (DakoCytomation, Carpinteria, CA, USA) with the EnVision system. The reaction was visualised by 0.01% hydrogen peroxide containing 3,3-diaminobenzidine tetrachloride, and histological counterstaining was performed with hematoxylin (38). Quantitative assessment of TRPA1 and TRPV1 immunopositivity was performed based on the % ratio of the immunopositive cells on 10 fields of vision/slide/animal by an expert pathologist blinded to the study.

3.2.8 *Trpv1* and *Trpa1* real-time qPCR analysis

Total RNA was purified by TRI Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) with Direct-Zol RNA MiniPrep isolation kit (Zymo Research, Irvine, CA) following the manufacturer's protocol. The quantity and purity of RNA samples were assessed by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and then treated with deoxyribonuclease I enzyme (Zymo Research, Irvine, CA, USA). Purified total RNA (100 ng) was reverse transcribed with Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Real-time qPCR was conducted on a Stratagene M×3000P qPCRSystem (Agilent Technologies, Santa Clara, CA, USA) using Luminaris HiGreen LowROX qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) to amplify transcripts of glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) (NM_017008.4) *Trpv1* (NM_031982.1), and *Trpa1* (NM_207608.1). The determination of relative mRNA expression levels was performed according to the comparative DDCt method using samples of control animals as calibrator.

3.2.9 Statistical analysis

Statistical analysis was performed by using GraphPad Prism v6 software. Values for all measurements are expressed as means ± SEM of n = 6 animals in each group. Evaluation of body weight change and fluid intake was performed by repeated-measures ANOVA followed by Bonferroni's modified t-test. Semiquantitative macroscopic scoring was analysed by the non-

parametric Kruskal–Wallis method followed by Dunn's multiple comparison test to observe intergroup differences at given timepoints, while Mann–Whitney test was performed to analyse intragroup differences by time. GSH measurements were analysed by one-way ANOVA, while TRPA1 and TRPV1 immunopositivities were evaluated by ordinary two-way ANOVA followed by Dunn's multiple comparisons test. qPCR measurements were evaluated by Student's unpaired t-test.

4 RESULTS

4.1 CHARACTERIZATION OF CS-INDUCED CHRONIC PULMONARY INFLAMMATION MODEL

4.1.1 Follow-up measurement of respiratory functions by unrestrained WBP

Pulmonary function parameters determined by this technique (RT, f, TV, MV, Ti, Te, PIF, PEF) were not different between the smoking and non-smoking groups at any time-points during the 6-month-period.

4.1.2 Invasive respiratory function parameters at the end of the 6-month-long protocol

At the end of the 6^{th} month, significant decrease of Rl, EEW, EEP, Te, as well as increase of EF50 and Ti/(Ti+Te) ratio were observed, whereas dynamic compliance did not change in response to chronic smoke exposure as compared to the non-smoking group.

4.1.3 Emphysema evaluation by micro-CT and mean linear intercept analysis

Emphysema formation was clearly observed on the reconstructed 3D images assessed by *in vivo* micro-CT. According to our morphometric analysis the LAA/TLV%, a quantitative indicator of emphysema showed significant increase by the end of the 5^{th} month that was further increased by the end of the 6^{th} month. Remarkable alveolar space enlargement (L_m) was observed on HE stained lung sections already after 1 month of CSE in comparison with the non-smoking group and progressively increased parallelly to our micro-CT findings. By the end of the 5^{th} month the distal air space was significantly expanded in smoking mice as compared to the L_m after the 1^{st} month.

4.1.4 Histopathological analysis

One month of CSE induced a minimal peribronchial and moderate perivascular oedema formation, and slightly increased numbers of granulocytes and macrophages in the lung parenchyma. After 2 months of smoking, there was an extensive perivascular and peribronchial oedema with large number of granulocytes, macrophages and lymphocytes infiltrating these regions. Inflammation was characteristic both to the interstitial and peribronchial areas, in addition, the bronchiolar epithelial cell layer became irregular, the bronchiolar and alveolar epithelium showed signs of damage, and the number of interepithelial mucus-producing cells was increased. The massive inflammatory reaction showed a decreasing tendency from the 3-month-timepoint, the peribronchial oedema was still present, but less extensive, the number of immune cells was reduced and were mostly lymphocytes, which moved from the peribronchial spaces to the interstitial regions. Meanwhile, the bronchiolar epithelial destruction was remarkably greater. At the 4-month CSE, the irregularity and damage of the bronchial epithelium was further aggravated. Tissue destruction became more severe, mild emphysema (enlargement of airspaces throughout the parenchyma) and atelectasis developed particularly on the peripheral regions. However, mild oedema was limited to the perivascular spaces and the number of inflammatory cells remarkably decreased. After 5-6 months emphysema dominated the histological picture, first mainly in the peripheral areas, then also in the central parts of the lung. Inflammatory reaction at this stage was minimal, only few macrophages and lymphocytes could be noticed in the remaining parenchyma, while irregularity of the bronchial epithelium and hyperplasia of the mucus producing cells could be observed.

4.1.5 Inflammatory cell count in the BALF

At the end of the 1st month flow cytometric analysis revealed no difference in the granulocyte, macrophage and lymphocyte numbers of BALF samples obtained from smoke-exposed and intact mice. In contrast, 2 months of CSE induced an enormous increase in the number of all these cells in the BALF, which gradually decreased afterwards. The total number and the composition of BALF cells did not differ from the values of the non-smoker mice from the 3rd month.

4.1.6 Chronic tobacco smoke increases MMP-2 and MMP-9 activities in the lung

Gelatine zymography showed a significant increase in pulmonary activity for MMP-2 as well as for MMP-9 in the lung samples of mice subjected to 6-month CSE as compared either to 1-month smokers or to non-smoker age-matched control mice.

4.1.7 Cytokine expressions in the lung and serum

Among the 40 investigated inflammatory cytokines and chemokines 26 proteins were detectable in lung homogenates throughout the 6-month experiment. At the end of the 1st month, interleukin-1β (IL-1β), IL-10 and monocyte chemoattractant protein-5 (MCP-5) increased significantly, but none of them were detectable later. The triggering receptor expressed on myeloid cells-1 (TREM-1) showed a peak expression at this time-point. The C5a complement component, interleukin-1 receptor antagonist (IL-1ra) produced by several immune cells and epithelial cells, interleukin-16 (IL-16), interferon-gamma inducible protein-10 (IP-10), keratinocyte chemoattractant (KC), macrophage colony-stimulating factor (M-CSF), monocyte chemoattractant protein-1 (MCP-1 or JE), monokine induced by gamma interferon (MIG), regulated on activation, normal T cell expressed and secreted (RANTES), and tissue inhibitor of metalloproteinase-1 (TIMP-1) cytokines and chemokines reached their maximum expression at the 2nd month. Meanwhile, the concentration of the soluble intercellular adhesion molecule-1 (sICAM-1) was high in the intact lung homogenate and remained at a similarly high level during the whole 6-month smoking period. In the serum of non-smoking mice B-lymphocyte chemoattractant (BLC), stromal cell-derived factor 1 (SDF-1), C5a, interleukin-1 alpha (IL-1α, IL-1ra, IL-16, JE, M-CSF, TIMP-1, TNF-α, and TREM-1 were detectable. The first two were not present in the intact lung, and they decreased by the end of the 6-month smoking period similarly to IL-16. KC remarkably, JE slightly increased by this time point, while the expression of the other cytokines remained unchanged in the serum.

4.2 INVESTIGATING THE ROLE OF TRPA1 IN CS-INDUCED CHRONIC AIRWAY INFLAMMATION

4.2.1 Comparison of basal airway function of *Trpa1* gene-deficient and wildtype mice

Under intact conditions, f, MV, PIF and PEF were significantly greater; TV, Ti, Te and RT were significantly smaller, while no difference was detected in the Penh value of *Trpa1*$^{-/-}$ mice compared to their wildtype counterparts, measured by unrestrained WBP**.**

4.2.2 Respiratory function alterations of *Trpa1*$^{-/-}$ and *Trpa1*$^{+/+}$ mice induced by 4 months of CSE

Respiratory functions were measured in a follow-up design before and at the end of each month in the 4-month long protocol of cigarette smoke exposure. CSE induced a gradual and significant decrease in TV, MV, PIF and PEF in *Trpa1*$^{+/+}$ mice with a peak at 3 months, which was not present in the *Trpa1*$^{-/-}$ animals. The significant differences in f, Ti, Te and RT measured in the *Trpa1*$^{-/-}$ mice were attributable to the significant differences between the wildtype and gene-deficient mice observed already in the intact animals, before CSE.

4.2.3 Histopathological analysis

After one month of CSE remarkable perivascular oedema developed that significantly decreased by time in both strains, and almost completely resolved at the end of the 4th month. However, the accumulation of perivascular and peribronchial, as well as interstitial neutrophil granulocytes, macrophages and lymphocytes remained moderately increased throughout the protocol. At the end of the 3rd month of CSE, structural destruction characteristic to emphysema already developed.

4.2.4 Emphysema evaluation by micro-CT and mean linear intercept analysis

Emphysema was quantified by *in vivo* micro-CT by the ratio of LAA and TLV before and after 2 and 4 months of CSE. The ratio correlating with the extent of air-filled regions did not show alterations either by CSE treatment or time. However, L_m measurement revealed that in *Trpa1*$^{+/+}$ mice emphysema already started to develop at an earlier timepoint compared to the gene-deleted counterparts; L_m was significantly increased in the wildtypes after 2 months of CSE, however, not in the *Trpa1*$^{-/-}$. At the end of the 4th month, L_m was elevated in both strains exposed to CS.

4.2.5 Inflammatory cell count in the BALF

CSE induced massive accumulations of granulocytes, macrophages and lymphocytes measured in the BALF of both *Trpa1*$^{+/+}$ and *Trpa1*$^{-/-}$ mice. In agreement with our previous findings (39), the number of inflammatory cells reduced by the end of the 3rd month of CSE. There was no biologically relevant difference between the wildtype and gene-deficient mice in either inflammatory cell components.

4.3 IAA-INDUCED CHRONIC GASTRITIS

4.3.1 Weight change and fluid consumption

In Wistar rats, IAA induced a concentration-dependent weight change. Similar to vehicle-treated animals, low concentration (0.05%) of IAA resulted in ~15% weight gain by the end of the 14-day experiment. Meanwhile, 0.1% and 0.2% IAA induced a concentration-dependent, gradual weight loss. The total water consumption of the 0.05% and the 0.1% IAA-treated groups was halved compared to the control group, and it was even more decreased in the case of 0.2% concentration.

4.3.2 Macroscopic evaluation of IAA-induced gastric lesions

Extensive hyperaemia, mucosal haemorrhage and several erosions or superficial ulcers were observed at both timepoints in all three examined concentrations. Semi-quantitative analysis showed significant hyperaemic areas and erosions in both 0.05% and 0.2% IAA-treated groups at day 7 compared to the controls. At day 14, lesions, especially the extent of the hyperaemic area in the 0.1% and 0.2% IAA-treated groups were significantly greater. Macroscopic changes showed no significant difference either by the increasing concentrations of ingested IAA or by time; however, ulcerations were more pronounced after 14 days of IAA-drinking.

4.3.3 Qualitative microscopic evaluation of gastric mucosa

Seven days of IAA treatment resulted in submucosal widening due to massive oedema. In higher concentrations, extensive inflammatory cell infiltration was also observed. After 14 days, focal epithelial cell sloughing/erosions, and in some areas, almost total mucosal necrosis involving the muscularis mucosae were seen, admixed with acute and chronic inflammatory cells, both in the mucosa and the submucosa.

4.3.4 Total glutathione concentration of rat gastric mucosa

There were no statistically significant differences in the GSH content of the rat gastric mucosa between the groups receiving 0.1% IAA for 14 days. IAA treatment did not increase tGSH remarkeably, whereas tGluCys showed significant increase.

4.3.5 Quantitative analysis of TRPA1 and TRPV1 immunohistochemistry

Mild TRPA1 and strong TRPV1 immunopositivity was detected on the epithelial cells in the intact control samples. The ratio of immunopositive cells revealed a significant upregulation of TRPA1 after both 0.05% and 0.2% IAA administration by day 14 in both the antrum and corpus. Although TRPV1 immunopositivity also increased in the corpus, but did not change in the antrum in the case of 0.05% IAA, it significantly decreased in both localizations after 0.2% IAA treatment.

4.3.6 *Trpa1* and *Trpv1* relative gene expression in the inflamed rat mucosa

In agreement with the TRPA1 protein expression, *Trpa1* mRNA was significantly upregulated in both 0.05% and 0.2% IAA-treated groups after 7 and 14 days as well, however, there was no detectable alteration in *Trpv1* relative gene expression either by time, dose, or localisation.

4.3.7 IAA-induced alterations in mice

In CD1 mice, we observed an IAA dose-dependent continuous, gradual weight loss; in the 0.3% and 0.5% IAA-treated groups, weight reduction was so severe at the end of the 7-day-long protocol, that a 14-day-long protocol could not be performed due to the ethical considerations of humane endpoints. Although water intake was significantly reduced in all IAA-treated groups, it showed no concentration-dependence, and could not explain the remarkable dose-dependent weight loss of these animals. C57Bl/6 mice proved to be more resistant to 0.3% IAA, which induced ~14% weight loss after 7 days, half as much as the same concentration in CD1 mice (~28%). Interestingly, adding 2% sucrose to 0.3% IAA significantly reduced both the fluid intake, as well as the weight (~21%) of C57Bl/6 mice compared to the 0.3% IAA-drinking group. Surprisingly, in contrast to Wistar rats, where we observed similar body weight change and decreased water intake, no macroscopic lesions or microscopic alterations were present in either of the mouse groups drinking IAA.

5 DISCUSSION

In the first part of our work we provided the first experimental evidence in a chronic mouse model that cigarette smoke induces characteristic pulmonary inflammation, emphysema and atelectasis. We proved with functional, morphological and immunological techniques that these well-defined pathophysiological alterations from the inflammatory reactions to the tissue destruction are

dependent on the duration of the smoke exposure and COPD-like structural and functional changes develop only after the fourth month.

Respiratory function determined by invasive WBP in anaesthetised, tracheotomised and mechanically ventilated mice showed a significant decrease in airway resistance, interestingly along with a decrease in the expiratory parameters, such as EF50 characteristic to bronchoconstriction, EEW, EEP and Te (40).

Inflammatory signs determined by the histopathological evaluation were clearly dependent on the duration of smoking. In the first two months peribronchial/perivascular oedema, neutrophil and macrophage infiltration were characteristic, from the third and fourth months macrophages and lymphocytes accumulated predominantly in the interstitial areas, and epithelial irregularity and hyperplasia developed. From the 5th month, the extent of inflammatory reaction decreased, and tissue destruction dominated as shown by remarkable development of emphysema and atelectasis. Vascular endothelial proliferation, destructed bronchi with desquamated epithelial cells, fibrosis and a loss of the alveolar structure were detected by the end of the 6-month experiment. The histologically observed peak of peribronchial inflammation at 2 months of smoking was strongly supported by the drastically elevated numbers of granulocytes, macrophages, and lymphocytes in the BALF. At later time-points cell counts in BALF were not changed, which is not surprising, since at this stage interstitial localisation of the inflammation (at month 3) and the destruction of the bronchial epithelium (from month 4) were observed on histology. The development of emphysema after 5-6 months of smoke exposure was also clearly detected by micro-CT in complete agreement with the histological picture. Therefore, one major message of our study is that duration of smoking strongly determines pathophysiological alterations that develop sequentially in the lung as a cascade from different types of inflammatory processes to tissue destruction. It is crucial to choose the correct experimental paradigm depending on which mechanisms and phase of the chronic disease model are aimed to be investigated (41).

Based on the well-established involvement of MMPs in COPD (42), we measured MMP-2 and MMP-9 activities in the mouse lung and found a significant increase after 6 months of smoking pointing out a similarity between the mechanisms in the mouse model and the human disease supporting its translational relevance.

The cytokine panel measured from the lung homogenates showed a 2-phase pattern during the 6-month-smoke exposure. The inflammatory burst at month 2 clearly suggests an IL-1-driven

cascade with the elevation of C5a, IL-1α, IL-1ra, IL-16, IP-10, M-CSF, KC, MIG, RANTES, TIMP-1 (43). In the tissue destruction phase at months 5-6 C5a, IFN-γ, IL-4, IL-7, IL-13, IL-17, IL-27, tumour necrosis factor-α (TNF-α), macrophage inflammatory protein-1α (MIP-1α), JE, TIMP-1, interferon-inducible T-cell chemoattractant (I-TAC) and TREM-1 cytokine levels increased.

The chronic moderate cigarette smoke exposure-induced mouse model therefore is appropriate to investigate smoking-induced time-dependent characteristic alterations and mechanisms in the lung. The pathophysiological alterations we described here appear to be similar to that observed in the clinics, which highlights the translational value of our model in relation to the human morbidity seen in COPD.

In the second part of our study we have investigated the involvement of TRPA1 in chronic airway inflammation this characterised and optimised model. We provided the first evidence that the TRPA1 channel has a complex role in basal airway function regulation and inflammatory mechanisms by triggering chronic CSE-evoked emphysema formation and respiratory deterioration, such as MV, TV, PIF and PEF decrease with a peak after 3 months.

Mostly *in vitro* data using cigarette smoke extract are available about the role of TRPA1 in CS-induced airway inflammation. TRPA1 is involved in CS extract-evoked alveolar and bronchial epithelial damage (44). Nicotine directly activates the TRPA1 receptor (45,46) which might mediate bronchoconstriction (47). Similarly, ROS and several lipid peroxidation products also stimulate TRPA1, which is likely to contribute to oxidative stress-evoked airway pathologies induced by CSE (8,48,49), such as emphysema, for which we provided the first data here.

The extent of perivascular/peribronchial oedema was the most severe after 1 month and gradually decreasing afterwards, and the inflammatory cell infiltration reached its maximum after 2 months of CSE in both groups, which is in agreement with our earlier findings in the same model (39). TRPA1 deficiency did not result in significant changes of the cellular components of these chronic inflammatory processes, as shown by both the histopathological results and BALF analysis.

The genetic deletion of the receptor does not directly predict prophylactic or therapeutic potential of TRPA1 agonists or antagonists. Therefore, further research is needed to determine TRPA1 potential as a pharmacological target in the lung.

In the third part of our work we investigated the potential role of TRPA1 and TRPV1 in a characterised and translationally relevant model of diffuse gastric injury. This was the first comprehensive and comparative acute and chronic diffuse gastritis model study, in which IAA-induced concentration- and duration-dependent changes were described in Wistar rats. IAA induced concentration-dependent weight loss and gastric erosions developed already after 7-day ingestion of IAA in drinking water accompanied by massive submucosal oedema and extensive infiltration of acute and chronic inflammatory cells, and subsequently, haemorrhagic erosions. After 14 days, ulcers were observed as deep necrosis involving the muscularis mucosae, which was more severe and more extensive in rats with high (0.2%) IAA concentration in their drinking water. IAA is a sulfhydryl alkylating agent, which inhibits free radical scavenging by depleting reduced GSH, thus inducing gastric injury (30). In our present study, we did not find GSH reduction in response to IAA application. This might be due to the fact that we measured it after 7 or 14 days of IAA ingestion when the lesions were already fully developed or started to heal. The small, but significant increase in tGluCys also supports the onset of the healing phase with the elevation of oxidative stress and/or GSH synthesis enzyme activity suggesting that at the time of lesion formation, the fine regulation is also activated to counteract the imbalance of the aggressive/defensive factors.

We did not observe significant changes in the severity of lesions regarding hyperaemia and erosions between 7 and 14 administration days in agreement with the literature (50), although ulcer formation was more pronounced after 14 days.

In this well-characterised gastric erosion/ulcer inflammatory model, our major finding is that both 0.05% and 0.2% IAA ingestion induced *Trpa1*, but not *Trpv1* mRNA upregulation in the rat antrum and corpus after 7 days—that remained elevated by the end of the 14-day period.

IAA being a cysteine-modifying alkylating compound is able to bind covalently to the reactive cytoplasmic cysteine residues, thus inducing TRPA1 activation (51). However, IAA-induced TRPA1 expression increase in the stomach is more likely due to the inflammatory cascade, which is further supported by its upregulation in water immersion restraint, stress-induced acute gastric mucosal ulcerations in rats (52).

TRPV1/A1 expression on capsaicin-sensitive peptidergic nerve endings and non-neural cells, such as gastric epithelial and inflammatory cells (14,15,17,18), also shown by our present results detecting *Trpa1* mRNA in the stomach, make the interpretation of their roles much more complex.

Several endogenous inflammatory mediators activating TRPV1 (protons, lipoxygenase products) are produced during the IAA-induced inflammatory reaction, which might also influence TRPA1 function and expression since their interactions have been described (3,11,17).

Surprisingly, mice (both CD1 and C57Bl/6 strains) proved to be resistant to all applied concentrations of IAA, even higher than the most damaging one in the rat. Although they also exhibited concentration-dependent weight loss similar to the rat, no macroscopic or microscopic changes have been found in the stomach. The few studies coming from one group point out the lack of IAA-induced macroscopic lesions in mice supporting our present results, but describe a mixed inflammatory infiltration, characteristic to mild gastritis (53). Interestingly, most of these studies showed that after an initial weight loss, mice recovered by the third day of administration, although their water intake was reduced by approximately 50% throughout the study (54). This is also in agreement with our observation, that body weight loss cannot be explained solely by less drinking in IAA-treated animals. The concentration-dependent reduction in fluid consumption suggests an oral aversion that might be due to the potential gastro-irritating effect of the colourless, odourless IAA solution.

Determining resistance mechanisms was beyond the scope of our study; however, it might provide valuable information on gastroprotective mechanisms yet not fully known.

TRPV1 and TRPA1 expression, and experimental data regarding its role in colitis appears to be virtually inconsistent (55). Activation of these receptors on sensory nerve terminals mediates neurogenic inflammation via the release of SP and CGRP, resulting in increased vascular permeability, plasma protein extravasation and inflammatory cell activation. Meanwhile, anti-inflammatory sensory neuropeptides, such as somatostatin and opioid peptides released simultaneously from the same nerve ending exert anti-inflammatory and analgesic actions both locally and systemically through getting into the circulation. Furthermore, these ion channels on vascular smooth muscle and inflammatory cells such as macrophages and T helper cells mediate both pro- and anti-inflammatory functions. Therefore, the overall role of TRPV1 and TRPA1 in experimental colitis is dependent on 1) the diversity of the expression of these ion channels on sensory nerves, immune cells, epithelial cells and vascular smooth muscle cells (3), 2) the consequent activation-induced release of broad range of pro- and anti-inflammatory mediators including sensory neuropeptides and cytokines exerting divergent mechanisms, 3) the complex

interactions of the co-expressed TRPV1 and TRPA1 receptors, 4) differences of the experimental models, protocols and paradigms (species, strain, concentration and composition of the chemicals, duration, intensity, complex mechanisms of the injury), as well as several limitations of the models.

6 SUMMARY OF NEW RESULTS, CONCLUSIONS

1) We provided evidence with functional, morphological and immunological techniques that chronic moderate cigarette smoke induces characteristic pulmonary inflammation, emphysema and atelectasis in a chronic mouse model. These well-defined pathophysiological alterations from the inflammatory reactions to the tissue destruction are dependent on the duration of the smoke exposure and COPD-like structural and functional changes develop only after the fourth month and are similar to that observed in the clinics, which highlights the translational value of our model in relation to the human morbidity seen in COPD. Therefore, this mouse model is appropriate to investigate smoking-induced time-dependent characteristic alterations and mechanisms in the lung.

2) We provided the first evidence that TRPA1 channel has a complex role in basal airway function regulation and inflammatory mechanisms by triggering chronic CSE-evoked emphysema formation and respiratory deterioration, such as MV, TV, PIF and PEF decrease with a peak after 3 months.

3) We provided the first results on the upregulation of the TRPA1 ion channel in a well-characterized translational gastric injury model in correlation with IAA-induced concentration- and duration-dependent macroscopic and microscopic lesions. These data will provide a good basis for evaluating the effect of TRPA1-targeting pharmacological interventions on the different components of the gastric injury.

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9 LIST OF PUBLICATIONS

9.1 Publications related to the thesis:

K. Csekő, D. Pécsi, B. Kajtár, I. Hegedűs, A. Bollenbach, D. Tsikas, I. L. Szabó, S. Szabó, Z. Helyes. Upregulation of the TRPA1 Ion Channel in the Gastric Mucosa after Iodoacetamide-Induced Gastritis in Rats: A Potential New Therapeutic Target. *Int J Mol Sci.* 2020; 21(16):5591. **IF: 5.924**

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