CARM1 regulates senescence during airway epithelial cell injury in COPD pathogenesis

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R.S.J.S., H.F. and A.Ö.Y. conceived the study and experimental design. R.S.J.S., C.M., B.S. and NK performed experiments. SEV prepared patient lung core samples. R.S.J.S., C.M., B.S., H.B., T.M.C. and A.Ö.Y. analyzed and interpreted the data. R.S.J.S., T.M.C. and A.Ö.Y. wrote the manuscript. All authors read and edited the manuscript.

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Abstract

Chronic obstructive pulmonary disease (COPD) is a life-threatening lung disease. Although cigarette smoke was considered the main cause of development, the heterogeneous nature of the disease leaves it unclear whether other factors contribute to the predisposition or impaired regeneration response observed. Recently, epigenetic modification has emerged to be a key player in the pathogenesis of COPD. The addition of methyl groups to arginine residues in both histone and non-histone proteins by protein arginine methyltransferases (PRMTs), is an important posttranslational epigenetic modification event regulating cellular proliferation, differentiation, apoptosis and senescence. Here, we hypothesize that coactivator-associated arginine methyltransferase-1 (CARM1) regulates airway epithelial cell injury in COPD pathogenesis by controlling cellular senescence. Using the naphthalene (NA)-induced mouse model of airway epithelial damage, we demonstrate that loss of CC10-positive club cells is accompanied by a reduction in CARM1 expressing cells of the airway epithelium. Furthermore, *Carm1* haploinsuffficent mice showed perturbed club cell regeneration following NA-treatment. In addition, CARM1 reduction led to decreased numbers of anti-senescent sirtuin 1-expressing cells accompanied by higher p21, p16 and beta-galactosidase-positive senescent cells in the mouse airway following NA-treatment. Importantly, CARM1-silenced human bronchial epithelial cells showed impaired wound healing and higher beta-galactosidase activity. These results demonstrate that CARM1 contributes to airway repair and regeneration by regulating airway epithelial cell senescence.

Keywords: airway epithelium, CARM1, COPD, senescence

Introduction

Chronic obstructive pulmonary disease (COPD) is a life-threatening lung disease, currently the third leading cause of death worldwide (28), characterized by chronic bronchitis, small airway remodeling and emphysema (20, 21, 50). It is a major global health problem and associated with high health-care costs. The staggering socio-economic burden that comes with COPD treatment is now surpassing any other disease (29) and thus necessitates a deeper understanding.

Airway epithelial cells function as the first host defense barrier against cigarette smoke or environmental pollutants (18). Club cells are progenitor/stem cells responsible for maintenance of the airway epithelium following an injury (41). However, extensive epithelial injury may disrupt the epithelial barrier integrity and cause cell death. Repetitive injury combined with a limited reservoir of progenitor cells leads to poor regeneration and repair processes resulting in abnormal wound healing. Contributing greatly to impaired repair processes is airway epithelial cell senescence (37, 52). These changes in the airway epithelium resulting from injury are early and key events in the development and progression of COPD (2, 31). Therefore, development of therapeutic strategies against COPD depends on unraveling the detailed mechanisms of airway injury.

Although cigarette smoke remains the greatest risk factor for COPD, epigenetic modification has recently emerged to be another key player in the pathogenesis of COPD (32, 45, 49). A lesser known epigenetic regulating event is the post-translational modification of proteins by the addition of methyl groups to arginine residues by a family of intracellular enzymes termed protein arginine methyltransferases (PRMTs) (5). Protein arginine methylation is a unique class of protein modification involved in cellular processes such as cell proliferation, differentiation, apoptosis and senescence (4, 5). Coactivator-associated arginine methyltransferase 1 (CARM1) or PRMT4 is a key family member. CARM1

72 is known to asymmetrically dimethylate arginine residues of histone H3 and various non-histone
73 proteins that play essential roles in transcriptional regulation (46, 47), RNA splicing (25, 35) and cellular
74 senescence (27, 36).

75 A recent analysis of PRMT expression in rat lung, heart, liver and kidney revealed the lung to be a major
76 source of CARM1, suggesting its possible role in maintaining lung homeostasis (19) . Indeed, CARM1
77 knockdown resulted in dysregulated proliferation and impaired trans-differentiation of alveolar
78 epithelial cells (34). Thus, abundant pulmonary expression accompanied by its ability to control cell
79 proliferation and differentiation make CARM1 a potential target for further investigation in COPD
80 development and progression. We previously investigated the regulation of CARM1 in the development
81 and progression of emphysema (44). CARM1 deficiency attenuated SIRT1-regulated anti-senescence,
82 and thus induced senescence in alveolar epithelial cells resulting in an increased susceptibility to
83 elastase-induced emphysema (44).

84 Here, we hypothesize that CARM1 also regulates airway epithelial cell injury by controlling cellular
85 senescence. As club cells are crucial to the homeostasis of distal airways in humans (6) and maintain the
86 airway following injury (41), we took advantage of the established mouse model of club cell targeted
87 naphthalene (NA)-induced airway ~~epithelial~~ damage (40, 51). We demonstrated that CARM1 expression
88 was downregulated in NA-treated murine lung accompanied by a loss of CC10-positive club cells from
89 the airways. *Carm1*$^{+/-}$ mice demonstrated impaired club cell regeneration following NA treatment
90 accompanied by enhanced levels of senescent airway epithelial cells. Moreover, CARM1-silenced human
91 bronchial epithelial (HBE) cells showed aberrant wound healing and significantly higher levels of beta-
92 galactosidase-positive senescent cells. Taken together, these findings suggested that CARM1 is
93 indispensable for airway epithelial regeneration and repair in murine lung by acting as a crucial regulator

94 of cellular senescence. We propose that the findings obtained from this study could help develop novel
95 therapeutic strategies to target the airway destruction observed in COPD.

96

Materials and Methods

Human Lungs

Explant lungs were collected in KU Leuven, Leuven Belgium following ethical approval by Institutional Review Board (S52174). These lungs were considered unsuitable for transplantation due to a variety of reasons (kidney tumor, microthrombi and logistics), but were histologically normal. Declined donor lungs can be used for research after second opinion examination under existing Belgian law. After excision, lungs were air inflated at 10cm water pressure and fixed under liquid nitrogen vapor, before being sliced into 2cm slices with a band saw and sampled with a core bore (diameter 1.4cm). Lung cores were sliced, fixed in 6% paraformaldehyde, embedded in paraffin and cut into 3 µm sections.

Study Patients and Bronchial Tissue.

Lung explants were obtained from 8 patients (all males), who were smokers and had COPD at stage 2 according to the guidelines for the global initiative for obstructive lung disease (1) and from 10 patients (4 females, 6 males), who were smokers but had no obstructive pulmonary disease. Patient's characteristics are summarized in Table 1. Before entering the study, the patients were clinically stable and had received no systemic antibiotics, steroids, cytostatic medications or radiotherapy. Patients were administered short-acting beta-2 agonists or anti-cholinergic treatments, as needed. None of the study subjects had upper or lower airway infections within the first month of the study. The study was approved by The Ethics Committee of Koc University, and informed written consents were taken from study volunteers. Bronchial tissue obtained from lung explants from patients, who had lobectomy or pneumonectomy for lung cancer or other reasons at Koc University, Research and Training Hospital (Turkey). The tissue confirmed as "normal" and macroscopically tumor free by a pathologist was

transferred to the molecular biology laboratory. The explant was stored at -80°C until being processed for qPCR studies. RNA was isolated using RNeasy kit (Qiagen).

Experimental animals

Pathogen-free female C57BL/6N mice aged between eight and ten weeks of age were purchased from Charles River Laboratories (Sulzfeld, Germany). The *Carm1*$^{+/-}$ mice were generously provided by Prof Mark Bedford (MD Anderson Cancer Center, University of Texas, Houston, TX) and maintained on a C57BL/6N background. Mice were housed in chambers maintained at constant temperature and humidity with a 12-hour light cycle and were allowed to access rodent laboratory chow and water ad libitum. All animal experiments were performed following strict governmental and international guidelines. The protocol was approved by the ethics committee of the regional Government for Upper Bavaria.

Experimental Protocols

C57BL/6N mice and *Carm1*$^{+/-}$ mice were intra-peritoneally injected once with 200mg/kg body weight of Naphthalene (NA) (Sigma, Munich, Germany) dissolved in Mazola corn oil (CO). Control mice received only CO. Wild type (WT) mice were analyzed on day 3, 7, 14 and 28 while CARM1 heterozygous animals were analyzed on day 14. Body weights of mice were taken at each time point. All mice weighed between 21-26g and were between 10-14 weeks of age at the time of administration. Mice were anaesthetized with intra-peritoneal injection of ketamine and xylazine, tracheostomized for

bronchoalveolar lavage collection and sacrificed by terminal exsanguination on the day of analysis. Experiments were repeated twice (n= 4-7/group in each experiment).

Bronchoalveolar lavage (BAL) collection

The lungs were lavaged by instilling with 3 x 0.5 ml aliquots of sterile PBS containing protease inhibitor (Roche). Cells were spun down at 400 g and total cell counts were determined in a hemocytometer via Trypan Blue exclusion method. Differential cell counts (200 cells/sample) were performed using morphological criteria following Giemsa staining (Merck, Darmstadt, Germany).

Lung processing and histology

Right lungs were snap frozen in liquid nitrogen, homogenized and total RNA isolated (peqGOLD Total RNA Kit, Peqlab, Erlangen, Germany) for gene expression analysis. The left lung was fixed at 20 cm H_2O pressure with 6% paraformaldehyde (PFA). Following an overnight fixation in PFA, the tissue was dehydrated, paraffin-embedded and cut in 3 µm sections. Tissues were stained with Hematoxylin and Eosin (H&E) (Merck, Darmstadt, Germany). High resolution histological images were taken using MIRAX Desk (Zeiss, Oberkochen, Germany) and were analyzed using Mirax Viewer software (Zeiss).

Immunohistochemistry

The paraffin embedded lung sections were de-paraffinized in xylene and rehydrated. The tissue was treated with 1.8% (v/v) H_2O_2 solution (Sigma, St. Louis, MO) for 20 minutes to block endogenous

peroxidase activity. Heat induced epitope retrieval (30 min at 125 °C; 10 min at 90 °C) was performed in HIER Citrate Buffer (pH 6.0, Zytomed Systems, Berlin, Germany) in a Decloaking chamber (Biocare Medical, Concord, CA). Nonspecific binding was inhibited with a blocking antibody (Biocare Medical). Tissue sections were incubated overnight at 4°C with primary antibodies against CC10 (1:1000, ab40873, Abcam, Cambridge, UK), CARM1 (1:250, ab87910, Abcam), SIRT1 (1:50, 07-131, Millipore, Darmstadt, Germany), p21 (1:200, sc-397, Santa Cruz, Dallas, TX), p16 (1:50, sc-1207, Santa Cruz) or beta galactosidase (1:250, A11132, ThermoFisher Scientific, Waltham, MA) followed by incubation with an alkaline phosphatase-labeled secondary antibody (Biocare Medical) for 1 hour at room temperature. Signals were amplified by a chromogen substrate Vulcan fast red (Biocare Medical). Tissues were counterstained with hematoxylin (Sigma), dehydrated in xylene and mounted with Entellan (Merck Millipore, Billerica, MA).

Immunofluorescence

De-paraffinized lung sections were rehydrated, heat-induced epitope retrieval was undertaken using HIER Citrate Buffer (pH 6.0, Zytomed Systems, Berlin, Germany) and blocked with 5% BSA in PBS for 30 min. Then the lung sections were incubated overnight at 4°C with primary antibodies against CC10 (1:2000, ab40873, Abcam, Cambridge, UK), β-tubulin (1:100, sc-5274, Santa Cruz Biotechnology, Heidelberg, Germany), keratin14 (1:50, ab7800, Abcam), SIRT1 (1:100, 07-131, Merck, Darmstadt, Germany) or beta-galactosidase (1:100, A-11132, Invitrogen, Thermo Fisher Scientific, Waltham, MA), followed by 1 hour incubation with goat anti-rabbit IgG Alexa Fluor 568 labeled secondary antibody (1:250, Invitrogen), goat anti-rabbit IgG Alexa Fluor 555 labeled secondary antibody (1:500, Invitrogen) or goat anti-mouse IgG Alexa Fluor 488 labeled secondary antibody (1:500, Invitrogen). To detect

CARM1 on the same section, the lung slices were washed in PBS and again blocked with 5% BSA. Next, the slices were incubated overnight at 4°C with a primary antibody against CARM1 (1:100, ab87910, Abcam) followed by 1 hour incubation with donkey anti-rabbit IgG Alexa Fluor 488 labeled secondary antibody (1:250, Invitrogen) or goat anti-rabbit IgG Alexa Fluor 555 labeled secondary antibody (1:500, Invitrogen) and with 4',6-diamidino-2-phenylindole (DAPI 1:2000, Sigma, St.Louis, MO) for nuclear counterstaining. Sections were mounted in fluorescent mounting medium (Dako, Agilent, Santa Clara, CA) and imaged with a fluorescent Olympus BX51 microscope running cellSens software (Version 1.14, Build 14116, Olympus, Hamburg, Germany).

Quantitative Morphometry

Airway epithelial cells positive for CC10, CARM1, SIRT1, p16, p21 or beta galactosidase were quantified by design-based stereology using a physical dissector. The stereology system consisted of an Olympus BX51 light microscope equipped with the new Computer Assisted Stereological Toolbox (newCAST) software (Visiopharm, Hoersholm, Denmark). The number of positively stained cells was determined as a percentage of the total number of cells positioned on the basal membrane of the airways.

Quantitative Real Time PCR

Reverse transcribed cDNA was synthesized using Random Hexamers and Reverse Transcriptase (Applied Biosystems, Darmstadt, Germany) from right lung isolated total RNA. cDNA was amplified with Platinum SYBR Green qPCR SuperMix (Applied Biosystems) on a StepOnePlus™ PCR System (Applied Biosystems)

using *Hprt1* as a reference gene. Primers are listed in Table 2. Relative gene expression presented as $2^{-\Delta CT}$ ($\Delta Ct = Ct_{target} - Ct_{reference}$) and relative change to control as $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct_{treated} - \Delta Ct_{control}$).

Western Blot Analysis

Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). 20 µg of protein was separated by SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Bio-Rad), blocked with 5% non-fat milk and immunoblotted overnight at 4°C with antibodies against CARM1 (1:1000, Cat. # 09-818, Sigma), asymmetrically dimethylated arginine (ADMA) (1:1000, Cat. # 07-414, Sigma), SIRT1 (1:1000, Cat. # 84695, Cell Signaling) and p53 (1:1000, Cat. # sc-126, Santa Cruz Biotechnology). Antibody binding was detected with HRP-conjugated secondary antibodies and developed using Amersham ECL Prime reagent (GE Healthcare). Bands were detected and quantified using the Chemidoc XRS system (Bio-Rad), and normalized to β-actin levels (anti-β-actin-peroxidase conjugated mouse monoclonal antibody, AC-15, Cat. # A3854, Sigma).

Wound healing assay

Human bronchial epithelial cell line 16HBE (ATCC, Rockville, MD) was seeded at a density of $4x10^4$ cells in 24 well plates and transfected 24h later with CARM1-specific siRNAs (Qiagen, Hilden, Germany) using SuperFect transfection reagent according to manufacturer's instructions (Qiagen) and incubated for 72 hours. Wound healing assay was performed on transfected cells using 200µl pipette tip to scratch the cell monolayer. In some experiments siCARM1 cells were additionally treated with 1µM Resveratrol (Cat. # R5010, Sigma) 24h before performing the scratch, and un-transfected cells were treated with 25

and 50µM Ex-527 (Cat. # E7034, Sigma) for 24h prior to performing the scratch. The size of the wound was determined at 0 and 18 hours using Axiovision software (Zeiss, Oberkochen, Germany).

Senescence-associated beta-galactosidase assay

A staining was performed on CARM1 siRNA transfected 16HBE cells to detect beta galactosidase activity at pH 6 using a senescence assay kit (Cell signaling, Frankfurt, Germany). Upon fixing the cells with a solution containing 2% fomaldehyde and 0.2% glutaraldehyde in PBS for 10 minutes at room temperature, the cells were incubated overnight with a staining solution containing 40 mM citric acid/sodium phosphate (pH 6.0), 150 mM NaCl, 2 mM MgCl2, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 1 mg/ml of X-gal. A percentage of positive cells was determined from 300 cells counted in 12-15 random fields/well.

Gene set enrichment analysis (GSEA)

The series matrix file comparing gene expression in the small airway epithelial cells of COPD patients compared with healthy smoking controls (GSE11784) was downloaded from the NCBI GEO database. To determine the enrichment of senescence genes (GO: 0090398, obtained from the GSEA-Molecular Signatures Database), GSEA software from the Broad Institute (http://www.gsea-msigdb.org/gsea/index.jsp) (33, 48) was used.

Statistical Analysis

242 Mean values ± S.D. were given unless stated otherwise. Student's un-paired t-test compared two
243 groups. One-way ANOVA followed by Bonferroni post-test compared more than two groups, if equal
244 variances and normal distribution was given. Analyses were conducted using GraphPad Prism 6
245 (GraphPad Software, La Jolla, CA).

Results

CARM1 expression is downregulated in naphthalene-induced mouse airway injury.

O'Brien et al. previously reported that CARM1 is expressed in alveolar type II (ATII) and club cells in lung tissue taken from E18.5 mouse embryos (34). Here, we demonstrate that CARM1 is expressed in healthy adult human airways as well as in adult mouse airways by club cells, indicated by the co-localization of the club cell marker CC10 and CARM1 following immunofluorescence analysis (Fig. 1A). To further define the role of CARM1 in airway epithelial cells and its potential contribution to epithelial repair processes, we took advantage of the NA-induced airway injury mouse model. Previous studies have shown that a single exposure to NA induces acute and club cell-specific injury in murine airway epithelium (51, 52). We validated our model by confirming that NA-induced injury, following a single application of 200mg/kg body weight NA i.p., led to a 73 ± 1.8% decrease ($p<0.001$ vs control) in CC10-positive club cells at day 3. The airway epithelial injury was followed by gradual club cell regeneration which was completely resolved by day 28 (Fig. 1B, C). *Scgb1a1* (CC10) expression analysis by quantitative RT-PCR (qRT-PCR) supported the histological findings of a time-dependent regeneration of club cells in NA-treated mouse lung (Fig. 1D). Having confirmed the reduction and subsequent regeneration of CC10 positive club cells in the NA injury model, we next investigated CARM1 expression levels during injury. Quantification of CARM1 positive cells in the airway showed a significant decrease at day 3 (58.4 ± 9.3% NA-treated vs 83.4 ± 1.6% controls, $p<0.01$) following NA application, with the number of CARM1 positive cells restored by day 28 (Fig. 1E, F). Analysis of whole lung homogenate revealed a constant downregulation of *Carm1* mRNA at all time points following NA injury (Fig. 1G). Taken together, these data demonstrate that airway injury is associated with a reduction in CARM1 expression in murine airway epithelial cells and support the idea that CARM1 ~~may~~ is supposed to be involved in airway injury.

CARM1 deficiency perturbs club cell regeneration following naphthalene treatment

To further define the function of CARM1 in the airway epithelium, WT and *Carm1*$^{+/-}$ mice were exposed to a single application of NA (200mg/kg i.p.). Mice with a complete knockout of CARM1 cannot survive beyond late embryogenesis (34). The lungs were examined on day 14 when in wild type (WT) mice club cell regeneration was ongoing but not yet completed. Fig. 2A clearly demonstrates that the lungs of *Carm1*$^{+/-}$ mice had reduced expression of CARM1 protein compared to WT controls following exposure to NA, and that this correlated to reduced levels of global asymmetrically dimethylated arginine residues in lung protein (Fig. 2B). Body weight measurement revealed that NA-treated CARM1 deficient mice significantly lagged in weight recovery compared to NA-treated WT mice (Fig. 2C). Quantitative morphometry of CC10-positive club cells demonstrated a significantly reduced percentage of CC10-positive club cells in *Carm1*$^{+/-}$ mouse airway at day 14 compared to WT mice following NA treatment (30.7 ± 2.5% vs 62.7 ± 7.6%, $p<0.01$) (Fig. 2D, E). The impairment in regeneration was further supported by a decreased level of expression of the proliferation marker *Pcna* in lung homogenate from NA-treated CARM1 deficient mice compared to WT controls (1.6 ± 0.04 vs 1.9 ± 0.1, $p<0.05$) (Fig. 2F). Together, these results indicate that CARM1 is involved in the regenerative response to naphthalene-induced airway epithelial cell injury.

CARM1 deficiency is not compensated by other closely related PRMT family members in mouse airway

To exclude the possibility of compensation for the loss of CARM1 (Fig. 3A) by overexpression of other PRMTs, we examined the expression levels of the relevant PRMT family members PRMT1, PRMT5 and

PRMT7. CARM1 and PRMT1 can act in cooperation to enhance gene transcription (24) and both CARM1 and PRMT5 have a role in the transcriptional regulation of cyclin E1 (12, 42). PRMT7 also functions in conjunction with PRMT5 (15). Our qRT-PCR data revealed no increase in the mRNA expression levels of these PRMTs in CARM1-deficient mouse lung compared to WT mice at day 14 following NA application (Fig. 3B-D).

Contribution of CARM1 to airway epithelial senescence in mouse lung

Club cell senescence plays an important role in the pathogenesis of COPD (2, 52). However, the underlying mechanism is not fully elucidated. We previously showed that CARM1 regulates alveolar epithelial senescence in an elastase-induced mouse model of emphysema (44). We thus hypothesized that CARM1 may also play an important role in the senescence of airway epithelial cells. We therefore performed immunohistochemical analysis for the anti-senescent protein SIRT1. This revealed a significantly reduced percentage of SIRT1-positive airway epithelial cells in *Carm1*$^{+/-}$ mice compared to WT controls at day 14 following NA application (73.5 ± 3.3% vs 87.8 ± 2%, $p<0.01$) (Fig. 4A, E). Next, we assessed lung sections for the senescence markers p21, p16 and beta-galactosidase activity. We observed that the basal level of airway epithelial cells positive for p21 (Fig. 4B, F) and p16 (Fig. 4C, G) were already significantly higher in *Carm1*$^{+/-}$ mice and the levels were not further increased by NA treatment. There was a significant increase in the number of beta-galactosidase positive cells in the airways of NA-treated *Carm1*$^{+/-}$ mice compared to NA-treated WT animals (31.8 ± 3.4% vs 8.2 ± 2.8%, $p<0.001$) (Fig. 4D, H).

Moreover, western blot analysis of whole lung homogenates from d14 mice treated with NA revealed a clear reduction in the level of SIRT1 protein in *Carm1*$^{+/-}$ mice compared to WT controls (Fig. 5A, B), with a

concomitant increase in p53 levels (Fig. 5A, B). In addition, we analyzed the mRNA expression of *Sirt1*, *Cdkn1a* (p21) and *Cdkn2a* (p16) in whole lung homogenate. NA application downregulated *Sirt1* in WT mice but no change was observed in *Carm1*$^{+/-}$ mice (Fig. 5C). *Cdkn1a* was significantly increased in CARM1 deficient mice following NA treatment (Fig. 5D). For *Cdkn2a*, no changes in expression were detected (Fig. 5E). With respect to the differences in protein and mRNA expression levels, one has to take into account that the clear picture seen in the immunohistochemical analysis of a defined tissue, i.e. airway epithelium, is most likely obscured in the qPCR analysis of whole lung homogenate being dominated by tissues other than airway epithelium which, however, also express the genes analyzed. To confirm that the impaired regeneration of airway epithelial cells in *Carm1*$^{+/-}$ mice was a consequence of enhanced senescence in the club cells, dual immunofluorescence analysis for the anti-senescent protein SIRT1 or beta-galactosidase activity with CC10 was undertaken. Fig. 5F clearly reveals the presence of SIRT1 positive club cells at day 14 following NA application in WT mice, that is not detectable in *Carm1*$^{+/-}$ mice. Consistent with this, the few CC10 positive cells in the airways of *Carm1*$^{+/-}$ mice at day 14 following NA application are beta-galactosidase positive (Fig. 5G). Together, this data demonstrates that CARM1 deficiency contributes to NA-induced airway epithelial cell injury by enhancing senescence in club cells, which is contributing to the impaired airway regeneration observed in *Carm1*$^{+/-}$ NA-treated mice.

CARM1 regulates wound healing and senescence in human airway epithelial cells

To translate our findings from the mouse model into humans, we examined the effects of a reduction of CARM1 in a human bronchial epithelial cell line (16HBE) following siRNA knock-down. The siRNA transfection significantly reduced *CARM1* mRNA expression by 72 ± 9.9% (Fig. 6A). We conducted a functional wound healing assay, where the siCARM1-transfected cell monolayer was scratched to induce

a wound. CARM1-silenced cells exhibited impaired wound healing compared with scrambled siRNA, as demonstrated by a larger wound area after 18h (Fig. 6B, C). To determine whether alterations in CARM1 expression regulates senescence in human bronchial epithelial cells, we analyzed the level of beta-galactosidase-positive cells. Consistent with mouse airway epithelial cells, the siCARM1-transfected human cells showed a significantly higher percentage of beta-galactosidase-positive cells compared with scrambled controls (13.2 ± 0.6% vs 3.7 ± 0.6%, $p<0.001$) (Fig. 6D, E). Mechanistically, CARM1-silenced cells demonstrated reduced expression of *SIRT1* (Fig. 6F), and treating CARM1-silenced cells with the SIRT1 activator resveratrol (39), reversed the impaired wound healing (Fig. 6G). In addition, blocking SIRT1 activity with the drug Ex-527 (14), impaired wound healing similar to that observed in CARM1-silenced cells (Fig. 6H). Thus, CARM1 reduction leads to cellular senescence in human airway epithelial cells. By undertaking gene set enrichment analysis (GSEA) upon publically available transcriptomics data of airway epithelial cells from COPD (n=22) versus healthy smoker (n=72) controls (GSE11784), we clearly identify airway epithelial cell senescence as a key component of COPD (Fig. 6I). Interestingly, we observe a clear reduction in the expression of *CARM1* in isolated bronchial epithelial cells from COPD patients compared to healthy smoking controls (Fig. 6J). Overall, this data implies that CARM1 regulates repair and regeneration in airway epithelial cells by affecting SIRT1 regulated cellular senescence.

Discussion

In physiological conditions, the airway epithelium has a rapid self-repair capacity following an insult that causes denudation of cells from the airway. However, repetitive exposure to cigarette smoke and/or other environmental particles can cause a chronic injury to the airway epithelium (11, 30). The injury can lead to altered migration, proliferation and re-differentiation processes which ultimately result in the airway remodeling observed in COPD pathogenesis (17). However, the underlying regulatory mechanisms of airway epithelial repair that are unique to COPD are still not well understood. Here, we investigated the function of the protein arginine methyltransferase CARM1 in airway epithelial repair. We demonstrated that CARM1 is expressed by CC10 positive club cells in both human and mouse airways and for the first time that it contributes to airway repair and regeneration by regulating airway epithelial cell senescence.

To simulate airway epithelial damage occurring in the lungs of patients with COPD, we used the widely accepted naphthalene (NA)-induced murine injury model. NA and several close structural analogs specifically target club cells, irrespective of the route of administration while alveolar epithelial type I or II cells remain uninjured in all animal species tested (7). Club cells, the progenitor cells in the airway epithelium, undergo cell death and exfoliation at 1–2 days following NA application (Fig. 1B-D) (9, 38) but are regenerated by d28 (Fig. 1B-D). The high susceptibility to NA is due to the high rate of metabolic activation leading to cytotoxicity catalyzed by the P450 monooxygenase CYP2F2 localized within the club cells (7). Interestingly, the loss in club cell number following injury was accompanied by a reduction in CARM1 expressing cells (Fig. 1E-G). Interestingly, a complete loss of CARM1 causes disrupted differentiation and proliferation of lung alveolar epithelial type-II cells (34). Previously, we showed that CARM1 contributes to ATII cell repair and regeneration by regulating cellular senescence (44). We therefore hypothesized that CARM1 plays a role in airway epithelial cell maintenance as well. Although

374 CARM1 is reported to be expressed in mouse club cells (34), here we additionally confirmed its
375 expression in club cells of human airways. Club cells are nearly absent in the proximal airway epithelium
376 of humans while 15% of proliferating airway epithelial cells in the terminal bronchioles are club cells (6).
377 This reflects the significant role of club cells in the homeostasis of distal airways in humans. Therefore,
378 we used the club cell targeted NA-induced injury model to understand the functional role of CARM1 in
379 the airway repair process.

380 Airway epithelial cell damage is usually followed by proliferation that repairs the airway epithelium by
381 day 10 after NA treatment and completes the reconstruction process by day 20 (22). We too, followed
382 histological changes until day 28 following NA exposure, and observed a complete recovery of airway
383 epithelium. The finding was corroborated by a significant downregulation of CARM1 expression in the
384 airway and whole lung homogenate. CARM1 was previously reported to regulate the proliferation of
385 neural cell lines (13). Loss of CARM1 has been linked to the developmental arrest in thymocyte
386 progenitor cells due to dysfunctional differentiation (23). However, it is not known whether CARM1
387 plays a role in the regeneration of airway epithelial cells. As mice with a complete knockout of CARM1
388 cannot survive beyond late embryogenesis (34), we investigated the repair of airways in *Carm1*$^{+/-}$ mice
389 following NA treatment. Heterozygous mice had significantly reduced *Carm1* mRNA expression in the
390 lungs, which was not compensated by an increase in the expression of other PRMT family members (Fig.
391 3). There are several reports of compensation by closely related PRMTs in other models. PRMT6 and
392 PRMT7 levels were elevated in an attempt to compensate for PRMT1 loss in embryonic fibroblasts (10).
393 *Carm1*$^{+/-}$ mice demonstrated impaired club cell regeneration following NA treatment accompanied by
394 enhanced levels of senescent airway epithelial cells. Our results therefore further strengthen CARM1s
395 role in maintaining cellular homeostasis by regulating cellular senescence which contributes to the
396 airway repair and regeneration process.

397 The induction of epithelial cell senescence is an established mechanism impairing the repair process
398 following airway injury (52). In COPD patients, senescence occurs in CC10-positive club cells (52).
399 Chronic LPS exposure triggers senescence in airway epithelial cells evident by increased senescence
400 associated beta galactosidase activity (43). Interestingly, CARM1 regulates cellular senescence. It is
401 down-regulated in various organs including testis, thymus, and heart of aging rats (19). Senescent
402 human diploid fibroblasts also expressed reduced levels of CARM1 (27). Mechanistically, CARM1
403 represses senescence by methylating the RNA binding protein HuR, which regulates the turnover of
404 SIRT1 mRNA (8, 36). SIRT1, a NAD1-dependent lysine deacetylase is known to suppress the senescence-
405 associated secretome (16), and SIRT1 pathway dysregulation has been observed in smoke-exposed
406 airway epithelium (3). Here we demonstrated that CARM1 deficiency led to lower levels of SIRT1
407 positive airway epithelial cells after an insult by NA. In addition, we observed increased basal levels of
408 p16 and p21 positive airway epithelial cells in CARM1 deficient mice which indicate an intrinsic pro-
409 senescent status. CARM1-dependent methylation has been shown to regulate *Cdkn2a* (p16) mRNA by
410 HuR-mediated stabilization (36). CARM1-dependent arginine methylation of HuD can also affect mRNA
411 turnover of *Cdkn1a* (p21) (13). However, HuD mRNA is uniquely expressed in brain tissue but not the
412 lungs. Therefore, in the lungs CARM1 might regulate *Cdkn1a* transcription by a different mechanism.
413 CARM1 methylates the transcriptional coactivator p300, which is preferentially targeted by BRCA1 to
414 induce expression of *Cdkn1a* (26). Further proof for CARM1-regulated cellular senescence in airway
415 epithelial cells, was demonstrated by an increase in the number of beta galactosidase-positive airway
416 epithelial cells in *Carm1*$^{+/-}$ mice compared to WT animals following NA treatment that localized to CC10
417 positive cells.

418 In support of our findings with the mouse model and to translate this into human disease, we
419 demonstrated by using human bronchial epithelial cells, that siRNA knock-down of CARM1 *in vitro* led to

reduced *SIRT1* expression, impaired wound healing and resulted in higher beta-galactosidase activity compared to scrambled-siRNA treated controls. CARM1 reduction therefore leads to cellular senescence in human airway epithelial cells. The 16HBE cells used are not a model for club cells suggesting CARM1 may have the potential to regulate senescence in other airway epithelial cell types. Our GSEA analysis of transcriptomics data from the airways of COPD patients compared to healthy smokers reiterates the contribution of airway senescence to COPD, and our own COPD data set confirms that this is accompanied by reduced *CARM1* expression. Taken together, our data implies that CARM1 is necessary for the repair and regeneration of airway epithelial cells by regulating cellular senescence. Thus, highlighting the potential of CARM1 as a novel therapeutic target in the treatment against airway injury observed in COPD patients.

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Disclosures

The authors declare no conflict of interest.

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627

628 **Figure legends**

629 **Figure 1. Naphthalene-induced bronchial epithelial injury down regulates CARM1 expression.** (**A**)
630 Representative immunofluorescence images of CC10 (Red) and CARM1 (Green) co-localization in airway
631 epithelial cells of human lung sections (upper panel, representative of 4 lungs) and mouse lung sections
632 (lower panel, representative of 4 lungs). Counterstained with DAPI (blue). (**B**) Airway-specific injury was
633 induced in wild type C57BL/6 mice by intraperitoneal (i.p.) application of naphthalene (NA) dissolved in
634 corn oil (CO) at a concentration of 200 mg/kg body weight. Control mice were treated with CO only.
635 Representative immunohistochemical analysis of lung at the time points indicated, stained to detect
636 CC10 (red) and haematoxylin counter stained. (**C**) Quantification of CC10 positive club cells by
637 stereological analysis of lung sections described in B. (**D**) mRNA expression level of *Scgb1a1* (CC10) in
638 mouse lung homogenate by qRT-PCR. (**E**) Representative immunohistochemical analysis of lung at the
639 time points indicated from mice described in B, stained to detect CARM1 (red) and haematoxylin
640 counter stained. (**F**) Quantification of CARM1 positive cells in the airways by stereological analysis of
641 lung sections described in E. (**G**) mRNA expression level of *Carm1* in mouse lung homogenate by qRT-
642 PCR. *P<0.05, **P<0.01, ***P<0.001, 1-way ANOVA followed by Bonferroni's multiple comparison post-
643 test, CO vs NA-treated animals. Data presented are mean ± s.d. The experiments were repeated twice
644 with 4-6 mice/group in each experiment.

645

646 **Figure 2. CARM1 deficiency impairs club cell regeneration following naphthalene-induced airway**
647 **injury**. Wild type (WT) or *Carm1*$^{+/-}$ mice treated with naphthalene (NA) or corn oil (CO) were assessed up
648 to day 14 for body weight analysis or on day 14 for all other analyses. (**A**) Western blot analysis of

CARM1 expression in the lungs of WT and *Carm1*$^{+/-}$ mice following NA treatment. Expression relative to β-actin shown, with each point indicating an individual mouse. (**B**) Western blot analysis of asymmetrically dimethylated arginine (ADMA) residues in protein isolated from the lungs of WT and *Carm1*$^{+/-}$ mice following NA treatment. Global expression relative to β-actin shown, with each point indicating an individual mouse. (**C**) Comparison of body weight between the mice described at the indicated time points. (**D**) Representative immunohistochemical analysis of lung stained to detect CC10 (arrows indicate positively stained red cells) and haematoxylin counter stained. (**E**) Stereological quantification of CC10 positive club cells from lung sections described in B. (**F**) mRNA expression level of *Pcna* in mouse lung homogenate by qRT-PCR. *P<0.05, **P<0.01 and ***P<0.001, 1-way ANOVA followed by Bonferroni's multiple comparison post-test (E and F) and two-tailed unpaired t-test between NA *Carm1*$^{+/-}$ and NA WT mice (A-C). Data presented are mean ± s.d. The experiments were repeated twice with 4-7 mice/group in each experiment.

Figure 3. CARM1 deficiency is not compensated for by other closely related PRMT family members. mRNA expression levels of *Carm1* (**A**), *Prmt1* (**B**), *Prmt5* (**C**) and *Prmt7* (**D**) in lung homogenate by qRT-PCR from wild type (WT) and *Carm1*$^{+/-}$ mice on day 14 post naphthalene (NA) or corn oil (CO) treatment. *P<0.05, 1-way ANOVA followed by Bonferroni's multiple comparison post-test. Data presented are mean ± s.d., from 4-7 mice/group.

Figure 4. CARM1 deficiency leads to airway epithelial cell senescence in mouse lung following naphthalene treatment. Wild type (WT) or *Carm1*$^{+/-}$ mice were treated with naphthalene (NA) or corn oil (CO) and analyzed on day 14. Representative immunohistochemical analysis of lung stained to detect

671 SIRT1 (**A**), p21 (**B**), p16 (**C**) and beta galactosidase (**D**), arrows indicate positively stained red cells.
672 Haematoxylin counter stained. Stereological quantification of SIRT1 (**E**), p21 (**F**), p16 (**G**) and beta
673 galactosidase (**H**) positive airway epithelial cells. *P<0.05, **P<0.01, ***P<0.001, 1-way ANOVA followed
674 by Bonferroni's multiple comparison post-test. Data presented are mean ± s.d., from 4-7 mice/group.

675

676 **Figure 5. Club cell senescence in the airways of *Carm1*$^{+/-}$ mice following naphthalene treatment.** Wild
677 type (WT) or *Carm1*$^{+/-}$ mice were treated with naphthalene (NA) or corn oil (CO) and analyzed on day 14.
678 (**A**) Western blot analysis of SIRT1 and p53 expression in the lungs of WT and *Carm1*$^{+/-}$ mice following NA
679 treatment. (**B**) SIRT1 and p53 expression relative to β-actin taken from (A), with each point indicating an
680 individual mouse. *P<0.05, two-tailed unpaired t-test. mRNA expression levels of *Sirt1* (**C**), *Cdkn1a* (p21)
681 (**D**) and *Cdkn2a* (p16) (**E**) in mouse lung homogenate by qRT-PCR. *P<0.05 1-way ANOVA followed by
682 Bonferroni's multiple comparison post-test. Data presented are mean ± s.d., from 4-7 mice/group. (**F**)
683 Representative immunofluorescence analysis of lung stained to detect SIRT1 (Red) and CC10 (Green),
684 counterstained with DAPI (blue). White arrows highlight CC10 positive cells lacking SIRT1 expression. (**G**)
685 Representative immunofluorescence analysis of lung stained to detect beta galactosidase (Red) and
686 CC10 (Green), counterstained with DAPI (blue). White arrows indicate CC10 and beta galactosidase
687 double positive cells. Images representative of 4 mice per group.

688

689 **Figure 6. CARM1 promotes repair and regeneration of human bronchial epithelial cells by regulating**
690 **senescence.** 16HBE cells were transfected with a cocktail of CARM1 specific siRNAs (siCARM1) or non-
691 specific scrambled siRNA (Sc) for 72 hours. Untreated cells were taken as medium control (CO). (**A**)
692 mRNA expression level of *CARM1* by qRT-PCR. (**B**) Wound healing assay was performed by scratching

confluent siRNA-transfected 16HBE cell monolayers, wound size was determined 18 h after injury and reported as percentage closure. (**C**) Representative images of wound size at time 0 h and 18 h. (**D**) siRNA-transfected 16HBE cells were incubated overnight with beta galactosidase staining solution and the number of positive cells quantified. (**E**) Representative images from the assay in D. (**F**) mRNA expression level of *SIRT1* by qRT-PCR. (**G**) siCARM1 cells were additionally treated with 1µM Resveratrol 24h before performing the scratch and wound size determined 18h after injury and reported as percentage closure. (**H**) 16HBE cells were treated with Ex-527 at the concentrations shown for 24h prior to performing a scratch assay, and wound size determined 18h after injury and reported as percentage closure. (**I**) Gene set enrichment analysis (GSEA) of cell senescence genes (GO: 0090398) in publically available array data from the small airways of COPD patients (n=22) v healthy smokers (n=72) (GSE11784), the normalized enrichment score (NES), P value and false discovery rate (FDR) are also indicated. (**J**) mRNA expression level of *CARM1* by qRT-PCR in isolated bronchial epithelial cells from COPD patients (n=8) compared to healthy smoking controls (n=10). Data presented (A-H) are mean ± s.d., representative of three independent experiments with n=2/3 per group in each experiment. *P<0.05, **P<0.01, ***P<0.001 student's two-tailed t-test.

709

	Smokers	COPD
Subjects (n)	10	8
Female/Male	4/6	0/8
Mean age years	61.5 ± 3.93	65.25 ± 3.21
Smoking (packs/year)	38.75 ± 13.11	44.00 ± 4.58
FEV1 (%)	103.8 ± 5.44	94.88 ± 3.93
FVC (%)	102.1 ± 5.63	75.88 ± 4.47 *
FEV1/FVC (%)	80.35 ± 1.98	62.31 ± 2.86 **
GOLD (mean, min-max)	NA	2 (2,2)

710 **FEV1:** Forced expiratory volume in the first second; **FVC:** Forced vital capacity.
711 *: p<0.01, **: p<0.001 COPD versus smokers.

712

713 **Table 1**. Clinical characteristics and demographics of subjects for the primary bronchial epithelial cell
714 cultures (Mean ± SEM).

715

716

717

Gene	Forward primer	Reverse primer
Carm1	GTGGGCAGACAGTCCTTCAT	GTCCGCTCACTGAACACAGA
Cdkn1a	CGGTGTCAGAGTCTAGGGGA	AGAGACAACGGCACACTTTG
Cdkn2a	TCGTGAACATGTTGTTGAGGC	CTACGTGAACGTTGCCCATC
Hprt1	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
Pcna	CCACATTGGAGATGCTGTTG	CCGCCTCCTCTTCTTTATCC
Prmt1	CGAACTGCATCATGGAGAAT	AGCGTTGGGCTTCTCCACTAC
Prmt5	TGACCAACCACATCCACACT	GTGTGTAGTCGGGGCATTCT
Prmt7	TACTGCAGGGGCTGACTTCT	TCACCTCAGTGGAGTGCTTG
Scgb1a1	ACCTCTACCATGAAGATCGCC	CTCTGATTCCATGAGGAGGGC
Sirt1	CCATTAATGAGGAAAGCAATAGGC	AATACAAGGCTAACACCTTGGG
CARM1	ACAGCGTCCTCAATCCAGTTC	GCTGGGACAGGTAGGCATAA
SIRT1	GCAGATTAGTAGGCGGCTTG	TCTGGCATGTCCCACTATCA
GAPDH	ATGGAAATCCCATCACCATCTT	CGCCCCACTTGATTTTGG

718

719 **Table 2.** Primers used for quantitative real time PCR.