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RESEARCH ARTICLE

Critical importance of dietary methionine and choline in the maintenance of lung homeostasis during normal and cigarette smoke exposure conditions

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[1]*Faculty of Medicine, Université Laval, Quebec City, Canada;* [2]*Quebec Heart and Lung Institute, Université Laval, Quebec City, Canada;* [3]*Department of Medicine, Université Laval, Quebec City, Canada; and* [4]*Centre de Recherche sur le Cancer de l'Université Laval, Quebec City, Canada*

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Jubinville É, Milad N, Maranda-Robitaille M, Lafrance MA, Pineault M, Lamothe J, Routhier J, Beaulieu MJ, Aubin S, Laplante M, Morissette MC. Critical importance of dietary methionine and choline in the maintenance of lung homeostasis during normal and cigarette smoke exposure conditions. *Am J Physiol Lung Cell Mol Physiol* 319: L391–L402, 2020. First published July 8, 2020; doi:10.1152/ajplung.00353.2019.—Genetic predispositions and environmental exposures are regarded as the main predictors of respiratory disease development. Although the impact of dietary essential nutrient deficiencies on cardiovascular disease, obesity, and type II diabetes has been widely studied, it remains poorly explored in chronic respiratory diseases. Dietary choline and methionine deficiencies are common in the population, and their impact on pulmonary homeostasis is currently unknown. Mice were fed choline- and/or methionine-deficient diets while being exposed to room-air or cigarette smoke for up to 4 wk. Lung functions were assessed using the FlexiVent. Pulmonary transcriptional activity was assessed using gene expression microarrays and quantitative PCR. Immune cells, cytokines, and phosphatidylcholine were quantified in the bronchoalveolar lavage. In this study, we found that short-term dietary choline and/or methionine deficiencies significantly affect lung function in mice in a reversible manner. It also reduced transcriptional levels of collagens and elastin as well as pulmonary surfactant phosphatidylcholine levels. We also found that dietary choline and/or methionine deficiencies markedly interfered with the pulmonary response to cigarette smoke exposure, modulating lung function and dampening inflammation. These findings clearly show that dietary choline and/or methionine deficiencies can have dramatic pathophysiological effects on the lungs and can also affect the pathobiology of cigarette smoke-induced pulmonary alterations. Expanding our knowledge in the field of "nutri-respiratory research" may reveal a crucial role for essential nutrients in pulmonary health and disease, which may prove to be as relevant as genetic predispositions and environmental exposures.

choline; cigarette; inflammation; lung; methionine

INTRODUCTION

Diet is critical to cardiovascular and metabolic health (8, 9, 19), but its impact on respiratory health is not as well established. The lung is a complex structure directly exposed to the environment and thus is subjected to continuous insult and injury from agents such as microbes, air pollution, or tobacco smoke. Therefore, the maintenance of lung homeostasis during health and disease is likely affected by changes in the supply of essential nutrients acquired through diet.

Our group has been investigating the interaction between smoking and pulmonary lipid homeostasis (13, 17, 25), progressively unraveling the critical role of the pulmonary surfactant in the response to cigarette smoke exposure. Phosphatidylcholine is a crucial pillar of the pulmonary surfactant, representing over 70% of its lipid content (1, 5, 10). During surfactant biogenesis, the required building blocks are captured by type II alveolar cells from the circulation, allowing for the synthesis of phosphatidylcholine species essential to a fully functional pulmonary surfactant (1, 11). Phosphatidylcholine is in high demand throughout the body as an essential component of cellular membrane bilayer and circulating lipoproteins. Therefore, a reduced capacity to produce phosphatidylcholine, in addition to the overall high demand, may jeopardize pulmonary surfactant homeostasis and, consequently, lung function.

Choline and methionine are two essential nutrients, which have to be acquired through diet to meet our daily requirements (16, 26). Choline is important for the synthesis of the neurotransmitter acetylcholine and the phospholipid phosphatidylcholine, among other crucial biological functions (26). Methionine is critical to protein synthesis, as it initiates the translation of every mammalian protein and can also be used to synthetize phosphatidylcholine from phosphatidylethanolamine (16). Therefore, these two essential nutrients contribute to phosphatidylcholine biosynthesis and can compensate for one another through shared metabolic pathways. Simultaneous dietary choline and methionine deficiency is known to have drastic effects. In mice, a diet deficient in methionine and choline rapidly leads to non-alcoholic hepatic steatosis followed by hepatic damage due to increased inflammation and fibrosis (14, 21, 23). It also markedly reduces very low-density lipoprotein (VLDL) biogenesis by the liver and decreases systemic lipid transporters such as high-density lipoproteins (HDLs) (22, 27). Dietary choline insufficiency also leads to lipid accumulation in the liver in humans (4). It is estimated that the majority of individuals do not acquire enough choline through the diet to meet daily requirements (28). Because circulating lipoprotein homeostasis and phosphatidylcholine availability are important factors that influence lung function, we hypothesized that dietary deficiency in choline and methionine would impact pulmonary homeostasis. Furthermore, as

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pulmonary lipid and surfactant homeostasis has been shown to be disrupted in cigarette smoke exposure (2, 7, 17, 20), the effects of dietary methionine and choline deficiency may be especially significant.

In this study, we used a mouse model to investigate the impact of a dietary methionine and choline deficiency on lung homeostasis during normal and cigarette smoke exposure conditions. We observed that dietary methionine and choline deficiency progressively and reversibly affected lung function in both room air- and cigarette smoke-exposed mice, leading to a restrictive pulmonary phenotype. The inflammatory response to cigarette smoke was also altered by this dietary deficiency, exhibiting a markedly reduced neutrophil infiltration. Finally, dietary deficiency in methionine and choline drastically reduced pulmonary surfactant levels, which partially explains the observed susceptibility to atelectasis conferred by the dietary deficiency.

METHODS

Animals, Cigarette Smoke Exposure, and Dietary Protocols

Mice. Female 6- to 8-wk-old C57BL/6 mice were purchased from Charles River Laboratories (Montréal, QC, Canada). Mice were housed following recommendations from the Guide for the Care and Use of Laboratory Animals of the Canadian Council on Animal Care (CCAC). Mouse protocols were approved by the Committee on the Ethics of Animal Experiments of Université Laval (#2017–101–1).

Cigarette smoke exposure. Mice were exposed every morning for 2 h, 5 days/week, for 2, 3, or 4 wk to either room air or the mainstream smoke of 3R4F reference cigarettes (University of Kentucky, Lexington, KY) with filters removed, using an established whole body exposure system (SIU24; Promech Laboratory, Vintrie, Sweden) (13, 24). Levels of total particulate matter in the cigarette smoke exposure chamber were not measured for the specific protocols presented in this study.

Diets. Mice were fed ad libitum a control diet (21% fat, 66% carbohydrate, 17% protein, 2 g/kg choline, and 3 g/kg methionine; Research Diets Inc. A02082003BY), a methionine-deficient diet (Research Diets Inc. A17091101), a choline-deficient diet (Research Diets Inc. A17091102), or a methionine/choline-deficient diet (Research Diets Inc. A02082002B) for 2, 3, or 4 wk.

Blood Collection and Processing

Prior to lung function assessment, orbital blood was collected under ketamine/xylazine anesthesia. Blood samples were then centrifuged (12,000 *g*, 4°C) and the serum stored at −80°C for future measurements.

Lung Function Assessment

Standard assessment. Lung function parameters were measured using the FlexiVent (SCIREQ, Montreal, QC, Canada) in mice with the chest cavity closed. Mice were anaesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, tracheotomized with an 18-gauge blunted needle, and mechanically ventilated at a respiratory rate of 150 breaths/min and a tidal volume of 10 mL/kg, with a pressure limit of 30 cmH_2O. Muscle paralysis was achieved using pancuronium (2 mg/kg, Sandoz, Boucherville, QC, Canada) to prevent respiratory efforts during maneuvers. The following sequence of measurements was repeated three times: Deep inflation, Snapshot-150, Quick Prime-3, and Pressure/Volume-loop (PV-loop) to obtain lung function parameters. For closed versus open chest assessment, aforementioned maneuvers were performed in mice with the chest closed first. The chest cavity was then opened by carefully cutting through the sternum and upper abdominal cavity. Aforementioned maneuvers were then repeated with the chest open.

Pre- and post-bronchoalveolar lavage assessments. These experiments were performed on a separate cohort of mice that were not included in any other assessment. Under the same anesthetic conditions of the "standard assessment" described above, the following sequence of measurements was repeated three times: Deep inflation, Snapshot-150, Quick Prime-3, and Pressure/Volume-loop (PV-loops) to obtain "Pre-bronchoalveolar lavage (BAL)" lung function parameters. The cannula was then removed and lungs were lavaged in situ once with 1 mL of warm PBS with an approximate 900 μL recovery to remove pulmonary surfactant. Mice were then reconnected to the FlexiVent and ventilated for 3 min. To assess "Post-BAL" lung function parameters, the following sequence of measurements was repeated three times: Deep inflation, Snapshot-150, Quick Prime-3, and PV-loop.

Bronchoalveolar Lavage, Lung Tissue Collection, and Lung Histology Assessments

After lung function assessment, mice were euthanized by exsanguination. Lungs were removed from the thoracic cavity, the trachea was cannulated, and right lobes were tied off. Bronchoalveolar lavage (BAL) was performed with a first injection of 250 μL of cold PBS and a second injection of 200 μL. The right lobes were then collected, immediately frozen in liquid nitrogen and kept at −80°C. For histological assessment, the left lung was inflated with 10% formalin and fixed for 3 days prior transfer to 70% ethanol and paraffin-embedding. Lung sections were stained with hematoxylin and eosin (H&E) as well as Masson's trichrome. Total cell count was performed on the fresh BAL using a hemacytometer. BAL cells were then centrifuged (800 *g*, 4°C), the supernatant (BAL fluid) collected and kept at −80°C, and the cells were resuspended in PBS for cytospins. Cytospins were prepared and stained using the Hema 3 protocol (Fisher) for differential cell counts. A total of 300 cells was counted per cytospin.

Alveolar Size Measurements

Hematoxylin and eosin-stained lung histological sections were scanned, and alveolar area was quantified on three sections per lung per mouse using ImageJ. Scale was set and calibrated to a known distance at ×2.5 (1 μm per pixel). The background was subtracted, despeckled, and the image was converted to 32-bit. Images were segmented by an inclusive threshold filtration creating a binary mask: tissue appears white and alveolar spaces black (empty). Analysis of particles allowed for each alveolar space to be traced and its area calculated, excluding areas smaller than 50 μm^2 and manually removing bronchioles, blood vessels, and other nonalveolar areas. Alveolar areas were exported and stratified into bins of logarithmically increasing range (e.g., bin 1 = 2^5–2^6 μm^2, bin 2 = 2^5–2^6 μm^2 . . . bin 13 = 2^{17}–2^{18} μm^2). Data were expressed as a percentage of the total area by taking the sum of the alveolar areas within a range and dividing it by the total alveolar area measured.

RNA Extraction, Quantitative PCR, and Gene Expression Microarray Analysis

RNA extraction. Lung total RNA was extracted from one frozen lobe using Trizol (Fisher) according to manufacturer's instructions. Lung RNA was resuspended in 50 μL of Tris-EDTA (Fisher) and quantitated using the BioTek Synergy H1 (BioTek, Winooski, VT). Lung RNA integrity and 28S/16S ratios were analyzed on agarose gels before cDNA synthesis.

Quantitative PCR. Lung cDNA was prepared with 1 μg of total RNA using the iScript cDNA synthesis kit following manufacturer's instructions (Bio-Rad, Mississauga, ON, Canada). Lung gene mRNA levels were assessed using the SsoAdvanced universal SYBR green Supermix (Bio-Rad, Canada). Two reference genes, *Rplp0* and *Hprt*,

and gene-specific primers were used to amplify *Col1a1*, *Col3a1*, *Eln*, *Cxcl5, Sftpa1, Sftpc*, and *Mmp-12* (IDT, Skokie, IL). All quantitative PCR (qPCR) reactions were composed of 10 μL of SsoAdvanced universal SYBR green Supermix 2X, 10 μM forward primer (0.6 μL), 10 μM reverse primer (0.6 μL), 5 μL of cDNA template, and were completed with RNAse-free water to the final volume of 20 μL (GE Healthcare, South Logan, UT). Thermo-protocols were as follow: 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 56–60°C for 30 s. All samples were run in duplicate and RNase-free water (GE Healthcare) was used as a no template control. Reactions were manually loaded into 0.1 mL PCR tube strips (Corning, Corning, NY). Amplifications were performed using a Rotor-Gene 6000 series (QIAGEN, Canada) and data acquired/analyzed with the on-board Rotor-Gene series Software version 1.7. Thermal gradients and efficiencies were all performed before qPCR assays. qPCR efficiencies were between 90 and 110%, with R^2 values ranging between 0.96 and 0.99. Melt curves and qPCR products on agarose gels were analyzed for specificity, and no template controls were detected past 35 Cq. Threshold from the efficiency assays were used to determine the Cq, and the ΔΔCq method was used to normalize all qPCR results.

Gene expression microarray analysis. Lung total RNA was sent to GenomeQuebec (Montreal, QC, Canada) to perform Affimetrix mouse Clariom D microarrays. The differential analysis was performed using the R software and packages *affy*, *oligo*, and *limma*.

BAL Fluid and Serum Biomarkers

IL-1α and CCL2 in BAL fluid. IL-1α and CCL2 levels were assessed in mouse bronchoalveolar lavage fluid using the mouse

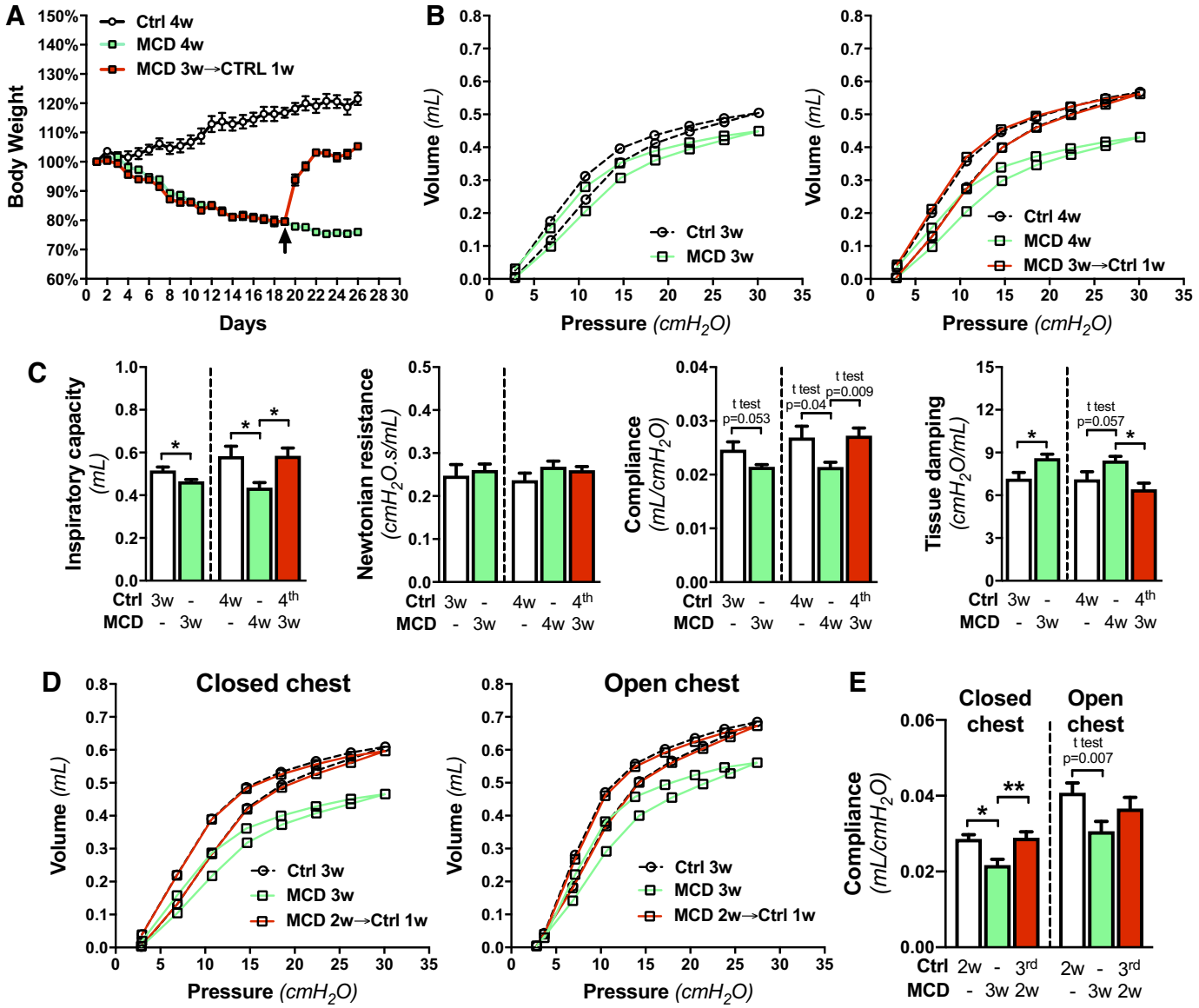

Fig. 1. Dietary methionine and choline deficiency progressively and reversibly affects lung functions. Female C57BL/6 mice were fed control (Ctrl) diet or methionine/choline-deficient (MCD) diet for 3 wk and switched back, or not, to control diet for an additional week to assess reversibility. *A*: body weight measurements over the 4 wk under control and MCD. Lung functions were assessed using the FlexiVent at 3 and 4 wk after initiation of the diet: the pressure-volume (P-V) loops (*B*) and inspiratory capacity, Newtonian resistance, compliance, and tissue damping (*C*). In a separate experiment, C57BL/6 mice were fed Ctrl or MCD diet for 2 wk and switched back, or not, to control diet for an additional week to assess reversibility. Lung functions were assessed using the FlexiVent at 3 wk after initiation of the diet and the P-V loops (*D*) and compliance were derived from the maneuvers performed first with the chest closed and then with chest open (*E*). Data are presented as means ± SE; $n = 5$ mice/group. *$P < 0.05$; **$P < 0.01$.

IL-1α and CCL2 DuoSet ELISA kits (R&D systems, Minneapolis, MN) following manufacturer's instructions.

Phosphatidylcholine levels in serum and BAL fluid. Serum and BAL fluid levels of phosphatidylcholine were measured using the phosphatidylcholine assay kit following manufacturer's instructions (STA-600; Cell Biolabs, San Diego, CA). Dilutions of 1:4 and 1:100 were used for BAL fluid and serum, respectively.

Statistical analysis

Statistical analyses were performed using GraphPad Prism Software V.6 (La Jolla, CA). Two group comparisons were made using an unpaired *t* test ($P \leq 0.05$). Experimental protocols with more than two groups were compared using a one-way ANOVA ($P \leq 0.05$) followed by a Bonferroni multiple comparisons post test. In some cases, two group comparisons were made within experiments with more than two groups using an unpaired *t* test as specifically stated.

RESULTS

Dietary Deficiency in Choline and Methionine Reversibly Affects Lung Function

To investigate the impact of dietary deficiency in choline and methionine on the lungs, we fed C57BL/6 mice a control or a methionine/choline-deficient (MCD) diet for 3 wk. To assess reversibility, we switched half of the mice on MCD diet to control diet while the other half remained on MCD diet for one additional week. As expected, MCD diet markedly reduced body weight (Fig. 1*A*) and reintroduction of control diet partially reversed the phenotype. We assessed pulmonary functions at 3 wk (before diet switch) and 4 wk (1 week after diet switch). We found that the MCD diet progressively affected pulmonary functions, causing a restrictive phenotype, as shown by the lowering of the PV-loop as well as the reduced inspiratory capacity and compliance (Fig. 1, *B* and *C*). Unchanged Newtonian resistance and increased tissue damping suggest that lung tissue, rather than conductive airways, is affected (Fig. 1*C*). Reintroduction of control diet completely reverses the lung function phenotype within 1 week (Fig. 1, *B* and *C*). Moreover, increased mechanical constraints of the chest wall were excluded as potential causes of the restrictive phenotype, because both closed and open chest respiratory maneuvers exhibited similar patterns (Fig. 1, *D* and *E*). This shows that the MCD diet rapidly and deeply affects lung functions, that this phenomenon is

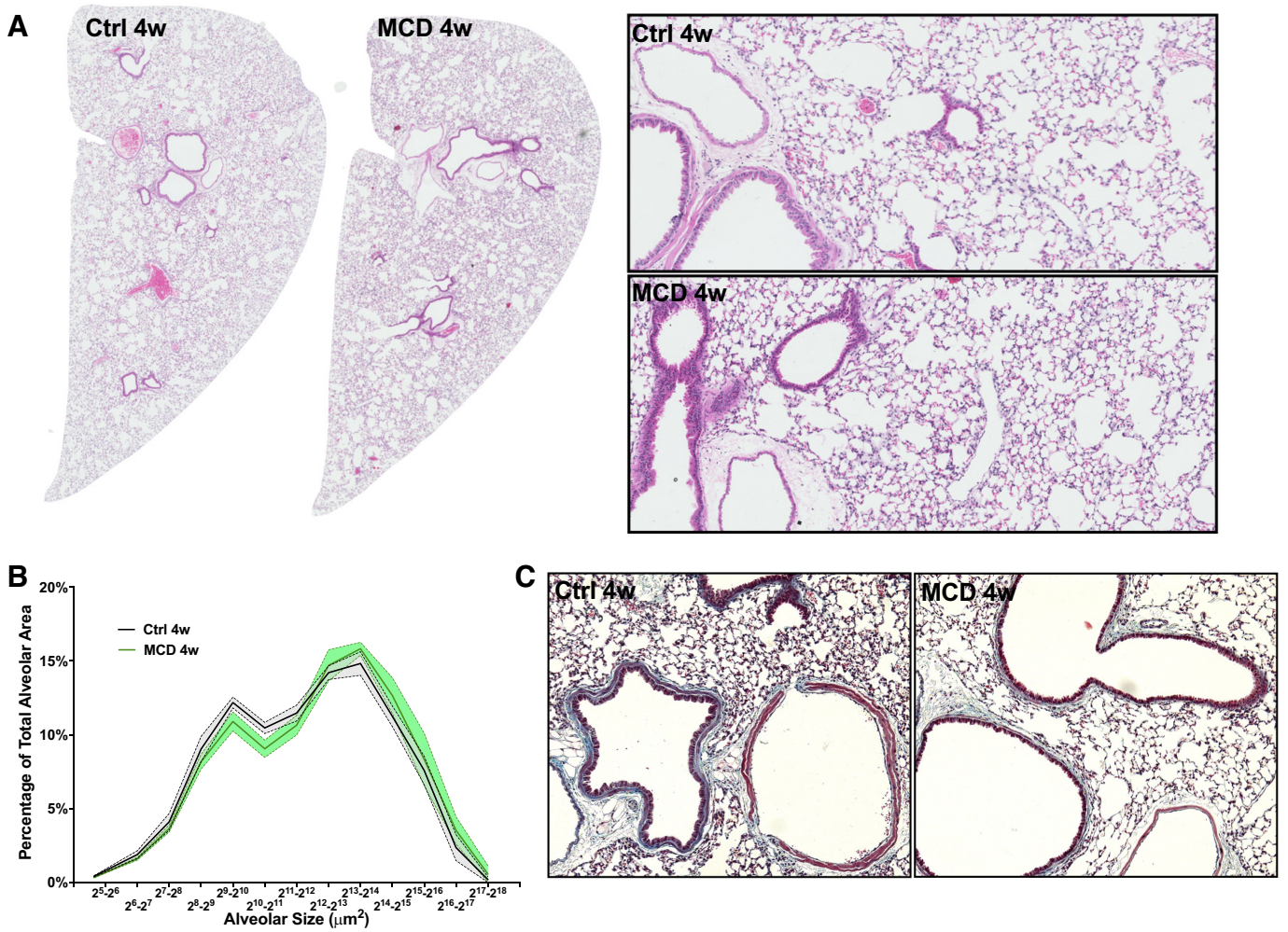

Fig. 2. Impact of dietary methionine and choline deficiency on alveolar size and collagen deposition. Female C57BL/6 mice were fed control (Ctrl) diet or methionine/choline-deficient (MCD) diet for 4 wk. *A*: hematoxylin and eosin (H&E) staining of formalin-fixed paraffin-embedded lung sections. *B*: quantification of alveolar size based on H&E-stained lung sections. Data are presented as means ± SE; $n = 5$ mice/group. *C*: Masson's trichrome staining of formalin-fixed paraffin-embedded lung sections.

reversible, and that it is caused by changes to the lung tissue itself. Since a restrictive pulmonary phenotype can result from pulmonary fibrosis, we investigated lung histology for potential changes. We found no evidence of alveolar enlargement or damage (Fig. 2, *A* and *B*), and no signs of established fibrotic remodeling were detected in the lung tissue of mice fed the MCD diet (Fig. 2, *A–C*), thus excluding development of lung fibrosis as the cause of the MCD diet-induced lung phenotype. This is also in accordance with the relatively rapid renormalization of lung functions upon reintroduction of the control diet, since lung fibrosis generally requires a longer period to resolve.

Dietary Choline and Methionine Deficiency Strongly Reduces Pulmonary Expression of Extracellular Matrix-Related Genes

To better understand the underlying mechanisms behind the impact of dietary choline and methionine deficiency on the lungs, we performed gene expression microarrays on lung tissue from mice fed control or MCD diet for 4 wk. We found that expression of genes encoding extracellular matrix proteins were markedly reduced by the MCD diet (Fig. 3*A*). We confirmed four important pulmonary extracellular matrix genes by quantitative PCR (Fig. 3*B*): *col1a1*, *col3a1*, and *elastin*, respectively, encoding collagen type I alpha 1 chain, collagen type III alpha 1 chain, and elastin. As observed in the case of lung function alterations (Fig. 1, *B* and *C*), mRNA levels of *col1a1*, *col3a1*, and *elastin* fully normalized after 1 week of control diet reintroduction (Fig. 3*B*). Altogether, this suggests that MCD diet rapidly limits mRNA expression of key genes encoding extracellular matrix proteins, potentially affecting extracellular matrix homeostasis in ways that cannot be observed histologically.

Dietary Choline and Methionine Deficiency Predisposes Mice to Small Airway Atelectasis Due to Reduced Pulmonary Surfactant Levels and Structural Alterations Independent of Pulmonary Surfactant

To investigate the role of pulmonary surfactant in lung function alterations caused by the MCD diet, we assessed phosphatidylcholine levels in the BAL fluid, which reflect the amount of pulmonary surfactant. We found that, similar to circulating phosphatidylcholine levels, phosphatidylcholine levels in the BAL fluid as well as lung *sftpa1* and *sftpc* mRNA levels were lower in mice fed the MCD diet (no variations in *sftpb* and *sftpd*; data not shown); phenomena that were fully reversed within a week on the control diet (Fig. 4, *A* and *B*). To assess whether lung dynamics re-

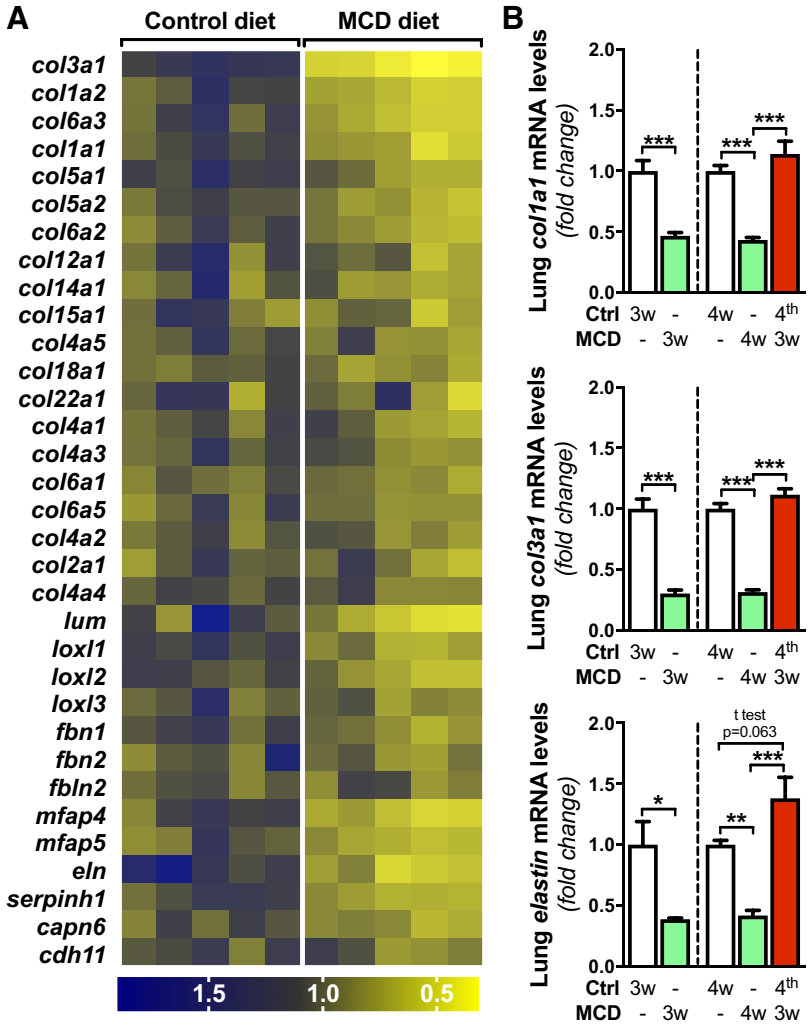

Fig. 3. Dietary methionine and choline deficiency reduces pulmonary expression of extracellular matrix genes. *A*: levels of extracellular matrix genes from gene expression arrays performed on lung tissue from female C57BL/6 mice fed control (Ctrl) diet or methionine/choline-deficient (MCD) diet for 4 wk. *B*: lung mRNA levels of *col1a1*, *col3a1,* and *elastin* assessed by quantitative PCR in female C57BL/6 mice fed control diet or MCD diet for 3 wk and switched back, or not, to control diet for an additional week. Data are presented as means ± SE; $n = 5$ mice/group. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

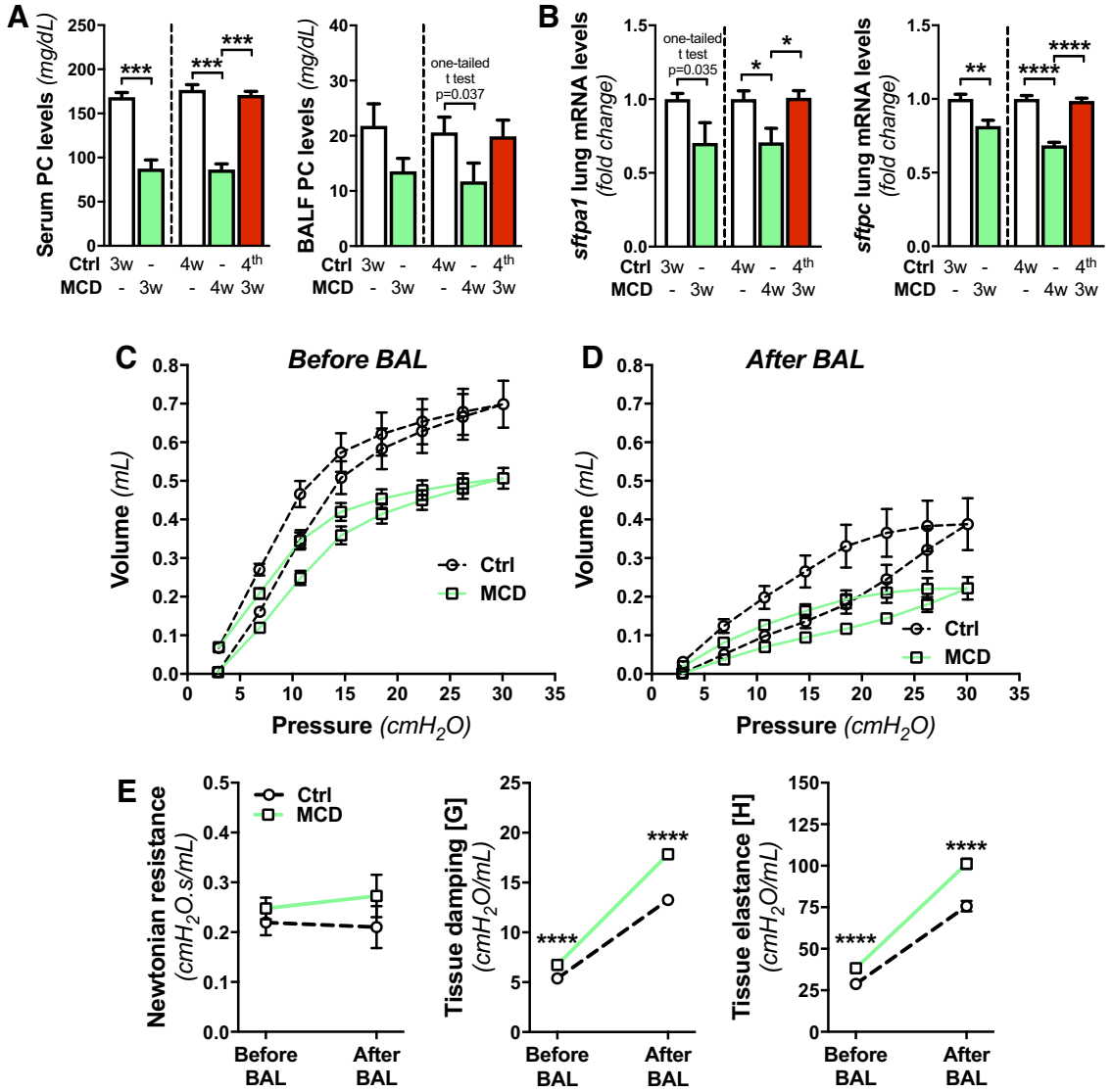

Fig. 4. Dietary methionine and choline deficiency reduces pulmonary surfactant levels as well as lung tissue mechanical properties. *A*: serum and bronchoalveolar lavage fluid (BALF) phosphatidylcholine (PC) levels were assessed in female C57BL/6 mice fed control (Ctrl) diet or methionine/choline-deficient (MCD) diet for 3 wk and switched back, or not, to control diet for an additional week. *B*: lung mRNA levels of *sftpa1* and *sftpc* were assessed by qPCR. *C–E*: lung functions were assessed using the FlexiVent before and after bronchoalveolar lavage (BAL) in female C57BL/6 mice fed Ctrl diet or MCD diet for 4 wk. Data are presented as means ± SE; $n = 5$ mice/group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

mained affected in the absence of pulmonary surfactant, we performed lung function tests before and after pulmonary surfactant removal via lavage. We found that the impact of pulmonary surfactant removal was worse in the lungs of MCD-fed mice compared with those fed control diet, as reflected by the lower PV-loop after the lavage (Fig. 4, *C* and *D*) and the more pronounced increase in tissue damping and tissue elastance (Fig. 4*E*). Of note, removal of pulmonary surfactant through lavage did not affect Newtonian resistance, supporting the notion that conducting airways were not affected by the procedure (Fig. 4*E*). Altogether, these data indicate that, although the MCD diet reduced pulmonary surfactant levels, surfactant dysfunction alone did not account for the entirety of the pulmonary phenotype and that structural alterations and/or impaired alveolar recruitment may also be present.

Dietary Choline and Methionine Deficiency Markedly Affects Cigarette Smoke-Induced Changes in Lung Function and Inflammation

Since dietary deficiency in choline and methionine markedly affected lung functions as well as the expression of genes associated to extracellular matrix, we investigated how this nutritional deficiency could impact the pulmonary response to cigarette smoke. A 2-wk exposure to cigarette smoke did not affect lung function, while the MCD diet-fed animals showed signs of early lung function changes (Fig. 5*A*). However, the MCD diet markedly blunted the neutrophilia induced by cigarette smoke exposure (Fig. 5*B*). BAL fluid IL-1α and CCL2 as well as tissue *cxcl5* and *mmp12* mRNA levels were all increased by cigarette smoke exposure, while only *cxcl5* mRNA levels were affected by the MCD diet (Fig. 5, *C* and *D*).

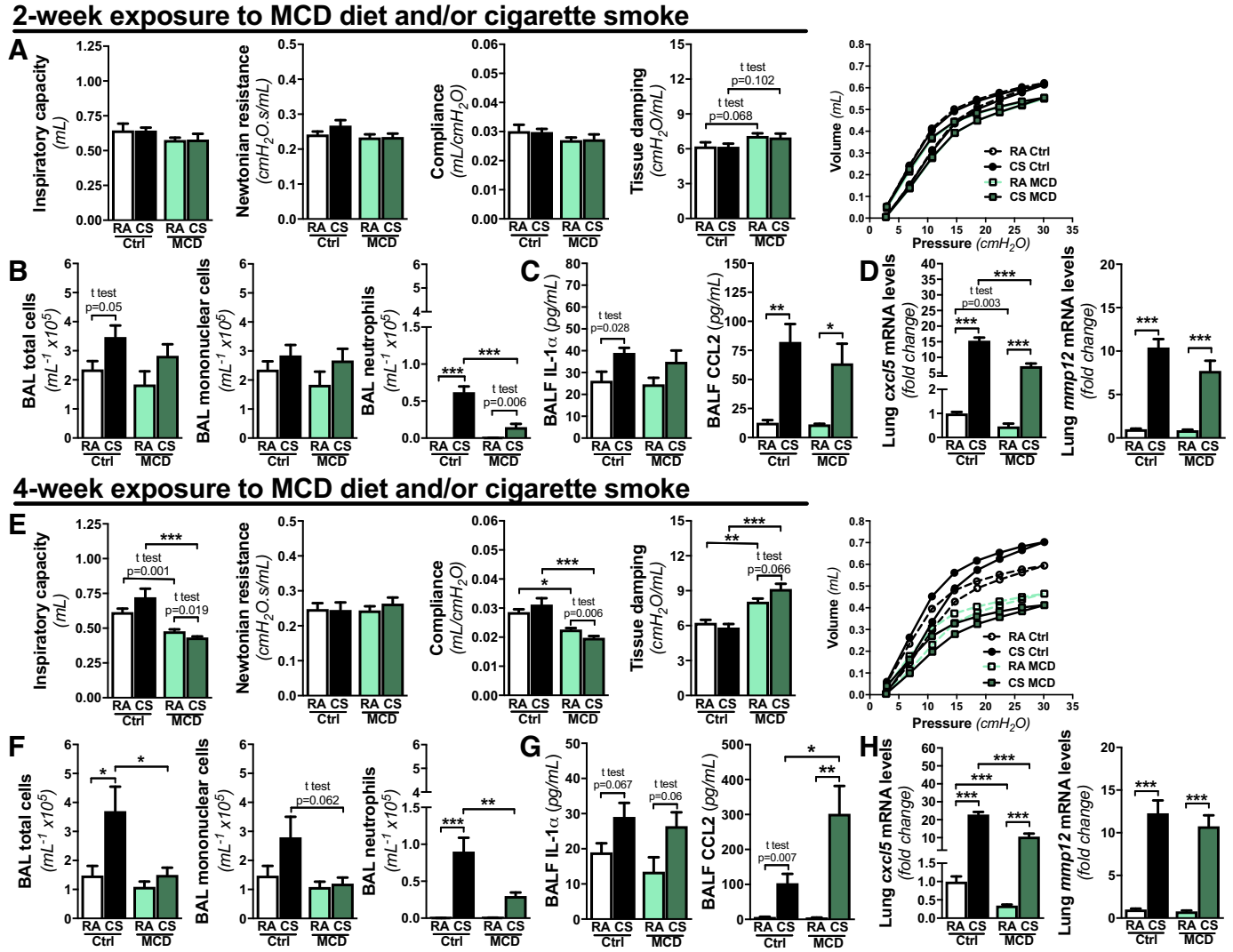

Fig. 5. Dietary methionine and choline deficiency progressively alters cigarette smoke-induced lung function changes and inflammation. Female C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for 2 wk (*A–D*) or 4 wk (*E–H*) and fed control (Ctrl) diet or methionine/choline-deficient (MCD) diet for the same period. *A* and *E*: lung functions were assessed using the FlexiVent. *B* and *F*: total, mononuclear, and neutrophil cell counts were performed on the bronchoalveolar lavage (BAL). *C* and *G*: protein levels of interleukin 1 alpha (IL-1α) and monocyte chemotactic protein 1 (CCL2) were assessed in the BAL fluid (BALF). *D* and *H*: lung mRNA levels of *cxcl5* and *mmp12* were assessed by qPCR. Data are presented as means ± SE; $n = 5$ mice/group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Regarding the 4-wk exposure to cigarette smoke, lung functions were altered as previously observed: increased inspiratory capacity but no effect on compliance or tissue damping (Fig. 5*E*). The MCD diet had a significant effect on the response to cigarette smoke exposure, leading to a discrepancy between cigarette smoke-exposed mice fed the control diet and those fed the MCD diet (Fig. 5*E*). The MCD diet maintained its blunting effect on cigarette smoke-induced neutrophilia, with an additional reduction in mononuclear cells and, consequently, total cell counts (Fig. 5*F*). BAL fluid IL-1α and lung tissue *mmp12* mRNA levels remained unaffected by the MCD diet, while BAL fluid CCL2 levels were increased and lung tissue *cxcl5* mRNA levels were reduced (Fig. 5, *G* and *H*). All these processes took place in the absence of marked lung tissue alterations with regard to alveolar enlargement or collagen deposition (Fig. 6, *A–C*). Interestingly, control diet-fed mice exposed to cigarette smoke at 2 and 4 wk exhibit markedly reduced *elastin* expression, with no or only a marginal effect on the *col3a1* and *col1a1* levels (Figs. 6, *D* and *E*). In MCD-fed animals, mRNA levels of all three genes were higher in the MCD-fed cigarette smoke-exposed group compared with the MCD-fed room air-exposed group (Fig. 6, *D* and *E*). Overall, the MCD diet not only progressively impacted lung functions during cigarette smoke exposure but also rapidly blunted cigarette smoke-induced inflammation, an effect that is observed before development of pulmonary function alterations.

Choline or Methionine Dietary Deficiencies Have Different Effects on Lung Function and Lung Inflammation

So far, we have documented the impact of concomitant methionine and choline deficiency. Here, we wanted to investigate the respective impact of methionine or choline deficien-

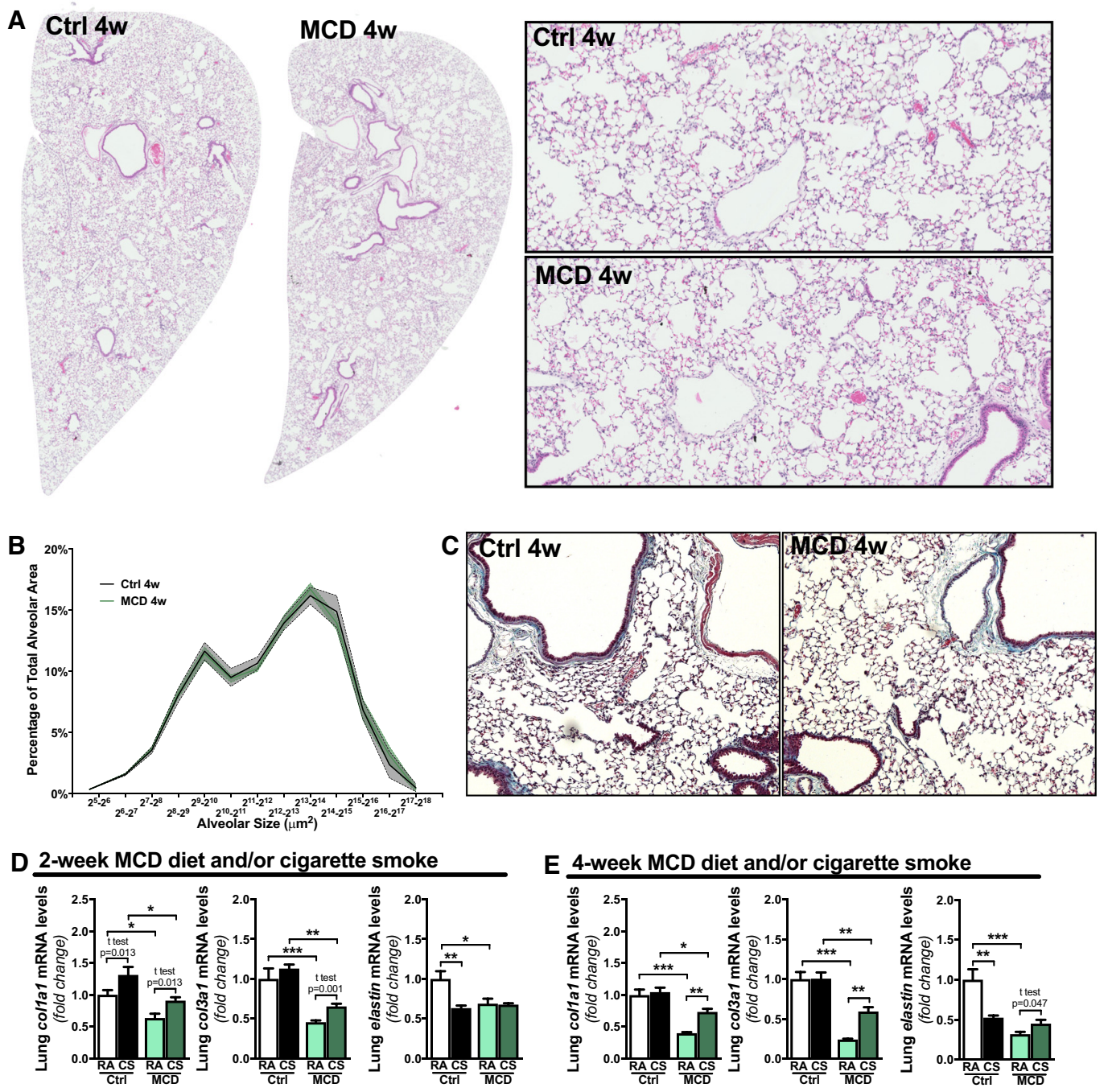

Fig. 6. Impact of dietary methionine and choline deficiency on alveolar size, collagen deposition, and extracellular matrix gene expression during cigarette smoke exposure. Female C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for 2 or 4 wk and fed control (Ctrl) diet or methionine/choline-deficient (MCD) diet for the same period. *A*: hematoxylin and eosin (H&E) staining of formalin-fixed paraffin-embedded lung sections. *B*: quantification of alveolar size based on H&E-stained lung sections. *C*: Masson's trichrome staining of formalin-fixed paraffin-embedded lung sections. Lung mRNA levels of *col1a1*, *col3a1*, and *elastin* assessed by qPCR in female C57BL/6 mice exposed to RA or CS for (D) 2 wk and (E) 4 wk and fed control diet or MCD diet for the same period, respectively. Data are presented as means ± SE; $n = 5$ mice/group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

cies on lung function and the response to cigarette smoke exposure. Mice were fed either a control, methionine-deficient (MD), or choline-deficient (CD) diet for 4 wk and exposed to room air or cigarette smoke during that period. Methionine deficiency alone caused a pulmonary phenotype similar to MCD diet, only milder, exhibiting a restrictive lung function pattern (Fig. 7*A*). To the contrary, choline deficiency led to the opposite phenotype: increased inspiratory capacity and compliance and a reduced tissue damping (Fig. 7*A*). Only MD diet caused reduction in body weight, while CD diet had no significant effects (Fig. 7*B*). With regard to gene expression, both MD and CD diets reduced *col1a1*, *col3a1*, and *elastin* levels, albeit to a greater extent, in MD diet-fed animals (Fig. 7*C*). Both MD and CD diets led to a similar reduction in circulating phosphatidylcholine levels, but only MD-fed animals had significantly reduced BAL fluid phosphatidylcholine levels (Fig. 7*D*).

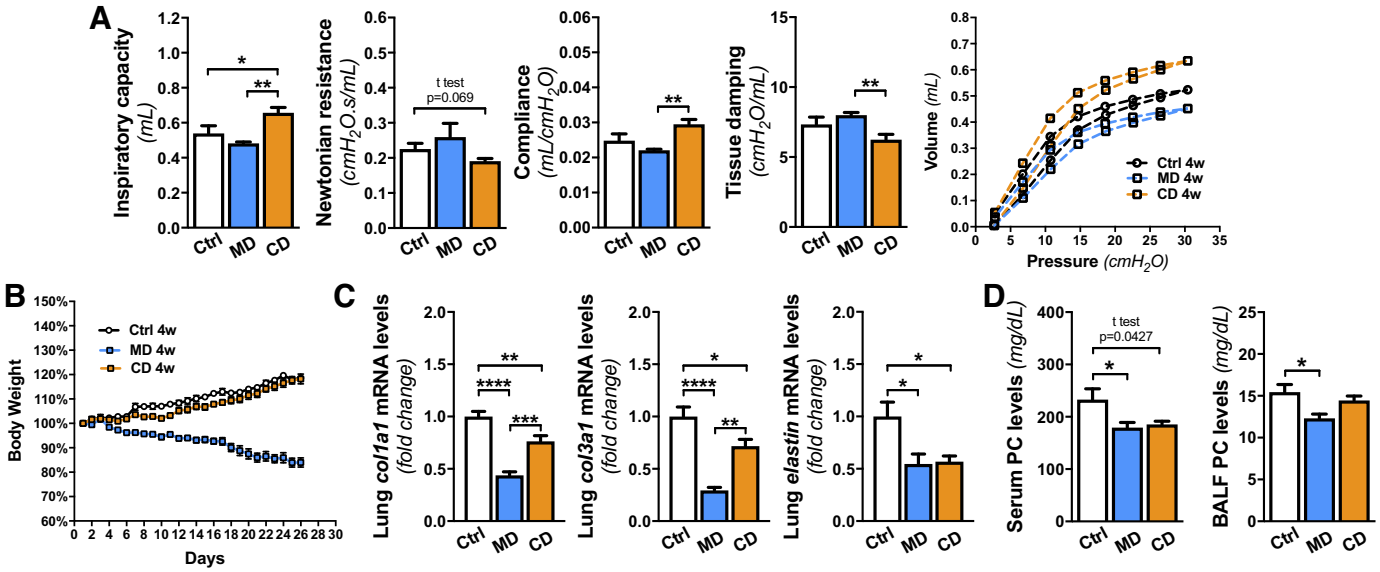

Fig. 7. Dietary deficiencies in choline or methionine affect pulmonary homeostasis differently. Female C57BL/6 mice were fed control (Ctrl) diet, methionine-deficient (MD) diet, or choline-deficient (CD) diet for 4 wk. *A*: lung functions were assessed using the FlexiVent. *B*: body weights were measured on a daily basis. *C*: lung mRNA levels of *col1a1*, *col3a1*, and *elastin* were assessed by quantitative PCR. *D*: phosphatidylcholine (PC) levels were assessed in the serum and bronchoalveolar lavage fluid (BALF). Data are presented as means ± SE; $n = 5$ mice/group. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

Regarding the impact of these diets on the pulmonary response to cigarette smoke exposure, the CD diet had no significant effect on lung function during cigarette smoke exposure compared with control diet-fed animals, while MD diet reproduced the effects observed in MCD-fed mice (Fig. 8*A*). As observed in room air-exposed mice, only MD diet caused body weight loss in cigarette smoke-exposed mice (Fig. 8*B*). Both MD and CD diets markedly reduced BAL total, mononuclear, and neutrophil cell counts (Fig. 8*C*). However, only the CD diet significantly reduced BAL fluid IL-1α and CCL2 levels (Fig. 8*D*) as well as lung *cxcl5* and *mmp12* mRNA levels (Fig. 8*E*). In cigarette smoke-exposed groups, CD diet had no significant impact on *col1a1*, *col3a1*, and *elastin* lung mRNA levels, while MD diet reduced *col1a1* and *col3a1* expression (Fig. 8*F*). Finally, only CD diet reduced circulating phosphatidylcholine levels and neither affected BAL fluid phosphatidylcholine levels in the context of cigarette smoke exposure (Fig. 8*G*). Altogether, MD and CD diets were found to have different effects on lung homeostasis, both interfering with the normal pulmonary response to cigarette smoke exposure.

DISCUSSION

This study reveals that dietary deficiencies in methionine and/or choline can affect lung function in normal homeostatic conditions and alter the pulmonary response to cigarette smoke exposure in terms of lung function and inflammation. We also document that these effects are both progressive in nature and rapidly reversible. Finally, mechanistic insights suggest that a reduced rate of lung extracellular matrix turnover and pulmonary surfactant biosynthesis are likely involved in the pathogenesis.

Dietary methionine and choline deficiency progressively affects lung function. These effects are also rapidly and completely reversible when the nutrients are reintroduced into the diet. This is also observed in the liver of mice fed the MCD diet, where steatosis, inflammation, and fibrosis develop progressively and sequentially over 2 to 6 wk with a high degree of reversibility (6, 12, 15, 18, 21, 22). While the adverse effects of dietary methionine and choline deficiency on the liver stem from reduced lipid transport from the liver to the circulation due to impaired VLDL and HDL synthesis, such mechanisms are unlikely to be involved in the pulmonary effects observed in this study. There are many potential mechanisms whereby this "essential nutrient starvation state" caused by dietary methionine and choline deficiencies can affect lung function and the response to cigarette smoke exposure.

The Pulmonary Response to an "Essential Nutrient Starvation State"

The MCD diet rapidly affected pulmonary transcription of genes associated with the extracellular matrix. This effect can also be observed in mice fed CD and MD diets, supporting the concept that both choline and methionine are important to maintain normal transcription of genes such as *col3a1*, *col1a1*, and *elastin*. However, methionine deficiency seemed to have a more pronounced impact. With regard to the mechanism, methionine is well known for its role in the initiation of protein synthesis. It is therefore possible that reduced methionine intake leads to reduced protein synthesis capability in the lungs. When looking at the histological sections of the lungs, we could not observe any reduction in cellularity. This suggests that, in the context of methionine and choline deficiency, lung extracellular matrix synthesis may have been sacrificed to prioritize synthesis of crucial pulmonary proteins and thus maintain the integrity of structural cells. This hypothesis would explain why the mechanical properties of the lung are rapidly affected by methionine and choline restriction, while the tissue itself remains relatively normal. The same concept can be applied to the pulmonary surfactant, where reducing extracellular

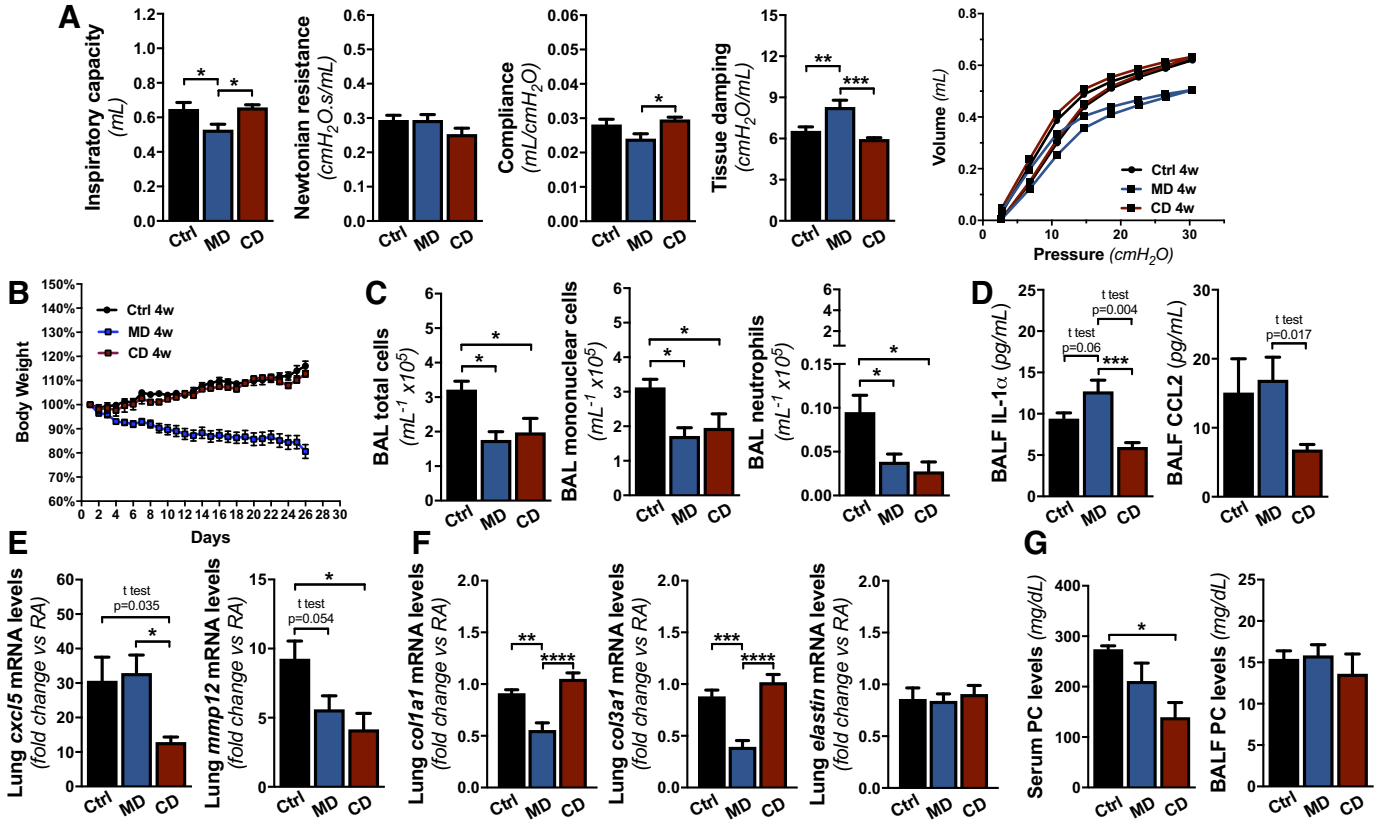

Fig. 8. Dietary deficiencies in choline or methionine affect the pulmonary response to cigarette smoke exposure differently. Female C57BL/6 mice were exposed to room air (RA) or cigarette smoke (CS) for 4 wk and fed control (Ctrl) diet, methionine-deficient (MD) diet or choline-deficient (CD) diet for the same period. *A*: lung functions were assessed using the FlexiVent. *B*: body weights were measured on a daily basis. *C*: total, mononuclear, and neutrophil cell counts were performed on the bronchoalveolar lavage (BAL). *D*: protein levels of interleukin 1 alpha (IL-1α) and monocyte chemotactic protein 1 (CCL2) were assessed in the BAL fluid (BALF). *E*: lung mRNA levels of *cxcl5* and *mmp12* were assessed by quantitative PCR (qPCR). *F*: lung mRNA levels of *col1a1*, *col3a1*, and *elastin* were assessed by qPCR. *G*: phosphatidylcholine (PC) levels were assessed in the serum and BALF. Data are presented as means ± SE; $n = 5$ mice/group. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

matrix and pulmonary surfactant production to a minimum to maintain the cellular integrity of the lung would also allow for rapid recovery upon reintroduction of methionine and choline in the diet. In fact, we did observe a very rapid return to homeostatic conditions after 1 week of return to the control diet, indicating that all the required cellular machinery was still functionally intact. While this hypothesis remains to be fully tested, it suggests an important adaptive pathway for the maintenance of lung integrity during times of essential nutrient restriction.

Interaction between Cigarette Smoke Exposure and Dietary Methionine/Choline Deficiency

Dietary methionine and/or choline deficiency also have very interesting effects on the pulmonary response to cigarette smoke exposure. First, the MCD and MD diets impact lung function. These diets led to a completely different pulmonary phenotype compared with control diet mice exposed to cigarette smoke, marked by reduced tissue damping and inspiratory capacity. Choline deficiency did not have this effect. These findings suggest that human smokers under methionine restriction could be more susceptible to atelectasis and air-trapping, likely exacerbating existing symptoms. A second noticeable impact of dietary methionine and/or choline deficiency during cigarette smoke exposure was the blunted inflammatory response and reduced neutrophil infiltration, in particular. Deficiencies in methionine, choline or both were associated with reduced pulmonary inflammation in response to cigarette smoke exposure and, in the case of MCD-fed mice, this blunted neutrophilia appeared before any effects on lung function. As we did not observe any change in circulating neutrophils (data not shown), it is therefore unlikely that the lungs of methionine- and/or choline-deficient mice were unable to mount a proper immune response. This question, therefore, remains open. A third interesting interaction between cigarette smoke exposure and dietary methionine and choline deficiency is the effect on extracellular matrix gene mRNA levels. Lungs from mice exposed to cigarette smoke and fed the MCD diet exhibited higher mRNA levels of *col3a1*, *col1a1,* and *elastin* compared with room air-exposed mice fed the MCD diet. This may indicate that, to maintain lung integrity, upregulation of extracellular matrix gene transcription was required during cigarette smoke exposure, thus interfering with the normal adaptation to dietary methionine and choline restriction observed in room air-exposed, MCD-fed mice.

Implications for Respiratory Health in Nonsmokers and Smokers

Since methionine and choline dietary insufficiency can impact the healthy lung, all lung diseases are likely impacted by

such dietary deficiencies. Daily intake of choline and methionine should therefore be considered when assessing pulmonary health. With regard to cigarette smoke exposure, dietary methionine and choline deficiency was found to affect three crucial aspects of the pulmonary response: lung function, extracellular matrix homeostasis and inflammation. While we do not know if methionine and choline deficiencies have significantly deleterious effects over time in smokers, it is likely that deficiency in these essential nutrients would affect pulmonary function and symptoms linked to inflammation such as exacerbations in patients with chronic obstructive pulmonary disease.

Study Limitations

The present study has limitations. We did not measure the concentration of smoke particulates in the chamber during the experimental protocols presented in this study, but we expect them to be similar to the levels reported by Botelho et al. (3) using a similar system.

As choline is a distinct essential nutrient with partial endogenous de novo synthesis, the observed effects of choline dietary deficiency on lung homeostasis are likely specific to choline. However, dietary methionine deficiency might not be restricted to this specific essential amino acid. In fact, it could reflect in its whole or in part the general presentation of essential amino acid deficiency. This aspect was not addressed in the study. There are eight other essential amino acids: valine, leucine, isoleucine, phenylalanine, tryptophan, lysine, histidine, and threonine. To the best of our knowledge, the specific impact of dietary deficiency on lung homeostasis for each essential amino acid has never been systematically addressed experimentally. However, given the breadth of extrapulmonary effects associated with specific essential amino acid deficiencies, it is likely that some of the observations made with methionine deficiency might also be observed with one or more the deficiencies in other essential amino acids. Therefore, this study cannot discriminate between methionine-specific effects and effects noticeable with dietary deficiencies in other essential amino acids.

This study adds to the emerging field of "nutri-respiratory" research and shows that some essential nutrients, such as choline and methionine, are crucial to pulmonary health. Further research is required to fully understand the mechanisms behind the impact of dietary choline and methionine deficiency on the lungs. Finally, given the drastic effect of methionine and choline deficiency on pulmonary health, more efforts should be devoted to studying the impact of dietary deficiencies in essential amino acids and other essential nutrients on lung biology and lung pathologies.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

E.J., M.L., and M.C.M. conceived and designed research; E.J., N.M., M.M.-R., M.-A.L., M.P., J.R., M.-J.B., and S.A. performed experiments; E.J., N.M., M.M.-R., M.-A.L., J.L., J.R., and M.C.M. analyzed data; E.J., N.M., M.-A.L., M.L., and M.C.M. interpreted results of experiments; E.J. and M.C.M. prepared figures; E.J. and M.C.M. drafted manuscript; E.J., N.M., M.M.-R., M.-A.L., M.P., J.L., J.R., M.-J.B., S.A., M.L., and M.C.M. approved final version of manuscript; N.M., M.M.-R., M.-A.L., M.P., J.L., J.R., M.-J.B., S.A., and M.L. edited and revised manuscript.

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