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RESEARCH ARTICLE

Inter-Organ Communication in Homeostasis and Disease

Increased exploration and hyperlocomotion in a cigarette smoke and LPS-induced murine model of COPD: linking pulmonary and systemic inflammation with the brain

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Abstract

Brain-related comorbidities are frequently observed in chronic obstructive pulmonary disease (COPD) and are related to increased disease progression and mortality. To date, it is unclear which mechanisms are involved in the development of brain-related problems in COPD. In this study, a cigarette smoke and lipopolysaccharide (LPS) exposure murine model was used to induce COPD-like features and assess the impact on brain and behavior. Mice were daily exposed to cigarette smoke for 72 days, except for *days 42*, *52*, and *62*, on which mice were intratracheally exposed to the bacterial trigger LPS. Emphysema and pulmonary inflammation as well as behavior and brain pathology were assessed. Cigarette smoke-exposed mice showed increased alveolar enlargement and numbers of macrophages and neutrophils in bronchoalveolar lavage. Cigarette smoke exposure resulted in lower body weight, which was accompanied by lower serum leptin levels, more time spent in the inner zone of the open field, and decreased claudin-5 and occludin protein expression levels in brain microvessels. Combined cigarette smoke and LPS exposure resulted in increased locomotion and elevated microglial activation in the hippocampus of the brain. These novel findings show that systemic inflammation observed after combined cigarette smoke and LPS exposure in this COPD model is associated with increased exploratory behavior. Findings suggest that neuroinflammation is present in the brain area involved in cognitive functioning and that blood-brain barrier integrity is compromised. These findings can contribute to our knowledge about possible processes involved in brain-related comorbidities in COPD, which is valuable for optimizing and developing therapy strategies.

behavior; cigarette smoke exposure; COPD; inflammation; lung-brain axis

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by chronic pulmonary inflammatory responses causing airway restriction that is not fully reversible. COPD is being acknowledged as a disease that affects more than solely the lungs (1). It is a heterogeneous and multicomponent disease as specific aspects of the disease, such as disease severity, exercise tolerance, and prevalence of comorbidities, can differ between patients who have a similar grade of airflow limitation (2). In recent years, the occurrence of extrapulmonary comorbidities in COPD has been demonstrated in various studies. These comorbidities include cardiovascular disease, osteoporosis, diabetes, mental health disorders, and cognitive impairment (3, 4). Among the extrapulmonary comorbidities in COPD, brain-related comorbidities are of increasing interest because of their impact on overall health and treatment outcomes. Brain-related comorbidities in COPD include mental health problems, such as anxiety and depression, and cognitive impairment (5–14). Although there are some inconsistencies between studies, most studies demonstrate an increased prevalence of mental health problems and cognitive impairment in patients with COPD, frequently two to three times higher than in healthy subjects (4). Brain-related comorbidities have been associated with an increased disease progression and mortality (15–17). In addition, patients with COPD

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suffering from mental health disorders often show poor treatment adherence and efficacy (18, 19). It is therefore of importance to increase the focus on and understanding of these comorbidities when treating patients with COPD.

An exacerbation, defined as a sustained worsening of a patient's condition (20), is a significant factor modulating disease progression and mortality (21). It is mostly caused by a bacterial and/or viral infection and often requires hospitalization and antibiotic treatment (20). In most cases, patients with COPD do not fully recover from an exacerbation. As demonstrated by Dodd et al. (16), cognitive impairment is one of the consequences of exacerbations requiring hospitalization and may not improve within 3 mo after recovery. Therefore, optimizing treatment strategies during and after exacerbations are vital to reduce the development of comorbidities and to improve overall health in these patients.

Inflammatory processes that are elevated in COPD or during COPD exacerbations might play a role in the development of brain comorbidities (22). In general, ongoing systemic inflammatory processes, but also local neuroinflammatory processes within the brain, are important triggers for developing depression and cognitive impairment (23). Correspondingly, it was demonstrated that depressive symptoms are associated with pulmonary obstruction, and that higher levels of interleukin-6 (IL-6) and C-reactive protein (CRP) partly account for this association (24). Although there are indications that (systemic) inflammation can play a role in the development of brain comorbidities, neuroinflammatory processes within the brain have not yet been demonstrated in COPD. Potentially, both sustained exposure to harmful components in tobacco smoke and inflammatory processes during and after an exacerbation may induce neuroinflammation and subsequently cause functional impairment of the brain.

Experimental models of COPD exacerbations allow us to carefully investigate processes in the brain that are relevant for brain-related comorbidities. Numerous studies with murine models of COPD exacerbations have been performed so far (25–35). Studies have mostly focused on the effects of cigarette smoke exposure on the cardiovascular system and skeletal muscle function (32, 36). However, focus on studying brain-related comorbidities in these models is very limited; hence, we used a mouse model of COPD to study the impact on behavior and the brain. Mice were exposed to cigarette smoke—the main inducer of COPD in humans—and lipopolysaccharide (LPS, mimicking a bacterial trigger) to induce emphysema and pulmonary inflammation, respectively. In this way, we can gain more insight into the relationship between emphysema and exacerbations in the lungs, and impairments in the brain. In addition, we can investigate which areas within the brain are affected and whether inflammation plays a role. This knowledge will contribute to the development of more accurate treatment strategies targeting brain-related comorbidities and with this, improve the overall health condition in COPD after exacerbations.

MATERIALS AND METHODS

Animals and Experimental Design

Female Balb/cByJ mice (n = 90; Charles River Laboratories, France), 11–13 wk old, were group-housed (n = 3 or 4/cage) in filtertop cages under controlled conditions with a 12-h day-night light cycle and ad libitum access to water and food (AIN-93M, SSNIFF, Soest, Germany). All procedures within the animal experiment were performed according to governmental guidelines and approved by the Ethical Committee for Animal Research of the Utrecht University, the Netherlands (AVD1080020184785).

Mice were divided into one of the experimental groups based on average body weight (Fig. 1). Mice were whole body exposed to cigarette smoke or air from *day 0* until *day 72*, except for *days 42*, *52*, and *62*. A Plexiglas box containing four metal cages each with four compartments was used to expose the mice to either cigarette smoke or air. Two mice from the same home cage were placed in each compartment. Before starting the cigarette smoke exposure, filters from the cigarettes were removed. Cigarette smoke of 3R4F reference cigarettes (University of Kentucky, Lexington, Kentucky) was run into the box using a peristaltic pump set at 35 rpm. A vacuum pump was attached to the bottom of the box to mix the cigarette smoke with air and to maintain a constant flow in the box. A carbon monoxide level between 200 and 400 ppm was sustained. Cigarette smoke exposure was gradually increased in the first 5 days from 4 to 14 cigarettes per day, corresponding to a duration of ~45 min. This was maintained for the remainder of the experiment.

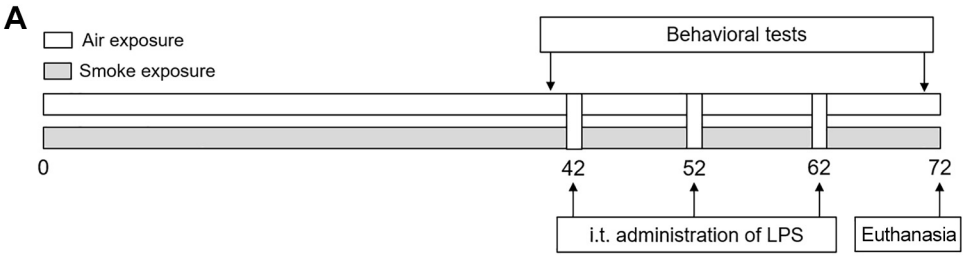
To mimic exacerbations, 50 μL of 2 or 10 μg/mL lipopolysaccharide solution (LPS; *Escherichia coli*, serotype O55:B5; Sigma-Aldrich, St. Louis, MO) was intratracheally injected on *days 42*, *52*, and *62*, as previously described (37). Briefly, mice were anesthetized with isoflurane and positioned vertically after which a cannula was inserted into the trachea. Using a Hamilton gastight syringe, the solution was injected followed by an equal volume of air to secure complete administration. The study design is presented in Fig. 1*A*. Behavioral tests were performed in air or cigarette smoke-exposed mice on *days 40–41*, before the first LPS/vehicle exposure, and *days 70–71*, at the end of the study. Food intake was monitored by weighing the remaining food and newly added food once per week.

Open Field Test

The open field test was performed on *days 40* and *70* (Fig. 1*C*) as previously described (38). Tests were performed in a brightly lit room under white light conditions during the light phase of the day. In short, mice (n = 11 or 12/group; randomly selected) were placed in the center of a black open field (45 × 45 cm) and allowed to explore for 5 min. After every run, the open field was cleaned with 70% ethanol. Recordings were analyzed using video tracking software (EthoVision XT 14.0, Noldus, Wageningen, the Netherlands). The inner zone was established digitally at 10 cm from the wall. Several parameters including total distance moved, and frequency and time spent in the inner zone and corners were determined with post-acquisition analysis.

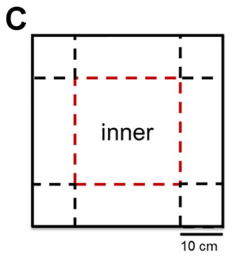
Social Interaction Test

On *days 41* and *71*, the social interaction test was performed (Fig. 1*D*) during the light phase as previously described (39). In short, mice (n = 11 or 12/group; randomly selected) were placed in a black 45 × 45 cm open field



B

	Air	Smoke
Vehicle	15	15
2 μg/mL	14	16
10 μg/mL	15	15



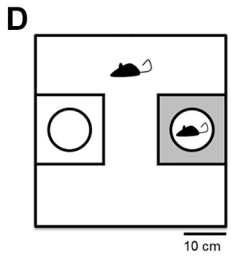


Figure 1. Study design. Mice were exposed to cigarette smoke or air throughout the study, except for *days 42*, *52*, and *62* (*A*). On these days, mice were intratracheally exposed to LPS or vehicle (*n* per group is listed in *B*). Two days before the first LPS/vehicle exposure and before euthanization, behavioral tests were performed. These consisted of the open field test (*C*) and social interaction test (*D*). For the open field test, a central square of 25 × 25 cm was considered the inner zone and the remaining area was considered the periphery. For the social interaction test, two Plexiglas transparent cages were put in the open field arena and an unfamiliar mouse was placed in one of the cages, after which the interaction of the test mouse with the unfamiliar mouse (gray square) was recorded and compared with the interaction with the empty cage (white square). LPS, lipopolysaccharide.

containing two perforated Plexiglas cages (10 cm diameter). The cages were located against opposite walls and allowed visual, olfactory and minimal tactile interaction (39). In the first 5 min, mice were habituated to the open field. Afterward, the mouse was returned to the home cage and an unfamiliar age-matched female mouse was introduced to one of the cages. The mouse was then reintroduced to the open field for an additional 5 min. Open fields and cages were cleaned with 70% ethanol and dried after each run. Using video tracking software (EthoVision XT 14.0, Noldus, Wageningen, the Netherlands), an interaction zone was established around each cage. A ratio of time spent in the interaction zone of the unfamiliar mouse cage versus the empty cage and total distance moved were obtained.

Assessment of Emphysema

Mice (n = 45) were euthanized by decapitation under anesthesia (ketamine-dexmedetomidine; 196.8 mg/kg and 1.32 mg/kg, respectively; intraperitoneal). Lungs were fixed with 10% formalin via a cannula at a constant pressure of 25 cmH_2O for at least 5 min. Afterward, left lungs were stored in 10% formalin for a minimum of 24 h followed by paraffin embedding. Sections of 5 μm were cut and stained with hematoxylin-eosin. Six random photomicroscopic images were used to determine the average inter-alveolar distance using a reference grid as previously described (40). This mean linear intercept (L_m) analysis was performed by dividing the grid length by the number of alveolar wall-grid line intersections.

Bronchoalveolar Lavage

After anesthesia with ketamine-dexmedetomidine, mice (n = 45) were euthanized by maximal blood collection from cardiac puncture. A cannula was inserted into the trachea and lungs were first lavaged with 1 mL 0.9% NaCl (37°C) supplemented with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Germany) for cytokine measurements. Subsequently, lungs were lavaged three times with 1 mL nonsupplemented 0.9% NaCl (37°C). To isolate the bronchoalveolar lavage (BAL) cells, all lavages were centrifuged (400 *g*, 5 min) and pellets were pooled and resuspended in Türk solution. A Bürker-Türker chamber was used to determine the total BAL cell counts. For the differentiation of BAL cells, cytospins were prepared and stained with DiffQuick (Merz and Dade A.G., Switzerland). Using standard morphology, an observer differentiated the cells into macrophages, neutrophils, and lymphocytes in each sample. Around 200 cells were counted in each sample, and the absolute number for each cell type was calculated using the total BAL cell counts of the corresponding sample (40).

Cytokines and Chemokines in BAL Fluid and Serum

Blood was collected in Mini collect tubes (Greiner Bio-One, Alphen aan den Rijn, the Netherlands) and centrifuged at 14,000 *g* for 10 min. Serum was stored at −20°C. A mouse multiplex assay (ProcartaPlex, Thermo Fisher Scientific, Austria) was used according to the manufacturer's instructions to measure IL-10, IL-12p40, IL-1β, IL-6, keratinocyte chemoattractant (KC), leptin, tumor necrosis factor-α, and vascular endothelial growth factor A (VEGF-A) in BAL fluid (BALF) and serum. For BALF, only results regarding IL-12p40, KC, and VEGF-A, and for serum, only results regarding KC, leptin and VEGF-A were included, because other markers were either below detection level or no differences between groups were observed (data not shown). In addition, C-reactive protein (CRP) levels were determined in BALF and serum using an enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer's instructions. Results of CRP, IL-12p40, KC, and VEGF-A levels in BALF were used for correlations only. For serum, the CRP, KC, and VEGF-A results were used for correlations only. In addition to correlation analyses, measurements of leptin levels in serum are presented in this study.

Immunohistochemistry and Image Analysis

After euthanization, brains of mice used for emphysema assessment and behavioral testing (n = 6 mice per group; randomly selected) were isolated and right hemispheres were fixed in 10% formalin for at least 24 h followed by 30% sucrose until further processing. Coronal sections of 40 μm were incubated overnight with blocking serum followed by

incubation with rabbit anti-iba-1 (1:1,000; 019–197410, Wako Chemicals, Germany) for 24 h. Next, sections were incubated with fluorophore-tagged donkey anti-rabbit secondary antibody (1:500; A-21207, Invitrogen) for 2 h. Finally, Prolong Gold mountant containing DAPI (Invitrogen) was added to the sections.

In the cingulate cortex—the equivalent of the anterior cingulate cortex (ACC)—amygdala, and hippocampal CA1 area, microglial cells were captured by imaging z-stacks with a step size of 1 μm using a confocal microscope (Leica TCS SP8 X) (41). In total, four z-stacks per brain area were captured in two brain sections. At least 12 cells per animal were included and only microglial cell somas that were fully captured were included in the analysis. Using Imaris 9.6.0 software, microglial cell numbers (occupancy) and soma volume, sphericity, oblate, and prolate ellipticity were determined in a blind manner by manually setting the threshold for accurate detection of each soma.

Microvessel Isolation

After euthanization, brains of mice used for BAL isolation were harvested. These brains together with the left hemispheres from the mice used for emphysema assessment were snap frozen using isopentane, and stored at −80°C. The protocol for microvessel isolation has been adapted from previously published protocols (42–44). Meninges and cerebella were removed from left hemispheres (n = 48 mice in total; n = 24 from BAL isolation animals; n = 24 from emphysema assessment animals). Hemispheres of two mice within one treatment group were pooled and homogenized in ice-cold DMEM (Thermo Fisher Scientific) using a glass tissue grinder with small clearance pistil (KIMBLE, Germany) for 30 up-and-down strokes. After adding 5-mL DMEM, samples were centrifuged for 20 min (4,300 g, 4°C). The pellet was resuspended in 7-mL 18% dextran (MW 100,000; Sigma-Aldrich) in DMEM, after which 5-mL DMEM with 31% dextran was added. The samples were then centrifuged for 20 min at 4,300 g (4°C, brake off). The interphase and lower phase of this resulting dextran gradient were collected and filtered using a 70-μm filter covered with a layer of glass beads (450–600 nm, Sigma-Aldrich). After collection in PBS, beads were centrifuged for 20 min (4,300 g) and resuspended in 0.5-mL PBS. The aqueous phase was collected and centrifuged for 10 min at 14,000 rpm. The pellet was resuspended in 100-μL lysis buffer (15% protease inhibitor solution in RIPA buffer) and the isolated microvessels were homogenized using a tissue homogenizer (Precellys 24, Bertin Instruments, France).

Western Blotting

Protein concentrations were determined using a Pierce BCA protein assay kit (Thermo Fisher Scientific). A total of 8 μg protein was loaded into a midi Criterion precast protein gel (4%–20%, Bio-Rad), separated by gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Bio-Rad) using the Trans-Blot Turbo system (Bio-Rad). Blots were blocked with 5% milk powder in PBST (0.1% Tween 20 in PBS) at room temperature for at least 1 h. Afterward, blots were incubated with primary antibodies for beta actin (1:2,000; Cell Signaling Technology), claudin-5 (1:500; 34-1600, Invitrogen), occludin (1:500; 40-4700, Invitrogen), or ZO-1 (1:750; 40-2200, Invitrogen). Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence substrate were used for detection. Quantification of band intensity was performed using ImageJ 1.52p. Peak ratios to β actin were calculated to compare relative expression levels for each protein.

Statistical Analysis

Two-way analysis of variance (ANOVA) was performed to identify effects of smoking and/or LPS, followed by a Šidák's multiple comparisons test. For effects of cigarette smoke exposure on body weight over time, a repeated-measures ANOVA test was used. Independent samples t tests were used to assess the effects of cigarette smoke exposure on behavior in the open field and social interaction tests at T1. When data were not normally distributed, the data were transformed, or alternative nonparametric analyses were applied. This was applied for the number of neutrophils and lymphocytes in BAL, the time spent in the inner zone, time spent in corners, frequency of entering the inner zone, and ratio of time spent in the inner zone to the periphery of the open field at T1 and T2, distance walked in the social interaction test at T1, microglia soma volume in ACC, and protein expression levels of the 22 kDa claudin-5 in brain microvessels. One animal in the air + LPS2 group was removed from the L_m analysis because of unforeseeable problems with processing of the tissue. One animal in the CS + vehicle group was removed from the BAL cell analyses as it was an outlier in the total cell number. For correlations, either Pearson's or Spearman's rank correlations were applied. GraphPad Prism 9 was used to perform statistical analyses. Statistical significance was considered when $P < 0.05$, and trends were considered when $0.05 < P < 0.08$. Data are presented as means ± SEM.

RESULTS

Lung Emphysema and Inflammation

Mean linear intercept (L_m) is a measure of alveolar size in the lungs and was used to determine the presence and severity of emphysema in histological lung sections (40). Histological sections show increased airspaces in cigarette smoke-exposed mice (Fig. 2, *A–F*). There was a significant effect of cigarette smoke on L_m values [$F(1, 38) = 67.11$; $P < 0.0001$]. Multiple comparisons analysis revealed a significant increased L_m in all cigarette smoke-exposed groups as compared with the respective air control group (Fig. 2*G*). No significant effects of LPS exposure on L_m were observed. L_m values correlated significantly with serum CRP levels (r = 0.47; Supplemental Table S1).

Ten days after the final LPS exposure, the number of inflammatory cells in bronchoalveolar lavage (BAL) was determined to assess the presence and degree of lung inflammation. There was a significant effect of both cigarette smoke [$F(1, 38) = 62.24$; $P < 0.0001$] and LPS [$F(2, 38) = 3.334$; $P < 0.05$] on total BAL cell numbers. In all cigarette smoke-exposed groups, the total cell number was significantly higher compared with the respective air-exposed group (Fig. 3*A*). In

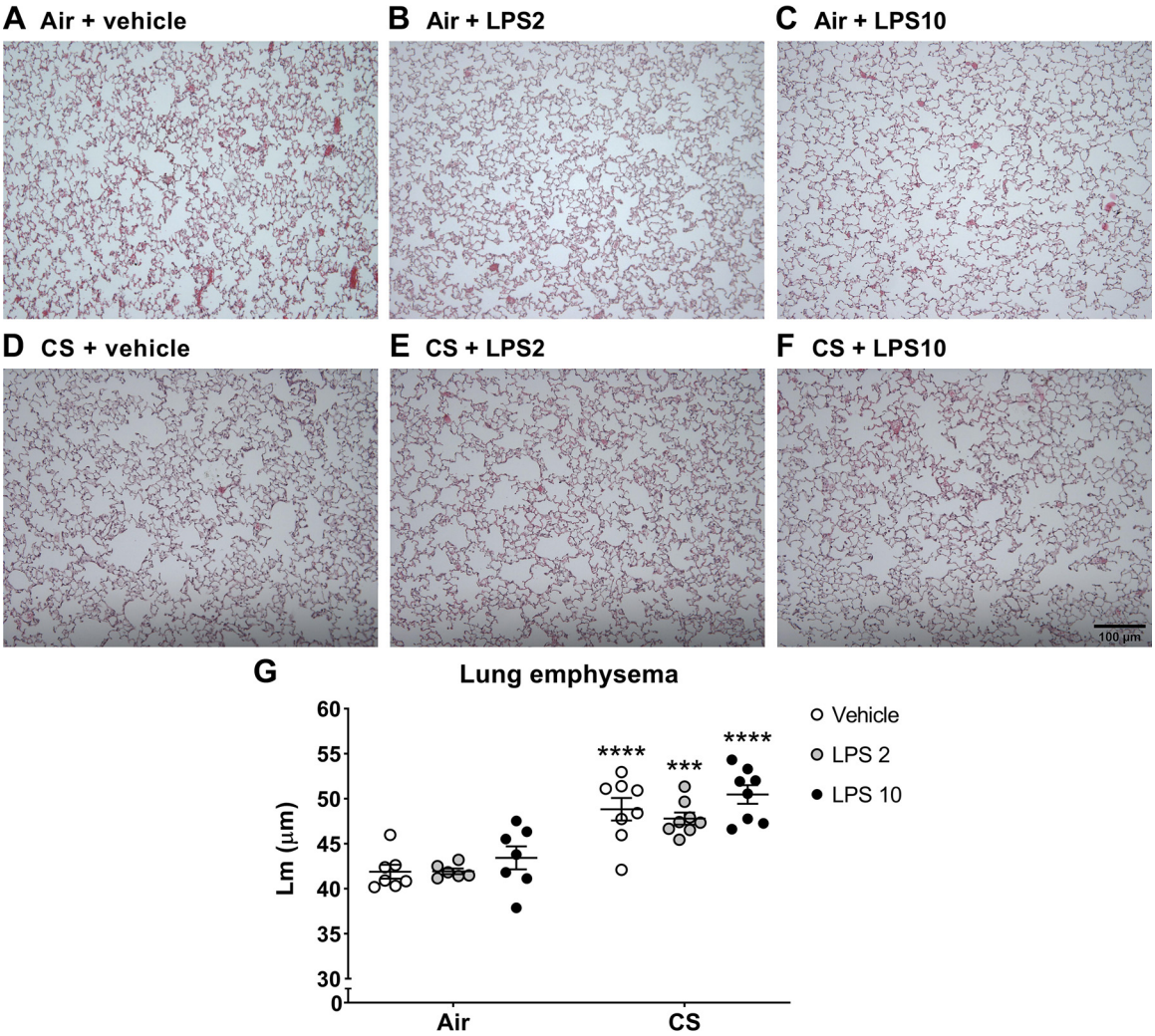


Figure 2. Photomicrographs of H&E-stained lung sections in the experimental groups (*A–F*; ×100 magnification; scale bar = 100 µm). Mean linear intercept (L_m; *G*) was determined in H&E-stained lung slices. There was a significant increase in L_m after cigarette smoke exposure independent of combination with LPS. Data are presented as means ± SEM. ***$P < 0.001$; ****$P < 0.0001$ compared with respective air control group. Air + vehicle: $n = 7$ mice; air + LPS2: $n = 6$ mice; air + LPS10: $n = 7$ mice; CS + vehicle: $n = 8$ mice; CS + LPS2: $n = 8$ mice; CS + LPS10: $n = 8$ mice. CS, cigarette smoke; H&E, hematoxylin-eosin; LPS, lipopolysaccharide.

addition, air-exposed mice treated with the highest LPS concentration showed significantly higher total inflammatory cell numbers as compared with vehicle-treated mice ($P < 0.05$).

Total BAL cells were differentiated to determine macrophage, neutrophil and lymphocyte cell numbers. There was a significant effect of cigarette smoke [$F(1, 38) = 25.6$; $P < 0.0001$] and LPS [$F(2, 38) = 7.348$; $P < 0.01$] exposure on macrophage numbers. In both air- and cigarette smoke-exposed animals, the highest LPS concentration significantly increased macrophage numbers (Fig. 3*B*).

Both cigarette smoke [$F(1, 38) = 136.8$; $P < 0.0001$] and LPS [$F(2, 38) = 4.281$; $P < 0.05$] had a significant effect on neutrophil numbers. In addition, a significant interaction effect of cigarette smoke and LPS exposure on neutrophil numbers was found [$F(2, 38) = 4.167$; $P < 0.05$]. Cigarette smoke exposure significantly increased neutrophil numbers whereas additional exposure to the highest LPS concentration resulted in a significant decrease in neutrophil numbers ($P < 0.01$; Fig. 3*C*).

Finally, a significant effect of cigarette smoke exposure on lymphocyte numbers was observed [$F(1, 38) = 7.7$; $P < 0.01$], which was only present in the vehicle treated group (Fig. 3*D*).

In addition, the number of total BAL cells, macrophages and neutrophils significantly correlated with levels of all inflammatory markers in BALF, including CRP, IL-12p40, KC, and VEGF-A (Supplemental Table S1). The number of lymphocytes only significantly correlated with IL-12p40 and KC levels in BALF. The increased number of total BAL cells, macrophages and neutrophils significantly correlated with the increase in KC serum levels. All pulmonary inflammatory cell numbers correlated negatively with serum leptin levels (Supplemental Table S1).

Body Weight and Leptin Levels

During the first 6 wk of the experiment, the effect of cigarette smoke exposure on body weight was assessed. In these weeks, there was a significant change in body weight over

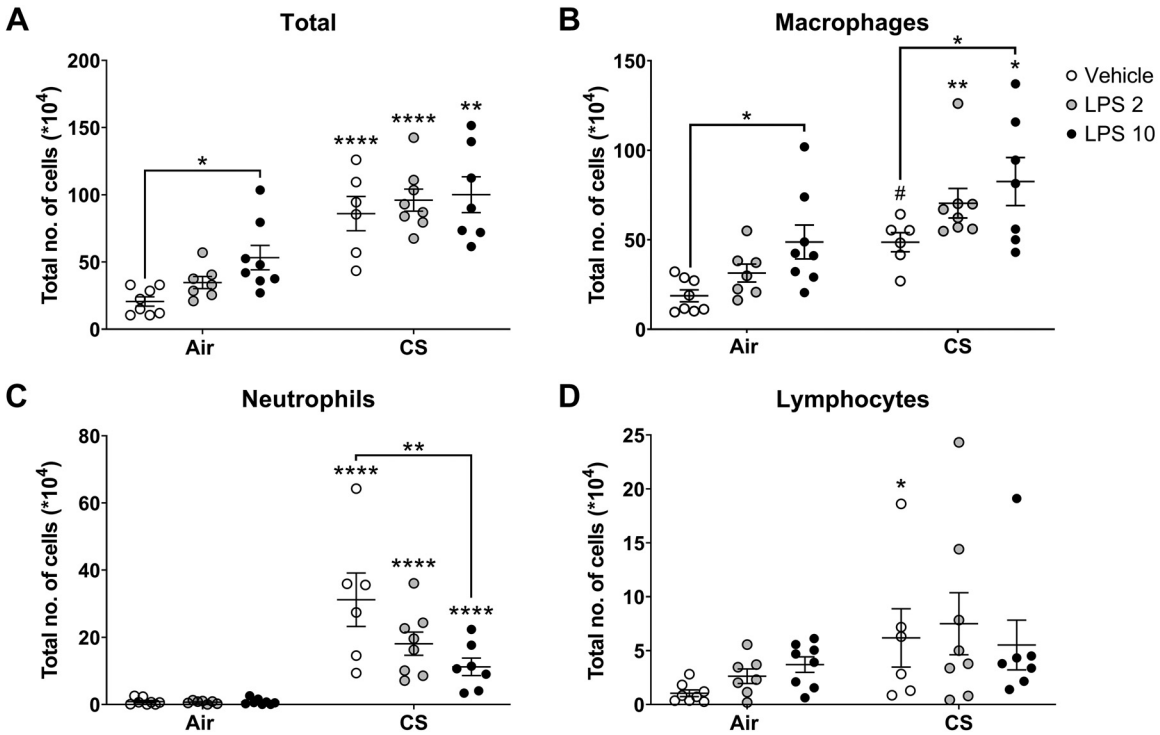


Figure 3. Inflammatory cell numbers in bronchoalveolar lavage (BAL) are increased after cigarette smoke and/or LPS exposure. Total number of cells (*A*) was determined from BAL fluid (BALF) samples and cells were differentiated into macrophages (*B*), neutrophils (*C*), and lymphocytes (*D*). Data are presented as means ± SEM. Asterisks above a group represent comparisons with the corresponding air exposed group. $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$; $\#P = 0.052$. Air + vehicle: $n = 8$ mice; air + LPS2: $n = 7$ mice; air + LPS10: $n = 8$ mice; CS + vehicle: $n = 6$ mice; CS + LPS2: $n = 8$ mice; CS + LPS10: $n = 7$ mice. CS, cigarette smoke; LPS, lipopolysaccharide.

time [$F(6, 528) = 53.87$; $P < 0.0001$; Fig. 4*A*] in the repeated measures ANOVA test. In addition, there was a significant overall effect of cigarette smoke exposure [$F(1, 88) = 44.14$; $P < 0.0001$] and of cigarette smoke exposure over time [$F(6, 528) = 96.55$; $P < 0.0001$]. Mean body weight significantly correlated with mean food intake per cage during these first 6 wk of cigarette smoke exposure (Supplemental Fig. S1). There was no additional effect of LPS exposure on body weight (data not shown).

In addition to body weight, we measured leptin levels in serum since leptin is one of the adipokines regulating appetite. A significant effect of cigarette smoke exposure on leptin levels was observed [$F(1, 70) = 36.56$; $P < 0.0001$; Fig. 4*B*]. In all cigarette smoke-exposed groups, the leptin levels were significantly lower as compared with the air-exposed control group. No additional effect of LPS on leptin levels was observed. There was a significant positive correlation between body weight and leptin levels in serum (Supplemental Fig. S1). Finally, a trend in the association between serum leptin levels and L_m values was present ($r = -0.34$).

For further parameters, we only analyzed LPS10-exposed groups.

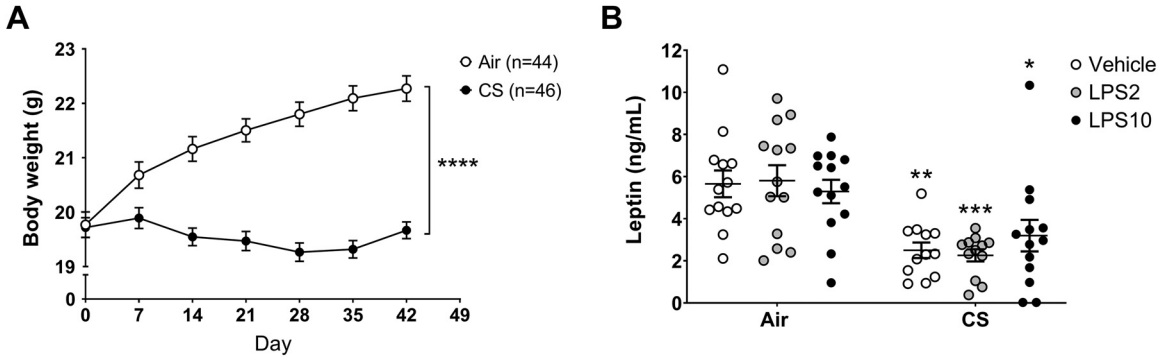


Figure 4. Body weight during the first 6 wk of the experiment (*A*), representing the effect of 6 wk of daily cigarette smoke exposure on body weight ($n = 44/46$ mice per group). At the end of the study, the effect on body weight was accompanied by lower levels of leptin in serum (*B*). Data are presented as means ± SEM. $****P < 0.0001$ with repeated measures ANOVA. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ compared with the respective air-exposed group. Air + vehicle: $n = 13$ mice; air + LPS2: $n = 13$ mice; air + LPS10: $n = 13$ mice; CS + vehicle: $n = 12$ mice; CS + LPS2: $n = 12$ mice; CS + LPS10: $n = 13$ mice. CS, cigarette smoke; LPS, lipopolysaccharide.

Open Field Behavior

The open-field test was performed twice to examine locomotion and exploratory behavior after cigarette smoke and/or LPS exposure throughout the study. After 6 wk of cigarette smoke exposure, a significantly higher total distance walked in the open field test was observed in cigarette smoke-exposed mice as compared with air-exposed mice [$t(43) = 3.765$; $P < 0.001$; Fig. 5*A*]. A trend toward increased time spent in the inner zone was observed in cigarette smoke-exposed mice (Welch's t test; $P = 0.068$; Fig. 5*B*).

At the end of the study, there was a significant effect of cigarette smoke exposure on total distance walked [$F(1, 43) = 8.509$; $P < 0.01$], but no significant overall effect of LPS was observed. Multiple comparisons revealed a significant higher total distance walked in cigarette smoke- and LPS-exposed mice as compared with air- and LPS-exposed mice (Fig. 5*C*). In addition, significant differences in time spent in the inner zone were observed (Kruskal–Wallis test; $P < 0.01$). Dunn's multiple comparisons revealed significant more time spent in the inner zone by both cigarette smoke-exposed groups as compared with the respective air-exposed groups ($P < 0.05$; Fig. 5*D*). Additional parameters including time spent in the corners, frequency of entering the inner zone, and the ratio of time spent in the inner zone to periphery are presented in Supplemental Fig. S2.

There was a significant positive correlation between time spent in inner zone and KC serum levels ($r = 0.37$; $P < 0.05$; Fig. 5*E*) and a negative correlation between time spent in inner zone and leptin serum levels ($r = -0.48$; $P < 0.01$; Fig. 5*F*). No significant correlations between distance moved and inflammatory markers in serum were observed (Supplemental Table S1).

Social Interaction Behavior

After 6 wk of cigarette smoke exposure, no differences in distance moved in the social interaction test were observed

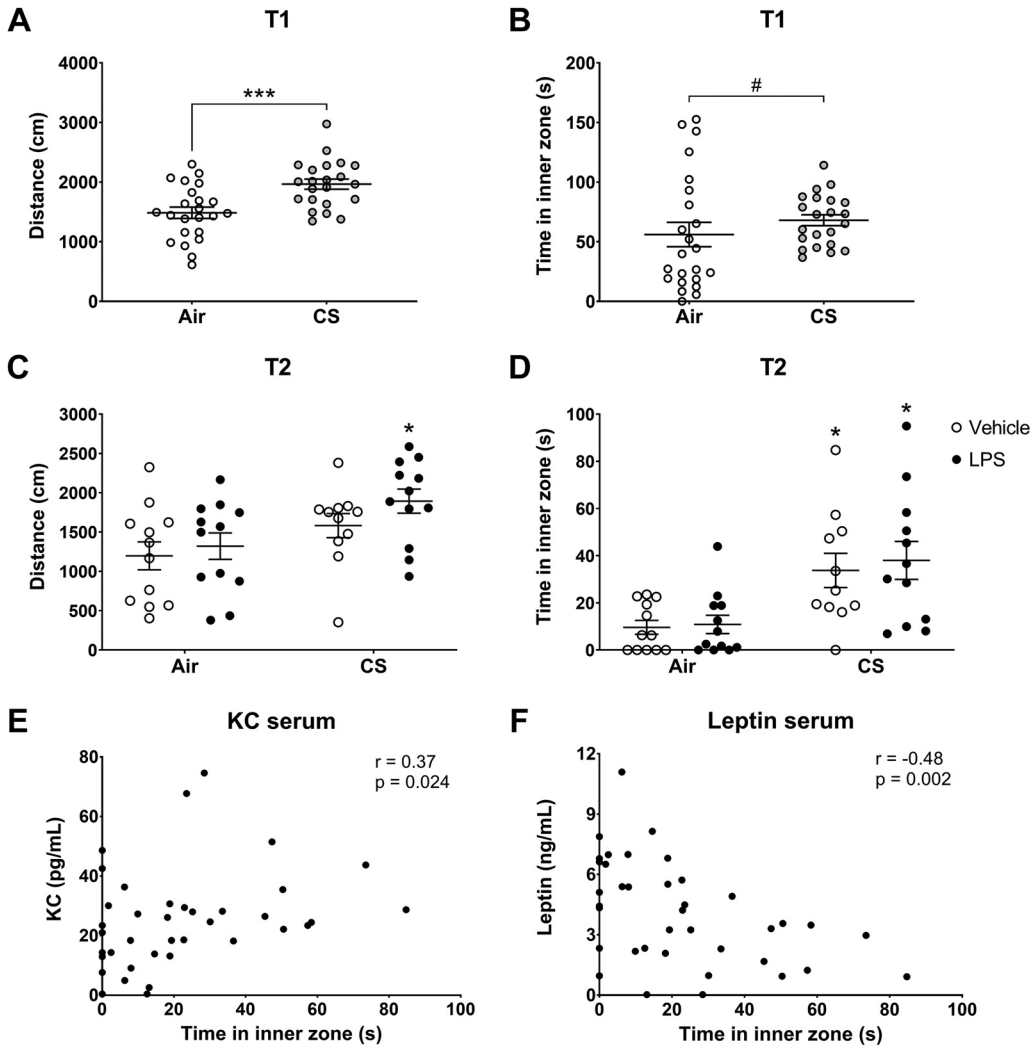


Figure 5. Parameters in the open field test after 6 wk of cigarette smoke exposure (T1; *A* and *B*; air: $n = 23$; CS: $n = 22$) and at the end of the study (T2; *C* and *D*). Depicted are distance walked (*A* and *C*) and time spent in the inner zone of the open field (*B* and *D*). Significant correlations were observed between the time spent in the inner zone and serum cytokine levels of KC (*E*) and leptin (*F*). Data are presented as means ± SEM. ***$P < 0.001$; #$P = 0.068$. *$P < 0.05$ as compared with the respective air-exposed group. Air + vehicle: $n = 12$ mice; air + LPS: $n = 12$ mice; CS + vehicle: $n = 11$ mice; CS + LPS: $n = 12$ mice. CS, cigarette smoke; KC, keratinocyte chemoattractant; LPS, lipopolysaccharide.

(Fig. 6*A*). Cigarette smoke-exposed mice spent more time within the unfamiliar mouse zone as compared with air-exposed controls [$t(43) = 2.495$; $P < 0.05$; Fig. 6*B*].

At the end of the study, there was a significant interaction effect between cigarette smoke and LPS [$F(1, 43) = 7.544$; $P < 0.01$] on distance moved but no significant effect of either of these factors was observed (Fig. 6*C*). In addition, no effects on the interaction with the unfamiliar mouse were observed (Fig. 6*D*).

Microglial Activation in the Brain

Within the brain, microglia cells are the main immune cells, comprising around 75%–80% of all brain immune cells (45). An increased activation and number, either by increased proliferation or expression of iba-1, are indications for the presence of neuroinflammation (46, 47). To assess the presence and degree of neuroinflammation, we measured the occupancy and soma volume of microglial cells in several brain areas: anterior cingulate cortex (ACC), amygdala, and hippocampus. Examples of merged z-stack immunohistochemical images in the hippocampus used for quantification are depicted in Fig. 7*A*. In both ACC and amygdala, there were no differences in occupancy or soma volume (Supplemental Fig. S3). No differences in the microglial cell occupancy were found in the hippocampus (Fig. 7*B*). There was a significant effect of cigarette smoke exposure on microglial soma volume in the hippocampus [$F(1, 20) = 9.428$; $P < 0.01$]. An increased soma volume in the cigarette smoke- and LPS-exposed mice was observed as compared with the air-exposed controls (Fig. 7*C*). Additional morphological parameters are included in Supplemental Fig. S4.

The microglial soma volume in any of the brain areas did not show any significant correlations with any of the inflammatory markers in serum (Supplemental Table S1). A significant positive correlation between microglial soma volume in the hippocampus and both distance walked ($r = 0.53$) and time spent in the inner zone ($r = 0.47$) in the open field test was observed (Supplemental Fig. S5).

Tight Junction Protein Expression in Brain Microvessels

Brain microvessels were isolated for tight junction protein expression measurements, as an assessment of blood-brain barrier (BBB) integrity. Two hemispheres were pooled and in total twelve hemispheres per group were included, leading to six measurements per group. Relative protein expression levels of claudin-5, occludin, and ZO-1 were compared between groups. A significant effect of cigarette smoke exposure was observed for both the 17 kDa claudin-5 [$F(1, 20) = 8.709$; $P < 0.01$] and 22 kDa claudin-5 [$F(1, 20) = 10.91$; $P < 0.01$], and occludin [$F(1, 20) = 7.975$; $P < 0.05$]. Multiple comparisons revealed a lower expression of claudin-5 in cigarette smoke-exposed mice as compared with air-exposed mice (Fig. 8, *A* and *B*). In addition, a significant lower occludin expression was present in cigarette smoke controls as opposed to air controls ($P < 0.05$; Fig. 8*C*). For both claudin-5 and occludin, no additional effect of LPS was present. No significant effects of cigarette smoke or LPS were observed on the expression levels of ZO-1 (Fig. 8*D*).

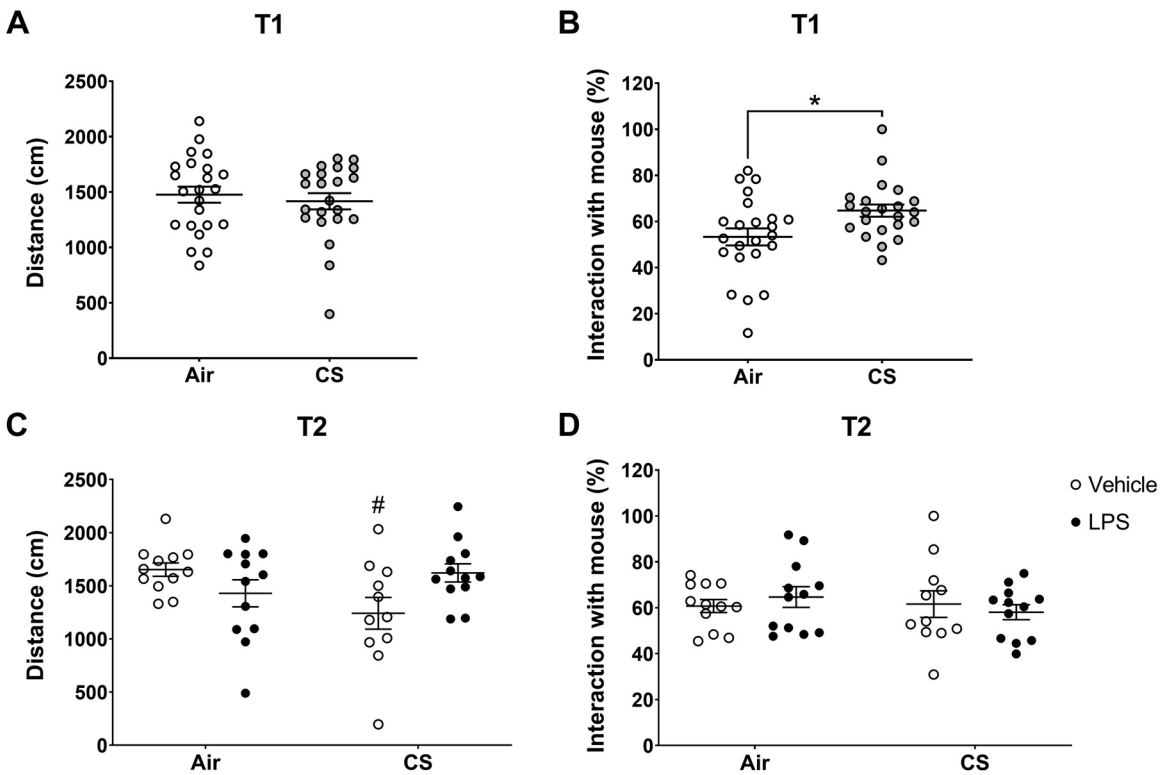


Figure 6. Outcomes of the social interaction test after 6 wk of cigarette smoke exposure (T1; *A* and *B*; air: $n = 23$; CS: $n = 22$) and at the end of the study (T2; *C* and *D*). Depicted are distance walked (*A* and *C*) and percentage of interaction with the unfamiliar mouse (*B* and *D*). Data are represented as means ± SEM. *$P < 0.05$. #$P = 0.071$ compared with respective air control group. Air + vehicle: $n = 12$ mice; air + LPS: $n = 12$ mice; CS + vehicle: $n = 11$ mice; CS + LPS: $n = 12$ mice. CS, cigarette smoke; LPS, lipopolysaccharide.

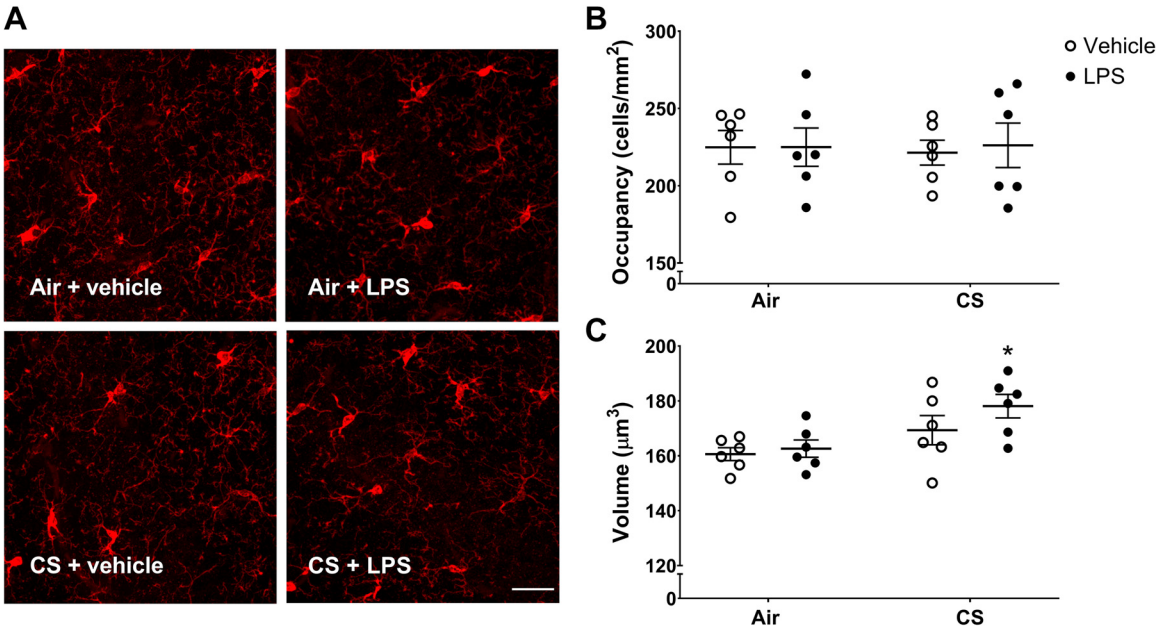


Figure 7. Two-dimensional representations of analyzed *z*-stacks for microglial activation measurements (*A*), scale bar = 30 μm. Microglial cell occupancy (*B*) and soma volume (*C*) in the CA1 area of the hippocampus. Data are presented as means ± SEM (n = 6 mice/group). *$P < 0.05$ as compared with air- and LPS-exposed animals. LPS, lipopolysaccharide.

The 17 kDa claudin-5 significantly and negatively correlated with VEGF-A levels in serum ($r = -0.63$; $P < 0.01$).

DISCUSSION

This study focused on examining the effects of cigarette smoke exposure combined with bacterial triggers on brain and behavior. Our study shows that the inflammation in the lungs observed in this COPD model is accompanied by changes in locomotion and exploratory behavior, possible compromised blood-brain barrier (BBB) and neuroinflammation specifically in the hippocampus. As presented by Wang et al. (48), the levels of CRP, IL-12p40, KC, and VEGF-A were increased in BALF, and only CRP and KC were increased in serum after cigarette smoke exposure. The additional LPS triggers decreased levels of IL-12p40, KC, and VEGF-A in the lungs. It is currently under debate whether (systemic) inflammation is actually the primary cause of extrapulmonary comorbidities in COPD; however, our observations show that inflammation might play an important role in the brain comorbidities observed in this murine model. First, serum KC and leptin levels correlated with increased explorative behavior. Second, although serum cytokines did not correlate with microglia activation in the hippocampus, VEGF-A levels in serum negatively correlated with claudin-5 protein expression in brain microvessels.

In the current study, alveolar enlargement after cigarette smoke exposure demonstrated significant emphysema, regardless of additional LPS trigger exposure. Interestingly, air space size in the lungs positively correlated with CRP levels in serum. This suggests that the degree of lung damage is related to systemic inflammation. This is in accordance with findings in patients, where patients in the highest quartiles of the BODE index showed higher levels of CRP in serum, and lung function was negatively correlated with serum CRP levels (49). It is unclear how systemic inflammation is precisely related to lung damage, although it is likely that pulmonary inflammation induced by cigarette smoke plays an important role, since the damage was caused by cigarette smoke and LPS did not affect this in the current model. As a result of pulmonary inflammation, cytokines from the lungs can enter the circulation and promote the production of CRP in the liver. CRP can then exert protective functions in innate immune responses, especially against bacteria (50). Several inflammatory markers in serum were increased by cigarette smoke exposure alone, including CRP and VEGF-A (48). We also found a significant positive correlation between inflammatory cell numbers in BAL and CRP levels in BAL fluid, suggesting that pulmonary inflammation might be related to translocation of CRP or even local production of CRP in the lungs (51).

Cigarette smoke and/or bacterial trigger exposure are known to significantly increase the inflammatory profile in the lungs (33). Our findings showed that LPS exposure on top of cigarette smoke exposure increased the number of macrophages and decreased the number of neutrophils. The long-term dose-dependent increase in macrophages of LPS on top of cigarette smoke exposure has not been observed before. It is very likely that this is also dependent on the dose of LPS. In the current study, we used a relatively low dose of LPS whereas in another study—with a similar experimental design—a higher dose of LPS was used, showing a maximum in total inflammatory cell influx since LPS alone elicited a similar response (25). The reduction in neutrophils observed after LPS exposures in cigarette smoke-exposed mice is contrary to previous observations (25, 27). It is worth noting that the timing of BAL cell harvest in the current study deviates from these studies, since BAL was sampled 10 days after the final LPS exposure. Thus, mice might have recovered from the LPS-induced inflammatory response. This suppressive

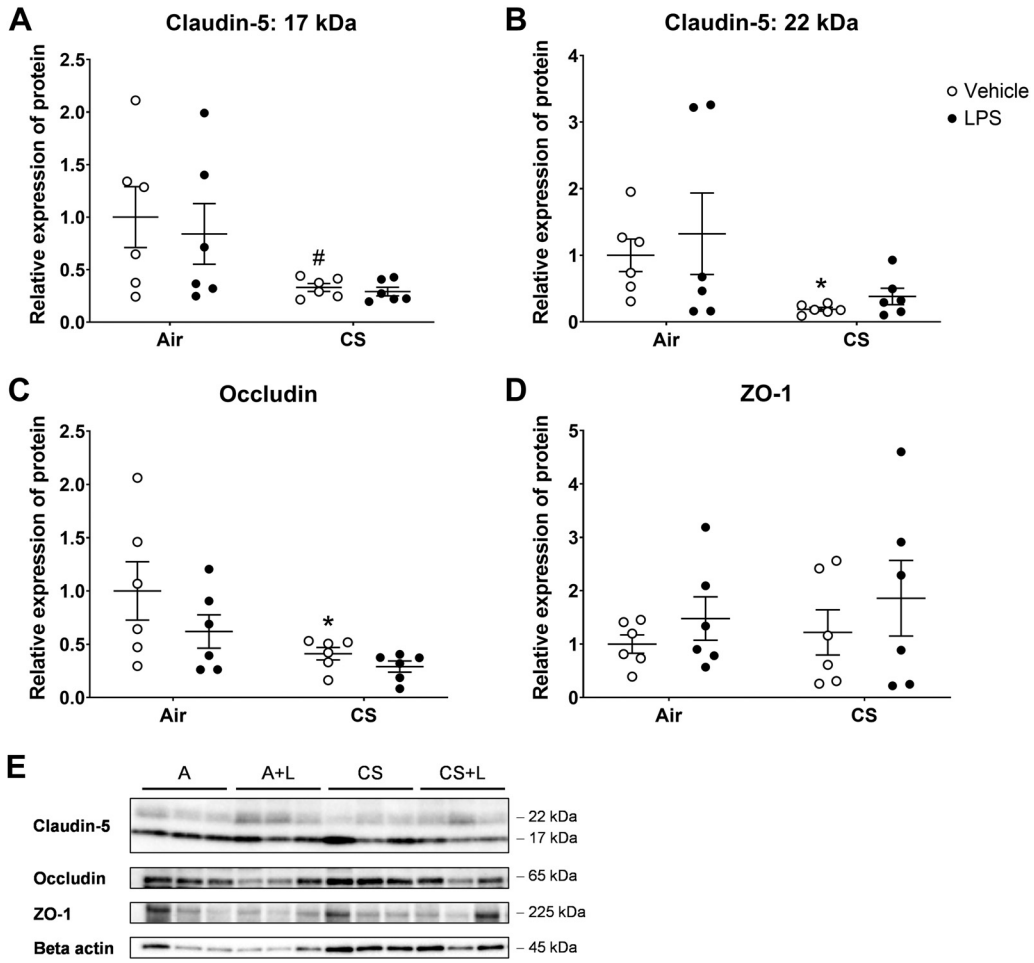


Figure 8. Protein expression levels of the 17 kDa claudin-5 (*A*), the 22 kDa claudin-5 (*B*), occludin (*C*), and ZO-1 (*D*) normalized to β-actin in isolated brain microvessels relative to the air + vehicle group. Data are presented as means ± SEM ($n = 6$ pooled samples per group). Examples of immunoblots are presented in *E*. *$P < 0.05$; #$P = 0.064$ as compared with air- and vehicle-exposed mice. A, air + vehicle; A + L, air + LPS; CS, cigarette smoke + vehicle; CS + L, cigarette smoke + LPS; LPS, lipopolysaccharide.

synergistic effect has been observed before (27); however, this was compared with LPS exposure alone suggesting a suppressive effect of cigarette smoke instead of LPS. Again, in the current study, we used a relatively low dose of LPS. This might have different effects on the inflammatory response, especially on top of cigarette smoke, as compared with high doses of LPS. We observed a similar pattern in KC levels in the BALF (48), which might explain the lower neutrophil numbers in the lungs as there is less KC to induce neutrophil recruitment into the lungs. It is unclear how LPS can reduce the cigarette smoke-induced KC production and neutrophil influx and it needs to be elucidated which mechanisms are involved.

We observed an increased locomotor activity and time spent in the inner zone in the open field test in cigarette smoke-exposed mice. This is contrary to our hypothesis that COPD induces anxiety. Our results suggest cigarette smoke-exposed mice display more explorative behavior. The observed increase in time spent in the inner zone of the open field is specifically dependent on cigarette smoke exposure as LPS exposure alone did not affect explorative behavior. Although there was no main effect of LPS, the effect of smoking on distance moved was only present in the LPS-exposed mice, suggesting a synergistic effect of LPS and cigarette smoke exposure on locomotion. There are several possible explanations for the increased exploratory behavior observed after cigarette smoke exposure. First of all, it is likely that nicotine in the cigarette smoke affected behavior. It is known that nicotine exhibits a strong effect on behavior; a study in mice by Liu et al. (52) demonstrated that subcutaneous administration of nicotine leads to an increased locomotion in the open field test. Furthermore, a study in rats showed that repeated exposure to nicotine decreases anxiety-like behavior, represented by an increased time spent in the inner zone of the open field (53). Similar to the findings of the current study, the authors showed that the increased time spent in the inner zone lasted until 24 h after nicotine exposure and was only long lasting when the exposure was repeated. It might be that the behavioral effects in the current study precede the psychological symptoms observed in patients with COPD. In this way, performing the behavioral experiments after cigarette smoke cessation may reduce the direct influence of nicotine on behavior. In addition, the specific effects of subsequent bacterial induced exacerbations

may be further investigated. Second, changes in the metabolic state due to cigarette smoke exposure may also affect behavior. We observed a decrease in food intake and fat mass after cigarette smoke exposure (48). In accordance, leptin serum levels were decreased in cigarette smoke-exposed mice and parameters in the open field test negatively correlated with leptin levels. Studies show that hypoleptinemia is associated with hyperactivity and other behavioral problems observed in anorexia nervosa (54, 55). Similarly, leptin administration in activity-based anorexia rats (56) and food-restricted rats (57) leads to a decrease in locomotor activity suggesting that leptin strongly regulates food-related locomotion. Therefore, the decrease in food intake and associated reduction in systemic leptin levels due to cigarette smoke exposure may have contributed to the increase in exploratory behavior. Finally, the presence of a bacterial trigger might also exacerbate the effects on locomotor activity. The increased locomotion was not observed in the social interaction test, which might be explained by the fact that an additional stimulus was introduced to the open field. With this, possible other behaviors beside the social behavior might be suppressed. Moreover, in control mice, the time spent in the inner zone of the open field at T2 as opposed to T1 was markedly lower. This suggests an increased anxiety-like behavior over time; however, it can also be a result of reduced locomotion. Although it is not likely that habituation due to multiple testing will occur, because of the large timespan between T1 and T2. However, we hypothesize that the extensive handling of mice required for this study resulted in a decreased interest for novel environments. Furthermore, the lighting conditions might have been a factor influencing behavior; the tests were performed during the light, or resting, phase in which mice are less active in behavioral tests as compared with the dark phase (58). Although these conditions were identical for all animals, the impact of these factors might be different for control as opposed to cigarette smoke exposed mice.

The BBB is crucial to prevent bacterial triggers from entering the brain. When the tightness of this barrier is compromised, circulating cytokines and toxic substances—chemicals in cigarette smoke or LPS—can enter the brain and induce both neurotoxicity and neuroinflammation by activating microglia. Various studies showed that among others nicotine in cigarette smoke can affect tight junctions in the endothelial cells composing the BBB (59, 60). The reduction in tight junction proteins in the vasculature of the brain is one of the indications that the BBB tightness might be compromised. In the current study, cigarette smoke exposure resulted in a decrease in claudin-5 and occludin protein expression in brain microvessels, suggesting a compromised BBB integrity. There are multiple possible explanations for this observation. First of all, the toxic components in cigarette smoke can enter the circulation and directly affect the BBB. In vivo studies that have focused on this are lacking, although several in vitro studies demonstrated that cigarette smoke can disturb the BBB integrity and increase its permeability (61–64). Second, as a result of cigarette smoke exposure and alveolar wall breakdown, cytokines can affect the BBB (65, 66). VEGF-A might be an important cytokine as its exposure can lead to reduced claudin-5 and occludin protein expression, and corresponding BBB breakdown (67). It is suggested that this BBB breakdown by VEGF-A is especially dependent on claudin-5 expression. In line with this, we observed a negative correlation between VEGF-A levels in serum and claudin-5 expression in brain microvessels. It is therefore possible that cigarette smoke exposure increases VEGF-A production in the lungs, and consequently leads to increasing systemic VEGF-A levels, which subsequently affects BBB junctional network and integrity. However, this significant correlation was only observed for the 17 kDa claudin-5, and not for the 22 kDa claudin-5 expression. Therefore, additional studies are necessary to determine whether the BBB integrity is mainly dependent on VEGF-A or other factors as well, and whether VEGF-A is mainly produced in the lungs or by reactive astrocytes in the brain. In addition, more functional assessments are required to demonstrate BBB leakage as a result of cigarette smoke exposure.

In the brain, microglia are the main immune cells regulating neuroinflammation. In the current study, microglial activation in the hippocampus of cigarette smoke- and LPS-exposed mice was increased. The hippocampus is involved in cognitive functioning, which is known to be frequently impaired in patients with COPD (68). An increase in microglial activation suggests presence of neuroinflammation in the brain area involved in cognition after both cigarette smoke and LPS exposure. A link between neuroinflammation in cortical regions and hyperlocomotion has been demonstrated in traumatic brain injury (69). Although the hippocampus is mainly involved in cognitive function, studies have demonstrated that it is also involved in explorative behavior since part of the exploratory activities are dependent on memory (70). Interestingly, downregulation of integrin β4—an important hallmark in asthma—leads to pulmonary inflammation as well as hyperlocomotion in the open field test (71). There was an increase in microglia and neuroinflammation in the hippocampus, which suggests that hippocampal function is important in explorative behavior and that this is partially dependent on microglial cells. This is further supported by a study in which hyperlocomotion was accompanied by an increased number of activated microglia in the hippocampus in an Alzheimer's disease mouse model (72). Although it is possible that the hippocampus is involved in the observed behavioral changes, the nicotine in cigarette smoke might also have an impact on behavior. It is known that nicotine can reduce inflammatory responses, among others by decreasing microglial activation through the α7 nicotinic acetylcholine receptors (73). Therefore, it is not very likely that nicotine is the primary contributor to the observed neuroinflammation in the hippocampus. Furthermore, additional research is important to relate the observed neuroinflammation in the hippocampus with impaired cognitive function as this is one of the main brain-related comorbidities in COPD. Additional parameters to confirm the presence of neuroinflammation in the hippocampus and other brain areas might be useful to support our observations. In addition, interpretation of these results relative to behavioral changes is limited since both BBB integrity and microglia activation were only assessed at the end of the study. Whereas the increased locomotion was already observed before exposure to the additional LPS triggers at T1. To relate these changes in the brain to behavior, it

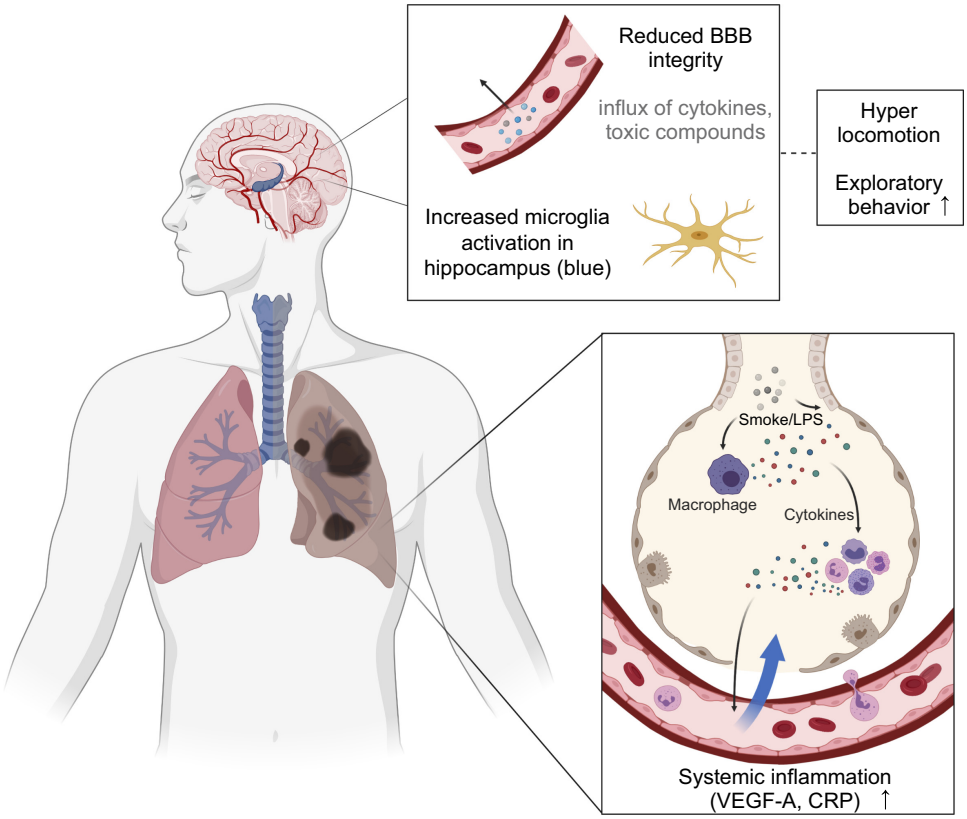


Figure 9. Proposed hypothesis based on the findings of this study. In cigarette smoke- and LPS-induced pulmonary inflammation, circulating inflammatory markers such as VEGF-A and CRP increase. These inflammatory markers together with LPS and toxic substances in cigarette smoke may reduce BBB integrity, leading to increased leakage of these compounds into the brain. This might result in increased microglial activation and subsequent neuroinflammation. Eventually, these changes are accompanied by behavioral changes such as increased explorative behavior and hyperlocomotion. These processes might play an important role in the brain-related comorbidities observed in COPD. BBB, blood-brain barrier; COPD, chronic obstructive pulmonary disease; CRP, C-reactive protein. [Created with BioRender.com.]

will be necessary to assess the BBB integrity and microglia activation at T1 in future experiments as well. Nevertheless, our findings suggest that neuroinflammation in the hippocampus may be one of the pathological processes within the brains of patients with COPD.

With respect to the behavioral deficits, the murine COPD model described in this study has its limitations. The effects on behavior are mainly caused by cigarette smoke exposure. The LPS exposures in control or cigarette smoke-exposed mice barely resulted in an additional effect on behavior. Therefore, this model does not fully reflect the human situation where the occurrence of exacerbations contributes to transient behavioral deficits (74, 75).

This study provides insights into the possible mechanisms involved in the brain-related comorbidities in COPD and COPD exacerbations. Our concluding hypothesis is depicted in (Fig. 9). Additional research is required to confirm and further elaborate this hypothesis. For example, inducing COPD by another trigger than cigarette smoke—for example elastase—might be of relevance to prevent the interference of nicotine in the behavioral assessments. Overall, this study can contribute to the development of novel intervention strategies for treating brain-related comorbidities in patients with COPD.

DATA AVAILABILITY

The data that support the findings of this study will be made available upon reasonable request from the corresponding author.

SUPPLEMENTAL DATA

Supplemental Table S1 and Supplemental Figs. S1–S5: https://doi.org/10.6084/m9.figshare.19704388.

GRANTS

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DISCLOSURES

J.G. is a part-time employee of Danone Nutricia Research, Utrecht, the Netherlands. A.v.H. is an employee of Danone Nutricia Research, Utrecht, the Netherlands. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

C.E.P., L.W., S.B., G.F., A.v.H., and A.D.K. conceived and designed research; C.E.P., L.W., L.N.P.M., S.K., I.v.A., and T.L.-M. performed experiments; C.E.P. and L.W. analyzed data; C.E.P. and A.D.K. interpreted results of experiments; C.E.P. prepared figures; C.E.P. drafted manuscript; C.E.P., L.W., L.N.P.M., S.K., I.v.A., T.L.-M., S.B., G.F., J.G., A.v.H., and A.D.K. edited and revised manuscript; C.E.P., L.W., L.N.P.M., S.K., I.v.A., T.L.-M., S.B., G.F., J.G., A.v.H., and A.D.K. approved final version of manuscript.

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