Marijuana smoke induces severe pulmonary hyperresponsiveness, inflammation and emphysema in a predictive mouse model not via CB1 receptor activation

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ABBREVIATIONS

BALF: bronchoalveolar lavage fluid
THC: tetrahydrocannabinol
Penh: enhanced pause
MPO: myeloperoxidase
MCP-1: monocyte chemoattractant protein-1
IFN-γ: interferon-gamma
TNF-α: tumor necrosis factor-alpha
IL: interleukin
CB1<sup>−/−</sup> mice: CB1 gene-deleted mice
L<sub>m</sub>: mean linear intercept (chord) length
Sporadic clinical reports suggested that marijuana smoking induces spontaneous pneumothorax, but no animal models were available to validate these observations and to study the underlying mechanisms. Therefore, we performed a systematic study in CD1 mice as a predictive animal model and assessed the pathophysiological alterations in response to 4-month-long whole body marijuana smoke with integrative methodologies in comparison with tobacco smoke. Bronchial responsiveness was measured with unrestrained whole body plethysmography, cell profile in the bronchoalveolar lavage fluid with flow-cytometry, myeloperoxidase activity with spectrophotometry, inflammatory cytokines with ELISA, and histopathological alterations by light microscopy. Daily marijuana inhalation evoked severe bronchial hyperreactivity after a week. Characteristic perivascular/peribronchial edema, atelectasis, apical emphysema, neutrophil and macrophage infiltration developed after one month of marijuana smoking, lymphocyte accumulation after 2 months, macrophage-like giant cells, irregular, destroyed bronchial mucosa, goblet cell hyperplasia after 3 months, and severe atelectasis, emphysema, obstructed, damaged bronchioles, endothelial proliferation at 4 months. Myeloperoxidase activity, inflammatory cell and cytokine profile correlated with these changes. Airway hyperresponsiveness and inflammation were not altered in mice lacking the CB1 cannabinoid receptor. In comparison, tobacco smoke induced hyperresponsiveness after 2 months, and significantly later developing inflammatory cell infiltration/activation with only mild emphysema. We provide the first systematic and comparative experimental evidence that marijuana causes severe airway hyperresponsiveness, inflammation, tissue destruction and emphysema, which are not mediated by the CB1 receptor.
INTRODUCTION

There is an ongoing debate on the sinister effects of marijuana on the airways including several controversial clinical reports describing chronic lung injury and even primary spontaneous pneumothorax. Observational case studies have described severe emphysema and airway inflammation attributed to marijuana smoking (1, 2, 4, 5, 7, 11, 16, 19, 22, 25, 30, 35, 42). The fact, that marijuana smokers are usually simultaneous nicotine abusers, also complicates the picture. Increased basal and goblet cell hyperplasia developed in the bronchiolar epithelium in the lung of marijuana smokers compared to tobacco cigarette smokers. Furthermore, squamous cell metaplasia was observed when both tobacco and marijuana smoking occurred simultaneously (12). The total number of inflammatory cells, but particularly neutrophils, in the bronchoalveolar lavage fluid (BALF) obtained from marijuana smokers remarkably increased. Marijuana potentiated cigarette smoke-induced macrophage accumulation (3). Tashkin and colleagues found a direct correlation between marijuana smoking and airway hyperresponsiveness (40, 41). Despite all these clinical data, the underlying mechanisms are poorly understood, predominantly due to the lack of reliable and predictive animal models for experimental investigation of the pathophysiological processes. Only few sporadic studies were performed in the 70s, which focused on the pulmonary effects of marijuana in animal experiments. They showed that marijuana smoke inhalation induced alveolitis, bronchiolitis and pneumonitis in a dose-dependent manner in dogs (38). Severe inflammatory histopathological changes developed after a year of marijuana smoke exposure in rats (8, 9), but toxicity signs were also seen after a period of 23-day inhalation or even less (37). Furthermore, marijuana smoke was found to dose-dependently impair the pulmonary antibacterial defense system \textit{in vivo} in rats (17), and to inhibit the antibacterial activity of isolated rat alveolar macrophages. In Rhesus monkeys marijuana smoke also induced
alveolitis, hyperplasia of alveolar cells and granulomatous inflammatory reaction (10). In the surprisingly low number of animal experiments different species, exposure durations and experimental paradigms were used and they mainly focused only on pulmonary morphology. Therefore, strong conclusions could not be drawn from these studies. Furthermore, there were no data available in mice so far.

Since the legalization and uses of marijuana in certain severe pain and vomiting conditions are important clinical questions and the available human data are contradictory due to a variety of individual influencing factors, we analysed the pulmonary effects of chronic marijuana smoke exposure in a predictive mouse model using a complex, integrative approach. Functional, morphological, biochemical and immunological techniques were applied and the actions were compared to tobacco smoke. Furthermore, the potential involvement of the CB1 cannabinoid receptor in the marijuana smoke-evoked pulmonary changes was also assessed with gene-deleted mice.
MATERIALS AND METHODS

Animals

The studies were performed with male CD1 mice, which were 8 weeks old (20-25 g) at the beginning of the experiments. Breeding pairs of CB1 receptor homozygote knockout mice (CB1-/-) were originally supplied by Catherine Ledent, Brussels, Belgium (IRIBHM, Université Libre de Bruxelles, Brussels, Belgium). The animals contained the DNA construct previously shown to produce genetic deletion of CB1 cannabinoid receptors [24]. All mice were provided with standard rodent chow and water ad libitum. The animal experimental procedures were carried out according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988) and complied with the recommendations of the Helsinki Declaration. The studies were approved by the Ethical Committee on Animal Research of the University of Pécs according to the Ethical Codex of Animal Experiments and licence was given (licence No.: BA 02/2000-5-2011).

Chronic whole body marijuana smoke exposure and experimental paradigm

The dried marijuana containing 0.35±0.02 w/w% tetrahydrocannabinol (THC) was provided by the Hungarian Police Superintendancy after obtaining the necessary permission from the Drug Licencing Department operating at the Office of Healthy Authorization and Administrative Procedures (Budapest, Hungary; licence number: 01174/2009/KÁB).

It was chopped and cigarettes containing 0.77±0.03 g marijuana were prepared. Mice were exposed to whole body marijuana smoke in a smoking system (TE2 Teague Enterprises, USA) from the age of 4 weeks for 1-4 months twice a day for 40-40 min (2 cigarettes per occasion, 70 mg total particulate material/m³). Kentucky research cigarettes (3R4F: 9.5 mg tar, 0.73 mg nicotin and 11.9 mg CO; University of Kentucky, USA) were used for
comparison according to the same protocol, in both cases the cigarettes were used without a filter.

We performed a longitudinal self-control chronic study, in which different outcomes could be measured in the same animals. Therefore, after measuring the airway responsiveness at the end of each month, the BAL was performed very carefully one day later under anaesthesia. Then the lungs were excised, the left half was immediately fast frozen in liquid nitrogen for cytokine and myeloperoxidase (MPO) measurement, the other half was put into formalin for histological processing. The advantage of this technique is that we have all information from the same mouse for better comparison and correlation, as well as help to keep the 3R regulations from the animal ethical point of view (15).

**Determination of cannabinoid concentrations in the inhaled smoke with HPLC**

The HPLC system consisted of a Dionex P680 gradient pump (Dionex Corp., Sunnyvale, CA, USA), a helium degassing system, a Rheodyne 8125 injector valve with a 20μL loop (Rheodyne Europe GmbH, Bensheim, Germany), and a Dionex 340D UV–vis diode array detector (Dionex Corp., Sunnyvale, CA, USA). The eluate was monitored at ambient temperature at range of 200 to 500 nm by DAD. The concentrations were calculated by using wavelengths where the investigated cannabinoids have their absorbance maximums. Chromatographic separations were achieved using Intersil ODS-4 C18 reversed phase column (3 μm, 2.1 mm x 150 mm i.d.). A Chromeleon data management software (Version 6.60 SP3 Build 1485, Dionex Corp., Sunnyvale, CA, USA) was used to control the equipment and for data evaluation. The mobile phase consisted of a mixture of methanol/water containing 50 mM of ammonium formate (adjusted to pH 5.2). Initial setting was 75% methanol (v/v), which was linearly increased to 90% methanol over 25 min, then increased to 100% in 1 min. After maintaining this condition for 3 min, the column was set to initial condition in 1 min.
and re-equilibrated under this condition for 6 min. The total runtime was 36 min. Flow rate was set to 0.3 ml/min, the injection volume was 20 μl. All experiments were carried out at 25°C.

Measurement of total cannabinoid concentration of the urine
Since cannabinoids have 97% plasma protein binding, penetrate to all tissues, have high accumulation and urinary excretion (32, 33), we measured total cannabinoid concentrations in the mouse urine in our chronic model. Urine samples (200-350 μl per mouse) were collected from day 5, once a week throughout the total duration of the 4-month experimental period and stored at +4°C until the measurement. The total cannabinoid concentration was determined with fluorimetric polarized immunassay within the calibrated detection range of 25–135 μg/ml compared to the control reagent. The mean recovery of the reagent was 102.8%. The precision values of the measurements (CV%) were 4.47%, 4.03% and 3.89% at 35 μg/ml, 50 μg/ml and 100 μg/ml concentrations, respectively, therefore, they fit in the required range below the permitted <5% analytical error. The reagents were sensitized separately for 25 ng/ml Δ9-THC-COOH, 11-OH-Δ9-THC, 8-β-OH-Δ9-THC, and 8-β-dOH-Δ9-THC, as well as 80 ng/ml CBN.

Determination of airway responsiveness
Airway responsiveness in conscious animals was measured by whole body plethysmography (Buxco Europe Ltd, Winchester, UK [28]) once every week. Bronchoconstriction was induced by nebulized carbamoyl-choline (carbachol, muscarinic receptor agonist; 5.5, 11 and 22 mM, 50 μl/mouse for 50 sec; Sigma, St. Louis, MO, USA) and the mean enhanced pause (Penh) curves were measured as an indicator of bronchoconstriction and consequent increase of airway resistance. Penh is a complex, calculated parameter ((expiratory time/ relaxation
time)-1): (max. expiratory flow/ max. inspiratory flow), which closely correlates with airway resistance measured by invasive techniques using ventilated animals. The mean Penh values and the percentage increase above baseline were calculated for each 15-min measurement period (15).

**Determination of inflammatory cell profile in the BALF**

Bronchoalveolar lavage was performed 4 times with PBS each time through the cannula inserted into the trachea of anaesthetized mice (100 mg/kg ketamine i.p. and 5 mg/kg xylazine i.m.). The retrieved BALF aliquots were pooled, their volume was measured and centrifuged. The pellet was resuspended in 0.5 ml staining buffer containing anti-mouse CD45 FITC leukocyte marker and propidium-iodine for staining non-viable cells. After an incubation period of 30 min, the samples were washed twice and the number of inflammatory cells (granulocytes, lymphocytes and macrophages) was determined with Partec CyFlow Space flow cytometer. Differentiation was made on the basis of the size and granulation, cell numbers were calculated by the FowMax Software (15).

**Histopathological evaluation: inflammatory cell counting, semi quantitative scoring and measuring acinar air space complex in the lung sections**

The whole right lung was fixed in 4% buffered formaldehyde. Evaluation of the morphological changes by semi quantitative scoring, inflammatory cell number count and mean linear intercept (chord) length (Lm) measurement of the lung sections at each timepoint were performed by an expert pulmonary pathologist who was blinded from the experimental setup.

The scoring parameters and values were determined after investigation of several representative microscopic fields on the basis of the characteristic inflammatory signs: 1) perivascular/peribronchial edema, 2) epithelial damage, 3) fibrosis, 4) inflammatory cell
infiltration and 5) goblet cell hyperplasia in the bronchial epithelium were scored from 0 to 3
(0: absent, 1: mild/some cells, 2: moderate/many cells, 3: severe/cell aggregations).
Neutrophil granulocytes, lymphocytes and macrophages were counted in thirty 10,000 μm²
interstitial regions per slides, where cells were distributed more evenly, therefore assessment
could be more precisely done.

The mean linear intercept (chord) length, a widely used parameter to quantify distal air space
enlargement, was measured to evaluate the size of the acinar air space complex related to
emphysema (21). Slides were scanned by a Pannoramic DESK scanner (3D HISTECH Ltd.,
Hungary) and the alveolar space or alveolar and ductal air space together were measured
along parallel test lines by using the Case Viewer software (3DHISTECH Ltd., Hungary). At
least 3 sections were evaluated from each mouse from different depths to obtain reliable
results, tissue shrinkage or integrity damage were not observed (n=80-100 measurements per
group).

**Determination of granulocyte and macrophage accumulation and function by MPO activity measurement**
Accumulation of neutrophils and macrophages in the lung homogenates was assessed by
MPO activity measurement with spectrophotometry. The lung pieces were weighed,
homogenized with a polytrone homogenizer (Kika Lab Techniques) in 4 ml 20 mM
potassium-phosphate buffer (pH 7.4), centrifuged at 10,000 g at 4 °C for 10 min. The pellet
was resuspended in 4 ml 50 mM potassium-phosphate buffer containing 0.5% hexadecyl-
trimethyl-ammonium-bromide (pH 6.0) and centrifuged again. MPO enzyme activity of the
pellets was compared to a human standard MPO preparation in 96-well microtitre plates using
H₂O₂-3,3’,5,5’-tetramethyl-benzidine (TMB/H2O2) (Sigma, St. Louis, MO, USA). The
optical density (OD) was measured with a microplate reader (Labsystems) at 620 nm, plotted
and MPO activity was determined with the help of a calibration curve.
Measurement of inflammatory cytokines in the lung homogenates

The lung samples were weighed, homogenized in 500 µl RPMI-1640 buffer (Biochrom Ltd., Berlin, Germany) containing 50 µl phenyl-methyl-sulphonyl-fluoride (PMSF; Sigma-Aldrich Ltd., Budapest, Hungary) mixture at 13,500 rpm for 2 min at 4 °C. The homogenates were then centrifuged for 10 min at 10,000 rpm and 4 °C, and the concentrations of 6 inflammatory cytokines (monocyte chemoattractant protein-1: MCP-1, interferon-gamma: IFN-γ, tumor necrosis factor-alpha: TNF-α, and interleukins (IL): IL-6, IL-10, IL-12) were determined by mouse cytokine cytometric bead array (CBA, Becton Dickinson Biosciences, catalog No. 552364; detection limit: 20 pg/ml) from the supernatants. The CBA kit contains 6 bead populations with distinct fluorescent intensities, that have been coated with capture antibodies specific for the measured cytokine proteins. 50 µl mixed capture beads, 50 µl homogenate and 50 µl PE-labelled detection antibody solution were added into an assay tube and counted by flow cytometry. Data were evaluated by the FCS Express Software version 3 and the cytokine concentrations were expressed as pg/g wet tissue.

Statistical analysis

Results are expressed as means±SEM of n=6-8 mice in each group and evaluated by one-way ANOVA followed by Bonferroni’s modified t-test. In all cases p<0.05 was considered to be significant. Evaluation of the semiquantitative histopathological scoring and inflammatory cell count (n=30 counts/slides) was analysed by Kruskal-Wallis followed by Dunn’s multiple comparison test to observe intergroup differences at given timepoints. The mean linear intercept length (n=80-100 measurements/slides) was analysed with two-way ANOVA followed by Sidak’s multiple comparison test.
RESULTS

The THC, cannabinol and cannabidiol concentrations of the inhaled smoke determined with HPLC were 0.04 mg/ml, 0.02 mg/ml and 7.48x10^{-4} mg/ml, which corresponded to 14.77%, 8.64% and 0.27% content of these components, respectively, per dry plant. The total cannabinoid concentration of the urine was 56.6±5.4 ng/ml on day 5, and then remained relatively stable from 57.1±4.3 ng/ml to 78.08±6.2 throughout the total 4-month-investigation period.

Airway hyperreactivity

Airway responsiveness to the inhalation of the muscarinic receptor agonist carbachol remarkably increased immediately after a week of marijuana smoke exposure, as shown by the mean Penh curves (Fig. 1A) and also by the percentage Penh increase values above baseline (Fig. 1E). This remained relatively stable during the total duration of the 4-month-study. In contrast, tobacco smoke did not induce broncial hyperreactivity until the ninth week of the experiment, but after this timepoint its action was similar to that of marijuana (Fig. 1A-E).

Histopathological alterations in the lung

One month after the beginning of marijuana smoke exposure remarkable perivascular/peribronchial edema (Figs. 2A, 6D), neutrophil, lymphocyte and macrophage infiltration (Figs. 2B, 6A-C, E), minimal epithelial irregularity (Figs. 2A, B, 6F) and alveolar space enlargement (Fig. 2C) developed in the marijuana-exposed group. Most of these signs were similar in tobacco-smoking mice, but edema formation was significantly increased only
in case of marijuana smoke exposure (Figs. 2D, 6D) and there were significantly more infiltrating granulocytes and lymphocytes than in response to tobacco smoke (Fig. 6A, B).

Two months of marijuana smoking induced vascular endothelial proliferation, definitive inflammatory cell infiltration with significantly higher number of neutrophils as compared to the tobacco smoke (Figs. 3A, 6A, E), bronchial goblet cell hyperplasia (Fig. 3B, 6G) and gradually increasing emphysema (Fig. 3C, 6I).

Three months after starting the marijuana smoke exposure, massive interstitial neutrophil and macrophage infiltration occurred including multinucleated giant cells being significantly greater than in case of tobacco-smoke, extensive lymphocyte accumulation around the vessels and bronchi (Figs. 4A, B, 6A-C), significantly greater epithelial damage and hyperplasia of goblet cells (Figs. 4C, 6F, G, I) could be seen.

Following 4 months of marijuana smoking, narrowed, obstructed, disintegrated and disrupted bronchial epithelium with desquamated epithelial cells (Fig. 5A, 6F), atelectasis, significantly increased number of lymphocytes among cell debris in the scattered alveolar lumens, microbleedings and remarkable fibrosis (Fig. 5B, 6H) together with very severe emphysema (Fig. 5C, 6I) were the characteristic histopathological alterations. In comparison, 4 months of tobacco smoke induced significantly less severe interstitial inflammation (Fig. 6E) and epithelial damage (Fig. 6F), no fibrosis (Fig. 6H) and milder emphysema (Fig. 5D, 6I).

**Inflammatory cell profile of the BALF**

Inflammatory cells did not increase in the BALF after one month of either marijuana or tobacco smoke exposure compared to the intact, non-smoking mice (Fig. 7A). In contrast, lymphocytes, granulocytes and macrophages significantly increased in both groups after 2 months (Fig. 7B). The absolute number of all these inflammatory cells remarkably decreased
by the end of the third month. Lymphocytes and granulocytes remained significantly elevated in the marijuana-smoking group, but the increase of only lymphocytes in the fourth month was significant in response to tobacco (Fig. 7C, D).

**MPO activity in the lung homogenates**

MPO activity, as a biochemical marker of neutrophil and macrophage accumulation, significantly increased in the lung after one month of marijuana smoke exposure. It showed an almost 5-fold elevation at the end of the second month, then it dramatically decreased, but still remained significantly higher than that of the intact lung at the three-month timepoint. This enzyme activity got back to the normal level after 4 months of marijuana smoking. In contrast, in the lung of the tobacco-smoking group, the only remarkable, 3-fold MPO activity elevation was observed at 2 months, but it proved to be significantly less than that measured in marijuana-smoking animals (Fig.8A).

**Cytokine concentrations in the lung homogenates**

At the 2-month timepoint where the intensity of the cellular inflammatory reaction showed its maximum, the concentrations of four important inflammatory cytokines (MCP-1, IFN-γ, TNF-α and IL-12), but interestingly also the anti-inflammatory IL-10, significantly increased in the lung of tobacco-smoking animals compared to non-smoking ones. Furthermore, they were significantly higher in the marijuana-smoking group than in the tobacco-smoking one. Significant increase of the IL-6 level was only detected in marijuana-smoking mice (Fig. 8B).

**Marijuana smoke-induced airway hyperresponsiveness, severe inflammation and tissue destruction similarly develop in CB1 receptor gene-deleted mice**
Since the maximal inflammatory response was observed 2 months after the beginning of the marijuana smoke exposure, the severity of the inflammation was investigated in CB1/− mice compared to their wildtype CD1 controls at this timepoint. Pulmonary hyperreactivity to inhaled carbachol developed similarly from the first week of marijuana smoking in both groups (Fig. 9A). The histopathological evaluation showed no significant difference either between the massive perivascular/peribronchial edema and inflammatory cell accumulation, or the moderate goblet cell hyperplasia in the bronchial epithelium and emphysema formation in the two groups (Fig. 9B, C, D).

**DISCUSSION**

In the present carefully designed comparative chronic experiment we provided several lines of evidence that marijuana smoke is more harmful to the lungs than tobacco smoke. The obvious advantage of using the cigarette smoke control group in our study is that it is the most clinically relevant stimulus, which triggers inflammatory pathways leading to lung destruction. The pathophysiological changes found in animal studies appear to be consistent to that observed in man (23, 28). These are the first experimental data in a predictive mouse model that marijuana smoke induces remarkable bronchial hyperresponsiveness very early, as well as severe pulmonary inflammation, emphysema and tissue destruction, which are independent of CB1 receptor activation. We proved with functional, morphological, biochemical and immunological techniques that these characteristic pathophysiological alterations are dependent on the duration of the smoke exposure. Airway hyperresponsiveness was also detected after 2 months of tobacco smoke, but more severe hyperreactivity developed in response to marijuana much earlier, 7 days after the first smoke inhalation. Since remarkable inflammatory signs are not likely to develop at this timepoint, it is suggested that
marijuana smoke directly increases airway reactivity. Although in clinical reports direct short-term bronchodilation was described in response to marijuana primarily through CB1 receptor activation in the lung (13), we clearly showed that after a longer period of time, it markedly increases airway reactivity to muscarinic receptor activation in a CB1-independent manner. Inflammation, as assessed by several histopathological parameters, such as perivascular/peribronchial edema, neutrophil and macrophage infiltration, epithelial irregularity and hyperplasia, atelectasia and emphysema, were also more severe and developed earlier in the marijuana-exposed group than in tobacco-smoking mice. Vascular endothelial proliferation, narrowed, obstructed and destructed bronchi with desquamated epithelial cells, massive interstitial lymphocyte infiltration, fibrosis and a remarkable loss of the alveolar structure could be seen after four months of regular marijuana smoke exposure. These severe morphological changes did not occur in response to tobacco smoke at this timepoint. These results support the data of a controlled clinical study providing evidence for a frequency- and duration dependent emphysema formation, focal alveolar edema, alveolar and bronchiolar destruction in regular marijuana smokers. The cell counts for granulocytes, macrophages and lymphocytes in the BALF significantly and similarly increased at month 2 in both the marijuana- and cigarette-smoking groups. This is explained by the histological picture showing that at month 1 the massive edema, but not cell influx dominated the picture, and the peribronchial/perivascular cellular inflammation peaked at the 2-month timepoint. At the 3- and 4-month timepoints the BALF cell counts markedly decreased, which is explained by the fact that inflammatory reaction decreases, it is localized to the lung parenchyma, and the bronchiolar-alveolar structure becomes disrupted. Human investigations demonstrated that there was no remarkable difference between the BALF cell counts in the marijuana smokers and tobacco smokers, which also supports our similar, non-significant findings regarding the BALF cell counts of marijuana- and cigarette-smoking mice (3).
MPO activity measured in the lung homogenates, which reflects to the number and function of the accumulated granulocytes and macrophages in the parenchyma, significantly increased after one month of marijuana smoke administration, but it showed an almost 5-fold elevation at the end of the second month. It dramatically decreased, but still remained significantly higher than that of the intact lung at the three-month timepoint. MPO activity got back to the normal level after 4 months of marijuana smoking, which is explained by lymphocyte dominance at this late timepoint. This explanation is well supported by the histopathological picture. In contrast, in the lung of the tobacco-smoking group, the only remarkable (3-fold) MPO activity elevation was observed at 2 months, but it proved to be significantly less than that measured in marijuana-smoking animals.

The concentrations of four important inflammatory cytokines (MCP-1, IFN-γ, TNF-α and IL-12), but interestingly also the anti-inflammatory IL-10, were significantly higher in the lung of marijuana-smoking animals compared to tobacco smoking ones at month 2 when the inflammatory reaction reached its maximum. However, there was no difference in IL-6 levels, which is predominantly secreted by T-cells and macrophages particularly in acute phase reaction when fever is a major symptom, but not in chronic pulmonary inflammation. IL-10 (cytokine synthesis inhibitory factor) is synthesized by macrophages and lymphocytes, and it decreases the production of several pro-inflammatory cytokines, such as IFN-γ and TNFα. Its elevation can suggest an endogenous counter-regulatory mechanism for the inflammatory reaction at the timepoint where the maximal inflammation develops.

MCP-1 is produced by macrophages and dendritic cells and has a potent chemotactic effect at the site of injury or infection, it induces basophil and mast cell degranulation (36). IFN-γ synthesized by natural killer and T lymphocytes plays an important role in antigen presentation, IgG and inducible nitric oxide synthase production. TNF-α enhances phagocytosis, IL-1 and PGE2 synthesis, as well as neutrophil accumulation (29). IL-12
produced by macrophages and B cells regulates T lymphocyte differentiation, natural killer cell activation, as well as IFN-γ and TNF-α synthesis. The activation of this cytokine cascade by chronic marijuana smoking clearly leads to massive inflammation and later tissue destruction. Besides these inflammatory mechanisms, the structural damage and severe emphysema might also be related to a direct action of the marijuana smoke to the epithelial cells by impairing their energetic processes (39). The concentrations of these cytokines in the lung of the intact control group were very low, which is in agreement with the literature also showing values around 10-40 pg/ml in the non-inflamed mouse lung homogenates determined by other immunassays (27, 31).

We showed more severe pulmonary damage in marijuana-smoking mice compared to tobacco smoke, which is in agreement with literature data. Several studies reported that the harmful effects of cannabis on lung function are different from those of tobacco, but it is very difficult to do reliable comparative clinical studies due to a range of modifying factors, such as variability in duration, amount and simultaneous use of both agents (4, 14). However, it was concluded that a single marijuana cigarette per day remarkably increases the risk of malignant lung tumors every year, which is comparable with the risk induced by one package of tobacco cigarette (1, 20, 25). The chemical compositions of marijuana and tobacco, as well as their smoke are basically different, marijuana does not contain nicotine, and tobacco lacks cannabinoid derivatives. Furthermore, marijuana smoke contains double concentrations of phenol, benzantracene, naphthalene, acetaldehyde, hydrogen-cianide and ammonia than tobacco (18). The higher amount of these irritants is likely to be responsible for its greater inflammatory actions in the airways, possibly through the activation of peptidergic sensory nerves. Furthermore, the blood carboxyhemoglobin level was 5 times higher and the amount of the inhaled tar was 3 times greater in marijuana smokers compared to tobacco smokers (43).
Cannabinoids act at the cannabinoid 1 and 2 receptors (CB1 and CB2), a 7TM orphan receptor and the transmitter-gated channels transient receptor potential vanilloid types 1-4 receptors. Recent evidence also showed that cannabinoids acting at members of the nuclear receptor family, peroxisome proliferator-activated receptors, regulate cell differentiation and lipid metabolism (6, 34). CB1 receptors are expressed predominantly at nerve terminals where they mediate inhibition of transmitter release.

Sporadic and contradictory clinical data and the lack of reliable and predictive animal models are the two main reasons for a poor understanding of the pathophysiological changes in the lung due to marijuana smoke. Our integrative, longitudinal and comparative mouse experiments monitored the effects of a long-lasting marijuana smoke exposure and provided the first evidence, that it induced duration-dependent characteristic changes in the lung, but not via CB1 receptor activation. Although the mediating targets, pathways and mechanisms still need to be identified, but at least one important candidate can be excluded.

Hyperresponsiveness develops earlier and the inflammatory alterations are all more severe than in case of tobacco smoke. Well-established scientific information regarding the impact of long-standing marijuana smoking on lung structure and gas exchange is imperative in order to support legal, political and medical decision making. Our results in a chronic mouse model provide reliable evidence against unrestricted use of marijuana smoking, but do not question the medical use of synthetic CB1 agonists.

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All authors have read the journal's authorship agreement.

All authors have read the journal's policy on conflicts of interest, and all authors declared no conflicts of interest.
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FIGURE LEGENDS

Fig. 1. Marijuana smoke-induced airway hyperresponsiveness
Panels A-D show 11 mM and 22 mM carbachol-evoked bronchoconstriction demonstrated by the mean Penh values calculated in a 15 min period after each stimulation. The four panels represent these data 1, 2, 3, and 4 months after the beginning of daily whole body marijuana smoke exposure in comparison with tobacco smoke. Panel E describes the 22 mM carbachol-induced percentage increase of Penh above baseline ((mean values in response to carbachol-baseline values/baseline values) x 100).
Data show means±S.E.M. of n=6-8 mice per group; +p<0.05, ++p<0.01 vs. the intact, non-smoking group; *p<0.05, **p<0.01 vs. tobacco smoke (one-way ANOVA + Bonferroni’s modified t-test).

Fig. 2. Histopathological alterations after 1 month of marijuana smoke exposure
Representative histopathological pictures of the lung samples obtained 1 month after the beginning of daily marijuana smoke exposure showing (A) remarkable perivascular edema formation, (B) interstitial macrophage accumulation, and (C) moderate emphysema in comparison with the (D) edema and mild inflammatory cell accumulation in response to the same duration of tobacco smoke (HE staining; a: alveolus, b: bronchioles, v: vessels; e: emphysema; asterisk: perivascular edema, arrows: macrophages).

Fig. 3. Histopathological alterations after 2 months of marijuana smoke exposure
Representative histopathological pictures of the lung samples obtained 2 months after the beginning of daily marijuana smoke exposure showing (A) perivascular and interstitial inflammation, (B) peribronchial edema and massive cellular inflammation, and (C) moderate
Fig. 4. Histopathological alterations after 3 months of marijuana smoke exposure

Representative histopathological pictures of the lung samples obtained 3 months after the beginning of daily marijuana smoke exposure demonstrating (A) massive interstitial inflammation with macrophage-like multinucleated giant cells, atelectasis, (B) perivascular and peribronchial lymphocyte accumulation, (C) bronchial irregularity and mucus producing goblet cell hyperplasia and emphysema, as compared to the (D) mild perivascular and interstitial inflammation in response to cigarette smoke (HE staining; b: bronchus, v: vessels; e: emphysema; asterisks: perivascular edema, black arrows: multinucleated macrophage-like giant cells; arrowheads: perivascular/peribronchial lymphocyte accumulation).

Fig. 5. Histopathological alterations after 4 months of marijuana smoke exposure

Representative histopathological pictures of the lung samples obtained 4 months after the beginning of daily marijuana smoke exposure showing (A) structural destruction, (B) fibrosis, atelectasis, microhaemorrhages and (C) extensive emphysema in comparison with the (D) interstitial inflammation and mild emphysema observed after tobacco smoke (HE staining; e: emphysema; black arrows: multinucleated macrophage-like giant cells).

Fig. 6. Histopathological evaluation of the lung sections

Box plots representing the medians with the 25 and 75 percentiles and whiskers showing the minimum and maximum values of (A) granulocyte, (B) lymphocyte and (C) macrophage cell
counts in 10,000 μm² interstitial regions per slides (n=30 counts/ slides, 3 slides per mouse, n=6-8 mice per group), as well as semiquantitative histopathological scoring (range: 0-3) of (D) perivascular/peribronchial edema, (E) inflammatory cell infiltration, (F) epithelial damage, (G) goblet cell hyperplasia and (H) fibrosis (+p<0.05, ++p<0.005, +++p<0.0005, ++++p<0.0001 vs. the intact, non-smoking group; *p<0.05, **p<0.005, ***p<0.0005 vs. tobacco smoke; Kruskal-Wallis followed by Dunn’s multiple comparison test). Panel I shows the evaluation of emphysema by the measurement of the mean linear intercept length (Lₘ). Columns show means±S.E.M. of n=6-8 mice per group (+p<0.05, ++p<0.005, ++++p<0.0001 vs. the intact, non-smoking group; **p<0.005, vs. tobacco smoke; n=80-100 measurements/slides, 3 slides per mouse, two-way ANOVA followed by Sidak’s multiple comparison test).

Fig. 7. Inflammatory cell profile and concentrations in the bronchoalveolar lavage fluid (BALF)

The number of lymphocytes, neutrophils and macrophages in the BALF after (A) 1, (B) 2, (C) 3 and (D) 4 months of marijuana smoke exposure as compared to the intact and respective tobacco-smoking groups. Columns show means±S.E.M. of n=6-8 mice per group; the dots show the individual values (●: intact; ■: tobacco; ▲: marijuana); +p<0.05, ++p<0.01 vs. the intact, non-smoking group; *p<0.05, vs. tobacco smoke (one-way ANOVA + Bonferroni’s modified t-test).

Fig. 8. Myeloperoxidase (MPO) activity and inflammatory cytokine concentrations in the lung homogenates

Panel A shows the MPO activities in the lung homogenates as an indicator of neutrophil and macrophage number and functions, at each timepoint in the marijuana smoking group.
compared to the non-smoking and tobacco-smoking groups. Panel B represents the inflammatory cytokine concentrations in the pulmonary tissue at the 2-month timepoint when the intensity of the cellular inflammation reaches its maximum. Columns show means±S.E.M. of n=6-8 mice per group; the dots show the individual values (●: intact; ■: tobacco; ▲: marijuana); +p<0.05, ++p<0.01, +++p<0.001 vs. the intact, non-smoking group; *p<0.05, **p<0.01, ***p<0.001 vs. tobacco smoke (one-way ANOVA + Bonferroni’s modified t-test).

Fig. 9. Airway hyperresponsiveness and inflammatory histopathological changes in CB1−/− mice

Panel A shows the 22 mM carbachol-induced percentage increase of Penh above baseline, panel B represents the composite semiquantitative histopathological scores (by adding score values for edema, inflammatory cell infiltration, epithelial damage and goblet cell hyperplasia) at the 2-month timepoint when inflammation reaction reaches its maximum. Box plots represent the medians with the 25 and 75 percentiles, the whiskers show the minimum and maximum values (n=5-6 mice per group; +p<0.05 vs. the intact, non-smoking group; Kruskal-Wallis + Dunn’s multiple comparison test). Panels C and D are representative histopathological pictures of (C) a wildtype CD1 and (D) a CB1−/− mouse lung samples obtained 2 months after the beginning of daily marijuana smoke exposure (HE staining; v: vessels; asterisk: perivascular edema).
Figure 1
Figure 2
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9