*Basic & Clinical Pharmacology & Toxicology*

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ORIGINAL ARTICLE OPEN ACCESS

# Differences in the Inflammatory Response and Corticoid Responsiveness of Human Lung Macrophages and Parenchymal Explants Exposed to Cigarette Smoke Extracts

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**Received:** 20 March 2025 | **Revised:** 18 April 2025 | **Accepted:** 22 April 2025

**Funding:** The authors received no specific funding for this work.

**Keywords:** chronic obstructive pulmonary disease | cigarette smoke extract | corticosteroid resistance | cytokine | glucocorticoid | lung explant | lung macrophage

## ABSTRACT

Smoking is the main cause of chronic obstructive pulmonary disease (COPD) and is associated with corticosteroid resistance. Given the paucity of data on human lung preparations, macrophages (LMs), and parenchymal explants (LPEs) were exposed to cigarette smoke extracts (CSE) in the presence or absence of lipopolysaccharide (LPS). Moreover, LMs and LPEs were treated with budesonide prior exposure to CSE or LPS. The levels of cytokines (TNF-α, IL-6) and chemokines (CCL2, CCL4, CXCL1, CXCL5, and CXCL8) in the supernatants were measured using ELISAs.

In LMs, exposure to CSE was not associated with significant difference in the production of cytokines and chemokines, with the notable exception of greater CXCL8 production. The results were generally the same for LPEs. CSE exposure did not potentiate the LPS-induced production of the cytokines and chemokines and even tended to reduce this production in LMs and LPEs. Lastly, CSE exposure inhibited budesonide's anti-inflammatory activity in LMs but not in LPEs.

This study extends the data on the CSE inflammatory effects and its inhibition of corticosteroid efficacy in human lung preparations. Our findings question the relevance of these preparations with regard to the long-term toxicity of smoking and the corticosteroid resistance observed in smokers and in patients with COPD.

Marion Brollo and Quentin Marquant Equal contributions.

**Summary**

Smoking is a risk factor for asthma outcomes and the leading cause of chronic obstructive pulmonary disease. There is no consensus on the effects of in vitro exposure to cigarette smoke extracts on both the inflammatory response and the corticoid responsiveness of human lung preparations. The present results show a weak inflammatory effect of two cigarette smoke extracts and the absence of corticoid resistance in whole lung tissue explants. Our findings question the relevance of these lung preparations with regard to the long-term toxicity of smoking and the corticosteroid resistance observed in smokers and in patients with COPD.

## 1 | Introduction and Background

The global prevalence of chronic obstructive pulmonary disease (COPD) is about 10%, and COPD causes around three million deaths annually [1]. Although it has been estimated that half of all cases of COPD worldwide are due to tobacco smoking, fewer than 50% of heavy smokers develop the condition [1]. The pharmacological treatment of stable COPD is based on inhaled bronchodilators and inhaled corticoids (ICSs) [1]. Treatment with ICSs improves lung function and reduces exacerbation rates in both current smokers and ex-smokers with COPD, although the magnitude of these beneficial effects is lower in heavy or current smokers than in light smokers or ex-smokers [1]. Furthermore, around half of adults with asthma are current or former smokers [2]. Smoking is a risk factor for poor asthma control; asthma exacerbation, the development of persistent airflow obstruction over time, and reduced effectiveness of inhaled and oral corticosteroids [2, 3] .

Cigarette smoke is a complex mixture of more than 4700 chemical compounds, and each cigarette puff contains an estimated $10^{14}$ free radicals [4, 5] . Exposure to cigarette smoke induces an increase in the number of immune cells within the lung; most of these are alveolar macrophages (AMs) [6–9].

Several studies have shown that AMs from smokers versus ex- or nonsmokers produce higher levels of interleukin (IL)-8 (CXCL8) under basal conditions or after exposure to lipopolysaccharide (LPS) [10–20]. However, the data are inconsistent for tumor necrosis factor alpha (TNF-α) and other cytokines. Yet other studies have shown that the production of CXCL8 and various cytokines (TNF-α, IL-1β, IL-6, CCL2, and CCL5) by AMs under basal conditions or after stimulation with LPS is the same in smokers as in nonsmokers or is even weaker in smokers [7, 8, 13, 17–19, 21–26].

Among smokers with asthma and people with COPD, there are large interindividual differences in the response to treatment with ICSs [2, 27]. The relative insensitivity of the LPS-induced production of CXCL8, TNF-α, and GM-CSF by AMs from smokers and from patients with COPD to the inhibitory effect of dexamethasone was first described 20 years ago [13, 14]. A similar result was subsequently reported for the LPS-induced production of CXCL8 and GM-CSF by AMs from patients with COPD and the LPS-induced production of CXCL8 by AMs from smokers but not for the LPS-induced production of IL-6 and CCL2 [20]. Furthermore, passive smoke exposure reportedly reduced the inhibitory effects of dexamethasone on CXCL8 release in AMs from children with uncontrolled, severe asthma [28]. However, a pooled analysis of seven studies failed to evidence low corticosteroid sensitivity of LPS-induced production of CXCL8 and TNF-α by AMs from patients with COPD or smokers [27]. Moreover, there were no significant differences between nonsmokers, smokers, and patients with COPD with regard to corticosteroid inhibition of the LPS-stimulated production of IL-6, CXCL1, CCL3, CCL5, and IL-10 by AMs [17, 24, 25, 27].

The reasons for these contrasting results are unclear but might include (i) heterogeneity among healthy smokers, as shown by the hierarchical clustering of the AM polarization-related genes (about one third of healthy smokers cluster with healthy nonsmokers) [8]; (ii) the rapid loss of tissue-specific signatures when macrophages are isolated and cultured [29, 30]; (iii) the lack of accurate measurements of tobacco smoke exposure [31]; and (iv) the considerable interindividual variations in the in vitro effect of corticosteroids, particularly at low concentrations [27, 32]. Therefore, an overall analysis of the data does not provide an objective view of the in vivo impact of smoking on AM activation and the AMs' sensitivity to corticosteroids.

In order to better assess the impact of smoking on lung cells in general and macrophages in particular, many research groups have studied the in vitro exposure of various human or murine primary lung cells, blood cells, or cell lines to cigarette smoke extract (CSE) [19]. Cytokine production by human lung macrophages (LMs) or monocyte-derived macrophages (MDMs) in response to CSE (alone or combined with LPS) has yielded conflicting observations, with the exception of CXCL8 (the production of which was typically increased by CSE exposure) [13, 26, 33–41]. In vitro exposure to CSE reduced the corticosteroid sensitivity of U937 cells [37, 42–45], peripheral blood mononuclear cells [44], and AMs [37] but not that of MDMs [36]. Again, an overall analysis of the data and the scarcity of data on AMs prevent an objective description of the in vitro impact of CSE on the activation of AMs and the cells' sensitivity to corticosteroids.

The primary objective of the present study was to assess the inflammatory responses induced by two different CSEs in human LMs and lung parenchymal explants (LPEs) since different extracts can lead to different results [19]. One extract was produced in-house by the classic method of bubbling cigarette smoke through tissue culture medium, and the other was prepared from the cigarette condensate produced by an engineered smoking machine [19]. Given that there are more than 50 different types of cell in the lung (which might respond differently to tobacco smoke), we decided to study LPEs too. These are explants in which the in situ parenchymal architecture and cell–cell communication are maintained [19, 46]. The secondary objective was to compare the response to budesonide (a corticosteroid used widely to treat both asthma and COPD) in LMs versus LPEs, in order to further document potential inhibition of the corticosteroid's anti-inflammatory effects by in vitro exposure to CSE.

## 2 | Material and Methods

### 2.1 | Materials

Bovine serum albumin and Roswell Park Memorial Institute 1640 (RPMI) medium were purchased from Eurobio Biotechnology (Les Ulis, France). Budesonide, antibiotics, dimethylsulphoxide, L-glutamine, Trypan blue dye, heat-inactivated fetal calf serum (FCS), and LPS (from *E. coli* serotype 0111:B4) were purchased from Sigma Aldrich (St. Louis, Missouri, United States). TNF-α was obtained from Biotechne (Minneapolis, USA). Budesonide was dissolved in 0.05% dimethylsulphoxide.

### 2.2 | Characteristics of the Sample Donor Patients

The research use of human lung tissue was approved by an institutional review board (DC 11 10 05 - *CPP Ile de France VIII,* Boulogne-Billancourt, France), and all patients gave their informed consent prior to surgery. The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies [47]. Lung tissues were obtained from 51 patients (31 males and 20 females; mean ± standard deviation age: 63.5 ± 8.1 years; 22 current smokers, 24 ex-smokers, and 5 never-smokers; mean ± standard deviation pack-years: 43.0 ± 21.9; FEV1 = 81.7 ± 16.2%; FEV1/FVC ratio: 0.74 ± 0.17; 12 patients with (nonsevere) COPD (FEV1/FVC < 0.7)) undergoing surgical resection for lung carcinoma and who had not received chemotherapy or radiotherapy before surgery. LMs and LPEs were isolated from lung parenchyma taken from areas lacking macroscopic signs of infection, necrosis, or ischemia and at some distance from the tumor. The samples were dissected free of pleura, visible airways, and blood vessels.

### 2.3 | Isolation and Culture of Human LMs

LMs were isolated from finely minced parenchyma, as described previously [48–52]. Briefly, the fluid collected from several washings of the minced lung tissues with sterile RPMI 1640 medium (supplemented with 100 μg.mL$^{-1}$ streptomycin, 100 U.mL$^{-1}$ penicillin, and 2 mM glutamine) was centrifuged at 300 × g for 10 min, and the cell pellet was resuspended in the culture medium supplemented with 10% FCS (complete medium). Resuspended, viable cells were then plated at $10^6$ cells mL$^{-1}$ per well in a 24-well plate. After a 90-min incubation at 37°C in a humidified 5% $CO_2$ atmosphere, nonadherent cells were removed by gentle washing. The adherent cells (≈200 × $10^3$ cells per well) were > 95% pure macrophages, as determined by May–Grünwald–Giemsa staining and CD68 immunocytochemistry. Cell viability exceeded 90%, as assessed by Trypan blue dye exclusion. Prior experiments (performed after the adherence step) with a CytoFLEX SRT flow cytometer (Beckman Coulter Life Sciences, Villepinte, France) showed that the vast majority (~90%) of the LMs were AMs (CD45+/CD169+/CD206+/CD43+/CD14low) and that the mean proportion of interstitial macrophages (CD45+/CD169+/CD206+/CD43-/CD14high) was ~10% [52].

### 2.4 | Preparation and Culture of LPEs

The procedure for preparation of LPEs has been described previously [46, 53, 54]. Briefly, parenchyma was finely chopped into ~3–5-mm$^3$ fragments, washed in complete medium (to prevent contamination by blood), and then maintained overnight at 4°C. On the day after isolation, the parenchymal fragments were washed again in complete medium. Five fragments (total weight ~50–100 mg) were distributed into 6-well plates (3 mL of medium per well).

### 2.5 | Production of CSEs

A classical CSE was prepared as previously described [13, 40]. Briefly, the smoke from two cigarettes (Marlboro Red, each containing 8.5 mg of tar (ISO 4387), 0.65 mg of nicotine (ISO 10315), and 8 mg of carbon monoxide (ISO 8454) on average) was bubbled through 20 mL of sterile RPMI 1640 medium (supplemented with 100 μg.mL$^{-1}$ streptomycin, 100 U.mL$^{-1}$ penicillin, and 2 mM glutamine) for ≈5 min per cigarette, with the aid of a peristaltic pump. The medium was then sterilized using a 0.2-μm filter.

A cigarette smoke condensate (CSC) was obtained using standard trapping and extraction procedures. It contained total particulate matter, water, nicotine, and "tar" and was a generous gift from SEITA Imperial Tobacco (Fleury-les-Aubrais, France). The condensate was obtained from 2R4F* reference cigarettes (University of Kentucky, Lexington, Kentucky, United States), using a standard analytical cigarette-smoking machine (ISO 3308) under ISO 4387 conditions (puff duration: 2 s; puff volume: 35 mL; 1 puff per minute). Ten cigarettes were smoked per filter. The trapped condensate was then diluted in DMSO to a "tar" concentration of 10 mg/mL, which corresponds roughly to 0.75 mg/mL nicotine.

To ensure standardization between experiments, the absorbance (Biochrom WPA Biowave DNA Life Science, Fisher Scientific, Illkirch, France) of CSE and CSC solutions was adjusted by dilution to a value of 1 at 320 nm (the optical density for nicotine/tar). The standardized CSE and CSC solutions were used in experiments within 30 min of preparation because it has been reported that CSE no longer optimally induced CXCL8 release from THP-1 cells more than 1 h after preparation [33]. The CSE and CSC were diluted to 1%, 5%, and 7.5% (final percentage) in culture medium. As reported previously [38, 40, 41, 55], the incubation of LMs or LPEs with the smoke extracts (up to 7.5%–10%) alone or in combination with LPS for 24 h did not affect cell viability as assessed in either a lactate dehydrogenase assay or a tetrazolium salt (3-[4.5-dimethylthiazol-2-yl]-5.5-diphenyltretrazolium bromide) assay (data not shown).

### 2.6 | Treatment of LMs and LPEs

The LM and LPE preparations were exposed to the vehicles (culture medium or DMSO) or the CSE or CSC dilutions for

24 h at 37°C in a humidified atmosphere (95% air, 5% (v/v) $CO_2$). In some experiments, suboptimal concentrations of LPS (10 ng/mL for LMs and 1 μg/mL for LPEs) vehicle (culture medium) were added to the culture wells 1 h after CSE or CSC exposure and remained present for the remainder of the 24 h incubation. The supernatants were then collected and stored at −80°C, prior to cytokine assays. The choice of a 24 h incubation period was based on our previous experiments on both LM and LPE preparations exposed to LPS [46, 48–51, 53] and to 7.5% CSE (data not shown). The maximal concentration of DMSO (0.3%) did not alter either the basal or the LPS-induced production of cytokines.

In order to evaluate the corticosteroid sensitivity of CSE-induced production of cytokines, the LMs and LPEs were pretreated with three concentrations of budesonide ($10^{-10}$, $10^{-9}$, and $10^{-8}$ M) for 45 min before exposure to CSE (7.5%) and/or LPS. After 24 h, supernatants were collected and stored at −80°C, prior to cytokine assays.

### 2.7 | Cytokine Assays

The concentrations of TNF-α, IL-6, CCL2, CCL4, CXCL1, CXCL5, and CXCL8 in the supernatants from LM and LPE experiments were measured with ELISAs (Duoset Development System, R&D Systems Europe, Lille, France), according to the manufacturer's instructions. The supernatants were diluted with reagent diluent as appropriate, and the optical density at 450 nm was determined using an MRX II microplate reader (Dynex Technologies, Saint Cloud, France). The limits of detection were 10 $pg.mL^{-1}$ for IL-6, 16 $pg.mL^{-1}$ for TNF-α, CCL2, CCL4, and CXCL5, and 32 $pg.mL^{-1}$ for CXCL1 and CXCL8. To determine whether the culture medium containing CSE or CSC at a final dilution of 7.5% affected the cytokine concentration measured in ELISAs, the assay standards were prepared in culture medium with or without CSE or CSC; the difference between the two conditions was not significant (data not shown).

### 2.8 | Statistical Analysis

Data were expressed as the mean ± standard error of the mean (SEM) cytokine production or as a percentage of the LPS- or CSE-stimulated production of cytokine per $10^6$ LMs or (for LPEs) per 100 mg of wet weight. The number of patients from whom LMs or LPE preparations were obtained is indicated by *n*. Budesonide's potency was estimated as the best fit values from the concentration-response curve and expressed as the -log of the concentration of budesonide that reduced the production of the cytokines by 50%, with respect to the maximum observed effect (i.e., -log$EC_{50}$ ± standard deviation). Log-transformed data were analyzed using an unpaired or paired Student's *t* test, a one-way repeated-measures analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons, a mixed-effects repeated-measures ANOVA followed by Tukey's test for multiple comparisons, or a two-way ANOVA followed by Tukey's test for multiple comparisons, as appropriate. The threshold for statistical significance was set to $p < 0.05$. All analyses were performed using GraphPad Prism software (Version 8.4.2, GraphPad Software Inc., San Diego, California, United States).

## 3 | Results

### 3.1 | Effects of CSE and CSC on Cytokine Production by LMs

As expected and in line with our previous report [50], LPS markedly induced the production of all the cytokines analyzed (Figure 1 and Table 1). Exposure to CSE or CSC significantly promoted CXCL8 production in a concentration-dependent manner in the absence of LPS but not in the presence of LPS (Figure 1). The basal production of CCL2 was weakly promoted both by CSE and CSC, although the relative increase was not statistically significant with CSC. The LPS-induced production of CCL2 was not changed by exposure to CSE or CSC (Figure 1). The basal production of TNF-α was not altered in any way, although the LPS-induced production was significantly reduced in presence of CSE or CSC (Figure 1). The basal and the LPS-induced productions of CCL4 (Figure 1), IL-6, CXCL-1, and CXCL5 (Table 1) were reduced by exposure to CSE or CSC.

### 3.2 | Effect of CSE and CSC on Cytokine Production by LPEs

As we reported previously for LPEs [46, 53, 54], LPS induced the production of the four cytokines assessed in the present study (Figure 2). In contrast to the LM experiments, exposure of LPEs to CSE or CSC was not associated with a difference in the basal production of CXCL8, TNF-α, or CCL2. The basal production of CCL4 was lower, as observed in the LM preparations. Exposure to CSE or CSC was associated with a significant reduction in the LPS-induced production of TNF-α only; the intercondition differences in the production of the other three cytokines were not statistically significant (Figure 2).

In both LM and LPE preparations, there was no apparent difference in the production of any measured cytokines between current smokers and ex-smokers or nonsmokers or between individuals with versus without COPD (data not shown).

### 3.3 | The Inhibitory Effect of Budesonide on Basal and CSE- and LPS-Induced Production of Cytokines

In LMs, budesonide significantly inhibited the basal and LPS-stimulated production of TNF-α, CCL2, CCL4, and CXCL8 in a concentration-dependent manner, with a higher inhibitory effect at $10^{-8}$ M for TNF-α and CCL2 than for CCL4 and CXCL8 (Figure 3 and Table 2). Budesonide also significantly inhibited the CSE-stimulated production of the four cytokines in a concentration-dependent manner, although the effects were significantly smaller than for basal and LPS-induced production (Figure 3 and Table 2).

In LPEs, budesonide had similar effects on the basal, LPS-induced, and CSE-induced production of TNF-α, CCL2, CCL4, and CXCL8. A small but significant difference was only observed

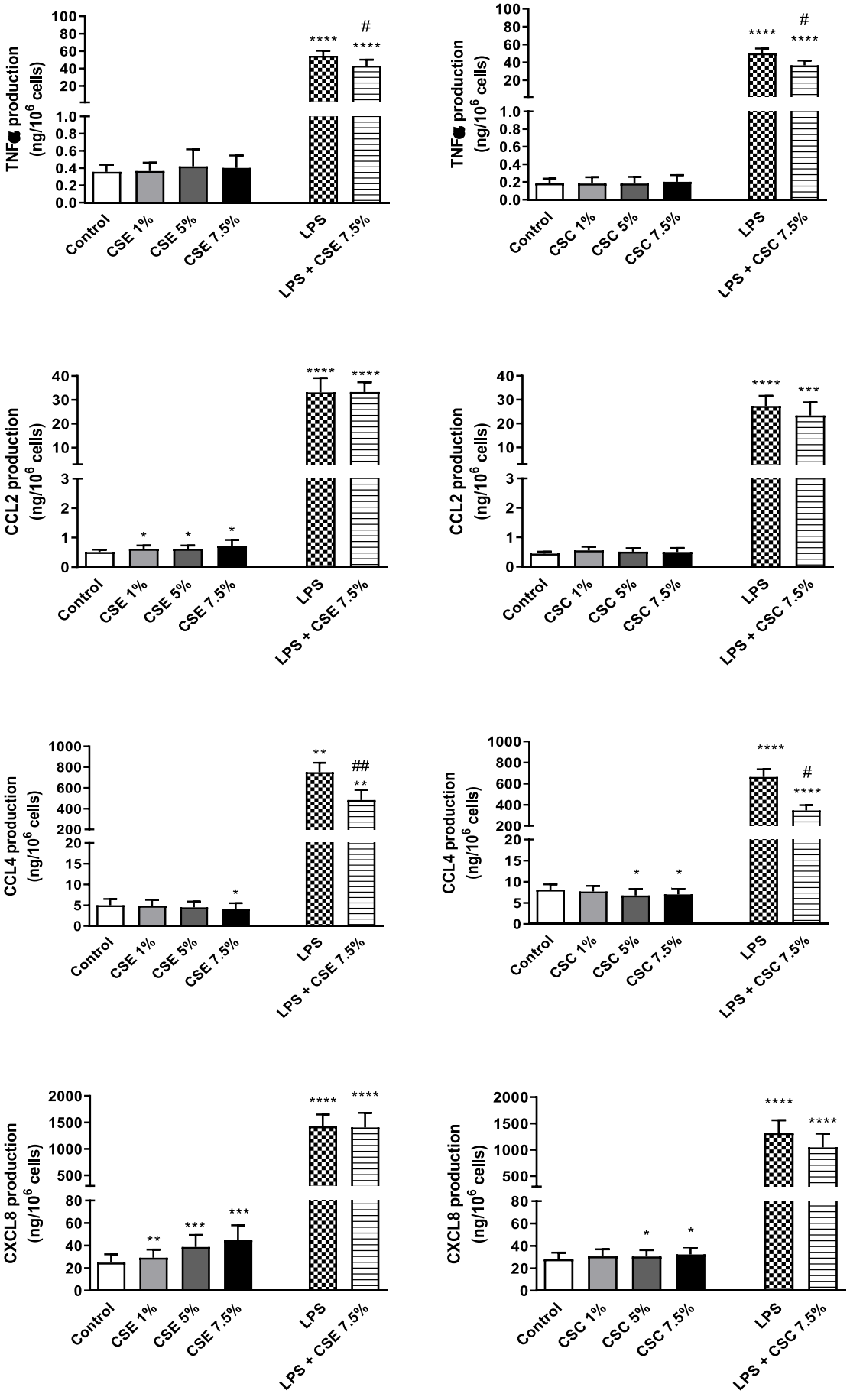


**FIGURE 1** | Legend on next page.

**FIGURE 1** | Effects of CSE and CSC on the basal production and LPS-stimulated production of cytokines by human LMs. The values are quoted as the mean ± SEM of 12–21 independent experiments (for CSE) or 7–11 independent experiments (for CSC).

**TABLE 1** | The effect of CSE and CSC (alone or combined with LPS) on levels of IL-6, CXCL1, and CXCL5 in the culture supernatant of human LMs.

| | Control | CSE 1% | CSE 5% | CSE 7.5% | LPS | LPS + CSE 7.5% |
|---|---|---|---|---|---|---|
| IL-6 | 0.22 ± 0.08 | 0.16 ± 0.06 (−14.7 ± 6.3%) | 0.16 ± 0.07** (−23.7 ± 4.6%) | 0.19 ± 0.10* (−26.4 ± 8.3%) | 326.32 ± 49.9 | 203.52 ± 59.73** (−43.2 ± 8.1%) |
| CXCL1 | 1.22 ± 0.15 | 1.35 ± 0.17 (13.7 ± 3.7%) | 1.15 ± 0.14 (−4.3 ± 5.9%) | 1.03 ± 0.14 (−18.8 ± 7.4%) | 390.20 ± 92.98 | 293.33 ± 67.02# (−17.2 ± 5.7%) |
| CXCL5 | 0.50 ± 0.12 | 0.40 ± 0.12 (−25.7 ± 6.4%) | 0.43 ± 0.16 (−24.5 ± 11.9%) | 0.53 ± 0.25 (−12.2 ± 20.4%) | 7.49 ± 1.79 | 5.08 ± 2.33$ (−38.7 ± 12.8%) |
| | **Control** | **CSC 1%** | **CSC 5%** | **CSC 7.5%** | **LPS** | **LPS + CSC 7.5%** |
| IL-6 | 0.18 ± 0.06 | 0.16 ± 0.06 (−1.9 ± 4.0%) | 0.14 ± 0.06 (−17.5 ± 6.7%) | 0.11 ± 0.04** (−27.7 ± 7.3%) | 312.88 ± 63.93 | 146.46 ± 40.68*** (−59.2 ± 5.9%) |
| CXCL1 | 1.20 ± 0.22 | 1.12 ± 0.20 (−4.9 ± 3.9%) | 0.92 ± 0.13 (−15.6 ± 7.1%) | 0.75 ± 0.11# (−25.7 ± 11.1%) | 359.49 ± 94.31 | 221.13 ± 32.09* (−26.1 ± 11.0%) |
| CXCL5 | 0.37 ± 0.15 | 0.40 ± 0.20 (23.1 ± 8.8%) | 0.21 ± 0.07** (−41.5 ± 4.9%) | 0.20 ± 0.07* (−38.6 ± 7.5%) | 10.97 ± 2.00 | 4.03 ± 1.92* (−67.2 ± 10.6%) |

*Note:* Lung macrophages were incubated with CSE or CSC in the presence or absence of LPS (10 ng/mL). Supernatants were harvested at 24 h and analyzed for cytokine levels with an ELISA. The results are expressed in ng/$10^6$ LMs. Values are quoted as the mean ± SEM per series of experiments performed on paired preparations from 7 to 15 different patients. The relative difference (%) is quoted in brackets as the mean ± SEM.
$$p = 0.066$.
#$p = 0.052$.
*$p \leq 0.05$ versus control experiments or (for the combination of LPS with CSE or CSC (7.5%)) vs. LPS only.
**$p < 0.01$ versus control experiments or (for the combination of LPS with CSE or CSC (7.5%)) vs. LPS only.
***$p < 0.001$ versus control experiments or (for the combination of LPS with CSE or CSC (7.5%)) vs. LPS only.

in the inhibitory effect of $10^{-9}$ M budesonide when comparing basal production of TNF-α with the LPS- or CSE-induced production of TNF-α (Figure 3 and Table 2).

## 4 | Discussion

The present study is the first to have compared the effects of the in vitro exposure of LMs and LPEs to two types of CSEs. The CSE contained the water-soluble components of cigarette smoke while the CSC contained the lipid-soluble components. In LMs, CSE and CSC did not significantly increase or decrease the production of TNF-α, IL-6, and chemokines such as CCL4, CXCL1, and CXCL5; the only exceptions were CXCL8 and (to a lesser extent) CCL2, the production of which was higher after exposure to CSE and CSC. In LPEs, the results were generally the same but a significant elevation in the production of CXCL8 was not observed. Furthermore, CSE and CSC did not accentuate the LPS-induced production of cytokines and even tended to reduce it in both LMs and LPEs. Moreover, CSE inhibited the anti-inflammatory activity of budesonide in LMs but not in LPEs.

### 4.1 | Effects of the Cigarette Smoke Extracts on Cytokine Production by Human LMs

According to the literature data, exposure to classic CSE obtained by bubbling cigarette smoke into culture medium is associated with greater basal production of CXCL8 by AMs or LMs from nonsmokers, smokers, or patients with COPD [13, 33, 37, 38]. However, the production of TNF-α was either not changed or increased to a lesser extent than for CXCL8 [37, 38]. The production levels of IL-6 and CCL2 were not significantly different, and that of CCL5 was even reduced. The expression of the CCL4 gene was also reduced by exposure to CSE [36–38]. The present study of human LMs not only confirms the previous findings but also extends them to the production of other cytokines (CCL4, CXCL1, and CXCL5) and the standardized CSC.

Although the MDM is a surrogate macrophage model that does not adequately recapitulate the biology of human LMs [56–58], exposure to CSE of MDMs from patients with COPD [35] or healthy volunteers [34, 40, 41, 59] was similarly associated with greater basal production of CXCL8 but not TNF-α, IL-6, CCL2, CCL5, or CXCL1. However, it has been reported that CSE increases the production of TNF-α, IL-6, and CXCL8 in a dose-dependent manner in MDMs from healthy blood donors [60]. These contradictory results for the response to CSE might be due to experimental differences in the way that blood monocytes are differentiated into MDMs.

Regarding the impact of CSE on LPS stimulation, Metcalfe et al. studied LM preparations from patients with COPD and from patients without COPD [38]. The latter LM preparations were probably similar to the one used in the present study. The researchers found that exposure to CSE had no effect on

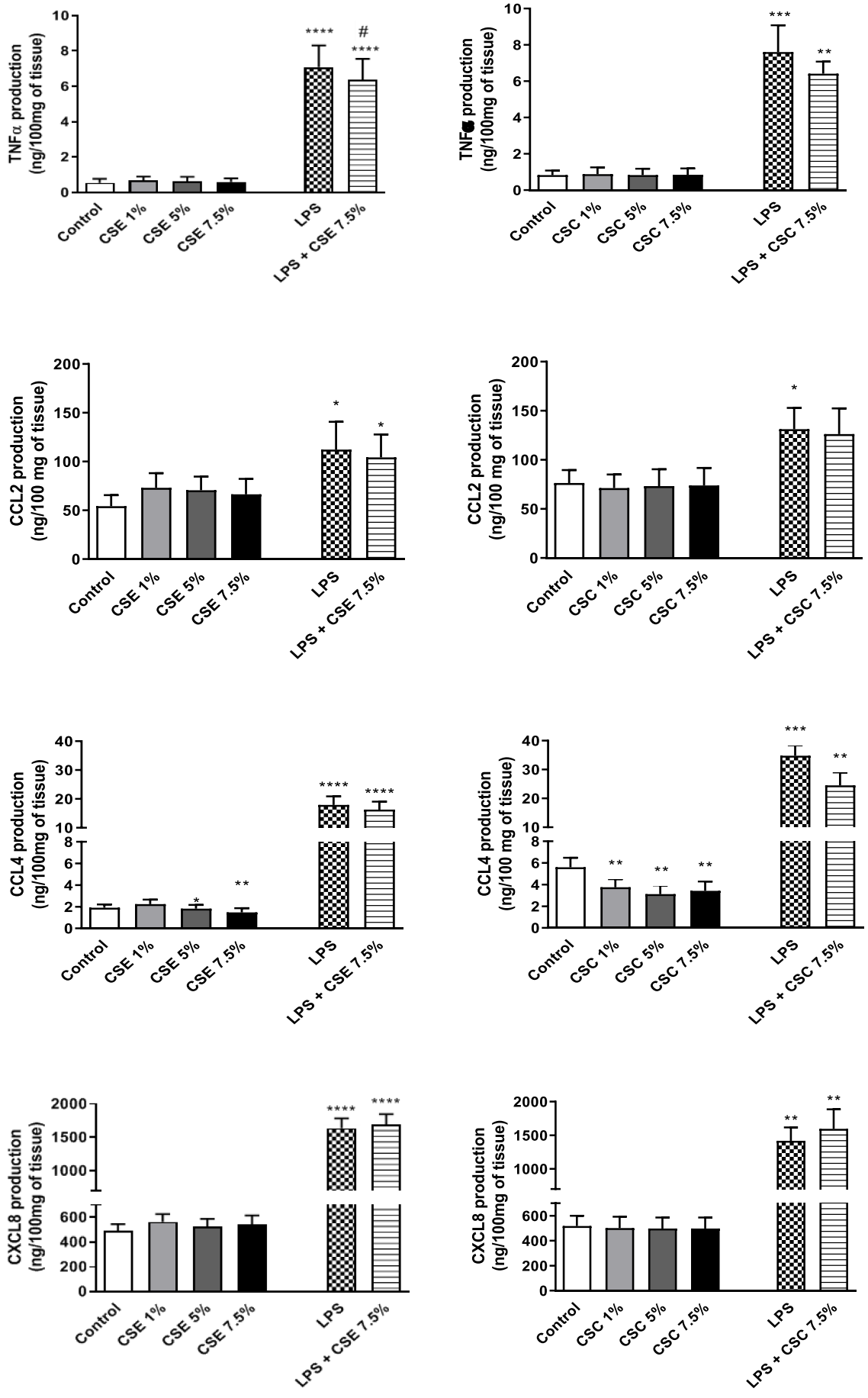


**FIGURE 2** | Legend on next page.

**FIGURE 2** | Effects of CSE and CSC on the basal production and the LPS-stimulated production of cytokines by human LPEs. The results are quoted as the mean ± SEM of 15 independent experiments (for CSE) and 7–10 independent experiments (for CSC).

CXCL8 expression induced by Toll-like receptor (TLR) ligands (including LPS) but suppressed the TLR-induced production of TNF-α, IL-6, and CCL5 [38]. Our present findings are in line with Metcalfe et al.'s results and extends the results to the LPS-induced production of other cytokines (CCL2, CCL4, CXCL1, and CXCL5) with two different cigarette smoke extracts. In contrast, exposure to CSE reportedly causes a significant increase in the LPS-induced production of CXCL8 and TNF-α in AM preparations from bronchoalveolar lavages. The differences in the results for CXCL8 and TNF-α might be explained (at least in part) by the fact that (i) the bronchoalveolar lavages came from nonsmokers with diffuse interstitial lung diseases, and (ii) the isolated AMs were exposed to a higher concentration of LPS (0.5 μg/mL) [37].

Two studies of MDMs from the same research group showed that CSE exposure potentiated the LPS-induced production of CXCL8 and CCL2, while the production of TNF-α was not changed and that of IL-6 and CXCL1 was suppressed [40, 41]. Furthermore, CSE reportedly inhibits the LPS-induced release of TNF-α, IL-1β, and IL-18 in MDMs [61].

Taken as a whole, our present findings and the literature data on the in vitro effects of CSE on macrophages suggest that CXCL8 is the only inflammatory cytokine whose production is consistently increased by short-term exposure to CSEs. For the other cytokines tested, levels of production or expression were either unchanged or (quite frequently) reduced. It has been suggested that the increase in CXCL8 protein and gene expression in COPD AMs is linked to the CSE-induced activation of p38 MAPK [38].

These in vitro results are in line with the ex vivo results for AMs from smokers. In the vast majority of published studies, the production of cytokines and chemokines (with or without stimulation with TLR agonists, including LPS) by AMs from smokers was the same as (or lower than) than that observed with AMs from non or ex-smokers; however, increases in the LPS-induced production of CXCL8 and CCL2 have been reported in AMs from smokers [7, 12, 17, 20, 22–26, 38, 62].

### 4.2 | Effects of the Cigarette Smoke Extracts on Cytokine Production by Human LPEs

Along with immune cells such as macrophages, human structural lung cells (including airway epithelial cells [63–65], pulmonary artery endothelial cells [37, 66], bronchial smooth muscle cells [67–72], and lung fibroblasts [73]) produce CXCL8 and some other cytokines in response to CSE. However, the cytokine expression profile depends on the CSE concentration and exposure time.

In integrated models of human lung tissues, an inflammatory response to CSE was weak or absent. In a model of human bronchial tissue with a fibroblast layer, a significant increase in the production of CXCL8 and CXCL1 was observed only after several days of CSE exposure [74]. In a native matrix-based human lung alveolar tissue model, no significant increases in IL-6, CXCL8, and TNF-α were observed in response to CSE [75]. The LPEs studied here contain the full set of lung cells, with a normal spatial configuration and normal cell-to-cell ratios; as such, they accurately approximate in vivo conditions in the lung [46, 53, 76]. To the best of our knowledge, the present study is only the second to have explored the effect of in vitro exposure to CSEs on human LPEs. In the first such study [55], the exposure of LPEs to 0.625%–20% *v*/*v* of CSE from 3R4F cigarettes for 24 h was not associated with an increase in the production of CXCL8 or IL-6. In our LPE model, the basal production and LPS-induced production of CXCL8 and of three other cytokines (TNF-α, CCL2, and CCL4) were not increased by exposure to CSE or CSC.

### 4.3 | Effects of CSE on the Anti-Inflammatory Activity of Budesonide in Human LMs and LPEs

Dexamethasone has been shown to weakly inhibit CSE-stimulated CXCL8 release by AMs from smokers and to have no effect on this release by AMs from patients with COPD. In AMs from smokers, dexamethasone was about 10-fold less potent in inhibiting CXCL8 production after exposure to CSE, while its maximum inhibitory effects at 10 μM were similar in the presence and absence of in vitro exposure to CSE [13]. In human AMs, exposure to CSE (5%) or $H_2O_2$ (500 μM) approximately halved the inhibitory effect of budesonide (100 nM) and dexamethasone (1 μM) on the LPS-induced production of CXCL8 and TNF-α [37]. The results of our present study of budesonide concentrations of up to 10 nM in human LMs suggests that the CSE-induced reduction in the inhibitory effect of corticosteroids is not limited to CXCL8 and TNF-α but also concerns CCL2 and CCL4.

The exposure of U937 macrophages to $H_2O_2$ (100 or 500 μM) reduced the ability of dexamethasone (10 nM or 1 μM) to suppress LPS-induced CXCL8 release [14, 37]. Furthermore, the treatment of U937 macrophages with CSE reportedly reduces the potency and the efficacy of dexamethasone with regard to TNF-α-induced CXCL8 production [42, 44, 77]. However, CSE (10%) exposure did not reduce the inhibitory effect of dexamethasone (100 nM) on mRNA and protein expression of CXCL8 by MDMs from patients with COPD [36]. These observations suggest that U937 macrophages, MDMs, and AMs differ in their corticoid responsiveness following in vitro exposure to CSE.

In sharp contrast, in vitro exposure to CSE did not alter the inhibitory effects of budesonide on LPEs. This finding is probably due to the variety of cell types present in LPEs that contribute to the budesonide response. It is noteworthy that budesonide's inhibition of the release of CXCL8 and IL-6 in response to IL-1β and TNF-α was not altered by exposure to CSE in fibroblasts from both normal and COPD lungs [78]. Moreover, exposure of primary bronchial epithelial cells from healthy, nonsmokers

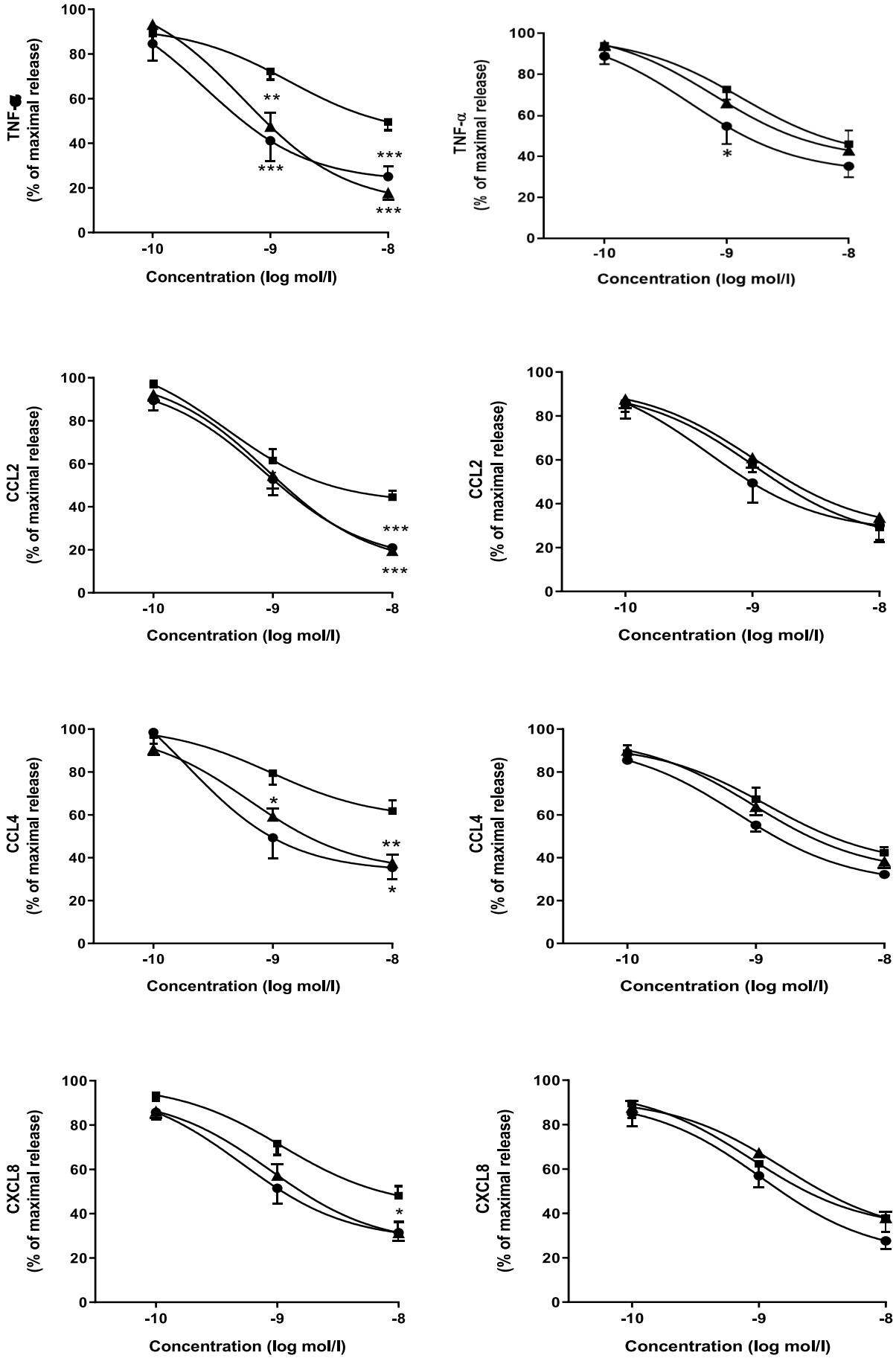


**FIGURE 3** | Legend on next page.

**FIGURE 3** | Effects of budesonide on the production of cytokines by human LMs (left column) and by LPEs (right column) not exposed to LPS or CSE (basal, ●) or exposed to LPS (▲) or CSE (■). The results are shown as the mean ± SEM of 7–15 independent experiments. The curves were fitted using nonlinear regression (least-squares fit).

**TABLE 2** | The potency (-log$EC_{50}$) and efficacy (% inhibition of cytokine production) of budesonide on basal cytokine production and LPS- or CSE-induced cytokine production in culture supernatants of human LMs and LPEs.

| | | LMs | | LPEs | |
|---|---|---|---|---|---|
| **Cytokines** | | **Potency** | **Efficacy** | **Potency** | **Efficacy** |
| TNFα | Basal | 9.55 ± 0.22 | 75.2 ± 3.9 | 9.30 ± 0.13 | 63.9 ± 3.6 |
| | LPS | 9.23 ± 0.15 | 83.4 ± 5.3 | 9.11 ± 0.12 | 57.6 ± 4.9 |
| | CSE | 8.69 ± 0.30 | 50.6 ± 6.8 | 8.88 ± 0.29 | 55.4 ± 7.2 |
| CCL2 | Basal | 9.07 ± 0.20 | 79.1 ± 4.1 | 9.34 ± 0.21 | 69.9 ± 4.8 |
| | LPS | 9.04 ± 0.14 | 81.8 ± 5.7 | 8.99 ± 0.18 | 67.3 ± 5.5 |
| | CSE | 9.38 ± 0.23 | 54.3 ± 4.6 | 9.01 ± 0.21 | 77.2 ± 6.7 |
| CCL4 | Basal | 9.72 ± 0.52 | 66.4 ± 5.7 | 9.14 ± 0.13 | 67.9 ± 3.4 |
| | LPS | 9.19 ± 0.19 | 61.5 ± 4.9 | 9.02 ± 0.18 | 62.1 ± 5.1 |
| | CSE | 9.02 ± 0.36 | 38.3 ± 3.8 | 8.95 ± 0.24 | 58.7 ± 6.1 |
| CXCL8 | Basal | 9.46 ± 0.16 | 68.2 ± 5.2 | 8.96 ± 0.22 | 73.4 ± 3.8 |
| | LPS | 9.06 ± 0.19 | 68.6 ± 3.7 | 8.81 ± 0.16 | 70.2 ± 4.7 |
| | CSE | 9.01 ± 0.27 | 51.2 ± 4.1 | 9.06 ± 0.30 | 67.2 ± 8.4 |

*Note:* The quoted potency ($EC_{50}$) and efficacy values are the best fit values from the curves shown in Figure 3. The $EC_{50}$ is the concentration required to elicit 50% of the maximum effect of budesonide for each cytokine.

to $H_2O_2$ did not reduce budesonide's concentration-dependent inhibitory effect on baseline and TNF-α-induced CXCL8 production but did significantly reduce the inhibitory effect of budesonide on the human bronchial epithelial cell line 16HBE; hence, primary cells and cell lines might differ in their responsiveness [79]. In contrast, CSE reduced the inhibitory effect of dexamethasone on the TNF-α -induced CXCL8 production by human primary pulmonary artery endothelial cells [66].

The literature data on the impact of smoking or COPD on the ex vivo response to corticosteroids are contradictory. In some studies, dexamethasone's ability to inhibit basal and LPS-induced production of CXCL8 was reduced in AMs from patients with COPD, when compared with AMs from smokers and/or to healthy nonsmokers [13, 14, 20]. Other studies did not show a difference between AMs from healthy nonsmokers, smokers, and patients with COPD, with regard to the effects of dexamethasone or beclometasone-17-monopropionate on LPS-induced production of TNF-α, IL-6, CCL2, CCL3, CXCL1, and CXCL8 [7, 17, 24, 25, 27, 32, 80]. These discrepancies (particularly for CXCL8) might be due to large interindividual differences in the influence of the stimulus (CSE alone or combined with LPS) on corticoid sensitivity [27, 36, 38].

### 4.4 | Strengths and Limitations of the Study

The study's strengths include the use of two types of cigarette smoke extracts, the measurement of levels of several cytokines other than CXCL8, and the use of preparations of human LMs and LPEs (rather than MDMs or macrophage cell lines) that more closely mimic the in vivo situation and provide greater translational value. Furthermore, we used a lengthy procedure for LM isolation because a short procedure is associated with lower corticosteroid inhibition of LPS-induced TNF-α and CXCL8 production [32].

The study also has some limitations. Firstly, CSE and CSC only capture finite fractions of the respectively water-soluble and lipid-soluble components of complex smoke mixtures. Secondly, we did not reproduce the experiments on the effects of CSE on the response to budesonide using CSC because (1) the data in the literature on the inhibition of the in vitro response to corticoids by cigarette smoke are based exclusively on the use of classical CSE, obtained by bubbling or $H_20_2$ as a surrogate, (2) exposure to CSC did not induce different effects than exposure to CSE on macrophage and explant preparations, and (3) there was difficulty in obtaining sufficient quantities of human lung tissue. Thirdly, acute in vitro exposure of LMs and LPEs might not accurately recapitulate a long-term exposure to the cigarette smoke mixture as a whole [81]. The limited clinical effectiveness of corticosteroids in asthmatic smokers and patients with COPD might be linked not only to the various molecular mechanisms associated with the reduced in vitro inhibitory effect of corticoids mainly observed in macrophages after acute exposure to CSE but also to the underlying, smoke-induced inflammation and epithelial remodeling (which is largely corticosteroid-resistant) and irreversible pulmonary lesions in COPD (such as

the changes in elastic fibers, emphysema, and fibrosis) [82–84]. Human lung organoids (particularly bronchial lung organoids) and lung-on-a-chip systems might better reflect the situation in vivo because they can be maintained for long periods of time. However, lung organoids and lung-on-a-chip systems do not model the entire organ, require standardization, and are expensive and complex to set up [85].

## 5 | Conclusion

In vitro exposure to CSEs induces a reduction rather than a stimulation of the innate immune response by LMs and LPEs, with the exception of an increase in CXCL8 production by LMs, and a reduced anti-inflammatory effect of corticoids on LMs but not on LPEs. Taken as a whole, our present findings and the literature data on the effects of acute in vitro exposure to CSEs question the relevance of these cell-based or tissue-based models of the lung with regard to the toxicity of smoking and the corticosteroid resistance observed in smokers and in patients with COPD.


### Acknowledgements

The authors thank David Fraser DPhil (Biotech Communication SARL, Ploudalmezeau, France) for copy editing assistance.


### Conflicts of Interest

Nicolas Roche has given paid lectures and/or done consulting for Boehringer Ingelheim, GlaxoSmithKline, Astra Zeneca, Sanofi, Chiesi, Pfizer, Novartis, Austral, Biosency, Zambon, MSD, and Menarini all unrelated to the present work; Nicolas Roche reports participation in research projects funded by Boehringer-Ingelheim, Pfizer, and GlaxoSmihLine, all with funds paid to the institution where he was employed (no personal fees) and with no relation to the work reported in this paper. Nicolas Roche is European Respiratory Society Science Council Chair. Martin Dres reports participation in research projects funded by GlaxoSmihKLine and Fisher & Paykel all with funds paid to the institution where he was employed (no personal fees) and with no relation to the work reported in this paper. Philippe Devillier has given paid lectures and/or done consulting for Boehringer Ingelheim, GlaxoSmithKline, Astra Zeneca, Chiesi, and Menarini all unrelated to the present work. The remaining authors report no potential conflicts of interest.

### Data Availability Statement

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

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