ORIGINAL ARTICLE

# Effect of cigarette smoke extract on the intestinal microenvironment of ulcerative colitis tissue

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## Abstract

**Background and Aim:** Ulcerative colitis (UC) is an autoimmune disease characterized by inflammation in the gastrointestinal tract. The severity of UC is higher in non-smokers than smokers; however, the biological mechanisms controlling this effect remain unknown. The aim of this study was to examine the effect of cigarette smoke extract (CSE) on inflamed and noninflamed colonic tissue from UC patients and to determine if inflammatory mediators, transcription factors, and T cell phenotypes are altered by CSE.

**Methods:** Blood and colonic biopsies were obtained from UC patients undergoing endoscopy. Biopsies were cultured in the presence or absence of CSE. Multiplex enzyme-linked immunosorbent assay (ELISA) measured secreted levels of inflammatory mediators. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Hypoxia-inducible factor 1-alpha (HIF-1α) expression were measured by DNA-binding ELISA. T cell phenotypes were assessed by flow cytometry in matched blood and biopsies.

**Results:** Secreted levels of interleukin 2 (IL-2), interleukin 6 (IL-6), tumor necrosis factor - alpha (TNF-α), chemokine (C-C motif) ligand 2 (CCL2), and interleukin 10 (IL-10) were significantly (all $P < 0.05$) decreased following treatment with CSE. This effect was specific to inflamed tissue and was not observed in noninflamed tissue. CSE did not alter the expression of NF-κB or HIF-1α. Assessment of T cell phenotypes in blood and tissue revealed that there were significantly more activated and exhausted T cells in the colonic tissue compared to matched blood. These profiles were not altered following CSE treatment.

**Conclusion:** These data suggest that observed effects of CSE in reducing inflammatory mediators *ex vivo* are specific to inflamed colonic tissue but are not due to the activation of NF-κB or HIF-1α and are not caused by alterations in subpopulations of T cells in these UC tissues.

## Introduction

Ulcerative colitis (UC) is a chronic inflammatory condition affecting the colon. Understanding of the etiology of UC remains limited, with the current model favoring a complex interplay of genetics, environmental factors, gut microbiota, and immune system dysregulation. Environmental factors such as diet, urban environment, and smoking status contribute to the pathogenesis of UC.[1–3] The most reproducibly robust example of the influence of the environment on UC behavior is that of tobacco use, particularly cigarette smoking.[4] Interestingly, smoking is well recognized as having a protective role in UC patients, with smokers reported as having fewer UC-related symptoms, fewer hospitalizations, and lower colectomy rates.[5,6] In addition to the association between UC diagnosis and smoking status, it has been

reported that smoking status may alter the disease course of UC with worsening of symptoms and a requirement of more frequent surgery in ex-smokers compared to smokers.[7,8] Despite a wealth of clinical evidence linking smoking status to UC behavior, the biological mechanism(s) controlling this effect remain poorly understood.

Cigarette smoking influences both cellular and humoral immunity. Smokers have been reported to have altered T cell profiles, with heavy smokers having an increased level of suppressor CD8^{+} cells, indicating that immune suppression may be associated with heavy smoking.[9] Invariant natural killer cells (iNKT) cultured with cigarette smoke extract (CSE) have a reduced capacity to secrete interferon (IFN)-γ and tumor necrosis factor (TNF)-α.[10] Generally, naïve T cells are excluded from nonlymphoid tissue,

except in the case of chronic inflammation, where naïve T cells are actively recruited to tertiary tissues by chemokines. This is a significant phenomenon as naïve T cells, once activated, can react to local insult or injury. It is now accepted that nonlymphoid tissue such as colonic mucosa possess naïve, effector, and memory T cell subpopulations, capable of secreting a wide range of chemokines and cytokines.[11] PD-1 (CD279), expressed on the surface of T and B cells following activation and during exhaustion, plays a key homeostatic role in T cell activation and termination of an inflammatory T cell response.[12]

The aim of this study was to examine the effect of CSE on the secretion of inflammatory markers in colonic tissue from UC patients and to examine T cell profiles in UC patients.

## Methods

***Ethic statement and patient recruitment.*** This study was approved by the St James's Hospital and Adelaide, Meath and National Children's Hospital Joint Research Ethics Committee (REC Reference 2012/35/06). All patients presenting to the endoscopy suite at St. James's Hospital between October 2012 and July 2015 with a confirmed diagnosis of UC were invited to participate. A peripheral blood sample was drawn prior to the colonoscopy using standard phlebotomy methods. Colonic tissue from inflamed (where present) and noninflamed tissue was taken using the standard endoscopic biopsy technique. The presence and absence of mucosal inflammation were determined by the endoscopist (FMacC, DK, and NM) during the procedure. All participating endoscopists were experienced in assessing UC patients endoscopically. Samples were processed immediately upon receipt.

*Clinical and demographic data.* Thirty-four patients with a diagnosis of UC were included in this study. Age at diagnosis, age at recruitment, gender, smoking status, and current therapies were recorded for all patients. For full demographic data and clinical data, please see Table 1.

***Generation of CSE.*** CSE was generated by lighting four Marlboro Reds, Class A cigarettes, through 30 mL of Roswell Park Memorial Institute 1640 (RPMI) (Lonza, Switzerland). The generation of CSE is based on a validated pump system.[13] Each cigarette was smoked for 10 puffs, each puff generating 35 mL of smoke, every 30 s, which burned approximately 75% of the cigarette. The volume of smoke generated was 350 mL. Each milliliter of CSE contains 0.133 (4/30) cigarette's worth of smoke-derived constituents. The resultant CSE was sterile-filtered through a 0.2-μm filter (Millipore), with pH adjusted to 7.4, and stored at −20°C. The CSE was diluted in RPMI to make 5%, 10%, and 20% solutions that were used for *ex vivo* human explant tissue experiments. Based on Bernhard's validated volumetric calculations,[13] a 5% solution of CSE equates to 40 cigarettes per day.

***Culturing of biopsies.*** Upon receipt, fresh biopsies were washed four times in explant wash buffer (phosphate-buffered saline [Lonza], 1% penicillin/streptomycin [Lonza], 0.1% gentamicin [Lonza], and 1% Fungizone® [Invitrogen, Carlsbad, CA, USA]). Biopsies were washed a fifth time in complete media (RMPI 1640 media [Lonza] supplemented with 10% fetal bovine

**Table 1** Demographic and clinical characteristics of patients

| | | |
|---|---|---|
| Age at diagnosis (years)[†] | 37.5 | (13–66) |
| Time since diagnosis (years)[†] | 13.36 | (0–40) |
| Age at recruitment (years)[†] | 50.5 | (21–76) |
| Gender | | |
| Female | 12 | 35% |
| Male | 22 | 65% |
| Smoking status | | |
| Nonsmoker | 11 | 32% |
| Ex-smoker | 16 | 47% |
| Current smoker | 5 | 15% |
| Other[‡] | 1 | 3% |
| Unknown | 1 | 3% |
| Current therapies[§] | | |
| 5ASA therapy | | |
| Yes | 27 | 79% |
| No | 7 | 21% |
| Oral prednisone | | |
| Yes | 2 | 6% |
| No | 32 | 94% |
| Thiopurine therapy | | |
| Yes | 10 | 29% |
| No | 24 | 71% |
| Anti-TNFα therapy | | |
| Yes | 7 | 21% |
| No | 27 | 79% |

[†]Represented as mean (range).
[‡]Tobacco in pipe.
[§]Current therapies at time of recruitment to study.

serum [Invitrogen], 1% penicillin/streptomycin, 0.1% gentamicin, and 1% Fungizone®). Biopsies were cultured in the presence of 0%, 5%, 10%, or 20% CSE for 24 h at 37°C and 5% $CO_2$. Following 24-h treatment, biopsies were snap frozen in liquid nitrogen and stored at −80°C. The tissue-conditioned media (TCM) was removed and stored at −80°C.

***Measurement of inflammatory mediators.*** Levels of inflammatory mediators were measured in the TCM. A multiplex ELISA for inflammatory mediators was custom designed and obtained from Mesoscale Discovery. Each well on the 96-well microplate detected CCL2, CCL20, CXCL1, IL-1β, IL-10, IL-2 IL-6, MMP2, MMP9, and TNF-α. Manufacturer's protocol was adhered to. All secretion data were normalized to total protein in matched biopsy tissue. Total protein was isolated from the biopsy using the TransAM® Nuclear Extract Kit (Active Motif, Belgium) using the manufacturer's guidelines. Protein was quantified using the Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA).

***NF-κB and HIF-1α assay.*** Protein isolated from human *ex vivo* biopsies was used to measure NF-κB p65 and HIF-1α expression by TransAM® DNA-binding ELISA (Active Motif), adhering to manufacturer's protocols.

***Tissue digestion for flow cytometric analysis.*** Biopsies were mechanically and enzymatically digested for T cell phenotyping. Biopsies were chopped finely using a scalpel and

subsequently incubated in collagenase buffer (Krebs-Ringer buffer, 4%[w/v] bovine serum albumin and 2 mg/mL collagenase type IV) for 25 min at 180 rpm in an orbital shaker. Following incubation, dissociated tissue was strained through a 70-μm filter and washed in wash buffer (Krebs-Ringer solution, 1%[w/v] bovine serum albumin). Cells were resuspended in complete media and immediately stained for flow cytometric analysis.

***Flow cytometry.*** Cells were stained with antibodies for CD3, CD4 (Biolegend, San Diego, CA, USA), CD8, CD69, CD45RA (BD bioscience, Franklin Lakes, NJ, USA), CD45RO (Immunotools, Friesoythe, Germany), CD62L (eBiosciences, San Diego, CA, USA), and PD-1 (eBiosciences, USA) for 30 min at 4°C. Cells were washed in flow buffer (phosphate-buffered saline [Sigma-Aldrich, St. Louis, MO, USA], 2%(v/v) fetal bovine serum [Lonza], 0.1%(v/v) sodium azide [Sigma-Aldrich]). Cells were immediately acquired using a CyAn ADP flow cytometer (Beckman Coulter, Brea, CA, USA) and Summit software (v4.3); Fort Collins, CO, USA. All data were analyzed using FlowJo v7.6.1 software (FlowJo, TreeStar, Ashland, OR, USA).

***Statistical analysis.*** Data were analyzed using GraphPad Prism (GraphPad Prism, San Diego, CA, USA) software and expressed as mean ± SEM. Statistical differences in mean values between two groups were analyzed using a paired, nonparametric *t*-test (Wilcoxon signed rank). Statistical differences in mean values between more than two experimental groups were determined by a nonparametric one-way ANOVA test (Kruskal-Wallis) with Dunn's posttest. Statistical significance was determined as $P \leq 0.05$.

## Results

***CSE alters the inflammatory microenvironment in inflamed UC tissue.*** To determine if CSE alters the secretion of inflammatory mediators from the UC microenvironment, the TCM was screened for levels of CXCL1, CCL20, MMP2, MMP9, IL-1β, IL-2 IL-6, TNF-α CCL2, and IL-10. Following treatment with 20% CSE, there was a significant reduction in the secretion of IL-2 ($P = 0.02$), IL-6 ($P = 0.004$), TNF-α ($P = 0.01$), CCL2 ($P = 0.006$), and IL-10 ($P = 0.003$) from inflamed tissue, compared to 0% CSE (Fig. 1a–e). This effect was specific to the inflamed UC tissue and was not observed in the matched noninflamed UC tissue (Fig. 1f–j). Levels of CXCL1 ($P = 0.07$), CCL20 ($P = 0.6$), MMP2 ($P = 0.5$), MMP9 ($P = 0.4$), or IL-1β ($P = 0.06$) were not altered following CSE treatment.

***The effect of CSE on the inflammatory microenvironment is not linked with NF-κB or HIF-1α.*** To determine if the alterations observed in the inflammatory mediator secretion were due to the activation of master transcription factors, NF-κB, and HIF-1α, CSE-treated inflamed and noninflamed tissue was examined. A previous study demonstrated that the activation of NF-κB and HIF-1α was associated with a reduction in the expression of inflammatory mediators.[14] Treatment with 5%, 10%, or 20% CSE did not significantly affect the activation of NF-κB or HIF-1α in inflamed or noninflamed tissue (Fig. 2a–d). These data suggest that the effect of CSE on the secretion of
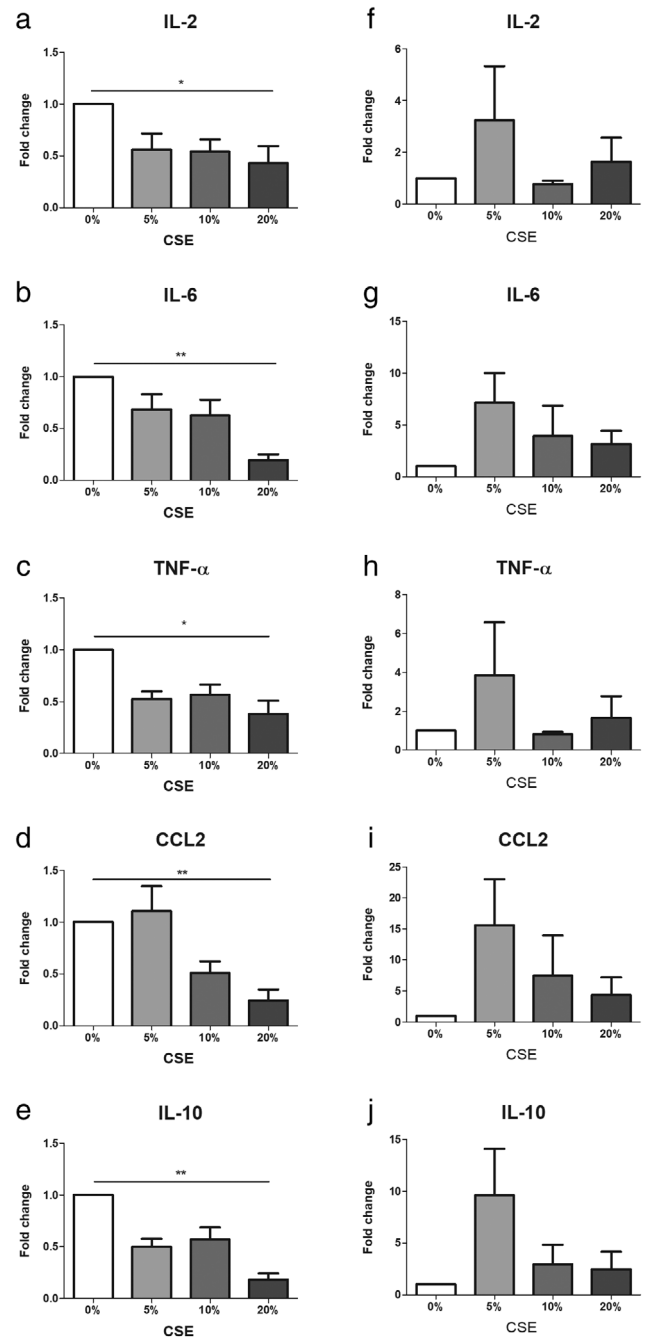


**Figure 1** Effect of CSE on inflamed and noninflamed UC tissue. (a–e) INFLAMED treatment with 20% CSE significantly decreased secretion of IL-2 ($P < 0.05$), IL-6 ($P < 0.01$), TNF-α ($P < 0.05$), CCL2 ($P < 0.01$), and IL-10 ($P < 0.01$) compared to matched untreated tissue (0% $n = 5$, 5% $n = 4$, 10% $n = 5$, 20% $n = 4$). (f–j) NONINFLAMED treatment with 5%, 10%, or 20% CSE did not significantly alter secretion of IL-2, IL-6, TNF-α, CCL2, or IL-10 compared to matched untreated tissue (all $P > 0.05$) (0% $n = 7$, 5% $n = 3$, 10% $n = 7$, 20% $n = 5$). All secreted data are normalized to total protein content of tissue. Data are presented as mean ± SEM. Kruskal-Wallis one-way ANOVA with Dunn's posttest *$P < 0.05$, **$P < 0.01$.
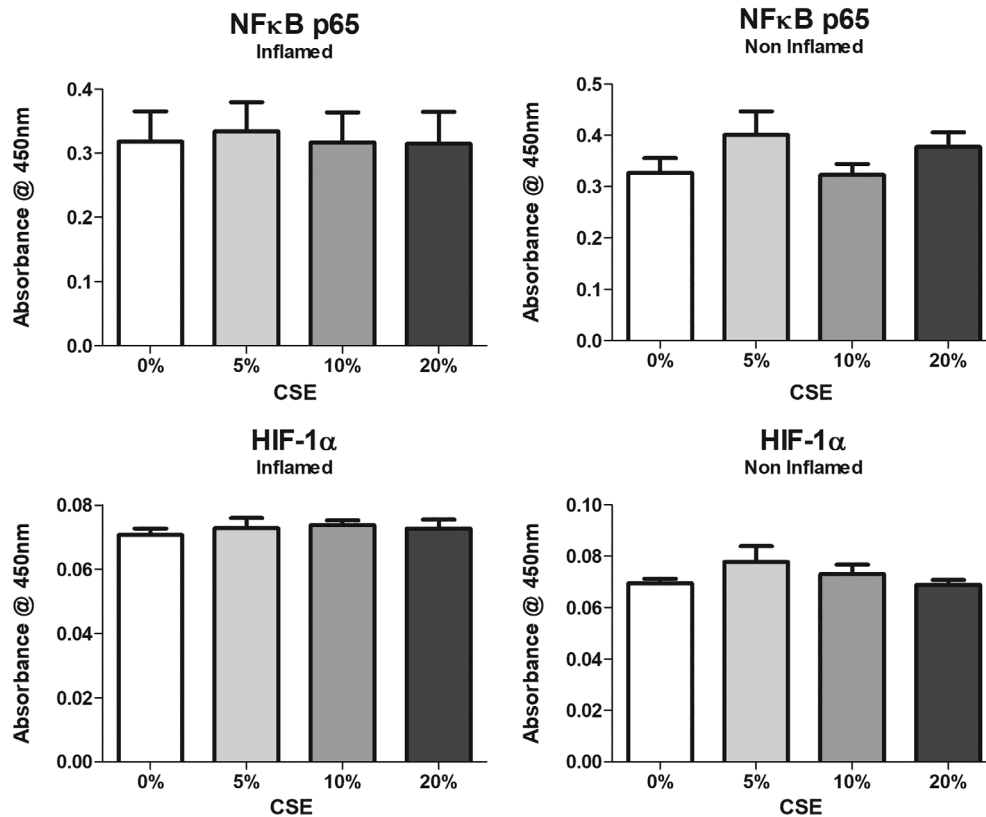
**Figure 2** Expression of NF-κB and HIF-1α in inflamed and noninflamed UC tissue. (a,b) Levels of the p65 subunit of NF-κB are not significantly different across CSE treatments in inflamed or noninflamed tissue (all, $P > 0.05$). (c,d) Levels of HIF-1α are not significantly different across CSE treatments in inflamed or noninflamed ex vivo tissue from UC patients (all $P > 0.05$). All secreted data are normalized to total protein content (0% $n = 5$; 5% $n = 4$; 10% $n = 5$; 20% $n = 4$). Data are presented as mea$n$ ± SEM. Kruskal-Wallis one-way ANOVA with Dunn's posttest.

inflammatory mediators in inflamed UC tissue is not due to NF-κB or HIF-1α activation.

***Inflamed and noninflamed colonic tissue is enriched with activated and exhausted T cells, but CSE does not alter these T cell profiles in UC tissue.*** Prior to determining if CSE alters T cell phenotypes in UC tissue, it was necessary to characterize the T cell subsets present in matched blood and UC tissue. The proportion of CD4+ cells expressing the activation marker, CD69, was significantly higher in inflamed ($P = 0.03$) and noninflamed ($P = 0.004$) tissue compared to blood (Fig. 3a). Similarly, there was a significantly higher proportion of CD8+ CD69+ T cells in inflamed and noninflamed tissue compared to blood ($P = 0.03$ and $P = 0.004$, respectively) (Fig. 3b).

There was no significant difference in the percentage of CD4+ CD45RO+ T cells between blood and tissue (inflamed and noninflamed) ($P > 0.05$) (Fig. 3c). A significant increase in the percentage of CD8+ CD45RO+ T cells was observed in the noninflamed tissue compared to blood ($P = 0.02$). There was no increase in CD8+ CD45RO+ cells in inflamed tissue compared to blood ($P > 0.05$) (Fig. 3d). Examining two markers of naïve T cells, CD45RA and CD62L, the percentage of CD4+ CD45RA+

T cells was significantly lower in the inflamed and noninflamed tissue compared to blood ($P < 0.05$ and $P < 0.01$) (Fig. 3e). The proportion of CD8+ CD45RA+ T cells was significantly lower in the noninflamed tissue compared to blood ($P < 0.05$). There was no significant difference in the percentage of CD8+ CD45RA+ T cells between inflamed tissue and blood ($P > 0.05$) (Fig. 3f). The proportion of CD4+ cells expressing the naïve marker CD62L was significantly lower in inflamed ($P = 0.03$) and noninflamed ($P = 0.004$) tissue compared to blood (Fig. 3g). In a similar manner, there was a significantly lower proportion of CD8+ CD62L+ T cells in inflamed and noninflamed tissue compared to blood ($P = 0.03$ and $P = 0.004$, respectively) (Fig. 3h).

To elucidate the level of T cell exhaustion due to prolonged activation, PD-1 expression on CD4+ and CD8+ T cells was assessed. There was a significantly higher proportion of PD-1+ CD4+ and PD-1+ CD8+ in noninflamed tissue compared to blood (both $P < 0.02$). In the inflamed tissue, there was a higher proportion of CD8+ T cells expressing PD-1 ($P < 0.03$) compared to blood; however, the proportion of PD-1+ CD4+ T cells did not differ between blood and inflamed tissue ($P > 0.05$) (Fig. 3i,j). For all markers examined (CD69, CD45RO, CD45RA, CD62L, and PD-1), there were no significant differences in the proportions between inflamed and noninflamed tissue (all $P > 0.05$).
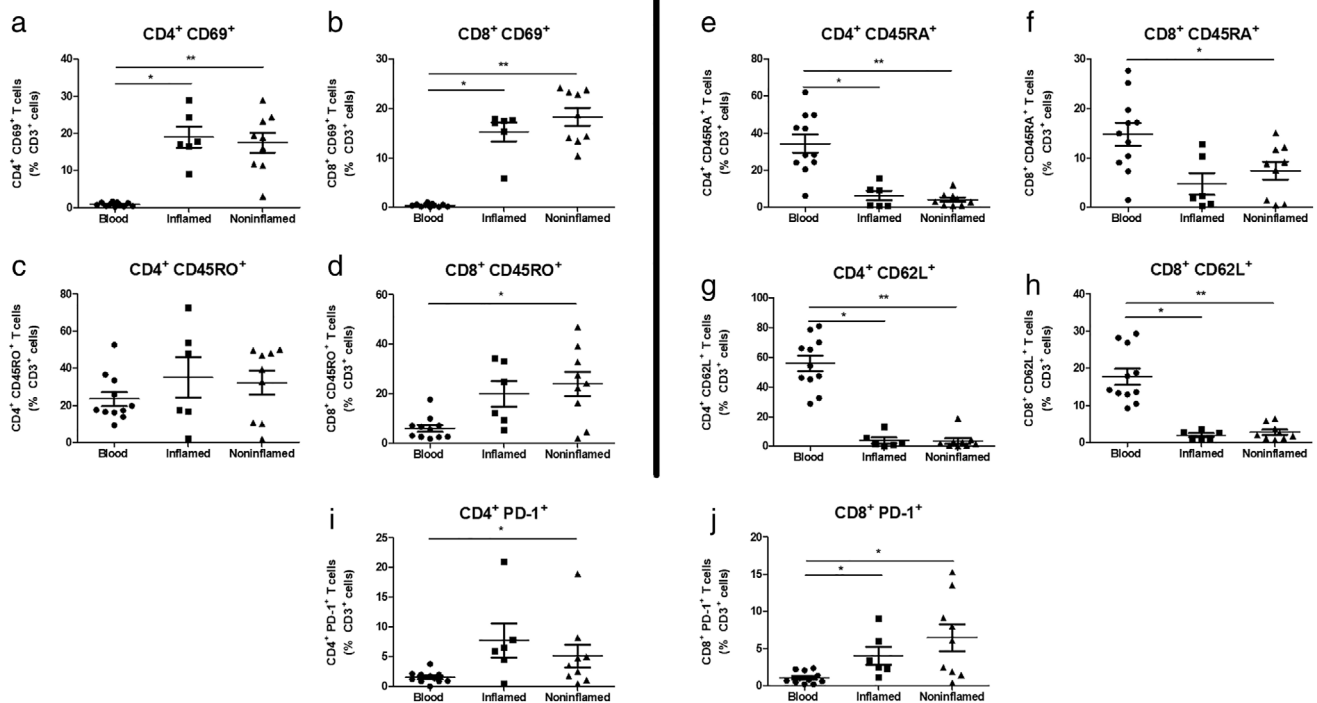
**Figure 3** T cell profiles in blood and tissue of UC patients. (a,b) There were significantly lower percentages of CD4+ CD69+ and CD8+ CD69+ T cells in the blood compared to matched inflamed and noninflamed tissue. (c,d) There was a significantly higher percentage of CD8+ CD45RO+ T cells in the noninflamed tissue compared to the blood in UC patients with no difference in the percentages of CD4+ CD45RO+ cells between blood and inflamed and noninflamed tissue from matched UC samples. (e,f) There was a significantly higher percentage of CD4+ CD45RA+ cells in the blood compared to matched inflamed and noninflamed tissue with a higher percentage of CD8+ CD45RA+ T cells in the noninflamed tissue only. (g,h) There was a significantly higher percentage of CD4+ CD62L+ and of CD8+ CD62L+ cells in the blood compared to matched inflamed and matched noninflamed tissue. (i,j) There were significantly more CD4+ PD-1+ and CD8+ PD-1+ T cells in the noninflamed tissue compared to matched blood of UC patients ($n = 11$ blood, $n = 6$ inflamed, $n = 9$ noninflamed). Data presented as mean SEM. Wilcoxon matched pairs test.*$P < 0.05$, **$P < 0.01$.

To determine if the observed effects of CSE on the secretome of the inflamed microenvironment were due to shifts in T cell phenotypes, CSE-treated biopsies were examined. In both the inflamed and noninflamed tissue, compared to matched untreated tissue, CSE treatment did not affect the percentages of activated CD4+ CD69+, CD8+ CD69+, CD4+ CD45RO+, or CD8+ CD45RO+ T cells (all $P > 0.05$) (Fig. 4a–d). Similarly, CSE did not affect the percentages of naïve CD4+ CD45RA+, CD8+ CD45RA+, CD4+ CD62L+, or CD8+ CD62L+ (all $P > 0.05$) (Fig. 4e–h). When examining the exhaustion status of the T cells present in the inflamed and noninflamed tissue, there was no difference in the proportion of CD4+ PD-1+ (both $P > 0.05$) or CD8+ PD-1+ (both $P > 0.05$) between untreated and CSE-treated samples (Fig. 4i,j).

## Discussion

Notwithstanding the clinical evidence supporting the protective effect of smoking in UC, there is little progress made toward understanding the biological mechanism(s) controlling this effect. To date, no studies have examined the effect of CSE using human *ex vivo* UC tissue.

We hypothesized that CSE would reduce the secretion of inflammatory mediators in human *ex vivo* tissue and that this may, in part, be caused by altered T cell phenotypes in the UC tissue microenvironment. To address whether the effect of CSE was pancolonic, we examined *inflamed* and *noninflamed* tissue. Levels of pro-inflammatory cytokines IL-2, IL-6, and TNF-α and the chemokine CCL2 are all reduced following treatment with CSE in the inflamed tissue only, indicating that the effect of CSE is specific to inflamed tissue and not a pancolonic effect. It is particularly remarkable that CSE reduced both IL-6 and TNF-α as they are two cytokines that are most strongly associated with autoinflammatory diseases.[15] Interestingly, neutralization of IL-6 signaling results in colonic T cell apoptosis and subsequent suppression of colitis in mice.[16] Wewers et al. demonstrated that, in individuals who smoke, there was a decrease in the secretion of TNF-α from bronchoalveolar lavage fluid lymphocytes following lipopolysaccharide (LPS) stimulation *ex vivo*.[17] It is noted that their studies were conducted on human immunodeficiency viruses (HIV), where a responsive immune system is paramount. In the case of UC, downregulation of inflammatory cytokines by CSE may help explain the protective mechanism of cigarette smoking in UC.
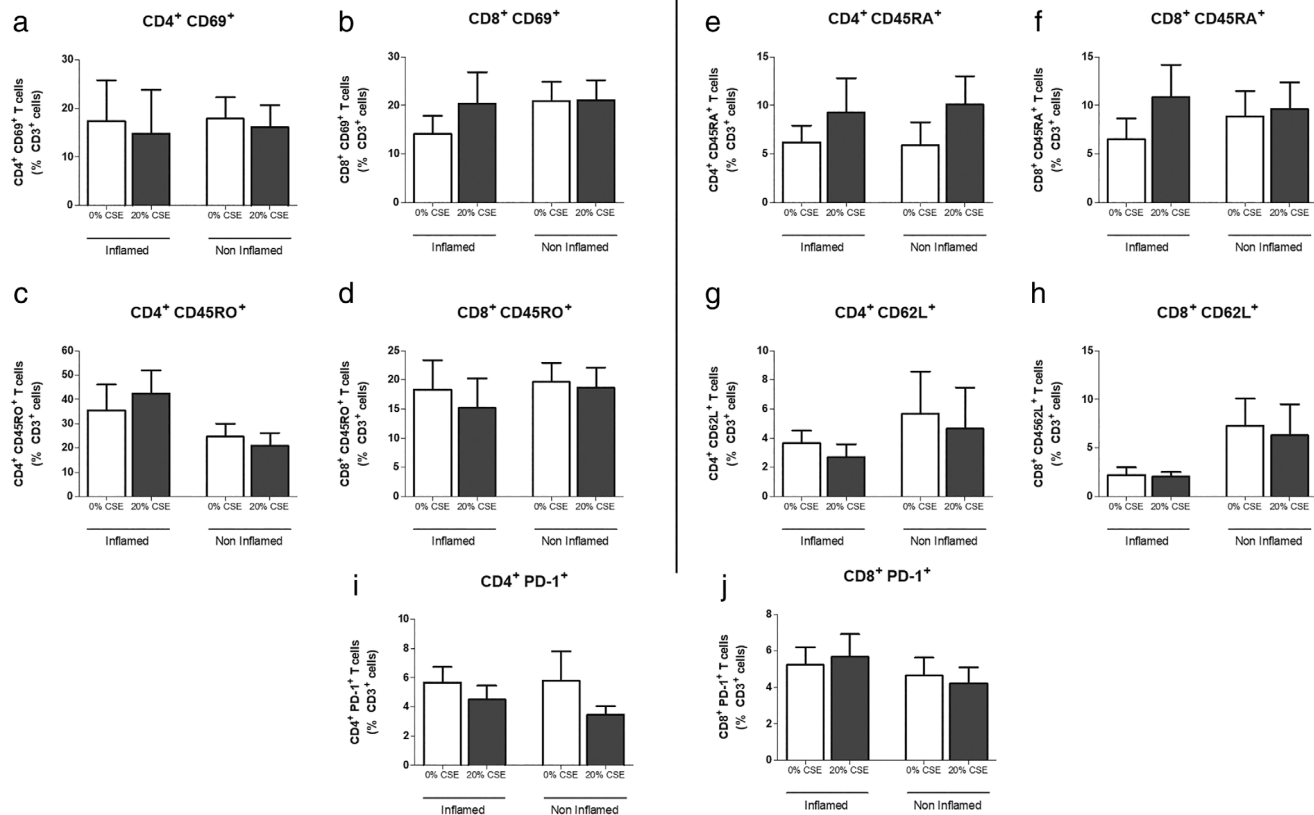
**Figure 4** Effect of CSE treatment on activation, naivety, and exhaustion status of T cells from inflamed and noninflamed UC tissue. Following treatment with 20% CSE, there was no significant difference in the percentage of activated (a–d), naïve (e–h), or exhausted (i,j) T cells from inflamed or noninflamed colonic tissue of UC patients ($n = 8$ inflamed, $n = 16$ noninflamed). Data presented as mean ± SEM. Kruskal-Wallis one-way ANOVA with Dunn's post-test.

In addition to the reduction in pro-inflammatory mediators, we also observed that CSE treatment reduced the secretion of IL-10. While IL-10 is largely considered an anti-inflammatory regulatory cytokine, there is evidence that IL-10 can also act in a pro-inflammatory manner. It is hypothesized that $CD4^+$ T cells produce IL-10, and only a subset of these IL-10-producing cells have regulatory activity. $CD4^+$ T cells incubated with IL-10 demonstrated a strongly enhanced ability to produce pro-inflammatory mediators such as IFN-γ, TNF-α, and IL-2.[18] In addition, Brockmann et al. demonstrated that patients with inflammatory bowel disease (IBD) had a deficiency in IL-10-producing $CD4^+$ T cells that have a regulatory capacity.[19]

NF-κB is a transcription factor for many inflammatory genes, including IL-6, IL-1β, and TNF-α, and HIF-1α is a transcription factor that plays an essential role in the cellular responses to hypoxia, as found in the colon.[20] In mice, previous studies have shown that upregulation of HIF-1α resulted in a protective effect from colitis.[14] We investigated if the anti-inflammatory effects of CSE we observed in the inflamed UC biopsies correlated with alterations in NF-κB and/or HIF-1α. We found that there was no difference in the levels of NF-κB or HIF-1α, suggesting that the mechanism of action of CSE in human *ex vivo* explant tissue is not via the NF-κB or HIF-1α

pathways. These findings conflict with the literature as there have been numerous studies which have shown that the activation of both NF-κB and HIF-1α pathways are protective in colitis.[21–23] Activation of NF-κB and HIF-1α occurs rapidly within the cell, and perhaps by 24 h (duration of CSE treatment), NF-κB and/or HIF-1α may have exerted their effects and returned to baseline.

As we observed differences in the secretion of inflammatory mediators with CSE treatment *ex vivo*, we investigated if the immunomodulatory effects of CSE may be linked to alterations in T cell phenotypes in the UC tissue microenvironment. It has been shown that cigarette smoke can induce an accumulation of $CD4^+$ and $CD8^+$ T cells, induce apoptosis of activated T cells, and suppress cytokine gene expression.[24–26] We examined activated, naïve, and exhausted T cells and the effect of CSE on these subsets in peripheral blood and inflamed and noninflamed colonic tissue from UC patients. In addition, we explored the effect of CSE on T cells in UC tissue. We have shown that the levels of naïve T cells were significantly lower in the tissue compared to matched blood as expected; however, we have also identified naïve T cells present in the inflamed tissue of UC patients. These results match those of Weninger *et al.*, who demonstrated similar percentages of $CD45RA^+$ $CD3^+$ colonic-infiltrating lymphocytes in inflamed mucosa from UC patients.[27]

Furthermore, these findings complement data from murine models where naïve CD4^{+} and CD8^{+} T cells have been shown to traffic directly into the intestinal mucosa as part of their normal migration.[28,29] While CD62L^{+} T cells were higher in the blood compared to the tissue, there was no difference in CD62L^{+} T cells between the inflamed and noninflamed tissue. It has been shown that the percentage of CD62L^{+} T cells is higher in the inflamed tissue of UC patients compared to healthy controls.[30] An explanation for this may be that CD62L is a lymphoid-homing receptor, and CD62L^{+} T cells are recruited to the mesenteric lymph nodes during acute inflammation.[31] CD69^{+} T cells were significantly higher in the colonic tissue of patients compared to blood. This finding is expected as CD69 is an early activation marker and is constitutively expressed at sites of chronic inflammation.[32] Studies in mice have shown that CD69^{+} T cells infiltrate extensively into the inflamed mucosa of the colon in colitis.[33]

We found a significant increase in PD-1^{+} T cells in the noninflamed tissue compared to the blood. The abundance of CD8^{+}PD-1^{+} T cells was higher in the noninflamed tissue compared to matched inflamed tissue (nonsignificant). In a mouse model of colitis, the ligand PD-L1 has been demonstrated to ameliorate disease activity.[34] However, caution is warranted as the expression of PD-1 is associated with dysfunctional T cells in cancer.[35] This is highlighted by the fact that anti-PD-1 therapy is a treatment option for several cancer types.

Following treatment with CSE, there was no significant alteration in the levels of any of the T cell surface markers examined. This suggests that the effects we detect in inflammatory mediators following CSE treatment are not associated with alterations in subpopulations of αβ T cells in the UC tissue microenvironment. While the focus of this study was the effect of CSE on CD3^{+} CD4^{+} and CD3^{+} CD8^{+} T cells, the effect of CSE on other immune cells is an important consideration. CSE may affect antigen-presenting cells (APCs) and may reduce their functional capacity to activate and direct T cells to sites of inflammation. Indeed, studies have shown that CSE can cause dendritic cells to change their chemokine profiles in chronic obstructive pulmonary disease.[36] Furthermore, Vassallo *et al.* demonstrated that CSE inhibited dendritic cell-mediated T cell priming in the lung by inhibiting the secretion of IL-12p70 in mature dendritic cells.[37] In addition, CSE treatment is associated with an inhibition of Dendritic cells (DC) capacity to activate antigen-specific T cell responses[38] *Ex vivo* treatment with CSE has been shown to inhibit the upregulation of CD107 on CD8^{+} T cells and natural killer and invariant natural killer T (iNKT) cells.[10] Furthermore, exposure to cigarette smoke induced a marked increase in recruitment of iNKT cells to the colon *in vivo*, and iNKT-deficient mice with colitis failed to display a protective effect on cigarette smoke exposure.[39] Furthermore, alveolar macrophages from cigarette smokers have an impaired bioenergetic immune response and reduced capacity to secrete inflammatory mediators such as TNF-α, IL-1β, and IFN-γ.[40,41]

Therefore, it is possible that CSE may exert an immunomodulatory effect on other immune cells in the UC microenvironment. Due to the whole-body harmful effects of cigarette smoking, smoking should not be encouraged for individuals with UC. Future studies may reveal if components of cigarette smoke could be a novel therapeutic option for UC. In conclusion, this study has shown, for the first time, that CSE alters inflammatory protein secretion exclusively in inflamed UC tissue, with no effect on the proportion of subsets of T cells within the UC tissue microenvironment. Furthermore, the immunomodulatory effect of CSE on the UC tissue secretome is not linked to the activation of NF-κB or HIF-1α. These data provide new insights into the biological processes controlling the protective effect of cigarette smoking on UC.

## Acknowledgments

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